Research Article

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Phytosynthesis of selenium nanoparticles using the costus extract for bactericidal application against foodborne pathogens

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Abstract: Selenium (Se) as a bioactive micronutrient could be augmented via transforming into nanoparticles (NPs), especially using biogenic protocols, for usage as an antimicrobial element. The reducing power of costus (*Saussurea costus*) root extract (SCE) was employed for phytosynthesis of Se-NPs through a simple and rapid protocol that included stirred mixing of 10 mM Na₂SeO₃ with 1.0% SCE solution for 4 h. The phytosynthesized SCE/Se-NP composite was obtained with a mean diameter of 6.13 nm and a zeta potential of −42.8 mV. Infrared analyses revealed the involvement of many SCE phytochemicals in Se-NP synthesis, whereas transmission microscopy displayed well distribution and spherical shapes of the phytosynthesized NPs. The antibacterial assessments against foodborne pathogens (*Escherichia coli*, *Salmonella typhimurium* and *Staphylococcus aureus*) revealed the superior powers of SCE/Se-NPs and the elevated potentialities of SCE and Se-NPs for inhibition of bacterial pathogens. The scanning micrographs indicated that SCE/Se-NPs were attached to bacterial cells and led to their complete lysis/explosion with exposure prolongation. The SCE/Se-NP composites are recommended for the effective control of

foodborne bacterial pathogens, applying a simple and eco-friendly phytosynthesis protocol.

Keywords: antimicrobial, green synthesis, mode of action, *Saussurea costus*, Se-NPs

1 Introduction

The ongoing scenario of the spread of foodborne outbreaks due to pathogenic bacteria, and the emergence of multiple antibiotic-resistance strains from these pathogens, enforced the search for effectual alternatives from novel sources to solve these obstacles [1]. The most promising agents for employment as antimicrobial alternatives are the plant extracts [2] and metal nanoparticles (NPs) [3]. Nanomaterials, including nanometals and their based drugs, were proposed as highly auspicious means for overcoming microbial infections and contamination endangering human health and food sectors [4,5]. The inorganic NPs and their based drugs (e.g., titanium dioxide, zinc oxide, silver, gold, copper and selenium) were verified as powerful antimicrobial agents toward numerous groups of zoonotic and foodborne pathogens; these NPs were deemed to be appropriate effectual antimicrobials' substitutes because of their extreme effectiveness at diminutive levels [3].

Selenium (Se), the important micronutrient found in human and animal bodies, is required to be acquired at ~40 µg/day with a maximum daily intake of ~300 µg [3]. The extreme daily intake of Se (administration of 3,200–6,700 µg) could cause toxicity in humans, but the extra loads of Se are normally excreted by urination [6].

Numerous outstanding biological features could be attained by Se through nanotechnology achievements, such as antitumor, antibacterial, antioxidant and antiviral characteristics [7].

Selenium nanoparticles (Se-NPs) were evidenced to possess extra bioactivities and functionalities as potent antioxidant, antibacterial, antifungal and anticancer agents [8–13].

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The biosynthesis of metal NPs, and specifically Se-NPs, was efficaciously achieved using numerous biological reducing agents, including plant extracts, resins and microbial metabolites [13–18]. Compared to physicochemical protocols of NP synthesis, biological green synthesis of Se-NPs has elevated eco-friendly, non-toxic and biosafety advantages [10]. From the biological green synthesis agents, the employment of plant extracts in NP synthesis (phytosynthesis) is reported to be a simple, effectual and cost-effective method, compared to other biosynthesis agents involving microorganisms and enzymes [11].

