Original Article

Preparation of Nano-Medicine to Eliminate *Helicobacter pylori* Infection

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ABSTRACT

Helicobacter pylori (H. pylori) is considered a challenging type of bacteria that is difficult to treat with the currently used antibiotics, such as amoxicillin, erythromycin, and metronidazole, which have proven ineffective against these bacteria. In this study, modern technology was used to treat these bacteria by converting the aforementioned antibiotics to their nano state using the lyophilization method and diagnosing them using scanning electron microscopy. A mixture of the three nano-antibiotics was prepared in the form of a nano-medicine, which was used to treat *H. pylori* bacteria in cultures and determine the effectiveness of nano-antibiotics and nano-medicine on these bacteria. The findings showed that nano-medicine was highly effective in inhibiting these bacteria at the lowest concentration (OD=0.042) and the highest concentration (OD=0.038), compared to the three micro-antibiotics individually. The OD values of amoxicillin, azithromycin, and metronidazole were 0.523, 0.521, and 0.453, respectively. The OD values of the three nano-antibiotics, including nano-amoxicillin, nano-azithromycin, and nano-metronidazole, were 0.386, 0.258, and 0.167, respectively. It was observed that the percentage of inhibition in each of the nano-antibiotics was higher than the inhibition in micro-antibiotics and that the nano-medicine had much higher inhibition than each of the three micro- and nano-antibiotics alike. The safety of using nano-antibiotics in the prepared medicine was confirmed using electrochemical technology and cyclic voltammetry to identify the electrochemical properties through oxidation and reduction in blood media. Based on the findings, only reduction peaks appeared, and there were no oxidation peaks in the prepared kit or for each of the three nano-antibiotics. It was found that they were all non-oxidants and could be used safely as good antioxidants in treatments. However, the same three micro treatments showed blood oxidation due to the appearance of oxidation peaks in all of them. The study proved that all *H. pylori* isolates are resistant to usable antibiotics. All of the antibiotics in the nano-medicine had an anti-bacterial effect, and the effect of the new form of antibiotic was proportional to the concentration of the antibiotic.

Keywords: Cyclic voltammetry, *H-pylori*, Nano-antibiotic, Nano-medicine

1. Introduction

Helicobacter pylori (H. pylori), as shown in figure 1, is a ubiquitous organism in about 50% of the world's population. Chronic infection with *H. pylori* causes atrophic changes and even gastric metaplasia and has an association with peptic ulcer disease. The most common way to contract *H. pylori* is through mouth-to-mouth or stool-to-mouth contact. *H. pylori* is resistant to routine treatments, and they do not completely eradicate it (1).

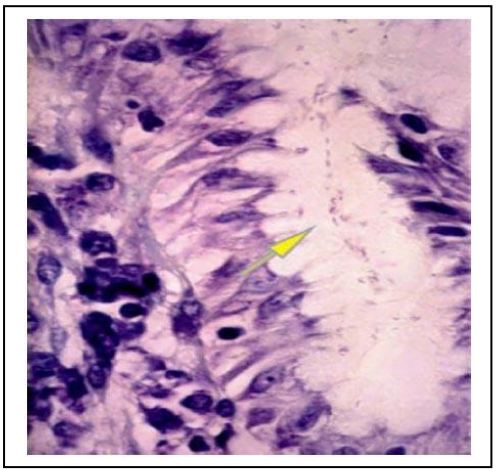

Figure 1. *Helicobacter Pylori* Infection. This image shows an antral gland of the stomach with a large Giemsa-stained colony of *Helicobacter pylori* in the lumen (arrow) at 250× power. Courtesy of Pantaleo Bufo, University of Foggia, Italy

The discovery of *H. pylori* in 1982 was the starting point of a revolution related to the concepts and management of gastrointestinal diseases. The most common stomach disease, peptic ulcer disease, is now recognized as an infectious disease, and all congresses agree that the causative agent, *H. pylori*, should be treated with antibiotics. Moreover, the concept emerged that this type of bacteria could be a catalyst for several malignant gastric diseases, which is now a model for chronic carcinogenic bacterial infection. Most techniques used in diagnosing *H. pylori* infection are performed in clinical microbiology laboratories (2).

