TÜRKİYE CUMHURİYETİ
ADANA ALPARSLAN TÜRKEŞ SCIENCE AND TECHNOLOGY UNIVERSITY

GRADUATE SCHOOL
BIOENGINEERING DEPARTMENT

GREEN SYNTHESIZED SILVER NANOPARTICLES EMBEDDED CHITOSAN EDIBLE FILMS FOR FOOD PACKING MATERIALS

DİDEM AKDOĞAN

M.Sc.

ADANA, 2024

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CERTIFICATION OF APPROVAL

(GREEN SYNTHESIZED SILVER NANOPARTICLES EMBEDDED CHITOSAN EDIBLE FILMS FOR FOOD PACKING MATERIALS)

This **M.Sc.** thesis, **completed under the conditions determined by the relevant regulations**, by **Didem Akdoğan with student number** 20800301017 in **Adana Alparslan Türkeş Science and Technology University, Institute of Graduate School, Bioengineering Department**, has been evaluated by the following academic committee, and approved in accordance with the relevant articles of the "Adana Alparslan Türkeş Science and Technology University Graduate Education Regulations".

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DECLARATION OF CONFORMITY

In this thesis study, which was prepared following the thesis writing rules of Adana Alparslan Türkeş Science and Technology University Institute of Graduate School, I declare that I provide all the information, documents, evaluations and results in accordance with scientific ethics and moral codes without resorting to any means or assistance that would be contrary to scientific ethics and traditions. I also declare that I refer to all of the articles I used in this study with appropriate references and accept all moral and legal consequences if a situation is found contrary to my statement regarding my work.

25/01/2024

[Signature]

Didem AKDOĞAN

ÖZET

GIDA AMBALAJ MALZEMELERİ İÇİN YEŞİL SENTEZLENMİŞ GÜMÜŞ NANOPARTİKÜLLER İÇEREN YENİLEBİLİR KİTOSAN FİLMLER

Didem AKDOĞAN
Yüksek Lisans, Biyomühendislik Anabilim Dalı
Danışman: Asst. Prof. Zeynep İyigündoğdu

Ocak 2024, 50 sayfa

Bu yüksek lisans tezinde, gıda ambalaj malzemesi olarak yeşil sentezlenmiş gümüş nanopartikül katkılı, antimikrobiyal, çevre dostu, sürdürülebilir, yenilenebilir kitosan-jelatin bazlı yenilebilir filmler üretmeyi amaçlanmıştır. Bu amaçla, ceviz (*Juglans regia*) yaprağı ekstraktı kullanılarak yeşil sentez yöntemi ile gümüş nanopartiküller sentezlenmiştir. Dört farklı koşulda sentezlenen nanopartiküllerin karakterizasyonu için XRD, SEM, FTIR ve DLS yöntemleri kullanılmıştır. Gümüş nanopartiküllerin antimikrobiyal aktivitesi 7 farklı mikroorganizmaya (*Escherichia coli, Pseudomonas aeruginosa,, Staphylococcus aureus, Salmonella typhi, Candida albicans, Penicillium italicum, Aspergillus nige*r) karşı disk difüzyon ve MIC yöntemleri kullanılarak test edilmiştir. Elde edilen verilere göre AgNPs-4 için MIC değerleri 3.9-15.625 µg/mL aralığındadır. Bu MIC değerlerine göre, AgNPs-4 maya ve mantarlara karşı bakterilerden daha fazla antimikrobiyal etki göstermiştir. Sentezlenen tüm AgNPlerin disk difüzyon inhibisyon çapları 9-11 mm aralığında olmakla birlikte tüm mikroorganizmalara karşı en yüksek inhibisyon çapı AgNP-4 örneğine aittir. Bu nedenle, AgNP-4'ler Kitosan-Jelatin bazlı yenilebilir filmlere antimikrobiyal aktif ajan olarak eklenmiştir. Üretilen yenilebilir filmler karakterize edilmiş ve antimikrobiyal testler yapılmıştır ve toplanan verilere göre nanopartikül eklenmiş yenilebilir filmlerin mekanik ve antimikrobiyal özellikleri tatmin edici düzeydedir.

Anahtar Kelimeler: Antimikrobiyal**,** biyolojik olarak parçalanabilir, yeşil sentezlenmiş gümüş nanopartiküller, yenilebilir film

ABSTRACT

GREEN SYNTHESIZED SILVER NANOPARTICLES EMBEDDED CHITOSAN EDIBLE FILMS FOR FOOD PACKING MATERIALS

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GRADUATE SCHOOL

BIOENGINEERING DEPARTMENT

Advisor: Asst. Prof. Zeynep İyigündoğdu

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In this master's thesis, it is aimed to produce green synthesized silver nanoparticle doped, antimicrobial, environmentally friendly, sustainable, renewable chitosan-gelatin based edible films as food packaging material. For this purpose, silver nanoparticles were synthesized by green synthesis method using walnut (*Juglans regia*) leaf extract. XRD, SEM, FTIR and DLS methods were used to characterize the nanoparticles synthesized under four different conditions. The antimicrobial activity of silver nanoparticles was tested against 7 different microorganisms (*Escherichia coli, Pseudomonas aeruginosa,, Staphylococcus aureus, Salmonella typhi, Candida albicans, Penicillium italicum, Aspergillus nige*r) using disk diffusion and MIC methods. According to the data obtained, the MIC values for AgNPs-4 were in the range of 3.9-15.625 µg/mL. According to these MIC values, AgNPs-4 showed more antimicrobial effect against yeasts and fungi than bacteria. Although the disk diffusion inhibition diameters of all synthesized AgNPs were in the range of 9-11 mm, the highest inhibition diameter against all microorganisms belongs to AgNP-4 sample. Therefore, AgNP-4s were incorporated into Chitosan-Jelatin based edible films as antimicrobial active agents. The produced edible films were characterized and antimicrobial tests were performed and according to the data collected, the mechanical and antimicrobial properties of the nanoparticle-added edible films were satisfactory.

Keywords: Anticmicrobial, biodegredible, edible film, green syhntesized silver nanoparticles

Dedication

First of all, I would like to thank the founding leader of our country, head teacher Mustafa Kemal ATATÜRK, and his comrades in arms for founding this country and giving us the right to education. My dear teacher and thesis advisor, who taught me countless things within the scope of this thesis study, always supported me and strived to be better every day. I would like to thank Asst. Dr. Zeynep İYİGÜNDOĞDU. Who always supported me financially and morally throughout my entire education life; I would like to thank my dear grandfather Enver AKDOĞAN, my grandmother Zeliha AKDOĞAN, my father Hasan AKDOĞAN, my uncle Mahmut AKDOĞAN, my aunt Margaret AKDOĞAN, my aunts Selma and Fatma AKDOĞAN.

Didem AKDOĞAN
25 JANUARY,2024

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LIST OF ABBREVIATIONS

$AgNO_3$	: Silver nitrate
AgNPs	: Silver nanoparticles
Sol	: Solution
MIC	: Minimum Inhibitory Concentration
MBC	: Minimum Bactericidal Concentration
MFC	: Minimum Fungucidal Concentration
mm	: Milimeter
g	: Gram
WVTR	: Water vapor permability rate
SEM	: Scanning Electron Microscopy
XRD	: X-Ray Diffraction
FTIR	: Fouirer Transform Infrared Spectrofotometer
dH_2O	: Distilled water
PBS	: Phosphate buffered saline

1. INTRODUCTION

Food packaging is one of the most important parts of the food industry as it protects food from contamination and spoilage. However, traditional food packaging materials such as plastics are not environmentally friendly and can have negative impacts on human health. Therefore, there is a growing demand for sustainable and environmentally friendly food packaging materials.

Edible films are a promising alternative to traditional food packaging materials. Edible films and coatings are substances derived from non-synthetic natural sources such as proteins, polysaccharides, lipids or mixtures thereof. Edible films and coatings are used by applying a thin layer on the surface of foods or between food components and protect foods from physical, chemical and microbial threats, delay spoilage, extend the shelf life of food and prevent quality losses.

The application of edible films and coatings can improve the physical properties of food products, reduce particle aggregation and improve the visual and tactile properties on the food surface, giving it an aesthetic appearance while increasing product appeal. It can also protect foodstuffs from moisture migration, microbial growth on the surface, light-induced chemical changes and oxidation. Edible films and coatings have attracted great interest in recent years compared to synthetic films due to their advantages such as reducing environmental pollution. Moreover, edible films and coatings can reasonably reduce the complexity compared to more harmful and traditional environmentally unfriendly packaging materials by acting as barriers, thus increasing the recyclability of packaging materials and replacing synthetic polymer films.

Increasing consumer demands for microbiologically safe foods and longer shelf life have driven the food industry to find new food processing, transportation and packaging strategies. Solid and ready-to-eat foods are often exposed to microbiological contamination after processing, resulting in shorter shelf life. Non-edible packaging materials are generally used in the food industry, and due to their harmful effects on the human body and the environment, researchers have turned to edible food packaging materials. With these materials being a barrier against various mass diffusions such as gas and moisture, edible films can be used as carriers for a wide

range of food additives, including antioxidants, vitamins and colorants. When various antimicrobial agents are incorporated into the product, it retards the growth of various pathogens in a wide range of products.

In this thesis, it was aimed to produce green synthesized silver nanoparticles (AgNPs) using walnut leaf extract and loaded into chitosan-based edible films that can be used as food packaging material. *Juglans regia* (walnut) leaf extract was used as a reducing agent for the production of AgNPs. It was aimed to investigate the antimicrobial activity of green synthesized silver nanoparticles added chitosan based antimicrobial edible film and the antimicrobial activity was tested by producing edible film. Various characterization procedures were performed on AgNPs and their shapes, sizes and structures were verified. Necessary antimicrobial tests were performed on silver nanoparticles and edible film. As a result of the tests, the antimicrobial activity of the product was determined.

2. LITERATURE REVIEW

2.1. Nanoscience and Nanotechnology

The Greek word "nano," which means "dwarf" or "very small," is used to represent one thousand millionth of a meter (10^{-9} m). Nanoscience is the study, engineering, and modification of matter, particles, and structures at the nanoscale, 1 to 100 nm, or the size of atoms and molecules. The way that molecules and atoms come together to form larger structures on the nanoscale determines important features of materials, including their electrical, optical, thermal, and mechanical properties (Bayda et al., 2019). Furthermore, because quantum mechanical effects become significant, these features are frequently different in nanoscale size structures than they are on the macroscale (Popescu et al., 2019). The application of nanoscience to create novel nanomaterials and nanoscale components for practical goods is known as nanotechnology (Mansoori & Soelaiman, 2005).

There are many criteria such as origin, morphology, geometry, dimensionality, chemical components, toxicity, agglomeration and so on, which are taken into account by researchers when classifying nanoparticles (Dolez, 2015). If nanoparticles are classified according to their origin, it can be classified as natural nanoparticles, incidental nanoparticles, and engineered nanoparticles. According to dimensional classification, they can be categorised into four groups such as zero-dimensional nanoparticles, one-dimensional nanoparticles, two-dimensional nanoparticles, and three-dimensional nanoparticles. Depending on the chemical composition, they can be categorised as metallic nanoparticles, semiconductor nanoparticles, carbon-based nanoparticles, ceramic nanoparticles, lipid-based nanoparticles, and polymeric nanoparticles (Khan & Hossain, 2022). These groups represent a wide variety of nanoparticles, each with unique characteristics that enable them to be used in a range of industries, including materials science, electronics, medical, and energy (Figure 1.) (Nath & Banerjee, 2013). Among them, metallic nanoparticles are appealing for a variety of applications due to their outstanding properties.

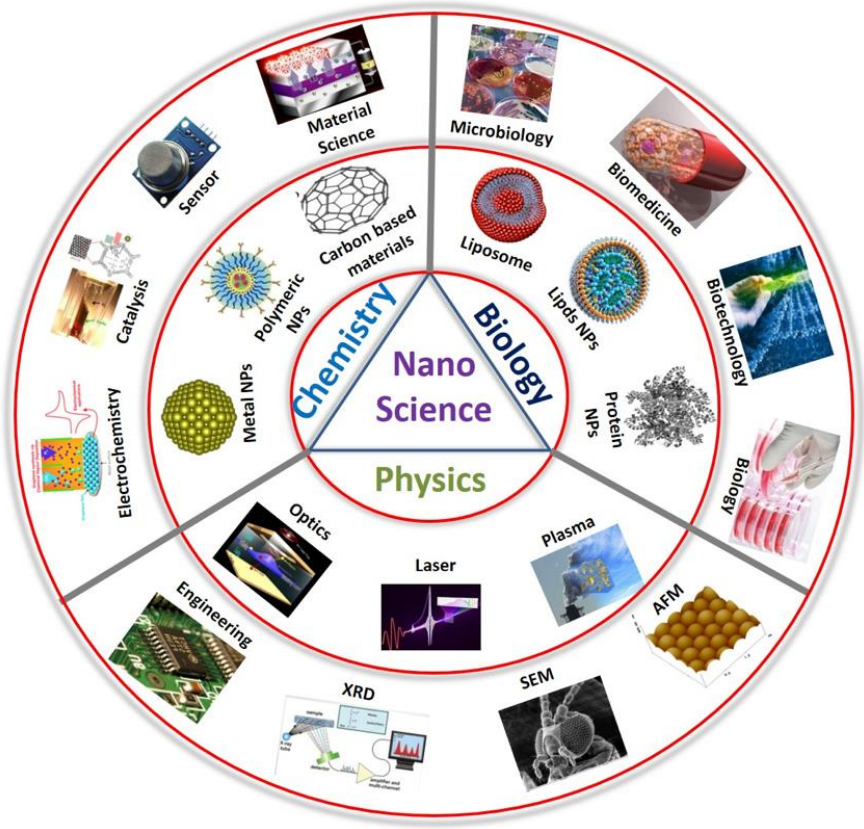

Figure 1. Some application areas of nanoscience (Bayda et al., 2019)

2.2. Nanoparticle Synthesis

Two different approaches, called top-down and bottom-up, can be used to produce nanoparticles (Alharbi et al., 2022). A simplified representation of the process is presented in Figure 2.

