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EVALUATION OF CHEMICALLY SYNTHESIZED SILVER NANOPARTICLES COATED ANTIBIOTICS AGAINST MULTI DRUG RESISTANT BACTERIA ISOLATED FROM OTITIS MEDIA PATIENTS

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Abstract

Silver nanoparticles which have well-known antimicrobial properties, are used extensively in various medical and general applications. They have unique physical, chemical and biological properties as well as antibacterial activity. In the present study, silver nanoparticles were synthesized chemically and then different analytical techniques such as UV-visible spectroscopy, Scanning Electron Microscopy (SEM), and X-Ray Diffraction (XRD), were performed for the characterization of AgNPs. UV-visible spectroscopy gave absorption peak at 430 nm which was in the prescribed range and confirmed the synthesis of AgNPs. SEM micrograph demonstrated the morphology of AgNPs, while the XRD peak gave information about the phase purity, size, internal crystalline structure and nature of the synthesized AgNPs. On the Other hand, various pathogens were isolated such as *S. aureus S. epidermidis Salmonella spp, Enterobacter spp, Provedencia spp, Proteus spp, Streptococcus pneumonia, Klebsiella spp, Edwardsiella terda ssp, Klebsiella oxytoca and Pseudomonas aeruginosa* from pus samples. Antibacterial activity of multiple coated antibiotics with AgNPs and non-coated antibiotics including Amoxicillin + Clavulanic acid, Imipinem, Amikacin, Ciprofloxacin, Polymixin, Cefoxitin, Tazobactum + Piperacillin Nitrofurantoin, Fosfomycin,and Gentamycin were recorded against these bacterial isolates. Most enhanced silver

nanoparticles activity was observed in combination with the antibiotics Ciprofloxicin (500% increase in potential) against *Provedencia Rettgeri* and *Proteus Mirabilis* while it was (100 % increase in potential) against *S. aureus* and *S. epidermis*, followed by (140% increase in potential) in combination with antibiotics Gentamycin against *Proteus Mirabilis*. Amoxicillin + Clavulanic acid and Fosfomycin in combination with silver nanoparticles also enhanced (100% increase in potential) against all Gram-negative bacterial species. Fractional Inhibitory Concentration (FIC) values for two bacterial isolates *S. aureus*, and *E. coli* showed synergistic effect.

INTRODUCTION

Small size particles which have 1 to 100nm size are considered nanoparticles. In consumer utilities silver nanoparticles are mostly utilized because of its excellent antimicrobial activities (Krug & Height *et al.*, 2011). In the science of nanotechnology scientists have many successful achievements in the fields of healthcare and medicine, nanoelectronics, biotechnology, national security and information technology. Nanoparticles have been synthesized through chemical reduction assay, chemical reduction through aqueous solution, chemical reduction through non aqueous solution, electrochemical reduction, photo- induced reduction, chemical synthesis with the help of micro waves, reduction through irradiation, biochemical method and micro emulsion method (Sathyavathi & Rao *et al.*, 2010).

Silver compounds have damaging effect to bacterial cells when they are used as antibacterial substance but silver nanoparticles are less toxic to normal human cells. Silver nanoparticles have antiseptictic and wound healing properties. Silver nanoparticles are highly effective antibacterial agents against a wide range of pathogens (Raji & Parikh *et al.*, 2012). Silver nanoparticles interact with the pathogenic microorganisms, ions of the silver are released and microorganism are damage and affect in different ways for example, these ions interrupted cell membrane permeability and dysfunction of cellular enzymes and attack on those microbes who has negatively charged components cell wall, and ultimately cell death and cell lysis take place (Choi & Hu *et al.*, 2008).

Silver nanoparticles are also have confirmed antimicrobial activity towards Gram negative and Gram-positive bacterial pathogens. It is also effective against pathogenic fungi (Karwowska *et al.*, 2017). Different methods are used for synthesis of silver nanoparticles in-vitro such as chemical, biological and physical. In biological method abundant number of sources- polymers, plants, proteins, microbes and chemical agents can be used to create silver nanoparticles by reducing agents. Nanoparticles are classified in different type - metallic, magnetic, quantum dots, dendrimer, liposomes, carbon nanotubes and polymeric (Bhatia *et al.*, 2016).

Middle ear infection is the major public health problem in the world both in teen-agers and adults (Bluestone & Klein *et al.*, 2007). Throughout the world 65–330 million people are affected due to ear inflammation, and 60% people are suffer hearing loss significantly (Woodfield & Dugdale *et al.*, 2008). Middle ear infection is also called Otitis Media (OM), the etiologic agents for middle ear infection are fungi, viruses and bacteria. Otitis media have three categories that's acute otitis media, effusion otitis media, and chronic otitis media (Coticchia & Mutchnick *et al.*, 2013). Research study revealed that chronic middle ear infection causes inflammation of mastoid cavity of middle ear with purulent discharge in various time intervals. Middle ear discharge comes through perforated ear drum called tympanic membrane. Otitis media is complicated health issue in the developing countries of the world (Juyal *et al.*, 2014).

Multi Drug Resistant (MDR) are life threatening bacteria and increase their resistivity to antibiotics with the passage of time. Currently antibiotics efficacy to MDR bacteria are very slow down, it is a public health problem in the world (Aslam & Baloch *et al.*, 2018). Pseudomonas aeruginosa is the causative agents of otitis media, surgical site infections, bloodstream, urinary tract, soft tissue, wound infections and ventilator associated pneumonia in ICUs and is showing resistance to multiple antibiotics (Barrios *et al.*, 2014).

The traditional antimicrobial medicines are being used in the current battle against antibiotic-resistant bacterial strains, certain metals and metal oxides, for example, were resurrected,

however in today's nanoparticle formula (NPs). The majority of research is focused on silver nanoparticles (AgNPs), either alone or in combination with antibiotics (Möhler & Ziora *et al.*, 2018). With the presence and spread of antibiotic-resistant bacteria, as well as the continued focus on health-care costs, numerous researchers have attempted to develop novel antibacterial reagents that are both effective and cost-efficient. Such issues and requirements have prompted a renaissance in the use of silver-based antiseptics, which may be connected to broad-spectrum activity and a lesser risk of germ resistance than antibiotics (Jones & Parsons *et al.*, 2004).

METHODOLOGY

CLINICAL SPECIMEN COLLECTION AND PROCESSING:

The pus samples were collected from Lady Reading Hospital (L.R.H) Peshawar, with sterile culture swab which was labelled for the patient indication, personal history like patient name, gender, age, collection time and date. After collection the pus samples were transported to the Microbiology Laboratory Abasyn University Peshawar and were inoculated on different media. The plates were incubated for 24 hours at 37°C. Bacterial growth on the plates were further processed for species identification and antibiotic sensitivity profiles. Nutrient agar, Muller Hinton agar, Eosin methylene blue (EMB), MacConkey agar, Mannitol salt agar (MSA) and Blood agar were used as common, differential and selective media respectively.

IDENTIFICATION OF BACTERIAL ISOLATES:

Gram staining of bacterial isolates was performed to differentiate between Gram positive and Gram-negative bacteria. Biochemical tests like Catalase, Coagulase, Oxidase, Urease, Citrate, Indole, Triple sugar iron (TSI), were performed for up to species level identification according to the methods of Gashe & Zeleke *et al.* (2018).

ANTIBIOTIC SUSCEPTIBILITY ASSAY:

After identification, antibiotic sensitivity of the bacterial isolates was checked on Mueller Hinton Agar (MHA) by using using Kirby Bauer method. The antibiotics i.e., Amikacin, Erythromycin, Imipenem, Polymycin, Fosfomycin, Amoxicillin/Clavulanic Acid, Doxycycline, Vanocomycin, Cefoxitin, Ciprofloxacin, Oxacillin, Nitrofurantoin, Ceftazidime Gentamycin and Tazobactum. The antibiotic susceptibility profiles and MDR screening were selected on basis of Kahlmeter & Sharp *et al.* (2019 guidelines).

SYNTHESIS OF SILVER NANOPARTICLES:

Materials including Tri-Sodium Citrate and Silver Nitrate (Ag NO3) were used in the chemical synthesis of Ag NPs. One hundred milliliter of AgNO3 solution (0.50, 1.00, and 1.50 mM) were heated in a water bath at 90℃ for five minutes. Drop by drop, while continually stirring, 12.5 mL of the ready-made TSC solution (0.50, 1.00, and 1.50%) was added to this. The color shifted from translucent to pale yellow, which indicated the creation of Ag NPs, and marked the beginning of the reduction process. For a further stability analysis, the nanoparticle solution was agitated for 10, 15, and 20 minutes on a magnetic stirrer at 90℃ and stored at 8±1℃ Chowdhury & Sulaiman *et al.* (2016).

CHARACTERIZATION OF SILVER NANOPARTICLES:

UV-VISIBLE SPECTROSCOPY:

UV-Visible spectroscopy is based on the idea of light absorption, which is dependent on the particle concentration in the solution. In this study, it generated a graph of absorption vs wavelength of nanoparticles by utilizing UV-Visible spectroscopy. It detailed the silver nanoparticles' wavelength range according to protocol used by Singh *et al.* (2014).

X-RAY DIFFRACTION (XRD) SPECTRUM:

The size and structure of the nanoparticles were determined and confirmed using XRD patterns

from a tiny sample. Furthermore, the three diffraction peaks, (111), (200), and (220) of face-centered cubic silver, were highlighted by the XRD pattern according to method used by Chandrappa *et al.* (2016).

