ACADEMIA ROMÂNĂ

Revue Roumaine de Chimie

https://www.icf.ro/rrch/

Rev. Roum. Chim.,
2024, *69*(10–12), 535–543
DOI: 10.33224/rrch.2024.69.10-12.02

Papers

GREEN SYNTHESIS OF COPPER OXIDE NANOPARTICLES USING *SYZYGIUM CUMINI* SEED EXTRACT AND ITS ANTIOXIDANT AND ANTIBACTERIAL ACTIVITY

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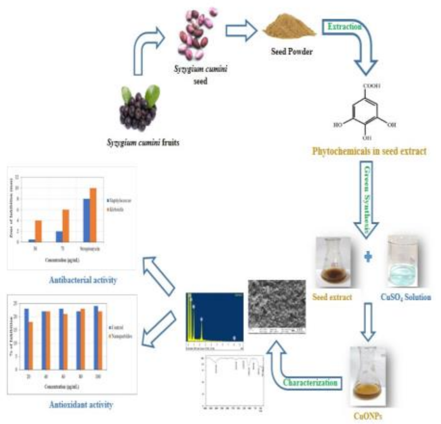
Received February 27, 2021

In this study, biosynthesis of copper oxide nanoparticles with new approach to enhanced the Antimicrobial properties against gram-negative and gram-positive and the antioxidant activity were performed by use of a new type of plant extract, *Syzygium cumini* seed, in an environmentally friendly, cost-effective, simple procedure way by adding $CuSO_4$ to aqueous *Syzygium cumini* seed extract followed by stirring. The resulted solution was heated at 40–45°C and CuONPs were synthesized. Disc diffusion method was applied to evaluation the Antimicrobial properties of the extract and nanoparticles towards resistance into *Klebsiella* (gram-negative) and *Staphylococcus* (gram-positive). DPPH assay was performed to evaluation the antioxidant activity. X-ray diffraction pattern result showed characteristic peaks of CuO that demonstrated a successful formation of pure crystalline CuONPs. In the Fourier transform infrared spectroscopy, there are two bands in the 609 cm^{-1} and 451 cm^{-1} which is corresponded to CuO. Also, a negative potential value around –10 mV obtained by Zeta potential analysis. Energy-dispersive X-ray spectroscopy analysis showed strong peaks for Cu and O, support supposition of CuONPs. Scanning Electron Microscope images indicated polydispersed spherical rounded particles with the size of average 42–90 nm. Antibacterial tests showed effective diameter about 6 and 2 mm for CuONPs against *Klebsiella* and *Staphylococcus* in agar disc diffusion method, respectively. DPPH assay found CuONPs to possess effective antioxidant properties when compared to ascorbic acid at all the concentrations tested. Biosynthesized CuONPs exhibited an excellent antibacterial and antioxidant activity against Gram-negative and Gram-positive bacteria and DPPH free radical, respectively. Hence, the CuONPs are expected to be used in future for effective biomedical applications.



INTRODUCTION

In recent years, Nanotechnology plays a vital role in several fields such as biotechnology, medical science, biochemistry, environment, biotechnology, material science and genetic engineering.[1] Many researchers have attracted by metallic nanoparticles because of their electronic chemical,[2] excellent physiochemical,[3] optical, catalytic, antibacterial, antifungal applications,[4,5]

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electrical, mechanical and thermal conduction properties[6] for past few decades much focus on copper nanoparticles (CuNPs). This noble metal CuNPs was manipulated by common methods such as exploding wire method,[7] metal vapours synthesis,[8] laser irradiation,[9] vacuum vapour deposition,[10] and microemulsion.[11] These methods are quite expensive and produce several hazardous materials, which are environment pollutants. Thus, to investigate all these adverse circumstances, researchers have attracted great attention for biosynthesis of metallic nanoparticles. This greener method are simple and low cost[12] and generating metal nanoparticles for being environmentally benign and easily available.[13,14] Biosynthesis of metal nanoparticles has been done using various source such as micro-organisms like bacteria, yeast, fungi, and enzyme. However, purification steps and yield are the major problem related to this biosynthesis. Hence, the usage of plant extracts is a most promising approach owing to their easy preparation, very cheap, very stable, and scalable compared to other biomolecules.[15] Moreover, CuNPs were synthesized using various plant extracts including, *Ficus religiosa*,[16] tea leaf,[17] *Calotropis procera*,[18] *Murraya Koenigii* leaves,[19] *Eclipta prostrata* leaves,[20] *Citrus medica Linn*[21] and *Punica granatum* peel extract,[22] etc.

