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One Pot Synthesis Of Colloidal Zirconium Oxide Nanoparticles Using Evolvulus Alsinoides Leaf Extract For Potential Bone Tissue Engineering Applications

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ABSTRACT

The number of people seeking alternative medicine is also growing exponentially. There is a growing interest in the field of phytomedicine not just because it's inexpensive but has lower side effects on normal cells when compared to allopathic medicine. Therefore, this research focuses on the synthesis of Zirconium oxide nanoparticles using Evolvulus alsinoides leaf extract and to find its efficiency in bone tissue engineering, optical engineering, photocatalysis, and high-κ dielectrics. Here we synthesized ZrO_2 NPs using an aqueous extract of Evolvulus alsinoides leaves, characterized the ZrO_2NPs using various techniques, evaluated its biocompatibility, and reported a facile one-pot method for the synthesis of colloidal stable Zirconium oxide nanoparticles. The results confirmed the ZrO_2 NPs formation by changing the color from greenish to dark yellow. The UV spectrum of NPs shows maximum absorbance at 285 nm, confirming the ZrO_2 formation, which is evidenced by the shift in maximum absorbance compared to the extract. The surface morphology was analyzed using SEM which revealed an aggregated morphology of NPs resulting from moisture in the sample. A biocompatibility study performed using Annexin V and Propidium iodide staining revealed the non-toxic nature

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of ZrO_2 NPs in Peripheral blood mononuclear cells (PBMC). Therefore, we conclude that eco-friendly ZrO_2 NPs can be used as potential candidates in various biomedical applications, including therapeutics and diagnostics, after validation via in-vitro and in-vivo application studies.

Keywords: Nanoparticles; Evolvulus alsinoides; green synthesis; Biocompatibility; SEM

INTRODUCTION

Alternative medicine has gained much attention for the treatment of various diseases and is used as a form of preventive medicine for decades. The number of people seeking alternative medicine is also growing exponentially. There is a growing interest in the field of phytomedicine not just because it's inexpensive but has lower side effects on normal cells when compared to allopathic medicine (1). Herbal medicines have been practiced for thousands of years, but very little is known about their mode of action. The recent finding also proved that herbal medicines could have severe consequences and such drugs are not tested for their efficacy and toxicity (2) (3). In recent years, nanobiotechnology has been explored in almost all fields due to its distinctive physiochemical property and wide range of applications (4). Nanomaterial is utilized in biomedical research, therapeutic and diagnostic purposes, etc, (5) and has recently been practiced in dental and bone tissue engineering. Synthesis of this nanoparticle by the chemical method is usually toxic and can cause an immune response, so an alternative approach and the best eco-friendly method would be green synthesis (6) (7). Production of nanoparticles by green technique has higher stability (8). Following the principle of green chemistry, nanoparticles can be prepared from different parts of plants such as a leaf, root, bark, stem, and even flowers, and extracted using solvents. Nanoparticles synthesized from plant sources can be produced much faster and are more stable (9). The plant extracts contain phytochemical constituents such as alkaloids, flavonoids, terpenoids, and triglycerides. reducing sugars, etc., which can act as capping agents and some compounds even possess reducing activity which can aid in the synthesis of nanoparticles (10) (11).

Zirconium oxide is a polymorphic material, a crystalline metal oxide, non-toxic, mechanical strength, high thermal resistivity, and optical properties, and can exist in three thermodynamically stable phases (12,13). Zirconium oxide is an inorganic nanoparticle and is a transitional metal element (14). Zirconium oxide is extensively known for its corrosion and microbial resistance, specificity, and

