



Antibacterial potential of synthesized silver nanoparticles from leaf extract of *Moringa oleifera*

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ABSTRACT

Silver nanoparticles (Ag-NPs) are among the most widely used nanoparticles that show a broad spectrum of antibacterial activity. Green synthesis of Ag-NPs from plant extract is the most popular method *in vitro*. In this study, Ag-NPs were biologically synthesized from the leaf extract of *Moringa oleifera* and the antimicrobial activity was observed. Furthermore, a comparative assessment of antimicrobial activity of biologically synthesized Ag-NPs and crude plant extract was performed. Initially, 11 pathogenic bacterial strains were used to evaluate the antibacterial potentiality of crude leaf sample. However, the result showed that the crude leaf extract exerted maximum potential against *Proteus vulgaris*. Additionally, the application of biologically synthesized Ag-NPs was assessed against the same pathogenic strains and observed enhanced antibacterial activities with larger inhibited zones. Thus, our results suggest that the biological synthesis of Ag-NPs significantly enhanced the antibacterial activity of *M. oleifera* leaf extract against selected pathogenic bacterial strains. *M. oleifera* could be a potential source of Ag-NPs for successful use as an antibacterial agent in pharmaceutical and cosmetic industries.

INTRODUCTION

Nanoparticles are very commonly used word which represents particles in nano scale size (1–100 nm) with increased surface area. Their structural conformation and organized distribution in solution confer enhancement of their physical and chemical properties which are beneficial for different biological areas. In recent times, silver nanoparticles (Ag-NPs) has gained enormous attention because of its diverse characteristics which are appropriate for various medical and biomedical applications such as catalysis [1], diagnostics [2], antimicrobial development [3] and targeting of drug [4].

Different conventional methods (both physical and chemical) for nanoparticle synthesis have very restricted use due to many limitations, especially for their toxicity, high-energy requirements and an expensive downstream processing. On the contrary,

biological synthesis of silver nanoparticles offers several advantages such as fast, high yields and importantly, the cheap downstream processing requirements compared to conventional methods. Thus, different biological methods of nanoparticles synthesis using microorganisms [5, 6], enzymes [7], and plants or plant extracts [8-13] have been suggested as possible ecofriendly alternatives to chemical and physical methods. In the process of nanoparticles synthesis using plant extracts involved chemical reaction with phytochemicals present in the plant extract and silver nitrate (AgNO₃) [14].

In the present study, we used leaf of *Moringa oleifera* Lam. (Moringaceae) plant, locally known as “Drumstick”, for synthesis of green Ag-NPs. The leaves of this plant are prominent for their natural healing properties and are gobbled up in varieties ways. Also, it possess high natural antioxidant properties and antibacterial activity against different

gram positive and gram negative pathogenic bacteria [15]. Moreover, it does not require addition of any external stabilizing agents during synthesis of nanoparticles which are really detrimental to the environment [16]. Thus, here we report the biosynthesis of Ag-NPs from *M. oleifera* leaf extract which are further assessed and compared for antibacterial efficacy.

MATERIALS AND METHODS

Collection of samples, processing and extraction of metabolites

The leaf of *M. oleifera* were collected from Khulna University campus, Khulna, Bangladesh. All samples were carefully washed and cleaned for surface sterilization. Undesired materials were removed from samples and finally cut into tiny pieces. After proper sun drying, the leaves were firmly grounded and immersed into 50% ethyl acetate (Loba Chemie Pvt Ltd, Mumbai, India). The soaked mixtures were maintained in dark with periodic shaking and stirring for next 7 days. Then the mixtures were filtered and the filtrates were incubated at 45 °C for 30 min in water bath. After that it was transferred to an incubator (at 37 °C for 72 hours). The crude leaf extract was quantified and stored in 4 °C. This extraction procedure is slightly modified from that of reported by Alinezhad et al. in 2012 [17].

Green synthesis of Ag-NPs

Silver nitrate salt (AgNO_3 , 1 mM) (Unichem Laboratories Ltd., Mumbai, India) was used for the synthesis of silver nanoparticles. This synthesis procedure was previously reported by Moodley et al., in 2018 [18]. In brief, 5 ml of Silver nitrate solution was mixed with 50 ml of plant extract and incubated into direct sunlight for 30 min. Subsequent change in color to dark brown indicated the formation of nanoparticles in the reaction tubes. To prevent agglomeration of the synthesized nanoparticles the respected tubes were removed from sunlight and kept in dark at room temperature. The synthesized Ag-NPs were further confirmed and quantified using a UV-vis spectrometer (Prolific Instruments, Mumbai, India) at 300-700 nm.

