

***In-vitro* Investigation of the Efficacy of chitosan-Supported Metal Nanoparticles in the Treatment of Multidrug-Resistant *Candida auris***

by

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A Dissertation Submitted to the  
Graduate School of Health Sciences  
in Partial Fulfillment of the Requirements for  
the Degree of  
Master of Science

in

Medical Microbiology



August 8, 2025

# **In-Vitro Investigation of the Efficacy of Chitosan-Supported Metal Nanoparticles in the Treatment of Multidrug-Resistant *Candida auris***

Koç University

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Date: August 8, 2025

## ABSTRACT

### **In-Vitro Investigation of the Efficacy of Chitosan-Supported Metal Nanoparticles in the Treatment of Multidrug-Resistant *Candida auris***

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**Master of Science in Medical Microbiology**

**August 8, 2025**

*Candida* species are a natural element of the human microbiome and cause fatal infections when the immune system is weakened and the right opportunity arises. *Candida auris* is one of the five most important microorganisms on the list of the most serious microorganisms threatening humanity published by the Centers for Disease Control and Prevention (CDC) in 2019. Its resistance to many known antifungal drugs, antiseptic and disinfectant substances, its thermotolerance and osmotolerance properties, its ability to secrete enzymes such as phospholipase and hemolysin, its ability to form biofilms, and to colonize living and non-living surfaces make it a remarkable microorganism. Previous studies have shown the potential antifungal activities of metal nanoparticles (MNPs) against *Candida* species. In this study, the in vitro activity of gold (Au) and iron oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles and their chitosan-supported forms against *C. auris* planktonic and biofilm cells was investigated.

In this study, a multidrug-resistant clinical *C. auris* isolate (K1575) and four reference isolates were used. The drug resistance profile of the isolates was revealed by broth microdilution testing. The genomic basis of the resistance profile of the clinical *C. auris* isolate was analyzed by whole genome sequencing. AuNP synthesis was carried out by citrate-reduction reaction, and chitosan-supported gold (Chi-AuNP) nanoparticles were synthesized by chitosan-supported reduction reaction. Fe<sub>3</sub>O<sub>4</sub>NP synthesis was carried out with oleyl amine and then colloidal stabilization was provided with citric acid and chitosan. The compatibility of all synthesized NPs with the literature was checked by X-Ray Diffraction (XRD), Fourier-Transform Infrared Spectroscopy (FT-IR), Scanning Electron Microscope (SEM), UV-Visible Spectroscopy (UV-Vis), Nanoparticle Tracking Analysis (NTA) and Dynamic Light Scattering (DLS) tests. For *in-vitro* efficacy tests, cytotoxicity tests were tested on the NIH 3T3 fibroblast cell line by the MTT method. Within the scope of in vitro efficacy tests, five selected isolates were subjected to colony forming unit (CFU), spectrophotometric analysis, biofilm thickness determination by the XTT/Menadione method, SEM, reactive oxygen species (ROS) determination by

H<sub>2</sub>DCFDA and demonstration of NPs on the biofilm formed by the isolates by confocal microscope experiments.

The azole and echinocandin resistance detected with the broth microdilution test was confirmed with genomic analysis. Mutations were detected in the FKS1, ERG11, CDR1, TAC1b and FCY1 genes. In the CFU experiments initiated by determining the cytotoxic concentrations (CC) of the nanoparticles synthesized and quality controlled in accordance with the literature, it was determined that AuNPs and Chi-AuNPs had an inhibitory effect on the planktonic and biofilm forms of the clinical isolate K1575 at every dose applied, and that there was an inhibitory effect depending on the isolate at the doses applied on the planktonic and biofilm forms of the reference isolates. In the ROS experiments conducted, it was shown that this effect was provided by the ROS created by the interaction of the nanoparticles with the isolates. It was determined that the Fe<sub>3</sub>O<sub>4</sub>NP and Chi-Fe<sub>3</sub>O<sub>4</sub>NPs applied in the CFU experiments had no inhibitory effect on any of the isolates used.

As a result, the genomic base of multidrug resistance in K1575 was shown by multiple mutations in the target antifungal genes. It was determined that K1575 and reference isolates showed inhibition in gold-based nanoparticles and that this inhibition was primarily related with ROS formation and fungal cell wall disruption. It was shown that gold-based nanoparticles should be considered as a potential for a new treatment method for multidrug resistant *C. auris* infections.

**Keywords:** *C. auris*, multidrug resistance, gold nanoparticles, iron nanoparticles, chitosan-supported metal nanoparticles

## ÖZETÇE

### Çok İlaça Dirençli *Candida auris* Tedavisinde Kitosan Destekli Metal

### Nanopartiküllerin Etkinliğinin *In-Vitro* Araştırılması

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8 Ağustos, 2025

*Candida* türleri, insan mikrobiyomunun doğal bir unsurudur ve bağışıklık sistemi zayıfladığında ve doğru fırsat doğduğunda ölümcül enfeksiyonlara neden olur. *Candida auris*, Hastalık Kontrol ve Önleme Merkezleri (CDC) tarafından 2019 yılında yayınlanan insanlığı tehdit eden en ciddi mikroorganizmalar listesindeki en önemli beş mikroorganizmadan biridir. Bilinen birçok antifungal ilaca, antiseptik ve dezenfektan maddeye karşı direnci, termotolerans ve ozmotolerans özellikleri, fosfolipaz ve hemolizin gibi enzimleri salgılayabilmesi, biyofilm oluşturabilmesi ve canlı ve cansız yüzeylerde kolonize olabilmesi onu dikkat çekici bir mikroorganizma yapmaktadır. Önceki çalışmalar, metal nanopartiküllerin (MNP'ler) *Candida* türlerine karşı potansiyel antifungal aktivitelerini göstermiştir. Bu çalışmada, altın (Au) ve demir oksit ( $Fe_3O_4$ ) nanopartiküllerinin ve kitosan destekli formlarının *C. auris* planktonik ve biyofilm hücrelerine karşı in vitro aktivitesi araştırılmıştır.

Bu çalışmada, çoklu ilaca dirençli klinik bir *C. auris* izolatı (K1575) ve dört referans izolat kullanıldı. İzolatların ilaç direnç profili, Klinik ve Laboratuvar Standartları Enstitüsü (CLSI) M27M44S kılavuzuna dayalı sıvı mikrodilüsyon testi ile ortaya kondu. Klinik *C. auris* izolatının direnç profilinin genomik temeli, tüm genom dizilemesi ile analiz edildi. AuNP sentezi sitrat indirgeme reaksiyonu ile, kitosan destekli altın (Chi-AuNP) nanopartikülleri ise kitosan destekli indirgeme reaksiyonu ile gerçekleştirildi.  $Fe_3O_4$ NP sentezi oleyl amin ile gerçekleştirildi ve ardından kolloidal stabilizasyon sitrik asit ve kitosan ile sağlandı. Sentezlenen tüm NP'lerin literatürle uyumluluğu X-Işını Kırınımı (XRD), Fourier Dönüşümlü Kızılötesi Spektroskopisi (FT-IR), Taramalı Elektron Mikroskobu (SEM), UV-Görünür Spektroskopisi (UV-Vis), Nanopartikül İzleme Analizi (NTA) ve Dinamik Işık Saçılımı (DLS) testleri ile kontrol edildi. İn vitro etkinlik testleri için, NIH 3T3 fibroblast hücre hattında MTT yöntemi ile sitotoksikite testleri yapıldı. İn vitro etkinlik testleri kapsamında, seçilen beş izolat üzerinde koloni oluşturma birimi (CFU), spektrofotometrik analiz, XTT/Menadione yöntemi ile biyofilm

kalınlığı tayini, SEM, H2DCFDA ile reaktif oksijen türleri (ROS) tayini ve izolatların oluşturduğu biyofilm üzerinde konfokal mikroskop deneyleri ile NP'lerin gösterilmesi işlemleri gerçekleştirildi.

Sıvı mikrodilüsyon testi ile tespit edilen azol ve ekinokandin direnci genomik analiz ile doğrulanarak FKS1, ERG11, CDR1, TAC1b ve FCY1 genlerinde mutasyonlar tespit edilmiştir. Literatür doğrultusunda sentezlenen ve kalite kontrolü yapılan nanopartiküllerin sitotoksik konsantrasyonlarının (CC) belirlenmesiyle başlatılan CFU deneylerinde, AuNP'lerin ve Chi-AuNP'lerin uygulanan her dozda klinik izolat K1575'in planktonik ve biyofilm formları üzerinde inhibitör etkiye sahip olduğu, referans izolatların planktonik ve biyofilm formlarına uygulanan dozlarda ise izolata bağlı olarak inhibitör etki gösterdiği belirlenmiştir. Yapılan ROS deneylerinde ise bu etkinin nanopartiküllerin izolatlarla etkileşimi sonucu oluşan ROS tarafından sağlandığı gösterilmiştir. CFU deneylerinde uygulanan Fe<sub>3</sub>O<sub>4</sub>NP ve Chi-Fe<sub>3</sub>O<sub>4</sub>NP'lerin kullanılan izolatların hiçbirinde inhibitör etkiye sahip olmadığı belirlenmiştir.

Sonuç olarak, K1575'te çoklu ilaç direncinin genomik temeli, hedef antifungal genlerdeki çoklu mutasyonlarla gösterilmiştir. K1575 ve referans izolatlarının altın bazlı nanopartiküllerde inhibisyon gösterdiği ve bu inhibisyonun öncelikli olarak ROS oluşumu ve fungal hücre duvarı bozulması ile ilişkili olduğu belirlendi. Altın bazlı nanopartiküllerin çoklu ilaca dirençli *C. auris* enfeksiyonları için yeni bir tedavi yöntemi potansiyeli olarak değerlendirilmesi gerektiği gösterildi.

**Anahtar kelimeler:** *C. auris*, çoklu ilaç direnci, altın nanopartikül, demir nanopartikül, kitosan destekli metal nanopartiküller

## AKNOWLEDGEMENTS

First of all, I would like to express my sincere gratitude to my very valuable advisor Assoc. Prof. Dr. Özlem DOĞAN. She enlightened my dark graduate adventure with her knowledge, experience, interest and tolerance. She saw me not only as a student but also as a researcher she was working with, and she valued and listened to every idea of mine, making me trust myself and the work I did.

Secondly, I would like to thank my valuable laboratory director Prof. Dr. Füsün CAN and Assoc. Prof. Dr. Mert Ahmet KUŞKUCU. I developed myself with the valuable information I learned from the meetings we held every week, and I benefited from their vast knowledge and experience in every step of this thesis. I would like to thank our center director Prof. Dr. Önder ERGÖNÜL for his smiling face and great leadership. I would like to thank my colleagues in the KUISCID team for showing me the value and beauty of working as a team during this process.

I would like to thank Assoc. Prof. Dr. Emel SOKULLU, with whom we worked together on this project's nanoparticle synthesis and characterization part, for her valuable knowledge and support, Buse KELEŞ, Cennet GÜNDÜZ and Elif Cemre ERYİĞİT for their help and support.

I would like to thank İsmail HANÇER for his unlimited love and support, for making me trust myself and helping me find the strength I needed.

I would like to thank my valuable colleagues Fatihan PINARLIK and Ghazal SHAHROKHI. I would like to thank them for providing me with all kinds of support throughout this process, always being there for me, sharing my good and bad days and holding my hand. Both their friendship and work friendship showed me what real support and friendship is on this path. I would like to thank my valuable friend Caner ABLAK for being with me starting from undergraduate and continuing graduate studies.

Finally, I would like to thank my valuable parents Saadet and Hakan ARMUTCUOĞLU, my sister Zeynep ARMUTCUOĞLU, my dear aunt Fatma ALTUNTAŞ and my dear grandmother Asiye ALTUNTAŞ. I would like to thank them for supporting me and being behind me in everything I do. The pride in their eyes and the endless love in their hearts illuminated my path during this process.

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## ABBREVIATIONS

|        |                                                            |
|--------|------------------------------------------------------------|
| AMR    | Antimicrobial Resistance                                   |
| CDC    | Centers for Diseases Control and Prevention                |
| HIV    | Human Immunodeficiency Virus                               |
| WHO    | World Health Organization                                  |
| FPPL   | Fungal Priority Pathogens List                             |
| FC     | Filamentation-Competent                                    |
| ECM    | Extracellular Matrix                                       |
| ABC    | ATP-Binding Cassette                                       |
| MFS    | Major Facilitator Superfamily                              |
| SFC1   | Surface Colonization Factor 1                              |
| HSP    | Heat Shock Protein                                         |
| MTL    | Mating Type-Like                                           |
| HS1    | Hot-Spot 1                                                 |
| RNA    | Ribonucleic Acid                                           |
| DNA    | Deoxyribonucleic Acid                                      |
| GlyNAc | N-acetylglucosamine                                        |
| MDR    | Multidrug Resistant                                        |
| XDR    | Extensive Drug Resistant                                   |
| TMRCA  | Time to Most Recent Common Ancestor                        |
| SNP    | Single Nucleotide Polymorphism                             |
| SAP    | Secreted Aspartyl Proteases                                |
| NET    | Neutrophil Extracellular Traps                             |
| SAPK   | Stress-Activated Protein Kinase                            |
| SDS    | Sodium Dodecyl Sulfate                                     |
| EV     | Extracellular Vesicles                                     |
| ROS    | Reactive Oxygen Species                                    |
| MALDI- | Matrix-Assisted Laser Desorption Ionization–Time of Flight |
| TOF MS | Mass Spectrometry                                          |
| IV     | Intravenously                                              |
| IP     | Intraperitoneal                                            |
| ITS    | Internal Transcribes Spacers                               |
| AFLP   | Amplified Fragment Length Polymorphism                     |

|        |                                                                            |
|--------|----------------------------------------------------------------------------|
| WGS    | Whole Genome Sequencing                                                    |
| FDA    | U.S. Food and Drug Administration                                          |
| PCR    | Polymerase Chain Reaction                                                  |
| RT-PCR | Real Time- Polymerase Chain Reaction                                       |
| LAMP   | Loop-Mediated Isothermal Amplification                                     |
| GPI    | Glucose Phosphate Isomerase                                                |
| TBP    | Transmission Based Precautions                                             |
| HCP    | Healthcare Personnel                                                       |
| PPE    | Personal Protective Equipment                                              |
| NNDSS  | The National Notifiable Disease Surveillance System                        |
| PAHO   | Pan American Health Organization                                           |
| CNISP  | The Canadian Hospital Infection Surveillance Program                       |
| SGSS   | The Second-Generation Surveillance System                                  |
| NP     | Nanoparticle                                                               |
| ATCC   | American Type Culture Collection                                           |
| UV-Vis | Ultraviolet-Visible Spectrophotometry                                      |
| XRD    | X-Ray Diffraction                                                          |
| FT-IR  | Fourier-Transform Infrared Spectroscopy                                    |
| SEM    | Scanning Electron Microscopy                                               |
| NTA    | Nanoparticle Tracking Assay                                                |
| DLS    | Dynamic Light Scattering                                                   |
| FITC   | Fluorescein Isothiocyanate                                                 |
| MWCO   | Molecular Weight Cut-Off                                                   |
| PBS    | Phosphate Buffered Saline                                                  |
| MTT    | (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)             |
| XTT    | (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) |
| CFU    | Colony Forming Unit                                                        |
| RPMI   | Roswell Park Memorial Institute                                            |
| SDA    | Sabouraud Dextrose Agar                                                    |
| TBHP   | Tert-butyl Hydroperoxide                                                   |
| FCC    | Face Centered Cubic                                                        |

## Chapter 1:

# INTRODUCTION

*Candida* species are ubiquitous commensal fungi, natural element of the human microbiome and predominantly inhabit human mucosal surfaces such as skin, oral cavity, gastrointestinal tract, and genital tract (Shiyadeh et al., 2024). *Candida* species are typically benign under the effect of immune system, but they are also opportunistic pathogens that can shift benign state to virulent state and cause infections such as superficial, submucosal and invasive infections in humans which affects almost every organ and has a high mortality rate and cause fatal infections in cases where the immune system is weakened, and a suitable opportunity arises (Huang et al., 2025; Amann et al., 2025). The predisposing factors of opportunistic *Candida* infections are open abdominal surgery, the usage of broad-spectrum antibiotics, treatment in the intensive care unit, human immunodeficiency virus (HIV) infections, chemotherapy, solid organ or stem cell transplantation, neutropenia and diabetes (Bhattacharya et al., 2020; Banda-Flores et al., 2025).

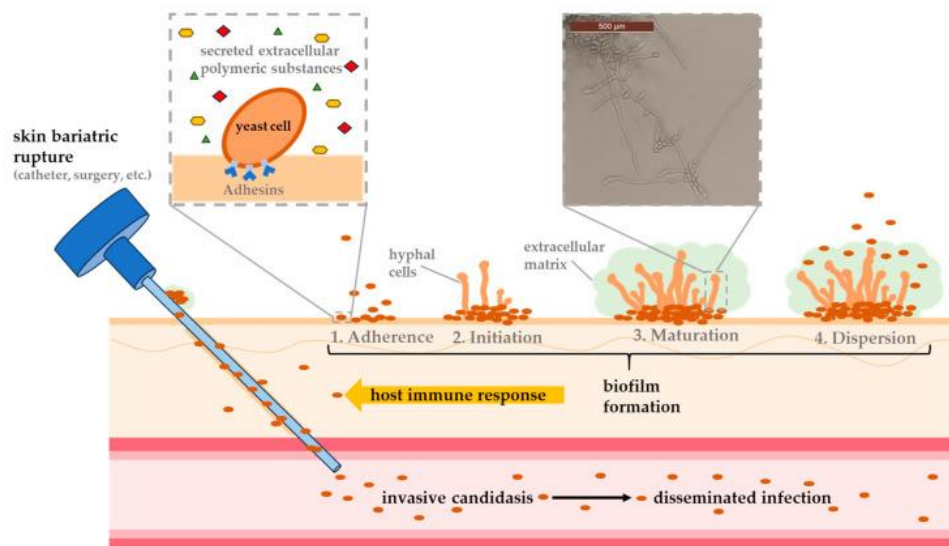


**Figure 1.1** Schematic illustration of invasive candidiasis infection caused by skin lesions the use of medical devices (Amann et al., 2025)

Candidemias are bloodstream infections caused by *Candida* species and can lead to dissemination to organs such as lung, liver, spleen, kidneys, bone and eye (Kullberg and Arendrup, 2015). Crude mortality in bloodstream infections caused by *Candida* species varies between 35-50% and fourth leading cause of nosocomial infections (Pappas et al., 2018; Papon et al., 2013).

The distribution of *Candida* species causing invasive infections, *Candida albicans* is found to be the most prevalent pathogenic species in the genus, approximately 37% reported cases (Huang et al., 2025). However, recent studies showed that the frequency of non-albicans *Candida* species has been increasing in recent years. *C. glabrata*, *C. tropicalis*, *C. parapsilosis*, *C. krusei*, *C. dubliniensis*, *C. lusitaniae* and *C. auris* species are associated with high mortality, primary resistance and reduced susceptibility to antifungal drugs due to the increased use of antifungal drugs in recent years (Bhattacharya et al., 2020; Husni et al., 2023; Papon et al., 2013). This change in the prevalence and epidemiology of invasive *Candida* infections in health care centers around the world constitutes a major public health problem (Parslow and Thornton, 2022).

### **1.1 *Candida auris***

*Candida auris* was first discovered in the ear discharge from the external ear canal of a 70-year-old woman from Tokyo Metropolitan Geriatric Hospital in Japan, 2009 (Sato et al., 2009). History of *C. auris* dates back to 1996, but it was misidentified as *C. haemulonii* because of its close relation with *C. haemulonii*, *C. duobushaemulonii*, *C. pseudohaemulonii*, *C. ruelliae* and *C. lusitaniae* (Lee et al., 2011; Sekyere, 2018). *C. auris* demonstrates a rapid global dissemination, now being reported in over 50 countries across six continents with 60% mortality rate (Valdez et al., 2025; Du et al., 2020). Many *C. auris* strains exhibit resistance to multiple classes of antifungal drugs, and some isolates have demonstrated resistance to all three main antifungal drug class (azoles, polyenes, and echinocandins). U.S. Centers for Diseases Control and Prevention (CDC) categorized it an “urgent antimicrobial resistance (AMR) threat” which is the highest category of concern for AMR pathogens (CDC, 2023). In 2022, the World Health Organization (WHO) included *C. auris* in its Fungal Priority Pathogens List (FPPL), assigning it as a “critical priority pathogen” with its rapid worldwide emergence, high prevalence of

multidrug resistance, and the severe morbidity and crude mortality rates reported between 30% and 60%, associated with its infections (Kim et al., 2024; WHO, 2022).

## 1.2 Taxonomy

Taxonomy of *Candida auris* is defined as: Superkingdom *Eukaryota*; Clade *Opisthokonta*; Kingdom *Fungi*; Subkingdom *Dikarya*; Phylum *Ascomycota*; Subphylum *Saccharomycotina*; Class *Pichiomycetes*; Order *Serinales*; Family *Debaryomycetaceae/Metschnikowiaceae*; CTG Clade; Genus *Candidozyma*; Species *Candida auris* (Satoh and Makimura, 2009).

## 1.3 General Properties

### 1.3.1 Phenotypic Features

*C. auris* cells are in the shape of paired and/or grouped round-to-ovoid or elongated shape, size of (2.0-3.0) x (2.5-5.0)  $\mu\text{m}$  with pale purple, pink or creamy smooth colonies on CHROMagar (CHROMagar, Paris, France) (Osei Sekyere, 2018). *C. auris* strains can grow on different media e.g. Sabouraud's dextrose agar, cornmeal agar, CS4 agar medium, CHROMagar, and Brilliance *Candida* agar.

*C. auris* shows significant morphologic plasticity to provide invasion by displaying three distinct cell types: typical yeast, filamentation-competent (FC) yeast, and filamentous cells (Bing et al., 2024). This phenotypic switching system is crucial for its pathogenesis and adaptation to diverse host environments. This morphogenesis is regulated by some transcription factors and genes. Yue et al. conducted a study about this phenotypic switching in *C. auris* by comparing *C. albicans* which has well-documented phenotypic switch. They compared differential gene expression of *C. auris* with *C. albicans*, and they concluded that they have homolog genes which are responsible this switching. And these morphological changes provide invasion, virulence, and antifungal resistance. *In-vivo* experiments on mammalian models showed that *C. auris* can switch its phenotype from typical yeast to FC/filamentous phenotype like *C. albicans* hyphae formation. These filamentous colonies can be observed in liver and kidney tissues, indicate pathogenicity. And also, lower temperatures (<25°C) can induce filamentous phenotype from representative colonies, while high temperatures (e.g. 37°C) and

synthetic media promote a switch back to yeast form which forms smooth colonies (Yue et al., 2018).

Another phenotypic feature is multicellular aggregates in some *C. auris* isolates which result from a defect in cell division or increased inter-cell adhesion. Among the *C. auris* clades, clade III isolates show the aggregative feature predominantly, but clade I and clade II can also show this feature (Forgács et al., 2020). These aggregates can be adhesin-dependent, which is strongly upregulated in the aggregate-forming clades and related with biofilm formation, or adhesin-independent, induced by sub-inhibitory concentration of echinocandins (Pelletier et al., 2024). Both forms of aggregations are resistant to immune clearance by macrophages.

Biofilm formation is a major virulence factor and crucial component of *C. auris* as a nosocomial pathogen. They have ability to colonize biotic (human skin, host tissues, and wounds) and abiotic (medical devices, hospital materials) surfaces by embedding itself in the polysaccharide-rich (mannan-glucan complex) extracellular matrix (ECM). ECM protects *C. auris* cells from penetration of antifungal drugs and reducing their efficacy by preventing drugs to reach their target and results drug resistance. Biofilm-producing isolates have unique properties such as upregulation of efflux pump encoding genes [ATP-binding cassette (ABC) (*MDR1* and *RDC3*) and major facilitator superfamily (MFS) (*CDR1*, *SNQ2* and, *YHD3*)] and adhesin-relates glycosylphosphatidylinositol (GPI)-anchored cell wall genes (*CSA1*, *IFF4*, *PGA26*, *HYR3*, *PLB3*, *PGA52*, *ALS1* and *ALS5*). First step of biofilm formation is adherence. Some adhesin genes are upregulated for maintaining adhesion such as *ALS4112*, *IFF4109* and *SCF1* (Kean et al., 2018; Rossato et al., 2018). *ALS4112* gene is significant for attachment to keratinocytes and direct colonization. Deletion of this gene can significantly reduce skin colonization and systemic virulence. Surface Colonization Factor (*SCF1*) specific for *C. auris*, necessary for robust attachment to polymer substance. *IFF4109* is conserved adhesin that mediated association with abiotic surfaces (Santana et al., 2023; Zhao et al., 2025).

*C. auris* has thermoresistance ability that allows to grow between 30-42°C, unique trait unseen in other *Candida* species (Satoh et al., 2009). Their thermoresistance also enhance their pathogenicity and persistence in the host by disseminating to organs. This unique feature has led to hypotheses linking its emergence to global climate change, suggesting that environmental warming may have selected it to adapt to higher temperatures. For maintaining this feature, *C. auris* overexpresses heat shock protein

(HSP90) (Jackson et al., 2019; Casadevall et al., 2019). And *C. auris* can live in high salinity conditions (10% NaCl wt/vol), but they showed elongated cell shape with incomplete cell division and pseudohyphae formation for adapting stressful conditions. (Welch et al., 2017; Wang et al., 2018).

### 1.3.2 Genomic Features

*C. auris* has haploid genome with ranged from 12.3-12.5 Mb genome size located in 5-7 chromosomes, 5500 protein coding genes and nearly 44.53%-44.8% guanine-cytosine (GC) (Osei Sekyere, 2018; Bravo Ruiz et al., 2019; Du et al., 2020). *C. auris* belongs to CTG clade (codes serine instead of leucine) which is characterized by translational mechanism (Yang et al., 2024). It has 6 different clades originated from different geographic regions. Clade I (South Asian), Clade II (East Asian), Clade III (South African), Clade IV (South American), Clade V (Iran), and Clade VI (Singapore/Bangladesh) are determined by single-nucleotide polymorphism (SNP) analysis, and each clade is separated from other clade with thousands of SNPs (Lockhart et al., 2017; Spruijtenburg et al., 2022; Khan et al., 2024). Clade I and Clade IV of *C. auris* have MTL $\alpha$  mating type, while Clade II, Clade III, Clade V and Clade VI have MTL $\alpha$  mating type (Muñoz et al., 2018; Gifford et al., 2024a; Suphavitai et al., 2024).

Karyotyping of *C. auris* genome showed that its chromosome numbers and sizes can vary between different geographic clades (Bravo Ruiz et al., 2019). Under stress condition, *C. auris* tends to rearrange its genome and chromosome contributing to drug resistance and virulence (Narayanan et al., 2021).

## 1.4 Antifungal Resistance of *Candida auris*

*C. auris* exhibits resistance to several major classes of antifungal drugs, for this reason effective treatment is significant challenge. Each clade has different resistance pattern, and this pattern also vary between geographics regions. According to CDC, 90% of *C. auris* strains have azole resistance, 30% have amphotericin B resistance, <5% have echinocandin resistance (Chaabane et al., 2019).

Azoles are the first line treatment of candidiasis by targeting fungal cytochrome P450-dependent 14- $\alpha$ -demethylase enzyme responsible for production of ergosterol which is important part of fungal cell wall (Lockhart et al., 2017). Resistance to azoles is

widespread in *C. auris* isolates and (e.g., fluconazole, voriconazole, posaconazole, itraconazole) vary between 87% to 100% (Kim et al., 2024). Azole resistance related mainly overexpression of 14- $\alpha$ -demethylase enzyme encoded *ERG11* gene or different point mutation such as Y132F, F126L and K143R on this gene (Chowdhary et al., 2018; Lockhart et al., 2017). These mutations are associated with reduced azole susceptibility (Healey et al., 2018). Azole resistance can also be maintained by overexpression of ABC and MFS transporter genes or point mutations on drug efflux pump coding genes (*CDR1*, *CDR2* and *MDR1*) (Li et al., 2021a). E709D and V704L (Hong et al., 2023; Jacobs et al., 2022) Increased azole resistance is also related with overexpression or point mutation of transcription factors, *TAC1* and *MRR1*. *TAC1b* is transcription factor of *CDR1* and *CDR2* genes, point mutations on this gene such as Q503E, A538S, D865N, A640V, R495G, A15T, or S195C can lead overexpression of *CDR1* and *CDR2* genes and result azole resistance (Rybak et al., 2020; Rybak et al., 2022a; Hong et al., 2023; Hirayama et al., 2024). *MRR1A* is transcriptional factor of *MDR1* gene and mutation on *MRR1* can result azole resistance such as N647T, A1940C (Mayr et al., 2020; Li et al., 2022). *UPC2* is transcription factor of *ERG11* and other ergosterol biosynthesis pathway genes in *C. auris* (Li et al., 2024). *UPC2* hyperactivation, deletion, or the point mutation on this gene affect azole resistance *MRR1/MDR1*-dependent or -independent way (Li et al., 2024).

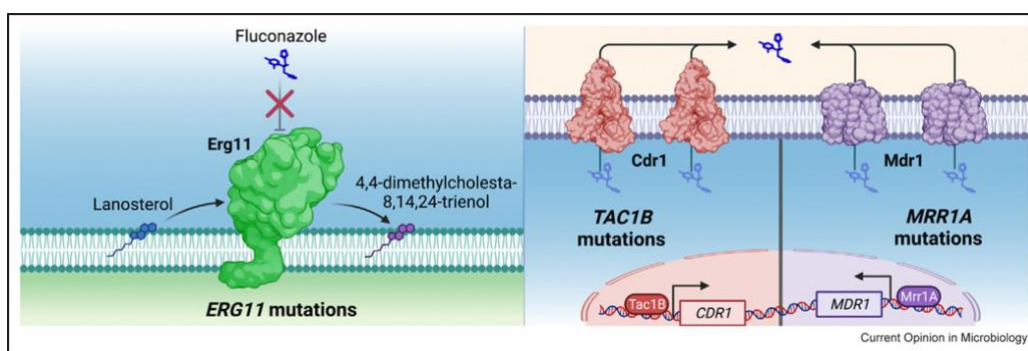


**Figure 1.2** Azole resistance in *C. auris* result of *ERG11*, *TAC1B*, and *MRR1A* mutations (Rybak et al., 2022a)

Echinocandins target fungal cell wall, and which encoded by *FKS1* and *FKS2* genes, and result in stopping glucan production. Echinocandin resistance is originated from

mutations on *FKS1* and *FKS2* genes that encodes 1,3-beta-D-glucan synthase enzyme's two subunits. Mutations can be seen on the highly conserved hot-spot 1 (HS1) region of *FKS1* gene as point mutation S639F, S639P and S639Y and reducing the enzyme's sensitivity to the drug (Berkow and Lockhart, 2018; Chowdhary et al., 2018). In previous studies showed that *FKS2* mutation have no role in echinocandin resistance (Jacobs et al., 2022).

Amphotericin B is a fungicidal drug in the class of polyene antifungal drugs and main action mechanism is binding as large aggregates and removing the major fungal membrane sterol, ergosterol, from cell membrane, thereby preventing ergosterol from performing many of its essential cellular functions (Anderson et al., 2014). Frame shift mutation on *ERG6* gene, encodes sterol-methyltransferase, (R97fs) is associated with high amphotericin B resistance (Rybak et al., 2022b). All isolates have Amphotericin B resistance shows G145D amino acid substitution on *ERG2* gene (Yadav et al., 2021). Two previous studies showed E39D mutation on *ERG2* gene, but effect of mutation on amphotericin resistance is uncertain (Biswas et al., 2020; Oladele et al., 2022).

5-Flucytosine (5-Fluorocytosine) is a pyrimidine analog type of antifungal drugs and inhibits RNA and DNA synthesis by producing toxic nucleotides in pathogenic fungi. Transportation of this drug is carried by membrane permeases, encoded by *FYC1* and *FYC2* gene, intracellular processing is carried by cytosine deaminase, encoded by *FUR1* gene, 5-aminoimidazole-4-carboxamide ribonucleotide transformylase, encoded by *ADE17* (Delma et al., 2021; Jacobs et al., 2022). 5-Flucytosine resistance coming from an amino acid substitution (F211I) or deletion (1Δ33) on *FUR1* gene, point mutation (G75V) on *ADE17*, point mutation (V119L) on *CrcB*, or frameshift mutation (M128fs) on *FYC2* gene (Rhodes et al., 2018; Jacobs et al., 2022).

