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Green Synthesis of Silver Nanoparticles Using Walnut Shell Powder and *Cynara* sp. and their Antibacterial Activities

Yeşil Sentezle Ceviz Kabuğu Tozu ve Enginar (*Cynara* sp.) Kullanarak Gümüş Nanoparçacıkların Hazırlanması ve Antibakteriyel Özellikleri

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ABSTRACT

The silver (Ag) is a well-known material with interesting properties (i.e. catalytic activity, antimicrobial, etc.). The nano-sized particles of silver propose enhanced properties due to having relatively higher surface areas. The green synthesis is a promising way of material preparation/production being relatively more environmentally friendly by utilization of less harmful materials. In this work, the plant extracts (Cynara & Walnut shell powder) were used as reaction media for the synthesis of silver nanoparticles (Ag NPs). The nanoparticles produced via two plant extracts were ~46 nm and ~109 nm in size, respectively. The antibacterial activities of the produced silver nanoparticles (against E. coli and S. aureus species) were determined and minimum effective concentrations (MIC) for antibacterial activity were investigated.

Key Words

Silver nanoparticle, green synthesis, antimicrobial activity.

ÖZ

Gümüş (Ag) ilgi çeken özellikleri ile (katalitik etkinlik, antimikrobiyal, vb.) bilinen bir malzemedir. Nano boyuttaki Gümüş artan yüzey alanı sebebiyle gelişmiş özellikler sunar. Yeşil üretim görece daha az zararlı malzemelerin kullanılması sebebiyle umut veren daha çevre dostu bir malzeme hazırlama/üretme yöntemidir. Bu çalışmada, gümüş nano parçacıkların (Ag NP) hazırlanması için bitki özütleri (enginar, ceviz kabuğu tozu) hazırlama ortamı olarak kullanılmıştır. Farklı iki bitki özütüyle hazırlanan nano parçacıkların boyutları, sırasıyla ~46 nm ve ~109 nm olmuştur. Hazırlanan Ag NP'ların E.coli and S.aureus suşlarına karşı antibakteriyel etkileri belirlenmiş ve minimum etkili yoğunlukları araştırılmıştır.

Anahtar Kelimeler

Gümüş nano parçacık, yeşil üretim, antimikrobiyal etkinlik.

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INTRODUCTION

Nanoparticles and Green Synthesis

Nanoparticles (NPs) are known as atomic or molecular aggregates [1] and/or groups having at least two dimensions from 1 to 100 nm [2, 3]. The definition may differ according to application areas [3]. There are diverse NPs in different shapes, surface areas, size distributions [4] and structures [3]. Depending on the properties of NPs, they can be used in commercial applications of medical products [5]. The high surface area with small dimension enables a high contact & reaction possibility of NPs at cellular level [3]. The nanoparticles (NPs) are generally produced by two different approaches, namely "top-down" and "bottom-up" [6]. In the former way, the "top-down" NPs synthesis, the nanoparticles (NPs) are produced via diminishing size by several methods (e.g. chemical or laser ablations, sputtering, and mechanical milling) after selection of a proper starting material [3]. Top-down approach may have less production efficiency and may be detrimental to the treated vulnerable material and the environment due to using relatively harsher treatments. The latter approach, called "bottom-up" NPs synthesis incorporates some other techniques (e.g. chemical or electrochemical precipitation, vapour deposition, atomic or molecular condensation, sol-gel and aerosol processes) which mainly builds nanostructures (NPs) from small entities [3, 7]. This approach is principally based on chemical and biological generation/synthesis of the nanomaterial.

NPs synthesis methods can also be classified as physical, biological [8] and chemical methods [9]. While physical methods include methods such as ball milling and spray pyrolysis, chemical techniques include methods such as catalysis, hydrolysis, sol-gel process, photochemical and sonochemical [10] methods. Biological methods, however, have a single-step bio-reduction, but demand less energy and enable eco-friendly synthesis. In the biological methods, enzymes, bacteria, yeast [11], fungi and versatile plant tissues [12,13] can be used. The NPs synthesized via biological methods are reported to be more biocompatible and better choices for medical applications with the presence of natural phytochemicals for suspending (capping) the NPs instead of synthetic chemicals (surfactants) which are hard to degrade, may be hazardous for health and less environmentally friendly [3]. It has been reported that different types of silver nanoparticles can be produced from different plant tissues [3, 14], more rapid and cost effective than the case using microorganisms [6, 15, 16].

Green chemistry/synthesis with its well-known 12 principles listed [17] is aiming to create/renew chemical processes in order to protect human health, to decrease the environmental impact and do this in an economically feasible way to assure sustainability. Biosynthesis of NPs via plants (tissues; leaves, roots, shells etc.) can be considered as a "green chemistry" or "green synthesis" approach [18, 19, 20], using non-toxic chemicals and ecologically benign solvents [16, 21], harmonizing nanotechnology and plant biotechnology [22]. At the stage of forming metal nanoparticles, bio-reduction occurs with ions coming from the plant. In these bio-reactions, some chemical compounds of plant such as terpenes, alkaloids, sugars, phenolic acids and proteins may play a major role in the reduction and stability (capping) of the nanoparticles [3, 20, 23]. Produced NPs via green synthesis may have versatile applications due to their diverse properties like catalytic and antimicrobial activity [20]. Copper (Cu), silver (Ag), gold (Au) nanoparticles are some examples for successfully prepared metallic nanoparticles (Me NPs) via biosynthesis [23].

