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Biogenic Synthesis of Silver Nanoparticles from *Piper cubeb* Corns with Potent Antioxidant and Antimicrobial Efficacy

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Abstract
Current research evaluated the aqueous extract of *Piper cubeb* corn as a reducing and stabilizing agent for sunlight-assisted eco-friendly synthesis of silver nanoparticles (Ag NPs). The synthesis is preferred over chemical methods due to the non-involvement of toxic reagents. The synthesized Ag NPs were assessed for the antioxidant and microbicidal potential. A distinct color change from colorless to light red and finally reddish brown, accompanied by a maximum surface plasmon resonance absorption at 440-445 nm, confirmed the successful synthesis of Ag NPs. The phytochemicals in the aqueous extract, with diverse functional groups, not only reduced but also stabilized Ag NPs. In contrast, SEM-EDS analyses confirmed that the particles were predominantly spherical, with an average diameter of 18 to 27 ± 0.4 nm (calculated using ImageJ) and an elemental composition. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays confirmed the significant free radical scavenging potential of Ag NPs. The Agar Well Diffusion method revealed prominent antimicrobial potential, including notable zones of inhibition (ZOI), minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) against gram-negative (*Escherichia coli* and *Salmonella typhi*) and gram-positive (*Bacillus subtilis* and *Bacillus licheniformis*) bacterial strains. Thus, the biosynthesized Ag NPs possess promising antioxidant and antimicrobial potential as eco-friendly alternatives to conventional antimicrobial and antibacterial agents.

Keywords: antibacterial, nanoparticles, antioxidant, *Piper cubeb*, DPPH

1. INTRODUCTION

Among NPs, Ag NPs have significant catalytic and biological applications, including antifungal, anti-inflammatory, antimicrobial, and antioxidant activities. Ag NPs are also used in the food industry, cosmetics, automobiles, electronics, and medical fields [1-4]. The antimicrobial characteristics of Ag NPs are favorable for biomedical equipment, such as dressings, antiseptic sprays, ointments, and creams [5]. Moreover, Ag NPs are of interest to researchers working in areas such as drug delivery, nanomedicine, the agricultural industry, dye degradation, the food and textile industries, data storage devices, and chemical sensing [6].

Ag NPs are synthesized by chemical and physical methods [7-9]. Chemical methods for synthesizing Ag NPs are considered more useful but involve using toxic, expensive, and environmentally unfriendly reagents. The alternative approaches for synthesizing Ag NPs are green methods, which are environmentally safe, economical, and do not involve any hazardous reagents [10]. Medicinal plants comprise phytochemicals such as alkaloids, terpenoids, steroids, carbohydrates, saponins, and flavonoids [11,12], which are responsible for pharmacological attributes [13-15]. The phytochemicals act as reducing agents for silver ions to silver NPs without external toxic reagents. Different plant parts (leaves, roots, fruits, flowers, stems, peels, and seeds) serve as a natural and rich source of reducing agents, including enzymes, terpenoids, carbohydrates, polyphenols, tannins, alkaloids, and vitamins [16,17].

P. cubeb (kabab chini and java pepper) belongs to the genus *Piper*, a genus of spices abundantly cultivated in Indonesia, India, and Africa [18,19]. *P. cubeb* is popular among Unani folk healers as an antiseptic, sedative, mouth freshener, disinfectant, antihypertensive, antispasmodic, stimulant, anti-rheumatic, analgesic, and anesthetic [20,21]. The *P. cubeb* is also reported to cure cancer in the old Chinese and Moroccan medicine systems. The green fruit of the plant turns black when exposed to sunlight, becomes bitter with an intense aroma, and is used as a spice [22,23]. The extract of *P. cubeb* not only reduces metal ions but also stabilizes Ag NPs due to certain bioactive compounds such as terpenoids, hydrocarbons, polyphenols, and alkaloids, as reported in the literature [24].

