# *HELICOBACTER PYLORI* DIAGNOSIS, VIRULENCE GENES, RESISTANCE TO CONVENTIONAL ANTIBIOTICS AND ANTIMICROBIAL ACTIVITY OF SELECTED MEDICINAL PLANTS ON ISOLATES OBTAINED FROM PATIENTS UNDERGOING ENDOSCOPY AT AGA KHAN UNIVERSITY HOSPITAL, KENYA

**STEPHEN KIBE NJOROGE**

**DOCTOR OF PHILOSOPHY**

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# *Helicobacter pylori* Diagnosis, Virulence Genes, Resistance to Conventional antibiotics and Antimicrobial activity of selected Medicinal plants on Isolates obtained from Patients undergoing Endoscopy at Aga Khan University Hospital, Kenya

**Stephen Kibe Njoroge**

**A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Infectious Diseases and Vaccinology of the Jomo Kenyatta University of Agriculture and Technology**

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## DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

Signature…… Date…….

**Stephen Kibe Njoroge**

This thesis has been submitted for examination with our approval as the university supervisors.

Signature…… Date…….

**Dr. Andrew Nyerere, PhD**

**JKUAT, Kenya**

Signature…… Date…….

**Prof. Gunturu Revathi, PhD**

**AKUH-N, Kenya**

Signature…… Date…….

**Dr. Smita Devani, PhD**

**AKUH-N, Kenya**

## DEDICATION

To my Mother who said where there is a way and always do the best.

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## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF APPENDICES

## LIST OF ABBREVIATIONS AND ACRONYMS

| | |
|---|---|
| **AKUHN** | Aga Khan University Hospital Nairobi |
| **AST** | Antimicrobial Susceptibility Testing |
| ***cagA*** | Cytotoxin associated gene A |
| ***dupA*** | Duodenal Promoting gene A |
| **GC-MS** | Gas Chromatography Mass Spectrometry |
| **JKUAT** | Jomo Kenyatta University of Agriculture and Technology |
| **MBC** | Minimum bactericidal concentration |
| **MHC** | Major Histocompatability Complex |
| **MIC** | Minimum inhibitory concentration |
| **OGD** | Oesphago-duodenoscopy |
| **RUT** | Rapid Urease Test |
| ***vacA*** | Vacuolating Cytotoxin Gene A |

## ABSTRACT

*Helicobacter pylori* colonizes the gut of persons of all ages, and is the etiologic agent for acute and chronic gastritis and a predisposing factor in peptic ulcer disease, gastric carcinoma and B-cell mucosa-associated lymphoid tissue lymphoma. It is a class 1 carcinogen. Prompt detection of *H. pylori* is imperative for effective treatment. Rapid urease test is the most routinely used diagnostic method. Culture, histology and molecular methods are the available alternatives. Diagnostic performances of these methods have never been evaluated in Kenya. The growing resistance of *H. pylori* to triple therapy antibiotics imposes an exploration for alternative treatment which could be naturally occurring, readily available, and cost effective. Hence, antimicrobial activity of aqueous and ethanol extracts of naturally occurring *Aloe secundiflora, Bridelia micrantha* and *Lippia javanica* herbal medicines to *H. pylori* was determined, while also identifying any known potential bacteriocidal active phytochemicals. To achieve these objectives, gastric biopsies were obtained from 274 patients suffering from gastritis. The biopsies were subjected to rapid urease test, histology and culture diagnostic procedures for diagnosis and for comparison of performance of the methods. Whole genome sequence for *H. pylori* isolates was performed to determine the presence of *cagA*, *vacA* and *dupA* virulence genes. These enabled the possible association between these genes and gastro-duodenal diseases to be determined. Phenotypic antimicrobial resistance profile of *H. pylori* isolates to five antibiotics such as clarithromycin, metronidazole, tetracycline, levofloxacin and amoxicillin, which are the commonly used conventional antibiotics, was determined through E-test strips. Antimicrobial susceptibility testing was also performed using agar well diffusion technique for *H. pylori* isolates on the aqueous and methanol extracts of *Aloe secundiflora, Brideria micrantha* and *Lippia javanica* medicinal plants. Zone of inhibition, minimum inhibitory concentration and minimum bactericidal concentration was determined. Post hoc comparisons using the Tukey HSD test was used to compare score, while independent-samples t-test was used to compare zones of inhibition. Presence of phytochemical in plant extract was determined through Gas Chromatography-Mass Spectrometry. A total of 274 antrum biopsy specimens were collected from patients, fifty two percent (52%) were female and the age 15-82) years. The Pronto Dry Rapid Urease® test out performed culture in sensitivity and NPV measures. Out of 274 gastric biopsies, 147(54%) were positive for Pronto dry rapid urease® test, 122(44%) for histology and 91(33%) for culture. Virulence genes *cagA, dupA, vacA* s1/m1 were detected in all disease conditions. Detection of *vacA s1* in 23 (55%) in isolates with gastric ulcer disease was significantly high (P = 0.036). All (100%) *H. pylori* isolates expressed no antimicrobial resistance to Tetracycline. This study demonstrated that the total resistance rates of *H. pylori* to metronidazole, amoxycillin and clarithromycin were 93,63, and 23%, respectively, There was a significant difference in the zones of inhibition for *A. secundiflora* $p<0.0001$), *B. mirantha*, $p<0.0001$) and *L. javanica*, $p<0.0001$). Post hoc comparisons using the Tukey HSD test indicated that the mean score for *A. secundiflora* was significantly different than *B. mirantha, L. Javanica*, $p<0.0001$ and ciprofloxacin $p<0.0001$). The aqueous extracts of *A. secundiflora* leaves had the highest MIC at 1.56 mg/ml. Key bioactive compounds by GC-MS identified included Butylated bydroxytoulene Phenol, 2,4-bis(1,1-dimethylethyl) Bibutyl

bhthalate benzyl butyl phthalate. The performance of Pronto dry rapid urease® was commendable as it was comparable to the ‘gold standard’. *cagA, vacA* and *dupA* virulent genes could possibly be associated with gastro-duodenal diseases. *H. pylori* faces serious resistance to most of the triple therapy antibiotics, except tetracycline, necessitating explorations that can lead to new anti-*H. pylori* agent . As such there is hope since extracts of *A. secundiflora, B. micrantha and L. javanica* have compounds with antimicrobial activities and possess inhibitory prospects to *H. pylori*.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background

*Helicobacter pylori* (*H. pylori*) is a Gram-negative, microaerophilic bacterium that colonizes human gastric mucosa. This organism is not only closely associated with active gastritis, but also chronic gastritis, peptic ulcers, gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma (Gisbert & Pajares, 2007). Most individuals infected with *H. pylori* remain asymptomatic. There is no foregone conclusion in the mode of transmission for *H. pylori* infection. Nevertheless, several epidemiological studies have made several claims that the primary mode of transmission is through person-to-person, and the risk of infection increases when the infectious agent arises from within the family (Brown, 2000). Hence, the bacterium strain in a child mostly has a genetic characteristic indistinguishable from that detected in their parents (Hunt *et al.*, 2010). The spread from one person to another has been linked to oral-oral, gastro-oral or fecal-oral routes. Therefore, the practice of good hygiene and improved living conditions are vital aspects in reducing the rate of spread of the infection (Goh *et al.*, 2011). Most of these infections occur during childhood, and once infected children maintain the bacterium throughout their life (Hunt *et al.*, 2010).

The prevalence of *H. pylori* infection varies worldwide, with the developing countries carrying the highest load (Kalali *et al.*, 2015; Tanih *et al.*, 2010). Population with acute gastritis and peptic ulcer diseases (PUDs) is about 90% in developing countries, and around 25% in developed countries. Reportedly, there is high prevalence observed in Africa at than 70% and Asia at 54% as compared to Northern America at less than 40% and Oceania at 24% (Hooi *et al.*, 2017). In Kenya, the prevalence of *H. pylori* infection is 56% (Kiman'ga *et al.*, 2010).

Virulence factors for *H. pylori* are associated with mucosal damage that contribute to various clinical outcomes. *H. pylori* adheres to columnar epithelial cells, and initiate an important phase in the development of gastroduodenal diseases. Duodenal ulcer promoting gene A antigen (*dupA*) permits adherence of *H. pylori* to the epithelial cells, which is succeeded by the transfer of cytotoxin associated gene A (*cagA*) and vacuolating cytotoxin (*vacA*) proteins near the gastric epithelium, enhancing gastric tissue damage (Torres *et al.*, 2009). The *cagA* protein is a recognized virulence factor, encoded by the *cagA* gene (cytotoxin associated gene A). Several studies have described this gene as present in more than 60% of *H pylori* isolates, and nearly all *cagA* gene positive strains express this protein. It has been described to be linked with gastroduodenal diseases (Andres, 2010). The *vacA* is another virulence factor present in all *H. pylori* strains. It's comprised of two areas that show significant sequence variability between strains; the s-region (signal) located at the 5′ end of the gene, and the m-region (middle) of the gene. Despite all *H. pylori* strains containing the *vacA* gene, they vary in terms of their ability to produce cytotoxin. Type m1 strains exhibit more toxin activity than m2. The *dupA* which is another virulence factor located within a plasticity region of the *H. pylori* genome has been reported to be a marker that contributes to the increased risk of duodenal ulcer and reduced risk for gastric atrophy and cancer. It has been reported to be prevalent in isolates from patients with gastritis, duodenal ulcer and gastric cancer (Kusters *et al*., 2006). *cagA, vacA* and *dupA* genes have been considered important virulence factors in relation to gastroduodenal diseases both in children and adults (Kusters *et al*., 2006).

Infection with *H. pylori* has been diagnosed by a variety of invasive and non-invasive tests. Nevertheless, the "gold standard" for *H. pylori* detection, as suggested by the Maastricht consensus report (Cutler *et al*., 1995) is positive culture or both a positive histologic examination and a positive rapid urease test. Negative histology results for gastric biopsy do not exclude the possibility of *H. pylori* infection in areas not sampled (Al-Sulami *et al., 2008)*. Growing *H. pylori* from gastric biopsy in laboratory by culture for microbial isolation remains the gold standard for linking the organism to the disease condition. However, growth cultures are slow to yield results due to the fastidious nature of the bacterium (Mégraud and Lehours, 2007).

Molecular methods often come in handy for the diagnosis of *H. pylori* infection, especially for the analyses of diversity, virulence, persistence and resistance patterns of these bacteria (Minami *et al*., 2010).

Infection with *H. pylori* is curable with antibiotic therapy in combination with proton pump inhibitors (PPI) or bismuth salts (Manyi *et al*., 2010). Current standard *H. pylori* eradication regimen, comprises two antibiotics (amoxicillin and clarithromycin or metronidazole) and a proton pump inhibitor (PPI) (Cheha 2018). To increase *H. pylori* eradication rate, different treatment strategies have been implemented including regimen, sequential and bismuth-based quadruple regimens, as well as the culture-based, susceptibility guided treatment approach (Lee & Kim, 2015). The bacterium has established several mechanisms of resistance to clarithromycin, amoxicillin, tetracycline, levofloxacin, and metronidazole (Dadashzadeh *et al.,* 2013). Clarithromycin resistance is due to substitution mutations involving the adenine residues at positions 2142 and 2143 normally detected in the 23 *rRNA* gene (H. et al., 2018). Amoxycillin resistance is also common and it has been ascribed to mutation in a penicillin-binding protein involved in cell wall metabolism (Bińkowska *et al.,* 2018). Metronidazole resistance is principally due to the inactivation of *RdxA*, an oxygen-insensitive NADPH nitroreductase (Vega *et al.,* 2010). Furthermore, it has been demonstrated that the level of metronidazole resistance attributed to *RdxA* inactivation can be enhanced more by *FrxA* nitroreductase (Bińkowska *et al.,* 2018). Amino acid substitutions at positions 87 and 91 in DNA gyrase subunit A (*GyrA*) resulted in *H. pylori* resistance against flouroquinolones (Gerrits, 2002).

Growing antimicrobial resistance, side effects, and falling eradication rates emphasizes the need of an updated guidelines on the management of *H. pylori* infections. Medicinal plants are a natural source of phytochemical compounds that possess therapeutic properties, and play an important role in treating many human diseases. Antimicribial activity of this medicinal herbal plants have been documented and considerable interest in the study of medicinal herbal plants as potential sources of new drugs against *H. pylori* is increasing. The high intricacy of bioactive compounds in medicinal plants coupled with their broad antimicrobial

activity may make it challenging for pathogenic bacteria, as well as *H. pylori* to acquire resistance during treatment. Medicinal herbal plants have been used as medicines for thousands of years (Mostafa *et al.,* 2018). Isolation and biochemical characterization of pharmacologically active compounds from medicinal plants continue today (Soejarto *et al.,* 2012). Significant point of research is the likelihood that medicinal plant components might produce synthetic or chemical drugs effective in *H. pylori* infections. So, the investigation for new drugs for the improvement of alternative therapies is essential.

### 1.2 Problem Statement

More than half of the world population is infected with *Helicobacter pylori* (*H. pylori*), which is recognized as the major cause of gastric cancer and other gastrointestinal diseases such as gastric ulcer, duodenal ulcer and peptic ulcer. Effective management of the infection and the associated outcomes is dependent on prompt diagnosis and treatment. Despite the current combined triple therapies being efficacious, at 80-90%, *H. pylori* infection is still challenging as the resultant treatment outcomes are often erratic owing to the perpetual emergence of strains of *H. pylori* that are resistant to the antibiotics. These recommended antibiotics for management of *H. pylori* have reportedly been misused, with more than 90% resistance being reported for agents such as metronidazole, which is rampantly used empirically (Smith *et al.,* 2014). The challenge is compounded by the inadequacies of clinical laboratories to conduct culture which is a requisite for AST useful in detection of antimicrobial resistance. Evidently, *H. pylori* resistance to conventional antibiotics is therefore a growing problem in Kenya (Kimang'a *et al.,* 2010) and the search for alternative antimicrobials is not keeping up with the pace, although medicinal plants are gaining prominence in medicine. Moreover, there is no literature available that shows plants have been evaluated in Kenya for antimicrobial activity on clinical isolates of *H. pylori.* Therefore, this study mainstreams the search of alternative treatment for *H. pylori* from the locally available medicinal plants, otherwise used by herbalists.

### 1.3 Justification of the study

Gastrointestinal diseases caused by *H. pylori* are preventable, through rapid diagnosis and effective therapeutic management. Knowledge on *H. pylori* diagnosis, virulence genes and antimicrobial resistance profile can assist in infection management, therefore reducing the incidence of *H. pylori*-associated diseases. The results of this study may lead to the identification and isolation of antimicrobial agents that can be purified to provide readily available therapeutic agents against clinical *H. pylori*

### 1.4 Research questions

i. What are the performances of diagnostic methods for *H. pylori* from gastric biopsies?
ii. What is the relationship between *H. pylori* virulence genes and gastroduodenal diseases among patients?
iii. What the antimicrobial resistance pattern of *H. pylori* is to commonly used conventional antimicrobial agents?
iv. What is the antimicrobial activity of *Aloe secundiflora, Bridelia micrantha* and *Lippia javanica* medicinal plants on *H. pylori*?
v. What are the potential active compounds of *Aloe secundiflora, Bridelia micrantha,* and *Lippia javanica* against on *H. pylori?*

### 1.5 General Objective

To compare *H. pylori* diagnostics methods, determine virulence genes, resistance to conventional antibiotics and antimicrobial activity for selected medicinal plants on isolates from patients undergoing endoscopy at Aga Khan University Hospital

#### 1.5.1 Specific Objectives

i. To compare *H. pylori* diagnostic methods for *H. pylori* in gastric biopsies
ii. To determine the relationship between *H. pylori* virulence genes and gastroduodenal diseases among patients

iii. To determine antimicrobial resistance pattern of *H. pylori* to commonly used conventional antibiotics

iv. To determine the antimicrobial activity of *Aloe secundiflora, Bridelia micrantha* and *Lippia javanica* herbal medicines on *H. pylori*

v. To identify potential active phytochemicals, present in *Aloe secundiflora, Bridelia micrantha,* and *Lippia javanica*

**1.6 Hypothesis**

There is association between virulence genes and gastroduodenal disease outcome. Medicinal plants possess antimicrobial activity against of *H. pylori* infections.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Introduction

In 1886, Thoedor Escherish was the first to observe *Helicobacter* in the colons of infants later known as *Campylobacter*. In 1972, Sebalt and Veron isolated the bacteria from human feaces and called it ―vibrio like organism and gave the name *Campylobacter* as genera based on shape and biological character (Samie *et al*., 2006). These bacteria permanently colonize gastric epithelial cells in approximately 25% of the population in developed countries and 70–90% in developing countries, whereas most infected individuals are asymptomatic. Chronic *H. pylori* infection in susceptible individuals is associated with a variable degree of mucosal damage ranging from mild gastritis and ulcer disease to gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Milani *et al*., 2012). Colonization with these bacteria is usually without clinical consequences, but increases the risk of developing peptic ulcer disease, gastric adenocarcinoma and lymphoma (Suerbaum & Michetti, 2004). A number of diagnostic tests can be performed to detect *H. pylori* infection, and can be optimally treated with antibiotics. Antimicrobial chemotherapy (a combination therapy) used for the management of *H. pylori* infections appears to be the most significant way to eradicate these infections. Nonetheless, antimicrobial therapy is loaded with a number of innate boundaries such as resistance, cost of treatment, unavailability of drugs in rural areas and undesirable side effects. These and other factors necessitate a need to search for an alternative approach from natural sources such as plants for reduction of the global burden of infectious diseases (Mulu *et al.*, 2004). These findings can shed light to efficient, large-scale, low cost and alternative solutions with fewer side effects to eradicate *H. pylori* infections.

## 2.2 Transmission of *H. pylori*

Although, the mode of transmission of *H. pylori* is not completely elucidated, most of the available evidence supports person-to-person transmission by fecal-to-oral,

oral-to oral and gastric-to-oral routes (Hardin & Wright, 2002; Asrat *et al.,* 2004; Dube *et al.,* 2009). In children, gastric inflammation could cause low gastric secretion which results in impaired "gastric barrier" associated with increased susceptibility to enteric infections, a major public health concern linked to diarrhoea, malnutrition and growth failure in developing countries (Thomas *et al.,* 2004).

Evidence of gastro-oral transmission has been suggested (Asrat, *et al* in 2004). Presence of *H. pylori* in the gastric juice of up to 58% of patients infected with *H. pylori* raises the possibility that refluxed gastric juice may represent a vehicle of transmission for this organism. It has been shown to be potentiated by specific eating habits, such as the premastication of food by mothers before feeding children in some African countries and the coating of nipples with saliva before breast feeding by Hindu mothers in Bangladesh (Fuller, 1991). This is very important considering that childhood appears to be the critical period during which *H. pylori* is acquired, especially in areas of over-crowding and socio-economic deprivation (Ahmed *et al.,* 2007).

### 2.3 Diseases Caused by *H. pylori*

Infection with *H. pylori* produces extremely variable representation which is influenced by variety of factors like dietary habits, lifestyle, host immunity, virulence of the infecting strain. Colonization of gastric mucosa with *H. pylori* invariably results in inflammatory response. Only in few patients this leads to severe diseases like peptic ulcer, MALT lymphoma and gastric adenocarcinoma among others. Rest others have mild asymptomatic gastritis which goes unnoticed.

Gastritis is of two major form type A and B. The type A is an autoimmune disease where antibodies are produced against acid secreting parietal cells. Inflammation is confined to corpus of stomach predominantly. The type B gastritis affects the gastric antrum involving the mucus secreting cells. Association of *H. pylori* and gastritis is found to be 70-90% (Salimzadeh *et al.,* 2015).

Majority of patients with duodenal and gastric ulcer are infected with *H. pylori*. Majority of the ulcers are in the first part of duodenum and the lesser curvature of

stomach (Wong & Mclean, 2016). Eradication of infection with combination triple therapy has shown to heal the ulcers (Moshi *et al.,* 2014). Peptic ulcers occur more commonly in men than in women with the average ratio of 18:1 in developing countries (Baghaei *et al.,* 2009). This indicates the influence of environmental factors on disease manifestations.

The prevalence of *H. pylori* infection in duodenal ulcer has consistently been found to be between 90% and 100% (Amieva & El-Omar, 2008). Studies have shown that *H. pylori* has a role in the multifactorial etiology of duodenal ulcer disease and there is interplay of many factors such as the acid attack and the mucosal defense (Adinortey *et al.,* 2018).

This is the second common cause of fatal malignancy in the world accounting for more than a half million deaths annually. *H. pylori* infection leads to a chronically inflamed state of the gastric milieu (Fathi *et al.,* 2013). This is found to be a significant risk factor contributing to the development of adenocarcinoma (Boehnke *et al.,* 2017) Gastric lymphoma is the proliferation of mucosa associated monoclonal B lymphocytes. There is a strong correlation between prevalence of gastric MALToma and *H. pylori* colonization. In-vitro studies on the proliferation of cells derived from these lymphomas show that, this change is dependent on *H. pylori* specific T cells and their products and not due to the bacteria themselves (Andres, 2010). Eradication of *H. pylori* results in regression of this tumor. Gastric MALT lymphoma is the only known malignancy which can be prevented by elimination of *H. pylori* infection (Boehnke *et al.,* 2017).

