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Comparative Study of Green and Chemical Synthesized Selenium Nanoparticles and Its Antibacterial Assay Against Fish Pathogens

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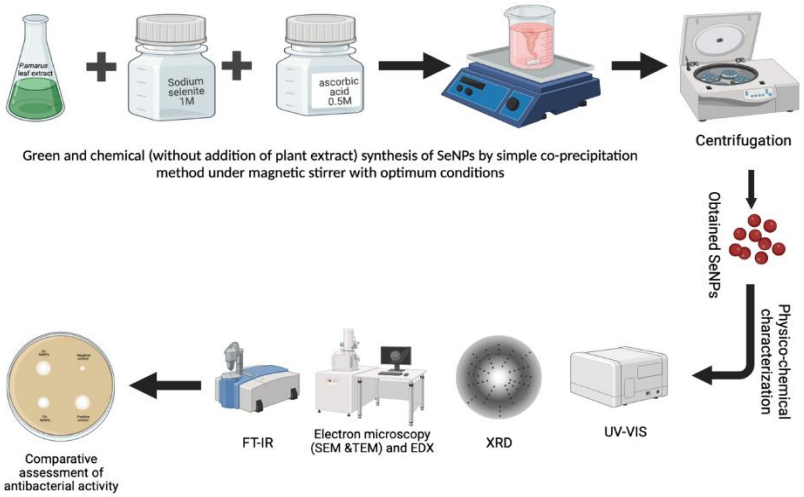
GRAPHYICAL ABSTRACT



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ABSTRACT

Nanometals are widely used in industries like engineering, medicine and the environment; but their synthesis is often chemically prone to environmental contamination, high energy usage, and health issues. Green synthesis, which uses plant extracts instead of chemical agents, addresses these challenges by being more cost-effective, reducing pollution, and protecting both the environment and human health. In this study, two different SeNPs were synthesized where one from sodium selenite using *Phyllanthus amarus* leaf aqueous extract and another one is via chemical mediated SeNPs. The synthesized SeNPs were characterized by UV-Vis Spectroscopy and exhibited a peak at 264 nm and 265 nm for Gr- SeNPs and Ch-SeNPs; XRD, EDX revealed the atom percentage at 89.07% for Gr-SeNPs and for Ch-SeNPs at 78.45%. The electron microscopy results revealed that synthesized SeNPs morphology was spherical in shape. XRD reports explain the sharp peaks indicate the crystallinity of the nanoparticles the crystalline size of Gr-SeNPs and Ch-SeNPs are 32.13 nm and 33.41 nm. FT-IR spectrum results describe the information about the interaction between functional groups of phytochemicals in the leaf extract and the SeNPs. This study also proved the antibacterial potential of green SeNPs against common fish pathogens comparable to chemical SeNPs. Based on these results, it is confirmed that leaf extract capped SeNPs may have potential bio-medicinal applications when compared to chemical synthesized SeNPs.

1. Introduction

When compared to bulk materials, nanoparticle's distinguishing characteristics, such as their larger size, enhanced shape, and greater surface area, are distinct and better. Because of their distinctive physical characteristics, chemical reactivity, and prospective uses in several research fields including antibacterial, antiviral, diagnostics, anticancer, and targeted drug administration, metal nanoparticles are widely used [1]. The most common way for creating metal nanoparticles is chemical reduction, which can be done via solvothermal techniques, electrochemical methods, photochemical reactions or hydrothermal methods [2]. Among the different synthesizing methods, chemical reduction is a highly used method [3]. According to earlier research, using a chemical-reducing agent produced larger particles and used more energy. Additionally, it was stated that more hazardous chemical methods produced harmful byproducts. Additionally, it was noted that the chemically produced nanoparticles had reduced stability and increased aggregation [4]. Therefore, it is necessary to create a green strategy that will use less energy while producing stable and dispersible nanoparticles of adjustable size [5]. As reducing agents, bacteria, fungi, and plant extracts are used in alternative methods to synthesis metal and metal oxide nanoparticles. These biological techniques, often known as "green synthesis techniques," are not only safe and environmentally friendly but also quicker, less challenging, readily expandable to large scales, and more

