International Journal of Scientific Research in Chemistry (IJSRCH)
© 2022 IJSRCH | Volume 7 | Issue 4 | ISSN : 2456-8457

Therapeutic Potential of Silver Nano Particles Synthezised from Azadirachta Indica Against Methicillin-Resistant Staphylococcus Aureus

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Article Info
Volume 7, Issue 4
Page Number : 14-26

Publication Issue :
July-August-2022

Article History
Accepted : 01 July 2022
Published : 05 August 2022

ABSTRACT

The increasing rate of drug resistance associated with methicillin resistant Staphylococcus aureus is not only a problem in the clinical sector but also in animal's disease treatment and management. Methicillin resistant Staph. aureus is now a leading cause of staphylococcal infections in human and animals. Silver is known for its antimicrobial activity, silver nanoparticles are gaining great importance due to their antimicrobial activities. "Green technology" is the use of various plant materials for the biosynthesis of nanoparticles, as it does not involve any harmful chemicals. Bioactive compounds such as flavonoids, terpenoids etc present in plant extracts have made them best material for the green synthesis of nanoparticles. The present work leads to the synthesis of silver nanoparticles from 1 mM AgNO3 solution through various concentration of aqueous leaf extract of Azadirachta indica reducing as well as capping agent and the evaluation of its therapeutic potential against MRSA. The leaf extracts of Azadirachta indica were prepared using water and is screened for antimicrobial active principles. The phytochemical screening of the leaf extracts revealed the presence of alkaloids, flavonoids and saponins. The activities were tested against bacterial pathogen. The results evidently show that the inclusion of Azadirachta indica extracts improves the solubility of AgNPs, which led to a significant enhancement in the toxicity of the NPs against the assessed microorganism All the three synthesized nanoparticles (S1, S2, and S3) inhibit the bacteria at various concentration of 0.1ml, 0.2ml, and 0.3ml. The result for S1 at the said concentration were 9mm, 10mm, and 11mm. the result for S2 were 11mm, 12mm, and 13mm. The result for S3 were 12mm, 13mm and 15mm. The minimum inhibitory concentration for AgNPs against MRSA were found to be at the highest concentration of 10-1(v/v) on all the three replica of the synthesized AgNPs i.e S1, S2 and S3. The zones of inhibition are 9mm, 11mm and 13mm respectively. Nanoparticles of silver could inhibit the growth of MRSA in low densities. In well diffusion method, results revealed that the increase in the concentration of the nanoparticles of silver enlarges the diameter of the inhibited growth zone of the MRSA. The diameter of the inhibited growth zone greatly depends on the dose of nanoparticles of silver

used.

Keywords : Antimicrobial Activity, Silver Nanoparticles, AgNPs, MRSA

INTRODUCTION

Methicillin resistant *Staphylococcus aureus* (MRSA) is an important pathogen in the healthcare sector that has not been eliminated from the hospital nor community environment. In humans, S. *aureus* causes superficial lesions in the skin and localized abscesses, central nervous system infections, osteomyelitis, invasive endocarditis, septic arthritis, septicemia, pneumonia, and urinary tract infections (Valezquez 2005). A bacteremia caused by S. aureus produces between 25% and 63% of mortality (Bustos *et al.,* 2006). In 1960, the first strain of MRSA was isolated in the UK, just 1 year after methicillin started to be used as an alternative to penicillin. Nowadays, MRSA strains have a wide range of drug resistances, including to more than 16 types of antibiotics. Resistance to methicillin is related to the gen mecA, which codifies the protein PBP2a that has low affinity to methicillin and to all β-lactamics (Valezquez 2005).

Investigations focused in the search of other alternatives for the treatment of MRSA infections are continuously being held. Among the range of compounds whose bactericidal activity is being investigated, silver nanoparticles rise as a promising new antibacterial agent that could be helpful to confront this and other drug-resistant bacteria. Antibacterial properties of silver are documented since 1000 B.C., when silver vessels were used to preserve water. Silver's mode of action is presumed to be dependent on Ag+ ions, which strongly inhibit bacterial growth through suppression of respiratory enzymes and electron transport components and through interference with DNA functions (Song *et al.,* 2006).

