Investigation of the effect of silver nanoparticles alone and their combination with clarithromycin on *H. pylori* isolates

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Abstract

Helicobacter pylori is a common bacterial pathogen responsible for gastrointestinal diseases worldwide. Clarithromycin has been considered the best tolerable and safe antibiotic in treating *H. pylori* infection, but increased levels of clarithromycin resistance have reduced the effectiveness of the recommended treatment regimens. So alternative treatment approaches such as nanotechnology have been considered recently. This study aimed to determine the effect of silver nanoparticles (Ag-NP) alone and their combination with clarithromycin on *H. pylori* isolates.

Gastric biopsy specimens were collected from 163 patients with different gastrointestinal signs referred to the endoscopy ward of Beheshti Hospital in Kashan, Iran. *H. Pylori* strains were isolated from 40 patients out of 163 (24.5%). Minimum inhibitory concentration (MIC) of clarithromycin on *H. pylori* isolates was determined by the Epsilometer test. The effect of the combination of Ag-NP with clarithromycin on the growth inhibition of clarithromycin-sensitive and resistant *H. pylori* isolates was determined by the checkerboard titration method.

The clarithromycin resistance rate to *H. pylori* was 42.5%. The MIC of Ag-NP in clarithromycin-sensitive was 31.25-125 µg/ml and resistant *H. pylori* isolates ranged from 62.5-250 µg/ml. Due to the combination of Ag-NP with clarithromycin, 70.58% of clarithromycin-resistant isolates and 78.26% of clarithromycin-sensitive isolates showed a synergistic effect.

A significant difference was observed in comparing the MIC of clarithromycin in combination with Ag-NP and clarithromycin or Ag-NP alone. The MIC clarithromycin was decreased in the presence of Ag-NP against clarithromycin sensitive, and resistant *H. Pylori* isolates.

Introduction

H. Pylori is one of the most common causes of gastrointestinal infection that affects humans worldwide (1–3). The prevalence of *H. Pylori* infection varies from 18.9–87.7% (4). The goal of eliminating *H. Pylori* is to treat and reduce the risk of stomach cancer (5). The standard eradication therapy plan in symptomatic patients includes PPI (proton pump inhibitors), amoxicillin, and clarithromycin (3). In Iran, the rate of clarithromycin resistance increased from 17–45%. Due to the presence of clarithromycin in many global guidelines, this antibiotic plays a vital role in the treatment of *H. Pylori*, and even bacterial resistance cannot remove it from the treatment lines (6). Recently, metal nanoparticles, known as antibacterial agents, have been used against isolates resistant to antibacterial drugs (7). The significant benefits of nanoparticles used as drug carriers are high stability, high carrying capacity, the possibility of combining hydrophilic and hydrophobic materials, and the possibility of utilization in various routes, including food and inhalation. These properties of nanoparticles improve the biological availability of the drug, reduce the dose of the drug, and solve non-compliance with the prescribed treatment (8). Currently, silver nanoparticles have become more and more critical in their uses in several fields, including antimicrobial abilities. Studies show that silver nanoparticles have multidimensional effects such as

antibacterial and anti-biofilm activity against *H. Pylori* (9). This study aimed to determine the effect of silver nanoparticles alone and their combination with clarithromycin on *H. Pylori* isolates.

Material And Methods

Sample collection

One hundred sixty-three patients with signs of abdominal pain or burning, nausea, vomiting, frequent burping, bloating, and weight loss with an average age of 51.5 years (ranged from 20 to 83) had undergone endoscopic investigations at Beheshti Hospital in Kashan, Iran, from May 2019 to November 2020. Patients, who received antibiotic therapy three months before endoscopy, including PPI, non-steroidal anti-inflammatory drugs, and clarithromycin, were excluded from the study. Written informed consent was obtained from the patients. In sum, 163 patients, 119 (73%) cases presented with non-ulcer dyspepsia and 44 (27%) cases with peptic ulcer diseases (including four peptic ulcers, five duodenal ulcers, and thirty-five cases with both gastritis and peptic ulcers.

H. Pylori Culture

Gastric biopsy specimens are transferred to the microbiology laboratory in two pieces, one in the Stuart transport medium and the other in the rapid urea medium. The biopsy sample was cultured on Brucella agar enriched with 10% horse serum and 5mg/l trimethoprim, 10 mg/l vancomycin, 5 mg/l amphotericin B, 5 mg/ml in cefsulodin (*H. pylori* selective supplement SR147) (OXOID, USA). The cultured plates were incubated at 37˚C under microaerophilic conditions (5% O2, 10% CO2, and 85% N2) for 5 to 7 days to obtain a single colony. *H. Pylori* are detected using gram staining, urease test, catalase test, and oxidase test.