Costus, *Saussurea costus* Falc. Lipschitz (syn. *Saussurea lappa* C. B. Clarke), is an important identified medicinal plant that was broadly applied in many ethnic medicines to treat various disorders, e.g., inflammatory diseases, asthma, ulcers, stomach problems and microbial infections [19,20]. Sesquiterpene lactones were the main phytoconstituents in *Saussurea costus* root extract (SCE); this phytochemical group possessed various pharmacological activities (anticancer, anti-inflammatory, antiulcer and hepatoprotective actions), which supported the rationales for SCE traditional uses [21]. Many bioactive constituents, exclusively found in the SCE, were isolated and identified (including costunolide, dehydrocostus lactone, costic acid, β -sitosterol, α -cyclocostunolide, alantolactone, β -cyclocostunolide, isoolantolactone, 12-methoxydihydrodehydrocostus lactone, 4 β -ethoxydehydrocostus lactone, saussure aldehyde, isodehydrocostus-lactone-15-aldehyde lactones and saussurea) [21–23]. Costunolide, the major sesquiterpene lactone constituent in the SCE, possessed effectual anti-inflammatory, antioxidative, antiallergic, neuroprotective, bone remodeling, antidiabetic and anticancer properties [24].

The reducing and antioxidative properties of SCE were effectually applied to synthesize metal (Ag) NPs via simple and direct methods [25].

Accordingly, the phytosynthesis of Se-NPs using SCE was intended with the evaluation of their antibacterial potentialities, against different foodborne pathogens, and the interpretation of their characteristics and potential antibacterial action modes.

2 Materials and methods

All used materials/reagents were certified analytical grades; sodium selenite (Na_2SeO_3), ethanol, *p*-iodonitrotetrazolium violet (INT), trypticase soy agar (TSA) and trypticase soy broth (TSB) media were attained from Sigma-Aldrich (St. Louis, MO, USA).

2.1 Phytosynthesis of Se-NPs

2.1.1 Plant extract preparation

Dried roots of *Saussurea costus* Falc. were obtained from the Aromatic and Medicinal Plants Unit, NRC, Egypt. Plant materials were washed with deionized water (DIW), redried with hot air and finely ground to homogenous powder. One hundred grams of grinded roots were immersed in 750 mL of 50% ethanol, agitated at 25°C for 24 h at $280 \times g$ speed (using orbital incubator shaker, IKA, KS 3000 I control, Germany) and filtered to exclude plant residues. The extract of *S. costus* (SCE) was vacuum dried at 42°C and the dried materials were kept in the dark at 4°C.

2.1.2 NP synthesis

For the Se-NP phytosynthesis, different concentrations of Na_2SeO_3 (1, 10, 25, 50 and 100 mM, in extract solution) and of the SCE (0.5, 1.0, 1.5, 2.0 and 2.5%, in DIW) were prepared and preliminarily evaluated for Se-NP phytosynthesis. The optimized concentrations (for obtaining the least NP homogenous size with least SCE concentration) were 10 mM Na_2SeO_3 in 1.0% extract solution. The applied synthesis conditions were the stirring of composite solution at $180 \times g$ for 4 h at 25°C in the dark. The color change to brownish orange (due to the synthesis of Se-NPs) was visually observed (Appendix: Figure A1); no further color variation was observed after 4 h. The phytosynthesized Se-NPs with SCE (SCE/Se-NPs) were separated by centrifugation at $12,000 \times g$ for 20 min (using SIGMA 2–16 KL centrifuge; Sigma Lab. GmbH, Germany), washed twice with DIW, recentrifuged and then subjected to characterization [13].

2.2 Characterization of the phytosynthesized SCE/Se-NPs

The characteristics of phytosynthesized SCE/Se-NPs were measured first with Fourier transform infrared spectroscopy (FTIR) analysis (Perkin Elmer™ FTIR-V. 10.03.08, Germany) for each of SCE and SCE/Se-NPs; the transmission mode (wavenumber range of $450\text{--}4,000\text{ cm}^{-1}$) was applied after intermixing of samples with KBr.

The distribution, size and charges of the phytosynthesized SCE/Se-NPs were measured by photon correlation spectroscopy using Zetasizer (Malvern™, UK), after dissolving and sonication of nanomaterials. The morphology, size and dispersity of SCE/Se-NPs were

further investigated using transmission electron microscopy (TEM; JEOL JEM IT-100, Japan) [16].