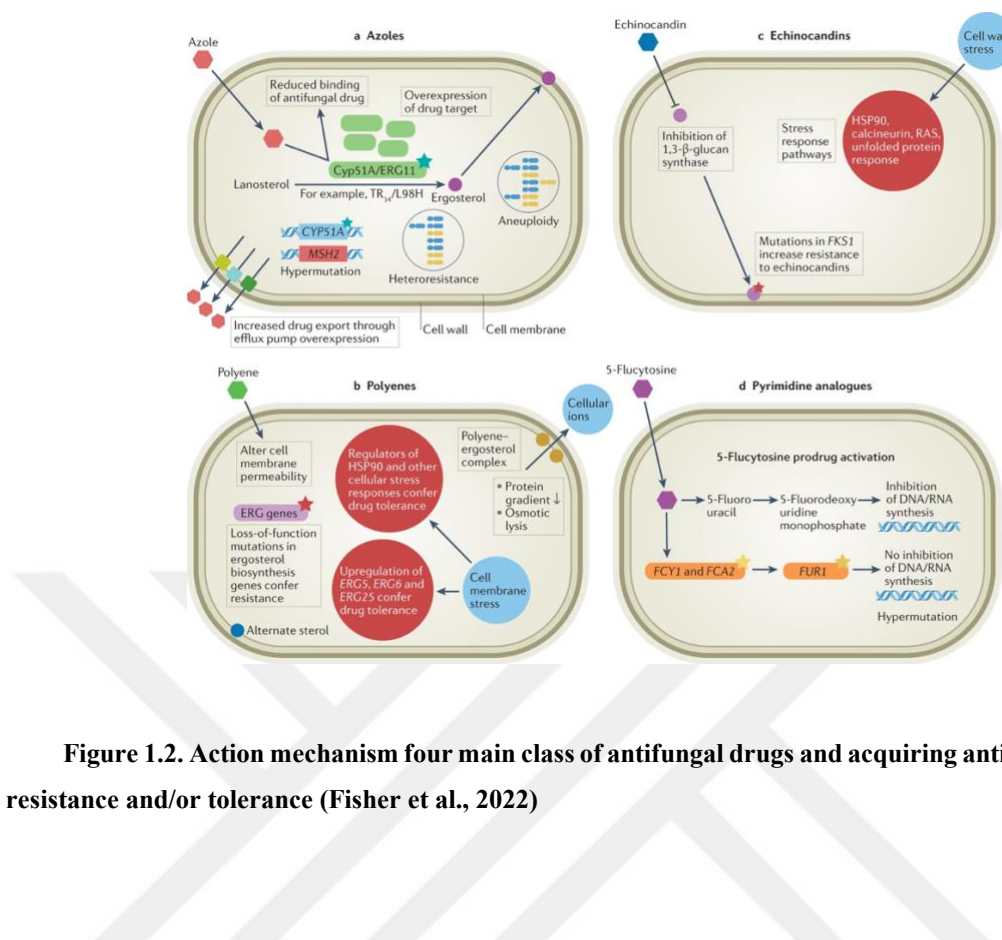


**Figure 1.2. Action mechanism four main class of antifungal drugs and acquiring antifungal drug resistance and/or tolerance (Fisher et al., 2022)**

### 1.5 Clade-Based Analysis of *Candida auris*

*C. auris* have six distinct genetic clades categorized primarily by their geographical origin: Clade I (South Asian), Clade II (East Asian), Clade III (South African), Clade IV (South American), Clade V (Iran), and Clade VI (Singapore/Bangladesh). Each clade has different characteristics resulting from geographical and genomic differences.

**Clade I (South Asian):** This clade found mostly in India and Pakistani and the most prevalent and widespread, having been identified in at least 17 countries including Canada, France, Germany, Kenya, Saudi Arabia, the United Kingdom, the United Arab Emirates, and the United States (Chow et al., 2020). Possible emergence of this clade dates back to 36 to 38 years ago (Chow et al., 2020). This clade demonstrates high resistance to chemicals, osmotic stressors (tolerates up to 8% NaCl) and high metabolic activity on N-acetylglucosamine (GlyNAc) (Brandt et al., 2023). Clade I has 97% fluconazole resistance which is the highest percentage compared to other clades, 47% amphotericin B. resistance which is the highest percentage compared to other clades, 6%

micafungin resistance (Chow et al., 2020). Overall analyze showed that this clade shows the highest rates of MDR (45%) and is the only clade reported to have extensive drug resistance (XDR) (3%) (Chow et al., 2020). Clade I has MTL $\alpha$  mating type (Muñoz et al., 2018). This clade is also associated with invasive infections, nosocomial transmissions, large-scale health epidemics (Kim et al., 2024).

**Clade II (East Asian):** Clade II which originates from Japan and South Korea, is less common worldwide, while isolates are found in Canada, Japan, South Korea and the USA (Chow et al., 2020). Results from the Time to Most recent Common Ancestor (TMRCA) analysis clade II is the oldest one among of all clades (Chow et al., 2020). This clade II is also the only clade shows the highest genetic diversity among of all clades (Chow et al., 2020). Clade II has MTL $\alpha$  mating type (Muñoz et al., 2018). This clade is also associated with ear infections (Kim et al., 2024). In terms of antifungal resistance patterns, Clade II shows considerably lower rates of azole resistance (14%) due to copy number variation of ERG11 gene instead of mutation, generally susceptible to antifungal drugs and shows no MDR or XDR patterns (Chow et al., 2020). This clade is generally the least able to utilize different carbon sources (only 16 substrates), unable to grow on GlyNAc but only sorbic acid can be used as carbon source, cannot grow higher salinity conditions (>3% NaCl) and sensitive to chemicals (Brandt et al., 2023).

**Clade III (South African):** This clade is reported in Australia, Canada, Kenya, Spain and the USA but first identification area is South Africa (Chow et al., 2020). This clade is also associated with invasive infections and large-scale outbreaks like Clade I (Kim et al., 2024). Clade III has MTL $\alpha$  mating type (Muñoz et al., 2018). Clade III emerged approximately 207.5 years ago (Chow et al., 2020). Antifungal resistance patterns of Clade III shows that this clade has 98% fluconazole resistance due to both SNP induced (F126L/V125A) and increased copy number of ERG11 gene, 8% micafungin resistance but no amphotericin B resistance, exhibits only MDR pattern (8%) not XDR (Chow et al., 2020). This clade forms aggregates leading to more extensive biofilm formation and higher expression of adhesins and upregulation of adhesin genes (Brandt et al., 2023). Isolates belong to this clade can use GlyNAc in a high manner, can live high salinity conditions (8% NaCl), but they are least versatile in dipeptide utilization (Brandt et al., 2023).

**Clade IV (South American):** This clade is reported in Colombia, Israel, Panama, Venezuela and the USA but first identification area is Venezuela (Chow et al., 2020). This clade shows the strongest phylogeographic substructures with isolates often clustering significantly by country (Chow et al., 2020). Clade IV emerged approximately 31.5 to 40.5 years ago, around 1982 (Chow et al., 2020). Like Clade I and Clade III, Clade IV is associated to invasive infections and outbreaks (Kim et al., 2024). Clade IV has MTL $\alpha$  mating type (Muñoz et al., 2018). Antifungal resistance patterns of Clade IV shows that this clade has 59% fluconazole resistance due to both SNP induced (Y132F) of ERG11 gene, 11% amphotericin b, 9% micafungin resistance, exhibits only MDR pattern (10%) not XDR (Chow et al., 2020). Clade IV shows high metabolic activity on GlyNAc, like Clade I and Clade III (Brandt et al., 2023). And also, this clade shows resistance to chemical, like Clade I (Brandt et al., 2023). But this clade has low growth in >3% NaCl concentration (Brandt et al., 2023).

**Clade V (Iran):** Origin area of this clade is Iran, differs from other isolates via exhibiting more than 200,000 SNPs and this situation gives this clade unique lineage (Chow et al., 2019). Confirmed via WGS data from a patient that has otomycosis in Iran, in 2017 (Abastabar et al., 2019). Clade V has MTL $\alpha$  mating type (Gifford et al., 2024a). Only two of the five isolates were resistant to fluconazole due to Y132F mutation on ERG11 gene, they were susceptible to other antifungal drugs, and they have no MDR or XDR patterns (Spurijenburg et al., 2022). This clade uniquely forms *In-vivo* filament formation due to upregulation of SCF1 adhesin gene and GPI-anchored gene include PGA31, IFF4, UME6, and HGC1 (Gifford et al., 2024b).

**Clade VI (Singapore/Bangladesh):** This clade is newly identified and originated from Bangladesh and Singapore, and it differs from other isolates via exhibiting more than ~42,447 SNPs (Khan et al., 2024). Isolated from a Bangladeshi patient in Singapore, in 2019 (Khan et al., 2024). Clade VI emerged approximately 35.6 years ago, around 1988 (Suphailai et al., 2024). Clade VI has MTL $\alpha$  mating type (Suphailai et al., 2024). This clade can be an emerging threat, and it has potential for international transmission (Suphailai et al., 2024). This clade has no known mutation in its genome and susceptible to all types of antifungal drugs except one isolate that has amphotericin b resistance (Khan

et al., 2024). Phenotypically, colonies of this clade have larger, whitish-blue single isolated colonies (Khan et al., 2024).

### **1.6 Virulence of *Candida auris***

*C. auris* has different virulence factors for maintaining infections beyond morphological traits and biofilm formation.

Secreted hydrolytic enzymes are the part of most important virulence factors of *C. auris*. *C. auris* uses secreted aspartyl proteases (SAPs) for cleaving host proteins. SAPs, lipases and phospholipases are secreted for aiding in adhesion, biofilm formation, tissue invasion/degradation, and disrupting immune responses (de Jong and Hagen, 2019). And these enzymes' production is elevated at 42°C, indicates these enzymes increase survivability of *C. auris* at higher temperatures. Likewise, lipases contribute to virulence of *C. auris* by supporting immune evasion, host cell damage, and biofilm formation. Hemolytic enzymes (Hemolysins) are another virulence factor of *C. auris* by efficiently sequester iron from host and promoting rapid growth, hyphal extension, and dissemination (Kumar et al., 2015).

*C. auris* uses unique ways to evade the host immune response by inhibiting neutrophil activity, specifically neutrophil extracellular traps (NETs), and by protecting itself with its outer mannan layer (Johnson et al., 2018). This outer mannan layer protects *C. auris* from phagocytosis and also reduce macrophages' glucose level, leading to their death without triggering inflammasome responses (Weerasinghe et al., 2023). And also, biofilm formation of *C. auris* protects it from immune clearance and shows robust defense mechanism. *C. auris* can reduce the effectiveness of immune response by modifying its mannans and  $\beta$ -glucans in biofilm (Fayed, 2025a).

*C. auris* has Hog1 stress-activated protein kinase (SAPK) as a unique stress response for maintaining virulence. This kinase contributes to the virulence of pathogen by increasing resistance to osmotic pressure, hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), sodium dodecyl sulfate (SDS), and other cell wall-damaging agents. cAMP/PKA pathway is also important for maintaining virulence. cAMP/PKA pathway involves in pathobiological virulence functions such as growth, stress resistance, pseudohyphae and biofilm formation, drug and disinfectant resistance, cell wall integrity, and ploidy switching (Kim et al., 2021).

Extracellular vehicles (EVs) are released by *C. auris* as a virulence factor for carrying molecules such as protein, lipids, carbohydrates, pigments, and RNA. These vesicles promote adherence to host tissue, contribute pathogenesis and increase survivability of *C. auris* (Zamith-Miranda et al., 2021).



Figure 1.3. *Candida auris* virulence factors (Bhargava et al., 2025)

## 1.7 Host Immune Response to *Candida auris*

Innate immune system is the first line of defense against pathogens. But *C. auris* has unique properties, such as biofilm formation, enhanced stress response, and lytic enzymes, that help it evade the immune system and evade immune clearance. Macrophages are recruited for phagocytosis of fungal pathogens which have pathogen-associated molecular patterns (PAMPs) such as mannans, glucans and chitins of fungal cell wall or opsonizing molecules of pathogens such as neutralizing antibodies or complement-derived molecules (Miramón et al., 2023). But *C. auris* can escape from macrophages and also it can kill macrophages by utilize its metabolic regulation (Weerasinghe et al., 2023). Even if they phagocytosed by macrophages, *C. auris* can divide itself in the macrophages and protect macrophages viability and integrity for the first 8-10 hours (Miramón et al., 2023). Elimination of macrophages normally triggers

NLRP3 inflammasome-dependent antimicrobial responses, but host macrophage glycolysis can be disrupted by *C. auris* and results preventing of NLRP3 inflammasome activation and reduce induction and expression of pro-inflammatory cytokines and chemokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , CXCL2, and CXCL2 (Wang et al., 2022; Weerasinghe et al., 2023). By hiding its  $\beta$ -glucans via mannosylation, *C. auris* can decrease recognition by immune system, failing to activate MAPK pathways that controls and pro-inflammatory cytokine and chemokine expression in macrophages (Wang et al., 2022; Miramón et al., 2023).

Neutrophils are also part of innate immunity, and they are recruited to fungal infection sites to immune clearance. They are the most effective *Candida* killers, and they are the only host cells that can prevent yeast-to-hyphae forming of *Candida*. But in case of infection, *C. auris* resists killing by neutrophils due to lack of NET formation (Johnson et al., 2018). *C. auris* cannot inhibit NET or ROS formation of neutrophils but rather evade these responses (Johnson et al., 2018). The patients with severe neutropenia, may face major risk factors of invasive fungal diseases (Netea et al., 2015).

*C. auris* construct its cell wall by using high density mannan layer and low structural complexity, in this way *C. auris* can reduce its recognition by innate immunity. However, if the N-mannan cell wall layer is disrupted or removed, the  $\beta$ -glucans of *C. auris* are exposed to Dectin-1 receptors (which is the  $\beta$ -glucan receptor) of macrophages, then the MAPK pathway is activated through host-pathogen interaction and proinflammatory cytokines and chemokines are induced, which restore innate immune recognition, neutrophil recruitment and immune clearance (Yadav et al., 2020; Wang et al., 2022).

*C. auris* can colonize and infect skin tissue of host. This infection can be classified as superficial or cutaneous mycoses. In terms of fungal infection, host cells such as macrophages, natural-killer (NKs) cells, dendritic cells (DCs), mast cells, T lymphocytes as well as melanocytes, keratinocytes and Langerhans cells (LCs) initiates immune response (Bombassaro et al., 2023). Previous studies about *C. auris*, skin colonization showed that yeast and filamentous forms of *C. auris* activate different immune response of the host's skin. And yeast and filamentous forms of *C. auris* and *C. albicans* can activate different host immune response due to their different forms of cell wall compositions (Bryak et al., 2024; Bruno et al., 2020). Interleukin 17 (IL17) response is critical for host defense against fungal infections (Datta et al., 2023). Skin infection of *C. auris* induces IL-17 receptor (IL-17R) responses with IL-17R-associated adaptor

molecule (Act1) and IL-17A/IL-17F responses with  $\alpha\beta$  T cells,  $\gamma\delta$  T cells and ILCs, these responses protect host from *C. auris* infection (Datta et al., 2023; Huang et al., 2021). *C. auris* skin infection triggers adaptive immune response includes T helper 1 (Th1), T helper 17 (Th17), and T helper 22 (Th22) but Th17 response due to unique outer mannan layer (Das et al., 2025; Balakumar et al., 2024). Humoral immunity also induces in terms of fungal infections such as IgG and IgA levels (Das et al., 2023).

## 1.8 Identification and Typing

*C. auris* infections are diagnosed by culturing body fluid like other *Candida* infections. Incorrect identification of *C. auris* is seen often by using biochemical testing methods and some automated identification tools such as VITEK 2, BD Phoenix, RapID Yeast Plus, API 20C (Chybowska et al., 2020). For identification clinical laboratories use different methods including automated and molecular methods.

*C. auris* has no distinctive features that would distinguish it from other *Candida* species. Chromogenic media are used for identification of *Candida* species by using their colony color on media. On CHROMagar™ *Candida* medium *C. auris* colonies give characteristic pale purple to pink after incubation 48 hours at 35°C and on CHROMagar™ *Candida* Plus *C. auris* colonies give characteristic white colonies with blue-green halo after incubation 72 hours at 35°C (Tamura et al., 2022). To differentiate *C. auris* from closely related species such as *C. haemulonii*, *C. pseudohaemulonii*, and *C. duobushaemulonii* further identification methods required. CHROMagar combined Pal's medium with incubation at 42°C for 48 hours (contains agar, creatinine, glucose, KH<sub>2</sub>PO<sub>4</sub> and sunflower seed extract) can also be used to differentiate *C. auris* from the *C. haemulonii* species complex (Kumar et al., 2017). In other studies, two non-commercial agar types were developed. The first one includes high NaCl (12.5%), and 9 mM ferrous sulfate (FeSO<sub>4</sub>) combined with 42°C (Das et al., 2021), other one includes 10% NaCl, peptic digest of animal tissue (5 g), pancreatic digest of casein (5 g), mannitol (20 g/l), and crystal violet (0.5 mg/l) combined with 40°C (Ibrahim et al., 2021). These media can be used for differentiating *C. auris* from other species but need to be further confirmation.

*C. auris* can be differentiate by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Bruker Daltonics, Bremen,

Germany) with FDA-cleared databases (Lockhart et al., 2022). MALDI-TOF MS uses species-specific protein fingerprints of microorganisms and compares them with its own database; it works with rapid recognition, high accuracy, low operating cost and easy-to-use technology (Zhu et al., 2024).

Sequencing of *C. auris* by using specific regions of ribosomal genes, such as D1/D2 domain of 18S rDNA, D1/D2 domain of 28S rDNA, or internal transcribed spacers (ITS), AFLP (amplified fragment length polymorphism) fingerprinting, microsatellite typing methods can also be used for identification and typing (Vatanshenassan et al., 2020). But whole genome sequencing (WGS) is the most reliable method for clade identification by analyzing microsatellite typing and short tandem repeat (STR).

*C. auris* can be identified by using PCR-based tests [conventional PCR, real-time PCR (RT-PCR), T2 magnetic resonance, or Loop-mediated Isothermal Amplification (LAMP)] by using ITS1, ITS2, GPI, 5.8S, 18S, and 28S rDNA and ITS regions are good molecular targets (Kordalewska and Perlin, 2019; Wong et al., 2025). These tests offer rapid and accurate identification of *C. auris*.

## **1.9 Clinical Manifestations, Prevention and Treatment**

*C. auris* causes a wide variety of infections, and transmission is most often hospital acquired. It can cause superficial infections such as skin and ear infections or bloodstream infections (candidemia) but most patients who colonized with *C. auris* do not develop invasive diseases (Mallick et al., 2025). But when it turns into the invasive candidiasis, it can disseminate other organs. *C. auris* commonly causes respiratory tract and urinary tract infections (La Bella et al., 2025). Patients with *C. auris* candidemia usually have multiple underlying medical conditions and a history of broad-spectrum antibiotic and antifungal medication use. And also, immunocompromised patients, long-term hospitalized and ICU patients, neutropenic patients, HIV/AIDS, surgical procedures, solid or bone marrow transplanted patients, and patients that have chronic kidney disease are risk factors of *C. auris* colonization (Watkins et al., 2022; Mallick et al., 2025). COVID-19 studies showed that patients who are hospitalized due to COVID-19 infections, at risk for *C. auris* infection (Khojasteh et al., 2022). Urinary and central venous catheters, medical devices, tracheostomies, on patient's room and bathroom materials, feeding tubes and medical ventilation can cause transmission to critically ill

patients (Ahmad and Alfouzan, 2021). Features of *C. auris* such as its ability to resist heat and osmotolerance, its ability to survive on biotic and abiotic surfaces, its ability to colonize asymptotically in patients, and its tolerance to disinfectants makes it hard to eradicate and it should be taken into consideration. Screening of *C. auris* colonization is important to protect critically ill patients. Main colonization areas are groin and axilla of patient's skin, urine, nose and throat, perineal and rectal swab and stool sample (Mallick et al., 2025; Ahmad and Alfouzan, 2021). In patients with *C. auris* candidemia, reported mortality rates generally range from 30% to 60% (Eix and Nett, 2025).

For prevention of *C. auris* colonization on patients, *C. auris* cases should be accurately diagnosed by MALDI-TOF MS or PCR-sequencing of rDNA, and *C. auris* isolates from sterile and, when indicated, non-sterile sites should be confirmed to the species level, especially in high-risk settings such as facilities with known cases or patients with international exposure. Screening should be performed for patients in close contact with *C. auris*-colonized positive individuals, those previously hospitalized in affected facilities, or those with a history of healthcare exposure abroad. Transmission-based precautions (TBPs) should be strictly followed by isolation in single rooms or, if necessary, cohorting to limit transmission.

Environmental cleaning should be performed using UV, peracetic acid, sodium hydrochloride, sodium hypochlorite (1000 ppm) or sporicidal disinfectants, and additional precautions such as cleaning and hydrogen peroxide vapor should be taken in outbreak settings (Ledwoch and Maillard, 2018). Attention should be paid to patient-related contacts of healthcare personnel (HCP), proper use of personal protective equipment (PPE), monitoring of hand hygiene compliance, and providing alcohol-based hand hygiene with soap/water. Although there is no definitive decolonization protocol, chlorhexidine-based interventions may help reduce colonization. Education and training in infection control practices, antifungal stewardship, and appropriate disinfection procedures by clinicians and clinical microbiologists for all HCPs is critical to minimize the spread of *C. auris* (Ahmad and Alfouzan, 2021).

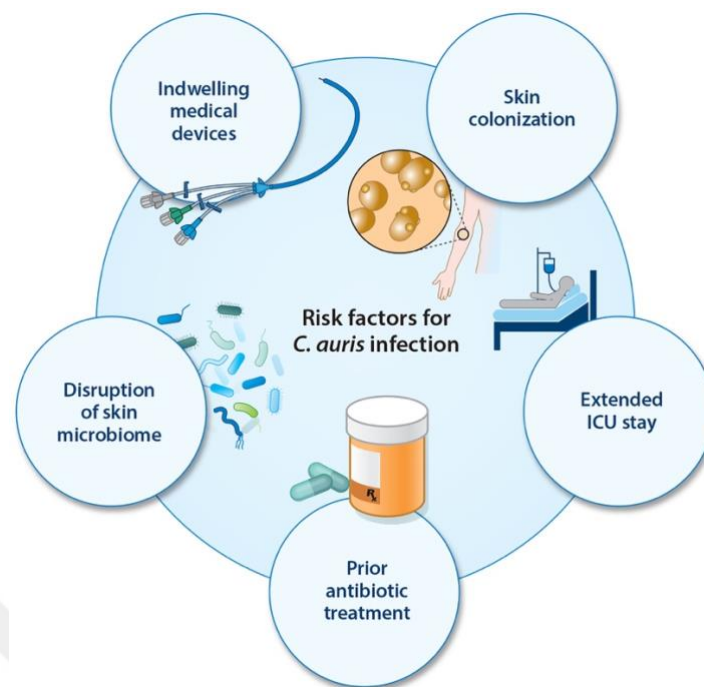


**Figure 1.3 Hospital-associated infection of *C. auris* (Eix and Nett, 2025)**

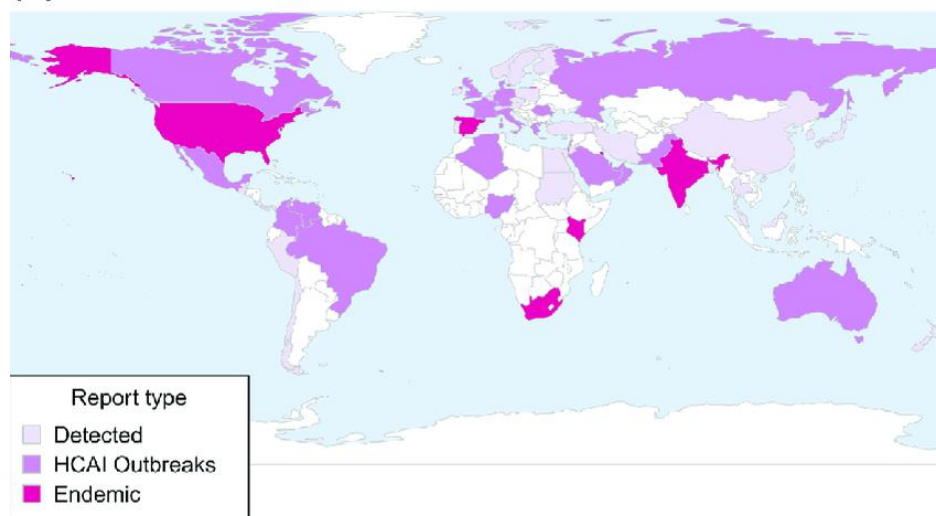
*C. auris* has intrinsic drug resistance that reduces treatment options. Because of rapidly evolving drug resistance, susceptibility testing is important to determine treatment approaches, and, in some cases, infection persists, and these tests should be repeated (CDC, 2024). Adults and children who are older than two months, can be treated by rezafungin, caspofungin and micafungin but anidulafungin is restricted to adults (CDC, 2024). Main problem of this drug type is *C. auris* isolates that have echinocandin resistance *FKSI* mutation. If the initial treatment does not work on adult patient or patient is a neonate, amphotericin B deoxycholate or liposomal amphotericin B can be used (CDC, 2024). Antifungal drug combinations for pan-resistant strains are a new option with *in-vitro* studies. No antagonistic effect of azole, amphotericin B and 5-flucytosine antifungals, which can be combined with echinocandins, has been observed, but synergistic or indifference effects have been demonstrated (Fakhim et al., 2017). The combination of flucytosine with amphotericin B, azoles or echinocandins was found to be highly effective and fungicidal (O'Brien et al., 2016; Aghaei Gharehbolagh et al., 2021). New antifungal drugs Ibrexafungerp which is glucan synthase inhibitor, Rezafungin which is second-generation echinocandin, and Fosmanogepix/Manogepix which targets GPI-anchored protein maturation and inhibits Gwt1 gene (Hoenigl et al., 2021). T-2307 is a novel arylamidine that targets respiratory chain enzymatic complexes

III and IV in the inner mitochondrial membrane and causes the collapse of the mitochondrial membrane potential of yeast (Wiederhold, 2021). VT-1598 is a novel CYP51 inhibitor that has broad-spectrum activity against fungal species (Gu et al., 2024).

### 1.10 Epidemiology

Many countries all over the globe are developing surveillance programs to ensure rapid detection and prompt implementation of infection control measures, and to adapt strategies from other multidrug-resistant organisms to identify and control *C. auris*. Between 2009 and 2020, 912 cases were reported from 44 countries worldwide. The most prevalent strain was the South Asian strain from 17 countries, followed by the South African strain from 8 countries, the East Asian strain from 8 countries, and the South American strain from 3 countries (Hu et al., 2021). According to CDC data, the number of cases doubled from its initial discovery in 2019 to 2021, with cases occurring on three continents (CDC, 2023). The European Centre for Disease Prevention and Control (ECDC) reported an increase in *C. auris* cases in Europe, with the most significant increases reported in the United Kingdom, Spain and Italy (Kohlenberg et al., 2022). While sporadic cases were detected in other European countries, the overall infection rate in the EU/EEA region was reported to have increased significantly. The number of cases doubled between 2020 and 2021, from 335 cases in eight EU/EEA countries to 655 cases in 13 countries (Kohlenberg et al., 2022).

(a)



(b)

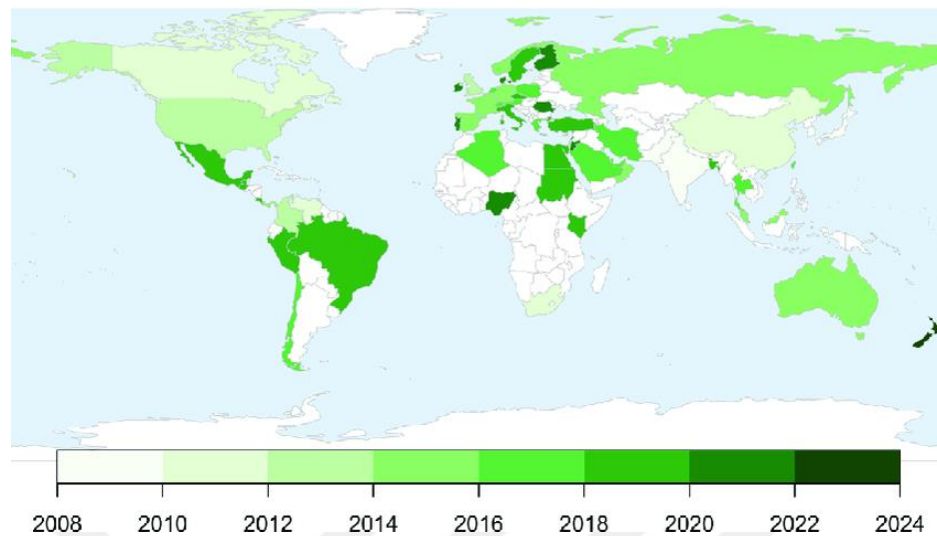


**Figure 1.4 Epidemiology of *C. auris* (a) Distribution of *C. auris* by healthcare associated infection outbreaks (HCAI) or country-based endemicity (b) Distribution of countries reporting the first case of *C. auris* by notification date (Jones et al., 2024)**

More than 1,812 cases of *C. auris* have been reported in EU/EEA countries since 2013 and outbreaks were reported from five country between 2019 and 2021 (Kohlenberg et al., 2022). In Italy, an outbreak affecting at least eight healthcare facilities occurred between July 2019-December 2022 in the Liguria region, resulting in 297 confirmed cases, 15 cases were recorded in two facilities in Emilia-Romagna, 48 cases in a hospital in Piedmont, and one case in a hospital in Veneto (Sticchi et al., 2023). In Germany, seven cases, six of which were imported, were detected in different hospitals between November 2015 and December 2017 (Hamprecht et al., 2019). The UK Health Security Agency reported that the first case in the UK was identified in 2013, followed by one case in 2014, 15 in 2015, 50 in the 2015-2016 outbreak, 34 in 2016, 70 in 2017, 26 in 2020, 38 in 2022, 93 in 2023, 178 in 2024 and total of 637 *C. auris* isolates were reported in the UK between January 2013-December 2024 (Borman and Johnson, 2020; UK Health Security Agency, 2025a; 2025b). In Greece, the first case of *C. auris* was detected in 2019, 13 cases in 2020, and 58 new cases in 2021 (Kohlenberg et al., 2022). In France, two cases were reported in 2015, one case in 2016, one case in 2017, three cases in 2019, four cases in 2020, and four cases in 2021 (Kohlenberg et al., 2022). In Spain, which is an endemicity region, four cases were recorded between April and June 2016, 41 of 140 *C. auris* cases between April 2016 and January 2017 were invasive fungemia, two cases

in September-October 2017, 203 cases, 30 of which were invasive, between September 2017 and August 2019, and 591 new cases were recorded between 2020 and 2021 (Ruiz-Gaitán et al., 2017; 2018; Kohlenberg et al., 2022; García et al., 2020). The first case in Türkiye was isolated from the blood culture of a patient in 2021, the second case, which resulted in death, was isolated from the blood culture of a patient, the third case was isolated from three patients in the same hospital between November 2020 and January 2021, and the last case was isolated from the wound area of a patient in Izmir in January 2023 (Kurt et al., 2021; Teke et al., 2021; Kömeç et al., 2021; Kulaklı et al., 2023).

The African continent, which is the source of clade III and accounts for 25% of all candidemia cases, has reported more than 2,500 *C. auris* cases from 6 countries and 4 African subregions, with a crude mortality rate of 42.7% (Osaigbovo et al., 2023). The first case was seen in Kenya in 2011, and it has caused hospital outbreaks in Nairobi, accounting for 29–38% of all candidemia cases (Adam et al., 2019). Colonization was observed in 26 patients in Sudan in 2019, in 7 patients in Algeria in 2022, and in the single *C. auris* case seen in Nigeria in 2021, the patient was recorded as having been treated in the United States (Badri and Sherfi, 2019; Zerrouki et al., 2022; Oladele et al., 2022). It has been stated that the cases seen in Egypt and Nigeria are sporadic.

After the first case was reported in the USA in 2016, the number of cases increased every year, reaching 171 in 2017, 330 in 2018, 477 in 2019, 764 in 2020, 1553 in 2021, 2928 in 2022 and 4514 in 2023, reaching a total of 10,788 cases and a total of 22,931 colonization cases detected through screening were recorded (CDC, 2024; CDC, 2022; Lyman et al., 2023; Vallabhaneni et al., 2016). In Canada, one of the countries where four different genomic clades are seen, the first case was seen in a patient arriving from abroad in 2012, but the case was reported in 2017 (Schwartz and Hammond, 2017). Of the 24 cases reported between 2012 and 2019, 12 were Clade 1, three were Clade 2, four were Clade 3, and five were Clade 4, and a total of 43 cases were seen between 2012 and 2022 (De Luca et al., 2022; Ontario Public Health, 2023).

In India, *C. auris* was first reported with 12 cases in New Delhi between 2009 and 2011 and *C. auris* causes 5.3% of candidemia cases, has a high prevalence rate of 30% and a mortality rate of 41.9% (Chowdhary et al., 2013; Chakrabarti et al., 2015; Rudramurthy et al., 2017). Analyses of three different *C. auris* isolates detected in the blood culture of a patient to Singapore in January 2018, in the blood culture of a patient previously treated in a hospital in Indonesia in April 2022 and in the blood culture of a

patient treated in a hospital in Singapore in April 2023 were compared with a previously sequenced isolate of Bangladeshi origin and the analyses showed that these isolates did not belong to the first five clades but belonged to a sixth new clade (Suphavitai et al., 2024). After the first 8 cases were reported in China in 2016, the number of cases increased every year, reaching 23 cases in 2017, 51 in 2018, 96 in 2019, 270 in 2020, 325 in 2021, 455 in 2022, 982 in 2023, and 1846 in December 2024, with Clade 3 being the dominant clade (Bing et al., 2025). In Japan, the first case was isolated in 2005 and reported in 2009, the second case in 2017, five cases in 2018, and two cases belonging to Clade 1 in 2023 (Sato et al., 2009; Iguchi et al., 2018a; Iguchi et al., 2018b; Ohashi et al., 2023). In South Korea, 15 of 23 patient samples isolated from two hospitals between May 2004 and December 2006 were reported to be *C. auris* (Kim et al., 2009; Oh et al., 2011). Three cases of *C. auris* were reported from blood cultures in 2011, and 111 cases of *C. auris* were reported in 79 patients at a single medical center between February 2016 and July 2018 (Lee et al., 2011; Jung et al., 2019). In Russia, the first case was detected in October 2017, followed by 49 cases between 2016 and 2017, 38 cases between 2017 and 2019, and 30 cases between 2017 and May 2020, totaling 350 cases during the COVID-19 pandemic (Pchelin et al., 2020; Barantsevich et al., 2019). In another study, 112 *C. auris* isolates were collected from 18 hospitals between May 2017 and March 2023 (Oganesyan et al., 2025). The first case in Taiwan occurred in 2018, and the first invasive infection occurred in a Taiwanese patient hospitalized in Vietnam (Tang et al., 2019; Tsai et al., 2022). Two of the 24 isolates analyzed between 2017 and 2023 were reported as *C. auris* (Li et al., 2025). In Thailand, the first case was detected in 2018, and the second was detected in 2021 (Pharkjaksu et al., 2021).

