Original Article

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Green Synthesis of *Terminalia Arjuna*-mediated Hydroxyapatite Nanoparticles, Its Morphological Assessment and Evaluation of Cytotoxic and Antioxidant Properties: An In-vitro Study

Pharmacology Section

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ABSTRACT

Introduction: *Terminalia Arjuna* (TA) has been used in the field of ayurvedic and unani medicine for quite some time now. It has been shown to possess several medicinal properties. Literature has highlighted the osteoinductive properties of TA and has been extensively been used for its fracture healing and bone forming potential. This would have a positive impact in the field of periodontics and orthodontics, which require bone grafts to maintain ideal periodontal conditions.

Aim: To synthesise TA-mediated Hydroxyapatite Nanoparticles (TA-HApNPs) and subject it to morphological assessment and evaluate its cytotoxic and antioxidant properties.

Materials and Methods: This in-vitro study was done under a laboratory setting within the city of Chennai, Tamil Nadu, India, extending for a period of one month from April 2022 to May 2022. A 1 g of TA plant bark was mixed with 100 mL distilled water and heated to derive the plant extract. A 20 mL of distilled water was mixed with 0.502 g of Hydroxyapatite (HAp). An 80 mL of TA extract was mixed with 20 mL of HAp solution. The mixture was placed in a magnetic stirrer and was subjected to an Ultraviolet Visible (UV-Vis) double beam spectrophotometer. The solution was dried and was subjected to Transmission Electron Microscope (TEM) analysis for morphological characterisation, 2,2-Diphenylpicrylhydrazyl (DPPH) assay for the assessment of antioxidant properties and brine shrimp lethality test to evaluate its cytotoxic properties. The results were obtained electronically, tabulated in a spreadsheet and were subjected to descriptive statistics using Microsoft excel 2019 Management Services Organisation (MSO) (Version 2202; Build 16.0.14931.20118).

Results: The UV-Spectroscopy revealed a sharp peak at 375 nm wavelength suggesting Nanoparticle (NPs) formation. Spherical shaped NPs with size ranging from 5-25 nm were seen under the TEM. At concentration of 48 µL also TA-HApNPs elicited good cytotoxicity as seven out of 10 brine shrimps were alive at the end of 24 hours. Good antioxidant properties at 50 µL concentration with a DPPH assay reading of 1.044 was noted.

Conclusion: The TA-HApNPs can be synthesised using simple and routinely used laboratory armamentarium in the size range of 5-25 nm. TA-HApNPs have minimal cytotoxic effect and a good antioxidant activity.

Keywords: Bone grafting, Brine shrimps, Spectrophotometry, Transmission electron microscopy

INTRODUCTION

The ideal bone substitute or grafting material should possess minimum cytotoxicity and antioxidant property since any grafting procedure involves the production of free radicals [1]. HAp is a common constituent of human bones and teeth, that occurs in the form of mineral calcium apatite consisting of calcium, oxygen and phosphorus, growing in hexagonal crystals [2]. It is an osteoconductive bone substitute material that is commonly used as a scaffold in bone graft materials. However, it is incapable of inducing the bone formation on its own [3].

The HApNPs have also been exclusively used in dentistry for dental implantology, dentin hypersensitivity, bleaching, and caries prevention [4]. HApNPs have been synthesised in several methods such as reverse micro-emulsion, precipitation, sol-gel, hydrothermal, microwave hydrothermal and solid state reaction [5]. HApNPs have previously been used as effective bone substitutes since the human bone primarily is made up of nano sized HA crystals, constituting upto 65% (by weight) of bone [6]. Several HApNPs formulations in combination with plant extracts have been formulated which combined the biological and medicinal properties of these plant extracts with the chemical stability and mechanical properties of HAp crystals [7]. Several plants such as cinnamomum camphora, garcinia mangostana, nelumbo nucifera, Azadirachta indica have been used to derive NPs [8]. TA plant extract is gaining recognition for its versatile properties and is yet to be explored in terms of bone formation and as a material for artificial bone grafting [9]. TA has been used in ayurvedic and unani medicine also it has shown promising activity against a wide spectrum of microbes such as viruses, bacteria, fungus, yeast and algae [10]. TA has also been extensively used in the green synthesis of several NPs as an alternative to the conventional method of NPs synthesis, since it is a simple, safe, economic and biological alternative to the chemical methods [11,12]. Recent studies have also shown that TA has an osteo-inductive property that promotes fracture healing and bone growth [13,14]. Apart from very mild side-effects such as constipation, headache, insomnia, bodyache, gastritis and nausea, there have been no major haematological, renal, or metabolic toxicity noted even after 24 months of TA administration [11].

