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Green synthesis and characterization of silver nanoparticles using leaf extract of *Aegle marmelos* (L.) and their antibacterial, antioxidant and anti-inflammatory activities

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Abstract

The present research comprises of green synthesis of silver nanoparticles (AgNPs) by *Aegle marmelos* leaf extracts their characterization and biological applications such as antimicrobial, antioxidant and anti-inflammatory activity. The synthesized AgNPs were characterized using UV–Vis spectroscopy, FTIR, SEM and XRD, which collectively confirmed the successful formation of stable and crystalline nanoparticles with defined particle sizes. The UV–Vis analysis showed a surface plasmon resonance (SPR) peak centered at 405 nm, indicating the presence of AgNPs. The FTIR spectrum revealed distinct absorption bands at 660.2 cm^{-1}, 639.83 cm^{-1}, 620.23 cm^{-1} and 605.47 cm^{-1} suggesting the involvement of functional groups in stabilization. The scanning electron microscope analysis demonstrated that the nanoparticles had an irregular morphology, with a broad size distribution and noticeable aggregation. Particle size analysis from selected regions indicates sizes of 93.5 nm, 136 nm, 178 nm, and 187 nm, yielding an average particle size of approximately 148.1 nm. Furthermore, XRD analysis exhibited five prominent diffraction peaks at 27.82°, 32.10°, 38.10°, 41.15°, 44.10°, 46.13°, 64.39°, and 77.32°, which correspond to the (111), (210), (122), (200), (231), (220), and (311) planes of face-centered cubic silver, confirming their crystalline structure. Bioactivity assays revealed that AgNPs from *A. marmelos* exhibited significant antimicrobial, antioxidant and anti-inflammatory activities. Zone of inhibition increases with concentrations 25- 200µg/ml. The maximum zone of inhibition 15 mm for *B. subtilis* and 17 mm for *E. mori* was observed at 200µg/ml. The minimum inhibitory concentration (MIC) assay of *A. marmelos* AgNPs demonstrated a dose dependent antimicrobial effect; the lowest concentration of 5 µg/ml was inhibition of 8.54% whereas a substantial inhibitory effect of 63.41% was recorded at the highest concentration of 160 µg/ml. The antioxidant activity of *A. marmelos* - derived AgNPs was evaluated using the DPPH radical scavenging assay, and concentration dependent increase in radical scavenging activity, with inhibition ranging from 11.5% at 25µg/ml to 46.9% at 200µg/ml. The anti-inflammatory activity of *A. marmelos* derived AgNPs increased in a concentration dependent manner with inhibition ranging from 14.45% at 25 µg/ml to 38.55% at 200 µg/ml. Based on our studies, further identification of the major compounds of leaf extracts is acceptable.

Keywords: *Aegle Marmelos;* Silver Nanoparticles; Antibacterial; Antioxidant, Minimum Inhibitory Concentration; Anti-Inflammatory

1. Introduction

Aegle marmelos is extensively described in the Vedic literature for the treatment of various diseases like jaundice, constipation, dysentery, stomachache, acute bronchitis chronic diarrhea stomachic, fever, asthma, inflammations, febrile delirium, snakebite, abdominal discomfort, acidity, burning sensation, smallpox, epilepsy, indigestion, leprosy, myalgia, spermatorrhoea, leucoderma, eye disorders, ulcers, mental illnesses, nausea, sores, swelling, thirst, thyroid disorders, tumors, ulcers and upper respiratory tract infections [1- 3]. *Aegle marmelos* belongs to the *Rutaceae* family

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and is widely used in India's traditional Ayurvedic system [4, 3]. It is distributed in the deciduous forests of India. *Aegle marmelos*, commonly known as bael, is a medicinally important plant and its leaves are particularly rich in diverse phytoconstituents with therapeutic potential. The therapeutic value of the plant has been referred to by almost all the ancient ayurvedic treatise like Siddha, Unani, Sushruta Samhita and Charaka Samhita, etc. [5, 4]. Nowadays, most drugs are obtained from natural sources or semi-synthetic derivatives of natural products, which are mostly used in traditional systems of medicine [6]. The *A. marmelos* is one of the primary medicinal and nutritious plants. Practically all parts of the plant are utilized therapeutically, valued for their multifaceted healing properties. Studies have shown that bael helps regulate blood glucose levels and supports digestive health [7-10].

It has also been traditionally employed in treating different diseases as it contains a rich matrix of phytochemicals, including tannins, essential oils, glycosides, polyphenols, alkaloids, terpenoids, phenylpropanoids, and coumarins [4, 11]. The recent research on crude extracts of different parts of *A. marmelos* shows that it has many broad therapeutic properties such as anti-inflammatory, antipyretic, antidiarrhoeal, antidiabetic, analgesic, antimicrobial, hepatoprotective, gastroprotective, anti-ulcerative, cardioprotective, antioxidative, radioprotective, anticancer, and antiviral activities [3, 4, 12-21]. More than 100 bioactive compounds have been identified and documented in *A. marmelos*, and the major ones include alkaloids, coumarins, terpenoids, flavonoids, phenolic acids, polyphenols, carotenoids, organic acids, fatty acids and amino acids [3, 17, 20]. Some of the key phytochemicals found in *A. marmelos* including glycosides, flavonoids have been identified in the fruit and leaves of *A. marmelos* and some of these compounds have been shown to have antinociceptive and anti-inflammatory activities [20, 22]. The leaves of *A. marmelos* contain high levels of tannins, which have been shown to have strong antioxidant and anti-inflammatory activities. Flavonoids are a group of compounds that are widely distributed in the plant kingdom and are known for their anti-inflammatory, anticancer, and antioxidant activities [8, 23].

Concurrently, the efficacy of conventional antibiotics is rapidly diminishing due to the widespread development and dissemination of antimicrobial resistance (AMR), rendering common bacterial and fungal infections increasingly difficult to treat [24]. In response to these demanding health challenges, nanotechnology has emerged as a transformative field, offering promising avenues for the development of advanced therapeutic agents [25]. In recent decades, increasing attention has been directed towards nanotechnology as a promising field for developing nanomaterials and nanoparticles for applications in catalysis, electrochemistry, medicine, sensors, food technology, and cosmetics [26, 27]. Nanotechnology encompasses the synthesis and application of nanomaterials within the 1–100 nm size range [28, 29]. It plays a crucial role in producing various biologically active, nanoscale drugs using nanoparticles based on metals or alloys, which exhibit novel optical, chemical, and physical properties [30, 31]. Several metals such as silver, copper, gold, palladium, nickel, cobalt can be synthesized in the form of nanoparticles; however, AgNPs have gained significant attention. Among the widely studied nanomaterials, AgNPs stand out due to their versatile applications in fields due to their well-documented broad-spectrum antimicrobial, antioxidant, and anticancer properties, making them a compelling candidate for addressing drug resistance and malignant proliferation [32-34]. Their unique physicochemical properties, including high surface area-to-volume ratio and ability to interact with biological molecules, contribute to their diverse bioactivity [35].

Nanoparticle synthesis methods are broadly categorized into chemical, physical, and biological, each with their own advantages and disadvantages [36]. Physical methods (mostly top-down approaches), typically do not involve chemical reagents or solvents, resulting in cleaner nanoparticles with high purity [37-39]. However, they often require specialized equipment and high energy consumption. Chemical methods, by far the most used, offer good control, scalability and many functionalization options, but usually require the use of toxic or environmentally hazardous reagents and solvents [40-43]. These conventional approaches raise significant concerns regarding environmental toxicity, the generation of hazardous by-products, and potential health risks associated with residual chemicals in the final product [44, 45]. However, traditional methods for synthesizing AgNPs, such as chemical reduction using sodium borohydride or hydrazine, often involve harsh chemicals, elevated temperatures, and high pressures [29, 46]. To circumvent the aforementioned drawbacks of conventional nanoparticle synthesis, the innovative concept of green synthesis has gained substantial traction [47].

