IN VITRO ACTIVITY OF BIOACTIVE COMPOUNDS OF SELECTED SOUTH AFRICAN MEDICINAL PLANTS ON CLINICAL ISOLATES OF *Helicobacter pylori*

By

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A dissertation submitted in fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Biochemistry and Microbiology

University of Fort Hare

2011

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DECLARATION

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

Name:…….

Signature:……

Supervisor's signature……

Date…….

ACKNOWLEDGEMENTS

My thanks first and foremost to God Almighty, who has been the source of my energy throughout my studies. I sincerely express my profound gratitude and appreciation to my supervisor, Professor R.N. Ndip for his supervision, patience, vision, encouragement and love, surely without him; this study would not have been possible.

I am grateful to the National Research Foundation South Africa (grant reference CSUR2008052900010), and the Govan Mbeki Research and Development Centre, University of Fort Hare, South Africa for funding. I would like to appreciatively acknowledge the effort and support of Dr. N. Mkwetshana, Dr. A. Clarke, Mr. E. Green and other members of the Microbial Pathogenicity and Molecular Epidemiology Research Group (MP&MERG). I am very grateful to Dr. Naido N and the staff of the GIT unit, Livingstone Hospital, Port Elizabeth, and to Dr. A. Samie, Dr. P.O. Bessong, Ms. N.F. Tanih, Mr. C. Njume and Mr. Tshikhawe P for technical assistance.

Thanks to my parents, Mr. & Mrs. G.O. Okeleye, my siblings, my aunt and the husband, Professor & Mrs. S.O. Fatoki for their love, vision and support, may God bless you. Lastly but not least, I would like to thank my family and friends who always stood close to me, faithfully and unconditionally, Mr. & Mrs. O.O. Osunkunle, Ms. N.A. Joseph, Ms. A.M. Enitan, Mr. E.A. Adewusi, Mr. S.S. Oyedemi, Ms. T. Vukubi, Mrs. A. Badmus, Mrs. R. Scott, Mrs. P. Rudman and Mr. & Mrs. B. English, for their care and moral support, also the staff and colleagues of the Department of Microbiology and Biochemistry for all the time and support they gave to me during the course of the study.

ABSTRACT

The stem bark of *Peltophorum africanum* and *Bridelia micrantha* are used in South Africa traditional medicine for treatment of intestinal parasites, relieve stomach problems and human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS). The growing problem of antibiotic resistance by *Helicobacter pylori* the major etiological agent in gastritis, gastric cancer, peptic and gastric ulcer demands the search for novel compounds from plant based sources. This study was aimed to determine the antimicrobial activity of five solvent (ethylacetate, acetone, ethanol, methanol and water) extracts of the stem bark of *P. africanum* and *B. micrantha* on clinical strains of *H. pylori* in a bid to identify potential sources of cheap starting materials for the synthesis of new drugs. *H. pylori* strains were isolated from patients presenting with gastric related morbidities at the Livingstone Hospital, Port Elizabeth for endoscopy and confirmed following standard microbiology procedures. The plant extracts including clarithromycin were tested against 31 clinical strains of *H. pylori* by the agar well diffusion method. The most potent extract was evaluated by the microdilution method to determine the Minimum Inhibitory Concentration ($MIC_{50\&90}$), followed by the rate of kill. Preliminary phytochemical analysis was carried out. The one way ANOVA test was used to statistically analyse the results. All the extracts demonstrated anti-*H. pylori* activity with zone diameters of inhibition that ranged from 0 to 23 mm for the extracts and 0 to 35 mm for clarithromycin. Marked susceptibility (100%) was recorded for the ethyl acetate extract of *P. africanum* (*P. afr*. EA) and the acetone extract of *B. micrantha* (*B. mic.* A), which were statistically significant ($P < 0.05$) compared to all other extracts and clarithromycin. For *B. micrantha* ethyl acetate extract, 93.5% susceptibility was observed while for the control

antibiotic, clarithromycin it was 58.1%. The MIC_{50} ranged from 0.0048 to 0.313 mg/mL for *P. afr*. EA, and from 0.0048 to 0.156 mg/mL for *B. mic.* EA; MIC_{90} ranged from 0.156 mg/mL to 0.625 mg/mL and 0.0048 to 2.5 mg/mL for *P. afr*. EA and *B. mic.* EA respectively. There was a significant statistical difference observed in potency of both *P. afr*. EA and *B. mic.* A compared to the two antibiotics ($P < 0.05$). One hundred percent killing by *P. afr* EA was observed at 0.05 mg/mL (½ x MIC) and 0.2 mg/mL (2 x MIC) in 66 h for strain PE466C and PE252C respectively. For *B. mic.* EA, 100% killing effect of both strains (PE430C and PE369C) was observed at 0.1 mg/mL (2 x MIC) in 66 h. Qualitative phytochemical analysis confirmed the presence of alkaloids, flavonoids, steroids, tannins and saponins in the ethyl acetate extracts of both plants, which could be a potential template of lead molecule for the design of new anti-*Helicobacter pylori* therapies.

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CHAPTER ONE

1.1 INTRODUCTION

Helicobacter pylori is one of the most common chronic bacterial pathogens of humans. Colonization is usually life long and may lead to chronic gastritis, duodenal and peptic ulceration and gastric cancer in later life (Konturek, 2004). This Gram-negative curved rod colonizes the gastric epithelial surface and withstands the stomach's hostile ambience by microaerophilic growth capacity (Lynch, 2007). In addition, it produces numerous virulence factors, the most important being urease, oxidase and catalase. The major pathogenic factor of this organism includes the cytotoxin *CagA* and *VacA* which may contribute to the pathogenesis of disease. In addition, host factors may also play a pathogenic role, because autoantibodies appear during *H. pylori* infection in certain patients (Pasceri *et al.*, 1998).

Infections have been reported to be higher in the developing than in developed countries and studies have documented high prevalences in Africa including South Africa (Samie, *et al;* 2007; Ndip *et al.,* 2008). The principal reservoir of infection is the human stomach (Go, 2002), drinking water (Bunn *et al.,* 2002) and transmission has been epidemiologically linked to person-to-person contact, and vehicle (e.g., water) (Go, 2002; Bunn *et al.,* 2002). The prevalence of *H. pylori* infection in a community is related to three factors: firstly, the rate of acquisition of infection, that is, incidence; secondly, the rate of loss of the infection; thirdly, the prolonged persistence of the bacterium in the gastroduodenal mucosa between infection and eradication. Variation in the prevalence is dominated by the great differences between communities in the incidence of infection during childhood (Pounder and Ng, 1995; Tkachenko *et al*., 2007).

The high prevalence of infection in developing countries is influenced by socioeconomic conditions, ethnic background, gender and age (Taverner and Becker, 2006). An average prevalence of 38.2% (range 20.0 - 82.0%) was reported in Europe and North America (Raghunath *et al.*, 2003). In Africa, 70 to 80% are infected with the organism, and 61 to 100% harbour the organism in sub-Saharan Africa (Holcombe, 1992; Ndip *et al.*, 2008). Infection occurs more often in younger individuals and among primary school children (50-80%) than in adults (50-69%) (Samie *et al*., 2007). Another study had indicated no significant difference in prevalence between rural and urban Africans (Sathar *et al.*, 1994).

It is known that *H. pylori* could be eradicated by a combination of therapeutic agents such as antibiotics, bismuth subsalicylate, proton pump inhibitors and H2-blockers which 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 (Hentschel *et al.*, 1993; Sepulveda and Coelho, 2002). However, the cure thus achieved is incomplete and undesirable side effects are known to occur (Stamatisa *et al*., 2003). Eradication failure rate remains as high as 5–20% along with frequent relapses of gastric ulcers even after the discerned 'complete healings' (Bazzoli *et al.*, 1994, Bell *et al.*, 1995; Bayerdörffer *et al.*, 1995). This situation could be attributed to a couple of reasons such as lack of penetration of antibiotics into the depth of gastric mucosa, drug resistance of the bacterium developed during repeated applications of anti-*Helicobacter pylori* agents and poor compliance on the part of the patient and/or ulcerogenic side-effects of some drugs co-administered imperatively for the management of other accompanying diseases (Adamek *et al.*, 1998); therefore, alternative available means of eradicating or preventing infections is required (O'Gara *et al.*, 2000).

Antibiotic resistance in *H. pylori* varies according to geographical region. Resistance to metronidazole and clarithromycin was reported to be significantly higher globally and mostly in females than in males (Glupczynski *et al.,* 2001; Mégraud, 2004; Ndip *et al.,* 2008). Until recently, resistance to amoxicillin was considered to be absent or very rare; however, amoxicillin-resistant *H. pylori* strains have now been identified in different countries (Kohanteb *et al.,* 2007). For example, amoxicillin resistant strains was reported to be up to 85.6% in Cameroon (Ndip *et al.,* 2008), 31% to 45% in Italy, and 41.2% in China (Wu *et al.,* 2000). Tetracycline which has also been used extensively as therapy in other types of infection, such as non-gonococcal urethritis, in recent years, was reported not resistant in *H. pylori* therapy not until 1996, when Midolo and colleagues observed tetracycline-resistant strains from Australia (Midolo *et al.,* 1996). The emerging resistance to antibiotics limits their use in the treatment of infections and this problem is encountered more in Africa (Smith *et al.,* 2001; Asrat *et al.,* 2004, Sherif *et al.,* 2004).

On the other hand, the anti-*H. pylori* activity of traditional herbal medicines has not been extensively studied. Accordingly, there is a growing need of finding new anti-*H. pylori* agents that can hopefully eradicate the invasion and presence of resistant strains of *H. pylori* to avoid relapses of gastric ulcer (Li *et al.,* 2005). Phytomedicine has shown great promise in the treatment of intractable infectious diseases in Africa (Iwu *et al.,* 1999). It is estimated that plant materials are present in or have provided the models for about 50% of Western drugs (Harbone, 1998), and herbal remedies continue to play a role in the cure of diseases (Tabuti *et al.,* 2003; Ndip *et al.,* 2007); however, most of these claims do not have any scientific backing, though continuous usage of these plants in the management of infections indicates that the plants may contain constituents, which have antibacterial activities. Medicinal plants such as *Xanthum*

brasilicum, *Thymus copticum*, *Ageratum conyzoides*, *Scleria striatinux* and *Lycopodium cernua* have shown promising anti-*Helicobacter pylori* activities (Nariman *et al.*, 2004; Mahady *et al.*, 2005; Ndip *et al.*, 2007).

Some anti-*Helicobacter pylori* natural products have been reviewed in previous studies, for example, oil-macerated garlic constituents, fatty acids and terpenes in *Aristolochia paucinervis*, costunolides in *Magnolia sieboldii* and some acidic and alkaline constituents in *Coptis japonica*, *Eugenia caryophyllata*, *Magnolia officinalis* and *Rhus javanica*. Several results, along with the observation, suggested that a wider range of phytochemicals could be anti-*Helicobacter pylori*, rationalizing the utility and efficacy of traditional medicines in the treatment of gastric ulcers (Li *et al*., 2005).

The anti-inflammatory properties of several phytomedicines' origin, that contain substances like phytoestrogens, flavonoids and its derivatives, phytosterol, tocopherol, ascorbic acid, curcumin, genistein, and others, can be inhibitors of the molecular targets of pro-inflammatory mediators in inflammatory responses (Iwalewa *et al*., 2007). There are other plants that contain alkaloids, tannin, saponins, anthraquinones, triterpenoids and other constituents which have been reported to possess a diverse range of bioactivities including anticancer, immunostimulatory, antibacterial, antimalarial and antituberculosis activities. Bearing in mind that some of the causative organisms such as *H. pylori* infections and factors responsible for initiating and promoting inflammation could be removed or neutralised to suppress the expression of pro-inflammatory agents and based on the molecular events that lead to inflammation, it has been suggested that the process of sustained inflammation that accompanies chronic diseases like

gastritis, ulcers and gastric cancer can be ameliorated and possibly even prevented by phytomedicines (Iwalewa *et al*., 2007).

Peltophorum africanum (Sond Fabaceae), also known as Weeping Wattle in English (Musese in Venda) is a semi-deciduous to deciduous tree of about 15m with a spreading, untidy canopy widespread in South Africa. The stem bark has been traditionally employed in South Africa to clear intestinal parasites, relieve stomach problems, treat human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS), infertility and a sore liver (Samie *et al.*, 2005; Bizimenyera *et al*., 2006).

Bridelia micrantha (Hochst., Baill., Euphorbiaceae), also known as Coast gold leaf in English (Munzere in Venda) is a semi-deciduous to deciduous tree up to 20 m tall with a dense rounded crown and tall, bare stem widespread in South Africa. The stem bark has been traditionally employed in South Africa to treat several clinical conditions including intestinal parasites, gastritis, salmonellosis and gastro-enteritis, stomach problems, HIV/AIDS, infertility, neurosis and psychosis (Bessong *et al.,* 2006; Iwalewa *et al.,* 2007). This plant has been reported to possess compounds like friedelin, a-amyrin, gallic acid, luteoforol and also being active against *Escherichia coli, Klebsiella pneumoniae, Shigella flexneri, Pseudomonas aeruginosa* and some other organisms (Samie *et al.,* 2005).

However, to the best of our knowledge, these plants have not been evaluated for their antimicrobial activity on clinical isolates of *Helicobacter pylori* isolated in South Africa, especially when this organism is known to exhibit profound heterogeneity compounded by an emerging trend of resistance to the current treatment regimen employed in South Africa (Tanih *et*

al., 2010); hence the search of potential sources of cheap starting materials for the synthesis of new drugs against the pathogen becomes imperative.

1.2 HYPOTHESIS

Peltophorum africanum and *Bridelia micrantha* can provide potent and cheap regimen with anti-*H. pylori* activity.

1.3 RESEARCH OBJECTIVES

1.3.1 Overall objective

This study was therefore aimed at investigating the anti-*Helicobacter pylori* activity of *Peltophorum africanum* and *Bridelia micrantha* on clinical isolates of *H. pylori.*

1.3.2 Specific objective

To test our hypothesis we proposed the following specific objectives

1. Screen the plant extracts for anti-*H. pylori* activity.
2. Determine the minimum inhibitory concentration ($MIC_{50\&90}$).
3. Determine the rate of kill of the plant extracts.
4. Identify the phytochemical compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 INTRODUCTION

The discovery of *Helicobacter pylori* sounds like a chapter taken from the exciting book, Microbe Hunters written in 1926 by Paul de Kruif. It marked the discovery of microbes causing infectious diseases that ravaged the world's population at the time (Konturek, 2004; Lynch, 2007). *H. pylori* is one of the most genetically diverse of bacterial species, and is a major cause of at least 90% of duodenal ulcers, 70% of gastric ulcers, non-ulcer dyspepsia, gastro-oesophageal reflux disease, adenocarcinoma of the distal stomach and mucosa-associated lymphoid tissue (MALT) lymphoma in many societies (Tanih *et al*., 2010).

Despite years of experience with *H. pylori* treatment, the ideal regimen for treating the infection remains elusive. The most effective eradication treatment is the combination of a proton pump inhibitor with 2 antibiotics, but many (10-20%) of the patients don't get complete eradication of the infection. Antibiotic resistance is a major factor affecting the outcome of treatment which is a growing global concern that needs public health attention (Mégraud, 2004). This demands the search for novel compounds from plant based sources. Medicinal plants have become important elements in indigenous medicinal systems and a number of scientific investigations have highlighted the importance and contribution of many plant families. Although there are several promising lead studies which are for the most part preliminary, additional work is needed before any of these treatments can be considered viable alternatives to conventional therapy (Ndip *et al*., 2007).

2.2 PATHOGENESIS AND CLINICAL MANIFESTATION OF *HELICOBACTER PYLORI* INFECTION

Colonization with *H. pylori* virtually always lead to infiltration of the gastric mucosa in both the antrum and corpus with neutrophilic and mononuclear cells. This chronic active gastritis is the primary condition related to *H. pylori* colonization; other *H. pylori*-associated disorders in particular result from intragastric distribution and severity of this chronic inflammatory process may depend on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production (Kusters *et al*., 2006). Understanding these factors is thus crucial for the recognition of the role of this organism in the etiology of upper gastrointestinal pathology.

