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Detection of Antimicrobial Properties of Green Synthesized Copper Nanoparticles Using *Hippophae rhamnoides* L.

POOJA DADHWAL, HARISH KUMAR DHINGRA* AND VINAY DWIVEDI[1]

Department of Biosciences, School of Liberal Arts and Sciences, Mody University of Science and Technology, Lakshmangarh-332 311 (Rajasthan), India
**(e-mail:harishdhingra2000@gmail.com; Mobile: 94615 36233)*

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ABSTRACT

Metallic-element like gold, copper and silver NPs has numerous applications in the field of bio-technology and bio-medicine. Copper nanoparticles (CuNPs) have exhibited significant properties like anti-microbial and anti-oxidant properties. The aim of this research was to synthesize copper nanoparticles by using *Hippophae rhamnoides* L. stem and to evaluate their anti-microbial properties. The anti-microbial activities of *H. rhamnoides* L. stem-CuNPs were analyzed by using growth zone inhibition methods against *Staphylococcus aureus* and *Escherichia coli* bacteria strains and *P. chrysogenum* and *C. albicans* fungal strains. The results indicated that plant-CuNPs had the maximum antifungal activity against *P. chrysogenum* with 12 mm zone of inhibition and antibacterial activity against *S. aureus* (11 mm) at 80 µl of concentration. These outcomes showed that such copper NPs made with *H. rhamnoides* L. stem extracts had significant anti-bacterial properties and could be used as possible medications to prevent diseases.

Key words: Copper nanoparticles (CuNPs), *Hippophae rhamnoides* L., sea buckthorn, antimicrobial

INTRODUCTION

Hippophae rhamnoides L. is an antique plant with contemporary qualities, used for land recovery and flora and fauna habitation as it advances the soil assembly, dropping soil loss and also recognized as 'cold desert gold'**.** *H. rhamnoides*, generally called as sea buckthorn, is a deciduous blossoming shrub and goes to the Elaeagnaceae family (Lee *et al.*, 2021). It is a very strong plant and can survive winter temp. up to -43°C and nurtures extensively in coldest area like China, Northern Europe and in several other countries (Husain *et al.*, 2018). Indian Himalayas and in hilly regions of India such as Badrinath region, H. P. Lahaul Spiti region, Sikkim, Chamoli and Kashmir, A. P., Ladakh and Uttarakhand, where sea buckthorn germplasm resources are present. In Raling (Lahaul-Spiti) and Sumur (Ladakh) varied population of *H. rhamnoides* ssp. Turkestanica has been found (Nawaz *et al.,* 2019).

H. rhamnoides has been stated to have an extensive spectrum of pharmacological properties like anti-atherogenic, radio-protective, anti-stress, antioxidant, hepato-protective and immune-modulatory, along with tissue repair promoting properties (Nawaz *et al.,* 2019). From ancient time, it was utilized as a cardiovascular disease, stomach mucous damages, increased blood circulation and skin damages (Su *et al.*, 2021). This plant extracts have been revealed to lessen blood lipids and enrich other risk aspects of heart disease, and pre-treatment of sea buckthorn was known to have its defensive property in contrast to porcine intestinal epithelial cells to lipo-polysaccharide induced damage. Its leaves have antioxidant, anti-inflammatory, anti-obesity and anti-diabetic activities (Tkacz *et al.,* 2019). Dadhwal and Dhingra (2022) described that *H. rhamnoides* contained high amount of poly-phenols and phyto-chemicals such as organic acid, tocopherol and carotenoids. These bioactive components contribute its vast use as a natural anti-oxidant. Its leaves consist of bioactive substances and nutrients which largely include isoprenols, flavonoid, triterpenols, free and esterified sterol and carotenoids. Its leaves contain anti-oxidant components like ellagic acid, catechins, vit. E, folic acid, and β-carotene, ferulic acid and also prominent amount of potassium, magnesium and

[1]Department of Biotechnology Engineering and Food Technology, Chandigarh University, Gharuan-140 413 (Punjab), India.

calcium. Sea buckthorn fruit consists of carotene, tocopherol, flavonoid, xanthophyll, phenolic compound, etc. (Dolkar *et al.*, 2017).

