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Curcumin Coated *Orthosiphon Stamineus* Leaf Extract Based Selenium Nanoparticle for Potential Tissue Engineering Applications

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Abstract

Curcumin is a type of polyphenol phytochemical that is bright yellow in colour and produced by the plant Curcuma longa. Despite its pharmacological properties, curcumin has low bioavailability, poor solubility, and undergoes rapid degradation. Nanoparticles (NPs) are used as a nanocarrier for drug delivery, to improve the stability and pharmacokinetics of the drug. Therefore, by coating curcumin over selenium NPs (SeNPs), the bioactivity, bioavailability, stability, and may increase the solubility of curcumin of SeNPs. This study aimed to synthesize the SeNPs from Orthosiphon stamineus leaf extract and coat it with curcumin and to characterize it and check its biocompatibility. Biosynthesis of SeNPs was carried out using plant extract of Evolvulus alsinoides and characterized using UV spectrophotometer, FT-IR, and SEM. Annexin V PI apoptotic and Hemolytic assay were used for checking biocompatibility. The UV-Vis spectrum gave a strong peak at 265 and 423 nm at various time intervals, indicating the SeNPs formation. Similarly, FT-IR has strong absorption bands at 3279, 1284, 1072, 1028, and cm^{-1} with wavelengths ranging from 4000-500 cm^{-1}. SEM analysis of biosynthesized SeNPs showed a spherical shape. Our results suggest that curcumin-coated SeNPs possess greater biocompatibility towards PBMCs which was evaluated by Annexin V - PI assay and erythrocytes by hemolytic studies. Curcumin-coated Selenium nanoparticles were successfully synthesized by the biological method using leaf extract of Orthosiphon stamineus and reported as biocompatible using Flow cytometry. But a more detailed study should be done for implementing it in tissue engineering.

Keywords: *Selenium nanoparticle, Curcumin, Orthosiphon stamineus, Tissue Engineering.*

Introduction

Nanotechnology has become a prominent area of research in recent years, and its implications have been explored in almost all fields including tissue engineering, regenerative medicine, cancer therapy, diagnosis, etc [1–3]. Nanotechnology usually involves the synthesis of nanoparticles (NPs) with sizes ranging from 10-100 nm and this size is known to be ideal for its application not only in the biomedical field but also in many industrial applications. One of the important applications of nanotechnology is target-specific drug delivery. NPs possess unique physiochemical, optical, and biological properties [4–8]. NPs are used as a nanocarrier for drug delivery, to improve the stability and pharmacokinetics of the drug, and are also used as an alternative for antimicrobial agents [9, 10] Selenium is a nutritional element that is required in trace amounts by the body for many metabolic pathways and also it exists in different oxidation states [10–12] Selenium has got innumerable applications in various fields such as metallurgical, medical, and chemical industries [13]. Selenium is an integral constituent that acts as an antioxidant and is also an important component of the glutathione peroxidase enzyme [14]. But selenium nanoparticles

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(SeNPs) differ from selenium elements. When compared with organic and inorganic selenium, SeNPS are found to be more biologically active [15]. SeNPs possess a high surface-to-volume ratio and unique physical, chemical, and biological properties [16–19]. SeNPs are potent bioactive NPs with low toxicity, exhibit some important activities, such as anti-oxidant, anti-microbial, anti-viral, and anti-cancer activities, higher free radical scavenging activity, and act as a chemopreventive agent [9, 20, 21]. SeNPs possess greater stability, bioavailability, and biocompatibility. Hence, SeNPs are known for their excellent biochemical properties and act as a carrier for drug delivery. But the major drawback of SeNPs is their poor cellular uptake. NPs can be synthesized by physical, chemical, or by biological methods. Among the well-known methods for the synthesis of nanoparticles, green synthesis has gained importance due to its advantages over other methods as it is non-toxic, eco-friendly, and low-cost [11, 22–26]. So green synthesis is employed for the synthesis of SeNPs. Fabrication of SeNPs from plant extracts is known as green synthesis.