2.2.1 Top-Down Approach

Top-down approach is a destructive method which can be described in general terms as dividing an entire material into nano-dimensions using techniques such as mechanical milling, nanolithography, laser ablation, sputtering and thermal decomposition (Alharbi et al., 2022).

2.2.2. Bottom-Up Approach

Bottom-up approach is a constructive method based on the principle of synthesizing nanoparticles from atoms to clusters to nanoparticles using techniques such as sol-gel, spinning, chemical vapour deposition, pyrolysis and biosynthesis (Alharbi et al., 2022).

Biological processes, called green synthesis and generally created using plant extracts, have many advantages over chemical and physical methods. These advantages are that it is cheap, environmentally friendly, and easily accessible (Alharbi et al., 2022).

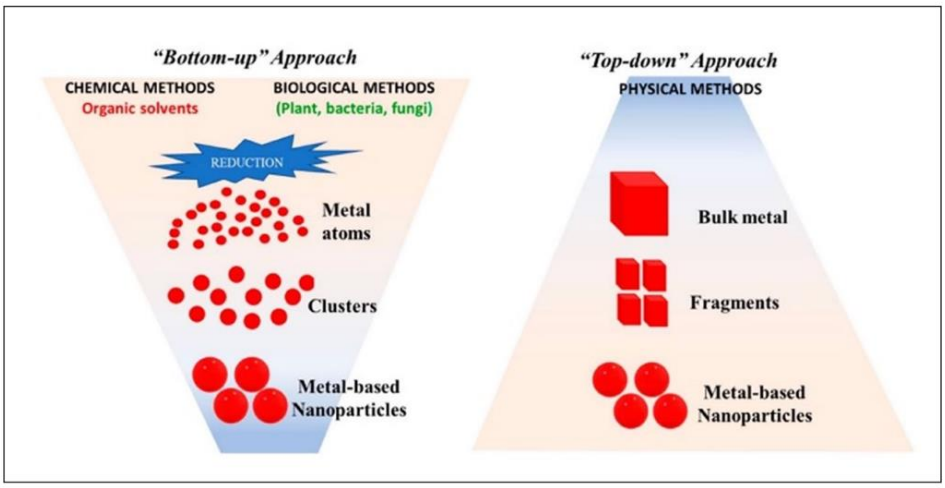

Figure 2. Synthesis methods for nanoparticles (Amiri et al., 2022)

2.3. Chemical vs. Biological Nanoparticle Synthesis

Chemical nanoparticle synthesis is a widely used method that involves the bottom-up approach of nanoparticles through chemical reactions. There are various advantages of chemical synthesis such as size and shape control. Many different chemical techniques, including sol-gel, precipitation, and reduction, are used to convert precursor materials into nanoparticles. Also, it is prefered for industrial large-scale production due to high reproducibility, cost-effective and high efficiency (Zhang et al., 2016). On the other hand, in order to synthesize nanoparticle by chemical methods might require hazardous chemicals, high pressure, or temperatures, which could be dangerous for the environment and human safety (Zhang et al., 2016). Biological synthesis is also known as green synthesis is an environmentally sustainable method of nanoparticle production. Green synthesis method uses biological contents such as, bacteria, fungus, or plants, to mediate the reduction of metal ions or the synthesis of nanomaterials (Noah & Ndangili, 2022). Compared to some chemical processes, green synthesis usually takes place at room temperature and atmospheric pressure, which reduces the environmental impact of this method. Since biological synthesis often produces naturally biocompatible and biodegradable nanoparticles, the materials produced by this method are

particularly suitable to be used in environmental science and medicine. Although it is difficult to control the shape and size of nanoparticles synthesised by biological methods, the properties of these nanoparticles, such as complex structures and the potential for natural surface functionalisation, provide unique advantages to these nanoparticles. Moreover, the use of biological ingredients as reducing agents instead of toxic chemicals in green synthesis is important in terms of the sustainability of nanoparticle synthesis(Yoksan & Chirachanchai, 2010) (Pallela et al., 2018, Guilger-Casagrande & de Lima, 2019, Nasrollahzadeh et al., 2019). Some advantages and disadvantages of using chemical and biological methods for the synthesis of nanoparticles are summarised in Table 1.

Table 1. Some advantages and disadvantages of using chemical and biological methods for the synthesis of nanoparticles (Kulkarni & Muddapur, 2014)

Biological Synthesis	**Chemical Synthesis**
Advantages	**Advantages**
Nonuse of expensive and toxic chemicals	Higher yield
Ambient temperature and atmospheric pressure	Short time of synthesis
Modification by genetic engineering	
Nanoparticles with:	**Disadvantages**
Greater commercial viability	Use of expensive and toxic chemicals
Greater specific surface area	Physical methods need high temperature
Large savings in reductants	Chemical methods need high pressure
Large savings energy costs	Chemical methods need a stabilizer
High production rate	
Higher catalytic reactivity	
Small nanoparticles in large-scale	
Disadvantages	
Time consuming	
Difficult to control (size, shape and crystallinity)	

2.4. Metallic Nanoparticles

Metallic nanoparticles have been of great interest to scientists for many years. Metallic nanoparticles are currently used for various reasons in fields such as biomedical sciences and engineering. They attract great attention due to their great potential in nanotechnology (Mody et al., 2010). Metal nanoparticles show antifungal effects due to effects such as ion release, oxidative and nitrosative stress, membrane and cell wall damage, and disruption of enzymatic activity (Carmo et al., 2023). Metal nanoparticles are generally synthesized by physical, chemical and biological methods (Saravanan et al., 2021). It is known that the best synthesis method is biological methods due to its cost performance and environmental friendliness (Saravanan et al., 2021).

Today, metal nanoparticles can be produced and modified with the help of various chemical functional groups. This allows them to be conjugated with antibodies, ligands and relevant drugs, opening up great potential application areas in biotechnology and magnetic separation (Mody et al., 2010). Optimizing the synthesis processes of metal nanoparticles, which have unique properties in terms of chemical and physical properties, in different ways can change the properties of these nanoparticles (Carmo et al., 2023).

2.4.1. Silver Nanoparticles

Silver nanoparticles (AgNPs) have attracted attention due to their exceptional stability and minimal chemical reactivity compared to other metals. Traditional synthesis methods use hazardous chemical reducing agents to convert metal ions into uncharged nanoparticles (Mustapha et al., 2022).

Recently, studies on the development of environmentally friendly green synthesis techniques have been concentrated in order to eliminate the use of harmful chemicals. Studies are being carried out on the use of natural biomolecules such as proteins/enzymes, amino acids, polysaccharides, alkaloids, alcoholic compounds and vitamins found in plants in AgNP synthesis (Mustapha et al., 2022). Silver nanoparticles with antimicrobial properties are easily synthesized from plant extracts with high stability and environmental friendliness (Sofi et al., 2022).

The use of silver nanoparticles (AgNPs) is widespread in industry and healthcare (Kalwar & Shan, 2018). AgNPs are the most common antimicrobial agent for bacteria than other species (Kalwar & Shan, 2018). Compared to the damage it causes to human cells and bacteria, it has a less toxic effect on human cells (Kalwar & Shan, 2018).

2.4.2 Advantages of Using Plant Extracts in Green Synthesis of Metallic Nanoparticles

Metal nanoparticles can be produced using various chemical, physical and biological synthetic methods. In the literature, some of disadvantages of these methods are stated as difficulties in adjusting the size, size distribution, shape, stability,and crystal size of nanoparticles. The method of synthesizing nanoparticles using plant extracts has many advantages over the synthesis method using microorganisms (Rafique et al., 2017). The most notable features and biggest advantages of the green synthesis method are that it is rapid, provides a one-step technique, is economical, non-pathogenic and does not harm the environment (Rafique et al., 2017).

It has been shown that metal nanoparticles obtained by using plants in the green synthesis method are more stable compared to those produced by other organisms. It has also been reported in the literature that metal ions can be reduced faster when plant externals are used instead of fungi or bacteria.

In addition, plant extracts are definitely better than plant biomass or living plants for large-scale production of metal nanoparticles by green synthesis (Iravani, 2011).

2.4.3. Application Areas of Green Synthesized Silver Nanoparticles

The green chemistry method, which brings an innovative and environmentally friendly breath to the field of nanotechnology, is based on a production process using plant extracts. Metal nanoparticles synthesized using plant extracts attract more attention because they provide more benefits compared to physicochemical methods. There are areas where silver nanoparticles produced by this method are applied as antimicrobial agents of certain microorganisms with scientifically proven activities (Castillo-Henríquez et al., 2020). Silver nanoparticles are used in many different areas such as deodorants, water filters, soaps, food preservation and air fresheners (Rafique et al., 2017). Silver nanoparticles (AgNPs) have demonstrated remarkable

properties in various applications, including superior coloration, biocidal activity, thermal stability, ultraviolet protection, and improved mechanical performance (Hasan et al., 2022).

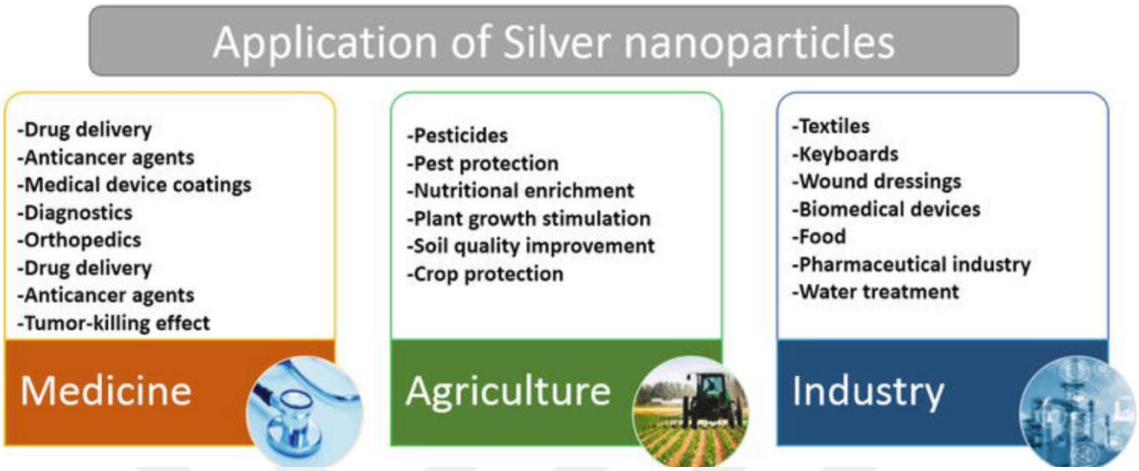

Figure 3. Applications of silver nanoparticles (Chobba et al., 2023)

2.4.4. Antimicrobial Mechanism Action of Silver Nanoparticles

Although the mechanism of the antibacterial effects of silver nanoparticles has not been fully explained today, there are some possible mechanisms that can be explained. Various antimicrobial mechanisms of AgNPs are given in Figure 4. It can be thought that silver nanoparticles develop an antimicrobial mechanism by killing microorganisms with their tendency to continuously release silver ions. These ions exhibit a strong affinity for the bacterial cell wall and cytoplasmic membrane, driven by electrostatic interactions and a specific binding preference for sulfur-containing proteins. This initial attachment disrupts membrane permeability, compromising the integrity of the bacterial envelope. When silver ions enter the cell, respiratory enzymes are disabled and reactive oxygen species can be produced, but adenosine triphosphate production may be interrupted. Reactive oxygen species may be the main agent in provoking cell membrane disruption and deoxyribonucleic acid (DNA) modification. Since sulfur and phosphorus are important components of DNA, the interaction of silver ions with sulfur and phosphorus of DNA can cause problems in DNA replication, cell proliferation, and may lead to the destruction of microorganisms. (Yin et al., 2020).

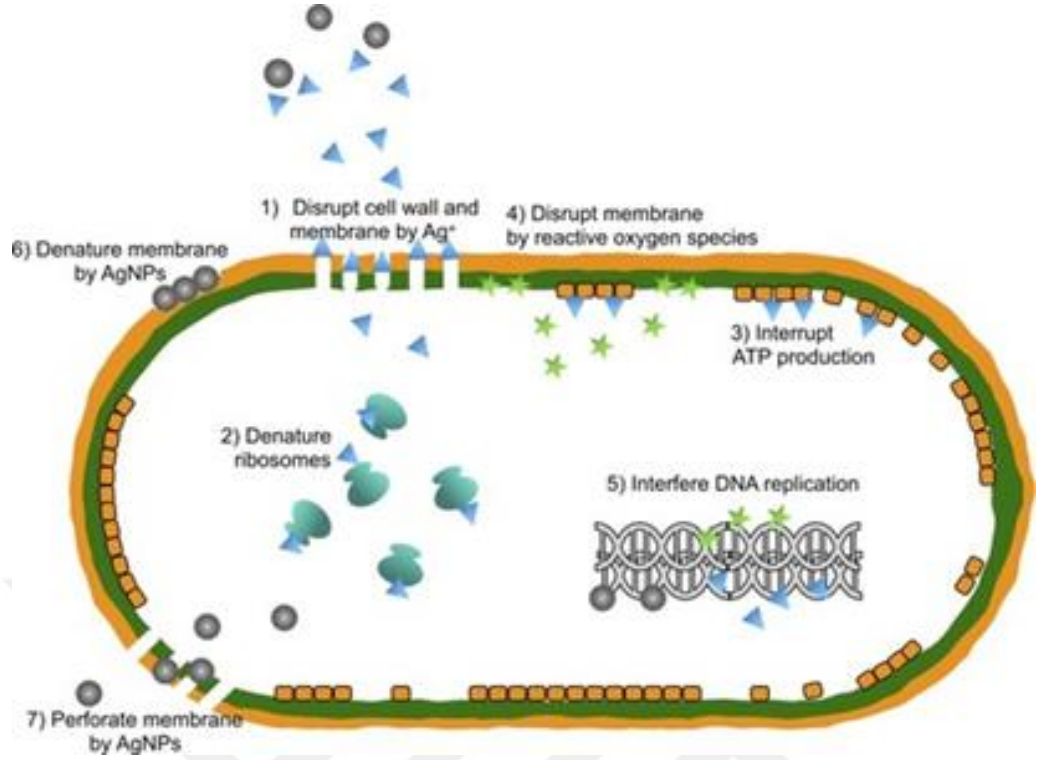

Figure 4. Antimicrobial mechanisms action of silver nanoparticles (Yin et al., 2020)

2.5. Walnut (*Juglans regia*) Leaves

Walnut (*Juglans regia*) is a species that is very popular worldwide, constituting a significant portion of deciduous trees primarily found in temperate regions and commercially grown in the United States, western South America and Asia (Bayazit et al., 2016). The *Juglans* genus includes several different species and is widely found throughout the world (Pereira et al., 2007a). It is known that walnut leaves, fruit and timber are used in different fields of industry.While green fruits are used in food and medicine industry, leaves, shells and roots in tannin and paint industry before the shell hardens, walnut oil is a precious oil that is sought in both technology and painting. In addition, walnut trees are an important source of raw materials in the furniture industry. Furthermore, the main reason why walnuts are grown worldwide is the richness of the nutritional content of their fruits. Almost all parts of the walnut are used in traditional and modern medicine. Surprisingly, according to data obtained from many studies, walnut consumption has been shown to reduce the risk factors of some cardiovascular diseases, cancer and type 2 diabetes (Jaćimović et al., 2020). Among the fruit types, walnut is the only

fruit type that contains silver. The organ that needs the silver ion is the brain. Silver is very effective in protecting the health of the human brain and in learning. Because silver ion is known to have antibacterial properties. Walnuts are among the rare foods that contain selenium. Selenium binds to proteins to make selenoproteins, which are important antioxidant enzymes (Bayazit et al., 2016). Figure 5 shows the parts of walnut trees and fruit.

