Annealing temperature influences the cytocompatibility, bactericidal and bioactive properties of green synthesised TiO₂ nanocomposites

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Abstract
Annealing is a crucial functional parameter relevant to the green synthesis and bactericidal properties of TiO₂ nanocomposites (TiO₂-NPs). In this work, the effect of the annealing temperature on the physicochemical, bactericidal and cytocompatibility properties of TiO₂-NPs obtained from Calotropis gigantea was comprehensively studied. Results indicated that amorphous-phase TiO₂-NPs were transformed into the anatase phase at 500 °C with a crystallite size of 40.9 nm and MIC of 100 mg/mL towards Staphylococcus aureus. Whereas TiO₂-NPs annealed at 400 °C demonstrated no bacterial reduction, TiO₂-NPs annealed at 500 °C showed a moderate zone of inhibition towards Escherichia coli and Pseudomonas aeruginosa. Findings from this study found that TiO₂-500C nanocomposites concentration at 100 mg/mL does not inhibit fibroblast cells proliferation activity after 24 h treatment. The plant-mediated nano-sized cubic and spherical anatase TiO₂-NPs encapsulated bioactive green elements, such as carbon, sodium, magnesium, chlorine, potassium, calcium and sulphur, from the C. gigantea extract, ultimately leading to versatile and eco-friendly bactericidal agents with wound-healing properties. Further studies involving in vivo are needed to support this work.

Keywords Calotropis gigantea · Bactericidal agent · Annealing temperature · Titanium dioxide nanoparticles · Cytocompatibility · Green synthesised nanoparticles

List of symbols
\( \bar{d} \) · Crystallite size (nm)
\( \lambda \) · X-ray wavelength of Cu Kα radiation (nm)
\( \theta \) · Bragg diffraction angle (°)
\( T \) · Temperature (°C)
\( t \) · Time (min)

Abbreviations
C. gigantea · Calotropis gigantea
TiO₂-NPs · TiO₂ nanocomposites
C · Carbon
Na · Sodium
Mg · Magnesium
Cl · Chlorine
K · Potassium
Ca · Calcium
S · Sulphur
MIC · Minimum inhibitory concentration
XRD · X-ray diffraction
MDR · Multi-drug resistant
E. coli · Escherichia coli
K. pneumoniae · Klebsiella pneumoniae

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Introduction

Recent progress in research on natural products has resulted in the development of many natural plant-mediated nanoparticles (NPs) with bactericidal properties; amongst these materials, green synthesised titanium dioxide NPs (TiO₂-NPs) have sparked great interest on account of their potential use in wound healing therapy. Green synthesis method represents an advance over conventional chemical and physical methods (Aravind et al. 2021; Ansari et al. 2022). It has shown multiple benefits such as eco-friendly, simple, economical, sustainable and safe (Ansari et al. 2022). Moreover, green synthesised TiO₂-NPs produces biocompatible and enhanced antibacterial stable nanoparticles for biomedical applications compared to the chemically synthesized TiO₂-NPs (Aravind et al. 2021; Ansari et al. 2022).

**Calotropis gigantea** is a traditional medicinal plant with antimicrobial properties that is often used to treat skin diseases (Kumar et al. 2010) and open wounds (Sangetha et al. 2020). Aqueous solutions of *C. gigantea* leaf extract function as excellent reducing and capping agents in the formation of green TiO₂-NPs. Indeed, given their promising bactericidal properties for addressing skin and wound infections due to pathogens, plant-mediated TiO₂-NPs are amongst the most extensively studied bactericidal agents in the biomedical field (Table 1). TiO₂-NPs exist in three phases, namely, anatase, rutile and brookite, under different processing conditions (Sugapriya et al. 2013; Tesfaye Jule et al. 2021). Scientists worldwide have sought to establish methods to control the size and morphology of TiO₂-NPs as the particle size exerts a massive influence on their bactericidal properties (de Dicastillo et al. 2020). Even slight variations in the annealing conditions could result in prominent effects on the phase and morphology of TiO₂-NPs (Sugapriya et al. 2013; Długosz et al. 2020). A significant improvement in degree of recrystallization, phase transition and uniform size distribution of TiO₂-NPs was seen when increasing the annealing temperature (Tesfaye Jule et al. 2021; Muthee and Dejene 2021). Therefore, a slight alteration on size of NPs and transformation of phase could result in dramatic improvement on antibacterial activity (Lin et al. 2014; Senarathna et al. 2017). The present work discusses the formation of bioactive elements of *C. gigantea* leaf extract and the morphology of the resultant TiO₂-NPs under the effect of different annealing temperatures (i.e., 400 and 500 °C). The physicochemical properties of the TiO₂-NPs were characterised using scanning electron microscopy (SEM), transmission electron microscopy (TEM), X-ray diffraction (XRD), energy-dispersive X-ray spectroscopy (EDAX), UV–Vis spectrophotometry and Fourier transform infrared (FTIR) spectroscopy. Additionally, bactericidal and cytocompatibility properties of green TiO₂-NPs were further investigated.

Materials and methods

**Green synthesis and characterisation of bioactive TiO₂-NPs**

**Green synthesis**

TiO₂-NPs was synthesised via green route by the reaction of *C. gigantea* leaves extract aqueous solution with titanium (IV) isopropoxide (Sigma-Aldrich). This grounded green synthesis method uses the following protocol (Govindasamy et al. 2021a, b). Green TiO₂-NPs samples calcined at different low temperatures 400 °C and 500 °C are designated as TiO₂-400C and TiO₂-500C, respectively. In this work, commercially available TiO₂ P25, anatase (Sigma-Aldrich, 99.7%) was used as a commercial control.

