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Evaluation of Drugs Candidates as Inhibitors of *Helicobacter pylori* Urease *in Silico* and *in Vitro*

A thesis

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Dedication

The woman who raised me as a seed of life and irritated me with her kindness, her dear and noble soul، my mother Najah، my deceased father is in the house of truth with God, Dr. Mohammed Ali

To the candles that light up the path of my life, my sisters Azal, Shahad, and my brother Dhualfiqar and his children Saif and Qamar,

My beautiful aunt Nadia, and the one who honored me with her prayers and encouragement, my aunt Umm Iman

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Summary

Peptic ulcer disease is a disorder that causes open ulcers in the stomach or upper section of the small intestine. *Helicobacter pylori*, the bacteria that cause most common peptic ulcers, generates an enzyme called urease, which propagate to the disease's progression. The urease degrades urea in the stomach, generating ammonia that aids in the neutralization of gastric acid. This process had given an advantage to colonizing *H. pylori*, however, damages the protective mucus lining and destroys the gastrointestinal barrier, result in inflammation and ulcer development. Furthermore, the immunological response elicited by *H. pylori* adds to tissue damage. Treatments are used to eradicate *H. pylori* and drugs to lower urease production and enhance ulcer healing.

seventy patients were enrolled in this study collected directly from the endoscopy unit in the Karbala Center for Gastroenterology and Hepatology at Imam Al-Hussein Medical-City, all patients were diagnosed by a stool antigen test for detection of *H. pylori*. The positive results were taken from biopsy samples. The results showed 58 (82.8%) patients from 70 patients suffering from *H. pylori* infection by stool antigen detection test while the culture of the biopsy showed 12 (17.14%) with *H. pylori*,

Moreover, this thesis investigated the optimal incubation time for enzyme activity in *H. pylori* isolates. The results demonstrated that the enzyme activity reached its peak at 72 hours of incubation, indicating an optimal period for enzyme activity before it started to decline.

Purification steps were carried out to obtain purified urease enzymes from both the reference strain and the local strain of *H. pylori.* The purification process involved, preparative high-performance liquid chromatography (HPLC). The specific activity of the purified enzyme

increased, indicating successful purification. The yield varied throughout the purification steps, with slight loses observed during certain stages.

Virtual screening was performed to identify potential *H. pylori* urease inhibitors. Molecular docking simulations were conducted using the Glide software, and several compounds from the zinc15 database were analyzed. Among the top-ranked compounds, paromomycin, tobramycin, amikacin, capreomycin, and gentamycin exhibited strong binding affinity to the *H. pylori* urease protein.

The inhibitory effect for purified urease was performed by using a drug candidate to bind the n active site other f urease. In silico results indicate that some of the aminoglycosides such as paromomycin, amikacin, gentamicin, tobramycin, and capreomycin can bind to urease enzymes at active sites. The experimental results indicate that paromomycin, amikacin, gentamicin, tobramycin, and capreomycin have inhibitory effects on urease activity.

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List of abbreviation

abbreviation	Definition
Å	Ångström
ATP	Adenosine triphosphate
BHI	Brain-heart infusion
BSA	Bovine Serum Albumin
C	Carbon
Cag A	Cytotoxin associated gene A
CO_2	Carbon dioxide
D.W	Distilled water
DNA	Deoxyribonucleic acid
DU	Duodenal ulcer
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and drug administration
FFPE	Formalin-fixed paraffin-embedded
g	grams
GIT	The gastrointestinal tract
GLIDE	Grid-Based Ligand Docking with Energetics
GU	Gastric ulcer
H. pylori	*Helicobacter pylori*
HCl	Hydrochloric acid
HPLC	High-performance liquid chromatography
hrs.	Hours
IARC	The International Agency for Research on Cancer
IC_{50}	Half maximal inhibitory concentration
ICA	Immunochromatography
IgA	Immunoglobulin alpha
IgG	Immunoglobulin gamma
IL	Interleukin

MALT	Gastric mucosa-associated lymphoid tissue
mRNA	Messenger ribonucleic acid
N_2	Nitrogen
NCTC	National Collection of Type Cultures
NGS	Next-generation sequencing
NH_3	Ammonia
Nm	Nanometer
NSAIDs	Non-steroidal anti-inflammatory drugs
O_2	Oxygen
OMPs	Outer membrane proteins
PCR	Polymerase chain reaction
PDB	Protein data bank
PPI	Proton pump inhibitors
PUD	Peptic ulcer disease
rRNA	Ribosomal ribonucleic acid
RUT	Rapid urease test
SATs	Stool antigen tests
TAE	Tris Acetate Ethylenediaminetetraacetic acid
TNF	Tumor necrosis factor
TNFα	Tumor necrosis factor alpha
U	Unit
UBT	Urea breath test
VacA	Vacuolating associated cytotoxin A
Cryo- EM	Cryogenic electron microscope
SPSS	Statistical package for social science
Conc	Concentration

Chapter one
Introduction and Literature Review

1. Introduction and Literature Review

1.1 Introduction

A peptic ulcer is a common gastrointestinal illness characterized by the formation of ulcer in the stomach, duodenum, or esophageal lining. It affects millions of individuals globally and is associated with considerable morbidity and mortality. The etiology of Peptic ulcer is numerous, including *Helicobacter pylori (H. pylori)* infection, use of nonsteroidal anti-inflammatory drugs, stress, Steroid, and smoking is among the most essential risk factors (Kuna, *et al.*, 2019).

Peptic ulcer pathogenesis includes a complicated interaction of variables that modify the balance between mucosal protection and aggressive forces. Then breakdown of the mucosal barrier, which allows acid and pepsin to enter the underlying tissue. As a result, there is inflammation, tissue damage, and the development of a peptic ulcer (Périco, *et al.*, 2020).

Gastric duodenal ulcers are open ulcers that form on the stomach lining (gastric ulcers) or the upper section of the small intestine called the duodenum (duodenal ulcers). These ulcers are often produced by an imbalance between stomach acid and the protective systems of the stomach and duodenum lining (Ravisankar, *et al.*, 2016).

Peptic ulcer disease due to *Helicobacter pylori* affects the high percentage of the population, with an estimated frequency of 50% in the global population. It is a breach in gastric or duodenal epithelium extending beyond the muscularis mucosa layer. This results from an imbalance between mucosal protective factors (mucous production, bicarbonate secretion, and blood flow) and mucosal aggressive factors (acid and pepsin), A reduction of acid output is an integral part of complications of this disease (Salih Barik A., 2009)

H. pylori has a characteristic spiral shape and can move by using 4 to 6 flagella present at one end. *H. pylori* can produce urease that can break down

urea into carbon dioxide and ammonia. It additionally serves to neutralize the gastric acidic pH around its cover, providing it with a more beneficial environment for bacterial survival and growth. (Da Costa, *et al.* , 2015).

H. pylori infection usually does not appear any noticeable symptoms, but it may represent a significant long-term risk to the human health. Chronic infection with *H. pylori* has been connected with the development of many site-specific diseases, including gastritis, peptic ulcer disease, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma(MALT lymphoma) (Bauer and Meyer, 2011).

Epidemiological investigations indicate that *H. pylori* infects about 50% of the popular worlds, with a significant number, about 10-15%, acquiring major digestive disorders (Pachathundikandi *et al.*, 2015). *H. pylori* is a major contributor to the development of stomach cancer (Cover, 2016).

The ability to generate urease, as well as mobility and motility, are vital characteristics of *H. pylori* which enable it to survive in the stomach's extremely acidic environment (Mommersteeg, *et al.*, 2018). *H. pylori* is special in that it can release a considerable quantity of urease into the stomach, which works as a defensive mechanism toward the acidic environment. Ammonia reacts with neutrophil metabolites, leading to a release of harmful substances and an increased risk of stomach cancer. Furthermore, the urease enzyme promotes the production of pro-inflammatory cytokines, which aid in the inflammatory response in the gastric mucosa (Bruna M Roesler,*et al*.,2014)

Since the discovery of nonspecific treatment for *H. pylori didn't present* and Antibiotic resistance in *H. pylori* impedes effective eradication therapy and leads to continued prevalence of *H. pylori*. (Kim and Sung Eun, 2023) The administration of clarithromycin and levofloxacin in the treatment of this disease is important, but the development of antibiotic-resistant *H. pylori* strains increased in the past few years. Global various guidelines have been

created concerning it, promoting novel techniques for managing *H. pylori* infection, with an emphasis on resolving the problem of antibiotic resistance (Zagari, *et al.*, 2018).

1.2 Aim of the study: -

- Evaluation of new drugs candidates approved by the Food and Drug Administration (FDA) as *Helicobacter pylori* urease inhibitors, to prevent urease catalysis action to convert urea to ammonia.
- Eradication of *Helicobacter pylori* and inhibition of bacterial resistance.
- Using of Molecular Docking which has the ability to analyze large number of molcules for their possibility of binding to specific proteins with low cost ,high efficiency for drug development by reducing the time and resource required for experimental testing.

1.3 Literature Review

1.3.1 *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) was first identified in 1983 by two researchers Barry Marshall and Robin Warren. Their discovery transformed our knowledge of the etiology of peptic ulcer disease. in 2005 they were awarded the Nobel Prize (Majumdar, *et al.* , 2011).

Helicobacter pylori (*H. pylori*) is a microaerophilic gram-negative bacterium that requires low oxygen levels for survival. Firstly, known as Campylobacter pylori, and its name comes from its spiral or helical structure. *H. pylori* produces many enzymes that are important in the development of peptic ulcer disease such as urease, catalase, and oxidase. To cultivate of *H. pylori* selective media and microaerobic conditions are required, such as an environment with 80-90% N_2, 5-12% CO_2, and 5-8% O_2 at 37°C for 3-7 days. *H. pylori* may be grown on both selective and non-selected agar, such as *H. pylori* special peptone agar, Columbia base agar, and brain-heart infusion (Kalali, *et al.*, 2015; Szymczak, *et al.*, 2017). *H. pylori* is a bacterium adapted to the acidic environment in the stomach and infects almost half of the world's population.

The bacteria can be spread orally, focally, or by contaminated water and food. *H. pylori* can be a cause of chronic gastritis and peptic ulcer disease. Furthermore, it has been revealed to be crucial in the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma and distal gastric adenocarcinoma. *H. pylori* can drive stomach cell growth in the absence of sufficient apoptosis, leading to gastric carcinogenesis. Depending on the patient, different stomach morphologies generated by *H. pylori* infection may have varying clinical effects. The outcome of the infection may be significantly influenced by bacterial virulence factors (such as CagA, urease, VacA, and babA2), environmental factors (such as socioeconomic conditions, nutrition, and exposure to toxic substances), and genetic substrates

of the host (such as IL1B gene cluster and tumor necrosis factor-α (TNFα) gene polymorphism) (Gravina, *et al.*, 2018).

1.3.1.1 *Helicobacter pylori* characteristics

H. pylori is a gram-negative, microaerophilic bacteria that may lead to stomach irritation and ulcers. *H. pylori* can thus be regarded as normal flora, which most humans acquire within the first few years of childhood and then carry for life. People infected with *H. pylori* may not show symptoms at first, but when gastritis and peptic ulcer disease develop, they may feel stomach discomfort, nausea, vomiting, and dyspepsia. This activity intends to offer an overview of *H. pylori* infection diagnosis and treatment, emphasizing the significance of a coordinated interprofessional approach in managing patients with these illnesses (Parikh and Ahlawat, 2021).

H. pylori-type gram-negative bacteria are distinguished by their distinctive S-shaped or curved structure. It can move, resist the stomach's periodic contractions, and puncture the gastric membrane thanks to its two to six flagella. *H. pylori* is between 0.5 and 1.0 µm wide and 2.4 to 4.0 µm long the antrum, is the main reservoir of *H. pylori* infection. However, it does not colonize areas of the stomach with intestinal metaplasia or dysplasia. By creating an alkaline environment, the significant enzyme protein urease present in *H. pylori* enables the bacterium to survive in the acidic stomach. One of *H. pylori*'s virulence components, vacuolating cytotoxin (vacA), may be connected to several illnesses. Approximately 10-20 percent of people are immune to *H. pylori*, and the establishment of chronic infection may be influenced by host genetic factors such as ABO blood group and Lewis blood-group antigen, as well as by differences in susceptibility to certain *H. pylori* strains (Alexander *et al.*, 2021).

1.3.2 Bacterial virulence factor

In terms of virulence, *H. pylori* strains exhibit genetic variability and possess various genetically determined virulence factors. The urease enzyme, cag pathogenicity island (cag PAI), and vacuolating cytotoxin A are the three most significant variables that influence the colonization of the stomach bacteria and the chance of developing severe *H. pylori*-associated disorders figure (1-1) (Rodriguez, Urrea and Prada, 2021).

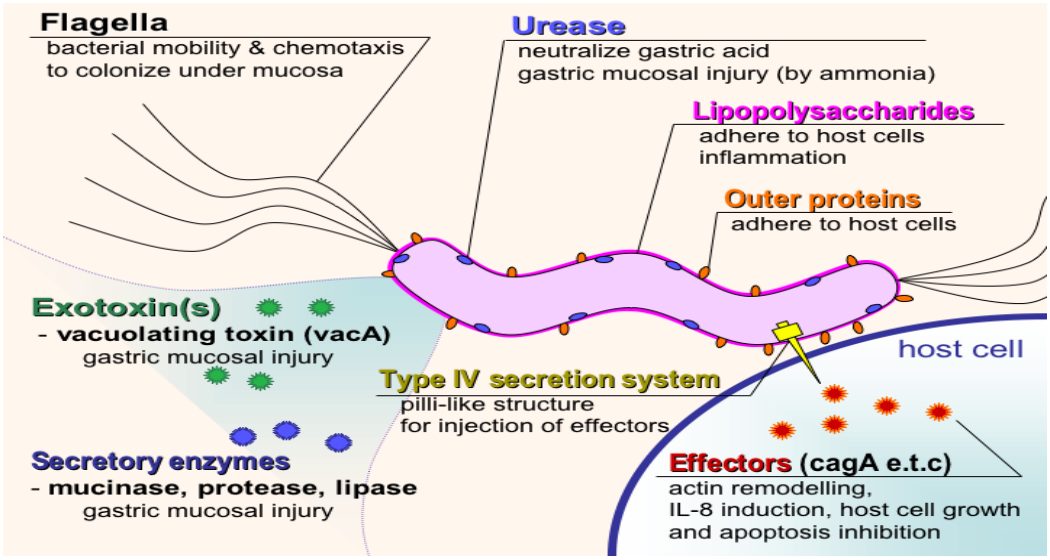


Figure 1-1: virulence, *H. pylori* (Kumar, *et al.*, 2018)

1.3.2.1 Urease enzyme

Urease is produced by numerous taxonomically diverse bacterial species, including normal flora and nonpathogens. Also, urease has been demonstrated as a potent virulence factor for some species, including Proteus mirabilis, Staphylococcus saprophyticus, and *Helicobacter pylori*. Urease is central to *H. pylori* metabolism and virulence, is necessary for its colonization of the gastric mucosa, and is a potent immunogen that elicits a vigorous immune response. *H. pylori* generates an enzyme known as urease that helps in its survival in the stomach's acidic environment. Urease converts urea to ammonia and carbon dioxide. According to research, this enzyme is required for *H. pylori* to colonize the stomach since bacteria without it cannot live in

the gastric environment. However, urease's synthesis of ammonia might be damaging to the organism. It also can harm the stomach lining and generate carcinogenic chemicals which may development of gastric cancer. Ammonia may also interfere with cellular functions such as vesicular membrane transport, protein synthesis, Adenosine triphosphate (ATP) generation, and cell cycle. Urease can also attract immune cells to the mucosa and stimulate the release of proinflammatory cytokines (Bruna M. Roesler, Rabelo-Gonçalves and Zeitune, 2014). *H. pylori* urease has a mass of 1.06 MDa and has 12 active sites containing two Ni2+ ions each. The enzyme has two subunits of masses 62 and 29.5 kDa, labeled α and β, respectively. These subunits make up the dimeric αβ subunit(Minkara, *et al.*, 2014).

The active site of the enzyme is found in the UreB subunit and comprises amino acid residues found throughout the primary structure that are brought into proximity in the tertiary structure. With a numbering specific for *H. pylori* UreB residues His-136, His-138, Lys-219, His-248, His-274, and Asp-362 come in direct contact with the two nickel ions, urea, or a water molecule within the active site. In addition, His-322 is near the active site and acts as a general base in the catalysis.

Urease (urea amidohydrolase:)catalyzes the hydrolysis of urea to yield ammonia and carbamate. The latter compound spontaneously decomposes to yield another molecule of ammonia and carbonic acid:

$$H_2N-CO-NH_2 + H_2O \xrightarrow{\text{Urease}} NH_3 + H_2N-C(O)OH$$

$$H_2N-C(O)OH + 2H_2O \rightarrow NH_3 + H_2CO_3$$

In aqueous solutions, the released carbonic acid and the two molecules of ammonia are in equilibrium with their deprotonated and protonated forms, respectively. The net effect of these reactions is an increase in pH.

$$H_2CO_3 \rightleftharpoons H^+ + HCO_3^-$$
$$2NH_3 + 2H_2O \rightleftharpoons 2NH_4^+ + 2OH^-$$

The researchers determined that the optimum pH for purified urease from *H. pylori* activity was approximately pH 8.0. However, the enzyme exhibited activity across a wide pH range, from pH 4.5 to 10.0. The study aimed to address concerns regarding the validity of the observed pH activity curve, including the pH 8.0 optimum and the absence of urease activity at pH 3.5 and below. The researchers sought to ascertain whether these results were artifacts of the enzymatic method employed to determine ammonium ion concentration.

(Rezaee, *et.al.*, 2019) study the optimization conditions of urease activity from *H. pylori* as following parameters urea concentration, pH, and temperature for maximum urease activity. The optimized parameters of 15% urea concentration, pH 5.8, and a temperature range of 46-55 °C.

The urease are inhibition by three strategy including:

- Active-Site Binding: Urease inhibitors can directly bind to the active site of the enzyme, preventing the substrate (urea) from binding and inhibiting its catalytic activity. These inhibitors often resemble the structure of the urea substrate or its hydrolysis products, such as ammonia or carbamate. By binding to the active site, they competitively inhibit the binding of urea and interfere with the enzymatic reaction (Svane, *et al.*, 2020).
- Nickel Depletion: Urease contains a nickel ion coordinated within the active site, which is essential for its catalytic activity. Some urease inhibitors work by chelating or sequestering the nickel ion, thereby depleting its availability for urease activation. Without the nickel cofactor, the enzyme is unable to perform its catalytic function effectively (Tsang and Wong, 2022).

- Allosteric Inhibition: Allosteric inhibitors bind to a site other than the active site of the enzyme, causing a conformational change that affects the active site's function. In the case of urease, allosteric inhibitors can induce structural changes that impair the enzyme's catalytic activity or substrate binding ability. These inhibitors modulate the enzyme's function by binding to allosteric sites and altering its overall conformation(Marques, *et al.*, 2017).

1.3.2.2 Vacuolating associated cytotoxin A (VacA)

Vacuolating cytotoxin A (VacA) is a key factor that plays an important role in *H. pylori* virulence and impacts various host cell pathways. This protein has been the most extensively studied cytotoxin in *H. pylori* (Matsunari *et al.*, 2016; Kabamba, Tuan and Yamaoka, 2018).

VacA is a protein that has a wide range of effects including mitochondrial failure, autophagy, membrane depolarization, activation of protein kinases leading to mitogen activation, suppression of T cell activity, and induction of apoptosis (Foegeding, *et al.*, 2016).

1.3.2.3 Cytotoxin associated gene A (cag A)

CagA is a well-studied virulence factor of *H. pylori* that is related to cytotoxins. It is encoded by the cag pathogenicity island, a 40-kb DNA segment that was horizontally acquired by the bacterium. This island encodes for a type IV secretion system, which delivers the CagA effector protein into host cells through a pilus structure (Ahn and Lee, 2015; Tegtmeyer, *et al.*, 2017). CagA contributed to the pathogenesis of *H. pylori*-associated gastric illnesses, particularly in more severe disease states like gastric cancer (Nagaich and Sharma, 2018).

1.3.2.4 Flagella Mediating Motility

Helicobacter pylori has a bundle of two to six sheathed flagella at one end of its body. Generally, either unipolar or lophotrichous, these flagella

have a terminal bulb-like structure that is a dilatation of the flagellar sheath. The length of each flagellum is about 3 µm long. The flagellar sheath, an extension of the outer membrane, shields the acid-labile flagellar structure from stomach acid. FlaA and FlaB, two flagellins that have been copolymerized, make up the distinctive sheathed flagellar filament of Helicobacter (Sycuro, *et al.*, 2010; Gu, 2017).

1.3.2.5 Adhesion Colonization of *H. pylori*

The gastrointestinal epithelium is a vital part of innate mucosal immunity, which serves as a barrier against invading pathogens. The gastric epithelium is a multilayer of mucus-covered cells that invade to form a functional stomach gland. The mucosal epithelial cells can protect the underlying tissue from infectious agents (Wroblewski and Jr, 2011).

Adhesins are key elements present on the surface of *H. pylori* and allow the bacteria to survive in the stomach environment. The adhesive molecules can attach to glycans presence on the surface of stomach epithelial cells and allowing bacteria to pass through the mucus gel and establish themselves within the host (Huang, *et al.*, 2016).

1.3.3 Diagnosis of *H. pylori*

Both invasive and non-invasive diagnostic techniques are used to identify *H. pylori* infection. Endoscopy may be utilized for invasive procedures to gather samples of stomach tissue or mucus. In invasive procedures, histology, culture, urease testing, and molecular methods are utilized, whereas non-invasive treatments use molecular analysis, urea breath testing, serology, and stool antigen testing on non-invasive material (Fonseca *et al.*, 2010; Talebi *et al.*, 2018).

1.3.3.1 Invasive test

1.3.3.1.1 biopsy-based tests

These tests involve an upper gastrointestinal endoscopy and are

typically utilized to examine gastric biopsy samples. The fast urease test, culture, histology, and polymerase chain reaction (PCR) were used for the identification of *H. pylori* infection before starting drugs intake. Endoscopic examination of treatment indications, such as ulcer disease, is also conceivable (Wang, *et al.*, 2015).

1.3.3.1.2 Rapid urease testing (RUT)

The rapid urease test (RUT) is a minimally invasive diagnostic technique used to identify *H. pylori* infection quickly. RUT is a simple, inexpensive, and easily accessible test. RUT detects the presence of the *H. pylori* urease enzyme in the biopsy sample. urease enzyme converts urea to ammonia, which increases the pH and changes the color (Eslaminejad *et al.*, 2020).

1.3.3.1.3 Bacterial culture

H. pylori culture is a specialized identification test that includes cultivating the bacterium using endoscopic biopsy samples. Rather than being used to regularly diagnose *H. pylori* infections, this method is more typically utilized for scientific study or when other diagnostic tests have failed. This test is indicated before beginning a subsequent treatment program to identify the bacterium's antibiotic resistance. Selective media and microaerophilic conditions (80-90% N2, 5-12% CO2, and 5-10% O2) are necessary for *H. pylori* to be cultivated. Bacteria typically require 5 to 7 days for incubation at 37°C on one type of culture media such as *H. pylori* agar, the Wang medium, the Wilkins-Chalgren medium, brain-heart infusion (BHI), trypticase agar bases, Columbia, and blood agar (Sabbagh, *et al.*, 2019).

1.3.3.1.4 Histological examination

Histological examination was the first method used for identifying *H. pylori* infection and continues as most effective way. The number, size, and position of the samples, staining techniques, proton pump inhibitors (PPI),

antibiotics, and the pathologist's experience can all have an impact on the accuracy of this test (Wang, *et al.*, 2015).

1.3.3.1.5 Molecular Methods

The PCR process is utilized for both invasive and non-invasive approaches to detect *H. pylori*. Molecular diagnostics have revolutionized the clinical management of many infectious diseases over the past few decades. Among these techniques, PCR is the most advanced and versatile as it has numerous clinical applications. It can identify specific or broad-spectrum pathogens, detect emerging new pathogens, perform surveillance, enable early detection of bio-threat agents, and provide antimicrobial resistance profiling (Kalali, *et al.*, 2015).

1.3.3.2 Non – invasive test

There are numerous ways to find out if you have *H. pylori* infection, including urea breath test, and serology analysis. These procedures test samples such as serum, whole blood, feces, expired air, saliva, or urine without the need for a gastroscopy (Dore and Pes, 2021).

1.3.3.2.1 Serological test

In clinical practice, detecting particular antibodies after exposure to various *H. pylori* antigens is widely regarded as a beneficial approach since it is a cost-effective, quick, and patient-acceptable method (Talebi Bezmin Abadi, 2018). When a person becomes infected with *H. pylori*, their immune system responds by producing antibodies against the bacterium. Initially, particular IgM antibody levels rise, followed by a steady rise in specific IgG and IgA antibody levels. Tests based on IgG antibodies are more accurate than tests based on IgA antibodies (Smirnova, *et al.*, 2022). *H. pylori* was detected in the blood by Enzyme-linked immunosorbent assay (ELISA), latex agglutination tests, and Western blotting can all be used to identify these antibodies. ELISA is the most widely utilized of these

procedures (Best, *et al.*, 2018).

1.3.3.2.2 Urea breath test (UBT)

The 13C-UBT is a non-invasive diagnostic approach for identifying *H. pylori* that works on a simple basis. Patients consume urea that has been tagged with either 13C or 14C. *H. pylori* creates an enzyme called urease after ingesting urea, which breaks it down into ammonia and 13C-labeled carbon dioxide. The 13C carbon dioxide created enters the circulation, travels to the lungs, and is expelled in the breath (Pichon, *et al.*, 2020).

1.3.4 Disease of the gastrointestinal tract caused by *H .pylori*

The capacity of *Helicobacter pylori* to establish and sustain long-term colonization in the human stomach is crucial for the pathogenesis of associated diseases. According to epidemiological data, *H. pylori* may be connected to several gastroduodenal conditions, including gastric cancer, gastric MALT, lymphoma, duodenal ulcers, and gastric ulcers (Sutton and Boag, 2019). An acute upper gastrointestinal disease brought on by an *H. pylori* infection can be characterized by upper abdominal discomfort that can last for 13-14 days. *H. pylori* infection has been related to the majority of stomach and duodenal ulcers. According to estimations, those infected with *H. pylori* have a 10 to 20% lifetime risk of developing ulcer disease and a 1-2% lifetime risk of developing gastric cancer (Stubljar, *et al.*, 2015).

1.3.4.1 Gastritis

The colonization of the gastric mucosa by *Helicobacter pylori* frequently triggers an immune response characterized by infiltration of inflammatory cells, leading to gastritis. (Sipponen and Maaroos, 2015). Patients often go through an acute phase of infection after first coming into contact with the bacteria, Acute inflammation spreads across the whole stomach as a result of the first immune response to *H. pylori* infection. Numerous immune cells, including neutrophils, lymphocytes, plasma cells,

and macrophages, are infiltrated during this inflammatory response. *H. pylori* binds to the surface of the gastric epithelium and produces urease, an enzyme that converts urea into ammonia and bicarbonate. These byproducts generate a protective cloud of acid resistance around *H. pylori*, enabling its survival within the gastric environment. In response to bacterial infection, the epithelial cells also release cytokines that attract inflammatory cells to the mucosa, leading to gastritis. The severity of clinical symptoms in a given population is influenced by the immune status and virulence characteristics of the infecting *H. pylori* strain. Therefore, environmental variables do not specifically influence the clinical outcome of *H. pylori* infection (Alam, *et al.*, 2020). In 10-20% of infected people, acute gastritis can evolve into chronic gastritis, which raises the risk of peptic illness and stomach cancer. Most chronic gastritis sufferers show a gradual buildup of lymphocytes, plasma cells, and B-cell proliferation that causes their condition to progress from acute to active chronic (Reyes and Peniche, 2019).

