Green Orange Peel-Mediated Bioinspired Synthesis of Nanoselenium and Its Antibacterial Activity against Methicillin-Resistant Staphylococcus aureus

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ABSTRACT: In this study, green orange peel (GOP) was feasibly evidenced in preparing selenium nanoparticles (SeNPs). Acting as reducing agents, polyphenolic compounds were extracted from GOP at the optimal extraction conditions (at 70 °C for 1.5 h, mass ratio of dried orange peel/distilled water of 5/100). The formation of SeNPs was observed at the wavelength range of 250−300 nm by ultraviolet−visible spectroscopy (UV−vis), and their highest yield could be reached at the following conditions: volume ratio of extract/selenious acid solution (V_{Ext}/V_{Sa}) of 40/10, synthesis duration of 4 h, selenious acid concentration (C_{Sa}) of 80 mM, and reaction temperature of 120 °C. The highly crystalline structure of SeNPs in the hexagonal phase was characterized by powder X-ray diffraction (XRD) with a lattice parameter of 4.3 Å; meanwhile, their spheres with an average crystal size of 18.3 nm were estimated by high-resolution transmission electron microscope (HR-TEM). The rationale of bioreducing agents extracted from green orange peel for the formation of SeNPs was also recognized by Fourier-transform infrared spectroscopy (FT-IR). The antibacterial investigation of the SeNP sample was assessed against antibiotic-resistant bacteria, typically methicillin-resistant Staphylococcus aureus (MRSA), by executing the zone of inhibition and the minimum inhibitory concentration (MIC) tests. The SeNP sample demonstrated excellent antibacterial activity with an average diameter of inhibition zones of 20.0 ± 0.7 mm and an MIC of 4.94 μg/L. A comparison of the physicochemical properties of SeNPs synthesized from GOP extract by the hydrothermal method with SeNP products from other green reducing agents and other methods as well as its antibacterial activity compared with other nanoparticles and some antibiotics was conducted to highlight the superiority of GOP-mediated green-synthesized SeNPs.

INTRODUCTION

Selenium is a practically useful element in the medical, antibacterial, antifungal, electronic, ceramic, metallurgical, and glass industries due to its unique chemical and physical properties.1−3 Compared with traditional selenium compounds, nanoselenium has more unique properties, higher activity, and lower toxicity.4 Especially, selenium nanoparticles (SeNPs) are investigated as new antibacterial agents since they are comparable in efficacy and even more powerful compared with conventional antibiotics.5,6 Besides, much fewer new antibiotics have been introduced by the pharmaceutical industry, and none of them have enhanced activity against multiresistant bacteria. Methicillin-resistant Staphylococcus aureus (MRSA) is an antibiotic-resistant kind of Staphylococcus aureus that is typically resistant to beta-lactam antibiotics such as cephalosporin and penicillin (oxacillin and methicillin).7 Various antibiotics, such as co-trimoxazole, fusidic acid, clindamycin, and mupirocin, are also utilized as a second-line option in treating MRSA.8 But, these antibiotics can only be prescribed when there is no other alternative available because of the resistance risk. Alternative treatments against MRSA without the use of different antibiotic classes are extremely required. So, SeNPs are considered an effective solution for inhibiting drug-resistant bacteria. Therefore, the synthetic strategies and biological effects of SeNPs have been constantly explored for antibacterial purposes.

In general, the SeNP preparation focuses on the physical method, chemical reduction, and biosynthesis.9,10 In the
physical method, mechanical measures are usually related to extrusion, friction, ultrasound, impact, shear on solid raw materials, or sublimation condensation is assumed to change the intermolecular force of selenium to obtain SeNPs. In spite of the fast and straightforward approach, it has strict requirements for equipment conditions, the obtained SeNPs are of low purity, and their crystalline particle sizes are poorly controlled. In the chemical reduction, external reducing agents were added to the seleniumic acid solutions or sodium selenite to deliver SeNPs, such as ferrous iron, hydroquinone, sodium thiosulfate, hydrazine, silk fibroin, ascorbic acid, glucose, and polysaccharides. Nevertheless, this method requires harsh conditions and multiple steps involved in the preparation. Besides, the reducing agent, stabilizer, and template agent used in the synthesis are frequently harmful, as well as the secondary pollutants generated from chemical reactions.