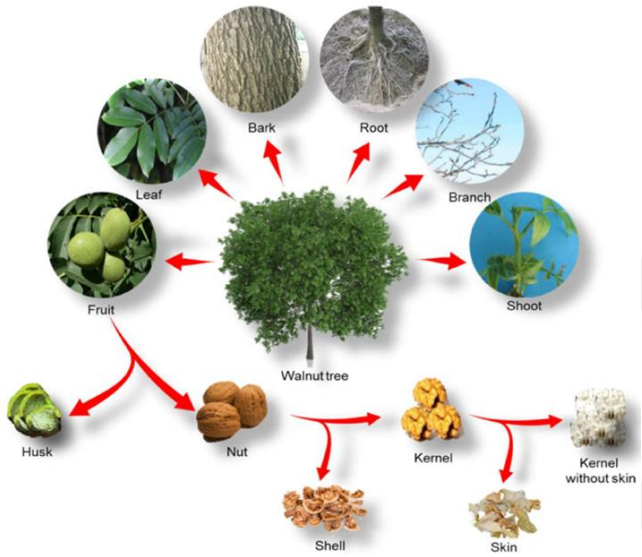

Figure 5. Different parts of the walnut tree and fruit (Jahanban-Esfahlan et al., 2019)

2.5.1. Application Areas of *Juglans regia*

Walnut leaves are widely used in alternative medicine in the light of traditional methods in the treatment of some diseases (Yiğit et al., 2009). Walnut leaves are used in traditional medicine to treat symptoms of venous insufficiency and hemorrhoids. It is known that walnut leaves have anti-diarrheal and anthelmintic properties (Moravej et al., 2016). Green walnuts, shells, kernels, and seeds, shell and leaves are widely used in the pharmaceutical and cosmetic industry. Leaves are easily and abundantly obtained, but the bark is scarce, and the life of the plant is endangered if collected (Bayazit et al., 2016; Yiğit et al., 2009). Dried walnut leaves are boiled then used for as a tea in some countiries (Keskin et al., 2012).

Walnut leaves are also used as a dye in some regions. Since walnut leaves and fruit are widely used, the trade circle is wide (Pereira et al., 2007b; Yiğit et al., 2009). For centuries, people living in rural areas have used walnut leaf to dye various objects and their hair (Yiğit et al.,

2009). Studies on walnut show us that each part of the walnut plant is valuable and useful separately (Bayazit et al., 2016).

2.5.2. Chemical Content of *Juglans regia* (Walnut)

Walnut fruit is rich in essential fatty acids. Linoleic acid, oleic, linolenic, palmitic, and stearic acid show a protective effect in cardiovascular diseases by decreasing LDL cholesterol and increasing HDL cholesterol. In addition, walnut fruit is a very useful fruit in nutrition due to its vegetable proteins, fibers, melatonin, plant sterols, folate, tannin ,and polyphenols (Keskin et al., 2012). Its active ingredient is juglone (5-hydroxy-1,4-naphthoquinone), which is abundant in green leaves, and this substance has strong antioxidant and antimicrobial properties (Yiğit et al., 2009). Green bark and leaf parts are rich in phenolic substances and flavonoids (Pereira et al., 2007b). Phytochemicals such as phenolic compounds are thought to be beneficial for health because they reduce the risk of disease by reducing oxidative stress and inhibiting macromolecular oxidation (Vieira et al., 2019).

2.5.3. Antimicrobial Properties of *Juglans regia* Leaves Extract

Antimicrobial activity is observed in aqueous solutions of walnut leaves. The antimicrobial effect of walnut leaves has been proven according to previous studies.Some studies have shown that walnut leaves as well as walnut green shell has significant antimicrobial activity (Keskin et al., 2012). As a result of some analysis, the aqueous solutions of walnut leaves were found to be inhibitory against some fungal species (*Candida albicans, Cryptococcus neoformans*) species, including gram negative (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumoniae*) and gram positive (*Bacillus cereus, Bacillus subtilis, Staphylococcus aureus*) bacteria (Vieira et al., 2019). Silver nanoparticles synthesized with walnut leaf extract can be used as natural antimicrobial agents in food packaging (Korbekandi et al., 2013). The antimicrobial activity of walnut (*Juglans regia*) leaf is due to the chemical composition of the walnut leaf (Keskin et al., 2012; Pereira et al., 2008).

2.6. Instruments for Determining Properties of Nanoparticles

Various tools and techniques are used to determine various properties of nanoparticles such as size, shape, surface charge, composition and concentration. Transmission Electron Microscopy (TEM), Scanning Electron Microscope (SEM), Atomic Force Microscopy (AFM), Dynamic

Light Scattering (DLS), X-Ray Diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR), Zeta Potential Analyzer, Nuclear Magnetic Resonance (NMR), UV-Visible Spectroscopy are some instruments used for nanoparticle characterization. The characterisation methods used in this thesis are briefly described below.

2.6.1. Scanning Electron Microscope (SEM)

SEM imaging can be used to determine the morphological properties of nanoparticle composites such as surface area, particle pore size, particle distribution, etc. SEM produces a detailed visualisation of particle shape by scanning a focused electron beam across the surface of a sample. While the maximum magnification in SEM imaging is usually determined by the size of the electron beam, it can be up to one million (Naito et al., 2018).

SEM can not resolve the internal structure, but it can give beneficial information relating to the purity and the volume of particle aggregation. With its high-resolution capabilities, the modern SEM has become a powerful tool for visualizing the shapes of nanoparticles as small as 10 nanometers and even smaller (Zhang et al., 2016).

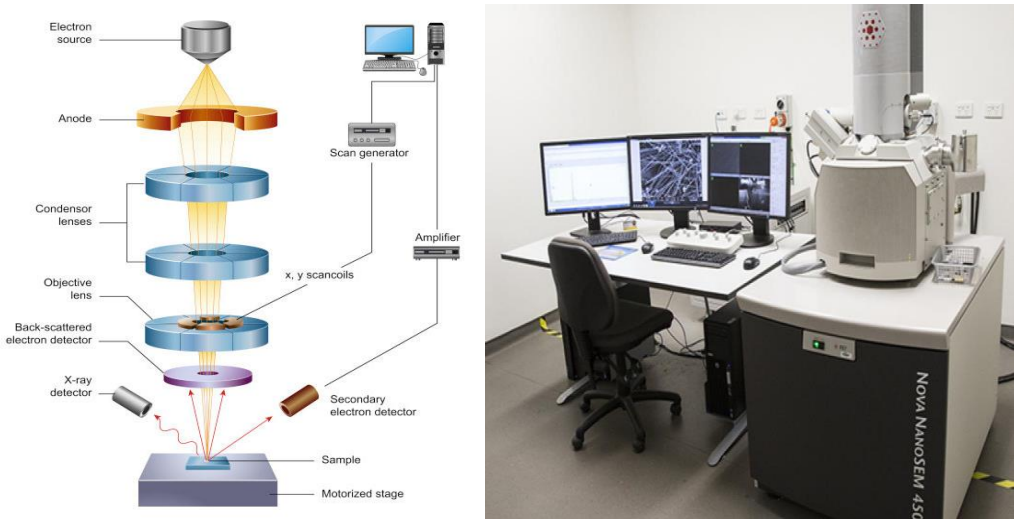

Figure 6. Working principle and visual image of a SEM machine (*Britannica*, n.d.; *SEM - FEI Nova NanoSEM 450 FE-SEM | UNSW Mark Wainwright Analytical Centre*, n.d.)

2.6.2. X-ray Diffraction

X-ray Diffraction (XRD) is a technique used for the characterization of materials and provides important information about the crystal structure and crystallographic properties of the materials. In XRD, a monochromatic X-ray beam is directed at the sample, leading to constructive interference of X-rays scattered by the crystal lattice (Figure 7).The resulting diffraction patterns is then recorded that gives various micro and macrostructural features of the sample. With the peak position, lattice parameters, space group, chemical composition, macro stresses or qualitative phase analysis research and observations can be made. XRD allows information about crystal structure, crystal size, lattice parameters depending on peak intensity (Epp, 2016).

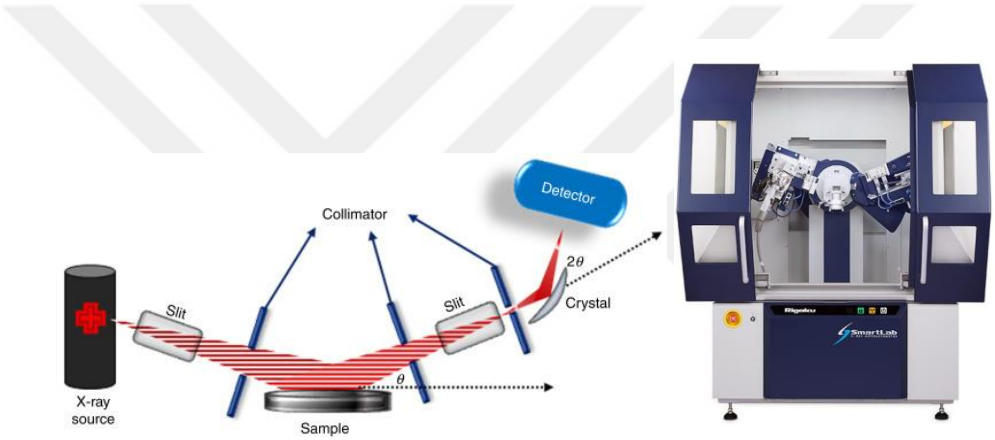

Figure 7. Working principle and visual image of a XRD machine (Jagadeesh et al., 2024; *Rigaku XRD User Meeting: Powder Diffraction*, n.d.)

2.6.3. Fourier-Transform Infared Spectrometer (FTIR)

Nanoparticles with small sizes and a saturated surface state are highly oblique to Van der Waals interactions. They can lead to the existence of aggregates, which is one of the stability conditions of nanoparticles. Van der Waals interactions affect the way nanoparticles chemically bond to stabilizers and protective coatings. FTIR device (Figure 8). is used to visualize data regarding chemical bonds formed due to nano-sized materials (Indiarto et al., 2022).

FTIR device is used to visualize data regarding chemical bonds formed due to nano-sized materials. Production of each bond peak curve is possible with the FTIR method. The electromagnetic properties of nanoparticles are detected using FTIR. The size of the resulting

nanoparticles can be affected by electromagnetic properties. Increases in the magnetic field are inversely proportional to particle size. As the magnetic field decreases, the particle size increases (Indiarto et al., 2022).

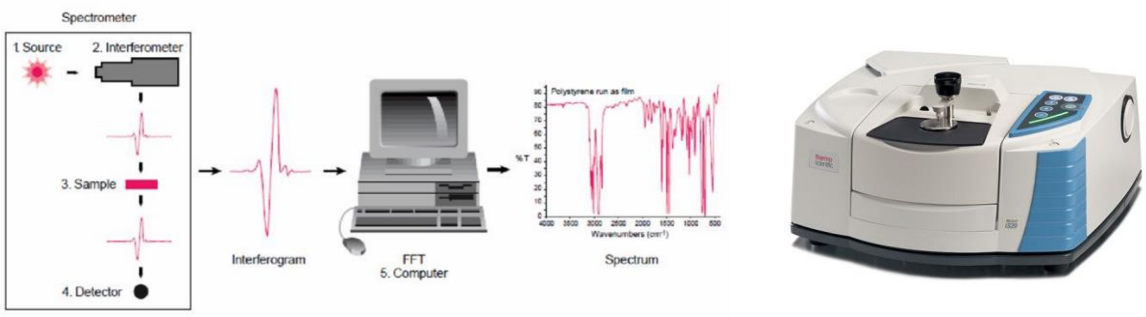

Figure 8. Working principle and visual image of a FTIR machine (Gurumurthy et al., 2017; *Nicolet FTIR Spectrometers | Nicolet iS20 | Thermo Fisher Scientific - AU*, n.d.)

