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Volume 04, Issue (1), Page: 91-100, 2022

Ministry of Science and Technology
Government of The People's of Bangladesh
Bangladesh Secretariat, Dhaka.

Evaluation of the Synergistic Effect of *Azadirachta indica*-based Silver Nanoparticles in Combination with Antibiotics and Hypoglycemic Drugs: *In vitro* Antimicrobial and *In vivo* Antidiabetic Activities

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Abstract

This study was conducted to evaluate the synergistic effects of the mixtures of green silver nanoparticles (AgNPs) with ampicillin and metformin separately in obviating the global threats of antimicrobial resistance and diabetes mellitus. AgNPs was synthesized using *Azadirachta indica* aqueous leaf extract at varying extract concentration and incubation time. The synthesis of AgNPs in aqueous solution was confirmed by the visual color change and by using a UV-Vis spectrophotometer. The biosynthesized AgNPs was further characterized using Scanning Electron Microscope (SEM) analysis. The experimental results revealed that the biosynthesized AgNPs were polydispersed, smaller in size and aggregated. The mean diameter of the formed AgNPs was 50 nm as evident from the SEM analysis. Additionally, the mixtures of AgNPs with ampicillin and metformin exhibited synergistic *in vitro* antimicrobial and *in vivo* antidiabetic effects i.e., being more efficacious than AgNPs, ampicillin and metformin alone.

Received: 31.08.2022
Revised: 27.03.2023
Accepted: 10.05.2023

DOI: https://doi.org/10.3329/jscitr.v4i1.67372

Keywords: *Azadirachta indica*; Silver nanoparticles; Characterization; Synergism; Antimicrobial and Antidiabetic activities.

Introduction

The antimicrobial resistance (AMR) and diabetes are among the leading causes of death worldwide. The development of resistance can be the result from inadequate selection, overdose and misuse of antimicrobial agents (Zhang *et al*., 2011). On the other hand, diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both (Guariguata *et al*., 2014). Commercially available hypoglycemic agents have various side effects which greatly limit their wide application in the clinic (Bonnefont *et al*., 2000). Thus, there is an urgent need to develop more effective and safe oral antimicrobial and antidiabetic agents with high degree of specificity as well as to achieve maximal therapeutic efficacy with minimal side effects (Etxeberria *et al*., 2012). Recently a new strategy called "Nanotechnology" has been developed to obviate the above two global threats.

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"Nanoparticles (NP)" are products of Nanotechnology having particle sizes of 1–100 nm (Chen *et al*., 2013). Metallic NPs are commonly synthesized by traditional chemical and physical methods (Nath and Banerjee, 2013) which require the use of hazardous chemicals and high energy. Biological synthesis of nanoparticles involves the use of natural materials such as plants, bacteria, fungi (Shawkey *et al*., 2013; Mollick*et al*., 2015; Donda *et al*., 2013). However, the use of plant extracts has advantages over other biological methods due to their viability, low cost, eco-friendliness etc (Iravani, 2011; Rath *et al.,* 2014). *Azadirachta indica* is the most widely used traditional medicine in Indian subcontinent due to its antibacterial (Ghonmode *et al*., 2013), antifungal and antidiabetic (Sher 2009) properties. Therefore, in this study, we have developed a protocol of AgNPs biosynthesis using *A. indica* aqueous leaf extract.

Synergistic action is now commonly used to describe an interaction of two or more drugs or occasionally more than two (Berenbaum 1978) in which the effect produced by the drugs in combination is greater than the sum of their individual effects when the drugs are used alone (Jawetz 1968). The synergistic antimicrobial activity of plant-based AgNPs in conjugation with antibiotics has been evaluated (Hari *et al*., 2014), however, no study has investigated yet the *in vitro* synergistic antimicrobial effect of *A. indica* based AgNPs in combination with antibiotics. Therefore, we have investigated the antimicrobial and antidiabetic activities of biogenic AgNPs alone and also the synergistic action of AgNPs with ampicillin and metformin against different pathogens and in Streptozotocin-induced diabetic rats, respectively.

Materials and Methods

Collection and preparation of plant extract

Fresh and healthy leaves of *Azadirachta indica* were collected from locally grown trees of the campus of Islamic University, Kushtia, Bangladesh. The leaf surfaces were cleaned under running tap water followed by double-distilled water to remove all the dust and unwanted visible particles. Leaves (2g) were finely incised after air drying at ambient temperature and separately taken in 250mL beakers containing 100mL double-distilled water and boiled for about 20min. The boiled solutions in each after cooling were filtered separately through Whatman filter paper no.1 and stored at 4°C for further study.