H. pylori is the cause of chronic gastritis, peptic ulcer disease, and stomach cancer. The increasing antibiotic resistance is one of the biggest challenges of the time due to its ever-expanding growth. To define an alternative or adjuvant strategy for standard antibiotic therapy, the *in vitro* activity of newly synthesized silver nano-clusters (SUNCs), which have an average size of <5 nm, was investigated against clinical strains of *H. pylori* with different antibiotic sensitivity. SUNCs showed potential synergy with metronidazole and clarithromycin. They also showed low toxicity to human cells and were effective in eliminating the mature biofilm produced by *H. pylori*. SUNCs could represent a new strategy for treating *H. pylori* infection either alone or in combination with metronidazole (3).

The prevalence of *H. pylori* infection remains significant worldwide and depends on many factors, such as gender, age, socioeconomic status, geographic region, diet, and lifestyle. All successful infectious disease treatments use antibiotic susceptibility testing, but this strategy is not currently practical for *H. pylori*, and typical cure rates are lower for *H. pylori* than for other bacterial infections. No treatment guarantees complete eradication of this pathogen. In the context of the alarming increase in antibiotic resistance (particularly to clarithromycin and metronidazole), alternative and complementary options and strategies are being considered. Since the success of antibacterial therapy depends not only on susceptibility to specific drugs but also on specific doses, formulations, adjuvant use, duration of treatment, and reinfection rates, this review discusses current *H. pylori* treatments, along with their advantages and limitations. As an alternative, this study presents a widely referenced approach to natural medicines against *H. pylori*, including the importance of nanotechnology in developing new strategies for treating *H. pylori* infection (4).

In the development of next-generation biosensors, nano-materials with outstanding thermal, mechanical, optical, and electrical properties have been identified as one of the most promising materials for opening new portals. This study discusses the latest developments in the identification of antibiotics by biosensors fabricated with nano-materials. The construction of biosensors for electrochemical signal transmission mechanisms has been used in various types of nano-materials, including

quantum dots, metal-organic frameworks, magnetic nanoparticles (NPs), metallic nano-materials, and carbon nano-materials. To provide an outline of future study directions, current problems and future opportunities in this field are also included (5).

The largest size of gold NPs is between 12 and 9 nm, and the maximum absorption is 522 nm. However, in a previous study, the maximum absorption was 540 nm, indicating an accumulation of drug-bound NPs in the conjugated state. Some changes indicated the binding of metronidazole to gold NPs. Antimicrobial testing of gold NPs and metronidazole did not affect *H. pylori*, and the combination of gold NPs and metronidazole had a growth inhibition area of 17 mm (6).

In bacterial survival rates by amoxicillin treatment and NP heating alone, the synergistic effect can be attributed in part to the heating-induced damage to the cell membrane and the protective biofilm, which may increase the permeability of bacteria to antibiotics. Our method provides a viable approach to the treatment of *H. pylori* infection, with the potential to reduce side effects and enhance the efficacy of combating drug-resistant strains (7).

The Brazilian consensus recommends a short-term course of clarithromycin, amoxicillin, and proton pump inhibitors to eradicate *H. pylori*. This course of treatment is characterized by good efficacy, but a significant part of the population cannot tolerate it. Azithromycin, amoxicillin, and omeprazole, for several purposes, are supported by the Brazilian federal government. Therefore, the short-term course of treatment with these drugs is low-cost, but their efficacy concerning the eradication of bacteria has not yet been proven (8).

A previous study aimed to determine the eradication rate of an extremely short treatment schedule for *H. pylori* infection in a population with peptic ulcers, using omeprazole, secnidazole, and azithromycin at a once-daily dose for three days. Patients who tested negative for *H. pylori* by the rapid urease test and histological examination were considered to have recovered. Despite the low rate of side effects and good compliance, the eradication index was low. A possible drawback of this treatment is that it reduces the effectiveness of macrolide and nitroimidazole compounds in subsequent treatments (9).

