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Effect of some antibiotics and alcoholic extracts of *Loranthus europaeus* on growth of *Helicobacter pylori* by using nanochitosan

A Thesis

Submitted to the council of College of Science/Al-Nahrain University as a partial fulfillment of the requirement for the Degree of Master of Science in Biotechnology

By

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Summary

This study was aimed to an attempt for early detection of *Helicobacter pylori* bacterium by relying the relationship of ammonia production with changing the color of the media, pH, bacterial growth density and the ulceration type (acute or hemorrhage). The study also aimed to use biodegradable synthetic nanomaterials as a mean of delivery for therapy as well as knowledge of their efficiency as a therapeutic agent.

H. pylori considered as the most causative agent of stomach and duodenum ulcer which may develop into stomach or duodenum cancer. New broth medium (ZAN-1) and modified media (Brain Heart Infusion BHI, and Tryptic Soybean Broth, TSB) were used to stimulate and support bacterial growth since it is difficult to be grown and isolated. Results of selection acute and hemorrhage isolates were based on ammonia production within 2 hours and changing the color of fast urea solution in addition to the histological tests, immunological tests, confirmation tests and genomic profile.

A nano carrier from chitin were developed to produce chitosan by ionic gelation method and used it as carrier and therapeutic agent.

Results obtained could be summarized as follows:

A total of 115 biopsies specimens were collected from gastroscopy department in Alkadumia hospital during June to September,2014 from adult aged from 40 to 60 years. Eighty of these samples for males and 35 for females, Ten isolates were selected for each acute and hemorrhage infection depending on the direct tests in hospital; and based on the laboratory and hospital tests , one acute isolate (A-1) and one hemorrhage isolate (H-1) were selected.

Genomic profile study revealed that acute and hemorrhage isolates (A-1, H-1) of *H. pylori* have only genomic DNA and absence of plasmids.

Susceptibility test showed that A-1 was less response to antibiotics than H-1.

Phenate method was selected to be the best method to estimate the concentrations of ammonia in culture media of this bacterium, results indicated that acute isolate is more active in ammonia production (0.41µg/ml) and changing the color of media comparing with hemorrhage isolate which was (0.38 µg/ml) ammonia.

Prepare nanochitosan and test its shape using SEM.

Prepare of *Loranthus europaeus* extract.

Preparation of nanochitosan loaded with antibiotic and *L. europaeus* extract and prepare lyophilized mixtures.

Semi- quantitative applications and results showed clear superiority thatA-1 isolate was more sensitive to the nanachitosan loaded with the antibiotic (ciprofloxacin) and give inhibition zone 48 mm after 48 hr incubation, while when used ciprofloxacin alone it give15 mm inhibition after 48 hr of incubation. Quantitative applications illustrated that the higher concentration of ammonia production could be detected by spectrophotometer was 5.2 µg/ml for A-1 isolate using inoculm 3×10^5 cell/ml for nanochitosan loaded with ciprofloxacin. *Invitro* application showed that the nanochitosan with ciprofloxacin have more inhibitory action on the cells. (Ammonia production was 4.5µg /ml compared with the ciprofloxacin alone 41µg/ml).

List of contents

List of Tables

List of Figures

List of Abbreviation

Abbreviations	*Full name*
CagA	Cytotoxin Associated Gene A
CD	Cluster of Differentiation
CHI	Chitosan
DD	Degree of Deacetylation
ELISA	Enzyme-Linked Immunosorbent Assay
FTIR	Fourier Transform Infrared spectroscopy
MALT	Mucosa-Associated Lymphoid Tissue
MHC	Major Histocompatibility Complex
OMPs	Outer Membrane Proteins
PCR	Polymerase Chain Reaction
SEM	Scanning Electro Microscope
TCC	Triphenyltetrazolium chloride
TPP	Tripolyphosphate
UreA	Urease Enzyme A
UreB	Urease Enzyme B
VacA	Vacuolating cytotoxine

Chapter One

Introduction and Literature Review

1.1 Introduction

Injuries of digestive system are considered as the most common diseases around the world, regardless of the level of development where it is related to poor nutrition and luxury as well as genetic and environmental impacts.

Ulcers were not infectious diseases attributed to bacterial infections of not long ago. It has been discovered by Warren *et al*., (1983) and regarded the starting point of a revolution concerning the concepts and management of gastroduodenal .

Helicobacter pylori is one of the most common bacterial pathogens that infects human around the worldwide which acquired in the early childhood and is carried throughout lifetime if not treated with antimicrobial agents (Weyermann *et al*., 2006).

H.pylori is one of the most successful pathogenic bacteria that induces gastritis in all infected patients and considered as class-I carcinogen that specifically colonizes the gastric epithelium of humans as a unique niche, where it can induce inflammatory disorders such as (ulceration and chronic gastritis) and malignant neoplastic diseases (mucosa-associated lymphoid tissue [MALT] lymphoma and gastric cancer)(Blaser *et al.,* 2004).

This bacterium possess multiple virulent mechanics and comes in the forefront of the urease enzyme which provides suitable microenvironment for the viability in the of stomach acid medium through the production of ammonia at high activity and quantity to protect it.

There are three main gastric phenotypes have been identified. The cases vary between chronic and acute infection and the causative is one and that depends on many circumstances.

The diagnosis of ulcers is varying in accuracy and speed in ways some of which needs to be an endoscopy which requires complicated procedures. Others are including the diagnosis of immune antibody in the blood and the proportion of

its presence is limited. Following the use of appropriate diagnosis and effective treatment.

Through our review of what is available from the references of our hands, it did not make any attempts to link the type and severity of infection and rapid diagnosis through the investigation on virulent factors and use their products as indicators. It is now well accepted that the most common stomach disease, peptic ulcer disease, is an infectious disease, and all consensus conferences agree that the causative agent, *H. pylori*, must be treated with antibiotics (Lee, 1997).

Due to the wide range of *H. pylori* infection and its strong effective virulence factor and because of the infection with *H. pylori* may be developed into a variety of diseases, this study was suggested to achieve the biggest goal of the early and rapid diagnosis of infectious ulcer using ammonia produced by *H. pylori* and to use the nano science to achieve active drug delivery nano biodegradable biopolymer for direct treatment. So the following steps was proposed to achieve the goal:

- Isolation and identification of *H. pylori* bacterium from acute and hemorrhage ulcer infection by using new and modified media.
- Comparison between acute and hemorrhage isolates for its growth, virulent factor and ammonia production in all stages of the study.
- Indirect measurement of the important virulence factor in the bacteria especially ammonia production by urease enzyme.
- Trying to reach relationship among the virulent parameters and type of infection.
- Preparation of drug delivery chitosan nanoparticles.
- In vitro studying the effect nanotherapy on bacterial suspension and biopsy.

1.2 Literatures review

1.2.1Historical background

Helicobacter pylori (*H. pylori*), was first discovered in the stomach of patients with gastritis in 1982 by Marshall and warren they described the successful isolation and culture of a spiral bacterial species. Later known as *Helicobacter pylori.* The bacterium was placed in the following classification:

Subdivision: Proteobacteria.

Order: Campylobacter.

Family: *Helicobacteraceae.*

Genus: *Helicobacter*.

Scientific Name: *Helicobacter pylori* (Kusters *et al*., 2006).

This family also includes the genera *Wolinella*, *Flexispira*, *Sulfurimonas*, *Thiomicrospira*, and *Thiovulum*. The genus *Helicobacter* consists of over 20 recognized species, with many species awaiting formal recognition (Fox, 2002).

1.2.2Characteristics of *H. pylori*

H. pylori is a Gram- negative spiral bacteria measuring 2-4 μm in length, 0.5-1 μm in width and has 2-6 sheathed flagella 3 μm in length. Members of the genus *Helicobacter* in most cases are catalase and oxidase positive, and many but not all species are also urease positive (Kusters *et al.,* 2006; Guo *et al*., 2011).

H. pylori have many biochemical and pathogenic properties components, such as Lipopolysaccharide, peptidoglycan, glycoproteins.

Lipopolysaccharide is a primary component of the bacterial outer membrane. It consists of three parts: the lipid A domain, a set of core sugars, and a long chain of oligosaccharide repeats known as the O-antigen (Raetz *et al.,* 2002). Peptidoglycan (PG) is a thin, mesh-like, found in the periplasmic space between inner and outer membranes; it is responsible for cell shape and integrity (Moran, 2007).

1.2.3 *H. pylori* growth requirements

H. pylori growth at optimal rang of temperature ranged from 34 to 40^{o}C, pH (5.5 to 8.0) but can survive at pH 4 and the key feature of *H. pylori* its microaeropholicity. Growth at optimal level of: 2 -5% oxygen, 5- 10% carbon dioxide and 85% nitrogen. *H. pylori* is a fragile organism, it must be protected from desiccation and contact with oxygen (Han, 1995). *H. pylori* is urease positive and highly motile via flagella (Solnick *et al*., 2001). Urease is allow short-term survival in the highly acidic gastric lumen and motility is thought to allow rapid movement toward the more neutral pH of the gastric mucosa; this may explain why both factors are prerequisites for colonization of the gastric mucosa (Schreiber *et al*., 2004; Schreiber *et al*., 2005). Upon entry, *H. pylori* display urea- and bicarbonate-mediated chemo-tactic motility toward the mucus layer. The spiral morphology and flagellar motility then help in penetration into the viscous mucus layer, where the more pH-neutral conditions allow growth of the bacteria (Yoshiyam *et al*., 2000).

H. pylori is a slow-growing organisms that requires rich culture media for sufficient growth, there are two main type of media (a) nonselective media such as brain heart infusion agar mixed with 7% sheep or horse blood and 1% Iso Vitale X (Goodwin,1985). Some investigator favored Colombia agar with lysed horse blood(Dent, 1988),(b) selective media based on supplemented nutrient agar containing antibiotics(Queiroz, 1987).

H. pylori strains are auxotrophic for several amino acids with some diversity exiting in these requirements. A genomic scale metabolic model, which takes into account the genome sequence annotation (Tomb et al 1997) and physiological data, calculates that 47 metabolites necessary for growth. Eight of these are amino acids with L-arginine and alanine thought to provide the major sources of carbon. However, the requirement for this element can be met other compounds including glucose, pyruvate, lactate, malate, and several amino acids. It seems *H. pylor*i may have evolved to selectivity utilize these since the host's nutritional needs, and the

subsequent proteolysis of food sources, would generally guarantee their presence in the human gastric environment (Schilling *et al.,*2002).

1.2.4 Transmission

There is a very large population of humans throughout the world that is colonized with *H. pylori*, relatively little is known about how it is transmitted. Epidemiologic studies of *H. pylori* transmission show that the majority of infections tend to occur within families through close person-to-person contact (Falush *et al*., 2003). Most transmission occurs in childhood, and maternal-to-child and sibling–sibling transmission seem most likely (Kivi *et al*., 2005; Weyermann *et al*., 2006).

It seems more likely that transmission occurs in situations in which gastric content can be transferred quickly from person to person. For example, gastric-oral transmission is suggested in association with gastroenteritis with vomiting (Brown, 2000). Fecal–oral transmission may also be possible. However, this bacterium has evolved to successfully colonize the hostile environment of the human stomach in the face of a constant innate and adaptive immune response (Kabir, 2004).

1.2.5 Bacterial virulence factor

1.2.5.1 Adhesion and colonization of *H. pylori*

The gastrointestinal epithelium has cells with features that make them a powerful line of defense in innate mucosal immunity, the gastric epithelium consists of a monolayer of cells covered by mucus and that invaginate in order to form functional gastric glands. A critical function of mucosal epithelial cells is to protect the underlying tissue from pathogenic microorganisms that may access the lumen (Wroblewski *et al*., 2011). In order to survive and maintain the chronic infection *H. pylori* employs an assortment of mechanisms that aid its adaptation to the tough environment of the stomach, there are multiple effects that *H. pylori* has on gastric epithelial cells, among which are induction of apoptosis, destruction of

epithelial cell junctions and cell proliferation (Xia HH *et al*., 2001).An essential step in the colonization by *H. pylori* and its ability to mediate effects on the gastric epithelium is its selective tissue tropism leading to the establishment of intimate interactions with the epithelial surface. These interactions are largely mediated via outer membrane proteins (OMPs) that serve as adhesions .*H. pylori* genome has more than 30 genes which encode OMPs that are divided into Hop (*Helicobacter* outer membrane proteins) and Hor (hop-related) subgroups (Backert *et al*., 2011).

1.2.5.2Urease enzyme

Urease enzyme is a cytoplasmic enzyme that consisting of two structural subunits (UreA and UreB).*H. pylori* contain a urease gene cluster which consists of seven conserved genes (*UreA–B* and *E–I*). It hydrolyze the urea to ammonia and carbon dioxide in order to neutralize the acidity of the stomach (Aguilar *et al.,*2001; Suerbaum and Michetti ,2002).UreA and UreB are nickel-containing enzyme that consists of 12 UreA and 12 UreB subunits (Ha *et al*., 2001). The UreA and UreB subunits have molecular masses of27 KD and 62 KD respectively, and the subunits are encoded by an operon containing the *ureA* and *ureB* genes. UreE, UreF, UreG and UreH are accessory proteins involved in nickel incorporation and enzyme assembly. Together with arginase, UreI is responsible for a continues supply of urea under acidic environmental conditions (Zanotti, 2010). Urea transport into the cell is controlled via the H-gated urea channel UreI resulting in increased urea transport in acidic conditions (Weeks *et al*., 2001).

Amino acids and urea are the two major sources of nitrogen in the gastric environment, since ammonia is a key component in nitrogen metabolism as well as acid resistance (Stingl *et al*., 2002). It is not surprising that *H. Pylori* can utilize several alternative sources of ammonia (Merrell *et al.,* 2003). The different pathways contributing to ammonia synthesis are regulated in response to different stimuli, which probably allows *H. pylori* to switch different pathways On or Off depending on the environmental conditions, the main route of ammonia production

is through the highly active urease enzyme, which functions in nitrogen metabolism but also in acid resistance and virulence.

H. pylori produce large amounts of urease and it has been estimated that up to 10% of the total protein content of *H. pylori* consists of urease. When excess ammonia is produced, this can be removed via the glutamate synthetase enzyme. *pylori* utilize different strategies for attachment to the gastric epithelium and downstream signaling during the course of infection by the binding of urease subunits to CD74 and CD46 receptors, Urease also acts as an adhesion as it binds directly to both class II major histocompatibility complex (MHC) molecules and CD74 (Beswick *et al*., 2006). *H. pylori* binding to the cells was increased when CD74 surface expression increased by treatment with interferon gamma (IFN-γ) or by fibroblast cells transfected with CD74 constructs (Beswick *et al.,* 2005).*H. pylori* was also shown to bind directly to affinity-purified CD74 protein even in the absence of MHC-II. long-term colonization with *H. pylori* significantly increase the risk of developing gastro-duodenal diseases, peptic ulcer disease, gastric adenocarcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (peek *et al*., 2006).

In addition, urease activity support flagellar motility through the mucus layer by changing the viscoelasticity properties of gastric mucins, at low pH, gastric mucins form a gel that effectively traps the bacteria, but urease stimulate production of ammonium ions raises the pH to near neutral and the mucus gel transitions to a viscoelastic solution through which *H. pylori* can swim (Celli *et al*., 2007;Celli *et al.*, 2009).

1.2.5.3Vacuolating cytotoxine A (VacA)

VacA could be the most sever toxin secreted by all *H. pylori* strains, it was named for its ability to induce numerous large vacuoles in host cell, VacA is associated with tissue damage by induce vacuolation, cytochrome release from mitochondria leading to apoptosis and responsible for initiation of pro-

inflammatory response (Palframan *et al*.,2012). VacA toxin is encoded by *vac A* gene which express in all *H. pylori* strains (Suzuki *et al*.,2012).

1.2.5.4Cytotoxin associated gene A(cagA)

CagA, encoded by cytotoxin-associated gene A (cagA), CagA-positive *H. pylori* strains are associated with greater inflammation and increased risk of ulcers and cancer in humans (Kim *et al*., 2011), CagA toxin is directly injected into the host cells via type IV secretion system (Miwa *et al.,*2002 ;Suzuki *et al.,*2005). Cardaropoli *et al.*(2011) were described CagA as a highly immunogenic toxin encoded by *cag A* gene which located at one end of Cag pathogenicity island (PAI) which encodes type IV secretion system through which Cag A toxin delivered to host cells(Kadhem, 2011).

1.2.6Genomic content of *H. pylori*

The size of the two sequenced *H. pylori* genomes is approximately 1.7 Mbp, with a G+C content of 35% to 40% (Boneca *et al*., 2003). In contrast to other pathogenic bacteria which are highly clonal, *H. pylori* is genetically heterogeneous which is possibly an adaptation of *H. pylori* to the gastric conditions of its host (Kuipers *et al*., 2000). Genetic heterogeneity is occurring via several methods of DNA rearrangement and the introduction and deletion of foreign sequences (Falush *et al*., 2003; Suerbaum *et al*., 2004). Diversity is also seen at the nucleotide level via several mechanisms including translational and transcriptional phase variation and mutation (Falush *et al.*, 2001).

1.2.7 Diagnosis of *H. pylori* infection

Infection with *H. pylori* is associated with an increase in gastric acid output and a reduction in the thickness of the mucous layer and in gastric mucosal hydrophobicity (Ernst and gold, 2000). Several invasive and non-invasive tests are

available to detect *H. pylori* infection (Koletzko, 2005;Anagnostopoulos *et al*., 2007).

The choice of test depends to a large extent on availability and cost, and includes a difference between tests used to establish a diagnosis of the infection and those used to confirm its eradication. The important factors are clinical situation, population prevalence of infection, pretest probability of infection, differences in test performance, and factors that may influence the test results, such as the use of anti-secretory treatment and antibiotics (Cutler *et al*., 1995).

1.2.7.1Invasive tests

1.2.7.1.1 Biopsies and histology

The definitive diagnosis of *H. pylori* and the evidence of the consequences of infection can be made reliably only by endoscopy with multiple biopsy specimens obtained in one or more regions of the stomach including antrum, body, and transition zones. Histology provides information regarding the presence of *H. pylori* and the severity and topographic distribution of gastritis including the presence of atrophic gastritis, intestinal metaplasia and MALT lymphoma, Identification of *H. pylori* in tissues can be easily done by examination of hematoxylin and eosin stained slides in about 70-80% of infected patients. Special stains including Gram stains, silver stains, Giemsa, Diff-quick, thiazine and immunohistochemical stains, can detect the remaining 10-20% (Singhal *et al*., 2005).There are drawbacks to diagnostic gastrointestinal endoscopy. It is a relatively invasive procedure requiring sedation or anesthesia, the test remain relatively expensive in many centers, and access to an endoscopist with specific pediatric expertise is limited in many geographic areas (Megraud, 1991; Dohil *et al.*, 1999).