2.3 Antibacterial property assessment

The assessment of antibacterial potentialities of SCE, Se-NPs and SCE/Se-NPs was performed (via qualitative and quantitative assays), targeting many identified foodborne bacterial strains, i.e., *Escherichia coli* ATCC-25922, *Salmonella typhimurium* ATCC-23852 and *Staphylococcus aureus* ATCC-25923. The bacterial propagation and challenging were conducted using TSA and TSB at 37°C, aerobically. The examined Se-NPs were obtained via recurrent centrifugation/washing with DIW to remove most of the detached SCEs with NPs.

2.3.1 Qualitative assay (inhibition zone, IZ)

For qualitatively evaluating the activity of produced agents toward foodborne bacterial pathogens, the IZ assay (using disc diffusion method) was conducted directly after spreading bacteria onto TSA plates. Sterile discs (Whatman No. 1 filter paper, 6 mm diameter) carried 100 µg of SCE, Se-NPs or SCE/Se-NPs, after dissolving them in DIW. The appeared IZs after plate incubation (for 24 h at 37°C) were measured and the mean values of triplicated trials were calculated.

2.3.2 Quantitative assay (minimum inhibitory concentration, MIC)

The microdilution technique was employed to estimate the MICs of SCE, Se-NPs or SCE/Se-NPs against challenged foodborne bacteria [1]. Bacterial suspension ($\sim 2 \times 10^7$ CFU/mL) was challenged with the examined agents, in tissue-culture microplates, at serial concentrations in TSA ranging from 2.5 to 100 µg/mL, then after microplate incubation, bacterial viability was appraised via chromogenic staining with INT (4% w/v) which transformed to formazan (red color) by survival cells (Appendix: Figure A2). A total of 100 µL from each challenged well were further plated and incubated on fresh TSA plates for confirming the bactericidal action. The least concentration from screened agents that prohibited bacterial development (in microplates and on TSA plates) was quantified as MIC, which quantifies the agents' bioactivity and the exact required NP doses for bacterial exterminating.

2.3.3 Scanning electron microscopy (SEM) imaging

The micrographs were captured via SEM (JEOL JSM-IT100, Tokyo, Japan) for detecting morphological and structural alterations in *E. coli* and *S. typhimurium* cells, after their exposure to SCE/Se-NPs, to elucidate the potential action mode of NPs. Bacterial imaging using SEM was performed after exposing cells to 25 µg/mL of SCE/Se-NPs (in TSB) for 0 (control), 5 and 10 h, with incubation at 37°C. The treated cells were collected and washed with DIW via centrifugation (at $4,600 \times g$ for 30 min) and then subjected to SEM preparation and imaging. The SEM micrographs were captured based on the alterations in cell morphology after exposure to NPs.

2.4 Statistical analysis

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

3 Results and discussion

3.1 Se-NP phytosynthesis

The bioreduction of Na_2SeO_3 (sodium selenite) to Se-NPs was achieved by SCE, which could be visually evidenced through the change in NP solution color from whitish yellow to deep reddish-orange color. The analysis of SCE phytosynthesized Se-NPs revealed that their size range was around 2.21–11.63 nm, with mean and median diameters of 6.13 and 7.39 nm, respectively. The Se-NPs carried a negative charge with a strong zeta potential of -42.8 mV (Appendix: Figure A3). The change in Se-NP solution color, after SCE reduction, is attributed to surface plasmon resonance effect, after the precursor salt reduction to elemental Se-NPs, by SCE biomolecules that acted as reducing and stabilizing agents [13,26].

The phytosynthesized SCE/Se-NPs generated highly negative Z-potential (-42.8 mV), which is suggested to increase the electrostatic stability and prevent aggregate formation [16]. Negative charge NPs tend to possess high stability and extended half-life in serum and do not

interact with cells/tissues in nonspecific manners [8]; these features are important for *in vivo* applications in antimicrobial perspectives.

The illustrated green phytosynthesis of Se-NPs is simple, cost effective and eco-friendly; the resultant NPs have non-toxic and high stability attributes [27].

