International Journal of Nanomedicine

Dovepress
Taylor & Francis Group

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ORIGINAL RESEARCH

Mechanism of Antioxidant Activity of Selenium Nanoparticles Obtained by Green and Chemical Synthesis

Anna Grudniak[1], Julia Folcik[1], Jakub Szmytke[1], Aleksandra Sentkowska[2]

[1]Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland; [2]Heavy Ion Laboratory, University of Warsaw, Warsaw, Poland

Correspondence: Anna Grudniak, Department of Bacterial Genetics, Institute of Microbiology, Faculty of Biology, University of Warsaw, Miecznikowa 1, Warsaw, 02-096, Poland, Tel +48 225541321, Email a.grudniak@uw.edu.pl

Background: Selenium nanoparticles (SeNPs) show high therapeutic potential. SeNPs obtained by green synthesis methods, using commonly available plants, are an attractive alternative to nanoparticles obtained by classical, chemical methods. The green synthesis process uses environmentally friendly reagents, which offer an eco-friendly advantage. Clarifying their mechanism of action is key to their safe use.

Methods: The study used SeNPs obtained using extracts of sage, hops, blackberry, raspberry, and lemon balm, without the use of additional stabilizers, and nanoparticles chemically obtained with ascorbic acid and gallic acid, stabilized with polyvinyl alcohol. The study was carried out on a model strain of Escherichia coli. In the study, the activities of the key enzymes catalase (CAT), superoxide dismutase (SOD), and the response of bacterial cells to osmotic shock were determined.

Results: One of the key mechanisms of action of SeNPs is related to the formation of ROS in bacterial cells. The SeNPs tested showed strong inhibition of CAT, an enzyme crucial for bacterial cells that is involved in the removal of hydrogen peroxide. The tested SeNPs also had an effect on reducing the activity of superoxide dismutase (SOD), which is also involved in the removal of reactive oxygen species from cells. Green SeNPs were also shown to be involved in the cellular response to osmotic shock, confirming their pleiotropic mechanism of action in bacterial cells.

Conclusion: NPs synthesized via green methods exhibit antibacterial activity against E. coli. The green synthesis process employs environmentally friendly reagents, offering a pro-ecological advantage. Notably, these nanoparticles are strongly stabilized by the post-reaction mixture, eliminating the need for toxic stabilizers. Their antimicrobial mechanism involves ROS generation, catalase (CAT) inhibition, and reduced SOD activity, affecting ROS defense and by disrupting the cellular response to osmotic shock.

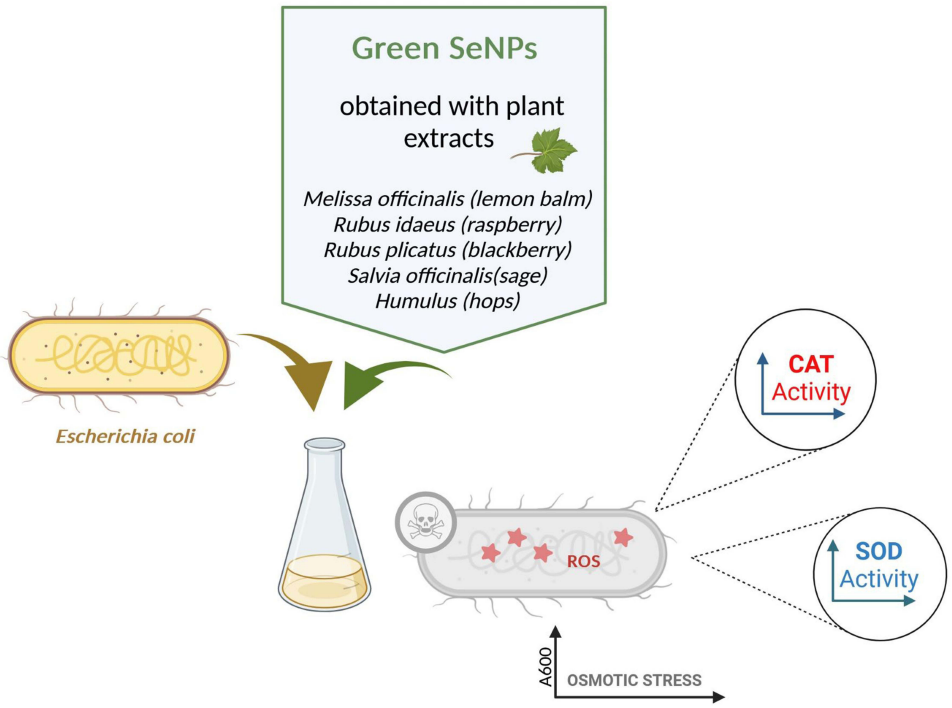
Keywords: selenium nanoparticles, catalase, dismutase, osmotic shock

Introduction

Nanoparticles are diverse organic and inorganic particles obtained by nanotechnology with sizes on the order of 1–100 nm.[1–3] They are widely used as therapeutics with high antibacterial potential, given growing antibiotic resistance, they serve as a promising substitute, reducing antibiotic consumption.[4,5] The antimicrobial properties of nanoparticles are based on mechanisms of action different from those of antibiotics, and they are usually not a single mechanism, making them an effective option for combating bacterial infections. Above that, nanoparticles can find applications in disease diagnostics, anticancer therapies, or as carriers for drugs, among others. Nanoparticles, efficiently phagocytosed by macrophages, can also be used to deliver vaccines.[1] Metals such as silver and copper have long been known as materials with antimicrobial properties, and nanoparticles of both metals and nonmetals and organic compounds are now being used. Commonly used silver nanoparticles show a broad spectrum of activity against viruses, bacteria, and other contaminants, and they can also be used in combination with other antimicrobial substances. Zinc oxide nanoparticles, being environmentally friendly and relatively safe, are used in dentistry and food industry. Titanium oxide nanoparticles are used in water and air purification. Magnesium nanoparticles can

Received: 4 December 2024
Accepted: 11 February 2025
Published: 6 March 2025

Graphical Abstract



be more easily degraded and excreted by the human body than other nanoparticles and are a potential plant protection agents against pathogens. There are also antimicrobial carbon-based nanostructures such as graphene, fullerenes, and carbon nanotubes. On the other hand, are ε-polylysine nanoparticles, which are biodegradable, easily soluble in water, and non-toxic. Many nanoparticles have shown efficacy against bacterial biofilms, making their potential applications more attractive.[1] Nanoparticles act on bacterial cells through a variety of mechanisms. They damage the cell membrane, a mechanism that is particularly effective against gramme-negative bacteria; positively charged ions released from metal nanoparticles are attracted to negatively charged elements of the cell membrane, leading to cell damage. Ions released from metal nanoparticles can also interact with cellular proteins, such as respiratory enzymes, impairing their function. By generating more reactive oxygen species (ROS), nanoparticles effectively damage bacteria. They are also responsible for disrupting the metabolism of bacterial cell.[1] Reactive oxygen species include superoxide anions, hydrogen peroxide, or hydroxyl radicals. Elevated concentrations of ROS have been observed in solutions of copper oxide nanoparticles, among others.[6] In the case of metal oxide nanoparticles, the activation of their antibacterial properties based on ROS production occurs under the influence of light[7] - this could be a potential limitation in the application of these types of nanoparticles. Reactive oxygen species cause bacterial cell death due to DNA damage, protein damage (carbonylation, oxidation of cysteine residues) and depolarization of the cell membrane.[8] Under the influence of ROS, there can also be a cascade of reactions in which the primary products created by the reaction of reactive oxygen species with cell components enter further reactions, generating even more ROS, resulting in extensive cellular damage.[8] Reactive oxygen species (ROS) production is part of the host's nonspecific immune defense against bacterial infections. In this respect, the action of nanoparticles mimics the natural defense mechanism of higher organisms that protects them from invasion by bacterial pathogens. Among others, phagocytic cells, such as neutrophils, are capable of producing ROS, and during phagocytosis, they release significant amounts of ROS to kill the invading bacteria.[9] ROS are also formed as intermediates of oxygen reduction in bacterial cell metabolism. Many bacteria have defence mechanisms to protect them from the effects of ROS, which can include enzymes that inactivate them, such as peroxidase, superoxide dismutase, or catalase, as well as cellular DNA damage repair mechanisms.[10] Strains with effective defense

