Phytochemical analysis and bioactivity of selected South African medicinal plants on clinical isolates of *Helicobacter pylori*.

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DECLARATION

The experimental work described in this thesis was conducted in the Department of Biochemistry and Microbiology, Faculty of Science, University of Fort Hare between March 2009 and July 2011 under the supervision of Prof. Roland N. Ndip and Prof. Anthony J. Afolayan.

The study is a result of my own investigations, except where the work of others is acknowledged, and has not been submitted in any other form to another university.

I declare the above statement to be true.

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GENERAL SYNOPSIS

Medicinal plants have been used as traditional medicine in the treatment of numerous human diseases for thousands of years in many parts of the world. In the developing world, especially in rural areas, herbal remedies continue to be a primary source of medicine. Scientifically, medicinal plants have proven to be an abundant source of biologically active compounds, many of which have already been formulated into useful therapeutic substances or have provided a basis for the development of new lead molecules for pharmaceuticals.

Antibiotic resistance, undesireable side effects and expences associated with the use of combination therapy in the treatment of *Helicobacter pylori* infections have generated a considerable interest in the study of medicinal plants as potential sources of new drugs against this organism. The high complexicity of bioactive compounds accumulated in plants coupled with their broad antimicrobial activity may make it difficult for pathogenic organisms, including *H. pylori* to acquire resistance during treatment. This study therefore evaluates the antimicrobial potential of selected South African medicinal plants employed in the treatment of *H. pylori*-related infections, and the subsequent isolation of the plant active principles.

An ethnobotanical survey of plants used in the treatment of *H. pylori*-related infections was conducted in the study area. Crude extracts of *Combretum molle*, *Sclerocarya birrea*, *Garcinia kola*, *Alepidea amatymbica* and 2 *Strychnos* species were screened against 30 clinical strains of *H. pylori* and 2 standard control strains (NCTC 11638 and ATCC 43526). In the preliminary stages of this study, ethyl acetate, acetone, ethanol, methanol and water extracts of the plants were tested against *H. pylori* by agar well diffusion and micro broth dilution methods. The plant crude extracts that exhibited anti-*H. pylori* activity with a

percentage susceptibility of 50% and above were considered for the rate of kill assays and the most active crude extracts selected for bio-assay guided isolation of the active ingredient.

Preliminary fractionation of the crude extract was achieved by thin layer chromatography (TLC) using different solvent combinations; hexane/diethylether (HDE), ethyl acetate/methanol/water (EMW) and chloroform/ethyl acetate/formic acid (CEF) in order to determine the most suitable combination for column chromatography (CC) and subsequent testing by indirect bioautography. The extract was then fractionated in a silica gel column using previously determined solvent combinations as eluent. Active fractions obtained from column chromatography separations were further fractionated and the compounds identified by gas chromatography/mass spectrometry (GC/MS) analysis.

All the plants exhibited antimicrobial activity against *H. pylori* with zone of inhibition diameters ranging from 0 - 38 mm and minimum inhibitory concentration (MIC) values ranging from 0.06 - 5.0 mg/mL. The most active plant extracts were the acetone extract of *C. molle* with a percentage susceptibility of 87.1%, acetone and aqueous extracts of *S. birrea* (71% each) and the ethanolic extracts of *G. kola* (53.3%). Except for the aqueous extract, these extracts also exhibited a strong bactericidal activity against *H. pylori* at different concentrations. TLC analysis revealed the presence of 9 components in the acetone extract of *S. birrea* with the EMW solvent system as opposed to 5 and 8 with HDE and CEF respectively. Bioassay-guided isolation led to the identification of 52 compounds from the acetone extract of *S. birrea* with n-octacosane being the most abundant (41.68%). This was followed by pyrrolidine (38.91%), terpinen-4-ol (38.3%), n-eicosane (24.98%), cyclopentane (16.76%), n-triacontane (16.28%), aromadendrene (13.63 %) and α-gujunene (8.77 %).

Terpinen-4-ol and pyrrolidine demonstrated strong antimicrobial activity against *H. pylori* at all concentrations tested.

These results may serve as preliminary scientific validation of the ethnomedicinal uses of the above mentioned plants in the treatment of *H. pylori*-related infections in South Africa. Terpinen-4-ol and pyrrolidine could be considered for further evaluation as therapeutic or prophylactic agents in the treatment of *H. pylori*-related infections. However, further investigations would be necessary to determine their toxicological properties, in-vivo potencies and mechanism of action against *H. pylori*.

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LIST OF ABBREVIATIONS

BabA	Blood adhesion binding antigen
CagA	Cytotoxin associated gene A antigen
CagPAI	Cag pathogenicity island
CC	Column chromatography
CEF	Chloroform, ethyl acetate, formic acid
CoNS	Coagulase negative *Staphylococcus aureus*
EMW	Ethyl acetate, methanol, water
FNQ	Furanonaphthoquinolone
GC/MS	Gas chromatography/mass spectrometry
H_2RA	Histamine receptor antagonist
HDE	Hexane, diethyl ether
HPLC	High performance liquid chromatography
MALT	Mucosa associated lymphoid tissue
MIC_{50}	50% minimum inhibitory concentration
MIC_{90}	90% minimum inhibitory concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
NADPH	Nicotinamide adenine dinucleotide phosphate
PASW	Predictive analytics software statistics

PBP	Penicillin binding protein
PHFA	Preferential homoduplex formation assay
PPI	Proton pump inhibitor
PUD	Peptic ulcer disease
QRDR	Quinolone resistant determining region
SPSS	Statistical package for the social sciences
TLC	Thin layer chromatography
VacA	Vacuolating cytotoxin

CHAPTER ONE

INTRODUCTION

1.1 **Introduction**

The increase of antibiotic resistance of microorganisms to conventional drugs has generated a considerable interest in the search for new, efficient and cost effective ways for the control of infectious diseases. There is a constant search for new organic molecules with antibacterial activity, which could be cheap and readily available to the local population as a means of improving primary health care. Two to three antibiotics derived from microorganisms are launched each year (Cowan, 1999). Scientists have realised that in order to cope with this slow pace, coupled with the fact that previously discovered drugs are rendered obsolete by resistant bacterial strains, plant based remedies would have to be considered as alternative sources of new drugs.

Ethno botanical studies carried out in South Africa and other parts of Africa have shown that medicinal plants constitute a great source for the isolation of active drugs (Ndip *et al*., 2007; Afolayan and Lewu, 2009; Ojewole *et al*., 2010). Traditional medicine is well recognized in South Africa and different communities use a wide variety of plants to treat gastrointestinal disorders (Samie *et al*., 2005). Stomach pain, gastritis and peptic ulcers are among a plethora of *H. pylori*-related illnesses that are treated traditionally with herbal remedies (Njume *et al*., 2011). These remedies are readily available and affordable and many used are considered suitable alternatives to western drugs often characterised with high pill burden and cost. Consequently, many rural dwellers in the developing world rely on ethno medicines for health care. There is therefore, an increasing trend to integrate folklore medicine with

primary health care considering that about 80% of the people in the developing world depend on it (Patra *et al*., 2009). Over the years, the World Health Organisation (WHO) has advocated the need for interaction between modern and traditional medicines with a view to exploiting and identifying compounds that could provide safe and effective remedies for ailments of both microbial and non-microbial origins (WHO, 1987). It is estimated that plant materials are present in or have provided the models for about 50% of Western drugs (Patra *et al*., 2009), and herbal remedies continue to play a role in the cure of diseases (Tabuti *et al*., 2003; Patra et al., 2009).

Considerable attention has been given to the screening of medicinal plants all over the world as a means to identify cheap sources of new drugs against *H. pylori*, a human gastric pathogen with high morbidity rate (Ndip *et al*., 2004). Infection with *H. pylori* is curable with antibiotic therapy in combination with proton pump inhibitors (PPI) or bismuth salts (Manyi-Loh *et al*., 2010a). The success rate of combination therapy is up to 90% (Ndip *et al*., 2007). However, success rates have decreased substantially during the last few years owing to development of resistant *H. pylori* strains. Post therapeutic resistance has been shown to increase dramatically, making repeated use of some of the antibiotics impossible (Bohr and Malfertheiner, 2009).

With very few exceptions, the most commonly recommended treatment regimen (PPI, amoxicillin and clarithromycin or metronidazole) now provides unacceptably low treatment successes (Graham and Fischbach, 2010). Approximately one in five patients now need second and third-line therapies due to eradication failure (Bohr and Malfertheiner, 2009). These therapies may require the use of bismuth-based combinations that produce satisfactory

eradication rates, but bismuth is not available in many areas of the developing world (Manyi-Loh *et al*., 2010a). Equally important is the fact that combination therapy is plagued with undesirable side effects such as epigastric pain, abdominal discomfort, diarrhoea, nausea and vomiting (Njume *et al*., 2009). As a result, patients most often do not complete the treatment course, thus generating suboptimal antibiotic blood concentrations that predispose to the selection and survival of resistant bacterial strains (Njume *et al*., 2009). The high pill burden which is at times associated with combination therapy may also reduce patient compliance and therapeutic efficacy. Consequently, there has been a continuous search for new compounds to replace or complement previously existing ones in a bid to circumvent the overall burden of antimicrobial resistance and other problems associated with the present treatment regimens.

1.2 **Infection with *Helicobacter pylori* and associated diseases**

Helicobacter pylori is a Gram negative microaerophilic helical bacillus that inhabits various areas of the human stomach (Ndip *et al*., 2008). The organism is pleomorphic and highly motile by means of lophotrichous flagella. It has the ability to convert the harsh acidic environment of the stomach, a bactericidal barrier that protects against many infections into a suitable ecological niche (Schreiber *et al*., 2005). It achieves this by producing urease, an enzyme that breaks down urea to ammonia and carbon dioxide, decreasing stomach acidity and making the environment suitable for its survival (Tanih *et al*., 2008).

Infection with *H. pylori* often begins in infancy and may last for years or decades (Ndip *et al*., 2004; Shi *et al*., 2008). It starts in the gastric antrum and spreads to the corpus, after extensive mucosal damage. Invasion of the mucosa causes damage that is eventually

worsened by the acid produced in the stomach and this may lead to complications (ulcers and cancers). Half of the world's population is infected by this organism (Atherton, 2006).

Marshall and Warren (1983) proved that infection with *H. pylori* is strongly associated with chronic gastritis, peptic ulcer and gastric cancer. These authors by using technologies generally available (fibre endoscopy, silver staining of histological sections and culture techniques for microaerophilic bacteria), made an irrefutable link between the bacteria and the above diseases.

H. pylori is now a confirmed cause of 90% of all duodenal ulcers, 75% of all gastric ulcers and two forms of stomach cancer; adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Jones *et al*., 2008). Evidence of its association with gastric cancer, the second most common cause of cancer-related death globally (Epplein *et al*., 2008), led to its classification as a class 1 carcinogen by the International Agency for Research on Cancer and the World Health Organisation (Mégraud and Lehours, 2007).

H. pylori infection confers around a two-fold increase in the risk of developing gastric cancer particularly strains expressing the cytotoxin-associated gene A antigen (*CagA*) which further causes a two-fold increase in gastric cancer risk (Enroth *et al*., 2000). Death from gastric cancer is second only to lung cancer in men and thus contributes to approximately 10% of all cancer deaths annually (Prinz *et al*., 2006). While 80–90% of *H. pylori*-infected individuals have clinically asymptomatic gastritis, 10–15% develop peptic ulcer, and 1–2% gastric malignancies (Wu *et al*., 2008; Dube *et al*., 2009). Until the discovery of Marshall and

Warren (1983), diet, stress and life-style factors were hitherto considered major causes of gastritis and peptic ulcer, and the stomach, a sterile environment (Khalifa *et al*., 2010).

1.3 **Reservoirs of infection and transmission**

H. pylori has a narrow host range and is found almost exclusively in humans and some non human primates (Hannula and Hanninen, 2007). Environmental and animal reservoirs have been under intense investigations as possible sources of infection. Food, animals and water sources have been suggested as reservoirs outside the human gastrointestinal tract and the organisms or their DNA have been detected in these sources (Dube *et al*., 2009). However, there is no definitive evidence that they are natural or primary vehicles of transmission.

The exact mechanisms by which the organism is acquired are still largely unknown (Janzon *et al*., 2009). However, transmission is thought to follow iatrogenic, oral-oral or faecal-oral routes (Janzon *et al*., 2009). Oral-oral transmission is regarded as a plausible route (Khalifa *et al*., 2010). It has been shown to be potentiated by specific eating habits, such as the premastication of food by mothers before feeding children in some African countries and the coating of nipples with saliva before breast feeding by Hindu mothers in Bangladesh (Frenck and Clemens, 2003). This is very important considering that childhood appears to be the critical period during which *H. pylori* is acquired, especially in areas of over-crowding and socio-economic deprivation (Ahmed *et al*., 2007; Manyi-loh *et al*., 2010a).

Repeated use of gastric tubes by endoscopists without proper sterilization is also considered a possible means of transmission. *H. pylori* infection has been shown to follow socio-

demographic factors such as occupation, family income level and living conditions (Tanih *et al*., 2008). A high prevalence of up to 90% has been reported in developing countries characterized by overcrowded homes and poor sanitation as opposed to 10-40% in western affluent societies, the developed world (Malaty, 2007; Castillo-Juarez *et al*., 2009; Haesebrouck *et al*., 2009). The status quo of *H. pylori* transmission routes should therefore be addressed in third world countries where the incidence is high.

1.4 **Pathogenesis of *H. pylori* infection**

H. pylori has adapted to the inhospitable conditions found at the gastric mucosal surface. Urease production and high motility are very vital for its survival in this environment (Tanih *et al*., 2008). Urease is thought to allow short-term survival in the highly acidic gastric lumen. This enzyme catalyses the breakdown of urea into ammonia and bicarbonates (Iizumi *et al*., 2005; Tanih *et al*., 2008). The ammonia which is naturally alkaline reduces the acidity of the stomach forming a protective alkaline cloud suitable for the survival of the organism.

Motility of the lophotrichous flagellated organism is also thought to allow rapid movement towards the more neutral pH of the gastric mucosa implying that both factors are prerequisites for colonization of the gastric mucosa. The spiral morphology also assists in penetration into the viscous mucus layer, where the more pH-neutral conditions allow growth of the organism (Tanih *et al*., 2008).

Adherence of *H. pylori* to epithelial cells is a relevant step in the development of gastro duodenal pathologies. Blood adhesion binding antigen (*BabA2*) allows for attachment to these cells followed by delivery of the vacuolating cytotoxin(*VacA*) and the cytotoxin-associated gene (*CagA*) proteins near the gastric epithelium, enhancing gastric tissue damage (Torres *et al*., 2009).

Studies have also demonstrated that some 18 *Cag* PAI-encoded proteins serve as building blocks of a type IV secretion apparatus, which forms a syringe-like structure capable of penetrating the gastric epithelial cells and facilitating the translocation of *CagA*, peptidoglycan, and possibly other bacterial factors into host cells (El-Omar, 2003; Robinson *et al*., 2007; Mcnamara and El-Omar, 2008). Once delivered inside the cell, the *CagA* protein is phosphorylated at tyrosine residues by kinases. Phosphorylated *CagA* then interacts with a range of host signaling molecules, such as the tyrosine phosphatase SHP-2, which results in morphological changes in the epithelial cells (El-Omar, 2003; 2006).

The vacuolating cytotoxin (*VacA*) is a highly immunogenic 95-kDa protein which has been reported to induce massive vacuolization in epithelial cells *in-vitro* (El-Omar, 2006). It is found in 50% of all *H. pylori* strains (Epplein *et al*., 2008). Its activities include membrane channel formation, disruption of endosomal and lysosomal activity, effects on integrin receptor-induced cell signaling, interference with cytoskeleton-dependent cell functions, induction of apoptosis, and immune modulation (El-Omar, 2003; 2006).

As a result of *H. pylori* infection, the epithelial cells release cytokines which recruit inflammatory cells to the mucosa. These cells consist of leukocytes that gather in the Lamina propria causing gastritis (Konturek *et al*., 2009). The primary disorder, which occurs after colonization with *H. pylori*, is chronic active gastritis (Romano and Cuomo, 2004; Kusters *et al*., 2006). This condition can be observed in all *H. pylori*-positive subjects (Enroth *et al*., 2000).

Data on the acute phase of infection are scarce and largely come from reports of subjects who deliberately or inadvertently ingested *H. pylori* or underwent procedures with contaminated material (Robinson *et al*., 2007). Acute infection in adults is usually symptomatic, with variable symptomatology including epigastric discomfort, nausea, malaise, belching and halitosis (Kusters *et al*., 2006). Symptoms usually resolve within two weeks. Histologically, it is characterised by a heavy neutrophilic infiltration and then gradual infiltration by all classes of inflammatory cells, predominantly lymphocytes, which may persist (Robinson *et al*., 2007).

Although infection with *H. pylori* almost always results in chronic active gastritis, most infected patients develop no other complications and are free of any obvious clinical symptoms of this infection (Epplein *et al*., 2008). The intra-gastric distribution and severity of a chronic inflammatory process resulting from *H. pylori* infection depends on a variety of factors, such as characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production (El-Omar, 2006; Shi *et al*., 2008).

Some strains may be more virulent than others particularly those expressing the highly immunogenic cytotoxin associated gene A protein (*cag A*) present in approximately 50 to 70% of these organisms (El-Omar, 2006; Epplein *et al*., 2008; Lee *et al*., 2008; Tanih *et al*., 2010a). Patients infected with $CagA^{+}$ strains usually have a higher inflammatory response and are significantly more at risk of developing a symptomatic outcome (Torres *et al*., 2009). *H. pylori*-induced ulcer disease, gastric cancer, and lymphoma are all complications of this chronic inflammation; ulcer disease and gastric cancer in particular occur in those individuals and at those sites with the most severe inflammation (El-Omar, 2006).

1.5 **Diagnosis of *H. pylori* infection**

Ample evidence now exists to link *Helicobacter pylori* to a number of gastro duodenal pathologies. Detection of this organism in gastric biopsies and vomitus becomes an important means of diagnosing gastro duodenal infections due to *H. pylori*. Culturing samples for microbial isolation remains the gold standard for linking the organism to the disease condition. However, culture tests are slow to produce results due to the slow growing nature of the organism (Mégraud and Lehours, 2007).

PCR based techniques are preferred for their rapidity, sensitivity and specificity but also limited by the likelihood of false positives arising from residual *H. pylori* DNA from poorly processed equipment (Mégraud and Lehours, 2007). Diagnosis of *H. pylori* can be divided into two groups; invasive and non invasive tests.

1.5.1 *Invasive Tests*

These are the initial tests that were used in the diagnosis of *H. pylori* infections. They include endoscopy, gastric mucosal biopsy, microscopic examination of histological sections and rapid urease tests (Tanih *et al*., 2008). Histology can reveal the presence of bacteria as well as the type of inflammation. Stains used to detect the bacteria include Warthine-Starry, Hp silver stain, Dieterle, Giemsa, Giminez, acridine orange, McMullen and immunostaining (Ndip *et al*., 2003), while haematoxylin and eosin are used to evaluate inflammatory cells.

Biopsy specimens are fixed in formalin, embedded in paraffin and stained with hematoxylin–eosin for histological examination under a light microscope and the organisms recognised by their appearance as helical or spiral bacilli resting on the epithelial surface or in the mucus layer (Gatta *et al*., 2003). Gastric biopsies can also be cultured in blood or serum-supplemented media for microbial isolation. Identification of cultured bacteria consists mainly of testing for the presence of certain enzymes: cytochrome oxidase, catalase, and urease (Samie *et al*., 2007) and eventually γ-glutamyl transpeptidase, leucine aminopeptidase, and alkaline phosphatase (Tanih *et al*., 2008).

- ***Urease biopsy test***

The high level of urease production by *H. pylori* is exploited in the detection of the organism directly on gastric biopsies by placing them in a gel containing urea and phenol red (a pH-indicating dye). If *H. pylori* is present, preformed urease will hydrolyze the urea, raise the pH, and change the colour of the phenol red from yellow to red (Mégraud *et al*., 2001). *H. pylori* cultured directly from endoscopic biopsy specimens on serum-based medium virtually

always gives a strong urease reaction, which, together with the presence of positive oxidase and catalase reactions, is diagnostic for this species (Mégraud and Lehours, 2007).

1.5.2 ***Non invasive tests***

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

- ***Urea breath test (UBT)***

Although the urease biopsy test reaction is simple, it requires obtaining biopsies by endoscopy, an invasive procedure. The urea breath test has been developed as a non-invasive procedure that serves as a sensitive and specific, although qualitative, indicator of infection (Mégraud *et al*., 2001). The patient is given an oral dose of labelled urea, either ^{13}C urea or ^{14}C urea. If the organism is present, urea will be hydrolyzed and $^{13}CO_2$ or $^{14}CO_2$ will be liberated.

The labelled CO_2 will enter the bloodstream, exchange in the lungs, and be exhaled. The exhaled CO_2 is trapped and quantified in a mass spectrometer for $^{13}CO_2$ or a scintillation counter for $^{14}CO_2$. A number of members of the normal anaerobic gut flora are urease positive and potentially could interfere with this test, but data collected thus far indicate that false-positive reactions are rare (Mégraud *et al*., 2001; Tanih *et al*., 2008).

1.6 **Treatment of *H. pylori* infections**

The discovery of *H. pylori* in 1983 led to a revolution in the management concepts of gastro duodenal infections. It is well accepted that the most common stomach disease, peptic ulcer, is an infectious disease which must be treated with antibiotics (Graham and Fischbach, 2010). The purpose of *H. pylori* treatment in any clinical situation therefore is the eradication of the organisms from the stomach. Eradication is defined as the presence of negative tests for *H. pylori* four weeks or longer after the end of antimicrobial therapy (Romano and Cuomo, 2004). It results in the effective healing of ulcers, prevents ulcer relapse, reduces recurrence of gastric cancer and potentially decreases the risk of progression to gastric carcinoma (Manyi-loh *et al*., 2010a).

Treatment regimens for *H. pylori* infection have been evolving since the early 1990s, when monotherapy was first recommended. Treatment generally involves a triple drug regimen; two antibiotics and an antisecretory drug, essentially a proton pump inhibitor (PPI) to which bismuth salt or H_2 antagonists can be added (Alarcón *et al*., 1999; Romano and Cuomo, 2004; Manyi-loh *et al*., 2010b).

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

1.7 **Components of *H. pylori* treatment regimen**

1.7.1 ***Proton pump inhibitors (PPI) and histamine H_2 receptor antagonists (H_2RA)***

These are generally known as acid reducers. They reduce the level of stomach acid in order to interfere with the physiology of *H. pylori*, an organism that is known to survive very well in an acidic milieu. Reducing stomach acid levels may also help prevent progression of an ulcer in an already damaged epithelial mucosa and improve the effectiveness of antibiotics whose activity has been shown to decrease at low pH (Mégraud and Lehours, 2007).

Apart from metronidazole which is very stable in the gastric juice at pH 2 and 7 with a half life of over 800 hours, amoxicillin is very unstable at low pH while clarithromycin is particularly sensitive to degradation with a half life of less than 1 hour at a pH of 2 (Vakil and Mégraud, 2007). Increasing the gastric pH with the use of histamine H_2 receptor antagonist (H_2RA) or PPI therefore improves the effectiveness of antibiotic therapy with possible synergistic interactions (Alarcon *et al*., 1999).

H_2 blockers work by blocking histamine, which stimulates acid secretion. They include cimetidine, ranitidine, ebrotidine, famotidine and nizatidine. PPIs suppress acid production by halting the mechanism that pumps the acid into the stomach. They include omeprazole, lansoprazole, rabeprazole, pantoprazole and esomeprazole (Manyi-Loh *et al*., 2010a). They are preferable in the presence of peptic ulcer disease because of their ability to provide more rapid pain relief and better control of pH (Romano and Cuomo, 2004). By reducing gastric emptying (Parkman *et al*., 1998) and mucus viscosity (Goddard and Spiller, 1996), PPI's also increase the gastric residence time and mucus penetration of antimicrobials (Pedrazzoli *et al*., 2001), thereby making them more effective.

1.7.2 ***Bismuth compounds (subcitrate or subsalicylate)***

These are stronger compounds which in combination with antibiotics are used in the treatment of recurrent peptic ulcer disease or infections caused by resistant *H. pylori* strains that have failed to respond to first line treatment regimens (Malfertheiner *et al*., 2007). They act by reducing the intracellular ATP levels and interfere with the activity of the urease enzyme of *H. pylori*, a key enzyme that is vital in the survival of the organism in the gastric mucosa. They also induce the formation of an ulcer-specific coagulum preventing acid back diffusion and inhibit protein and cell wall synthesis as well as membrane function (Romano and Cuomo, 2004; Manyi-loh *et al*., 2010a).They are specifically known to disrupt the integrity of bacterial cell walls by accumulating in the periplasmic space and along membranes.

Bismuth compounds may cause an increase in the synthesis of prostaglandin E_2 (Sandha *et al*., 1998), detachment of *H. pylori* from the gastric epithelium and a reduction in capsular polysaccharide production (Meurer and Bower, 2002; Romano and Cuomo, 2004). The properties of bismuth compounds are bactericidal for *H. pylori* (Larsen *et al*., 2003). These compounds are extremely potent cytotoxic agents when attached to a monoclonal antibody as these can target leukemia, lymphoma and other tumours. This property is consistent with their usage in the treatment of infections due to *H. pylori*, a major cause of mucosa associated lymphoid tissue (MALT) lymphoma.

Although bismuth compounds have proven to be effective second and third line therapies in the management of *H. pylori* infections, they are lacking in most developing countries. Apart from that, bismuth based therapies are characterised by high pill burden, undesirable side

effects and poor patient compliance (Bohr and Malfertheiner, 2009). Therefore, PPI-based triple therapies that have less pill burden, are easier to take and believed to have fewer side-effects have been evaluated as alternative treatment options.

It is worth mentioning that the suppression of gastric acid may not be physiological considering that low gastric acid levels may promote the growth of swallowed and enteric flora in the proximal gut, and these bacteria may be aspirated during episodes of physiological reflux (Talley, 2009). However, the antibiotic component of the treatment regimen is there to minimise the chances of such infections.

1.7.3 ***Antibiotics***

Infection with *H. pylori* will persist for life and may result in severe gastro duodenal complications without the intervention of antimicrobial therapy (Kusters *et al*., 2006; Lee *et al*., 2008). Consequently, a number of antibiotics are used in the management of *H. pylori* infections. The most commonly used antibiotics include metronidazole, clarithromycin, amoxicillin and tetracycline as mentioned earlier (Cameron *et al*., 2004; Bonacorsi *et al*., 2009; Tanih *et al*., 2010a).

Like any infectious agent, *H. pylori* can acquire resistance to these drugs, leaving clinicians with a limited list of drugs to choose from. As a result, treatment regimens are often modified with new entries of more effective antibiotics whenever the need arises. The susceptibility of *H. pylori* to these drugs has been reported to change with time, ethnicity, ulcer status, geographical location and test method (Mégraud, 2004). These factors therefore have to be considered in making a prescription for the eradication of the infection.

H. pylori is intrinsically resistant to glycopeptides, cefsulodin, polymyxins, nalidixic acid, trimethoprim, sulfonamides, cycloheximide and a few antifungal drugs some of which are used as selective agents in isolation media (Mégraud and Lehours, 2007). These exposures particularly in suboptimal concentrations give the bacteria a chance to develop resistance to the drugs (Cowan, 1999).