The present study reported a novel and green approach to biosynthesize Ag NPs from *P. cubeb* extract, which serves as a reducing and capping agent with the assistance of diffused sunlight. The synthesis of Ag NPs using the aqueous extract of *P. cubeb* as reducing and capping agent is not reported yet. The prepared Ag NPs were characterized via UV-Visible Spectroscopy, FTIR, SEM, and EDS analysis. The antimicrobial potential of Ag NPs was estimated by determining ZOI, MIC, and MBC against Gram-negative (*E. coli* and *S. typhi*) and Gram-positive (*B. subtilis* and *B. licheniformis*). The antioxidant potential of prepared Ag NPs was evaluated by DPPH and ABTS assays.

2.0. MATERIALS AND METHODS

2.1 Materials

Dried *P. cubeb* corns were purchased from a local market in Lahore, Pakistan. The reagents DPPH, ABTS, and silver nitrate were acquired from Merck. Trolox and butylated hydroxyanisole (BHA) were procured from Sigma Aldrich. Gram-negative (*E. coli* and *S. typhi*) and Gram-positive (*B. subtilis* and *B. licheniformis*) bacterial isolates were gifted by the Institute of Biochemistry and Biotechnology Laboratory, University of the Punjab, Lahore. All the solutions and dilutions were prepared in doubly deionized water.

2.2. Extract Preparation

The dried piper corns (10.0 g) were heated in water at 80°C on a hot plate for 30 min to prepare the extract. The extract was cooled and filtered to remove insoluble particles, then stored at 4°C and used as a reducing and capping agent for Ag NPs.

2.3. Preparation of Ag NPs

The prepared *P. cubeb* extract (10.0 mL) was added to the $AgNO_3$ solution (10.0 mL), which was then exposed to sunlight (UV index 10+) at room temperature. A rapid color change was observed, turning brownish-red [3] within 15 minutes.

2.4. Isolation of Ag NPs

The freshly prepared Ag NPs were centrifuged at 11,000 rpm for 15 minutes. The Ag NPs thus settled were separated by decantation. The Ag NPs were washed three times with deionized water and dried at 80°C in a vacuum oven. The dried Ag NPs were scratched from the petri dish and stored in a vacuum desiccator before characterization and biological applications.

2.5 Characterization of BioNPs

The maximum absorption, active functionalities, particle size, and surface morphology were assessed using various characterization techniques. The reduction of silver ions by bioactive compounds in the extract to Ag NPs was scanned in the 200-800 nm range after fixed time intervals (5, 10, and 15 min) using a UV-spectrophotometer (Cary-60). The bioactive functionalities in the extract and Ag NPs were examined via Bruker 27 Tensor FTIR Spectrophotometer in the 4000-400 cm^{-1} wavenumber range. The surface morphology of the synthesized Ag NPs was studied using FEI Nova 450 Nano SEM. SEM-energy-dispersive X-ray spectroscopy (EDS) was used to further analyze the elemental composition of phytochemical-capped Ag NPs [33].

2.6. Antioxidant Activity

The antioxidant activity of Ag NPs was assessed by DPPH and ABTS assays.

2.6.1. DPPH Assay

The absorbance of the DPPH solution blank was recorded using a UV-visible spectrophotometer (Cary-60) at 517 nm. The violet-colored DPPH solution (2.5 mL) was shaken thoroughly with Ag NPs solution (1.0 mL). The solution was kept in the dark, and absorbance was recorded after fixed time intervals for 30 min. Each experiment was performed thrice, and values are reported as the average. Using the absorption data, the antioxidant potential was calculated using the following equation (i)[25,33];

$$\%\ \text{Free radical scavenging} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad \text{(i)}$$

2.6.2. ABTS Assay

ABTS solution (3.0 mL) was prepared in phosphate buffer, then mixed with Ag NPs solution (1.0 mL), and the absorbance was measured at 734 nm using the previously mentioned UV-Vis spectrophotometer [4]. The mixture was incubated in the dark at room temperature for 16 hours. The antioxidant potential of Ag NPs was compared with that of the Trolox standard. Each experiment was performed thrice, and values are reported as the average. The antioxidant potential of the sample Ag NPs was calculated using the following equation (ii);

$$\%\ \text{Inhibition} = \frac{\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}}{\text{Absorbance}_{\text{control}}} \times 100 \quad \text{(ii)}$$

2.6.3. Antimicrobial Activity

The Agar Well Diffusion method was followed to assess the antimicrobial potential of Ag NPs. The study used two gram-positive bacterial strains, *B. subtilis* and *B. licheniformis*, and two gram-negative strains, *E. coli* and *S. typhi*. For this purpose, Muller-Hinton agar was evenly distributed on the Petri dishes, and the microbial cultures were applied to the plates. The Ag NPs suspension was applied to the wells of agar medium prepared with a sterile cork borer. The Petri dishes thus prepared were kept at 37°C for 24 h. The positive control (rifampicin) and negative control (dimethylsulfoxide) were used to compare the results of antimicrobial activity. Each experiment was carried out thrice, and an average ZOI (Zone of Inhibition) was measured [25].

