In vitro bioactivity of crude extracts of *Lippia javanica* on clinical isolates of *Helicobacter pylori*: Preliminary phytochemical screening.

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A dissertation Submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Biochemistry and Microbiology

University of Fort Hare

September 2010

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DECLARATION

I declare that this dissertation submitted to the University of Fort Hare for obtaining the degree of Master of Science (Microbiology) and the work contained herein is original unless cited and has not been submitted at any other university for any degree.

Name……..

Signature**.**……..

Supervisor's signature……

Date……

ACKNOWLEDGMENTS

My thanks first and foremost to God almighty, who has been the source of my energy through out my studies; without Him none of this would be possible. I thank my supervisor, Professor R.N. Ndip for his supervision, encouragement, version, editing, patience and love; surely without him this study would not have been achieved.

My special thanks to my family, sisters, cousins, and my grandmother, Gladys Vuyiswa Nkomo. My mother in particular, Nonceba Diagracia Nkomo, Dr Bongiwe Nkomo, Vuyo Nkomo, Lisakhanya Nkomo as well as Mzingisi Magwanya and the entire family who always stood by me faithfully and unconditionally with their ever-lasting love and support throughout this period. I would also like to thank the University of Fort Hare and the Department of Biochemistry and Microbiology for allowing me to do this study; I would not have the knowledge that I have without them.

My sincere gratitude to the National Research Foundation for financial support on the study. I would also like to thank my colleagues of the Microbial Pathogenecity and Molecular Epidemiology Research Group (MP&MERG) for the time and support they gave me during the course of study.

ABSTRACT

Helicobacter pylori classified as a class 1 carcinogen is a common human pathogen implicated in certain gastrointestinal diseases. *Helicobacter pylori* infection is acquired mainly in childhood, especially in developing countries. *H. pylori* infection causes peptic ulcer, duodenitis, gastritis and cancer. The growing resistance of *H. pylori* to antibiotics used in its treatment as well as other innate limitations of the triple therapy has necessitated a search for alternative treatment from natural sources which could be readily available, less cost effective. The antimicrobial activity of solvents (acetone, ethanol, methanol, chloroform and water) crude extracts of *Lippia javanica* were investigated against 31 *H. pylori* strains by the agar well diffusion technique. The minimum inhibitory concentration (MIC) was determined by spectrophotometric analysis at 620 nm using the broth micro dilution method and the rate of kill by broth dilution method. Phytochemical analysis was also performed. *H. pylori* standard strain NCTC 11638 was included as a positive control. Metronidazole and amoxicillin were used as positive control antibiotics. The ANOVA test was used to analyze the results using SPSS version 17.0. The strains were inhibited by all the extracts with inhibition zones of diameter ranging from 0-36 mm and 0-35 mm for the control antibiotic, clarithromycin. The MIC_{90} ranged from 0.039- 0.625 mg/mL for acetone; 0.039-1.25mg/mL for methanol, 0.00195-0.313 mg/mL for ethanol; 0.01975-2.5 mg/mL for metronidazole and 0.0048-2.5 mg/mL for amoxicillin. Acetone extract completely inhibited strain PE369C at MIC (0.1 mg/mL) and 2× MIC (0.2 mg/mL) in 18h and at ½× MIC (0.05 mg/mL) in 36h. Strain PE466C was completely inhibited at 4× MIC in 72h. Phytochemical analysis revealed the presence of flavonoids, saponins, tannins, steroids and alkaloids. The results indicate that the extracts of the leaves of *L. javanica* may contain compounds with anti-*H. pylori* activity and merits further study to identify the compounds.

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CHAPTER ONE

1.0 INTRODUCTION

Helicobacter pylori is a helical shaped, Gram-negative, microaerophilic bacterium (2-4µm long with diameter of 0.5µm) that infects the stomach and duodenum (Sasaki *et al.*, 1999). Infection with the organism may cause peptic ulcers, gastritis, duodenitis, and gastric cancer (Figueiroa *et al*.; 2002; Figueiredo *et al*., 2002; Ahmad *et al.*, 2009). *H. pylori* chronically infects billions of people worldwide, is one of the most common chronic bacterial pathogens of humans and genetically diverse bacterial species. *H. pylori* infection with other bacteria is unclear however, *H. pylori* genes increases the risk of disease development, including those genes that encode exotoxins. The disease seems to result from an interaction between the bacterium, the host and the environment. Infections have been reported to be higher in the developing than in developed countries, especially in Africa (Rothenbacher and Brenner, 2003; Ndip *et al.,* 2007; Tanih *et al.,* 2010). Interestingly, the organism has been reported to be prevalent in South Africa. For example, Pelser *et al.* (1997) documented a high prevalence of *H. pylori* antibodies (67-84%) in children in Bloemfontein, while Mosane *et al*. (2004) also documented *H. pylori* IgG antibodies in South African mothers and their children.

H. pylori infection is often treated with a triple therapy, such as metronidazole (Mtz), clarithromycin or amoxicillin and a proton pump inhibitor (Biradar *et al*., 2008). Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high risk populations (Ali *et al*., 1991; Vaezi *et al*., 2000; Tanih *et al*., 2009). However, resistance of the organism to antibiotics is a growing global concern which needs public health attention especially in developing countries. In Africa, an outsized amount of people die daily of avertable and treatable diseases because there is lack of basic health care. Even with the massive

development in health care made in the last half century, infectious diseases still account for 25% of mortality globally and 45% in under-developed countries (Diallo and Zougran, 2008; Doughari and Manzara, 2008). Anti-infective drugs are crucially imperative in reducing the worldwide burden of infectious diseases.

Medicinal plants are sources of enormous quantity of chemical substances which are able to initiate biological activities. These phytochemicals can enhance the treatment of human diseases. For example; the active principles of many drugs obtained from plants are secondary metabolites (Kubmarawa *et al.,* 2007). Therefore, basic phytochemical investigation of plant extracts for their major phytoconstituents is vital. Different solvent extracts of plants have different phytochemical properties (Hassan *et al.,* 2007). Many naturally occurring compounds found in medicinal plant extracts have also been shown to posses antimicrobial activities that inhibit the growth of microorganisms (Katsvana and Chigwaza, 2004; Ndip *et al.,* 2008).

Scientific investigations of medicinal plants used in folk medicine have attracted increased attention in the medical world, in a bid of finding solutions to the problems of multiple resistances to the existing synthetic antimicrobials. Most of the synthetic antibiotics now available in the market have major setback due to the accompanying side effects on the patients and resistance developed by the pathogenic microorganisms against these drugs. Thus, there is a need to search for new and more potent antimicrobial compounds of natural origin, especially from plants to overcome this problem.

Lippia javanica is widely distributed throughout South Africa where it is used extensively in traditional herbal preparations, and commonly used as a decongestant for colds and coughs as well as diarrhoea (Viljoen *et al.,* 2005). Its use has excelled in the treatment of respiratory aliments traditionally, which may either be fungal or bacterial as well as the treatment of

Gram-negative and yeast borne respiratory ailments (Viljoen *et al.,* 2005). However, some studies have shown that acetone and chloroform extracts of *L. javanica* are not active against *E. coli* and other bacteria (Samie *et al*., 2005). The acetone and methanol extracts of *L. javanica* were found to be active against most bacteria including *S. aureus, B. subtilis, P. mirabilis and S. marcescens* (Samie *et al.,* 2005). Thus, the present study sought to evaluate the antimicrobial activity of *L. javanica* in a bid to identify active compounds of the plant that could be a source of potent antimicrobials.

PROBLEM STATEMENT

Several studies have related a high prevalence of *H. pylori* to low-economic status, a situation common in the Eastern Cape Province in particular and other areas of the country in general. In fact, *H. pylori* infections have been shown to have a high prevalence in South Africa, being present in both asymptomatic and symptomatic individuals, as expected in developing countries (Pelser *et al*., 1997; Mosane *et al*., 2004; Samie *et al.,* 2006; Dube *et al*., 2009).

H. pylori infection is treated with potent combination therapies; a proton pump inhibitor and two antibiotics. These have a success rate of 80-90%, but problems including high levels of antibiotic resistance, undesirable side effects, poor patient compliance, are associated with significant levels of treatment failure and contraindications for some patients. In addition, the cost of combination therapy is also high. Some of these antibiotics are at times not readily available in rural areas thus compounding the problem of patients. Studies on anti-*H. pylori* activity of medicinal plants are lacking in South Africa. To the best of our knowledge, the plants has not been evaluated for its antimicrobial activity on clinical isolates of *H. pylori* circulating in South Africa, especially so when these organisms are known to exhibit profound heterogeneity.

HYPOTHESIS

Extracts if *L. javanica* can provide potent and affordable antibacterial agents for the treatment of *H. pylori.*

OVERALL OBJECTIVE

To assess the antimicrobial potential of extracts of *Lippia javanica* against *H. pylori*

Specific objectives

The specific objectives of this study are to:

1. Screen the plant acetone, chloroform, ethanol, methanol, ethyl-acetate or water extracts for anti-*H. pylori* activity.
2. Determine the minimum inhibitory concentration.
3. Determine the rate of kill.
4. Identify potential active compounds in the extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction

In developing countries a number of people die on a daily basis due to preventable and curable infectious diseases because there is lack of basic health care and financial support (Mulu *et al*., 2005). Although there is substantial management of infections in developing countries, infectious diseases still account for more than 13 million deaths per year globally and are continuing to be the major concern in health institutions. One in every two deaths in developing countries is caused by infectious diseases (Diallo and Zougran, 2008; Doughari and Manzara, 2008). However, for centuries bacteria have been known to colonise the stomach, yet known to be contaminants digested with food in patients presenting with gastric and stomach ulcers (Warren and Marshal, 1983; Bizzozero, 1983; Williamson, 2001).

Nonetheless, scientists continued to investigate *Camphylobacter pyloridis*, being difficult to culture. Warren and Marshall (1983), came up with a conclusive suggestion after successfully isolated and cultured the spiral species; that most of the stomach ulcers and gastritis caused by infection were due to a bacterium which was later known as *H. pylori* (Warren and Marshal, 1983). Self-ingestion experiments performed by Marshall (Marshall *et al.,* 1985) and later on volunteers (Morris *et al*., 1991) demonstrated that these bacteria can colonize the human stomach, thereby inducing inflammation of the gastric mucosa (Blaser and Atherton, 2004). These findings suggest that *H. pylori* has its primary reservoir in the stomach of humans and animals.

After self-ingestion, Marshal developed symptoms that were associated with persistent gastritis, this was determined after sequential therapy with antibiotics including doxycycline

and bismuth subsalicyclate. However, this data inspired further investigation which documented that gastric colonization with *H. pylori* can lead to various upper gastrointestinal disorders namely: peptic ulcer disease, gastric cancer, chronic gastritis, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma. Robin Warren and Barry Marshal were awarded the 2005 Nobel Prize in Physiology/Medicine for the discovery of *H. pylori* and its effects on gastritis and peptic ulcer disease. According to the findings by Marshal and Warren, this brought an insight to the major clinical impact considering the management of infectious diseases and persistence of this pathogen in an environment which for some time was thought to be sterile and marks within reach the pathogenesis of chronic diseases.

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). The organism is known for its helical or rod shape, microaerophilic, Gram negative, flagellated (2-6 flagella) bacterium, 2-4µm long with a diameter of 0.5µm; it is a slow growing organism with a low DNA composition and non fermentative metabolism (Fritz *et al*., 2006).

A growing interest on *Helicobacter* has been noted due to an increase of peer-reviewed publications over the past few years. Studies have shown the bacteria's morphology, decreasing prevalence in the Western world, and other important facts about *H. pylori* although its transmission route is still poorly understood. *H. pylori* is still known to be prevalent in underdeveloped than in developed countries. 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 less side effects to eradicate *H. pylori* infections.

2.2 Morphology

H. pylori is a pleomorphic, microaerophillic spiral Gram-negative motile rod bacterium which has been recognised as the causative factor of peptic ulcer disease, and an etiologic agent in the development of gastric cancer (Hoffman *et al*., 2004; Yang *et al*., 2005; Tiwari *et al.*, 2005; Njume *et al*., 2009). *H. pylori* exhibits four to six unipolar sheathed flagella, which has led the bacterium to be adapted to live and survive in an acid environment (Kusters *et al*., 2006) of the stomach in humans resulting in diseases or infections (Modena *et al*., 2007). However, the organism lacks fimbrial adhesions, differentiating the bacterium from other pathogens of the gastrointestinal tract. Nonetheless, the flagella confer mortility, thus allows rapid movement in viscous solutions including mucus layer found in the gastric epithelial cells (O'toole *et al*., 2000).

H. pylori possess five major outer membrane protein (OMP) families (Kusters *et al.*, 2006). The largest family includes putative adhesins. The other four families include porins, iron transporters, flagellum-associated proteins and proteins of unknown function. Like other typical Gram-negative bacterium, the outer membrane of *H. pylori* consists of phospholipids and lipopolysaccharide (LPS). The O antigen of LPS may be fucosylated and mimic Lewis blood group antigens found on the gastric epithelium (Sherif *et al*., 2004; Kusters *et al.*,

2006). The outer membrane also contains cholesterol glucosides, which are found in few other bacteria (Kusters *et al.,* 2006). Gram-negative bacteria including *H. pylori,* and mycobacteria both possess thick outer membranes that are highly hydrophobic, providing these organisms with a permeability barrier especially towards hydrophilic compounds such as macrolide antibiotics like erythromycin. This in part explains the greater resistance observed by Gram-negative bacteria as opposed to Gram-positive organisms (Cooper *et al*., 2002; Starvi *et al*., 2007).