Wild type strains are susceptible to β-lactams (except cefsulodin), fosfomycin, macrolides, aminiglycosides, tetracycline, chloramphenicol, rifampin, flouroquinolones, 5-nitroimidazoles and nitrofurans (Mégraud, 1998). With the exception of chloramphenicol (because of toxicity) and aminoglycosides (because of diffusion), they have all been used in *H. pylori* eradication regimens (Choung *et al*., 2006; Malfertheiner *et al*., 2007; Fuccio *et al*., 2007; Tanih *et al*., 2010a).

1.7.4 **Rationale of the study**

Despite the potency of combination therapy, treatment of *H. pylori* infection is still a complex issue as the results from combination treatments are often unpredictable due to the continuous evolution of resistant bacterial strains. Stronger agents particularly of the flouroquinolone group may be included in the treatment regimen, but *H. pylori* is developing resistance to these drugs too and is still a difficult infection to eradicate (Adrienne *et al*., 2007). The side-effects and cost associated with combination therapy are contraindications for some patients. Medicinal plants may constitute a natural reservoir of therapeutically useful compounds against *H. pylori*. A search for new organic molecules with antibacterial activity, which could be cheap and readily available to the local population, therefore becomes important as it offers the potential of improving primary health care. It is hoped that the results of this study

may lead to the identification and isolation of antibacterial compounds that can be concentrated into more potent, cheap and readily available therapeutic agents against *H. pylori*, with minimal chances of resistance.

1.8 **Hypothesis of the study**

Medicinal plants in South Africa can provide potent and affordable antibacterial agents for the treatment of *H. pylori* infections.

1.9 **Objectives of the study**

1.9.1 *Overall aim*

The broad aim of this study was to determine the antimicrobial potential and isolate the active components of medicinal plants used in the treatment of *H. pylori*-related infections in South Africa.

1.9.2 *Specific objectives*

i. Conduct an ethnobotanical survey of plants used in the treatment of *H. pylori*-related infections in the study area.

ii. Screen the plant crude extracts for anti- *H. pylori* activity

iii. Determine the minimum inhibitory concentration (MIC) of the active plant extracts.

iv. Determine the rate of kill of the most active plant extracts and compounds.

v. Fractionate and isolate the phytocomponents of active plant extracts by chromatographic methods (TLC/CC)

vi. Identify the active ingredients in the plant crude extract by gas chromatography/mass spectrometry (GC/MS) and high performance liquid chromatography (HPLC) analysis.

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CHAPTER TWO

LITERATURE REVIEW

2.1 **Antibiotic activity and resistance mechanisms**

H. pylori resistance to the most commonly used antimicrobial agents is a major cause of treatment failure in most eradication regimens (Ndip *et al*., 2009; Manyi-loh *et al*., 2010b). Therefore, efforts have to be made to understand the development of resistance as a means of getting clues that would be helpful in curtailing the spread and facilitate rapid testing of resistance strains.

H. pylori, like a few other bacteria such as *Mycobacterium tuberculosis*, acquires resistance mainly by mutation to the antibiotics used in the treatment regimens (Mégraud and Lehours, 2007). The mechanism may not involve plasmids, transposons or integrons which could be transmitted horizontally but point mutations which are transmitted vertically (Gerrits *et al*., 2006).

Genes involved in point mutation or other genetic events leading to antibiotic resistance in *H. pylori* include *rdxA frxA*, *rm*16S, *rm*23S, *gyrA*, *rpoB* and *pbp1* for metronidazole, tetracycline, macrolides, quinolones, rifampins and amoxicillin resistance respectively (Tankovic *et al*., 2003; Moore and Salama, 2005; Boyanova, 2009). However, some authors have also suggested that *H. pylori* can naturally acquire resistance to some antibiotics by exchange of DNA through transformation and conjugation particularly when two or more strains are present simultaneously in the stomach (Ndip *et al*., 2008; Buta *et al*., 2010). The consequence is a progressive increase in the resistance rate due to the selection pressure.

As in many bacteria, drug efflux proteins can contribute to natural insensitivity to antibiotics and to emerging antibiotic resistance. However, Bina *et al*. (2000) evaluated the relevance of three putative efflux systems in *H. pylori* resistance to antibiotics and concluded that, in contrast to what is usually described for Gram-negative bacteria such as *Escherichia coli* or *Pseudomonas aeruginosa*, efflux systems did not play a role in the intrinsic resistance to antibiotics.

2.1.1 *Resistance to Nitroimidazoles (primarily metronidazole)*

Metronidazole (Mtz), also known as1-(2 hydroxyethyl)-2-methyl-5-nitroimidazole, is administered as a pro-drug. It enters the cell by passive diffusion and is activated in the cytosol of the bacterium to a toxic metabolite (Moore and Salama, 2005). Unstable Mtz radicals react rapidly with proteins, RNA, and DNA. This may lead to DNA strand breakage, inhibited repair and disrupted transcription which may eventually result in cell death (Jones *et al*., 2008).

The means by which *H. pylori* acquires resistance to this drug has been widely studied but still remains the most controversial topic in the field of antibiotic resistance. Early studies showed that resistant strains accumulated metronidazole at a slower rate and to a lesser extent than sensitive strains, suggesting a role for transporters or efflux systems in resistance (Moore *et al*., 1995). Goodwin *et al.* (1998) showed that metronidazole's toxicity depends on its reduction by the oxygen-insensitive, nicotinamide adenine dinucleotide phosphate (NADPH) nitroreductase (*rdxA*), and that resistance arises from mutations that inactivate *rdx*A genes.

Normally, in the presence of low-redox potential in anaerobic organisms, drug activation can be catalyzed by nitroreductases such as pyruvate flavodoxin reductase via a single electron transfer event. *H. pylori* contains this enzyme, but owing to its microaerophilic nature, molecular oxygen is also present and can compete with the Mtz radical for electrons in a futile cycle that restores the prodrug along with superoxide (Buta *et al*., 2010). Instead, a separate mechanism seems to account for most Mtz sensitivity in *H. pylori*. A non-oxygen-sensitive NADPH nitroreductase encoded by the *rdxA* gene reduces Mtz by a two-electron transfer step into a toxic metabolite that cannot be retransformed to its parent by molecular oxygen (Buta *et al*., 2010).

The vast majority of clinically isolated or experimentally induced Mtz-resistant clones contain a mutation somewhere in the *rdxA* coding sequence (Jones *et al*., 2008). However, Mtz-resistant *H. pylori* strains devoid of mutations on the *rdx*A have also been reported (Moore and Salama, 2005). Such cases show low levels of resistance which may be due to deletion mutations resulting in truncations of *rfx*A gene (Moore and Salama, 2005; Buta *et al*., 2010). High Mtz-resistance is thought to be conferred when both the *frxA* and *rdxA* genes are altered. The result is a synergistic effect on resistance.

2.1.2 *Resistance to Macrolides (primarily clarithromycin)*

Clarithromycin is one of the first line therapy antibiotics used against *H. pylori* and is part of a class of broad spectrum antibiotics called macrolides (Malfertheiner *et al*. 2007). Macrolides function to prevent protein translation. Specifically, these antibiotics interact with the bacterial ribosome and promote the premature release of peptidyl-tRNA from the acceptor site (Jones *et al*., 2008).

The first-line therapy against *H. pylori* is primarily based on the use of clarithromycin in combination with either metronidazole or amoxicillin. In this regard therefore, resistance to clarithromycin is an important predictor of treatment failure. Resistance to clarithromycin is reported to be about 3 % in untreated patients and up to 50 % after treatment failure (Moder *et al*., 2007). Point mutations in two positions (an adenine to guanine transition at either position 2142 or position 2143, or an adenine to cytosine transversion at position 2142) in 23S rRNA domain V are associated with macrolide resistance (Moder *et al*., 2007; Tanih *et al*., 2011). *H. pylori* resistance is therefore a consequence of point mutations which lead to a conformational change and a decrease in macrolide binding (Mégraud and Lehours, 2007). As high as 91.4% of clarithromycin resistant strains contain the A2142G or the A2143G mutation (Mansour *et al*., 2010).

2.1.3 *Resistance to beta-lactams (primarily amoxicillin)*

Beta-lactams are one of the most effective antibiotics clinicians have against *H. pylori* (Mégraud, 2004). They include penicillins, cephalosporins, carbapenems, monobactams, and clavams. All β-lactam antibiotics act by inhibiting the synthesis of the peptidoglycan layer of bacterial cell wall. They do so by targeting penicillin binding proteins (PBPs) on the cytoplasmic membrane. PBPs are enzymes that carry out carboxypeptidation and transpeptidation, which are the terminal steps of peptidoglycan synthesis.

Generally, resistance to β-lactams in many bacteria may arise by decreased membrane permeability of the drugs, increased efflux of the drug from the bacterial cell, modification of

the PBPs that diminish the affinity of the drug for the protein, and the presence of β-lactamases that inactivate the antibiotic by hydrolyzing its ring structure (Jones *et al*., 2008).

H. pylori acquires resistance to this drug by mutations on the PBP1 genes (Njume *et al*., 2009). Amino acid substitution Ser414→Arg appears to be involved, leading to a blockage of penicillin transport (DeLoney and Schiller, 2000; Mégraud and Lehours, 2007). Mutations of two different outer membrane proteins have been proven to be sufficient to cause amoxicillin resistance (Co and Schiller, 2006). These mutations include changes in amino acids 116-201 of *hopB* or a stop codon at amino acid 211 of *hopC*. Exposures to sub inhibitory amoxicillin concentrations *in vivo* during treatment as a result of poor compliance to therapy may cause resistance (Matteo *et al*., 2008).

2.1.4 *Resistance to tetracyclines*

Tetracyclines are often used as a second line therapy when *H. pylori* infections are not cured by the first line drug regimen. The tetracyclines include tetracycline, chlortetracycline, oxytetracycline, doxycycline, and tigecycline (Jones *et al*., 2008). Their favourable antimicrobial properties and the absence of major adverse side-effects have led to their extensive use in the therapy of human and animal infections. Tetracyclines function by inhibiting bacterial protein synthesis (Chopra and Roberts, 2001). They do this by disrupting codon-anticodon interactions at the ribosome level, specifically, by binding to the 30S ribosomal subunit, preventing attachment of aminoacyl-tRNA to the acceptor site (Wu *et al*., 2005). This effectively stops synthesis of bacterial peptides.

H. pylori resistance to tetracycline is rare but can result from multiple mutations in the 16S rRNA-encoding genes that affect the binding site of the drug, development of tetracycline efflux systems, decreased membrane permeability, enzyme degradation of the drug and production of ribosomal protection proteins that reduce the affinity of ribosome for tetracycline or release the bound tetracycline from the ribosome (Wu *et al*., 2005; Njume *et al*., 2009).

2.1.5 *Resistance to fluoroquinolones*

Fluoroquinolones are used as rescue or salvage therapy when both first and second line therapies have failed to eradicate *H. pylori* infection (Chisholm and Owen, 2009). They function by inhibiting the A and B subunits of the gene encoding DNA gyrase (*gyr*A or *gyr*B) in the bacterial cell (Wang *et al*., 2010), automatically interfering with DNA replication.

H. pylori resistance to these drugs has been attributed to specific mutations on these genes. Mutations in the quinolone resistance-determining region (QRDR) of *gyrA* have been identified in *H. pylori* (Jones *et al*., 2008). The amino acid positions concerned are mainly 87 and 91(Wang *et al*., 2010).

2.1.6 *Resistance to Rifamycins*

Rifampicin and rifabutin are included in the treatment regimen as rescue therapies of recurrent *H. pylori* infections (Jones *et al*., 2008). They are bactericidal due to their irreversible blockage of DNA-dependent RNA polymerase. They inhibit the β-subunit of the polymerase encoded by the *rpo*B gene and abrogate both the RNA and protein synthesis of

bacteria (Mégraud and Lehours, 2007; Chisholm and Owen, 2009). *H. pylori* resistance to these drugs is still low and has been attributed to point mutations in the *rpo*B gene at positions 530, 540 and 545 (Glocker *et al*., 2007).

2.1.7 *Resistance to nitrofurans*

These include furazolidone and nitrofurantoin. They are administered as pro-drugs activated when the nitro group is reduced by an oxygen insensitive nitroreductase (Jones *et al*., 2008). This activation results in the production of electrophilic intermediates that cause DNA damage and attack bacterial ribosomal proteins, thus blocking protein synthesis and causing cell death. While the mechanism of action of these drugs are similar to 5-nitroimidazoles, resistance is not acquired through the same mechanisms. While in 5-nitroimidazole (such as metronidazole), resistance is thought to arise from mutations within *rdxA* and *frxA*, knockouts of these genes did not produce resistance to furazolidone or nitrofurantoin (Kwon *et al*., 2001). Instead, a low level resistance to nitrofurantoins is thought to be due to mutations of pyruvate flavodoxin oxidoreductase and 2-oxoglutarate oxidoreductase genes (Sisson *et al*., 2002). This suggests that the nitrofurans could be used as an alternative to metronidazole. Substituting nitrofurans for metronidazole could produce better eradication rates with first line therapies considering the high level of metronidazole resistance, especially in the developing world (Ndip *et al*., 2008).

2.2 **Detection of Antibiotic resistance in *H. pylori***

Numerous techniques have been developed to detect antibiotic resistance in *H. pylori*. Phenotypic methods of susceptibility testing can be applied, but because resistance is

essentially due to point mutations, genotypic methods are also used (Ladeiras-Lopes *et al*., 2004; Njume *et al*., 2009). Methods for detecting resistance can be divided into two categories; culture and nucleic acid based methods.

Culture-based techniques include agar well or disc diffusion, agar dilution, broth microdilution and epsilometer (E)-test (Kohanteb *et al*., 2007). Agar diffusion tests are economical and simple to perform. They are the easiest and most economical methods for susceptibility testing of single isolates in routine practice (Mégraud and Lehours, 2007). In the disc diffusion test for example, up to six discs can be placed on an agar plate; after incubation, the zone of inhibition is measured and the isolate is recorded as resistant or susceptible on the basis of a cut off point validated in the laboratory. A similar procedure is carried out for the agar well only that the discs are now replaced by wells punched on the medium and filled with the antimicrobial agent at a predetermined concentration. The discs and agar well diffusion methods are not usually recommended for bacterial species requiring long incubation periods, because of the unstable patterns of antibiotic release from the discs or wells (Mégraud *et al*., 2001).

Studies comparing the disk diffusion method to agar dilution and/or E-test have produced conflicting results for metronidazole (Alarcon *et al*., 1998; Wouden *et al*., 1999). This could be expected, as *H. pylori* is a slow-growing microorganism that needs specific growth conditions and thus methodological problems may often be encountered. For example, the choice of the medium, the age of the colonies, the inoculum size, the duration of incubation, and the threshold at which the interpretative breakpoint is set may all influence the outcome of the susceptibility tests. Nevertheless, an excellent agreement has usually been found

between disc diffusion and the other testing methods for most classes of antibiotics other than the nitroimidazoles (Lang and Garcia, 2004)

2.2.1 *Agar dilution method*

Agar dilution is a reliable technique, which is usually carried out as the reference method for evaluating the accuracy of other testing methods. Although this method proves well suited in large studies on stored strains, it is laborious, time-consuming, and thus not very practicable in routine laboratories. Recently, the National Committee for Clinical Laboratory Standards (NCCLS) now known as the Clinical Laboratory Standard Institute (CLSI) approved the agar dilution method as the test of choice for susceptibility testing of *H. pylori* to clarithromycin (Megraud and Lehours, 2007).

2.2.2 *Broth micro dilution*

Broth dilution methods were previously considered inadequate because of the difficulties in growing *H. pylori* in broth. Supplementation of brucella, Mueller-Hinton and Brain Heart Infusion broths with horse serum or fetal calf serum has led to luxuriant growth of the organisms in these media (Njume *et al*., 2011). The advantage of this method over agar dilution is that it is more adaptable to automation and therefore decreases the workload. Correlations between the MICs determined by the broth micro-dilution method and the Epsilometer (E)-test have generally been found to be excellent for amoxicillin and clarithromycin (Mégraud *et al*., 2001).

2.2.3 *Epsilometer (E)-test*

The E-test is a quantitative variant of the disc diffusion method that yields a quantitative determination of susceptibility to antimicrobial agents (Mégraud, 2001). The E-test method has the advantage of being a quantitative method with a direct expression of MICs, and furthermore, it is adapted to slow-growing bacteria like *H. pylori*. A good correlation has been found between this method and the agar dilution method (Mégraud and Lehours, 2007). Several studies have reported an excellent correlation between agar dilution and E-test MIC results for most antibiotics but not for metronidazole for which controversial results have also been reported (Kohanteb *et al*., 2007). From the available data, it can be said that the rate of metronidazole resistance may be overestimated by 10 to 20% by the E-test in comparison to the agar dilution method (Mégraud, 2001). This difference could be due to the lack of anaerobic preincubation of the plates with the E-test.

Part of the variations in metronidazole resistance rates observed between tests may also be due to differences in methodology in different studies. The E-test appears as the most consistently reproducible method for routine testing of *H. pylori* and it has the advantage (over disc diffusion) to yield an MIC; but with the drawback of being costly (Mégraud and Lehours, 2007).

2.2.4 *Breakpoint susceptibility testing*

This is a simplified method of the agar or broth dilution methods mentioned above. Colonies of *H. pylori* are inoculated on an agar plate or in broth containing the critical concentration of antibiotic necessary to define resistance. For example, 1g/mL for clarithromycin, or two

different concentrations (0.25 and 1g/mL) to classify the strains as susceptible, intermediary, or resistant (Mégraud and Lehours, 2007). This test is easy to perform, and theoretically excellent. It has been used in comparison to the agar diffusion method (E-test and disc) for metronidazole susceptibility testing with a 94% correlation (Mégraud, 2001). This method is interesting because, being a direct measure of antimicrobial activity; it avoids errors associated with extrapolating disc diffusion zone sizes and MIC results. It is also much easier to perform than the standard agar dilution method. However, additional validation studies are required before this method can be recommended for susceptibility testing of *H. pylori*.

2.3 *Nucleic acid-based methods*

H. pylori resistance is essentially due to chromosomal mutations, and for the most part, a limited number of punctual mutations are present which can be easily detected with molecular techniques (Mégraud and Lehours, 2007). Different variations of the polymerase chain reaction including nested/semi nested PCR, reverse transcription PCR, PCR-RFLP and real time PCR have been used in the identification and quantification of specific genes associated with antimicrobial resistance (Xing *et al*., 2005).

PCR-based methods can be used to detect *H. pylori* from biopsy, stool, and vomit specimens. Modern techniques like oligonucleotide ligation assay, DNA enzyme immunoassay, preferential homoduplex formation assay (PCR-PHFA), microelectronic chip array, electrocatalytic detection, 3´-mismatched PCR and, 3´-mismatched reverse PCR have been specifically employed in the detection of macrolide resistance in *H. pylori* (Xing *et al*., 2005; Vakil and Mégraud, 2007).

2.3.1 *Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)*

PCR-RFLP is a PCR-based assay that is commonly used to detect mutational changes in *H pylori* obtained from biopsy samples or faeces (Gerrits *et al*., 2006). It is based on the fact that mutations reveal restriction sites within the amplicon obtained with primers specific to the *H. pylori* 23S rRNA gene. These restriction sites are recognized by the enzyme BsaI for the A2142G mutation and BsbI for the A2143G mutation, so two bands will be present on the gel if one or the other of these mutations is present (Mégraud and Lehours, 2007). More recently, a third enzyme (BceAI) has been proposed to detect the A2142C mutation (Vakil and Mégraud, 2007). Just like most PCR based methods, it is expensive, requires special laboratory facilities and there is a delay in the manipulation of the amplicons obtained. This method may not be suitable for clinical application.

A PCR-RFLP method has also been developed to amplify part of the 16S rRNA gene for the detection of tetracycline resistance (Mégraud and Lehours, 2007). The amplicon is then submitted to restriction by Hinft. The strains which harbour the triple mutation leading to a high tetracycline resistance level exhibit three bands, while only two are present for susceptible strains or for strains with a low resistance level. Comparison of amino acid sequences of *pbp1*A genes in both susceptible and resistant *H. pylori* strains have also been used to detect resistance due to amoxicillin (Matteo *et al*., 2008).

2.3.2 *Allelic specific polymerase chain reaction (AS-PCR)*

Reduced susceptibility of *H. pylori* to fluoroquinolones is due to mutations at either codon 87 or 91 of the *gyr*A gene (Nishizawa *et al*., 2007). A rapid test to detect such mutations is

allelic-specific PCR (AS-PCR) with a high degree of specificity and sensitivity (Mégraud and Lehours, 2007). Point mutations can be identified easily within a short period of time by PCR amplification alone, without direct sequencing or digestion with restriction enzymes. In the AS-PCR analysis, PCR amplification is performed using a specific primer in which the second nucleotide from the 3′ end is designed to match the site of the point mutation and the third nucleotide is designed to produce a mismatch in order to yield allele-specific PCR amplification. The point mutations can be identified by determining whether or not the PCR amplicons corresponding to the specific primers can be observed (Nishizawa *et al*., 2007).

Amino acid sequencing of *rdx*A and *frx*A genes (mutated and non mutated) have been widely studied to attempt an explanation on the development of resistance to metronidazole. This aspect is still a controversial issue as other factors are thought to be responsible for resistance and not just alterations of the above mentioned genes (Vakil and Mégraud, 2007; Njume *et al*., 2009). Research is still under way to find a suitable molecular method for detecting metronidazole resistance.

2.3.3 *Fluorescence in situ hybridisation (FISH)*

FISH is used to detect clarithromycin resistance without performing PCR. It has been reported to produce rapid and accurate results within short time intervals (Vegas *et al*., 2007; Caristo *et al*., 2008). It is applied directly on fresh shock frozen tissue or formalin fixed paraffin-embedded biopsies. It consists of an rRNA-based whole cell *in situ* hybridisation using a set of fluorescent labelled oligonucleotide probes. Labelling of intact single bacteria is monitored by fluorescence microscopy. This method allows detection of *H. pylori* with a 16S rRNA probe labelled with the fluorochrome Cy3 (red) and of resistant mutants with a

23S rRNA probe labelled with fluorescein (green) simultaneously. It is highly recommended because of its specificity and sensitivity compared with standard methods of culture and susceptibility testing (Caristo *et al*., 2008).

2.4 **Prevalence of *H. pylori* resistance to antibiotics**

The literature is full of studies on *H. pylori* antimicrobial resistance. However, most of these studies are limited by the number and representativeness of the strains tested (Njume *et al*., 2009). In most of these studies, samples were gotten from reference hospitals where patients who experienced previous treatment failures were explored, thus improving the chances of finding resistant strains. These studies may not be representative of the general population as a whole. They however give an indication of the situation.

Even though it is difficult to get healthy patients volunteer to donate gastric biopsies, attempts should be made to recruit patients that are selected at random. The development of new methods that don't make use of gastric biopsies for the rapid detection of resistance would also ease the way for such studies. Considering the ever evolving nature of resistant bacteria to the drugs, only the most recent studies (for the most part since the year 2000) are discussed.

2.4.1 *Prevalence of H. pylori resistance to metronidazole*

Resistance to metronidazole is the most common form of antimicrobial resistance in *H. pylori* (Gerrits *et al*, 2004; 2006). It ranges from 29-52% in the developed countries (Jone *et al*., 2008) and up to 100% in some countries of the developing world (Njume et al., 2009; Tanih et al., 2010). These differences in prevalence have been attributed to differences in local

antibiotic prescription practices and usage in various regions of the world. There is a high level of general use of metronidazole in the developing countries, which are mostly tropical and this inexpensive drug also sold in the street corners is used to treat parasitic infections such as amoebiasis (Alarcon *et al*., 1999; Mégraud, 2004).

In the developed countries, these drugs are also used in the treatment of dental infections (Gerrits *et al*., 2004). There seem to be some segregation also in resistance prevalence between the sexes. Women have been reported to be more likely to habour Mtz-resistant *H. pylori* strains than men and more likely to have a history of previous nitroimidazole ingestion (Vakil and Mégraud, 2007; Mansour *et al*., 2010). Patients previously exposed to either metronidazole or tinidazole are more likely to habour resistant strains (Mégraud and Lehours, 2007). Thus, the high prevalence of Mtz-resistant *H. pylori* in females is probably due to previous consumption of metronidazole or other nitroimidazoles in the treatment of gynaecological or genital infections such as trichomoniasis. Countries in which the resistance prevalence is more than 90% should consider replacing metronidazole with other nitroimidazoles in the treatment regimen (Njume *et al*., 2009).

2.4.2 *Prevalence of H.pylori resistance to Clarithromycin*

Clarithromycin is the most attractive compound in the treatment of *H. pylori* infection (Mégraud and Lehours, 2007). It has one of the best efficacies *in vitro* and is moderately affected by pH. Good concentrations can also be obtained in the gastric mucosa. As earlier mentioned, the prevalence of clarithromycin resistance varies amongst nations; 17% in Japan, and 10.6-25% in North America (Ahmad *et al*., 2009). A prevalence of 35.6% was recently reported amongst children in Spain (Agudo *et al*., 2010).

It is known that clarithromycin resistance in *H. pylori* has sharply decreased the success of eradication by 40 to 50% (Njume *et al*., 2009). Consumption of macrolides (erythromycin or clarithromycin) for the treatment of other infections is a risk factor for this resistance. The high level of clarithromycin resistance among *H. pylori* strains from children compared to adults suggests the importance of macrolide use in children especially in respiratory infections (Kalach *et al*., 2001). The use of amoxicillin and metronidazole as first-line eradication therapy in combination with a proton pump inhibitor is good as it will help to reduce the spread of clarithromycin resistance (Cameron *et al*., 2004), and is considerably cheaper.

2.4.3 *Prevalence of H. pylori resistance to other antibiotics*

Until the 20th century, *H. pylori* resistance to amoxicillin and tetracycline was very rare. The prevalence for amoxicillin is <2% in the United States and Europe (Njume *et al*., 2009), 29% in Brazil and up to 100% in some African countries (Smith *et al*., 2001). The incidence of amoxicillin and tetracycline resistance in *H pylori* seems to increase in geographic regions where these antibiotics can be obtained without prescription. Resistance rates of 72% and 59% have been reported for amoxicillin and tetracycline respectively (Gerrits *et al*., 2004). However, these estimates are based on a single report and thus require further confirmation. The prevalence of *H. pylori* resistance to rifampins is virtually absent, given that these antibiotics have a limited use (Mégraud and Lehours, 2007).

Among the fluoroquinolones, levofloxacin has recently become a good second or third choice treatment in case of eradication failure in adults (Bohr and Malfertheiner, 2009). However, this antibiotic is increasingly used for other infections, and, consequently, the prevalence of

primary resistance is already high: 8.8% in Alaska, 15% in Japan, 16.8% in Belgium and 17.2% in France (Vakil and Mégraud, 2007).

2.5 **Factors affecting the eradication of *H. pylori* infections**

2.5.1 *Genetic differences in the metabolism of drugs*

PPIs are an important part of many current treatment strategies for *H. pylori*. Some of the currently available PPIs are metabolized by the cytochrome P450 system in the liver, and genetic polymorphism of the cytochrome (CYP) 2C19 can affect *H. pylori* eradication (Ozdil *et al*., 2010). Poor metabolic activity is genetically determined and results in high plasma concentrations of the PPI and a prolonged effect. Poor metabolizers are found in 3%–5% of populations of Western origin, but prevalence rates of 18%–23% have been reported in China, Vietnam, Thailand, and Japan where differences in the outcome of therapy may be more relevant (Vakil and Mégraud, 2007). It has recently been suggested that alternative remedies or dietary adjuncts be included in the treatment regimens of these populations to improve *H. pylori* eradication rates (Ozdil *et al*., 2010).