The first case in Iran was reported in 2017 (Ahmadi et al., 2023). The *C. auris* isolate in Babol that emerged in 2017 and was reported in 2018, was identified by WGS as belonging to Clade 5 (Abastabar et al., 2018; Chow et al., 2019; Spruijtenburg et al., 2022). One case was reported in August 2020, one case diagnosed in 2017 and reported in 2021, and one case was reported in Tehran the same year (Armaki et al., 2021; Safari et al., 2022; Mirhendi et al., 2022). The first case of *C. auris* was seen in Bahrain in 2021 (Alagha et al., 2024). The first case in Lebanon was observed in 2020 (Allaw et al., 2021). A hospital outbreak affecting 32 patients was reported in Oman between April 2018-April 2019, with a crude mortality rate of 53% (Al Maani et al., 2019). A blood culture-isolated case was reported in the United Arab Emirates in 2018 (Alatoom et al., 2018). The first

three cases in Saudi Arabia were detected and reported between December 2017 and February 2018 (Abdalhamid et al., 2018). The first case in Kuwait occurred in 2014, and the 2018 outbreak resulted in the detection of 17 candidemia patients and 54 colonized patients, with a crude mortality rate of ~50% (Alfouzan et al., 2020). The first case in Qatar was detected in November 2018, the first outbreak was detected in 13 patients between December 2018 and August 2019, 331 cases were detected between May 2019 and December 2022, and 76 cases were detected in 9 major hospitals between March 2020 and June 2021 (Shaukat et al., 2020; Visan et al., 2020; Al Ajmi et al., 2025; Ben Abid et al., 2023). The first outbreak in Pakistan resulted in 30 cases between April 6 and 17, 2015, and November 16, 2015, and January 6, 2016. There were 92 cases in an outbreak between September 2014 and March 2017. Colonization was detected in another outbreak in an intensive care unit in 2018, and in a pediatric intensive care unit patient in 2022 (Farooqi et al., 2020; Sayeed et al., 2019; Imtiaz et al., 2022). During the COVID-19 pandemic, four cases of *C. auris* were reported in COVID-19 patients and six in non-COVID-19 patients (Moin et al., 2021).

The first outbreak in Venezuela was detected in blood cultures of 18 patients between March 2012 and July 2013, and 5 patients died (Calvo et al., 2016). In Brazil, the first case was reported from an intensive care unit in 2020, with 15 cases and 2 deaths from the first outbreak occurring between December 2020 and December 2021, a new case in the second outbreak in December 2021, two cases in the third outbreak in January 2022, and the most dangerous outbreak, with 47 cases between December 2021 and March 2022 (Agência Nacional de Vigilância Sanitária, 2020; ANVISA, 2022; de Melo et al., 2023). The first case in Colombia Between January 2015 and September 2016, a total of 40 cases from four hospitals, a total of 1720 cases Between March 2016 and December 2020, 541 cases in 2019, 404 cases of COVID-19 were reported (Armstrong et al., 2019; Escandón et al., 2022). The first case in Panama Between July and October 2016, 14 *C. auris* isolates, 124 cases, 108 of which were related to COVID-19, have been reported since the beginning of 2021, 239 cases have been reported (Rodriguez et al., 2017; PAHO, 2021; Lanna et al., 2025). The first case in Chile occurred in a patient arriving from India in December 2018, and an outbreak of 12 cases subsequently occurred in the same hospital in May 2020 (Moreno et al., 2019; Villanueva-Lozano et al., 2021). The first case in Peru was reported as the colonization of three patients in a hospital during COVID-19 (Paucar-Miranda et al., 2021). In Mexico, the first case was reported in May

2020, and three months later, in 12 patients hospitalized in the COVID-19 ward of the same hospital, 34 *C. auris* colonizations and ten *C. auris* infections were reported on November 27, 2020, and a total of 37 cases were reported between September 2020 and December 2023 (Ayala-Gaytán et al., 2021; Villanueva-Lozano et al., 2021; Dirección General de Epidemiología, 2020; la Garza et al., 2024).

The first case in Australia was reported in 2015, belonging to Clade 3. Nine of the 13 isolates emerged belonged to Clade 3 and four to Clade 1, and finally, two cases were reported in 2018 (Australian Pathogen Genomics Program, 2025; Worth et al., 2020).

### **1.11 Nanomaterials**

*C. auris*, with its antifungal resistance and other remarkable virulence properties, has been isolated with increasing frequency all over the world in a short period of 10-12 years since it was first identified, and its outbreaks by forming biofilms on hospital equipment have increased the search for new effective treatments against this fungus (Hayes, 2024). Since fungi are eukaryotes, they share many common biochemical and molecular properties with human cell structure. This situation makes it difficult to find new drug targets that are effective against fungi. The thick cell wall around the fungal cells, the different virulence properties it develops to escape the immune system, and the intrinsic resistance mechanism it develops against conventional antifungal agents make it difficult to discover new lethal agents that can be effective against fungi (Chowdhary et al., 2017; Fisher et al., 2022). The long development time, high cost, and the fact that they need to undergo numerous in-vitro, in-vivo, and phase tests before use have brought the use of old drugs with drug repositioning techniques in new important infections such as *C. auris* and COVID-19 to the agenda, but the results obtained from the studies have shown that in vitro data are not very consistent with in-vivo data and that the drugs cause high toxicity (Maziarz and Stencel, 2022; de Oliveira et al., 2022). Another method used to break the drug resistance of *C. auris* is the combined use of existing drugs. Although synergy has been shown with some combinations in in vitro experiments conducted for this purpose, the combined use of these drugs in the clinic also brings up the possibility of high side effects and toxicity in this fragile patient group with many comorbidities, and these factors make the use of combined drugs difficult in the clinics (Caballero et al., 2021).

With the wide use of nanomaterials in medicine and many other fields, the potential antimicrobial activities of these materials have recently been investigated. Many studies have been conducted using metallic, polymeric and lipid-based nanoparticles (NPs) as an alternative treatment method, especially in the treatment of infectious diseases (Fayed, 2025a). Metal NPs are substances made of pure metals (e.g., gold, platinum, silver, titanium, zinc, cerium, iron and thallium) or their compounds (e.g., oxides, hydroxides, sulfides, phosphates, fluorides and chlorides) with sizes ranging from 10 to 100 nm (Yaqoob et al., 2020). According to the results of these studies, metal nanoparticles in particular attract attention with their significant antifungal activities against *Candida* species (Monteiro et al., 2012; Ahmad et al., 2016). Nanomaterials can also be used in the delivery of antifungal drugs. In this way, it enables the controlled application of antifungal drugs at lower doses with increased localization, while improving the pharmacokinetic properties of the drugs and increasing their effectiveness (Zhi et al., 2021). In particular, metal nanoparticles have been shown to cause toxic effects by increasing reactive oxygen molecules in the cell and cause changes in the morphological structure of the fungus (Seong and Lee, 2018). Therefore, metal nanoparticles stand out as a new hope in the treatment of multidrug-resistant *C. auris* (Billamboz et al., 2021, Fernandes et al., 2022).

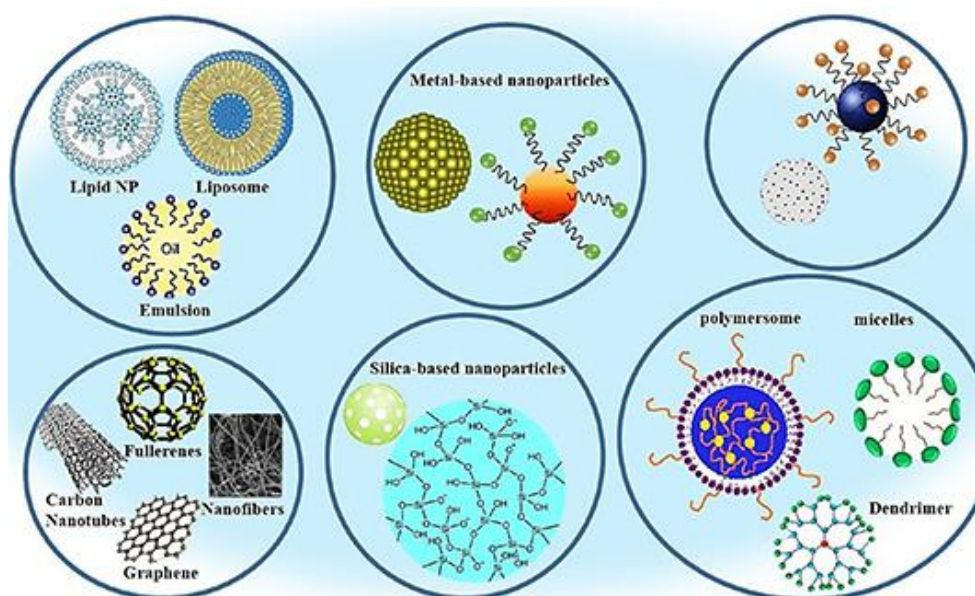


Figure 1.5 Different types of nanomaterials that can be used against *C. auris* (Izadi et al., 2024)

### 1.11.1 Action Mechanisms of Nanoparticles

Nanoparticles have physicochemical properties such as high surface-to-volume ratio and surface modifiability. Due to these properties, they can facilitate interactions with fungal cells, unlike traditional drugs. Nanoparticles have different action mechanisms against fungal cells. The main purpose is to break drug resistance and achieve treatment success by attacking fungal cells or their biofilms forms through different strategies and several different mechanisms at the same time. The one of these mechanisms is the leakage of cytoplasmic content and disruption of membrane potential by disintegration of fungal cell membranes and walls (Izadi et al., 2024). In a study conducted on *C. albicans*, it was shown that silver nanoparticles (AgNPs) adhered to the surfaces of *C. albicans* cells, increased membrane permeability, and triggered apoptosis through ROS production (Hwang et al., 2012). In addition, when liposomal forms of nanoparticles are used as drug delivery systems, they can penetrate the protective extracellular matrix (ECM) and prevent biofilm formation, increasing the stability of antifungal agents regardless of whether they are hydrophilic or hydrophobic, and supporting their delivery (Fayed, 2025a). In addition, the use of liposomal nanoparticles as a drug delivery system minimizes systemic toxicity and supports effective delivery targeting infection sites (Jaromin et al., 2024). They can cause isolates to regain susceptibility to drugs to which they are resistant by reducing the activities of drug efflux pumps in the cell membrane (Carmo et al., 2023). Trimetallic nanoparticles formed in different combinations have shown synergistic antifungal effects by inducing mitochondrial dysfunction, DNA fragmentation and cell cycle arrest in *Candida* cells (Bandara and Samaranayake, 2022; Ganeshkumar et al., 2024).

### 1.11.2 Types of Nanoparticles

Metallic (gold, silver, bismuth, iron, copper, nickel, platinum, titanium, and zinc nanoparticles), metal oxide (iron oxide, zinc oxide, magnesium oxide, titanium dioxide, cupric oxide, cuprous oxide, zirconium dioxide), bimetallic (silver-iron nanoparticles, silver chromite nanocomposites, silver-nickel nanoparticles, gold-platinum nanoparticles) nanoparticles and trimetallic (silver-copper-cobalt nanoparticles, gold-platinum-silver nanoparticles) have attracted attention due to their strong antibacterial and antifungal properties (Carmo et al., 2023; Skłodowski, 2023; Wu et al., 2025). Their sizes

range from 10-100 nm and have unique physical and chemical properties (Yaqoob et al., 2020). AgNPs are the most studied nanoparticles in the literature and have long been used for their broad-spectrum antibacterial and antifungal activity, and they show their effect by interacting with basic cell components; their sizes, usually smaller than 10 nm, enter the fungal biofilm and facilitate direct contact with cells, thus increasing membrane permeability, causing membrane damage, causing oxidative stress, leading to DNA damage, and eventually leading to apoptosis and death of the cell (Bandara and Samaranayake, 2022; Vazquez-Munoz et al., 2020a). It has also been shown in the literature that it works synergistically with antifungal drugs such as voriconazole, fluconazole, amphotericin b, and nystatin, increasing their efficacy, decreasing the drug concentration required for inhibition, and reducing toxicity (Carmo et al., 2023). Gold nanoparticles (AuNPs) are known to have antibacterial and antifungal properties and have been used in the treatment of different diseases for a long time. Similar to AgNPs, they have action mechanisms such as fungal outer membrane damage, increased permeability of *Candida* cells, leakage of cytoplasmic content, and ROS production (Piktel et al., 2020). Nanoparticle complexes formed with gold(I)-phosphine and chiral square-planar gold(I) complexes have shown antifungal activity against *C. auris* at low MIC concentrations, but host cell cytotoxicity causes limitations in the use of these nanoparticles for therapeutic purposes (Dennis et al., 2019). Bismuth (MIC 1-4 µg/ml for *C. auris*) and gallium (MIC 128-256 µg/ml for *C. auris*) nanoparticles also disrupt the morphology and biofilm formation of fungal cells in the literature, but they have a moderate effect (Vazquez-Munoz et al., 2020b; Bastos et al., 2019). Trimetallic nanoparticles combining Ag, Cu and Co play an effective role in combating *Candida* species with antifungal drug resistance by increasing their fungicidal activity and stability by working with synergistic and high catalytic effect (Kamli et al., 2021).

Nanoparticles formed with lipid-based formulations are used in the literature as an antifungal drug delivery strategy. Solid lipids (SLNs), liposomal nanoparticles and nanostructured lipid carriers (NLCs) can encapsulate both hydrophilic and lipophilic drugs, increase bioavailability, reduce the natural toxicity of strong antifungal drugs such as amphotericin B, increase the stability of drugs and optimize the controlled drug release kinetics (Metha et al., 2023; Wu et al., 2025).

Polymeric and organic nanoparticles are nanoparticles that are usually synthesized with biocompatible materials such as chitosan and poly (lactic-co-glycolic acid) (PLGA)

and can be designed to release the loaded drug content in response to specific stimuli such as pH or redox sensitive conditions common in the microenvironment of fungi, thus providing more sensitive and effective drug delivery (Li et al., 2021b). Polysaccharide-supported metal nanoparticles are used in vaccine technology and magnetic resonance imaging methods as a new research area (Zhao et al., 2018). Chitosan  $[(C_6H_{11}NO_4)_n(C_8H_{13}NO_4)_m]$  is a polymeric heteropolysaccharide formed by deacetylation of chitin and is a biomaterial that attracts attention for researchers with its completely natural (organic) structure, high biocompatibility, biodegradation capacity, and increased mucoadhesive properties (Desai et al., 2023). The hydroxyl (-OH) and amino (-NH<sub>2</sub>) groups in chitosan are the main functional groups responsible for antioxidant activity because they react with unstable free radicals, transforming them into stable molecules and binding metal ions (Xie et al., 2001). Chitosan is a linear biopolymer that has the ability to chelate reactive amino (-NH<sub>2</sub>), acetylamino (-NH-CO-CH<sub>3</sub>) and hydroxyl (-OH) groups and many metal ions (Dutta et al., 2004). Chitosan is a biocompatible, biodegradable and non-toxic polysaccharide with intrinsic antifungal properties and facilitates drug penetration by interacting with negatively charged fungal membranes (Ing et al., 2012). It has also been reported that chitosan exhibits antibacterial and antifungal activity by developing a synergistic effect when combined with other nanoparticles (Dhillon et al., 2014). Despite the antibacterial and antifungal activities of metallic nanoparticles shown in the literature, the biggest obstacle to their use in the treatment of infectious diseases is the possible side effects and toxicity of these particles on eukaryotic cells in systemic use (Skłodowski et al., 2023; Zhu et al., 2023). Another important factor is the very rapid degradation of metal nanoparticles and their low bioavailability in animal models, especially in intravascular use (Gustafson et al., 2015; Zhang et al., 2016). Studies using chitosan-supported nanoparticles in the treatment of infectious diseases have focused more on the surface use of chitosan-supported silver and gold hybrid NPs in burn bandages and the antibacterial activity of these particles (Li et al., 2017; Lozano Chamizo et al., 2021). Singh et al. demonstrated the antifungal activity of chitosan against *Candida* species (Shih et al., 2019). In another study, researchers investigated the efficacy of chitosan against 105 *Candida* species, showing significant antifungal activity in 89.9% of the isolates and finding complete inhibition in 68.6% of the isolates (Alburquerque et al., 2010).

In this study, our aim was to reveal the genomic resistance mechanisms of the clinically originated *Candida auris* (K1575) isolate with multidrug resistance and to determine the effectiveness of the synthesized and characterized Au and Fe<sub>3</sub>O<sub>4</sub> nanoparticles and their chitosan-supported forms on this isolate.

## Chapter 2:

### METHODOLOGY

#### 2.1 Isolates, Culture Media and Growth Conditions

In this thesis study, multidrug-resistant *C. auris* clinical isolate (*C. auris* K1575) was used in all experiments. This isolate is available in the strain stocks of Koc University Faculty of Medicine, Koc University-Is Bank Infectious Diseases Research Center (KUISCID). The source of the relevant strain was a bloodstream infection and was isolated in a tertiary education and research hospital in our country in 2022. The *C. auris* clinical strain to be used in the study was identified at the species level using the Matrix assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS) method, and the species-level identification of the isolate was confirmed by sequencing the 18S rRNA ITS1-2 regions using the Sanger sequencing method and comparing them with the NCBI database. In this study, *C. auris* ATCC MYA-5002 isolate, *C. auris* NR-52717 (AKU-2019-34), *C. auris* NR-52714 (AKU-2018-257) and *C. auris* NR-52713 (AKU-2017-385) isolates will be used as standard control isolates. All isolates were stored in tryptic soy broth (TSB) (Sigma-Aldrich, Merck, USA) with 10% glycerol (Sigma-Aldrich, Merck, USA) at -80 °C.

#### 2.2 Antifungal Susceptibility Tests

In this thesis study, antifungal susceptibility testing was performed for confirmation of the isolate whose multidrug resistance was confirmed in previous tests. Antifungal susceptibility tests were performed by broth microdilution method according to CLSI references at Koc University Faculty of Medicine, KUISCID, Mycology Research Laboratory (Istanbul, Turkey). *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as reference strains in this test for confirmation of test correction. The

interpretation of this test was performed according to CLSI methodology M27M44S and CDC tentative breakpoints (CLSI, 2017; CLSI, 2022; CDC, 2024).

### 2.2.1 Medium Preparation

RPMI-1640 (with glutamine, without bicarbonate, and with phenol red as pH indicator) (Sigma-Aldrich, Merck, USA) has been used for antifungal susceptibility tests. RPMI-1640 (D-glucose rate is 0.2%) medium buffered with 0.165 mol/l MOPS (Sigma-Aldrich, Merck, USA) was prepared by adding 10.4 g of RPMI 1640 medium powder (with glutamine and phenol red, without bicarbonate) and 34.53 g of MOPS (3-[N-morpholine] propane sulfonic acid) buffer. Powdered medium is dissolved in 900 ml dH<sub>2</sub>O, and MOPS is added, then pH is adjusted to 7.0 with 1 M sodium hydroxide (NaOH), final volume arranged to 1 L. Sterilized by using a 0.22 µm pore-sized filter and stored 4 °C.

### 2.2.2 Preparation of Antifungal Agent Stocks

In this study, fluconazole (Sigma Aldrich, USA), voriconazole (Sigma Aldrich, USA), posaconazole (Sigma Aldrich, USA), itraconazole (Sigma Aldrich, USA), anidulafungin (Sigma Aldrich, USA), amphotericin b (Sigma Aldrich, USA) antifungals were used. Stock solutions are prepared according to the formula below:

$$Weight (mg) = \frac{Volume (ml) \times Concentration \left(\frac{\mu g}{ml}\right)}{Assay Potency \left(\frac{\mu g}{ml}\right)}$$

Antifungal agents are diluted in RPMI-1640 to prepare gradually decreasing concentrations of drugs. Two-fold serially diluted 50 µl medium is plated to 96 well plate which ranged from 64 µg/ml to 0.125 µg/ml for fluconazole and ranged from 16 µg/ml to 0.313 µg/ml for voriconazole posaconazole, itraconazole, anidulafungin and amphotericin b.

### 2.2.3 Preparation of Fungal Inoculum

*C. auris* isolates, obtained from clinical culture collections grown on Sabouraud's dextrose agar by incubating at 37°C temperature for 48 hours. Fungal cell suspensions were prepared in sterile Phosphate Buffer Saline (PBS) (EcoTech Biotechnology,

Turkey) solution. McFarland turbidity standard was used to determine the density of the suspension by using BD PhoenixSpec nephelometer (BD, USA). 0.5 McFarland standard is equivalent to  $1-5 \times 10^6$  CFU/ml for fungal cells.

#### 2.2.4 Inoculation of Microdilution Plates

Each well of the microdilution plate was filled with 50  $\mu$ l diluted 0.5 McFarland fungal inoculum and 50  $\mu$ l RPMI-1640 medium. Fungal inoculums were diluted 1:1 with the final inoculum  $1-5 \times 10^6$  CFU/ml. The plate was then incubated at 35-37 °C temperature for 48 hours.

#### 2.2.5 Interpretation of Results

Minimum inhibitory concentrations (MIC) were assessed visually 24<sup>th</sup> and 48<sup>th</sup> hours' time points. The evaluation of the results was made according to the tentative breakpoints published by the CDC and EUCAST Epidemiological Cutoff Values (ECOFF) from public consultation on proposed new breakpoints (CDC, 2024; EUCAST, 2025). For second generation triazole susceptibility assessment, fluconazole breakpoint can be applied. Isolates resistant to fluconazole may also be resistant to other triazoles. *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 strains are used as reference control strains in all experiments of the study. Evaluation of reference isolates was made according to CLSI ECOFF (CLSI, 2022). Table 2.1 shows all tentative breakpoints and cutoff values applied in this interpretation.

**Table 2.1 Interpretive Guidelines for In-Vitro Antifungal Susceptibility Tests for *Candida auris***

| Species              | Antifungal Agent | MIC Range ( $\mu$ g/ml) |        |                 |               |
|----------------------|------------------|-------------------------|--------|-----------------|---------------|
|                      |                  | CDC                     | EUCAST |                 |               |
|                      |                  | Resistant (R)           | ECOFF  | Susceptible (S) | Resistant (R) |
| <i>Candida auris</i> | Fluconazole      | $\geq 32$               | -      | -               | -             |
|                      | Voriconazole     | NA                      | -      | -               | -             |
|                      | Posaconazole     | NA                      | -      | -               | -             |

|  |                |          |      |       |      |
|--|----------------|----------|------|-------|------|
|  | Itraconazole   | NA       | -    | -     | -    |
|  | Anidulafungin  | $\geq 4$ | 0.25 | 0.25  | 0.25 |
|  | Amphotericin B | $\geq 2$ | 2    | 0.001 | 2    |

## 2.3 Whole Genome Sequencing

In this part of the study, it was aimed to reveal the genomic resistance mechanism of clinical isolate K1575 to antifungal drugs and to reveal its placement in the phylogenetic tree by determining the clade to which clinical isolate K1575 belongs.

### 2.3.1 DNA Isolation

For degradation of fungal cell wall Lyticase (Sigma-Aldrich, Merck, Germany) and Proteinase K were used at the first step. 95  $\mu$ l nuclease free (NF) (EcoTech, Turkey) water, 95  $\mu$ l solid tissue buffer (Zymo Quick DNA Miniprep Plus Kit, Catalog Number: D4068), and 50  $\mu$ l 2500 U/ml (125 U) Lyticase were added in an Eppendorf tube and one loopful of K1575 isolate colonies are added into the mixture and gently pipetted. Mixture was incubated at 37°C for one hour. After incubation, the mixture was centrifuged at 10,000 xg for one minute 30 seconds at room temperature (RT). The supernatant was removed and 95  $\mu$ l NF water, 95  $\mu$ l solid tissue buffer, and 10  $\mu$ l Proteinase K were onto the cell pellet, this cell mixture was incubated at 55°C for one hour. Then, centrifuged at 10,000 xg for one minute 30 seconds at RT. DNA isolation was continued with commercial DNeasy Ultraclean Microbial Kit (QIAGEN, USA) according to manufacturer's instructions with slight modifications. Extra washing steps are added to the procedure for obtaining pure DNA. From 50  $\mu$ l elution, the sample quality was assessed through DNA concentration and the 260/280 and 260/230 ratios using a NanoPhotometer NP80 spectrophotometer (Implen, Germany). 260/280 ratio is used for confirming purity of DNA and acceptable range for 260/280 is  $\sim 1.8$ . 260/230 is used for confirming purity of nucleic acids and acceptable range for 260/230 is 2.0-2.2. Confirmed pure DNA was sent to Eurofins Genomic Laboratory (Ebersberg, Germany) for whole genome sequencing.

### 2.3.2 Whole Genome Sequencing with Illumina

Library preparation and whole genome sequencing were performed at Eurofins Genomic Laboratory. Whole genome sequencing was performed with 150 base pair (bp) paired end reads using the Illumina NovaSeq 6000 (Illumina, California, USA) device. Quality controls of the obtained sequences were performed using the FastQC program (GitHub, FastQC v0.12.1).

### 2.3.3 Phylogenetic Tree and SNP/InDel Analysis

*C. auris* whole genome sequencing analysis study was carried out as a multi-stage process including preparation and analysis of genomic data. First, raw data obtained after sequencing were cleaned and edited with FastP (Github, FastP, v0.24.1) software; low quality data and adapters were removed at this stage. Then, genome assembly was performed using SPAdes (Github, SPAdes, v4.2.0) software and scaffolding of the assembly was completed with Redundant software and gaps were filled. In the genome annotation stage, a customized pipeline was created using RNAseq data obtained from NCBI; functional annotation was performed using various software such as InterProScan, EggNog, SignalP, Phobius, TMbed, CAZymes, MEROPS, KOs (with BlastKoala) and AntiSmash. In the variant calling stage, K1575 read data were aligned to the NCBI B11220 (GCA\_003013715.2) reference genome and variants were identified using bwa, sambamba markdup, samtools and freebayes software. In order to investigate the potential effects of these variants, K1575 variants were analyzed against B11220 using SNPeff software. In order to evaluate the phylogenetic relationships, SNP-based phylogenetic analysis of Clade 1 (B8441): GCA\_002759435.3, Clade 2 (B11220): GCA\_002759435.2, Clade 3 (strain B11221): GCA\_030122265.1, Clade 4 (strain B11243): GCA\_003014415.1, Clade 5: GCA\_016809505.1 and K1575 retrieved from the NCBI database were performed and the connections between K1575 and the B11205 reference strain (Clade 1) were examined. Finally, amino acid sequence differences among B11220, B8441 and K1575 were investigated manually using Aliview software. In addition, SNP-InDel analysis was performed using Lofreq and SnpEff programs to investigate gene regions that may cause multidrug resistance and mutations in these regions. CDR1, CDR2, ERG1, ERG2, ERG3, ERG6, ERG7, ERG8, ERG11, ERG24, ERG27, MDR1, MRR1, TAC1a, TAC1b, FKS1, CIS2, FUR, FYC1, FLO8, PEP1,

RIM15, WOR2, EDC3 sequences were extracted from the *C. auris* B11221 genome and used as reference. Then, the reference genes were mapped against the sequences generated in the study using BLAST + v2.11.0 (Camacho et al., 2009) and the relevant genes were determined. Protein products were compared, and relevant amino acid changes were identified. Phylogenetic analysis was performed with VCF2PopTree using the genetic drift algorithm and a MEGA-based distance matrix was generated (Subramanian S. et al., 2019). This matrix was loaded into MEGA11, and a phylogenetic tree was generated using the Neighbor-Joining tree method (Tamura K. et al., 2021).

## 2.4 In-vitro Biofilm Model of *Candida auris*

In order to establish a *C. auris* biofilm model at this stage of the study, multidrug-resistant (MDR) *C. auris* clinical isolates and *C. auris* quality control reference isolates were passage into SDA and incubated at 37°C for 48 hours. To create the biofilm model, *Candida parapsilosis* isolates known to form high (K875 and K802) and medium (K1102) level biofilms in a previous study were added as controls (Dogan et al., 2021). Matured colonies were placed in falcon tube with RPMI-1640 (Sigma-Aldrich, USA, catalog code: R6504) broth containing 2% glucose, buffered with L-glutamine and bicarbonate-free and incubated at 200 rpm and 30°C for 24 hours. Then, yeast cells adjusted to a density of 0.5 McFarland were transferred to 96-well flat-bottom polystyrene microplates (SARSTEDT, Nümbrecht, Germany) with 100 µl in each well. Three wells without yeast were used as negative controls at the end of each series. Microplates were incubated at 75 rpm and 37°C to take measurements at three different time points: 24, 48 and 72 hours.

The XTT (2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide)/Menadione method was used to measure biofilm density. XTT solution (Sigma-Aldrich, USA) was prepared as one mg/ml with PBS and mixed with Menadione solution (Sigma-Aldrich, USA) prepared as one mg/ml with chloroform (Sigma-Aldrich, Merck, USA) (final concentration 1 mM) and sterilized by passing through filters with 0.45 µm pore size. At the end of plate incubation, the wells were washed twice with PBS to remove the medium and planktonic cells and clean the biofilm surface. In order to prevent the XTT/Menadione solution from being affected by light, 200 µl of solution was added to the wells in the dark and incubated at 37°C for two hours

in the dark. At the end of the incubation period, the solutions in the wells were placed in clean wells and measurements were taken on a MultiSkan Go Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) with an Optical Density (OD) value set at 490 nm (Park et al., 2014). The results were normalized by taking the average values of three wells at each measurement point and subtracting the blank values from these values.

## 2.5 Synthesis of Metal Nanoparticles

All nanoparticles were synthesized in KUTTAM laboratories, in Asst. Prof. Emel SOKULLU Laboratory group. All experiments were performed in KUTTAM laboratories and characterized in KUYTAM laboratories by graduate students Buse KELEŞ and Cennet GÜNDÜZ under the supervise of Prof. Emel SOKULLU.

### 2.5.1 Synthesis of Gold Nanoparticles

For synthesis of AuNPs, 1g  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  (tetrachloro auric acid) (Sigma-Aldrich, Merck, USA) was dissolved in 250 ml of distilled water to prepare a stock solution (10 mM) for 25 synthesis. For the 1 mM  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  solution to be used in the synthesis, 2.5 ml of the 10 mM solution was taken into a 50 ml beaker and mixed with 22.5 ml of distilled water. For the beginning of the synthesis, 20 ml of 1 mM  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  solution was taken into a 50 ml beaker and heated to 70°C with a magnetic stirrer. 1%  $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7$  (trisodium citrate) (Sigma-Aldrich, Merck, USA) solution was prepared fresh in each synthesis. 0.5 g of powdered trisodium citrate was mixed with 50 ml of distilled water to be used in the synthesis. 2 ml of 1% trisodium citrate solution was quickly added to the boiled  $\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  solution and the mixing was continued. When the solution reached cherry red color, the reaction was completed, and the beaker was taken from the heater and left to cool for measurements. In order to eliminate large-sized particles, the prepared gold nanoparticle solution was centrifuged at 3750 rpm for 30 minutes. In order to calculate the gold concentration obtained as a result of the synthesis, UV-Vis measurement was taken at 520 nm wavelength, and the gold concentration was determined by drawing the calibration curve. It was stored at room temperature and in the dark for later use (McFarland et al., 2004). Fourier Transform Infrared Spectroscopy (FTIR), X-ray Diffraction (XRD), Dynamic Light Scattering (DLS), Zeta Sizer, Scanning

Electron Microscopy (SEM) and Nanoparticle Tracking Assay (NTA) analyses were performed for the characterization of the synthesized gold nanoparticles.

### 2.5.2 Synthesis of Chitosan Capped Gold Nanoparticles

For the synthesis of chitosan-capped gold nanoparticles (Chi-AuNP), 0.33% (w/v) chitosan was prepared in 1% acetic acid solution and stirred at 75°C for 30 minutes. After dissolving chitosan, 1 mM HAuCl<sub>4</sub>·3H<sub>2</sub>O was added and the mixture was continued to be stirred at 90°C until it turned dark red. The mixture was centrifuged at 14,000 rpm for 30 minutes, then the pellet was washed and stored at +4°C for subsequent experiments (Franconetti et al., 2019). FTIR, XRD, DLS, SEM, Zeta Sizer, and NTA analyses were performed for the characterization of the synthesized chitosan-capped gold nanoparticles.