2.6.4. Dynamic Light Scattering Techniques

Light scattering techniques called laser diffraction (LD) and dynamic light scattering (DLS) are used to study the properties of nanoscales. Laser diffraction focuses the blue laser on the smallest particles and the red laser on the largest particles when measuring particle size (Figure 9). With this technique, a narrower particle size range of 20 nm–2000 µm is obtained. Morever, DLS can be used to find out their zeta potential value (Indiarto et al., 2022).

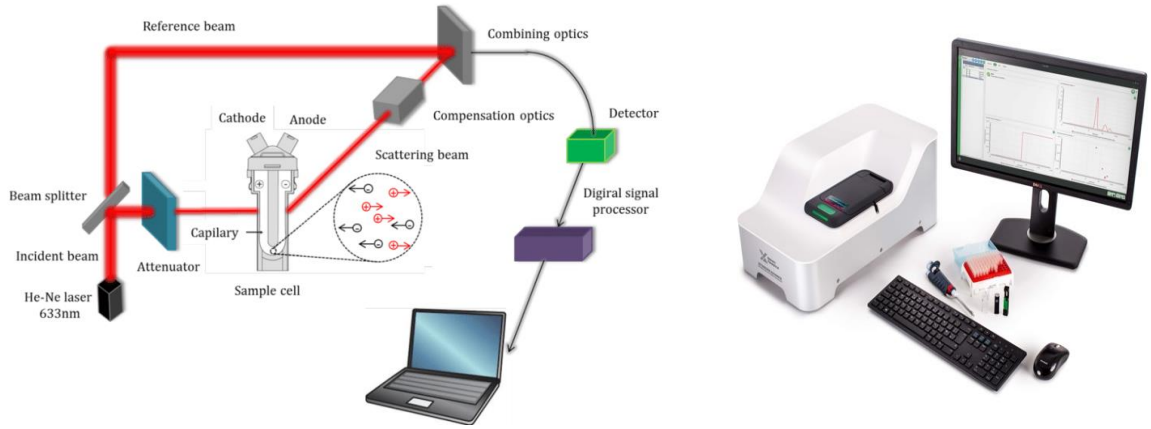

Figure 9. Working principle of DLS and visual image of a Zetasizer machine (*Zetasizer Ultra - Zeta Potential Analyzer | Malvern Panalytical*, n.d.)

3. MATERIALS AND METHODS

3.1. Materials

Juglans regia (Walnut) leaves were collected from walnut trees from Pozanti, Adana, Türkiye. Ultrapure water was used all experiments obtained from ATU, Department of Bioengineering laboratories. Silver nitrate ($AgNO_3$), acetic acid (CH_3COOH), methanol (CH_3OH), glycerol ($C_3H_8O_3$), low molecular weight chitosan, were purchased from Sigma-Aldrich (Germany). Gelatine was purchased from SERVA Electrophoresis GmbH (Invitrogen, USA). Nutrient agar (NA), nutrient broth (NB), potato dextrose agar (PDA), and potato dextrose broth (PDB) were purchased from Sigma-Aldrich (Germany). Blank, oflaxacin, and nystatin antimicrobial susceptibility testing discs were purchased from Oxoid (ThermoFisher, USA). Microbial species, except *Penicillium italicum,* used in antimicrobial activity tests, are given in Table 2 were obtained from American Type Culture Collection (ATCC). *P. italicum* was kindly obtained from Yeditepe University culture collection.

Table 2. Microbial species used in antimicrobial studies

Bacteria	**Yeast**	**Fungus**
Eschericia coli (ATCC 25922)	*Candida albicans*	*Aspergillus niger*
*Pseudomonas aeruginosa (*ATCC 27853)	(ATCC 10231)	(ATCC 16404)
Salmonella typhi (ATCC 19430)		*Penicillium italicum*
Staphylococcus aureus (ATCC 29213)		

3.2. Methods

3.2.1. Collection of *Juglans regia* (Walnut) Leaves and Extraction

Juglans regia (Walnut) leaves were, collected from Pozanti region of Adana, Türkiye. First, the leaves were cleaned with distilled water, crushed into small pieces with a mortar and dried in an oven at 37 °C for 24 hours as shown in Figure 10. Walnut leaf extracts were obtained by four different parameters to find the best condition. The extraction conditions are given in Table 3. As an example; 10 g of leaves were mixed with 100 mL of distilled water and 100 mL of

methanol in a heated magnetic stirrer at 50 °C for 60 min. The extract was allowed to cool to room temperature and centrifuged at 9000 rpm for 10 min. The final extract was obtained using Whatman No. 1 was filtered using filter paper and the filtrate was collected in a 250 mL Erlenen flask as shown in Figure 11. Alcohol was removed form the filtrate using rotary evaporated which was operated at 50 ^{o}C and 120 rpm for 30 min. The alcohol-removed extract obtained was poured into glass Petri dishes, covered with parafilm, and frozen by keeping it in the freezer at -80 ^{o}C for 24 hours. Then the frozen extract was placed into freeze dryer to obtain powdered extract.

Figure 10. Fresh and ground walnut leaves

Table 3. Conditions used to obtain walnut leaves extract

	Walnut leaves (g)	dH_2O (mL)	MetOH (mL)	Temperature (^{o}C)	Mixing (h / rpm)	Centrifuge (min / rpm)
Extract-1	10	100	100	25	1 / 150	10 / 9000
Extract-2	10	200	-	25	1 / 150	10 / 9000
Extract-3	10	200	-	50	1 / 150	10 / 9000
Extract-4	10	100	100	50	1 / 150	10 / 9000

Figure 11. Extract-4 preparation and filtered extract

3.2.2. Green Synthesis of Silver Nanoparticles (AgNPs)

First, 0.05 g each of 4 different powdered extract obtained under the conditions specified in Table 3 were dissolved in 100 mL distilled water in a beaker. In another beaker, 0.05 g of $AgNO_3$ salt for each extract was dissolved in 200 mL of water, then the two solutions were mixed and stirred at 50 ^oC for 1 h. Conditions used to obtain AgNPs using walnut leaves extract weresummirized at Table4. After stirring the mixture for 1 hour, a color change was observed in the form of darkening of the color. The color changes and final AgNPs are shown in Figure 12 and 13.

The resulting mixture was centrifuged at 9000 rpm for 5 min at room temperature, and the nanoparticles were precipitated, and then the precipitated nanoparticles were washed twice, and then removed from the water at 9000 rpm for 5 min. The pellet obtained at the end of the second wash was suspended with 5 mL of distilled water and frozen at -20°C and then dried with the help of a freeze dryer to obtain 4 different AgNPs.

Table 4. Conditions used to obtain AgNPs using walnut leaves extract

	Extract number	Powdered extract (g/100 mL dH_2O)	$AgNO_3$ (g/100 mL dH_2O)	Mixing (^oC / h)	Centrifuge (min / rpm)
AgNP-1	Extract-1	0.05	0.05	50 / 1	10 / 9000
AgNP-2	Extract-2	0.05	0.05	50 / 1	10 / 9000
AgNP-3	Extract-3	0.05	0.05	50 / 1	10 / 9000
AgNP-4	Extract-4	0.05	0.05	50 / 1	10 / 9000

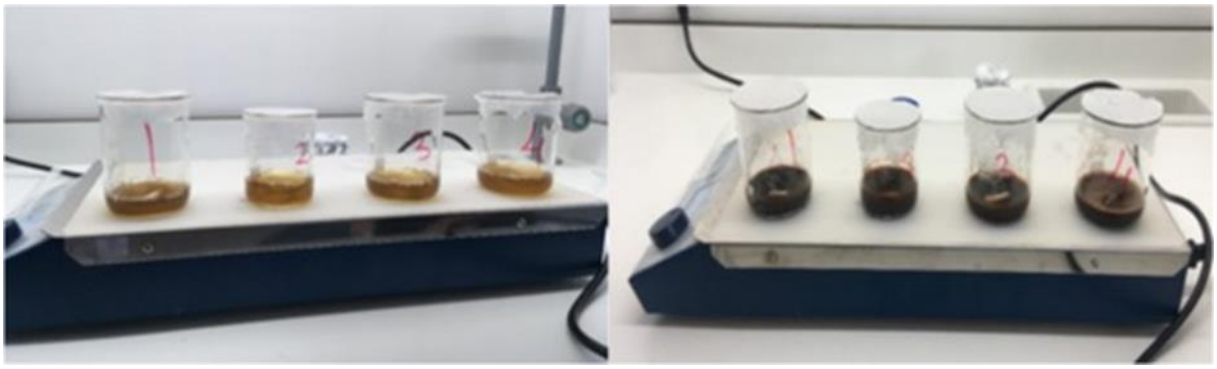

Figure 12. The color change of the solution before and after 1 hour of mixing which is an indicator of AgNP formation

Figure 13. AgNP solutions

3.2.3. Characterization of Green Synthesized AgNPs'

3.2.3.1. XRD Analysis

XRD analysis was applied to find out whether there is any silver presence in the extracts and to confirm the crystal structures of AgNPs. X-ray diffraction (XRD) pattern of AgNPs was found using a powder diffractometer (X-ray diffractometer Ultima IV, Rigaku, Japan) with Kα radiation (λ= 1.54059 nm) in the 2θ scope from 20º to 90º.

3.2.3.2. SEM Analysis

Surface morphologies of AgNPs and were characterized by scanning electron microscopy (SEM, FEI, Quanta 650, USA) analysis. The samples are attached on aluminium stumps covered with gold in a sputtering equipment before examining under the SEM. The SEM data were collected at the magnification of 200Kx for monitoring.

3.2.3.3. FTIR Analysis

FTIR analysis was applied to determine the bond structures and chemical groups of the silver nanoparticles. The presence of specific chemical groups in the AgNPs were analyzed by Fourier transform infrared spectroscopy (FTIR, PerkinElmer, USA) in the 400– 4000 cm^{-1} wavelength range.

3.2.3.4. Zeta Sizer Analysis

The size distribution of the nanoparticles (refractive indices of silver and water are 0.15 and 1.333, respectively, measurement temperature 25 °C; medium viscosity 0.8872 mPa s^{-1};) were determined by dynamic light scattering (DLS, Malvern, Zetasizer Nano S90) analysis under 633 nm laser light. All measurements were performed in a quartz microcuvette.

3.2.4. Antimicrobial Activity of AgNPs

To evaluate the antimicrobial activity, 7 different microorganisms, including bacteria, yeast and fungus, given in Table 2 were used. Disc diffusion method and minimum inhibitory concentration assays were used to determine antimicrobial activity. Bacteria were incubated at 37 ºC for 24 h on nutrient agar and yeast and fungus were incubated on PDA at 28 ºC for 48 h.

3.2.4.1. Disc Diffusion Method

Disc diffusion assay (Lalitha, 2005) was modified and used in the study to assess the antimicrobial efficacy against individual microorganisms (Figure14). Initially, a 100 µL suspension comprising 10^8 colony forming units (CFU)/mL of bacteria, 10^6 CFU/mL of yeast, and 10^4 spores/mL of fungi was prepared using freshly cultivated cultures. This suspension was then spread evenly on NA for bacteria and PDA for fungi. Blank disks with a diameter of 6 mm were impregnated with 10 µL of sterile distilled water containing 0.005 g of silver nanoparticles (AgNPs). These disks loaded with AgNPs were then placed on the inoculated plates. Ofloxacin disks (5 µg/disc) were used as positive control for bacteria, while nystatin disks (100 units/disc) were used for yeasts and fungi. Following inoculation, the plates were incubated at 37 °C for 24 hours for bacterial strains, 48 hours for yeast strains and 72 hours at 28 °C for fungal strains. Evaluation of antimicrobial activity in the modified disc diffusion assay was performed by measuring the zone of inhibition against the test microorganisms (Kalaycı et al., 2013).

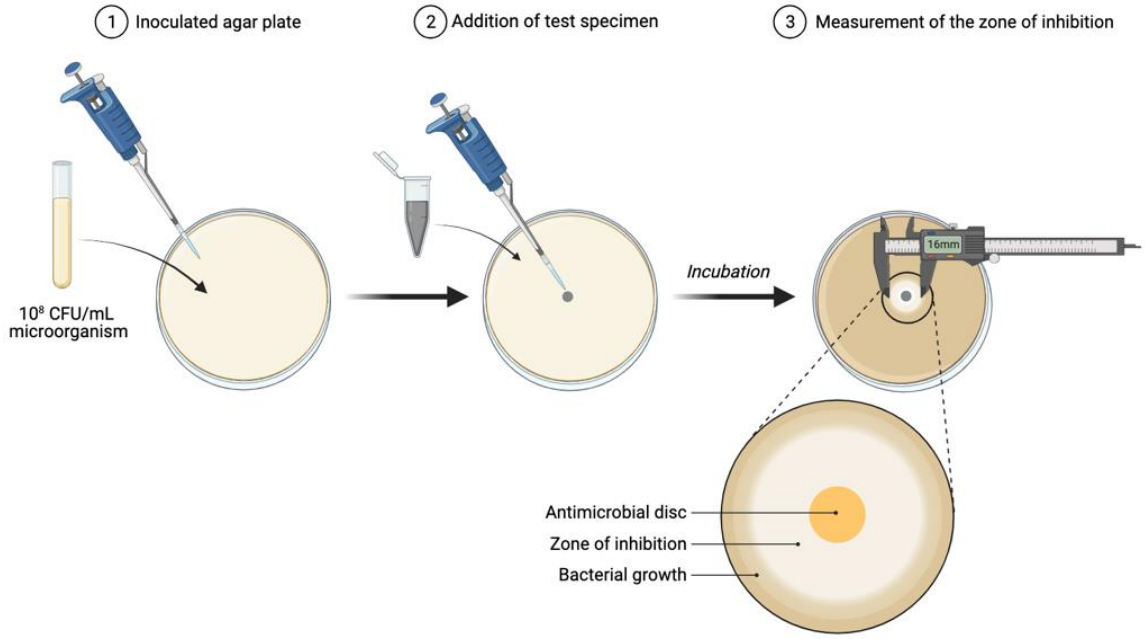

Figure 14. Schematic representation of disc diffusion method

3.2.4.2. Minimum Inhibitory Concentration of Green Synthesized AgNPs

Microbial species listed in Table 2 were inoculated onto nutrient agar (NA) for bacterial strains and onto PDA for yeast and fungal species. The inoculated plates were then incubated at 36±1 °C for 24 hours for bacterial strains, 36±1 °C for 48 hours for yeast, and at 27±1 °C for 72 hours for fungal isolates. Fresh cultures were employed to optain 0.5 McFarland microbial suspensions. Bacterial and yeast suspensions were formulated in sterile PBS, while fungal suspensions were prepared in a solution of 0.5% (v/v) Tween 80 with PBS. Silver nanoparticles (AgNPs) were dissolved in sterile ultrapure H2O within sterile testing tubes, achieving a concentration of 500 µg/µL. For MIC tests, 96-well plates were employed. Initially, 200 µL from the stock solutions of AgNPs, prepared at a concentration of 500 µg/µL, was dispensed into the first well. Subsequently, 100 µL of sterile ultrapure water was poured to the following nine wells. Then, 100 µL from their serial dilutions was sequentially transferred into the next nine wells. Finally, 95 µL of broth and 5 µL of each inoculum were introduced into every well. Nutrient broth (NB) was utilized for bacterial species, while potato dextrose broth (PDB) was employed for yeast and fungal species. In the 11th wells, 200 µL of broth served as the positive control, while the 12th wells contained 195 µL of broth and 5 µL of the inoculum, acting as the negative control. Plates were placed in a shaker incubator set at 180 rpm and maintained at

