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SYNTHESIS OF BENIGN SILVER NANOPARTICLES TO ENHANCE ANTIBACTERIAL ACTIVITY OF AZADIRACHTA INDICA

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ABSTRACT

Biologically synthesized nanomaterials and their industrial utilization have become an integral part of bionanotechnology. There is an increasing demand for silver nanoparticles (Ag-NPs) due to its wide properties in various fields of biomedical science including bio-sensing, drug delivery etc. Thus, the aim of the current study was to synthesize Ag-NPs by reducing the silver ions present in the silver nitrate (AgNO3) solution by green method to enhance antibacterial activity of a common medicinal plant *Azadirachta indica*. Initially, pure cultures of four gram positive (gm +ve) and eight gram negative (gm -ve) pathogenic bacteria were evaluated against the crude plant (leaf and bark) extracts and based on their susceptibility, two gm +ve and four gm -ve bacterial strains were finally selected. In case of crude extract, the highest zone of inhibition (13.00±1.00 mm) was observed against *Staphylococcus aureus* when treated with leaf samples, whereas it was 14.83±2.26 mm when treated with barks. Afterwards, Ag-NPs has been synthesized following greener method and the formation of Ag-NPs was confirmed by visual observation (colour change from pale yellow to dark brown) followed by Ultra-Violet Visible (UV-VIS) spectroscopy. Later, plant extracts with synthesized Ag-NPs were tested against same six bacterial strains and the obtained zone of inhibitions were compared with those of crude extract. As expected, both leaf and bark extracts showed increased zone of inhibition than previous. In this case, the diameter of the highest zone of inhibition was found 27.33±1.36 mm against *S. aureus* when treated with leaf extracts containing Ag-NPs and it was30.00±2.60 mm against the same bacterial strain. In case of bark extract with Ag-NPs. Finally, minimum inhibitory concentration (MIC) test was performed using both crude and Ag-NPs containing plant extracts. Significant differences were observed in MIC values between both types of plant extracts. The MIC value was found 0.312 mg/ml for both crude leaf and bark extracts and 0.078 mg/ml and 0.039 mg/ml for leaf and bark extract containing Ag-NPs, respectively. So, it could be concluded that the eco-friendly *A. indica* extracts (leaf and bark) can be used as an effective reducing agent for the synthesis of Ag-NPs and thus the antimicrobial activity of the selected medicinal plant can be enhanced by synthesized nanoparticles.

Keywords: Bionanotechnology, Silver nanoparticles (Ag-NPs), *Azadirachta indica*, Antibacterial activity, Zone of inhibition (ZOI).

1. INTRODUCTION

Silver (Ag) is familiar worldwide for its antimicrobial activity which was first identified in the 19th century (Hugo WB *et al*., 1982 & Teot L *et al*., 2005). In recent times, silver nanoparticles

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(Ag-NPs) became one of the most investigated and commercialized nano-materials as it has auspicious characteristics appropriate for various biomedical applications (Lugli P *et al*., 2005;Karni TC *et al*., 2012 & Ahmed S *et al*., 2014). Different preparation approaches have been devised and applied in the synthesis of nanoparticles of several materials, such as metallic (Murphy CJ *et al*., 2005; Ledwith D M *et al*., 2009 &Yu CH *et al*., 2009), dielectric (Nelson JK., 2007 & Baklanov MR, 2012), semiconductor (Zhang JZ *et al*., 2004 & Weber C *et al*., 2008) and magnetic (Lu A *et al*., 2007 & Koksharov YA, 2009). However, the rigorous usage of solvents and synthetic reactants is detrimental for the environment. For this reason, it is very prudential to devise alternative methods of nanomaterial preparation that use environment-friendly reactants. Different bioactive compounds (flavonoids, terpenoids etc.) found in plant extracts have made them suitable for the green synthesis of nanoparticles (Atawodi SE *et al*., 2009). The synthesis of Ag-NPs using different plant extracts can be an alternative and effective choice as it is simple, cost-effective, environment friendly and relatively reproducible and often results in more stable materials (Ahmed S *et al*., 2015 & Mittal J *et al*., 2014).

In recent times, there is a growing interest in the synthesis of metal nanoparticles by using extracts from different plants (Niraimathi KL *et al*., 2013; Sharma VK *et al*., 2009; Vankar PS *et al*., 2012; Narayanan KB *et al*., 2011; Singh S *et al*., 2013; Das S *et al*., 2013; Suriyakalaa U *et al*., 2013; Mohanpuria P *et al*., 2008; Gardea-Torresdey JL *et al*., 2008; Armendariz V *et al*., 2004; Kumar VG *etal*., 2011; Ghoreishi SM *et al*., 2011; Dubey SP *et al*., 2011; Lahtinen M *et al*., 2010; Shankar SS *et al*., 2003; Rai A *et al*., 2004; Parsons JG *et al*., 2007; Dubey SP *et al*., 2010 & Noruzi M *et al*., 2012). In our study, extracts from *Azadirachta indica* were used as reducing agents. The use of *A. indica* plant for the production of silver nanoparticles has drawn our attention as it is one of the most common medicinal plants in Bangladesh and other regions of South Asia. Also, it does not require addition of external stabilizing agent during synthesis of nanoparticles (Tripathi A *et al*., 2009). Moreover, the plant is rich in quercetin and ß-sitosterol, polyphenolic flavonoids, and well known to have antibacterial and antifungal properties (Govindachari TR *et al*., 1998; Girish, 2008 & Alzohairy MA, 2016).

