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Research Article

Characterization and Antibacterial Activity of Green Copper Nanoparticles Synthesized by *Saponaria officinalis* L., a Plant with High Saponin Content

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Abstract

Evaluation of antimicrobial metal ions and nanoparticles generally concludes that metallic nanoparticles are more stable and active than their metal ion counterparts from which they were synthesized. The antimicrobial effect of these metal ions and nanoparticles typically covers gram-positive, gram-negative bacteria along with fungi. And unlike antibiotics that target specific essential synthetic cellular pathways, antimicrobial actions of metallic nanoparticles are nonspecific. Due to this fact, NPs with antibacterial properties are foreseen as potential agents against stubborn bacterial infections that show multidrug resistance. In this work, we report copper nanoparticle (CuNP) synthesis through the green route, its' structural characterization, and evaluation of its' antibacterial properties. The source of a reducing agent selected was soapwort (*Saponaria officinalis* L.) root extracts known to be high in saponin content. The main aim of generating green nanoparticles from copper with *S. officinalis* root extracts was to combine the antibacterial effects of both CuNPs and bioactive phytochemicals from soapwort. Structural characterizations of the Green-CuNPs were performed through scanning electron microscopy (SEM), UV-Visible spectroscopy (UV-Vis), Fourier transform infrared spectrophotometer (FTIR), and X-ray diffraction (XRD) analyses. Green-CuNPs, probably coated with saponins and other active phytochemicals, were tested for their antimicrobial activities against two common bacteria (*Escherichia coli* and *Staphylococcus aureus*). The bulk of the generated NPs were pure nanocrystalline structures (XRD analysis) with an average diameter of 17 nm. FTIR analysis data has confirmed both CuNP formation and functionalization with aromatic hydrocarbon structures (possibly saponins). In the evaluation of antimicrobial properties, it was observed that NPs had weak to moderate but statistically significant antimicrobial effects against *E. coli* and *S. aureus* when used at high doses. Further diagnostics and experimentations may reveal the actual mechanisms of antimicrobial activity of Green-CuNPs synthesized with *S. officinalis*.

Keywords: CuNPs, *Saponaria officinalis*, Antibacterial, Green-synthesis.

Yüksek Saponin İçerikli *Saponaria officinalis* L. ile Sentezlenen Bakır Nanopartiküllerin Karakterizasyonu ve Antibakteriyel Aktivitesi

Öz

Antimikrobiyal metal iyonlarının ve nanoparçacıkların değerlendirilmesi genel olarak metalik nanoparçacıkların sentezlendikleri metal iyon karşılıklarından daha kararlı ve aktif olduğu sonucuna varır. Bu metal iyonlarının ve nanopartiküllerin antimikrobiyal etkisi genellikle mantarlarla birlikte gram pozitif, gram negatif bakterileri de kapsar. Ve spesifik temel hücresel yolakları veya enzimleri hedefleyen antibiyotiklerin aksine, metalik nanopartiküllerin antimikrobiyal etkileri spesifik değildir. Bu nedenle antibakteriyel özelliklere sahip NP'ler, çoklu ilaç direnci gösteren inatçı bakteriyel enfeksiyonlara karşı potansiyel ajanlar olarak öngörülmektedir. Bu çalışmada, yeşil yoldan bakır nanoparçacık (CuNP) sentezini, yapısal karakterizasyonlarını ve antibakteriyel özelliklerinin değerlendirilmesini rapor ediyoruz. Seçilen indirgeyici ajanın kaynağı, saponin içeriği yüksek olduğu bilinen çöğür (*Saponaria officinalis* L.) kök özütleridir. *S. officinalis* kök ekstreleri ile bakırdan yeşil nanopartiküller üretmenin temel amacı, hem CuNP'lerin hem de sabun otundan elde edilen biyoaktif fitokimyasalların antibakteriyel etkilerini birleştirmektir. Bu amaçla sentezlenen Yeşil-CuNP'lerin yapısal karakterizasyonları, taramalı elektron mikroskobu (SEM), UV-Görünür spektroskopi (UV-Vis), Fourier dönüşümlü kızılötesi spektrofotometre (FTIR) ve X-ışını kırınımı (XRD) analizleri yoluyla gerçekleştirilmiştir. Muhtemelen saponinler ve diğer