Silver Nanoparticle characterization

Silver Nanoparticle (Ag-NP) with an approximate size of 5 to 8 nm purchased in solution from Pishgaman Iranian Nanomaterials Company, Mashhad, Iran. True density was 10.9 g/cm^3. The purity of Ag-NP was 99.99%. Also, the color of Ag-np was black, and Morphology was spherical. Specific surface area (*SSA*) was ~25-42 m^2/g. Figure 1 shows the size distribution report by the intensity measured by Zetasizer Version 6.00 from Malvern Instruments Ltd. Figure 2 illustrated a micrograph of silver nanoparticles obtained by transmission electron microscopy. The XRD pattern of silver nanoparticles showed in Figure 3.

Antimicrobial susceptibility tests

E-test

The pattern of sensitivity and resistance to clarithromycin is determined by the E-test method on enriched Muller Hinton Agar medium with 10% horse serum. The H. pylori culture is prepared with turbidity equivalent to 3 McFarland standard, and after inoculation on the medium, the E-test strips (LIOFIL CHEM,

Italy) are placed on the surface of the cultured medium. The plates were incubated at a temperature of 37˚C for 72 hours under microaerophilic conditions. After the incubation period, MIC was determined by the oval aura formed around the strip. If the MIC was ≤1µg/ml, the isolate was sensitive to clarithromycin, and if ≥1µg/ml, the isolate was resistant to clarithromycin (10, 11).

Determination of MIC of silver nanoparticle (Ag-Np)

Determination of MIC was carried out in 96-well microtitre plates using a standard twofold broth microdilution method of the antibacterial agents in Mueller–Hinton broth following Clinical and Laboratory Standards Institute (CLSI) guidelines (11). Broth microdilution was performed in Mueller–Hinton broth supplemented with 5% horse serum. Twofold dilutions of silver nanoparticles ranging from 3.90 to 2000 µg/ml were used. The standardized inoculum was diluted to achieve a final inoculum concentration of approximately 5*105 CFU per well. Each test was performed in triplicate. The microtiter plates were incubated at 37°C under microaerophilic conditions. MICs were read after 72 h of incubation. The MIC was defined as the lowest concentration of silver nanoparticles inhibiting visible growth (11, 12).

Combination assay

Standard powder forms of Clarithromycin (C9742 Sigma-Aldrich Inc., Germany) were stored at 2 to 8°C until use. The stock solutions and serial twofold dilutions of each drug to at least double the MIC were prepared. The MICs of Clarithromycin and silver nanoparticles alone or in combination were determined by broth microdilution method in a 96-well plate by CLSI standards using MH broth supplemented with 5% horse serum. For the double treatment, a 2D checkerboard with twofold dilutions was used to test the different combinations. The checkerboard method was adjusted by twofold dilutions of Ag-Np and clarithromycin for combination treatment. Growth control wells containing the medium were included in each plate. Each test was performed in triplicate. The index of fractional inhibitory concentration (FICs) was calculated as follows: (13, 14)

FIC of Clarithromycin: MIC clarithromycin in combination/ MIC clarithromycin alone

FIC of Ag-Np: MIC Ag-Np in combination/ MIC Ag-Np alone

FICi is calculated as the sum of each FIC and is interpreted as follows:

FICi <0.5, synergy; 0.5 ≤ FICi<1, partial synergy; FICi=1, additive; 2≤FICi<4, indifferent; FICi>4, antagonism

Statistical analysis

The statistical analysis of data was conducted using SPSS software version 16 (SPSS, Inc.). Kolmogorov–Smirnov test was used for all analyzes. Tests such as Chi-square, T-Test, Fischer Exact Test, and Mann Whitney were used for comparison. The p-Values < 0.05 were considered statistically significant.

Results

Characteristics of patients

H. Pylori strain was isolated from 40 patients out of 163(24.5%). Patient's demographic and clinical characteristics are presented in Table 1.

Results of clarithromycin resistant

The clarithromycin resistant rate to *H. pylori* was 42.5% (MICs ≥1 µg/ml) (Figure 4). According to resistance to clarithromycin, there was no significant difference between age, sex, and type of disease (Table 2).

Results of MIC of silver nanoparticles

The frequency percent of MIC (µg/ml) of silver nanoparticles in clarithromycin-sensitive and resistant *H. Pylori* isolates illustrated in Figure 5. Nano-Ags showed antibacterial activity against both clarithromycin sensitive, and resistant *H. Pylori* isolates with MIC values of 31.25-250 µg/ml. The dispersion of the MIC of silver nanoparticles in clarithromycin sensitive isolates was 13.04% -65.21%, and in clarithromycin resistant isolates was 17.64% -52.94%. In general, the MIC of nanoparticles insensitive and resistant to clarithromycin isolates was in the range of 31.25-250 µg/ml. The MIC dispersion of silver nanoparticles in clarithromycin-sensitive isolates was in the range of 31.25-125 µg/ml compared to 62.5-250 µg/ml in clarithromycin-resistant isolates. The highest MIC frequency was 125 µg/ml, and this value was applied to both groups.

