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ANTIBACTERIAL ACTIVITY OF SILVER NANOPARTICLES AGAINST MULTIDRUG RESISTANT (MDR) ENTERIC HUMAN PATHOGEN

KEYWORDS

Antibacterial Activity, Combinational Effect, Silver Nanoparticles, Multidrug Resistant (MDR), E. coli, Klebsiella, Salmonella, Shigella.

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ABSTRACT Silver Nanoparticles (AgNPs) have been used as an antibacterial agent due to its unique physical, chemical and biological properties. The present study was aimed to investigate antibacterial activity of silver nanoparticles in combination with conventional antibiotics against MDR enteric human pathogens viz. *E. coli*, *Klebsiella*, *Salmonella* and *Shigella* species respectively. The results indicate high prevalence of *E.coli* species. 10 (55%) in all the clinical and environmental samples tested followed by *Klebsiella* species 4 (22%), *Salmonella* species 2 (11%), and *Shigella* species 2 (11%) respectively. In Antibiotic Sensitivity/Resistant pattern it was observed that out off eighteen enteric bacterial isolates, isolates E7, K3 and Sa1 showed resistant against all the antibiotics tested, with MAR index equal to 1 which indicates the presence of multiple drug resistance (MDR) in them. MIC values of AgNPs against MDR isolate E7and K3 was establish to be 80µg/ml whereas, for isolate Sa1 the MIC value was 70µg/ml. The synergistic effect of antibiotics in conjugation with biologically synthesized AgNPs induce the susceptibility among the tested bacterial cultures; viz. *Salmonella* followed by *Klebsiella* and *E.coli.* The susceptibility was induced in bacterial cultures to near about 20 to 50 percent analyzed antibiotics.

Introduction

"Nano" is a Greek word synonymous to dwarf meaning extremely small. Nanoparticles are simply defined as particles in the 10^{-9} nm range (Aitken et al., 2004). Globally due to outbreaks of infectious diseases caused by different pathogenic bacteria and the development of antibiotic resistance, the pharmaceutical companies and the researchers are now searching for new antibacterial agents. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their unique chemical and physical properties (Morones et al., 2005; Kim et al., 2007).

Silver nanoparticles (AgNPs) have emerged as an arch product from the field of nanotechnology. The current study throws light on applications of silver nanoparticles which is being exploited in medicine for antibacterial therapy.

Antibiotic resistance profiles lead to fear about the emergence and reemergence of multidrug-resistant (MDR) pathogens. This is largely the result of over-use and misuse of antibiotics in hospitals and the community generally. The Indian scene is particularly grim due to various factors. Generally, there is little control on the use of antibiotics. Community awareness of the issues involved in antibiotic therapy is poor and this is compounded by over-the-counter availability. We focused on antibiotic-resistant enteric bacteria because these represent the most immediate urgent global concern (WHO report 2013 and WHO report 2014) and diarrheal diseases are among the most common causes of morbidity and mortality in low income nations, disproportionately affecting children under the age of five (Okeke et al., 2007 and APUA final report 2011).

The use of Silver-Nanoparticles to potentiate antibiotic activity against Multidrug resistant enteric human pathogens has received minor attention and with few published citations. Hence, in the proposed studies, Antibacterial activity of biologically synthesized silver nanoparticles against Multidrug resistant enteric human pathogen has investigated.

Material and Method

Isolation and Identification of Enteric Human Pathogens

Total fifteen samples @ 5 each, viz. urine, stool and sewage samples respectively were obtained from Government Civil Hospital, Washim (MH) in sterile containers (Span diagnostics). For the isolation of frequently reported enteric human pathogens viz. *E.coli, Klebsiella, Salmonella* and *Shigella* species correspondingly, 0.5ml of enriched broth of each sample was spread separately on plate's of Eosin methylene blue (EMB), MacConkey agar and Hektoen Enteric (HE) Agar, Salmonella Shigella agar and Bismuth Sulfite agar (Forbes et al., (2007)) and further incubated for 24 hours at 37°C temperature past incubation, the plates were screen for the development of typical colonies.

The isolates were examined for, cultural characteristics, microscopic and biochemical characteristics, enzyme assay and identification was done as per Bergey's Manual of Determinative Bacteriology ninth edition. The identified isolates were further used for the purpose of antimicrobial sensitivity/ resistance pattern.