**X-ray diffraction**

X-ray diffractometer (XRD; Bruker D8) was used to study crystalline nature and size of green TiO₂-NPs. The X-ray diffraction peaks were captured via Cu Kα radiation with wavelength of \( \lambda = 0.1541 \) nm and step scan mode with step size of 0.030° in the range of 10° to 90°. The equipment was operated at a voltage of 40 kV and current of 30 mA. Scherrer’s equation was applied to determine the average crystallite size of green TiO₂ which is as under

\[
d = \frac{K\lambda}{\beta \cos \theta}
\]

where \( d \) represents the crystallite size, \( K = 0.9 \) is the shape factor, \( \lambda \) stands for the X-ray wavelength of Cu Kα radiation (1.541 Å), \( \theta \) is used as a Bragg diffraction angle, and \( \beta \) symbolize the full-width at half-maximum (FWHM) of the respective diffraction peak (Govindasamy et al. 2021a).

**SEM observation**

The morphology of TiO₂-NPs was further investigated using scanning electron microscopy (SEM Fei Quanta FEG 650). Samples were placed on a stub with carbon tape and coated with Pt for 1-min prior to imaging and elemental analysis. The TiO₂-NPs and bioderived elements composition and their percentage from green TiO₂-NPs was further confirmed with EDAX.
| Precursor                  | Reducing agent            | Calcination temperature (°C) | Size (nm) | Shape                  | Antimicrobial activity                                                                 | Killing mechanism                                                                 | Toxicity | Application                      | References                          |
|---------------------------|---------------------------|-----------------------------|-----------|------------------------|----------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|----------|----------------------------------|-------------------------------------|
| Titanium tetra chloride    | *Orange peel*             | 500                         | Crytallite: 17.30 | Porous angular         | ZOI: *E. coli*: 9 mm, *P. aeruginosa*: 14 mm and *S. aureus*: 12 mm                    | Nanoparticle’s attachment and generation of ROS                                 | Nil      | Antibacterial, antican and humidity sensor | Amanulla and Sundaram (2019)          |
| TiO(OH)₂                   | *Euphorbia prosstrata*    | Nil                         | Grain: 83.22  | Circular and irregular | *Leishmania* parasites                                                                  | ROS production                                                                   | Nil      | Antileishmanial agents           | Zahir et al. (2015)                 |
| Titanium isoprotopoxide    | *Aloe vera*               | 500                         | Grain: 60–80 | Irregular              | Nil                                                                                   | Nil                                                                              | Nil      | Semiconductor material            | Khadar et al. (2016)                |
| TiO₂ solution              | Lemon-fruit               | Nil                         | Grain: 20–200 | Near-spherial and irregular | *Dickeya dadantii*: 60% reduction at 50 µg/mL                                           | Generation of oxidative stress                                                   | Nil      | Phytopathogenic agent            | Hossain et al. (2019)               |
| TiCl₄                      | *Cicer arietinum* L.      | 500                         | Crytallite: 14 | Spherical              | Nil                                                                                   | Nil                                                                              | Nil      | Lithium Ion Battery              | Kashale et al. (2016)               |
| TiO₂ solution              | *Vigna unguiculata*       | Nil                         | Oval       | ZOI: *E. coli*: 2 cm, *Salmonella*: 4 cm, *Enterobacter*: 4 cm, *P. aeruginosa*: 3.5 cm and *S. marcescens*: 4 cm at 75 µL | Nil                                                                                   | Nil                                                                              | Nil      | Anticancer treatments            | Chatterjee et al. (2017)            |
| TiO₂ solution              | *Vigna radiata* legumes   | Nil                         | Oval       | ZOI: *S. aureus*: 2.8 cm, *E. coli*: 3.3 cm, *Enterobacter sp.*: 3.2 cm, *S. marcescens*: 3.6 cm, *Salmonella sp.*: 2.8 cm, *P. aeruginosa*: 3.2 cm, *K. pneumonia*: 3 cm, *P. mirabilis*: 3.4 cm and *Shigella sp.*: 3.6 cm 100 µL | Hydroxyl groups                                                                  | Nil                                                                              | Nil      | Antibacterial and anticancer      | Chatterjee et al. (2016)            |
### Table 1 (continued)