Results of combination assay

The synergistic effect of silver nanoparticles with clarithromycin in clarithromycin-sensitive and resistant *H. Pylori* isolates shown in Figure 6. This combination showed a 70.58% synergistic effect against clarithromycin resistant isolates compared to 78.26% against clarithromycin sensitive isolates. Comparison of the MIC of clarithromycin, silver nanoparticles, and the combination of both in clarithromycin sensitive and resistant *H. Pylori* isolates described in Table 3. There was a significant difference between the clarithromycin sensitive and resistant groups (p-value = 0.003). Also, there was a significant difference in comparing the MIC of silver nanoparticles between two clarithromycin sensitive and resistant groups concerning p-value = 0.039. A significant difference was observed in the comparison of the MIC of clarithromycin and clarithromycin in combination with silver nanoparticles and the comparison of the MIC of Nano-Ags and the combination of nanoparticles with clarithromycin according to p-value = 0.001. Comparison of the MIC of clarithromycin alone and the combination of clarithromycin with silver nanoparticles in *H. Pylori* isolates are shown in Table 4. Comparing the MIC of silver nanoparticles alone and the combination of clarithromycin with silver nanoparticles in all *H. Pylori* isolates are described in Table 5.

Discussion

During the last years, a rise in clarithromycin resistance rates has been seen worldwide, which affects the effectiveness of treatment (10, 15, 16). In this study, the resistance to clarithromycin in *H. Pylori* isolates was 42.5%. Increased clarithromycin resistance over several years in the area highlights the need for new treatments (10, 17). Today, due to the lack of treatment for the complete eradication of *H. Pylori* infection and increased antibiotic resistance, attention to alternative approaches, including nanotechnology, has increased (18). The mechanisms of action of silver nanoparticles are the binding of silver nanoparticles to the surface of cell walls and membranes, the penetration of silver nanoparticles into cells, and damage to intracellular structures and biomolecules, causing toxicity and cellular oxidative stress by producing oxygen free-radicals (19). In this study, the MIC range of silver nanoparticles was 31.25–250 µg/ml. Most of the MIC for clarithromycin-sensitive *H. Pylori* isolates at 125 µg/ml was 65.2% versus 53% for resistant ones. The effect of silver nanoparticles on gram-negative bacteria was stronger than gram-positive bacteria, and MIC was similar between antibiotic-resistant and sensitive bacteria, and the lethal effect was greater in antibiotic-sensitive isolates (20). Saravanakumar et al. found that nanoparticles at a concentration of 18.14 µg/ml inhibit *H. Pylori* (21). The study of Muhammad Amin et al. showed that the MIC of silver nanoparticles in clarithromycin-resistant *H. Pylori* isolates was 1–16 µg/ml and in clarithromycin-sensitive *H. Pylori* isolates was 4–16 µg/ml (22). Nazari et al. investigated the antibacterial effect of bismuth nanoparticles against various clinical isolates and the standard strain of H. Pylori (ATCC 26695). The MIC between clinical isolates varied between 60–100 µg /ml. Exposure of *H. Pylori* to an inhibitory concentration of bismuth nanoparticles (100 µg/ml) results in some metabolites release such as acetate, formic acid, glutamate, valine, glycine, and uracil from the bacteria into their supernatants. This result indicates that these nanoparticles interfere with the Krebs cycle, nucleotide, and amino acid metabolism (23). Gurunathan et al. showed that silver nanoparticles have multidimensional effects such as antibacterial and anti-biofilm activity against *H. Pylori* and *Helicobacter felis*, as well as cytotoxic effects against human cancer cells. This study found that silver nanoparticles reduce the formation of biofilms and increase the production of reactive oxygen species (ROS) and DNA fragmentation in *H. Pylori* and *Helicobacter felis* (9). Physicochemical properties of nanoparticles, including size, zeta potential, surface morphology, and crystal structure, are important elements in regulating the function of nanoparticles on bacterial cells. Also, environmental conditions, bacterial strains are other factors affecting the antibacterial effects of nanoparticles (24). In this study, silver nanoparticles were 5–8 nm in size and spherical. Higher inhibition can be due to the smaller size of the nanoparticles (25). Smaller nanoparticles have larger surface areas that lead to greater contact and passage through the bacterial cell membrane (26). Nanoparticles in different shapes can cause varying degrees of bacterial cell damage by interacting with periplasmic enzymes (27). Silver nanoparticles in cubic form show stronger antibacterial activity than silver nanoparticles in the spherical form (28). A synergistic effect of the combination of clarithromycin and silver nanoparticles was 70.58% in clarithromycin resistant isolates and 78.26% of sensitive ones. Porntip Pan-In et al. found that the combination of antibiotics with nanoparticles increased the inhibition of different isolates of *H. Pylori* two to four times (29). The combination of nanoparticles and antibiotics could reduce the MIC from 125

