Phytosynthesis of Copper Nanoparticles Using Extracts of Spices and Their Antibacterial Properties

Gayathri Vijayakumar, Hindhuja Kesavan, Anisha Kannan, Dhanalakshmi Arulanandam, Jeong Hee Kim, Kwang Jin Kim, Hak Jin Song, Hyung Joo Kim, and Senthil Kumaran Rangarajulu

1. Introduction

In modern years, there has been a continuing renewal of interest pertaining to the utilization of therapeutic and aromatic plants to produce antimicrobial drugs as plant-derived drugs are assured to be harmless and without side-effects [1]. People in different countries have a long-standing custom of employing a wide range of herbal products to heal diseases. Spices are indispensable components of Indian cuisines. They are also rich sources of dynamic antimicrobial compounds [2]. Nanoparticles have fascinated many researchers because they have unique characteristics such as size, shape, distribution, and morphology compared to bulk materials [3–5]. In all science fields, including the therapeutic field, metallic nanoparticles are being utilized. They are still alluring scientists to investigate new scopes due to their small sizes. These metal nanoparticles have advantages due...
to their small sizes, large surface areas, excellent chemical and optical properties, and good electrical conductivities [6]. Among several metallic-element nanoparticles, copper nanoparticles have been under an exceptional spotlight. Plant-based molecules are of great interest to the scientific community because they have a wide range of sizes and structures with various biological functions [7]. Copper nanoparticles are broadly used in batteries, optical devices, polymers, and multilayer metal ceramics. They are also used for drug delivery and as antimicrobials or antioxidants [8].

The potential for developing an antimicrobial into a medication appears gratifying from perspectives of medicine improvement and phytomedicine. Spices are known to possess various beneficial properties that are beneficial for human health due to their anti-oxidative, anti-inflammatory, anti-hepatotoxicity, anti-tumor, and anti-microbial effects. These biological activities of spices are due to the presence of various antimicrobial compounds. Inhibiting microbial growth and synthesizing antimicrobial compounds using spices have been topics of several studies [9]. Several spices such as oregano, clove, cinnamon, cumin, and thyme have been practiced for treating communicable diseases and protecting food as they have been theoretically demonstrated to have antimicrobial actions toward disease-causing organisms [10–12]. Energetic plant-based chemicals obtained from spices have a molecular basis for these activities. Furthermore, these antimicrobial activities are due to secondary metabolites of spices, the bulk of which are normally not dangerous materials for food or have side effects due to unfavorable properties [13].

Copper nanoparticles are tremendously minute. They typically have high surface-to-volume ratios. They play roles as antibacterial and antifungal agents. Their antimicrobial actions are brought by their near-proximity contact with microbial membranes and their metal ions released into solutions because nanoparticles undergo oxidization gradually in solutions with cupric ions liberated from them. They can generate lethal hydroxyl free radicals when the lipid membrane is adjacent to it. Free radicals can deconstruct lipids in cell membranes through oxidation and collapse membranes, leading to the leakage of the intracellular substances of cells through collapsed membranes. Such cells may not be able to maintain essential metabolic reactions, leading to modification within cells and necrobiosis [14].

Recently, green synthesis approaches have been utilized for the biosynthesis of nanoparticles using phyto-formulated bioactive products [3,15]. Biological compounds can act as strong reducers and stabilizers in the process of biogenic nanosynthesis, which is highly preferred to have an ecofriendly environment [16]. Spices are important plant condiments with many bioactive agents having various applications [11,12]. The objective of this study was to evaluate the efficacy of using extracts of three different spices (star anise, nutmeg, and mace) for the synthesis of CuNPs. Although several studies have been conducted on these spices, green-synthesized CuNPs using these spices and their antibacterial properties are still largely unexplored. Phyto-formulated copper nanoparticles obtained from these three spices were then characterized with various analytical methods. The antimicrobial activities of these extracts and synthesized nanoparticles were assayed with standard approaches.

2. Materials and Methods
2.1. Biosynthesis of Copper Nanoparticles

All chemical reagents used in this experiment were of analytical grade. The three spices (Illicium verum—star anise, Myristica fragrans—nutmeg, and mace (lacy coating of the nutmeg seed)) were collected from an herbal medicine shop located in Chennai, India. These three different spices were dried at 45 °C in an incubator. Dried spices were crushed to fine particles using a grinder in the dark at indoor temperatures (35 °C), placed into containers, and sealed. Typically, 5 g of each dried spice material was dissolved in 50 mL of water. The aqueous solution containing spice material was heated in a heating mantle at 60–80 °C for 20 min and filtered using Whatmann No.1 paper. After 0.5 M of copper sulfate solution was prepared freshly, it was then added to each spice extract at a 1:1 ratio
and kept in the dark at 37 °C for 24 h. A color change of the solution from bluish green to brownish red was noted by visual examination. This confirmed the synthesis of CuNPs using spice extracts [17,18].