Its helicoidal shape and the action of flagella allow it to cross the thick layer of mucus lining the stomach. It then binds to Lewis antigens present on host gastric cells, and secrete factors that attract and stimulate inflammatory cells, as well as the multifunctional toxin *vacA*. *H. pylori* produces chemical components in their cell walls that are very much like molecules made by the stomach cells of the host which creates a problem for the immune system (Lynch, 2007). Among the clinical consequences of this *H. pylori*-initiated autoimmune process are achlorhydria with secondary hypergastrinemia, with or without pernicious anemia, and increased risk for gastric enterochromaffin-like cell carcinoids and cancer (Tanih *et al*., 2008).

Acute infections with *H. pylori* may cause a transient clinical illness, characterized by nausea and abdominal pain that may last for several days. After these symptoms resolve, the majority of people progress to chronic infection. However there is no recognizable symptom complex or syndrome that can be ascribed to chronic gastritis, whether or not due to *H. pylori*. Peptic ulcer

often present with dyspepsia. There may be pain at night; some patients report relief of pain with food or antacids and a recurrence of pain in two to four hours (Kusters *et al*., 2006; Tanih *et al*., 2008).

A possible association between *H. pylori* infection and idiopathic sideropenic anemia has been suggested with resolution of anemia observed in some cases after cure of the infection. Another interesting clinical manifestation related to *H. pylori* infection is growth retardation. A Danish study involving 1756 women showed that the infection is related to late menarche and has also been linked to several skin diseases (Realdi *et al.,* 1999). An association with chronic idiopathic urticaria has been proposed with the pathogenic mechanism related to an increase in gastric vascular permeability during *H. pylori* infection, resulting in a greater exposure of the host to alimentary allergens. Infection has also been linked to functional vascular diseases; primary headache has recently been implicated as related to *H. pylori* infection (Realdi *et al.,* 1999).

Respective immunological studies of systemic and local T- and B-cell responses in patients chronically infected with *H. pylori* have shown that the numbers of CD4+ and CD8+ T cells are increased in the gastric mucosa of patients with *H. pylori* gastritis and that T cells isolated from the antrum region are able to produce gamma interferon (IFN-) (Appelmelk *et al*., 1997). Very recently, the technique of intracellular staining of cytokines was used to demonstrate that CD4+ and CD8+ cells from the antrum region of patients with *H. pylori* gastritis produce IFN-γ but nearly no interleukin-4 (IL-4) (Bamford *et al*., 1998). Far less characterized are immune responses in the corpus region of the stomach. Because corpus-predominant *H. pylori* gastritis is associated with the appearance of autoantibodies and atrophic changes, the T-cell response in the corpus region may be different (Bamford *et al.*, 1998; Sommer *et al*., 1998). Studies have shown

that infection by more virulent *CagA*-positive strains of *H. pylori* is significantly associated with ischemic heart disease. These findings strongly suggest that the association between *H. pylori* and ischemic heart disease is related to the virulence of this bacterium (Pasceri *et al*., 1998).

2.2.1 Gastritis

Gastritis is mostly due to *H. pylori* colonization which may be associated with transient nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting, and with considerable inflammation of both the proximal and distal stomach mucosa, or pangastritis. This acute phase is often associated with hypochlorhydria, which can last for months. It is unclear whether this initial colonization can be followed by spontaneous clearance and resolution of gastritis and, if so, how often this occurs. When colonization does become persistent, a close correlation exists between the level of acid secretion and the distribution of gastritis (chronic gastritis) (Kusters *et al*., 2006). Gastritis is closely related with the activity of free radicals and these oxygen-derived free radicals can initiate membrane damage by lipid peroxidation which plays a major pathological role in the pathogenesis of gastritis. Natural antioxidants had been justified in the treatment of this disease (Demir *et al.,* 2003).

2.2.2 Peptic ulcer disease

Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are defined as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Gastric ulcers mostly occur at the transition from corpus to antrum mucosa while duodenal ulcers usually

occur in the duodenal bulb, which is the area most exposed to gastric acid. Initial reports from all over the world in the first decade after the discovery of *H. pylori*, indicated that approximately 95% of duodenal ulcers and 85% of gastric ulcers occurred in the presence of *H. pylori* infection. Complications of ulcer disease include bleeding, perforation, and stricture formation. Bleeding is the most common complication of ulcer disease and is estimated to occur in 15 to 20% of ulcers (Demir *et al.*, 2003; Kusters *et al.*, 2006).

2.2.3 Non-ulcer dyspepsia

H. pylori plays a role in the etiology of dyspeptic symptoms of upper gastrointestinal distress which may have a reflux-like character, with heartburn and regurgitation as predominant signs; may appear dysmotility-like, with early satiety and nausea; or may be ulcer-like, with pain and vomiting. Thirty to 60% of patients with functional dyspepsia carry *H. pylori* and for patients with uninvestigated dyspepsia, *H. pylori* test-and-treat strategy may be an appropriate option (Talley and Hunt 1997).

2.2.4 Gastric cancer

Gastric cancer is one of the most common cancers worldwide, ranking fourth in overall frequency, and accounting for over 870,000 new cases and over 650,000 deaths annually, which is second only to lung cancer and more frequently in men than in women (Carl-McGrath *et al*., 2007). Tumors of the stomach may be either malignant (cancerous) or benign (non-cancerous) and can be classified based on gross morphological and histopathological features. Almost all

malignant tumors of the stomach are epithelial in origin and can thus be classified as adenocarcinomas. Several factors are suspected to play a role in gastric carcinogenesis, including the effects of diet, exogenous chemicals, intragastric synthesis of carcinogens, genetic factors, infectious agents and pathological conditions in the stomach (such as gastritis) (Stadtlander and Waterbor, 1999). Evidence that *H. pylori* increases the risk of gastric cancer development via the sequence of atrophy and metaplasia originates from various studies and it has been estimated that this organism colonization increases the risk approximately 10-fold and therefore designated a class I carcinogen by the WHO (Kusters *et al*., 2006). These bacteria play a role in 53% of gastric cancer cases in developing countries and in 60% in developed countries (Stadtlander and Waterbor, 1999).

2.2.5 Gastric MALT lymphoma

Gastric low grade B cell lymphomas arising from mucosa associated lymphoid tissue (MALT) are the most frequent lymphomas among those located in the primary digestive tract. There is now evidence supporting the role of *H. pylori* infection in the development of these gastric lymphomas (GLs). Indeed, *H. pylori* provide the antigenic stimulus which is mediated by mucosal T cells for sustaining growth of gastric MALT lymphoma. The morphological hallmark of MALT GL is proliferation of centrocytic-like cells and epithelial destruction that causes lymphoepithelial lesions detected on histological examination and the initial stage of tumour may also affect the results of treatment (Ruskoné-Fourmestraux *et al*., 2001). The histological criteria for the diagnosis of MALT lymphoma and the differentiation from polyclonal reactive infiltrates remain controversial. In particular, diagnosis is based on histological appearance during routine

microscopy and on demonstration of clonality by immunohistochemistry or molecular techniques, such as PCR; nearly all MALT lymphoma patients are *H. pylori* positive (Parsonnet *et al*., 2004; Kusters *et al*., 2006).

2.2.6 Gastroesophageal reflux disease (GERD)

Helicobacter pylori has been demonstrated as the causative factor of various gastrointestinal diseases; nevertheless, the relationship between its infection and gastroesophageal reflux disease is still debated (Grande *et al*., 2008). However, further studies suggested that *H. pylori* might protect against the development of this disease and as such also be of benefit to their hosts. This slowly emerging concept came from repeated observations of a low prevalence of *H. pylori* among GERD patients, particularly of more virulent strains (Kusters *et al*., 2006). It was suggested that *H. pylori* could contribute to GERD through different mechanisms: cardias inflammation causing sphincter weakness; increased acid secretion due to antral gastritis; delayed gastric emptying and cytotoxin production causing esophageal epithelium injury (Grande *et al*., 2008).

2.3 EPIDEMIOLOGY

2.3.1 Prevalence of *Helicobacter pylori*

The prevalence of *H. pylori* infection in a community is related to three factors: firstly, the rate of acquisition of infection, that is, incidence; secondly, the rate of loss of the infection; thirdly, the prolonged persistence of the bacterium in the gastroduodenal mucosa between infection and eradication. The onset of infection is from childhood, which once established may persist

throughout life (Ndip *et al*., 2004; Tanih *et al*., 2009; Dube *et al*., 2009). However, from a study in Tanzania, seropositivity rose steeply with age from 76% in children aged 0 - 4 years to 99% in adults (Mbulaiteye *et al*., 2006). In another study in Egypt, a high prevalence (72.38%) of infection was noted among school children (Mohammad *et al*., 2008).

In Bloemfontein, South Africa, a study detected a high prevalence (67 - 84%) of *H. pylori* antibodies in children (Dube *et al*., 2009). In yet another study, *H. pylori* IgG antibodies were also detected from South African children and their mothers (Mosane *et al*., 2004) and in KwaZuluNatal, 76% of Africans were seropositive and more than 50% of African children were infected by the age of 10 years. In Ivory Coast, 55% of children aged less than 10 years have been reported to be infected. In Cameroon, Nigeria and the Gambia, 50% of children less than 5 years are infected (Ndip *et al*., 2004; Tanih *et al*., 2009).

The prevalence of infection is greater in developing countries and is influenced by socioeconomic conditions, ethnic background, gender and age (Taverner and Becker, 2006). An average prevalence of 38.2% (range 20.0 - 82.0%) was reported in Europe and North America (Raghunath *et al.,* 2003). In Africa, 70 to 80% are infected with the organism and 61 to 100% harbour the organism in sub-Saharan Africa (Holcombe, 1992; Ndip *et al.,* 2008). In Nigeria, a prevalence of 73% - 84% was reported as opposed to 92.2% in Cameroon (Mustapha *et al.,* 2007; Ndip *et al.,* 2008). Prevalence rates of 75 - 85% were reported in Ghana, Kenya and Zimbabwe (Mustapha *et al*., 2007). In the Republic of South Africa, a prevalence of 83.3% was reported in Mpumalanga and 50.6% in Venda (44.5% in female and 58.6% in male) (Fritz *et al*., 2006; Samie, *et al*; 2007).

2.3.2 Risk factors and transmission of *H. pylori*

Transmission of *H. pylori* probably occurs mostly by the faecal- oral and oral- oral routes and via recently contaminated food and water and unclean hands. Variation in the prevalence of infection between and among populations suggest that parameters such as age, cultural background, genetic predisposition, socio-economic status and environmental factors all play a role in the acquisition and transmission of the organism (Tanih *et al*., 2008). The first, and most frequent, mode of transmission is iatrogenic, in which tubes or endoscopes that have been in contact with the gastric mucosa of one individual are used for another patient (considered to be marginal) and usually occupationally transmitted from a patient to endoscopists and gastroenterologists themselves (van Duynhoven and de Jonge, 2001).

H. pylori has been detected in saliva, vomitus, dental plaque, gastric refluxate and feces. DNA fingerprinting studies have confirmed the similarity of strains from children and those from their mothers thereby suggesting a possibility of mother-to-child transmission. Intraspecific transmission from mother to child has been linked to premastication of food (Kusters *et al*., 2006; Dube *et al*., 2009). Several studies have reported the presence of *H. pylori* DNA in environmental water sources, but this probably reflects contamination with either naked DNA or dead *H. pylori* organisms. To our knowledge there is only a single report of *H. pylori* being successfully cultured from water, but this involved wastewater and as such may well represent fecal contamination of the water source (Kusters *et al*., 2006).

Increasing consumption of food items has been linked as a risk factor in the prevalence rates of *H. pylori* infection. For instance a marginal association was found between consumption of chili and *H. pylori* prevalence as it is commonly believed in some regions that those who eat chili are

prone to develop peptic ulcer disease (Rodolfo *et al.,* 1998). Evaluation of dietary factors suggested that eating food from street vendors might be implicated in the acquisition of the infection. Fruits that are peeled shortly before consumption are specially recommended for travelers as a means to decrease the risk of acquiring intestinal infections (Rodolfo *et al.,* 1998; Herbarth *et al*., 2001; Cardinale *et al*., 2005).

Studies of the potential of an animal reservoir led to investigation of risk factors in persons working with animals, which suggested that abattoir workers are more frequently seropositive than other people working in the same environment. However, studies of meat as a possible vehicle showed that vegetarians and populations that abstain from eating either pork or beef show no difference in seropositivity than those with less restricted diets (Feldman *et al*., 1998).

2.4 LABORATORY DIAGNOSIS

2.4.1 Collection of biopsy specimens

The best specimens to culture *H. pylori* from are gastric biopsies obtained during endoscopy. The number of biopsies needed to diagnose infection is a subject of controversy. A single biopsy specimen taken from the antrum (2 cm from the pylorus) gives good sensitivity but is not sufficient for a reliable diagnosis. Indeed, *H. pylori* may have a patchy distribution, and the more biopsy specimens analyzed, the higher the chance of detection. The recommendation is, therefore, to take two biopsy specimens from the antrum as well as two specimens each from the anterior and posterior corpus. There are some rare cases where the infection lies only in the corpus, but usually, *H. pylori* is present in all sites. After consumption of antisecretory drugs, the

corpus may be the only site which remains positive. Biopsy specimens for culture must be taken before specimens for histological examination, the latter being introduced in a fixative, otherwise there is a risk of transferring small amounts of fixative to the container for biopsy specimens to be used for culture (Megraud and Lehours, 2007).

Antibiotics and bismuth, when used in suboptimal therapy, can suppress but not eliminate the organism. Therefore, the patient should not have used these agents for several weeks prior to the test. Following inadequate therapy, the organism may regrow in a patchy manner and may not be detected by random biopsies. Care must be taken to ensure that the patients did not receive antibiotics or antisecretory drugs, especially proton pump inhibitors (PPI), omeprazole, as this drug has been shown to inhibit growth of the organism. Although PPI have no direct antimicrobial effect at the concentration present in the gastric mucosa (Megraud *et al*., 1991), they indirectly interfere with their distribution in the stomach by changing the pH of its niche, leading to its disappearance in the antrum. It is recommended not to consume these drugs 2 weeks prior to endoscopy. Obviously, the impact also depends on the dose and length of treatment; a single dose will not be as detrimental as a substantial acid suppression. Other ulcer drugs, e.g., sucralfate, have not shown any effect (Megraud and Lehours, 2007).

2.4.2 Microbiological isolation of the organism

In most instances, the bacteria are not distributed homogeneously in the biopsy specimens, therefore grinding of biopsy is the first and mandatory step in *H. pylori* isolation. Comparison of culture performed with or without grinding showed a higher number of colonies after grinding, although the number of positive specimens did not change (Goodwin *et al*., 1985). In growing

this organism, the media components should include an agar base, growth supplements, and selective supplements. Most agar bases e.g., brain heart infusion agar, Columbia agar, and Wilkins Chalgren agar are satisfactory for growing the organism (Ndip *et al.*, 2003).

For growth supplement, it is mandatory to add blood or serum, which includes numerous nutrients (vitamins and oligoelements, etc.) that enhances growth. The proportion of blood or serum can be 5, 7, or preferably, 10%. Red blood cells can be lysed for these growth substances to be more readily available. Animal blood, e.g., sheep and horse blood, can be added. Different selective supplements containing antimicrobial compounds have been proposed: vancomycin or teicoplanin to inhibit Gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim, or cefsulodin to inhibit Gram-negative rods; and nystatin or amphotericin B to inhibit fungi. The Dent supplement, a modification of Skirrow's formula in which cefsulodin replaces polymyxin and amphotericin B is added, is commercially available (Ndip *et al* 2003; Megraud & Lehours, 2007).

Lack of nutrients can result in *H. pylori* losing its spiral shape and becomes progressively coccoidal, which most investigators believe these forms are both nonculturable and nonviable. However, others claim that some of them may be viable but nonculturable and constitute a resistant form of the bacterium (Benaissa *et al*., 1996; Kusters *et al*., 1997). Colonies can survive on plates for a week provided they are kept in a microaerobic atmosphere at 4°C. For long-term preservation, bacteria must be frozen at a low temperature (–70°C freezer or liquid nitrogen). Different broth media have been used, always with a cryoprotective agent, such as glycerol. The freezing-thawing process is always deadly for the bacteria, and only a small proportion survives. For this reason, it is mandatory to use bacteria in their exponential growth phase, as they are

more likely to survive. Frozen *H. pylori* specimens can be maintained for decades at –70°C (Spengler *et al*., 1992; Ndip *et al.,* 2003; Tanih *et al.*, 2010).