2.6.4. MIC and MBC Determination

The MIC (Minimum Inhibitory Concentration) was determined by preparing bacterial cultures and then transferring the cultures (30.0 µL) to the sterile test tubes. Different dilutions (20) of Ag NPs, ranging from 10 to 0.5 mg mL^{-1}, were prepared and then shifted to individual test tubes containing bacterial cultures. The test tubes were incubated for 24 hr at 37°C, and the optical density of the resultant samples was examined at 523 nm. After assessing MIC values, 100 µL of

the samples were transferred to agar plates for 24 hr incubation to determine MBC (Minimum Bactericidal Concentration) values [25].

3.0 RESULTS AND DISCUSSION

Ag NPs were synthesized by mixing aqueous extract of corns with silver nitrate solution. The phytochemicals in the aqueous extract rapidly reduced silver ions (Ag^{+}) to Ag^{o}. The reduction is confirmed by a swift color change from light yellow to reddish brown, which became persistent after 15 minutes, confirming the completion of the reaction (Fig. 1). The observed color change (reddish brown) is the characteristic color shown by most of the Ag NPs given in the literature [26-29]. Phytochemicals in plants act as reducing agents, including flavonoids, alkaloids, polyphenols, steroids, carbohydrates, saponins, and amino acids, which are naturally occurring and responsible for color changes. Excitation of surface plasmon vibrations also contributes to the reddish-brown color of Ag NPs [28,30].

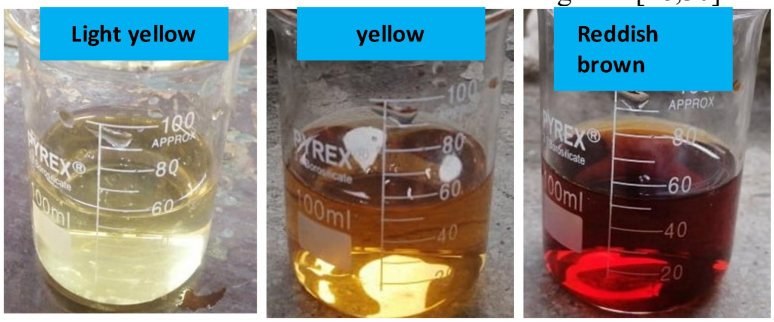

Figure 1: Color of nanoparticle solution after (a) 5 min (light yellow), (b) 10 min (yellow), and (c) 15 min (reddish brown)

The absorption maximum of Ag NPs was observed using a spectrophotometer at varying time intervals (5, 10, and 15min). A definite and similar peak around 440 to 445 nm was observed for all the test samples, confirming the nanoparticle synthesis (Fig. 2). Maximum absorption (440-445 nm) could be related to the average particle size of 25 to 40 nm. The particle size increases over time due to Ag NP agglomeration. The diameter of Ag NPs changes with the maximum absorption wavelength of light. The diameter changes from 25-30 nm to 35-40 nm as the wavelength increases from 440 to 445 nm, followed by an increase in absorption of radiation [31, 32].

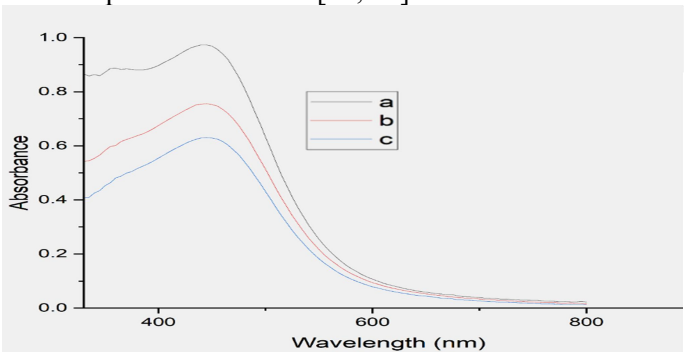

Figure 2: Spectral Analysis of the Ag NPs at varying intervals: a. 5, b. 10 c. 15 min, showing an increase in absorption of radiation in the range of 440 to 445 nm.

