

Green-Synthesized Zinc Oxide Nanoparticles using *Stevia rebaudiana* Leaves: *In Vitro* Antioxidant and Antimicrobial Evaluation

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ABSTRACT

Background: Green synthesis of metal oxide nanoparticles using medicinal plants offers a sustainable route to bioactive nanomaterials. **Objectives:** This study aimed to synthesize zinc oxide nanoparticles (ZnO NPs) using *Stevia rebaudiana* aqueous leaf extract and evaluate their *in vitro* antioxidant, antibacterial, and antifungal activities. **Materials and Methods:** Aqueous *S. rebaudiana* leaf extract served as both a reducing and stabilizing agent to produce SRZnO NPs. The nanoparticles were characterized by X-ray diffraction (XRD), energy-dispersive X-ray spectroscopy (EDX), transmission electron microscopy (TEM), and dynamic light scattering (DLS). Antioxidant activity was assessed using DPPH free radical scavenging, ferric reducing antioxidant power (FRAP), and phosphomolybdenum (PM) assays. Antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger*, and *Candida albicans* was evaluated by the agar well diffusion method. **Results:** TEM revealed predominantly round to quasi-spherical nanoparticles with slight aggregation; DLS indicated a hydrodynamic size of ~107–130 nm. XRD confirmed a crystalline hexagonal wurtzite ZnO structure, and EDX verified zinc as the major element. SRZnO NPs exhibited markedly higher antioxidant activity than the crude extract, with a DPPH IC₅₀ of 12.04 µg/mL compared with 83.30 µg/mL. FRAP and PM assays further demonstrated superior reducing power and total antioxidant capacity at all tested concentrations. The nanoparticles exhibited concentration-dependent antimicrobial effects, producing inhibition zones of 14–17 mm at 100 µL, whereas the leaf extract showed minimal activity. **Conclusion:** *Stevia rebaudiana*-mediated ZnO nanoparticles display enhanced antioxidant and broad-spectrum antimicrobial properties compared with the parent leaf extract, supporting their potential as promising bioactive nanomaterials for pharmaceutical and biomedical applications.

Keywords: *Stevia Rebaudiana*, Zinc Oxide Nanoparticles, Green Synthesis, Antioxidant Activity, Antimicrobial Activity

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INTRODUCTION

The rapid emergence of multidrug-resistant (MDR) microorganisms and the increasing prevalence of oxidative stress-related disorders have intensified the search for novel therapeutic agents possessing both antimicrobial and antioxidant properties. Nanotechnology has emerged as a promising approach to address these challenges through the development of multifunctional nanomaterials. Among various metal oxide nanoparticles, zinc oxide (ZnO) nanoparticles have gained considerable attention owing to their excellent biocompatibility, low toxicity, cost-effectiveness, and unique optical, electrical, and catalytic properties (Singh, 2021). According to Elabbasy et al. (2025) ZnO nanoparticles exhibit broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria, including MDR

pathogens, while also possessing significant antioxidant potential through free radical scavenging and reactive oxygen species (ROS) modulation. These multifunctional properties make ZnO nanoparticles attractive candidates for biomedical, pharmaceutical, and food safety applications.

Conventional methods for synthesizing ZnO nanoparticles, including chemical and physical approaches, often require toxic reducing agents, elevated temperatures, sophisticated instrumentation, and high energy consumption, leading to environmental pollution and hazardous waste generation. In this context, Singh (2021) described plant-mediated green synthesis as an environmentally friendly, economical, and scalable approach in which naturally occurring phytochemicals, such as polyphenols, flavonoids, alkaloids, terpenoids,

and glycosides, function as reducing, stabilizing, and capping agents. The resulting nanoparticles exhibit enhanced stability, improved biocompatibility, and superior biological activity because plant-derived compounds remain adsorbed on the nanoparticle surface.

Stevia rebaudiana Bertoni, a perennial medicinal herb belonging to the family Asteraceae, is widely recognized for its abundance of steviol glycosides and diverse bioactive phytochemicals. As reported by Sabirov et al. (2024), the leaves are rich in phenolic compounds, flavonoids, diterpenoids, and polyphenols that possess antioxidant, antimicrobial, antiviral, anti-inflammatory, and antidiabetic activities. These phytochemicals efficiently reduce zinc ions during nanoparticle synthesis while simultaneously acting as stabilizing and capping agents, thereby influencing nanoparticle size, morphology, stability, and biological performance.

Recent studies have demonstrated the successful biosynthesis of ZnO nanoparticles using *Stevia rebaudiana* leaf extracts. Elabbasy et al. (2025) synthesized *Stevia rebaudiana*-mediated ZnO nanoparticles with an average particle size of 15.7 nm and demonstrated potent antibacterial activity against multidrug-resistant *Staphylococcus aureus*, reporting inhibition zones of up to 24.4 mm, MIC values ranging from 6.25 to 25 µg/mL, MBC values of 12.5–50 µg/mL, and biofilm inhibition reaching 92.1%. Likewise, Tran et al. (2024) successfully employed *Stevia rebaudiana*-derived ZnO nanoparticles for the removal of antibiotics from aqueous solutions, illustrating the versatility of this green synthesis approach for both biomedical and environmental applications.

Although these studies have confirmed the feasibility of synthesizing ZnO nanoparticles using *Stevia rebaudiana*, most investigations have focused primarily on nanoparticle synthesis, antibacterial activity against selected microorganisms, photocatalytic performance, or environmental remediation. In particular, studies simultaneously investigating antioxidant activity using standardized assays such as DPPH and Ferric Reducing Antioxidant Power (FRAP), together with antimicrobial efficacy against bacterial and fungal pathogens and mechanistic investigations, are scarce.

The antioxidant potential of biosynthesized ZnO nanoparticles is commonly assessed using DPPH and FRAP assays, which evaluate free radical scavenging activity and reducing power, respectively. Singh et al. (2021) reported that plant-mediated ZnO nanoparticles exhibit remarkable antioxidant activity, with DPPH IC₅₀ values as low as 38 µg/mL and radical scavenging activity exceeding 66% at higher concentrations. According to Sabirov et al. (2024), this enhanced antioxidant performance is largely attributed to phytochemicals

adsorbed onto the nanoparticle surface, which facilitate electron donation and hydrogen atom transfer.