2.4.3 Biochemical characterization

Identification of cultured bacteria consists essentially of testing for the presence of certain enzymes: cytochrome oxidase, catalase, and urease (Konturek, 2004; Samie *et al;* 2007) and eventually {gamma}-glutamyl transpeptidase, leucine aminopeptidase, and alkaline phosphatase. Aminopeptidases and esterases (C4 to C12) are also present. The family Helicobacteraceae is comprised of the genera *Helicobacter* and *Wolinella*. Helicobacteraceae and Campylobacteraceae are in the Epsilonproteobacteria, according to the latest edition of Bergey's Manual. Cytochrome oxidase is present in all members of the Epsilonproteobacteria. It is usually detected with special reagents (Tetramethyl-p-phenylenediamine hydrogen chloride), or a strip with coated end, which when rubbed on the colony produced colour changes after 10 to 15 seconds. Catalase is also present in all Helicobacters and most members of the Campylobacteraceae and is detected by introducing a loopful of bacteria into a drop of hydrogen peroxide and observing abundant production of bubbles. Nevertheless, catalase-negative mutants of *H. pylori* have been discovered (Westblom *et al*., 1992). Urease is definitely the most important enzyme for identification. *H. pylori* produces large amounts of urease. To survive in its particular ecological niche, *H. pylori* produces large amounts of this enzyme to buffer the acidic medium and creates a microenvironment. When a loopful of the organism is put in contact with a few drops of urease medium, the color change occurs instantaneously regardless of the formulation. Other diagnostic tests are indeed either strictly based on urease, like the rapid urease

test and urea breath test, or partially based, like serology and PCR, which may target urease genes (Megraud & Lehours, 2007).

2.5 TREATMENT AND CONTROL

2.5.1 Antibiotic resistance patterns

Numerous treatment trials for *H. pylori* have been described using different antimicrobials, either singly or in combination, along with other drugs such as bismuth compound, ranitidine or omeprazole. Early treatment studies showed that many antibiotics were able to clear *H. pylori*, however, long term follow-up studies showed that the organism was extremely difficult to eradicate. Certainly, eradication of the organism would be considered beneficial in preventing gastritis, ulcers, and cancer; there may be other, as yet unknown adverse effects on infected children, such as retarded growth or loss of energy (Megraud, 2004). Furthermore, if antibiotics are used so extensively, it is very likely that the target bacteria would develop resistance to effective drugs within a few years. Prolonged treatment alters the normal microbial population of the gastrointestinal tract, eliminating some beneficial bacteria as well as pathogens (Abdul *et al.*, 2001; Megraud, 2004).

Antibiotic resistance in *H. pylori* varies according to geographical region. During recent years, resistance to antibiotics, especially metronidazole, has emerged as the major cause of treatment failure in most developing countries (Mégraud, 2004) and in South Africa, 96% resistance has been observed in metronidazole (Tanih *et al.*, 2010). In general, combined therapy is used to eradicate *H. pylori* infection (Suerbaum & Michetti, 2002), however, increase in the resistant

isolates highlights the need for susceptibility testing of *H. pylori* isolates prior to treatment (Mendonca *et al*., 2000). Resistance to metronidazole and clarithromycin was reported to be significantly higher globally and mostly in females than in males, while resistance to clarithromycin were significantly higher in children and teens (Glupczynski *et al.*, 2001; Ndip *et al*., 2008).

Amoxicillin is the only β-lactam used to treat *H. pylori* infection and it is included in most current therapeutic regimens. Until recently, resistance to amoxicillin was considered to be absent or very rare; however, amoxicillin-resistant *H. pylori* strains have now been identified in different countries (Kohanteb *et al.*, 2007). Tetracycline resistance in *H. pylori* was not reported until 1996, when Midolo and colleagues reported tetracycline-resistant strains from Australia (Midolo *et al*., 1996). Tetracycline was a component of the bismuth-based triple-therapy regimen recommended in the 1990s for treating *H. pylori* infection, and has also been used extensively as therapy in other types of infection, such as non-gonococcal urethritis, in recent years. Whether the mechanism of tetracycline resistance is related to carriage of resistance plasmids, originating from other bacterial species, remains to be determined (Wu *et al.*, 2000).

Susceptibility testing of *H. pylori* strains reported globally revealed that sensitivity for clarithromycin, tetracycline, amoxicillin and metronidazole ranged from 24 - 100% (Megraud, *et al*., 1999; Mégraud, 2004). Global resistance to these antibiotics was reported to be 0 to 100% for clarithromycin, 9 to 100% for metronidazole, 0 to 87.1% for tetracycline and 0 to 100% for amoxicillin. In Africa, resistance ranged from 60 - 100%, 4 - 100%, 2.5 – 100%, 1.9 – 87.1% and in Europe 14.5 - 46%, 1.7 – 28%, 0 – 1.1% and 0 – 5.6% for metronidazole, clarithromycin, amoxicillin and tetracycline respectively. In Asia and the Middle East, it was reported to range

from 9 – 62.7%, 0 – 27%, 0 – 36.1%, 0 – 5.3% while in South and North America, it ranged from 18 – 80%, <4 – 50%, 0 – 18% and 0% for metronidazole, clarithromycin, amoxicillin and tetracycline respectively (Njume *et al*., 2009).

2.5.2 Alternative approaches to treatment

Treatment of *H. pylori* infection using antibiotic is not without risk. Antibiotic therapy can lead to the development of pseudomembranous colitis, a potentially severe infection caused by *Clostridium difficile*. In addition, antibiotics frequently enable the overgrowth of *Candida albicans*, which can result in vaginitis, gastrointestinal disturbances, or other complaints. Moreover, it could lead to the overgrowth of antibiotic-resistant strains of *H. pylori*, making further attempts at eradication more difficult. Because of these risks, most physicians elect to treat *H. pylori* infection only in situations in which the benefits of doing so have been proven (e.g., peptic ulcer). If safer alternatives were available to eradicate *H. pylori*, then a case could be made that all such infections should be treated because of the possibility that the risk of gastric cancer would be reduced or that nutritional status would be improved.

Several naturally occurring substances have been investigated as potential alternatives for the treatment of *H. pylori* infection. Although there are several promising leads, these studies are for the most part preliminary, and additional work is needed before any of these treatments can be considered viable alternatives to conventional therapy (Gaby, 2001; Ndip *et al*., 2007).

Bismuth, although not an antibiotic per se, is capable of suppressing the growth of *H. pylori*. In addition, antibiotic resistance does not occur with bismuth therapy, as it can with metronidazole

and clarithromycin (Gaby, 2001). The use of pharmacogenomics as an individualize medication regimen had also been suggested and demonstrated (TEC, 2008). Preventive care and Making lifestyle changes, such as avoiding the long-term use of irritants (aspirin, anti-inflammatory drugs, coffee, and alcohol) will go a long way to preventing gastritis and its complications like an ulcer.

Vaccination has become one of the most promising approaches against *H. pylori* infection (Li *et al*., 2007). At present, most vaccines against *H. pylori* in animal models have been studied using whole-cell preparation, native or recombinant proteins from *H. pylori* and mucosal adjuvant. In general, these vaccines are based on a 'natural' form of the pathogen by lysating and inactivating the organism (Del Giudice *et al*., 2001). In response to *H. pylori* infection, the host triggers vigorous humoral and cellular immune response. Though *H. pylori* specific antibodies have been detected at high titers both in inflamed gastric mucosa and in serum, the infection is still persistent and even for a life time, suggesting that *H. pylori* can evade both innate and adaptive immune responses and the immune responses triggered by *H. pylori* in nature are not optimal to eliminate the pathogen (Baldari *et al.,* 2005). Important protective antigens of *H. pylori*, such as *VacA*, Lpp20, Catalase, *CagA*, and Urease have been selected and identified which have been reportedly used to produce monoclonal antibodies by the hybridoma technique using recombinant Catalase–GST as the immunogen and therefore immunoscreened against phage-displayed random dodecapeptide library. More mimotopes need to be identified and characterized in order to optimize the combination presentation of multi-mimotopes to develop an efficient vaccine for *H. pylor*i (Li *et al*., 2007).

Stress reduction through relaxation techniques including yoga, tai chi, and meditation can also be quite helpful. Eating a diet (Allium vegetables, particularly garlic (*Allium sativum* L.), Capsaicin (hot pepper) consumed as a flavoring spice, Cranberry (*Vaccinium macrocarpon*) natural fruit, (native to North America) high in fiber may not only cut the risk of developing ulcers in half, but fiber-rich foods may also speed the healing of ulcers. Fruits and vegetables are particularly protective sources of fiber and seem to reduce the amount of inflammation in the lining of the stomach; fruit juice appears to have this benefit as well. Fat in the diet may also increase the risk of gastritis (Alternative Medicine, 2008; Manyi-Loh *et al*., 2010).

Honey-derived remedies constitute a potential source of new compounds that may be useful in the management of *H. pylori* infections (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010). It contains approximately, 35% glucose, 40% fructose, 5% sucrose, 20% water, enzymes, amino acids, organic acids, polyphenols (flavonoids and phenolic acids) and carotenoid-like substances, Maillard reaction products, vitamins, ascorbic acids, ά-tocopherol and minerals. However, honey contains these natural antioxidants which exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions (Manyi-Loh *et al*., 2010).

2.5.3 Medicinal plants: potential alternative approach to the treatment of *H. pylori* infections

Plant cells fundamentally are chemical factories and many possess a rich supply of therapeutically useful constituents with significant biological effects on humans. The use of medicinal plants in the treatment of diseases is an ancient tradition that has co-existed with

human habitation especially where modern drugs are not affordable, inaccessible or unacceptable (Njume *et al*., 2009). There are many herbs, some of which may be recommended by an herbal specialist for symptoms of gastritis, ulcers and other gastrointestinal disorders majorly caused by *H. pylori* infections. Some of such herbs are: Astragalus (*Astragalus membranaceus*), Barberry (*Berberis vulgaris)*, Bilberry (*Vaccinium myrtillus*), Cat's Claw (*Uncaria tomentosa*), Cranberry (*Vaccinium spp*.), Ginger (*Zingiber officinale*), Green Tea (*Camellia sinensis*), Licorice (*Glycyrrhiza glabra*) e.t.c. They should, therefore, be used with caution and only under the guidance of a professionally trained and qualified herbalist as herbs may cause side effects or interact with medications (Ndip *et al*., 2007; Alternative Medicine, 2008). Herbal medicines form a significant part of culture and traditions of rural people in developing countries and as a result, there is an increasing trend to integrate traditional medicine with primary healthcare (Njume *et al*., 2009).

2.5.3.1 Peltophorum africanum (Sond, Fabaceae)

Peltophorum africanum also known as Weeping Wattle, is a semi-deciduous to deciduous tree of about 15m with a spreading, untidy canopy widespread in South Africa. All parts of this plant has been reported to constitute flavonoids, gallic and chlorogenic acid with reported medicinal uses. Leaves and bark have been traditionally employed to clear intestinal parasites and relieve stomach problems; the stem bark is used to treat colic, diarrhoea, dysentery, helminthosis, human immunodeficiency virus/ acquired immune deficiency syndrome (HIV/AIDS), venereal diseases and infertility; and roots are used to heal sore throat, wounds and toothache (Iwalewa *et al.,*

2007; Theo *et al.,* 2009). It has also been reported to be active against *E. coli, S. aureus, A. hydrophila, S. sonei* and *C. jejuni* (Samie *et al.,* 2005).

The roots of this plant traditionally give a very effective remedy for pregnant women when the foetus kicks heavily and causes pain to the mother. But this remedy is prepared only if the problem lasts at least for two to three days. The outer skin of the roots is scraped away, the roots are cut into pieces, put into cold water and heated until it cooks, then the roots are removed. One cup of the cooled down infusion is drunk. The same infusion also stops heavy bleeding while giving birth. The roots are also used for treating cough with blood and tuberculosis (TB). They are soaked in water and slightly heated. The bark provides a cure for treating a sore liver. The bark is boiled and the decoction is then drunk. This will induce vomiting that is said to clean the liver and relieve pain. It is also used to treat eyes (Bizimenyera *et al.,* 2006).

2.5.3.2 Bridelia micrantha (Hochst., Baill., Euphorbiaceae)

Bridelia micrantha (Euphorbiaceae), native to sub-Saharan Africa is a semi-deciduous to deciduous tree up to 20 m tall with a dense rounded crown and tall, bare stem. All parts of the plant are used in traditional medicine in the treatment of stomach ailments and diseases such as gastritis, salmonellosis, gastro-enteritis, diarrhea and constipation, tapeworms and as an emetic for poisons (causes vomiting) (blackherbals.com). Recent studies show that *Bridelia micrantha* root, stem bark and leaf constitutes friedelin, epi-friedelin, flavonoids, tannins, gallic acid, sterol, saponin, anthocyanidin, caffeic acid, taraxerone and taraxerol (a phytochemical found in dandelions and seaweed to name a few), which is used in producing anti-cancer drugs. Taraxerol-3B-acetate improves tolerance of exercise, removes fatigue and is an anti-aging, anti-

tumour, hepatoprotective, an antineoplastic agent and an immune stimulator (Bessong *et al.,* 2005; Iwalewa *et al.,* 2007). This plant has been reported to be active against *E. coli, K. pneumoniae, S. flexneri,* and *P. aeruginosa* (Samie *et al.,* 2005).

Scientific studies have shown that extracts of the whole stem demonstrate antimicrobial activity by inhibiting the growth of *Campylobacter jejuni/coli*. It is used as a treatment for skin problems such as ulcers, boils and rashes; for respiratory problems such as persistent cough, TB, pneumonia, bronchitis and pleurisy; as an analgesic (pain reliever); as an antimalarial; for toothache and gum diseases; for painful menstruation; to prevent abortion; as a stimulant and restorative tonic (alternative) for fortifying pregnant women; for sickle cell anemia, HIV/AIDS; and also used traditionally in Africa to treat psychological problems such as neurosis and psychosis and protection against enemies. Preliminary research on medical properties of *B. micrantha* has shown this herb to be beneficial in treating HIV/AIDS as it cures diarrhea and stomach discomfort, which are common illnesses in AIDS and contributes to the well-being of the patient. It has also been shown to be a possible principle inhibitor to HIV-1 reverse transcriptase (FAO, 1986; Hines and Eckman, 1993; Bessong *et al.,* 2005).

The stem bark decoction is reportedly taken as a remedy for stomach-ache and tapeworm. The bark is also boiled to make a soup for treating diarrhoea in children, or is mixed with milk and drunk as a tonic. A decoction of roots is drunk to cure aching joints. The leaf sap is used as an application to sore eyes and, in a decoction with a number of other plants, for the treatment of conjunctivitis. The root is used as a remedy for severe epigastric pain and is applied to the scalp to relieve headache. A decoction of the root is drunk as a purgative, an anthelmintic or an antidote for poison, as it causes vomiting or diarrhoea that gets rid of the poison. An infusion

made from the root is taken orally for coughs. The powdered bark is applied to burns to speed healing (FAO, 1986; Hines and Eckman, 1993).

2.5.3.3 Solvents mostly used in plant extractions

Previous studies have shown that the solvents used in extraction affect the antimicrobial activities of plants. A variety of extractants are used for their ability to solubilize anti-microbials and also other factors from plants. The type of solvent used may have an effect on the nature of the compounds extracted and the resulting bioactivity of the extract, therefore implying that some bioactive components can only be extracted by polar solvents while others by less polar and yet some by non-polar solvents (Bizimenyera *et al*., 2006; Ndip *et al*., 2007). Methanol extracts of *Ekebergia senegalensis*, a common Senegalese plant widely used in folk medicine, showed marked antibacterial activity than the petroleum spirit extracts. Water extracts of *Plumbago zeylanica* have also demonstrated lower anti-*Helicobacter pylori* activity than that of organic solvents (Ndip *et al*., 2007; Njume *et al*., 2009).

Many studies have revealed that the ethyl acetate extract of plants is the most active against *H. pylori* and other organisms compared with the other solvents used. For example, the study of Theo *et al*. (2009) demonstrated a good activity of the ethyl acetate extract of *P. africanum* against HIV-1. Also, Maoela *et al*. (2009) worked on *Carpobrotus mellei* and *Carpobrotus quadrifidus*; common South African plants widely used in folk medicine and reported marked activity with the ethyl acetate extract compared to the chloroform and butanol extracts. On the other hand, acetone was mostly selected as a suitable extractant as it extracts compounds of a wide polarity range, is miscible with organic and aqueous solvents, and is non-toxic to test

organisms. It has been noted that organic solvents extract more plant material than water. Besides, acetone extracts the larger amounts of material from plants compared to ethanol, dichloromethane, methanol, hexane and water (Bizimenyera *et al*., 2006) which was also the case in this study. Djipa *et al*. (2000) and Asha *et al*. (2008) reported best antimicrobial activity by acetone extract against pathogenic bacteria compared to other solvent used.