2.2. Analytical Characterization of Biogenic CuNPs

Biogenically synthesized CuNPs were initially analyzed by UV-Vis spectroscopy (U2900, Hitachi, Tokyo, Japan). The reduction of copper ions was evaluated using 1 mL of biogenically synthesized test samples along with a control (copper sulfate solution without plant extract). The absorbance was recorded with a resolution of 1 nm at wavelengths from 150 to 600 nm. A scanning electron microscopy (SEM) study was carried out to examine the size and surface morphology of the synthesized copper particle. For sample preparation, a thin gold layer was sputter-deposited onto the synthesized copper particle using an ion sputter coater with a gold target instrument. The thin gold layer produced was air-dried under ambient conditions. Images were then obtained using a Hitachi S-3400N instrument with an electron beam accelerated at 300 V to 30 kV. Signals were detected by secondary electron (SE)/backscattered electron (BSE) detectors with enlargement up to 300,000. Energy-dispersive X-ray (EDAX) spectroscopic analysis was performed to identify the existence of copper in synthesized particles. Elemental compositions for synthesized particles were determined in spot profile mode using an EDAX instrument coupled with SEM analysis. The existence of copper was determined using a detection graph. Elemental composition was estimated as a weight percentage. A dried pellet of synthesized copper nanoparticles was analyzed using a Perkin Elmer Fourier-Transform Infrared (FTIR) Spectroscopy C100599 Instrument with a resolution of 0.4 cm⁻¹ at a working range of 350–7800 cm⁻¹ [8]. The GC–MS technique was adopted for the detection and identification of phytochemical compounds present in copper nanoparticles of spices. It was carried out using a PerkinElmer GC Clarus 500 system (Waltham, MA, USA). The GC–MS detection was executed using electron impact mode with an ionization energy of 70 eV. The carrier gas was helium (99.999%) with a constant flow rate of 1 mL/min and the injection volume was 1 µL. Temperature settings were: injector temperature, 250 °C; ion-source temperature, 200 °C; and oven temperature, 110–280 °C [19].

2.3. Antibacterial Activity of Biogenic Copper Nanoparticles

Biogenically synthesized CuNPs at a concentration of 10 µg/mL were dispersed in sterile deionized water and used for an antimicrobial activity study using the well diffusion method [19]. Briefly, Mueller-Hinton agar plates were prepared and 100 µL of Staphylococcus aureus culture was spread evenly onto surfaces of these agar plates. Wells of comparable sizes (6 mm) were made on these plates and then loaded with as-synthesized CuNPs in triplicate. These plates were incubated at 37 °C for 24 h. The antimicrobial activity was determined based on the formation of the zone of inhibition (ZOI) in millimeters [20].

2.4. Antibacterial Activity of Spice Extract

One gram of granulate of plant material was immersed in 5 mL of hexane (1:5) for 24 h at 32 °C in an orbital shaker (120 rpm) incubator. The mixture was strained with Whatmann no. 1 paper. The filtrate was kept in petri dishes and the solvent was allowed to evaporate at 37 °C. For the evaluation of antimicrobial activity, samples were prepared by dissolving 100 mg of each extract in 1 mL of dimethyl sulfoxide (DMSO). The antimicrobial activity of each test extract was determined with the disc diffusion method [21]. For the antibacterial assay, the bacterial culture of S. aureus was inoculated into a nutrient agar broth medium and grown at 37 °C. Plates of agar media were prepared. Each plate was inoculated with an aliquot (100 µL) of bacterial suspension, which was spread evenly on the surface of the medium of the plate. After 15 min, discs were dipped in 2 µg/mL of test samples and placed on these plates. The positive control was prepared with gentamicin. All tests were carried out in triplicate. Plates were incubated at 37 °C for 24 h. The antimicrobial
activity was assessed by measuring the diameter of ZOI in millimeters. The experiment was repeated three times for every extract against the test organism [21].

2.5. Extract Preparation and Chromatography

To prepare nanoparticles with spice extracts for chromatographic separation, 0.5 g of each nanoparticle sample was added to 1 mL of petroleum ether. Samples were separated on aluminum-backed thin-layer chromatography (TLC) plates using a mixture of chloroform/ethyl acetate/formic acid (5:4:1). Separated chemical compounds were detected using iodine vapor. After incubation at room temperature, compounds were observed with an ultraviolet-visible (UV-Vis) spectroscope [22].