The rationale of conducting the present study was to green synthesise a plant mediated NPs, that could serve as a bone grafting substitute, with the potential to induce bone formation by virtue of its osteoinductive properties. This novel combination of the aforementioned plant extract and NPs was the first of its kind to be synthesised and the method described in the present study will provide the readers an easy method of synthesising HA NPs in a simple laboratory set-up. Hence, the aim of the present study was to synthesise TA-HApNPs followed by its characterisation, evaluation of cytotoxicity and antioxidant properties.

MATERIALS AND METHODS

This in-vitro study was done under a laboratory setting within Saveetha Dental Collge and Hospital, Chennai, Tamil Nadu, India., extending for a period of one month from April 2022 to May 2022.

Study Procedure

Preparation of plant extract: Freshly retrieved bark extract of the TA plant was washed in distilled water and then dried in an incubator. It was then ground into coarse particles with a mortar and one gram of the grounded powder was mixed homogeneously with 100 mL distilled water [15]. This solution was heated at 50-60ºC until vapours were seen emerging from the beaker kept in a heating mantle. Purification of solution was done by filtration using a Whatman filter paper no.1. Residue collected in the filter paper was discarded and 80 mL of the supernatant was collected in a conical flask [Table/Fig-1].

Preparation of Hydroxyapatite Nanoparticles (HApNPs): A 20 mL of distilled water was mixed with 0.502 g of HAp powder to obtain HAP solution [16]. A 20 mL of this HAP solution was mixed with the 80 mL of TA bark extract and kept overnight on an orbital shaker for homogenous mixing of all particles. The reaction mixture was stirred continuously on a REMI 2MLH magnetic stirrer set at 600 rotations per minute (rpm) for 72 hours and was monitored for colour change [Table/Fig-2].

[Table/Fig-1]: Collection of purified plant extract post filtration using Whatman filter paper no.1.
[Table/Fig-2]: TA-Hap solution kept in a magnetic stirrer till colour change was observed. (Images from left to right)

UV-Vis spectroscopy was performed using a UV-Vis spectrophotometer (SL-159 single beam microprocessor based scanning UV-Vis Spectrophotometer, ELICO) to determine the rate of absorption of light and thereby detect the formation of NPs [15]. The UV-Vis spectroscopy was first switched on and kept for 20 minutes to let the lamp heat sufficiently. A 5 mL sample of the TA-HApNps was taken and kept in the first chamber. A standard control was kept on the other chamber to act as the control, to compare the formation of NPs. At hourly intervals, the particulates were monitored using UV spectroscopy to see when the NPs were formed. When the graph reading reaches a sharp peak, it is indicative of NPs formation. Colour change of TA-HApNPs indicated its formation at a certain wavelength, that was measured on a UV-Vis spectrophotometer. Postspectroscopic analysis, the mixture was collected in five test tubes and TA-HApNps were separated from solution by centrifugation for 20 minutes.

Characterisation of synthesised TA-HApNp's: The UV-Vis absorption peak of the synthesised TA-HApNps was recorded using UV-Vis spectroscopy. The scanning range of the samples was between 250 to 650 nm [16]. All UV-Vis absorption spectra were read against distilled water. The size and shape of the NPs were measured using High Resolution Transmission Electron Microscopic analysis (HR-TEM) Model: FEI-TECNAI G2-20 TWIN) with an operating voltage of 200 kV.

Cytotoxic activity: The obtained TA-HApNps solution was subjected to brine shrimp lethality assay to estimate its cytotoxic activity [17]. Enzyme Linked Immuno Sorbent Assay (ELISA) plates containing 12 wells were taken and 8 mL of water mixed with iodine free rock salt was added to each well, to simulate the alkaline sea water environment, to form a favourable sea water environment for the brine shrimps [Table/Fig-3].

[Table/Fig-3]: ELISA plates containing nauplii shrimp exposed to differing concentrations of TA-HApNPs.

It was made sure that the rock salt had completely dissolved into the water to form a homogeneous solution that simulated the alkaline sea water environment for the brine shrimps. Ten nauplii shrimps were added to each well of the ELISA plate. TA-HApNPs solution was then added to each well in differing concentrations (5 mL, 10 mL, 20 mL, 30 mL, 48 mL). The solution was incubated for 24 hours and the mortality rate of the nauplii shrimps were checked by counting the number of nauplii shrimps that had survived out of the 10 nauplii shrimps present in each well.