These may be due to the fact that the active compounds against bacteria strains (e.g. *H. pylori*) in most plants were less polar as non-polar solvents dissolve non-polar compounds best and different solvents extract different compounds. Organic solvents were therefore observed to be better for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents (Abu-Shanab *et al*., 2006). To ascertain the value of each extractant therefore, several parameters, including the rate of extraction, the quantity extracted, the diversity of compounds extracted, the diversity of inhibitory compounds extracted, the ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and the potential health hazard of the extractants have to be evaluated. The efficiency of extraction has to be optimized to ensure that as many of the potentially active constituents as possible are extracted (Njume *et al*., 2009).

2.5.3.4 Potential anti-H. pylori compounds mostly isolated from plants

Flavonoids (or bioflavonoids), also collectively known as Vitamin P and citrin are synthesized by the phenylpropanoid metabolic pathway in which the amino acid phenylalanine is used to produce 4-coumaroyl-CoA. They have been referred to as "nature's biological response modifiers" because of strong experimental evidence of their inherent ability to modify the body's

reaction to allergens, viruses, and carcinogens (Justesen and Knuthsen, 2001). They show anti-allergic, anti-inflammatory, anti-microbial and anti-cancer activity. The huge increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is not caused directly by the flavonoids themselves, but most likely is due to increased uric acid levels that result from expelling flavonoids from the body and the process of gearing up to get rid of unwanted compounds induces so-called Phase II enzymes that also help to eliminate mutagens and carcinogens, and therefore may be of value in cancer prevention. Flavonoids could also induce mechanisms that help kill cancer cells and inhibit tumor invasion (Filippos *et al*., 2007).

Gallic acid is an organic acid, also known as 3,4,5-trihydroxybenzoic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark e.t.c. and mostly found both free and as part of tannins. Gallic acid is commonly used in the pharmaceutical industry. It is used as a standard for determining the phenol content of various analytes by the Folin-Ciocalteau assay; results are reported in gallic acid equivalents. Gallic acid can also be used as a starting material in the synthesis of the psychedelic alkaloid mescaline and with anti-fungal and anti-viral properties. As an antioxidant, it helps to protect our cells against oxidative damage and also found to show cytotoxicity against cancer cells, without harming healthy cells. Gallic acid is used as a remote astringent in cases of internal haemorrhage, to treat albuminuria and diabetes; also some ointment used to treat psoriasis and external haemorrhoids contain gallic acid (Pathak *et al*., 2004).

Chlorogenic acid is a family of naturally occurring organic compounds, esters of cinnamic acids and (-)-quinic acid. It is an important biosynthetic intermediate (lignin) and one of the phenols found in coffee, in the bamboo *Phyllostachys edulis* as well as many other plants and long

known as an antioxidant, it also slows the release of glucose into the bloodstream after a meal. It is also an inhibitor of the tumor promoting activity of phorbol esters and might contribute to the prevention of Type 2 diabetes mellitus and cardiovascular disease. It is claimed to have antiviral, antibacterial and antifungal effects with relatively low toxicity and side effects, alongside properties that do not lead to antimicrobial resistance (Clifford *et al*., 2003).

Many plant species are rich in sterols and sterolines which have been credited to have immuno-modulatory effects and boost the vitality of AIDS patients. *B. micrantha* and *P. africanum* investigated herein are generally used among the VhaVendas of South Africa to cure diarrhea and stomach discomfort, common illnesses in AIDS (Bessong *et al*., 2006). Bioassay-guided fractionation carried out on the roots and stem-bark of *Peltophorum africanum* afforded the known compounds bergenin and catechin and a red coloured gallotannin. Structural information gathered by MALDI-Tof, LC/MS, silylation and acid hydrolysis of the gallotannin indicated that it is composed of meta-depside chains of gallic and protocatechuic acids esterified to a 1-O-isobutyroly-β-d-glucopyranose. Gallotannins are examples of plant polyphenols, and some researchers have proposed that polyphenols and tannins are responsible for apparent inhibitory properties in enzyme systems, since polyphenols precipitate proteins from solution, and therefore the activity of such compounds against enzymes may be non-specific (Bessong *et al*., 2005).

Some of these naturally occurring bioactive substances with antioxidant properties, such as plant phenols, vitamins, carotenoids, phytoestrogens and terpernoids also have been shown to have anti-inflammatory activity and may play an important role in disease prevention and immune promotion, especially in chronic inflammatory diseases. Based on the molecular events that lead to inflammation, it has been suggested that the process of sustained inflammation that

accompanies chronic diseases can be ameliorated and possibly even prevented by phytomedicines (Iwalewa *et al*., 2007).

The study previously conducted by Li *et al*. (2005) on *Eugenia caryophyllata, Abrus cantoniensis* and *Saussurea lappa* had led to the characterization of volatile oil (mainly contains eugenol and caryophyllen), a chromone C-glucoside, elagitennins, phytosterols and among these plant constituents, eugenol was disclosed to have prominently antibacterial properties (anti-*Helicobacter pylori*). In these plants had also been found compounds like triterpenic saponins, anthraquinones, alkaloids, flavonoids, and some other constituents such as abrin, abrussic acid, uric acid, glabrolide sesquiterpenes, monoterpenes, triterpenes, and aromatic compounds.

CHAPTER THREE

MATERIALS AND METHODS

3.1 BACTERIAL STRAINS

A total of 31 strains of *H. pylori* in addition to a control strain, NCTC 11638 were subjected to antimicrobial assays in this study. Strains were isolated from patients presenting with gastric related morbidities at the Livingston Hospital, Port Elizabeth for endoscopy and confirmed following our previously reported scheme (Ndip et al., 2008). Informed consent was obtained from the patients and ethical approval (Protocol number EcDoH-Res 0002) from the Eastern Cape Department of Health, and the institutional review board of the University of Fort Hare (GMRDC).

3.2 PREPARATION OF PLANT EXTRACTS

The stem bark of *P. africanum* and *B. micrantha* were selected based on ethnobotanical information. The plants were collected in Limpopo Province and identified in collaboration with botanist at the University of Venda, Limpopo Province where voucher specimens (number BP01 & BP03 respectively) have been deposited.

The method described by Ndip *et al*. (2007) was employed with modifications. Briefly, the plants were harvested, washed, air dried for 2 weeks and ground to fine powder using a blender (ATO MSE mix, 702732, England). Technical grade ethylacetate, acetone, ethanol, methanol (100%) and water were employed for extraction. Dried plant materials, 2 kg were macerated in

five fold excess of the solvent in extraction bottles such that the level of the solvent was above that of the plant materials. The slurry was put in a shaker incubator (Edison, N.J., USA) regulated at room temperature (RT) for 48 h then centrifuged at 3000 rpm for 5 mins (Model TJ-6 Beckman, USA) and filtered using filter paper of pore size 60^{Å}. The process was repeated twice for a total of three extractions (exhaustive extraction) for each solvent. The combined extracts were concentrated in a rotavapor (BUCHI R461, Switzerland) and transferred to appropriately labeled vials and allowed to stand at RT to permit evaporation of residual solvent. A 6.2 gram sample of each plant extract was used for the preliminary bioassay, and 13.8g kept in the extract bank. Stock solutions were prepared by dissolving the extracts in 10% Dimethyl Sulphoxide (DMSO) which we established to be non inhibitory to *H. pylori*.

3.3 SCREENING OF CRUDE EXTRACTS FOR ANTI – *H. PYLORI* ACTIVITY

The agar-well diffusion method was used as previously described (Boyanova *et al*., 2005; Ndip *et al*., 2007). Brain Heart Infusion (BHI) agar (Oxoid, England) supplemented with 7% horse blood (Oxoid, England) and Skirrow's supplement (Oxoid, England) was used. *H. pylori* inoculum was prepared from subcultures of bacteria as follows: four to five colonies of the isolates were emulsified in sterile distilled water 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. The plates were allowed to dry for 3 to 5 min. Wells were punched in the plates using a sterile stainless 6 mm cork borer. The wells were filled with 30 µL of the extract (50 mg/mL). DMSO (10%) was used as a negative control and 0.05 µg/mL clarithromycin as a positive control. The tests were

repeated in duplicate and the plates incubated microaerophilically at 37 °C for 72 h (Anaerocult Basingstoke, England). The diameters of the zones of inhibition were measured in millimeters. *H. pylori* control strain NCTC 11638 inoculated plate was included in all the experiments.

3.4 DETERMINATION OF MIC_{50} & MIC_{90}

Test for MIC was carried out as described by Banfi *et al*. (2003) with modifications. Extracts that gave a zone of inhibition ≥14 mm were chosen for MIC determination by the microdilution test method in 96-well plates. Two-fold dilutions of the most potent extracts; ethyl acetate extracts of *Peltophorum africanum* (*P. afri* EA) and *Bridelia micrantha* (*B. mic.* EA), acetone extract of *Bridelia micrantha* (*B. mic.* A) and antibiotics, metronidazole and amoxicillin were prepared in the test wells in complete BHI broth (Oxoid, England) supplemented with 7% horse serum (Oxoid, England) and Skirrow's supplement (Oxoid, England); the final extracts and antibiotics concentrations ranged from 0.0048 - 10mg/mL respectively. Each strain was sub-cultured in 2mL 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 microliters 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. Also included was culture medium and extract only at different concentrations. The plates were sealed and incubated under microaerophilic condition at 37°C for 3 days. After incubation, 32µL of resazurin solution was added per well, colouring them blue. Plates were incubated at 37°C for additional 1 h. 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). The lowest concentration of the extract resulting in inhibition of 90% & 50% of bacterial growth were recorded as the MIC_{90} & MIC_{50} respectively.

3.5 DETERMINATION OF THE RATE OF KILL

Assay for the time and extent of killing of bacterial isolates by *P. afri* EA and *B. mic.* EA were determined in accordance with previously established methods (Ali *et al*., 2005; Akinpelu *et al*., 2009). Briefly, the 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 7% horse serum and Skirrow's supplement (Oxoid, England) and then adjusted for the inclusion of different concentrations of the solution of the extracts (½ x MIC, MIC, 2 x MIC and 4 x MIC). These were incubated at 37^{o}C in a microaerophilic cabinet shaking at ~120 rpm and the killing rate determined over a period of 72 h at 6 h interval (0, 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72 h). Exactly 0.5 mL volume of each suspension was withdrawn at time intervals and transferred to 4.5 mL of BHI broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carryovers from the test organisms. The suspension was then serially diluted and plated out for viable counts. The plates were later incubated microaerophilically at 37^{o}C for 72 h. The control plates contained the bacterial cells without the extract. The emergent bacterial colonies were counted and compared with the counts of the culture control.

3.6 PHYTOCHEMICAL ANALYSIS

Adopting the methods of Adegboye *et al*. (2008), the qualitative phytochemical analysis of the ethyl acetate extract of *B. micrantha* and *P. africanum* were carried out as follows:

3.6.1 Test for alkaloids

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

3.6.2 Test for flavonoids

A 0.2 g 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 appearance of a red or orange colouration was indicative of the presence of flavonoids.

3.6.3 Test for tannins

About 1.0 g of the plant extract was dissolved in 10 mL of distilled water and filtered (Acrodisc syringe filter, Pall USA). Two to three drops of 10% $FeCl_3$ was added to 2 mL of the filtrate. A blackish-blue colouration was indicative of tannins. To another 2 mL of the filtrate was added 1 mL of bromine water. A precipitate was also taken as positive for tannins.

3.6.4 Test for steroids

A small sample of ethyl acetate extract was dissolved in methanol, spotted on a Thin Layer Chromatography (TLC) silica gel 60 F_{254} plate (Merck) and developed in chloroform: methanol (4:1) solvent system. The plate was air-dried and sprayed with concentrated sulphuric acid before heating and the development of black spots was recorded as positive for steroids.

For more confirmation of the presence of steroids, approximately 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered (Acrodisc syringe filter, Pall USA). To the filtrate was added concentrated H_2SO_4 to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

3.6.5 Test for saponins

Two grams of the extract was boiled in 20 mL of distilled water in a water bath and filtered (Acrodisc syringe filter, Pall USA). Approximately 10 mL of the filtrate was mixed with 5mL of distilled water and shaken vigorously for a stable persistent froth. The frothing was mixed with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion.

For confirmation of the presence of saponins, Columbia blood agar medium supplemented with 7% blood was freshly prepared and wells were made in it. The extract dissolved in ethyl acetate (and methanol) was applied. Distilled water, methanol and ethyl acetate were used as negative control while commercial saponin (BDH) solution (dissolved in distilled water, methanol and ethyl acetate) was used as positive control. The plates were incubated at 37^0C for 6 hrs. Complete haemolysis of the blood around the extract was indicative of the presence of saponins.

3.7 STATISTICAL ANALYSIS

Analysis was performed using SPSS Version 17.0 (Illinois USA, 2009). The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of zones of inhibition of the plant extracts and clarithromycin; the MIC of the most active extracts (*P. afri* EA, *B. mic.* EA and *B. mic.* A) and the control antibiotics (metronidazole and amoxicillin). P-values <0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 ANTI-*HELICOBACTER PYLORI* ACTIVITIES

Our results on *P. africanum* showed that the zones of inhibition ranged from 17 to 23 mm for ethyl acetate extract; 0 to 21 mm for acetone; and 8 to 15 mm for methanol (Tables 1 & 2), while for *B. micrantha* it was 12 to 20 mm for the ethyl acetate extract; 16 to 23 mm for acetone; and 0 to 15 mm for water (Tables 3 & 4); the control antibiotic, clarithromycin had a zone diameter of 0 to 35 mm (Tables 2 & 4). An inhibition zone of ≥14 mm was chosen as representative of bacterial susceptibility to the extracts and antibiotic. DMSO (10%) and ethyl acetate (20%) used as negative control, showed no activity.

Of the 31 strains subjected to the plant extracts and antibiotic, 100% susceptibility was recorded for the *P. afr*. EA and *B. mic.* A. For *P. afr*., susceptibility to acetone, methanol and water extracts were 6.5%, 16.1%, and 25.8%, respectively while for *B. mic.* 93.5%, 3.2%, and 12.9% were noted for ethyl acetate, methanol and water extracts respectively. For the control antibiotic, clarithromycin it was 58.1% (Figures 1 and 2). The mean difference of the ethyl acetate extract of *P. africanum* and acetone extract of *B. micrantha* were statistically significant ($P < 0.05$) compared to the other extracts (except *B. mic.* EA.) and clarithromycin at 95% confidence interval.

Table 1: Antibacterial activity of plant extracts (*P. africanum*) against *Helicobacter pylori* strains.

H. pylori strains	Zone diameter of inhibition of growth (mm)					
	***P.afr.* (EA)**	***P.afr.* (A)**	***P.afr.* (E)**	***P.afr.* (M)**	***P.afr.* (W)**	**Clr.**
PE11A	20	9	10	8	9	10
PE26A	18	9	11	8	9	18
PE93A	17	8	10	13	0	28
PE93C	20	0	12	9	9	8
PE102C	20	0	8	8	9	16
PE115A	19	0	11	8	11	10
PE155A	19	8	9	13	9	15
PE162A	20	8	10	8	9	35
PE219C	18	0	0	10	15	12
PE252C	20	8	10	12	11	15
PE258C	18	8	12	8	15	10
PE265C	19	8	8	8	14	13
PE296C	17	0	12	13	8	0
PE308C	18	9	13	13	0	15
PE369A	18	10	8	11	14	20
PE369C	21	9	7	14	12	18

PE402A	23	11	11	14	13	12
PE406C	19	21	10	14	12	27
PE407C	19	9	8	11	12	13
PE411C	19	9	9	9	13	17
PE430A	22	9	11	11	12	21
PE430C	18	8	10	9	15	31
PE436A	19	8	11	12	15	23
PE436C	22	10	11	12	12	20
PE462A	18	8	8	12	12	18
PE462C	20	10	10	12	12	17
PE466C	18	9	8	12	12	25
PE467A	20	8	10	11	14	0
PE467C	20	14	12	15	12	0
PE469C	21	12	10	14	15	8
PE471A	20	8	10	13	9	0

P.afr., Peltophorum africanum; EA, Ethyl acetate; A, Acetone; E, Ethanol; M, Methanol; W, Water; Clr., Clarithromycin.