Phytochemicals are secondary metabolites produced by plants as a mechanism of defense against pathogens [27–29]. The compounds have been a part of traditional medicines for decades. Therefore, they are being explored for their medicinal properties and are now used in pharmaceutical, industrial, and food industry applications as well. The most studied and used phytochemicals are flavonoids, alkaloids, terpenoids, saponins, carotenoids, and other aromatic and organic acids. Such chemicals possess anti-microbial, anti-fungal, anti-carcinogenic, anti-mutagenic, anti-inflammatory, anti-oxidative, and much more properties [30].

Curcumin is a type of polyphenol phytochemical that is bright yellow in colour and produced by the plant *Curcuma longa* species which belongs to the family Zingiberaceae [31]. They are practiced as a part of Indian and Chinese traditional medicine for centuries and are still being explored for their various medicinal applications. *Orthosiphon stamineus* commonly known as *misai kucing* and *kumis kucing* belonging to the family Lamiaceae is a widely used plant in tropical countries. The leaves of this plant commonly called “Java Tea” are extensively used for their ability to treat conditions such as diuretic, arthritis, abdominal pain, kidney and bladder inflammation, edema, and hypertension [32]. It is also reported to possess pharmacological properties, such as antibacterial, antifungal, anti-tumor, immunoregulation, antidiabetic, and antioxidant activities [33, 34]. Phytochemical analysis of *Orthosiphon stamineus* interpreted the presence of bioactive constituents such as lipophilic flavonoids, alkaloids, glycosides, terpenoids, polyphenols, essential oil, sterols, etc. Among all, flavonoids are the most important constituents in the leaf, and the presence of almost 20 different phenolic compounds has been reported [35, 36]. Due to its immense active compounds, *Orthosiphon stamineus* is used for the green synthesis of selenium nanoparticles. The phytochemicals such as flavonoids, and phenolic compounds, in orthosiphon stamineus act as bioreduction, while carbohydrates, and proteins act as stabilisers for the synthesis of SeNPs. SeNPs is used as a drug carrier for curcumin. Despite its pharmacological properties, curcumin has low bioavailability, poor solubility, and undergoes rapid degradation [37].To enhance the activity of curcumin, it is coated over SeNPs. The coating of curcumin over SeNPs will increase the bioactivity of SeNPs and also increase the bioavailability, stability, and solubility of curcumin.

Materials and Methods

Collection of Sample

The leaf extract of *Orthosiphon stamineus* was used for the NPs preparation. The plant’s leaves were obtained from the nearby village in Thiruvallur District, India, and authenticated by a botanist.

Chemicals and Reagents

Dulbecco's Modified Eagle Medium (DMEM), antibiotic/antimycotic solution, fetal bovine serum, and Trypan Blue were all procured from Himedia. Propidium iodide and Annexin V were purchased from Sigma Aldrich, India. Sodium selenite was procured from SRL. All other reagents used were of analytical grade and MilliQ water was used throughout the study.

Preparation of Extract

The leaves of the collected *Orthosiphon stamineus* were washed several times with distilled water to remove residual impurities from the leaves.

The plant was dried at room temperature and ground into a coarse powder. 2 g of coarse powder (*Orthosiphon stamineus*) was mixed with 50 ml of double distilled water and boiled for 30 minutes. Then the extract was filtered using Whatman filter paper. This filtrate was then used for further synthesis of selenium nanoparticles.

Synthesis of SeNPs

5 ml of extract was added to 45 mL of 20 mM of sodium selenite and stirred at 40°C for 3 h. A pale green colour solution turns to ruby red followed by 24 h incubation. The SeNPs formed was separated by cold precipitation followed by lyophilization. The dried powder was used for further curcumin functionalization and characterization studies.

Functionalization of Curcumin

About 500 µL 0.4 % PEG was added to the 10 mL solution of selenium nanoparticles (2 mg/mL) and stirred for 1 h at room temperature. Then the curcumin solution (5 mg/mL) was added to this and stirred for 30 minutes. Then the precipitate was centrifuged for 10 minutes at 10,000 rpm and the curcumin functionalized nanoparticles were collected. This precipitate was subjected to freeze drying and used for further characterization and biocompatibility studies.