### 2.5.3 Synthesis of Fe<sub>3</sub>O<sub>4</sub> Nanoparticles

Synthesis of Fe<sub>3</sub>O<sub>4</sub> nanoparticles was prepared in a four-neck reactor with nitrogen gas as iron source in tris (acetylacetonate) iron (III) benzyl ether, oleyl amine at a concentration of 3 mM. The reaction was prepared by stirring at 120°C for one hour and 300°C for one hour. After the impurities were removed, the concentration of the obtained substance was determined, and the prepared nanoparticles were then coated with citric acid in order to perform cytotoxicity experiments. In order to overcome the colloidal problem, the nanoparticles were subjected to surface modification with citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>) containing carboxyl groups (-COOH). Before starting the coating process, the concentration of the synthesized iron oxide nanoparticles was determined and then they were brought to powder form by drying. This drying process was carried out in a hot air dryer at 60°C. In the first step for the coating process, citric acid solution was prepared. For this, 2.1 grams of solid citric acid was dissolved in 100 ml of pure (distilled) water and the pH of the solution was adjusted to be neutral (pH 7). The Fe<sub>3</sub>O<sub>4</sub> nanoparticles, calculated as 176 mg in total, were divided into two groups, 76 mg of which would be coated with citric acid. The amounts were determined to be 4.32 ml of the prepared citric acid solution according to the coating rates. The citric acid coating process was initiated by suspending iron oxide nanoparticles in hexane. pH-adjusted citric acid solution was added dropwise to this hexane suspension in a 1:1 volume ratio. The mixture was placed in a sonication bath and sonicated for 30 minutes. After this process, the mixture was kept

at 60°C for two hours. After the process was completed, the mixture was brought to room temperature and centrifuged at 12,000 rpm for 10 minutes. After centrifugation, the supernatant was discarded, the remaining nanoparticles were washed with pure water and then dried in a hot air dryer and turned into powder form again. Nanoparticles obtained from citric acid coating processes were characterized using FT-IR, XRD, DLS, Zeta Sizer and SEM techniques. Concentration determinations of the obtained nanoparticles were made and stored under appropriate conditions to be used in cytotoxicity experiments.

#### 2.5.4 Synthesis of Chi-Fe<sub>3</sub>O<sub>4</sub> Nanoparticles

Synthesis of Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles was prepared in a four-neck reactor with nitrogen gas as iron source in tris (acetylacetonate) iron (III) benzyl ether, oleyl amine at a concentration of 3 mM. The reaction was prepared by stirring at 120°C for one hour and 300°C for one hour. After the impurities were removed, the concentration of the obtained substance was determined, and the prepared nanoparticles were then coated with citric acid in order to perform cytotoxicity experiments. Before starting the coating process, the concentration of the synthesized iron oxide nanoparticles was determined and then they were brought to powder form by drying. This drying process was carried out in a hot air dryer at 60°C. The Fe<sub>3</sub>O<sub>4</sub> nanoparticles, calculated as 176 mg in total, were divided into two groups, 100 mg of which would be coated with chitosan. For the preparation of the chitosan solution, 50 mg of chitosan was dissolved in 30 ml of 1% acetic acid and this solution was kept at room temperature overnight on a magnetic stirrer until it became homogeneous. The nanoparticles to be coated with chitosan were mixed in a sonication bath with the prepared chitosan solution for 5 hours. Then, nanoparticles were centrifuged in the same way, the supernatant was removed, washed with distilled water and dried in a hot air dryer and turned into powder. Nanoparticles obtained from chitosan coating processes were characterized using FT-IR, XRD, DLS, Zeta Sizer and SEM techniques. Concentration determinations of the obtained nanoparticles were made and stored under appropriate conditions to be used in cytotoxicity experiments.

#### 2.5.5 Coating of MNPs with Fluorescently Labeled Chitosan

Chitosan was dissolved in 1% (v/v) acetic acid solution at a concentration of 1% (w/v). This dissolution process was carried out overnight (approximately 16–18 hours)

on a magnetic stirrer at RT. The obtained chitosan solution was filtered through a 0.45  $\mu\text{m}$  filter in order to ensure homogeneity and to remove insoluble particles. This step helped to obtain a more homogeneous and clear solution in the labeling of chitosan. After the filtration process, FITC solution prepared in methanol at a concentration of 0.5 mg/mL was added to the obtained chitosan solution. The amount of FITC was kept low due to the limited stock available. The mixture was kept in the dark since FITC is light sensitive and was continuously stirred at room temperature for 3 hours. At this stage, it was aimed to form covalent bonds between the isothiocyanate ( $-\text{N}=\text{C}=\text{S}$ ) group of FITC and the free amine ( $-\text{NH}_2$ ) groups in the chitosan chain. After the conjugation process, the pH value was adjusted to pH 10 by adding 0.2 M NaOH solution in order to precipitate chitosan in the mixture. Since chitosan becomes insoluble in basic environment, its physical precipitation was provided in this step. Thus, the separation of FITC covalently bound to chitosan and unbound free FITC was facilitated. The precipitated chitosan-FITC conjugate was collected by centrifugation at 10,000  $\times g$  for 10 minutes. The resulting precipitate was washed several times with 70% methanol solution in order to remove the fluorescent dye molecules remaining free on its surface or in its structure. Methanol ensured the dissolution of free FITC while preserving the structural stability of chitosan. After the washing processes, dialysis step was started to make the conjugate purer. The sample was dialyzed in pure water for 3 days using a dialysis membrane with appropriate molecular weight cut-off (MWCO). During dialysis, water was changed every 12 hours to remove low molecular weight impurities (such as free FITC, methanol residues, NaOH residues). As a result of this method, pure chitosan sample covalently labeled with FITC via amine groups was obtained. In order to prevent photodegradation of FITC throughout the process, all stages were worked in the dark and continuous stirring was provided for homogeneous mixture of reagents. Nanoparticles obtained from chitosan coating processes were characterized using FT-IR, XRD, DLS, Zeta Sizer, UV-Vis and SEM techniques. Concentration determinations of the obtained nanoparticles were made and stored under appropriate conditions to be used in cytotoxicity experiments.

#### 2.5.6 Cytotoxicity of Metal Nanoparticles

For AuNP, Chi-AuNP,  $\text{Fe}_3\text{O}_4$  and Chi- $\text{Fe}_3\text{O}_4$  cell compatibility tests, the response of nanoparticles to NIH 3T3 cells was evaluated with the cytotoxicity MTT assay. Toxicity

was analyzed at the end of 24-, 48- and 72-hours using methyl thiazolyl tetrazolium (MTT), which is based on mitochondrial dehydrogenase activity. Cell viability was assessed by measuring mitochondrial NADH/NADHP-dependent dehydrogenase activity. Before the experiment, Fe<sub>3</sub>O<sub>4</sub> and Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles were prepared in phosphate buffered saline (PBS) solution at a concentration of 1 mg/mL to form the main stock solution. This stock solution was kept in a sonication bath at room temperature overnight in order to disperse the particles smoothly and homogeneously. Therefore, the solution prepared for chitosan-capped nanoparticles was dissolved in distilled water containing 1% acetic acid and the nanoparticles were sonicated in this medium overnight at RT. Since the solvent medium was acidic, a control group containing 1% acetic acid solution was also included in the experimental design in order to control the effect of the pH of the medium on the cells as well as the effect of the nanoparticles. Thus, the viability of the cells without nanoparticles but in the same solvent medium was analyzed and it was clearly evaluated whether the observed cytotoxic effect was due to the nanoparticles alone or the acidic nature of the solution. MTT experiments were performed in 96-well plates; NIH 3T3 cells (approximately 10<sup>5</sup> cells per well) were treated with a fixed amount of nanoparticles sterilized with UV for two hours and incubated for 24, 48 and 72 hours. At the end of the determined time points, the supernatants were removed and 10 µl of MTT solution was added to each well. The plates were incubated for 3.5 hours at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub> and in the dark. MTT was taken up by active cells and reduced to insoluble purple formazan granules in mitochondria (Saruchi et al., 2022). Afterwards, the supernatant was removed, and the precipitated formazan was dissolved in dimethyl sulfoxide (DMSO) (100 µl per well) and the optical density of the solution was measured using a microplate spectrophotometer (Kayto RT-2100C) at a wavelength of 570 nm. The % live cell value was calculated by comparing with the control wells using the formula:

$$\text{Cytotoxicity} = \frac{\text{OD490 Value of Exposed Cells}}{\text{OD490 Value of Unexposed Cells}} \times 100$$

The data obtained as a result of the test were calculated with the [aatbio.com](http://aatbio.com).

## 2.6 Effect of Metal Nanoparticles on *Candida auris* Biofilm and Planktonic Cells

### 2.6.1 Demonstration of the Effectiveness of Metal Nanoparticles on *Candida auris* Planktonic Cells

In order to determine the effectiveness of AuNPs against planktonic cells, AuNPs prepared at concentrations of 250/100/50/10 µg/ml, Chi-AuNPs prepared at concentrations of 30/15/5/2.5 µg/ml, Fe<sub>3</sub>O<sub>4</sub>NPs prepared at concentrations of 100/50/10/5 µg/ml, and Chi-Fe<sub>3</sub>O<sub>4</sub>NP prepared at concentrations of 100/50/10/5 µg/ml in RPMI-1640 medium. *C. auris* planktonic cells adjusted to a density of 0.5 McFarland in PBS and 100 µl of inoculum and 100 µl of nanoparticle were added in 96-well flat-bottom microplates and incubated at 37°C for 48 hours. The results were evaluated visually and spectrophotometrically. Then, serial dilutions (1/10) were prepared from each well and these dilutions were seeded onto SDA plates in triplicate from each well, the plates were incubated at 37°C for 72 hours and the colonies that grew at the end of the incubation period were counted. The growth results of *C. auris* isolates exposed to different nanoparticle concentrations were recorded as mean colony forming unit (CFU) and percent (%) growth inhibition compared to the control without NPs.

### 2.6.2 Demonstration of the Effectiveness of Metal Nanoparticles on *Candida auris* Biofilm Cells

The effectiveness of metal nanoparticles on the *C. auris* biofilm model was shown spectrophotometrically and by colony counting. 250/100/50/10 µg/ml AuNP concentrations, 30/15/5/2.5 µg/ml Chi-AuNP concentrations, 100/50/10/5 µg/ml Fe<sub>3</sub>O<sub>4</sub>NP concentrations, and 100/50/10/5 µg/ml Chi-Fe<sub>3</sub>O<sub>4</sub>NP concentrations were applied on *C. auris* biofilms prepared in RPMI-1640 medium. The microplates were incubated at 37°C for 48 hours. At the end of the incubation period, the wells were washed with PBS and 100 µl PBS was added to the wells to remove and homogenize the biofilm. 1/10 serial dilutions were made from the wells and seeded onto SDA plates. The plates were incubated at 37°C for 72 hours. The results were evaluated visually and spectrophotometrically. Then, serial dilutions (1/10) were prepared from each well and these dilutions were seeded onto SDA plates in triplicate from each well, the plates were

incubated at 37°C for 72 hours and the colonies that grew at the end of the incubation period were counted.

### 2.6.3 Demonstration of Reactive Oxygen Radicals Formed by Nanoparticles

In order to demonstrate that the antifungal activities of AuNPs and Chi-AuNPs occur due to the formation of intracellular reactive oxygen radicals, fluorescently labeled H<sub>2</sub>DCFDA 2',7'-dichlorodihydrofluorescein diacetate (ABCAM DCFDA/H<sub>2</sub>DCFDA - Cellular ROS Assay Kit, catalogue no: ab113851) was used. Yeast cell suspension prepared at a density of 1 McFarland was washed with PBS and distributed to 96-well microplates in 100 µl (v/v) volume and incubated with 250 µg/ml AuNP and 30 µg/ml Chi-AuNP diluted with 100 µl RPMI-1640 for four hours at 37°C. For positive control wells, 50 µM Tertbutyl hydroperoxide (TBHP) was prepared and incubated with 100 µl of fungal cells. After the incubation period, the solutions taken into Eppendorf tubes were centrifuged at 13,000 rpm for 5 minutes and the supernatant was discarded. 100 µl of H<sub>2</sub>DCFDA was added to the pellet and vortexed and incubated in the dark for 45 minutes. Reactive oxygen species formed by fungal cells were measured by Beckman Coulter CytoFLEX SRT Cell Sorter Flow Cytometry (California, USA) (Seong and Lee, 2018; James et al., 2015).

### 2.6.4 Demonstration of Morphological Changes on the Surfaces of *Candida auris* Cells Due to Antifungal Activity of Nanoparticles by SEM

In order to observe the morphological changes caused by AuNPs and Chi-AuNPs in *C. auris* cells, SEM images were taken. For this purpose, 100 µl of isolates adjusted to a density of 1 McFarland in RPMI-1640 medium buffered with MOPS containing 2% glucose were added to 12-well IBIDI (IBIDI, Germany) slides placed on a 12 mm slide and incubated at 37°C in an 80-rpm shaking incubator. At the end of the 72-hour incubation, the biofilms were washed twice with PBS and 100 µl of 250 µg/ml AuNPs and 100 µl of 30 µg/ml Chi-AuNPs were added. The prepared slide was incubated for 24 hours at 37°C in an 80-rpm shaking incubator. At the end of the incubation period, 2.5% glutaraldehyde was added as 100 µl to fix the samples and incubated at 37°C in the dark. At the end of the incubation, the samples fixed on the slide were kept in ethyl alcohol prepared at 50%, 75%, 95% and 100% for 15 minutes and then left to dry (Bilici et al.,

2020). The slide was kept at +4°C until the measurement was taken. The images were evaluated with the help of Zeiss Evo LS15 Microscope with 20.000X magnification (Zeiss, Oberkochen, Germany) in Koç University Surface Research Center (KUYTAM) laboratory.

#### 2.6.5 Demonstration of Antifungal Activity of Nanoparticles in *Candida auris* Cells by Confocal Microscopy

Confocal microscopy was used to demonstrate the ability of nanoparticles to adhere to *C. auris* biofilms. Previously prepared and characterized FITC-labeled chitosan-coated gold nanoparticles were used at the concentration at which viability was finally observed, determined in the cytotoxicity test. For this purpose, 100 µl of isolates adjusted to a density of 1 McFarland in RPMI-1640 medium buffered with MOPS containing 2% glucose were added to 12-well IBIDI (IBIDI, Germany) slides placed on a 12 mm slide and incubated at 37°C in an 80-rpm shaking incubator. At the end of the 72-hour incubation, the biofilms were washed twice with PBS and 100 µl of 30 µg/ml FITC tagged Chi-AuNPs was added. The prepared slide was incubated for 24 hours at 37°C in an 80-rpm shaking incubator. At the end of the incubation period, 3.5% formaldehyde was added as 100 µl to fix the samples and incubated at RT for 15 mins in the dark. At the end of the incubation, the samples fixed on the slide were kept in PBS to protect biofilm. The slide was kept at +4°C until the measurement was taken. Slide was stained with Calcofluor White stain. One drop of stain and one drop of 10% potassium hydroxide were dripped. The images were evaluated at 40x magnification with the help of Leica DMI8 SP8 CS/DLS Microscope (Leica, Wetzlar, Germany) in Koç University Research Center for Translational Medicine (KUTTAM) laboratory.

## 2.7 Statistical Analysis

In all statistical analyses and preparation of graphs performed within the scope of the thesis, GraphPad Prism application version 10.4.2 was used. In the analyses performed, normality tests were applied when the number of data was sufficient, and the Shapiro-Wilk test was used to examine the distribution of the data. 4 different nanoparticle concentrations were compared with the control group using the multiple comparison

method. While the analysis of data sets with normal distribution was performed using the ANOVA test, data sets without normal distribution were evaluated using the Kruskal-Wallis test. In nanoparticle concentration groups with different distribution types within the same group, tests were analyzed using the one-to-one comparison method. If the two compared groups were normally distributed, “Student T Test” was used, and if they were not normally distributed, Mann-Whitney U Test was used. The p value was taken as 0.05 for the evaluation of the test results. Asterisks were used to indicate the different p values of the statistically significant nanoparticle concentrations on the graph. “\*” means  $P < 0.05$ , “\*\*” means  $P < 0.01$ , “\*\*\*” means  $P < 0.001$ , “\*\*\*\*” means  $P < 0.0001$ .

## 2.8 Ethical Statement

Ethics committee approval numbered 2023.HADYEK.036 for this thesis has been received from the Ethics committee of Koç University.

This thesis is part of the "In-vitro and In-Vivo Evaluation of the Efficacy of Chitosan-Supported Metal Nanoparticles Carried by Erythrocytes in the Treatment of Multidrug-Resistant *Candida auris*" project supported by the TÜBİTAK 1001 Scientific and Technological Research Projects Support Program.

## Chapter 3:

## RESULTS

### 3.1 Antifungal Susceptibility Test

According to the antifungal susceptibility test results, shown in Table 3.1, the MIC values of isolate K1575 was found to be resistant to fluconazole and echinocandin class antifungal drugs and susceptible to amphotericin B. MIC values of other quality control isolates were also determined, and resistance to fluconazole was detected in all isolates. High MIC values against voriconazole and posaconazole were found for strains numbered NR-52713 and NR-52714 of the quality control isolates, while MIC values against other azoles were lower in isolate numbered NR-52717. All quality control isolates were found to be susceptible to anidulafungin, an echinocandin class of antifungal drugs, and amphotericin B, a polyene class antifungal drug, used in the study.

**Table 3.1 MIC values and antifungal susceptibility test results of *C. auris* clinical (K1575) and reference isolates**

|                 | <b>Fluconazole</b>                         |           | <b>Voriconazole</b>                        |             | <b>Posaconazole</b>                        |             |
|-----------------|--------------------------------------------|-----------|--------------------------------------------|-------------|--------------------------------------------|-------------|
|                 | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |           | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |             | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |             |
| <b>K1575</b>    | 64                                         | Resistant | 0,125                                      | -           | 0,06                                       | -           |
| <b>NR-52717</b> | 64                                         | Resistant | 2                                          | -           | 0.125                                      | -           |
| <b>NR-52714</b> | 64                                         | Resistant | 16                                         | -           | 16                                         | -           |
| <b>NR-52713</b> | 64                                         | Resistant | 16                                         | -           | 16                                         | -           |
| <b>MYA-5002</b> | 64                                         | Resistant | 1                                          | -           | 0.125                                      | -           |
|                 | <b>Itraconazole</b>                        |           | <b>Anidulafungin</b>                       |             | <b>Amphotericin B</b>                      |             |
|                 | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |           | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |             | 48 <sup>th</sup> hour<br>MIC value (µg/ml) |             |
| <b>K1575</b>    | 0.25                                       | -         | 8                                          | Resistant   | 1                                          | Susceptible |
| <b>NR-52717</b> | 1                                          | -         | 2                                          | Susceptible | 1                                          | Susceptible |
| <b>NR-52714</b> | 0.25                                       | -         | 0.25                                       | Susceptible | 1                                          | Susceptible |
| <b>NR-52713</b> | 0.25                                       | -         | 0.25                                       | Susceptible | 1                                          | Susceptible |
| <b>MYA-5002</b> | 0.06                                       | -         | 0.5                                        | Susceptible | 1                                          | Susceptible |

## 3.2 Whole Genome Sequencing

### 3.2.1 Phylogenetic Tree of *Candida auris* K1575 Isolate

SNP-based phylogenetic tree was constructed using variant calling and SNP analysis with reference genomes belonging to 5 different clades (Figure 3.1). The obtained SNP-based phylogenetic tree showed that K1575 belongs to clade 1, defined as the South Asian Clade.

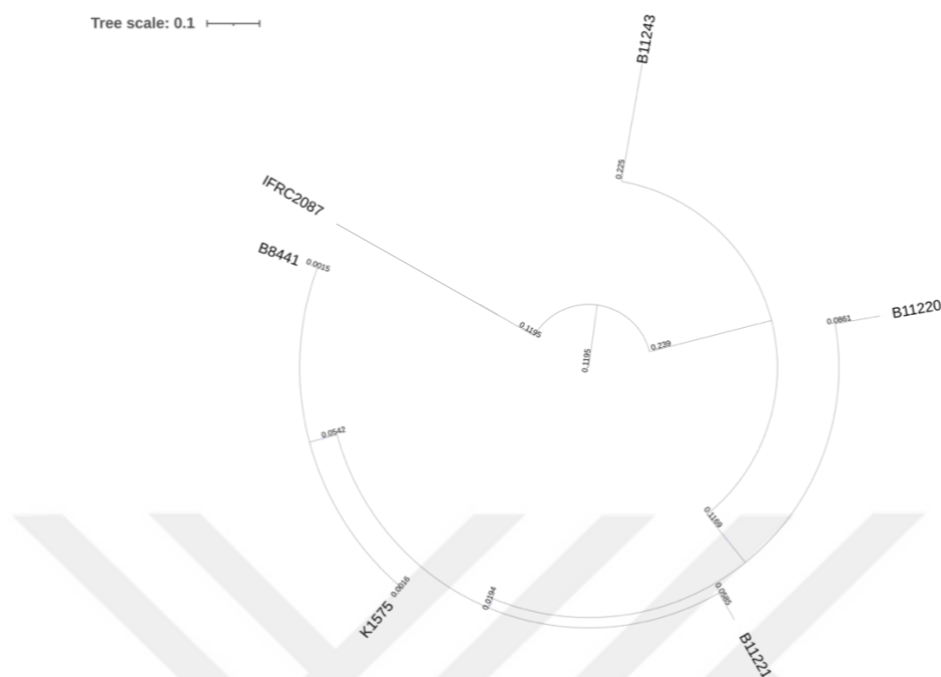


**Figure 3.1 SNP-based maximum likelihood phylogeny tree of K1575**

### 3.2.2 SNP/InDel Analysis of *Candida auris* K1575 Isolate

As a result of single nucleotide polymorphism analysis, shown in Table 3.2, Y132F mutation in the ERG11 gene; A15T, S195C and L328S mutations in the TAC1b gene and E709D mutation in the CDR1 gene were detected to be related to fluconazole resistance. In addition to these mutations, E39D mutation in the ERG2 gene, which is related in fluconazole resistance, was also detected in isolate K1575. F635del mutation was shown in the “hot-spot 1” region of the FKS1 gene, which is the most important gene region for echinocandin resistance. In addition to these resistance genes, the S70R mutation was detected in the FCY1 gene in association with flucytosine resistance.

**Table 3.2 Resistance genes found in clinical isolate of *C. auris* K1575**

| Gene Name    | Mutations in clinical <i>C. auris</i> isolate (K1575) |
|--------------|-------------------------------------------------------|
| <b>ERG11</b> | Y132F                                                 |
| <b>CDR1</b>  | E709D                                                 |
| <b>TAC1b</b> | A15T, S195C, L328S                                    |
| <b>ERG2</b>  | E39D                                                  |
| <b>FKS1</b>  | F635del (Hot-Spot 1)                                  |
| <b>FCY1</b>  | S70R                                                  |

### 3.2.3 In-vitro Biofilm Model of *Candida auris*

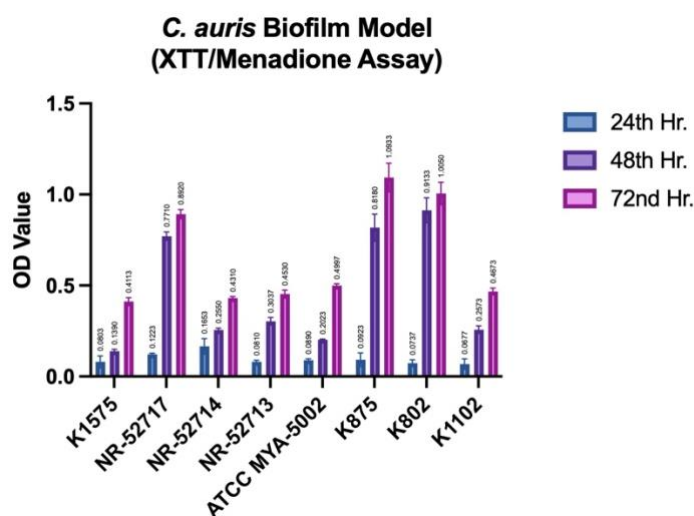


Figure 3.2 In-Vitro *C. auris* biofilm model measured via XTT/Menadione assay

Measurements of biofilm model were taken three different time points to determine exact time point for further experiments. For determining of the biofilm density of isolates, three *C. parapsilosis* isolates were used. K802 and K875 isolates have high biofilm capacity while K1102 has moderate biofilm capacity. Pattern of maturation in all isolates followed same path, means assay shows accurate and reliable results. OD values of XTT/Menadione assay showed that 72<sup>nd</sup> time point is the exact time for obtaining mature biofilm forms of all isolates.

### 3.3 Characterization of Synthesized Nanoparticles

XRD (X-ray Diffraction), FT-IR (Fourier Transform Infrared Spectroscopy), DLS (Dynamic Light Scattering), NTA (Nanoparticle Tracking Assay), Scanning Electron Microscopy (SEM) and Zeta-Potential measurements were taken of the obtained nanoparticles.

3.3.1 Characterization of AuNPs

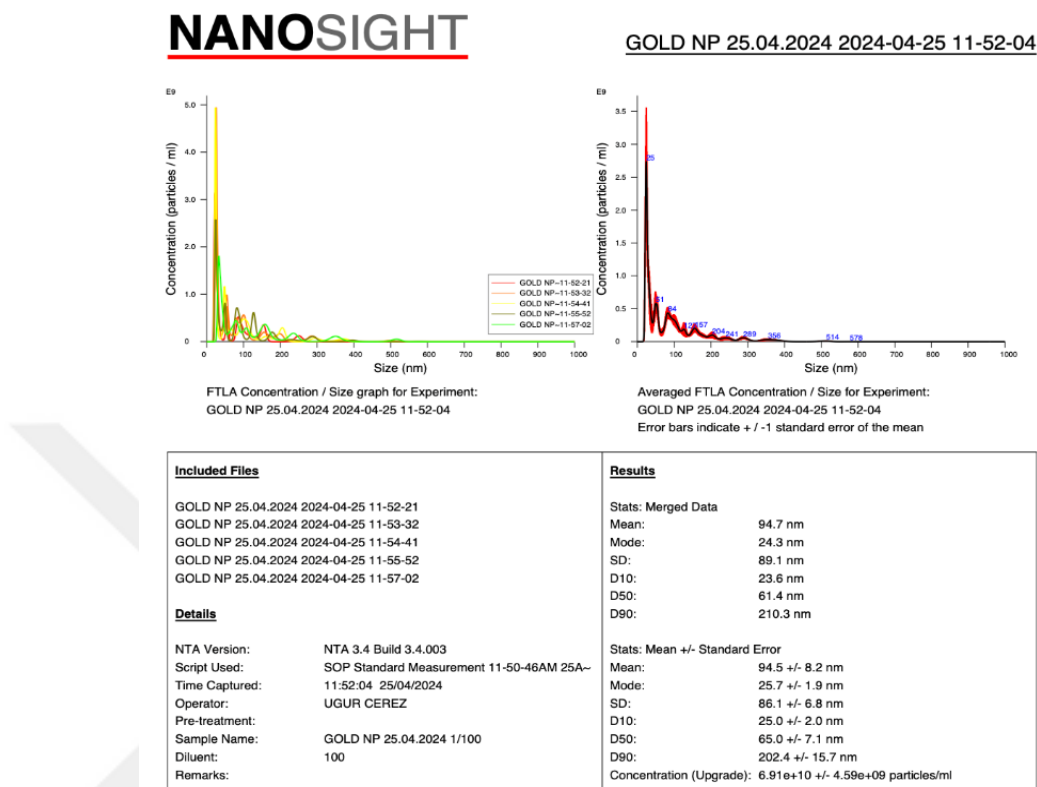


Figure 3.3 AuNPs Nanoparticle Tracking Assay (NTA) result report

Figure 3.3 shows the NTA result displaying the dimensions and average power of the synthesized gold nanoparticles. According to the result, the mode of synthesized nanoparticle size was found 24.3 nm. Mean size is around 94-95 nm, and median size is about 61-65 nm. Sample concentration is approximately  $6.9 \times 10^{10}$  particles/ml.

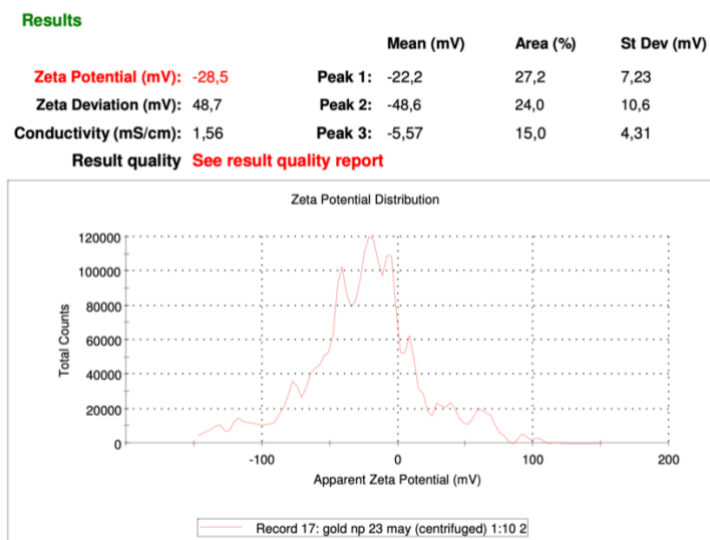


**Figure 3.4 AuNPs Zeta-Potential result report**

Figure 3.4 shows the result of Zeta-potential result of AuNPs. Main zeta potential result is -28,5 mV with 48.7 mV zeta deviation. The first peak indicates 27,2% of the population of nanoparticles have the mean charge of -22.2 mV with 7,23 standard deviations. Second peak indicates 24.0% of the population of nanoparticles have the mean charge of -48.6 mV with 10,6 standard deviations. Third peak indicates 12,0% population of nanoparticles have mean charge of -5,57 mV with 4,31 standard deviations.

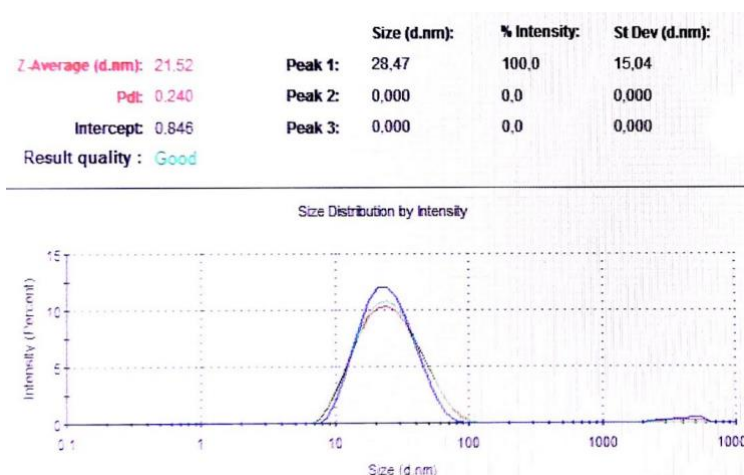


**Figure 3.5 AuNPs Dynamic Light Scattering (DLS) result report**

Figure 3.5 shows the result report of the DLS test performed for the forward progress of the NTA result. Z-Average (d.nm) hydrodynamic size is ~21.5 nm and PdI is 0.240. PdI value means nanoparticles have moderate polydispersity.

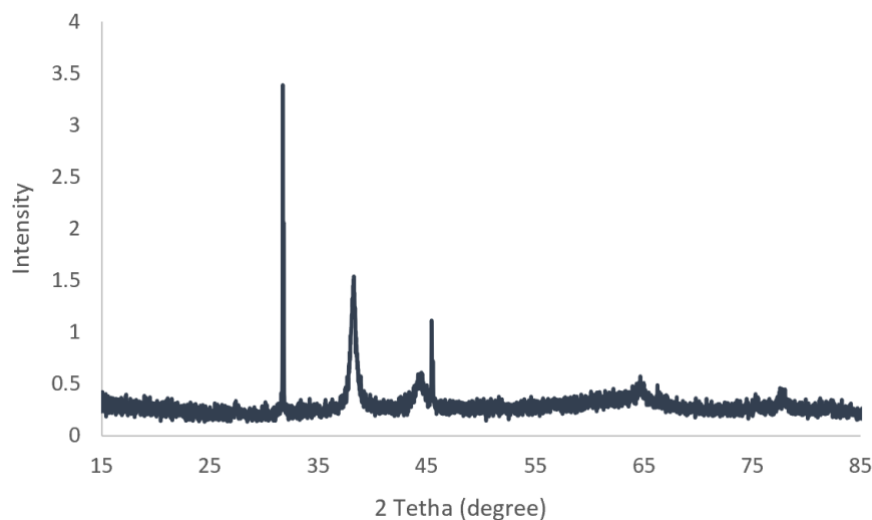


**Figure 3.6 AuNPs X-Ray Diffraction (XRD) result graph**

The XRD measurement graph in Figure 3.6 was performed to display the structure, crystal orientation, chemical composition and physical properties of the synthesized gold nanoparticle.  $\sim 38.2^\circ(2\theta)$  peak is the most intense peak in the pattern. This intense peak at  $\sim 38.2^\circ(2\theta)$  perfectly matches the Au (111) crystallographic plane. The peak at  $44.5^\circ(2\theta)$  corresponds to the Au (200) crystallographic plane. The peak at  $64.8^\circ(2\theta)$  corresponds to the Au (220) crystallographic plane. The peak at  $77.8^\circ(2\theta)$  corresponds to the Au (311) crystallographic plane.