2.3 Pathogenisis and clinical manifestation

H. pylori cells characteristically attach to surface mucous cells and colonize the mucous gel layer covering the gastric mucosa. *H. pylori* infection increases proliferation activity of gastric epithelial cells without adhering to the proliferating epithelial cells which are localised around the neck of the normal gastric mucosa (Masayoshi *et al.,* 2005). *H. pylori* infection is probably one of the most common bacterial infections worldwide (Sherif *et al*., 2004; Tiwari *et al*., 2005). The organism has also been documented as one of the most genetically diverse bacterial species and its infection persists for life with inflammation of the gastric mucosa leading to a wide variety of upper gastrointestinal tract diseases such as atrophic gastritis, gastric/duodenal ulcer, gastric cancer, gastric MALT lymphoma, and hyper-plasmic gastric polyps (Tanih *et al*., 2010; Asaka *et al*., 2010). Approximately 20% of persons infected with *H. pylori* develop related disorders during their life time (Hoffman *et al*., 2004). Irrespective of their histology, most gastric cancers arise from mucosa infected *H. pylori,* and these tumours vary rarely arise from gastric mucosa without inflammation which therefore, makes *H. pylori* to be considered as the contributing factor that is strongly related to the development of gastric cancer (Asaka *et al*., 2010).

2.3.1 Gastritis

H. pylori is genetically versatile, diverse and adapt readily to stress conditions such as gastric acid (Konturek *et al*., 2000). Their ability to adhere, invade, evade host defences and cause tissue damage, is prominently due to their production of colonization and virulence factors. The bacterium has flagella and moves through the stomach lumen and drills into the mucoid lining of the stomach (Scheiman and Cutler, 1999; Ottemann and Lowenthal, 2002; Michael and John, 2006). Many bacteria can be found deep in the mucus, which is continuously secreted by mucous cells and removed on the luminal side. To avoid being carried into the lumen, *H. pylori* senses the pH gradient within the mucus layer by chemotaxis and swims away from the acidic contents of the lumen towards the more neutral pH environment of the epithelial cell surface (Falsafi *et al*., 2005; Asaka *et al.*, 2010).

It produces adhesins (Petersen and Krogfelt, 2003) which binds to membrane-associated lipids and carbohydrates that assists the bacterium to remain on epithelial cells. On the other hand, a huge amount of urease is produced which breaks down urea to carbon dioxide and ammonia that is converted into ammonium ion by absorbing hydrogen from water upon its breakdown into hydrogen and hydroxyl ions (Mobley *et al*., 1995; Ilver *et al.*, 1998). The essential role of urease as a virulence factor is shown by the fact that urease-defective *H. pylori* mutants cannot colonize the stomach, thus the survival of *H. pylori* in the acidic environment is dependent on urease, and would eventually die without the enzyme (Smoot, 1997; Nariman *et al*., 2004; Tanih *et al*., 2009). However, urea is toxic to the bacterium at neutral pH because a hostile alkaline environment is generated (McNamara and El-Omar, 2008).

Colonization of the stomach by *H. pylori* results in chronic gastritis, resulting in an inflammation of the stomach lining. The severity of the inflammation is likely to underlie *H. pylori*-related diseases (Perez-Perez *et al*., 2000; Ndip *et al*., 2008; Dube *et al*., 2009).

Duodenal and stomach ulcers are mainly caused by an inflammation thus, allowing the acid and pepsin in the stomach lumen to engulf the mechanisms that safe guards the stomach and duodenal mucosa from these caustic substances. The type of ulcer that develops depends on the location of chronic gastritis, which occurs at the site of *H. pylori* colonization (Hoffman *et al.,* 2004; Asaka *et al*., 2010). The acidity within the stomach lumen affects the colonization pattern of *H. pylori* and therefore ultimately determines whether a duodenal or gastric ulcer will form. In people producing large amounts of acid, *H. pylori* colonizes the antrum of the stomach to avoid the acid-secreting parietal cells located in the corpus of the stomach (Kato *et al*., 2007; Tanih *et al*., 2009).

The inflammatory response to the bacteria induces G-cells in the antrum to secrete the hormone gastrin, which travels through the bloodstream to the corpus (Uemura *et al*., 2001; Nasu *et al*., 2005). Gastrin stimulates the parietal cells in the corpus to secrete even more acid into the stomach lumen. Chronically increased gastrin levels eventually cause the number of parietal cells to also increase, further escalating the amount of acid secreted (Hoffman *et al*., 2004). The increased acid load damages the duodenum, and ulceration may eventually result. In contrast, gastric ulcers are often associated with normal or reduced gastric acid production, suggesting that the mechanisms that protect the gastric mucosa are defective (Boyanova *et al*., 2000). In these patients, *H. pylori* can also colonize the corpus of the stomach, where the acid-secreting parietal cells are located. However chronic inflammation induced by the bacteria causes further reduction of acid production and, eventually, atrophy of the stomach lining, which may lead to gastric ulcer and increases the risk for stomach cancer (Asaka *et al*., 2010).

2.3.2 Gastric cancer

Sero-invasion rates ranging from 5%-10% in infected persons with *H. pylori* have been reported to be spontaneous as the majority of individuals remain asymptomatic (Lacy *et al*., 2001; Wong *et al*., 2004). The progression of atrophic gastritis in infected patients often begins with chronic superficial gastritis to cause disease. This may be due to development of gastric carcinoma resulting from the cellular cascade. In patients with severe, multifocal atrophic gastritis, the data shows a 90-fold increase in rates of gastric carcinoma (Occhialini *et al*., 2000; Hoffman *et al*., 2004; Fukase *et al*., 2008). The formation of the tumour involves DNA damage induced by different cytokines and free radicals released in the setting of chronic inflammation in susceptible persons (Kato *et al*., 2005; Nasu *et al*., 2005; McClain *et al*., 2009).

H. pylori is linked with the progression and development of adenocarcinoma of the antrum and body of the stomach including: gastric mucosa associated lymphoid tissue lymphomas (MALT) (Parkinson *et al*., 2000; Hoffman *et al.,* 2004; Masayoshi *et al*., 2005; Argent *et al*., 2008). On the other hand, the organism stimulates lymphocytic infiltration of the mucosal stroma which acts as a focus for cellular alteration and proliferation, eventually resulting in neoplastic alteration to lymphoma (Fukase *et al*., 2008). According to Morgner *et al.* (2000), it has been documented that deterioration of low-grade gastric MALT lymphoma can be accomplished in 70% to 90% of patients with eradication of *H. pylori* infection. An ultrasound endoscopic investigation revealed an invaluable grade of MALT lymphoma and predicted the effectiveness of treating *H. pylori* infection to acquire regression of the lymphoma (Argent *et al.,* 2008).

2.3.3 Peptic ulcer

Previous studies have shown that *H. pylori* is the causative agent of peptic ulcers found mostly in the human stomach worldwide. Eradicating the infection can alter the natural course of peptic ulcer disease by dramatically reducing its recurrence rate in treated patients, compared with untreated patients (Van der Hulst *et al.*, 1997; Williamson *et al*., 2001; Njume *et al*., 2009). Elimination of the bacteria occurs in patients with duodenal and gastric ulcers who have no history of nonsteroidal anti-inflammatory drug use. Eradication of *H. pylori* slows down the progression of gastric cancer but re-occurrence may occur at site different from the resection site (Asaka *et al.*, 2010) However, the mechanism by which *H. pylori* induces peptic ulcer disease is moderately implicit but most probable entails a combination of genetic intolerance of the host, virulence factors of the organism (eg, VacA and CagA proteins), mechanical damage to the mucosa, and modification of gastric and duodenal secretions (Albert *et al*., 2005; Tanih *et al*., 2009).

2.3.4 Non-ulcer Dyspepsia

Non-ulcer dyspepsia encompasses a collection of diverse symptoms including: dysmotiliy, ulcer, and reflux-like symptoms. Studies have revealed that non-ulcer dyspepsia is caused by a number of factors like altered visceral sensation, way of life, stress, altered visceral sensation, alterations in gastric acid secretion, gastric clearance and increased serotonin sensitivity, as well as *H. pylori* infection (Tanih *et al*., 2009; Asaka *et al*., 2010). Olden and Drossman (2000) in their review highlighted the role played by psychosocial impairment in patients with non-ulcer dyspepsia. On the other hand, studies also showed a link between *H. pylori* infection and non-ulcer dyspepsia; thus patients with the latter condition were twice as likely to be positive for the organism (Vaira *et al*., 2000). Nonetheless, regardless of epidemiologic confirmation, treatment studies have failed to consistently confirm that

eradication of *H. pylori* results in enhancement of non-ulcer dyspepsia symptoms (Hoffman *et al*., 2004; Lee *et al*., 2008). Therefore, suppression of the organism cannot be considered the standard of care in all patients with non-ulcer dyspepsia, because *H. pylori* infection is only a single part of the mutifactorial etiology of the disease.

2.3.5 Gastroesophageal Reflux Disease (GERD)

A lot of interest has been focused on the feasible connection between infection with *H. pylori* and gastroesophageal reflux disease (GERD) in its diverse manifestations (eg, esophagitis, Barrett's esophagus). Various researchers have recommended an association between the existence of *H. pylori* and reduced threats for developing esophagitis and Barrett's esophagus (Loffeld *et al.*, 2000; Willian *et al*., 2004; Biradar *et al*., 2008); even though this opposite relationship is maintained/ sustained by various prevalence studies, whilst others failed to verify it (Dube *et al.*, 2009; Tanih *et al*., 2009; Asaka *et al*., 2010). Investigations have also specified that certain strains of *H. pylori*, particularly the CagA positive strains, may be protective against the development of Barrett's esophagus (Vaezi *et al.*, 2000; Tanih *et al*., 2009). In addition, Labenz and colleagues revealed that the frequency of esophagitis may, in fact, increase after eradication of the organism (Labenz *et al.*, 1997).

H. pylori treatment can lead to exacerbation of GERD in several patients, prompting many gastroenterologists to suspend endoscopic antral biopsies in patients with significant GERD and absence of ulcer (Labenz *et al.*, 1997; Parsons *et al*., 2001). Conversely, other studies using endoscopic findings, pH probe measurements, and histology to determine the presence of *H. pylori* did not find any association between GERD and infection with *H. pylori* (Oberg *et al.*, 1999; Graham and Shiotani, 2005).

2.3.6 Virulence proteins

The genome of *H. pylori* is about 1.7 Mbp, with a G+C content of 35 to 40%. Several strains of *H. pylori* have been identified, including: *H. pylori* strain 26695 with 1,587 genes, and J99 strain with 1,491 genes (Tomb *et al.*, 1997; Baltrus *et al.*, 2009). Two copies of 16S, 23S, and 5S rRNA genes are contained in both genes, whereas various strains carry one or more cryptic plasmids, that do not carry antibiotic resistance or virulence factors/genes (Albert *et al*., 2008; Baghaei *et al*., 2009). At some instance most of these plasmids outlines the foundation of *H. pylori*, *E. coli* shuttle vectors used in molecular cloning experiments (Baghaei *et al*., 2009). Detailed characterisation of *H. pylori* is lacking, though the existence of *H. pylori*-infecting bacteriophages has been well documented (Masayoshi *et al.*, 2005).

The majority of the *H. pylori*-infected individuals world-wide are either asymptomatic or only have gastritis, associated with epigastric pain, abdominal distention or bloating, belching, nausea, flatulence, and halitosis (Meurer and Bower, 2002; Ndip *et al.*, 2008). Disease outcome may be the result of a number of factors including host factors, environmental factors and differences in the prevalence or expression of bacterial virulence elements (Ahmad *et al*., 2009). Most of the clinical isolates of *H. pylori* from patients suffering from peptic ulcer or malignant disease express virulence-associated factors including: urease, the vacuolating cytotoxin VacA, as well as the cytotoxin-associated geneA antigen CagA (Asahi *et al.*, 2000; Mukhopadhyay *et al*., 2008). Although these virulence factors have been proposed, the best studied virulence factor is the *cag* pathogenicity island (PAI) which is an approximately 40 kilobase pair region (Asahi *et al*., 2000; Baghaei *et al.*, 2009; Tanih *et al*., 2010).

The *cag* PAI encodes a type IV secretion system, where CagA is delivered into host cells. After the delivery into gastric epithelial cells, CagA is mainly tyrosine-phosphorylated at Glu-Pro-Ile-Tyr-Ala (EPIYA) motifs located in the 3' region of *cagA* gene (Masayoshi *at al*., 2005; Argent *et al*., 2008; Baghaei *et al.*, 2009). It also causes inflammation by activating the NF-kB and secretion of cytokines and chemokines such as interleukin 8 (IL-8), and facilitates the translocation of CagA into the cytosol of epithelial cells, where it becomes tyrosine phosphorylated by Src kinases. Phosphorylated CagA interacts with SHP-2 phosphatase (Higashi *et al*., 2002; Figueroa *et al.*, 2002; Hocker *et al.*, 2003; Ko *et al*., 2008) and results in the formation of long needle like cellular protrusions referred to as the 'hummingbird' phenotype. This phenotype is considered to be proliferative, so may be important in carcinogenesis (Argent *et al*., 2008), hence *H. pylori* has been recognized as a class I carcinogen.

2.4. LABORATORY DIAGNOSIS OF *H. PYLORI*

The significant burden of *H. pylori*-related sequelae indicates that there is a need for the exact diagnosis of the infection (Nakamura *et al*., 2001; Nguyen *et al*., 2009) with advantages and limitations. Numerous invasive and non- invasive diagnostic tests are accessible for determining the presence of *H. pylori* infections. Invasive tests require endoscopy and these include; the biopsy urease test, histology, fluorescent *in situ* hybridization, culture and polymerase chain reaction (PCR), whilst non- invasive tests are more convenient and do not require endoscopy. These include; ^{13}C-urea breath tests, serology and stool antigen enzyme immunoassay tests (faeces, dental plaque) (Gramley *et al*., 1999; Bravos *et al*., 2003; Tanih *et al.*, 2008).

