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Research Article **Botany**

Titanium nanoparticle synthesized from *Piper betle* L. and evaluation of antioxidant, antiinflammatory and antimicrobial activity

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ABSTRACT

In the present study aimed to Synthesis, characterization and evaluation of its antioxidant, anti-inflammatory and antibacterial activity of titanium nanoparticles from *Piper betle* leaf extract. Titanium nanoparticles exhibit brown colour in aqueous solution due to excitation of surface plasmon vibrations in titanium nanoparticles. The appearances of brown colour in the reaction vessels suggest the formation of titanium nanoparticles. UV-Visible and FTIR spectroscopy are further confirmed the structural characterization and functional group identification of titanium nanoparticles. The SEM analysis showed the particle size 70-140nm as well face-centred cubic structure of the nanoparticles. Titanium nanoparticles might be useful for the development of newer and more potent antibacterial agents. All the above data's represented in our study contribute to a novel and unexplored area of nanomaterials as medicine.

Keywords: Nanoparticles, Titanium nanoparticles, Antibacterial activity, Antioxidant,
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INTRODUCTION

Nanoparticles exhibit completely new or improved properties based on specific characteristics such as size, distribution and morphology. The titanium dioxide nanoparticles have various and important applications. Historically, titanium dioxide has been known to has a disinfecting effect and has been found in applications ranging from traditional medicines to culinary items. It has been reported that titanium dioxide nanoparticles (TiNPs) are non-toxic to humans and most effective against bacteria, virus and other eukaryotic micro-organism at low concentrations, without any side effects (Jeong *et al.*, 2005). Moreover, several salts of titanium dioxide and their derivatives are commercially manufactured as antimicrobial agents (Krutyakov *et al.*, 2008). In small concentrations of titanium dioxide is safe for human cells, but lethal for microorganisms (Sharma *et al.*, 2009). Nanotechnology is now creating growing sense of

excitement in the life sciences especially biomedical devices and Biotechnology (Prabhu *et al.*, 2010).). Titanium dioxide has received great attention due to its unique photocatalytic activity in the treatment of environmental contamination. But for practical application, the photocatalytic activity of TiO_2 needs further improvement. An efficient way to improve the TiO_2 photoactivity is to introduce foreign metal ions (surface modifications) into TiO_2, which is also called heterogeneous photocatalysis. The sol-gel process is the most attractive method to introduce foreign metal ions into TiO_2 powders and films. Several different methods have been developed for generating titanium nanoparticles.

Plant derived medicines have been used in traditional health care systems for the treatment of various ailments and diseases since time immemorial. According to the World Health Organisation (WHO), it has been estimated that 80% of the world's population is still dependant on traditional medicines for maintaining their health and combating various diseases. Besides, 56% of world's populations in the rural areas rely chiefly on herbal medicine and supplementation for their primary health care needs. Today bacterial infections, fungal infections, hypertension, diabetes, malaria and cancer are the common health problems in rural communities throughout the world. A huge number of traditionally important medicinal plants have been known to be biologically effective against these diseases. One such potential plant is *Piper betle***.** In the present study, synthesis of titanium dioxide nanoparticles from aqueous extract of *Piper betle*.

MATERIALS AND METHODS

Collection of plant materials

The leaves of *Piper betle* were collected from MelaThirupanthuruthi, Thanjavur district, Tamil Nadu and India. The leaves were rinsed with water thrice followed by distilled water to remove the fine dust materials and then, the leaves were dried under direct sun light for one week to completely remove the moisture.

Preparation of alcoholic extract:

The powdered leaves of *Piper betle* were extracted with 70% methanol for 24 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in a refrigerator for further use.

Phytochemical screening

Chemical tests were carried out on the aqueous extract of *Piper betle* using standard procedures to identify the constituents as described by Harborne (1973) Trease and Evans (1989) Sofowara (1993).

