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ANTIBACTERIAL AND ANTICANCER ACTIVITY OF GREEN SYNTHESISED TITANIUM DIOXIDE NANOPARTICLE FROM *TERMINALIA CHEBULA*

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ABSTRACT

Objectives: To synthesize titanium nanoparticles from *Terminalia* and compare the antibacterial and anticancer effectiveness of biologically synthesized titanium nanoparticles to crude extracts. **Methods:** Green synthesis of titanium dioxide nanoparticles (NP) is carried out with the fruit rind extract of *Terminalia chebula* The NP was characterized by FTIR and SEM. Antibacterial activity of the NP was checked against clinical pathogens. The antioxidant activity was determined by DPPH assay. Cytotoxic activity was done on vero cell lines and toxic free concentrations were estimated. Anti cancer activity was done against A549 cell lines by MTT assay. **Results:** Titanium nanoparticles were biologically synthesized. The particles showed antibacterial activity and exhibited antioxidant nature higher than the extracts. Cytotoxicity assay revealed the nanoparticles were nontoxic and were capable of inhibiting proliferation of A549 cell lines. **Conclusions:** *T. chebula* was found to be a good source for biosynthesis of titanium nanoparticles which exhibited significant activity than the extract. Hence its concluded that nanoparticles are more suitable for drugs than crude extracts.

KEYWORDS: Green synthesised nanoparticles, Titanium dioxide, *Terminalia chebula*, DPPH, MTT assay.

1.0 INTRODUCTION

Nanotechnology has shown a rapid growth due to their application in medical field in form of drug delivery, therapy, diagnostics, tissue regeneration, cell culture etc.[1] Nanomaterial of different sizes and shapes are used for the research purposes because of their selectivity and physico-chemical properties compared to that of bulk materials**.** Drugs bound to nanoparticles have been reported to be more advantage when compared with conventional form of drug.

Synthesis of nanoparticles from plant extract from leaves, bark, stem, shoots, shreds, seeds, latex, secondary metabolites, roots, twigs, peel, fruit, seedlings, essential oils, tissue cultures and gum were proved.[2] The nanoparticles produced by the plants were found to be more stable and the rate of synthesis was also faster than chemically synthesized nanoparticles. Extracts from plant may act both as reducing and capping agents in case of synthesizing nanoparticles.

Titanium dioxide is not classified as hazardous according to the United Nations (UN) Globally Harmonized System (GHS) of classification and labeling of chemicals.[3] The aqueous metal ion precursors from metal salts are reduced and as a result a colour change occurs in the reaction mixture[4]**.** The colour change indicates the formation of nanoparticles. According to several studies, it's believed that the metal oxides carry the positive charge while the microorganism carry negative charge, this causes electromagnetic attraction between microorganism and the metal oxides which leads to oxidization and finally death of microorganism.[5,6] Titanium dioxide nanoparticles are known to react with O_2 and –OH adsorbed on the surface to obtain oxygen free radical and hydroxyl free radical which are capable of directly attacking the cell wall and cell membrane of the microorganisms.

Terminalia chebula Reitz. is a plant species belonging to family Combretaceae. *T. chebula* is referred to black myrobalan in English. It is native to Indian subcontinent. *T. chebula* has been extensively used in Ayurvedha, Unani and Homeopathic medicines and has become a cynosure of modern medicine.[7]

The increasing incidence of resistance developed by bacteria against antibiotics, human sensitivity towards antibiotics and the adverse effect of the present synthetic antibiotics highlight the need of newer drugs. Hence the present investigation was designed to evaluate the antibacterial activity of green synthesised TiO_2 nanoparticles using the aqueous extract of fruit rind of *Terminalia chebula* to crude extract.

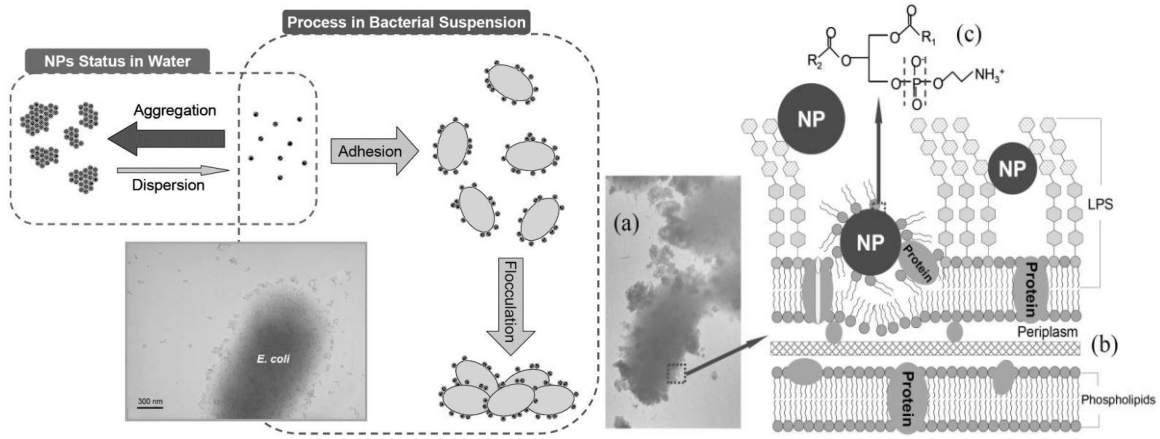


Fig 1: The mechanism of binding and effects of TiO_2 nanoparticles on cell walls of *E.coli* (adopted from W. Jiang 2011, Elnaz Babaei *et al.*, 2016).[8,9]

2.0 METHODOLOGY

Dry fruit of *Terminalia chebula* were collected locally and identified Plant Anatomy Research Centre, Tambaram, Tamil Nadu. Titanium IV isopropoxide and other solution were obtained from Sigma Aldrich.