| Precursor         | Reducing agent                  | Calcination temperature (°C) | Size (nm) | Shape            | Antimicrobial activity | Killing mechanism | Toxicity | Application                  | References                  |
|-------------------|---------------------------------|------------------------------|-----------|------------------|------------------------|---------------------|----------|-------------------------------|-----------------------------|
| TiO$_2$ solution  | *Azadirachta indica*            | 60                           | 15–50     | Spherical        | ZOI: *E. coli*: 27.67 mm, *B. subtilis*: 24 mm, *S. typhi*: 21.63 mm, *S. aureus*: 27 mm and *K. pneumoniae*: 25.33 mm at 200 μg/mL; MIC: *E. coli*: 10.42 μg/mL, *B. subtilis*: 25 μg/mL, *S. typhi*: 10.42 μg/mL, *S. aureus*: 20.83 μg/mL and *K. pneumoniae*: 16.66 μg/mL; MBC: *E. coli*: 166.67 μg/mL, *B. subtilis*: 166.67 μg/mL, *S. typhi*: 100 μg/mL, *S. aureus*: 133.3 μg/mL and *K. pneumoniae*: 83.33 μg/mL | Nil                  | Nil                  | Nil                        | Antimicrobial agents | Thakur et al. (2019) |
| TiO$_2$ solution  | *Cinnamomum tamala*             | Nil                          | 23        | Irregular        | Nil                    | Nil                  | Nil                  | Nil                        | Anticancer drug treatment | He et al. (2017)           |
| Titanium oxysulphate | Polyvinyl pyrrolidone          | 300–500                      | Grain: 10–20 | Irregular        | Nil                    | Nil                  | Nil                  | Nil                        | Photocatalytic              | Khade et al. (2015)        |
| TiO$_2$ solution  | *Cinnamomum*                    | 500                          | Grain: 70–150 | Spherical        | Nil                    | Nil                  | Nil                  | Solar cells                | Nabi et al. (2019)          |
| TiO$_2$ solution  | *Solanum trilobatum L.*         | Nil                          | Grain: 70  | Spherical and oval | Effective against *P. h. capitis*, *H. a. anatolicum*, and *A. subpictus* | Nil                  | Nil                  | Nil                        | Antiparasitic               | Rajakumar et al. (2013)    |
| Precursor                        | Reducing agent | Calcination temperature (°C) | Size (nm) | Shape                                      | Antimicrobial activity | Killing mechanism | Toxicity | Application            | References                  |
|---------------------------------|----------------|-------------------------------|-----------|--------------------------------------------|-------------------------|-------------------|----------|-------------------------|--------------------------------|
| TiO(OH)_2                        | Mangifera indica | Nil                           | Grain: 30 | SEM: Spherical and oval; TEM: round        | Effective against R. microplus, Hyalomma anatolicum anatolicum, Haemaphysalis bipinosa, A. subpictus, and Culex quinquefasciatus | Nil               | Nil     | Antiparásitico          | Rajakumar et al. (2015)          |
| TiO(OH)_2                        | Glycosmis cochinchinensis | Nil                           | Grain: 40 | Spherical                                   | ZOI: S. saprophyticus: 21 mm, B. subtilis: 19 mm, E. coli: 23 mm and P. aeruginosa: 25 mm at 100 µL | Nil               | Nil     | Photocatalítico y antimicrobial | Rosi and Kalyanasundaram (2018) |
| Titanium (IV)-iso-propoxide      | Carica papaya   | 400                           | Grain: 15.6 | Cages                                      | Nil                     | Nil               | Nil     | Photocatalítico         | Kaur et al. (2019)             |
| Titanium (IV) isopropoxide       | Ocimum basilicum L. | 500                           | Grain: 50  | Hexagonal                                   | Nil                     | Nil               | Nil     | Nil                     | Salam and Sivaraj (2014)        |
| Titanium tetra-isopropoxide      | Pomegranate Peels | 90                            | Grain: 75–90 | Nil                                        | Nil                     | Nil               | Nil     | Pharmaceutical           | Dubey and Singh (2019)          |
| Titanium Chloride                | Aloe vera       | 500                           | Grain: 32  | Irregular                                   | Effective against S. aureus | Nil               | Nil     | Nil                     | Rao et al. (2015)               |
| Titanium sulfate                 | Acacia catechu  | 700                           | Grain: 17.90 | Spherical and hexagonal-shaped             | Nil                     | Nil               | Nil     | Photocatalítico y antimicrobial | Chand et al. (2020)             |
| Titanium oxysulfate              | Gum Kondagogu   | 500–900                       | Grain: 8–13 | Spherical                                   | Nil                     | Nil               | Nil     | Photocatalítico         | Saranya et al. (2018)           |
| Titanyl hydroxide                | Euphorbia heteraden Jaib | Nil                           | Grain: 20  | Spherical                                   | Nil                     | Nil               | Nil     | Biocientífico y farmacéutico | Nasrollahzadeh and Sajadi (2015) |
| TiO_2 solution                   | Sesbania grandiflora | Nil                           | Grain: 43–56 | Triangular, square and spherical          | Nil                     | Lethal to zebrafish at concentration < 2.5 mg/L | Drug    |                         | Srinivasan et al. (2019)        |
| Precursor          | Reducing agent          | Calcination temperature (°C) | Size (nm)        | Shape      | Antimicrobial activity | Killing mechanism | Toxicity | Application          | References               |
|--------------------|-------------------------|------------------------------|------------------|------------|------------------------|-------------------|----------|-----------------------|--------------------------|
| TiO(OH)$_2$       | Artemisia haussknechtii | Nil                          | Grain: 92.58 ± 56.98 | Spheres   | ZOI: Lack of growth for S. epidermidis and S. marcescens and no inhibition for E. coli and S. aureus | Nil               | Nil                  | Antibacterial          | Alavi and Karimi (2017) |
| TiO(OH)$_2$       | Protoparmelopsis muralis Lichen | Nil                          | Grain: 133.32 ± 35.33 | Spherical | ZOI: S. aureus: 10.00 mm, E. coli: 11.66 mm and P. aeruginosa: 9.66 mm; MIC: 80 µg/mL; MBC: 100 µg/mL | Nil               | Nil                  | Antibacterial          | Alavi et al. (2019)     |
| Titanium tetraiso-propoxide | Trianthema portulacastrum | 450                          | Crytallite: 6–8 | Different | Effective against wheat Rust (Ustilago tritici) | Nil               | Nil                  | Antifungal            | Irshad et al. (2020)   |
| Titanium tetraiso-propoxide | Chopodium quinoa | 450                          | Crytallite: 6–8 | Round     | Effective against wheat Rust (Ustilago tritici) | Nil               | Nil                  | Antifungal            | Irshad et al. (2020)   |
