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Biogenic Synthesis of Silver Nanoparticles and Study their Effect with Amoxicillin against *Salmonella typhi* and *S. typhimurium*

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Abstract: The emergence of antibiotic- and /or multidrug-resistant bacteria is recognized as a crucial challenge for public health. The potential of AgNps as an anti-microbial agent is greatly investigated which considered as an alternative method to decrease multi-drug resistance microbes. AgNPs can offer a new strategy to tackle multidrug-resistant bacteria. The present study discusses the novel approach to synthesize nanoparticles involving biogenic synthesis of AgNPs using *Klebsiella pneumoniae* and study their impact as antimicrobial spectrum .The combined effect of silver nanoparticles with Amoxicillin against *S. typhi* and *S. typhimurium* was also observed . These tactics are used to promote antimicrobial efficacy of silver nanoparticles by modification of surfaces and to survey activity of antibiotic acceptance.

Keywords: Nanomedicine, *S. typhi*, Synergism, Biomaterial, Amoxicillin, Transmission Electron Microscope. Atomic force microscope, Nano Biomed, XRD

There is a growing requirement for using the ecofriendly nanoparticles (NPs) that don't produce any toxic waste in their protocol of synthesis (Vahabi et al 2011). Several approaches were designed for synthesizing NPs and the most essential aspects of nanotechnology depend on the sizes, shapes and controlled mono dispersity (Pugazhenthiran et al 2009). The biotechnological method has elicited as an alternative and safe process in the formation of NPs by employing ambient bio resources (Baker et al 2013). The combination of biosynthesis AgNPs with any antibiotic has potent anti-bacterial efficacy. In fact, the ampicillin breaks down the bacterial cell wall and assists the internalization of AgNPs in cells of bacteria. The AgNP binds with DNA and inhibits its unwinding, which leads to the death cells and naturally interacts with the bacterial membrane and disrupts the integrity of membranes, and the ion of silver binds to nitrogen, sulfur, and oxygen, of main biomolecules and inhibits the growth of bacteria (Juan 2010). Biological approaches describe the inexpensive and ecofriendly method for the formation of NPs. The synthesis of NPs was demonstrated from some microorganisms, among them the gram negative and gram positive bacteria (Sunkar and Nachiyar 2012). *S. enterica* represents a main animal and human pathogen. *S. enterica serovar typhi* and *typhimurium* are the best described serovars, with the first being included in localized gastro-enteritis in several hosts and the latter causing the systemic human-specific disease (Pauline 2016). Expressively, the biofilm cell resistance to anti-microbial significantly increased in comparison with the same cells being planktonic (Gilbertand Mcbain 2003). Therefore, the formation of biofilms improves the ability of pathogenic *Salmonella enterica* for surviving stresses that are generally encountered both within food processing, in addition to during host infection(Al-Khafaji 2017). The current study aimed to produce AgNPs biologically using *Klebsiella pneumoniae* and study their synergistic effect with amoxicillin.

MATERIAL AND METHODS

Chemicals and media: Silver nitrate (AgNo3) was purchased from Appli Chem, Germany and all media used throughout this study were purchased from Oxoid Ltd., England

Isolation and identification of *Salmonella* spp.: A total of 95 clinical specimens have been taken from blood, stool and mid-stream urine, from patients attending AL-Ramadi Hospital and special private laboratories. Specimens were cultured on MacConkey, XLD and blood agar and incubated overnight at 37°C. Afterward, the grown colonies were identified by Vitek 2 compact system using GN card depending on instructions of the BioMérieux manufacturer.

Synthesis of AgNPs: Faria et al (2016) method with some modification which included the use of supernatant solution of bacteria *Klebsiella pneumonia* instead seed extract of *Cydonia oblonga* and used glucose solution 10mM (bio-chemical reduction method) (Zamin 2015). All modification were adopted for preparing the colloidal of the silver nanoparticles at a concentration of 10mM as follow

Solution A: The stock solution of AgNo3 at concentration 100mM has been made by dissolving 0.8493 gram was dissolved from AgNO3 powder in 50 ml of de ionized distilled water. It was heated at 50c^0 for 15-30 mint by magnetic stirrer, then where getting stock solution with concentration 100Mm were used for synthesis AgNPs as needed. Stock solutions kept in dark bottle in refrigerators at 2-8^{O}C.