2.1 Preparation of the seed rind extract

Potential and healthy fruit were collected. The outer coat of seed was removed and powdered well and then it was sieved well to get a fine powder.

Fig 2: *Terminalia chebula* dried fruit.

20 grams of the powdered fruit rind were weighed and soaked in 100ml of distilled water and incubated overnight at 4ºC, centrifuged and filtered through whattman filter paper. The method was repeated more times and the resultant extracts were further sterilised through 0.45µm millipore filter.[10]

2.2 Preparation of 0.1 mm titanium dioxide

2.2.1 Synthesis of Titanium dioxide nanoparticles using aqueous extract of *Terminalia chebula*

To synthesize the TiO_2 NPs, the erylenmeyer flask containing 80mL of Titanium(IV) Propoxide was stirred for 2 hours. Twenty ml of the aqueous extract of *Terminalia chebula* was added in 80mL of Titanium (IV) Propoxide at room temperature under stirred condition for 24 hour. After incubation, the synthesized nanoparticles turned light green in color.

2.2 Lyophilisation

The synthesised nanoparticle was transferred into sterile lyophilisation flask and frozen at -80 ºC in a deep freezer. The frozen extract was loaded to lyophilizer. The lyophilised nanoparticle was stored in -20ºC till bioevaluation.[10]

2.3 Qualitative phytochemical screening of *terminalia chebula*

The different qualitative chemical tests were performed to determine the phytoconstituents of the extract as per standard procedure.

2.4 UV-Visible Spectra Analysis

To monitor the formation and completion of bioreduction of Titanium dioxide ions in aqueous solution UV-visible analysis was done by using Shimadzu UV visible spectrophotometer. The bioreduction was monitored by periodical sampling of aliquots and the UV-visible spectra of these aliquots were monitored in 200-800nm range operated at a resolution of 1nm. Titanium IV Isopropoxide solution was used as a blank.

2.5 FTIR Analysis

FTIR spectroscopy was used to investigate surface chemistry and identify surface residues such as functional groups like carbonyls and hydroxyls moieties that attach to the surface during nanoparticle synthesis.

2.6 Scanning Electron Microscopy

Scanning electron microscope was done to observe clear images of the particles in colloid and also to know the size.

2.7 Anti-Bacterial Activity

Aqueous extract of *T. chebula,* and synthesized titanium dioxide nanoparticles were tested or their antibacterial activity against *Staphylococcus aureus, Escherichia coli, Pseudomonas aeroginosa, and Salmonella typhi.* Streptomycin was used as a standard.

2.7.1 Preparation of inoculum

The working bacterial strains were maintained in nutrient agar slants at 4ºC. Active cultures for experiment were prepared by inoculating a loop full of organism from the stock cultures into test tubes containing nutrient broth and incubated at 24hrs. The turbidity was adjusted to 0.5 Mac Farland standards.

2.7.2 Agar disc diffusion method

Antibacterial screening was carried out using the standard disc diffusion test[11, 12]. Samples were diluted for 1000µg/ml. 20µl of the nanoparticle suspension were incorporated in 6-mm-diameter sterile discs, and dried. The discs were placed on a 90-mm Mueller Hinton agar (MHA) plate seeded with test bacteria. Streptomycin is used as standard antibiotic disc. After overnight incubation at 37ºC, the agar plates were observed for zones of inhibition.

2.8 Anticancer Activity

2.8.1 Cell line and culture

A549 Cell line was obtained from NCCS, Pune. The cells were maintained in DMEM with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 50 µg/ml CO_2 at 37°C.

2.8.2 *In Vitro* assay for Anti Cancer activity (MTT assay)

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37^0C with 5% CO_2 condition. After the cell reaches the confluence, the sample was added and incubated for 24hrs. After incubation, the sample was removed from the well and washed with phosphate-buffered saline (pH 7.4) or DMEM without serum. 100µl/well (5mg/ml) of 0.5% 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl--tetrazolium bromide (MTT) was added and incubated for 4 hours. After incubation, 1ml of DMSO was added in all the wells .The absorbance at 570nm was measured with UV- Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

% cell viability = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments.[13]