2.6. TLC-Direct Bioautography

TLC-direct bioautography is suitable for the rapid chemical and biological testing of plant extracts. It is mainly based on the antimicrobial properties of evaluated substances. Briefly, developed plates were dried under a stream of fast-paced air and then incubated at 90 °C for 30 s. Bacterial cultures were prepared in a liquid medium. Prepared chromatograms were dipped in bacterial suspension. The TLC-chromatogram was then incubated overnight at 37 °C. For visualization of microbial growth, tetrazolium salts were employed. These salts could be converted to an intensely colored formazan by dehydrogenases of living microorganisms. These salts were sprayed onto the chromatogram and incubated at 37 °C for 3 to 4 h. The formation of clear white areas aligned with the purple backdrop on the TLC plate, indicating an antimicrobial effect of the test sample [23,24].

3. Results and Discussion

3.1. Nanoparticle Synthesis

The synthesis of copper nanoparticles was performed by adding copper sulfate solution (CuSO$_4$·5H$_2$O) to the extract at a 1:1 ratio. A color change of the aqueous mixture was observed after 24 h of incubation. Visual confirmation of the color change from the initial color to a brown color was recorded (Figure 1). An increase in the intensity of color with time indicated an enhanced production of CuNPs. In addition, the synthesis was confirmed by UV-Vis spectroscopy with a characteristic peak recorded at 250 nm without peaks recorded between 150 nm and 600 nm for the control (copper sulfate solution), as shown in Figure 2. This showed the reduction of Cu ions to CuNPs with a characteristic peak in the absorption range from 200 nm to 300 nm. In this study, all three test samples (star anise, nutmeg, and mace) showed the existence of a single plasma resonance peak at 250 nm, indicating the synthesis of biogenic CuNPs in the test solution, similar to CuNPs synthesized by using Caesalpinia bonducella seed extract via a green synthetic pathway [6], which also showed the maximum absorption peak at 250 nm.

3.2. SEM Analysis

The structures and shapes of biogenic CuNPs were revealed by scanning electron microscopy. The maximum size of these CuNPs was 270 nm. Their structures were found to have distinct shapes. The dimensions of these nanoparticles were found to be within the nanoscale range. Biogenic CuNPs prepared with extracts of star anise, nutmeg, and mace had sizes of 210–270 nm, 170–210 nm, and 150–220 nm, respectively (Figure 3). Thus, typical sizes of copper nanoparticles synthesized in this study ranged from 150 nm to 270 nm. Green-synthesized CuNPs with aloe barbadensis leaf extract have been reported to have sizes of 80 nm to 120 nm [25]. CuNPs prepared with clove bud extract have sizes of ~12 nm [26]. Cuprous oxide nanoparticles (Cu$_2$O NPs) produced by the chemical reduction of copper sulfate salt in water-in-oil microemulsion solution using NaBH$_4$ as a reductant have sizes of ~250 nm to ~550 nm [27] based on SEM analysis.
Figure 1. Phyto-formulated biosynthesis of CuNPs. (A) Plant materials of star anise (1), nutmeg (2), and mace (3) used for making extracts; (B) a mixture of copper sulfate and plant extract of star anise (1), nutmeg (2), or mace (3); (C) synthesis of CuNPs with star anise (1), nutmeg (2), or mace (3) and characteristic color change after 24 h of incubation; (D) copper sulfate aqueous solution (control).

Figure 2. UV-Vis spectroscopic analysis of test samples after 24 h of incubation, showing phytogenic synthesis of CuNPs with the maximum absorption at 250 nm.
Figure 3. SEM images of biogenic CuNPs prepared with extracts of spices. (A) Nutmeg (150 nm to 220 nm), (B) star anise (210 nm to 270 nm), and (C) mace (150 nm to 220 nm).

3.3. EDAX Analysis

For elemental identification and quantitative composition determination, EDAX analysis was performed. It confirmed the presence of copper (Cu) and oxygen (O) along with a meager impurity of sulfur in trace amounts (Figure 4). All of the three tested spices samples showed the existence of very similar profiles in its elemental composition. Weight compositions of copper (Cu), oxygen (O), and sulfur (S) in these synthesized nanoparticles were found to be 88.20%, 11.78%, and 0.02% by mass, respectively. The stoichiometric fraction of the Cu:O molar ratio was found to be 1.8851, which is very close to 2:1 (molar ratio), proving that the obtained copper nanoparticles were in the form of cuprous oxide (Cu$_2$O), consistent with results of previous studies [27,28]. Recently, researchers have shown increasing interest in finding the mechanism involved in the synthesis of CuNPs with green plant sources. Biomolecules such as flavonoids, proteins, tannins, phenols, and terpenoids in plants have been reported to be good reducing and stabilizing agents for CuONPs synthesis [29]. The existence of phenols and terpenoids in green-synthesized CuNPs with spices was confirmed in the present study. Several studies have discovered that phytochemicals in plant extracts can initially form complexes with ion salts and then reduce ions to form nanoparticles [18]. Biomolecules in plant extracts can usually react with copper ions to cause reduction, which subsequently transforms into copper oxide nanoparticles (CuONPs) [30]. A similar type of reaction was also observed in the present study, where EDAX analysis proved the presence of CuONPs.

