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Biosynthesis of selenium nanoparticles as a potential therapeutic agent in breast cancer: G2/M arrest and apoptosis induction

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ABSTRACT

The drawbacks and adverse reactions of conventional breast cancer (BC) medications have prompted researchers to seek novel therapeutic approaches. This study aimed to study the impact of biosynthesized selenium nanoparticles by yeast on breast cancer (MCF-7) cells and to find potential underlying mechanisms. Therefore, marine yeast isolates were screened for their ability to biosynthesis selenium nanoparticles (SeNPs). The most potent isolate was identified as *Candida pseudojiufengensis* based on 18 S rRNA gene sequencing. Incubation of cell-free extract with 0.8 mM of SeO_2 for 48 h at 40°C in pH of 7.0 were optimal conditions for the biosynthesis of SeNPs. The biosynthesized SeNPs were characterized by UV–Vis spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM), and dynamic light scattering (DLS) measurements including average particle size distribution and average zeta potential. The results showed that the biosynthesized SeNPs displayed a maximum absorbance peak in the UV-Vis spectrum at 560 nm due to surface plasmon resonance. TEM image elevated spherical shape particles with an average size of 12 nm. SRB assay, flow cytometry, and other biochemical methods were employed to assess SeNPs anti-proliferative effects on MCF-7 cells. SeNPs showed superior anti-cancer efficacy against MCF-7 cells compared to colon (HCT-116) and liver (HepG2) cancer cells, as evidenced by lower IC50 values (19.59 μg/ml) against 36.36 μg/ml and 27.81 ±1.4 μg/ml, respectively. However, SeNPs demonstrated no cytotoxic effects against HSF cells. Moreover, treatment with SeNPs induces G2/M arrest along with triggering apoptosis in MCF-7 cells. Furthermore, MCF-7 cells treated with SeNPs showed increased oxidative stress, as indicated by observable rises in LPO and 8-OHDG, accompanied by considerable exhaustion in antioxidant enzyme activity. These findings demonstrated that Se nanoparticles synthesized from yeast have therapeutic promise in BC treatment.

1. Introduction

Nanobiotechnology is an arm of biotechnology in which microbes are used for the production of medically and/or industrially important nanoparticles (NPs). It has drawn great attention for people who look for green technology instead of using chemical, toxic, and expensive reagents [15,47].

Selenium (Se) is an important micronutrient required for many vital biological activities in the human body and for most of living organisms since it represents a main part of selenoenzymes [21,32,61]. Such Se-enzymes are important in many different metabolic pathways such as thyroid hormones metabolism, antioxidation systems and in immune functions ([35]; X. [86,43]). As an essential dietary element, selenium intake is important for reduction of cancer development and infestation [25,87].

Selenium nanoparticles (SeNPs) have potential physical and chemical properties make them unique for different industrial applications such as semiconductors, photoelectric applications [76]. In medicine, progress of many diseases and cancers are related to the oxidative stress [25,69]. Application of new efficient SeNPs as potential antioxidants helps in the detoxification process and conversion of such harmful free radicals and will be a novel tool for control of different diseases [78]. Therefore, SeNPs have gained attention as potential chemo preventive agents in a variety of cancers, including melanoma, breast, colon, prostate, and liver cancer [19,26,36]. Breast cancer is the world's second most widespread and fatal cancer among women due to its

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aggressive characteristics, high risk of recurrence and metastasis, and poor response to traditional treatments [2,51]. The clinical and epidemiological data showed that Se's status corresponds with breast cancer survival. As a result, one strategy to reduce breast cancer mortality is through Se supplementation, particularly in individuals with significantly depleted Se status [18].

SeNPs synthesis mediated by living organisms has benefits due to the increasing need for non-toxic, environmentally friendly and low-cost methods, offering a promising alternative to expensive, and possibly hazardous physical and chemical techniques [11,44,82]. Yeasts are characterized by their ability to grow in simple media and tolerate to different concentrations of selenium. Due to their high protein content, they are able to incorporate high concentrations of inorganic and organic forms of selenium for amino acids biosynthesis [40,56]. For these reasons, it was useful for us to investigate the SeNPs biosynthesis using marine yeast and focus on the application of the biosynthesized SeNPs as anticancer agent.

The bio-reduction of selenium ions to a zerovalent nano-selenium can be done intracellular or extracellular according to the environmental conditions [13,22]. Intracellular biosynthesis of SeNPs is achieved through a detoxification process via different membrane-enzymes [20]. Also, when the cells are exposed to a high concentration of selenium that exceed their threshold, they defend themselves by secretion of redox components such as quinines and thiols that reduce selenium ions to the elemental form [71]. For extracellular biosynthesis of SeNPs, secreted enzymes in the cell-free extract with high redox potential such as NADH and NADPH-dependent nitrate reductase enzymes are doing so by acting as electron transporters [20]. The last-mentioned enzymatic mechanism of bio-reduction has been reported for both intracellular and extracellular biosynthesis of nanoparticles [48].

To the best of our knowledge, few studies investigated the anticancer mechanisms of SeNPs in breast cancer. Therefore, the aim of this study was to optimize the biosynthesis of SeNPs as a potential therapeutic agent by a locally isolated marine yeast. The biosynthesized SeNPs were characterized by different techniques including UV–Vis spectroscopy, XRD, TEM and DLS measurement. Finally, the possible mechanisms of SeNPs as a promising therapeutic approach in MCF-7 cancer cells, relying on the observed growth inhibitory activity, have been evaluated.

2. Material and methods

2.1. Chemicals and materials

YPD ready-made broth medium (Yeast-Peptone-Dextrose) used for inoculum preparation, Selenium Dioxide was purchased from Loba Chemie Pvt. Ltd., India. All other chemicals were of the highest analytical grade from NRC laboratory. Apple peals and Molasse were obtained from the local Egyptian market.

2.2. Marine samples collection and isolation

Marine samples were obtained from different locations (beach sand and sea water) of the Red-Sea / Giftun island, Hurghada- Egypt (27∘13′N 33∘56′E). Isolation of marine microbiota was conducted by serial-dilution-plate method using YMP medium (Yeast extract, 3.0; Malt extract, 3.0; Peptone, 5.0 and Glucose, 10 in g/L). The seawater sample (200 ml) was filtered with sterilized 0.45 μm Millipore membrane filter, which was aseptically placed face-up on a YMP-Agar plate. The marine sand samples (1.0 g) were dissolved in sterile seawater (9 ml), serially diluted and streaked in YMP-Agar plates in duplicates. Growing colonies were picked up and inoculated in YPD slants at 30°C for 48 h. Inoculated slants were finally preserved in the fridge at 4°C and renewed every two weeks (Y. H. [9]).

2.3. Molecular identification

The selected marine yeast isolate was identified using an 18S RNA-based molecular technique. Genomic DNA extraction, PCR amplification, purification, and sequencing were performed using a protocol of Macrogen Company (Seoul, South Korea; https://www.macrogen.com). The 18 S rRNA gene was amplified using the universal primers, NS1 and NS8 with the sequences "GTAGTCATATGCTTGTCTC" and "TCCGCAGGTTCACCTACGGA", respectively. The PCR was carried out under the following conditions: initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 45 s and an extension step at 72°C for 5 min. The purified PCR product was sequenced, using the ABI 3730 DNA Analyzer (Applied Biosystems). The obtained sequences were aligned and compared with the sequences deposited in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool (BLAST). To determine the taxonomic position of the isolate, a phylogenetic tree was constructed with MEGA version 5.0 and the sequences of identified phylogenetic neighbors were aligned with the sequences of representative strains [73].