3.2. Scanning Electron Microscopy of the Ag NPs

The SEM analysis is carried out to confirm the morphology of the synthesized NPs [33]. The results obtained from the FEI Nova 450 Nano SEM showed spherical, well-dispersed Ag NPs with minimal agglomeration, as indicated by a single, symmetrical UV peak. The uniform, spherically shaped Ag NPs were 18-27 nm in diameter (calculated using ImageJ), which is consistent with UV-visible data. The uniform distribution of Ag NPs revealed controlled nucleation and growth, supporting the use of plant extracts as reducing and capping agents, which falls within the ideal range according to the literature (Fig. 3) [32,34].

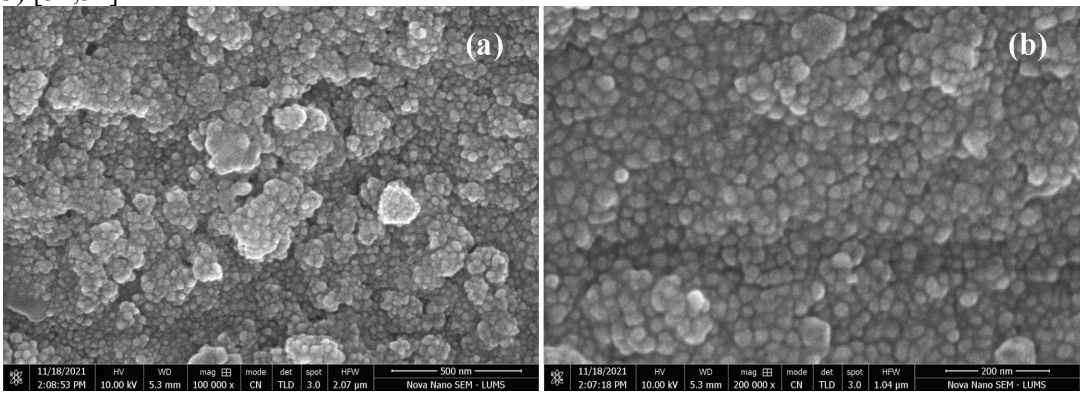

Figure 3: SEM images of Ag NPs at different magnifications (a) 500 nm and (b) 200 nm

3.3. EDS Analysis

The elemental composition of Ag NPs was investigated using EDS analysis (Fig. 4). A substantial and significant peak at 3.0 keV is attributed to Ag NPs, confirming the successful reduction and stabilization of Ag NPs by the phytochemicals in the plant extracts [35,36]. The spectrum also showed the carbon and oxygen peaks, due to the adsorption of phytochemicals on the surface of Ag NPs. Literature also confirmed such results for Ag NPs [37,38].