3.2 FTIR analysis

The FTIR analysis indicates functional biochemical groups in compounds and their interaction with each other. From the IR spectrum of SCE (Figure 1 – upper curve), many sharp indicative peaks were observed at 896 cm^{-1} (aromatic stretching), $1,113\text{ cm}^{-1}$ (C–O stretching), $1,321\text{ cm}^{-1}$ (SO_4 -functionalities), $1,431\text{ cm}^{-1}$ ($-\text{CH}_2$ groups), $1,489\text{ cm}^{-1}$ (aromatic double bonds) and at $1,602\text{ cm}^{-1}$ (aliphatic double bonds). The broad strong peak at around $3,408\text{ cm}^{-1}$ wavenumber authenticated the –OH existence (within acidic, phenolic or alcoholic groups).

Most of these characteristic peaks were shifted to closing values, in the SCE/Se-NP spectrum (Figure 1 – lower curve), e.g., to $3,412$, $1,619$, $1,457$, $1,332$ and 912 cm^{-1} ; this suggests the biochemical interaction of Se-NPs with these groups. The sharp peak at 586 cm^{-1} is assumingly accredited to Se–O interaction; it could also validate Se-NP formation [18]. The characteristic –OH peak at around $3,408\text{ cm}^{-1}$ became much broader after Se-NP synthesis, which indicates the potential action of Se-NPs with these groups. Also, the disappearance of the SCE spectral peaks at 871 , $1,756$ and $3,087\text{ cm}^{-1}$ in the SCE/Se-NP spectrum validated the Se interaction with the extract groups in this range. The peak at $1,113\text{ cm}^{-1}$ became sharper and had higher intensity due to Se interaction with stretched C–O groups in the SCE.

The analysis of SCE/Se-NP FTIR spectrum evidenced the association of Se-NPs with the SCE constituents from proteins, carbohydrates and lipids [17]. Additionally, these results could advocate the capping of phyto-synthesized Se-NPs with the combined mixtures from polyphenols, proteins and flavonoids in the SCE [28,29]. The Se-NP surface coating with SCE biomolecules is supposedly responsible for a high negative Z-potential value of the SCE/Se-NPs [18].

3.3 TEM analysis

The TEM micrographs of the phytosynthesized SCE/Se-NPs indicated their homogenous distribution and

particle sizes (in the range of 2.86 – 8.73 nm). The Se-NPs appeared with spherical shapes with no aggregation forms (Figure 2). Minute SCE particles were appeared in combination with Se-NPs in the SCE/Se-NP matrix. The miniature NP sizes and their well-dispersity nature indicated the higher reducing and stabilizing activities of SCE to generate Se-NPs. The antioxidant and radical scavenger potentialities of SCE were documented, mainly because of its contents of chlorogenic acid and many other phytoconstituents [30,31].

Thus, these compounds could force the reduction of Se ions to the nanoscale and also stabilize the formed Se-NPs and prevent their aggregation. Former studies could achieve diverse Se-NP sizes and shapes depending on the employed biogenic reducing agents, e.g., extract of fenugreek seed-generated Se-NPs with smooth oval shape and 50 – 150 nm size [8], dried raisin extract gave 3 – 18 nm selenium nanoballs [14], *Bougainvillea spectabilis* flower enforced spherical Se-NP synthesis with 18 – 35 nm size [15] and spherical NPs with sizes of 102 – 170 were obtained after green microbial synthesis [16]. The biomolecules and organic compounds in the plant extract, e.g., SCE, are evidenced to cause NP reduction and stabilization and also prevention of their aggregation [13].

3.4 Antibacterial activity of NPs

The antibacterial activities of SCE, Se-NPs and SCE/Se-NP composites were validated (qualitatively and quantitatively) against the entire foodborne pathogens under investigation (Table 1); the SCE/Se-NP composite was the most forceful. The qualitative zone of inhibition (ZOI) and quantitative minimal inhibitory concentration (MIC) assays revealed remarkable antimicrobial action of the prepared agents, with significant sorting as $\text{SCE} < \text{Se-NPs} < \text{SCE/Se-NPs}$. *S. typhimurium* was significantly more sensitive to SCE than the other species, whereas the most significantly resistant species to SCE/Se-NP action was *S. aureus*. Generally, the sensitivity sorting of the examined bacterial species toward the screened agents is Gram-negative < Gram-positive in the following order: *S. typhimurium* < *E. coli* < *S. aureus*.