The antimicrobial activity of ZnO nanoparticles involves several complementary mechanisms. Singh (2021) explained that direct interaction of ZnO nanoparticles with microbial cell membranes increases membrane permeability, resulting in leakage of intracellular contents. Furthermore, Singh et al. (2021) demonstrated that ZnO nanoparticles generate reactive oxygen species that induce oxidative stress, lipid peroxidation, protein oxidation, and DNA damage, ultimately leading to microbial cell death. In addition, the release of Zn²⁺ ions interferes with essential cellular enzymes and metabolic pathways, thereby enhancing antimicrobial efficacy. Plant-derived phytochemicals present on the nanoparticle surface further improve microbial cell interaction and ROS generation, resulting in superior antimicrobial performance compared with chemically synthesized ZnO nanoparticles (Singh, 2021).

Despite increasing interest in green-synthesized ZnO nanoparticles, comprehensive investigations evaluating both antioxidant and antimicrobial activities of *Stevia rebaudiana*-mediated ZnO nanoparticles using standardized pharmacological assays remain limited. Therefore, a systematic evaluation of their dual biological activities is warranted.

ZnO nanoparticles synthesized using aqueous *Stevia rebaudiana* leaf extract would exhibit enhanced antioxidant and antimicrobial activities because the phytochemicals present in the extract function as natural reducing and capping agents, improving nanoparticle stability and biological performance.

Accordingly, the present study aimed to synthesize ZnO nanoparticles using aqueous *Stevia rebaudiana* leaf extract through a green synthesis approach, characterize the synthesized nanoparticles by X-ray diffraction (XRD) and transmission electron microscopy (TEM), evaluate their antioxidant activity using DPPH and FRAP assays, assess their antimicrobial activity against selected bacterial and fungal pathogens using the agar well diffusion method, and investigate the possible antimicrobial mechanism through bacterial membrane and DNA damage studies. By providing a comprehensive pharmacological evaluation of both antioxidant and antimicrobial properties, this study extends previous work on *Stevia rebaudiana*-mediated ZnO nanoparticles and supports their potential application as environmentally friendly alternatives for future biomedical and pharmaceutical use.

MATERIALS AND METHODS

Materials

Loba Chem Pvt. Ltd. supplied DPPH, ethanol, ascorbic acid, potassium ferricyanide, trichloroacetic acid, ferric

chloride, and the molybdate reagent. Fluconazole was procured from Sigma-Aldrich in Bangalore, India; Brain Heart Agar (BHA) and Sabouraud Dextrose Agar were purchased from Hi Media Laboratories Pvt. Ltd. in Mumbai, India. Distilled water and GraphPad Prism were used. All compounds were of analytical quality and utilized without purification.

Method

Aqueous leaf extract of *Stevia rebaudiana* (SR)

The extract of *Stevia rebaudiana* (SR) was prepared by washing leaves twice with tap water, followed by doubledistilled water, air-drying for 4–6 days, shredding, and grinding to a fine powder. Ten grams of powder were extracted in 100 mL MilliQ water at 70°C with stirring for 20 minutes, cooled, and filtered through muslin, filter paper, and Whatman No.1. The filtrate was stored at 4°C.

Synthesis of zinc oxide nanoparticles (SR-ZnO nanoparticles)

Stevia rebaudiana leaf extract was used to biosynthesize zinc oxide nanoparticles. A 0.1 M $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution was prepared by dissolving 29.75 g of $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ in ~800 mL distilled water, transferring to a 1L volumetric flask, and bringing to volume. Aqueous SR leaf extract served as the reducing and stabilizing agent. The extract and zinc solution were reacted at $70 \pm 2^\circ\text{C}$ with continuous stirring, yielding a brownish–yellowish–white precipitate. The precipitate was centrifuged, washed, and calcined at 400°C to obtain pure ZnONPs.

Characterization of SR-ZnO nanoparticles

SR-ZnO nanoparticles were characterized using particle size, zeta potential (ZP), X-ray diffraction (XRD), Transmission Electron Microscopy (TEM), and energy-dispersive X-ray (EDX) analysis.

In Vitro Evaluation of Antioxidant Activity of Synthesized SR-ZnO Nanoparticles

DPPH (2,2-Diphenyl-1-2-picrylhydrazyl) Free Radical Scavenging Assay

The free radical scavenging activity of aqueous *Stevia rebaudiana* leaf extract and synthesized SR–ZnO nanoparticles was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay with slight modifications to previously reported methods (Gulcin, 2023; Apak et al., 2016; Blois, 1958). A stock solution (0.5 mg/mL) of aqueous SR leaf extract and SR–ZnO nanoparticle suspension was prepared and serially diluted to obtain different concentrations. One milliliter of each sample solution was mixed with 4 mL of DPPH solution. The blank consisted of ethanol in place of the DPPH solution, while the control contained all reagents except the test sample. The reaction mixtures were incubated in the dark at room temperature for 30 min, after which the absorbance was measured at 517 nm using a UV–Visible spectrophotometer. Ascorbic acid was used as the

reference antioxidant. The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ Radical Scavenging Activity} = [(\text{control} - \text{sample}) / \text{A control}] \times 100$$

Where *control* is the absorbance of the control, and *sample* is the absorbance of the test sample. The IC_{50} value (concentration required to scavenge 50% of DPPH radicals) was determined by nonlinear regression analysis using GraphPad Prism software.