Silver Nanoparticles (Ag NPs)

Silver has been used as a disinfection preventing/healing material since ancient times before the utilization of antibiotics (e.g. silver sulfadiazine salt) [3]. Metallic nanoparticles (Me NPs) (e.g. Ag NPs) may have distinctive physical, optical, electronic and chemical properties, in comparison to metal in block form, which enables utilization of them for different applications in various fields (e.g. nanomedicine, electronics) [24, 25]. Different parts of the plants can be used as raw material (e.g. leaves, roots, fruits and/or seeds) for the preparation of nanoparticles [6, 26]. The utilization of plant parts for preparation of Me NPs via green synthesis was reported to be advantageous since the process is eco-friendly, less time-consuming and cost effective [6, 25]. It has been reported that many different plant extracts can successfully be used for the production of silver nanoparticles (Ag NPs) via the "green synthesis" method. Some reported plants used for formation of silver nanoparticles (Ag NPs) are: *Curcuma longa* [27], *Bryonia laciniosa* [28], *Nardostachys jatamansi* [29], *Ocimum sanctum* [30], and more mentioned in the literature [3, 6, 31, 32, 33].

Silver nanoparticles are reported to have possible diverse pharmacological applications like applications demanding antimicrobial effect on bacterial strains [34, 35], cancer therapy [3, 25, 31, 36] and antifungal practices [37, 38]. The preparation of metallic nano particles for cancer therapy via green synthesis makes them more reliable in terms of minimizing the possible side effects and less threatening the patient's health [3]. The Ag NPs are reported to be used in preparation of mesoporous filters for water purification [19]. The Ag NPs were also investigated for developing wound dressing biomaterials with antibiotic property against anti-biotic resistant microorganisms especially for patients with diabetic ulcer for whom the infection on wounds may lead limp amputation [39]. The research on using Ag NPs for preparing antimicrobial catheters resulted in an efficient antimicrobial activity [40]. Thanks to their antibacterial property utilization of biogenic Ag NPs for preparation of gels for treating the burn wounds were also investigated [41]. The biogenic Ag NPs as catalysts were investigated and reported to be an effective catalyst for degradation of synthetic dyes [42, 43]. Another possible application of green synthesized Ag NPs was reported as insecticide (larvicidal effect on mosquitos) reported to be more effective than sole plant extracts [44]. Having potential applications for many purposes, the preparation (green synthesis) and possible antimicrobial activity of Ag NPs is a focus of interest in the current research.

Characterisation of AgNPs

The presence and characteristics of the nanoparticles produced via green synthesis can be done via various methods/equipment like FTIR, DLS, UV-visible spectrophotometer, TEM, SEM, XRD, XPS, AFM in order to characterize them with respect to surface chemistry, size, size distribution, shape, particle morphology and particle composition [37]. The presence and the size properties of the synthesized nanoparticles can be determined either using microscopic techniques (e.g. SEM, TEM, etc.) or light scattering techniques (e.g. DLS (dynamic light scattering)). The DLS technique, considering relatively a higher number of nanoparticles during the analysis at once, has the advantage of presenting information on particle size distribution without any extra effort to consider different zones/portions of the sample as for the microscopic techniques.

The aim of this study was to investigate the possibility of green synthesis of silver nanoparticles (Ag NPs) using the aqueous extracts of different plant origin raw materials, namely *Cynara* sp. leaves (as was reported for other plant leaves in literature [46]) and walnut shell powder (very probably for the first time in the literature) and determine the possible differences in their properties. The antimicrobial activity of silver nanoparticles (Ag NPs) produced by green synthesis via these two plant extracts was also investigated via antimicrobial assays to determine their potential for antimicrobial practices.

MATERIALS and METHODS

Preparation of the plant extracts

Cynara sp. was obtained from a local market in Kayseri (Turkey). To prepare *Cynara* aqueous extract, initially fresh plant leaves were thoroughly washed by distilled water, then allowed each of them to dry at room temperature. Dried leaves were cut into pieces and a portion of 5 grams were taken and mixed into 25 ml distilled water. The mixture was placed on magnetic stirrer and boiled for 15 minutes being partially sealed to prevent to excess evaporation of water. After cooling the extract was filtered through Whatman No.2 filter paper. The extract was centrifuged (5,000 rpm, 5 minutes, Hettich Rotina 380) to remove undesirable rough plant fragments. Then 2.5 ml of light yellowish supernatant was taken and stored at 4°C. Unlike plant leaves, walnut shell was first grounded as a fine powder (<400 micron) via ball milling (Retsch MM400). 3 grams of powder was mixed into 15 ml distilled water and as for the leaves it was boiled while stirring. Then 2.5 ml of the extract supernatant was taken and stored at 4°C.

Biosynthesis of AgNPs

In order to synthesize Ag NPs, 2.5 ml of plant extracts individually were added to 1 M 22.5 ml aqueous solution of silver nitrate ($AgNO_3$, Sigma 85228). The addition of the plant extract to the silver nitrate solution, being stirred, was performed dropwise. The mixture was kept stirring until the colour change from light yellowish colour to amber (more brownish) colour either at room temperature or elevated temperature. The silver ions (Ag^+) in the reaction medium were reduced by the electrons donated by chemical species in the plant extract (phytochemicals like flavonoids, phenols, tannins, etc. depending on the plant) to form metallic silver (Ag^0). This reaction results in a colour change in the sample

from yellowish to brownish (i.e. amber). This colour change was also reported in the literature as the sign of metallic Ag NPs formation, having surface plasmon resonance activity which results in colour formation [16, 25, 41]. The colour change was observed in order to monitor the progress of the reaction forming metallic silver. It took place spontaneously in general and if not, the samples were heated and kept at elevated temperature stirring for some minutes to complete the amber colour formation. The yellowish and amber colour samples are shown in Figure 1. The Ag NP suspensions were stored at 4° C for antimicrobial assays.