TRANSMISSION ELECTRON MICROSCOPY:

Citrate-AgNPs were prepared in TEM micrographs at various magnifications by reducing citrate. The particles range in size from 15 to 60 nm in diameter, with a 40 nm average. In the first procedure, trisodium citrate was added to an aqueous $AgNO_3$ solution and heated to near boiling temperature. These particles were separated and not homogenous, as seen by the TEM features. Due to the deposited citrate ions, the silver nanoparticles had a negative charge. A repulsive force acted on the particles, preventing them from aggregating. As a result, without the use of any extra stabilising agents, the particles in the solution remained stable according to method used by Singh *et al.* (2014).

SCANNING ELECTRON MICROSCOPY:

An electron beam interacts with a sample and produces a variety of signals during the surface imaging process known as SEM which showed the atomic structure and contour of the surface. SEM used backscattering electrons and secondary electrons produced by the sample to provide a three-dimensional image of the substance being investigated. Once these electrons had left the sample's surface, a photomultiplier was used to find them. However, due to their inability to properly deflect the electron beam, many nanoparticles were invisible to electron microscopes. As a result, the sample was coated with a tiny layer of metal to generate a conductive layer (Devika *et al.* (2012).

ANTIBACTERIAL ASSESSMENT OF AG-NPS COATED ANTIBIOTICS:

A stock solution of silver nanoparticles having concentration of 20µg/µL was set by mixing 20 mg of AgNPs residue in 1mL of distilled water. After this about 5µL (100µg Ag- NPs) of suspension was taken out from stock solution and was poured on antibiotics disc within petri plates and then was placed in room temperature for 1 hr to dry them completely and this method of coating was repeated for each antibiotic disc according to the methods of Aruna & lori *et al.* (2013).

ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES:

Disc diffusion assay was used to assess the antimicrobial activity of silver nanoparticles. Both Gram positive and Gram negative isolated MDR bacteria were used for the activity. The sterile discs were placed on the nutrient agar plate, coated with AgNPs 5, 10, or 15g/mL, and incubated for 24 hrs at 37°C. A cleared area was seen around the wells after inhibitory activity. The studies were carried out three times, and the average values of zone diameter were calculated Jeong & Yi *et al.* (2005).

PHENOTYPIC DETECTION OF EXTENDED SPECTRUM BETA-LACTAMASE:

Phenotypic detection of extended spectrum beta-lactamase producing Gram negative bacteria was determined through double disc synergy method. Two antibiotic discs amoxicillin/clavulonic acid and ceftazidime were used synergistically. Then culture plates were incubated at 37℃ for 24 hours and zone of inhibition was observed for phenotypic detection of ESBL production according to the modified protocol of Yarima & Gurama *et al.* (2020).

MINIMUM INHIBITORY CONCENTRATION:

The antibacterial activity of silver nanoparticles was investigated according to standard broth dilution method. The sequential two-fold dilutions of antibiotic and AgNPs in various concentrations was used together with corrected bacterial turbidity (10^8 CFU/ml, 0.5 McFarland's standard). The inoculated broth was utilized as

positive control while un-inoculated broth was kept as negative control. Then were incubated for 24 hours at 37℃. The MIC of silver nanoparticles and antibiotic was the minimum concentration of AgNPs and antibiotic where no observable growth in the tubes was observed. The turbidity of MIC tubes was noted before and after incubation period along with positive and negative controls according to the modified methodology of (Estrela *et al.*, 2001; Kowalska-Krochmal *et al.*, 2021).

Synergistic potential of Silver Nanoparticles:

Synergistic potential of silver nanoparticles was determined by comparing the minimum inhibitory concentrations of antibiotic and silver nanoparticles. Furthermore, comparison of the percent fold increase in the zone of inhibition against test microorganisms for antibiotics and antibiotics coated with silver nanoparticles was also performed (Barapatre & Aadil *et al.* 2016).

FRACTIONAL INHIBITORY CONCENTRATION

Fractional Inhibitory Concentration and FIC indices (FICI) were determined using the following formulae to evaluate synergistic, additive and antagonistic effects. The results of the preceding formulas were used to classify the combinations as per evaluation criteria.

$$FIC\ index = \frac{MIC\ of\ gentamicim\ with\ SNP}{MIC\ of\ gentamicin}$$

THE FIC VALUES HAVE BEEN INTERPRETED AS FOLLOWS:

FIC ≤ 0.5 - synergistic effect
0.5 and ≤ 1 - additive effect
1 and <4 - no action
≥ 4 - antagonistic effect according to the protocol of Buyck *et al.* (2015).

RESULTS

Out of 120 pus samples of (O.M) patient's hundred (100%) samples were positive for bacterial growth. Among 100 positive samples, the infected males count was 52 (52%) and that of females was 48 (48%). According to age wise distribution, highest infection was observed between 1-10 years followed by 11-20 years and 50 years. Out of 100 patients, 43 (43%) had left, 37 (37%) had right, and 20 (20%) had bilateral ear discharge. All the patients had 100% ear discharge problem, 90% ear pain, 68% hearing problem, 52% itching and 40% had fever problems. Twelve different bacterial infections were discovered on the basis of biochemical testing, of which 55% were Gram negative bacteria and 45% were Gram positive bacteria. *S. aureus* had the highest occurrence (32.5%), followed by *S. epidermidis* (10%) and then *Salmonella spp.*, (7.5%). While *E. coli* (7.5%), *Enterobacter spp.* (7.5%), *Provedencia spp.* (5%), *Proteus spp.* (5%), *S. pneumonia* (2.5%), *Klebsiella spp.* (2.5%), *Edwardsiella tarda* (2.5%), *Klebsiella oxytoca* (2.5%) and *P. aeruginosa* (2.5%), respectively, as shown in table 1, 2, 3, 4 and figure 1, 2, 3, and 4.

TABLE: 1 AGE WISE DISTRIBUTION OF PATIENTS (N=100)

S.No	Age	No of isolates	Percentage
1	<10 year	55	55 %
2	11-20	20	20 %
3	21-30	10	10 %
4	31-40	10	10 %
5	41-50	5	5 %

