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Green synthesized silver nanoparticles promote macrophage activation and antibacterial immunity

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Urgently needed new antimicrobial strategies due to antibiotic resistance. This study aimed to develop and evaluate green-synthesized silver nanoparticles (AgNPs) with A*loe vera* leaf extract as a low-cytotoxic, safe method to boost innate immunity. The nanoparticles were characterized by TEM, showing spherical particles 15–25 nm in size with a zeta potential of −28.6 mV, indicating high colloidal stability. UV–Vis analysis displayed a clear surface plasmon resonance peak at 420 nm. The murine macrophage cell line RAW 264.7 was used to assess cytocompatibility and immunostimulatory activity through nitric oxide release and cytokine (TNF-α, IL-6) production. Assays for macrophage clearance, minimum bactericidal concentration (MBC), and minimum inhibitory concentration (MIC) were conducted to evaluate antibacterial effectiveness against *Staphylococcus aureus* and *Escherichia coli*. The biosynthesized AgNPs maintained macrophage viability, increased pro-inflammatory cytokine and nitric oxide production at non-toxic levels, and exhibited strong antibacterial activity with low MIC and MBC values. Green-synthesized AgNPs can serve as effective, low-toxicity agents combining antimicrobial and immune-stimulating properties to help combat antibiotic resistance, as demonstrated by the enhanced bacterial clearance shown by treated macrophages.

Keywords: *Aloe vera* extract, biosynthesis, RAW 264.7 macrophages, nitric oxide, cytokine modulation, antibacterial activity

Aloe vera is a popular industrial and medicinal plant that is grown extensively in tropical areas, especially in China, India, and Mexico. It grows on roughly 50,000 hectares in India alone, producing 12–15 tonnes of leaves per hectare on average each year. Its bioactive ingredients, particularly polysaccharides and phenolic compounds, are being utilized more in nanotechnology, cosmetics, and pharmaceuticals[1]. According to recent reports, green-synthesized silver nanoparticles (AgNPs) derived from plants like *Aloe vera* have significant antibacterial activity and can boost immune responses[2,3].

Silver nanoparticles' wide range of antimicrobial activity against bacteria, fungi, and viruses has drawn a lot of interest in biomedical research[4,5]. However, their biological applications are limited by the high energy, toxic reagents, and hazardous byproducts that are frequently required by traditional physical and chemical synthesis routes[6]. In contrast, a low-cost, biocompatible, and environmentally friendly alternative that produces stable nanoparticles with regulated size and shape is plant-mediated synthesis employing phyto chemicals as reducing and stabilizing agents[7,8]. Their biological activity and potential for treatment are significantly influenced by structural characteristics[9].

AgNPs can alter immune responses in addition to their antimicrobial function. They engage in interactions with macrophages and promote the release of pro-inflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), as well as nitric oxide (NO), which are crucial mediators of innate immunity[10,11]. New therapeutic approaches that combine direct bactericidal action with immune activation are required in light of the global rise of multidrug-resistant pathogens such as *Staphylococcus aureus* and *Escherichia coli*[12–14]. To (i) synthesize and characterize green silver nanoparticles using *Aloe vera* extract, (ii) assess their cytocompatibility and immunostimulatory effects on RAW 264.7 macrophages through the production of nitric oxide and cytokines, and (iii) evaluate their antibacterial potential against *E. coli* and *S. aureus* using both direct and macrophage-mediated assays[15].

Materials and Methods

Plant material authentication

The agricultural geneticist Ammar Majeed Chaloop, who works at Debbane Modern Agriculture Company (Iraq), scientifically verified the *Aloe vera* (common name: Aloe) plant used in this study. In Karbala, Iraq, local farms provided fresh, healthy leaves, which were promptly cleaned and used to prepare an aqueous extract.