mechanisms against reactive oxygen species (ROS) show greater resistance to oxidative stress. A separate group of nanoparticles is known as selenium nanoparticles (SeNPs), which exhibit broad antibacterial, antifungal, antiviral, anticancer, antioxidant, and antidiabetic activities. SeNPs inhibit the growth of pathogenic bacteria such as *E. coli, S. aureus* (including strains resistant to methicillin and vancomycin), *Enterococcus faecalis, Bacillus cereus, Streptococcus agalactiae*, and *Listeria monocytogenes*.[11,12] Selenium nanoparticles also exhibit antiparasitic activity against protozoa, nematodes, and tapeworms. The antiviral effect of SeNPs increases as their size decreases.[13] It is possible to use SeNPs as an antimicrobial coating for medical equipment. Coating polyvinyl chloride and polyurethane with a layer of SeNPs inhibited the growth on their surface of bacteria such as *Streptococcus pneumoniae, E. coli, S. aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa*, and fungi such as *Saccharomyces cerevisiae, Candida albicans, Cryptococcus neoformans, Rhodotorula rubra*.[13] Other promising properties shown by SeNPs include immunomodulatory effects against neutrophils or enhanced action of the anticancer drug sorafenib. SeNPs are also widely used in the production of cosmetics.[13] To use them safely, it is crucial to understand their mechanisms of action. Naturally occurring selenium in organic form shows more toxic effects compared to SeNP.[14] Scientists have been focussing on elucidating the mechanism of action of selenium nanoparticles, with studies focussing on their antioxidant and antimicrobial effects.[15,16] Green methods for synthesising selenium nanoparticles in addition to classical chemical methods have recently gained great popularity.[17–20] Plant extract-based methods are among the most environmentally friendly. This method requires cheap, nontoxic, easily accessible reagents, which makes this synthesis not harmful to the environment. Many methods of SeNP synthesis using plant extracts have been described in the literature. However, their frequent drawback is the use of plants that occur only locally, in a small area, eg avaram (*Cassia auriculata*) or arauna (*Terminalia arjuna*), both growing in India and Sri Lanka.[21,22] This makes it much more difficult to reproduce such synthesis by other research groups, eg from Europe. In addition, if the obtained nanoparticles could be used in biomedical applications, they must undergo a series of clinical trials, analogous to drugs. Then each stage of the procedure for obtaining them will be subject to optimization, so the limitation cannot be the acquisition of the material itself. Our goal was to use known and commonly cultivated herbs, so that the developed method could be repeatable and reproducible not only in our laboratory but also by other scientists. n our opinion, in order to achieve a breakthrough in scientific research, cooperation and constructive discussion are necessary, and for this to occur, all interested parties must have the opportunity to conduct similar research in their laboratories. The SeNPs used in this work were obtained using the following plants: raspberry (*Rubus idaeus*), blackberry (*Rubus plicatus*), lemon balm (*Melissa officinalis*), hops (*Humulus*) and sage (*Salvia officinalis*). Their polyphenolic profile, as well as antioxidant and antibacterial properties of obtained SeNPs were determined in vitro.[18] The methods of obtaining and the detailed characterisation of the selenium nanoparticles under study are described in Sentkowska et al, 2024. Selenium nanoparticles produced via green synthesis have demonstrated antibacterial and antibiofilm activity.[18] Plants used for the synthesis of nanoparticles are well-known herbs that have therapeutic potential, which has been widely reported in the literature.[18,23–26] The obtained nanoparticles were characterized by high stability in the post-reaction mixture, they did not show the ability to aggregate, which often occurs in chemical synthesis. It is therefore not necessary to use a stabilizer, which is usually another, potentially toxic chemical substance. Recent studies confirm significant antibacterial and antioxidant properties of selenium nanoparticles. However, research on their mechanism of action is ongoing. Some of the reports on this subject are contradictory. Zhang et al reported that ROS induced by bio-SeNPs were the main mechanism for antibacterial activity.[27] However, Filipović claimed that interaction with cell barrier or inhibition of the synthesis of proteins and DNA also can play an important role in the overall mechanism of action of SeNPs. In our previous work, we investigated the antioxidant and antibacterial properties of the obtained nanoparticles.[18,28] In this study we want to determine the dominant mechanism behind these properties. In the presented work, we hypothesised that SeNPs obtained by green synthesis methods using plant extracts induce oxidative stress in bacterial cells, affect the amount of ROS in bacterial cells by disrupting the activity of enzymes key to their removal from bacterial cells, which directly contributes to their antibacterial activity. We decided to verify this hypothesis in in vivo studies on cells of the model microorganism *Escherichia coli*. Our hypothesis proposes that SeNPs synthesized via plant extracts exert antibacterial effects by inducing oxidative stress and increasing ROS levels through the disruption of key detoxifying enzymes in bacterial cells. We decided to verify this hypothesis in in vivo studies on cells of the model microorganism *Escherichia coli*.

Materials and Methods

Strains of Microorganisms Used in the Work

The bacterial strain used in this study were *Escherichia coli* 439 (Polish Collection of Microorganisms, Institute of Immunology and Experimental Therapy Polish Academy of Sciences, Wrocław) from the collection of the Institute of Microbiology, Department of Biology, UW. LB medium - Lysogeny Broth (BioMaxima, Poland) was used for the bacterial culture. Solid medium was obtained after solidifying the LB with 1.5% agar (BioMaxima, Poland). For oxidative stress experiments, Davies' medium was used (K_2HPO_4 - 1.4 g/L, KH_2PO_4 – 0.6 g/L, $MgSO_4$ $7xH_2O$ 0.02 g/L, $(NH_4)_2SO_4$ – 0.2 g/L, Sodium citrate 0.1g/L, enriched with glucose (final concentration 0.4%), casein hydrolysate (final concentration 0.2%), tryptophan and thiamine (final concentrations 0.01%).

Herbal Samples

Dried herbs used to prepare the extracts were obtained from Kawon (Gostyn, Poland). According to information obtained from the producer, the plants came from last year's harvest, did not come from genetically modified seeds, and were dried with warm air. To prepare the extract, 5 g of herbal material previously ground in a ball mill was poured with 50 mL of boiling water. The brewing process was carried out for 30 min using a mixing intensity of 200 rpm. After that, the extract were filtered through a paper filter and used for nanoparticle synthesis. The green synthesis involved extracts from the following plants: lemon balm (*Melissa officinalis*), raspberry (*Rubus idaeus*), sage (*Salvia officinalis*), blackberry (*Rubus plicatus*), and hops (*Humulus*).