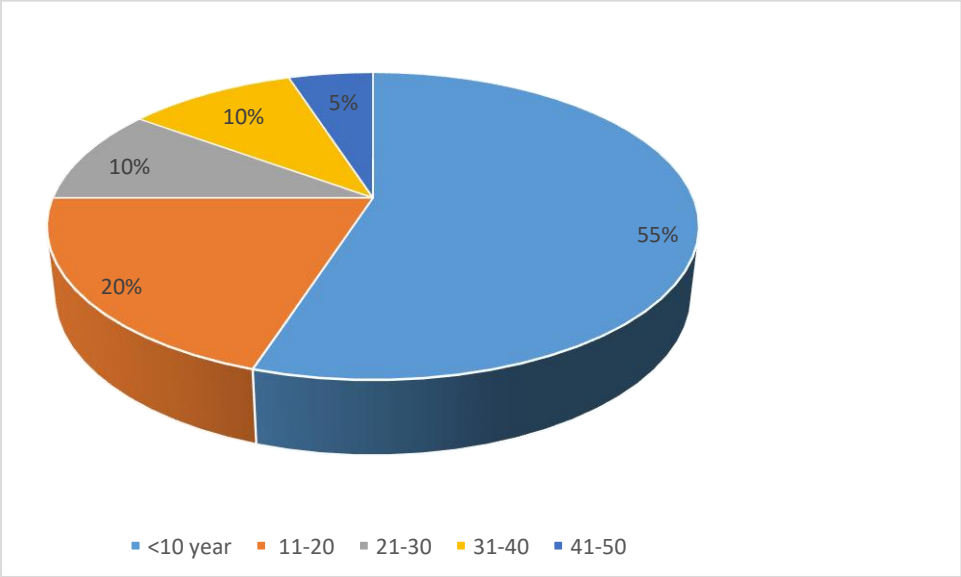


FIGURE: 1. AGE WISE DISTRIBUTION OF O.M PATIENTS BACTERIAL INFECTION PREVALENCE

TABLE: 2. GENDER WISE DISTRIBUTION OF 100 PATIENTS

S.No	Gender	No. of isolates	Percentage
1	Male	52	52%
2	Female	48	48%

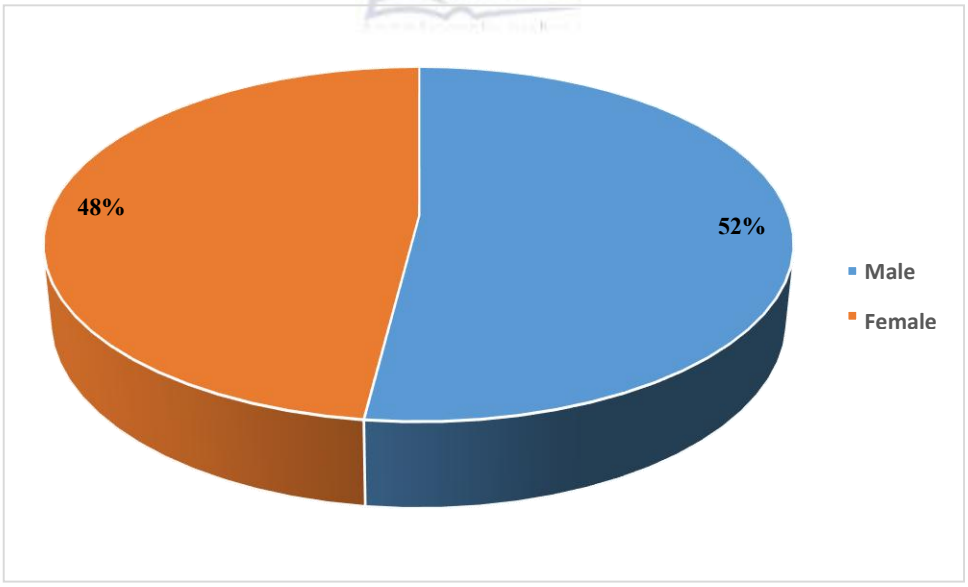


FIGURE: 2 GENDER WISE DISTRIBUTION OF PATIENTS

TABLE: 3 SITE DISTRIBUTION OF 100 PATIENTS

S.No	Site	Percentage
1	Left	43 %
2	Right	37 %

3	Bilateral	20 %

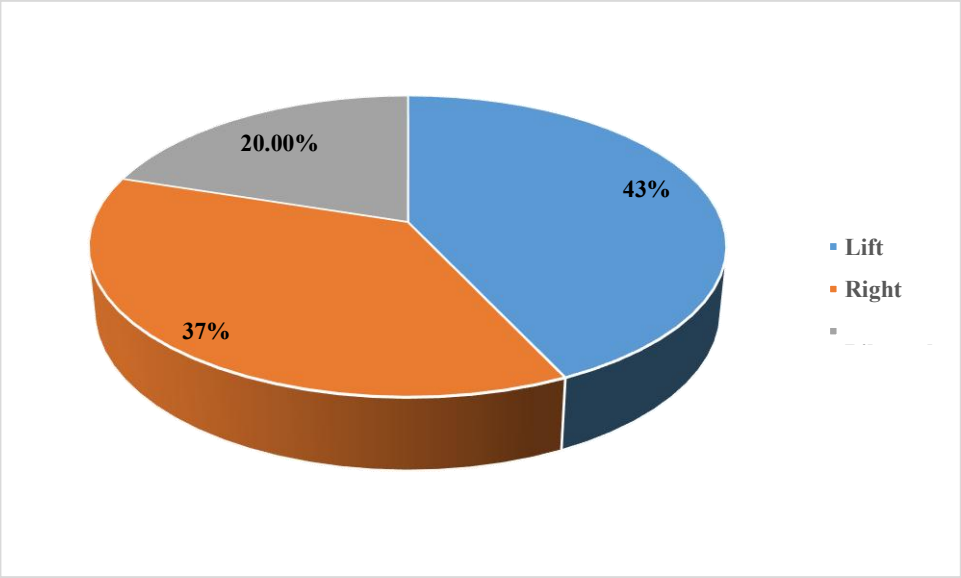


FIGURE: 3 SITE DISTRIBUTION OF 100 PATIENTS

TABLE: 4 CLINICAL/SIGNS AND SYMPTOMS OF ALL THE PATIENTS

Clinical/signs and symptoms	Yes	No
Ear discharge	100 %	0 %
Ear pain	90 %	10 %
Hearing problems	68 %	32 %
Itching	52 %	48 %
Fever	40 %	60 %

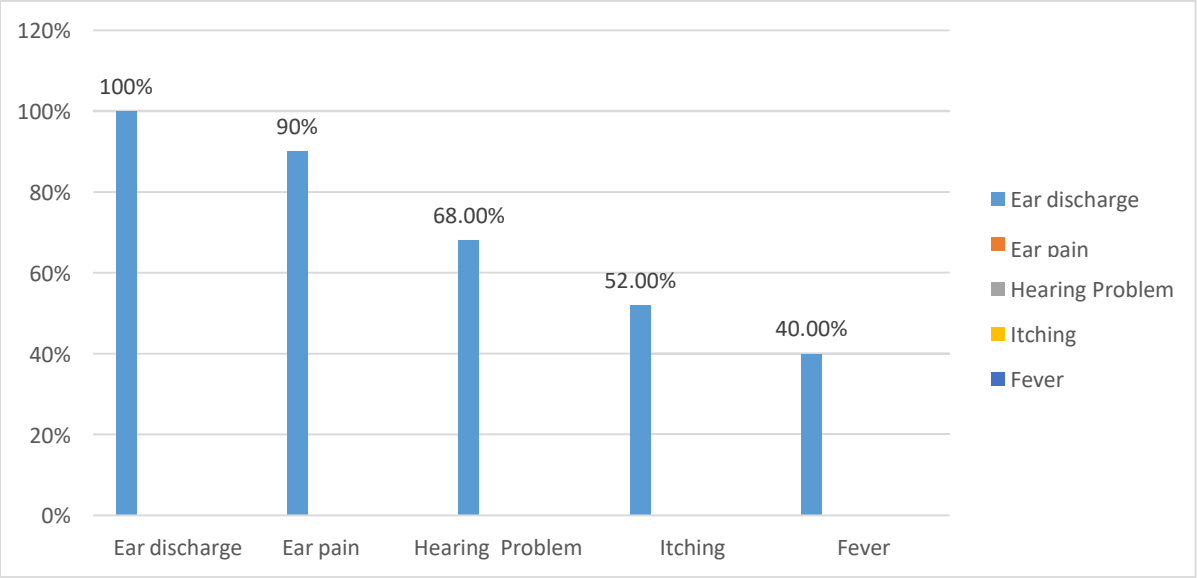


FIGURE: 4 CLINICAL SIGN AND SYMPTOMS OF ALL THE 100 PATIENTS.

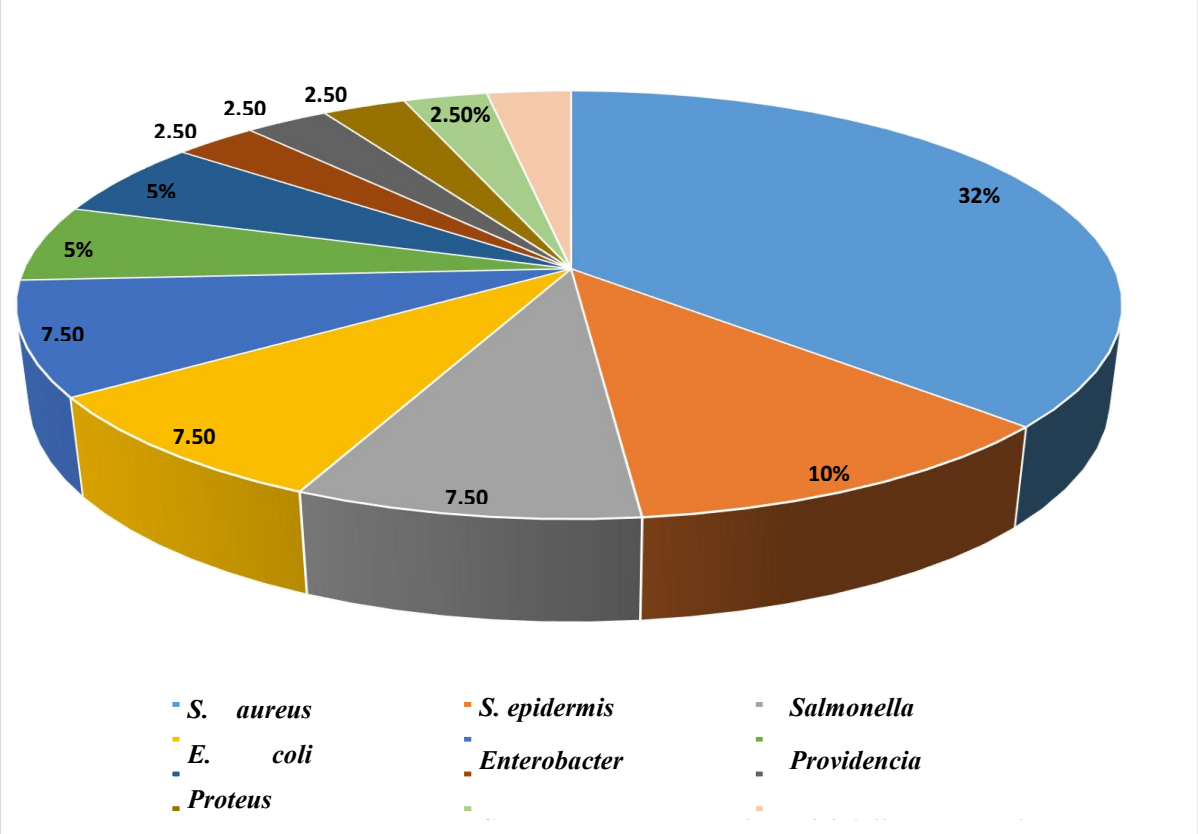


FIGURE: 5 PERCENTAGE OF ALL THE BACTERIAL ISOLATES

IDENTIFICATION OF BACTERIA:

Bacterial identification through Gram staining, different biochemical tests and culture characteristics.

A total of 12 bacterial species were identified by using standard procedures of Gram staining and biochemical tests. Gram positive bacteria such as *S. aureus*, *S. epidermidis* and *S. pneumonia* and Gram-negative bacteria such as *E. coli*, *Salmonella spp..*, *Enterobacter spp.*, *Providencia retgerri*, *Proteus spp.*, *K. pneumonia*, *Klebsiella oxytoca*, *Edwardsiella tarda* and *P. aeruginosa* were identified, respectively. The results of the Gram staining and biochemicaltests are shown in the table.