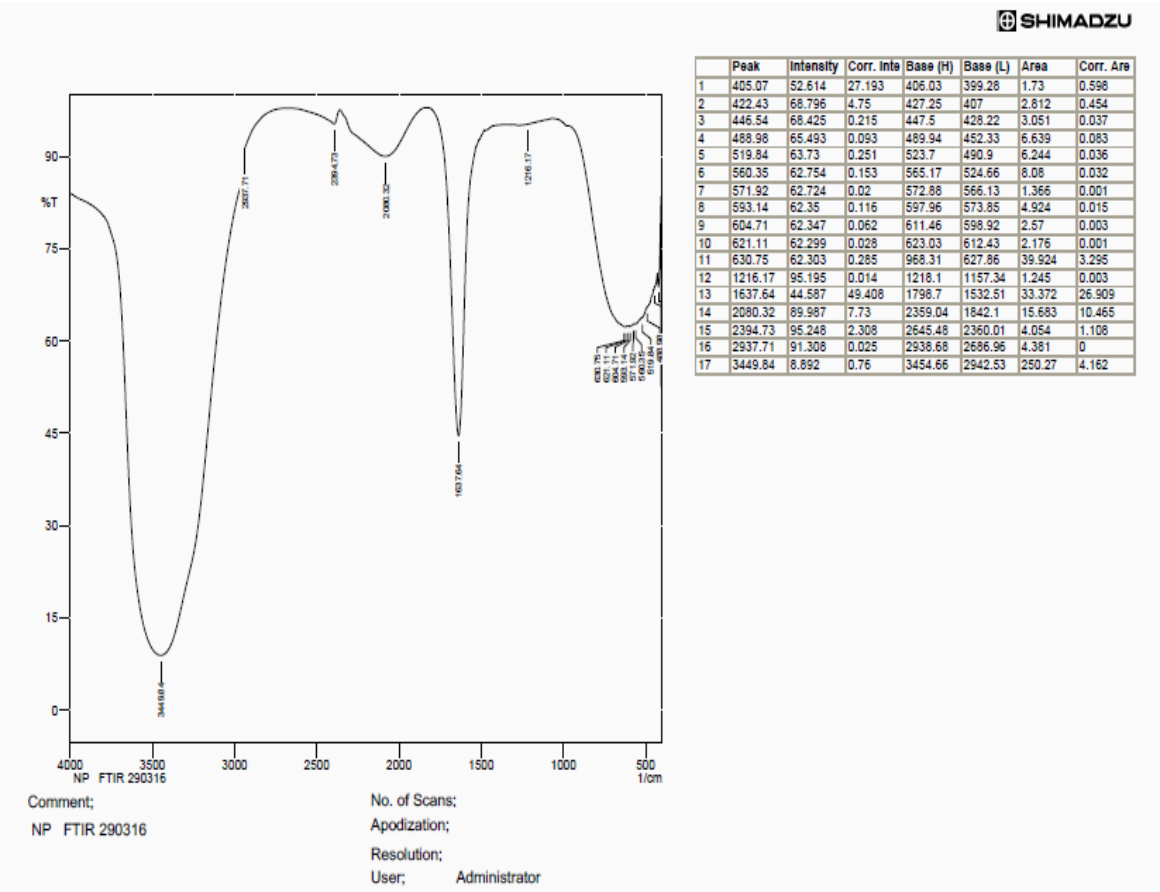
2.9 Anti-Oxidant Analysis

DPPH assay was performed to determine the antioxidant activity of the biologically synthesized titanium nanoparticles.[14] DPPH (1,1-diphenyl-2-picrylhydrazyl) is characterised as a stable free radical. When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of violet colour (although there would be expected to be a residual pale yellow colour from the picryl group still present). Aliquot 3.7 ml of absolute methanol in all test tubes and 3.8ml of absolute methanol was added to blank. Add 100µl of BHT to tube marked as standard and 100µl of respective samples to all other tubes marked as tests. 200µl of DPPH reagent was added to all the test tubes including blank. Incubate all test tubes at room temperature in dark condition for 30 minutes. The absorbance of all samples was read at 517 nm.

Calculation

$$\% \text{ Antioxidant activity} = \frac{\text{(Absorbance at blank) - (Absorbance at test)}}{\text{(Absorbance at blank)}} \times 100$$

3.0 RESULTS



	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	405.07	52.614	27.193	406.03	399.28	1.73	0.598
2	422.43	68.796	4.75	427.25	407	2.812	0.454
3	446.54	68.425	0.215	447.5	428.22	3.051	0.037
4	488.98	65.493	0.093	489.94	452.33	6.639	0.083
5	519.84	63.73	0.251	523.7	490.9	6.244	0.036
6	560.35	62.754	0.153	565.17	524.66	8.08	0.032
7	571.92	62.724	0.02	572.88	566.13	1.366	0.001
8	593.14	62.35	0.116	597.96	573.85	4.924	0.015
9	604.71	62.347	0.062	611.46	598.92	2.57	0.003
10	621.11	62.299	0.028	623.03	612.43	2.176	0.001
11	630.75	62.303	0.285	968.31	627.86	39.924	3.295
12	1216.17	95.195	0.014	1218.1	1157.34	1.245	0.003
13	1637.64	44.587	49.408	1798.7	1532.51	33.372	26.909
14	2080.32	89.987	7.73	2359.04	1842.1	15.683	10.465
15	2394.73	95.248	2.308	2645.48	2360.01	4.054	1.108
16	2937.71	91.308	0.025	2938.68	2686.96	4.381	0
17	3449.84	8.892	0.76	3454.66	2942.53	250.27	4.162

Fig 3: FTIR analysis of the synthesised Nanoparticle.

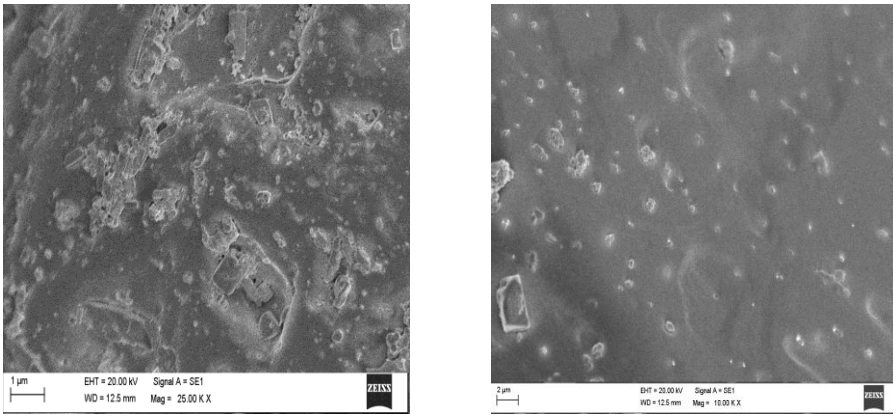


Fig 4: SEM Micrograph of biosynthesized TiO_2 nanoparticles.