37°C for 24 hours for bacterial species, while yeast required 48 hours, and fungal strains were cultured for 72 hours at 28°C. Microbial growth inhibition was assessed by measuring absorbance at 600 nm for bacteria and 530 nm for fungal isolates using a microplate reader.For determining the Minimum Bactericidal/Fungicidal Concentration (MBC/MFC), the lowest concentration of AgNPs inhibiting microbial growth was identified from each well for each microorganism. Subsequently, aliquots from these wells were spread onto NA and PDA for bacteria and fungi, respectively. Following incubation for 24 hours at 37°C for bacteria and 48 hours at 28°C for fungi, microbial colonies were enumerated, and MBC/MFC values were established. Schematic illustrations of MIC and MBC assays wer given in Figure 15.

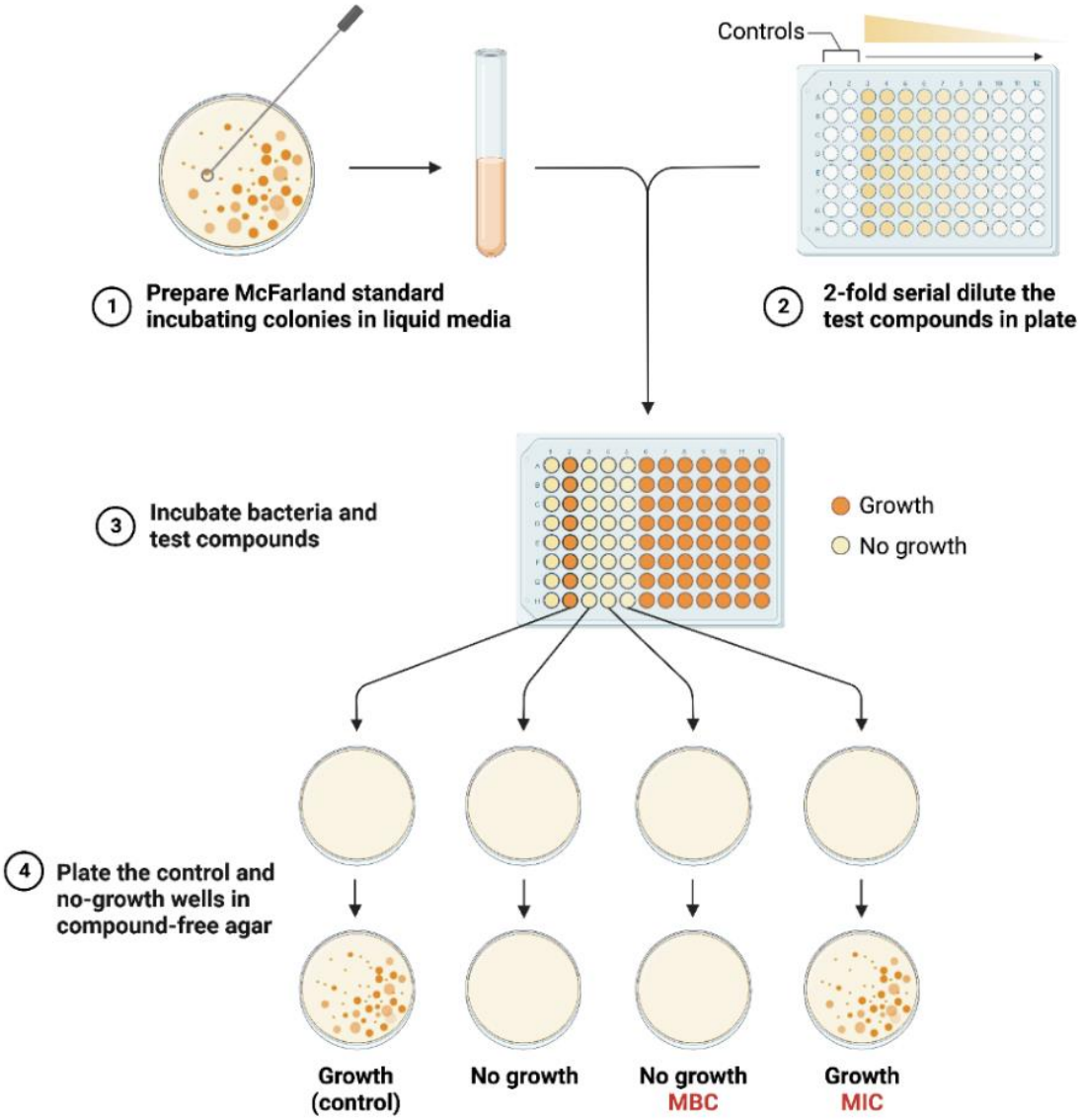

Figure 15. Schematic representation of minimum inhibitory concentration assay

3.3. Preparation of Chitosan-Gelatin-AgNP Edible Films

3.3.1. Chitosan Preparation

First of all, 99 mL of distilled water was poured into the beaker. The beaker flask on the magnetic stirrer was mixed at 10000 rpm and the magnetic fish was placed into the beaker flask. While mixing the water, 1 mL of acetic acid was added with an automatic pipette. Then, acetic acid and water were mixed at room temperature for 1 min, and 2 g of chitosan was slowly poured out. The beaker was covered with parafilm and the chitosan solution was stirred at room temperature for 24 hours.

3.3.2. Gelatin Preparation

After adding 2 g gelatin to 100 mL distilled water, the beaker was covered with parafilm and the gelatin solution was prepared by stirring in a thermoshaker at 50 oC for 30 min. Gelatin-AnNP mixtures (Solution Bs) obtained by adding 4 different amounts of AgNP-4 (NPs prepared using Extract-4) to the obtained gelatin solution were used for the preparation of edible films. Solutions B1, B2, B3, B4 were weighed in the order of AgNPs using the amounts in Table 5, added to beakers containing 100 mL of gelatin each and mixed on a magnetic stirrer at 10000 rpm for 30 min. Solution B4 was designated as a control sample and no AgNPs were added.

3.3.3. Preparing AgNP Loaded Edible Films

First, 5.25 g of Solution A was poured into each of 4 different beakers and 1.75 g of Solutions B1, B2, B3 and B4 were poured over each of them. The prepared mixtures were placed on a magnetic stirrer and stirred at 1000 rpm for 30 min. 0.1 g of glycerol was added to each sample and mixed at 1000 rpm for 2 hours until homogenized. 0.1 g of glycerol was added to each sample and mixed at 1000 rpm for 2 hours until homogenized. The contents of the components of the film solution are given in Table 5. The homogenized film solutions were poured into plastic petri dishes and allowed to dry in a controlled atmosphere in a fume hood for 5 days. The preparation of the film solution is briefly shown in Figures 16 and 17, while the resulting edible films are shown in Figure 18.

Table 5. Components of membrane solutions and quantities of components used in membrane preparation

	Chitosan	Acetic Acid	Water
Chitosan Sol (A)	2 g	1 mL	99 mL

Gelatin Sol (B)	Gelatin	Water	AgNP
B1	2 g	100 mL	0.010787 g
B2	2 g	100 mL	0.021574 g
B3	2 g	100 mL	0.032361 g
B4	2 g	100 mL	-

Sample Name	Sol A	Sol B	Glycerol
Y1	5.25 g	1.75 g	0.1 g
Y2	5.25 g	1.75 g	0.1 g
Y3	5.25 g	1.75 g	0.1 g
Y4 (Control)	5.25 g	1.75 g	0.1 g

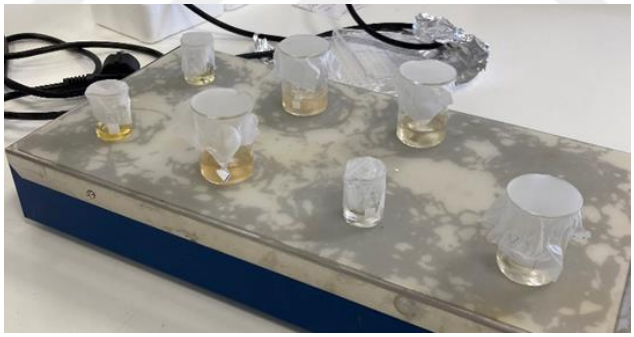

Figure 16. Preparation of edible films

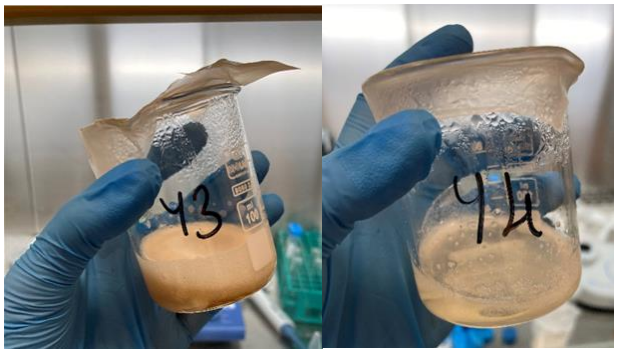

Figure 17. After mixing Y3 and Y4 film forming solutions

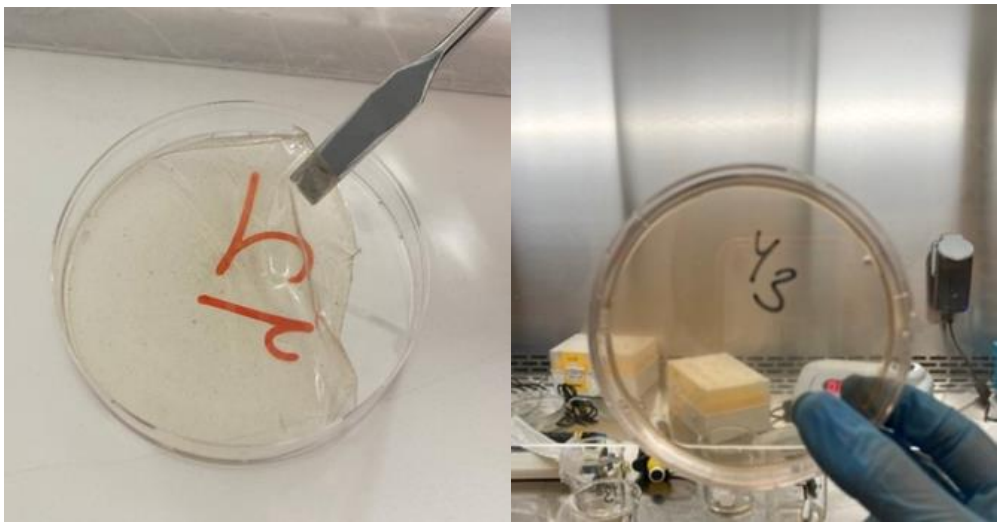

Figure 18. Y2 and Y3 after 5 days dried edible films

3.3.3.1. Antimicrobial Activity of Chitosan-Gelatin-AgNP Edible Films

The dynamic shaking flask method was used to calculate the antimicrobial activity of AgNP loaded edible films. 0.2 g of each sample was cut and the samples were subjected to UV sterilization for at least 30 min on both sides. NP-free control films were used as negative controls. Microbial suspensions of 0.5, 1 and 1 McFarland were prepared for bacteria, yeasts and fungi, respectively. 9 mL PBS and 100 µL microbial suspension were mixed with 900 µL of NB for bacteria and 900 µL of PDB for yeast and fungi. The suspensions were poured into previously sterilized edible films which were placed in sterile test tubes. Test tubes containing microorganism, media and edible film samples were incubated at 37 °C, 37 °C and 28 °C for bacteria, yeasts and fungi, respectively, for 24 hours with shaking at 200 rpm. At the end of 24 h, 10-fold serial dilutions of the suspensions containing edible films were made and 100 µL of each diluted solution was spread onto the relevant agar and incubated at 37 °C for 24 h for bacteria and yeasts and at 28 °C for 48 h for fungi. As a final step, colony numbers were counted and antimicrobial activity was determined as logarithmic and percent reduction.

Equation (1) was used to calculate logarithmic reduction and equation (2) was used for percentage reduction.

$$R = U_t - A_t \quad (1)$$

where;

R is the antimicrobial activity as logarithmic reduction

U_t is the average of the common logarithm of the number of viable cells recovered from the Chitosan-Gelatin-Control edible film after 24 and 48h for bacteria and fungi, respectively.

