One Pot Synthesis of Colloidal Zirconium Nanoparticles using Orthosiphon Stamineus Leaf Extract for Potential Bone Tissue Engineering Applications

Abraham Sabu, Kavitha Sankaran, Gayathri Rengasamy, Vishnu Priya Veeraraghavan* Centre of Molecular Medicine and Diagnostics (COMManD), Department of Biochemistry, Saveetha Dental College, and Hospitals, Saveetha Institute of Medical and Technical Sciences, Saveetha University, Chennai, India

Abstract

Zirconium (ZrO_2) is a metal oxide nanoparticles (NPs) possessing antimicrobial, antifungal, antioxidant, biosensing, biocompatibility, and anticancer activities. Due to their unique properties, ZrNPs can be used for multiple biomedical applications. Orthosiphon stamineus (OS) is a perennial medicinal herb with potent bioactive constituents. Traditionally, it was used in treating rheumatism, epilepsy, jaundice, hepatitis, etc, Hence OS could be used as a capping agent for synthesizing ZrO₂NPs. The current study aimed to synthesize ZrO₂NPs using a green source like Orthosiphon stamineus(OS) leaves extract and characterized using UV spectrophotometry, FTIR, SEM, EDX, and their biocompatibility was tested using Annexin V apoptosis assay. Milky precipitation formed, followed by the addition of OS extract to the aqueous solution of Zirconium oxychloride Octahydrate, revealed the formation of ZrO₂NPs was further confirmed by the maximum absorbance at 296 nm in the UV-Vis spectrum. The peaks in the fingerprint region of FTIR revealed the presence of the functional groups of the phytoconstituents, confirming the capping. Apoptosis assay revealing the strong biocompatibility of ZrO₂NPs towards peripheral blood mononuclear cells with 77.64% cell viability. From the apoptosis assay, it was evident that ZrO₂NPs are less cytotoxic, indicating their applicability for medicinal applications. However further studies are required to validate its actions on bone tissue engineering.

Keywords: Green synthesis, Leaf extract, Nanoparticles, Cytotoxicity.

Introduction

One of the developing field in material science is nanotechnology. It describes the applications of the materials, especially at the nanoscale. The unique property of the nanomaterial makes it a key element in the biological field for disease diagnosis, for research and molecular-level therapeutics, etc. [1]. Zirconium oxide, also known as Zirconia, is one of the most important metal oxide nanoparticles used extensively due to their high stability, conductivity, etc, [2]. Since it is a transitional metal ion, it is monoclinic, tetragonal, or cubic [3]. Zirconium oxide is a neural bioceramic material, and recently, it has gained a wide range of applications in the

biomedical field. It is also a carrier in drug delivery [3, 4]. Studies interpreted that zirconium oxide possesses antimicrobial, antifungal, antioxidant, biosensing, biocompatibility, photodegradation, and anticancer activities [5]. Plants possess the property of reducing metal ions. Green synthesis of nanoparticles from plants is considered ecofriendly and is an alternative and prominent approach to physical and chemical synthesis [6-10]. The synthesis of nanoparticles from plants is mediated by bioactive compounds such as tannins, flavonoids, polyphenols, alkaloids, etc [6, 11]. Phytochemical constituents present in orthosiphon stamineus, such as flavonoids, saponins, polyphenols, etc, are known as potent

Received: 10.12.2023 Accepted: 16.12.2023 Published on: 30.12.2023 *Corresponding Author: vishnupriya@saveetha.com antioxidants and are important in synthesizing zirconium oxide nanoparticles by the process of biocapping, biostabilizing, etc. Orthosiphon stamineus is a perennial herb and is an important traditional folk medicine popular in southeast Asia [12–17], especially in Indonesia, Malaysia, Thailand, Vietnam and Myanmar [18]. It is also known as 'Misai Kucing' or 'Kumis Kucing' and Java tree. This plant is traditionally used to treat multiple diseases such as rheumatism, epilepsy, jaundice, hepatitis, renal calculus, tonsillitis. gonorrhea, eruptive fever, and [19]. Studies revealed influenza that Orthosiphon stamineus possess diuretic. hypouricemia, anti-inflammatory, analgesic, antipyretic, antioxidant, hepatoprotective, nephroprotective, gastroprotective activity, antihypertensive, antiproliferative, cytotoxic, antiangiogenic, and antibacterial activities [18, 20-25].