Table 2: Mean zone diameter of inhibition of the crude extract (*P. africanum*) and antibiotic.

Extract / control antibiotic	Mean zone diameter (mm)	Inhibition diameter range
P.afr. (EA)	19.35±1.450	17 – 23 mm
P.afr. (A)	8.00±4.359	0 – 21 mm
P.afr. (E)	9.68±2.329	0 – 13 mm
P.afr. (M)	11.13±2.262	8 – 15 mm
P.afr. (W)	11.10±3.664	0 – 15 mm
Clr.	15.32±8.852	0 – 35 mm

P.afr., Peltophorum africanum; EA, Ethyl acetate; A, Acetone; E, Ethanol; M, Methanol; W, Water; Clr., Clarithromycin.

Table 3: Antibacterial activity of plant extracts (*B. micrantha*) against *Helicobacter pylori* strains.

	Zone diameter of inhibition of growth (mm)					
***H. pylori* strains**	***B.mic.* (EA)**	***B.mic.* (A)**	***B.mic.* (E)**	***B.mic.* (M)**	***B.mic.* (W)**	**Clr.**
PE11A	16	22	0	0	8	10
PE26A	17	22	8	8	10	18
PE93A	18	20	0	0	0	28
PE93C	18	20	0	8	9	8
PE102C	16	20	0	0	11	16
PE115A	17	20	0	0	11	10
PE155A	16	20	0	0	9	15
PE162A	12	22	0	8	10	35
PE219C	18	20	0	0	11	12
PE252C	20	20	0	0	11	15
PE258C	15	21	8	8	10	10
PE265C	16	22	0	0	9	13
PE296C	14	21	0	0	9	0
PE308C	19	21	0	0	10	15
PE369A	13	20	0	12	13	20

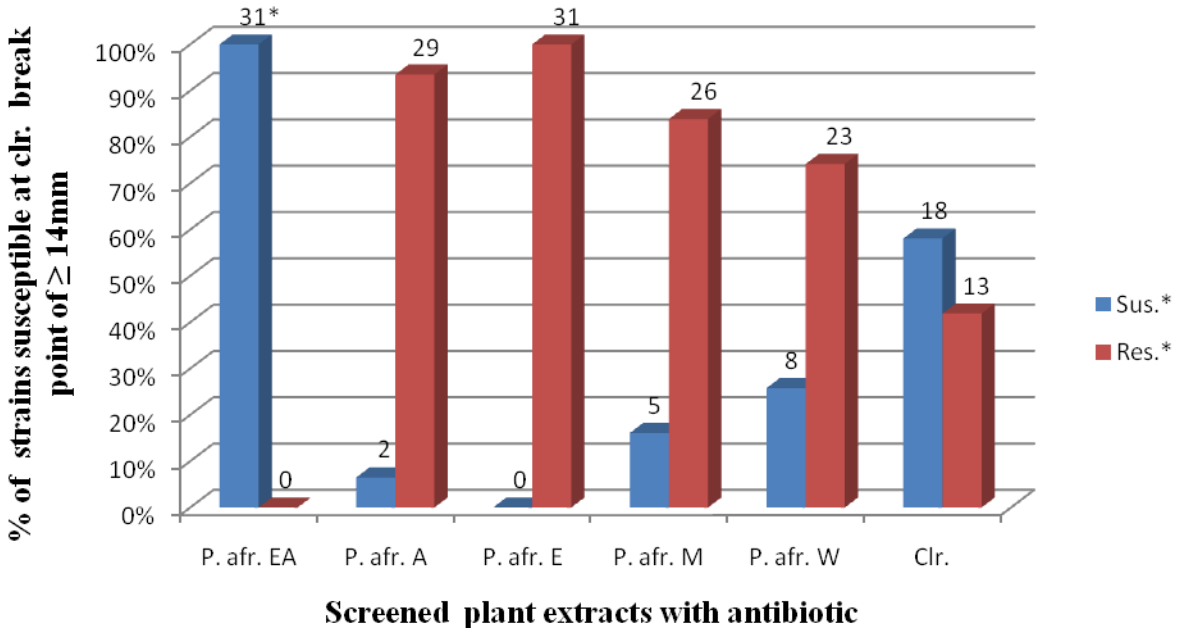
PE369C	16	20	7	11	12	18
PE402A	14	22	0	11	15	12
PE406C	16	21	7	10	13	27
PE407C	17	23	0	10	12	13
PE411C	15	20	0	0	13	17
PE430A	18	18	10	0	12	21
PE430C	17	19	0	8	15	31
PE436A	14	16	0	0	10	23
PE436C	15	22	8	8	14	20
PE462A	15	21	8	12	13	18
PE462C	16	21	8	8	10	17
PE466C	17	17	0	10	13	25
PE467A	16	20	7	10	14	0
PE467C	16	20	0	12	13	0
PE469C	17	20	7	8	13	8
PE471A	16	21	7	14	9	0

B.mic., *Bridelia micrantha;* EA, Ethyl acetate; A, Acetone; E, Ethanol; M, Methanol; W, Water; Clr., Clarithromycin.

Table 4: Mean zone diameter of inhibition of the crude extract (*B. micrantha*) and antibiotic.

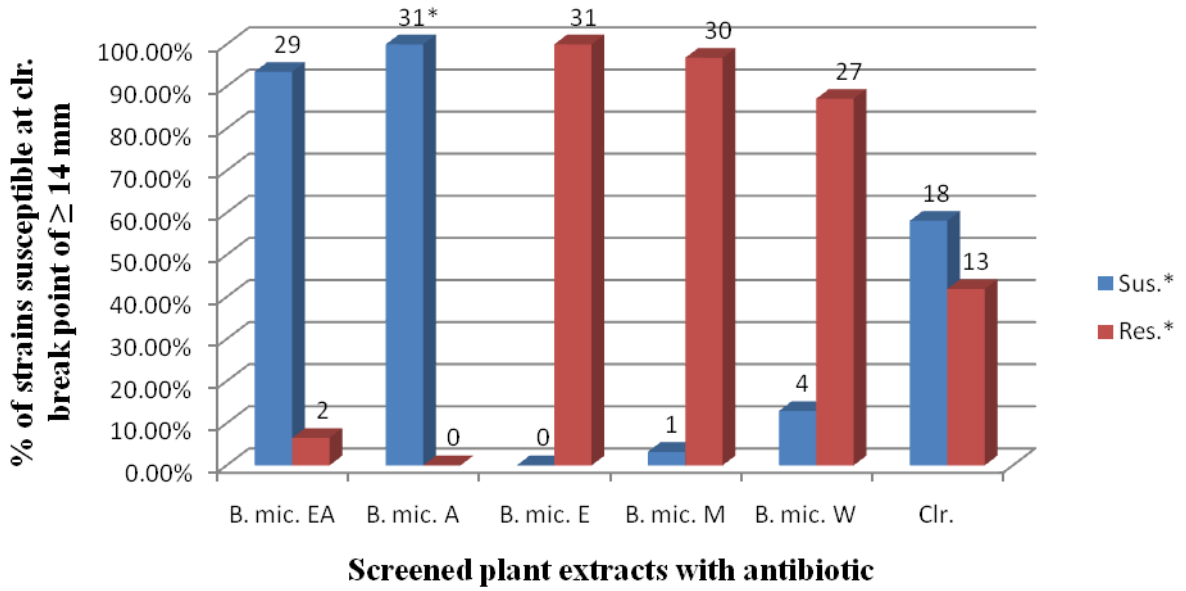
Extract / control antibiotic	Mean zone diameter (mm)	Inhibition diameter range
B.mic. (EA)	16.13±1.708	12 – 20 mm
B.mic. (A)	20.39±1.476	16 – 23 mm
B.mic. (E)	2.74±3.794	0 – 10 mm
B.mic. (M)	5.68±5.108	0 – 14 mm
B.mic. (W)	11.03±2.811	0 – 15 mm
Clr.	15.32±8.852	0 – 35 mm

B.mic., *Bridelia micrantha;* EA, Ethyl acetate; A, Acetone; E, Ethanol; M, Methanol; W, Water; Clr., Clarithromycin.



*, No. of strains; * Sus, Susceptible; * Res, Resistance

Figure 1. Plant extracts (*P. africanum*) and antibiotic susceptibility against 31 strains of *H. pylori*.



*, No. of strains; * Sus, Susceptible; * Res, Resistance

Figure 2. Plant extracts (*B. micrantha*) and antibiotic susceptibility against 31 strains of *H. pylori*.

4.2 MIC_{50} AND MIC_{90} DETERMINATION

Since *P. afr.* EA, *B. mic.* EA and *B. mic* A. were the most potent, their MIC_{50} and MIC_{90} were determined against the 31 strains alongside the antibiotics. The MIC_{50} for *P. afr*. EA & *B. mic.* A ranged from 0.0048 mg/mL - 0.313 mg/mL, and 0.0048 - 0.156 mg/mL for *B. mic.* EA, metronidazole and amoxicillin respectively. On the other hand, the MIC_{90} for *P. afr*. EA ranged from 0.156 mg/mL - 0.625 mg/mL, 0.0048 to 2.5 mg/mL for *B. mic*. EA, 0.078 to >0.625 mg/mL for *B. mic* A, 0.0098 to >5 mg/mL for metronidazole and 0.078 to >2.5 mg/mL for amoxicillin (Tables 5 & 6).

At a concentration of 0.0098 mg/mL (MIC_{50}), 10 (32.3%) of the 31 strains were inhibited by metronidazole followed by *P. afr.* EA, 8 (25.8%), and amoxicillin, 5 (16.1%). At 0.313 mg/mL, 6 (19.4%) strains were inhibited by *P. afr.* EA and no activity was observed for both metronidazole and amoxicillin (Figure 3). Approximately, 48.4% of the strains were inhibited by *B. mic*. EA followed by amoxicillin, 12 (38.7%) and metronidazole, 11 (35.5%) at a concentration of 0.0048 mg/mL (MIC_{50}). At 0.078 mg/mL, 11 (35.5%) strains were inhibited by *B. mic* A followed by amoxicillin, 6 (19.4%) and metronidazole, 5 (16.1%) (Figure 4).

There was a statistical significant difference observed with potency of *P. afr* EA and *B. mic.* A compared to the two antibiotics at MIC_{50} ($P < 0.05$), but not for the antibiotics compared to each other ($P > 0.05$). The activity of *B. mic.* EA was not statistically significant ($P > 0.05$) to the two antibiotics tested at 95% Confidence Interval (appendix 4).

Table 5: *In-vitro* anti-*H. pylori* activities of *P. africanum* EA extract and antibiotics at MIC_{50} and MIC_{90} (mg/mL).

	***P.afr.* (EA)**		**Metronidazole**		**Amoxicillin**	
***H. pylori* strains**	**MIC50**	**MIC90**	**MIC50**	**MIC90**	**MIC50**	**MIC90**
PE11A	0.078	0.313	0.156	0.625	0.156	ND*
PE26A	0.078	0.156	0.156	>0.625*	0.039	>0.313
PE93A	0.078	ND	0.0098	ND	0.039	ND
PE93C	0.313	ND	0.0195	ND	0.0048	>0.625
PE102C	0.078	0.313	0.078	0.156	0.039	0.156
PE115A	0.0098	>0.313	0.0098	ND	0.0098	ND
PE155A	0.313	ND	0.0048	ND	0.0048	0.313
PE162A	0.0098	>0.313	0.0048	ND	0.0048	0.078
PE219C	0.0098	ND	0.0048	ND	0.078	0.313
PE252C	0.313	>0.313	0.0098	ND	0.0098	ND
PE258C	0.078	0.625	0.078	0.156	0.078	0.156
PE265C	0.0048	ND	0.0048	ND	0.0048	1.25
PE296C	0.0098	ND	0.0098	0.625	0.0048	0.156
PE308C	0.0048	ND	0.0048	ND	0.0048	>0.625
PE369A	0.156	ND	0.078	>5	0.078	>2.5
PE369C	0.313	ND	0.0098	ND	0.0098	ND
PE402A	0.078	>0.313	0.156	>0.156	0.078	>0.156
PE406C	0.313	ND	0.0098	>5	0.0195	ND

PE407C	0.078	0.313	0.078	>0.156	0.039	0.625
PE411C	0.0048	>0.313	0.0098	ND	0.0048	0.156
PE430A	0.156	ND	0.0195	ND	0.039	0.156
PE430C	0.156	ND	0.078	>5	0.078	>0.313
PE436A	0.0098	ND	0.0048	ND	0.0048	ND
PE436C	0.0098	ND	0.0048	0.0098	0.0048	0.625
PE462A	0.313	ND	0.0098	ND	0.0098	ND
PE462C	0.156	ND	0.0098	ND	0.0195	ND
PE466C	0.039	>0.039	0.0098	ND	0.0098	ND
PE467A	0.0048	>0.313	0.0048	ND	0.0048	0.625
PE467C	0.0098	ND	0.0048	ND	0.078	0.313
PE469C	0.0098	0.156	0.0048	1.25	0.0048	0.313
PE471A	0.156	>0.313	0.0048	ND	0.0048	0.313
Average	0.108		0.034		0.031	

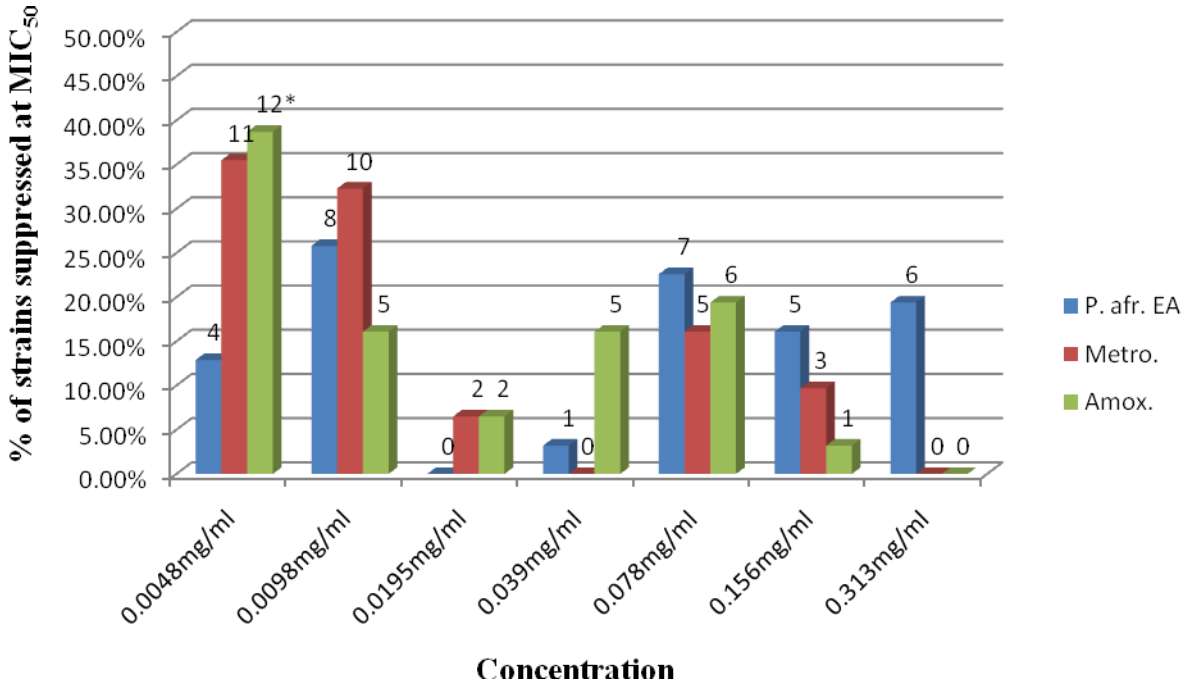
*ND, Not determined; *>, Closer but not exact.

Table 6: *In-vitro* anti-*H. pylori* activities of *B. micrantha* EA extract and antibiotics at MIC_{50} and MIC_{90} (mg/mL).