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Green synthesis of AgNPs

The *Aloe vera* (common name: Aloe) leaves used were fresh ones gathered in Karbala province, Iraq. A water extract of leaves was obtained and used as a reducing and stabilizing agent of silver nitrate ($AgNO_3$). Because of its abundance of phytochemicals (flavonoids, phenolic compounds, ascorbic acid, and polysaccharides) that promote the bio-reduction of silver ions and offer robust capping for nanoparticle stability, *Aloe vera* was selected as the reducing and stabilizing agent. Compared to many other plant extracts, *Aloe vera* is a great option for green synthesis because it is non-toxic, readily available, and environmentally friendly. To confirm the role of the *Aloe vera* extract, a control reaction was performed using 1 mM $AgNO_3$ mixed with distilled water only, under identical conditions. No visible color change was detected, indicating the absence of nanoparticle formation in the absence of the plant extract.

Green synthesis of silver nanoparticles (AgNPs)

The aqueous extract of *Aloe vera* leaves served as the stabilizing and reducing agent for the environmentally friendly synthesis of AgNPs. In short, 90 mL of 1 mM $AgNO_3$ solution was mixed thoroughly with 10 mL of freshly made *Aloe vera* extract, and the mixture was then left at 60 °C for 30 minutes until the solution turned brown, signifying the formation of silver nanoparticles. Instead of using the plant extract ($AgNO_3$ + distilled water), a control reaction was conducted under the same conditions using 1 mM $AgNO_3$ mixed with distilled water to verify the role of the *Aloe vera* extract. This control showed no discernible color change, indicating that the extract was charged with the Ag^+ ion reduction and nanoparticle formation. To confirm that no nanoparticle formation took place in the absence of both extract and silver ions, additional blank control tubes filled solely with distilled water were also made.

Nanoparticle characterization

The biosynthesized AgNPs are described by a surface plasmon resonance (SPR) band of about 420 nm, which was verified by UV-Vis spectra. Transmission electron microscopy (TEM) was used to give information about the morphology of the particles and core size distribution. Hydrodynamic diameter and polydispersivity index (PDI) were measured by using dynamic light scattering (DLS), and zeta potential was used to determine surface charge and colloidal stability. These have been used extensively to measure the size, shape, and stability of green-synthesized nanomaterials.

Cytocompatibility (Viability)

Murine macrophages (RAW 264.7) cells were grown in standard conditions and planted in a uniform number in a series of plates. Cells were subsequently incubated using the graded concentrations of biosynthesized AgNPs (0, 5, 10, 15, and 20 µg/mL), vehicle controls, and a positive control of cytotoxicity (doxorubicin, 10 µg/mL). The colorimetric MTT assay was used as a surrogate viability measure of cell metabolic activity during the exposure period. Percent viability was determined using spectrophotometers by dividing the formazan signal by the vehicle control. To directly compare the antibacterial activity of biosynthesized AgNPs, Polymyxin-B was employed as a positive control due to its well-established membrane-permeabilizing and bactericidal effects on Gram-negative bacteria. These conditions were parallel to polymyxin-B; these conditions were also added in case of possible endotoxin interference. The pre-determined non-toxic working range of the downstream immunological assays was pre-determined concentrations that would retain viability of the results ≥80-90% to control. All experiments had biological and technical replicates, were presented form of mean and standard deviation, and were analyzed as mentioned in the section on statistics.

Macrophage activation

Non-cytotoxic levels of green-synthesized AgNPs were used in RAW264.7 macrophages in accordance with the determination in the viability test. The production of nitric oxide (NO) was detected in the culture supernatants by the Griess reaction [16], and the production of cytokine interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) was measured using the ELISA kits according to the instructions. These experiments only contained non-toxic levels. Biological triplicates are done, and the results give the mean SD of NO and cytokine levels in relation to the untreated controls.

Antibacterial assays

AgNPs were assessed as antibacterial agents against the gram-negative bacterium, *Escherichia coli*, and the gram-positive bacterium, *Staphylococcus aureus*. Broth microdilution was used to establish minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using CLSI guidelines (12). Time-kill kinetics were conducted whereby bacterial suspensions were exposed to 0.5×, 1×, and 2× of MIC, and CFU counts were determined with time[17]. Besides, bacterial clearance assays through macrophages were performed where RAW264.7 cells were infected with bacteria at a specified multiplicity of infection (MOI) and then subjected to AgNPs at a non-toxic concentration. Following the intracellular CFU lysis and plating. Independent cell cultures or inoculation of bacteria were also conducted in each experiment using three biological replicates (independent cell cultures) and three technical replicates (parallel measurements of a sample) to guarantee the reproducibility and statistical accuracy.