FRAP Assay: Ferric Reducing Antioxidant Power

The antioxidant capacity of SR–ZnO nanoparticles and aqueous SR extract was measured by slightly modified versions of previously published techniques Rajakumar et al., 2018; Gul et al., 2024; Pulido et al. 2000). SR extract and SR–ZnO nanoparticle suspension were produced at varying doses (12.5–200 $\mu\text{g/mL}$). After treating each sample with 2.5mL of potassium ferricyanide solution (1% w/v) and 2.5 mL of phosphate buffer (20 mM), the samples were incubated at 50°C for 30 minutes. After the addition of 0.5 mL of ferric chloride solution (0.1% w/v) and 2.5 mL of trichloroacetic acid (10% w/v), the mixture was incubated for a further 10 minutes. A UV-visible spectrophotometer was used to check the absorbance at 593 nm. Greater antioxidant capability was indicated by increased absorption. Ascorbic acid was used as the standard reference when calculating the p-value.

Phosphomolybdenum (PM) Assay

The total antioxidant capacity of aqueous *Stevia rebaudiana* (SR) leaf extract and green-synthesized SR–ZnO nanoparticles was determined using the phosphomolybdenum (PM) assay as described by Prieto et al. (1999) with slight modifications. Aqueous SR leaf extract and SR–ZnO nanoparticle suspensions were prepared at concentrations ranging from 12.5 to 200 $\mu\text{g/mL}$. Briefly, 0.3 mL of each sample was mixed with 3.0 mL of phosphomolybdenum reagent and incubated at 95°C for 90 min (Rajakumar et al., 2018). The reaction mixtures were then cooled to room temperature for 20–30 min, and the absorbance was measured at 695 nm using a UV–Visible spectrophotometer (Pulido et al., 2000). Ascorbic acid was used as the reference standard, and higher absorbance values indicated greater total antioxidant capacity (Vargas Ramella et al., 2026).

Statistical Analysis

All experiments were performed in triplicate, and the results are expressed as mean $\pm$ standard deviation (SD). Statistical analyses were carried out using GraphPad Prism version 11 (GraphPad Software). Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value < 0.05 was considered statistically significant.

Ethics Statement: This study involved only *in vitro* experiments and did not involve human participants, animals, or human tissues. Therefore, ethical approval and informed consent were not required.

Assessment of Antibacterial and Antifungal Activity *in Vitro*

Agar well diffusion test

The antibacterial and antifungal activities of aqueous *Stevia rebaudiana* leaf extract and green-synthesized SR-ZnO nanoparticles were evaluated using the agar well diffusion method with slight modifications (Ranjani et al., 2024). Bacterial and fungal strains were cultured overnight in nutrient broth to obtain a microbial suspension of approximately 1×10^6 CFU/mL (Gul et al., 2024). The microbial suspension was uniformly spread onto sterile nutrient agar and potato dextrose agar plates using a sterile cotton swab. Sterile wells (8 mm in diameter) were prepared using a sterile cork borer, and 50 μ L and 100 μ L of aqueous SR leaf extract, SR-ZnO nanoparticle suspension, or the respective standard antimicrobial agents were dispensed into the wells. Tetracycline and ketoconazole were used as the positive controls for antibacterial and antifungal studies, respectively. The inoculated plates were incubated at 37°C for 24 h for bacterial strains and 48 h for fungal strains. Following incubation, the diameters of the zones of inhibition were measured in millimeters (mm), and the antimicrobial activity was assessed by comparing the inhibition zones with those of the standard drugs Pulido et al., 2000).

RESULTS

Synthesis of ZnO nanoparticles from *Stevia rebaudiana* using aqueous leaf extract.

A brownish–yellowish–white precipitate was obtained, indicating ZnO nanoparticle formation. The product was recovered by centrifugation, washed, and calcined at 400 ± 2 °C for 2 h to yield a white ZnO powder.

Characterization of SR-ZnONPs

The comprehensive physicochemical characterization of the biosynthesized ZnO nanoparticles is presented in Figure. 1. TEM analysis (Figure. 1a) demonstrated predominantly spherical to quasi-spherical nanoparticles with slight agglomeration, indicating successful nanoparticle formation at the nanoscale. The DLS particle size distribution (Figure. 1b) revealed a relatively narrow and uniform hydrodynamic particle size ranging from approximately 107–130 nm, suggesting low polydispersity and good colloidal stability. XRD analysis (Figure. 1c) confirmed the highly crystal-like nature of the nanoparticles, exhibiting typical diffraction peaks consistent to the hexagonal wurtzite crystal structure of ZnO without any detectable impurity phases. Furthermore, EDX analysis (Figure. 1d) verified the elemental composition of the synthesized nanoparticles, showing prominent zinc (Zn) and oxygen (O) peaks with no significant contaminant elements.