2.5.2 *Smoking*

The risk of gastric cancer is increased by 60% in male smokers and 20% in female smokers, compared to non smokers (Lopez *et al*., 2008). A meta-analysis of 22 studies including 5538 suggested that smoking was associated with a reduced rate of eradication (8.4%; 95% CI: 3.3%–13.5%), particularly in patients who had non-ulcer dyspepsia. In this analysis, smokers had a higher likelihood of failed eradication (Suzuki *et al*., 2006; Vakil and Mégraud, 2007). In another study carried out by Camargo *et al*. (2007), it was observed that smokers had a 2-fold higher probability of failure in *H. pylori* eradication than non-smokers (OR: 2.0; 95% CI:

1.01-3.95). Smoking cessation may benefit *H. pylori* eradication rates (Lunet *et al*., 2008). It is not clear how smoking influences eradication therapy but it is thought to cause an increase in stomach acid, thus working in antagonism with many treatment regimens.

2.5.3 *Adverse effects, complexity of the regimen and cost*

Adverse effects are frequent with *H. pylori* eradication therapy and increase with the number of previous therapies (Gisbert *et al*., 2008). While first line-therapies lead to approximately one quarter to one-third of patients reporting adverse advents, this increases to more than half of the patients who receive third-line therapy. When selecting a therapy to eradicate *H. pylori*, duration of treatment, pill burden and adverse effects should be considered due to the fact that patient adherence and efficacy of the therapy have been shown to be strongly dependent on these (Bohr and Malfertheiner, 2009).

The recommended duration of therapy for *H. pylori* eradication is 7 to 14 days (Malfertheiner *et al*., 2007). Although not proven, potential benefits of shorter regimens with smaller pill burdens include better compliance, fewer adverse drug effects, and reduced cost to the patient. Undesirable effects include diarrhoea, nausea, vomiting, taste perversion with clarithromycin, darkening of oral cavity and stool with bismuth and metallic taste with metronidazole (Manyi-Loh *et al*., 2010a). A clear explanation to the patient of the anticipated adverse effects and the potential adverse consequences of discontinuing therapy may improve adherence. Therapy additives, for example probiotics or lactoferrin, can significantly also reduce side effects and improve eradication rates (Bohr and Malfertheiner, 2009).

2.5.4 *The effect of pH*

Changes in pH have been shown to affect the efficacy of some antibiotics against *H. pylori in-vitro* (Sjostrom and Larsson, 1996; Aydemir *et al*., 2005; Mégraud and Lehours, 2007). The susceptibility of *H. pylori* to the antibiotics is reduced, with high MICs under acidic conditions (Sjostrom and Larsson, 1996). Susceptibility to amoxicillin, erythromycin and clarithromycin has been shown to reduce remarkably at pH 5.9 (Sjostrom and Larsson, 1996). For this reason, antibiotics are administered with PPIs or bismuth compounds. As mentioned earlier, PPIs and bismuth compounds increase the pH in the vicinity of *H. pylori* and thus improve conditions for antibiotic therapy with possible synergistic effects on the organism. Novel therapies must be able to remain active in the acidic gastric niche of *H. pylori*.

2.5.5 *Development of resistant bacterial strains*

Antimicrobial resistance is a major cause of treatment failure and is responsible for the declining rates of *H. pylori* eradication seen in many countries (Njume *et al*., 2009). Post therapeutic antibiotic-resistant *H. pylori* reduces the cure rate of infection by up to 66% (Bohr and Malfertheiner, 2009). Metronidazole resistance reduces effectiveness by an average of 37.7%, whereas clarithromycin resistance reduces it by an average of 55% (Dore *et al*., 2000). Resistance increases with the number of unsuccessful treatments. After two eradication failures one can assume that almost all patients carry *H. pylori* strains that are resistant to metronidazole and clarithromycin (Cammarota *et al*., 2004). A systematic review of *H. pylori* therapy reported a 70% decline in eradication rates if clarithromycin resistance was present and a clarithromycin containing regimen was used (Vakil and Mégraud, 2007). This reiterates the necessity for the development of new therapies against *H. pylori* infections.

2.6 **Plants used in the treatment of *H. pylori*-related infections in South Africa.**

South Africa is a rich source of medicinal plants and members of different indigenous communities consult traditional herbalists for the treatment of numerous infections (Afolayan and Lewu, 2009; Green *et al*., 2010). Plant natural products have been very useful in the discovery of new drugs against many infections (Taylor, 2005; Singh and Lal, 2008; Green *et al*., 2010). Some examples of plant compounds that have been formulated into useful chemotherapeutic agents are represented below (Table 2.1).

Table 2.1: **Plant-derived products used in Western medicine (Taylor, 2005)**

Drug/Chemical	**Action/Clinical Use**	**Plant Source**
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Aesculetin	Anti-dysentery	*Frazinus rhychophylla*
Agrimophol	Anthelmintic	*Agrimonia supatoria*
Ajmalicine	Circulatory Disorders	*Rauvolfia sepentina*
Allantoin	Vulnerary	Several plants
Allyl isothiocyanate	Rubefacient	*Brassica nigra*
Anabesine	Skeletal muscle relaxant	*Anabasis sphylla*
Andrographolide	Baccillary dysentery	*Andrographis paniculata*
Anisodamine	Anticholinergic	*Anisodus tanguticus*
Anisodine	Anticholinergic	*Anisodus tanguticus*
Arecoline	Anthelmintic	*Areca catechu*
Asiaticoside	Vulnerary	*Centella asiatica*
Atropine	Anticholinergic	*Atropa belladonna*
Benzyl benzoate	Scabicide	*Several plants*
Berberine	Bacillary dysentery	*Berberis vulgaris*
Bergenin	Antitussive	*Ardisia japonica*
Acetyldigoxin	Cardiotonic	*Digitalis lanata*
Adoniside	Cardiotonic	*Adonis vernalis*
Aescin	Anti-inflammatory	*Aesculus hippocastanum*
Aesculetin	Anti-dysentery	*Frazinus rhychophylla*
Agrimophol	Anthelmintic	*Agrimonia supatoria*
Ajmalicine	Circulatory Disorders	*Rauvolfia sepentina*

Allantoin	Vulnerary	Several plants
Allyl isothiocyanate	Rubefacient	*Brassica nigra*
Anabesine	Skeletal muscle relaxant	*Anabasis sphylla*
Betulinic acid	Anticancerous	*Betula alba*
Chymopapain	Proteolytic, mucolytic	*Carica papaya*
Cocaine	Local anaesthetic	*Erythroxylum coca*
Emetine	Amoebicide, emetic	*Cephaelis ipecacuanha*
Hemsleyadin	Bacillary dysentery	*Hemsleya amabilis*
Kaibic acud	Ascaricide	*Digenea simplex*
Morphine	Analgesic	*Papaver somniferum*
Neoandrographolide	Dysentery	*Andrographis paniculata*
Nicotine	Insecticide	*Nicotiana tabacum*
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata*
Morphine	Analgesic	*Papaver somniferum*
Neoandrographolide	Dysentery	*Andrographis paniculata*
Nicotine	Insecticide	*Nicotiana tabacum*
Nordihydroguaiaretic acid	Antioxidant	*Larrea divaricata*
Papain	Proteolytic, mucolytic	*Carica papaya*
Quinine	Antimalarial, antipyretic	*Cinchona ledgeriana*
Qulsqualic acid	Anthelmintic	*Quisqualis indica*
Rotenone	Piscicide, Insecticide	*Lonchocarpus nicou*
Rotundine	Analagesic, sedative	*Stephania sinica*
Sanguinarine	Dental plaque inhibitor	*Sanguinaria canadensis*
Santonin	Ascaricide	*Artemisia maritma*
Thymol	Antifungal (topical)	*Thymus vulgaris*

Our previous studies have identified 17 species used in the treatment of infections symptomatic of *H. pylori* in the Eastern Cape Province (Njume *et al*., 2011b). Other plants such as *Combretum molle* and *Sclerocarya birrea* have found good medicinal uses among the Vhavendas, Xhosas, Zulus and Sothos of South Africa while *Garcinia kola* seeds are mostly used by city dwellers and vendors in traditional medicine (unpublished findings). The medicinal properties of most of these plants have been a subject of numerous investigations (Eloff, 2001; Eloff *et al*., 2005; Akinpelu *et al*., 2008) with interesting results.

2.6.1 *Combretum molle*

The genus *Combretum* belongs to the family Combretaceae which includes 20 genera and about 600 species of plants distributed especially in the tropical and subtropical regions (Ponou *et al*., 2008). *Combretum molle* is a small graceful deciduous tree (3-13 high) used in African traditional medicine for the treatment of abdominal pains and worm infections (Bessong *et al*., 2004). According to the Agro Forestry Tree Database, boiled root decoction of *C. molle* is used to induce abortion and treat constipation, stomach pains and dysentery. The leaves are chewed or pounded, soaked in water and the juice drunk for chest complaints or used as an inhalant in hot steam bath (Gronhaug *et al*., 2008). An infusion of the inner bark is taken orally or as an enema to relieve various stomach ailments (Njume *et al*., 2009).

Although many members of the Combretaceae have been widely studied, very few antibacterial compounds have been isolated from *C. mole*. Ojewole (2008) found analgesic, anti-inflammatory and cardiovascular effects of mollic acid glucoside isolated from *C. molle* leaves. Asres *et al*. (2001) reported that the acetone extract of leaves from *C. molle* has antiprotozoal activity. In an investigation of the biological activity of different *Combretum*

species, McGaw *et al*. (2001) found *C. molle* to have both anti-inflammatory and antischistosomal activity. These findings indicate that *C. molle* has good medicinal potentials. However, knowledge on the antibacterial activity of the plant or its compounds is lacking, especially against *H. pylori*.

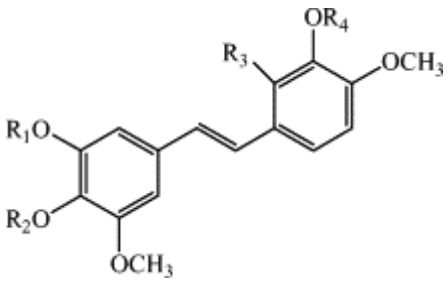
- *Examples of compounds isolated from C. molle*

A large repertoire of compounds have been isolated from the Combretaceae but the biological activities of some of these compounds are yet to be discovered (Eloff *et al*., 2008). The Combretaceae is the source of a wide range of tannins, flavonoids, terpenoids and stilbenoids (Eloff *et al*., 2005). The latter have generated immense interest in this family because of their chemical simplicity, which belies their therapeutic potential.

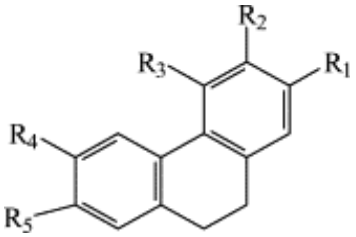
Compounds isolated from *C. molle* include stilbenoids and phenanthrenes. Stilbenoids from Combretaceae are referred to as Combretastatins and are designated A, B, C and D (Eloff *et al*., 2008). Combretastatin A which possesses an ethylene bridge between the two benzene rings (i.e stilbene) and combretastatin B with an ethane bridge (dihydrostilbene) have been isolated from different species of the Combretaceae including *C. molle* (Eloff *et al*., 2005; 2008). Flavanones and flavones have also been isolated and appear to be ubiquitous constituents in the leaf extracts of *Combretum* species. As for terpenoids, the Combretaceae have yielded mainly pentacyclic triterpenoids varying from oleanoic and ursanoic acids to friedelins, cycloartanes and dammaranes.

Arjunolic acid and glycosides are the main terpenoids isolated from *C. molle* (Panzini *et al*., 1993). Other oleanene-type pentacylic triterpenoids bearing 29-carboxy and 1α-hydroxy

substituents have been isolated from *C. molle.* Co-occurrence of tetracyclic and pentacylic classes of these triterpenoids is unusual but *C. molle* contains both (Panzini *et al*., 1993).

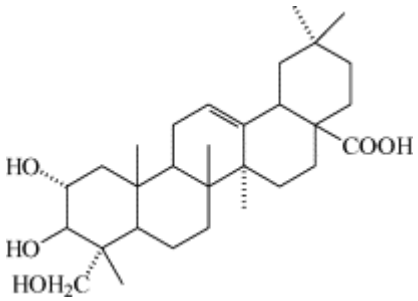


Combretastatin A

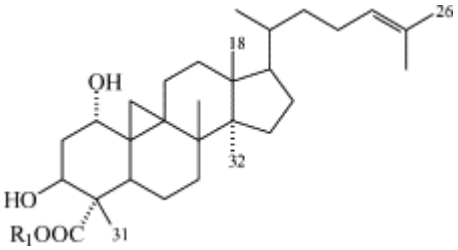


9,10-Dihydrophenanthrenes

(Letcher and Nhamo, 1972; Eloff *et al*., 2008)



Arjunolic acid



Molic acid and derivatives (Panzini *et al*., 1993)

(Panzini *et al*., 1993)

Primary structures of compounds isolated from C. molle

2.6.2 *Sclerocarya birrea*

Sclerocarya birrea (A. Rich) Hochst., subspecies *cafra* (Sond) Kokwaro belongs to the family Anacardiaceae (Eloff, 2001). It is an important cultural and ethno medicinal plant in South Africa and other parts of the African continent (Njume *et al*., 2011c). It is a medium size to large deciduous tree with an erect trunk and rounded crown. The tree is commonly

found in semi-arid, deciduous and savannah regions of sub-Saharan Africa. In South Africa, the tree is mostly found in Limpopo province and is commonly referred to as 'Marula' (Masoko *et al*., 2008).

S. birrea produces greenish-yellowish fruits with a vitamin C-rich pulp that is delicious and sought after by humans and animals (Afolayan and Sunmonu, 2010). Its medicinal uses are popular among the Xhosa and Zulu people who use bark decoctions as enemas for diarrhoea and the Vhavenda people who use the same parts for treating fevers, stomach ailments and ulcers (Ojewole *et al*., 2010). A number of researchers have reported on the anti-diabetic and antifungal activities of this plant with interesting results (Masoko *et al*., 2008; Afolayan and Sunmonu, 2010).

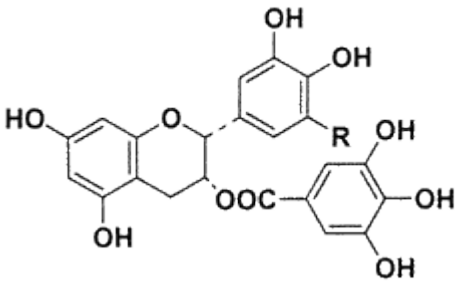
- *Examples of compounds isolated from S. birrea*

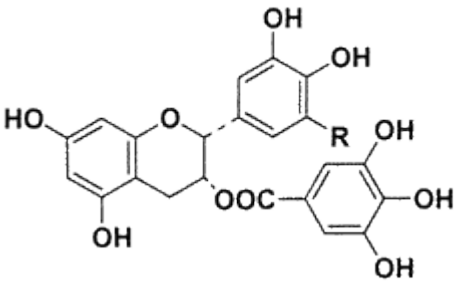
The fruits of *S. birrea* are rich in K, Na, Ca, Mg, Fe, Zn, Mn, crude oils, carbohydrates, crude proteins, fibre, saponins, minerals and ascorbic acid (Borochov-Neori *et al.*, 2008; Ojewole *et al*., 2010). The fruit also contains phenolic compounds; mainly hydrolizable tannins, catechins and hydroxycinnamic acid (Borochov-Neori *et al.*, 2008). The kernels yield 54–60% of non-drying oils, and contain as much as 28% proteins, and some iodine. The oil-rich seeds contain 64% oleic acid, myristic, stearic and amino acids with a predominance of glutamic acid and arginine (Ojewole *et al*., 2010).

Viljoen *et al.* (2008) documented the presence of esters and hydrocarbons in the fruits. Caffeic acid, vanillic acid, p-hydroxybenzaldehyde, ferulic acid, p-hydroxybenzoic acid, and p-coumaric acid have also been isolated from the fruits (Ndhlala *et al.*, 2007). A study carried

out by Braca *et al.* (2003) revealed the presence of gallic acid, (-)-epicatechin 3-*O*-galloyl ester, and (-)-epigallocatechin 3-*O*-galloyl ester from the leaves of *S. birrea*. Other compounds isolated from the leaves include polyphenols, tannins, flavonoids (quercetin and its derivatives), alkaloids, anthocyans, and saponosides (Ojewole *et al*., 2010).

Literature reports have also indicated that *S. birrea* stem-bark yields 3.5–20.5% tannins, 10.7% tanning matter, and traces of alkaloids (Eloff, 2001). Other compounds including gallotannins, flavonoids, steroids (including β-sitosterol), coumarins, triterpenoids, sesquiterpene hydrocarbons, ascorbic acid, oleic acid, myristic, stearic and amino acids with a predominance of glutamic acid and arginine have also been isolated (Ojewole *et al*., 2010). However, none of these studies has evaluated the antimicrobial activity of the plant extracts or the compounds against *H. pylori*.





R =H R=OH

(-)-Epicatechin 3-*O*-galloyl ester (-)-Epigallocatechin 3-*O*-galloyl ester

(Braca *et al*., 2003)

Examples of antimicrobial compounds isolated from S. birrea.

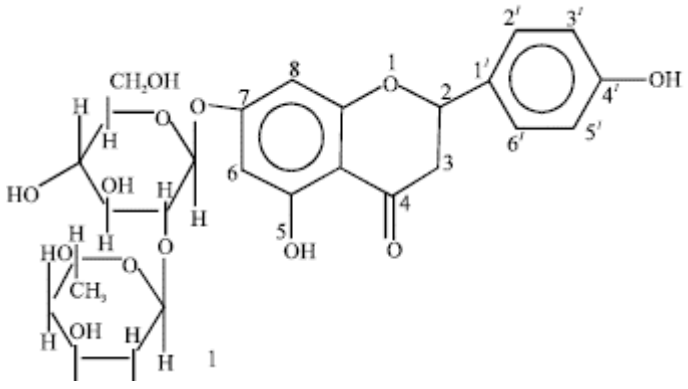
2.6.3 *Garcinia kola*

Garcinia kola is a medium sized tree about 12-28 m high and mostly found in moist tropical forests (Njume *et al*., 2011a). It belongs to the family Guttiferae. Its medicinal uses are

mostly popular in the West and Central African countries. The seeds of *G. kola* are used in the treatment of diarrhoea, stomach ache, gastritis, constipation and other stomach-related morbidities in West and Central African countries. The seeds are habitually chewed during social gatherings. They have a bitter taste and are referred to as 'bitter kola' (Akoachere *et al*., 2002; Adeboye *et al*., 2008). The medicinal properties of this plant have been a subject of numerous investigations (Akoachere *et al*., 2002; Akinpelu *et al*., 2008).

- *Examples of compounds isolated from G. kola*.

The seeds of *G. kola* are rich in flavonoids, steroids, saponins and tannins, compounds with good medicinal potentials and health promoting abilities (Cowan, 1999; Okwu and Morah, 2007; Akinpelu *et al*., 2008). Other compounds isolated from the seeds include kolaflavone, 2-hydroxybiflavonols, anthraquinones, polyphenols and chromanols (Adegboye *et al*., 2008). A flavone; flavanone glycoside 4´, 5, 7-trihydroxyflavonone rhamnoglucose (naringin-7-rharmnoglucoseside) isolated from *G. kola* seeds is thought to be responsible for its bitter taste and antimicrobial activity (Okwu and Morah, 2007). The structure of this compound is represented below.



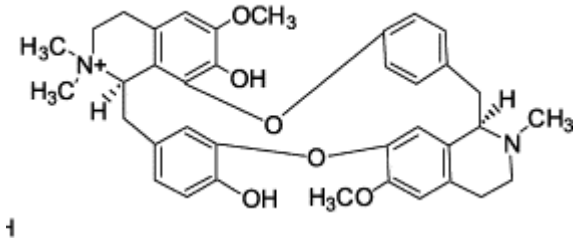
Structure of flavanone glycoside 4´, 5, 7-trihydroxyflavonone rhamnoglucose isolated from G. kola seeds (Okwu and Morah, 2007).

2.6.4 *Strychnos species (S. henningsii and S. decussata)*

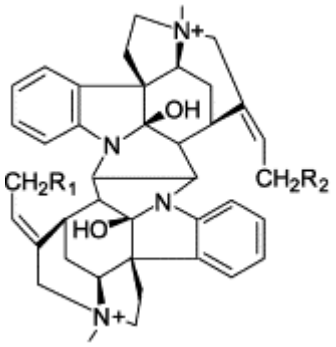
The genus *Strychnos* is very well known as the plants providing one of the most famous poisons called strychnine, a property that is consistent with their usage as arrow poisons in west and central Africa (Philippe *et al*., 2004). *Strychnos* belongs to the family Loganiaceae. *S. henningsii* is one of the most widely distributed species of *Strychnos* in east and southern Africa (Oyedemi *et al*., 2010). It is a small evergreen tree or shrub, with a stem bark that is crown compact with dark green, glossy foliage and leathery leaves (Oyedemi *et al*., 2010). *S. decussata* on the other hand is a small to medium garden tree 3-12m high also found in Southern Africa (Philippe *et al*., 2004). The stem barks of both species are used in the treatment of abdominal pains, gastrointestinal pains, ulcers and diarrhoea in several parts of Africa (Philippe *et al*., 2004).

- *Examples of compounds isolated from Strychnos species*

The genus *Strychnos* is rich in poisonous alkaloids, specifically strychnine or strychnine-like compounds with tetanising and curarine properties. Examples of tetanising alkaloids isolated from *Strychnos* species include C-toxiferine and C-dihydrotoxiferine while C-curarine and C-calebassine are curarine alkaloids (Philippe *et al*., 2004). Another curarine alkaloid; (+)-tubocurarine is a useful neuromuscular relaxant in all forms of anaesthesia (Philippe *et al*., 2004). The antibacterial activities of most of these compounds are still largely unknown.



(+)-tubocurarine

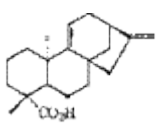


C-calebassine; R1=R2=H

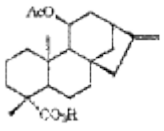
Structures of some curarizing alkaloids isolated from Strychnos species (Philippe *et al*., 2004).

2.6.5 *Alepidea amatymbica*

A. amatymbica is a herbaceous perennial plant with dark green leaves arising from a single or branched rhizome. It belongs to the family Apiaceae (Njume *et al*., 2011b). The flowering stalk is hollow and up to two meters in height, with numerous small flowers arranged in dense, rounded heads (Afolayan and Lewu, 2009). The roots and rhizomes of this plant are used in the Eastern Cape province of South Africa for the treatment of diarrhoea, constipation, stomach pains and ulcers (Wintola and Afolayan, 2010). The rhizomes and roots contain high concentrations of several diterpenoids of the kaurene type (Samova *et al*., 2001). The major compounds are dehydrokaurenoic acids and kaurenoic acids, of which ent-16-kauren-19-oic acid is usually present in the greatest quantity (Samova *et al*., 2001; Dold and Cocks, 2002). Below is a structural representation of some of these compounds.



ent-16-kauren-19-oic acid



Acetoxy-ent-kauren-16-en-19-oic acid

Structures of some kaurenoic acids isolated from A. amatymbica (Samova *et al*., 2001).

2.7 **Phyto chemicals as anti-*Helicobacter pylori* agents**

Phyto chemical compounds may inhibit bacterial growth by mechanisms different from presently used treatment regimens, and could therefore be of clinical value in the treatment of resistant bacteria, including *H. pylori*. Tannins, flavonoids, alkaloids, essential oils and many phenolic compounds serve as plant defence mechanisms against predation by insects, herbivores and infection by microorganisms (Cowan, 1999). It is therefore not surprising that these compounds have been found to exhibit profound antimicrobial activities *in-vitro* against a wide array of organisms. Some of these compounds are discussed below vis-à-vis *H. pylori*.

2.7.1 *Quinones*

Quinones are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are characteristically highly reactive (Cowan, 1999; Njume *et al*., 2009). Quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of the protein and loss of function (Cowan, 1999). For that reason, the potential range of quinone antimicrobial effects is great. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Njume *et al*., 2009). The inner stem bark of the South American trumpet tree (*Tecoma ipe* Mart) has been an important source of an active quinone compound against *H. pylori*.

Nagata *et al*. (1998) successfully isolated furanonaphthoquinone (FNQ) from the above mentioned plant and found it active against a number of microorganisms including *H. pylori*, (MIC 0.1µg/mL). Considering that the MICs of some antibiotics are known to decrease by 1/10 or 1/100 in acidic culture medium (pH 5.5), Nagata *et al*. (1998) evaluated the activity

of FNQ at pH values 5.5 and 7.2, and found no change in the activity of this compound against *H. pylori*.

Other quinones; idebenone, duroquinone, menadione, juglone and coenzyme Q1 have also been reported to be inhibitory to *H. pylori* at low concentrations of 0.8 to 3.2 µg /mL (Inatsu *et al*., 2006). The potential usefulness of quinones as antimicrobial agents against *H. pylori* is great. However, the mechanism of action for most of these compounds against *H. pylori* as well as their toxicities on humans would have to be thoroughly examined.

2.7.2 *Flavonoids, flavones and flavonols*

Flavonoids are a large classs of naturally occurring phenolic compounds mostly responsible for the colours of flowers and fruits (Bylka *et al*., 2004). The word flavonoid comes from the Latin word *flavus* which means yellow; however some flavonoids are red, blue, purple, or white. They can be isolated from red wine, apples, blueberries, bilberries, onions, soy products and tea (Bylka *et al*., 2004). Chemically they are C6-C3-C6 compounds in which the two C6 groups are substituted benzene rings, and the C3 is an aliphatic chain which contains a pyran ring (Robinson, 1991). Flavonoids occur as O- or C-glycosides or in the free state as aglycones with hydroxyl or methoxyl groups present on the aglycone (Mills and Bone, 2000). They include; flavones, flavonols, flavonones, chalcones, xanthones, isoflavones, and biflavones (Cowan, 1999; Bylka *et al*., 2004). Since they are known to be synthesized by plants in response to microbial infection (Schinor *et al*., 2007), it should not be surprising that they have been found *in-vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Njume *et al*., 2009).

The anti-*H. pylori* activity of a number of flavonoids has been reported. For example; the chloroform extract of *Cistus laurifolius* flower buds used traditionally in folk medicine in Turkey to treat gastric ailments have been shown to possess significant anti-*H. pylori* activity with the flavonoid; quercetin 3-methyl ether (isorhamnetin) as the active component (Ustün *et al*., 2006).The mechanism of action of some flavonoids notably; hesperidin, phloroglucinol, and resorcinol has been established to lie in the inhibition of *H. pylori* urease production by 60-70% (Bylka *et al*., 2004). This is very important considering that the urease enzyme is very vital for the survival of this organism in the stomach. It has also been found that aglycones inhibit the growth of *H pylori*, whereas glycosides are inactive (Bylka *et al*., 2004).