| Ti plate           | H. rosa-sinensis        | 550                          | Nil              | Nil       | Nil                    | Nil               | Nil                  | Wastewater treatment   | Zamri and Sapawe (2018) |
| Precursor                          | Reducing agent                  | Calcination temperature (°C) | Size (nm)          | Shape                        | Antimicrobial activity | Killing mechanism                        | Toxicity | Application            | References                |
|----------------------------------|---------------------------------|------------------------------|--------------------|------------------------------|------------------------|------------------------|----------|------------------------|---------------------------|
| Titanium isopro-poxide (IV)      | Pristine pomegranate peel       | 200–1100                     | Grain: 1–5 µm      | Randomly oriented            | ZOI: *S. aureus*: 22 mm, *E. coli*: 19 mm and *P. aeruginosa*: 17 mm at 2 wt.%; MIC: *S. aureus*: 189.1 µg/mL, *E. coli*: 304.7 µg/mL and *P. aeruginosa*: 309.2 µg/mL at 90 µg/mL; MBC: *S. aureus*: 200 µg/mL, *E. coli*: <310 µg/mL and *P. aeruginosa*: <315 µg/mL | Production of reactive oxygen species | Nil                     | Water disinfection     | Abu-Dalo et al. (2019) |
| Titanium-isopro-poxide           | *Syzygium cumini*               | 570                          | Grain: 11          | Spherical and irregular      | Nil                    | Nil                     | Nil                   | Wastewater treatment      | Sethy et al. (2020)       |
| Precursor               | Reducing agent       | Calcination temperature (°C) | Size (nm)     | Shape                        | Antimicrobial activity                                                                 | Killing mechanism                     | Toxicity | Application        | References              |
|------------------------|----------------------|------------------------------|---------------|------------------------------|----------------------------------------------------------------------------------------|----------------------------------------|----------|---------------------|-------------------------|
| Titanium (IV) oxide    | Withania somnifera   | 120                          | Grain: 50–90  | Spherical and square         | MIC: *E. coli*: 32 µg/mL, *P. aeruginosa*: 32 µg/mL, *MRSA*: 32 µg/mL, *L. monocytogenes*: 60 µg/mL, *S. marcescens*: 8 µg/mL, and *C. albicans*: 60 µg/mL; MBC: *E. coli*: 32 µg/mL, *P. aeruginosa*: 60 µg/mL, *MRSA*: 60 µg/mL, *L. monocytogenes*: 128 µg/mL, *S. marcescens*: 16 µg/mL, and *C. albicans*: 128 µg/mL | Reactive oxygen species production     | Nil       | Anti-infective agent| Al-Shabib et al. (2020) |
| Titanium isopropropoxide | *Sonchus asper*       | 500                          | Grain: 9–22   | Spherical                    | ZOI: *E. coli*: 12 mm, *S. aureus*: 12 mm and *K. pneumoniae*: 10 mm                      | ROS generation                        | Nil       | Biomedical          | Babu et al. (2019)      |
| Precursor         | Reducing agent                  | Calcination temperature (°C) | Size (nm) | Shape     | Antimicrobial activity | Killing mechanism | Toxicity                      | Application                  | References                  |
|-------------------|---------------------------------|------------------------------|-----------|-----------|------------------------|-------------------|-------------------------------|-----------------------------|-----------------------------|
| TiO₂ solution     | *Allium eriophyl-lum* Boiss     | Nil                          | Grain: 22 | Spherical | ZOI disc diffusion: C. *guilliermondii*: 39.6 mg/mL, C. *krusei*: 40.6 mg/mL, *C. albicans*: 36.2 mg/mL, C. *glabrata*: 36.4 mg/mL, *P. aeruginosa*: 35 mg/mL, *S. typhimurium*: 35 mg/mL, *E. coli*: 36.6 mg/mL, *S. aureus*: 36.8 mg/mL, *S. pneumoniae*: 38.8 mg/mL, and *B. subtilis*: 39.8 mg/mL; MBC for Gram (+) and Gram (−): 2–4 mg/mL; MBC for Gram (+) and Gram (−): 4 mg/mL | ROS generation | No cytotoxicity toward human umbilical vein endothelial cells | Industrial and remedial | Seydi et al. (2019) |
| TiO₂ solution     | Fruit’s peel agrowaste: plum, kiwi and peach | 400–500               | Grain: plum: 47.1–63.2, kiwi: 54.1–85.1 and peach: 200 | Cylindrical | ZOI ≥ 2 and ≤ 20 mm for *E. coli*, *S. aureus*, *P. aeruginosa* and *B. subtilis* at 12.5–100 μg/mL | HO and O₂ radicals | Nil | Biomedical | Ajmal et al. (2019) |
| Precursor                  | Reducing agent           | Calcination temperature \(^{\circ}\)C) | Size (nm)                          | Shape                     | Antimicrobial activity | Killing mechanism                                                                 | Toxicity          | Application            | References            |
|---------------------------|--------------------------|----------------------------------------|------------------------------------|---------------------------|------------------------|----------------------------------------------------------------------------------|-------------------|------------------------|------------------------|
| TiO(OH)\(_2\)            | Cola nitida              | Nil                                    | Grain: 25.00–191.41                | Near spherical            | 49.2–73.4\% growth inhibition of *S. aureus*, *P. aeruginosa*, *E. coli*, *K. pneumoniae* at 80 µg/mL using broth method | Ti\(^{4+}\), generation of reactive oxygen species and hydroxyl radicals | Nil                | Catalyst and biomedical | Akinola et al. (2020) |
| Titanium Oxy-sulfate       | *Hibiscus rosa-sinensis* L. | 100                                    | Nil                                | Spherical                | ZOI: *V. cholerae*: 17 mm, *P. aeruginosa*: 14 mm, *S. aureus*: 14.5 mm at 20 µg/mL | Nanoparticle’s penetration | Nil                | Biomedical             | Kumar et al. (2014)   |
| Titanium tetra isopro-   | *Terminalia Catappa*     | 450                                    | Crytallite: 10–21                  | Spherical                | Nil                    | Nil                                                                            | Nil               | Therapeutic agent      | Rajendhiran et al. (2020) |
| oxide isopro-             | *Carissa Carandas*       | 450                                    | Crytallite: 10–21                  | Spherical                | Nil                    | Nil                                                                            | Nil               | Therapeutic agent      | Rajendhiran et al. (2020) |
| TiO\(_2\) solution        | *Echinacea purpurea* Herba | Nil                                    | Grain: 120                         | Spherical clusters       | Nil                    | Nil                                                                            | Nil               | Photocatalytic agent   | Dobrucka (2017)         |
| Titanium isopro-          | *Acadirachta indica*     | Nil                                    | Grain: 124                         | Interconnected spherical | Nil                    | Nil                                                                            | Nil               | Photocatalytic          | Sankar et al. (2015)    |