Free radicals are a group of detrimental molecules formed during the regular breakdown of cells in the human physique. The damaging properties of free radicals may also cause harm to DNA, membrane and enzymes, which play a great role in numerous human disease such as neurodegenerative, malaria, rheumatoid arthritis, atherosclerosis, coronavirus disease (COVID-19 (Alsammarraie *et al.*, 2018). Though, nanoparticles displayed a variety of applications, including catalyst, anticancer, antioxidant and antimicrobial, electrical and optical activity (Ahani and Attaran, 2022).

Green synthesized nanoparticles play important part in *in vitro* diagnostic application, medicines, and clinical application (Ahani and Attaran, 2022). Some green synthesized material can be used as dispersant and end capping agent all together, which decreases energy utilization, and also dodges the usage of harmful and toxic reagents. Nanoparticles synthesized via green methods display brilliant antimicrobial effects (Nate, 2018). Among the nanoparticles, copper nanoparticles have boundless consideration because of its conductivity, anti-fungal, optical, catalytic, anti-bacterial, anti-cancerous and high electrical properties. So, the motive to conduct this study was to assess the anti-microbial and anti-oxidant properties of CuNPs synthesized via sea buckthorn stem extract.

MATERIALS AND METHODS

The investigational plant material i.e. *H. rhamnoides* L. stem was collected in October 2021 from Zanskar Ranges, Village - Rangrik near Kee Monastery, Dist. Lahaul-Spiti, Himachal Pradesh, India. *H. rhamnoides* stem was eroded carefully 2 to 3 times with tap water and then dehydrated out in air in shade at room temperature of 32 to 37°C for about seven days. The dehydrated plant samples were crushed into powder form by using a homogenizer. These powdered samples were used for further experiments.

The plant extracts were used to analyze alkaloids, flavonoids, phenol, tannin, terpenoids, steroids and amino acids (Dadhwal *et al.*, 2022).

In the sample extract, 4 o 5 drops of ninhydrin reagent were added. It was properly assorted. The solution mixture was boiled for 2 to 3 min in water. Presence of amino acids was shown by the dark blue-black colour. Five ml of distilled water was added to 10 mg of stem extract. 5% $FeCl_3$ solution was presence of phenolic compound was shown by dark colour. Seven ml of 1% HCl was added to 0.5 g of stem extract. It was warmed and filtered out. With 2 ml of filtrate was titrated with Mayer's reagent. Yellow precipitate specified the presence of alkaloids (Dadhwal *et al.*, 2022).

The 20 mg extract was mixed in distilled water. Three ml of 10% $Pb(C_2H_3O_2)_2$ solution were added. Tannin presence was visualized by the formation of bulky white precipitate. Thirty mg of plant sample in 10 ml of $C_4H_8O_2$ was steamed in water bath for 2 to 3 min and was then filtered. Then 4 ml of filtrate was mixed with 1 ml of diluted NH_3 solution, yellow colour specified the flavonoids occurrence. Two ml of chloroform 5 ml (1 mg/ml) of stem extract was mixed and concentrated sulphuric acid (3 ml) was cautiously added to make a layer. The positive result was confirmed by the appearance of reddish-brown colouration (Dadhwal *et al.*, 2022).

The synthesis of copper nanoparticle using *H. rhamnoides* L. stem (HRS) was finished using the method of modified Hussain *et al.* (2016). In a separate beaker, 5 g of stem part was weighed. Then plant part was added in 100 ml of distilled water and heated at 60°C for 10 min. Later it was filtered out by using different sized membrane filter (0.45 µm Millipore membrane filter and 0.2 µm Millipore membrane filter). For synthesis of copper nanoparticles, 60 ml of plant extract was added in 40 ml of $CuSO_4$ in Erlenmeyer flask at RT. The mixture was stirred using a magnetic stirrer and changes in colour of the solution were observed (Fig. 1). The produced CuNPs were categorized by different methods such as FTIR, Scanning Electron Microscope, Ultraviolet-visible spectra and X-ray diffraction.