1.3.4.2 Peptic ulcer disease (PUD)

Peptic ulcer disease (PUD) is a chronic disorder that affects the stomach and small intestine. The illness can be recognized by the formation of ulcers and sores in the interior layer of the digestive tract mainly the outcome of the acid in the stomach and digestive enzymes damaging the mucosal layer. PUD is a lesion in the stomach or duodenal mucosa which affects many people in the world. The PUD may be leading to morbidity and mortality. *H. pylori* is a kind of bacteria that infects around 50% of the globe's people. *H. pylori* infects more than 70% of gastric ulcer (GU) patients and 80% of duodenal ulcer (DU) patients (Sanaei, *et al.*, 2021).

Causes of peptic ulcer is *H pylori* which is directly transmitted from person to person, the infection remains throughout life if not treated.

The bacteria may spread from mouth-to-mouth contact, GI tract illness

(particularly when vomiting occurs), contact with stool (fecal material) contaminated food and water, (Morgan DR and Crowe SE., 2016)

PUD caused by *H. pylori* requires various stages. Firstly, the bacteria adhere to the digestive epithelium and rupture the mucosal barrier. Environmental factor for *H. pylori* which include smoking alcohol NSAID caused of chronic gastritis, low acid production lead to pangastritis and atrophic gastritis which lead to gastric cancer. High acid production lead to antral-predominant gastritis and peptic ulcer disease as show in figure (1-2). (Dawood and Mamdooh, 2021)

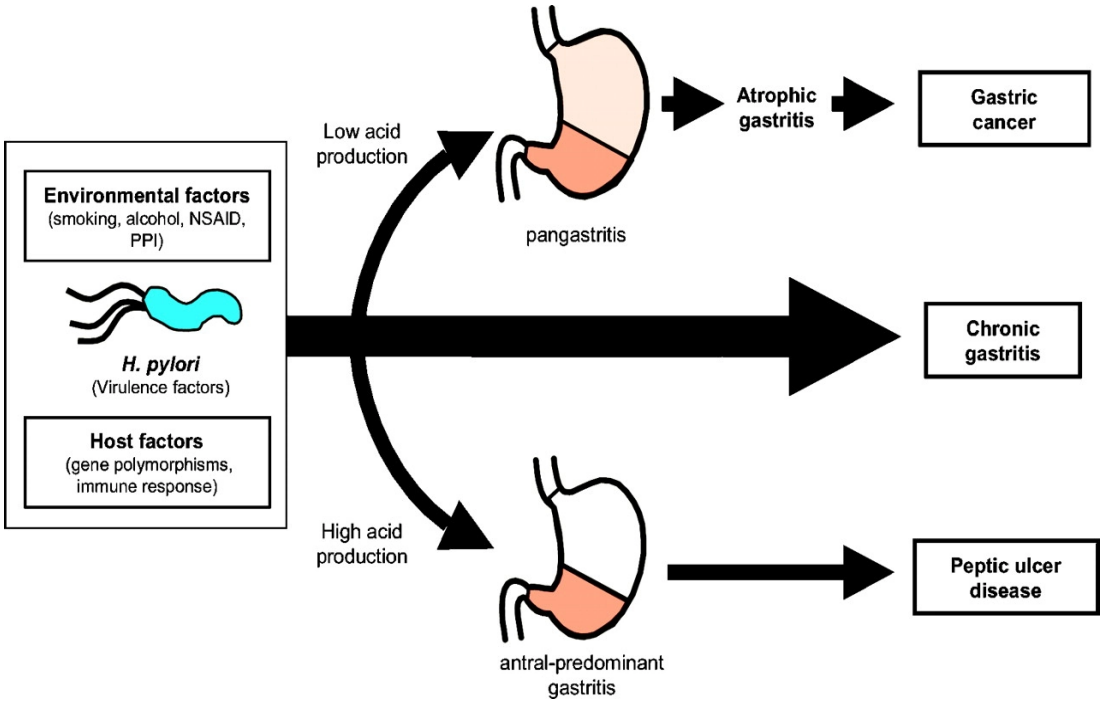


Figure 1-2 representation of the factors contributing to gastric pathology and disease outcomes in *H. pylori* infection (Dawood and Mamdooh, 2021).

The cytokines attract neutrophils and other immune cells to the site of infection, which leads to inflammation. Chronic inflammation induces damage to the stomach mucosa, resulting in ulcers formation which can cause bleeding, perforation, or obstruction. Moreover, *H. pylori* was shown to prevent the formation of prostaglandins, which are important to the preservation or repair of stomach mucosal integrity (Kishikawa, *et al*., 2020 .

H. pylori can survive in stomach acid by secreting enzymes that neutralize the acidic environment (Shamsuddeen, *et al.*, 2011). The distribution of gastritis in the stomach during *H. pylori* infection plays a critical role in the progression of stomach ulcer disease. Duodenal ulcers have been linked to high acid secretion and antrum-predominant gastritis, whereas stomach ulcers and cancer are linked to low acid production and corpus-predominant gastritis. Long-term use of Non-steroidal anti-inflammatory drugs (NSAIDs) like aspirin and ibuprofen may cause or exacerbate ulcers. *H. pylori* infection is a substantial risk factor for the development of peptic illness. While neither stress nor spicy food directly causes peptic ulcers, they might worsen their symptoms. Alcohol use and smoking can both exacerbate ulcers and slow the recovery process (Lanas and Chan, 2017).

1.3.5 Clinical features of peptic ulcer

Peptic ulcer disease (PUD) can cause a range of clinical features, which can vary in severity depending on the location and extent of the ulcer (Shell, 2021).

(PUD) is characterized by symptoms including epigastric pain, upper abdominal discomfort, gas, and feeling of fullness nausea and vomiting, (Shapiro,*et al* 2023) , Complications from PUD include bleeding, perforation, Bleeding can cause hematemesis (blood vomiting) or melena (black, tarry stools). Perforation can cause severe stomach discomfort, fever, and indications of peritonitis, the ulcer can develop and perforate, when gastric juice and gas enter the peritoneal cavity and cause chemical peritonitis, which leads to suffering extreme pain and increasing heart rate (Chung and Shelat, 2017).

1.3.6 peptic ulcer Treatment

Acid suppressants and antibiotics can be used together to completely eradication the bacteria, but antibiotic resistance has become a serious issue

that may impair the effectiveness of treatment (Godavarthy and Puli, 2023).

Drugs used for treatment of peptic ulcer included:

1- Antibiotics: Chronic infection with *H pylori* is present in most patients with recurrent peptic ulcers. Eradication of this organism greatly reduces the rate of recurrence of ulcer in these patients. One regimen of choice consists of a proton pump inhibitor plus a course of clarithromycin and amoxicillin (or metronidazole in patients with penicillin allergy). (katzung, 2018)

This combination of the drug is highly cost, broad spectrum use when occurring of resistant to other medication, this regimen has multiple side effects such as an increase in the liver enzyme ratio (AST/ALT) Asthenia, abdominal pain, nausea, and diarrhea, Abnormal feces, Dysgeusia, vaginal infection (Graham, D.Y., Dore, M.P. and Lu, H., 2018).

2. Antacids: Antacids are weak bases that neutralize stomach acid by reacting with protons in the lumen of the gut and may also stimulate the protective functions of the gastric mucosa. When used regularly in the large doses needed to significantly raise the stomach pH, antacids reduce the recurrence rate of peptic ulcers.

Popular antacids include **magnesium hydroxide** ($Mg[OH]_2$) and **aluminum hydroxide** ($Al[OH]_3$). Neither of these weak bases is significantly absorbed from the bowel. Magnesium hydroxide has a strong laxative effect.

3. ***H_2-receptor antagonists:*** **Cimetidine** and other H_2 antagonists (**ranitidine**, **famotidine**, inhibit stomach acid production, especially at night. They are effective in the treatment of, peptic ulcer disease, Although they are still used widely, their clinical use is being supplanted by the more effective and equally safe proton pump inhibitors.

4. Proton pump inhibitors: **Omeprazole** and other proton pump inhibitors (**esomeprazole, lansoprazole, pantoprazole,** and **rabeprazole**) are lipophilic

weak bases that diffuse into the parietal cell. There they undergo conversion to compounds that irreversibly inactivate the parietal cell H^+/K^+ ATPase, the transporter that is primarily responsible for producing stomach acid. Oral formulations of these drugs are enteric coated to prevent acid inactivation in the stomach. After absorption in the intestine, they are rapidly metabolized in the liver, with half-lives of 1–2 h. However, their durations of action are approximately 24 h, and they may require 3–4 d of treatment to achieve their full effectiveness.

Proton pump inhibitors are more effective than H_2 antagonists for peptic ulcer. Chronic treatment with proton pump inhibitors may result in hypergastrinemia. The use of these drugs increases the incidence of carcinoid or colon cancer.

Proton pump inhibitors may decrease the oral bioavailability of vitamin B_{12} and certain drugs that require acidity for their gastrointestinal absorption (eg, digoxin, ketoconazole). Patients taking proton pump inhibitors may have a small increase in the risk of respiratory and enteric infections.

5. ***Sucralfate*****—**An aluminum sucrose sulfate, sucralfate is a small, poorly soluble molecule that polymerizes in the acid environment of the stomach. The polymer bind to injured tissiue and forms a protective coating over ulcer beds. Sucralfate accelerates the healing of peptic ulcers and reduces the recurrence rate. Unfortunately, sucralfate must be taken 4 times daily. Sucralfate is too insoluble to have significant systemic effects when taken by the oral route; toxicity is very low.

6. ***Misoprostol*****—**An analog of PGE_1, misoprostol increases mucosal protection and inhibits acid secretion. It is effective in reducing the risk of peptic ulcers but is not widely used because of the need for multiple daily

dosing and poorly tolerated adverse effects (gastrointestinal upset and diarrhea

***7. Colloidal bismuth*—**Bismuth has multiple actions, including formation of a protective coating on ulcerated tissue, stimulation of mucosal protective mechanisms, direct antimicrobial effects, and sequestration of enterotoxins. Bismuth subsalicylate, a nonprescription formulation of bismuth and salicylate, reduces stool frequency and liquidity in infectious diarrhea. Bismuth causes black stools.

1.3.7 Molecular docking

Molecular docking is an automated technique for simulating the interaction between a drug and a receptor molecule. It is a crucial method in the development and discovery of drugs, it allows researchers to find possible drug candidates that may bind to and influence the activity of a certain target protein. The molecular docking process depends on the binding mechanism and affinity of a ligand molecule with a target receptor molecule.

This is accomplished by modeling the interaction of two molecules using many of theoretical techniques and software tools (Naqvi, *et al.*, 2018).

The benefits of molecular docking are the ability to analyze a large number of molecules for their possibility of binding to specific proteins. the molecular docking is considered a low-cost, high-efficiency approach for drug development by reducing the time and resources required for experimental testing. (García-Ortegón, *et al.*, 2022).

The docking method for predicting the interactions of a small molecule (the ligand) with a larger molecule (the receptor), such as a protein. It may be a valuable method for discovering prospective urease inhibitors. Docking-based screening entails docking a library of compounds into the urease active site and assessing each compound's binding affinity. Overall, molecular docking is a useful method for finding possible urease inhibitors and driving

the development of novel antimicrobial medicines (Kafarski and Talma, 2018).

1.3.8 Aminoglycosides as *H. pylori* urease Inhibitors *in silico*

1.3.8.1 Paromomycin sulfate

The Paromomycin sulfate, an aminoglycoside antibiotic produced through Streptomyces rimosus fermentation, is employed to treat specific bacterial infections by inhibiting bacterial protein synthesis. It has a molecular formula of $C_{23}H_{45}N_5O_{14}$ and a molecular weight of approximately 615.6 g/mol. exhibit the spectrum of activity that is often associated with aminoglycoside antibiotics, are stable, and are not absorbed after oral administration. For the pre- or postoperative decrease of the intestinal flora, can be administered orally to control intestinal infections by susceptible organisms. These antibiotics function as an efficient supplementary therapy for hepatic coma because they lower the number of ammonia-forming bacteria in the digestive tract. (Shilling and Erin N., 2021)

Paromomycin sulfate is a white crystalline powder, readily soluble in water but insoluble in alcohol and ether (Chen, *et al.*, 2023). Pharmacokinetically, paromomycin has limited distribution in the body, primarily localized in the gastrointestinal tract(Leder and Weller, 2015). It's not orally absorbed and requires non-oral administration methods. It is not metabolized in the liver and is primarily eliminated unchanged through glomerular filtration in the kidneys, with a half-life of approximately 1-2 hours (Kip, *et al.*, 2018).

1.3.8.2 Tobramycin

Tobramycin, an aminoglycoside antibiotic derived from streptomyces tenebrous, is used to treat bacterial infections, particularly those caused by gram-negative bacteria. It acts by inhibiting protein synthesis in bacterial cells, leading to their demise(Tsakou, *et al.*, 2020). Tobramycin is a complex

organic compound with a molecular formula of $C_{18}H_{37}N_5O_9$, consisting of a six-membered ring with amino groups, hydroxyl groups, and a glycoside bond connecting a sugar molecule. It's a water-soluble white crystalline powder with a slightly bitter taste, and a molecular weight of 467.5 g/mol, (da Silva Medeiros, *et al.*, 2021).

Pharmacokinetically, it's quickly absorbed after intramuscular injection, reaching peak serum concentrations within 30 to 90 minutes. Tobramycin is primarily eliminated by the kidneys, with a serum half-life of 2 hours in adults and 4.5 to 8.7 hours in newborns(Reyhanoglu and Reddivari, 2019).

1.3.8.3 Capreomycin

Capreomycin, a.cyclic peptide derived from Streptomyces capreolus, serves as a crucial antibiotic for treating multidrug-resistant tuberculosis (MDR-TB) and various mycobacterial infections. It is typically employed alongside other medications like ethionamide and fluoroquinolones to combat MDR-TB, a drug-resistant form of tuberculosis. Additionally, Capreomycin finds application in addressing infections caused by Mycobacterium avium complex and Mycobacterium kansasii (Liu, *et al.*, 2018). This polypeptide consists of four amino acids in a cyclopeptide ring, boasting a chemical formula of $C_{25}H_{44}N_{14}O_8$ and a molecular weight of 668.7 g/mol(Information National Center for Biotechnology, 2023). It effectively hinders bacterial protein synthesis by binding to specific ribosomal subunits. (Vianna, *et al.*, 2019).

Administered intramuscularly or intravenously, Capreomycin is rapidly absorbed, widely distributed throughout the body, and primarily excreted in the urine, with renal function affecting clearance(Zuur, *et al.*, 2018; Bahuguna and Rawat, 2020).

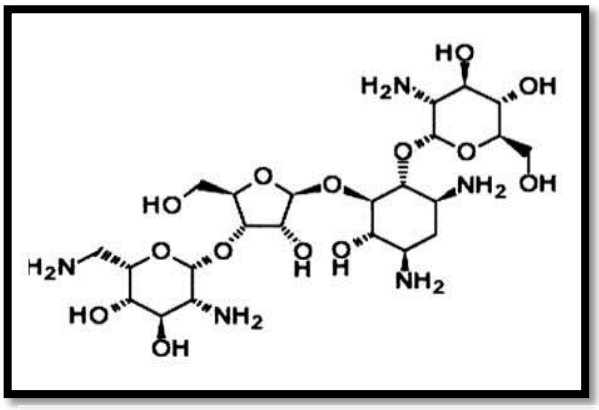
1.3.8.4 Amikacin

Amikacin, a synthetic aminoglycoside antibiotic, is utilized for treating a wide range of bacterial infections affecting the respiratory system, urinary

tract, bones, joints, skin, and soft tissues. It plays a crucial role in combating multidrug-resistant infections due to its broad-spectrum activity (Anandabaskar, 2021). This antibiotic is soluble in water, heat-stable up to 60°C for brief periods, but sensitive to alkaline solutions (Martínez Illamola, 2013; Corrêa *etal.*, 2020; Dagur, *et al.*, 2023). Its pharmacokinetics involve parenteral administration, wide distribution in the body, and primarily renal elimination. The chemical formula of Amikacin is $C_{22}H_{43}N_5O_{13}$ and its molecular weight is 585.60 g/mol (sadeghi, *et al.*, 2018)

1.3.8.5 Gentamicin

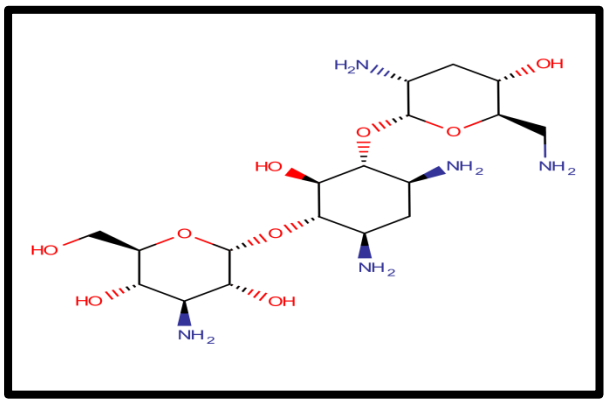
Gentamicin, an aminoglycoside antibiotic derived from Micromonospora purpurea, is employed in the treatment of bacterial infections, particularly aerobic gram-negative bacteria (Byrn, *et al.*, 2022). Administered primarily through parenteral routes due to minimal gastrointestinal absorption, (Choubey,2022). chemical formula of $C_{21}H_{43}N_5O_7$ and a molecular weight of 477.60 g/mol Pharmacokinetically, it exhibits rapid distribution but limited penetration of certain tissues due to the blood-brain barrier, with renal elimination and a half-life of 2-3 hours in healthy individuals (Crcek, *et al.*, 2019; He Sha, *et al.*, 2022). Gentamicin is mostly used parenterally to treat severe gram-negative infections brought on by sensitive organisms, while it is also available in formulations (0.1%) for topical application.



Paromomycin

IC_{50} for local strain=66.85uM

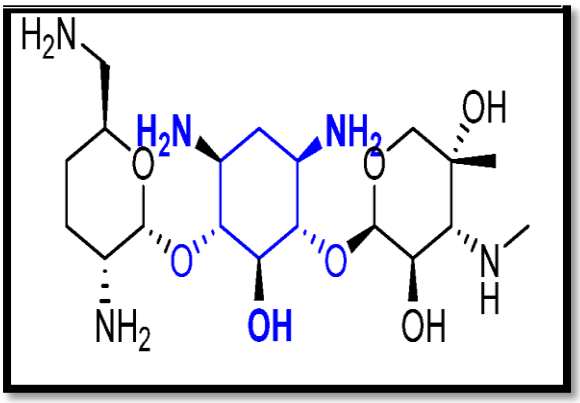
IC_{50} for Reference strain=21.55uM



Tobramycin

IC_{50} for local strain=82.7uM

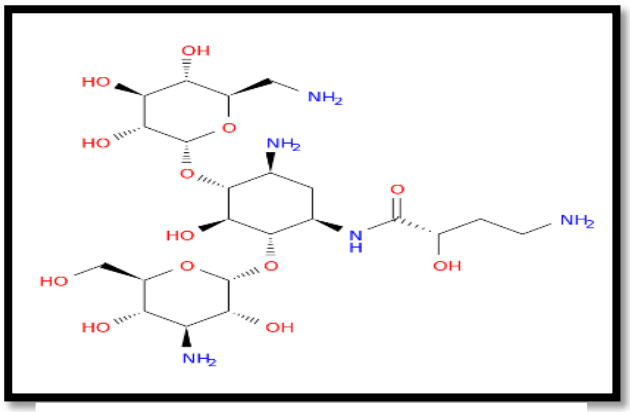
IC_{50} for Reference strain=35.8uM



Gentamicin

IC_{50} for local strain=85.97uM

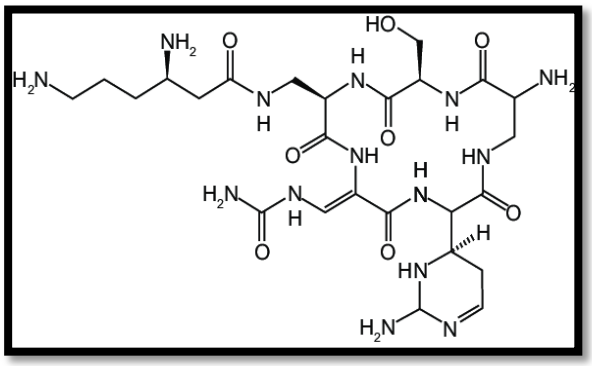
IC_{50} for Reference strain=41.91uM



Amikacin

IC_{50} for local strain=51.7uM

IC_{50} for Reference strain=22.14uM



Capreomycin

IC_{50} for local strain=48.09uM

IC_{50} for Reference strain=19.35uM

Figure 1-3: The Chemical structures of Aminoglycosides (Wilson,2004)

1.3.9 Aminoglycoside mechanism of action

Aminoglycosides have a wide antibacterial spectrum, rapidly acting bactericidal activity, and concentration dependent killing involves irreversible binding to the bacterial ribosomal 30S subunit. Specifically, they are believed to bind to the A-site (aminoacyl) on the 16S rRNA, a component of the ribosomal 30S subunit Through this binding, the genetic code gets misread, and the translation is disrupted, leading to the bacterial inhibiting protein synthesis, aminoglycoside also has post antibiotic effect in which residual bactericidal effect persists even after serum concentration has fallen below minimum inhibitory concentration aminoglycoside diffuse across the outer coat of gram negative bacteria through porin channel entry cytoplasmic membrane (passive diffusion), further transport across cytoplasmic membrane takes place by active transport by proton pump an oxygen dependent process (Schwarz*, et al., 2022).*

1.3.10Aminoglycoside Adverse Effects

1. Ototoxicity: Ototoxicity (vestibular and auditory) is directly related to high peak plasma concentrations and the duration of treatment. Aminoglycosides accumulate in the endolymph and perilymph of the inner ear. Deafness may be irreversible and has been known to affect developing fetuses. Vertigo (especially in patients receiving streptomycin) may also occur. The important risk factors for ototoxicity are:

a. Elderly patients.

b. Repeated courses of aminoglycosides.

c. Patients with pre-existing auditory impairment.

d. Concurrent use of other ototoxic drugs such as cisplatin, vancomycin, minocycline, loop diuretic.(Leis JA *et al* 2015).

2. Nephrotoxicity: Retention of the aminoglycosides by the proximal tubular cells disrupts calcium-mediated transport processes. This results in kidney

damage ranging from mild, reversible renal impairment to severe, potentially irreversible acute tubular necrosis. (Wargo KA and Edwards JD,2014).

3. Neuromuscular paralysis: This adverse effect is associated with a rapid increase in concentration (for example, high doses infused over a short period) or concurrent administration with neuromuscular blockers. Patients with myasthenia gravis are particularly at risk. Prompt administration of calcium gluconate or neostigmine can reverse the block that causes neuromuscular paralysis.(LiuM,*et al* 2001).

1.3.11 Aminoglycoside clinical use

- Serious, life threatening gram negative bacteria such as pseudomonas, enterobacter,acinebacter, *Escherichia coli, Klebsiella pneumoniae*
- Complicated skin, bone, or soft tissue infection
- Have good activity against pseudomonas aeruginosa especially in combination with pencicillins
- Sepsis
- Endocarditis
- Mycobacterium *tuberculosis,*
- Ocular infection (topical)
- Otitis externa (topical)
- Complicated urinary tract infection
- Peritonitis and other sever intraabdominal infection.(Pagkalis S, *et al.*, 2011)

Chapter two
Materials and methods

2. Materials and Methods

2.1. Materials, Equipment, and Instruments

2.1.1. Equipment and Instruments

The equipment used in this study is listed in Table (2-1)

Table (2-1): Equipment and instruments

No	Equipment and instruments	Company/ Origin
1	Anaerobic jar	Local
2	Autoclave	Haramaya / Japan
3	Deep freezer	Stuart Scientific / UK
4	Digital camera	Sony / Japan
5	Dialysis bags	Viskase / USA
6	Electrophoresis	Thermo /USA
7	Eppendorf tube	Sterillin LTD/UK
8	Eppendorf centrifuge	Hitich /Germany
9	Eppendorf tubes	Eppendorf /Germany
10	Hood	Fisher scientific/ Germany
11	Incubator	Memmert/ Germany
12	Light microscope	Olympus /Japan
13	Microwave	Binder /Germany
14	Millipore filters 0.20 μm	Sartorious membrane /Germany
15	Oven	Memmert/ Germany
16	Petri dish	Sterilin/ England
17	PH meter	Radiometer/ U.K
18	Plain tubes	DMD-DIDPO / Syria
19	Quantus Florometer	Promega /USA
20	Refrigerator	Concord/ Lebanon
21	Binary high-pressure gradient	Knuaer / Germany

	pump	
22	Diode array detector	Knuaer / Germany
23	Sample loop (1000 µl) and injector	Knuaer /Germany
24	Fraction collector	Teledyne Isco/ USA
25	Analyses and system control software	Dataapex/ Czech Republic
26	Sensitive balance	Sartorius / Germany
27	Ultrasonic bath	Bandelin/ Germany
28	Spectrophotometer	Hitachi/Japan
29	Microplate reader	Biotek/Germany
30	Thermal Cycler	BioRad/ USA
31	UV- Tran illuminator	Accuris/USA
32 a	Vortex	Quality Lab System/ England
33	Water bath	Memmert / Germany
34	Water distillater	GFL/ Germany

2.1.2. Chemical and Reagents

Chemical and reagents that are used in this study are listed in Table (2-2)

Table (2-2) Chemical and Reagents

No	Chemical and reagents	Company/ Origin
1.	Agarose	AppliChem / Germany
2.	Ammonium sulfate	BDH / England
3.	Bovine Serum Albumin (BSA)	Sigma-Aldrich /USA
4.	Coomassie Brilliant Blue	Sigma-Aldrich /USA
5.	Disodium dihydrogen Phosphate	BDH / England
6.	DNA Ladder	Bionet / Korea
7.	DNA loading dye	Bionet / Korea
8.	EDTA	BDH / England

9.	Ethanol 90%	NHF(National health factory) /Iraq
10.	Ethidium bromide (10mg / ml)	Promega/U.S.A
11.	Microaerophilic bag	Oxoid/ Japan
12.	Glycerol	Fluka/Switzerland
13.	Normal saline	Pioneer/Iraq
14.	Nuclease free water	Promega /USA
15.	phosphoric acid	Merck/ Germany
16.	Primers	Macrogen Company /Korea
17.	sodium dihydrogen Phosphate	BDH / England
18.	sodium hydroxide	Panreac / Spain
19.	sodium hypochlorite	Fluka / Switzerland
20.	sodium nitroprusside	Merk/ Germany
21.	sodium salicylate	Fluka / Switzerland
22.	TAE 40X	Promega/ U.S.A
23.	Urea supplement	Himedia , India

2.1.3 kits

The Kit that is used in this study are listed in Table (2-3)

Table (2-3) kit used in this study

No	Kit	Company/origin
1.	Master mix	Promega / USA
2.	*H. pylori* antigen rapid test kit	LimiQueq, USA
3.	DNA Extraction kit	ABIOpure / USA

2.1.4 Cultures Media

The culture media used in this study is listed in Table 2-4

Table 2-4: Culture media used in this study

No	Culture media	Company/origin
1.	Blood agar base	**Himedia /India**
2.	Brain–heart infusion broth	**Himedia / India**
3.	Brain–heart infusion agar	**Himedia / India**
4.	Urea agar base	**Himedia / India**

2.1.5 Antibiotics

All antibiotics used in this study are listed in Table (2-4).

