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**ANTIBACTERIAL AND PHYTOCHEMICAL STUDIES OF SELECTED SOUTH
AFRICAN HONEYS ON CLINICAL ISOLATES OF *HELICOBACTER PYLORI***

BY

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**A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE
AWARD OF THE DEGREE OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY**

**FACULTY OF SCIENCE AND AGRICULTURE
DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY**

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APRIL, 2012

DECLARATION

I, Christy E. Manyi-Loh declare that the thesis for the award of a Doctor of philosophy degree in Microbiology at the University of Fort Hare, hereby submitted by me, has not been previously submitted for a degree at this or any other university and that it is my original work in design and execution, and that all the reference materials contained therein have been appropriately acknowledged.

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DEDICATION

This work is dedicated to my;

Beloved husband, Mr. Stephen Loh Tangwe

Lovely and wonderful kids, Alexia-Augusta and Gad-Manuel Loh

Precious mother, Ms. Cecilia Ntongho Echakachi

ACKNOWLEDGEMENTS

My immense gratitude to God Almighty for the knowledge, wisdom, and guidance and above all, love bestowed upon me during this period of my life. If not of Him I wouldn't be where I am because He is my strength and at any point in time that I thought I was down to nothing God was up to something. I am equally very grateful to my supervisor, Dr. Anna M. Clarke and co-supervisor, Prof. Roland Ndip, for the conception and design of this study, their close supervision as well as their immeasurable suggestions that culminated to the success of this work. I will forever be grateful.

Special thanks to Govan Mbeki Research and Development Centre, University of Fort Hare, Alice for their financial support and also to the Department of Biochemistry and Microbiology for the logistics made at my disposal. Also, a big thank you to, Dr. Green Ezekiel for imparting me with knowledge on packing of the glass column and Dr. Mkwetshana Noxolo for her positive suggestions during the research group meetings. Not leaving out the assistance granted to me by the members of the Microbial Pathogenicity and Molecular Epidemiology Research Group (MPMERG), I equally appreciate it.

My sincere gratitude goes to relatives and friends, the Echakachis', Tangwes', Issas', Ngandas', Longkongs', Lifongos' and Ms. Maria Smith, Ms. Munzhelele Tsillivhali for their inspiration, words of encouragement and prayers. May God bless them abundantly and also those who have contributed in one way or the other to the success of this project.

REMAIN BLESSED IN JESUS NAME, AMEN!!!

LIST OF ABBREVIATIONS

Abs	Antibodies
Ags	Antigens
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
<i>Cag A</i>	Cytotoxin-associated gene
CBA	Columbia Blood Agar
CFU/mL	Colony Forming Units per Millilitre
CLO	<i>Campylobacter-like</i> organism
DNA	Deoxyribonucleic acid
DMSO	Dimethyl Sulphoxide
ELISA	Enzyme Linked Immunosorbent Assay
EtOH	Ethanol
FISH	Fluorescent <i>in situ</i> Hybridization
G+C	Guanine plus cytosine
GC-MS	Gas Chromatography linked Mass Spectrometry

GERD	Gastroesophageal Reflux Disease
GIT	Gastrointestinal Tract
HE	Hematoxylin-eosin
HMF	Hydroxymethylfurfural
<i>H. pylori</i>	<i>Helicobacter pylori</i>
Ice A	Induced by contact with epithelium factor Antigen
IL	Interleukin
Ig G	Immunoglobulin G
iNOS	Inducible Nitric Oxide Synthetase
MALT	Mucosa-associated lymphoid tissue
MBC	Minimum Bactericidal Concentration
Mg/mL	Milligram per millilitre
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin Resistant <i>Staphylococcus aureus</i>
PCR	Polymerase Chain Reaction
PPI	Proton Pump Inhibitor
RBC	Ranitidine Bismuth Citrate
Rf	Retention Factor

rRNA	Ribosomal Ribonucleic Acid
Rpm	Revolutions per minute
SPSS	Statistical Package for Social Sciences
TLC	Thin Layer Chromatography
UBT	Urea Breath test
<i>Vac A</i>	Vacuolating cytotoxin gene
VOC's	Volatile Organic Compounds

GENERAL ABSTRACT

Infection with *Helicobacter pylori* has been associated with the pathogenesis of numerous stomach and gastroduodenal diseases that pose threats to public health. Eradication of this pathogen is a global challenge due to its alarming rate of multidrug resistance. Consequently, to find an alternative treatment, the search is increasingly focused on new antimicrobial product from natural sources including honey. Honey has been used as medicine in several cultures since ancient time due to its enormous biomedical activities. Its beneficial qualities have been endorsed to its antimicrobial, antioxidant, anti-inflammatory properties added to its phytochemicals. In this study, the anti-*H. pylori* activity of South African honeys and their solvent extracts as well as the phytochemicals present in the two most active honeys were evaluated. Agar well diffusion test was used to investigate the antimicrobial activity of six honey varieties obtained from different locations in the country. Subsequently, the honeys were extracted with four organic solvents viz n-hexane, diethyl ether, chloroform and ethyl acetate employed in order of increasing polarity. The antibacterial activity of the different solvent extracts of each honey was evaluated by agar well diffusion; broth micro dilution and time kill assays. Different chromatographic techniques (Thin layer & column chromatography) were employed to enumerate the phytochemical constituents in the most active solvent extracts of Pure Honey (PH) and Champagne Royal Train (CRT); and were identified by gas-chromatography linked mass-spectrometry. Linalool pure compound was equally evaluated for anti-*H. pylori* activity in a bid to trace the antibacterial agent among the variety of compounds identified. Data were analyzed by One-way ANOVA test at 95% confidence interval. Crude honeys and their solvent extracts demonstrated potent anti-*H. pylori* activity with zone diameter that ranged from [16.0mm (crude) to 22.2mm (extract)] and percentage susceptibilities of test isolates between 73.3% (crude) and 93.3% (extract). The chloroform extracts of PH and CRT were most active with MIC₅₀ in the ranges 0.01-

10%v/v and 0.625-10%v/v respectively, not significantly different from amoxicillin ($P > 0.05$); and efficient bactericidal activity (100% bacterial cells killed) at 1/2MIC and 4xMIC over different time intervals, 36-72hrs and 18-72hrs respectively. The appearance of bands on the thin layer chromatography (TLC) chromatogram spotted with the chloroform extracts of PH and CRT; and developed with hexane: ethyl acetate: acetic acid (HEA) and methanol: acetic acid: water (MAAW) solvent systems indicated the presence of compounds. Purification of the compounds contained in these extracts over silica gel column yielded numerous fractions which were evaluated for antibacterial activity and purity. PHF5 was the most active fraction with a mean MIC₅₀ value of 1.25mg/mL. Volatile compounds belonging to different known chemical families in honey were identified in all the active fractions obtained from PH. Conversely, only four compounds were identified in the active fractions obtained from CRT hence the non volatile constituents could be of prime relevance with respect to antibacterial activity of this honey. Of novelty was the presence of thiophene and N-methyl-D3-azirdine compounds, essential precursors used for the synthesis of natural products and pharmaceuticals with vital biomedical properties. Linalool demonstrated potent inhibitory (MIC₉₅, 0.002- 0.0313mg/mL) and bactericidal activity (0.0039-0.313mg/mL) against the test isolates. On the other hand, a significant difference was recorded ($P < 0.05$) in comparing the activity of linalool compound to the fractions. PH could serve as a good economic source of bioactive compounds which could be employed as template for the synthesis of novel anti-*H. pylori* drugs. However, further studies are needed to determine the non volatile active ingredients in PH and CRT as well as toxicological testing.

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CHAPTER ONE

GENERAL INTRODUCCION

1.1 INTRODUCTION

The human gastric pathogen *Helicobacter pylori* (*H. pylori*), is a spiral, Gram-negative and microaerophilic bacterium that colonizes the stomach of more than 50% of the world's population and can persist for a lifetime if not completely eradicated (Ahmed and Sechi, 2005). It is one of the most genetically diverse of all bacterial species and has been incriminated as the main etiological agent in many diseases of the upper gastrointestinal tract (GIT) such as chronic gastritis, gastric and duodenal ulcers and as a risk factor for mucosa-associated lymphoid tissue lymphoma as well as adenocarcinoma (Appelmeik *et al.*, 2000).

However, majority of the infected patients remain asymptomatic; with only a small subset of infected people experiencing *H. pylori*-associated illnesses, in view of the fact that the outcome of infection depends on a variety of bacterial, host genetic and environmental factors that mostly relate to the pattern and severity of gastritis (Krusters *et al.*, 2006). Several *H. pylori* virulence genes have been identified, of these, *oipA*, *vacA*, *cagA* and *babA* appear to play a major role in pathogenicity (Yilmaz *et al.*, 2006).

The prevalence of *H. pylori* shows geographical, age and socio-economic variations, with rates ranging from 20% - 50% in developed countries and over 80% in the developing countries. In Western countries, *H. pylori* infection is on a steep decline mainly because of the success of combination therapies and improved personal hygiene and community sanitation to prevent re-infection (Ahmed and Sechi, 2005). On the other hand, in developing countries, infection is usually acquired in childhood and is associated with low socio-

economic status, overcrowding, poor sanitation and unclean water supplies (Feldman, 2001; Dube *et al.*, 2009a). *H. pylori* has been reported to be ubiquitous in Africa. Ndip *et al.* (2008a) conducted a study in Cameroon and documented a high prevalence of 92.2%. Interestingly, the organism has been reported to be prevalent in South Africa; Samie *et al.* (2007) reported an *H. pylori* prevalence of 50.6% in their study in Venda, North of the country. Equally, Dube *et al.* (2009b) presented a high prevalence (85.6%) of *H. pylori* antigens in fecal samples obtained from asymptomatic individuals in the Nkonkobe municipality, in the Eastern Cape Province of South Africa. Moreover, Tanih *et al.* (2010) reported a prevalence of 66.1% in patients who presented with gastric-related morbidities at Livingstone Hospital, Port Elizabeth, in the Eastern Cape Province of the country.

H. pylori is thought to be indigenous to the human population and is well adapted to existing in the gastric mucosa, so humans are the major reservoir. While it exists as normal flora in some people, in others it causes serious clinical manifestations. In spite of its implications in the above-mentioned diseases and its high prevalence rate worldwide, available literature states that the mode of its transmission is poorly understood (Suzuki *et al.*, 2008); although, person-to-person transmission is suggested either by fecal-to-oral, oral-to-oral and gastric-to-oral routes (van Duynhoven and de Jonge, 2001; Asrat *et al.*, 2004) in the general population.

Due to the public health importance of *H. pylori* since its discovery and coupled with its role in the pathogenesis of many stomach diseases, effective diagnostic methods are mandatory. The available methods could be classified as invasive or non invasive tests (Mégraud and Lehours, 2007). Invasive tests are biopsy-based tests, thus require endoscopy and include the biopsy urease test, histology, fluorescent *in situ* hybridization, culture and polymerase chain

reaction (PCR) (Liu *et al.*, 2008). To evade endoscopy, non invasive tests such as ¹³C-urea breath tests, serology and stool antigen enzyme immunoassay tests have been used in various clinical setting to detect the pathogen (Oderda *et al.*, 2000). These different diagnostic procedures employed in the identification of *H. pylori* are usually associated with different degrees of specificity as well as sensitivity.

Strategies to limit the burden of *H. pylori* infections and its complications are needed, and prevention by treatment of *H. pylori* infection at childhood has been suggested. Conventional treatment of *H. pylori* infections is by triple therapy which composes of a PPI plus two antibiotics (i.e. clarithromycin or amoxicillin with metronidazole); that has been accepted as the first-line treatment of choice (Hardin and Wright, 2002) and administered for 7-14days. Eradication of the bacterium from the foregut grades to effective healing of ulcers (Meurer and Bower, 2002), prevents ulcer relapse (Ables *et al.*, 2007), reduces recurrence of gastric cancer (Lee *et al.*, 2008) and potentially decreases the risk of progression to gastric carcinoma (Bytzer and O'Morain, 2005; Ndip *et al.*, 2008a). On the other hand, there are shortcomings associated with treatment regimens for the bacterium's eradication; some of which have been presented elsewhere (Manyi-Loh *et al.*, 2010).

These conundrums go beyond finding the correct combination of antibiotics and manipulation of gastric pH to ensure eradication and include avoiding the development of antimicrobial resistance and ensuring compliance with prescribed treatment (O'Connor *et al.*, 2009). Accordingly, the standard 7-14 days triple therapies fail to eradicate *H. pylori* infections in up to 20-25% of patients (Fuccio *et al.*, 2007).

Many *H. pylori* strains show resistance to the limited range of antibiotics used in its treatment *in vitro*. Antibiotic resistance of *H. pylori* strains is prevalent world-wide and varies widely by geographic location and socio-economic status (Alarcón *et al.*, 1999). Moreover, *H. pylori* has been reported to develop resistance to clarithromycin, a key antibiotic in the triple therapy as well as metronidazole an important antibiotic used in its treatment (Mégraud, 2004). The prevalence of such resistant strains is highest in the developing countries. In South Africa, Tanih *et al.* (2010a) documented a resistance rate of 95.5% for metronidazole among the isolates circulating in the Eastern Cape Province. In addition, there is emerging resistance of the bacterium to tetracycline, fluoroquinolones, and rifampicins, which are alternative antibiotics with known anti-*H.pylori* activity (Mégraud, 2004).

Therefore, there is the need to seek alternative compounds from other sources with proven antimicrobial activity. This has led to the search of active antibacterial agents from natural products, with the aim of discovering potentially useful active ingredients that can serve as source and template for the synthesis of new antimicrobial drugs (Aibinu *et al.*, 2002). Several authors have documented the anti-*H. pylori* activity of different natural products; plants (Zhang *et al.*, 2005; Zeyrek and Oguz, 2005; Ndip *et al.*, 2008b; Zaidi *et al.*, 2009;), probiotics (Lesbros-Pantoflickova *et al.*, 2007) and honey (Nzeako and Al-Namaani, 2006; Ndip *et al.*, 2007).