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aktif fitokimyasallarla kaplanmış olan Yeşil-CuNP'ler, iki yaygın bakteriye (*Escherichia coli* ve *Staphylococcus aureus*) karşı antimikrobiyal aktivitesi açısından test edilmiştir. Üretilen NP'nin büyük kısmı, ortalama çapı 17 nm olan saf nanokristal yapılardır (XRD analizi). FTIR analiz verileri, hem CuNP oluşumunu hem de aromatik hidrokarbon yapıları (muhtemelen saponinler) ile işlevselleşmeyi doğrulamıştır. Antimikrobiyal özelliklerin değerlendirilmesinde, NP'lerin yüksek dozlarda kullanıldığında *E. coli* ve *S. aureus*'a karşı zayıf ila orta ancak istatistiksel olarak anlamlı antimikrobiyal etkilere sahip olduğu gözlendi. Daha fazla teşhis ve deneyler, *S. officinalis* ile sentezlenen Green-CuNP'lerin antimikrobiyal aktivitesinin gerçek mekanizmalarını ortaya çıkarabilir.

Anahtar Kelimeler: CuNPs, *Saponaria officinalis*, Antibakteriyel, Yeşil-sentez.

1. Introduction

So far, no single accepted definition covers all the nanomaterials (Abdulazeem[1], Hussien[2], Al-Gburi[3], & Jassani[4], 2020). A European Commission definition addresses the issue from the size distribution perspective. In this definition, a mixture of particulate matter is categorized as a nanoparticle if at least half of the particles' size in one dimension is not more than 100 nm. Based on this view, the literature associated with this definition states the nanoscale as the range 1-100 nm (L. Wang, Hu, & Shao, 2017). In this context, only a few hundred atoms are held together in a single nanoparticle structure since the mean sizes of atoms and molecules fit into the 0.1 nm range, meaning that a few hundred ions from the solution can collapse together to form a nanoscale structure. There is another somewhat rough definition for nanomaterials; related literature mentions particulate matter as nanomaterials if at least one of the dimensions measures below (or equal to) the 100 nm range.

The size restriction to 100 nm and the structure being composed of several hundred atoms in an array pattern, ideally approximate the particle structure of nanomaterials to that of perfect crystal lattices at the supra-atomic level. Structural convergence of nanomaterials into these super crystalline forms might reveal the nano-level electronic, optical, physical, and kinetic properties otherwise masked as background noise in their bulky structures.

Along with their cost-effective synthesis being prominently eased through the green route, the versatile nature of nanoparticles being synthesized has potentiated their uses in many industrial, medical, environmental, and research areas; from the manufacture of novel nanomaterials (Velmurugan et al., 2014a) to self-cleaning surfaces and nano-catalysis (Hussain, Singh, Singh, Singh, & Singh, 2016; Mott, Galkowski, Wang, Luo, & Zhong, 2007) and even till nano diagnostics (Abdulazeem[1] et al., 2020).

Accelerating research in nanosciences in the last decade - from electronics to optics (Ananda Murthy, Abebe, C H, & Shantaveerayya, 2018a; Olajire, Ifediora, Bello, & Benson, 2018a; Shashanka, 2021) and the fight against intractable nosocomial infections (Aderibigbe, 2017; Iram et al., 2016; Sathyanarayanan, Balachandranath, Genji Srinivasulu, Kannaiyan, & Subbiahdoss, 2013) to antimicrobial (as well as anti-corrosive) nanosurface coatings on medical devices and implants (Beyth, Houri-Haddad, Domb, Khan, & Hazan, 2015) as well as from nano-sensors to biosensors (Allaf & Hope-Weeks, 2014; Ananda Murthy, Abebe, C H, & Shantaveerayya, 2018b; Beyth et al., 2015; Murthy, Desalegn, Kassa, Abebe, & Assefa, 2020; Shah, Fawcett, Sharma, Tripathy, & Poinern, 2015; Shashanka, 2021)- made nanomaterials a joint scientific research topic. As a result, science extensively evaluates the value of nanoparticles used in medicine, biology, cosmetics, electronics, food industry, chemical industry, etc. (Virkutyte & Varma, 2011a)

When we shift our focus to a more confined set of nanomaterials, so to say metallic nanoparticles, we observe more prominent optical, magnetic, electronic properties. The causative fact for the sudden appearance of these properties at the nanoscale is conceptualized within quantum confinement (Shashanka, 2021). As in the case of nanodots, material properties can be enhanced or altered with a change in nanoparticle size or atomic composition. In the case of metallic nanoparticles, as the size is confined (limited) to the quantum scale, electro-optic properties of these nanoparticles and hence excitation-emission patterns are also confined at this quantum level size. Compared to the bulk metallic mass, metallic nanoparticles holding a few crystal lattices exhibit clearer and wider band gaps of excitation-emission approximating a single metaloxide. Several crystal lattices combine into a single metallic nanoparticle unit, possessing an extremely high surface area to volume ratio. From the quantum confinement viewpoint, these crystal lattices are bound into a nanoparticle unit and express their distinct (electronic) excitation-emission patterns in harmony. From this perspective, metallic nanoparticles can be regarded as electronic nano-catalysts that can shuttle electrons to and from the surrounding (organic) milieu consequnetly catalysing ROS generation.

