RESEARCH ARTICLE

Green Synthesis of TiO_2 Nanoparticles Using Azadirachta Indica with Multifunctional Bioactivity

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Abstract

In this study, titanium dioxide nanoparticles (TiO_2-NPs) were synthesized via a green, cost-effective method using Azadirachta indica leaf extract as a natural capping and reducing agent. Characterization techniques including UV-vis, FTIR, XRD, SEM, EDX, and PL spectroscopy confirmed successful synthesis. UV-vis and FTIR confirmed surface functionalization by organic residues, while XRD revealed a well-crystalline anatase phase with average crystallite sizes of 42.2–54 nm. SEM analysis showed predominantly spherical particles (70–90 nm), and EDX confirmed high purity with only Ti and oxygen present. PL spectra exhibited emission peaks at 420, 468, 493, and 539 nm. The green TiO_2-NPs demonstrated multifunctional biomedical activities. In vitro anti-inflammatory assays showed significant human red blood cell membrane stabilization, with maximum inhibition of 74.7% and 71.3% in heat-induced hemolysis tests. Compared to diclofenac sodium standard, TiO_2-NPs achieved 87.3% and 76.3% inhibition at high concentrations. Anti-diabetic assays revealed up to 71.4% inhibition of glucose uptake by yeast cells (vs. 86.6% for standard drugs), while glucose adsorption ranged from 0.45 to 7.3 mg/g. Antibacterial activity against Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) strains showed inhibition zones of 22.2 mm and 21.2 mm, respectively, comparable to standard drugs (23.3 mm and 21.7 mm). These results highlight green TiO_2-NPs as promising candidates for biomedical applications.

Keywords: green TiO_2-NPs, azadirachta indica leaf extract, anti-inflammatory, anti-diabetic, anti-bacterial efficacy.

1 Introduction

Nanotechnology is transformative in many industries, from energy and electronic to agriculture, textiles and food [1]. Its impact is especially significant in medicine, where it enables advances in targeted drug deliver, bio sensing, medical imaging, and wound healing gene therapy anti-bacterial treatments. NP features, such as their size, shape, biocompatibility, surface chemistry, surface-to-volume ratio, stability, reactivity, magnetic behavior, and low hazardous

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nature, can initiate the biomedical application [2–6]. Presently, huge amounts of NPs have been fabricated from silver (Ag), copper (Cu), zinc (Zn), titanium (Ti), alg (alginate), magnesium (Mg) and their oxides, respectively, via chemical, physical, and green synthesis techniques [7–13]. Metal and metal oxides NPs have predominantly application in the health and medicine sector, because they have approximately nil toxicity when interacting with the cells of mammals. Gold (Au)-NPs have the capability of damaging cancer cells with the assistance of heat generated by the light through a technique called photo thermal radiation therapy [14]. Silver (Ag) and ZnO-NPs possessed the strength to kill the bacterial cells, reduce inflammation, and facilitate the regeneration of tissues [15, 16]. Out of all these materials, TiO_2 has attracted great attention from researchers currently because of its unique characteristics, including high refractive index, stability, versatility, and biocompatibility. TiO_2 is a well-known n-type, wide-bandgap semiconductor with diverse applications, including as pigments, in photocatalysis and solar cells, in supercapacitors and coatings, and in medical products such as sunscreens and cosmetics [17–20]. It is normally existed in three fundamental forms like rutile, anatase and brookite. The anatase form of TiO_2 in the photocatalytic and cytotoxic domain are more efficient than the rutile form [21]. Existing research has reported that the mixed crystalline form of TiO_2 (rutile : anatase) in 1:4 ratio demonstrated prominence biomedical performance compared with the single crystal phase [22, 23]. Recently, researchers have reported the, anti-cancer, anti-skin infection, anti-viral, anti-fungal, and anti-microbial pharmacological potentials of TiO_2-NPs [24–27]. TiO_2-NPs produce reactive oxygen species such as OH and O_2^- which can effectively damage the cancer and microbial cells [28]. The crystal structure and morphology TiO_2 NPs is very important for their use in biomedical applications and to achieve the desired functionality. Therefore, TiO_2-NPs can be achieved in different shapes and morphologies depending the choice of synthesis route. Various synthesis methods have been reported in the literatures. Buraso et al. [29], synthesized TiO_2-NPs by co-precipitation method and investigated their photocatalytic potential. Sadek et al. [30] manufactured TiO_2-NPs adopting the method known as sol-gel. Arthi et al. [31], employed the solvothermal technique for TiO_2 nanostructures and fabricated dye-sensitized solar cells from them. Ansari et al. [32], used the biosynthesis method to synthesized TiO_2 NPs using A. calamus leaf extract and investigated their anti-microbial and photolytic activities. Aravind et al. [33], used the extract of jasmine flowers in a green technique and efficiently fabricated TiO_2-NPs and studied their multifunctional biomedical properties.

Diseases such as inflammation, bacterial infections, and diabetes are highly prevalent and necessitate the development of innovative and effective therapeutics. Inflammation is the body's immune system response to external injurious stimuli, including toxic or chemical irritants, pathogens, and cell damage, characterized by redness, swelling, pain, warmth, and loss of function [34, 35]. A small period of time of inflammation is called acute, which basically extends and converts into chronic inflammation [36]. Chronic inflammation performs an important character in the cellular remodeling and encourages a considerable amount of reactive oxygen species production and cell proliferation [36]. The enlargement of chronic inflammation leads to diseases including obesity, sugar, metabolic disorder ,cardiac diseases , rheumatoid arthritis, and cancer [37].

A chronic disorder of carbohydrate breakdown that includes insufficient production of insulin or inability to respond appropriately to insulin, leading to higher glucose levels in the blood (refer as diabetes). It is the tenth leading cause of death worldwide [41]. Diabetes has been divided into three primary types, including gestational Diabetes, Type 1 Diabetes, and the most common Type, type 2 Diabetes [38, 39]. Long-lasting and untreated diabetes may cause microvascular and macrovascular problems that cause death and disability in diabetic humans [40]. International estimates reports that around 638.6 million people worldwide will live with suffering from diabetes by the year 2045 [42]. Diabetes has such an extremely dangerous consequence that researchers are eager to work hard and produce safe, efficient, and cost-effective medication to treat it precisely.

In this study, we present green synthesized TiO_2-NPs using Azadirachta indica leaf extract for biomedical application. Azadirachta indica, which is also known as neem, is the evergreen, fast-growing, approximately 20-25m tall plant of the Meliaceae family and is native to the Indian subcontinent and Africa [43]. The Aza plant has historically served a vital part in therapeutics owing to more than 60 bioactive ingredients discovered in its leaves, seeds, flowers, and trunks, comprising nimbin, nimbidin, azadirachtin, nimbolinin, and many more [44].