More recently, there is renaissance of interest in the use of honey for treatment of microbial infections and it constitutes a potential source of new compounds that may be useful in the management of *H. pylori* infections (Manyi-Loh *et al.*, 2010). Honey is the natural sweet substance produced by honeybees from plant nectar, plant secretions and from excretions of plant sucking insects (aphids) (Williams *et al.*, 2009). It has been consumed since ancient

times as a high nutritive value food distinguished by its characteristic aroma and pleasant sweet taste. The antimicrobial potential of honey has been endorsed to its high osmolarity, low acidity, hydrogen peroxide content as well as non-peroxide components.

Notwithstanding, hydrogen peroxide is the major antibacterial factor in honey, produced by glucose oxidase enzyme from the oxidation of glucose in the presence of water (French *et al.*, 2005). On the other hand, the non-peroxide components (phytochemicals) which are derived from plant or bee constitute of flavonoids, non flavonoid phenolic compounds and volatiles. Volatile organic compounds in honey originate from numerous biosynthetic pathways (Guyot-Declerck *et al.*, 2002) and could be classified as hydrocarbons, alcohols, aldehydes, ketones, acids, norisoprenoids, benzene derivatives, terpenes and its derivatives, furan and pyran derivatives (Barra *et al.*, 2010).

Ndip *et al.* (2007) documented the anti-*H. pylori* activity of four honey varieties (Mountain, Manuka™, Capillano®, Eco-honey) obtained from different geographical locations. The strongest inhibitory activity was demonstrated by Mountain honey with MIC and MBC concentrations in the ranges 0.117 - 0.938 µg/mL and 0.366 - 2.965 µg/mL respectively. This preliminary study confirmed the use of these honeys in folk medicine and provides the basis for a more detailed evaluation.

However, it is evident that honeys obtained from different floral sources, different regions and countries vary in their antimicrobial potency due to differences in their chemical composition which is greatly influenced by the prevailing climatic conditions and soil characteristics in the different geographical areas (Castro-Várquez *et al.*, 2010). So, plant

originated bioactive components can be transferred to honey by the foraging bees (Baltrusaitytė *et al.*, 2007). Even honeys obtained from the same floral source but in different geographical locations have shown variation in their chemical composition therefore, could influence their antimicrobial activity. Undoubtedly, the chemical composition of honey depends on the floral source used to collect nectar, seasonal and environmental factors, as well as processing and storage conditions (Yao *et al.*, 2003).

As a result, a survey of honey varieties obtained from the vast floral biodiversity in South Africa, might unearth a honey with good antibacterial activity against this medically important pathogen. Against this background, the present study sought to evaluate the antibacterial and phytochemical properties of selected South African honeys including Pure Honey (PH), Champagne Royal Train (CRT), Goldcrest (GC), Citrus Blossom (CB), Honeyline (HL) and Heritage (HH) on clinical isolates of *H. pylori* obtained from patients with gastric related pathologies attending Livingstone Hospital, Port Elizabeth, in the Eastern Cape Province of South Africa.

1.2 Rationale

H. pylori infection is one of the most chronic bacterial infections of mankind (Ndip *et al.*, 2004). Several studies have related a high prevalence of this pathogen to low socio-economic status, a situation common in the Eastern Cape Province in particular and other areas of the country in general. Its infections have been shown to have a high incidence in South Africa, being present in both asymptomatic and symptomatic individuals, as expected in developing countries (Dube *et al.*, 2009b; Tanih *et al.*, 2010a). Unsuccessful eradication of the pathogen (failure rates between 10 & 25%) (Suerbaum and Michetti, 2002) as well as increase in its

resistance to antibiotics implemented in the triple therapy is a public health issue of global, national and local dimension.

Furthermore, undesirable side effects of the drugs, poor patient compliance and the significant cost of combination therapy are added challenges. Also, some of the key antibiotics are at times not readily available in rural areas thus in addition compounding the problems for patients. Moreover, immunization of individuals which could be the best method of prevention of this infection at childhood, the age at which most infections start is still pending. These challenges associated with significant levels of treatment failure and contraindications for some patients therefore, require the exclusive need to search alternative approaches of eradicating or preventing *H. pylori* infections (O’Gara *et al.*, 2000).

Phytomedicine has proven to be an unexploited treasure for the discovery of model compounds to cure different diseases including gastrointestinal (GI) disorders (Thompson and Ernst, 2002). This has led to the re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products including honey. At present, a number of honeys are sold with standardized levels of antibacterial activity. For instance, Manuka and Capillano produced in Australia are sold as therapeutic honeys suitable for use in ulcers, infected wounds and burns (Lusby *et al.*, 2005).

Therefore, the search for other honeys with antimicrobial activity from different regions and countries continues. The observation that honey in New Zealand and Saudi Arabia at concentrations approximating 20% v/v can inhibit the growth of *H. pylori in vitro* and the

finding that Medihoney and Manuka honeys have been shown to have *in vivo* activity and are suitable for use in ulcers, infected wounds and burns are important findings (Lusby *et al.*, 2005), which could be exploited clinically.

Consequently, several studies have been focused on the anti-*H. pylori* activity of honey and their solvent extracts in different parts of the world (Ndip *et al.*, 2007; Nzeako and Al-Namaani, 2006). Nonetheless, to the best of my knowledge there is no existing data in the literature on the antibacterial activity of honey on clinical isolates of *H. pylori* circulating in South Africa, more especially since this organism and its antibiotic resistant strains have been reported to be prevalent in the country (Mosane *et al.*, 2004; Samie *et al.*, 2007; Tanih *et al.*, 2010b). According to Pubmed there is no evidence of such study.

Owing to the fact that the pathogen exhibits profound heterogeneity and that variation occurs in the antimicrobial activity of honey obtained from different regions and countries (Basson and Grobler, 2008; Manyi-Loh *et al.*, 2010), it becomes necessary to evaluate the antibacterial activity of selected honeys and their solvent extracts on clinical isolates of *H. pylori* circulating in South Africa in an attempt to identify new lead molecules, which could be used in the development of new drugs against the pathogen to circumvent the problem of increasing drug resistance. These new organic molecules with antibacterial activity, which could be inexpensive and readily available to the local population would therefore lead to an improvement in primary health care.

1.3 Hypothesis

Honey can provide potent and affordable antibacterial agents for the treatment of *H. pylori* infections.

1.4 Objectives

1.4.1 Overall objective

To validate scientifically the use of honey in folk medicine.

1.4.2 Specific objectives

- i) To screen the honey varieties for anti-*H. pylori* activity.
- ii) To extract the components responsible for this activity.
- iii) To determine the Minimum Inhibitory Concentration (MIC) of honey extracts displaying anti-*Helicobacter* activity.
- iv) To ascertain the rate of kill of these active honey extracts.
- v) To isolate and purify the active compounds from the selected honey extracts.
- vi) To determine the volatile constituents in the active fractions of selected honey extracts.
- vii) To identify the volatile compounds responsible for anti-*H. pylori* activity in the most active solvent extracts.

1.5 Chapter overviews

This thesis comprises of six chapters. Chapter one represents a general introduction to the topic of the thesis.

Chapter two; literature review. It encompasses materials on the physiology and growth conditions of *H. pylori*, its genes and genomes, pathogenesis and clinical manifestations,

diagnosis of *H. pylori* infections, epidemiology, and treatment (conventional), factors implicated in treatment failure, antimicrobial resistance, prospective natural remedies and an overview of honey as an alternative source of treatment for *H. pylori* infections.

Chapter three covers the general antibacterial activity (inhibitory) of all the honeys studied and their solvent extracts. Pure Honey (PH), Citrus Blossom (CB) and Goldcrest (GC) honeys were most active at 75% v/v concentration whilst Champagne Royal Train (CRT), Heritage Honey (HH) and Honeyleine (HL) honeys were most active at 50% v/v concentration. The four most active honeys i.e. PH, GC, CRT & HL were subjected to solvent extraction and the resulting solvent extracts were equally evaluated for their anti-*H. pylori* activity.

Chapter four focuses on the bactericidal activity (time kill assay) of the most active solvent extract of PH, GC, CRT and HL honeys that were previously reported as the most active honeys. These included the chloroform extract of PH, n-hexane extract of GC, chloroform extract of CRT and the ethyl acetate extract of HL.

Chapter five contains materials on the phytochemical studies of the chloroform extract of the two most active honeys at different concentrations (i.e. PH at 75% v/v and CRT at 50% v/v concentration) via thin layer chromatography, column chromatography and identification of volatile compounds by gas chromatography linked mass spectrometry. However, each of the above-mentioned chapters has its own introduction, materials and methods, results, discussion, conclusions and references. The chapters were arranged in accordance with the order of the bioassays.

Chapter six assembles the general discussion, conclusions and recommendations.

1.6 References

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CHAPTER TWO

LITERATURE REVIEW

2.1 Physiology and growth conditions of *H. pylori*

In 1984, Marshall and Warren isolated a curved bacterium from the stomach of patients with gastritis and peptic ulcer and classified it under the genus *Campylobacter*. Later, this bacterium was placed under the genus *Helicobacter* due to differences in flagellation, cell wall composition and antimicrobial susceptibility, presence of sheath, urease enzyme and bulb as well as formation of glycocalyx *in vitro* (Velázquez and Feirtag, 1999).

H. pylori is a Gram-negative, S-shaped or curved rod-like bacterium with 1 to 3 turns, 0.5 x 5µm in length, with a tuft of 5 to 7 polar sheathed flagella. However, the bacterium could exist in coccoid forms owing to prolonged incubation or adaptation to marginal or hostile environment (Andersen *et al.*, 1997). The response to altered environment leads to a change in morphology, metabolism and growth behaviour. Coccoid forms have been implicated as possible reservoirs of bacteria exposed to harsh conditions and may be involved in transmission of infection or as *in vivo* cells could be responsible for treatment failures (Worku *et al.*, 1999).

Coccoid forms of *H. pylori* are viable but non-culturable and can revert to the spiral morphology and re-grow once an appropriate environment is encountered (Shirai *et al.*, 2000). Generally, it is considered that the spiral morphology and flagella are essential for colonization of gastric and intestinal mucus as well as for pathology. In addition, *H. pylori* is a fastidious micro-organism that requires complex growth media supplemented with blood or serum for its growth in the presence of O₂ levels of 2-5% but with an additional need of 5-

10% CO₂, high humidity (96-100%) (microaerophilic environment) and a pH near 7.0 at a temperature of 30-37°C. It is equipped against oxidative stress with enzymes such as superoxide dismutase, peroxidases and highly active catalase (Kelly *et al.*, 2001). Besides, it is urease, catalase and oxidase positive, characteristics which are often explored for identification by biochemical testing (Krusters *et al.*, 2006). Urease enzyme appears to be vital for the organism's survival and colonization; its activity is clinically important since it forms the basis for several invasive and non invasive tests to diagnose infection (Weeks *et al.*, 2000).

2.2 Genes and Genome

H. pylori possesses a small, circular, open pan-genome with plasticity zones, a special cluster of DNA rearrangements that protects the essential complement of genes by acting as a bed for foreign DNA insertion or abrogation (Fischer *et al.*, 2010). The plasticity region encodes the type IV secretion system involved in the pathogenesis of this bacterium (Ahmed and Sechi, 2005). Israel *et al.* (2001) discovered that the organism undergoes microevolution and give rise to sub-species during prolonged colonization in a single host. Also, genetic heterogeneity occurs with strains evolving separately of one another and coupled with the fact that the organism is genetically diverse, distinct genotypes have been found to be associated with particular geographic regions (Kersulyte *et al.*, 2000).

Accordingly, *H. pylori* is known as one of the most genetically diverse species due to its high mutation rates and frequent recombination events (Fischer *et al.*, 2010). In addition to its natural transformation competence, conjugative transfer of genomic islands has to be considered as an important source of genetic diversity (Fischer *et al.*, 2010). The diversity may facilitate adaptation of the bacterium to its new hosts and to the distinct patterns of the

host-mediated immune response as well as persistence for decades despite a changing gastric milieu, consequently contributing to the high prevalence of the infection worldwide (Kivi *et al.*, 2007).

Furthermore, the genome sequence of two different patients isolates (J99 and 26995) has been characterized and compared (Tomb *et al.*, 1997; Alm *et al.*, 1999). The genome of the strain 26695 includes 1,587 genes whereas the genome of strain J99 comprises of 1,491 genes, both with a G + C content of 35- 40%. Notwithstanding, *H. pylori* genome has an approximately 40kb long Cag pathogenicity island which contains over 40 genes, some of which codes for the complex type IV secretory system. Also, the genome contains two copies of 16S, 23S and 5S rRNA genes which are known as house-keeping genes (Baldwin *et al.*, 2007).

Several virulent genes have been described in *H. pylori* such as cytotoxin-associated gene (*cag A*), vacuolating cytotoxin (*vacA*), the induced by contact with epithelium factor Antigen (*ice A*), the outer membrane protein (*oipA*) and adhesins (*BabA* and *BabB*) (Mansour *et al.*, 2010; Tanih *et al.*, 2010c; Tanih *et al.*, 2011). In addition, there are other genes that are essential to the bacterium for motility and metabolism e.g. flagellin A and urease genes.

Furthermore, the organism has been considered as part of the indigenous biota of human stomach, thus identification and characterization of the precise roles of genes and their products in its virulence, is extremely important in the prediction of clinical outcomes (Proença-Modena *et al.*, 2009).

2.3 Pathogenesis and Clinical manifestations

2.3.1 Pathogenesis

H. pylori is a well-recognized human pathogen that chronically infects the gastric mucosa of up to 50% of the world's population and has been associated with serious diseases with significant morbidity and mortality; however, it represents a serious public health concern (Aguilar *et al.*, 2001). A hallmark of *H. pylori* is its ability to maintain an infection throughout the lifetime of an individual however, only 10-15% of those infected actually present with symptoms of disease conditions (Frenck and Clemens, 2003).