Biosynthesis, optimization and separation of AgNPs

The biosynthesis of AgNPs was carried out by following the procedures of Asimuddin *et al*.,(2020). The optimization of AgNPs biosynthesis was carried out by mixing the aqueous extract (1 to 7mL) with silver nitrate ($AgNO_3$) solution keeping the final concentration of the mixtures always to 1mM. The mixtures were subsequently incubated at 85°C in a hot air performance incubator (AP120, Froilabo, France) at different time intervals such as 10, 20, 30, 40, 50, 60, 120, 180, 240, and 300min, respectively. The UV-Vis spectrum of each of the mixture at each time interval was carried out in order to confirm the optimum extract concentration and incubation time. Furthermore, the color progression of the biosynthesized AgNPs using 1 to 7mL extract incubated at 85°C for 180min was observed to optimize the extract concentration. The separation and purification of the synthesized AgNPs from the reaction mixtures was performed by continuous centrifugation (9000 rpm; 20min; 4°C) with sterile double-distilled water. The obtained pellets were repeatedly washed (3-4 times) with water to ensure better separation of the AgNPs from other contaminants. After drying the pellets at 60°C in an oven, the dried AgNPs were kept at 4°C for further characterization.

Characterization of AgNPs

The average particle size, shape and morphology of the produced AgNPs were studied by the high-resolution images of nanoparticles recorded by field emission scanning electron microscope (FESEM) (JSM-7610F, JEOL Ltd. Japan) using an acceleration voltage of 15 kV.

Antimicrobial activity evaluation

The Kirby-Bauer test was used to evaluate the antimicrobial activity against *Klebsiella pneumoniae*, *Bacillus subtilis*, and *Serratia marcescens* following the procedure as described previously (Garibo *et al*., 2020). At first Whatman No 1 cellulose filter paper was cut into discs (6mm), soaked with 50µL of the test solutions: (i) disc with extract; (ii) disc with 1mM $AgNO_3$ solution; (iii) discs with AgNPs; (iv-v) mixtures of AgNPs and ampicillin; and air dried for 10 min in sterile condition. An inoculum of 0.1mL (0.5 McFarland standards) of overnight-grown test microorganisms was uniformly spread on plates containing nutrient agar media. The previously dried five types of test discs in addition to the standard antibiotic disc (vi) ampicillin (25µg), were placed on the surface of each plate. Subsequently, the plates were incubated at 37°C for 24h in a bacteriological incubator followed by measuring the zone of inhibition (ZOI) against each type of test microorganism. The standard antibiotic was used as a positive control.

In vivo investigation of antidiabetic activity

Experimental design

Healthy long Evan rats (120–180g) were obtained from the Animal House of the Dept. of Applied Nutrition and Food Technology, Islamic University, Kushtia. Rats were housed in hygienic polypropylene cages maintained in a well-ventilated room at 27°C. Rats were divided into five groups having six animals in each and were treated as follows: Group I, normal control group, administered 0.5mL of 0.1mol/L citrate buffer (pH 4.5) by gavage; Group II, diabetic control group, with diabetes induced by a single i.p. injection of 50mg/kg streptozotocin (STZ); Group III, STZ-diabetic rats treated with AgNPs (10mg/kg body weight): Group IV, STZ-diabetic rats treated with AgNPs (10mg/kg body weight) and metformin (30mg/kg body weight): Group V, STZ-diabetic rats treated with metformin (30mg/kg body weight). Blood glucose levels were measured by a glucometer (Gluco Leader Enchance, UK) for all the animals. The rats with blood glucose levels of 14mmol/L or above were chosen for the study. The treatment protocol was continued for 28 days.

Determination of blood glucose level

The experimental animals were made to fast for 12–16h and bloodsamples were collected by "Rupturing tail vein method" in the experimental animals. Blood samples were collected on days 1, 7, 14, 21, and 28.