Figure 4. EDAX analysis of green-synthesized CuNPs from star anise showing peaks representing the presence of copper (indicated by arrows).
3.4. Fourier-Transform Infrared (FTIR) and Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

3.4.1. FTIR Characterization of CuNPs

FTIR investigation depicted possible biomolecules in plant extracts accountable for the stabilization of CuNPs and to examine functional groups of phytochemicals on NPs as adsorbent materials.

As shown in Figure 5, FTIR spectra for green-synthesized nanoparticles with star anise revealed bands at 3004.89 cm\(^{-1}\) corresponding to =CH stretching, proving the presence of aromatic –H; 2923.88 cm\(^{-1}\) corresponding to OCH\(_3\) stretching; 2866.46 cm\(^{-1}\) corresponding to –CH stretching proving the presence of aromatic C–H; and 1745.4 cm\(^{-1}\), 1608.52 cm\(^{-1}\), and 1100.06 cm\(^{-1}\) corresponding to C=O stretching, C=C bending, and C–O stretching, respectively, proving the presence of an ester group, aryl C=C group, and aliphatic C–O group, respectively. The FTIR spectrum attributed to OCH\(_3\) stretching and –CH stretching has revealed the existence of a benzene ring in the obtained green-synthesized Cu nanoparticles of star anise [31]. The presence of these functional groups in green-synthesized nanoparticles with star anise indicates that it contains known structures such as anethole and methyl ester compounds such as stearate [32,33]. The existence of an ether group and aromatic cycle structure in star anise has been proven by earlier studies [34,35].

![Figure 5. FT-IR spectra of green-synthesized CuNPs of star anise showing probable functional groups in synthesized nanoparticles.](image)

FT-IR spectra attained from green-synthesized nanoparticles of nutmeg (Figure 6) revealed bands at 3194.92 cm\(^{-1}\) corresponding to –OH stretching, proving the presence of carboxylic acid –OH; 2939.39 cm\(^{-1}\) corresponding to the C–H stretching of alkane C–H; 1704.54 cm\(^{-1}\) corresponding to C=O stretching of the ester group; 1430.48 cm\(^{-1}\) corresponding to C–H bending of alkane C–H; and 1264.88 cm\(^{-1}\) corresponding to C–O–C stretching of an ether group. The existence of these functional groups in green-synthesized nanoparticles of nutmeg depicted the presence of esters, alcohols, and phenolic compounds, similar to results of a previous study [36]. In one earlier study, the FTIR spectra on methanolic solvent extract and ethyl acetate extract of Myristica fragrans seeds also revealed the presence of a carbonyl group, alkane group, and ether group as functional groups of biological compounds such as alkaloids, steroids, tannins, flavonoids, phenolics, and glycosides, known to be secondary metabolites with antimicrobial properties [37]. It has been concluded that these functional groups are responsible for their antimicrobial properties [38].
FTIR spectra of green-synthesized nanoparticles of mace (Figure 7) disclosed bands at 3385.65 cm\(^{-1}\) corresponding to the O–H stretching, proving the presence of carboxylic acid –OH; 2922.84 cm\(^{-1}\) and 2852.79 cm\(^{-1}\) corresponding to C–H stretching; 1704.82 cm\(^{-1}\) corresponding to C=O stretching, confirming the presence of an ester group; 1632.3316 cm\(^{-1}\) and 1415.16 cm\(^{-1}\) both corresponding to aromatic C–C stretching; and 1024.28 cm\(^{-1}\) and 810.90 cm\(^{-1}\) corresponding to C–O–C stretching and the deformation vibration of C–H bonds in phenolic rings, respectively. These results of FTIR analysis of green-synthesized nanoparticles of mace indicated a unique set of biochemical markers such as aromatic rings, aldehydes, alkanes, alkenes, and phenols [39]. These were similar to previous studies that depicted the presence of ester, methyl ester, and phenolic compounds [40,41].
3.4.2. GC–MS Analysis

Gas chromatography–mass spectrometric (GC–MS) analysis showed sharp and broad peaks after 10 min of retention time. These obtained peaks of copper nanoparticles of spices were compared with the NIST library (ADMIS 2005). A few of them produced matching results (Tables 1–3).