2.7.3 *Tannins*

Tannins are a group of phenolic substances capable of tanning leather or precipitating gelatine from solution. They occur in almost every part of the plant including bark, wood, leaves, fruits and roots (Hoste et al., 2006). They occur in two forms; condensed tannins and hydrolysable tannins (Cowan, 1999). They react with proteins, forming complexes through hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Scalbert, 1991). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc.

Hydrolyzable tannins have been reported to have good potential as new and safe therapeutic regimens against *H. pylori* infection *in vitro* (Funatogawa *et al*., 2004). These compounds; tellimagrandin 1 (TG1) and tellimangrandin 2 (TG2) from a group of medicinal plants used in many countries in Asia did not affect the viability of MKN-28 cells derived from human

gastric epithelium indicating that they could provide good treatment alternatives with minimal effects on the host.

2.7.4 *Coumarins*

Coumarins are phenolic substances made of fused benzene and α-pyrone rings. They are responsible for the characteristic odour of hay (Cowan, 1999). There are three main classes; hydroxycoumarins, furanocoumarins and pyronocoumarins. In a study carried out by Kawase *et al*. (2003), it was found that a number of hydroxycoumarins; 7- hydroxy-4-methylcoumarin, 6,7-dihydroxy-4-methylcoumarin, 6-hydroxy-7-methoxy-4-methylcoumarin and 5,7- dihydroxycyclopentanocoumarin showed comparable anti-*H. pylori* activity with metronidazole. This is very important given the continuous evolution of *H. pylori* resistance to metronidazole in the developing world (Banatvala *et al*., 1994).

A recent study has also documented the antimicrobial activity of coumarins isolated from the roots of *Ferulago campestris* against *H. pylori* isolates in Italy (Basile *et al*., 2009). Generally, data about specific antibiotic properties of coumarins against *H. pylori* are scarce.

2.7.5 *Terpenoids and essential oils*

The fragrance of plants is carried in the so-called *quinta essentia*, or essential oil fraction. They are called terpenes, their general chemical structure is C10H16 and they occur as diterpenes, triterpenes and tetraterpenes (C20, C30 and C40), as well as hemiterpenes (C5) and sesquiterpenes (C15). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999). More than 20,000 of these compounds have been described from plant sources. Examples include; camphor, limonene, abscissic acid, aucubin,

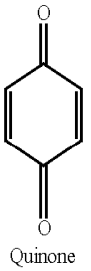
gossypol, gibberellic acid, menthol, euginol, β-carotene and the recently reported terpinen-4-ol (Njume *et al*., 2011d).

The anti-*H. pylori* activity of essential oils is being studied with the hope that people with asymptomatic gastritis would certainly benefit from a nutritional approach to help them manage the infection and therefore decrease the risk of development of associated pathologies. These compounds have shown good activity *in vitro* (Bergonzelli *et al*., 2003; Njume *et al*., 2011d). They can be used as food additives to complement present therapies. However, more research is needed to clarify the use of terpenoids and essential oils as potential anti-*H. pylori* agents.

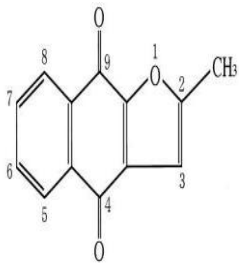
2.7.6 *Alkaloids*

Heterocyclic nitrogen compounds called alkaloids are often found in methanolic and ethanolic plant crude extracts (Njume *et al*., 2009). Examples include; codeine, atropine, morphine, vincristine etc. Crude extracts of the fruits of *Evodia rutaecarpa*, used in Chinese medicine has been reported to contain two quinolone alkaloids; 1-methyl-2-[(Z)-8-tridecenyl]-4-(1H)-quinolone and 1-methyl-2-[(Z))-7- tridecenyl]-4-(1H)-quinolone. The minimum inhibitory concentration (MIC) of these compounds against reference strains and clinically isolated *H. pylori* strains were less than 0.05 µg/mL, which was similar to the breakpoint MIC values of amoxicillin and clarithromycin that are used worldwide for the clinical eradication of *H. Pylori* (Hamasaki *et al*., 2000). Furthermore, these investigators noted that the antimicrobial activity of these compounds was highly selective against *H. pylori* and almost non-active against other intestinal pathogens. The results showed that these alkyl methyl quinolone alkaloids could be useful for the eradication of *H. pylori* without affecting other intestinal flora.

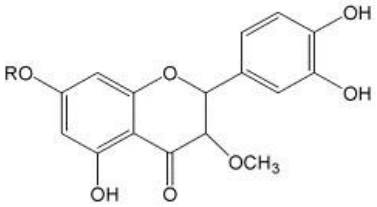
In this study, we have also documented the potent antimicrobial activity of pyrrolidine, an alkaloid identified in the stem bark of *S. birrea* against *H. pylori* strains expressing a clarithromycin and metronidazole-resistant phenotype (Njume *et al*., 2011d). The activity of this compound was comparable to amoxicillin, one of the antibiotics recently reported to be highly active against *H. pylori* in South Africa (Tanih *et al*., 2010). The structures of some of these phytochemicals or their monomers are represented below (Fig. 2.1).



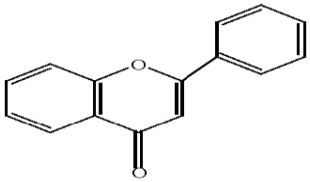
Quinone



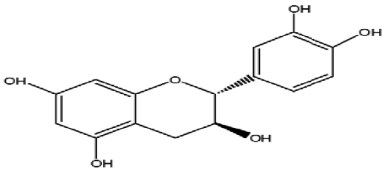
Furanonaphthoquinone



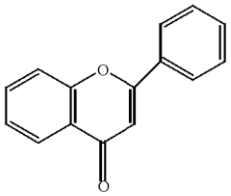
R=H; Quercetin-3-methyl ether
R=CH3; Quercetin-3, 7-dimethyl ether



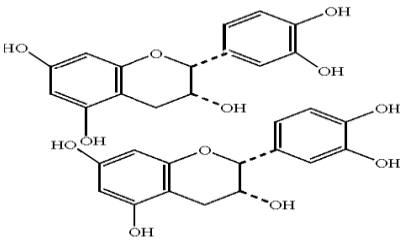
Flavone



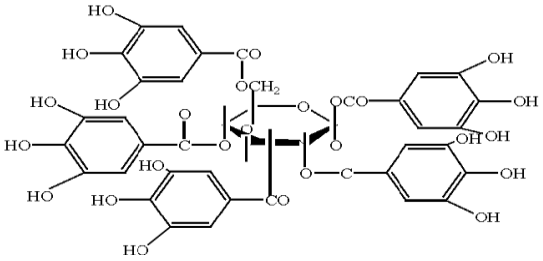
Catechin



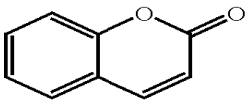
Flavone



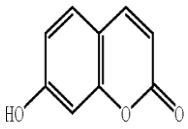
Procyanidin B-2
(Condensed tannin)



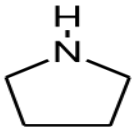
Hydrolyzable tannin



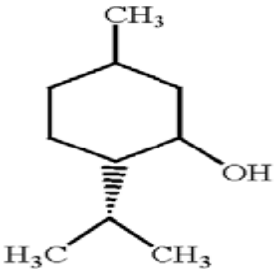
Coumarin



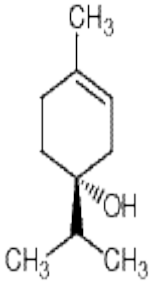
Hydroxycoumarin



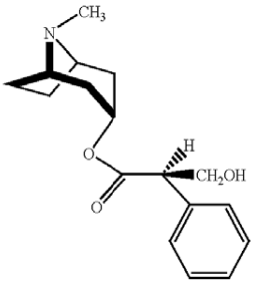
Pyrrolidine



Menthol



Terpinen-4-ol



Atropine

Fig. 2.1: **Structures of some anti-*H. pylori* phytochemicals and/or their monomers**
(Funatogawa *et al*., 2004; Eloff *et al*., 2008)

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CHAPTER THREE

DIVERSITY OF PLANTS USED IN THE TREATMENT OF *HELICOBACTER PYLORI*-ASSOCIATED MORBIDITIES IN THE NKONKOBE MUNICIPALITY OF THE EASTERN CAPE PROVINCE OF SOUTH AFRICA.

ABSTRACT

Helicobacter pylori is a major cause of gastro-duodenal pathologies. Expenses associated with combination therapy and the adverse effects of the treatment regimens have led to increased usage of ethnomedicines in the management of infections. Despite the usage of plants in the management of infections in the Nkonkobe municipality, empirical studies to document the specific plant species used by traditional doctors are lacking. This study was conducted to document the various plant families and species used in the management of *H. pylori*-associated morbidities in the Nkonkobe municipality. A semi-structured questionnaire was used to interview the local dwellers including traditional doctors, herbalists and hawkers in traditional medicine. The plant parts used, preparation, mode of administration and dosages were recorded. Seventeen plant species belonging to 13 genera and 11 families were collected and identified by their vernacular and scientific names. The Asphodelaceae was the most represented family (4 species), followed by Apocynaceae (3 species) and Loganiaceae (2 species). The plant parts most frequently used were the roots (35.3%), followed by the leaves and stem barks (23.5% each). Further research is needed to scientifically correlate treatment claims with folkloric uses and to isolate the plants' active components, determine their *in-vivo* potencies and toxicity.

3.1 INTRODUCTION

Helicobacter pylori, a Gram-negative microaerophilic helical bacillus has been implicated in the pathogenesis of a number of digestive tract disorders such as chronic active gastritis, peptic ulcer, gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma (Ndip *et al*., 2008; Manyi-Loh *et al*., 2010). The organisms are found to be suspended in the stomach mucosa or attached to epithelial cells of the stomach which happens to be the only known reservoir of infection (Atherton, 2006; Correa and Piazuelo, 2008).

Half of the world's population is infected by this organism and a high prevalence of up to 90% has been reported in the developing countries (Adrienne *et al*., 2007), as opposed to 20-50% in the developed nations (Castillo-Juarez *et al*., 2009). A high prevalence of infection has been found almost always to correlate with low socio-economic status and poor sanitary conditions (Ndip *et al*., 2004; Kusters *et al*., 2006).

Infections are treated with potent combination therapies; a proton pump inhibitor (PPI) or bismuth in combination with two antibiotics most commonly metronidazole, clarithromycin, amoxicillin and tetracycline with an expected success rate of 80 – 90% (Njume *et al*., 2009). However, eradication of *H. pylori* is still a difficult problem as treatment failure rate remains at 10 – 40% (Lai *et al*., 2006). Equally important is the increasing prevalence of antimicrobial resistant strains in South Africa which jeopardize the success of therapeutic regimens aimed at eradication of infections (Tanih *et al*., 2010; Njume *et al*., 2011).

In the Nkonkobe municipality just like many areas of the developing world, the use of medicinal plants in the treatment of stomach-related morbidities is an old custom that has co-existed with the people for many years (Wintola and Afolayan, 2010). This municipality is

located within the Eastern Cape Province, home to 15.5% (6.3 million) of South Africa's total population and incorporates two of the former 'homelands' of the apartheid period (i.e.Ciskei and Transkei), where many aspects of traditional culture are still part of everyday life (Dold and Cocks, 2002). Consequently, the people of the Eastern Cape tend to be more traditional and rural than those in other parts of South Africa.

The use of medicinal plants in the treatment of diseases has occupied a pivotal position in the socio-cultural and spiritual lives of rural and tribal families in this municipality (Oyedemi *et al*., 2009). This region abounds in its ethnic diversity, in which many aboriginal cultures have retained traditional knowledge concerning the medicinal utility of the native flora.

Herbal remedies are commonly used in this municipality to treat gastritis, peptic ulcer, stomach cancer and other *H. pylori*-associated pathologies, as a means to evade the high cost and adverse effects associated with Western drugs. Contrary to these drugs, most of the herbal remedies used are readily available with almost no resistance reported (Wintola and Afolayan, 2010). Ethno botanical studies to identify potential sources of new drugs have become very necessary considering the ever evolving nature of *H. pylori* resistance to current antimicrobial agents (Njume *et al*., 2009). Equally important is the potential of such studies to bring to lime light numerous plants having significant medicinal properties hitherto unknown to the scientific world.

More than 80% of rural African people depend on plant based remedies for primary health care (Afolayan and Lewu, 2009). In order to preserve valuable plant information and prevent loss of traditional values, it is imperative for studies covering the folkloric use of these plants to be assembled into regional or provincial pharmacopoeias. This becomes very important

considering that 25% of prescription drugs issued in the USA and Canada today contain bioactive compounds that are derived from or modelled after plant natural products (Singh and Lal, 2008). This study was carried out as a means to preserve the enormous wisdom and traditional knowledge associated with treatment of infections symptomatic of *H. pylori* in the Nkonkobe municipality. This becomes imperative considering that most of the traditional doctors are old and may die with their libraries of knowledge.

3.2 MATERIALS AND METHODS

3.2.1 Study area

Nkonkobe Municipality, Eastern Cape province of South Africa is located within 32° 47' 0" South, 26° 50' 0" East (Wintola and Afolayan, 2010). The area is bounded by the sea in the east and drier Karroo in the west (Erasto et al., 2005) with an altitude of approximately 1300 m above sea level and vegetation veld type 7 (Masika and Afolayan, 2003). It is inhabited by the Xhosa-speaking people with farming as their main source of income. A great majority of these people use plants either alone or as supplements to western therapies in the treatment of numerous gastro-duodenal infections.

3.2.2 Collection of information

A reconnaissance survey was carried out in Fort Beaufort, Alice, King William's Town, Dimbaza, Middledrift and Keiskammahoek to identify possible traders or dealers in traditional medicine in April 2009. Field trips were made between May and July 2009 and participation of the locals was voluntary.

Ethno botanical information was collected using the method of Jovel *et al*. (1996), consisting of general conversation and questionnaires. General questions on the local names of the plants, the parts used, methods of preparation, mode of administration, dosage, other medicinal values of the plants and the perceived efficacy of the remedies on stomach complains were mostly asked directly and the responses filled on the questionnaires (Appendix 2). One of our research group members, a Xhosa lady; did the translation from

Xhosa to English and vice versa during our interactions with the locals. The respondents were traditional healers, herbalists, farm owners, hawkers of traditional medicines and owners of "amayeza yesiXhosa" (Xhosa medicine) stores in Alice, King William's Town, Middledrift, Dimbaza and Keiskammahoek. Villagers who had practical knowledge on medicinal plants used to treat stomach-related morbidities were also recruited in the study.

The participants were motivated by giving them financial incentive to provide samples of the plant specimens used in the treatment of stomach complaints but when this was not possible, they optionally accepted to walk the researchers into the bushes where the samples were harvested. Samples were also purchased from the street vendors, "amayeza yesiXhosa" stores and traditional medicine markets in Alice and King William's Town. Plant specimens were initially identified by their vernacular names. Authentication of plant material was done by the Giffen herbarium, School of Biological Sciences, University of Fort Hare where voucher specimens were deposited.

3.2.3 Intellectual property agreement

Prior to the interviews, the healers and traditional doctors were given information about the project, the participants in the survey and the objectives. The conversations with the locals were built on trust with the common goal of increasing the knowledge on medicinal plants used in the treatment of stomach problems in the municipality. Ethical clearance was obtained from the Eastern Cape Department of Health and the Govan Mbeki Research and Development Centre, University of Fort Hare (Appendix 1).

3.3 RESULTS

A total of 52 informants participated in the study. The majority of them were traditional medicine sellers, 36/52 (69.2%) and villagers, 8/52 (15.4%) who had practical knowledge on medicinal plants used to treat gastritis and other stomach problems. Very few traditional doctors; 4/52(7.7%) and herbalists; 4/52(7.7%) accepted to participate in the study. The number of informants who participated in the study is presented below (Table 3.1).

Table 3.1: **Summary of questionnaire on people interviewed**

Locality	**Traditional doctors**	**Herbalist**	**Traditional medicine sellers**	**Villagers**	**Total**
Fort Beaufort	0	0	2	0	02
Alice	3	3	2	1	09
Middle drift	0	0	1	2	03
Keiskammahoek	1	1	0	3	05
Dimbaza	0	0	3	2	05
King Williams Town	0	0	28	0	28
Total	04	04	36	08	52
Percentage	7.7	7.7	69.2	15.4	100

3.3.1 Types of plants sampled

A total of 17 plant species belonging to 13 genera and 11 families were sampled. The Asphodelaceae was the most represented family (4 species), followed by Apocynaceae (3 species) and Loganiaceae (2 species). Stomach pain was the most frequent manifestation that was treated with most of the plants. Except for one plant, treatment was given orally for all stomach related-illnesses. Further details about the plants sampled and preparation are represented below (Table 3.2).

Table 3.2: **Plants used in the treatment of *Helicobacter pylori*-related morbidities in the Nkonkobe municipality of the Eastern Cape province of South Africa**

Scientific name/Family	**Vernacular/Local name(s)**	**Herbarium vouchers**	**Parts used /State**	**Type of stomach illness**	**Preparation**	**Mode of administration and dosage**
Hypoxis hemerocallidea (Hypoxidaceae)	Inongwe (African potato)	CNUFH07	Tuber (Fresh)	Gastritis	Crushed in warm water and sieved	Taken orally, three glasses a day
Rubia petiolaris (Rubiaceae)	Ubulawu	CNUFH08	Roots (Fresh)	Severe stomach and chest pains	Infusion	Taken orally until symptoms subside
Strychnos henningsii (Loganiaceae)	Umnonono	CNUFH04	Stem bark (Dry)	Stomach pains	Infusion	Taken orally
Strychnos decussata	Umnonono	CNUFH06	Stem bark	Stomach pains	Infusion	Taken orally

(Loganiaceae)			(Dry)			
Aloe arborescence (Asphodelaceae)	Unomawenii	CNUFH09	Leaves (Fresh)	Stomach pains	Decoction	Taken orally
Aloe ferox (Asphodelaceae)	Ikhalaah	CNUFH10	Leaves (Fresh)	Stomach pains	Decoction	Taken orally
Aloe tenuior (Asphodelaceae)	Intelezi	CNUFH11	Leaves (Fresh)	Stomach pains	Decoction	Taken orally
Alepidea amatymbica (Apiaceae)	Iqwili	CNUFH03	Rhizome/Roots (Fresh or dry)	Chest pain, belching	Boiled , infusion	Steam inhaled until symptoms subside
Hydnora africana (Hydnoraceae)	Umavumbuka	CNUFH12	Whole plant (dry)	Gastritis, stomach cramps, ulcers	Infusion	Taken orally
Pachycarpus concolor	Itshongwe	CNUFH13	Roots (Dry)	Stomach ache, gastritis	Infusion	Taken orally

(Apocynaceae)						
Xysmalobium orbiculare (Apocynaceae)	Itshongwe	CNUFH14	Roots (Dry)	Stomach ache, gastritis	Infusion	Taken orally
Xysmalobium undulatum (Apocynaceae)	Itshongwe	CNUFH15	Roots (Dry)	Stomach ache, gastritis	Infusion	Taken orally
Elephantorrhiza elephantine (Fabaceae)	Intolwane	CNUFH16	Roots (Fresh)	Stomach pains	Boiled	Taken orally, three glasses daily
Ilex mitis (Aquifoliaceae)	Isidumo	CNUFH17	Stem bark (Dry)	Bloated painful stomach	Infusion	Taken orally
Cissampelos	Umayisake	CNUFH18	Leaves (Fresh	Stomach pains	Crushed in hot	Taken orally

capensis (Menispermaceae)			and dry)		water	
Bulbine abyssinica (Asphodelaceae)	Uyakayakana	CNUFH19	Roots (Fresh)	Stomach pains	Infusion	Taken orally
Trichilia dregeana (Meliaceae)	Umkhuhlu	CNUFH20	Stem bark (Fresh and dry)	Pains and stability of the stomach	Infusion	Taken orally

The roots were the most commonly used plant parts, constituting 35.3% of all medicinal preparations. This was followed by the stem bark and leaves (23.5% each), rhizome, tuber and whole plant each having a percentage representation of 5.9%. The plant parts used are represented below (Fig. 3.1).

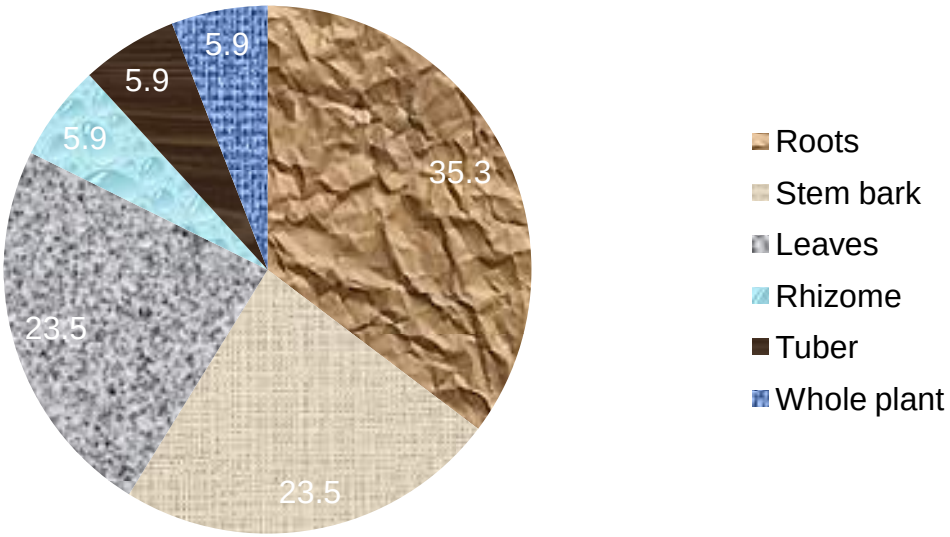

Fig 3.1: **Percentage of plant parts used in the treatment of *Helicobacter pylori*-associated morbidities**

3.4 DISCUSSION

The folkloric use of plants in the treatment of *H. pylori*-associated diseases is becoming more and more popular due to expenses associated with antimicrobial chemotherapy as well as the many adverse effects which reduce the quality of life and discourage patients from observing medication protocols (Bohr and Malfertheiner, 2009). Numerous plants, 17 of which are enlisted herein are used in the treatment of stomach-related morbidities in the Nkonkobe municipality of South Africa and other parts of the developing world (Ndip *et al*., 2007; 2009). However, this study is the first to document plant species used in the treatment of infections symptomatic of *H. pylori* in this municipality.

The results of this study indicate that three species; *Aloe arborescence*, *A. tenuior*, *A. ferox* from the Asphodelaceae family are commonly used in the treatment of stomach problems in the study area. Our results are consistent with the findings of Wintola and Afolayan (2010) who also reported the use of these species in the treatment of constipation in this municipality. It is worth mentioning that the Aloes are widely distributed in Africa and have been reported to be useful medicinal plants with antibacterial properties (Mbanga *et al*., 2010). However, little is known about their anti-*H. pylori* activities.

Thong-Ngam and Chatsuwan (2007) evaluated the antimicrobial activity of *Aloe vera* on *H. pylori* and found no activity. However, aloe-emodin, a compound that is found in some *Aloe* species has been shown to elicit a dose-dependent growth inhibition of *H. pylori* cultures (Wang *et al*., 1998). *Aloe arborescence* was also recently reported to exhibit strong antimicrobial activities against a wide variety of organisms including *Staphylococcus aureus*, *Bacillus substilis*, *Escherichia coli*, *Salmonella typhimurium*, *S. gallinarum*, *Klebsiella*

species, *Proteus* species and *Candida albicans* in Zimbabwe (Mbanga *et al*., 2010). The antimicrobial activity of plant extracts may vary across different regions and may depend on the age of the plants as well as the parts used (Ndip *et al*., 2007). More studies to evaluate the antimicrobial activities of these plants against *H. pylori* would shed light on their potential usefulness as possible sources of novel therapies against this notorious pathogen.

Information from literature indicates that almost all of these plants have been used in the treatment of other illnesses in humans or animals in the Eastern Cape province of South Africa and other countries in Africa (Dold and Cocks, 2002; Cocks and Dold, 2006). Five of the plants studied herein have been recently reported in our study area as remedies for the treatment of constipation. They include; *Strychnos henningsii*, *Aloe arborescence*, *A. tenuior*, *A. ferox* and *Alepidea amatymbica* (Wintola and Afolayan, 2010). Two of these; *Strychnos henningsii* and *A. ferox* are also used for treating diabetes mellitus (Oyedemi *et al*., 2009), while *Alepedia amatymbica* is used in the treatment of diarrhoea (Afolayan and Lewu, 2009).

This is not surprising considering that a single plant species may contain several chemical compounds that may be active against a wide variety of diseases. Plant cells fundamentally are chemical factories and many possess a rich supply of therapeutically useful constituents (Akinpelu *et al*., 2009). Some of these constituents; tannins, catechins, alkaloids, steroids, triterpenoids, essential oils and polyphenolic acids have antibacterial properties and health promoting abilities with therapeutic effects on multiple disease conditions (Cowan, 1999; Iwalewa *et al*., 2007; Adeboye *et al*., 2008).

Our results show that traditional naming of plants in the Nkonkobe municipality is a little controversial considering that different plants may be given the same names. For example,

Strychnos henningsii and *S. decussata* are both referred to as 'Umnonono' while 'Itshongwe' can be used to mean *Pachycarpus concolor*, *Xysmalobium orbiculare* or *X. undulatum* (Table 2). This phenomenon has been reported in many traditional societies and is probably due to the fact that most of these plants are named after their uses (Bussmann *et al*., 2006; Focho *et al*., 2009), which may cut across a number of plants. The results also demonstrate that dosage was dependent most of the time upon the disappearance of symptoms. This may give a false impression of treatment considering that clinical presentations may vary amongst patients and there is also the risk of over-dosage.

In this study, the most used plant parts are roots (35.3%), leaves (23.5%) and stem barks (23.5%) (Fig.3.1). The frequent harvesting of roots and barks may destroy the plants, but they are preferable, probably due to the fact that they contain larger quantities of antibacterial components and they are easier to transport or store for longer periods as previously suggested (Eloff, 2001). It would be much more sustainable if traditional medicine practitioners and dealers in traditional medicine are encouraged to use leaves.

The Apocynaceae was also one of the most represented families in this study (Table 3.2). A World Wide Web search indicates that none of the three members represented herein; *Pachycarpus concolor*, *Xysmalobium orbiculare* and *X. undulatum* have been evaluated against *H. pylori*. This calls for more research on these plants which could be possible sources of new compounds for the eradication of *H. pylori* infections. Equally important is the need to document their toxicological properties and *in vivo* potencies.

Although the use of traditional medicine to manage *H. pylori*-associated morbidities is gaining interest in this municipality, information on the effectiveness as well as the

toxicological and pharmacological properties of most of these plants are lacking. The locals claim they are efficient and safe because of their natural origin. As a consequence, the inhabitants often use these remedies without medical advice, and in some cases combined with medically prescribed drugs. This constitutes a health risk because some of these plants may contain several constituents which could interact with the prescribed drugs and affect drug metabolic pathways.