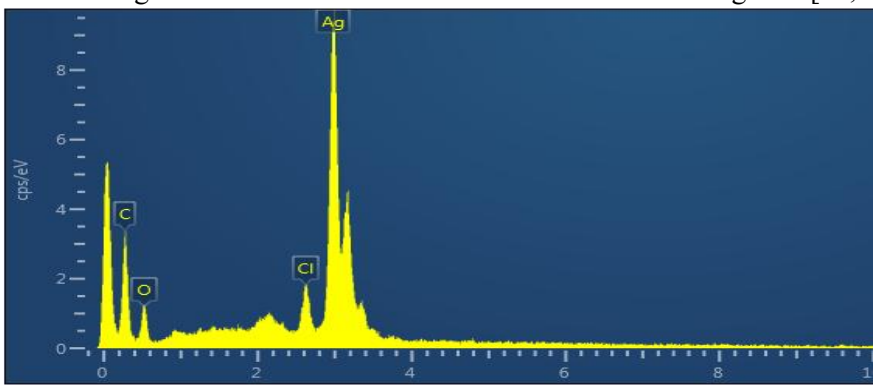

Figure 4: EDS spectrum of Ag NPs showing a prominent peak of Ag NPs at 3 keV

3.4. FTIR Analysis

Phytochemicals in the plant extract not only reduce silver ions to silver atoms but also cap Ag NPs [5]. FTIR spectrum confirmed the successful synthesis of Ag NPs due to the appearance of a band at 478 cm^{-1} due to the Ag-O bond, while other bands are due to the adsorption of phytochemicals on the surface of Ag NPs. A broad band observed at 3281 cm^{-1} is attributed to N-H or O-H stretching vibrations in the extract's spectrum, which shifted to a slightly higher wavenumber of 3287 cm^{-1} in the FTIR spectrum of Ag NPs. The functional groups, such as N-H and O-H, indicated the presence of flavonoids and polyphenols [39]. The stretching vibration of the amide carbonyl group appeared at 1638-1630 cm^{-1} in the extract and Ag NPs [40]. The peak observed at 1522 cm^{-1} is assigned to NH bending vibrations. This peak shifted to a higher wavenumber of 1539 cm^{-1} in the FTIR spectrum of Ag NPs [26]. Different bands in FTIR spectra of both the extract and Ag NPs indicated phytochemicals, such as polyphenols and flavonoids, reduced and capped Ag NPs (Fig. 5) [41,42].

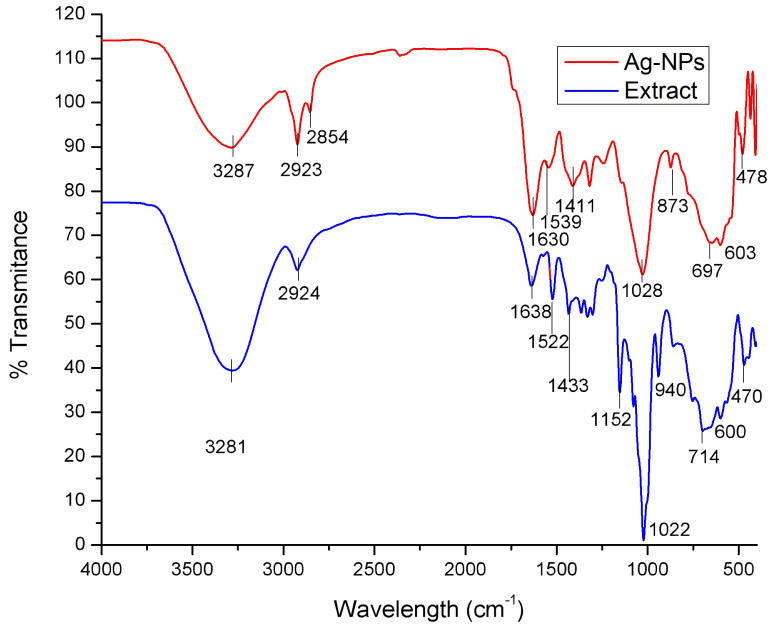

Figure 5: FTIR spectra of *P. cubeb* extract and Ag NPs capped by aqueous extract

3.5. Antioxidant Activity

3.5.1. DPPH Assay

The antioxidant potential of Ag NPs, Trolox, BHA, and blank was evaluated using a widely accepted approach, i.e., DPPH assay over 30 minutes [43]. The percentage of free radical scavenging was used as a measure of antioxidant potential. The scavenging activity was 100% with blank, which served as a baseline to compare the antioxidant potential of Ag NPs, BHA, and Trolox. The DPPH solution (a violet-colored free radical) turned yellow with the addition of Ag NPs solution because Ag NPs capture DPPH free radicals in a time-dependent manner and act as antioxidants. Ag NPs demonstrated strong antioxidant potential, 92% scavenging over 30 minutes. The capturing of free radicals was maximum for Ag NPs, followed by BHA and Trolox, which were taken as standards (Fig. 6) [44].