Consequently, there has been increasing interest in developing alternative synthesis methods that are more efficient, cost-effective, and environmentally sustainable, such as the biosynthesis of AgNPs [48, 49]. Biological methods (biosynthesis), which use complex extracts from plants or supernatants from microorganisms, require less energy and avoid hazardous chemicals, reducing their environmental and economic impact, though they require more laborious isolation protocols [50, 51]. This eco-friendly approach leverages natural biological resources, primarily plant extracts, microorganisms, or biomolecules, to reduce metal ions into nanoparticles, thereby eliminating the need for toxic chemicals and reducing energy consumption [29, 52]. Biosynthesis utilizes plant-derived secondary metabolites that function as both reducing and stabilizing agents during nanoparticle formation [53, 54]. Plant-derived bioactive

compounds such as alkaloids, saponins, flavonoids, carbohydrates, terpenoids, proteins, and glycosides play an important role in reducing silver ions (Ag^{+}) into metallic silver nanoparticles (Ag^{0}) and stabilizing the formed nanoparticles [55, 56]. The characteristics of the synthesized nanoparticles are influenced by factors such as physical conditions, adsorption of stabilizing agents, and the interaction rate between metal ions and reducing compounds. The presence of strong reducing agents in plant extracts can accelerate reaction rates and facilitate the formation of smaller nanoparticles [26, 57]. In the present study, an eco-friendly approach was developed for synthesizing silver nanoparticles (AgNPs) using aqueous leaf extract of *Aegle marmelos*. The biosynthesized AgNPs were characterized using UV–Vis spectroscopy, FTIR, XRD, and SEM to determine their physicochemical properties and functional properties. Also we evaluated the biomedical potential of the synthesized AgNPs by evaluation of their antibacterial, antioxidant and anti-inflammatory potencies.

2. Materials and Methods

2.1. Collection and preparation of plant extract

Fresh leaves of *A. marmelos* were collected from Jnanabharathi Campus, Bengaluru. Plant authentication was performed by the Central Ayurveda Research Institute (Ministry of AYUSH, Govt. of India), affiliated with the Central Council for Ayurvedic Science, Bengaluru.

For further study, disease free leaves were carefully harvested and initially cleaned by rinsing under running tap water for 2–3 times, followed by double distilled water to remove surface impurities. The cleaned leaves were then oven dried at 60 °C for 24 h and ground into a fine powder using an electric blender [7]. Aqueous extraction was prepared by mixing 10 g of leaf powder with 100 ml of water and subjecting the mixture to reflux at 70 °C for 2 h with continuous stirring, to maximize solubilization of bioactive compounds. Following extraction, the mixture was filtered using Whatman No. 1 filter paper to obtain a clear filtrate, which was collected in a clean beaker [58].

2.2. Qualitative phytochemical analysis

The aqueous leaf extracts were subjected to preliminary phytochemical screening following standard methods [59]. Qualitative phytochemical analyses of both the extracts were performed by following the protocol of Adetuyi and Popoola [60], Trease and Evans [61], and Sofowora [62].

Test for phenols: (Ferric chloride test): The 2 ml of 5% solution of $FeCl_3$ was added to 1 ml plant leaf extracts. A black color of the reaction mixture was developed which confirms the presence of phenol.

Test for Alkaloids: (Mayer's reagent test): The 3 ml of plant leaf extract was added to 3 ml of 1% HCl solution and it was heated for 20 min on a water bath. The resulting reaction mixture was cooled at room temperature and used to perform Mayer's test. To the cooled reaction mixture in a test tube, 1 ml of Mayer's reagent was added drop by drop. The formation of a greenish colored cream precipitate indicated the presence of alkaloids.

Test for Flavonoids: (Alkaline reagent test): The 3 ml of plant leaf extract were treated with 1 ml of 10% NaOH solution. The formation of an intense yellow color confirms the presence of flavonoids.

Test for anthraquinones: (Bontrager's test): The 0.2 ml of plant leaf extract, 5 ml of chloroform and 5 ml of ammonia solution was added. The presence of bright pink color in the aqueous layer indicated the presence of anthraquinones.

Test for terpenoids and steroids: (Salkowski test): The 5 ml of plant leaf extract solution were mixed in 2 ml of chloroform, and 3 ml of concentrated sulfuric acid to form a layer. The reddish-brown coloration of the interface was formed to indicate the presence of terpenoids. Red color at the lower surface indicates the presence of steroid.

Test for tannins: (a) Ferric chloride test: The 0.5 ml plant leaves extract solution, 1 ml distilled water and 2 drops of ferric chloride solution was added to it and blue-black coloration was observed which indicates the presence of tannins.

(b) Lead acetate test: The 10 % lead acetate solution was added to 0.5 ml of plant leaf extract solution and white precipitation was observed which confirms the presence of tannins.

Test for saponins: (Frothing test): The 0.2 ml of the plant leaf extracts was mixed with 5 ml of distilled water and then heated to boil. The frothing shows the presence of saponin.

2.3. Biosynthesis of silver nanoparticles from *Aegle marmelos* leaf extract

The 1 mM silver nitrate ($AgNO_3$) solution was prepared by dissolving 0.169 g of analytical grade $AgNO_3$ in 1000ml of deionized water. The solution was stored in an amber colored glass bottle to minimize photodegradation and prevent self-oxidation of silver ions. Silver nanoparticles were synthesized using a 1:2 volume ratio of silver nitrate solution to plant extract (100 ml:200 ml), where the plant extract acted as both reducing and capping agent and silver nitrate served as the precursor. For the reduction of Ag^+ ions, pH adjusted volumes of plant extract were mixed separately with the optimized volume of 1 mM $AgNO_3$ solution. The reaction mixture was incubated at optimized temperature of 40°C for 90 min in a closed incubator. The visual color transition from pale yellow to dark brown indicated the formation of AgNPs, a change attributed to surface plasmon resonance (SPR). The successful reduction of silver ions and formation of nanoparticles were further confirmed by UV–Vis spectrophotometric analysis, which showed characteristic absorption peaks for AgNPs [63, 64].

2.4. Characterization of synthesized nanoparticles

The characterization studies of synthesized silver nanoparticle from the plant extract were done by UV- Vis spectral analysis, FT IR analysis, and SEM and XRD analysis.

2.5. UV -Vis Spectroscopy analysis of synthesized nanoparticles

The synthesis of AgNPs was initially confirmed by the visual color change of the reaction mixture from pale yellow to brown, which indicates the excitation of surface plasmon vibrations in AgNPs. The reduction of silver ions (Ag^+) to metallic AgNPs was further validated using UV–Visible spectroscopy. The absorption spectra of the colloidal solutions were recorded in the range of 300–600 nm using a UV–Vis spectrophotometer, where a distinct peak corresponding to the surface plasmon resonance (SPR) band of AgNPs was observed [63, 64].

