Research Article

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Potent antibacterial action of phycosynthesized selenium nanoparticles using *Spirulina platensis* extract

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Abstract: Selenium nanoparticles (SeNPs) are reinforced safe forms of the essential micronutrient selenium (Se) which take a lead in countless biotechnological and biomedical applications. The phycosynthesis of SeNPs was successfully investigated using cell-free extract of the microalgae, *Spirulina platensis*. The phycosynthesized *S. platensis*-SeNPs (SpSeNPs) were characterized using several characterization techniques such as UV-Visible, transmission electron microscopy, Fourier transform infrared spectroscopy, X-ray diffraction, and energy dispersive X-ray. They were effectively achieved using different concentration from sodium selenite (Na$_2$SeO$_3$) (1, 5, and 10 mM) to give size means of 12.64, 8.61, and 5.93 nm, respectively, with spherical shapes and highly negative zeta potentialities. The infrared analyses revealed the involvement of many phycochemicals in SpSeNPs production. The antibacterial properties of SpSeNPs were confirmed, qualitatively and quantitatively, against foodborne microorganisms (*Staphylococcus aureus* and *Salmonella typhimurium*); the antibacterial activity was correlated and increased with SeNPs’ size diminution. The scanning micrographs of *S. typhimurium* cells treated with SpSeNPs indicated the severe action of nanoparticles to destroy bacterial cells in time-dependent manners. The innovative facile phycosynthesis of SeNPs using *S. platensis* is recommended to generate effectual bioactive agents to control hazardous bacterial species.

Keywords: antimicrobial, green synthesis, microalgae extract, *Spirulina platensis*, selenium nanoparticles

1 Introduction

Multidrug-resistant bacteria (MDRB) are well acknowledged to be one of the most vital recent public health problems. The World Health Organization (WHO) identifies antimicrobial resistance (AMR) as “a threat for the effective prevention and treatment of infections” [1]. In this way, growing rates of MDRB have an impact on all responses to global-leading antibiotics such as methicillins, penicillins, and cephalosporins, which in turn cause the development of life-threatening diseases including sepsis, pneumonia, meningitis, and bacteremia [2]. Clinical effectiveness of the emerging drugs is vulnerable leading to even higher rates of MDRB, thus creating a serious predicament that prompted WHO to create an action plan in 2015 [3]. MDRB are showing AMR by drug uptake limitation, drug sites inactivation, and drug efflux [4]. According to Centers for Disease Control and Prevention, more than 2.8 million MDR infections occur in the USA each year, and more than 35,000 people die as a result [5]. Furthermore, WHO stated that if efficient drugs are not provided, the death caused by MDR infections may increase globally to ~10 million by 2050 [6].

Metallic nanoparticles (NPs) have a high potential to confront these predicaments and have been extensively exploited in different biomedical applications such as...
2 Materials and methods

All used materials/reagents were certified analytical grades; Na₂SeO₃ (≥90.0%), ethanol, Sterilized MilliQ Water (MQW), INT 95% (p-iodonitrotetrazolium violet), Nutrient broth (NB), and Nutrient agar (NA) were attained from Sigma-Aldrich (St. Louis, MO, USA).

2.1 Phycosynthesis of SpSeNPs

2.1.1 Collection of algal material and preparation of S. platensis extract

Dry powder of blue–green microalgae, S. platensis, was attained from the Research Algal Farm, Kafrelsheikh University, Egypt. Exactly, 5.0 g of S. platensis dried powder was extracted in 50 mL distilled water for 20 min at 80°C. The pasty extract was vacuum filtered and centrifuged (SIGMA, 2-16 KL Germany) at 6,430× g for 10 min [32]. The resulting extract was vacuum evaporated at 42°C, and then dried powder was used in further experiments.