Synthesis of titanium nanoparticles (TiNPs)

Two different conical flasks were taken and add 20 ml of *Piper betle* extract separately. 80 ml of 1 mM aqueous TiO_2 solution added to the each conical flask. The flask was then incubated in the dark at 24 hrs (to minimize the photo activation of titanium dioxide) at room temperature. A control setup was also maintained without leaves extract. The TiO_2 nanoparticle solution thus obtained was purified by repeated centrifugation at 10,000 rpm for 15 min followed by re-dispersion of the pellet in de-ionized water. Then the TiO_2 nanoparticles were freeze dried using SEM analysis (Thirunavukkarasu *et al*., 2014).

UV-Visible Spectrum analysis

The extracts were examined under visible UV-Visible spectrum. The sample is dissolved in same solvent. The extracts were scanned in the wavelength ranging from 330-920 nm using Systronic Spectrophotometer. These solutions were scanned in turn at intervals of 50 nm and the characteristic peaks were detected. The peak value of the UV-Visible was recorded.

SEM analysis of titanium nanoparticles

Scanning Electron Microscopic (SEM) analysis was done using JSM 6701F – 6701 machine (Japan). Thin films of the sample were prepared on a carbon coated copper grid by just dropping a very small amount of the sample on the grid. Extra solution was removed using a blotting paper and then the films on the SEM grid were allowed to dry by putting it under a mercury lamp for 5 min.

In vitro antioxidant activity

DPPH radical-scavenging activity was determined by the method of Shimada, *et al.*, (1992).

In-vitro anti-inflammatory activity

In vitro anti-inflammatory activity was carried out by the method of Sangita Chandra *et al*.,(2012).

DETERMINATION OF ANTIMICROBIAL ACTIVITY

Preparation of plant extract, titanium and Standard solutions for the experiment

The leaves extract of *Piper betle* was weighed (10mg/10ml) and dissolved in sterile distilled water. TiNPs (1mM titanium dioxide was added to plant extract to make up a final solution 200 ml and centrifuged at 18,000 rpm for 25 min. The collected pellets were used in this study) and Standard solution as Chloromphenical (25mg/ml distilled water). They were kept under refrigerated condition unless they were used for the experiment.

Preparation of dried filter paper discs

Whattman filter paper (No:1) was used to prepare four discs approximately 6 mm in diameter, which are placed in hot air for sterilization After sterilization, each discs were loaded with 30µl of *Piper betle* extract, TiNPs and Standard solution as Chloromphenical respectively and again kept under refrigeration for 24 hrs.

Application of discs to inoculated agar plates

Previously prepared paper discs were dispensed onto the surface of the inoculated agar plate. Each disc was pressed down firmly to ensure complete contact with the agar surface. The discs were placed on the medium suitably apart and the plates were incubated at 5°C for 1 hr to permit good diffusion and then transferred to incubator at 37°C for 24 hrs. After completion of 24hrs, the plates were inverted and placed in an incubator, for 24 hrs.

Antimicrobial assay

Antibiogram was done by disc diffusion method (Awoyinka *et al.*, 2007) using plant the leaf extracts of *Piper betle.* Petri plates were prepared by pouring 30 ml NA medium for bacteria. The test organism was inoculated on solidified agar plate with the help of micropipette and spread and allowed to dry for 10 minutes. The surfaces of media were inoculated with bacteria from a broth culture. A sterile cotton swab is dipped into a standardized bacterial test suspension and used to evenly inoculate the entire surface of the nutrient agar plate. Briefly, inoculums containing *Escherichia coli* were spread on nutrient agar plates for bacteria. Using sterile forceps, the sterile filter papers (6 mm diameter) containing each 30µl of leaf extract, TiNPs and Standard solution as Chloromphenical were laid down on the surface of inoculated agar plate. The plates were incubated at 37°C for 24 h for the bacteria and at room temperature (30±1) for 24-48 hr. Each sample was tested in triplicate. The antimicrobial potential of test compounds was determined on the basis of mean diameter of zone of inhibition around the disc in millimeters. The zones of inhibition of the tested microorganisms by the extracts were measured using a millimeter scale.