Statistical analysis

All experiments were carried out in 3 (biological) and 3 (technical) replicates unless otherwise mentioned. Data are displayed in the form of mean and standard deviation (SD). One-way analysis of variance (ANOVA) was used to determine statistical significance, and then the Tukey test of multiple comparisons was used. A p-value of below 0.05 was deemed to be statistically significant. All the analyses and graphical representations were done using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA).

Results

Green synthesis of AgNPs

The addition of silver nitrate to the *Aloe vera* plant extract observed the occurrence of a visible change, as the yellowish color changed to brown, which indicated that the Ag^{+} was reduced to Ag^{0} nanoparticles. Neither extract nor silver ions were used in control tubes, and no color shift was observed (Fig. 1). UV Vis spectral analysis revealed an article of surface plasmon resonance (SPR) around 420 nm that is typical of nanoscale silver formation [18]. The characteristic SPR was not observed in control preparations with no plant extract or silver ions, which indicated that the synthesis of nanoparticles required both the contents. The measured SPR peak of 420 nm is the size range of 10 to 30 nm in the report of AgNPs prepared by a green method[19,20].

Nanoparticle characterization

UV-Vis spectrum

The AgNPs green-synthesized had a clear surface plasmon resonance (SPR) band of 420 nm (Fig. 2), which is associated with the creation of nanoscale silver particles.

Transmission electron microscopy

Showed predominantly spherical particles with an average diameter of 15-25 nm (Fig. 3A).

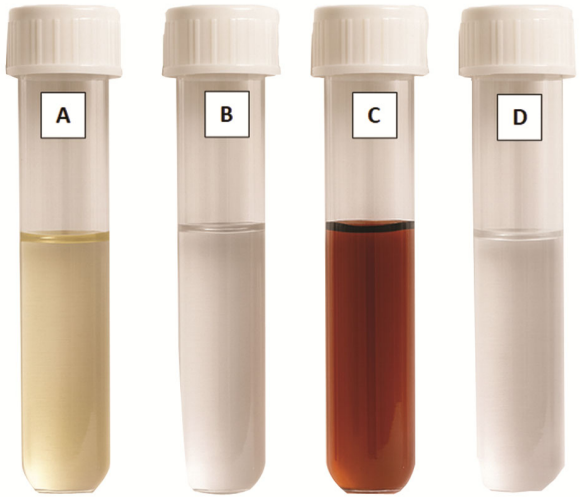

Fig. 1 — Optical monitoring of biosynthesis of silver nanoparticles. (A) Aqueous leaf extract; (B) Silver nitrate solution ($AgNO_3$); (C) Brown colloid, which indicates the formation of green-synthesized silver nanoparticles; (D) Distilled water control. Nanoparticle formation is confirmed by the change of color from light yellow to dark brown.

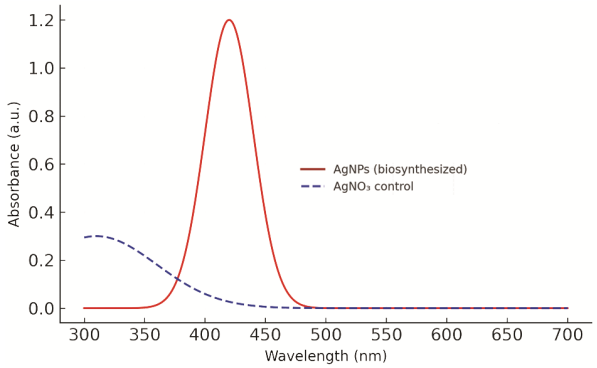

Fig. 2 — UV-Vis absorption spectrum of green-synthesized silver nanoparticles (AgNPs) with a resonant surface plasmon resonance (SPR) band at 420 nm.