2.6. Fourier transform infrared spectroscopy analysis of synthesized nanoparticles

Fourier Transform Infrared (FTIR) spectroscopy was used to investigate the functional groups responsible for the reduction and stabilization of the biosynthesized AgNPs. The spectrum was recorded in the range of 4000–450 cm^{-1} using a PerkinElmer Spectrum. Two spectrophotometer with a resolution of 4 cm^{-1}. Prior to analysis, the nanoparticle sample was dried and finely ground with potassium bromide (KBr) to form a pellet, which was then scanned for infrared absorption. The obtained FTIR spectra provided information about the characteristic vibrational modes of biomolecules involved in capping and stabilization of nanoparticles, including hydroxyl, carboxyl, amine, and aromatic groups. Such functional groups from phytochemicals act as reducing and stabilizing agents, preventing agglomeration and enhancing nanoparticle stability, as widely reported in green synthesis approaches [64, 65].

2.7. Scanning electron microscopy analysis of synthesized nanoparticles

The surface morphology of the biosynthesized AgNPs was examined using a scanning electron microscope (SEM, Hitachi S-3400N). For sample preparation, a thin layer of dried AgNPs was mounted on a carbon-coated aluminum stub and sputter-coated with a thin conductive layer to enhance surface conductivity and reduce charging effects during imaging. The analysis was performed at an accelerating voltage of 10.0 kV with a working distance of 5.7 mm, and micrographs were obtained at a magnification of 6000× under secondary electron (SE) mode. The particles were observed in the submicron range with rough surface topography, indicating successful nanoparticle formation and aggregation behavior [63, 64].

2.8. X- Ray Diffraction analysis of synthesized nanoparticles

The crystalline structure of the biosynthesized AgNPs was characterized using X-Ray Diffraction (XRD) analysis. The diffraction pattern was recorded on a Rigaku Smart Lab diffractometer equipped with Cu Kα radiation (λ = 1.5406 Å) operated at 40 kV and 30 mA. The scanning was carried out over a 2θ range of 20°–80° with a step size of 0.02° and a scan speed of 2°/min. The obtained diffractogram was analyzed using X-ray powder diffraction software for phase identification, peak indexing, and crystallite size determination. The average crystallite size of the nanoparticles was calculated using the Debye–Scherrer equation, where D is crystallite size, K is the Scherrer constant, λ is X-ray wavelength, β is the full width at half maximum (FWHM), and θ is the Bragg's angle [65].

$$D = \frac{K\lambda}{\beta\cos\theta}$$

2.9. Anti-bacterial activity of synthesized nanoparticles

The antimicrobial activity of biosynthesized AgNPs, as well as aqueous leaf extracts of *Aegle marmelos* was assessed using the agar well diffusion method. Mueller–Hinton agar plates were prepared and inoculated with test pathogens, including Gram positive bacteria (*Bacillus subtilis*) and Gram negative bacteria (*Enterobacter mori*). The bacterial cultures were sub cultured into freshly prepared nutrient broth and incubated at 37°C for 24 h to produce fresh cultures. Under aseptic condition, bacterial inoculum was uniformly spread on the surface of solidified Mueller Hinton agar. A sterile cork borer was used to create wells with a diameter of 5 mm diameter in the Mueller Hinton agar medium and the plates were allowed to dry for 20 min to remove any excess moisture. Different concentrations of AgNPs (25, 50, 75, 100, 150, and 200 µg/ml) 100 µl were loaded into wells and plant extracts, to evaluate antimicrobial efficacy. Streptomycin served as a positive control while distilled water served as the negative control. Plates were incubated at 37 °C for 24 h, after incubation, the antimicrobial activity was determined by measuring the diameter of the inhibition zones in millimeters (mm) around the wells [66].

2.10. Minimum inhibitory concentration of synthesized nanoparticles

The antimicrobial activity of biosynthesized AgNPs was assessed using the minimum inhibitory concentration (MIC) assay based on the broth microdilution method. Serial dilutions of AgNPs were prepared in sterile microtiter plates and inoculated with bacterial suspensions. The MIC was recorded as the lowest concentration of AgNPs that inhibited visible bacterial growth when compared with the untreated control. Briefly, 50 µl of AgNPs suspensions prepared in Luria–Bertani (LB) broth were added to 96 well plates in decreasing concentrations (160, 80, 40, 20, 10, and 5 µg/ml). Bacterial cultures were grown in LB broth and adjusted to a 0.5 McFarland standard (1×10^8 CFU/ml). A volume of 50 µl of this standardized inoculum was added to each well containing AgNPs dilutions. Streptomycin Ampicillin served as positive control, while sterile deionized water was used as the negative control. The plates were sealed and incubated at 37 °C for 24 h. After incubation, 10 µl of Alamar Blue dye was added to each well and the plates were further incubated for 3 h in the dark. Viable bacteria reduced the non-fluorescent resazurin to the pink, fluorescent resorufin, and thus the fluorescence intensity was directly proportional to the number of metabolically active cells. The MIC was defined as the lowest concentration of AgNPs that prevented color change, indicating complete inhibition of bacterial viability. The fluorescence signal was measured using a spectrophotometer at excitation/emission wavelengths of 530-560/590 nm [67].

2.11. Antioxidant activity of synthesized silver nanoparticles

The antioxidant property of biosynthesized AgNPs was evaluated using the DPPH radical scavenging assay, a widely applied method for assessing antioxidant potential. Varying concentrations (25, 50, 100, 150, and 200 µg/ml) of each nanoparticle preparation were mixed with 1.0 ml of freshly prepared DPPH solution (0.1 mM in methanol) agitated, and incubated at room temperature for 30 min in the dark [66]. The absorbance was measured at 517 nm, corresponding to the reduction of DPPH radicals. While a DPPH solution without any test sample served as the control. The percentage of scavenging activity was calculated using the formula:

DPPH scavenging activity (%) = [A control – A sample]/A control×100.

Where, A sample indicated the absorbance of sample, while A control indicated the control. Ascorbic acid was used as standard antioxidant. Results of triplicate experiments were presented as scavenging percent.

2.12. Anti-inflammatory activity of synthesized silver nanoparticles

The anti-inflammatory activity of biosynthesized silver nanoparticles (AgNPs) was evaluated in vitro using the heat-induced protein denaturation assay as described by Mizushima and Kobayashi [68] with slight modifications. Silver nanoparticles were prepared in different concentrations (25, 50, 75, 100, 150 and 200µg/ml) using phosphate buffered saline (PBS, pH 6.4) as solvent, while diclofenac sodium (100µg/ml) served as the standard drug. The assay mixture consisted of 0.5 ml of AgNPs solution, 0.5 ml of 0.2% bovine serum albumin (BSA) prepared in PBS, and the pH adjusted to 6.4 using 1 N HCl. A control was prepared containing BSA and PBS without nanoparticles. All test solutions were incubated at 37°C for 20 minutes followed by heating at 70°C for 5 minutes to induce denaturation, then cooled to room temperature. Absorbance was recorded at 660 nm using a UV–Vis spectrophotometer [69]. The percentage inhibition of protein denaturation was calculated using the formula:

% Inhibition of protein denaturation = [(A1-A2)/A1] x100

Where, A1 is the absorbance of the control, A2 is the absorbance of the sample.

3. Results and Discussion

3.1. Qualitative phytochemical analysis of leaf extracts of *A. marmelos*

Qualitative phytochemical analysis was assayed using leaf extracts of *Aegle marmelos.* The presence and/or absence of beneficial bioactive compounds such as alkaloids, tannins, flavonoids, terpenoids, phenols, saponins and anthraquinones in leaf extracts were discovered by the assenting tests, involving color changes, precipitate formation and other confirmations (Figure 1 and Table 1). The results revealed that bioactive compounds like phenols, tannins, flavonoids, alkaloids, terpenoids, phenols and saponins are present and absence of anthraquinones from *A. marmelos* leaf extract. These findings are in close agreement with earlier reports on *A. marmelos*. For instance, Sabu and Kuttan [70] and Giri [71] confirmed the occurrence of tannins, flavonoids, phenols, terpenoids, saponins, and alkaloids in *A. marmelos* leaves, with anthraquinones not detected.