RESULTS AND DISCUSSION

Medicinal plants are the nature's gift to human being to make disease free healthy life. It plays a vital role to preserve our health. India is one of the most medico-culturally diverse countries in the world where the medicinal plant sector is part of a time-honoured tradition that is respected even today. Hence, the main traditional systems of medicine include Ayurveda, Siddha and Unani (Kotnis *et al.*, 2004). In the present study was carried out on the plant sample revealed the presence of medicinally active constituents. The phytochemical characters of the *P. betle* investigated and summarized in Table-1. The phytochemical screening of leaves *P.betle* showed that the presence of flavonoids, phenolics, tannin, saponins, protein, glycosides, alkaloids, steroids and while phlobatannins and anthroquinones were absent. (Table 1) Pradhan *et al*., (2013) reported the *P. betle* leaf contains Water (85-90%), Proteins (3-3.5%), Carbohydrates (0.5-6.1%), Minerals (2.3- 3.3%), Fat (0.4-1%), Fibre (2.3%), Essential oil 0.08-0.2%), Tannin (0.1-1.3%), Alkaloid arakene). It also contains different vitamins like Vitamin-C (0.005-0.01%), Nicotinic acid (0.63-0.89mg/100gms), Vitamin-A (1.9- 2.9mg/100gms), Thiamine (10-70µg/100gms), Riboflavin (1.9-30µg/100gms) beside this it contains minerals such as Calcium (0.2-0.5%), Iron (0.005-0.007), Iodine (3.4µg/100gms), Phosphorus (0.05-0.6%) and Potassium (1.1-3%). Lakshmi (2011) investigated the phytochemicals on on leaves revealed the presence of Alkaloids, Carbohydrate, Amino acids, Tannins and Steroidal components.

Table 1: Phytochemical screening of the leaves of *Piper betle*

S.No	Phytochemical analysis	Observation
1	Tannin	+
2	Phlobatannins	-
3	Saponin	+
4	Flavonoids	+
5	Steroids	+
6	Terepenoids	+
7	Triterpenoids	+
8	Alkaloids	+
9	Carbohydrate	++
10	Protein	+
11	Anthroquinone	-
12	Polyphenol	+
13	Glycoside	+

(+) Presence (-) Absence (++) High concentrations

Synthesis of titanium nanoparticles

The green synthesis of titanium nanoparticles through plant extracts were carried out. titanium dioxide is used as reducing agent as titanium has distinctive properties such as good conductivity, catalytic and chemical stability. Applications of such eco-friendly nanoparticles in bactericidal, wound healing and other medical and electronic applications, makes this method potentially exciting for the large-scale synthesis of other inorganic materials like nanomaterials. The aqueous titanium ions when exposed to herbal extracts were reduced in solution, there by leading to the formation of titanium hydrosol. The time duration of change in colour varies from plant to plant. The phytochemicals present in the leaves extract were considered responsible for the reduction of titanium ions. It is well known that titanium nanoparticles exhibit yellowish - brown colour in aqueous solution due to excitation of surface plasmon vibrations in titanium nanoparticles (Thirumurgan *et al.*, 2010). The appearances of yellowish-brown colour in the reaction vessels suggest the formation of titanium nanoparticles (SNPs) (Shankar *et al.*, 2004).

Titanium nanoparticles are being extensively synthesized using many different biological sources including fungi, bacteria and plants (Shivaji *et al.*, 2011; Shaligram *et al.*, 2009). Among them the plant mediated nanoparticles synthesis is getting more popular because of the high reactivity of plant extract and easy availability of plant materials. This method of nanoparticles synthesis involves no toxic chemicals and termed as green chemistry procedure. In this present study leaves of *Piper betle* extract was used for the synthesis of titanium nanoparticles. The aqueous TiO_2 solution turned to brown colour after 4 hours (Plate 1 shows- TiO_2 and TiNPs), indicating the formation of TiNPs in the reaction solution probably as a result of the excitation of Surface Plasmon Resonance (SPR) bands (Mulvaney, 1996). The control tubes (Tio_2) showed no change in colour when incubated in a similar condition. (Plate 1)

SEM analysis

SEM analysis was carried out to understand the topology and the size of the Ti-NPs, which showed the synthesis of higher density polydispersed spherical Ti-NPs of various sizes. The SEM image showing the high density titanium nanoparticles synthesized by the leaf extract was further confirmed the development of titanium nanostructures. Most of the nanoparticles aggregated and only a few of them were scattered, and observed under SEM. The SEM analysis of *Piper betle* synthesized titanium nanoparticle showed the particle size between 70–140 nm as well the cubic, face-centred cubic structure of the nanoparticles (Plate 2).