photocatalytic activity and is used in biological and healthcare fields, such as dental coating (12,13). Zirconium oxide nanoparticles have gained more attention in material sciences as they possess excellent biodegradable and optical properties. Due to its antifungal and antibacterial activity, Zirconium oxide is also used in food packing industries (14). *Evolvulus alsinoides* is a perennial herb found commonly in East Asia and is often used in the olden systems of medicine such as Ayurveda and Siddha to treat several neurodegenerative disorders, amnesia, as a brain tonic and epilepsy and a potent medicine for brain disorders (15). Each part of the plant possesses different medicinal properties (16,17). Phytochemical screening of *Evolvulus alsinoides* interpreted the presence of various bioactive constituents such as alkaloids, carbohydrates, tannin, terpenoids, triterpenoids, phenols, flavonoids, and volatile oil. The leaf extract is also reported to contain metals in them namely, sodium(Na), potassium (K), Iron(Fe), copper(Cu), Zinc(Zn), and calcium(Ca). The presence of these secondary metabolites marks them an excellent anticancer, antioxidant, antidiabetic, adaptogenic (anti-stress), anti-amnesic, antiulcer, anti catatonic activity, gastroprotective, and immunomodulatory activity (18).
Orthopedic surgery and dental prosthetics are much developed in recent years which provide personalized dental treatments and bioengineered materials for tissue repair and regeneration. Materials that are commonly used in dental restorations and restorations are porcelain crowns (19) and implants (20). High-purity ZrO_2 is a common material used in dental procedures. ZrO_2 has a good natural white color, higher strength, and stable chemical properties, and it is corrosion resistant as well (21); (22) Zirconium is extensively used to make prosthetics due to its good mechanical and chemical properties. When zirconium is exposed to oxygen, it becomes biocompatible with zirconium dioxide (ZO, chemically ZrO_2) (23,24). Zirconium oxides have a very natural ivory color which is similar to that of natural teeth (25), making it aesthetically important in the areas of dental aesthetic procedures. Most importantly its light transmittance makes it an ideal object in cosmetic prosthetics (26). Recently, ZrO_2 nanopowder is used to promote the biological and mechanical properties of dental porcelain as well as in tissue engineering scaffolds. Recent studies have shown that the combination of ZrO_2 nanopowder can significantly enhance the flexural strength, fracture strength, and shear strength of dental materials (27); (28)

MATERIALS AND METHOD

Collection of Sample

The plant *Evolvulus alsinoides* was used for the present investigation. The plant's leaves were obtained from the nearby gardens in Chennai, 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. Zirconium oxychloride octahydride was procured from SRL. All other reagents used were of analytical grade. MilliQ water was used throughout the study.

Plant extract preparation:

The leaves of the collected *Evolvulus alsinoides* 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. 1 gram of *Evolvulus alsinoides* powder was mixed with 25 mL of double distilled water and boiled for 30 minutes. Then the extract was cooled at room temperature and filtered. This filtrate was then used for further synthesis of zirconium oxide nanoparticles.

Synthesis of Zirconium oxide: 10 ml of extract mixed with 100 mmol of zirconium oxychloride octahydride was stirred using a magnetic stirrer at 40°C for 3 h. The color changed from green to dark yellow. The pH of the solution was neutralized by adding an ammonia solution. A cloudy precipitate was obtained and the precipitate was centrifuged at 6000 rpm for 20 minutes. The precipitate was then washed three times with water and dried overnight under a hot air oven at 60°C. This dried powder was used for further analysis. A schematic representation of ZrO_2 NPs is given in Figure 1.

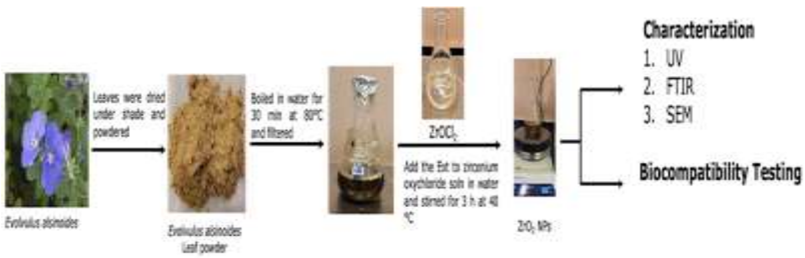

Figure 1 Schematic representation of synthesis and characterization of ZrO_2

Characterisation of Zirconium oxide nanoparticle:

One-pot synthesis of Zirconium oxide nanoparticles using *Evolvulus alsinoides* plant extract was characterized using a UV-Visible spectrophotometer, Fourier-transform infrared spectroscopy (FTIR), and Scanning Electron Microscopy (SEM). The maximum absorbance of ZrO_2 NPs was determined using a UV-Visible spectrophotometer (Jasco) by screening between 350-700 nm. The presence of a functional group in ZrO_2 NPs 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 ZrO_2 NPs. The elemental composition of synthesized ZrO_2NPs was determined by Energy-dispersive X-ray analysis (EDX).

Biocompatibility of ZrO_2 NPs in Peripheral blood mononuclear cells (PBMC):

For determining the biocompatibility of ZrO_2 NPs 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 ZrO_2 NPs 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.

RESULTS

In this current study, one-pot synthesis of Zirconium oxide nanoparticles was synthesized using *Evolvulus alsinoides* leaf extract. The color change from greenish yellow to deep yellow indicates the synthesis of ZrO_2 NPs.

Characterization of ZrO_2 NPs:

The UV-visible spectral analysis for ZrO_2 NPs showed a typical surface plasmon resonance (SPR) peak with maximum absorbance at 285 and 364 nm. Figure 2 represents the UV spectra of ZrO_2 NPs. The UV-visible spectral analysis for ZrO_2 NPs showed a typical surface plasmon resonance (SPR) peak with maximum absorbance at 285 nm, confirming the ZrO_2 NPs formation, which is evidenced by the shift in maximum absorbance compared to the extract.

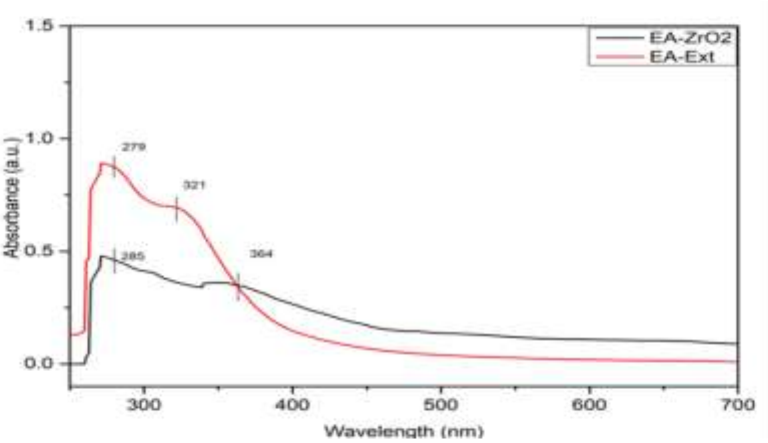

Figure 2. UV spectra of ZrO_2NPs

FTIR spectra were recorded between 4000 to 500 cm^{-1}. Figure 3 represents the result of the synthesized ZrO_2 NPs. The FT-IR spectrum of ZrO_2 NPs showed strong absorption bands at 3275, 1636, 1428, 1276, 1076, and 608 cm^{-1}. Characteristic absorbance at 3258 cm^{-1} confirms the ZrO_2 formation, which is evidenced by the shift in maximum absorbance compared to the extract. Peaks at 1636 cm^{-1} correspond to aromatic ring stretch. The other peak at 1076 cm^{-1} corresponds to the Zr-O-Zr stretching.