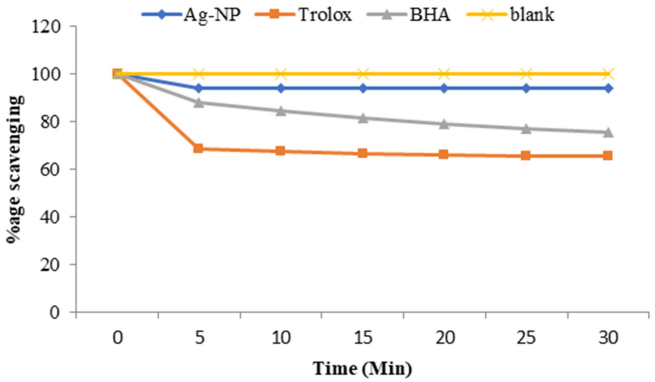

Figure 6: Antioxidant potential of Ag NPs, Trolox, and BHA using DPPH assay

3.5.2. ABTS Assay

The antioxidant activity was further assessed using the ABTS assay, and results are expressed as Trolox Equivalent Antioxidant Capacity (TEAC) values. The ABTS assay measures the ability of antioxidants to capture ABTS free radicals. The ABTS assay is suitable for both hydrophilic and hydrophobic antioxidants. Potassium persulfate or manganese dioxide is used as a buffer along with ABTS. The addition of Ag NPs to an ABTS solution fades its blue color, indicating its antioxidant potential [45]. The reasonable antioxidant capability of Ag NPs can be observed from the Trolox equivalent antioxidant capacity (TEAC) value, i.e., 4.609. The standard BHA demonstrated a TEAC value of 7.46. Thus, BHA is more potent in capturing free radicals than Ag NPs; however, Ag NPs still exhibit considerable antioxidant activity, supporting their use as complementary antioxidants in food, pharmaceuticals, and nanomedicine[2,3,46] (Fig. 7).

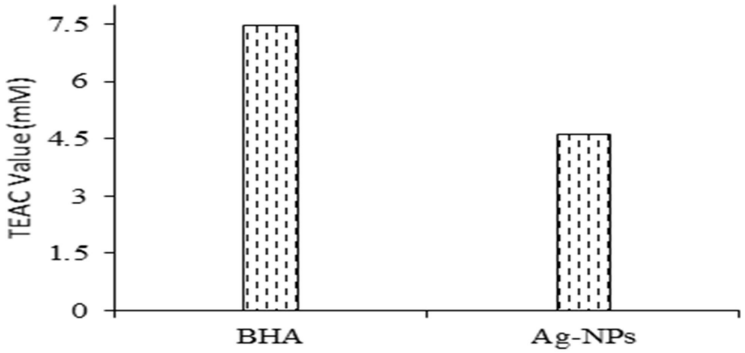

Figure 7: Antioxidant potential of Ag NPs using ABTS assay.

3.5.3. Antibacterial Assay

Different concentrations of Ag NPs were assessed for antibacterial activity using the agar well diffusion method against *E. coli, B. licheniformis, B. subtilis*, and *S. typhi*. Ag NPs exhibited prominent antibacterial activity, as evidenced by the zone of inhibition. The Ag NPs interact with the nitrogen and sulphur atoms of the cell wall peptidoglycan, disrupting the cell wall. After that, NPs denature proteins, leading to mitochondrial dysfunction and DNA disintegration, which in turn kills bacteria. Ag NPs revealed significant bactericidal potential against both the Gram-negative and Gram-positive bacteria, despite differences in cell wall structures. No antibacterial activity was shown by the negative control (NC), confirming that antibacterial activity is due to Ag NPs. The ZOI by Ag NPs against bacterial strains is comparable to that of standard rifampicin (Fig. 8). It was further observed that NPs are more effective against *Bacillus species* than other strains. Literature revealed that Ag NPs prepared from different plant extracts inhibited the growth of Gram-positive and Gram-negative bacteria. Polar extract of *Cotoneaster nummularia* inhibited the growth of *E. coli* with a ZOI of 10 mm [3]. Similarly, mucilage-capped Ag NPs from cress seeds displayed significant antibacterial activity against *B. cereus* (27 mm), *E. coli* (25 mm), and *E. aerogenes* (22 mm) at a concentration of 100 µg/mL [46]. Glucuronoxylan-capped Ag NPs depicted prominent growth reduction of *B. subtilis* (10 mm) and *E. coli* (18 mm) at 100 mmol concentration [2]. Thus, Ag NPs from plant extracts could be potential substitutes for commercial antibacterial agents in antimicrobial coatings and wound dressings as potent therapeutic agents. The calculated values of respective inhibition zones and the images of each strain are shown in Figure (9).