The Fourie transform infrared spectroscopy of the sample was analyzed on the IR Affinity-1 FTIR Shimadzu Spectrometer (class-1 laser product, Japan) in the diffuse reflectance operating mode at a tenacity of 4/cm. This instrument required small amount of dried sample grinded with KBr pellets. Ny doing this

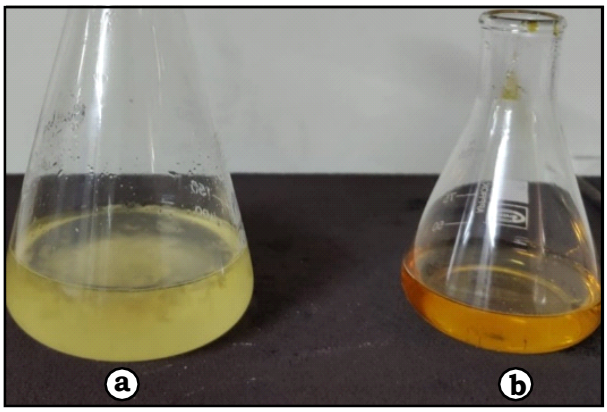

Fig. 1. Picture of *Hipphophae rhamnoides* L. stem extract with CuNPs solution (a) before and (b) after the synthesis of CuNPs.

one depicted the functional group present in it. CuNPs were detected by UV–Vis spectrophometer for which the reaction mixture was subjected to optical analysis and the spectra (Beckman - Model No. DU-50, Fullerton, CA, USA) were obtained at the resolution of 1 nm from 200 to 800 nm for each sample.

The actual sizes and agglomeration state of the materials (nano and bulk) were examined using SEM (Carl ZEISS EVO^{R}-18, Germany), operating at an extra high tension or accelerating voltage (EHT) of 20 kV, where WD was 8.5 mm. Minute amounts of the test materials were loaded one by one on the sample discs. Sputter coating (gold coating) was applied on the materials for better imaging under SEM in Quorum Q150RS rotary pumped sputter coater before putting on specimen stage.

In labanti-bacterial activity of the HRS-CuNPs was examined in contrast to *Staphylococcus aureus* and *Escherichia coli* by the agar well diffusion (AGD) method of Ningthoujam and Dhingra (2021). Mueller Hinton agar no. 2 (Hi Media, India) was used as the microbiological medium. A standardized inoculum of 1.5×10^8 CFU/ml in 0.9% NaCl solution was used. Wells were organized in the agar plates. The HRS-CuNPs solution was introduced in the 6 mm well with the concentration of 20, 40, 60 and 80 µl. The plates were kept for 24 h. at 37°C temperature. The anti-microbial spectrum of the extract was determined for the microbial species in terms of inhibition zone sizes around each well. The diameters of zone of inhibition produced by the compound were linked with those formed by the standard antibiotics i.e. 40 µl Ciprofloxacin. The standard zones were deducted from the tested compound zones and the subsequent zone diameter was calculated with anti-biotic zone reader to nearest mm.

Anti-fungal activity of the HRS-CuNPs was inspected against *Penicillium chrysogenum* (MTCC 5108) and *Candida albicans* (MTCC 183) by AGD method of Ningthoujam and Dhingra (2021). The fungi were sub-cultured onto SDA media (Merck, Germany) and individually incubated at 25°C for 2-5 days. Inoculums of fungal spores were prepared in sterile phosphate buffer saline and adjusted to a conc. of 10^6 cells/ml. Dipping a disinfected swab into the fungal inoculums and spread on the agar medium surface. 20, 40, 60 and 80 µl of HRS-Copper nanoparticles solution was directed to fullness for each well. The positive control was used as Ketoconazole (40 µl). Plates were incubated at 37°C. After growth period of 24 h, bio-activities were determined by calculating the span of zone of inhibition in mm.