Solution B: The stock solution of glucose at concentration 100mM was prepared by dissolving 0.45 gram glucose powder in 25 ml of de ionized distilled water and mixed by stirrer without heating for 15-30 mint. The stock solution with concentration 100Mm were used for synthesis AgNPs as reducing agent at needed. The solutions were sterilized by Millipore filter 0.2 mm stock solutions and kept in dark bottle in refrigerator at 2-8^{O}Cat ready to use.

Solution C: This solution was prepared according to the procedure described by (Srinath and Rai (2015**).** The overnight culture of *Klebsiella pneumoniae* was inoculated in 250 ml-flask containing 100ml Luria–Bertani broth and incubated at 37°C for 24 hr. Subsequently, the turbidity of the culture was adjusted to 0.5 McFarland unit (1.5 × 10^8 cfu/ml) using densi check. Thereafter, the cultures were centrifuged and at 4000 rpm for 15 min. The supernatant obtained after centrifugation was applied to produce solution B. Solution A was put to heating at 70°C with stirring vigorously and with regular time 40-45 mint to obtain a homogeneous volume solution of AgNo3.Optimization of PH was used to adjustment (pH = 9.5). About 5 ml of solution C was added into the vortex of the solution A and after 5 mint solution B was added into the vortex of the solution reaction. The color of solution changed from pale yellow to brown. Then, heating was removed and stirring was continued at 15 minutes. When the solution cools to the room temperature, it is filtered through a 0.8µm a micro filter paper. The made solution was preserved in the refrigerator at 4 °C and measured using ultraviolet -visible at Shimadzu UV/vis 1800 spectrophotometer), Transmission electron microscope, XRD and Atomic force microscope spectrum.

Antibacterial activity of AgNPs against *Salmonella* spp.: The anti-bacterial efficacy of the biosynthesized AgNPs was estimated by disk diffusion method (Lipa and Jarosz 1991). The purer culture of the *Salmonella* spp. was separately sub cultured in Luria bertani broth (LB) at 37$^{\backslash O}$C for 18 hr. About 20 milliliter of medium of the Muller Hinton agar has been poured in each petri dish and each *Salmonella* isolate has been swabbed in a uniform way in dishes by using a sterile swab. The 6 mm disk were prepared from what man No.3, sterilized and soaked with a silver nanoparticle solution for 1 hour, then put for drying. These disks were separately, put on all bacteria inoculated dishes by using a sterile loop **(**Badawy et al 2010). The bactericidal efficacy has been assumed by a clear zone of inhibition around the disks after dishes incubation 18-24 hr at 37^{O}C.

Synergism effect of AgNPs and antibiotic against *Salmonella* spp.: Synergism effect of AgNPs and antibiotics (Amoxicillin) against *S. typhi and S. typhimurium* were evaluated using disk diffusion method (Al-Taee 2018). The bacterial suspension was prepared and compared with the standard McFarland No. 0.5. Five ml from *Luria bertani* broth inoculated with a loop full of *Salmonella spp.* from overnight culture grown on MacConkey and XLD agar, was incubated for 24hr at 37°C. The 20 ml of medium of Mueller Hinton Agar was poured in each Petri dish and each strain has been swabbed in a uniform way in dishes by using a sterile swab. Amoxicillin disk soaked with the AgNPs solution for 1hr, then placed for drying. Disks placed onto each bacterium inoculated agar plate by using sterile plastic forceps. The bactericidal efficacy was assesses by a clear zone of inhibition around the disks after dishes incubation overnight at 37°C (Fayaz et al 2010).