2.4. 1 Histology

Histology has been traditionally used to diagnose the presence of bacteria or *H. pylori* infection as well as any type of infection present in a specimen. A need for an endoscopy to obtain a tissue disadvantages this technique. However, limitations arises at times because of an insufficient amount/number of biopsy specimens obtained or failure to obtain specimens from different areas of the stomach (Choe *et al*., 2001). On the other hand, many stains can be used to detect *H. pylori* including: Warthine-Starry, Hp silver stain, Dieterle, Giemsa, Giminez, acridine orange, McMullen and immunostaining (Gatta *et al*., 2003; Ndip *et al*.,2004); these involve longer processing times and higher costs. Normally the stains used for the evaluation of inflammatory cells are haematoxylin and eosin as well as Giemsa stain which is used to detect *H. pylori* because of its simplicity, low cost and technical simplicity (Gatta *et al*., 2003). However, histologic sampling does allow for definitive diagnosis of infection, as well as of the degree of inflammation or metaplasia and the presence/absence of MALT lymphoma or other gastric cancers in high-risk patients.

2.4.1.2 Culture

Helicobacter pylori can be cultured from gastric biopsies (Kel *et al*., 2002; Lopez-Vidal *et al*., 2008). However, *H. pylori* is a fragile organism. It must be protected from desiccation and contact with oxygen and room temperature. It is mandatory not to expose the biopsy specimens to air and to place them either in a saline solution for short-term transport (4 h maximum) or in a transport medium, usually consisting of semisolid agar, maintained at 4°C. Therefore, the biopsies specimens are kept in a transport medium (Stuart's transport medium) for 24 h at 4 ^{0}C, then cultured on agar (Columbia or brainheart infusion), generally with added antibiotics and albumin. The agar plates are incubated in a micro-aerobic environment,

using a jar with a gas-generating kit for a micro-aerobic atmosphere (5% oxygen and 5-10% CO_2) and incubated for at least 5 days at 37 ^{0}C. The growth may start to appear after 3 days (Yang and Seo, 2005). The organisms are identified as *H. pylori* when they form characteristic visible colonies which show typical cell morphology on Gram staining and biochemical tests (Yang and Seo, 2005; Salehi *et al*., 2008). The colonies are Gram negative, urease, oxidase, and catalase positive. The disadvantage about culture is that the organism is fragile out of the gastric environment and yet inexpensive (Kel *et al*., 2002; Yang and Seo, 2005). Therefore, the role of culture in diagnosis of the infection is limited mostly to research and epidemiologic considerations.

2.4.1.3 Rapid urease test

The test is based on the activity of the *H. pylori* urease enzyme, which splits urea to form ammonia. Ammonia enhances pH, which is identified by the indicator phenol red (Molbey *et al*., 1988). Various urease tests are commercially accessible, together with gel-based (CLOtest, HpFast), paper-based (PyloriTek, ProntoDry HpOne) and liquid-based tests (CPtest, EndoscHp). Results are obtaibed within 24 hours, depending on the format of the test and number of *Helicobacter* in the biopsy specimen (Kaklikka *et al*., 2006). This test is advantageous in the sense that it is cheap, rapid, and extensively accessible. Limitations maybe due to false positive results; decreased urease activity, caused either by current intake of antibiotic agents, bismuth compounds, proton pump inhibitors, or sucralfate or by bile reflux, can contribute to these false-positive results (Midolo *et al*., 1999), as well as the increased luminal pH which can lead to extremely high pH adjacent to the organism, such that the organism can be destroyed by the action of its own urease (Kaklikka *et al*., 2006).

2.4.1.4 Polymerase Chain Reaction

The polymerase chain reaction (PCR) has increasingly been described as the latest gold standard for detecting some microbes. PCR is the most commonly used nucleic acid amplification technique for the diagnosis of infectious disease, surpassing the probe and signal amplification methods with marked sensitivity and specificity (Frenck *et al*., 2006). It allows identification of the organism in small samples with few bacteria present and entails no special requirements in processing and transport. Moreover, PCR can be performed rapidly and cost- effectively, and it can be used to identify different strains of bacteria for pathogenic and epidemiologic studies. PCR has been used extensively for the diagnosis of *H. pylori i*nfection from gastric biopsy specimens, saliva, faeces and archived specimens (Bravos *et al.,* 2003; Tiwari *et al*., 2005; Dube *et al*., 2009; Tanih *et al*., 2009).

This technique has been used successfully to detect *H. pylori* DNA in gastric tissues by amplifying genes such as the adhesin genes, the urease gene and the 16S rRNA gene (Tiwari *et al.,* 2005). The 16S rRNA gene of *H. pylori* is a highly specific target for amplification and has been used previously to help reclassify the organism. The 16S rRNA is one of the 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. PCR yields information on the presence of potential virulence markers in the strain (Vaira *et al*., 2000), which might have implications for the development of severe disease or efficacy of eradication (Bravo *et al*., 2003). Different primers have been utilised and some have been developed into commercial kits. Different loci have been used as target for amplification: 16S rRNA; A-, B- and C-urease; flaA; cagA; vacA and heat-shock protein (hsp). Real-time PCR results can be obtained using light-cycle technology (Ricci *et al*., 2007).

It also requires special laboratory conditions with separate facilities. In addition, because PCR can detect segments of *H. pylori* DNA in the gastric mucosa of previously treated patients, false-positive results can occur, and errors in human interpretation of bands on electrophoretic gels can likewise lead to false-negative results (Ko *et al*., 2008; McClain *et al*., 2009; Tanih *et al*., 2009).

2.4.1.5 Urea Breath Test

Urea breath test correspondingly relies on the urease activity of *H. pylori* to detect the presence of active infection. Patients with suspected infection ingest either ^{14}C- labeled or ^{13}C- labeled urea. The ^{13}C- labeled urea has an advantage of being nonradioactive and thus theoretically safe for children and women of childbearing age. Urease, if present, splits the urea into ammonia and isotope-labeled carbon dioxide; the carbon dioxide is absorbed and eventually expired in the breath, where it is detected. Besides being excellent for documenting active infection, this test is also valuable for establishing the absence of infection after treatment, an important consideration in patients with a history of complicated ulcer disease with bleeding or perforation (Gatta *et al*., 2003). In addition, a urea breath test is relatively inexpensive (whichever isotope is used), is easy to perform, and does not require endoscopy. However, if the patient has recently ingested proton pump inhibitors, antibiotic agents, or bismuth compounds, a urea breath test can be of limited value. Therefore, at least 1 week should separate the discontinuing of antisecretory medications and testing for active infection (Frenck *et al*., 2006; Njume *et al*., 2009), and 4 weeks should separate treatment of *H. pylori* infection and testing for eradication of the organism. Moreover, except for major medical centers or tertiary referral centers where results are usually available in fewer than 24 hours, a urea breath test may be further limited by a turn around time of several days (or

longer) required for transport of samples and analysis by specialized laboratories not present in many community settings.

2.4.1.6 Serologic Tests

The test is used for diagnosis of antigen infection and antibodies can be detected long after a successful treatment. The most widely used test in the assay uses polyclonal anti-*H. pylori*-capture antibodies absorbed to microcells (Ricci *et al*., 2007). These antibodies can be detected in serum or whole-blood samples. The presence of IgG antibodies to *H. pylori* can be detected by use of a biochemical assay commercially available. The test is highly specific and sensitive like the urea breath test (Tanih e*t al*., 2009). However, this method is not a useful means of confirming eradication of *H. pylori*; several different samples and changes in titers of specified amounts over time would be needed (Lerang *et al*., 1998). In addition, few patients become truly seronegative, even after eradication of the organism (Momoyo *et al*., 2006). In low-prevalence populations, serologic tests should be a second-line methodology because of low positive predictive value and a tendency toward false-positive results. Serologic tests may be useful in identifying certain strains of more virulent *H. pylori* by detecting antibodies to virulence factors associated with more severe disease and complicated ulcers, gastric cancer, and lymphoma.

2.4.1.7 Stool antigen testing

Stool antigen testing uses an enzyme immunoassay to detect the presence of *H. pylori* antigen in stool specimens. A cost effective and reliable means of diagnosing active infection and confirming cure, such testing has a sensitivity and specificity comparable to those of other noninvasive tests (Monteiro *et al.*, 2001; Ndip *et al*., 2004; Frenck *et al*., 2006; Ricci *et al*., 2007; Dube *et al*., 2009). Questions remain regarding possible cross reactivity with other *Helicobacter* species present in the intestines, but definitive studies are lacking.

2.5 EPIDEMIOLOGY

2.5.1 Prevalence

H. pylori infection is probably one of the most common bacterial infections worldwide (Sherif *et al*., 2004; Falsafi *et al*., 2005; Tiwari *et al*., 2005). It is responsible for a significant cause of morbidity and mortality imposing a major burden on health care systems worldwide. The prevalence of *H. pylori* infection varies from 20-50% in industrialized countries to over 80% in developing countries (Feldman, 2001; Ndip *et al*., 2004).

The organism has been reported to be prevalent in South Africa. For example, Pelser *et al.* (1997) documented a high prevalence of *H. pylori* antibodies (67-84%) in children in Bloemfontein, while Mosane *et al*. (2004) also documented *H. pylori* IgG antibodies in South African mothers and their children. Recently, Samie *et al*. (2006) reported an *H. pylori* prevalence of 50.6% in a study in Venda, North of South Africa. *H. pylori* prevalence's of 84%, 83%, 70%, and 50.6% have been respectively reported in Gauteng, Mpumalanga, Western Cape, and Limpopo Provinces of South Africa(Fritz *et al*., 2006). Childhood appears to be the critical period during which *H. pylori* is acquired, especially in areas of

overcrowding and socio-economic deprivation (Feldman, 2001; Horiuch *et al*., 2001; Ndip *et al*., 2004; Dube *et al*., 2009).

As prevalence in developing countries remains moderately stable, it is constantly declining in developed countries (Ndip *et al*., 2004; Asrat *et al.,* 2004 Salehi *et al*., 2008). However, improved hygiene, sanitation and activation of carriership via antimicrobial treatment may be the contributing factor to the reduction of the infection in children. Studies show that in developing countries infections due to *H. pylori* are still persistent in the first 5 years of childhood (Fegueiredo *et al*., 2002; Ndip *et al*., 2004; Kandulski *et al*., 2008; Reyberg *et al*., 2008) and increases with age in under-developed countries. This increase results only to a small extent from *H. pylori* acquisition at later age. *H. pylori* infection appears to be less than 0.5% in adults in the Western world and higher prevalence of infection among the elderly has been noted, thus reflecting a birth cohort effect with higher infection rates in the past (Kaklikkaya *et al*., 2006; Parsonnet *et al*., 2009). Eradication of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium (Salehi *et al*., 2008; Dube *et al*., 2009; Torres *et al*., 2009). Generally, new-fangled infections usually arise within childhood and endure for life not unless treated.

2.5.2 Transmission of *H. pylori* infection

In Africa, *H. pylori* infection in early life is strongly associated with pernicious anaemia, growth faltering and increased susceptibility to other food and waterborne pathogens, especially in young children from the poorest societal strata (Argent *et al*., 2008). *H. pylori* transmission remains poorly understood, however it is believed to spread from person to person through oral-to-oral or faecal-to-oral routes (Thomas *et al*., 2004; Ahmadu *et al*., 2007; 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; Hoffman *et al*., 2004; Tanih *et al*., 2009).

Acquisition of *H. pylori* in children is believed to be related to close family members (Van der Ende *et al.*, 1996; Raymond *et al.*, 2004; Alborzia *et al*., 2006; Rowland *et al.*, 2006; Ko *et al*., 2008). Such observations suggest that these infections may be associated with low socioeconomic status and overcrowded living conditions (Delport *et al*., 2006). *H. pylori* has been detected in saliva, vomitus, gastric refluxate, and faeces (Ferguson *et al.*, 2001; Parsonnet *et al*, 1997;Sugiyamea *et al.*, 1998; Sharara *et al*., 2002; Ricci *et al.*, 2007), but there is lack of convincing evidence for the principal transmission route.

The existence of *H. pylori* DNA in environmental water sources, probably reflects contamination with either naked DNA or dead *H. pylori* organisms (Dube *et al*., 2009). Li *et al*. (2002) successfully cultured *H. pylori* from wastewater that represented faecal contamination of water. Hence, an increased faecal contaminants is sustained by the occurrence of *H. pylori* infections among institutionalized young people during outbreaks of gastroenteritis (Lopez-Vidal *et al.*, 2008; Dube *et al.*, 2009).

2.5.3 **Treatment of *Helicobacter pylori***

Antibiotic resistance is a major cause of treatment failure. *H. pylori* infection is often treated with a triple therapy, such as metronidazole, clarithromycin, amoxicillin and a proton pump inhibitor (PPI) (Alarcon *et al*., 2001; Al-Qurahi *et al*., 2001; Kato *et al*., 2002; Sharara *et al*., 2002; Asaka *et al*., 2010). Eradication therapy of symptomatic *H. pylori* infection substantially reduces the recurrence of associated gastro-duodenal diseases. Therapy entails complicated regimens of several antimicrobial agents for at least two weeks. In general, the triple therapy regimens entail two of the following antibiotics: metronidazole, clarithromycin, amoxicillin and tetracycline with a proton pump inhibitor or bismuth (Hoffman *et al*., 2004; Duck *et al.*, 2004; Njume *et al.*, 2009).

Combination therapy may have a success rate of 80-90%, but can be the cause of undesirable side effects including: vomiting, epigastric pain, abdominal discomfort and poor patient compliance (Bina *et al*., 2000; Njume *et al*., 2009); all these factors are associated with the major high levels of treatment failure. The most common cause of treatment failure are patient noncompliance and antimicrobial resistance of the infecting *H. pylori* strain. Quadruple regimens are used as a salvage therapy when triple therapy regimens have failed. Pre-treatment resistance of the organism has been reported to compromise the efficacy of treatment (Gatta *et al*., 2001; Adrienne *et al*., 2007; Asaka *et al*., 2010). Other treatment regimens can be a 7-days treatment with a double dose of PPI plus clarithromycin and metronidazole, or a double dose of PPI plus amoxicillin and metronidazole (Boyanova *et al*., 2009; Njume *et al*., 2009). According to studies in the literature, there is evidence revealing that natural antimicrobials can act as resistant microbial inhibitors (Okunade *et al.*, 2002; Adebolu, 2005; Aboderin *et al*., 2007; Baghaei *et al*., 2009). There is an increased need for the identification of new antimicrobials that are capable of inhibiting and treating a wide range of microorganisms including multi-drug resistant strains of bacteria.