Acute infection is most commonly asymptomatic and may be associated with epigastric pain, abdominal distention or bloating, belching, nausea, flatulence, and halitosis (Meurer and Bower, 2002). Prolonged infection and inflammation due to bacterial virulence and host genetic factors will lead to gastritis, peptic ulcers (gastric ulcers and duodenal ulcers), gastric atrophy and B cell mucosa associated lymphoid tissue lymphoma (Módena *et al.*, 2007). It is noteworthy that the clinical manifestations of *H. pylori* infection are dependent on the interplay between pathogen and host, which, in turn, are dependent upon strain-specific bacterial factors and/or inflammatory responses governed by host genetic diversity as well as environmental factors (Clyne *et al.*, 2007).

a) Virulence determinants of the pathogen

Virulence determinants encompass all the factors contributing to the evolutionary success of the bacterium and the development of the disease in the host (Aguilar *et al.*, 2001). Several microbial virulence factors have been identified in *H. pylori* including *cagA* and its related pathogenicity island (*cag* PAI), *vacA*, *BabA* and *BabB*. They have been linked to enhance pathogenicity of the bacterium. The *cag* PAI represents a disease-associated locus because it

contained approximately 41 putative genes important for increased inflammation and the secretion of virulence-related products including IL-8 induction, neutrophil recruitment, tyrosine phosphorylation and protein secretion (Peek, 2005).

Some of the genes in *cag* PAI region encode type IV bacterial secretory apparatus, which can translocate CagA protein into the host target cells (Ahmed and Sechi, 2005). Of prime importance, *cagA* encodes the immunoreactive 120-145kDA protein, CagA (Das and Paul, 2007). The CagA protein has been reported as a marker for more severe disease since it is almost always associated with increased inflammation and with strains obtained from patients with peptic ulcers and gastric cancer. However, individuals infected with *cag A*⁺ strains are more likely to develop gastroduodenal ulcers than those infected with *cagA*⁻ strains (Chan and Leung, 2000).

Virtually, all *H. pylori* strains have vacuolating cytotoxin *vacA*, which encodes secreted bacterial toxin (VacA). VacA induces epithelial cell vacuolization and its expression is determined by variations in the signal sequence (s1a, s1b, s1c, s2) and mid-region (m1, m2) of the *vacA* gene (Peek, 2005). Coupled to its well-known vacuolating activity, *vacA* has been reported to induce apoptosis in epithelial cells, affect B lymphocyte antigen presentation, inhibit the activation and proliferation of T lymphocytes, and to modulate the T cell mediated cytokine response (Ahmed and Sechi, 2005).

The adhesin, BabA is an outer-membrane bound protein encoded by the *H. pylori* strain-selective gene *babA2*, interacting with the blood-group Lewis antigen on gastric epithelial cells (Das and Paul, 2007). Strains possessing *bab2* are associated with increased risk of gastric carcinoma. Another outer membrane protein that might mediate disease is a

proinflammatory protein encoded by *oipA*. The presence of *oipA* in any strain of this bacterium has been linked to more severe inflammation, higher bacterial colonization, enhanced mucosal level of IL-8 and duodenal ulceration (Yamaoka *et al.*, 2002).

An independent strain-specific *H. pylori* locus associated with disease is *ice A* which exists in two allelic forms, *iceA1* and *iceA2* (Mansour *et al.*, 2010). *IceA1* has been suggested to be a marker for strains that induce more severe gastric inflammation and injury due to its ability to increase tissue damage and disease, its allelic distribution on a minority of clinical strains, its linkage disequilibrium with *cag A* and *vacAs1* alleles plus its induction by physiologic events that provoke pathologic responses (Peek, 2005). Pathogenic *iceA1* strains are significantly associated with the presence of peptic ulceration and distal gastric adenocarcinoma (Kidd *et al.*, 2001).

b) Host factors

Host genetic factors might affect *H. pylori* colonization and development of disease. Genetic polymorphism of cytokines and other related ligands, receptors and enzymes might influence infection (Das and Paul, 2007). Interleukin1 β is a pro-inflammatory cytokine endowed with a powerful inhibiting activity against gastric acid secretion. Therefore, host genetic factors that may affect IL- 1 β may determine the quantity of acid secreted which in turn may verify why some individuals have gastric cancers or ulcers whilst others do not (Hocker and Hohenberger, 2003).

2.3.2 Clinical manifestations of *H. pylori* infections

After being ingested, *H. pylori* has to thrive in its ecological niche within the host. The gastrointestinal tract (GIT) of humans is a very hostile environment, therefore, in order for the bacterium to colonize the gastric mucosa; it must survive the acidic pH of the lumen. *H. pylori* metabolizes urea with its abundant urease enzyme producing ammonia and bicarbonate that neutralises the gastric acid (Go, 2002).

Alternatively, VacA permeabilizes the host epithelial cells to urea by functioning as a transmembrane pore, thereby allowing the bacterium to manipulate the pH of its environment by generating ammonia (Peek, 2005). In addition, several alternative routes for ammonia production exist; via enzymatic degradation of diverse amides and amino acids, the bacterium expresses two paralogous amidases, AmiE and AmiF, which together with four amino acid deaminases probably serve as sources for ammonia in low urea environment. Apparently, ammonia is vital to the metabolism and virulence of *H. pylori* as justified by the numerous sources of its supply (Krusters *et al.*, 2006).

The pathogen possesses flagella that allow it to burrow across the viscous gastric mucus to reach its niche, close to the stomach's epithelial cell layer. This motility enables the bacterium to resist the muscular contractions (peristalsis) that regularly empty the stomach (Ottemann and Lowenthal, 2002). Adhesion of the bacterium to the intestinal epithelium is a critical step in enteric colonization. It could use several adherence mechanisms (adhesins) to establish contact with the surface of the epithelial cells (Aguilar *et al.*, 2001). Adhesion is most likely important for acquisition of nutrients from the host and/or for resistance to shedding of the mucus gel layer.

Following attachment, the CagA protein is inserted into the host epithelial cell via type IV secretion system at the point of contact between *H. pylori* and the host cell. The translocated Cag A protein undergoes tyrosine phosphorylation by Src family kinases and induces rearrangements of the host cell cytoskeleton (so called “hummingbird phenotype”). This causes disruption of the cytoskeleton, adherence to adjacent cells, intracellular signalling, cell polarity and other cellular activities (Backett and Selbach, 2008). The presence of *H. pylori* in the host triggers a series of inflammatory and immune response in the host (Go, 2002).

The initial migration and activation of inflammatory cells into the gastric mucosa is reliant on the production of various proinflammatory cytokines namely IL-8 (Figueirido *et al.*, 2005). The innate immune system is the first line of defence against invading pathogens. CD4⁺ T-lymphocytes are the major subset lymphocyte involved in immunity against *H. pylori* and are divided into Type 1 T helper (Th1) and Type 2 T helper (Th2) phenotypes. The predominance of IL-12 in the human stomach favours the development of a subset of helper T cells that tend to produce interferon gamma, which is associated with Th1 cells and enhanced cell-mediated immunity (Zevering *et al.*, 1999).

Despite, the vigorous immune responses mounted against *H. pylori*, the pathogen persists within its host by evading immune clearance and subsequently causing disease conditions. It could evade immune response via one of the following mechanisms: by siphoning L-arginine from a host competitive enzyme, inducible nitric oxide synthetase (iNOS) thereby limiting the production of nitric oxide. It also causes an increased level of IL-10, an anti-inflammatory peptide that inhibits the secretion of proinflammatory chemokines from macrophages and neutrophils (Peek, 2005) added to the fact that it could replicate within epithelial cells and

macrophages. Moreover, *H. pylori* can vary the antigenic repertoire of surface exposed proteins (Suerbaum and Michetti, 2002).

Accordingly, the pathogen successfully colonises its host. Combination of *H. pylori*'s virulent attributes, host genetic factors and environmental factors causes a series of changes that gradually present with different clinical conditions as shown in Figure 2.1 (Das and Paul, 2007). However, a typical course of disease begins with chronic superficial gastritis, eventually progressing to atrophic gastritis; this appears to be a key event in the cellular cascade that results in the development of gastric carcinoma. Other disease conditions in which *H. pylori* has been incriminated include gastroesophageal reflux disease (GERD), non-ulcer dyspepsia, cardiovascular disease and iron deficiency anaemia (Hardin and Wright, 2002).

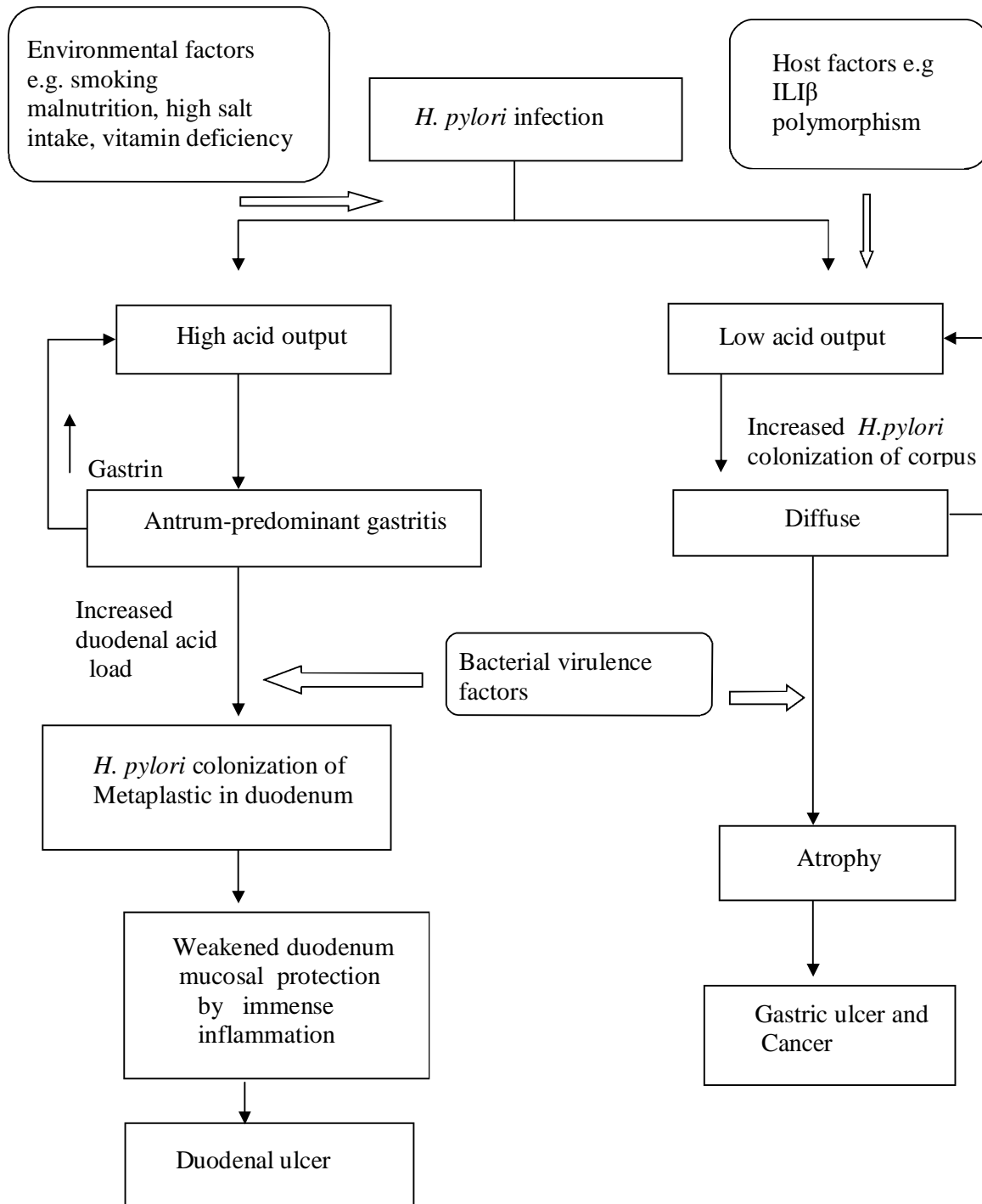


Figure 2.1: Proposed interaction between host, environment and *H. pylori* infection in the development of gastric and duodenal ulcers (Das and Paul, 2007).

2.4 Diagnosis of *H. pylori* infections

Numerous invasive and non-invasive diagnostic tests of different sensitivity and specificity have been developed to diagnose *H. pylori* infections. However, each has its limitation in clinical applications (Tiwari *et al.*, 2005). Still, no single test is absolutely reliable to detect *H. pylori* and if feasible, a combination of two tests is recommended (Krogfelt *et al.*, 2005). The tests used should depend on clinical circumstances, the likelihood ratio of positive and negative tests, cost-effectiveness of the testing strategy and the availability of the tests (Vaira *et al.*, 2002; Krogfelt *et al.*, 2005).

2.4.1 Invasive tests

These tests detect the micro-organism in a biopsy sample of the upper gastric mucosa obtained at upper gastro-intestinal endoscopy (Lin *et al.*, 2004). These biopsy-based tests which require an endoscopic procedure are uncomfortable to the patients. Also, there is the possibility of false negative results due to sampling error because *H. pylori* infection in the stomach maybe patchy. This could be as a result of intestinal metaplasia or re-growth after failed eradication (Kato *et al.*, 2001).

Furthermore, long acid suppressive treatment may decrease the number of *H. pylori* colonizing the gastric antrum, with an increase in the numbers colonizing the fundus (Logan and Walker, 2002). To salvage this situation, multiple biopsies have to be taken both from gastric antrum and corpus in order not to miss the bacterium. On the other hand, these tests are able to detect active infections while being highly specific with a very high predictive value.

a) Rapid Urease Test

This is a simple, inexpensive, fast and widely available test whose principle rests on the powerful urease activity of *H. pylori* and its exclusive ecological niche in the gastric mucosa protected from competition with other bacteria (Mödena *et al.*, 2007). Urease activity in the biopsy sample is detected by the rise in pH (seen as colour change) which follows ammonium ion production from urea (Dönmez-Altuntas and Güven, 2002).