| oxide isopro-             | *Origanum vulgare*       | Nil                                    | Grain: 341                         | Spherical                | Nil                    | Nil                                                                            | Nil               | Therapeutic agent      | Sankar et al. (2014)    |
| TiO(OH)\(_2\) solution   | *A. alissima*            | Nil                                    | Grain: 60–100                      | Spherical                | Nil                    | Nil                                                                            | Nil               | Photocatalytic agent   | Ganesan et al. (2016)   |
| Titanium (IV) isopro-     | *Phyllanthus niruri*     | 350                                    | Grain: 30–50                       | Spherical                | Nil                    | Nil                                                                            | Nil               | Photocatalytic material | Shanavas et al. (2020)  |
| Precursor                | Reducing agent                  | Calcination temperature (°C) | Size (nm)                        | Shape             | Antimicrobial activity                                      | Killing mechanism                                      | Toxicity | Application       | References                           |
|-------------------------|---------------------------------|------------------------------|---------------------------------|-------------------|------------------------------------------------------------|--------------------------------------------------------|----------|-------------------|-------------------------------------|
| Titanium oxy sulphate   | *Trigonella foenum-graecum*     | 700                          | Grain: 20–90                    | Spherical         | ZOI: *S. aureus*, *E. faecalis*, *K. pneumoniae*, *S. faecalis*, *P. aeruginosa*, *E. coli*, *P. vulgaris*, *B. subtilis*, *Y. enterocolitica* and *C. albicans*; 8.5–11.6 mm at 10 mg/mL | Reactive Oxygen Species (ROS), mainly hydroxyl radicals (–OH) | Nil      | Biomedical        | Subhapriya and Gomathipriya (2018) |
| Titanium tetraisopropoxide | Soluble starch                  | 500                          | Grain: 20–70                    | Irregular spherical | Nil                                                        | Nil                                                   | Nil      | Photocatalytic      | Muniandy et al. (2017)              |
| Titanium tetra-chloride | *M. citrifolia*                 | 400                          | Grain: 15–19                    | Quasi-spherical   | ZOI: *S. aureus*: 13 mm, *E. coli*: 10 mm, *B. subtilis*: 12 mm, *P. aeruginosa*: 9 mm, *C. albicans*: 13 mm and *A. niger*: 6 mm at 150 μg/mL | Ti⁴⁺, generation of reactive oxygen species             | Nil      | Biomedical        | Sundraraj et al. (2017)            |
| Titanium (IV) isopropoxide | *Fomes fomentarius*            | Nil                          | Grain: 100–120                  | Irregular         | ZOI: *E. coli*: 15 mm and *S. aureus*: 11 mm at 100 μg/mL | Nil                                                   | Nil      | Antibacterial and anticancer | Rehman et al. (2020a)               |
| Titanium (IV) isopropoxide | *Fomitopsis pini-cola*         | Nil                          | Nil                             | Irregular         | MIC/MBC: 62.5/125 μg/mL for *E. coli* and *S. aureus*     | Nil                                                   | Nil      | Biomedical        | Rehman et al. (2020b)               |
| Titanium chloride       | *Jatropha curcas* L.            | 450                          | Grain: 10–20                    | Spherical         | Nil                                                        | Nil                                                   | Nil      | Wastewater        | Goutam et al. (2018)                |
| Titanium tetraisopropoxide | *Piper betel*                  | 400                          | Grain: 6.6                      | Round             | Nil                                                        | Nil                                                   | Nil      | Photocatalytic     | Pushpamalini et al. (2021)          |
| Titanium tetraiso-propoxide | *Ocimum tenuiflorum*           | 400                          | Grain: 7.0                      | Round             | Nil                                                        | Nil                                                   | Nil      | Photocatalytic     | Pushpamalini et al. (2021)          |
| Titanium tetraiso-propoxide | *Moringa oleifera*            | 400                          | Grain: 6.6                      | Round             | Nil                                                        | Nil                                                   | Nil      | Photocatalytic     | Pushpamalini et al. (2021)          |
| Precursor                  | Reducing agent             | Calcination temperature (°C) | Size (nm) | Shape         | Antimicrobial activity | Killing mechanism       | Toxicity | Application          | References                           |
|---------------------------|----------------------------|------------------------------|-----------|---------------|------------------------|------------------------|----------|----------------------|--------------------------------------|
| Titanium tetraiso-propoxide | *Coriandrum sativum*      | 400                          | Grain: 6.8 | Round         | Nil                    | Nil                    | Nil       | Photocatalytic        | Pushpamalinii et al. (2021)           |
| TiO(OH)$_2$                | *Psidium guajava*          | Nil                          | Grain: 32.58 | Spherical     | ROS generation         | Nil                    | Biomedical |                      | Santhoshkumar et al. (2014)           |
| TiO$_2$ solution           | *Moringa oleifera*         | Nil                          | Grain: 100  | Spherical     | Nil                    | Nil                    | Nil       | Skin infection treatments | Sivaranjani and Philominathan (2015) |
| Titanium tetraiso-propoxide | *Moringa Oleifera*         | 500                          | Crytallite: 12.22 | Nil          | Nil                    | Nil                    | Nil       | Nil                  | Patidar and Jain (2017)               |
| Titanium tetraiso-propoxide | *Mentha arvensis*          | 500                          | Grain: 20–70 | Spherical     | ROS generation         | Nil                    | Antibacterial and antifungal | Ahmad et al. (2020)                  |
| Titanium tetraiso-propoxide | *Acanthophyllum Laxiusculum* | 400                          | Grain: 20–25 | Nanospheres   | Nil                    | Nil                    | Nil       | Nil                  | Madadi and Lotfabad (2016)            |
| TiO$_2$ solution           | *Cassia fistula*           | Nil                          | Nil        | Spherical     | Effective against S. aureus and E. coli | Nil                    | Nil       | Nil                  | Swathi et al. (2019)                 |
TEM analysis