The emerging and hasty spread of bacteria conveying multidrug resistance (MDR) has called for the invention of new antimicrobials and resistance-modifying agents, particularly in nosocomial situations (Ngemenya *et al*., 2006). For these microorganisms to resist the lethal effects of environmental toxins, they have devised a diverse system for resistance including that of natural antimicrobial agents, of which some have been developed into anti-fungal drugs (Gblade *et al*., 1999; Perron *et al*., 2005; Fernandez *et al*., 2008; Truong-Bolduc *et al*., 2005). For this reason, there is one way or an initial step to stop these toxic compounds, that is, by actively reducing their entry into the cells through the action of efflux pumps located in the cytoplasmic membrane (Truong-Bolduc *et al*., 2005).

2.6 Prevention

H. pylori is a major cause of diseases of the upper gastrointestinal tract. Hence, an understanding of the route of *H. pylori* transmission is important if public health measures to prevent its spread are to be implemented. Researchers are studying different adjuvants, antigens, and routes of immunization to ascertain the most appropriate system of immune protection, with most of the research only recently moving from animal to human trials (Alborzia *et al*., 2006; Kabir, 2007). Improved home sanitation, decreasing family size, less household crowding, changes in dietary habits, improvements in refrigeration and hygiene at home are beneficial.

For gastric cancer, the unique opportunity is a preventive strategy against the disease (Graham and Shiotani, 2005). Eradication therapies are either used to reverse the inflamed mucosa to normal or to prevent further progression of advanced chronic changes (atrophic gastritis, intestinal metaplasia). Although *H. pylori* infection is a significant risk factor for the development of gastric cancer, there are few controlled clinical trials showing that eradication

can prevent the progression from normal gastric mucosa to gastric cancer (Smith *et al*., 2007).

The clinical usefulness of eradicating *H. pylori* requires further investigations as current regimens are expected to achieve greater than 85 to 90 percent rates of eradication after one to two weeks of treatment. Once treated, re-infection rates are less than 0.5 percent per year. Management of peptic ulcers in some patients may require maintenance treatment (Graham and Shiotani, 2005; Malfertheiner *et al*., 2008).

2.7 Factors affecting *H. pylori* treatment.

Some of the factors known to induce treatment failure include drug expenses and accessibility, side effects (Alarcon *et al*.,1999), the need of infiltration of antibiotics into the depth of gastric mucosa (Wong *et al.,* 2004), antibiotic inactivation by pH, and misuse of drugs by patients (Oderd *et al*., 2001; Bytzer and O'Morain, 2005). The lack of correlation between *in vitro* susceptibility test and *in vivo* efficacy and the presence of *H. pylori* strains with primary or secondary resistance to the antimicrobial agents used (Bytzer and O'morain, 2005), duration of treatment and antibiotics dosage (Njume *et al.,* 2009) are also important. Antimicrobial resistance is escalating and regional deviations in susceptibility and resistance patterns maybe attributed to differences in local antibiotic prescriptions, antibiotic usage in the community and mass eradication programmes for *H. pylori* (Ndip *et al*., 2008).

The recommended duration of therapy for *H. pylori* eradication is 10 to 14 days (Njume *et al*., 2009). Potential benefits for shorter regimens include: better compliance, fewer adverse drug effects, and reduced cost to the patient (Meurer and Bower, 2002; Fernandez *et al*., 2008). Low gastric pH seems to affect the activity of antibiotics since most are active at

neutral pH. However, the effect that the gastrointestinal environment may have on these orally consumed extracts is usually not explored even though it is well-known and accepted in biopharmaceutics that the gastrointestinal system plays a considerable part in the ultimate bioavailability of conventional medicines (O'Gara *et al*., 2000; Hassan *et al*., 2007). The human gastrointestinal tract transforms orally consumed substances into and waste products by means of a digestive process.

The fluids present in the gastrointestinal system contain various substances and enzymes which aids in the digestion process. The stomach has a pH between 1 and 2 due to the secretion of hydrochloric acid and contains the digestive enzyme pepsin. The pH of the small intestine ranges between 5.1 and 7.5 and contains numerous digestive enzymes (Mader, 1996; Hassan *et al*., 2007). It is well documented that some active pharmaceutical ingredients have to be protected, e.g. through enteric coating, against this harsh environment in order to be effective.

The presence of hydrochloric acid and enzymes in the stomach can easily cause acid or enzyme catalysed hydrolysis of orally consumed compounds thereby resulting in chemical modification and inactivation. Presystemic metabolism and degradation in the gastrointestinal tract primarily results in reducing the bioavailability of medicines. However, in the case of pro-drugs these processes are essential for the active parent drug to become available for absorption. Medicines such as erythromycin stearate depend on degradation in the gastrointestinal tract to release the therapeutically active parent molecule (Boargets *et al*., 2006). It exhibits limited solubility in gastric fluid but dissolves and dissociates readily in the intestinal fluid, liberating the free base which is absorbed (Duck *et al.,* 2004; Adeniyi *et al*., 2009).

It has been well documented that minimal bactericidal concentrations (MBC) and minimal inhibitory concentrations (MIC) of most active antibiotics against *H. pylori* excluding metronidazole and tetracycline, are dependent on the pH of the environment (Malfertheiner *et al.*, 2007; Argent *et al*., 2008). At pH values lower than 7 or 7.4, the MIC increases. This is why PPI's are used in therapy so as to increase the pH of the stomach, to allow better antimicrobial activity (Alarcon *et al*., 1999; Choe *et al*., 2001). In patients who are acid hyper secretors, the pH remains low; consequently, antimicrobial activity may be insufficient to eradicate the bacteria. As a result, increasing the dosage of PPI in the treatment regimen may have beneficial effects (Malekzadeh *et al.*, 2004).

The most important causes of treatment failure are poor patient compliance and the development of bacterial resistance to antimicrobial agents (Ndip *et al*., 2007). Patient compliance can only be improved by choosing a simple and well tolerated treatment regimen (Steenkamp *et al*., 2007). In addition, patients should be educated on the importance of eradication therapy

2.8 Medicinal plants

Phytomedicine has shown great promise in the treatment of intractable infectious diseases. 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 South Africa. These plants are ingested as decoctions, teas and juice preparations to treat respiratory infections (Clarkson *et al*., 2004; Birdar *et al*., 2008; Njume *et al*., 2009). They are also made into a poultice and applied directly on the infected wounds or burns. When people from these remote communities get an infectious disease, they are usually treated by traditional healers and "shamans" because of their expertise in such procedures as

making diagnoses, treating wounds, setting bones and making herbal medicines (Mulu *et al*., 2004; Hassan *et al*., 2007; Centikaya *et al*., 2008).

Traditional healers claim that their medicine is cheaper and more effective than modern medicine. They claim that patients of these communities have a reduced risk to get infectious diseases from resistant pathogens than people from urban areas treated with traditional antibiotics because of the accessibility and affordability of medicinal plants. However, if they are treated in a hospital the chance of contracting a nosocomial infection is increased (Adjanohoun *et al*., 1996; Hassan *et al*., 2007; Temaru *et al*., 2007). 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; Rojas *et al*., 2006). Traditional practitioners also claim that some medicinal plants such as *Lippia spp* are more efficient to treat infectious diseases than synthetic antibiotics. It is necessary to evaluate, in a scientific base, the potential use of folk medicine for the treatment of infectious diseases produced by common pathogens. Reports have documented the existence of many plants in Africa used by traditional medical practitioners which they claim are efficient in the management of complaints symptomatic of peptic ulcer diseases; a major outcome of *H. pylori* infection. However, the claims are not substantiated with scientific facts, though continuous usage of these plants in the management of infections indicates that the plants may contain constituents, which have antibacterial activities.

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 (Huang *et al*., 2004; Umeh *et al*., 2005). Hence, a more detailed search for new antimicrobial drugs is needed.

As antimicrobial resistance to antibiotics increases globally, development of novel drugs is imperative and there has been an improved interest in ethnopharmacological research globally. According to studies in the literature, there is evidence that natural antimicrobials can act as resistant microbial inhibitors (Sofowera *et al.,* 1993; Adebolu, 2005). Studies are generally of a screening nature, and investigate biological activities such as antioxidant, antibacterial, anti-inflammatory and antifungal properties of extracts from medicinal plants usually based on ethnobotanical leads.

Plants have been documented to have compounds that contribute to their antimicrobial activity including phytochemals such as flavanoids, phenolics, and propolis (Harbourne, 1983; Cushnie and 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-defence mechanisms by protecting the plant against bacteria, fungi and viruses. They have been described as non-peroxide antimicrobial factors. It is believed that these pytochemical factors are composed of many complex phenols and organic acids.

Some of these compounds like polyphenols have been shown to exert their antibacterial action through membrane perturbations. This perturbation of the cell membrane coupled with the action of β-lactams on the transpeptidation of the cell membrane could lead to an enhanced antimicrobial effect of the combination (Esimone *et al*., 2006). It has also been shown that some plant derived compounds can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site (that is, peptidoglycan) in the cell wall (Aiyegoro *et al*., 2009).

The use of plant as an antimicrobial may inhibit bacteria by a mechanism different from an antibiotic, thus may contribute to the treatment of resistant microbial pathogens. As a result

of this, a number of investigations have been reported by authors on the screening of plants as natural antimicrobials (Adebiy *et al*., 2004; Adebolu, 2005; Viljoen *et al*.,2005; Ndip *et al*., 2008). Medicinal plants have had a great 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, and thus offers potential benefits.

2.8.1 *Lippia javanica*

The genus *Lippia* (Houst.), a member of the Verbenaceae family is represented by approximately 200 herbs, shrubs and small trees which are often of an aromatic nature (Terblanche and Kornelius, 1996). The species are widely distributed in tropical and Southern Africa as well as throughout South and Central America with the exception of the Western Cape (Velasco-Negeureula *et al.*, 1993; VanWyk *et al*., 1997). *Lippia javanica* (Burm. f.) Spreng is one of the four indigenous *Lippia* species in South Africa where it occurs as an erect woody shrub approximately 2m in height. The stems have a square appearance when looked at in cross-section. The leaves are hairy with noticeable veins and when crushed gives off a strong lemon-like smell. The small cream flowers can be found on the shrub from summer to autumn in some areas and in others are produced all year. These flowers are arranged in dense, rounded flower heads. The fruit are rather inconspicuous, small and dry.

The plant is used extensively in traditional medicine by both lay people and traditional healers to treat minor ailments (Pascual *et al*., 2001). Many of its uses relate to microbial infections, e.g. coughs or colds and also for skin infections or wounds. The leaves and stems are often used and in some cases the roots as well (Van Wyk *et al*., 1997). Strong leaf infusions are made which are commonly used as inhalants. The leaf infusions are also used

topically for scabies and lice (Gelfand *et al*., 1985;VanWyk *et al*., 1997). An infusion of the leaves is commonly used as a decongestant for colds and coughs as well as diarrhoea (Viljoen *et al.,* 2005). Its use has excelled in the treatment of respiratory aliments traditionally, which may either be fungal or bacterial and also in the treatment of Gram-negative and yeast borne respiratory aliments (Viljoen *et al.,* 2005). More commonly, leaf and stem infusions are used as a tea, and this is taken to treat coughs, colds, fever and bronchitis (Hutchings, 1996).

The plant has also been used for bronchial ailments and influenza (Hutchings, 1996). The Vhavenda people use leaf infusions as anthelmintics, for respiratory and febrile ailments and as prophylactic against dysentery and malaria (Mabogo, 1990). Roots are used as antidotes for suspected food poisoning and for bronchitis and sore eyes (Hutchings, 1996). *L. javanica* has also been used as a mosquito repellent by the population in Southern Africa for a long time and previous studies in Zimbabwe have shown that essential oils from *L. javanica* have very strong and lasting repellent activity against starved female *Anopheles arabiensis* (Omolo *et al*., 2005). However, other studies in Durban and Venda showed that the extracts of *L. javanica* were not active against *E. coli* and other bacteria (McGaw *et al*., 2000; Samie *et al*., 2005).

The acetone and methanol extracts of *L. javanica* were found to be active against most bacteria including *B. subtilis, P. mirabilis and S. marcescens* and *S. aureus*. (Same *et al.,* 2005). The antimicrobial activities of this plant are said to be due to some chemotypes, including limonene, germacrene-D, camphon, linalool, β-caryophyllene, myrcene and genanial (Velasco-Negeureula *et al*., 1993; Terblanche and Kornelius, 1996). *L. javanica* is administered in the form of thin leaves and teas, it is most probable that the non-volatile compounds could be acting in a synergistic/ additive manner to produce enhanced chemical properties (Viljoen *et al.,* 2005).

Neidlein and Staehle (1974) reported the presence caryophyllene, linalool and *p*-cymene while Chagonda *et al*. (2000) documented variations in major essential compounds in the plant in samples taken from the same location in Zimbabwe e.g. linalool, which had a range between 1.7 and 27%. Also, Ngassapa *et al*. (2003) reported a geranial and neral chemotype from Tanzania. Essential oils are known for their antimicrobial properties.