The synthesized green TiO_2-NPs were characterized by XRD, SEM, EDX, FTIR, UV-VIS and PL to study their structural, morphological, elemental, Optical and functional group composition. Biomedical activities such in vitro anti-diabetic, anti-inflammatory and antibacterial activities using TiO_2-NPs were performed. In vitro assays revealed strong anti-inflammatory activity through effective stabilization of human red blood cell (HRBC) membranes, indicating their role in protecting cells against lysis under stress conditions. Simultaneously, anti-diabetic potential was validated via glucose adsorption assays, where the NPs exhibited significant glucose uptake efficiency. Moreover, TiO_2-NPs showed pronounced anti-bacterial effects against both Gram-positive and Gram-negative (E-coli & Staphylococcus aureus) strains, highlighting their broad spectrum antimicrobial action.

2 Materials and Methods

2.1 Materials

The fresh, healthy Azadirachta indica (Neem) leaves were obtained from M. Anis Nursery Farm, Guli Bagh, Mardan KPK, Pakistan. The chemicals isopropoxide ($C_{12}H_{28}O_4Ti$), sodium hydroxide (NaOH), sodium chloride (NaCl), and glassware were purchased from Sigma Aldrich. The healthy blood for anti-inflammation activity was collected from a healthy individual following the standard protocol. The Department of Zoology, AWKUM provided the E. coli bacterial strain (ATCC8739) for antibacterial activity. The levofloxacin, metformin, and diclofenac drugs were obtained from the Pharmacy Department, AWKUM. Furthermore, two-way ANOVA method is used for statistical analysis.

2.2 Azadirachta indica leave extract collection

The Azadirachta indica leaves were first resirinsed with tap water, then with distilled water. A total of 20g of leaves was added to 100mLof distilled water in a beaker and heated on hot plate at 60°C, for half an hour with continuous stirring. A yellowish Azadirachta indica extract was obtained which was stored at 4°C until further use in TiO_2 synthesis.

2.3 Synthesis of TiO_2-NPs

TiO_2-NPs were synthesized with some modifications using the standard synthesis procedures [45]. In the very first step double-distilled water is used as solvent to prepare 0.1 M titanium isopropoxide. Azadirachta indica leaf extract was added drop wise to obtain a pH7. The solution was stirred at a constant frequency at 50°C for 4h. The dense yellowish solution was then resin with distilled water and ethanol for 10 min at 3000rpm three times using centrifugation. The obtained precipitate was kept in oven at 60°C for 60 min to dry. Finally, the samples were annealed at 450°C for 2h. Figure 1 shows synthesis flowchart of green TiO_2 –NPs. The green TiO_2 samples were synthesized by treating it with two different concentrations of Azadirachta indica extract (20mL & 30mL) respectively and pure TiO_2 sample was prepared by synthetic route.

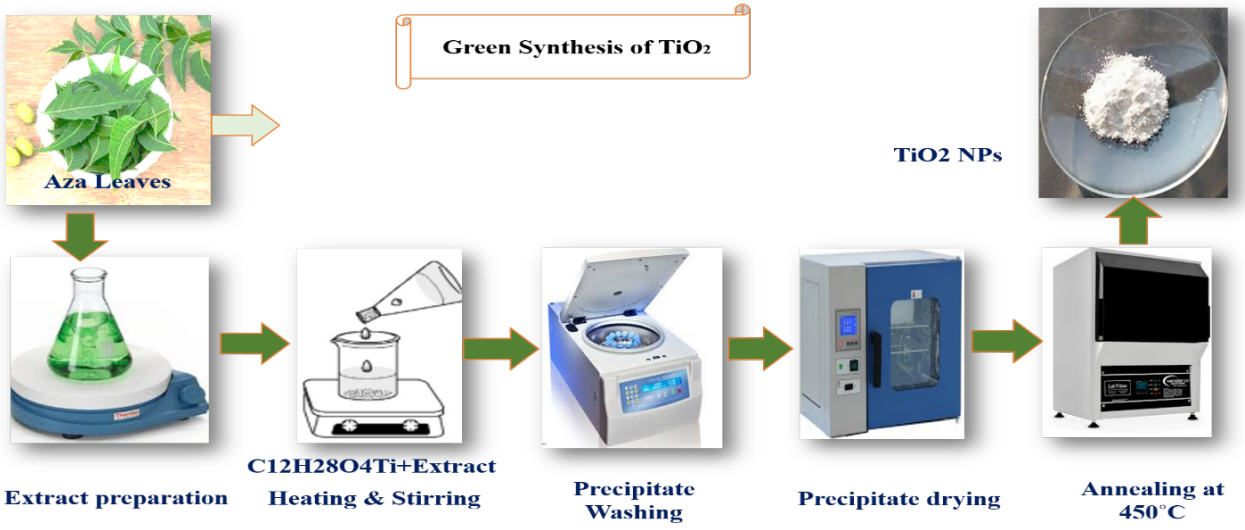

Figure 1. Flowchart of synthesis of TiO_2-NPs with the Azadirachta indica extract.

3 In vitro anti-inflammatory activity

3.1 Suspension preparation for HRBC membrane stabilization assay

The anti-inflammatory potential of TiO_2-NPs were examined implementing the HRBCs membrane stabilization assay. The fresh and healthy blood was obtained from an individual in good health in an EDTA tube who had not taken any medicines for 14 days. The blood was centrifuged many times for 20 min at ambient temperature at 4000rpm. The supernatant was discarded, and the pellet containing HRBCs was used. The normal saline solution 0.9% (w/v) was employed for washing the HRBCs. A 10% (v/v) suspension of RBCs has been prepared in normal saline solution. Then the 10% HRBC suspension in phosphate-buffered saline was prepared and treated with different concentrations (10μm/mL-70μm/mL) of TiO_2-NPs, such as pure-TiO_2, TiO_2-20mL, TiO_2-30mLand standard drug (diclofenac sodium). The HRBCs were later exposed to hypotonic saline (0.25–0.5% NaCl) to trigger hemolysis. The resultant mixture underwent incubation at 37°C for half an hour and was then centrifuged. The absorbance of the supernatant was determined using spectrometer at the wavelength (λ=540 nm), The percentage inhibition was calculated by using equation (1) [12].

$$\%\text{inhibition} = \frac{\text{Control absorbance} - \text{Treated absorbance}}{\text{Control absorbance}} \times 100 \quad (1)$$

3.2 Suspension preparation for heat-induced hemolysis assay

To study the anti-inflammatory capability of pure-TiO_2, TiO_2-20mL, and TiO_2-30mL, diclofenac sodium was tested in comparison with TiO_2 samples. A 10% HRBC suspension was prepared with TiO_2-NPs and diclofenac sodium at various concentrations (10 μg/mL–70 μg/mL). To induce hemolysis, the resulting mixture was incubated at 54 °C for approximately 20 min. At room temperature, the reaction mixtures were allowed to cool down, and then the samples were centrifuged at 4000 rpm for 5 min. The absorbance was measured using a spectrometer at a wavelength $\lambda = 540$ nm, and the corresponding percentage inhibition was calculated using equation (2).

$$\%\text{ inhibition rate} = \frac{\text{Control Absorbance} - \text{Treated absorbance}}{\text{Control absorbance}} \times 100 \quad (2)$$

3.3 Suspension preparation for Glucose adsorption assay

To investigate the glucose adsorption potential of TiO_2-NPs, an *in vitro* anti-diabetic assay for glucose adsorption was conducted. A 100 mL glucose solution (5–25 mM glucose) was prepared and mixed with the test samples, using 0.5 g of each TiO_2 sample. The resultant solution was then incubated at 37 °C for 1 h. The supernatant was separated by repeated centrifugation. Using a glucometer, the absorbance values G1 (before the reaction, representing initial glucose concentration) and G6 (after 6 h, representing final glucose concentration) were determined. The amount of adsorbed glucose was calculated using equation (3).

$$\text{Bounded Glucose} = \frac{G1 - G6}{\text{Control absorbance}} \times \text{Volume of sample} \quad (3)$$

3.4 Suspension preparation for Glucose uptake by yeast cells

The aim of this experiment was to investigate the anti-diabetic potential of TiO_2-NPs using an *in vitro* antidiabetic assay based on glucose uptake by yeast cells. Metformin was used as a standard drug for comparison with the tested samples.