Different studies have established the bactericidal effect of Nano-silver in Gram negative and Gram positive bacteria, but the bactericidal mechanism of this compound has not been clearly elucidated. Morones *et al.,* (Yoon *et al.,* 2008) defined the antibacterial activity of silver nanoparticles in four types of Gram negative bacteria: Escherichia coli, Vibrio cholera, *Pseudomonas aeruginosa*, and *Salmonella typhi* and suggested that silver nanoparticles attach to the surface of the cell membrane and disturb its function, penetrate bacteria ,and release silver ions (Morones *et al.,* 2005). Other groups determined a similar antibacterial activity in Gram positive bacteria, such as *Bacillus subtilis* (Yoon *et al.,* 2008), S. aureus (Shrivastava *et al.,* 2007), and *Enterococcus faecalis* (Panaek *et al.,* 2006). Silver nanoparticles have also been found to exert antibacterial activity against some drug-resistant bacteria (Birla *et al.,* 2006 and Inoue *et al.,* 2009).

Furthermore, the antiviral capability of silver nanoparticles against the human immunodeficiency virus type and hepatitis B virus (Lut *et al.,* 2008) has been established. Current applications of silver nanoparticles include antimicrobial bandages for burns (Tredget *et al.,* 1998), water filters (Jain *et al.,* 2005), and others. Toxicity of silver nanoparticles has been studied in different mammalian cell systems, including rat liver cells (Hussein *et al.,* 2005), human keratinocytes and fibroblasts cultures, and human spermatogonial stem cells (Braydich *et al.,* 2005). In vitro, an elevated dose of Nano silver induces oxidative stress (liberation of reactive oxygen species) as a mechanism of cytotoxicity (Arora *et al.,* 2008). But, what happens at Nano-silver concentrations that are nontoxic? Can they be used for a therapeutic purpose? At an innocuous concentration range, silver nanoparticles have been described to exert anti-inflammatory effects as: acceleration of wound healing (Tian *et*

al., 2007), modulation of cytokine production and induction of peripheral blood mononuclear cells proliferation (Shin SH *et al.,* 2007), inhibition of allergic contact dermatitis in mice, suppression of the expression of TNF- α and IL-12, and induction of apoptosis of inflammatory cells (Bhol *et al.,* 2005).

In this work, we will report the antimicrobial effect of silver nanoparticles using a plant extract of *Azadirachta indica* (Indian Neem).

MATERIALS AND METHODS

Study area: The research was conducted in microbiology laboratory, Federal university Dutse and samples were collected from Dutse general hospital.

Study Population: The subject of study were patients with pyogenic ailments visiting the emergency care unit of general hospital Dutse.

Study Design: Random sample collection

Ethical approval: Consent was sought from the patients before study started.

Sample size: 20 samples were collected

Materials

Culture media

Nutrient agar (NA), Nutrient broth (NB), Mannitol salt agar (MSA), all media were prepared according to manufacturer's instruction, sterilized at 121^{0}C for 15 minutes and stored at 4^{0}C until required.

Chemical reagents

Silver nitrate, Hydrogen peroxide, Lugols, Iodine, crystal violet, Ethanol, safranin.

Collection Of Sample

The pus samples were collected from patients of pyogenic ailments visiting the emergency care unit of general hospital Dutse, Jigawa state. Samples were collected using a sterile swab and spread on Mannitol Salt agar plates (Singh *et al.,* 2018)

Phytochemicals Analysis

Preparation of plant extracts

The leaves were washed with distilled water to remove dust and particles, followed by shade-drying at room temperature. The leaves were then grinded to make fine powder using a blender. Five-gram of the leaf powder was mixed in 100 ml of distilled water and heated for one hour at 100°C. The extract was then centrifuged for 10 min at 10,000 rpm and filtered using the Whatman filter. The extract was stored at –20°C for further use (Faisal *et al.,* 2019).

Phytochemical analysis

The phytochemical tests were conducted using standard procedures to identify the constituents as described by Edeoga *et al.,* (2005) and Harborne (1973). Tests for the presence of the alkaloids, saponins, tannins, terpenoids, flavonoids, glycosides, reducing sugar polysaccharides, phytosteroles and phenols were carried out (Marinova *et al.,* 2005).

