RESEARCH PAPER

Antioxidant, Cytotoxic, Antimicrobial Activities And Time Kill Kinetics Of Selenium Nanoparticles Synthesized From *Picrorhiza Kurroa* Root Extract

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ABSTRACT

Background: The present study addresses with the antioxidant, cytotoxic and antimicrobial activities and time kill kinetics of selenium nanoparticles synthesized through green methods employing extracts from *Picrorhiza kurroa.*

Methodology: The antimicrobial activity of the synthesized nanoparticles from *Picrorhiza kurroa* root was determined via the agar well diffusion method to evaluate the consistency of the inhibition zones against the tested organisms. A time kill curve assay was used to measure the response of organisms to exposure to selenium nanoparticles (Se-NPs) overtime and evaluate variations in sensitivity. The cytotoxic potential and the antioxidant activity were assessed via the Brine Shrimp Lethality Assay (BSLA) and the DPPH Radical Scavenging Assay (DPPH Assay), respectively.

Results: The selenium nanoparticles synthesised using *Picrorhiza kurroa* root extract exhibited significant antimicrobial activity against tested the microorganisms, with inhibition zones observed for *Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, Aspergillus fumigates and Aspergillus niger* at the highest concentration of 100 µl. In the time kill curve assay, 100µg/mL was the most effective concentration across all pathogens. Notably, its efficacy in both bacterial and fungal systems highlights the broad-spectrum antimicrobial potential of PK-SeNPs. The PK-Se-NPs had effective antioxidant potential, with a percentage of 90.3% in the DPPH assay at a maximum concentration of 50 µl. The result revealed a very minimal cytotoxic activity at a concentration of 80 µg/ml, which indicates biocompatibility of the synthesized nanoparticles.

Conclusion: The results of this study indicate that PK-Se-NP nanoparticle from *Picrorhiza kurroa* root exhibits antimicrobial and variable sensitivity, antioxidant, and cytotoxic properties, with their therapeutic potential enhanced by increasing concentrations, suggesting that they could be a promising drug delivery tool and serve as powerful alternatives for regular pharmaceutical drugs.

Keywords: Green synthesis of selenium nanoparticles, *Picrorhiza kurroa* root extract, Time kill kinetics assay

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1. Introduction:

Recently, nanotechnology has significantly advanced across various fields, including drug discovery and development. This increases and paves the way for new findings in disease pathophysiology and treatment options [1]. NPs are synthesized from various elements such as silver, gold, copper, zinc, selenium, and titanium. Different approaches such as physical, chemical and biological methods are used for the synthesis of metallic NPs.

Recently, selenium has attracted increasing attention and has been utilized in various applications. Selenium is an essential micronutrient and is present as a cofactor for various antioxidant enzymes, thus protecting cells

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from oxidative stress and reducing the damage caused by various diseases such as diabetes, cancer, hyperlipidaemia, and cardiovascular disorders [2]. It plays an important role in thyroid hormone metabolism and immune functions [3]. Green fabricated selenium nanoparticles (Se NPs) are more popular because of their ease of synthesis and use, eco-friendliness and bio-degradability.

Se-NPs have attracted increasing because of their excellent bioavailability, and relatively high adsorptive ability, which enables them to interact with numerous functional groups (i.e., N-H, C-O, COO and C-N) in humans [4, 5]. Comparatively, the use of plant extracts for nanoparticle synthesis is much easier and more cost effective than the use of microbes (such as bacteria and fungi) [6].

Picrorhiza kurroa is a tiny perennial herb that grows between 3,000 and 5,000 m above sea level and is a member of the Scrophulariaceae family. *Picrorhiza kurroa* has a long rootstock, creeps and grows in rock crevices and moist, sandy soil. The shapes of the leaves of *Picrorhiza kurroa* are flat, oval, and sharply serrated. The flowers are white or pale purple and bear on tall spikes, which appear from June through August, and the plant is harvested by hand from October through December. The roots and rhizomes contain many secondary metabolites such as Kutkin, apocynin, drosin, and nine cucurbitacin glycosides [7]. Kutkin is composed of kutkoside and iridoid glycoside picrosides I, II, and III. Apocynin is a catechol that has been shown to be a powerful anti-inflammatory agent whereas the cucurbitacins have been shown to be highly cytotoxic and possess antitumor effects [8].

The roots of *Picrorhiza kurroa* are traditionally used to cure various medical conditions such as diarrhea, nausea, vomiting, jaundice, eye infection, fever, skin problems, asthma, arthritis, scorpion sting, cancer, diabetes and gastrointestinal problems [9]. Root extracts of *Picrorhiza kurroa* scavenge oxygen-free radicals [10] such as superoxide and hydroxyl radicals, and inhibit lipid peroxidation induced by the Fe^{2+}ascorbate system in rat liver homogenates. Ethanol extracts of rhizomes accelerate the healing of gastric ulcerated stomach walls via free radical scavenging [11].

Thus, this study assessed the antioxidant, cytotoxic, antimicrobial and time-kill kinetics of selenium nanoparticles synthesized from roots of *Picrorhiza kurroa* in selected organisms. The agar-well diffusion method was used to identify the zone of inhibition and a time kill curve test was used to measures the rate and extent of microbe killing over time after exposure to an antimicrobial agent and to identify optimal dosages. Several physical and chemical techniques have been developed to synthesize Se NPs; however the use of the chemical compounds and residues of these chemicals limits the applications of the formed Se NPs in the pharmaceutical and medicinal fields. Although prior findings on SeNPs mediated by microbes and plant extracts are available, scientific literature on SeNPs fabricated from the roots of *Picrorhiza kurroa* is scarce. Therefore, this study proposes a novel approach for the biosynthesize SeNPs via *Picrorhiza kurroa* root extract and to exploration of various pharmacological properties.

2. Materials and Methods:

2.1 *Picrorhiza kurroa* collection process:

Picrorhiza kurroa was procured from regional herbal center in Chennai, Tamil Nadu, India. It was then identified and authenticated. The roots were cleaned, shade dried, blended into powder and retained for future research.