Table (2-5) types of antibiotic and supplement

Group	Purpose	Name	Company/origin
1.	Antibiotic used for inhibition of urease enzyme of *Helicobacter pylori*	Paromomycin	Macklen/China
		Topramycin	Macklen/China
		Amikacin	Macklen/China
		Capreomycin	Macklen/China
		Gentamycin	Macklen/China
2.	Skirrow selective supplement	Vancomycin	Himedia
		Trimethoprim	
		Cefsulodin	
		Amphotericin B	

2.2. Experimental design

The experimental design was illustrated in Figure (2 – 1)

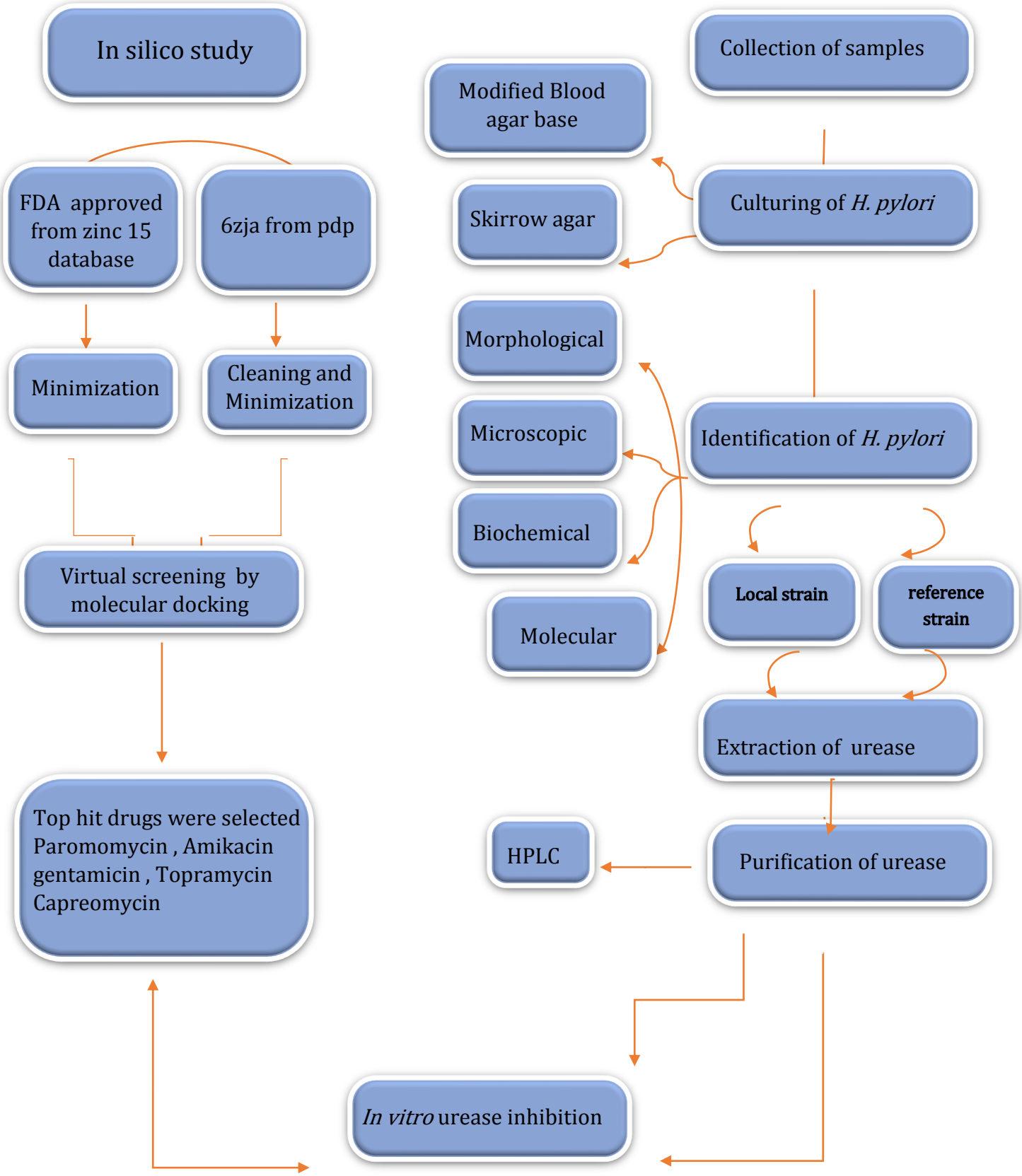


Figure (2 – 1) scheme of experimental design

2.3 Methods

2.3.1 Sterilization methods

Moist heat sterilization is used for the sterilization of media and other solutions not affected by 121°C autoclave heating at a 15 bar/in2 pressure for 15 minutes. Sterilization of the oven at 160-180 ºC for 3-2 hours, used for dry sterilization of glassware (Harrigan and McCance, 2014).

2.3.2 Solutions and Reagents

2.3.2.1 Solutions

2.3.2.1.1 Solution for urease assay

The urease assay solution was prepared according to (Richmond and Yep, 2019)

A) Chromogen Buffer

The chromogen buffer contains : (phosphate buffer 20 mmol/L ,EDTA 2 mmol/L ,sodium salycilate 60mmol/L ,sodium nitroprusside 3.4 mmol/L)

It was prepared as follows; a 100 ml volumetric flask was chosen to accommodate 80 mL of distilled water. The initial step involved adding precisely 30.98 mg of sodium dihydrogen Phosphate to the distilled water. Subsequently, 11.7 mg of Sodium Phosphate was carefully introduced into the solution 58.45 mg of Ethylenediaminetetraacetic acid (EDTA) was added 960.66 mg of sodium salicylate was added finally add 10.13 mg of sodium nitroprusside wait until fully dissolved. Distilled water was then added incrementally until the total volume reached 100 mL. (Richmond and Yep, 2019).

b) alkaline Hypochlorite

Comprising sodium hypochlorite (10mmol/L) and NaOH (150 mmol/L)

Weigh out 74.44 mg of sodium hypochlorite and 600 mg of NaOH using an analytical balance. Then transfer to a volumetric flask. Add distilled

water to a volumetric flask and stir the mixture until is fully dissolved. Then complete volume to 100 mL.

2.3.2.1.2 Solutions Used for Estimation of Protein Concentration

(Bradford, 1976)

A. Phosphoric Acid (85%)

To prepare phosphoric acid (85%), 85 ml of phosphoric acid was combined with 15 ml of distilled water**.**

B. Sodium Hydroxide (1M)

A solution of sodium hydroxide (1M) was obtained by dissolving 4g of sodium hydroxide in 100ml of distilled water.

C. Coomassie Brilliant Blue G-250 Stain

To prepare the Coomassie Brilliant Blue G-250 stain, 0.1g of Coomassie Brilliant Blue G-250 was dissolved in a mixture of 100 ml of 85% phosphoric acid and 50 ml of absolute ethanol. The solution was then brought to a total volume of 1 liter using distilled water. The resulting solution was filtered through Whatman No.1 filter paper and stored in a dark container.

D. Bovine Serum Albumin (BSA) (1 mg/ml)

To prepare a Bovine Serum Albumin (BSA) solution with a concentration of 1 mg/ml, 0.1g of BSA was dissolved in 100 ml of distilled water. From this stock solution, various concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 mg/mL) were prepared by diluting the stock solution with distilled water.

2.3.2.1.3. Phosphate Buffer

Was prepared from Solution (A) It was prepared by dissolving 2.78 g of sodium dihydrogen Phosphate (NaH2PO4) in 200ml D.W.

Solution (B) It was prepared by dissolving 7.16 g of Disodium hydrogen phosphate (Na2 HPO4 .12H2O) in 200 ml D.W.

Then 78 ml of solution A were mixed with 122 ml of solution B and the volume was completed to 400 ml with D.W. The pH was adjusted to 7.2. It was subsequently autoclaved for 15 minutes and stored at 4ºC

2.3.2.2 Reagent

2.3.2.2.1 Catalase reagent

The reagent for recognizing bacterial capability to produce the catalase enzyme was prepared in a 3% concentration. This was achieved by adding 3% of H2O2 (hydrogen peroxide) to 100 ml of distilled water (D.W.). The resulting solution was then stored in a dark bottle to protect it from light (Forbes, *et al.*, 2007)

2.3.2.2.2. Oxidase reagent

The reagent was promptly prepared by dissolving 0.1g of tetramethyl-p-phenyl diamine dihydrochloride in 10 ml of distilled water. The resulting solution was then transferred to a dark bottle and stored in a cold place to maintain its stability and integrity (Yılmaz, *et al.*, 2019).

2.3.3. Preparation of Culture Media

2.3.3.1. Ready-Culture Media

Ready-made culture media were prepared according to the manufacturing company instructions, while the other culture media were prepared in the laboratory according to the scientific references. All these media (with some exceptions) were autoclaved at 121°C for 15 minutes at 15 pounds per square inch (Brown and Smith, 2014).

2.3.3.2 Laboratory Prepared Culture Media

2.3.3.2.1 Modified blood agar base (selective media)

Forty-five (45) grams of blood agar media were taken and suspended in one litter of D.W and sterilized in an autoclave (121 C° for 20 minutes), and after that left to cool to 45 C° and Skirrow selective supplement was added to it (figure 2-1) which consist of antibiotic.

- Amphotericin B 2.5 mg/ ml
- Trimethoprim 5 mg/ml
- Vancomycin 10 mg/ml
- And 10% of human blood was added, mixed well then poured into a petri dish (25 ml/one petri dish), leaving the media to cool and then saved in the refrigerator for use.

Figure 2-2 Modified blood agar base

2.3.3.2.2 Skirrow media

This media was prepared by dissolving 37 gm of brain heart infusion broth in 1000 ml of D.W, sterilized by autoclave (121C° for 20 min), left to cool then added Human blood (5%), Skirrow supplement consisting of Amphotericin B 2.5 mg/ ml, Trimethoprim 5 mg/ml and Vancomycin 10

mg/ml and poured into sterile Petri dishes, which were then stored at 4°C until used

2.3.3.2.3 Chocolate agar

This medium was made by dissolving 40 grams of blood agar base in 1000 ml of sterile water pH was adjusted to 7 and sterilizing it at 121 C° for 15 min, according to the manufacturer's instructions. After cooling to 45°C, 5% human blood was added to the media and then heated with continued swirling until the color shifted to dark brown. and poured into sterile Petri dishes, which were then stored at 4°C until used (MacFaddin, 2000).

2.3.3.2.4 Urea agar base

It was prepared by dissolving 60 gm of urea agar base in 100 ml of D.W, sterilized by autoclave (121C° for 20 min), left to cool and one vial of urea supplement (5 ml) was added to it, mixed well, and poured in the sterile plain tube (5 ml/ tube) and saved in the refrigerator until its used. This media was used to test the ability of bacteria to produce urease enzymes (Forbes, *et al.*, 2007).

2.3.3.2.5 Brain heart infusion broth

This media was prepared by dissolving 37 gm of brain heart infusion broth in 1000 ml of D.W, sterilized by autoclave (121C° for 20 min), left to cool, mixed well, and poured into the sterile plain tube (5 ml/ tube), and saved in the refrigerator until it's used. This media was used to activate bacteria as well as preserve of biopsy through taken in endoscopic unit for culturing in the microbiology lab (MacFaddin, 2000).

2.3.3.2.6 Brain heart infusion broth supplemented with 10% blood

This media was prepared by dissolving 37 gm of brain heart infusion broth in 1000 ml of D.W, sterilized by autoclave (121C° for 20 min), left to cool to 45 C° then 10% of human blood was added mixed well saved in refrigerator until its used (Matongo and Nwodo, 2014).

2.3.3.2.7 Preserved media

This media was prepared in the same way as brain heart infusion broth 3.7 g in 80 ml od D.W then adding of glycerol (20 ml) and poured in the sterile plain tube (5 ml/ tube) and saved in the refrigerator until it was used. This media was used to preserve bacteria for a long time (MacFaddin, 2000)

2.3.4 Ethical approval

This study was appended by the committee of publication ethics at the College of medicine, University of Babylon, Iraq. The study protocol and the subject information and the consent form were reviewed and approved by a local ethics committee according to the document number 4-3, at 06/07/2022 to get this approval.

2.3.5 Collection of samples

During the period (August 2022 to November 2022). A total number seventy 70 patients' samples (biopsy and stool) were collected directly from persons in endoscopic unit in Karbala center for Gastroenterology and Hepatology in Karbala at Imam Al-Hussein Medical-City. The collected data include the *H. pylori antigen* test. This study includes collection of biopsy samples to isolation and identification of *H. pylori* .

2.3.5.1 Stool samples

stool samples (70 samples) were collected in plastic sterile containers.

2.3.5.1.1 Stool antigen test

Stool antigen tests (SATs) are noninvasive diagnostic modules for *H. pylori* infection Early SATs use immunochromatography (ICA) based on polyclonal antibodies as in the following steps:

1) A Proximally 0.25 gram of stool was transported by a wooden stick into a tube containing the buffer solution to prepare a suspension which is used to mitigate the sample
2) The sample was mixed well before being dispensed in a card

3) The outer cover of the card was removed upward before the direct use.
4) Two drops of suspension solution are added to the circular region of the card.
5) The results read within 15 minutes

Figure (2-3) Rapid antigen detection test for *H. pylori*, C represents the control band and T represents the test band

2.3.5.2. Biopsy samples:

Small pieces of the gastric biopsy were collected directly in an endoscopic unit in the tube containing brain heart infusion broth. The tube was preserved in cool pox and transported to the microbiology lab within two hours, the tube was used to activate and cultivation bacteria.

2.3.5.3 Culturing of *H. pylori* local isolate

The antral biopsy specimens were transported to the microbiology laboratory immediately within less than two hours in 2-2.5 mL brain heart infusion broth as a transport medium. Incubated at 37 C° for 24 hours then subculture on modified blood agar base and Skirrow agar incubation at 37C° in microaerophilic condition by microaerophilic bag (figure 2-4) generate for 3-7 days (Colle, *et al.*, 1996).

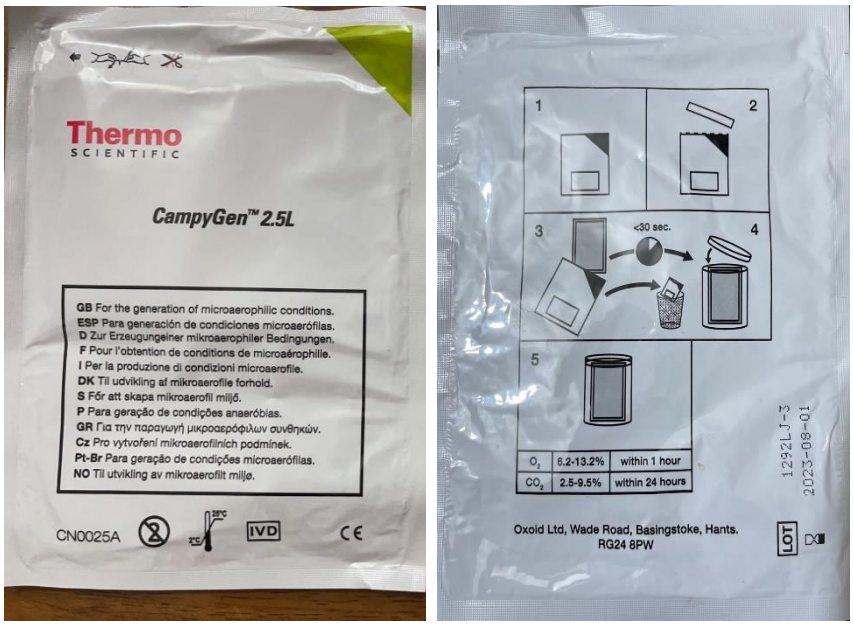


Figure 2-4: Microaerophilic bag generated of microaerophilic condition for culturing of *Helicobacter pylori*

2.3.5.4 Culturing of one Reference strain *H. pylori* NCTC 11916

The growth promotion of reference strain NCTC11916 according to as recommended manufacturer.(Chauhan.,*et al.* 2020)

- The outside of the ampoule (Figure 2-5) was cleaned using tissue soaked in 70% alcohol.
- The ampoule was wrapped in a tissue soaked in 70% alcohol.
- Then, it was wrapped with paper towels, tissue, or gauze to act as a padding layer. This was done to prevent accidental injury when the ampoule was snapped open.
- The ampoule was snapped while wrapped at the score mark.
- The ampoule was carefully unwrapped as fragments of glass may have been present in the tissue. The tissue and the ampoule tip were discarded into a sharps bin.
- If the plug did not come away with the tip of the ampoule before reconstituting the culture, the plug was removed with forceps.

- To reconstitute, approximately 0.5ml of broth (figure 2-6), when necessary enriched with blood, was transferred to the ampoule.
- The microorganisms were allowed to rehydrate for 5-10 min. They were mixed very carefully.
- Finally, subculture was performed onto modified blood agar.

Figure 2-5 The reference strain *H. pylori* NCTC 11916 ampoule

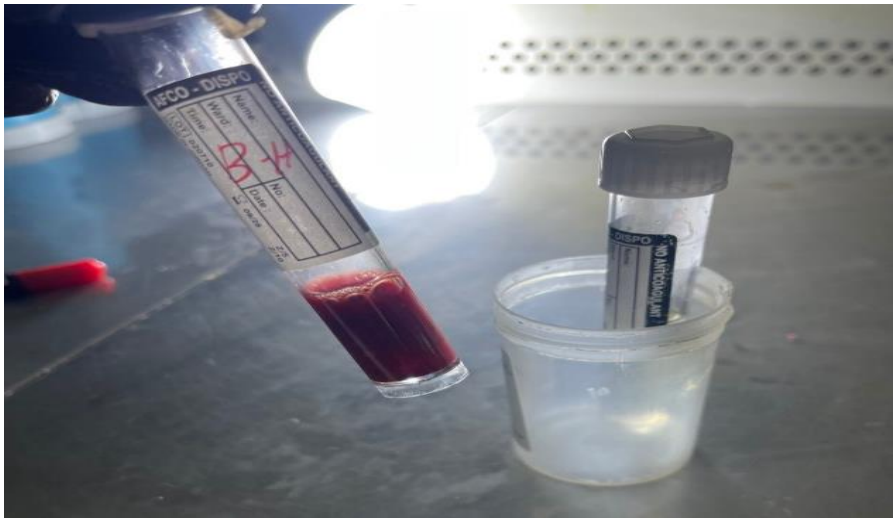


Figure 2-6: Blood which was added to the reference strain

2.3.6 Identification of *H. pylori*

2.3.6.1.Morphological identification of *H. pylori*

This is dependent on the physical characteristics of colonies, such as color, size, form, and growth conditions, as well as microscopic examinations such as Gram staining, bacterial cell size and shape, and cell arrangement. The results were compared with Brooks, *et al.*, (2010)

2.3.6.2.Biochemical diagnostic test of *H. pylori*

2.3.6.2.1.Gram stain

The procedure for the Traditional Gram-Stain Technique described by Brooks, *et al.* (2013) is as follows:

- One isolated colony was placed on a microscopic slide
- Heat-fixed smears of bacteria are prepared.
- The slides are placed on the staining rack.
- The smears are flooded with crystal violet and left to stand for 30 seconds.
- Water is rinsed over the smears for 5 seconds.
- Gram's iodine mordant is covered over the smears and left to stand for 1 minute.
- Water is rinsed over the smears for 5 seconds.
- The smears are decolorized with 95% ethanol for 15 to 30 seconds. The decolorizer is added drop by drop until the crystal violet fails to wash from the slide. Alternatively, the smears may be decolorized for 30 to 60 seconds.
- Water is rinsed over the smears for 5 seconds.
- The smears are counterstained with safranin for about 60 to 80 seconds.
- Water is rinsed over the smears for 5 seconds.
- The smears are blotted dry with paper and examined under oil immersion.

- Gram-positive organisms are stained blue to purple and gram-negative organisms are stained pink to red.

2.3.6.2.2.Oxidase test

A small drop of reagent was placed on filter paper and then a small portion of bacterial colonies was spread on this paper by a wooden stick, the positive results appeared as a color change to purple, while the negative results appeared as no color change. his test relies on the detection of specific bacterial oxidase enzymes that play a crucial role in facilitating the transfer of electrons between electron donors within bacteria. These oxidase enzymes are responsible for catalyzing the redox reaction between the electron donors and a redox dye called Tetramethyl-p-phenylene-diamine dihydrochlorid (Yılmaz, *et al.*, 2019).

2.3.6.2.3 Catalase test

On a clean microscope slide this test was performed

1. By using the end of a wooden swab, some cells from the bacterial culture were transferred to the surface of a clean microscope slide.
2. Two to three drops of 3% hydrogen peroxide were added to the cells, then mixed with the wooden stick, and vigorous bubbles were observed (Brown and Smith, 2014).

2.3.6.2.4 Urease test

The urea medium was made up of 2.4g of urea agar base in 95 ml D.W., the PH of the medium was neutralized to 6.8, and after it was autoclaved, a 5ml of 40 percent urea that had been sterilized with Millipore filter paper (0.22µm) was applied to it and divided into test tube volume. Bacteria were added to a tube of urea agar medium, and the tube was then cultured under microaerophilic conditions for 24 hours at 37 °C. A favorable outcome was attained when the medium's color shifted from pale pink to dark pink (Brown and Smith, 2014).

2.3.6.3. Molecular detection of *H. pylori*

The PCR assay was performed to detection of *H. pylori* based on the 16S rRNA gene, genomic DNA was extracted directly from bacteria and using ABIOpure extraction kit.

2.3.6.3.1.Protocol of bacterial DNA extraction

Genomic DNA was isolated from bacterial growth according to the protocol of ABIOpure extraction kit (ABIOpure Company/ USA) as the following steps:

- A volume of 1ml of overnight culture was centrifuged at 13000 rpm for 2min and the supernatant was discarded to get cells pellet.
- Aliquots of 20µl of Proteinase K solution (20 mg/ml) and 200µl of buffer CL were added into a sample tube which contained cell pellet for protein digestion and cell lysis then the tube was mixed vigorously using vortex and incubated at 56˚C for 30 minutes, for further lysis incubated 30 minutes at 70 ˚C.
- Aliquot of 200µl of buffer BL was added into the sample tube then mixed vigorously and incubated at 70˚C for 30 min.
- Aliquot of 200µl of absolute ethanol was added to the lysate sample and the sample was mixed thoroughly.
- After mixing, all of the mixtures were transferred to the Eppendorf tube carefully and centrifuged at 8,000 rpm for 1 minute, and the collection tube was replaced with a new one.
- Aliquots of 600µl of buffer BW were added to the Eppendorf tube and centrifuged at 8,000 rpm for 1 minute then the collection tube was replaced with a new one.
- Aliquot of 700µl of TW was applied and centrifuged for 1 min at 8,000 rpm.

- The pass-through was discarded and the mini-column was reinserted back into the collection tube.
- Residual wash buffer was removed by centrifugation of the minicolumn at full speed 13,000 rpm for 1 min then the mini-column was placed into a fresh 1.5 ml tube.
- Aliquot of 50µl of buffer AE was added and incubated for 1 min at room temperature, then centrifuged at 5,000 rpm for 5min.

2.3.6.3.2. Polymerase chain reaction

All primers were used in this study were illustrated in Table (3-4)

2.3.6.3.3.Primer pairs preparation

The lyophilized form of the primers was provided by Macrogen Company (Korea). To prepare the primers for use, they were dissolved in nuclease-free water to create a stock solution with a final concentration of 100 pmol/µl. For convenience, a working solution of the primers was made by combining 10 µl of the stock solution (which was stored at -20°C in a freezer) with 90 µl of nuclease-free water. This resulted in a working primer solution with a concentration of 10 pmol/µl. The specific primers used in the study are detailed in the accompanying tables (2-6).

Table (2-6): Primers and probe used in this study

Primers	Sequence 5`---------- 3`	Product Size (bp)	Annealing Temp. (C^o)	Reference
16S rRNA	**27F:** AGAGTTTGATCCTGGCTCAG **1492R:**TACGGTTACCTTGTTACGACTT	1500	60	(Srinivasan *et al.*, 2015)

2.3.6.3.4 Quantitation of DNA (Tran, *et al.*, 2019)

The concentration of the extracted DNA was determined using the Quantus Fluorometer, which is a device used to measure DNA concentration.

To detect the concentration, 1 µl of the extracted DNA was mixed with 199 µl of diluted Quanty Fluor Dye. The mixture was then incubated at room temperature for 5 minutes. Following the incubation period, the DNA concentration values were measured using the Quantus Fluorometer. This process allowed for the assessment of the DNA integrity in the samples, which was crucial for subsequent applications (Hussain, *et al.*, 2022).

2.3.6.3.5 Amplification of *16S rRNA H. pylori*

- According to the manufacturer company information, diluted of primer by adding sterile deionized nuclease-free water.
- Before use, the contents of the master mix were thawed at room temperature. The GoTaq® Green Master Mix contains various components, which are listed in Table (2-7).
- ABIOpure extraction kit (ABIOpure Company/ USA) listed in Table (2-8).

Table (2-7): Components of *Taq* master mix.

Component	Concentration
Taq polymerase	2.5µl
dNTP(dATP, dCTP, dGTP, dTTP)	250 mM
Tris – BASE (pH 9.0)	10 mM
KCl	30 mM
MgCl2	1.5 mM

Table (2-8): Components of the PCR reaction mixture for amplification of *16S rRNA H. pylori*

Components	Volume(µl)
Master Mix	12.5
Forward Primer	1
Reverse Primer	1
Nuclease - Free Water	8.5
DNA Template	2
Final Volume	25

- All the PCR components were mixed in PCR tubes under aseptic conditions and vortexed at low speed, and then placed into the thermos-cycler PCR machine in their right location.
- Amplification was performed using a thermal cycler programmed as shown in Table (2-9).

Table (2-9): Program used to amplify the universal *16S rRNA* genes

Steps	C°	m:s	Cycle
Initial Denaturation	95	05:00	1
Denaturation	95	00:30	30
Annealing	60	00:30	
Extension	72	01:00	
Final extension	72	07:00	1
Hold	4	10:00	

- The program was held at 4 C°, and then the PCR products were visualized by gel electrophoresis.