A_t is the average of the common logarithm of the number of viable cells recovered from the AgNP-Chitosan edible film after 24 and 48 h for bacteria and fungi, respectively.

$$P = (1 - 10^{-R}) \times 100 \quad (2)$$

where;

P is the per cent reduction;

R is the logarithmic reduction

3.3.3.2. Surface Morphology and Mechanical Properties of Chitosan-Gelatin-AgNP Edible Films

Surface characterization of the Chitosan-Gelatin-AgNP edible films was performed using field emission scanning electron microscopy (FE-SEM) on an FEI Quanta 650 instrument. This analysis provides detailed information about the morphology of the films at the nanoscale. For the mechanical characterization of the Chitosan-Gelatin-AgNP edible films, tensile testing was performed using a Stable Microsystems TA.XT Plus texture analyzer. Rectangular specimens (40 x10 mm) were subjected to a crosshead speed of 1 mm/s with a 5 kg load cell under dry conditions. Elongation at break, tensile strength and Young's modulus were then calculated.

3.3.3.3. Water Vapor Transmission Rate

Y3 and Y4 edible films were cut in three replicates with a diameter of 15 mm. 10 mL of water was added to 6 test tubes of 13 mm diameter, covered with the cut films and fixed with parafilm to keep them upright and stable on the tube stand. Each tube was weighed and the initial measurement results were recorded. The samples were kept in a controlled atmosphere for 24 h in 3 different conditions at 4 ^{o}C, 25 ^{o}C and 37 ^{o}C. After 24 h, final measurements were taken.

Equation (3) was used to calculate the water vapor transmission rate of edible films (Ceylan, 2021)

$$WVTR = \frac{Mk-Mt}{A \times t} \quad (3)$$

where;

Mk	weights of the tubes filled with wate
Mt	weights of the membrane sample at the beginning
A	cross sectional area of tube
t	time passed between initial and final measurements

4. RESULTS AND DISCUSSION

4.1. Characterization of Green Synthesized AgNPs

4.1.1. XRD Analysis of AgNPs and Walnut Leaves Extract

XRD analysis was performed to determine the presence of silver in the extracts and to confirm the crystal structures of AgNPs. Also crystallinity and purity of the synthesized nanoparticles were verified with XRD analysis. As shown in Figure 19, no presence of silver was found in the extracts obtained using walnut leaves. The XRD spectrum of AgNPs synthesized using the extract is in agreement with the Ag reference spectrum as shown in Figure 20. The XRD results are similar to those obtained in a previous study (Mohan & Panneerselvam, 2021). This result confirms the successful synthesis of silver nanoparticles with walnut leaf extract. When the XRD data of the synthesized AgNPs were analyzed, it was observed that the sample with the best crystallinity was AgNP-4. Figure 20 shows the diffraction peaks of the green synthesized AgNPs and the peaks were raisen at 37.88, 43.98, 64.36 and 77.31^{o} corresponding to the crystal planes (111), (200), (220) and (311) of the face-centered cubic structure of Ag, respectively (Nasar et al., 2019).

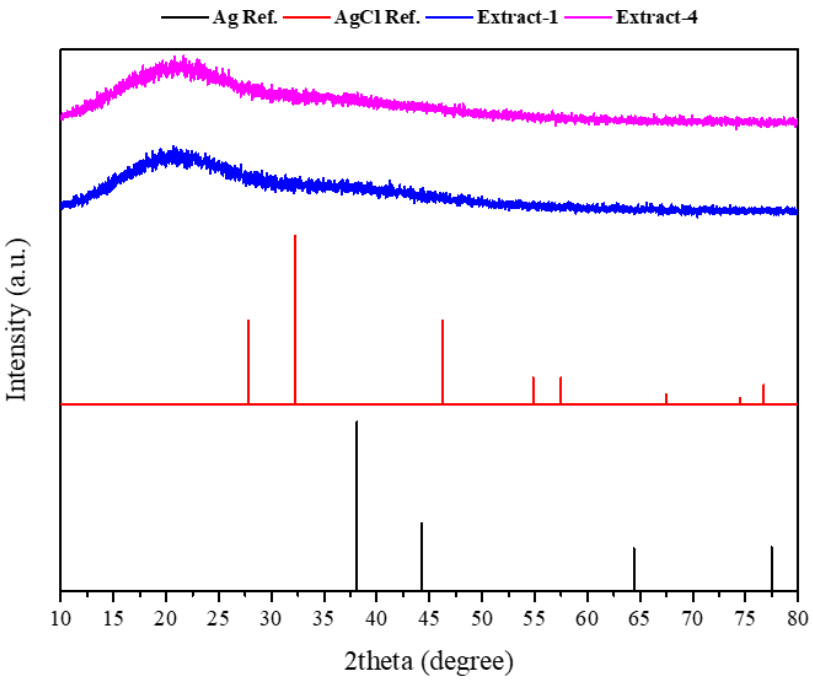

Figure 19. Extract 1 and 4 XRD paterns

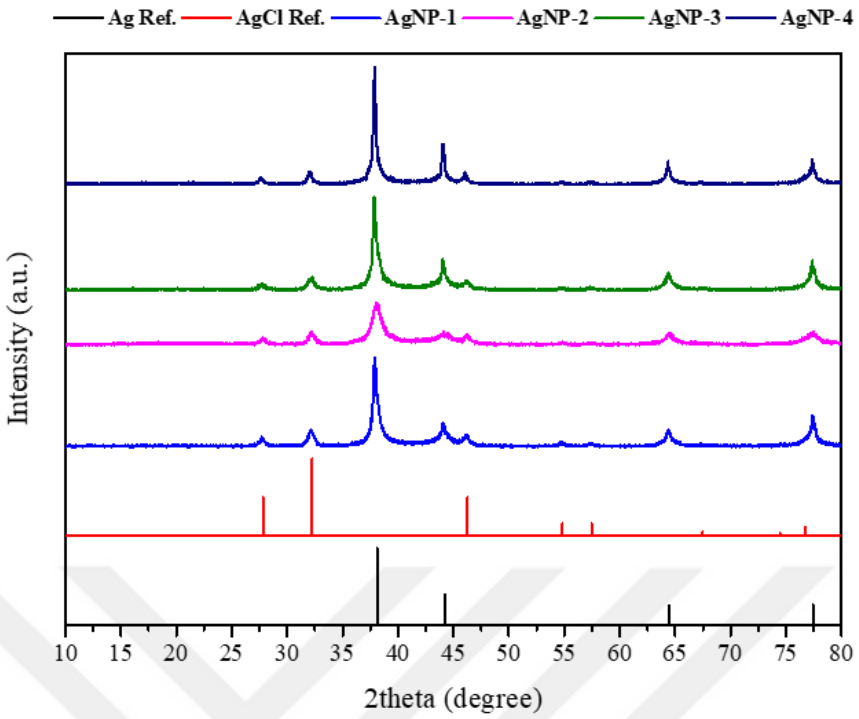

Figure 20. XRD Patterns of AgNP-1, AgNP-2, AgNP-3, AgNP-4

4.1.2. FTIR Analysis of AgNPs

FTIR analysis was applied to determine the bond structures of the synthesized nanoparticles. According to the information obtained from FTIR data, it is seen that 4 different nanoparticles give similar peaks. Extract-4 and 4 different AgNPs gave peaks between 1750 and 1500 cm^{-1}. Although these values are known as aromatic compounds or the amide group of proteins containing benzene rings, they can also be interpreted as phenolic and flavonoid compounds as they emit strong vibrations in this range of values. The peak belonging to the -OH group at 3000-3500 cm^{-1} seen in Extract-4 obtained from walnut leaf may belong to phenolic and flavonoid compounds that play a very important role in nanoparticle synthesis (Jabbar et al., 2020).

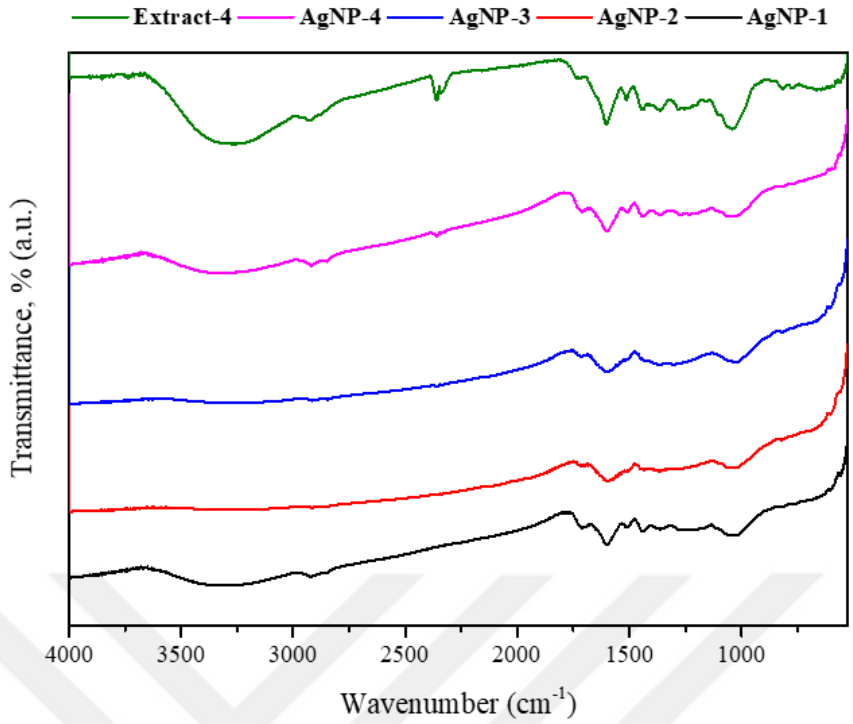

Figure 21. FTIR analysis of Extract-4 and leaf extract mediated syhntesized AgNP-1, AgNP-2, AgNP-3, AgNP-4

4.1.3. SEM Analysis of AgNPs

The morphological properties of AgNPs synthesized green using walnut leaf extract were examined with the help of SEM images. In the image given in Figure 22, no merging or aggregation is observed on the surfaces of the nanoparticles and average particle size was around 50 nm. In addition, it is seen in Figure 22 that AgNPs have a spherical shape and there are no defects or signs of deformation on their surfaces. The morphological properties of the obtained nanoparticles are consistent with previous studies in the literature (Baran, 2019). It was observed that the AgNP in the SEM image was homogeneously dispersed. This feature may allow the nanoparticles to be evenly distributed into the bacterial cells and thus kill the bacteria more effectively.

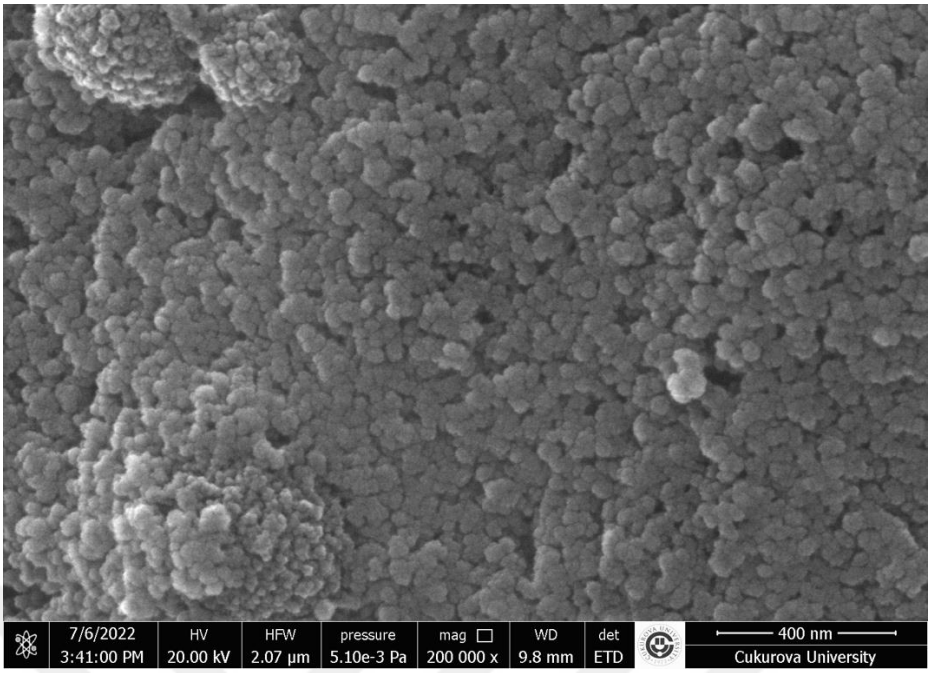

Figure 22. Scanning Electron Microscopy (SEM) image of AgNPs

4.1.4. Zetasizer Analysis of AgNPs

Zetasizer analysis was performed to determine the size distribution of the green synthesized AgNPs using walnut leaves. According to the results, the hydrodynamic diameter measured by volume was determined to be 228.9±49.1 nm, while the hydrodynamic diameter based on intensity was measured more or less the same with a size of 230.3+41.3 nm. These results suggest a relatively narrow and uniform size distribution, which is very important for the consistent performance of nanoparticles used in various application areas.

The polydispersity index (PdI) is used to assess the homogeneity of the particle size distribution. High PdI indicates heterogeneity in particle size (Clayton et al., 2016). The PdI value of the synthesized nanoparticles was found to be 0.547, indicating a wide size distribution, but it is noteworthy that the obtained PdI value remains within an acceptable range for certain applications. Further research on optimizing the green synthesis process to reduce the PdI value can be carried out to synthesize nanoparticles with more homogeneous size distribution.

The results show that the Z-average value is larger than the average particle size which indicates a heterogeneous size distribution (Clayton et al., 2016). Heterogeneous size distribution is an expected result in nanoparticles obtained by green synthesis method using plant extracts.

During nanoparticle synthesis, many different components present in the extract can reduce silver ions with different mechanisms and resulting nanoparticles can form at various sizes. Thus, since the synthesized nanoparticles will be obtained in different sizes, there may be a heterogeneous distribution between the sizes of the nanoparticles, which may explain the difference between the Z-average value and the average particle size (Clayton et al., 2016). In the literature, it has been determined that AgNPs of varying sizes have been synthesized in studies using different plant extracts (Al-Sahli et al., 2024).