Syzygium cumini Linn. (Synonym: *Eugenia jambolana*) (Myrtaceae) commonly known as 'Jamun', is a large evergreen tree which grows widely in the Indo Gangetic plains and in the Cauvery delta of Tamil Nadu. *Syzygium cumini* is a species native from tropical Asia and well adapted in Brazil, which has been used for more than 100 years to control diabetes, it is rich in polyphenol and polysaccharides and saccharides that are associated to its antidiabetic effect.[23] *Syzigium cumini Linn.* is a, medium sized to large tree, and it has been attributed to several medicinal properties.[24] The bark of the plant is sweet, astringent, carminative, refrigerant, digestive, diuretic, anthelminthic, febrifuge, stomachic, constipating, and antibacterial. The fruits and seeds are used to treat, pharyngitis, diabetes, urethrorrhea, spleenopathy and ringworm infection. The leaves are anti-bacterial and used to strengthen gums and the teeth.[24] The leaves have been extensively used to treat constipation, diabetes,[25] stomachalgia, leucorrhoea, fever, strangury, gastropathy, dermopathy[24] and to inhibit blood discharges in the faeces.[25] In addition, compounds such as ellagic acid, quercetin, isoquercetin, myricetin and kaempferol etc. isolated from *Syzigium cumini* have also found to possess antioxidant and free radical scavenging activities.[26,27] Herein, the diverse medicinal properties attributed to the plant and the presence of antioxidant and free radical scavenging compounds in the 'Jamun' stimulated us to investigate it's in nanoparticles synthesis. Therefore, the aim of the present study was to evaluate the antibacterial and antioxidant activity of green synthesized CuONPs by using *Syzygium cumini* seed extract as a reducing agent against pathogens bacteria and 2,2-diphenylpicrylhydrazyl (DPPH) free radical.

In this study, biosynthesis of CuONPs with new approach to enhanced the Antimicrobial properties against gram-negative and gram-positive and the antioxidant activity were performed by use of a new type of plant extract, derived from *Syzygium cumini* seeds, in an environmentally friendly, cost-effective, simple procedure way.

EXPERIMENTAL

Materials

Green seeds of *Syzygium cumini* were collected from Vellore District Tamil Nadu India. Analytical graded Copper sulphate ($CuSO_4$), Ascorbic acid, DPPH (2,2-Diphenyl-1-Picrylhydrazyl), Dimethyl Sulfoxide (DMSO), Phosphate Buffer Solution (PBS) and Streptomycin antibiotics were purchased from (Sigma-Aldrich Chemicals, Bangalore, India). Nutrient agar and Muller Hinton agar were purchased from (Hi-Media, Mumbai, India). The overall experiment was performed with Deionized (DI) water with 18.2 M Ω cm (Millipore Co., USA).

Preparation of *Syzygium cumini* seed extract

Seeds of collected *Syzygium cumini* were washed thoroughly using deionized water to remove the dust particles on their surface and dried under shade for 1 week. Dried seed was ground using a kitchen blender to obtain a fine powder. 1 g of seed powder boiled with 100 mL of deionized water for 5 min at 60 °C and allowed to cool at room temperature. The mixture of the extract was filtered through filter paper (Whatman No.1) and stored at 4 °C for further use.

GC-MS analyses

The *Syzygium cumini* seed extract was analyzed using an Agilent-Technologies 6890 N Network GC system. A sample of 1.0 µL was injected, using split mode (split ratio, 1:100). The composition was reported as a relative percentage of the total peak area. The identification and authentication of the *Syzygium cumini* seed extract compounds was realised using a comparison of their retention times and their mass spectra to the published data and spectra of authentic compounds.

Synthesis of CuONPs using *Syzygium cumini* seed extracts

Twenty mL of *Syzygium cumini* seed extract was added into 80 mL of 10 mM of $CuSO_4$ kept under constant stirring using magnetic stirrer at 45–50 °C for 10 h. At the end of the step brownish black colour was obtained after centrifugation process (at 8000 rpm for 10 min) the product was washed twice with deionized water and dried in hot air oven at 100 °C for 3 h. Finally, the dried powder was stored in properly labelled and used for further analysis.