2.2 Preparation of *Picrorhiza kurroa* root extract:

One gram of powdered *Picrorhiza kurroa* root sample was combined with 100ml of deionized water in a beaker, and the mixture was boiled for 20 min. The extract was then allowed to reach room temperature and filtered through filter paper (Whatman filter paper No.1).

2.3 Biosynthesis of *Picrorhiza kurroa* Mediated Selenium Nanoparticles

Sodium selenite (Na_2SeO_3) (30 mM) was dissolved in 50 ml of distilled water, and 50 ml of *Picrorhiza kurroa* root extract was slowly added. The reaction mixture was then kept on a magnetic stirrer at 650-700 rpm for 48-72 hours to attain a uniform dispersion of nanoparticles, which is mandatory in nanoparticles synthesis [12]. A Change in the color of the solution was observed, which indicates the generation of nanoparticles (Figure 1).The solution was then investigated periodically using UV-vis-spectroscopy at 250–650 nm.

Figure 1: Biosynthesis of *Picrorhiza kurroa* root mediated selenium nanoparticles

2.4 Susceptibility testing

The agar well diffusion is a widely used preliminary screening method for determining antimicrobial activity. Selenium nanoparticles synthesized from *Picrorhiza kurroa* root extract were tested against selected organisms for comparison with standard antibiotics to identify the efficacy of nanoparticles [13]. For this experiment, 24hr freshly prepared microbial cultures were used. Muller-Hinton agar (MHA) was prepared and autoclaved for 30min at 121°C to sterilize it, after which it was transferred to sterile Petri dishes and allowed to solidify [14,15]. To prepare the wells, a 6-mm sterile metallic borer was placed 40 mm apart from one another and sealed. Different concentrations 25µg/mL, 50 µg/mL and 100 µg/mL of selenium nanoparticles were loaded into separate wells on agar plates in triplicate and tested against *Staphylococcus aureus, Streptococcus mutans, Enterococcus faecalis Aspergillus niger, and Aspergillus fumigatus*[16,17]. The plates were incubated for 24 h at 37°C. As a zone of inhibition, the diameters of the clear zones surrounding each well were measured. These results indicated the antimicrobial effectiveness of the PK-SeNPs. The antibiotics amoxicillin and fluconazole were used as the standards for bacterial and fungal cultures respectively [18].

2.5 Antioxidant activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to test the antioxidant activity of the biosynthesized selenium nanoparticles. The ability to transfer hydrogen bonds was assessed by observing the decolorization of a DPPH-containing methanol solution[19]. A total of 450 µL of 50 mM TrisHCl buffer (pH 7.4) and 1 mL of 0.1 mM DPPH in methanol were combined with varying quantities (10–50 µg/mL) of selenium nanoparticles interceded by *Picrorhiza kurroa* root extract, and the mixture was incubated for 30 minutes. The decrease in the amount of DPPH free radicals was subsequently evaluated on the basis of the absorbance at 517nm. To combat DPPH free radicals, BHT (butylated hydroxyl toluene) was used as a standard.

The percentage of inhibition was determined from the following equation:

$$\%\text{inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100$$

2.6 Brine Shrimp (Artemiasalina) Lethality Bioassay

The Brine Shrimp Lethality Assay (BSLA), also known as the Artemiasalina assay or simply the brine shrimp assay, is a widely used bioassay for preliminary assessment of the cytotoxic and pharmacological activities of various compounds.It is often utilized as a simple and inexpensive screening tool to assess the general toxicity of compounds. The toxicity of various concentrations of PK-Se-NPs. 200 ml of filtered water was used to dissolve 2g of iodine-free salt and Six-well ELISA plates were loaded with 10–12 ml of saline [22]. 10 nauplii were added to the wells (5, 10, 20, 40,80µl) and the 6th well was used a control. The nanoparticles were then introduced at the desired concentration level and incubated for 24 hours [23].After the incubation period; the number of surviving brine shrimp nauplii in each well was counted. If the compound is considered toxic, it causes a significant reduction in the number of surviving nauplii compared with that in the control wells as in Figure 2. The data obtained from the assay, typically expressed as the LC50 were analyzed to determine the concentration of the test sample required to kill 50% of the brine shrimp nauplii.