Statistical analysis

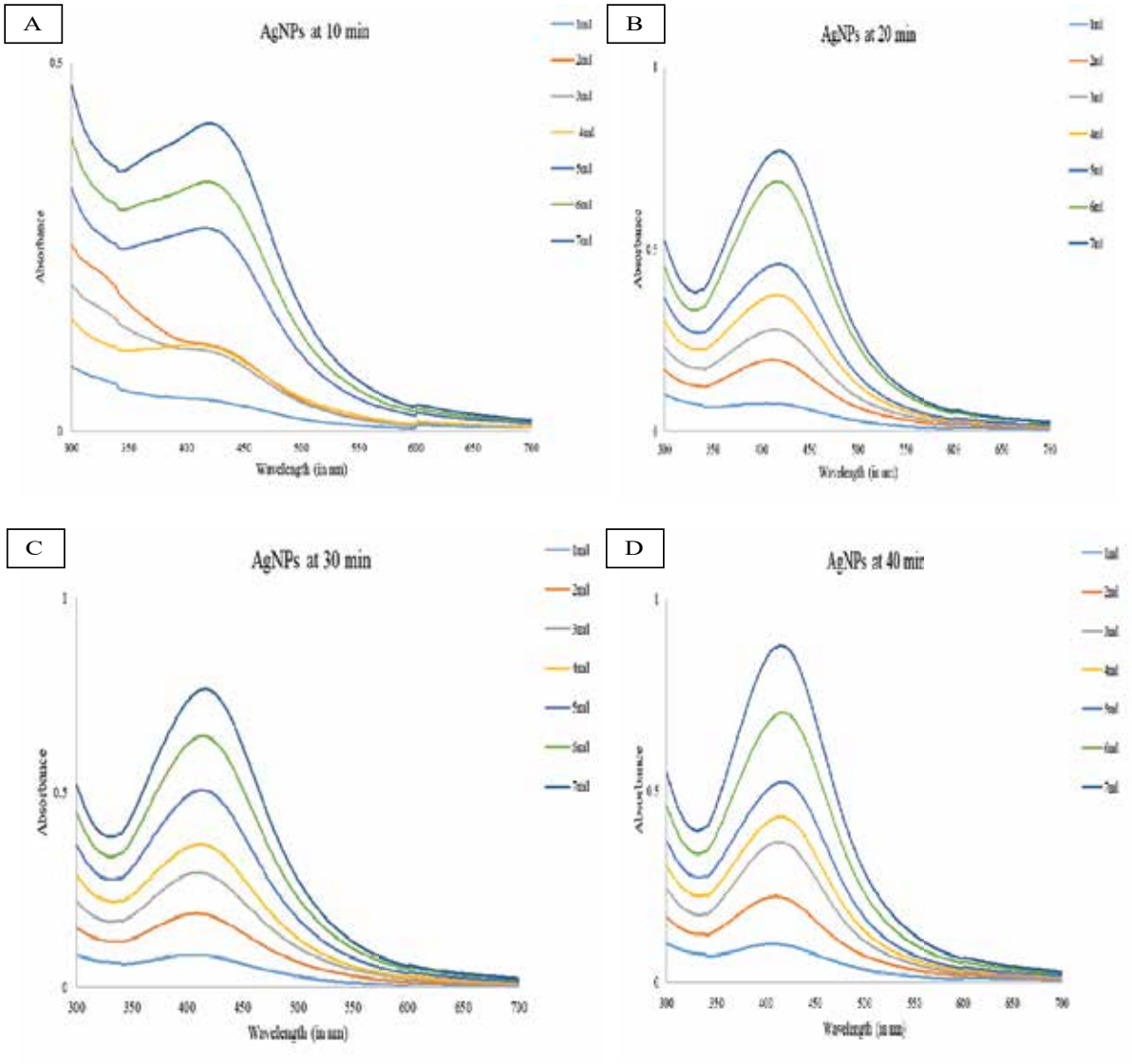
All data are expressed as Mean ± standard deviation (mean of three determinations).

Results and Discussion

Confirmation of the biosynthesized AgNPs

The formation of *A. indica*-mediated AgNPs in aqueous solution was primarily confirmed by the visual color change of the reaction mixtures as well as by UV-Vis spectrophotometer. The biogenic AgNPs initially showed yellowish color due to the excitation of surface plasmon vibrations in the metal nanoparticles (Veerasamy *et al*., 2011). Previous studies (Ahmed *et al*., 2016; Roy *et al*., 2017) have also reported similar changes in color due to the reaction between leaf extract and $AgNO_3$.