Characterization of CuONPs

The double beam UV-vis spectrophotometer –2800 (Shimadzu, Kyoto, Japan) was used to characterize the CuONPs synthesis, the particles morphology was analysed using Scanning Electron Microscope JSM-6700F SEM (Jeol, Tokyo, Japan) and atomic force microscopy (AFM), the elements composition were analysed using EDX spectrum attached in the SEM instrument. Powder X-ray diffractometer (Philips-PW 1830 instrument) was used in order to analyse the crystalline nature and further the evaluation of chemical groups responsible for CuONPs synthesis was investigated using Fourier transform infrared spectroscopy (FT-IR) (Perkin-Elmer). Malvern Nano-Zetasizer ZS (Malvern, UK) was used to analyse the zeta potential (ZP) of CuONPs synthesis.

Application of CuONPs

Antibacterial activity

The well diffusion method was performed to examine the antibacterial activity of seed extract of *Syzygium cumini* synthesized CuONPs against two different pathogens bacteria such as *Staphylococcus* (Gram-positive) and *Klebsiella* (Gram-negative). The CuONPs powder was sterilized in an oven at 120 °C for 20 min. The test bacteria were grown in Luria-Bertani (LB) medium, at 37 °C. Fresh overnight culture of each bacterial strain (log phase) was swabbed uniformly onto the individual plates containing sterilized and cooled Muller Hinton Agar (MHA). Then, the sterilized CuONPs solution with various concentrations (50 and 75 µg/mL, respectively) were added into each well and incubated for 24 h at 37 °C. Commercial antibiotic discs (Streptomycin) was placed as control. After incubation, different levels of zonation formed around the discs were measured.

Antioxidant activity

The green synthesized CuONPs was tested for the scavenging effect on DPPH (1,1-Diphenyl-2-Picryl hydroxyl) radical methods, as reported in Rajeshkumar and Rinitha.[14] Briefly, the synthesized CuONPs was dissolved in methanol at different concentrations (20–100 µg/mL). About 0.5 mL of dissolved plant mediated nano copper oxide particles were added to 3 ml of 0.5 mM DPPH in methanol solution and this reaction mixture was incubated for 30 min in the dark conditions at room temperature. The optical intensity of the peak 517 nm was measured for each mixture, which allowed to estimate the antioxidant activity. Ascorbic acid was used as a standard to calibrate the resultant activity. The percentage of inhibition was calculated using the following formula:

Scavenging activity % = (1 – Absorbance of sample/ Absorbance of control) × 100

RESULTS AND DISCUSSION

GC-MS analysis

The presence of phytochemicals in *Syzygium cumini* seed extract was characterized using GC-MS analysis. The GC-MS chromatogram results revealed 15 peaks, which are corresponding to 15 phytoconstituents. The plants are the major sources of phytochemicals, which play an important role in various biological applications listed in Table 1.

Table 1

Phytochemical compounds obtained in *Syzygium cumini* seed extract and their different biological activities

Compound name	Activity	References
Alpha-Amyrin	Antioxidant activity	28
Beta-Amyrin	Anti-inflammatory activity	29
Betulin	Antitumour activity	30
Ether	Anticancer activity	31
Ethyl acetate	Antihemolytic activity	32
Acetic acid	Antibacterial activity	33
Oleanane	Anti-inflammatory activity	34
Oleanolic acid	Anticancer activity	35
Enol	Antitumor activity	36
Enoic acid	Antimicrobial activity	37
Methyl ester	Antimicrobial activity	38
Benzopyranone	Antiproliferative activity	39
Lupenone	Anticarcinogenic activity	40
Cycloartenol	Anti-immunosuppressive activity	41
Isopropanol	Antimutagenic activity	42

UV-vis Spectroscopy of CuONPs

Green synthesis of CuONPs using *Syzygium cumini* seed extract was initially confirmed by visual observation. The aqueous copper sulphate solution was added to the *Syzygium cumini* seed extract forming a yellowish green to brownish black colour mixture indicating the formation of CuONPs.[14] Further UV-vis spectroscopy was performed to confirm the biosynthesis of CuONPs and to determine the surface plasmon resonance band (SPR). The (Fig. 1) shows a clear SPR with a maximum absorbance at 300 nm, which is characteristics peak for CuONPs and this seems to be like the results reported by Ashtaputrey *et al.*[19]