The percentage of live nauplii was calculated [24] as,

(Number of live nauplii/Number of dead nauplii + Number of living nauplii) * 100

Figure 2: Brine shrimp lethality assay setup for PK-Se-NP

3. Results:

3.1 Susceptibility Testing

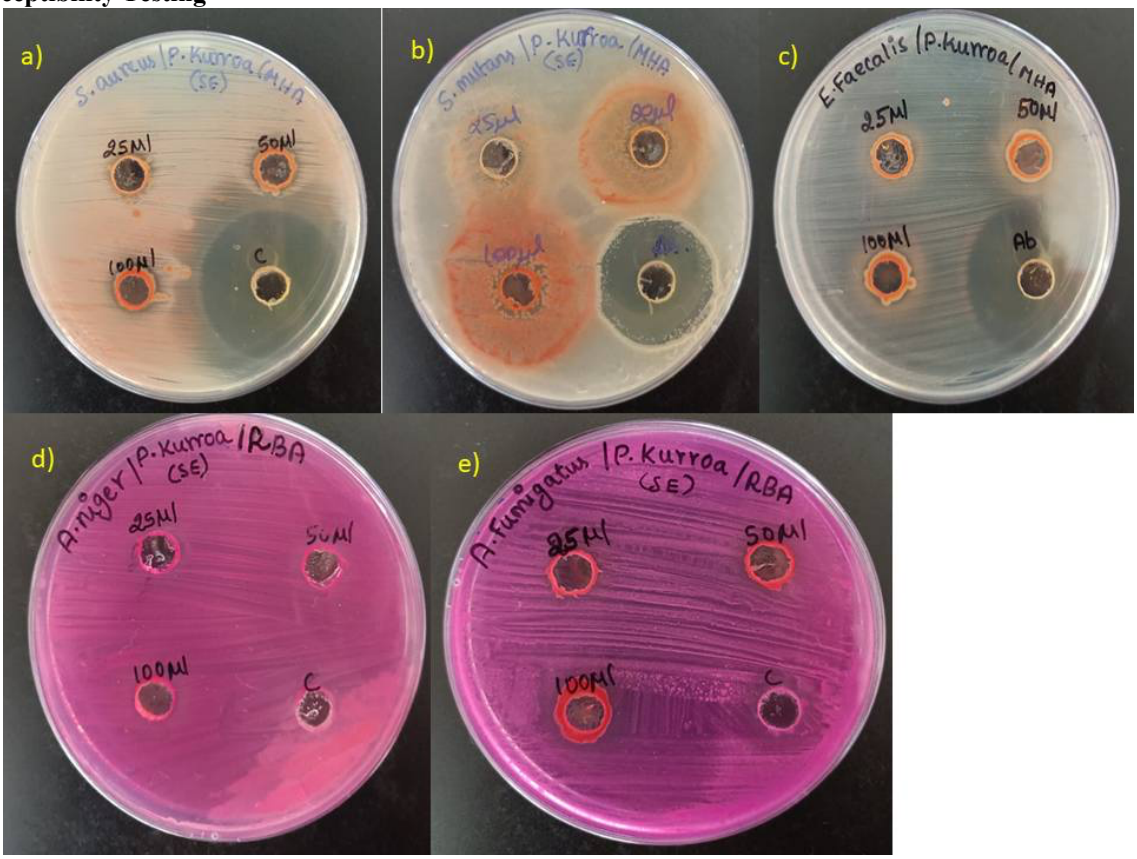

Figure 3: Antimicrobial susceptibility test of PK-SeNPs. Images showing the antimicrobial activity of *Picrorhiza kurroa* root mediated selenium nanoparticles against *a) Staphylococcus aureus b) Streptococcus mutans c)Enterococcus faecalis d)Aspergillus niger, e)Aspergillus fumigatus* at three different concentrations 25µg/mL, 50 µg/mL and 100 µg/mL

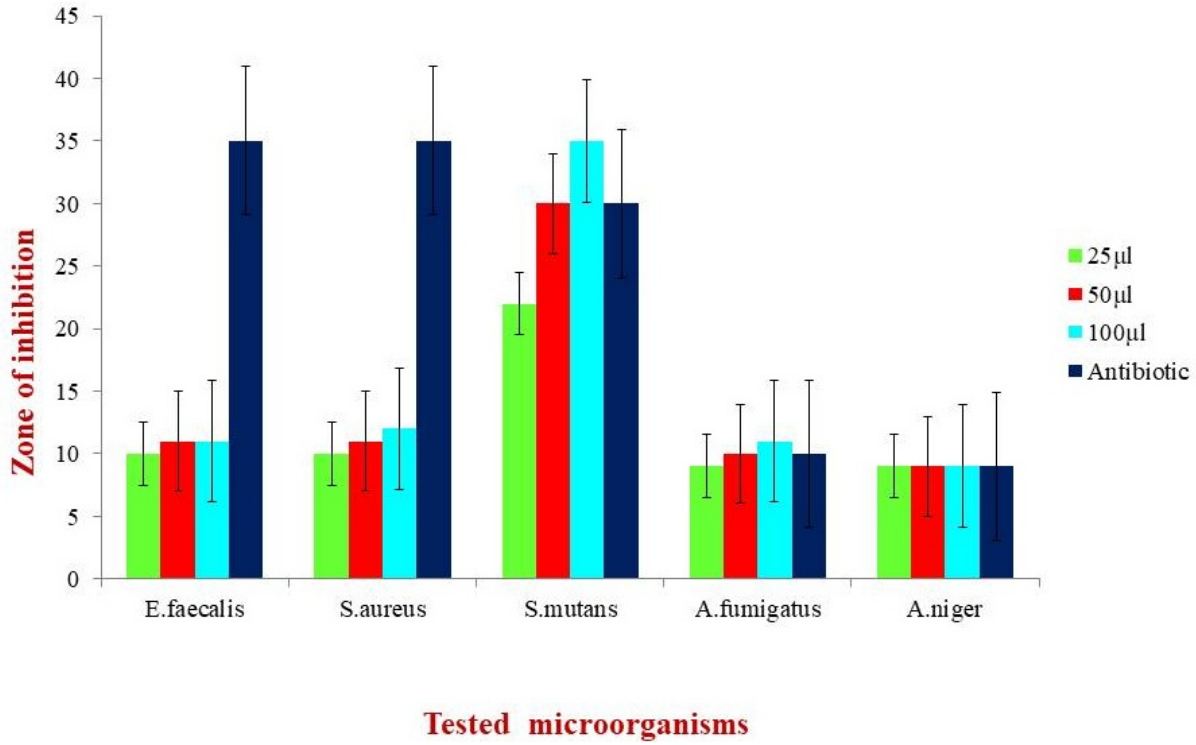

Figure 4: Agar well diffusion method to assess the antimicrobial activity of PK-Se-NPs

The synthesized PK-Se-NPs were tested against bacterial and fungal strains such as the agar well diffusion method. The antimicrobial activity of the prepared Se-NPs against the tested organisms is shown in Figure 3 & 4. The highest susceptibility was observed in the zone from 22mm (25µL) to 35mm (100 µL) which indicates that the potential antibacterial activity was observed against Streptococcus mutans, and that showed PK-Se-NPs were even more effective than antibiotics. However, the zone of inhibitions of Enterococcus faecalis and Staphylococcus aureus, showed modest inhibition. There was not much antifungal activity produced by the synthesized SeNPs. These clinical findings show that, selenium nanoparticles from *Picrorhiza kurroa* roots exhibit strong antimicrobial activity, especially against Streptococcus mutans, suggesting potential oral care formulations. Additionally, the antimicrobial efficacy was dose dependent, increasing with increasing SeNP concentration. However, more investigations are needed to optimize and evaluate the antimicrobial properties in various settings.