Table 1. GC-MS analysis of CuNPs of star anise.

| S.No. | Retention Time | Compound Name                      | Molecular Formula |
|-------|----------------|------------------------------------|-------------------|
| 1     | 4.159          | Pentanoic acid, 2-ethylhexyl ester  | C_{13}H_{26}O_{2} |
| 2     | 4.043          | D-Limonene                         | C_{10}H_{16}      |
| 3     | 6.58           | α-pinene                           | C_{10}H_{16}      |
| 4     | 6.854          | Anethole                           | C_{10}H_{15}O_{2} |
| 5     | 12.403         | Chloroacetic acid, tetradeyl ester | C_{16}H_{31}ClO_{2} |
| 6     | 21.02          | Trans-Anethole                      | C_{10}H_{20}      |
| 7     | 26.166         | Methyl stearate                     | C_{19}H_{36}O_{2} |

Table 2. GC-MS analysis of Cu nanoparticles of nutmeg.

| S.No. | Retention Time | Compound Name                      | Molecular Formula |
|-------|----------------|------------------------------------|-------------------|
| 1     | 4.067          | 2-Decenal                          | C_{2}H_{10}O      |
| 2     | 5.224          | 7-Decen-2-one                       | C_{10}H_{18}O_{2} |
| 3     | 10.458         | 2-Methoxy-4-(1-propenyl)phenol     | C_{14}H_{13}O_{2} |
| 4     | 11.913         | Myristic acid                       | C_{14}H_{28}O_{2} |
| 5     | 13.551         | Myristic acid methyl ester         | C_{15}H_{30}O_{2} |
| 6     | 26.162         | Heptadecanoic acid, 16-methyl-, methyl ester | C_{19}H_{36}O_{2} |
| 7     | 26.458         | Cyclododecane                       | C_{12}H_{22}O     |

Table 3. GC-MS analysis of Cu nanoparticles of mace.

| S.No. | Retention Time | Compound Name                      | Molecular Formula |
|-------|----------------|------------------------------------|-------------------|
| 1     | 4.043          | D-Limonene                         | C_{10}H_{16}      |
| 2     | 5.264          | Butanedioic acid, diethyl ester    | C_{8}H_{14}O_{4}  |
| 3     | 10.964         | Eugenyl methyl ether               | C_{11}H_{16}O_{2} |
| 4     | 11.913         | Myristic acid                      | C_{14}H_{28}O_{2} |
| 5     | 13.551         | Myristic acid methyl ester         | C_{15}H_{30}O_{2} |
| 6     | 16.41          | Methyl eugenol                     | C_{12}H_{16}O_{2} |
| 7     | 26.162         | Heptadecanoic acid, 16-methyl-, methyl ester | C_{19}H_{36}O_{2} |

About 57 compounds were identified by GC-MS analysis of Cu nanoparticles of star anise. Of these compounds, pentanoic acid, 2-ethylhexyl ester, D-limonene, α-pinene, anethole, chloroacetic acid, tetradeyl ester, trans-anethole, and methyl stearate were identified (Table 1) with reference to previous studies in the literature [12,42]. Most of these compounds had an alkane bond (hydrocarbons containing a single bond), a phenyl parent ring, and an alkene bond (containing one or more carbon–carbon double bonds). D-Limonene, α-pinene, anisole, chloroacetic acid, tetradeyl ester, and methyl stearate were also evident in the FTIR analysis of the present study. The same results have been revealed by other researchers [43]. Anethole and estragole compounds have been detailed by earlier studies [44,45]. Gholivand et al. [46] detected volatile constituents of star anise by GC-MS and disclosed that its key components are trans-anethole (81.4.0%), limonene (6.5%), chavicol (2.10%), and anisaldehyde (1.81%). The dried star anise fruit has nearly 8–12% essential oil, primarily anethole and fatty oil [47,48].

The GC-MS analysis of CuNPs of nutmeg revealed the presence of 78 compounds. Several compounds present in nutmeg were responsible for its biological activity and antimicrobial activity (Table 2). The major biological compounds in Cu nanoparticles of nutmeg based on the GC–MS analysis were 2-methoxy-4-(1-propenyl) phenol (RT: 10.458), octadecanoic acid (RT: 14.360), and cyclododecane (RT: 26.458). These compounds
have nutritional, pharmacological, and therapeutic importance [49–51]. FTIR analysis of the tested spices also showed the presence of esters, alcohols, and phenolic compounds. These organic compounds are responsible for the antimicrobial effect of green-synthesized nanoparticles [52,53]. In one earlier study, metal oxide nanoparticles synthesized with the aqueous seed extract of *Myristica fragrans* (nutmeg) were characterized by gas GC–MS. The results showed the existence of bioactive components that play an efficient role in reducing and capping mediators for transferring AgNO$_3$ to AgNPs, thereby illuminating itself as an effective drug against multi-drug-resistant bacteria [54].

