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Research Article

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Biosynthesis and characterization of Cu-Se bimetallic nanoparticles: an effective approach as anticancer agents

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Abstract

Bimetallic nanoparticles (BNPs) have garnered a great interest rather than monometallic nanoparticles (NPs) in terms of biotechnological applications due to their enhanced properties. They have a growing interest as promising biomedical agents for drug delivery, antibacterial, and anticancer treatments due to their significant permeability and retention effects. Both of nano-size copper (Cu) and selenium (Se) exhibited a significant activity in biological treatment and drug delivery systems. Consequently, the present work aimed to focus on the novel biogenic synthesis of Cu-Se BNPs using cell-free extract of a marine bacterium, and studying their anticancer and antioxidant activities. Based on 16S rDNA sequencing, the isolated marine bacterium was identified *Bacillus amyloliquefaciens* by phylogenetic analysis. Biogenic synthesis of Cu-Se BNPs was optimized by studying the effect of some physiological factors such as reaction time, pH, and temperature. Based on the reaction conditions, the biologically synthesized Cu-Se NPs was obtained with different particle size that ranged from 15.7-106.0 nm. The spherical small sized (15.7 nm) bimetallic Cu-Se BNPs displayed anticancer activity against HepG2 and MDA cell lines, while the cell viability was reduced by 87 % and 81 %, respectively. The estimated IC_{50} values were 696.4 µg/ ml for HepG2 and 273.9 µg/ ml for MDA, while scavenging capacity (SC) of Cu-Se BNPs (SC_{50} = 305.3 µM) showed lower ability to 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) scavenging compared to ascorbic acid (SC_{50} = 95.9 µM).

Keywords: Nanoparticles, Cu, Se, Bimetallic, Marine bacterium, Anticancer activity

1. Introduction

Nanobiotechnology is the application of biological entities in biosynthesis of NPs (Jamil *et al*., 2024), which has attracted more attention due to its low toxicity, economic viability, ecofriendly, and simplicity offering a possible substitute for costly and dangerous chemical processes (Zhang *et al*., 2023). NPs have been used in different fields of science such as cosmetics, biology, biomedicine, catalysis, and drug-gene delivery (Chan *et al*., 2017). Utilization of nanomaterials in the treatment of cancer has gained interest due to its unique physicochemical properties and ability to overcome certain current restrictions regarding drug delivery and imaging techniques (Li *et al*., 2023).

Multi-metallic and bimetallic NPs have been significantly applied in many fields, including biological, catalysis, and water treatment (Emam *et al*., 2021). Recently, BNPs have been recognized as promising biological agents in the long run, because of the synergistic interaction between the two metals used, which improves the properties of the bimetal nanostructure compared to their monometallic counterparts (Medina-Cruz *et al*., 2020). In general, two distinct aqueous metal solutions are mixed with an eco-friendly reducing agent to create BNPs. The metals with greater reduction potential undergo faster reduction than metal ions with weaker reduction potential (Jamil *et al*., 2024). Modification of the size and shape of BNPs presents a major advantage in improving their catalytic and optical properties and increasing their antibacterial and anticancer activities (Cusimano *et al*., 2020). It has been suggested that a wide range of BNPs can be synthesized by microorganisms used as bio-factories (Li *et al*., 2018). They have been found to biosynthesis NPs through conversion of metal ions into zero-valence metal NPs, which can occur either intracellularly or extracellularly. The advantage of extracellular synthesis of BNPs compared to intracellular synthesis is that the recovery of NPs is easier (Alam *et al*., 2020). In addition, Li *et al*., (2018) used protein extracts of *Deinococcus radiodurans* as reducing and capping agents for biosynthesis of Au-Ag BNPs. Reduction of Se to SeNPs may occur inside the bacterial cell or by enzymes released in cell-free extracts such as NADH-dependent reductase (Zhang *et al*., 2023).

Metal NPs, such as Cu and Se are attractive nanomaterials for application in medicine and therapeutics owing to their antimicrobial and anticancer properties (Hassabo *et al*., 2022). In addition, biosynthesized SeNPs are used as potential chemo-preventive and chemotherapeutic agents in a variety of cancers, including colon, breast, prostate, and liver due to their broad range of biological activity, minimal toxicity, and prolonged effects (Lesnichaya *et al*., 2022). The anticancer mechanisms of biosynthesized SeNPs include induction of cellular and mitochondria-mediated apoptosis, triggering cellular and mitochondrial death, and inhibition of tumor cell invasion (Li *et al*., 2023). Moreover, a previous study demonstrated the antioxidant and hepatoprotective activities of Cu and SeNPs (Akçay and Avcı, 2020).

The physicochemical properties of MNPs such as shape, charge among others, size, and composition are the main factors influencing their biological performance and interaction with the cancer cells (Medina-Cruz *et al*., 2020). Recent findings have demonstrated that combinations of various metal compounds can improve their applications as anticancer agents and drug delivery systems (Padilla-Cruz *et al*., 2021). BNPs with an Ag core enclosed in an Au shell demonstrated potent efficacy in inhibiting Lewis Lung Carcinoma (LLC) tumor growth and metastasizing compared to all the analyzed NPs (Shmarakov *et al*., 2017). While much researches have been done on monometallic NPs; however, few studies

have been conducted on biosynthesis of Cu-Se bimetallic NPs using microbiota.

The objective of this study was to exploit the use of both Cu and Se as potential nano-precursors for biological formation of Cu-Se BNPs; using cell-free extract of a marine bacterium, and their use as anticancer agents.

2. Material and methods

2.1. Materials

Yeast-Peptone-Dextrose ready-made broth medium (YPD) was obtained from Merck (Darmstadt, Germany), Selenium dioxide was purchased from Loba Chemie Pvt. Ltd., India and Copper sulfate was supported by Rasayan Laboratories Pvt. Ltd., India. All other chemicals were of the highest analytical grade.