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successful than traditional techniques [6]. Additionally, green synthesis is also beneficial by being used in more biomedicine, pharmaceutical industries and biotechnological applications. Additionally, since they don't employ hazardous chemicals for synthesis, it is suitable for a variety of biomedical and pharmaceutical applications, which is advantageous to us. Moreover, green synthesis produces nanoparticles with a high degree of dispersion, a high degree of stability, and narrow size distribution [7]. Selenium (Se) nanoparticles are greatly important and have grown in the biomedical field as a replacement for the rising bacterial resistance to antibiotics and fighting bacterial drug resistance [8]. Se NPs are also essential microelements in the human body for subsistence the growth [9]. Additionally, Se NPs are a trace and an essential metal, promising antimicrobial material in both suspended and immovable types [10]. Previous research has synthesized Se NPs using *Cassia auriculate* [11], *Azadirachta indica* [12], *Vitis vinifera* [13], *Moringa oleifera* [14], *Petroselinum crispum* [15]. The plant extract has a variety of biological compounds such as phenols, alkaloids, flavonoids, and terpenoids itself. These biological compounds can act as reducing agents of metal and oxide nanoparticles, preventing their aggregation, being highly monodispersed and increasing the stability of the nanoparticles [16].

Phyllanthus amarus, commonly called Keezhanelli is known as a traditional medicinal plant. It contains many therapeutic benefits, like anti-malaria, anti-viral, antibacterial, anti-diarrheal, as well as used for a few skin treatments, anti-inflammatory, anti-carcinogenic and antidiabetic properties due to being rich in phytochemicals [17]. Hence, this study aimed for the first time use of *Phyllanthus amarus* and standardized the protocol for the synthesis of selenium nanoparticles. we characterized the synthesized Se nanoparticles to study the morphology, size, crystalline structure, surface-capped functional groups and stability of the green and chemical synthesized nanoparticles. This study also investigated the antibacterial activity against 8 fish pathogens such as *Enterococcus faecalis, Micrococcus luteus, Staphylococcus aureus, Streptococcus pyogens, Aeromonas hydrophilia, Pseudomonas aeroginosa, Klebsiella pneumonia, Stenotrophomonas maltophilia.* We examined the characterization profile and the antibacterial properties of green and chemical synthesized selenium nanoparticles to support the method that we optimized.

2. Experimental Methods

2.1 Materials

All the chemicals and reagents used for the synthesis of selenium nanoparticles were purchased from Modern Scientific Company (Madurai, India), Sigma Aldrich, Merck India Ltd and Hi Media. The standard strains viz., *Streptococcus pyogens* (NCT 289), *Enterococcus faecalis* (NCT 34), *Micrococcus luteus* (NCT 24), *Staphylococcus aureus* (NCT 45), *Stenotrophomonas maltophilia* (NCT 259), *Aeromonas hydrophilia* (NCT 337), *Klebsiella pneumonia* (NCT 40) and *Pseudomonas aeruginosa* (NCT 114.2) used for the antibacterial activity were obtained from National Culture Collection Centre (www.ncccc.in), Department of Biotechnology and Microbiology, National College (Autonomous), Trichy, Tamilnadu, India. The plant samples were collected from Thoppampatti, Palani, Dindigul district, Tamilnadu, India.

2.2 Preparation of P.amarus Aqueous Extract

About 10 grams of powdered leaves were taken in 100 mL of distilled water. The mixture was allowed to boil on the hotplate at 60 °C for 20 minutes and kept to cool down to room temperature. The produced solutions were separated from powdered leafy components by filtering them using common filter paper. The filtrate was once more passed through Whatman No. 1 filter paper for a clear solution. The filtrate was used for the green selenium nanoparticles [18].

2.3 Preparation of Green and Chemical Selenium Nanoparticles

For the synthesis of green selenium nanoparticles 10 mL of *Phyllanthus amarus* aqueous leaf extract was added dropwise into the 90 mL of 100 mM concentrated sodium selenite precursor solution under the magnetic stirring condition at room temperature with average rpm. The pH of the solution was maintained at 7 by adding the required volume of sodium hydroxide. With that solution, 10 mL of 50 mM concentrated ascorbic acid was added slowly, which acts as a reducing agent to initiate the selenium nanomaterial synthesizing. This stirring process was continued for 90 minutes. The solution colour turns into a dark ruby-red colour representing and confirming the formation of selenium nanomaterial. This colloidal solution was kept in a resting stage for a period overnight to settle down the nanoparticles. After synthesizing the nanoparticles, the solution was centrifuged at 6000 rpm for 20 minutes. The pellets were subjected to the purification process using distilled water and ethanol several times, washed by vortexing and centrifugation until obtaining pure dark pellets. The same protocol was used to synthesis chemical selenium nanoparticles without the addition of leaf extract.