The use of medicinal plants in the management of *H. pylori*-related morbidities and other infections needs to be regulated considering that some of these plants contain pesticides or heavy metals. Equally important is the fact that a misidentified plant might be used or a different plant than the one originally used for the treatment may be substituted for the same treatment. Not only could plant components affect the efficacy of current *H. pylori* treatment regimens, their interactions could lead to toxicity. However, despite the above mentioned problems, some of these plants are already showing promising results as potential sources of novel anti-*H. pylori* compounds. One of these plants, *Hydnora africana*, studied in our group, has been embraced as a potential reservoir that may contain a large repertoire of new anti-*H. pylori* compounds (Nethathe and Ndip, 2011).

3.5 CONCLUSION

Ethno botanical studies of plants used to manage *H. pylori*-associated morbidities can help in identifying cheap sources of new efficient compounds that would be used to better manage the infection. In this study, 17 species belonging to 11 families were identified. Our next sequence of experiments will be to investigate the antimicrobial activity of these plants and a few others used in different parts of Africa against *H. pylori* in order to be able to scientifically correlate treatment claims with plant usage.

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CHAPTER FOUR

AQUEOUS AND ORGANIC SOLVENT EXTRACTS OF SELECTED SOUTH AFRICAN MEDICINAL PLANTS POSSESS ANTIMICROBIAL ACTIVITY AGAINST *HELICOBACTER PYLORI*: INHIBITORY AND BACTERICIDAL POTENTIAL

ABSTRACT

The aim of this study was to identify new sources of cheap starting materials for the synthesis of new drugs against *Helicobacter pylori*. Solvent extracts of selected medicinal plants; *Combretum molle*, *Sclerocarya birrea*, *Garcinia kola*, *Strychnos* species and *Alepidea amatymbica* were investigated against 30 clinical strains of *H. pylori* alongside a reference control strain (NCTC 11638) using standard microbiological techniques. Metronidazole and amoxicillin were included in these experiments as positive control antibiotics. All the plants demonstrated anti-*H. pylori* activity with zone diameters of inhibition between 0 – 38mm and 50% minimum inhibitory concentration (MIC_{50}) values ranging from 0.06 – 5.0mg/mL. MIC_{50} values for amoxicillin and metronidazole ranged from 0.001 – 0.63mg/mL and 0.004–5.0 mg/mL respectively. The acetone extracts of *C. molle* and *S. birrea* exhibited a remarkable bactericidal activity against *H. pylori* killing more than 50% of the strains within 18 hours at 4xMIC and complete elimination of the organisms within 24 hours. Their antimicrobial activity was comparable to the control antibiotics. However, the activity of the ethanol extract of *G. kola* was lower than amoxicillin ($P<0.05$) as opposed to metronidazole ($P>0.05$). These results demonstrate that *S. birrea*, *C. molle* and *G. kola* may contain compounds with anti- *H. pylori* activity.

4.1 INTRODUCTION

Helicobacter pylori, a Gram negative microearophilic helical bacillus inhabits the stomach of approximately half of the human population, in whom it may persist for a life time, making it one of the most successful human pathogens (Ndip *et al*., 2008a). Infection with this organism is strongly associated with chronic active gastritis, peptic ulcer and mucosa associated lymphoid tissue (MALT) lymphoma (Atherton *et al*., 2006). The organism is classified by the World Health Organisation and the International agency for Research on Cancer as a class 1 carcinogen (Adeniyi *et al*., 2009).

Eradication of the organism from the stomach results in significant remission from the above diseases (Megraud and Lehours, 2007). Current eradication regimens involve the use of combination therapies; a proton pump inhibitor (PPI) or bismuth compounds and two antibiotics, most commonly clarithromycin and metronidazole or amoxicillin (Boyanova, 2009), with an expected success rate of 80 – 90% (Njume *et al*., 2009). However, *H. pylori* infection is still difficult to eradicate as eradication failure rate remains at 10 – 40% (Perna *et al*., 2007; Abbas *et al*., 2009).

Antimicrobial resistance to current regimens is increasingly recognised as a major cause of treatment failure (Boyanova and Mitov, 2010). With primary susceptibility patterns becoming less predictable, it is not uncommon to find other stronger antibiotics particularly of the flouroquinolone group being part of the treatment regimen (Malfertheiner et al., 2007), but *H. pylori* is developing resistance to these drugs too (Eisig *et al*., 2009). Other problems including undesirable side effects (nausea, vomiting, diarrhoea, stomach ache) and poor

patient compliance are associated with significant levels of treatment failure and contraindications for some patients (Abbas *et al*., 2009). In addition, combination therapy is not readily affordable and some of the drugs are not available in some rural settings in the developing world. These factors and others have necessitated the search for alternative treatment regimens with highly selective activity against the organism and without the risk of resistance or other untoward effects.

The use of medicinal plants for the treatment of diseases is a common phenomenon in most African countries particularly in areas where medical health facilities are not readily accessible or affordable (Afolayan and Lewu, 2009). Thus, plants would seem to be a logical source of new anti-*H. pylori* compounds. In fact, our previous studies have documented that some medicinal plant extracts have antibacterial activity against *H. pylori* (Ndip *et al*., 2007; Ndip *et al*., 2008b; Njume *et al*., 2011).

Combretum molle, *Sclerocarya birrea*, *Garcinia kola*, *Alepidea amatymbica* and some *Strychnos* species have found good medicinal uses in the treatment of infections symptomatic of *H. pylori* in South Africa and other African countries (Dold and Cocks, 2002; Philippe *et al*., 2004; Abbas *et al*., 2009). The medicinal properties of most of these plants have been a subject of numerous investigations (Eloff, 2001; Eloff *et al*., 2005; Akinpelu *et al*., 2008). However, reports on their anti-*H. pylori* activity are lacking. This is surprising, given that *H. pylori* antimicrobial resistance to current treatment regimens is increasingly recognised as a major cause of treatment failure. This study was therefore carried out to evaluate the antimicrobial activity of the selected medicinal plants on clinical isolates of *H. pylori* as part

of an ongoing effort to identify potential sources of cheap starting materials for the synthesis of new drugs against this carcinogenic organism.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

A total of thirty strains of *H. pylori* were isolated from gastric biopsies of patients with recurrent peptic ulcer infection undergoing endoscopy at the Livingstone hospital, Port Elizabeth. Isolation and identification was done following our previously reported scheme (Ndip *et al*., 2008a). Briefly, biopsies were homogenised under aseptic conditions in 0.2 g/L of cysteine and 20% glycerol in Brain heart infusion broth (CM0225, Oxoid, England). A loop-full of the homogenate was plated on freshly prepared Columbia agar base (CM0331, Oxoid, England) supplemented with 6% horse blood and Skirrow's supplement (SR0147E, Oxoid, England) containing trimethoprim (2.5 mg), vancomycin (5 mg), cefsulodin (2.5 mg) and amphotericin (2.5 mg). Inoculated plates were incubated at 37 ºC for 5 days under microaerophilic conditions (5–6% O_2, 10% CO_2, 80 – 85% N_2) (BR0056A, Anaerocult Basingstoke, Hampshire, England). The isolates were identified based on colony morphology, positive oxidase, urease and catalase tests. Pure cultures were suspended in eppendorf tubes containing 1 mL of Brain Heart Infusion (BHI) broth and 20% glycerol and stored at -80 ^{o}C (Ilshin®, Model DF 9007, Sanyo, Osaka, Japan) for future experiments.

Ethical clearance was obtained from the Eastern Cape Department of Health and the Govan Mbeki Research and Development Centre, University of Fort Hare (Appendix 1). As inclusion criteria, specimens were only collected from patients who had given consent and had not received antibiotics or PPIs for at least a week.

4.2.2 Preparation of plant material

The plants were selected based on ethno botanical information. The stem barks of *C. molle* and *S. birrea* were harvested in the vicinity of the University of Venda, Limpopo Province. The stem bark of *Strychnos* species commonly referred to as 'Umnonono' and the roots/rhizomes of *A. amatymbica* referred to as 'Iqwili' were purchased from the traditional medicine market in King William's Town and amayeza yesiXhosa (Xhosa medicine) stores in Alice. The seeds of *G. kola* were purchased from a local market in Cameroon. *C. molle* was identified by botanists at the University of Venda where vouchers have been deposited. The rest of the plants were identified by botanists in the phytomedicine research unit at the University of Fort Hare and vouchers deposited in the Giffen herbarium. The plant materials were washed, separately chopped into small pieces and dried in a hot air oven (Schwabach, Germany) at 40ºC for 48 hours. The plant material was powdered (ATO mix, Torrington, CT, USA) and stored in air tight containers in a dark cupboard for future use.

4.2.3 Preparation of plant extract

Exactly 300 g of dried powdered plant material was macerated separately in 600 mL of concentrated ethyl acetate, acetone, ethanol and methanol (Merck, Wadeville, Gauteng, South Africa) in large glass bottles (SIMAX, Sazava, Czech Republic). Aqueous extracts were also prepared by soaking same amount of plant material in tap water. The bottles were labelled and put in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) for 48 hours. The plant extracts were centrifuged at 1006.2 g for 5 min, and filtered using a fritted filter funnel of pore size 60 Å (Merck, Gauteng, South Africa). The procedure was repeated twice and the three extracts combined and evaporated to dryness under vacuum in a rotary evaporator (BUCHI rota vapour, R-461, Switzerland) set at temperatures depending on the solvent in

use. The filtrate obtained from aqueous extracts was lyophilized (Castillo-juarez *et al*., 2009). The dried crude extracts were collected in clean universal bottles and left open in a bio-safety class 11 cabinet (Vivid Air, VA 1211, Durban, South Africa) for complete evaporation of residual solvents. A 2-g sample of each extract was used for the preliminary bioassay, and where possible, another 2 g was kept in the extract bank. Stock solutions were prepared by dissolving the extracts in dimethyl sulphoxide (DMSO) or acetone; 10% and 80% respectively (neither DMSO nor acetone were inhibitory to the *H. pylori* strains at the tested concentrations).

4.2.4 Screening of crude extracts for anti-*H. pylori* activity

This was achieved by the agar well diffusion method as previously reported (Boyanova *et al*., 2005). Briefly, *H. pylori* inocula prepared at McFarland's turbidity standard 2 was plated onto Mueller- Hinton agar (CM0337, Oxoid, England) supplemented with 5% horse blood and Skirrow's supplement (Oxoid, England). The inocula was evenly spread on the plate and allowed to dry for 15 min. Wells (6 mm in diameter) were punched into the agar using a sterile stainless steel borer and filled with 65 µL of the extract at 100 mg/mL. Sixty-five micro litres of 0.05 µg/mL clarithromycin and 10% dimethyl sulphoxide (DMSO) were included in all experiments as positive and negative controls respectively. The plates were incubated under microaerophilic conditions (Anaerocult, Oxoid, UK) at 37 ^{o}C for 72 hours after which the diameters of zones of inhibition were measured in millimetres. The experiment was repeated once and mean zones recorded. *H. pylori* control strain, NCTC 11638 inoculated plate was included in all the experiments.

4.2.5 Determination of 50% Minimum Inhibitory Concentration (MIC_{50})

Active extracts that had given a percentage susceptibility of ≥50% by agar well diffusion were selected for further determination of MICs by the micro broth dilution method (Bonacorsi *et al*., 2009), performed in 96-well plates. The test extracts were prepared at a concentration of 5.0 mg/mL and filtered through a 2.0 µm filter (Acrodisc Pall, MI, USA). Two-fold dilutions of each extract were made in the test wells in BHI broth supplemented with 5% horse serum and Skirrow's supplement (Oxoid, England). The final extract concentrations ranged from 0.002 – 5.0 mg/mL. Twenty microlitres of an 18-hour-old broth culture of *H. pylori* (McFarlands turbidity standard 2) suspension was added to 180 µL of extract-containing culture medium. Control wells were prepared with culture medium only and bacterial suspension and broth only respectively. Metronidazole and amoxicillin were run alongside each batch of extracts at 0.002 – 5.0mg/mL and 0.0005 – 5.0 mg/mL concentration ranges respectively. An automatic ELISA microplate reader (Biorad, Tokyo, Japan) adjusted to 620 nm was used to measure the absorbance of the plates before and after 72-hour incubation at 37 °C under microaerophilic conditions. The absorbencies were compared to detect an increase or decrease in bacterial growth and the values plotted against various extract concentrations. The lowest concentration of the test extract resulting in inhibition of 50% of bacterial growth was recorded as the MIC.

4.2.6 Determination of Rate of Kill of crude extracts

The most active plant crude extracts with mean MIC values ≤ 1.0 mg/mL were considered for the rate of kill experiments. The rate and extent of killing of *H. pylori* by the acetone extracts of *C. molle* and *S. birrea* as well as the aqueous extract of the latter were determined as described by Akinpelu *et al*. (2009) with slight modifications. The turbidity of an 18-hour old

broth culture of *H. pylori* was standardized to 10^8 CFU/mL. One milliliter of the suspension was added to 9 mL of BHI broth supplemented with 5% horse serum and Skirrow's reagents containing the extract at 1/2xMIC, MIC, 2xMIC and 4xMIC in McCartney bottles (Oxoid, England). A negative control bottle was prepared with bacterial suspension and broth only.

A 0.1mL sample was plated from these bottles before incubation at 37 °C under microaerophilic conditions. Exactly 0.5 mL volume of each suspension was withdrawn at 6 hour interval for 72 hours and transferred to 4.5 mL of BHI broth recovery medium containing 3% 'Tween 80' to neutralize the effects of the antimicrobial extract carry-overs from the test organisms. The suspension was 10-fold serially diluted in sterile saline (0.9% w/v sodium chloride) and plated in triplicates. The plates were incubated at 37 °C for 72 hours under microaerophilic conditions and emergent bacterial colonies were counted. The counts were multiplied by the dilution factor and recorded as CFU/mL. Counts obtained from extract-containing culture bottles were compared with those of the negative control bottle and the percentage of bacteria killed was determined.

4.2.7 Statistical analysis

The statistical packages used for analysis were Excel (Microsoft, Redmond, WA, USA) and SPSS version 17.0 (2009, SPSS, Inc., Chicago, IL, USA). One-way ANOVA was used to compare the mean difference in inhibitory activities of extracts and antibiotics, followed by Turkey's *post-hoc* test. Differences were considered significant at $P< 0.05$.

4.3 RESULTS

4.3.1 Extract yield

The total amount of crude extract obtained with the different solvents shows that methanol was quantitatively the best solvent for all the plants while ethyl acetate had the lowest yields in four of the five plants studied (Table 4.1).

Table 4.1: **Crude extract yield**

		Percentage of plant crude extract obtained with different solvents				
Plant	**Family**	**EA**	**A**	**E**	**M**	**W**
Combretum molle R.Br. ex G. Don	Combretaceae	0.4	1.5	1.1	1.7	1.0
Sclerocarya birrea A. Rich Hochst	Anacardiaceae	0.9	3.3	4.2	5.2	1.9
Garcinia kola Heckel	Guttiferae	0.5	1.5	1.5	3.2	1.8
Strychnos species Gilg.	Loganiaceae	0.4	0.6	0.7	4.7	2.3
Alepidea amatymbica Eckl. and Zeyh	Apiaceae	4.6	1.9	1.2	5.6	2.9

EA, Ethyl acetate; A, Acetone; E, Ethanol; M, Methanol; W, Water

4.3.2 Antimicrobial susceptibility testing and MIC_{50} determination

All the plant crude extracts tested demonstrated anti-*H. pylori* activity with zone diameters of inhibition between 0 – 38mm. The highest mean zone diameter of 17.4±5.0mm was recorded for the acetone extract of *C. molle* while the least (1.0±2.6) was recorded for the aqueous extract of *G. kola*. Further information about zone of inhibition diameters is summarised below (Table 4.2).

Table 4.2: **Screening of crude extracts against *H. pylori* isolates**.

Plant	**Solvent extract**	**Mean zone diameter ± SD (mm)**	**Inhibition diameter range (mm)**
C. molle	Ethyl acetate	10.7±4.7	0 – 21
	Acetone	17.4±5.0	10 – 38
	Ethanol	12.9±4.7	7 – 35
	Methanol	13.1±5.3	7 – 32
	Water	2.7±5.5	0 – 20
	Positive control	13.5±8.7	0 – 32
S. birrea	Ethyl acetate	13.2±2.8	8 – 20
	Acetone	14.7±2.5	11 – 21
	Ethanol	3.3±5.0	0 – 16
	Methanol	3.0±4.4	0 – 11
	Water	15.0±2.7	10 – 20
	Positive control	16.6±7.4	0 – 32
G. kola	Ethyl acetate	5.1±4.6	0 –13
	Acetone	8.8±5.2	0 – 25
	Ethanol	9.2±7.2	0 – 19
	Methanol	7.1±5.8	0 – 20
	Water	1.0±2.6	0 – 8
	Positive control	16.1±8.3	0 – 32

Strychnos sp	Ethyl acetate	10.1±6.4	0 – 26
	Acetone	8.8±6.8	0 – 26
	Ethanol	4.9±6.2	0 – 18
	Methanol	5.5±5.9	0 – 15
	Water	11.9±5.6	0 – 23
	Positive control	15.5±7.3	0 – 32
A. amatymbica	Ethyl acetate	8.5±4.8	0 – 17
	Acetone	7.0±6.5	0 – 20
	Ethanol	6.7±6.7	0 – 20
	Methanol	6.1±6.4	0 – 15
	Water	8.0±8.2	0 – 25
	Positive control	14.4±7.7	0 – 30

Data are mean±SD of 31 determinations for each plant extract or control antibiotic.

The acetone extract of *C. molle* recorded the highest percentage susceptibility of 87.1%, followed by the acetone and aqueous extracts of *S. birrea* with a percentage of 71% each. *Strychnos* species and *A. amtymbica* gave percentage susceptibilities of less than 50%. Further details about the activity of the plant extracts based on agar well diffusion are represented below (Fig. 4.1).

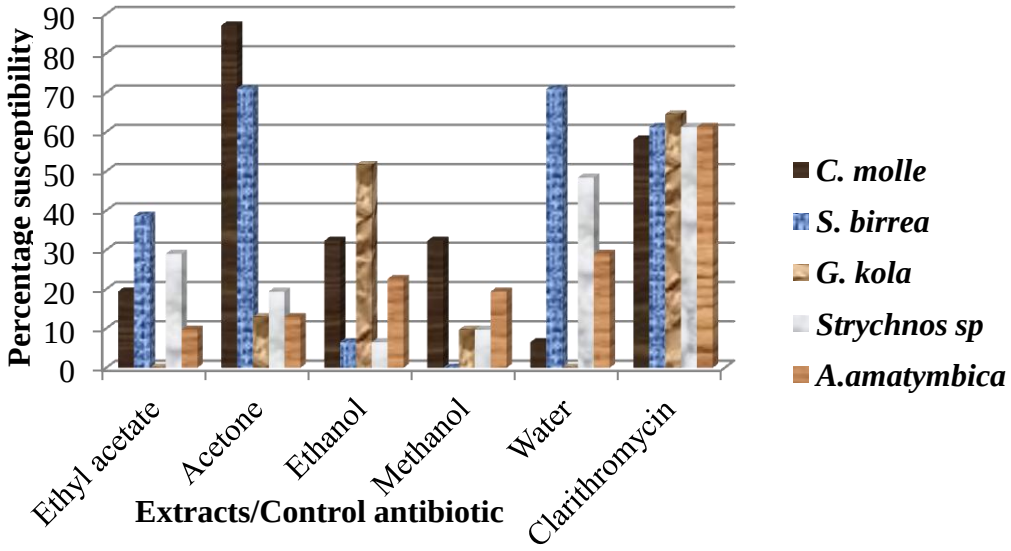

Fig. 4.1: **Activity of plant crude extracts on *H. pylori* by agar well diffusion method.**

Percentage susceptibility is representative of the number of strains whose zone of inhibition diameter is ≥14mm

MIC values of the plant extracts ranged from 0.06 – 5.0 mg/mL and those of amoxicillin and metronidazole ranged from 0.001 – 0.63 and 0.004 – 5.0 mg/mL respectively. Of all the extracts analysed, the acetone extracts of *S. birrea* and *C. molle* had the best activities with MIC values ranging from 0.06 – 2.50 mg/mL and 0.08 – 2.50 mg/mL respectively with no significant differences between these values and those of amoxicillin ($P>0.05$) as opposed to metronidazole ($P<0.05$). However, the activity of the ethanol extract of *G. kola* was significantly different from amoxicillin ($P<0.05$) as opposed to metronidazole ($P>0.05$). The MIC values of the plant crude extracts and control antibiotics are presented below (Table 4.3).

Table 4.3: **Minimum inhibitory concentrations (MIC_{50}) of plant crude extracts and control antibiotics (mg/mL)**

	Crude extracts/Antibiotics					
***H. pylori* strains**	**AC**	**AS_1**	**AS_2**	**EG**	**AMX**	**MTZ**
PE2A	0.312	0.06	0.47	5.0	0.32	1.25
PE5A	0.625	0.12	0.63	1.25	0.63	1.25
PE9C	1.25	0.08	0.63	1.25	0.01	2.5
PE11A	0.156	1.25	0.16	5.0	0.04	5.0
PE11C	0.156	0.08	0.63	1.25	0.04	1.25
PE14C	0.156	0.06	0.47	1.25	0.31	1.25
PE26A	0.312	0.63	1.25	1.25	0.63	1.25
PE70A	0.625	0.06	0.94	1.25	0.32	2.5
PE76A	1.25	2.5	2.5	5.0	0.31	5.0
PE84C	0.625	1.25	–	1.25	0.63	1.25
PE93A	0.078	0.06	0.63	1.25	0.32	2.5
PE93C	1.25	0.16	0.47	1.25	0.63	1.25
PE102C	0.156	0.31	2.5	1.25	0.63	1.25
PE115A	0.312	0.63	1.25	1.25	0.31	5.0
PE155A	2.5	0.08	0.94	1.25	0.16	2.5

PE219C	1.25	0.94	–	1.25	0.31	1.25
PE252C	0.312	1.25	2.5	1.25	0.63	2.5
PE258C	0.312	0.94	2.5	1.25	0.08	3.75
PE369A	0.312	0.63	0.31	1.25	0.31	0.08
PE369C	0.625	0.31	0.31	1.25	0.31	2.5
PE397C	0.312	0.08	1.5	5.0	0.01	5.0
PE402A	1.25	0.06	0.49	5.0	0.32	2.5
PE411A	0.625	0.08	0.63	1.25	0.08	2.5
PE411C	1.25	0.63	0.63	1.25	0.31	1.25
PE430A	0.312	1.25	–	1.25	0.31	1.25
PE430C	0.625	0.31	0.47	1.25	0.31	1.25
PE435A	0.625	0.63	1.25	0.63	0.31	1.25
PE436A	1.25	0.08	0.63	0.63	0.31	0.04
PE436C	0.312	1.25	2.5	1.25	–	1.25
PE466C	0.312	0.36	2.5	1.25	0.31	1.25
NCTC 11638	0.312	0.63	2.5	2.5	0.001	0.004
Mean ± SD	**0.63±0.53**	**0.54±0.56**	**1.0±0.85**	**1.85±1.42**	**0.30±0.2**	**2.1±1.4**

AC, Acetone extract of *C. molle*; AS_1, Acetone extract of *S. birrea*; AS_2, Aqueous extract of *S. birrea*; EG, Ethanol extract of *G. kola*; AMX, Amoxicillin; MTZ, Metronidazole; –, Value

not within susceptible range; SD, Standard deviation. The results shown are representative of duplicate determinations for each strain and 31 determinations for each extract or control antibiotic. The strains bearing the letter 'A' were isolated from the antrum while those bearing the letter 'C' were isolated from the corpus. The resistant breakpoint for amoxicillin and metronidazole was >0.002mg/ml and >0.008mg/ml respectively.

4.3.3 Bactericidal activity of crude extracts

The acetone extracts of *C. molle* and *S. birrea* exhibited a remarkable bactericidal activity against the *H. pylori* strains at all concentrations tested. At a concentration of 1.2mg/mL (2xMIC) and 2.4mg/mL (4xMIC), the acetone extract of *C. molle* killed 35.0 and 55.0% of *H. pylori* respectively within 18 hours. A killing rate of 100% was maintained from the 24th hour to the end of the assay at the same concentrations (Fig 4.2a).

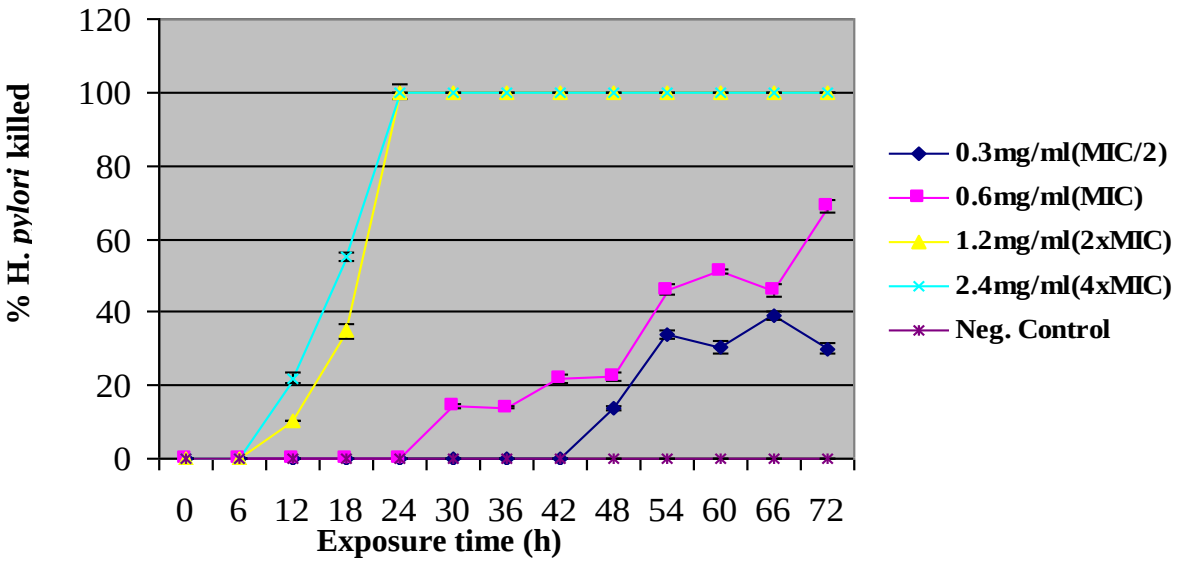

Figure 4.2a: **Rate of killing of *H. pylori* by the acetone extract of *C. molle*.**

Percentage of *H. pylori* killed represents mean values of triplicate determinations for the strains PE 2A, PE 14C and PE 93A. Error bars represent the standard deviations (SD). Very small error bars have been obscured by data symbols. Neg. Control; extract-free cells.

The acetone extract of *S. birrea* exhibited a stronger killing rate with 54.4% and 93.0% of the strains killed within 12 h at 1.2 mg/mL (2xMIC) and 2.4 mg/mL (4xMIC) extract concentrations respectively. A killing rate of 100% was maintained from the 18th hour to the end of the assay at the same concentrations (Fig. 4.2b).