The use of synthesized compounds in the treatment of chronic diseases is rendered ineffective due to the alarming resistance of microorganisms to antibiotics, however, the potential use of plant based medicine is more effective and a cheaper alternative, which is probably responsible for the fast growing industry of herbal medicine (Ghaemi *et al.,* 2007; Hassan *et al.,* 2007). Plants are used medicinally in different countries and are a source of many potent and powerful drugs (Kubmarawa *et al.,* 2007). Many natural occurring compounds found in medicinal plant extracts have also been shown to posses antimicrobial activities that inhibit the growth of microorganisms (Katsvana and Chigwaza, 2004; Ndip *et al.,* 2008). The active principles of many drugs found in plants are secondary metabolites (Kubmarawa *et al.,* 2007). Therefore, basic phytochemical investigation of these extracts for their major phytoconstituents is also vital. However, different solvent extracts of plants have different phytochemical properties (Hassan *et al*., 2007).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacterial strains

A total of 31 *H. pylori* strains isolated and characterized based on our previously reported schemes (Ndip *et al*., 2008; Tanih *et al*., 2010) from patients presenting with upper gastrointestinal complaints at the Livingstone hospital in Port Elizabeth were used after informed consent and ethical clearance from the Department of Health, Eastern Cape (Protocol number EcDoH-Res 0002) and institutional review board of the University of Fort Hare. A reference strain of *H. pylori* (NCTC 11638) was included as a control.

3.2 Preparation of plant extracts

The plant was selected based on ethnobotanical information and identified in collaboration with botanists of the University of Venda, Limpopo Province, where voucher specimens have been deposited (number AS 19). *Lippia javanica* leaves were harvested, air dried for 2 weeks and ground to fine powder. Dried plant material, 2.5 - 2.8 kg, was macerated in five fold excess of solvent (ethanol, ethylacetate, methanol, chloroform or water) in extraction pots such that the level of the solvent was above that of the plant material. The slurry was allowed to stand at room temperature (RT) for 48h and filtered using filter paper of pore size 60Å. The process was done 3 times. The combined extracts were concentrated in a rotavapor (BUCHI R461, Switzerland) and transferred to appropriately labelled vials and allowed to stand at RT to permit evaporation of residual solvents. A stock solution was prepared by dissolving the extracts in 10% Dimethyl Sulphoxide (DMSO) (Okeleye *et al*., 2010).

3.3 Antimicrobial susceptibility testing

The agar well diffusion method, which conforms to the recommended standards of the Clinical and Laboratory Standards Institute (CLSI), was used as previously described (Ndip *et al.,* 2007). Brain heart infusion (BHI) agar (Oxoid,UK) was used. Wells were made in each plate using a sterile cork borer. The bacterial inoculum was prepared from subcultures of bacteria as follows: four to five colonies of the isolates were emulsified in sterile normal saline and the turbidity adjusted to 1.5×10^8 CFU/mL (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the BHI agar plates. Plates were allowed to dry for 3 to 5 minutes. Thereafter, 0.1 mL of the plant extracts (10-20 mg/mL) were filled into each well separately. Clarithromycin (0.05 µg/mL) was used as the positive control. DMSO was used as negative control. The plates were incubated at 37 °C for 2–5 days. They were then examined and the diameter of the zone of inhibition measured in millilitres. *H. pylori* control strain NCTC 11638 was included in all the experiments. Each experiment was replicated three times.

3.4 Determination of minimum inhibitory concentrations (MIC)

Extracts that gave a zone of inhibition ≥ 15mm were chosen for MIC determination. The MIC was determined using a microdilution test (Banfi *et al*., 2003; Samie *et al*., 2005) with modifications. Two-fold dilutions of each extract was prepared in the test wells in complete BHI supplemented with 5% horse serum (Oxoid, England) and Skirrow's supplement (Oxoid, England). The final extract concentrations ranged from 0.0048 –10 mg/mL. Each strain was sub-cultured in 2 mL of BHI broth for 2 days and the turbidity adjusted by adding 0.5mL to 4.5mL of normal saline and then serially diluted to correspond to 0.5 McFarland standards. Twenty five microlitres of each bacterial suspension was added to 175 µL of extract-

containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. Control antibiotics included amoxicillin and metronidazole. The plates were sealed and incubated under microaerophilic condition for 3 – 5 days at 37°C, after which 32 µL of resazurin solution was added per well, colouring them blue. Plates were incubated at 37°C for an additional 1hour. Plates were observed for colour change from blue to pink in live *H. pylori* -containing wells and then read with a microtiter plate reader adjusted to 620nm (Model 680 Bio-Rad, Japan). MIC was defined as the lowest concentration of extract that prevented resazurin solution colour change from blue to pink resulting in inhibition of 90% & 50% of bacterial growth (MIC_{90} & MIC_{50} respectively). Each MIC was determined three times.

3.5 Determination of rate of kill

The method described by Akinpelu *et al*. (2009) was used to determine the rate of kill with modifications. Turbidity of an 18 h old test organism was first standardized to 10^8cfu/mL. A 0.5 mL volume of known cell density from each strain suspension was added to 4.5 mL of BHI broth supplemented with 5% horse serum and Skirrow's supplement (Oxoid, England) this was then incorporated with the extract at ½× MIC, MIC, 2× MIC and 4× MIC and incubated at 37^{o}C on an orbital shaker at 120 rpm. The rate of kill was determined over a period of 72 h at 6 h interval. A volume of 0.5 mL of each suspension was removed from cultures at time intervals and transferred to 4.5 mL of BHI broth and recovery medium containing 3% Tween-80 to neutralize the effects of the crude extracts carryovers from the test suspensions. The suspension was then serially diluted and plated out for viable counting. The plates were later incubated microaerophilically at 37^{o}C for 72 h. The control plates

included extract free BHI broth seeded with the test inoculum. Visible bacterial colonies were counted and compared with the counts of the culture control.

3.6. *Phytochemical Analysis*

A small portion of the dry extract was subjected to phytochemical test using the method of Adegboye *et al*. (2008) to test for alkaloids, tannins, flavonoids, steroids, saponins.

3.6.1 Test for alkaloids

Approximately 0.5g of the plant extract was dissolved in 5 mL of 1% HCl on steam bath. A millilitre of the filtrate was treated with drops of Dragendorff's reagent. Turbidity or precipitation was taken as indicative of the presence of alkaloids.

3.6.2 Test for tannins

One gram of the extract was dissolved in 20 mL of distilled water and filtered. Two to three drops of 10% $FeCl_3$ was added to 2 mL of the filtrate. The production of a blackish-blue or blackish-green colouration indicated the presence of tannins. To another 2 ml of the filtrate was added 1 ml of bromine water. A precipitate was taken as positive for tannins.

3.6.3 Test for flavonoids

Approximately 0.2g of the extract was dissolved in 2 mL of methanol and heated. A chip of magnesium metal was added to the mixture followed by the addition of a few drops of concentrated HCl. The occurrence of a red or orange colouration indicated the presence of flavonoids.

3.6.4 Test for saponins

Agar well diffusion was used to test for saponins. A freshly prepared 7% blood agar medium was used. The extract in methanol was added to distilled water; methanol was used as a negative control while commercial saponin (BDH) solution served as a positive control. The plates were incubated at 35^0 C for 6h. Complete haemolysis of the blood around the extract was an indicative of saponins.

3.6.5 Test for steroids

About 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered. Concentrated Sulphuric acid was added to the filtrate to form a lower layer. A reddish brown colour was taken as a positive for steroids.

3.7 Statistical analysis

Analysis of data was performed using SPSS Version 17.0 (Illinois USA, 2009). The ANOVA test was used to determine if there was any statistically significant between plant extracts and antibiotics; the MIC of the most active extract and the control antibiotics. P-values <0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 Antimicrobial susceptibility testing

A total of 31 strains were screened for susceptibility to the crude extracts of *L. javanica* (Table 1). A zone of inhibition of ≥15mm of the crude extract was chosen as representative of susceptibility to the tested strains. All the extracts demonstrated antimicrobial activity with zone diameter ranging from 0 - 36mm. The acetone extract exhibited the best activity with a zone diameter of 15 - 36mm. All the test strains were susceptible to the acetone extract (100%), whilst the least activity (16%) was observed for water extract at 10 - 20 mg/mL (Table 1). The concentration of 10% DMSO had no effect on the bacterial growth. The mean zone diameter of inhibition the acetone extract was statistically significant ($P<0.05$) compared with the other extracts and the control antibiotic, clarithromycin (Table 2).

Table 1 Zone inhibition diameter (mm) of the crude extracts of *L. javanica* and antibiotic.

	mg/ml					
Bacterial strains	Acetone	Ethanol	Methanol	Chloroform	Water	Claritromycin
PE2A	25	16	12	24	9	14
PE5A	26	16	12	13	10	19
PE9C	23	15	12	0	8	32
PE11A	20	13	11	15	0	23
PE11C	21	15	12	12	0	11
PE14C	24	13	12	10	0	8
PE252C	23	15	13	13	8	29
PE369A	23	20	15	18	0	20
PE 369C	18	12	15	7	12	18
PE397C	34	27	19	20	9	0
PE402A	15	8	8	7	8	12
PE406C	22	13	24	10	18	12
PE407C	19	12	15	10	0	22
PE411A	22	15	15	15	10	14
PE411C	21	12	15	13	10	16
PE430A	24	16	15	10	30	23
PE430C	15	15	15	7	12	35
PE435A	22	15	10	15	10	20
PE435C	24	13	11	8	11	8
PE436A	20	15	19	18	13	13
PE436C	24	12	15	13	10	12
PE462A	19	10	8	7	11	18
PE462C	19	13	15	7	8	17
PE466A	36	15	15	16	20	15
PE466C	26	25	24	15	15	19
PE 467A	25	19	24	18	8	0
PE467C	26	20	24	7	19	0
PE469A	18	15	15	16	12	12
PE469C	20	15	13	18	13	8
PE 471A	23	12	17	12	11	0
PE471C	24	12	13	11	12	0
Average	100%	58.00%	60.00%	39%	16%	55%

Average was calculated as number of strains that gave the zone diameter of inhibition ≥15 mm divided by the total number of strains. This then showed the number of strains that were inhibited by extracts and the control antibiotic.

Table 2 Mean zone diameter of inhibition of the crude extracts (*L. javanica*) and antibiotics.

Extract / control antibiotic	Mean zone of diameter of inhibition (mm)	Inhibition diameter range	P-value
L.jav. (A)	22.61±4.432	15 – 36 mm	0.041
L.jav. (E)	14.97±3.920	8 – 27 mm	0.062
L.java. (M)	14.94±4.36	8 – 24 mm	0.061
L.jav. (Chl)	12.92±4.985	0 – 24 mm	0.082
*L.jav. (*W)	10.23±6.39	0 – 30 mm	0.091
Control antibiotic (Clr)	15.42±8.732	0 – 35 mm	0.060

L.jav= Lippia javanica; A=Acetone, E=Ethanol, M=Methanol, Chl=Chloroform, W=Water, Clr=Clarithromycin

4.2 Minimum Inhibitory Concentration (MIC) determination

The MIC of the extracts ranged from 0.00195 - 0.625 mg/mL for acetone at MIC_{50} and 0.0048 - 0.625 mg/mL (MIC_{50}) for metronidazole and amoxicillin respectively. On the other hand MIC_{90} ranged from 0.039 - 0.625 mg/mL for acetone, 0.0097 - 5 mg/mL for metronidazole and 0.039 -> 2.5 mg/mL for amoxicillin. The activity of the acetone extract was statistically insignificant ($P > 0.05$) compared to the other crude extracts and antibiotics.

Table 3 *In-vitro* anti-*H. pylori* activity of *L. javanica* extracts and antibiotics at MIC_{50} and MIC_{90} in mg/mL.

Bacterial strains	Acetone		Methanol		Ethanol		Metronidazole		Amoxicillin	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
PE2A	0.078	0.156	0.078	0.156	>0.039	0.313	0.039	ND	0.078	0.313
PE5A	0.25	ND	0.039	0.625	0.039	>0.313	0.0048	>2.5	0.0048	0.625
PE9C	0.078	0.625	ND	0.156	0.039	0.156	0.125	0.625	0.156	ND
PE11A	0.078	0.313	0.078	0.156	0.039	0.625	0.125	0.625	0.078	0.25
PE11C	1.25	0.313	1.25	1.25	1.25	0.0078	0.039	0.156	0.039	0.313
PE14C	0.039	ND	0.078	ND	0.039	0.313	ND	2.5	0.625	0.078
PE252C	0.078	0.156	0.078	0.156	0.039	0.313	0.00975	ND	0.00975	ND
PE369A	ND	0.313	0.625	1.25	ND	0.313	ND	ND	1.25	>0.125
PE 369C	0.039	0.156	0.039	0.156	0.00195	0.156	0.00975	>5	0.078	>2.5
PE397C	0.039	0.156	0.078	0.156	0.0095	0.313	ND	ND	0.078	0.039

PE402A	0.039	0.156	0.039	0.156	0.039	0.078	0.313	0.625	0.039	0.078
PE406C	0.039	1.25	0.039	0.625	0.00195	0.313	0.00975	>2.5	0.00975	ND
PE407C	1.25	0.625	1.25	0.625	0.625	0.0078	0.078	>0.156	0.039	0.625
PE411A	0.078	0.156	0.039	0.078	ND	ND	ND	ND	0.0048	0.313
PE411C	0.625	1.25	0.313	1.25	2.5	0.313	0.00975	ND	0.0048	0.156
PE430A	0.0048	0.039	1.25	0.625	ND	0.039	0.00975	ND	0.039	0.156
PE430C	0.039	0.625	0.039	0.625	ND	0.039	0.078	>2.5	0.078	>0.313
PE435A	0.156	ND	0.039	0.156	0.156	ND	ND	ND	0.0048	1.25
PE436A	0.00195	ND	0.078	0.625	0.00195	0.078	0.0048	ND	0.0048	ND
PE436C	0.0095	0.156	0.625	0.313	0.00195	0.078	0.0048	0.00975	0.0048	0.625
PE462A	0.039	2.5	0.039	0.156	0.00195	0.313	0.00975	ND	0.00975	ND
PE462C	0.039	0.625	0.039	0.156	0.00195	0.156	0.00975	ND	0.00975	ND
PE466A	0.156	ND	0.078	0.156	0.156	ND	1.25	2.5	0.078	0.039
PE466C	0.00195	0.625	0.039	0.156	0.00195	0.078	0.00195	ND	0.00195	ND
PE467A	0.00195	0.156	0.039	0.313	0.0095	0.078	0.0048	ND	0.0048	0.625
PE467C	0.039	0.156	0.00195	0.078	0.078	0.00975	0.0048	ND	0.078	0.313
PE469A	0.039	0.078	0.039	0.156	0.00195	0.039	0.625	ND	0.078	0.039
PE469C	0.039	0.313	0.156	0.039	0.078	0.00195	0.0048	1.25	0.0048	0.125
PE471A	0.078	0.156	0.039	0.156	0.039	0.078	0.0048	ND	0.0048	0.313
PE471C	0.039	0.078	0.0048	0.156	0.0048	0.156	0.0048	1.25	0.0048	0.313
Average	**0.173**	**0.45**	**0.225**	**0.29**	**0.21**	**0.152**	**0.126**	**1.06**	**0.12**	**0.26**