Statistical analysis: All data were analyzed by test of Chi-square (Cross tabulation) or Mann-Whitney.

RESULTS AND DISCUSSION

Isolation and Identification of *S. typhimurium* and *S. typhi*

A total of 239 clinical specimens were taken from mid-stream urine, blood, and stool, which included sixty-three(63) patients, (66.3%) were positive for *S. typhimurium* and thirty-two (32) patients (33.7%) were positive for *S. typhi* (Table 1). The isolates obtained from XLD and MacConkey agar were identified by VITEK® 2 system. *Salmonella isolates* appeared on MacConkey agar as small, pale and lactose non fermenter colonies while appeared on XLD pink color colonies with black center on XLD agar.

Synthesis of AgNPs: *K. pneumoniae* biomass (solution C) was added to the solution A after heating at 70 °C at 45 mint with stirring and then added glucose 10mM. The color of reaction mixture changed from pale-yellow to brown color. Such color change indicates AgNO3 reduction to AgNPs (Fig. 1). The results exhibited that the formation of AgNPs by extracellular enzymes and biomolecules secreted from *K. pneumoniae* as reduction agent and glucose as reducing agent of the silver nitrate solution and appearance of brown color. These results are in agreement with study of Sabaa et al 2019).

Optimization parameters of synthesis of silver nanoparticles conditions: In the present study, silver ions reduction was occurred and examined at temperature of 70-80^{O}C considered the optimum temperature and synthesis

silver nanoparticles .This agreement with previous studies which showed a characteristics band for silver nanoparticles with mono dispersity which meant he maximum peak intensity was detected at increase in reaction temperature (Ibrahim 2015). The results are in agreement with Zamin (2015). An alkaline pH favored the synthesis of AgNPs. The color of the solution was noticed at varying pH values. As the nanoparticles aren't developed in extremely acidic media there was no reaction at a pH value ranged 6-7, but mono dispersive AgNPs were obtained at pH equal to 9. The results are in agreement with Faria et al (2016). The AgNP formation under optimal conditions as a function of time. After 45-50 min, the reduction was 100% completed. The reduction was quite rapid and was much faster than 40- 50^{0}C (Vivekanandhan et al 2014). The results are agreement with (Yin et al 2014). The Glucose solution (10mM concentration) was employed as a reducing agent of $AgNO_3$ because encapsulating effect and trapping effect the silver nanoparticles in side in glucose (Elkhenshen et al 2012) and may be glucose concentration exposed more aldehyde group which interacted electrostatically with silver and resulted in high rate of reduction of AgNO3 to Ag ions using glucose (Zamin 2015, Peng et al 2016).

Table 1. Isolation and identification of *S. typhi* and *S. typhimurium*

Samples	Total *Salmonella* isolates (%)	Total *S. typhi* (%)	Total *S. typhimurium* (%)
Stool	71	8	63
Blood	23	23	0
Urine	1	1	0
	95 (100)	32 (33.7)	63 (66.3)

Total samples: 239(100)% , Total samples of positive culture of *Salmonella* isolates= 95(39.7)%
Negative culture showed higher percentage rather than positive culture of *S. typhi* and *S. typhimurium*. These results are in agreement with Seraj (2018)

Fig. 1. Color change of AgNo3 (pale-yellow to brown color) denoting the synthesis of AgNPs

Characterization of Synthesis Sialver Nanoparticles

Ultra violet (UV) –Visible spectroscopy analysis: UV-visible spectrum indicated the absorption peak at wave length 420 nm (Fig. 2), which shows the absorption peak of silver. The result of current study is in agreement with Ekbal et al (2017).

Transmission electron microscope analysis : Analysis of TEM (Transmission Electron Microscope) defined the shape and size of the bio-synthesized AgNPs using *K. pneumoniae* and distribution of AgNPs. The present study revealed that this AgNPs is mono-dispersed and size range was between 17-100 nm (Fig. 3). These results are in agreement with Nanda and Saravanan (2009).