Purification of synthesized Ag-NPs and spectral analysis

The mixture of Ag-NPs was centrifuged at 10,000 rpm for 1 hour at 4 °C using a centrifuge machine (Thermo Fisher Scientific, Massachusetts, USA). The supernatant was discarded and the pellet was washed with distilled water to remove the adulterated plant material. The pellets were again centrifuged at 10,000 rpm for 30 minutes at 4 °C. This wash step was repeated twice to remove water soluble biomolecules such as proteins and cellular metabolites. Finally, the pellets containing the silver nanoparticles were kept at 37 °C for 24 hours to obtain a concentrated mixture of nanoparticles. For further confirmation of Ag-NPs UV-Vis spectral analysis was performed after sunlight exposure of 0, 30 and 90 min. The absorbance bands of the samples were fixed in 300 to 700 nm.

Agar disc diffusion assay

Agar disc diffusion assay was performed to evaluate the antibacterial potential of extracted samples. The assay procedure was previously reported by Biemer in 1973 [19]. Briefly, overnight incubated bacterial subcultures were inoculated in nutrient broth (Himedia Laboratories Pvt. Ltd., Bengaluru, India) and mix properly. Then the broth was flooded into petri dishes containing Muller-Hinton agar media (Himedia Laboratories Pvt. Ltd., Bengaluru, India) and was spread throughout the media gently with a sterile glass spreader. The 5 mm sterile filter paper disc were impregnated with 50 µg of the test stuffs and dried under restrained condition to vaporize residual solvent. Standard ciprofloxacin (30µg/disc) were used as positive control and blank discs were used as negative control. The sample discs, antibiotic discs, negative control discs were gently placed on to inoculated agar plates. Then the plates were transferred to the incubator (at 37°C for 24 hours) and kept inversely. After incubation the antibacterial activities were quantified by measuring the diameters of zone of inhibitions. An overall representation of the methodology is plotted in Figure 1 for better understanding.

Statistical analysis

All data were analytically scrutinized using SPSS version 21. In the case of inhibited zone measurement ANOVA test was performed at 95% confidence level.

Differences were considered significant at the 0.05 level and where possible, means were separated by a Tukey post-hoc test.

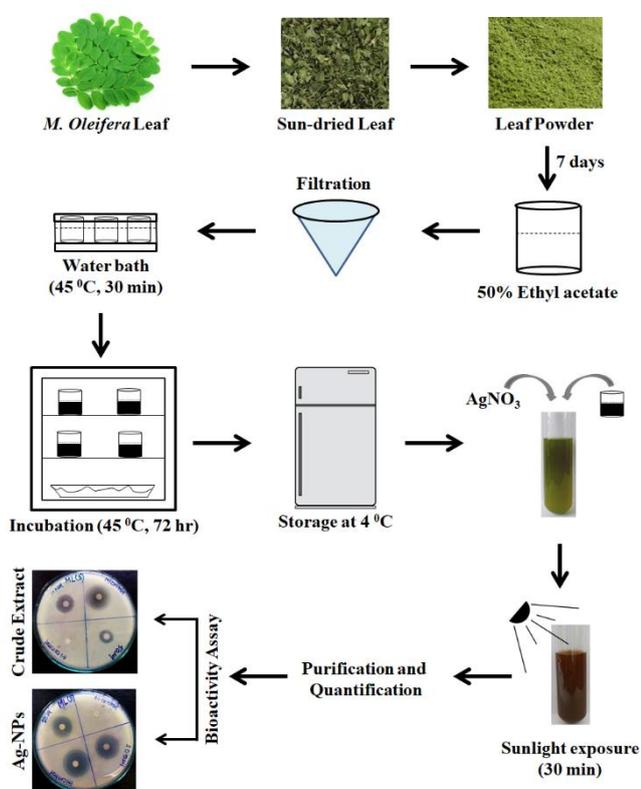


Figure 1. Overview of the green synthesis procedure of Ag-NPs using sunlight as the primary energy source. The leaves were dried and powdered to dissolve in 50% of ethyl acetate for preparing crude extract. After seven days of incubation the mixture was filtered and evaporated in water bath. The concentrated extract was then incubated for 72 h at 45 °C. The extract was ready for further analysis and can be stored at 4 °C. The biological synthesis of Ag-NPs involved the mixture of silver nitrate solution with plant extract followed by the direct sunlight exposure for 30 min. Subsequent change in color to dark brown indicated the formation of nanoparticles in the reaction tubes. After purification the synthesized Ag-NPs were further confirmed using a UV-vis spectrometer at 300-700 nm. Then, both of the crude extract and Ag-NPs were subjected to evaluate their antibacterial activities against pathogenic bacterial strains.