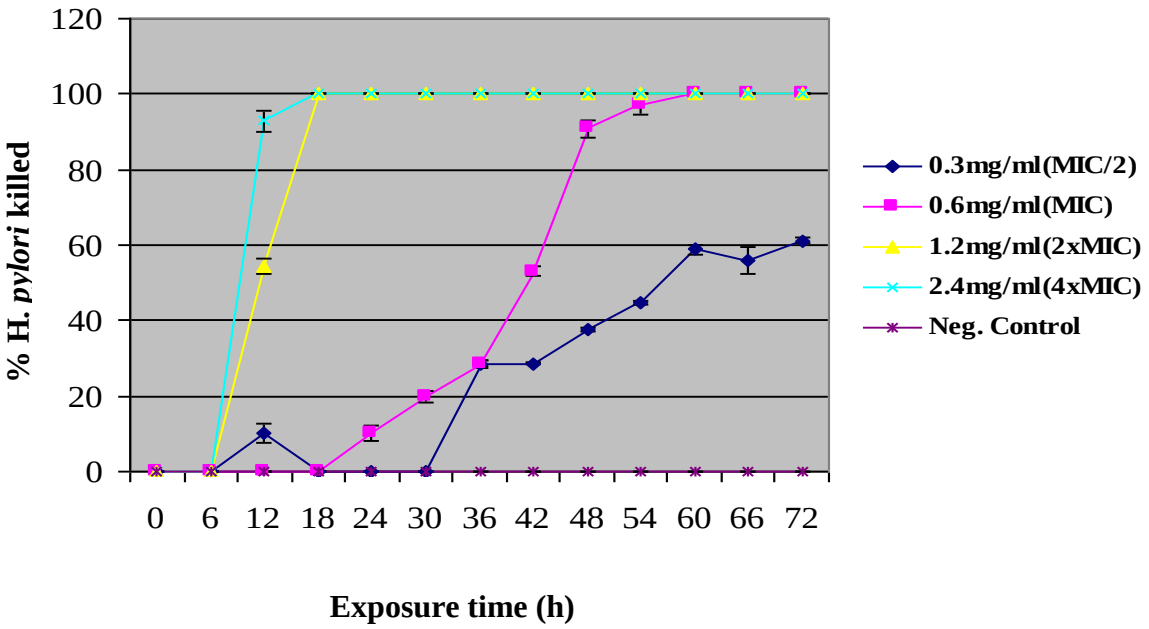

Figure 4.2b: **Rate of killing of *H. pylori* by the acetone extract of *S. birrea*.**

Percentage of *H. pylori* killed represents mean values of triplicate determinations for the strains PE 2A, PE 14C and PE 93A. Error bars represent the standard deviations (SD). Very small error bars have been obscured by data symbols. Neg. Control; extract-free cells.

The aqueous extract of *S. birrea* however exhibited a weaker bactericidal activity with less than 30% of the strains killed after 72 hours at all concentrations tested. However, there seemed to be a killing rate of almost 50% at the 36th hour of the assay for the 1.0(MIC), 2.0(2xMIC) and 4.0mg/mL (4xMIC) extract concentrations which was not maintained to the end of the assay (Fig. 4.2c).

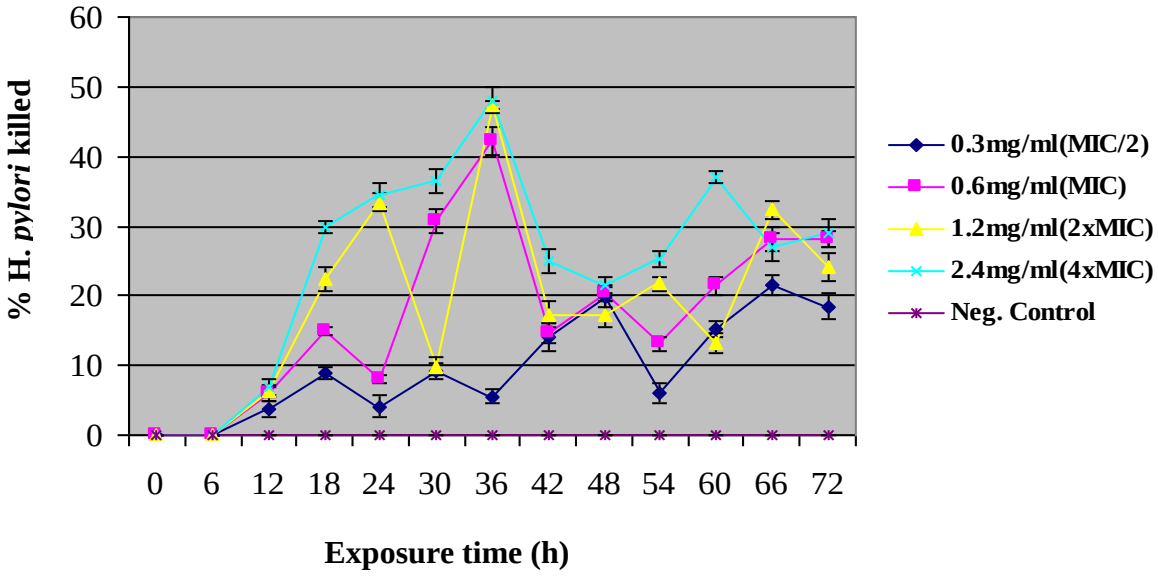

Figure 4.2c: **Rate of killing of *H. pylori* by the aqueous extract of *S. birrea*.**

Percentage of *H. pylori* killed represents mean values of triplicate determinations for the strains PE 2A, PE 14C and PE 93A. Error bars represent the standard deviations (SD). Neg. Control; extract-free cells.

4.4 DISCUSSION

The efficiency of methanol in the extraction of phytochemicals has been reported in other studies (Ndip *et al*., 2007; Masoko et al., 2008; Ezekiel *et al*., 2009). The results of this study are in line with others confirming methanol as a good extractant of bioactive compounds from plants as it gave the highest yield in all five plants. However, the extract yield may not always relate proportionally with its activity as revealed by the activity of the methanol extract herein (Fig. 4.1). Nevertheless, the activity demonstrated by the methanol extracts gives an indication of their potential as useful bioactive substances.

The acetone extracts of *C. molle* and *S. birrea* exhibited strong anti- *H. pylori* activity recording the highest mean zone diameters (Table 4.1) and percentage susceptibilities of >70% (Fig. 4.1). This suggests that acetone could be a better solvent for the extraction of anti- *H. pylori* compounds from both plants. Eloff (1998) in a comparison of acetone, ethanol, methanol, methylenedichloride, methanol/chloroform/water and water observed acetone to be the best in terms of the diversity of compounds extracted. Such diverse compounds may act in synergy and produce a greater antimicrobial effect on *H. pylori*, thus resulting in high susceptibility patterns and low MIC values observed for the acetone extracts of *C. molle* and *S. birrea* in this study (Table 4.3).

The acetone extracts of *C. molle* and *S. birrea* seem to possess significant inhibitory activity against *H. pylori* when compared with metronidazole and amoxicillin. Previous findings have reported a 97.5% susceptibility of the *H. pylori* strains in South Africa to amoxicillin and 95.5% resistance to metronidazole (Tanih *et al*., 2010). Crude extracts of other plants have

been shown to have comparable activity with amoxicillin and better activity when compared with metronidazole against *H. pylori* in other studies (Ndip *et al*., 2008a; Castillo-juarez *et al*., 2009). This is quite remarkable particularly as standard antibiotics are in the purified and concentrated form whereas the extracts are crude and may harbour both pharmacologically and non-pharmacologically active compounds with the chance of some compounds having a masking effect over others. This is an indication that the acetone extracts of *C. molle* and *S. birrea* may contain therapeutically useful compounds against *H. pylori* infections.

There seemed to be a linear relationship exhibited by the bactericidal activity of the extracts with time and concentration. For example, at 1.2 mg/mL a killing rate of 54.4% of *H. pylori* was recorded for the acetone extract of *S. birrea* after 12 hours and complete eradication of the organisms after 18 hours. When this concentration was doubled (2.4mg/mL), the killing rate was more than 90% after 12 hours with complete eradication of the organisms after 18 hours (Fig. 4.2b). The relationship was almost the same for the acetone extract of *C. molle* (Fig. 4.2a). These findings corroborate the observations of Akinpelu *et al*. (2009) who reported the rate of killing of *Escherichia coli* and *Bacillus subtilis* by the aqueous fractions of *Afzelia africana* to be dependent on time and extract concentration. Unlike in Akinpelu's study where fractions were used, our extracts are still crude with better activity expected upon fractionation and purification from pharmacologically inactive substances that may interfere with the assay.

A relatively lower bactericidal activity was noticed with the aqueous extract of *S. birrea*. Even though there was a consistent increase in the percentage of *H. pylori* killed with time at

MIC, 2xMIC and 4xMIC concentrations, this was not maintained to the end of the assay (Fig. 4.2c). The peak bactericidal activity exhibited by the extract at the 36th hour coincides with the exponential growth phase of the organisms, a time during which they are most sensitive to antimicrobial agents. It is likely that some of the organisms that survived this period were already becoming resistant to the extract after 36 hours. From all indications, the aqueous extract of *S. birrea* is only weakly bactericidal.

It is worth mentioning that the literature is rich with information on the very limited activity or complete non responsiveness of microorganisms to plant aqueous extracts (Madamombe and Afolayan, 2003; Eloff *et al*., 2005; Moghaddam *et al*., 2009). Our study lays credence to this as aqueous extracts of 4 of the 5 plants studied recorded percentage susceptibilities of less than 50%. However, these results also provide evidence that water could still be a good solvent in the extraction of anti-*H. pylori* compounds from *S. birrea* as it gave an activity of 71% in the screening (Fig. 4.1). This is particularly important in the study area and other rural settings in Africa where organic solvents reported to be better extractants (Ndip *et al*., 2007; Eloff *et al*., 2008) may not be within the reach of the traditional healers, as most of them make use of aqueous extracts in their practice. Equally important is the fact that the use of water is environmentally safer than most organic solvents.

Crude ethanol extracts of *G. kola* seeds exhibited a better activity when compared to metronidazole ($P>0.05$), a pure compound. This is not surprising given the high *H. pylori* resistance to metronidazole in the study area. Equally important is the fact that the seeds of this plant are reported to be rich in flavonoids and tannins, substances with great antimicrobial potentials and health promoting abilities (Cowan, 1999; Adeboye *et al*., 2008).

The antimicrobial activity demonstrated by crude extracts of *Strychnos* species and *A. amatymbica* was relatively low. It is likely that the amount of active ingredients in these plant extracts may not occur in quantities large enough to produce significant activity. Such extracts upon fractionation, purification and concentration of the active ingredients may still lead to the isolation of therapeutically useful compounds against *H. pylori* or other infectious organisms. Moreover, extracts with little or no activity *in-vitro* may have properties similar to pro-drugs which are administered in an inactive form. Their metabolites could be active *in-vivo*.

4.5 CONCLUSION

All the plants studied herein demonstrated considerable anti-*H. pylori* activity, thus validating their ethno botanical use in the treatment of infections symptomatic of this organism. *C. molle*, *S. birrea* and *G. kola* may contain compounds mostly in the acetone, acetone/aqueous and ethanol crude extracts respectively that could be used as lead molecules for the synthesis of novel drugs against *H. pylori* infections. Our next series of experiments is geared towards the isolation and characterization of the active principles in the acetone and aqueous crude extracts of *S. birrea*.

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CHAPTER FIVE

PRELIMINARY PHYTOCHEMICAL SCREENING AND *IN-VITRO* ANTI-*HELICOBACTER PYLORI* ACTIVITY OF ACETONE AND AQUEOUS EXTRACTS OF THE STEM BARK OF *SCLEROCARYA BIRREA* (ANACARDIACEAE)

ABSTRACT

Helicobacter pylori antibiotic resistance and other problems associated with combination therapy have generated a considerable interest in the search of alternative therapeutic agents. In order to identify phytocomponents with antimicrobial activity, the acetone and aqueous extracts of the stem bark of *Sclerocarya birrea* were fractionated by thin layer chromatography (TLC) using different solvent combinations; ethyl acetate/methanol/water (EMW); chloroform/ethylacetate/formic acid (CEF) and hexane/diethyl ether (HDE). The phytocomponents were tested against 30 clinical strains of *H. pylori* and a standard control strain (NCTC 11638) by agar-overlay bioautography. A total of 35 components were separated, 22 of which were revealed in the acetone extract and 13 in the aqueous extract. Most of the active phytocomponents were also located in the acetone extract; $R_f \leq 0.62$ with more than 90% inhibition. The aqueous extract was highly inhibitory at the origin (R_f 0). These results demonstrate that the acetone and aqueous extracts of *S. birrea* may contain compounds with therapeutic activity, therefore potential sources of new anti- *H. pylori* regimen.

5.1 INTRODUCTION

Helicobacter pylori is a Gram negative, microaerophilic helical bacillus with lophotrichous flagella (Ndip *et al*., 2008). The organisms inhabit various areas of the stomach and duodenum and are specifically adapted to colonise and survive the hostile acidic gastric environment (Tanih *et al*., 2010). More than half of the world's population harbours this organism in the upper gastrointestinal tract and over 80% of individuals infected with the bacterium are asymptomatic (Go, 2002).

The organism is strongly associated with chronic gastritis, peptic ulcer disease (PUD), duodenal ulcer and mucosa associated lymphoid tissue (MALT) lymphoma (Go, 2002; Ndip *et al*., 2008). Infections are treated with potent combination therapies; a proton pump inhibitor (PPI) or bismuth and two antibiotics most commonly metronidazole, clarithromycin, amoxicillin and tetracycline with an expected success rate of 80 – 90% (Mégraud and Lehours, 2007). However, eradication of *H. pylori* is still a difficult problem as treatment failure rate remains at 10 – 40% together with frequent relapses of gastric ulcer (Lai *et al*., 2006). Equally important is the increasing prevalence of antimicrobial resistant strains which jeopardize the success of therapeutic regimens aimed at eradication of infections (Malfertheiner *et al*., 2007; Njume *et al*., 2009). These factors and others including; cost of combination therapy, the non availability of the drugs in some rural settings in Africa, side effects of the drugs and poor patient compliance have necessitated the search for alternative treatment regimens with highly selective activity against the organism and without the risk of resistance or other untoward effects.

The use of medicinal plants for the treatment of diseases is a common phenomenon in Africa particularly in areas where medical health facilities are inadequate or inaccessible (Afolayan and Lewu, 2009). Plants would therefore seem to be a logical alternative to turn to for new therapeutic compounds against *H. pylori* infections. The stem bark of *Sclerocarya birrea*, a medium size to large deciduous tree with an erect trunk and rounded crown is widely used for the treatment of stomach-related morbidities and other illnesses in South Africa (Eloff, 2001; Masoko *et al*., 2008). Its medicinal uses are popular among the Xhosa and Zulu people who use bark decoctions as enemas for diarrhoea and the Vhavenda people who use the same parts for treating fevers, stomach ailments and ulcers (Masoko *et al*., 2008).

The anti-bacterial and antifungal activities of this plant have been reported (Eloff, 2001). However, reports on its anti- *H. pylori* activity are lacking even though the plant is locally used in the treatment of diseases symptomatic of infections caused by this organism (Njume *et al*., 2009). It was therefore necessary to investigate the anti- *H. pylori* activity of *S. birrea* in an attempt to identify potential sources of cheap starting materials for the synthesis of new drugs against this organism.

5.2 MATERIALS AND METHODS

5.2.1 Bacterial strains

A reference strain of *H. pylori* NCTC 11638 and resistant strains we isolated from gastric biopsies of patients with recurrent peptic ulcer infection undergoing endoscopy at the Livingstone Hospital, Port Elizabeth were used (Tanih *et al*., 2010). Isolation and identification of the strains was done following our previously reported schemes (Njume *et al*., 2011). The study was approved by the Eastern Cape Department of Health and the Govan Mbeki Research and Development Centre, University of Fort Hare. Gastric biopsies were only collected from patients who had given consent and had not been on any form of anti-*H. pylori* regimen for at least a week. Cultures were purified by sub-culturing twice on Columbia Blood Agar (CBA) supplemented with 7% horse blood and skirrows supplements (Oxoid, England).

5.2.2 Preparation of plant material

The stem bark of *S. birrea* was harvested from different trees at Nzhelele, 12 km from the University of Venda in August 2009 and transported to the University of Fort Hare. The plants were identified by botanists at the University of Fort Hare, School of Biological Sciences, Alice with vouchers deposited at the school's herbarium (GEUFH01). The plant material was washed with tap water, chopped into small pieces and dried at 40º C for one week in a hot air oven (Memment 854, Western Germany). The dried plant material was powdered using a blender (ATO MSE mix, England).

5.2.3 Preparation of plant extracts

Exactly 300 g of dried plant material was macerated separately in 600 mL of concentrated ethyl acetate, acetone, ethanol and methanol in large glass bottles (SIMAX, Czech Republic). Aqueous extracts were also prepared by soaking same amount of plant material in tap water. The bottles were put in an orbital shaker for 48 hours. The plant extracts were centrifuged at 1006.2 g for 5 minutes, and filtered using a fritted filter funnel of pore size 60 Å. The procedure was repeated twice and the three extracts combined and concentrated to dryness under vacuum (BUCHI rota vapour, Switzerland). The filtrate obtained from the aqueous extract was lyophilized (Castillo-Juarez *et al*., 2009). The dried crude extracts were collected in universal bottles and left open in a bio-safety class II cabinet (Vivid Air, Durban, South Africa) for complete evaporation of residual solvents. A 3-g sample of each extract was used for the preliminary bioassay and the rest kept in the extract bank. Stock solutions were prepared by dissolving the extracts in 10% dimethyl sulphoxide (DMSO) or 80% acetone; concentrations we established to be non inhibitory to the *H*. *pylori* strains.

5.2.4 Thin layer chromatography (TLC) and bioautography

Preliminary identification of the active components of the crude extracts was achieved by TLC and indirect bioautography (Nalina and Rahim, 2007), with slight modifications. Fifteen micro litres of neat plant extract at 100 mg/mL was applied 2 cm from the base of aluminium-backed silica plates (Merck $60F_{254}$, Germany) cut to size (10x5 cm). The plates were dried for 15 minutes at room temperature and separately developed in the following solvent combinations; ethyl acetate/methanol/water (20:2.7:2.5); chloroform/ethyl acetate /formic acid (5:4:1) and hexane/diethyl ether (2:1) (Masoko *et al*., 2008). The plates were prepared in duplicates for each solvent combination (‘A’ and ‘B’) and developed in glass

tanks closed with aluminium foil. Plate 'A' was used as a reference chromatogram to visualize the separated spots under visible light and UV irradiation at 365 nm (Alliance 4.7, Cambridge, UK) and sprayed with vanillin (Eloff, 2001). The plate was carefully heated at 105 ºC for optimal colour development. The R_f values (Retention factor) of the spots on the plate were computed and recorded. Plate 'B' was left in a bio-safety class II cabinet for 72 hours under a stream of air for complete evaporation of residual solvents. The plate was cut to size (5x5 cm) and aseptically placed in sterile Petri-dishes. Two hundred micro litres of an 18-hour broth culture of *H. pylori* (Mcfarland's turbidity standard 2) was added to 15 mL of molten BHI agar cooled to about 45 ºC and supplemented with 5% horse serum and Skirrow's supplement (Oxoid, England) in a conical flask. The flask was swirled to mix and the mixture poured over the TLC plate in a manner just enough to cover the plate in the Petri-dish, and allowed to set. The Petri-dishes were incubated under microaerophilic conditions (Anaerocult, Oxoid, UK) at 37 ^{o}C for 72 hours and examined for bacterial growth inhibition. The R_f of the zones of inhibition on plate 'B' was compared with the R_f of the reference chromatogram (plate 'A').

5.3 RESULTS

5.3.1 Detection of active components in the extracts by indirect bioautography

TLC analysis and vanillin staining revealed the presence of 22 (R_f: 0.98, 0.96, 0.8, 0.71, 0.62, 0.57, 0.54, 0.47, 0.44, 0.32, 0.67, 0.52, 0.3, 0.75, 0.93, 0.91, 0.53, 0.33, 0.26, 0.22, 0.18, 0) components in the acetone extract and 13 (R_f: 1.0, 0.92, 0.89, 0.81, 0.73, 0.64, 0.56, 0.54, 0.42, 0.3, 0.2, 0.1, 0) in the aqueous extract. The most active components were revealed in the acetone extract ($R_f \leq 0.62$) with more than 90% of the strains inhibited (Table 5.1). As for the aqueous extract, a majority of the strains were inhibited at the origin (*Rf* 0) (Fig.5.1). Further details on the components and their antimicrobial activity are presented in Table 5.1.

Table 5.1: **Degree of inhibition of acetone and aqueous crude extracts of *S. birrea* by agar-overlay bioautography.**

H. pylori strain	**EMW**				**CEF**				**HDE**			
	A. Ext Rf	*d*	*W. Ext Rf*	*d*	*A. Ext Rf*	*d*	*W. Ext Rf*	*d*	*A. Ext Rf*	*d*	*W. Ext Rf*	*d*
PE2A	0.31	++	n	0	0	+++	n	0	0	0	0	0
PE5A	n	0	n	0	n	0	n	0	n	0	0	0
PE9C	0.32	++	n	0	n	0	n	0	n	0	0	0
PE11A	≤ 0.32	+++	0	+++	0	+++	0	+	n	0	0	0
PE11C	≤ 0.44	++	0	++	0	++	0	+	0	+	0.64	+
PE14C	0.33	++++	≤ 0.64	++++	≤ 0.54	+	0	+	0.22	+	0.56	+
PE26A	0.34	++	0.89	++	0.89	+	0.73	++	0.96	++	0.71	+
PE70A	0.33	++	0.89	+	0.62	+	0.20	+	0.62	+	0.20	+
PE76A	≤ 0.54	++++	0.92	+	0.98	++	0.20	+	0.98	+	0.20	+
PE84C	≤ 0.54	++++	0.92	+	0.96	+	0.10	+	0.44	+	0.10	+
PE93A	0.32	+	0	++	0	++	0	++	0	+	0	+

PE93C	≤ 0.4	++++	0	++	0	++	0	++	0	++	0	+
PE102C	0.31	+++	0	++	0	++	0	++	0	+	0	+
PE115A	0.3	+++	0	++	0	++	0	+	0	+	0	+
PE219C	0.33	++	0	++	0	++	0	++	0	++	0	+
PE252C	≤ 0.35	+++	0	++	0	++	0	++	0	++	0	+
PE258C	0.3	+++	0	++	0	++	0	++	0	++	0	+
PE265C	0.3	++++	0	+++	0	+++	0	++	0	+	0	+
PE369A	0.32	++++	0	++	0	++	0	++	0	++	0	+
PE369C	0.3	++	0	++	0	++	0	++	0	+	0	+
PE397C	0.3	++	0	++	0	++	0	+	0	+	0	+
PE402A	0.34	++++	0	++	0	++	0	++	0	++	0	+
PE411A	0.33	++++	0	++	0	++	0	++	0	+	0	+
PE411C	≤ 0.57	++	n	0	n	0	n	0	n	0	n	0
PE430A	≤ 0.62	++	0	+++	0	+++	0	++	0	++	0	+
PE430C	≤ 0.44	++	0	+++	0	+++	0	++	0	++	0	+
PE435A	≤ 0.43	++	0	+	0	+	0	+	0	+	0	+

PE436A	≤ 0.55	++	0	+++	0	+++	0	++	0	+	0	+
PE436C	≤ 0.52	++	0	++++	0	++	0	++	0	++	0	+
PE466C	≤ 0.47	++	0	+	0	+	0	+	0	+	0	+
NCTC 11638	0.31	+++	0	++	0	++	0	++	0	++	0	+

A. Ext, Acetone extract; *W. Ext*, Water extract; *A. Ext Rf*, Retention factor values of the acetone extract components; *W. Ext Rf*, Retention factor values of the water extract components; *d*, degree of inhibition; n, No active component was found; +, Slight inhibition; ++, Good inhibition; +++, Very good inhibition; ++++, Very high inhibition; EMW, ethyl acetate/methanol/water; CEF, chloroform/ethyl acetate/formic acid; HDE, hexane/diethyl ether

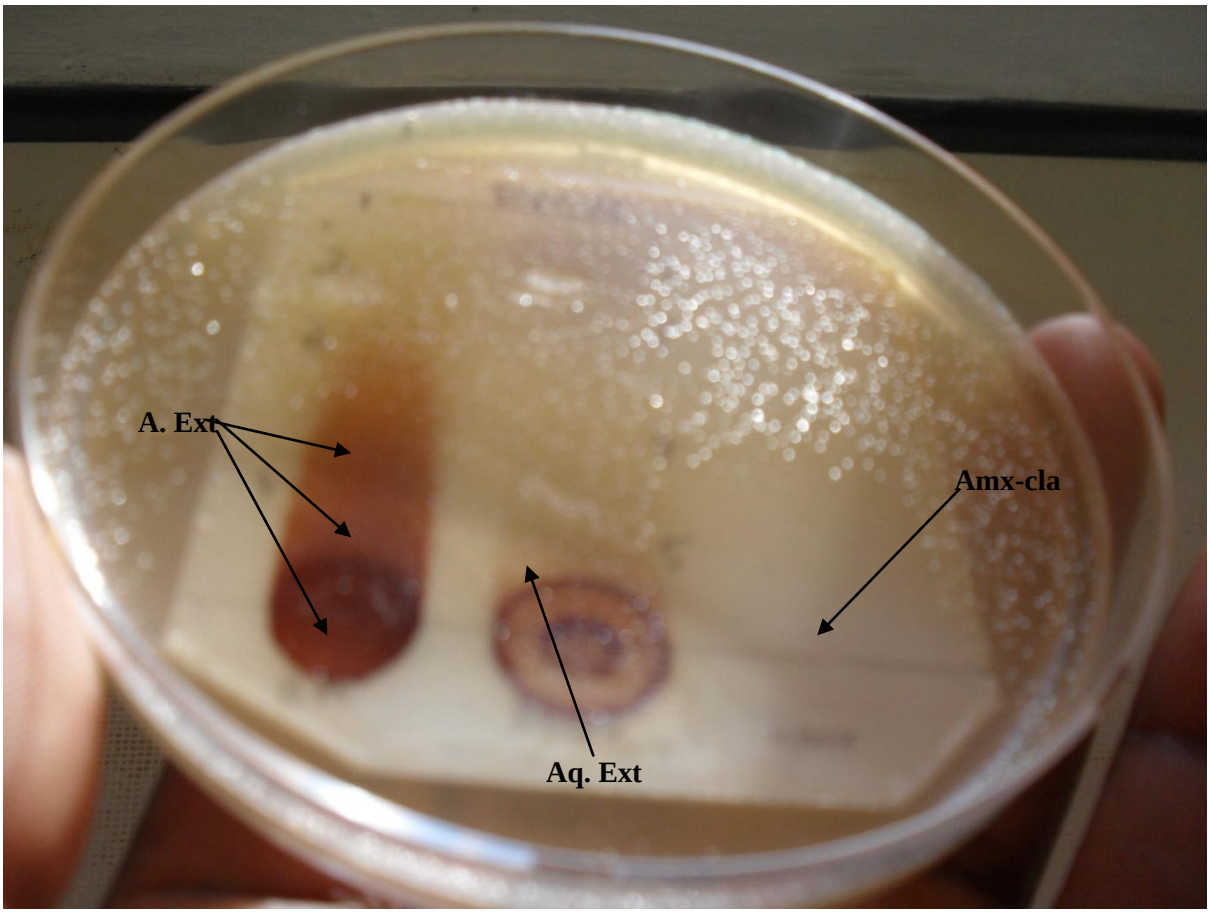

Fig. 5.1: **A typical plate of the agar-overlay bioautography method.** The plate was spotted with the plant crude extracts and developed in a solvent mixture of ethyl acetate/methanol/water (EMW) in the ratio of 20:2.7:2.5. The plate was inoculated with 20 µL of *H. pylori* strain PE 252C. A. Ext, Acetone extract of *S. birrea*; Aq. Ext, Aqueous extract of *S. birrea*; Amx-cla, Amoxicillin-clavulanate (positive control antibiotic). Arrows indicate areas of bacterial growth inhibition. Medium was enriched with 5% serum instead of blood in order to enhance visibility.