3.2 Time kill kinetics assay

A Time kill kinetics assay was conducted to evaluate the antibacterial activity of the green synthesized SeNPs,the results of which are shown in Figure 5. Three different concentrations of PK-Se-NPs (25 µg/mL, 50 µg/mL and 100 µg/mL) were tested and the results were compared with those of standards and control. The optical density (OD) values were measured at various time points (1-5hrs) to assess the growth inhibition of *Staphylococcus aureus.*

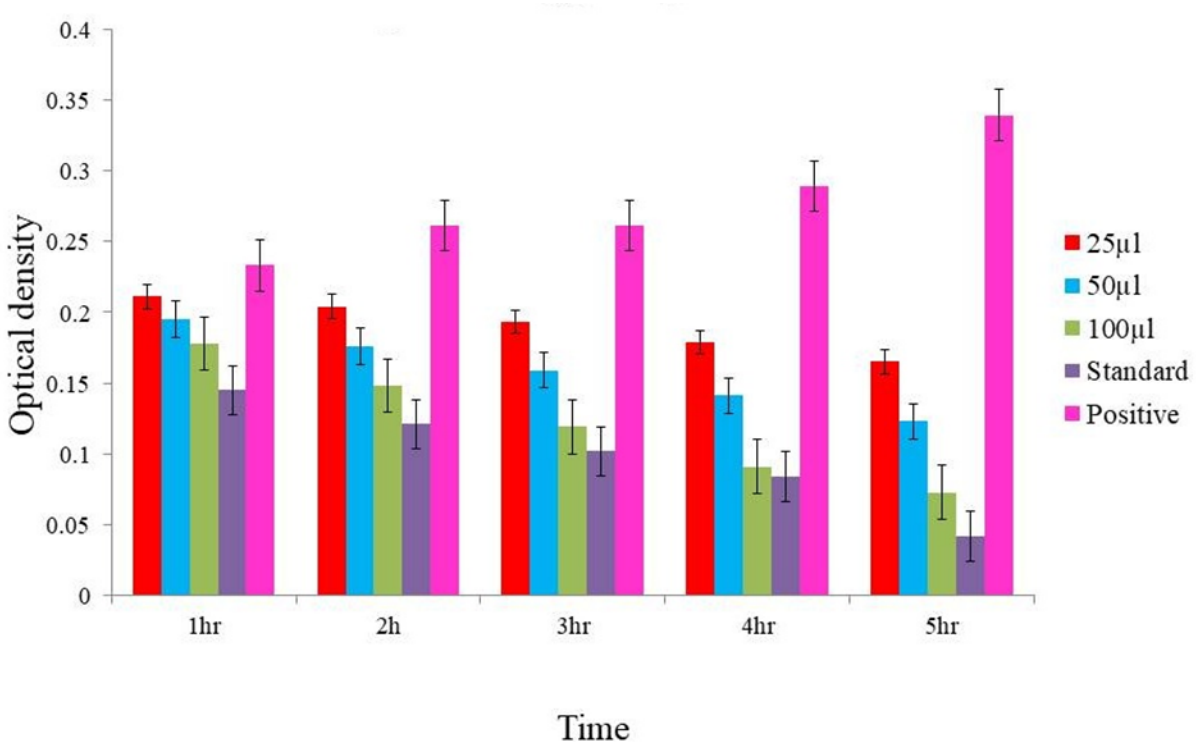

Figure 5: Time kill curve of green synthesized selenium nanoparticles against *Staphylococcus aureus*

At all tested time points, the OD values were recorded for each concentration of PK-Se-NPs, Standard and Control. The optical density values gradually decreased over time, indicating a time dependent inhibition of *Staphylococcus aureus* growth. The 100 µg/mL extract consistently resulted in the lowest OD across all time points, which indicates that the strongest inhibition of bacterial growth occurred at this highest concentration. The 50µg/mL SeNPs treated group presented moderate reduction; the 25µg/mL SeNPs treated group presented the least inhibition among the SeNP-treated groups, but was still significantly better than the control. All the SeNP treated groups showed a gradual decline in the OD over time (from 1hr to 5hr) confirming time dependent bactericidal activity which disrupted bacterial metabolism or the cell wall over prolonged exposure.

In this assay, amoxicillin was used as the standard antibiotic, which showed a very strong inhibitory effect, compared with to 100 µg/mL SeNPs at later time points (4-5hrs). The positive control group, which represented untreated *Staphylococcus aureus,* increased over time, indicating unrestricted growth. The OD values ranged from 0.2333 to 0.339.

The time kill curve assay results demonstrated that the PK-Se-NPs exhibited both concentration and time dependent antibacterial activity against *Staphylococcus aureus*. The 100 µg/mL dose was the most effective, showing significant growth inhibition as early as 2 hrs, sustained through 5hrs.Thus, the antibacterial efficacy of PK-Se-NPs at relatively high concentrations is comparable to that of the standard antibiotics, suggesting their potential for therapeutic use.