2.3.6.3.6 Detection of amplified products by gel electrophoresis

Agarose gel electrophoresis was used following PCR amplification to confirm the presence of amplification.

a) Agarose Preparation

The agarose preparation according to (Armstrong and Schulz, 2015)

In a beaker, 100 ml of 1X Tris Acetate EDTA (TAE) buffer was added. Then, 1.5 gm (for a 1.5% concentration) of agarose was added to the buffer. The solution was heated to boiling, using a microwave, until all the gel particles were dissolved. Next, 1 µl of Ethidium Bromide (10 mg/ml) was aliquoted and added to the agarose. To ensure proper mixing and to prevent

the formation of bubbles, the agarose was stirred gently. Finally, the solution was allowed to cool down, reaching a temperature between 50-60 C°.

b) Casting of the horizontal agarose gel

Once the agarose solution was prepared, it was carefully poured into a gel tray. Before pouring, both ends of the tray were sealed with cellophane tapes to create a barrier. The agarose was then allowed to solidify at room temperature for approximately 30 minutes to harden and create a gel. After the gel had hardened, the comb used to make sample wells was carefully removed from the gel. The gel was then put back into the gel tray, with the wells pointing in the direction of the gel migration. The gel tray was filled with 1X TAE-electrophoresis buffer to aid the electrophoresis process. The buffer was added until it reached a height of about 3-5 mm above the surface of the gel (Armstrong and Schulz, 2015).

c) DNA loading

Each 5 µL of DNA sample received 3 µL of loading dye before the samples were properly inserted to the wells (Armstrong and Schulz, 2015). By adding 10 µL of ladder straight to the well, PCR products were loaded (Morovat,

et al., 2009). Power was turned on for 45 minutes at 80 volts per minute. From the cathode to the positive anode poles, DNA travels. Gel bands stained with Ethidium Bromide were seen using a UV transilluminator.

2.3.7 Determination of Protein Concentration

Protein concentration was determined according to the method of (Bradford, 1976) using bovine serum albumin standard curve as follows:

A standard curve was generated using different concentrations of bovine serum albumin (BSA), which included 0,10,20,30,40,50,60,70,80,90,100 µg/mL. These concentrations were prepared from a stock solution of BSA (100 µg/mL) as shown in Table (2-10).

Next, 2.5 mL of Coomassie brilliant blue G-250 dye was added to the different BSA concentrations and the mixture was allowed to stand for 2 minutes at room temperature. The absorbance was measured at 595 nm, with the blank being prepared from 0.45 mL of 0.05 M phosphate buffer pH 7 and 2.5 mL of the dye reagent. A standard curve was plotted between the BSA concentrations and the corresponding absorbance at 595 nm (Fig. 2-7).

To estimate the protein concentration of the test samples, 0.05 mL of the sample was mixed with 0.45 mL of phosphate buffer and 2.5 mL of Coomassie brilliant blue G-250. The mixture was then allowed to stand for 2 minutes at room temperature before measuring the absorbance at 595 nm. The protein concentration was calculated using the standard curve generated with the BSA concentrations and their corresponding absorbance values at 595 nm.

Table (2-10): Preparation of Bovine serum albumin (BSA) concentration from a stock solution of BSA (100µg/ml)

Tube No.	Volume of BSA solution (ml)	Volume of D.W. (ml)	Final volume (ml)	Final concentration (µg/ml)
1	0	1.0	1	0
2	0.1	0.9	1	10
3	0.2	0.8	1	20
4	0.3	0.7	1	30
5	0.4	0.6	1	40
6	0.5	0.5	1	50
7	0.6	0.4	1	60
8	0.7	0.3	1	70
9	0.8	0.2	1	80
10	0.9	0.1	1	90
11	1	0	1	100

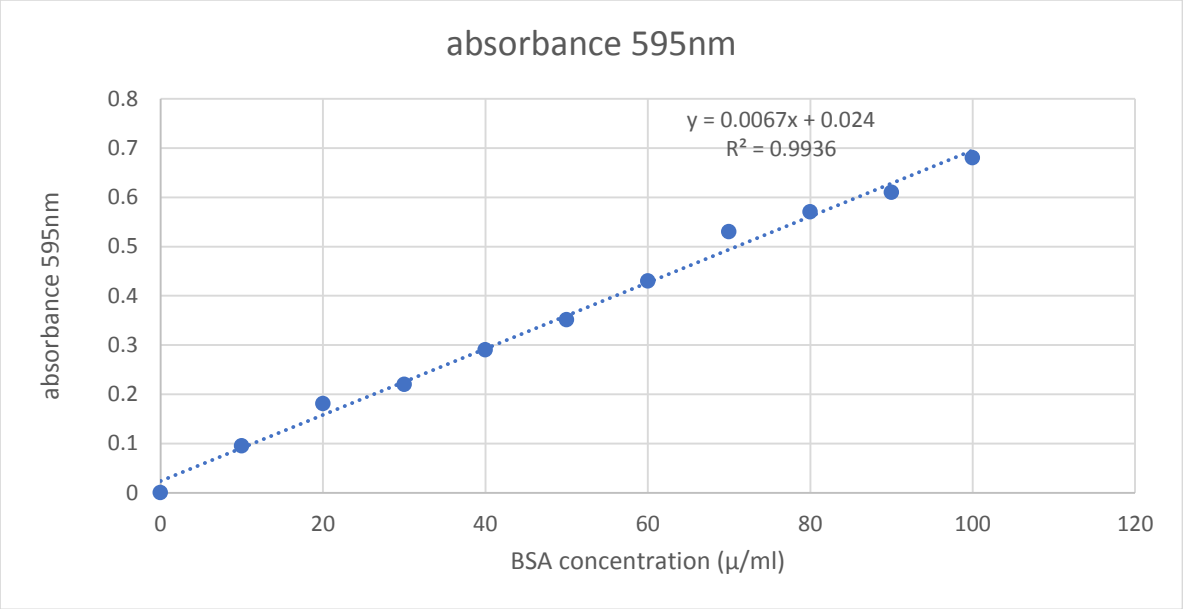


Figure (2-7): Standard curve of bovine serum albumin for determination of protein concentration

2.3.8 Urease assay

Urease activity was assessed by quantifying the release of ammonia from urea using a modified Berthelot reaction. This reaction involves the enzymatic hydrolysis of urea, resulting in the liberation of ammonia. The liberated ammonia then undergoes a chemical reaction with salicylate and hypochlorite, forming a distinctive green-colored indophenol compound (Saem, *et al.*, 2015). The enzymatic assay procedure is done by the following steps

- 100 microliter (µl) of chromogen buffer was added to the microplate
- 100 µl of purified enzyme was added
- 10 µl of urea was added and waiting for 5 minutes.
- 100 µl of alkaline hypochlorite was added which contained and waiting for 5 minutes until occur changing in color.
- The absorbance of the samples and the standard was read at 578 nm against the reagent blank.
- Calculate enzyme activity plotting absorbance on ammonia standard curve

- Two types of control solutions were used: chromogen solution with all the additives and with the same steps without the enzyme and the enzyme solution with all the additions and with the same steps without urea.

One unit of urease activity is defined as the amount of purified enzyme required to release 1 µmol of ammonia after 5 min incubation at room temperature (Richmond and Yep, 2019)

2.3.9 Ammonia Standard curve preparation

The standard curve prepared according to (Saem, *et al.*, 2015) with some modification

- 100 microliter (µl)of buffered chromogen which contains: (phosphate buffer 20 mmol/L, EDTA 2 mmol/L, sodium salycilate 60 mmol/L, sodium nitroprusside 3.4 mmol/L) was added to the microplate.
- 100 µl of ammonium sulphate was added at different concentrations (0, 5,10,15,20,25,30 µM)
- 100 µl of alkaline hypochlorite which contains: (sodium hypochlorite 10mmol/L, NaOH 150 mmol/L) was added and wait for 5 mint until occur color change.
- The absorbance of the samples and the standard were read at 578 nm against the reagent blank. (Figure 2-8)

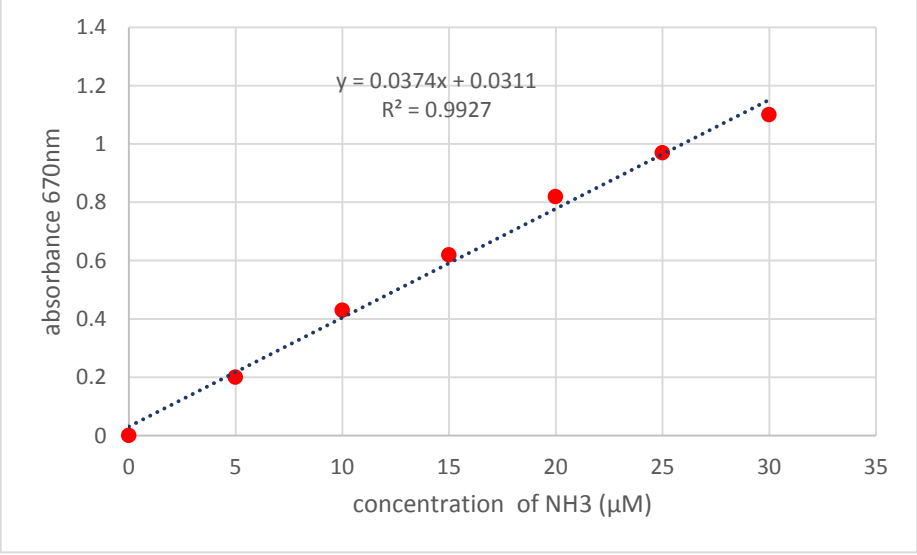


Figure 2-8 Ammonia standard curve

2.3.10 Extraction of Urease

Cultivation and preparation of crude cell-free extracts of *Helicobacter pylori* for both local and reference strains (NCTC 11916) for the isolation of intracellular urease according to (Matongo and Nwodo, 2014). The strains were grown in brain heart infusion broth supplemented with 10% blood for 24-96 hours at 37C under microaerophilic conditions (Figure 2-9). Urease activity was assessed every 24 hours, and the culture times with the optimum activity were used for the preparation of crude cell-free extracts.

Figure 2-9: microaerophile container and gas bottle

The cells were harvested by centrifugation at 5,000 g for 20 minutes at 4°C. The resulting cell pellets were then washed twice with phosphate-buffered saline (pH 7.4) to remove any extracellular contaminants. These washed pellets were used for the subsequent isolation of intracellular urease. To begin the urease isolation process, the cells were resuspended in a buffer supplemented with protease inhibitors. The cell suspension was divided into 20 Falcon tubes to facilitate further processing. After that make freezing -20°C (24 hrs.) and thawing 25°C (2 hrs.) cycle and repeated two times. Bacterial cell disruption was achieved using an ultrasonic homogenizer, which effectively disrupted the cell structure. Following cell disruption, the homogenized samples were subjected to centrifugation at 15,000 g for 20

minutes at 4°C. This centrifugation step aimed to remove any cell debris present in the samples.

The resulting supernatant obtained after centrifugation, known as the cell-free extract (CFE), was collected for further analysis. The CFE obtained from the sonicated cells was subjected to protein concentration and urease activity assays.

2.3.11 Purification of urease

2.3.11.1 Ammonium sulphate precipitation

The supernatant which was obtained from both local and reference strains (NCTC 11916) previously (2.3.10), a specific weight of ammonium sulfate was added to the crude enzyme gradually in an ice bath with continuous stirring for 60 min to get a saturation percentage of 70%. Then centrifuged at 8000 rpm at 4 Co for 20 min, then the precipitate was harvested and dissolved in a minimal volume of 0.1M phosphate buffer (Singh, *et al.*, 2017).

2.3.11.2 Dialysis

The dialysis process for partial purified for both local and references (NCTC 11916) strains have been carried out in dialysis bags with 16000 MW cutoff after the Precipitation step (2.3.11.1) against 0.1M phosphate buffer for 24 hrs. at 4 C° with continuous stirring and then concentrated by sucrose until it reached a volume of (10ml) then kept in the refrigerator for further purification (Brayman,*et al.*, .1996).

2.3.11.3 Purification by preparative HPLC

- Prepare the C3 column (Figure 2-10) according to the manufacturer's instructions. Equilibrate the column with a phosphate buffer (10 mM, pH 7) at a flow rate of 4 ml/min for at least 30 minutes to ensure column stability.

- Clarify and concentrate the urease sample to the desired concentration. Load partially purified urease enzyme from both local and references (NCTC 11916) strains onto the sample loop (1000 µl) separately and inject it into the column using the injector. (Mant, 2007)
- Start the binary high-pressure gradient pump with the following conditions:

❖ Mobile phase A: phosphate buffer (10 mM, pH 7)

❖ Mobile phase B: acetonitrile

❖ Initial conditions: 100% mobile phase A

❖ Gradient conditions: increase to 100% mobile phase B over 30 minutes

❖ Total run time: 60 minutes

❖ Flow rate: 4 ml/min

- Monitor the separation of urease using the diode array detector at a wavelength of 280 nm. Set the detection threshold to a level of 10 mAU and slope to 0.2 AU/min.
- Collect the protein-containing fractions using a fraction collector. Set the fraction size to 5 ml.
- Analyze the collected fractions using the system control software to determine the fractions containing protein.
- Pool the fractions containing protein and measure protein concentration and urease assay for each fraction.

Figure 2-10 column C3 used for urease purification by HPLC

2.3.12 Candidate Urease Inhibition Drug in Silico

2.3.12.1 Generation of FDA approved drugs library

All FDA approved drugs structure were retrieved from the zinc15 database Applying the FDA filter on all zinc15 database substances as follows, a collection of 1615 different drug structures was retrieved. (Kadioglu.,*et al.*,2021)

The retrieved SDF structures were consolidated and stored in a single SDF file. This file served as a repository for all the obtained structures before further preparation for docking.

2.3.12. 2 Retrieval of protein

The protein data bank (PDB) ID 6ZJA which represents *Helicobacter pylori* urease with inhibitor bound in the active site, the resolution of this native structure was 2.0Å, and includes both alpha and beta sub unit and covers 100% of the protein. Within this, all amino acids that are involved in the active site are presented.

2.3.12.3 Preparation of ligands for docking

The ligand structures were prepared for docking using the Grid-Based Ligand Docking with Energetics (GLIDE) software's ligand preparation script. Conformers were generated based on ionic states, stereochemistry, and ring conformations. The OPLS4 force field was used to calculate partial atomic charges. A maximum of thirty-two low-energy conformers were generated per ligand, and the ligand size was limited to 500 atoms. The pH range for conformer generation was set to 7.0 ± 2.0. The resulting conformers were saved in a single file and used for downstream virtual screening. (Kaur, *et al.,* 2019).

2.3.12.4. Preparation of protein for docking

The Protein Preparation Wizard script was utilized to optimize and refine the structure of *Helicobacter pylori* urease. Several efforts were taken to improve the quality and dependability of the protein structure for future computer analysis.

The protein structure was first given bond instructions to ensure correct atom connection. Hydrogen atoms were introduced into the protein at their predicted places. In addition, zero-order bonds for the metal ions in the framework were formed. Water molecules beyond a distance of 5 from the protein were eliminated to increase the efficiency of future computations. This narrowed the scope of the study to the immediate protein environment.

The root means square deviation (RMSD) cutoff value of 0.30 was used to do energy minimization at a pH of 7.0. The Optimized Potentials for Liquid Simulation 4 (OPLS4) force field was used to do the optimization. A more precise depiction of the protein's behavior at the specified pH is possible thanks to this force field, which takes into consideration the interactions and dynamics of the protein. these steps ensured that the *H. pylori* urease structure was refined and optimized for subsequent computational analyses, providing a reliable foundation for further investigations. (Sandor, *et al.,* 2010).

2.3.12.2.5 Docking protocol

Following the findings of Cunha, *et al*., (2021) nineteen specific amino acid residues from the beta subunit were identified as active site participants in *Helicobacter pylori* urease. These residues include HIS13, ILE137, HIS138, ALA169, ILE220, HIS221, GLU222, ASP223, HIS274, ALA278, GLY279, HIS314, MET31, CYS321, ARG338, ASP326, ALA365, and MET366 as depicted in (Figure 2-11)

To facilitate ligand docking, these residues were selected as potential binding sites using the GLIDE receptor grid generation function. The van der Waal radii of the receptor atoms were scaled by 1.00 Å, while the partial atomic charge was set to 0.25 Å. A grid box with coordinates X, Y, Z = 30 Å was generated at the centroid of the *Helicobacter pylori* urease active site. For the subsequent docking analysis, the extra precision docking protocol was employed utilizing the Glide software, which is integrated into the Schrodinger package Maestro Version 12.8.117, Mshare Version 5.4.117, Release 2021-2. This protocol allows for a more accurate assessment of ligand binding within the active site of the *Helicobacter pylori* urease protein.

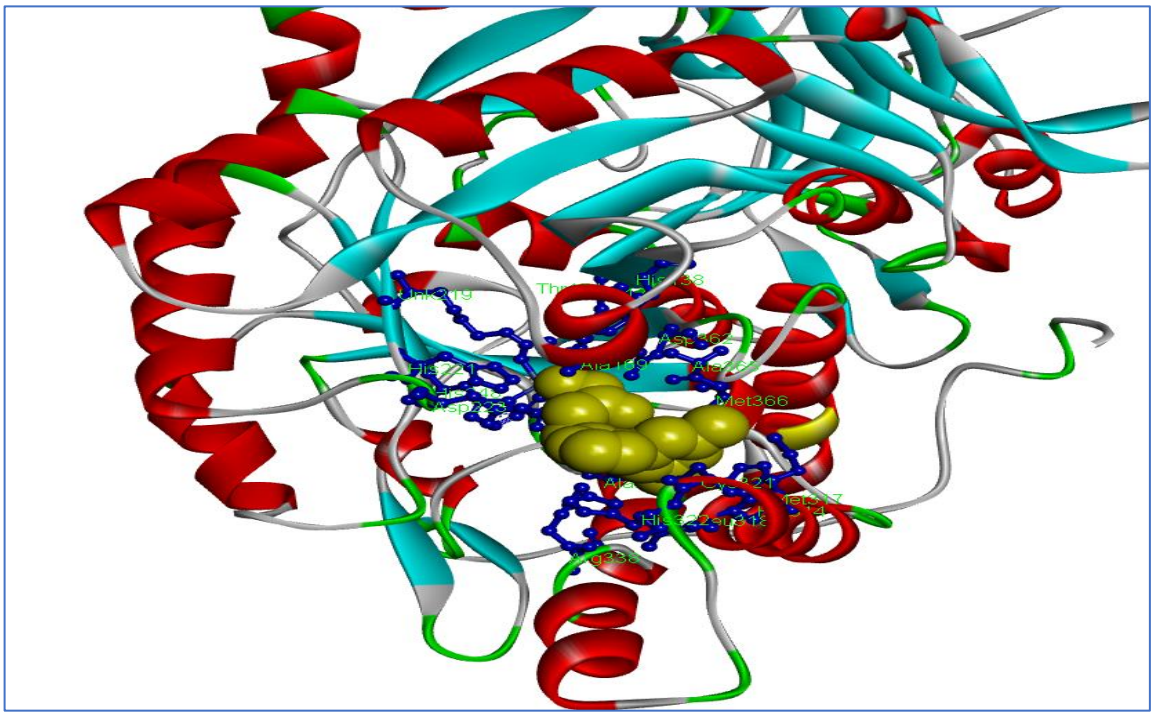


Figure 2-11 ***H. pylori*** **urease with inhibitor (yellow CPK structure) bound in the active site, the active site amino acids represented as blue ball and sticks structures.**

2.3.13 Inhibition of urease *in vitro* study

The inhibition of urease by drug was done according to Sharaf, *et al.*, (2022) with some modifications as follows:

1. A volume of 10 µl of phosphate buffer and 10 µl of D.W was added to the microplate.
2. To initiate the enzymatic reaction, 20 µl of purified enzyme from each strain was added to the plate
3. Subsequently, 20 µl of each drug including Paromomycin sulfate, tobramycin, gentamicin, amikacin, and Capreomycin sulfate, at a concentration of 3.2mg/ml and subjected to serial dilution (third dilution), was added to their respective wells on the plate.
4. Following the enzymatic reaction, 40 µl of urea was added to the plate, and a waiting period of 10 minutes at 37℃ was observed.
5. The amount of liberated ammonia from the reaction mixtures was detected in the same procedure of urease activity as follows:

- 100 µl of chromogen buffer was added to the microplate
- 100 µl of alkaline hypochlorite was added to the microplate and waited for 5 minutes until occur color change.
- Read the absorbance of the at 578 nm.
- Two types of control solutions were used: chromogen solution with all the additives and with the same steps without the enzyme and the enzyme solution with all the additions and with the same steps without urea

6. Following this the IC_{50} (half maximal inhibitory concentration) was measured. The IC_{50} serves as an indicator of the drug's potency in inhibiting the urease enzyme.
7. The same aforementioned steps were performed for the standard strain urease sample (NCTC 11916), Additionally, control samples were prepared on the plate, consisting of the local and standard strains for urease with all additives without inhibitor, and inhibitor without enzyme. These control samples were employed for comparative analysis.

2.3.14 Statistical Analysis

Statistical calculation was performed by the using of SPSS software (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp. USA) and Microsoft Excel (2010, Microsoft Corp. USA). All the results were expressed as a mean P-value ≤ 0.05 was considered statistically significant. LSD was carried out to find the significant difference in mean concentrations comparing all the drug's diluted concentrations using genstat software, (Payne,*et al.*, 2002) .one-way analysis of variance (ANOVA) was conducted to determine if there were significant differences in group variance (De jesus souza,2019). MyCurvefit (online software) used for curve fitting and IC_{50} calculation.

Chapter Three

Results

3. Results

3.1 Bacterial Isolation and Identification

The study samples included seventy patients who were collected directly from persons in the endoscopic unit at Karbala center for Gastroenterology and Hepatology in Karbala at Imam Al-Hussein Medical-City, all patients were diagnosed with stool antigen detection test for *H. pylori* then the positive results took biopsy samples. The results showed 58 (82.8%) patients of 70 patients suffering from *H. pylori* infection by Stool antigen detection test while the culture of the biopsy showed only 12 (17.14%) from 70 with *H. pylori*.

3.2 Results of *H. pylori* Isolate Identification

3.2.1. Conventional identification (Gram stain and Urease test) for *H. pylori*

The *Helicobacter pylori* under light microscopic appears as Gram negative bacteria, spiral shape or the shape of the letter S or rod curved and filamentous forms is also noted in Figure (3-1).

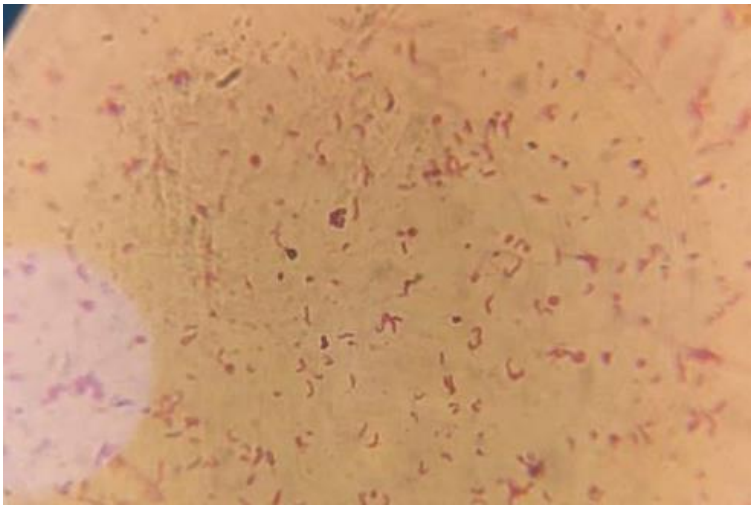


Figure (3-1): Smear of *H. pylori* Colony Stained by Gram Stain (100x).

By using Biochemical test e.g. Urease test 10 isolates from 12 (83.33%) gave positive results for bacterial culture, samples changing in color

from yellow to pink through (24 hours) under micro-aerophilic conditions figure. (3-2) All 10 (100%) isolates gave positive results for catalase and oxidase test.

Figure 3-2: urease test of *H. pylori* in present study

Helicobacter pylori infection was diagnosed by culture on chocolate agar Figure (3-3). Out of 58 only 10 (17.24%) isolates were pronounced visible colonies on chocolate agar. The colonies were translucent and 1-2 mm in diameter, which was confirmed by a typical appearance on a direct smear that showed the curved or spiral organisms.

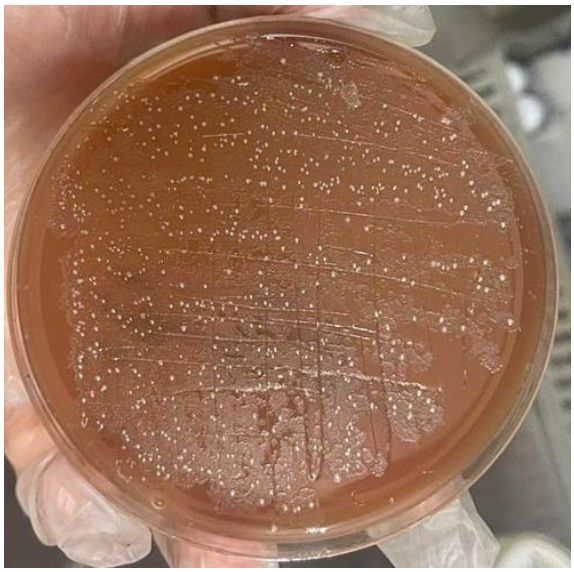


Figure 3-3: *H. pylori* colonies on chocolate agar in present study

3.3 Molecular Identification of the Suspected *H. pylori* Isolates

Six isolates from a total of 10 isolates with a percentage of 60% that gave positive results through conventional identification *H. pylori* (Gram stain and urease test) were selected and subjected to molecular analysis through the amplification of universal bacterial 16S rRNA gene. The product size genes for six isolates were 1500 bp which indicated these isolates were *H. pylori* depending on primers sequence yielding.