5.4 DISCUSSION

Twenty two of the 35 phytocomponents reported in this study were revealed in the acetone extract. Nine (*Rf*; 0.96, 0.8, 0.71, 0.62, 0.57, 0.54, 0.47, 0.44, 0.32) were revealed with the EMW solvent system, 8 (*Rf*; 1.0, 0.98, 0.93, 0.91, 0.53, 0.33, 0.26, 0.18) with CEF and 5 (*Rf*; 0.98, 0.71, 0.67, 0.52, 0.37) with HDE. This shows that acetone extracted a larger amount of compounds from the plant material than water. These findings are in consonance with the observations of Eloff (1998) and other researchers (Masoko *et al*., 2008; Green *et al*., 2010) who observed acetone to be the best in its ability to extract a large variety of compounds from plants.

Using the EMW solvent system led to better separation of the phytocomponents compared to CEF and HDE. Both the acetone and aqueous extracts recorded the highest number of component-separation in the EMW solvent system. The most active antimicrobial component (*Rf*; 0.3) revealed in the acetone extract was also well separated in this system (Table 5.1).

A literature survey indicates that most compounds isolated from the stem bark and pulp of *S. birrea* have been identified as tannins, flavonoids, alkaloids, steroids, sesquiterpene hydrocarbons, ascorbic acid, oleic acid, myristic acid, stearic acid, coumarins, catechins and epicatechins (Ojewole *et al*., 2010). It is likely that some of these compounds may be responsible for the anti-*H. pylori* activity reported herein.

All attempts to grow *H. pylori* on the TLC plate during bioautography analysis were futile due to the very fastidious nature of the organism. We therefore adopted the agar-overlay method of Nalina and Rahim (2007). In order to improve visibility of the zones of inhibition

of the plant active components, Columbia blood agar was enriched with 5% serum instead of blood in order to be able to identify areas of inhibition which for the most part were concentrated somewhere between the bottom and the middle of the plate for the EMW solvent system (Fig.5.1), indicating that most of the anti-*H. pylori* activity is very likely to be present in the polar component of the extract.

Vanillin staining of this component revealed a deep red to ox-blood-coloured compound (*Rf* 0.32). These observations seem to be consistent with the reports of Eloff (2001) and Masoko *et al*. (2008) who reported on the presence of a deep red-coloured component in the acetone extract of *S. birrea*.

Bioautography analysis also revealed that the activity of the aqueous extract components was stronger at very low *Rf* values (Rf 0). This may be an indication that most of the components were not separating well in the solvent combinations employed in the TLC analysis. Although not investigated in this study, some researchers have attributed the activity of plant aqueous extracts to water soluble tannins (Akiyama *et al*., 2001; Funatogawa *et al*., 2004; Kim *et al*., 2008), whch are not readily soluble in hexane, diethyl ether or chloroform used herein as part of the separating system.

Toxicology studies on the aqueous and methanolic extracts of *S. birrea* stem bark carried out on mice and brine shrimp suggest that the plant is relatively safe compared to most other plants (Ojewole *et al*., 2010). In one such study, *S. birrea* extracts gave negative results in the Ames test, suggesting that they are devoid of mutagenic properties (Verschaeve *et al*., 2008).

5.5 CONCLUSION

The activity of various plant components in the acetone and aqueous extracts of *S. birrea* suggest that this plant contains a large number of compounds with anti- *H. pylori* activity, and therefore may constitute a good and readily available source of compounds for the synthesis of new drugs against this notorious pathogen. Most of the antimicrobial components are found in the acetone extract which may account for its antimicrobial activity earlier reported. Generally, the antimicrobial components were more separatable in the EMW solvent system. Our next objective is the column chromatographic separation of the extract and identification of the compounds responsible for activity by gas chromatography/mass spectrometry (GCMS) analysis.

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CHAPTER SIX

VOLATILE COMPOUNDS IN THE STEM BARK OF *SCLEROCARYA BIRREA* (ANACARDIACEAE) POSSESS POTENT ANTIMICROBIAL ACTIVITY AGAINST DRUG-RESISTANT STRAINS OF *HELICOBACTER PYLORI*.

ABSTRACT

The aim of this study was to isolate and identify phytochemicals with anti-*Helicobacter pylori* activity from the stem bark of *Sclerocarya birrea*. The plant crude extract was initially fractionated by silica gel column and thin layer chromatography techniques. Subsequent fractionation and identification of the phyto-constituents was achieved by gas chromatography and mass spectrometry (GC/MS) analysis. The antimicrobial activity of the fractions and compounds was evaluated against 5 metronidazole and clarythromycin-resistant strains of *H. pylori* and a standard control strain (ATCC 43526) using micro-broth dilution technique and time kill assays. Amoxicillin-clavulanate was included in these experiments as a positive control antibiotic. Thirty four of the 38 fractions collected demonstrated anti-*H. pylori* activity with minimum inhibitory concentration (MIC_{90}) values ranging from 310–3750 µg/mL. The most abundant compound was n-octacosane (41.68%), followed by pyrrolidine (38.91%), terpinen-4-ol (38.3%), n-eicosane (24.98%), cyclopentane (16.76%), n-triacontane (16.28%), aromadendrene (13.63 %) and α-gujunene (8.77 %). MIC_{90} (µg/mL) ranges for two of the compounds; terpinen-4-ol and pyrrolidine were 0.04 – 0.12 and 0.01–12.6 respectively, while those for amoxicillin-clavulanate were 0.001 – 0.12. Terpinen-4-ol and pyrrolidine demonstrated time- and dose-dependent bactericidal activity against *H. pylori* with complete elimination of the organisms within 12 hours at a concentration (µg/mL) of 0.32 and 0.8, respectively. Their antimicrobial activity was similar to amoxicillin-clavulanate

(P>0.05). Most of these compounds are being reported in this plant for the first time, thus a new source of therapeutically useful compounds against *H. pylori*.

6.1 INTRODUCTION

Helicobacter pylori colonizes the gastric mucosa of half the world's population (Go, 2002). Infection may result in a number of gastro duodenal pathologies including peptic ulcer, gastric carcinoma and mucosa associated lymphoid tissue (MALT) lymphoma (Kusters *et al*., 2006; Ndip *et al*., 2008). The use of combination therapy involving antibiotics with proton pump inhibitors or bismuth compounds has proven effective in the eradication of *H. pylori* infections (Megraud and Lehours, 2007). However, combination therapy is plagued with undesirable side effects (e.g. epigastric pain, abdominal discomfort, diarrhoea, nausea and vomiting) (Bohr and Malfertheiner, 2009). As a result, patients most often do not complete the treatment course, thus generating suboptimal antibiotic blood concentrations that predispose to the selection and survival of resistant bacterial strains (Njume *et al*., 2009). Equally important is the fact that *H. pylori*-colonization of gastric epithelial cells and mucus gel layer may result in the formation of protective biofilms reported to be more resistant to killing by antibiotics (Cammarota *et al*., 2010). It becomes imperative therefore for combination therapy to be modified with new entries of stronger agents capable of penetrating or dislodging these structures to enhance the efficacy of the therapy.

Antibiotic resistance to commonly used drugs has become a major cause of treatment failure in the eradication of *H. pylori* infections with failure rates of up to 40 % (Lai *et al*., 2006). These factors and others including cost of combination therapy and the non availability of some of the drugs in rural areas (especially in the developing world) have necessitated the search for new prophylactics and therapeutic alternatives (Manyi-Loh *et al*., 2010).

The folkloric use and anecdotal evidence of some plants have shown great promise in the discovery of novel therapies against *H. pylori* infections (Ndip *et al*., 2007; Njume *et al*.,

2011a). One such plant is *Sclerocarya birrea*, a popular medicinal plant amongst the Zulus, Vhavendas, Xhosas and Sothos of South Africa (Masoko *et al*., 2008). The leaves and stem barks of this plant are widely used in treating a plethora of stomach illnesses including gastritis, peptic ulcers and stomach cancer in South Africa and other African countries (Eloff, 2001; Afolayan and Sunmonu, 2010).

Despite its numerous medicinal uses, the activity of this plant has rarely been evaluated against *H. pylori*, a risk factor and causative agent of gastritis, peptic ulcer and stomach cancer (Marshall and Warren, 1983; Ndip *et al*., 2008). An internet search revealed a dearth of information on the antibacterial activity of *S. birrea* and specifically on its anti-*H. pylori* activity. There is a critical need for new therapies against *H. pylori* considering that susceptibility patterns are changing globally and current therapies are rendered obsolete by resistant bacterial strains. Interestingly, our previous studies demonstrated that crude acetone extracts of *S. birrea* are active against *H. pylori* (Njume *et al*., 2011b). It was therefore necessary to identify the active principle in these extracts as a continuation for the search of potent therapies against this notorious pathogen in a bid to circumvent the overall burden of antimicrobial resistance.

6.2 MATERIALS AND METHODS

6.2.1 Bacterial strains

A reference strain of *H. pylori* ATCC 43526 (Rockville, MD, USA) and 5 clinical strains isolated from gastric biopsies of patients presenting with gastro duodenal pathologies at the Livingstone hospital, Port Elizabeth, South Africa were used in this study. Strains were selected from a stock of over 500 stored at -80 ºC (Ilshin®, Model DF 9007, Sanyo, Osaka, Japan) in the Medical Microbiology Laboratory, Department of Biochemistry and Microbiology, University of Fort Hare. The selection was based on their resistance to clarithromycin and metronidazole, two drugs widely used as first line therapies in the eradication of *H. pylori* infections worldwide (Tanih *et al*., 2010). The strains were revived on Columbia Blood Agar (Oxoid, England) supplemented with 7 % defibrinated sheep blood and Skirrow's supplement.

6.2.2 Fractionation of crude extract by Column chromatography analysis

Three 40 cm long x 2.5 cm diameter glass columns were packed to a height of 31 cm with a slurry of silica gel 60 (Merck, Germany; particle size 0.063 – 0.2mm/70 – 230mesh). The columns were equilibrated with concentrated hexane for 30 min. Ten grams of the extract and 20 g of gel were mixed in concentrated hexane to slurry. The mixture was left to dry under a stream of air in a bio safety class II cabinet and loaded on top of the packed columns each to a height of 7.5 cm. The columns were initially eluted with hexane at 45 drops/min and the hexane fractions collected. One of the columns was then eluted with a mixture of hexane/diethyl ether (HDE) in the ratio of 2:1; the other initially with chloroform (C) and subsequently with a combination of chloroform/ethyl acetate/formic acid (CEF) in the ratio of

5:4:1; and the third initially with ethyl acetate (EA) and subsequently with a solvent combination of ethyl acetate/methanol/water (EMW) in the ratio of 20:2.7:2.5 (Masoko *et al*., 2008). A standard eluent volume of 200 mL was collected for each fraction. Thirty-eight fractions were collected, concentrated to dryness in a rotary evaporator and transferred to glass Petri dishes. The dishes were left under a stream of air for 48 hours for complete evaporation of residual solvents. The number of phyto-components in each fraction was checked by running them on TLC plates as previously described (Nalina and Rahim, 2007).

6.2.3 Identification of compounds by gas chromatography and mass spectrometry (GC/MS) analysis

GC/MS analysis was performed on a Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column, 30 m × 0.25 mm i.d., and 0.25 µm film thickness. The sample was introduced through an injector working in the split mode, with helium as the carrier gas, initial flow 0.7 mL per minute and average linear velocity of 32 cm/s. The temperature program was as follows: injector temperature 220 ºC, initial oven temperature was 70 ºC with a purge flow of 30 mL per minute and purge time, 0.2 minutes, ramp 4 ºC per minute. The final temperature was 240 ºC, pressure of 8.27 psi. The compounds were identified by matching their mass spectra and retention indices with those of the Wiley 275 library (Wiley, New York) in the computer library and literature (Adams, 1995). The percentage composition of each compound was calculated from the summation of the peak areas of the total fraction composition.

6.2.4 Determination of 90% minimum inhibitory concentration (MIC_{90}) of fractions and compounds

The MIC of fractions and compounds was determined by micro broth dilution method performed in 96-well plates (Njume *et al*., 2011a). Test fractions were prepared at a concentration of 5.0 mg/mL while terpinen-4-ol and pyrrolidine (Augsburg, Germany) were prepared at a concentration of 2 µg/mL and 200 µg/mL respectively. Two-fold dilutions of each fraction or compound were made in the test wells in brain heart infusion (BHI) broth supplemented with 5 % horse serum and Skirrow's supplement (Oxoid, England). The final concentrations ranged from 2 – 5000 µg/mL, 0.001 – 2 µg/mL and 0.003 – 200 µg/mL for fractions, terpinen-4-ol and pyrrolidine respectively. Twenty microlitres of an 18-hour old broth culture of *H. pylori* (McFarlands turbidity standard 2) suspension was added to 100 µL of fraction or compound-containing culture medium. Control wells were prepared with culture medium only and bacterial suspension and broth only respectively. Amoxicillin-clavulanate was used as a positive control antibiotic and run alongside each batch of tests at a concentration range of 0.0002 – 2 µg/mL. An automatic ELISA microplate reader (SynergyMx, Biotek® USA) adjusted to 620 nm was used to measure the absorbance of the plates after which they were incubated for 72 hours at 37 °C under microaerophilic conditions and the absorbance read again at the same wavelength. The initial and post-incubation absorbencies were compared to detect an increase or decrease in bacterial growth and the values plotted against concentration. The lowest concentration of the fraction or compound resulting in inhibition of 90 % bacterial growth was recorded as the MIC.

6.2.5 Determination of Rate of kill of compounds

The rate and extent of killing of *H. pylori* by terpinen-4-ol and pyrrolidine was determined as described by Akinpelu *et al*. (2009) with modifications. The turbidity of an 18-hour old broth

culture of *H. pylori* was standardized to 10^8 CFU/mL. One millilitre of this suspension was added to 9 mL of BHI broth supplemented with 5% horse serum and Skirrow's reagents in McCartney bottles (Oxoid, England). Terpinen-4-ol, amoxicillin-clavulanate and pyrrolidine were added in the bottles at a concentration (µg/mL) of 0.04, 0.08, 0.16, 0.32; 0.02, 0.04, 0.08, 0.16 and 0.1, 0.2, 0.4, 0.8, respectively. A negative control bottle was prepared with bacterial suspension and broth only. A 0.1-mL sample was plated from these bottles before incubation in an orbital shaker at 37 °C under microaerophilic conditions. Exactly 0.5 mL volume of each suspension was withdrawn at 6-hour intervals for 72 hours and transferred to 4.5 mL of BHI broth recovery medium containing 3 % 'Tween 80' to neutralize the effects of the antimicrobial compound carry-overs from the test organisms. The suspension was 10-fold serially diluted in sterile saline (0.9 % w/v sodium chloride) and plated in triplicates. The plates were incubated at 37 °C for 72 hours under microaerophilic conditions and viable counts determined.

6.2.6 Statistical analysis

The statistical packages used for analysis were Excel (Microsoft, Redmond, WA, USA) and SPSS version 19.0 (2010, SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to compare the mean difference in inhibitory activities of fractions, compounds and antibiotic, followed by Turkey's *post-hoc* test. Differences were considered significant at $P< 0.05$.

6.3 RESULTS

6.3.1 TLC analysis of fractions

According to the TLC analysis of the fractions, there were 2 prominent components in the EMW fractions; a non polar purple-coloured component (R_f: 0.96) that migrated almost at the same speed with the solvent combination, and a polar deep red or ox blood component (R_f: 0.32) that migrated only slightly from the initial point of spotting on the plate and was very much abundant in EMW fractions 3, 4 and 5. When viewed under UV at 365 nm, the non polar component produced a strong bright fluorescence. TLC analysis of the ethyl acetate (EA) fraction1 revealed an abundance of this component.

6.3.2 Chemical and percentage composition of volatile compounds in the stem bark of *S. birrea*

A total of 52 volatile compounds were identified from the fractions; 34 of them from the EA fraction. EMW fractions 3 and 5 were devoid of them. The major volatile compounds detected were n-octacosane (41.68%), followed by pyrrolidine (38.91%), terpinen-4-ol (38.3%), n-eicosane (24.98%), cyclopentane (16.76%), n-triacontane (16.28%), aromadendrene (13.63 %), α-gujunene (8.77 %) and 2, 3-dimethyl pentane (4.22%). Further details of the compound composition are represented below (Table 6.1).

Table 6.1

Volatile compounds isolated from the stem bark of *S. birrea* as revealed by GC/MS analysis

Fraction	Compound	Rt (mins)	Area%
EA fraction 1			
	Terpinen-4-ol	14.61	38.3
	α-Terpineol	14.91	1.66
	β-Caryophyllene	17.11	2.71
	Calarene	18.10	0.3
	Cyclohexasiloxane	19.65	0.2
	α-Gurjunene	22.84	8.77
	Aromadendrene	23.20	13.63
	Tetradecamethyl-cycloheptasiloxane	23.38	0.89
	Naphthalene	24.60	3.94
	δ-Cadinene	25.35	6.37
	Globulol	26.11	1.9
	Epiglobulol	26.51	3.93
	1H-Indene	26.69	0.81
	Viridiflorol	26.92	1.41
	Cadina-1,4-diene	27.38	3.0
	α-Copaene	27.85	1.22
	Octadecamethyl-cyclononasiloxane	31.67	0.12
	Hexadecanoic acid	34.64	0.11

	α-Pinene	9.74	0.22
	Benzene	10.49	0.13
	α-Terpinene	12.00	0.47
	1,8-Cineole	12.44	0.74
	γ-Terpinene	13.20	1.17
	α-Terpinolene	14.06	0.18
	Hexadecanoic acid	34.89	1.14
	9-Octadecenoic acid	38.26	1.72
	Octadecanoic acid	38.52	0.21
	Eicosamethyl-cyclodecasiloxane	34.76	0.08
	3-Octyne	35.77	0.05
	Silane	37.55	0.41
	9-Octadecenoic acid	38.00	0.19
	Ethyl linoleate	38.21	0.05
	Pentasiloxane	40.35	0.04
EMW fraction 6	Pyrrolidine	3.43	38.91
	1,2-benzene-dicarboxylic acid	30.94	3.34
	Hexadecanoic acid	35.16	0.94
	Carbonic acid	38.59	3.60

	Octadecanoic acid	38.92	0.47
HDE fraction 4	Cyclopentane	3.25	16.76
HDE fraction 5	Methyl cyclopentane	3.29	3.61
	4-Methyl-1-pentene	3.30	3.52
	2,3-dimethyl pentane	3.71	4.22
HDE fraction 6	n-Octacosane	40.96	41.68
	n-Triacontane	42.78	16.28
	n-Eicosane	42.64	24.98
CEF fraction 3	Cyclopentane	5.92	9.63
	Toluene	7.79	0.56
	Xylene	10.12	0.54
CEF fraction 4	Toluene	8.37	1.09
	n-Octane	8.89	0.25
	Benzene	10.70	1.30

EA, Ethyl acetate; EMW, Ethyl acetate/methanol/water; HDE, Hexane/diethyl ether; CEF, Chloroform/ethyl acetate/formic acid; Rt (mins), Retention time in minutes; Identification of compounds is based on retention index followed by comparison of mass spectra relative to C_9-C_{28} alkane standard.

6.3.3 Minimum inhibitory concentration (MIC_{90}) of fractions and compounds

Thirty four of the 38 fractions collected demonstrated considerable anti-*H. pylori* activity with MIC(µg/mL) values ranging from 310 – 3750. MIC (µg/mL) values for terpinen-4-ol and pyrrolidine ranged from 0.04 – 0.12 and 0.01 – 12.6 respectively, while those for amoxicillin-clavulanate ranged from 0.001 – 0.12 (Table 6.2). The inhibitory activities of terpinen-4-ol and pyrrolidine were similar to amoxicillin-clavulanate ($P>0.05$).

Table 6.2

Minimum inhibitory concentration (MIC_{90}) of fractions, terpinen-4-ol, pyrrolidine and amoxicillin (µg/mL)

	***H. pylori* strains**					
	PE252C	**PE219C**	**PE102C**	**PE26A**	**PE14C**	**ATCC43526**
F^n/Cpd/Amx-cla						
EA 1	310	310	310	310	310	310
EA2	–	–	–	–	–	–
H	630	630	630	630	630	630
CHL	na	na	na	na	na	na
EMW 1	630	630	310	310	1250	630
EMW2	630	630	630	630	2500	630
EMW3	630	310	630	630	2500	1250
EMW4	630	310	310	310	–	630
EMW5	630	310	310	630	630	310
EMW6	310	630	310	630	310	310
EMW7	630	630	630	630	630	310
EMW8	630	630	630	1250	630	630
EMW9	630	310	630	–	630	630
EMW10	310	310	630	630	630	630
EMW11	630	310	1250	630	630	1250
EMW12	310	310	310	310	630	630
EMW13	310	310	310	310	630	310

EMW14	–	–	–	–	–	–
EMW15	310	630	630	310	310	310
EMW16	310	310	310	310	310	310
HDE 1	-	na	-	na	na	na
HDE 2	3750	3750	3750	-	3750	-
HDE 3	630	630	630	630	1250	630
HDE 4	630	630	630	630	2500	630
HDE 5	630	630	630	630	630	630
HDE 6	630	630	630	630	630	1250
HDE 7	na	na	na	na	na	na
HDE 8	na	na	na	na	na	na
CEF 1	630	630	630	630	630	630
CEF 2	630	3750	-	630	-	-
CEF 3	3750	3750	-	-	-	3750
CEF 4	3750	-	630	630	630	1250
CEF 5	na	-	na	-	na	na
CEF 6	na	na	630	-	na	-
CEF 7	na	3750	na	-	na	na
CEF 8	na	na	na	na	na	na

CEF 9	na	na	na	na	na	na
CEF 10	630	na	na	3750	3750	-
Cpds						
Terpinen-4-ol	0.12	0.04	0.04	0.04	0.04	0.04
Pyrrolidine	12.6	0.01	6.2	6.2	0.1	6.2
Amoxicillin	0.06	0.001	0.04	0.12	0.02	0.06

F^n, fraction; Cpds, Compounds; Amx-cla, Amoxicillin-clavulanate; EA, Ethyl Acetate; EMW, Ethyl acetate/methanol/water; na, no activity; –,Value not within susceptible range. The results shown are mean values of duplicate determinations for each strain.

6.3.4 Bactericidal activity of compounds

Terpinen-4-ol and pyrrolidine exhibited a remarkable bactericidal activity against *H. pylori* at all concentrations tested over 72 hours with the greatest decrease in cell viability occurring between 12 and 36 hours (Figs. 6.1 and 6.2). Both compounds also exhibited an early bactericidal activity with complete elimination of the organisms within 12 hour-exposure at 8xMIC.

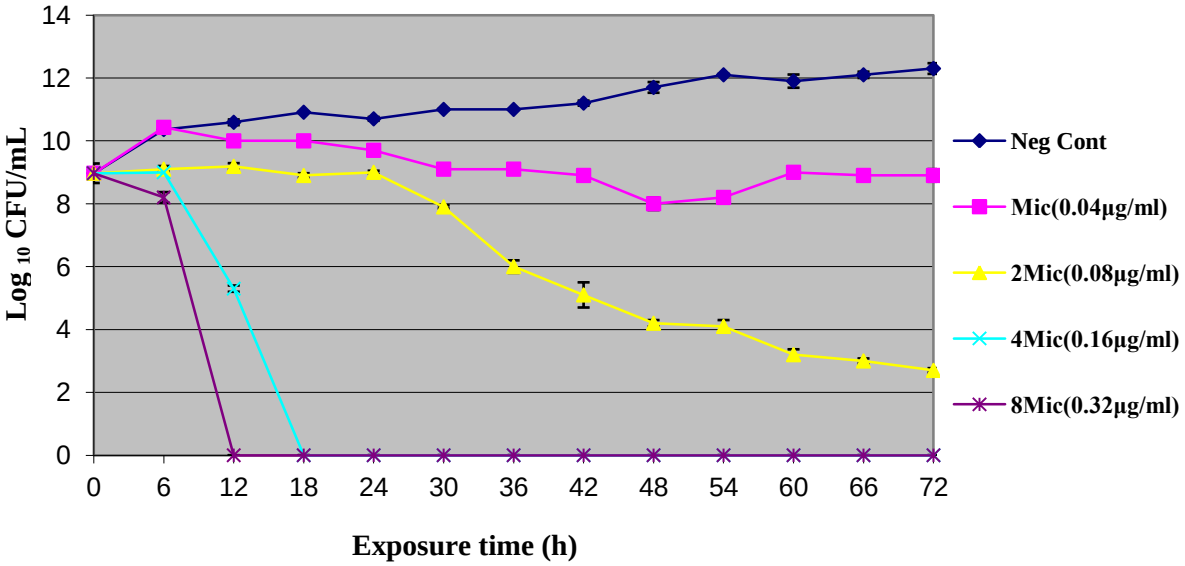

Fig. 6.1: **Effect of terpinen-4-ol on the viability of *H. pylori*, strain PE 14C.**

Neg cont, Negative control (compound-free cells). Data are mean ± SD of viable counts at 6 h-intervals. Error bars represent the standard deviation (SD) of triplicate plate counts. Very small error bars have been obscured by data symbols.

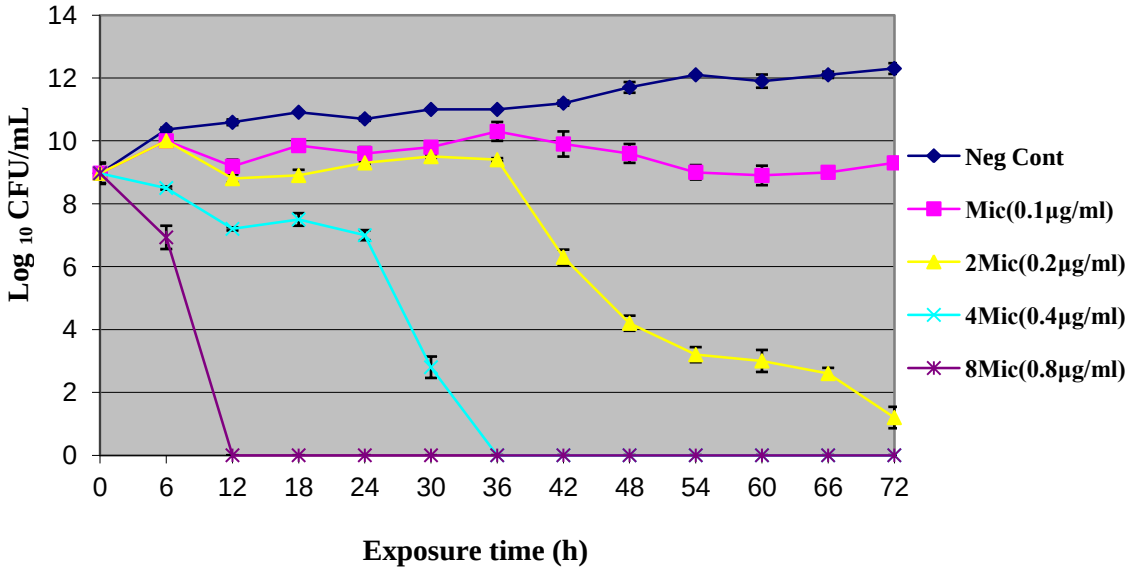

Fig. 6.2: **Effect of pyrrolidine on the viability of *H. pylori*, strain PE 14C.**

Neg cont, Negative control (compound-free cells). Data are mean ± SD of viable counts at 6 hour-intervals. Error bars represent the standard deviation (SD) of replicate experiments (n=3). Very small error bars have been obscured by data symbols.