Determination of Antibiotic Sensitivity / Resistance Pattern of isolated human enteric pathogens.

The isolates were subjected to antibiotic susceptibility test as per the protocols suggested by Bauer and Kirby (1966). The bacterial lawn was prepared on Muller-Hinton agar plates by swabbing overnight broth culture. Standard antibiotic discs viz. Ampicillin (10 µg), Amoxicillin-clavulanate(20 µg), Ceftazidime(30 µg), Ceftriaxone(30 µg), Imipenem(10 µg), Amikacin(30 µg), Gentamycin(30 µg), Co-Trimoxazole(30µg), Azithromycin(15 µg), Nitrofurantoin (300 µg), Chloramphenicol (30 µg), Ciprofloxacin (5 µg), Ofloxacin (5 µg), Tetracyclin(30 µg) obtained from Himedia laboratories, Mumbai were used in the study. The disc were aseptically impregnated on the surface of the agar plate and left undisturbed for an hour. The plates were then incubated at 37°C for 24 - 48 hours. Post incubation, the zone of inhibition was measured using Hi Antibiotic zone scale (Hi-media) and recorded in mm diameter. The assays were implemented in triplicate and express in terms of Mean values. The readings obtained were then compared with "Disc diffusion supplemental table" given by CLSI (2013), and the S/R blueprint of the isolates was determined. MAR (Multiple Antibiotic resistance) index was also calculated adopting standard formula suggested by Sarter et al., (2007) and Wei et al., (2010). The isolate showing MAR index equal to 1 was selected for further analysis.

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$$\text{MAR Index} = \frac{\text{Number of antibiotics to which isolates showed resistance}}{\text{Total number of antibiotics tested.}} \quad \text{.....(1)}$$

Combined Efficacy of antibiotics and Silver Nanoparticles against test pathogens

Combined Efficacy of antibiotics and AgNPs against selected MDR isolates was carried out using disk-diffusion method suggested by Ali et al., (2013), with slight modifications. The concentration of biologically synthesized AgNPs was considered as MIC value and the concentrations of antibiotics were in use as per CLSI guide lines. The inoculums of each test pathogen viz., E7, K3 and Sa1 was prepared by inoculating the test pathogen in nutrient broth and incubated at 37°C for 3 to 4 hours. After incubation, turbidity of the inoculum was match with 0.5% McFarland standard which represents 10^6 CFU/ml culture and further used for antibacterial susceptibility test (CLSI 2009).

Determination of MIC of biologically synthesized Silver Nanoparticles:

The Antibacterial activity of biologically synthesized AgNPs against selected MDR bacteria viz. E7, K3 and Sa1 was done using the disk-diffusion method (Bauer and Kirby 1966). Muller–Hinton agar plates were inoculated separately with 0.5 ml of inoculum of the test bacterial culture viz., E7, K3 and Sa1, using sterile swabs. Plates were allowed to set and used for antibacterial studies. Standard stock solution of different concentration ranging from 100µg/ml-10µg/ml of silver nanoparticles was prepared using autoclaved deionized water. The control was maintained without AgNPs. The suspensions were sonicated for 20 minutes to avoid deposition of AgNPs. Sterilized blank discs (Hi Media Lab.) were further dip in different stock solutions and air dried, this disc were placed aseptically in prepared MH agar plates using sterile forceps and incubated at 37^0C for 16 to 18 hours. After incubation the zone of inhibition was observed and MIC of AgNPs was recorded and used in synergistic study. The assays were implemented in triplicate and express in terms of Mean values.

Combined Antibacterial activity of an Antibiotics with Silver Nanoparticles :

For determining synergistic effects, each standard antibiotic disc was impregnated with minimum inhibitory concentration of Silver nanoparticles against selected MDR bacteria viz., E7, K3 and Sa1. The disc were aseptically impregnated on the surface of the agar plate and left undisturbed for an hour. All the plates' were then incubated at 37°C for 24 hrs. After incubation, the zone of inhibition were measured around the respective antibiotic disc and recorded in mm diameter. The assays were implemented in triplicate and express in terms of Mean values. The readings obtained were then compared and expressed in terms of fold area increase in antibacterial activity, by using the formula. (Birla et al., 2009), Where a and b are the zone of inhibition (mm) obtained for antibiotic alone and antibiotic in combination with AgNPs, respectively.