2.1.2 SpSeNPs phycosynthesis

MQW was applied for experiments solution preparation. According to Gunti et al. [17], three glass vials containing 10 mL of 1, 5, and 10 mM of Na₂SeO₃ (Sigma-Aldrich) solutions were kept on magnetic stirrer for 30 min to study the effect of Na₂SeO₃ concentration on size and zeta potential of SpSeNPs. Drop-wise addition of freshly prepared S. platensis extract solution (2 mL, 1.0% concentration, w/v) was made in each vial. Mixtures were kept under stirrer for 72 h in dark conditions at 25 ± 2°C until the color of sodium selenite solution changed to orange-red, indicating the formation of phycosynthesized SpSeNPs. The pH value was measured for each vial (AD1200, pH meter, Adwaa, Romania).

2.2 Characterization of phycosynthesized SpSeNPs

2.2.1 Surface plasmon resonance (SPR) characteristic

The UV-Visible spectrum of SpSeNPs solution was recorded using a spectrophotometer (model UV-2450, Shimadzu, Japan). The absorbance was measured in the range 200–800 nm.

2.2.2 Fourier transform infrared spectroscopy (FTIR) analysis

Briefly, SpSeNPs solution was dried and ground into a homogeneous powder, and spectra were achieved at
2.2.5 X-ray micrographs were taken.

EDX spectroscopy

The elemental analysis of SpSeNPs was performed by EDX spectroscopy (JSM-IT100, JOEL, Japan).

2.2.6 Energy dispersive X-ray (EDX) analysis

The elemental analysis of SpSeNPs was performed by EDX spectroscopy (JSM-IT100, JOEL, Japan).

2.3 Antibacterial potentiality evaluation

The antibacterial potentialities of S. platensis extract and SpSeNPs were evaluated, qualitatively and quantitatively, against the challenged bacterial strains. Salmonella typhimurium (ATCC 14028) and Staphylococcus aureus (ATCC 25923) bacterial strains were used as challenged models. The cultures were propagated and examined in NB and NA media at 37 ± 1°C.

2.3.1 Qualitative assay: Inhibition zone (IZ)

The qualitative assay (using disc diffusion method) was mostly applied in dark to exclude the potential light effect on NPs activity. Bacterial cultures (24 h old) were spread onto NA plates, and then sterile discs (from Whatman No.1 filter paper, 6 mm in diameter) were loaded with 30 µL of S. platensis extract or SpSeNPs solutions (each with 100 µg/mL concentration) and positioned on the surfaces of the inoculants. After incubation (for 24 h at 37°C), the appeared IZ diameters were measured, and their triplicates mean was calculated. Clear zone of 25–40 mm, 15–25 mm, 10–15 mm, and <10 mm in diameter was classified as very strong inhibition, strong inhibition, moderate inhibition, and weak inhibition [33]. Ampicillin was used as a standard antibiotic disc for the comparative antibacterial analysis.

2.3.2 Quantitative assay: minimum inhibitory concentration (MIC)

The described microdilution technique [34] was used to determine the MICs of S. platensis extract and SpSeNPs against examined foodborne bacteria. In 96-well microplates, the bacterial cultures (~2 × 10^7 CFU/mL) were challenged with serial concentrations from examined agents (in the range of 1–200 µg/mL), then microplates were incubated as mentioned above, and the viability of cells was assessed using chromogenic indicator p-iiodonitrotetrazolium violet aqueous solution (4% w/v), which produces red-formazan color by active biological cells. Portions from wells containing inhibited cells were plated onto fresh NA plates and incubated to confirm the inhibitory action. The MIC was quantified as the least concentration that prevented bacterial growth in microplates and on NA plates.

2.3.3 Scanning electron microscopy (SEM) imaging

The SEM imaging was used to detect morphological alterations in S. typhimurium cells, after exposure to SpSeNPs, for potential elucidation of NPs action mode. The SEM (Hitachi S-500, Tokyo, Japan) bacterial imaging
was conducted using standardized protocol [35]. Grown bacterial cells in NB for 24 h were treated with SpSeNPs (100 μg/mL) for 0 (control), 6, and 12 h at 37°C, then bacterial cells were collected with centrifugation (4,500×g for 30 min), washed with saline buffer, re-centrifuged, and subjected to SEM preparation. Dehydrated samples were mounted onto SEM stubs and coated using gold/palladium, and then micrographs were captured.