2.4. Culture conditions

To prepare the pre-culture, a loopful of each isolate was taken from preserved agar slants to inoculate Erlenmeyer flasks containing 50 ml of YPD broth medium. The flasks were incubated for 24 h at 30°C at 200 rpm. One milliliter of pre-culture was used as inoculum to inoculate new Erlenmeyer flasks containing 50 ml of YMP broth medium to obtain the main culture. The flasks were incubated for 48 h at 30°C and 200 rpm. To obtain the cell-free extract, a 48h-old culture was centrifuged at 4°C, 15,000 ×g for 10 min. (Hettich Universal, 320 R, Tuttlinger, Germany), washed twice with sterile distilled water and resuspended in 5 ml sodium citrate buffer, pH 7.0. The cell suspension is then treated by mechanical disruption by sonication using Ultrasonic Probe Sonicator (Bandelin electronic GmbH, SonopulsGM2200, Germany). Samples were sonicated for three cycles, 30 s each cycle while the power was set to 50 W and frequency at 60 MHz. The cell-free extract was finally clarified by cooling centrifuge at 4°C, 15,000 ×g for 10 min. [23].

2.5. Biosynthesis of selenium nanoparticles

The biosynthesis process of the selenium nanoparticles was schematically presented in Fig. 1.

Biosynthesis of SeNPs was accomplished according to a modified recent method described by Akçay & Ayşe Avcı [4] started by incubation of the cell-free extract with selenium metal solution containing SeO_2 (0.8 mM), unless otherwise indicated. The control sample contains only the cell-free extract without selenium precursor. The reaction mixture was incubated at 37°C and 200 rpm. After 72 h of incubation, the reaction mixture was collected and centrifuged at 3000 rpm for 15 min to collect the SeNPs. The media debris and bulk selenium were precipitated and the supernatant containing the SeNPs was collected, dried at 95°C for 6 h and calcinated at 300°C for 3 h. SeNPs samples were finally kept in the fridge for further analysis and application.

2.6. Characterization of the biosynthesized SeNPs

The green synthesis of SeNPs was characterized using different instrumental tools to determine the molecular structure and size of the biosynthesis of SeNPs. The physicochemical analysis included UV-Vis spectroscopy (Hitachi U-2900) in the range of 200–800 nm for each sample against distilled water was measured as blank [4]. XRD diffraction was investigated at room temperature by using X-ray diffraction (Shimadzu 7000, Japan). The topographical investigation was performed by TEM (Model JEM2010, Japan). The DLS

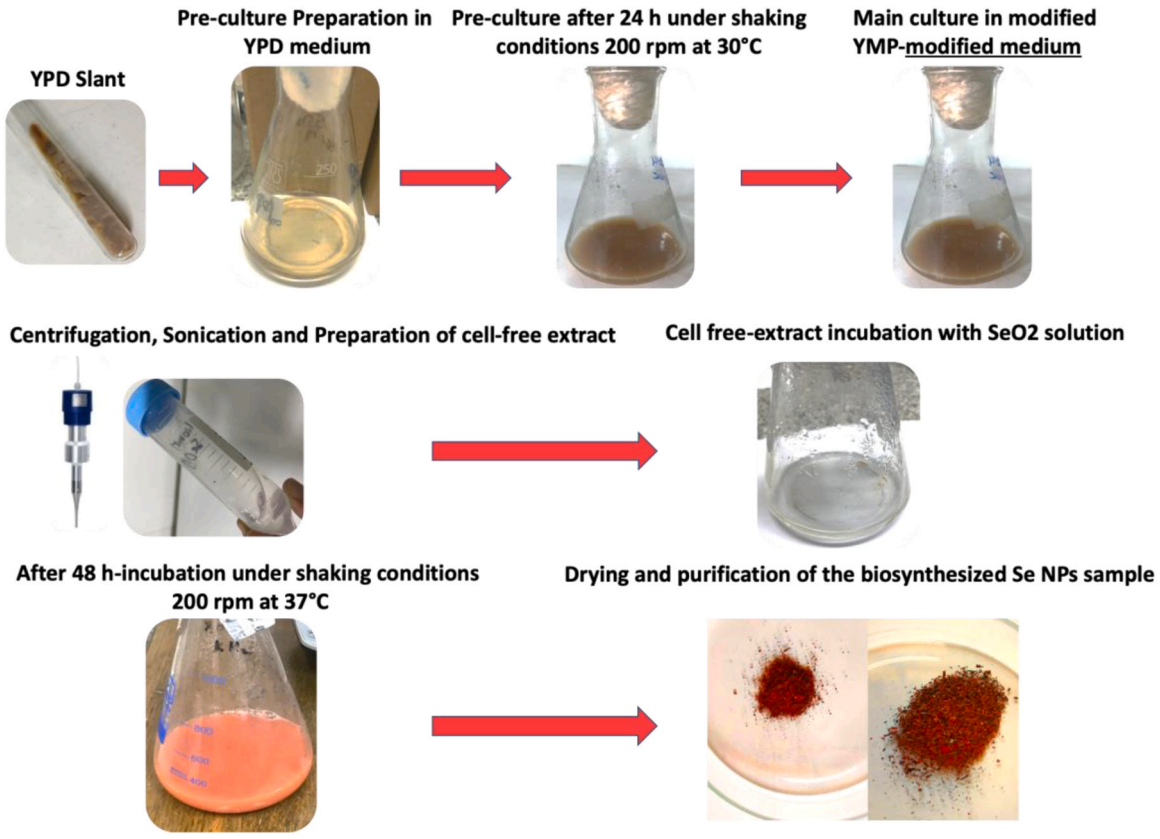

Fig. 1. Schematic workflow for the biosynthesis of selenium nanoparticles by marine yeast cells. The YMP-modified medium contains 0.5 % molasse instead of glucose.

measurements included particle size distribution and average zeta potential was measured via Nano-ZS, Malvern Instruments Ltd., UK.

2.7. Optimization of the SeNPs production

Different physical and chemical factors have been studied to optimize the SeNPs biosynthesis in a similar fashion described in Section 2.5. These factors are summarized in Table 1. The reaction mixture was subjected to detection and their UV-Vis spectra were obtained using a spectrophotometric analysis of the biosynthesized SeNPs.

2.8. Cell culture

The human hepatocellular carcinoma cell line (HepG2), human breast cancer cell line (MCF-7), human colon cancer cell line (HCT-116), and the human skin fibroblast cell line (HSF) were obtained from Nawah Scientific Inc. (Mokatam, Cairo, Egypt). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) with 100 µg/ml streptomycin (Lonza GmbH, Köln, Germany), 100 units/ml penicillin (Lonza GmbH, Köln, Germany), and 10 % fetal bovine serum (FBS; Gibco, NY, USA) at 37°C and 5 % CO2 [16].

Table 1
Effect of Physical and Chemical factors on SeNPs biosynthesis.

Fig. (1)	Factor	Variables
(a)	**Substrate**	Apple peal and Molasse
(b)	**Se concentration**	0.6 and 0.8
(c)	**Cell-extract concentration**	1:1, 2:1 and 3:1 of Se-reaction volume
(d)	**Reaction time with Se**	24, 48 and 72 h
(e)	**Reaction temperature**	5, 6, 7 and 8
(f)	**Reaction pH**	30°C and 40°C

2.9. Cytotoxicity using SRB assay

We used the SRB assay to assess the cell viability after treating cancer and normal cells with SeNPs and blank formula in 96-well plates for 72 h. Then, cells were fixed with 10 % trichloroacetic acid (Merck), washed five times with distilled water, stained with sulforhodamine (SRB) solution (Sigma-Aldrich) (0.4 % w/v) for 10 min in the dark, and washed three times with 1 % acetic acid (Chem-Lab). Then, the excess dye was dissolved in Tris pH 10.5 (Chem-Lab) (10 mM), and the absorbance was measured at 540 nm using a BMG LABTECH®-FLUOstar Omega microplate reader (Ortenberg, Germany). IC50 values were reported as mean ± SD. IC50 values were calculated using GraphPad Prism 8 software by applying the inhibitor vs normalized response method. The IC50 concentration gives 50 % viability. Cell viability (%) was calculated using the following equation: Absorbance of treated cells / Absorbance of untreated cells (Control) X 100 [68].