Phytochemical tests were done to find the presence of the active chemical constituents such as alkaloid, flavonoids, glycosides, terpenoids, steroids, tannin and phenols, reducing sugar, carbohydrates and protein and amino acids by the following procedure. (Prashanth Tiwari *et al.,* 2011).

Tests for alkaloids; Mayer's reagent test

To the extract, dilute hydrochloric acid was added, shaken well and filtered. With the filtrate, the following tests were performed. To 3 ml of filtrate, few drops of Mayer's reagent were added along sides of tube. Formation of creamy precipitate indicates the presence of alkaloids (Harborne 1973).

Tests for carbohydrates; Molisch test

2 ml of aqueous extract was treated with 2 drops of alcoholic α-naphthol solution in a test tube and then 1 ml of concentrated sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of carbohydrates (Harborne 1973).

Tests for reducing sugars; Benedict's test

Equal volume of Benedict's reagent and extract were mixed in a test tube and heated on a water bath for 5-10 minutes. Solution appears green, yellow or red depending on the amount of reducing sugar present in the test solution which indicates the presence of reducing sugar (Harborne 1973).

Test for Flavonoids.

A colorimetric method for detection of flavonoids was adopted. Dilute ammonia solution (5 ml) was mixed with the aqueous filtrate of the extract, and concentrated H_2SO_4 was added. The development of yellow colour indicates the presence of flavonoids that disappeared on standing. One-hundred microlitres of NH_3 solution (1%) was added to a portion of filtrate. The yellow colour again appeared, which an indicator for the presence of flavonoids (Dash *et al.,* 2017).

Test for Glycosides.

A small amount of the plant extract was dissolved in water. 1 ml of % NaOH solution was added. Appearance of yellow colour confirmed the presence of glycosides (Odebiyi *et al.,* 1978).

Tests for tannin and phenolic compounds; ferric chloride test

A small amount of extract was dissolved in distilled water. To this solution 2 ml of 5% ferric chloride solution was added. Formation of blue, green or violet color indicates presence of phenolic compounds (Dash *et al.,* 2017).

Test for saponin; Froth test

The extract was diluted with distilled water and shaken in a graduated cylinder for 15 minutes. The formation of layer of foam indicates the presence of saponins (Dash *et al.,* 2017).

The Synthesis Of Silver Nano Particle From The Neem Extract

Preparation of leaf extracts from *Azadirachta indica* (neem) leaves

Fresh neem leaves were collected from University Campus in the month of August. Leaves were thoroughly washed in running water to remove the dirt and dust on the surface of the leaves. Twenty grams of finely chopped neem leaves were added to 100 ml of double-distilled water and boiled for 10 min. The extract was cooled and filtered and store for further use. This solution was used for green synthesis of silver nanoparticle (AgNP) or reducing the silver ions (Susheela 2013).

Synthesis of silver nanoparticles

Silver nitrate was used to prepare 1000 ml of 1 mM solution of silver nitrate. Then 1, 2, and 3 ml of neem extract was added separately to 5 ml of silver nitrate solution. This set up was incubated in dark chamber to

minimize photo-activation of silver nitrate at room temperature. The colour change from colourless to brown in colour confirms the reduction of silver ions (Susheela 2013).

Preparation of nutrient agar, manitol salt agar and nutrient broth

All the petri dishes, conical flasks and beakers were cleaned with detergent and autoclaved then dried in hot air oven. Nutrient Agar (Hi-Media), Manitol salt agar, Nutrient broth were prepared as per manufacture's instruction (Cheesbrough 2000).

Isolation And Identification Of MRSA

3.6.1 Isolation and Identification of *Staphylococcus aureus*

The specimens was inoculated on mannitol salt agar and incubated aerobically at 37°C for 48 hours. The strains of *Staphylococcus aureus* was identified on the basis of colony morphology, Gram's stain, and different biochemical tests (coagulase and citrate tests) (Forbes *et al.,* 2007).

Gram staining

Gram staining was carried out using the method described by (Cheesbrough 2000). A smear of the culture of the test organism was made evenly on a clean glass slide and heat-fixed. The smear was stained with crystal violate, fixed with Lugol‟s iodine and decolorized rapidly with acetone-alcohol, after which it was counter-stained with dilute carbol fuchsin solution. The stained slides were microscopically examined under the oil immersion objective. Sample colonies that appeared as purple/violet cocci, predominantly in cluster were selected. Sample colonies were inoculated into sterile NB and incubated at 37^0C for 18 hrs. Each sample was sub-cultured in duplicate onto slants and incubated at 37^0C for 18 hrs. Slants were kept at 4^0C until required.