2.4 Statistical analysis

Triplicated trials were performed and their mean values and standard deviation (SD) were calculated (using Microsoft Excel 2010). Statistical significance calculation at \( p \leq 0.05 \) was determined using one-way ANOVA using MedCalc software V. 18.2.1 (MedCalc, Mariakerke, Belgium).

3 Results and discussion

3.1 Phycosynthesis of SpSeNPs

Initially, \( \text{Na}_2\text{SeO}_3 \) solution was colorless. After addition of \( \text{S. platensis} \) cell-free extract, the reaction mixture possessed a pale green color. After 24 h, the color of the mixture turned into brownish-orange indicating initial reaction of sodium selenite with the extract. Gradually, the reaction mixture turned into orange-red color which was an indication for SpSeNPs formation (Figure 1). These observations were similar to those stated recently [36].

The cell-free extract was added to \( \text{Na}_2\text{SeO}_3 \) solution; after 72 h, the color changes from green to orange-red color indicating the reduction of \( \text{SeO}_3^{2-} \) into red \( \text{Se}^0 \), and it is suggested that the color change was because of the excitation of the SPR. Because of SPR, the reaction mixture color changed from green (before phycosynthesis) to orange-red (after phycosynthesis). Those findings are similar to the results of Faramarzi et al. [37]. They also reported that by SeNPs formation using \( \text{Saccharomyces cerevisiae} \) yeast, the mixture color converted from pale yellow to dark orange. Algal extracts consist of carbohydrates, proteins, minerals, oil, fats, and polyunsaturated fatty acids in addition to bioactive molecules like antioxidants (polyphenols, tocopherols), and pigments like carotenoids (carotene, xanthophyll), chlorophylls, and phycobilins (phycocyanin, phycoerythrin) [38]. From available reports, these potentially active compounds have been elucidated as reducing and stabilizing agents [24]. It has been recorded that \( \text{Spirulina} \) extracts contain potent biomolecules such as small peptides, proteins, alcohols, phenols, phycocyanins, esters, and amines, which can act as reducing and stabilizing agents [39]. These biomolecules, definitely, facilitated and participated in the reaction with \( \text{SeO}_3^{2-} \) to produce SpSeNPs, which is insoluble in water [18].

3.2 Characterization of phycosynthesized SpSeNPs

3.2.1 SPR characteristic

UV-Visible analysis was used to confirm the formation of SpSeNPs. SpSeNPs formation was visually recognized by color changing of the reaction mixture from colorless into orange-red. The absorption peak at 270 nm because of the SPR of SpSeNPs is shown in Figure 2. According to literature, because of SPR of SeNPs, they had a broad absorption peak \( \lambda_{\text{max}} \) at UV-Visible wavelength ranged from 270 to 400 nm [37,40].

3.2.2 FTIR analysis

According to Figure 3a, the cell-free extract of \( \text{S. platensis} \) showed strong transmission peaks at 3,441, 2,848, 2,930,
The peak at 3,441 was referred as O–H stretching vibration because of the presence of alcohol and phenol groups. The appeared peaks at 2,848 and 2,930 cm$^{-1}$ are due to the C–H stretching vibration of alkenes. The sharp peak at 1,650 and 1,541 cm$^{-1}$ was responsible for C=O stretching and N–O asymmetric stretching vibration of nitro compounds, respectively. The bands at around 1,408 and 1,450 cm$^{-1}$ are attributed to C–C stretch and methylene scissoring vibrations present in the proteins, respectively. Moreover, the C–N stretching vibration of aliphatic amines and the N–H stretching of the primary and secondary amines could be responsible for peaks at 1,081 and 1,242 cm$^{-1}$, respectively. Comparable results were formerly reported regarding these functional biochemical bonds [27].