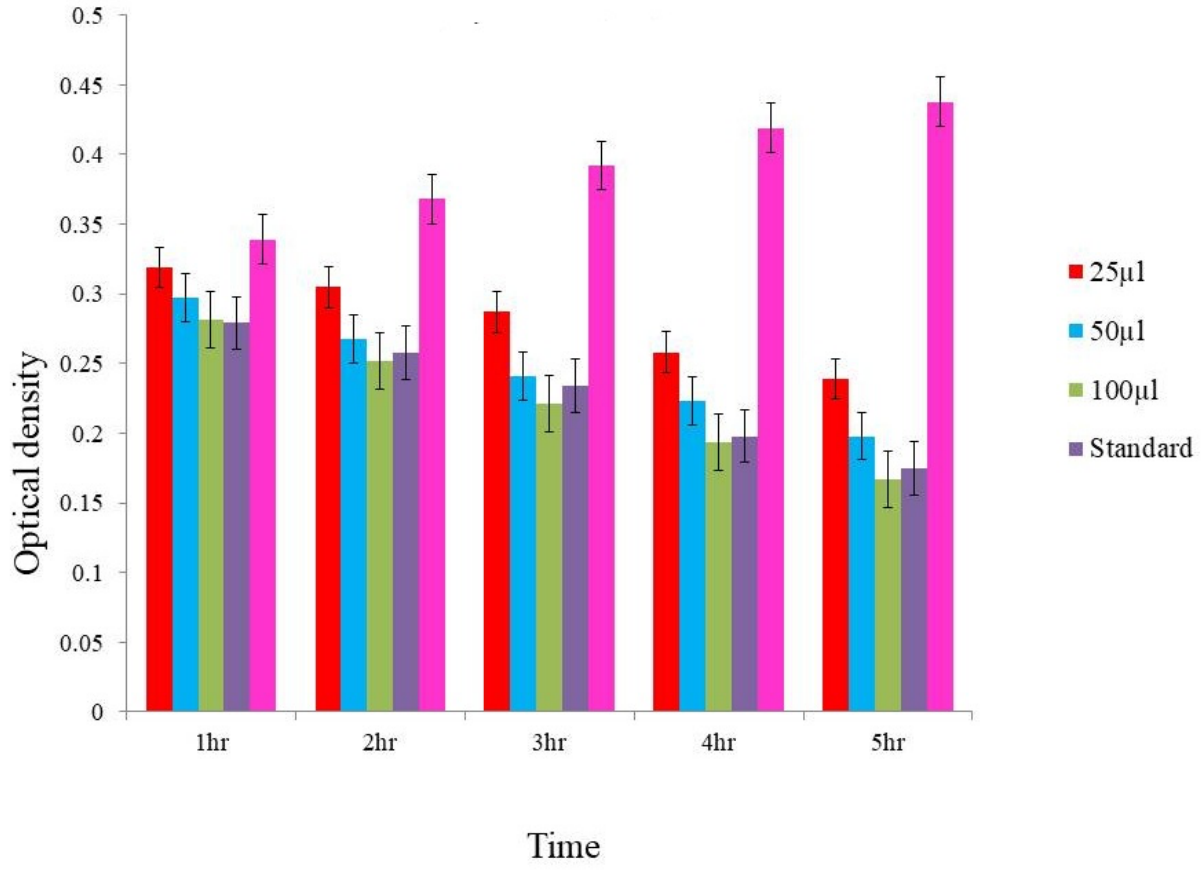

Figure 6: Time kill curve of green synthesized selenium nanoparticles against *Streptococcus mutans*

Figure 6, shows the time-kill curve of green mediated PK-Se-NPs against *Streptococcus mutans*. Three different concentrations of PK-Se-NPs (25µg/mL, 50µg/mL and 100 µg/mL) were tested and the results were compared with those obtained using a standard and control. The optical density (OD) values were measured at various time points (1-5hrs) to assess the inhibition of *Streptococcus mutan* growth. Compared with 25 and 50µg/mL, 100µg/mL consistently resulted in a lower OD compared to 25 and 50 µg/mL especially from 3 to 5 hrs, which indicates increased bacterial inhibition at higher PK-Se-NPs concentration. The 50 µg/mL extract had moderate inhibitory effect, whereas the 25µg/mL extract was the least effective. The OD values of the PK-Se-NP treated groups showing gradually decreased from 1 to 5 hours, indicating progressive bacterial suppression. The Most significant inhibition was observed between the 3rd and 5th hr at relatively high concentrations. The growth status of *Streptococcus mutans* is represented by the reported OD values at each time point. Greater inhibition of bacterial growth was indicated by lower OD values, which demonstrated the antibacterial activity of the tested compounds against the Streptococcus mutans, and confirmed their dose-dependent and time dependent antibacterial activity.