RESULTS AND DISCUSSION

Copper is a significant micro nutrient and a crucial constituent of numerous enzymes and proteins. The production of copper nanoparticle using plant extracts is of high importance due to its environmental friendliness, good antioxidant properties, low cytotoxicity, economic prospect, biocompatibility and antibacterial activity.

In preliminary test performed on CuNPs of *H. rhamnoides* stem revealed the strong presence (++) of phenolic compound and tannin, whereas the amino acid, alkaloid, flavonoid and terpenoid indicated weak presence (+). The results confirmed the existence of tannins, alkaloids, glycosides, flavanols, phenols, ascorbic acid, cardiac glycosides, flavonoids and terpenoids in the leaves of this plant and these compounds were accountable for the anti-oxidant capacity of sea-buckthorn (Table 1).

For CuNPs materials, two broad band emergences in range of 3600-3400 and 1200-1000/cm was observed along with a peak at 3424/cm representing functional group (OH group, H-bonded OH stretch) and 1087/cm signified aliphatic fluoro compounds, C-F stretch (Fig. 2). Another band was recorded in a narrow range of FTIR sample containing HRS-CuNPs showed a six additional absorption

Table 1. Preliminary phytochemical analysis of *H. rhamnoides* stem sample

Phytochemicals tested	Test performed	Test result
Amino acids	Ninhydrin test	+
Phenolic compounds	Ferric chloride test	++
Alkaloids	Mayer's test	+
Tannins	Lead acetate test	++
Flavonoids	Alkaline reagent test	+
Terpenoids	Salkowski test	+

++indicates strong presence and + indicates weak presence.

peaks in the range of 3000-1200 and 1000-600/cm with peaks at 2937, 1628, 1446, 1383, 1264, 816 and 608/cm. Absorption peak at 2937/cm was assigned to Methyne (>CH-). Similarly, 1628/cm represented Alkenyl (C=C stretch), 1446/cm signified Methyl (C-H asymmetric/symmetric bend), 1383/cm characterized about gem-Dimethyl or 'iso' (doublet), 1264/cm primary and secondary OH in plane bend, 816/cm was assigned to C-H 1,4-Disubstitution (para) which was of aromatic ring (aryl), 608/cm, represented Aliphatic bromo compounds (C-Br stretch). FTIR spectra for green-synthesized copper NPs with star anise exposed to =CH stretching corresponded by the bands at 3004.89/cm, which proved the presence of aromatic –H; OCH_3 enlarging corresponding to 2923.88/cm; –CH stretching proving the presence of aromatic C–H; corresponding to 2866.46 and 1745.4/cm, 1608.52/cm, and C=O stretching, C=C bending and C–O stretching which corresponded to 1100.06/cm, 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 revealed the existence of a benzene ring in the obtained green-synthesized CuNPs of star anise.

The synthesis of CuNPs was established by obtaining a distinguishing peak at 270 nm (Fig. 3). Nate (2018) observed that the colour of CuNPs solution was changed from blue to brown as shown by exterior plasmon resonance of copper nanoparticle. The peak of CuNPs was 421, 425, 425 and 448 nm, respectively for chitosan, *C. molle*, *C. sinensis* and *M. azedarach* Linn. by using UV–vis spectroscopy (Nate, 2018). Hussain *et al.* (2016) revealed absorption peak of synthesized CuNPs (yellow colour) for *Punica granatum* leaves at 450 nm.

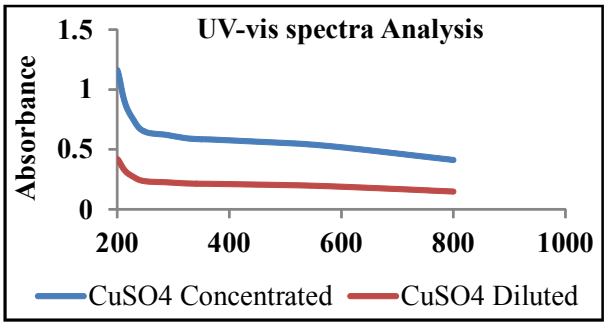

Fig. 3. UV-vis spectra of HRS-CuNPs.