This perspective above, combined with the historical use of precious metals such as gold and silver for health problems, makes it possible for researchers to use nanoparticles for treating infectious diseases, preserving food, autocatalytic degradation of toxic chemicals, etc. Today, Ag, Au, Pt, Cu, Zn nanoparticles and their metal oxides are used for many purposes.

Synthesis of nanoparticles is another issue (is a factor of art and science in this decade) where synthesis is made through physical, chemical, and biological (green synthesis) methods. When producing nanoparticles through physical and chemical synthesis, comparably higher costs and generation of toxic end-products along with nanoparticles make these routes questionable. With these synthesis routes, it is also a reasonably challenging task to control the surface chemistry, size, and structure of nanoparticles synthesized (Gudikandula & Charya Maringanti, 2016; Taş, Köroğlu, & Çelebioğlu, 2021). Contrary to these two routes, a cheaper, environmentally friendly, and non-toxic synthesis method (the Green-NP synthesis) comes to the fore (Manikandan et al., 2017; Olajire et al., 2018a). Simply stated, green synthesis utilizes organic agents as reducing power for bonding metal ions into nanoparticles in a solution. During this process, particle size, shape, and active surface chemistry are directly affected by the composition of the biological extract used. And during this bottom-up type of synthesis, metal ions are reduced and capped into various dimensions and structures in the presence of active phytochemicals ranging from alkaloids, phenolic acids, polyphenols, terpenoids to sugars (Shah et al., 2015).

Nanoparticles produced through the green route (by reduction and capping with cellular components and metabolites) possess features from both nanomaterials' and phytochemicals' nature. And hence nanoparticles generated through the green route exhibit a plethora of properties; antibacterial (Beyth et al., 2015; Chinnappan et al., 2018; Desalegn, Murthy, Ravikumar, & Nagaswarupa, 2021; Gholami,

Azarbani, Hadi, & Murthy, 2021; Gudikandula & Charya Maringanti, 2016; Hussain et al., 2016; Tovar-Corona et al., 2018; Virkutyte & Varma, 2011a), antioxidant (Ananda Murthy et al., 2018a; Jayarambabu, Akshaykranth, Venkatappa Rao, Venkateswara Rao, & Rakesh Kumar, 2020; Shah et al., 2015), photo-catalytic and chemo-catalytic (Allaf & Hope-Weeks, 2014; Ananda Murthy et al., 2018a; Beyth et al., 2015; Hussain et al., 2016; Olajire et al., 2018a; Shah et al., 2015) properties.

These properties further potentiate nanoparticle use in diverse application areas such as drug delivery systems (Abdulazeem[1] et al., 2020; Shah et al., 2015; Velmurugan et al., 2014b), cosmetics, pharmaceuticals (Ananda Murthy et al., 2018a; Jayarambabu et al., 2020; Saranyaadevi, Subha, Ernest Ravindran, & Renganathan, 2014; Shah et al., 2015; Virkutyte & Varma, 2011b) and food industry (Beyth et al., 2015; Gholami et al., 2021; Hussain et al., 2016; Manikandan et al., 2017; Raghunath & Perumal, 2017; Velmurugan et al., 2014b)

Utilization of nanoparticles (NP) in medicine is still hot, especially in treating infectious diseases, where exceptional infectious agents with multidrug resistance phenotypes are emerging. As noted elsewhere in the text above, the antimicrobial effects of nanoparticles (especially metal oxides) are attractive solutions for such difficult-to-eradicate nosocomial infections (Abdulazeem[1] et al., 2020; Iram et al., 2016; Lee, Ko, & Hsueh, 2019a; Z. Wang et al., 2017). Current (non-nano particle type) antimicrobials exert their effects by interfering with the cell wall, protein synthesis, nucleic acid synthesis, or blocking any metabolic pathway (Beyth et al., 2015; Raghunath & Perumal, 2017; Tenover, 2006).