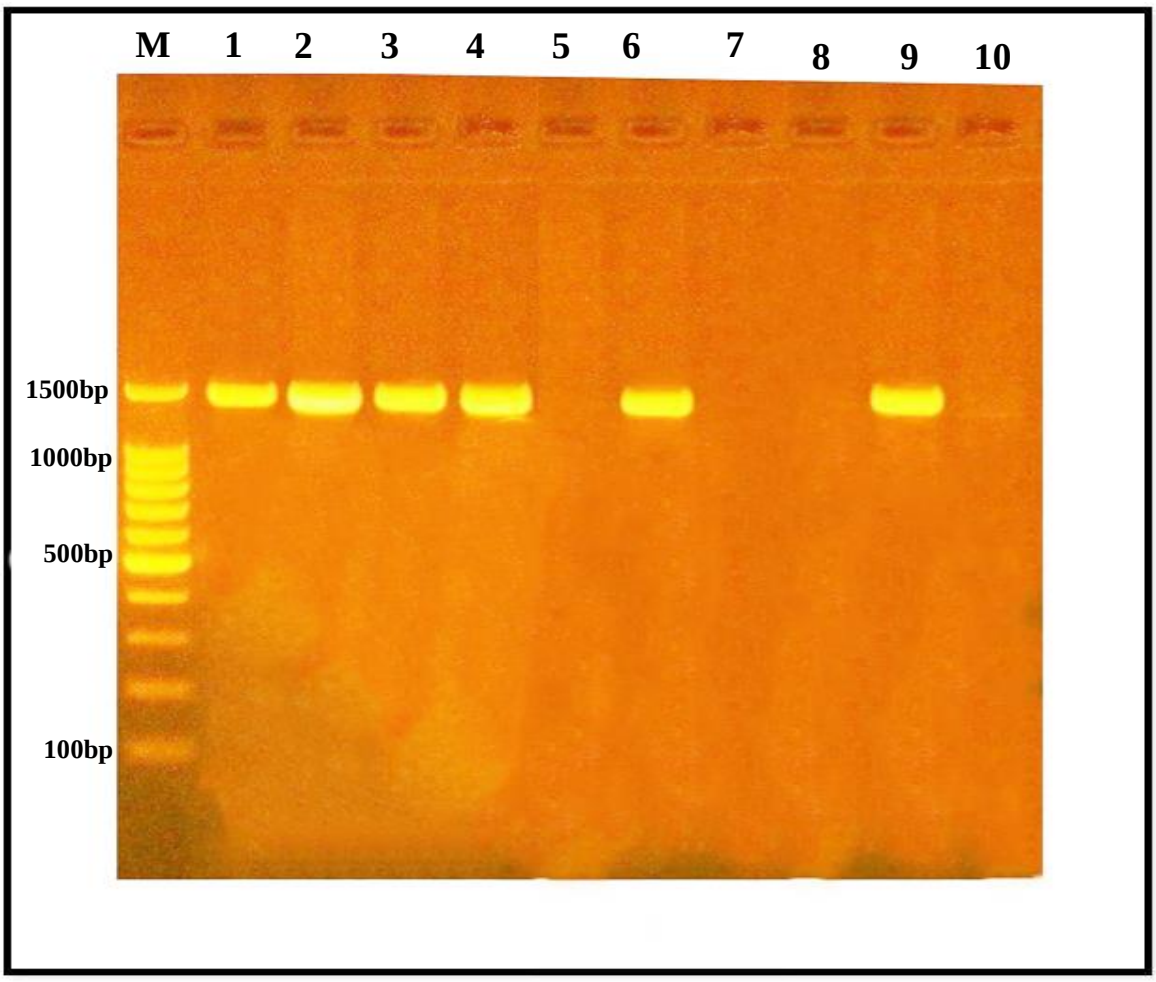


Figure (3-4): The amplification of the 16S rRNA gene of suspected bacterial species was fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. Lane M: 100bp DNA marker for 45 min at 80 volts per min.

3.4 Optimum incubation time

To determine the optimum incubation time for enzyme activity. the enzyme activity was measured at different incubation times (24, 48, 72, and 96 hours) at 37 °C and pH 7.0 for both a local strain and the NCTC 11916 strain.

The results showed that for both strains, the enzyme activity increased from 24 to 72 hours and then decreased at 96 hours. This suggests that there is an optimal time period during which the enzyme activity reaches its peak as shown in Table 3-1.

For the local strain, the enzyme activity was 0.25 U/ml at 24 hours, increased to 0.68 U/ml at 48 hours, reached its highest value of 0.86 U/ml at 72 hours, and slightly decreased to 0.71 U/ml at 96 hours. Similarly, for the NCTC 11916 strain, the enzyme activity was 0.16 U/ml at 24 hours, increased to 0.36 U/ml at 48 hours, reached its maximum of 0.41 U/ml at 72 hours, and decreased to 0.32 U/ml at 96 hours.

The results indicate that the optimum incubation time for enzyme activity in both strains is 72 hours. This is the time point at which the enzyme activity is highest before it starts to decline.

Table 3-1: Optimum incubation time for enzyme activity

Incubation time	Enzyme activity U/ml	
	Local strain	Reference strain
24 hrs.	0.25	0.16
48hrs	0.68	0.36
72hrs	0.86	0.41
96hrs	0.71	0.32

3.5. Purification method results

3.5.1 Ammonium sulphate precipitation results

Ammonium sulfate $(NH_4)_2SO_4$ was used at variable concentrations (40-80%) as a preliminary step in the purification of the urease enzyme, these salts are used to get rid of a high percentage of water by precipitation of protein. saturation rates of ammonium sulphate 70% was the best concentration for precipitation of urease for both local and reference strains. The urease activity for both local and reference strains were 0.81 U\ml and 0.38U\ml respectively, while the specific activity was 0.13 U/mg and 0.12 U/mg.

The enzyme solution was dialyzed after precipitation with ammonium sulphate against 0.1M phosphate buffer for 24 hrs. at 4 C° with continuous stirring and then concentrated by sucrose until it reached a volume of (10ml), then kept in the refrigerator for further purification. (Sing, *et al*., 2007)

3.5.2 Purification of urease by preparative HPLC

This study, conducted purification by using preparative HPLC, C3 column using a 280 nm detector to purify urease from both the reference strain and the local strain. For the reference strain, six distinct peaks were observed, and their retention times were determined. Among these peaks, the first peak at a retention time of 4.8 min exhibited urease activity, confirming the presence of active urease within fraction 7. The fraction was collected and determined the urease activity and protein concentration. The results showed specific activity 0.8 (u/mg protein). However, another peak in the analysis did not show any urease activity (Figure 3-5). (Mant, 2007)

Similarly, for the local strain, only two distinct peaks were observed during the HPLC purification. The retention times of these peaks were determined, and the first peak at a retention time of the same time 4.8 min exhibited urease activity with specific activity 2.25 (u/mg protein). However, the second peak, as shown in Figure 3-6, did not show any urease activity. These findings provide valuable insights into the purification of urease.

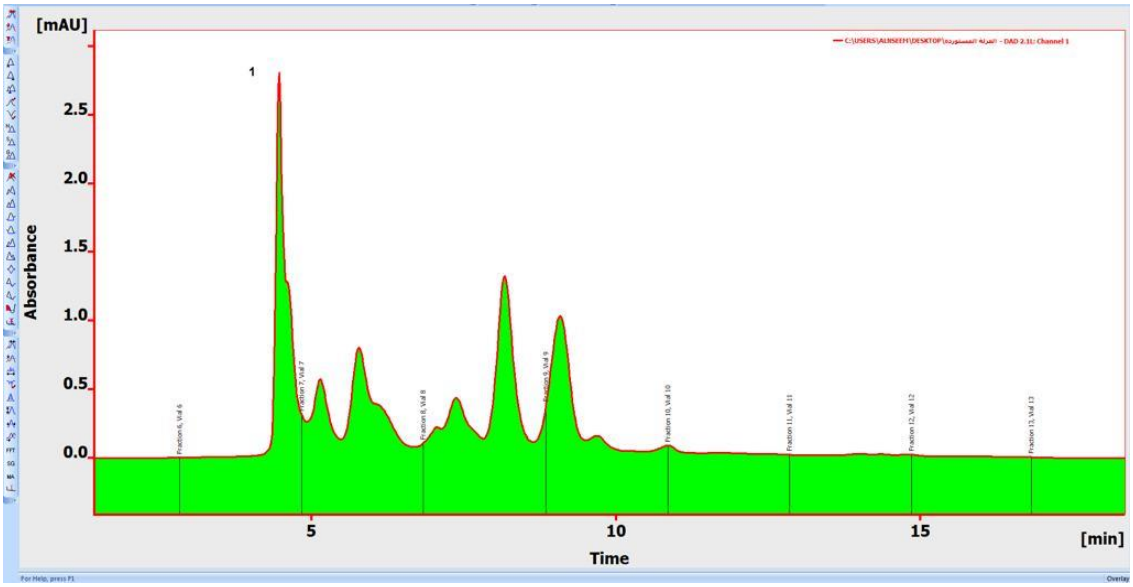


Figure 3-5: Urease purification from *H. pylori* NCTC 11916 by using preparative HPLC C3 column in present study

3.5.4 Purification of the Reference Strain

As shown in Table 3-2 the initial step, "Crude," involved a volume of 10 ml with specific activity at this stage was 0.10 U/mg protein. The purification fold was calculated to be 1.00, indicating that no purification occurred at this stage. The yield was 100.00%, suggesting that all the protein present in the crude sample was recovered.

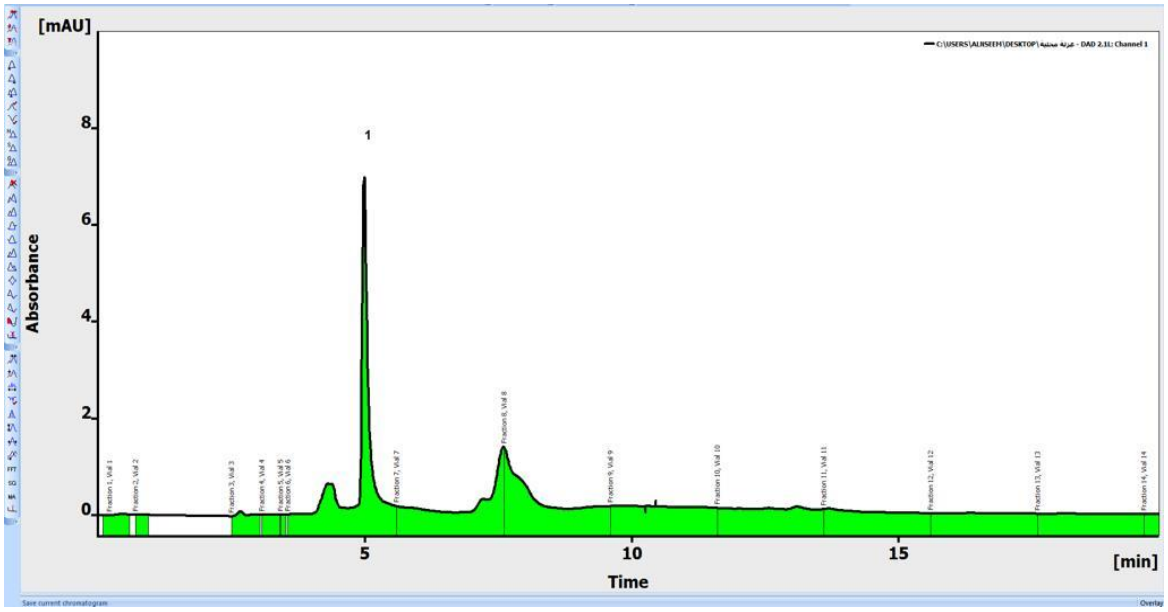


Figure 3-6: Urease purification from local strain by using preparative HPLC In present study

The "Preparative HPLC" step involved a volume of 50 ml. The total protein content significantly decreased to 4.16 mg, indicating a higher degree of purification. The total activity also decreased to 3.32 U. The specific activity increased to 0.80 U/mg protein, indicating a higher enzyme activity per unit protein. The purification fold increased to 7.68, demonstrating a substantial purification effect. The yield decreased to 84.91%, suggesting some loss during the HPLC purification process.

The final step, "Vacuum Concentration," involved a volume of 10 ml with specific activity increased to 1.18 U/mg protein, suggesting further concentration of enzyme activity. The purification fold increased to 11.33, indicating the highest level of purification achieved throughout the process. The yield decreased to 78.26%, likely due to losses during concentration

Table 3-2: Purification steps of urease enzyme from the reference strain

Steps	Volume (ml)	Total Protein Mg	Total Activity U	Specific Activity (U/Mg	Purification Fold	Yield %
Crude	10	37.65	3.91	0.10	1.00	100.00
preparative HPLC	50	4.16	3.32	0.80	7.68	84.91
vacuum concentrate	10	2.6	3.06	1.18	11.33	78.26

$$\text{Specific activity (u/mg)} = \frac{\text{total urease activity U}}{\text{total protein mg}}$$

$$\text{purification fold} = \frac{\text{Specific activity } \left(\frac{\text{u}}{\text{mg}}\right) \text{ of purification step}}{\text{Specific activity } \left(\frac{\text{u}}{\text{mg}}\right) \text{ of crude extract}}$$

$$\text{yield \%} = \frac{\text{total activity of purification step}}{\text{total activity of crude extract}} \times 100$$

3.5.5 Purification of Local Strain

As shown in Table 3-3 The initial step, "Crude," involved a volume of 10 ml with specific activity at this stage was 0.13 U/mg protein. The purification fold was calculated to be 1, indicating no purification occurred.

The yield was 100%, suggesting all the protein in the crude sample was recovered. The subsequent step, "Ammonium Sulfate Precipitation," involved a volume of 10 ml with specific activity remained the same at 0.13 U/mg protein. The purification fold decreased slightly to 0.97, indicating a minor decrease in purity. The yield was 98.18%, indicating efficient recovery of the protein.

The "HPLC" step involved a larger volume of 50 ml with specific activity increased to 2.25 U/mg protein, indicating a higher enzyme activity per unit protein. The purification fold increased significantly to 17.17, demonstrating a substantial purification effect. The yield decreased to 74.42%, suggesting some loss during the HPLC purification process.

The final step, "Vacuum Concentration," involved a volume of 10 ml with specific activity increased to 2.64 U/mg protein, suggesting further concentration of enzyme activity. The purification fold increased to 20.67, indicating the highest level of purification achieved throughout the process. The yield decreased to 70.30%, likely due to losses during concentration.

Table 3-3:Purification steps of urease enzyme from local strain

Steps	Volume (ml)	Total protein mg	Total activity U	Specific activity (U/mg)	Purification fold	Yield %
Crude	10	62.97	8.25	0.13	1	100
HPLC	50	2.73	6.14	2.25	17.17	74.42
vacuum concentrate	10	2.2	5.8	2.64	20.67	70.30

$$\text{Specific activity (u/mg)} = \frac{\text{total urease activity U}}{\text{total protein mg}}$$

$$\text{purification fold} = \frac{\text{Specific activity } \left(\frac{\text{u}}{\text{mg}}\right) \text{ of purification step}}{\text{Specific activity } \left(\frac{\text{u}}{\text{mg}}\right) \text{ of crude extract}}$$

$$\text{yield \%} = \frac{\text{total activity of purification step}}{\text{total activity of crude extract}} \times 100$$

3.6 Virtual Screening

The compounds is most likely to act as the *H. pylori* urease inhibitor 6ZJA from the zinc15 database molecular docking simulations for each compound in the library using programs were carried out.

the molecular docking found 331 compounds that can bind to 6ZJA, this study used the five drugs with the highest docking scores by the program including paromomycin, tobramycin, amikacin, capreomycin, and gentamicin (Table 3-4) Figure (3-7 to 3-16).

In this study, molecular docking was performed using the Glide software to predict the binding affinity and ligand efficiency of these drugs. The Glide docking scores represent the predicted binding affinity, where lower scores indicate stronger binding to the target protein.

Comparing the docking scores, paromomycin achieved the highest negative score of -15.339, suggesting a potentially strong binding affinity with the target protein. gentamicin and tobramycin followed with docking scores of -13.864 and -13.035, respectively. Amikacin and capreomycin exhibited slightly lower docking scores of -12.928 and -12.192, respectively, indicating comparatively weaker binding affinities in this study

Table 3-4: Docking Scores of Selected Drugs

Drug name	Chemical structure	Zinc Id	Glide docking score
Paromomycin	C23H45N5O14	ZINC000060183170	-15.339
Gentamycin	C21H43N5O7	ZINC000242437514	-13.864
Tobramycin	C18H37N5O9	ZINC000008214692	-13.035
Amikacin	C22H43N5O13	ZINC000008214483	-12.928
Capreomycin	C25H44N14O8	ZINC000150338698	-12.192

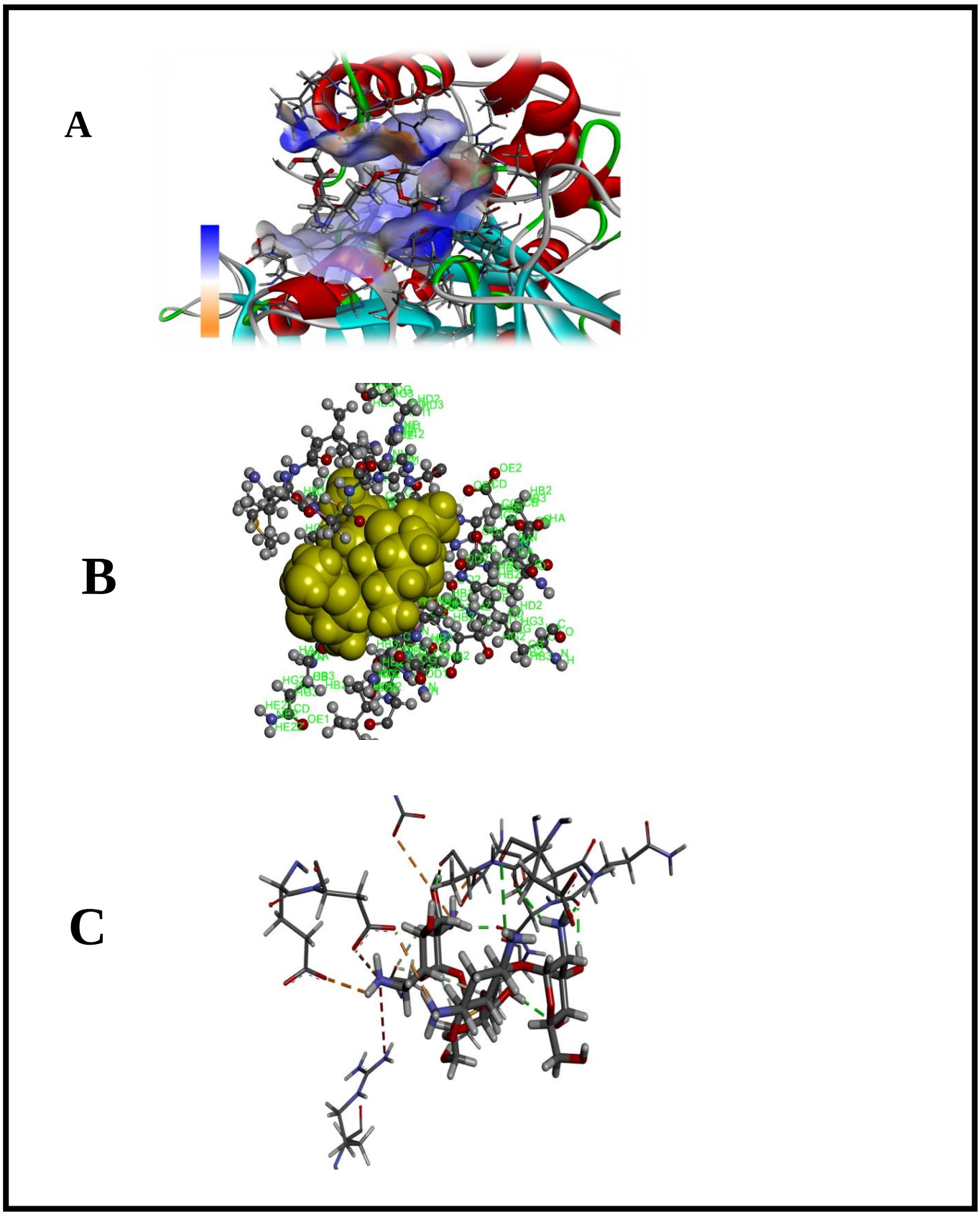


Figure 3-7: A:3- dimensional *H. pylori* urease with paromomycin (CPK structure) bound in the active site, B: the active site represented as ball and sticks structures yellow color paromomycin interaction with urease enzyme, C: three 3- dimensional *H. pylori* urease with paromomycin

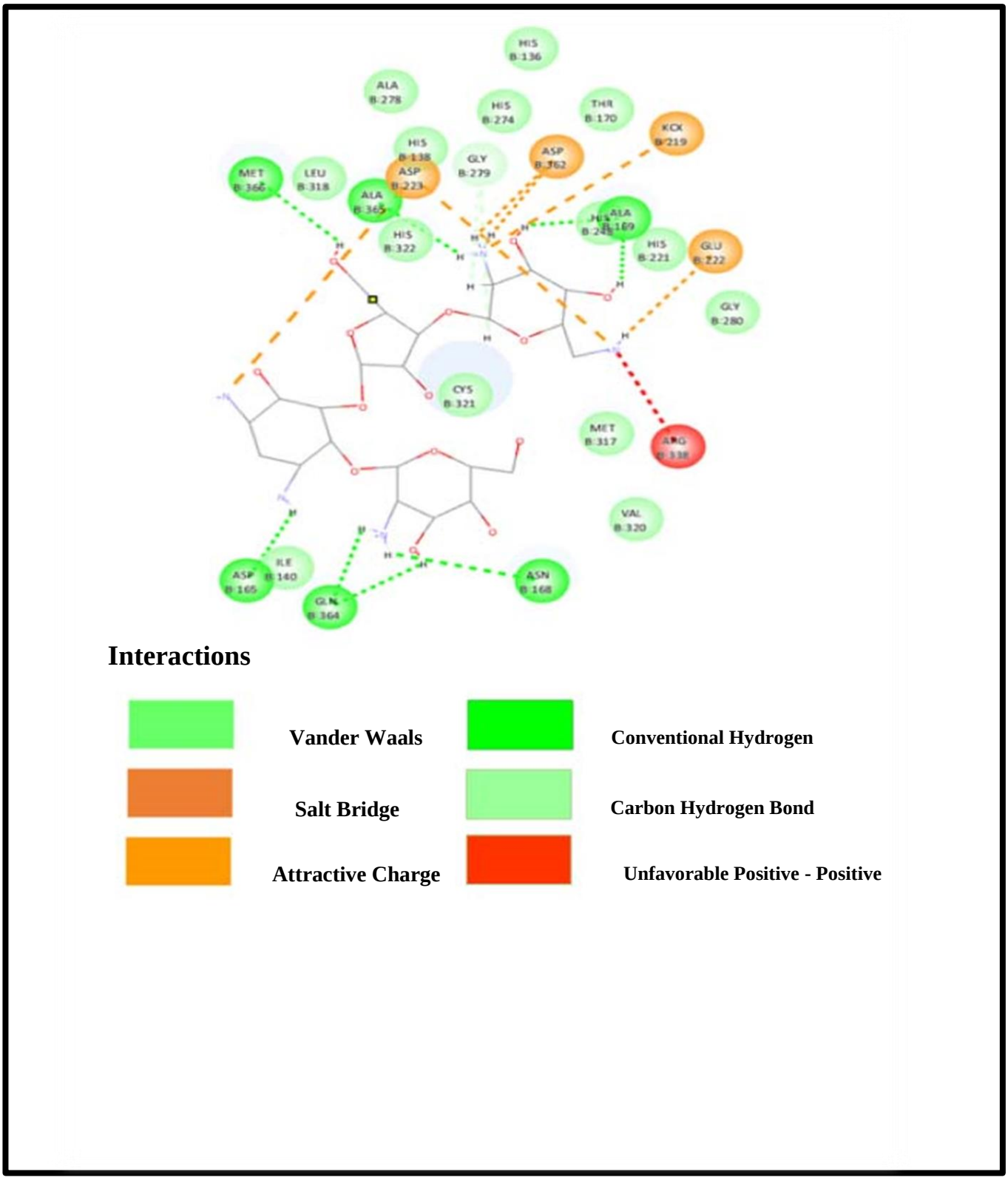


Figure 3-8 : 2-dimensional interactions between Paromomycin and urease interactions with amino acids in active site

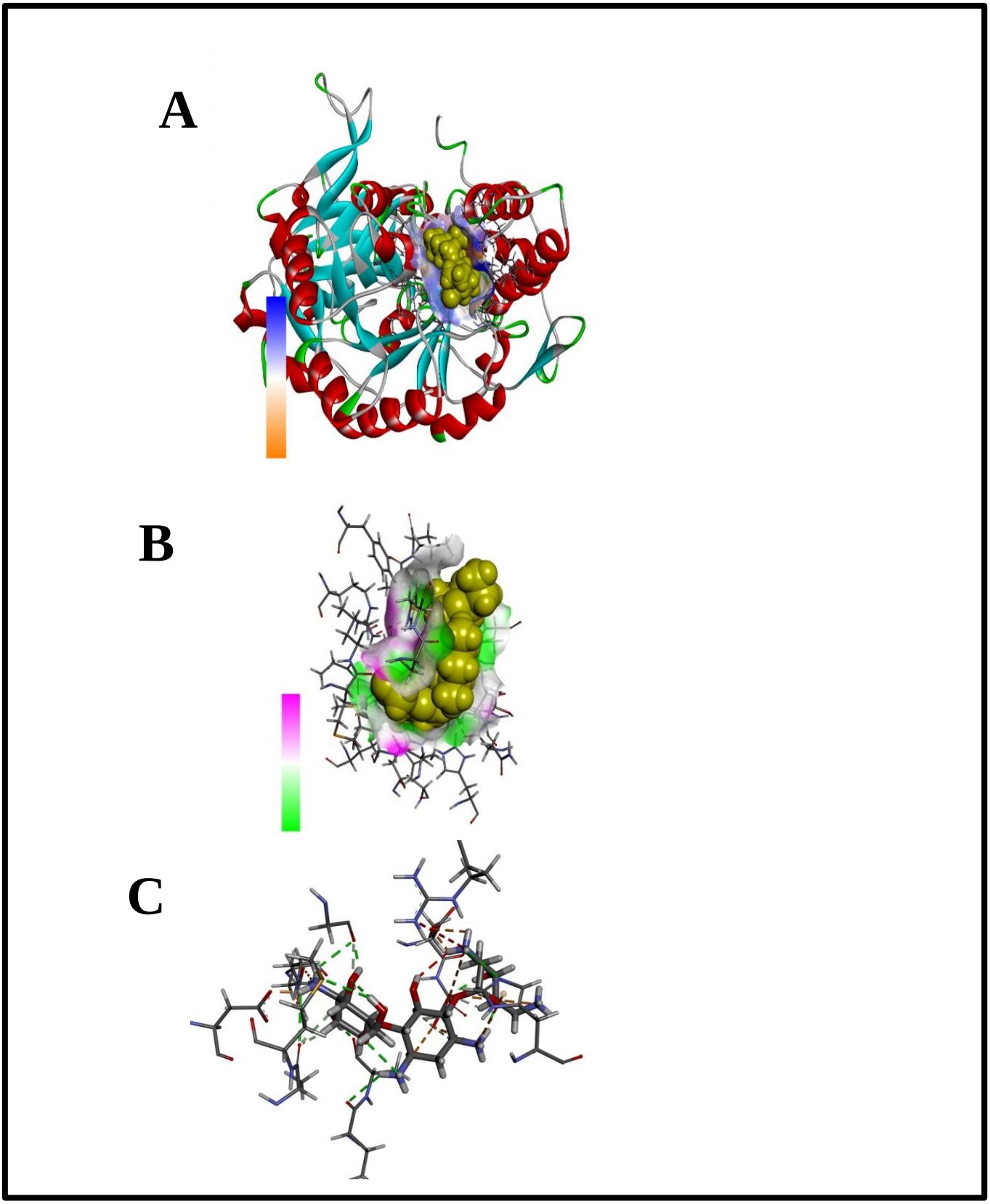


Figure 3-9 :A: 3- dimensional *H. pylori* urease with tobramycin (CPK structure) bound in the active site, B: the active site represented as ball and sticks structures yellow color tobramycin interaction with urease enzyme, C: 3- dimensional *H. pylori* urease with tobramycin