Though majority of MALT lymphoma patients are positive for *H. pylori* infection, only less than 1% of those who tested positive for *H. pylori* developed MALT lymphoma (Amieva & El-Omar, 2008). The eradication of the bacteria in MALT patients can lead to complete remission in 60%–80% of patients with stage 1 low-grade gastric MALT lymphoma(Wong & Mclean, 2016) In Uemura *et al* study gastric cancer was seen in approximately 3% of infected patients, compared to none of the uninfected patients (Uemera *et al.,* 2003). This study also supported the fact that eradication of infection would decrease the risk of gastric cancer in infected

individuals without premalignant lesions (Tovey *et al.*, 2006). Taken together, these studies support an unequivocal role for *H. pylori* in the development of gastric carcinoma and that the anti-*H. pylori* therapy will be an effective means of prevention.

### 2.4.1 Pathogenesis of *H. pylori* infection

It is estimated that more than 50% of the world's population is infected with *H. pylori*, but the factors associated with different outcomes, such as non-ulcer dyspepsia (NUD), peptic ulcer disease (PUD) or gastric carcinoma, are not well understood (Kim *et al.*, 2001). Frequently, infected individuals display only asymptomatic gastritis; however, a small population may develop severe disease, including peptic ulceration and gastric malignancy. Although the factors that govern the outcome of the infection are not well understood, *H. pylori* virulence factors to play important roles (Suerbaum &Michetti, 2002).

Prevalence of *H. pylori* is not evenly distributed there is significant difference between developed countries and developing countries. The general trend shows that there is high prevalence of *H. pylori* infection in developing countries compared to developed countries. It is present in 20 to 40% of the population in developed countries and more than 80% of the population in developing countries (Robertson *et al.*, 2003). Increased infection rate with *H. pylori* has been associated with poor hygiene, overcrowding and low social economic status (Moayeddi *et al*., 2002). Normal acquisition of *H. pylori* infection ensues primarily in the early years of life (Malaty *et al.*, 2007). Successful colonization in the gastric mucosa *H. pylori* has developed a number of approaches to overcome hindrances that it faces in the human gastrointestinal tract. *H. pylori*, survival and development in the gastric mucosa it converts urea to ammonia and bicarbonate (Strugatsky *et al.*, 2013). Motility and attachment are important for *H. pylori* to overcome the effects of peristaltic movements, shedding of the mucus layer, and low pH. High motility rate and production of urease enzyme has been associated with its adaptability to the gastric mucosa (Tanih *et al.*, 2008). Urease enzyme catalyzes the breakdown of urea into ammonia and bicarbonates (Lizumi *et al.*, 2005). Ammonia reduces the acidity

of the stomach forming a protective alkaline cloud suitable for the survival of *H. pylori*. The flagellation *H. pylori* allow rapid motility to more neutral pH of the gastric mucosa indicating that both factors (motility and urease production are fundamentals for establishment of the bacteria in the gastric mucosa. The helical morphology also contributes in the penetration into the mucoid layer, where more neutral pH allows proliferation of the *H. pylori* (Tanih *et al.*, 2008). *H. pylori* adherence to epithelial cells is an important step in the development of gastro duodenal disease. The *dupA* allows adherence to mucosal region followed by transfer of the vacuolating cytotoxin (*vacA*) and the cytotoxin associated gene (*cagA*) near the gastric lining, enhancing gastric tissue damage (Torres *et al.*, 2009).

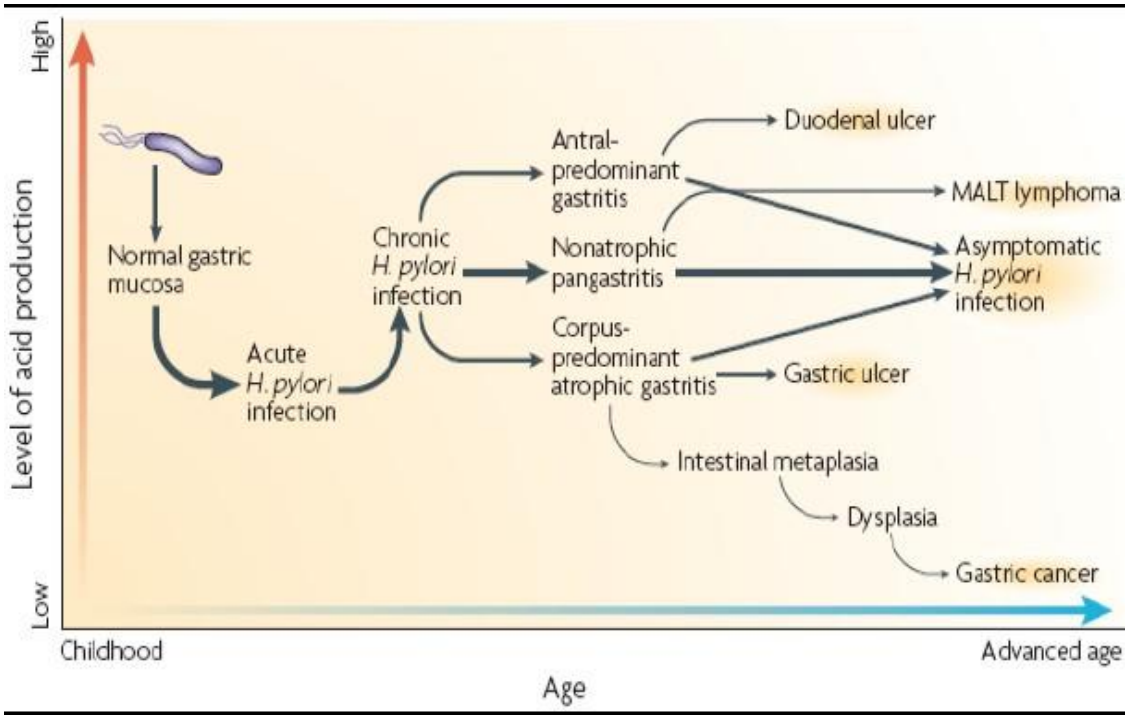



**Figure 2.1: Occurrence of disease in *H. pylori* Infection** (Suerbaum & Josenhans 2007)

### 2.4.2 Virulence Determinants Associated with Pathological Damage of the Gastric Mucosa

#### 2.4.2.1 Cytotoxin Associated Gene antigen (Cag A) and Cag Pathogenicity Islands (PAI)

One of the most extensively studied pathogenic gene of the *H. pylori* virulence factors is the 40kb cag pathogenicity island (PAI). The cag PAI contains 27 to 32 genes that code the effector protein *CagA,* a type IV secretion system (TFSS) and other proteins with unknown functions (Nguyen-Hoang *et al.,* 2019). This protein encoded by this gene varies from 128 kDa to 140 kDa according to the number of copies of 102 to 108 bp motif that is repeated with specific strains (Xiang *et al.,* 1995). The *CagA* protein was the first component of the cag PAI to be identified, its identification and characterization observed that the highly immunogenic *CagA* was present in more50% of *H. pylori* strains, and that antibodies to *CagA* were higher in patients with peptic ulcer disease and that an association occurred with vacuolating activity (Crabtree, 2018). *H. pylori* strains with the *cagA* gene are able to induce epithelial cells to secrete Interleukin 8 (IL-8) and cause mucosal inflammation (Orsini *et al*., 2013). Several reports have established an association of presence of *cagA* and peptic ulceration in adults (Arents *et al.,* 2001). *The cagA* within *cag*-PAI accounts for the virulence variability concerning to the bacterium (Morshedzadeh *et al.,* 2018). However, reports from countries such as Singapore, Japan, Korea and Hong Kong showed that *cagA* positive strains which were predominantly present were not associated with any specific clinical outcome (Wong *et al.,* 2001). It has also been established that possession of *cagA* gene by the *H. pylori* supplements the risk of a number of diseases including adenocarcinoma (Covacci *et al.,* 1993). According to a recent study *H. pylori* eradication can significantly reduce peptic ulcers and gastric cancer. The study also acknowledged that presence of *cagA* can lead to more severe gastritis (Shiota *et al.,* 2014).

#### 2.4.2.2 Vacuolating Cytotoxic gene A (*vacA*)

Another major virulence factors associated with *H. pylori* is the vacuolating cytotoxin gene A (*vacA*), which causes cytoplasmic vacuolization in gastric

epithelial cells). The vacuolating cytotoxin gene (*vac* A) is present in most *H. pylori* strains, although the *vac* A toxin may not be expressed in all cases (Cover *et al.*, 1994). The *vacA* gene encodes a vacuolating toxin (~88 kDa) produced by *H. pylori* that destroys mammalian cell and causing epithelial damage. A range of further effects on gastric epithelial and immune cells have been attributed to *vacA,* including the initiation of apoptosis, modification of antigen presentation by B cells and inhibition of T cell activation (Clyne, Dolan, & Reeves. 2007). *vacA* is composed of three primary allelic segments, the signal sequence (s1 and s2), middle region (*m1* and *m2*) and the recently identified intermediate region (*i1* and *i2*) (Atherton, 2006). The signal region exists as an *s1* or *s2* allele while middle region occurs as an *m1* or *m2* allele. *H. pylori* strains isolated from different regions display diverse distributions of *vacA* alleles (Zheng *et al.,* 2000). The *s1m1* strains are the most virulent and are linked with severe vacuolating activity, whereas *s1m2* strains have a lower vacuolating activity and *s2m2* lack vacuolating activity. In North America and Western Europe, *s1 vacA* allele is linked with severe peptic ulcer disease (Atherton *et al.,* 1997). Although *s1* alleles are predominate in Asia, there is no association of the *vacA* genotypes with any clinical manifestation has been observed (Zheng *et al.,* 2000). Both s1/m1 and *cag* A-positive strains have been reported to be associated with PUD and gastric carcinoma (Miehlke *et al.,* 2000).

#### 2.4.2.3 Duodenal Ulcer Promoting Gene (*dupA*)

The duodenal ulcer promoting gene A (*dupA*) is another important virulence determinant of *H. pylori* that has been associated with duodenal ulcer disease. Presence of *dupA* has been found to be associated to be neutrophil infiltration and production of interleukin-8 (IL-8) production by epithelial cells (Hussein *et al.,* 2010). Moreover, *dupA* is classified into two main subtypes *i.e.* the functional *dupA* with an extended open reading frame (ORF) within *jhp0917-19* (*dupA1*), and non-functional *dupA* with an early stop codon to truncate the ORF (*dupA2*) which are located in the plasticity region of *H. pylori* genome. This has been reported to be an indicator that contributes to the amplified risk of duodenal ulcer and reduced risk for gastric atrophy and cancer (Romo *et al.,* 2015). The gene *dup A* is reported to be more prevalent in Western strains than in Asian strains (Yamaoka, 2012).

Worldwide prevalence of *dupA* in patients with gastritis is reported to be more than 40%, *H. pylori* isolates from South America are significantly more likely to possess *dupA* than those from East Asian, Middle Eastern or European countries (Hussein *et al.,* 2008). A recent report resolved that the effects of *dupA* may be population specific, predisposing to duodenal ulcer in some populations and gastric cancer and gastroduodenal ulcer in others (Hussein, 2010).

### 2.5 Diagnosis of *H. Pylori* Infection

The isolation of *H. pylori* is primarily essential for diagnosis of gastroduodenal infections associated to this bacterium. Various tests are available for the diagnosis of *H. pylori*. They include invasive and non- invasive diagnostic tests are available for ascertaining the presence of *H. pylori* infections. Invasive tests require endoscopy and collection of gastric biopsy tissue. They involve direct microscopy with appropriate staining, Rapid urease tests, bacterial culture, polymerase chain reaction (PCR) and immunohistochemistry for the diagnosis of the infection in biopsy tissue sample. On the other hand, non- invasive tests do not require endoscopy and are more convenient. They comprise of serology, urea breath test and stool antigen test. Owing to the uneven distribution of *H. pylori* bacteria in the stomach, gastric biopsy- based invasive tests have a limitation of missing the diagnosis of infection as opposed to noninvasive tests. However, endoscopy allows the evaluation of disease state and the need for treatment (Gramley *et al.,* 1999; Tanih *et al.,* 2008).

#### 2.5.1 Invasive Tests

The Invasive tests such as rapid urease test, culture, histological examination, and molecular methods such as PCR require endoscopy. The endoscopy and biopsy collection for invasive tests diagnosis must be performed before treatment or at least 4 weeks after the last treatment with antibiotics and proton pump inhibitor. Gastric biopsy tissues are taken from the antrum and corpus (body of stomach). For isolation of *H. pylori* the gastric biopsy tissues should be processed immediately or stored at -20$^{o}$C for one hour or frozen at -8$^{o}$C for subsequent processing (Peretz, 2015).

#### 2.5.1.1 Rapid urease test

Rapid Urease test (RUT) is an invasive test that requires gastric biopsy tissue sampling. Urease tests have been widely used because they are simple, cheap, and easy to carry out and can be performed readily in the endoscopy unit and give a rapid result. The RUT provide indirect evidence of *H. pylori* infection by identifying presence of enzyme urease in or on the gastric mucosa *H. pylori* has a very high urease activity that can be utilized for diagnosis of infection (Marshal *et al.,* 2010).The RUT test was first described by Mc Nulty and Wise by using Christensen's urea broth incubated up to 24 hours (Fischbach *et al.,* 2004). Numerous variations have been made to make the test rapid. Presently numerous RUT like Pronto dry rapid test, CLO test, Stuart's urease test broth, Pylori-tek are commercially available which are designed to provide results in few seconds or minutes (Uotani *et al.,* 2015). They have high positive predictive value and have a sensitivity of between 85- 95% and specificity of 90-95% and many versions have been approved for use in humans. Gastric biopsy is embedded in the RUT gel and the results are read by colour change. False positive results are rare if the RUT contains antibacterial agent that prevent urease containing contaminants. Use of antibacterial agent and proton pump inhibitors may result in false positive test (Journal, 2019). The RUT can also be used to provide informal assessment of the precision of histopathology result (Yakoob *et al.,* 2005). The limitation to the urease test is the number of *H. pylori* bacteria required to obtain sufficient positive results. At least $10^4$ CFU organisms are required to give a positive result. Treatment management with proton pump inhibitor (PPI) may also affect the result.

#### 2.5.1.2 Culture

Culture of *H. pylori* from gastric biopsy tissues is one of the ways of diagnosis of *H. pylori* associated infections. Antibiotic susceptibility testing determination of *H. pylori* isolates becomes important in the management of *H. pylori* associated infections. The prominence of *H. pylori* culturing exist in the awareness gained about the *H. pylori* growth characteristics and likelihood of determining its resistance profile to antibiotics used in treatment (Bayona Rojas, 2013). Culturing

of *H. pylori* is most specific method to establish diagnosis, sensitivity of the test depends on transport media used from endoscopy and the time used to process the specimen, experience of laboratory personnel, culture media used and provision of microaerophilic environment during incubation at 35-37ºC (Mégraud & Lehours, 2007). An important point to note in the transport of biopsy specimen from the endoscopy is that the specimen must be protected from drying and interaction with oxygen and room temperature. It is important not to expose the biopsy specimens to air and to place them either in a saline solution for 4°C maximum or in a transport medium, usually consisting of semisolid agar (Thirunavukkarasu *et al.,* 2017). If transport conditions cannot be sustained, it is important to freeze the gastric biopsy specimens at -81°C and transport them frozen state to the laboratory (Mégraud & Lehours, 2007). In most instances *H. pylori* is not evenly distributed in the biopsy specimen therefore there is need to macerate biopsy specimen before streaking on the culture media plate. This can be achieved by use of mechanical grinder in small volume of featal bovine serum broth or normal saline (Goodwin *et al.,* 1985).

Primary culture takes 5-7 days to achieve optimal growth. Colonies of *H. pylori* are small, round and appear translucent against blood agar background. Identification of colonial growth of *H. pylori* can be made by confirming the presence of urease, catalase and oxidase enzyme and microscopic evidence of motility helical curved morphology (Peretz *et al.*, 2015).

#### 2.5.1.3 Histology

Histology plays an important role in the diagnosis of *H. pylori* associated infections, as it provides information about degree of inflammation, associated pathology and presence of gastric cancer. Diagnosis of *H. pylori* could be accomplished by staining biopsy tissue specimen with hematoxylin and eosin (H&E). Hematoxylin and eosin (H&E) stained smears can evaluate the degree of inflammation in a high-magnification of microscopic field. However, specificity can be improved by use of Giemsa stain, Warthin-Starry silver stain, and immunohistochemical (IHC) stain to 90-100% (Laine *et al.,* 1997). Histology has the benefit of providing information on the degree of inflammation and related pathology, such as, atrophic gastritis (AG),

intestinal metaplasia (IM), gastric cancer, or lymphoma (Lee & Kim, 2015). The drawbacks associated with histology diagnostic a procedure is that its time consuming, high cost, dependence on the skills of the pathologist reading the slides and inter-observer variability in assessment. The results may also be affected if the patient has been taking proton pump inhibitor (PPI) or antibiotic therapy. Histological results must be reported according to Sydney system guidelines. The presence of bacteria in the corpus and or in the antrum is stated semi-quantitatively on a scale of 0 to 4, characteristics of the gastric mucosa such as inflammation activity, atrophy, intestinal metaplasia must also be reported (Dixons *et al.,* 1991). The correctness of histology depends on the quality of the histological slide preparation, which must allow examination of the surface epithelium (Mégraud & Lehours, 2007).

#### 2.5.1.4 Molecular Methods

*H. pylori* possess remarkable degree of genetic diversity, closely related to its epidemiological and pathological characteristics and dynamics of transmission. Molecular diagnostic methods are used in the diagnosis of *H. pylori* infections due to their ability to amplify virulence and drug resistance genes analysis. Their sensitivity and specificity depend on factors such as the density of bacteria present in each biopsy, the presence of *H. pylori* in endoscopic material and the presence of microorganisms besides *H. pylori.* The application of molecular methods such as polymerase chain reaction (PCR) has transformed the analytical methods for the identification of *H. pylori*. Molecular methods are also able to tracks the genetic variation in *H. pylori* for the understanding the virulence genes and antibiotic resistance characteristics (Fasciana, *et al.,* 2015). The molecular tests currently available for diagnosis, including those targeting 16S rRNA genes, are focused on *H. pylori* and considered as specific targets to confirm *H. pylori* infection, and positive amplification of *H. pylori* specific DNA may be considered as a direct evidence of the presence of the pathogen (Hoshina *et al.,* 1990). Sequence analysis of the 16S rRNA gene has led to current understanding of prokaryotic phylogeny and *H. pylori* 16S rRNA gene sequence analysis.

### 2.5.2 Non-invasive tests

Non-invasive tests are based on the analysis of samples of breath, blood, or stool. They can be divided into two categories; active and passive tests. Active tests such as urea breath and stool antigen tests detect the presence of *H. pylori* and provide evidence of a current infection while passive tests including serological, near patient, saliva and urine tests are based on the detection of antibodies to *H. Pylori* (Tanih *et al.,* 2008).

#### 2.5.2.1 Urea Breath Test

Urea breath test (UBT) is a rapid diagnostic test used to identify the presence of active infection through *H. pylori* urease production. It's based upon the ability of *H. pylori* to convert urea to ammonia and carbon dioxide which is then absorbed from the stomach and eliminated in the breath (Yakoob *et al.,* 2005). Use of urea with a labeled carbon (C) isotope, the exhaled $CO_2$ can be quantified for the diagnosis of *H. pylori infection* (Kalari *et al.,* 2015). Patients ingests urea labeled non-radioactive isotope carbon$^{13}$ (Breath Tek UBT for *H. Pylori*, Meretek Diagnostics, Inc, Lafayette, CO) or the radioactive isotope carbon$^{14}$ (14C), (PY test, Kimberley – Clark Corp, Draper, UT) (Mackay, *et al.,* 2003). If *H. pylori* are present in the stomach, hydrolysis occurs and labeled carbon dioxide is produced which is detectable within a few minutes in the breath. However, antibiotics, PPI can reduce urease activity and produce false negative results therefore before UBT test the patient discontinue antibiotics and PPI medication for at least four weeks (Thirunavukkarasu *et al.,* 2017).

#### 2.5.2.2 Stool Antigen Test (SAT)

Stool antigen test (SAT) is another non- invasive test used to detect presence of *H. pylori* in stool. The SAT utilizes the patient's own immune response to confirm the presence of active infection. The test is able to establish the presence of an active infection by analyzing the stool for *H. pylori* antigens. These are antigens that are produced by body immune system in order to fight the *H. pylori* infection (Khalifehgholi *et al.,* 2013). The SAT is non-invasive, fast, has good sensitivity,

specificity and reliable. This test can be used both for diagnosis of the infection and for monitoring therapy effectiveness, already four weeks after the test the end of treatment (Pajares *et al.,* 2007). It is cheap, easy use and the possibility to collect samples and perform SAT utilizes polyclonal anti-*H. pylori* capture antibody adsorbed to micro wells. The Premier Platinum HpSA EIA (Meridian Diagnostics, USA) was the first commercially available SAT which utilizes an affinity purified polyclonal antibody attached to micro well plates to which is added a stool suspension followed by a peroxidase conjugated antibody and substrate (Vaira *et al.,* 2000).

### 2.6 Treatment of *H. pylori* Infections

The purpose of treatment of *H. pylori* infection in any clinical situation is to eradicate the bacterium from the stomach (Harris & Misiewicz, 2002). Eradication is defined as a negative test for the bacterium four weeks or longer after treatment (Harris & Misiewicz, 2002; Romano & Cuomo, 2004). It results in the effective healing of ulcers (Meurer & Bower, 2002), prevents ulcer relapse (Leodotler *et al.,* 2001; Ables *et al.,* 2007), reduces recurrence of gastric cancer (Steinbach *et al.,* 1999; Lee *et al.,* 2008) and potentially decreases the risk of progression to gastric carcinoma (Bytzer & O'Morain, 2005; Ndip *et al.,* 2008). For successful eradication of the bacterium, it is imperative that the clinician be aware of the current antimicrobial susceptibility profiles of the isolates within the region (Sherif *et al.,* 2004). Development of a successful treatment for *H. pylori* infection has been fraught with difficulty. Its location within the stomach various anatomical stomach sites including the mucus lining the surface epithelium, deep within the mucus-secreting glands of the antrum, attached to cells and even within the cells) provides a challenge for antimicrobial therapy (Ricci *et al.,* 2002; Romano & Cuomo, 2004).