TEM (FEI TECHNAI F20 G2) is used for analysing shape and grain size of TiO$_2$-NPs. At first, TiO$_2$-NPs was dispersed in absolute ethanol and then sonicated for 30 min. After that, a single drop of TiO$_2$-NPs solution was added onto a lacey carbon film-coated copper grid (300 mesh) and then dried at room temperature for 30 min. At last, it was kept in desiccator prior to TEM imaging.

FTIR study

The functional groups of green TiO$_2$-NPs were recorded by FTIR spectroscopy (PerkinElmer) within the range of 4000–400 cm$^{-1}$ through the KBr pellet method. The FTIR samples were prepared by dispersing small dosage of TiO$_2$-NPs uniformly in a KBr matrix which was then compressed to thin transparent disc.

UV–visible spectroscopy

The absorption spectrum of TiO$_2$-NPs was determined using UV–Vis spectrophotometer (Varian) in wavelength range between 200 and 700 nm.

Antibacterial tests

Kirby-Bauer disc diffusion test and minimum inhibitory concentration (MIC) were determined after 24 h of contact with the prepared green samples according to following methods (Govindasamy et al. 2021a, b, c). Four overnights cultured ATCC bacterial suspensions of $P$. aeruginosa 27853, $K$. pneumoniae 700603, $E$. coli 25922 and $S$. aureus 29213 were employed to study antibacterial effect of TiO$_2$-NPs. 30 µg of Cefoxitin discs used as a positive control. 10% (v/v) DMSO act as negative control in this study.

Cytocompatibility assay

The cytocompatibility assay was performed on fibroblast cells lines model, L929 obtained from American Type Culture Collection (ATCC, USA) and is maintained in RPMI-1640 media (Gibco, Life technologies) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) PenStrep (Gibco, USA), sodium bicarbonate, 12.5 g/mL HEPES and 1% (v/v) l-glutamine at 37 °C in a 5% CO$_2$ humidified atmosphere. 10% (v/v) of dimethyl sulfoxide (DMSO) was adapted as strong cytotoxic material (negative control) while fibroblast cells lines without administration of TiO$_2$ as blank control. The in vitro cytocompatibility of green-synthesised TiO$_2$ on fibroblast cells lines was performed by direct contact method according to the protocol recommendations in ISO 10993-5 (2009) (Harun et al. 2021; Chellappa et al. 2015).

The cell viability percentage was determined using (1:10) alamarBlue™ Cell viability reagent DAL1025 (Invitrogen, United Kingdom). At first, cells were subcultured, trypsinized and seeded at a density of 1 × 10$^4$ cells/well (100 µL/well) on 96 well plate and grown for 24 h in CO$_2$ incubator at 37 °C. After that, different concentrated TiO$_2$ (0, 25, 50 and 100 mg/mL) were added in the culture media and kept overnight. Then, the serially diluted test sample solution ($n=3$) was incubated with monolayer cells at 37 °C for 24 h. The proportion of live and healthy cells after treatment were further estimated quantitatively through colour change from blue to pink using Alamar blue assay where the treated cells were incubated as a minimum 20 h before measuring the absorbance at wavelength 570 nm and 600 nm using a microplate reader (Bio-Tek Instruments, USA). The cytocompatibility of test sample was compared with the toxic material (10% (v/v) DMSO) and blank control. The live and dead cells were examined microscopically via Olympus CKX41 optical light microscope at magnification of 10× and 20×.

Statistical analysis

The different calcination temperature treated group of samples were analysed using analysis of two-way ANOVA implemented in the GraphPad Prism software package. Results were considered statistically significant if $p$ value is less than 0.05. Data are presented as mean ± standard deviation (SD).

Results and discussion

Physicochemical characterisation of the TiO$_2$-NPs

The SEM images in Fig. 1a, b show that the TiO$_2$-400C and TiO$_2$-500C NPs are spherical in shape with a nano-sized and agglomerated morphology (Fig. 1a, b, insets). The EDAX images of the TiO$_2$-NPs confirm the presence of Ti and O, which make up approximately 29.28 wt% and 36.72 wt%, respectively, of TiO$_2$-400C and 33.55 wt% and 41.91 wt%, respectively, of TiO$_2$-500C (Table 2, Fig. 1c, d). The TEM images in Fig. 1e, f reveal that the NPs produced at a low calcination temperature are nearly spherical with a large, agglomerated morphology whilst those calcined at a high temperature are spherical and cuboidal in shape with a partly agglomerated morphology.

EDAX analysis successfully identified an abundance of bioactive elements in $C$. gigantea, such as carbon (C), calcium, chlorine, sodium, magnesium, potassium and sulphur,
in agreement with a previous research (Govindasamy et al. 2021a, b) (Table 2). These elements are by-products of the green synthesis of the NPs. The combination of these bio-derived elements with high concentrations of C and antimicrobial species, such as hydroxyl radicals, hydrogen peroxide, superoxide anions and titanium (IV), may arrest the growth and development of skin pathogens (Cheng et al. 2009).