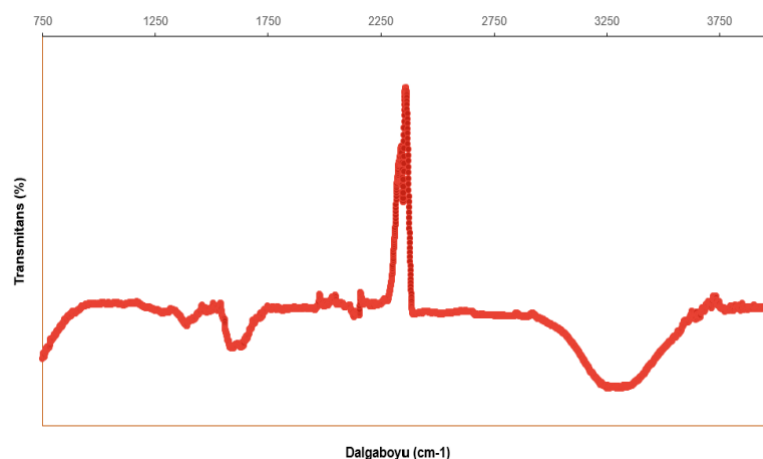


**Figure 3.7 AuNPs Fourier Transform Infrared Spectroscopy (FT-IR) result graphic**

The FT-IR measurement graph in Figure 3.7 shows the chemical bonds of the gold nanoparticle molecules. As a result of the FTIR analysis of the synthesized gold

nanoparticles, peaks appeared at  $3250\text{ cm}^{-1}$ ,  $1635\text{ cm}^{-1}$ , and  $1300\text{ cm}^{-1}$  indicating the presence of O-H, C=C, C-O, C-C groups in the sample. Broad band at  $3200\text{--}3400\text{ cm}^{-1}$  shows O-H stretching vibrations. Peaks in the  $1600\text{--}1400\text{ cm}^{-1}$  are critical for identifying the presence of citrate. Significant peak at  $1600\text{--}1650\text{ cm}^{-1}$  corresponds asymmetric stretching vibration of the carboxylate ( $\text{COO}^-$ ) group.  $1400\text{ cm}^{-1}$  less distinct peak is attributed to the symmetric stretching vibration of the carboxylate ( $\text{COO}^-$ ) group. The peak observed at  $2350\text{ cm}^{-1}$  can be attributed to the presence of atmospheric  $\text{CO}_2$  present in the environment during the measurement.

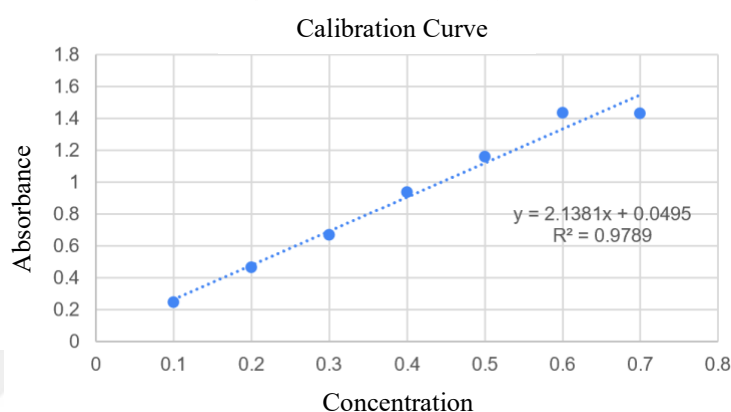


**Figure 3.8 Calibration curve of AuNP**

The gold concentration in the solution obtained as a result of synthesis and centrifugation was calculated as a result of the equation obtained with the graph drawn with UV-Vis results. The obtained graph is shown in Figure 3.8.

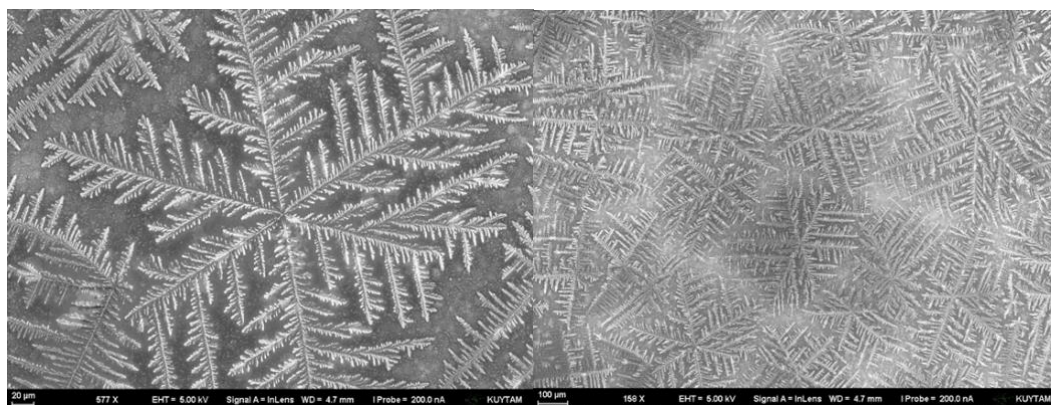


**Figure 3.9 Scanning Electron Microscopy images of gold nanoparticles**

This SEM images show big salt crystals belong to citric acid in the sample. AuNPs are barely seen but if we look closer to the images, little star-like dots of AuNPs can be seen in Figure 3.9.

### 3.3.2 Characterization of Chi-AuNPs

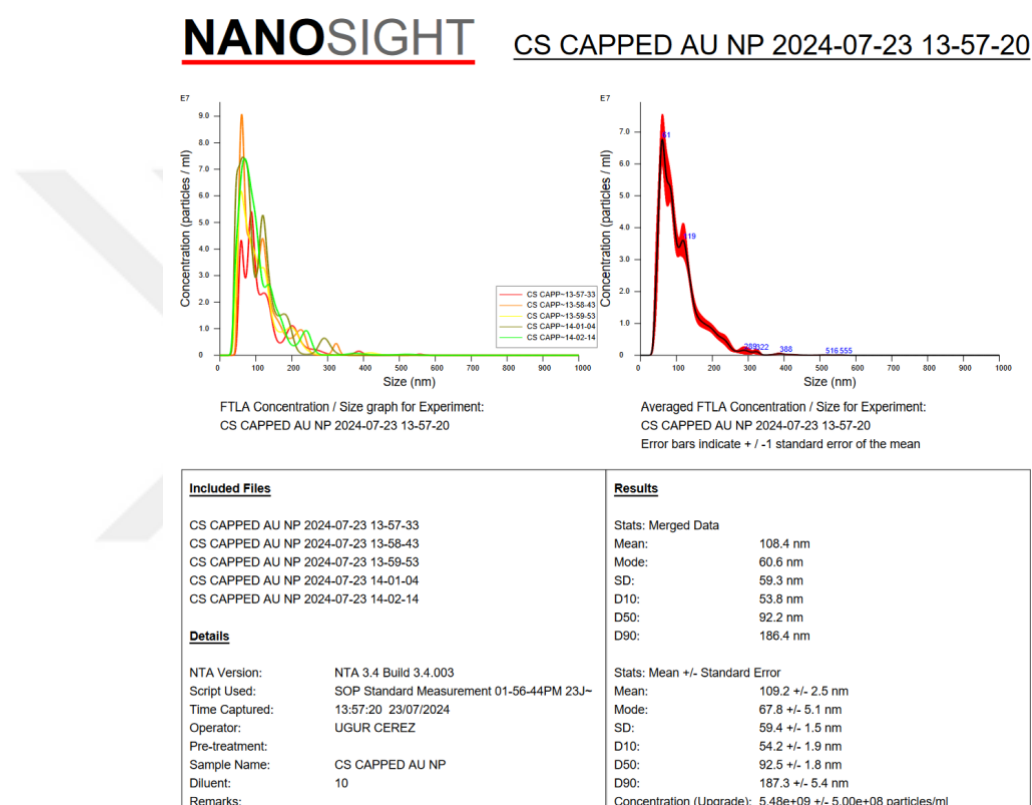


**Figure 3.10 Chitosan-capped gold nanoparticles Nanoparticle Tracking Assay (NTA) result report**

Figure 3.10 shows the NTA result displaying the dimensions and average power of the synthesized chitosan-capped gold nanoparticles. According to the result, the mode of synthesized nanoparticle size was found 60.6 nm. Mean size is around 109.2 nm, and median size is about 92.2 nm, shown as D50 value in result part. Sample concentration is approximately  $5.48 \times 10^9$  particles/ml.

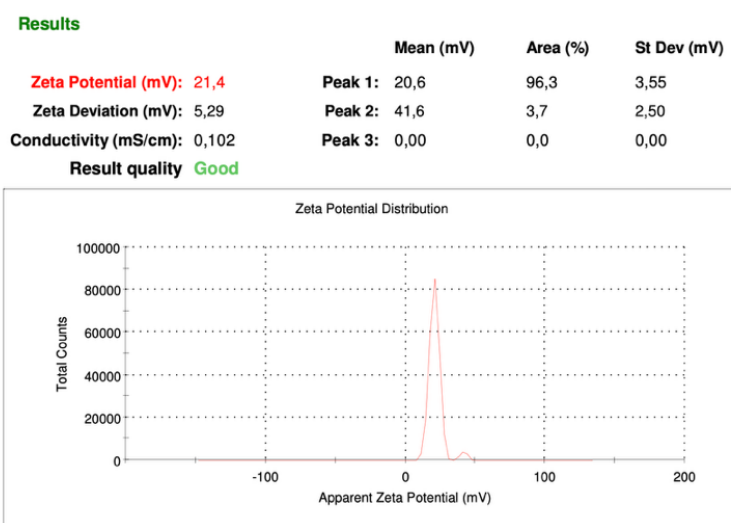


**Figure 3.11 Chi-AuNPs Zeta-Potential result report**

Figure 3.11 shows the Zeta-Potential of Chi-AuNPs. The first peak shows 21.4 mV zeta potential with the zeta deviation of 5.29 mV. The first peak indicates major population (96.3%) of nanoparticles have the mean charge of 20.6 mV. The second peak which is smaller one, indicates smaller population (3.7%) of nanoparticles have the mean charge of 41.6 mV.

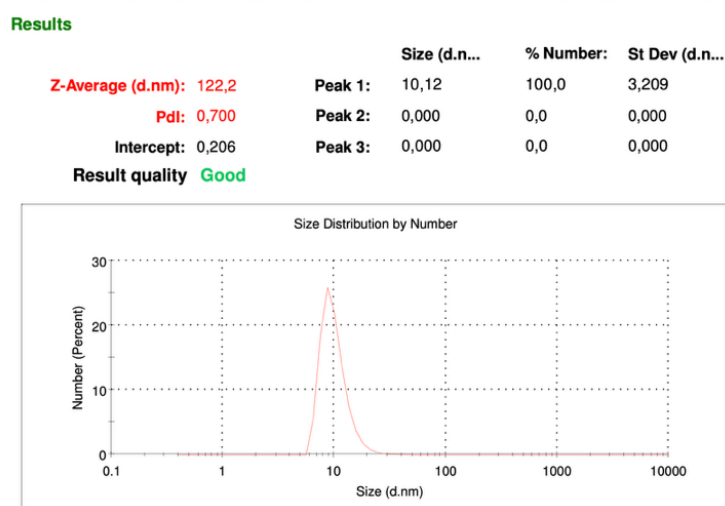


**Figure 3.12 Chi-AuNPs Dynamic Light Scattering (DLS) result report**

Figure 3.12 shows DLS result of Chi-AuNPs. Z-Average (d.nm) hydrodynamic size is ~122.2 nm and PdI is 0.7, PdI value means nanoparticles have moderate polydispersity.

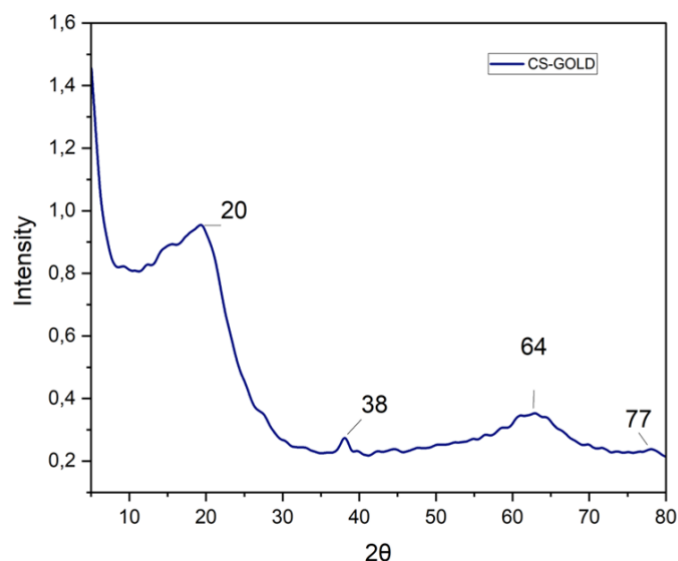


**Figure 3.13 Chitosan-capped gold nanoparticles X-Ray Diffraction (XRD) result graph**

Figure 3.13 shows XRD pattern of Chi-AuNPs to show up its crystal structure of gold nanoparticles and the effects of chitosan coating. The broad peak around  $\sim 20^\circ$  ( $2\theta$ ) shows the effect of the amorphous or semi-crystalline nature of chitosan. The peak around  $38^\circ$  ( $2\theta$ ) is the characteristic diffraction peak of the (111) crystal plane of gold nanoparticles. The peak around  $64^\circ$  ( $2\theta$ ) is associated with the (220) plane and reflects the accuracy and order in the structure of gold nanoparticles. The peak around  $77^\circ$  ( $2\theta$ ) belongs to the (311) plane supports the order in the crystal structure of gold nanoparticles.

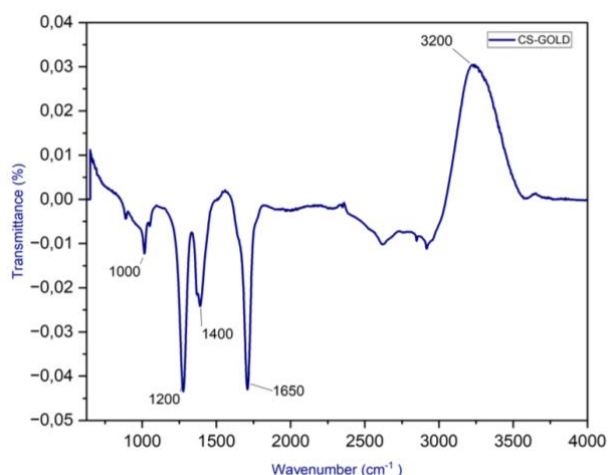


**Figure 3.14 Chitosan- capped gold nanoparticle Fourier Transform Infrared Spectroscopy (FT-IR) result graphic**

Figure 3.14 shows FT-IR. The broad peak at  $\sim 3200\text{ cm}^{-1}$  indicates the stretching vibrations of N-H (amine) and O-H (hydroxyl) groups of chitosan and this broadness reflects the interaction with hydrogen bonds. The peak around  $\sim 1650\text{ cm}^{-1}$  assigned to the C=O stretching vibration of amide in chitosan and indicates the interaction between chitosan and the surface of gold nanoparticles. The peak at  $1400\text{ cm}^{-1}$  reflects the C-H bending vibrations, indicating the presence of the hydrocarbon backbone of the chitosan polymer. The peak around  $1200\text{ cm}^{-1}$  reflects the C-O-C stretching vibrations of glycosidic linkages of chitosan, indicating that the structural components of chitosan provide stabilization by binding with gold nanoparticles. Finally, the peak at  $1000\text{ cm}^{-1}$  confirms the presence of glycosidic bonds of chitosan and their involvement in the interaction with gold nanoparticles.

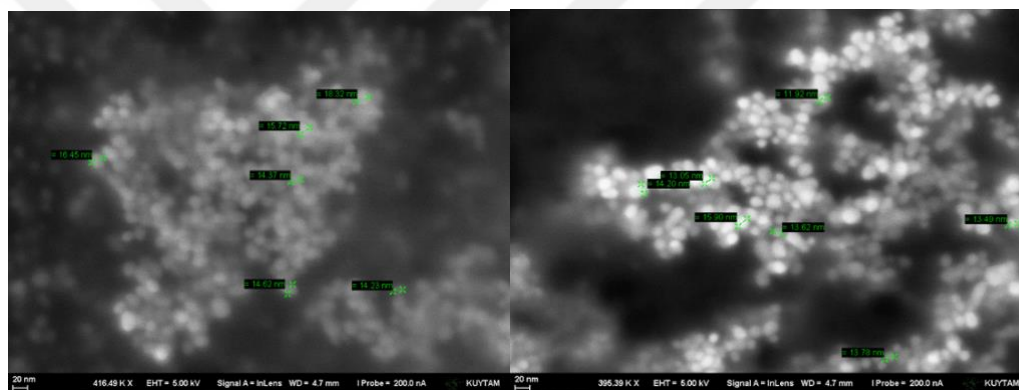


**Figure 3.15 SEM images of chitosan-capped gold nanoparticles**

Figure 3.15 shows SEM image of Chi-AuNPs. The images show discrete roughly spherical nanoparticles. Individual particle core size is in the range of approximately 12-18 nm.

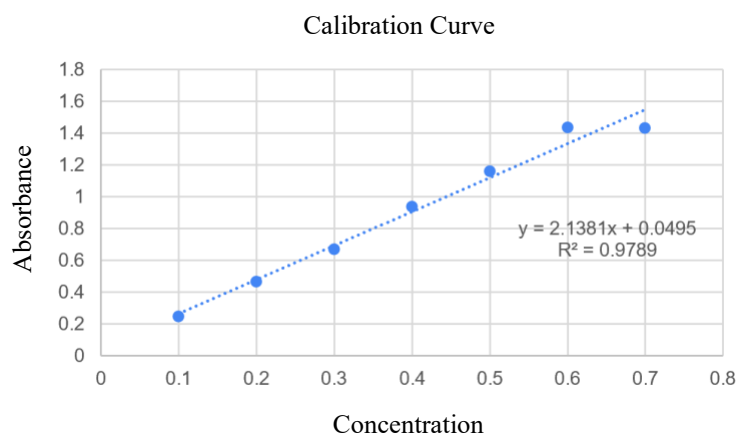


Figure 3.16 Calibration curve of Chi-AuNPs

The nanoparticle concentration in the solution obtained as a result of synthesis was calculated as a result of the equation obtained with the graph drawn with UV-Vis results, same graphic as gold nanoparticle's UV-Vis result. The graph obtained is shown in Figure 3.16.

### 3.3.3 Characterization of CA-Fe<sub>3</sub>O<sub>4</sub>NPs

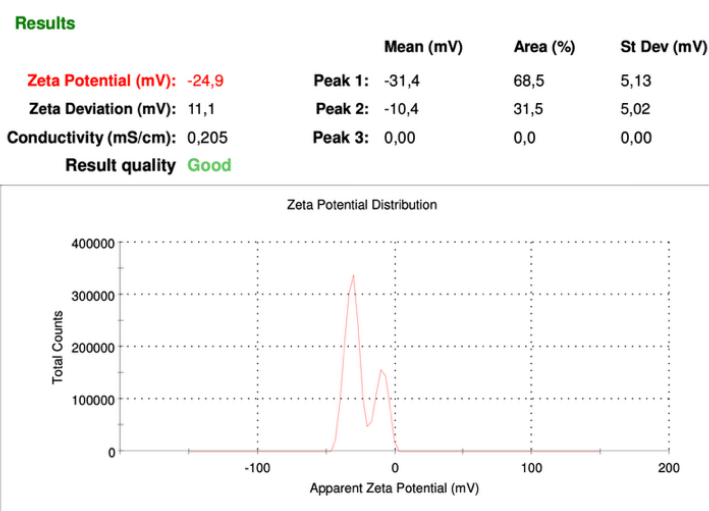


Figure 3.17 Zeta Sizer result of CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles

Figure 3.17 shows the zeta potential results which measures liquid suspension's charge repulsion/attraction between particles. Mean zeta potential of CA-Fe<sub>3</sub>O<sub>4</sub> is -24.9 mV. Zeta deviation of -10.4 mV and the single, relatively narrow peak in the Zeta

Potential Distribution graph indicates that the nanoparticles have two different surface charge distribution. The first peak indicates the major population (67.9%) of nanoparticles have the mean charge of -31.8 mV, the means more stability. Second peak which is smaller one, indicates smaller population (32.1%) of nanoparticles have mean charge of -10.4 mV, means low stability.

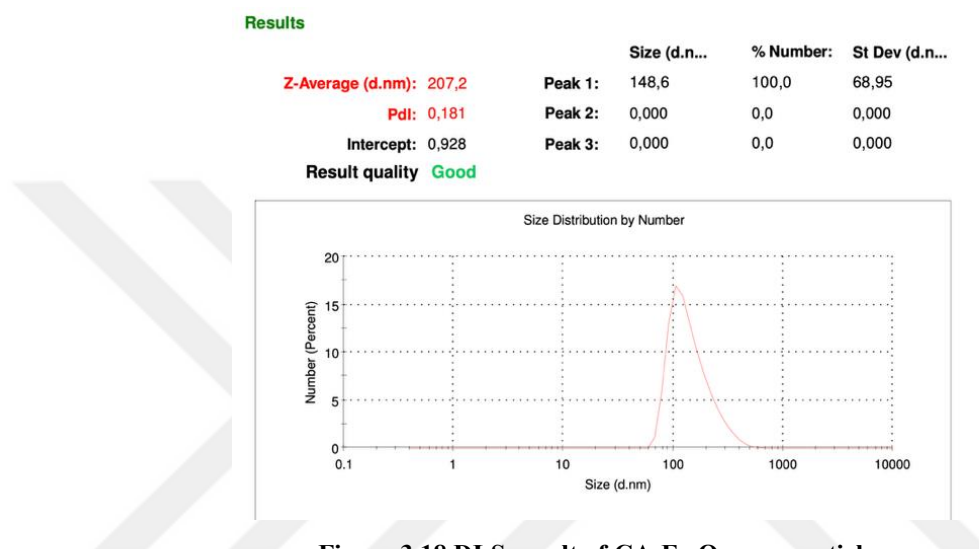


Figure 3.18 DLS result of CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles

DLS measurements were taken for validation of nanoparticle size. Figure 3.18 shows the result report of the DLS test performed. Z-Average (d.nm) hydrodynamic size is ~207.2 nm and PdI is 0.181, PdI value means nanoparticles are monodispersed.

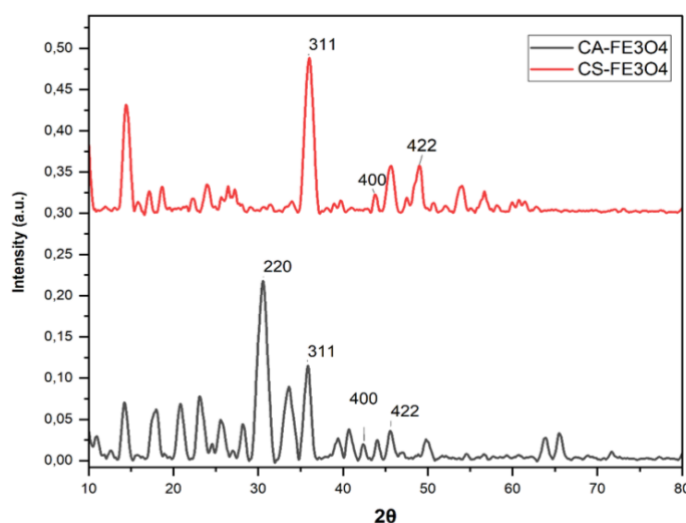


**Figure 3.19 Citric acid-coated  $\text{Fe}_3\text{O}_4$  nanoparticles X-Ray Diffraction (XRD) result graph (black line)**

For CA- $\text{Fe}_3\text{O}_4$  nanoparticles (Figure 3.19), prominent peaks are typically observed at  $2\theta \approx 30^\circ$ ,  $2\theta \approx 35.5^\circ$ ,  $2\theta \approx 43^\circ$ ,  $2\theta \approx 53.5^\circ$ . These peaks are associated with the (220), (311), (400), and (422) crystal planes of CA- $\text{Fe}_3\text{O}_4$ . In XRD pattern peaks are in good agreement with the standard diffraction pattern for CA- $\text{Fe}_3\text{O}_4$ .  $2\theta \approx 30^\circ$  and  $2\theta \approx 35.5^\circ$  peaks are associated with (220) and (311) planes of  $\text{Fe}_3\text{O}_4$  and indicate that citric acid minimally changes the crystal structure of  $\text{Fe}_3\text{O}_4$ .

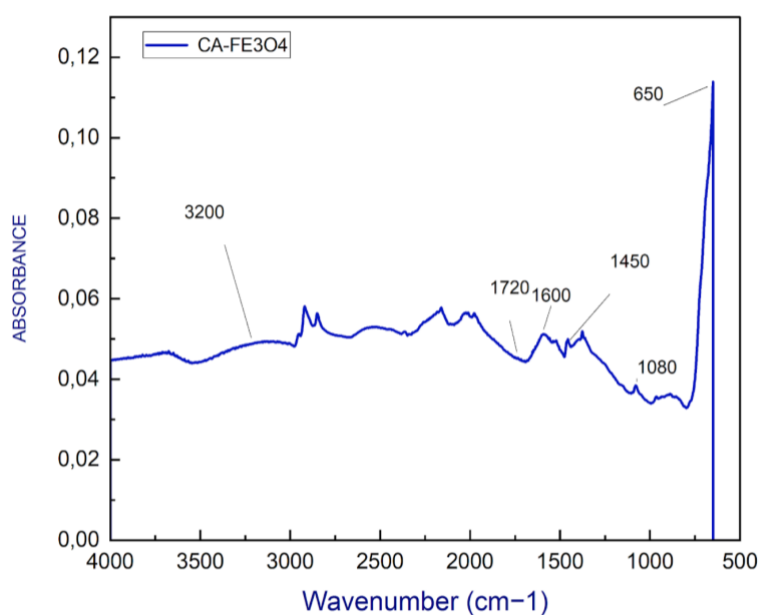


**Figure 3.20 Citric acid-coated  $\text{Fe}_3\text{O}_4$  nanoparticle Fourier Transform Infrared Spectroscopy (FT-IR) result graphic**

As a result of FTIR analysis of CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles (Figure 3.20), the characteristic peak observed in the range of 580–650 cm<sup>-1</sup> represents the Fe–O (metal-oxygen) stretching vibrations of the Fe<sub>3</sub>O<sub>4</sub> core. The weak broad peak appearing around 1720 cm<sup>-1</sup> is the characteristic stretching vibration of the carboxyl (C=O) groups of the citric acid molecule, confirming the presence of surface-bound carboxyl groups (-COOH) associated with the Fe<sub>3</sub>O<sub>4</sub> nanoparticles likely as stabilizing agent. ~1600 cm<sup>-1</sup> peak can represent asymmetric COO<sup>-</sup> stretching vibration of carboxylate groups. The signals observed in the 1400–1450 cm<sup>-1</sup> band originate from the symmetric stretching vibrations of the COO<sup>-</sup> groups and indicate that the bonding with the surface has occurred. The broad band observed in the 3200–3500 cm<sup>-1</sup> range represents the O–H stretching vibrations originating from the hydrogen bond interactions of the free hydroxyl (–OH) groups of absorbed water molecules on the surface. The weak signals observed in the 1100–1200 cm<sup>-1</sup> region can be associated with the C–O (alkoxy or carboxylate) stretching vibrations within the citric acid molecule.

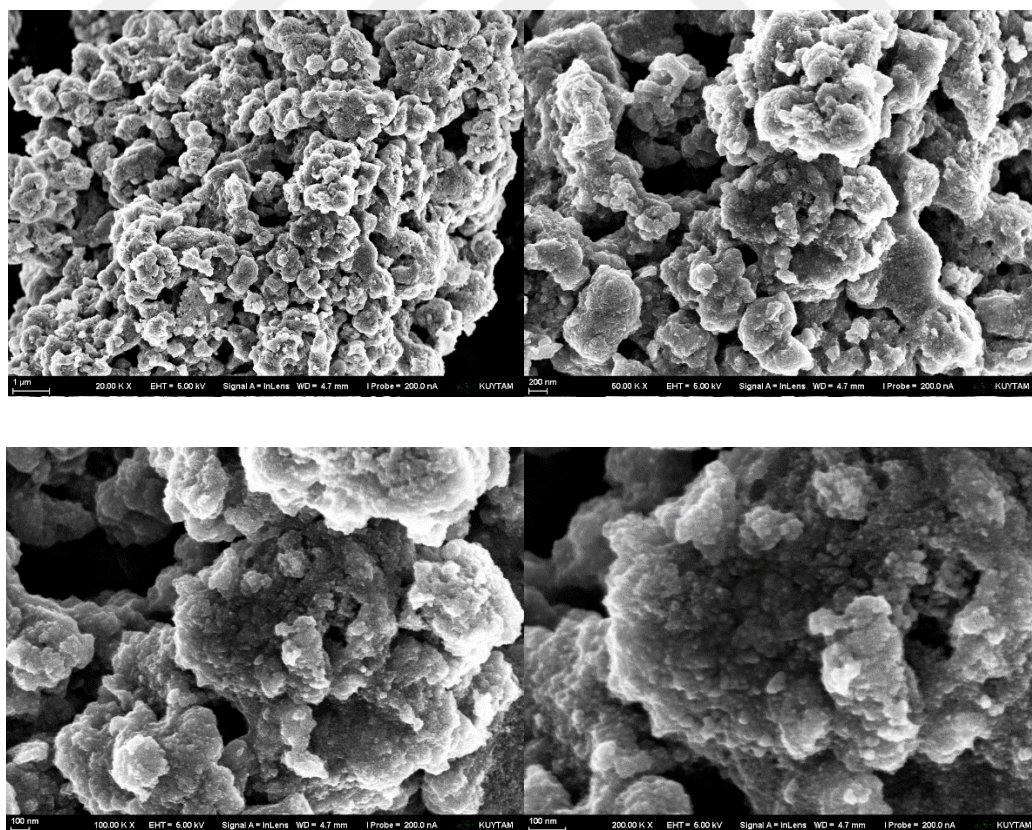


**Figure 3.21** SEM images of citric acid-coated iron oxide (Fe<sub>3</sub>O<sub>4</sub>) nanoparticles

Scanning electron microscope (SEM) analysis was performed to determine the morphological properties of the surface modified  $\text{Fe}_3\text{O}_4$  nanoparticles. In the SEM images of the  $\text{Fe}_3\text{O}_4$  nanoparticles coated with citric acid, an amorphous, irregular layer formation was observed around the particles (Figure 3.21).

### 3.3.4 Characterization of Chi- $\text{Fe}_3\text{O}_4$ NPs



Figure 3.22 Zeta-Potential result of Chi- $\text{Fe}_3\text{O}_4$  nanoparticles

For measuring liquid suspension's charge repulsion/attraction between particles Zeta potential was used, can be seen in Figure 3.22. The measured mean zeta potential of Chi- $\text{Fe}_3\text{O}_4$  is 54.8 mV. Zeta deviation of 4.89 mV and the single and narrow peak in the Zeta Potential Distribution graph indicates the nanoparticles have a uniform surface charge.

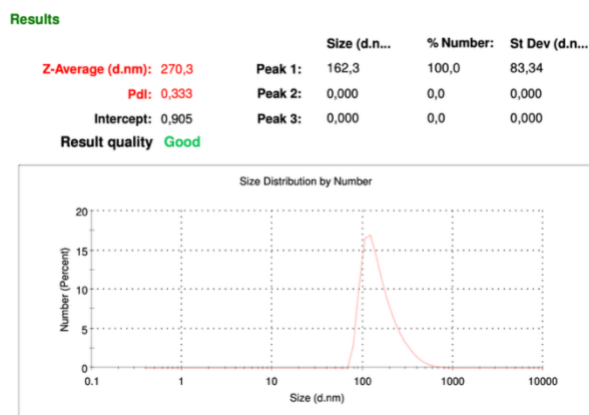


Figure 3.23 DLS results of Chi- $\text{Fe}_3\text{O}_4$  nanoparticles

DLS measurements were taken for validation of nanoparticle size. Figure 3.23 shows the result report of the DLS test performed. Z-Average (d.nm) hydrodynamic size is  $\sim 370.3$  nm and Pdl is 0.333 Pdl value means nanoparticles have moderate polydispersity.

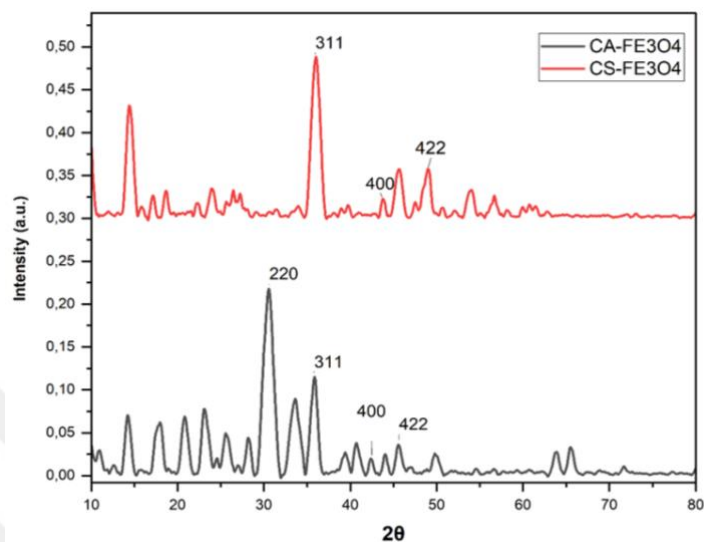


**Figure 3.24** Chitosan-coated  $\text{Fe}_3\text{O}_4$  nanoparticles X-Ray Diffraction (XRD) result graph (red line)

On the other hand, in Chi- $\text{Fe}_3\text{O}_4$  XRD pattern (Figure 3.24), peaks at  $2\theta \approx 30.1^\circ$ ,  $2\theta \approx 35.5^\circ$ ,  $2\theta \approx 43.1^\circ$ ,  $2\theta \approx 57.0^\circ$ ,  $2\theta \approx 62.6^\circ$  labeled (220), (311), (400), (511), and (422) planes.  $2\theta \approx 30.1^\circ$  peak is placed (220) crystallographic plane of magnetite, and this peak is strong indicator of  $\text{Fe}_3\text{O}_4$  formation.  $2\theta \approx 35.5^\circ$  is the most intense peak in this XRD pattern of  $\text{Fe}_3\text{O}_4$  (311) plane and confirms the magnetite phase. Peak at  $2\theta \approx 43.1^\circ$  (400) plane also shows identification of the  $\text{Fe}_3\text{O}_4$  phase.