Mujeeb et al. [59] investigated phytochemical screening of aqueous and methanolic leaf extracts of 18 varieties of *A. marmelos*. The crude extracts of *A. marmelos* revealed the presence of several biologically active phytochemicals with the highest quantity of alkaloids, flavonoids, and phenols in Pant Aparna variety. Janarthanan et al. [72] studied the qualitative phytochemical analysis reflects the presence of carbohydrates, alkaloids, flavonoids, tannins, terpenoids, saponins, quinones, glycosides, coumarins in the *Aegle marmelos* leaf extracts [73]. Veer and Singh [74] identified the phytochemicals and antioxidant properties in different extracts derived from leaves of *A. marmelos*-a value addition to waste bael-patra. The phytochemical screening of *A. marmelos* dried leaves revealed the presence of alkaloids, carbohydrates, flavonoids, phenols, saponins, steroids, and tannins.

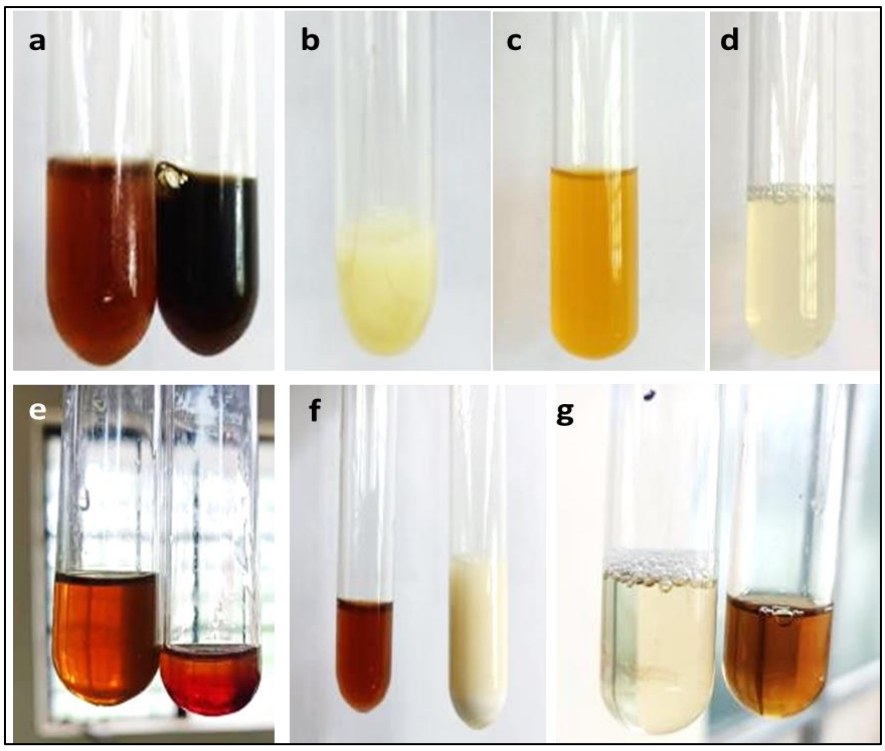

Figure 1 Phytochemical test for *A. marmelos* leaf extract. a- Phenols, b- Alkaloids, c- Flavonoids, d-Anthraquinones, e- Terpenoids and steroids, f- Tannins, g- Saponins

Table 1 Phytochemical screening from *A. marmelos* leaf extract

Phytochemicals	Test	Result
Alkaloids	Mayer's reagent test	+
Flavonoids	Alkaline reagent test	+
Saponins	Frothing test	+
Tannins	Ferric chloride test Lead acetate test	+ +
Terpenoids and Steroids	Salkowski test	+
Phenols	Ferric chloride test	+
Anthraquinones	Bontrager's test	-

+ Present, - Absent

3.2. Green synthesis of silver nanoparticles from *A. marmelos* leaf extract

The primary indication of nanoparticle synthesis was visually observed through the gradual color change of the reaction mixture. The reduction of aqueous $AgNO_3$ solution by *A. marmelos* leaf extracts led to a transformation from brown to blackish brown, a characteristic change attributed to the surface plasmon resonance (SPR) of AgNPs. The phytochemicals present from the plant extracts, including flavonoids, phenolics, alkaloids, and terpenoids, represented both as reducing agents for Ag^+ ions and as stabilizing/capping agents, thereby preventing agglomeration and enhancing nanoparticle stability.

3.3. Characterization of silver nanoparticles

3.3.1. UV- Visible spectroscopy analysis

The optical properties of the synthesized AgNPs were analyzed using UV–Visible spectroscopy in the wavelength range of 300–600 nm. The *A. marmelos* AgNPs exhibited a distinct SPR peak at 405 nm with a maximum absorbance of 0.611 suggesting enhanced nanoparticle formation efficiency (Figure 2). The observed SPR bands in the visible region confirm the successful reduction of Ag^+ ions to Ag^+ nanoparticles. Biomolecules, such as polyphenols, flavonoids, saponins, terpenes, sugars, and alkaloids, act as reducing and stabilizing agents. Functional groups of these molecules like hydroxyl (—OH), carbonyl (C═O), and carboxyl (—COOH) facilitate Ag^+ reduction to Ag^0 and adsorb onto nanoparticle surfaces, providing capping and stability, which control size, morphology, and biological activity of the resulting silver nanoparticles [75]. Additionally, presence of phenolic and amine groups has been associated with enhanced nanoparticle stability and biocompatibility [76]. Mogensen and Kneipp [77] explained that based on the AgNPs size, the shift of the plasmon resonance was changed. In large AgNPs, the SPR band shift towards the red region and, in small size bands shifted to the blue region.