According to Figure 3b, phycosynthesized SpSeNPs showed that the broad intense peak at 3,441 cm$^{-1}$ of cell-free extract of S. platensis was shifted to 3,421 cm$^{-1}$ of SpSeNPs, which suggested that Se has interacted with the hydroxyl group from aqueous cell-free extract of S. platensis through hydrogen bonding and facilitated phycosynthesis of SpSeNPs [17]. Likewise, the peak 1,450 cm$^{-1}$, which corresponds to methylene scissoring vibrations present in the proteins of cell-free extract, has disappeared in the phycosynthesized SpSeNPs, which imply that methylene scissoring vibrations present in the proteins have enabled the synthesis of SpSeNPs [52]. Similarly, the sharp peaks at 1,630 and 1,541 cm$^{-1}$ in the S. platensis extract, which were responsible for C=O stretching and N–O asymmetric stretching vibration of nitro compounds, were both shifted to higher frequencies 1,650 and 1,592 cm$^{-1}$ in phycosynthesized SpSeNPs, respectively, which shows the interaction of carbonyl C=O stretch and nitro compounds of cell-free extract of S. platensis with Se. Two strong absorption peaks of C–N stretching vibration of aliphatic amines and the N–H stretching of the primary and secondary amines at 1,081 and 1,242 cm$^{-1}$, respectively, are indicative of protein character of phycosynthesized SpSeNPs that may be responsible for reduction and stabilization [31]. From the FTIR, it can be indicated that the bio-organics like proteins, esters, amino acids, and carbonyl β-unsaturated ketone amides phycochemicals from S. platensis extract served as a strong capping and reducing agents on SpSeNPs [39].

### 3.2.3 Zeta potential (ζ)

ζ is not an actual measurement of the individual molecular surface charge; rather, it is a measurement of the electric double layer produced by the surrounding ions in solution. Typically, NPs with ζ values greater than +30 mV or less than −30 mV exhibit high degrees of stability because of the inter-particle electrostatic repulsion [48]. The photosynthesized NPs SpSeNPs were confirmed to be negatively charged, which indicates higher stability of the NPs without forming aggregates at pH range of 8.5 ± 0.5 for the three reactions. Particularly, increasing the sodium selenite concentrations increased the zeta potential values of the phycosynthesized SpSeNPs (Table 1). These
results indicate that increasing the precursor concentration leads to increase in \( \zeta \) values, which will eventually cause colloidal instability and irreversible aggregation \[49\]. Zeta values are also known to be influenced by several parameters including pH \[50\] and extract concentration \[51\]. The negative charge \( \zeta \) value could be measured because of the reducing agents of the Spirulina extract (e.g., phycocyanin), which reveals the existence of electrostatic forces with the phycosynthesized SpSeNPs \[47\]. The best nanocomposite (SpSeNPs-A) resulted from the evaluated Na\(_2\)SeO\(_3\) concentrations (Table 1), with the least particle size, was subjected for further experiments and characterization.

### 3.2.4 TEM imaging

In Figure 4, the NPs (SpSeNPs-A) are spherical, uniformly distributed, measured size from 2.8 to 38.9 nm, and approximately crystalline in nature. The stoichiometric ratio of the extract and metal precursor is a factor that affects the size and shape of phycosynthesized NPs. Therefore, the concentrations of sodium selenite precursor mainly affected the size of NPs \[44\].

Particularly, increasing the sodium selenite concentrations increased the particle size range of the phycosynthesized SpSeNPs (Table 1). This indicates that increasing the precursor concentration leads to increase in particle size. These results were coordinated with those mentioned by Kumar et al. \[42\]. Similarly, the particle sizes of AgNPs and AuNPs were found to be larger at higher metal ion concentrations \[43\], and that also lately confirmed \[44-46\]. Particle size is also known to be influenced by other parameters such as extract concentration. The sizes and shapes of SeNPs were dependent on the extract used, which may be attributed to the reduction potential of the extract. It also depends on the capping ability of the phycochemicals that exist in the cell-free extract of S. platensis \[47\].