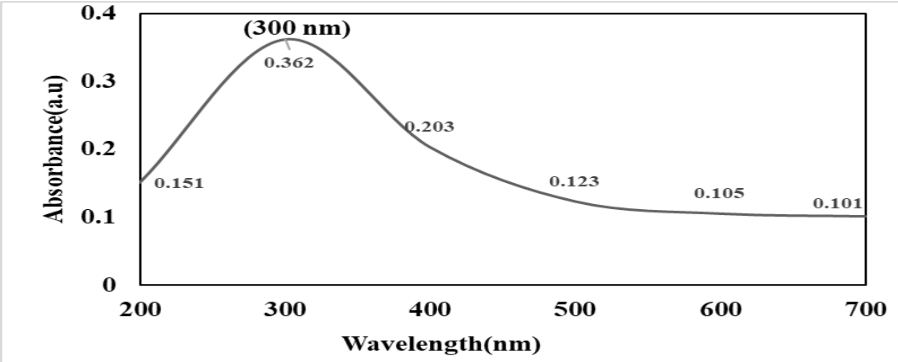

Fig. 1 – UV–vis Spectrum of CuONPs synthesized using *Syzygium cumini* seed extract.

Fourier transforms infrared (FT-IR) spectroscopy of CuONPs

In order to gain more information (the functional groups and the types of the existing bends) on the structure of the biosynthesis CuONPs, we have undertaken a vibrational study using FT-IR. The FT-IR spectra of green synthezed CuONPs were recorded in the range of 500–4000 cm^{-1} are shown in (Fig. 2). The peak at 1022 cm^{-1}, 1072 cm^{-1}, 1328 cm^{-1}, 1635 cm^{-1} and 3135 cm^{-1} corresponds to the C–O stretch ethers or esters, N–H bends primary amines, C–C–C symmetric stretch of alkenes, H–C–H asymmetric and symmetric stretch alkanes and hydrogen bonded O–H stretch alcohols and phenols respectively. The peak at 866 cm^{-1} denoted to the aromatic bending vibration of C–H group.[43] Prominent peaks at 609 cm^{-1} and 451 cm^{-1} characteristic the presence of Cu–O vibrations in the synthesized CuONPs.[44] Thus, FT-IR results clearly confirmed the availability of the phytochemicals of plant extract for CuONPs biosynthesis. In biological green synthesis, phytochemicals usually play an important role in reducing as well as stabilizing agent in nanoparticles synthesis.[45]

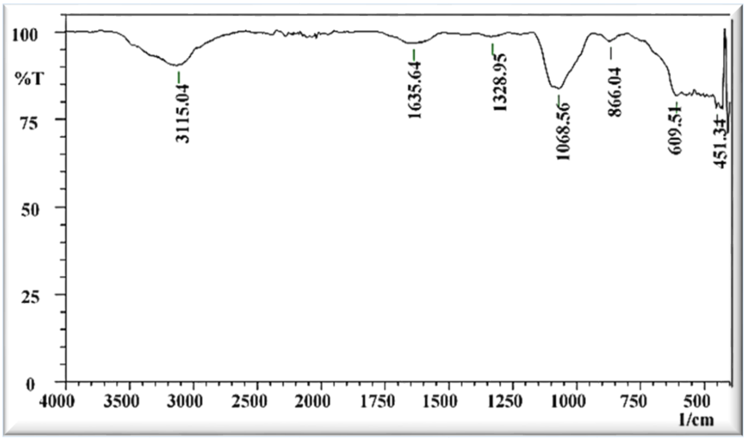

Fig. 2 – FT-IR spectrum of CuONPs synthesized using *Syzygium cumini* seed extract.

XRD pattern of CuONPs

X-ray diffraction analysis was performed to examine the crystal phases and crystallinity of the biosynthesized CuONPs. The nano copper synthesized using *Syzygium cumini* seed extract and the oxidation state were confirmed by X-ray diffraction analysis (Fig. 3). The diffraction indicates the presence of crystalline face-centered cubic (FCC) CuO that related to the angles 24°, 30 °, 38 ° and 42° associated respectively with the planes (111), (200), (210) and (222). No impurity peaks other than CuO was observed in the XRD pattern confirming that all products have high phase purity. Moreover, the obtained XRD pattern indicates clearly a successful formation of pure crystalline CuONPs. The average crystallite size of CuONP was calculated and found to be in range 42–90 nm by Scherrer formula applied to calculate FWHM value get from the diffraction peaks.[14]