2.4 Characterization of Green and Chemical Selenium Nanoparticles

The following techniques characterized the green and chemically synthesized selenium nanomaterial's physical and chemical properties.

2.4.1 UV-Visible Spectroscopy

The absorption spectra of the green and chemical synthesized selenium nanoparticles were recorded by UV-Vis spectroscopy. 4 mL of supernatant was used for this analysis. The samples were recorded between 200 and 800 nm. Data was collected and analyzed on a Thermo Scientific GENESYS 180 UV-Vis Spectrophotometer [19].

2.4.2 X-Ray Diffraction

X-ray diffraction, a rapid analysis technique, is widely used to determine the phase of nanocrystals. In addition, detailed information about crystalline materials can be provided. XRD was performed to confirm the crystallite structure of green and chemically synthesized selenium nanoparticles. A Panalytical/X pert 3 X-ray powder diffractometer was used to record the sample. During analysis, the nanomaterial is homogenized and finely ground before an average bulk composition evaluation is made. The system was operated with CuK radiation in a 45 kV voltage and 30 mA current configuration. The crystallite size of SeNPs was identified using Scherrer estimation based on the width of the XRD peaks. The size of the domesticated crystallites was calculated using Debye-Scherrer's equation, $D=K\lambda/\beta.\cos\theta$, where k is the aspect ratio with a constant value of 0.94, "D" appears to be the average size of the crystallites on the reflecting planes, λ is the wavelength of the Cu radiation k, and the Bragg diffraction angle is measured at half maximum full-width radians (FWHM) [20].

2.4.3 Electron Microscopy

Electron microscopy is a surface morphology imaging technique that provides information about sample size, shape, and morphology. It can study the surface morphology of nanoparticles as it detects electrons scattered on the particle surface and passes through the sample with the help of a Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM). The synthesized green and chemical selenium nanoparticles were observed with the SEM of the VEGA 3 system from Tescan (Czech Republic). To conduct this test, a small amount of thin sample layers were applied to a carbon-coated copper grid. An acceleration voltage of 10 kV was used. The contrast and brightness of the photos were adjusted to ideal levels to separate the particles from the background. The SEM grid film was dried under a mercury lamp for five minutes after removing the excess solution with a paper towel [21].

TEM is a vital characterization technique for direct imaging of nanoparticles to obtain quantitative measurements of particle size and distribution. A 5 μL of synthesized nanomaterial colloidal sample was placed on carbon-coated copper mesh and followed by solvent evaporation under vacuum. The morphology of the selenium nanoparticles was characterized by TEM(device name) at an acceleration voltage of 200 kV [22].

2.4.4 Energy Dispersive X-Ray Analysis

In the energy-dispersive X-ray analysis technique (EDX), nanoparticles are activated and analyzed with an energy-dispersive X-ray spectrophotometer. This method was performed with Bruker Nano, GmbH, D-12489 (Germany) and, when properly instrumented, provides accurate results for elemental detection and concentration determination of green and chemically synthesized selenium nanoparticles [23].

2.4.5 Fourier Transform Infra-Red Spectroscopy

Two milligrams of air-dried green and chemically synthesized selenium nanomaterial were combined with KBr and ground into pellets. The pellets were tested by placing the samples in a sample holder and recording the spectrum in the 400 to 4000 cm^{-1} wavelength range. The Jasco FT/IR - 4700 type "A" detector was used to record the FT-IR spectrum [24].