Fig. 1 (A-J) shows the UV-visible absorption spectra of the reaction mixtures with varying quantities of *A. indica* leaf extract (1 to 7mL) were recorded after time intervals of 10, 20, 30, 40, 50, 60, 120, 180, 240, and 300min, respectively from the initiation of reaction. AgNPs usually exhibit a SPR band due to the free electron excitation in the visible range of 400–500 nm by UV-Vis absorption spectroscopy (Sastry*et al*., 1997), however, not below than 390 nm. In all the cases, the absorption bands were exhibited within the range, therefore, confirmed the formation of AgNPs. Moreover, as the quantities of the plants extract and the reaction time increased, the absorbance intensity of the reaction mixtures also increased.



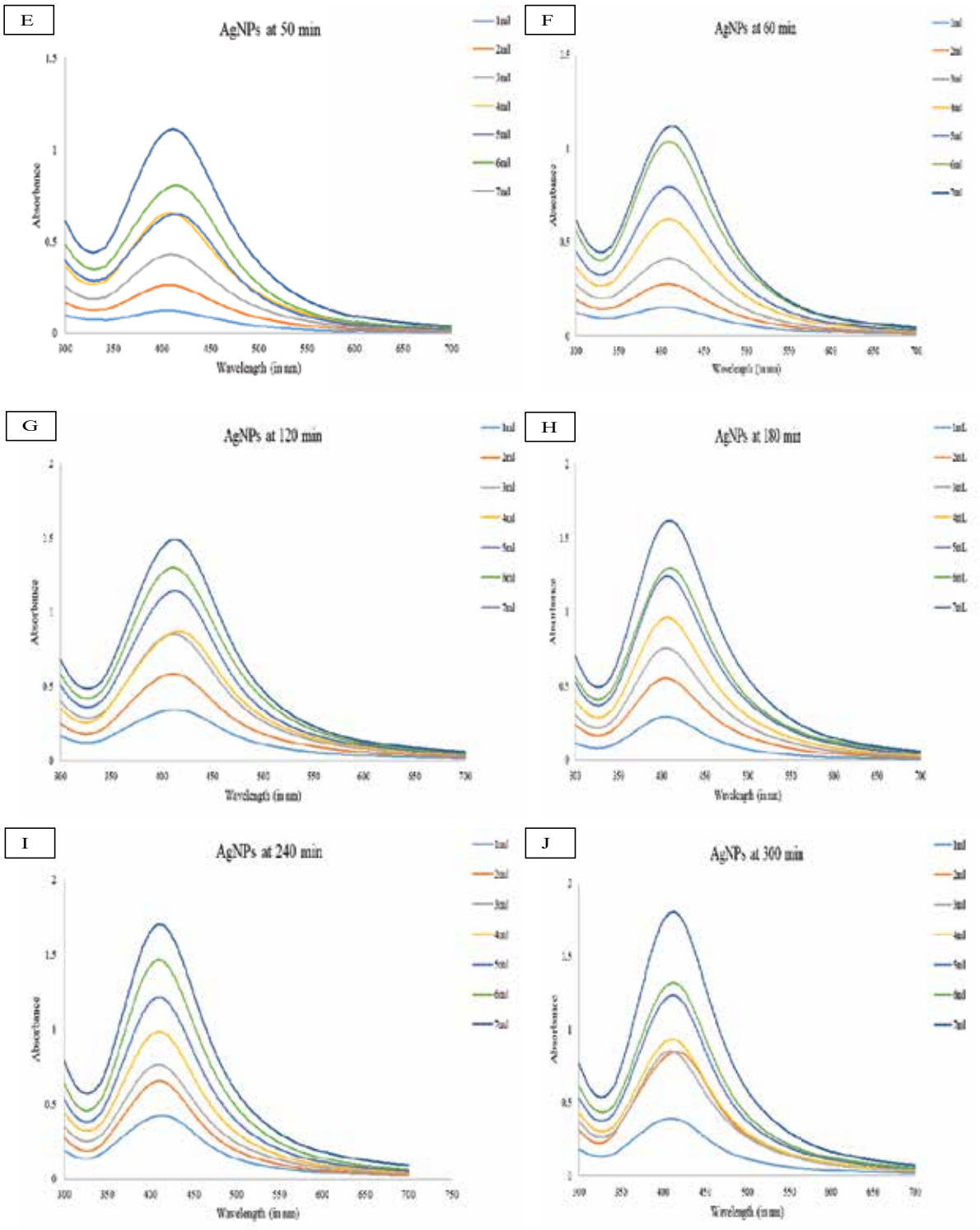


Fig. 1 (A-J). The absorption spectra of AgNPs formed from different concentrations of *A. indica* leaf extract (1 to 7mL) at different time intervals of 10, 20, 30, 40, 50, 60, 120, 180, 240, and 300min, respectively incubated at 85°C.