While the antibacterial action modes of nanoparticles are mentioned in scientific studies, the contact of nanoparticles with the cell wall and the formation of free radicals by nanoparticles at the contact points, and the subsequent local cell wall damage and DNA chain breaks due to these free radicals, DNA replication and DNA repair are all discussed issues (Abdulazeem[1] et al., 2020; Aderibigbe, 2017; Beyth et al., 2015). Unlike the mechanisms of action of antibiotics that target subcellular structures in specific ways, nanoparticles disrupt their subcellular targets mostly non-specifically and affect them through ROS generation and physically shreds or disjoint the structural integrity. And consequently, if the microorganism does not possess nanoparticle deactivating, modifying mechanisms (unlike their antimicrobial chemical inactivating enzymes), the organism may not develop resistance against the actions of nanoparticles (Abdulazeem[1] et al., 2020; Lee et al., 2019a; Raghunath & Perumal, 2017; Z. Wang et al., 2017) Nanoparticles, synthesized with active phytochemicals or with antibiotics or synthesized as standard (plain, bald) nanoparticles, hold the premise for the fight against stubborn microbial infections (Iram et al., 2016; Tenover, 2006).

Copper itself and its nanoparticle counterparts (Cu and CuO NPs) are known to be effective against both bacterial and fungal infections. CuNPs are in the metallic nanoparticles group and withhold the features like the high surface area to volume ratio, producing an almost complete set of reactive oxygenic species and possessing excellent catalytic yield. It is also cited that the antimicrobial activity of CuNPs is associated with metal ions released from nanoparticles (Jayarambabu et al., 2020). Its close interaction with microbial membranes further increases this antimicrobial action due to its small size and high surface area to volume ratio (Mott et al., 2007).

Besides these properties (stated above in the previous paragraph) and lower preparation costs, ease of accessibility to different forms of Cu raw materials, and availability of different CuNP synthesis methods highlight Cu and CuO NPs use as prominent antimicrobial agents (Aderibigbe, 2017; Beyth et al., 2015; Lee, Ko, & Hsueh, 2019b; Olajire, Ifediora, Bello, & Benson, 2018b).

In this work, we have utilized *Saponaria officinalis* root extracts as reducing and capping agents for CuNP synthesis. *S. officinalis* plant parts are among the agents traditionally used in folk medicine. Roots of the plant have diuretic properties and are utilized for curing respiratory tract infections, bronchitis, gastrointestinal illnesses. While the leaves of *S. officinalis* are consumed as a tea against liver diseases, it is also used as an insect repellent by applying the leaf extracts to the skin among the folk. The most prominent active phytochemical of the plants' roots is saponins with ribosome function inhibition and with anticancer, antimicrobial and insect repellent effects (Chandra, Rawat, & Bhatt, 2021).

Here, we also want to draw attention to the use of *S. officinalis* root saponins as a stable foaming agent in the food industry, as we encountered this foaming phenomenon during nanoparticle synthesis (Jurado Gonzalez & Sörensen, 2020). Throughout the scope of the broad literature search we have done, to our knowledge, NP synthesis with *S. officinalis* extract has not been touched yet.

In this study, we evaluated whether the reduction of copper ions to CuNPs in the presence of *S. officinalis* extracts showed significant antibacterial effects against two common bacteria, *Escherichia coli* and *Staphylococcus aureus*. Since aqueous extracts of *S. officinalis* have been shown to have vigorous antimicrobial activity against many bacteria (Sengul et al., 2011), we aimed to see if both CuNPs and *S. officinalis* root extracts could have a combinatorial antibacterial effect.

2. Material and Method

2.1. Preparation of the Plant Extract

Commercially sold soapwort (*Saponaria officinalis* L.) roots, the Bartin region (Turkey) was collected. *Saponaria officinalis* L. roots were washed several times with distilled water to remove dust and soil debris and dry blotted on filter paper. After drying, the roots were cut into small pieces and placed in mortar for grinding. Root phytochemical extraction was made with 10 gr of powdered root in 100 ml of distilled water at ambient room temperature overnight on a magnetic stirrer (500 rpm). During sample preparation, the mixture was covered to prevent any possible photo-oxidation. In the end, the plant extract obtained was filtered, bott dry, and stored at 4° C for later use.