μg/ml to 15.6 μg/ml (30). The MIC of zinc oxide-polyethyleneimine nanoparticles on *H. Pylori* was 100 μg/ml, and the inhibition of bacteria by nanoparticles alone was 40%. However, combining nanoparticles and ampicillin increased the inhibition of bacteria to 80%. This combination reduced the MIC of ampicillin from more than 5μg/ml to 1μg/ml (31). The combination of nanoparticles and antibiotics has a higher inhibitory effect on bacteria than the use of nanoparticles and antibiotics alone (32). The increased bacterial resistance to antibiotics due to the use of nanoparticles is reported (33). Nanoparticles are highly mutagenic, and they increase the bacterial resistance to antibiotics by enhancing stress tolerance through intracellular ROS induction (34). Resistance to nanoparticles is unlikely because multiple simultaneous gene mutations are required in a microbial cell (35). The combination of nanoparticles with antibiotics not only reduces the toxicity of both agents to human cells at lower doses but also enhances their antimicrobial properties (36). Besides, nanoparticles combined with antibiotics increase the concentration of antibiotics at the site of interaction between bacteria and antibiotics, and it facilitated the binding of antibiotics to microorganisms (37).

Conclusion

Compared to using nanoparticles and antibiotic alone, when combining nanoparticles with clarithromycin, an increase in the synergistic effect of bacterial inhibition and the lack of antagonism was observed. This study showed that the combination of Ag-NP with clarithromycin reduced the MIC in different isolates of *H. Pylori* up to two times. Data obtained from in vivo studies carried out to test the toxicity and efficacy of the AgNPs was the limitation of this study. We conclude that using this combination is a new approach in the treatment of *H. pylori* infection. Future In-vivo studies can determine the toxicity of the nanoparticles. Further In-vivo investigations through well-defined studies and clinical trials will lead to applications of AgNPs in treatments of *H. pylori* infection.

Declarations

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Author Contributions

Rezvan Moniri designed the study, and analyzed the data, wrote, and edited the manuscript. Fateme Mansouri collected and analyzed the data and wrote the manuscript. Mahmood Saffari and Hosein Sedaghat designed the study and collected the data. Mohsen Razavizade and Mohamadreza Molaghanbari collected the samples. Gholam Abbas Moosavi analyzed the data.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Ethical approved

Kashan University of Medical Sciences Research Ethics Committee approved this study (#9771).

Availability of data and materials

The participants have consented for availability of data and materials in the journal

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Tables

Table1. Demographic and clinical characteristics of patients in this study

Characteristics of patients	*H. Pylori* positive NO. (%)	*H. Pylori* Negative No. (%)	P-value	Odds ratio	CI
Age (year)					
50≥ (78)	19 (24.4)	59 (75.6)	0.95	0.98	0.48-2.005
50< (85)	21 (24.7)	64 (75.3)			
Sex					
Female (108)	24 (22.2)	84 (77.8)	0.33	1.4	0.68-3.003
Male (55)	16 (29.1)	39 (70.9)			
Disease					
Non ulcer dyspepsia (119)	17(14.3)	102 (85.7)	0.001	6.57	3.002-14.38
Peptic ulcer (44)	23 (52.3)	21(47.7)			

Table 2. Frequency percent of patients based on age, sex and type of gastric disease and clarithromycin resistance pattern

	Resistant No. (%)	Sensitive No. (%)	P-value	Odds ratio	CI
Age (year)					
50≥ (19)	7 (36.8)	12 (63.2)	0.49	1.5	0.43-0.52
50< (21)	10 (47.6)	11(52.4)			
Sex					
Female (24)	11 (45.8)	13 (54.2)	0.6	1.4	0.38-5.1
Male (16)	6 (37.5)	10 (62.5)			
Disease					
Non ulcer dyspepsia (17)	6 (35.3)	11 (47.8)	0.42	0.59	0.16-2.1
Peptic ulcer (23)	11 (64.7)	12 (52.2)			

Table 3. Comparison of the MIC of clarithromycin, silver nanoparticles, and the combination of the both in clarithromycin sensitive and resistant *H. Pylori* isolates