Yeast cells were separated from the yeast solution through repeated centrifugation, and a colloidal suspension of yeast cells in distilled water was prepared. Test samples (TiO_2-NPs) were dissolved in ethanol, and a glucose solution (5 mM, 1 mL) was also prepared. A mixture containing all three solutions was prepared and incubated for 30 min at 37 °C. Various concentrations (20–80 µg/mL) of TiO_2-NPs and metformin were incubated under the same conditions.

Glucose uptake was measured before and after the experiment using a spectrometer at a wavelength of 520 nm. The percentage increase in glucose uptake was calculated using equation (4).

$$\%\text{ increase in glucose uptake} = \frac{\text{Abs(control)} - \text{Abs(sample)}}{\text{Abs(control)}} \times 100 \quad (4)$$

3.5 In vitro antibacterial activity

The antibacterial activity of TiO_2 nanoparticles (TiO_2-NPs) was evaluated using the agar well diffusion method against Gram-negative *E. coli* and

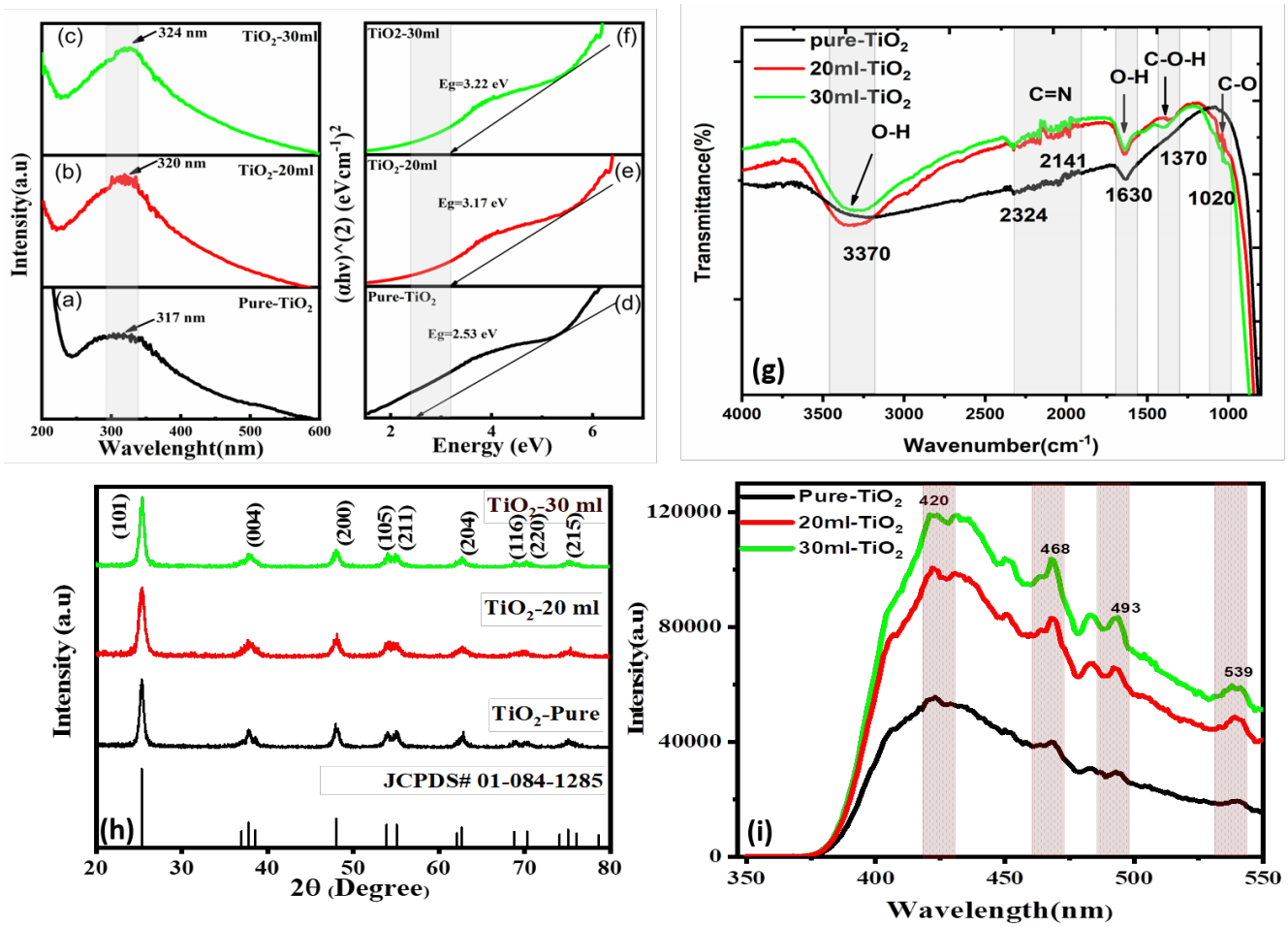

Figure 2. (a-c) shows UV-vis profiles of TiO_2-NPs, (d-f) shows bandgap profile of each sample, (g) shows FTIR spectra of TiO_2-NPs, (h) XRD pattern and (I) photoluminescence spectrum of TiO_2-NPs.

Gram-positive *S. aureus* bacterial strains, following the procedure described by Buraso et al. [29].

Sterile Mueller Hinton Agar (MHA) plates were prepared and inoculated with bacterial suspensions standardized to 0.5 McFarland. Approximately 100 µL of each suspension was evenly spread over the agar surface. A sterile cork borer was then used to punch five wells, each 6 mm in diameter, into the agar.

For *E. coli*, three wells were filled with 50 µL of TiO_2 suspensions (pure TiO_2, TiO_2-20 mL, and TiO_2-30 mL), one with the standard antibiotic levofloxacin (positive control), and one with 10% sterile DMSO (negative control). For *S. aureus*, separate plates were prepared, and two concentrations (30 µL and 60 µL) of each TiO_2 suspension (pure TiO_2, TiO_2-20 mL, and TiO_2-30 mL) were tested.

The plates were incubated at 37 °C for 24 h. After incubation, the inhibition zone diameter of each well was measured in triplicate using a Vernier caliper to ensure precise and accurate results.