RESULTS

Visible color change of crude extract indicating the formation of nanoparticles

The formation of nanoparticles in the crude extract is usually detected by observing the color change from clear (transparent) to dark brown in the presence of sunlight. During this study, the leaf extract changed its color to dark brown after 30 min of exposure in sunlight. Whereas, the control (distilled water and

silver nitrate solution) did not show any color difference after sunlight exposure. The color change was easily noticeable and satisfactory to confirm the presence of Ag-NPs in a significant amount as presented in Figure 2A-C.

The UV-vis spectral analysis showed that the absorbance of samples was increased gradually following sunlight exposure. At the beginning, the absorbance of the reaction mixture was found nil/zero as there were no particles formed at that time. The increased absorbance indicates the formation of Ag-NPs with an increased concentration. The surface plasmon resonance of Ag-NPs showed a peak near 440 nm to 450 nm for leaf extract (Figure 2D). The symmetrical shape of the plasmon bands suggesting well-dispersed and uniform-sized nanoparticle synthesis.

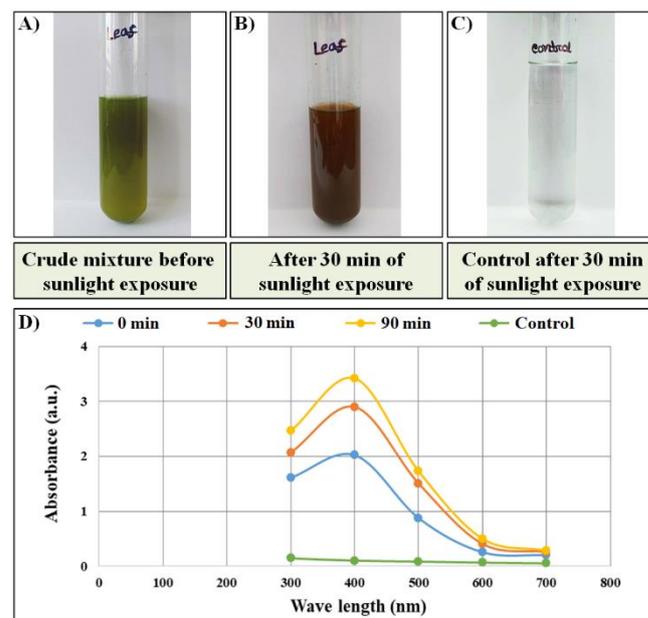


Figure 2. Synthesis of Ag-NPs and its confirmation. A-C represents confirmation of nanoparticle formation by visual observation. Silver nitrate solution was mixed with leaf extract and exposed to sunlight for 30 min. During the incubation period the color of the crude extracts were gradually changed to brown color. After 30 min of sunlight exposure the color reached to the dark brown. The change of color indicates the formation of nanoparticles in the reaction tubes. D showing the UV-vis absorption spectra of synthesized silver nanoparticles as a function of time. The control represents the reaction mixture of AgNO₃ solution with distilled water.

Antibacterial activities of crude extract of *M. oleifera*

The crude extract from leaves was applied to observe its antibacterial potentials on 11 different pathogenic bacterial strains. Among the 11 strains 3 were gram positive (*Staphylococcus aureus*, *Micrococcus* and *Mycobacterium*) and 8 were gram negative (*Escherichia coli*, *Vibrio cholera*, *Salmonella typhi*, *Salmonella paratyphi*, *Proteus vulgaris*, *Shigella dysenteriae*, *Shigella flexneri* and *Campylobacter*) bacteria. However, only 6 strains showed countable susceptibility against crude extract (50 µg/disc). The maximum zone of inhibition (7.93±1.68 mm) was recorded for *P. vulgaris* against *M. oleifera* leaf extract. The detailed information of the 6 susceptible strains with their inhibited zone diameters is presented in Table 1.