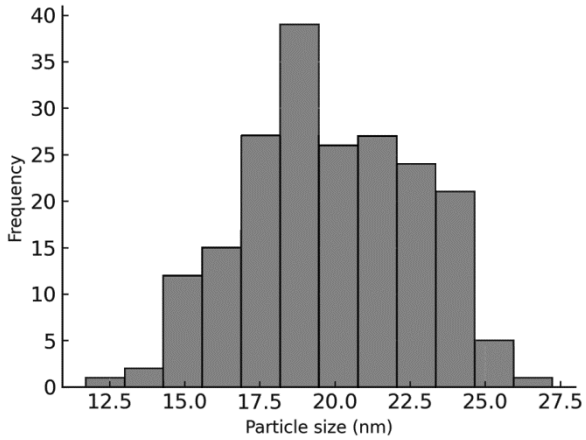

Fig. 3 — TEM observation of biosynthesized silver nanoparticles (AgNPs) showing spherical morphology with particle size ranging from 15 to 25 nm, and observation of biosynthesized AgNPs was used to obtain a histogram of particle size distribution (15-25 nm) schematic graph of the spherical silver nanoparticles (AgNPs) to show their morphology.

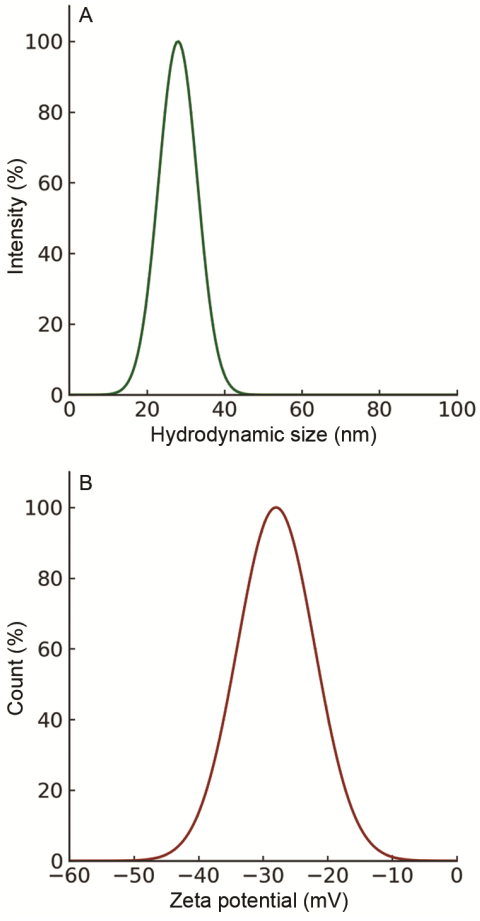

Fig. 4 — (A) Dynamic light scattering (DLS) profile of AgNPs with a mean hydrodynamic diameter of 28.4 ± 3.6 nm and polydispersity index (PDI) of 0.21. (B) Zeta potential distribution curve of AgNPs, which shows only one peak at -28.6mV, indicating colloidal stability.

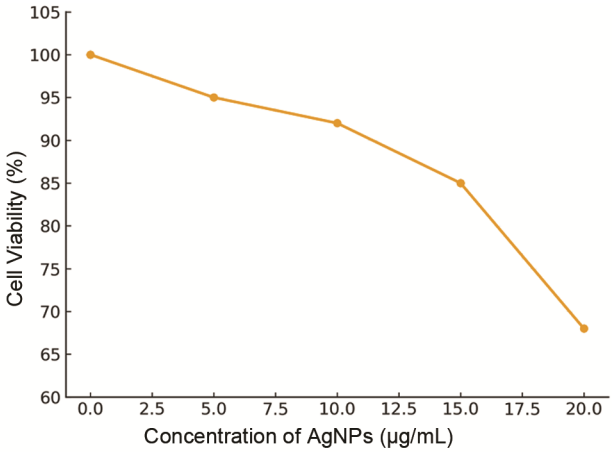

Fig. 5 — Effect of biosynthesized silver nanoparticles (AgNPs) on the viability of RAW264.7 macrophages. Cells were treated with increasing concentrations of AgNPs (0, 5, 10, 15, and 20 µg/mL) for 24 h. Cell viability remained above 90% at ≤10 µg/mL, indicating minimal cytotoxicity, whereas higher concentrations (≥15 µg/mL) led to a dose-dependent decrease in viability, with a marked reduction at 20 µg/mL. Data are presented as a percentage of control.