The human hepatocellular carcinoma (HepG2) cells and epithelial human breast cancer cell line (MDA-MB-231) have been obtained from American Type Culture Collection (ATTC, Rockville, MD, USA). MDA cells had been cultured in Dulbecco, Modified Eagle's Medium (DMEM), while HepG2 cells had been cultured in RPMI 1640 media supplemented with 10 % Fetal Bovine Serum (FBS), 2 mM 1-glutamine and 100 units/ ml (1%) penicillin/streptomycin antibiotic (100 µg/ ml). Cells have been maintained in 37 °C humidified air containing 5 % CO_2. At 70 %-80 % confluence, monolayer cells had been harvested by Trypsin/ EDTA medium and supplements were obtained from Sigma-Aldrich, USA.

2.2. Microbial strain and culture conditions

The marine bacterial isolate used in this study was isolated from marine environments of the Red Sea, Sharm El-sheikh, Egypt (27°54′54″N 34°19′39″E). Using MYP medium (Malt extract, 3.0; Yeast extract, 3.0; Peptone, 5.0; and glucose, 10 g/ l dist. water) using the dilution plate technique. Using sterile dist. water, 2 ml of seawater were serially diluted and 0.5 ml of each dilution was streaked in triplicate in MYP petri plates. After incubation at 30 °C for 48 h, the isolated bacteria were preserved in MYP slants at 4 °C (Chen *et al*., 2012).

A bacterial culture was prepared using 250-ml Erlenmeyer flask containing 50 ml of yeast peptone dextrose (YPD) broth medium. The medium was inoculated with 1 ml of a 24 h bacterial suspension of 3.0-4.0×10^5 cfu/ ml and incubated at 30 °C for 48 h under shaking at 200 rpm (Hassabo *et al*., 2022).

2.3. Biogenic synthesis of Cu-Se bimetallic nanoparticles (BNPs)

At the end of incubation, bacterial cells were harvested by centrifugation at 5000 rpm for 15 min., washed twice with dist. water and re-suspended in 50 mM phosphate buffer (pH 7.4) (Hassabo *et al*., 2022). The cell suspension was disrupted by sonication in ice using a Sonicator (Vibra-Cell 72,405, Bioblock Scientific, Illkirch, France). The samples underwent three cycles of sonication for 2 min., with a power setting of 60 W and a frequency setting of 60 MHz. The homogenate was centrifuged under cold conditions at 5000 rpm for 20 min., and the obtained clear supernatant was used as cell-free extract.

For biogenic synthesis of Cu-Se BNPs, a total of 10 ml of cell-free extract was mixed with 10 ml of metal solution containing SeO_2 (0.8 mM) and $CuSO_4$ (2.0 mM). The reaction mixture was incubated with shaking at 200 rpm) at 37 °C in pH 7.0 for 72 h. Control sample contained only the cell-free extract without metal precursors. After incubation, the reaction mixture was centrifuged at 3000 rpm for 15 min. and the supernatant containing the Cu-Se BNPs was collected, dried for 8 h at 70 °C, calcinated for 3 h at 300 °C, and kept finally at 4 °C for further analysis.

2.4. Molecular identification

The bacterial isolate was identified using molecular characterizations, and deposited in the NCBI/Gene Bank nucleotide sequence databases. Molecular identification of the most promising

bacterial isolate, recording maximum SPR observed at UV-vis spectrum, was carried out based on 16S rDNA sequencing method. Using a Gene Jet Plant genomic DNA purification kit methodology (Thermo Scientific, Lithuania), DNA extraction was conducted. Two primers were used during polymerase chain reaction (PCR) analysis; mainly 8F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1492R (5′-GGG CGG GGT GTACAA GGC-3′). First, denaturation was carried out at 96 °C for 3 min., followed by 25 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, and an extension step at 72 °C for 1 min. were conducted throughout the PCR (Tamura *et al*., 2011). Following purification of the resulting amplified PCR 16S rDNA product, it was extracted from the agarose gel using a promega Wizard Genomic DNA Purification Kit. The amplified PCR product was purified and sequenced at Macro gene, Korea. Raw data of sequencing were edited (contig and peak chromatogram verification) using the Finch T.V 1.4.0 program. Analysis of 16S rDNA sequences of the bacterial isolate was performed using the BLAST (N) program of the National Center of Biotechnology Information (NCBI) (Rockville Pike, Bethesda MD, USA) (Tamura *et al*., 2011). Multiple sequence alignment was done using the ClustalW 2.1 program. The phylogenetic trees were created using the neighbor joining technique adopted by MEGA. X. (Tamura *et al*., 2011).

2.5. Optimization of the biogenic synthesis of Cu-Se BNPs

An optimization study had been done by observing the effect of several reaction conditions such as temperature, reaction time, and pH of medium on the biogenic synthesis of Cu-Se BNPs as summarized in Table (1). To study the effect of the reaction pH, the reaction mixture was adjusted at different pH values (6, 7, and 8) using potassium phosphate buffer (50 mM). Similarly, the effect of reaction temperature (30 and 37 °C) and reaction time (24, 48, 27 h) on the biogenic synthesis of bimetallic NPs was also investigated. For comparison, the monometallic NPs of the Cu and Se were individually biosynthesized under the same processes and reaction conditions.

2.6. Characterization of the biosynthesized Cu-Se BNPs

Absorbance of the bio-synthesized Cu-Se BNPs was investigated using UV-Vis spectrophotometer (Hitachi U-2900). The geometrical features of the Cu-Se NPs were measured and tested using the high-resolution transmission electron microscope (HRTEM, JEOL-JEM-1200 from Japan) with Oxford electron beam. A 400-copper coated carbon grid was immersed in the BNPs solution for few seconds and then left in the air at room temperature for solution vaporization before measurement. Zetasizer analyzer (Nano ZS, from Malvern-UK) was used to measure the polydispersity index (PdI), particle size distribution, mean size, and zeta potential for the bio-synthesized Cu-Se BNPs. These measurements were performed using dynamic light scattering (DLS) at a wavelength of 633 nm in an isolated room with He-Ne laser lamp (0.4 mW), while 10 runs were carried out at two different measurements positions and the average values only were considered (Emam *et al*., 2020). The crystalline feature of the Cu-Se BNPs was investigated using X'PertPRO diffraction from PANalytical diffractometer, while, the used monochromatized was Cu (Kα X-radiation at 40 kV and λ = 1.5406 Å). The angle of diffraction (2θ) for the samples was measured in the diffraction range of 10-80° (Emam *et al*., 2020).