The influential antimicrobial power of *S. costus* (*lappa*) was evidenced against numerous pathogenic bacterial and yeast species [19,32,33]. Copious bioactive constituents in the SCE were purified and identified with strong microbicidal potentiality toward frequent zoonotic pathogens; their phytochemical groups were mostly terpenoids, glycosides, flavonoids and lactones [20]. The controlling bioactivities of

Figure 1: FTIR spectra of SCE and the phytosynthesized Se-NPs with this extract.

Figure 2: TEM micrograph of the phytosynthesized Se-NPs with SCE.

many SCE phytochemicals, e.g., isodihydrocostunolide, cynaropicrin and costunolide, were verified and proposed

as excellent bases to develop novel antimicrobial medications and drugs [34].

The SCE exhibited potent *in vitro* anti-*Helicobacter pylori*, with an MIC of 40 µg/mL; the antibacterial potentiality was supposedly attributed to its volatile oil contents [35].

Regarding biogenic synthesized Se-NPs, the antimicrobial activities of NPs were documented and confirmed; *Aloe vera*-synthesized Se-NPs were able to inhibit two species (*S. aureus* and *E. coli*), with larger ZOI around *S. aureus* [12]. Former investigations also indicated the bactericidal influences of the biosynthesized Se-NPs against both mentioned bacterial strains, but with varied potentialities [36,37]. The variation in bactericidal activities from different biosynthesized Se-NPs is supposedly attributed to the reducing biogenic agents used for synthesis, as their constituents from bioactive phytochemicals could influence

Table 1: Antimicrobial powers of phytosynthesized Se-NPs with SCE

Examined agents	Antibacterial activity*					
	<i>E. coli</i>		<i>S. typhimurium</i>		<i>S. aureus</i>	
	ZOI** (mm)	MIC (µg/mL)	ZOI (mm)	MIC (µg/mL)	ZOI (mm)	MIC (µg/mL)
SCE	10.5 ± 0.7 ^{a+}	42.5	11.1 ± 0.7 ^{a&}	40.0	9.8 ± 0.6 ^{a+}	47.5
Se-NPs	13.8 ± 1.3 ^{b+}	32.5	15.2 ± 1.4 ^{b+}	27.5	13.1 ± 1.1 ^{b+}	32.5
SCE/Se-NPs	20.8 ± 1.2 ^{c+}	20.0	22.3 ± 1.5 ^{c+}	17.5	18.6 ± 1.1 ^{c&}	25.0

* Dissimilar superscript symbols (in the same row) and letters (in the same column) indicate significant difference (at $p \leq 0.05$). ** IZs impart triplicates' diameter mean ± SD, assay discs (diameter 6 mm) carried 100 µg from SCE or SCE/Se-NPs.

the bactericidal powers of the resulting nanocomposite. The excellent properties of Se-NPs for inhibiting microbial growth, colonization and biofilm development were illustrated in numerous investigations [38–40]; the main suggested factor for this is the high NP oxidative stress [41]. The effect of size of NPs on their antibacterial activity was also illustrated [40]; they revealed that the biocidal properties of metal NPs are size dependent (i.e., smaller sized NPs of ≤50–100 nm are more forceful than bigger sizes of 200–300 nm). Accordingly, the extremely minute SCE/Se-NPs and the action of SCE phytochemicals could be the explanations for the powerful bactericidal activity of the synthesized nanocomposite.

The effects of exposing bacterial strains (*S. typhimurium* and *E. coli*) to SCE/Se-NPs on the morphology and deformation of bacterial cells are illustrated in Figure 3. Both bacterial cells appeared with normal, smooth, healthy and contacted shapes at zero exposure time (control, Figure 3 – 0). After 5 h of exposure to SCE/Se-NPs, the bacterial cells became puffed and the NPs appeared attaching the cell walls; many NPs are supposed to penetrate and enter the cells in this stage. Also, many cells had observable lyses/explosion signs after their interaction with SCE/Se-NPs (Figure 3 – 5 h). By the SCE/Se-NP exposure elongation for 10 h, the comprised NPs with bacterial cells became more apparent with higher numbers. The bacterial cells were mostly lysed/explored at this time; their released interior components were apparently attached with SCE/Se-NPs.