The DLS analysis

Revealed a hydrodynamic diameter of 28.4 ± 3.6 nm and a polydispersity index (PDI) of 0.21, a narrow size distribution, and good monodispersity (Fig. 4A). Zeta potential analysis showed that there was only one peak at -28.6 mV, which was evidence of an electrostatic stabilization and high colloidal stability of the suspension (Fig. 4B).

Cytocompatibility (Viability)

The viability of RAW264.7 macrophages treated with biosynthesized AgNPs was found to have a definite dose-dependent effect, as shown in Fig. 5. Cells at the control (0 µg/mL) were in normal morphology and metabolism. No significant viability reduction was observed at 5 µg/mL and 10 µg/mL of treatment, where the values were always more than 90. At 15 µg/mL, a small decrease in viability (the form of about 85% viability) was observed. Nevertheless, cell viability significantly reduced (below 70 percent) at 20 µg/mL.

Macrophage activation

Biosynthesized AgNPs at concentrations of ≤10 µg/mL increased nitric oxide (NO) release in the RAW264.7 macrophages significantly over untreated controls (Table 1). It was also coupled with dose-dependent elevation of pro-inflammatory cytokines IL-6 and TNF-α (Fig. 6).

Fig. 7 shows that biosynthesized AgNPs significantly raised macrophage activation markers in

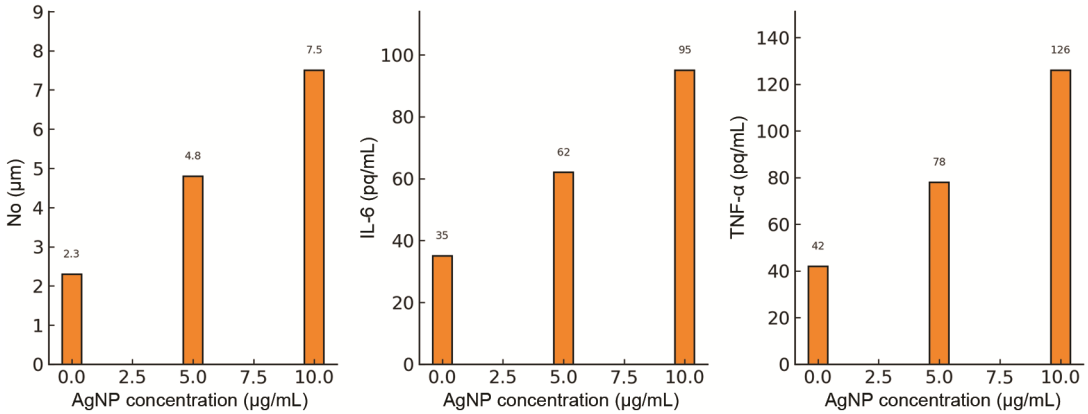

Fig. 6 — Biosynthesized AgNPs have immunostimulatory effects on RAW264.7 macrophages. Nitric oxide was added to water and reacted using the Griess assay. ELISA Cytokine secretion (IL-6, TNF-α). Data are reported in terms of mean SD of three experiments; $p < 0.05$ compared to control.

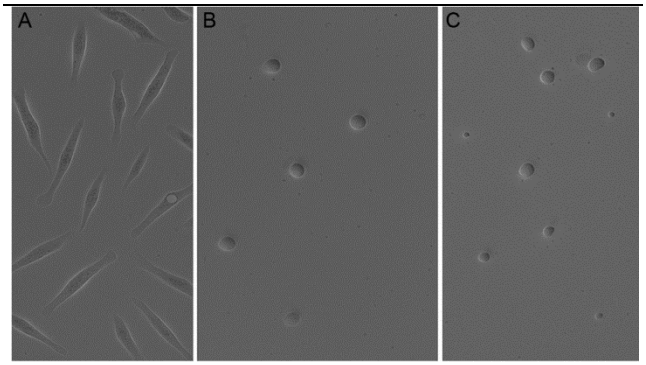

Fig. 7 — Biosynthesized silver nanoparticles (AgNPs) and their dose-dependent immunostimulatory effects on RAW 264.7 macrophages. (A) Production of nitric oxide (NO). (B) Secretion of interleukin-6 (IL-6) and (C) Secretion of tumor necrosis factor-alpha (TNF-α).

Table 1 — AgNPs influence NO and cytokines production in RAW264.7 macrophages.