### 3.2.5 XRD analysis

The XRD pattern revealed the formation of crystalline phycosynthesized SpSeNPs. The XRD spectrum showed strong diffraction peaks at 23.22° (100), 29.52° (101), 41.14° (110), 43.60° (102), 45.50° (111), 51.48° (201), 55.50° (202), 61.40° (112), 64.88° (210), and 71.40° (113), respectively (Figure 5). All the diffraction peaks in the 20 range correspond to the hexagonal structure of Se with lattice constants \( a = 4.357 \, \text{Å} \) and \( c = 4.945 \, \text{Å} \) and have good agreement with the standard JCPDS data (JCPDS No. 06-0362). However, some noise background was noticed, which may be attributable to the existence of bioactive compounds present in the S. platensis extract.

### 3.2.6 EDX analysis

Figure 6 shows the EDX of SpSeNPs which reveals the presence of 55.21%, 20.43%, 19.26%, and 5.19% for C, N, O, and Se, respectively. This indicates the phycosynthesis of SpSeNPs which contain other elements because of the presence of bioactive compounds of the S. platensis extract. However, Zhang et al. \[53\] attributed the increased proportion of C to the copper mesh of the electron microscope, which is a typical carbon support film.

### 3.3 Antibacterial potentiality evaluation

The antibacterial potentiality of the cell-free extract of S. platensis (S) and phycosynthesized SpSeNPs (SpSeNPs-A,

<table>
<thead>
<tr>
<th>Nanoparticles</th>
<th>Na(_2)SeO(_3) concentration (mM)</th>
<th>Size range (nm)</th>
<th>Median diameter (nm)</th>
<th>Mean diameter (nm)</th>
<th>Z-potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpSeNPs-A</td>
<td>1</td>
<td>2.8–38.9</td>
<td>9.7</td>
<td>5.93</td>
<td>−37.4</td>
</tr>
<tr>
<td>SpSeNPs-B</td>
<td>5</td>
<td>5.7–41.1</td>
<td>11.8</td>
<td>8.61</td>
<td>−36.1</td>
</tr>
<tr>
<td>SpSeNPs-C</td>
<td>10</td>
<td>8.2–50.8</td>
<td>14.2</td>
<td>12.64</td>
<td>−34.6</td>
</tr>
</tbody>
</table>
SpSeNPs-B, and SpSeNPs-C) was investigated toward *S. aureus* (Gram positive) and *S. typhimurium* (Gram negative) strains.

### 3.3.1 Qualitative assay: IZ

The results indicate that the smaller the mean diameter of NPs, the wider the diameter of IZ. Therefore, the highest antibacterial potentiality of SpSeNPs against the two strains (15.3 mm for *S. typhimurium* and 18.7 mm for *S. aureus*) was for SpSeNPs-A which possess the smallest particle mean diameter (Table 2). There is a robust relationship between the size of the NPs and their biological activity [45,54]. In the aspect of size-dependent antibacterial activity, the smaller-sized NPs have extra surface area that interact with the bacterial cell membrane, and thus have the greatest interaction with bacteria. This interaction may lead to the increase in penetrability of the outer membrane, which leads to the entry of NPs into cells and affects the cellular response [55]. The effect of many sizes of Ag NPs on the antibacterial activity against *E. coli* was studied [56]; the order of antibacterial activity of Ag NPs of diverse diameter was 10 > 20 > 70 nm.