2.7. Cytotoxicity

In this study, the cytotoxic behavior of different Cu-Se BNPs concentrations (100, 50, 25, 12.5 µM) on HepG2 and MDA cancer cell lines was investigated in triplicates. HepG2 and MDA cell lines were cultured in tissue culture flasks and maintained in Dulbeco,s Modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 2 % µmol/ ml L-glutamine, 250 ng/ ml fungizone, 100 units/ ml penicillin, and 100 units/ ml streptomycin at 37 °C in a humidified 5 % CO_2 atmosphere.

Table 1: Different conditions used during biosynthesis of Cu-Se BNPs samples

Sample	SeO_2 (M)	$CuSO_4$ (M)	Reaction time (h)	pH	Temperature (°C)
Cu-Se BNPs-1	0.8	2.0	24	7	37
Cu-Se BNPs-2	0.8	2.0	48	7	37
Cu-Se BNPs-3	0.8	2.0	72	7	37
Cu-Se BNPs-4	0.8	2.0	48	6	37
Cu-Se BNPs-5	0.8	2.0	48	8	37
Cu-Se BNPs-6	0.8	2.0	48	7	45
CuNPs	0.8	2.0	48	7	37
SeNPs	0.8	2.0	48	7	37

Where; Column represent Cu-Se BNPs (1-6 samples) in addition to the two metallic NPs, while rows represent CuNPs and SeNPs conditions needed for biosynthesis of monometallic NPs

The culture's cells were harvested by trypsinization using 1.5 ml Trypsin/ EDTA solution for 5 min. at 37 °C. This assay is based on the ability of active mitochondria; in living cell lines, to break down the tetrazolium rings using 40 µl of MTT 3-[4, 5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (yellow) through hydrogenase enzyme, forming formazan crystals (Dark Blue), which are insoluble and impermeable to the cell membrane as reported by Hansen *et al*., (1989). These crystals are then dissolved using 160 µl acidified isopropanol and measured using a FLUO star optima multi-detection system at 570 nm (McHale and McHale, 1988). The half maximal inhibitory concentration (IC_{50}) has been calculated from the dose-dependent curve for 24 h time point.

2.8. Estimation of antioxidant potential by DPPH assay

The free radical-scavenging activity of Cu-Se BNPs concentrations (100, 50, 25, 12.5 µM) was investigated using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging method reported by MacDonald-Wicks *et al*., (2006). DPPH; a deep violet radical with an unpaired electron, which was used in this assay due to its ability to abstract hydrogen in presence of antioxidant radical scavenger and changes its color into pale yellow. The assay was performed in a 96-well plate with DPPH (0.004 µg) and sample aliquots at a series of concentrations ranging from 1.7 to 100 µM with a total volume of 200 µl. DPPH solutions were used as controls with the same serial concentrations but without the tested samples. Plates were incubated at 25 °C for 30 min. and then absorbance was read at 520 nm. Absorbance of samples was plotted against each Cu-Se BNPs concentration that was relative to DPPH radicals. The concentration causing 50 % DPPH scavenging (SC_{50}) was calculated.

2.9. Statistical analysis

Microsoft excel was used in computation of the results reported in this study. The mean of ten runs was used to calculate the particle size recorded by the

zetasizer. Using 4 pi software, the particle size observed via TEM micrograph was computed. The data have been analyzed using student's unpaired t-test and one-way analysis of variance test (ANOVA). The mean values of three tests were applied for the obtained data of the anticancer assay.

3. Results and Discussion

3.1. Identification of the isolated bacterium using a molecular technique

The 16s rDNA gene sequence revealed that the marine bacterium belongs to the genus *Bacillus amyloliquefaciens* (GenBank accession no. PQ373570), using BLAST in database of the GenBank. The phylogenetic tree was created using the neighbor-joining method. It disclosed that the isolated bacterium was intimately connected to *B. amyloliquefaciens* (Fig. 1). Therefore, *B. amyloliquefaciens* was proposed as its name.

3.2. Optimization conditions and characterization of the bio-synthesized Cu-Se BNPs

The biogenic Cu-Se BNPs production was optimized through studying various factors, including reaction time, medium pH values, and applied temperatures of reaction, as summarized in Table (1). For quite good comparison, the monometallic (Cu and Se) was individually bio-synthesized at the optimal conditions. Reduction of the metal ions (Cu^{+2} and Se^{+4}) was carried out through the action of the reductive functional groups in the bacteria and the color was then changed indicating the formation of Cu-Se BNPs. The primary indication for BNPs formation was the change in color of the solution to faint greenish color and consequently the absorbance was measured as presented in Fig. (2). The individual monometallic SeNPs and CuNPs exhibited an absorbance peaks at 282 nm and 737 nm; respectively, which are in harmony with the previous studies conducted by Hassabo *et al*., (2022); Khudier *et al*., (2023). The surface plasmon resonance (SPR) for SeNPs was recorded around at 280 nm (Cittrarasu *et al*., 2021; Khudier *et al*., 2023), while the SPR for CuNPs was formerly obtained as a broader band at 730 nm, in agreement with Hassabo *et al*., (2022). In case of the biogenic Cu-Se BNPs-2, both absorbance peaks for the individual monometallic NPs were disappeared and new absorbance peak at 295 nm was recorded, reflecting the formation of Cu-Se BNPs instead of the individual monometallic NPs.