$$\text{Increase in fold area} = \frac{(b^2-a^2)}{a^2} \quad \text{... (2)}$$

Result and discussion

Isolation and Identification of Enteric human pathogens

The isolation of enteric human pathogens viz. *E.coli, Klebsiella, Salmonella* and *Shigella* species respectively, was done using differential and selective enteric media. From all the 15 samples tested total (18) isolates were obtained. From the results of conventional identification, it was observed that all the isolates were Gram negative bacilli. The results on the morphological and biochemical properties indicated that the isolated pathogens belong to *E.coli, Klebsiella, and Salmonella* and *Shigella* species respectively. The identified pure cultures of enteric human pathogens were further labeled as E1 to E10 for *E.coli,* K1 to K4 for *Klebsiella*, Sa1 and Sa2 for *Salmonella* and Sh1, Sh2 for *Shigella* species respectively**.** It was observed that there was high prevalence of *E.coli* species 10 (55%) in all the samples tested followed by *Klebsiella* 4 (22%), *Salmonella* 2 (11%), and *Shigella* 2 (11%) species respectively. Similar findings were also reported by the other researchers. Mamuye et al., (2015), Ballal et al., (2014), Manikandan and Amsath (2013). They had also reported the presence of the above cited pathogens in different samples viz. urine, feces and sewage with predominance of *E.coli.* All these isolates were further processed for determination of Antibiotic S, R, I rating respectively.

Determination of Antibiotic Sensitivity/Resistant pattern of Isolated Human enteric pathogens

The findings on antimicrobial susceptibility testing are presented in Table (1) and Photoplate (1). From the table, it was observed that Maximum resistance in isolates (88%) among tested pathogens was found to Gentamycin, Co-Trimoxazole and Tetracyclin followed by Nitrofurantoin and Ceftriaxone which showed 83% resistance in isolates. Whereas, in case of Azithromycin and Chloramphenicol the (72%) isolates among the test pathogens showed at par resistance against both the antibiotics. The resistance was exhibited by only 22-50% of isolates under study against Ampicillin, Amoxicillin-clavulanate, Ceftazidime, Imipenem, Amikacin, Ciprofloxacin, Ofloxacin. The research findings on Antibiotic susceptibility testing of enteric human pathogens were in concordance with the findings of Ballal et al., (2014), Ikpeme Emmanuel et al., (2011) and Sumathi et al., (2009). They had also reported the existence of antibiotic resistance in enteric human pathogens with same antibiotics which may be due to the consistence and overuse of antibiotics as well as it may be due to resistant gene transfer from animals to man via Food chain.

The results on MAR index of test isolates are presented in Table (1) .From the table it was found that out of eighteen isolated tested, isolates E7, K3 and Sa1 showed the MAR index equal to 1 which indicates the presence of multiple drug resistance (MDR) in these isolates and their origin from a high risk source of contamination where antibiotics are often used (Wei and Wee 2011).Hence, only E7, K3, and Sa1 isolates were used for further investigation

Combined Efficacy of antibiotics and AgNPs against test pathogens Determination of MIC of biologically synthesized AgNPs

The MIC values of AgNPs against test pathogens viz. E7, K3 and Sa1are depicted in Table (2), Photoplate (2). MIC values for isolate E7and K3 was recorded to be 80µg/ml whereas, for isolate Sa1 MIC value was 70µg/ml. Our findings are similar with the results given by Humberto et al., (2010) and Kim et al., (2007).They had reported the lethal effect of silver nanoparticles against different pathogens with MIC values in the range of 50 to 75 µg/ml.

Combined antibacterial activity of antibiotics with AgNPs against test pathogens.

The Combined antibacterial activity of antibiotics with AgNPs against three test pathogens viz., E7, K3, Sa1was determined and compared with the individual antibacterial activity of antibiotics and AgNPs respectively. In combined antibacterial activity increase in zone of inhibition was expressed in terms of average fold-area inhibition in antibacterial activity. The results obtained are depicted in Table (3), photoplate (3). From the table it was observed that, in case of antibacterial activity of antibiotics alone all the selected MDR isolates exhibited resistance(R) against conventional antibiotics. In case of antibacterial activity of AgNPs alone, mild bactericidal activities were observed in terms of zone of inhibition ranging from 10-11 mm.