Biochemical tests

Catalase Test

The ability of the isolates to produce an enzyme catalase was demonstrated by the addition of 1ml of 3 % w/v hydrogen peroxide solution to 24 hr culture of the isolate. Rapid evolutions of gas bubbles indicated the breakdown of hydrogen peroxide into oxygen and water by catalase peroxidase enzyme present a positive result.

$$2H2O2 \rightarrow H2O + O2$$

Coagulase Test

The tube coagulase method was carried out as described by Cowan (1985) and Cheesbrough (2000). A positive coagulase test differentiates *Staph. aureus* (which produce free coagulase and converts fibrinogen to fibrin by activating a coagulase-reacting factor present in plasma) from the coagulase-negative *staphylococci* species. Pooled, EDTA-anticoagulated human plasma was diluted 1 in 10 with sterile normal saline. Aliquots of 0.5 ml were pipetted into sterile test tubes into which 0.1ml of 24 hrs NB culture of the isolate was added, mixed and incubated in water bath thermostated at 37^0C for 4 hrs. At intervals of 30 minutes, the test tubes were observed for clotting of tube contents or fibrin clot in tubes. Two different test tubes were set up for positive control test (containing 24 hrs culture of a known *Staph. aureus*) and negative control (containing only sterile NB). Incubation period may be extended for 24 hr to take care of delayed reactions since *Staph. aureus* is not prone to spontaneous clumping of plasma which usually accompanies the utilization of citrate present in the anticoagulant.

Determination of methicillin (oxacillin) resistance using cefoxitin disc

CLSI recommends using cefoxitin instead of oxacillin when using disk diffusion method to determine methicillin resistance in *Staph.aureus* (CLSI, 2007). Cefoxitin results are easier to interpret and are thus more sensitive and enhance induction of PBP2a for the detection of mecA mediated resistance than oxacillin results. The gene is located on the staphylococcal chromosome cassette mec and encodes penicillin binding protein (PBP2a) (BergerBachi and Rohrer 2002). A direct colony suspension of each *Staph.aureus* isolate was prepared equivalent to 0.5 McFarland standards and plated on Mueller Hinton agar surfaces). Cefoxitin 30µg disc was placed on the plate. Plates were incubated at 36^0C for 24h. The zones of inhibition were measured and compared to that of CLSI interpretative chart (CLSI, 2007). Inhibition zones diameter of 21mm was reported as methicillin resistant and >22mm was considered as methicillin sensitive.

Antimicrobial Susceptivility Assay

The silver nanoparticle synthesized was tested for antimicrobial activity by agar well diffusion method against the MRSA. The pure cultures of bacteria were sub cultured on nutrient broth. Each strain were swapped homogeneously onto the individual plates using sterile cotton swabs. Wells of 10 mm diameter were made on Nutrient agar using gel puncture. Different concentration of silver nanoparticle 0.1ml, 0.2ml and 0.3ml of each synthesized silver nanoparticle were poured on each well. After 24 hours incubation the various levels of zone of inhibition were measured. Three replicates of experiments were carried out i.e with S1 (1ml neem extract to 5ml silver nitrate), S2 S(2ml neem extract to 5ml silver nitrate), S3 (3ml neem extract to 5ml silver nitrate).

Minimum Inhibitory Concentration

Serial dilution

Each of the three synthesized silver nanoparticles of varying concentration were subjected to a serial dilution using sterile distilled water as a diluent.1ml from each crude extract was added into a tube containing 9ml of sterile distilled water, from this tube, a serial dilution was done and covered a dilution range of 10^{-1} to 10^{-5}. This helped to determine the minimum inhibitory concentration (MIC) of each silver nanoparticle on the MRSA strain (Uwimbabazi *et al.,* 2015)

RESULT

Sample Population

A total of 20 samples collected were screened for the presence of *Staph. aureus*. After 48 hours incubation, 17 of the bacterial culture ferment the sugar manitol present in the manitol salt agar. Isolates that were Gram positive cocci, catalase positive and coagulase positive were presumptively characterized as *Staph. aureus.*

Detection Of Penicillin Binding Protein (PBP2a)

Table 1; detection of methicillin resistant isolates

Isolate	PBP2a positive	PBP2a negative	% positive
Staph aureus	16	1	94%

Key: =MRSA; methicillin resistant *Staphylococcus aureus*. =PBP2a; penicillin binding protein

The presence of mecA gene product PBP2a which is responsible for methicillin resistance was determined in isolates that showed phenotypic resistance to cefoxitin. 17 isolates were tested, 16 methicillin resistant *Staph. aureus* were PBP2a positive and 1 was negative as represented in table 1 above.