Antioxidant activity: Differing concentrations of the solutions (5 mL, 10 mL, 20 mL, 30 mL, 50 mL) were taken in five different clear test tubes [15]. These test tubes were subjected to 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) assay to evaluate the antioxidant activity possessed by the NPs. A 1 mL of 2,2-DPPH was added to each test tube, followed by which 1990 µL, 1980 µL, 1970 µL, 1960 µL and 1950 µL of 50% methanol was added to the respective test tubes containing 5 mL, 10 mL, 20 mL, 30 mL, 48 mL of the NPs solution, respectively. The test tubes were then incubated in a dark room for 10 minutes. Ascorbic acid was used as a standard and absorbance was measured at 517 nm in the UV Spectrophotometer.

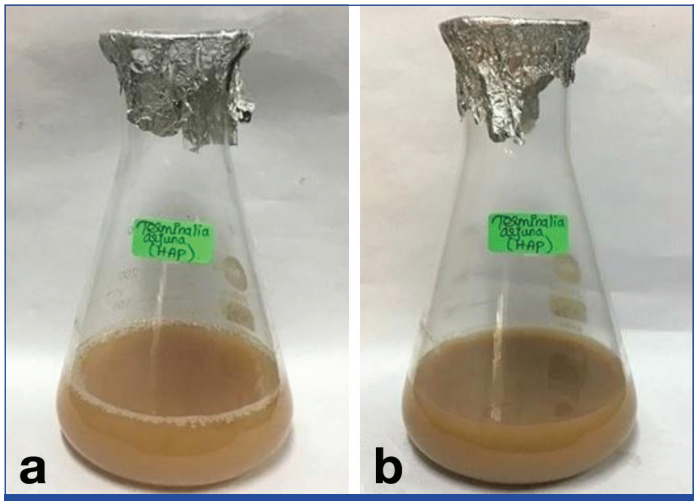
STATISTICAL ANALYSIS

Descriptive statistics were used to analyse the data.

RESULTS

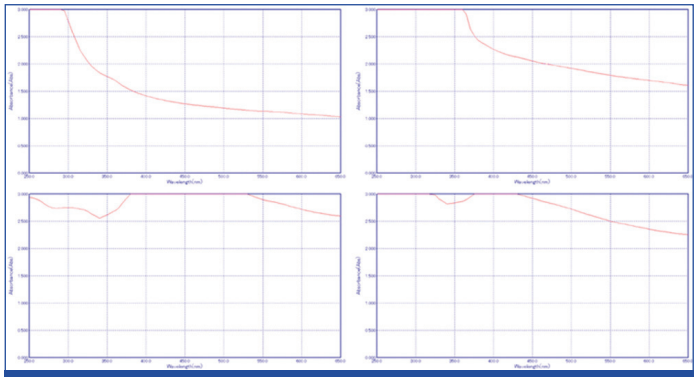
Visual observation of Nanoparticles (NPs): Colour change was observed after stirring TA-HApNp's continuously on a magnetic

stirrer. The change in colour from pale brown to dark brown indicated the formation of TA-HApNp's [Table/Fig-4].



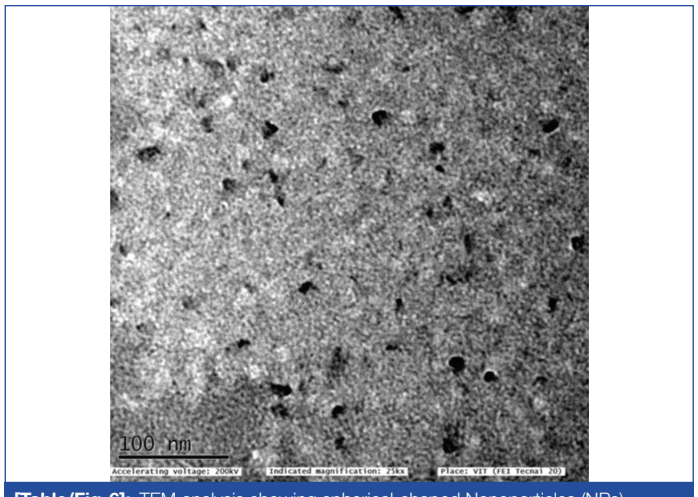
[Table/Fig-4]: a) Pale brown colour of the TA-HAp solution; b) Colour changing to dark brown indicating Nanoparticle (NPs) formation.