The present study aims to investigate the synthesis of Zirconium oxide nanoparticles using Orthosiphon stamineus leaf extract and characterize the nanoparticles using UV Spectrometry, FTIR analysis, EDX spectroscopy, and visualize the surface morphology Scanning Electron using Microscopy (SEM). Also, to check the biocompatibility of the synthesized nanoparticles conducting Annexin by V apoptosis assay on Peripheral Blood Mononuclear Cells (PBMC) with the help of flow cytometry.

Methodology

Preparation of Extract

The leaves of *Orthosiphon stamineus* was collected from a village near Thiruvalluvar district and dried in the shade, after which it was coarse powdered using a mixer grinder. 1 g of the coarse powder is mixed with 25 ml of distilled water and boiled for 30 mins at 80°C. The extract was then filtered using Whatman filter paper. The filtrate was then prepared as a plant extract.

Preparation Of ZrO2 NPs

10 ml of the leaf extract was added to 25 ml of 50 mM of Zirconium oxychloride Octahydrate. The pH was adjusted to 9 using an ammonia solution. The resulting mixture was stirred continuously for 3 hours at 40°C using a magnetic stirrer. The milky precipitate formed was centrifuged and washed with distilled water, and obtained NPs were dried in a hot air oven at 60°C overnight.

Characterisation of ZrO2 NPs

The *Orthosiphon stamineus* Zirconium oxide nanoparticles (OS-ZrO₂ NPs) was characterized using UV Spectrophotometry, FTIR, EDX, and SEM. To confirm the formation of OS- ZrO₂ NPs, optical absorbance was measured in a range of 200 to 700 nm using UV-Vis spectrum.

presence functional The of groups corresponding to the leaf extract in the green synthesized OS-ZrO₂ NPs was determined using Fourier Transform Infrared (FTIR) Spectroscopic analysis. Element analysis of OS-ZrO₂ NPs was performed using Energy Dispersive X-ray (EDX) Spectroscopy. The size, shape, and morphology of OS-ZrO₂NPs were analyzed using Scanning Electron Microscopy (SEM) (JEOL JSM-IT800 FE-SEM, JEOL Ltd, Tokyo, Japan).

Apoptosis Assay

The biocompatibility of the particles was checked using PBMC by flow cytometer (BD FACSLyric, BD Biosciences, USA). The blood was collected from healthy donors after appropriate ethical committee approval from the ethical approval committee. The PBMC cells using were isolated density gradient centrifugation under 2000 rpm for 40 minutes using histopaque. After centrifugation, a buffy coat was formed over the histopaque layer, which was taken for culturing. The culturing was done in a medium consisting of RPMI media, 10% FBS (Fetal Bovine Serum), 1% amino acid L-glutamine, and 1% PenStrep.

The culturing was done for 24 hours in triplicate. The cells were then collected and stained using Annexin V FITC (5 μ l), and Propidium Iodide (5 μ l) and kept at room temperature for 15 minutes.

After incubation, 400 microlitres of 1x binding buffer was added to all the tubes and acquired (10000 events) using BD FACSLyric flow cytometer, treated cells were observed for apoptosis. The analysis was performed using FACSuite 4.1 software.

Results

Addition of *Orthosiphon stamineus* leaf extract to Zirconium oxychloride Octahydrate results in a milky solution, indicating the formation of ZrO_2NPs .

The precipitate was separated using centrifugation and repeatedly washed with water to remove the impurities and dried under a hot air oven. The dried powder was used for further characterization studies.

Characterization of Nanoparticles

UV-Vis spectrum of ZrO₂NPs gave an intense maximum peak at 296 nm (Figure 1) was specific to ZrO₂NPs. Figure 2 represents the FTIR spectrum of OS-ZrO₂NPs, showing peaks at 3277, 1589, 1482, 1418, 1262, 1030, and 814 cm⁻¹. 3277 cm⁻¹ corresponds to O-H / C-H stretch, indicating the alcohol group / carboxylic group/alkyne group. 1589 cm⁻¹ corresponds to N-O stretching indicating the nitro compound. The 1482 cm⁻¹ and 1262 cm⁻¹ peaks correspond to C-O stretching, indicating an alkyl group. 1030 cm⁻¹ indicates the sulfoxide group, and 814 cm⁻¹ indicates halo compounds. These peaks confirm the presence of the different functional groups in bioactive compounds which plays a role in the capping of nanoparticles. The SEM micrograph of green synthesized ZrO₂ NPs showed spherical particles with aggregated morphology (Figure 3). The elemental analysis confirmed the presence of ZrO₂ as shown in Figure 4.