AgNP (mcg/mL)	NO (mcM)	IL-6 (pg/mL)	TNF-α (pg/mL)
0 (Control)	2.3 ± 0.4	35 ± 6	42 ± 7
5	4.8 ± 0.6	62 ± 9	78 ± 10
10	7.5 ± 0.8	95 ± 11	126 ± 14

Values are mean ± standard deviation (n = 3). NO: nitric oxide; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α.

a dose-dependent manner. As AgNP concentrations rose, nitric oxide (NO) levels progressively rose relative to the untreated control (Fig. 7A). Similarly, pro-inflammatory cytokines TNF-α and IL-6 were secreted in a concentration-dependent manner by RAW264.7 macrophages subjected to ≤10 µg/mL of AgNPs (Fig. 7B and 7C). Every measured parameter showed statistically significant increases ($P < 0.05$) when compared to the control group.

Table 2 — The antimicrobial effect of AgNPs biosynthesized against E. coli and S. aureus.

Strain of bacteria	MIC (µg/mL)	MBC (µg/mL)
E. coli	4	8
S. aureus	6	8
AgNO3 control	128	256

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration.

Antibacterial activity

AgNPs synthesized using biosynthesis showed a strong antibacterial effect against *Escherichia coli* (Gram-negative) and against *Staphylococcus aureus* (Gram-positive). The lowest possible concentrations of the inhibitor were defined as the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), which corresponded to the 4-8 µg/mL range (Table 2).

Fig. 8 shows the biosynthesized AgNPs' antibacterial activity in both extracellular and intracellular models. When exposed to 2× MIC doses in the time-kill experiment, colony-forming units (CFUs) for both *S. aureus* and *E. coli* dropped by more than 90% in 6 hours (Fig. 8A). In the macrophage infection experiment, treatment with biosynthesized AgNPs dramatically decreased intracellular bacterial counts when compared to the infected untreated control group (Fig. 8B). Each CFU decline was statistically significant ($P < 0.05$).

Discussion

Physicochemical properties

The present study demonstrates that the silver nanoparticles (AgNPs) produced using the green technology possess a unique combination of

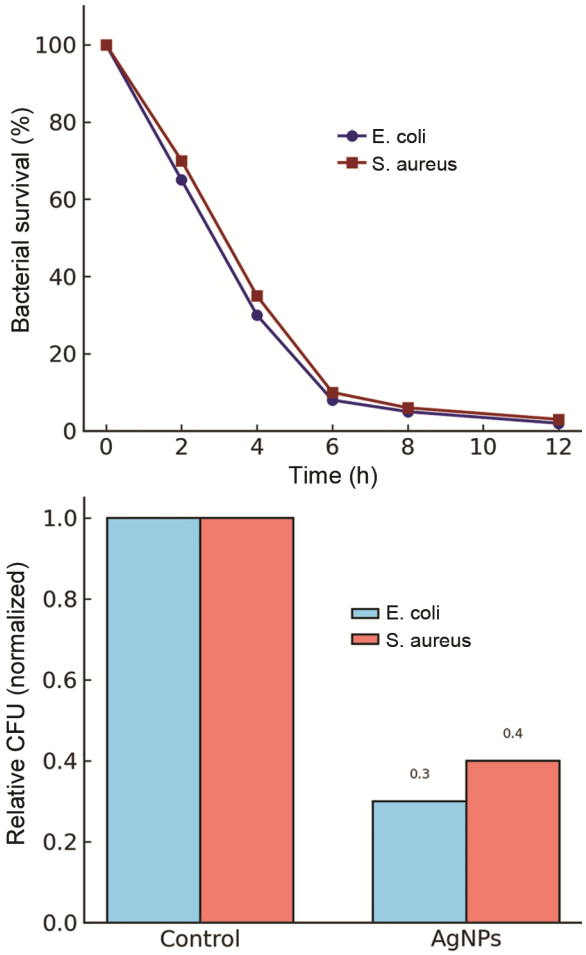

Fig. 8 — The silver nanoparticles (AgNPs) biosynthesized showed high antibacterial activity. (A) Time-kill kinetics of *E. coli* and *S. aureus* at 2x MIC, which reveal a fast bacterial clearance in 6 h. (B) AgNPs on intracellular bacterial survival of a macrophage infection assay, in terms of colony-forming units (CFUs). Data is the mean ± SD; $p < 0.05$ compared to control.