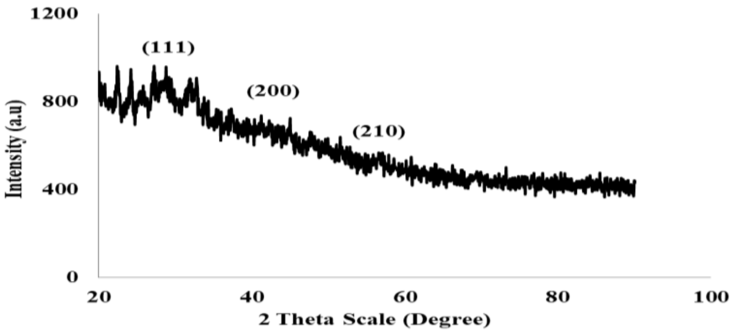

Fig. 3 – XRD-Spectrum of CuONPs synthesized using Syzygium cumini seed extract.

AFM analysis of CuONPs

AFM characterization stated about morphological structure of green synthesized CuONPs in three-dimensional forms.[46,47] Analysis of the surface structure and topography roughness of the synthesized CuONPs was achieved by AFM. (Fig. 4) shows three-dimensional images in the particles were mostly spherical shaped with aggregations and some particles with undefined shape were noticed.

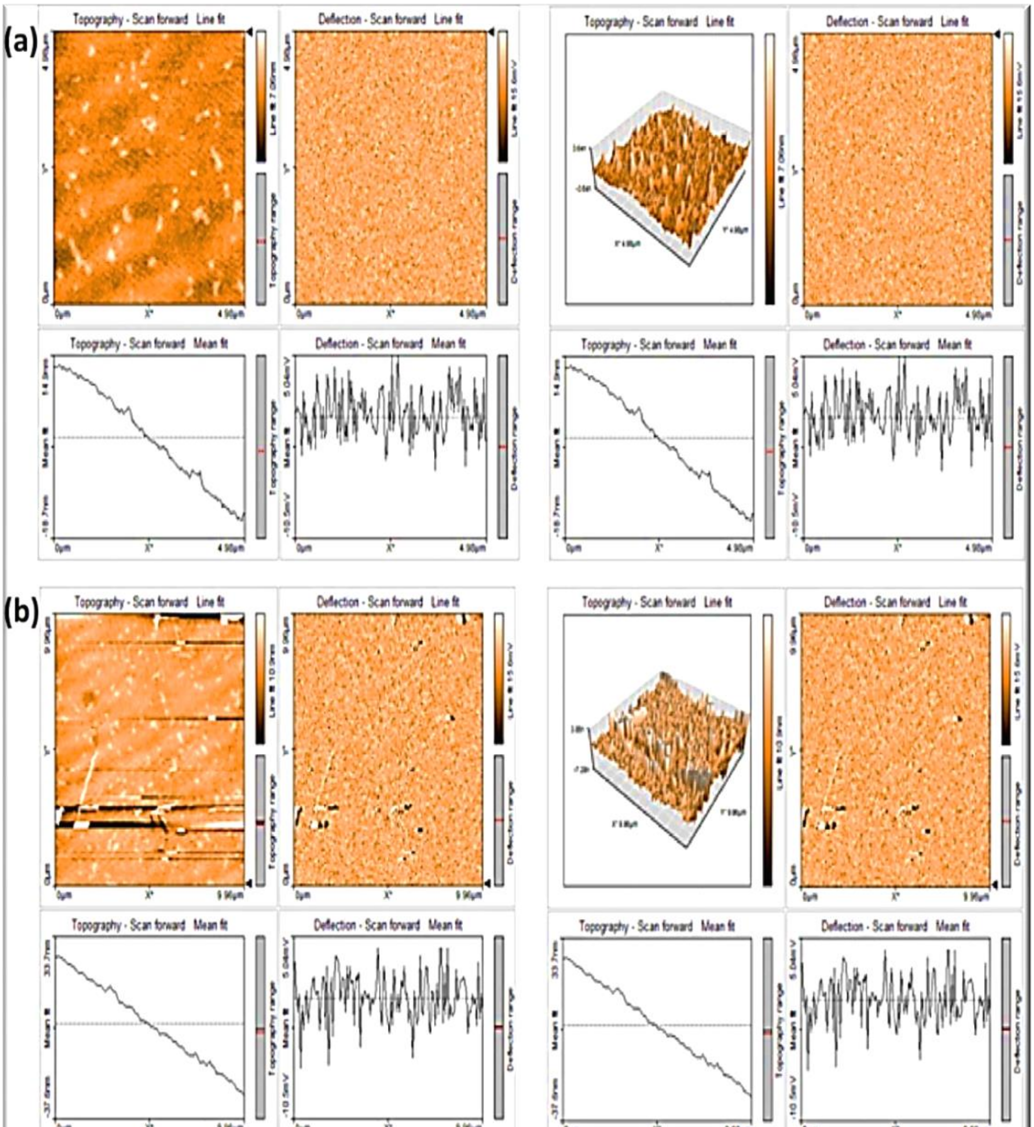

Fig. 4 – AFM Images of CuONPs synthesized using *Syzygium cumini* seed extract.
Particle size with an average diameter: a) 4.90 µm; b) 9.96 µm are observed.

Scanning Electron Microscope (SEM) analysis of CuONPs