The SCE antimicrobial action was stated and supposed to involve complex mechanisms, including suppression of nucleic acid metabolism and activity, restraint of cell wall/membrane synthesis and deactivation of intracellular components and proteins [32,33]. Furthermore, the Se-polymeric coating was suggested to possess innovative antibacterial action via the reduction of biological functions of microbes [38].

The shape and size of the phytosynthesized SCE/Se-NPs augmented their antibacterial action, as it was

recently proposed that the spherically shaped and smaller sized Se-NPs could easily access the bacterial cell/membrane and hinder their biological activities [13]. Se-NPs exhibited more inhibitory action toward

Figure 3: SEM micrographs of exposed *S. typhimurium* and *E. coli* to phytosynthesized Se-NPs with costus extract after 0, 5 and 10 h of treatment. Arrows indicate some attached Se-NPs to compromised bacterial cells.

Gram-negative bacterial species here and in previous investigation (including *Proteus* sp. and *Serratia* sp.), which was explained by the lesser surface charges of NPs that effectually enable them to bind with bacterial membranes [9]. Additionally, the antimicrobial action of Se ions was advocated to depend on their absorption and accumulation onto microbial cells, which derive the cytoplasm membrane shrinkage and cell bioactivity inhibition [42].

Customarily, the β -lactam antibiotics (e.g., cephalosporins) are the golden standard classes for the treatment of *E. coli* and most common bacterial infections. Compared to them, the combined natural antimicrobial agents could be more effective, especially for ordinary antibiotic-resistant strains [2,4,43].

The phytosynthesized Se-NPs in combination with SCE are supposed to possess powerful antimicrobial action, even against multidrug-resistant microbes, because of the presence of a wide variety of microbicidal substances in this composite. Microbial cells cannot resist several combined antimicrobial agents, especially with varied antimicrobial action from each of them [2,43–45].

The specific microbicidal mechanisms of Se-NPs are still somewhat ambiguous, but several studies claimed that the production of reactive oxygen species (ROS) and free radicals is the main cause of bacterial cell destruction by Se organic compounds [41,46]. The microbicidal action of metallic NPs was due to their interaction with vital cellular components (RNA, DNA and ribosomes) to alter and deactivate their intracellular processes [47].

Supporting preceding study demonstrated that SEM and TEM imaging of the treated *S. aureus* and *E. coli* with Se-NPs revealed sinking, deformation and damaging of compromised cell walls [48]; they implied that Se-NPs could kill bacteria via penetrating bacterial membranes with increase in ROS production.

4 Conclusion

Toward the control of pathogenic foodborne bacteria, using eco-friendly green synthesized NPs, the phytosynthesis of Se-NPs was effectually achieved using the SCE, with a diminutive particle size of ~6.13 nm mean diameter, spherical shape and high stability. SCE, Se-NPs and SCE/Se-NPs composite exhibited potent bacterial inhibitory action toward different foodborne pathogens (*E. coli*, *S. typhimurium* and *S. aureus*); the bactericidal action was confirmed by imaging and validated the efficiency of the

phytosynthesized NPs; the SCE/Se-NPs were attached to bacterial cells and led to their complete lysis/explosion with exposure prolongation. The SCE/Se-NP composites are recommended for effective control of foodborne bacterial pathogens, applying a simple and eco-friendly phytosynthesis protocol.

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Appendix

Figure A1: Visual appearance of phytosynthesis solutions that contained 10 mM Na_2SeO_3 and 1.0% from *Saussurea costus* extract, after incubation for 0, 2 and 4 h.

Figure A2: Assessment of bacterial viability via chromogenic staining with INT (4% w/v), in 96-well microplates. The transformation to formazan color (red color) indicates viable cells.

Figure A3: Zeta potential of the phytosynthesized Se-NPs with *Saussurea costus* extract = -42.8 mV .