It is limited, however, by false negative results due to decreased urease activity, caused either by recent ingestion of antibiotics, bismuth compounds and proton pump inhibitors, gastric atrophy and contamination with the bile (Meurer and Bower, 2002). Commercially available test kits include CLO test (*Campylobacter-like organism* test) that has been proven to be of high sensitivity and specificity after an hour (Krogfelt *et al.*, 2005).

b) Histology

Histological evaluation has traditionally been the “gold standard” method for diagnosing *H. pylori* infection (Orhan *et al.*, 2008). It allows for the definitive diagnosis of infection, as well as the degree of inflammation or metaplasia and the presence/absence of MALT lymphoma or other gastric cancers in high risk patients (Tanih *et al.*, 2008). In clinical practice, knowledge of the microscopic appearance of the gastric mucosa aids in therapeutic decision making and in the planning of follow-up arrangements (Sipponen *et al.*, 1997). *H. pylori* colonized with high density can be observed in routine Hematoxylin-eosin (HE)-stained section (Orhan *et al.*, 2008).

However, to show the characteristic morphology and to detect lower density of the organism, special stains are to be employed such as Gram, Giemsa, Genta or Warthin-starry stains (Ndip *et al.*, 2003). The speed and simplicity of the modified Giemsa stain technique makes it the method of choice in routine clinical practice (Gatta *et al.*, 2003). However, none of these stains are specific for *H. pylori*, and the *Helicobacter*-like organisms may cause false positive results with these stains (Orhan *et al.*, 2008). Its accuracy depends on the stain selected and to a great extent on the experience of the observer (Tiwari *et al.*, 2005).

i) Immunohistochemical staining

Immunohistochemical staining with specific polyclonal antibodies to visualize *H. pylori* in gastric biopsies has also been available since 1988. Contrary to histology, this method helps to identify the organism at low magnification, even in small numbers (Gental *et al.*, 1994) and also visualizes its coccoid forms. It could be better than Urea Breath test (UBT) since coccoid forms of *H. pylori* cannot produce urease, its activity on which UBT is based (Orhan *et al.*, 2008). It is simple, sensitive, specific, reproducible and easy to perform.

ii) Fluorescent *in situ* hybridization (FISH)

FISH is a genotype-based technique that can detect *H. pylori* and its macrolide-resistant strain in formalin-fixed, paraffin-embedded specimens (Jüttner *et al.*, 2004) and also in deparaffinized gastric biopsies (Yilmaz *et al.*, 2007). It involves rRNA-targeted fluorescence-labeled oligonucleotide probes binding to either 16S rRNA (Can *et al.*, 2005) or

23 rRNA sequences (Caristo *et al.*, 2008). This technique is very valuable in situations in which results from conventional culturing and antibiotic susceptibility testing are not available for clinical decision making (Rüssman *et al.*, 2003). Of major advantage is the fact that, it can be used for accurate determination of macrolide susceptibility, thus providing the clinician with important information with which to make a proper treatment recommendation. It is promising because it provides rapid, easy-to-implement, reliable, cost-effective and culture-independent method for routine diagnosis (Can *et al.*, 2005).

c) Culture

Microbiological isolation of the organism is the theoretical “gold standard” for the detection of *H.pylori* infections. Cultures could be recovered from gastric biopsies, brushings, aspirate, feces, vomitus, dental plaque and saliva, which have been used as potential specimen sources (Ndip *et al.*, 2003). Gastric mucosal biopsy materials are currently considered the easiest and best source to culture the organism. They can be collected and transported in 0.9% physiological saline, commercially available Portagerm pylori (Biomerieux), Stuart’s transport medium (Ndip *et al.*, 2003) and Brain Heart Infusion broth+20% glycerol+ cysteine (Tanih *et al.*, 2010).

Culture can be performed on both solid and liquid media, e.g. Columbia blood agar medium supplemented with Skirrow’s antibiotics (10mg vancomycin, 2.500 units polymyxin B, 5mg trimethoprim) and 7% defibrinated horse blood to select specifically for *H. pylori* (Megraud and Lehours, 2007). Since it contains blood, the medium must be used within two weeks, if optimum culture conditions are to be maintained (Ndip *et al.*, 2003). Other media that could

also be used include; Chocolate Agar, Marshall Brain Heart Infusion medium (BHI) and Wilkins-Chalgren Agar with horse blood. Incubation of inoculated plates is done under microaerophilic conditions (5% O₂, 10% CO₂, and 85% N₂) which could be produced by Campy Pak (Oxoid Ltd, UK) at 37°C for 4-7days (Velapatino *et al.*, 2006).

The bacterium is identified based on colony morphology, gram staining, and positive reaction to oxidase, catalase, and urease assays (Nguyen *et al.*, 2009). Various conditions can affect the survival rate of *H.pylori* e.g. the time lapse between sampling and culture, transport temperature, transport media and duration of exposure to air (Yuen *et al.*, 2005). The success rate of isolation also depends on the clinical expertise of the microbiology laboratory ranging from 30% to 73% (Destura *et al.*, 2004). Although bacteriological culture procedure is costly, time consuming and labour intensive, it gives insight into the antibiotic susceptibility of strains, a factor important to the clinician for the effective management of *H. pylori* infections. It also provides information regarding the virulence factors of the organism especially *cag A* and *vac A* alleles (Módena *et al.*, 2007).

d) Polymerase Chain Reaction (PCR)

PCR is an extremely sensitive and specific method of detecting *H.pylori* in gastric biopsy, saliva, feces and gastric juice samples (Dönmez-Altuntas and Güven, 2002). Minute quantities of target DNA are amplified with the PCR technique thus maximizing the sensitivity of detection. The 16SrRNA gene of *H.pylori* is a highly specific target for amplification and has been previously used to help reclassify the organism (Tiwari *et al.*, 2005).

This molecular technique is advantageous in identifying virulence genes of the infecting strain (*cagA* being the main determinant), which might have implications for the development of severe disease or efficacy of eradication (Tanih *et al.*, 2008). Among the draw backs are i) the possible existence of *Taq* polymerase inhibitors which can decrease the sensitivity of the reaction ii) the possible contamination of the specimen by exogenous *H. pylori* DNA which alters specificity. Notwithstanding, several methods have been proposed to increase the sensitivity of PCR (Mégraud and Lehours, 2007). A positive result detected by any of the molecular techniques does not indicate current infection as it can also detect the DNA of dead organisms (Ricci *et al.*, 2007).

2.4.2 Non-invasive tests

The non-invasive tests are more convenient and equally accurate in diagnosing *H.pylori* infections. They obviate the need for endoscopy which is invasive and discomforting in nature (Kato *et al.*, 2001), and thus are appropriate for large scale community based studies or routine screening of children, the group in which most infections start (Mackay *et al.*, 2003). These tests are relatively inexpensive and rapid, all are proxy measurements of infections; thus validation typically using histology or culture as the gold standard must be performed initially (Frenck *et al.*, 2006). These tests can either be active or passive (Oderda *et al.*, 2001).

a) ¹³C-Urea Breath test (UBT)

¹³C- UBT is the gold standard in non-invasive diagnostic testing for *H.pylori* infection (Zambon *et al.*, 2004). It is based on the principle that, a solution of urea labeled with ¹³Carbon or ¹⁴ Carbon is rapidly hydrolyzed by the urease enzyme of *H. pylori*. ¹³C- labeled urea has the advantage of being non radioactive and thus safer for children and women of child bearing age. The resulting CO₂ is absorbed across the gastric mucosa and hence via the systemic circulation, excreted as ¹³CO₂ in the expired breath (Logan and Walker, 2002).

Differences in ¹³CO₂ expired may reflect the extent of the infection. This may also be affected by the strain-dependent urease activity, duration of contact between ¹³C-labelled urea and the infected gastric mucosa that may vary because of individual differences in gastric acid secretion and motility (Nigel Bird, www.packardinstrument.com). UBT is theoretically subjected to false positives from urease positive bacteria present in the oral cavity. It is a relatively inexpensive, reliable and valuable test for establishing absence of infection after treatment (Krogfelt *et al.*, 2005).

b) Serology

H. pylori infection induces an immune response through the production of immunoglobulins to organism-specific antigens. These antibodies can be detected in serum or whole blood samples. Circulating IgG antibodies can be detected by Enzyme Linked Immunosorbent Assay (ELISA) or latex agglutination (Logan and Walker, 2002). ELISA relies on the hydrogen bond formed between the antibodies (abs) and antigen (ags) which is monitored by a colour change when there is a reaction of ag-ab (Oderda *et al.*, 2001). Individuals vary

considerably in their antibody response to this organism and no single antigen is recognized by sera from all subjects.

The accuracy of serological tests therefore depends on the antigens used in the test, making it essential that *H. pylori* ELISA is locally validated (Logan and Walker, 2002). These tests are generally simple, reproducible, and inexpensive and can be done on stored samples. Furthermore, they are useful in identifying certain strains of more virulent *H.pylori* by detecting antibodies to virulent factors associated with more severe disease and complicated ulcers, gastric cancer and lymphoma (Hardin and Wright, 2002). Since antibody titres fall only slowly after successful eradication, serology cannot therefore be used to determine *H.pylori* eradication (Lin *et al.*, 2004).

c) Stool Antigen test

The stool antigen test (Premier Platinum Hp SA Meridian Diagnostics, Inc., Cincinnati, Ohio) has been successfully used to detect the presence of *H.pylori* in stool samples (Lin *et al.*, 2004). It is well standardized, easy to perform, less expensive and relies on the detection of *H.pylori* antigens in feces by enzyme immunoassay (Ndip *et al.*, 2004). It utilizes an immunoaffinity purified polyclonal anti-*H. pylori* rabbit antibody absorbed to micro wells. The fecal samples can be stored at -70°C until the test is performed.

Results of the test are assigned to positive, negative or equivocal value on the basis of the manufacturer's recommended cut off values (Shepherd *et al.*, 2000). Highly sensitive and

specific, the stool antigen test reverts to negative from 5 days to a few months after eradication of the organism. Hence, it is useful in confirming eradication (Meurer and Bower, 2002).

2.5 Epidemiology of *H. pylori* infections

H. pylori infections in humans have a worldwide distribution. Investigation of the incidence of the organism has been scarce because acute infection does not present with diagnostic symptoms, hence it is impossible to pinpoint the time a person acquires the infection (Logan and Walker, 2001). Notwithstanding, incidence of the infection can be determined by extrapolation from the prevalence data.

2.5.1 Prevalence and geographical distribution.

The prevalence of infection seems mostly to depend on the rate of acquisition but also on rate of loss of infection and the length of the persistence period between acquisition and loss (Malaty *et al.*, 1999). Based on these factors, prevalence of infection varies, both among countries and between different racial groups' resident within the same country (Segal *et al.*, 2001; Krusters *et al.*, 2006). The highest rates of *H. pylori* prevalence are in Eastern Europe, Asia, and many developing countries and developing populations in developed countries (e.g. Native Americans) (Tkachenko *et al.*, 2007).

In industrialized countries, 40-50% of the population is infected and infection is low early in childhood and slowly rises with increasing age; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past (Gold, 2001).

On the other hand, the prevalence of infection in developing countries is higher about 90%, acquired early in childhood and infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter. Although, high prevalence rates have been reported in developing countries, these rates are lower in some developing countries with better industrialization, public health and living conditions; however, the rate is still several times much higher than that reported in developed countries (Aguilar *et al.*, 2001).

2.5.2 Factors correlated with prevalence

Ethnicity, genetic predisposition, age, sanitation, educational level, socio-economic status, and living conditions are important determinants of *H. pylori* prevalence (Mittal and Mathew, 2003; Khalifa *et al.*, 2010; Tanih *et al.*, 2010a).

a) Age

Acquisition of *H. pylori* infection appears on the basis of age-specific prevalence to be relatively constant over time in some population. Age is positively correlated with *H. pylori* prevalence and has been demonstrated both in developed and developing countries (Huang *et al.*, 2004; Rodrigues *et al.*, 2004). Consistently, the prevalence of infection has been found to be higher in adults than in children, and this may be attributable either to new acquisition of infection among the population or the effect of birth cohorts, each with a different rate of acquisition in childhood (Malaty, 2007).

However, there is little age-related increase in prevalence in developing countries given that the transmission of infection already occurs at a high rate in children. This is due to the fact that children are more susceptible to infection because of their lack of knowledge with

reference to hygiene and their increased susceptibility during episodes of achlorhydria induced by gastroenteritis (Luman, 2002).

b) Ethnic and Genetic predisposition

The prevalence of *H. pylori* infection varies between geographical locations i.e. between developed and developing countries. Although, the organism is acquired at childhood in both countries, black children have been found to have a higher rate of initial acquisition, a lower rate of loss of infection and a higher rate of reinfection than the white children (Malaty *et al.*, 2002). Marked differences in seroprevalence of *H. pylori* have been observed between various ethnic and racial groups living in the same region (Bardhan, 1997). For example, in USA the overall prevalence of *H. pylori* infection is estimated at 30-40%, but it remains much higher in certain ethnic groups such as the African-American and Hispanics (Go, 2002).

In South Africa, increase in *H. pylori* prevalence with age as well as among coloreds (68.4%) than in whites (59.5%) has been noted in the Eastern Cape Province (Tanih *et al.*, 2010a). Notwithstanding, variation in acquisition of infection among ethnic and racial groups appears to be primarily related to differential exposure (e.g. cultural background, social, dietary and environmental factors) and not to differences in genetic predisposition although, genetic predisposition to infection is also feasible (Aguilar *et al.*, 2001).

c) Socioeconomic factors

Apparently, the variation in prevalence rates between developed and developing countries have purportedly been associated with the socioeconomic status. A lower socioeconomic status has been indicated as a cause for the higher age-specific *H. pylori* prevalence in developing countries. Socioeconomic status has been reported to be positively correlated with

H. pylori prevalence and could be described in terms of occupation, income level, social class, living standards, sanitation, urbanization and educational level (Lehours and Yilmaz, 2007; Dube *et al.*, 2009b).