TABLE: 5 IDENTIFICATIONS OF GRAM-POSITIVE BACTERIA THROUGH BIOCHEMICAL TESTS AND CULTURE CHARACTERISTICS

Name ofBacteria	Culture Characteristics	Gram Staining	Biochemical Tests		
			Catalase	Coagulase	Oxidase
S. aureus	Medium, irregular, light yellow and transparent	Gram +ive cocci cluster (Bunch shape)	+	+	-
S. epidermidis	Small, round, Bright- white, Creamy colonies	Gram +ive cocci cluster (Bunch shape)	+	-	+

S. pneumoniae	Grayish-white, creamy, mucoid, narrow zone of β-hemolysis	Gram +ive cocci chains (pairs)	-	+			

TABLES: 6 IDENTIFICATION OF GRAM-NEGATIVE BACTERIA THROUGH BIOCHEMICALTESTS AND CULTURE CHARACTERISTICS

Name ofBacteria	Culture Characteristics	Gram Staining	Biochemical Tests				
			Catalase	Oxidase	Urease	Citrate	Indole
E. coli	Small, smooth, rhizoid, flat, dry, yellow pink, Lactose fermenting colonies with spreading growth	Gram – ive Rods	+	-	-	-	+
Salmonella	Pale, colorless, smooth, transparent, raised colonies, non-lactose fermenting,	Gram – ive Rods	+	-	-	+	-
Enterobacterspp..	Large, Light pink, Lactose fermenting, mucoid colonies	Gram – ive Rods	+	-	-	+	-
Providencia retgerri	Smooth, pale or colorless, non-lactose fermenting	Gram – ive Rods	+	-	-	+	+
Proteus	Smooth, pale or colorless, non-lactose fermenting, swarming (Blood agar) foul smell colonies	Gram – ive Rods	+	-	+	+	-
K. pneumonia	Large, Dark pink Lactosefermenting (LF) mucoidString forming colonies	Gram – ive Rods	+	-	-	+	+
K. oxytoca	Large, Dark pink Lactosefermenting (LF) mucoidString forming colonies	Gram – ive Rods	+	-	+	-	+
E. tarda	Transparent whitish non-Lactose fermenting, irregular dense colorlesscolony	Gram – ive Rods	+	-	-	+	+
P. aeruginosa	Large, flat, spreading colonies, fruity odor or earthy smell, non-lactose fermenting, producing blue-green pigment	Gram – ive Rods	+	+	-	+	-

TABLE: 7 GRAM NEGATIVE BACTERIAL IDENTIFICATION THROUGH TSI

Name ofBacteria	Triple Sugar Iron			
	Slope	Butt	Gas	H_2S
E. coli	+	+	+	-
Salmonella	+	-	-	+
Enterobacterspp.	+	+	+	-
P. rettgeri	-	-	-	-
Proteus	-	-	-	+
K. pneumonia	+	+	-	-
K. oxytoca	+	+	-	-
E. tarda	-	-	+	+
P. aeruginosa	-	-	-	-

CHARACTERIZATION OF SYNTHESIZED SILVER NANOPARTICLES:

UV–VIS SPECTROSCOPY

UV–vis spectroscopy was used to investigate the optical characteristics of AgNPs at room temperature. After 48 hours of incubation, the color of the treated solution of silver nitrate and tri sodium citrate changed to yellow, a sign that silver nanoparticles have formed. The absorbance of produced AgNPs was measured using a spectrophotometer with a wavelength range of 200-800nm. Due to the presence of Ag ions, a prominent peak was found in the spectrum at 430nm. There was no additional peak in the spectrum, indicating that AgNPs have good optical characteristics. The material is highly efficient and optically active, with good chemical and optical properties, as shown in figure 7.

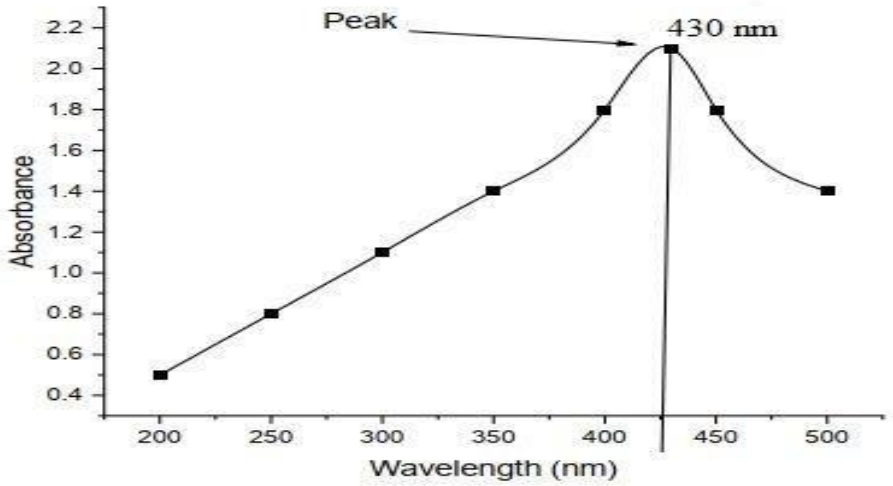


FIGURE: 6 UV-VISIBLE SPECTRA OF CHEMICALLY SYNTHESIZED AGNPS SHOWING PEAK AT 430NM

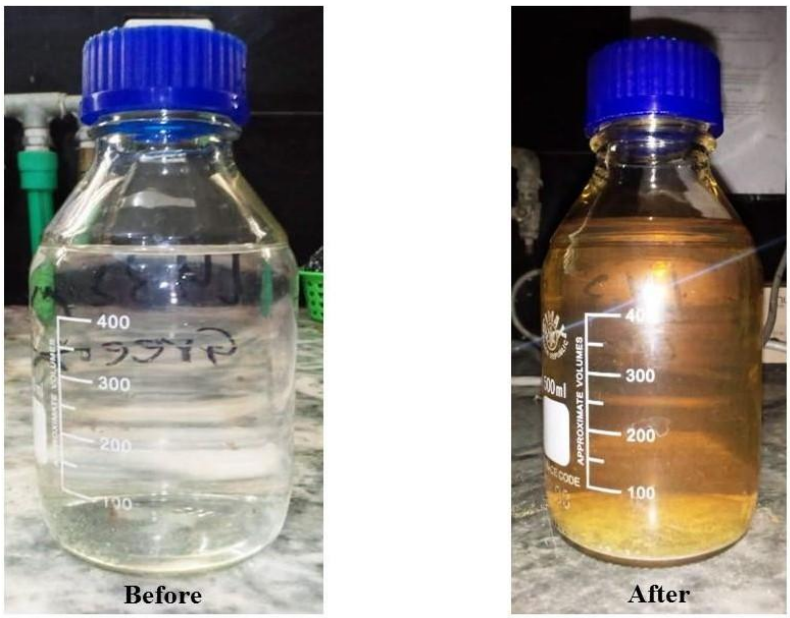


FIGURE: 7 COLOUR CHANGE OF THE TREATED SOLUTION BEFORE AND AFTER 48 HOURS OF INCUBATION

X-RAY DIFFRACTION SPECTRUM ANALYSIS

To determine the nature of AgNPs, XRD was performed for analysis of synthesized AgNPs.This showed that manufacted nano particles were crystalline in nature and produced distinct peaks at 2. The XRD spectrum revealed various brag peaks at 15 different values: 5.33, 5.36, 31.88, 32.42, 38.16, 44.32, 47.92, 64.46 which were indexed to cubic structural planes (50), (80), (90), (100), (111), (390), and (420) showed strong agreement of AgNPs as shown in figure 10.

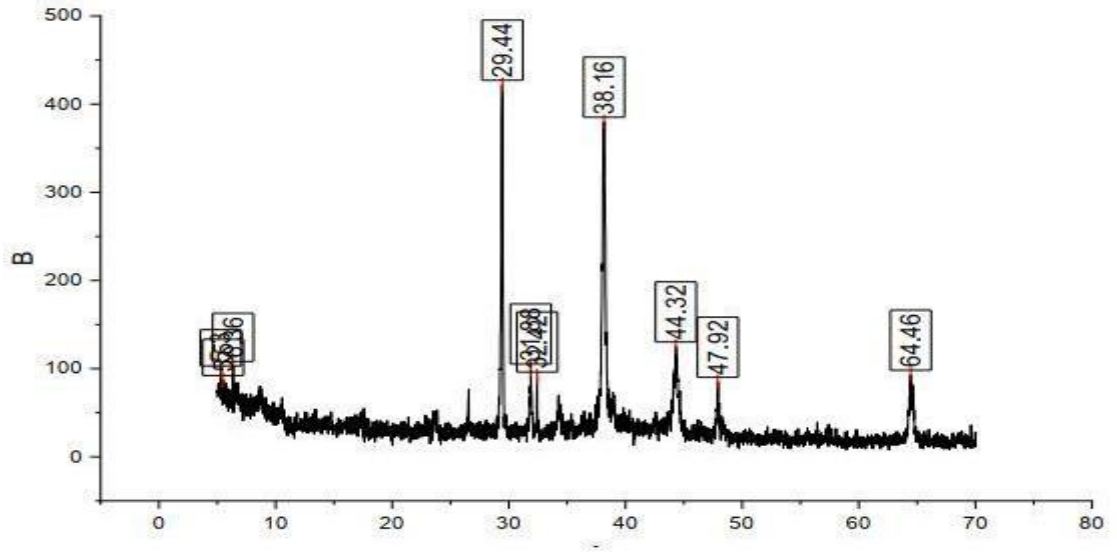


FIGURE:10 XRD SPECTRUM OF SYNTHESIZED NANOPARTICLES SHOWING CRYSTALLINE NATURE OFNANOPARTICLE

SEM ANALYSIS OF SYNTHESIZED NANOPARTICLES

The morphological properties of produced AgNPs were assessed using SEM. At 50,00X resolution, a low-magnification picture of AgNPs revealed tiny spherical nanostructures dispersed across the whole surface. The NPs were well-organized, and they looked to be connected to each other and forming spherical structure. To provide further confirmation of the individual

particle shape and size, a high-magnification image with 60,000X resolution was acquired and is presented below. The diameter of each individual particle was estimated to be in the range of ~13±1 nm based on the acquired data, as shown in figure 11.