Treatment regimens for *H. pylori* infection have been evolving since the early 1990s, when monotherapy was first recommended. Treatment generally involves a triple drug regimen; two antibiotics and an anti-secretory drug, essentially a proton pump inhibitor (PPI) to which bismuth salt or $H_2$ Antagonists can be added (Alarcón *et al.,* 1999; Manyi-loh *et al.,* 2010). The most commonly used combination

worldwide is a double dose of PPI plus clarithromycin comprising 500 mg twice a day (b.i.d) and amoxicillin (1 g b.i.d.) for 7 days (treatment 1). Other 7-day regimens include a double dose of PPI plus clarithromycin (500 mg b.i.d.) and metronidazole (500 mg b.i.d.), Treatment 2 or a double dose of PPI plus amoxicillin (1 g b.i.d.), metronidazole (500 mg b.i.d.), Treatment 3, with the latter being mostly used as a second choice treatment for 14 days in the case of failure of treatment 1 (Njume *et al.*, 2009).

### 2.7 Antimicrobial Resistance of *H. pylori*

Antimicrobial resistance is a major cause of eradication failure on *H. pylori* infections. Resistance of *H. pylori* to the limited range of antibiotics that have efficacy in its treatment can severely affect attempts to eradicate the bacteria. Resistance to useful antimicrobials agents, especially Metronidazole and Clarithromycin, has been a major problem even among people not previously treated for their *H. pylori* infections. Such resistance is generally related to unintentional *H. pylori* exposure during treatment for other conditions (Secka *et al.*, 2013). Bacterial resistance to antimicrobials, however, could be either primary (that is, present before therapy) or secondary (that is, develop as the result of failed therapy (Romano & Cuomo, 2004). Primary resistance in *H. pylori* has been reported in metronidazole (6 - 95%), clarithromycin (0 - 17%), and tetracycline (0-6%) in different countries (Boyanova *et al.,* 2000; Huynh *et al.,* 2004). Therefore, clinicians should choose the appropriate combination of drugs based on sensitivity patterns provided by a local reference centre (Meurer & Bower, 2002). Ideally, in cases of treatment failure, the antibiotic sensitivity pattern of the organism should be established before the second line therapy is chosen (Destura *et al.,* 2004).

Clarithromycin (Cla), a bacteriostatic antimicrobial agent binds in a reversible manner to the peptidyl transferase positioned in dominion V of the 23S *rRNA* gene, inhibiting protein synthesis in *H. pylori* (Versalovic *et al.,* 1996). Antimicrobial resistance associated clarithromycin (Cla), is usually accomplished by point mutations at either of two adjacent sites in 23S rRNA that weakens macrolide binding to the ribosome (Peretz *et al.,* 2015). Single nucleotide mutations in

positions A2142G and A2143G are the most common variations describe ( Xuan *et al.,* 2016). Tetracyclines are bacteriostatic antimicrobial agents that utilize their antimicrobial effect on the 30S subunit of the ribosome and block the binding of aminoacyl-tRNA, resulting in impaired protein biosynthesis. Therefore resistance of *H. pylori* to tetracyclines is described to be caused by mutations in the16S *rRNA* (Dadashzadeh *et al.,* 2013) Prevalence of tetracycline resistance *H. pylori* also has continued to remain low; it has been reported to be less than 2% in most studies (Nishizawa & Suzuki, 2014). The triple point-mutations at positions 965–967 are mainly responsible for tetracycline resistance. Single and double point-mutations are related with low and intermediate MIC values, respectively.

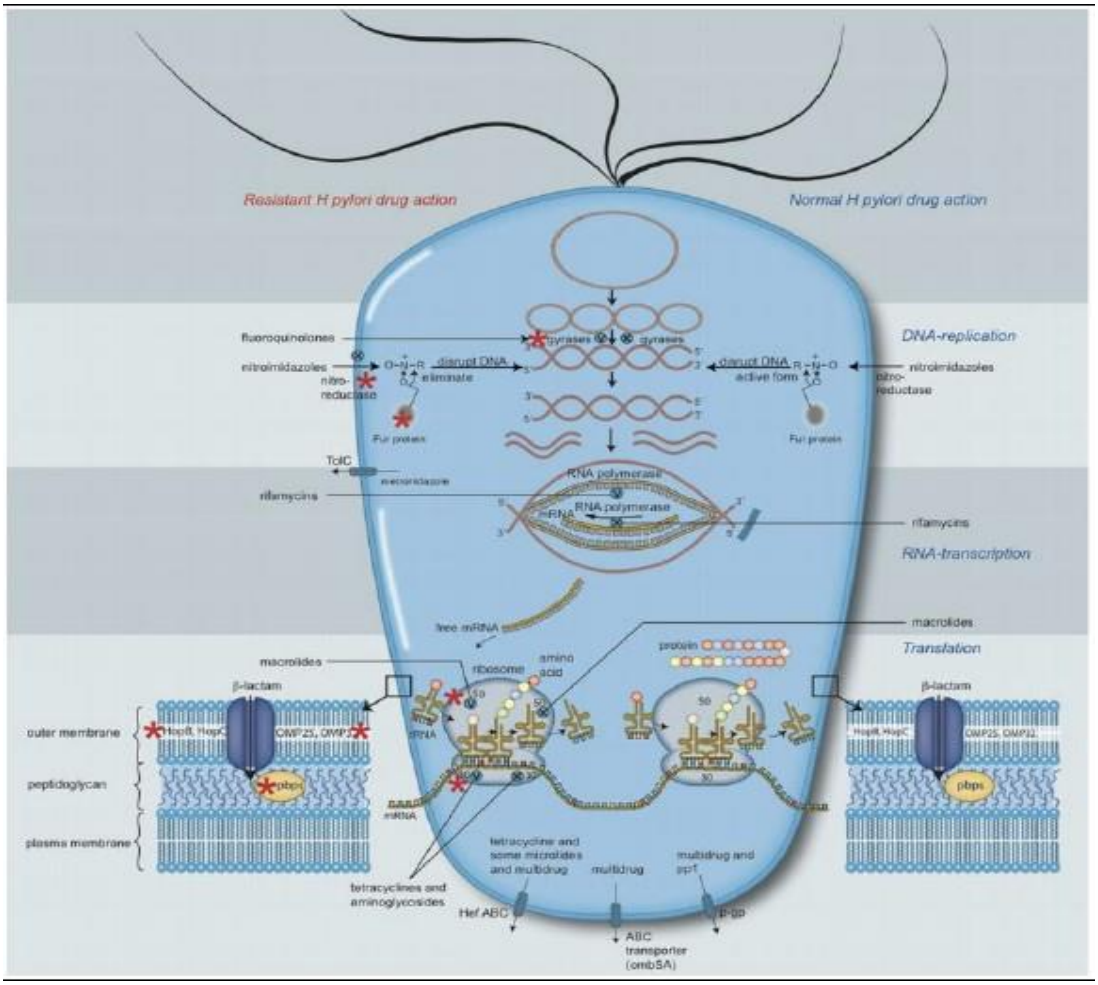



**Figure 2.2: Genes Linked to Antibiotic Resistance in *H. pylori* (Yue *et al., 2016)***

The prevalence of *H. pylori* resistance to metronidazole has been described to be between more than 70% in different regions of the world (Suzuki *et al.,* 2010). Metronidazole is a pro-drug that requires to be activated by reduction of the nitro group attached to the imidazole ring. The reduction step leads to the creation of DNA-damaging nitroso- and hydroxylamine containing compounds. The reduction of metronidazole is mainly mediated by oxygen-insensitive NADPH nitro-reductase (*RdxA*), NADPH-flavin-oxido reductase (*FrxA*), and ferredoxin like enzymes (FrxB) in *H. pylori* (Mé Graud *et al.,* 1999). Point mutations in *frxA* and *frxB* increases *H. pylori* resistance exclusively in the presence of mutations in the rdxA gene (Nishizawa & Suzuki, 2014). Diverse mutations related to rdxA gene include frame shift mutations, insertions and deletions of transposons, and missense and could be present simultaneously. These mutations are known to be the main mechanisms that confer *H. pylori* metronidazole resistance (Klesiewicz *et al.,* 2014).

The mode of action of amoxicillin is by interfering with peptidoglycan synthesis, blocking transporters, namely penicillin binding proteins (PBP). Multiple mutations in *pbp1* gene are the main reason for amoxicillin resistance (Gosciniak *et al.,* 1998). The resistance of *H. pylori* to amoxicillin is related to a point mutation on pbp1, and the increase of pbp1 mutations could lead in a gradual increase in amoxicillin resistance.

Similarly the resistance *H. pylori* to levofloxacin has been described to be more than 20% in different regions of the world (Suzuki *et al.,* 2010). Levofloxacin exert their antimicrobial activity by inhibiting the function of the enzyme DNA gyrase. The enzyme DNA gyrase is a tetramer consisting of two A and two B subunits encoded by the *gyrA* and *gyrB* genes, respectively. The mechanism of levofloxacin resistance in *H. pylori* has been linked to mutations in the quinolone resistance determining regions of the *gyrA* and *gyrB*, coding of the DNA gyrase. The resistance to levofloxacin results from mutations in the quinolone resistance determining region (QRDR), where the genes *gyrA* and *gyrB*, encoding subunits of bacterial gyrase. The point mutations which occur in the *gyrA* subunit are located in the codons position 86, 87, 88, and 91 while *gyrB* subunit is mainly at position 463, 438, 484 (Bińkowska *et al.,* 2018).

## 2.8 Medicinal Plants and H. pylori

Phytomedicine involve in the use of plants or plant extracts for medicinal purposes and has shown great promise in the treatment of intractable infectious diseases such as gastrointestinal infections, malaria, skin diseases among others. It is estimated that plant materials are present in or have provided the models for about 50% of synthesized drugs and herbal medicine continue to play a role in the cure of diseases. Traditional medical practitioners play important roles in health care delivery in Africa, including Kenya. One way to prevent antibiotic resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents (Bessong *et al.,* 2005).

Medicinal plants represent a rich source form which antimicrobial agents may be obtained. Plant extracts have a positive impact on the treatment of gastroenteritis and other infectious diseases caused by bacteria. Exploration of newer antimicrobials in plants brings about a different approach in minimizing antibiotic resistance (Umeh *et al.*, 2005). Hence, a more detailed search for new antimicrobial drugs is needed.

Plants have been documented to have compounds that contribute to their antimicrobial activity including phytochemals such as flavanoids, phenolics, and propolis (Cushnie & Lamb, 2005; Xuan *et al.,* 2005) which are not fully characterized but possess antimicrobial activity against bacterial pathogens. Phytochemicals are substances that are produced naturally by plants. They contribute to the plant self-defense mechanisms by protecting the plant against bacteria, fungi and viruses. They have been described as non-peroxide antimicrobial factors. It is believed that these phytochemical factors are composed of many complex phenols and organic acids.

### 2.8.1 *Aloe secundiflora*

The herbal plant of *Aloe secundiflora* are perennial monoecious with a variety of sizes that include the miniature herbs such as the grass aloes, stemless rosette-forming species, stemmed and sprawling shrubs and small trees. The leaves in the

stemmed aloes may be borne in terminal rosettes or may be spaced along the stem, which in some cases emerges to about 15 meters in height.

In Kenya *Aloe secundiflora* is known locally as kiruma, Thugurui (kikuyu), mukuni (Embu), Echuchuka (Turkana), kiluma (Kamba) and sukuroi (Maasai, Samburu). It is predominantly found in areas just above sea level in Kenya e.g. Mombasa through Voi. it contains important phytochemicals compounds including aloenin A, aloenin B, isobarbaloin, and other aloin derivatives among them are chromones and phenylpyrones with a low content of polysaccharides and aliphatic compounds. The high percentage of anthrones in the exudate could explain the use of the species in ethno veterinary practices (Rebbeca, 2003).

The herb, *Aloe secundiflora* has been used to treat both human and veterinary diseases that include conjuctivitis, gall,wounds, gastro-enterites, salmonellosis and gastiritis; as a laxative and purgative; and as a cathartic (Politz, 1998). A lot of research has also revolved on its anti-malarial activity against plasmodium falciparum (Oketch, 1999).

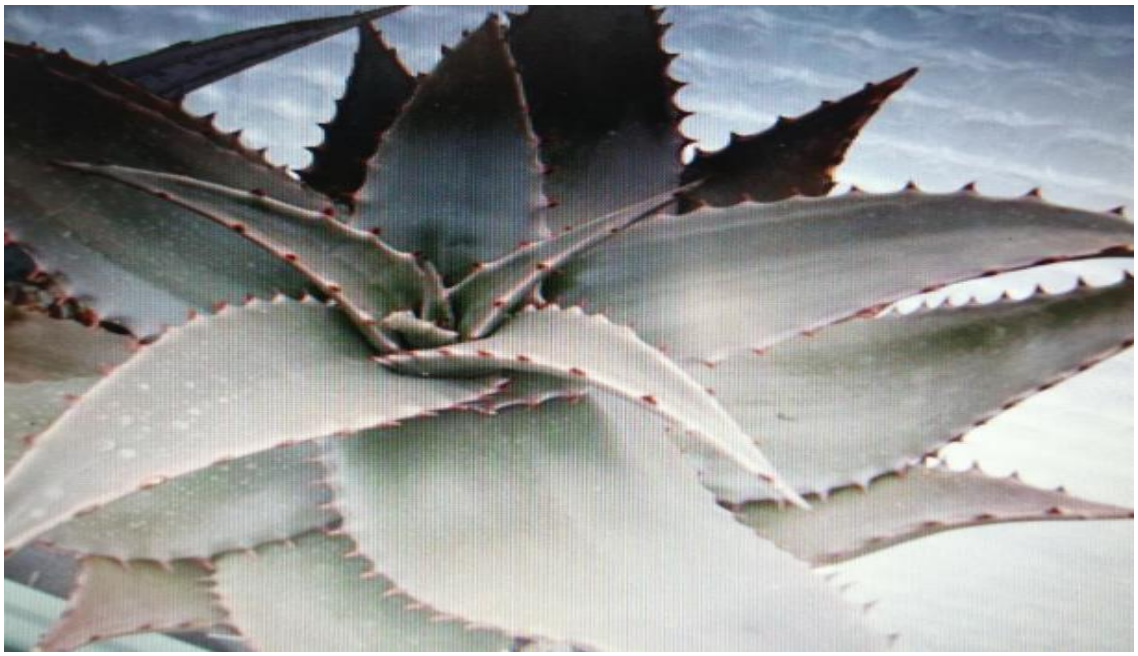


**Figure 2.3: Leaves of *Aloe secundiflora***

### 2.8.2 *Bridelia micrantha*

The herbal plant also referred as *Bridelia micrantha*, the Mitzeeri or the Coastal Golden-leaf, is a tree in the phyllanthaceae family and is native to tropical and southern Africa as well as to the Island of Réunion in the Indian Ocean and Kenya it is mainly found in the Eastern and Western parts of the country. Locally it is known as *Mukoigi* in central Kenya *Mukwego* in Eastern Kenya (Gakuya *et al.*, 2013) and S*hikagania* or *Kamulandangombe* in Western Kenya (Ochwang'i *et al*., 2014).

It is medium to tall tree up to 20 m, with a dense widely spreading crown. The leaves are large, alternate and simple. The tree may be deciduous or evergreen. A bark decoction is taken as a remedy for stomach-ache and tapeworm. The root is used as a remedy for severe epigastric pain and is applied to the scalp to relieve headache. A decoction of the root is drunk as a purgative, an anthelmintic or an antidote for poison, as it causes vomiting or diarrhoea that gets rid of the poison. An infusion made from the root is taken orally for coughs. The powdered bark is applied to burns to speed healing (Katende *et al.*, 1995).

**Figure 2.4:** ***Brideria micrantha***

### 2.8.3 *Lippia javanica*

The herbal plant *Lippia javanica* (Burm.f.) Spreng. (Verbenaceae) has a long history of traditional uses in tropical Africa as indigenous herbal tea or tisane refreshing beverage, or food additive based on its perceived health and medicinal properties (Lukwa *et al.,* 2009*).* It is used medicinally in Africa and many herbalists and herb gardeners. Different parts (the leaves, twigs and occasionally the roots) of the plant are used for different reasons. The most important traditional applications include its uses as herbal tea and ethnomedicinal applications for diarrhoea, chest pains, bronchitis, colds, cough, fever or malaria, wounds, repelling mosquitos, and asthma (Endris, 2016).

**Figure 2.5: *Lippia javanica***

## 2.9 Prevention and Control of *H. pylori* infection

The spread of *H. pylori* infection within the family members can be controlled by training the members of the family about the sanitary measures and importance of early diagnosis and treatment of infected persons. Good hygienic practices, consuming properly cooked food and clean safe drinking water can cut down the

rate of infection rate to great extent. The strict enforcement of good hygienic measures assists in alleviating infections in the family as well as community. Currently there is no vaccine available to prevent *H. pylori* infection.

### 2.10 *H. pylori* vaccine

Recently a number of new *H. pylori* vaccine development programs have started, predominately by research institutions (Sutton & Boag, 2019). All *H. pylori* vaccines currently in development are in preclinical. These vaccines are predominantly composed of purified or recombinant components of *H. pylori* antigens with an adjuvant (Sutton & Boag, 2019).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1 Study Site

The study was conducted from December 2017 to November 2019 a study was conducted at the **Aga Khan University Hospital** Nairobi (**AKUHN**), which is a private institution located in Nairobi County, Kenya. Aga Khan University Hospital was established in 1958; the hospital currently is a 254-bed long-term care facility offering general medical services, specialist clinics and diagnostic services such as laboratories, radiology/imaging and endoscopy among other specialized services. The catchment population for the AKUHN is approximately 600,000 persons. Services at the AKUHN are accessed by middle- and upper-income population in Nairobi and the adjacent areas Machakos, Kiambu and Muranga Counties (Figure 3.1). In the year prior to this study, the AKUHN had an average of 2300 in-patients and 12,000 out-patients. Nairobi County lies 1,795m above sea level and has a tropical climate. The average annual temperature is 25 °C while the rainfall is 14 mm annually. Nairobi County has a population of approximately 4 million people and a density of 239,000per $km^2$ (Census, 2019).

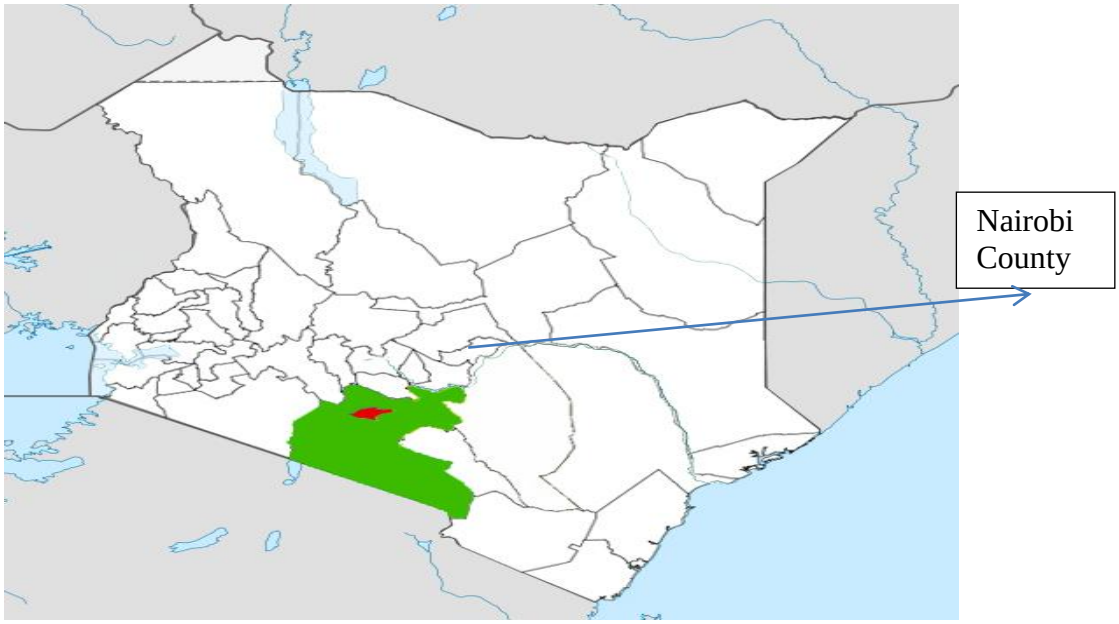



**Figure 3.1: Kenya map showing Nairobi County**

### 3.2 Study Design

This was a cross-sectional, descriptive study.

### 3.3 Study population

All dyspeptic patients undergoing oesphago-duodenoscopy (OGD) at the Endoscopy unit at (AKUNH) from December 2017 – November 2019.

### 3.4 Inclusion and Exclusion Criteria

Any dyspeptic patient undergoing OGD at the endoscopy unit of AKUH was included. Any of the dyspeptic patients who had been on triple therapy antibiotics or acid suppressive drugs within 2 weeks prior to the OGD were excluded. Those who declined to give informed written consent were also excluded.

### 3.5 Sample Size Determination

$$N = \frac{Z^2 1 - \alpha/2 \ (1 - P)}{d2}$$

Where n = sample size

Z = the standard normal deviate

P = estimated prevalence of the characteristic

d = degree of precision or accuracy, 0.1; (10%)

α = significance level

The confidence level is 95%

Z (1 – α/2) is the corresponding value to the 95% confidence level.