In addition, Goldman *et al.* (2006) in their study indicated that *H. pylori* prevalence was positively associated with number of siblings, presence of pet cats and birds in the household and antecedents of gastritis among family members, in symptomatic children population in Buenos Aires, Argentina. Other poverty-related factors such as overcrowding, bed sharing, large sibling size, lack of domestic hot water have been reported to influence the prevalence rate (Mitipat *et al.*, 2005). Combined, these factors are likely to increase the risk of infectious diseases in general.

2.5.3 Transmission of *H. pylori* infections

2.5.3.1 Reservoirs

The human gastric mucosa appears to be the most suitable environment for the survival of this organism (Van Duynhoven and de Jonge, 2001) thus it is the major reservoir. Other environmental or animal reservoirs have been suggested which could be involved in indirect transmission, such as food, animals (pigs, sheep, cats), water sources and the midgut of housefly (Khalifa *et al.*, 2010).

2.5.3.2 Possible routes of transmission

H. pylori is one of the human pathogens with the highest prevalence around the world; yet its exact modes of transmission are still uncertain. Despite the poor knowledge on how the bacterium is being transmitted, the extensive prevalence, the clustering of cases in the families, the higher prevalence of *H. pylori* seropositivity rates among persons living in

institutions or other crowded conditions and the lack of a plausible environmental reservoirs strongly suggest that direct transmission by person-to-person contact is the primary mode of transmission (Khalifa *et al.*, 2010).

Transmission via person-to-person contact is dependent on the degree of contact between susceptible and infected individuals, in tandem with the degree of crowding and age-distribution among those susceptible to infection and those that are infected (Das and Paul, 2007). It may occur by one or combination of three means; oral-oral, gastro-oral or fecal-oral routes but determining a dominant route is not an easy task. These different routes of transmission may be predominant in different geographical settings (Engstrand, 2001).

More elaborately, the oral-oral transmission pathway has been suggested as the prevailing route in developed countries characterized with improved sanitation, and proper water treatment (Brenner *et al.*, 1999). Saliva from the oral cavity could also act as a vehicle of transmission since *H. pylori* can be isolated from saliva and dental plaques (Chitsazi *et al.*, 2006). On the other hand, studies from developing countries with low socio-economic status and poor management of drinking water such as the rural areas, advocate that environmental factors are essential (Dube *et al.*, 2009a).

Feces-contaminated water may be a source of infection. Successful identification of the pathogen from the feces of Cameroonian and Iranian children though by different diagnostic methods proved that *H. pylori* can survive in the intestinal tract and be shed into the environment along with feces (Ndip *et al.*, 2004; Falsafi *et al.*, 2009). Diarrhoea is a common childhood ailment and, coupled with reduced awareness of hygiene in children and the liking

for putting objects into their mouths, the faecal-oral route of transmission can take place through sharing of food or toys touched by contaminated fingers (Luman, 2002).

However, transmission by oral-oral route is also feasible as it can be potentiated by specific eating habits for instance, the pre-mastication of food by mothers before feeding their children in some African countries and the use of a common spoon by the mother and child (Luman, 2002). This is because transient colonization in the mouth could occur in mothers with free reflux (Luman, 2002). It is therefore reasonable to suggest a direct gastro-oral route of transmission mediated by refluxed gastric juice (Luzza *et al.*, 2000). This mode of transmission has been postulated in young children among whom vomiting and gastro-esophageal reflux is common. Vomitus could act as a vehicle of transmission and infection could occur through ingestion of food or exposure to objects contaminated with vomitus colonized by *H. pylori* (Luman, 2002).

2.6 Treatment of *H. pylori* infections: Mitigating factors and prospective natural remedies

H. pylori infection is a serious, chronic, progressive and transmissible infection associated with significant morbidity and mortality, which alone emphasizes the priority of developing adequate prophylactic or therapeutic measures (Scarpignato, 2004). Development of a successful treatment for *H. pylori* infection has been fraught with difficulty. Its location within the stomach (that is, the mucus lining the surface epithelium, deep within the mucus-secreting glands of the antrum, attached to cells and even within the cells) provides a challenge for antimicrobial therapy (Romano and Cuomo, 2004).

In addition, the gastric mucosa is a hostile environment for antimicrobial therapy because drugs must penetrate thick mucus and may need to be active at pH values below neutral (Malekzadeh *et al.*, 2004; Manyi-Loh *et al.*, 2010a). Moreover, emerging bacterial resistance presents an added challenge (Hardin and Wright, 2002).

The purpose of treatment of *H. pylori* infection in any clinical situation is eradication of the bacterium from the fore gut or stomach. Eradication is defined as a negative test for the bacterium four weeks or longer after treatment (Harris and Misiewicz, 2002). For successful eradication of the bacterium, it is imperative that the clinician be aware of the current antimicrobial susceptibility profiles of the isolates within the region (Sherif *et al.*, 2004). Consequently, antibiotic recommended for patients may soon differ across regions of the world because different areas have begun to show resistance to particular antibiotics. Such regional variation in resistance patterns probably reflects geographical variation in local antibiotic-prescription practices, and antibiotic use and abuse, as drug control is much tighter in some areas than others (Ndip *et al.*, 2008).

2.6.1 Drugs used for treatment

H. pylori infections are treated with drugs that kill the bacteria (antibiotics), reduce stomach acid [H₂ blockers and proton pump inhibitor (PPI)] and protect the stomach lining (bismuth compounds).

a) Bismuth compounds

The discovery of *H. pylori* in 1983 led to renewed interest in bismuth compounds, because these were found to successfully treat the infection in combination with antibiotics (Ford *et al.*, 2008). Bismuth compounds (colloidal bismuth sub citrate and bismuth subsalicylate) may reduce the development of resistance to co-administered antibiotics and are also effective at treating *H. pylori* strains with established resistance to other antibiotics (Andersen *et al.*, 2000). These compounds act by reducing intracellular ATP levels and interfere with the activity of urease enzyme, a key enzyme of *H. pylori* (Romano and Cuomo, 2004). Also, they induce the formation of an ulcer-specific coagulum, preventing acid back diffusion (Meurer and Bower, 2002) and inhibit protein and cell wall synthesis as well as membrane function (Bland *et al.*, 2004).

Furthermore, they cause an increase in the synthesis of prostaglandin E₂ (Sandha *et al.*, 1998), detachment of *H. pylori* from the gastric epithelium and a reduction in capsular polysaccharide production (Romano and Cuomo, 2004). Therefore, the properties of bismuth compounds are bactericidal for *H. pylori* (Larsen *et al.*, 2003).

The bismuth compounds are extremely potent cytotoxic agents when attached to a monoclonal antibody as these can target leukemia, lymphoma and other tumors. Interestingly, *H. pylori* is incriminated in mucosa associated lymphoid tissue lymphoma, thus there is a clear connection between anti-tumor activity and bismuth compounds (Wotherspoon, 1998). Side effects encountered with this drug include darkening of oral cavity and stool, nausea and gastrointestinal upset (Stenström *et al.*, 2008). Recently, bismuth containing regimen has

been recommended as a potential first line therapy, because there have been concerns expressed that PPI-based triple therapies for *H.pylori* do not lead to a satisfactory eradication rate (Ford *et al.*, 2008).

b) Acid Reducers

Two types of acid-suppressing drugs might be used: H₂ blockers and PPI. H₂ blockers work by blocking histamine, which stimulates acid secretion. They include; cimetidine, ranitidine, famotidine and nizatidine. On the other hand, PPI suppress acid production by halting the mechanism that pumps the acid into the stomach. They also include; omeprazole, lansoprazole, rabeprazole, pantoprazole and esomeprazole (Gendull *et al.*, 2003).

H. pylori has evolved multi-faceted acid-adaptive mechanisms (Wen *et al.*, 2003). Increasing the gastric pH with the use of histamine H₂ receptor antagonist (H₂RA) or PPI has been shown to improve the effectiveness of antibiotic therapy. In the presence of ulcer disease, PPI have largely replaced H₂RA because of their ability to provide more rapid pain relief and better control of pH (Meurer and Bower, 2002). PPI's also display several pharmacological actions that promote eradication. These include:

- i) An antibacterial action against *H. pylori* (Nakao and Malfertheiner, 1998).
- ii) By increasing intra gastric pH, they allow the microorganisms to reach the growth phase and become more sensitive to antibiotics such as amoxicillin and clarithromycin (Farthing *et al.*, 1998; Scott *et al.*, 1998).
- ii) They increase antibiotic stability and efficacy (Erah *et al.*, 1997).

iv) By reducing gastric emptying and mucus viscosity, they increase the gastric residence time and mucus penetration of antimicrobials (Pedrazzoli *et al.*, 2001).

In addition, some of these anti-ulcer drugs show *in vitro* synergy when tested with some antibiotics. Seemingly, the increased absorption and tissue penetration of antimicrobial agents that occur with elevated gastric mucosal levels caused by omeprazole may contribute to the observed synergy (Calafatti *et al.*, 2000).

c) Antibiotics

H. pylori is susceptible *in vitro* to commonly used antibiotics such as; amoxicillin (AMOX), tetracycline (TET), metronidazole (MET) and clarithromycin (CLR) (Alarcón *et al.*, 1999). Of the *H. pylori* isolates collected in Cameroon, Ndip *et al.* (2008) found 56.1% to be susceptible to TET, 55.3% to CLR, 14.4% to AMOX and only 6.8% to MET. Currently, these antibiotics are administered in combination, with no single agent ever used as monotherapy because of lack of efficacy and the potential development of resistance (Hardin and Wright, 2002).

Metronidazole (prodrug) is highly active against *H. pylori*, and requires activation by bacterial nitroreductases (Meurer and Bower, 2002). The bacterium possesses a number of enzymes with the potential to reduce MET. The reduced nitroimidazole cause loss of the helical structure of bacterial DNA, break strands and thus, impair bacterial infection (Romano and Cuomo, 2004). However, other studies have reported high rates of resistance to this drug (Ndip *et al.*, 2008; Tanih *et al.*, 2010b). MET can have unpleasant adverse effects (e.g.

nausea, a metallic taste, dyspepsia) and a disulfiram-like reaction with alcohol consumption (Stenström *et al.*, 2008).

Clarithromycin is recognized as the key antibiotic for *H. pylori* treatment because of its powerful bactericidal effect *in vitro* compared with the other available molecules (De Francisco *et al.*, 2007). Co-administration with PPI significantly increases the concentration of CLR in the antral mucosa and the mucus layer (Meurer and Bower, 2002; Njume *et al.*, 2009). Results of studies showing approximately 90% *H. pylori* eradication with triple therapy regimens using CLR have led to the widespread use of this antibiotics (Romano and Cuomo, 2004), though it is more expensive than other antibiotic agents. Adverse effects with CLR include a bitter metallic taste, gastrointestinal upset and diarrhea (Ables *et al.*, 2007; Stenström *et al.*, 2008).

Amoxicillin, semi synthetic penicillin is an effective antibiotic for *H. pylori* infection. Its action against *H. pylori* is pH dependent and therefore requires co-administration with a PPI (Meurer and Bower, 2002). It inhibits the synthesis of bacterial cell wall after absorption into the bloodstream and subsequent delivery into the gastric lumen (Romano and Cuomo, 2004). Side effects include gastrointestinal upset, diarrhea and headache (Stenström *et al.*, 2008).

Tetracycline has demonstrated *in vitro* efficacy against *H. pylori* and is active at low pH (Romano and Cuomo, 2004). It is inexpensive and is a close derivative of the polycyclic naphthacene carboxamides. Therefore; it inhibits protein synthesis and bind specifically to the

30S ribosomal subunit. Tetracycline can cause discoloration of teeth in children, photosensitivity reaction and gastrointestinal upset (Hardin and Wright, 2002).

Furazolidone, a synthetic nitro furan appears to be effective for many *H. pylori* strains which are resistant to MET (Meurer and Bower, 2002). However, it is described as an alternative to MET; patients should be warned against using alcohol or monoamine oxidase inhibitors (Gendull *et al.*, 2003).

2.6.2 Treatment Strategy

The treatment of *H. pylori* infection can be likened to the treatment of tuberculosis because it is a multidrug regimen and an adequate length of time is needed to eradicate the organism (Meurer and Bower, 2002). Combination drug regimens are essential to minimize the risk of promoting antimicrobial (namely to MET and or CLR) resistance (De Boer and Tytgat, 2000). The only conditions, for which such treatment is strongly recommended on the basis of unequivocal evidence, are peptic ulcer disease and low grade gastric MALT lymphoma (Tanih *et al.*, 2009). Monotherapy in which antibiotic agents alone were used in the eradication of the bacterium was inefficient, making it imperative to use dual, triple or even quadruple therapy (Alarcón *et al.*, 1999).

Dual therapy regimen refers to the combination of PPI's or Ranitidine bismuth citrate (RBC) and one antibiotic usually AMOX or CLR (Wu and Sung, 1999). The first dual therapy combining omeprazole with AMOX had unpredictable efficacy ranging from 20% to 90%

(Laine *et al.*, 1997). The dual therapy is, however, more reproducible when AMOX is replaced with CLR (Wu and Sung, 1999).

Triple therapy regimen is the most popular and standard treatment regimen for the cure of *H. pylori* infection (De Boer and Tytgat, 2000). It consists of an acid-suppressant (PPI or RBC) and two antimicrobials (Wu and Sung, 1999). The first PPI- based triple therapy was described by Bazzoli *et al.* (1993) and its good efficacy (eradication rate of more than 80%) has been supported in several studies in Europe (Malfertheiner *et al.*, 1999) and Canada (Zanten *et al.*, 1999). The choice of antibiotics determines the efficacy of the PPI-based triple therapy, thus CLR being included in the therapy ensures high efficacy and reproducible results (Wu and Sung, 1999).