2.10. Cell cycle analysis

The cellular DNA content of MCF-7 cells was examined using an ACEA Novocyte™ flow cytometry (ACEA Biosciences Inc., San Diego, CA, USA) after the treatment for 48 h with the IC_{50} value of SeNPs and blank formula. Doxorubicin was used as a positive control. Following trypsinization, the cells were pelleted, washed twice, and fixed in 60 % ethanol in the refrigerator for at least 2 h. After washing with PBS, the pellets were stained with a staining mixture (RNase (Sigma-Aldrich) + Propidium Iodide (PI, Sigma-Aldrich)) for 30 minutes in the dark, and the results were assessed using the ACEA NovoExpressTM program (ACEA Biosciences Inc., San Diego, CA, USA) [62].

2.11. Annexin V/PI apoptotic/necrotic assay

The impact of SeNPs and blank formulaon the apoptotic/necrotic cell death of MCF-7 cells was investigated using the Annexin V-FITC Apoptosis Staining/Detection kit (ab14085; Abcam, Cambridge, MA). Doxorubicin was used as a positive control. In brief, cells were planted in six-well plates at a density of 1×10^4 cells per well for 24 h and exposed for treatment for another 48 h to the set IC50 values of each extract. After that, the cells were trypsinized and washed twice with PBS. The cells were then resuspended in 500 µl of 1X Binding Buffer and stained with Annexin V-FITC and PI for 30 min. at room temperature in the dark [1].

2.12. Determination of oxidative stress/ antioxidant markers

MCF-7 cells (3×10^5 cells/ml) were treated for 24 h with SeNPs, Dox, and blank formula, then harvested, lysed in a buffer, and centrifuged at 12,000× g for 1 min at 4°C. The supernatant was collected and utilized to identify lipid peroxidation (LPO) and deoxyguanosine (8-OHDG) levels using the colorimetric method as previously reported [52] and ELISA techniques (ELISAKITS. CO., London, UK) according to the manufacturer's protocol, respectively. For antioxidant markers, Catalase (CAT) activity and Superoxide dismutase (SOD) activity were measured at 240 nm [3] and at 560 nm [45], respectively.

2.13. Statistical analysis

The results presented in this work was figured out using Microsoft Excel. The particle size from zetasizer was measured as mean of 10 runs. The particle size from TEM micrograph was calculated by 4 pi software. Results of the anticancer experiments were mean values of three tests and the error bars were showed in the data.

3. Results

3.1. Screening of different isolated marine microbiota for SeNPs biosynthesis

Different isolated marine strains were grown on YDP-Agar supplemented with selenium dioxide (1 mM) for 48 h. Positive colonies which able to grow in selenium were picked up and used as inoculum for screening of SeNPs biosynthesis. The selected isolates showed substantial growth in YMP medium supplemented with SeO_2 in concentration 1 mM. The most potent isolate exhibited significant red color intensity and consequently a maximal surface plasmon resonance (SPR) peak after 48 h of growth at 30°C (Fig. 2). The color change indicates the metabolic activity of selenium-exposed cells for bio-reduction of colorless selenium ions in SeO_2 to elemental zerovalent Se^0 through their reductive enzymatic systems.

3.2. Molecular identification of the isolated marine yeast

According to the 18S rRNA gene sequence with BLAST in the GenBank database, the marine yeast strain used in this study belong to the genus ***Candida*** and have been deposited in the GenBank nucleotide sequence database under the accession number MT762177. By construction of the phylogenetic tree using the neighbor- joining method, results disclosed that the isolate is closely related to ***Candida pseudojiufengensis*** (Fig. 3). Thus, ***C. pseudojiufengensis*** was proposed as the strain of the study.

3.3. Optimization of the growth and biosynthetic conditions for SeNPs production

The effective reduction of selenium ions to elemental Se was first observed by a color change in the reaction mixture, *as mentioned before*,

Fig. 2. Color change of the reaction solutions at different conditions; (a) Control on the right and biosynthesized SeNPs solution on left.

to an orange-red color which indicates the formation of zerovalent selenium as SeNPs. A summary of the UV-Vis Spectra of the biosynthesized SeNPs at different conditions is presented in Fig. 4.

Based on the fact that using the agro-industrial by-products is worthy for such bioprocesses to target economic production of valuable biomolecules; sugarcane molasse, apple peal and mixture of both with different ratios have been used as substrates for NPs synthesis. The SeNPs obtained in the presence of molasse as the sole substrate exhibited the highest SPR peak (Fig. 4-a). In addition, SeNPs have been biosynthesized by two different methods; **1.** by direct exposure the growing cells to selenium ions through supplementation of SeO_2 to the growth medium or 48 h (Figs. 4-a), 2**.** by exposure of the cell-free extract to an aqueous solution of SeO_2 for 48 h (Fig. 4(b - f)). In this latter case, the UV-Vis spectra showed that the SPR corresponded to the SeNPs is at 550–600 nm with broad peak. Noticeably, the λ_{max} value was shifted backward to 270 nm when SeNPs were synthesized inside the growth medium from the early beginning. This shift comes back to the interference of the growth medium components and the living cells with nanoparticle absorbance. Therefore, the optimization study for SeNPs biosynthesis was continued using the aqueous solution of the cell-free to emphasize that the color change and the SPR peak is only verify the SeNPs formation and not any other extracellularly secreted substances from the microbial growth.

To investigate the effect of SeO_2 concentration on the biosynthesis of SeNPs, cell-free extract was supplemented with different concentrations of SeO_2, the optimal SeO_2 concentration used for SeNPs synthesis was 0.8 mM with higher SPR intensity and lower peak broadening (Fig. 4-b). This revealed that nanoparticles synthesis started in this concentration due to the availability of aforementioned functional biomolecules required for the synthesis and stability of nanoparticles. While the concertation of the functional groups will be unequal compared to the higher concentration of the precursor which affects the bio-reduction of selenium to stable/capped selenium nanoparticles as it was pointed before [12,89]. It could be concluded that the amount of bio-generated SeNPs is highly affected by SeO_2 concentration in the cell-free extract solution. Moreover, *C. pseudojiufengensis* cells were able to tolerate up to 2.0 mM SeO_2 which was noticed by visual observation, data are not shown. Since that our goal was to obtain SeNP by using less concentration of SeO_2, 0.8 mM was used further.

On the other hand, the effect of cell-extract concentration on the SeNPs biosynthesis has been studied (Fig. 4-c). Thus, cell-free extract of different culture volumes (30, 60 and 90 ml) was incubated with 30 ml of SeO_2 solution (final concentration of 0.8 mM), which equivalent to

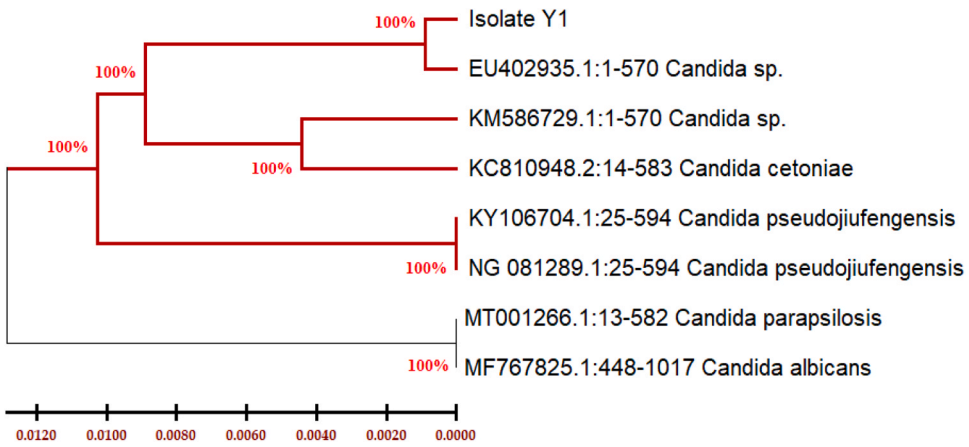

Fig. 3. Phylogenetic tree based on partial 18S rRNA sequences, showing the relationship between isolate *Candida pseudojiufengensis* and other species belong to the genus ***Candida***.