Thus, in the present study, at first we synthesized silver nanoparticles using leaf and bark extracts of *A. indica.* We have characterized the resulting nanoparticles by ultraviolet-visible (UV-Vis) spectroscopy and minimum inhibition test (MIC).Finally, we evaluated and compared antimicrobial activity of both plant extracts with and without Ag-NPs. To the best of our knowledge, this is the first report in the literature on nanoparticle synthesis for enhancing antibacterial activity using this plant.

2. MATERIALS AND METHODS

2.1 Collection and preparation of plant extracts

The fresh *A. indica* leaves and barks were collected from Khulna University campus, Khulna, Bangladesh and safely taken to laboratory for further experimental analysis. Collected leaves and barks were carefully cleaned and sliced into small pieces. The sliced plant materials were dried under sunshade and air for several weeks. After the completion of drying, the plant materials were grounded into powder with a grinder machine. The powder was weighted and soaked into 50% ethyl acetate (50g powder within 100ml ethyl acetate) individually. They were then sealed

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and kept for 168 hours in a dark room accompanying occasional shaking and stirring. Finally, the mixtures were filtered with meracloth and stored at 4^0C until for next experiment.

2.2 Green synthesis of Ag nanoparticles

Silver nitrate salt (AgNO3 M.W. 169.88) was used for the synthesis of silver nanoparticles. For preparation of 300 ml silver nitrate solution, 0.0507g silver salt was dissolved in distilled water by continuous stirring with the help of magnetic stirrer for 5 min. Then the solution container was sealed with foil paper and kept into a dark place to avoid reduction reaction. 50ml of freshly prepared 1mM silver nitrate solution was added to 5ml of each sample solution (leaf and bark extracts). Then the mixture was exposed into direct sunlight to drive nanoparticle formation for about 30 min until visible colour change (brown colour) was observed.

2.3 Characterization by UV-Vis Spectral analysis

The UV–VIS spectroscopy was used to examine the reduction of Ag+ from $AgNO_3$ and finally to confirm the biosynthesis of silver nanoparticles and the absorbance was measured from 0min of preparing the reaction mixture to 120 min with the regular interval of 30 minutes at different wavelengths (300nm to 700nm).

2.4 Antibacterial activity tests

The *in vitro* antibacterial activity of plant extracts with and without silver nanoparticles was assessed quantitatively by disc diffusion assay and the results were validated by determining the Minimum Inhibitory Concentration (MIC) values of test samples. For disc diffusion assay, 24hrs old grown bacterial subcultures were inoculated in nutrient broth and poured into petri plates containing Muller Hinton Agar (MHA) media (HiMedia Laboratories) and were spread gently with a sterile spreader. Antibacterial activity of the samples was tested against four gram positive and eight-gram negative pathogenic bacterial isolates (Table 1). These bacterial cultures were previously isolated and characterized by Microbiology Laboratory, Biotechnology and Genetic Engineering Discipline, Khulna University, Bangladesh. Then the sample discs (concentration for leaf were 45µg/disc and 90µg/disc and for bark were 30µg/disc and 60µg/disc), antibiotic disc (20µl/disc) as a positive control and blank disc (negative control) were inserted on the spread plates and kept into incubator for 24hrs at 37°C. The diameter of the zone of inhibition (ZOI) was measured and the mean value was calculated.

To support the results obtained from disc diffusion assay, minimum inhibitory concentration (MIC) assay was performed by micro broth dilution method. Briefly, at first stock solution was prepared by dissolving plant extract in 10% dimethyl sulfoxide (DMSO) to make a final concentration of 20mg/ml. Eight Eppendorf tube (2 ml) were taken for MIC assay. In the first tube 1ml of stock solution were taken and in other tubes 0.5ml of broth supplemented with glucose and phenol red. For micro dilution, 0.5 ml of stock from the first tube was transferred to 0.5 ml broth on the next tube, and then serially diluted to rest of the tubes. Then, 10µl of bacterial suspension (4.5×10^5cfu/ml) was added to each tube and placed in an incubator at 37°C for 18–24 hrs. After the incubation period, colour change of the mixture tube was assessed visually. Any colour changes from red to yellow or colourless were recorded as positive. The lowest

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concentration at which colour change occurred was taken as the MIC value. The average of three values was calculated and that was the MIC for the test material and bacterial strain.