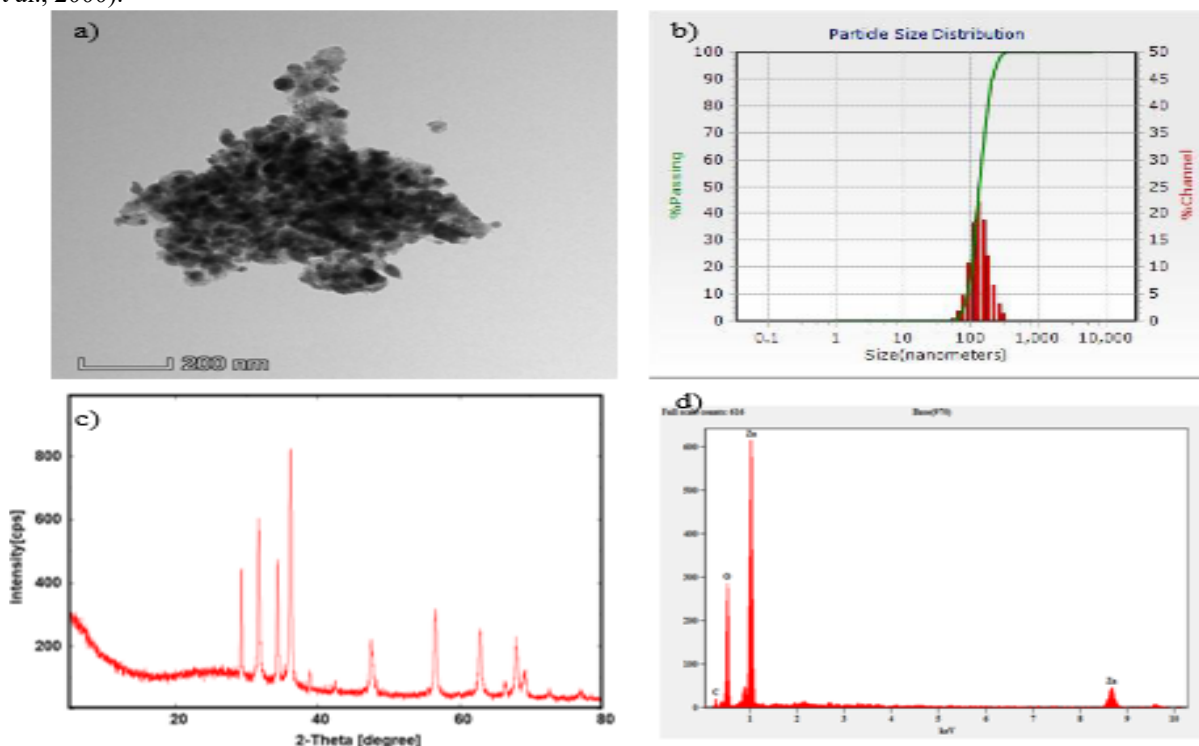


Figure.1.a) Transmission Electron Microscopy (TEM) Analysis, b) Particle Size Distribution, c)X-ray Diffraction (XRD) Analysis, d)Energy Dispersive X-ray Spectroscopy (EDX) Analysis

In Vitro Evaluation of Antioxidant Activity of Synthesized SR-ZnO Nanoparticles DPPH (2,2-Diphenyl-1-picrylhydrazyl) Free Radical Scavenging Assay

The antioxidant activity of *Stevia rebaudiana*-mediated zinc oxide nanoparticles was evaluated using the DPPH free radical scavenging assay. As shown in Figure 2 and Table 1, both the aqueous *Stevia rebaudiana* leaf extract and SR-ZnO nanoparticles exhibited concentration-dependent DPPH radical scavenging activity, with the percentage inhibition increasing as the concentration increased. However, the SR-ZnO nanoparticles demonstrated significantly greater free radical scavenging activity than the crude leaf extract at all tested concentrations. The synthesized SR-ZnO nanoparticles

exhibited an IC₅₀ value of 12.04 µg/mL, whereas the aqueous leaf extract showed an IC₅₀ value of 83.3 µg/mL, indicating the superior antioxidant potential of the nanoparticles. Similar findings have been reported by Maind et al (2025), Kingori et al (2024), who demonstrated that green-synthesized ZnO nanoparticles exhibited strong DPPH radical scavenging activity owing to their enhanced surface reactivity and phytochemical-mediated stabilization. Likewise, Islam et al. (2024) observed significantly higher antioxidant activity of plant-mediated ZnO nanoparticles than the corresponding plant extract, while Elabbasy et al. (2025) reported that *Stevia rebaudiana*-derived ZnO nanoparticles possess excellent biological activity attributable to the synergistic effects of ZnO and surface-bound phytochemicals.

Table 1. Comparative *in vitro* DPPH radical scavenging activity of ascorbic acid, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations.

Sr. No.	Concentration (µg/ml)	Ascorbic Acid	SR ZnO nanoparticles	SR leaf Extract
1	12.5	62.32±1.21	50.37±2.02	39.06±3.08
2	25	64.18±2.27	58.46±3.3	42.09±2.65
3	50	69.76±1.09	62.79±2.96	43.72±1.87
4	100	73.25±2.38	68.41±1.99	52.58±1.99
5	150	76.74±3.02	70.13±1.85	58.83±2.39
6	200	81.16±2.99	76.39±2.27	64.55±2.85

Note: The values presented are the arithmetic mean ±SD of three determinations.

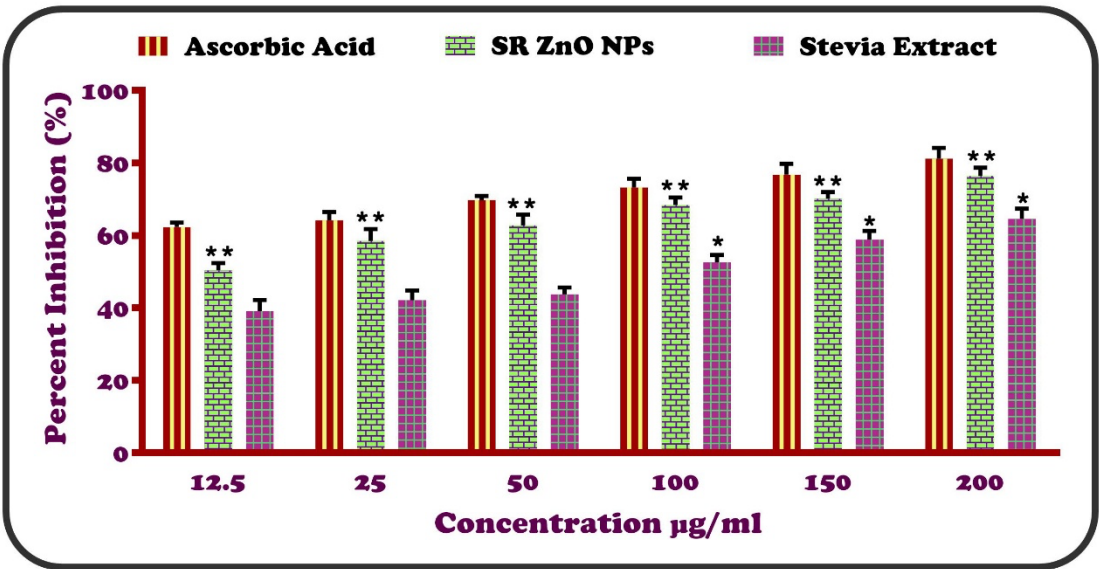


Figure 2. In vitro DPPH scavenging activity of ascorbic acid, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations. Data are presented as mean $\pm$ SD (n = 3). Statistical significance was determined by comparing SR-ZnO nanoparticles and leaf extract groups; ns = not significant, $p < 0.05$, $p < 0.01$, $p^* < 0.001$.