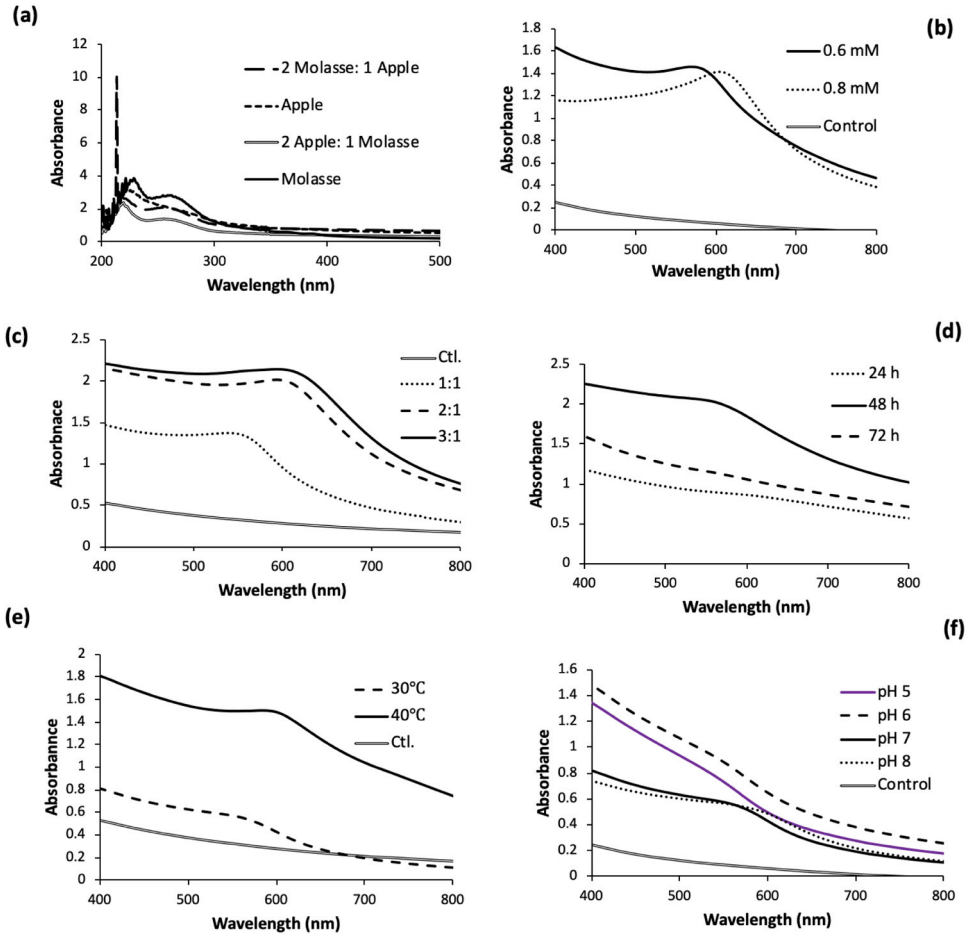

Fig. 4. UV–Vis spectra of the biosynthesized SeNPs under different (a) substrates, (b) selenium concentrations, (c) cell-extract concentrations, (d) reaction times, (e) incubation temperatures and (f) pH values.

1:1, 2:1 and 3:1 of Se-reaction volume, respectively. Expectedly, SPR peak intensity increased proportionally with the cell concentration used for SeNPs synthesis with a maximal absorption peak obtained by using cell-free extract of 90 ml culture (3:1). The obtained results imply the importance of cell concentration on the biosynthesis of SeNPs. It was reported that the concentration of biomolecules in the cell-free extract, in terms of cell densities of the culture supernatant, has a considerable effect on NP synthesis [66].

Investigation of the optimal reaction time, temperature and pH of the reaction mixture for SeNPs synthesis are shown in Fig. 4(d - f), respectively. Incubation of the reaction mixture (cell-free extract with 0.8 mM of SeO_2) for 48 h at 40°C and pH of 7.0 were the optimal conditions for the biosynthesis of SeNPs. This was visualized by a color intensity change and detected in UV-Vis spectra by maximal SPR intensities with lower peak broadening at these conditions. In light of all these results, it concluded that 1. reaction temperature has an important role in bio-

reduction of selenium ions Se^{4+} to the elemental form [84]; 2. high temperatures (>40°C) hinder the biosynthesis of stable SeNPs which makes the biological synthesis of SeNPs an energy-saving method [4]. Also, a maximum absorbance of SeNPs was achieved at 33°C which is regarded as an optimized temperature with no color change at 45°C [4]. The pH of the reaction mixture has significant effect on the size, shape and composition of the nanoparticles synthesized chemically or biologically [5,7]. Therefore, many studies made a pH control for the reaction medium to neutral or weak alkaline pH before initiation of the reduction reaction to obtain a maximum synthesis of SeNPs, like what have been done in our study [30,67].

3.4. Characterization of the optimized biosynthesized SeNPs

Fig. 5 illustrates the TEM images at low and high magnifications (a and b) as well as the selected area electron diffraction pattern (c). The TEM image at low magnification (Fig. 5-a) presented assembled spherical particles that represented a universal spherical shape. Moreover, a high magnification image (Fig. 5-b) observed spherical particles arranged on top of each other with average nanosized around 12 nm. In this context, the SAED pattern (Fig. 5-c) observed a polycrystalline behavior of the SeNPs that is a convention pattern of biosynthesis nanoparticles. Moreover, the dynamic light scattering (DLS) measurements showed an average particles size around **14 nm** (Fig. 5-d) with PDI 0.15 which referred to homogenous particle distribution and this is in a nice agreement with the TEM study. Additionally, the average zeta potential (Fig. 5-e) was recorded at around 34 mV indicating the high stability of biosynthesized SeNPs. Conspicuously, biosynthesis SeNPs showed excellent stability where absolute values of zeta potential about 30 mV and $\leq$ 60 mV indicate outstanding good and excellent stability, respectively. These findings affirmed that the biosynthesis of SeNPs is in the nanoscale with homogeny particles as well as excellent stability as well.

XRD crystallographic pattern is shown in Fig. 6. A typical XRD pattern of Se was observed at diffraction peaks 31.6, 34.2, 36.1, 39.3, 47.4, 56.5, 62.7, 66.2, 67.8, 68.8, 72.02 and 76.7°. All the sharp and strong diffraction peaks were readily correlated to the trigonal structure of Se nanoparticles, which consistently yield an average lattice constant following X-ray analysis. This is in good agreement with the data documented in the JCPDS standard card (No. 06–0362) for SeNPs [63].