H. pylori DNA voltammetric assay was investigated using a bismuth-stabilized carbon nanoelectrode. The peak current-limit potential appeared at 0.4 V, at which there was an optimal diagnostic square wave stripping working range of 0.72-7.92 μg/ml of helical DNA. Developed sensors can be used for clinical applications where the patient's peak current increases a hundred times more than that of passively healthy tissues (10).

Amoxicillin is one of the most successful antibiotics used in human therapy. It is widely used to prevent or treat bacterial infections in humans and animals. However, its widespread distribution and excessive use can be an environmental and health hazard due to the potential risks associated with liquid pharmaceutical industry residues. In addition, its extensive use in animal food production may lead to some unwanted residues in food products, such as meat, eggs, and milk. Therefore, when sufficiently high concentrations are present in biological fluids, they may be responsible for various diseases, such as nausea, vomiting, rash, and colitis, associated with antibiotics. For this reason, the detection and quantification of amoxicillin in pharmaceuticals, biological fluids, environmental samples, and food products requires new electrolysis techniques with sensitive and rapid measurement capabilities (3).

A sensitive and selective electrochemical method has been developed for the determination of amoxicillin using a paste electrode made from natural carbon tubes modified with a carboxylic acid. The modified electrode showed good electroactivity for the electrochemical oxidation of amoxicillin at pH 10.5 phosphate stock solution. The electrocatalytic oxidation peak current of amoxicillin showed two linear dynamic ranges with a detection limit of 8.7 nmol/L-1 amoxicillin. Linear calibration ranges were 0.03-0.35

μmol/L and 0.50-32.70 μmol/L amoxicillin using the square wave voltmeter method. Finally, this modified electrode was also examined for the determination of amoxicillin in real urine samples (11).

2. Materials and Methods

2.1. Synthesis of Antibiotic Nanoparticles by Lyophilization (Deep Freezing Method)

The first step was to prepare a solution of each of the three antibiotics (amoxicillin, erythromycin, and metronidazole) at a concentration of 0.1 M. The suspension cooled, and ice crystals were formed from pure water at -18°C. The second step involved sublimating the ice from the frozen product by passing convection air from the lyophilizer rack into the frozen solution in the vial, as shown in figure 2. The ice sublimated, and the resulting water vapor passed through the dried part of the product to the surface of the sample. The water surface of the product passed through the chamber into the condenser, and the condensed water vapor was formed on the condenser. At the end of the sublimation step, a porous plug was formed. Its pores corresponded to the spaces occupied by ice crystals. The third step was drying, which involved removing the absorbed water from the product. All steps were continued for approximately 48-72 h.

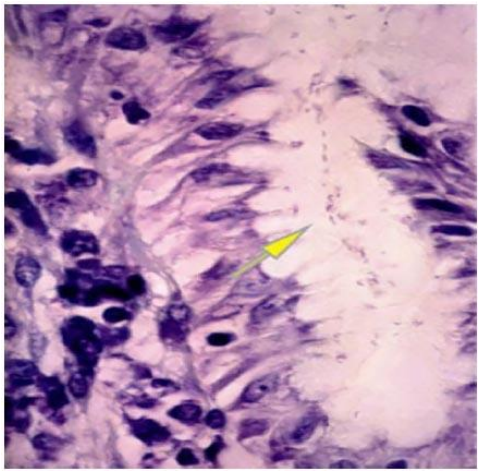

Figure 2. Lyophilization instrument, LABCONCO Company (U.S.A.)

2.2. Characterization of Nanoparticles

2.2.1. Scanning Electron Microscopy

Scanning electron microscopy (SEM) is an imaging technology that can examine the shape of NPs and measure their size at the nanometer level.

Figure 3 presents the SEM images of nano-antibiotics, showing measurements of 37.77, 179.61, and 102.08 nm for nano-amoxicillin, nano-azithromycin, and nano-metronidazole, respectively. The details of the surface shape of the antibody NPs revealed the shapes of the surfactants that accepted the results of the conversion of antibiotics into NPs.