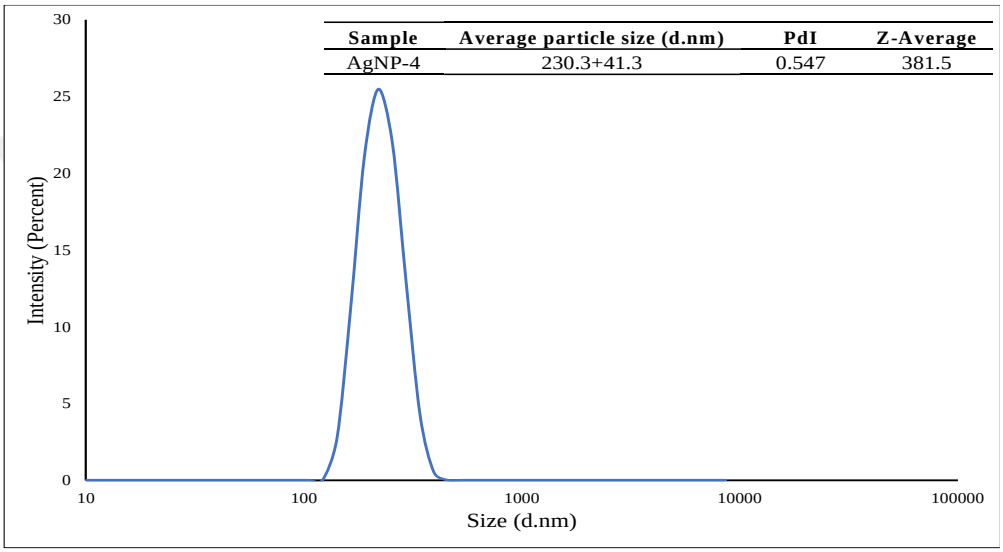

Figure 23. AgNP-4's particle size distribution by intensity obtained by DLS analysis

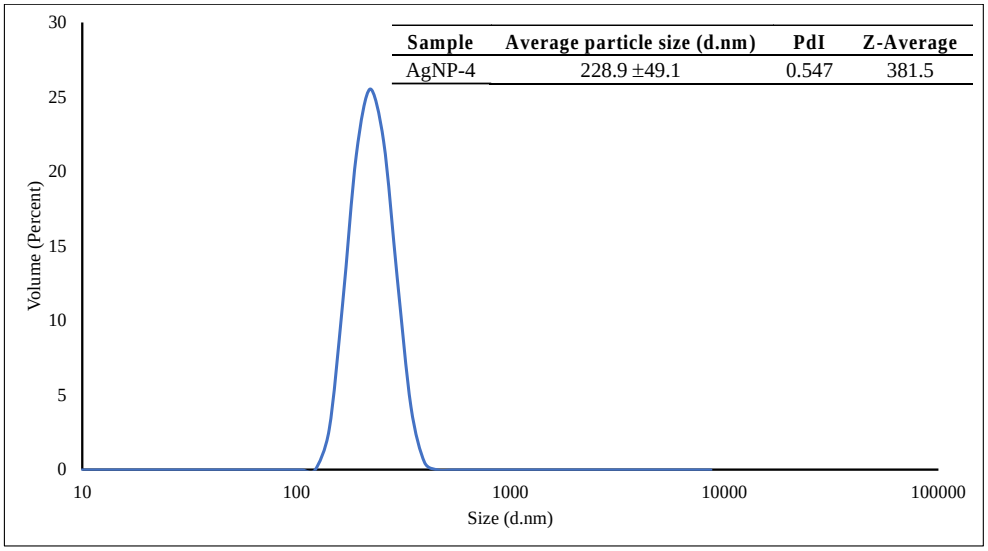

Figure 24. AgNP-4's particle size distribution by volume obtained by DLS analysis

4.2. Antimicrobial Activity Studies

4.2.1. Modified Disc Diffusion Assay

The antimicrobial activity of AgNPs synthesized using walnut leaf extract was evaluated by Modified Disk Diffusion Assay. As shown in Figure 25, clear zones of inhibition were observed around the AgNPs containing disks, indicating antimicrobial activity against the tested microorganisms. The increase in the inhibition diameter indicates the success of AgNPs in inhibiting the growth of the test microorganism.

According to the test resuls shown in Table 6 AgNPs exhibited remarkable antimicrobial activity against all microorganisms used in the experiment. According to the results, none of the AgNPs showed a significant superiority in antimicrobial activity against any of the gram-positive, gram-negative, yeast or fungi. The inhibition diameters showed similar results among the all strains tested. However, AgNP-4 showed slightly higher antimicrobial activity against all microorganism among all AgNPs. Zone diameter of AgNP-4 was changing between 9 – 11 mm. In a study conducted by Nasar et al., AgNPs were also synthesized using walnut leaf extracts.

The antimicrobial activity of AgNPs was explained by three different mechanisms in the literature (Dakal et al., 2016; Marambio-Jones & Hoek, 2010; Qing et al., 2018). The first one is that AgNPs increase cell membrane permeability and cause leakage of cellular contents and subsequent death (Ivask et al., 2014; Seong & Lee, 2017). Studies have also shown that AgNPs can cause structural damage by interacting with sulfur-containing proteins in the cell wall of bacteria, leading to cell wall rupture (Bruna et al., 2021). In the second mechanism, nanoparticles interact with sulfur or phosphorus groups in intracellular contents such as DNA and proteins, changing their structure and function. In addition, they interact with thiol groups in proteins and enzymes and induce the formation of reactive oxygen species (ROS) and free radicals, causing damage to intracellular mechanisms. The third mechanism is proposed to occur in parallel with the other two mechanisms. This mechanism suggests that silver ions released from nanoparticles interact with cellular components including the cell's metabolic pathways, cell membrane and even genetic material due to their size and charge (Gomaa, 2017; Ivask et al., 2014; Quinteros et al., 2016; Webster & Seil, 2012). The literature suggests that

the mechanisms by which AgNPs exert their antibacterial effects may work together or separately (Bruna et al., 2021). Although it can be said that the mechanisms underlying the antimicrobial activity of AgNPs obtained by the green synthesis method in this study can be said to be one of these three, further research is required to determine which mechanism or mechanisms are used in particular.

Table 6. Syhntesized AgNPs disc diffusion inhibition zones (mm)

	AgNP-1	AgNP-2	AgNP-3	AgNP-4
Escherichia coli	9	10	10	10
Pseudomonas aeruginosa	9	9	8	9
Salmonella typhi	8	8	8	10
Staphylococcus aureus	9	9	9	10
Candida albicans	9	9	9	11
Penicilium italicum	10	10	10	11
Aspergillus niger	9	8	10	10

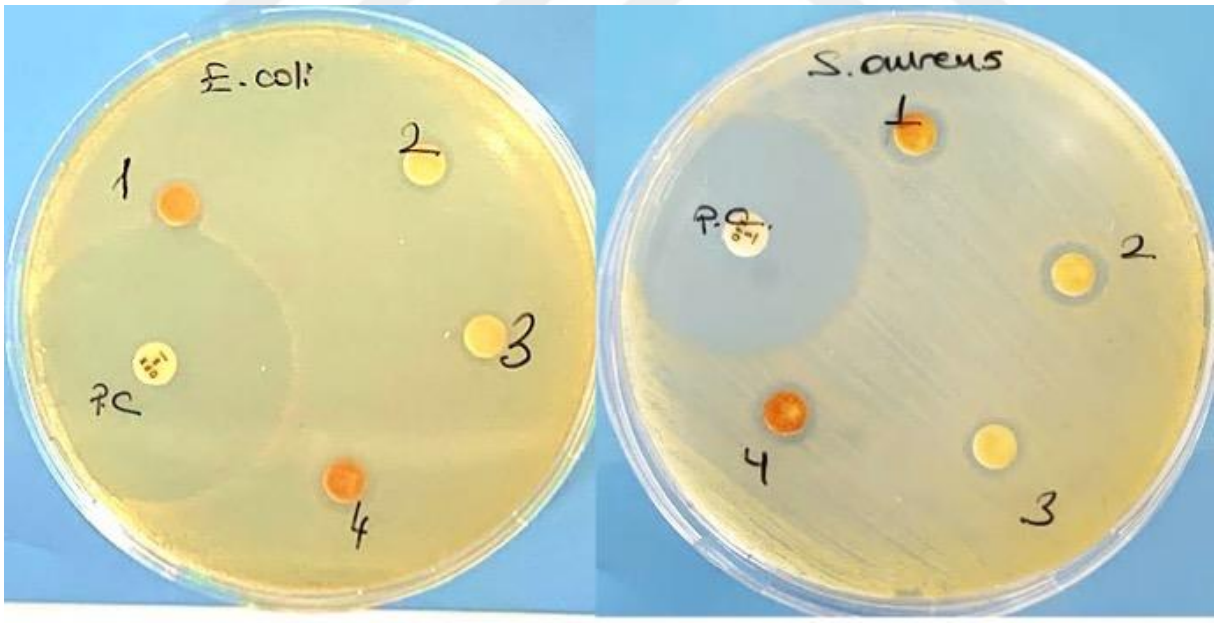

Figure 25. Disk diffusion test results of AgNPs against one selected gram-negative and one gram-positive bacteria

4.2.2. Minimum Inhibitory/Bactericidal (Fungucidal) Concentration of AgNPs

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungucidal Concentration tests were performed to evaluate the antimicrobial potency of AgNPs synthesized using walnut leaf extract against the tested microorganisms and the results obtained were given in Table 7. The results demonstrate the remarkable antimicrobial potency of green synthesized AgNPs using walnut leaf extract over a wide range of microorganisms in the range of 3.9 - 31.25 μg/mL. This broad spectrum activity demonstrates the potential applicability of these AgNPs to combat various infectious diseases. The results reveal comparable and even lower MIC values for AgNPs against fungi among the tested microorganisms which means AgNP-4 can be used at lower concentrations to kill fungal species.

While determining the minimum inhibition concentration, the minimum concentration was calculated by selecting the well with the least microbial growth and no turbidity among the wells as a result of the experiment.

Table 7. MIC and MBC values of AgNP-4

Microorganism	**MIC (μg/mL)**	**MBC (μg/mL)**
Escherichia coli	15.625	62.5
Pseudomonas aeruginosa	15.625	31.25
Salmonella typhi	31.25	31.25
Staphylococcus aureus	31.25	250
Candida albicans	3.9	3.9
Aspergillus niger	3.9	3.9
Penicillium italicum	3.9	3.9

However, effectiveness may vary depending on bacterial species and AgNPs concentration. Our study shows that AgNP-4 has antimicrobial activity against those 7 different microorganisms. More research is needed to better understand the potential applications of AgNPs-4. A study reported that AgNPs have strong antimicrobial activities even when used at low concentrations (Hatipoğlu, 2021). A previous study concluded that AgNPs showed greater antimicrobial activity against *Bacillus subtilis* than $AgNO_3$ and antibiotics. (Hatipoğlu, 2021).

In contrast to walnut leaves, a study concluded that silver nanoparticles showed resistance against *Listeria monocytogenes*, *E. coli* O157:HO7, *Salmonella typhimurium, Vibrio parahaemolyticus* and the MIC values was determined 3.12, 6.25, 3.12, 3.12 µg/mL respectively while all the microorganisms showed the same MBC values of 6.25 µg/mL (Zarei et al., 2014).

4.3. Surface Morphology and Mechanical Properties of Chitosan-Gelatin-AgNP Edible Films

4.3.1. SEM Analysis of Edible Films

Figure 26. shows the surface morphology of an edible film based on chitosan and gelatin. The image shows a distribution of homogeneously dispersed chitosan and gelatin microfibrils with a smooth surface. According to the conclusion drawn from the image, no defects were observed in the edible film. There were no crack or pores on the surface of the AgNPs loaded edible film, the SEM image of which is shown in Figure 26. We see that the nanoparticles added to the films are distributed homogeneously on the film surface.

We can conclude that the Y4 surface, which does not contain AgNPs, and the Y3 surface, which contains AgNPs, have a smooth and homogeneous structure. No defects or damage to the film structure were observed on the surface of the edible film loaded with nanoparticles called Y3. Similar results were obtained in a past study (Dotto et al., 2021).