In recent years, eco-friendly and nontoxic approaches have been studied for SeNP biosynthesis using various microorganisms such as yeast, bacteria, and fungi as reducing agents. The size, morphology, and properties of SeNPs can be controlled by the incubation reaction duration, temperature, metallic ion concentration, initial pH solution, and the type of used microorganism. Practically, SeNP biosynthesis was performed on different bacteria such as Lactobacillus acidophilus, Bacillus subtilis, and probiotic lactic acid bacteria. Besides, plant extracts derived from leaves, roots, fruits, and peels containing polyphenols, flavonoids, ascorbic acid, citric acids, carotenoids, etc., were also utilized as natural reducing and stabilizing agents for SeNP biosynthesis, such as broccoli, fenugreek extract, parsley leaf, dried Vitis vinifera, Terminalia arjuna leaves, Clausena dentata, lemon juice, and Aloe vera leaf.

Green orange is one of the world’s largest fruit crops, with a global production of millions of tons. A large portion of this production is used to extract citrus juice, which leads to vast amounts of residues, including peel and segment membranes. Peels represent between 40 and 50% of the total weight of the fruits and remain as the primary byproduct. This byproduct is rich in polyphenolics, bioflavonoids, proteins, etc., that have been evidenced as reducing and stabilizing agents for nanomaterial synthesis. In particular, polyphenolic compounds have been paid more and more attention due to their important biological activities, such as antibacterial, antioxidant, and anti-inflammatory activities. Therefore, it is necessary to optimize the extraction conditions, obtaining the highest polyphenol content in the green orange peel (GOP) to enhance the efficiency of the SeNP biosynthesis, which has been less explored.

In this study, SeNPs were synthesized by reducing the Se⁴⁺ ion from a selenious acid solution using GOP extract as a reducing agent. The effect of conditions of GOP extraction, including duration and temperature, on the obtained polyphenol concentration was investigated. The operation parameters on the SeNP formation and its antibacterial activity against MRSA were also evaluated.

### EXPERIMENTAL SECTION

**Synthesis of Selenium Nanoparticles.** After washing and shredding, GOP was dried to a constant weight at a temperature of 60 °C for 12 h. Then, 50 g of dried GOP was blended with 1 L of distilled water and heated to different temperature levels (60–80 °C) for various durations (1–3 h) under stirring. Finally, the orange peel extract was filtered and kept at 4 °C for further experiments. Selenious acid (H₂SeO₃ >99.9%), gallic acid (C₆H₅(OH)₂COOH, >97.5%), and the Folin–Ciocalteu phenol reagent were purchased from Merck.

The polyphenol concentration in the GOP extract was determined by the colorimetric method using the Folin–Ciocalteu reagent with gallic acid selected as the calibration standard. The presence of polyphenol in the solution was determined by a UV–vis spectrophotometer (UV-1800, Shimadzu) at a wavelength of 760 nm.

To synthesize SeNPs, V_ext (mL) of the GOP extract was mixed with V_se (mL) of H₂SeO₃ solution with C_se (mM) under stirring at 300 rpm for 30 min at room temperature to deliver a homogeneous solution. After stirring, the solution was transferred to a Teflon autoclave for hydrothermal treatment at T °C for t h. Effects of the volume ratio of extract/seleniumic acid solution (V_ext/V_se), the seleniumic acid concentration (C_se), the duration time (t), and the synthesis temperature (T) on the formation of SeNPs were investigated to determine the best conditions.

**Characterization of Selenium Nanoparticles.** A UV–vis spectrometer (UV-1800, Shimadzu) was used to observe SeNP formation at 200–800 nm. The samples were diluted five times before being analyzed. The crystalline structure of the SeNP powder was determined by XRD analysis using a Bruker D2 Phaser powder diffractometer with Cu Kα radiation of 1.5406 nm wavelength at 40 kV and 30 mA in the range of 2θ angles 10–80° and a step size of 0.02°/s. The functional groups in the GOP extract on SeNPs were explored by FT-IR spectroscopy using an active Tensor 27-Bruker spectrometer with a scanning angle from 400 to 4000 cm⁻¹. The morphology and nanosize of SeNP samples were estimated by HR-TEM analysis on a JEOL JEM2100 instrument. The element analysis of SeNP samples was performed by energy-dispersive X-ray (EDX) spectroscopy on a Horiba-7593 instrument.