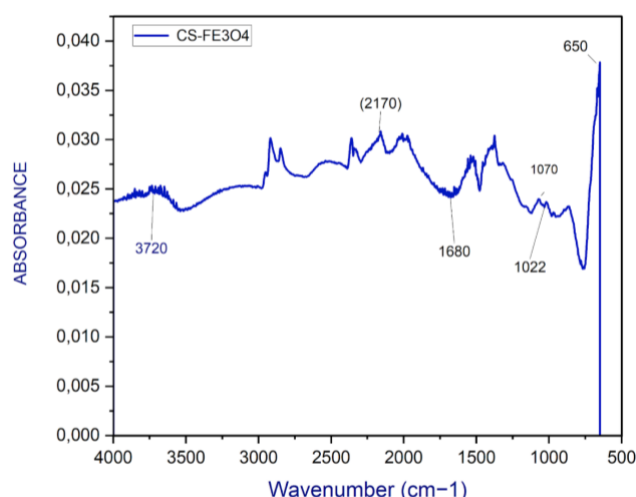


**Figure 3.25 Chitosan-coated Fe<sub>3</sub>O<sub>4</sub> nanoparticle Fourier Transform Infrared Spectroscopy (FT-IR) result graphic**

As a result of FTIR analysis in Figure 3.25, characteristic peaks were observed at 650, 1022, 1070, 1680, 2170 and 3720 cm<sup>-1</sup> wavelengths from Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles. The prominent peak observed at 650 cm<sup>-1</sup> region corresponds to the vibration mode of Fe–O bonds and confirms the presence of Fe<sub>3</sub>O<sub>4</sub> core (around 570-585 cm<sup>-1</sup>). The peaks around 1022 and 1070 cm<sup>-1</sup> originate from C–O–C and C–O stretching vibrations in the chitosan structure and indicate that chitosan coating is present on the nanoparticle surface. The broad and slightly noisy band observed at 1680 cm<sup>-1</sup> indicates the C=O stretching region of carboxylic acids. The weak peak recorded at 2170 cm<sup>-1</sup> may belong to C≡N (nitrile) groups or a side band of small structural changes from synthesis process of chitosan. The broad and wavy band observed in the 3720 cm<sup>-1</sup> region corresponds to the stretching vibrations of the free O–H (hydroxyl) and N–H (amine) groups on the surface of chitosan.

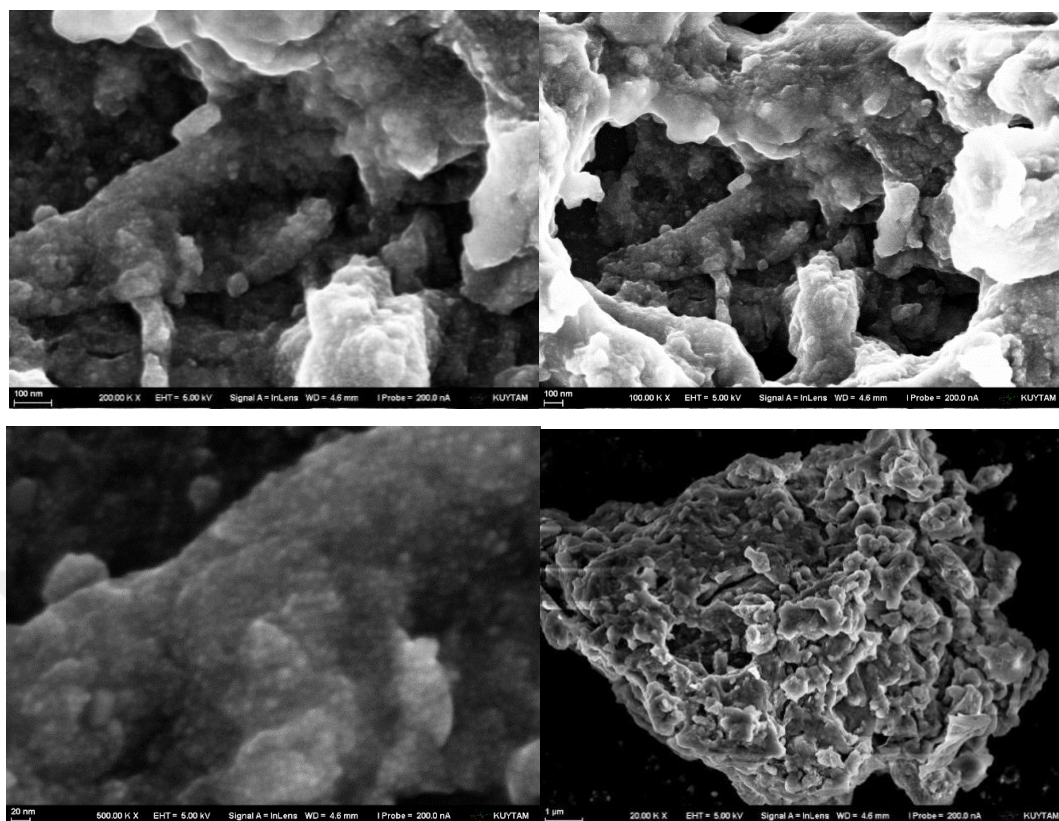


**Figure 3.26 SEM images of chitosan-coated iron oxide ( $\text{Fe}_3\text{O}_4$ ) nanoparticles**

In Figure 3.26, a relatively thick and homogeneous layer with smoother contours was detected surrounding the particles in chitosan-modified nanoparticles. Chitosan, due to its polymeric structure, covers the particle surface more clearly and this situation is reflected in the SEM images with a more dense and smooth coating form.

### 3.3.5 Cytotoxicity Assay on NIH 3T3 Fibroblast Cell Line

In the analysis results, the highest nanoparticle dose that showed viability in NIH 3T3 cells at 24 hours for bare AuNPs was determined as 250  $\mu\text{g}/\text{ml}$  and for Chi-AuNPs as 60  $\mu\text{g}/\text{ml}$ . IC50 calculation program and the gold nanoparticle concentration that reduced the viability to 50% was determined as 183  $\mu\text{g}/\text{ml}$  (Figure 3.27), and the chitosan-coated gold nanoparticle concentration was determined as 22  $\mu\text{g}/\text{ml}$  (Figure 3.28). According to the IC50 calculations made with the data obtained from MTT assay, the IC50 value of citric acid-coated  $\text{Fe}_3\text{O}_4$  nanoparticles was found to be 44.74  $\mu\text{g}/\text{mL}$  (Figure

3.29) and the IC<sub>50</sub> value of chitosan-coated Fe<sub>3</sub>O<sub>4</sub> nanoparticles was found to be 50.72 µg/ml (Figure 3.30).

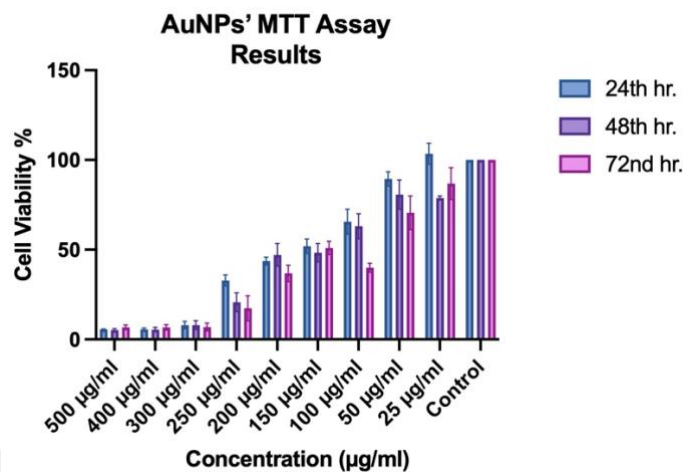


Figure 3.27 MTT result graphic of AuNP treated NIH 3T3 cell line

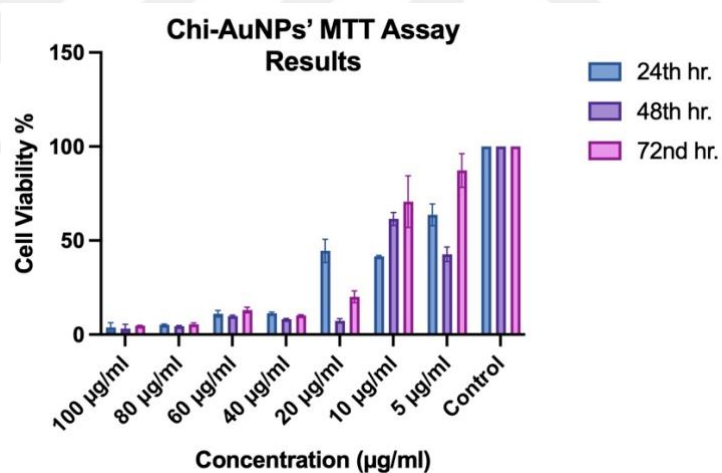


Figure 3.28 MTT result graphic of Chi-AuNP treated NIH 3T3 cell line

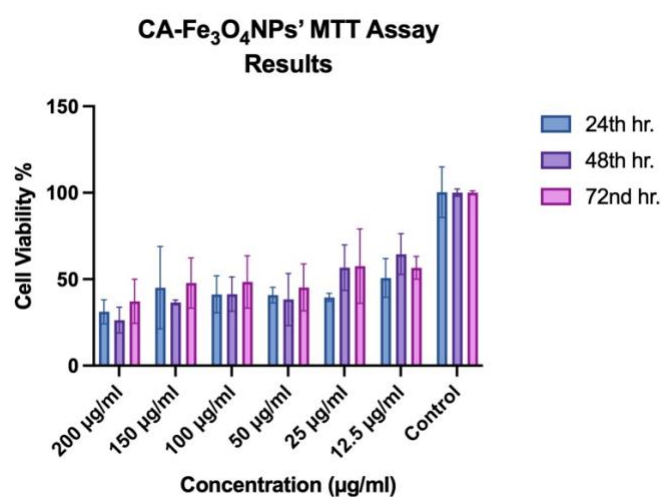


Figure 3.29 MTT result graphic of CA-Fe<sub>3</sub>O<sub>4</sub>NP treated NIH 3T3 cell line

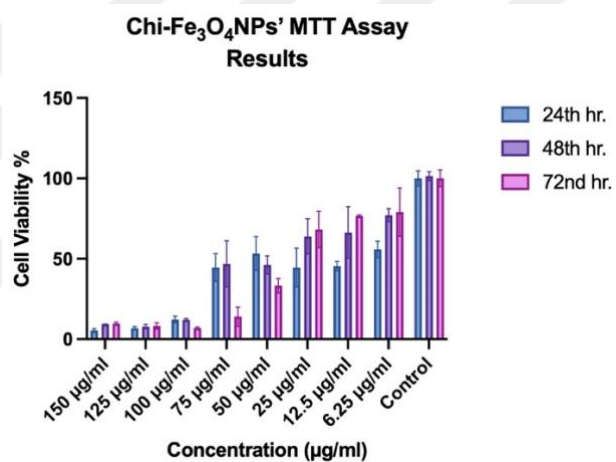


Figure 3.30 MTT result graphic of Chi-Fe<sub>3</sub>O<sub>4</sub> NP treated NIH 3T3 cell line

### 3.4 Effect of Metal Nanoparticles on *Candida auris* Biofilm and Planktonic Cells

#### 3.4.1 Demonstration of the Effectiveness of Metal Nanoparticles on *Candida auris* Planktonic Cells

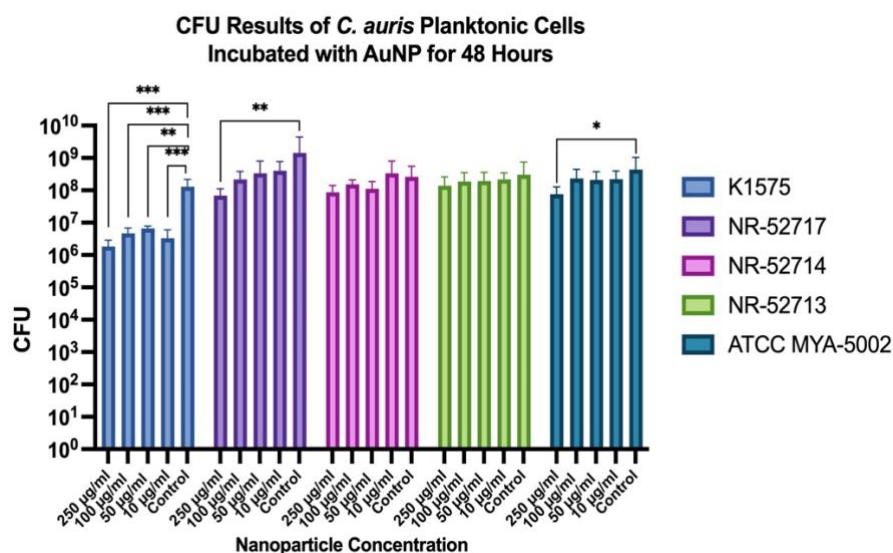


**Figure 3.31 Results of growth inhibition in terms of colony forming units (CFU) after 48-hour incubation of planktonic cells of *C. auris* isolates with AuNP**

In clinical isolate K1575, a statistically significant decrease was observed at 250 µg/ml AuNP concentration compared to the growth control (p value = 0.0001, 0.0002, 0.0017 and 0.0001 respectively). Similarly, in isolates NR-52717 and ATCC MYA-5002, the decrease at 250 µg/ml AuNP concentration was also statistically significant (p value = 0.0046 and 0.0218, respectively). Isolates NR-52714 and NR-52713 did not show a statistically significant decrease compared to the control at any AuNP concentration used (Figure 3.31).

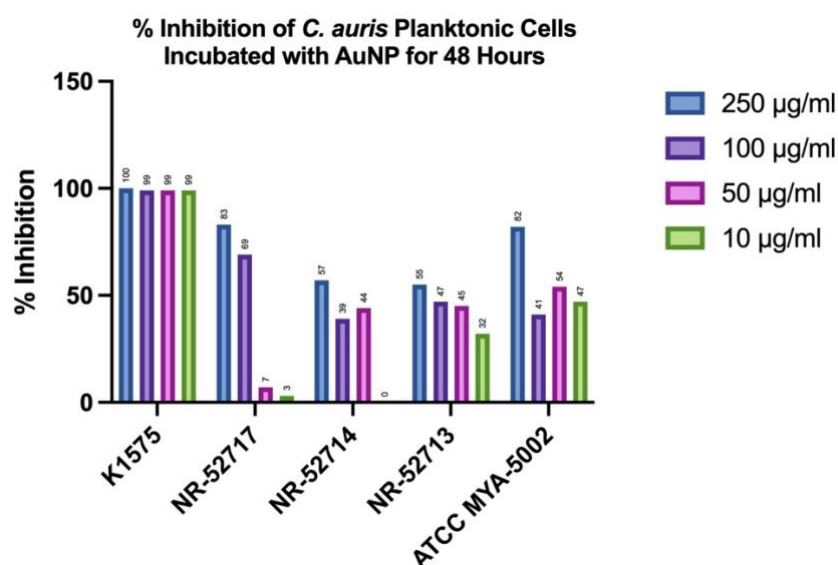


**Figure 3.32 Results of growth inhibition in terms of percentage inhibition (%) after 48-hour incubation of planktonic cells of *C. auris* isolates with AuNP**

When the results were evaluated as % inhibition, clinical isolate K1575 showed high inhibition at all concentrations (99%-100%), while isolates NR-52717 and ATCC MYA-5002 showed inhibition at 250 µg/ml concentration (83% and 82%, respectively). At the highest concentration of 250 µg/ml nanoparticle, the moderate inhibition shown by isolates NR-52714 and NR-52713 of 57% and 55%, respectively, and the low inhibition rates shown in both isolates afterwards indicate that gold nanoparticles are partially effective for these two isolates (Figure 3.32).

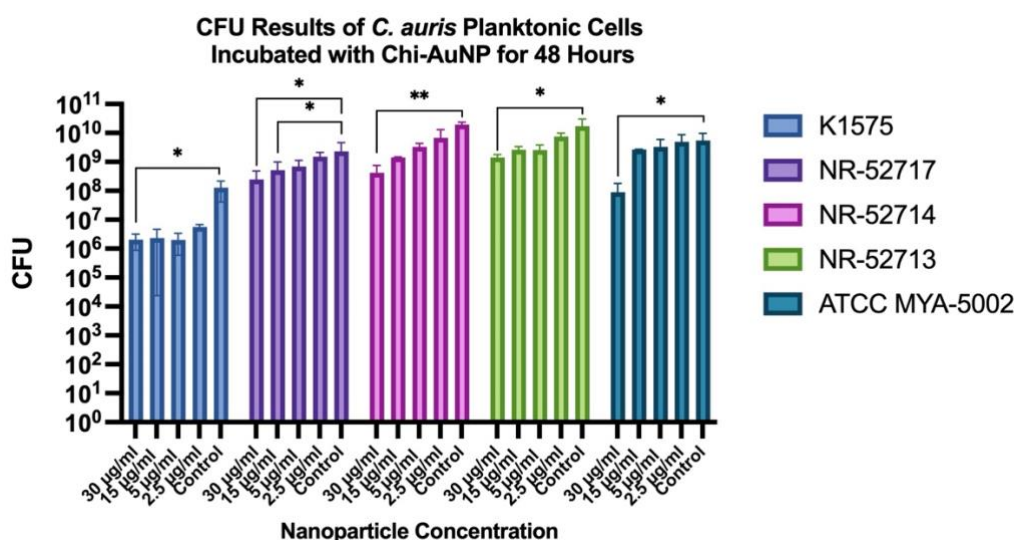


**Figure 3.33 Results of growth inhibition in terms of colony forming units (CFU) after 48-hour incubation of planktonic cells of *C. auris* isolates with Chi-AuNP**

As seen in Figure 3.33, it is seen that clinical isolate K1575 and NR-52717 isolates show a statistically significant inhibition at 30  $\mu\text{g/ml}$  nanoparticle concentration ( $p$  (K1575) = 0.0164,  $p$  (NR-52717) = 0.0201 and 0.0481 respectively), and both of the isolates show decreasing trend. On the other hand, a statistically significant decrease was shown in NR-52714, NR-52713 and ATCC MYA-5002 isolates at 30  $\mu\text{g/ml}$  nanoparticle concentration compared to the control ( $p$  values = 0.0014, 0.213 and 0.0187, respectively).

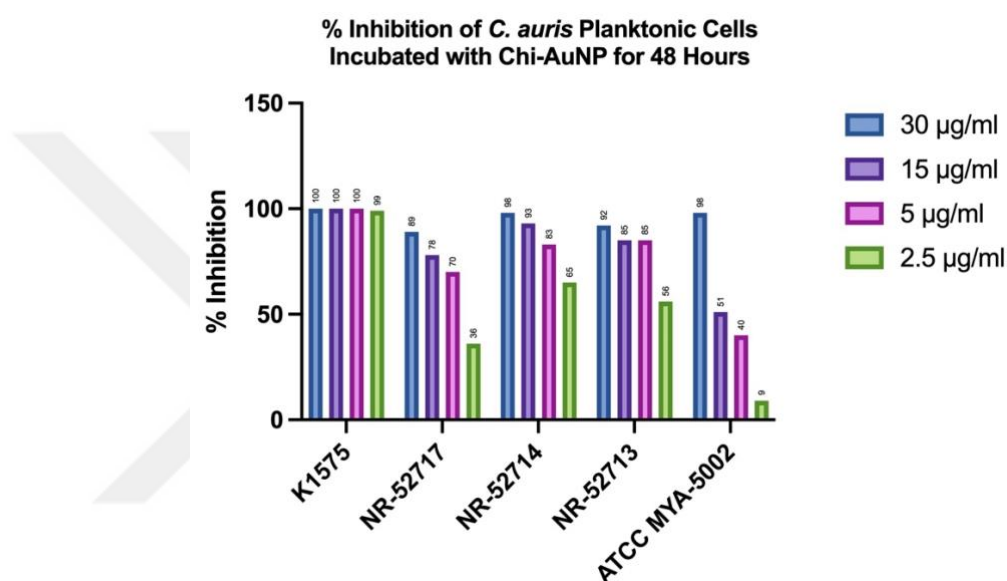


**Figure 3.34 Results of growth inhibition in terms of percentage inhibition (%) after 48-hour incubation of planktonic cells of *C. auris* isolates with Chi-AuNP**

Evaluation of the results in terms of percent inhibition in Figure 3.34, it was determined that the isolate for which Chi-AuNPs were most effective was K1575 (> 99.9% for all Chi-AuNP concentrations). A decreasing trend was shown in %inhibition graphic, parallel with the CFU results in NR-52717, NR-52714 and NR-52713 isolates. All reference isolates showed high inhibition at a concentration of 30  $\mu\text{g/ml}$  (89, 98, 92 and 98 inhibitions respectively) and this inhibition was found to be statistically significant.

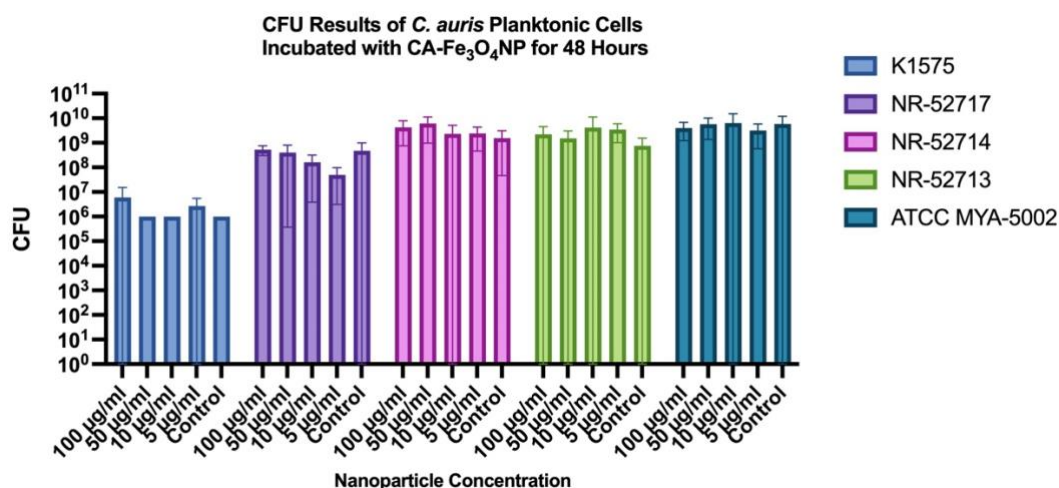


**Figure 3.35** Results of growth inhibition in terms of colony forming units (CFU) after 48-hour incubation of planktonic cells of *C. auris* isolates with CA-Fe<sub>3</sub>O<sub>4</sub>NP

As seen in Figure 3.35, CA-Fe<sub>3</sub>O<sub>4</sub>NPs did not cause statistically significant growth inhibition on any isolate. For this reason, the % inhibition evaluation of CFU results was not performed. When the K1575 isolate was evaluated on its own, no growth inhibition was observed even when treated with the highest dose of citric acid-coated iron nanoparticles.

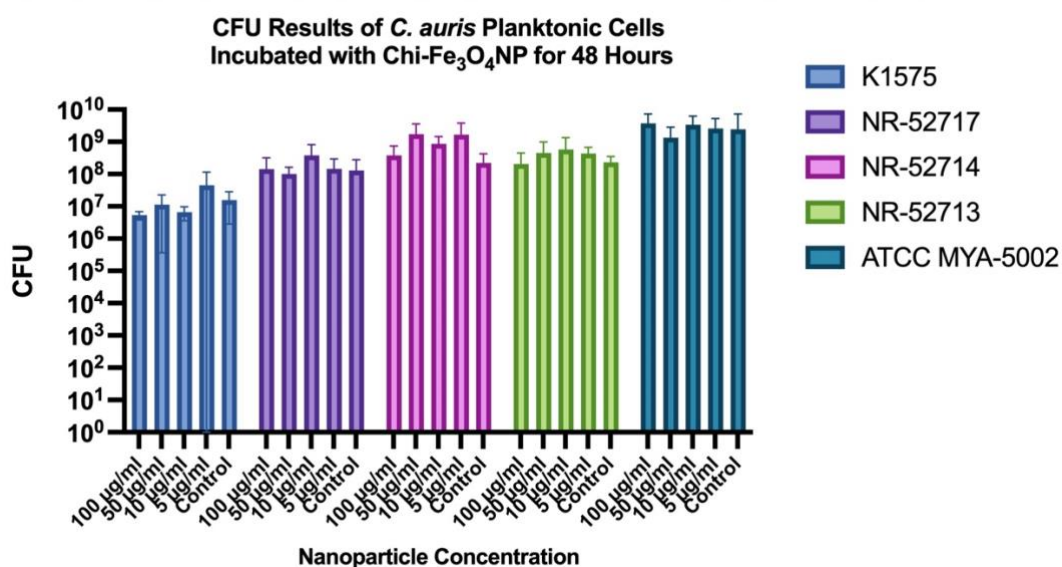


**Figure 3.36** Results of growth inhibition in terms of percentage inhibition (%) after 48-hour incubation of planktonic cells of *C. auris* isolates with Chi-Fe<sub>3</sub>O<sub>4</sub>NP

When the CFU graph of the Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles in Figure 3.36 is evaluated, it did not cause statistically significant growth inhibition in any of the isolates tested. For

this reason, the % inhibition evaluation of CFU results was not performed. When the K1575 isolate was evaluated on its own, no growth inhibition was observed even when treated with the highest dose of chitosan-coated iron nanoparticles.

### 3.4.2 Demonstration of the Effectiveness of Metal Nanoparticles on *Candida auris* Biofilm Cells

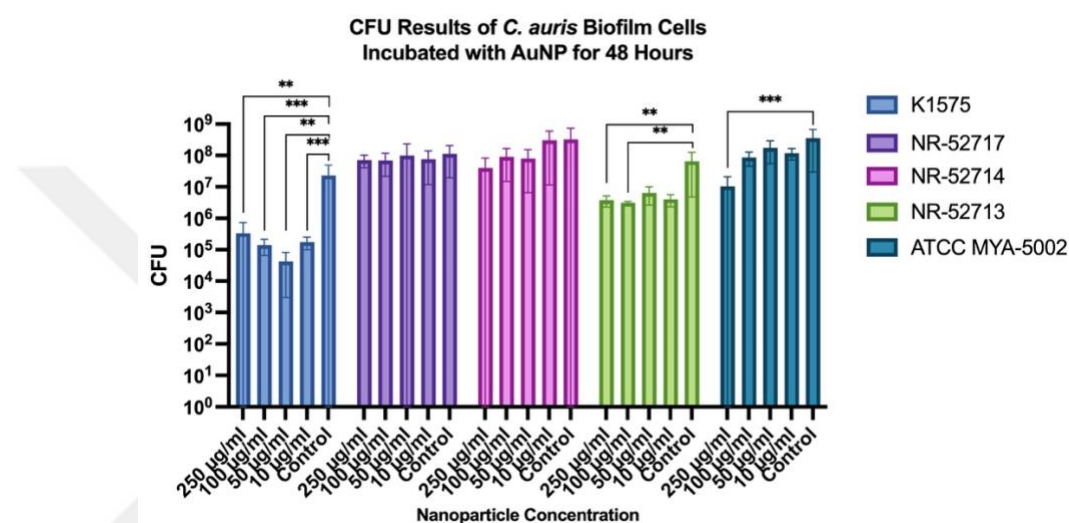


**Figure 3.37 Results of growth inhibition in terms of colony forming units (CFU) after 48-hour incubation of biofilm cells of *C. auris* isolates with AuNP**

According to CFU results, inhibitory effect was detected on the biofilm at all AuNP concentrations used in the study on clinical *C. auris* isolate K1575 compared to control biofilms not treated with AuNP (Figure 3.37). In K1575 biofilm cells, a statistically significant decrease was observed at all gold nanoparticle concentration compared to the growth control (p value = 0.0091, 0.0010, 0.0028 and 0.0004 respectively). No statistically significant growth inhibition was found in NR-52717 and NR-52714 reference isolates. Statistically significant growth inhibition was detected at 250 µg/ml and 100 µg/ml in NR-52713 isolate and at 250 µg/ml ATCC MYA-5002 (NR-52713 p=0.0073 and 0.0035, ATCC MYA-5002 p= 0.0002).

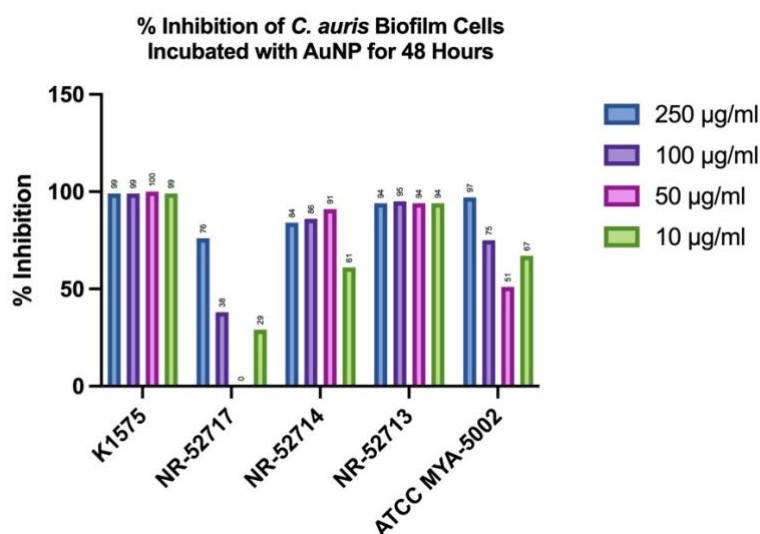


**Figure 3.38** Results of growth inhibition in terms of percent inhibition (%) after 48-hour incubation of biofilm cells of *C. auris* isolates with AuNP

The trend shown by all isolates in the % inhibition graph seen in Figure 3.38 has similar trends with the CFU result graphic. Biofilm formation of clinical isolates K1575 and NR-52713 caused growth inhibition at all concentrations incubated with AuNPs (for K1575 99-100%, for NR-52713 94-95%). Isolates NR-52717, NR-52714 and ATCC MYA-5002 show a dose-dependent inhibition trend.

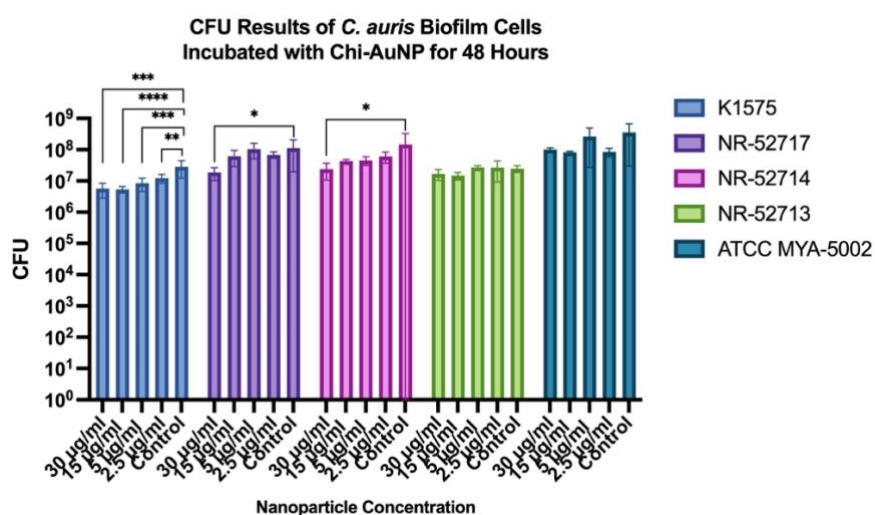


**Figure 3.39** Results of growth inhibition in terms of colony forming unit (CFU) after 48-hour incubation of biofilm cells of *C. auris* isolates with Chi-AuNP

As seen in Figure 3.39, while clinical isolate K1575 showed high and significant growth inhibition at increasing nanoparticle concentrations ( $p = 0.0001$ ,  $<0.0001$ ,  $0.0005$  and  $0.0049$  respectively), isolates NR-52717 and NR-52714 showed a decreasing trend

and this decrease was found to be statistically significant at 30  $\mu\text{g/ml}$  nanoparticle concentration (p values= 0.0135 and 0.0180, respectively). However, no statistically significant decrease was found in isolates NR-52713 and ATCC MYA-5002.