UV-Vis spectroscopy: UV-Vis spectroscopic analysis was used to monitor the formation of TA-HApNp's at zero hour, 24 hours, 48 hours and 72 hours. Sharp peak was seen at 375 nm wavelength, which corresponded to the Surface Plasmon Resonance (SPR) band of the TA-HApNps. This confirmed the formation of the NPs [Table/Fig-5].



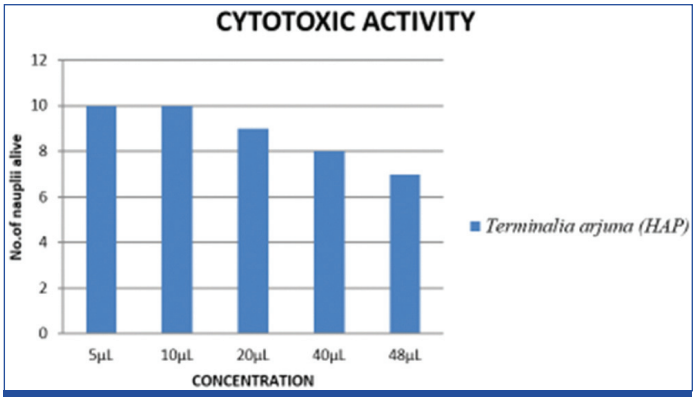
[Table/Fig-5]: UV-Vis spectroscopic analysis showing sharp peak at 375 nm wavelength in the bottom left graph.

Transmission Electron Microscope (TEM): TEM analysis was employed for characterisation of size and shape of NPs. Some spherical shaped NPs ranging from 5-25 nm in size were observed in TEM analysis, which appeared white in colour. The black dots are indicative of the voids in between the NPs [Table/Fig-6].



[Table/Fig-6]: TEM analysis showing spherical-shaped Nanoparticles (NPs).

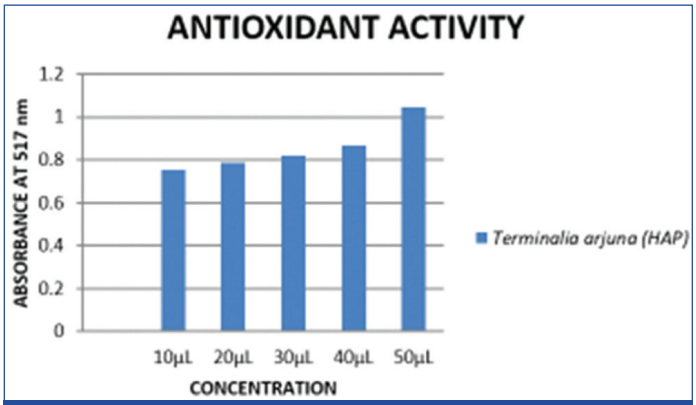
Cytotoxic activity: The cytotoxic activity of TA-HApNPs is depicted in [Table/Fig-7].



[Table/Fig-7]: Number of live nauplii shrimps at different concentrations of TA-HApNPs.

At 5 mL and 10 mL concentrations of the TA-HApNPs diluted with 8 mL of rock salt mixed water, all 10 nauplii shrimps survived at the end of 24 hours. At 20 mL concentration of TA-HApNPs, nine out of 10 nauplii shrimps survived. At 30 mL concentration, eight out of 10 nauplii shrimp had survived and at 48 mL concentration, seven out of 10 nauplii shrimps had survived at the end of 24 hours. This showed that the cytotoxicity of the NPs increased slightly, as the concentration was increased.

Antioxidant activity: The antioxidant activity of TA-HApNPs is depicted in [Table/Fig-8]. Even at 50 mL concentration, TA-HApNPs showed increased antioxidant property. The antioxidant activity of the NP increased with increasing concentration of the NP solution.



[Table/Fig-8]: Increasing absorbance at 517 nm wavelength with increased concentration of TA-HApNPs.

DISCUSSION

The HApNPs are extensively used as a synthetic bone graft material in the field of Periodontics, Prosthodontics, Oral and Maxillofacial Surgery. However, the HApNPs are osteoconductive in nature and do not possess osteoinductive potential [13,14]. In the present investigation, an attempt was made to synthesise HApNPs mediated with TA as TA is reported to possess osteoinductive potential [13,14]. Following the synthesis of TA-HApNPs, morphological characterisation was done using TEM analysis and chemical characterisation was done using brine shrimp assay and DPPH assay.