Polyphenolic Profile of the Herbal Extracts

The used herbal extracts as well as post-reaction mixture containing selenium nanoparticles were tested for their content of polyphenolic compounds. For this purpose hydrophilic interaction liquid chromatography (HILIC) coupled with MS/MS was used. The separation was carried out using sulfobetaine ZIC-HILIC (100 × 2.1, 3 μm) column purchased from Merck. Gradient elution was used, where the mobile phase consisted of ACN and water, delivered at 0.2 mL/min. The gradient profile was, as follows: 0–4 min 98% B, 6–7 min 90% B, 8–84 min 80% B, 8.4–12 min 50% B, and 13–20 min 98% B. Polyphenolic compounds were identified based on the comparison of their retention times and m/z values obtained by MS and MS2 with the mass spectra for the standards.

Green and Chemical Synthesis-Obtained SeNPs

The protocol of selenium nanoparticle preparations - SeNPs with the participation of plant extracts (green synthesis) and chemical method was described in Sentkowska et al, 2024. In green synthesis, the reduction of selenium salt (Na_2SeO_3) was done using herbal extracts. In detail, 15 mL of water was added to 2.5 mL of Na_2SeO_3 solution (0.1 mol L^{-1}), and the mixture was placed on a magnetic stirrer. The stirring intensity was set to 1000 rpm. After a while, variable volumes of the extract were added dropwise, 2.5, 5, or 7.5 mL, respectively. The reaction mixture was stirred for 1 hour. The assumption of the method was to conduct the synthesis with a variable ratio of herbal extract to a constant concentration of selenium. The plant extracts used contain both natural selenium compound reducers and stabilizers of the resulting nanoparticles. Therefore, the concentration of these compounds may potentially affect the properties of the obtained SeNPs. Therefore, it was decided to conduct three parallel syntheses using variable volumes of plant extract. Conventional chemical synthesis of selenium nanoparticles was also performed using reducers considered health-promoting substances, such as ascorbic acid and gallic acid. In this approach, 20 mL of sodium selenite solution (5 x 10^{-3} mol L^{-1}) was placed in a biker with magnetic stirrer. The stirring intensity was similar to that used in green synthesis (1000 rpm). Then, 10 mL of reductant solution (4 x 10^{-2} mol L^{-1}) was added dropwise. After one hour of mixing, 70 mL of Mili-Q water was added.

Characterization of Obtained SeNPs

The obtained SeNPs were characterized using transmission electron microscopy (TEM) with a TALOS F200 model (Thermo Fisher Scientific, Waltham, MA, USA) working at an accelerating voltage of 200 kV. Before the measurement

the drop of selenium nanoparticle suspension was dropped on a copper grid and then air-dried before the examination. All the obtained results were processed in the iTEMprogram. Simultaneously, obtained nanoparticles were studied using dynamic light scattering (DLS), involving Mastersizer 2000 (Malvern, Panalytical, UK) with a wet sample dispersion unit (Hydro 2000 MU, Malvern, Panalytical, UK). To perform the studies of SeNPs surface, scanning electron microscopy (SEM) was also involved, using a field-emission SEM (Merlin Zeiss, Montreal, Canada). As part of preparation for measurement, samples were plasma-sputtered with a few-nanometers-thick Au/Pd layer.

Preparation of Cultures for Enzymatic Assays

Overnight *E. coli* were run on LB medium in 37°C with shaking. The next day, after the cultures were rejuvenated to A600= 0.05, the cultures were run for 1 hour under similar conditions. The cultures were then split by adding the selenium nanoparticles under study to a final concentration of 12.5%; the control sample did not contain the addition of nanoparticles. Cultures were continued at 37°C, with shaking for another 2.5 h until they reached a maximum value not exceeding A600= 1.2. The cultures were then centrifuged for 10 min at 10,000–12,000 x g for 10 min). The resulting pellet was resuspended in 1/10 volume of cooled 0.05 M phosphate buffer. For CAT assay experiments, the suspension obtained was sonicated (20 sec, amplitude 25% - 9 repetitions), (Sonics, Vibra-cell) and then centrifuge the homogenate at 4 °C (14,000 x g for 5 minutes) and transfer the supernatant to a new tube. With experiments determining SOD activity, cell should be lysed in ice-cold 0.1 M Trizma®-HCl, ph 7.4, containing 0.5% Triton X-100, 5mM mercapthoethanol and protease inhibitors. Centrifuge the homogenate at 4°C (14,000 x g for 5 minutes) and transfer the supernatant to a new tube. The obtained lysates were used to determine enzymatic activity according to the protocols recommended by the manufacturers.

Determination of Superoxide Dismutase (SOD) Activity

The activity of superoxide dismutase (SOD) was determined using Sigma-Aldrich's original Activity Assay Kit, No. CS0009, according to the manufacturer's recommended procedure. The Superoxide Dismutase (SOD) Activity Assay Kit offers a straightforward and sensitive method to measure SOD enzymatic activity across various samples. It quantifies activity by detecting the reduction of superoxide anions generated by xanthine oxidase (XO). These anions react with the WST dye to produce a color at 450 nm, where reduced color intensity indicates SOD inhibition activity. The SOD activity was measured by following the reduction of cytochrome C at 550 nm. Data are presented as the mean of three (n = 3) independent experiments. Analysis readings were taken on multimode plate reader TECAN Infinite® Nano$^+$ (Tecan Sales Switzerland AG).

Determination of Catalase (CAT) Activity

Catalase (CAT) activity was determined using Sigma-Aldrich's original Catalase Assay Kit, No. CAT100, according to the manufacturer's recommended procedure. Catalase activity was measured by monitoring H_2O_2 degradation at 240 nm. Data are presented as the mean of three (n = 3) independent experiments. Analysis readings were taken on multimode plate reader TECAN Infinite® Nano$^+$ (Tecan Sales Switzerland AG).

Osmotic Adaptation

The experiments followed the method described by Meury and Kohiyama (1991). The *E. coli* strain were grown at 37°C in minimal medium (MM Davis) supplemented, with glucose 0.4%, casein hydrolysate 0.2%, tryptophan 0.01%, and thiamine 0.01%. In the morning, the cultures were rejuvenated bringing them to A600 = 0.05. The cultures were then separated into 10 mL parts. Test selenium nanoparticles obtained by green synthesis and chemical methods were added to each part to a final concentration of 6%, the control of the experiment was *E. coli* cultured without the presence of nanoparticles. Cultures were conducted for 2h at 37°C. After reaching A600 = 0.2, the reaction was started by adding to the culture NaCl or KCl to a final concentration of: 0, 1%, 2%, 3%, 4% and NaCl or NaOH. The samples were taken periodically (every 15 min via 2 hours) and absorbance at 600 nm (A 600) was measured with TECAN-Sunrise spectrophotometer (Tecan Sales Switzerland AG).[29]