Characterization of Nanoparticles

Curcumin-coated selenium nanoparticles prepared using *Orthosiphon stamineus* plant extract was characterized using a UV-Visible spectrophotometer, Fourier-transform infrared spectroscopy (FT-IR) and Scanning Electron Microscopy (SEM). The maximum absorbance of SeNPs was determined using a UV-Visible spectrophotometer (Jasco) by screening between 350-700 nm. The presence of a functional group in SeNPs was determined by FT-IR (Bruker) at a scan range of 3500 to 500 cm^{-1} with a scanning speed of 4 cm^{-1} in ATR mode. Scanning electron microscopy (SEM) (JEOL JSM –IT800 SEM, Japan) was used to examine the surface morphology of SeNPs.

Biocompatibility of SeNPs in Peripheral Blood Mononuclear Cells (PBMC)

For determining the biocompatibility of SeNPs in PBMCs, an Annexin V - PI apoptosis assay was performed. Following the approval of the Institute Human Ethical Committee, blood was collected from healthy donors. 2 ml of blood was added over 2 ml of HiSepTM LSM 1077 medium and centrifuged to isolate PBMCs. The viability of PBMCs was confirmed using Trypan Blue Assay. Equal volume PBMCs were seeded in six-well plates and treated with 100 µg of biogenic SeNPs and incubated for 12 h. Untreated cells were used as control and were incubated for 12 h. After incubation, cells were harvested, centrifuged, and the supernatant was discarded and resuspended in a binding buffer. The cells were then stained using Annexin V FITC (5 µl) and Propidium Iodide (5 µl) and incubated at room temperature for 15 minutes. After incubation, 400 µl of 1X binding buffer was added and acquired (10000 events) using BD FACS Lyric flow cytometer, and the treated cells were observed for apoptosis. The analysis was performed using FAC suite 4.1 software.

Hemolytic Assay

To determine the toxicity of functionalized SeNPs, the hemolytic assay was performed. The

amount of hemoglobin released from erythrocytes after treatment with functionalized SeNPs determines its toxicity. Protocol for this assay was carried out as per the previously reported protocol by [38]. Human blood was centrifuged at 1500 ×g for 5 minutes and plasma was discarded to collect the erythrocytes (RBCs). Phosphate-buffered saline (PBS) with pH 7.4 was used to wash the isolated RBCs thrice. After washing, the obtained RBCs were diluted to 10 % of their initial concentration using PBS to get erythrocyte suspension. 200 µl of erythrocyte suspension was added to samples of varying concentrations (12.5, 25, 50, 100, and 200 µg/mL) and the sample was made up to 1 ml using PBS. The contents were incubated for 1 h at 37°C and centrifuged at 1500 ×g for 5 minutes. After centrifugation, the supernatant was loaded in 96 well plates and the absorbance was measured at 540 nm in an ELISA plate reader. Cells treated with PBS were taken as negative control and the cells treated with deionized water were taken as a positive control. The procedure was done in triplicates. The percentage of hemolysis was calculated using the formula [39],

Results

In this current study, the synthesis of curcumin-coated selenium nanoparticles using *Orthosiphon stamineus* plant extract was performed and the characterization of SeNPs using UV-Visible Spectroscopy, FT-IR, and SEM analysis was analyzed and the biocompatibility of SeNPs was analyzed by Hemolytic and Annexin V PI apoptotic assay.

Characterization of SeNPs

The UV- Visible spectral analysis for SeNPs showed a typical surface plasmon resonance (SPR) peak with maximum absorbance at 265 and 423 nm. Figure 1 represents the UV spectra of SeNPs. The UV-visible spectral analysis for SeNPs showed a typical surface plasmon resonance (SPR) peak with maximum absorbance at 265 nm, confirming the SeNPs formation, which is evidenced by the shift in maximum absorbance compared to the extract.