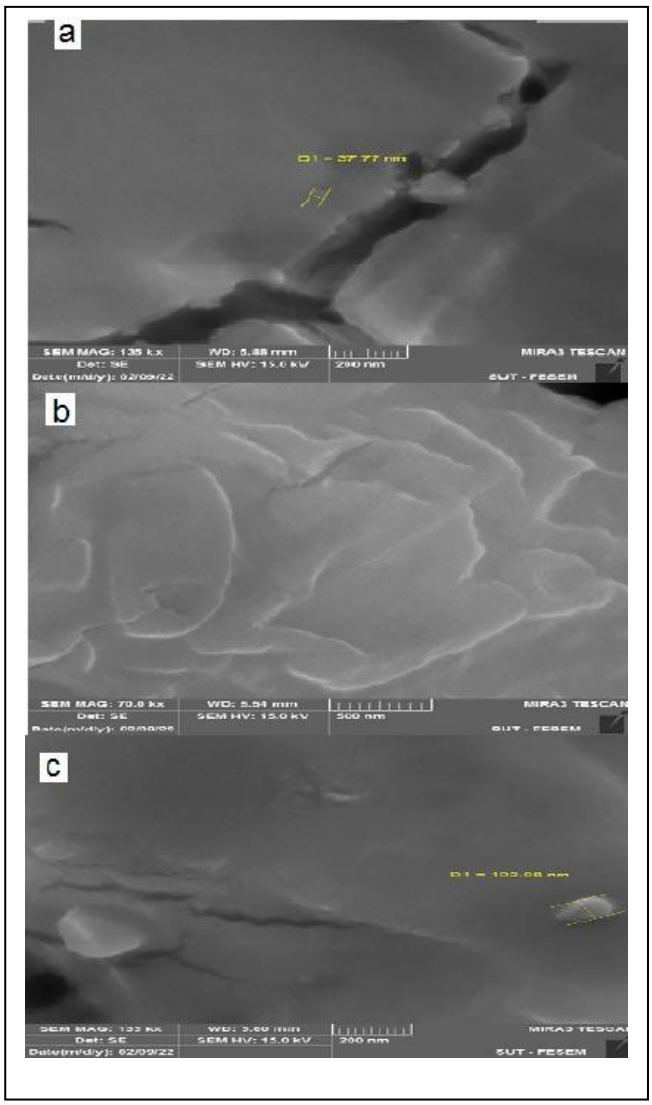

Figure 3. SEM for the **(a)** amoxicillin NPs, **(b)** azithromycin NPs, and **(c)** metronidazole NPs

2.3. Cyclic Voltammetry Study of the Nano-Antibiotics

Cyclic voltammetry (CV) is a powerful electrochemical method often used to study the reduction and oxidation processes of nano-antibiotics in the blood and investigate their effects on blood composition (Figure 4).

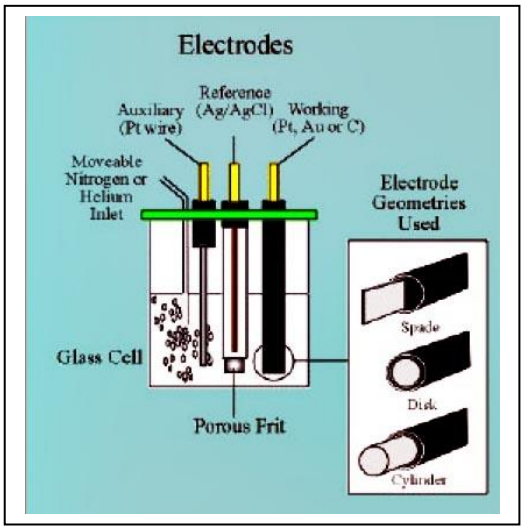

Figure 4. Cyclic voltammetric quartz cell with electrodes

2.4. Biomedical Study

2.4.1. *H. pylori* Sample Collection

H. Pylori samples were collected in the Hospital of Gastroenterology and Liver in Medical City, Baghdad, Iraq, through three endoscope biopsies for patients with *H. pylori* infection diagnosed with gastric and duodenal ulcers.