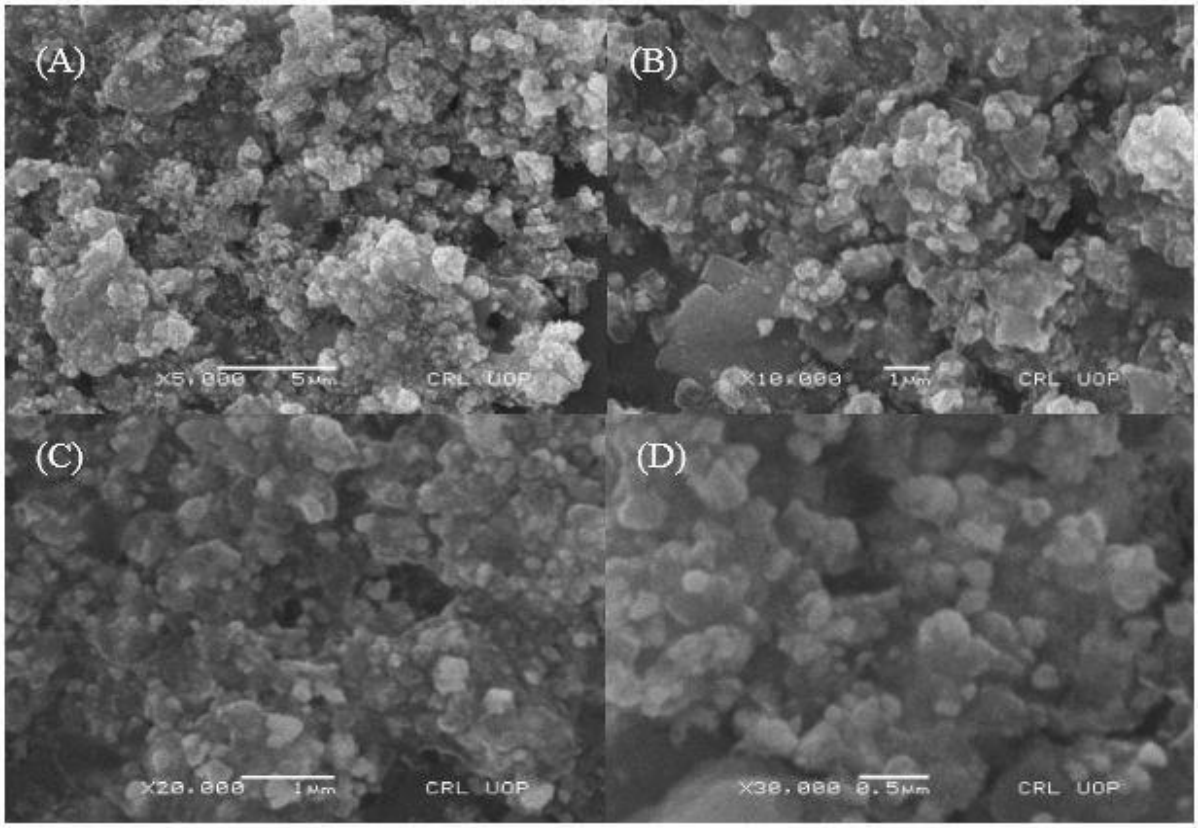
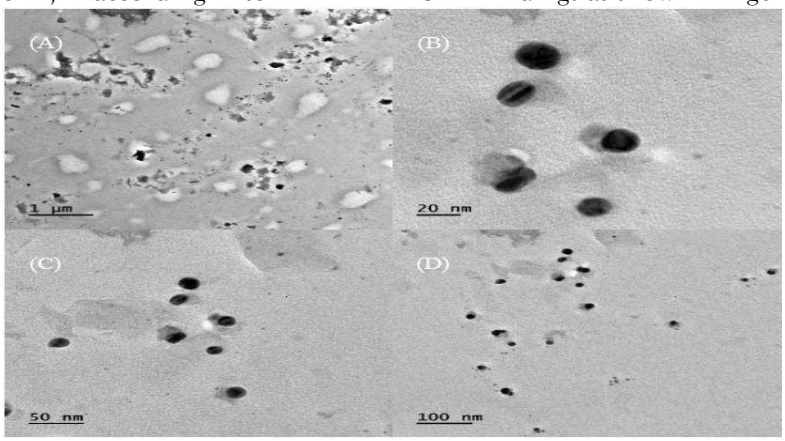


FIGURE: 11 (A, B, C, AND D) SHOWING SCANNING ELECTRON MICROSCOPY OF CHEMICALLY SYNTHESIZED SILVER NANOPARTICLES

TEM ANALYSIS OF SYNTHESIZED NANOPARTICLES

To determine the size and shape of the produced silver nanoparticles, TEM analytical techniques were used. The produced silver nanoparticles had a size range of 20 to 65nm and were mainly spherical in form, according to TEM investigation of synthesized silver nanoparticles. The acquired image revealed many nanometers (nm)-sized particles NPs, with individual NPs measuring ~13±1 nm in size. The smooth and clear surfaces of the spherical NPs were confirmed by TEM pictures, which matched the SEM findings as shown in figure 12.



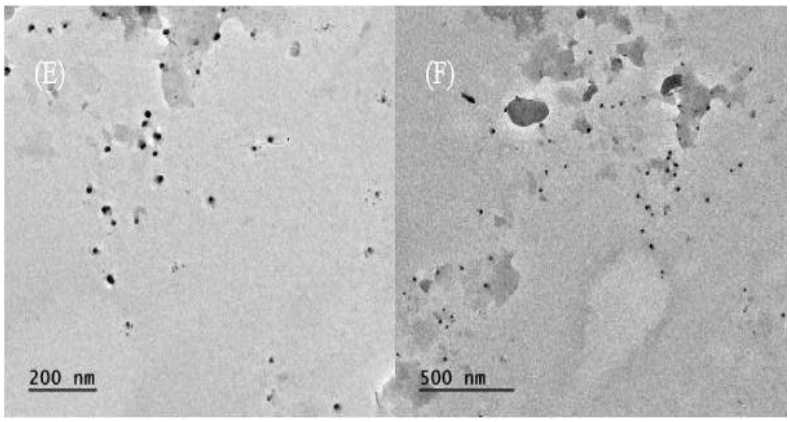


FIGURE: 12 (A, B, C, D, E, AND F) SHOWING TRANSMISSION ELECTRON MICROSCOPY OF CHEMICALLY SYNTHESIZED SILVER NANOPARTICLES

ACTIVITY OF SILVER NANO PARTICLES AGAINST OTITIS MEDIA PATIENTS

Activity of different concentrations of silver nanoparticle i.e., 5mg, 10mg, and 15mg was recorded against *S. aureus*, *P. rettgeri*, *E. tarda*, *K. oxytoca*, *P. mirabilis*, *S. epidermis* and *P. aeruginosa*. The 15mg particle of AgNPs showed excellent efficacy against all the bacterial species used. Maximum of 20mm and a minimum of 15mm inhibition zone was recorded by 15mg nanoparticle solution followed by a maximum of 18mm and minimum of 14mm in 10mg nanoparticle solution, while maximum of 18mm and minimum of 12mm of inhibition zones were recorded for 5mg of nanoparticle solution. Detailed values for each size of particle against every bacterial specie are given in table 8.

TABLE: 8 ACTIVITY OF SILVER NANO PARTICLES AGAINST OTITIS MEDIA PATIENTS (MM)

Species	5 µg (mm)	10 µg (mm)	15 µg (mm)
S. aureus	16	16	20
P. rettgeri	16	18	20
E. tarda	14	14	18
K. oxytoca	14	16	18
P. mirabilis	18	18	20
S. epidermis	12	15	15
P. aeruginosa	12	16	20

TABLE: 9 ANTIBIOTIC SENSITIVITY AGAINST GRAM NEGATIVE BACTERIA (MM)

Species	AMC	IPM	AK	CIP	PB	FOX	TZP	F	FOS	CN
E. tarda	R	R	R	20	R	R	R	R	R	12
P. rettgeri	R	12	R	5	5	R	R	R	R	R
K. oxytoca	R	18	14	18	12	R	14	16	R	14

P. mirabilis	R	14	8	5	5	R	R	R	R	5
P. aeruginosa	R	R	R	12	R	R	R	R	R	10

In the present research work antibiotic sensitivity against Gram-positive as well as Gram-negative microorganisms was recorded. Multiple antibiotics were used including Amoxicillin + Clavulanic acid, Imipinem, Amikacin, Ciprofloxacin, Polymixin, cefoxitin, Tazobactum + Piperacillin, Nitrofurantoin, Fosfomycin, and Gentamicin. In gram negative bacteria Ciprofloxacin showed 20mm of inhibition zone against *E. tarda* while all the other antibiotics used were resistant to *E.tarda*. Imipinim showed 12mm of inhibition zone against *P. Rettgeri* followed by both Ciprofloxacin and Polymixin 5mm, respectively. 18 mm of zone of inhibition was recorded by Imipinem and Ciprofloxacin against *Klebsiella oxytoca* followed by 16mm for Nitrofurantoin and 14mm of zone of inhibition for Amikacin, Tazobactum + Piperacillin and Gentamicin respectively. 14mm of zone of inhibition was recorded by Imipinem followed by Amikacin 8mm, Ciprofloxacin 5mm, Polymixin 5 mm and Gentamicin 5mm against *P. mirabilis*. While 12mm of zone of inhibition was recorded by Ciprofloxacin and 10mm by Gentamicin against *Pseudomonas*. Ciprofloxacin was found to be the most effective antibiotic as it showed excellent efficacy against all the Gram-negative bacteria, followed by Imipinem, Gentamicin and Amikacin.