Statistical Analysis

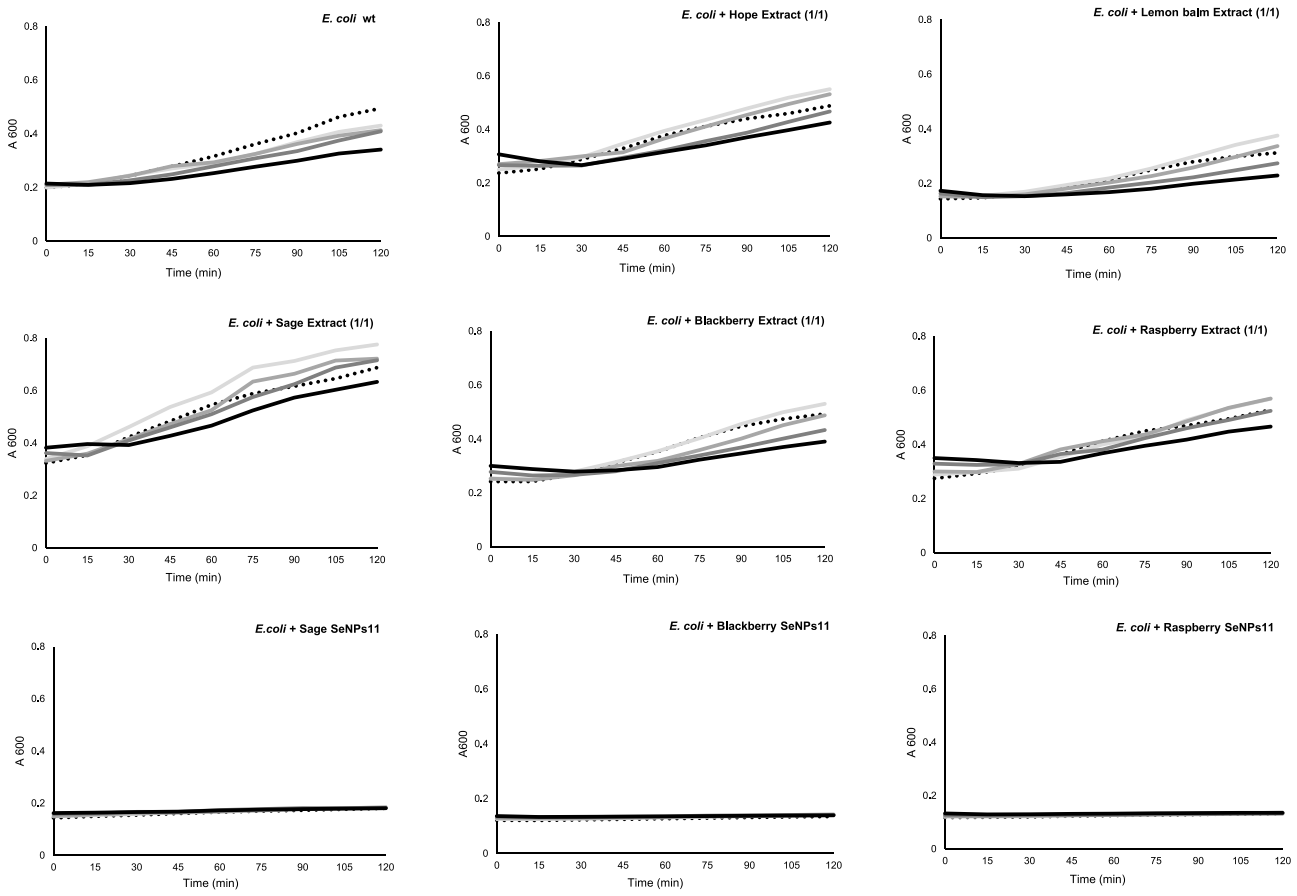
The results were compared by Student's *T*-test for independent samples, using Statistica 13.3 software. The p-value was considered statistically significant when it was less than 0.05.

Results and Discussion

Chemical compounds naturally occurring in plant extracts such as flavonoids, alkaloid saponins, carbohydrates, proteins, tannins, and steroids act in green synthesis as natural reducers and/or stabilizers. Marslin[30] reported, that the mechanism of their action seems to be complex. The first step of the process is the dissociation of selenium salts and saturation of obtained cations resulting in the formation of hydroxyl complexes. Then, the crystallite growth of metal with oxygen species starts to originate. This stage of the synthesis is promoted by heating. This stage continues until activation of the capping agent from the plant extract, which eventually stops the growth of high-energy atomic growth planes. This process ends with the formation of nanoparticles. Plant extract is the source of reducing agents, which are electron donors to metal ions that are converted to NPs. On the other hand, polyphenolic compounds can also stabilize the obtained nanoparticles and prevent their aggregation. Plant extracts used in all green synthesis described in this work are a great source of flavonoids and polyphenolic acids, what is presented in Table 1 (Supplement). The concentration and type of specific polyphenolic compounds depends on the plant from which the extract was obtained. Not all of polyphenols are consumed in the SeNPs synthesis process, thus ensuring surprisingly high stability of the obtained nanoparticles. The observed stability is so high that conventional methods of separating nanoparticles from the post-reaction solution are impossible using classical methods, eg centrifugation.

Bacteria have developed several mechanisms that enable them to respond rapidly to changing, stressful environmental factors. ROS such as hydroxyl radicals, and hydrogen peroxide cause stress in bacterial cells. Hydrogen peroxide, superoxide anion, and hydroxyl radical ($OH\cdot$) are reactive forms of oxygen that can be generated during the normal course of aerobic metabolism by incomplete reduction of oxygen to water during respiration. These are particles that appear in cells as a result of many factors, including toxic substances with antibacterial potential, including nanoparticles. Sudden changes in environmental conditions such as an increase in osmolarity are highly damaging to bacteria, often resulting in growth inhibition and cell death. A decrease in cellular turgor leads to water loss, resulting in the activation of adaptive pathways to counteract this situation. Various membrane transporters like the *proU* operon are involved in this process. The first target we decided to investigate was the effect of selenium nanoparticles on the bacterial response induced by NaCl and KCl, which are responsible for bacterial osmotic stress. In this study, we used SeNPs obtained by the green synthesis method using extracts of sage, hope, blackberry, raspberry, lemon balm, and obtained by the chemical method using ascorbic acid (AA), gallic acid (GA), which were optionally additionally stabilised with polyvinyl alcohol (pvA). The results obtained are presented in Figures 1–2. The osmotic shock in cells was induced using NaCl at concentrations of 1–4% and KCl at concentrations of 1–4%. The control was a culture conducted without compound pressure. The control strain of *E. coli* showed the fastest growth under control conditions, the addition of NaCl and KCl significantly affected the inhibition of bacterial growth in proportion to the concentration used, and the weakest bacteria grew in the presence of 4% NaCl and 4% KCl (The inhibition reached 31% for NaCl and 20% KCl). *E. coli* bacteria cultured in the presence of pure plant extracts (1/1), which were used to obtain SeNPs, and in the presence of NaCl during 2 h of culture showed growth comparable to that of the control. In the presence of 1–2% NaCl for selected extracts, cultures showed slightly better growth than in the control (extract sage (1/1) increase 40% −80% compared to control, raspberry increase by 7–35% compared to control), only the highest concentrations of NaCl had a slowing effect on *E. coli* but did not completely inhibit it. *E. coli* cultures conducted in the presence of green synthesised selenium nanoparticles (SeNPs11), for all extracts tested both in the presence of NaCl and without, showed strong growth inhibition - 100% growth inhibition, this effect was due to the presence of synthesized SeNPs. To demonstrate the effectiveness of green nanoparticles, analogous experiments with chemically derived nanoparticles. In all experimental variants, *E. coli* was inhibited (in 100%) in the presence of SeNPsAA, SeNPsGA also those stabilised by pVA regardless of the presence and concentration of NaCl. The results show that the obtained selenium nanoparticles completely inhibit the growth of bacteria in the presence of factors that cause osmotic shock, consequently leading to their death. Osmotic