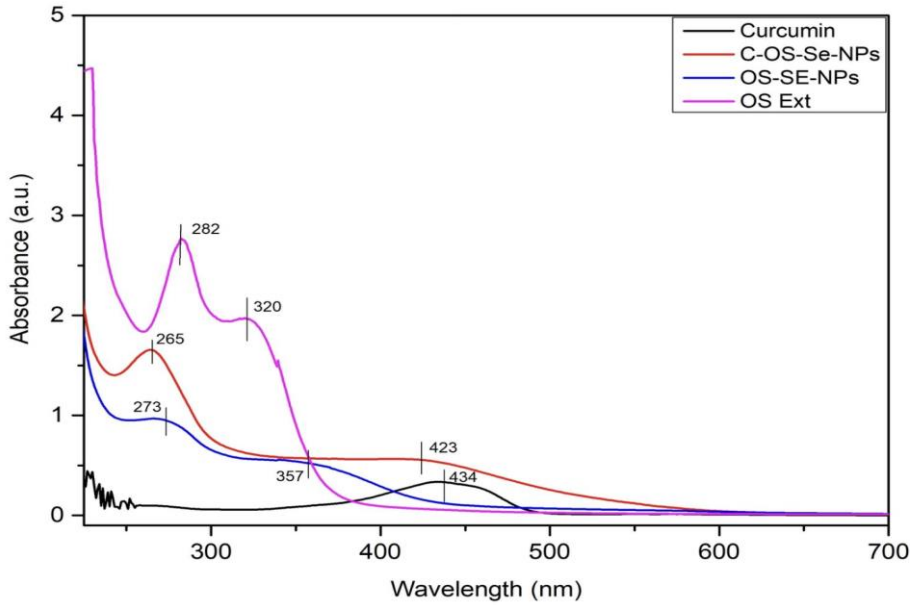

Figure 1. UV Spectra of SeNPs

FT-IR spectra were recorded between 4000 to 500 cm^{-1}. Figure 2 represents the result of the synthesized SeNPs. The FT-IR spectrum of SeNPs showed strong absorption bands at 3279, 1284, 1072, 1028, and cm^{-1}. Characteristic stretching at 3263 cm^{-1} confirms the OH group present in the extract used for SeNPs formation, which is evidenced by the shift in maximum absorbance compared to the extract.

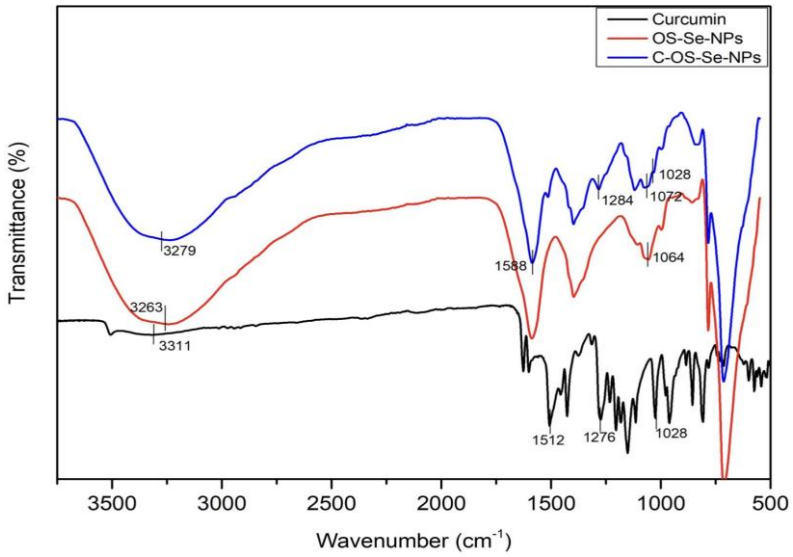

Figure 2. FT-IR Spectrum of SeNPs

The morphology of the synthesized nanoparticles was determined by scanning electron microscopy. The SeNPs exhibit agglomerated spherical shape with a size range of around 100 nm. On the other hand, the coating of curcumin increases the particle size to 140 nm. Figure 3 represents the morphology of SeNPs and curcumin coated Se NPs