These results are in agreement with previous studies that reported the enhancement of absorbance intensity of reaction mixtures with increasing the quantities of the plant extracts and the reaction time (Ahmed *et al*., 2016; Roy *et al*., 2017). The increase in absorbance intensity with increasing plant extracts indicates a higher abundance of phytochemicals in the reaction solutions and consequently, an increased reducing power (Altemimi *et al*., 2017). Moreover, AgNPs generated using 5mL of extract at180min (3h) of reaction showed that the maximum absorbance was 1.241 at 408 nm. However, further increasing the extract concentration and reaction time resulted in the SPR band shifted towards red suggesting that 5mL *A. indica* leaf extract and incubation for 180min may be the optimum extract concentration and reaction time for the formation of small-sized AgNPs. However, these findings could be further confirmed by visual observation of the color progression of the biosynthesized AgNPs using 1 to 7mL extract incubated at 85˚C for 180 min. Moreover, it has been reported that the peak position of the spectra can be used to roughly predict the size of AgNPs such as a maximum absorption peak near at 408 nm wavelength indicates a size of approximately 30-40 nm.

Fig. 2. Color progression of AgNPs using 1 to 7mL of *A. indica* leaf extract incubated at 85°C for 180min.

Fig. 2 shows the photographs of the reduction of Ag^+ to Ag^0 by means of color development of the reaction mixtures between 1-7mL extract incubated at 85˚C for 180min. The visible color changes occur from colorless to light yellow followed by light reddish brown which further changed to reddish brown and finally to deep red as the quantity of plant extract increased. Therefore, the color intensity increased as the concentration of leaf extract (1-7mL) increased. Moreover, the deep red color seems to be stayed unchanged in the reaction mixtures from 5mL to 7mL extract which indicates the complete bioreduction of Ag^+ to Ag^0. Therefore, 5mL *A. indica* leaf extract is the optimum concentration for small-sized AgNPs formation.

Scanning electron microscopic (SEM) analysis

Fig. 3 shows the morphology of the biosynthesized AgNPs through SEM. AgNPs were polydispersed, smaller in size and aggregated. The average size of the synthesized AgNPs was in the range of 30-50 nm determined by SEM which is almost the same as predicted by the UV-spectra.

Determination of antimicrobial activity

The results of antimicrobial activities of *A. indica* leaf extract, 1mM $AgNO_3$ solution, *A. indica*-mediated AgNPs, standard ampicillin antibiotic disc, and *A. indica*-mediated AgNPs mixed with ampicillin against three different bacterial strains are given in Fig. 4 and Table 1. The table shows that AgNPs exhibited excellent antimicrobial activity against all strains as compared to its respective leaf extract (Ibrahim

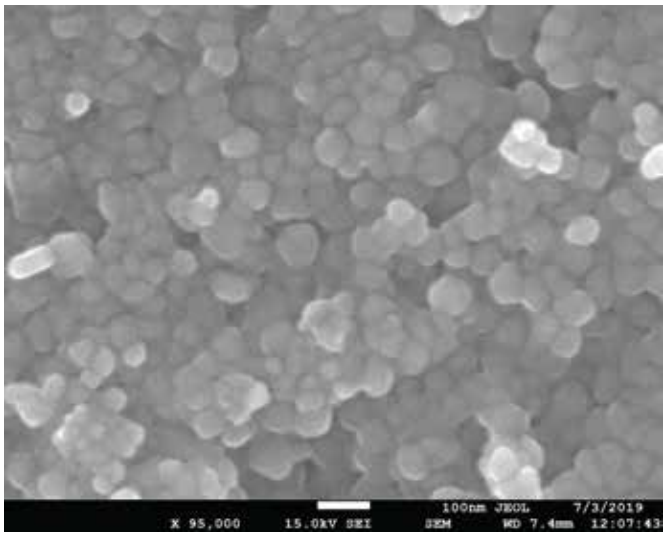


Fig. 3. SEM photograph of AgNPs.