2.5 Assessment of Antibacterial Assay

Green and chemical synthesized selenium nanoparticles were comparatively tested for antibacterial activity against four gram positive and four gram negative fish pathogens using the usual agar well diffusion technique. The green and chemical SeNPs concentration for antibacterial

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activity was 100 µg/mL. First, a loop of inoculum of chosen microorganisms was introduced to sterilized nutritional broth. The inoculum was allowed to incubate for 18-24 hours after the development of microorganisms and optical density (OD) was measured at 600 nm using a UV-visible spectrophotometer (Thermoscientific GENESYS 180). Using a cotton swab, the inoculum was spread on the autoclaved nutrient agar plates (Miller-Hinton Agar). Four wells were created with 6 mm diameter using a well cutter on the agar plates and different volume of leaf extract was loaded into the wells. The positive control was streptomycin (0.5 mg/mL), and DMSO was used for negative control. The prepared plates were allowed for incubation at 37 °C for 24 h to measure the inhibition zone in millimetres [25].

3. Results and Discussion

3.1 Synthesis of Green and Chemical SeNPs and Characterization

In this study, green SeNPs were synthesized from aqueous leaf extract of *P. amarus*, and chemical SeNPs were synthesized without the addition of plant extract, as represented in Fig. 1. For green, synthesized SeNPs from pale green into brick red colour indicates the formation of selenium nanoparticles, and for chemical SeNPs, colourless solution to brick red colour formation [26]. The synthesized both green and chemical nanoparticles were characterized using UV-Vis spectroscopy, SEM, EDX, TEM, XRD, FTIR, Zeta potential and examined for antibacterial activity against 8 strains of fish pathogens in comparison with chemical synthesized SeNPs.

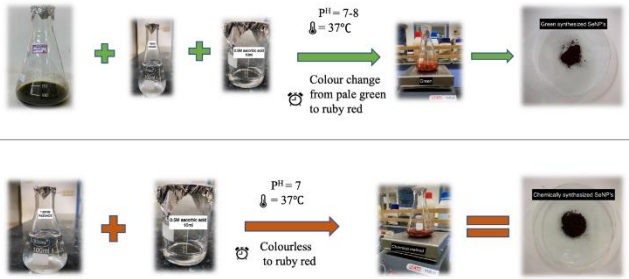

Fig. 1 a) Green synthesis of SeNPs and b) chemical synthesis of SeNPs

3.1.1 UV-Vis Spectroscopy

The UV spectrum of plant extract, green and chemical synthesized SeNPs was observed between the range of 200 to 800 nm which was shown in Fig. 2. For plant extract high-intensity peak was observed at 222 nm, meanwhile for Gr-SeNPs an absorbance peak was recorded at 264 nm. For Ch-SeNPs high-intensity peak was found at 265 nm. Srivastava et al., have reported a peak at 270 nm which confirms the formation of SeNPs [27]. Boroumand et al., have studied that a strong absorption peak of polyvinyl alcohol-SeNPs was observed at 264 nm [28]. Vahdati et al., reported that fenugreek-mediated SeNPs showed a strong peak recorded at 265 nm [29].

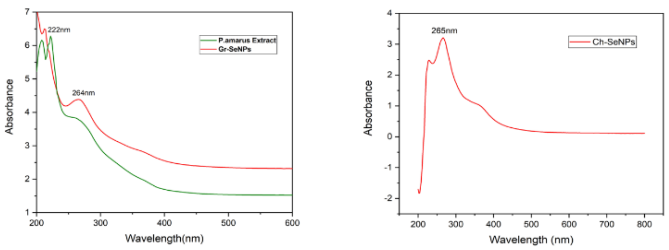

Fig. 2 UV spectrum of a) *P. amarus* aqueous leaf extract and green synthesized SeNPs; and c) chemical synthesized SeNPs