shock in *E. coli* cells was also induced by KCl; the results obtained were analogous to those presented for NaCl. Selenium nanoparticles obtained by green synthesis methods using extracts of sage, hope, blackberry, raspberry, and lemon balm, similar to SeNPs obtained chemically using AA, GA in all variants completely in 100% inhibited the growth of *E. coli*. The results indicate a strong growth retarding effect on *E. coli* obtained by green synthesis of selenium nanoparticles in the presence of osmotically active compounds. The presence of selenium nanoparticles aggravates the osmotic shock, blocking the cell defence mechanisms, which consequently leads to cell death. Selenium nanoparticles block the activation of adaptive mechanisms in bacterial cells, whose function is to restore proper turgor. The mechanism of this action may involve blockade in the action of major membrane transporters. This mechanism of action of nanoparticles is known and consists in their interaction with the negatively charged surface of bacteria. The consequence of this action is the disruption of the integrity of bacterial cellular envelopes as a result of their depolarization and changes in membrane potential can lead to a loss of proper cell turgor. Selenium nanoparticles also work by exploiting these mechanisms.[31] The results made it possible to raise another hypothesis that green nanoparticles can induce the formation of increased amounts of reactive oxygen species in cells. This hypothesis was tested by measuring the levels of two key antioxidant enzymes in *E. coli* that help remove reactive oxygen species. Catalase, found in both bacterial and mammalian aerobic cells, contains four ferrihemoprotein groups per molecule and has a molecular mass of 240 kDa. *Escherichia coli* produces two distinct catalases, hydroperoxidase I (HPI) and HPII, differing in structure and catalytic properties from each other and other catalases. HPI, encoded by the *katG* gene, is a bifunctional catalase-peroxidase with two protoheme IX groups in a tetramer of identical subunits.[32] HPII, encoded by the *katE* gene and regulated by the *katF* gene, is a monofunctional catalase with a high Km for H_2O_2.[33,34] It features one cis-heme d isomer per subunit (MW 84,200 Da) and forms an apparent hexameric structure[33,35]. The larger subunit size and unique heme d component



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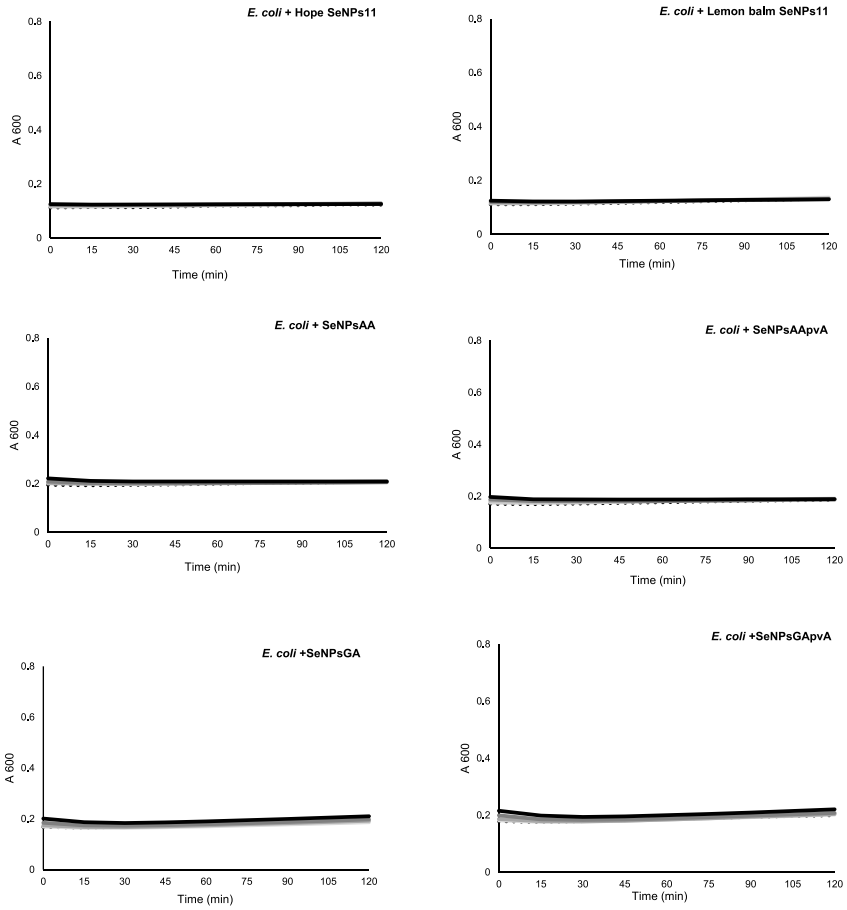

Figure 1 NaCl-induced osmotic shock in *E. coli* cells treated with nanoparticles (dashed black line- 0% NaCl, — 1% NaCl, — 2% NaCl, — 3% NaCl, — 4% NaCl).

suggest HPII's uniqueness.[35] However, its primary structure shows striking similarities to catalase sequences from plants, mammals, and fungi.[33,36] Catalase catalyzes the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. H_2O_2, a by-product of oxidase and superoxide dismutase reactions, is highly damaging, causing oxidation of cellular components like DNA, proteins, and lipids, which can lead to mutagenesis and cell death.[37–40] By removing H_2O_2, catalase protects cells from oxidative damage and plays a crucial role in oxidative stress-related diseases.[37,41] The Sigma-Aldrich CAT100 colorimetric assay was used to determine catalase activity in bacterial cells cultured in the presence of selenium nanoparticles obtained by classical chemical methods and the green synthesis method. The results obtained are presented in Figures 3–4. When *E. coli* cells were treated with pure plant extracts, a significant decrease in CAT activity was observed for all pure extracts, CAT activity decreased from 1.5x for hops to 4x for blackberries. Green selenium nanoparticles obtained with the tested extracts significantly reduced CAT activity in *E. coli* cells, which directly resulted in the accumulation of H_2O_2. CAT activity in *E. coli* cells in the presence of selenium nanoparticles was inhibited even more strongly, raspberry SeNPs11 inhibition 5.4x, hop SeNPs11 5.3x for blackberry SeNP11 4.5x, Sage SeNP11 4.6x and lemon balm SeNPs11 4.8x against *E. coli* wt. In comparison, the use of chemically derived selenium nanoparticles produced similar effects. SeNPsAA had a 4x reduction in CAT activity, regardless of the use of a stabiliser, while SeNPsGA reduced CAT activity ~1.5x. CAT activity in E. coli cells cultured in the presence of green SeNPs was inhibited by 78% - 81%. Selenium nanoparticles obtained chemically from AA inhibited catalase activity by ~ 75%, while those obtained with GA by 30% - 40%. The green SeNPs obtained in this work show activity comparable to nanoparticles obtained by chemical methods. Reduced CAT activity in *E. coli* cells in the presence of SeNPs influences the accumulation of hydrogen peroxide, which is highly damaging to bacterial cells and leads to the oxidation of many cellular targets including DNA, lipids, and proteins which consequently contributes to cell death. In the case of selenium

nanoparticles obtained by green synthesis, this may be one of the main mechanisms of their bactericidal action. Nanoparticles have been studied in terms of their interactions with enzymes in eukaryotic cells. The interaction between enzyme and nanoparticles is determined by the properties of the nanoparticles, such as their size, shape, structure, and charge. Nanoparticles can affect the structure and thus the function of the enzyme. Most human diseases are associated with changes in enzyme activity, so modulation of enzyme activity by nanoparticles may have significant therapeutic potential.[42] Studies of silver nanoparticles (AgNPs) conducted by Marinho et al on *Danio rerio* showed a decrease in the activity of the following enzymes, acetylcholinesterase (AChE) in muscle and brain and catalase activity (CAT) in liver and gill.[43]