Another example confirmed that smallest NPs exhibit the best antibacterial action against *E. coli* and *S. aureus* [57]; because of their size, they can simply reach the nuclear content of bacteria when compared to 29 and 89 nm. In addition, a 100 μg of SeNPs produced from cow urine extract showed a weak IZ of 8.4 ± 5.7 mm for *S. aureus* [58], whereas a 100 μL of SeNPs produced from *Allium sativum* extract showed strong IZ of 27 mm for *S. typhimurium* [59]. The IZs of SpSeNPs of the current study were wider than those reported by Fardsadegh and Jafarizadeh-Malmiri [60]. They used *Aloe vera* leaf extract to synthesize SeNPs which showed IZ of 10 mm against *S. aureus*. Moreover, the use of such low concentration of Na<sub>2</sub>SeO<sub>3</sub> was found to have no antibacterial effect which is
Ampicillin 8.2

Compared to the control SEM imaging

3.3.2 Quantitative assay: MIC

After challenging the bacteria with 1–200 µg/mL serial concentrations of SpSeNPs, the results indicate that the smaller the particle size of SpSeNPs, the smaller the MIC value (Table 2). This shows that SpSeNPs-A, which possessed the smallest particle size, can inhibit bacteria at a lower MIC (22.5 µg/mL for S. typhimurium and 17.5 µg/mL for S. aureus), whereas SpSeNPs-C which possess the largest particle size can inhibit bacteria at a higher concentration for the two strains (30.0 µg/mL for S. typhimurium and 27.5 µg/mL for S. aureus). These findings were in line with those recently mentioned [63]. In addition, it has been reported that the higher the SeNP concentration, the higher the inhibition effect on the various bacterial strains [64]. SeNPs produced from propolis extract inhibited S. aureus and S. typhimurium at MIC of 250 µg and 1,000 µg, respectively [36].

3.3.3 SEM imaging

Compared to the control (Figure 7a), SEM micrographs manifested that the treated bacterial cell walls of S. typhimurium had been deformed after 6 h of exposure to SpSeNPs-A (100 µg/mL); many cells were lysed and their internal constituents started to leak (Figure 7b). After 12 h of exposure, most of the treated S. typhimurium cells were lysed, and the few residual intact cells were observed in a pond of leaked internal constituents (Figure 7c).

Numerous reports suppose that Se may attach to the cell membrane surface disturbing penetrability and respiratory role of the cell. Probably, SeNPs not only interact with the surface of membranes but can also penetrate inside the bacteria [36,65].

The cell membrane damage of S. typhimurium was found to be because of increasing oxidative enzyme activities after treatment with SpSeNPs-A and therefore increasing generation of reactive oxygen species [66–68].

### Table 2: Antimicrobial potentialities of SpSeNPs phycosynthesized by S. platensis extract

<table>
<thead>
<tr>
<th>Examined agents</th>
<th>Antibacterial activity**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>S. typhimurium</td>
</tr>
<tr>
<td>IZ (mm)*</td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>8.2 ± 1.5</td>
</tr>
<tr>
<td>(S) extract</td>
<td>ND</td>
</tr>
<tr>
<td>SpSeNPs-A</td>
<td>15.3 ± 1.4b@</td>
</tr>
<tr>
<td>SpSeNPs-B</td>
<td>13.1 ± 1.1b@</td>
</tr>
<tr>
<td>SpSeNPs-C</td>
<td>12.7 ± 0.9b@</td>
</tr>
</tbody>
</table>

* Inhibition zones present means of triplicates ± standard deviation, including diameter of disc assay (6 mm) that loaded with 100 µg from nanoparticles, standard drug, and extract. ** Dissimilar superscript symbols (in the same row) and letters (in the same column) indicate significant difference (at p < 0.05).
activity is dependent on the time of exposure of bacterial cells to \( \text{SpSeNPs} \). Therefore, this research work has further recognized that environment-friendly phycosynthesis of \( \text{SpSeNPs} \) with enhanced phycochemical functionalities using \( \text{S. platensis} \) extract would be an economical and viable alternative to classical procedures.

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