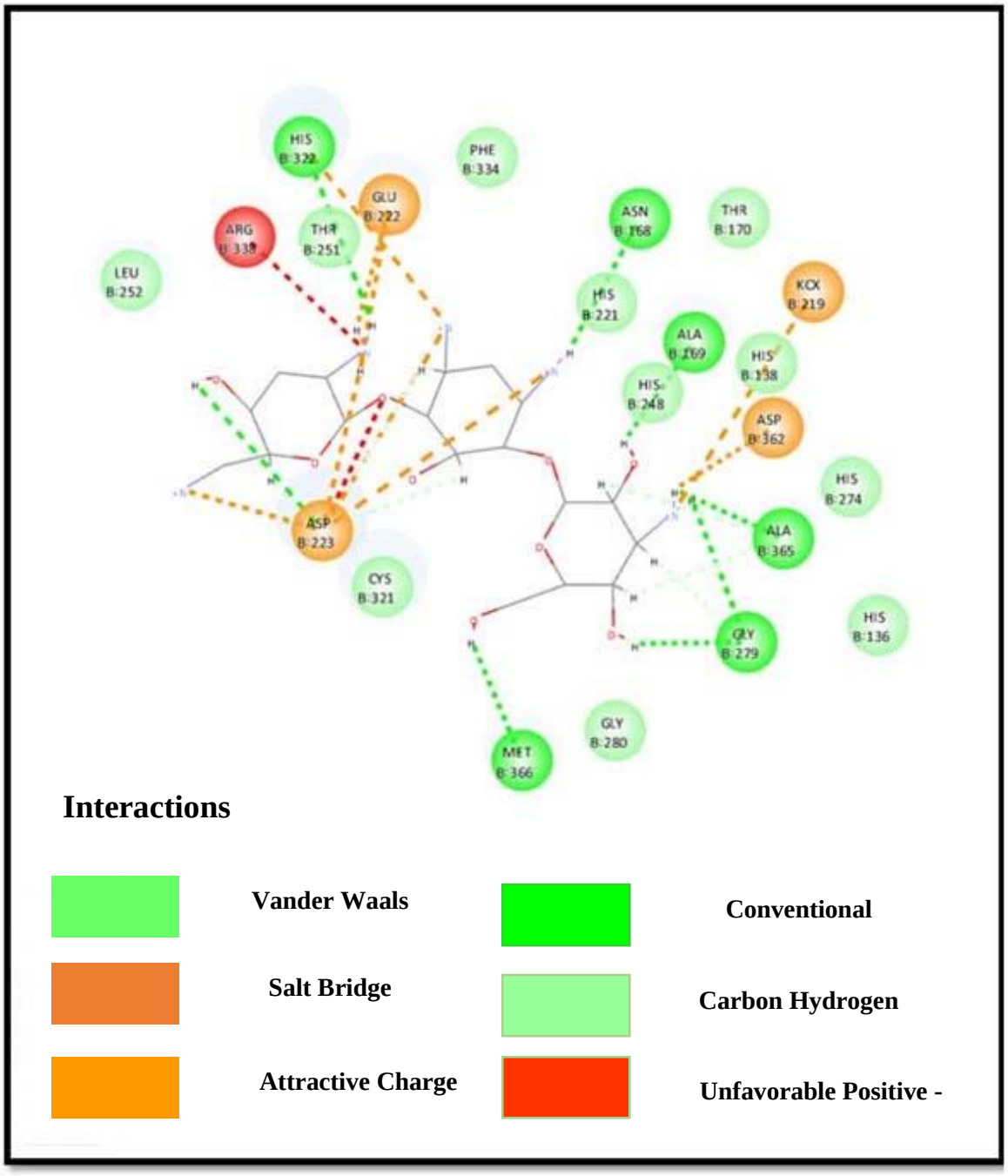


Figure 3-10: 2-dimensional interactions between Tobramycin and urease interactions with amino acids in active site

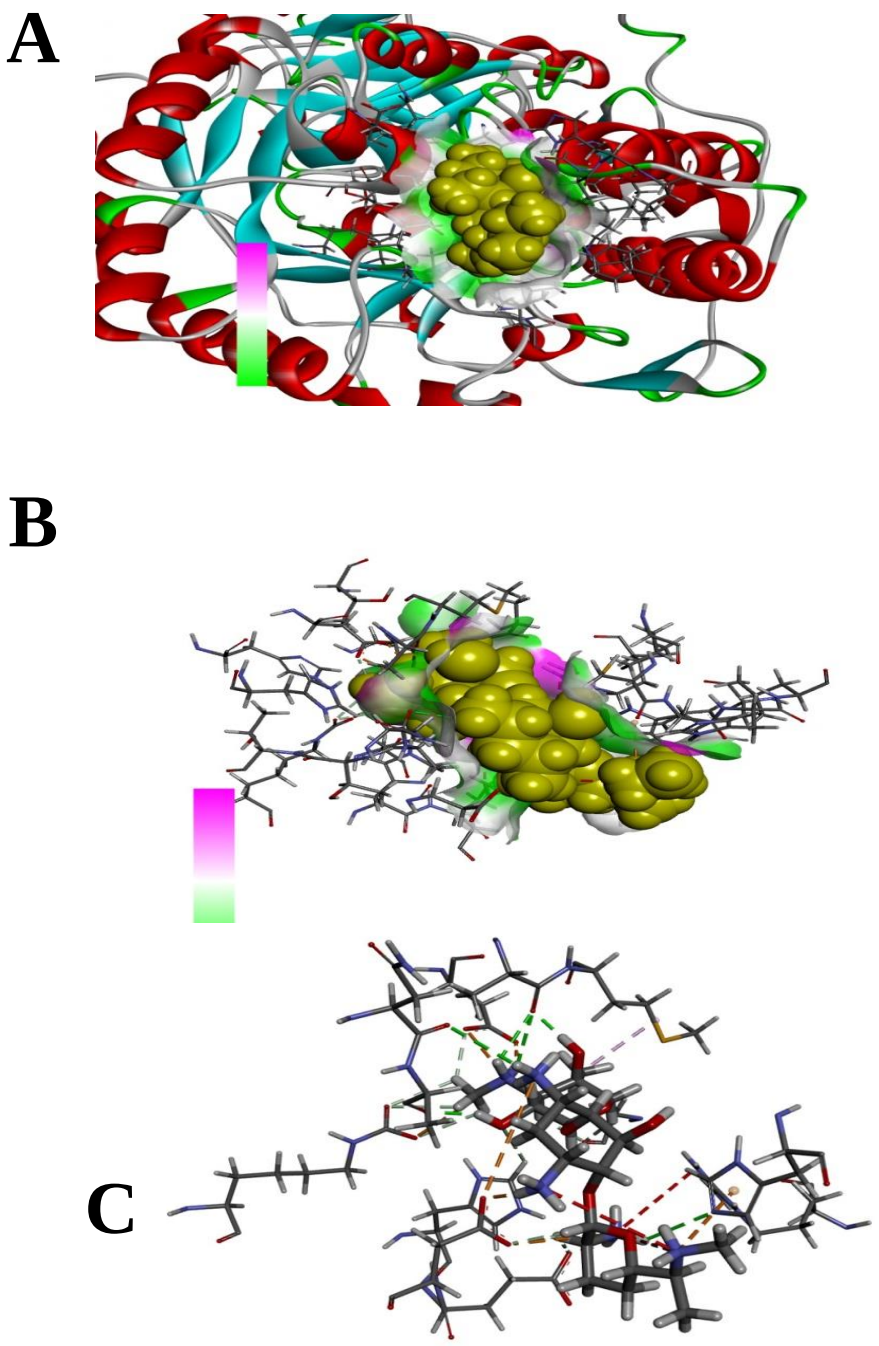


Figure 3-11 : A: 3-dimensional *H. pylori* urease with gentamycin (CPK structure) bound in the active site, B: the active site represented as ball and sticks structures yellow color gentamycin interaction with urease enzyme, C: 3- dimensional *H. pylori* urease with gentamycin

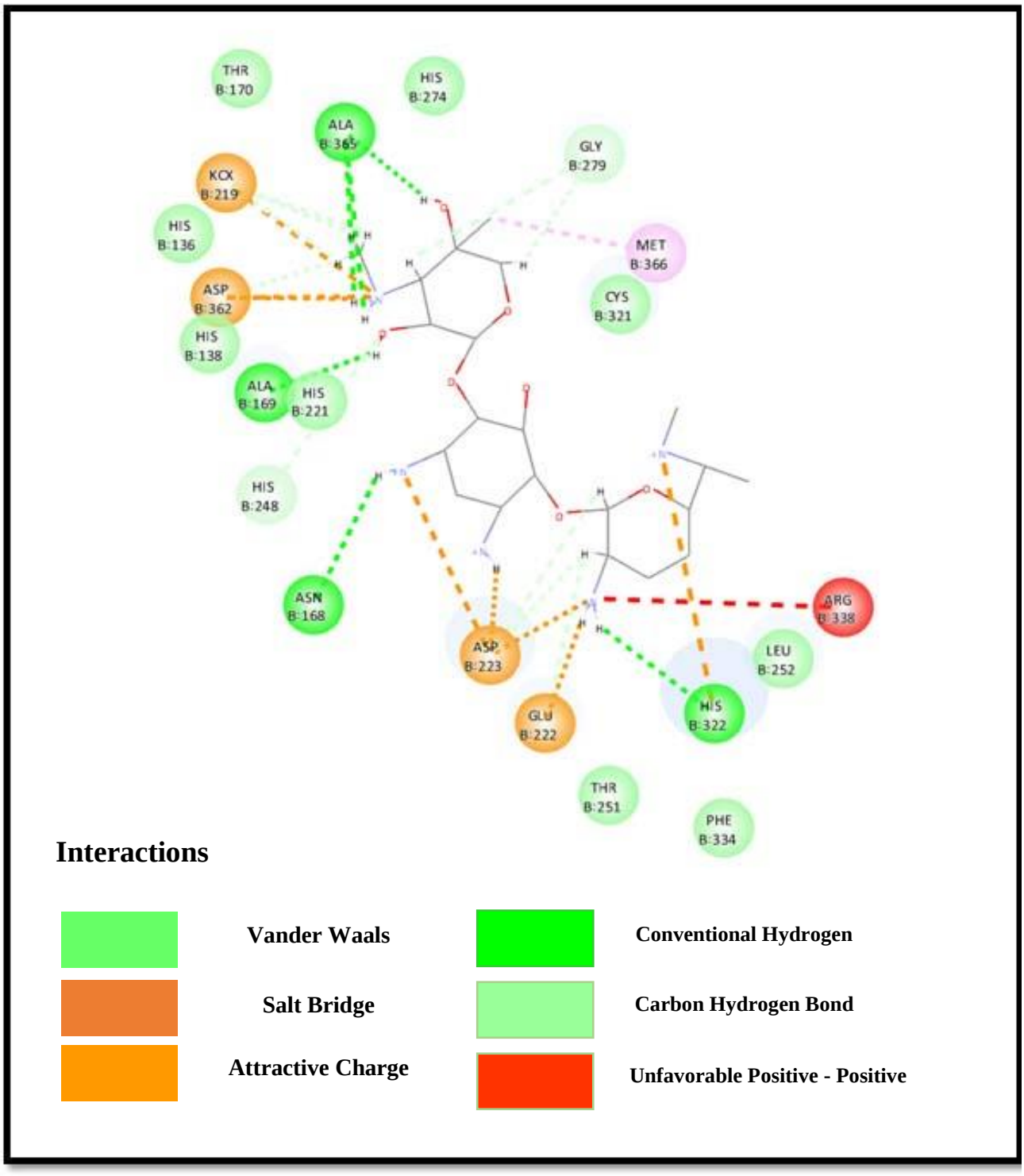


Figure 3-12; 2-dimensional interactions between gentamycin and urease interactions with amino acids in active site

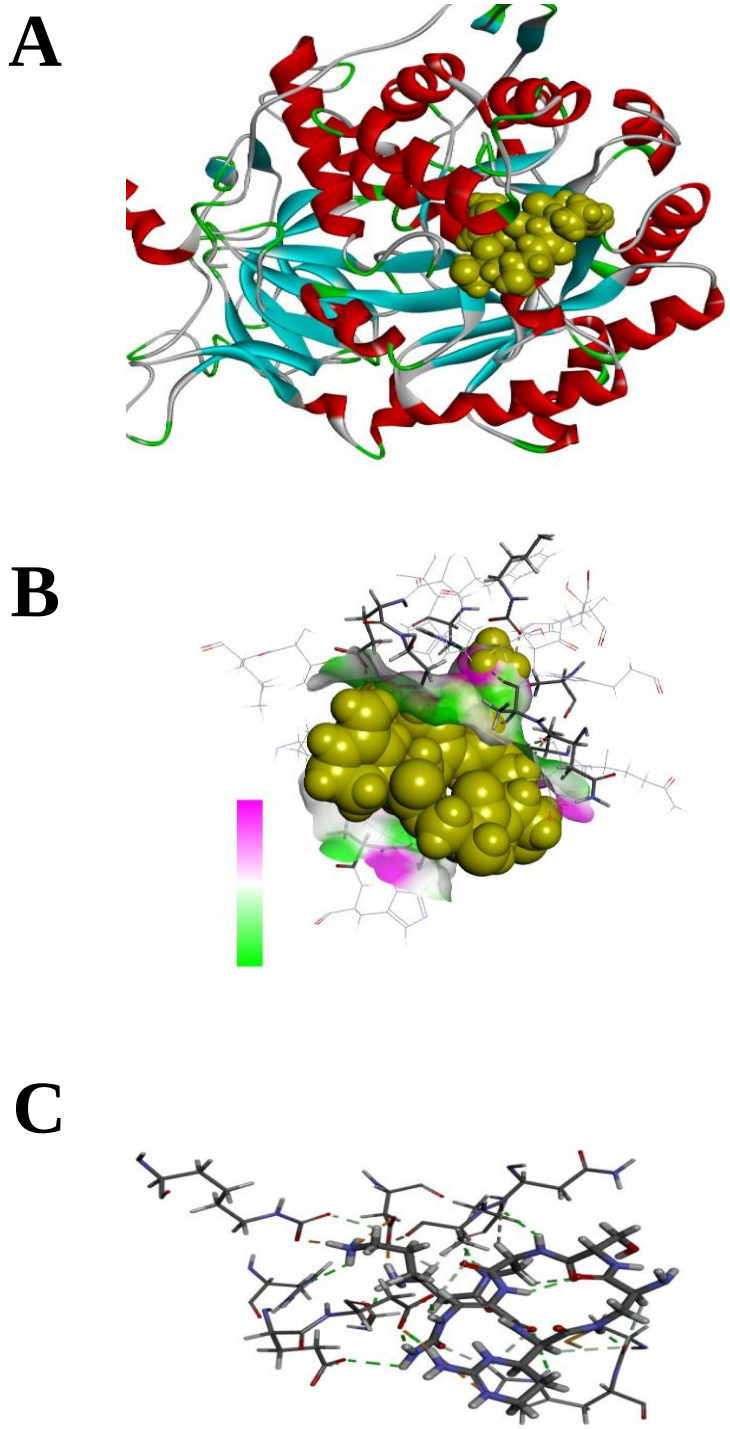


Figure 3-13: A:3- dimensional *H. pylori* urease with Capreomycin (CPK structure) bound in the active site, B: the active site represented as ball and sticks structures yellow color Capreomycin interaction with urease, C: 3dimensional *H. pylori* urease with Capreomycin

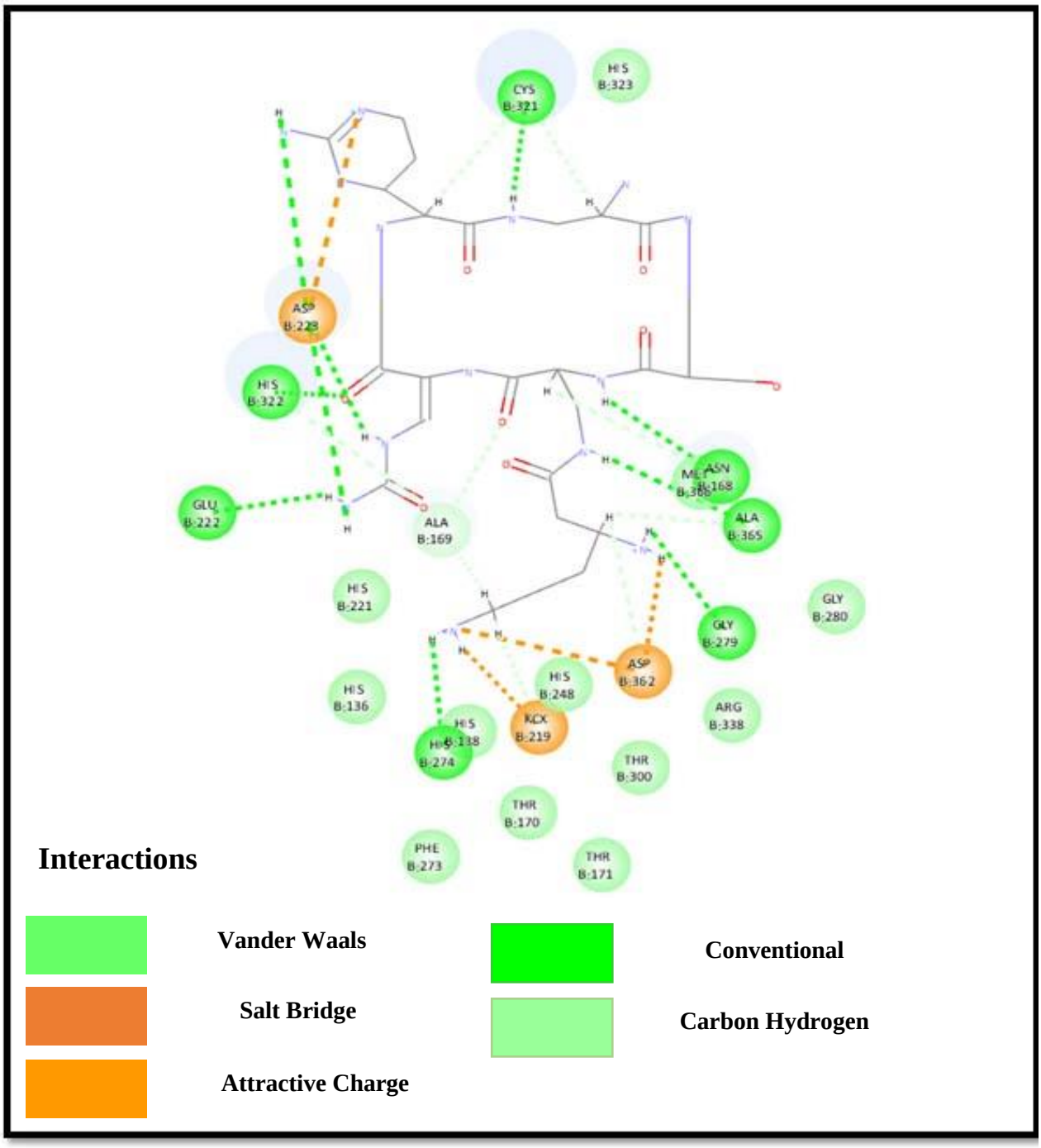


Figure 3-14: 2-dimensional interactions between capreomycin and urease interactions with amino acids in active site

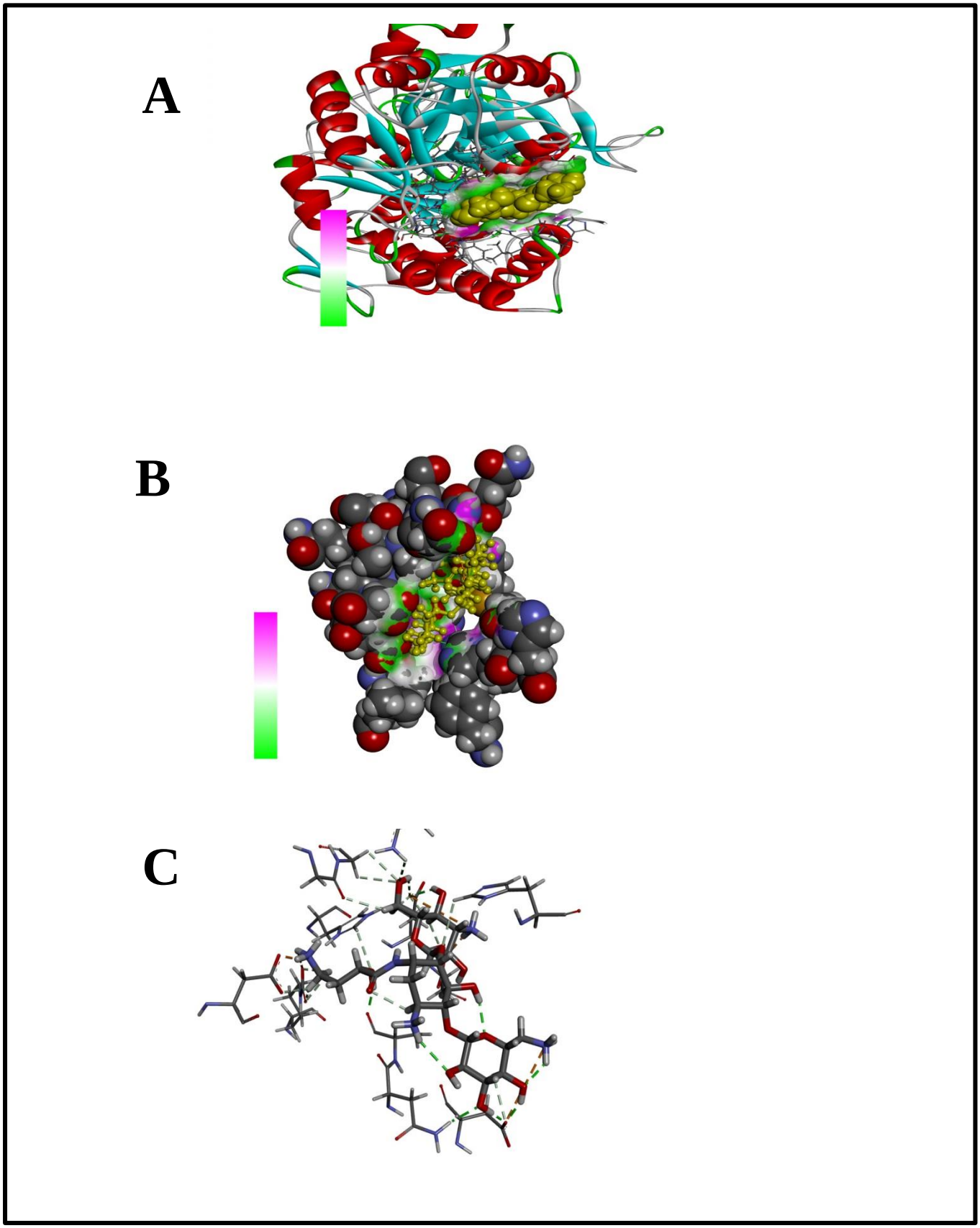


Figure 3-15: A:3- dimensional *H. pylori* urease with amikacin (CPK structure) bound in the active site, B: the active site represented as ball and sticks structures yellow color amikacin interaction with urease, C: 3- 3-dimensional *H. pylori* urease with amikacin

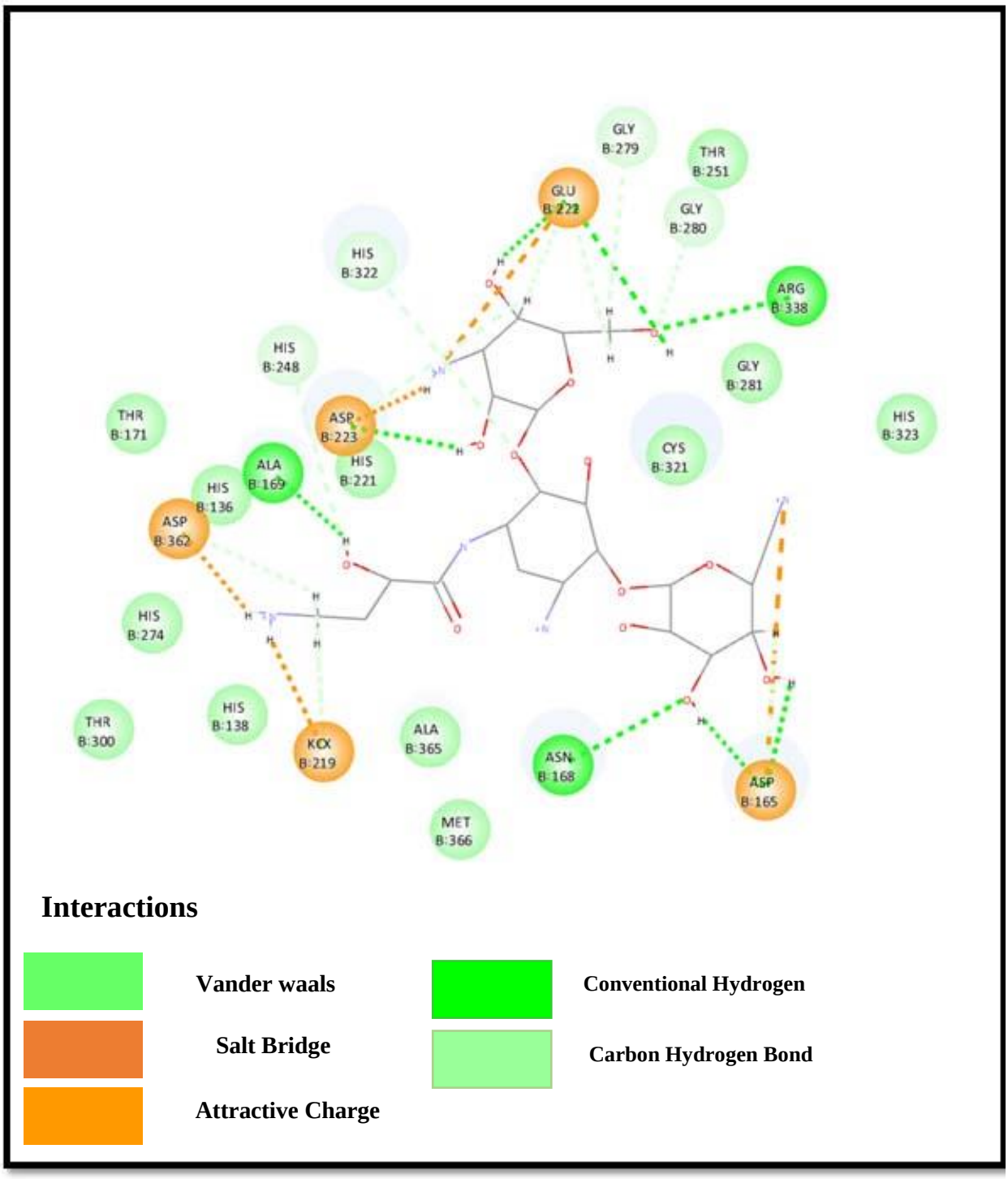


Figure 3-16: 2-dimensional interactions between amikacin and urease interactions with amino acids in active site

3.7 *In vitro* assessment of the inhibitory performance of drug candidate upon urease

This study investigated the effect of FDA approved candidate drugs Aminoglycosides on the urease activity for local and reference strains (NCTC 11916) of *H. pylori* Then a comparative study regarding the effect of inhibitory performance was performed.

3.7.1 Paromomycin

The IC_{50} values for paromomycin were reported as 66.85 µM and 21.55 µM for the local and reference strains, respectively IC_{50} calculated by using online software MyCurveFit for curve fitting. There was a significant difference at $P \leq 0.05$ between the inhibitory performance on local compared to the reference strain figure as shown in Table 3-5, Figure 3-17.