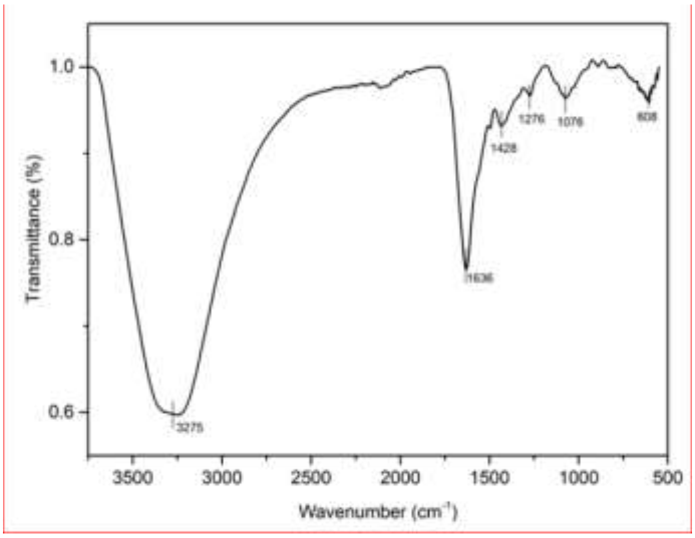

Figure 3 FT-IR spectrum of ZrO_2NPs

The morphology of the synthesized nanoparticles was determined by scanning electron microscopy. The ZrO_2 NPs exhibit agglomerated spherical shape with a size range of around 100 nm. (Figure 4). EDX analysis was carried out to verify the compositional elements. The results revealed the presence of Zr (Zirconium), O (Oxygen), and C

(Carbon) materials. The presence of these elements confirms the ZrO_2 structure thus demonstrating the purity of the sample.