3. RESULTS AND DISCUSSION

Present study was conducted with a view to synthesize Ag-NPs by means of a biological method (green method) and then assess its impact on the antibacterial activity of a medicinal plant.

3.1 Synthesis and confirmation of nanoparticles

For small scale formation of Ag-NPs, plant (leaf and bark) extracts were prepared using 50% ethyl acetate and then kept under sunlight for the formation of Ag-NPs. The formation of nanoparticles was initially confirmed by visual observation (colour change) and finally by UV-Vis Spectroscopy. After 30 min exposure, visible colour change (from transparent to dark brown) was observed which indicated the formation of silver nanoparticles (Figure 1) (Syafiuddin A *et al*., 2017). This colour change occurred due to the reduction of silver ions by terpenoids (Azadirachtin, nimbin, nimbidin) and flavonoids (quercetin) present in the plant extracts (Atawodi SE *et al*., 2009). Since the leaf and stem of *A. indica* contains polyphenols, it was anticipated that the plant extracts would serve as reducing agent for nanoparticle synthesis. Actually, the same molecular mechanisms that give antioxidant properties to these molecules must promote the reduction of Ag+ ions to Ag atoms (Ericka RL *et al*., 2013). The main mechanism is hydrogen abstraction (Sivaraman SK *et al*., 2009) due to the OH groups in the polyphenol molecules. Finally, after 120 minutes, there was no change in the intensity of colour development, which indicated the completion of reduction reaction.

The change in colour, and thus the formation of silver nanoparticles, was confirmed by the UV-Vis experiments. For UV-VIS spectral analysis, absorbance bands of the samples were recorded in the range of 300 to 700 nm at five time intervals (0, 30, 60, 90 and 120 min). At the beginning of the reaction, the absorbance of the mixture was 0 as there were no synthesis occurred. With the increase of time, the absorbance of samples also increased gradually indicating the formation and increasing concentration of Ag-NPs. The intensity of the peaks (for both leaf and bark extracts) were recorded highest around 400nm. The UV-Vis peak is more pronounced for higher AgNO3 concentrations (Fig 2 & 3), indicating that more nanoparticles per unit volume are formed when this concentration increases (Ericka RL *et al*., 2013). The UV-VIS absorption spectrum gave a characteristic surface plasmon resonance absorption band centred at 400nm. The shape of the plasmon bands were almost symmetrical suggesting well-dispersed and uniform-sized nanoparticle synthesis (Tran QH *et al*., 2013). The overall band patterns indicated that the Ag-NPs prepared by leaf extract were very stable without aggregation.

3.2 Antibacterial activity of plant extracts with and without Ag-NPs:

Initially 12 bacterial isolates (Table 1) were used to assess antibacterial activity of crude leaf and bark extracts. Among these isolates, two gram positive (*Micrococcus* and *Staphylococcus aureus*) and four gram negative (*E. coli, Vibrio cholerae, Salmonella typhi and Proteus vulgaris*) isolates were selected based on their susceptibility against the test plant samples. The selected bacterial isolates showed prominent and better zone of inhibition compared to others. These 6 bacterial isolates were then used for further assessment of plant extracts with and without Ag-NPs.

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Among the 6 bacterial isolates, *S. aureus* (gram negative) showed highest zone of inhibition in case of both plant extracts. The diameter of zone of inhibition was 13.00±1.00 mm when it (*S. aureus*) treated with leaf extracts and 14.83±2.26 mm with bark extracts. Later, antibacterial activity of both plant extracts and synthesized Ag-NPs were tested against previously used 6 bacterial isolates. Enhancement of antimicrobial activity by addition of Ag-NPs was also reported before with other plant extracts (Tahany GM *et al*., 2015; Prasad TN *et al*., 2011; Moyo M *et al*., 2015; Satish V Patil *et al*., 2012 & Dobrucka R *et al*., 2015). In this experiment, synthesized Ag-NPs significantly enhanced the antibacterial activity of both plant extracts against all tested bacteria. Among all tested bacteria, *S. aureus* showed highest zone of inhibition compared to others when treated with plant extracts. In case of leaf extract, Ag-NPs increased the diameter of zone of inhibition by 14.33±0.36 mm. Similarly, in case of bark extract, nanoparticles increased the diameter of zone of inhibition by 15.17±0.34 mm. However, in comparison to leaf and bark extracts, bark extract was found more effective than leaf against all tested microorganisms (Figure 5 & 6).

For further confirmation of the above-mentioned effects of Ag-NPs, MIC test was performed with all samples against the same bacterial isolates. MIC value was significantly low (0.078 mg/ml and 0.039 mg/ml for leaf and bark extract respectively) containing nanoparticles compared to that (0.312 mg/ml) of only crude plant extracts (Table 4). Thus, presence of Ag-NPs in both plant samples enhanced their antibacterial activity to a significant level.