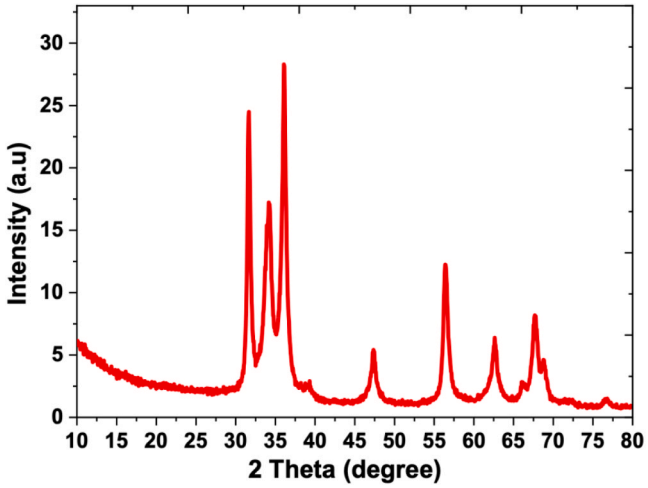

Fig. 6. XRD crystallographic pattern of biosynthesized SeNPs.

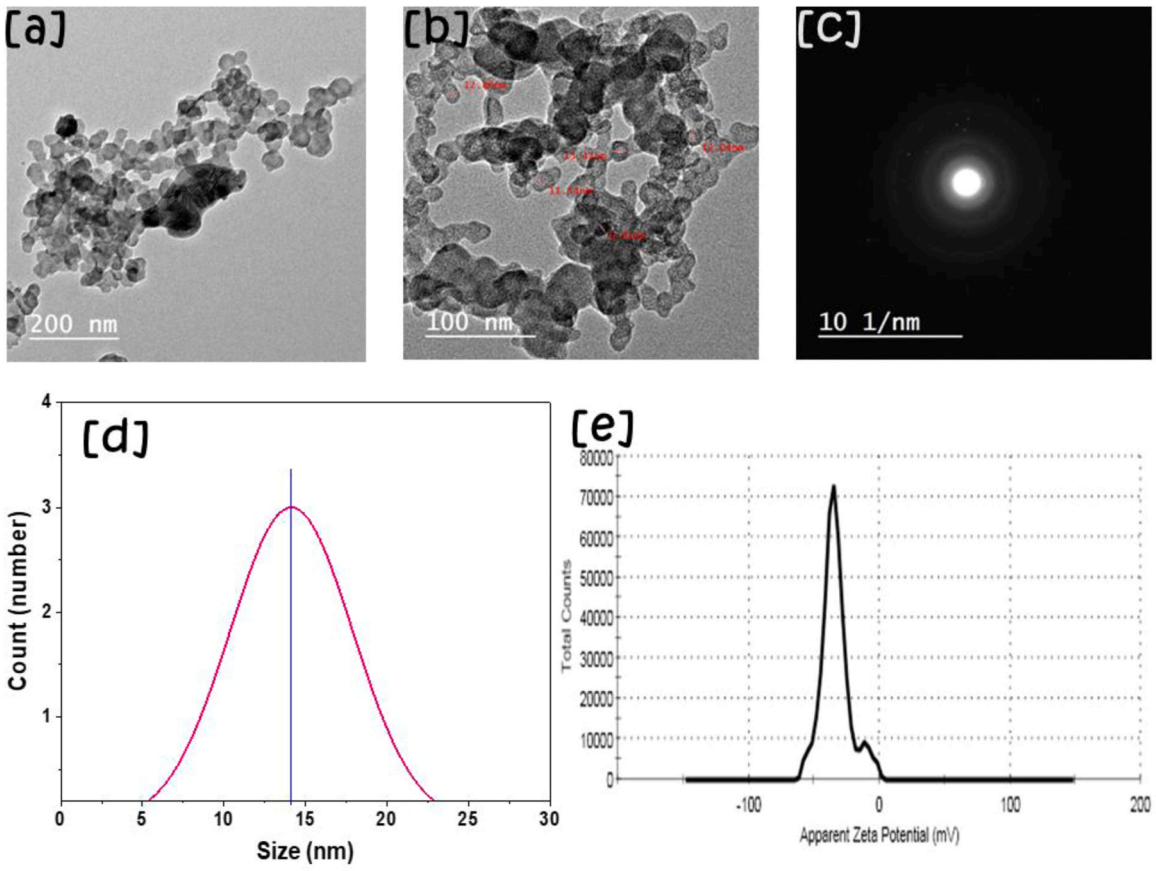

Fig. 5. TEM image of biosynthesized SeNPs with low (a) and high (b) magnifications as well as SAED pattern (c) (upper). Particle size distribution (d) and average zeta potential value (e) (lower).

3.5. Cytotoxicity assay

Selenium nanoparticles (SeNPs) have received substantial interest for their outstanding biological activities [27,64]. Therefore, the anti-cancer activity of SeNPs was screened on the survival rates of liver (HepG2), breast (MCF-7), and colon (HCT-116) cancer cells using the SRB method treated with different concentrations of SeNPs versus its blank formula (Blank). As shown in Fig. 7-A, the half-maximal inhibitory concentration (IC50) of SeNPs in HepG2 cells was 27.81 ±1.4 µg/ml, in MCF-7 was 19.59± 1.67 µg/ml (showed better anti-tumor effect) and that in HCT-116 was 36.36±2.41 µg/ml (Showed least anti-tumor effect), while the exposed blank formula IC50 values were much higher than 100 µg/ml in all tested cell lines (Fig. 7-B). Interestingly, in non-cancerous HSF cells, we found that the IC50 values of both SeNPs and blank formula were much higher than 100 µg/ml (Fig. 7 A & B). The most prominent inhibitory effect of SeNPs was observed in the breast cancer cell line (MCF-7). Subsequently, it was selected to assess SeNPs' anticancer mechanism in detail.

3.6. Effect of SeNPs on cell cycle of MCF-7 cells

Targeting cell-cycle machinery is a crucial aspect of studying the anti-tumor mechanisms [53,72]. Here, flow cytometry was used to detect the influence of SeNPs on the cell cycle distribution of MCF-7 cells. As shown in Fig. 8 (A & B), SeNPs treatment significantly increased the proportion of S phase and G2/M phase cells to 30.88 % ± 1.1 and 57.94 % ± 1.7, respectively, compared to 16.51 % ± 0.7 and 21.05 % ± 1.4 of control untreated cells. As cells accumulated during these two stages, the pre-G1 population increased from 2.29 % ± 0.1–6.63 % ± 0.5, indicating cell death. Reciprocally, after treatment with SeNPs, the G0/G1 population decreased significantly from 62.44 % ± 1.7–11.18 % ± 1.1, compared to the control group. However, there was not much variation in the distribution of all phases of MCF-7 after treatment with blank formula compared to untreated cells.

The observed modification of cell cycle phases following SeNPs treatment was more potent than DOX therapy. Dox treatment significantly reduced the G0/G1 population to 40.6 %±0.25, increased the G2M population to 42.6 %±1.4, and slightly increased the pre-G1 population to 4.42 %±0.1, without affecting the S phase population (Fig. 8 A & B). These findings indicated that these two agents mainly targeted the G2/M phase of cell cycle arrest in breast cancer, implying that SeNPs mediated cancer cell inhibition via G2/M phase arrest. Antimitotic medicines are among the most influential chemotherapeutic medications available to oncologists to treat most solid malignancies. These drugs may offer more effective techniques for leveraging mitotic catastrophes in cancer prevention and treatment [77]. Furthermore, this technique may enhance sensitization to alkylating chemicals because the G2 checkpoint is critical for cancer cells exposed to them, as cells require more time to fix DNA damage and safely enter the mitotic phase. As a result, G2/M arrest is commonly regarded as a therapeutic target for inhibiting cancer cell development [37].