Characterization of Ag NPs

The nanoparticles were characterized by determining their particle size distribution using the dynamic light scattering (DLS) technique (Nano ZS, Malvern Co., UK, He-Ne laser with wavelength: 633 nm). The nanoparticles (Ag NPs) performing Brownian motion in the suspension scatters the laser light while passing through the laser beam, with respect to their sizes, and the intensity of the scattered light was used in Schmoluchowsky Equation to determine the particle size distribution (PSD) and the average (hydrodynamic) diameter (i.e. average particle size, APS) for the nanoparticles in the suspension. The utilization of DLS techniques for particle size determination has the advantage of considering relatively higher number of particles (which are passing through the laser light path during analysis), in comparison to microscopic techniques in which relatively fewer static particles can be considered in the restricted area of interest.

The scanning electron microscope with energy dispersive X-ray spectroscopy device (SEM-EDX, Zeiss Gemini) was used for taking micrographs and elemental analysis of biosynthesized Ag NPs. The samples for SEM-EDX analysis were prepared by addition of some drops of Ag NP suspensions on Carbon tape on a stab and drying at room temperature.

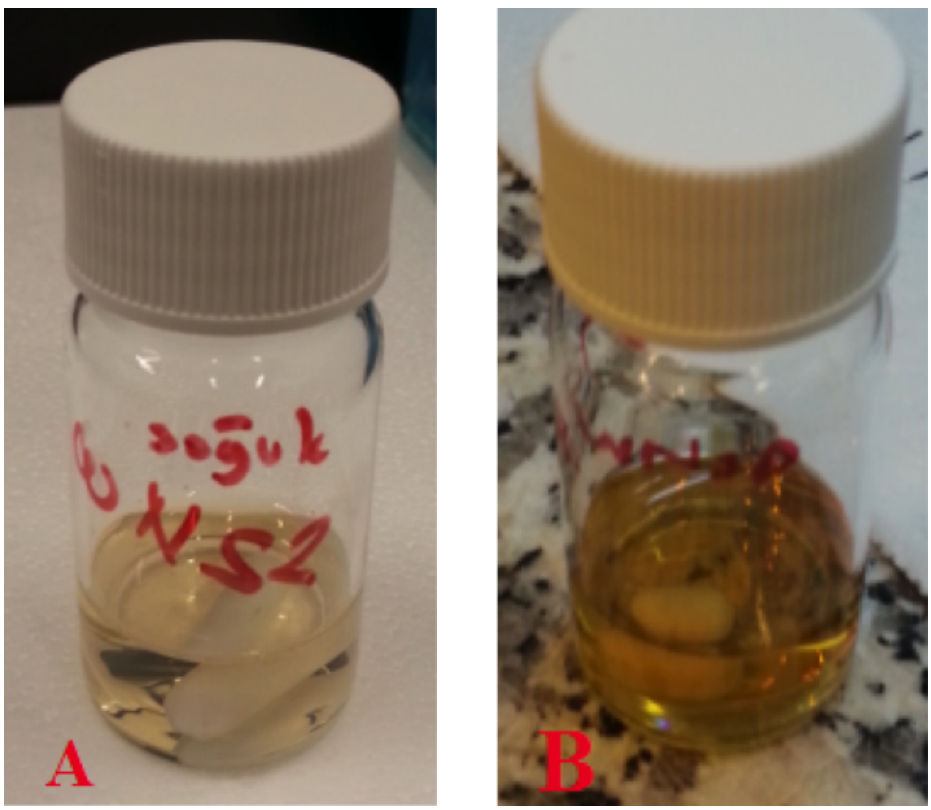

Figure 1. Heat treated (B) and untreated (A) Ag NP suspensions prepared via walnut shell powder (WNSP).

Preparation of Bacteria Culture and Antimicrobial Tests

The dehydrated TSA (Tyriptic soy agar, Oxoid CM 0131, 40g/L) medium was dissolved in 400 mL of distilled water, as 16 g/L, by heating on a magnetic stirrer. TSB (Tyriptic soy broth, Oxoid CM 0129, 30 g/L) medium was similarly dissolved in 400 mL of distilled water as 12 g/L. Both prepared mediums have clear and yellowish-brown colour.

0.9% NaCl solution was prepared (1.8 g in 200 mL) for dilution and absorbance measurement. It was shared to 10 mL glass tubes. All utensils, NaCl solutions and mediums were sterilized by autoclaving at 121° C for 15 min. After autoclaving TSA medium was poured into sterile petri dishes in a biosafety cabinet (Faster, SafeFast Elite), cooled till TSA solidifies.

E. coli is a bacterium normally present in the intestines of animals. *S. aureus* is one the most common causative agent of many of human infections. The antimicrobial tests were performed via *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) strains. The freshly incubated colonies were transferred to the NaCl solutions (preparation was described above) to have an absorbance value of 0.1 at a wavelength of 620 nm (Shimadzu UV-1800). The necessary inoculations and dilutions were performed by using these stock bacteria suspensions.

Antibacterial activity assay of *Cynara* leaf and walnut shell powder (WNSP) extracts mediated Ag NPs was performed against the selected bacterial pathogens by disc diffusion method. For this method, Whatman no.2 filter paper was punched in disc form to have a diameter of 6 mm. 5 µL of each sample (Ag NPs formed in extracts) was added on these discs in the biosafety cabinet and dried before placing on the inoculated TSA petri dishes. The petri dishes were fragmented into different regions and coded. Then they were inoculated using fresh bacteria suspension (with 0.1 Abs. at 620 nm wavelength), the discs with and without Ag NPs were placed on the coded regions and bacterial growth was monitored while incubating at 37° C for 48 hours (Memmert, INE 400).