1.2.7.1.2Rapid urease testing

Urease testing provides indirect identification of *H. pylori* infection within a few hours of endoscopy. The finesse of the test is dependent on the number of tissue specimens tested, the location of biopsy sites, bacterial load and previous usage of antibiotics and proton pump inhibitors, as well as the prevalence of *H. pylori* in the population tested. Sensitivity of detection depends on organism load in the mucosal biopsy specimen and the number of biopsy samples; it exceeds 95% in patients with gastric ulcer disease if the corpus is sampled, whereas the sensitivity is diminished markedly if only antral samples are obtained from these patients (Bermejo *et al.*, 2002).Rapid urease tests enable convenient detection of *H pylori* infection within 3 hours in most cases, although the agar gel–based tests usually require 24 hours of incubation for maximal sensitivity and specificity (Elitsur *et al*., 1998; Elitsur and Neace, 1999).

1.2.7.1.3Bacterial culture

Culture of *H. pylori* from the gastric mucosa provides an opportunity to obtain a profile of antibiotic sensitivity that could identify potential treatment failure due to antibiotic resistance. Culture also provides a bacterial strain for use in epidemiologic studies to examine associations of virulence characteristics with disease outcome. However, bacterial culture for *H. pylori* is relatively expensive (Holton, 1997; Vander *et al*., 1998).

1.2.7.1.4Polymerase chain reaction of bacterial DNA

Polymerase chain reaction (PCR) is a highly sensitive technique that can be used to detect the presence of *H. pylori* in body fluids (e.g., gastric juice and stool), tissues (e.g., gastric mucosa), and water. Testing of *H. pylori* genomic DNA by PCR can be used to advance knowledge at the molecular level, for example, by providing information about point mutations conferring resistance to antibiotics and about putative bacterial virulence factors. However, PCR is expensive, the

assay is difficult to set up, specificity may be compromised by inadvertent contamination, and it is not widely available outside the research laboratory (Westblom, 1997).

1.2.7.2Non-invasive tests

1.2.7.2.1Serum and whole blood antibody

The detection of multiple antibodies in serum by protein array has been used for *H. pylori* diagnosis. This array is comprised of three recombinant *H. pylori* antigens: UreB, VacA and CagA immobilized on nitrocellulose membranes. Bound IgG are detected using staphylococcus protein A labeled with colloidal gold. Sensitivity and specificity were above 90% compared to ELISA (Han *et al*., 2006).

1.2.7.2.2Saliva antibody and Urine antibody

Similar to serologic tests, saliva-based tests also detect the presence of *H. pylori*–specific IgG antibodies. The tests are easy to perform, painless, and inexpensive. Saliva tests are less sensitive than assays of serum or whole blood. The protein concentration of saliva appears to affect the accuracy of test results. Urine-based assays are easy to perform, require minimal labor for collection, and are painless. These assays are highly variable and are not yet commercially available. Therefore, saliva and urine assays for the detection of *H. pylori* antibodies cannot be recommended (Fallone *et al.,* 1996).

1.2.7.2.3Stool antigen

Testing of *H. pylori* antigens in stools has shown promising results in adults for the noninvasive diagnosis of gastric infection using a commercially available kit testing for *H. pylori* antigens in faeces. This test was based on the intensity of color developed, results were reported as *H pylori* antigen detected or not detected (Baronand Thompson, 2011).

A positive result (antigen detected) is indicative of *H. pylori* presence. A negative result (antigen not detected) indicates absence of *H. pylori* or an antigenic level below the assay limit of detection. The test has a sensitivity and specificity of 96% for detecting *H pylori* infection. False-negative results may occur on patients receiving antimicrobials, proton pump inhibitors, and bismuth preparations. If a negative result is obtained on a patient receiving these compounds, the test should be repeated on a new specimen obtained two weeks after discontinued treatment. However, patients may be reluctant to collect stool specimens. In addition, refrigerated stools are more difficult to test. Additional pediatric studies evaluating the accuracy of stool antigen testing for both initial diagnosis and post treatment follow-up are required before specific recommendations can be considered (Oderda *et al*., 2000; Asfeldt AM *et al*., 2004)

1.2.7.2.4 Urea breath testing

Urea breath tests are noninvasive and have high sensitivity and specificity (>95%) both in adults and children. Test results may be influenced by concurrent use of antibiotics and acid-suppressing medications and by the presence of other urease-producing organisms present in the oral cavity. Test parameters are currently laboratory-specific (e.g., dosages for differing ages of children, cut off values, duration of fasting, use of a test meal, times of sampling, and timing of post therapy testing) and have not been well standardized for children. In addition, urea breath testing is technically more difficult to perform in small children and infants, with failure rates in collection up to 10%, especially outside the clinical research setting (Braden and Caspary,2001; Rockville, MD.,2013)

1.2.8 Determination of ammonia concentration

Phenate method for ammonia determination was proposed by many researchers. It was included the mechanism of reaction consisted of the formation indophenols by reaction of ammonia with phenol and hypochlorite reagents (Rileyand Sinhaseni, 1957; Rossum and Villarruz, 1963 and Mann, 1963).

The phenate method was a very effective method for measuring ammonia concentration in the aqueous solution (Ga-eun Park *et al*., 2009).This method was used the principle that ammonia react with hypochlorite base and phenol to change into indophenol blue, as the following reaction:

$$2NH_4^+ + OCL^- + 2C_6H_5OH \rightarrow O{=}C_6H_4{=}N{-}C_6H_4{-}NH_2$$

The reaction speed can be faster by increase pH rapidly by adding phenate reagent solution that transfer ammonia ion generated in the urea decomposition process into indophenol blue (Riley and Sinhaseni, 1957).

1.2.8.1 Ammonia production by *H. pylori*

Ammonia is a key component of bacterial nitrogen metabolism, because it is considered as source of nitrogen for the synthesis of amino acid, purines and pyrimidines. Ammonia plays a central role in pathogenesis and metabolism of *H. pylori* by contributes to epithelial cell damage and apoptosis (Igarashi *et al*., 2001; Nagahashi *et al*., 2002). *H. Pylori* has several adaptation for an acid environment of the stomach, one of them is urease which converts urea that physiologicaly present in the stomach into ammonia and bicarbonate (Montecucco *et al*., 2001). The ammonium hydroxide formed raises pH of gastric juice and enables *H. pylori* colonization of gastric mucosa, urease is essential for gastric colonization. The urease enzyme test is very efficient method uses the fact that *H .pylori* produce urease enzyme which has a much higher degree of activity compared to other microorganisms, when ammonia produced will cause pH to be increase making a

pH indicator react and change color. The urea decomposition process by urease is presented as following reaction formula (Follmer, 2008; Krajewska, 2009).

1.2.9 Antibiotics Sensitivity of *H. pylori*

H. pylori are quite susceptible to most antibiotic in vitro, but treatment of *H. pylori*- infected individuals is difficult (Dunn *et al*,. 1997). Single-antibiotic therapy has not been very effective, successful treatment usually requires two or three antibiotic give in a combination.

Amoxicillin acts by interfering with the peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP) (Gerrits *et al*., 2002). While Tetracyclines interfere in the protein synthesis at the ribosome level by binding to the 30S subunit (Gerrits *et al*., 2002). Ciprofloxacin is a highly active fluoroquinolone antibacterial agent with a broad spectrum of activity it is act by binding two of the four topoisomerases of bacteria (Gendrel *et al*., 2003).

H. pylori can acquire resistance to the antimicrobial agents used to treat the infection, and therefore, susceptibility testing is important in the management of the infection. The susceptibility of *H. pylori* to antibiotics can be quite variable in particular geographical areas of the same country as well as among different countries (Graham, 1998).The success of a scheme of treatment in a community does not enable the generalization of the results it would be ideal to provide a therapy based on previous knowledge of the microbial resistance rate in a local community, which has been difficult in most centers of developing countries (Han *et al*., 1999).

1.2.10 Treatment of *H. pylori*

The current recommended treatment for *H. pylori* eradication includes two antibiotics and an antisecretory drug (Malfertheiner *et al*., 2006). Administer triple therapies for 10-14 days. These regimens are also known as triple therapies and have reported cure rates from 85-90% .The treatment regimens are omeprazole, amoxicillin, and clarithromycin (OAC) for 10 days; bismuth subsalicylate, metronidazole, and tetracycline (BMT) for 14 days; and lansoprazole, amoxicillin, and clarithromycin (LAC), which has been approved for either 10 days or 14 days of treatment(Liou *et al.*, 2010; Greenberg *et al*., 2011).

1.2.11 Chitosan

Chitosan was first discovered in 1811 by Henri Braconnot a French chemist and pharmacist, he observed that a certain substance (chitin) found in mushrooms did not dissolve in sulfuric acid (Labrude, and Becq**,**2003). Later, chitin was found in the exoskeleton, in marine diatoms and algae, in some fungal cell walls and the internal structure of invertebrates.

Chitin is a hard, white, inelastic, nitrogenous polysaccharide. It is the second most natural polysaccharide after cellulose on earth and is consist of β(1→4)-linked 2-acetamido 2-deoxy-β-D-glucose (N-acetylglucosamine). It is often considered as cellulose derivative but it does not occur in organisms producing cellulose. It is structurally identical to cellulose, but it has acetamide groups (−NHCOCH3) at the C-2 positions. Similarly the principle derivative of chitin (Dutta *et al.,* 2002). The term 'chi-tosan' describes a heterogenous group of polymers combining a group of physicochemical and biological characteristics, which allow for a wide scope of applications that are both attractive and as yet unknown. (Raafat *et al.,*2008).

Chitosan is a linear polymer of α(1→4)-linked 2-amino-2-deoxy-β-D-glucopyranose and is easily derived by N-deacetylation, to a varying extent that is

characterized by the degree of deacetylation, and is consequently a copolymer of N-acetylglucosamine and glucosamine as shown in figure (1-1).

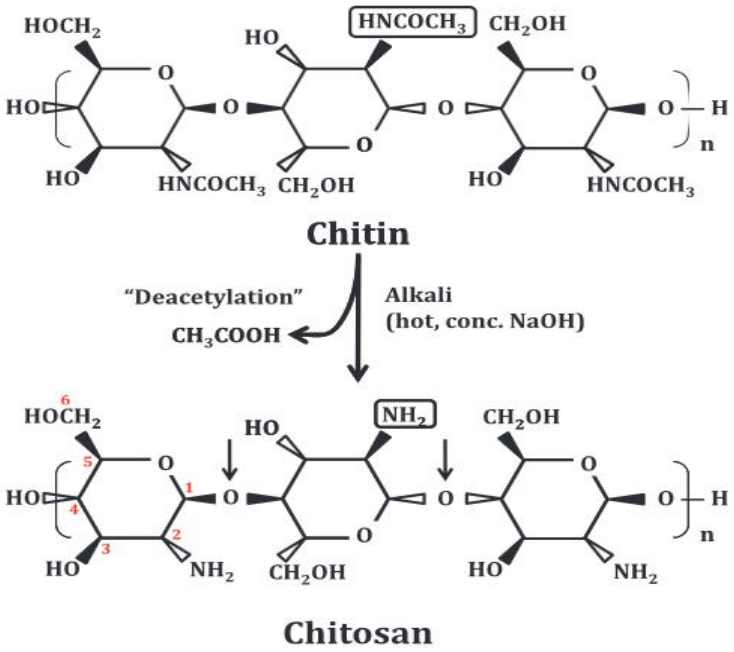


Figure (1-1): **Structure of chitin and chitosan** (Pradip *et al*., 2004)

Chitin and chitosan have excellent properties such as biodegradability, bio-compatibility, non-toxicity and adsorption. Physical and chemical properties of chitosan depend mainly on its molecular weight and degree of deacetylation. The main difference between chitin and chitosan lies in their solubility, chitosan is soluble in acidic condition and the free amino groups on its polymeric chains protonates and donate to its positive charge (Phaechamud, 2008). Pure chitosan is insoluble in water, in alkaline medium and even in organic solvents. However, water- soluble salts of chitosan may be formed by neutralization with organic acids (e.g. 1–10% aqueous acetic, formic, succinic, lactic, glutamic and malic acids) or inorganic acids such as hydrochloric acid (Henriksen *et al.*, 1996; Singla and Schawla, 2001).

Upon dissolution, chitosan forms viscous solutions, which could function as thickeners, stabilizers or suspending agents, chitosan solutions show pseudoplastic and viscoelastic properties; their viscosity is affected by chitosan's molecular weight (MW), concentration, concentration and types of solvents, the prevailing solution pH and ionic strength, temperature (Chen and Tsaih, 1998; Singla and Chawla,2001), as well as DD (Degree of deacetylation) which is an important parameter to examine chitosan, i.e. the ratio of GlcNAc to GlcN structural units.The DD of chitosan is influenced by the preparation procedure, a number of analytical devices have been used to define the DD, such as FTIR spectroscopy, UV spectrophotometry, as well as thermal analysis (Kumar, 2000).

1.2.12 Chitosan nanoparticles

1.2.12.1Nanotechnology

Nanotechnology can be defined as the science and engineering involved in the design, synthesis, characterization, and application of materials and devices whose smallest functional organization, in at least one dimension, is on the nanometer scale or one billionth of a meter. (Silva GA, 2004 ; Wang and brain, 2008)

Nanotechnology based drug delivery systems include nanoemulsions, nanoparticles, liposomes, lipid or polymeric and nanofibers. Polymeric nanoparticular drug delivery systems have the advantages of cheaper cost, scalability, targeted delivery, biodegradability, biocompatibility, sustainability in release of encapsulated drug and improved efficacy. The biopolymers of carbohydrate origin such as Chitosan, Alginate and proteinous origin such as gelatin, albumin and silk proteins have added advantage over the synthetic polymers when there can be a compromise for long lasting stability (Chang and Yeh, 2012). At the same time there are many synthetic polymers that are biocompatible and less biodegradable in comparison with natural polymers, which include polyglycolides (PGA), poly(lactide-co-glycolides) (PLGA) , polylactides

(PLA), polyorthoesters and polyanhydrides. These nanoparticulate drug delivery systems regulate the normal pharmacokinetic profile of encapsulated therapeutic drug and help in marked and sustained release of drug (Gulsun. *et al.*, 2009; Yokoyama, 2010).

The nano-therapeutics can confirm the efficacy and add to the commercial value of the health-care products and sustained release of drugs. The polymer carry the drug to target, reduces the metabolic drug degradation, increases the activity of the active pharmaceutical ingredient and reduces the side effects of the drug (The Nanotech Revolution in Drug Delivery. Cientifica Ltd., 2007).

1.2.12.2 Chitosan nanoparticles production

Nanoparticles are solid colloid particles of 1 to 100nm. Compared with micron-grade particles, it has strong mobility because of their small size and can enter cells easily to accumulate at the lesion site. Chitosan nanoparticles are formed spontaneously on the conjunction of polyanion such as tripolyphosphate (TPP) in chitosan solution under continuous stirring condition (Singh and Lillard, 2009).

Ionic gelation method is the major method in preparation nanochitosan. Chitosan nanoparticles can be prepared by the interaction of oppositely charged macromolecules. Tripolyphosphate (TPP) has often been used to prepare chitosan nanoparticles because TPP is nontoxic, multivalent and able to form gels through ionic interactions. The interaction can be controlled by the charge density of TPP and chitosan, which is dependent on the pH of the solution(Zhao *et al.,* 2011).

Nasti *et al.* (2009) studied the influence of a number of factors, such as pH, concentration, ratios of components, and method of mixing, on the preparation of chitosan/TPP nanoparticles.

1.2.13 Chitosan nanoparticles applications

1.2.13.1 Chitosan antimicrobial activity

Antimicrobial activity of chitosan and its derivatives extends to include filamentous fungi, yeasts and bacteria (Jeon *et al.,* 2001; No *et al*., 2002).

In common with many cationic polymers, chitosan has obvious antimicrobial effects due to destabilization of the outer membrane of Gram-negative bacteria and permeabilization of the microbial plasma membrane (Li *et al*., 2011; Tang *et al*., 2010).Many factors present in the chitosan molecule or its environment can influence the antimicrobial properties such as (molecular weight (MW), Degree of deacetylation (DD), viscosity, solvent, concentration), the environmental conditions such as (test strain, its physiological state and the bacterial culture medium, pH with higher activity observed at lower pH Value, temperature, ionic strength, metal ions, EDTA and organic matter) respectively (No *et al*., 2002). The physical state of the chitosan can present very different antimicrobial properties, such as whether the chitosan is present in the form of coatings, films, hydrogels, in solutions or in combinations with other materials, interaction between positively charged chitosan molecules and negatively charged microbial cell membranes leads to the disruption of microbial membrane, and then the leakage of proteinaceous and other intracellular constituents (Li P *et al*., 2011; Rabea *et al*., 2003 ; Kong *et al*., 2010; Raafat *et al*., 2008).

1.2.13.2Chitosan antimicrobial mode of action

The overall mechanism of action of an antimicrobial may be defined according to the target component of the bacterial cell against which it has its main activity. Three levels of interaction can be described: (i) interaction with outer cellular components, (ii) interaction with the cytoplasmic membrane and (iii) interaction with cytoplasmic constituents. The properties and structure of the bacterial cell envelope play an important role in chitosan's antimicrobial activity,

Gram-positive bacteria are markedly more sensitive to the antimicrobial activity of chitosan, compared with Gram-negative ones this difference in sensitivity is largely ascribed to the different architectures of their cell envelopes(Rhoades and Roller, 2000; Jeon *et al*., 2001; No *et al*.,2002).Chitosan loses its antimicrobial activity at pH 7.0,assumabledue to the deprotonation of amine groups, as well as poor solubility in water at this pH (Liu *et al*., 2001), The antimicrobial activity of chitosan was found to be directly proportional to its DD(Park *et al*., 2004).

1.2.13.3Nanochitosan drug delivery system

Nanochitosan has become of great interest for nanomedicine , biomedical engineering and development of new therapeutic drug release systems with improved bioavailability, increased specificity and sensitivity, and reduced pharmacological toxicity (Jayakumar *et al*., 2010).There is another procedure used by Ghadi *et al*.,(2014) including the use of sonication instead of TTP treatment and adjust the pH and filtarate, the resulting solution was clear.

Chitosan nanoparticles have been a subject of interest for application to wounds and burns not for their intrinsic antimicrobial and wound-healing effects only, but also because of their properties as versatile drug delivery vehicles that can enhance antimicrobial and wound-healing effects (Rossi *et al*.,2007; Aoyagi *et al*.,2007; Noel *et al*., 2008).Drugs carried by chitosan nanoparticles can be released through degradation and corrosion of chitosan, leading to a clear sustained-release effect. Because of varied degradation rate and time of chitosan of different molecular weight and degree of deacetylation degree, different types of nanoparticles can be used to regulate drug-release rate (Yuan *et al*., 2010).

1.2.14Phytomedicine

Phytomedicin described as herbal therapy by using plants or plant extract for medicinal purposes. Herbal products include whole or parts of the plant such as stems, flowers, leaves, seeds and roots. Due to complex mixture of organic chemicals phytotherapy products and application is still very limited (Vitor *et al.*, 2011). The risk and benefits of herbal medicine are complex and incomplete; there is a need for more controlled clinical trials addressing the possible efficacy of herbal medicine (Ke *et al.*, 2012). Even before the identification of *H. pylori* herbs have been used to deal with diseases that today are known to be associated with *H. pylori* infections. Treatment failure of *H. pylori* associated with antibiotic resistance can be reduced by using phytomedicine product which have anti- *H. pylori* activity and gastroprotective action (Dubois *et al.*, 2007).