Another key enzyme for bacterial cells involved in ROS removal is superoxide dismutase (SOD). The enzyme's activity was measured in *E. coli* cells using Sigma-Aldrich's CS0009 assay, which allows quantitative determination of the active enzyme. The Superoxide Dismutase (SOD) Activity Assay Kit offers a simple and sensitive method for measuring SOD activity across various sample types. The kit quantifies SOD activity using a provided enzyme standard, with results shown in Figures 5–6. SODs are metal ion-containing enzymes found in organisms ranging from bacteria to mammals, catalyzing the dismutation of superoxide (produced during aerobic respiration) into molecular oxygen and hydrogen peroxide.[44] Superoxide dismutases (SODs) are the first line of defense against reactive oxygen species (ROS)-mediated damage, decomposing superoxide radicals to protect against oxidative stress. *Escherichia coli* contains two homologous SODs: a manganese-containing enzyme (Mn-SOD) and an iron-containing enzyme (Fe-SOD), differing in cellular localization, metal cofactors, and responses to oxygen levels. The Fe-SOD, localized in the periplasm, is released

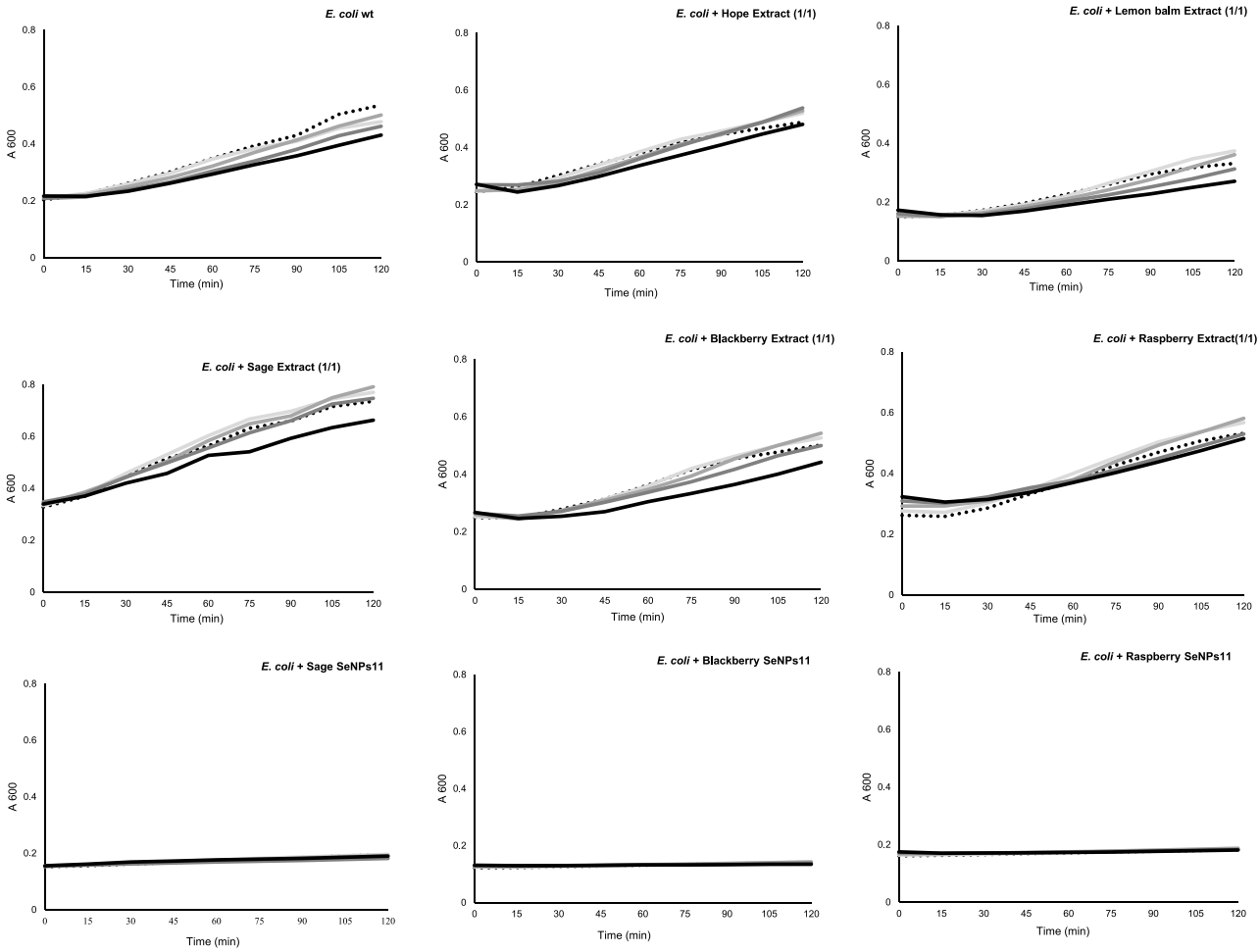

Figure 2 Continued.

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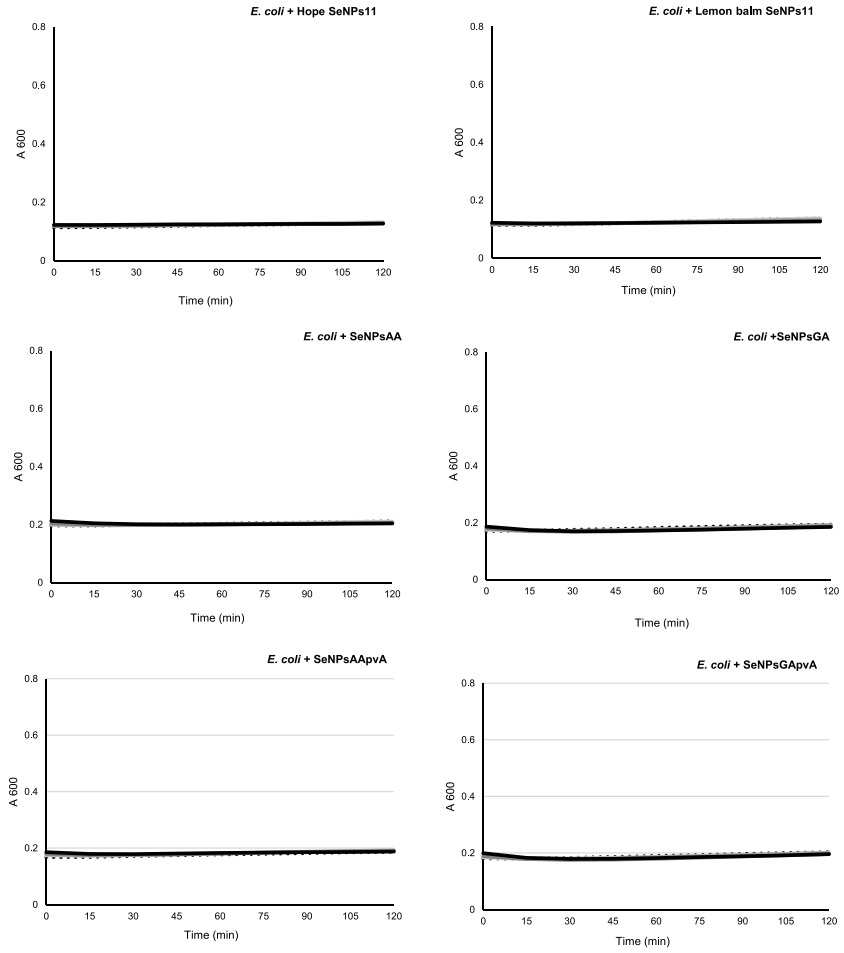

Figure 2 KCl-induced osmotic shock in *E. coli* cells treated with nanoparticles (dashed black line- 0% KCl, — 1% KCl, — 2% KCl, — 3% KCl, — 4% KCl).