Analysis of atomic force microscopy (AFM): AFM was achieved for colloidal solution of the AgNPs to observe the AgNPs, size and size distribution. The present study revealed that the maximum particle size ranges between

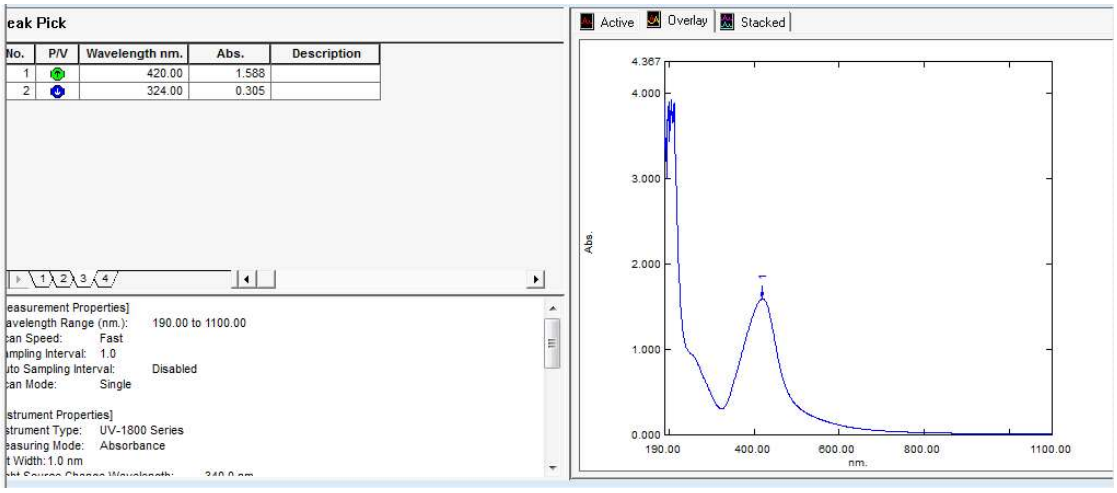

Fig. 2. Absorbance peak for nanoparticles at 420 nm

22–50 nm (Fig. 4). These results are in agreed with Sabaa et al (2019).

XRD analysis: XRD spectrum of synthesis AgNPs exhibited one high peak at 2θ(38.5^0) which corresponds to direction (111) plane of standard data of XRD of nanosilver crystals (Fig. 5). Silver nanoparticle size ranged between 12-44 nm. The results are in agreement with Maria et al (2018).

Antimicrobial activity of AgNo3 against *S. typhimurium* and *S. typhi*: AgNPs and antibiotics were tested for antibacterial activity against pathogenic *S. typhimurium* and *S. typhi* at concentrations of 1,2, 3, 4,5 and 10 mM of AgNPs which were loaded on the sterile disks. AgNPs concentrations were observed slightly of zone inhibition (4, 3.2.1 mM) (Table 2).

The inhibition zones was 28 and 21 at 10 and 5mM concentration of AgNPs for *S. typhi* .The inhibition zones of *S.typhimurium* ranged from 24.6 mm, at 10mM concentration of AgNPS and 19.4 mm at 5mM concentration of AgNPs. These concentrations of AgNPs solution were compared with AgNo3 solution. In the latter case no zone inhibition was observed. The AgNPs in present study showed excellent antibacterial activity against all tested bacterial strains at 5, 10 mM concentration compared to silver nitrate solution. Silver nanoparticles have antibacterial activities more than silver nitrate. These results are in agreement with Nanda and Saravanan (2009) and Ekbal et al (2017).