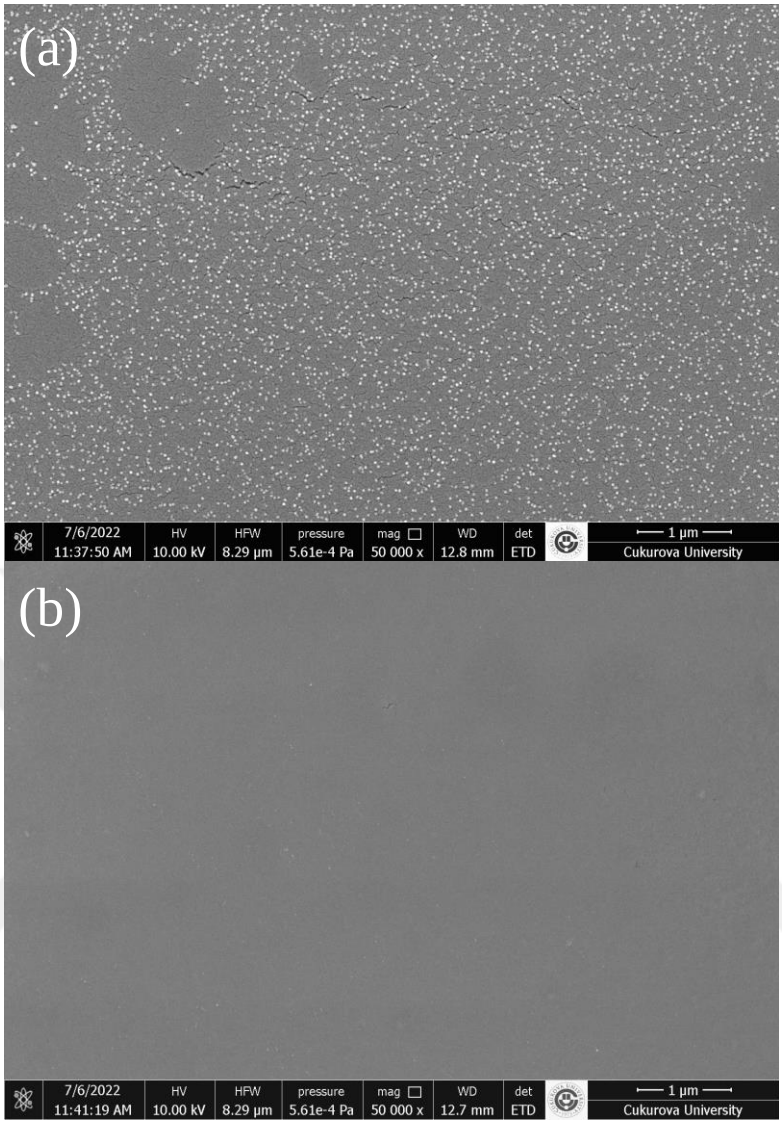

Figure 26. SEM images of (a) AgNP-4 embedded chitosan-gelatin edible film (Y3) and (b) control chitosan-gelatin edible film (Y4)

4.3.2. Mechanical Properties of Chitosan-Gelatin-AgNP Edible Films

The data obtained from texture analysis are shown in Table 8. As can be seen from the data, the addition of AgNPs increased the tensile strength, toughness, Young's modulus and elongation at break of the edible film. The addition of AgNPs to chitosan films increased the stiffness of the membrane by restricting the movement of the chitosan matrix, which explains the increase in tensile strength (Mohamed & Madian, 2020). The results show a strong interaction in

molecular linkages between AgNPs and the Chitosan-Gelatin membrane, leading to stronger and stiffer films with tensile ability (Kadam et al., 2019).

The increase in the mechanical properties of the film is significantly important for films intended to be used as food coating materials and has a positive effect on shelf life. Contrary to our results, a previous study reported that the addition of AgNPs did not significantly affect the mechanical properties of the edible films (Bizymis et al., 2023). According to another study, the addition of AgNPs caused a decrease in mechanical properties (Khater et al., 2023). The addition of AgNPs to chitosan-gelatin edible films can have different effects on their mechanical properties, depending on various parameters such as size, and shape of the nanomaterial, nanoparticle concentration, incorparation method and overall composition of the film (Kadam et al., 2019).

Table 8. Mechanical properties of Chitosan-Gelatin-AgNP edible films

	Tensile Strength (MPa)	**Toughness (MJ/m^3)**	**Young Modules (MPa)**	**Elongation at Break (%)**
Y3	97.285	2.218	7043.370	3.265
Y4 (Control)	58.882	0.624	3614.100	2.05

4.4. Antimicrobial Activity of Chitosan-Gelatin-AgNP Edible Films

The data obtained as a result of the experiment conducted to determine the antimicrobial activity of edible films are shown in Table 9 below. The conclusion from the numerical results obtained is that the edible film named Y3 has the highest antimicrobial effect. Y4, which was chosen as the control sample, did not show any antimicrobial activity.

Based on the data obtained, it can be interpreted that as the silver nanoparticle concentration increases, the antimicrobial activity of the product increases. In literature there are similar results as our study, addition of AgNPs improve the films antimicrobial activity (Kadam et al., 2019). Figure 27 shows the antimicrobial activity results of edible films in terms of percent reduction.

Table 9. Antimicrobial activity of Chitosan-Gelatin-AgNP edible films

Microorganism name	**Y1**	**Y2**	**Y3**	**Y4**	**Y1**	**Y2**	**Y3**	**Y4**
					(At)	***(At)***	***(At)***	***(Ut)***
E. coli	86	10	0	105000	1.93	1.00	0	5.02
P.s aeruginosa	200	140	80	290000	2.30	2.15	1.90	5.46
S. aureus	160	120	0	270000	2.20	2.08	0	5.43
S. typhi	88	0	0	280000	1.94	0.00	0	5.45
C. albicans	3	2	0	63	0.48	0.30	0	1.80
A. niger	5	1	0	10	0.70	0	0	1.00
			R				**P**	
Microorganism name	**Y1**	**Y2**	**Y3**	**Y4**	**Y1**	**Y2**	**Y3**	**Y4**
E. coli	3.09	4.02	5.02	-	99.92	99.99	100	-
P. aeruginosa	3.16	3.32	3.56	-	99.93	99.95	99.97	-
S. aureus	3.23	3.35	5.43	-	99.94	99.96	100	-
S. typhi	3.50	5.45	5.45	-	99.97	100	100	-
C. albicans	1.32	1.50	1.80	-	95.24	96.83	98.41	-
A. niger	0.30	1	1	-	50.00	90.00	90.00	-

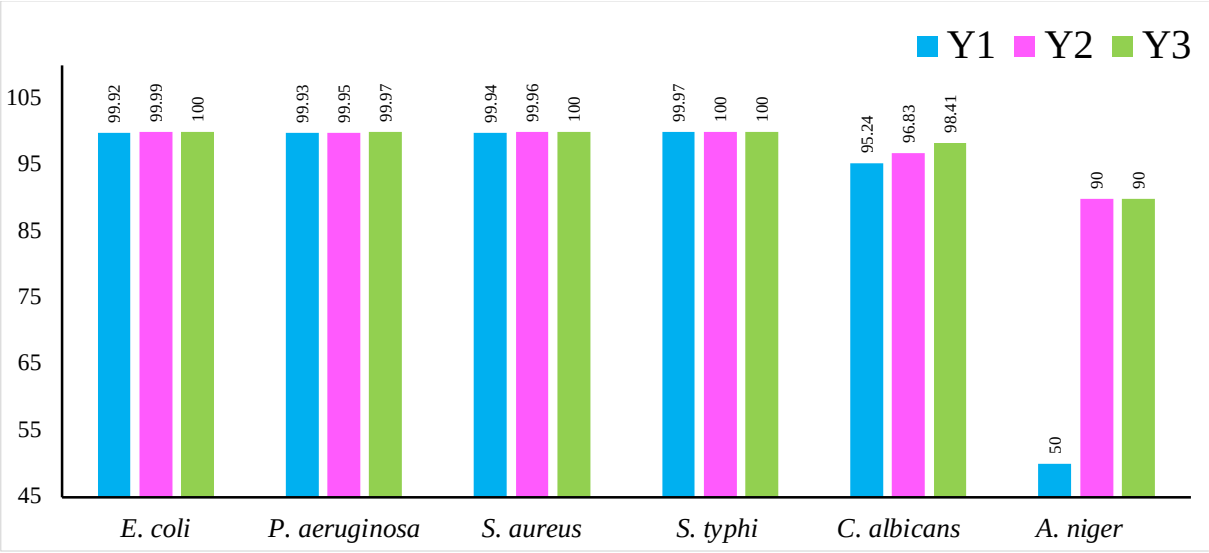

Figure 27. Antimicrobial activity results of edible films in terms of percent reduction

4.5. Water Vapor Transmission Rate

The water vapor transmission rate (WVTR) of the control Y4 film without AgNp was 2420.38 $g.m^{-2}.day^{-1}$; with the addition of silver nanoparticles, the WVTR increased slightly to 2675.16 $g.m^{-2}.day^{-1}$ after 24 hours at 37 ^{o}C. The data obtained as a result of the experiment are shown in Table 10. Similar results were obtained in a previous study and an increase in the water vapor transmission rate (WVTR) of films containing AgNPs was observed (Yoksan & Chirachanchai, 2010).

Table 10. Water vapor transmission rate (WVTR) of AgNP-loaded chitosan-gelatin based films (Y3) and neat chitosan–gelatin based film (Y4) at different conditions

Conditions	**Sample name**	**First measurement (g)**	**Last measurement (g)**	**WVTR ($g.m^{-2}.day^{-1}$)**
37^{o}C for 24 h	Y3	19.76	19.55	2675.16
	Y4	19.55	19.36	2420.38
25^{o}C for 24 h	Y3	19.79	19.67	1528.66
	Y4	19.93	19.83	1273.88
4^{o}C for 24 h	Y3	19.56	19.53	382.17
	Y4	21.72	21.70	254.78

4.6. Shelf-Life Experiment With Strawberry Dip Coating Experiment

To investigate the effect of edible films on food shelf life, strawberry fruits were divided into groups. The first group was immersed in Y3 solution containing AgNPs, the second group was immersed in Y4 solution without AgNP and the third group was kept at room temperature for 7 days without any coating process. The condition of the fruits was monitored and photographed for 7 days. When the changes in the appearance of the fruits at the end of 7 days were observed, no deterioration was observed in the appearance of the strawberries coated by immersion with Y3 and Y4 solution, while some shrinkage and water loss were observed in the uncoated control fruits. As shown in this experiment, the edible films produced have positive effects on the shelf life of foods. Figure 28 shows photographs of strawberries with and without film solution.

The findings of this study are in line with the studies in the literature and show that chitosan-based films can protect fruits physically and microbiologically.(Elsabee & Abdou, 2013; Pavinatto et al., 2020).

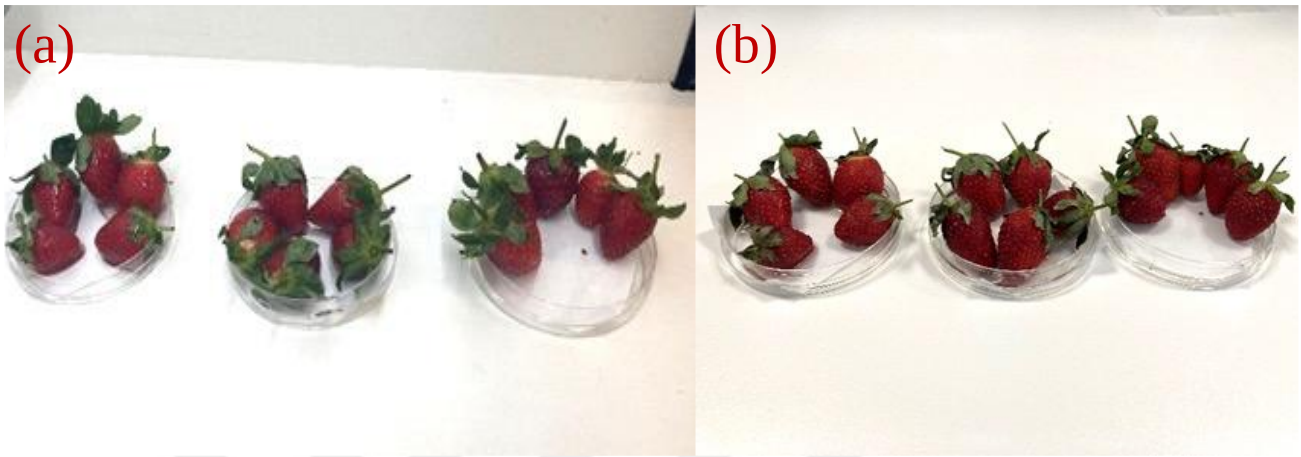

Figure 28. Shelf life experimens of strawberries coated with Y3, Y4, and uncovered strawberried on (a) day 1 and (b) day 7

5. CONCLUSIONS

Within the scope of this master's thesis, green synthesis of AgNPs was carried out using walnut leaf extract and these synthesized AgNPs were incorporated into Chitosan-Jelatin based films and an edible film that can be used in food applications was developed. The antimicrobial properties of the green synthesized AgNPs were determined by disc diffusion method and MIC assay against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhi, Candida albicans, Aspergillus niger, Penicillium italicum* and it was determined that AgNP-4 was the most active among the synthesized nanoparticles. The synthesized AgNPs were characterized using various methods. When XRD data were analyzed, it was observed that AgNP-4 had the best crystallinity. SEM micrographs showed that the average size of AgNPs was around 50nm and they had a spherical shape. Zetasizer results showed that the hydrodynamic diameters of AgNP-4 were measured as 230.3±41.3 nm and 228.9±49.1 nm, depending on intensity and volume, respectively.

Then the AgNP-4 was incorporated into Chitosan-gelatin films in varying rations and antimicrobial activity of these films were determined by using dynamic shake flask method. The antimicrobial activity of edible films containing AgNP increased with increasing AgNP concentration and the highest antimicrobial activity was obtained with the Y3 formulation. The presence of AgNP in the membranes was confirmed by SEM imaging. Mechanical test data revealed that the addition of AgNPs to chitosan-gelatin films increased the tensile properties, Young's modulus, toughess and elongation at break of the membranes. Moreover, AgNP addition increased the water vapor transmission rate of the membranes. Finally, Strawberry fruit was used for the shelf-life experiment and the change in its physical properties was visually observed for 1 and 7 days. According to the results, the physical properties of strawberry fruits coated with an edible film containing AgNPs were significantly better preserved after 7 days. Considering all the results, green synthesized AgNPs added to Chitosan-gelatin films can be considerd as new active food packaging materials to extend the shelf life of foods.

6. RECOMMENDATIONS

Within the scope of the thesis, green synthesized silver nanoparticles were added to the edible film as an antimicrobial agent. Walnut leaf extract was used as a precipitating agent in the production of green synthesized AgNPs. For further studies, experiments can be made by increasing the amount of extract used and decreasing the amount of $AgNO_3$ salt. Also the effect of pH on AgNP synthesis can be investigated. The European Food Safety Authority recommends that AgNPs should not exceed a limit of 0.05 mg/kg in food (Kumar et al., 2018). Therefore, it is important to conduct Ag migration studies to determine the amount of Ag migrating from the developed edible packaging into food. The cytotoxicity of the synthesized AgNPs can be determined. Different food materials can be used for shelf life experiments and the duration of the experiment can be extended. Further studies are needed to determine, the possibility of how much AgNP is removed from the fruits by washing the edible films containing AgNPs just before consumption and the potential impact of AgNPs on the sensory properties of coated fruits.

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