A Phytotherapy product that's used for *H. pylori* are subdivided in two groups, the first is based on *in vitro* testing by using pure culture of *H. pylori* obtained from clinical isolates, the second is based on *in vivo* test in which the herbal products are administered to animal models. The first study is more abundant because of its simplicity and cost (Wang *et al*., 2005). Plant extract is usually prepared by drying and reduce to fine powder which is then dissolved in a solvent such as aqueous ethanol or methanol, sonicated, filtered or centrifuged and the solvent evaporated. Different concentration of the plant extracts are mixed with a bacterial suspension of *H. pylori* and plated in standard *H. pylori* medium. Herbal extract embedded paper discs is another option, the inhibitory action is evaluated by determination of the clear zone around each well or disc (Zaidi *et al.*, 2009).

1.2.14.1 Phytotherapy antimicrobial mode of action

The most common active compound that has anti-inflammatory, antioxidant, antiallergic and anticarcinogenic activity is flavonoids.

Flavonoids antibacterial mode of action for *H. pylori* can be done through the inhibitory effect on bacterium growth (Pastene *et al.*, 2010), on *H. pylori* DNA gyrase (Asha *et al.*, 2013), on urease enzyme and vaculation activity , many flavonoids may inhibit the apoptotic signaling induced by *H. pylori* VacA toxin (Quilez *et al.*, 2010; Paulo *et al.,* 2011).

1.2.14.2 *Loranthus eurpaeus*

Kingdom:*Plantae.*

Subkingdom:*Tracheobionta*.

Super division: *Spermatophyta.*

Division:*Magnoliophyta.*

Class: *Magnoliopsida.*

Subclass: *Rosidae*.

Order: *Santalales.*

Family: *Loranthaceae.*

Genus: *Loranthus* .

Scientific Name: *Loranthus europaeus.*

Common Name: Mistletoe.

The family Loranthaceae, is a large family that includes about 75 genus belong to 1000 species , some of these species are parasites on the roots while the remaining parasites on branches and stems trees and known as Mistletoe (Waly *et al.,* 2012*)*. The flowers of *L. europaeus* are small, green, usually have four to six parts and may be either unisexual or bisexual (Fleming, 2002). Generally the studies on some species of the Loranthaceae family have indicated the presence of several chemical compounds. The quantitative phytochemical screening of the aqueous extract revealed that the plant contains 0.30% flavonoids (Olatunde and

Dikwa, 2014). The species *L. micranthus* was studied for its antimicrobial activities at various concentrations using agar diffusion method, and was tested on *Staphylococcus aureus*, *Escherichia coli* and *Klebbsiella pneumonia,* the results of inhibition was concentration dependent so increase in concentration results in wider zone of inhibition (Egbuonuand Nwankwo, 2011).

Chapter Two

Materials and Methods

2.1 Materials

2.1.1 Apparatus:

The following apparatus were used in this study**:**

Apparatus	Company	Origin
Anaerobic gar	BBL	Canada
Analytical Balance	Sartorius	Germany
Autoclave	Express	Germany
Cooling Incubator	Sanyo	Japan
Cooling box for the biopsies	Locally	
Deep freezer	GFL	Germany
Digital Camera	Sony	Japan
Gel electrophoresis unit	Consort	Ireland
Heat magnetic stirrer	SCO tech	India
High speed cooling centrifuge	Eppendrof	Germany
Hood	ESCO	USA
Lyophilizer	Christ	UK
Maxwell for DNA extraction	Promega	USA
Microscope	Olympus	Japan
Gel decumentation	Bio Rad	USA
Nanodrop	Agilent	USA
pH meter	Hana	Italy
SEM microscope	INSPECT S50	UK
UV-Visible Spectrophotometer	BUCK	USA
Vortex	Bohemia	Czech
Water bath	Memmert	Germany

2.1.2- Chemicals and Biological materials :

The following chemicals and Biological were used in this study**:**

Chemicals	Company	Origin
Agar	Hi-media	India
Ammonium chloride (NH_4Cl)	BDH	England
Beef extract	Hi-media	India
Chitin	Xi'an Lyphar Biotech Co., Ltd.	China
Chitosan		
Commercial bleach	Local market	
Glacial acetic acid	Scharlau	Spain
Glycerol	BDH	England
KH_2PO_4		
L-Cystein		
$MnSO_4.H_2O$		
Na_2HPO_4		
NaOH		
Phenol (C_6H_5OH)		
Phenol Red		
2,3,5-Triphenyltetrazolium chloride		
Tri-polyphosphate		
Urea ($NH_2.CO.NH_2$)	Thomas Baker	India
Yeast extract	Hi-media	India

2.1.3-Kits

The following kits were used in this study:

Kit	Company	Origin
Rapid anti -*H.pylori* test kit	INTEC	China
Grams color staining kit	CDH	India
Maxwell 16 Tissue DNA purification kit	Promega	USA
H. pylori Ag Rapid Test Cassette (Feces)	HEALGEN	China

2.1.4Antibiotics Discs

The following Antibiotic Discs were used in this study:

Antibiotic	Symbol	Concentration (µg/disc)	Origin
Amikacin	AK	30	Hi-media / India
Amoxicillin	A	10	
Ciprofloxacin	CIP	5	
Clarithromycin	CLA	15	
Erythromycin	E	15	
Levofloxacin	LEV	5	
Metronidazole	MZ	5	
Tetracycline	T	30	

2.1.5 Antibiotics powder

These antibiotics include Naldixic acid, Ampicillin, Ciprofloxacin and Tetracycline which were supplied from local pharmacy and they were prepared as required.

2.1.6 Culture media

2.1.6.1 New prepared Urea Broth (ZAN-1)

A new media proposed in this study was prepared from the following ingredients (gm/L): Urea, 20; Di-Potassiumphosphate (Na_2HPO_4),9.5; Mono-Potassium phosphate (KH_2PO_4), 9.1;Phenolred, 0.01; Yeast extract, 0.1; Ampicillin, 0.1; Naldixic acid,0.02; L-Cystein, 1. Thesecomponents were dissolved in 800 ml distilled water and adjusted pH to 6.4 then complete the volume to 1000 ml with DW and filtrated through sterilized Millipore (0.22µm). Filtrate medium was distributed in sterile screw cupped test tubes under aseptic conditions.

2.1.6.2Modified Brain Heart Infusion Agar Medium (BHI Agar)

BHI agar was used as mentioned by (Westblom TU, 1997). The modified BHI medium prepared by adding these ingredients (gm/L) to the BHI agar which was prepared according the manufacturer (Hi-media) :urea, 10; L-cystine, 1; Phenol red, 0.01;Ampicillin,0.1; Naldixic acid,0.02 and 2, 3, 5-Triphenyltetrazolium chloride, 0.04. PH was adjusted to 6.4.These ingredients were added to the warm autoclaved BHI and sterilized by Millipore filter (0.22 µm) and poured into petridishes. It was kept at 4^{o} C until use.

2.1.6.3 Modified Tryptic Soyabeen Broth (TSB Broth)

This modified media used for preservation and prepared by adding these ingredients (gm/L) to the TSB broth which was prepared according the manufacturer (Hi-media):beef extract, 5; Urea, 10; L-cystine, 1; Ampicillin, 0.1; naldixic acid,0.02.pH was adjusted to 6.4then sterilized by sterile Millipore filter (0.22 µm).

2.1.6.4 Modified Tryptic Soyabeen Agar Medium (TSB Agar)

TSB medium was prepared according to the manufacturer (Hi-media) while modified TSB agar medium was prepared by adding these ingredients (gm/L) to it: beef extract, 5 ; 2,3,5-Triphenyltetrazolium chloride, 0.04; Agar, 20. pH was

adjusted to 6.4 and autoclaved at 121° C for 15 minutes while these components urea, 10; L-cystine, 1; Phenol red, 0.01; Ampicillin, 0.1; naldixic acid ,0.02 were added after sterilizing by Millipore filter (0.22µm).

2.1.6.5 Egg Yolk emulsion Agar Medium (EYE Agar) (Ulfwestblom *et al.,* 1991).

This Medium was prepared by mixing 30 ml of TSB broth with 20gm agar and sterilized in autoclave at 121° C for 15 minutes and after cooling to 50°C;100 ml of egg yolk was added under aseptic conditions and mixed well then poured into petridishes.

2.1.6.6 Blood agar medium (Queiroz *et al*., 1987)

It was prepared by addition of 5% sheep blood to warm autoclaved blood agar base, mixed and poured in to plate and kept at 4 °C until use.

2.1.7 Reagents and indicators

2.1.7.1 Fast Urea solution (Finegold, 1986)**.**

It was prepared by dissolving urea(10gm) and phenol red(0.002gm) in 100 ml of distilled water then pH was adjusted to 6.4 and sterilized by sterile Millipore filter 0.22µm. This reagent was stable for 2 weeks at 4-8°C.

2.1.7.2 Fast urea solution without phenol red

It was prepared by dissolving urea(10gm) in 100 ml of distilled water then pH was adjusted to 6.4 and sterilized by sterile Millipore filter 0.22µm. This reagent was stable for 2 weeks at 4-8°C.

2.1.7.3 Oxidase reagent (Collee *et al*., 1996)

This reagent was prepared by dissolving p-aminodimethylaniline in distilled water. (The kit of oxidase test was supplied from CDH, India) It was used to detect the production of oxidase enzyme.

2.1.7.4Catalase reagent (H_2O_2)

It was supplied from pharmacy.

2.1.7.5 Hypochlorous acid reagent ($HClO_4^-$) (APHA, 1998)

It was prepared by mixing 40 ml distilled water with 10 ml of 5% commercial bleach then pH was adjusted to 6.5 with HCl. This reagent was unstable, therefore it was prepared weekly.

2.1.7.6 Manganous sulfate solution (APHA, 1998)

Dissolve 50 mg $MnSo_4.H_2O$ in 100 ml distilled water.

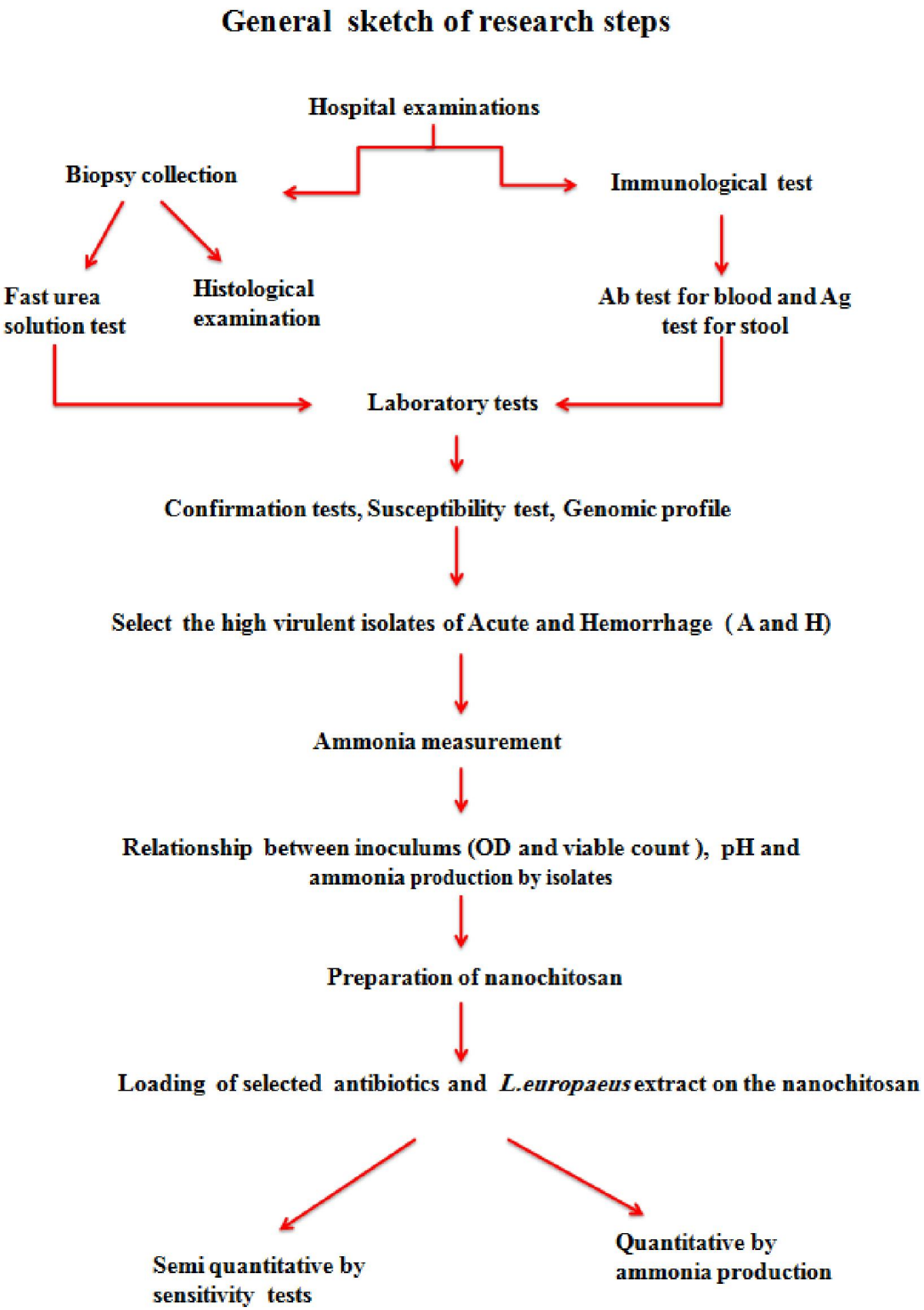
2.1.7.7 Phenate reagent (APHA, 1998)

This reagent was prepared by dissolving 2.5 gm NaOH and 10 gm phenol (C_6H_5OH) in100 ml ammonia-free water. It was prepared weekly, because this reagent was stand on darkness.

2.1.8 Sterilization Methods

- Autoclaving method by using the autoclave at 1.5 b, 121 ºC for 15 minutes
- Filtration by sterilized Millipore filter (0.22 µm and 0.45 µm).

2.2 Methods


General sketch of research steps
Hospital examinations
Biopsy collection
Immunological test
Fast urea solution test
Histological examination
Ab test for blood and Ag test for stool
Laboratory tests
Confirmation tests, Susceptibility test, Genomic profile
Select the high virulent isolates of Acute and Hemorrhage (A and H)
Ammonia measurement
Relationship between inoculums (OD and viable count), pH and ammonia production by isolates
Preparation of nanochitosan
Loading of selected antibiotics and L.europaeus extract on the nanochitosan
Semi quantitative by sensitivity tests
Quantitative by ammonia production

2.2.1 Detection of *Helicobacter pylori*

2.2.2. Hospital examination

These tests included:

2.2.2.1 Immunological test (Han *et al*., 2006)

a. Antibody detection in blood

One drop of blood from suspected patients with stomach ulcer was tested with rapid anti-*H. pylori* test kit. This kit gives direct result of presence or absence of *H .pylori* infection through their antibody presence in the blood of patient. Rapid Anti-*H. pylori* test is a colloidal gold enhanced rapid immunochromatographic assay for the qualitative detection of antibodies to *H. pylori* in human whole blood serum or plasma .

b. Antigen detection in faeces specimens (Baronand Thompson, 2011)

It was difficult to achieve this test with patients visiting the hospital .So it was done randomly on suspected cases outside the hospital. One gram of fresh stool collected in clean and dry plastic container and using *H. pylori* Ag Rapid test Cassette (Feces) to detect the presence of *H. pylori* antigen.

2.2.2.2 Biopsies collection

A total of 115biopsies specimens, 80 of these samples for males and 35 for females were collected from gastroscopy department in Alkadumia hospital during June to September 2014. Tree to four biopsies were collected from each patient (40-60 years) whom suffered from acute and hemorrhage stomach and duodenum ulcer. Care must be taken to insure that the patients not received antibiotics or antisecretory drugs especially proton pump inhibitors (PPI). One biopsy is inoculated directly in fast urea solution and one biopsy is inoculated in formalin for histological examination as mention bellow , other biopsies were stored at -70^{o}C in deep freezer after inoculating urea broth medium with 40% glycerol.

a. Fast urea solution test (Finegold, 1986)

Part of the biopsy specimen was inoculated into fast urea solution. Result appears within 2 hr by changing the color from yellow to pink.

b. Histological examination (Singhal *et al*., 2005)

Histological examination was conducted in private laboratory. There are five important stages in histology slide production:

a. Tissue fixation

Slide preparation begins with fixation of the tissue specimen. The purpose is to prevent tissue autolysis and putrefaction. For best results, the biological tissue samples should be transferred into fixative immediately after collection. There are many types of fixative, most specimens are fixed in 10% neutral buffered formalin. The optimum formalin-to-specimen volume ratio should be at least 10:1 (e.g., 10ml of formalin per 1cm^3 of tissue). This will allow most tissues to become adequately fixed within 24-48 hours.

b. Specimen transfer to cassettes

After fixation, specimens are trimmed using a scalpel to enable them to fit into an appropriately labelled tissue cassette. Specimens should not be so big that they fill the cassette – they are trimmed so as not to touch the edges. Additionally, they must not be too thick (ideally they should be less than 4mm), otherwise they risk being "waffled" when the cassette lid is closed. The filled tissue cassettes are then stored in formalin until processing begins.

c. Tissue processing

Processing tissues into thin microscopic sections is usually done using a paraffin block, as follows:

- *Dehydration* is the first step, which involves immersing your specimen in increasing concentrations of alcohol to remove the water and formalin from the tissue.
- *Clearing* is the next step, in which an organic solvent such as xylene is used to remove the alcohol and allow infiltration with paraffin wax.
- *Embedding* is the final step, where specimens are infiltrated with the embedding agent – usually paraffin wax. The tissue becomes surrounded by a large block of molten paraffin wax, creating what is referred to as the "block". Once the block solidifies, it provides a support matrix that allows very thin sectioning.

d. Sectioning

The tissue specimen is now ready to be cut into sections that can be placed on a slide. Wax is removed from the surface of the block to expose the tissue. Blocks are chilled on a refrigerated plate or ice tray for 10 minutes before sectioning. A microtome is used to slice extremely thin tissue sections off the block in the form of a ribbon. The microtome can be pre-set to cut at different thicknesses, but most tissues are cut at around 5 µm. After that the tissue ribbons are carefully transferred to a warm water bath. Here they are allowed to float on the surface, and can then be scooped up onto a slide placed under the water level. Charged slides work best for this process – they improve tissue adhesion to the glass, and help to reduce the chance of sections washing off the slide during staining. Slides should be clearly labelled, and then allowed to dry upright at 37^{o}C for a few hours to gently melt the excess paraffin wax, leaving the tissue section intact.

e. Staining

Most cells are transparent, and appear almost colourless when unstained. Histochemical stains (first, combined Hematoxylin with Eosin and second the Giemsa stain). are used to provide contrast to tissue sections, making tissue structures more visible and easier to evaluate. Following staining, a cover slip is mounted over the tissue specimen on the slide, using optical grade glue, to help protect the specimen.