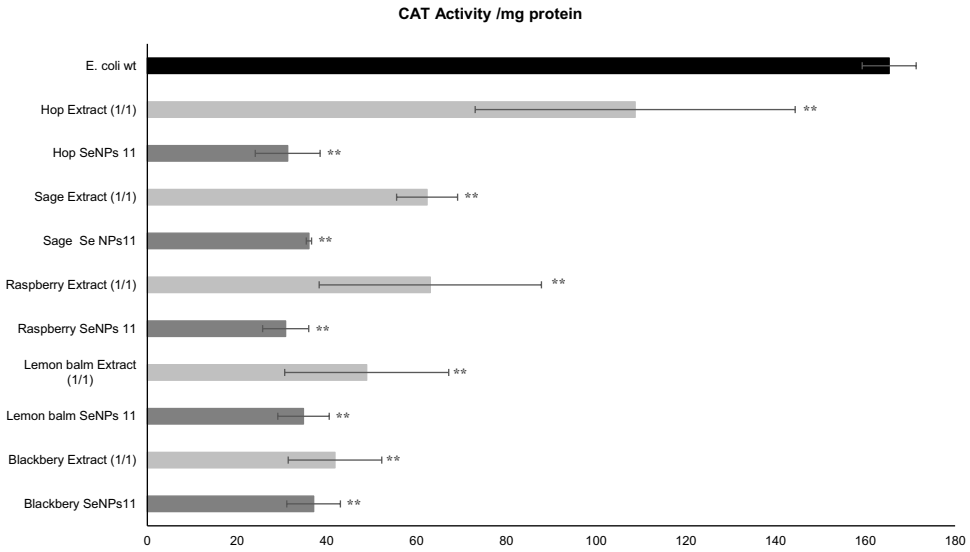

Figure 3 CAT activity in *E. coli* cells treated with plant extracts of hop, sage, raspberry, lemon balm and blackberries and SeNPs obtained with them. Unit definition: One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM hydrogen peroxide. Statistical significance was calculated with a *T*-test in relation to *E. coli* wt, *p >0.05.

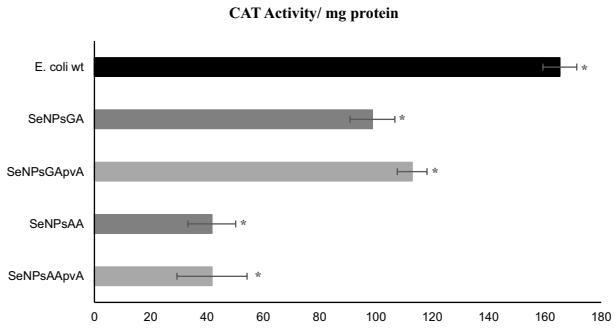

Figure 4 CAT activity in *E. coli* cells treated with SeNPs obtained by chemical methods. Unit definition: One unit of catalase will decompose 1.0 micromole of hydrogen peroxide to oxygen and water per minute at pH 7.0 at 25 °C at a substrate concentration of 50 mM hydrogen peroxide. Statistical significance was calculated with a *T*-test in relation to *E. coli* wt, **p> 0.0001.

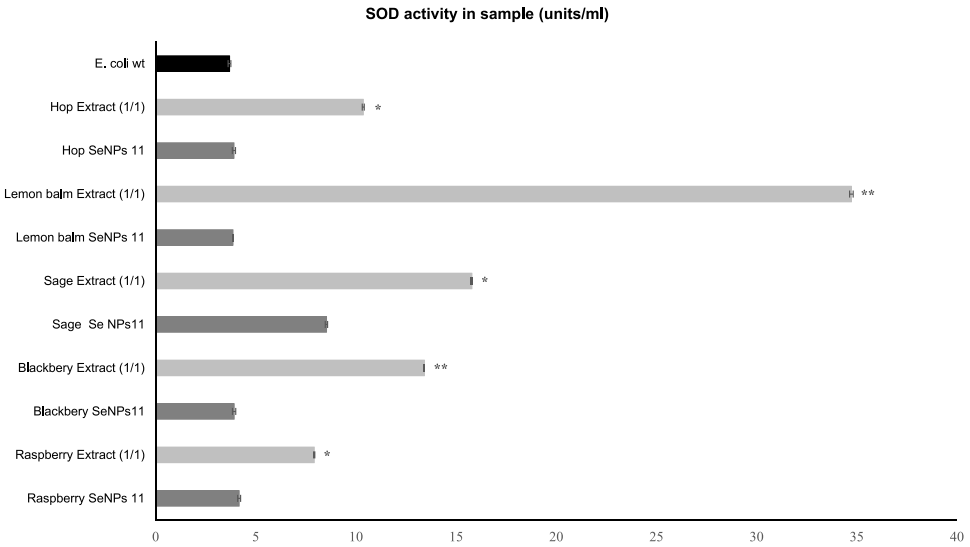

Figure 5 The graphs show the total SOD enzyme activity in the sample calculated according to the protocol in *E. coli* cells treated with plant extracts of hop, sage, raspberry, lemon balm and blackberries and SeNPs obtained with them. Unit Definition of the provided SOD Enzyme: One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase, at pH 7.8 at 25 °C in a 3.0 mL reaction volume. Statistical significance was calculated with a *T*-test in relation to *E. coli* wt, *p <0.05, **p<0.0001.

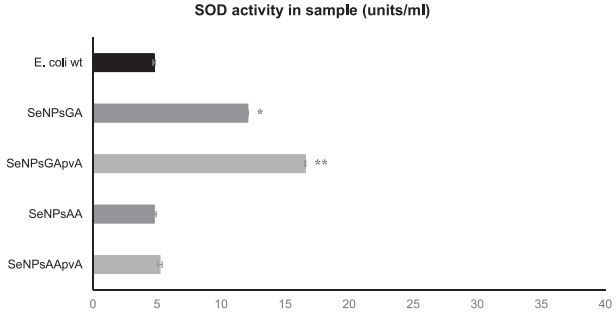

Figure 6 The graphs show the total SOD enzyme activity in the sample calculated according to the protocol in *E. coli* cells treated with SeNPs obtained by chemical methods. Unit Definition of the provided SOD Enzyme: One unit will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase, at pH 7.8 at 25 °C in a 3.0 mL reaction volume. Statistical significance was calculated with a *T*-test in relation to *E. coli* wt, *p <0.05, **p<0.0001.

by osmotic shock and maintains constant levels regardless of pO_2, protecting against exogenous superoxide. In contrast, Mn-SOD, found in the cytoplasm, is not solubilized by osmotic shock and increases in response to high pO_2, counteracting endogenous superoxide toxicity.[45,46] In *E. coli*, Fe-SOD (encoded by *sodB*) is constitutively synthesised and may

https://doi.org/10.2147/IJN.S507712

be more important for protecting cytoplasmic enzymes from metabolic oxidative damage.[47] In contrast, manganese-containing enzyme synthesis (encoded by *sod*A) is induced in response to environmentally induced oxygen stress and may have additional potential in preventing oxygen-dependent DNA damage.[47] The expression of the Mn-SOD gene is also markedly increased by exposure to redox-cycling agents,[47] heat shock [48], DNA binding drugs [49], ethanol [50], high salt concentrations [50] and metals [51.52].