**Antibacterial Activity.** The agar disk diffusion method was employed to determine a zone of inhibition against MRSA. The agar medium after autoclaving and drying was inoculated with liquid overnight MRSA culture to a cell density of 5 × 10⁸ CFU/mL. Once the agar was solidified, the wells were punched in the agar and filled with 10 μL of the SeNP solution. The plates then were photographed, and the inhibition zone diameter was measured after incubation for 24 h at 37 °C. All the investigations were run in triplicate, and the average result was taken.

The MIC value of the SeNP samples against MRSA was determined using the dilution method. To evaluate the MIC, various concentrations (N/2, N/4, N/8, N/16, N/32, N/64, N/128, N/256, N/512, N/1024, and N/2048, with N being the initial concentration, N = 1.263 g/L) of the SeNP solution were tested against MRSA. Then, 10 μL of MRSA was added to different plates containing different concentrations of the SeNP solutions mentioned above, and the plates were incubated at 37 °C for 24 h. The lowest concentration inhibiting visual growth of MRSA, indicated by increased turbidity, is considered the MIC value.

### RESULTS AND DISCUSSION

**The Suitable Extraction of Green Orange Peel.** The effects of temperature and duration on the polyphenol yield were examined, showing the highest polyphenol yield of 13.5 ±
0.2 mg/g extracted at 70 °C for 1.5 h using the mass ratio of dried orange peel/distilled water of 5/100. At lower temperatures and shorter times, polyphenolic compounds were not absolutely extracted. Theoretically, plant tissues are softened, and weak interactions affect cell membranes under high temperatures; thus, polyphenol compounds can be easily extracted into water. However, if the process takes place at high temperatures for too long, these compounds are susceptible to chemical changes due to their unstable nature, leading to decreasing process efficiency. Therefore, the extraction conditions to obtain polyphenols were determined as 70 °C for 1.5 h.

The Green Synthesis of Selenium Nanoparticles. The influence of the operation parameters on the SeNP synthesis yield was investigated by UV−vis spectral analysis (Figure 1), showing the SeNP formation observed in the wavelength range of 250−300 nm. When the $V_{\text{Ext}}/V_{\text{Se}}$ ratio increased from 30/20 to 35/15 and 40/10, the yield of SeNP formation increased; however, the absorbance of SeNPs decreased when this ratio increased up to 45/5 (Figure 1a). As analyzed above, polyphenols contained in the green peel extract played an important role in reducing Se$^{4+}$ toward Se$^0$. Therefore, an increase of the extract fraction in agreement with an increase of polyphenols facilitated the reduction of metal ions, achieving a higher efficiency of SeNP formation. However, when this ratio increased to a certain level (45/5), the selenium concentration in the survey sample dropped, resulting in a decrease in the SeNP formation efficiency. Therefore, the $V_{\text{Ext}}/V_{\text{Se}}$ ratio was chosen to be 40/10 for the subsequent investigations.

When the initial Se$^{4+}$ concentration ($C_{\text{Se}}$) increased from 40 to 80 mM, the SeNP formation efficiency increased. At higher concentrations (100 and 120 mM), the intensity of SeNP plasmon sharply decreased (Figure 1b). At low concentrations, metal nanoparticles appeared with very low intensity; when increasing Se$^{4+}$ concentration, the amount of SeNPs formed also increased due to the increase of Se active sites. However, a higher Se$^{4+}$ concentration can lead to larger-sized particles due to their agglomeration and thus a decrease in process efficiency. When increasing the synthesis time from 2 to 4 h, the SeNP formation increased; after 4 h, the maximum absorbance of the SeNP solution insignificantly changed (Figure 1c). Up to 6 h, the SeNP formation efficiency decreased, and the plasmon band shifted to a higher wavelength region attributed to the agglomeration of SeNPs toward larger particles. Therefore, the synthesis time of 4 h should be chosen for further conditions. The biosynthesis of selenium nanoparticles was investigated at different temperatures (90−130 °C) (Figure 1d), indicating the suitable temperature of 120 °C with the highest absorbance intensity and lowest absorbance wavelength.