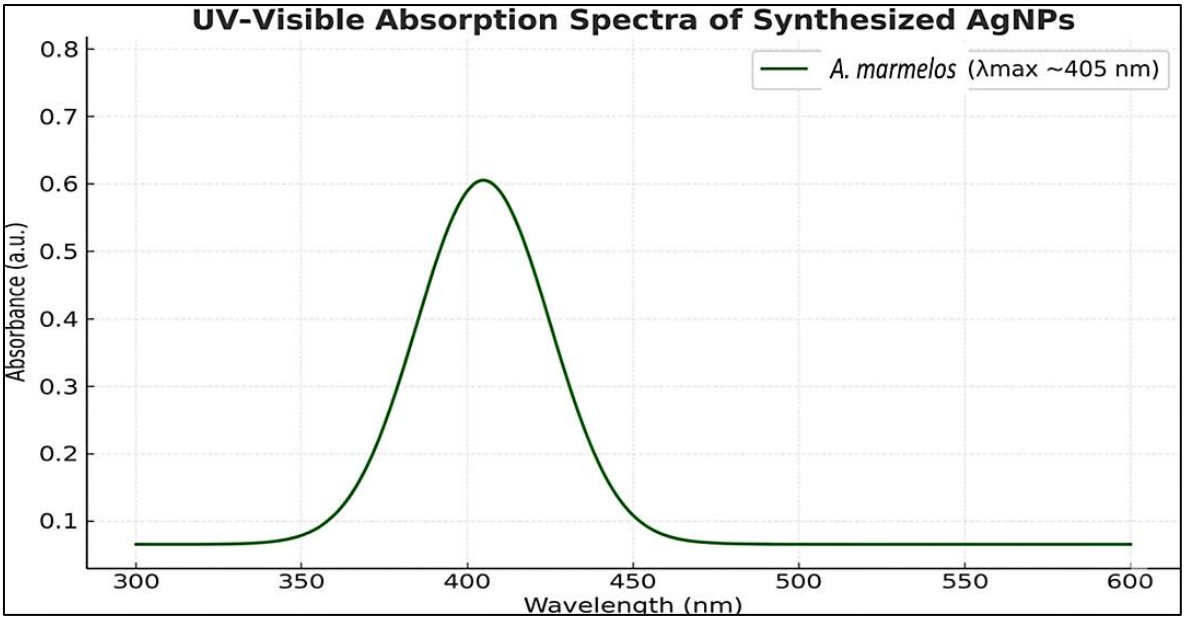

Figure 2 UV- Visible spectroscopy analysis of *A. marmelos* silver nanoparticles

3.4. Fourier Transform Infrared analysis of *A. marmelos* – AgNPs

The FTIR spectrum of the synthesized *A. marmelos* AgNPs was obtained in the range of 4000–600 cm^{-1} using a PerkinElmer Spectrum.Two spectrophotometer with a resolution of 4 cm^{-1} and 8 scans. The analysis revealed distinct absorption bands at 660.2 cm^{-1}, 639.83 cm^{-1}, 620.23 cm^{-1} and 605.47 cm^{-1}. These bands are characteristic of metal oxygen stretching vibrations and are commonly associated with AgO linkages, confirming the binding of silver with oxygen-containing functional groups during nanoparticle synthesis (Figure 3). The FTIR spectroscopy was used for identification of functional groups of phytochemicals responsible for reduction of silver ions and capping/stabilization of synthesized AgNPs [78, 79]. The peak at 660 cm^{-1} resembles the polyphenolic compound [80]. The absence of prominent peaks in the high wavenumber region suggests that most volatile organic residues were eliminated, leaving behind primarily inorganic and capping-associated vibrations [81].

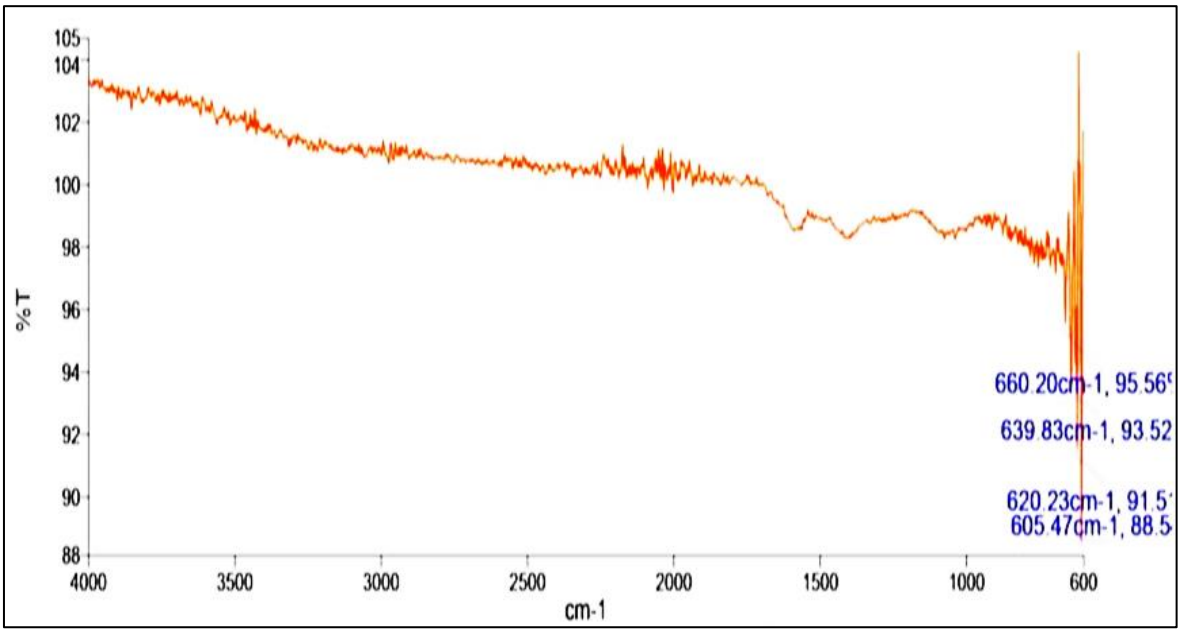

Figure 3 Fourier Transform Infrared analyses of *A. marmelos* silver nanoparticles

3.5. Scanning electron microscopy analysis of *A. marmelos* AgNPs

The SEM micrograph illustrates the morphology and size distribution of AgNPs synthesized using *A. marmelos* leaf extract as a natural reducing and stabilizing agent (Figure 4). The nanoparticles predominantly exhibit irregular and flake like morphologies, with certain areas displaying agglomeration. Particle size analysis from selected regions indicates sizes of 93.5 nm, 136 nm, 178 nm, and 187 nm, yielding an average particle size of approximately 148.1 nm. The observed aggregation may result from phytochemicals such as flavonoids, tannins and phenolics present in *A. marmelos* extract, which cap and stabilize the nanoparticles in solution but may facilitate clustering upon drying for SEM imaging. The results have been reported by Patil et al. [82], who synthesized AgNPs using *A. marmelos* leaf extract and observed predominantly spherical nanoparticles in the size range of 15–30 nm. Similarly, Rao and Paria [80] described nanoparticles averaging 60 nm with uniform morphology. In contrast, our study revealed irregular, flake-like nanoparticles with an average size of 148.1 nm, along with noticeable agglomeration. The plant extract mediated synthesis often leads to particle clustering due to phytochemicals acting as both stabilizers and bridging agents, supporting the aggregation seen in our SEM images [83].

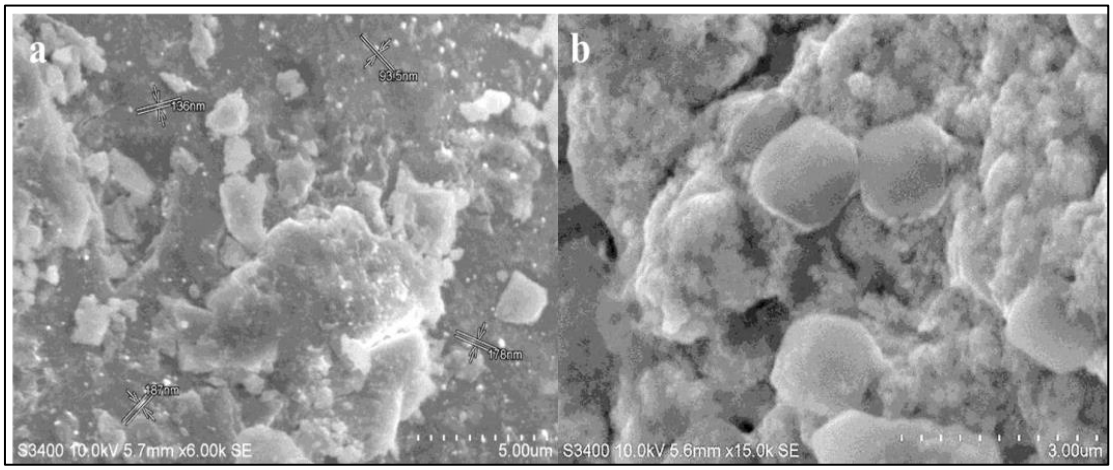

Figure 4 Scanning electron microscopy analysis of *A. marmelos* silver nanoparticles