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) assay was employed to evaluate the antioxidant activity of aqueous *Stevia rebaudiana* leaf extract and SR-mediated ZnO nanoparticles by measuring the reduction of ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions, resulting in the formation of a colored complex that was quantified spectrophotometrically (Gülçin & Alwasel, 2023). As shown in Figure 3 and Table 2, both the SR leaf extract and SR-ZnO nanoparticles exhibited concentration-dependent ferric reducing activity over the concentration range of 12.5–200 $\mu\text{g/mL}$, with absorbance increasing progressively as the concentration increased. The SR-

ZnO nanoparticles consistently exhibited higher absorbance values than the crude leaf extract at all tested concentrations, indicating superior reducing power. At 200 $\mu\text{g/mL}$, the SR-ZnO nanoparticles exhibited an absorbance of 0.1685 ± 0.0036 , whereas the SR leaf extract showed an absorbance of 0.1455 ± 0.0026 . Similar concentration-dependent increases in ferric reducing activity have also been reported for green-synthesized ZnO nanoparticles by Pulido et al., (2000). Furthermore, the enhanced reducing power of biosynthesized ZnO nanoparticles has been attributed to the synergistic interaction between ZnO and phytochemical capping agents, which facilitate electron donation and improve antioxidant efficiency (Elabbasy et al., 2025).

Table 2. Comparative *in vitro* ferric reducing antioxidant power of Standard, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations.

Sr. No.	Concentration ($\mu\text{g/mL}$)	Ascorbic Acid	SR ZnO NPs	SR leaf Extract
1	12.5	0.1164 ± 0.0030	0.0630 ± 0.0024	0.0330 ± 0.0021
2	25	0.1329 ± 0.0041	0.0745 ± 0.0032	0.0655 ± 0.0026
3	50	0.1416 ± 0.0040	0.1175 ± 0.0026	0.0710 ± 0.0019
4	100	0.1548 ± 0.0032	0.1235 ± 0.0036	0.1005 ± 0.0026
5	150	0.1689 ± 0.0023	0.1465 ± 0.0021	0.1189 ± 0.0024
6	200	0.1825 ± 0.0034	0.1685 ± 0.0036	0.1455 ± 0.0026

Note: The values presented are the arithmetic mean $\pm$ SD of three determinations.

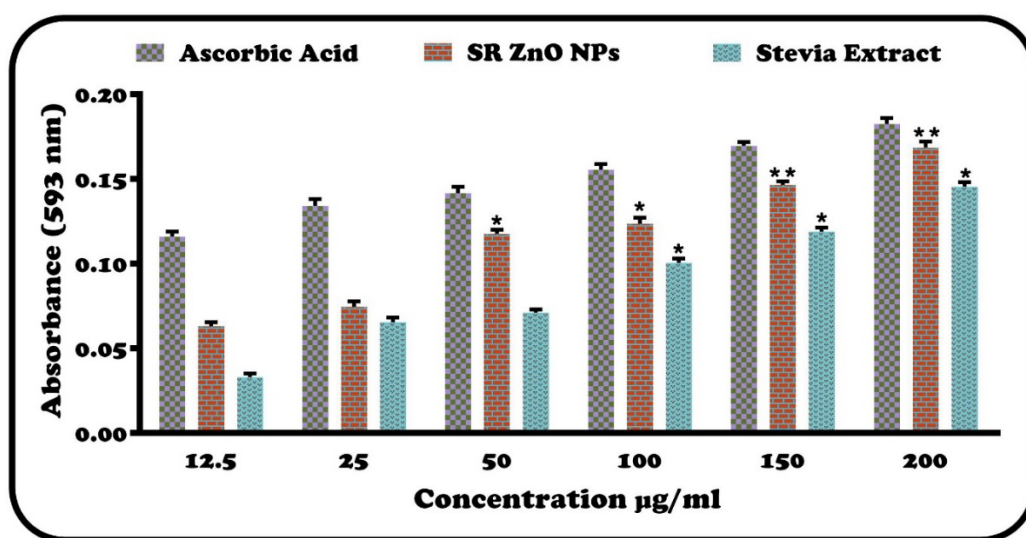


Figure 3. In vitro ferric reducing antioxidant power of Standard, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations. Data are presented as mean $\pm$ SD (n = 3). Statistical

significance was determined by comparing SR-ZnO nanoparticles and leaf extract groups; ns = not significant, $p < 0.05$, $p < 0.01$, $p^* < 0.001$

Phosphomolybdenum (PM) Assay

The total antioxidant capacity of aqueous *Stevia rebaudiana* leaf extract and SR-mediated ZnO nanoparticles was evaluated using the phosphomolybdenum (PM) assay, which is based on the reduction of molybdenum (VI) to molybdenum (V) and the subsequent formation of a green phosphate/Mo(V) complex under acidic conditions (Prieto et al., 1999; Vargas Ramella et al., 2026). As presented in Figure 4 and Table 3, both the SR leaf extract and SR-ZnO nanoparticles exhibited concentration-dependent antioxidant activity over the concentration range of 12.5–200 $\mu\text{g/mL}$, with absorbance increasing progressively as the concentration increased. Similar concentration-dependent increases in total antioxidant capacity have been reported by Islam et al. (2024). The SR-ZnO

nanoparticles consistently exhibited higher absorbance values than the crude SR leaf extract at all tested concentrations, indicating superior antioxidant capacity. At 200 $\mu\text{g/mL}$, the SR-ZnO nanoparticles showed an absorbance of 0.1669 ± 0.0030 , compared with 0.1491 ± 0.0020 for the SR leaf extract. Likewise, at 12.5 $\mu\text{g/mL}$, the SR-ZnO nanoparticles exhibited nearly two-fold greater antioxidant activity than the crude extract. The enhanced antioxidant activity of biosynthesized ZnO nanoparticles has been attributed to the synergistic effects of phytochemical capping agents and the high surface area of ZnO nanoparticles, which facilitate electron transfer and improve antioxidant efficiency (Maind et al., 2025). Comparable findings were also reported by Pulido et al. (2000), who observed significantly greater antioxidant activity of plant-mediated ZnO nanoparticles than the corresponding crude plant extracts.