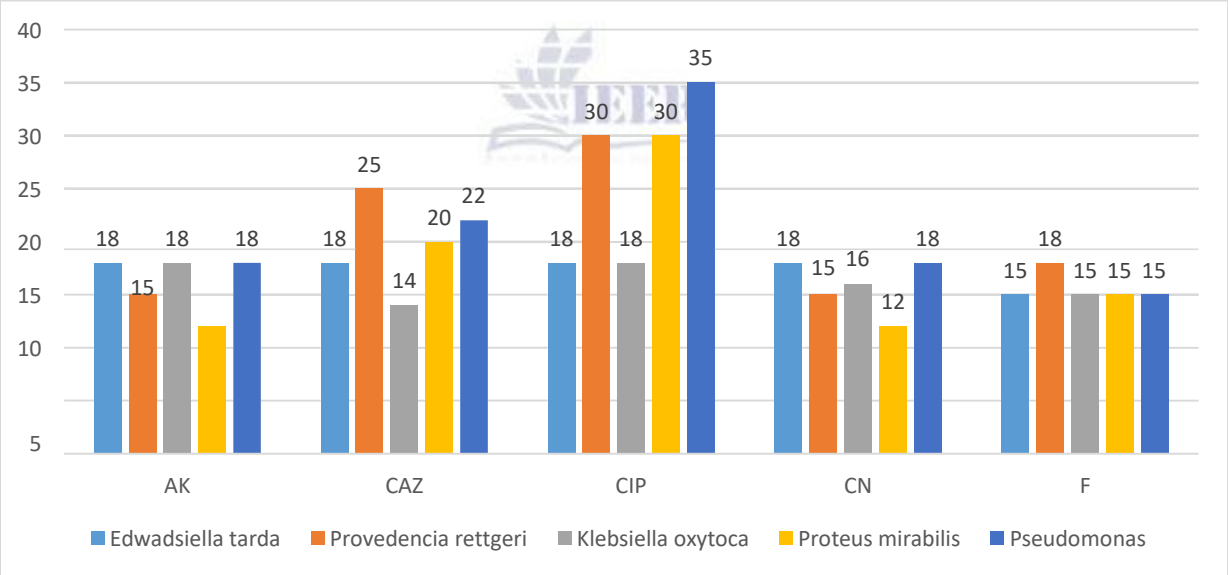


FIGURE: 11 (A) SILVER NANO PARTICLES COATED ANTIBIOTIC AGAINST GRAM NEGATIVE SPECIES

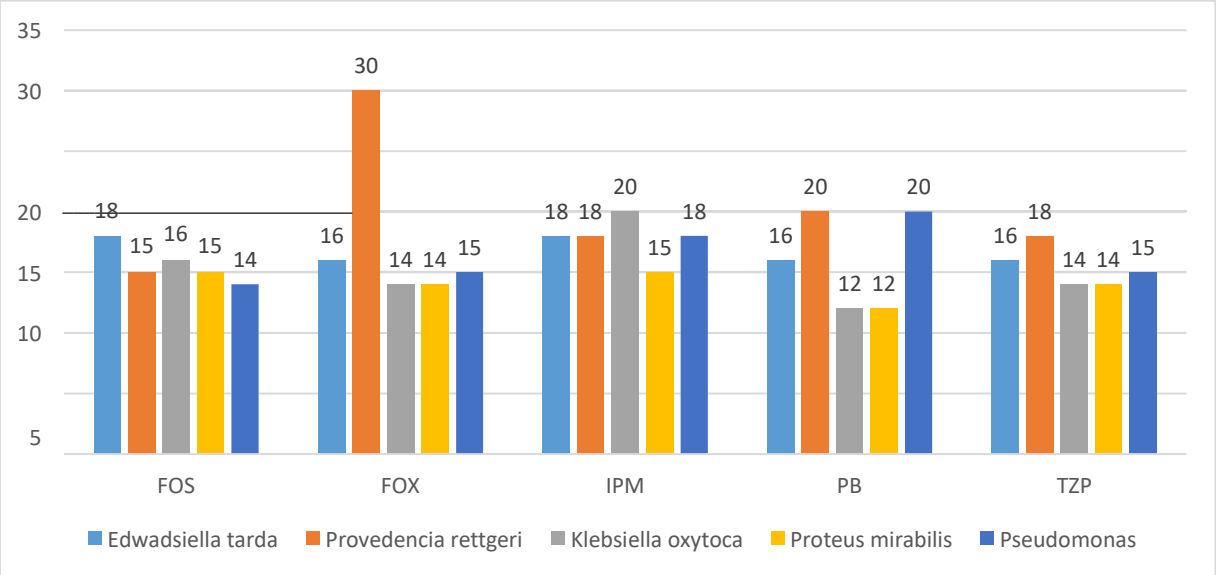


FIGURE: 5.11 (B) SILVER NANO PARTICLES COATED ANTIBIOTIC AGAINST GRAM NEGATIVE SPECIES

Antibiotics coated with AgNPs produced significant inhibition zones. Antibiotic Amoxicillin + Clavulanic acid alone was resistant to all the isolated Gram-negative bacteria but in combination with AgNPs, its. potency was recorded up to 100% against *E. tarda*, *P. rettgeri*, *K. oxytoca*, *P. mirabilis*. Effect of Imepinem in combination with silver nanoparticles was increased to 100% (Inc. potency) against *E. tarda*, followed by 50% (Inc. potency) against *P. Rettgeri*, 11.10% (Inc. potency) against *K. oxytoca*, and 7.10% (Inc. potency) against *P. mirabilis*. Effect of Amikacin was increased to 100% (Inc. potency) againstboth *E. tarda*, *P. rettgeri*, followed by 50% (Inc. potency) against *P. mirabilis* and 28.50% (Inc. potency) against *K. oxytoca*. Effect of Ciprofloxacin in combination with silver nanoparticle was increased up to 500% (Inc. potency) against *P. rettgeri*, *P. mirabilis* followed by 11% (Inc. potency) against *E. tarda* while no increase was seen in *Klebsiella oxytoca*.

Effect of Polymixin in combination with AgNPs was increased up to 300% (Inc. potency) against *P. rettgeri*, followed by 140% (Inc. potency) against *Proteus Mirabilis*, 100% (Inc. potency) against *E. tarda*, and 0% against *K. oxytoca*. Effect of cefoxitin in combination with AgNPs was increased up to 100% (Inc. potency) against all the bacteria used. Effect of Tazobactum + Piperacillin, in combination with AgNPs was increased up to 100% (Inc. potency) against all the bacteria used except 0% against *K. oxytoca*. Effect of Nitrofurantoin in combination with silver nanoparticle was increased up to 100% (Inc. potency) against all the bacteria used except 6.60% (Inc. potency) against *K. oxytoca*. Effect of Fosfomycin in combination with AgNPs was also increased up to 100% (Inc. potency) against all the bacteria used. Effect of gentamicin in combination with AgNPs was increased up to 140% (Inc. potency) against *P. mirabilis* followed by 100% (Inc. potency) against *P. rettgeri*, 50% (Inc. potency) against *E.tarda* and 14.2% (Inc. potency) against *K. oxytoca*. Most enhanced AgNPs activity 500% (Inc. pot) was observed in combination with the antibiotic Ciprofloxacin against *P. rettgeri* and *P. mirabilis* while it was 100% (Inc. pot) against *S. aureus* and *S. epidermis* followed by (140% inc pot) in combination with antibiotic gentamicin against *P. mirabilis*. Amoxicillin + Clavulanic acid and cefoxitin in combination with AgNPs also enhanced 100% (Inc. pot) against all Gram-negative bacterial species. Detailed values for each AgNPs coated

and non-coated antibiotic along with Inc. potency percentage against each Gram-negative bacteria used, are given in table 10.

TABLE: 10 ACTIVITY OF COATED AND NON-COATED ANTIBIOTICS AGAINST GRAM-NEGATIVEBACTERIA

Antibiotic used in activity Measured in mm		*Edwardsiella tarda*	*Providencia rettgeri*	*Klebsiellaoxytoca*	*Proteus mirabilis*
AMC	Uncoated AgNPs	R	R	R	R
	Coated	18 mm	25 mm	14 mm	20 mm
	Inc %				
	Potency	100%	100%	100%	100%
IPM	Uncoated AgNPs	R	12 mm	18 mm	14 mm
	Coated	18 mm	18 mm	20 mm	15 mm
	Inc %				
	Potency	100%	50%	11.10%	7.10%
AK	Uncoated AgNPs	R	R	14 mm	8 mm
	Coated	18 mm	15 mm	18 mm	12 mm
	Inc %				
	Potency	100%	100%	28.50%	50%
CIP	Uncoated AgNPs	18 mm	5 mm	18 mm	5 mm
	Coated	20 mm	30 mm	18 mm	30mm
	Inc %				
	Potency	11%	500%	0%	500%
PB	Uncoated AgNPs	R	5 mm	12 mm	5 mm
	Coated	16 mm	20 mm	12 mm	12 mm
	Inc %				
	Potency	100%	300%	0%	140%
FOX	Uncoated AgNPs	R	R	R	R
	Coated	16 mm	30 mm	14 mm	14 mm
	Inc %				
	Potency	100%	100%	100%	100%
TZP	Uncoated AgNPs	R	R	14 mm	R
	Coated	16 mm	18 mm	14 mm	14 mm
	Inc %				
	Potency	100%	100%	0%	100%
F	Uncoated	R	R	16 mm	R

	AgNPs Coated	15 mm	18 mm	15 mm	15 mm
	Inc % Potency	100%	100%	6.60%	100%
FOS	Uncoated	R	R	R	R
	AgNPs Potency	18 mm	15 mm	16 mm	15 mm
	Inc % Potency	100%	100%	100%	100%
CN	Uncoated	12 mm	R	14 mm	5 mm
	AgNPs Coated	18 mm	15 mm	16 mm	12 mm
	Inc % Potency	50%	100%	14.2	140%