Table 3-5; urease activity and paromomycin sulfate concentration for both Local and Reference strain

Well	Paromomycin sulfate con. µM	Urease activity (U/ml) for Local strain	Urease activity (U/ml) for the Reference strain
1	0	2	2
2	528.24	0	0
3	176.08	0.31	0.15
4	58.69	1.11	0.47
5	19.56	1.75	1.04
6	6.52	1.96	1.58
7	2.17	2	1.77
8	0.72	2	1.84

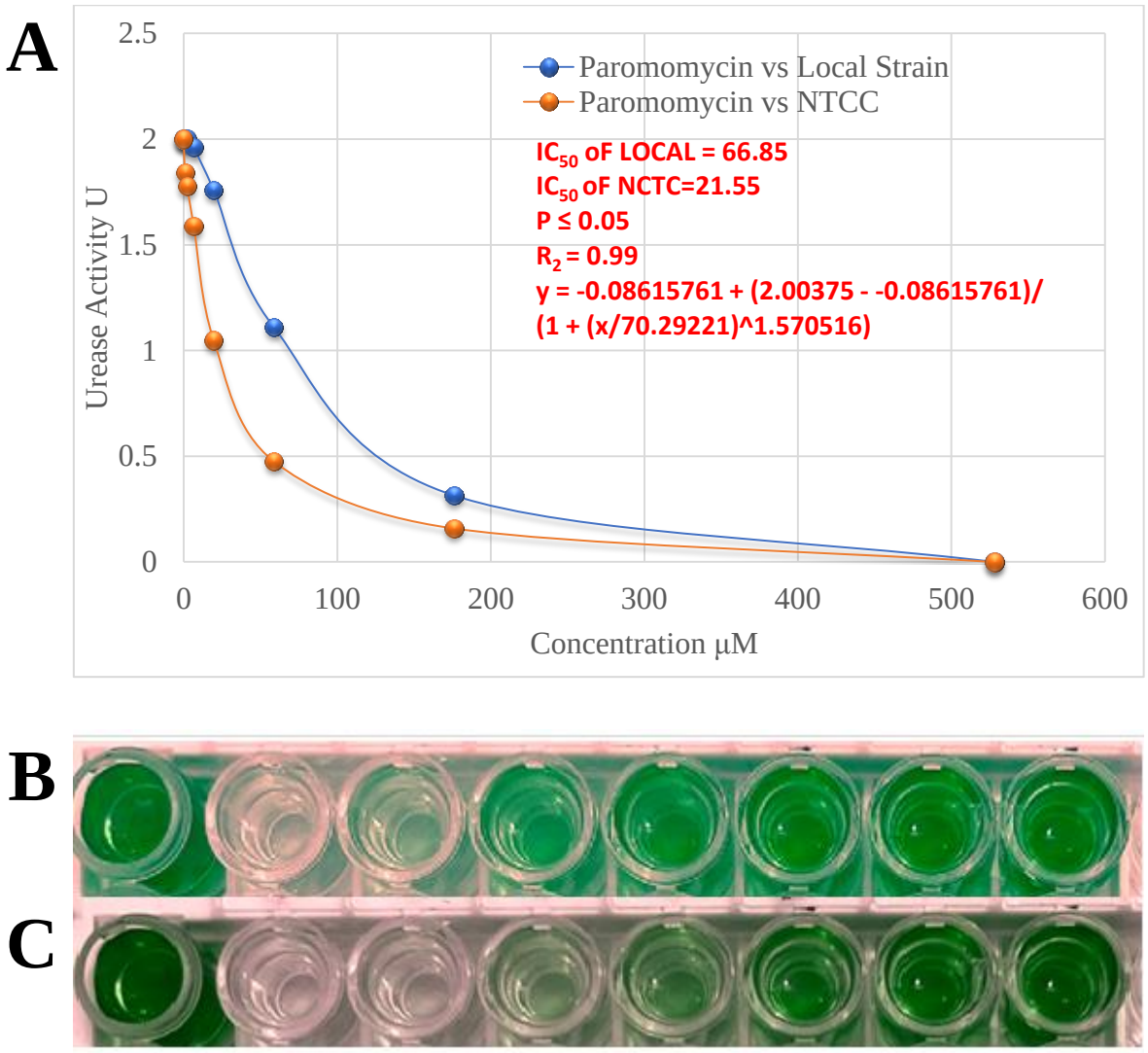


Figure (3-17): The inhibitory effect of paromomycin on urease activity. A: IC_{50} of paromomycin against urease of *H. pylori* local strain and NCTC 11916, B; inhibition of urease activity from local strain by paromomycin, C: inhibition of urease activity from reference strain by paromomycin

Results demonstrated a clear dose-dependent response for both strains. Increasing concentrations of paromomycin caused a gradual decrease in urease activity for both strains. At the lowest concentration (0 µM), urease activity was 2 U/ml for both the local and reference strains. As the paromomycin concentration increased, the urease activity decreased steadily.

At the highest concentration tested (528.24 µM), the urease activity dropped to 0 U/ml, indicating effective inhibition of urease activity by

paromomycin. Furthermore, as the paromomycin concentration decreased, the urease activity started to recover. At concentrations of 176.08 µM, 58.69 µM, and 19.57 µM, the urease activity increased to 0.16 U/ml, 0.47619 U/ml, and 1.05 U/ml, respectively, for the local strain. Similarly, for the reference strain, the urease activity increased to 0.32 U/ml, 1.11 U/ml, and 1.76 U/ml, respectively.

As the concentration of paromomycin decreased further to 6.52µM, 2.17 µM, and 0.72 µM, the urease activity reached a plateau at 1.96 U/ml and remained constant until the lowest concentration of paromomycin (0.72 µM), where the urease activity remained at 2 U/ml for local strain, in reference strain As the concentration of paromomycin decreased further to 6.52 µM, 2.17 µM, and 0.72 µM, the urease activity continued to rise, reaching 1.59 U/ml, 1.78 U/ml, and 1.84 U/ml, respectively.

3.7.2 Tobramycin

The IC_{50} values for tobramycin were reported as 82.7 µM and 35.80 µM for the local and reference strains, respectively there was a significant difference at $P \leq 0.05$ between the inhibitory performance on Local compared to the Reference strain as shown in Table (3-6), Figure (3-18).

Table 3-6 urease activity and tobramycin concentration for both local and reference strain

well	Tobramycin con. uM	Urease activity (U/ml) for local strain	Urease activity (U/ml) for the reference strain
1	0	2	2
2	695.6	0	0
3	231.9	0.49	0.20
4	77.3	1.14	0.82
5	25.8	1.20	0.85
6	8.6	2	1.92
7	2.7	2	2
8	0.95	2	2

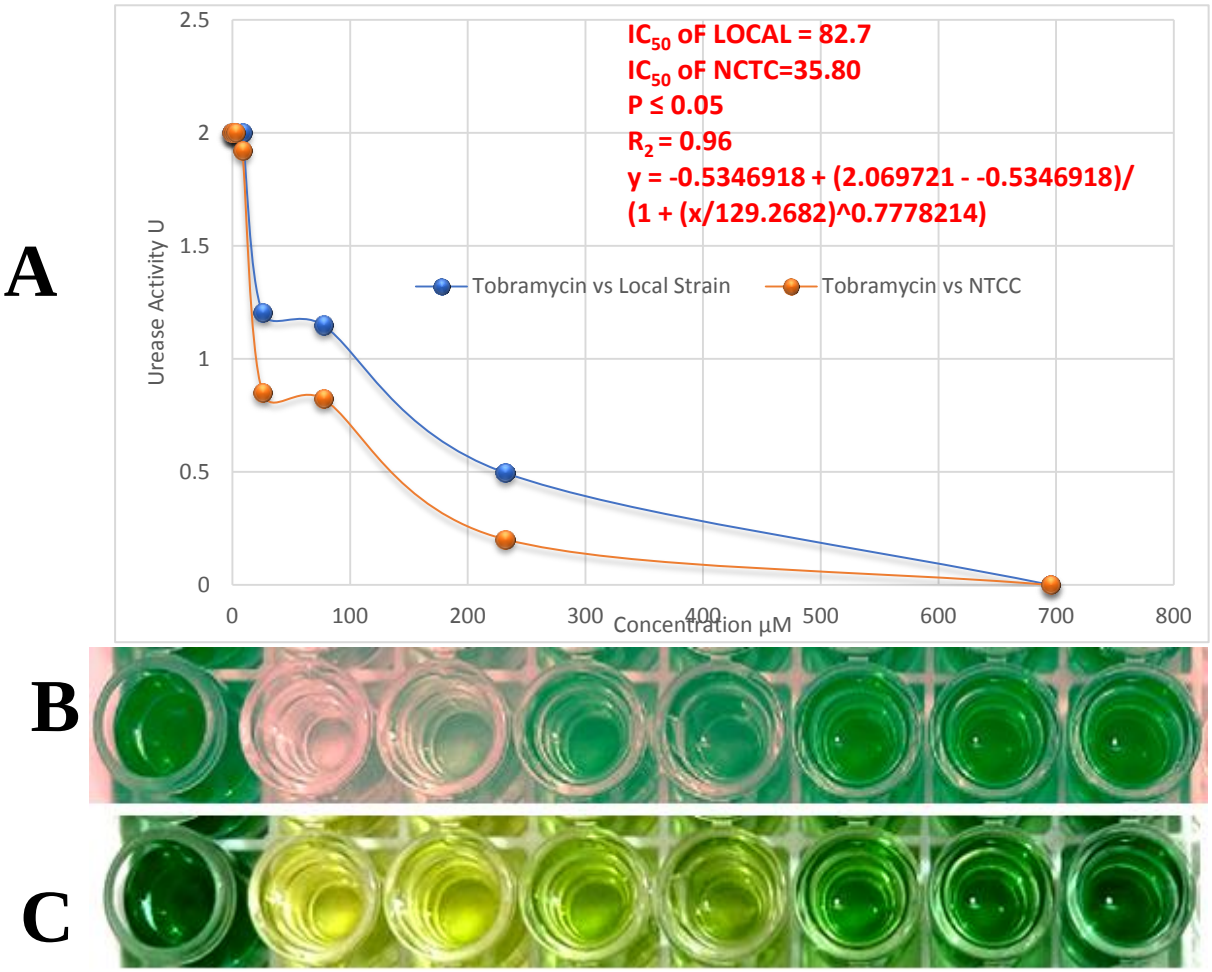


Figure (3-18) The inhibitory effect of tobramycin on urease activity. A: IC_{50} of tobramycin against urease of *H. pylori* local strain and NCTC 11916, B; inhibition of urease activity from local strain by tobramycin, C: inhibition of urease activity from reference strain by tobramycin

The experimental results revealed a concentration-dependent effect of tobramycin on urease activity for both strains. As the Tobramycin concentration increased (ranging from 0 µM to 695.60 µM), the urease activity gradually declined. At the lowest concentration tested (0 µM), the urease activity was measured as 2 U/ml for both strains. At the highest concentration (695.60 µM), Tobramycin effectively inhibited urease activity, resulting in a complete loss of activity (0 U/ml). Further analysis demonstrated that decreasing the tobramycin concentration led to the recovery of urease activity. At concentrations of 231.87 µM, 77.29 µM, and 25.76 µM,

the urease activity increased to 0.50 U/ml, 1.15 U/ml, and 1.2 U/ml, respectively, for the local strain. Similarly, for the reference strain, the urease activity increased to 0.20 U/ml, 0.82 U/ml, and 0.85 U/ml, respectively, at the corresponding concentrations of 231.87 µM, 77.29 µM, and 25.76 µM.

As the concentration of tobramycin decreased further to 8.587 µM in both strains, tobramycin did not have a significant impact on the urease activity.

3.7.3 Gentamicin

The IC_{50} values for gentamicin were reported as 85.97 µM and 41.91 µM for the local and reference strains, respectively there was a significant difference at $P \leq 0.05$ between the inhibitory performance on local compared to the reference strain as shown in Table (3-7), Figure(3-19).

Table 3-7 Urease activity and gentamicin concentration for both Local and Reference strain

Well	Gentamicin uM con.	Urease activity (U/ml) for local strain	Urease activity (U/ml) for the reference strain
1	0	2	2
2	680.95	0	0
3	226.98	0.39	0.31
4	75.66	1.09	0.75
5	25.22	1.62	1.14
6	8.40	2	1.71
7	2.80	2	1.93
8	0.93	2	2

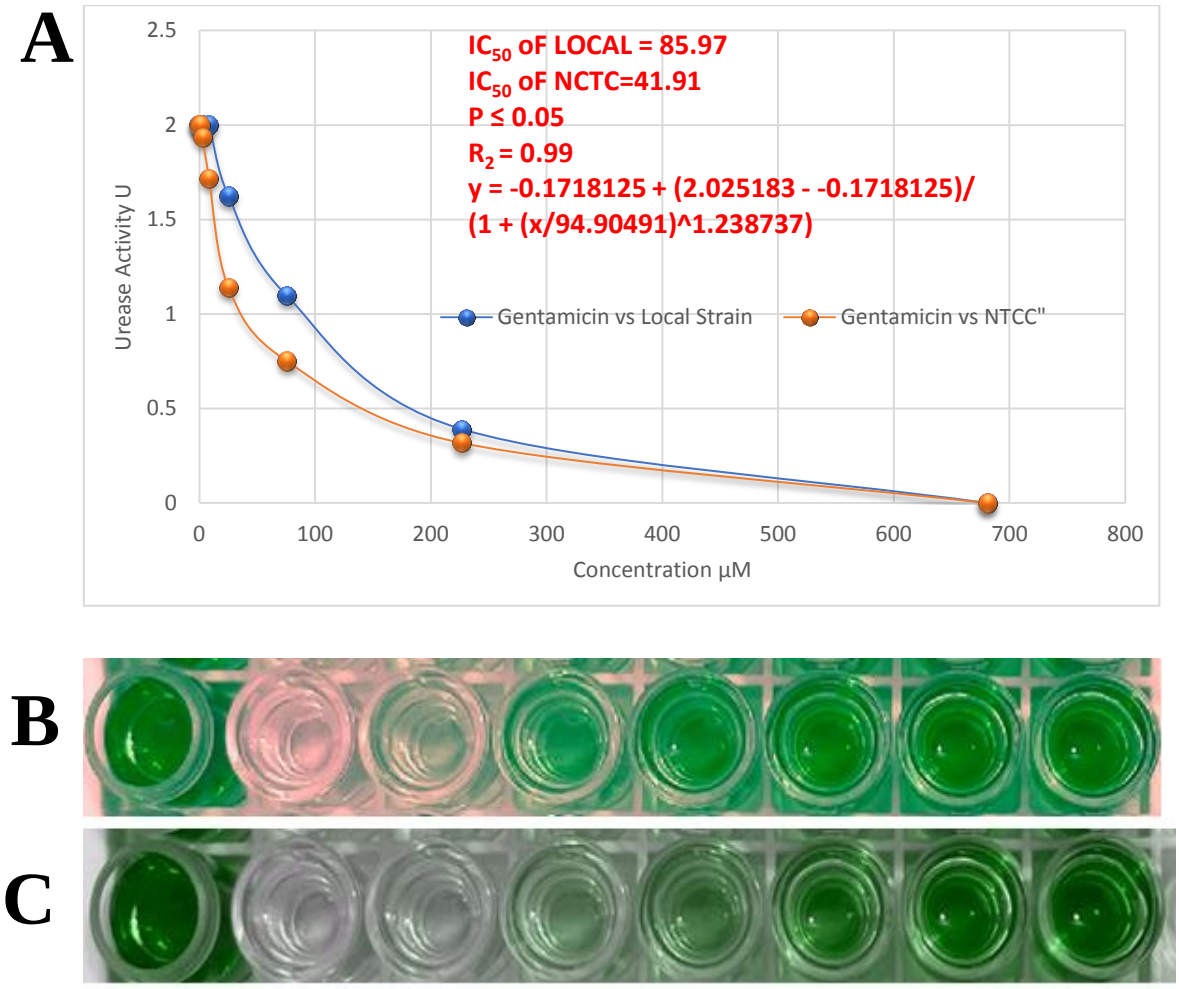


Figure (3-19) The inhibitor has the effect of gentamicin on urease activity. A: IC_{50} of Gentamicin against urease of *H. pylori* local strain and NCTC 11916, B; inhibition of urease activity from local strain by gentamicin, C: inhibition of urease activity from reference strain by gentamicin.

The experimental results revealed a concentration-dependent impact of gentamicin on urease activity for both strains. Increasing concentrations of gentamicin (ranging from 0 µM to 680.96 µM) led to a gradual decrease in urease activity. At the lowest concentration tested (0 µM), the urease activity was measured as 2 U/ml for both strains. As the gentamicin concentration increased, the urease activity steadily declined.

At the highest concentration tested (680.96 µM), gentamicin effectively inhibited urease activity, resulting in a complete loss of activity (0 U/ml) for both the local and reference strains. Further analysis demonstrated that as the

Gentamicin concentration decreased, the urease activity started to recover. At concentrations of 226.98 µM, 75.66 µM, and 25.22 µM, the urease activity increased to 0.39 U/ml, 1.10 U/ml, and 1.62 U/ml, respectively, for the local strain. Similarly, for the reference strain, the urease activity increased to 0.31 U/ml, 0.75 U/ml, and 1.14 U/ml, respectively, at the corresponding concentrations of 226.98 µM, 75.66 µM, and 25.22 µM.

As the concentration of gentamycin decreased further to 8.40 µM, 2.80 µM, and 0.93µM, the urease activity of the local strain remained constant at 2 U/ml, while the urease activity of the reference strain continued to rise, reaching 1.718412 U/ml, 1.935018 U/ml, and 2 U/ml, respectively.

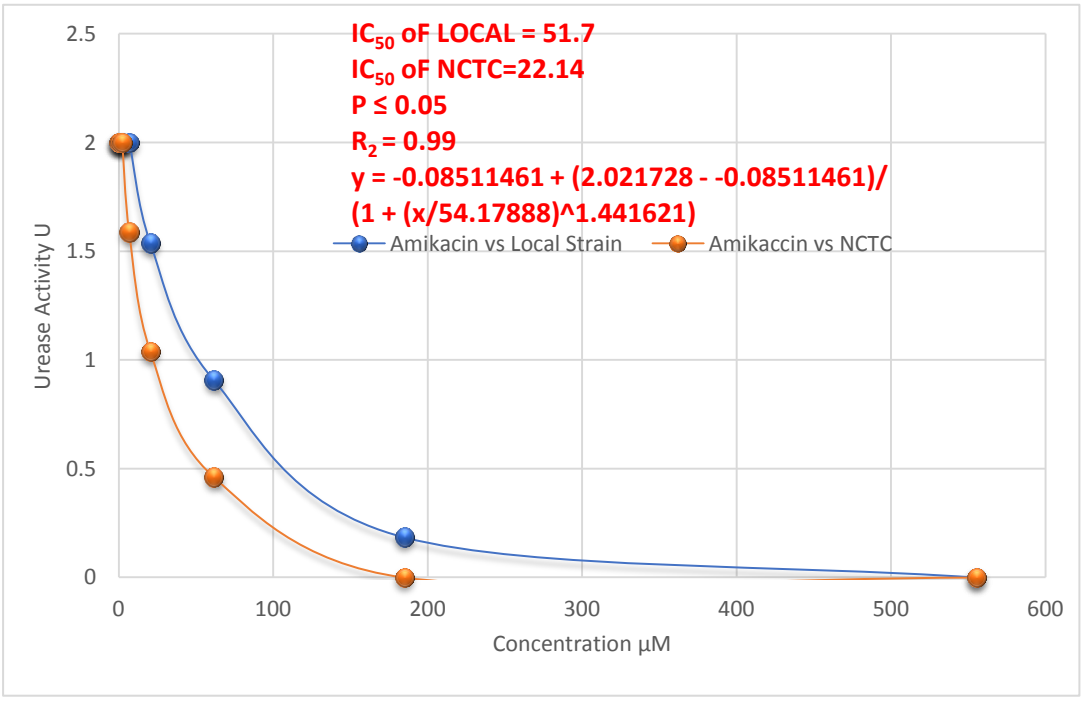
3.7.4 Amikacin

The IC_{50} values for amikacin were reported as 51.7 µM and 22.14 µM for the local and reference strains respectively, there was a significant difference at $P \leq 0.05$ between the inhibitory performance on local compared to reference strain Table (3-8), Figure (3-20).

Table 3-8 Urease activity and amikacin concentration for both local and reference strain

Well	Amikacin µM con.	Urease activity (U/ml) for local strain	Urease activity (U/ml) for the reference strain
1	0	2	2
2	555.33	0	0
3	185.11	0.19	0
4	61.70	0.91	0.46
5	20.56	1.54	1.03
6	6.85	2	1.58
7	2.28	2	2
8	0.76	2	2

A



B

C

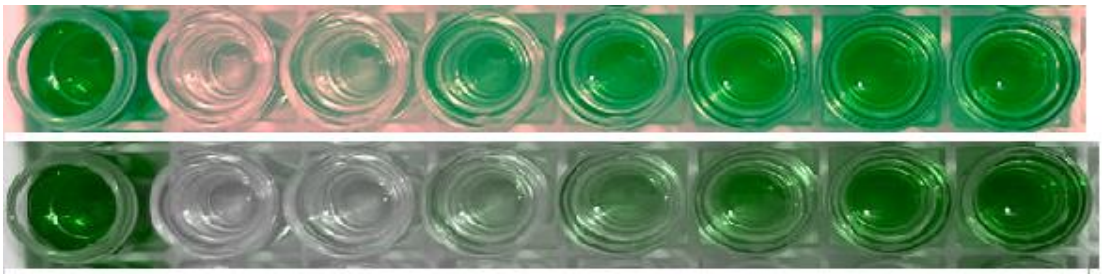


Figure (3-20) The inhibitory effect of amikacin on urease activity. A: IC_{50} of amikacin against urease of *H. pylori* local strain and NCTC 11916, B; inhibition of urease activity from local strain by amikacin, C: inhibition of urease activity from reference strain by Amikacin.

The experimental findings revealed that increasing concentrations of amikacin (ranging from 0 µM to 555.33 µM) caused a concentration-dependent decrease in urease activity for both strains. At the lowest concentration tested (0 µM), the urease activity was measured as 2 U/ml for both strains. As the concentration of amikacin increased, the urease activity gradually declined. At the highest concentration tested (555.33 µM), amikacin effectively inhibited urease activity, resulting in a complete loss of activity (0 U/ml) for both the local and reference strains. Further analysis demonstrated that as the Amikacin concentration decreased, the urease activity started to recover. For the local strain, at concentrations of 185.11 µM, 61.70 µM, and

20.57 µM, the urease activity increased to 0.19 U/ml, 0.91 U/ml, and 1.54 U/ml, respectively.

As the concentration of amikacin decreased further to 6.85 µM, 2.28 µM, and 0.76 µM, the urease activity of the local strain remained constant at 2 U/ml, while the urease activity of the reference stretched each 1.58 U/ml, 2 U/ml, and 2 U/ml, respectively.

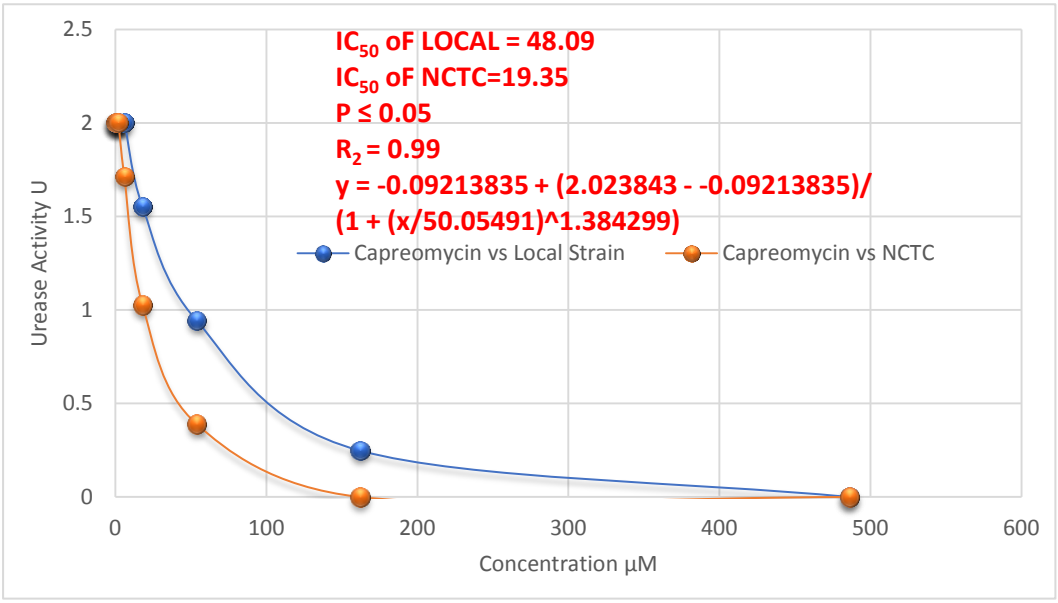
3.7.5 Capreomycin.

The IC_{50} values for capreomycin were reported as 48.09 µM and 19.35 µM for the local and reference strains, respectively There was a significant difference at $P \leq 0.05$ between the inhibitory performance on local compared to the reference strain as shown in Table (3-9), Figure (3-21).

Table 3-9 urease activity and capreomycin concentration for both local and reference strain

Well	Capreomycin µM con.	Urease activity (U/ml) for local strain	Urease activity (U/ml) for the reference strain
1	0	2	2
2	486.31	0	0
3	162.10	0.24	0
4	54.03	0.94	0.38
5	18.01	1.55	1.02
6	6.00	2	1.71
7	2.00	2	2
8	0.66	2	2

A



B

C

Figure (3-21) The inhibitory effect of capreomycin on urease activity. A: IC_{50} of capreomycin against urease of *H. pylori* local strain and NCTC 11916, B; inhibition of urease activity from local strain by capreomycin, C: inhibition of urease activity from reference strain by capreomycin.

The experimental results indicated that increasing concentrations of capreomycin (ranging from 0 µM to 486.32 µM) caused a concentration-dependent decrease in urease activity for both strains. At the lowest concentration tested (0 µM), the urease activity was measured as 2 U/ml for both the local and reference strains. However, as the concentration of capreomycin increased, the urease activity gradually declined. At the highest concentration tested (486.32 µM), capreomycin effectively inhibited urease activity, resulting in a complete loss of activity (0 U/ml) for both the local and reference strains. Further analysis revealed that as the Capreomycin concentration decreased, the urease activity started to recover. For the local

strain, at concentrations of 162.11 µM, 54.03 µM, and 18.01 µM, the urease activity increased to 0.25 U/ml, 0.94 U/ml, and 1.55 U/ml, respectively.

In the case of the reference strain, at concentrations of 162.11 µM and 54.03 µM, the urease activity remained at 0 U/ml, indicating complete inhibition. However, as the capreomycin sulfate concentration decreased further to 18.01 µM, 6.01 µM, and 2.00 µM, the urease activity started to recover, reaching 1.03 U/ml, 1.71 U/ml, and 2 U/ml, respectively.

the IC_{50} values of different drugs, including paromomycin, tobramycin, gentamicin, amikacin, and capreomycin sulfate, on urease activity in both local strains and reference strains as shown in Figure (3-22).