The SEM analysis was carried out to identify the size and shape of CuONPs. Herein, the *Syzygium cumini* seed extract synthesized CuONPs are polydisperse which include relatively spherical grain shape with the size range from 42 to 90 nm (Fig. 5). The aggregation of small sized nanoparticles might have been due to the thermodynamic stability of CuO preventing oxidation, without protection of the Cu^{+} ions.[48]

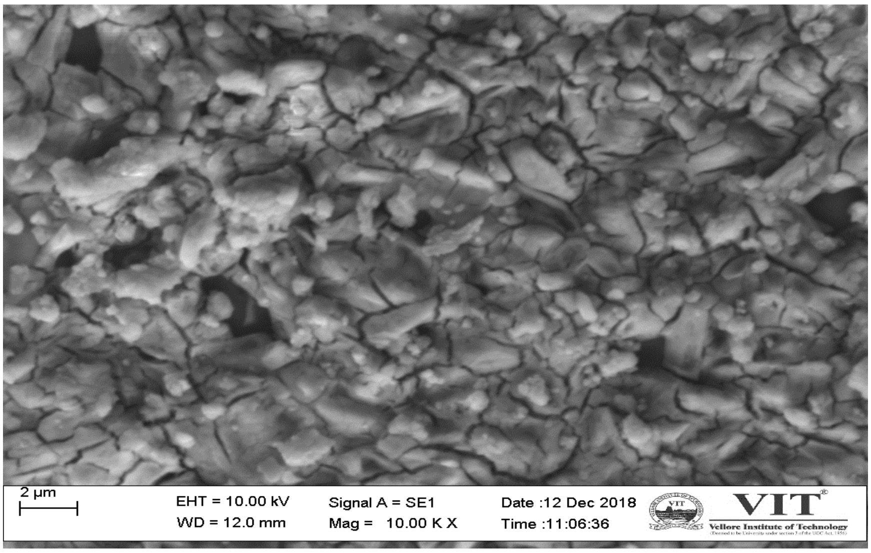

Fig. 5 – SEM images of CuONPs synthesized using *Syzygium cumini* seed extract at a scale of 2 µm.

EDX analysis of CuONPs

The elemental composition of the CuONPs was determined by EDX analysis. The energy dispersive X-ray study shows strong copper (Cu) and Oxygen (O) signals indicating the copper in the form of oxide or dioxide. Other weak signals of sulphur and carbon was revealed in EDS spectrum might be due to the presence of phytochemicals in the seed extract of *Syzygium cumini*. The weight composition (wt%) of Cu and O obtained from EDX is 57.84% and 26.06% respectively (Fig. 6).[49]