2.2.2.3 Laboratory tests (Isolation and Identification)

These tests included the morphological (shape, motility, Gram, s stain) and confirmation tests and as follow:

a. One loop full of biopsy culture was inoculated in new urea broth (ZAN-1) prepared in (2.1.6.1) and streaking the modified BHI and incubated at 37^{o}C for (24-72hr) under anaerobic conditions using anaerobic jar and gas generation kit (H2 and CO2 libration).

b. Gram,s stain was used to stain the cells and examining under microscope for morphological studying.

c. Urease test (Finegold, 1986).

Pure colony was selected from modified TSB agar culture and inoculated into 5 ml of Fast urea solution (2.1.7.1) and incubated at 37^{o}C for 2hr. changing in color from yellow to red indicated to the positive result. pH number for the culture was raised to alkalinity.

d. Catalase test (Collee *et al*., 1996)

Pure colony was selected from modified TSB agar culture and smeared on a microscope slide. One drop of 3% hydrogen peroxide was added. If plenty bubbles were observed, catalase test was positive.

e. Oxidase test (Collee *et al.,* 1996)

Pure colony was transferred to filter paper and one drop of oxidase reagent was added. Developing of deep blue color in and around the colony was indicated to the presence of cytochrome oxidase enzyme.

f. Lecithenase test (Ulf westblom *et al.,* 1991).

Pure colony streaked on modified egg yolk agar and incubated under anaerobic condition at 37^{o}C for 24 hr. Positive result shows a clear zone surrounding the grow colonies due to lecithin neutralization.

g. 2, 3, 5-Triphenyltetrazolium chloride hydrolysis test (Queiroz *et al*., 1987).

Pure colony was inoculated by streaking into modified TSB agar supplemented with 0.04 gm/L of 2, 3, 5-Triphenyltetrazolium chloride (as mention in 2.1.6.4) and incubated under anaerobic condition. Positive result shows a change in the color of the media from yellow to red due to 2, 3, 5-Triphenyltetrazolium chloride reduction.

h. Blood hemolysis (Queiroz *et al*., 1987)

Hemolysis was detected by streaking of pure colony on a blood agar plate.

i. Scanning Electron Microscope (SEM) image

This test was conducted in Physical department / College of Sciences / Al Nahrain University.

j. Antibiotic susceptibility test

Pure active colony was inoculated into modified TSB agar by spreading and then distributed antibiotic discs on the surface of culture, and then plates were incubated under anaerobic conditions at 37^{o}C for 24 to 48 hr. The zone of inhibition was measured in mm. The susceptibility detection according to the table (2-1). *(* Xing JZ *et al.,*2005; Lawson AJ *et al*., 2005)

Table (2-1) : MIC breakpoint for *H. pylori (* Xing JZ *et al.,*2005; Lawson AJ *et al*., 2005)

Antimicrobial	MIC breakpoint (µg/ml)		
	S*	**I***	**R***
Amoxicillin	≤ 0.5	NA*	≥ 1
Ciprofloxacin	≤ 0.5	NA	≥ 1
Clarithromycin	≤ 0.5	NA	≥ 1
Levofloxacin	≤ 0.5	NA	≥ 1
Metronidazole	≤ 1	2-4	≥ 8
Tetracycline	≤ 1	2-8	≥ 16

* NA = not applicable
* S = susceptible
* I = intermediate
* R = resistance

k. Genomic profile

Maxwell ® 16 Tissue DNA Purification Kit and Maxwell device for genomic DNA extraction were used. Active and pure colonies of bacteria were selected from modified TSB agar culture and extracted their DNA in Maxwell device. DNA purity was measured in nanodrop device by using DNA extracted.

Gel electrophoresis was prepared as follows: (Maniatis *et al.,* 1982)

- 2% agarose gel was prepared by dissolving 2 gm agarose in 100ml of 1X TBE buffer in glass bottle with heating and mixing of magnetic stirrer.
- This solution was cooled to 70ºC, and then2µl of ethidium bromides was added from stock solution and mixed thoroughly.
- The clean glass tank (17×12×4 cm) was set on a horizontal section of the bench. The comb was set in position 0.5-1.0 mm above the surface of the tank so that a complete well was formed when agarose was added.

- The warm agarose-solution was poured into the tank.
- After the gel was completely set (20-30 min. at room temperature), the comp was carefully removed and the gel mounted in the electrophoresis tank which contain previously small amount of 1X TBE buffer.
- A volume of 600ml of 1X TBE was added to cover the gel to depth about 1 mm.
- A volume of 10 µl of the sample of DNA was added slowly into the slots of the submerged gel using an automatic micropipette.
- A volume of 5 µl of DNA marker was mixed with 1 µl of loading buffer. The mixture was set slowly into the slots of the submerged gel using an automatic micropipette.
- The gel was subjected to electrophoresis at 70 volts until the bromophenol blue tracking dye, migrated at least two-thirds of the way down the gel.
- The gel was examined by ultraviolet light using UV trans illuminator then photographed.
- DNA bands were visualized using the molecular imager device.

2.2.3 Determination of ammonia

a. Preparation of Ammonia standard curve (APHA , 1998)

In phenate method, a blue compound (indophenol) is formed by the reaction of ammonia, hypochloride and phenol catalyzed by manganous salt .The maximum absorption of blue color was observed at 630_{nm}. Standard curve was prepared as follows:

- **Preparation the stock solution**

A stock solution was prepared by dissolving 381.9 mg of NH_4Cl in suitable volume of deionized water and complete to 1 litter (L) with same water .This stock solution contains 1000 µg/ml of (NH_3- N).

- **Preparation of standard ammonia solution (5µg/ml NH_3 – N)**

Five ml of stock solution was diluted in 1 L of fast urea solution without phenol red (2.1.7.1). To prepared standard solution curve, according to the procedure, serial concentrations of ammonia confined from 0.5 µg/ml to 5 µg /ml (NH_3 – N) were prepared in fast urea solution without phenol red. The final volume of each dilution was 10 ml.

Table (1-1) showed the preparation of the standard curve according to the method of (Ga-eun Park *et al*., 2009 ; APHA , 1998) but using fast urea solution instead of water.

Procedure

- To 10 ml of sample add 1 drop (0.05 ml) $MnSO_4$ solution then add (0.5 ml) hypochlorous acid solution and shake well using vortex.

-Immediately add a drop (0.6ml) phenate reagent, add the reagents without delay using bulb pipette for convenient delivery. The change in

color noticed within 10 min. pH and OD were measured using spectrophotometer and wave length 630_{nm} .

- For the colorimetric determination of ammonia, spectrophotometer was used at 630_{nm} with a light path of 1 cm. The absorbance of indophenol measured and the calibration curve was obtained by plotting absorbance (630_{nm}) against ammonia concentrations (0 to5µg/ml). Figure (2-2) showed the standard curve of ammonia.

Table (2 -2): Ammonia (NH_3–N) concentrations used for the preparation of standard curve of ammonia.

No. of tube	Volume of Stock ammonia solution (ml)	Volume of the fast urea solution* (ml)	Final volume (ml)	Absorbance at 630 nm	Ammonia (NH_3–N) concentration (µg/ml)
1	0	10	10	0	0.0
2	1	9	10	0.005	0.5
3	2	8	10	0.011	1
4	3	7	10	0.016	1.5
5	4	6	10	0.025	2
6	5	5	10	0.028	2.5
7	6	4	10	0.033	3
8	7	3	10	0.059	3.5
9	8	2	10	0.145	4
10	9	1	10	0.299	4.5
11	10	0	10	0.023	5

*Fast urea solution without phenol red.

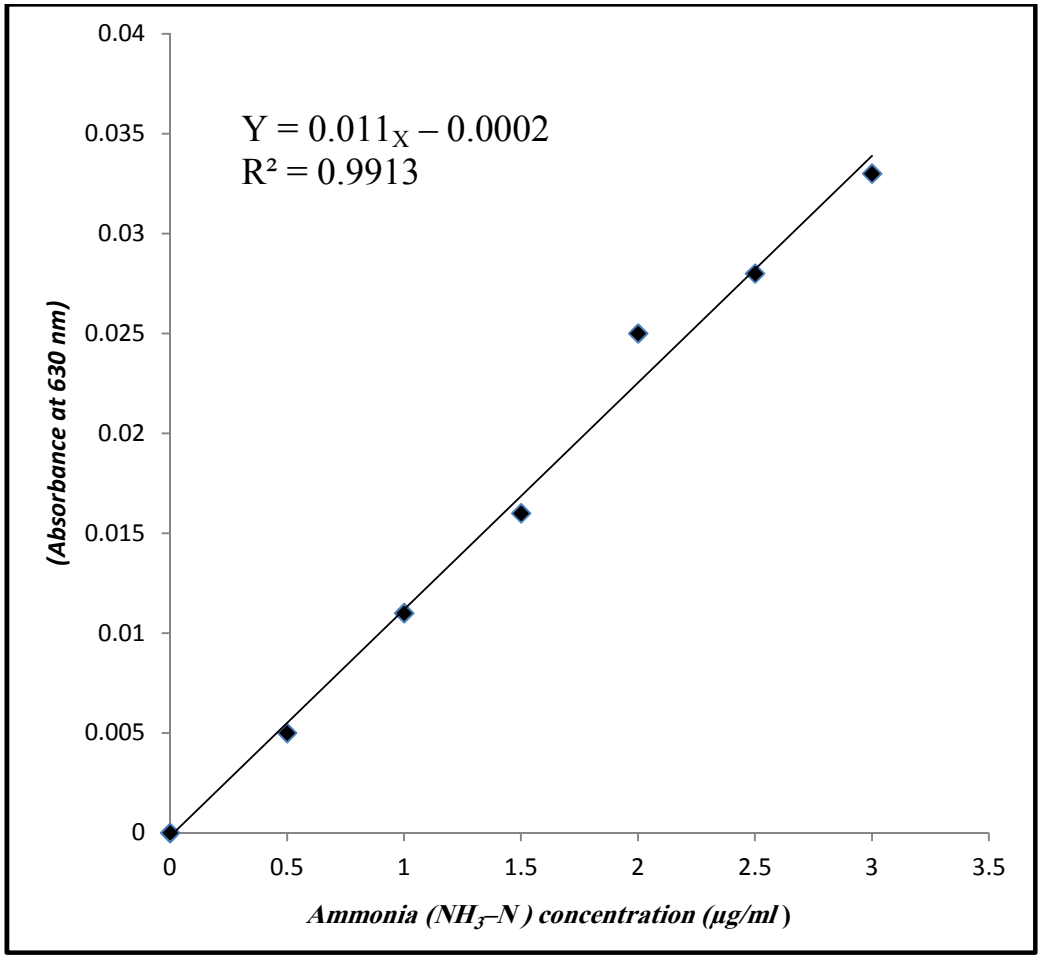


Figure (2-1): Standard curve of ammonia – Nitrogen using phenate method.

According to the standard carve we can get a formula to determine the amount of ammonia produced by the bacteria depending on its absorbance at 630_{nm}.By getting the slop from figure (2-1) its value was determine by using the following equation:

$$\text{Slope} = \frac{\Delta\ \text{Abs}\ (630_{\text{nm}})}{\Delta\ \text{Ammonia concentration}}$$

$$\text{Slop}e = \frac{0.033}{3} = 0.011$$

b. To the serial concentrations of standard curve, one drop of 0.02% phenol red solution was added and imaged the change in color.

c. pH of serial concentrations of standard curve was measured using pH meter.

2.2.4 *H. pylori* inoculums tests

2.2.4.1 Cells suspension

- Pure and active colonies of *H. pylori* (H and A) were selected from modified TSB agar culture (after incubation for 24 hr at 37ºC under anaerobic condition) and re-suspended in sterile screw capped tubes containing sterile normal saline then mixed well .
- Suspension density was Adjusted to 1 OD $575_{nm}$with normal saline .
- Serial dilutions of cells suspension (H and A) were prepared in range 0.1-1 OD using normal saline.
- Ten sterile screw capped tubes containing 9.9 ml of fast urea solution (pH 6.4) without phenol red were prepared using sterile Millipore filter (0.22µm).
- Ten sterile screw capped tubes of fast urea solution with phenol red contain 9.9 ml were prepared using sterile Millipore filter (0.22µm).
- Inoculated the fast urea tubes with 0.1 ml of bacterial suspension from each dilution and incubated at the same conditions.
- Color, pH changing and ammonia concentrations were measured after 2 and 4 hours of incubation at the same conditions.

2.2.4.2 Biopsy preparation

Two of biopsies A and H in urea broth and glycerol that stored in deep freezer were transferred to refrigerator and mix well in vortex to make suspension. This suspension was used in applications.

2.2.4.3Determination of cells viable count

Cells viable count of cultures were determined at zero time, 2hr and 4hr after inoculation in the fast urea solution. Serial dilutions were prepared from the fast urea cultures and using pouring plate technique. Decimal dilutions were used by transferring 1 ml from fast urea culture into 9 ml of sterile normal saline and complete the subsequent serial dilutions. One ml of dilutions 10^{-4} and 10^{-5}were transferred into sterile plates and poured the modified TSB agar plate. After solidification the plates were incubated at the same conditions.

2.2.5 *Loranthus europaeus* extract (Arokiyaraj *et al*., 2007 ; Rajab,2015)

a. This fruit plant was bought from Iraqi market and air-dried at room temperature. It was crashed by blunder for extraction.

b. Fifty grams of dried plant fruits were extracted overnight in 250 ml of methanol by maceration, using shaker incubator 25°C. The extract solution was filtered by Buchner funnel then concentrated at 40°C by rotary evaporator. Crude powder was kept at -20 until use.

c. 0.1 mg of the powder Dissolved in 5 ml of 20% ethanol and completed the volume to 100 ml with DW to obtain 100 µg / ml as stock solution.

2.2.6 Antibiotic preparation

- Commercial Ciprofloxacin- HCl and Tetracycline – HCl (500 mg */capsule for each antibiotic)* were supplied from pharmacy.
- One mg of each antibiotic was dissolved in 100 ml of DW. The final concentration of stock solution became 100 µg / ml**.** These stocks were prepared as required.

2.2.7 Preparation of nanochitosan (Ko *et al.,* 2002)

Chitosan nanoparticles were prepared by ionic gelation method. Cross linking of chitosan solution with Tripolyphosphate (TPP) was formed .

a. By Dissolving0.2mg of chitosan in 200 ml of 2% glacial acetic acid and mix for 30 minutes at 400 rpm using heat magnetic stirrer .

b.TPP(17.8 mg) were added ,The ratio of mixing was 5 : 2 (Chitosan : TPP). Mixing was continuing for 2 hours.

c. After mixing, the reactants was centrifuged at 8000 rpm for 15 minutes in high speed cooling centrifuge. Supernatant discarded and the deposit was washed twice with DW.

d. The precipitate was lyophilized in lyophilizer.

e. General characteristics of chitosan nanopartical were examined under Scanning Electron Microscope(SEM) such as shape, Size and surface morphology. Particle sizes of the nanochitosan was evaluated using oclumeter slide optical microscope. Table (2-3) showed the general physiochemical characteristics of chitosan (Al-Qadi *et al.,* 2012).

f. Nanochitosan particles was examined under light microscope using 40× lenses and particlesizemeter slide. One drop of prepared nanochitosan was spread over the known size dot in slide and test directly by microscope.

Table (2-3): Physiochemical characteristics of chitosan (Protasan® HCl).

Properties	Chitosan
Molecular weight, Mw (KDa)	150 – 400
Viscosity (mpa.s, 25 °C)	20 – 200
Deacetylation degree	75 – 90

2.2.8 Loading the antibiotic and *L.europaeus* extract on the nanochitosan

Stock antibiotics solution (as mentioned in 2.2.6) was used. The antibiotics and *L.europaeus* extract (as mention in 2.2.5)were added to the chitosan after the step of addition the TPP and mixed well using heat magnetic stirrer for 15 minutes at room temperature. The reactant was centrifuged using high cooling centrifuge at 8000 rpm and 1º C and lyophilized the precipitate.

a. To obtain 100µg /ml of the antibiotic and 1mg/ml of nanochitosan as a final concentration, 2 ml of 1mg/ml of the antibiotic stock solution (2.2.6) were added to 18 ml of nanochitosan (2.2.7),This addition was repeated for *L. europaeus* extract. These solutions were used as stock solution for subsequent steps.

b. A sample of 16 tubes containing fast urea solution was divided into two groups, The first group consist of 8 sterile screw cupped tubes, each tube was filled with 8.9 ml of fast urea solution with phenol red and the other 8 sterile screw cupped tubes, each tube was filled with 8.9 ml of fast urea solution without phenol red.

c. The following prepare 7 treatments were added as 1 ml for each tube of the fast urea solution with and without phenol red:

- Nanochitosan stock solution.
- Nanochitosan and ciprofloxacin.
- Nanochitosan and Tetracycline.
- Nanochitosan and *L. europaeus*.
- Ciprofloxacin.
- Tetracycline.
- *L.europaeus*.

The final concentration became 1µg/ml for the nanochitosan and 10µg/ ml for each antibiotics (ciprofloxacin and tetracycline) and for *L. europaeus* .

d. pH , $OD_{575}$and color change for each tube were measured and observed before inoculation . The blank was the fast urea solution only.

2.2.9 Semi quantitative sensitivity tests of *H.pylori*

2.2.9.1 Preparation of antibiotic stock solution by using Agar wells diffusion method (Perez *et al*. 1990)

a. For ciprofloxacin antibiotic every 250µl should contain 5µg of ciprofloxacin as in commercial ciprofloxacin disks and 0.1mg of nanochitosan. 4.75 ml of ciprofloxacin (100µg/ml) was completed to 19 ml using DW. Then 1 ml of nanochitosan stock solution which contains 1 mg /ml of nanochitosan was added. The final concentrations of ciprofloxacin and nanochitosan solution became 5µg and 0.1mg / 250 µl respectively.

b. For tetracycline antibiotic every 250µl should contain 30µg of tetracycline as in commercial tetracycline disks and 0.1mg of nanochitosan. 1 ml of tetracycline (2.4 mg/ml) was completed to 19 ml with DW. 1 ml of nanochitosan (1mg/ml) was added. The final concentration of tetracycline and nanchitosan became 30µg and 0.1 mg/250 µl respectively.

2.2.9.2 Preparation of *L. europaeus* stock solution by using Agar wells diffusion method (Perez *et al*. 1990)

- 4.75 ml of *L. europaeus* (100µg/ml) was completed to 19 ml with DW and 1 ml of nanaochitisan (1mg/ml)was added .The final concentration of *L. europaeus* and nanochitosan became 5 µg and 0.1mg / 250 µl respectively.
- Modified TSB agar plates was prepared as mention in (2.1.6.4). The medium was streaked with active colonies of A-1 and H-1 using sterile swabs.
- Numbers of wells were made using a sterile cork borer .
- Add 250µl from (nanochitosan), (nanochitosan and ciprofloxacin), (nanochitosan and Tetracycline), (nanochitosan and *L. europaeus*) , (ciprofloxacin), (Tetracycline) and *(L. europaeus*) to the wells respectively. The final concentration in this 250 µl should be 0.1 mg/ml for the nanochitosan , 5µg for ciprofloxacin , 30 µg for tetracycline and 5µ for *L. europaeus*.