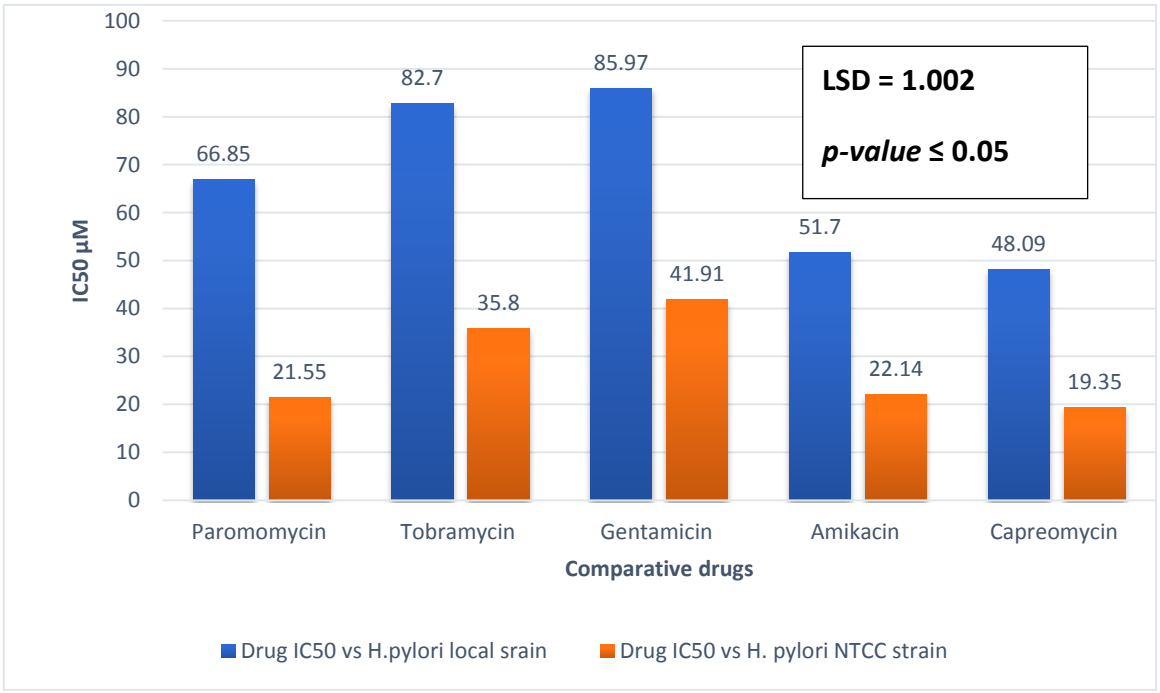


Figure 3-22: Concerning the IC_{50} values of aminoglycosides on *H. pylori* urease between the local and reference strains

Figure 3-22 illustrated that the urease from the local *H. pylori* strain emerges relatively high resistance towards the inhibitory action of aminoglycoside in general when compared to NCTC 11916 strain in

which capreomycin showed the significantly strongest p-value ≤ 0.05 inhibitory effect among the all aminoglycosides (from the lowest IC_{50}) meanwhile capreomycin manifested the same significantly strongest p-value ≤ 0.05 inhibitory effect on urease of the NCTC 11916 strain compared to other aminoglycosides although all of these aminoglycosides gave remarkable results.

Chapter Four

Discussion

4. Discussion

4.1 Bacterial isolation and Identification

Helicobacter pylori is difficult to isolate in a suitable media due to its micro-aerophilic requirements fragile nature of the organism, the unavailability of adequate transport media, and the presence of contaminants in the culture medium a fact recorded by (Sarma, *et al.*, 2016).

H. pylori is considered a fastidious bacterium which has difficult to grow in laboratory due to their complex ,decrease neutrition culture and environment with a special requirement for incubation represented by 10 % CO_2 and high humidity; therefore, it is difficult to grow and shows a low growth rate. several selective agar media have been used for the isolation of *H. pylori* from fresh biopsies, one of the frequently used media is Columbia agar (Chomvarin, *et al.*, 2006). Many factors are involved in the successful culture of *H. pylori* including incubation time, tissue processing procedure (cutting of the tissue biopsy), composition of culture media that include antibiotic supplements and growth supplements, environment conditions (CO_2 and humidity), potential patchy distribution of microorganisms on the gastric mucosa, contamination of biopsy forceps, and loss of viability of *H. pylori* during transport. Cultural methods for *H. pylori* isolation display a limited sensitivity and now decreased due to the small population of *H. pylori* that colonizes the stomach, and two or more biopsy samples from different locations are needed for accurate diagnosis (Miftahussurur and Yamaoka, 2016).

The results of cultivation were found to be obtained by (Salih Barik A., *et al.*, 2002) who recorded a low percentage (14.9%) for the isolation of *H. pylori* from 87 patients through conventional cultural methods. Closely results were recorded by other researchers in 2016 during the isolation of *H. pylori* from a biopsy obtained from 100 patients, and only 9 specimens showed positive culture (Sarma, *et al.*, 2016). On the other hand, as reported by Saadi

and Saeed in 2019, the bacteria was isolated with a high ratio (32.7%) from gastric biopsies of 86 patients using conventional cultural methods (Saadi and Saeed, 2019). Also (Ibrahim, 2011) recorded a high ratio that reached (60%) for the isolations of *H. pylori* from 25 patients with peptic ulcer. In addition, a study by Al Sulami, *et al.* in 2008 reported the isolation of *H. pylori* in 92 patients from a total of 136 with a ratio of (67.6%)(Al Sulami, *et al.*, 2008).

In a study by Shiota *et al.*, 2015 took 135 Biopsies sample in the USA. The results indicated (20.6%) were positive for *H. pylori.* In another study by (Idowu, *et al.*, 2019) 444 patients detected *H. pylori* in 115 (25.9%) by culture analysis and 217 (48.9%) by direct PCR method.

In recent years, there has been a noticeable decline in *H. pylori* isolation by culture from biopsy samples due to many difficulties, such as culture maintenance, a time-consuming cultivation process that requires specific physical conditions and microbiologist skilled experts to confirm the diagnosis (De Francesco, *et al.*, 2010). Some authors using Chocolate, Brucella, and Brain Heart Infusion (BHI) agars containing 5% horse blood provided effective culture conditions for the growth of *H. pylori* (Lee, *et al.*, 2017).

4.2 Molecular detection of *H. pylori*

Molecular identification using *H. pylori* housekeeping genes for accurate identification and to evaluate the causes of gastroduodenal diseases (Suzuki, *et al* ., 2012).

The sensitivity and specificity of the molecular methods for the identification of bacteria in the infectious diseases were confirmed by a huge number of studies. Moreover, the use of the PCR diagnosis method by amplifying the conserved 16S rRNA gene which represents the most common housekeeping gene marker for the identification of *H. pylori* in addition to its rule to detect other species that are close to a phylogenetic cluster of

Helicobacter bacteria. *H.pylori*-specific polymerase chain reaction was detected in the 16S rRNA gene and It's more reliable due to the misidentification of microorganisms when using cultural technique (Srinivasan *et al.*, 2015).

The results we obtained were confirmed by a previous study by Al-Sulami and his team when they used 16S rRNA for the detection of *H. pylori* as they recorded 7 positive isolates out of 14 that were positively diagnosed as *H. pylori* through morphological and biochemical methods (Al-Sulami, *et al* ., 2012).

The negative results that were obtained might be due to several factors as described by a previous study (Al-wendawi, 2020) in which they attributed the negative results to the mistakes that occurred during the collection of the biopsy sample from a site other than the exact stomach ulcerative area or the collected bacteria from the ulcer biopsy is not *H. pylori*. Another explanation for the negative diagnosis through molecular methods might be the contaminated media with other types of bacteria such as Proteus spp. and Klebsiella spp; The source of contamination might acquired during obtaining the biopsy, transporting the sample and preparation of the blood added to the Columbia or Brucella agar (Clayton, *et al.*, 1992).

4.3 Optimum incubation time for urease production

The results of this study indicate that both the local strain and the NCTC 11916 strain exhibit a similar pattern of urease activity over time. The enzyme activity grew gradually from 24 to 72 hrs. and then decreased at 96 hours at 37c pH 7.0 media. These data point to a best incubation period of 72 hrs. for both strains, urease activity peaks appear at 72 hrs. before dropping.

The observed increase in enzyme activity at this incubation time can be attributed to the bacteria's growth and metabolic activity. The production and release of the enzyme by microbial cells may result in a buildup of urease,

resulting in the observed increase in activity. Other factors, such as enzyme denaturation or substrate depletion, may contribute to the drop-in urease activity after 72 hours.

The results of this study are compatible with the findings of Matongo and Nwodo (2014). They also found that throughout the growth phase of *H. pylori*, urease activity increased at an ideal peak at 72 hours, which is related to the late exponential phase. This finding contributes to the growing body of information demonstrating *H. pylori* urease is mostly found in the cytoplasm during the exponential growth phase.

4.4 Purification of urease

4.4.1 Ammonium sulphate precipitation

The findings of this study indicate that the optimal concentration of ammonium sulfate for urease precipitation was 70% for both the local strain and the reference strain. This concentration allowed for the efficient precipitation of proteins.

The specific activity of urease, determined as 0.13 U/mg for the local strain and 0.12 U/mg for the reference strain, indicates the enzyme's catalytic efficiency per unit of protein. The comparable specific activity values before and after ammonium sulfate precipitation suggest that the purification process did not significantly affect the enzyme's specific activity. This further supports the suitability of 70% ammonium sulfate saturation for urease purification.

Because proteins have hydrophilic amino acid side chains provided by essential amino acids, they are soluble in water solutions. Ammonium sulfate interferes with these interactions between amino acid side chains and water by decreasing the available water, reducing the protein's solubility. this lead protein exits from the solution. Various proteins would separate at various ammonium sulfate saturation levels depending on their hydrophobicity. To

disrupt the protein-water interactions, higher hydrophobicity, and a greater ammonium sulfate concentration would be necessary to enable its precipitation (Khwen, 2021).

These findings align with previous studies that have utilized ammonium sulfate precipitation for enzyme purification, demonstrating its efficacy in preserving enzymatic activity (Singh, *et al*., 2017). The use of this technique allows for a significant reduction in the complexity of the enzyme mixture, facilitating downstream applications and analysis

The study conducted by (Cho, *et al.*, 2002) confirmed that the majority of *H. pylori* urease was indeed precipitated when utilizing a 60% and 80% saturated solution of ammonium sulfate. This suggests that the urease enzymes of *H. pylori* tend to undergo precipitation under these specific conditions.

4.4.2 Purification of urease by preparative HPLC

The use of preparative HPLC in purifying urease from *H. pylori* allows for the separation and isolation of individual components based on their physicochemical properties. The C3 column, known for its hydrophobic interaction chromatography properties, was employed to achieve a high degree of separation for urease from other molecules in the *H. pylori* extract (Mant, *et al.*, 2007).

The urease activity observed in this fraction confirms that the purification steps effectively enriched the target enzyme and removed unwanted protein. The retention time of 4.8 min suggests that urease has a relatively low affinity for the C3 stationary phase and can be eluted relatively early in the chromatographic run.

4.5 Docking study for urease inhibition

The reported cryo-electron microscopy (EM) structure of *H. pylori* urease enzyme with an inhibitor (3,5-dimethyl imidazolyl-sulfanyl-N-hydroxy

acetamide) (pdp code: 6ZJA),6ZJA represent *Helicobacter pylori* urease with inhibitor bound in the active site is selected as a target for this study

The docking scores obtained from the molecular docking simulations provide insights into the potential binding affinities of the drugs towards *H. pylori* urease inhibition. Among the drugs investigated, paromomycin exhibited the highest docking score of -15.339, indicating a strong binding affinity with the target protein. gentamicin followed closely with a docking score of -13.864, suggesting favorable interactions as well. amikacin, tobramycin, and capreomycin demonstrated slightly lower docking scores but still exhibited notable binding affinities toward the target protein.

There are no reports in the literature citing the activity of tobramycin, capreomycin, paromomycin, amikacin, and gentamycin as urease inhibitors, which makes this work the first one to associate anti-uratolytic activity with these compounds.

Kafarski and Talma, (2018) mentioned the two popular Levofloxacin and Ciprofloxacin, as well as their analogs, appeared to be quite promising inhibitors of *Helicobacter pylori* enzymes. Molecular modeling suggests their binding with the carboxylic group interacting with active site nickel ions. However, the mechanism of additional covalent interaction with the enzymatic cysteine similar to this observed for simple quinones, cannot be ruled out.

Several studies used 6zja as an active site for inhibiting urease enzyme Abd Elghani *et al.*, (2023) indicating the mechanism of the anti-*Helicobacter pylori* activity, An in silico study on D-Limonene has been performed, using *H. pylori* urease enzyme 6zja, which revealed that D-Limonene showed promising binding scores.

The study by de Paula, *et al.*, (2023) about the effect of urease Activity of kaempferol aberrative as a urease inhibitor by using the 6zja model for urease showed promising binding scores.

4.6 Effect of aminoglycoside antibiotic on urease activity *In vitro* study

The findings of this study provide valuable insights into the inhibitory effects of aminoglycoside antibiotics, specifically paromomycin, tobramycin, gentamicin, amikacin, and capreomycin, on the urease activity of *H. pylori*. These antibiotics exhibited concentration-dependent inhibition, suggesting their potential as therapeutic agents for targeting urease activity in *H. pylori* infections. The results conformed to the docking study which showed paromomycin, tobramycin, gentamicin, amikacin, and capreomycin can bind to the active site of urease and then inhibit urease activity.

paromomycin demonstrated a dose-dependent inhibitory effect on urease activity, with IC_{50} values of 66.85 µM and 21.55 µM for the local and reference strains, respectively. As shown in figure (3-17) The higher IC_{50} value of the local strain indicated that it was less susceptible to paromomycin inhibition than the reference strain. This discrepancy might be due to mutations or modifications within the local strain, which could affect the structure or degree of expression of the urease enzyme and bacterial resistance .

Tobramycin inhibited urease activity in a concentration-dependent manner, with higher doses resulting in more significant inhibition. tobramycin's IC_{50} values for the local and reference strains were 82.7 µM and 35.80 µM, respectively. As shown in figure (3-18) The discrepancy in IC_{50} values between strains showed that was susceptible to tobramycin in different ways, probably due to variations in the urease gene and bacterial resistance.

Gentamicin inhibited urease activity in a concentration-dependent way, with larger dosages completely inhibiting it. as shown in figure (3-19) the IC_{50} values for gentamicin were reported as 85.97uM and 41.19 uM for local and reference strains respectively. at lower doses, urease activity was recovered, especially in the reference strain. This recovery at lower doses may

be attributable to gentamicin's reversible binding mechanism with the urease enzyme, which allows the enzyme to reactivate once the antibiotic concentration drops below a specific threshold.

Amikacin inhibited urease activity in both the local and reference strains. amikacin at higher doses significantly suppressed urease activity,as shown in figure (3-20) IC_{50} values were reported as 51.7uM and 22.14uM for local and reference strain respectively, although lower quantities allowed for partial recovery, especially in the local. The variable responses to amikacin among the strains might be due to genetic differences or adaptations within the local strain .and bacterial resistance .

Capreomycin inhibited urease activity concentration independently in both the local and reference strains, with IC_{50} values of 48.09 µM and 19.35 µM, respectively as shown in (figure 3-21) .However, as compared to the reference strain, the local strain required a greater dose of capreomycin for equal inhibition, indicating probable genetic changes or adaptations in the local strain and bacterial resistance .

Several discrepancies may be found when comparing the inhibitory actions of various aminoglycoside antibiotics. paromomycin, tobramycin, gentamicin, amikacin, and capreomycin all demonstrated concentration-dependent inhibition of urease activity in *H. pylori*. However, their potencies, as indicated by the IC_{50} values, varied among the antibiotics and strains. Additionally, these antibiotics belong to the class of aminoglycosides, which have broad-spectrum antimicrobial activity. This characteristic provides potential advantages in cases of *H. pylori* coinfections or polymicrobial gastric diseases, as these antibiotics can target other bacterial pathogens in addition to inhibiting urease activity. Such as proteus mirabilis and staphylococcus saprophyticus which has ability to produce urease as *H.pylori.*

This study suggested according to the docking study the inhibition of urease enzyme by tobramycin, capreomycin, paromomycin, amikacin, and gentamicin due to direct binding to the active site of urease enzyme.

Urease inhibition holds significant therapeutic potential due to its broad range of applications. Inhibition of urease activity has been explored as a strategy for treating infections causd by urease-producing bacteria, such as *Helicobacter pylori*, which is implicated in gastric ulcers and stomach cancer (Kafarski and Talma, 2018).

One antibiotic that has been reported to exhibit inhibitory effects on urease activity is acetohydroxamic acid (AHA). AHA is a drug. It works by inhibiting the enzyme urease, which is responsible for the hydrolysis of urea to ammonia and carbon dioxide. By inhibiting urease activity, AHA reduces the production of ammonia, thereby creating an unfavorable environment for urease-producing bacteria to thrive (Yang *et al.*, 2022).

The effect of antimicrobials on urease activity was related to the nitrogen use efficiency of urea. Sulfamethazine, enrofloxacin, and tetracycline, on the activity of urease and its mechanism (Wang, *et al.*, 2020). The effect of sulfamethazine, enrofloxacin, and tetracycline on urease activity within a certain concentration range (0-800 μmol/ml) was determined by sodium phenol sodium hypochlorite colorimetry by a study by (Wang, *et al.*, 2020).

The high concentration of tetracycline significantly inhibited the activity of urease ($P \leq 0.05$) and the influence degree increased with the increase of benzene ring number. sulfamethazine, enrofloxacin, and tetracycline could form a 1:1 complex with urease, resulting in static quenching of intrinsic fluorescence of urease and changing the micro-environment of amino acid residues in the urease active center resulting in the hydrophilicity of the active site to change, thus affecting the activity of

urease. sulfamethazine, enrofloxacin, and tetracycline combined with amino acid residues in the urease active center produced hydrogen bonds.

Urease inhibition of amikacin and its derivatives was mentioned by (Anwar, *et al.*, 2020). In this study, Schiff base derivatives of amikacin were synthesized and evaluated for their anti-urease activity. The urease inhibitory assay revealed that the tested compounds exhibited potent activity, with the standard amikacin displaying an IC_{50} value of 3.992 ± 1.638 µM. The Schiff base derivatives exhibited IC_{50} values ranging between 1.955 ± 0.832 µM to 5.696 ± 1.927 µM, surpassing the inhibitory potential of the standard drug. These findings were consistent with computational analysis, suggesting specific binding of the Schiff base derivatives to the active site of the enzyme, resulting in enhanced enzyme inhibition.

4.7 Comparative study between traditional anti-urease drugs and tobramycin, capreomycin, paromomycin, amikacin, and gentamycin.

Unlike the drug used in this study, Acetohydroxamic acid (AHA) was a synthetic urea derivative with half live 5-10 hr. AHA is act as reversible antagonist of bacterial enzyme urease has no direct antimicrobial action used in the treatment of certain types of urinary tract infections and conditions associated with excessive ammonia production infection of urinary tract (chronic urinary tract infection) , also used as adjunctive therapy with antibiotic for treatment of blader infection ,not commonly used as a treatment specifically for *H. pylori* infection .over dose lead to anorexia ,malaise,lethargy, (Milo, *et al.*, 2021).

While elemental ions such as mercury (Hg2+) and silver (Ag+), humic acid derivatives, and thiols have shown inhibitory effects on urease activity, their use as therapeutic agents for *H. pylori* infection in humans is limited.

Elemental ions like (Hg2+) and (Ag+) are toxic and can have harmful effects on human health (Zhao, *et al.*, 2018). Therefore, they are not used as treatments for *H. pylori* infection due to their potential for toxicity.

enrofloxacin is a compound that originates from a group of fluoroquinolones that is widely used in veterinary medicine as an antibacterial agent (this antibiotic is not approved for use as a drug in humans). It reveals strong antibiotic activity against both Gram-positive and Gram-negative bacteria. The high efficacy of this molecule has been demonstrated in the treatment of various animals on farms and other locations. However, the use of enrofloxacin causes severe adverse effects, including skeletal, reproductive, immune, and digestive disorders, Grabowski, *etal.,* 2022,unlike aminoglycosides which included tobramycin, capreomycin, paromomycin, amikacin, and gentamicin can be used for humans.

Humic acid derivatives was organic molecules which play role in plant growth ,increase soil physical and biochemical activities by improving texture ,water holding capacity .have shown inhibitory effects on urease activity in laboratory studies, but their use as specific treatments for *H. pylori* infection in humans is still under investigation. Further research is needed to determine their safety, effectiveness, and optimal dosage in human clinical settings. The effectiveness of essential oils in inhibiting urease activity in vivo and their clinical relevance in treating *H. pylori* infections is still being explored. While tobramycin, capreomycin, paromomycin, amikacin, and gentamicin was FDA approved drugs furthermore have antimicrobial activity and direct anti-urease effect on urease produced by *H. pylori*.

Conclusions and Recommendations

Conclusions :-

1. The most effective drugs on urease inhibition was paromomycin with the highest negative score (glide docking score) which give spontaneous reaction and suggesting a potentially strong binding affinity with urease for *Helicobacter pylori*.
2. *In silico* results indicate that paromomycin, amikacin, gentamicin, tobramycin, and capreomycin can bind to urease at the active site.
3. The experimental results indicate that paromomycin ,amikacin, gentamicin, tobramycin, and capreomycin have inhibitory effects on urease activity by using molecular docking technique.
4. The finding of this study have shown dose dependent response increasing concentration of aminoglycosides gradually decrease in urease activity for both local and reference strains.
5. Urease activity measured by using spectrophotometer was 2 unit/ml for both local and reference strains.

Recommendations:-

1. Highlighting for further experiment and researches for the purpose of developing these aminoglycosides for best and convenient clinical use as well as study of absorption, distribution and metabolism of them for this purpose.
2. *In vivo* studies and clinical trials are warranted to assess the potential of aminoglycosides as a therapeutic agents for Helicobacter pylori infection.
3. Investigation about the metronidazole and clarithromycin resistance genes

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الخلاصة:

مرض القرحة الهضمية هو اضطراب يسبب تقرحات مفتوحة في المعدة أو الجزء العلوي من الأمعاء الدقيقة. تفرز بكتيريا الحلزونية البوابية *H. pylori*، وهي البكتيريا التي تسبب معظم القرحة الهضمية ، إنزيمًا يسمى اليورياز ، مما يزيد من تطور المرض. يحلل اليوريا اليوريا في المعدة ، وتنتج الأمونيا التي تساعد في معادلة حامضية المعدة. تعطي هذه العملية ميزة لاستعمار الحلزونية البوابية

H. pylori و تدمر بطانة المخاط الواقية وتدمر الحاجز المعدي المعوي ، مما يؤدي إلى حدوث التهاب وتطور القرحة. علاوة على ذلك ، فإن الاستجابة المناعية التي تسببها الحلزونية البوابية تزيد من تلف الأنسجة. تسُتخدم العلاجات لإزالة الحلزونية البوابية ، وكذلك الأدوية لتقليل إنتاج اليورياز وتعزيز التئام القرحة.

شملت عينات الدراسة سبعين مريضًا تم جمع العينات مباشرة من الأشخاص في وحدة المنظار في مركز كربلاء لأمراض الجهاز الهضمي والكبد في مستشفى الحسين التعليمي. شخصت جميع العينات بواسطة اختبار كشف مستضد سريع لـ*H. pylori* ، ثم تم أخذ عينات الخزعة في حالة ظهور نتيجة إيجابية. أظهرت النتائج أن ٥٨ (٨٢.٨%) مريضًا من بين ٧٠ مريضًا يعانون من عدوى *H. pylori*وفقًا لاختبار كشف مستضد سريع، بينما أظهرت نتائج زرع الخزعات ١٢ (١٧.١٤%) يعانون من اصابة بالبكتريا *H. pylori*.

علاوة على ذلك، اوضحت هذا الدراسة الوقت المثلى لانتائج الإنزيم في عزلات *H. pylori*. أظهرت النتائج أن نشاط الإنزيم يصل إلى ذروته بعد ٧٢ ساعة في درجة حرارة ٣٧ مؤوية ، مما يشير إلى الفترة الزمنية المثلى لنشاط الإنزيم.

استخدم عدد من خطوات التنقية للحصول على إنزيم اليورياز المنقى من كل من السلالة القاسية والسلالة المحلية من الحلزونية البوابية. تضمنت عملية التنقية الكروماتوجرافيا السائلة عالية الأداء التحضيري (HPLC). زاد الفعالية النوعية للإنزيم المنقى ، مما يشير إلى نجاح عملية التنقية اختلفت النسبة المئوية للحصول على الإنزيم المنقى طوال خطوات التنقية، مع وجود خسائر طفيفة في بعض المراحل.

تم إجراء فحص افتراضي للكشف عن مثبطات إنزيم اليورياز في *H. pylori*. بواسطة إجراء محاكاة رصد الجزيئات با ستخدام برنامج Glide، وتم تحليل العديد من المركبات من قاعدة بيانات zinc15.

من بين المركبات ذات التصنيف الأعلى، أظهر ت paromomicin و tobramycin و amikacinو capreomycinو gentamycin ارتباطاً قوياً لانزيم اليورياز المنتج بواسطة بكتريا *H.pylori*

تم إجراء اختبار تأثير التثبيطي للإنزيم المنقى باستخدام الادوية التي رشحت بواسطة المحاكاة . أظهرت النتائج أنه مع زيادة تركيز المضادات الحيوية، تنخفض نشاطية اليورياز بشكل مستمر. تشير النتيجة إلى أن paromomycin وamikacin وgentamicin وtobramycin وcapreomycin لها تأثيرات تثبيطية على فعالية انزيم اليورياز.

تتمتع الامينوكلايكوسيدات بطيف واسع مضتد للجراثيم ونشاط مبيد للجراثيم سريغ المفعول وخصائص كيميائية وخلركية دوائية مناسبة تجعلها فئة مفيدة سريريا من الادوية عبر العديد من أنواع العدوى وقد أدى فهم المحركات السمية والفعالية الى تنفيذ أنظمة جرعات محسنة تعمل على تحسين السلامة مع الحفاظ على الفعالية تحمل الأمينوغليكوزيدات مزايا كبيرة مقارنة بالفئات الأخرى من المضادات الحيوية. تتضافر تأثيراتها المبيدة للجراثيم مع أدوية أخرى، وخاصة بيتا لاكتام، وهي مضادات حيوية قوية واسعة النطاق؛ على عكس البنسلين، فإنها تظهر فعالية كبيرة ضد مسببات الأمراض الهوائية سلبية الغرام، بما في ذلك البكتيريا المعوية والزائفة .

جمهورية العراق
وزارة العليم العالي والبحث العلمي
جامعة بابل/ كلية الطب
قسم الادوية

تقييم الادوية المرشحة كمثبطات لإنزيم اليورياز لهيليكوباكتر بيلوري في الحاسوب وفي المختبر

رسالة

مقدمة الى مجلس كلية الطب ولجنة الدراسات العليا/ جامعة بابل

كجزء من متطلبات نيل درجة الماجستير في الادوية/ الادوية والسموم

من قبل:

تماضر محمد علي عبد الحسن احمد

(بكالوريوس صيدلة ٢٠١٢ – ٢٠١٣)

كلية اليرموك الجامعة

باشـــراف

الاستاذ المساعد
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ماجستير في علم الادوية

المحاضر
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