3.1.2 X-Ray Diffraction

The XRD pattern of green and chemical SeNPs is shown in Fig. 3. A sharp and highly intense peak was observed in both green and chemical synthesized SeNPs. The lattice index obtained was close and consistent with standard data for SeNPs (JCPDS 96-901-2502). For green synthesized SeNPs recorded reflection peaks are 23.43°, 29.63°, 41.25°, 43.57°, 45.29°, 51.64°, 56.03°, 61.59° and 65.14°. While the reflection peaks for chemical SeNPs represent 23.42°, 29.62°, 41.27°, 43.56°, 45.29°, 51.61°, 56.02°, 61.41° and 65.12°Bragg's lattice planes for both were similar at (100), (101), (110), (102), (111), (201), (112), (202) and (210). The average crystallite size of synthesized SeNPs was calculated by using Scherrer's formula. The calculated crystallite size was found to be 32.13 nm and 33.41 nm for green and chemical synthesized SeNPs. Overall, the XRD profile explains that the green synthesis of SeNPs using *P. amarus* leaf extract is reasonable for the production of bio-stabilized SeNPs with possible bio-medicinal effects. Shar et al., studied that synthesized SeNP's reflection peaks at 23.5°, 29.7 °, 41.4°, 43.6°, 45.4°, 51.7°, 55.9° and 61.5° corresponded to the Bragg's planes of (100), (101), (110), (102), (111), (201), (112) and (202) [30]. This report is similar to our results. Previous reports have also utilized XRD patterns as a piece of evidence for the confirmation of SeNPs [31].

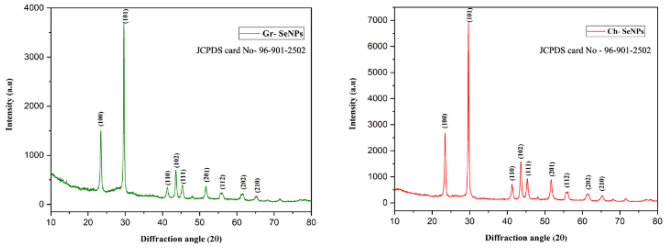

Fig. 3 XRD pattern of a) Green synthesized SeNPs and b) chemical synthesized SeNPs

3.1.3 Electron Microscopy

Figs. 4 and 5 represents the SEM and TEM images of green and chemical synthesized SeNPs. These images explain the surface morphology of the SeNPs. The green synthesized SeNPs explain mono-dispersion without aggregation when compared to that chemical synthesized SeNPs. This is because of the capping of *P. amarus* leaf extract with the SeNPs. The particles were precise spherical with distinct edges and without aggregation. The average particle size of green synthesized SeNPs was 23.14 nm and 44.86 nm for chemical synthesized SeNPs. The green synthesized SeNPs size was smaller than the chemical synthesized, thus indicating the presence of phytochemical constituents in the leaf extract coated on the surface of SeNPs, resulting decrease in size. Surface coating of biological compounds results in an internal strain in the particles consequently reducing the signal and noise [32]. Zhang et al., reported that a biosynthesized SeNPs surface morphology revealed that spherical shape with a uniform size distribution [33]. Earlier reports that plant biomolecules functionalized on nanoparticles to avoid aggregation and had good stability with dispersibility [34].

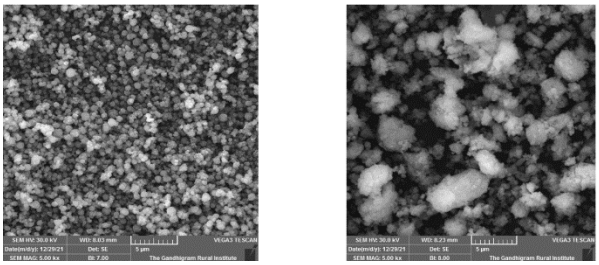

Fig. 4 SEM images of a) Green synthesized SeNPs and b) Chemical synthesized SeNPs

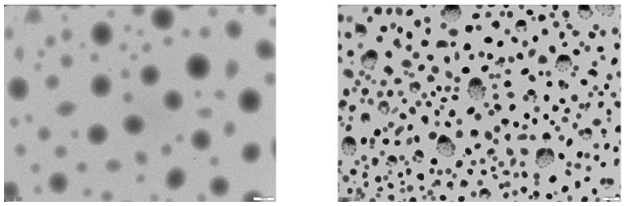

Fig. 5 TEM images of a) Green synthesized SeNPs and b) Chemical synthesized SeNPs

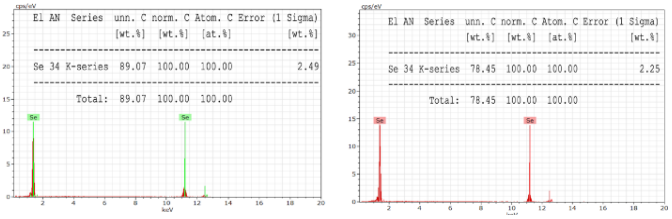

Fig. 6 EDX spectrum of a) Green and b) Chemical synthesized SeNPs

3.1.4 Energy Dispersive X-Ray analysis

The EDX spectrum of green and chemical synthesized SeNPs was analysed between 0 to 10 KeV represented in Fig. 6. The elemental composition of green SeNPs was 89.07%; meanwhile, for chemical SeNPs was 78.45% by the presence of a strong signal from Se atoms. Kokila et al.,

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reported that in biosynthesized SeNPs, Se atom's elemental composition was 94.44% [35]. Yu et al., reported that the Se atom's composition level was 85.5% [36].