The MIC (minimum inhibitory concentration) assay was performed via 96 well-plate. The different dilutions of Ag NPs prepared either by using walnut shell powder (WNSP) or *Cynara* were prepared in 15 mL sterile conical tubes with TSB. The inoculum was 20 µL and the total volume per well was 2 mL. The analysis was done with a pair of wells for each concentration. Last two columns were left with no bacteria inoculation as control groups. The growth in the wells after incubation at 37° C for 24 hours was determined by measuring the absorbance via a plate reader (Varian).

RESULTS and DISCUSSION

Particle Size Distribution (PSD) analysis

Particle size measurements of nanoparticles in solutions were performed by Malvern Nano ZS. The average particle size (APS) for heat treated (amber) nanoparticle solution prepared via walnut shell powder (WNSP) was determined as 109.3 nm (polydispersity index (PDI): 0.204). The APS for the similar solution without heat treatment (light colour) was 92.9 nm (polydispersity index (PDI): 0.083). The reduction reaction of silver seems to affect the APS of Ag NPs (in ionic or metallic form) slightly. The particle size distribution of Ag NPs via WNSP extract with or without heat treatment can be seen in Figure 2.

The average particle size (APS) for heat treated (amber) Ag NPs via *Cynara* extract was 46.3 nm (polydispersity index (PDI): 0.364), of which particle size distribution can be seen in Figure 3.

The particle size distribution was not in a wide range as indicated with low PDI (polydispersity index) values. The PDI values can be in the range of 0 to 1. The lower PDI values indicate the homogeneity of the particle size distribution, i.e. having most of the particles in a smaller size range (with similar sizes). The prepared Ag NPs are all with low PDI values ensuring the particles in the suspension have similar sizes. The APS for Ag NPs prepared with *Cynara* extract was around half of the APS of Ag NPs prepared via walnut shell powder (WNSP), 46 nm vs. 109 nm, respectively, with applied synthesis parameters. The PDI values were not high for both samples (0.204 and 0.364) showing the nanoparticles have monodisperse, relatively homogeneous size distribution (i.e. having relatively similar sizes). The PDI value before formation of metallic Ag from ionic Ag was found to be smaller (0.083 vs 0.204, respectively) indicating the formation of metallic Ag results formation of NPs with wider size distribution.

SEM-EDX Analysis

The exemplified SEM (scanning electron microscope) micrograph is shown in Figure 4 which is for Ag NPs

prepared via *Cynara* extract. The Ag NPs were within a particle size range of around 7 to 44 nm in diameter having sphere-like shape. This result is in accordance with previous records in the literature, where different size ranges (e.g. 30-40 nm, 50-150 nm) and shapes (e.g. spherical, quasi-spherical, triangular) were reported [5, 7, 14, 21, 32, 45]. The results of SEM analysis are in accordance with the results of PSD (particle size distribution) analysis via DLS (dynamic light scattering, APS: 46 nm). The slightly higher values for PSD analysis via DLS may be a result of possible partial coagulation of Ag NPs resulting determination of relatively larger particles during DLS analysis.

The EDX (energy dispersive X-ray spectroscopy) analysis was performed to confirm the presence of metallic Ag in the form of nano particles and result is shown in Figure 5. The formation of Ag NPs was confirmed with the characteristic Ag peak around 3 keV. There is a considerable C peak due to the Carbon tape holding the Ag NPs on the metal stab.