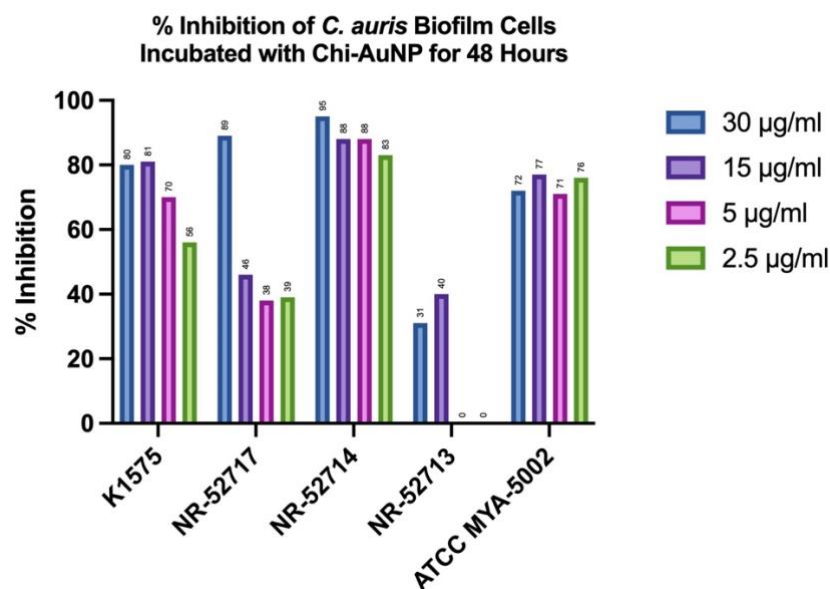


**Figure 3.40** Results of growth inhibition in terms of percent inhibition (%) after 48-hour incubation of biofilm cells of *C. auris* isolates with Chi-AuNP

In parallel with the CFU graphic in Figure 3.40, clinical isolate K1575 showed the same decreasing trend in the % inhibition graphic. A similar trend to the CFU graphic is observed in isolates NR-52717 and NR-52714 in the % inhibition graphic. While the inhibition of growth that was not seen in the CFU graph in the NR-52713 isolate was reflected in the same way, the ATCC MYA-5002 isolate showed a decrease in absorbance at 30  $\mu\text{g/ml}$  and 15  $\mu\text{g/ml}$  nanoparticle concentrations, this decrease in % inhibition shown by the ATCC MYA-5002 isolate was not reflected as a significant inhibition of growth in the CFU graphic.

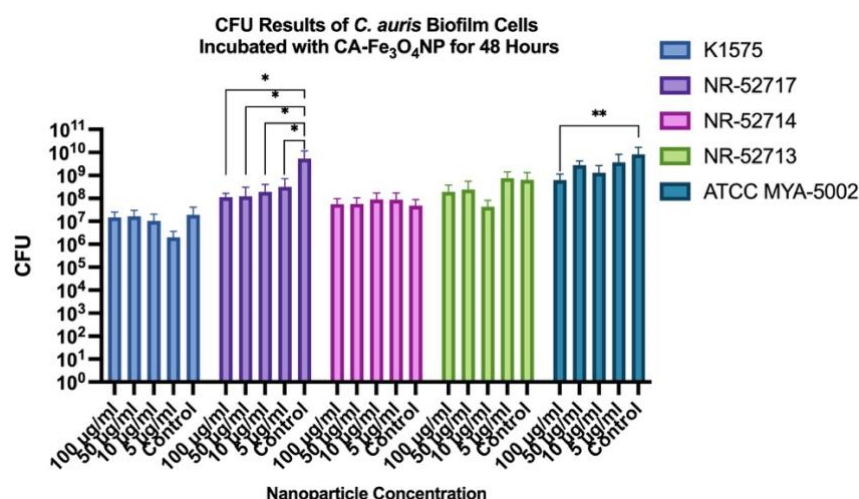


Figure 3.41 Results of growth inhibition in terms of colony forming unit (CFU) after 48-hour incubation of biofilm cells of *C. auris* isolates with CA-Fe<sub>3</sub>O<sub>4</sub>NP

As seen in the graphic in Figure 3.42, the activity was found to be statistically significant at all concentrations in NR-52717 isolate ( $p = 0.0256, 0.0371, 0.0212$  and  $0.0250$ ) and at  $100 \mu\text{g/ml}$  nanoparticle concentration in ATCC MYA-5002 isolate ( $p = 0.0038$ ). However, in multidrug-resistant K1575, NR-52714 and NR-52713 showed no activity against planktonic cells as well as biofilm cells. Therefore, the % inhibition graph was not plotted.

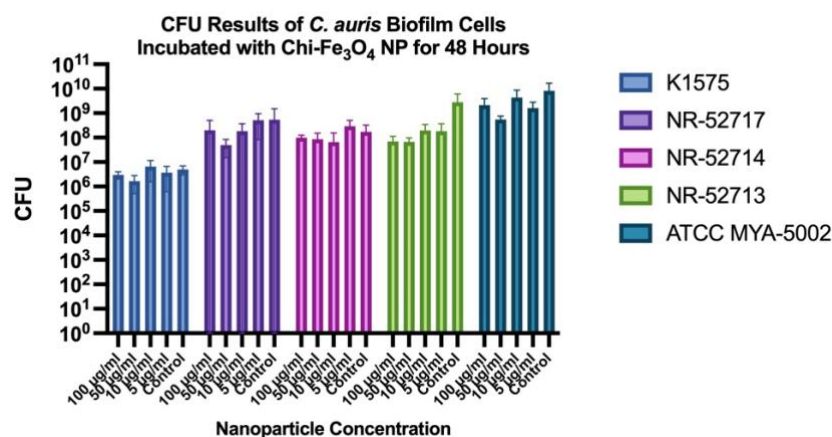


Figure 3.42 Results of growth inhibition in terms of colony forming unit (CFU) after 48-hour incubation of biofilm cells of *C. auris* isolates with Chi-Fe<sub>3</sub>O<sub>4</sub>NP

Figure 3.43 shows the CFU graph of Chi-Fe<sub>3</sub>O<sub>4</sub>NPs. As seen in the CFU graph, no significant growth inhibition was detected in K1575, NR-52717, NR-52714 and ATCC

MYA-5002 isolates. The growth inhibition seen in the graph in the NR-52-713 isolate is not statistically significant.

### 3.4.3 Demonstration of Reactive Oxygen Radicals Formed by Nanoparticles

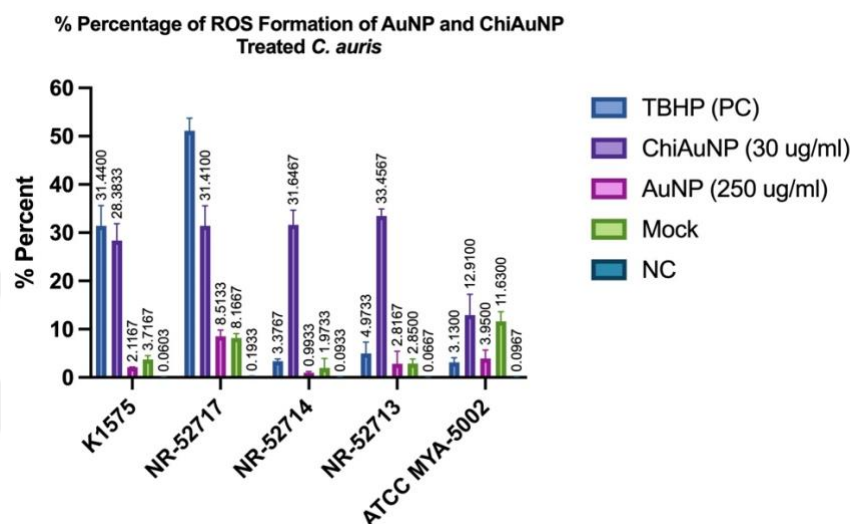
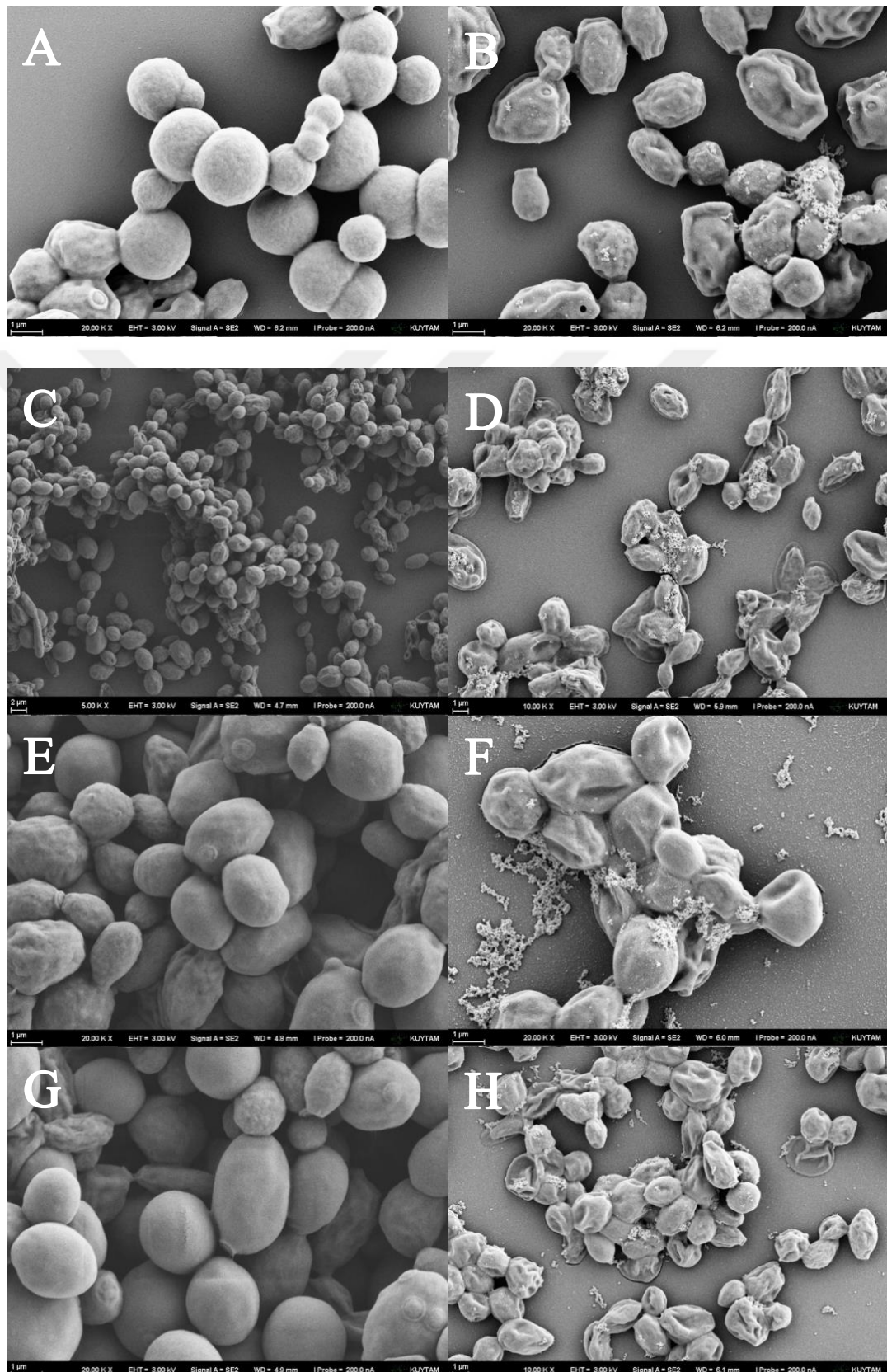


**Figure 3.43** Percentage values of reactive oxygen radical formation after treatment of *C. auris* isolates with AuNP and Chi-AuNP

Figure 3.47 shows % percent ROS formation of the isolates included in the study treated with 250 ug/ml AuNPs and 30 ug/ml Chi-AuNPs, the *C. auris* isolate that generated the highest amount of reactive oxygen radicals was seen in the Chi-AuNP treated isolates (K1575: 28.38%, NR-52717: 31.41%, NR-52714: 31.64% NR-52713: 33.45%, ATCC MYA-5002: 12.91%) compared to the controls after incubation for four hours. AuNP treated groups did not generate ROS compared mock to the group.

### 3.4.4 Demonstration of Morphological Changes on the Surfaces of *Candida auris* Cells Due to Antifungal Activity of Nanoparticles by SEM



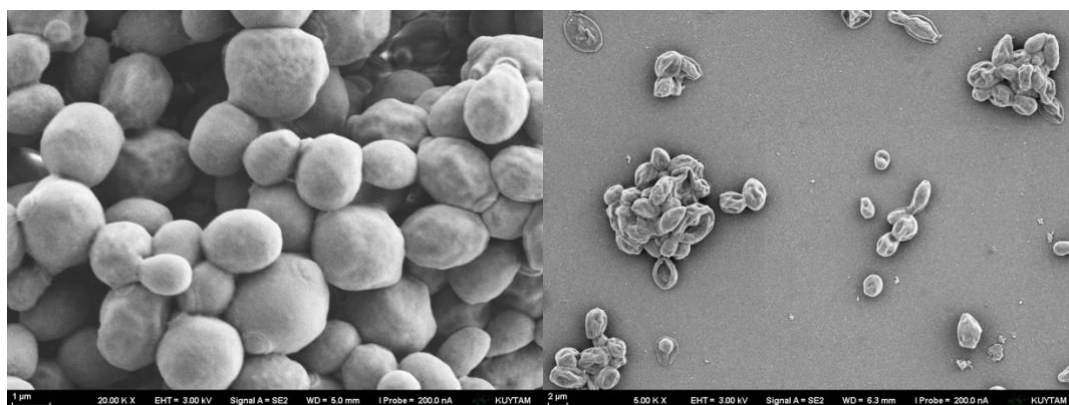
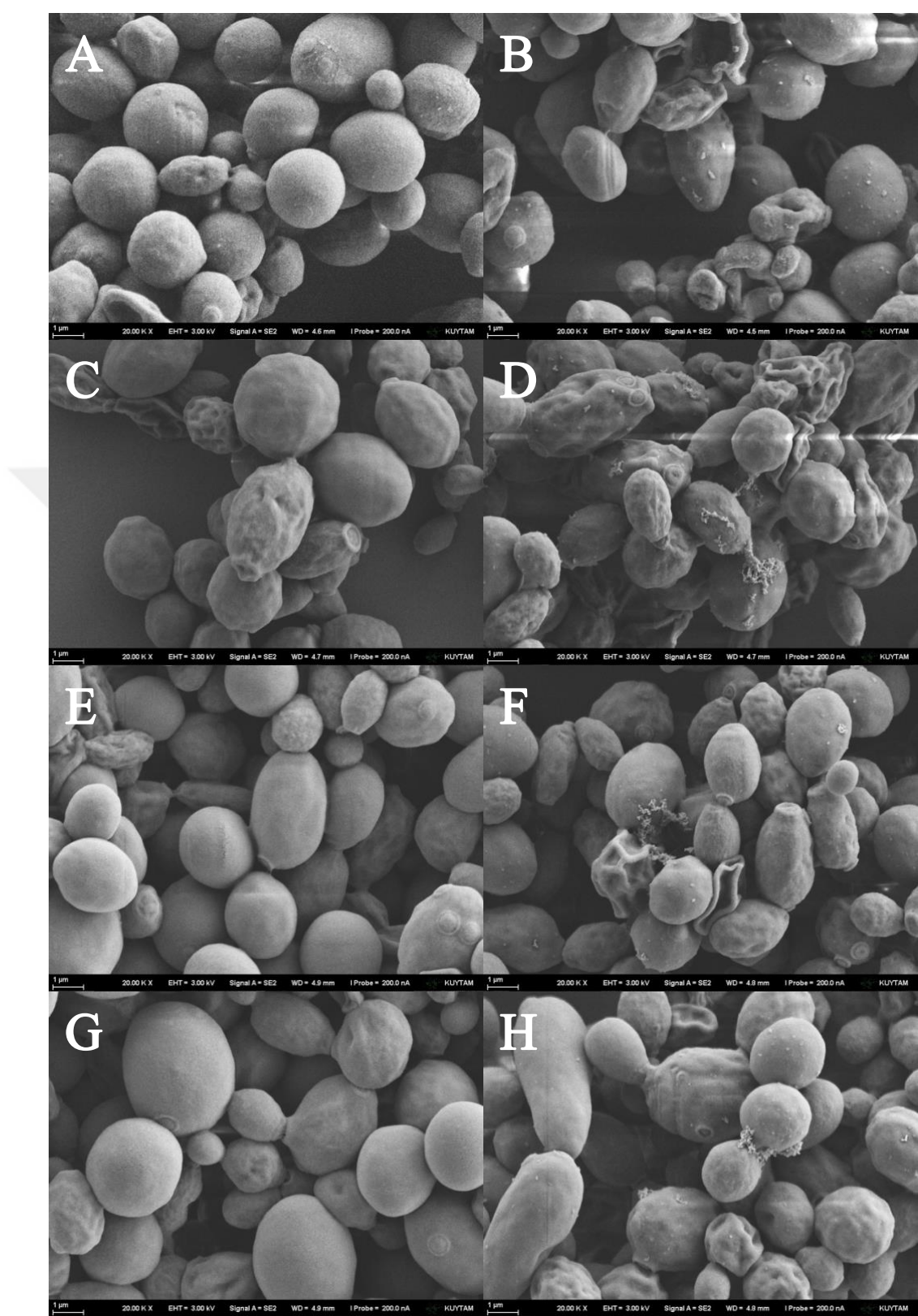


**Figure 3.44 SEM images of 250  $\mu\text{g/ml}$  AuNP treated *C. auris* isolates. A) Untreated K1575, B) Treated K1575, C) Untreated NR-52717, D) Treated NR-52717, E) Untreated NR-52714, F) Treated NR-52714, G) Untreated NR-52713, H) Treated NR-52713 I) Untreated ATCC MYA-5002 J) Treated ATCC MYA-5002**

Figure 3.48 shows 250  $\mu\text{g/ml}$  AuNP treated *C. auris* isolates' SEM images. Non-treated *C. auris* cells display healthy, typical yeast morphology with smooth surfaces and active budding. In contrast, the AuNP treated cells exhibit signs of damage, including roughened and irregular surfaces, cellular deformation, shrinkage, and lysis, indicating a potent antifungal effect of the nanoparticles. These observations align with established mechanisms of nanoparticle-mediated damage to fungal cells.



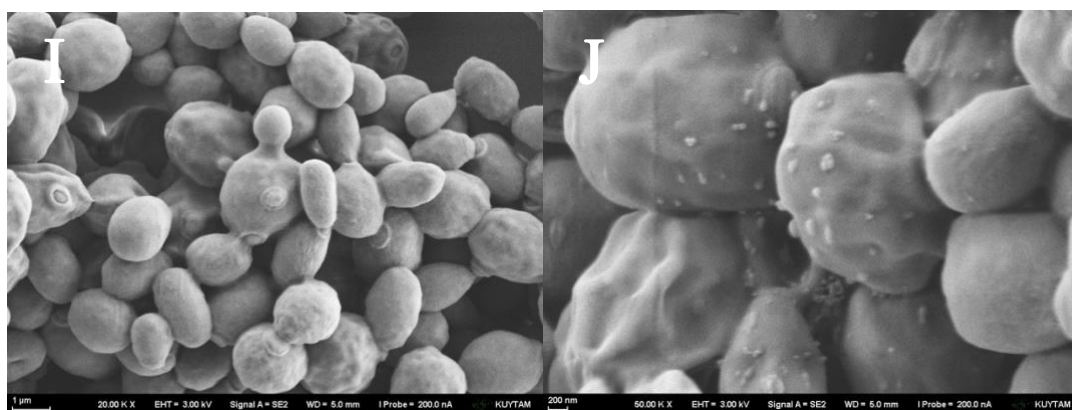


**Figure 3.45** SEM images of 30 µg/ml Chi-AuNP treated *C. auris* isolates. A) Untreated K1575, B) Treated K1575, C) Untreated NR-52717, D) Treated NR-52717, E) Untreated NR-52714, F) Treated NR-52714, G) Untreated NR-52713, H) Treated NR-52713 I) Untreated ATCC MYA-5002 J) Treated ATCC MYA-5002

Figure 3.49 shows 30 µg/ml Chi-AuNP treated *C. auris* isolates' SEM images. Like AuNP treated cells, the Chi-AuNP treated cells exhibit signs of damage, including roughened and irregular surfaces, cellular deformation, shrinkage, and lysis, indicating a potent antifungal effect of the nanoparticles.

#### 3.4.5 Demonstration of Antifungal Activity of Nanoparticles in *Candida auris* Cells by Confocal Microscopy

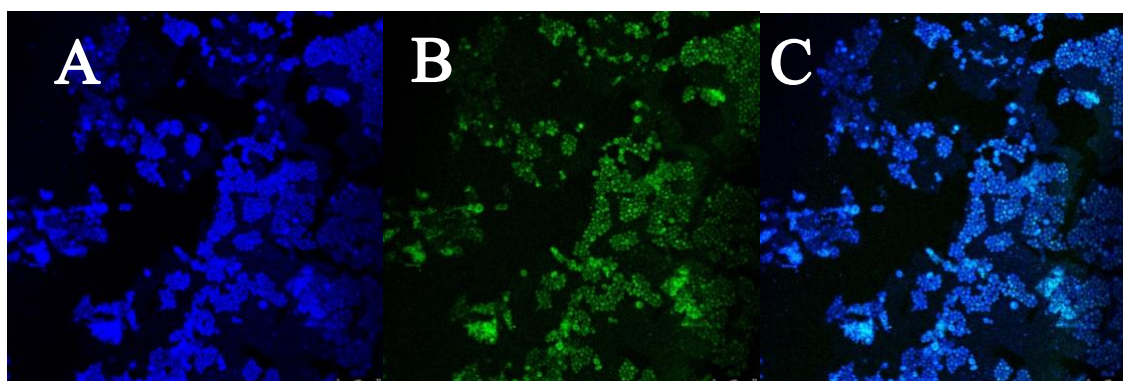


**Figure 3.46** Confocal microscopy images of K1575 treated with FITC tagged Chi-AuNPs (A) CFW stained K1575 biofilm (B) FITC tagged Chi-AuNP with biofilm cells (C) Merged confocal microscopy image of CFW stained K1575 biofilm and FITC tagged Chi-AuNPs

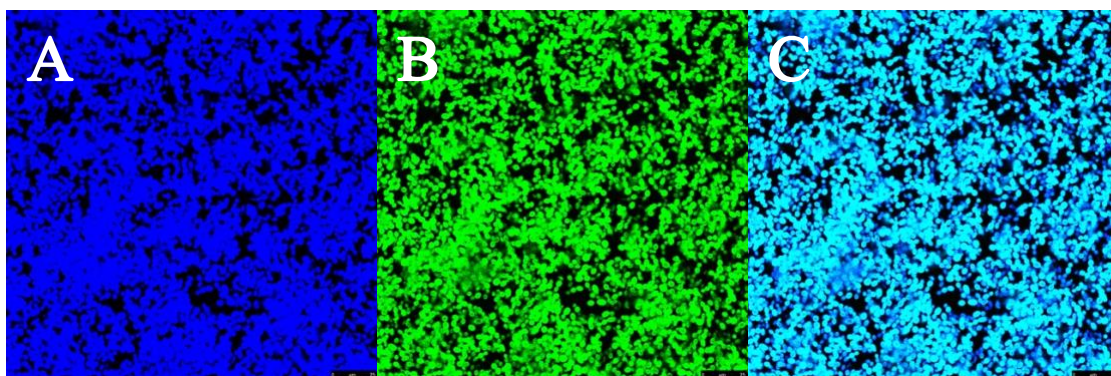


**Figure 3.47** Confocal microscopy images of NR-52717 treated with FITC tagged Chi-AuNPs (A) CFW stained NR-52717 biofilm (B) FITC tagged Chi-AuNP with biofilm cells (C) Merged confocal microscopy image of CFW stained NR-52717 biofilm and FITC tagged Chi-AuNPs

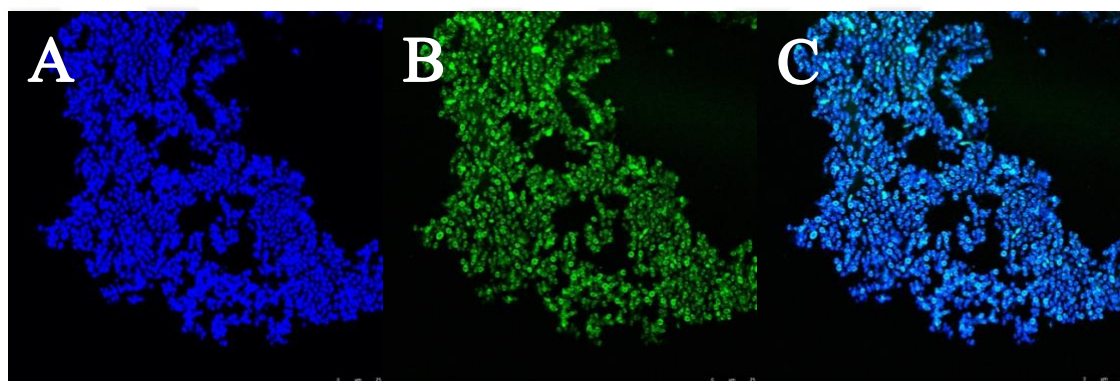


**Figure 3.48** Confocal microscopy images of NR-52714 treated with FITC tagged Chi-AuNPs (A) CFW stained NR-52714 biofilm (B) FITC tagged Chi-AuNP with biofilm cells (C) Merged confocal microscopy image of CFW stained NR-52714 biofilm and FITC tagged Chi-AuNPs

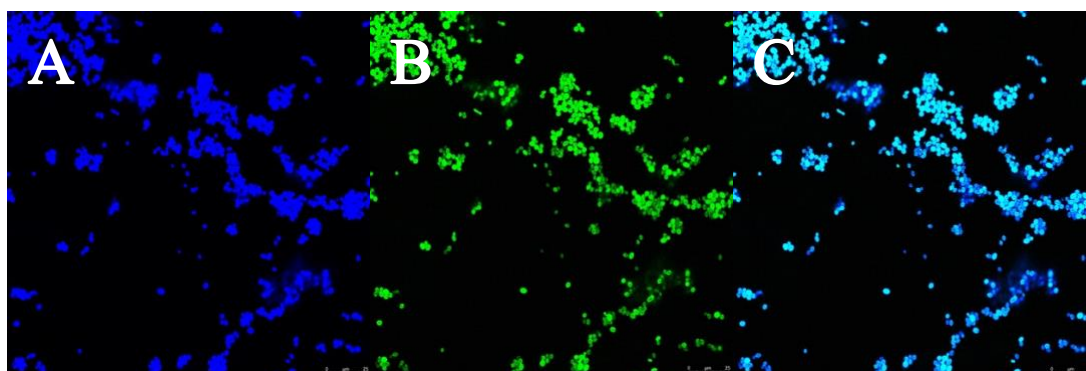


**Figure 3.49** Confocal microscopy images of NR-52713 treated with FITC tagged Chi-AuNPs (A) CFW stained NR-52713 biofilm (B) FITC tagged Chi-AuNP with biofilm cells (C) Merged confocal microscopy image of CFW stained NR-52713 biofilm and FITC tagged Chi-AuNPs

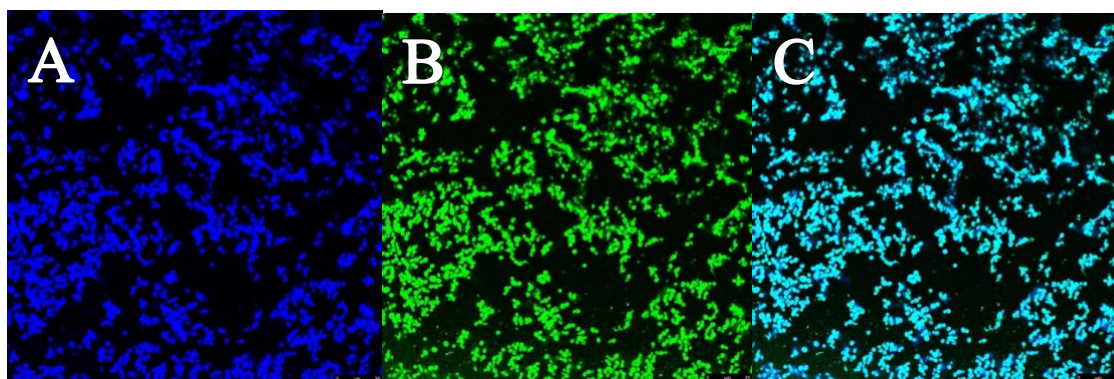


**Figure 3.50** Confocal microscopy images of ATCC MYA-5002 treated with FITC tagged Chi-AuNPs (A) CFW stained ATCC MYA-5002 biofilm (B) FITC tagged Chi-AuNP with biofilm cells (C) Merged confocal microscopy image of CFW stained ATCC MYA-5002 biofilm and FITC tagged Chi-AuNPs

As shown in Figure 3.50, 3.51, 3.52, 3.53, 3.54, oval-shaped fungal cells stained with Calcofluor White are visible. Second images of the isolates taken with confocal microscopy show that FITC-labeled nanoparticles have coated the biofilm layer and are successfully bound. The third image, which is the intersection of the two images, shows the light blue color of the fungal cells resulting from the combination of the green FITC fluorescence of the nanoparticles and the blue fluorescence of Calcofluor White, confirming this binding.

## Chapter 4:

### DISCUSSION

Candidemia is one of the serious bloodstream infections with 30-50% mortality rate and can disseminate to other organs (Kullberg & Arendrup, 2015; Pappas et al., 2018). *C. albicans* is the most common one among all other species (Huang et al., 2025) but non-albicans species like *C. glabrata*, *C. tropicalis*, and *C. auris* are increasingly reported showing high resistance to antifungal drugs (Bhattacharya et al., 2020; Husni et al., 2023; Papon et al., 2013). This epidemiologic change leads to growing health concern (Parslow & Thornton, 2022).

*Candida auris* was first identified from a patient's ear discharge in Japan 2009 (Satoh et al., 2009), but retrospective analyses suggest that it was present as early as 1996 but was misidentified because of its similarity to other *Candida* species (Lee et al., 2011;

Sekyere, 2018). Now *C. auris* incidents spread all around the world, reported over 50 countries with 60% mortality rate (Valdez et al., 2025; Du et al., 2020). *C. auris* has 6 different clades and some of them resistant to more than one class of antifungal drugs and some of them resistant to all three major drug classes. Because of its multidrug resistance and high mortality, CDC categorized *C. auris* as “urgent AMR threat” (CDC, 2023) and WHO listed as a “critical priority pathogen” (WHO, 2022; Kim et al., 2024).

*C. auris* has a haploid genome that includes ~12.3–12.5 Mb placed in 5-7 chromosomes with ~44.5% GC content (Bravo Ruiz et al., 2019; Du et al., 2020). It belongs to CTG clade, which translates serine instead of leucine and has 6 different clades (I to VI) with different mating types (MTLa and MTL $\alpha$ ) (Yang et al., 2024; Lockhart et al., 2017; Muñoz et al., 2018; Suphavitai et al., 2024). Chromosome number and size vary among clades and stress-induced genomic rearrangements contribute to drug resistance and virulence. *C. auris* has different phenotypic features that maintain its virulence properties, persistence on surfaces, and immune evasion. *C. auris* is thermo and osmotolerant, has high phenotypic and genomic plasticity, and form switching enables it to build drug-resistant biofilms (Osei Sekyere, 2018; Yue et al., 2018; Forgács et al., 2020). Growth at 30–42°C and high salinity conditions is associated with stress adaptation and climate-induced emergence.

*C. auris* has intrinsic multidrug resistance with different resistance pattern to multiple classes of antifungal drugs that makes the treatment challenging. According to the CDC, ~90% of isolates are resistant to azoles, ~30% to amphotericin B, and <5% to echinocandins (Chaabane et al., 2019).

ERG11 gene encodes lanosterol 14 $\alpha$ -demethylase which is an essential enzyme in ergosterol synthesis pathway. Azoles are targeting this enzyme but mutation on this gene reduces drug-binding efficacy. Y132F, K143R and F126L (VF125AL) mutations on ERG11 gene are linked to azole resistance (Chowdhary et al., 2018; Lockhart et al., 2017; Healey et al., 2018). Li et al. identified a new novel mutation F444L on ERG11 (Li et al., 2021). R157S, A298G, Q357K, I466L, L43H, G459S, I466M, Y501H and E468V mutations were also detected on ERG11 gene (Byun et al., 2023; Rybak et al., 2022). CDR1 gene encodes an ABC drug efflux pump transporter. This gene’s overexpression is the one of the hallmarks of multidrug resistance in *Candida* species. E709D, V704L and D709E (Hong et al., 2023; Jacobs et al., 2022; Reslan et al., 2022). Tac1b is a zinc cluster transcription factor that regulates CDR1 gene’s expression. F124S, F214L,

A640V, A657V, A15T, S192N, S195C, K247E, R495G, A583S, P595L/H, S611P, A651T, M653V, Q503E and F682\_N866del frameshift deletion (Rybak et al., 2022; Hong et al., 2023; Rybak et al., 2020). ERG2 gene is also involved in ergosterol synthesis pathway. In literature, the association of ERG2 gene mutations with amphotericin B resistance in *C. auris* has limited details. In two articles, E39D mutation was found on ERG2 gene (Oladele et al., 2022; Biswas et al., 2020). FKS1 gene encodes catalytic subunit of  $\beta$ -(1,3)-D-glucan synthase enzyme and echinocandin class antifungal drugs target this enzyme. Several studies showed different point mutations on this gene specifically on HS1 site of this enzyme. F635Y, F635L, F635C, F635 $\Delta$ , S639F, S639Y, S639T and D642Y were shown in literature (Sharma et al., 2022; Tian et al., 2024; Spruijtenburg et al., 2023; Alfouzan et al., 2020). FCY1 gene is encoding cytosine deaminase enzyme which has a critical role in the activation of the prodrug 5-flucytosine. S70R point mutation was defined in literature but this mutation does not impact 5-FC resistance, and its clinical significance remains unclear (Jacobs et al., 2022).