4. CONCLUSION

Synthesis of nanoparticles is of current interest for applications such as catalysis, electronics (Lu W*et al*., 2007; Lugli P *et al*., 2010; Karni TC *et al*., 2012 & Mitin VV *et al*., 2008), photonics (Shen Y*et al*., 2000; Zalevsky Z *et al*., 2009 & Taylor A, 2008), catalysis (Kalidindi SB *et al*., 2012; Serp P et al., 2013 & Kung HH *et al*., 2007), medicine (Shomura Y, 2011; Jotterand F *et al*., 2011; Lee H *et al*., 2007; Etheridge ML *et al*., 2013 & Mata A *et al*., 2012) etc. This increasing demand must be accompanied by "green" synthesis methods to reduce globally generated hazardous wastes. Here in this experiment, we strived to synthesize Ag-NPs by means of biological (green) method. We have prepared silver nanoparticles using extracts of *A. indica*, a plant abundantly found in Bangladesh, as reducing agent. The results are very promising since the extract promotes the formation of nanoparticles at room temperature with a fast kinetics and with no harmful chemicals. Our method is easy to perform in a single step. UV-Vis spectroscopy experiments show that *A. indica* is a plant rich in polyphenols, such as catechines and stilbenes, molecules that have antioxidant activity. The same molecular mechanisms responsible of the antioxidant activity allow the use of these molecules as reducing agents and stabilizing effects for silver nanoparticles. The silver nanoparticles synthesized by this method are strong candidates for its use in biological systems. The silver nanoparticles obtained by this method definitely opened a new route to study catalytical activity, antimicrobial properties, and the optical response of this nanomaterial.

Emergence of resistance to multiple antimicrobial agents in pathogenic bacteria has become a significant public health threat as there are fewer, or even sometimes no, effective antimicrobial agents available for infections caused by these bacteria. Gram-positive and Gram-negative

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bacteria are both affected by the emergence and rise of antimicrobial resistance (Govindachari TR *et al.*, 1998). The synthesized silver nanoparticles showed efficient antimicrobial activities against both gram positive and negative bacterial isolates. Benefits of using plant extract for synthesis is that it is energy efficient, cost effective, protecting human health and environment leading to lesser waste and safer products. This eco-friendly method could be a competitive alternative to the conventional physical/chemical methods used for synthesis of silver nanoparticle and thus has a potential to use in biomedical applications and will play an important role in opto-electronics and medical devices in near future.

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Author's contribution

AH designed the experiments, guided the entire study, participated in data analysis, wrote and extensively revised this manuscript. SK performed the research and participated in drafting this manuscript. AA initiated the project, guided the study and revised the manuscript. All authors participated in the research and approved the final manuscript.

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Table 1: List of bacterial isolates primarily used to test antibacterial activity of crude plant samples

Gram positive bacteria	Gram negative bacteria
Staphylococcus aureus	*Vibrio cholera*
Micrococcus	*Salmonella paratyphi*
Mycobacterium	*S. typhi*
Epidermidis	*Escherichia coli*
	Proteus vulgaris
	Shigella dysenteriae
	S. flexneri
	Campylobacter

Table 2: Antibacterial activity of leaf and bark extracts without Ag-NPs against six bacterial isolates

List of bacteria	Gram Staining	Zone of Inhibition					
		Neem Leaf (Crude)		Neem Bark (Crude)		Control	
		45µg/disc	90µg/disc	30µg/disc	60µg/disc	Positive control (Ciprofloxacin) (20µg/disc)	Negative control (DW*)
Escherichia coli	-ve	7.42±0.72	10.17±0.76	9.17±0.76	11.50±2.18	23.00±2.00	n.d**
Micrococcus	+ve	6.17±0.76	10.00±1.00	7.72±1.8	13.50±2.18	28.00±3.60	n.d
Vibrio cholera	-ve	5.72±0.85	9.5±1.80	8.25±1.96	13.83±1.50	30.00±1.00	n.d
Salmonella typhi	-ve	7.33±1.04	11.33±1.53	5.33±0.76	9.50±1.04	30.67±2.30	n.d

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Staphylococcus aureus	+ve	8.33±0.76	13.00±1.00	9.50±1.50	14.83±2.26	34.33±2.08	n.d
Proteus vulgaris	-ve	7.83±1.76	13.00±2.00	7.17±0.76	12.83±1.26	30.33±1.53	n.d

* Distilled Water

** No Dimension

Table 3: Antibacterial activity of leaf and bark extract with Ag-NPs against six bacterial isolates

List of bacteria	Gram Staining	Zone of Inhibition					
		Ag-NPs From Leaf extract		Ag-NPs From Bark extract		Control	
		45µg/disc	90µg/disc	30µg/disc	60µg/disc	Positive control (Ciprofloxacin) (20µg/disc)	Negative control (DW*)
Escherichia coli	-ve	20.67±2.3	22.00±1.6	22.00±1.00	28.00±2.08	32.00±2.00	n.d**
Micrococcus	+ve	20.33±1.7	25.00±1.00	25.25±0.58	28.33±1.00	28.00±3.60	n.d
Vibrio cholera	-ve	24.67±1.19	26.00±1.73	24.33±0.58	28.67±0.58	33.00±5.19	n.d
Salmonella typhi	-ve	17.33±1.33	19.67±0.33	24.00±1.00	27.33±0.58	30.67±2.30	n.d
Staphylococcus aureus	+ve	24.33±1.20	27.33±1.36	28.00±2.64	30.00±2.60	34.33±2.08	n.d
Proteus vulgaris	-ve	19.50±0.50	26.75±0.50	24.00±1.00	25.67±2.41	27.33±1.00	n.d