Therefore N= 1.962 (1- 0.675) 0.12

=274 this was the minimum sample size required.

### 3.6 Sampling procedure

Consecutive patients with history of dyspepsia and who had been referred for OGD for assessment at the endoscopy unit of the Aga Khan University Hospital Nairobi (AKUHN) were enrolled into the study.

### 3.7 Diagnostic Methods for H. pylori from Gastric Biopsies

#### 3.7.1 Collection of gastric biopsies by upper Endoscopy Procedure

Patients were instructed to fast for a minimum of 12 hours before endoscopy. The Fibreoptic gastroduodenoscope was rinsed thoroughly with water and disinfected by placing in a solution of 2% gluteraldehyde for 30 minutes. The endoscope was rinsed in sterile physiological saline just before the procedure.

Patient's throat was sprayed with lignocaine spray to reduce gagging and they were made to lie down in left lateral position comfortably. The end of the endoscope was lubricated with lignocaine jelly before inserting into the mouth. A mouth piece was introduced into the mouth and oesophageal Gastro Duodenoscopy (OGD) was performed in all our patients in the endoscopy section using Olympus (GFIXQ150, SN: 2206196, Olympus corporation, Tokyo) forward viewing EGD under intravenous sedation using a video-gastroscope. All the patients were examined for findings suggestive of endoscopic gastritis, such as erythema, hyperemia, atrophy, and mucosal nodularity according to the criteria of the Sydney grading system. A thin, lighted tube with a camera on the end was eased carefully into the mouth and down the throat to the stomach and duodenum. The lining of the oesophagus, stomach and duodenum was observed. Gastric antrum biopsies, about 2-3 mm in length were collected. Six biopsies specimens were obtained for Rapid urease test which was done in Endoscopy room, culture specimens was placed in Brain heart infusion (BHI) broth containing 1.5% glycerol and histology specimens were immediately fixed in buffered formalin. The biopsy specimens for bacterial culture were transported to the laboratory within one hour of collection, while samples for histological analysis were transported to at the histopathology laboratory for processing.

### 3.7.2 Rapid Urease Test

Pronto Dry Rapid Urease® results was performed on the bed side after collection of gastric biopsies and the results were read between 5minutes and one hour after sampling as directed by the manufacturer. Biopsy specimen was introduced in the test well. The colour change was read by comparing with the chart provided on the back of the test kit (yellow, pink, orange Dark pink and fuchsia) with the colour change from yellow to pink was considered positive result and no color change (yellow) as negative for Pronto Dry Rapid Urease® (Figure 3.2).


**Figure 3.2: Positive Pronto Dry Rapid Urease®.**

### 3.7.3 Bacterial Culture

The biopsy specimens collected for bacterial culture were transported in Brain Heart Infusion (BHI) broth containing 1.5% glycerol. The biopsy specimen tissues were macerated in a sterile mortar with the aid of a sterile fine glass rod to form a homogenate and inoculated afterward onto Brucella blood agar. The inoculated culture media were incubated at 37 °C for 72h under microaerophilic atmosphere 10%$CO_2$, 5%$O_2$, and 85% N2 using Anoxomat system. The incubation jar was opened on 3$^{rd}$, 5$^{th}$ and 7$^{th}$ day and checked for growth. Inoculated culture plates which had no growth were discarded after 7$^{th}$ day. The isolated bacteria were initially identified as *H. pylori* by colonial morphology as tiny (0.5-1 mm), moist, convex and watery colonies (translucent).

For Gram reaction, single colonial growth was emulsified in a drop of normal saline on a clean glass slide. The smear was air dried and methanol fixed. On Gram stain's 'reaction seagull shaped Gram negative helical shaped (Figure 3.3).

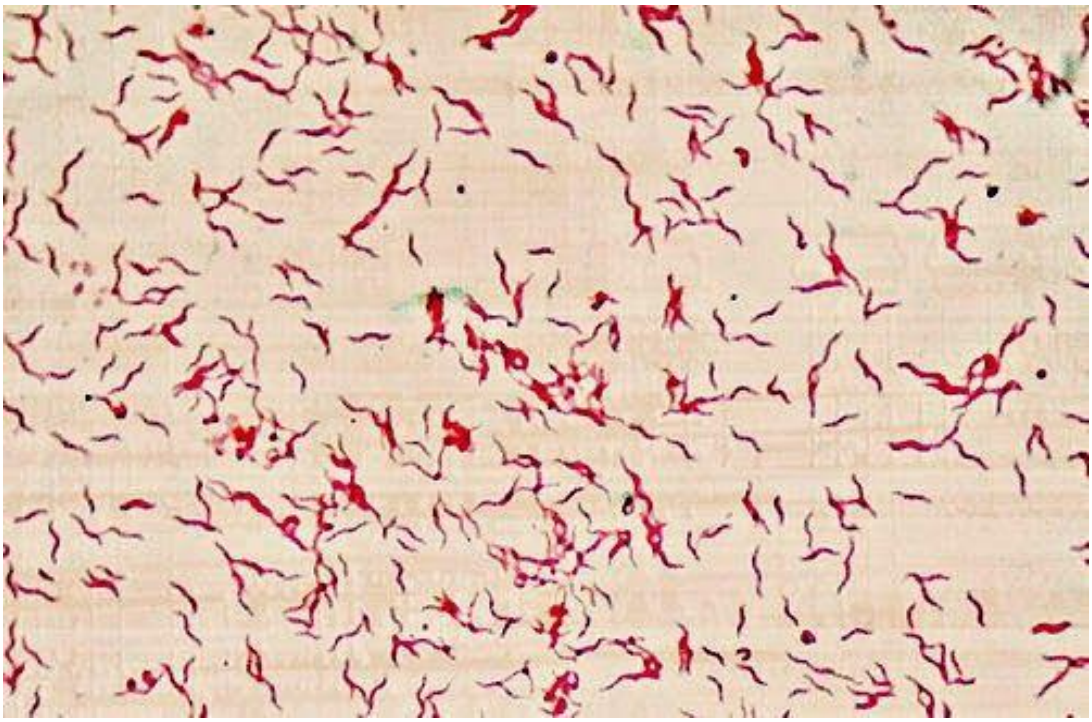


**Figure 3.3: Gram reaction of *H. pylori***

The catalase test involved picking a colony was with sterile wire loop and emulsified into 3% hydrogen peroxide solution drops on a sterile glass slide. Rapid bubbling indicates of strong catalase positive of *H. pylori*.

The oxidase test involved picking suspected colony with sterile wire loop and smeared on to a dry filter paper impregnated with Tetramethyl para-phenylene diamine dihydrochloride. The development of from white to purple colour within 10 seconds indicating oxidase positive *for H. pylori*.

The urease test reaction involved picking few colonies and streaking them on the slant of urease agar, and incubating for 5-10 minutes a rapid colour change from yellow to pink demonstrated the presence of urease thereby indicating the presence of *H pylori.*

### 3.7.4 Histopathology

The microscopic examination of biopsy specimen tissues in order to study the manifestations of disease was carried out and scored as indicated in the figure 3.4. Assessment of Gastritis A histopathological classification of gastritis was used using updated Sydney system (Strote, 2001) which had a scale of 0 – 3 for scoring the features of chronic gastritis, corresponding to none, mild, moderate or severe, respectively. Histology was used as the gold standard to evaluate performance of culture and Pronto dry rapid urease test.

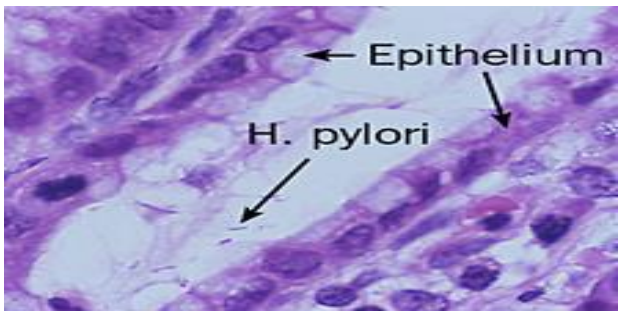



**Figure 3.4: Haematoxylin and Eosin Staining of *H. pylori* infected gastric Mucosa**

### 3.8 Determination of Virulence Genes of Clinical Isolates *H. pylori.*

### 3.8.1 Preparation of genomic DNA, Library Preparation and Whole-Genome Sequencing

The detection of three virulence genes *cagA*, *vacA* and *dupA* was performed as follows; total DNA of bacterial isolates was extracted from isolated pure *H. pylori* colonies with the DNeasy Blood and Tissue Kit according to manufacturer's guideline (Qiagen, Hilden, Germany). DNA concentration of each sample was quantified using the Quantus Fluorometer (Promega, Madison, WI, USA). A <300-bp libraries of 1 ng DNA was prepared, clean up and normalized with the Nextera XT DNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA). Normalized DNA libraries were pooled and used for paired-end sequencing of 300-bp reads with the

Reagent Kit using 300 cycles into a Miseq platform (Illumina, Inc., San Diego, CA, USA). Fluorescent images were assessed using the MiSeq Control Software, and FASTQ-formatted sequence data was created with MiSeq Reporter Analysis Software. A density cluster and Q-score≥30 of sequenced reads ranged respectively from 1185 to 1196 k/mm$^3$ and from 85 to 88%, attesting good quality in sequencing runs.

### 3.9 Determination of Antimicrobial Resistance Pattern to the Five Conventional Antimicrobial Agents used to treat *H. pylori.*

#### 3.9.1 Antimicrobial Susceptibility Testing by Epsilometer test (E -Test)

Antimicrobial susceptibility testing was performed on Mueller Hinton agar (OXOID Ltd, Basingstoke Hampshire, U.K) supplemented with 10% sheep blood for the following five antibiotics metronidazole (8µg/mL), clarithromycin (0.75µg/mL), amoxicillin (0.25µg/mL), tetracycline (2µg/mL), and levofloxacin (1µg/mL). The MiC values were obtained by Epsilometer test (E-test, AB Biodisk, USA) in accordance with Manufacturer instructions. Bacterial suspension was prepared in Mueller Hinton broth and adjusted to correspond to four McFarland turbidity standards and inoculated on Mueller Hinton Blood agar 10% (OXOID Ltd, Basingstoke Hampshire, U.K) using sterile swab. The E-test strips for the antibiotics were aseptically placed onto the dried surface of inoculated agar plates (Mueller Hinton agar, Merck, Germany) and sheep blood (10% v/v), according to the EUCAST guidelines 2017. The plates were incubated under microaerophilic condition at 37°C for 72h. MIC values were read as the intercept of the elliptical zone of inhibition with the graded strip for the E-test. EUCAST (2017) breakpoints defined resistance as follows: MIC >0.12mg/L and ≥2mg/L for amoxicillin, >8mg/L and ≥8mg/L for metronidazole, >0.5mg/L and ≥1mg/L for clarithromycin, and >1mg/L and ≥4mg/L for tetracycline and >1mg/L levofloxacin. The E-test results of the *H. pylori* isolates were compared with a standard susceptible strain of *H. pylori* (ATCC 43504) for the quality control of antibiotic susceptibility testing.

### 3.10 Bioinformatics analyses

The data obtained with the Illumina Miseq platform were either exploited as high-throughput short reads or assembled into a draft genome of each isolate. The genomics Workbench Software v8.5.1 (CLC bio, Aarhus, Denmark) was used to filter and pair short reads, to assess their quality and to trim low-quality bases (<Q30) and adapters from individual sequences. In further analyses, following the recommendations of Illumina, samples with more than 80% reads at quality ≥Q30 were selected. Draft WGS were de novo assembled from Illumina paired-end short reads with SPAdes v3.14.0. Then, after quality assessment of the obtained draft WGS with QUAST v5.0.2, gene sequences were identified and annotated using the RASTtk pipeline in the Rapid Annotation using Subsystem Technology v2.0.

### 3.11 Determination of Antimicrobial Activity of Medicinal Plants

#### 3.11.1 Collection of Medicinal Plants

Ethno-botanical survey for the collection of plant material involved the conversation with local herbalists with established health facilities within Nairobi. Questions involved the local names of the plants used to treat gastrointestinal infections, the parts of the plant used, methods of preparation, mode of administration and dosage, other medicinal values of the plants and the perceived efficacy of the remedies on stomach complains. Plant materials used in the current study were collected during the month of May 2017. The following plants were collected leaves *A. secundiflora*, bark of *B. mirantha*, and leaves of *L. javanica* from Mwingi Kitui County, Kenya. The plants were packed separately in a bag and transported to National Museum of Kenya Botany Department where Taxonomic identification of the plants was confirmed. The voucher specimens from each plant were deposited at the Department of Botany.

#### 3.11.2 Preparation of Medicinal plants

The plant materials were then air-dried at room temperature and ground into a fine powder. Preparation of plant extract exactly 500 g of dried powdered plant material

was macerated separately in 600 mL of methanol solution in large conical flasks. The aqueous extracts were also prepared by soaking 500g of powered plant material in distilled water. The bottles were labeled and put in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) for 72h. The plant extracts were filtered using a fitted filter funnel of pore size 60 Å. The procedure was repeated twice and the aqueous extracts evaporated to dryness under vacuum using freeze drier whereas methanol extract was evaporated to dryness under vacuum in a rotary evaporator (BUCHI rota vapour, R-461, Switzerland) set at temperatures depending on the two solvent in use. The dried crude extracts were collected in clean universal bottles and left open in a bio-safety cabinet for complete evaporation of residual solvents. Methanol stock solutions were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO) whereas aqueous extracts were dissolved in distilled water.

### 3.12 Agar Well Diffusion

The antimicrobial susceptibility testing of *H. pylori* to the methanol and aqueous extracts was performed by agar well diffusion technique. The Mueller Hinton Blood agar supplemented with sheep blood 10% (MHBA) (OXOID Ltd, Basingstoke Hampshire, UK) were carefully swabbed with *H. pylori* broth suspension corresponding to four McFarland turbidity standards. The inoculated plates were allowed to dry at room temperature for 3-5 minutes. Five wells were punched using a sterile glass rod with 6mm diameter. Using a micropipette three wells were filled with 100ml of crude extracts of medicinal plants crude extracts 50 mg/mL. One well was filled with 100ml of Ciprofloxacin 2.5 µl/mL as positive control while the other well was filled with 10% Dimethyl sulfoxide (DMSO) as negative control for methanol extracts and distilled water for aqueous extract. The plates were incubated at $37^0$C for 72h under microaerophilic 10%$CO_2$, 5%$O_2$, and 85% $N_2$ using Anoxomat system conditions after which the diameters of zones of inhibition were measured in millimeters. The experiment was repeated thrice and means zones were recorded. A standard *H. pylori* control strain, ATCC 43504 inoculated plate was included in all the experiments.

### 3.13 Determination of Minimum inhibitory concentration (MIC)

The MIC was carried out in accordance with the method of (Banfi *et al.*, 2003). Minimum inhibitory concentration (MIC) was determined using the agar dilution method. The MIC was assessed on plant extracts that showed antimicrobial activity by producing inhibition zones (≥14mm) during the agar well diffusion assay. About 100 µl of extracts (50 mg/ml in 5 % DMSO) were added to a multi well plate containing 100 µl of freshly prepared broth and serially diluted, yielding 12.5 mg/ml in the first well. Two-fold serial dilution method of the methanol and aqueous extracts were prepared. The final extracts and ciprofloxacin concentrations ranged from 0.0049–12.5 mg/mL respectively and the tests were carried out in duplicate. One hundred microliters of inoculum prepared from 72h colonies growth on Brucella agar supplemented with 5% sterile sheep blood to correspond to four at McFarland turbidity standards was added to 100 $\mu$L of the extract-containing culture medium. Control wells were prepared with culture medium and bacterial suspension and broth, only respectively. Ciprofloxacin was used as positive control while DMSO and normal saline were used as negative control. The plates were covered with a sterile plate sealer and incubated at 37ºC for 72h under microaerophilic conditions. After incubation 40 µl of 0.2 mg/µl of iodonitrotetrazolium dye (INT) was added in each of the wells and the plates examined after an additional 45 minutes of incubation. Viable *H. pylori* reduced the yellow dye to pink reddish color (conversion of INT to formazan). The lowest concentration at which the color change was apparently invisible as compared to the next dilution was taken as the minimum inhibitory concentration.

### 3.14 Minimum Bactericidal Concentration (MBC)

The Minimum bactericidal concentration (MBC) was determined by taking 50 µl of suspension from plate wells that demonstrated no growth and inoculating on Brucella agar supplemented with 5% sterile sheep blood. The plates were incubated at 37°C for 72h under microaerophilic conditions. In circumstances where there was no *H. pylori* growth and lowest concentration of extract, which did not produce any growth, was considered as MBC.

### 3.15 Preliminary Phytochemical Tests

To determine the presence of phytochemical compounds in the crude extracts of medicinal plants they were subjected to the following phytochemical screening (Parekh, 2008).

**Alkaloids**

A weight of 200gm of the extracts was stirred with 2ml of 1%HCl on water bath. One milliliter of the filtrate was treated with five drops of Mayer's reagent and another 1ml was equally treated with Dragendorff's reagent. Formation of an orange precipitate indicated preliminary evidence for the presence of alkaloids.

**Flavonoids**

A weight of 200 mg of extracts was dissolved in 10 ml distilled water and then filtered using Whatman filter No.1. 10mg magnesium turnings were added into 1 ml of the filtrate, followed by the addition of 0.05 ml concentrated HCL acid. The presence of pink red color observed within three minutes confirmed the presence of flavonoids.

**Saponins**

A weight of about 5 g of extract was reacted with 10ml water and shaken properly in a test tube. Samples showing froth were warmed. The stable froth of more than 1.5 cm and persisting for at least 30 min indicated the presence of saponins.

**Steroids**

Two millilitres of acetic anhydride was added to 200 mg extracts of each sample with 2 ml of 0.1 M $H_2SO_4$. The colour change from violet to blue or green ring indicated the presence of steroids.

**Tannins**

A weight of 200mg of the extract was dissolved in 10 ml of distilled water and filtered. Two drops of 2% (w/v) ferric chloride was added to the filtrate. A blue, black precipitate indicated the presence of tannin.

### 3.16 Determination of the Potential Bio active Compounds Gas Chromatography-Mass Spectrometry (GC–MS) Analysis to Detect Presence of Phytochemicals

The mass spectrometer examines the compounds eluted at different times to identify the nature and structure of the components. The large compound fragments to small compounds giving rise to appearance of peaks at different m/z ratios. The presence of bioactive compounds of medicinal crude extract was analyzed using GC–MS (SHIMADZU QP2010). The GC specifications were as follows: Column oven temperature set at 70$^{o}$C, injector temperature 200$^{o}$C, injection mode-Split, Split Ratio-40, Flow control mode Linear velocity, Column flow 1.51 ml/min, Carrier Gas-Helium 99.99% purity. The MS specifications were as follows: Ion source temperature 200$^{o}$C, interface temperature 24$^{o}$C, scan range was 40–1000 m/z, event time-0.5 s, solvent cut time was 5 min, start time was 5 min, end time was 35 min, and ionization was EI (−70 ev).

### 3.17. Data analysis

Statistical analysis was performed using SPSS software (Statistical Product and Services Solutions, version 17, SPSS Inc, Chicago, Il, USA) to analyze data. Association between *H. pylori* infection and virulence genes was tested independently using Pearson's chi$^2$ test and Fischers exact test. Data on zones of inhibition and MIC and MBC were collected and coded from where it was summarized into means and standard deviations.

The independent-samples t-test was conducted to compare the zones of inhibition by plant extracts dissolved in methanol and aqueous solvents.

# CHAPTER FOUR

# RESULTS

## 4.1 Demographic Data of Participants

A total of 274 antrum biopsy specimens were obtained from patients and analyzed. These included 156 (52%) females. The median age of the patients was 37 (Range=15-82) years (Figure 4.1).

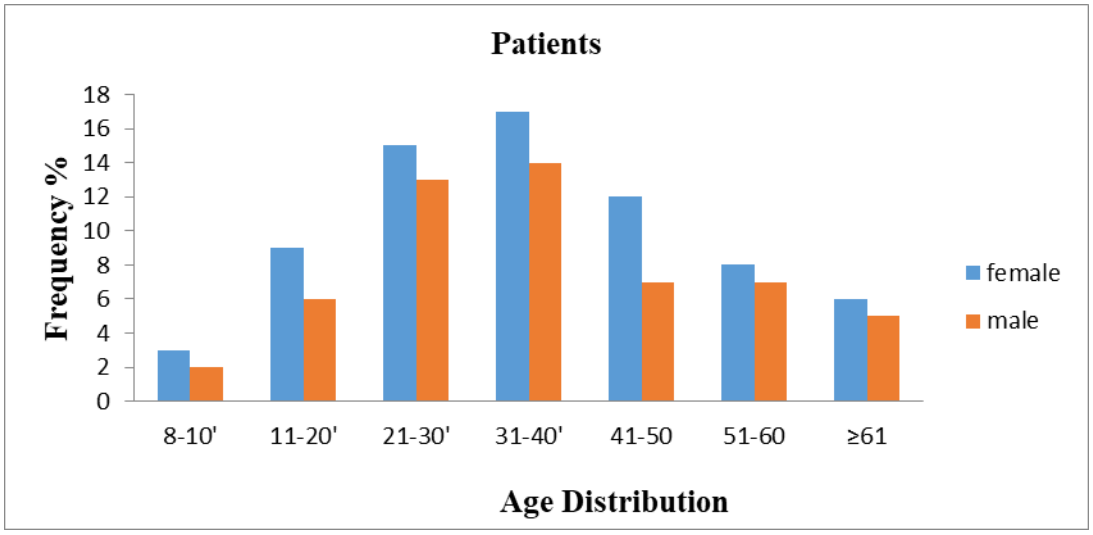



**Figure 4.1: Graph showing age distribution of study participants**

Most participants were drawn from 31-40 years age group.