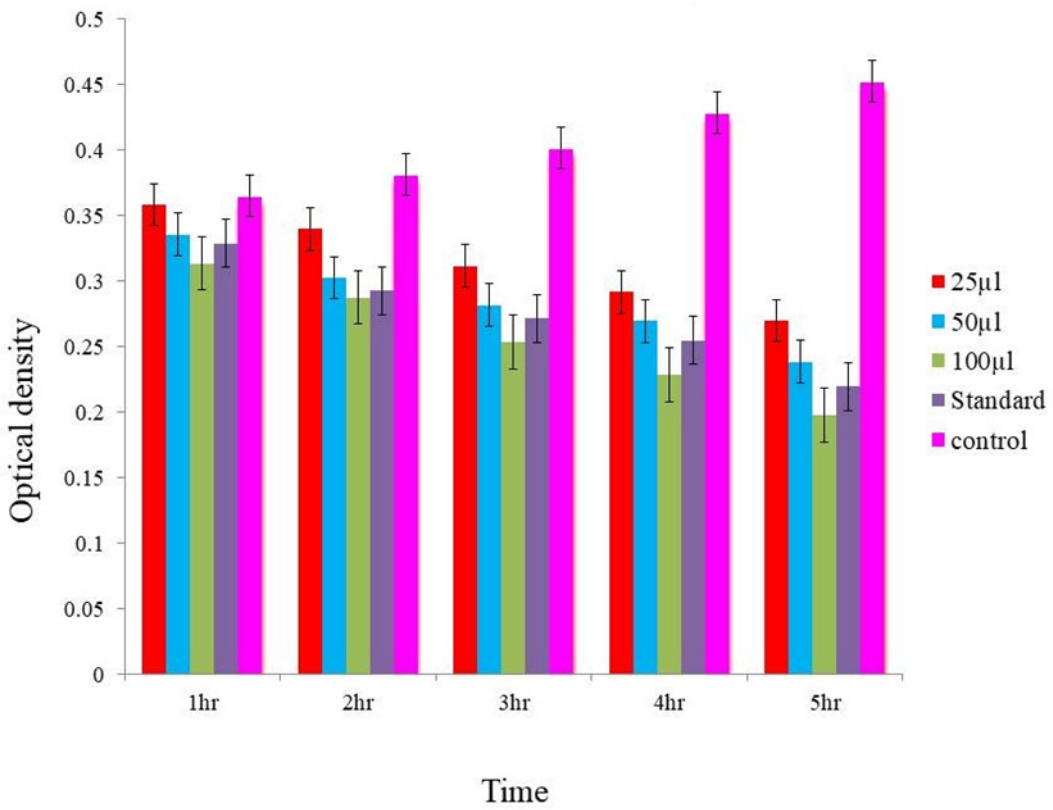

Figure 7: Time-kill curve of green synthesized selenium nanoparticles against *Enterococcus faecalis*

The anti-bacterial efficacy of green-mediated selenium nanoparticles against *Enterococcus faecalis* was examined using a time-kill curve experiment, as shown in Figure 7. Selenium nanoparticles at three different concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL) were assessed, and their effects were compared with those of a control and a plant extract. At several intervals (1-5hrs), optical density (OD) values were taken to evaluate the growth of *Enterococus faecalis.* The growth status of *Enterococcus faecalis* is represented by the reported OD values at each time point. Greater inhibition of bacterial growth was indicated by lower OD values, which demonstrated antimicrobial efficacy of the tested compounds' against *Enterococcus faecalis*.

The 100 µg/mL mixture showed the strongest antibacterial activity with the steepest decline in the OD, at the 3rd hour indicating significant antibacterial efficacy, closely matching the effect of amoxicillin. However, 50µg/mL resulted in moderate inhibition, and 25µg/mL, resulted in the least inhibition, although it was still significantly better than that of the control. These findings support the potential of PK-Se-NPs as novel green antibacterial agent for treating resistant gram positive organisms such as *Enterococcus faecalis.*

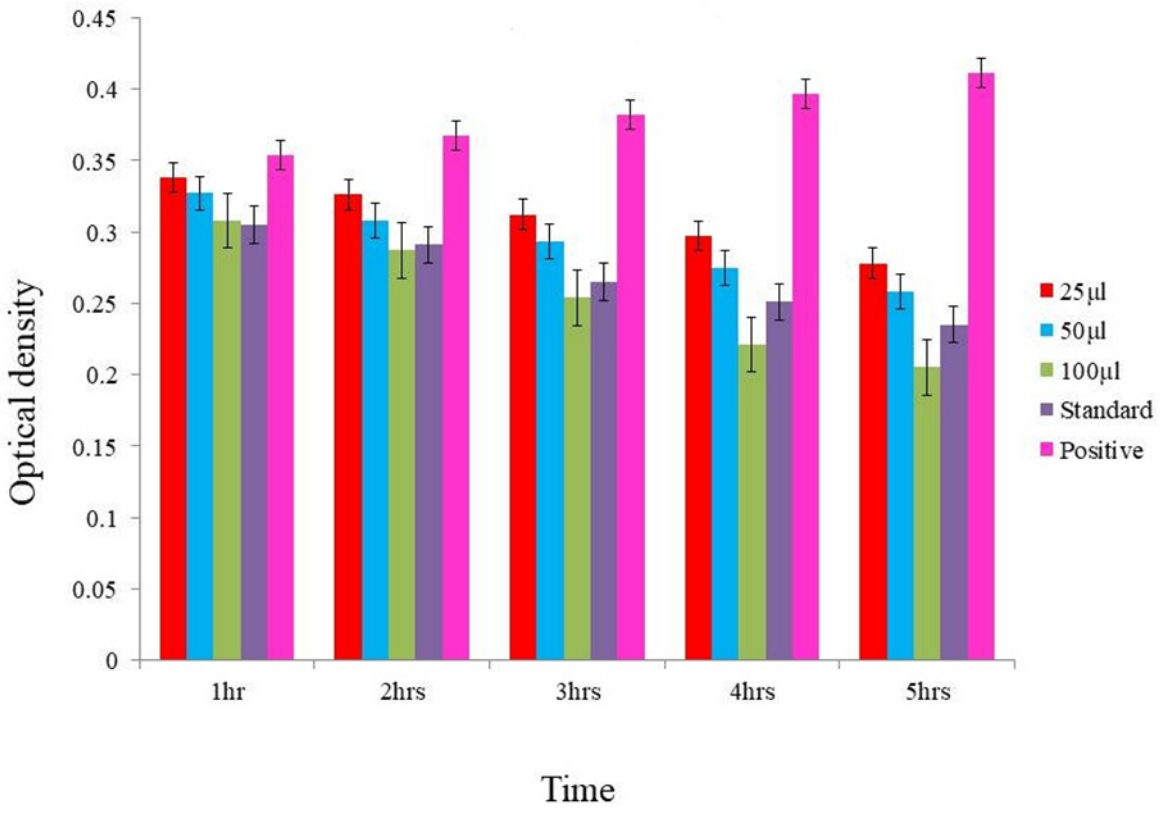

Figure 8: Time-kill curve of green synthesized selenium nanoparticles against *Aspergillus niger*

The antifungal activity of green-synthesized PK-SeNPs at different concentrations (25 µg/mL, 50 µg/mL, 100 µg/mL) over a 5-hour period. The results were compared with those of a standard antifungal (fluconazole) and a positive control**,** and the optical density (OD) was used to assess fungal growth inhibition as shown in Figure 8. A progressive decrease in the OD was used over time was observed in all the treated groups, indicating the inhibition of fungal growth. Among the PK-SeNP treatments,100 µg/mL exhibited the greatest antifungal effect, with a sharp decline in the OD over time. The 50µg/mL treatment resulted in moderate inhibition in the least, while the 25 µg/mL treatment resulted in the least amount of inhibition. This dose-dependency clearly supports the increasing efficacy with increasing PK-Se-NP cconcentrations.