- The plates were left at room temperature for 2 hours to allow diffusion of test sample and incubated at the same condition.
- The diameter of inhibition zone was measured in mm.

2.2.10 Quantitative sensitivity tests of *H.pylori*

2.2.10.1 Fast urea solution without phenol red:

2.2.10.1.1 Bacterial suspension

- Active pure colonies of bacteria were collected and resuspended in sterile normal saline. The optical density (OD_{575nm}) was adjusted to 0.3 for A-1 and 0.8 for H-1.
- Fast urea solution Inoculated as mentioned in (2.2.8-c) with the 0.1 ml from the suspension of A-1 (OD , 0.3) and H-1 (OD ,0.8) separately .
- The treatments were Incubated for 2hr at the same conditions.
- The pH and the growth were measured at OD_{575} after 2hours**.**
- Ammonium concentrations were determination (2.2.3–a) after centrifugation the treatments at 8000 rpm for 15 minutes.

2.2.10.1.2Biopsy specimens

Steps in (2.2.10.1.1) were repeated, but the inoculation was substituted with 0.1 ml of biopsy suspension as mentioned (2.2.4.2) and incubated at the same condition for 2 hours.

2.2.10.2 Fast urea solution with phenol red

2.2.10.2.1 Application on acute (A-1) and hemorrhage (H-1) bacterial suspension

- Repeat the steps in (2.2.10.1.1).
- After 2hr of incubation, pH and the change in color were observed.

2.2.10.2.2 Application on acute and hemorrhage bacterial biopsy specimens

- Steps in (2.2.10.1.1) were repeated, but the inoculation was substituted the suspension inoculums with 0.1 ml of biopsy specimens as mentioned in (2.2.4.2).
- After 2hr of incubation, pH and the change in color were observed.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Sample collecting

A total of 115 biopsies specimens for adult patients age category ranged from 40 to 60 years suffering from acute and hemorrhage stomach and duodenum ulcer were collected, 80 of the samples were for males while 35 of them for females. Biopsies were collected by endoscopy which is considered as one of the invasive tests and in which care must be taken to insure that the patient didn't receive antibiotic or proton pump inhibitors (PPI) (Megraud, 1991; Dohil,1999).

Samples were specified according to the severity of infection. It was found that 83of the samples were acute ulcer while 27 of them have hemorrhage ulcer and 5 samples were obtained from normal stomach as illustrated in table (3-1).Samples were also specified according to the age of the patients as shown in table (3-2).

Table (3-1): Distribution and percentage of cases according to the gender and severity.

Cases	Number of cases / % of cases	
	Male	**Female**
Normal stomach	3 / 2.6%	2 / 1.7%
Acute ulcer	58 / 50.4%	25 / 21.7%
Hemorrhage ulcer	21 / 18.2%	6 / 5.2%

Table (3-2):Distribution and percentage of acute and hemorrhage ulcer according to the age of patients.

Age	Type of infection / % of infection		
	Normal	**Acute ulcer**	**Hemorrhage ulcer**
40-50	4 / 3.4%	36 / 31.3%	9 / 7.8%
50-60	1 / 0.8%	47 / 40.8%	18 / 15.6%

In general, results showed that the acute cases were more than hemorrhage for both age categories and the male represent the largest number.

3.2 Detection of *Helicobacter pylori*

3.2.1 Hospital Direct tests

a. **Immunological tests**: The other direct test that considered as invasive test made in the hospital was the rapid anti *H. pylori* test that developed to positive or negative in the in presence or absence of *H. pylori* antibody in the blood specimens of the patients . As well as for stool Ag detection, the kit that developed into two lines mean the result is positive for the presence of *H.pylori*, while the kit that developed into only one line mean that the result is negative for the presence of *H.pylori.* Figure (3-1) showed the positive and negative reaction.

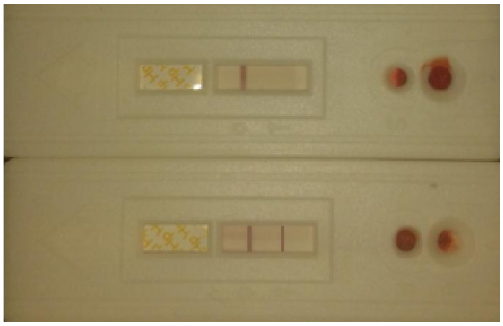


Figure (3-1): Rapid anti *H. pylori* test kit for human blood. (Lower slide, +ve result; Upper slide, -ve result).

b. Fast urea solution tests: Selected biopsies should be changed the fast urea solution color from yellow to pink color within 2- 6 hours after inoculation with the biopsy specimens due to the urea utilization (Hydrolysis) and production of ammonia as shown in figure (3-2) and table (3-3)and (3-4).

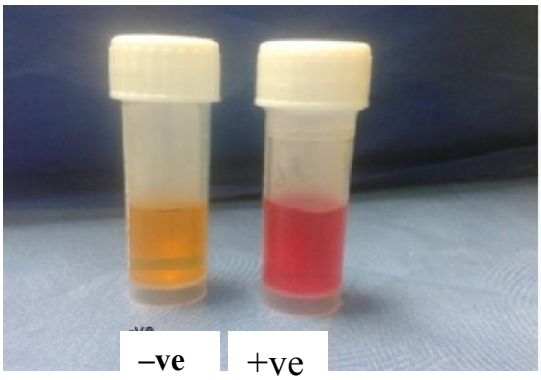


Figure (3-2): Fast urea solution test for the biopsy collection of peptic ulcer cases.

c. Histological tests

Histological tests showed the difference in tissue level between the normal stomach and the infected tissue as illustrated bellow:

- Staining with hematoxylin and eosin (H&E) showed that gastric body mucosa with normal looking gastric gland that contain oxyphiles cells with normal looking lamina propria and the tissue that infected with *H. pylori* section showed gastric body mucosa with increased lamina propria inflammatory cells with disorganization of gastric glands .No atrophic changes seen as shown in figure(3-3a,b,c,d and f)
- Giemsa stain were also used as a part of histological examination to distinguish the different between acute ulcer and hemorrhage ulcer. As shown in figure (3-4). Detection of *H. pylori* can be easily done in many biopsy spacemen by using hematoxylin and eosin (H&E) stain and Giemsa stain as shown in figure (3-5).
- There is a huge difference in cell level between normal stomach cells and cells that infected with *H.pylori*. Also the stage of infection is differ between acute and hemorrhage ulcer, cells in hemorrhage ulcer were more disintegration than cells in acute ulcer as shown in figure (3-3 and 3-4).
- *H.pylori* can be found as individuals or as clusters as shown in figure (3-5).

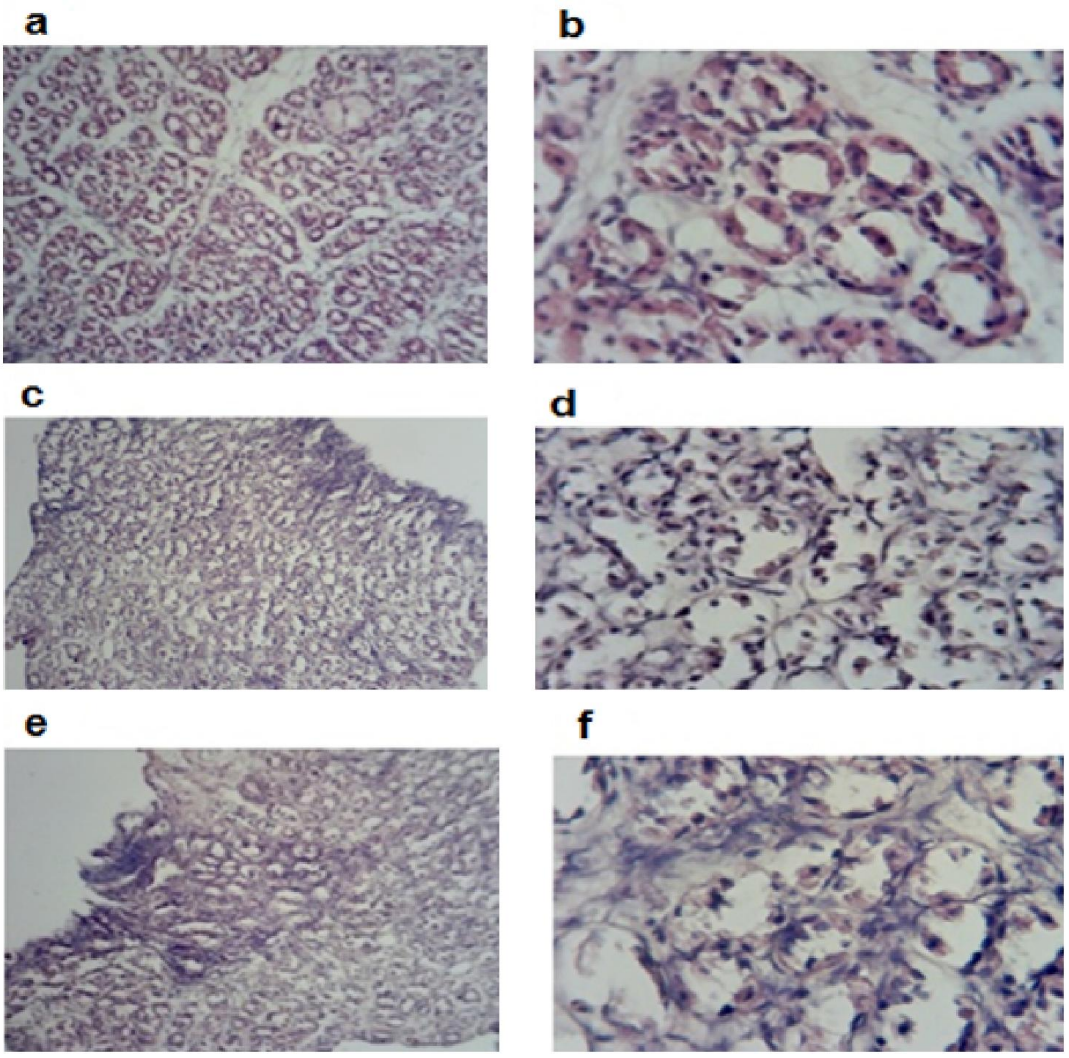


Figure (3-3): Section a and b stained with H and E stain show gastric body mucosa with normal looking gastric gland that contain oxyphiles cells with normal looking lamina propria. c and d for acute ulcer and e, f for hemorrhage ulcer (section stained with H and E stain show gastric body mucosa with increased lamina propria inflammatory cells with disorganization of gastric glands , no atrophic changes seen).

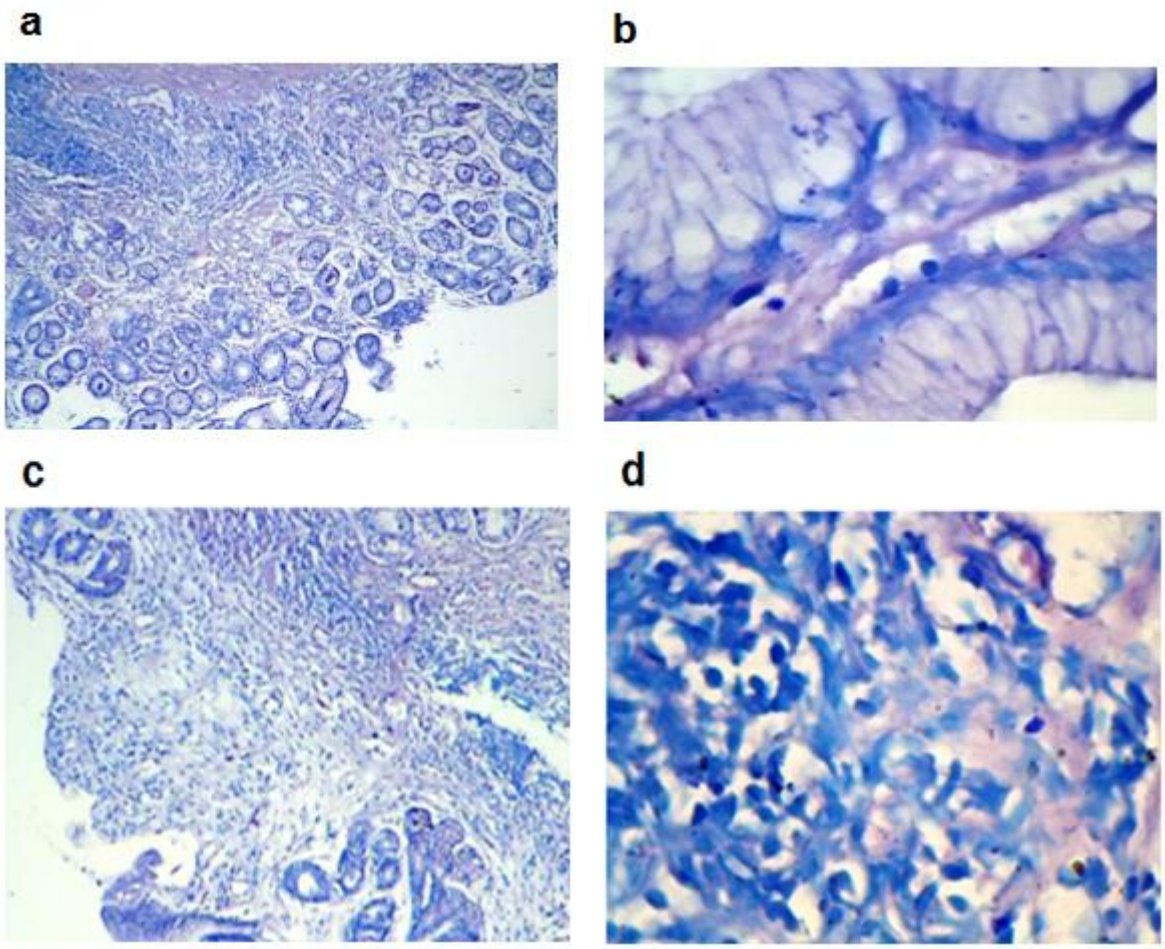


Figure (3-4): Section a, b for (acute stomach ulcer stained with Giemsa stain). Section c, d for (hemorrhage stomach ulcer stained with Giemsa stain).

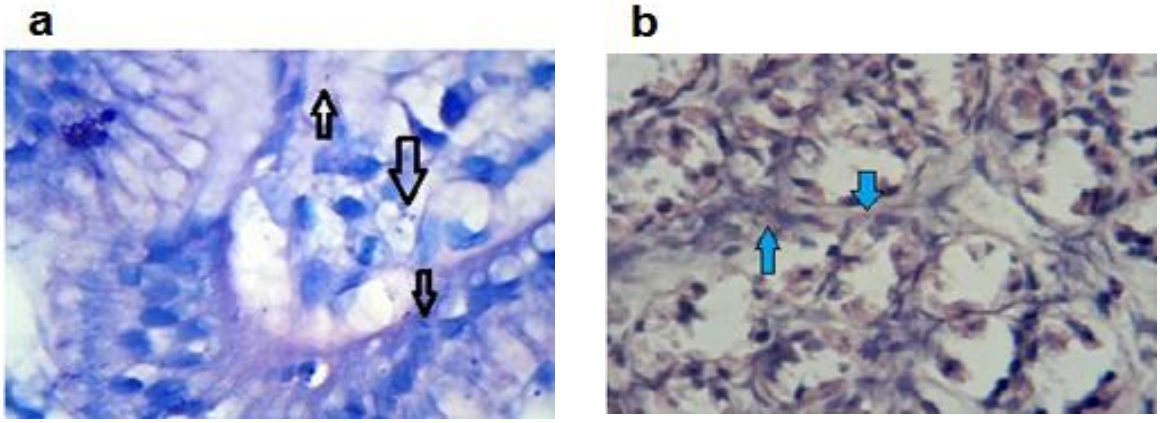


Figure (3-5): a: *H. pylori* in a section stained with Giemsa stain, b: *H. pylori* in a section stained with H and E stain.

All biopsies specimens were then transferred to the lab under suitable conditions and store at - 4ºC in urea broth medium containing 20% or 40 % glycerol (Han ,*et al*.1995).

3.2.2 Isolation and Identification of *H.pylori*

According to the results of hospital tests, ten isolates were selected from acute ulcer specimens (A-1 to A-10) and ten isolates from hemorrhage ulcer (H-1 to H-10). The selection was depended on the results of fast urea and serological tests for biopsies which gave a result within 6 h.

When the biopsy or isolates were cultured into new urea broth medium (ZAN-1), It was noticed, as showed in figure (3-6), the clear and fast change in color from yellow to pink and appearance of the large colonies in comparison with another media due to the composition of this new medium.

In both modified BHI agar and TSB agar media, part of the biopsies were cultured and gave us colonies with red zone due to 2,3,5-triphenyltetrazolium chloride reduction by the activity of this bacteria as shown in fig.(3-7) and fig.(3-8).

The detection tests with direct observation of the endoscopy test were enough for our study and not needed more than tests. Tables (3-3) and table (3-4) showed the confirmation tests for acute and hemorrhage isolates.

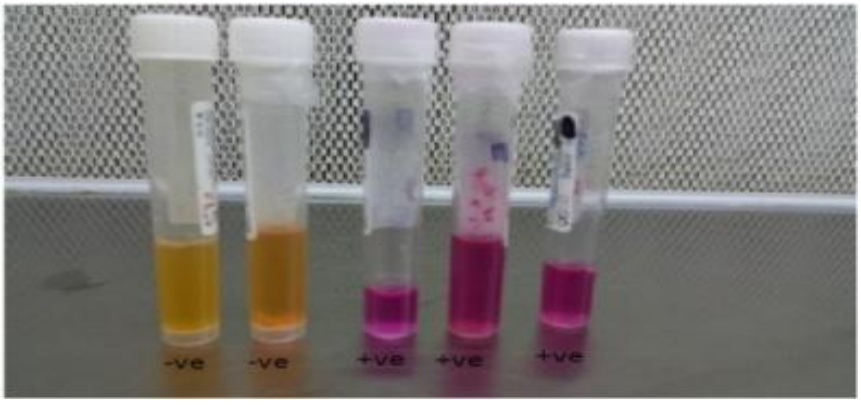


Figure (3-6): Growth of *H. pylori* in urea broth medium (ZAN-1).

The hydrolysis of urea during this short period was the most important indicator for the ability of *H. pylori* in production high active and multi copies of urease enzyme which regarded as important virulent factor as mentioned by (Elitsur and Hill, 1998; Elitsur, 1999).