3.7. Effect of SeNPs on apoptosis/necrosis of MCF-7 cells

Triggering apoptosis in tumor cells is a promising cancer therapeutic strategy [54,88]. In this study, the effect of SeNPs on MCF-7 cells was assessed using the Annexin V/PI double staining technique coupled with flow cytometry. As seen in Fig. 9 (A and B), after 48 h, Se-NP treatment resulted in a higher percentage of early (31.12 % ± 1.69) and late apoptotic cells (8.76 % ± 0.19) compared to control cells (0.02 % ± 0.004 and 0.15 % ± 0.007, respectively) ($p < 0.05$), indicating that apoptosis is the primary cell death mode of se nanoparticles in MCF-7. Only slight necrotic cells were observed after SeNPs (2.97 % ± 0.99) compared to control cells (1.21 % ± 0.02).

Furthermore, there was no discernible distinction in the apoptotic and/or necrotic impact between the blank formula treated and the control group. In contrast to the SeNPs group, Dox treatment substantially enhanced the proportion of late apoptotic cells (77.91 % ± 2.38) and necrotic cells (15.42 % ± 1.44) ($p < 0.05$), indicating a distinct mechanism of cell death than SeNPs in MCF-7 cells. Compared with the SeNPs group, the late apoptosis in the Dox group accounted for a large proportion.

Anticancer medications trigger both apoptotic and non-apoptotic cellular death in diverse cancer types. For more than three decades, targeting apoptotic signaling pathways has been the main objective of clinical oncology to boost the sensitivity of cancer cells to therapy [8], implying that SeNPs can be engaged in clinical trials [74].

3.8. Effect of SeNPs treatment on the oxidative stress in MCF-7

ROS generation and antioxidant exhaustion are pivotal mechanisms underlying the apoptotic effect and cell cycle arrest of anticancer medicines on tumor cells [50]. As a result, LPO and 8-OHDG levels were evaluated as oxidant biomarkers, whereas the activity of antioxidant enzymes (SOD and CAT) was determined as antioxidant biomarkers in MCF-7 cells treated with SeNPs, DOX, and blank formula.

As shown in Fig. 10, considerable decreases were observed in SOD and CAT enzyme activity from 7.8 ± 0.3 U/mg to 2.89 ± 0.13 U/mg and from 0.79 ± 0.04 U/mg to 0.24 ± 0.06 U/mg, respectively. This was paralleled by a significant increase in LPO and 8-OHDG levels in the SeNPs (1.38 ± 0.06 nmol/mg and 139.71 ± 2.88 ng/mg) and DOX-treated groups (1.31 ± 0.013 nmol/mg and 134.82 ± 3.7 ng/mg) compared with the control group (0.52 ± 0.04 nmol/mg and 29.57 ± 1.36 ng/mg), respectively.

4. Discussion

Because of its aggressive nature and poor response to conventional treatments, breast cancer is the second most common and deadly cancer in the world to affect women. [2,51]. Recently, selenium nanoparticles have drawn interest as possible chemo-preventive medicines for a number of malignancies, including as liver, breast, colon, prostate, and melanoma [19,26]. Thus, optimizing the production of SeNPs from

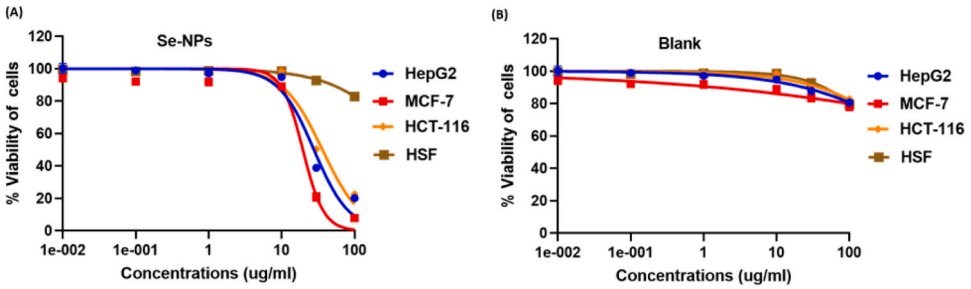

Fig. 7. Cytotoxic activity of (A) SeNPs and (B) Blank formula on cancerous HepG2, MCF-7, and HCT-116 and normal HSF cells. Cells were treated with serial dilution of both treatments for 72 h. Cell viability was evaluated by SRB assay. Data are expressed as mean ± SD (n = 3).

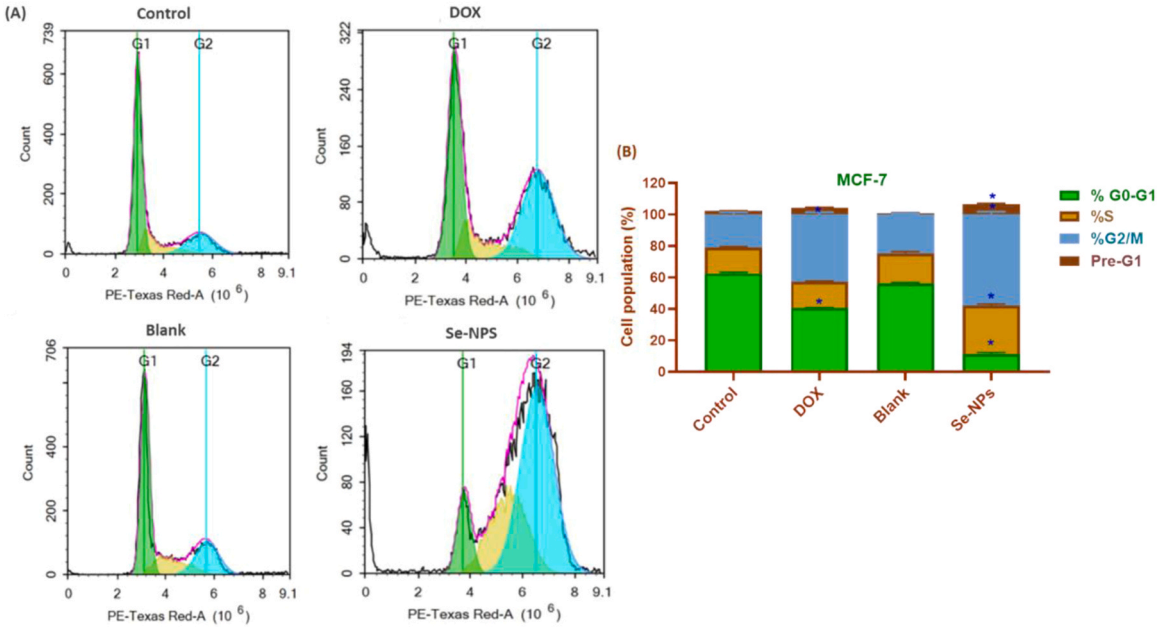

Fig. 8. (A) Cell cycle distribution using DNA cytometry analysis in MCF-7 cells after treatment with SeNPs compared with the standard chemotherapy (Doxorubicin) for48h. (B) Quantification of the percentage of cells in each phase of the cell cycle, including pre-G1, depicted as a bar graph of the mean proportion of total events ± SD; n = 3. * Statistically significant difference from the control untreated cells ($p < 0.05$).