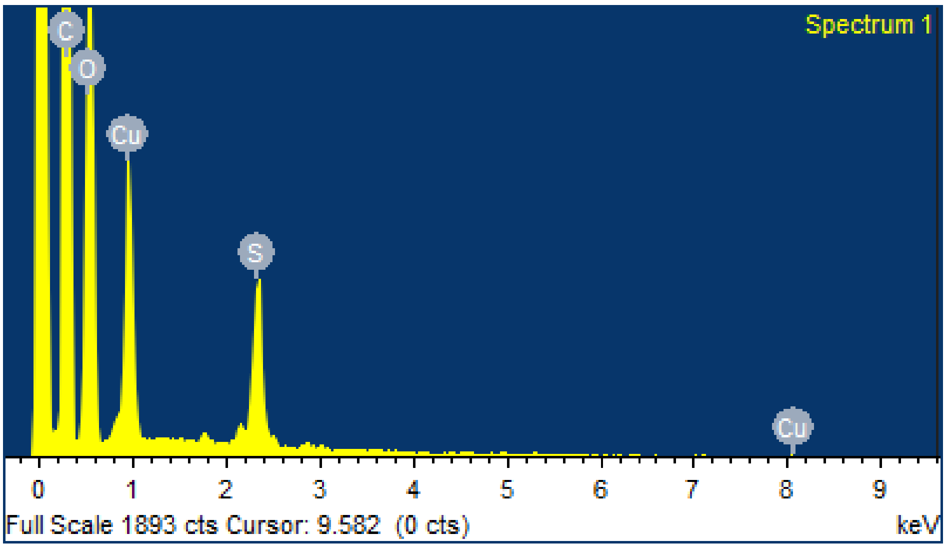

Fig. 6 – EDX data for CuONPs synthesized using *Syzygium cumini* seed extract.

Zeta potential analysis of CuONPs

The Zeta potential play an important indicator of the surface charge of nanoparticles in colloidal dispersion. The zeta potential analysis of CuONPs suspended in deionised water, shows a negative potential value around –10 mV, which indicates the stability of CuONPs (Fig. 7). Higher negative zeta potential related the strong repulsion force between the particles indicating quality and stability.[50]

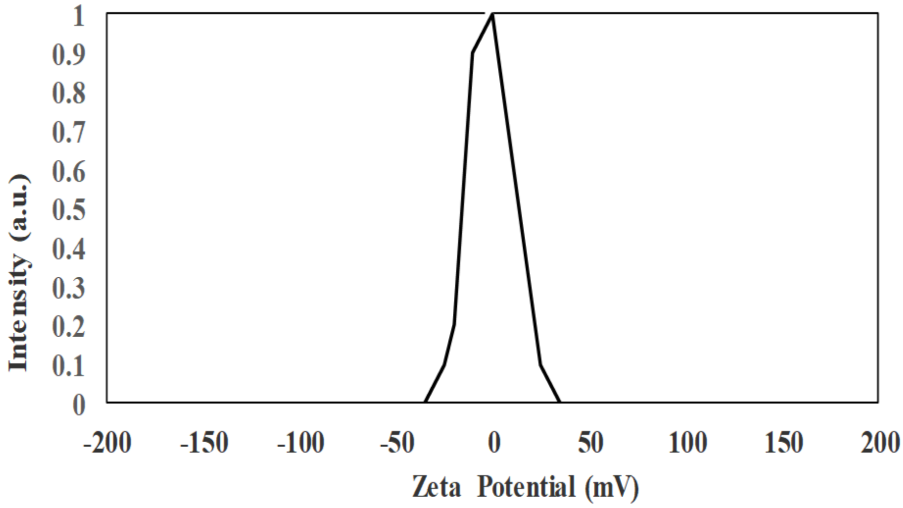

Fig. 7 – Zeta potential for CuONPs synthesized using *Syzygium cumini* seed extract.

Antibacterial activity of CuONPs

The antimicrobial activities of the CuONPs were evaluated against Gram-negative bacteria (*Klebsiella*) and Gram-positive bacteria (*Staphylococcus*) using agar disc diffusion method. The results clearly showed that CuONPs formed distinct zones around the bacterial strains. CuONPs exhibited maximum bactericidal activity against the Gram-negative bacteria *Klebsiella* (6 mm) followed by Gram-positive bacteria *Staphylococcus* (2 mm) at the highest concentration of 75 µg/mL CuONPs compared to the Streptomycin standard drug were represented in the form of bar graph as shown in (Fig. 8). The mechanism of the antibacterial properties of the biosynthesized CuONPs have been proved in previous reports, that is based on the strong interaction between the bacteria cell wall and the Cu^+ ions, which resulted in rupturing the cell walls.[51,52,53] Therefore, application of CuO in nanoforms could be used as a new remedy for treating infectious diseases resulting from bacterial pathogens.