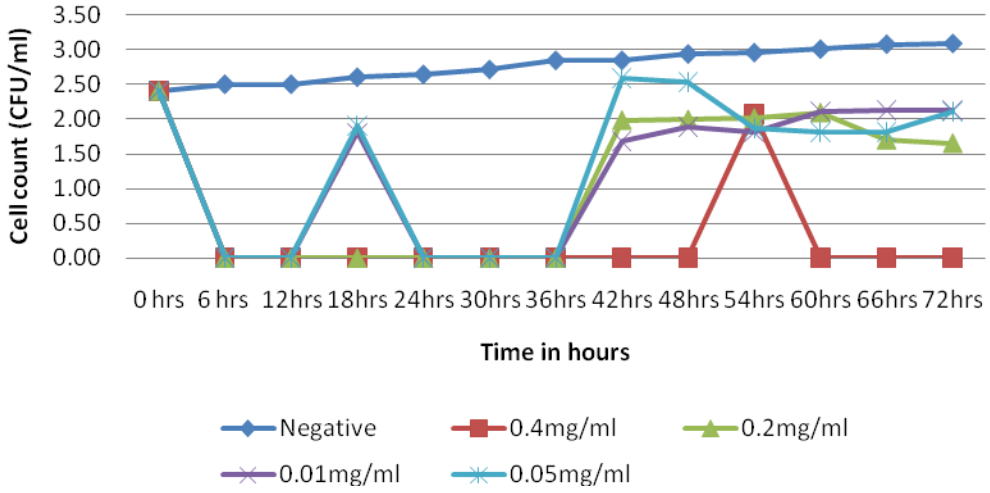
*ND- Not determined

4.3 Determination of rate of kill

The bactericidal activity of extracts against the *H. pylori* strains was determined using time-kill essay. A significant decrease in mean viable count of isolates at each time interval was observed.

The acetone extract was bactericidal against PE369C at MIC and 2× MIC after an 18h interaction period. At 4× MIC, the extract was bactericidal to PE466C at 0h-72h interaction (Figure 1a and 1b) period; an increase in colony count was observed at the 54h interval, and then a sudden decrease was observed at 60h-72h interaction period. At ½× MIC, a remarkable decrease in colony count was observed after a 24h interaction period; and an increase was then observed after the 36h. On the other hand, the ethanol extract exhibited bactericidal effect against PE369C and PE466C at 2× MIC and 4× MIC during the 6h-72h interaction period with complete inhibition of the bacterial strains (Figure 2a and 2b). Complete inhibition was also observed for PE369C at ½× MIC and MIC at 6h-36h interaction period. The ethanol extract also showed remarkable results by further inhibiting the growth of PE252C at 2× MIC and MIC at 0hr-72hr interaction period, whilst at ½× MIC and MIC, the bactericidal effect was observed after the 12h and 36h interaction periods respectively.

(a)



(b)

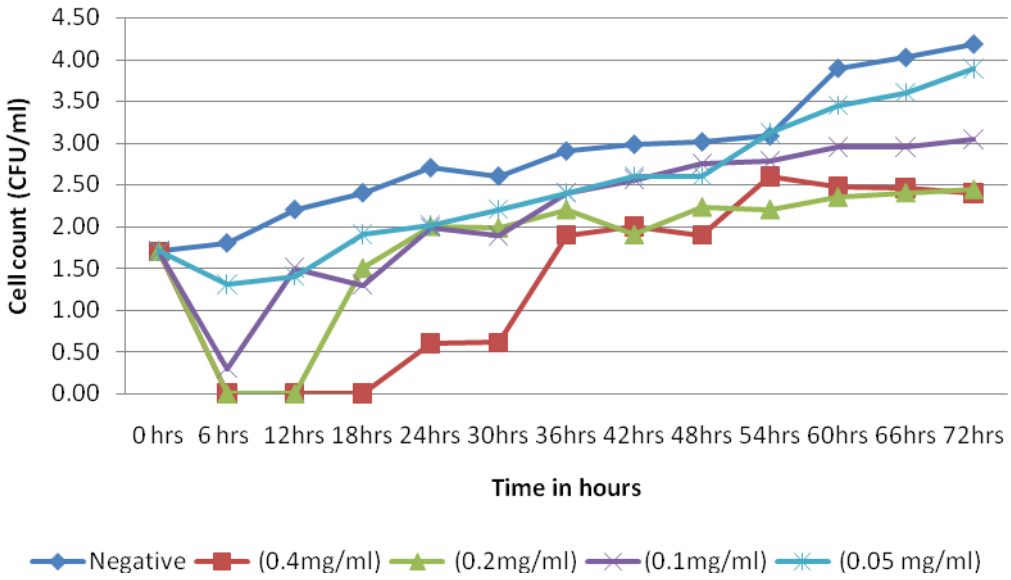
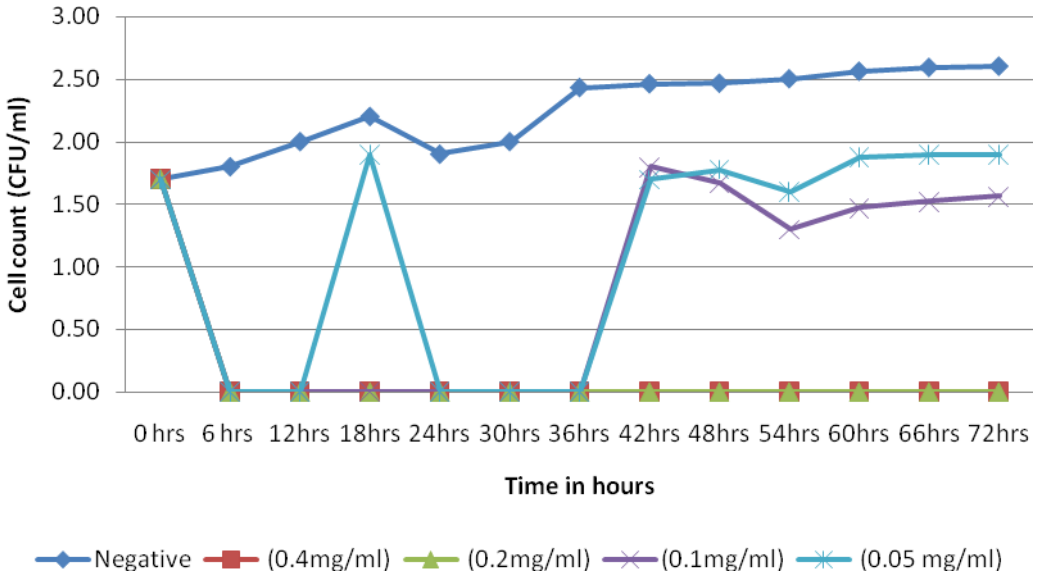


Figure 1: Profile of rate of kill of *H. pylori* strain (a) PE 466C and (b) PE 369C by acetone extract of *L. javanica* as compared to negative control (*H. pylori* strain). The concentration of the extracts used in these experiments were 4× MIC (0.4 mg/ml), 2× MIC (0.2 mg/ml), 1×MIC (0.1 mg/ml) and ½× MIC (0.05 mg/ml).

(c)



(d)

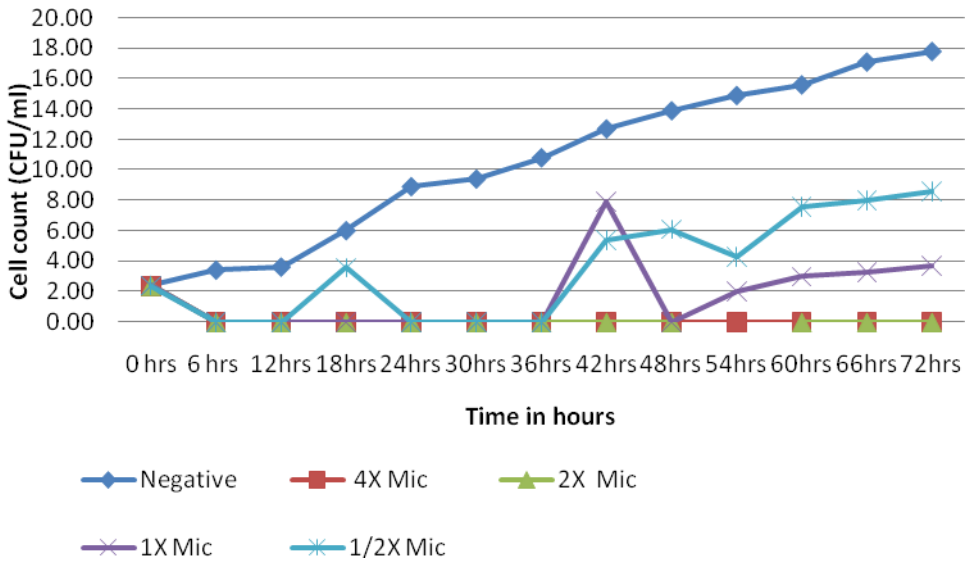


Figure 2: Profile of rate of kill of *H. pylori* strain (c) PE 369C and (d) PE 252C by ethanol extract of *L. javanica* as compared to negative control (*H. pylori* strain). The concentration of the extracts used in these experiments were 4× MIC (0.4 mg/ml), 2× MIC (0.2 mg/ml), 1×MIC (0.1 mg/ml) and ½× MIC (0.05 mg/ml).

4.4 Phyto-chemical analysis

Phytochemical analysis of the acetone and ethanol leaf extract of *L. javanica* revealed the presence of the following secondary metabolites: flavanoids, saponins, tannins, steroids and alkaloids (Table 4). The presence of precipitate or turbidity was an indicative for the presence of alkaloids, tannins.

Table 4 Phytochemical screening of the acetone and ethanol extract of *L. javanica.*

		Acetone	Ethanol
Components	Reaction	Reactivity	
Alkaloids	Turbid/Precipitate	+++	+++
Flavanoids	Red/Orange color	+++	+++
Tannins	Blackish-green/Precipitate	++	++
Steroids	Reddish Brown Color	+++	+++
Saponins	Complete Haemolysis	+++	+++

Key: +++; Strongly present; ++; Moderately present; +; weakly present

CHAPTER FIVE

5.1 DISCUSSION AND CONCLUSION

Antibiotic resistance is a worldwide phenomenon. Currently, antibiotic resistance by bacteria has become a worrisome phenomenon in developing countries, especially in sub-Saharan Africa. This situation is more likely related to the recurrent use or misuse of drugs (Mulu *et al*., 2004). The antimicrobial effects of various plants have been well documented for centuries, especially in traditional medicine (Nostro *et al*., 2000; Gislene *et al*., 2000; Nostro *et al.,* 2005). Findings from previous studies have documented plant based substances known to exhibit a very good inhibitory effect. This has led to a search for antimicrobials that could inhibit various drug-resistant microorganisms (Li *et al*., 2005; Steenkamp *et al.,* 2007). This study investigated the anti-*H. pylori* activity of *Lippia javanica,* a South African medicinal plant, in a bid to find potential source of active anti-*H. pylori* agents that could be cheap, readily available.

The plant was investigated for anti-*H. pylori* properties. Most of the extracts exhibited antimicrobial activity with an inhibition zone diameter of ≥15 mm by agar well diffusion assay. Precisely the zones ranged from 0 – 36 mm (Table 1). Our result corresponds with the findings of Atapour *et al*. (2009), who also reported an inhibition zone diameter of ≥15 mm of their medicinal plant extracts of *Bunium persium*, *Heracleum persium*, *myrtus communis*, *Nigella sativa*, *Pimpella anisum*, *Salvia mirzayanii*, *Teucrium polium*, *Zataria multifllora* against clinical isolates of *H. pylori*. Among the plants tested, *Salvia mirzayanii*, *Heracleum persicum*, *Zataria multiflora*, and *Teucrium polium* exhibited the strongest anti-*H. pylori* activities with a diameter range of 20 – 30 mm. This is also in line with a study conducted by

Ndip *et al*. (2007), that revealed the antimicrobial activity of plant extracts of *Ageratum conyzoides*; *Scleria striatinux*; *Lycopodium cernua*; *Emilia coccinea*; *Eryngium foetidium*; *Euphorbia hirta*, *Tapienachilus ananassae*; *Aulatandria kamerunensis*; *Acanthus montanus* and *Scleria verucossa* against the tested isolates with the zone of inhibition ranging from 0-30 mm. Samie *et al.* (2009) screened 16 South African medicinal plants including *L. javanica* against *Campylobacter* isolates; and reported an MIC ranging from 1.25 - 10 mg/mL. According to their findings, *L. javanica* and *Ptenocarpus angolensis* extract exhibited the strongest inhibitory activity compared to other plant extracts tested. To achieve effective treatment, antimicrobials that can penetrate a cell at high concentrations should be chosen and the duration of the therapy should be set properly (Huang *et al*., 2004). The results revealed that *L. javanica* extracts were able to inhibit the growth of *H. pylori* at concentrations ranging from 10 - 20 mg/mL.