2.4.2. Preparation of Cultures

Bacteria were cultured on Columbia agar for 7.5 days at a constant temperature of 37°C under anaerobic conditions in a Candle Jar at 5-10% CO_2 . In the initial diagnosis, the biopsy sample was extracted by laparoscopy, which was previously diagnosed by urease examination. In the confirmative diagnosis, the following tests were conducted after the samples were transplanted: 1) Columbia agar 2) urease, as shown in figure 5, 3) gram stain, as shown in figure 6, 4) catalase, as shown in figure 7, 5) oxidase, as shown in figure 8, and 6) molecular PCR diagnostics.

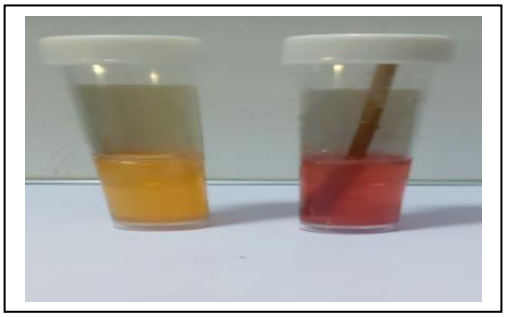

Figure 5. Urease test positive for color change from yellow to pink urease + ve

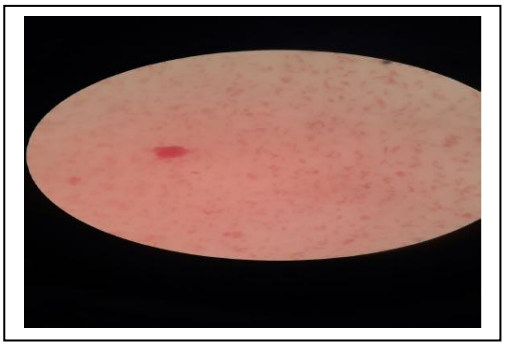

Figure 6. Gram-negative Bacillus Gram ve . stain

Figure 7. The catalase test positive for the appearance of catalase + ve. air bubbles

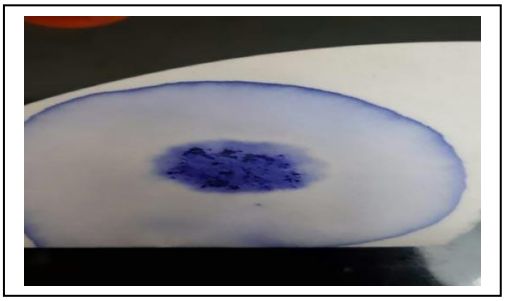

Figure 8. Assay of the oxidase enzyme positive, changing the colour to violet, oxidase + ve

2.4.3. Determination of Minimal Inhibitory Concentration

The lowest inhibitory concentration of bacteria was determined after the bacteria were cultured on Columbia agar. A confirmatory examination of these bacteria was performed to determine the effective antibiotics according to the method of spectrophotometry and Optical Density (OD).

2.4.3.1. Use of Antibiotics

The antibiotics used in the treatment of *H. pylori* were amoxicillin, azithromycin, and metronidazole.

2.4.3.2. Spectrophotometer Calculation

The method of using antibiotics on the agar did not result in any sensitivity to the penetration of the antibiotic into the agar, so the OD was used by the spectrophotometric method to determine the Minimal inhibitory concentration value using a Jasco spectrophotometer with a wavelength of 600 nm, as shown in figure 9.

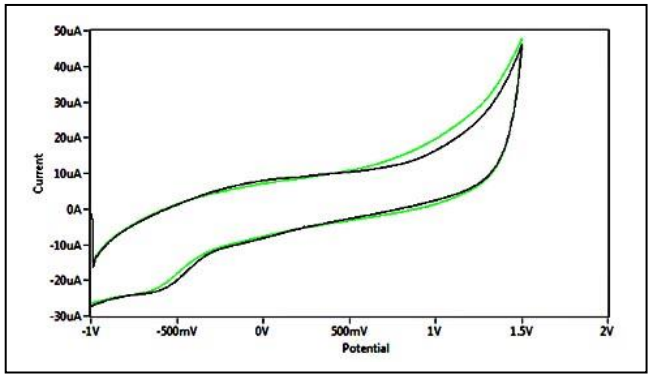

Figure 9. Cyclic voltammogram of azithromycin nanoparticles at different concentrations in serum blood medium on GCE as working electrode against Ag/AgCl as reference electrode at a scan rate of 0.1 Vsec-1

The absorbance was read at the indicated wavelength of bacteria after incubation for three days at a temperature of 37°C in anaerobic conditions. Two sizes from the conveyor were taken and then implanted in a tube inside it.