Table 1. The antibacterial activity of crude extract from *M. oleifera* leaves.

Bacterial Strain	Diameter of the zone of inhibition		
	Crude leaf extract (50 µg/disc)	Positive control (Ciprofloxacin, 20 µg/disc)	Negative control (Distilled Water)
<i>Escherichia coli</i>	6.00±0.67 mm	23.00±2.00 mm	No Dimension
<i>Micrococcus</i>	5.16±0.76 mm	28.00±3.60 mm	No Dimension
<i>Vibrio cholera</i>	4.46±0.85 mm	30.00±1.00 mm	No Dimension
<i>Salmonella typhi</i>	5.00±1.00 mm	30.67±2.30 mm	No Dimension
<i>Staphylococcus aureus</i>	5.67±1.52 mm	34.33±2.08 mm	No Dimension
<i>Proteus vulgaris</i>	7.93±1.68 mm	30.33±1.53 mm	No Dimension

Antibacterial activities of the synthesized Ag-NPs

To observe the antibacterial activities of the synthesized Ag-NPs agar disc diffusion assay was performed again. The same amount of synthesized Ag-NP (50 µg/disc) was used against all of the six pathogenic strains. The Ag-NPs from leaf extract showed highest antibacterial potential against *V. cholera* and *S. aureus* with the zone diameter of 21.67±4.72 mm and 21.67±1.53 mm, respectively. The detailed information is presented in Table 2.

Table 2. The antibacterial activity of synthesized Ag-NPs from *M. oleifera* leaf extract.

Bacterial Strain	Diameter of the zone of inhibition		
	Ag-NPs from leaf extract (50 µg/disc)	Positive control (Ciprofloxacin, 20 µg/disc)	Negative control (Distilled Water)
<i>Escherichia coli</i>	20.33±2.30 mm	23.00±2.00 mm	No Dimension
<i>Micrococcus</i>	18.33±0.57 mm	28.00±3.60 mm	No Dimension
<i>Vibrio cholera</i>	21.67±4.72 mm	33.00±5.19 mm	No Dimension
<i>Salmonella typhi</i>	19.00±1.15 mm	30.67±2.30 mm	No Dimension
<i>Staphylococcus aureus</i>	21.67±1.53 mm	34.33±2.08 mm	No Dimension
<i>Proteus vulgaris</i>	18.33±3.21 mm	27.33±1.00 mm	No Dimension

Synthesized Ag-NPs significantly enhanced the antibacterial potential than crude extract

It was observed that the similar dose of either crude extract or synthesized Ag-NP produced different zone diameters. In the all cases the antibacterial activity of Ag-NPs were enhanced compared to that of the crude extract. The highest fold enhancement of antibacterial activity with 50 µg/disc was observed against *V. cholera* (4.98 fold) when compared with leaf extract. The detailed result is plotted in Figure 3.

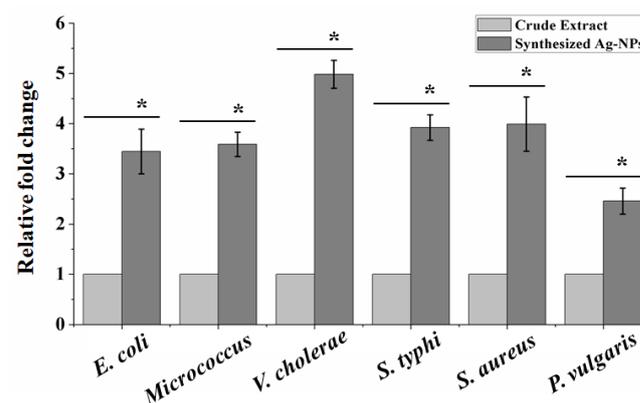


Figure 3. Fold enrichment of antibacterial activities of synthesized Ag-NPs when compared to crude extracts. These bar graphs showed the relative fold enhancement of Ag-NPs from crude leaf extract. Values are represented as the average fold change ± SEM bars, n = 3 replicates. Asterisks indicate statistically significant changes based on adjusted p values < 0.05.

Higher dose of the synthesized Ag-NPs showed higher antibacterial activity

From the above results it was observed that synthesized Ag-NPs were more potent than crude extract. However, it was not clear that whether the increased dose of synthesized Ag-NPs also show increased activity. To conclude this point we used a higher dose of 100 µg/disc to evaluate the antibacterial capacity against *V. cholera*, and *S. aureus*. The result showed that this increased dose was more potent as expected. The detail result is presented in Table 3.