4 Results and discussions

The optical, structural and spectroscopic analysis of pure and green TiO_2-NPs samples provided a comprehensive insight into the extract induced modifications in the physiochemical behavior of the samples. UV-Vis absorption spectra as shown in Figure 2(a-c), which revealed a distinct redshift from 317 nm, 320 nm and 324 nm for pure, 20 mL and 30 mL samples respectively. It indicates enhanced visible light absorption and defect formation due to extract mediated surface modification in green and chemically synthesized TiO_2-NPs. This shift signifies improved photon harvesting ability and narrowed particle size distribution, which promote charge excitation and reactive oxygen species generation (ROS), crucial for anti-bacterial, anti-diabetic and anti-inflammatory mechanism [46, 47].

The corresponding Tauc's plots given in Figure 2(d-f), estimated optical bandgaps of 2.53 eV for pure, increasing to 3.17 and 3.22 eV for the 20 mL and 30 mL samples, suggesting extract assisted crystalline

refinement and organic functionalization that alter electronic transition [48].

FTIR spectra given in Figure 2(g) further confirmed the successful incorporation of phytochemicals from the extract showing characteristic O-H stretching around 3370 cm^{-1}, C=O and C-O vibrations at 1630 and 1020 cm^{-1} and C-N at 2141 cm^{-1} [49]. These functional groups attached due to bioactive compounds such as phenols, terpenoids and alkoids etc, which enhanced surface hydrophilicity and biocompatibility, enabling better bimolecular interactions of TiO_2-NPs in biomedical assays.

Furthermore, Figure 2(h) presents the XRD patterns of green-synthesized and chemically synthesized TiO_2-NPs. The XRD of TiO_2 reported the reflective planes at Miller indices (101), (004), (200), (105), (211), (204), (116), (220), and (215) corresponding to the 2θ angle at 25.2°, 37.6°, 47.9°, 54.0°, 54.9°, 62.6°, 68.7°, 70.2°, and 75.0°, respectively. The existence of sharp and intense peaks in the XRD of TiO_2 revealed their well crystalline nature. The intensive peak (101) at 25.2° 2θ value represents the TiO_2 characteristic peak. The XRD of TiO_2-NPs matches well with JCPDS card no. (01-084-1285) and confirmed the anatase phase of TiO_2-NPs with tetragonal arrangement of atoms.

The average crystalline size was measured from XRD by Debye Scherer formula,

$$D = \frac{k\lambda}{\beta \cos\theta} \tag{5}$$

where λ is the wavelength of the X-ray, β represents the full width at half the maximum (FWHM) intensity, θ is assigned for Bragg's angle, and k is called the Scherer constant. Equation (5) gives an average crystallite size of 27.5 nm of TiO_2-NPs calculated from XRD peaks. The XRD of TiO_2-NPs did not show any shift or secondary peak.

Photoluminescence characterization was employed at room temperature, with 350nm exciton wavelength for the study of electronic structure and optical properties of prepared TiO_2-NPs. Figure 2(i) shows that the TiO_2-NPs exhibited several peaks in the PL spectra, showing an identical profile with only variation in the intensity levels. The visible peak at a wavelength of 420 nm describes blue-violet emission observed in all three samples, indicating the self-trapped-electron-hole pair in the TiO_2 nanostructures [50]. The emission peak located at 468 nm corresponds to the emission of blue light might be associated with the transition of charged entities at a higher level [51, 52]. The less intense and broad peaks at 493nm (blue-green region) and 539 nm (green region) are assigned to the transition of electrons in the states that are formed due to the oxygen vacancies at the time of synthesis of TiO_2 [53, 54]. The higher PL intensity in green synthesized TiO_2-NPs describes the greater electron-hole recombination rate as compared to chemically synthesized ones [40]. the phytochemicals (tannins, phenols, terpenoids, alkaloids and flavonoids etc) remains with TiO_2 after treating with green extract play an active role in shaping its electronic structure. These organic compound act as stabilizing and reducing agents, which partially reduce Ti^{4+} to Ti^{3+} and create oxygen vacancies in the TiO_2 lattice. Such defects are well known to serve as radiative recombination centers, where electrons and holes recombine with photon emission, giving rise to PL intensity. Additionally these organic residues from Azadirachta indica often bound to the TiO_2 surface, introducing shallow surface states, that favor radiative over non radiative transition, this phenomena helps in smaller particle sizes and larger surface areas further confirming defect related and surface state emission [55–57]. Overall the enhanced PL intensity reflects the direct influence of phytochemicals, present in the extract treated samples which tailors optical properties.

The SEM micrographs of TiO_2-NPs given in Figure 3(a-c) exhibit highly agglomerated, roughly spherical NPs. The elemental compositions of TiO_2-NPs were revealed by EDX analysis. EDX reported exclusively the constituent elements of the prepared product such as Ti and Oxygen (O_2) with no extra peaks of any other elements. The EDX profiles along with atomic and weight percentages of Ti and O_2 are given Figure 3(e-f), The narrow and intense EDX peaks are defining the well-crystalline and highly pure nature of the prepared product. The EDX confirms a significant increase in the amount of oxygen in the resulting product, which may be due to the organic compounds present in the Aza extract mediating the increase. Moreover, The average particle size was calculated using the ImageJ software. The estimated particle sizes for pure, 20mL-TiO_2, and 30mL-TiO_2 samples are 91.8nm, 84.1nm, and 72.4nm, respectively. The corresponding histograms given in Figure 4(a-c) highlight the numerical details on the distribution of TiO_2-NPs. The particle sizes of the synthesized NPs are significantly decreased with the incorporation of extract, which is evidence of the capping and reducing capability of phytochemicals in