In case of combined activity of Antibiotics with AgNPs on test pathogens viz.E7, K3, Sa1. In case of isolate E7, maximum increase in

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fold area inhibition (2.3) was recorded against Cotrimoxazole - AgNPs combination followed by Tetracyclin - AgNPs combination (1.7). The remaining combinations showed increase in fold area inhibition in the range of (0.1) to (0.9). However, in case of Amikacin - AgNPs and Azithromycin - AgNPs conjugates, no increase in fold area inhibition was observed. Similarly, in case of Isolate K3 maximum increase in fold area inhibition (3) was observed in Tetracyclin - AgNPs combination followed by Cotrimoxazole - AgNPs (1.8), Amoxicillin-clavulanate - AgNPs (1.13) and Nitrofurantoin - AgNPs (1.08) combinations respectively. The remaining combinations showed increase in fold area inhibition in the range of (0.5) to (0.1) and Azithromycin - AgNPs showed no change in increase fold area inhibition.

Isolate Sa1 showed maximum increase in fold area inhibition (9.03) with Azithromycin - AgNPs combination followed by Gentamycin-AgNPs (9.02). Chloramphenicol - AgNPs and Ofloxacin - AgNPs combination showed at par increase in fold area inhibition of (8). Cotrimoxazole - AgNPs and Tetracyclin - AgNPs combination showed at par results (7.03), all of the remaining combinations showed increase in inhibition fold area inhibition greater than (1) except Nitrofurantoin - AgNPs and Ciprofloxacin - AgNPs combination which showed increase in fold area inhibition of (0.21). Hence, Maximum synergistic antibacterial activity of Cotrimoxazole - AgNPs combination was observed against isolate E7, Tetracyclin - AgNPs combination against K3 and Azithromycin - AgNPs combination against Sa1.

In combinational treatment, it was also observed that the synergistic action of antibiotics with AgNPs had altered the resistance pattern of the test pathogens. In case of isolate Sa1, it was observed that the pathogen was initially found to be resistant to all the antibiotics tested. However, it in synergistic activity of AgNPs with antibiotics, it become susceptible to seven antibiotics viz. Imipenem, Gentamycin, Co-Trimoxazole, Azithromycin, Chloramphenicol, Ofloxacin, Tetracyclin. Similarly while testing isolate K3, it showed susceptibility against three antibiotics viz. Amoxicillin-clavulanate, Ceftazidime and Imipenem. Isolate E7 exhibited intermediate status against three antibiotics viz. Ampicillin, Imipenem and Ofloxacin though; it showed resistant when tested against antibiotics alone.

Consequently, the results enlightened that the synergistic effect of antibiotics in conjugation with biologically synthesized AgNPs increased the susceptibility among the tested bacteria in following sequence; viz. *Salmonella* species followed by *Klebsiella* species and E.coli species respectively. The susceptibility was induced in bacterial cultures to near about 20 to 50 percent analyzed antibiotics. These results are in line with the findings of Birla et al., 2009 who mentioned increasing efficacies percentage of antibiotics like vancomycin, gentamycin, streptomycin, ampicillin, and kanamycin when used in combination with AgNPs against *P. aeruginosa*, *S. aureus*, and *E. coli*.

Conclusion

In conclusion, the present work demonstrates the potential of silver nanoparticles which on combination with Antibiotics has a synergistic antibacterial efficiency on isolates of MDR Enteric Human Pathogens viz. *E.coli, Salmonella* and *Klebsiella* species respectively. This research, though very preface, provides helpful insights to the development of novel antimicrobial agents. To elucidate the mechanism of this synergistic antibacterial effect, more elaborate experimental evidences will be needed. Focus may also be given towards the Toxicity studies of Silver nanoparticles on human pathogenic in relation to human physiology which may open a door for new range of antibacterial agents.**References**

Table 1 :- Antimicrobial Susceptibility Pattern of the isolated human enteric pathogens