Further confirmation of formation of the biogenic Cu-Se BNPs and optimization of their biosynthesis process was done by measuring the zetasizer analyzer using dynamic light scattering technique as concluded in Table (2). The DLS particle size, zeta potential, and polydispersity index (PdI) values were all varied according to the applied conditions during the biogenic preparation process. Regardless of the applied conditions, zeta potential (ZP) for all tested samples recorded negative values that ranged from -4.23 mV to -18.1 mV. These recorded values reflected stability of the all-biogenic NPs in accordance with Ahmed and Emam, (2020); Emam *et al*., (2021). The obtained particle size significantly changed according to the applied production conditions and ranged from 15.7 to 106.0 nm (Fig. 3). Prolongation of the reaction time from 24 h to 48 h resulted in significant decrement in the particle size of the biogenic Cu-Se BNPs from 50.7 nm to 15.7 nm. Further prolongation of the reaction duration to 72 h was accompanied with aggregation of the smallest NPs size forming agglomerated particle size of the biosynthesized Cu-Se BNPs with a recorded size of 58.8 nm. The data showed that the bacteria require quite prolonged period of time (48 h) in order to secrete the enzymes responsible for reducing the functional groups, which are required for metal ions (Cu^{+2} and Se^{+4}) reduction, thus forming Cu-Se BNPs.

This interpretation of the obtained results is supported by the reported data in previous studies involving synthesis of the BNPs using different microorganisms, including *Rhodotorula mucilaginosa* (Hassabo *et al*., 2022), *Streptomyces* MHM38 (Bukhari *et al*., 2021), and *Shewanella oneidensis* (Kimber *et al*., 2018), which were carried out at longer time periods. Similarly, more recent studies

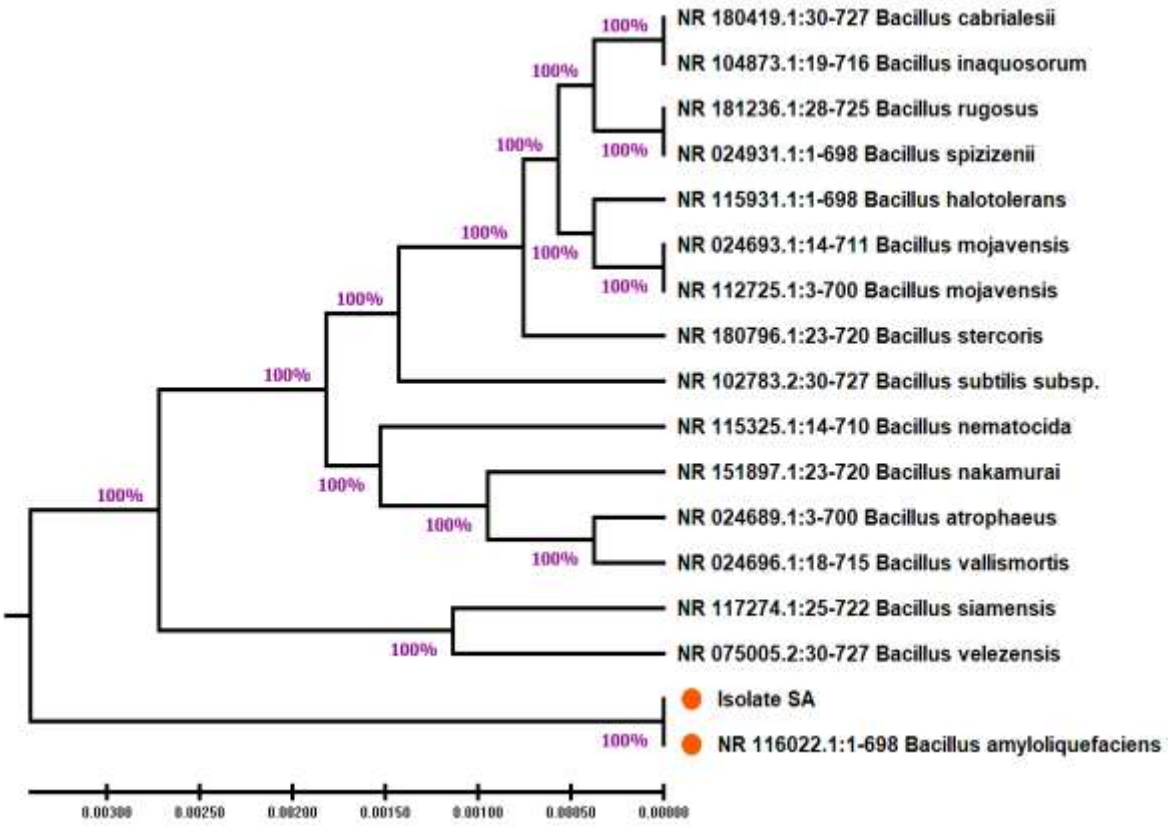

Fig. 1: Phylogenetic tree based on partial 16S rDNA sequences, showing the relationship between isolated *B. amyloliquefaciens* strain and other bacterial spp. belonging to the genus *Bacillus*. The tree was constructed using the MEGA11 and neighbor-joining method

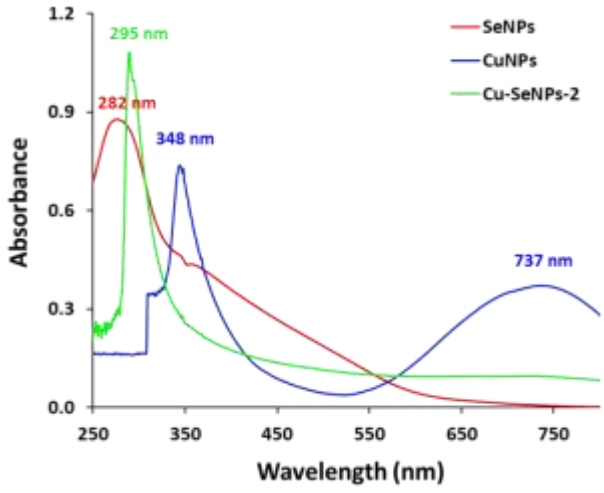

Fig. 2: UV-visible absorbance spectra for the bio-synthesized Cu-Se BNPs

Table 2: Data of zetasizer analyzer used for the biosynthesized Cu-Se BNPs samples

Sample	Particle size (Zetaszier, nm)	Average size (TEM, nm)	Zeta potential (mV)	PdI
Cu-SeNPs-1	50.7	--	-5.61	0.51
Cu-SeNPs-2	15.7	9.7	-8.58	0.41
Cu-SeNPs-3	58.8	--	-7.83	0.49
Cu-SeNPs-4	68.1	--	-4.23	0.43
Cu-SeNPs-5	78.8	--	-8.52	0.69
Cu-SeNPs-6	106.0	--	-18.1	0.66
CuNPs	--	8.9	--	--
SeNPs	--	71.5	--	--

Where; TEM: Transmission electron microscope, PdI: Polydispersity index

summarized the effective role of reaction time in controlling the stability and size of the obtained NPs such as Emam *et al*., (2020).