SEM micrographs of sample revealed a high level of aggregation of their particle (Fig. 4). The observed sizes of the NPs were less than 100 nm in the range of 13.1 to 34.9 nm. The shape of HRS-CuNPs was spherical. Thus, the dimensions of these NPs were found to be within the nano-scale range. It reported 80-120 nm sized of green-synthesized copper oxide nanoparticles with leaf extract of *Aloe barbadenses*. The size of ~12 nm was

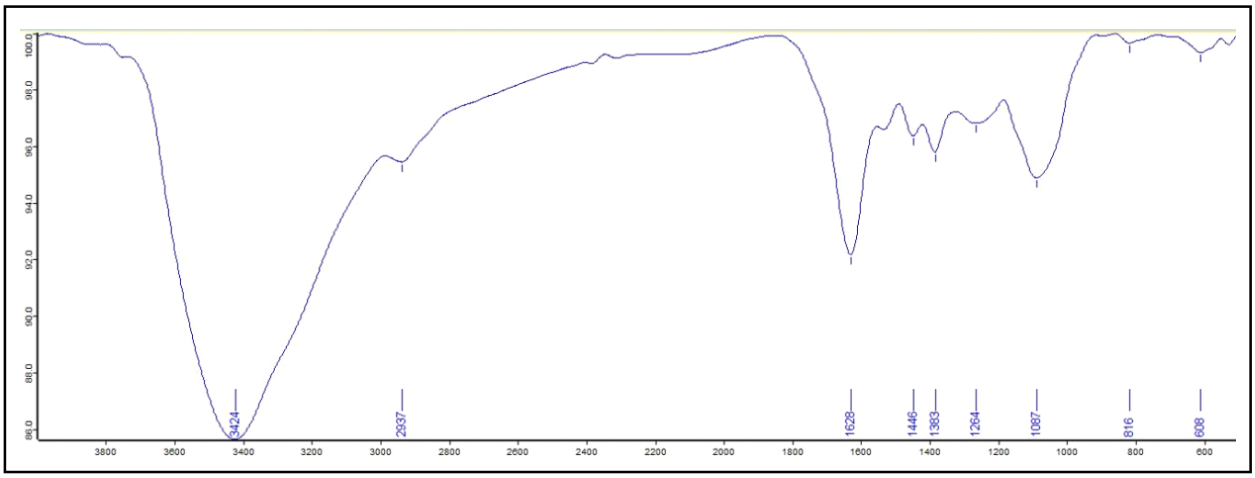

Fig. 2. FTIR spectrum of plant-CuNPs.

characterized by Ahani and Attaran (2022) from CuNPs of clove bud extract. Ahani and Attaran (2022) revealed green synthesized copper oxide nanoparticles were in spherical form and finely dispersed without accumulation with size from 40 to 80 nanometer of range in SEM analyses. The green produced $CuSO_4$ were spherical form and well dispersed without gathering at a place with size ranging from 38 to 94 nm in Dakal *et al.* (2016) study. They concluded that the shape of nanoparticles development was reliant on the kind of bio-molecules present in the plant extract.

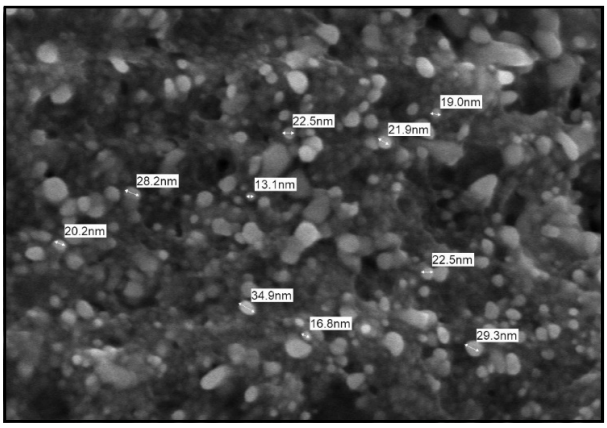

Fig. 4. Scanning electron micrograph (SEM) of HRS-CuNPs.