3.6. X ray diffraction analysis of synthesized nanoparticles

The XRD pattern of *A. marmelos*- AgNPs exhibited characteristic diffraction peaks at 2θ values of 27.82°, 32.10°, 38.10°, 41.15°, 44.10°, 46.13°, 64.39°, and 77.32°. The most intense peak was observed at 38.10° corresponds to the (111) plane, confirming the face-centered cubic crystalline structure of silver nanoparticles. Other peaks were indexed to (210), (122), (200), (231), (220), and (311) planes, which are consistent with the standard JCPDS file No. 04-0783 for metallic silver. The relatively sharp and intense diffraction peaks confirm the crystalline nature of the nanoparticles, while the broadening of the peaks indicates nanometer-sized crystallites. The average crystallite size estimated using the Debye–Scherrer formula was found to be in the nanoscale range 38.10° nm suggesting successful synthesis of silver nanoparticles (Figure 5).

The XRD patterns clearly showed that the obtained *A. marmelos* -AgNPs are crystalline; the crystalline size was calculated as 38.10° nm in range. These results are in good agreement with previously synthesized AgNPs from *Rheum ribes* plant extract, which has a similar range of diffraction peaks [84]. However, the present synthesized Am-AgNPs have a small crystalline size in nature while comparing to the other report, Sundeep et al. [85] synthesized AgNPs from *Mangifera indica* which showed 32.4 nm crystal size. Similar findings of face-centered cubic crystal formation and predominant (111) orientation have been reported by Ahmad et al. [86] in biogenic silver nanoparticles synthesized using plant extracts. The additional weak peaks at 27.82° and 32.10° may be attributed to organic phytoconstituents or secondary crystalline phases from *A. marmelos* metabolites acting as capping and stabilizing agents. Such minor variations have also been observed in earlier studies of Mittal et al. [81] and Saini et al. [87], where bioactive molecules from plant extracts influence lattice strain and particle stability. The peaks around 2θ values of 38 (111), 44 (200), 64 (220), and 77 (311) confirm the diffraction planes of AgNPs related to the crystalline silver face-centered cubic (FCC) phase as indexed in JCPDS Repot No: 04-0783 [88]. However, additional peaks in XRD patterns could be attributed to phytochemicals or the formation of silver oxide on the surfaces of AgNPs which were in agreement with the results of Alamier et al. [89].

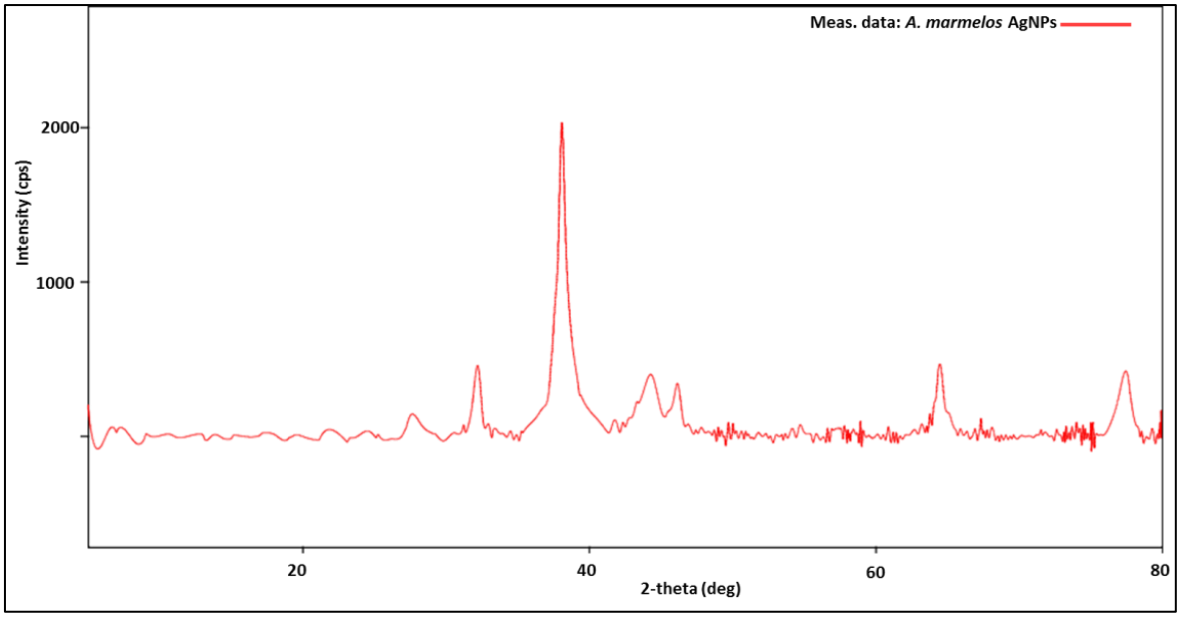

Figure 5 XRD analysis of *A. marmelos* silver nanoparticles

3.7. The antibacterial activity of synthesized nanoparticles

The antibacterial activity of synthesized *A. marmelos* silver nanoparticles against pathogenic microbes was shown in table 5. Zone of inhibition increases with concentrations 25, 50, 75, 100, 150, 200µg/ml. The maximum zone of inhibition 15 mm for *B. subtilis* and 17 mm for *E. mori* was observed at 200µg/ml. While the lowest zone of inhibition 7 mm for both bacteria was observed at 25µg/ml (Figure 6). For instance, *Aegle marmelos* methanolic leaf extracts show potent antimicrobial activity against Gram positive (*Bacillus cereus, Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram negative (*Enterobacter aerogens*) bacteria [90]. The AgNPs and released Ag^{+} catalyze the formation of reactive oxygen species (ROS), resulting in oxidative damage to lipids, proteins, and nucleic acids. Finally, silver species can interact with DNA and ribosomal subunits, inhibiting replication and protein synthesis. Current assessments emphasize that these mechanisms often act simultaneously, making AgNP antibacterial action inherently multifactorial [91-93]. The synthesized AgNPs biologically in an environmentally friendly way using *S. quercetaroensis* peel aqueous extract and reported the potent antimicrobial activity of AgNPs against Gram positive bacteria and a methicillin resistant strain of *S. aureus*. Gram-positive *S. aureus* and methicillin-resistant *S. aureus* (MRSA) showed minimum inhibitory concentration (MIC) values of 0.313 ppm [94]. Riazunnisa [95] studied the effect of AgNPs synthesized using *Bauhinia racemosa* leaf extracts on *E. coli*, *K. pneumonia*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. Significant inhibition of all the cultures was observed with maximum zone of inhibition was reported against *K. pneumonia* and *S. aureus* i.e., 24 mm and 21 mm. It is followed by *E. coli*, *P. aeruginosa* and *B. subtilis* with aqueous extract. The antibacterial efficiency of AgNPs green-synthesized from the leaf extract of *Vernonia amygdalina* against both Gram-positive, *S. aureus* and *Streptococcus pyogenes*, and Gram-negative, *E. coli* and *Pseudomonas aeruginosa* [96]. The AgNPs from *Rubus discolor* leaf extract and highlighted their biological activities against various bacteria, including *E. coli*, *P. aeruginosa* and their MDR strains, AgNPs showed immense bactericidal activity with inhibition zones of 16.55 mm against *E. coli* and 18 mm against *P. aeruginosa* [97]. Patil et al. [82] have also reported the effectiveness of AgNPs synthesized from *A. marmelos* leaf extract for *E. coli* (7–10 mm at 25–100 µl), *P. aeruginosa*, (7–10 mm) and *S. aureus* (6–9 mm) which are comparable to the results obtained in this study.