Fig. 4. Antimicrobial activities of *A. indica* leaf extract, AgNPs, ampicillin, and AgNPs mixed with ampicillin against; left: *Klebsiella pneumoniae*, middle: *Serratia sp*., and right: *Bacillus subtilis.*

2015). Silver nitrate solution and leaf extract did not exhibit any antimicrobial effect alone probably due to their low concentration applied during experimentation. Furthermore, in case of positive control (Ampicillin), no ZOI was observed indicating all strains have become resistant to the commercial antibiotics. The measured ZOI for AgNPs was 12± 0.23mm, 10± 0.56mm, and 11±0.15mm against *Klebsiella pneumoniae*, *Serratia sp.*, and *Bacillus subtilis*, respectively. However, the measured ZOI of AgNPs mixed with ampicillin were 14± 0.6 and 14± 0.5mm; 13± 0.5 and 12± 0.4mm; and 13±0.7 and 14±0.2mm against *Klebsiella pneumoniae*, *Serratia sp.*, and *Bacillus subtilis*, respectively. Interestingly, AgNPs mixed with ampicillin exhibited higher antimicrobial activities compared to AgNPs and ampicillin alone. The variation in the antimicrobial potential might be due to the synergistic effect between AgNPs and ampicillin. The results are in agreement with Aabed *et al*., 2021 who also reported the synergistic effects of biogenic AgNPs in combination with commercial antimicrobial antibiotics.

Table 1. Zone of inhibition of *A. indica* leaf extract, 1 mM $AgNO_3$, AgNPs, ampicillin, and AgNPs mixed with ampicillin against different bacterial strains.

Microorganisms	*A. indica* extract (mm)	$AgNO_3$ solution (mm)	Positive control (ampicillin) (mm)	*A. indica*-mediated AgNPs (mm)	Ampicillin impregnated with AgNPs(mm)	Ampicillin impregnated with AgNPs(mm)
Klebsiella pneumoniae	NZ	NZ	NZ	12± 0.23	14± 0.6	14± 0.5
Serratia sp.	NZ	NZ	NZ	10± 0.56	13± 0.5	12± 0.4
Bacillus subtilis	NZ	NZ	NZ	11±0.15	13±0.7	14±0.2

Table 2. Fasting blood glucose levels (mmol/L) in control and experimental groups of rats.

Groups	Groups Name	Fasting Blood Glucose Levels (mmol/l)				
		1st day	7th day	14th day	21th day	28th day
I	Normal control	5.5±0.02	5.3±0.01	5.4±0.06	5.3±0.03	5.5±0.04
II	Diabetic control	14.9±0.06	15.0±0.03	14.4±0.04	14.1±0.04	14.7±0.1
III	*A. indica* -based AgNPs (10 mg/kg body weight)	11.2±0.05	10.3±0.04	9.0±0.03	7.9±0.02	6.8±0.02
IV	*A. indica* -based AgNPs (10 mg/kg body weight) + metformin (30 mg/kg body weight)	8.3±0.01	7.8±0.03	6.2±0.02	6.1±0.01	5.4±0.02
IV	Metformin (30 mg/kg body weight)	10±0.02	8.9±0.05	8.1±0.01	7.2±0.04	6.3±0.02

Determination of in vivo antidiabetic activity

Effects on blood glucose level

The effect of AgNPs, mixture of AgNPs and metformin, and metformin in reducing blood glucose level of STZ-induced diabetic ratsare shown in Table 2. The blood glucose levels of AgNPs treated diabetic rats gradually decreased to normal after 28 days. Metformin also resulted in the reduction of blood glucose level to normalcy after 21 days. However, oral administration of the combination of AgNPs and metformin for a period of 14 days produced a significant reduction in blood glucose levels as compared to AgNPs and metformin treated diabetic rats. The synergistic anti-diabetic effect of the mixture of AgNPs with metformin clearly explains the results.

Conclusions

The mixtures of AgNPs with ampicillin and metformin separately offer novel approaches in nanomedicine for antimicrobial resistance and diabetes management.

Contributions

Z.S., S.A., and M.M.R., collected samples and carried out the experiments of synthesizing and evaluating the antimicrobial and antidiabetic potential of AgNPs. A.U.D., M.A.K.T., D.K.P., B.S.A., and M.S.R. performed the experimental characterization of AgNPs. A.T.M.M.R. and A.S.M.A.H.A. designed the research work. A.U.D. and A.T.M.M.R. were involved in writing the original draft of the manuscript and all authors read and approved the final manuscript.

Acknowledgement

This research project was supported by a grant from the "Ministry of Science and Technology" Project No:39.00.0000.009.14.011.20-ID-458/1794, Bangladesh.

Statement of conflict of interest

The author has declared no conflict of interest.

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