The alteration in pH of the biogenic reaction mixture to 6 or 8 resulted in the formation of larger particle size of Cu-Se NPs to 68.1 nm and 78.8 nm, respectively. Furthermore, rise in the temperature of the reaction to 45 °C was accompanied by obtaining agglomerated Cu-Se BNPs with a size of 106.0 nm. These data declared sensitivity of bacterium towards pH and temperature of the reaction mixture. Increasing the temperature of biogenic medium to 45 °C led to an increment in the internal kinetic energy of BNPs. Subsequently, the possibility of collision rate and sedimentation was increased, thus producing much larger Cu-Se BNPs. Such obtained results are in agreement with the previously reported studies that recorded that increasing the pH and temperature of the microorganism medium led to enlargement in particle size of the BNPs (Cuevas *et al*., 2015). Similarly, a previous study reported that pH 7 is the optimal reaction pH for biosynthesis of CuNPs using different microorganisms such as the case of Cu-Se BNPs (Cuevas *et al*., 2015). In accordance, smaller size of AgNPs and CuNPs was obtained at temperature of 37 °C similar to that for Cu-Se BNPs (Pantidos *et al*., 2018).

For the all the biogenic Cu-Se BNPs samples, the obtained PdI values changed from 0.41-69. Based on the reported data in previous studies and principles of uniformity, the best dispersed and homogenous NPs showed PdI index around 0.3 (Ahmed and Emam, 2020). Therefore, the applied bacterium acted in the biogenic synthesis of quite stable, uniform sized, and homogeneous shaped BNPs for Cu-Se BNPs-2 sample with PdI = 0.41 (Okita *et al*., 1968). Therefore, our results revealed that pH 7 in 37 °C for 48 h are considered as the optimal conditions for successful biogenic synthesis of the smallest homogenous sized Cu-Se BNPs, and subsequently Cu-Se BNPs-2 was selected for the next measurements and applications.

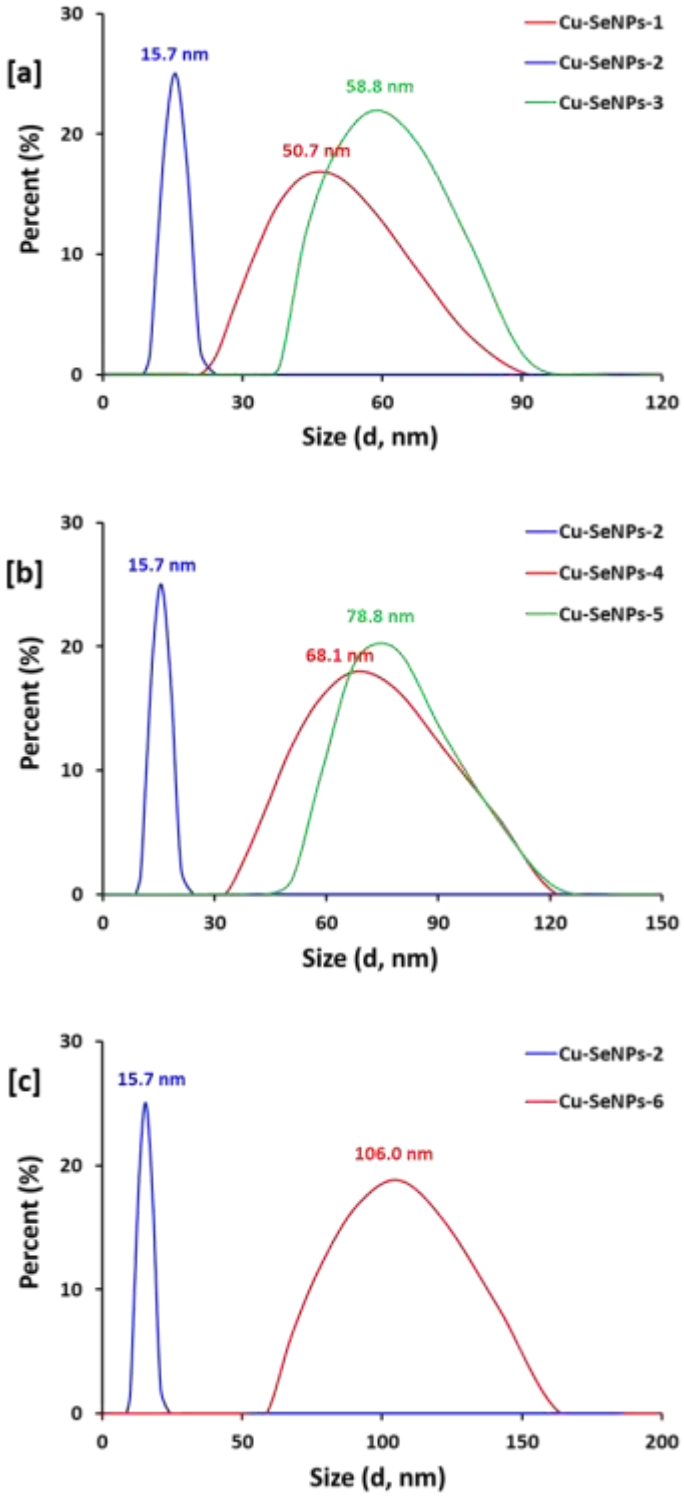

Fig. 3: Zetasizer analysis for the biogenic Cu-Se BNPs

Where; [a] Effect of time, [b] Effect of pH, and [c] Effect of temperature

The biogenic Cu-Se BNPs were examined under the TEM as observed in Fig. (4), while the individual monometallic (Cu and Se) NPs were tested for comparison. Size of the NPs in the measurement areas was estimated using 4 pi software. The geometrical features of the individual monometallic CuNPs and SeNPs were spherical with the estimated mean size of 8.9 nm and 71.5 nm, respectively. Meanwhile, the biogenic Cu-Se BNPs were spherical in shape with an average size of 9.7 nm. The obtained Cu-Se BNPs were observed in a colonial form.