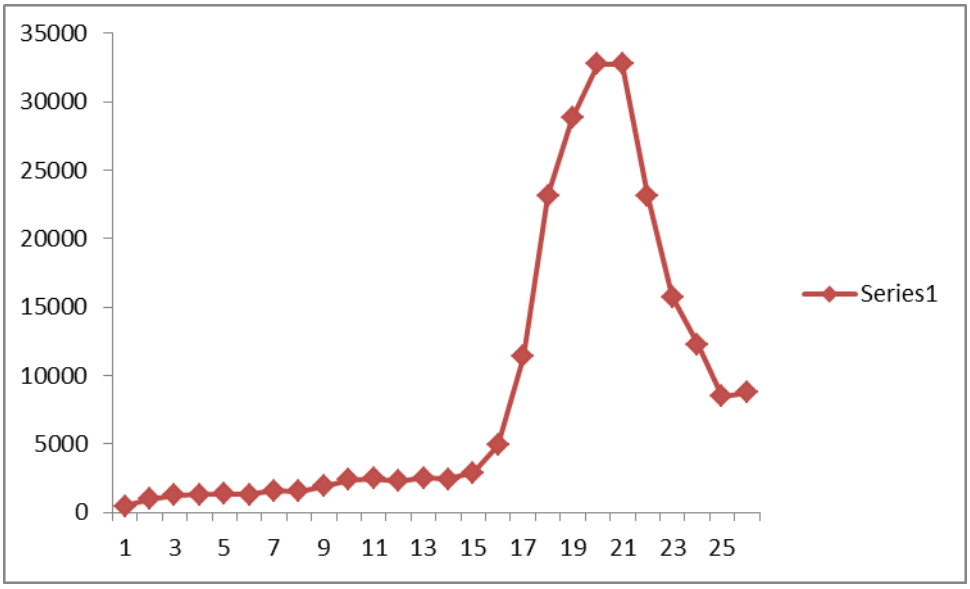


Fig 5: UV–VIS Spectrum Analysis of TiO_2 Nanoparticles reduced by *T.chebula* at 450 nm.

Table 2: Antibacterial activity of synthesised nanoparticles.

Organisms	Zone of inhibition (mm) Concentration(1000µg/ml)		Antibiotic (1mg/ml)
	Nanoparticle	Extract	
Staphylococcus aureus	23	11	35
Escherichia coli	11	8	19
Shigella flexneri	9	6	15
Bacillus subtilis	28	14	36
Salmonella typhi	14	10	26

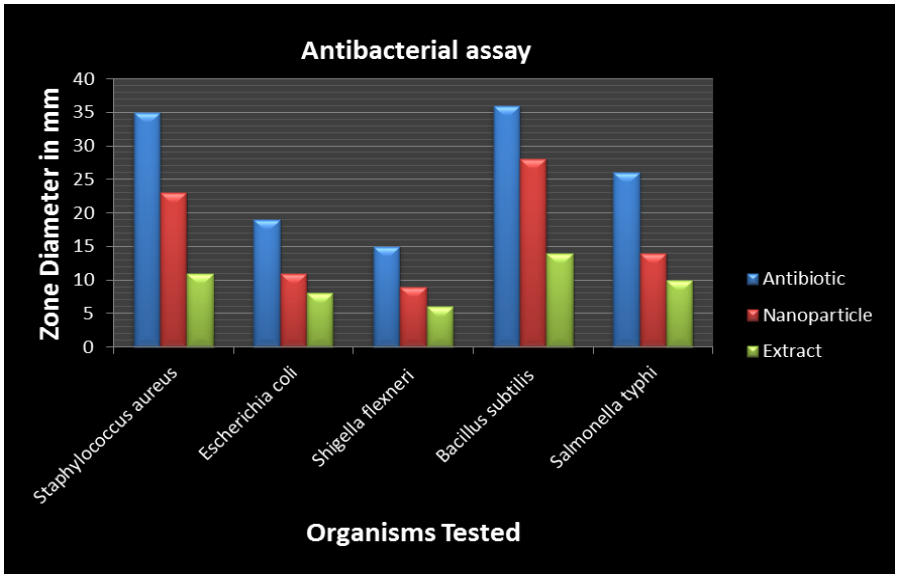


Fig 4: Antibacterial activity of synthesised nanoparticles in comparison with extracts.

Table 3: Cytotoxicity effect of extract on *Vero cell* line.

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.932	51.63
2	500	1:1	1.090	60.38
3	250	1:2	1.215	67.31
4	125	1:4	1.364	75.56
5	62.5	1:8	1.496	82.88
6	31.2	1:16	1.654	91.63
7	15.6	1:32	1.734	96.06
8	7.8	1:64	1.803	99.88
9	Cell control	-	1.805	100