2.2. Synthesis of Copper Nanoparticles

As described below, *S. officinalis* root extracts prepared then stored at four °C were used for CuNP synthesis. A 0.1 M 100 ml stock solution of $Cu(NO_3)_2.3H_2O$ in distilled water (99.99%, Sigma-Aldrich) was used as a Cu^{+2} ion source. A 10 ml of this Copper (II) nitrate solution was titrated into 100 ml of plant root extract (solution prepared from 10 gr of plant material) with a slow phase, in a dropwise manner while, the whole solution was mixed at 500 rpm on a magnetic stirrer. The development of Cu nanoparticles was maintained on the same setup on a magnetic stirrer for 24 hours at room temperature. The expected gradual color change as the indication of NP synthesis was observed during 24 hrs incubation period. Color development was recorded as a transition from light yellow to brown, as shown in Figure 1. At the end of 24 hours of NP synthesis, the whole sample was centrifuged at 8000 rpm for 20 minutes at room

temperature to harvest the nanoparticles. After the centrifugation step, the supernatant was gently poured out, and the pellet containing the nanoparticles was washed several times with distilled water. The CuNPs handled at the end of this process were dried in a vacuum oven at 70° C for 24 hours and finally stored in sterile tubes until later usage.

2.3. Characterization of Copper Nanoparticles

Cu nanoparticles generated with *Saponaria officinalis* L. root extracts were analyzed through UV–vis spectroscopy (THERMO Multiscaner Spectrophotometer), FTIR spectroscopy (Shimadzu Iraffinity-1), SEM (TESCAN, MAIA3 XM), EDAX, and XRD (Rigaku, Smartlab) methods and characterized according to the protocols frequently cited in the literature (Rajaganesh et al., 2016)

2.4. Antibacterial Activity Tests

Broth Micro-dilution Assay was used to investigate antibacterial activities of the nanoparticles synthesized (Brandt et al., 2010). For this, frozen stocks (-20º C) of Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus* were inoculated into Nutrient Broth (NB) as starter cultures. Following culture development at 37ºC for 24 hours, bacteria were inoculated into new media to achieve 0.5 McFarland Unit bacterial concentration. Bacterial product at 0.5 McFarland Unit bacterial concentration was utilized as the test bacterial stock solution. For testing the antibacterial effects of CuNPs, 200 µL microtiter plate wells were inoculated with 20 µL of test bacterial stock solution and 160 µL of NB. The final 20 µL empty volume of the microtiter plate was filled with varied (0-30 mM) concentrations of CuNPs dissolved in 50% DMSO. The final DMSO concentration of all preparations was around 5% maximum.

A negative control group was devised with only LB media without any bacterial inoculation to test any unintended microorganism contamination during the culture period. Also, another negative control group was devised for discriminating the NPs' effect, where LB media without NPs but with 5% DMSO was utilized.

Bacterial growths were measured through absorbance values at 600 nm (optical densities at 600 nm; OD_{600}). Measurements were made with the micro-plate reader (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer) at culture start (hour zero) and after 24 hours. A comparison of the effects of NPs was made by evaluating OD_{600} growth score measurements. Comparisons of OD_{600} scores from NP administration groups and OD_{600} scores from negative control groups with only LB and DMSO were used to measure the antibacterial effects of CuNPs. Based on these measurements, bacterial growth inhibitions were stated as the percentages of recorded optical densities concerning the negative controls (bacterial growths of negative control groups were taken as 100%).

2.5. Statistical Analysis

Statistical analysis of antibacterial tests was performed through one-way ANOVA or Students' t-test. Observed score differences that fit into probabilities below 0.05 ($p<0.05$) were considered statistically significant.

3. Results and Discussion

3.1. UV–Visible Absorbance Spectrum of NPs Generated

UV-Vis spectrum of CuNPs synthesized by the green synthesis method from *Saponaria officinalis* L. is shown in Figure 1. Synthesis of the nanoparticles (CuNPs) was confirmed by UV-visible spectrum and surface plasmon resonance analysis. UV-visible absorbance spectrum scanning performed between 200 and 800 nm revealed an absorption maximum at 555 nm (Figure 1). This absorbance is consistent with the literature cited CuNPs particles obtained by similar methods (Lv et al., 2018). Absorbance value at 555 nm was related to the $\pi \rightarrow \pi^*$ transitions of CuNPs.