The cellular proteins of bacteria become inactive after treatment with silver nanoparticles. Silver nanoparticles had inactivated their enzymes after penetration into the bacteria, producing hydrogen peroxide and causing bacterial cell death. Heavy metals are toxic and associate with proteins, thereby binding protein molecules as a result cellular metabolism is disrupted causing microorganism death (Kim et al 2007). The high affinity of silver to sulfur and phosphorus,was the main element of the antimicrobial, effect due to the abundance of sulfur-containing proteins on the bacterial cell membrane. The silver nanoparticles can react with amino acids containing sulfur within or outside the cell membrane, which affects the viability of bacterial cells. Similarly nanoparticles may interact with phosphorus molecules in DNA, resulting in DNA replication, inactivation, or may react with proteins containing sulfur, leading to enzyme function inhibition (Ekbal et al 2017). The sensitivity of AgNps activity was almost of gram negative and gram

Table 2. Impact of AgNPs on growth of *S. typhi and S. typhimurium* growth (Mean ± SD)

AgNPs conc.	Inhibition zone of *S. typhi*	Inhibition zone of *S. typhimrium*
10Mm (470 µg ml^{-1})	28 ±0.63	24.6 ± 0.85
5mM (235 µg ml^{-1})	21 ± 0.91	19.4 ± 0.87
4mM (188 µg ml^{-1})	11.2 ±0.13	13.2 ± 0.74
3mM (141 µg ml^{-1})	9.0 ±0.49	11.5 ±0.43
2mM (94 µg ml^{-1})	8.6 ±0.36	10.2 ±0.51
1mM (47 µg ml^{-1})	5.8 ±0.61	7.1 ±0.14

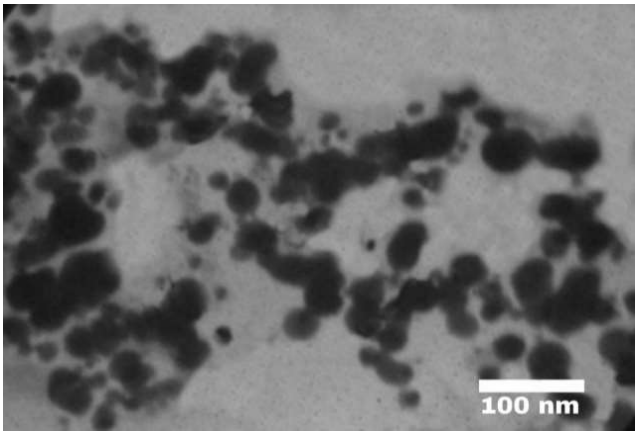

Fig. 3. Transmission electron micrograph of silver nanoparticles (size range between 17-100 nm)

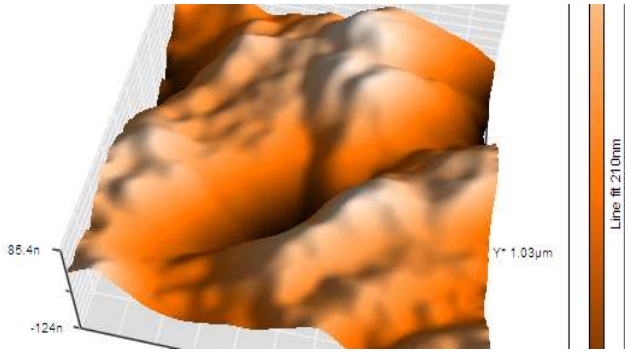

Fig. 4. AFM image of silver nanoparticle having a size of 22nm

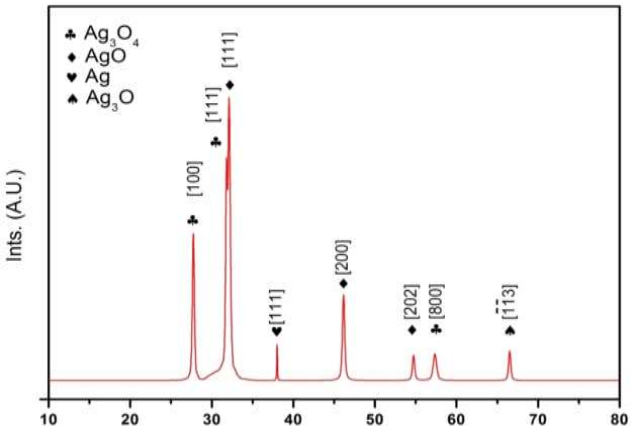

Fig. 5. XRD spectrum of synthesis silver nanoparticles

Positive bacteria (Nanda and Saravanan 2009).