Synthesis Of Sliver Nanoparticles

After the addition of Neem leaf extract to AgNO3 solution a visible color change from transparent to dark brown was observed which indicates the formation of silver nanoparticle. This occurred due to the reduction of silver ions present in the solution due to terpeniods present in neem leaf extract. After 60 minutes there was no change in the intensity of color developed, which indicates the completion of reduction reaction. The reduced silver particles are in the range of nano size. The result is presented in table 4.3.

Table 2; synthesized silvernano particles

Silver nano particles	Time of incubation	Colour change
5ml $AgNO_3$ + 1ml plant extract (S1)	60 minutes	Dark brown
5ml $AgNO_3$ + 2ml plant extract (S2)	60 minutes	Dark brown
5ml $AgNO_3$ + 3ml plant extract (S3)	60 minutes	Dark brown

Key: =$AgNO_3$; silver nitrate

Phytochemical Analysis Of *Azadirachta indica* Leave Extract

The phytochemical analysis of plant extract using Aqueous was showed in Table 4.4. From phytochemical analysis, reducing sugar were found in *Azadirchta indica*. Aqueous extract of *Azadirchta indica* showed the presence of alkaloids, flavanoides, glycoside reducing sugar, polysaccharides and phenols but there was absence of tannins, saponins and terpenoides.

Table 3 : phytochemical analysis; phytochemical screening of leaf aqeous extract A. *indica*

Phytochemicals	Result
Alkaloid	+
Carbohydrate	++
Flavonoid	+
Saponin	++
Tannin	++

Key: + Positive

Antimicrobial Assay

The silver nanoparticles with different concentrations of neem extract 1ml plant extract 5mL silver nitrate, 2mL plant extract 5mL nitrate, 3mL plant extract 5mL silver nitrate, was added 0.1ml, 0.2ml and 0.3ml on these culture plates. These plates were kept overnight in incubator at 37^0C. The next day, zone size was measured. In sample S1, the zone size for 0.1ml, 0.2ml and 0.3ml was 9mm, 10mm and 11mm, respectively. For sample S2, the zone size for 0.1ml, 0.2ml and 0.3ml were 11mm, 12mm and 13mm. In sample S3, for 0.1ml it was 12mm, 0.2ml it was 13mm and 0.3ml it was 15mm. Sensitivity of each was determined on MRSA and the result is presented in table 4.5.

Table 4: antimicrobial assay of the silvernano particles

Synthesized silvernano particle	Zones of inhibition		
	0.1ml	0.2ml	0.3ml
5ml $AgNO_3$ + 1ml plant extract (S1)	9mm	10mm	11mm
5ml $AgNO_3$ + 2ml plant extract (S2)	11mm	12mm	13mm
5ml $AgNO_3$ + 3ml plant extract (S3)	12mm	13mm	15mm

Key: =$AgNO_3$; silver nitrate. =mm; millimeter =ml; milliliter

Minimum Inhibitory Concentration (MIC)

The result of the Minimum Inhibitory Concentration (MIC) of the silvernano particles (S1, S2 and S3) showed that MRSA is susceptible or sensitive at a concentration of 10^{-1} which is the lowest concentration of the extract which inhibited bacterial growth resulting in visually clear zones after 24 hours incubation (Table 4.6a, 4.6b and 4.6c).

Table 5a: minimum inhibitory concentration of S1

Concentration (v/v)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Zones of inhibition (mm)	9	0	0	0

Table 5b : minimum inhibitory concentration of S2

Concentration (v/v)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Zones of inhibition (mm)	11	0	0	0

Table 6c: minimum inhibitory concentration of S3

Concentration (v/v)	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Zones of inhibition (mm)	13	0	0	0

DISCUSSION

This study used the bactericidal effects of nanoparticles of rolled silver synthesized from neem leaves against methicillin-resistant *Staphylococcus aureus*.