The XRD patterns of TiO$_2$-400C and TiO$_2$-500C (Anatase ICDD No. 98-003-7543) are illustrated in Fig. 2a. The XRD spectrum of TiO$_2$-500C showed characteristic peaks at 25.32°, 37.86°, 48.06°, 53.96°, 55.09°, 62.76°, 68.87°, 70.33°, 75.14° and 82.76°, which respectively correspond to the (011), (004), (020), (015), (121), (024), (116), (220), (125) and (224) crystal planes of anatase-phase TiO$_2$-NPs (Nasrollahzadeh et al. 2016). The XRD pattern of TiO$_2$-400C exhibited a single broad peak, which confirms its amorphous character (Sugapriya et al. 2013). The phase transformation and differences in physicochemical properties of inorganic oxide compound (TiO$_2$) at different annealing temperature were successfully captured and it was further validated by previous reported works (Xu et al. 2020; Guo et al. 2020). The green TiO$_2$-500C NPs had a tetragonal crystalline structure with space group of number 141. The crystallite size of green TiO$_2$-NPs obtained at a high calcination temperature was calculated from the strongest XRD peak at 25.32° using Scherrer’s equation (Ahmad et al. 2020) and found to be 40.9 nm. The XRD patterns of the TiO$_2$-NPs confirm the presence of pure TiO$_2$ along with C. gigantea bioderived elements, in agreement with the SEM-EDAX analyses (Fig. 1c, d).

The functional groups responsible for the formation of green TiO$_2$-NPs were determined by FTIR analysis. The FTIR spectrum of the green nanocomposites shown in Fig. 2b reveals a broad peak at 3438 cm$^{-1}$, which could be assigned to the O–H stretching vibrations of flavonoids from C. gigantea (Govindasamy et al. 2021a, b). The peak at 1633 cm$^{-1}$ could be attributed to C=C (carbonyl group), and the peaks at 1361 and 1119 cm$^{-1}$ could respectively be attributed to the O–C–O stretching vibrations of esters and the C–O stretching vibrations of bio-derived elements from the C. gigantea leaf extract (Govindasamy et al. 2021a, b). The peak at 595 cm$^{-1}$ confirms the presence of TiO$_2$-NPs with Ti–O–Ti framework bonds (Nasrollahzadeh et al. 2016; Zhou et al. 2021).

UV–Vis spectrophotometry was applied to measure the bandgap of the green synthesised TiO$_2$-NPs, and the results obtained are shown in Fig. 2c. The absorbance peak at 228 nm is due to the dispersion of natural C in deionised water (Govindasamy et al. 2021a, b), and the small peak at 327 nm confirms the formation of green synthesised TiO$_2$-NPs (Zhou et al. 2014). The UV–Vis spectrum of the control C. gigantea leaf extract showed two prominent absorbance peaks; the sharp peak at 206 nm corresponds to the carbonyl group, whilst the low broad peak at 269 nm represents phenolic compounds (Govindasamy et al. 2021a, b).

### Assessment of minimum inhibitory concentration

A preliminary minimum inhibitory concentration (MIC) study performed in this research focused on Staphylococcus aureus.

| Sample     | C  | O  | Na | Mg | Cl | K  | S  | Ca | Ti  |
|------------|----|----|----|----|----|----|----|----|-----|
| TiO$_2$-400C | 27.13 | 36.72 | 0.42 | 0.26 | 0.47 | 4.84 | –   | 0.89 | 29.28 |
| TiO$_2$-500C | 9.41  | 41.91 | 0.51 | 0.47 | 3.87 | 9.56 | 0.71 | –   | 33.55 |

*Fig. 1 Morphology of bioactive green TiO$_2$ nanocomposites; a SEM image of TiO$_2$-400C (5 µm), b SEM image of TiO$_2$-500C (5 µm), c EDAX spectra of TiO$_2$-400C, d EDAX spectra of TiO$_2$-500C, e TEM images of TiO$_2$-400C (20 nm) and f TEM images of TiO$_2$-500C (20 nm)*
aureus because staph skin infections are most commonly observed in open wounds. The MIC of TiO2-500C for S. aureus was 50.0 mg/mL. TiO2-500C exhibited strong bactericidal activity at a high concentration of 100 mg/mL (Fig. 3e). However, the results for TiO2-400C showed no bacterial reduction despite these NPs having a low MIC of approximately 25.0 mg/mL. A sharp decrease in colony count from 4.3 log10 (control S. aureus) to 1.01 log10 was observed for TiO2-500C at a concentration of 100 mg/mL (Fig. 3). Here, * indicates statistically significant differences (** p ≤ 0.0001) between the different calcination groups for each measurement. There is significant difference between control strain group and anatase TiO2-500C group at concentration of 100 mg/mL.

The stronger bactericidal effect of TiO2-500C compared with that of TiO2-400C may be attributed to the anatase crystalline structure of the former, as confirmed by the XRD pattern shown in Fig. 2a (Fu et al. 2005; Senarathna et al. 2017). Inhibition of bacterial colonies was observed only at a high dosage of the NPs. Thus, green TiO2-500C nanocomposites may be considered an excellent bactericidal agent against S. aureus, a gram-positive bacterium (Behera et al. 2017). Table 3 and Fig. 3a–e illustrates the MICs of the green TiO2-NPs for S. aureus.

**Determination of zone of inhibition**

The bactericidal activity of the TiO2-NPs was further evaluated against S. aureus, the gram-negative bacterium Escherichia coli and the antibiotic-resistant bacteria Klebsiella pneumoniae and Pseudomonas aeruginosa via the Kirby–Bauer disc diffusion method. In this study, TiO2-500C showed a moderate zone of inhibition (ZOI) for E. coli and P. aeruginosa (Table 4 and Fig. 4). By comparison, commercially available anatase TiO2 (P25) and TiO2-400C showed poorer ability to interfere with the cell wall of multi-drug resistant (MDR) and non-MDR strains and disrupt their biochemical processes at a concentration of 100 mg/mL. Cefoxitin antimicrobial discs showed a large ZOI against all four bacterial strains. Researchers found that green TiO2-NPs synthesised from Trigonella foenum-graecum extract have a ZOI of approximately 11.2 mm against S. aureus (Subhapriya and Gomathipriya 2018).