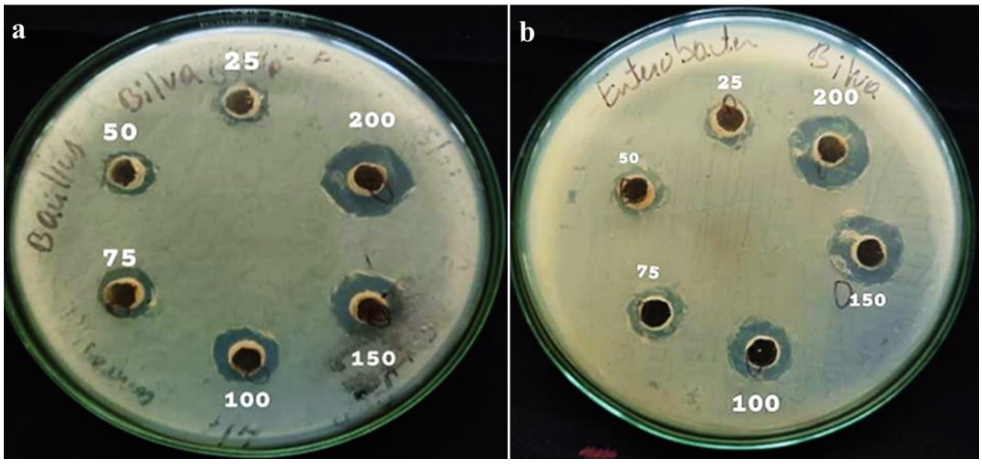

Figure 6 Antibacterial activity of *A. marmelos* silver nanoparticles. a – *B. subtilis*, b – *E. mori.*

3.8. Minimum inhibitory concentration assay of synthesized nanoparticles

The MIC assay of *A. marmelos* AgNPs demonstrated a concentration- dependent inhibition of microbial growth, as evident from the progressive increase in percentage inhibition with rising concentrations. At the lowest concentration tested of 5 µg/ml, inhibition was relatively low 8.54%, whereas a substantial inhibitory effect of 63.41% was recorded at the highest concentration of 160 µg/ml. This trend suggests that *A. marmelos* AgNPs possess significant antimicrobial potential, with efficacy improving proportionally to nanoparticle dosage (Table 2, Figure 7). The present MIC assay shows inhibition increasing from 8.54% at 5µg/ml to 63.41% at 160µg/ml, with a calculated IC_{50} of approximately 55µg/ml.

Table 2 Minimum inhibitory concentration assay of synthesized silver nanoparticles from *A. marmelos*

Sample	Concentration in µg/ml	O.D at 540nm	% of inhibition
A. marmelos AgNPs	Control	0.82	0.00
	5	0.75	8.54
	10	0.63	23.17
	20	0.52	36.59
	40	0.44	46.34
	80	0.36	56.09
	160	0.30	63.41

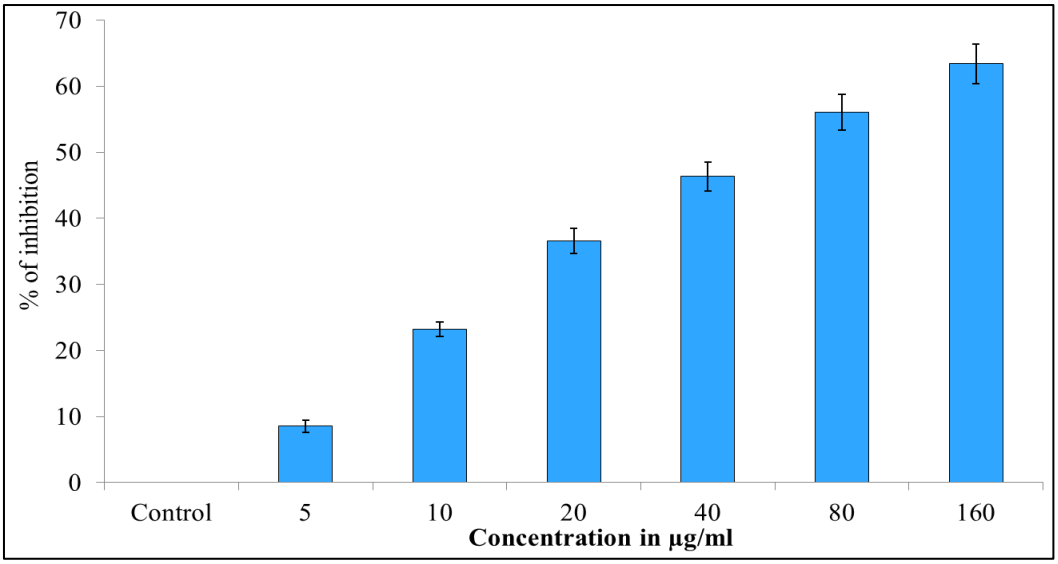

Figure 7 Minimum inhibitory concentration of silver nanoparticles from *A. marmelos* leaf extracts.

3.9. Antioxidant activity of *A. marmelos* silver nanoparticles

The antioxidant activity of ascorbic acid (standard) was assessed using the DPPH radical scavenging assay, and the results revealed a strong dose-dependent increase in free radical inhibition (Figure 8). At lower concentrations (25 µg/ml), ascorbic acid exhibited 24.78% inhibition, which progressively increased to 67.76% at 200 µg/ml. The IC_{50} value of ascorbic acid in the DPPH assay was calculated to be 81.6 µg/ml. The antioxidant potential of *A. marmelos* AgNPs was evaluated using the DPPH radical scavenging assay, and the results are presented in Figure 8. The nanoparticles exhibited a dose-dependent increase in radical scavenging activity, with inhibition ranging from 11.5% at 25µg/ml to 46.9% at 200µg/ml. Although the observed scavenging activity was very lower compared to standard antioxidants such as ascorbic acid, the steady increase in inhibition with concentration indicates the effective hydrogen-donating ability of the AgNPs, which may be attributed to the phytochemicals from *A. marmelos* extract acting as capping and reducing agents.

Priya et al. [98] reported the antioxidant activity of green synthesized AgNPs form *Pongamia pinnata* extract and found significant free radical scavenging potential. The synthesized AgNPs using an aqueous leaf extract of *Costu* safer and reported that nanoparticles showed high scavenging of free radicals by the DPPH method as compared to the leaf extract [99]. Menon et al. [100] reported antioxidant activity of silver nanoparticles from *Acalypha indica* by DPPH and reducing power assay that showed more antioxidant activity as compared to standard ascorbic acid. The antioxidant activity of AgNPs may be due to the bioactive compound on the surface of the AgNPs. Biogenic AgNPs had a better DPPH free radical scavenging activity with lower IC_{50} of 13.83 µg/ml as compared to that of the *Rubus ellipticus* with IC_{50} of 15.86 µg/ml [101]. The silver nanoparticles prepared by Rajput et al. [102] were better DPPH scavengers than the aqueous leaf extract of *Atropa acuminate*. Similar concentration dependent antioxidant effects of plant mediated AgNPs have been reported earlier, highlighting their potential as eco-friendly therapeutic agents with free radical scavenging properties by Ahmad et al. [86]; Chinnasamy et al. [103]. The AgNPs from leaf extract of the medicinal plant *Blumea lacera*, which exhibited a sharp SPR band at 430 nm. These AgNPs possessed good antioxidant activity (DPPH free radical scavenging activity) with an IC_{50} value of 6 µg/ml [104].