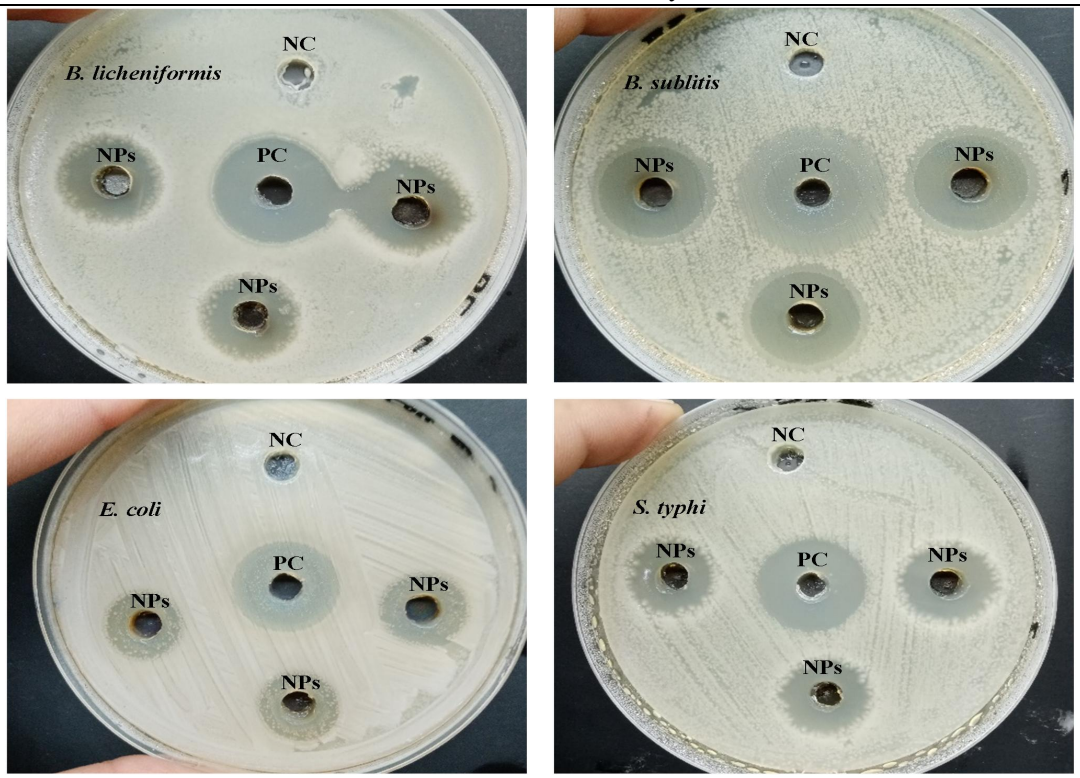

Figure 8: Images of bacterial growth inhibition by Ag NPs, positive control (rifampicin), and negative control (deionized water)

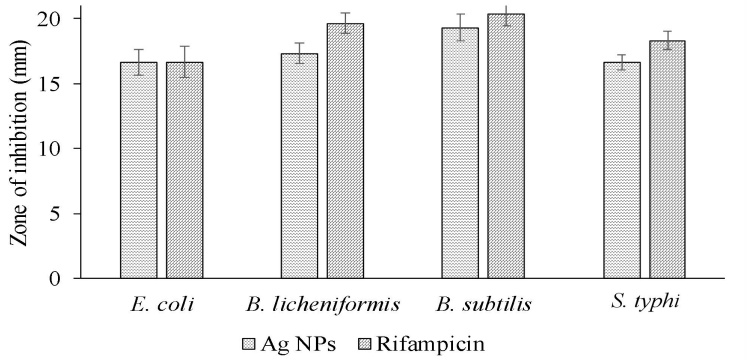

Figure 9: Inhibition Zones by Ag NPs and rifampicin against both the Gram-positive and Gram-negative bacteria