Table 3. Comparative in vitro phosphomolybdenum reducing activity of ascorbic acid, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations.

Sr. No.	Concentration ($\mu\text{g}/\text{ml}$)	Ascorbic Acid	SR ZnO nanoparticles	SR leaf Extract
1	12.5	0.1300 ± 0.0063	0.0600 ± 0.0024	0.0335 ± 0.0038
2	25	0.1415 ± 0.0064	0.0760 ± 0.0028	0.0530 ± 0.0042
3	50	0.1505 ± 0.0063	0.1145 ± 0.0021	0.0701 ± 0.0049
4	100	0.1605 ± 0.0049	0.1235 ± 0.0049	0.0985 ± 0.0021
5	150	0.1758 ± 0.0061	0.1472 ± 0.0014	0.1220 ± 0.0014
6	200	0.1945 ± 0.0030	0.1669 ± 0.0030	0.1491 ± 0.0020

Note: The values presented are the arithmetic mean $\pm$ SD of three determinations.

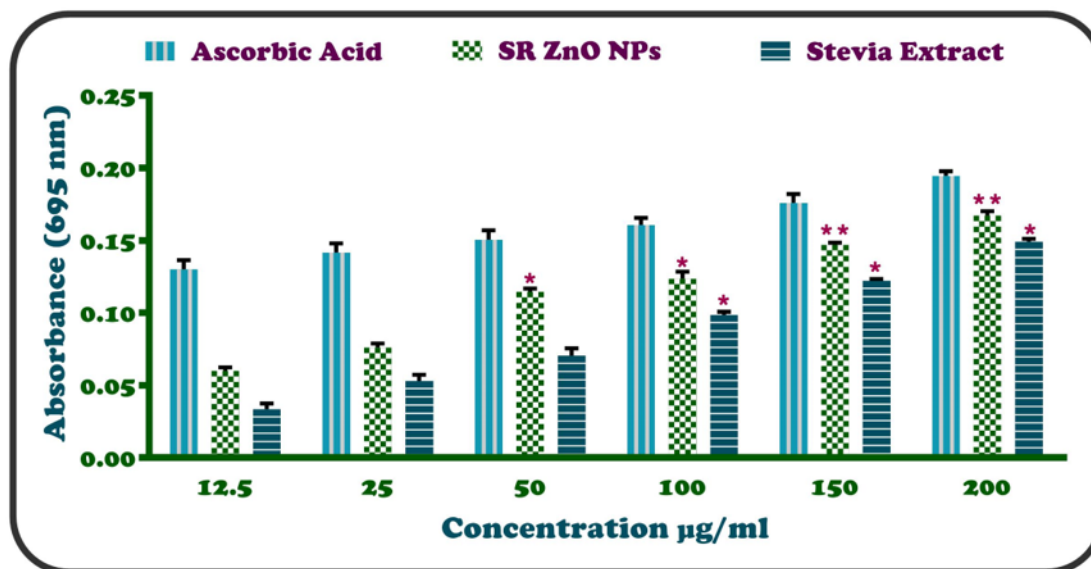


Figure 4. In vitro phosphomolybdenum reducing antioxidant activity of ascorbic acid, SR-ZnO nanoparticles, and *Stevia rebaudiana* leaf extract at different concentrations. Data are presented as mean $\pm$ SD (n = 3). Statistical significance was determined by comparing SR-ZnO nanoparticles and leaf extract groups; ns = not significant, $p < 0.05$, $p < 0.01$, $p^* < 0.001$.

In Vitro Evaluation of Antibacterial and Antifungal Activity

The activity of *Stevia rebaudiana*-mediated ZnO nanoparticles and *Stevia rebaudiana* leaf extract was assessed against *C. albicans*, *A. niger*, *E. coli*, and *S. aureus* using the agar well diffusion method. The results are in Table 4, with representative images shown in Figure 5 and Figure. 6. The SR-ZnO nanoparticles exhibited concentration-dependent antimicrobial activity against all the tested microorganisms. At 50 μ L, the zones were 9, 10, 12, and 10 mm against *C. albicans*, *A. niger*, *E. coli*, and *S. aureus*, respectively. Increasing the concentration to 100 μ L enhanced the antimicrobial activity, with

inhibition zones of 17, 14, 16, and 14 mm, respectively. Similarly, the *Stevia rebaudiana* leaf extract also exhibited antimicrobial activity, although with comparatively smaller inhibition zones. At 50 μ L, the inhibition zones measured 10, 7, 10, and 11 mm against *C. albicans*, *A. niger*, *E. coli*, and *S. aureus*, respectively, which increased to 13, 10, 14, and 14 mm at 100 μ L. The positive control produced the largest zones of inhibition at 100 μ L, measuring 27 mm against *C. albicans*, 30 mm against *A. niger*, 22 mm against *E. coli*, and 18 mm against *S. aureus*. No inhibition was detected for the negative control (distilled water), confirming the absence of antimicrobial activity.

Table 4. Zone of inhibition of SR-ZnO nanoparticles and *Stevia rebaudiana* extract against test microorganisms.

Concentration (in μ L)	<i>C. albicans</i>		<i>A. niger</i>		<i>E. coli</i>		<i>S. aureus</i>	
	ZnO NPs	Extract	ZnO NPs	Extract	ZnO NPs	Extract	ZnO NPs	Extract
50 (Sample)	9	10	10	7	12	10	10	11
100 (Sample)	17	13	14	10	16	14	14	10
100 (+ve Control)	27	—	30	—	22	—	18	—
100 (–ve Control: DW)	NZI	NZI	NZI	NZI	NZI	NZI	NZI	NZI

Note: NZI- No zone of inhibition.