Antibacterial agent	Nano-Ag + CLR		Nano-Ag		CLR	
Statistical index	R	S	R	S	R	**S**
$\bar{X}$	0.84	0.82	1.50	99.2	46.32	0.43
SD	1.06	1.09	70.08	37.19	54.82	0.27
P-value	0.665		0.039		0.003	

(S= Sensitive, R= Resistant)

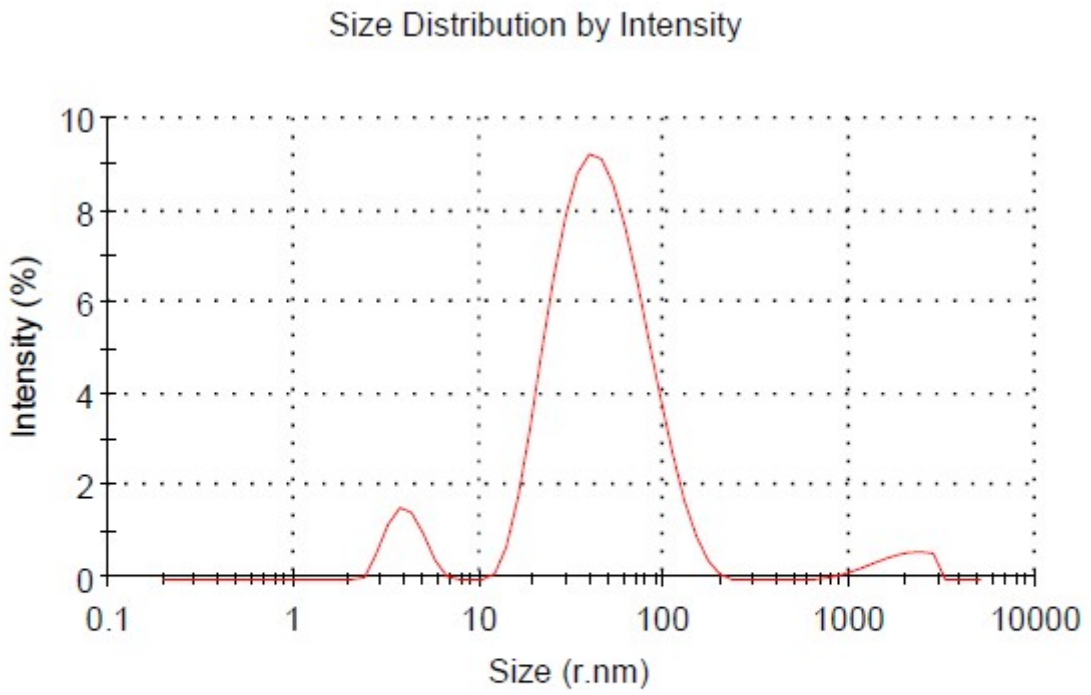
Table 4. Comparison of the MIC of clarithromycin alone and the combination of clarithromycin with silver nanoparticles in *H. Pylori* isolates in this study

MIC		
Statistical index	**Clarithromycin + Nano-Ag**	**Clarithromycin**
$\bar{X}$	8.68	19.93
SD	25.5	41.96
P-value	0.001	

Table 5. Comparison of the MIC of silver nanoparticle alone and the combination of clarithromycin with silver nanoparticles in all *H. Pylori* isolates

MIC		
Statistical index	Clarithromycin + Nano-Ag	Nano-Ag
$\bar{X}$	58.03	121.1
SD	102.17	58.8
P-value	0.001	

Figures



	Diam. (nm)	% Intensity	Width (nm)
Peak1	51.24	90.2	28.99
Peak2	4.085	6.2	0.8742
Peak3	1906	3.6	573.2