Amoxicillin was most active at 0.16 µg/mL (8xMIC) concentration with a $3.3\log_{10}$ reduction in bacterial count after 12 hours and complete elimination of the organisms after 18 hours at the same concentration (Fig. 6.3).

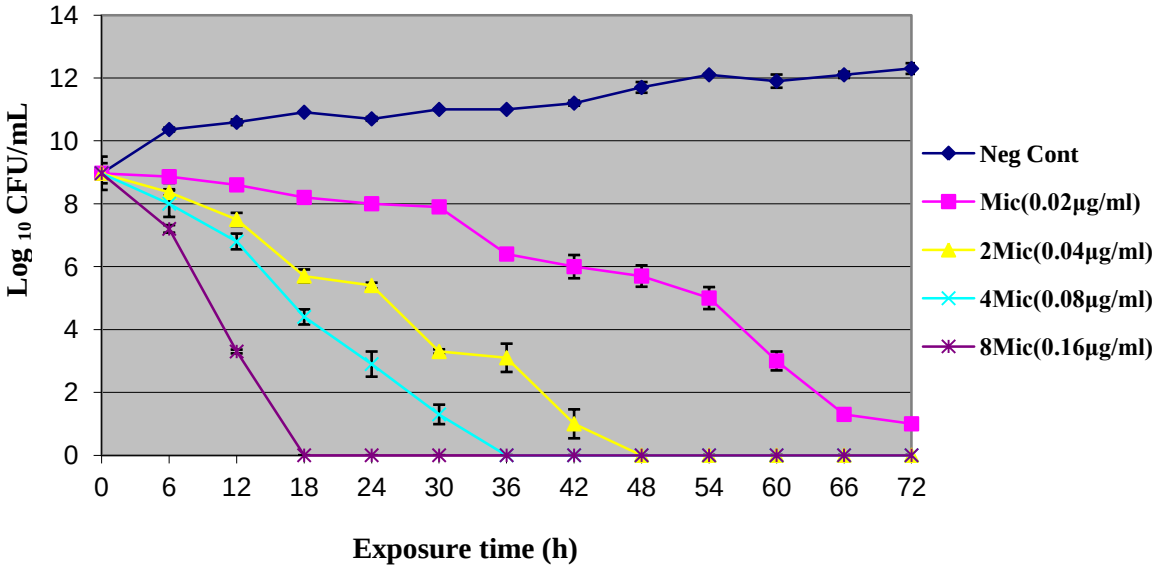

Fig. 6.3: **Effect of amoxicillin-clavulanate on the growth of *H. pylori*, strain PE 14C.**

Neg cont, Negative control (antibiotic-free cells). Data are mean ± SD of viable counts at 6 h-intervals. Error bars represent standard deviation (SD) of replicate experiments (n=3). Very small error bars have been obscured by data symbols.

6.4 DISCUSSION

Excessive use of antibiotics in the treatment of infections is limited by the development of resistant bacterial strains which increase the chances of treatment failure, thus necessitating the search for novel and potent therapeutic agents (Ndip *et al*., 2008; Bohr and Malfertheiner, 2009). The results of this study indicate that the stem bark of *S. birrea* may provide a good and unexplored source of such agents.

Most of the fractions demonstrated good anti-*H. pylori* activity (Table 6.2). However, the low activity demonstrated by some of the fractions could probably be due to insufficient amount of active ingredients as the main aim of fractionation was to isolate the active ingredient. The activity of each fraction is expected to be related to the chemical composition of its components, the proportions in which they are present and their interactions with the test bacteria as earlier suggested (Saidana *et al*., 2008). There is the possibility that individually or synergistically active components might have been disrupted by the solvent combination used during fractionation, and this might also have caused a decrease or lost of activity of some of the fractions or compounds.

Our data suggest that terpinen-4-ol and pyrrolidine may be the major mediators of the antimicrobial activity reported for the acetone crude extract of *S. birrea* shown in previous studies (Eloff, 2001; Masoko *et al*., 2008; Njume *et al*., 2011). The activity of these compounds may have been working alone or synergistically with other unknown compounds present.

Our results are consistent with the reports of Edris (2007) and Loughlin *et al*. (2008) who reported on the strong antimicrobial properties of terpinen-4-ol, an essential oil and oxygenated monoterpine. Even though *H. pylori* was not among the organisms tested in those studies, the antimicrobial activity of terpinen-4-ol demonstrated herein and earlier reports of its anti-carcinogenic properties (Calcabrini *et al*., 2004) can be exploited for the treatment of infections caused by *H. pylori*, a class one gastric carcinogen (Megraud and Lehours, 2007).

The inhibitory activity of terpinen-4-ol in this study was similar to amoxicillin-clavulanate ($P>0.05$), one of the most effective drugs used in the eradication of *H. pylori* infections worldwide especially in South Africa (Tanih *et al*., 2010), indicating that this compound could be used as a possible substitute in the management of drug-resistant *H. pylori* infections. Its activity was significantly different from EMW fractions 4, 5, 10 and 13 ($P<0.05$). This was not surprising considering that TLC analysis revealed that these fractions still had other components, some of which could have a diluting effect on the potency of the active ingredients. The activity of these fractions is therefore expected to improve with purification from these components.

Previous studies on the cytotoxicity of terpinen-4-ol on human fibroblast cells by Loughlin et al. (2008) showed that this compound did not affect the cell viability at concentrations ≤100 µg/mL, thus validating its safety within this range. It is interesting to note that in our study, the compound was active at concentrations <1 µg/mL (Table 6.2). Another study by Itzhak et al. (1996) however reports a much higher inhibitory concentration (2.0 mg per disc) against *H. pylori* (US patent 5560912) using the disc diffusion method. Although not a very suitable

method for a slow-growing bacterium such as *H. pylori*, which requires long incubation periods, and the unstable release of antimicrobial agent from the disk (Megraud and Lehours, 2007), their study and ours highlighted the inhibitory activity of terpinen-4-ol against *H. pylori*.

The antimicrobial activity of terpinen-4-ol is thought to be as a result of its favourable hydrophobic/hydrophilic character, in that it possesses sufficient hydrophilicity to diffuse through surrounding water to the bacterial cell wall, and sufficient hydrophobicity to diffuse to the cytoplasmic membrane where it gets portioned between the lipid bilayer of the membrane causing loss of membrane integrity and leakage of vital cell contents (Carson *et al*., 2002). Its mechanism of action is thus believed to lie in its ability to compromise the structural and functional integrity of the bacterial cell membrane by increasing cytoplasmic membrane fluidity and permeability (Carson *et al*., 2006). Many essential oils have the ability to disrupt or penetrate lipid structures and may show great antibacterial properties in the stomach since they are more active at low pH (Ohno *et al*., 2003). This is very important as there is a critical need for drugs that will remain active in the acidic gastric niche of *H. pylori*.

The percentage composition of terpinen-4-ol (38.3 %) in the stem bark of *S. birrea* in this study is similar to that recently reported in tea tree oil (36.71 %) by Garozzo et al. (2009). To the best of our knowledge, this compound is being reported in *S. birrea* for the first time at such high percentage. It is worth mentioning that because of the good anti-*H. pylori* activity demonstrated by essential oils (Ohno *et al*., 2003; Vila *et al*., 2010), they are being studied with the hope that people with asymptomatic gastritis would benefit from a nutritional

approach to help them manage the infection, and therefore decrease the risk of developing severe pathological conditions (Ohno *et al*., 2003; Mégraud and Lehours, 2007).

Terpinen-4-ol was highly bactericidal against *H. pylori* at concentrations as low as 0.04µg/mL (Fig. 6.1). A consistently stronger bactericidal activity was recorded when this concentration was quadrupled (4MIC) and octupled (8MIC) with complete elimination of the organisms within 18 hours and 12 hours respectively, indicating that the extent of killing is concentration-dependent and more rapid at higher concentrations. These findings seem to be consistent with earlier reports on its antibacterial properties, especially against methicillin-resistant *Staphylococcus aureus* (Carson *et al*., 2006; Loughlin *et al*., 2008).

Another compound of high percentage abundance isolated in this study was pyrrolidine (Table 6.1), a strong basic liquid and secondary amine commonly found in tobacco leaves (Higashio and Shoji, 2004). Some of its derivatives are used in the pharmaceutical industry, especially as modifiers of quinolone antibacterial agents, while others are used as fungicides and insecticides (Higashio and Shoji, 2004; Mital, 2009). Its anti-*H. pylori* activity which was also similar to amoxicillin-clavulanate ($P>0.05$) should therefore not be surprising. However, just like many other plant-derived antimicrobials, toxicological studies of this compound are necessary before any therapeutic application.

Despite the critical need for new anti-*H. pylori* therapies, the isolation of pyrrolidine (a toxic chemical), and naphthalene (an environmental human carcinogen) (Jia and Batterman, 2010) in this study calls for caution in the ethno-medicinal uses of *S. birrea*. Various plant parts

(leaves, stem barks and roots) used as remedies for stomach morbidities in South Africa are considered safe, but this may not always be the case considering that most of their dosages are usually not standardized (Njume *et al*., 2011c).

Viljoen *et al*. (2008) reported β-caryophyllene as the major constituent (91.3 %) in the fruit pulp of *S. birrea*. We equally reported β-caryophyllene, aromadendrene, calarene, δ-cadinene, cadina-1, 4-diene and α-copaene in the stem bark of the same plant (Table 6.1). These compounds share the same chemical formula and even though they were eluted at different times, they seem to be isomers of the same compound. In any case, the compound composition would most likely depend on the nutritional status of the plant, parts used in the analysis, location (regional), age, soil composition and possibly, weather conditions (Ndhlala *et al*., 2007).

6.5 CONCLUSION

The stem bark of *S. birrea* is a promising new source of anti-*H. pylori* compounds; with some of the major volatile constituents being terpinen-4-ol and pyrrolidine, two widely prominent compounds in the pharmaceutical industry. Their anti-*H. pylori* activity demonstrated herein would be useful to this industry as novel agents against this notorious pathogen, or provide a basis for chemical synthesis of active analogues. However, more studies on their toxicity, *in-vivo* potency and mechanism of action against *H. pylori* will shed more light on their potential usefulness.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

7.1 GENERAL DISCUSSION

A paradigm shift in the management of infectious diseases is necessary to prevent antibiotics becoming obsolete, and where possible, alternatives to antibiotics ought to be considered (Carson *et al*., 2006). In this study, we evaluated the anti-*H. pylori* activity of five medicinal plants namely; *Combretum molle*, *Sclerocarya birrea*, *Garcinia kola*, *Alepidea amatymbica* and *Strychnos* species extensively used in the treatment of stomach-related morbidities in South Africa.

The use of these plants in the treatment of stomach problems is made popular with their availability, low cost and the understanding that some of the drugs prescribed in hospitals are derived from plants or modelled after similar products (Patra *et al*., 2009). However, while Western drugs are well defined with isolated active principles formulated into standardized dosages, the plants on the other hand can be regarded as a reservoir of crude drugs which, if properly explored, may lead to the improvement of primary health care, especially in the management of *H. pylori* infections. It is therefore essential to isolate and study medicinally useful plant components considering that valuable medicinal plants are often over exploited and this might lead to extinction. There is the fear that the multitude of potentially useful phytochemical components which could be synthesized chemically may be at risk of being lost irretrievably.

In order to enhance the extraction of the plant active components, we used five different solvents namely; ethyl acetate, acetone, ethanol, methanol and water to exhaustively extract the compounds starting with the least polar of them and ending with water, the most polar solvent. Different solvents generally extract slightly different plant components and the extract yield in each case is different, so too is the activity. The varied level of activity between solvent extracts may not only depend on the type of component but also on the concentration of the active ingredient in the extract. Other researchers have also made these observations (Eloff, 2001; Masoko *et al*., 2008).

Interestingly, all the plant solvent extracts in this study demonstrated considerable antimicrobial activity against *H. pylori* which is not surprising considering that plants have a natural ability to accumulate or produce low molecular weight compounds (secondary metabolites) such as alkaloids, flavonoids, terpenoids, tannins and polyacetylenes which in many cases serve as defence mechanisms against predation by insects, animals and infection by microorganisms (Cowan 1999; Njume *et al*., 2009). It is therefore not surprising that these compounds have been found to exhibit great antimicrobial activity against a wide range of microorganisms, including *H. pylori*.

Of interest is the fact that most organic solvent extracts demonstrated a better activity than the aqueous extracts. Our findings are consistent with the results of other researchers who have also reported on the low antimicrobial activity of plant aqueous extracts (Eloff *et al*., 2005; 2008; Nkomo and Kambizi, 2009). Organic solvents may have a better capacity to penetrate deeply into the plant material and dislodge potentially useful compounds. However, because water is readily available to the traditional doctors and considerably safer than most

organic solvents, most herbal remedies are prepared as decoctions in water (Bessong *et al*., 2005; Njume *et al*., 2011a).

The highest antimicrobial activity in this study was revealed by the acetone extracts of *C. molle* and *S. birrea*. However, it was interesting to note that both aqueous and acetone extracts of *S. birrea* demonstrated similar anti-*H. pylori* activity ($P>0.05$), contrary to the relatively low activity of aqueous extracts recorded with the other plants. Upon further investigations (MIC determination, time kill assay and bioautography), it was evident that the acetone extract of *S. birrea* demonstrated a better activity against *H. pylori*.

Apart from its ability to extract a wide variety of compounds, its high volatility and lack of inhibition of the strains under investigation at the required concentrations made acetone a better solvent to work with. These observations seem to corroborate the findings of Eloff (1998) and Green *et al*. (2010) confirming acetone as the best solvent among many tested in the extraction of antimicrobial compounds from plants. However, *H. pylori* was not among the organisms tested in those studies. To the best of my knowledge, this study appears to represent preliminary evidence of the anti-*H. pylori* activity of the above mentioned plants.

Thin Layer Chromatography (TLC) analysis revealed more components in the acetone extract of *S. birrea* than in the aqueous extract with the most active of the components (*Rf* 0.32) revealed in the acetone extract after bioautography analysis. It was therefore logical to proceed with the acetone extract for further analysis. This is not to say that extracts that demonstrated minimal activity against *H. pylori* are worthless. Studies of such extracts have

demonstrated that they could still be potentially useful sources of biologically active compounds after fractionation and bioassay-guided isolation (Akinpelu *et al*., 2008; Ndip *et al*., 2009). Some of the compounds in such extracts may also be active against other organisms not investigated in this study.

Bioassay-guided fractionation of the acetone extract followed by GC/MS analysis led to the identification of 52 antimicrobial compounds; two of the constituents tested (terpinen-4-ol and pyrrolidine) demonstrated remarkable activity against *H. pylori*. Others such as hexadecanoic acid, 9-Octadecenoic acid, α-terpineol, α-terpinene, γ-terpinene, and 1,8-cineole are well documented for their *in-vitro* antimicrobial activity against a wide range of bacteria as well as some yeast (Carson *et al*., 2006; Kose *et al*., 2007; Garozzo *et al*., 2009). These findings support the traditional medicinal uses of *S. birrea* stem barks in the treatment of gastritis and other stomach-related morbidities in South Africa. However, due to lack of logistics, only the non-polar volatile compounds were identified in this study.

Terpinen-4-ol is an essential oil and volatile monoterpene alcohol (Carson *et al*., 2006; Edris, 2007). It is the principal antimicrobial and most active component of *Malaleuca alternifolia* (tea tree) oil, an Australian native plant (Carson *et al*., 2006; Loughlin *et al*., 2008). However, this compound has also been isolated from many other plants including *Cupressus sempervirens* (cypress), *Juniperus communis* (juniper), *Origanum marjorana* (majoram sweet) and *Lavendula latifolia* (lavender) (Ohno *et al*., 2003; Camp, 2004). To the best of our knowledge it is being reported from *Sclerocarya birrea* for the first time (Njume *et al*., 2011b). Its antibacterial, antimycotic, antiviral and anticancer activities have been widely studied *in-vitro* (Carson *et al*., 2002; 2006; Garozzo *et al*., 2009). Reports on its antibacterial

activity against methicillin-resistant and coagulase negative *Staphylococcus aureus* (MRSA and CoNS) have generated interesting results that have been quite useful to the pharmaceutical and cosmetic industries. The compound is now incorporated in face and hand washes, pimple gels, vaginal creams, foot powders, shampoos and verterinary skin care products (Cox *et al*., 2001).

There is also the possibility that this compound might inhibit respiration by interfering with the functioning of membrane-embeded enzymes in *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* cells considering that it is the main antimicrobial component in tea tree oil that has been reported to inhibit respiration in these organisms (Cox *et al*., 2000). However, its mechanism of action vis-à-vis *H. pylori* is lacking. Nevertheless, its demonstrated activity against *H. pylori* coupled with earlier reports of its anticarcinogenic properties may provide a basis for further evaluation as an alternative treatment option or simply, a chemo-preventive agent in the management of infections due to *H. pylori*.

Some of the fractions that demonstrated anti-*H. pylori* activity in this study were devoid of volatile compounds while others were mostly made of alkanes, many of which to the best of our knowledge are not known to exhibit any antimicrobial activity against *H. pylori*. The major antimicrobial principles in these extracts are most likely to be polar compounds not detectable by GC/MS analysis. More studies would be required to elucidate their identities and chemical structures.

It is worth mentioning that for the last few decades, ethnopharmacological studies have greatly forcused on the search for single ‘active principles’ in plants, with the hope that a single active compound can be easily formulated into a potent therapeutic agent (Eloff *et al*.,

2008). However, traditional doctors usually administer their treatment in the form of decoctions which are composed of numerous constituents with the knowledge that each component is very crucial in potentiating the efficacy of the therapy. They may not be far from the fact as scientist have also realised that plant antimicrobials most of the time seem to be acting in synergism (Ellof *et al*., 2008).

This is supported by the fact that libraries of single bioactive compounds isolated worldwide only yield moderate results (Ulrich-Merzenich *et al*., 2010), which may partially explain the scarcity of plant-derived antimicrobial agents in the pharmacy. However, in this study, we were able to show that a single plant compound demonstrated better anti-*H. pylori* activity than when in the library of other compounds in the crude extract. Therefore, an indication that some of the components in the crude extract may be non active against the target organism, in which case their presence may have a diluting effect on the potency of the active ingredient. Its isolation would consequently lead to improved activity. Equally important is the fact that not all the plant components in the crude extract may act in synergism.The activity of some may be antagonistic to others, thus limiting the activity of the crude extract as a whole.

H. pylori lives in the mucus layer overlying the human gastric epithelium. The bacterial density, pH of gastric juice and location of the organism within the mucosa are important factors that potentially affect drug activity. Therapies must be able to specifically target the organisms by direct inhibition, killing or interfere with their survival mechanisms. For this to be achievable, therapies must remain active in the unique gastric mucus niche of *H. pylori*. This is not possible with some of the drugs in the treatment regimen (e.g. amoxicillin and

clarithromycin) as their activity is reduced at low pH. Terpinen-4-ol has the potential for further studies as an alternative therapeutic agent against this oraganism. Being an essential oil, it has the potential to be more active at low pH and could therefore be of clinical value in the eradication of *H. pylori* from the stomach.

It is likely that with a terpinen-4-ol-based treatment regimen, PPIs, H_2RA, and bismuth compounds may not be very necessary to eradicate the infection. This may also go a long way to reduce the pill burden associated with combination therapy which may in tend improve therapy adherence and consequently therapy efficacy and success. However, considering that novel therapies should be easy to take, easily tolerated and safe, there is a critical need to evaluate the toxicity and *in-vivo* potency of this compound prior to subsequent clinical trials.

Pyrrolidine, a plant alkaloid isolated herein is also worth considering for further investigations as an alternative or complementary adjunct to existing therapies against *H. pylori* infections.

7.2 GENERAL CONCLUSIONS

- Ethno medicinal plants used in the treatment of stomach morbidities in South Africa may represent cheap sources of therapeutically active compounds against *H. pylori* infections. In this study, 17 species belonging to 11 families were identified from the Eastern Cape Province while 3 species belonging to 3 families were identified from the North and Northwest provinces.
- Three of the plants studied herein (*C. molle*, *S. birrea* and *G. kola*) exhibited strong antimicrobial activity against *H. pylori* with zone diameters of inhibition ranging from 0 – 38 mm and minimum inhibitory concentration values (MIC_{50}) ranging from 0.06 – 5.0 mg/mL. The acetone extracts of the stem barks of *C. molle*, *S. birrea* and the ethanolic extract of *G. kola* seeds were strongly bactericidal to *H. pylori*. These results may serve as preliminary scientific validation of their ethno medicinal uses in the treatment of gastritis, peptic ulcer, gastric cancer and other *H. pylori*-related morbidities in South Africa.
- Chromatography methods (TLC, CC and GC/MS) were successfully used to isolate and identify volatile compounds from the acetone extract of *S. birrea*. Two of the compounds (terpinene-4-ol and pyrrolidine) were strongly inhibitory and bactericidal to *H. pylori* with complete elimination of the organisms within 12 hours at 8xMIC. Therefore, terpinen-4-ol and pyrrolidine may be considered for further investigations regarding the development of novel therapies against *H. pylori*.
- Both terpinen-4-ol and pyrroilidine may also serve as templates for chemical synthesis of more active analogues against *H. pylori*.

7.3 RECOMMENDATIONS

- More studies are required to elucidate the mechanism of action of the isolated compounds against *H. pylori*.
- Toxicology studies to evaluate safety parameters of the isolated compounds are necessary prior to clinical trials.
- *In-vivo* studies to determine the therapeutic potential of the isolated compounds would be very necessary prior to subsequent clinical evaluation.

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APPENDICES

APPENDIX 1

ETHICAL CLEARANCES

University of Fort Hare
Together in Excellence

GOVAN MBEKI RESEARCH AND DEVELOPMENT CENTRE
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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 "Genotypes of *Helicobacter pylori* circulating in South Africa: Understanding disease and transmission", 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

ECDoH-Res0002_

Eastern Cape Department of Health

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Date: 12th June 2008
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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

APPENDIX 2

QUESTIONNAIRE

ETHNOBOTANICAL SURVEY OF PLANTS USED IN THE TREATMENT OF *H. PYLORI*-RELATED MORBIDITIES IN THE NKONKOBE MUNICIPALITY OF THE EASTERN CAPE PROVINCE OF SOUTH AFRICA.

Date.....

Number.....

Answer the questions by writing in the space provided.

1. Where do you live?.....
2. What is your occupation?.....
3. Do you use medicinal plants to treat stomach problems?.....
4. If yes, what kind of stomach problems do you treat with medicinal plants?.....
5. What is the name of the plant(s).....
6. Which parts do you used?.....
7. Can you help me get the plant?.....
8. How is it prepared?.....
9. How is it administered?.....
10. What is the dosage?.....
11. How efficient is the treatment?.....
12. Is the medicine safe?.....
13. If yes, how do you know?.....

Thank you for participating in this study.

APPENDIX 3

LIST OF PUBLICATIONS

Njume, C., Afolayan, A.J., Green, E., Ndip, R.N. (2011). Volatile compounds in the stem bark of *Sclerocarya birrea* (Anacardiaceae) possess antimicrobial activity against drug-resistant strains of *Helicobacter pylori*. *International Journal of Antimicrobial Agents*. **38**: 319-324.

Njume, C., Afolayan, A.J., Ndip, R.N. (2011). Aqueous and organic solvent-extracts of selected South African medicinal plants possess antimicrobial activity against drug-resistant strains of *Helicobacter pylori*: inhibitory and bactericidal potential. *International Journal of Molecular Sciences*. **12** (9): 5652-5665.

Njume, C., Afolayan, A.J., Ndip, R.N. (2011). Preliminary phytochemical screening and in vitro anti-*Helicobacter pylori* activity of acetone and aqueous extracts of the stem bark of *Sclerocarya birrea* (Anacardiaceae). *Archives of Medical Research*. **42**: 252 – 257.

Njume, C., Afolayan, A.J., Clarke, A.M., Ndip, R.N. (2011). Crude ethanolic extracts of *Garcinia kola* seeds Heckel (Guttiferae) prolong the lag phase of *Helicobacter pylori*: inhibitory and bactericidal potential. *Journal of Medicinal Food*. **14** (7/8): 822–827.

Njume, C., Afolayan, A.J., Samie, A., Ndip, R.N. (2011). In-vitro anti-*Helicobacter pylori* activity of acetone, ethanol and methanol extracts of the stem bark of *Combretum molle* (Combretaceae). *Journal of Medicinal Plants Research*. **5** (14): 3210-3216.

Njume, C., Afolayan, A.J., Ndip, R.N. (2011). Diversity of plants used in the treatment of *Helicobacter pylori*-associated morbidities in the Nkonkobe municipality of the Eastern Cape province of South Africa. *Journal of Medicinal Plants Research*. **5** (14): 3146-3151.

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Njume, C., Afolayan, A.J., Samie, A., Ndip, R.N. (2011). Inhibitory and bactericidal potential of crude acetone extracts of *Combretum molle* (Combretaceae) against drug-resistant strains of *Helicobacter pylori*. *Journal of Health Population and Nutrition*. **29**(5): 438-445.

Manuscripts in preparation

Njume, C., Afolayan, A.J., Ndip, R.N. *In-vitro* anti-*Helicobacter pylori* activity of crude acetone and aqueous extracts of the stem bark of *Sclerocarya birrea* (A. rich.) Hochst. subsp. *caffra* (Sond.) Kokwaro (Anacardiaceae)

Njume C, Afolayan AJ, Ndip RN. *In-vitro* anti-*Helicobacter pylori* activity of *Alepidea amatymbica* (Apiaceae) and *Strychnos* species (Loganiaceae): medicinal plants used in the treatment of stomach-related morbidities in the Eastern Cape Province of South Africa.

Njume, C., Afolayan, A.J., Ndip, R.N. (2011). Bactericidal activity and marked susceptibility of metronidazole-and clarithromycin-resistant strains of *Helicobacter pylori* to terpinen-4-ol and pyrrolidine.

APPENDIX 4

WORKSHOP AND CONFERENCE PRESENTATION

Njume, C., Afolayan, A.J., Ndip, R.N. (2010). In-vitro anti-*Helicobacter pylori* activity of selected medicinal plants employed in the treatment of stomach-related morbidities in South Africa: inhibitory and bactericidal potential. Presented at the traditional medicine conference, 28-30 July 2010, ICC Durban. Poster and oral presentations.

GMRDC Statistical training workshop on Quantitative research methodology using SPSS (Part 1), 19-23 February 2011, University of Fort Hare, Alice, South Africa.

Njume, C., Afolayan, A.J., Ndip, R.N. (2011). Crude acetone and aqueous extracts of the stem bark of *Sclerocarya birrea* (Anacardiaceae) possess antimicrobial activity against *Helicobacter pylori*. Presented at the South Africa Society for Microbiology (SASM) conference, Southern Sun Cape Sun Hotel, Cape Town, South Africa**.** 6-9 November, 2011. Poster presentation.