GC-MS analysis of copper nanoparticles of mace (Table 3) revealed many components similar to those of nutmeg. This is because mace is the fleshy red, net-like skin covering the kernel of nutmeg. These compounds included D-limonene (RT: 4.043), myristic acid (RT: 11.913), myristic acid methyl ester (RT: 13.551), and methyl eugenol (RT: 16.41). Most of these analyzed compounds were found to be essential oils and phenolic compounds [55]. Key compounds such as D-limonene, methyl eugenol, eugenyl methyl ether, myristic acid, myristic acid methyl ester were categorized as essential oils [56]. The presence of limonene [57], α-pinene, and myristene [58] in mace has been found by earlier analysis. CuONPs of spices exhibited catalytic activities in the 1,3-dipolar cycloaddition reaction between azides and terminal alkynes to form 1,2,3-triazoles. These triazole compounds have been reported to possess excellent antimicrobial activities [59,60]. They were proven to be responsible for the antimicrobial activity of synthesized nanoparticles. The antimicrobial action of essential oils is due to their solubility in the phospholipid bilayer of cell membranes [61].

Copper nanoparticles as a nano-encapsulation of essential oils show improved antimicrobial activities [62]. In the present study, the GC-MS analysis of green-synthesized copper nanoparticles of spices also revealed the presence of essential oils. An indispensable characteristic of essential oils is their hydrophobicity, which permits them to be separated into the lipid of the cell membrane of bacteria, thus affecting the membrane structure and making it leakier [63]. This can be the origin of the seepage of ions and other cellular molecules [64]. The seepage of numerous biomolecules into the cytoplasm such as proteins, amino acids, and carbohydrates is the major cause for the cell death of bacteria due to the inclusion of nanoparticles [65]. CuNPs can intermingle with the microbial cell wall because they are attracted by the carboxyl group present on the microbial exterior [66]. In the present study, the presence of carboxyl groups was proved by FTIR and GC-MS analysis. The production of reactive oxygen species (ROS), enzyme activity loss, membrane damage, protein dysfunction, and so on are responsible for the antimicrobial action of nanoparticles [67]. It has been discovered that when CuNPs come in contact with a bacterial cell, copper ions are released and engrossed on the cell wall, resulting in the generation of ROS and loss of membrane integrity [68].

3.5. Antibacterial Activity of Spice Extract by Disc Diffusion Method

Antibacterial activities (in terms of zone of inhibition) of spice extracts were assessed against *S. aureus* by the disc diffusion method (Figure 8). In the present study, extracts of star anise, nutmeg, and mace at a concentration of 100 mg/mL were tested. Results of the disc diffusion assay revealed that extracts of star anise, nutmeg, and mace had inhibitory effects on *S. aureus*. Inhibitory effects of extracts of *Illicium verum, Myristica fragrans* (nutmeg), and *Myristica fragrans* (mace) on *S. aureus* showed ZOIs of 1.03 ± 0.2 cm, 0.9 ± 0.2 cm, and 0.9 ± 0.1 cm, respectively (Table 4). The presence of essential oil in star anise [69], nutmeg, and mace [70] has already been reported. The solubility in water possessing essential oil components is precisely associated with the capacity to go through the cell walls of a bacterium [71]. It is additionally influenced by the cell wall of each kind of bacteria, with Gram-positive bacteria having thicker peptidoglycan layers than Gram-negative bacteria do [72]. The process of inhibiting a bacterial system includes damage to the cell membranes, inhibition of protein synthesis, and disruption of the biological functions through specific enzymes [73]. Terpenoids in essential oils of spices
are characterized by their lability that can cause swelling of the cytoplasmic membrane to a greater extent, thereby exhibiting antimicrobial effects [61]. They can also disintegrate the cellular integrity and inhibit respiration and ion transportation activity [74]. Phenolic compounds in spice extracts might contribute to their antibacterial effects the most, whilst other constituents might contribute very little to such effects [75]. Compounds such as myristicin and macelignan [76] isolated from the plant seed exhibit good antibacterial activities against selected Gram-positive bacteria [77].

![Image](image-url)

**Figure 8.** Growth of *S. aureus* showing the zone of inhibition (circled in red) in the presence of CuNPs formulated with spice extract: (A) star anise, (B) nutmeg, (C) mace, and (D) gentamycin, a positive control, by disc diffusion method.