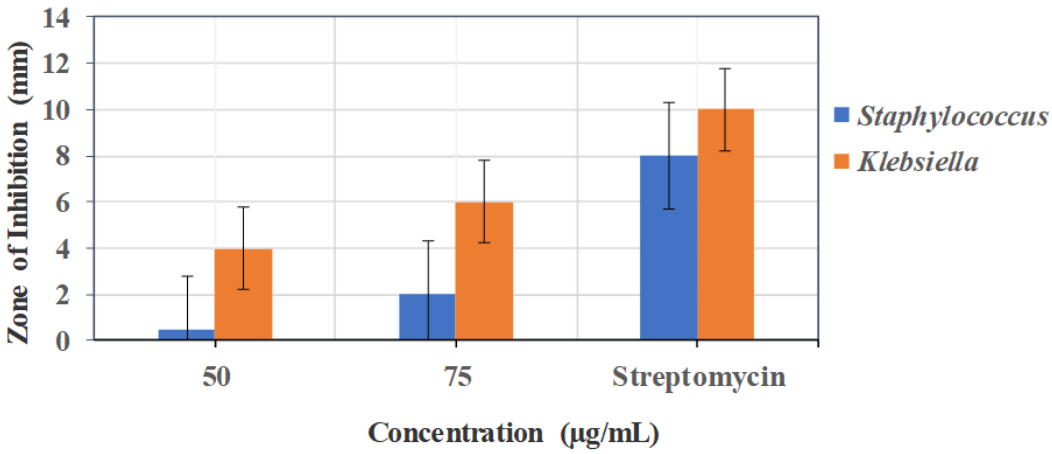

Fig. 8 – Antibacterial activity of CuONPs synthesized using *Syzygium cumini* seed extract.

Antioxidant activity of CuONPs

DPPH is a dark-colored free-radical molecule which could be used as an antioxidant.[54] DPPH was used based on a characteristic absorption at 517 nm for radical scavenging study of CuONPs prepared using *Syzygium cumini* seed extract. The decrease in absorption is considered as a measure of the extent of radical scavenging by the acceptance of hydrogen or electron.[14] DDPH assay found biogenic CuONPs to possess effective antioxidant properties when compared to ascorbic acid at all the concentrations tested (Fig. 9). Moreover, the free radical scavenging activity of CuONPs are very close to the standard ascorbic acid. Hence, the synthesized CuONPs holds great potential as an antioxidant and could be used effectively in several medical applications.

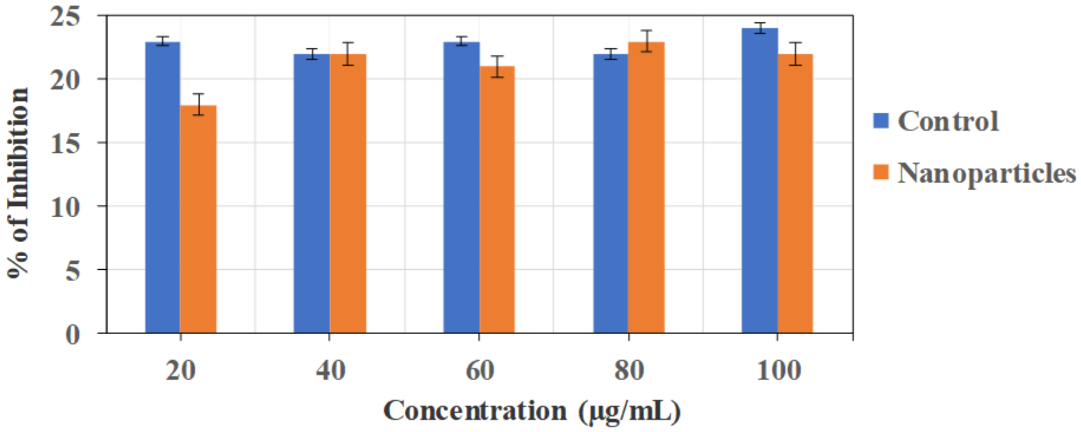

Fig. 9 – Antioxidant activity of CuONPs synthesized using *Syzygium cumini* seed extract by DPPH assay.

CONCLUSIONS

In this work, CuONPs have been successfully synthesized via a simple, low cost, and green method using *Syzygium cumini* seed extract as a reducing and stabilizing agent. The characterization of synthesized CuONPs was initially confirmed by the position of SPR band at 300 nm in UV-vis spectra. XRD spectrum shows highly stable and crystalline CuONPs and AFM techniques revealed the spherical shape of CuONPs with aggregation tendency. SEM image shows polydispersed CuONPs which include relatively spherical grain shape with the size range from 42 to 90 nm. The CuONPs exhibited an excellent antibacterial and antioxidant activity against Gram-negative and Gram-positive bacteria and DPPH free radical, respectively. Hence, the CuONPs are expected to be used in future for effective biomedical applications.

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