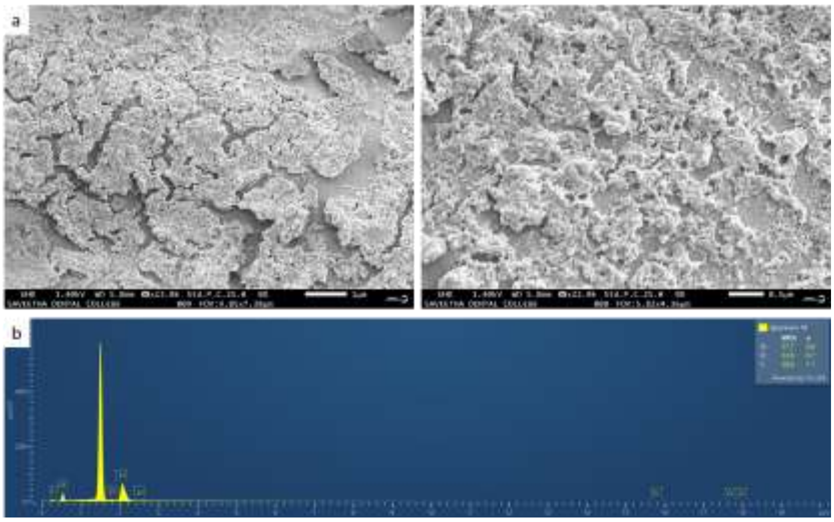

Figure 4 SEM micrograph (a) and EDX (b) of ZrO_2NPs

Biocompatibility study

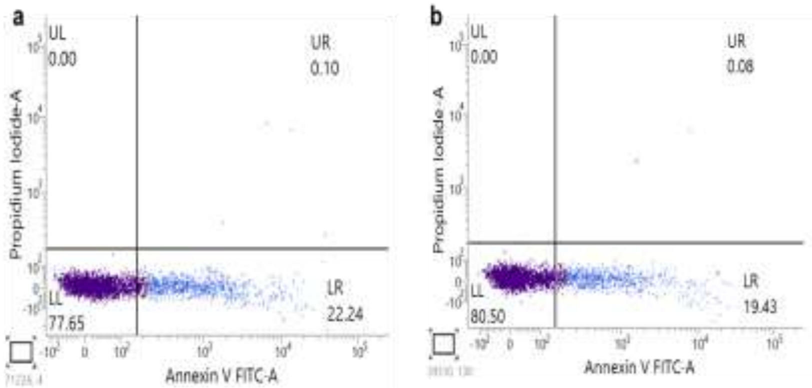

Figure 5. Annexin V and PI assay of a) Control, b) ZrO_2 NPs

Figure 5 represents the Annexin V-PI study of PBMC treated with ZrO_2NPs. The Annexin V-PI assay results showed the maximum viability of 80.5% with the ZrO_2NPs treated PMBCs, while 19.43% were found to be in the early apoptotic stage, 0.08% were found to be in the late apoptotic stage and non of the cells showed necrosis. The viability results were significantly comparable 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 ZrO_2 NPs in PBMC.

DISCUSSION

Nanobiotechnology plays a multi-strategic technique in various fields such as biomedical research, tissue engineering, regenerative medicine, etc (29). 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. (30). Considering this, we have concentrated on the synthesis of bio-friendly ZrO_2 NPs using a traditional medicinal plant *Evolvulus alsinoides,* and reported its biocompatibility towards PBMC. Green synthesis of ZrO_2 NPs was previously reported with various natural sources including *Helianthus annuus* (sunflower) seeds (31), fruit peels of *Punica granatum* (Pomegranate) (32), etc.

ZrO_2 NPs synthesized using *Evolvulus alsinoides* showed a time-dependent color change from greenish yellow to deep yellow red after 24 hours of incubation at room temperature. This result supports our present findings. UV- visible spectrum of ZrO_2 NPs showed a peak at 285 nm. The shifting of the peak corresponding to the leaf extract indicates its role in the bio-reduction of zirconium. FT-IR spectrum results showed strong peaks stretching at 3275, 1636, 1428, 1276, 1076, and 608 cm^{-1} revealing the presence of flavonoids and phenolic compounds in the extract. The peak absorbed at 3840.27cm^{-1} corresponds to intermolecular bonded O-H stretching of alcohol. The alkenyl C=C stretching confirms the monosubstituted alkene from the absorption peak of 1636 cm^{-1}. O-H bending of alcohol was confirmed by the peak formation at 1426 cm-1. A strong C-O stretching of alkyl aryl ether is confirmed at 1276 cm^{-1}. A string C-I stretching absorption peak of 608 cm-1 indicates a halo compound. The results concluded the presence of functional groups such as phenolic group, aromatic group, etc.

The SEM results of ZrNPs 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_2 NPs synthesized using the reducing power of fenugreek seed extract (33). Apoptosis assay revealed that almost 80.5% of cells were alive after treatment with 100 µg of ZrO_2 NPs, comparable with that of untreated control showing 77.65% viable cells; this confirmed the biocompatibility of biogenic ZrO_2 NPs and its non-toxic nature. Hence, ZrO_2 synthesized using *Evolvulus alsinoides* could be used for medicinal and therapeutic applications.

CONCLUSION:

We have synthesized ZrO_2 NPs, synthesized using *Evolvulus alsinoides* using the green synthesis method, and characterized their properties using techniques such as UV-Visible Spectral analysis, FT-IR, and SEM, and toxicity study was evaluated on PBMCs using Annexin V-PI apoptosis assay. ZrO_2 NPs synthesized were spherical in shape which was confirmed by SEM and was found to have a size range of around 100 nm. Our *in-vitro* study showed that ZrO_2 NPs do not induce significant apoptosis and showed no necrosis and hemolysis at 100 µg. Furthermore, the viability of ZrO_2 NPs treated PBMCs was similar to that of untreated control. Our findings showed that ZrO_2 NPs were non-toxic to PBMCs and could be used for biomedical applications. However, further investigation must be carried out to evaluate the *in-vivo* toxicity nature of the synthesized ZrO_2 NPs. Therefore, we concluded that the biogenic synthesis of ZrO_2 is economic. Because of its biocompatibility and high mechanical strength as per previous reports, it could be a key component in bone tissue engineering applications.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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