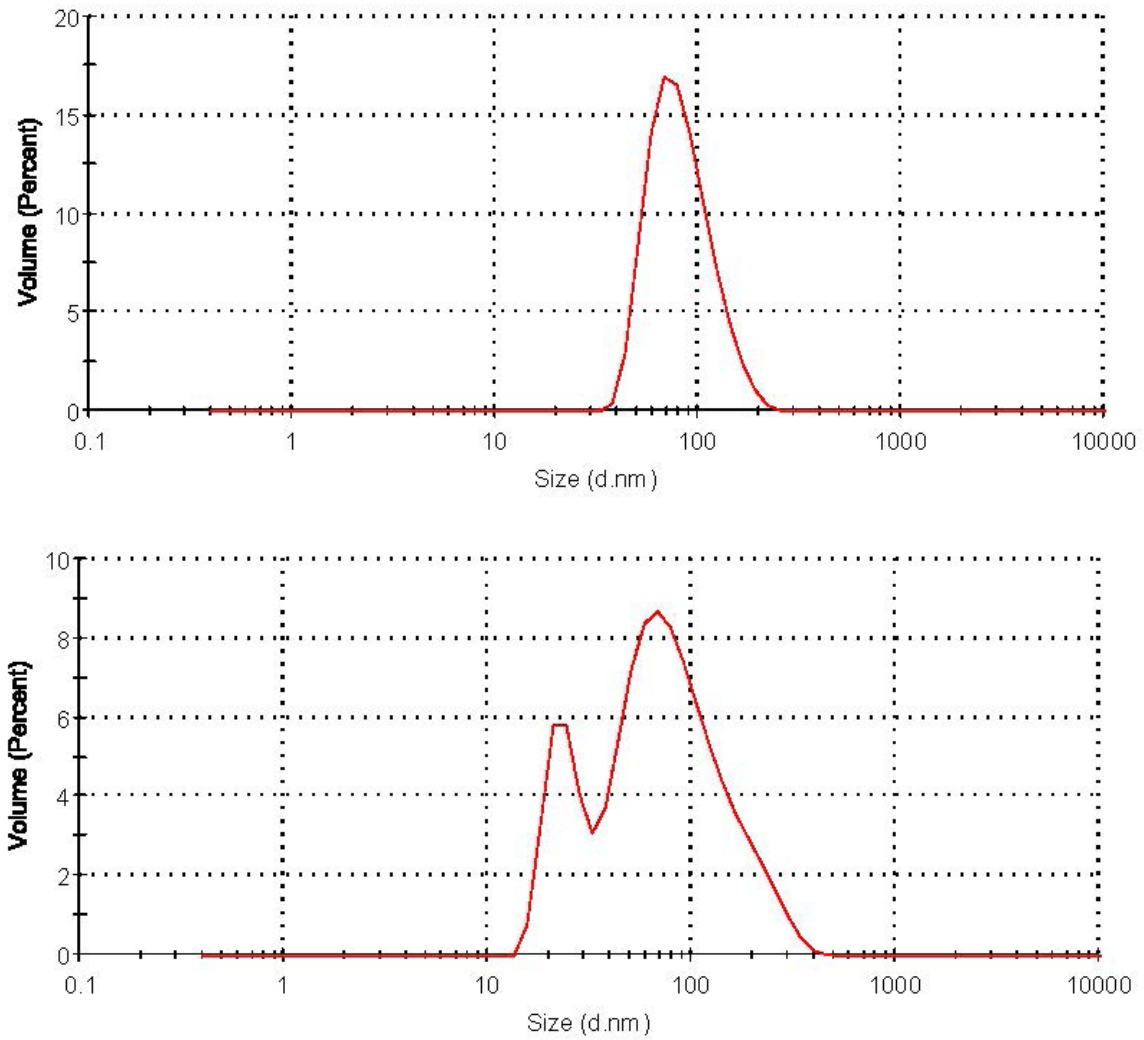

Figure 2. The PSD graph for Ag-NPs via walnut shell powder (WNSP) before heat treatment (light colour) (upper graph) and after heat treatment (amber colour) (lower graph).

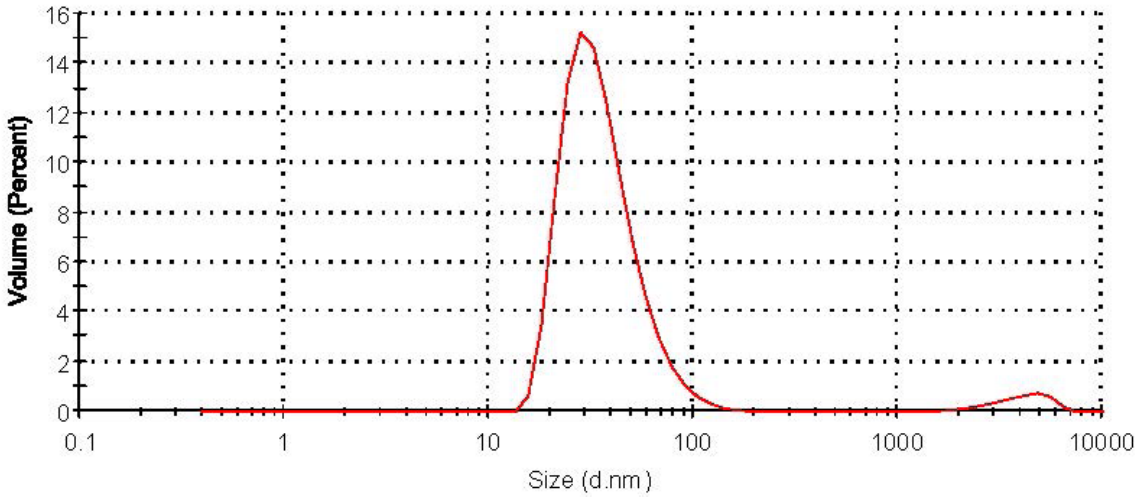

Figure 3. The PSD graph for Ag-NPs via Cynara after heat treatment (amber colour).

Antibacterial Activity Tests

Disc Diffusion Assay

Slight zone formations were observed for the discs, which were with Ag NPs suspension addition. It was more evident for petri dishes with Ag NPs via walnut shell powder (WNSP) as shown in Figure 6. The loaded amount of the Ag NP suspension on the discs may be the main parameter effecting the zone formation (zone size) without bacterial growth.

Minimum Inhibitory Concentration (MIC) Assay

The results of MIC assay are given below in Figure 7. For two samples (via *Cynara* and walnut shell powder (WNSP) extracts) containing 25 % (volumetric) extract with nanoparticles for *E. coli*, the absorbance is almost zero; bacteria development was obviously inhibited. Growth of *S. aureus* at these concentrations was unhindered. The MIC for *E.coli* can be calculated as 375 ng Ag / mL for both samples via *Cynara* and WNSP. This concentration is far more lower than the concentration reported by Nayak, *et al.* [31] which was 12.5 to 25 µg / mL (i.e. 12,500 to 25,000 ng / mL) and also by Jadhav, *et al.* [41] which was 0.99 to 7.93 µg / mL (i.e. 990 to 7,930 ng / mL). Nakkala, *et al.* investigated the bacteriostatic/ bactericidal effect of biogenetic Ag NPs prepared by using *Ficus religiosa* leaf extract against *E. coli* by following growth kinetics in the presence of different Ag NPs concentration, and reported that the concentrations of 10 & 30 µg / mL were bacteriostatic (postponing the logarithmic growth) and only 60 & 100 µg / mL were bactericidal (preventing the growth) [25]. These values are also higher than the values determined as MIC for the Ag NPs suspensions prepared in the current research (via *Cynara* or walnut shell powder (WNSP)). The considerable difference may be a result of different bacteria strain & initial bacterial load (initial CFU) for varying research, but also it may possibly indicate a synergistic effect of biomolecules (which were not washed away) in the present research for a better antibacterial efficiency, which should be further investigated. The possible synergistic effect of co-presence of some phytochemicals (like essential oils) with Ag NPs on microbial cell interruption and cell leakage was reported [32]. Ahmad, *et al.* [46] reported the possible effect of surface characteristics of Ag NPs on their antibacterial activity. The surface of biogenic Ag NPs was modified by chitosan to have different surface characteristics leading better interaction between Ag NPs and the microorganism, and lower MIC values. The MIC values for *E.coli* were 25 µg / mL via biogenic Ag NPs, which decreased to 6.25 µg / mL after surface modification of the nanoparticles with chitosan. The phytochemicals not washed away in the current research (from WNSP & *Cynara*) may also have influenced the surface characteristics (mainly zeta potential), which may be another possible explanation for lower MIC values. Chahar *et al.* also reported higher antimicrobial activity for Ag NPs via green synthesis (via various phytochemicals) than the ones prepared via synthetic chemical stabilizers (e.g. PVA, PVP), may probably indicating the positive contribution of phytochemicals [35].