Treatment of *E. coli* cells with pure plant extracts resulted in a very high increase in SOD activity; for lemon balm extract the activity increased more than 9.4x. For raspberry 2x, hop 2.8x, blackberry 3.6x, and Sage 4.3x extracts. All pure plant extracts caused a significant increase in SOD activity in *E. coli* cells, which may indicate the large amount of ROS formed in cells in the presence of plant extracts and the need to increase the expression of the sod gene. The enzyme activity in *E. coli* cells treated with SeNPs obtained with the use of plant extracts tested was slightly higher than that observed in the control *E. coli* strain (1 to 2.3x) and significantly lower compared to that observed for pure plant extracts. The presence of selenium nanoparticles in the tested samples significantly reduced the activity of the SOD enzyme in *E. coli* cells treated with pure plant extracts. *E. coli* cells in the presence of chemically synthesised SeNPs showed high SOD activity, especially in the case of SeNPsGA this activity further increased for those SeNPs obtained after the addition of a stabiliser. SeNPsAA showed SOD activity comparable to that of the control strain of *E. coli*. Superoxide dismutases (SODs) are important components that protect cells from oxidative stress by breaking down the superoxide radical. In a paper by Geslin, 2021, it was shown that a lack of SOD is associated in *E. coli* with an increased sensitivity to cadmium, nickel, and cobalt.[45]

Despite ongoing medical advances, microbial infections remain a significant factor in morbidity and mortality worldwide. Nanotechnology has provided many tools that aid us in the fight against bacteria. Unfortunately, recent data suggest the possibility of bacterial resistance to metal nanoparticles and metal oxides [53]. The search for potential alternative antibacterial therapies has naturally been redirected towards nonmetallic nanoparticles, which are also characterised by strong antimicrobial and antioxidant properties. Such nanoparticles are SeNPs. In this study, we used selenium nanoparticles obtained by green synthesis methods, for which extracts of hops, raspberry, sage, blackberry and lemon balm were used, as well as classically obtained SeNPs by the chemical method with AA and GA. Our previous research confirms that SeNPs obtained by the green synthesis method with the participation of plant extracts of hops, raspberry, sage, blackberry, and lemon balm are great nanoantioxidants. In addition, they exhibit strong antibacterial and antibiofilm activities which is similar to the effect of chemically obtained selenium nanoparticles. The results of these analyses were presented in our previous work.[18] In that work, it was also shown that in many cases, the ability of SeNPs to neutralise hydroxyl radicals was increased in comparison to that of the extract used for their synthesis. In the case of raspberry and sage, the NPs had a three-fold higher antioxidant capacity in comparison to the corresponding extracts. The green SeNPs that we used showed strong antibacterial activity; to use them safely it is necessary to clarify their mechanism of action. The results presented here confirm that the action of green SeNPs is pleiotropic and that one of the mechanisms is the strong osmotic stress that they induce in bacterial cells and the formation of an increased number of reactive oxygen species. Selenium nanoparticles have previously been investigated for various disorders related to oxidative stress and inflammation, such as arthritis, cancer, diabetes, and nephropathy, with potential therapeutic benefits [54]. The mechanisms involved in ROS scavenging in *E. coli* cells treated with green SeNPs of hops, raspberry, sage, blackberry, and lemon balm are impaired, the key enzyme whose activity is strongly inhibited is catalase involved in the removal of hydrogen peroxide from cells, superoxide dismutases also show reduced activity. A convergent mechanism of action was reported in the work of Seyedeh et al, 2023 where green synthesis-derived selenium nanoparticles (C@SeNP) with an aqueous extract of *Crocus caspius* exhibited strong antioxidant and antimicrobial and antifungal properties and inhibited the growth of MCF-7 and AGS cancer cells [55]. Studies conducted by Zhang et al on *Staphylococcus aureus, Bacillus subtilis, Bacillus cereus, E. coli, Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* showed that the increase in ROS concentration is responsible for the antibacterial activity of the tested bio-SeNPs, which resulted in the inhibition of the growth of the tested bacteria. Bio-SeNPs of 120 nm in size were obtained with the participation of *Providencia* sp. In addition to the increase in ROS concentration, significant changes in the permeability of the bacterial cell membranes were also observed.[27] In the study by Yuan et al, the antibacterial efficacy of selenium nanoparticles obtained with ascorbic acid was demonstrated against *Listeria monocytogenes, S. aureus, Staphylococcus epidermidis,*

Vibrio alginolyticus, and *Salmonella enterica*. These bacteria can be transmitted through food, and the high antibacterial efficacy of selenium nanoparticles may result in their use in preventing food spoilage.[12] At this stage of research, it is possible to talk about the enormous potential that selenium nanoparticles obtained by green synthesis in industrial and medical applications. On the basis of the improved properties of SeNPs compared to those of Se, they have already been studied in various states of the disease. SeNPs offer improved bioavailability with the added advantage of reduced toxicity. Pro-oxidant and anti-oxidant activities provide different opportunities for research in various pathological conditions. SeNPs have also been suggested to be used for various therapeutic purposes, including conventional antimicrobial, anticancer, antidiabetic and anti-inflammatory activities [54]. It seems that the selenium nanoparticles studied in this paper, obtained with hops, raspberries, sage, blackberries, and lemon balm, have tremendous therapeutic potential.

Conclusion

SeNPs obtained by the green synthesis method using extracts of sage, hope, blackberry, raspberry, lemon balm, and obtained by the chemical method using ascorbic (AA) and gallic (GA) acids show antibacterial activity against *E. coli* cells. The green SeNPs synthesis process involves the use of natural, environmentally friendly reagents, which makes it pro-ecological. However, the great advantage of these nanoparticles is their strong stabilization by the post-reaction mixture, which significantly facilitates their further use. It is not necessary to use a toxic stabilizer. The mechanism of this action is related to ROS formation of ROS in bacterial cells and strong inhibition of the activity of catalase (CAT) activity, a key enzyme for *E. coli* cells involved in the removal of hydrogen peroxide from cells. The tested SeNPs also had an effect on reducing the activity of SODs, which are also involved in the removal of ROS from cells. The tested green SeNPs impaired the osmotic shock response in *E. coli*, leading to cell death and confirming their pleiotropic mechanisms of action. The antimicrobial mechanisms of action of green SeNPs we investigated are consistent with those described by Dmitry et al, 2023. Most of the described mechanisms are related to the surface action of selenium nanoparticles, for example, degradation of proteins, interaction with functional groups of proteins and enzymes -SH, NH, or COOH, inactivation of natural transport mechanisms, overproduction of ROS, inhibition of dehydrogenase enzyme, inhibition of biofilm formation.[13] Many applications of SeNPs can be expected in the future. Due to their low toxicity and high biocompatibility, they can be used as food additives and dietary supplements. Their potential will also be used in medicine to improve diagnosis, treatment and delivery of drugs to diseased tissue. However, their most interesting potential application seems to be as radioprotectors, which can be used in radiotherapy. Such radioprotective agents provides the protection for healthy tissue and organs, with simultaneous minimizing toxicity and targeting healthy cells than the cancer cells.

Abbreviations

AA, ascorbic acid; GA, gallic acid; SeNPs, selenium nanoparticles; SeNPsAA, selenium nanoparticles obtained by synthesis method involved ascorbic acid; SeNPsGA, selenium nanoparticles obtained by synthesis method involved gallic acid; SeNPs11, selenium nanoparticles obtained by green synthesis performed in 1 to 1 Se/extract ratio; SeNPs12, selenium nanoparticles obtained by green synthesis performed in 1 to 2 Se/extract ratio; SeNPs13, selenium nanoparticles obtained by green synthesis performed in 1 to 3 Se/extract ratio; pVA, polyvinyl alcohol.

Funding

This research was funded by University of Warsaw Nowe Idee 3b POB I grant number 501-D355-20-1004310.

Disclosure

The author(s) report no conflicts of interest in this work. Graphical abstract Created in BioRender. Grudniak, A. (2025) https://BioRender.com/l45p084.

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