### 4.1.1 Comparison of Diagnostic Methods for *H. pylori*

The frequency of positive specimen of 274 biopsies positive for histology 122(44%) were. Five (3%) histology negative specimens were positive for Pronto dry rapid urease® test, while 2 (1%) histology negative specimens were positive for culture. There was significant difference in sensitivity and NPV between Pronto dry rapid urease® test and culture. However, there was no significant difference in specificity and PPV between Pronto dry rapid urease® test (Table 4.1).

**Table 4.1: Comparison of performance of Dry® Rapid Urease Test and Culture against Histology**

| Test | Sensitivity(95%Cl) | Specificity(95%Cl) | PPV(95%Cl) | NPV(95%Cl) |
|---|---|---|---|---|
| **RUT** | 100%(97.5%-100% | 96.1% (91.1-98.7%) | 96.7(92.5%-98.9% | 100% (97%-100%) |
| **Culture** | 73.6%(64.8%-81.3% | 98.7% (95.4-99.8%) | 97.8% (92.3-99.7%) | 82.5% (76.2-87.7%) |

**Table 4.2: Comparison between Pronto Dry® Rapid Urease Test and Culture against Histology**

| Test | Result | Histology | | P-value |
|---|---|---|---|---|
| | | Positive (%) | Negative (%) | |
| RUT | Positive | 122 (96.0%) | 5(4%) | <0.001 |
| | Negative | 0 (0.0%) | 147 (100%) | |
| Culture | Positive | 151 (98.7%) | 32 (26.5%) | <0.001 |
| | Negative | 2 (1.3%) | 89 (73.6%) | |

There was no false positive for Pronto dry rapid urease® compared to 2(1.3%) for culture. Likewise false negative results for Pronto dry rapid urease® were 5(4%) compared to 32 (26.5%) for culture. There was significant difference between Pronto dry rapid urease® and the reference histology, P< 0.001 (Table 4.2).

### 4.1.2 Agreement between Diagnostic Tests for *H. pylori* Among Studied Participants

Diagnostic concordance was significantly between the comparative methods at p<0.005. Concordance highest between RUT and Histology at k=0.908 and lowest between culture and Histology at k=0.784 (Table 4.3).

**Table 4.3: Agreement between diagnostic tests for *H. pylori* among studied participants**

| Diagnostic test | Kappa coefficient | *P* Value |
|---|---|---|
| RUT-Histology | 0.908 | <0.005 |
| RUT-Culture | 0.846 | 0.005 |
| Culture-Histology | 0.784 | <0.005 |

## 4.2 Relationship between *H. pylori* Virulence Genes and Gastro-duodenal Diseases

The endoscopy grading for the 274 patients showed normal mucosa in 51 (19%) individuals, gastritis in 120 (44%), gastric ulcer in 52 (19%), duodenal ulcer in 36 (13%) gastric cancer in 3 (1%) and polyps in 7(3%). The highest number (57) of *H. pylori* positive cases, representing 48% of all participants was obtained among gastritis patients, followed by 16 (31%) cases with gastric ulcer. Virulence genes were determined for all 91 confirmed *H. pylori* positive isolates obtained from culture isolates. Virulence genes *cagA, dupA, vacA* s1/m1 were detected in all disease conditions. Detection of *vacA s1* in 23 (55%) in isolates with gastric ulcer disease was significantly high (P = 0.036). The gene *cagA* gene was detected in 47% (43/91), *vacAs1* in 46% (42/91), *vacAs2* in 33% (30/91), *vacAm1* in 35% (32/91), *vacAm2* in 24% (22/91) and *dupA* in 43% (39/91) isolates. In this study, virulent allelic combination s1/m1 was predominant and found in 37 (41%) of *the H. pylori* strains, the s1/m2 was detected in 10 (11%) and the other genotypes, s2/m1 and s2/m2 were recorded in 7 (8%) and 12 (13%) strains respectively (Table 4.4).

**Table 4.4: Virulence Gene Detection and Endoscopic Conditions in *H. pylori* Positive Patients**

| | Gastritis | Gastric ulcer | Duodenal ulcer | Gastric Cancer | Normal | Polyps | Antral nodularity | Total |
|---|---|---|---|---|---|---|---|---|
| | N=120 (44%) | N=52(19%) | N=36(13%) | N=3(1%) | N=50(19%) | N=7(3%) | N=4(1%) | 274 |
| **Positive *H. pylori*** | **57(48%)** | **16 (31%)** | **10(28%)** | **1(67%)** | **3(6%)** | **2(28%)** | **2(0%)** | **91** |
| **Cag A** | 19 | 11 | 9 | 1 | 1 | 1 | 1 | 43 |
| **Vac As1** | 8 | 23 | 8 | 1 | 0 | 2 | 0 | 42 |
| **Vac As2** | 9 | 12 | 9 | 0 | 0 | 0 | 0 | 30 |
| **Vac Am1** | 15 | 12 | 5 | 0 | 0 | 0 | 0 | 32 |
| **Vac A m2** | 11 | 10 | 1 | 0 | 0 | 0 | 0 | 22 |
| **VacA s1m1** | 22 | 6 | 4 | 1 | 2 | 1 | 1 | 37 |
| **VacA s1m2** | 5 | 3 | 1 | 0 | 0 | 0 | 1 | 10 |
| **Vac As2m1** | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 7 |
| **VacAs2m2** | 8 | 4 | 0 | 0 | 0 | 0 | 0 | 12 |
| **DupA** | 16 | 12 | 7 | 1 | 1 | 1 | 1 | 39 |

### 4.2.1 Sequence of Virulence genes *cagA vacA* alleles and *dupA*

Figures below shows 4.2, 4.3, and 4.4 show the sequence of virulence genes.

**Figure 4.2: Virulence genes sequences *cagA***

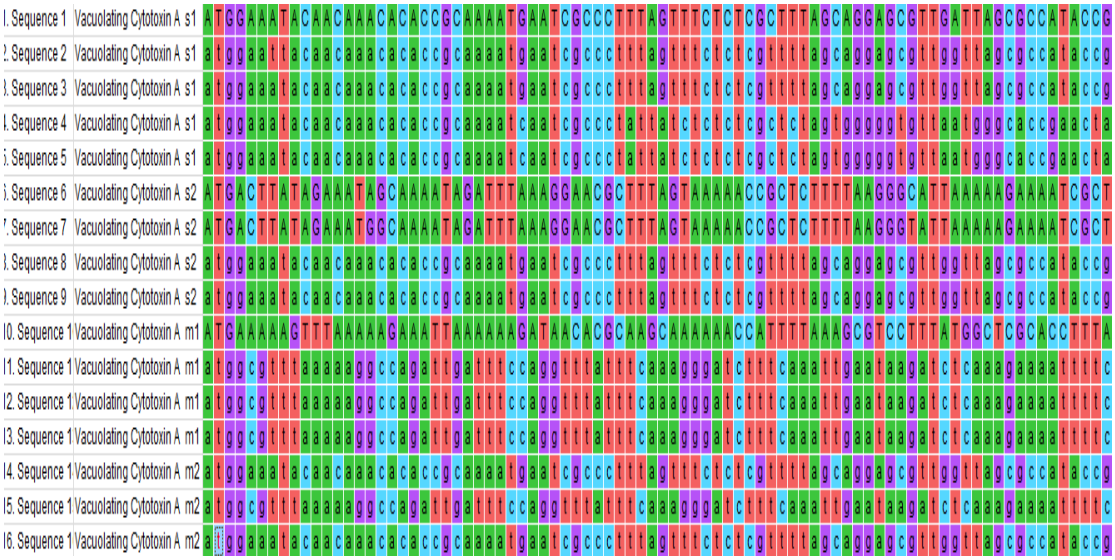



**Figure 4.3: Virulence genes sequences *vacA***

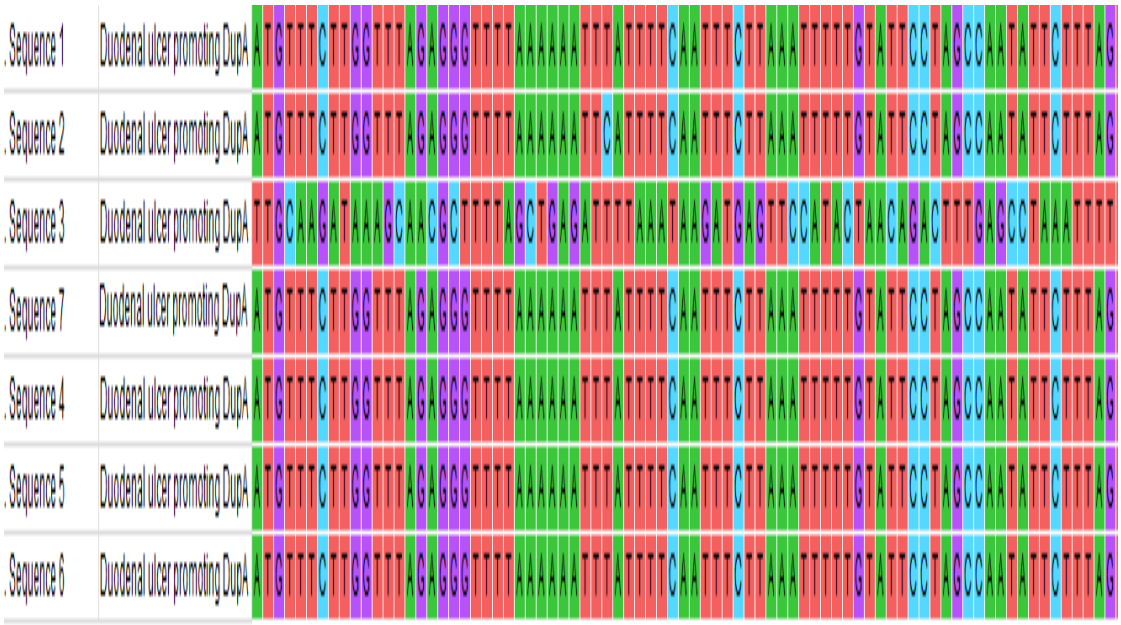



**Figure 4.4: Virulence genes sequences *dupA***

### 4.3 Antimicrobial Resistance of *H. pylori* to Commonly Used Conventional Antimicrobial Agents

In this study, resistance of the 91 *H. pylori* isolates to five conventional antimicrobial agents that is,

Clarithromycin, Levofloxacin, Amoxicillin, Metronidazole and Tetracycline were tested using the E-test. EUCAST (2017) breakpoints defined resistance as follows: MIC >0.12mg/L and ≥2mg/L for amoxicillin, >8mg/L and ≥8mg/L for metronidazole, >0.5mg/L and ≥1mg/L for clarithromycin, and >1mg/L and ≥4mg/L for tetracycline and >1mg/L levofloxacin. Resistance profile of *H. pylori* isolates to the five antibiotics were recorded for Metronidazole was 85 (93%), followed by Amoxicillin at 57 (63%), Clarithromycin at 23 (25%) and Levofloxacin at 12% (13%). No Tetracycline resistance was identified among the isolates. Only two isolates (2%) were susceptible to all antibiotics tested. Notably, 34 isolates (37%) were single drug resistance (SDR), and all of them had MTZ resistance. The rate of MDR was high, up to 53%. Among all clinical isolates, dual drug resistance to LVX + MTZ and MTZ + CLR was found in 22 (24%) and 35(38%) of isolates, respectively. Triple resistance to AMX + LVX + MTZ and CLR + LVX + MTZ accounted for 5% and11% respectively Table 4.5 below.

**Table 4.5: The antibiotic Resistance profile of 91 *H. pylori* isolates**

| Resistance Pattern | Number of Strains | Percentage % |
|---|---|---|
| Susceptible to all | 2 | 2 |
| All resistance | 89 | 98 |
| CLR | 23 | 25 |
| LVX | 12 | 13 |
| AMX | 57 | 63 |
| MTZ | 85 | 93 |
| TET | 0 | |
| Mono Resistance | 34 | 37 |
| MTZ ONLY | 34 | 37 |
| Multiple resistance | 48 | 53 |
| LVX + MTZ | 22 | 24 |
| CLR + MTZ | 35 | 38 |
| AMX + LVX + MTZ | 5 | 5 |
| CLR + LVX + MTZ | 10 | 11 |

Key: CLR; clarithromycin, LVX; levofloxacin, AMX; amoxicillin, MTZ; metronidazole, TET; tetracycline.

### 4.4 Determination of Antimicrobial Activity of Medicinal Plants

#### 4.4.1 Agar Well diffusion Results of Plants Extracts against *H. pylori*

There was a significant difference in the zones of inhibition for *A. secundiflora* (M=5.99, SD=4.78; t (91)=12.01, p<0.0001), *B. mirantha* (M=5.03, SD=4.03; t(91)=11.97, p<0.0001) and *L. Javanica* (M=4.22, SD=4.70; t(91)=8.59, p<0.0001). These results suggest that methanol is a more effective solvent for extracting antibacterial compounds from plants compared to aqueous. The results show greater zones of inhibition in methanol dissolved extracts. This is summarized in figure below (Figure 4.5).

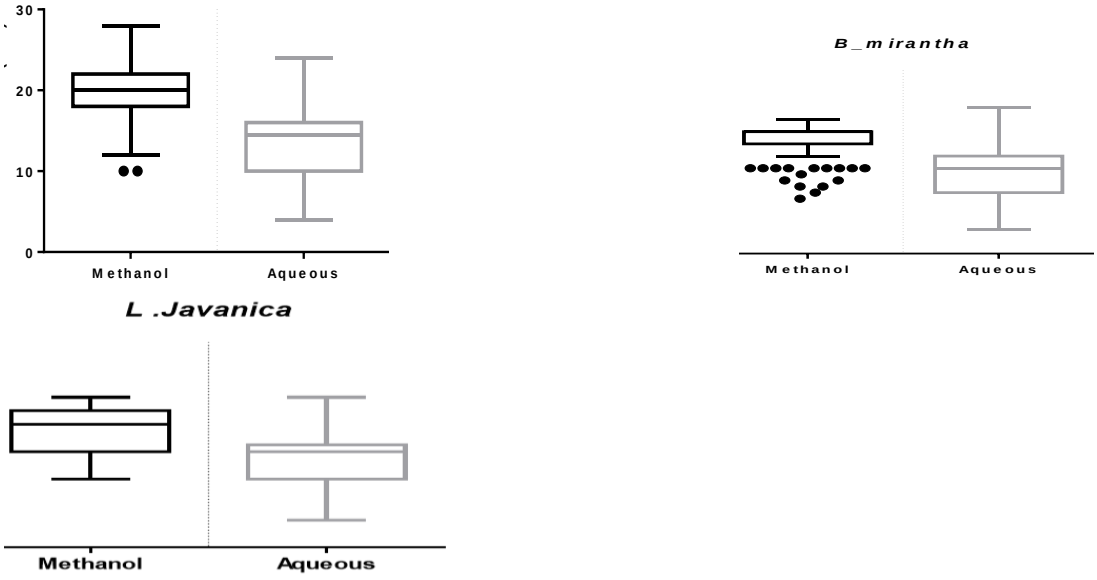



**Figure 4.5: Zones of inhibition for methanol and aqueous solvents in *A.secundiflora, B.mirantha L.javanica* extracts**

A one-way between subjects' ANOVA was conducted to compare the effect of three different plant extracts and ciprofloxacin on the zones of inhibition in *H. pylori*. For methanol-based extracts, there was a significant effect of the type of plant extract used on the zones of inhibition at the p<.05 level for the four conditions [F (3, 364)

=9.809, p<0.0001]. Post hoc comparisons using the Tukey HSD test indicated that the mean score for *A. secundiflora* (M = 19.73, SD = 3.78) was significantly different than *B. mirantha* (M = 18.41, SD = 3.02, p=0.0412), *L. Javanica* (M = 17.34, SD = 3.35, p<0.0001) and ciprofloxacin (M = 19.49, SD = 3.24, p<0.0001). However, zones of inhibition for ciprofloxacin did not significantly differ from that of *B. mirantha* and *A. secundiflora* methanol extracts. Contrastively, significant differences in the zones of inhibition were observed in aqueous extracts [F (3, 364) =61.24, p<0.0001]. Post hoc comparisons showed that the mean score for ciprofloxacin (M = 19.49, SD = 3.24) was significantly different than *L. Javanica* (M = 13.12, SD = 3.74, p<0.0001), *B. mirantha* (M = 13.38, SD = 3.96, p<0.0001) and *A. secundiflora* (M = 13.74, SD = 3.96, p<0.0001). However, the mean zones of inhibition for aqueous extracts in *L. javanica, B. mirantha* and *A. secundiflora* were insignificant. This is summarized in figure below (Figure 4.6).

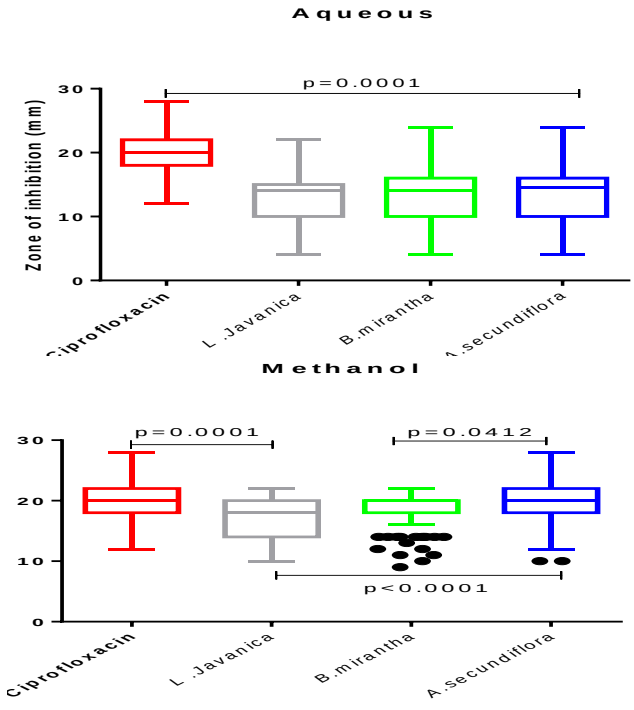
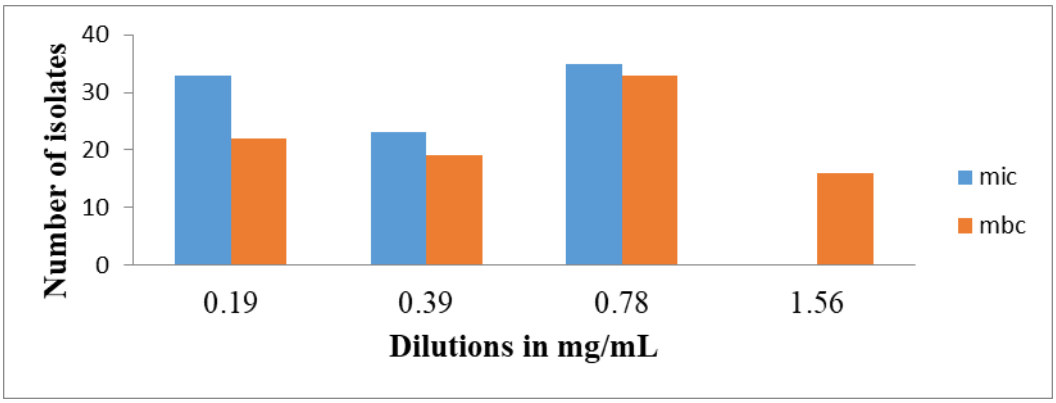



**Figure 4.6: Mean Zones of inhibition for methanol and aqueous solvents among medicinal plants**

### 4.4.2 Minimum Inhibitory concentration and Minimum bactericidal concentration of plant crude extracts

The aqueous extracts of *A. secundiflora* leaves had the highest MIC at 1.56 mg/ml against 0.78mg/ml for methanol and 0.32mg/ml for Ciprofloxacin. The lowest MIC for methanol and aqueous extracts were similar at 0.19mg/ml. This was higher than the lowest MIC for Ciprofloxacin at 0.02mg/ml. The aqueous extracts of *A. secundiflora* leaves had the highest MBC at 3.12mg/ml against 1.56mg/ml for methanol and 0.32mg/ml for Ciprofloxacin. The lowest MBC for methanol at 0.19mg/ml and aqueous at 0.39mg/ml were higher than MBC for Ciprofloxacin at 0.02mg/ml. Methanol extracts of *B. micrantha* had a MIC of 0.19mg/ml compared to *L. javanica* similar MIC (Figure 4.24).