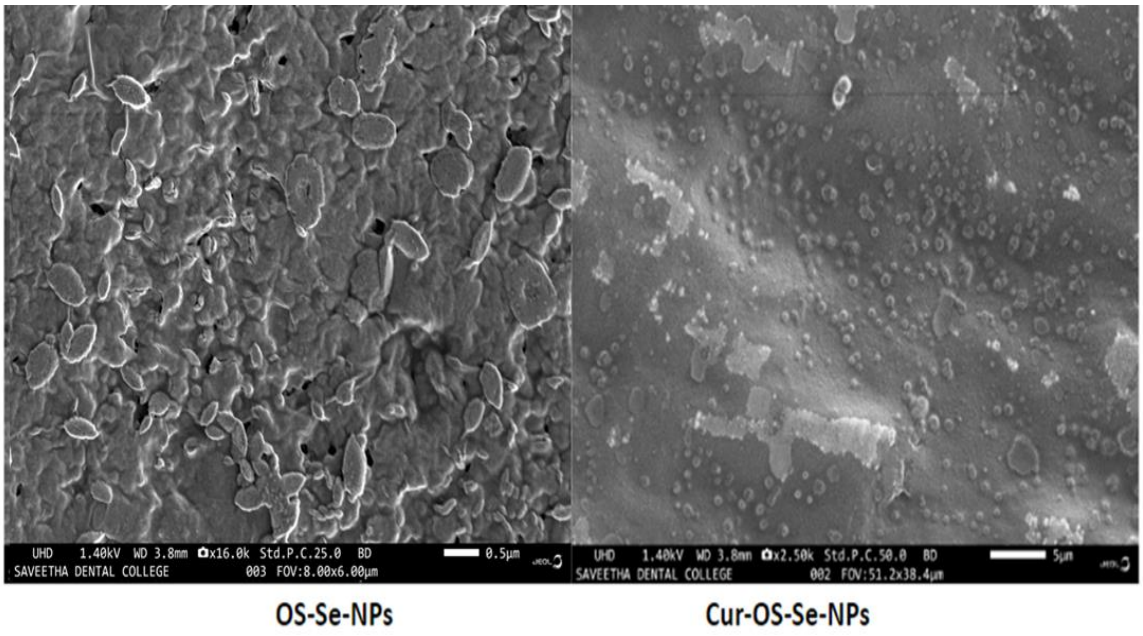

Figure 3. SEM Micrograph of OS-SeNPs and Cur-OS-SeNPs

Biocompatibility Study

Figure 4 represents the Annexin V-PI study of PBMC treated with SeNPs. Results were interpreted in four quadrants Lower left (LL), Lower right (LR), Upper left (UL), and upper right (UR) with annexin V in the X axis and Propidium iodide in the Y axis. LL represents the percentage of viable cells, LR represents the percentage of cells in early apoptosis, UR represents the percentage of cells in late apoptosis and UL represents the percentage of necrotic cell death. The Annexin V-PI assay results showed the maximum viability of 81.87

% with the SeNPs treated PMBCs, while 18.11 % were found to be in the early apoptotic stage, 0.02 % were found to be in the late apoptotic stage and none of the cells showed necrosis. The viability results were significant when compared with that of the untreated cells. Thus, this biocompatibility study performed using Annexin V and Propidium iodide staining method revealed the non-toxic nature of SeNPs in PBMC.