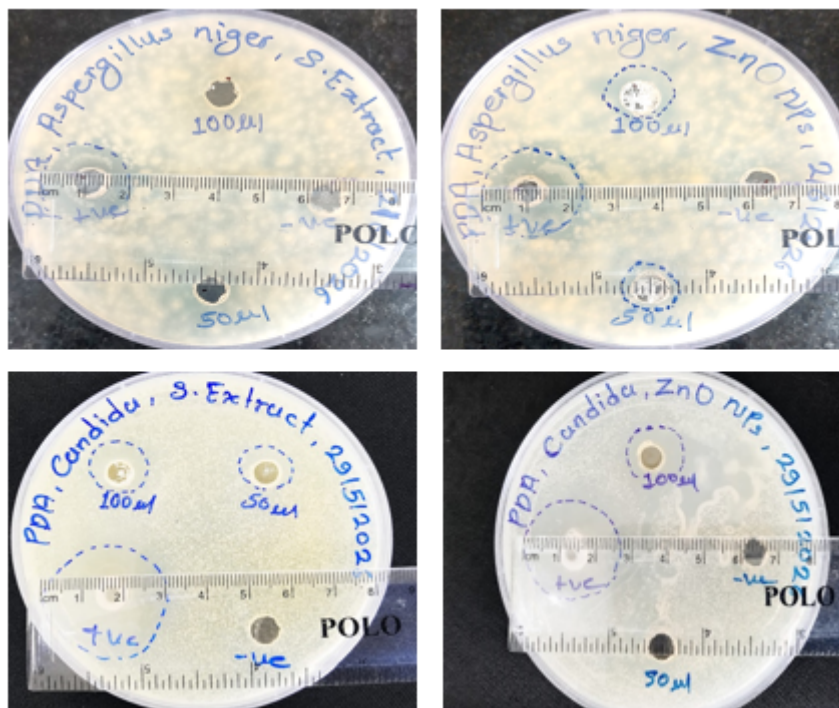


Figure 5. The antifungal activity of SR extract and SR-ZnO nanoparticles at various concentrations against *Aspergillus niger* and *Candida albicans*.

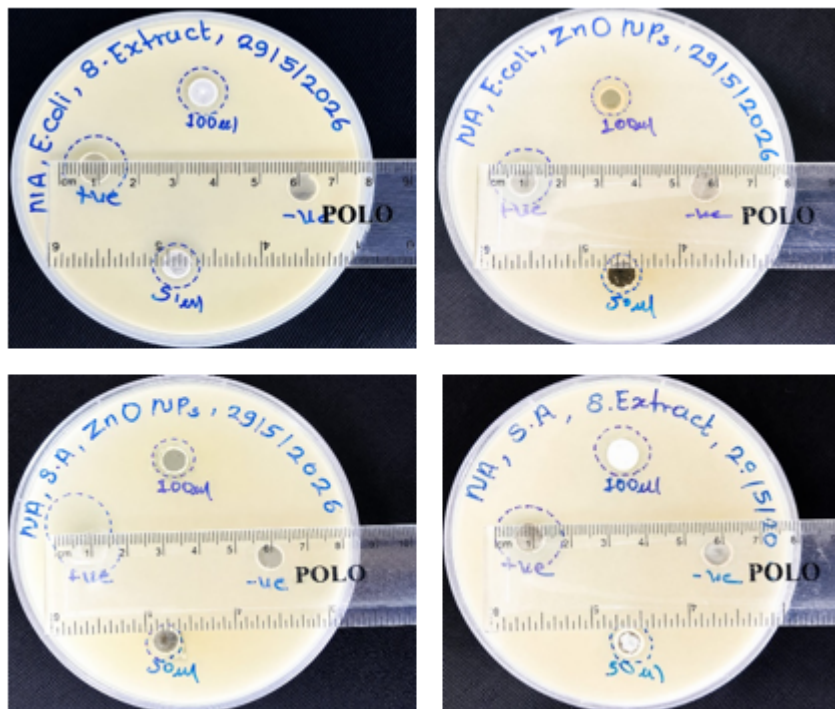


Figure 6. The Antibacterial activity of SR extract and SR-ZnO nanoparticles at various concentrations against *Escherichia coli* and *Staphylococcus aureus*.

DISCUSSION

The present study successfully demonstrated the eco-friendly synthesis of zinc oxide nanoparticles (ZnO NPs) using an aqueous leaf extract of *Stevia rebaudiana*. Green synthesis has emerged as a sustainable alternative to conventional physical and chemical methods because it eliminates the use of hazardous reagents while employing plant-derived phytochemicals as reducing, stabilizing, and capping agents. The successful formation of crystalline ZnO nanoparticles observed in the present investigation was confirmed by X-ray diffraction (XRD), transmission electron microscopy (TEM), dynamic light scattering (DLS), and energy-dispersive X-ray spectroscopy (EDX). The nanoparticles exhibited a hydrodynamic particle size ranging from 100–150 nm with predominantly spherical, oval, and hexagonal morphologies, demonstrating good structural integrity and colloidal stability. Similar physicochemical characteristics have been reported for *Stevia rebaudiana*-mediated ZnO nanoparticles and other plant-based ZnO nanostructures, confirming the reproducibility and reliability of green synthesis approaches (Elabbasy et al., 2025; Singh, 2021; Pulido et al. 2000). The enhanced biological activity of the synthesized nanoparticles can be attributed to the rich phytochemical composition of *S. rebaudiana*, which contains flavonoids, phenolic acids, diterpene glycosides, tannins, and other antioxidant metabolites. These

phytoconstituents not only facilitate nanoparticle synthesis but also remain adsorbed on the nanoparticle surface, improving stability and contributing synergistically to biological activity. Previous studies have shown that phytochemical-functionalized ZnO nanoparticles exhibit greater bioactivity than chemically synthesized nanoparticles because of improved surface reactivity and enhanced interaction with biological molecules (Elabbasy et al., 2025; Singh, 2021).