3.2. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis of NPs Generated

FTIR analysis performed to characterize the surface structure of CuNP is shown in Figure 2. The numbered pits in the graph seen are the percent depressions in infrared transmission. These depressions are signs of intra-atomic bond vibrations triggered at the infrared range. At the fingerprint region of the infrared spectrum (below 1500 cm^{-1} frequency), transmission depression at 864 cm^{-1} infrared vibration refers to Cu-Cu bond length that confirms the formation of CuNPs. At higher IR vibration frequencies, transmission depression at 1434 cm^{-1} is cited as C=C bond stretches. And at 1692 cm-1 vibration frequency, transmission depression refers to C=O bond stretches. And also, at 2329 cm^{-1}, IR vibration frequency transmission depression refers to C-N bond stretches. These three transmission depressions at the aforementioned vibration frequencies are signs of aromatic amines. In scientific literature, similar IR transmission depression patterns are cited as indications of flavonoids, terpenoids, and polyphenols in structure (Saranyaadevi et al., 2014; Velmurugan et al., 2014b)

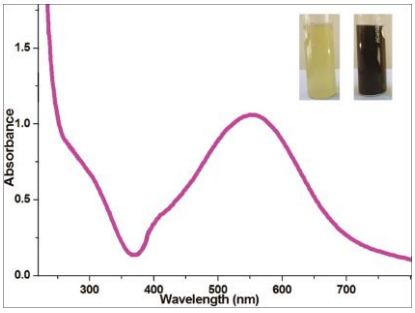

Figure 1: UV-visible spectrum data of CuNPs synthesized with *Saponaria officinalis* L. extract

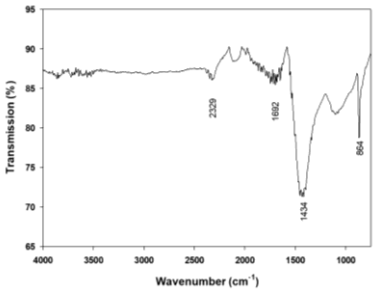

Figure 2: FTIR spectra of CuNPs synthesized with *Saponaria officinalis* L. Extract

3.3. XRD Analysis of NPs Generated

XRD is considered as an essential key tool for evaluating the crystallinity of nanoparticles synthesized and the presence of tertiary structures at molecular levels. XRD patterns of CuNPs biosynthesized with *Saponaria officinalis* L. are shown in Figure 3. Distinct crystal phases observed in CuNP by X-ray diffraction pattern belong to the metallic Cu structures. X-ray diffraction patterns of CuNP were obtained in the 2θ = 20-80

angle range. Diffraction peaks were observed at 32.43°, 35.48°, 44.15°, 51.84°, 56.51°, 61.29°, and 64.64° (Figure 3). Diffraction peaks at 2θ of 35.48°, 44.15°, and 51.84° correspond to the (002), (111) and (200) planes, respectively. These findings confirm the formation of single-phase CuNP in the fcc crystal structure. In addition, the crystal structures of the nanoparticles we obtained are in harmony with the crystal structures of the nanoparticles obtained similarly in the literature (Nasrollahzadeh, Sajadi, & Hatamifard, 2016; Valodkar et al., 2012). In addition, all peaks comply with JCPDF Card No: 01-089-5899. The asterisk sign (*) assigned peaks at 32.43°, 56.51°, 61.29°, and 64.64° in the graph are unidentified peaks. These unidentified peaks may be due to surface docking of biomolecules from *Saponaria officinalis* L. extract. Crystal size measurement for synthesized CuNPs was calculated using the Debye-Scherrer equation (Equation 1) (Aladpoosh & Montazer, 2015). The average crystal size of CuNPs was estimated to be approximately 17.81 nm according to the Debye-Scherrer formula

$$D = \frac{K\lambda}{\beta . \cos\theta} \qquad (1)$$

D: crystal size,
K: Debye Scherrer constant (0.94),
λ: Cu-Kα radiation (1.54 A),
β: half-length width of maximum peak (FWHM),
θ: it is the Bragg angle value obtained from the 2θ value of the maximum peak in the XRD diffraction pattern.

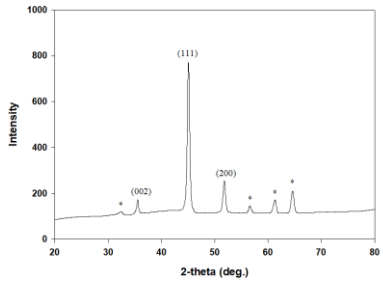

Figure 3: XRD patterns for CuNPs biosynthesized with *Saponaria officinalis* L.