Acetone and methanol extracts of several plants show promise as templates for the design of new anti-*H. pylori* therapies. In this study, the acetone crude extract was observed to be the most active against all the tested strains of *H. pylori*. The crude extract exhibited a 100% inhibition, followed by ethanol extract (60%) and methanol (58%). The least activity was observed for the aqueous extract (16%). This is in line with previous studies, which also documented the inhibitory effects of methanolic extracts of medicinal plants (Ndip *et al.*, 2008).

Also, the acetone and methanol extracts of *L. javanica* were found to be active against most bacteria responsible for respiratory aliments (Samie *et al.*, 2005), which is in line with our results. The results of this study revealed that water extract exhibited poor antimicrobial activity as compared to other extracts at 10 - 20 mg/mL where only 16% of the strains were inhibited with a zone of inhibition ranging from 0 - 30 mm. However, Steenkamp *et al.* (2007) reported that aqueous extracts of 16 plant species including *Afzelia quanzensis*,

Asparagus falcalus Thumb, Bruckenridgea zanguebarica, Bridelia macarantha, Burkea Africana, Combretum molle, Ficus sycomorus L, and *Peptoforum africanum sond* inhibited *Candidiasis* growth.

Organic solvents are better for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents (Kubmarawa *et al*., 2007; Arif *et al*., 2009). Literature documents that *Ficus sycomorus* organic stem extracts exhibited higher antifungal activity than the aqueous extracts (Kubmarawa *et al*., 2007; Hassan *et al.,* 2007; Meda *et al*., 2008). However, Arif *et al*. (2009) documented that chloroform extract of *Caesalpinia bonduc* seed exhibited antimicrobial activity against all tested bacterial strains except *Staphyloccocus aureus* and *Pseudomonus aeruginosa*. According to our results, although chloroform extracts showed an inhibitory effect against *H. pylori* strains with a zone diameter of inhibition ranging from 0 - 18 mm, compared to other extracts, the tested isolates were 39% susceptible to chloroform extract. This plant has been used in folk medicine as a mosquito repellent in various parts of South Africa for centuries as well as in Zimbabwe where a significant activity of the essential oils have been shown with a lasting repellent activity against starved *Anopheles arabienis* (Katsvanga and Chigwaza, 2004; Samie *et al*., 2005). Bergonzelli *et al*. (2003) reported the antimicrobial effects of essential oils of *C. carvi* which however remained weakly active against *H. pylori.*

The differences in the observed activities of the various extracts may be due to varying degrees of solubility of the active constituents in the five solvents used. It has been documented that different solvents have diverse solubility capacities for different phyto-constituents. However, previous studies have shown that the solvents used in extraction affect the antimicrobial activities of plants. It is known that different solvents extract different compounds, implying that some bioactive components can only be extracted by polar

solvents while others by less polar and yet some by non-polar solvents (Ogundare *et al.*, 2006; Ndip *et al*., 2007; Njume *et al*., 2009).

Compared with clarithromycin, the extracts showed inhibitory effects on the growth of *H. pylori* at higher concentrations; however, the results suggest that the extracts have a considerable antibacterial activity against *H. pylori.* The results revealed that the tested strains were 55% susceptible to clarithromycin. Tanih *et al.* (2010) recently documented that, *H. pylori* strains were 80% susceptible to clarithromycin. However, microorganisms have been reported to be resistant to clarithromycin by other investigators (Franzin *et al*., 2000; Wang *et al* 2000; Pace *et al*., 2007). Although clarithromycin is an expensive macrolide, cross-resistance linked with the use of other less expensive macrolides may be responsible for this resistance, since it is not used frequently by patients. However, clarithromycin-susceptible and resistant strains have been identified from patients with no history of exposure to macrolides, which may suggest that administration of clarithromycin may select for the resistant strains and therefore must be guided by empirical treatment (Tanih *et al*., 2010). The difference in the antimicrobial activities of plants and antibiotics can be a result of difference in quality and variety of compounds found in a plant. As well as for antimicrobials to be effective, they must attain a sufficient high concentration at target site in order to exert their antibacterial action (Adeleke *et al*., 2004).

In the current study, it was revealed that amongst the extracts evaluated for MIC; acetone, methanol and ethanol extracts exhibited the strongest antimicrobial activity; the least was observed for chloroform and water extracts (Table 3). It was observed that the MIC ranged from 0.00195 - 0.25 mg/mL for acetone extracts (MIC_{50}), while the methanol extract ranged from 0.00195 - 0.625 mg/mL. Ndip *et al*. (2007) reported a similar finding with an MIC range of 0.1698 - 0.2336 mg/mL for the methanol extracts of some plants tested against *H. pylori* strains. Atapour *et al*. (2009) also documented strongest antimicrobial activity of *S.*

mirzayanii plant extract against clinical isolates of *H. pylori* with an MIC ranging from 32 – 64 µg/mL. In another study, the methanol extract of the seeds of *C. carvi* had a MIC value of 100 µg/mL(Mahady *et al*., 2005; Atapour *et al*., 2009). The results also correspond with the investigation conducted by Same *et al*. (2009) on *Campylobacter* isolates; acetone and methanol extracts of the plant were active at a MIC range of 0.09-6 mg/mL. Aiyegoro *et al*. (2009) also reported activity for acetone and methanol crude extract of *Helichrysum longifolium* with a MIC range of 0.5 - 5 mg/mL on two isolates of *Pseudomonas aeruginosa* ATCC 19582 at a concentration of 0.1 mg/mL.

The MIC values of the extracts and antibiotics were statistically compared to determine any variation in their efficacy against the isolates. No statistically significant difference ($P > 0.05$) was observed between the MIC and antibiotic values of the five extracts. Ndip *et al.* (2007) compared MIC and MBC values of three plant extracts which revealed no statistically significant difference ($P > 0.05$) against *H.pylori* isolates. Li *et al*. (2005) recorded anti- *H. pylori* actions with MIC range of 60µg/mL on ethanol extracts of *Hippophae rhamnoides, Fritillaria thunbergii, Magnolia officinalis* and *Schisandra chinensis*, *Corydalis yanhusuo, Citrus reticulata, Bupleurum chinense* and *Ligusticum chuanxiong*. The results recorded MIC_{50} of ethanol extract ranging from 0.00195 - 2.5 mg/mL. On the other hand, MIC_{90} of ethanol extract ranged from 0.00975 - 0.313 mg/mL. High MIC values however, is an indication of lack of efficacy of the plant extracts against the test bacteria and/or the possibility that the bacteria may possess the capacity to develop resistance against the plant extracts. Thus, variable consequent activity of a plant and its components have been identified according to geographical strata of the plant at a time, as plant material may be collected at different times as well as different regions that might have contributed to different activities of the plant on the tested bacteria (Samie *at al*., 2005; Viljoen *et al*., 2005; Atapour *et al*., 2009; Oliver *et al*., 2009).

The varied activities reported in the different studies may be attributed to the type of bacterial species studied, the extracts, and the potential difference in plant chemistry of different samples as well as the season in which the plants were collected. Climate changes are amongst other factors that have been reported to affect antimicrobial activity of plants (WHO, 1992). Also, the antimicrobial effects of *L. javanica* might be due to some phytochemicals present in the plant including; linalool, β-carophyllene, and myrcene. It has been documented that essential oils of this plant are active against most bacterial species tested (Samie *et al*., 2005; Viljoen *et al.*, 2005). The antimicrobial activity of the plant may be directly related to the specific composition of the oil, therefore, activity of the plant may also fluctuate (Viljoen *et al*., 2005). It has also been reported that three compounds from *L. javanica* are able to inhibit the HIV-1 transcriptase enzyme (Fritz *et al*., 2006; Oliver *et al*., 2009).

The time-kill assay was performed to investigate the bactericidal activity of *L. javanica* on selected *H. pylori* strains. *L. javanica* was tested on the basis of MIC's obtained in the study. Comparison of the time kill plots of the ethanol and acetone extracts studied showed that the killing rate was greater for ethanol extract and very little reduction of microbial population was observed for the acetone extracts. Akinpelu *et al*. (2009) reported the bactericidal activities of the fractions of *G. kola* using viability studies. As the concentration of the fractions increased, the microbial population decreased. However, in the results obtained the efficacy of acetone and ethanol crude extract showed a killing rate within 0 - 12 h at all concentrations being tested. A greater effect of inhibition was shown with ethanol crude extract having the ability to inhibit the growth of *H. pylori* at 4× MIC as well as 2× MIC for a period of 72 h. To a lesser extent, the growth of *H. pylori* decreased at concentrations of ½× MIC, MIC, and 2× MIC within a period of 18h and a sudden increase in colony count was observed at 36h. This might be due to the fact that *H. pylori* is a slow grower. A reduction in colony count was then observed at ½× MIC and MIC after the 36h.

These results are interesting and justifies the use of *L. javanica* in the treatment of aliments' that are due to bacterial infections. The bactericidal activities of the crude extracts on the clinical isolates used in this study are worth noting, because these strains were isolated from gastric presenting patients. The inhibitory levels of the crude extracts of the plant was bacteriostatic or bactericidal. Also, the rate of kill of the extract appeared to be both concentration and time dependent which corresponds with other studies (Same *et al*., 2005; Ali *et al*., 2005; Akinpelu *et al*., 2009).

The phytochemical analysis of the extract of *L. javanica* revealed the presence of flavonoids, tannins, steroids, saponins and alkaloids. The presence of alkaloids which turned out to be strongly active, is a manifestation that this plant species (*Lippia javanica*) can be a very good source of medicine. Since some of the 30 known alkaloids are used in medicine today, we think that, it is important for the specific type of alkaloid present in the leaves of the *L. javanica* plant be isolated in the near future. Further, flavonoids present in the leaves are an indication of the plant's antiviral, antifungal, anti-inflammatory and cytotoxic activities (Ezeifeka *et al*., 2004). These phytochemical compounds are known to play important roles in bioactivity of medicinal plants and as such, produce definite physiological actions on the human body (Kamboj *et al*., 2008; Kamboj *et al*., 2009). Several reports on flavonoids, triterpenoids and steroids, which are part of the phytochemical constituents of *L. javanica*, showed that these molecules exhibit a wide range of biological activities, one of which is their ability to scavenge for hydroxyl radicals (free readicals), and superoxide anion radicals, and thus health promoting in action (Ferguson, 2001; Tasleem, 2009).

Literature has also documented the antimicrobial and resistance modulating potentials of naturally occurring flavonoids and polyphenolic compounds (Just *et al.*, 1998; Mansouri *et al*., 2001; Hodeck *et al*., 2002; Foroumadi *et al*., 2002; Sato *et al*., 2004; Cushnie and Lamb,

2005; Li *et al*., 2005; Ahmad *et al*., 2007; Aiyegoro *et al*., 2009). Some of these compounds like polyphenols have been revealed to exert their antibacterial activity through membrane perturbations. This perturbation of the cell membrane together with the action of β-lactams on the transpeptidation of the cell membrane could lead to an improved antimicrobial effect of the combination (Esimone *et al*., 2006; Sibanda and Okoh, 2008). Studies have also shown that some plant derived compounds can improve the *in vitro* activity of some peptidoglycan inhibiting antibiotics by directly attacking the same site in the cell wall (Zhao *et al*., 2001; Vu *et al.,* 2002). The presence of flavonoids in plant extract could be responsible for their anti-*H. pylori* activity.

Saponins, tannins and steroids are also very important compounds, thereby making this plant species a very good source of medicines and other important products. Another phytochemical compound observed to be present in *L. javanica* leaf extract is tannins. Tannins exert antimicrobial activities by iron deprivation, hydrogen binding or specific interactions with vital proteins, such as enzymes in microbial cells (Dharmananda, 2003; Manenzhe *et al*., 2004; Njume *et al*., 2009). According to literature, herbs that contain tannins are mordant in nature and are used for curing intestinal disorders, such as diarrhoea, dysentery and gastritis (Samie *et al*., 2009). Tannins have been observed to have an extraordinary activity in cancer deterrence (Li *et al*., 2003). In addition, Motal *et al*. (1985) revealed tannins to be useful in the treatment of inflamed or ulcerated tissues.

Consequently, the existence of tannins in *L. javanica* supports the traditional medicinal use of this plant in the treatment of diseases caused by microorganisms. It was also documented by Just *et al*. (1998) that sapononis revealed inhibitory effects on inflamed cells. The leaves of *L. javanica* could be a potential source of active antimicrobial agents, and a detailed assessment of their *in vivo* potencies needs to be investigated as antibiotic resistance of microorganisms to infectious diseases persists.

5.2 CONCLUSION

Based on the results obtained, the following conclusions can be drawn:

1. The study demonstrated *in vitro* activities of the crude extracts of *L. Javanica*, acetone, methanol and ethanol extracts gave a diameter zone of inhibition of extract ranging from 0 – 36 mm.
2. The MIC_{50} of the acetone extract of the leaves of *L. javanica* ranged from 0.00195 - 0.625 mg/mL; 0.0048 - 0.625 mg/mL for metronidazole and 0.0048-1.25 mg/mL for amoxicillin. On the other hand, MIC_{90} ranged from 0.039 - 0.625 mg/mL for acetone, 0.0097 - 5 mg/mL for metronidazole and 0.039 - 2.5 mg/mL for amoxicillin.
3. Complete inhibition of growth of *H. pylori* by *L. javanica* ethanol extract was observed at 2XMIC and 4X MIC for a period of 72 hours.
4. Phytochemical studies revealed the presence of several compounds including: alkaloids, flavanoids, tannins, saponnins and steroids, which could be potential sources for novel drugs to support the traditional medicine health care systems.