Figure 1

Size distribution of Nano-Ag

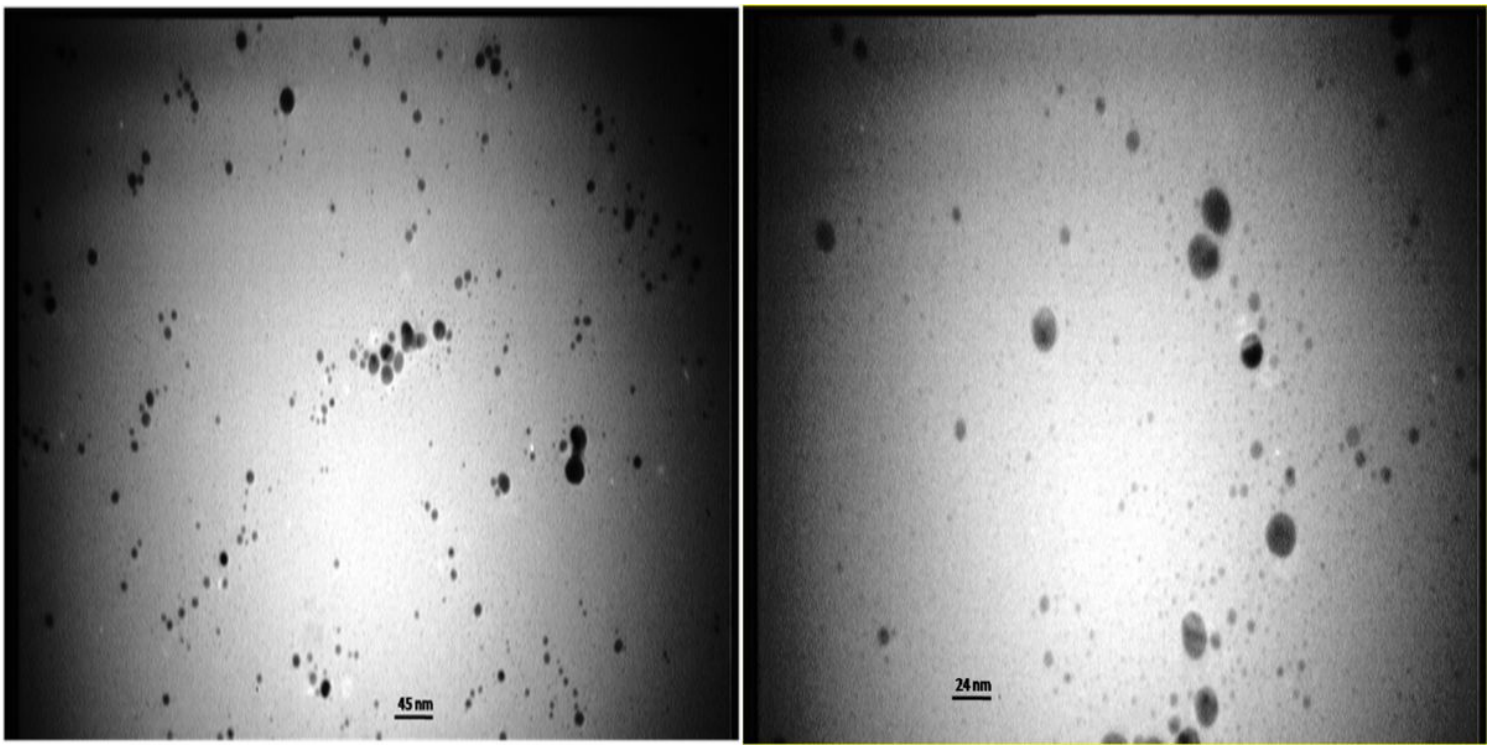


Figure 2

Transmission electron micrograph of Nano-Ag showing the spherical morphologies and size range