Because bacterial resistance to MET or CLR results primarily from previous treatment failure, first choice treatment should never combine CLR and MET in the same regimen (Romano and Cuomo, 2004). In fact, even though this combination is highly effective, patients who are not cured will have at least single, and usually double, resistance (Peitz *et al.*, 2002) and no viable empirical treatment remains afterwards. RBC-triple therapy has proven to be effective in eradicating *H. pylori* with cure rates ranging from 80% to 96% (Laine *et al.*, 1997). One week RBC based triple therapy is now increasingly considered as an effective regimen for *H.pylori* eradication (Savarino *et al.*, 1997).

Quadruple therapy regimen consists of bismuth, a PPI and two antibiotics (Malekzadeh *et al.*, 2004). Currently, quadruple therapy is mainly reserved as a second line regimen in cases of

treatment failure and even as an alternative first-line treatment for eradicating *H. pylori* infection, especially in areas of high prevalence of antibiotic resistance (Mirsattari *et al.*, 2010). Most common putative quadruple therapies currently in use include bismuth-based and levofloxacin-based therapies (Gisbert and Javares, 2002).

In addition to *H. pylori* eradication therapy, patient's education about the need for effective eradication therapy and the necessity of completing the initial drug regimen is critical. Confirmation of the eradication of the bacterium from the fore gut is necessary after treatment.

2.6.3 Factors affecting the effectiveness of treatment regimens

Factors implicated in treatment failure include drug costs and availability, number of *H. pylori* cells in the gastric mucosa, treatment side effects, lack of penetration of antibiotics into the depth of gastric mucosa (Wong *et al.*, 2002), antibiotic inactivation by pH, lack of compliance by patients, lack of correlation between *in vitro* susceptibility test and *in vivo* efficacy and the presence of *H. pylori* strains with primary or secondary resistance to the antimicrobial agents used (Bytzer and O'Morain, 2005), duration of treatment and antibiotics dosage (Gendull *et al.*, 2003).

Antimicrobial resistance is increasing and regional variations in susceptibility and resistance patterns maybe ascribed to differences in local antibiotic prescription practices, antibiotic usage in the community and mass eradication programmes for *H. pylori* (Destura *et al.*,

2004). Until recently, the recommended duration of therapy for *H. pylori* eradication was 10 to 14 days (Ables *et al.*, 2007). Potential benefits for shorter regimens include better compliance, fewer adverse drug effects, and reduced cost to the patient (Meurer and Bower, 2002). Low gastric pH seems to affect the activity of antibiotics since most are active at neutral pH.

The minimal bactericidal concentrations (MBC) and minimal inhibitory concentrations (MIC) of most antibiotics against *H. pylori* (except MET and TET), are dependent on the pH of the environment (Mégraud and Lamouiatte, 2003; Njume *et al.*, 2009). At pH values lower than 7 or 7.4, the MIC increases. This is why PPI's are used in therapy so as to increase the pH of the stomach, to allow better antimicrobial activity (Farthing *et al.*, 1998). In patients who are acid hyper secretors, the pH remains low. Consequently, antimicrobial activity may be insufficient to eradicate the bacteria. As a result, increasing the dosage of PPI in the treatment regimen may have beneficial effects (Malekzadeh *et al.*, 2004).

The most important causes of treatment failure are poor compliance on the part of the patients and the development of bacterial resistance to antimicrobial agents (Huynh *et al.*, 2004). Patient compliance can only be improved by choosing a simple and well tolerated treatment regimen. Also, patients should be educated on the significance of eradication therapy (Stenström *et al.*, 2008).

2.6.4 Antimicrobial resistance

Resistance of *H. pylori* to the limited range of antibiotics that have efficacy in its treatment can severely affect attempts to eradicate the bacteria. The prevalence of bacterial resistance in certain geographical areas can influence the selection of first line eradication regimen in those regions (Malekzadeh *et al.*, 2004). Bacterial resistance to antimicrobials, however, could be either primary (that is, present before therapy) or secondary (that is, develop as the result of failed therapy (Romano and Cuomo, 2004).

Primary resistance in *H. pylori* has been reported in MET (6-95%), CLR (0-17%), and TET (0-6%) in different countries (Boyanova *et al.*, 2000; Huynh *et al.*, 2004). The issue of resistance primarily concerns MET and CLR, however, acquired resistances to AMOX and TET have also been reported, although they are extremely rare (Kwon *et al.*, 2000; Kim *et al.*, 2003). Recently, an alarming rise in resistance of *H. pylori* to AMOX has been reported, especially in African countries namely, Cameroon, Nigeria and South Africa where stringent control of drugs is lacking (Ndip *et al.*, 2008).

Metronidazole-containing regimens have recently been shown to have limited effectiveness because of the increasing prevalence of resistance to this drug. Its prevalence varies from 10% to 90% in different countries (Wu and Sung, 1999). For example, a resistance rate of 28.6% for MET was reported by Boyanova *et al.* (2000) in their attempt to assess the primary resistance of four antimicrobials against clinical isolates of *H.pylori* circulating in Sofia, Bulgaria.

Furthermore in Cameroon, Ndip *et al.* (2008) documented a very high resistance to MET while Tanih *et al.* (2010b) reported a rate of 95.5% in South Africa. Seemingly, Mollison *et al.* (2000) in their study conducted in Australia registered a resistance of 36% of *H. pylori* isolates against MET. Increasing the dosage of MET administered generally improves the result of the therapy when treating MET-resistant *H. pylori* strains (Stenström *et al.*, 2008). The generally high prevalence of metronidazole resistance, for example, is probably as the result of the frequent, uncontrolled use of nitroimidazole derivatives for the treatment of protozoan infections and gynecological problems (Ndip *et al.*, 2008).

Primary clarithromycin resistance is increasing worldwide and it has been regarded as the main factor reducing the efficacy of eradication therapy (De Francisco *et al.*, 2007). However, the prevalence rate of CLR resistance is 12.9% in the U.S and it is as high as 24% in some European countries (Huynh *et al.*, 2004). Acquired resistance to CLR frequently develops in individuals after initial treatment failure (Wu and Sung, 1999). These CLR-resistant strains of *H. pylori* can be treated using a regimen containing levofloxacin (Hsu *et al.*, 2008). Nevertheless, there is a trend of rising resistance due to widespread use of CLR in the treatment of upper respiratory tract infections.

The increasing prevalence of antimicrobial resistance jeopardizes the success of therapeutic regimens aimed at the eradication of infection (Deltenre, 1997). Therefore, clinicians should choose the appropriate combination of drugs based on sensitivity patterns provided by a local reference centre (Meurer and Bower, 2002). Ideally, in cases of treatment failure, the

antibiotic sensitivity pattern of the organism should be established before the second line therapy is chosen (Destura *et al.*, 2004).

2.6.5 Prospective natural remedies

The current increasing prevalence of antimicrobial resistance and its negative impact on the eradication treatment regimens has brought forth the quest for novel therapeutic approaches. A non-antibiotic agent, which is readily available, inexpensive, and effective and free from side effects, might be of utmost importance for the eradication of *H. pylori* (Ndip *et al.*, 2007). Natural foods can be attractive as an alternative treatment for *H. pylori*.

Dietary approaches that would keep *H. pylori* density and infection-mediated inflammation on a low level could be of considerable interest in developing low-cost, large-scale alternative solutions to prevent or decrease *H. pylori* colonization (Calvet *et al.*, 2000). In this respect; probiotics may close the therapeutic gap. A probiotic is defined as a living microbial species that, on administration, may have a positive effect on bowel micro-ecology and improve health conditions (Fuller, 1991). Probiotics have been proven to be useful in the treatment of several gastrointestinal diseases and can be beneficial in *H. pylori*-infected subjects for several reasons.

At present, the most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* and *Bifidobacterium species* (Gottenland *et al.*, 2006), but others include *Weissella confusa* and *Bacillus subtilis* (Nam *et al.*, 2002). The action of inhibition of *H.*

pylori by probiotics could be nonimmunologically and immunologically mediated (Lesbros-Pantoflickova *et al.*, 2007).

Allium vegetables, particularly garlic (*Allium sativum L*) exhibit a broad antibiotic spectrum against both Gram-positive and Gram negative bacteria. It has been demonstrated *in vitro* that *H. pylori* is susceptible to garlic extract at a fairly moderate concentration. Even *H. pylori* antibiotic resistant strains are susceptible to garlic (Sivam, 2001).

Capsaicin (hot pepper) consumed as a flavoring spice, has pharmacological, physiological and antimicrobial effects (Molina-Torres, 1999). Zeyrek and Oguz (2005) demonstrated *in vitro* anti-*H. pylori* activity of capsaicin at a concentration of 50µg/ml against metronidazole-resistant and metronidazole-susceptible clinical isolates. There is lower ulcer prevalence in people consuming higher amount of pepper compared to controls. It may be advisable to consume raw pepper, since it is known that cooking can alter some chemical features of *Capsicum* species and by this way, their antibacterial effects may decrease (Cichewicz and Thorpe, 1996).

Cranberry (*Vaccinium macrocarpon*) is a natural fruit, native to North America and composes of a good source of vitamin C, fructose, and bioflavonoids with anti-oxidant properties, which may contribute to the bacteriostatic effect of its juice. Zhang *et al.* (2005) in their preliminary study in Linqu County of Shandong Province, China, suggested that dietary consumption of cranberry juice may reduce *H. pylori* infections in adults, which remains an important public health issue worldwide.

Currently, there is renaissance of interest in the use of honey and honey products by the general public. This alternative branch of medicine is called apitherapy (Gosh and Playford, 2003). Honey is the natural sweet substance produced by honey bees of the genera *Apis* and *Meliponini*, from nectar or blossoms or from the secretion of living parts of plants or excretions of plants, which they collect, transform, and combine with specific substances of their own to ripen and mature. It can also be defined as the nectar and saccharine exudation of plants, gathered, modified and stored as honey in the honeycomb by honeybees (Chauhan *et al.*, 2010).

Based on the source of nectar, it can be grouped into floral and non-floral honey. Honeys can either be unifloral or multifloral, depending whether the honey collected is from the nectar of the same flower or from nectar of flowers of various types. Non floral honey (honey dew) is made by bees that extract sugars from the living tissues of plants or fruits, and/or scavenge the excretions of insects (aphids) that tap the veins of higher plants (Subrahmanyam, 2007).

2.6.6 An overview of honey: therapeutic properties and contribution in nutrition and human health.

Because honey inherits plants properties, its color, aroma, flavor, density, and physical and chemical properties depend on the flowers used by bees, although weather conditions as well as processing also influences its composition and properties (Ramirez and Montenegro, 2004). As a result, the nutritional values and profiles vary accordingly and can thus influence the value of a specific honey for health promoting purposes. Wholly, honey is made up of about 181 compounds classified as carbohydrates, enzymes, amino acids, minerals, vitamins,

trace elements, volatiles, and polyphenols; all of these dissolved in water (Alvarez-Saurez *et al.*, 2009). Yao *et al.* (2003) reported that the chemical composition of honey depends on the floral source used to collect nectar, seasonal and environmental factors, as well as processing and storage conditions.

2.6.6.1 Therapeutic properties of honey

Meda *et al.* (2004) reported that honey is becoming acceptable as a reputable and effective therapeutic agent by practitioners of conventional medicine and by the general public. Its beneficial role has been endorsed to its antimicrobial, anti-inflammatory and anti-oxidant activities as well as boosting of the immune system.

a) Antimicrobial activity

The antimicrobial activity is very important therapeutically, especially in situation where the body's immune response is insufficient to clear infection. In other words, it has shown powerful antimicrobial effects against pathogenic and non- pathogenic micro-organisms (yeasts and fungi) even against those that developed resistance to many antibiotics (Zaghloul *et al.*, 2001). The antimicrobial effects could be bacteriostatic or bactericidal depending on the concentration that is used. However, such activity has been attributed to certain factors like high osmolarity (low water activity), acidity (low pH), and hydrogen peroxide and non-peroxide components (Taormina *et al.*, 2001).

Furthermore, honey is a supersaturated sugar solution; these sugars have high affinity for water molecules leaving little or no water to support the growth of micro-organisms (bacteria and yeast). Consequently, the micro-organisms become dehydrated and eventually die (Malika *et al.*, 2004). In addition, the natural acidity of honey will inhibit many pathogens. The usual pH range of most of the pathogens is around 4.0- 4.5. However, the major antimicrobial activity has been found to be due to hydrogen peroxide, produced by the oxidation of glucose by the enzyme glucose-oxidase, when honey is diluted (Temaru *et al.*, 2007). As hydrogen peroxide decomposes, it generates highly reactive free radicals that react and kill the bacteria. In most cases, the peroxide activity in honey can be destroyed easily by heat or the presence of catalase.

Notwithstanding, some honeys have antibacterial action separate to the peroxide effect, resulting in a much more persistent and stable antibacterial action (non-peroxide activity). They are however called “non-peroxide honeys. Manuka honey (*Leptospermum scoparium*) from New Zealand and jelly bush (*Leptospermum polygalifolium*) from Australia are non-peroxide honeys which are postulated to possess unidentified active components in addition to the production of hydrogen peroxide. They retain their antimicrobial activity even in the presence of catalase (Snow and Harris, 2004).

Weston (2000) suggested that the main part of this activity might be of honeybee origin, while part may be of plant origin. The compounds exhibiting this activity can be extracted with organic solvents (e.g. n-hexane, diethyl ether, chloroform, ethyl acetate) by liquid-liquid (Manyi-Loh *et al.*, 2010a) or solid phase extraction methods (Aljadi and Yusoff, 2003). The

extracted compounds have been reported to include flavonoids, phenolic acids, volatile compounds (ascorbic acid, carotenoid-like substances, organic acids, neutral lipids, and Maillard reaction products), amino acids and proteins (Vela *et al.*, 2007).