From the UV−vis spectral analysis, the suitable conditions for SeNP biosynthesis using orange peel extract as a reducing agent were determined, as follows: synthesis duration of 4 h, $V_{\text{Ext}}/V_{\text{Se}}$ ratio of 40/10, Se$^{4+}$ concentration of 80 mM, and reaction temperature of 120 °C.

The Characteristics of Selenium Nanoparticles. XRD analysis was performed to ensure the crystalline nature of the as-synthesized SeNPs at the optimized conditions (Figure 2a). The diffraction peaks at 2θ = 23.8, 29.5, 41.1, 45.1, 46.5, 48.0, 57.1, 61.1, 64.5, 65.7, 72.3, and 76.7° were recorded, corresponding to the (100), (101), (110), (102), (111), (200), (112), (202), (210), (211), (113), and (301) planes in the hexagonal phase of selenium crystals, being perfectly consistent with the pure phase of selenium (JCPDS Card No. 86-2246). Besides, a broad peak in the range of 20−30° was also detected, indicating the presence of carbon, related to...
phytochemical components of the orange peel extract (such as polyphenols, flavonoids, etc.), which are responsible for the reduction and stabilization of the formed SeNPs. The lattice parameter of as-prepared SeNPs was estimated via interplanar spacing (d-spacing) of the (101) plane at $2\theta = 29.5^\circ$, as follows:

$$
\alpha = d\sqrt{h^2 + k^2 + l^2}
$$

(1)

where $\alpha$ is a lattice parameter [Å]; $d$ is d-spacing [Å]; and $h$, $k$, and $l$ are the Miller indices of the (101) crystal plane. A d-spacing value of 3.1 Å and a lattice parameter of 4.3 Å for the Miller indices corresponding to (101) plane matched well with the standard lattice parameter ($\alpha = 4.36$ Å). The average

Figure 2. (a) XRD pattern, (b) FT-IR spectrum, (c) size distribution, (d) HR-TEM image, and (e) EDS spectrum of selenium nanoparticles synthesized at the optimized conditions.

Figure 3. The synthesis mechanism of SeNPs using the GOP extract as a reducing agent.
crystal size of SeNPs calculated by the Debye–Scherrer formula at $2\theta = 29.5^\circ$ of the (101) plane is about 18.9 nm.

Figure 2b shows the FT-IR spectrum of SeNPs synthesized at the optimal condition using the GOP extract as a reducing agent. The absorbent peak at 1380 cm$^{-1}$ was related to the stretching vibrations of the O–H groups. The absorption peak at 2925 cm$^{-1}$ was attributed to the C–H stretching of the aromatic compounds of the orange peel extract, the strong band at 1625 cm$^{-1}$ corresponded to the stretching vibrations of the C=C aromatic bond of phenolic groups, and the peak at 3081 cm$^{-1}$ was related to the bending vibrations of the CH$_2$ groups. The results confirmed the existence of polyphenols and flavonoid compounds in phytochemical compositions of the GOP extract. The presence of SeNPs was confirmed by the stretching vibrations of the terminal Se–O and O–Se–O groups at 1075 and 540 cm$^{-1}$, respectively. The mechanism by which phytochemical compositions performed their reduction function to form SeNPs is described in Figure 3. A single selenium atom is tiny, but its surface free energy is very high. The aggregation of multiple selenium atoms can significantly reduce the surface free energy and become stable after the formation of particles. Besides, polyphenol groups of the GOP have a strong steric effect on selenium and selenium ions; the biomolecules wrap around the surface of SeNPs and play an important role in their dispersion and protection. As the essence of protection, the encapsulation of phytochemicals reduces the surface free energy of SeNPs; the SeNPs become stable, preventing their agglomeration. The particle size distribution of SeNPs synthesized under optimal conditions is presented in Figure 2c, showing a range of 5–40 nm with the highest average intensity at 18.3 nm.