The PK-SeNPs demonstrated prominent antifungal activity against *Aspergillus niger*. The standard antifungal showed consistent and effective inhibition**,** although 100 µg/mL PK-SeNPs approached this efficacy by 5 hours. The 100 µg/mL dose is nearly as effective as fluconazole is, making it a strong candidate for antifungal therapy. Both dose- and time-dependent inhibition were observed, with 100µg/mL being the most effective. These results indicate that PK-SeNPs have potential as an eco-friendly, effective alternative to conventional antifungals such as fluconazole.

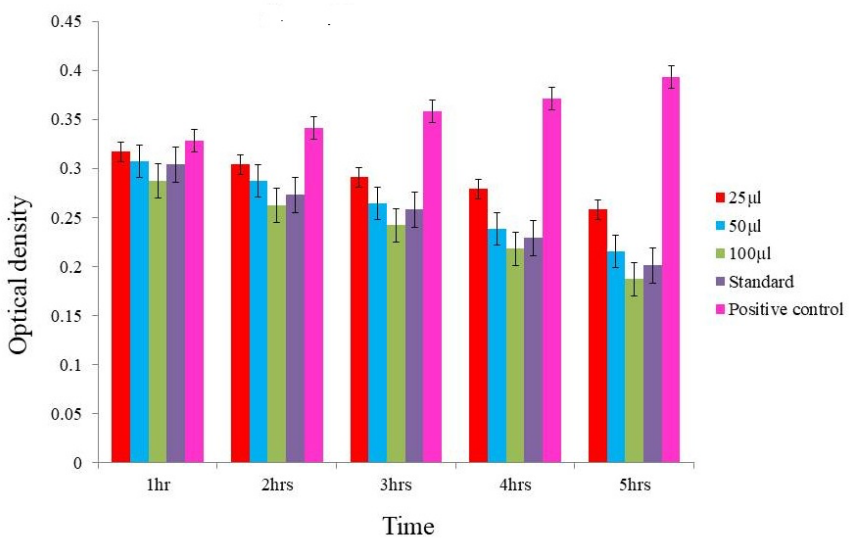

Figure 9: Time-kill curve of green synthesized selenium nanoparticles against *Aspergillus fumigatus*

Figure 9 shows the antifungal activity of green synthesized selenium nanoparticles (PK-SeNPs) at various concentrations (25 µg/mL, 50 µg/mL, and 100 µg/mL) against *Aspergillus fumigatus*. Optical density (OD) measurements over 1–5 hours were used to monitor fungal growth. OD values decrease over time in all treated groups (PK-SeNPs and fluconazole), indicating effective fungal growth inhibition**.** 100 µg/mL PK-SeNP shows the maximum inhibition, with OD decreasing significantly from 1hr to 5hrs.50 µg/mL shows moderate inhibition, whereas, 25 µg/mL has the least inhibition among the PK-SeNPs.

The performance of 100 µg/mL SeNPs approaches that of standard, particularly by the 5th hour**,** indicating comparable antifungal activity**.** The standard antifungal agent consistently and strongly inhibited *Aspergillus fumigatus* at all-time points. PK-SeNPs exhibit potent antifungal activity against *Aspergillus fumigatus* with both concentration- and time-dependent effects**.**

3.3 Antioxidant activity

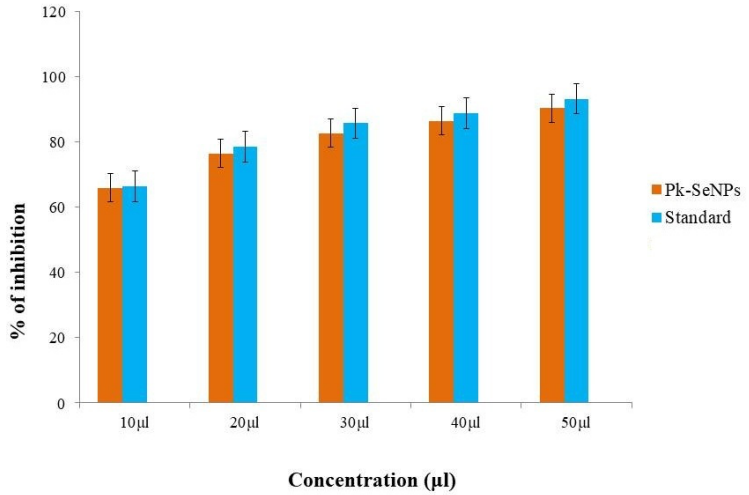

Figure 10: DPPH antioxidant activity of PK-Se-NPs at various concentrations compared with standards

In present study, a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was used to examine the antioxidant capacities of selenium nanoparticles *from Picrorhiza kurroa* root. The percentages of inhibition at various doses (10–50 µg/ml) are shown in Figure 10. The absorbance values gradually decreased as the concentration increased. The Percentage of inhibition of DPPH free radicals was 65.89 at 10µl, 76.37 at 20µl, 82.63 at 30µl, 86.38 at 40µl, and 90.26 at 50µl. A comparison of the extract and standard revealed that the lowest concentration (10µl) PK-Se-NPs inhibited 65.89%, whereas at the highest concentration (50µl), the inhibition reached 90.26%. The proportion of inhibition increased with increasing concentration, whereas the activity increased with increasing dosage volume. Thus, this study supported the antioxidant ability of selenium nanoparticles induced by *Picrorhiza kurroa* root extract.