* Distilled Water

** No Dimension

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Table 4: MIC value (mg/ml) of leaf and bark extracts with and without Ag-NPs against 6 test organisms

Organisms	Leaf extracts Crude	Leaf extracts with Ag-NPs	Bark extracts Crude	Bark extracts with Ag-NPs	Positive Control (Ciprofloxacin)
Escherichia coli	1.25	0.312	0.625	0.156	0.00030
Micrococcus	2.5	0.625	2.5	0.312	0.00098
Vibrio cholera	1.25	0.156	0.625	0.078	0.00015
Salmonella typhi	10.0	2.5	5.0	0.312	0.00061
Staphylococcus aureus	0.312	0.078	0.312	0.039	0.00013
Proteus vulgaris	0.625	0.156	1.25	0.078	0.00049

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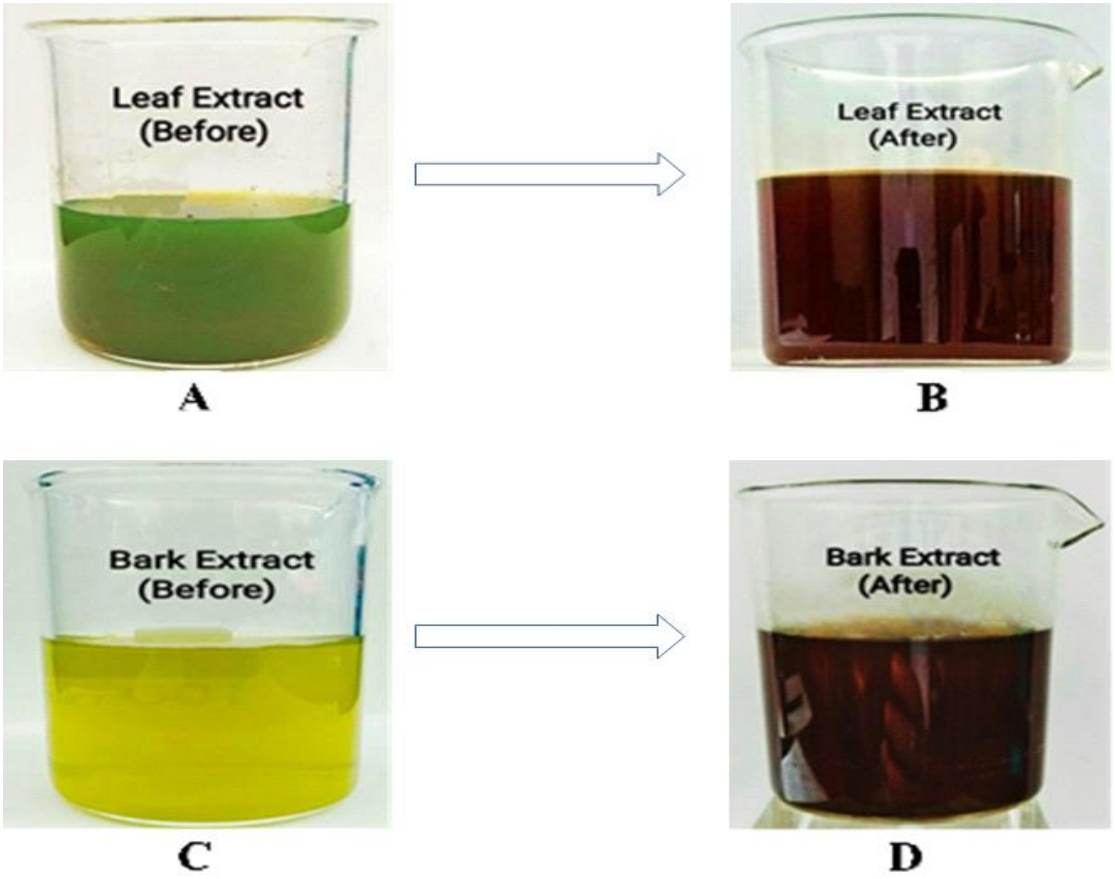

Figure 1: Confirmation of nanoparticle formation by visual observation: Leaf extract before exposure to sunlight (A) and after 30 min of exposure (B). Similarly bark extract before exposure (C) and after 30 min exposure (D).