The *Staphylococcus aureus* recovered were from patients of pyogenic ailments in Duste general hospital. Cefoxitin 30.0µg was used and it is the best for determining mecA mediated resistance in *Staph aureus* (CLSI, 2007). Cefoxitin will only detect MRSA with mecA mediated resistance mechanism (Swenson *et al.,* 2007). Sixteen (94%) out of the 17 *staph aureus* were found to be resistant isolates and carried mecA gene which contained the mecA gene product (PBP2a) determined by MRSA cefoxitin disc test as represented in table 4.2. Resistance has been known to be caused by a gene called mecA gene, which codes for resistance to methicillin in *Staph aureus*, and the gene is located on the staphylococcal chromosomes cassette mec. CLSI, (2010) has recommend that oxacillin be replaced by cefoxitin, a more potent inducer of mecA expression that is less affected by test condition and hyper production of penicillinase (Brown *et al.,* 2005). The gold standard for the detection of MRSA is the polymerase chain reaction (PCR) that detects mecA gene or alternatively detecting the mecA gene product PBP2a by latex agglutination test (Berger Bachi and Rocher 2002).

Bio-reduction of silver ions into AgNPs after addition of aqueos neem extract was confirmed with change in colour. Initially, after addition of aqueous neem extract, the colour was pale yellow with the increase in

incubation time the colour changed from pale yellow to light brown and after 24 h incubation it was deep brown in colour as represented in table 4.3. The brown colour was due to the excitation of the surface plasmon resonance (SPR), very much a characteristic property of silver nanoparticle (Banerjee *et al.,* 2014). According to Amendola (Amendola *et al.,* 2010) when conducting similar research said that the SPR band is depended on the particle size and refractive index of the solution. The flavenoids and terpenoids present in neem extract act like natural reducing agent which are responsible for reducing silver salts to silver nanoparticles (Verma and Singh Mehata 2016).

As represented in table 4.4 aqueous extract of the neem plant was found to have maximum number of phytoconstituents in saponins and sugar and have low flavonoids concentration. The result obtained in this table; showed the presence of alkaloids, flavonoids and saponins in all of the leaf extracts which corresponds with the work of (Timothy *et al.,* 2008). According to the research carried out by (Imran, *et al.,* 2011); the presence of these phytochemicals identified was not realized. This may be due to the fact that, ecological or geographical differences may bring about a difference in the presence of such phytochemicals of neem trees isolated. Secondly, neem leaf collected may also vary qualitatively because of different climatic conditions in the study areas, thereby bringing variations in the chemical constituents of the neem leaves.

The antimicrobial activity of silvernano particles was investigated against methicillin resistant *S. aureus* using the agar well diffusion assay. The zones of inhibition (mm) around each well containing AgNPs solution are represented in table 4.5. The AgNPs were found to be effective against the MRSA. All the three synthesized nanoparticles (S1, S2, and S3) inhibit the bacteria at various concentration viz 0.1ml, 0.2ml, and 0.3ml. The result for S1 at the said concentration were 9mm, 10mm, and 11mm. the result for S2 were 11mm, 12mm, and 13mm. The result for S3 were 12mm, 13mm and 15mm. The enhanced antibacterial activity of AgNPs solution is attributed to their large surface area that provides more surface contact with microorganisms (Logeswari *et al.,* 2015). Another important reason of enhanced antibacterial activity of AgNPs as documented in the literature is the synergistic effect between particles and natural compounds (Dur'an *et al.,* 2016). Cardozo *et al.,* found that synergy between phenazine-1-carboxamide and AgNPs increased the antibacterial effect by 32-fold against MRSA strains, causing morphological alterations to the cell wall of bacteria (Cardozo *et al.,* 2013). The mechanism of action of the antibacterial activity of AgNPs is attacking the respiratory chain and cell division that ultimately leads to cell death. The silver nanoparticles have also been reported to release silver ions inside the bacterial cells, further enhancing their bactericidal activity (Morones *et al.,* 2005).