Antibiotics	No. of isolate and %Sensitive	No. of isolate and %Intermediate	No. of isolate and %Resistant	Isolated Enteric Bacterial Species	MAR Index
AMP(10µg)	12(66)	2(11)	4(22)	E1	0.5
AMC(20µg)	2(11)	10(55)	6(33)	E2	0.5
CAZ(30µg)	3(16)	9(50)	6(33)	E3	0.5
CRO(30µg)	3(16)	0(0)	15(83)	E4	0.714
IPM(10µg)	12(66)	0(0)	6(33)	E5	0.5
AK (30µg)	14(77)	0(0)	4(22)	E6	0.5
CN (30µg)	2(11)	0(0)	16(88)	E7	1
COT(25µg)	0(0)	2(11)	16(88)	E8	0.5
AZM(15µg)	0(0)	5(27)	13(72)	E9	0.571
F (300µg)	1(5.5)	2(11)	15(83)	E10	0.5
C (30µg)	5(27)	0(0)	13(72)	K1	0.428
CIP (5µg)	1(5.5)	8(44)	9(50)	K2	0.714
OFX (5µg)	11(61)	0(0)	7(38)	K3	1
TE(30µg)	2(11)	0(0)	16(88)	K4	0.5
AMP-Ampicillin , AMC- Amoxicillin-clavulanate, CAZ- Ceftazidime, CRO-Ceftriaxone, IPM- Imipenem, AK- Amikacin, CN- Gentamycin, COT- Co-Trimoxazole, AZM- Azithromycin, F- Nitrofurantoin, C-Chloramphenicol, CIP- Ciprofloxacin, OFX-Ofloxacin, TE- Tetracyclin. NI- No Inhibition. Values in parenthesis is % sensitivity, %Intermediate and %Resistant.				Sa1	1
				Sa2	0.714
				Sh1	0.142
				Sh2	0.142

Photoplate (1) Antibiotics Sensitivity/ Resistance against clinical enteric isolates

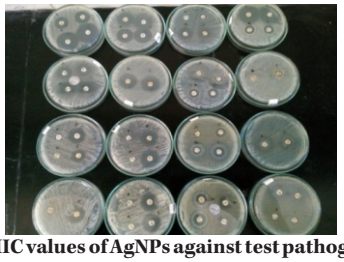

Table 2: The MIC values of AgNPs against test pathogens

NI- No Inhibition

Sr no.	Concentration of AgNPs	Mean Zone diameter in mm		
		E7	K3	Sa1
	Control	NI	NI	NI
1.	100µg/ml	13	13.5	15
2.	90µg/ml	11	12	13.5
3.	80µg/ml	10	10	13
4.	70µg/ml	NI	NI	11
5.	60µg/ml	NI	NI	NI
6.	50µg/ml	NI	NI	NI
7.	40µg/ml	NI	NI	NI
8.	30µg/ml	NI	NI	NI
9.	20µg/ml	NI	NI	NI
10.	10µg/ml	NI	NI	NI

Photoplate (2) Minimum Inhibitory Concentration of AgNPs; (a)AgNPs against *E coli,* (b)AgNPs against *Salmonella* species and (c)AgNPs against *Klebsiella* species

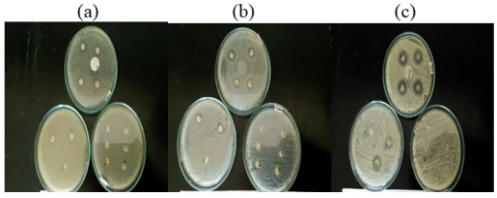

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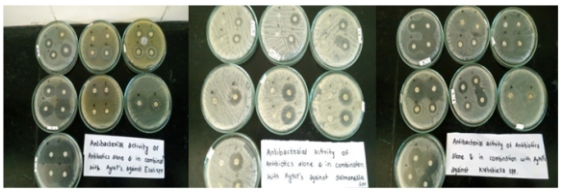
Table 3: Combine and Individual efficacy of antibiotics and AgNPs against selected test pathogens .