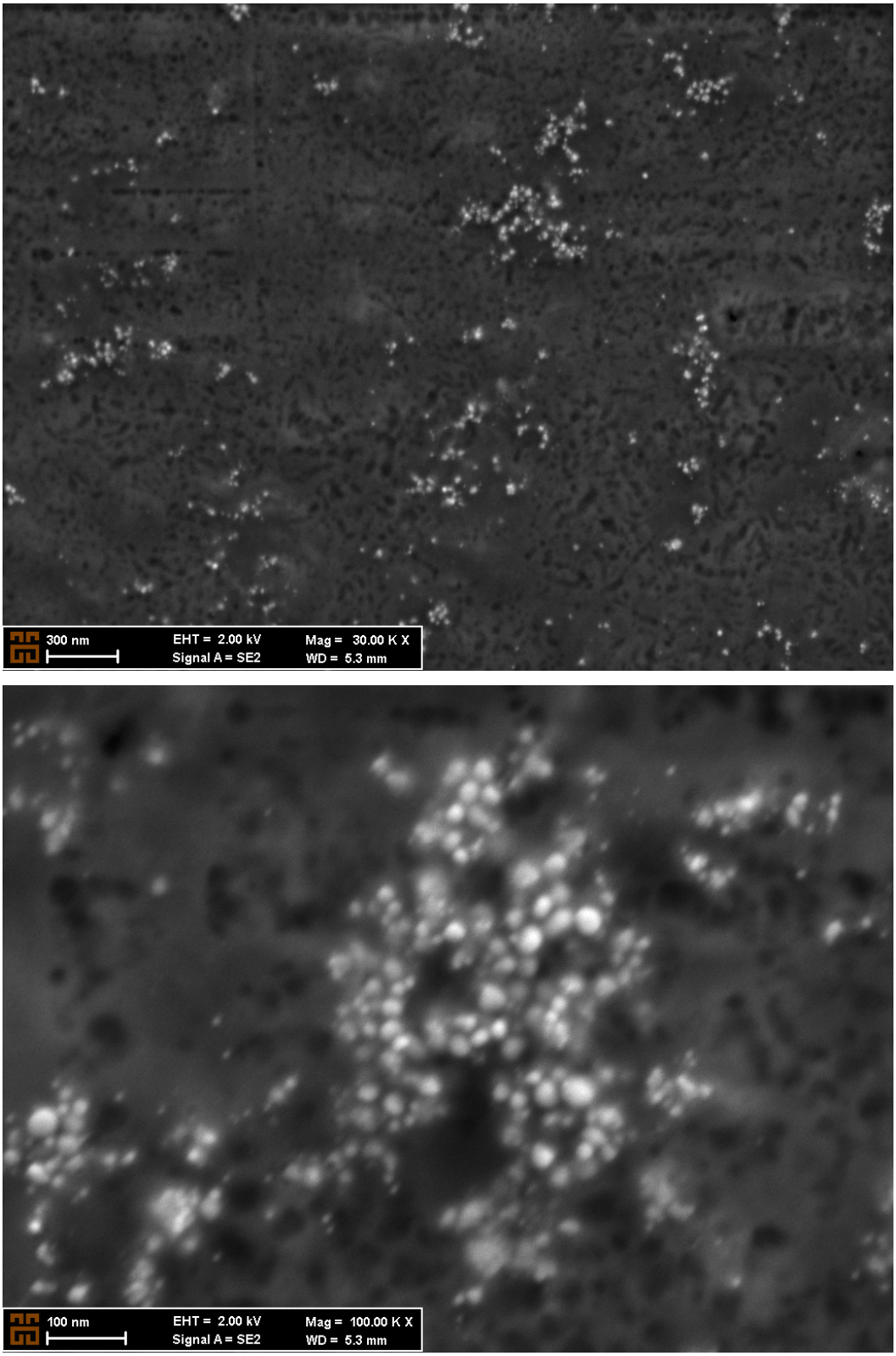

Figure 4. SEM micrograph of biosynthesized Ag NPs (prepared via Cynara extract, after heat treatment (amber colour)) at 30k & 100k magnifications.