2.4.3.3. Tryptic Soy Broth

Samples were implanted for three in Tryptic Soy Broth (TSB) culture and then in the tube consisting of TSB, 50 µL bacteria, and antibiotic.

A total of 50 µg of bacteria were taken from the base tube with antibiotics in different concentrations with the liquid culture medium in five sterile tubes with a volume of 10 mL. It was then incubated for three days in anaerobic conditions, and the lowest inhibitory concentration of bacteria with the antibiotic was read using a spectrophotometer according to the appropriate wavelength. The readings were recorded.

2.5. Study of Nano-Antibiotics

Dilute solutions were prepared from the three nano-antibiotics, including nano-amoxicillin, nano-azithromycin, and nano-metronidazole. Five concentrations were diluted and cultivated for three days, after which the bacterial growth was studied in a spectrophotometer at a wavelength of 600 nm, and the readings were recorded.

2.6. Study of the Three Nano-Antibiotics

The nano combination of the three antibiotics with the lowest and highest concentrations against bacteria was studied by spectrophotometry.

3. Results and Discussion

3.1. Study of Cyclic Voltammetry

3.1.1. Effect of Nano-Medicines (the Three Antibiotic Nanoparticles) on Blood Components

Analytical experiments were conducted on the nano-kit (for the three nano-antibiotics, including nano-amoxicillin, nano-azithromycin, and nano-metronidazole) in blood medium using a ring voltmeter to measure the toxicity level of these antibiotics on blood and its components through the peaks of the redox current. The observations from the analysis of each of the three nano-antibiotics (nano-amoxicillin, nano-azithromycin, and nano-metronidazole), as well as the mixture of the three antibiotics represented by nano-kits, are shown in figures 9, 10, 11, and 12. As can be seen from this cyclic voltammogram in the aforementioned figures, there were no oxidation peaks in all the NPs; rather, there were reduction peaks for each of these three nano-antibiotics and the NPs as well. Therefore, these nano-antigens can be considered non-oxidizing (antioxidants) to the composition of blood and can be used safely.

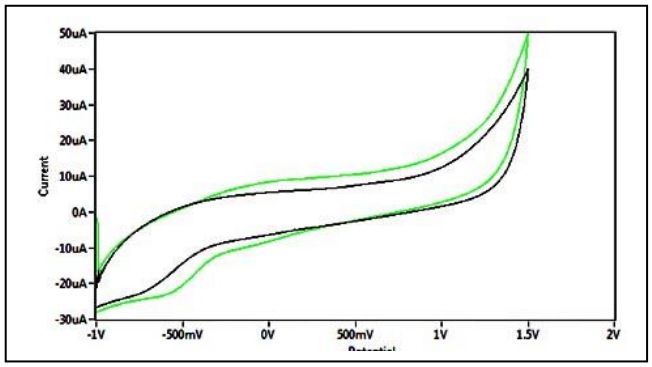

Figure 10. Cyclic voltammogram of amoxicillin nanoparticles at different concentrations in serum blood medium on GCE as working electrode against Ag/AgCl as reference electrode at a scan rate of 0.1 Vsec-1