	***B.mic.* (EA)**		***B.mic.* (A)**		**Metronidazole**		**Amoxicillin**	
***H. pylori* strains**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**	**MIC_{50}**	**MIC_{90}**
PE11A	0.156	>0.625*	0.078	0.313	0.156	0.625	0.156	ND*
PE26A	0.078	>0.156	0.078	0.313	0.156	>0.625	0.039	>0.313
PE93A	0.039	ND	0.078	ND	0.0098	ND	0.039	ND
PE93C	0.0048	>0.0048	0.078	>0.313	0.0195	ND	0.0048	>0.625
PE102C	0.156	>0.625	0.078	0.313	0.078	0.156	0.039	0.156
PE115A	0.0195	ND	0.313	ND	0.0098	ND	0.0098	ND
PE155A	0.0048	>0.0048	0.156	ND	0.0048	ND	0.0048	0.313
PE162A	0.0048	>0.0048	0.078	>0.625	0.0048	ND	0.0048	0.078
PE219C	0.0048	>0.0048	0.0048	>0.156	0.0048	ND	0.078	0.313
PE252C	0.156	ND	0.313	ND	0.0098	ND	0.0098	ND
PE258C	0.156	2.5	0.078	0.313	0.078	0.156	0.078	0.156
PE265C	0.0048	>0.0048	0.156	ND	0.0048	ND	0.0048	1.25
PE296C	0.0048	0.0048	0.0048	0.625	0.0098	0.625	0.0048	0.156
PE308C	0.0048	>0.0048	0.078	ND	0.0048	ND	0.0048	>0.625
PE369A	0.156	ND	0.156	ND	0.078	>5	0.078	>2.5
PE369C	0.0098	>0.156	0.156	ND	0.0098	ND	0.0098	ND

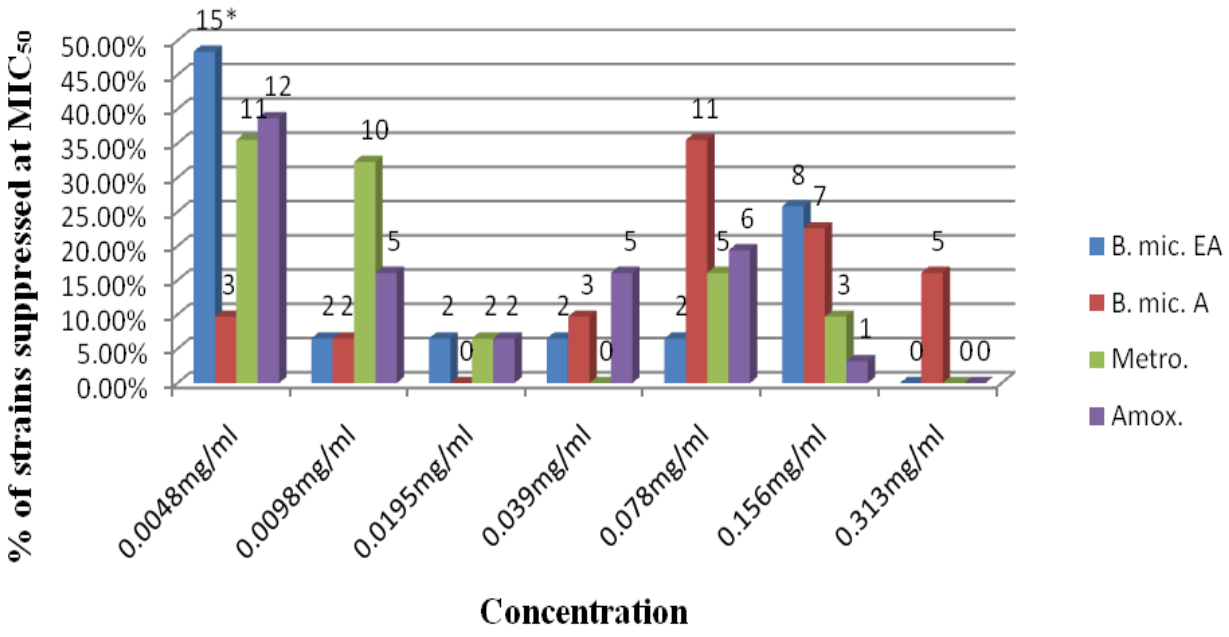
PE402A	0.156	>1.25	0.078	0.625	0.156	>0.156	0.078	>0.156
PE406C	0.078	ND	0.039	ND	0.0098	>5	0.0195	ND
PE407C	0.156	1.25	0.078	0.313	0.078	>0.156	0.039	0.625
PE411C	0.0048	>0.0048	0.313	ND	0.0098	ND	0.0048	0.156
PE430A	0.0048	ND	0.313	>0.625	0.0195	ND	0.039	0.156
PE430C	0.156	>0.156	0.156	ND	0.078	>5	0.078	>0.313
PE436A	0.0048	>0.156	0.0098	>0.078	0.0048	ND	0.0048	ND
PE436C	0.0048	0.0048	0.0098	0.625	0.0048	0.0098	0.0048	0.625
PE462A	0.039	ND	0.313	ND	0.0098	ND	0.0098	ND
PE462C	0.0195	ND	0.039	ND	0.0098	ND	0.0195	ND
PE466C	0.0098	ND	0.156	ND	0.0098	ND	0.0098	ND
PE467A	0.0048	>0.0098	0.039	>0.078	0.0048	ND	0.0048	0.625
PE467C	0.0048	>0.0048	0.156	ND	0.0048	ND	0.078	0.313
PE469C	0.0048	0.0048	0.0048	0.313	0.0048	1.25	0.0048	0.313
PE471A	0.0048	>0.0048	0.078	>0.313	0.0048	ND	0.0048	0.313
Average	0.052		0.118		0.034		0.031	

*ND, Not determined; *>, Closer but not exact.



*, No. of strains.

Figure 3. Antibacterial profile (MIC_{50}) of the tested extract (*P. africanum*) and antibiotics (metronidazole and amoxicillin) against 31 strains of *H. pylori*.



*, No. of strains.

Figure 4. Antibacterial profile (MIC_{50}) of the tested extract (*B. micrantha*) and antibiotics (metronidazole and amoxicillin) against 31 strains of *H. pylori*.

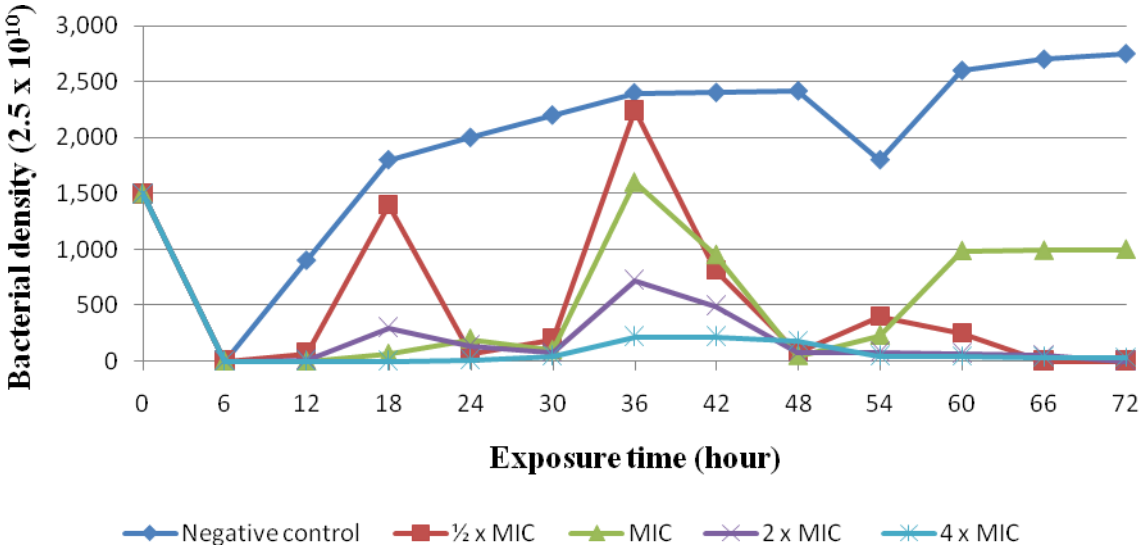
4.3 RATE OF KILL

P. afr EA was active at a concentration of 0.05 mg/mL (½ x MIC), 0.2 mg/mL (2 x MIC) and 0.4 mg/mL (4 x MIC) against strains PE466C and PE252C, while *B. mic.* EA was active at a concentration of 0.1 mg/mL (2 x MIC) and 0.2 mg/mL (4 x MIC) against strain PE430C and PE369C.

We examined the killing curve time course of the extract at different concentrations. *P. afr* EA completely inhibited the growth of *H. pylori* strain PE466C at 0.1 mg/mL in 12 h and 0.4 mg/mL in 12 and 18 h of incubation. Growth was later observed from 24 to 60 h before permanent killing at 0.05 mg/mL in 66 h and 0.05 mg/mL & 0.2 mg/mL in 72 h respectively. For strain PE252C, complete inhibition was observed at 0.1 mg/mL and 0.4 mg/mL in 12 h, and at 0.2 mg/mL in 24 h. One hundred percent killing effect was observed at 0.2 mg/mL in 66 h and at 0.05 mg/mL, 0.1 mg/mL & 0.4 mg/mL in 72 h (Figures 5a and b).

B. mic. EA completely inhibited the growth of *H. pylori* strain PE430C at all MIC concentrations in 12 h and at 0.1 mg/mL & 0.2 mg/mL in 18 & 24 h of incubation respectively. Growth and inhibition were later observed from 30 to 60 h before 100% killing at 0.1 mg/mL & 0.2 mg/mL in 66 and 72 h. Like strain PE430C, strain PE369C was totally inhibited at all the concentrations in 12 h. Inhibition was also observed at 0.1 mg/mL and 0.2 mg/mL in 54 & 66 h. One hundred percent killing was however observed at 0.1 mg/mL (2 x MIC) in 66 & 72 h (Figures 6a and b).

(a)



(b)

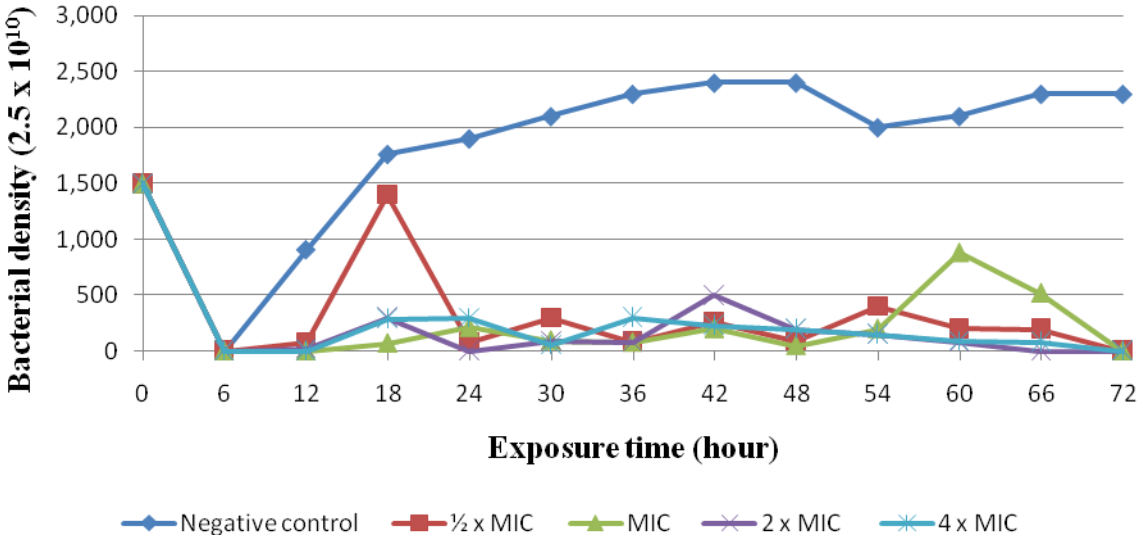
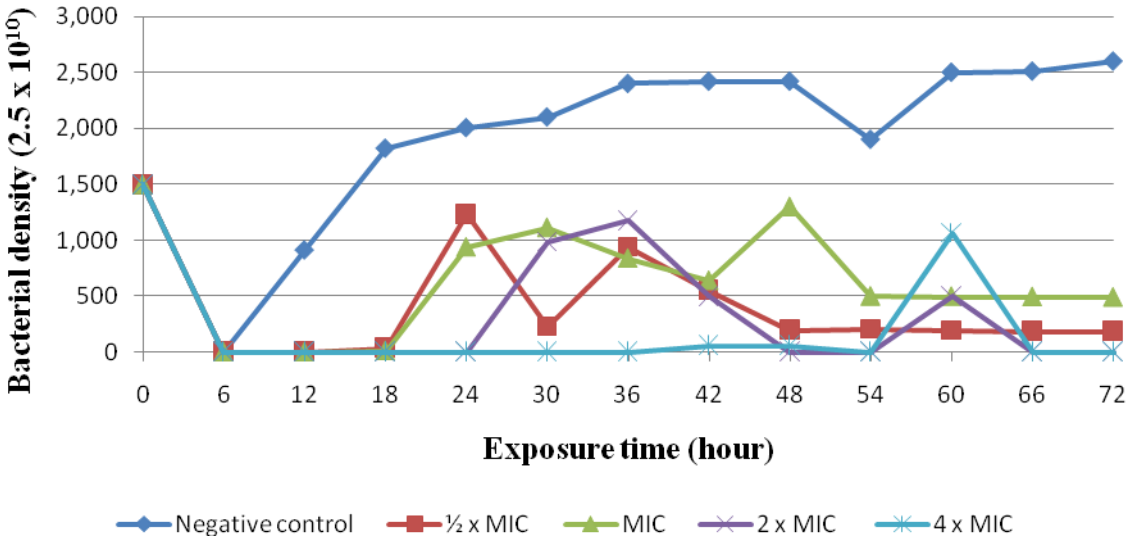


Figure 5. Profile of rate of kill of *H. pylori* strains **(a)** PE466C and **(b)** PE252C by ethyl acetate extract of *P. africanum* stem bark.

(a)



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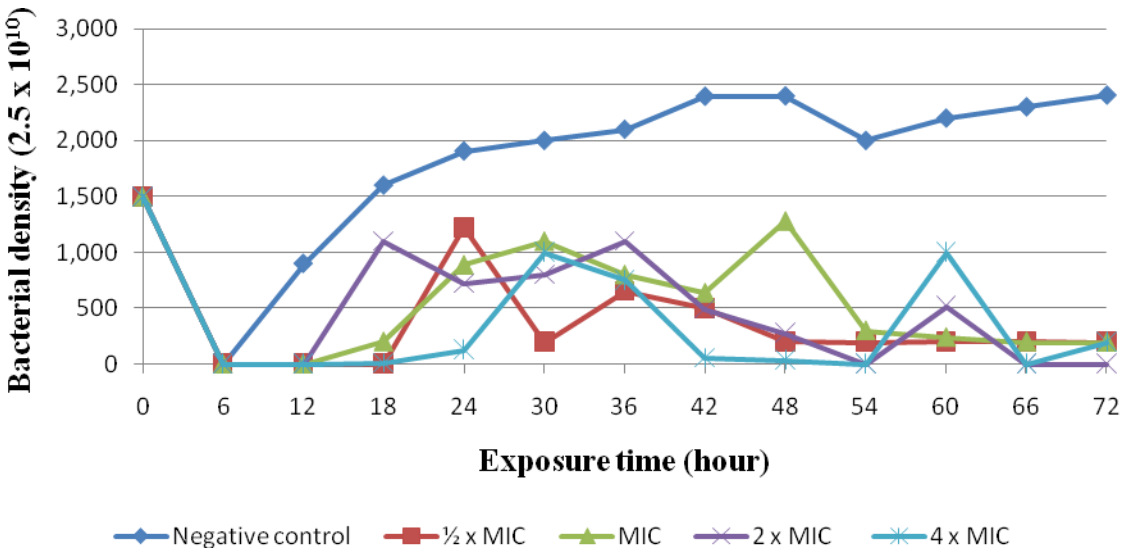

Figure 6. Profile of rate of kill of *H. pylori* strains **(a)** PE430C and **(b)** PE369C by ethyl acetate extract of *B. micrantha* stem bark.

4.4 PHYTOCHEMICAL COMPOUNDS

Qualitative phytochemical analysis revealed the presence of flavonoids, steroids, tannins, alkaloids, and saponins. It was observed from the reactions (colours, heamolysis, turbidity, layers, emulsification and precipitation) that there may be much more of steroids, tannins, flavonoids, and saponins present in *P. afr* EA and *B. mic.* EA compared to alkaloids (table 7).

Table 7: Qualitative analysis of the phytochemicals in the ethyl acetate extract of *Peltophorum africanum* and *Bridelia micrantha*.