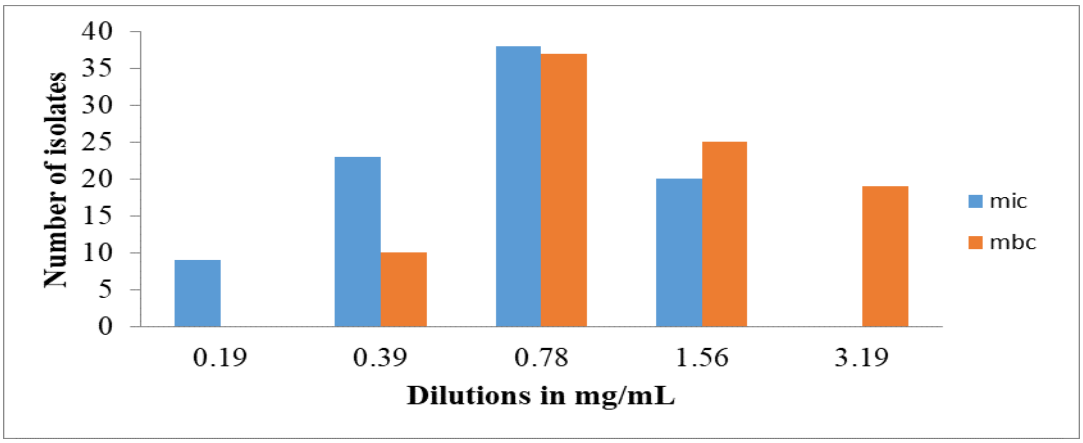
Among the crude plant extracts analysed, the methanol extracts of *A. secundiflora B. micrantha and L. javanica* had the best activities with MIC values ranging from 0.19-0.78 mg/mL with no significant differences between these values and those of ciprofloxacin (P>0.05).




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.7: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *A. secundiflora* Methanol Crude Extracts**

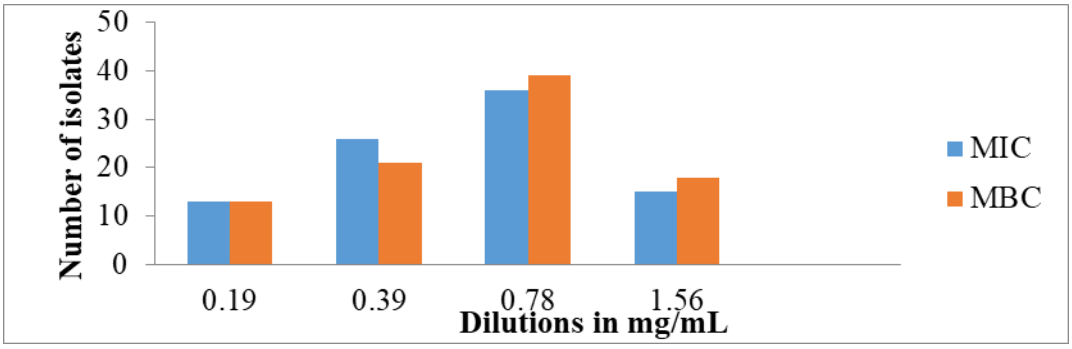
The Minimum Inhibitory Concentration (MIC) values ranged between 0.19 – 0.78 mg/mL, High number of isolate had both MIC and MBC at 0.78 mg/mL. No isolate had MIC values at 1.56 mg/mL.




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.1: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *A. secundiflora* Aqueous Crude Extracts**

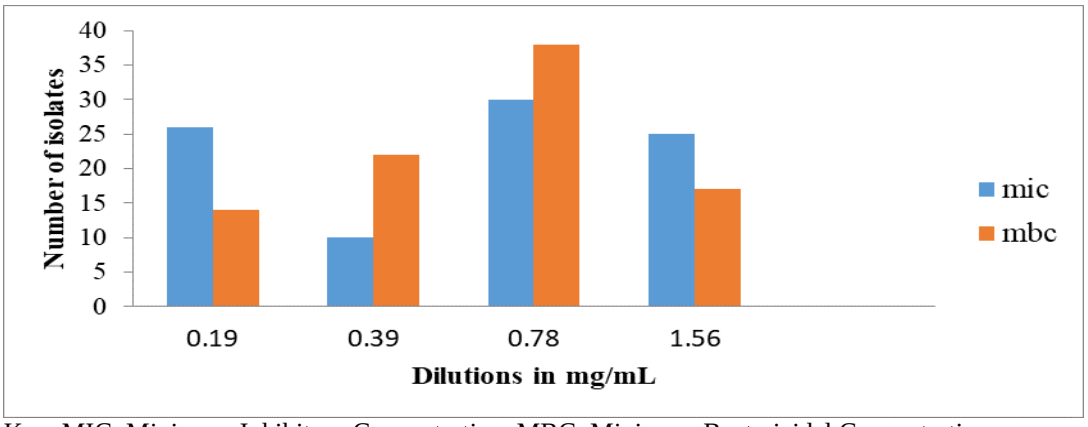
The Minimum Inhibitory Concentration (MIC) values ranged between 0.19 – 1.56 mg/mL. The high number of isolate had both MIC and MBC at 0.78 mg/mL. No isolate had MIC values at 3.19mg/mL.




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.9: Minimum Inhibitory concentration and Minimum Bactericidal Concentration of *B.mirantha* Methanol Crude Extracts**

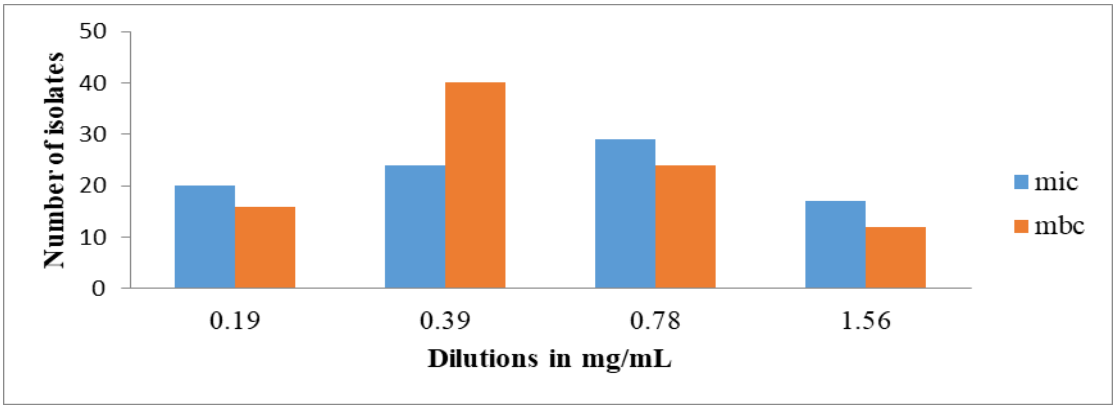
The Minimum Inhibitory Concentration MIC and Minimum Bactericidal Concentration (MBC) values ranged between 0.19 – 1.56 mg/mL with High number of isolate having both MIC and MBC at 0.78 mg/mL.




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.10: Minimum Inhibitory concentration and Minimum Bactericidal Concentration of *B.mirantha* Aqueous Crude Extracts**

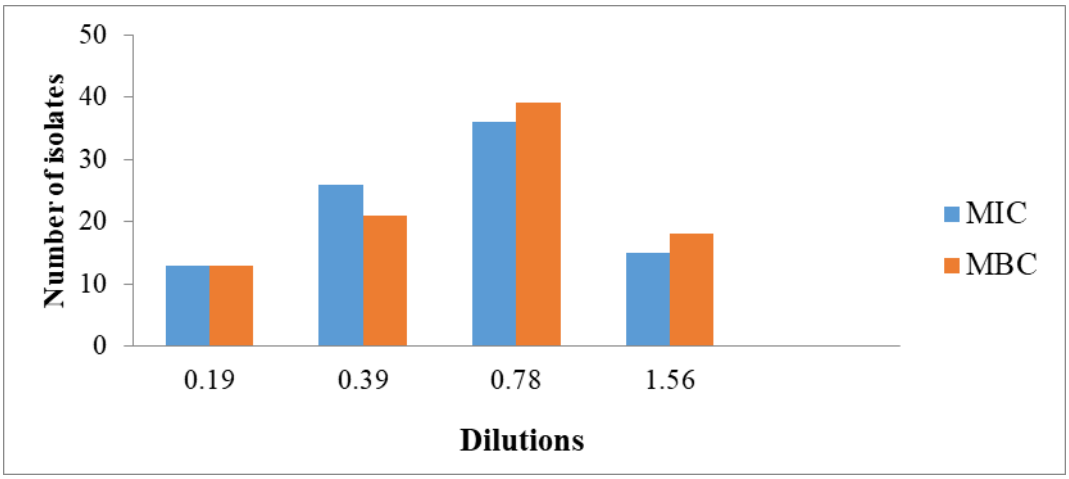
The Minimum Inhibitory Concentration MIC and Minimum Bactericidal Concentration (MBC) values ranged between 0.19 – 1.56 mg/mL with High number of isolate having both MIC and MBC at 0.78 mg/mL.




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.11: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *L. javanica* Methanol Crude Extracts**

The Minimum Inhibitory Concentration MIC and Minimum Bactericidal Concentration (MBC) values ranged between 0.19 – 1.56 mg/mL with High number of isolate having MBC at 0.39 mg/mL.




Key: MIC; Minimum Inhibitory Concentration, MBC; Minimum Bactericidal Concentration

**Figure 4.12: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *l.. javanica* Aqueous Crude Extracts**

### 4.5 Phytochemical screening of methanol and aqueous extract *A. secundiflora B. micrantha and L. javanica*

The results of phytochemical screening of methanol and aqueous extract of the three medicinal plants presence indicates presence of alkaloids saponin tannin, flavonoids, steroid in *A. secundiflora.* Steroids were only present in *A. secundiflora* (Table 4.6).

**Table 4.6: Phytochemical screening of methanol and aqueous extract *A. secundiflora, B. micrantha* and *L. javanica***

| ***A. secundiflora*** | | |
|---|---|---|
| Phytochemical | Methanol Extract | Aqueous Extract |
| Alkaloids | ++ | ++ |
| Saponins | +++ | + |
| Tannins | + | + |
| Flavonoids | ++ | + |
| Steroids | ++ | + |
| | | |
| *B. micrantha* | | |
| Alkaloids | + | + |
| Saponins | + | + |
| Tannins | + | + |
| Flavonoids | + | + |
| | | |
| *L. javanica* | | |
| Alkaloids | ++ | + |
| Saponins | ++ | + |
| Tannins | + + | + |
| Flavonoids | ++ | + |

Key: + Phytochemical intensity

### 4.5.1 Determination of the Potential Bioactive Compounds of *A. secundiflora, B.micrantha,* and *L. Javanica* by Gas Chromatography and Mass Spectrometry (GC/MS) analysis.

Various biological activities of *A.secundiflora* leaf extract, *B.mirantha bark and L. javanica leaf extract* were established by investigating the chemical composition of each extract using GC-MS analysis. The GC-MS spectrum established the presence of various compounds with different retention times as demonstrated. These mass spectra are fingerprint of that compound which can be identified from the data library (Figure 4.13).

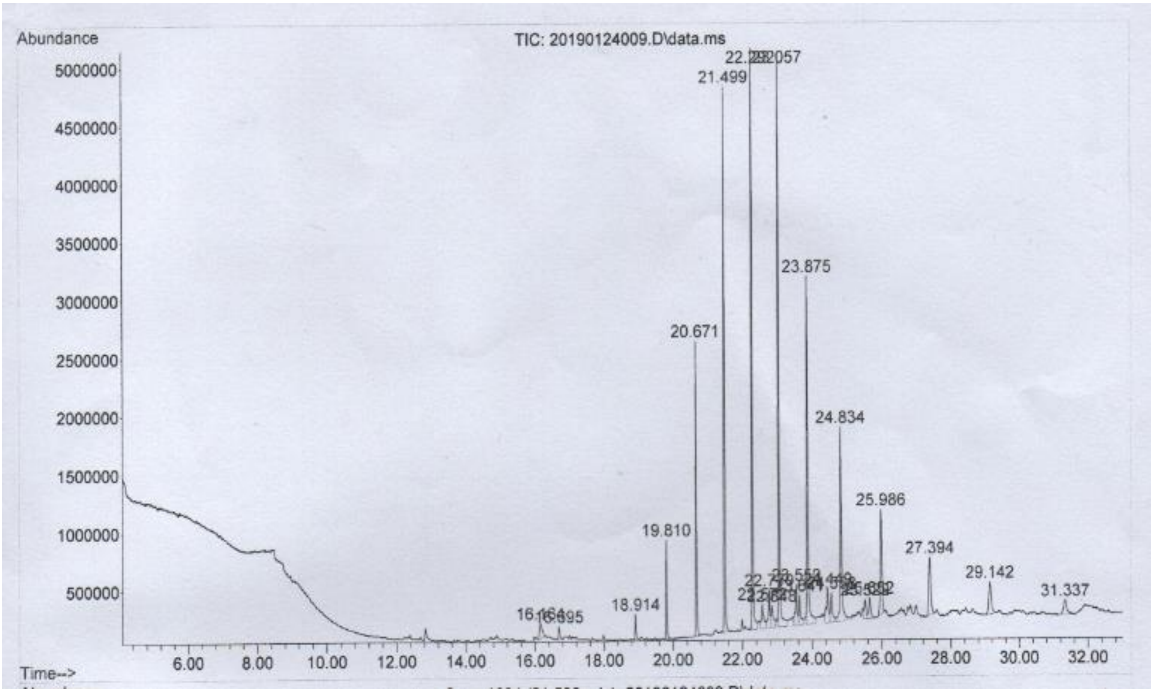



**Figure 4.13: The GC-MS profile of *A. secundiflora* plant extracts**

**Table 4.7: Bioactive Compounds identified in the methanol extract of *A. secundiflora* by GC-MS analysis**

| Compound Name | Bioactivity | Retention Time (minutes) |
|---|---|---|
| Camphor | Antibacterial | 7.9006 |
| Hexadecane | Antifungal, Antibacterial Essential oil, | 12.3806 |
| Butylated Hydroxytoulene | Antimicrobial, antioxidants | 12.6832 |
| Phenol, 2,4-bis(1,1-dimethylethyl) | Antibacterial , Anti-inflammatory | 12.8334 |
| Tridecane | AntiInflammatory, Anti-microbial activities and anti-cancerous | 14.8916 |
| 1,2 BenzeneDicarboxylicacid,bis(2-methylpropyl) ester | Antimicrobial agents Anti-cancer | 16.6906 |
| Dibutyl Phthalate | Antifungal,Antimicrobial agent, antimalarial and antifungal | 17.6564 |
| Eicosane | Antioxidant Antifungal, antibacterial,antitumor and cytotoxic effects | 17.9756 |
| Heneicosane | Antibacterial activity | 18.9125 |
| Docosane | Antibacterial activity | 19.8078 |
| Tricosane | Antibacterial activity | 20.668 |
| Benzyl butyl phthalate | Antimicrobial activity | 21.2276 |
| Tetracosane | Antimicrobial activity | 21.496 |
| Hexacosane | Antimicrobial activity | 23.0536 |
| Heptacosane | Antimicrobial activityAntioxidant activity | 23.8723 |
| Octacosane | Antimicrobial activity Antioxidant activity | 24.8319 |
| Triacontane | Anti-bacterial, Anti-diabetic, Antitumor | 27.3926 |

The methanol extract of *B. mirantha* bark GC-MS analysis identified nine compounds. Of this compound 9- Octadecanoic acid ethyl ester, Hexadecanoic acid, ethyl ester and Bis(2-ethylhexyl) Pthalate had the highest retention time (Figure 4.14).

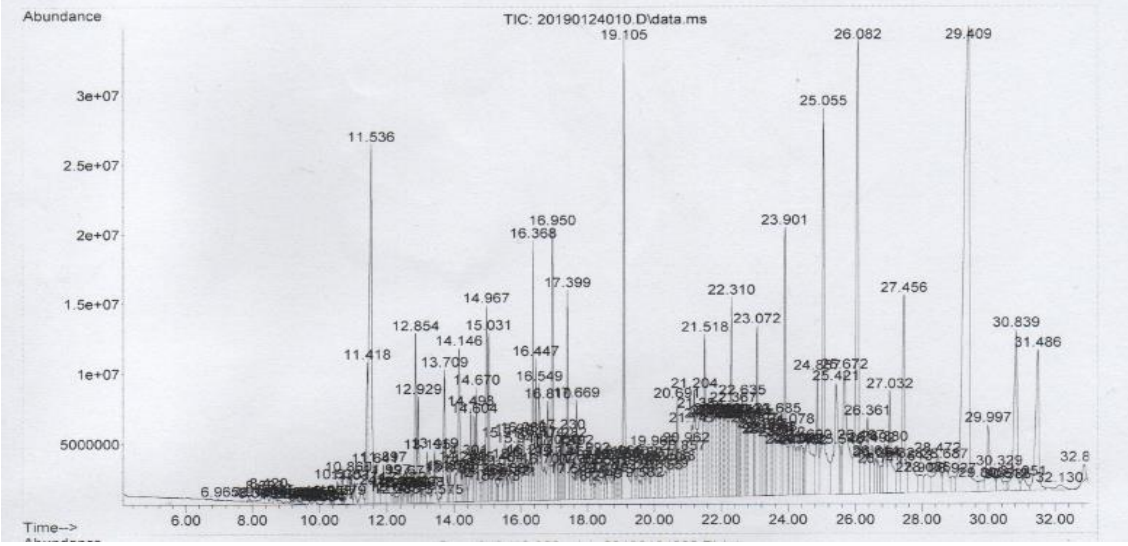



**Figure 4.14: The GC-MS profile of *B. micrantha* plant extracts**

**Table 4.8: Bioactive Compounds identified in the methanol extract of *B. micrantha* by GC-MS analysis**

| Compound Name | Bioactivity | Retention Time (minute) |
|---|---|---|
| Camphor | Antibacterial | 22.009 |
| Phenol, 2,4-bis(1,1-dimethylethyl) | Antibacterial , Anti-inflammatory | 23.269 |
| Dibutyl Phthalate | Antifungal,Antimicrobial agent, antimalarial and antifungal | 23.355 |
| Eicosane | Antifungal, | 23.755 |
| Hexadecane | Antifungal,Antimicrobial agent, Anti-inflammatory anti-cancer | 25.14 |
| Tetradecanoic acid, | Antioxidant, Antimicrobial,antiCancer | 26.682 |
| 9-Octadecenoic acid(Z)-, methyl ester | Antimicrobial ,Cancer preventive, Anti-inflammatory | 28.419 |
| Hexadecanoic acid | Antimicrobial | 29.92 |
| Bis(2-ethylhexyl)Pthalate | Antimicrobial ,Cancer preventive, Anti-inflammatory | 32.69 |

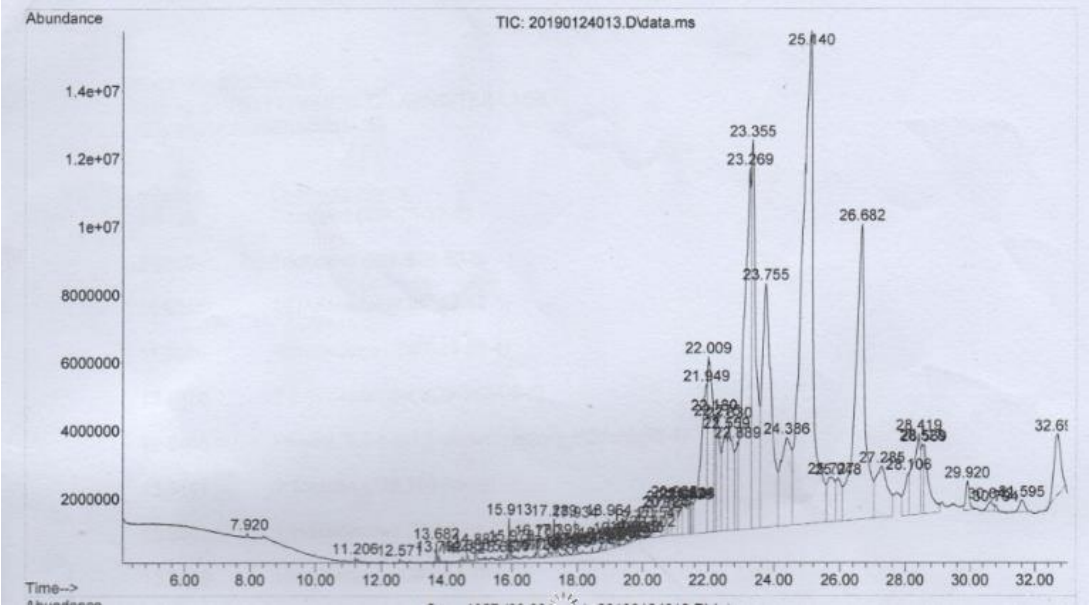



**Figure 4.15: The GC-MS profile of *L. javanica* plant extracts**

**Table 4.9: Bioactive Compounds identified in the methanol extract of L. javanica by GC-MS analysis**

| Compound Name | Bioactivity | Retention Time (minutes) |
|---|---|---|
| Phenol,2,4bis(1,1-dimethyl) | Antibacterial , Anti-inflammatory | 11.536 |
| Docosane | Antibacterial activity | 16.95 |
| Tricosane | Antibacterial activity | 19.105 |
| Trimethylamine | Hypercholesterolemic | 23.072 |
| Bis(2-ethylhexyl)Pthalate | Antimicrobial ,Cancer preventive, Anti-inflammatory | 23.901 |
| Hexacosane | Antimicrobial activity | 25.055 |
| Heptacosane | Antimicrobial activity Antioxidant activity | 26.082 |
| Octacosane | Antimicrobial activity Antioxidant activity | 27.456 |
| Squalene | | 29.409 |
| Triacontane | Anti-bacterial, Anti-diabetic, Antitumor | 30.839 |
| Eicosane | Antioxidant Antifungal, antibacterial, antitumor and cytotoxic effects | 31.489 |

# CHAPTER FIVE

# DISCUSSION

## 5.1 Comparison of diagnostic methods for *H. pylori*

This study found out that Pronto dry rapid urease® out performed culture in sensitivity and NPV measures. These findings are in agreement with similar studies performed in elsewhere (Uotani, Graham & DeBakey, 2015; Selgrad 2017). Moreover, sensitivity of Pronto dry rapid urease® according to the manufacturer is similar with our findings (Lee & Kim, 2015). The observed high sensitivity makes Pronto dry rapid urease® appropriate for screening ahead of the more time-consuming confirmatory tests. Furthermore, WHO recommends 'test and treat' strategy (Perna *et al.,* 2004) and this would come in handy due to its reasonable cost. Likewise, the observed high NPV implies that persons presenting at the endoscopy unit of the hospital for *H. pylori* testing who actually do not have the infection are likely to be appropriately classified as such by Pronto dry rapid urease® test. Pronto dry rapid urease® detects urease enzyme production by *H. pylori*. However, interpretation of result can be complicated by other urease producing bacteria are present in gastric mucosa Recent treatment with antibiotics, bismuth containing compounds and proton pump inhibitors decreases density of bacteria producing urease enzyme activity, can reduce sensitivity of the Pronto dry rapid urease® (Morio *et al.,* 2004).