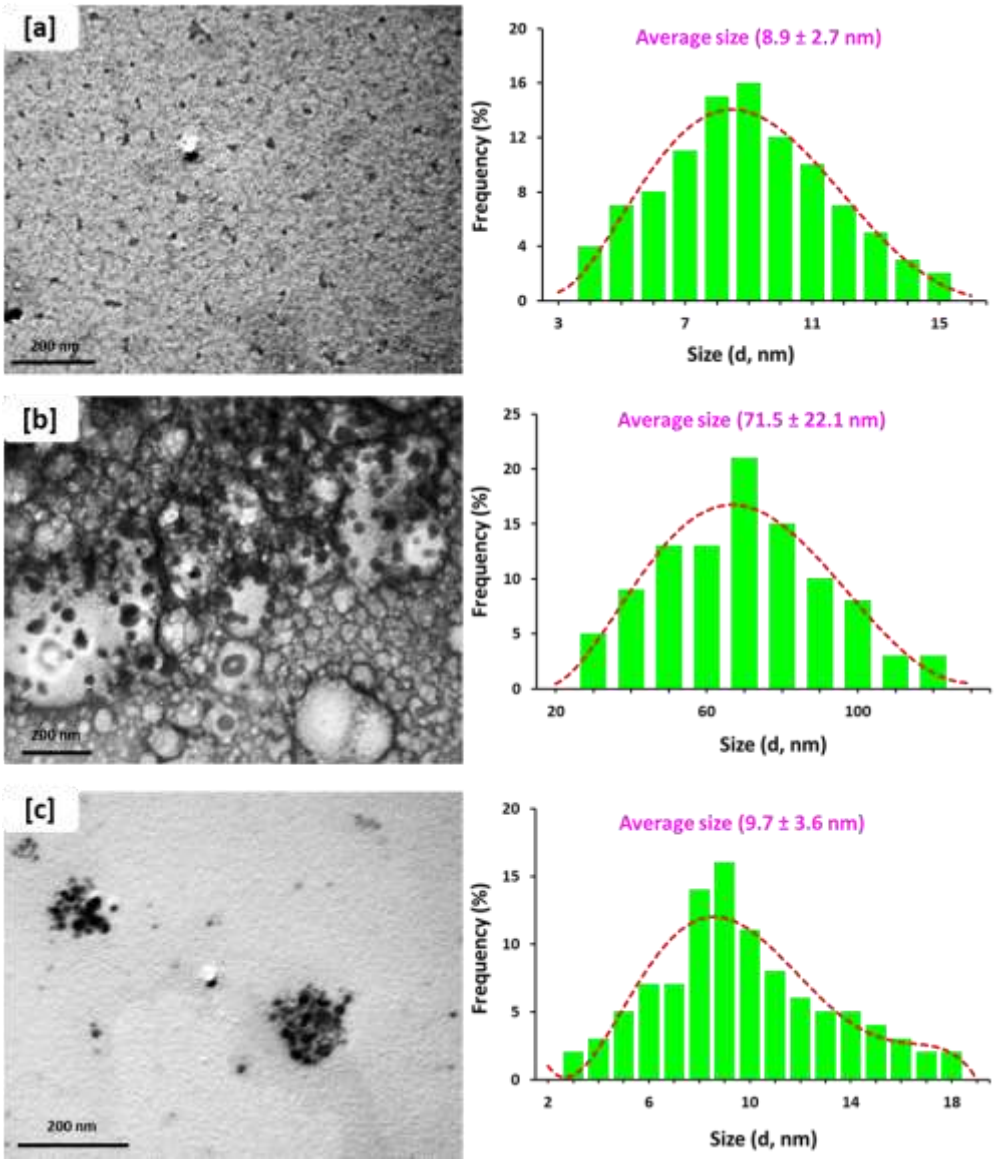

Fig. 4: Transmission electron micrographs (TEM) for the biogenic NPs, including [a] CuNPs, [b] SeNPs, and [c] Cu-Se BNPs-2

The X-ray diffraction (XRD) pattern was investigated for the biogenic samples of monometallic (Cu and Se) NPs and those of the Cu-Se BNPs, where the obtained results are demonstrated in Fig. (5). The biogenic CuNPs exhibited several diffraction bands, while the characteristic diffraction bands were recorded at 2θ° = 30.4°, 36.4°, and 50.6°, which are attributed to the crystal lattice of (110), (111), and (200); respectively, for the Cu_2ONPs (Hassabo *et al*., 2022).

The biogenic SeNPs displayed three significant diffraction bands at 2θ° = 23.6°, 30.2°, and 44.3° which are assigning for the index of (100), (101) and (102); respectively, for the metallic SeNPs (Cittrarasu *et al*., 2021; Khudier *et al*., 2023). In case of the biogenic BNPs, both diffractions for the individual monometallic NPs were shown together confirming successful biosynthesize of the Cu-Se BNPs.