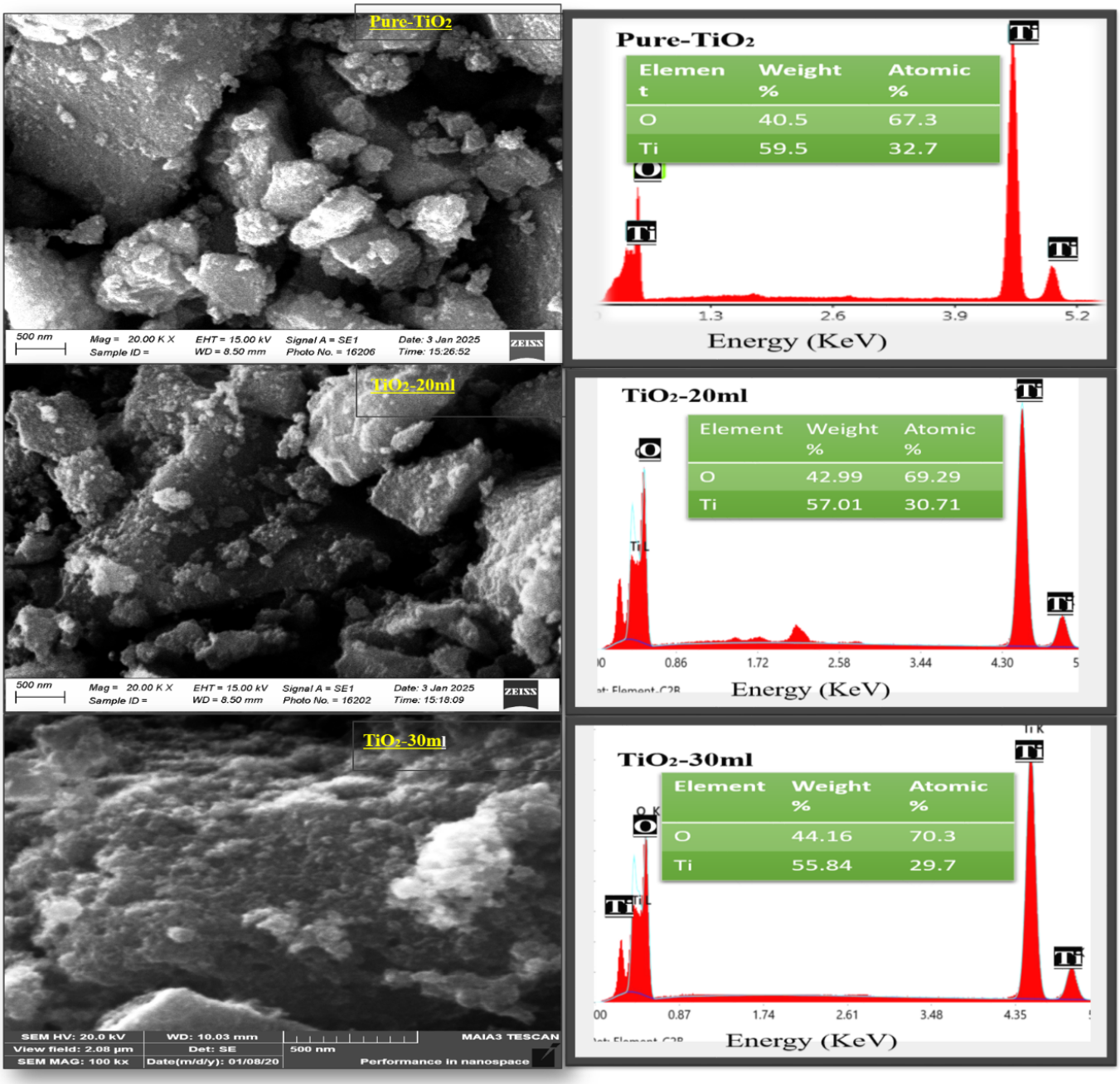

Figure 3. SEM micrographs of TiO_2-NPs and corresponding nanoparticles particles distributions histograms & EDX profiles of TiO_2-NPs.

the Aza leaves extract.

4.1 In vitro anti-inflammatory Activity

4.1.1 HRBC membrane stabilization assay

The anti-inflammatory potential of TiO_2-NPs and the standard drug was tested in seven concentrations from 10μg/mL to 70μg/mL. The activity has reported a dose-dependent behavior of the tested sample, as shown in Figure 5(a). At a concentration of 70μg/mL, the four samples (pure, 20mL, 30mL, and standard) displayed the maximum inhibition and the lowest at 10μg/mL. The maximum inhibition percentage shown by pure, 20mL, 30mLand standard are 54.6%, 69.7%, 74.7% and 87.3% respectively, where the minimum inhibition for each tested sample is 5.6%1 10.9%, 15%, and 20.2%. The green synthesized TiO_2-NPs showed a significant anti-inflammatory potential in contrast to the standard drug used in this study. Notably, TiO_2-30mL suggests that increased extract volume during synthesis enhances bioactivity of TiO_2-NPs, likely due to improved surface functionalization and phytochemical

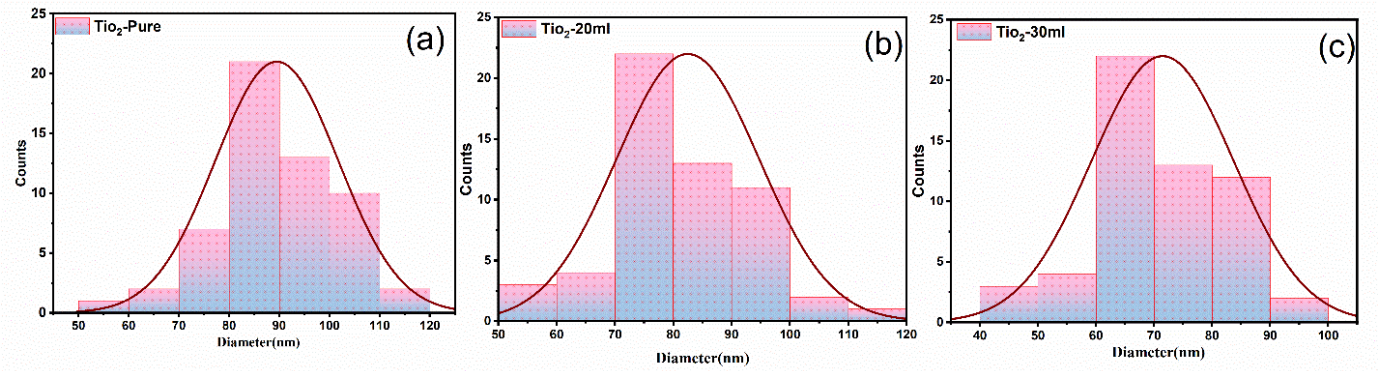

Figure 4. Histograms of TiO_2-NPs, particle size calculated from SEM micrographs using ImageJ software.

capping. Specifically, phenolics and flavonoids stabilize lysosomal and RBC membranes, suppressing the leakage of inflammatory mediators. They also downregulate lipoxygenase and cyclooxygenase enzymes, reducing the production of leukotrienes and prostaglandins [58]. The high amount of these organic residues might be the reason why the 30mL sample closely followed the standard drug result in each concentration and shows effective anti-inflammatory behavior. These findings align with the prior studies reported on green synthesized TiO_2-NPs using as an anti-inflammation agent [59, 60].

4.1.2 HRBC heat induced hemolysis

The high temperature treatment caused the hemolysis in human red blood cells. To inhibit the lysis of the cell membrane, TiO_2-NPs and a standard drug were used. In this activity, seven different concentrations from 10μg/mL to 70μg/mL of each sample were tested. The hemoglobin amount was measured with a spectrometer. The maximum inhibition% demonstrated by 70 concentrations in a standard, 30mL, 20mL, and pure samples are 76.3%, 71.3%, 62.3%, 58.3% respectively and the minimum inhibition% reported in 10 concentrations are 18.1%,14.2%, 7.55%, and 3.6%. The TiO_2-30mL sample effectively stabilized the HRBC membrane in contrast with the other two samples and closely followed the standard drug in each concentration, as shown in Figure 5(b). Additionally, a dose-dependent anti-inflammatory potential was noticed by each sample, dominated by 30mL-TiO_2, confirming the role of high amount of organic residues present on the surface of the TiO_2 framework. In conclusion, the green synthesized TiO_2-NPs reported dominant anti-inflammatory activity over pure ones, which signifies the anti-inflammatory nature of the extract used as a capping and stabilizing solvent for the synthesis of TiO_2 [61].