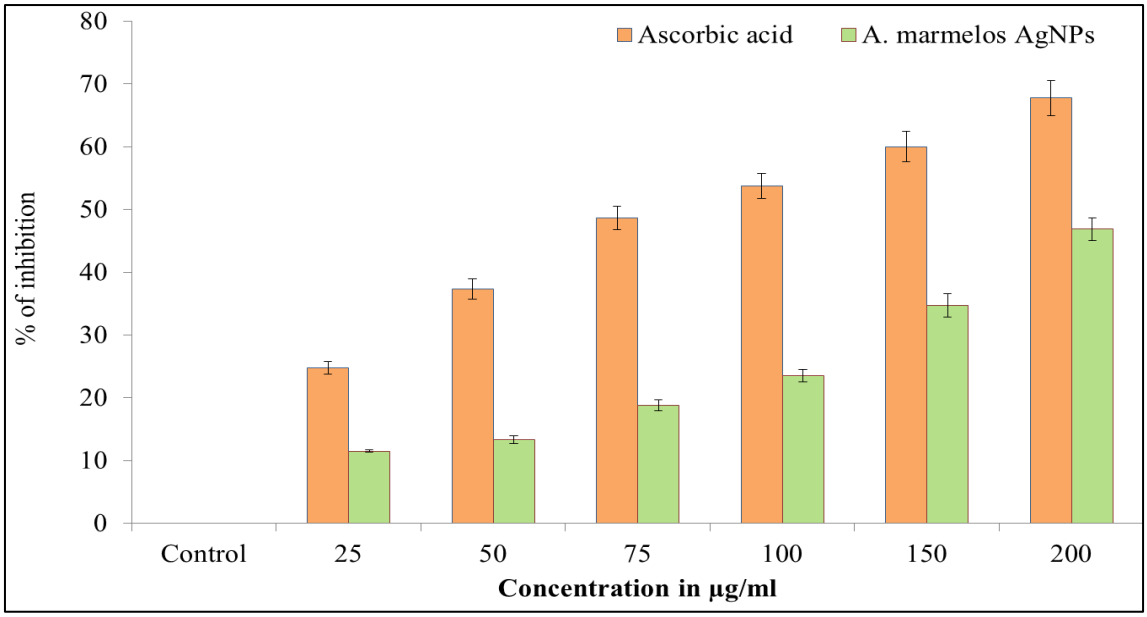

Figure 8 Antioxidant activity of *A. marmelos* silver nanoparticles

3.10. Anti-inflammatory activity of *A. marmelos* AgNPs

The anti-inflammatory activity of *A. marmelos* silver nanoparticles (AgNPs) showed a concentration-dependent increase with inhibition rising from 14.45% at 25 µg/ml to 38.55% at 200 µg/ml (Figure 9). This indicates that the nanoparticles effectively stabilized proteins against denaturation, a key mechanism in anti-inflammatory response. These findings suggest that the bioactive phytochemicals of *A. marmelos* coupled with the nanoscale properties of AgNPs contribute synergistically to their anti-inflammatory efficacy, highlighting their potential as natural alternatives for anti-inflammatory therapeutics. The assay showed a concentration-dependent inhibition, reaching 38.55% at 200 µg/ml, with the IC_{50} not achieved within the tested range; extrapolation suggests an IC_{50} of approximately 284 µg/ml. The AgNPs have gained significant attention in the field of regenerative medicine, regarding their significant antimicrobial activity and potential in anti-inflammatory applications [105, 106]. The AgNPs not only offer a potent antimicrobial activity that inhibits any pathogenic activity in the wound area, but also possess the ability to module cellular and molecular pathways that influence tissue repair and modulate anti-inflammatory mechanisms [107-109].

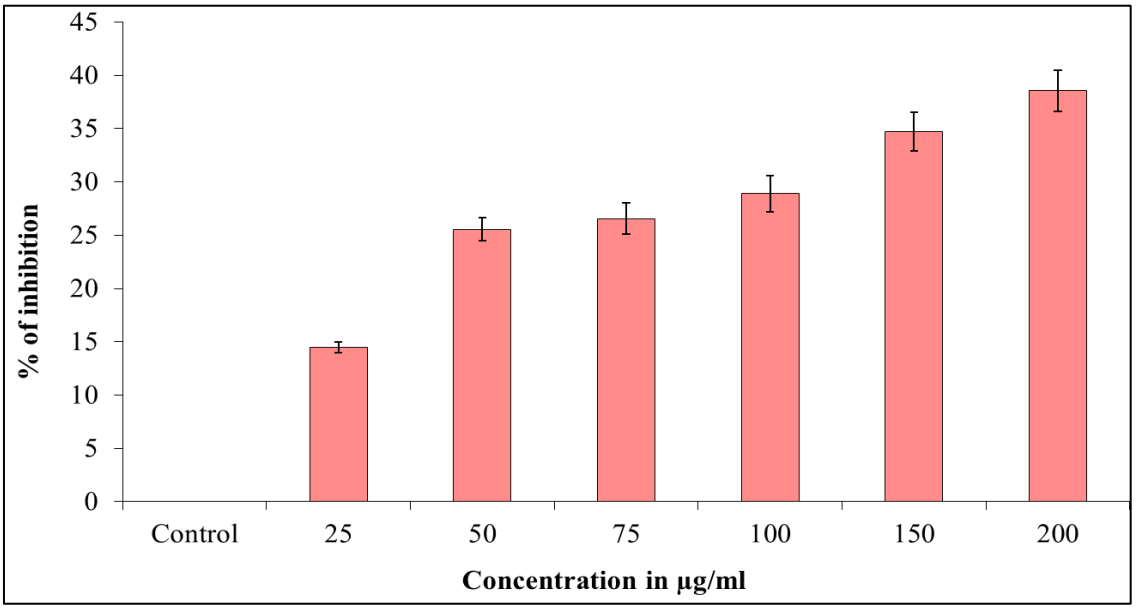

Figure 9 Anti-inflammatory assay of *A. marmelos* silver nanoparticles.

4. Conclusion

This study confirms the biomedical relevance of *Aegle marmelos* leaf-mediated AgNPs but also highlights the strategic advantage of employing combined phytochemical sources for enhanced bioactivity. Compared with conventional chemical AgNPs synthesis, which often poses toxicity risks and environmental concerns, the present green synthesis approach is cost-effective, biocompatible, and scalable. Hence, this study emphasizes that *Aegle marmelos* AgNPs represent a promising candidate for future applications in antimicrobial therapeutics, antioxidant nutraceuticals and anti-inflammatory formulations. AgNPs are being investigated as potential antimicrobial agents to block the growth of resistant strains through a variety of mechanisms, including the generation of oxidative stress, inhibition of DNA replication, and interactions with enzymes and proteins. The unique properties of nanoparticles and adsorbed secondary metabolites of leaf extracts on their surfaces may be the cause of their excellent biological activities. AgNPs may be used as antibiotics in the future because they are non-toxic, cheap, eco-friendly, and highly effective against bacteria. Moving forward, mechanistic studies, nano toxicological profiling, and in *vivo* validations are essential to bridge the gap between laboratory synthesis and clinical or pharmaceutical translation.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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