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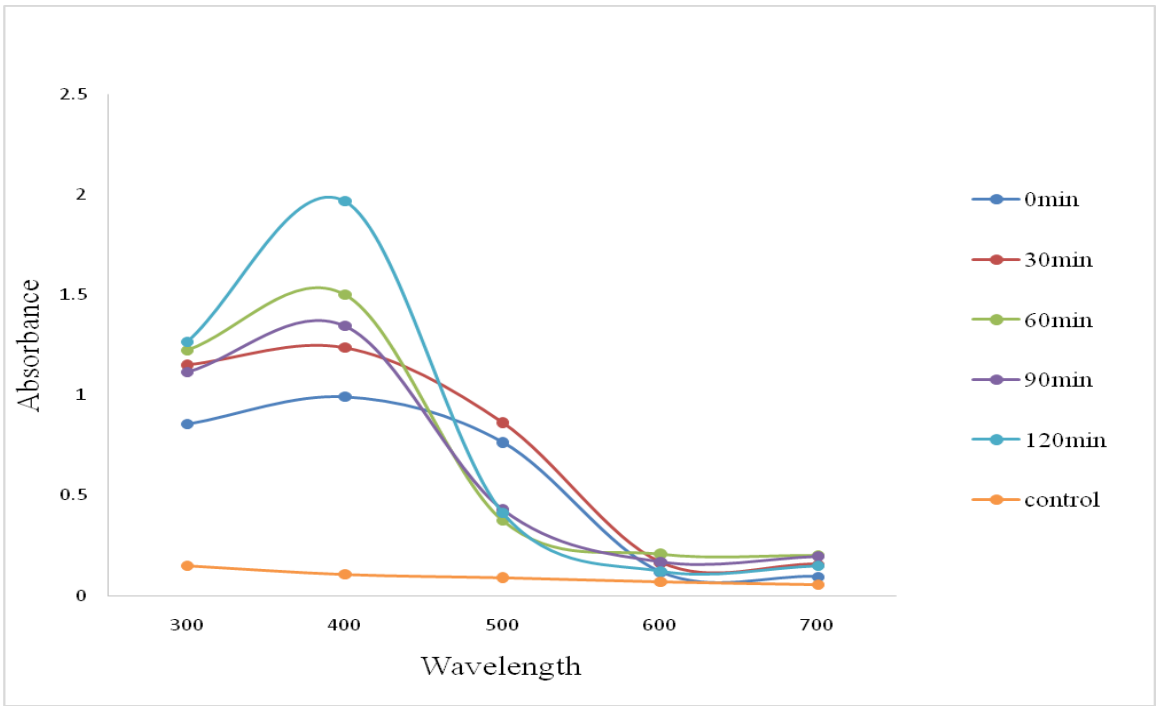

Figure 2: UV-VIS spectral analysis of *A. indica* leaf extracts and control.

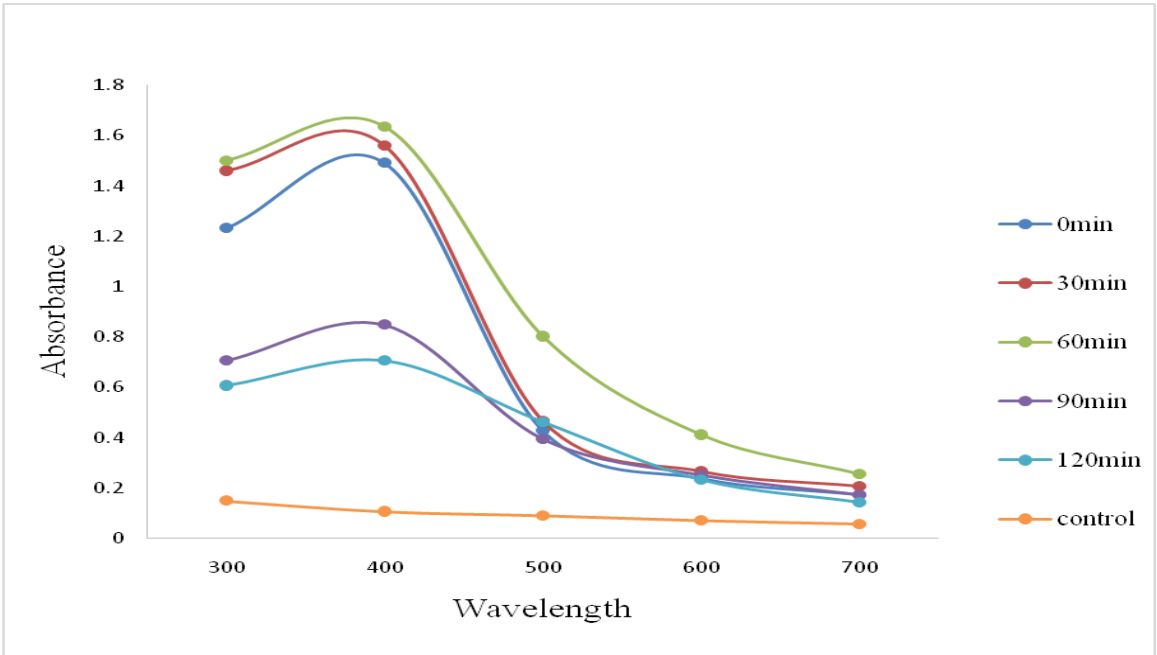

Figure 3: UV-VIS spectral analysis for *A. indica* bark extracts and control.