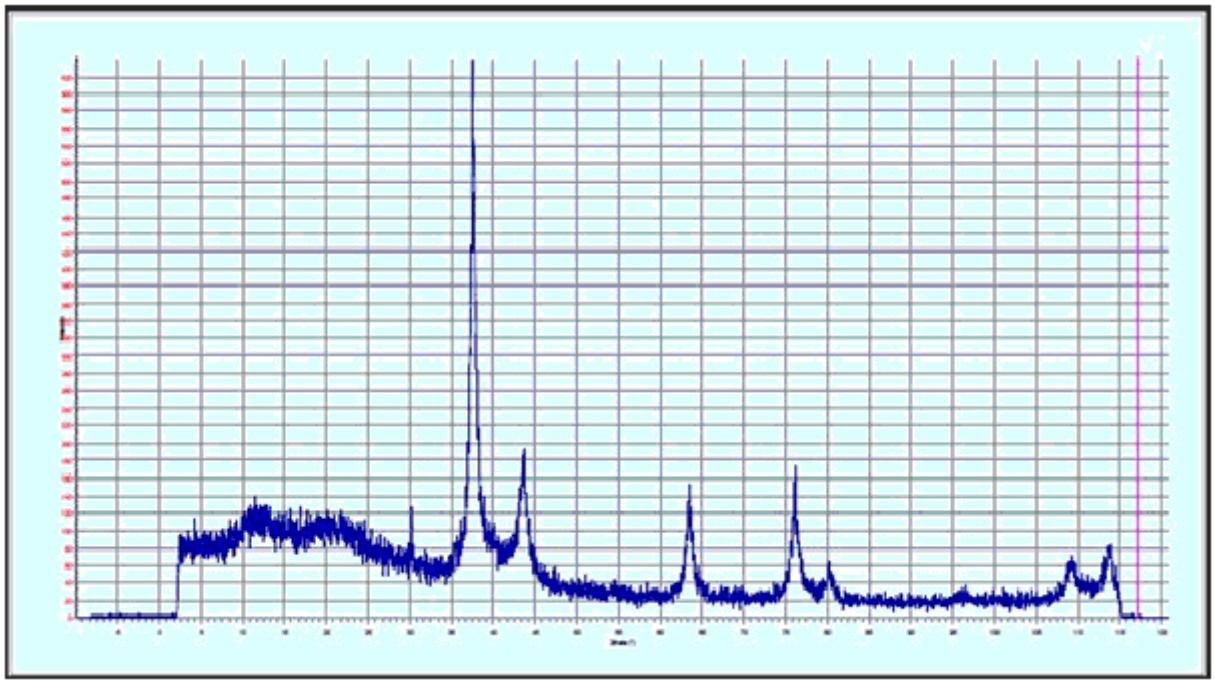


Figure 3

XRD pattern of silver nanoparticle

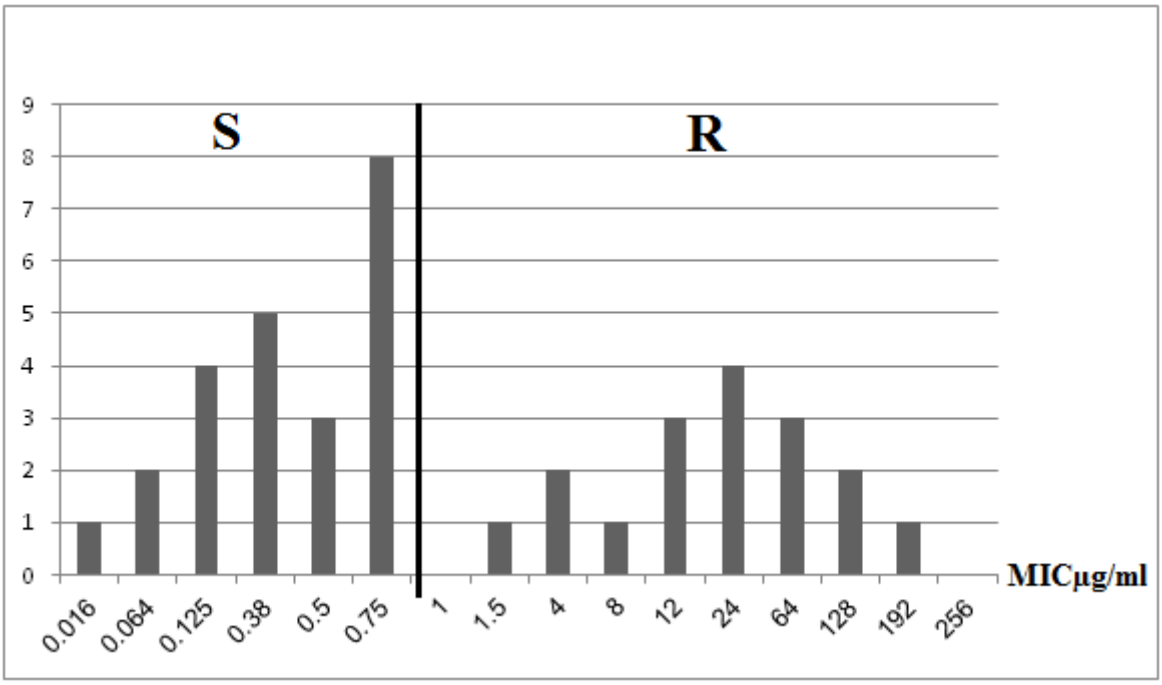


Figure 4

MIC of clarithromycin in *H. pylori* isolates in this study.