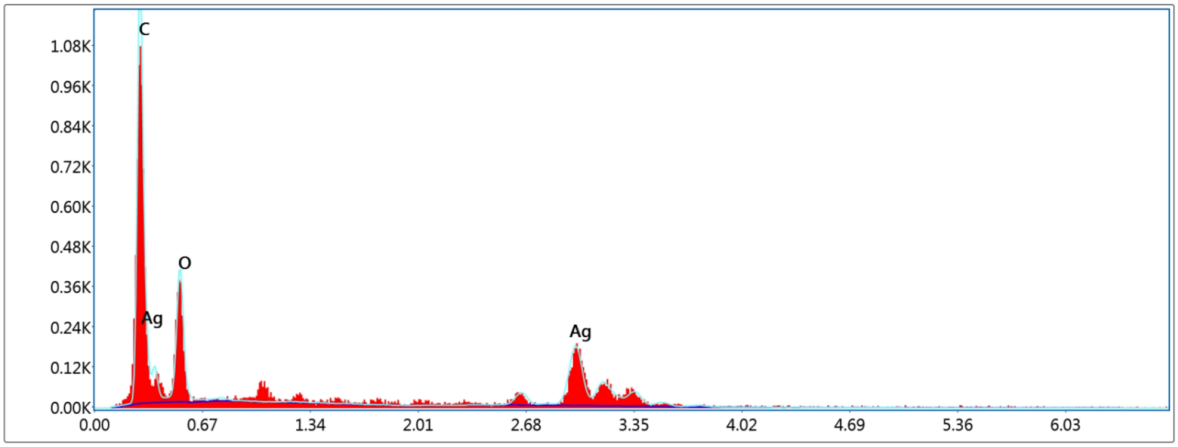

Figure 5. The EDX analysis for Ag NPs (prepared via Cynara extract, after heat treatment (amber colour)).

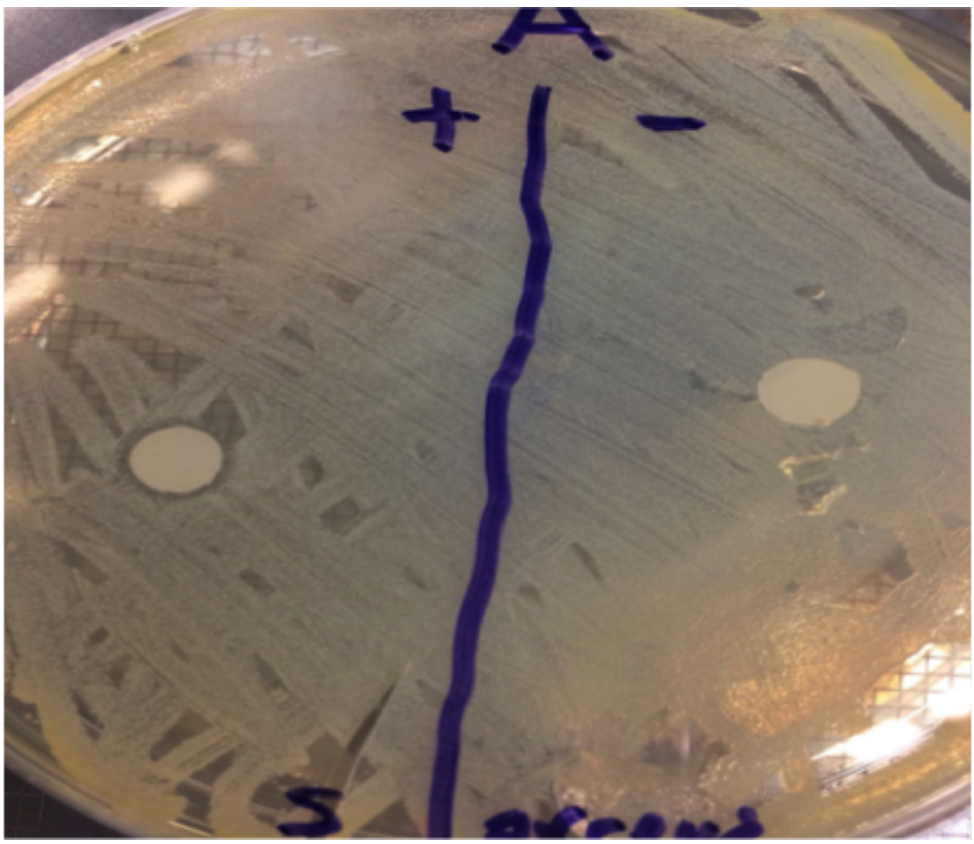

Figure 6. Disc diffusion assay for Ag NPs via walnut shell powder with S. aureus. The (+) sign indicates the disc was with added on Ag NPs, while (-) sign indicates the absence of Ag NPs.