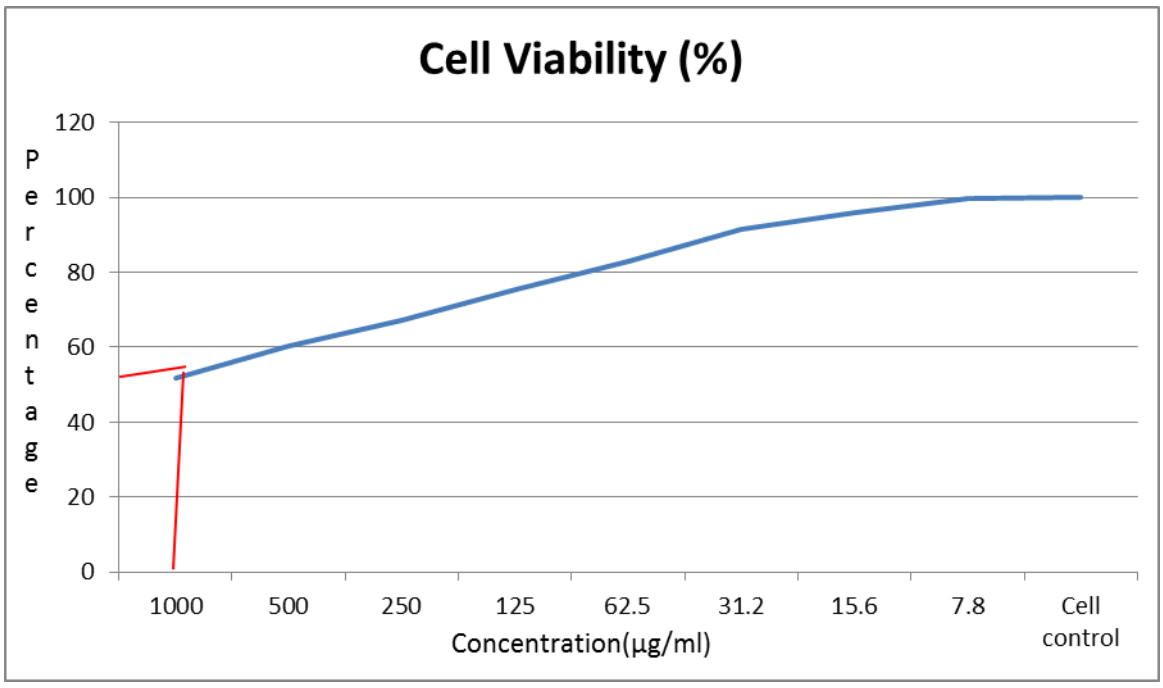


Fig 5: Cell viability of extract on *Vero cell* line.

Table 4: Cytotoxicity effect of Nanoparticle on *Vero cell* line.

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.832	46.09
2	500	1:1	0.914	50.63
3	250	1:2	1.093	60.55
4	125	1:4	1.155	63.98
5	62.5	1:8	1.343	74.40
6	31.2	1:16	1.486	82.32
7	15.6	1:32	1.593	88.25
8	7.8	1:64	1.754	97.17
9	Cell control	-	1.805	100

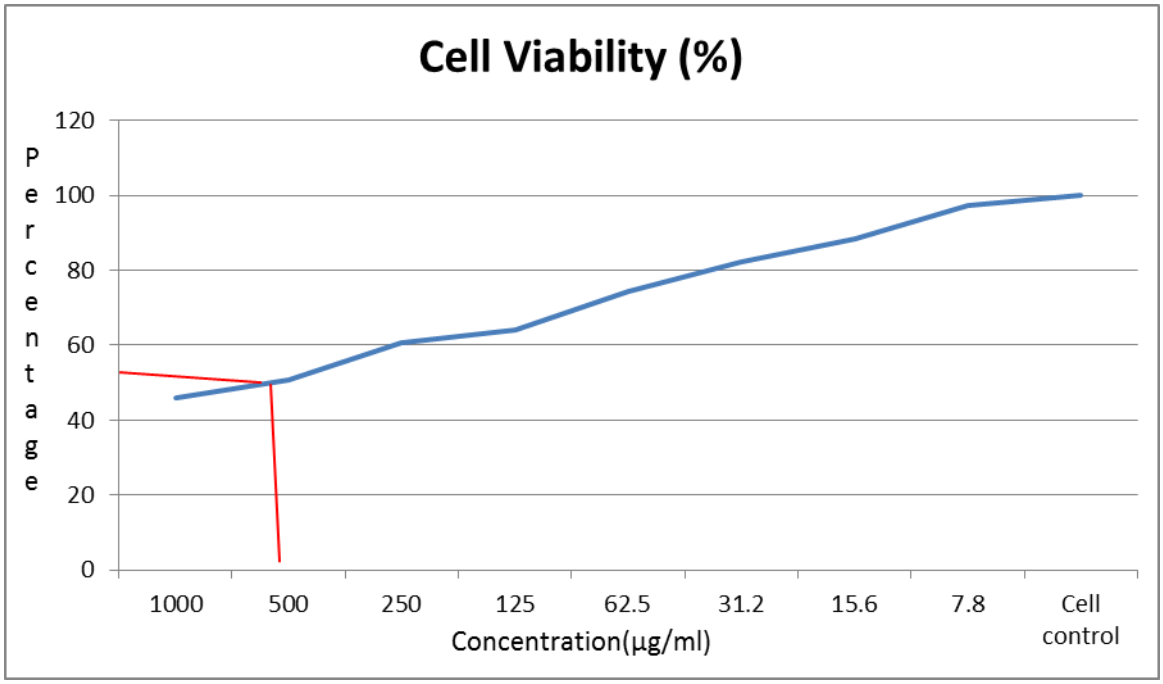
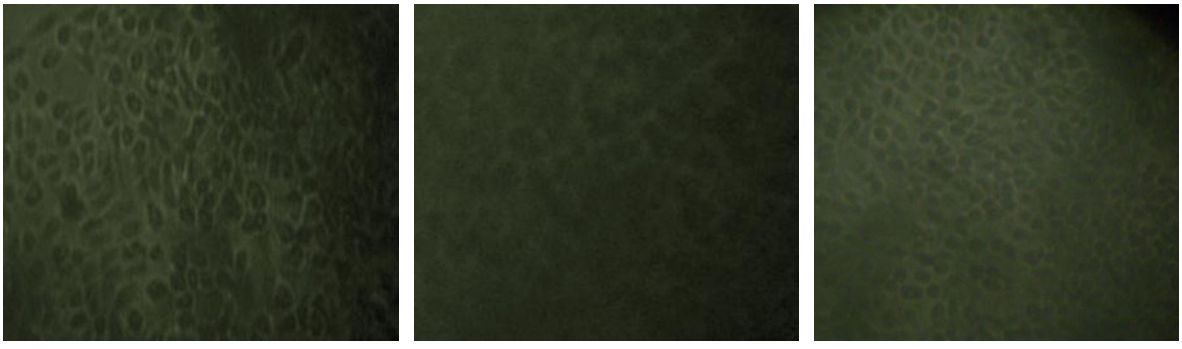
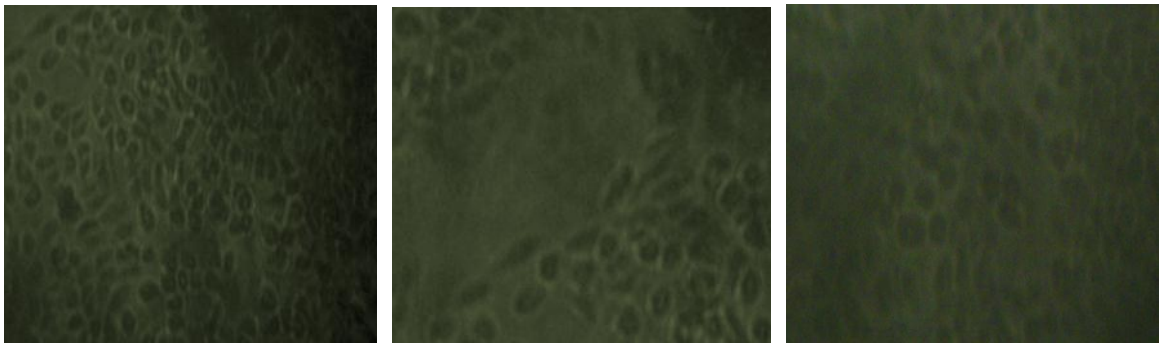