The antibacterial activities of green synthesized CuNPs from *H. rhamnoides* stem were tested in contrast to both the gram positive and negative bacteria with significant diameter zone of inhibition at 80 µl. Highest inhibitory activity was observed against *S. aureus* with 11 mm thereafter *E. coli* with 9 mm inhibition zone (Table 2 and Fig. 5). The inhibition zone showed the killing of pathogenic bacteria due to the presence of an antibacterial substance in the sample. The antimicrobial activity of copper oxide NPs synthesized by leaf extract of *E. indica* was detected significantly by Dakal *et al.* (2016) at 75 µg concentration against *A. niger*, *S. typhimurium*, *S. epidermidis* and *B. subtilis*. The antibacterial properties of CuNPs synthesized by Bambusa leaves extract were determined against, *B. subtilis, S. aureus, E. coli* and *P.. vulgaris*. Highest inhibition zone was identified against *E. coli* and *B. subtilis*, whereas moderate zone was recognized against *P. vulgaris* and *S. aureus* (Ahani and Attaran, 2022).

Table 2. Antibacterial activity of *H. rhamnoides* CuNPs against bacterial pathogens

Pathogens	Inhibition zone (mm)				
	Standard	20 µl	40 µl	60 µl	80 µl
E. coli	30	Nil	7	8	9
S. aureus	30	Nil	Nil	8	11

The results of antifungal activity of *H. rhamnoides* stem-CuNPs against four different fungal strains in terms of zone of inhibition were analyzed. And it was seen that *P. chrysogenum* gave the best activity at 80 µl with 12 mm diameter of zone of inhibition followed by *C. albicans* with 9 mm zone of inhibition (Table 3 and Fig. 6). Madiha *et al.* (2018) determined anti-fungal properties of CuNPs synthesized by *C. paniculatus* at 0.12, 0.18 and 0.24% concentrations against *F. oxysporum on the basis of* mycelial radial growth. Plant-CuNPs

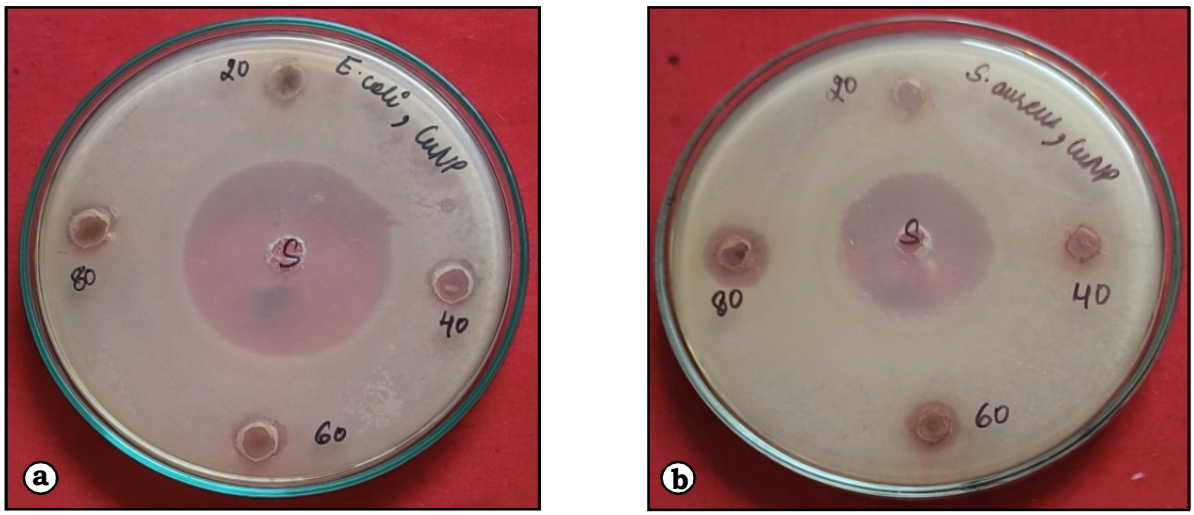

Fig. 5. Antibacterial activity of *H. rhamnoides* L. CuNPs against (a) *E. coli* and (b) *S. aureus*.