Plate 1. Aqueous solution of 1mM Tio_2 with *Piper betle* extract

(A) (B)

(A) After addition of extract at 4 hr. (B) Before adding the extract

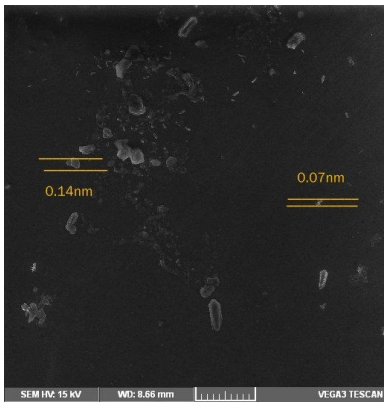


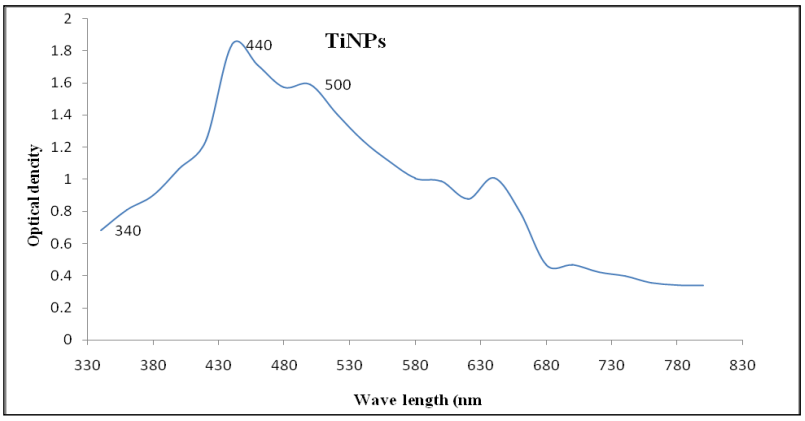
Plate 2 High resolution Scanning Electron Microscopic (SEM) image of titanium nanoparticles (AgNPs) synthesized from . *Piper betle* Extract Polydispersed (Cluster) TiNPs ranged between 70–140 nm.

Ultraviolet (UV) spectroscopy

Scattering from a sample is typically very sensitive to the aggregation state of the sample, with the scattering contribution increasing as the particles aggregate to a greater extent. For example, the optical properties of titanium nanoparticles change when particles aggregate and the conduction electrons near each particle surface become delocalized and are shared amongst neighbouring particles. When this occurs, the surface plasmon resonance shifts to lower energies, causing the absorption and scattering peaks to red-shift to longer wavelengths. UV-Visible spectroscopy can be used as a simple and reliable method for monitoring the stability of nanoparticle solutions.

The absorption spectrum of titanium nanoparticles changes as the particles transition from a well-dispersed state to an aggregated state following the addition of a concentrated salt solution; as aggregation proceeds, the plasmon peak at 420 nm decreases, a secondary peak at 620 emerges, and the baseline elevates due to scattering by aggregates. It is observed that the maximum absorbance of *Piper betle* titanium nanoparticles occurs at 440 nm. As the particles destabilize, the original extinction peak will decrease in intensity (due to the depletion of stable nanoparticles), and often the peak will broaden or a secondary peak will form at longer wavelengths due to the formation of aggregates (Tian, 2005).

Fig 3 UV-Visible spectrum of TiNPs synthesized from *Piper betle* extract



The rapid and irreversible change in the extinction spectrum clearly demonstrates that the nanoparticles are agglomerating. UV spectroscopy can be used as a characterization technique that provides information on whether the nanoparticle solution has destabilized over time. In the present investigation the peak was decreased due to destabilization of nanoparticles (Fig 3).