In this thesis study, we evaluated the MIC results of six different antifungal drugs by broth microdilution test to determine the drug resistance of our clinical isolate K1575. In the obtained results, we determined that the clinical isolate was resistant to fluconazole (64  $\mu$ g/ml) and anidulafungin (8  $\mu$ g/ml), but we did not find resistance to amphotericin B (1  $\mu$ g/ml). SNP/InDel analysis was performed to investigate the genomic reflection of the resistance pattern obtained from the broth microdilution test, and a phylogenetic tree was created using reference genomes belonging to five known clades to determine the clade to which the isolate belongs. As a result of whole genome sequencing, Y132F mutation was found in ERG11 gene, E709D mutation in CDR1 gene, L328S, A15T and S195C mutations in TAC1b gene, E39D mutation in ERG2 gene, F635 $\Delta$  mutation in HS1 region of FKS1 gene and S70R mutation in FYC1 gene. These mutations seen in ERG11, CDR1 and TAC1b genes explain azole resistance of the isolate. Similarly, mutation seen in FKS1 gene explains echinocandin resistance. Although we found a mutation in the ERG2 gene, which was also found in our clinical isolate and associated with polyene resistance in the literature, resistance of the isolate to amphotericin B could not be detected in the broth microdilution test. It has been shown that the S70R mutation found in the FYC1 gene in parallel with the literature does not contribute to 5-FC drug resistance. The fact that the *C. auris* isolates previously reported from Turkey predominantly belong to Clade I indicates that the prevalent clade in Turkey may be Clade I (Erköse Genç et al., 2024).

This clade, which is predominantly found in India and Pakistan, is included in the literature with its ability to cause hospital infections, invasive infections, MDR and XDR resistance patterns, and epidemics (Chow et al., 2020; Kim et al., 2024).

Using the XTT/Menadione assay, we determined the time point at which the isolates formed the best biofilm for future experiments. To determine the proper functioning of the experiment and the biofilm levels of the isolates, we used three *C. parapsilosis* isolates. Two had a high biofilm capacity (K802 and K875), and the other (K1102) had an intermediate biofilm capacity. All isolates generally showed low biofilm formation at 24 hours. The biofilm formation at 72 hours was selected as the appropriate time point for future experiments. When the two isolates with high biofilm capacity were compared with *C. auris* isolates, NR-52717 was recorded as the isolate with the highest biofilm formation. Similar to the high biofilm capacity *C. parapsilosis* isolates, this isolate showed a visible increase in biofilm formation at 48 hours and formed a mature biofilm at 72 hours. Isolates NR-52714, NR-52713, and ATCC MYA-5002 produced the same level of biofilm as the intermediate-biofilm-forming *C. parapsilosis* isolate. The biofilm formation in these isolates gradually increased until hour 72. Clinical isolate K1575 was classified as an intermediate-biofilm-capable isolate, producing biofilms close to intermediate-biofilm capacity. The clinical isolate formed a better biofilm at hour 72 compared to hours 24 and 48. In the literature, the results obtained from studies conducted with *C. auris* biofilm have noted that this species produces lower biofilm compared to *C. albicans* (Hernando-Ortiz et al., 2021).

For the characterization of nanoparticles, we took XRD, FT-IR, DLS, NTA, SEM and Zeta-Sizer measurements and compared their compatibility with the literature. After synthesis, we first tested whether the nanoparticles we synthesized had the desired size and density by checking the overall dimensions of the nanoparticles and the number of nanoparticles per milliliter with the NTA test. The mode size of the gold nanoparticles we synthesized with the Turkevich citrate-reduction method was 24.3 nm and the polydispersity index (PDI) was found to be 94.7 nm (SD 89.1 nm) on average. In the literature, the polydispersity index (PDI) of gold nanoparticles synthesized between 15-30 nm was recorded as 0.2 (Dong et al., 2020). Although it was dimensionally compatible with the literature, it showed moderate polydispersity. The average size and polydispersity index were recalculated with the DLS test. The NTA result was confirmed with the ~21.5 nm average size and 0.240 PDI found by the DLS test. Zeta-Potential of

AuNPs showed more than two peaks in the means the nanoparticles have more than two different surface charge distributions. It may be the result of polydispersity of nanoparticles. In the XRD analysis, we compared the locations of the atoms contained in the nanoparticle with the literature. The diffraction pattern shown by the gold nanoparticles and the peaks given in the graph are compatible with the literature (Fazaludeen et al., 2012). The XRD pattern of the gold nanoparticle published in the ICDD (International Center for Diffraction Data) database is compatible with face-centered cubic (FCC) gold (JCPDS file: 04-0784) (Ren et al., 2015). In FTIR analysis, we investigated the absorption bands given by specific chemical bonds to gold nanoparticles. We compared the specific bond pattern of nanoparticles reduced by citrate stabilization with literature. In line with literature, we reported characteristic carboxylate bands in the regions of  $1580\text{--}1610\text{ cm}^{-1}$  (asymmetric) and  $1390\text{--}1400\text{ cm}^{-1}$  (symmetric), confirming the adsorption of citrate as a stabilizing agent (Mohan et al., 2013). O-H stretching band is also commonly observed and  $\text{CO}_2$  artifact is a known experimental difficulty in FTIR (Smith, 2011). In SEM analysis of gold nanoparticles, big salt crystals were observed mainly. But these crystals are not AuNP, but they may be citric acid crystals for capping agent. These crystals may be formed while sample drying for SEM microscopy. Little star-like dots are AuNPs, but they are too small to observe them clearly. In literature, AuNPs defined as spherical shapes, if it is synthesized via Turkevich method (Dong et al., 2020).

In the NTA analysis, the mode size of the gold nanoparticles we synthesized with the chitosan-reduction method was 60.6 nm and the polydispersity index (PDI) was found to be 108.4 nm (SD 59.3 nm) on average. In the literature, the polydispersity index (PDI) of Chi-Au nanoparticles synthesized between approximately 30.6 nm was recorded as 0.2 (Mohan et al., 2019). The larger size compared to the literature indicates that the nanoparticles form small aggregates or larger pieces, but it differs between different synthesis methods. The average size and polydispersity index were recalculated with the DLS test with the  $\sim 122.2$  nm average size and 0.7 PDI. Zeta-Potential of Chi-AuNPs showed two peak means the nanoparticles have two different surface charge distribution. The first peak shows that the major population of nanoparticles have +21.4 mV zeta potential with moderate colloidal stability, second peak shows that smaller population of nanoparticles have +41.6 mV zeta potential with more stability. These results are parallel with literature (Benamer Oudih et al., 2023). In the XRD analysis, we compared the

locations of the atoms contained in the chitosan-capped gold nanoparticles with the literature. The diffraction pattern shown by the chitosan-capped gold nanoparticles and the peaks given in the graph are compatible with the literature. The XRD pattern of the chitosan-capped gold nanoparticles centered gold structure has same pattern published in the ICDD (International Center for Diffraction Data) database is compatible with face-centered cubic (FCC) gold with definitive peaks for FCC gold at approximately  $38^\circ$ ,  $44^\circ$ ,  $64^\circ$ , and  $77^\circ$  peaks (JCPDS file: 04-0784) (Ren et al., 2015). Except for 200 diffraction planes, our nanoparticle's XRD analysis is the same with literature (Saravanakumar et al., 2020). As a result, the XRD pattern of chitosan-capped nanoparticles presents distinct pixels, which confirms the regular crystal structure of gold nanoparticles and the amorphous effect of chitosan coating. These findings indicate that chitosan-capped nanoparticles were successfully synthesized, and chitosan coating affects the surface properties of nanoparticles. In FTIR analysis, we compared the specific bond pattern of nanoparticles reduced by chitosan stabilization with literature. In line with literature, The FTIR spectrum unambiguously confirms the successful capping of gold nanoparticles with chitosan. The characteristic absorption bands of chitosan, especially the broad O-H/N-H stretching band at  $\sim 3200\text{ cm}^{-1}$ , amide I band at  $\sim 1650\text{ cm}^{-1}$ , C-H bending band at  $\sim 1400\text{ cm}^{-1}$ , and C-O stretching bands between  $1000\text{-}1200\text{ cm}^{-1}$ , are clearly visible. This spectroscopic evidence strongly supports the role of chitosan as both a reducing and stabilizing agent in the synthesis of gold nanoparticles and agrees well with the findings reported in literature. In conclusion, the FTIR spectrum provides important information for the stabilization and functionalization of Chi-AuNPs and clearly reveals the interactions of this structure with the surface of gold nanoparticles. In NTA and DLS analysis, it was confirmed that Chi-AuNPs forming aggregates formed aggregates in SEM images. Nanoparticle sizes of approximately 12-18 nm confirmed that nanoparticles formed by chitosan with gold were similar to nanoparticles synthesized around 15.51 nm in the literature (Saravanakumar et al., 2020). In the same article, it was stated that chitosan caused polydispersity in synthesized nanoparticles. In the SEM analysis of Chi-Au nanoparticles, it was observed that nanoparticles have uniform size with aggregation. Spherical shape of these nanoparticles common for gold nanoparticles, means synthesizing process successfully completed. The size of nanoparticles are falling to the typical size range, but aggregation can affect its property, surface area and biological interactions. The reason why these aggregates formed can be high particle concentration,

surface charge, drying effect during sample preparation for SEM or the nature of chitosan itself.

In the DLS analysis, the mode size of the CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles we synthesized with the citric acid-reduction method was 55.1 nm and the polydispersity index (PDI) was found to be 0.314 means moderately polydisperse nanoparticle solutions. This nanoparticle showed two different peaks that means population nanoparticles were separated into two different nanoparticle size. Primary peak was found at 88.41 nm and secondary peak was found at ~323.6 nm. Secondary peak indicates aggregation or agglomeration in the sample. Citric acid provides thermodynamically stable colloidal nanoparticle solutions but, in our sample, citric acid could not completely prevent some aggregation (Mikelashvili et al., 2023). In the literature, synthesized CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles' size smaller than 20 nm which is much smaller than our result size (Laurent et al., 2008; Li et al., 2013). Two other articles showed -25.94 mV and -29.4 mV zeta potential result respectively but ours was +51.9 mV with uniform surface charge distribution (Mikelashvili et al., 2023; Mohammed et al., 2024). This difference is coming from synthesizing difference. In literature, CA-Fe<sub>3</sub>O<sub>4</sub> was synthesized neutral or slightly alkaline pH results deprotonation of carboxylic acid groups and negative zeta potential (Mikelashvili et al., 2023; Laurent et al., 2008). In the XRD analysis, we compared the locations of the atoms contained in the CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles with the literature. The diffraction pattern shown by the CA-Fe<sub>3</sub>O<sub>4</sub> and the peaks given in the graph are compatible with the literature except two peaks. The XRD pattern of the CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles is same with literature except 440 and 511 diffraction peaks (JCPDS file no. 19-0629) (Saravanakumar et al., 2020). CA-Fe<sub>3</sub>O nanoparticles present distinct (220), (311), (400), and (422) crystal planes of CA-Fe<sub>3</sub>O<sub>4</sub>. (220) and (311) planes of Fe<sub>3</sub>O<sub>4</sub> and indicate that citric acid changes the crystal structure of Fe<sub>3</sub>O<sub>4</sub>. The sharpness and intensity of the peaks imply that citric acid does not destroy the crystalline structure but only weakens the densities by interactions on the surface and causes broadening. This broadening indicates that citric acid tends to form amorphous structures, and amorphous regions are formed on the surface of Fe<sub>3</sub>O<sub>4</sub>. These findings indicate that CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles were successfully synthesized, and citric acid coating has little effect on the surface properties of nanoparticles. In FTIR analysis, we compared the specific bond pattern of nanoparticles reduced by citric acid with literature. Peaks at 580–650 cm<sup>-1</sup>

indicate the Fe–O, strong indicator of the formation of crystalline Fe<sub>3</sub>O<sub>4</sub> core structure (Li et al., 2013). The peak near 1720 cm<sup>-1</sup> indicates C=O groups of citric acid while ~1600 cm<sup>-1</sup> and 1400–1450 cm<sup>-1</sup> bands indicate asymmetric and symmetric COO<sup>-</sup> stretching, evidencing surface bonding (Li et al., 2013). The 3200–3500 cm<sup>-1</sup> broad band represents the O–H stretching vibrations from the hydrogen bond interactions and the absorbed water molecules. The weak 1100–1200 cm<sup>-1</sup> signals is associated with the C–O stretching in citric acid means that this nanoparticle capped with citric acid (Al Shiaa et al., 2016). The SEM analysis of CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles showed a layer formed as a result of the binding of citric acid molecules to the nanoparticle surface by electrostatic or covalent interactions (El-Khawaga et al., 2020). The decrease in the clear boundaries between the particles and the irregular contours on the surface support the fact that citric acid forms a coating on the surface. These observations are consistent with the assumption that the coating increases colloidal stability by surrounding the particle surface.

In the DLS analysis, the mode size of the Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles we synthesized with the chitosan-reduction method was 207.2 nm and the polydispersity index (PDI) was found to be 0.181 means monodisperse nanoparticle solutions. Chitosan provided thermodynamically stable colloidal nanoparticle solutions. In the literature, synthesized Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles' size ranged between 10-80 nm which is much smaller than our result size (Laurent et al., 2008). But in some articles showed that size of Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles can be lower than 10 nm (Abdollahi et al., 2015; Sun et al., 2019; Zeinali et al., 2016). The Zeta Potential Distribution graph indicates that the nanoparticles have two different surface charge distribution. Majority (67.9%) have mean charge of -31.8 mV, indicating more stability. Smaller fraction (32.1%) has a mean charge of -10.1 mV, indicating low stability. In literature, Chi-Fe<sub>3</sub>O<sub>4</sub> was synthesized neutral or slightly alkaline pH negative zeta potential and acidic media results positive zeta potential, same as gold nanoparticles (Abdollahi et al., 2015). In the XRD analysis, we compared the locations of the atoms contained in the Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles with the literature. The diffraction pattern shown by the Chi-Fe<sub>3</sub>O<sub>4</sub> and the peaks given in the graph are compatible with the literature. Chi-Fe<sub>3</sub>O nanoparticles present distinct (220), (311), (400), (511), and (422) crystal planes. (311) plane and (400) plane confirm magnetite phase of Fe<sub>3</sub>O<sub>4</sub> and indicate that chitosan changes the crystal structure of Fe<sub>3</sub>O<sub>4</sub> (Dung et al., 2009). This also indicates that the chitosan coating disrupted the crystalline structure of Fe<sub>3</sub>O<sub>4</sub>.

and formed amorphous structures. Chitosan coating caused major changes in the crystalline structure of  $\text{Fe}_3\text{O}_4$  nanoparticles, causing the peaks to spread and their intensity values to decrease. The amorphous structure of chitosan leads to a more pronounced peak broadening in XRD because chitosan is generally an organic polymer and disrupts the crystalline structure and forms amorphous regions. The FT-IR spectrum of Chi- $\text{Fe}_3\text{O}_4$  nanoparticles showed characteristic peaks at 650, 1022, 1070, 1680, and 3720  $\text{cm}^{-1}$ . The 650  $\text{cm}^{-1}$  peak corresponds to the Fe–O bonds and confirms  $\text{Fe}_3\text{O}_4$  core (around 570–585  $\text{cm}^{-1}$ ) (Abdollahi et al., 2015). The peaks at 1022 and 1070  $\text{cm}^{-1}$  indicate C–O–C and C–O stretching in the chitosan structure and indicate that chitosan coating is present on the nanoparticle surface (Garcia et al., 2024; Abdollahi et al., 2015). The broad at 1680  $\text{cm}^{-1}$  indicates the C=O stretching and hydrogen bonds between the  $\text{Fe}_3\text{O}_4$  surface (Sun et al., 2019). The broad and wavy band observed in the 3720  $\text{cm}^{-1}$  region corresponds to the stretching vibrations of the free O–H (hydroxyl) and N–H (amine) groups on the surface of chitosan (Hasan et al., 2019; Abdollahi et al., 2015). The broad spectral structure in this region is explained by the formation of hydrogen bonds and shows that the chitosan coating maintains its polymeric nature. In SEM analysis of Chi- $\text{Fe}_3\text{O}_4$ , it was observed that the distance between particles increased and clustering decreased in chitosan coated particles. These findings show that chitosan increased the electrostatic repulsive forces between particles by changing the surface interactions and reduced the aggregation tendency. The environmental layers observed in the SEM images for both coating types show that the surface modifications were successfully achieved, and the morphology of the particles changed with these coatings. These morphological changes are an important indicator that the coating materials are attached to the nanoparticle surface and that the surface modification can be confirmed at the microscopic level.

After characterization, we tested our nanoparticles' cytotoxicity on NIH 3T3 cell line. The highest dose that cell line showed viability at 24 hours for AuNPs was determined as 250  $\mu\text{g}/\text{ml}$  and for Chi-AuNPs as 60  $\mu\text{g}/\text{ml}$  and their IC50 values was found 183  $\mu\text{g}/\text{ml}$  for AuNPs, 22  $\mu\text{g}/\text{ml}$  for Chi-AuNPs, 44.74  $\mu\text{g}/\text{ml}$  for CA- $\text{Fe}_3\text{O}_4$  and, 50.72  $\mu\text{g}/\text{ml}$  for Chi- $\text{Fe}_3\text{O}_4$ . In literature, cytotoxicity value of AuNPs was found 60  $\mu\text{g}/\text{ml}$  on CEF cells, cytotoxicity value (approximately 60% inhibition) of Chi-AuNPs was found 125  $\mu\text{g}/\text{ml}$ , CA- $\text{Fe}_3\text{O}_4$  and Chi- $\text{Fe}_3\text{O}_4$  nanoparticles have no cytotoxicity value (Ambwani

et al., 2016; Lorenzo-Anota et al., 2021; Alili et al., 2015; Lotfi et al., 2019; Luo et al., 2017).

In literature different types of nanoparticles have gained attention due to their antifungal effects and some of them tested on *C. auris* isolates to combat with its multidrug resistance. The studies reported the antifungal activity of silver nanoparticles showed that these nanoparticles can inhibit more than 80%, both planktonic and biofilm forms of *C. auris* with 6.25 µg/ml (AlJindan and AlEraky, 2022). In other study Fayed et al. used zinc oxide nanoparticles against *C. auris* planktonic and biofilm cells. They found that these nanoparticles had strong antifungal effect against *C. auris* planktonic cells (151 µg/ml) and biofilm cells (51.6%) (Fayed et al., 2025b). Vazquez-Munoz et al. showed anticandidal effect of bismuth nanoparticles with 1-4 µg/ml MIC values (Vazquez-Munoz et al., 2020b). Ag-Cu-Co trimetallic nanoparticles were also used against *C. auris*, showed potent antifungal activity and induced apoptosis and cell cycle arrest with MIC values ranging from 0.39 to 0.78 µg/ml and Minimum Fungicidal Concentration (MFC) was found ranging from 0.78 to 1.56 µg/mL (Kamli et al., 2021). Metallic nanoparticles can also be combined with antifungal drugs to enhance their efficacy. Fayed et al., synthesized caspofungin-loaded zinc oxide nanoparticles, with  $10.34 \pm 0.7$  ng/ml MIC value (Fayed et al., 2021). Polymeric nanoparticles functionalized with metallic nanoparticle, liposomal nanoparticles, drug repurposing, photodynamic therapies and antimicrobial peptide-based therapies also have been used to overcome with this resistance (Alanís-Ríos et al., 2025; de Alteriis et al., 2021; Silva et al., 2023; Eldesousky et al., 2020; Ganeshkumar et al., 2024). Since there is no study in the literature showing the antifungal activity of Au and Fe<sub>3</sub>O<sub>4</sub> nanoparticles against *C. auris* planktonic and biofilm cells, we measured the activity of these nanoparticles against multidrug-resistant *C. auris* isolates by percentage inhibition calculated with CFU assay.

After that we evaluated effect of our nanoparticles on five different isolates. This thesis project is the first in literature that examines gold nanoparticles with *C. auris* isolates. First of all, we started with AuNP on planktonic cells of *C. auris*. Our clinical isolate K1575 showed near-complete growth inhibition (99-100%) and a statistically significant reduction in CFU across all tested concentration (10-250 µg/ml). This high vulnerability suggests that K1575 is particularly susceptible to fungicidal or fungistatic effects of these nanoparticles. Then we evaluate effect of AuNPs on biofilm cells of *C. auris*. Clinical isolate K1575 exceptional sensitivity of AuNPs with near-complete

biofilm inhibition (99-100%) across all tested concentrations, same as planktonic cells' response. This profile suggests that while high concentrations of gold nanoparticles are effective against this isolate's biofilm. In the literature, AuNPs were used against different types of microorganisms, including bacteria. These chemically inert nanoparticles have in-vitro and in-vivo biocompatibility, and their antimicrobial activity can be enhanced by surface modifications (Balderrama-González et al., 2021). The antibacterial and antifungal activity of AuNPs depends on the surface modifications, shape, and concentration of the nanoparticle. In a study published by Zheng et al., AuNPs <2 nm in size were tested against both Gram-positive and Gram-negative bacteria, and high antibacterial activity was recorded (Zheng et al., 2017). In another study, they were used to test the antibacterial activity against *E. coli* and *S. aureus*, and the antifungal activity against *C. albicans*. As a result of this study, it was recorded that gold nanoparticles exhibited high antibacterial activity against both *E. coli* and *S. aureus* and were active against *C. albicans* at concentrations of 20 and 40 µg/ml (López-Lorente et al., 2019). In another study, it was shown that as the size of the synthesized gold nanoparticles decreased, their antifungal activity increased on *C. albicans*, *C. tropicalis*, and *C. glabrata* (Ahmad et al., 2013). In a study conducted by Wani and Ahmad in 2013 with gold nanoparticles, the average MIC<sub>80</sub> value of synthesized 25 nm nanoparticles on different *Candida spp.* were in the range of 16-32 µg/ml (Wani and Ahmad, 2013). When we compare our results with previous studies, the effect we see, including the lowest concentration, is at the same level as literature.

After examining AuNPs against *C. auris* cells, we used chitosan to enhance its antifungal effect against planktonic and biofilm cells separately. Clinical isolate K1575 demonstrates high sensitivity to this nanoparticle near-complete (99-100%) inhibition of planktonic growth and highly statistically significant reduction in CFU across all tested concentrations. We tested Chi-AuNPs on biofilm cells of our isolates. Clinical isolate K1575 demonstrated high susceptibility against all tested concentrations. It also showed significant percentage inhibition (80-66%). With AuNP results, Chi-AuNPs are strongly effective against K1575 biofilm. In a study conducted with Chi-AuNPs in the literature, both antibacterial and antifungal activities of these nanoparticles were tested (Hashem et al., 2022). Of the bacteria studied, *P. aeruginosa* showed MIC values at concentrations of 1.56 µg/ml, *B. subtilis* 6.25 µg/ml, *S. aureus* 6.25 µg/ml, and *K. oxytoca* 3.12 µg/ml, and this value was much higher than the effects of gold and chitosan when applied

separately. In the same study, in the antifungal activity test of the nanoparticle, *C. albicans* showed MIC values of 62.5 µg/ml, *A. terreus* 250 µg/ml, *A. niger* 125 µg/ml, and *A. fumigatus* 125 µg/ml. In another study, the antifungal activity of this nanoparticle was found to be 3 mg/ml for *C. albicans*, *A. niger*, and *F. solani* (Ing et al., 2012). Comparing our study with the two studies, our results indicate that lower concentrations successfully inhibited the MDR *C. auris* isolate and demonstrated fungicidal activity.

Next step, we tested CA-Fe<sub>3</sub>O<sub>4</sub> against our isolates' planktonic cells and we did not find any significant reduction in terms of CFU results. Then we tested this nanoparticle against biofilm cells, K1575, NR-52714 and NR-52713 isolates showed no significant inhibition and CFU reduction against CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles, but NR-52717 isolate showed statistically significant reduction at every nanoparticle concentration. ATCC MYA-5002 showed statistically significant CFU reduction at the highest concentration of CA-Fe<sub>3</sub>O<sub>4</sub>. These results suggest that this nanoparticle has no effect on this isolate except NR-52717 and ATCC MYA-5002 isolates. This nanoparticle has fungistatic effect against these isolates. In a study conducted in the literature using this nanoparticle, *E. coli* and *B. subtilis* showed the best results with nanoparticles synthesized at a concentration of 20 µg/ml and with 0.5 and 0.3 M citric acid concentrations, respectively (Khan et al., 2020). Since there are no other studies on fungi comparable to ours in literature, more detailed studies are needed to determine the effectiveness of this nanoparticle. The nanoparticle was observed to be effective against isolates NR-52717 and ATCC MYA-5002, which have high biofilm capacity. The lower biofilm capacity of the other isolates compared to these two isolates suggests that the nanoparticle interacts with the biofilm and may be more effective against isolates with high biofilm capacity.

And then we tested Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles against planktonic and biofilm cells. This nanoparticle did not show any statistically significant CFU reduction and inhibition on both planktonic and biofilm cells. *Candida* cell wall and our nanoparticle includes negative charge, and they may repel each other. This situation may prevent the nanoparticles act on both planktonic and biofilm cells. For this reason, we decided to continue our experiments with AuNPs and Chi-AuNPs. According to previous studies, the antifungal effect of this nanoparticle was evaluated, and it was found to be that the overall nanoparticle showed antifungal activity against *C. glabrata* and *C. tropicalis* (61 µg/ml), but the same effect was obtained at much higher doses (59-475 µg/ml) for *C. parapsilosis*, *C. krusei* and *C. lusitaniae* (Abdulrassol et al., 2022). When the antibacterial

and antifungal activity of the chitosan capped iron oxide nanoparticles was evaluated according to the inhibition diameter types, inhibition varied as *F. solani*/*A. niger* (15 mm) < *C. albicans*/*E. coli* (16 mm) < *B. subtilis* (18 mm) (Nehra et al., 2017). In the same article, chitosan-coated iron oxide nanoparticles were shown to be more effective than bare Fe<sub>3</sub>O<sub>4</sub> nanoparticles. In another article, MIC values of *C. albicans* 1.25 µg/ml, *B. subtilis* 0.625 µg/ml, *S. aureus* 2.5 µg/ml, *P. aeruginosa* 2.5 µg/ml and *E. coli* 0.625 µg/ml were recorded and, in this article, as in the previous one, chitosan cap reduced the MIC values compared to bare Fe<sub>3</sub>O<sub>4</sub> nanoparticles (El-Khawaga et al., 2021). When comparing our findings with the efficacy reported in the literature, the same effect was not observed with the nanoparticles we synthesized. The lack of antifungal activity is believed to be due to the synthesis protocol of the nanoparticles. It is thought to be due to the aggregation of the nanoparticles, as seen in the SEM images.

For ROS assay, we used AuNPs and Chi-AuNPs with their highest concentrations (250 and 30 µg/ml respectively). When we compared effect of nanoparticles with positive control, Chi-AuNPs consistently induced significant ROS formation against all our strains, often comparable to or slightly lower than positive control. These results suggest that Chi-AuNPs can effectively induce oxidative stress in *C. auris*. But AuNPs induces very low level of ROS formation between different *C. auris* isolates. These results show that AuNPs cannot significantly contribute ROS formation in *C. auris*. Clinical isolate also showed high ROS induction compared TBHP. Multiple mechanisms of action for nanoparticles are described in the literature. The most important mechanism of action is their ability to generate ROS, which contributes to their antimicrobial properties by causing oxidative stress, disrupting membrane integrity, and leaking intracellular materials (Saed et al., 2024). This property has been shown in the literature to generate ROS for both gold and chitosan-coated gold nanoparticles (Saed et al., 2024; Hashem et al., 2022). In our ROS assay, chitosan-coated gold nanoparticles were observed to kill isolates by generating ROS, while gold nanoparticles did not. The inhibition of isolates may also be due to their ability to adhere to the fungal cell walls and outer cell membrane without generating ROS, damaging them and causing cellular contents to leak.

We performed SEM analysis to visualize effect of nanoparticles on *C. auris* biofilm cells. All isolates' biofilm cells are treated with the highest concentration, like ROS assay. Untreated biofilms images demonstrate characteristics of yeast growth and filamentation without any damage. *C. auris* cells show more uniformly spherical to oval cells forming

distinct pseudohyphal chains, suggest active growth and a specific adaptive form. But all treated isolates show small clusters of oval/elongated yeast cells. Presence of nanoparticles cause morphological changes, irregular shapes, aggregation and stress. When we compared these results with ROS assay, these morphological changes were caused by ROS production of nanoparticles. In a confocal microscopy experiment, the fungal cell walls were stained with Calcofluor White, which emits blue light (Sautter and Kwee, 1987). We expected the green light emitted by the FITC-labeled nanoparticles to produce an overlapping color. In this way, we aimed to visualize whether the nanoparticles remained on the surface after PBS washing using the confocal microscope's lasers. Our results demonstrated that Calcofluor White successfully stained the cell walls. We clearly demonstrated the oval shapes of the cells. FITC-labeled nanoparticles added to the formed biofilms similarly adhered to the cells and emitted light. When the two images were combined, the overlapping light emitted by Calcofluor White and FITC-labeled nanoparticles demonstrated that the nanoparticles adhered to the biofilm and initiated the action mechanism. It has been noted in the literature that different types of synthesized nanoparticles were labeled with FITC and treated with Calcofluor White stained *Candida* cells to determine and visualize their effectiveness (Galdiero et al., 2020).

## Chapter 5:

### CONCLUSION

This thesis extensively investigated the drug resistance pattern of a clinical *Candida auris* isolate (K1575) and investigated the antifungal activity of AuNPs, Chi-AuNPs, CA-Fe<sub>3</sub>O<sub>4</sub> NPs and Chi-Fe<sub>3</sub>O<sub>4</sub> NPs against five different *C. auris* isolates' both planktonic and biofilm cells.

Antifungal susceptibility test results revealed that our clinical isolate was resistant to fluconazole (64 µg/ml) and anidulafungin (8 µg/ml) but susceptible to amphotericin b (1 µg/ml). Genomic investigations, in the guise of SNP/InDel analysis, played key role in determining the molecular mechanism involved with this resistance. ERG11, CDR1 and TAC1b gene mutations were identified, and these mutations effectively explain the observed azole resistance. In a similar manner, FKS1 gene mutation was at the root of the

echinocandin resistance. Lastly, one FYC1 gene mutation which is S70R was found in *C. auris*' genome, but this mutation has no impact on 5-FC resistance pattern which previously identified in literature. This research highlighted the utility of whole genome sequencing in supporting phenotypic patterns of resistance, illustrating its use in clinically significant isolates. Furthermore, the phylogenetic analysis showed that our isolate would likely fall under Clade I, which agrees with the most predominant clade reported among *C. auris* isolates in Turkey and identifying the predominance of a clade known for its association with hospital infections and multidrug/extensively drug-resistant types.

The synthesized nanoparticles were well characterized by XRD, FT-IR, DLS, NTA, SEM, and Zeta-Sizer measurements, ascertaining their successful synthesis and offering in-depth information about their physical and chemical properties. AuNPs prepared using the Turkevich process were characterized by a mode size of 24.3 nm (DLS average  $\sim$ 21.5 nm) with moderate polydispersity (PDI 0.240). Chi-AuNPs were 60.6 nm in mode size (NTA), and SEM images confirmed uniformity of size and aggregation, and the DLS data were consistent with these findings. AuNPs and Chi-AuNPs both exhibited face-centered cubic (FCC) gold crystalline structure, as confirmed by XRD, with the FTIR studies confirming successful stabilization by citrate and chitosan, respectively. In the case of iron oxide nanoparticles, CA-Fe<sub>3</sub>O<sub>4</sub> nanoparticles exhibited a mode size of 55.1 nm with two distinct populations indicating some aggregation despite citric acid stabilization and a characteristic positive zeta potential (+51.9 mV) that was different from literature. Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles were bigger in size with a mode size of 207.2 nm but showed good monodispersed (PDI 0.181), with XRD and FTIR both confirming successful chitosan coating and its influence on the crystalline structure.

With respect to antifungal activity, AuNPs and Chi-AuNPs provided promising results against *C. auris* planktonic cells and biofilm cells. Our clinical isolate K1575 was extremely susceptible to AuNPs and Chi-AuNPs, with close-to-total growth inhibition and biofilm inhibition at tested concentrations. Chi-AuNPs preferentially exhibited augmented antifungal activity when compared with AuNPs and caused sharp inhibition and statistically significant CFU reductions in the majority of isolates, frequently demonstrating a dose-response association. Conversely, CA-Fe<sub>3</sub>O<sub>4</sub> and Chi-Fe<sub>3</sub>O<sub>4</sub> nanoparticles showed little or no significant antifungal activity against most isolates by possible electrostatic repulsion of the negatively charged *Candida* cell wall by the nanoparticles. Hence, further experiments focused on AuNPs and Chi-AuNPs.

Mechanism of action experiments indicated that Chi-AuNPs consistently induced significant ROS generation in all *C. auris* strains, typically comparable to the positive control, reflecting their capacity to be effectively induced for oxidative stress. AuNPs, however, generated very minimal ROS levels. SEM imaging of treated *C. auris* biofilm cells provided visual evidence of changes in morphology, including irregular shape, aggregation, and stress, which were consistent with the ROS generated by the nanoparticles. These findings show that part of the antifungal action of Chi-AuNPs is due to the induction of oxidative stress, which leads to cellular damage and morphology alteration.

Lastly, this thesis was successful in characterizing the drug resistance of a pivotal *C. auris* isolate and proving the efficiency of chitosan-gold nanoparticles as effective antifungal agents against *C. auris* planktonic and biofilm cells, with the induction of ROS as a key mechanism. The study presents novel insights into the genomic derivation of *C. auris* resistance in a Turkish isolate and opens the door for the creation of nanoparticle-based strategies to combat this emerging fungal pathogen. Future research will focus on tailoring nanoparticle preparations towards improved stability and targeting, investigating their in-vivo efficacy, and exploring combination therapies to overcome *C. auris* resistance.

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