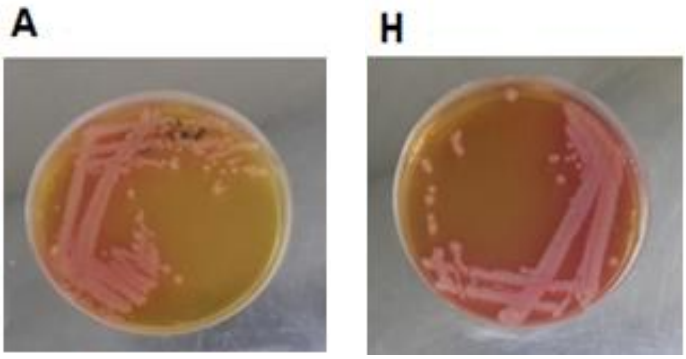


Figure (3-7): Growth of *H. pylori* on modified BHI agar after 24 hours (A for acute and H for hemorrhage), red zone around the colonies shows the distinct characters test for *H. pylori*.

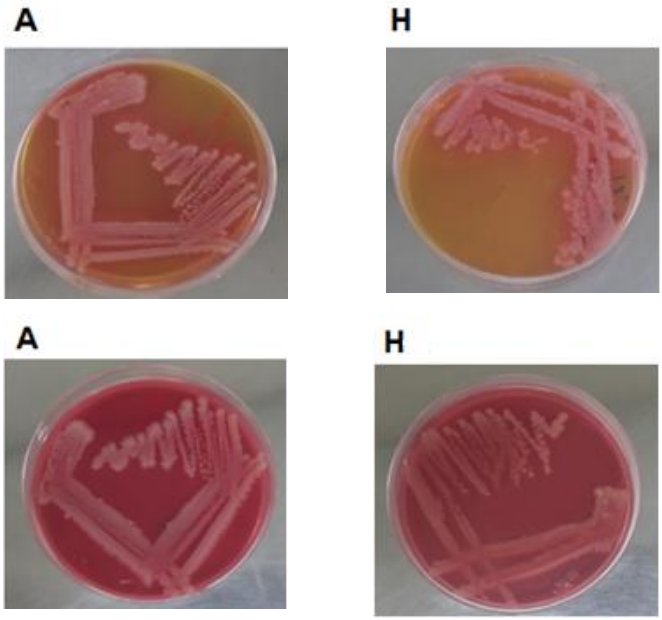


Figure (3-8): Growth of *H. pylori* on modified TSB agar. (Upper one: A for acute and H for hemorrhage after 24 hours while the lower one after 48 hours).

Table (3-3): Biological and serological tests for Isolation and Identification of *H. pylori* from acute ulcer infection.

Test / Sample	Antibody test	Antigen test	Motility	Catalase	Oxidase	Gram stain	Urease ≤ 4 hours	Urease ≥ 4 hours	Blood hemolysis	Lecithenase	TCC* hydrolysis
A-1	+	+	+	+	+	G-ve	+	-	-	+	+
A-2	+	-	+	+	+	G-ve	+	-	-	+	+
A-3	+	-	+	+	-	G-ve	-	+	+	+	-
A-4	+	+	-	+	+	G-ve	-	+	-	-	+
A-5	-	+	+	-	+	G-ve	-	+	-	+	+
A-6	+	-	+	+	+	G-ve	-	+	+	-	+
A-7	+	-	-	+	+	G-ve	-	+	-	+	+
A-8	-	+	+	+	-	G-ve	-	+	-	+	-
A-9	+	+	+	-	+	G-ve	-	+	-	+	+
A-10	+	-	-	+	+	G-ve	-	+	+	-	+

* Triphenyltetrazolium chloride

Table (3-4): Biological and serological tests for Isolation and Identification of *H. pylori* from hemorrhage ulcer infection.

Test / Sample	Antibody test	Antigen test	Motility	Catalase	Oxidase	Gram stain	Urease ≤ 4 hours	Urease > 4 hours	Blood hemolysis	Lecithenase	TCC* hydrolysis
H-1	+	+	+	+	+	G-ve	+	-	+	+	+
H-2	+	-	+	-	+	G-ve	+	-	-	+	+
H-3	+	-	-	+	+	G-ve	-	+	+	+	-
H-4	-	+	+	+	-	G-ve	-	+	+	-	-
H-5	+	-	-	-	+	G-ve	-	+	-	+	+
H-6	+	-	-	+	+	G-ve	-	+	+	-	+
H-7	-	+	+	+	+	G-ve	-	+	+	+	+
H-8	+	-	+	+	-	G-ve	-	+	-	+	+
H-9	+	+	-	+	+	G-ve	-	+	+	+	-
H-10	-	-	+	+	-	G-ve	-	+	+	-	+

* Triphenyltetrazolium chloride

Two isolates were taken to complete the aim of this study; one for acute infection (A-1) and the other for hemorrhage infection (H-1) based on the conformation test and the ability of its urease hydrolysis within less than 4 hours as mention by (Kusters *et al.,* 2006; Guo *et al*., 2011).

Results illustrated in figure (3-9) showed that *H. pylori* was a gram negative helical shape bacteria according to optical microscopic examination.

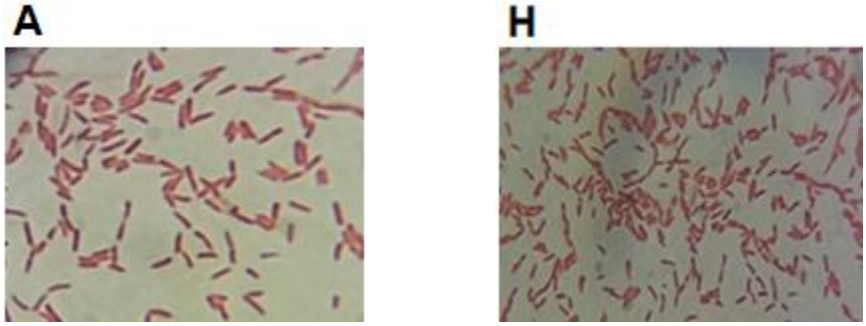


Figure (3-9): Morphological characteristics of *H. pylori*. (A) for acute, (H) for hemorrhage.

Oxidase, catalase and Lecithinase which all of them give positive result as shown in (figure 3-10).There were no morphological differentiation but the only different appeared with blood hemolytic Figure (3-10,D). It was found that hemorrhage isolate (H) produced the strange colonies with some alpha hemolysis which was completely different from (A).

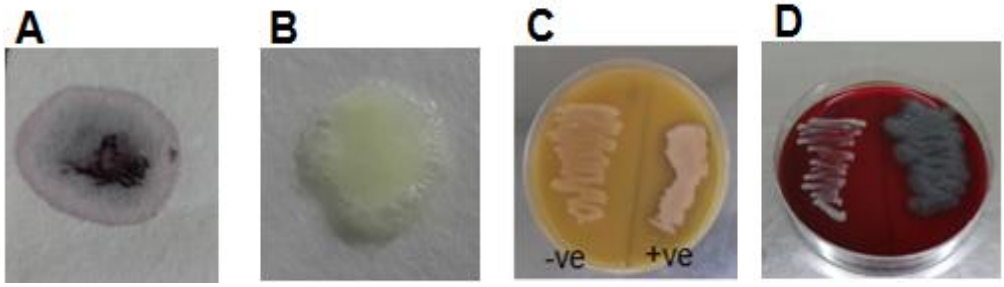


Figure (3-10): Positive result for confirmation tests for *H. pylori* isolates (A: Oxidase ; B: Catalese; C: Lecithinase ; D: Beta hemolysis for blood agar (Left side of plate represents acute case while the right one represents hemorrhage case.

3.2.3 Scanning electron microscope (SEM)

After examination in SEM using different magnification, results showed that *H. pylori* was flagellated curved cells bacteria and also it seems that there was no morphological differentiation between acute and hemorrhage bacteria isolates as pointed in figure (3-11).

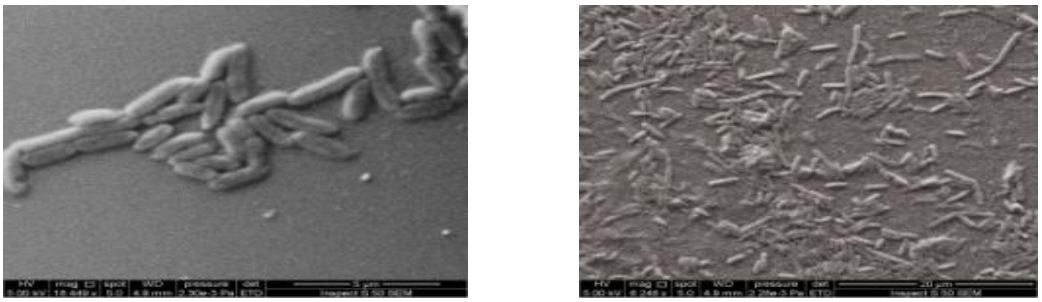


Figure (3-11): Flagellated shape of *H. pylori* in SEM under magnification (Left: 5µm and Right: 20µm).

3.3 Susceptibility test

Susceptibility tests showed that these isolates had the differences in response to the antimicrobial agents as mentioned in table (3-5). The antibiotics (Ciprofloxacin, Tetracycline, Levofloxacin, Amikacin) were the effective antibiotics against acute and hemorrhage isolate as shown in figure (3-12). The most active antimicrobial agent was the Ciprofloxacin and Amikacin for the hemorrhage isolate (H-1). Ciprofloxacin and tetracycline were selected to complete the study*(*Xing JZ *et al.,*2005; Lawson AJ *et al*., 2005).

Table (3-5): Inhibition zone formed by different types of antibiotic disc on acute and hemorrhage *H. pylori* isolates.

Antibiotic disc	Inhibition zone (mm)	
	Acute isolate (A-1)	Hemorrhage isolate (H-1)
Ciprofloxacin (cip5)	18 (s)	25 (s)
Tetracycline (T30)	17 (s)	14 (s)
Levofloxacin (Lev5)	22 (s)	24 (s)
Amikacin (Ak30)	23 (s)	25 (s)
Amoxicillin (A10)	(r)	(r)
Erythromycin (E15)	(r)	(r)
Metronidazole (MZ5)	(r)	(r)
Clarithromycin (CLA15)	(r)	(r)

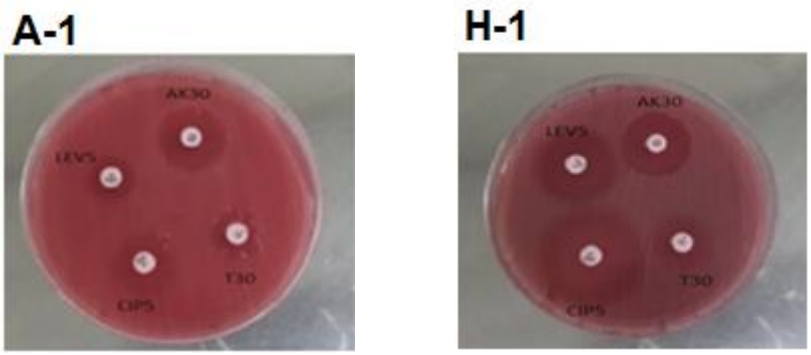


Figure (3-12): **Susceptibility tests for antibiotic test of *H. pylori* isolated by using modified TSB media (A-1 for acute infection, H-1 for hemorrhage infection).**

3.4 Genomic profile

Genomic study revealed that profile of acute and hemorrhage isolates (A-1, H-1) of *H. pylori* have only genomic DNA and the absent of plasmids as showed in figure (3-13).

These results confirmed that the virulent genes and other characteristics were carried on the DNA chromosomal of *H. pylori* and this may be undergoing more explanations about the peptic ulcer infection and their complications in the patients.

In conformity with what Labigne and de Reuse (1996) mentioned about the analysis of *H. pylori* whole genome, It was appeared a new look into its pathogenesis, acid tolerance , antigenic variation and microaerophilic characters. The availability of the complete genome sequence will allow further assessment of *H. pylori* genetic diversity. This Is an important aspect of *H. pylori* epidemiology as allelic polymorphism within several loci has already been associated with disease outcome.

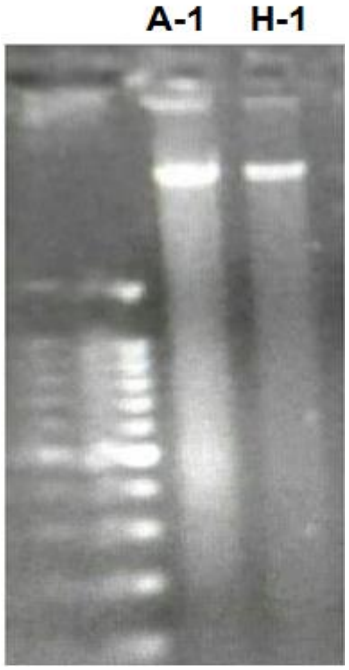


Figure (3-13): DNA profile for different acute and hemorrhage infectious cases of *H. pylori.* (From the left: Lines 1and 2, lader; line 3, acute isolate; line4, hemorrhage isolate. (Agarose, 2% ,100 V and 70 minutes).

3.5 Production of ammonia by *H. pylori*

3.5.1 Ammonia standard curve

The most important virulence factor in *H. pylori* is urease enzyme which founds in multi copies as impeded extracellular enzyme in the cell membrane. The specification of this enzyme is to hydrolysis the urea into ammonia and CO_2 at high turnover number in the acidic condition of the stomach .This activity is to avoid the high acidity of the stomach and

Providing suitable neutral microenvironment for bacteria to survive and hidden in the groves of lining layer.

Production of ammonia by urease used as indicator for virulent potential, stage of infection, primary identification of presence *H. pylori* bacteria and to put suitable relationship for onset detection of this bacteria. To do above relationships, it should be started with determination of ammonia standard curve in fast urea solution and the change in pH and color of this solution in presence or absent phenol red indicator. Phenate method proposed by (APHA 1998) was found to be the suitable procedure for our experiments conditions. The slope (Absorbency) of standard curve that have been prepared previously in (2.2.3) was 0.011 and this value was useful in calculation the ammonia production by the bacteria in the culture media or any fluid collected from the patients.

3.5.2 Color and pH

The gradual change in color of ammonia in the fast urea solution with phenol red was illustrated in the figure (3-14). The presence of ammonia serial concentrations were released the pH of the solution and changed the color. The change in the color was regarded as qualitative indicator for the virulence and the stage of infection of bacteria in relationship with the other parameters will be explained subsequent.

Color										
pH	6.4	6.7	7.01	7.25	7.34	7.47	7.53	7.56	7.6	7.62	7.7
Concentrations (µg/ml)	0	0.5	1	1.5	2	2.5	3	3.5	4	4.5	5

Figure (3-14): Color and pH change in the fast urea solution with phenol red containing ammonia concentration ranged from 0 to 5 µg/ml.

3.6 Bacterial growth parameters and ammonia production

These parameters were introduced to complete the relationship between the bacterial viable count and their density with ammonia production for the onset recognition the stage and type of *H. pylori* infection. Fast urea solution was selected to the measurement of the growth and ammonia production by isolates of *H. pylori* because this solution used in primary detection of presence this bacterium in biopsy collected. So, it was used for construction the relationship among the selected parameters.

3.6.1 Density of cells

The relation of ammonia- nitrogen production against optical density (OD $_{575\ nm}$) of serial suspension dilutions of cells (as mentioned in 2.2.4, for A-1 and H-1 isolates) showed in figures (3-15) and (3-16). Time of experiment 2h in order to simulate the real time for monitoring the biopsy testing in the hospital. It was excluded time 4 hours because its results outside the boundaries of experiment and methods of measurement. Isolate A-1 more active from H-1 in ammonia production during 2 h under the same conditions of inoculums density and others.

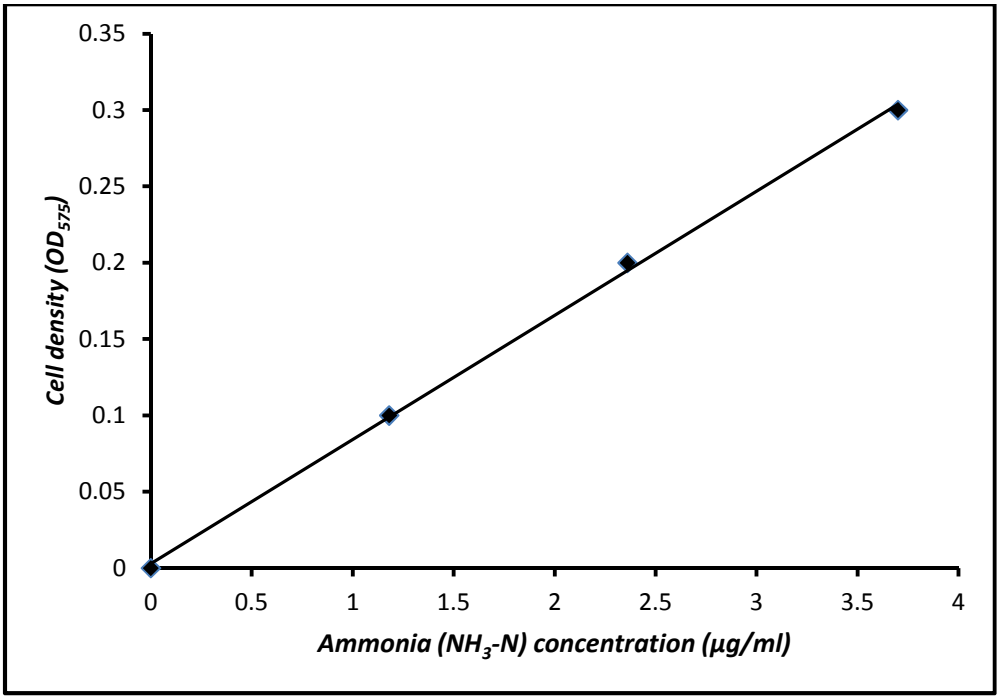


Figure (3-15): Ammonia-Nitrogen product concentration (µg/ml) for acute sample after 2 hours of incubation in fast urea solution.

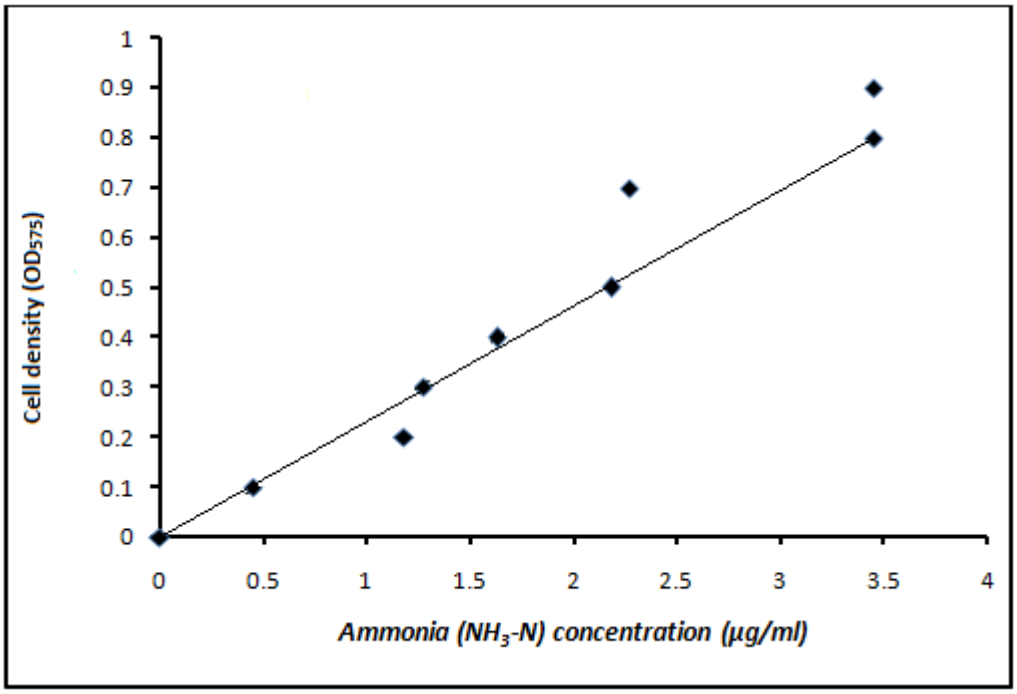


Figure (3-16): Ammonia-Nitrogen product concentration (µg/ml) for hemorrhage sample after 2 hours of incubation in fast urea solution.