3.5.4. MIC and MBC Values

The efficacy of Ag NPs was further investigated by determining the minimum inhibitory concentration and minimum bactericidal concentration. The concentration that minimally inhibits bacterial growth is the MIC. Both the MIC and MBC provided qualitative assessments of the bacteriostatic and bactericidal potential of Ag NPs, supporting the data from the zones of inhibition. The average MIC values of Ag NPs against *E. coli* (2.16 mg), *B. licheniformis* (2.83 mg), *B. subtilis* (1.33 mg), and *S. typhi* (3.66 mg) are low, indicating a significant bacteriostatic potential. MIC values discovered that Ag NPs are more effective against *B. subtilis,* followed by *E. coli*, *B. licheniformis,* and *S. typhi* (Fig. 10). The lower MIC values supported the inhibitory effect of Ag NPs against both the Gram-positive and Gram-negative bacteria.

The minimum bactericidal concentration needed to kill 99.9% of the bacterial population is the MBC. The Ag NPs exhibited minimum bactericidal concentrations of 3.0, 3.25, 2.25, and 4.0 mg against *E. coli*, *B. licheniformis*, *B. subtilis*, and *S. typhi*, respectively. It is confirmed from MBC values that *B. subtilis* is more susceptible to Ag NPs, followed by *E. coli, B. licheniformis*, and *S. typhi*. MIC and MBC values not only indicated bacteriostatic but also bactericidal potential of Ag NPs. The difference in susceptibility between Gram-positive and Gram-negative bacteria may be due to differences in cell wall composition. Galactomannan-stabilized Ag NPs revealed minimum MIC values against *B. cereus* 5.5 µg/mL), *E. coli* (9.0 µg/mL), and *E. aerogenes* (15 µg/mL), confirming their inhibitory potential. Similarly, Ag NPs killed bacteria such as *B. cereus* (10 µg/mL), *E. coli* (14 µg/mL), and *E. aerogenes* (14.5 µg/mL) with lower MBC values [46]. Literature revealed the similar inhibitory potential of Ag NPs prepared using plant materials such as glucuronoxylan from *Mimosa pudica* seeds [2], polar extract of *C. nummularia* [3], *Benincasa fistulosa* leaves [10], and *Citrus limon* zest extract [16]. Thus, it can be concluded from ZOI, MIC, and MBC values that Ag NPs could be a potential substitute for antibiotics, as they inhibit bacterial growth or kill bacteria.

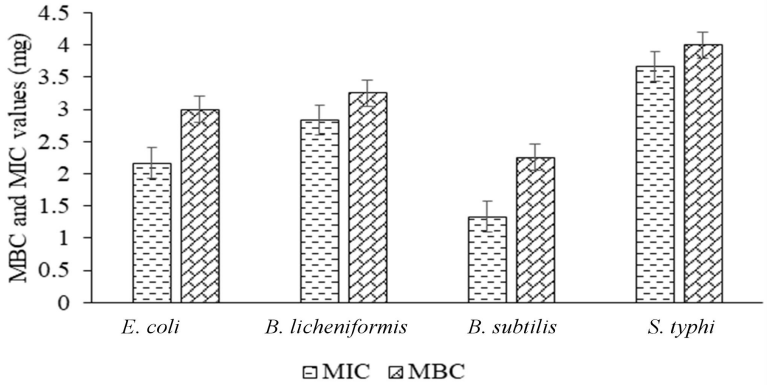

Figure 10: Comparison of MBC and MIC values against Gram-positive and Gram-negative bacteria

CONCLUSION

It was concluded that the *P. cubeb* corn extract was suitable for the biosynthesis of Ag NPs, which reduced silver ions within 15 min under sunlight. This was confirmed by a rapid color change, indicating the synthesis of Ag NPs. Moreover, it was established that using the aqueous extract of P. cubeb corns as a non-toxic, novel reducing and capping agent was eco-friendly and was effective in enhancing antioxidant and antibacterial activities of the NPs. The bioactive constituents polyphenols, terpenoids, and amino acids played dual roles as capping and reducing agents, enabling rapid and active biosynthesis of Ag NPs. It is recommended that more research is required on the biosynthesis of Ag NPs and on the evaluation of the mechanisms underlying the antioxidant and antimicrobial activity of Ag NPs in the medicinal field.

Acknowledgment

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