3.1.5 Fourier Transform Infra-red Spectroscopy

The functional groups that could cap the surface of the nanoparticles were confirmed by the FT-IR shown in Fig. 7. The figure shows the FT-IR spectrum of leaf extract (a), green synthesized SeNPs (b) and chemical synthesized SeNPs (c). In green synthesized SeNPs a broad band was observed between 3500 cm^{-1} to 3000 cm^{-1} which is due to (O-H) hydroxyl stretch indicating the water as moisture. The band at 2919 cm^{-1} explains the O-H stretch of a carboxylate group. 1613 cm^{-1} to 1234 cm^{-1} due to N-H bending of amines. 1020 cm^{-1} happens due to the stretching of the C-N stretching of amine. The short intense peaks between 780 cm^{-1} to 420 cm^{-1} depict the Se stretching bands. However, in chemical synthesized SeNPs a broad absorption peak of around 3410 cm^{-1} was due to the stretching of the vibration of O-H groups on the surface of SeNPs. The short peak at 3010 cm^{-1} and 2919 cm^{-1} is because of the N-H stretching of alkane. 1484 cm^{-1} found the O-H bending of molecularly absorbed water molecules. The bands between 800 to 540 cm^{-1} were described as the strong vibrations of Se bonds. FT-IR spectrum explains the information about the interaction between the functional groups of the plant biochemicals and the nanoparticles. From these results, we can get an idea of the different groups involved in surface functionalization. Shifts were identified from 1613 cm^{-1} to 764 cm^{-1} in green synthesized SeNPs respectively, which corresponds to amines and phenolic groups capping around the SeNPs. Phenol and amide-capped nanoparticles were reported to express significant biomedicinal values than uncapped nanoparticles [37]. These phenolic and amide groups of leaf extract act as a capping agent on the surface of the SeNPs and prevent them from aggregation. Hence, we could justify a supportive correlation between the electron microscopy (SEM & TEM) and the FT-IR report of the green synthesized SeNPs, both results confirming the stability and dispersibility of the nanoparticles. Previous reports of FT-IR were also used as evidence for the confirmation of SeNPs [38, 39]. Taken together the UV-Vis, XRD, Electron microscopy, EDX and FT-IR results show that the optimized protocol has generated SeNPs stabilized with phytochemicals of leaf extract and hence this can be referred to as green synthesized SeNPs.

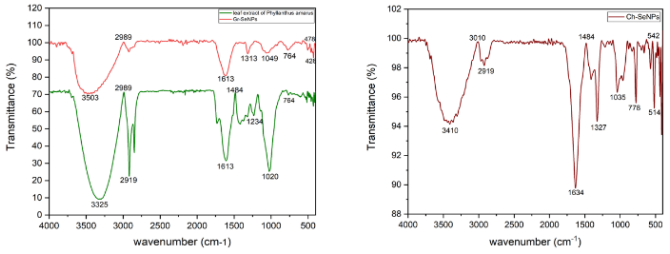


Fig. 7 FTIR spectrum of a) Green and b) Chemical synthesized SeNPs

3.2 Anti-Bacterial Assay

SeNPs testing concentration was fixed based on the earlier reports [38-41]. A single concentration (100 µg/mL) was chosen to test for antibacterial activity using 8 fish pathogens namely *S. pyogens, E. faecalis, M. luteus, S. aureus, S. maltophilia, A. hydrophilia, K. pneumonia* and *P. aeruginosa* by well diffusion method. The antibacterial activity of SeNPs was compared with the positive control streptomycin and the negative control DMSO. The inhibition zone is represented in Fig. 8 and Graph 1. The bactericidal activity of green SeNPs was higher in *S. aureus* (26.2 mm) and low in *P. kneumonia* (13.5 mm). However, green synthesized SeNPs were near to the positive control and higher than chemical SeNPs in all pathogens studied.