Other important effects of honey have been linked to its oligosaccharides. They have prebiotic effects, similar to that of fructo oligosaccharides (Sanz *et al.*, 2005). The oligosaccharides have been reported to cause an increase in population of bifidobacteria and lactobacilli, which are responsible for maintaining a healthy intestinal microflora in humans. As a matter of fact, *Lactobacillus* spp. protect the body against infections like salmonellosis; and *Bifidobacterium* spp restrict the over-growth of the gut walls by yeasts or bacterial pathogens and, perhaps reduce the risk of colon cancer by out-competing putrefactive bacteria capable of liberating carcinogens (Kleerebezem and Vaughan, 2009).

b) Anti-inflammatory activity

Although inflammation is a vital part of the normal response to infection or injury, when it is excessive or prolonged it can prevent healing or even cause further damage. The most serious consequence of excessive inflammation is the production of free radicals in the tissue. These free radicals are initiated by certain leucocytes that are stimulated as part of the inflammatory process (van den Berg *et al.*, 2008), as inflammation is what triggers the cascade of cellular events that give rise to the production of growth factors which control angiogenesis and proliferation of fibroblasts and epithelial cells (Simon *et al.*, 2009).

They can be extremely damaging and break down lipid, proteins and nucleic acids that are the essential components of the functioning of all cells. However, the anti-inflammatory properties of honey have been well established in a clinical setting (Subrahmanyam *et al.*, 2003) and its action is free from adverse side effects.

c) Anti-oxidant activity

Antioxidant capacity is the ability of honey to reduce oxidative reactions within the human body. It has been found to have a significant antioxidant content measured as its capacity to scavenge free radicals (Gheldof *et al.*, 2002). This anti-oxidant activity may be at least partly what is responsible for its anti-inflammatory action because oxygen free radicals are involved in various aspects of inflammation (Henriques *et al.*, 2006). Even when the antioxidants in honey do not directly suppress the inflammatory process, they can be expected to scavenge free radicals in order to reduce the amount of damage that would otherwise have resulted.

Honey exerts its anti-oxidant action by inhibiting the formation of free radicals, catalyzed by metal ions such as iron and copper. Flavonoids and other polyphenols, common constituents of honey have the potential to impound these metal ions in complexes, preventing the formation of free radicals in the first place (Makawi *et al.*, 2009).

d) Boosting of the immune system

As well as having a direct antibacterial action, honey may clear infection through stimulating the body's immune system to fight infections. It has been reported that honey stimulates B-

lymphocytes and T-lymphocytes in cell culture to multiply, and activate neutrophils (Tonks *et al.*, 2003). Furthermore; Jones *et al.* (2000) in their study reported the stimulation of monocytes in cell cultures to release the cytokines TNF-alpha, IL-1 and IL-6, the cell “messengers” that activate the many facets of the immune response to infection. Recently, Tonks *et al.* (2007) discovered a 5.8k DA component of Manuka honey which stimulates the production of TNF- α in macrophages via Toll-like receptor. In addition, honey provides a supply of glucose, which is essential for the “respiratory burst” in macrophages that produce hydrogen peroxide, the dominant component of their bacteria-destroying activity (Molan, 2001).

Moreover, it provides substrates for glycolysis, the major mechanism for energy production in the macrophages, and thus allows them to function in damaged tissue and exudates where the oxygen supply is often poor. The acidity of honey may also assist in the bacteria-destroying action of macrophages, as an acid pH inside the phagocytic vacuole is involved in killing ingested bacteria (Molan, 2001).

2.6.6.2 Health benefits of honey

Since ancient times, honey has been used for its medicinal properties to treat a wide variety of ailments. It may be used alone or in conjunction with other substances and administered orally or topically for the eradication of certain ailments (Williams *et al.*, 2009). The beneficial actions of honey have been established in wounds, gastrointestinal diseases and others. In wounds, it provides a moist environment that allow skin cells to re-grow across the wound, stimulates the formation of new blood capillaries (angiogenesis), the growth of

fibroblasts that replace connective tissue of the deeper layer of the skin and produce the collagen fibers that give the strength to the repair as well as it provides a protective barrier that helps safeguard patients by preventing cross contamination (Rozaini *et al.*, 2004).

On the account of the nutritional value (303kcal/100g honey) and fast absorption of its carbohydrate, honey is a food suitable for humans of every age (Blasa *et al.*, 2006). Simply, when orally consumed, its carbohydrates are easily digested and quickly transported into the blood and can be utilized for energy requirements by the human body. It is for this reason that honey is particularly recommended for children and sportsmen because it can help to improve on the efficiency of the system of the elderly and invalids (Alvarez-Saurez *et al.*, 2009).

2.6.6.2.1 *H. pylori* and honey

Honey-derived remedies constitute a potential source of new compounds that may be useful in the management of *H. pylori* infections (Manyi-Loh *et al.*, 2010b). The anti- *H. pylori* activity of honey has been demonstrated by the peroxide and non-peroxide mechanism. The peroxide mechanism explores factors like hydrogen peroxide, osmolarity and acidity whilst non-peroxide mechanism encompasses all the phytochemicals (Weston, 2000). Some of these phytochemicals are natural anti-oxidants and can be extractable by organic solvents (Aljadi and Yusoff, 2003). The amount of these components may be small or diluted in the honey but when extracted, they become concentrated and therefore exhibit activity.

Almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage but insufficient to protect them entirely (Oboh, 2005). However, honey contains these natural anti-oxidants which exhibit a wide range of biological effects, including antibacterial, anti-inflammatory, anti-allergic, antithrombotic and vasodilatory actions (Gómez-Caravaca *et al.*, 2006). They are reported to scavenge for free superoxide and other reactive oxygen metabolites liberated during respiratory burst in *H. pylori* induced mucosal damage (Li *et al.*, 2001).

Honeys from different countries and regions have a wide variability in their antimicrobial activity (Basson and Grobler, 2008). This is as a result of climatic variation which affects the distribution of flowers and plant species, from which honey bees gather nectar and sweet plant deposits (Ndip *et al.*, 2007). As a result of profound heterogeneity exhibited by *H. pylori*, in combination with the regional variation in the antimicrobial components present in honey, there is a difference in the concentration of honey that would inhibit *H. pylori* in specific locations (Manyi-Loh *et al.*, 2010a)

2.6.6.3 Volatile phytochemicals in honey

The non-peroxide activity of honey is usually attributed to its phytochemicals (carbohydrates, flavonoids, non flavonoid phenolic compounds & volatiles), which are derived from plant or bee origin (Yao *et al.*, 2004). More than 600 compounds have been identified as honey volatiles in different chemical families, originated from various biosynthetic pathways (Montenegro *et al.*, 2009). Generally, volatile organic compounds (VOCs) could be derived from the plant or nectar source, from the transformation of plant compounds by the

metabolism of a bee, from heating or handling during honey processing and storage, or from microbial or environmental contamination (Guyot-Declerck *et al.*, 2002).

The chemical families into which the volatile compounds in honey belong include hydrocarbon, aldehyde, alcohol, ketone, acid, ester, benzene and its derivatives, furan and pyran derivatives, norisoprenoids, terpenes and its derivatives and sulphur and cyclic compounds (Barra *et al.*, 2010). Though present in low concentration in honey, VOCs could contribute in the biomedical activities of honey especially antioxidant effect due to their natural radical scavenging potential. For example, ascorbic acid, hydroxymethylfufural (HMF) and maillard reaction products have been reported to demonstrate antioxidant activity and also other compounds such as terpenes, organic acids, benzene derivatives and carotenoid have presented potential antimicrobial as well as antioxidant activities (Turkmen *et al.*, 2006; Jerković *et al.*, 2010).

Aside the beneficial therapeutic properties of volatile compounds, they could be used to assess the botanical/floral origin of honey as well as in determining the aroma profile. Knowledge about the floral origin of a honey will help to prevent fraud, standardize its quality and authenticate the honey thus allowing it to be competitive in the market (Castro-Várquez *et al.*, 2010).

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CHAPTER THREE

SELECTED SOUTH AFRICAN HONEYS AND THEIR SOLVENT EXTRACTS POSSESS *IN VITRO* ANTI- *HELICOBACTER PYLORI* ACTIVITY

ABSTRACT

The growing problem of antibiotic resistance by *Helicobacter pylori* demands the search for novel compounds, especially from natural sources. The anti-*H. pylori* activity of six local honeys Pure Honey (PH), Citrus Blossom (CB), Goldcrest (GC), Champagne Royal Train (CRT), Honeyline (HL) & Heritage (HH)) at four different concentrations [10, 20, 50 & 75 (% v/v)] as well as their solvent extracts (n-hexane, diethyl ether, chloroform & ethyl acetate) were evaluated by agar well diffusion method. The minimum inhibitory concentration (MIC₅₀) of the two most active extracts of each honey was determined by the broth microdilution technique and absorbances were recorded by ELISA microtitre plate reader adjusted at 620nm. Data were analyzed by one-way ANOVA test at 95% significance level. All the honey varieties as well as their solvent extracts demonstrated varying levels of antibacterial activity based on different mean zone diameters [16.0mm (crude) to 22.2mm (extract)] and percentage susceptibilities [73.3% (crude) to 93.3% (extract)] of the test isolates. The chloroform extracts of PH and CRT recorded MIC₅₀ ranges of 0.01-10% and 0.625-10 % (v/v) respectively; that were not significantly different (P > 0.05) from amoxicillin (0.001-1.25mg/mL), the positive control. In conclusion, all honeys demonstrated anti-*H. pylori* activity at concentrations $\geq 10\%$, so also did the solvent extracts. Therefore, these honeys and solvent extracts possess potential compounds with therapeutic activity which could be exploited further as lead molecules in the treatment of *H. pylori* infections.

3.1 INTRODUCTION

Helicobacter pylori (*H.pylori*) is a curved or s-shaped gram-negative, non-capsulated, non-spore forming bacillus (Ghany, 2005) that infects at least 50% of the world's human population (Czinn, 2005). It is incriminated as the main aetiological agent of gastritis (Módena *et al.*, 2007); an essential factor in the pathogenesis of peptic ulcer as well as a risk factor in the genesis of gastric carcinoma and mucosa associated lymphoid tissue (MALT) type gastric lymphoma (Roszczenko and Jagusztyn-Krynicka, 2006). The significance of these infections and the need for effective therapeutic agents have led to the development of several drug treatment regimens including dual, triple and quadruple therapy.

However, the first line regimen for eradication of the organism includes a triple therapy, which combines two known antibiotics (clarithromycin or amoxicillin and metronidazole) with a proton pump inhibitor (Mégraud and Lehours, 2007). Nevertheless, because triple therapy fails to eradicate infection in 10-20% of patients, quadruple therapy was introduced as a new treatment modality as well as a rescue treatment for antibiotic-resistant strains of *H. pylori* (Suerbaum and Michetti, 2002).

Although, most of these therapeutic modalities are approximately 90% effective, they are usually associated with high levels of antibiotic resistance (Ndip *et al.*, 2008; Tanih *et al.*, 2010a), undesirable side effects (diarrhea, nausea, abnormal taste, dyspepsia, abdominal pain/discomfort, and headache), poor patient compliance (Bytzer and O'Morain, 2005), high cost, unavailability of antibiotics especially in rural areas and the pH of gastric juice, an important factor that

potentially affects drug activity (Malekzadeh *et al.*, 2004). These therefore, result in significant levels of treatment failure and contraindications for some patients.

Accordingly, there is a strong demand for compositions with beneficial properties of current therapeutic agents that could achieve eradication of the bacterium with minimal side effects, at reduced cost, without the risk of resistance and improved efficacy of the therapy. Antimicrobial substances other than antibiotics would be very useful in the treatment of *H. pylori* infections if they are shown to be effective against both the antibiotic-resistant and susceptible strains. Over the years, there has been a lot of interest in the investigation of natural products as sources of new antibacterial agents (Shmueli *et al.*, 2007).

Several natural products have demonstrated *in vitro* antibacterial activity against *H. pylori* (Lesbros-Pantoflickova *et al.*, 2007; Ndip *et al.*, 2008; Njume *et al.*, 2011). The vast amount of data about honey's therapeutic properties, along with the rapidly increasing interest in and research into natural health remedies and supplements, has led to a resurgence in interest in honey's therapeutic uses. Honey-derived remedies constitute a potential source of new compounds that may be useful in the management of *H. pylori* infections. Honey has been recognised for its medicinal properties since antiquity (Namias, 2003). It has been shown to be active against a diverse range of micro-organisms including Gram positive and negative organisms, aerobic and anaerobic bacteria (Zaghloul *et al.*, 2001; Ndip *et al.*, 2007), and *Candida albicans* as well as inhibiting the germination of the spores of *Bacillus cereus* (El-Toun and Yagoub, 2007).

The principal antibacterial factor has been reported to be hydrogen peroxide produced by the oxidation of glucose by the enzyme glucose oxidase, which is activated by successive dilutions of honey (Iurlina and Fritz, 2005). Other antimicrobial factors include its osmolarity, acidity, low protein content and non-peroxide components (Malika *et al.*, 2004). These non-peroxide factors (flavonoids and phenolic acids) are derived from plant origin. The amount of these components may be small or diluted in the honey but when extracted with organic solvents (Aljadi and Yusoff, 2003); they become concentrated and therefore exhibit more activity. Flavonoids, phenolic and organic acids in honey are known to scavenge for free superoxide and other reactive oxygen metabolites liberated during respiratory burst in *H. pylori* induced mucosal damage (Li *et al.*, 2001).