S, sensitive; R, resistance

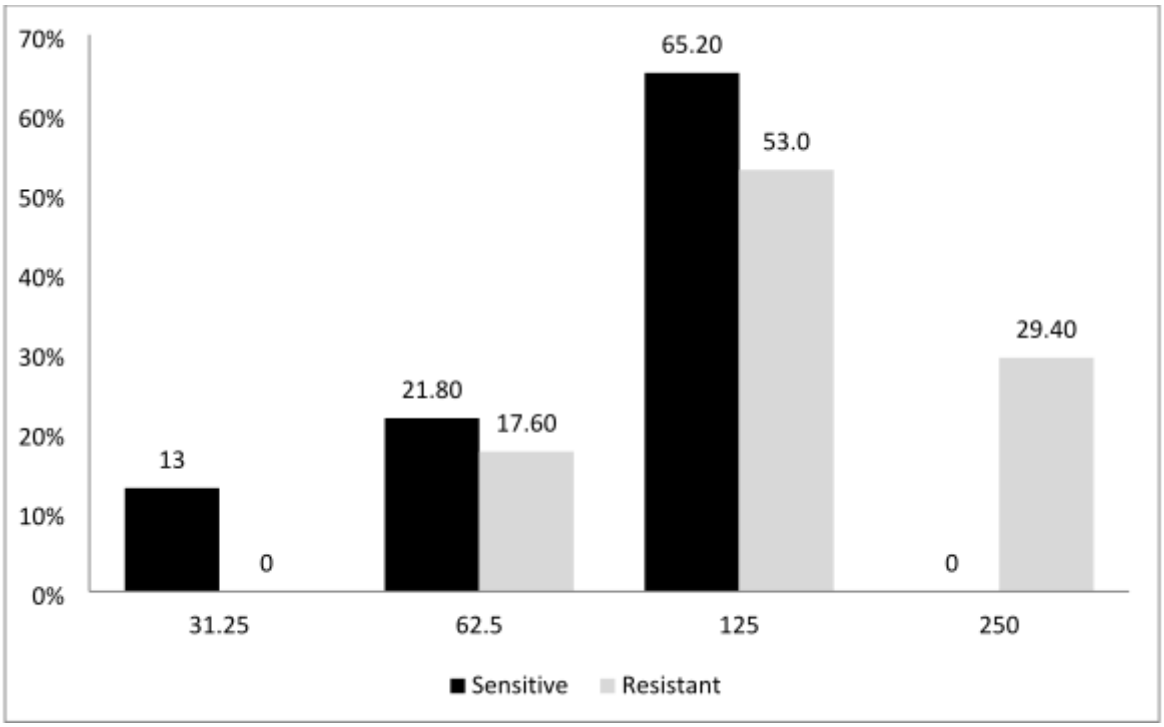


Figure 5

The frequency percent of MIC (µg/ml) of silver nanoparticles in clarithromycin-sensitive and resistant *H. Pylori* isolates

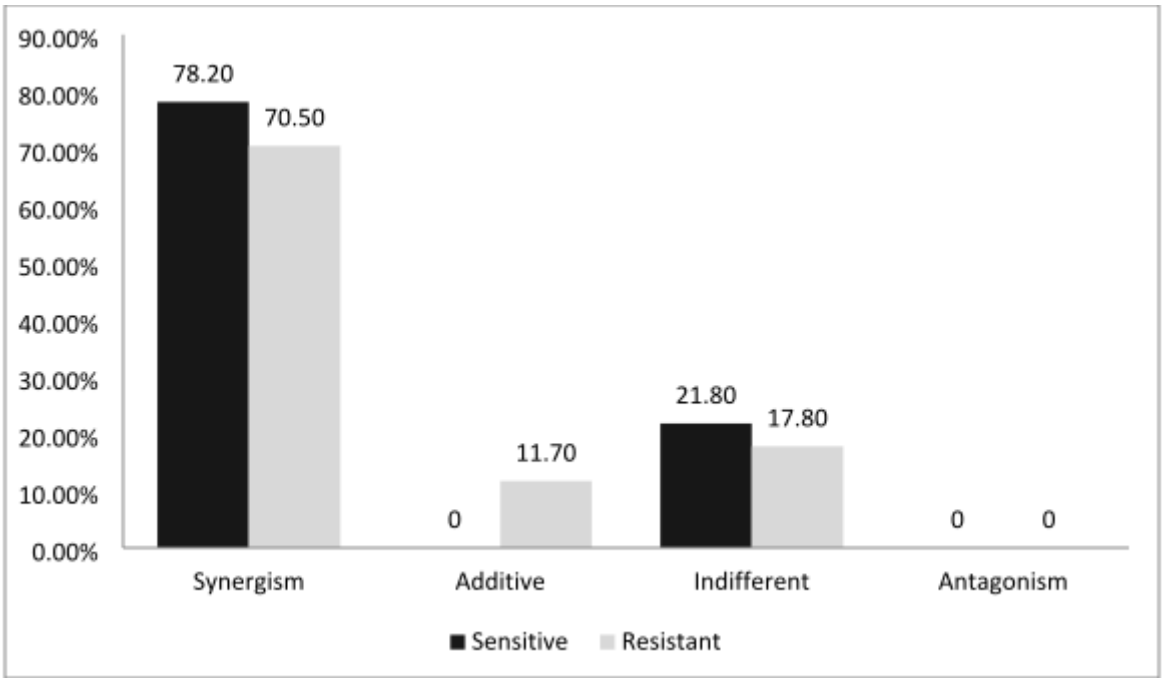


Figure 6

The synergistic effect of silver nanoparticles with clarithromycin in clarithromycin-sensitive and resistant *H. Pylori* isolates