Mechanism of bactericidal action against *S. typhi*: The morphological changes after silver ion treatment characterized by a cytoplasm, membrane detachment from cell walls and the presence of silver nanoparticles on the side of the *Salmonella* cell in the celli wall that possibly created pores and caused cell death (Figs 6, 7)**.** The result is in agreement with Feng et al (2000).

The presence of silver ions and sulfur in the thick electron granules found in the bacterial cells after the treatment of silver nanoparticles indicates an association with nucleic acids that is likely to hinder the replication of DNA (Feng et al 2000). Ag- nanoparticles exposure to *S. typhi, S. typhmurium* had altered membrane function and heat shock protein expression (Nelson et al 2010, Shekhar et al 2014). The TEM micrograph showed that AgNPs were present on the cell membrane and appeared to be bound to the lipopolysaccharide layer in the gram negative cell wall. AgNPs were found within the bacterial cell and this internalization of AgNPs was observed (Eby et al 2009). The AgNP – bacterial can be described through three mechanisms. The first, method during their interactions could be the electrostatic attraction between, negatively charged AgNPs and positively charged residues, on the bacterial surface of the integral membrane proteins (Eby et al 2009). The second is by altering the bacterial cell's osmoregulation which can induce extrusion. The third method, AgNPs appear to penetrate through, bacterial membranes via the, creation of pits / holes and disruption of the bacterial cell wall, and can promote their internalization inside the cell block cellular respiration (Morones et al 2005). These mechanisms contribute towards quick antibacterial effect (Shekhar et al 2014).

Synergism activity of Silver nanoparticles combination with Amoxicillin against *S. typhi and S. typhimurium*: Synergism activity was tested with disc diffusion system to evaluate the region inhibition of AgNPs (18 mm) and Amoxicillin (11 mm) (Fig. 8A, 8B). The silver nanoparticles combination with Amoxicillin revealed that the efficiency to 33 mm diameter inhibition zone against *S typhi* (Figure 8C).The zone inhibition of *S. typhimrium* of AgNPs was 20 mm and Amoxicillin was 14mm .The zone inhibition of silver nanoparticles combination with Amoxicillin was 25mm