**Table 4.** ZOI values of CuNPs synthesized with spices by disc diffusion method.

| Scheme 1.          | Bacterial Stain | ZOI in cm |
|--------------------|-----------------|-----------|
| Star anise         | *S. aureus*     | 1.03 ± 0.2|
| Nutmeg             | *S. aureus*     | 0.9 ± 0.1 |
| Mace               | *S. aureus*     | 0.9 ± 0.1 |
| Gentamycin (positive control) | *S. aureus* | 2.2 ± 0.06 |

3.6. Antibacterial Activity of CuNPs by Well-Diffusion Method

Antimicrobial activities of spice extracts and synthesized Cu nanoparticles against *S. aureus* and ZOIs were determined using a well-diffusion process (Figure 9). ZOIs of CuNPs synthesized with *Illicium verum* (star anise), *Myristica fragrans* (nutmeg), and *Myristica fragrans* (mace) at concentrations of 10 μg/mL against *S. aureus* were 1.33 ± 0.089 cm, 1.06 ± 0.073 cm, and 1.26 ± 0.039 cm, respectively (Table 5). Gentamicin was employed as a positive control. It showed a ZOI of 2.12 ± 0.047 cm. These results revealed that biosynthesized CuNPs utilizing a spice extract displayed improved inhibition of bacteria than the extract itself [78]. A comparable result has been reported for CuNPs synthesized using *Pinus merkusii* plant extracts. ZOIs of CuNPs of pine flower extract against *S. aureus* were in the range between 0.608 cm and 1.221 cm [79]. Utilizing green-nanotechnology for the production of CuNPs has also been explored by Parikh and coworkers [80]. They revealed that the antibacterial activities of CuNPs against *Escherichia coli*, *Bacillus megaterium*, and *Bacillus subtilis* were higher than those of the extract. The investigators distinguished that this phytophenic method of nanoparticle synthesis was an inexpensive and eco-friendly technology. Hence, it might be valuable in the production of cost-effective nanomaterials, which will be potentially exploited for water purification, air-quality management, and antibacterial packaging [18]. The possible mechanism of the bactericidal impact of CuNPs is the delivery of Cu ions from nano copper and their entrance to bacterial cells with the consequent disruption of biochemical activities [81]. Bogdanovic et al. [82] suggested that bacterial cell wall action can stimulate the oxidation of CuNPs to release Cu\(^{2+}\) ions, further reducing Cu\(^{2+}\) ions to Cu\(^+\), and consequently leading to electrostatic attraction.
with plasma membrane-based reductases. Cu⁺ ions simply move through the lipid-bilayer into the cytosol and produce reactive oxygen species (ROS), leading to lipid peroxidation and oxidation of proteins. Another study has also stated that the antibacterial action of nanoparticles is due to the high-level conductivity of treated cells and discharge of cellular components [83].

![Image of growth inhibition zones](image-url)

**Figure 9.** Growth of *S. aureus* showing zones of inhibition (circled in red) of CuNPs formulated with spice extract: (A) star anise, (B) nutmeg, (C) mace, and (D) gentamycin (positive control) with a well-diffusion method.

| Spices       | Bacterial Stain | ZOI in cm |
|--------------|-----------------|-----------|
| Star anise   | *S. aureus*     | 1.33 ± 0.089 |
| Nutmeg       | *S. aureus*     | 1.06 ± 0.073 |
| Mace         | *S. aureus*     | 1.26 ± 0.039 |
| Gentamycin   | *S. aureus*     | 2.12 ± 0.047 |

Table 5. ZOI values of CuNPs synthesized with extracts of spices using a well-diffusion method.

Results indicated that CuNPs synthesized with star anise possessed higher antibacterial activities against the test organism (*S. aureus*) than CuNPs synthesized with nutmeg and mace. Similar results have been reported for the antimicrobial activity of CuNPs synthesized with *Syzygium aromaticum* (clove) bud extract against different bacterial species including *Bacillus subtilis* (ZOI = 4.2 cm) and *Escherichia coli* (ZOI = 3.3 cm) at a concentration of 200 μg/mL [26].

3.7. Thin-Layer Chromatography

The TLC separation of petroleum ether extracts of spices showed relevant spots. Chemical compounds of these spots were viewed under UV illumination. Spots were observed under short-wave. There was no spot under the long-wave of UV light [84]. *R*₇ values and the colors of bands of petroleum ether extracts of spices were separated by TLC (Table 6). *R*₇ values of star anise, nutmeg, and mace extracts were found to be 0.75, 0.41, and 0.47, respectively. These *R*₇ values of nutmeg and mace indicate that an equivalent compound could be present in both spices as mace is the lacy coating of nutmeg seed. These spice extracts have been analyzed by previous studies. When spots are observed, similar *R*₇ values have been reported [85], confirming their bioactive contents [86].

| Spices       | *R*₇ Value |
|--------------|------------|
| Nutmeg       | 0.41       |
| Star anise   | 0.75       |
| Mace         | 0.47       |

Table 6. *R*₇ values of nutmeg, star anise, and mace by TLC.
3.8. TLC–Direct Bioautography

TLC–bioautography is an accessible and easy means of analyzing plant extracts and pure elements for their impacts on infective microorganisms. It allows the straightforward detection of functional fractions. Antimicrobial activities of spice extracts were assessed by TLC–direct bioautography. The results showed the presence of white spots on TLC plates. These spots indicated the presence of antimicrobial activities of chosen spices (Figure 10).