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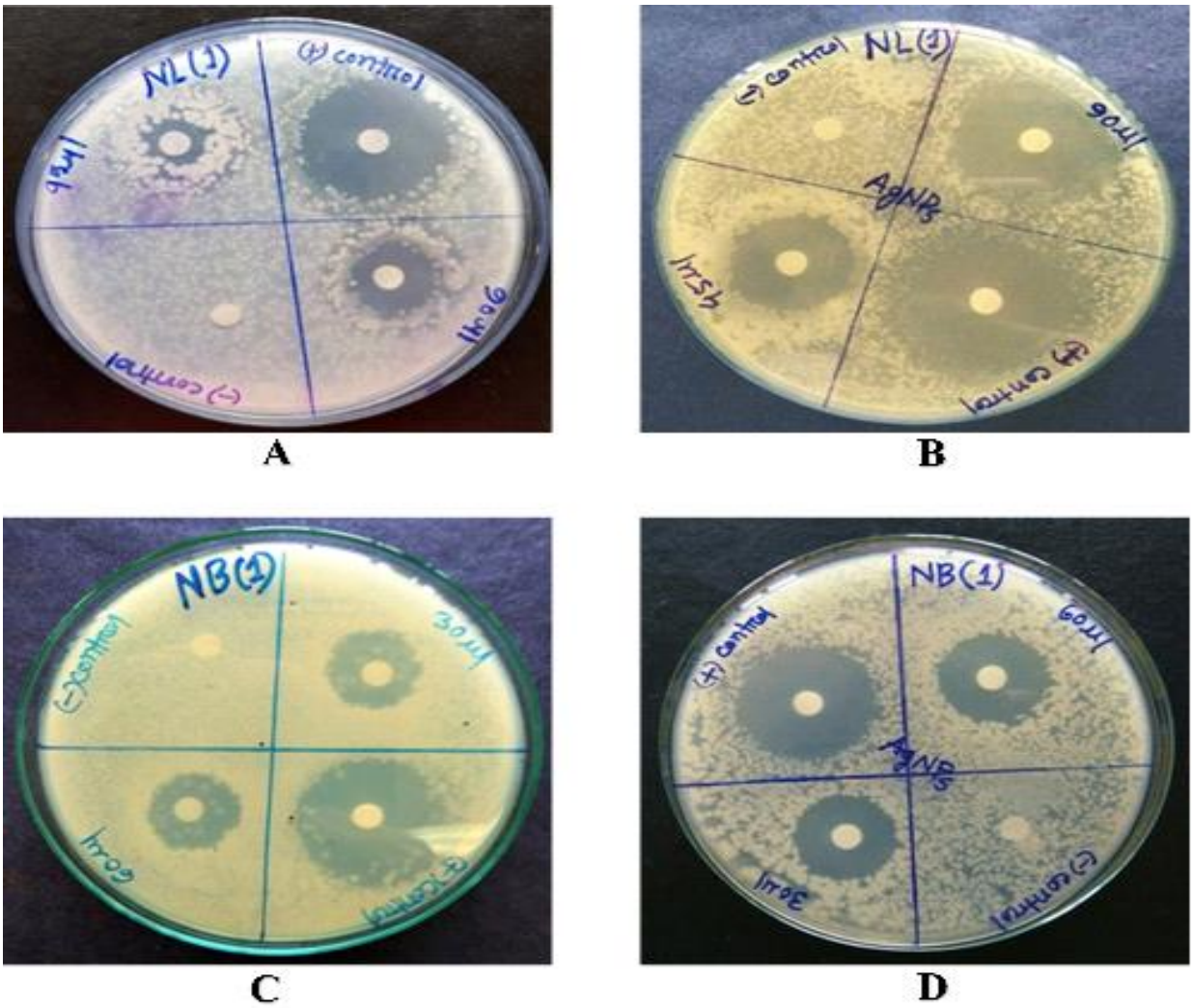

Figure 4: Antibacterial activity of leaf and bark extracts of *A. indica* against *E. coli* by disc diffusion method: A) Crude leaf extracts; B) Leaf extracts with Ag-NPs; C) Crude bark extracts and D) Bark extracts with Ag-NPs.