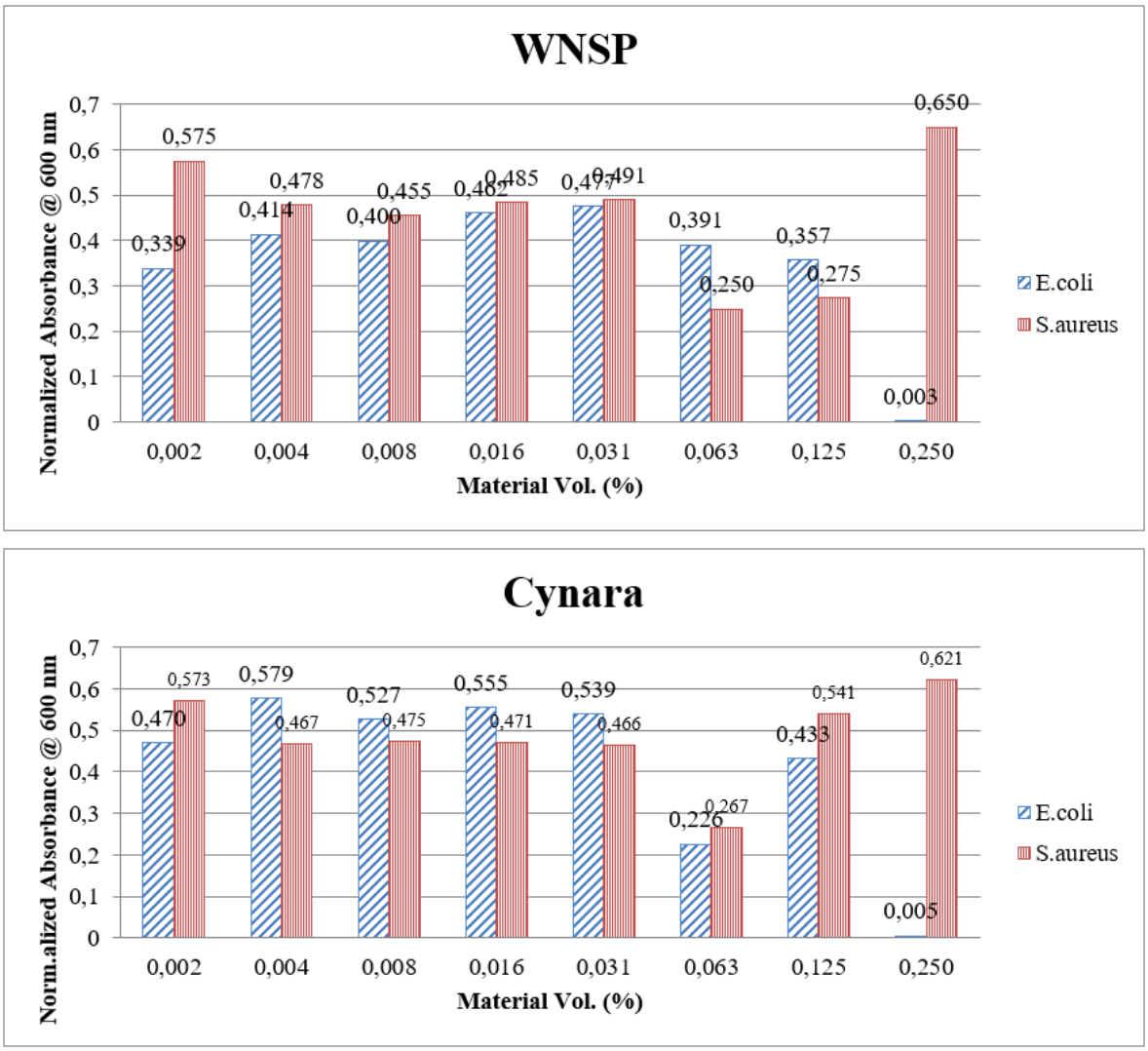

Figure 7. The normalized absorbance values pathogens incubated at various concentrations of Ag-NP suspensions via walnut shell powder (WNSP) and Cynara extracts.

A higher concentration of Ag NPs may also be effective on *S. aureus*. The thick peptidoglycan layer present at Gram positive bacteria membrane may be acting as a stronger barrier demanding a higher concentration of Ag NPs for cell growth inhibition [31]. The MIC values for *S. aureus* were reported to be higher than *E. coli* in other works using different plant sources, e.g. 99.26 µg / mL vs. 28.43 µg / mL, 31.2 µg / mL vs. 7.8 µg / mL, 125 µg / mL vs. 15.63 µg / mL, respectively [32], supporting the idea a higher concentration as MIC is necessary for *S. aureus*.

Conclusion

Green synthesis is an efficient route to synthesize Ag NPs in an environmentally friendly [47-53], relatively simple and cost-efficient procedure. Ag NPs, which have an important place in nanotechnology [54-57], finding applications in many fields such as physics, chemistry, electronics, food, health and biomedicine, were prepared via green synthesis. Silver nanoparticles, which are found in the group of metallic nanoparticles and exhibit antibacterial properties, are used in food shelf-life extension, food packaging, medical, biomedical and cosmetic industries. In the last ten years in the world, comprehensive studies have been focused on the antimicrobial properties [58] of nanoparticles, especially metal nanoparticles (Me-NPs) of iron and silver [23, 59]. Nowadays, Ag NPs having strong antimicrobial activity are also used in combination with antimicrobial agents to increase their activity [58, 60].

In this study, the biogenic Ag NPs, successfully prepared via green synthesis using the extracts of *Cynara* and walnut shell powder (WNSP) (for the first time in the literature), showed considerable antibacterial effect against *E.coli*, and also observed to be potentially effective on *S.aureus* at higher concentrations. Thick and more durable cell wall of *S. aureus*, could be a reason for the necessity of higher concentrations of Ag NPs. The MIC for *E. coli* was determined as 375 ng silver ions per mL, while it is expected to be a higher value for *S. aureus*. The results show possibility of using the prepared Ag NPs for biomedical applications demanding antibacterial properties (e.g. burn wound treating gel, wound dressings, etc.). The gained resistance of pathogenic microorganisms against current antibiotics which is threatening the public health may be overcome by utilization of Ag NPs with necessary concentration [56, 58, 61]. The nosocomial infections by biofilm forming pathogenic microorganisms are responsible for considerable health problems and expenditures [62]. These infections mainly related with usage of medical devices (e.g. catheters [40], wound dressings) may be partially decreased with surface modification/treatment of devices with biogenic Ag NPs.

The plant extracts used (*Cynara* and walnut shell powder (WNSP)) were effective on preparation of Ag NPs with different average particle sizes (APS 46 nm and 109 nm, respectively) that may affect the kinetics for activity/efficiency of Ag NPs, which should be further investigated. These particles were prepared relatively uniform in size (having low PDI (polydispersity index) values, 0.204 and 0.364, respectively) making the preparation procedure promising for further utilization. One possible utilization of the prepared Ag NPs (with relatively uniform APS) may be for selective inactivation of cancerous cells in which the particle" size may be a considerable parameter. The utilization of different plant extracts and process parameters of green synthesis of Ag NPs (e.g. ratio of silver nitrate and plant extract) and their possible effects on average particle size should be further investigated for the applications in which the average particle size is crucial. The phytochemicals responsible for the reduction of silver and also possible higher antimicrobial activity can further be investigated by using HPLC analysis and/or other methods to reveal the possible mechanisms and the contribution of the components in the plant extracts to these mechanisms.

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