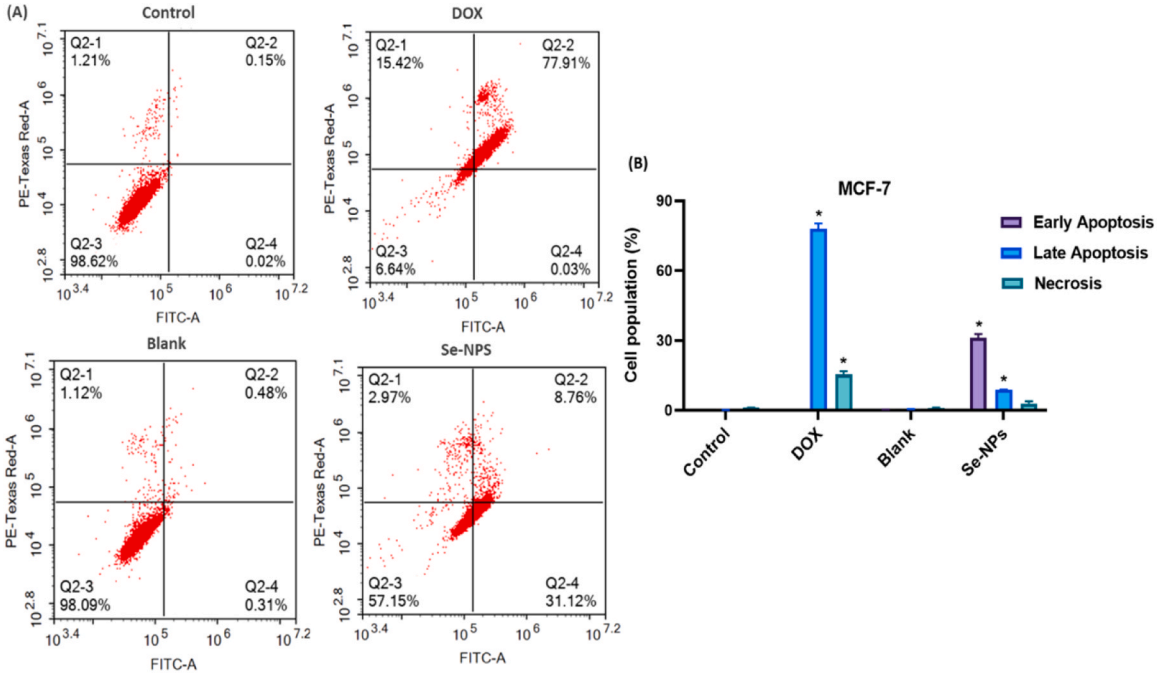

Fig. 9. (A) Evaluation of cell death modality (Apoptosis/necrosis) after treatment of MCF-7 cells with the SeNPs, blank formula, and Dox for 48 h followed by double staining with Annexin-FITC/PI. (B) Different cell populations (early apoptosis, late apoptosis, and necrosis) were plotted as a percentage of total events and presented as mean ± SD; n=3. * Statistically significant difference from the control ($p < 0.05$).

marine yeast as a possible anticancer drug was the goal of this work.

To accomplish this goal, marine yeast isolates were screened for their ability to biosynthesis SeNPs. The most potent isolate exhibited significant red color intensity and consequently a maximal SPR peak after 48 h of growth at 30°C. Based on 18 S rRNA gene sequencing, the marine yeast isolate was identified as *Candida pseudojiufengensis*. The UV-Vis spectra showed that the SPR corresponded to the SeNPs is at 550–600 nm with broad peak. The color change indicates the metabolic activity of selenium-exposed cells for bio-reduction of colorless selenium ions in SeO_2 to elemental zerovalent Se^0 through their reductive enzymatic systems. In a similar fashion, Musarrat et al., reported the potential of redox agents like ADH and ADH reductase of strain JS-11 for biomimetic reduction of SeO_3^{2-} to Se^0 nanospheres [14]. Thus, the color change indicates a positive biosynthesis of SeNPs. Similar color change from yellow to red-color upon exposure of isolate *Bacillus* sp. to 2 mM of SeO_2 was reported by Akçay and Avcı who determined the maximal absorbance of their biosynthesized SeNPs at 568 nm [4].

Utilization of agro-industrial by-products is worthy for such

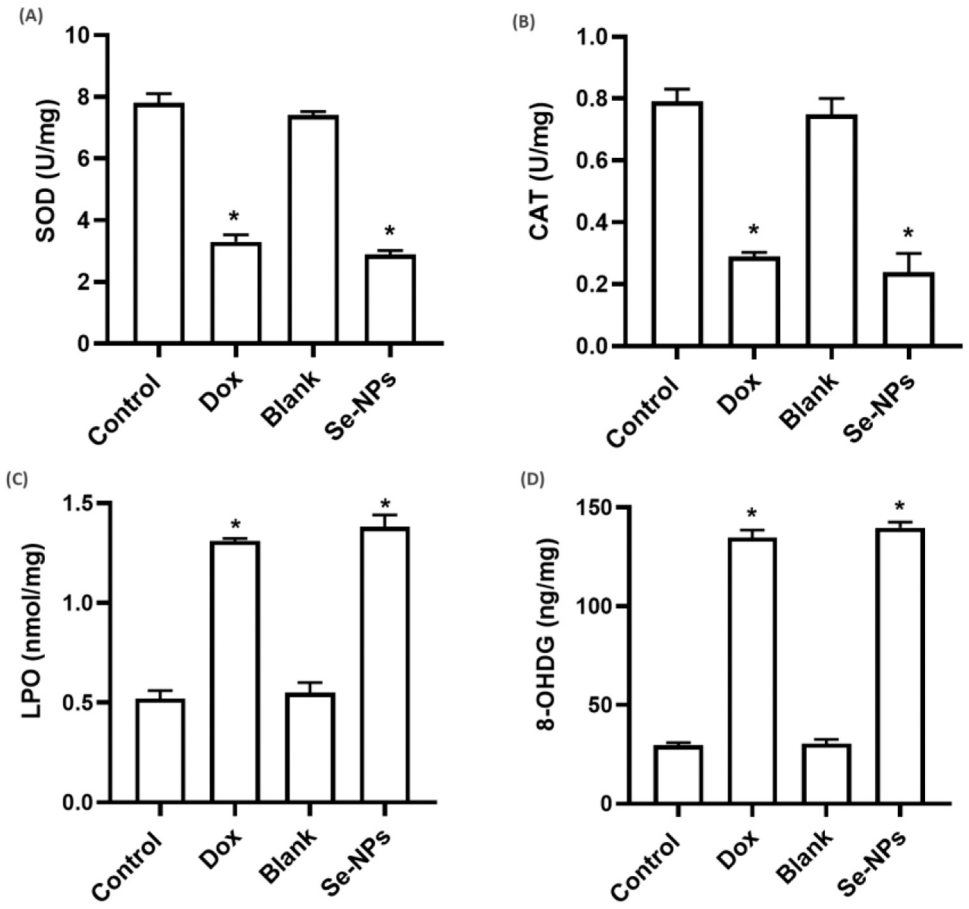

Fig. 10. MCF-7 cells were treated with SeNPs, DOX, and blank formula for 24 h, then antioxidants markers (A) SOD, (B) CAT, and oxidants markers (C) LPO, and (D) 8-OHDG were detected. Values represented as mean ± SD; n=3. * Statistically significant difference from the control ($p < 0.05$).

bioprocesses target economic production of valuable biomolecules. Sugarcane molasse proved to be the best substrate for biosynthesis of SeNPs by *C. pseudojiufengensis* exhibiting the highest SPR peak. This observation makes perfect sense with the rich composition of sugarcane molasses, regardless of their origin, since it contains more than 50 % sugars (Sucrose, glucose and fructose) [41]. Presence of such sugars with other natural biomolecules and plant secretions work as reducing, stabilizing and capping agents which required for nanoparticles fabrication [24,81]. Moreover, proteins, enzymes and other cofactors that have a redox potential are considered as electron shuttles useful in metal reduction [58].