Fig 6: Cell viability of extract on *Vero cell* line.



Normal Vero Cell line **Nontoxic – 1000 µg/ml** **Nontoxic – 7.8µg/ml**

Fig 7: Cytotoxicity effect of Extract on *Vero cell* line.



Normal Vero Cell line **Toxicity – 1000 µg/ml** **Nontoxic – 500 µg/ml**

Fig 8: Cytotoxicity effect of Nanoparticle on *Vero cell* line.

Table 5: Anticancer effect of extract on A549 cell line.

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.378	29.88
2	500	1:1	0.459	36.28
3	250	1:2	0.524	41.42
4	125	1:4	0.603	47.66
5	62.5	1:8	0.665	52.56
6	31.2	1:16	0.771	60.94
7	15.6	1:32	0.868	68.61
8	7.8	1:64	0.945	74.70
9	Cell control	-	1.265	100

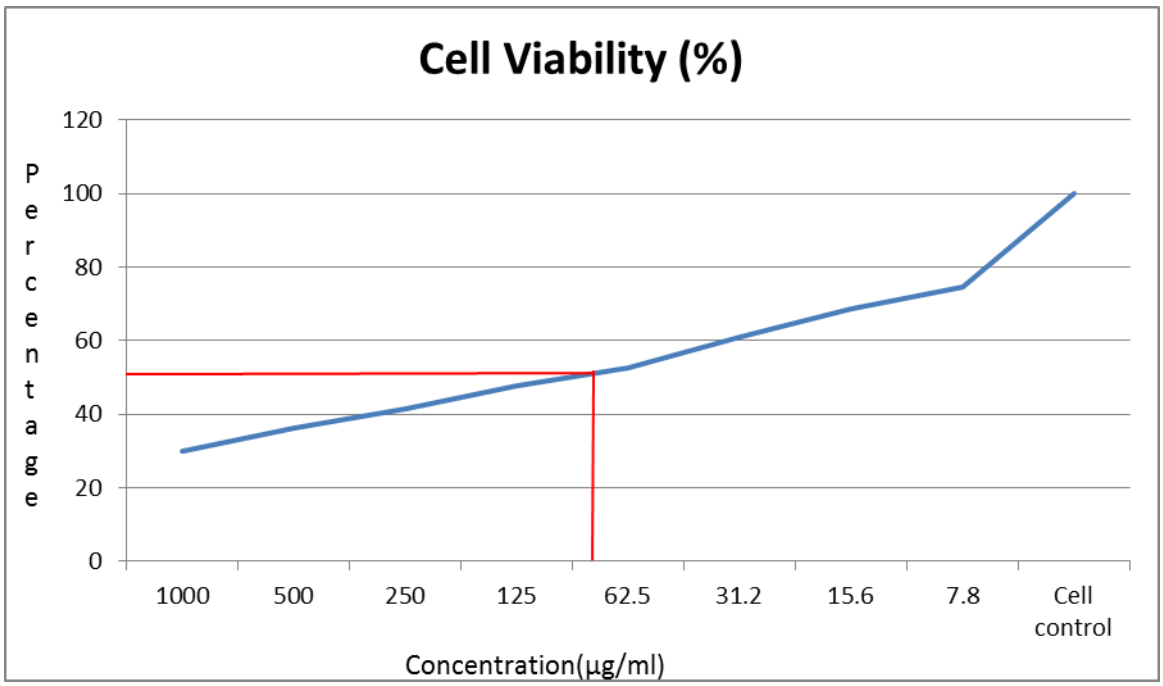


Fig 9: Anticancer effect of extract on A549 cell line.

Table 6: Anticancer effect of Nano Particle *on A549 Cell* line.