Sr No.	Antibacterial agents used	Bacterial Isolates		
		Mean Zone of Inhibition (mm)		
		E7	K3	Sa1
1. A	Ampicillin(a)(APM10ug)	13(R)	12(R)	NI(R)
B	AgNPs	10	10	11
C	Ampicillin+AgNPs(b)	14(I)	15(I)	13(R)
D	**Increase in fold area**	**0.16**	**0.56**	**3.7**
2. A	Amoxicillin-clavulanate(a)(AMC20ug)	10(R)	13(R)	NI(R)
B	AgNPs	10	10	11
C	Amoxicillin-clavulanate +AgNPs (b)	13(R)	19(S)	12(R)
D	**Increase in fold area**	**0.7**	**1.13**	**3**
3. A	Ceftazidime(a(CAZ30ug))	11(R)	17(R)	NI(R)
B	AgNPs	10	10	11
C	Ceftazidime +AgNPs(b)	12(R)	19(S)	14(R)
D	**Increase in fold area**	**0.2**	**0.22**	**4.4**
4. A	Ceftriaxone(a)(CRO30ug)	8(R)	18(R)	NI(R)
B	AgNPs	10	10	11
C	Ceftriaxone +AgNPs(b)	11(R)	20(I)	14(R)
D	**Increase in fold area**	**0.9**	**0.2**	**4.4**
5. A	Imipenem(a)(IPM10ug)	13(R)	13(R)	NI(R)
B	AgNPs	10	10	11
C	Imipenem +AgNPs(b)	15(I)	16(S)	16(S)
D	**Increase in fold area**	**0.33**	**0.5**	**6.1**
6. A	Amikacin (a)(AK30ug)	14(R)	14(R)	NI(R)
B	AgNPs	10	10	11
C	Amikacin+AgNPs(b)	14(R)	15(R)	11(R)
D	**Increase in fold area**	**0**	**0.1**	**2.3**
7. A	Gentamycin (a)(CN10ug)	11(R)	10(R)	NI(R)
B	AgNPs	10	10	11
C	Gentamycin +AgNPs(b)	12(R)	11(R)	19(S)
D	**Increase in fold area**	**0.2**	**0.21**	**9.02**
8. A	Cotrimoxazole(a)(COT30ug)	NI(R)	NI(R)	NI(R)
B	AgNPs	10	10	11
C	Cotrimoxazole +AgNPs(b)	11(R)	10(R)	17(S)
D	**Increase in fold area**	**2.3**	**1.8**	**7.03**
9. A	Azithromycin(a)(AZM15ug)	11(R)	11(R)	NI(R)
B	AgNPs	10	10	11
C	Azithromycin + AgNPs (b)	11(R)	11(R)	19(S)
D	**Increase in fold area**	**0**	**0**	**9.03**
10.A	Nitrofurantoin(a)(F300ug)	11(R)	9(R)	10(R)
B	AgNPs	10	10	11
C	Nitrofurantoin +AgNPs(b)	13(R)	13(R)	11(R)
D	**Increase in fold area**	**0.4**	**1.08**	**0.21**
11.A	Chloramphenicol(a)(C30ug)	10(R)	12(R)	NI(R)
B	AgNPs	10	10	11
C	Chloramphenicol+AgNPs(b)	12(R)	14(I)	18(S)
D	**Increase in fold area**	**0.4**	**0.36**	**8**
12.A	Ciprofloxacin(a)(CIP5ug)	13(R)	13(R)	10(R)
B	AgNPs	10	10	11
C	Ciprofloxacin +AgNPs(b)	15(R)	15(R)	11(R)
D	**Increase in fold area**	**0.33**	**0.33**	**0.21**
13.A	Ofloxacin(a)(OFX5ug)	12(R)	12(R)	NI(R)
B	AgNPs	10	10	11
C	Ofloxacin +AgNPs(b)	14(I)	13(I)	18(S)
D	**Increase in fold area**	**0.36**	**0.17**	**8**
14.A	Tetracyclin(a)(TE30ug)	NI(R)	NI(R)	NI(R)
B	AgNPs	10	10	11
C	Tetracyclin +AgNPs(b)	10(R)	12(R)	17(S)
D	**Increase in fold area**	**1.7**	**3**	**7.03**

Notes: In the absence of bacterial growth inhibition zones (NI), the disc's diameter (6mm)was used to calculate the fold increases (Birla *et al.*, 2009), Increase in fold area = $(b^2-a^2)/a^2$. (R)- Resistance, (S)- Sensitive, (I)- Intermediate

Photoplate (3) In vitro Combine and individual activity of antibiotics and AgNPs



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