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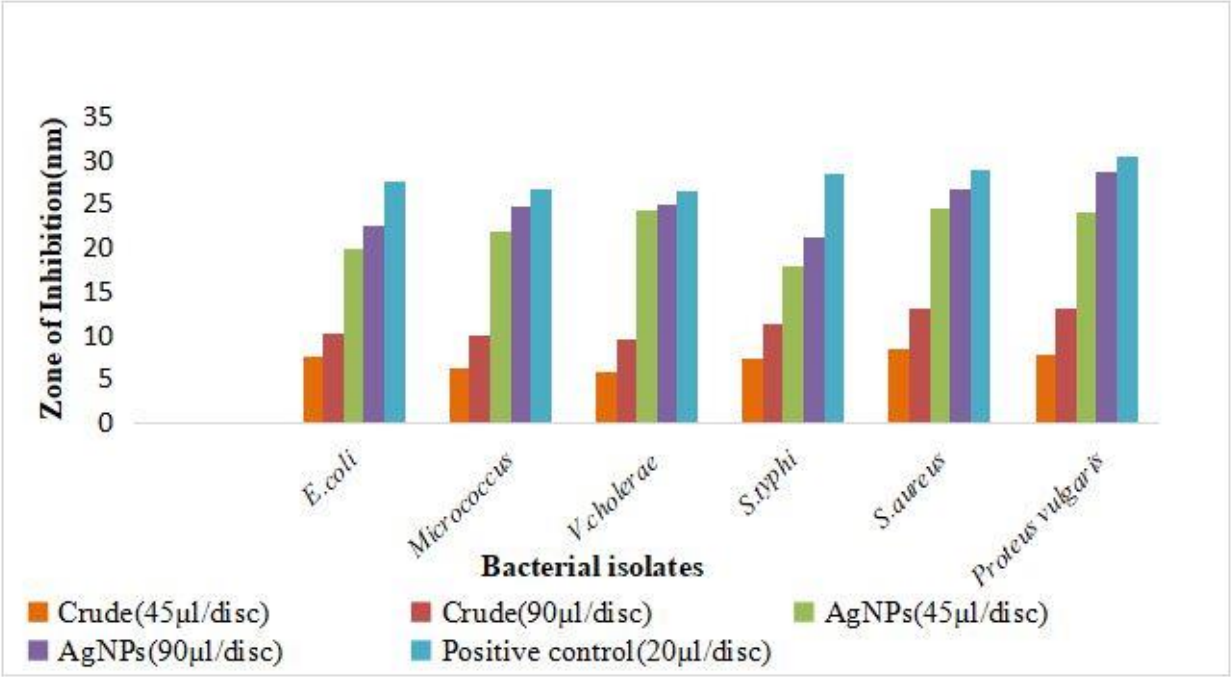

Figure 5: Comparison of the antibacterial activity between crude leaf extract and leaf extract with synthesized Ag-NPs against six bacterial isolates.

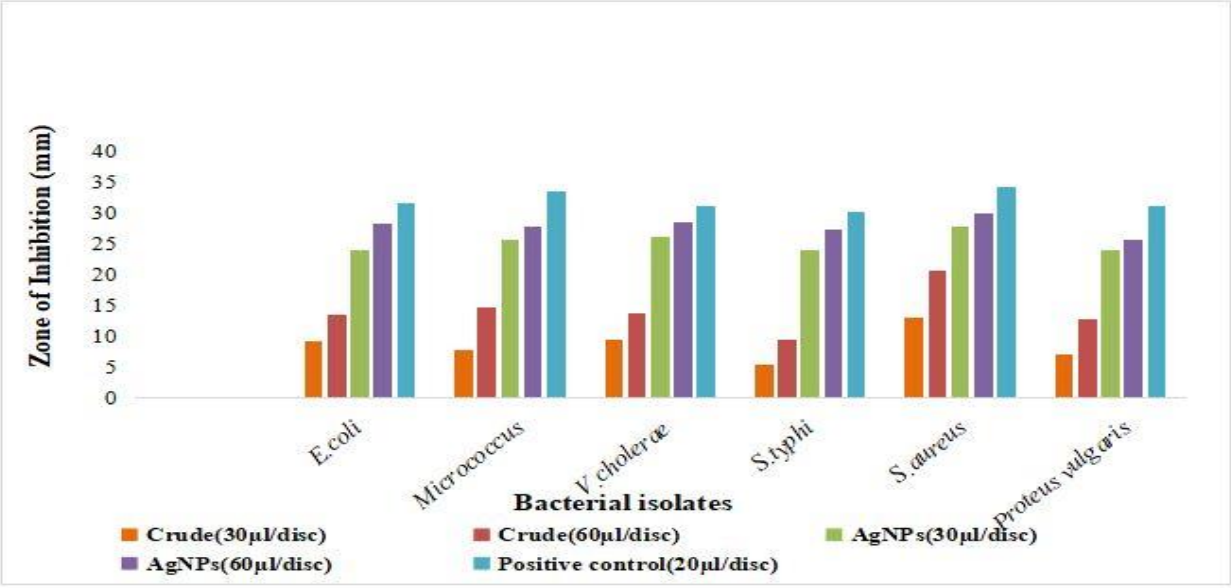

Figure 6: Comparison of the antibacterial activity between crude bark extract and bark extract with synthesized Ag-NPs against six bacterial isolates.