![Figure 10. Antimicrobial activities of phyto-formulated CuNPs by TLC–direct bioautography, showing the appearance of antibacterial spots in the presence and absence of DMSO.](image)

The microbial growth was seen unswervingly on the surface of a TLC plate, eliminating spots of antimicrobials. Antibacterial activities of green-synthesized nanoparticles from spices were perceived by overlaying TLC plates with agar comprising *S. aureus* and then spraying with tetrazolium salt [87]. Oxidoreductases of living microorganisms can convert tetrazolium salt into a pinkish purple formazan [88,89]. The so-called inhibition zone was a creamy spot appearing against a purple background indicating the presence of antimicrobial agents. It was observed when green-synthesized nanoparticles were tested for antimicrobial activities. This method allowed for the finding of spots of growth inhibition of cultures immediately in the extract. The TLC plate was previously distributed with a broth culture comprising microorganisms [90]. In earlier studies, antifungal activities of nutmeg [91] and star anise [92] have been proven by TLC–bioautography.

Furthermore, after crossing the membrane, biogenic nanoparticles can work together with some important sites inside the cell, resulting in the antibacterial activity. It has been reported that CuNPs are liable for the disruption of metabolic pathways, formation of membrane pits, and oxidative stress development known to cause cell death [93], thereby showing antibacterial activity. In addition, metal NPs may put off bacterial replication and protein binding by countering with soft bases such as phosphorous and sulfur molecules of cellular deoxyribonucleic acid (DNA) [94]. Cu ions (Cu$^{2+}$) can bind to DNA and engage in cross-linking of nucleic acid strands, leading to the incompetence in making helical structures. In a comparable way, these free CuNPs can attach to the cell membrane and enter into the bacterium via endocytosis [95].

Many plant-based CuO/CuNPs with established antibacterial effects tend to have antioxidant properties [96]. In an earlier study, *Cissus vitiginea*-mediated CuNPs have demonstrated antioxidant activities, which contribute to growth inhibition of urinary-tract-infection pathogens [97]. Likewise, CuO/CuNPs synthesized with spice extracts of star
anise, nutmeg, and mace exhibited antibacterial activities in the present study possibly due to their antioxidant properties [98].

4. Conclusions

In this investigation, we instituted a phytoformulation technique for the green synthesis of CuNPs using extracts of spices (star anise, nutmeg, and mace), which showed strong antibacterial activities against *S. aureus*. These biogenic copper nanoparticles of 150–200 nm in size were further characterized with UV-Vis, SEM, and EDAX analysis. These copper nanoparticles showed stronger antibacterial activities through zone-of-inhibition studies. The results proved that these synthesized copper nanoparticles were more competent than spice extracts alone. The antimicrobial activities of nanoparticles were evaluated with TLC–bioautography. It also deep-rooted the presence of antimicrobial compounds in these extracts with comparatively similar Rf values, especially in copper nanoparticles of nutmeg and mace. This was again sturdily emphasized by the FTIR and GC-MS analysis of biosynthesized CuNPs of spices, revealing the presence of phenolic compounds, esters, alkanes, and other essential oils with the ability to damage the cell wall of a microorganism, thereby acting as powerful antimicrobial agents. The overall results of this study revealed that the green-biosynthesis of CuNPs from spices could be exploited for the development of novel herbal-formulated antibacterial agents with prospective applications in antimicrobial therapy.

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**Abbreviations**

| CuNPs | Copper nanoparticles |
| CuO   | Copper oxide         |
| NPs   | Nanoparticles        |
| SEM   | Scanning electron microscopy |
| EDAX  | Energy-dispersive X-ray analysis |
| FTIR  | Fourier-transform infrared spectroscopy |
| GC-MS | Gas chromatography–mass spectrometry |
| TLC   | Thin-layer chromatography |
| ZOI   | Zone of inhibition   |
| DMSO  | Dimethyl sulfoxide   |
| UV-vis| Ultraviolet-visible |
| ROS   | Reactive oxygen species |
| RT    | Retention time       |
| NIST  | National Institute of Standards and Technology |
| SE    | Secondary electron   |
| BSE   | Backscattered electron |
| *S. aureus* | *Staphylococcus aureus* |
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