Phytochemical	***P. afr* EA**	***B. mic.* EA**
Alkaloids	++	++
Flavonoids	+++	+++
Steroids	+++	+++
Tannins	+++	+++
Saponins	+++	+++

++, moderately present; +++, strongly present.

CHAPTER FIVE

DISCUSSION AND CONCLUSION

5.1 DISCUSSION

In spite of the high prevalence of *H. pylori* infection among gastric related morbidity patients in the Eastern Cape Province of South Africa, as well as the increasing trend of antibiotic resistant strains of this pathogen to the current treatment regimen (Tanih *et al*., 2010), few studies have examined the activities of medicinal plants used in Africa to treat infections symptomatic of this organism (Ndip *et al*., 2007; Njume *et al*., 2009). We are not aware of any study which evaluated the anti-*H. pylori* activity of *P. africanum* and *B. micrantha* in spite of its demonstrated antimicrobial potential against several microorganisms including *Campylobacter* spp with similar physiological characteristics to *H. pylori* (Samie *et al*., 2005).

Resistance to metronidazole, clarithromycin and other antibiotic has been observed in different parts of the world and continue to increase; a major concern in the treatment of *H.* pylori infection which was also noted in this study. For example, strain PE296C observed to be resistant to metronidazole (Tanih *et al*., 2010) was also resistant to clarithromycin (0 mm) in the present study. The Gram-negative bacterial cell wall outer membrane and genetic make up are thought to act as a barrier to many substances including antibiotics. In contrast *P. afr* EA, *B. mic.* EA, *B. mic.* A, demonstrated activity against PE296C (and others) with high diameter zone of inhibition; 17 mm, 14 mm and 21 mm respectively and therefore makes these plants the subject of further studies.

Clarithromycin resistance to *H. pylori* observed in this study (41.9%) was similar to the finding reported by Gadhi *et al*. (2001), but higher compared to the study conducted by Cogo *et al.* (2010). The MIC_{50} of 0.034 mg/mL and 0.031 mg/mL were observed in this study for metronidazole and amoxicillin respectively (commercial antibiotics). This is expected to be more active compared with the crude extracts (*P. afr* EA 0.108 mg/mL, *B. mic.* EA 0.052 mg/mL and *B. mic.* A 0.118 mg/mL) used. The extracts were active with low MIC values which is in contrast with the study of Shikov *et al*. (2008) and Atapour *et al.* (2009) who reported high MIC values of their extracts.

In the present study, the ethyl acetate and acetone extracts were observed to be the most active against all the *H. pylori* strains tested (*P. afr* EA, 100%; *B. mic.* EA, 93.5%; *B. mic.* A, 100%) compared with the other solvents used (figures 1 & 2). This is similar to the study of Theo *et al*. (2009) who demonstrated a good activity of the ethyl acetate extract of *P. africanum* against HIV-1. Maoela *et al*. (2009) worked on *Carpobrotus mellei* and *Carpobrotus quadrifidus*, common South African plants widely used in folk medicine and also reported marked activity with the ethyl acetate extract compared to the chloroform and butanol extracts. Djipa *et al*. (2000) and Asha *et al*. (2008) reported potent antimicrobial activity of the acetone extract against pathogenic bacteria compared to other solvents used. This may be due to the fact that the active compounds against *H. pylori* strains in the plant were less polar, as ethyl acetate was the least polar followed by acetone among the solvents used. Non-polar solvents dissolve non-polar compounds best and different solvents extract different compounds. Organic solvents are better for more consistent extraction of antimicrobial substances from medicinal plants compared to other solvents (Abu-Shanab *et al*., 2006).

In the present study, these plants (stem bark) were observed to contain flavonoids, tannins, saponins, steroids and alkaloids which are the common compounds present in many active plants that had been reported by many investigators to possess antimicrobial property (Adegboye *et al*., 2008). These phytochemical compounds are known to be biologically active and thus aid in the antimicrobial activities of many plants. Tannins for example, act by iron deprivation, hydrogen bounding or specific interactions with vital proteins such as enzymes and has been revealed for the treatment of inflamed or ulcerated tissues and remarkable activity in cancer prevention and anticancer (Li *et al*., 2003; Akinpelu *et al*., 2009). Flavonoids in the human diet may reduce the risk of various cancers, as well as preventing menopausal symptoms (Hodek *et al*., 2002). The huge increase in antioxidant capacity of blood seen after the consumption of flavonoid-rich foods is due to increased uric acid levels and so-called Phase II enzymes that also help to eliminate mutagens and carcinogens (Filippos *et al*., 2007).

Saponins are known to produce an inhibitory effect on inflammation (Iwalewa *et al*., 2007) while alkaloids have been reported to have analgesic, anti-spasmodic and bactericidal effects; one of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines (Adegboye *et al*., 2008). All these facts support the usefulness of *P. africanum* and *B. micrantha* in folklore remedies and may be the reason these plants are widely used for the treatment of many diseases among many tribes in Africa. Ali *et al*. (2005) had also documented compounds including eugenol and cinnamaldehyde which at a concentration of 2 μg/ml completely inhibited all their *H. pylori* strains. It may therefore be the effects of these compounds that are responsible for the activities observed in the ethyl acetate and acetone extracts of *P. africanum* and *B. micrantha* tested in the present study.

Although the stem bark of *P. africanum* had not been previously investigated for its anti-*H. pylori* activity, it has been shown to be active against a wide variety of bacteria including *Campylobacter spp, Bacillus cereus, Enterococcus fecalis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa* and *Proteus mirabilis* (Samie *et al*., 2005; Bessong *et al*., 2006; Samie *et al*., 2009). In the present study, the ethyl acetate extract of *P. africanum* demonstrated a zone of inhibition that ranged from 17 mm to 23 mm. On the other hand, the ethyl acetate extract of *B. micrantha* recorded a zone of inhibition that ranged from 12 mm to 20 mm, while 16 mm to 23 mm was demonstrated by the acetone extract against this pathogen. This is remarkable and lays credence to similar diameter of zones of inhibition for ethyl acetate and other solvent extracts of some selected medicinal plants from Cameroon against *H. pylori* (Ndip *et al*., 2007). However, Adeleye *et al*. (2008) reported an inhibition zone diameter of 0 mm for the water extract of the leaves of *B. micrantha* against 0 mm to 15 mm reported in our study. This may be due to the fact that the different parts of the plant may contain varied phytochemicals.

The MIC_{50} of the *P. afr*. EA extract ranged from 0.0048 – 0.313 mg/mL with a mean of 0.108 mg/mL which is similar to the mean value of *B. mic.* A (0.118 mg/mL) but higher than the mean of *B. mic.* EA extract (0.052 mg/mL). Samie *et al*. (2005), found that *P. africanum* was active against *B. cereus, E. faecalis, S. aureus, E. coli* and *P. aeruginosa* with a MIC value that ranged between 1.5 - 12 mg/mL and that *B. micrantha* was mostly active against *S. flexneri* (1.5 mg/mL) and most other organisms tested with a MIC value that ranged between 3 to >12 mg/mL. This was less active compared to the result of this study. Adeleye *et al*. (2008) reported no activity for the water extract of the leaves of *B. micrantha* against *H. pylori* and a MIC of the ethanol extract of 40 mg/mL which is relatively high. We may ascribe these discrepancies to the different organisms used, season in which the plants were collected as well as storage conditions amongst

others as these factors have been reported to affect antimicrobial activity of even plants within the same species (Evans, 1996; WHO, 1992).

However, in a similar study conducted by Ndip *et al*. (2007), MIC values which ranged from 0.1698 mg/mL - 0.2336 mg/mL were obtained for the methanol extracts of the plants tested against *H. pylori.* For all the isolates tested, the MIC_{50} of the control antibiotics ranged from 0.0048 mg/mL (4.8 µg/mL) - 0.156 mg/mL (156 µg/mL) for amoxicillin and metronidazole respectively, which is similar to a recent study in the same locality conducted by Tanih *et al*. (2010) who reported a MIC of 2.5 µg/mL - 5.0 µg/mL for amoxicillin. Metronidazole and amoxicillin which served as the positive control showed a significant difference ($P < 0.05$) in activity compared to *P. africanum* ethyl acetate & *B. micrantha* acetone extracts, but not ($P > 0.05$) to *B. micrantha* ethyl acetate extract.

Among all the strains investigated at $MIC_{90,}$ only strain PE258C had a concentration value of 0.625 mg/mL (*P. afr*. EA); the others ranged between 0.0048 mg/mL to >0.313 mg/mL which reflects the highest concentration of killing effect observed at 0.4 mg/mL (4 x MIC). For *B. mic.* EA extract, apart from 5 strains with MIC_{90} >0.5 mg/mL others ranged from 0.0048 to >0.156; and therefore falls in line with the highest concentration of 0.2 mg/mL (4 x MIC) observed to have killed the organisms. This is in line with the study of Ali *et al*. (2005); and more potent compared to the observation reported by Gadhi *et al*. (2001). The organism was also noted to be completely killed when exposure time was increased to 66 and 72 h. From the observation, the rate of kill exhibited by the extract against the test strains were both concentration and time dependent which is in line with previous observations (Ali *et al*., 2005; Akinpelu *et al*., 2009).

The findings of this study revealed similar patterns of inhibition between the plant extracts and commercial antibiotics. For example, amoxicillin and metronidazole were more potent at the lowest concentration of 0.0048 mg/mL, than at higher concentrations. This also applied to *P. afr*. EA, *B. mic*. EA and *B. mic* A at 0.0098 mg/mL, 0.0048 mg/mL and 0.078 mg/mL respectively which is similar to the results of other investigators (Gadhi *et al*., 2001; Stamatis *et al*., 2003; Samie *et al*., 2009).

Although the two plants tested in this study possessed anti-*H. pylori* activities, the index of their activities varied and may due to the solvents and crude extract used. For example, based on zone diameter of inhibition and susceptibility pattern, *B. mic* A (20.39±1.476; 100%) was more active than *B. mic*. EA (16.13±1.708; 93.5%). However, for MIC values, *B. mic*. EA extract was more active at a lower concentration, (0.0048 mg/mL) than *B. mic* A, (0.078 mg/mL) by inhibiting 48.4% and 35.5% *H. pylori* strains respectively; this turn around in activity may due to the fact that the activity of extracts can be concentration dependent. This varied index in activity was also noted between *B. mic*. EA and *P. afr*. EA (zone diameter of inhibition, MIC and rate of kill). According to Uyub *et al*. (2010), it may not be known if the biological action, inhibitory and/or toxic activity is due to one or several compounds, although the isolation of pure compounds does not necessarily lead to an increase in inhibition as expected.

According to Meining *et al.* (1998), like some other drugs, treatment with lansoprazole had no significant influence on the density of *H. pylori* colonization in the corpus but a significant decrease in the antrum was observed. Tanih *et al.* (2010) also observed a high resistance to most antibiotic tested against *H. pylori* strains in the corpus than antrum. Based on the results of this study, the most potent extracts were active to all the strains of *H. pylori* tested irrespective of

their site; antrum or corpus, even those resistant to the control antibiotics were susceptible. Hence these extracts may contain acid suppressing drug properties which has been suggested to correlate with efficacy in increasing gastric pH and therefore, results in reduction or eradication of *H. pylori* strains at the antrum (Meining *et al*., 1998). On the other hand the extracts may also possess properties using other mechanisms of action apart from acid suppressing property; as it has been reported that in the corpus there is a positive correlation between increasing pH and aggravation of inflammation, while in the antrum the correlation is the inverse of this (Meining *et al*., 1998).

5.2 CONCLUSION

Based on the findings of this study the following conclusions can be drawn:

1. All the plants tested demonstrated anti-*H. pylori* activity with zone diameters of inhibition that ranged from 0 – 23 mm.
2. The most potent extracts *P. afr*. EA, *B. mic.* EA and *B. mic.* A inhibited all the strains at a concentration of 0.108, 0.052 and 0.118 mg/mL respectively.
3. The bactericidal activity investigated by the rate of kill showed *P. afr*. EA to be active at 0.2 & 0.4 mg/mL and *B. mic.* EA at 0.1 & 0.2 mg/mL respectively. This was time and concentration dependent.
4. Phytochemical analysis revealed the presence of flavonoids, tannins, saponins, steroids and alkaloids in *P. afr*. EA and *B. mic.* EA.

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Appendix 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

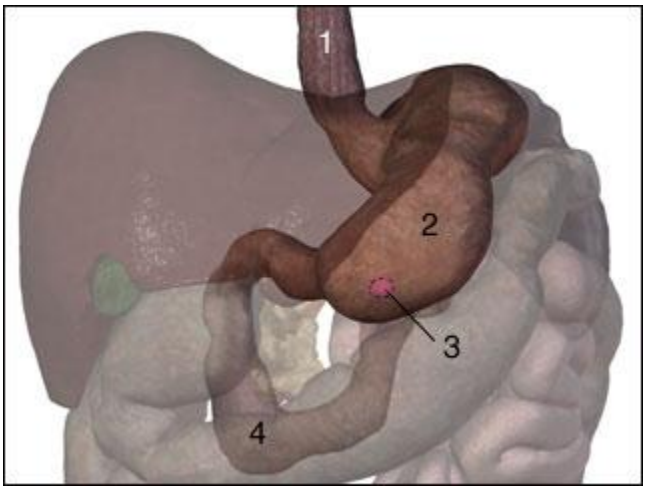
Dr Petrus DF Strijdom
Acting Dean of Research & Development