physicochemical, immunological, and antimicrobial properties. The spectroscopic and microscopic analysis of AgNPs produced by plants demonstrated that stable, uniformly dispersed AgNPs in a spherical form with a favourable zeta potential could be developed, as was once reported 36. The significance of these properties lies in the fact that the size of particles and the surface charge directly impact biological interactions and therapeutic potential. A small, symmetric absorption band means that there is a fairly uniform distribution of particle sizes and a stable colloid formation [21,22].

The phytochemical-assisted procedure also provides enhanced biocompatibility and eliminates toxic reducing agents compared to the chemically synthesized nanoparticles [22]. In general, these findings indicate that the chosen plant extract was successful in mediating the reduction and stabilization of silver nanoparticles, and generated stable AgNPs with optical characteristics associated with green-synthesized nanomaterials. This is the basis of further characterization, cytocompatibility study, and immunomodulatory-antibacterial activity assessment.

This confirmed the achievement of biosynthesis of stable and well-distributed AgNPs with physic chemical properties that can be used in biomedical applications. The determined value is consistent with previous studies, which report that plant-mediated AgNPs exhibit SPR peaks of 410–430 nm, a spherical morphology, and colloidal stability with a zeta potential of less than –25 mV [23–26].

Cytocompatibility

AgNPs are safe at concentrations below 10 µg/mL, according to cytocompatibility tests, which offers a biological insight that needs more research. This finding is consistent with earlier research that, while higher concentrations of AgNPs damage cell integrity through oxidative stress and apoptosis, lower concentrations maintain macrophage viability [27]. Our results highlight the necessity of carefully adjusting the dose of nanoparticles to produce positive effects without causing cytotoxicity.

It was discovered that 5–10 µg/mL was the ideal concentration range for biosynthesized AgNPs to produce effective immunostimulation without cytotoxicity. While retaining over 85% viability, macrophages within this range showed strong activation markers like increased nitric oxide and cytokine secretion. At concentrations above 15 µg/mL, partial cell shrinkage and decreased metabolic activity were noted, indicating the upper safety threshold for macrophage activation. The cytocompatibility profile indicates that biosynthesized AgNPs are safe in the low dose ranges and have a reliable foundation to be used in determining their immunostimulative and antibacterial effects [28].

Immunomodulation

The levels of NO, IL-6, and TNF-α in the macrophages were markedly increased by the immunostimulation activity of biosynthesized AgNPs. These mediators are necessary for both innate and adaptive immune responses to eradicate inflammation and bacteria. These immune-stimulating activities demonstrated the antimicrobial and immunomodulatory characteristics of AgNPs. An

increase in TNF-α and IL-6 is observed at low and moderate AgNP concentrations (≤10 µg/mL), which is suggestive of beneficial immunostimulation rather than harmful inflammation. Both macrophage activation and innate immune defense depend heavily on these cytokines. Increased NO and cytokine production, which supports the growth of innate and adaptive immune responses, are hallmarks of classical macrophage activation. While IL-6 and TNF-α coordinate the inflammatory response and draw in additional immune effector cells, high NO is the primary mediator of antimicrobial action, directly damaging pathogenic microorganisms[29,30].

These findings are consistent with earlier research showing that silver nanoparticles activate macrophages through pattern-recognition receptor signaling, which in turn activates NF-kB and cytokine release [31]. Similar cytokine-stimulating behavior has been demonstrated by plant-derived AgNPs [32,33], indicating that controlled elevation of TNF-α and IL-6 enhances macrophage-mediated immune defense without causing cytotoxicity.

RAW 264.7 cells retain essential macrophage traits, including the capacity to generate nitric oxide and release pro-inflammatory cytokines such as TNF-α and IL-6, making them suitable for assessing immunomodulatory effects *in vitro*. Cell viability above 85% at these doses indicates that AgNPs induce a balanced immune activation within a non-toxic range. Only at higher concentrations was a slight reduction in viability observed, highlighting the significance of appropriate dosage to optimize immunostimulatory effects while avoiding excessive inflammation [34-37].