3.4. Scanning Electron Microscopy (SEM) Study of CuNPs

SEM images of CuNPs obtained from *Saponaria officinalis* L. extract with green synthesis method are given in Figure 4. SEM images show that the particles possess variances in diameters and sizes. It has been determined that the shapes of CuNP are spherical, and particle sizes fit well below 100 nm. Elemental analysis of Cu nanoparticles was performed using energy-dispersive X-ray spectroscopy (EDX). Figure 4 shows the EDX image of Cu nanoparticles prepared by *Saponaria officinalis* L. extract. The synthesized nanoparticles' EDX spectrum shows strong signals belonging to elemental copper and confirms the presence of copper in nanoform. This indicates that the prepared CuNPs contain only metallic copper, not any other form of copper. Also, the EDX spectrum clearly shows that CuNPs are in crystalline form. Typically the stoichiometric mass percentage of Cu and O in CuO is 50.57% and 16.86%, respectively. But with our prepared nanoparticles, Cu content revealed is approximately 50.5%, whereas the remaining 49.5% are organic molecules composed of carbon and oxygen. This stochiometry based on weight percent also signs phytochemicals that surround the nanoparticles.

3.5. Antibacterial activity of CuNPs

In the test of the antibacterial effects of CuNPs biosynthesized with *Saponaria officinalis* L. aqueous root extracts, an increase in antibacterial effect was observed with the rise of applied NP concentration. Antibacterial tests against the common bacterium *E. coli* showed that gradual increase in the concentration of applied CuNP yielded 13% inhibition (OD_{600} absorbance score comparisons) in bacterial growth at maximum (Figure 5A). The maximum CuNP concentration used in the experimental setup was 2500 µg/mL where we observed the most bacterial growth inhibition. OD_{600} score at this concentration significantly differentiates from the rest of the application and positive control groups (Turkey's test $p<0,05$/ Figure 5.A).

Like the antibacterial scores seen with *E. coli* tests, a gradual increase in CuNP concentration against *Staphylococcus aureus* showed enhanced depression in bacterial growth. Antibacterial activity scores measured were 13% and 16% at CuNP concentrations of 1250 µg/mL and 2500 µg/mL respectively (Figure 5B). Both 1250 µg/mL and 2500 µg/mL applications were significantly different from the rest of the application groups (Turkey's test $p<0,05$/ Figure 5.B).

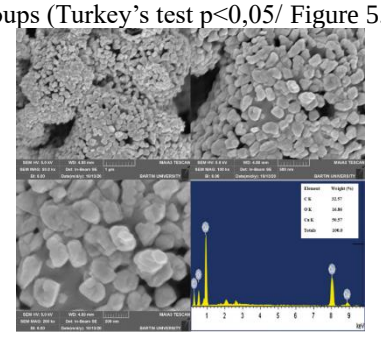

Figure 4: SEM graph of CuNPs biosynthesized using the *Saponaria officinalis* L. extract, and EDS spectrum showing the chemical composition of nanoparticles

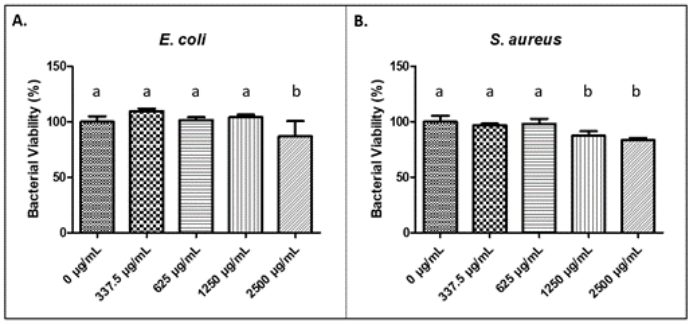

Figure 5. Anti-bacterial activity of Green-CuNPs against (A) *E. coli* and (B) *S. aureus*. Different lower-case letters indicate $p<0.05$, according to Tukey's test.

4. Conclusions and Recommendations

CuNPs synthesized through the green route exhibit antibacterial properties against a plethora of microbes (Ananda Murthy et al., 2018a; Gholami et al., 2021; Jayarambabu et al., 2020; Kaur, Thakur, & Chaudhury, 2016; Lv et al., 2018; Tovar-Corona et al., 2018). These antibacterial properties of Green-NpPs can be further potentiated in combination with different plant extracts. In this sense, we are all aware of the (rich) parental legacy of folk remedies, which is passed down from old to young.