To study the morphology and size of the as-prepared SeNPs, the HR-TEM image is shown in Figure 2d, evidencing the formation of crystalline SeNPs with a spherical shape in the size range of 10–20 nm. Besides, a thin layer of biomolecules in the GOP extract around SeNPs could be observed, taking the role of preventing their agglomeration and enhancing their stability. Furthermore, SeNPs exhibited three crystal planes, including (101), (100), and (102) with d-spacing of 0.303, 0.290, and 0.300 nm, respectively. These results are completely consistent with the above analysis.

The EDS result (Figure 2e) revealed the presence of Se, C, O, and Cu in the SeNP samples with high intensities. The appearance of the O and C signals could be contributed by phytochemicals in the GOP extract covering the surface of SeNPs. Also, the C and Cu peaks could be related to the use of the carbon tape as a substrate. The mass percentages of Se, C, O, and Cu elements in SeNP samples were 59.6, 14.4, 1.4, and 24.4%, respectively.

The average particle size of as-prepared SeNPs using the GOP extract as a reducing agent was compared to those of other studies with similar protocols (Table 1). In the previous reports, various plants with their respective portions have been exploited, showing polydisperse SeNPs with wide ranges of variation. Compared with other green reducing agents and precursors, the SeNP sample synthesized from the GOP extract and H$_2$SeO$_3$ by the hydrothermal method also has a spherical shape, but the synthesis duration is shortened significantly, and at the same time, it has smaller size and higher uniformity.

Antibacterial Activity against MRSA. Figure 4 shows the inhibition zone of the as-synthesized SeNP sample against MRSA bacteria. It can be observed that there is almost no difference in inhibition diameter between the three investigations. The average inhibition zone diameter of the SeNP

| origin of reducing agents | selenium precursor | synthesis conditions | particle size, nm | refs |
|---------------------------|--------------------|---------------------|------------------|-----|
| green orange peel         | H$_2$SeO$_3$       | hydrothermal at 120 $^\circ$C for 4 h | 5–40 | this work |
| Diospyros montana         |                    | incubated at RT for 24 h | 4–16 | 28 |
| Vitis vinifera            |                    | refluxed for 15 min | 3–18 | 32 |
| Allium sativum            |                    | incubated at RT for 48 h | 205 | 45 |
| Capsicum annum            |                    | stirred at RT for 15 h | 200–500 | 46 |
| Acacia senegal            |                    | stirred at RT for 6 h | 34.9 | 47 |
| Brassica                  |                    | stirred at RT for 12 h | 50–150 | 48 |
| orange peel               | Na$_2$SeO$_3$      | incubated at RT for 3 h | 16–95 | 49 |
| glucose                   |                    | incubated at 90 $^\circ$C for 12 h | 32.3 ± 5.6 | 6 |
| bee propolis              |                    | stirred at RT for 24 h | 52–118 | 50 |
| Aloe vera                 |                    | shaked in the dark for 72 h | 7–48 | 51 |
| Terminalia arjuna         |                    | incubated at 30 $^\circ$C for 72 h in dark conditions | 10–80 | 52 |
| Undaria pinnatifida        |                    | sonication condition for 5 min | 44–94 | 53 |
| Citrus limon              |                    | incubated at 30 $^\circ$C for 24 h in the dark | 60–80 | 54 |
| Terminalia                |                    | shaked at 30 $^\circ$C for 72 h in the dark | 10–80 | 52 |
| Citrus reticulata         |                    | stirred at 40 $^\circ$C | 70 | 55 |
| Zingiber officinale       |                    | shaked at 30 $^\circ$C for 72 h in dark conditions | 10–20 | 56 |
| parsley                   | NaHSeO$_3$         | stayed overnight at RT | 50–100 | 31 |
| Bacillus licheniformis     | SeO$_3$            | incubated at 37 $^\circ$C for 48 h | 10–50 | 57 |
| Aspergillus oryzae and gamma rays |     | incubated at RT for 24 h | 55.0 | 58 |
| chitosan and              |                    | stirred at RT (240 ± 2 $^\circ$C) for 20 min | 27.35 | 30 |
| Pleurotus ostreatus       |                    |                          |                  |     |

Note: RT, room temperature.
Sample against MRSA bacteria was 20.0 ± 0.7 mm. For the MIC test (Figure 5), the delayed exponential phase of MRSA

in the presence of the SeNP solution was observed, and this phenomenon was more prominent with the growth in the SeNP concentration. The SeNP solution could inhibit the exponential phase of bacteria and completely inhibit the MRSA bacteria at an MIC of $N=256$ (4.94 μg/L).