Culture performance on sensitivity and NPV was comparatively lower, which may be due to the fastidious nature of the bacterium and strict microaerophilic environment requirement (Yakoob, *et al.,* 2005). Only live bacterium can be propagated using culture. The bacterium viability is also known to be easily lost during transportation ahead of culture. For this study the challenge was mitigate this by using FBS for transport and promptly processing specimens for culture. Use of antibiotics by patients can also influence the outcome of culture results as it decreases the density of bacteria from the gastric mucosa. This culture findings are however not unique to this study, since even a study conducted by Selgrad (Selgrad,

2017) had similar results. Culture is imperative for antimicrobial susceptibility profiling.

Histology as used in this study would detect both dead and live bacilli in the gastric mucosa. This might explain the positive cases obtained by culture and Pronto dry rapid urease® which were negative with histology. Histology is dependent on interpersonal ability of the pathologist to morphologically identify the bacterium, which might limit the extent to which the method can be used as a 'gold standard'. However, histology has been used in method validation in a number of studies. Histology has the advantage of able to identify *H. pylori* and provide more information on the degree of inflammation and associated pathology ( Peretz *et al*.,2014).

## 5.2 Relationship between *H. pylori* virulence factors and gastro-duodenal diseases

The present study reported the significance between some virulence genes (*cagA*, *vacA* and *dupA*) of *H. pylori* and the clinical status. The *cagA* is one of the most studied virulence genes of *H. pylori* and this toxic protein has a molecular size of 120to 145 kDa and is found on the cag-PAI. The strains that carry the PAI are known to be more virulent than those lacking it (Fischer, 2011).

In this study, *cagA* was found in 46% of *H. pylori*-infected dyspeptic patients. This prevalence is lower campared to other reports who have shown *cagA*-positive *H. pylori* in more in Tunisia and Morocco where 61.6 and 61.2% *cagA* were reported respectively El Khadir *et al.* (2017). However, recent studies have reported the importance of the diversity of *cagA* in relation to gastrointestinal diseases. They have indicated that the diversity of the tyrosine *cagA* phosphorylation occurs at the unique Glu— Prol—Ile—Ala (EPIYA) motifs present in the C-terminal region, may induce abnormal proliferation and movement of gastric epithelial cells and be associated with the mortality rate of gastric cancer (Mukhopadhyay *et al.,* 2000).

Vacuolating cytotoxin (*vacA*) has been implicated to play a major role in gastroduodenal disease progression. The gene is a pore-forming toxin secreted

through an auto transporter. The mechanism of its toxigenic effect occurs by binding to the receptor of the eukaryotic cell lipid sphingomyelin. All strains of *H. pylori* comprise of the *vacA* gene, but vary in terms of their ability to produce cytotoxin (Podzorski *et al.,* 2003). Type s1 and m1 strains demonstrate more toxin activity than s2 and m2 strains (Atherton 2000). The presence of vacAs1 and *cagA* has been shown to be significantly associated with peptic ulcers (Ribeiro, 2003). This study showed *vacA* s1 to be predominant, which is similar to findings reported elsewhere such as Eastern South Africa (Tanih *et al.,* 2010). All vacA genotypes from our 91 *H. pylori*-infected dyspeptic patients contained the s1 signal region while 54% while 43% of *H. pylori* strains possessed the m1. The finding of this study are in agreement with previous reports that show a predominance of s1 the s1 and m1 were predominant among the *vacA* subtype of *H. pylori* strains (Yamaoka *et al.,* 1999).

Duodenal ulcer promoting gene (*dupA*) has been considered as a marker for the gastric ulcers by some Authors. In line with this study, some authors were unable to regard *dupA* as a predictor of duodenal ulceration in South Africa (Argent, 2007). In this study 52% *dupA* positive isolates were reported, while the lower prevalence of *dupA* (18.8%) was reported in Northern Iraq (Dadashzadeh, 2017) and as well as 39% in Iran (Miftahussurur, 2015).

Establishment of *H. pylori* infection is related to a spectrum of gastroduodenal pathologies. Although infection is usually associated with gastritis, the development of clinically significant disease appears to depend on a number of factors, including the virulence of the infecting strain, the susceptibility of the host and environmental co-factors, which varies geographically. There are studies correlating different *H. pylori* genotypes and the severity of the disease in adults and children (Arents *et al.,* 2001). This study, hence, centered on the important virulence genes causing gastroduodenal diseases among patients. The *cagA* and *vacA* have been connected with causing gastric adenocarcinoma, mucosal associated lymph tissue (MALT)-lymphoma, guodenal ulcer and peptic ulcer disease (PUD) in patients (Chang *et al*., 2018).

### 5.3 Antimicrobial resistance of *H. pylori* to commonly used conventional antibiotics

All the ninety-one isolates of *H. pylori* from patients with gastric disorders were evaluated for resistance patterns using E-test with five conventional antibiotics; clarithromycin, amoxicillin, tetracycline, Metronidazole and levofloxacin. . Resistance rate of metronidazole by E test was 98%, which is consistent with a studies in developing countries that place resistance to metronidazole at> 90% (Aboderin *et al*., 2007; Mergraud, 2013).This high resistance rate to metronidazole in developing countries could be attributed to frequent prescription and use of the drug in management of other parasitic, periodontal and gynecological infections. The abuse and misuse of this drug in developing countries could also explain this increased resistance (Pandya *et al.,* 2014).

There is a strong data indicating that eradication of *H. pylori* reduces the risk of peptic ulcer dyspepsia gastritis and the risk of gastric cancer and if it is treated early thus reducing cost of *H. pylori* associated diseases (Stollman, 2016). Appropriate selection of antibiotics regimen for treatment of *H. pylori* decreases the rate of *H. pylori* infections from occurring. This highlights the need for susceptibility testing of *H. pylori* isolates proceeding to the eradication of infection. Eradication failure of *H. pylori* associated infections is concerning by the recommended treatment regime. This could due to pre-existing antimicrobial resistant strains of *H. pylori* or new emerging resistant strains from a susceptible host. Prevalence of *H. pylori* resistant strains vary in different geographical area and increase with time in many countries. Studies from Africa indicate that *H. pylori* antimicrobial resistance rate is more than 15% for clarithromycin, 26% for metronidazole, 11% for amoxicillin and more than 15% for tetracycline (De Francesco *et al.,* 2010).

Amoxycillin is the only beta-lactam drug used to treat *H. pylori* infections, which is included in the current regimen for the treatment of *H. pylori* infections. There was significant resistance to amoxicillin by E test at 53% of the *H. pylori* isolates, which is in agreement with a study by Okimoto and Murakami in Japan that reported resistance rate of this drug at more than 35% (Okimoto & Murakami 2009). In

contrast, John Albert *et al.,* 2006 reported in an earlier study that amoxicillin resistance among *H. pylori* isolates was rare or non-existing (John Albert *et al.*, 2006). Ndip *et al*, 2008 in Cameroon reported 85.6% which is slightly higher than our finding in this study. This variation in reporting among different studies could be attributed to different prescription practices of amoxicillin antibiotic.

Resistance rate to tetracycline, which is currently used in the treatment of *H. pylori* infection as part of quadruple therapy was 2% by E-test method. The current low resistance rate of the tested clinical isolates to tetracycline (2%) shows the importance of this drug in eliminating *H. pylori* strains circulating in Kenya. However, tetracycline is not normally used in *H. pylori* eradication regimens, therefore reporting such a low resistance rate is not surprising. The present resistance rate is in agreement with previous studies Asia (2.4%), Europe (2.1%), and America (2.7%). reporting low resistance rate of *H. pylori* to tetracycline (Wolle *et al.,* 2002). However, a study showed that *H. pylori* tetracycline resistance was significantly higher in individuals from Cameroon 43.9%, Korea 8.8%, Chile 26.8%, and Africa 43.9% (De Francesco *et al.,* 2010). The differences between the resistance rates may reflect the difference in tetracycline usage between our regions.

There is dramatic development of resistance in clarithromycin resistance in this study setting (12.5%) compared to previously reported prevalence by in Kenya (Kimang'a *et al.*, 2010) where there was 100% susceptibility. This is worrying as it signifies the evolution of antibiotic-resistant *H. pylori*. However, this resistance rate is lower compared to what Ndip *et al.,* reported in Cameroon 43% (Ndip *et al.*, 2008). The resistance rates between regions could reflect the differences in clarithromycin usage between countries. Although clarithromycin resistance in this study was found to be low, more than of the isolates were found to have high MIC resistance to both clarithromycin and metronidazole, suggesting that administration of triple therapy as first line of treatment might no longer be effective production of antimicrobial and its unwarranted use in treatment of respiratory tract infections has vital role in *H. pylori* resistance to clarithromycin this region. Clarithromycin has widely been used to treat *H. pylori* infections eradication in combination with a proton pump inhibitor (PPI) with or without a secondary antibiotic.

In this study there was low resistance of isolated *H. pylori* to Levofloxacin at 8% of the total isolates. Levofloxacin has been reported as a salvage therapy with the first line treatment failure. This low resistance among *H. pylori* isolates contradicts other studies done in other developing countries where it was reported high resistance rate 20.9% among *H. pylori* isolates (Kuo *et al.,* 2017). This low resistance levels of levofloxacin could be explained by the fact that it's not used as the first line antibiotic in *H. pylori* associated infections.

Multidrug resistance of *H. pylori* was present in more than 50% of the patients. Factors associated with the multidrug resistance include increasing antibiotic practice, foregoing treatment failures, and bacterial mutations, efflux pumps (Boyanova *et al.,* 2019). Since most triple therapies generally comprise a proton pump inhibitor and two different antibiotics, synchronous resistance to two or more antibiotic agents could be a serious clinical problem, by undermining the efficacy of these treatments. This result recommends the need to always antimicrobial susceptibility tests (AST) to find the determine combination of antibiotic agents for *H. pylori* eradication.

With increased resistance to antibiotics of *H. pylori* strains, attention should focus on the choice of therapy taking in to consideration regional resistance profile and history of consumed antibiotics. The greatest challenge in the treatment of *H. pylori* associated infection is the resistance of clarithromycin and metronidazole.

### 5.4 Antimicrobial activity of *Aloe secundiflora, Bridelia micrantha and Lippia javanica*

Medicinal plants selected for this study was based on ethnobotanical information, the plant has been widely used ethnomedically to treat gastrointestinal infections (Kokwaro, 1993). The medicinal plants of *A. secudiflora, L. javanica* and *B. micrantha* were evaluated for anti-*H. pylori in-vitro* activity. Herbalists generally use water to extract active compounds from medicinal plant, because water is safe to humans and is easily available. Nevertheless, efficacious extraction of bioactive compound present in medicinal plants depends upon the type of solvents used. The total amount of crude extract obtained with the both methanol and aqueous solvents

showed that methanol was quantitatively the best solvent for extraction in all the three plants. The results of this study appear to be in agreement with others studies (Ndip *et al.*,2007; Ezekiel *et al.*, 2009) confirming methanol is a good solvent for crude extraction of phytochemical compounds from medicinal plants as it provided the highest yield in all the three medicinal plants under this study. The leaves extract of *A. secundiflora* methanol extract showed significant zone of inhibitions in all tested *H. pylori* isolates, followed by *B. micrantha* bark extracts.

The methanol extract *A. secundiflora* was the most active of all the extracts, showing activity against all the *H. pylori* isolates tested at the lowest concentration (25 mg/ml) used in the study with a MIC value that ranged from 0.19 to 0.78mg/ml. The aqueous extract also demonstrated activity but a higher concentration of the extract was required; the MIC value ranged from 0.19 to 1.56 mg/ml. These results agree with previous study where the methanol extracts demonstrated inhibitory and potent antimicrobial activities against different gastrointestinal bacterial species (Adefuye *et al.*, 2011).

The antimicrobial activity of the methanol extracts of *A. secundiflora* and *B. micrantha* may imply that methanol could be a better solvent for the extraction of anti-*H. pylori* compounds from both plants. The methanol extracts of *A. secundiflora* and *B. micrantha* appeared to have significant inhibitory activity against *H. pylori* when compared with ciprofloxacin. This is fairly outstanding bearing in mind that the antibiotics are in the purified and concentrated form whereas the medicinal plant extracts are crude and may possess both active and non-active bioactive compounds with which may accidentally of mask effectiveness of bioactive compounds over others. This is a sign that the methanol extracts of *A. secundiflora* and *B. micrantha* may contain therapeutically useful compounds against *H. pylori* infections.

The significant activity of this plant extract against *H. Pylori* in particular put this medicinal plant extracts as an important target for further purification and identification of bioactive constituents for treatment of *H. pylori* infections. The difference in antibacterial activity of these medicinal plants against *H. pylori* is possibly attributable to the variation in the quality and quantity of bioactive

secondary metabolites in the extracts of targeted morphological part. Phytochemicals compounds play an important part in plant defense against microorganism, stress as well as inter-species protections and therefore these plant compounds have been used as antimicrobial for treatment of infections. Hence, phytochemical compound screening serves as the first step in determining the varieties of potentially active compounds from medicinal plants (Harborne, 1998). In this study, *A. secundiflora B. micrantha* and *L. javanica* were confirmed to consist of phytochemical compounds. This finding corroborated with previous report on phytochemical screening of *A. secundiflora B. micrantha* and *L. javanica* (Rachuonyo *et al.*, 2016)

### 5.5 Potential bioactive compounds present in *Aloe secundiflora, Bridelia micrantha, and Lippia Javanica*

Gas chromatography mass spectrometry an important tool because of its ability to supply the qualitative and quantitative information on molecules based on their structural compositions. Gas chromatography is attached to a Mass Spectrometer (GC-MS) enables mixture of small molecules mainly organic compounds of low molecular weight (<600) to be analyzed.

GC-MS analysis identified bioactive compounds with antimicrobial activity from *A. secudiflora, L. javanica* and *B. micrantha* methanol solvent extracts. The highest number of compounds (17) was evidenced in the methanol leaf extract of *A. secundiflora* followed by *B. mirantha* bark extract. Most of the identified compounds are known to exhibit various pharmacological activities (Yue *et al.*, 2017) . In this study, the higher antimicrobial, antifungal and antioxidant activities of the methanol extract of *A. secundiflora* leaves could have been due to high number of bioactive compounds including eicosane, Phenol, dibutyl phthalate and butylated hydroxytoulene. On the other hand, Phenol, which was in this extracts, is an important aromatic compound that possesses antimicrobial, antioxidant, and anticancer activities (Firenzuoli & Gori, 2007). Hexadecane, found in the extract, is known to exhibit strong antimicrobial and anti-inflammatory activity (Radulović *et al.*, 2010). As such, butylated Hydroxytoulene and Phenol, 2,4-bis(1,1-

dimethylethyl) that act as natural antioxidants and possess various pharmacological values (Altemimi *et al.,* 2017). Tridecane, which was also in the extracts, is a good anti-inflammatory, Anti-microbial activities and anti-cancerous compound (Radulović *et al.,* 2010). Similarly, the bioactive compounds from plant extracts are known to possess anti-inflammatory, and anti-cancer activities (Endris *et al.,* 2016). The methanol extract of *B. mirantha* bark GC-MS analysis identified nine compounds, which have been reported to possess antibacterial, antioxidant and anti-inflammatory activities (Salman *et al.,* 2006, Yayli *et al.,* 2006). Key among these compounds are phenol, 2,4-bis(1,1-dimethylethyl), camphor, 9-Octadecenoic acid(Z)-, methyl ester andBis(2-ethylhexyl) Pthalate.

The GC-MS analysis shows the presence of eleven compounds in the Methanol extract of the *L. javanica*. These compounds were reported with antioxidant, antimicrobial, cancer preventive, and hypercholesterolemic activity (Harborne and Baxter, 1983). The antimicrobial antifungal mechanisms of the *L. javanica* chemical constituents may be related to their general properties of destroying the development of bacterial, fungi's cells wall and cells membranes (Isman and Machial, 2006). Among these bioactive compounds are…Phenol, 2,4 bis (1,1-dimethyl), Docosane, Hexacosane and Triacontane.

# CHAPTER SIX

# CONCLUSION AND RECOMMENDATIONS

## 6.1Conclusion

- The performance of Pronto dry rapid urease® was commendable. It outperformed culture in a number of performance measures, and is evidently useful in H. pylori detection especially where histology is untenable and antimicrobial profiling which require culturing the bacterium is needless.
- Virulence genes were rarely present in normal gut, and gastritis was realized in about half of the participants from whom *H. pylori* isolates were obtained. Presence or absence of *cagA, vacA* and *dupA* virulent genes could be used to explain the resultant gastro-duodenal diseases since they would occur in definite patterns in some and not in some of the diseases
- Tetracycline is the only conventional antibiotic for which *H. pylori* isolates did not exhibit resistance and almost all isolates of *H. pylori* were resistant to metronidazole. It is therefore evidently clear that inclusion of tetracycline and metronidazole play no or very little if any therapeutic role in the combination therapy. Future management of *H. pylori* infection is becoming grim as the bacterium still exhibited significant resistance to the other combination therapy antibiotics which include Amoxicillin, Clarithromycin and Levofloxacin.
- Extracts of *A. secundiflora, B. micrantha and L. javanica* possess inhibitory prospects against clinical isolates of *H. pylori.* Choice of solvent for crude extraction favour methanol to aqueous since extracts obtained using methanol have higher spectra of antimicrobial activity than aqueous extracts for the three medicinal plants. The differences observed in the antibacterial activities of the plants species could be due to the differences in their chemical constituents and in the mode of action of their bioactive compounds. The presence of various bio-active compounds detected after GC-MS analysis using the methanol extracts of the three medicinal plants

justifies the use of these plants for various gastrointestinal infections including *H. pylori* associated infections by herbalists.

- Methanol extracts *A. secundiflora B. mirantha* and *L javanica* possess various bio-active compounds with antimicrobial activities.

## 6.2 Recommendations

- There is need for the Ministry of Health to mainstream Pronto dry rapid urease® in the diagnosis of *H. pylori* infection in health facilities where endoscopy services are offered, particularly where histology is untenable and prompt treatment is required
- The Ministry of Health should review the combination therapy for *H.* pylori, since it's evident that these current regimens cannot be reliable to predictably achieve effective treatment for patients with *H. pylori*-associated gastro-doudenal diseases, which are often severe
- Results of this study complement the ethnobotanical use of *A. secundiflora, B. micrantha and L. javanica* in the treatment of *H. pylori* infections that need to be explored as a source of new anti-*H. pylori* agent.

- Further analysis is required for the pharmacological activity of specific compound of *A. secundiflora B. mirantha* and *L javanica.* Hence, there is need to conduct more studies aimed at purifying crude extracts identify and characterize the active compounds in the tested plants, which may serve as novel compounds for development of new and more effective antimicrobial drugs.

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# APPENDICES

## Appendix I: Informed Consent form

**Title: *Helicobacter pylori* Diagnosis, Virulence Genes, Resistance to Conventional antibiotics and Antimicrobial activity of selected Medicinal plants on Isolates obtained from Patients undergoing Endoscopy at Aga Khan University Hospital, Kenya**

**Principal investigator: Stephen Njoroge**

You are being asked to be part of a research study. The study will be carried out at The Aga Khan University Hospital. Please take your time deciding whether to join the study. Carefully consider the following information and ask any questions you may have.

You will be requested to give permission for collection of gastric biopsies sample. This sample shall be analyzed to detect presence of *Helicobacter pylori***.** Research findings shall be made available. You will also be asked to complete a short questionnaire or the questionnaire will be read for you and your response shall be recorded. Questions will include mainly details about your demographic background. Please take your time deciding whether to join the study. Carefully consider the following information and ask the study investigator (Stephen Njoroge) any questions you may have. If you agree to be in the study, you will be asked to sign on this consent form, and will be given a copy to keep.

**Participant Selection**

Participation in this research study is voluntary and you have the right to refuse to participate or withdraw.

**Risks/Discomforts**

There are no risks for participation in this study. However, you may feel physical discomfort during collection of gastric biopsy sample.

**Benefits**

If you are found to be suffering from *H. pylori* infection, you will be given treatment accordingly. **Confidentiality**

All information provided will remain confidential and will only be reported as group data with no identifying information. All data will be kept in a secure location and only those directly involved with the research will have access to them.

**Questions about the Research**

If you have questions regarding this study, you may contact:

Stephen Njoroge

PO Box 65761- 00607

Nairobi.

Tel. Mob: 0721 412674

Email: dsznjoroge@gmail.com

OR,

The Secretary, Kenyatta National Hospital/University of Nairobi- ERC

PO Box 20723 – 00202

Nairobi.