3.6.2 Total Viable Count (TVC)

Total viable count of serial cells suspension density was important parameter in relation with cells density, pH and ammonia production .Table (3-6) and (3-7) were explained these relationships.

3.6.3 pH number

It was monitoring the change in pH number as a result of effect the suspension density on hydrolysis of urea. Within 2 h of incubation, the pH could be measured but after 4 h it was not responding. At high suspension density, it was found that pH not responding. In general, pH value was negligible if not accompanied with color changing or may be
needed more accurate instrument. Tables (3-6) and (3-7) illustrated the relationship of parameters (pH, OD, TVC and ammonia production) with each other.

Table (3-6): Parameters relationships of acute *H. pylori* isolate A-1 in fast urea solution.*

No.	OD_{575}^{**}	Viable cell count/ml	pH		Ammonia concentration (µg/ml)	
			After 2 hours	After 4 hours***	After 2 hours	After 4 hours****
1	0.1	1×10^5	7.17	> 7.5	1.18	> 5
2	0.2	2×10^5	7.28		2.36	
3	**0.3**	**3×10^5**	**7.31**		**3.7**	
4	0.4	4×10^5	7.02		1	
5	0.5	5×10^5	7.02		0.9	
6	0.6	6×10^5	7.0		0.81	
7	0.7	7×10^5	7.2		1.45	
8	0.8	8×10^5	7.15		1.18	
9	0.9	9×10^5	7.03		0.9	
10	1	1×10^6	7.38		4	

* Fast urea solution without phenol red.

** The optical density of bacteria at 575_{nm}.

*** The pH is higher than 7.5.

****Ammonia production is too high to be read by the spectrophotometer.

Table (3-7): Parameters relationships of hemorrhage *H. pylori* isolate H-1 in fast urea solution.*

No.	OD_{575}^{**}	Viable Cell count /ml	pH		Ammonia concentration (µg/ml)	
			After 2 hours	After 4 hours***	After 2 hours	After 4 hours****
1	0.1	1×10^5	7.0	> 7.5	0.45	> 5
2	0.2	2×10^5	7.09		1.18	
3	0.3	3×10^5	7.09		1.27	
4	0.4	4×10^5	7.19		1.63	
5	0.5	5×10^5	7.32		2.18	
6	0.6	6×10^5	7.35		2.27	
7	0.7	7×10^5	7.39		2.72	
8	**0.8**	**8×10^5**	**7.4**		**3.45**	
9	0.9	9×10^5	7.41		3.45	
10	1	1×10^6	7.1		-1	

* Fast urea solution without phenol red.

** The optical density of bacteria at 575 nm.

*** The pH is higher than 7.5.

****Ammonia production is too high to be read by the spectrophotometer.

3.7 Preparation of nanochitosan

There were a huge difference in the microscopic level between chitin and chitosan. Figure (3-17- a, b) illustrated SEM images of chitin and chitosan which reflected the clear differences between them using the same magnification power (20 µm). Chitosan was appeared as smooth hairy structure, meanwhile, chitin looked like leave and this returned to the chemical method of preparation the chitosan in removing acetyl group from chitin and these results similar to observation of Trung *et al*., 2006and Toan, 2009.

First examination of nanochitosan under light microscope was preformed by using particalsizemeter slide. It was able to detect the partical size with 70 µm because of the limitation of the dot size of particlesizemeter slide that not less than 70µm . Figure (3-17,c) showed the nanochitosan in spherical structure using SEM according to the method of preparation by (Ko *et al.,* 2002).These nanosphere particles was not clearly characterized by SEM if not coated the nanometels to give their discrete shape and reached 10 -50 nm diameter (Nasti *et al*., 2009).

It is clearly that all chemical methods of nanochitosan production are similar. It was difficult to screen under UV-visible because of gelatinous form of nanochitosan solution and the turbidity appearance which not similar to the nanoparticals of metals that give high adsorption at 300-400 nm. FTIR screening of nanochitosan may be revealed only the high concentration of active groups that formed after chemical treatment with TPP and may be not useful for indication the formation of nanochitosan. So these characterization methods in addition to FAM were not sutable to study the proparties of nanochitosan alone without incorporated with nanometel to give it the distinct structure. (Singh and Lillard, 2009; Nasti *et al.* 2009 and Zhao *et al.,*2011).

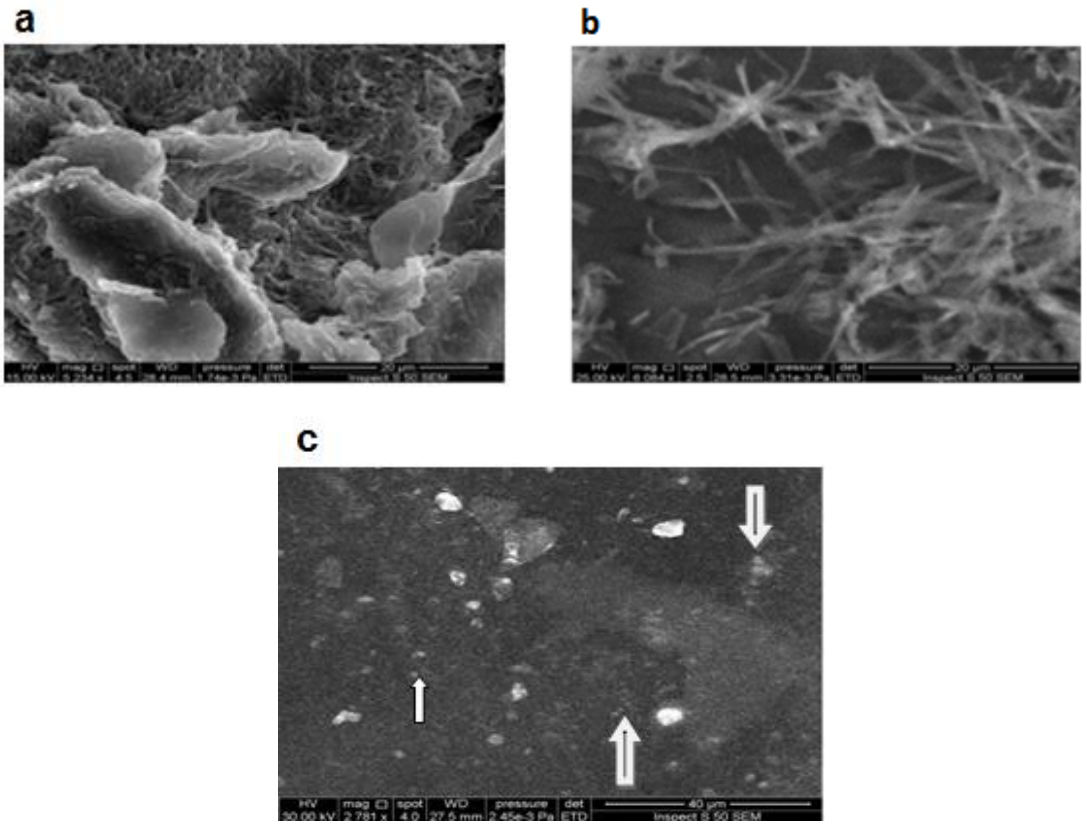


Figure (3-17): SEM image.(a) Chiten structure with magnification (20µm) using SEM (b) Chitosan structure with magnification (20µm) using SEM. (c) Nano-chitosan with magnification (40µm) using SEM.

3.8 Effect of antibiotic loaded on nanochitosan

Two types of antibiotic Ciprofloxacin and Tetracycline with *L. europaeus* extract were loaded on nanochitosan as mention in (2.2.8), the samples were then tested by using SEM as shown in figure (3-18).

It appeared that anitibiotics and *L. europaeus* were loading on the surface of nanochitosan particles. Quantities used in loading was 10µg/ml, as mentioned in 2.2.8, which equal to the mean concentration used by human has average weight 75 kg when used one dose every12 hr according to the instruction of physician.

The loading quantity could not be measured because no precipitation appeared at high speed centrifugation due to their low density and size.

In order to complete these experiments, the composite materials were used in liquid state. Figure (3-18) showed the loaded nanochitosan with antibiotic. It was seemed that abroad range of nanoparticles, part is visible and other not.

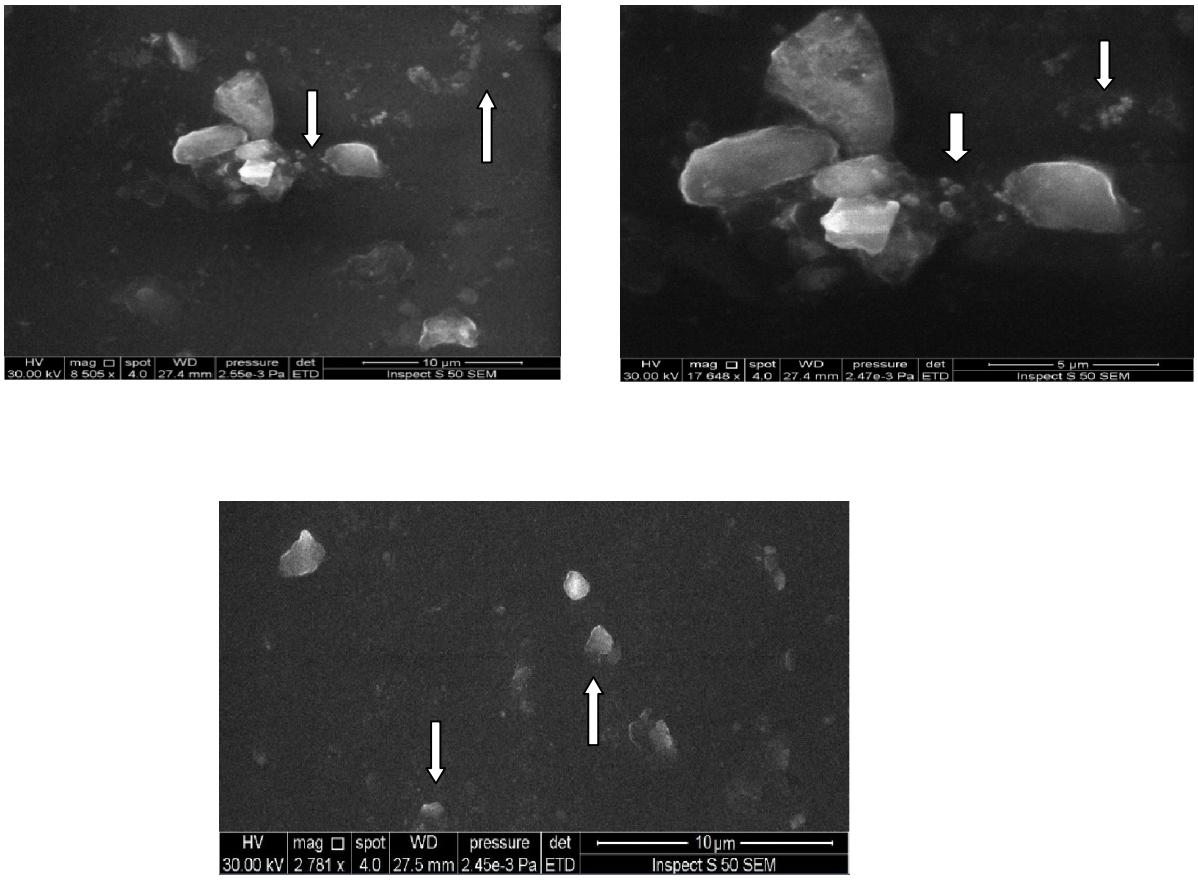


Figure (3-18): SEM image. (a) antibiotic congecated to nano-chitosan with magnification (10 µm), (b)antibiotic congecated to nano-chitosan with magnification (5 µm), (c) *L. europaeus* congecated to nanochitosan with magnification (10µm).

3.9 Application of antibiotic and *L. europaeus* loaded on chitosan nanopartical on *H. pylori*

3.9.1 Semi qualitative tests (Agar well diffusion method)

By using wells technique, as mentioned in 2.2.9, there were no effect of 0.1mg/ml for the nanochitosan and *L. europaeus* extract, but there was an inhibition zone for nanochitosan loaded with tetracycline and ciprofloxacin on acute and hemorrhage isolates (A-1 and H-1) as shown in tables (3-8, 3-9) and figures (3-19 and 3-20). It was found no effect of free nanochitisan and *L. europaeus* loading on nanochitosan on A-1 and H-1 growth after 24 and 48 h of incubation. A-1 isolate more sensitive to tetracycline and ciprofloxacin loaded on nanochitosan compared with isolate H-1. It was found that A-1 isolate more resistant to the free antibiotics. In general, it was found the ability of nanochitosan to increase efficiency of antibiotics and maintaining their activity.

Table (3-8): Effect of loaded nanochitsan with agents on *H. pylori* (A-1) isolate.

No .	Treatment	Inhibition zone (mm)	
		After 24 hours	After 48 hours
1	Nano CHI	(r)	(r)
2	Nano CHI+ *L. europaeus*	(r)	(r)
3	NanoCHI + CIP	30 (s)	48 (s)
4	Nano CHI+T	18 (s)	24 (s)
5	T	17 (s)	17 (s)
6	CIP	15 (s)	15 (s)

Table (3-9): Effect of laoded nanochitsan with agents on *H. pylori* (H-1) isolate.

No .	Treatment	Inhibition zone (mm)	
		After 24 hours	After 48 hours
1	Nano CHI	(r)	(r)
2	Nano CHI+*L. europaeus*	(r)	(r)
3	NanoCHI + CIP	28 (s)	42 (s)
4	Nano CHI+T	15 (s)	18 (s)
5	T	18 (s)	18 (s)
6	CIP	18 (s)	19 (s)

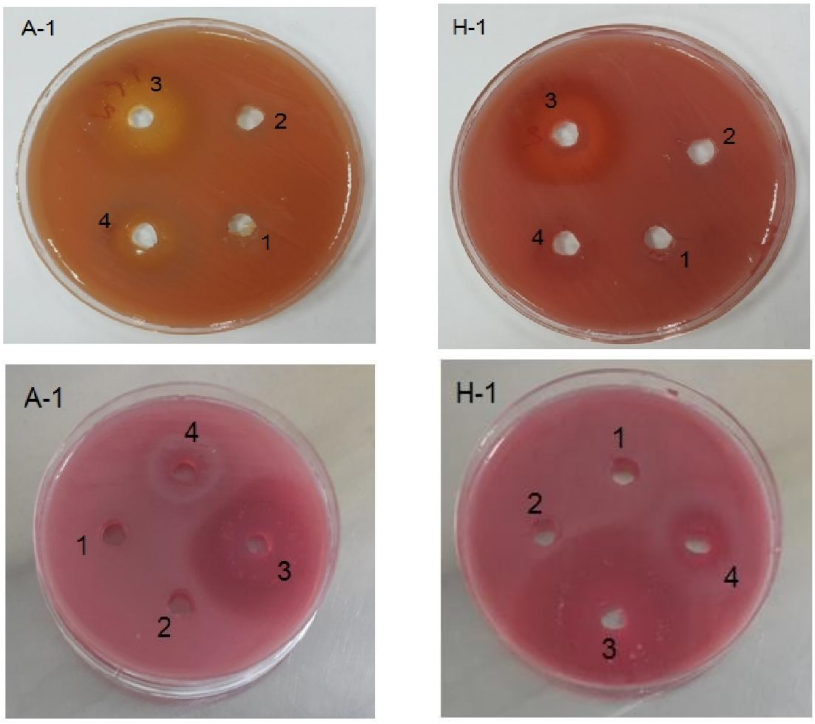


Figure (3-19): Inhibition zone for each 30 µg tetracycline loaded on chitosan nanoparticles and 5µg ciprofloxacin loaded on chitosan nanoparticalson (A-1 and H-1) bacterial culture , the tope is for the culture after 24 hours , down is for the culture after 48 hours.

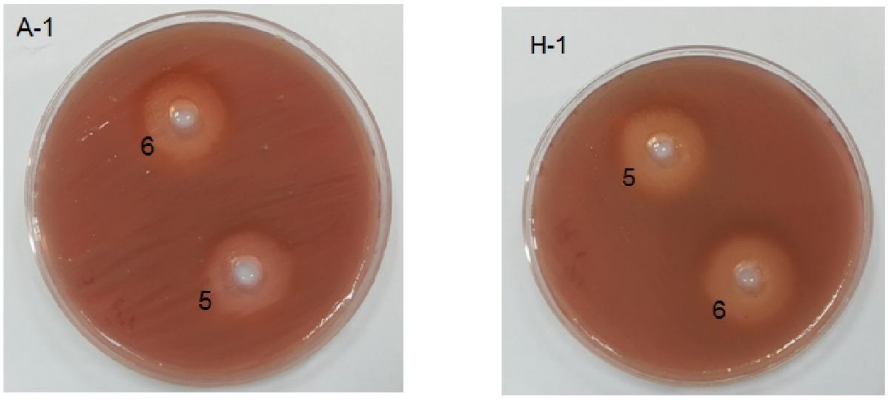


Figure (3-20): Effect of Inhibition on (A-1 and H-1) isolates after 48hours, No.(5)Tetracycline; No. (6) Ciprofloxacin

3.9.2 Quantitative tests

3.9.2.1 Fast urea solution without phenol red

Considering the body weight is 75 kg, a serial diluted suspension of active cell were prepared for each isolates as mentioned in (2.2.10.1.1). Dilution 0.3 for A-1 and 0.8 for H-1 were selected according to the results of tables (3-6, 3-7). For biopsies, it was homogenized using vortex after thawing and used them for inoculation in urea solution without phenol red (as mentioned in 2.2.10.1.2).

Table (3-10) and figures (3-21, 3-22) were illustrated the results of above applications.

Table(3-10):Ammonia production by bacteria suspension of the active cells and biopsies of A-1 and H-1.