4.2 In vitro Anti-diabetic activity

4.2.1 Glucose adsorption assay

To study the Glucose adsorption capability of TiO_2-NPs, Various concentrations from (5-30mM) of TiO_2 NPs (30mL-TiO_2, 20mL-TiO_2, and pure-TiO_2) were tested in the glucose adsorption assay. The activity demonstrated a maximum glucose adoption percentage at 30mM concentration are 7.5, 6.55, and 5 for 30mL-TiO_2, 20mL-TiO_2 and pure-TiO_2-NPs respectively, where in comparison the minimum adsorption percentages exhibited by TiO_2 samples at 5mM concentration, numerically equal to 1.8,1.3 and 0.4 respectively as displayed in Figure 6(a). Furthermore, the findings of the activity showcased a dose-dependent increase, as glucose amount effectively influenced the adsorption percentage. Similar trends have been reported in prior studies, where phytochemically functionalized metal oxide-NPs, demonstrated improved glucose uptake due to enhanced surface activity and affinity for glucose molecules [62, 63].

4.2.2 Glucose uptake by yeast cells

The anti-diabetic properties of TiO_2-NPs were investigated with the assay of glucose uptake by yeast cells. The assay registered a dose-dependent increase in uptake efficiency in all tested samples with significant findings. Moreover, numerically, the maximum inhibition was demonstrated by each sample at the maximum concentration 70μg/mL and the minimum was reported in the minimum 10μg/mL concentration, as shown in Figure 6(b). The standard drug, 30mL-TiO_2, 20mL-TiO_2, and pure-TiO_2 displayed the maximum inhibition of 86.6%, 71.4%, 59.7%, and 52.65%, respectively, and the minimum inhibition of 17.6%, 12.5%, 8.67%, and 5.54%. The findings of the activity revealed that TiO_2-NPs and standard drug efficiently control the rate of glucose transport in the yeast cells in a

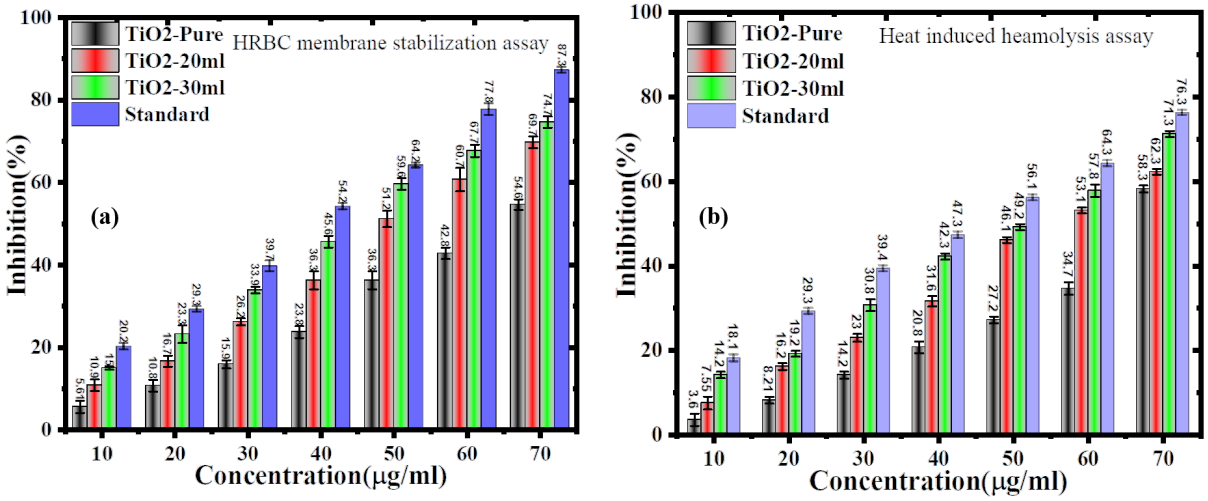

Figure 5. Anti-inflammatory activities. (a) HRBC membrane stabilization and (b) Heat induced haemolysis assay) of TiO_2-NPs.

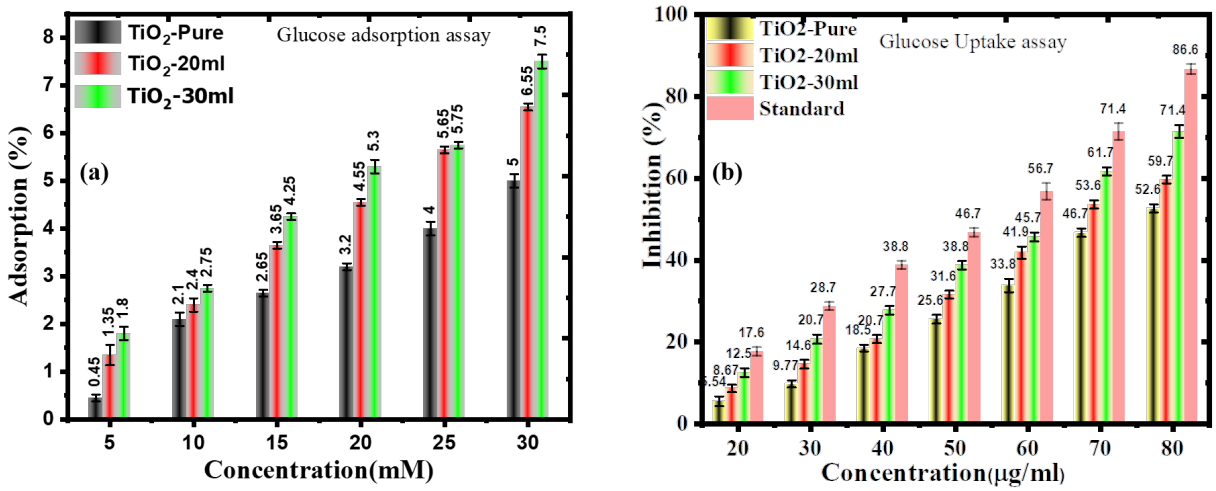

Figure 6. In vitro anti-diabetic activities of TiO_2, including (a) Glucose adsorption assay and (b) Glucose uptake by yeasts cells.

dose-dependent pattern (especially 30mL extract). These enhanced results are attributed to the hydroxyl, carbonyl and phenolic groups which form hydrogen binds with glucose molecules, facilitating adsorption on the nanoparticle surface, moreover terpenoids and flavonoids are reported to enhance glucose uptake (cellular) by stimulating glucose transporter pathways and enhancing insulin mimetic activity [64]. Overall green-synthesized TiO_2-NPs demonstrated relatively good results as compared to pure ones synthesized through chemical route because of the organic residues (phytochemicals)in the green extract treated samples.