TABLE: 11 ANTIBIOTIC SENSITIVITY AGAINST GRAM POSITIVE BACTERIA

SPECIES	AMC	IPM	AK	VA	DO	E	CIP	OX	FOS	CN
S. *aureus*	R	R	12	R	R	10	R	R	R	R
S. *epidermidis*	R	R	R	10	12	R	R	R	8	R

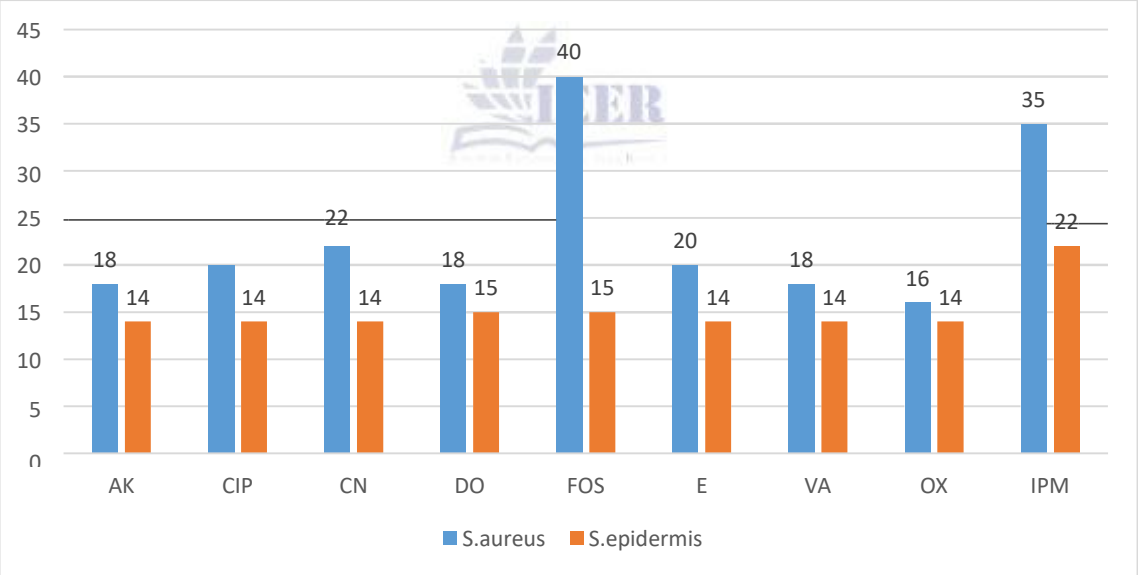


FIGURE :12 SILVER NANO PARTICLES COATED ANTIBIOTIC AGAINST GRAM POSITIVE SPECIES

Effect of Imipinem, Erythromycin, Ciprofloxacin, Oxacillin, and Gentamicin in combination with silver nanoparticle was increased up to 100% against both S. *aureus* and S. *epidermis*. Effect of Amikacin in combination with silver nanoparticle was increased up to 100% against S. *epidermidis* and 50% against S. *aureus*. Effect of vancomycin in combination with silver nanoparticle was increased up to 100% against S. *aureus* 40% against S. *epidermidis*. Effect of Doxycycline in combination with silver nanoparticle was increased up to 100% against S. *aureus* and 25% against S. *epidermidis*. Effect of Fosfomycin in combination with silver nano

particle was increased up to 100% against *S. aureus* and 87.50% and *S. epidermidis*. Detailedvalues for each AgNPs coated and non-coated antibiotic along with Increase in potential percentage against both *S. aureus* and *S. epidermidis* are given in table 12.

TABLE: 12 ACTIVITY OF COATED AND NON-COATED ANTIBIOTICS AGAINST GRAM-POSITIVE BACTERIA

Antibiotic used in activity	Measured in mm	*S.aureus*	*S.epidemis*
IPM	Uncoated	R	R
	AgNPs Coated	35 mm	22 mm
	Inc % Potency	100%	100%
AK	Uncoated	12 mm	R
	AgNPs Coated	18 mm	14 mm
	Inc % Potency	50%	100%
VA	Uncoated	R	10 mm
	AgNPs Coated	18 mm	14 mm
	Inc % Potency	100%	40%
DO	Uncoated	R	12 mm
	AgNPs Coated	18 mm	15 mm
	Inc % Potency	100%	25%
E	Uncoated	10 mm	R
	AgNPs Coated	20 mm	14 mm
	Inc % Potency	100%	100%
CIP	Uncoated	R	R
	AgNPs Coated	20 mm	14 mm
	Inc % Potency	100%	100%
OX	Uncoated	R	R
	AgNPs Coated	16 mm	14 mm
	Inc % Potency	100%	100%
FOS	Uncoated	R	8 mm
	AgNPs Coated	40 mm	15 mm
	Inc % Potency	100%	87.50%
CN	Uncoated	R	R
	AgNPs Coated	22 mm	14 mm
	Inc % Potency	100%	100%

DETECTION OF ESBL PRODUCING BACTERIAL ISOLATES

A total of 10 bacterial isolates including 7 *E. coli* and 3 *Klebsiella spp.* were used for the detection of ESBL producing bacterial isolates. Out of 7 *E. coli* and 3 *Klebsiella spp.* only 1 bacterial *spp.* from each bacterium used was ESBL Positive detected. Percentage of *Klebsiella* was higher than *E. coli.*

TABLE: 13 DETECTION OF ESBL PRODUCING BACTERIAL ISOLATES

S.No	Gram -ive Isolates	Total	ESBL Positive	Percentage
1	*E. coli*	7	1	14.20
2	*Klebsiella spp.*	3	1	33.30

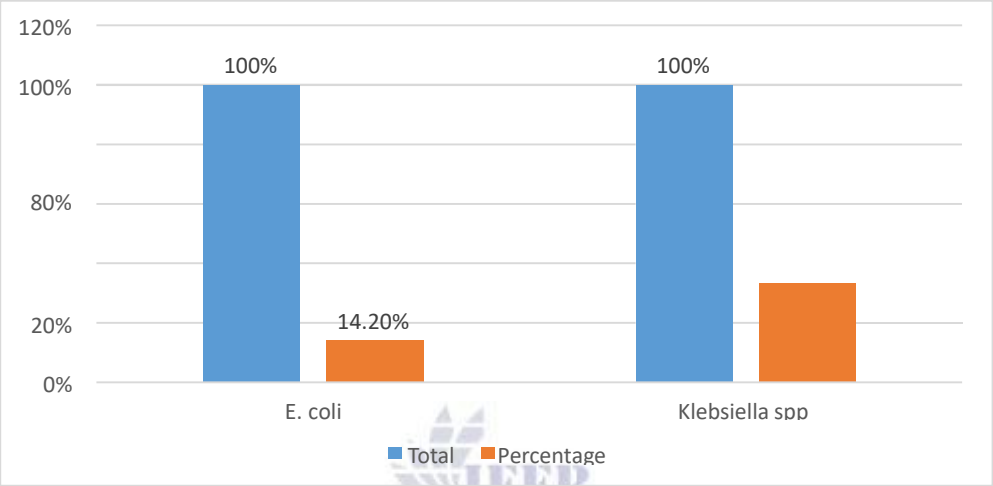


FIGURE: 13 PERCENTAGE OF ESBL PRODUCING BACTERIAL ISOLATES

MIC AND FIC OF AGNPS

The FIC index was used to analyse the synergistic effects of AgNPs and conventional antibiotics. Synergistic interactions of AgNPs and Gentamicin were observed against, *S. aureus*, and *E. coli*. Gentamicin was alone ineffective against *S. aureus* however, when combined with AgNPs it enhanced antibacterial effects and showed synergistic effects against *S. aureus*, and *E. coli* as shown in table no. 14 and 15.

TABLE: 14 MINIMUM INHIBITORY CONCENTRATION OF BACTERIAL ISOLATES

	MIC		
Bacteria	**Gentamicin**	**AgNPs**	**Antibiotic + AgNPs**
S. aureus	24	8	25
E. coli	26	12	30

TABLE: 5.15 FIC OF BACTERIAL ISOLATES

	MIC				
Bacteria	**Gentamicinn**	**AgNPs**	**Antibiotic +AgNPs**	**FIC**	**Effect**
S. aureus	24	8	25	0.3	Synergistic
E. coli	26	12	30	0.4	Synergistic

DISCUSSION

Nanoparticle act as a medium and carriers for antibiotic and natural antimicrobial complexes (Wang *et al.*, 2017). Among all metal nanoparticles, silver nanoparticles are regarded as the most significant and trustworthy.

AgNPs have a wide range of applications in the treatment of various bacterial and fungal infections, as well as serving as a drug carrier with the least amount of cytotoxicity (Prabhu *et al.*, 2012).

Out of 100 pus samples infected male were 52 (52%) and female were 48 (48%). By age wise distribution, highest infection was observed in age between 1- 10 year followed by 11-20 year and 50 years. Out of 100 patients, 43 (43%) had left, 37 (37%) had right, and 20 (20%) had bilateral ear discharge. Twelve different bacterial infections were discovered on the basis of biochemical testing of which (55%) were Gram negative bacteria and (45%) were Gram positive bacteria. *S. aureus* had the highest occurrence (32.5%), followed by *S. epidermidis* (10%) and then *Salmonella spp.* (7.5%). *E. coli* (7.5%), *Enterobacter spp.* (7.5%), *Provedencia spp.* (5%), *Proteus spp.* (5%), *S. pneumonia* (2.5%), *Klebsiella spp.* (2.5%), *Edwardsiella tarda spp.* (2.5%), *K. oxytoca* (2.5%) and *P. aeruginosa* (2.5%).