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APPENDIX 1

1.1 Ethical clearance

ECDoH-Res0002_

Eastern Cape Department of Health

Enquiries:	Zonwabele P. Merile	Tel No:	040 609 3408
		Fax No:	040 609 3784
Date:	12th June 2008		
e-mail address:	zonwabele.merile@impilo.ecprov.gov.za		

Dear Prof. Roland N. Ndip

Re: Prevalence and transmission of Helicobacter pylori in the Eastern Cape Province: Impact of water sources and household hygiene

The Department of Health would like to inform you that your application for conducting a research on the abovementioned topic has been approved based on the following conditions:

1. During your study, you will follow the submitted protocol and can only deviate from it after having a written approval from the Department of Health in writing.
2. You are advised to ensure observe and respect the rights and culture of your research participants and maintain confidentiality and shall remove or not collect any information which can be used to link the participants. You will not impose or force individuals or possible research participants to participate in your study. Research participants have a right to withdraw anytime they want to.
3. The Department of Health expects you to provide a progress on your study every 3 months (from date you received this letter) in writing.
4. At the end of your study, you will be expected to send a full written report with your findings and implementable recommendations to the Epidemiological Research & Surveillance Management. You may be invited to the department to come and present your research findings with your implementable recommendations.

Your compliance in this regard will be highly appreciated.

DEPUTY DIRECTOR: EPIDEMIOLOGICAL RESEARCH & SURVEILLANCE MANAGEMENT

University of Fort Hare
Together in Excellence

GOVAN MBEKI RESEARCH AND DEVELOPMENT CENTRE
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E-mail: pstrijdom@ufh.ac.za or rflanegan@ufh.ac.za

16 January 2008

TO WHOM IT MAY CONCERN

I declare that I have reviewed the attached Research Protocol with attachments of Prof Roland N Ndip, entitled "Prevalence and transmission of *Helicobacter pylori* in the Eastern Cape Province: Impact of water sources and household hygiene", which will be conducted under the auspices of the University of Fort Hare, Alice, South Africa.

The research, which does involve subjugation of humans as research objects, has been judged to be relevant, designed in accordance with accepted scientific practices and norms, as well as – particularly – in harmony with universally accepted international standards and ethical practice in its use of human persons as subjects of research and is in the opinion of the reviewer likely to be successful in achieving its objective.

The researcher has designed purpose-specific informed consent forms which are simple, properly designed and user-friendly in order to protect the interests of human subjects, enabling their understanding of all implications of consent to participate.

Yours sincerely

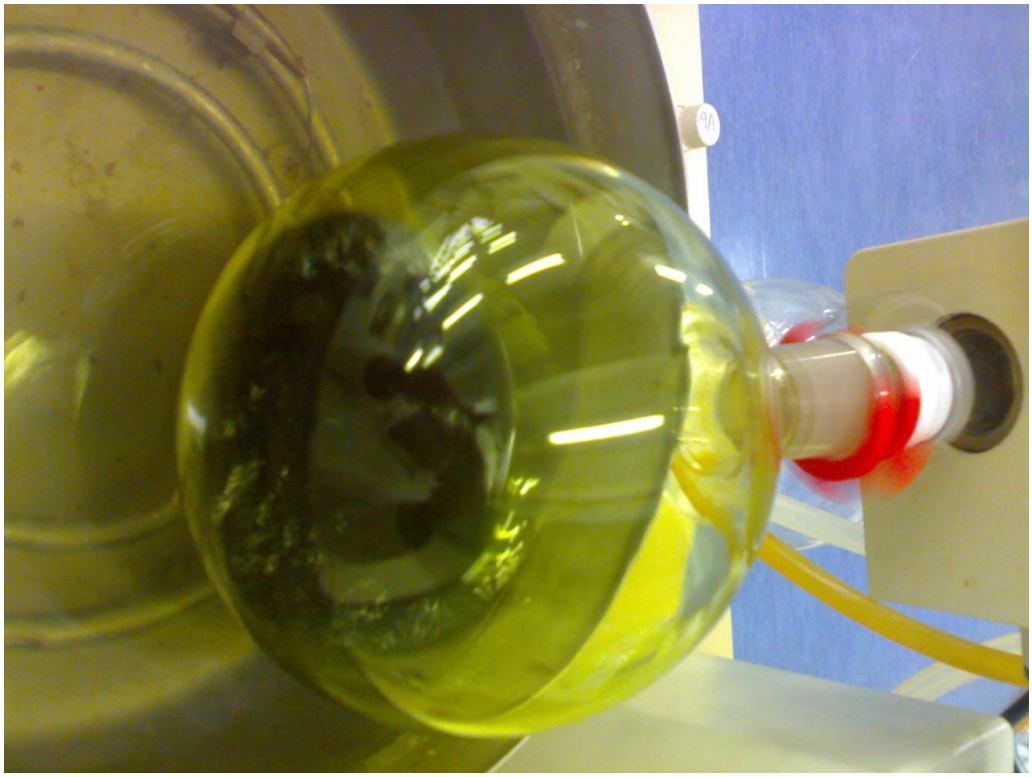
Dr Petrus DF Strijdom
Acting Dean of Research & Development

APPENDIX 2

2.1 Photographs of plant species, laboratory preparations & susceptibility plates

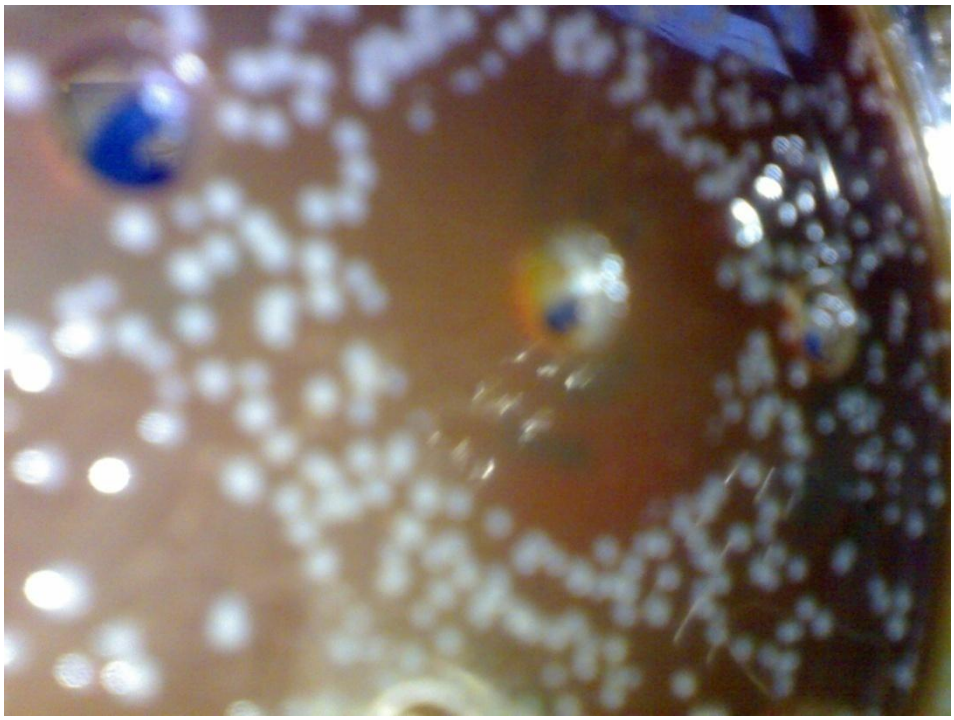
Lippia javanica plant species.

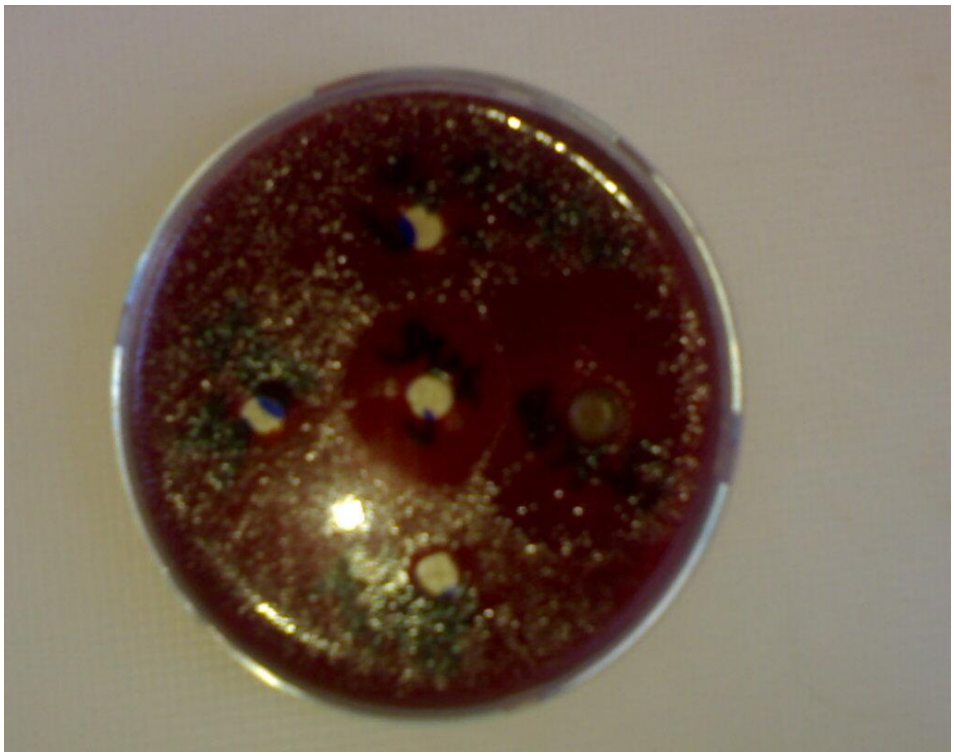
Dissolved plant extracts in organic solvents and water.



Extraction process of *L. javanica* using a Rotavapor system.

Susceptibility plates





Zone diameter of inhibition in agar plates.

APPENDIX 3

Statistical observation

Descriptives

MIC $_{50S}$

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	29	.17305	.334951	.062199	.04564	.30046	.002	1.250
2	29	.22513	.386543	.071779	.07810	.37216	.002	1.250
3	26	.20040	.537861	.105483	-.01684	.41765	.002	2.500
4	29	.11974	.267412	.049657	.01802	.22146	.005	1.250
5	25	.11193	.272764	.054553	-.00067	.22452	.005	1.250
Total	138	.16687	.368602	.031378	.10482	.22892	.002	2.500

1=acetone, 2=methanol, 3=ethanol, 4=amoxicillin, 5=metronidazole

Multiple Comparisons

MIC

Tukey HSD

(I) Extracts	(J) Extracts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.052081	.097533	.984	-.32181	.21765
	3	-.027356	.100307	.999	-.30476	.25005
	4	.053309	.097533	.982	-.21642	.32304
	5	.061122	.101359	.974	-.21919	.34144
2	1	.052081	.097533	.984	-.21765	.32181
	3	.024725	.100307	.999	-.25268	.30213
	4	.105390	.097533	.816	-.16434	.37512
	5	.113203	.101359	.797	-.16711	.39352
3	1	.027356	.100307	.999	-.25005	.30476
	2	-.024725	.100307	.999	-.30213	.25268
	4	.080664	.100307	.929	-.19674	.35807
	5	.088478	.104031	.914	-.19923	.37618
4	1	-.053309	.097533	.982	-.32304	.21642
	2	-.105390	.097533	.816	-.37512	.16434
	3	-.080664	.100307	.929	-.35807	.19674
	5	.007814	.101359	1.000	-.27250	.28813
5	1	-.061122	.101359	.974	-.34144	.21919
	2	-.113203	.101359	.797	-.39352	.16711
	3	-.088478	.104031	.914	-.37618	.19923
	4	-.007814	.101359	1.000	-.28813	.27250

1=Acetone, 2=Methanol, 3=ethanol, 4= Amoxicillin, 5= Metronidazole

MIC 90s

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean		Minimum	Maximum
					Lower Bound	Upper Bound		
1	25	.44528	.540840	.108168	.22203	.66853	.039	2.500
2	29	.36762	.362861	.067382	.22960	.50565	.039	1.250
3	27	.17298	.152287	.029308	.11274	.23322	.002	.625
4	23	.43048	.534530	.111457	.19933	.66163	.039	2.500
5	14	2.12120	2.035323	.543963	.94604	3.29636	.010	5.000
Total	118	.55984	.975791	.089829	.38194	.73774	.002	5.000

Multiple Comparisons

MIC

Tukey HSD

(I) Extracts	(J) Extracts	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.077659	.217013	.996	-.52397	.67929
	3	.272299	.220703	.732	-.33956	.88416
	4	.014802	.229744	1.000	-.62212	.65173
	5	-1.675916*	.265434	.000	-2.41178	-.94005
2	1	-.077659	.217013	.996	-.67929	.52397
	3	.194639	.212653	.890	-.39490	.78418
	4	-.062858	.222022	.999	-.67837	.55266
	5	-1.753576*	.258779	.000	-2.47099	-1.03616
3	1	-.272299	.220703	.732	-.88416	.33956
	2	-.194639	.212653	.890	-.78418	.39490
	4	-.257497	.225630	.784	-.88302	.36802
	5	-1.948215*	.261881	.000	-2.67423	-1.22220
4	1	-.014802	.229744	1.000	-.65173	.62212
	2	.062858	.222022	.999	-.55266	.67837
	3	.257497	.225630	.784	-.36802	.88302
	5	-1.690718*	.269545	.000	-2.43798	-.94345
5	1	1.675916*	.265434	.000	.94005	2.41178
	2	1.753576*	.258779	.000	1.03616	2.47099
	3	1.948215*	.261881	.000	1.22220	2.67423
	4	1.690718*	.269545	.000	.94345	2.43798

*. The mean difference is significant at the 0.05 level.

1=acetone, 2=methanol, 3=ethanol, 4=amoxicillin, 5=metronidazole

APPENDIX 3

Manuscript submitted.

Nkomo LP, Mkwetshana NT, Green E, Clarke AM, Ndip RN (2010). *In vitro* bioactivity of crude extracts of the leaves of *Lippia javanica* against clinical isolates of *Helicobacter pylori.* African Journal of Pharmacy and Pharmacology.