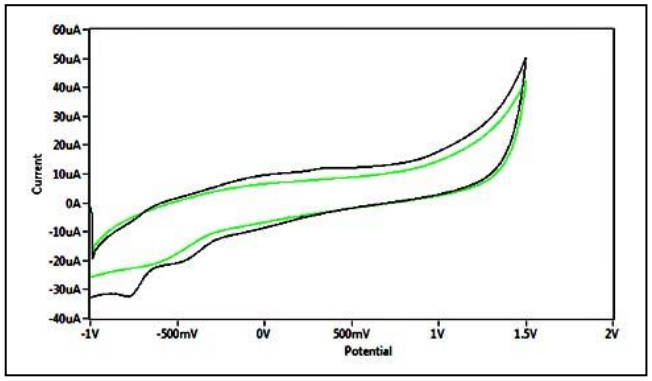

Figure 11. Cyclic voltammogram of metronidazole nanoparticles at different concentrations in serum blood medium on GCE as working electrode against Ag/AgCl as reference electrode at a scan rate of 0.1 Vsec-1

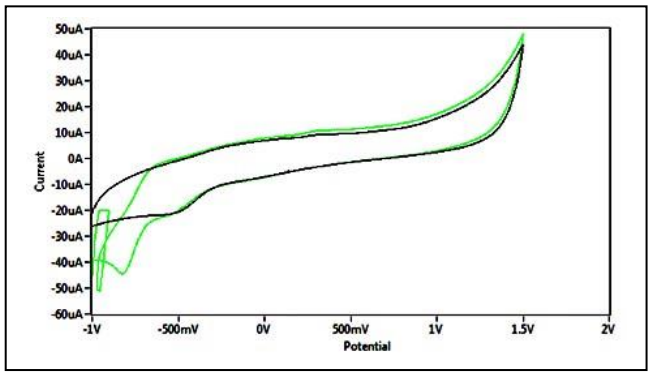

Figure 12. Cyclic voltammogram of a mixture of amoxicillin NPs, azithromycin NPs, and metronidazole NPs at different concentrations 0.02-0.2 mM in serum blood medium on GCE as working electrode against Ag/AgCl as reference electrode at a scan rate of 0.1 $Vsec^{-1}$

3.2. Study of the Effect of Antibiotics on *H. pylori*

3.2.1. Micro-Antibiotics

Different concentrations of the three micronized antibiotics (amoxicillin, azithromycin, and metronidazole) were used, from 0.03 to 0.2 µg, f0.05 to 0.3 µg, and 0.05 to 0.3 µg, respectively. *H. pylori* was used to draw the baseline of the inhibition pattern (the effect of the micro-antibiotic). Each of the three antibiotics (amoxicillin, azithromycin, and metronidazole) inhibited bacteria at the highest concentrations of 0.2, 0.3, and 0.3, respectively (Tables 1-3).

Table 1. Represents the highest effective doses of micro-amoxicillin against *H. pylori* bacteria

Amoxicillin Concentration in microgram	O.D
0.03	0.784
0.05	0.712
0.08	0.716
0.1	0.675
0.2	0.523

Table 2. Represents the highest effective doses of micro-metronidazole against *H. pylori* bacteria

MetronidazolConcentration in microgram	O.D
0.05	0.724
0.1	0.667
0.15	0.552
0.2	0.541
0.25	0.520
0.3	0.453

Table 3. Represents the highest effective doses of microflora azithromycin against *H. pylori* bacteria

Azithromcin Concentration in microgram	O.D
0.05	0.797
0.1	0.745
0.15	0.635
0.2	0.625
0.25	0.613
0.3	0.521

After determining the lowest and highest concentrations of the three antibiotics in inhibiting *H. pylori*, the concentration of 0.02 ng of amoxicillin was chosen and diluted to five concentrations, as shown in table 4. The concentration of 0.03 ng of metronidazole and the bacterial inhibition study for five dilute solutions are shown in table 5. In the same way, five dilute solutions of azithromycin were used with a concentration of 0.03 ng, as shown in figure 6. The inhibition rate of each of the micro-antibiotics against *H. pylori* was directly proportional to the concentration, as shown in tables 4, 5, and 6.