Appendix 2

Photographs of plant species, stomach ulcer and susceptibility plates

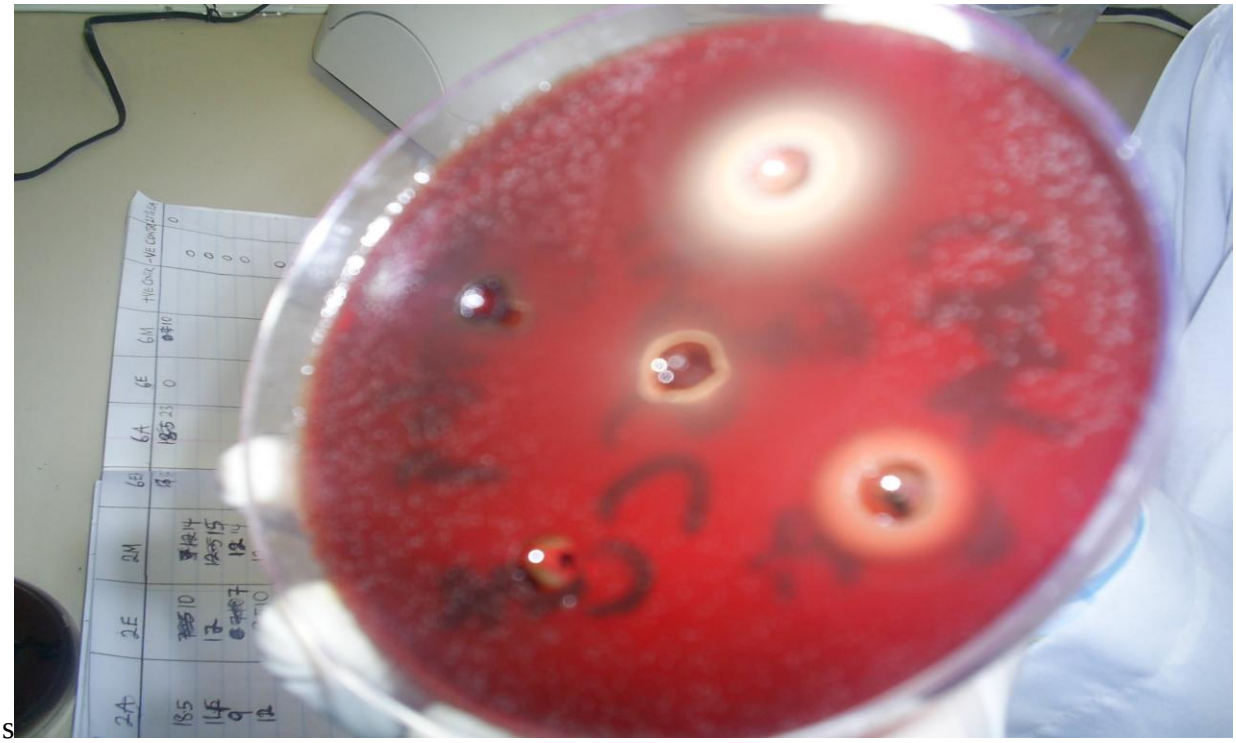
Peltophorum africanum

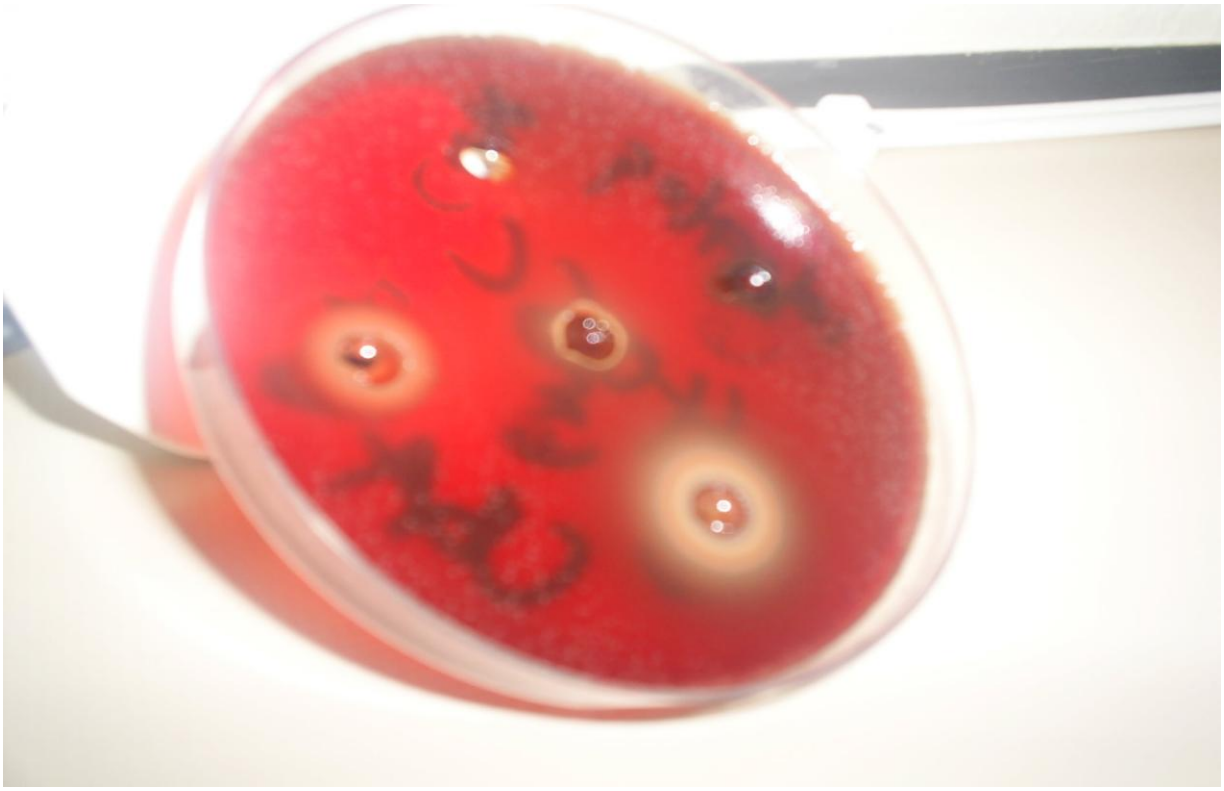
Bridelia micrantha



1. Esophagus
2. Stomach
3. Stomach ulcer
4. Duodenum (small intestine)

Stomach ulcer





Susceptibility plates demonstrating zone of inhibition

Appendix 3

Preparation of media used in the study

Preparation of transport media

The transport medium was composed of Brain Heart Infusion broth, glycerol and L-cysteine. Brain Heart Infusion broth was prepared following manufacturer's instruction which indicated 37g/l of the broth. Glycerol (20%) was added and sterilized in an autoclave at 121^0C for 15minutes, then allowed to cool after which 2g/l of L-cysteine, non-animal source was added.

Preparation of culture media

The culture medium was composed of Columbia Blood Agar Base, selective supplement and horse blood. Columbia Blood Agar Base was prepared following manufacturer's instruction which indicated 39g/l of the agar. The mixture was boiled to dissolve and sterilized in an autoclave at 121^0C for 15minutes. It was allowed to cool (50^0C), after which 2ml and 35ml (7%) of selective supplement and horse blood were added respectively.

Preparation of Urea Agar Base media

The Urea Agar Base medium was composed of Urea Agar Base and urea. Urea Agar Base was prepared following manufacturer's instruction which indicated 25.3g/l of the agar and sterilized in an autoclave at 121^0C for 15minutes. It was allowed to cool (50^0C) and an ampoule of urea (40%) was added.

Preparation of Brain Heart Infusion Agar

The culture medium was composed of Brain Heart Infusion Agar, selective supplement and horse blood. Brain Heart Infusion Agar was prepared following manufacturer's instruction which indicated 47g/l of the agar. The mixture was boiled to dissolve and sterilized in an autoclave at 121^0C for 15minutes. It was allowed to cool (50^0C), after which 2ml and 35ml (7%) of selective supplement and horse blood were added respectively.

Preparation of storage media

Storage media was composed of Brain Heart Infusion Broth (BHI), Glycerol and Distilled water. This was prepared by diluting 20% glycerol in distilled water containing BHI broth following manufacturer instructions as described above and then sterilized in an autoclave at 121^0C for 15minutes. It was stored at -80^0C until use.

Appendix 4

Statistical observations

P. africanum and antibiotic: comparison of zone diameters (SPSS Version 17.0, ONE WAY ANOVA)

Multiple Comparisons

ZD

Tukey HSD

(I) ET	(J) ET	Mean Difference (I-J)	Std. Error	Sig.*	95% Confidence Interval	
					Lower Bound	Upper Bound
1*	2	11.355*	1.152	.000	8.04	14.67
	3	9.677*	1.152	.000	6.36	13.00
	4	8.226*	1.152	.000	4.91	11.54
	5	8.258*	1.152	.000	4.94	11.58
	6	4.032*	1.152	.008	.71	7.35
2	1	-11.355*	1.152	.000	-14.67	-8.04
	3	-1.677	1.152	.693	-5.00	1.64
	4	-3.129	1.152	.077	-6.45	.19
	5	-3.097	1.152	.083	-6.42	.22
	6	-7.323*	1.152	.000	-10.64	-4.00
3	1	-9.677*	1.152	.000	-13.00	-6.36
	2	1.677	1.152	.693	-1.64	5.00
	4	-1.452	1.152	.806	-4.77	1.87
	5	-1.419	1.152	.821	-4.74	1.90
	6	-5.645*	1.152	.000	-8.96	-2.33
4	1	-8.226*	1.152	.000	-11.54	-4.91
	2	3.129	1.152	.077	-.19	6.45
	3	1.452	1.152	.806	-1.87	4.77
	5	.032	1.152	1.000	-3.29	3.35

	6	-4.194*	1.152	.005	-7.51	-.87
5	1	-8.258*	1.152	.000	-11.58	-4.94
	2	3.097	1.152	.083	-.22	6.42
	3	1.419	1.152	.821	-1.90	4.74
	4	-.032	1.152	1.000	-3.35	3.29
	6	-4.226*	1.152	.004	-7.54	-.91
6	1	-4.032*	1.152	.008	-7.35	-.71
	2	7.323*	1.152	.000	4.00	10.64
	3	5.645*	1.152	.000	2.33	8.96
	4	4.194*	1.152	.005	.87	7.51
	5	4.226*	1.152	.004	.91	7.54

*, The mean difference is significant at the <0.05 level; 1, *P. afr*. EA; 2, *P. afr*. A; 3, *P. afr*. E; 4, *P. afr*. M; 5, *P. afr*. W; 6, Clar.

B. micrantha and antibiotic: comparison of zone diameters (SPSS Version 17.0, ONE WAY ANOVA)

Multiple Comparisons

ZD

Tukey HSD

(I) ET	(J) ET	Mean Difference (I-J)	Std. Error	Sig.*	95% Confidence Interval	
					Lower Bound	Upper Bound
1*	2	-4.258*	1.191	.006	-7.69	-.83
	3	13.387*	1.191	.000	9.96	16.82
	4	10.452*	1.191	.000	7.02	13.88
	5	5.097*	1.191	.000	1.67	8.53
	6	.806	1.191	.984	-2.62	4.24
2	1	4.258*	1.191	.006	.83	7.69
	3	17.645*	1.191	.000	14.22	21.08
	4	14.710*	1.191	.000	11.28	18.14
	5	9.355*	1.191	.000	5.92	12.78
	6	5.065*	1.191	.000	1.63	8.49
3	1	-13.387*	1.191	.000	-16.82	-9.96
	2	-17.645*	1.191	.000	-21.08	-14.22
	4	-2.935	1.191	.140	-6.37	.49
	5	-8.290*	1.191	.000	-11.72	-4.86
	6	-12.581*	1.191	.000	-16.01	-9.15
4	1	-10.452*	1.191	.000	-13.88	-7.02
	2	-14.710*	1.191	.000	-18.14	-11.28
	3	2.935	1.191	.140	-.49	6.37
	5	-5.355*	1.191	.000	-8.78	-1.92
	6	-9.645*	1.191	.000	-13.08	-6.22
5	1	-5.097*	1.191	.000	-8.53	-1.67

	2	-9.355*	1.191	.000	-12.78	-5.92
	3	8.290*	1.191	.000	4.86	11.72
	4	5.355*	1.191	.000	1.92	8.78
	6	-4.290*	1.191	.005	-7.72	-.86
6	1	-.806	1.191	.984	-4.24	2.62
	2	-5.065*	1.191	.000	-8.49	-1.63
	3	12.581*	1.191	.000	9.15	16.01
	4	9.645*	1.191	.000	6.22	13.08
	5	4.290*	1.191	.005	.86	7.72

*, The mean difference is significant at the <0.05 level; 1, *B. mic.* EA; 2, *B. mic.* A; 3, *B. mic.* E; 4, *B. mic*. M; 5, *B. mic.* W; 6, Clar.

P. africanum and antibiotic: comparison of MIC_{50} (SPSS Version 17.0, ONE WAY ANOVA)

Multiple Comparisons

MI

Tukey HSD

(I) ET	(J) ET	Mean Difference (I-J)	Std. Error	Sig.*	95% Confidence Interval	
					Lower Bound	Upper Bound
1*	2	.073961*	.019015	.001	.02865	.11928
	3	.076645*	.019015	.000	.03133	.12196
2	1	-.073961*	.019015	.001	-.11928	-.02865
	3	.002684	.019015	.989	-.04263	.04800
3	1	-.076645*	.019015	.000	-.12196	-.03133
	2	-.002684	.019015	.989	-.04800	.04263

*, The mean difference is significant at the <0.05 level; 1, *P. afr*. EA; 2, Metro; 3, Amox.

B. micrantha and antibiotic: comparison of MIC_{50} (SPSS Version 17.0, ONE WAY ANOVA)

Multiple Comparisons

MI

Tukey HSD

(I) ET	(J) ET	Mean Difference (I-J)	Std. Error	Sig.*	95% Confidence Interval	
					Lower Bound	Upper Bound
1*	2	-.066239*	.016970	.001	-.11045	-.02202
	3	.018219	.016970	.706	-.02599	.06243
	4	.020903	.016970	.608	-.02331	.06512
2	1	.066239*	.016970	.001	.02202	.11045
	3	.084458*	.016970	.000	.04024	.12867
	4	.087142*	.016970	.000	.04293	.13136
3	1	-.018219	.016970	.706	-.06243	.02599
	2	-.084458*	.016970	.000	-.12867	-.04024
	4	.002684	.016970	.999	-.04153	.04690
4	1	-.020903	.016970	.608	-.06512	.02331
	2	-.087142*	.016970	.000	-.13136	-.04293
	3	-.002684	.016970	.999	-.04690	.04153

*, The mean difference is significant at the <0.05 level; 1, *B. mic*. EA; 2, *B. mic.* A; 3, Metro; 4, Amox.

B. micrantha and *P. africanum*: comparison of MIC_{50} (SPSS Version 17.0, ONE WAY ANOVA)

Multiple Comparisons

MI

Tukey HSD

(I) ET	(J) ET	Mean Difference (I-J)	Std. Error	Sig.*	95% Confidence Interval	
					Lower Bound	Upper Bound
1*	2	.055742	.024248	.061	-.00204	.11353
	3	-.010497	.024248	.902	-.06828	.04729
2	1	-.055742	.024248	.061	-.11353	.00204
	3	-.066239*	.024248	.021	-.12402	-.00845
3	1	.010497	.024248	.902	-.04729	.06828
	2	.066239*	.024248	.021	.00845	.12402

*, The mean difference is significant at the <0.05 level; 1, *P. afr*. EA; 2, *B. mic.* EA; 3, *B. mic.* A.

Appendix 5

Publications and manuscript submitted

Okeleye BI, Samie A, Bessong PO, Mkwetshana NF, Green E, Clarke AM, Ndip RN. (2010). Crude Ethyl Acetate Extract of the Stem Bark of *Peltophorum africanum* (Sond, Fabaceae) possesses *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori.* Journal of Medicinal Plants Research. 4 (14): 1432-1440.

Okeleye BI, Mkwetshana NF, Samie A, Bessong PO, Green E, Ndip RN. (2010). *In vitro* anti-*Helicobacter pylori* activities of crude ethyl acetate extract of the stem bark of *Bridelia micrantha* (Hochst., Baill., Euphorbiaceae): Preliminary phytochemical screenings. Submitted to African Journal of Traditional, Complementary and Alternative Medicines.

Journal of Medicinal Plants Research Vol. 4(14), pp. 1432-1440, 18 July, 2010
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Crude ethyl acetate extract of the stem bark of *Peltophorum africanum* (Sond, Fabaceae) possessing *in vitro* inhibitory and bactericidal activity against clinical isolates of *Helicobacter pylori*

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Accepted 10 June, 2010

***Helicobacter pylori* is the most important risk factor in gastritis, peptic ulcer and gastric cancer. This study explored the antimicrobial activity of the stem bark of *Peltophorum africanum* on *H. pylori* isolated in South Africa. Strains were isolated from patients presenting with gastric related morbidities at the Livingston Hospital, Port Elizabeth for endoscopy. Five extracts of *P. africanum* and clarithromycin were tested for the zone diameters of inhibition against 31 clinical strains of *H. pylori*, followed by the minimum inhibitory concentration (MIC) using metronidazole and amoxicillin as control antibiotics; and thereafter the rate of kill. Zone diameters of inhibition which ranged from 0 - 23 mm were observed for all the five extracts and 0 - 35 mm for clarithromycin. Marked susceptibility of strains (100%) was recorded for the ethyl acetate extract ($P < 0.05$). The MIC_{50} ranged from 0.0048 - 0.313 mg/mL and MIC_{90} from 0.156 - 0.625 mg/mL for the ethyl acetate extract (*P. africanum* EA). There was a significant statistical difference observed in potency with *P. africanum* EA compared to metronidazole and amoxicillin ($P < 0.05$) at MIC_{90}. Complete killing of strain PE466C by *P. africanum* EA was observed at 0.05 mg/mL (½ x MIC) in 66 and 72 h and 0.2 mg/mL (2 x MIC) in 72 h. For strain PE252C, total killing was observed at 0.2 mg/mL (2 x MIC) in 66 h and at 0.05 mg/mL (½ x MIC), 0.1 mg/mL (MIC), 0.2 mg/mL (2 x MIC) and 0.4 mg/mL (4 x MIC) in 72 h, respectively. Our findings demonstrate the *in vitro* activity of the crude extracts of *P. africanum* and therefore provide evidence to justify the use of this plant in traditional medicine.**

Key words: *Helicobacter pylori*, *Peltophorum africanum*, medicinal plant, antibacterial activity, MIC, rate of kill, South Africa.

INTRODUCTION

Helicobacter pylori is one of the most common chronic bacterial pathogens of humans. Colonization is usually life long and may lead to chronic gastritis, peptic ulceration and gastric cancer in later life (Konturek, 2004). It colonizes the gastric epithelial surface and withstands the stomach's hostile environment by microaerophilic growth capacity (Lynch, 2007), and the production of numerous virulence factors (Tanih et al., 2008).

Infections have been reported to be higher in the developing than in developed countries. In Africa, 70 - 80% is infected with the organism and 61 - 100% harbours the organism in sub-Saharan Africa (Holcombe, 1992; Ndip et al., 2008). In the Republic of South Africa, a prevalence of between 50 - 87% has been reported (Fritz et al., 2006; Samie et al., 2007; Dube et al., 2009). *H. pylori* could be eradicated by a combination of therapeutic agents such as antibiotics, bismuth

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