Tel: 726300-9

Email: uonknh_erc@uonbi.ac.ke

**Appendix II: Assent Form for persons aged below 18 years and are unable to understand the purpose of the study**

***Helicobacter pylori* Diagnosis, Virulence Genes, Resistance to Conventional antibiotics and Antimicrobial activity of selected Medicinal plants on Isolates obtained from Patients undergoing Endoscopy at Aga Khan University Hospital, Kenya**

I, being a patient/guardian _____ (name of patient/child) have had the research explained to me. I have understood all that has been read and had my questions answered satisfactorily. I understand that I can change my mind at any stage and it will not affect the benefits due to me/my child:

I agree to allow myself/my child to take part in this research.

**Patient/guardian's signature**: _____ Date _____

**Patient/guardian's name: _____**Time _____

(Please print name)

**I certify that I have followed all the study specific procedures in the SOP for obtaining informed consent.**

**Designee/investigator's signature: _____** Date_____

**Designee/investigator's name: _____** Time _____

**Only necessary if the Patient/guardian cannot read:**

I *attest that the information concerning this research was accurately explained to and apparently understood by the Patient/guardian and that informed consent was freely given by the patient/guardian.

**Witness' signature**: _____ Date_____

**Witness' name:** _____ Time

---

*A witness is a person who is independent from the trial or a member of staff who was not involved in gaining the consent.

Thumbprint of the parent as named above if they cannot write.

**Appendix III: Questionnaire on Demographic Data**

Patient ID: _____ Date: _____/_____/_____

dd/ mm /yyyy

1. How old are you? _____ Years

- 10-19 years
- 20 - 29 years
- 30 – 39 years
- 40 – 49 years
- 50 – 59 years
- >60 years

**2. Gender**

Male ☐

Female ☐

3. **Race/ethnicity**

How do you describe yourself? (please check the one option that best describes you)

- Asian
- Black African
- White

4. **Marital status**

- Married
- Divorced
- Widowed
- Separated
- Never been married

- A member of an unmarried couple

5. **Employment status**

- Employed for wages
- Self-employed
- Out of work for more than 1 year
- Out of work for less than 1 year
- A homemaker
- A student
- Retired
- Unable to work

6. **Education completed**

-What is the highest grade or year of school you completed?

- Never attended school or only attended kindergarten
- Primary Education Class 8
- Secondary Education
- College
- University

7. **Family size**

- How many children live in your household who are...?

- Less than 5 years old?
- 5 through 12 years old?
- 13 through 17 years old

8. How would you describe your current work situation?

- Unemployed

- Housewife
- CSW
- Casual worker

**Criteria for enrolment:**

- Read/understood/signed consent
- Past or present history of dyspepsia and have been referred for endoscopy presentation
- Have not been on any antibiotics PP1 or H2 blockers within 4 weeks prior to

**Appendix IV: Abstracts for published journal**

*29(12): 1-7, 2019; Article no.JAMMR.49477*
*ISSN: 2456-8899*
*(Past name: British Journal of Medicine and Medical Research, Past ISSN: 2231-0614, NLM ID: 101570965)*

# Comparing Diagnostic Performance of Pronto Dry Rapid Urease® and Culture to Histopathology among Endoscopy Patients at the Aga Khan University Hospital, Nairobi-Kenya

**Stephen Njoroge[1,3*], Catherine Mwangi[1], Kimang'a Nyerere[1], Gunturu Revathi[2], Smita Devani[2], Allan Rajula[2], Rose Kamenwa[2], Nyamongo Onkoba[3] and Fredrick Odhiambo[4]**

*[1]Jomo Kenyatta University of Agriculture and Technology, P.O.Box 62000-00200, Nairobi, Kenya.*
*[2]Aga Khan Hospital University, P.O.Box 37002-00100, Nairobi, Kenya.*
*[3]Technical University of Kenya, P.O.Box 52428, Nairobi, Kenya.*
*[4]National Public Health Laboratory, P.O.Box 20750-00200, Nairobi, Kenya.*

***Authors' contributions***

*This work was carried out in collaboration among all authors. Author SN designed the study, collected data, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author CM performed the laboratory component of the study and managed the analyses of the study. Authors KN, GR, SD, AR, RK, NO and FO coordinated the study and managed the literature searches. All authors read and approved the final manuscript.*

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*Original Research Article*

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## ABSTRACT

**Aim:** This study sought to evaluate Pronto dry rapid urease® diagnostic test and compare its performance with culture.
**Study Design:** Cross-sectional study.
**Place and Duration:** From September 2017 to July 2018, across-sectional study was conducted at the Aga Khan University Hospital.

East African Medical Journal Vol. 96 No. 3 March 2019

ANTIMICROBIAL ACTIVITY OF *ALOE SECUNDIFLORA* AGAINST CLINICAL ISOLATES OF *HELICOBACTER PYLORI*

Stephen Njoroge, Department of Medical Microbiology, Jomo Kenyatta University of Agriculture & Technology, P. O. Box 62000-00200, Nairobi, Kenya, Catherine Mwangi, Department of Medical Microbiology, Jomo Kenyatta University of Agriculture & Technology, P. O. Box 62000-00200, Nairobi, Kenya, Kimang'a Nyerere, Department of Medical Microbiology, Jomo Kenyatta University of Agriculture & Technology, P. O. Box 62000-00200, Nairobi, Kenya, Fredrick Odhiambo, Department of Monitoring and Evaluation, National Public Health Laboratory, P. O. Box 20750-00200, Nairobi, Kenya, Justin Tirimba, Department of Biomedical Science, Technical University of Kenya, P. O. Box 52428, Nairobi, Kenya, Gunturu Revathi, Department of Pathology, Aga Khan Hospital University, P. O. Box 37002-00100, Nairobi, Kenya.

# ANTIMICROBIAL ACTIVITY OF ALOE SECUNDIFLORA AGAINST CLINICAL ISOLATES OF HELICOBACTER PYLORI

S. Njoroge, C. Mwangi, K. Nyerere, F. Odhiambo, J. Tirimba and G. Revathi

## ABSTRACT

*Objective:* To evaluate the antimicrobial activity of methanol and aqueous crude extracts of *Aloe secundiflora (A. secundiflora)* against clinical isolates of *Helicobacter pylori* which is the most prevalent cause of gastrointestinal infections.

*Methods:* The agar diffusion method was used to determine the susceptibility of 23 clinical isolates of *H. pylori* to the methanol and aqueous crude extracts of *A. secundiflora*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) was determined by micro well dilution method. The presence of secondary metabolites was determined. The potential bioactive compounds were identified by Gas Chromatography-Mass Spectrometry (GC-MS).

*Results:* Both methanol and aqueous crude extracts of *A. secundiflora* demonstrated antimicrobial activity with highest mean zone diameter of 28 ± 0.47 mm for methanol extracts and 11±0.81mm for aqueous extracts. Lowest recorded MIC and MBC was between 0.19-0.39mg/ml for methanol and aqueous extracts respectively. There was no statistically significance difference ($p>0.05$) in potency of the extracts in different isolates of *H. pylori* tested both in MIC and MBC. Phytochemical screening of methanol and aqueous crude extracts of *A. secundiflora* showed presence of secondary metabolites such as alkaloids, saponin, tannins, flavonoids, and steroids. A total of 8 bioactive compounds were identified GC-MS analysis.

**Methodology:** Patients attending endoscopy unit at the hospital were randomly sampled to provide gastric biopsy specimen. One specimen was tested for presence or absence of H. pylori using Pronto dry rapid urease® test and another specimen subjected to in vitro culture test which were then compared with histology reference results. Test validity and reliability was determined using Graph Pad Prism v5.01.

**Results:** Of 274 study specimens, 121(44%) were positive for histology. Ninety-one (33%) of the study specimen were positive for culture compared to 147(54%) for Pronto dry rapid urease®. Pronto dry rapid urease® test had sensitivity of 100% (97.5%-100%) against 73.6% (64.8%-81.3%) for culture. Specificity was 96.1% (91.1%-98.7%) for Pronto dry rapid urease® compared to 35.3% (95% CI 24.1%-47.8%) for culture. Positive predictive value was 96.7% (92.5-98.9%) for Pronto dry rapid urease® compared to 97.8% (92.3%-99.7%) for culture. Negative predictive value was 100% (97%-100%) for Pronto dry rapid urease® against 82.5% (76.2%-87.7%) for culture. There was significant difference between both Pronto dry rapid urease® and culture test performance with histology in all validity measures, P< 0.001. On the other hand, there was no significant difference between Pronto dry rapid urease® and culture in all validity measures due to overlapping confidence intervals.

**Conclusion:** Pronto dry rapid urease® out-performed culture in sensitivity and NPV. It would be the method of choice in H. pylori detection where histology is untenable and antimicrobial profiling which require culturing the bacterium is needless.

*Keywords: Helicobacter pylori; diagnosis; pronto dry rapid® urease test; biopsy; rapid urease; diagnostic tests.*

## 1. BACKGROUND

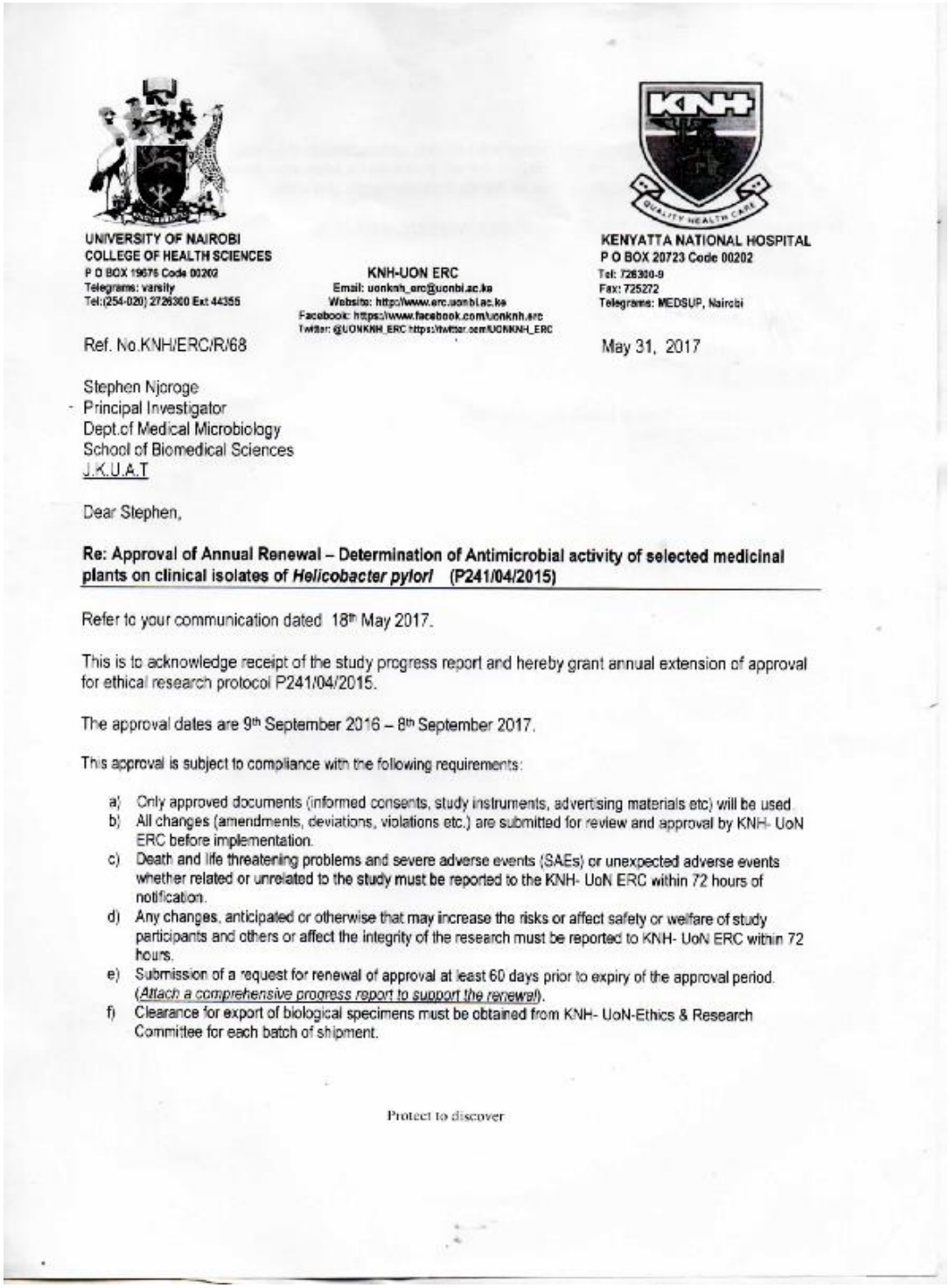
Approximately 50% of the world population is known to be infected with *Helicobacter pylori*. *Helicobacter pylori* infection is the major cause of gastric cancer, which accounts for >720000 annual deaths globally [1]. It is also the primary causes of other upper gastrointestinal diseases, including dyspepsia, peptic ulcer diseases, heartburn, gastroesophageal reflux disease and even malignant transformation [2]. Prevalence of the infection in industrialized countries seems to be decreasing, while in the developing countries it is still high, with Fig. of up to 90% being reported[3]. In Kenya prevalence of *H. pylori* is about 55% in adult and >70% children [4]. The burden of the named *H. pylori*-related disease in Kenya is unknown. *Helicobacter pylori* is transmitted from person-to-person through oral–oral and faecal–oral routes [5]. Inadequacies in sanitation practices and wanting sewerage systems common in low social economic populations are associated with *H. pylori* infection [3].

and disadvantages [6]. Invasive diagnostic techniques requiring endoscopy are usually preferred in patients with a higher prevalence of gastrointestinal disorders, as well as for their superiority in analyzing the severity of gastritis and detecting premalignant lesions [7].

Histopathological determination of gastrointestinal endoscopy is the most commonly performed invasive test and is particularly sensitive for revealing peptic ulcers [8]. However, expert pathologists are required for an accurate examination of the samples. It is also intense and requires a well-equipped histopathology laboratory for tissue processing.

Bacterial cultivation is another invasive technique available, though not commonly used in clinical diagnosis of *H. pylori*. Cultivation of *H. pylori* through this method requires specific selective culture agar and specific atmospheric conditions that hinder its routine use in the laboratory as a diagnostic method. Biopsy cultures are the most widely used methods for anti- microbial

## Appendix V: Ethical clearance



UNIVERSITY OF NAIROBI
COLLEGE OF HEALTH SCIENCES
P O BOX 19676 Code 00202
Telegrams: varsity
Tel:(254-020) 2726300 Ext 44355

KNH-UON ERC
Email: uonknh_erc@uonbi.ac.ke
Website: http://www.erc.uonbi.ac.ke
Facebook: https://www.facebook.com/uonknh.erc
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KENYATTA NATIONAL HOSPITAL
P O BOX 20723 Code 00202
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP, Nairobi

Ref. No.KNH/ERC/R/68

May 31, 2017

Stephen Njoroge
Principal Investigator
Dept.of Medical Microbiology
School of Biomedical Sciences
J.K.U.A.T

Dear Stephen,

**Re: Approval of Annual Renewal – Determination of Antimicrobial activity of selected medicinal plants on clinical isolates of *Helicobacter pylori* (P241/04/2015)**

Refer to your communication dated 18th May 2017.

This is to acknowledge receipt of the study progress report and hereby grant annual extension of approval for ethical research protocol P241/04/2015.

The approval dates are 9th September 2016 – 8th September 2017.

This approval is subject to compliance with the following requirements:

a) Only approved documents (informed consents, study instruments, advertising materials etc) will be used.
b) All changes (amendments, deviations, violations etc.) are submitted for review and approval by KNH- UoN ERC before implementation.
c) Death and life threatening problems and severe adverse events (SAEs) or unexpected adverse events whether related or unrelated to the study must be reported to the KNH- UoN ERC within 72 hours of notification.
d) Any changes, anticipated or otherwise that may increase the risks or affect safety or welfare of study participants and others or affect the integrity of the research must be reported to KNH- UoN ERC within 72 hours.
e) Submission of a request for renewal of approval at least 60 days prior to expiry of the approval period. (*Attach a comprehensive progress report to support the renewal*).
f) Clearance for export of biological specimens must be obtained from KNH- UoN-Ethics & Research Committee for each batch of shipment.

Protect to discover

g) Submission of an *executive summary* report within 90 days upon completion of the study
This information will form part of the data base that will be consulted in future when processing related research studies so as to minimize chances of study duplication and/or plagiarism.

For more details consult the KNH- UoN ERC website **http://www.erc.uonbi.ac.ke**

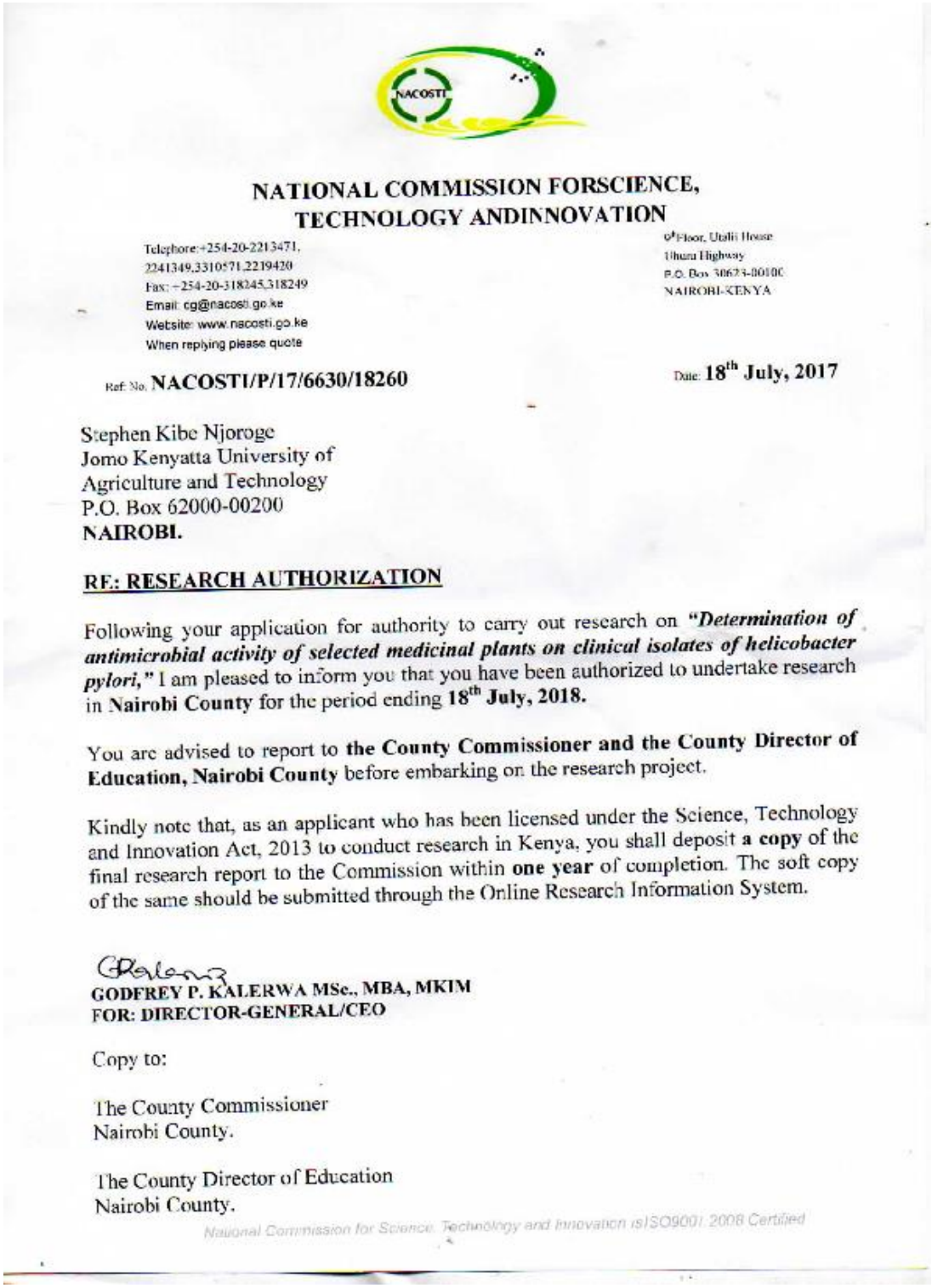
Yours sincerely,

**PROF. A.N. GUANTAI**
**CHAIR, KNH-UON ERC**

c.c. The Principal, College of Health Sciences, UoN
The Director CS, KNH

Protect to discover

## Appendix VI: NACOSTI Permit



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Email: cg@nacosti.go.ke
Website: www.nacosti.go.ke
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9th Floor, Utalii House
Uhuru Highway
P.O. Box 30623-00100
NAIROBI-KENYA

Ref: No. **NACOSTI/P/17/6630/18260**

Date: **18th July, 2017**

Stephen Kibe Njoroge
Jomo Kenyatta University of
Agriculture and Technology
P.O. Box 62000-00200
**NAIROBI.**

**RE: RESEARCH AUTHORIZATION**

Following your application for authority to carry out research on ***"Determination of antimicrobial activity of selected medicinal plants on clinical isolates of helicobacter pylori,"*** I am pleased to inform you that you have been authorized to undertake research in **Nairobi County** for the period ending **18th July, 2018.**

You are advised to report to **the County Commissioner and the County Director of Education, Nairobi County** before embarking on the research project.

Kindly note that, as an applicant who has been licensed under the Science, Technology and Innovation Act, 2013 to conduct research in Kenya, you shall deposit **a copy** of the final research report to the Commission within **one year** of completion. The soft copy of the same should be submitted through the Online Research Information System.

**GODFREY P. KALERWA MSc., MBA, MKIM**
**FOR: DIRECTOR-GENERAL/CEO**

Copy to:

The County Commissioner
Nairobi County.

The County Director of Education
Nairobi County.

National Commission for Science, Technology and Innovation is ISO9001: 2008 Certified