Visual examination of TA-HApNps solution revealed a colour change from pale brown to dark brown, which was indicative of the formation of the NPs. This was confirmed by means of a UV-Vis spectroscopy. SPR is the coherent oscillation of electrons in the presence of an electromagnetic field. The resonance of the HApNPs was seen at 375 nm wavelength, which initiated the colour change, thereby confirming the formation of TA-HApNPs [18]. The results from UV-Vis spectroscopy also confirmed the redox reaction between TA plant extract and HA precursor, leading to the formation of the NP. Here, the TA plant extract acts as a reducing agent, whereas the HAP precursors help stabilising the NP compound [19]. On TEM analysis, spherical shaped NPs ranging from 5-25 nm in size were observed. This was in accordance with other studies which saw similar spherical shaped NPs raging within the size ranges of the present

study observed result [20,12]. The results from the cytotoxicity test using brine shrimps showed that even at a higher concentration of 48 mL, almost seven out of 10 nauplii shrimp had survived at the end of 24 hours. This accounts to about 70% survival rate of nauplii shrimps. DPPH assay was employed in the present study to test the antioxidant activity of the TA-mediated HApNPs, which revealed that the free radical scavenging property as assessed by the UV spectroscopic reading, increased with increase in concentration of the NP solution. This indicated that the NP solution had a really good antioxidant activity.

Smaller sized NPs have an extended half-life period, longer circulation time and carry increased drug concentration [8]. NPs have accelerated adsorption rate due to their high surface to volume ratio [21]. Their ultra-fine size also contributes to their toxicity free behaviour making them excellent vehicles in drug delivery systems [22]. Ultra-small size (1-100 nnm) and large surface area-to-mass are major advantages of using TA-HApNps [23]. There are studies describing the synthesis of this plant extract mediated NPs, which have been as effective as the conventional chemical means [12]. Furthermore, the biological advantages that the TA plant extract possesses, can be effectively incorporated into the NPs [24].

The conventional method of NP synthesis involves the usage of certain chemicals as a capping agent for the stability of the NPs [25]. This in turn increases the toxicity of the obtained NPs. The green synthesis of NPs, thereby utilises naturally occurring plant extracts, which act as the reducing agent in the process of formation of NPs. The present study was conducted in order to test the cytotoxic and antioxidant activity of the newly synthesised TA-mediated HApNPs.

The brine shrimp lethality test is a reliable test employed to detect the cytotoxicity of any drug in its prototype stages [26]. It is a good indicator in identifying the cytotoxicity of any biological component in an in-vitro set-up [27]. DPPH assay was employed in the present study to test the antioxidant activity of the TA-mediated HApNPs. This test is an economical and easy to assess, free radical scavenging method that can be employed to test the antioxidant activity of any compound or any biological extract [28]. The results from this study revealed that the antioxidant property increased with higher concentration of the NP solution. This was in accordance with the study conducted by Viswanatha GLS et al., where he reported that the alcoholic extract of bark stem of *Terminalia arjuna* (ALTA) showed potent antioxidant property in DPPH assay [29]. Gaikwad D and Jadhav N also reported positive results in terms of the free radical scavenging property of TA using DPPH assay [30]. Mohammad S et al., compared five different extracts of TA and reported that the Methanol extract of TA had the highest antioxidant property, followed by ethanol and ascorbic acid, respectively [31].

Based on the findings of this study, it can be observed that the TA-mediated HApNPs has decent cytotoxic and antioxidant properties. However, further confirmatory tests are to be employed if the component is to be further taken ahead for an in-vivo setting. An interesting field of application would be on the osteogenic activity of TA plant. TA plant extract has shown promising results in wound healing and new bone formation as reported in several animal studies [9,14]. Future scope of the present study, would be to formulate a biological component, making use of this combination of NPs, that would mimic the property of a bone substitute, which would also be capable of inducing bone formation.

Limitation(s)

Although the employed tests for evaluation of cytotoxicity and antioxidant properties showed positive results, they remain primitive and simple laboratory tests. The newly synthesised TA-HApNPs needs to be further tested under an in-vivo animal study design, to determine its systemic effects.

CONCLUSION(S)

The TA-HApNPs can be synthesised, using simple and routinely used laboratory armamentarium in the size range of 5-25 nm. The TA-HApNPs have minimal cytotoxic effect and a good antioxidant activity.

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All authors contributed equally to the present study.

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