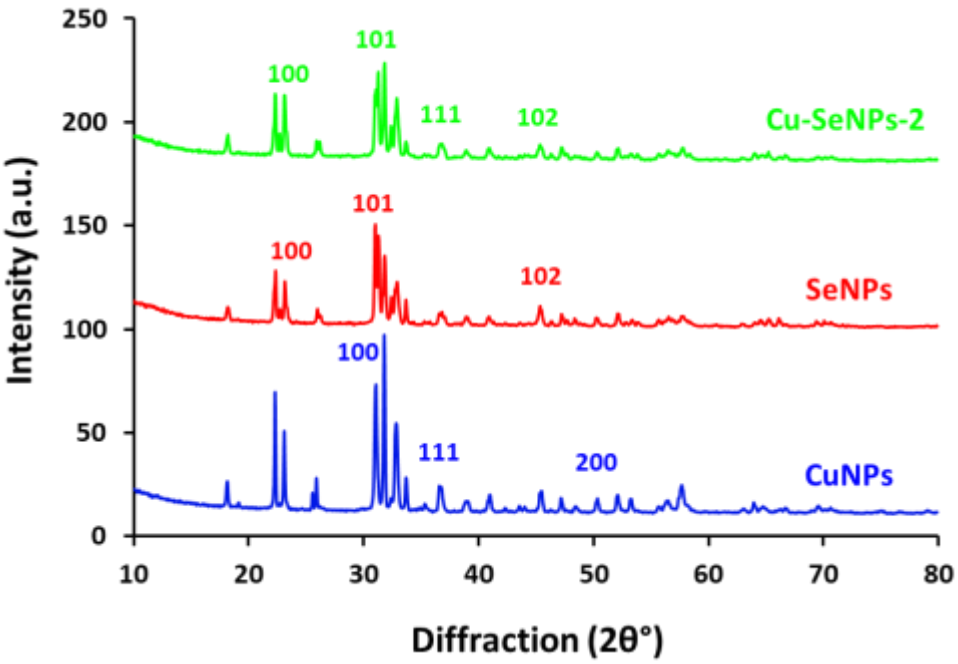

Fig. 5: X-ray diffraction (XRD) patterns for the biogenic synthesized CuNPs, SeNPs, and Cu-Se BNPs-2.

Where; a.u. = atomic unit

3.3. Bioactivity studies

Nano biotechnology is the application of biological entities in biosynthesis of NPs. It has attracted more attention due to low toxicity and economic viability. Utilization of nano materials in the treatment of cancer has gained interest due to its ability to overcome certain current restrictions regarding drug delivery (Chen *et al*., 2018; Li *et al*., 2023). Therefore, bioactivity of the biogenic Cu-Se BNPs was studied through testing the cytotoxicity and antioxidant activities. The cytotoxicity study was tested using MTT method at 24 h against two different cell lines; mainly HepG2 and MDA. Results represented in Fig.

(6) indicate that Cu-Se BNPs were able to induce a cytotoxic effect with significant reduction in viability (%) of both cell lines. Reduction in cell viability was linearly enlarged by increment in concentration of the applied Cu-Se BNPs. At high concentration of Cu-Se BNPs (100 µg/ ml), the recorded reduction in cell viability was 87 % for HepG2 and 81 % for MDA. The IC50 values were estimated to be 696.4 and 273.9 µg/ ml for HepG2 and MDA, respectively.

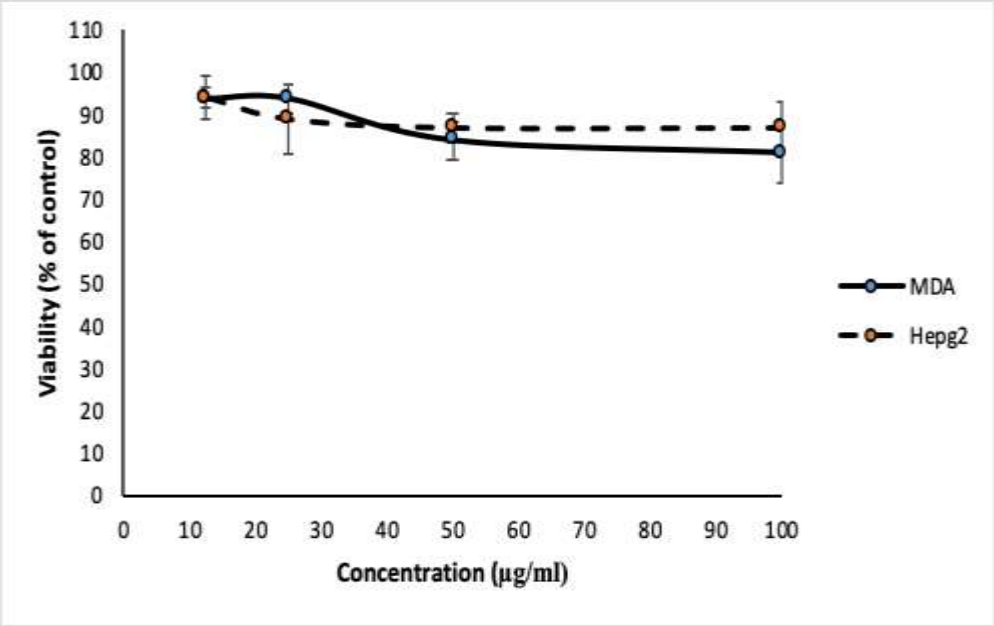

Fig. 6: Anticancer activity using triplicate repeats of different concentrations (100. 50. 25, 12.5 µg/ml) of bio-synthesized Cu-Se BNPs, according to MTT method in HepG2 and MDA cancer cell lines after 24 h of incubation. The viability is expressed as percentage of control (mean ± SD)

Antioxidant activity of the biogenic Cu-Se BNPs was evaluated by measuring the DPPH artificial radical scavenging activity, while the ascorbic acid was used as an antioxidant reference. The scavenge free radicals assay was carried out using different concentrations of bimetallic Cu-Se BNPs. As demonstrated in Fig. (7), the biogenic Cu-Se BM NPs exhibited low ability to scavenge DPPH at a high concentration of 100 µg/ ml. The estimated SC_{50} was 305.3 µM, which is quite higher than that for the reference of ascorbic (95.9 µM). Significant increment in the scavenging of DPPH may be needed at higher concentrations of the biogenic Cu-Se BNPs. These observations are in line with several previous studies that investigated the cytotoxic behavior of CuONPs and SeNPs on the human cancer cell lines. The cytotoxic activity of substances containing selenium varied depending on the chemical forms, dosages, level of selenium bioavailability, and kind of cancer (Kieliszek *et al*., 2020), while the four-valent selenite (Se^{+4}) showed efficiency in cancer treatment (Kieliszek and Lipinski, 2018). Selenium could be able to oxidize the vicinal sulfhydryl groups which in turn have a significant role in the treatment of cancer cells (Lipinski, 2017). Moreover, the selenite can retard the formation of the protect coating (*i.e*., fibrinogen–albumin) on the surface of cancer cell (Lipinski, 2005).