Improvement of the biosynthesis conditions is the best strategy for maximum synthesis of SeNPs. Results showed that the optimal SeO_2 concentration used for SeNPs synthesis was 0.8 mM with higher SPR intensity and lower peak broadening. Similarly, Akçay and Avcı confirmed that the reduction of selenium ions to SeNPs by either fresh culture or cell-free extract is directly related to the SeO_2 concentration [4]. In this context, Mollania et al. successfully obtained a maximal nano-selenium by cell-free extract of *Enterobacter* sp. through a reduction process of up to 3 mM sodium selenite [46]. The obtained results in this study imply the importance of cell concentration on the biosynthesis of SeNPs. This was investigated by the SPR peak strength which rose proportionately with the cell concentration utilized for SeNPs production with maximum absorption peak achieved using a cell-free extract of a 90 ml culture. Similarly, higher cell densities of *Synechococcus leopoliensis* resulted in the production of more SeNPs relative to lower cell densities because the solution contained more reducing molecules at higher cell densities [57]. Also, Wadhwani et al. [83] reported biosynthesis of SeNPs increased with increase in cell densities; the obtained polygonal-shaped SeNPs (79 nm) using the supernatant of *Acinetobacter* sp. SW30 with the maximum synthesis using 4 mg/ml of total cell protein [83].

The data revealed that the optimal biosynthesis of SeNPs by *C. pseudojiufengensis* was achieved when the reaction mixture (cell-free extract with 0.8 mM of SeO_2) was incubated at 40°C and pH 7.0 for 48 h. The efficiency of selenium NPs biosynthesis is most likely related to both; the concentration of bacterial cells and incubation time [79]. Our results are similar to Kora, A.J. 2018 who found that the absorption bands intensities of SeNPs increased from 24 to 48 h and remained more or less same at 72 h at Se concentration (1 mM selenite) [34]. In addition, *Bacillus megaterium* strongly reduced selenite to SeNPs after 40 h of incubation [59]. Fernández-Llamosas et al., 2016 obtained spherical SeNPs after 24 h of incubation using cellular extract from *Azoarcus* sp. CIB [17]. Wang et al. observed an optimal synthesis of SeNPs from cell pellets of *Acinetobacter* at up to 40°C; while reduction of selenium ions was stopped above this value [85]. This points out the enzymatic nature of the reductive enzymes which show a functional thermal stability beyond 40°C. Vanaja et al. also reported higher reduction rate at high temperature due to the metal ions consumption in the formation of nuclei while a second reduction reaction occurs on the surface of these nuclei [80]. Numerous researches have examined the significance of pH and how it affects the size, shape, and makeup of chemically or biologically synthesized nanoparticles. The significant effect of pH on size and shape of the nanoparticles is due to the direct impact of the hydrogen concentration on the chemical and physical reactions between selenium ions and the functional groups of the reductive enzymes (such as carboxyl and hydroxyl groups) [5]. Lian, Shengyang, et al. reported highest SPR peak intensity at pH 7.0 with color change observation from

yellowish orange at pH 7.0 to dark red at pH 8.0 which has been attributed to big-size nano-seleium [38]. Neutral or weakly alkaline pH were also addressed by Akçay and Avcı for reduction of 6 mM SeO_2 to SeNPs by secretions of Bacillus sp. EKT1 [4].

Transmission electron microscopy (TEM), X-ray diffraction (XRD), UV-Vis spectroscopy, and dynamic light scattering (DLS) studies, including average particle size distribution and average zeta potential, were used to characterize the biosynthesized SeNPs. The findings demonstrated caused the biosynthesized SeNPs to exhibit a maximum absorbance peak in the UV-Vis spectrum at 560 nm due to the surface plasmon resonance. TEM image elevated spherical shape particles with average size of 12 nm and these observations are in a well agreement with another previous finding [65]. The SAED pattern observed a polycrystalline behavior of the SeNPs that is a convention pattern of biosynthesis nanoparticles that is in a good agreement with other literature [6]. Additionally, average zeta potential was recorded around 34 mV where this value is referred to high stability of biosynthesis SeNPs in colloidal solutions [55].

Moreover, the possible mechanisms of SeNPs as a promising therapeutic approach in MCF-7 cancer cells, relying on the observed growth inhibitory activity, have been evaluated. The anticancer activity of SeNPs was screened on the survival rates of HepG2, MCF-7 and HCT-116 cancer cells. Results revealed that SeNPs showed superior anticancer efficacy against MCF-7 cells compared to colon (HCT-116) and liver (HepG2) cancer cells as evidenced by lower IC50 values. The most prominent inhibitory effect of SeNPs was observed in the breast cancer cell line (MCF-7). The observed mild toxicity of the blank could be attributed to the composition of the used buffer. Interestingly, in non-cancerous HSF cells, we found that the IC50 values of both SeNPs and blank formula were much higher than 100 µg/ml. This was consistent with previous research demonstrating that SeNPs have high selective toxicity for cancer cells [86,39] while having minor side effects on normal cells [28,75]. SRB assay, flow cytometry, and other biochemical methods were employed to assess SeNPs anti-proliferative effects on MCF-7 cells. Moreover, treatment with SeNPs induces G2/M in breast cancer arrest along with triggering apoptosis in MCF-7 cells [49,10]. It appears that triggering apoptosis is the predominant cell death approach used by selenium nanoparticles in killing cancer cells [31,70]. ROS generation and antioxidant exhaustion are pivotal mechanisms underlying the apoptotic effect and cell cycle arrest of anticancer medicines on tumor cells [29,33,50]. Moreover, ROS plays an integral role in selenium nanoparticles' ability to kill tumor cells [42,60]. MCF-7 cells treated with SeNPs showed increased oxidative stress, as indicated by observable rises in LPO and 8-OHDG, accompanied by considerable exhaustion in antioxidant enzyme activity. These findings were consistent with the cytotoxicity results, demonstrating that the increase in ROS is one mechanism by which SeNPs exert anticancer activity against breast cancer cells. It can be, therefore, hypothesized that the unique biological uses of SeNPs with anticancer activity are due to their redox modulatory capabilities. Given that many current chemotherapy medicines, including doxorubicin, generate ROS as one of their modes of action [50]. Based on these potential biological impacts, the biosynthesized SeNPs by *C. pseudojiufengensis* have biomedical potential applications, especially as anticancer agent.

5. Conclusion

In the current study, marine yeast strain was isolated and identified as *Candida pseudojiufengensis* based on 18S rRNA gene sequencing. Results demonstrated the ability of *C. pseudojiufengensis* to biosynthesize spherical shaped SeNPs with average size around 12 nm. The biosynthesized SeNPs were characterized and confirmed by UV–Vis spectroscopy, X-ray diffraction (XRD), transmission electron microscopy (TEM) and zeta potential measurement. The biosynthesis process was affected by reaction conditions such as SeO_2 concentration, pH and temperature. Consequently, *C. pseudojiufengensis* can be exploited as a prospective nanofactory for the environmentally friendly biosynthesis of selenium nanoparticles. Further detailed studies on possible mechanisms of SeNPs as therapeutic anticancer agent were investigated. SeNPs showed superior anticancer efficacy against MCF-7 cells compared to other cancer cell lines. Moreover, treatment with SeNPs induces G2/M arrest along with triggering apoptosis in MCF-7 cells. Furthermore, MCF-7 cells treated with SeNPs showed increased oxidative stress. Based on these potential biological impacts, the biosynthesized SeNPs by *C. pseudojiufengensis* have enormous potential for use in pharmaceutical and biomedical applications especially as anticancer agent.

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CRediT authorship contribution statement

Basant A. Ali: Writing – original draft, Methodology. **Rasha Mosa Allam:** Writing – original draft, Resources, Formal analysis, Conceptualization. **Mohamed S. Hasanin:** Writing – review & editing, Methodology, Data curation. **Amany A. Hassabo:** Writing – original draft, Methodology, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

All authors conceptualized and planned the research. Experiments were done, and data were evaluated by all authors. The manuscript was written by BAA, RMA, MSH and AAH. The article was reviewed by all authors. All authors read and approved the final manuscript.

Declarations

The protocol for the study was approved by the Medical Research Ethics Committee of the National Research Center, Egypt with approval number: Ex-09421223. The manuscript does not contain clinical studies or patient data.

Consent for publication

Not applicable.

Data Availability

Data will be made available on request.

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