AgNPs were found to be an effective antibacterial agent against the test bacteria. The recorded MIC values at which no visible growth of test bacterial strains is found are presented in table 4.6a, 4.6b, and 4.6b. The minimum inhibitory concentration for AgNPs against MRSA were found to be at the highest concentration of 10^{-1} on all the three replica of the synthesized AgNPs i.e S1, S2 and S3. The zones of inhibition are 9mm, 11mm and 13mm respectively. However, no defnite criteria have been established so far to consider the MIC breakpoints on the resistance level. Similar results have been documented earlier where MIC values of AgNPs synthesized using *Eucalyptus globulus* against *E. coli* were found to be at 10^{-1}(v/v) and 10^{-2} (v/v) (Ali *et al.,* 2015). Moreover, Ansari and Alzohairy have reported. MIC values of AgNPs prepared using seed extracts of *Phoenix dactylifera* as 10.67g/ml and 17.33g/ml, respectively, against methicillin-resistant *S. aureus* (Ansari *et*

al., 2018). These variations might be due to the different intrinsic tolerance levels of test strains used in the assays, size and nature of nanoparticles, and methods adopted for the determination of the MIC.

A similar result conducted by Faisan *et al.,* 2019 showed the dose-dependent effect of AgNPs against MRSA, MSSA, *and E. coli*. The growth was monitored at different time intervals by measuring absorbance at 620 nm using a microplate reader. The treatment with nanoparticles to all the bacterial strains was given in two fold dilution, and the wells without any amendment of AgNPs were taken as the control group. The extent of growth inhibition on MRSA by AgNPs was found to be 31.4%, 53.3%, and 85.6% at 8, 16, and 32 g/ml, respectively and the concentration above 32 g/mlwas found to be inhibitory to more than 90% of the bacteria. The treatment with 8, 16, and 32 g/ml of AgNPs resulted in 37.0%, 48.5%, and 90.8% inhibition on MSSA1, the concentration exceeding 32 g/ml checked the MSSA1 growth by more than 95%. A similar pattern of inhibition was obtained for MSSA2. For the *E. coli* strain, the reduction in viability was observed to be 19.8%, the concentration above 16 g/ml was found to inhibit *E. coli* by more than ninety percent. The toxicological impact of silver nanoparticles depends not only on its size but also on the test organisms (Azam *et al.,* 2012). It has been documented by Brunner *et al.,* that toxicity of nanoparticles may either be due to release of metal ions from nanoparticles such as Ag+ or production of reactive oxygen species or direct interaction and disruption of biological macromolecules such as intercalation to DNA (Brunner *et al.,* 2006).

A study has demonstrated that silver nanoparticles synthesized from the green approach with an average size of 18 nm exhibited the MIC value against *E. coli* and *P. aeruginosa* as 36 μg/ml and 27 μg/ml, respectively (Ali *et al.,* 2015).

The study conducted by Humberto et., al in 2010, the inhibitory effect of nanoparticles of silver were investigated on MRSA and *Escherichia coli* resistant to Ampicillin and the *Streptococcus pyogenes* resistant to Erythromycin. They approved of the bacteriostatic effects of nanoparticles of silver on bacteria. MRSA has several antibiotic resistant factors due to its plasmid and chromosome making its antibiotic treatment very difficult. Thus, new antibiotics failed to stop the mortality and morbidity it causes. The low penetrability of exterior membrane of the bacteria and the presence of drug efflux pumps are among the mechanisms causing its drug resistance. Furthermore, in 2005, Morones *et al.,* investigated the bactericidal effects of nanoparticles of silver on some bacteria. They discovered that nanoparticles of silver stick to the cell membrane of the bacteria degenerate the membrane and disrupt the penetrability of membrane through releasing silver ion.

Considering the results in this study, it could be said that nanoparticles of silver could inhibit the growth of MRSA in low densities. This concords with the results from Sondi *et al.,* working on *Escherichia coli*.

Conclusion

In this study, *Azadirachta indica* conjugated silver nanoparticles were synthesized using their leaves extract. The biosynthesized silver nanoparticles were proved to have excellent antimicrobial performance against pathogenic methicillin resistant *Staphylococcus aureus* using *A. indica* leaves extract. Therefore, AgNPs producing *A. indica* may be potentially utilized for the economical production of AgNPs for many pharmaceutical applications.

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