Previous studies demonstrated enhancements in the photocatalytic properties of C-decorated TiO2-NPs for water purification (Nasrollahzadeh et al. 2016; Sharma et al. 2018; Shah et al. 2012; Atchudan et al. 2017) and solid rocket propellants (Dey et al. 2013). None of these works,
however, have highlighted the bactericidal activity of natural C-encapsulated TiO$_2$-NPs. The present study is the first to report that TiO$_2$-500C nanocomposites could encapsulate the bioactive elements of C. gigantea leaf extracts and possess strong inhibitory effects against the tested organisms. This plant-mediated anatase TiO$_2$-based bactericidal agent may be a promising eco-friendly and non-hazardous biomaterial for future pharmaceutical applications.

**Cytocompatibility profiles**

The cytocompatibility of TiO$_2$-400C and TiO$_2$-500C with different concentration (0, 25, 50 and 100 mg/mL) were investigated by fibroblast cells lines model as described in Fig. 5 with the blank control group and 10% (v/v) DMSO. Blank control group set as 100% viability. Based on ISO 10993-5 (2009) recommendation it can be concluded that all different-concentrated green TiO$_2$ nanocomposites is considered as cyto-compatible since the cell viability is higher than 70%. Comparatively, DMSO exhibited significant cytotoxicity to the tested cell lines with cell viability percentage of approximately 31%. It strongly indicates statistically significant differences (****p value ≤ 0.0001) between different-concentrated green TiO$_2$ group and DMSO treated group. In addition, more healthy live cells with elongated filopodia (the leg like of the cell) between 95 and 129% were seen in all TiO$_2$-400C (Fig. 6b, c, d, f, g, h; Supplementary material) and TiO$_2$-500C (Fig. 6j, k, l, n, o, p; Supplementary material) treated nanocomposites, respectively. The outcome showed green TiO$_2$ had ability in promoting proliferation and viability of fibroblast cells lines.

However, TiO$_2$-500C caught much more attention in this study due to its strong bactericidal activity against Gram-positive S. aureus (Fig. 3e) and high proliferation of fibroblast cells lines at concentration level of 100 mg/mL (Fig. 5). The sedimentation of agglomerated green TiO$_2$ on bottom plate was picturized through yellow circle (Fig. 6; Supplementary material). Previously, researchers have investigated the effect of TiO$_2$ nanoparticles concentration related to cytotoxicity and cytocompatibility against MG63 cell lines.

### Table 3: MIC of green TiO$_2$-NPs against S. aureus

| Samples   | MIC (mg/mL) |
|-----------|-------------|
| TiO$_2$-400C | 25          |
| TiO$_2$-500C | 50          |

### Table 4: ZOI (mm) of green TiO$_2$-NPs against skin pathogens

| Strain          | TiO$_2$-400C | TiO$_2$-500C | Commercial P25 | Negative control | Positive control |
|-----------------|--------------|--------------|----------------|------------------|------------------|
| S. aureus       | 6 ± 0.00     | 6 ± 0.00     | 6 ± 0.00       | NA               | 10 ± 0.00        |
| E. coli         | 6 ± 0.00     | 6.83 ± 0.29  | 6 ± 0.00       | NA               | 10 ± 0.00        |
| K. pneumoniae   | 6 ± 0.00     | 6 ± 0.00     | 6 ± 0.00       | NA               | 9 ± 0.00         |
| P. aeruginosa   | 6 ± 0.00     | 6.33 ± 0.14  | 6 ± 0.00       | NA               | 9 ± 0.00         |

*“NA” be a sign of no antimicrobial activity observed for this sample*
and discovered that rapid cell proliferation and enhanced viability were seen at different concentrations without any adverse toxicity (Chellappa et al. 2015). Although, proliferation and survival of fibroblast cell lines slightly decreased when the concentration of green TiO$_2$ is further increased. It is ascribed to the higher level of ROS generation at high concentration level of TiO$_2$ (Behera et al. 2017). Future works on ROS release quantification and long-term cytocompatibility properties of green TiO$_2$ on fibroblast cells lines model is needed for further understanding of the molecular level.

**Conclusions**

Nano-sized cubic and spherical anatase TiO$_2$-500C decoated bioactive green elements nanocomposites with cytocompatible behaviour was successfully synthesised from C. gigantea leaf extract solution. Differences in annealing temperature exerted remarkable impacts on the crystalline phases, morphology, cytocompatibility and bactericidal activities of the green TiO$_2$-NPs towards S. aureus, E. coli and P. aeruginosa. Green TiO$_2$-NPs was found to be cytocompatible on fibroblast cells lines with increased cell viability (≥ 116%). Thus, the TiO$_2$-NPs developed in this work can address current limitations related to pathogen-induced open wound skin infections and wound healing characteristic. However, further investigation is needed to determine the detailed bactericidal mechanism of bioderived anatase TiO$_2$-NPs.

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**Author’s contribution** GAG mainly contributes in writing this manuscript and carried out all experimental works. NNH, WNFWEE, and SS assist in the procedures. RBSSNM is the principal investigator contributes in the concept idea, experimental design, writing process and gave final approval of this paper for publication. All authors have given approval to the final version of the manuscript.
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Availability of data and materials The datasets generated and/or analysed during the current study are not publicly available due to the patent application for methods of making and using of titanium dioxide nanocomposites formed by green synthesis but are available from the corresponding author on reasonable request.

 Declarations

Conflict of interest The authors declare no conflict of interests.

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