4.3 Anti-bacterial activity

The antibacterial activity of TiO_2-Samples was evaluated against gram positive S. aureus bacterial Strain using the agar well diffusion method, with test concentrations of 30 μL and 60 μL of each TiO_2 sample alongside a standard drug (levofloxacin). All samples exhibited distinct inhibition zones, confirming their antibacterial efficacy against S. aureus. Pure TiO_2, TiO_2-20 mL, and TiO_2-30 mL effectively inhibited bacterial growth within their respective zones. At 60 μL, Pure TiO_2 showed an inhibition zone of 17.84 mm, TiO_2-20 mL exhibited 19.33 mm, and TiO_2-30 mL demonstrated the highest activity with 22 mm,

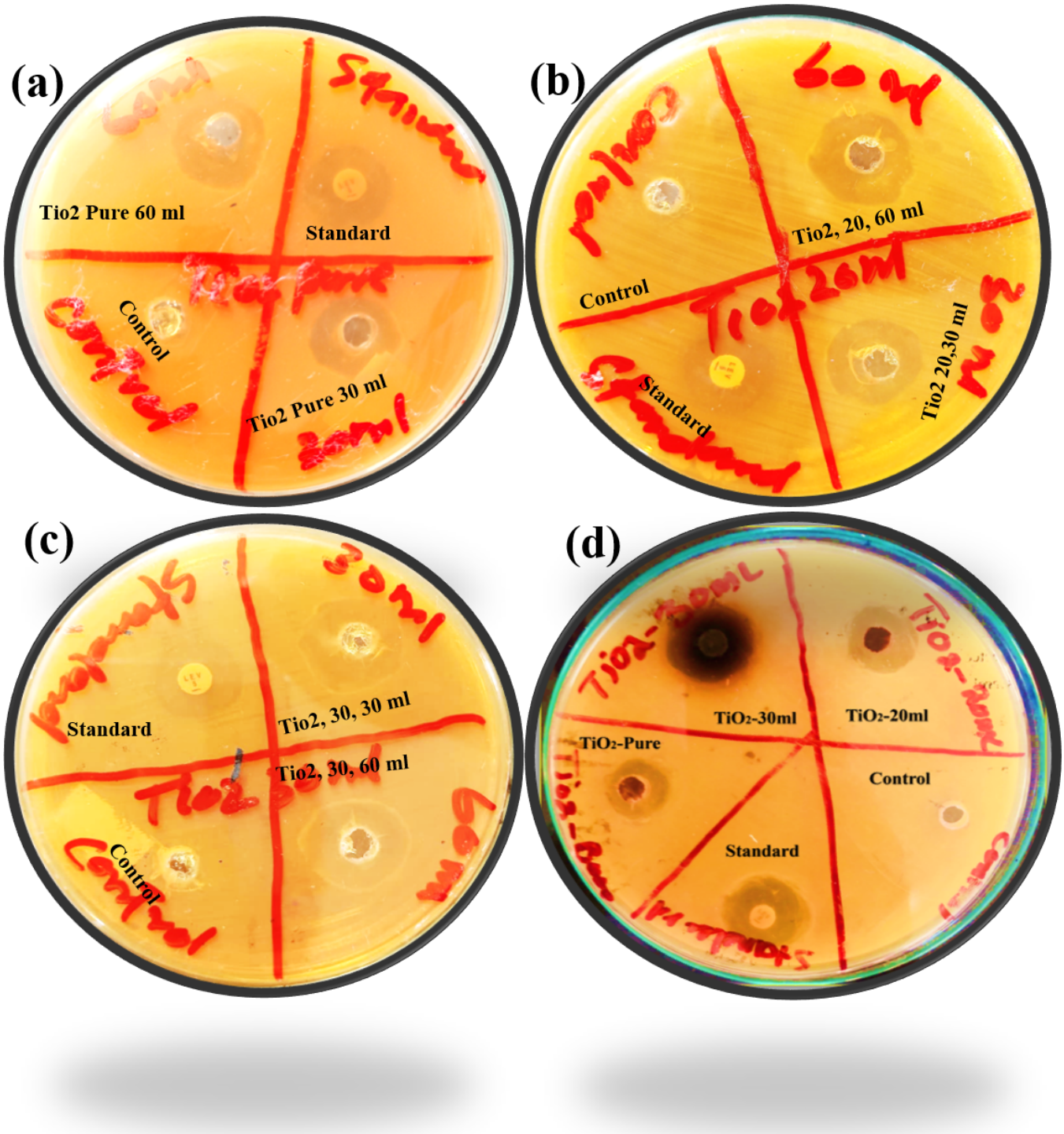

Figure 7. The well diffusion activity of TiO_2-NPs against both gram positive bacteria, S. aureaus (a-c) anti-bacterial response of each sample TiO_2-pure, TiO_2-20mL and TiO_2-30mL at two different concentrations 30mL and 60 mL with standard levofloxacin and (d) shows inhibition zones of TiO_2 samples against gram negative E.coli.

whereas the standard drug shown a zone of 23.33 mm, given in Figure 8(b), Measurements were recorded using a Vernier caliper. The results highlight the dose-dependent antibacterial potential of TiO_2-NPs, with TiO_2-30 mL displaying comparable or superior activity to the reference drug, suggesting that TiO_2 may serve as a promising antibacterial candidate antibacterial efficacy of TiO_2-NPs and the standard drug was studied employing the Furthermore, against gram negative bacteria E. coli, a 50μL dose of each TiO_2 sample and reference drug is used in the test. The activity showed clear inhibition zones, confirming

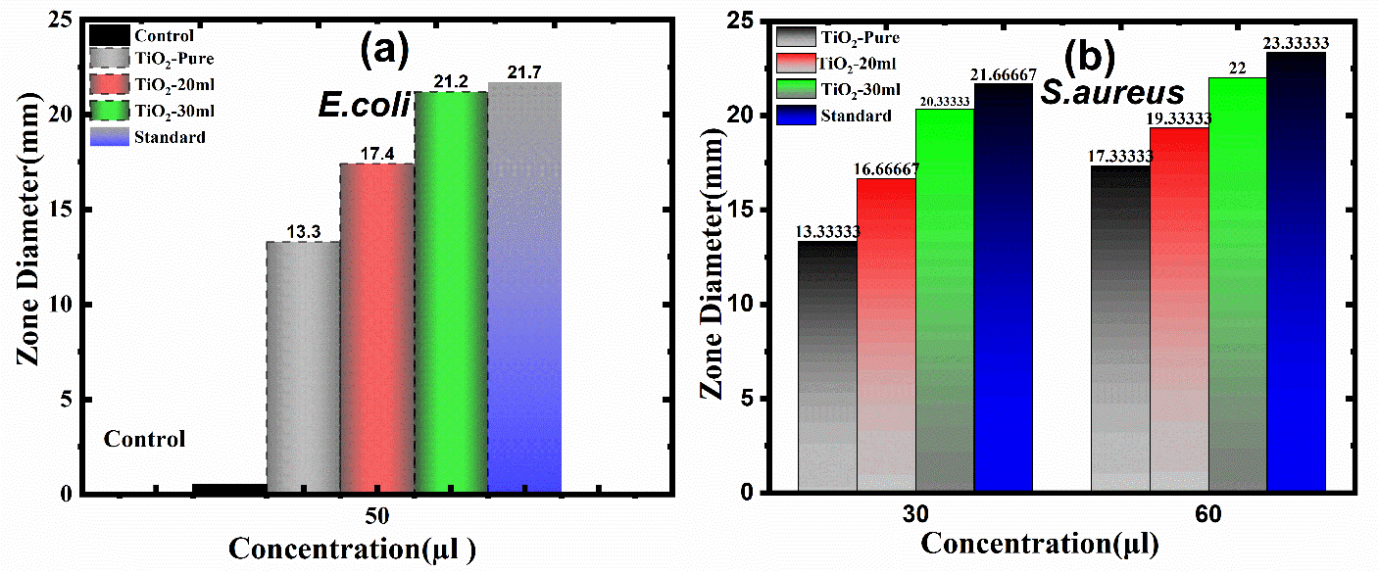

Figure 8. Zone diameter calculated using Vernier caliper (a) against E.coli at 50 μl concentration, (b) shows the zone diameter against S.aureus bacteria at two different concentration 30 and 60 μl.