The antioxidant activity of the synthesized SR–ZnO nanoparticles was significantly greater than that of the aqueous *S. rebaudiana* extract in all three antioxidant assays, namely DPPH, ferric reducing antioxidant power (FRAP), and phosphomolybdenum (PM). In the DPPH assay, SR–ZnO nanoparticles exhibited a markedly lower IC₅₀ value than the crude extract, indicating superior free radical scavenging efficiency. This observation is consistent with previous reports demonstrating enhanced antioxidant potential of plant-mediated ZnO nanoparticles due to increased surface area, improved electron-donating capacity, and effective stabilization by bioactive phytochemicals (Islam et al., 2024; Pulido et al., 2000; Rana, M.S. et al 2024). Similarly, the higher reducing power observed in the FRAP assay reflects the improved electron transfer capability of SR–ZnO nanoparticles, supporting earlier findings that ZnO nanoparticles synthesized through green methods possess enhanced

reducing potential compared with crude plant extracts (Maind et al., 2025). Furthermore, the phosphomolybdenum assay demonstrated significantly greater total antioxidant capacity for SR-ZnO nanoparticles, indicating efficient reduction of Mo(VI) to Mo(V), which has likewise been reported for phytochemical-capped ZnO nanoparticles (Vargas Ramella et al., 2026). The statistically significant differences observed between SR-ZnO nanoparticles and the aqueous extract ($p < 0.05$) confirm that nanoparticle formation substantially enhances the antioxidant potential of *S. rebaudiana*.

The enhanced antioxidant activity may be explained by several complementary mechanisms. The nanoscale dimensions provide a substantially larger surface-to-volume ratio, increasing the availability of active sites for electron donation and radical neutralization. Moreover, surface-bound phenolics and flavonoids act synergistically with ZnO nanoparticles by facilitating hydrogen atom transfer and single-electron transfer reactions. Zinc ions themselves also contribute indirectly to antioxidant defense by participating in cellular antioxidant enzyme regulation and maintaining redox homeostasis (Singh, 2021; Pulido et al., 2000).

The synthesized SR-ZnO nanoparticles also demonstrated broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria as well as fungal pathogens, with inhibition zones increasing in a concentration-dependent manner. The antimicrobial efficacy of the nanoparticles was considerably greater than that of the aqueous extract alone, suggesting that nanoparticle synthesis enhanced the intrinsic antimicrobial properties of *S. rebaudiana*. Similar findings have been reported for green-synthesized ZnO nanoparticles prepared using medicinal plant extracts (Elabbasy et al., 2025; Ranjani et al., 2024).

Several mechanisms may explain the superior antimicrobial activity observed. ZnO nanoparticles readily adhere to microbial cell surfaces owing to electrostatic interactions, leading to membrane destabilization and increased permeability. Subsequently, intracellular accumulation of Zn^{2+} ions and excessive generation of reactive oxygen species (ROS), including hydroxyl radicals, superoxide radicals, and hydrogen peroxide, induce oxidative stress, resulting in lipid peroxidation, protein oxidation, DNA damage, enzyme inhibition, and ultimately microbial cell death. Additionally, the small particle size enables greater penetration through microbial cell walls, thereby enhancing bactericidal and fungicidal activity (Ranjani et al., 2024). The synergistic interaction between ZnO nanoparticles and phytochemical capping agents may further improve microbial inhibition by disrupting

multiple cellular targets simultaneously, thereby reducing the likelihood of resistance development.

Overall, the findings of the present study demonstrate that *Stevia rebaudiana*-mediated ZnO nanoparticles possess significantly enhanced antioxidant and antimicrobial activities compared with the corresponding aqueous leaf extract. The superior biological performance can be attributed to the combined effects of nanoscale particle size, high surface area, crystalline ZnO structure, and phytochemical surface functionalization. These characteristics suggest that SR-ZnO nanoparticles represent promising candidates for pharmaceutical, nutraceutical, antimicrobial coating, wound-healing, and biomedical applications. Nevertheless, although the present in vitro findings are encouraging, comprehensive in vivo pharmacological investigations, toxicity profiling, pharmacokinetic studies, molecular mechanism elucidation, and long-term biosafety evaluations are essential before clinical translation and therapeutic application can be recommended.

CONCLUSION

The present study successfully demonstrated the synthesis of ZnO nanoparticles using *Stevia rebaudiana* leaf extract. Characterization studies confirmed the formation of stable, nanosized SR-ZnO nanoparticles with effective phytochemical capping. The synthesized nanoparticles exhibited enhanced antioxidant activity compared with the crude extract across DPPH, FRAP, and phosphomolybdenum assays, indicating improved radical scavenging and reducing potential. In addition, SR-ZnO nanoparticles showed promising antimicrobial activity against the tested microbial strains, supporting their broad biological potential. These findings suggest that SR-ZnO nanoparticles may serve as a promising natural nanomaterial for pharmaceutical and biomedical applications; however, further in vivo studies are required to confirm their safety and therapeutic efficacy.

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Abbreviation

A. niger: *Aspergillus niger*; **BHA:** Brain Heart Agar; **CFU:** Colony-Forming Units; **DLS:** Dynamic Light Scattering; **DPPH:** 2,2-Diphenyl-1-picrylhydrazyl; **DNA:** Deoxyribonucleic Acid; **E. coli:** *Escherichia coli*; **EDX:** Energy-Dispersive X-ray Spectroscopy; **FRAP:** Ferric Reducing Antioxidant Power; **IC₅₀:** Half-Maximal Inhibitory Concentration; **MDR:** Multidrug-Resistant; **MBC:** Minimum Bactericidal Concentration; **MIC:**

Minimum Inhibitory Concentration; **PM**: Phosphomolybdenum; **ROS**: Reactive Oxygen Species; **S. aureus**: Staphylococcus aureus; **SD**: Standard Deviation; **SR**: *Stevia rebaudiana*; **SR-ZnO NPs**: *Stevia rebaudiana*-Mediated Zinc Oxide Nanoparticles; **TEM**: Transmission Electron Microscopy; **UV**: Ultraviolet; **XRD**: X-ray Diffraction; **Zn**: Zinc; **Zn²⁺**: Zinc Ion; **ZnO**: Zinc Oxide; **ZnO NPs**: Zinc Oxide Nanoparticles; **ZP**: Zeta Potential;

Conflict of Interest

The authors declare no conflict of interest.

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