Figure 1. UV Spectra of OS-ZrO2 NPs and OS Leaf Extract







Figure 3. Scanning Electron Microscopy (SEM) Images of ZrO2 NPs



Figure 4. EDX Spectrum of the ZrO2 Nanoparticle Sample

Apoptosis Assay

Results of the apoptosis assay are shown in Figure 5. The samples stained with Annexin V-FITC and Propidium Iodide are represented by the four contour plots of Propidium Iodide vs Annexin V. The lower left quadrant includes cells that are propidium iodide negative and Annexin V negative, which indicates that the cells are undamaged. The lower right quadrant includes cells that are Annexin V positive and propidium iodide negative, showing cells undergoing early apoptosis. The upper left quadrant is Annexin V negative and propidium iodide positive, showing cells that undergo necrosis and the upper right quadrant is Annexin V positive and propidium iodide positive, showing cells that are undergoing necrosis. The control group showed 78.57% of viable cells and 21.40% of cells undergoing early apoptosis. Whereas in the ZrO₂NPs treated group there are 77.64% of viable cells and 22.28% of cells undergoing early apoptosis. Since there is no significant difference between the control group and the ZrO₂NPs treated group, we can say that green synthesized Zirconium oxide the nanoparticles are biocompatible.



Figure 5. Annexin V Apoptosis Assay Viability of PBMC Cells (a) Control Group (b) Treated Group

Discussion

In recent years, green synthesis of NPs gained importance over physical and chemical methods due to their cost effective, less toxic, and biocompatible properties. On the other hand, NPs generated through chemical methods are costly, toxic and importantly they are least biocompatible[26–28]. The bioactive/ phytochemical compounds present in plant act as reducing agents for NPS synthesis and interestingly they can be synthesized in one pot [29]. A wide variety of plant extracts have been previously reported for synthesizing ZrO₂ NPs.

UV Spectra of ZrO₂ NPs showed the maximum peak at 296 nm is due to the Surface Plasmon Effect (SPR) effect, which confirms the formation of ZrO₂ nanoparticles. The shift in the maximum wavelength observed in the ZrO₂ revealed the role of OS extract in the synthesis [30]. Previous studies on ZrO2 nanoparticles prepared using plant extracts as reducing agents have reported peaks at similar wavelengths. FTIR data of the ZrO₂ NPs obtained shows a prominent peak at 3277 cm⁻¹ and minor peaks around 1589, 1482, 1418, and 1262 cm^{-1,} which indicates the presence of stretching of hydroxyl (OH-) groups. The band present at 814 cm⁻¹ indicates the presence of ZrO₂ asymmetric stretching. The different functional groups in bioactive compounds act as capping and reducing agents in NPs synthesis. These data roughly correspond to previous studies conducted on ZrO₂NPs synthesized from Acalypha indica [31].

The morphology of the particles was analyzed using SEM. The particles are observed to have irregular spherical morphology with a size range around 150 nm. and exhibit mediocre levels of aggregation due to moisture. This aggregation is expected to occur as the nanoparticle sample did not undergo high-temperature cindering, which helps in obtaining perfect morphology. The elemental analysis showed an increased carbon content confirming the capping of phytoconstituents. Our results align with the previous findings [5, 32, 33].

Annexin V apoptosis assay is an effective method of analysis of the apoptotic stages of the cells [34]. Results from the assay showed no significant variation between the cells of the control group and the cells of the treated group. Hence, we can infer that the green synthesized ZrO₂ NPs were biocompatible. Based on our analysis, ZrO₂ NPs synthesized in an ecofriendly way, were found to be biocompatible and less toxic to PBMCs, suggesting its applications used in the medical field. Because of its size, high surface-to-volume ratio, greater biocompatibility, and other distant properties, ZrO₂ NPs can be used in Bone tissue engineering. But further research must be conducted to evaluate its action on bone tissue engineering.

Conclusion

In this present study ZrO₂ NPs was synthesized using Orthosiphon stamineus leaf extract by one-pot synthesis method. The formation of the nanoparticles was confirmed using UV spectrophotometry. A clear shift of peaks in the UV spectrophotometry graph and FTIR spectra indicates the formation of ZrO₂ NPs. Elemental analysis was also performed which confirms the presence of nanoparticles. The morphology of the nanoparticles was visualized using SEM. Flow cytometer data showed a negligible difference in cell apoptosis in the treated group when compared to the control group. Thereby affirming the biocompatibility of the ZrO₂ NPs. The asprepared ZrO₂ NPs were biocompatible and could be used for dental tissue engineering applications.

Conflict of Interest

There is no conflict of interest.

Funding

This Project was supported by Kamala Dental Speciality Hospital, Thiruvananthapuram.

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