S. No.	Concentration (µg/ml)	Dilutions	Absorbance (O.D)	Cell Viability (%)
1	1000	Neat	0.150	11.85
2	500	1:1	0.270	21.34
3	250	1:2	0.352	27.82
4	125	1:4	0.442	34.94
5	62.5	1:8	0.587	46.40
6	31.2	1:16	0.680	53.75
7	15.6	1:32	0.755	59.76
8	7.8	1:64	0.824	65.13
9	Cell control	-	1.265	100

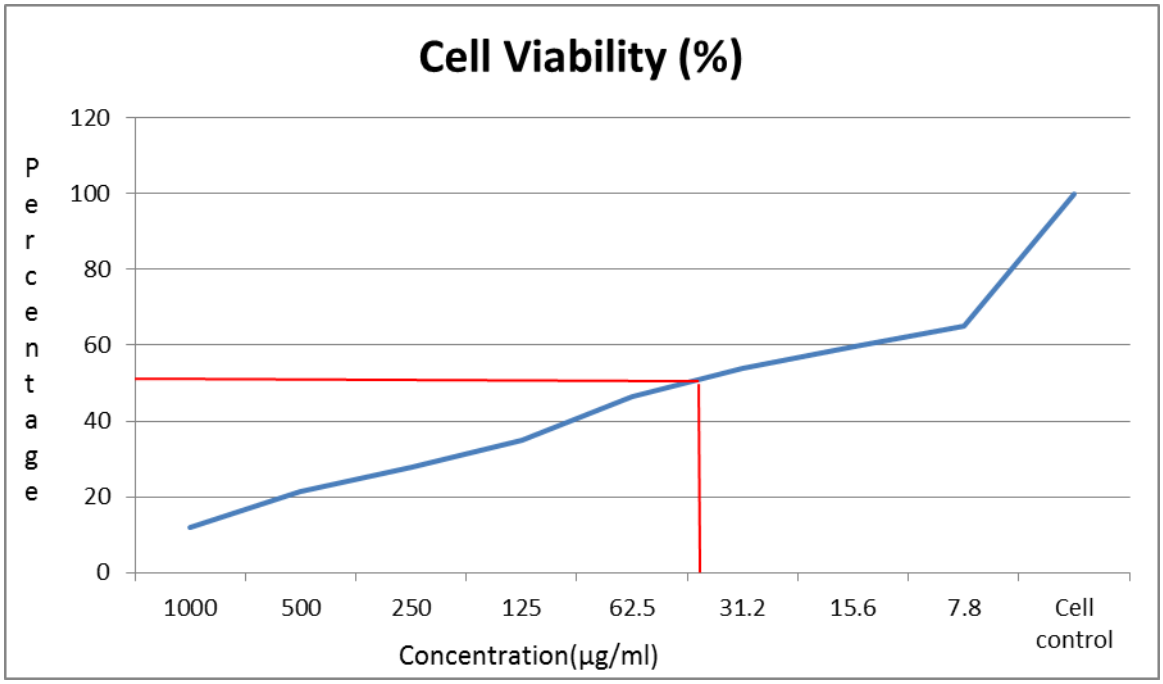
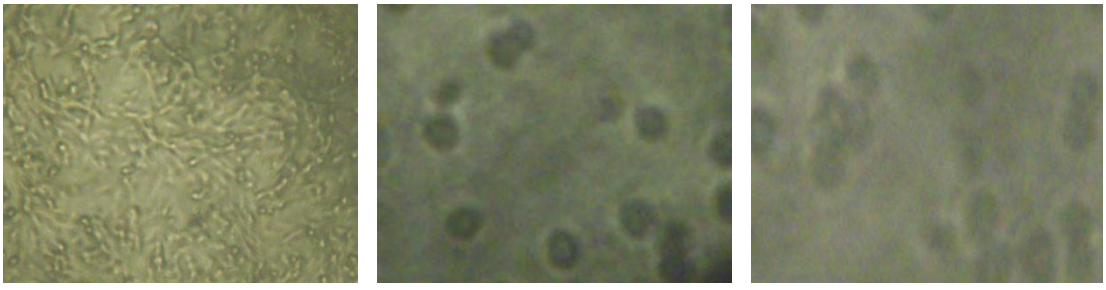
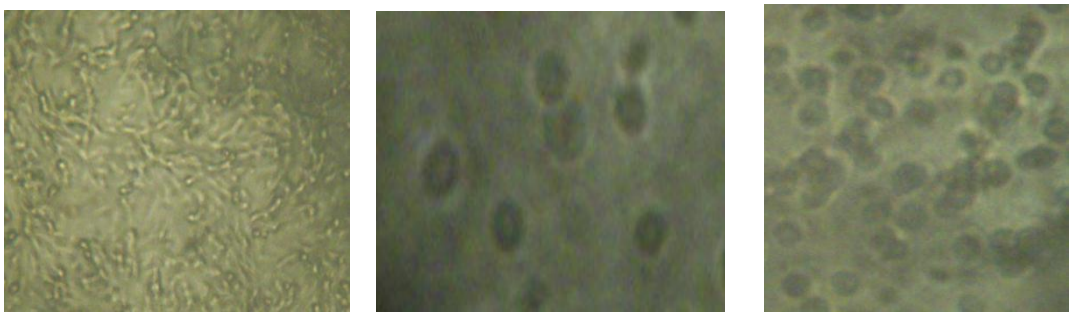


Fig 10: Anticancer effect of Nanoparticle on A549 cell line.



Normal A549 Cell line **Toxicity – 1000 µg/ml** **Nontoxic – 62.5µg/ml**

Fig 11: Anticancer effect of extract sample on *A549 cell* line.



Normal A549 Cell line **Toxicity – 1000 µg/ml** **Toxicity –62.5 µg/ml**

Fig 12: Anticancer effect of Nano Particle on *A549 cell* line.

Table 7: DPPH Assay Results.

S.no	Sample	Absorbance at 517 nm	%DPPH Activity
1.	Nanoparticle	0.104	83.72
2.	Extract	0.125	80.30

Control O.D: 0.639

4.0 DISCUSSION

Synthesis using non-toxic and environmentally friendly simple biological methods are gaining more attention recently. Microorganisms, whole plants and extracts from various plant parts and algae have been used to produce nanoparticles. Biosynthesis routes can actually provide NPs of a better defined size and morphology than some of the physico-chemical methods of production.