***In vitro* antioxidant activity**

The imbalance between ROS production and antioxidant leads to 'oxidative stress'. Any compound, natural or synthetic with antioxidant properties might contribute towards the partial or total alleviation of this type of damage. The harmful effect of ROS is neutralized by a broad class of protective agents termed antioxidants, which prevents oxidative damage by reacting with free radicals before any other molecules can become a target. Antioxidants are probably now regarded as the new generation 'superheroes' to maintain the health (Aruoma, 1994).

Research in the recent past has accumulated enormous evidences revealing that enrichment of body systems with natural antioxidants may correct the vitiated homeostasis and can prevent the onset as well as treat diseases caused and/or fostered due to free-radical mediated oxidative stress. Natural antioxidants strengthen the endogenous antioxidant defense from ROS ravage and restored the optimal balance by neutralizing the reactive species. In the present study to investigate the *in vitro* antioxidant activity of TiNPs through DPPH radical scavenging activity. Antioxidant activity TiNPs compared with standard as ascorbic acid

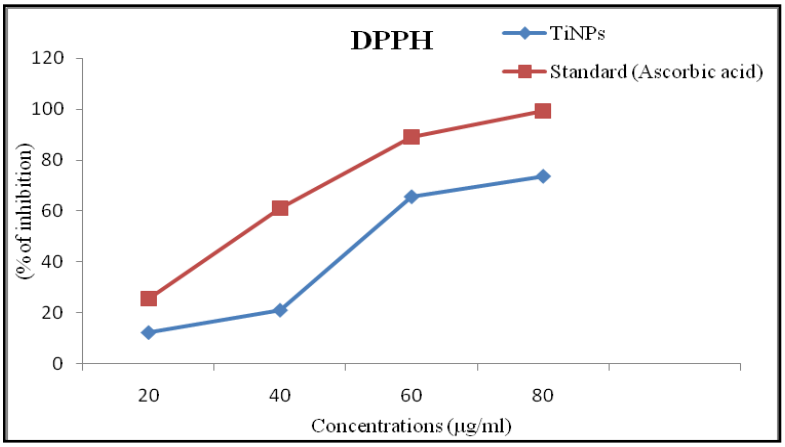
DPPH Assay

Recently, the use of the DPPH$^{\bullet}$ reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH$^{\bullet}$ free radical by a scavenger causes a decrease in absorbance at 517 nm, which can be followed by a common spectrophotometer set in the visible region. The effect of antioxidants on DPPH$^{\bullet}$ is thought to be due to their hydrogen donating ability (Sindhu and Abraham, 2006). DPPH radical scavenging activity of TiNPs and standard as ascorbic acid are presented in Table 2. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants (Nuutila *et al.,* 2003). The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

Table 2 DPPH Radical scavenging activity of leaf extract *P. betle*

Concentrations (µg/ml)	TiNPs	Standard (Ascorbic acid)
20	12.41±1.52	25.6±2.04
40	21.20±2.77	61.26±4.90
60	65.75±2.21	88.98±7.11
80	73.78±3.34	99.34±7.94

Fig 5 DPPH Radical scavenging activity of leaf extract P. *betle*



***In vitro* anti-inflammatory activity**

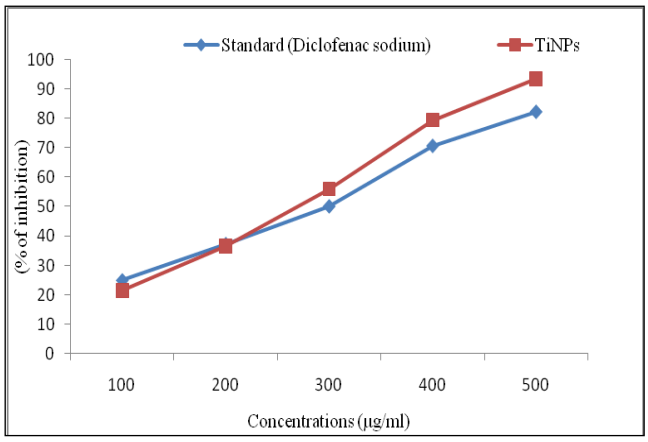
There are certain problems in using animals in experimental pharmacological research, such as ethical issues and the lack of rationale for their use when other suitable methods are available or could be investigated. Hence, in the present study the protein denaturation bioassay was selected for *in vitro* assessment of anti-inflammatory property TiNPs. Denaturation of tissue proteins is one of the well-documented causes of inflammatory and arthritic diseases. Production of auto antigens in certain inflammatory diseases may be due to *in vivo* denaturation of proteins. The mechanism of denaturation probably involves alteration in electrostatic, hydrogen, hydrophobic and disulphide bonding (Grant *et al*., 1970). Agents that can prevent protein denaturation therefore, would be worthwhile for anti-inflammatory drug development. The increments in absorbance of test samples with respect to control indicated stabilization of protein (Egg albumin) denaturation by and reference diclofenac sodium. TiNPs exhibited anti-inflammatory activities in dose dependent manner (Table 3 and fig 6).