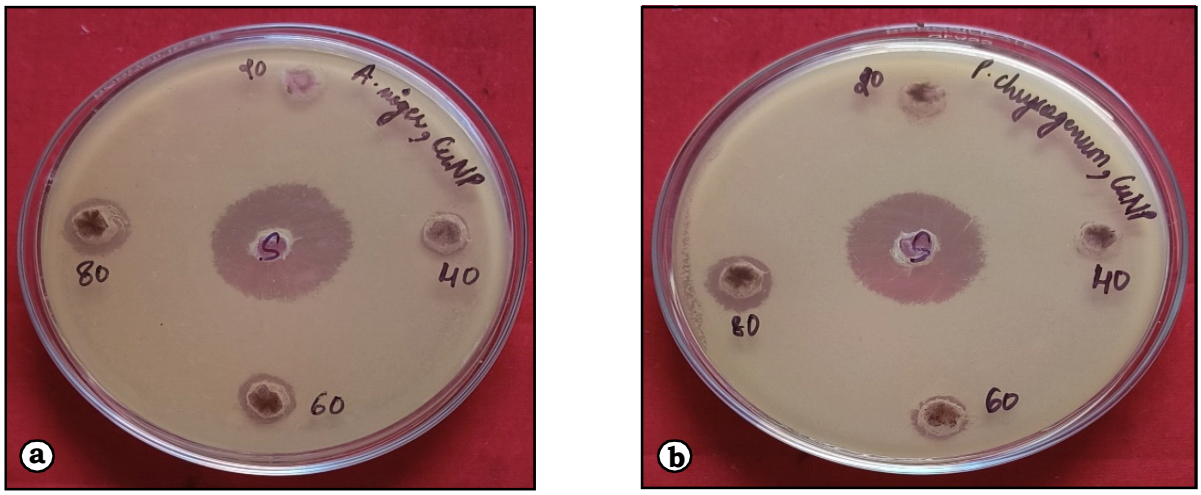

Fig. 6. Antifungal activity of *H. rhamnoides* L. stem CuNPs against (a) *A. niger* and (b) *P. chrysogenum*.

Table 3. Antifungal activity of *H. rhamnoides* L. CuNPs against fungal pathogens

Pathogens	Inhibition zone (mm)				
	Standard	20 µl	40 µl	60 µl	80 µl
Candida albicans	25	Nil	Nil	Nil	9
Penicillium chrysogenum	25	Nil	Nil	9	12

showed mycelial growth inhibition at 76.29 (0.24% of concentration), 73.70 (0.18% of concentration) and 59.25% (0.12% of concentration) in which highest mycelial growth inhibition was at 0.24% HRS-CuNPs.

CONCLUSION

Chemical solvents utilized to prevent pathogenic microbial growth are dangerous to human well-being with low anti-microbial and antioxidant properties. Therefore, in this investigation, *H. rhamnoides* stem was used to prepare CuNPs by using green technique that are ecofriendly method. The preliminary test qualitative estimations showed the strong presence of phenolic compound and tannin and other in a weak concentration. The physio-chemical properties were determined by SEM, UV–Vis spectroscopy and FTIR techniques that showed optimum characteristics of plant-CuNPs. Synthesized CuNPs from stem extract showed strong antifungal and antibacterial activities against both fungal and bacterial pathogens. The complete outcomes of this research exposed that the green-produced copper nanoparticles from *H. rhamnoides* stem could be subjugated for the progress of novel natural drugs with potential applications in anti-microbial activity.

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