AgNPs interact with toll-like receptors (TLRs) on macrophage membranes and other pattern-recognition receptors to activate the NF-κB and MAPK signaling pathways downstream. This cascade maintains homeostasis within a non-toxic threshold while increasing the transcription of pro-inflammatory mediators like TNF-α and IL-6. Thus, AgNPs can alter innate responses without causing excessive oxidative stress, acting as mild immune adjuvants [38].

4.4 Antibacterial activity

Time-kill assays showed that bacteria were cleared quickly, and more than 90% viable count reduction was found after 6 h at the concentrations of 2 × MIC (Fig. 7A). Infective microphage tests revealed that AgNP treatment drastically decreased the number of CFUs in the inner cells compared to the infected controls (Fig. 7B). This suggests that AgNPs stimulate the host innate immune response by boosting macrophages' bactericidal potential in addition to their direct antimicrobial effects. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), which ranged from 4 to 8 µg/mL, were the lowest concentrations of the inhibitor (Table 2). The superior antibacterial ability of the biosynthesized AgNPs was demonstrated by the fact that the $AgNO_3$ control needed much higher concentrations (MIC = 128 µg/mL; MBC = 256 µg/mL) to achieve comparable inhibition. There was also good activity of *E. coli* and *S. aureus* in the antibacterial assays, with MIC and MBC values in the low µg/mL range, and time-kill kinetics indicating high bactericidal activity. Moreover, AgNPs optimized the destruction of bacteria carried out by macrophages, demonstrating their potential in combination with host immunity. These results align with previous research indicating that AgNPs disrupt bacterial membranes and improve the activities of immune effectors [39].

Implications and future work

They may be useful adjuncts to conventional antibiotics due to their dual bactericidal and immune-stimulating properties. Such cooperation may lessen the need for antibiotic dosages and delay the emergence of resistance. Together, this dual-action, AgNPs possess direct bacterial inactivation and immune-potentiating effects, making them worthy of consideration in infectious disease treatment, particularly in the context of rising antimicrobial resistance. The molecular pathways of macrophage activation in the presence of AgNPs and the efficacy and safety of AgNPs *in vivo* investigation. Our results are consistent with recent studies demonstrating that plant-based AgNPs can induce regulated macrophage activation while preserving cytocompatibility [40].

AgNPs made from *Aloe vera* have potential as next-generation nano-immunotherapeutic candidates, according to the current research. Their small size, negative surface charge, and phytochemical capping support their stability and biocompatibility; their ability to suppress bacterial growth and stimulate macrophage cytokine release demonstrates their dual therapeutic mechanism. Facilitate translational applications in immune regulation and infection control, future *in vivo* research should examine biodistribution, pharmacokinetics, and long-term immunological safety.

Conclusion

In this work, silver nanoparticles (AgNPs) were green-synthesised using *Aloe vera* leaf extract and were obtained as stable, spherical, and uniformly dispersed particles with good colloidal properties. The biosynthesised AgNPs were non-toxic to macrophages at concentrations up to 10 µg/mL, where the cells remained metabolically active and viable. The nanoparticles improved macrophage activation within this safe range, resulting in higher secretion of the pro-inflammatory cytokines TNF-α and IL-6 as well as increased nitric oxide release. The AgNPs demonstrated robust antibacterial activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria in addition to their immunostimulatory effect. They also enhanced bacterial clearance in models of macrophage infection. Together, these results demonstrate that the biosynthesized AgNPs have both immunomodulatory and antimicrobial capabilities. Their potential as supplemental or alternative therapeutic tools for infection control and host immune defense enhancement is supported by their biogenic origin, safety, and dual mechanism.

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Conflict of Interest

The authors state that they have no conflict of interest as far as the publication of this article is concerned.

Funding Statement

No particular grant was provided by funding agencies, either in the public, commercial, or not-for-profit sectors, to support this research.

Ethical Approval Statement

The experiment was conducted with the help of a murine macrophage cell line, RAW264.7 that was acquired in a certified cell bank (ATCC, USA). The guidelines of the international standards of ethical guidelines in the study of cell culture *in vitro* were adhered to. There were no experiments with humans and living animals.

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