Copper ions are long known to have natural antibacterial and antimycotic effects in a dose-dependent manner. Elevating concentration to achieve a therapeutic effect is usually not suitable since the gap between toxic and therapeutic doses is so narrow and changes from one patient to another. To get around this problem, CuNPs synthesized through the green route with *Saponaria officinalis* root extracts can be utilized. Our scientific approach was to question the combinatorial antibacterial effect of Cu and *Saponaria officinalis* root extracts. So Green-CuNPs that deliver sub-toxic doses of Cu ions were utilized as antibacterial agents.

Antibacterial activities of the Green NPs can be attributed back to both metallic ions liberated from the nanoparticles and the presence of both redox states (in our case, Cupric and Cuprous states) within the nanoparticles. Nucleation and capping with various phytochemicals may generate distinct zones of redox centers in a nanoparticle unit (Jayarambabu et al., 2020). Possible antibacterial factors of green NPs can be discussed under four headings; (1) higher catalytic activities at the nanoscale combined with higher nano-kinetics, (2) release of metallic ions while in interaction, (3) holding a range of redox states in each NP units or NP mixture, and (4) antimicrobial phytochemical coatings borrowed from plant extracts. Due to such properties, Green-NPs continuously generate reactive oxygenic species and damage bacterial cell walls, proteins, DNA (Lv et al., 2018), etc.

CuNPs synthesized in our study through the green route with *Saponaria officinalis* L. root extracts were analyzed first with a UV-Visible spectrophotometer. The color transition of the reaction mixture from dirty-yellow to dark brown and the maximum peak absorbance value at 555 nm were regarded as the signature of reducing metal ions into CuNPs (Figure 1).

To detect the functional groups on the Green-CuNPs surface, we have focused on excitation frequencies spanning from 3000 to 1500 cm-1 in the FTIR spectrum scan (Figure 2). The transmission depression pits around 1600 cm-1 are usually denoted as aromatic hydrocarbons in the scientific literature. In our study, these regions can be annotated as the surface saponin content of the NPs. Also, in the fingerprint region, transmission depression pit around 864 cm-1 excitation frequency is literature cited as CuNP presence.

In the XDR analysis graph, the diffraction peaks formed in the 2θ scan (from 20 to 80 degrees) yielded a bulk peak (111) at 44.15°. This peak is indicative of the bulk crystal Cu structures residing in the core of CuNP generated. The unidentified peaks tagged with asterisks (*) may be attributed to the follow-up leftover saponin foams or crystals from the initial synthesis step. The average size calculation from the XRD data revealed an approximate NP size of 17 nm.

SEM image analysis of the nanoparticles shows that the particles generated were non-uniform crystalline structures, while EDS spectrum analysis revealed that the typical compositions were Cu but not CuO.

Throughout these analyses, we can conclude that the generation of CuNP using *Saponaria officinalis* L. root extract was successful, as evident from SEM, EDS, XRD, FTIR, and UV-Vis Spectra analysis.

The bactericidal effects of the NP generated were scored in terms of depression in growth and measured as optical density scores of bacterial growths. Different concentrations of CuNP applications introduced meaningful differences as bacterial growth inhibitions ranging from 10 to 15% growth depression. The resulting bactericidal effects are surprisingly low compared to similar reports (Raffi et al., 2010). Depending on the experimental data we have shared, we can conclude that we have in hand CuNPs functionalized with saponins. And saponins are regarded as antioxidant molecules. As we expect that the bactericidal effects of metallic nanoparticles are through the generation of a plethora of ROS agents, and saponins are antioxidant molecules in their nature, antioxidant saponins on the outer shell might counteract the ROS generated by the CuNP core. This scenario can explain why Green CuNPs synthesized in our work showed lower bactericidal effects than similar scientific reports. Controversially, the pronounced antibacterial effects seen at high CuNP applications can be attributed to eradicating bacterial membrane potential due to the extreme saponin load moved to bacterial surfaces through NPS.

Saponaria officinalis root extracts are already known in folk and used in many health issues and food preparations. The strong foamification in any preparations using *Saponaria officinalis* extracts is prominent. It should be diagnosed in another study if this saponification (also seen in our experimental setup during NP synthesis) is interfering with the complex formation between bioactive phytochemicals (other than saponins) and NPs.

As a result, we can conclude that CuNPs successfully synthesized with *Saponaria officinalis* root extracts showed moderate to low antibacterial activities against the two common bacteria, namely *E. coli*, and *S. aureus*. Further studies can diagnose the mechanisms behind this unexpectedly low antibacterial activity. We may shed light on why some combinations of bioactive phytochemicals and NPs are far more effective in eradicating microbial growth and hence infection.

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