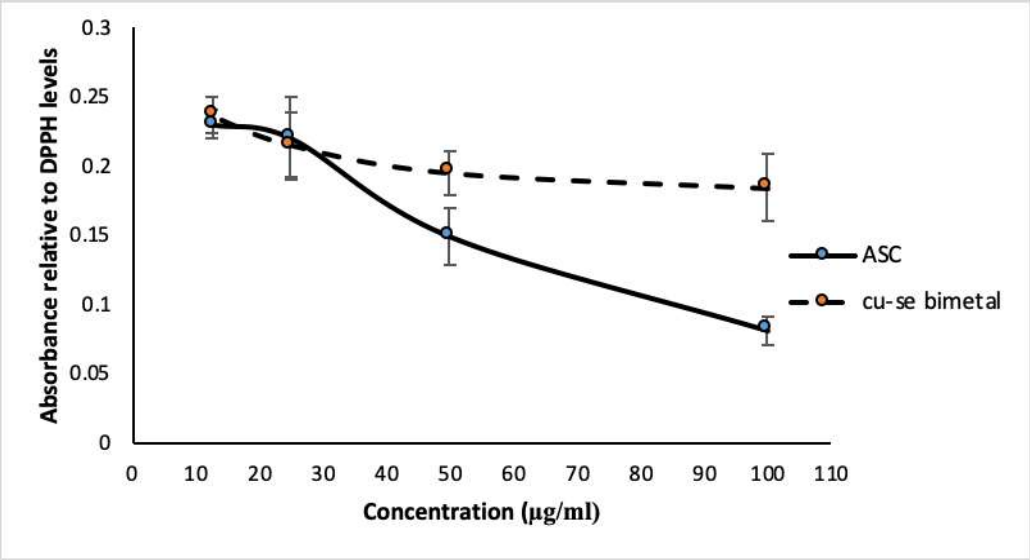

Fig. 7: Anti-oxidant activity through DPPH free radical-scavenging in triplicate repeats of different concentrations (100, 50, 25, 12.5 µg/ml) of biogenic Cu-Se BNPs and ascorbic acid (ASC)

Rehana *et al*., (2017) demonstrated that CuONPs has anticancer effect on a wide range of cancer cell lines such as human breast (MCF-7), cervical (Hela), epithelioma (Hep-2), and lung (A549) (Rehana *et al*., 2017). Siddiqui *et al*., (2013) determined the cytotoxic impacts of CuONPs on liver cancer cells and concluded that CuONPs result in death of the HepG2 cell line. Cu_2O NPs have the ability to bind and change their surface characteristics through conjugating with different biomolecules, including proteins and enzymes, which makes them useful as DNA-cleavage agents and powerful anticancer treatments (Halevas and Pantazaki, 2018). Furthermore, programmed cell death could be carried out by induction of apoptosis, which is considered as the much favorable anticancer action (Arkan *et al*., 2018). Promotion of the apoptosis takes place through activation of the Caspase protein (Elmore, 2007). The caspase-3 has the main role of deterioration of the cancer cells cellular structures by destroying their cytoskeletal proteins and/or the DNA fragmentation (Elmore, 2007).

Based on the previous studies reported by Elemike *et al*., (2019); Elsayed *et al*., 2022), anticancer activity of the Cu-Se BNPs in the current study could be attributed to the fact that Cu-Se BNPs can basically act in caspase-3 activation of the cancer cell lines, which consequently helped in mortality of the apoptotic cells. The biogenic Cu-Se BNPs consisted of free mobile electrons that can prevent rapid growth of the carcinogenic cells through release of the reactive oxygen species (ROS). Meanwhile, the released ROS can easily promote the caspase-3 activity, and subsequently deteriorate the cancer cells by apoptosis induction. Therefore, the Cu-Se BNPs display cytotoxic actions as indicated by a recorded decrease in cancer cell viability proportional with the increase in NPs concentration (100 µg/ ml). Additionally, cell proliferation was decreased in a time-dependent manner at the same dose. The study's findings imply that Cu-Se BNPs may find usage as biological applications in cancer chemotherapy and chemoprevention.

Conclusion

In the current study, biologically active Cu-Se BNPs were bio-synthesized using cell-free extract of a marine bacterium, identified as *B. amyloliquefaciens* by phylogenetic analysis. Biogenic synthesis of Cu-Se BNPs was successfully investigated using UV-Vis spectrophotometry, TEM, zetasizer, and XRD. Particle size, PDI, and ZP were changed according to changing the physiological factors prevailing during biosynthesis. Small sized (15.7 nm) Cu-Se BNPs were obtained with spherical shapes. The biogenic NPs showed good anticancer activity, where the cell viability of HepG2 and MDA cell lines was diminished by 87 % and 81 %, respectively. Meanwhile, the antioxidant activity of the biogenic NPs was much lower compared to that for ascorbic acid as a reference, while the estimated SC_{50} was 95.9, 305.3 µM in case of ascorbic acid and Cu-Se BNPs, respectively. Based on the obtained results, the bio-synthesized Cu-Se BNPs had a significant cytotoxicity and subsequently can be exploited as anticancer agents. Therefore, Cu-Se BNPs could be used for biomedical applications as promising anticancer agents with application in cancer chemotherapy and chemoprevention.

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Conflict of interests

The authors declare that they have no competing interests.

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Ethical approval

Non-applicable.

Author's Contributions

Conceptualization, A.A.H. and B.A.A.; Data curation, H.E.E. and A.A.H.; Investigation, B.A.A., S.A.T. and A.A.H.; Supervision, A.A.H., B.A.A. and H. E. E.; Validation, H.E.E. and S.A.T.; Roles/ Writing - original draft, A.A.H., H.E.E., S.A.T. and H.E.E.; Writing - review & editing, H.E.E. and B.A.A.

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