The SeNP colloid synthesized from GOP has quite high antibacterial activity against MRSA compared to the metallic nanoparticles decorated on supports, the colloids of AgNPs and CuNPs, as well as the extract of Quercus infectoria Olivier nutgalls and leaves, leaves of Melianthus comosus Vahl, Nymphaea lotus Linn., and Dodonaea angustifolia (L.f.) Benth (shown in Table 2). Besides that, when the antibacterial zone sizes of the SeNPs and the antibiotics are compared, it is noticeable that the zone size of the SeNP sample is larger than that of the antibiotics including clindamycin, erythromycin, and ciprofloxacin and approximately equal to that of linezolid and gentamicin (as shown in Table 2). But the MIC value of SeNPs green-synthesized using the GOP extract is much lower than that of all five antibiotics. In contrast to commercial antibiotics, nanoparticles may be described by their primary benefits as antibacterial agents since they can operate multiple mechanisms, while bacteria cannot gain resistance to these indicated action methods.69 Furthermore, these SeNPs demonstrated minimal or limited cytotoxicity against human dermal fibroblast, easing some of the safety concerns connected with the manufacturing procedure.60 As a result, SeNPs appear to be trustworthy candidates for safe medicinal uses to prevent MRSA development.60

The antibacterial mechanism of SeNPs against MRSA is illustrated in Figure 6, including the direct adhesion of SeNPs to the bacterial surface and the effect on the structural integrity of the membrane.73,74 Then, SeNPs enter the bacterial cell and interact with its internal components, causing it to become damaged to the point where it can no longer execute important cellular operations.75 SeNPs could also generate reactive oxygen species and free radicals, causing irreversible oxidative damage to bacteria.76 SeNPs impact critical signaling pathways, which are essential for the bacterial life cycle.

The attachment of SeNPs to the membrane drastically changes both the membrane’s permeability and structural integrity.74 Electrostatic forces are created when positively charged SeNPs interface with negatively charged bacterial cell membranes.78 The presence of the amino, carboxyl, and phosphatase groups contribute significantly to explain why the membrane is negatively charged.79 Several experiments on bacteria using transmission electron microscopes have shown that SeNPs permeate the cells, supporting this claim.80,81 Furthermore, in the presence of oxygen and proton, SeNPs produce a high reactive oxygen species, which can result in DNA denaturation and shearing, translation process to cease.82 The interaction renders the bacteria incapable of cell division and reproduction, ultimately leading to death.

The reactive oxygen species (ROS) are also the perpetrators of bacterial growth suppression.80 SeNPs produce a high quantity of ROS, causing oxidative stress in the cell.80 The destruction of major cellular components such as proteins, DNA, and ribonucleic acid (RNA) caused by oxidative stress results in altered membrane permeability and increased biological component leakage from the cell.80 The bacteria will suffer permanent oxidative damage and cell death as a

![Figure 5. The minimum inhibitory concentration (MIC) of the synthesized SeNP solution against MRSA ($N=1.263$ g/L).](https://doi.org/10.1021/acsomega.2c05469)
SeNPs are thought to be effective against MRSA because they demonstrate several modes of action on bacteria.

**CONCLUSIONS**

Green nanotechnology is gaining importance, eliminating harmful reagents and providing cost-effective protocols of expected products. This study established a rapid, simple, inexpensive, and eco-friendly approach to produce SeNPs using the GOP extract as reducing and stabilizing agents (byproduct of the orange manufacturing industry). At the optimal conditions, the as-prepared SeNPs were found to have a highly crystallized spherical shape and an average size of 18.3 nm, with high stability thanks to the encapsulation of phytochemicals in the green orange peel extract and the prevention of agglomeration. The wide inhibition zone and low MIC values against MRSA of SeNP samples indicated their good antibacterial activity. Green-synthesized SeNPs in this work could be a promising approach for the treatment of diseases caused by drug-resistant strains.

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**Notes**

The authors declare no competing financial interest.

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