Table 1. Comparative analysis of anti-bacterial activities of TiO_2-NPs.

TiO_2 Synthesis routes	NP's Size (nm)	Tested bacteria	Volume (μl)	Inhibited Zone (mm)	Antimicrobial method	Medium used	Ref.
Electromagnetic synthesis (Pure TiO_2)	25-30 (TEM)	*E. coli*	50	10.5	Well-diffusion method	Nutrient agar	[68]
Solution casting (Chitosan-TiO_2 composite)	30	*E. coli*	100	9.86	Well-diffusion method	Nutrient agar	[69]
Sol gel (Pure TiO_2)	68 (SEM)	*S. aureus*	100	16	Disc- diffusion method	Muller-Hin-ton Agar	[70]
Sol gel(Pure TiO_2)	68 (SEM)	*E. coli*	100	14	Disc- diffusion method	Muller-Hin-ton Agar	-
Green NPs (*Azadirachta indica*)	15-50 (TEM)	*S. aureus*	66	18	Well-diffusion method	Muller-Hin-ton Agar	[71]
Green synthesis (*Psidium guajava*)	33 (FESEM)	*S. aureus*	20	25	Disc- diffusion method	Muller-Hin-ton Agar	[72]
TiO_2 (*Ledebouria revolute*)	47	*B. cereus*	60	4	Well-diffusion method	Nutrient Agar	[73]
Sol-gel (Pure TiO_2)	78 (FESEM)	*E.coli &S. aureaus*	50 30-60	17.413-17	Well-diffusion method	Nutrient Agar	This-work
Sol-gel_ *Azadirachta indica*- 20mL-TiO_2	72 (FESEM)	*E.coli &S. aureaus*	50 30-60	21.216-19	Well-diffusion method	Nutrient Agar	This-work
Sol-gel_ *Azadirachta indica* -30mL-TiO_2	63 (FESEM)	*E.coli &S. aureaus*	50 30-60	21.720-22	Well-diffusion method	Nutrient Agar	This-work

dose independent but extract enhanced antibacterial efficacy against E. coli, of the TiO_2-NPs. They have effectively reduced or inhibited the growth of the E. coli bacterial strain in their corresponding zones. The affected zone diameter of the standard drug was measured using Vernier caliper, is to be 21.7mm; closely followed by 30mL sample at 21.2 mm, as shown in Figure 8(a), indicating comparable antibacterial activity. Hence such a close characteristic with the standard drug(levofloxacin) makes TiO_2 a potential anti-bacterial candidate in the field of biomedicine. Additionally, TiO_2-20mL and the pure samples showed 17.4 mm and 13.3 mm inhibited zone diameters, respectively, as shown in Figure 7(d). the surface-bound phytochemicals, not only stabilize NPs but also impart additional bioactivity. These compounds enhances membrane interaction, reactive oxygen species generation, and bacterial membrane disruption [65, 66]. The strong anti-bacterial response in this work is resulted from the synergism of phytochemicals mainly (terpenoids, tannins) and TiO_2 generated ROS. ROS directly damages microbial membranes while phytochemicals disrupt cell wall integrity, induce protein leakage and inhibit enzyme activity. This dual mechanism explains strong bacterial inhibition zones in the tested samples. Similar findings were reported on green synthesized TiO_2-NPs which exhibited effective bactericidal effects due to ROS-mediated membrane damage. Another study emphasized the role of biological agents, which enhance their affinity in anti-bacterial effects [67].Thus, the near-equivalent inhibition by the 30mL sample to that of the standard drug underscores the potential of green-synthesized

TiO_2-NPs as an effective and sustainable anti-bacterial agent, particularly in biomedical and health care applications. Table 1 shows the comparative analysis of TiO_2-NPs synthesis with different route and with different particle sizes.

5 Conclusion

In this study, TiO_2-NPs were successfully synthesized via a green synthesis approach using the Azadirachta indica leaf extract, as a reducing agent. The elemental, structural, morphological, and optical properties of the synthesized green TiO_2-NPs were characterized using UV, FTIR, XRD, EDX, SEM, and PL techniques. XRD confirmed the well-crystalline anatase phase, with average crystallite size ranging from 42nm to 55nm. SEM shows the spherically shaped agglomerated particles with average size of 70nm to 90nm. EDX confirm the Ti and oxygen at different ratios, with no other impurities. Optically visible light emission green TiO_2-NPs observed at 420nm, 468nm, 493nm, and 539 nm. The biomedical activities, such as anti-inflammatory, anti-diabetic, and anti-bacterial activities, of the prepared green TiO_2-NPs were conducted. In vitro anti-inflammatory investigation (HRBC membrane stabilization and heat-induced hemolysis) of TiO_2-NPs reported a maximum of 74.7% and 71.3% inhibition in comparison with 87.3% and 76.3% inhibition of the standard drug, respectively. Furthermore, TiO_2-NPs demonstrated 71.4% glucose uptake in yeast cells, in contrast to the standard drug, which displayed 86.6% in the in vitro anti-diabetic activity. The TiO_2-NPs (30mL) showed a maximum of 6.55 glucose adsorption and a minimum of 0.45 in pure TiO_2. The anti-bacterial activity using well diffusion methods, displayed a well-matched, well-aligned result with the reference drug. Compared to reference drug (an inhibition of 21.7 mm), the green TiO_2-NPs (30mL) showed a slightly larger inhibition zone of 21.2 mm, indicating their comparable antibacterial efficacy. These findings validate the potential of the green TiO_2-NPs as a multifunctional biomedical agent to treat diabetes, inflammation, and bacterial infections, providing a sustainable alternative to chemically produced Nano medicine.

Data Availability Statement

Data will be made available on request.

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Conflicts of Interest

The authors declare no conflicts of interest.

AI Use Statement

During the preparation of this work, the author(s) used ChatGPT to assist with English language improvements. The author(s) reviewed and edited the content as necessary and take(s) full responsibility for the final version of the publication.

Ethical Approval and Consent to Participate

All procedures involving human participants were approved by the Institutional Ethical Review Committee of Abdul Wali Khan University, Mardan, Pakistan and conducted in accordance with institutional ethical and biosafety guidelines. Informed consent was obtained from a healthy adult blood donor prior to sample collection.

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