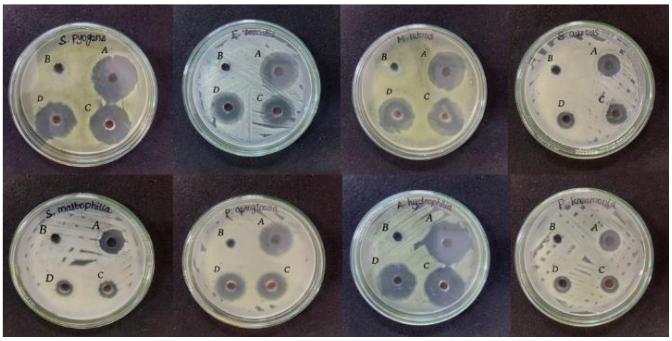
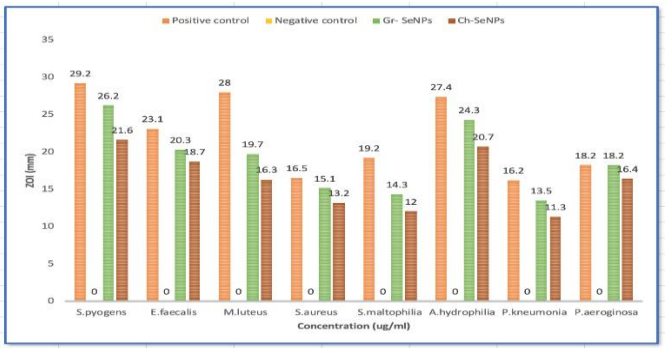


Fig. 8 Green and chemical synthesized SeNPs comparative antibacterial activity



Graph 1 Anti-bacterial assay of green and chemical synthesized SeNPs

The results of the antibacterial activity of SeNPs clearly suggest that green SeNPs using *P. amarus* leaf aqueous extract have a better antibacterial activity when compared to the chemical synthesized SeNPs. Numerous research conducted in recent years has demonstrated how selenium nanoparticles have anticancer, antioxidant, antibacterial, and anti-biofilm effects. These nanoparticles have demonstrated exceptional antibacterial action so far against pathogenic bacteria, fungi, and yeasts [29]. Filipovic et al., studied that SeNPs have the potential for antimicrobial activity as well as anti-biofilm activity against numerous pathogens [42]. The phenolic compounds effects on bacterial cells have been, to a certain extent, referred to as damage to the bacterial membrane, inhibition of virulence factors such as enzymes and toxins, and suppression of bacterial biofilm formation [43]. Bouarab et al., reported that many phenolic compounds can express high antibacterial activity [44]. With these studies, we suggest that the green synthesized SeNPs capped by phenolic compounds have better activity than chemical synthesized SeNPs to induce membrane damage and cause cell death against selective fish pathogens.

4. Conclusion

In this study, we have prepared SeNPs by i) using *P. amarus* leaf extract as a capping agent and ii) chemical method. Both nanoparticles were characterized by using UV-Vis Spectroscopy and exhibited absorbance peaks at 264 nm and 265 nm for green and chemical SeNPs. Electron microscopy found their morphology as spherical and size about 23.14 nm and 44.86 nm for green and chemical SeNPs. Elemental composition for green and chemical SeNPS are 89.07% and 78.45% by EDX analysis, crystallinity using XRD revealed 32.13 nm and 33.41 nm for green and chemical SeNPs and different functional groups which were present in the synthesized green and chemical SeNPs were confirmed by FT-IR. The synthesized green and chemical SeNPs, which were capped and stabilized by phenols and amides of leaf extract, were found to be less in size and more dispersed than chemical SeNPs. Furthermore, the leaf extract-capped SeNPs exhibited significant antibacterial activity against fish pathogenic bacteria, which was comparable with the standard antibiotic than chemical mediated SeNPs. Based on these results, concluded that the green synthesized SeNPs may have efficient bio-medicinal applications when compared to the chemical SeNPs due to their enhanced dispersibility, stability and surface coating.

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