Since there are only a few study reported in antimicrobial activity of green synthesised TiO2 Nanoparticles it was proposed to synthesise TiO_2 nanoparticle from plant extracts. *Terminalia chebula* fruit rind extract was used to synthesis Titanium oxide nanoparticles and was tested for its antimicrobial, antioxidant, cytotoxic and anticancer activities because of its wide usage in traditional system of medicine and availability in the rural area.

Aqueous extract of the plant was subjected to phytochemical analysis. Phytochemical analysis revealed the presence of tannin, alkaloid and steroid. The plant extracts was mixed with Titanium IV isopropoxide for the synthesis of NPs. After overnight stirring, colour changed to light green. The observed colour change can be attributed to the reduction of metal ions in the mixture. The results are in conformity with Thirunavukkarasu SanthoshKumar *et al.*, 2014[15] who reported colour change after 24 hrs of incubation from *Psidium guajava* aqueous leaf extract.

UV-visible analysis was done to monitor the completion of bioreduction of Titanium dioxide ions in aqueous solution. Reduction of Titanium IV isopropoxide to Titanium dioxide nanoparticles during exposure to plant extracts is followed by a gradual increase in colour development from clear to green. The formation and completion of titanium dioxide nanoparticles was characterized by using Shimadzu UV visible spectrophotometer. The UV-Vis Spec analysis of the sample showed maximum absorbance at 440-450nm which confirmed the presence of TiO_2 NPs. This was in conformity with Valli and Geetha 2015.[16]

In order to identity the functional groups of the plant extract with titanium dioxide, FT-IR spectra of extract (Fig. 3) and titanium dioxide nanoparticles were recorded. In the present study, the stretching at the wave number 3200 to 3600 cm-1 shows the presence of O-H functional group, alcohol, strong and broad peak. The sharp peak was observed in the range 2850-3000cm-1 shows the C-H stretch free. And a C=C stretch ranging from 1620-1680 cm-1 shows the alkenes. A comparison of these results with earlier reports indicated that alcohols, alkanes and alkenes may be participating in the process of nanoparticle synthesis. The results are similar to Ankita *et al.*, 2016[17] peaks were observed at 1631.78/cm and 1641.42/cm indicated O-Ti-O bond. The peak around 3000/cm was due to the -OH stretching and the Ti-O stretching vibration was confirmed by the peak at the region of 1400-1460/cm.

The NPs sample was lyophilised. The resultant powdered sample was further used for SEM analysis. From the SEM images, it was observed that most of the TiO2 nanoparticles were showing irregular particles structure. The size was ranging from 80 to 100nm.

The antimicrobial activity of the synthesized SNPs were carried out against gram positive *Staphylococcus aureus, Bacillus subtilis* and gram negative bacteria *Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri* using agar disc diffusion method. The synthesized TiO_2 nanoparticle exhibited intermediate sensitivity against *B. subtilis, S. aureus S. typhi.*

The diameter of inhibition zone (mm) around each well with TiO_2 nanoparticles and fruit rind extract is represented in the (Table 2). The zone of inhibition of synthesized TiO_2 nanoparticles were found to be greater than that of the plant fruit rind extract (Fig 4).

Estimation of *in vitro* maximal cytotoxic free concentration was done on Vero cell line. The extracts (Table 3) (Fig. 5 & 7) and the synthesized TiO_2 NPs (Table 4) (Fig. 6 & 8) were

evaluated for cytotoxicity at different concentration. The synthesized nanoparticles were found to be non toxic from 500 µg/ml concentration and was recorded as micrographs.

The extract showed anticancer effect till a concentration of 125µg/ml on A549 cell line (Table 5) (Fig. 9 &11). However the synthesized TiO_2 NPs showed a potent anticancer activity against A549 cells. The synthesized TiO_2 nano particles could inhibit the proliferation of A549 cells till a concentration of 62.5µg/ml (Table 6) compared to that of positive control. The effect of the TiO_2 nanoparticles on A549 cell line at different concentration was recorded as micrographs (Fig. 10 & 12). Ankita chatterjee et al. *2016*[17] carried out green synthesis of titanium dioxide nanoparticles from extract of *V. radiata* legumes and estimated the anticancer activity on osteosarcoma cell line. Cytotoxicity assay revealed the nanoparticles were capable of inhibiting proliferation of osteosarcoma cell lines.

The stable compound DPPH gets reduced by gaining a hydrogen or electron. The change in color in the test sample after 15 minutes incubation indicates the nature of the nanoparticles to be antioxidant and hence the reducing activity of the nanoparticles. The reducing activity of the particles qualifies them as good antioxidant compounds (Table7).

Based on these results it's found that the TiO_2 nanoparticles of fruit rind of *T. chebula* may have potential biomedical applications when compared to extracts due to its significant antibacterial, antioxidant and anticancer activity than the extracts.

5.0 CONCLUSION

To summarize, the present method is capable of producing TiO_2 nanoparticles with aqueous extract of *T. chebula* which is simple, cheap and environmental friendly. The biological reduction of the nanoparticles was carried out in appropriate condition and characterization of synthesized nanoparticles was carried out by UV–Vis spectroscopy, FT IR and SEM. The phytochemicals present in the extract of *T. chebula* reduced Titanium IV isopropoxide to TiO2 nanoparticles. The synthesized TiO2 nanoparticle exhibited intermediate antibacterial activity but greater than extracts against *B subtilis* and *S. aureus*. The synthesised NPs exhibited potential anticancer activity to A549 Cells and were also non toxic on mammalian Vero cell line. Hence the TiO2 NPs from fruit rind extract of *Terminalia chebula* may be used as a lead molecule.

CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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