Table 4. Represents the highest effective diluted doses of micro-amoxicillin against *Helicobacter pylori*

Amoxicillin Concentration in Nanogram	O.D
0.02	0.167
0.002	0.452
0.0002	0.685
0.00002	0.773
0.000002	0.870

Table 5. Represents the highest effective diluted doses of metronidazole metronidazole against *Helicobacter pylori*

Metronidazol Concentration in Nanogram	O.D
0.03	0.386
0.003	0.411
0.0003	0.528
0.00003	0.632
0.000003	0.789

Table 6. Represents the highest effective dilute doses of microflora azithromycin against Helicobacter pylori

Azithromycin Concentration in Nanogram	O.D
0.03	0.258
0.003	0.308
0.0003	0.441
0.00003	0.614
0.000003	0.713

3.2.2. Nano-Antibiotics

The previous method was applied by taking the highest concentration in determining the inhibition values of nano-antibiotics after converting micro-antibiotics using the lyophilization method (deep freezing) (10). The inhibition value of micro-amoxicillin was OD=0.523 at a concentration of 0.02 µg. There was also a difference in inhibition in the case of using the nano-antibiotic represented by nano-metronidazole, which caused the inhibition of bacteria (OD=0.386) at a concentration of 0.03 ng, compared to the inhibition of metronidazole, micro-inhibition (OD=0.453) at a concentration of 0.03 µg. It was also true in the case of using nano-azithromycin because of its high inhibition of bacteria (OD=0.258) at a concentration of 0.03 ng, compared to the inhibition caused by microencapsulation (OD=0.521) at a concentration of 0.03 µg and the previous method by taking the lowest concentration, as shown in tables 7, 8, and 9.

Table 7. Represents the lowest effective dilute doses of nano-amoxicillin against *Helicobacter pylori*

Amoxicillin Nanoparticles, nanogram	O.D
0.03	0.177
0.003	0.542
0.0003	0.615
0.00003	0.655
0.000003	0.777

Table 8. Represents the lowest effective diluted doses of metronidazole nanoparticles against *Helicobacter pylori*

Metronidazol Nanoparticles, nanogram	O.D
0.05	0.426
0.005	0.531
0.0005	0.615
0.00005	0.786
0.000005	0.891

Table 9. Represents the lowest effective diluted doses of Azithromycin nanoparticles against *Helicobacter pylori*

Azithromycin Nanoparticles, nanogram	O.D
0.05	0.278
0.005	0.388
0.0005	0.471
0.00005	0.665
0.000005	0.839

3.2.3. Nano-Medicine (the Three Nano-Antibiotics Together)

A nano-pharmaceutical composition was prepared consisting of the three nano-antibiotics, including nano-

amoxicillin, nano-azithromycin, and nano-metronidazole, in a mixture and analyzed by spectrophotometer for the highest and lowest concentrations (11). A higher inhibition value was obtained, compared to all three nano-antibiotics individually. One nano-medicine gave impressive results in eliminating bacteria, with an inhibition rate of nearly 100%. The values recorded for the micro- and nano-antibodies separately were distinct (9). Therefore, the nano-pharmaceutical composition can be adopted in the final eradication of the bacteria (Table 10).

Table 10. Represents the highest effective dilute doses of several nanoparticles against *H. pylori* bacteria

Mix Nano-antibiotic (NanoKit)	O.D
Amoxicillin NPs, metronidazole NPs,azithromycin NPs /low concentration	0.042
Amoxicillin NPs, metronidazole NPs, azithromycin NPs /high concentration	0.038

The nano-antibiotics were characterized by the nano-pharmacological composition of nano-amoxicillin, nano-azithromycin, and nano-metronidazole, which gave outstanding results in inhibiting *H. pylori* up to 100%.

The nano-kit is characterized by its safe use and the absence of side effects because the nano-antibiotics are non-oxidative to the blood. The nano-kit is highly efficient in inhibiting bacteria at a higher rate than all current treatments. Another advantage is the ease of preparation by converting the micro-antigens of amoxicillin, azithromycin, and metronidazole to NPs.

Authors' Contribution

Study concept and design: S. A. H. A. and M. M. R.
Acquisition of data: S. A. H. A.
Analysis and interpretation of data: Z. N. H.
Drafting of the manuscript: Z. N. H.
Critical revision of the manuscript for important intellectual content: M. M. R.
Statistical analysis: M. M. R.
Administrative, technical, and material support: M. M. R.

Conflict of Interest

The authors declare that they have no conflict of interest.

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