Table 3. The antibacterial activity of synthesized Ag-NPs with a higher dose (100 µg/disc).

Bacterial Strain	Diameter of the zone of inhibition			
	Crude leaf extract (100 µg/disc)	Ag-NPs from leaf extract (100 µg/disc)	Positive control (Ciprofloxacin, 20 µg/disc)	Negative control (Distilled Water)
<i>Escherichia coli</i>	10.13±2.0 5 mm	26.00±3.6 0 mm	28.33±1.53 mm	No Dimension
<i>Micrococcus</i>	11.00±1.0 0 mm	24.67±2.5 2 mm	30.00±1.00 mm	No Dimension
<i>Vibrio cholera</i>	13.00±2.0 0 mm	27.33±5.0 3 mm	26.83±1.25 mm	No Dimension
<i>Salmonella typhi</i>	12.67±2.0 8 mm	24.33±2.0 8 mm	30.33±1.53 mm	No Dimension
<i>Staphylococcus aureus</i>	13.00±2.6 4 mm	26.00±1.7 3 mm	28.67±2.08 mm	No Dimension
<i>Proteus vulgaris</i>	15.17±2.2 5 mm	24.33±3.2 1 mm	32.33±2.52 mm	No Dimension

DISCUSSION

In recent years, scientists are focusing on the invention of different synthesis procedures of Ag-NPs. Among the strategies biological or green synthesis of Ag-NPs are considering safe and simple as the use of conventional methods are associated with toxic environmental substances [20]. In the current manuscript the antibacterial potential of synthesized Ag-NPs from the leaf extract of *M. oleifera* were evaluated. The synthesis of Ag-NPs reported here is a green and cost effective synthetic approach. The green synthesis was performed under sunlight irradiation for nanoparticle formation within minimal time duration. It was reported that reduction of Ag⁺ by the leaf extract occurred in the presence of direct sunlight within 1 hour [18]. The completion of reduction process was indicated by the change of color due to the surface plasmon phenomenon from green to dark

brown [21-23]. In this study the color change was observed after 30 min of direct sunlight exposure. Thus, it indicates the suitability and accuracy of used synthesis procedure for Ag-NP formation. Furthermore, the use of renewable energy sources would be a promising alternative for silver nanoparticle production.

From the results it was observed that the green synthesis of Ag-NPs enhanced the bioactivity against different pathogenic bacteria. Enhancement of antimicrobial activity by addition of Ag-NPs was also reported before with other plant extracts [24-26]. From the different published reports it is observed that the antibacterial activity of Ag-NPs depend on the particle size of the synthesized Ag-NPs. The small-sized Ag-NPs exerting stronger bioactivities than large-sized Ag-NPs [27, 28]. In this study the size of the synthesized Ag-NPs were not measured. However, it was previously published that the use of direct sunlight favored the formation of small sized Ag-NPs. Moodley et al., showed that Ag-NPs synthesized from *M. oleifera* leaf extract using direct sunlight produced particles with an average diameter range of 9-11 nm [18]. Previous reports showed that Ag-NPs with oxidized surfaces (presence of silver, oxygen, carbon and nitrogen on the surface of Ag-NPs) enhanced their bioactivity by inducing holes on the bacterial surface [29-31]. Usually, biologically synthesized Ag-NPs carry silver, oxygen, carbon and nitrogen on their surfaces [18]. A major limitation of this study is that we did not perform any characterization of our synthesized Ag-NPs. However, we believe that the enhancement of antibacterial activity of the synthesized Ag-NPs was due to the smaller particle size and the oxidized particle surfaces as mentioned by previous reports.

CONCLUSION

In this study the antibacterial activity of synthesized Ag-NPs from *M. oleifera* leaf extract were evaluated against several pathogenic bacterial strains. The result showed a broad-spectrum of antibacterial susceptibility of the synthesized Ag-NPs. Thus, the green synthesis of Ag-NPs has promising antibacterial potential and could be used as a potent biomedicine.

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AUTHOR CONTRIBUTIONS

AH was involved in conception and design of the experiments. AI contributed to perform the experiments and also analyzed data. CM contributed to manage entire manuscript drafting including preparation of figures and tables, critical revising of the manuscript. Finally, AH approved the current version of manuscript for publishing.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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