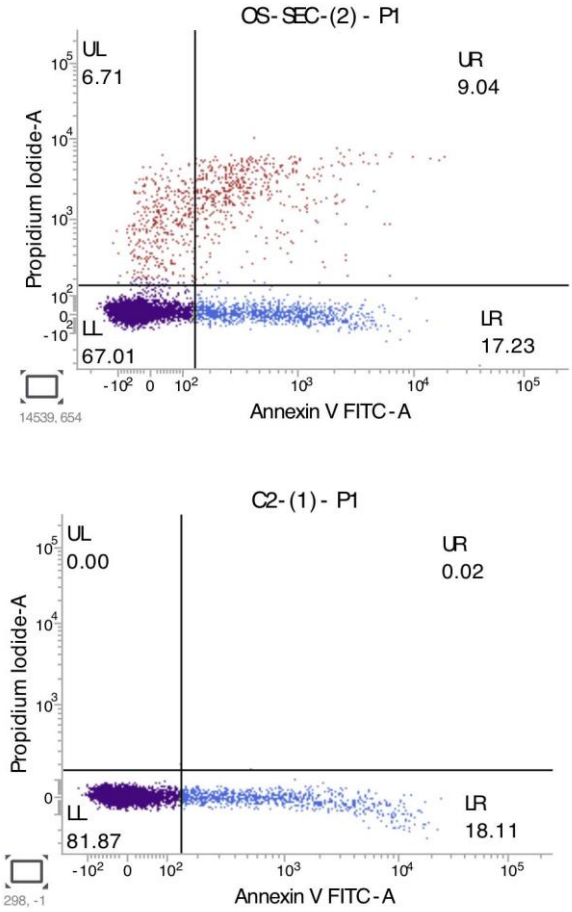

Figure 4. Annexin V and PI Assay of a) Control, b) SeNPs

Hemolytic Assay

Cur-OS-SeNPs showed less than 5 % hemolysis in erythrocytes at various concentrations from 200, 100, 50, 25, and 10 µg/mL in comparison with the control. Figures 5 and 6 represent the Hemolytic assay test results of SeNPs.

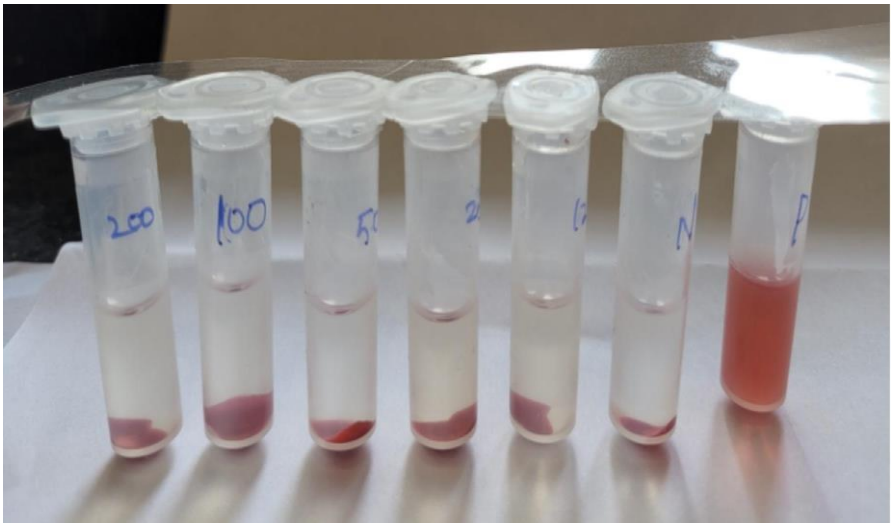

Figure 5. Visual Image of the Hemolysis Induced by Various Concentration of Cur-OS- SeNPs (200, 100, 50, 25 and 12.5 µg/mL) and Positive Control (p)