Table 3 *In vitro* anti-inflammatory activity of TiNPs from *Piper betle* (Egg albumin)

Doses (µg/ml)	TiNPs	Standard (Diclofenac sodium)
100	24.92±0.07	21.37±1.98
200	37.25±0.06	36.45±2.37
300	50.07±0.06	55.94±3.47
400	70.62±0.07	79.45±4.65
500	82.19±0.04	93.45±6.84

Values are expressed as Mean ± SD for triplicates

Fig 6 *In vitro* anti-inflammatory activity of TiNPs from *P. betle* extract



ANTIMICROBIAL ACTIVITY

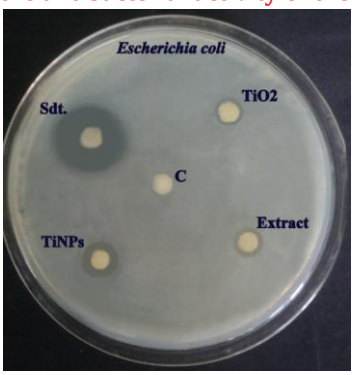
Toxicity studies on pathogen opens a door for nanotechnology applications in medicine. Biological synthesis of metal NPs is a traditional method and the use of plant extract has a new awareness for the control of disease, besides being safe and no phytotoxic effects (Torresdey *et al*., 2003). The biologically synthesized titanium nanoparticles using medicinal plants were found to be highly toxic against different pathogenic bacteria of selected species. The TiNPs of *Piper betle* was observed against *E. coli*. The inhibitory activities in culture media of the Ti nanoparticles reported in Table 4 were comparable with standard antimicrobiotic viz. chloromphenical. In this study, the antimicrobial effect of Ti nanoparticles was evaluate against the *E. coli*. When Ti nanoparticles were tested they effectively inhibited bacterial growth. The results show that Ti nanoparticles having antimicrobial activity against *E. coli* that was similar to that found by Sharma et al.,2009.The present study included the bio-reduction of titanium ions through *P. betle* leaf extract and testing for their antimicrobial activity. The aqueous titanium ions exposed to the extract, the synthesis of titanium nanoparticles were confirmed by the change of colour of leaf extract of *P. betle*. These environmentally being titanium nanoparticles were further confirmed by SEM analysis.

Table 4 Antimicrobial activity of TiNPs

Micro Organism	TiNPs	*Piper betle* Extract	TiO_2	Std.
Escherichia coli	4.60±0.32	2.10±0.14	0.50±0.03	7.30±0.51

Values were expressed as Mean ± SD.
TiNPs = titanium Nanoparticles; TiO_2 = Titanium dioxide
Bacterial standard - Chloromphenical

Plate 7 shows the antibacterial activity of the leaves of *Piper betle* TiNPs



CONCLUSION

The present study exhibit a simple environmentally being method safe for the synthesis of titanium nanoparticles from a novel primitive plant source. This method can be further used for industrial production of nanoparticles at room temperature and with a single step. Since the nanoparticles thus synthesized shows antioxidant and antimicrobial activity, they can be used in various fields such as pharmaceutical industry and so on. Titanium nanoparticles might be useful for the development of newer and more potent antimicrobial agents. The data represented in our study suggested that titanium nanoparticle synthesized from *Piper betle* extract has controlled nanoparticle synthesized and possess potential biological activity.

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