3.4 Cytotoxic activity

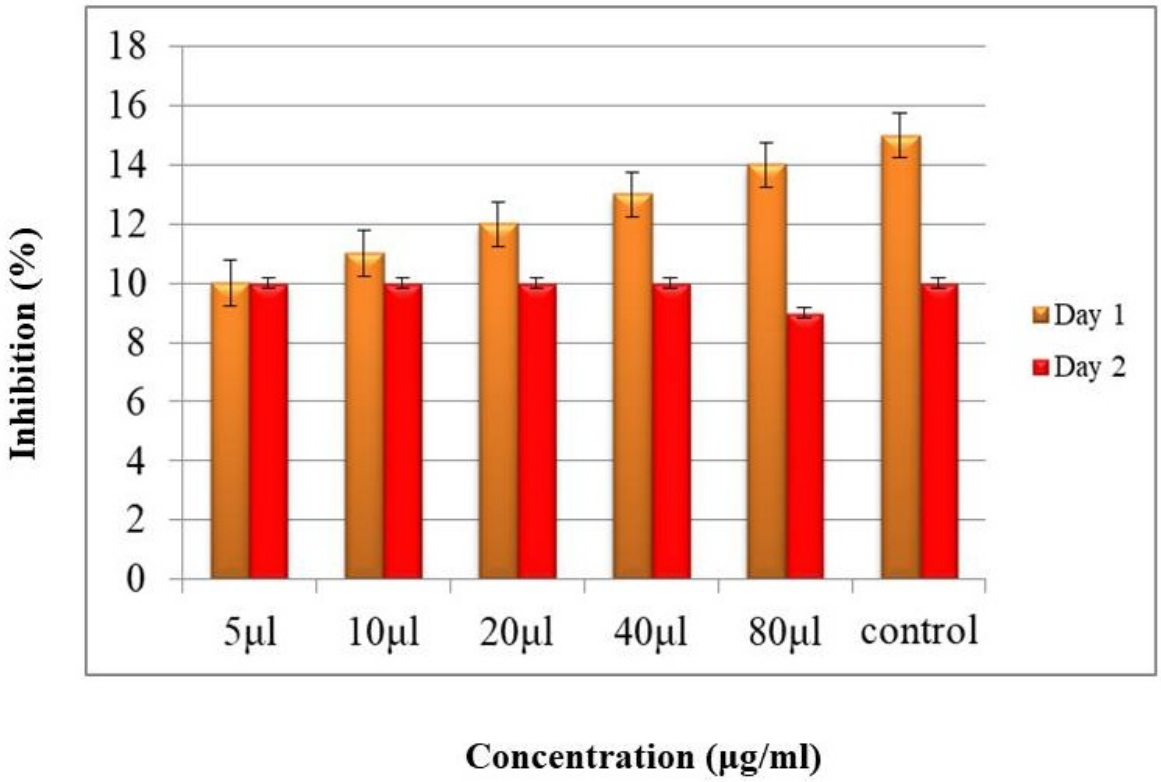

Figure 11: Graph showing the cytotoxic activity of PK-Se-NP

The cytotoxic effect of the PK-Se-NPs was evaluated using a brine shrimp lethality assay as shown in Figure 11. By examining the different fractions, at increasing concentrations, the naupliis were killed and their growth was inhibited; however, at a concentration of 80µg/ml, the resulting cytotoxicity demonstrated very little cytotoxic activity, suggesting that their use in ethno medicine could be performed with fewer adverse effects.

4. Discussion:

Green synthesis, a sustainable method of nanoparticle synthesis, uses plant extracts to reduce metal ions and stabilize nanoparticles, promoting environmental sustainability and reducing the ecological footprint[25,26].Compared with chemical methods,plant extracts offer cost-effective, biocompatible nanoparticles, with unique properties such as stability, solubility, and functionalization potential [27,28]. These plant-derived nanoparticles are more biocompatible and suitable for medical and pharmaceutical applications.

In a previous study, selenium nanoparticles synthesized from *Solanum lycopersicum* exhibited significant antimicrobial activity *against Escherichia coli, Bacillus subtilis ,Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus and Micrococcus leuteus.* It is also exhibited potent antioxidant activity with high a absorbance value [29].

The antibacterial activity of selenium nanoparticles from *Picrorhiza kurroa* extracts was tested against *Enterococcus faecalis, Staphylococcus aureus, Streptococcus mutans, Aspergillus fumigatus and Aspergillus niger.* Antibacterial activity against Staphylococcus aureus and Enterococcus faecalis was detected at all concentrations of the extracts. At a concentration of 100µl, the PK-Se-NPs exhibited the maximum antimicrobial activity *against Streptococcus mutans(35mm), Aspergillus fumigates (11mm), Staphylococcus aureus(12mm),* with no inhibition against *Aspergillus niger*. However, compared with the standard amoxyrite and fluconazole, the prepared PK-Se-NPs demonstrated slightly lower antimicrobial activity, except *Streptococcus mutans*. The zone of inhibition may be due to the adherence of Se-NPs to the bacterial cell wall where these nanoparticles release and destroy the bacterial cells.

5. Conclusion:

Numerous pathogens are known to cause a wide range of severe infections in humans. Therefore, there is a need for therapeutic agents for such infections. Recently, plant derived nanoparticles have attracted the attention of exploitation of several methods. The green synthesis method has attracted increasing interest because of its size dependent physiochemical properties, non-toxicity, moderate temperature, availability, and cost. Selenium nanoparticles may serve as a better alternative to conventional treatments with greater efficacies. Herbal formulations with *Picrorhiza kurroa* mediated selenium nanoparticles seem to be better alternatives with good antimicrobial

activity. Although many studies have been conducted, this study explored the pharmacological properties of PK-Se-NPs, and demonstrated their effectiveness and further studies to determine their effects on pathogens are needed. Additionally exploring the mechanism at the molecular level is the need of the hour. Hence, a blend of phytocompounds and nanoparticles effectively contributes to its diverse range of pharmacological effects.

CRediT authorship contribution statement

Subbulakshmi Packirisamy: Investigated and drafted the manuscript, and final structure and compilation of the draft. **Deepa Rajendiran**: Draft-writing and data analysis. **R.J.Vijayashree:** Editing, data analysis. **Bhavani Ganapathy** reviewed and visualization.

Declaration of competing interest

The authors hereby declare that they have no conflicts of interest.

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