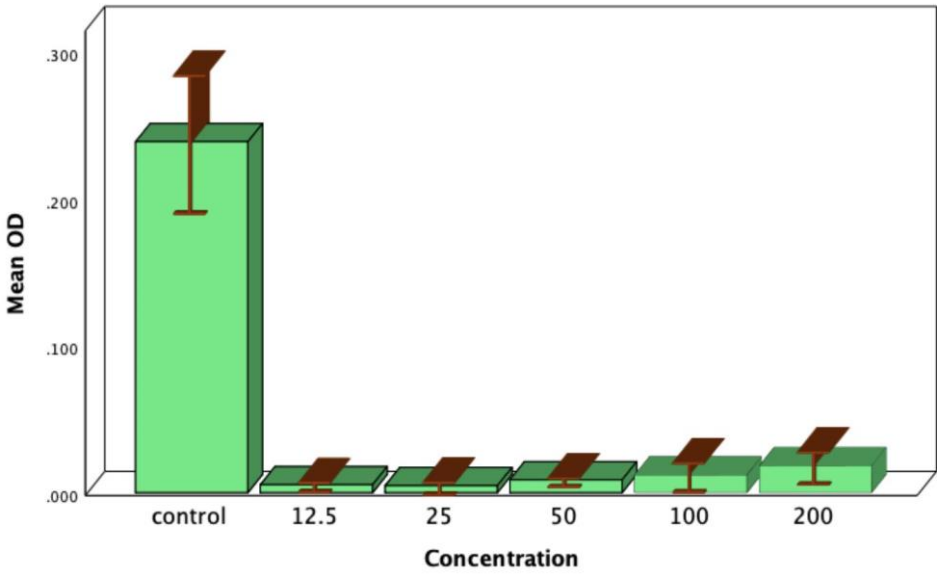

Figure 6. Hemolytic Activity of Cur-OS- SeNPs

Discussion

Nanobiotechnology plays a multi-strategic technique in various fields such as biomedical research, tissue engineering, regenerative medicine, etc. A well-defined nanosystem can be able to perform various cellular functions in a controlled dimension. However, the safety issues raised by using such nanoparticles are also alarming due to the trigger of cytotoxic pathways, the toxicity of the materials used, etc. [40]. Considering this, we have concentrated on the synthesis of biofriendly SeNPs using a traditional medicinal plant *Orthosiphon stamineus* and reported its biocompatibility towards PBMC. Green synthesis of SeNPs was previously reported with various natural sources including *Ceropegia bulbosa* (lantern flower) [41], fruit extract of *Emblica officinalis* (Indian Gooseberry) [42], etc. SeNPS are being used as a drug carrier for curcumin. Despite its pharmacological properties, curcumin has low bioavailability, poor solubility, and undergoes rapid degradation [37]. Therefore, to enhance the activity of curcumin, we have coated curcumin over SeNPS and our results show that this has substantially increased the bioactivity of SeNPs

and also increased the bioavailability, stability, and solubility of curcumin.

Characterization of curcumin-coated SeNPs synthesized using *Orthosiphon stamineus* was done by UV-Vis spectroscopy, FT-IR, and SEM. The result obtained supports our present findings. The UV-visible spectrum of SeNPs showed a peak at 265 nm. The shifting of the peak corresponding to the leaf extract indicates its role in the bio-reduction of selenium. FT-IR spectrum results showed strong peaks stretching at 3279, 1284, 1072, and 1028 cm^{-1} revealing the presence of flavonoids and phenolic compounds in the extract. The peak absorbed at 3279 cm^{-1} corresponds to medium C-H stretching of alkane. The peak absorbed at 1284 cm^{-1} corresponds to strong C-O stretching of alkyl aryl ether. A strong S=O stretching of sulfoxide was confirmed by the peak formation at 1072 cm^{-1}. The results concluded the presence of functional groups such as phenolic group, aromatic group, etc.

The SEM results of SeNPs revealed were found to be spherical-shaped surface morphology with a size range of around 100 nm. Similar spherical-shaped surface morphology was reported in ZrO_2NPs synthesized using the reducing power of fenugreek seed extract [43].

Apoptosis assay revealed that almost 81.87 % of cells were alive after treatment with 100 µg of SeNPs, comparable with that of untreated control showing 67.01 % viable cells. From the hemolysis assay, it was evident that the amount of hemolysis was directly linked to the concentration of curcumin coated SeNPs, as the maximum hemolysis was found to occur in the highest concentration used which is 200 µl; this confirmed the biocompatibility of biogenic SeNPs and its non-toxic nature. Hence, curcumin-coated selenium synthesized using *Orthosiphon stamineus* could be used for medicinal, therapeutic applications, as well as tissue engineering applications. However, further analysis needs to be done to find the efficacy as well as the toxicity of these SeNPs using *in-vivo models*.

Conclusion

The prepared selenium nanoparticles using an aqueous extract of *Orthosiphon stamineus*. The particles are characterised using UV-Visible Spectroscopy, FT-IR, and SEM. The biocompatibility of the nanoparticles is investigated and confirmed by Annexin V- PI apoptotic and Hemolytic assay. Our present study was done in the in-vitro condition in a small sample size. Further research must be done in a large sample size to provide better results. Further research targeting animal models to screen its toxicity, as well as its efficiency in in-vivo conditions, would substantially show that curcumin-coated SeNPs would be a better drug to be used in tissue engineering and regenerative medicine.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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Author Contribution

Shanmugam S B and Kavitha Sankaran conducted the literature collection, study design, experimental work and writing the manuscript. Vishnu Priya Veeraraghavan and Gayathri R, review and editing of the manuscript.

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