From UV– visible Spectroscopy the tested sample had absorbed energy at 430 nm which was the characteristic peak value of the silver nanoparticles. Different researcher such as Farsi & Farokhi, 2018, Gudikandula *et al.*, 2017 and Ingle *et al.*, 2008 observed absorption peak of silver nanoparticles in the range of 400 – 460nm which showed agreement to our findings of UV – visible Spectroscopy. The possible reasons for the variations in absorption peaks according to Route *et al.*, 2012 might be differences in the chemical, physical and biological methods of synthesizing silver nanoparticles.

To determine the nature of AgNPs, XRD was used to analyse synthesized AgNPs and they were found crystalline and produced distinct peaks at 2θ. The XRD spectrum revealed various bragg peaks at 15 different values: 5.33, 5.36, 31.88, 32.42, 38.16, 44.32, 47.92, 64.46 which were indexed to cubic structural planes (50), (80), (90), (100), (111), (390), and (420) showed strong agreement of AgNPs. XRD analysis of Anbazhagan *et al.*, (2017) showed four clear bragg's peaks at 38.68, 46.1, 64.11 and 77.4 corresponding to (111) (200) (220) and (311) planes of the face-centered cubic (FCC) silver planes of the face-centered cubic (FCC) silver while in the study of Husseiny *et al.*, (2015), XRD spectrum showed four distinct diffraction peaks at 38.15°, 44.18°, 64.63° and 77.50° corresponding lattice plane value was indexed at (111), (200), (220) and (311) planes of face centered cubic (FCC) silver with a lattice parameter of a = 4.08 Å, and Singh *et al.*, 2013 also reported the same findings with minute distinctions to our XRD analysis of synthesized silver nanoparticles. These variations might be due to biological, chemical, incubation, temperature,and handling applications.

Moreover, SEM and TEM analysis techniques were used to know the morphology andsize of the synthesized silver nanoparticle. The morphological properties of AgNPs were assessed using scanning electron microscopy (SEM). At 5000X resolution, a low- magnification picture of AgNPs reveals tiny spherical nanostructures dispersed across the whole surface. The NPs were well-organized, and they looked to be connected to other particles and forming spherical formations. To provide further confirmation of the individual particle shape and size, a high-magnification image with 60,000X resolution was obtained. The diameter of each individual particle was estimated to be in the range of ~13±1 nm based on the acquired data. To determine the size and shape of the produced silver nanoparticles, TEM analytical techniques were performed. The produced silver nanoparticles had a size range of 20 to 65 nm and were primarily spherical in form, according to TEM investigation of synthesised silver nanoparticles. The acquired image revealed many nanometres sized particles (NPs), with individual NPs measuring ~13±1 nm in size. The

smooth and clear surfaces of the spherical NPs were confirmed by TEM pictures, which matched the SEM findings. SEM analysis of Rahimi *et al.*, (2016) revealed that size of synthesized silver nanoparticles was 20
– 80 nm. Devika *et al.*, (2012) revealed that biosynthesized silver nanoparticles were dispersed and were about 50 nm in size. The study of Sagar and Ashok *et al.*, (2012) revealed that synthesized silver nanoparticles were polydisperse globular in shape about 1 – 20 nm in size. The studies of these researchers showed good similarities to our current study.

In the current research project various pathogens were isolated such as *S. aureus, S. epidermidis Salmonella spp.*, *Enterobacter spp.*, *Provedencia spp.*, *Proteus spp.*, *S. pneumonia*, *Klebsiella spp.*, *E. tarda spp.*, *K. oxytoca* and *P. aeruginosa* from pus samples that were collected from otitis media patients by using standard microbiological procedures. These isolated bacteria were identified by various biochemical tests including Gram staining, catalase,oxidase, coagulase, urease, citrate, indole and triple sugar iron reactions. Whereas, Ahmad *et al.*, (2013) also isolated multiple bacterial organisms from middle ear culture from which most common isolated organism was Methicillin-sensitive *S. aureus* (MSSA) (45.1%) was the most common, followed by *P. aeruginosa* (19.5%), Methicillin-resistant *S. aureus* (MRSA), Coagulase-negative *Staphylococcus, P. mirabilis*, and *E. coli.* However, in a similar study conducted by Malkappa *et al.*, (2012), *P. aeruginosa* (45.24%) was predominant followed by *Staphylococcus aureus* (22.22%). This study showed minute distinctions to our research in isolated pathogens from otitis media patients which is might be due to species variations and host distributions.

In the present research work antibiotic sensitivity against Gram-positive as well as gram-negative microorganisms was recorded. Multiple antibiotics were used including Amoxicillin + Clavulanic acid, Imipinem, Amikacin, Ciprofloxacin, Polymixin, Cefoxitin, Tazobactum + Piperacillin, Nitrofurantoin, Fosfomycin, and Gentamycin. In gram negative bacteria Ciprofloxacin showed 20mm of inhibition zone against *E. tarda* while all the other antibiotics used were resistant to *E. tarda.* Imipinem showed 12mm of inhibition zone against *P. rettgeri* followed by both Ciprofloxacin and Polymixin 5mm, respectively. 18mm of zone of inhibition was recorded by Imipinim and Ciprofloxacin against *K. oxytoca* followed by 16mm for Nitrofurantoin and 14mm of zone of inhibition for Amikacin, Tazobactum + Piperacillin, and Gentamicin respectively. 14 mm of zone of inhibition was recorded by Imipinem followed by Amikacin 8mm, Ciprofloxacin 5mm, Polymixin 5 mm and Gentamicin 5mm against *P. mirabilis*. While 12mm of zone of inhibition was recorded by Ciprofloxacin and 10mm by Gentamycin against *Pseudomonas*. Ciprofloxacin was found to be the most effective antibiotic as it showed excellent efficacy against all the Gram-negative bacteria, followed by Imipinem, Gentamycin and Amikacin, while Doxycycline and Amikacin showed good efficacy against gram positive bacterial isolates. The study of Gulati *et al.*, (1997), Varshney *et al.*, (1999) and Malkappa *et al.*, (2012) also revealed that Ciprofloxacin and Amikacin were found most effective as compared to other antibiotics used in their study.

AgNPs are renowned for their remarkable antibacterial activity against human microorganisms and exhibit synergistic action when provided in conjunction with various adjuvants. In the current study, the efficacy of AgNPs in combination with various antibiotics against a variety of bacteria was examined to see if the antibacterial activity of a particular antibiotic is boosted. Most enhanced silver nanoparticle activity (500% increase in potential) was observed in combination with the antibiotic Ciprofloxacin against *P. rettgeri* and *P. mirabilis* while it was (100% increase in potential) against *S.*

aureus and *S. epidermidis*, followed by (140% increase in potential) in combination with antibiotic Gentamycin against *P. mirabilis.* Amoxicillin + Clavulanic acid and Fosfomycin in combination with AgNPs also enhanced (100% increase in potential) against all Gram-negative bacterial species. Synergistic antibacterial effect of synthesized AgNPs with antibiotic Ciprofloxacin was also reported by Nikparast *et al.*, (2018). Mala, *et al.*, 2012 also stated that, the anti-phytopathogenic activity against *Pseudomonas solanocearum*, *Pseudomonas syringae*, *Xanthomonas malvacearum*, and Xanthomonas *campestris* was significantly boosted when 0.2mm silver nanoparticle was combined with 1g of ciprofloxacin. Smekalova, *et al.*, 2016 stated that the antibacterial activity of amoxycillin combined with different sizes of AgNP was synergistic and the additive effects of amoxycillin in combination with AgNPs was observed against Gram Positive *S. aureus*.

In the current investigation, 10 different bacterial isolates, including *E. coli* (7) and *Klebsiella spp.* (3) were used for the detection of ESBL producing bacterial isolates. Out of 7 *E. coli* and 3 *Klebsiella spp.* only 1 bacterial *spp.* from each bacterium used was ESBL Positive detected. Percentage of *Klebsiella* was higher than *E. coli*. Somily *et al.*, (2014), Shakya *et al.*, (2017) and Surgers *et al.*, (2019) also reported ESBL positive strains of *E. coli* and *Klebsiella spp.* in their research study. The Fractional Inhibitory Concentration (FIC) index was used to analyse the synergistic effects of AgNPs and conventional antibiotics. Synergistic interactions of AgNPs and Gentamicin were observed against *S. aureus*, and *E. coli*. Gentamicin was alone resistant to *S. aureus* however, when combined with AgNP it enhanced antibacterial effects and showed synergistic effects against both *S. aureus*, and *E. coli*. Some other researchers also reported the same findings with minute distinctions to our findings. Smekalova *et al.*, (2016) also achieve the synergistic effect of gentamicin in combination with silver nano particle. Birla *et al.*, (2009) shown that gentamicin's effectiveness against *S. aureus* and *E. coli* was boosted in the presenceof AgNPs.

CONCLUSION

It was concluded that *S. aureus, S. epidermidis Salmonella spp.*, *Enterobacter spp.*, *Provedencia spp.*, *Proteus spp.*, *S. pneumonia*, *Klebsiella spp..*, *E. tarda spp.*, *K. oxytoca* and *P. aeruginosa* were common bacterial pathogens isolated from otitis media patients. Moreover, it was also concluded that AgNPs enhanced the antimicrobial activity of all tested antibiotic. Most enhanced AgNPs activity was observed in combination with the antibiotic Ciprofloxacin(500% increase in potential) against *P. rettgeri* and *P. mirabilis* while it was (100% increase in potential) against *S. aureus* and *S. epidermidis*, followed by (140% increase in potential) in combination with antibiotic Gentamycin against *P. mirabilis.* Amoxicillin + Clavulanic acid and Fosfomycin in combination with AgNPs also enhanced (100% increase in potential) against all Gram-negative bacterial species.

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