No.	Treatment	Ammonia- N production (µg/ml) by			
		Bacterial suspension* (A-1)	Bacterial suspension (H-1)	Biopsy (A-1)	Biopsy (H-1)
1	nano-CHI	33.2	73	17.8	21.8
2	nano-CHI + CIP	5.2	28.5	4.5	8
3	nano-CHI +T	20.7	32	12.3	17.6
4	nano-CHI+*L.europaeus*	13.6	39.4	22.5	21.2
5	CIP	28.7	35.2	41	39
6	T	44.3	40.7	84	34.5
7	*L. europaeus*	31.4	39	32.9	24.9

* The inoculums were 0.1 ml for bacterial suspension (A-1) and (H-1) and Biopsies (A-1) and (H-1)

It was concluded From the values in the table that the less amount of ammonia production (µg/ml) were obtained from ciprofloxacin loaded on nanochitosan for (A-1) suspension and biopsies (A-1) and (H-1), followed by tetracycline loaded on nanochitosan. These values represent the final concentrations for ammonia production after subtract the zero time values Figures (3-21,3-22).

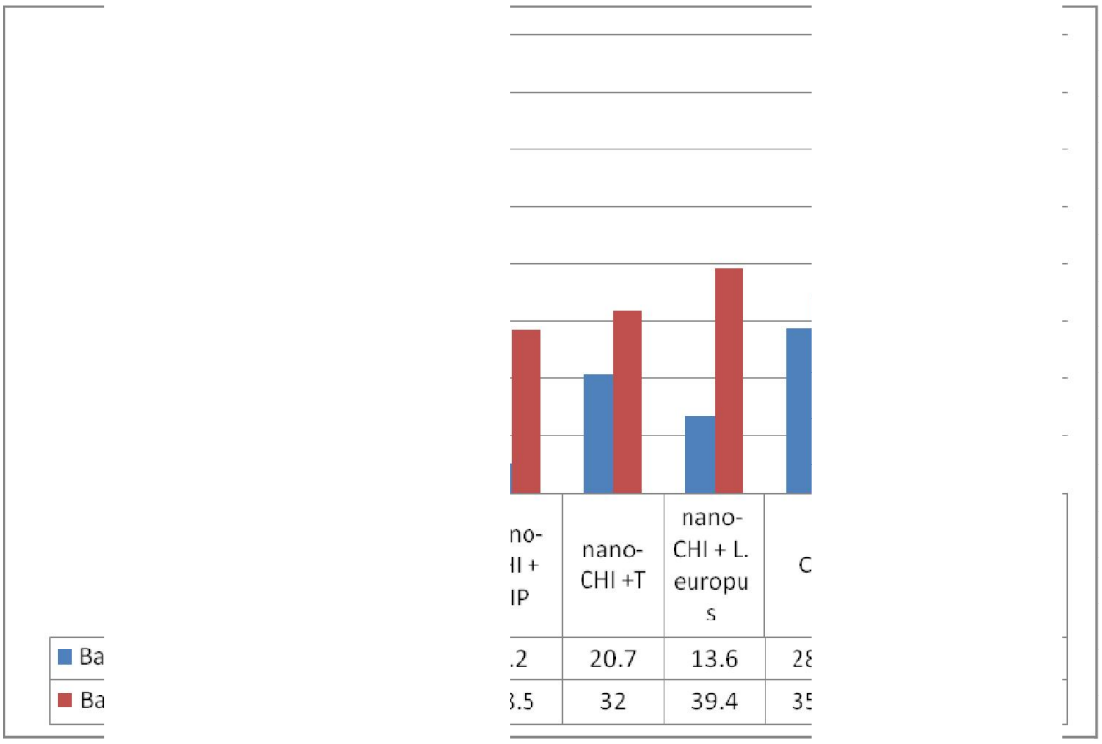


Figure (3-21): Ammonia production by *H.pylori* A-1 and H-1 suspension after 2 hr of incubation at 37ºC in Fast urea solution without phenol red.

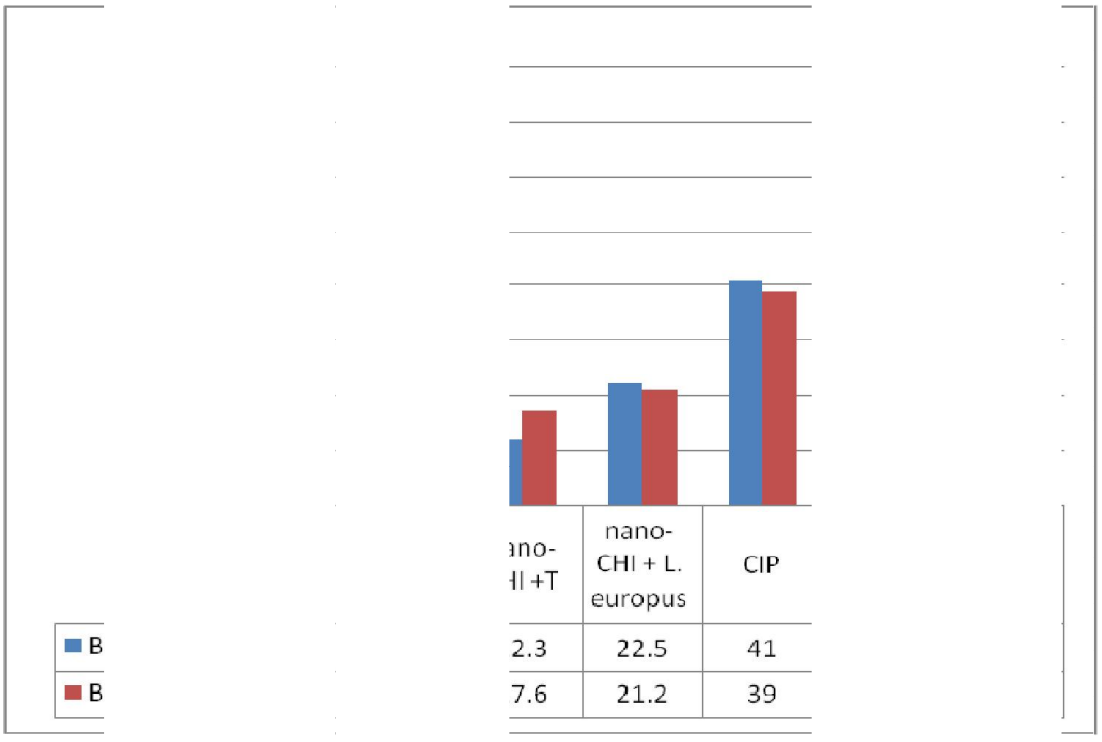


Figure (3-22): Ammonia production by *H.pylori* A-1 and H-1 biopsy after 2 hr of incubation at 37ºC in Fast urea solution without phenol red.

Table (3-11) illustrated the net values of growth optical density (OD_{575nm}) and ammonia production by A-1 and H-1 isolates after 2h of incubation in fast urea solution with different treatments of nanochitosan, nanochitosan + ciprofloxacin, nanochitosan + tetracycline, nanochitosan+ *L. europaeus*, ciprofloxacin, tetracycline and *L. europaeus*. These values mean that the bacteria protect them self from the severe environmental condition surrounded it by utilizing the urea in the medium and produce ammonia to survive. The table clearly shows that the net growth is very limited .There has been no change to growth within two hours of incubation and for all treatments including the control treatment. But it was found a clear activity of the urease activities and production of ammonia and almost for all treatments except for nano-CHI + CIP followed nanochitosan with tetracycline for and acute hemorrhagic biopsies. Weak growth due to the nature of the ammonia solution which is not the nutrition medium as much as fast detection solution. It was evident from table (3-11) that nanochitosan was not suited to be alone inhibiter to the growth of or urease activity for both types of bacteria (A-1 and H-1) and this also applies to ciprofloxacilin (CIP) antibiotics.

The presence of each of the nanochitosan and CIP antibiotic together has increased dramatically the inhibition of urease activity and ammonia production. This synergistic inhibitory action of nanochitosan with antibiotic is very important to prevent the infection of this bacterium to cause ulcers.

Table (3-11): Bacterial growth propotion and ammonia production after two hours of incubation .

Treatment	OD_{575} and Ammonia- N production (µg/ml)			
	Bacterial suspension (A-1)	**Bacterial suspension (H-1)**	**Biopsy (A-1)**	**Biopsy (H-1)**
nano-CHI	0.006 (OD) 33.2 (NH_3)	0.001 73	0.005 17.8	0.001 21.8
nano-CHI + CIP	0.008 5.2	0.009 28.5	0.006 4.5	0.008 8
nano-CHI +T	0.008 20.7	0.008 32	0.005 12.3	0.008 17.6
nano-CHI + *L. europaeus*	0.002 13.6	0.00 39.4	0.003 22.5	0.00 21.2
CIP	0.008 28.7	0.00 35.2	0.001 41	0.001 39
T	0.005 44.3	0.006 40.7	0.007 84	0.006 34.5
L. europaeus	0.001 31.4	0.00 39	0.001 32.9	0.003 24.9
Bacterial growth	0.001 35.2	0.001 32	0.002 36.6	0.002 35.9

3.9.2.2 Fast urea solution with phenol red

A serial dilutions of suspension as mentioned in tables (3-6, 3-7) for each isolates (prepared in 2.2.10.1.2) was used.

For biopsies, it was homogenized using vortex after thawing and used them for inoculation in urea solution with phenol red (as mentioned in 2.2.10.1.2). The results of the color change after 2 hours of incubation were showed in figures(3-23, 3-24).

There were direct correlations in explanation the results .If ammonia concentration was increased the pH number increased and color became pink. All these relations can be facilitated the onset of determination the infection type and stage in the biopsy or other fluid of patient. For the tubes that contain nanochitosan its color remain yellow while there is an ammonia production as mention in table (3-11) and that is due to the acidic nature of the nanochitosan that effect on the color.

A-1

H-1

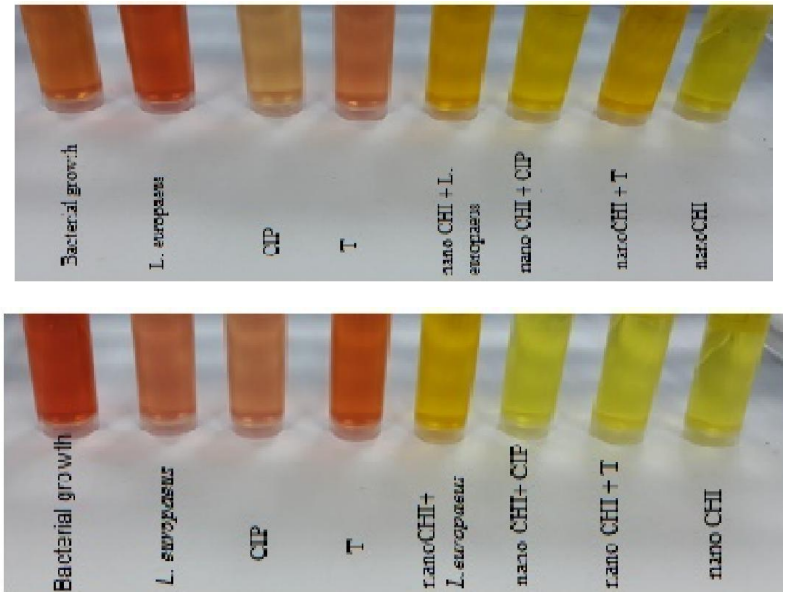
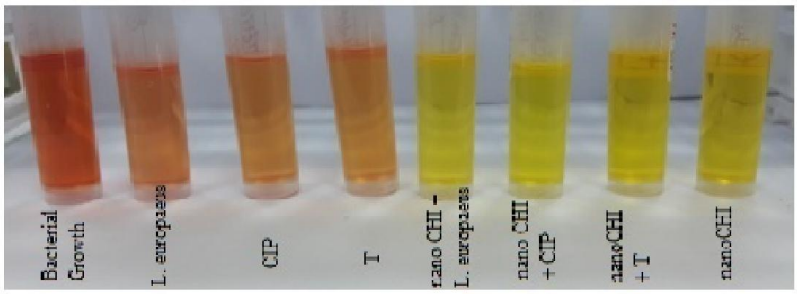


Figure (3-23): Color change of fast urea solution inoculated with A-1 and H-1 Bacterial solution, in addition to the control that contain only bacteria growth.

A-1



H-1

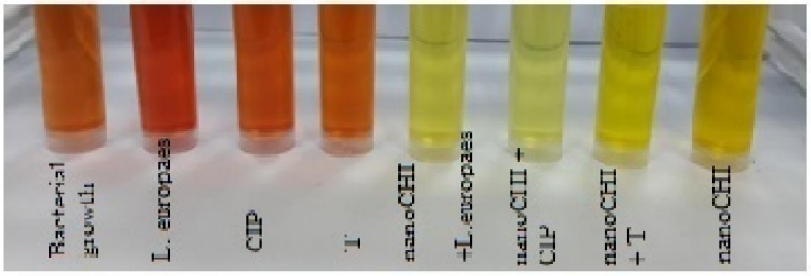


Figure (3-24) Color change of the fast urea solution inoculated with (A-1) and (H-1) biopsies, in addition to the control that contain only bacterial growth.

Chapter Four

Conclusions and Recommendations

4.1 Conclusions

- The new (ZAN-1) media and modified (BHI Agar, TSB Broth and TSB Agar) media was effectively used to accelerate growth and identification of *H. pylori*.
- Ammonia is a key of fast identification to this bacterium during 2 hr.
- Genetic profile showed that the virulent genes located on chromosome and not carried on the plasmid.
- A-1 was more virulent than H-1 according to the relation curve of OD, TVC and ammonia production.
- Cells suspension density that could be monitored for their ammonia producing during 2 h were 0.3 for A-1 and 0.8 - 0.9 for H-1.
- pH value change was negligible if not accompanied with color changing.
- Success nanochitosan as drug delivery by loading the antibiotic and plant extract.
- Nanochitosan loaded with antibiotics was more effective than free antibiotics against *H. pylori*.
- Efficiency of nanochitosan to maintain the activity of antibiotics.

4.2 Recommendations

- Test the product on laboratory animals because nanochitosan is harmless and have no damage on human body.
- Continuing in fast diagnosis of ulcer by suing saliva, gastric juice and plasma samples.
- Use nanochitosan to deliver other types of therapy for stomach ulcer or digestive system injuries.
- More studying on the mechanism of increasing the efficiency of therapy loaded on nanochitosan in treatment of diseases.

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الخلاصة

الهدف من هذه الدراسة هو محاولة الكشف المبكرعن بكتريا الملوية البوابية *Helicobacter pylori* من خلال الاعتماد على العلاقة بين إنتاج الأمونيا مع تغيير لون الاوساط الزراعية و درجة الحموضة و كثافة نمو البكتريا ونوع التقرح (حادة أو نزيفي) . كما تهدف الدراسة أيضا إلى استخدام المواد النانوية الصناعية القابلة للتحلل الحيوي كوسيلة لإيصال العلاج وكذلك معرفة كفاءتها كعامل علاجي .

تعتبر بكتريا الملوية البوابية *H. pylori* من المسببات الرئيسية لقرحة المعدة والاثنى عشر والتي قد تتطور الى سرطان المعدة او الاثنى عشري. تم تحضير وسط زراعي جديد (مرق اليوريا الجديد ZAN-1) و تطوير اوساط زراعية اخرى مهمه مثل (Tryptic Soyabean Broth ، TSB و Brain Heart Infusion Broth، BHI) من اجل دعم وتحفيز هذه البكتريا على النمو الجيد كونها من البكتريا صعبة النمو والعزل . اعتمدت النتائج على انتاج الامونيا خلال 2 ساعة وتغيير لون محلول اليوريا السريع خلال هذا الوقت لعزل البكتريا كونه صفة مميزة لها ولضراوتها فضلاعن الفحوصات التأكيدية والمناعية والنسق الوراثي . تم تطوير حامل نانوي من مادة الكايتين والمعروف بالنانوكايتوسان وقد حضر بالطريقة الجلاتينية الايونية واستخدم كمادة حاملة فضلا عن اختباره كمادة علاجية.

يمكن ايجاز النتائج كما يلي :

تم جمع 115 عينة لوخزات اصابات قرحة المعدة للاشخاص ضمن الفئة العمرية (40 – 60) من مستشفى الكاظمية التعليمي للفترة من حزيران الى تشرين الاول /2014 . ثمانون (80) للذكور و35 للاناث ، تم انتخاب 10 وخزات استنادا الى الفحوصات المباشرة في المستشفى لكل من الاصابات الحادة والنزفية ، استناد الى الفحوصات المختبرية والمستشفى ، تم انتخاب عزلة واحدة لاصابة حادة (A-1) وعزلة نزفية (H-1).

بين النسق الجينومي للعزلات الحادة والنزفية (A-1 , H-1) لبكتريا *H. pylori* وجود دنا المجين فقط وعدم وجود الدنا البلازميدي.

بينت نتائج الحساسية للمضادت الحياتية ان العزلة A-1 هي اكثر مقاومة من العزلة النزفية H-1 .

انتخبت طريقة الفينت لتكون افضل طريقة لتعين تراكيز الامونيا في اوساط تواجد هذه البكتريا، دلت النتائج الى ان العزلة الحادة اكثر فعالية في انتاج الامونيا وتغيير لون الوسط (3.7 مايكروغرام /مل) مقارنة ب (3.45 مايكرو غرام /مل) للعزلة النزفية خلال ساعتي الاختبار .

تم انتاج نانوكايتوسان بدمج عدة طرق للتحضير وباقطار اقل من 1 مايكرون استنادا الى صور المجهر الماسح الالكتروني .

تحضير خلاصة نبات حب الدبج بالاستخلاص بالميثانول وتجفيفها .

تم تحضير نانوكايتوسان محمل بالمضادات الحيوية سبروفلاكسين و تتراسايكلين وخلاصة نبات حب الدبج واختبارهم بحالتهم السائلة لصعوبة ترسيب النانو المحمل بالمضادات والخلاصة لانخفاض كثافتهم وقلة اوزانهم الجزيئية. كما تم تحضير خلطات مجفدة Lyophilized mixtures.

دلت نتائج التطبيقات شبه الكمية ان العزلة البكتيرية A-1 هي الاكثر حساسية للنانوكايتوسان الحامل للمضاد الحيوي سبروفلاكسين وبقطر تثبيط 48 ملم مقارنة بالسبروفلاكسين لوحده 15 ملم بعد حضن لمدة 48 ساعة ، كما دلت النتائج التطبيقية الى ان اعلى تركيز لانتاج الامونيا تم التحسس له من قبل جهاز المطياف هو 5.2 مايكروغم/ مل بلقاح 3×10^5خلية /مل بالنسبة للنانوكايتوسان المحمل بالمضاد سبروفلاكسين ، اما نتائج خارج خلوي للوخزات فقد دلت ان للنانوكايتوسان مع السبروفلاكسين تاثير فعال على تثبيط نمو الخلايا حيث اعطى اقل تركيز الامونيا (4.5 مايكروغرام/مل) مقارنة مع السبروفلاكسين لوحده الذي اعطى تركيز (41 مايكروغرام/مل) من الامونيا .

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قسم التقانة الاحيائية

تاثير بعض المضادات الحياتية والمستخلص الكحولي لحب الدبج *Loranthus europaeus* على نمو بكتريا الملوية البوابية *Helicobacter pylori* باستخدام الكايتوسان النانوي

رسالة
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كجزء من متطلبات نيل درجة الماجستير في علوم التقانة الاحيائية

من قبل
زينب سامي علي
بكالوريوس 2006

إشراف

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