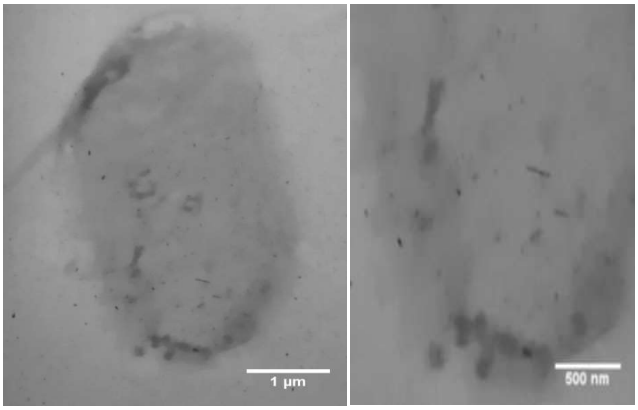

Fig. 6. *Salmonella typhi* treated with AgNPs

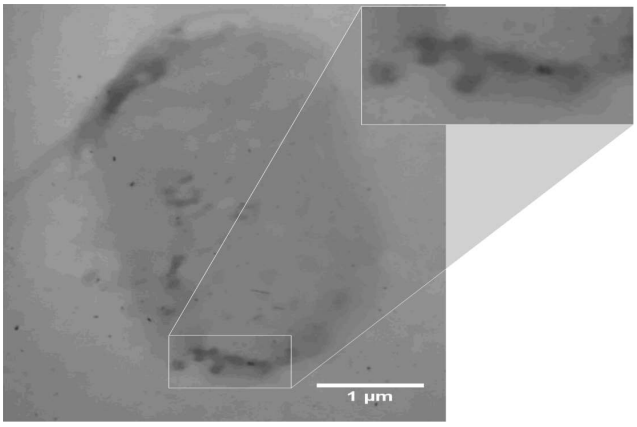

Fig. 7. AgNPs penetrated cell wall of *Salmonella typhi*

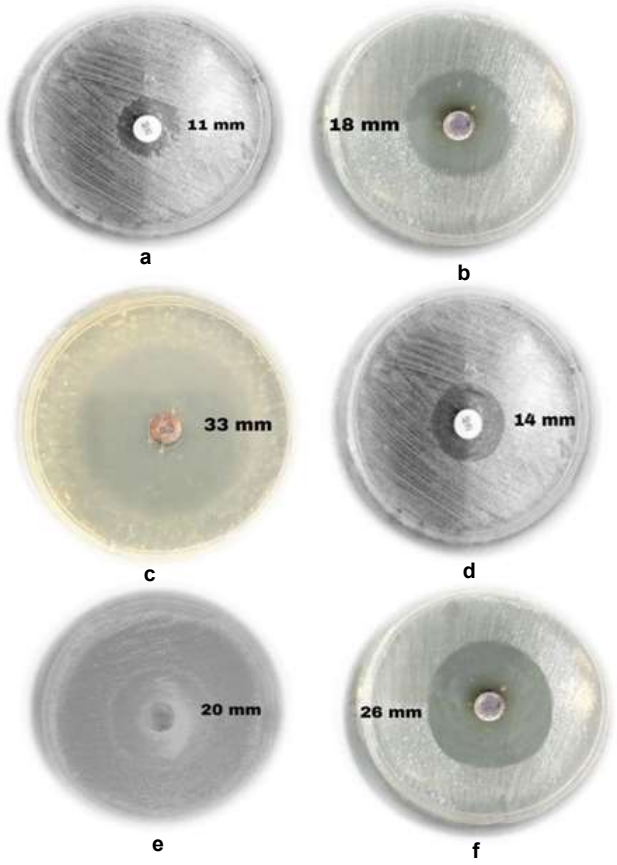

Fig. 8. A: *S.typhi* treated with Amoxicillin, B: AgNPs, C: combination of Amoxicillin and AgNPS, D: *S. typhimurium* treated with Amoxicillin, E: AgNPs F: combination between Amoxicillin and AgNPs

(Figures 13D,13E,13F). These results are in agreement with Fayaz et al (2010) and Marcelo et al (2018).

The combinations of silver nanoparticles and Amoxicillin actively inhibit biofilm formation to, varying degrees, but with nano Ags usually displayed greater inhibitory activity. The effective, ant biofilm effect can be achieved at lower, antibiotic concentrations. These results are agreement with Marcelo et al (2018). Silver nanoparticles and amoxicillin show different action mechanisms. The binding reaction between amoxicillin and silver nanoparticles possibly triggered the synergism, as amoxicillin molecules exhibit groups such as hydroxyl and amino groups that can react easily with silver nanoparticles. Silver nanoparticles will serve as a carrier of antibiotics (Nelson et al 2010). In the presence of, silver nanoparticles, the antibacterial activities of amoxicillin were increased against gram negative and gram positive bacteria (Shahverdi et al2007) .

CONCLUSION

The synthesis of AgNPs with some modification using *K. pneumonae* and glucose as reducing agent is good method to produce AgNps and was highly effective against *S. typhi* and *S. typhimurium* isolates. The present study showed the efficient and low-cost biological way to produce metallic nanoparticles and provided helpful insight to develop novel anti-microbial agents with the synergistic increase efficacy toward of the anti-microbial mechanism resistant toward the pathogenic microbes**.** The bio-synthesized Ag nanoparticles have potent anti-microbial efficacy to multi-drugs resistant bacteria.

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