Honeys from different countries and regions have a wide variability in their antimicrobial activity, as a result of different vegetative flowers and plant species blooming in different seasons (Ndip *et al.*, 2007; Basson and Grobler, 2008; Manyi-Loh *et al.*, 2010). Interestingly, South Africa has a large floral biodiversity with many unique plants indigenous to the region, from which various honeys are being produced. The inhabitants of the country consume these honeys with the belief that it boosts the immune system and is good for wound healing and stomach ailments. In the Eastern Cape Province of the country, the 'Xhosas' use their honeys to produce "iqhilika" (Mead), which is a cultural wine and is said to be of great medicinal value and can be used in the treatment of coughs, kidney and stomach ailments.

In addition, *H. pylori* has been reported to be prevalent and multidrug resistant strains are present in the study area (Tanih *et al.*, 2010a), a situation which merits attention. In spite of the wide body of research on the antibacterial activity of honey against several medically important pathogens including *H. pylori*, in different parts of the world, there is paucity of information, on the possible antibacterial activity of South African honeys against *H. pylori*.

Consequently, this study was designed to screen South African honey varieties and their solvent extracts for anti-*H. pylori* activity and to determine the minimum inhibitory concentration (MIC₅₀) of the two most active extracts of each honey.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains

A total of thirty clinical isolates of *H. pylori* were employed which were recovered from gastric biopsies obtained from patients presenting with gastro duodenal pathologies at Livingstone Hospital, Port Elizabeth, South Africa. This was done after informed consent was obtained as per previously reported schemes (Ndip *et al.*, 2008; Tanih *et al.*, 2010b). Confirmed isolates were stored in Brain Heart Infusion broth + 20% glycerol at -80°C for subsequent bioassays. *H. pylori* ATCC (American Type Culture collection) 43526 was used as the control strain.

3.2.2 Source and dilution of honey

In this study, six honey varieties obtained from different floral sources and localities in South Africa were used (Table 3.1). To the best of my knowledge, these honeys were raw, not processed. Different concentrations of each honey constituting, 10% v/v, 20% v/v, 50% v/v and 75% v/v were made in sterile distilled water. This was done by dissolving the respective volumes: 0.1mL, 0.2mL, 0.5mL, 0.75mL of each honey into corresponding volumes of sterile distilled water to give a 1mL final volume. The different dilutions of each honey were sterilized by filtering through a 0.22µm membrane filter (Acrodisc, Pall Corporation, USA) (Al-Somal *et al.*, 1994) into separate sterile bijoux bottles.

Table 3.1: Floral sources of honeys and their geographical locations

Honey types	Floral source	Physical property (color)	Geographical Location
Pure honey(PH)	Citrus	Dark brown	Eastern cape
Goldcrest (GC)	Citrus	Dark brown	Gauteng
Champagne royal train (CRT)	Vineyards	Dark brown	Western cape
Honeyleine (HL)	Strawberries	Dark brown	KwaZulu Natal
Heritage (HH)	Eucalyptus, fynbos, wildflower	Dark brown	Western Cape.
Citrus blossom (CB)	Berry orchards	Pale brown	Western Cape

3.2.3 Antibacterial screening of honey varieties

The agar well diffusion method was adopted according to the method of Dastouri *et al.* (2008) to assess the antibacterial activity of the crude honeys. Brain Heart Infusion(BHI) agar (Oxoid, UK) was prepared following the manufacturer’s instructions, supplemented with 7% laked horse blood (Oxoid, England) and Skirrow’s antibiotics (SR 0147E, Oxoid, UK). An inoculum of each clinical strain was prepared from a subculture of bacterial suspension and the turbidity adjusted to 1.8×10^8 CFU/mL (corresponding to 0.5 McFarland standards). A sterile cotton swab was dipped into the standardized bacterial suspension and used to evenly inoculate the BHI agar plates. The plates were allowed to dry for 3-5 minutes.

Five wells were cut in each agar plate with a flamed, cooled, cork borer of 6mm diameter, and the agar plugs removed with a sterile needle. One hundred μ L of the different concentrations of

each honey was dispensed separately into each well, in each plate. Clarithromycin (0.05µg/mL) was used as the positive control. The plates were incubated at 37°C for 2-5 days under microaerophilic conditions (CampyGen BR0056A, Oxoid, UK). Three replicates were carried out for each strain. After incubation, plates were examined and the diameters (in millimetres) of the zones of inhibition were measured not including the diameter of the well, averaged and the mean values recorded. *H. pylori* control strain ATCC 43526 was included in all the experiments.

3.2.4 Solvent extraction of crude honey

Observing that the percentage susceptibilities of the test isolates to honey varieties were greater than 50 based on zone diameter ≥ 14 mm, all honeys (except Heritage honey and Citrus Blossom) were extracted using different organic solvents. This was done using the method of Zaghloul *et al.* (2001) with modifications. One hundred grams of crude honey was placed in a 500mL separating funnel, diluted with 150mL of sterile distilled water and extracted with 150mL of the different organic solvents (n-hexane, diethyl ether, chloroform and ethyl acetate). This was performed as three successive extractions using 50mL of solvent each time.

The shaking time for each extraction process was 15minutes, after which the mixture was allowed to stand to permit the solvent layer to separate. The three successive layers were collected, mixed and concentrated by evaporation under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Padua, Italy) at 40°C for n-hexane, 30°C for diethyl ether, 50°C for chloroform, and 60°C for ethyl acetate. Water contaminating layers was removed by

filtration over anhydrous sodium sulphate. The complete extraction plan is as shown on the flow diagram (Fig.3.1).

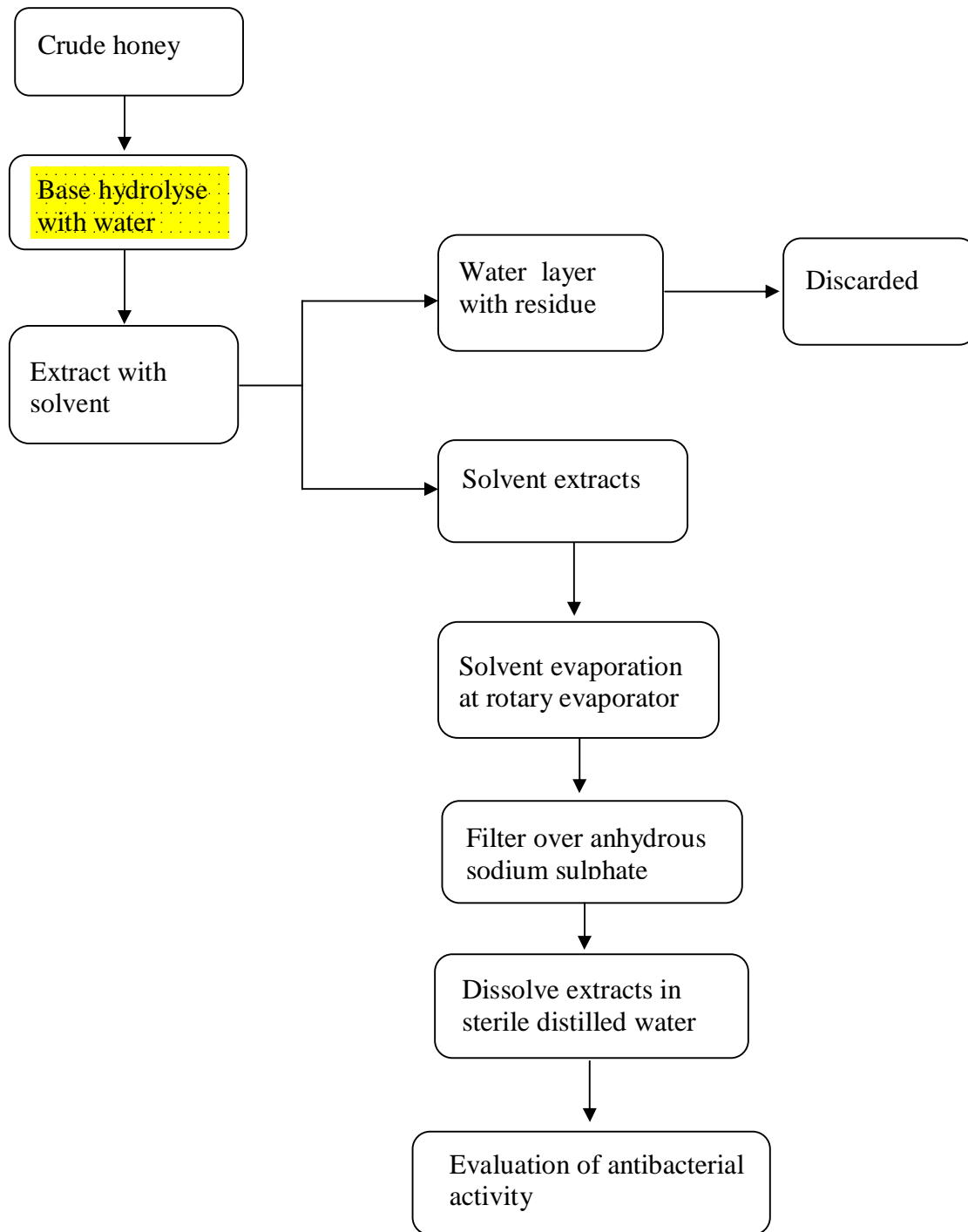


Fig.3.1. A flow chart for solvent extraction of honey

3.2.5 Antimicrobial susceptibility testing of honey solvent extracts.

The different solvent extracts of each honey at its most active concentration (50% v/v for CRT, HL; 75% v/v for PH, GC) were tested against the isolates using the method described for crude honey above. The respective pure solvent used for the extraction was tested side by side with its extract. Diameters of zones of inhibition of extracts were measured; averaged and mean values recorded in millimeters.

3.2.6 Determination of the minimum inhibitory concentration (MIC₅₀)

The two most active extracts of each honey were employed in broth microdilution assay to determine their MICs against the test isolates, according to the method of Njume *et al.* (2011). Two-fold dilutions were prepared in 96-well-round-bottom microtitre plates (Greiner Bio-One, Frickenhausen, Neuburg, Germany) in BHI broth (Oxoid, England); the final extract concentration was 0.01-10%v/v. Similarly, amoxicillin (0.0012 - 1.25 mg/mL) and metronidazole (0.01-10 mg/mL) were two-fold diluted and tested on the same plate with the honey solvent extracts as reference antimicrobials. Control wells were also prepared with culture medium only, culture medium with honey extract and culture medium with bacterial isolate only.

The inoculum of each strain was diluted tenfold in sterile normal saline. Twenty µL of the bacterial suspension (10^8 CFU/mL) was aliquoted into each well. The final volume in each well of BHI broth, honey extract, and inoculum was 120µL. The absorbencies were read using an ELISA microtitre plate reader (Model 680, S/N 19138, Biorad, Japan) adjusted at 620nm. The micro plates were sealed and incubated at 37°C under microaerophilic conditions for 2-3days,

agitated and the absorbencies were again read at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect any increase or decrease in bacterial growth. The lowest concentration of the extract resulting in inhibition of bacterial growth by 50% was taken as the MIC₅₀.

3.2.7 Statistical analysis

Diameters of zones of inhibition (excluding the diameter of the well) were expressed as mean \pm standard deviation using Excel. One-way ANOVA test was used while employing SPSS (version 18.0, Illinois, USA) to determine any statistically significant difference by comparing zone diameters of the different honeys at various concentrations; zone diameters of clarithromycin to different solvent extracts, zone diameters of different extracts as well as the MIC values of these extracts to amoxicillin and metronidazole at 95% significance level.

3.3 RESULTS

3.3.1 Antibacterial screening of honey varieties

All the honey samples at the various concentrations (10, 20, 50, 75%v/v) exhibited varying levels of antibacterial activity against the test isolates as indicated by mean \pm S.D of zone of inhibition and percentage susceptibilities (Table 3.2).The susceptibilities of the isolates to the honey varieties are shown on figure 3.2. PH, CB and GC honeys were most active at 75% v/v concentration whilst CRT, HL and HH were most active at 50% v/v concentration. Consequently, there was no statistically significant difference ($P > 0.05$) recorded when the mean zone diameters of these honeys obtained at their most active concentrations were compared to that of clarithromycin (positive control).

Table 3.2: Analysis of the anti-*Helicobacter pylori* activity of honey varieties by agar well diffusion assay

Honey types	Zones of inhibition (mm) ^a and Percentage susceptibility (%)			
	Concentrations of honey(% v/v)			
	10	20	50	75
Pure honey(PH)	12.0 ± 8.9 16/30(53.3)	13.3 ± 7.8 19/30 (63.3)	12.7 ± 9.9 16/30(53.3)	16.0 ± 7.5 22/30 (73.3)
Citrus blossom (CB)	13.3 ± 7.5 17/30 (56.7)	12.6 ± 8.0 17/30 (56.7)	15.1 ± 8.7 20/30 (66.7)	15.5 ± 8.5 21/30 (70.0)
Goldcrest(GC)	13.3 ± 7.8 18/30(60.0)	12.9 ± 7.9 16/30(53.3)	14.4 ± 9.0 18/30(60.0)	13.7 ± 10.0 20/30(66.7)
Champagne royal train(CRT)	11.0 ± 8.6 16/30 (53.3)	12.5 ± 9.1 15/30 (50.0)	12.7 ± 8.6 17/30 (56.7)	16.0 ± 7.4 16/30 (53.3)
Honeyleine(HL)	11.0 ± 8.7 14/30 (46.7)	11.7 ± 8.5 17/30 (56.7)	13.0 ± 9.2 19/30 (63.3)	14.8 ± 8.6 18/30 (60.0)
Heritage(HH)	11.6 ± 9.2 16/30 (53.3)	12.4 ± 8.4 17/30 (56.7)	13.1 ± 9.2 18/30 (60.0)	15.1 ± 6.1 16/30 (53.3)
Clarithromycin (CLR);0.05µg/mL		18.0 ± 7.3 23/30(76.7)		

^a mean of triplicate assay ± standard deviation; Zone of sensitive isolate ≥ 14mm.

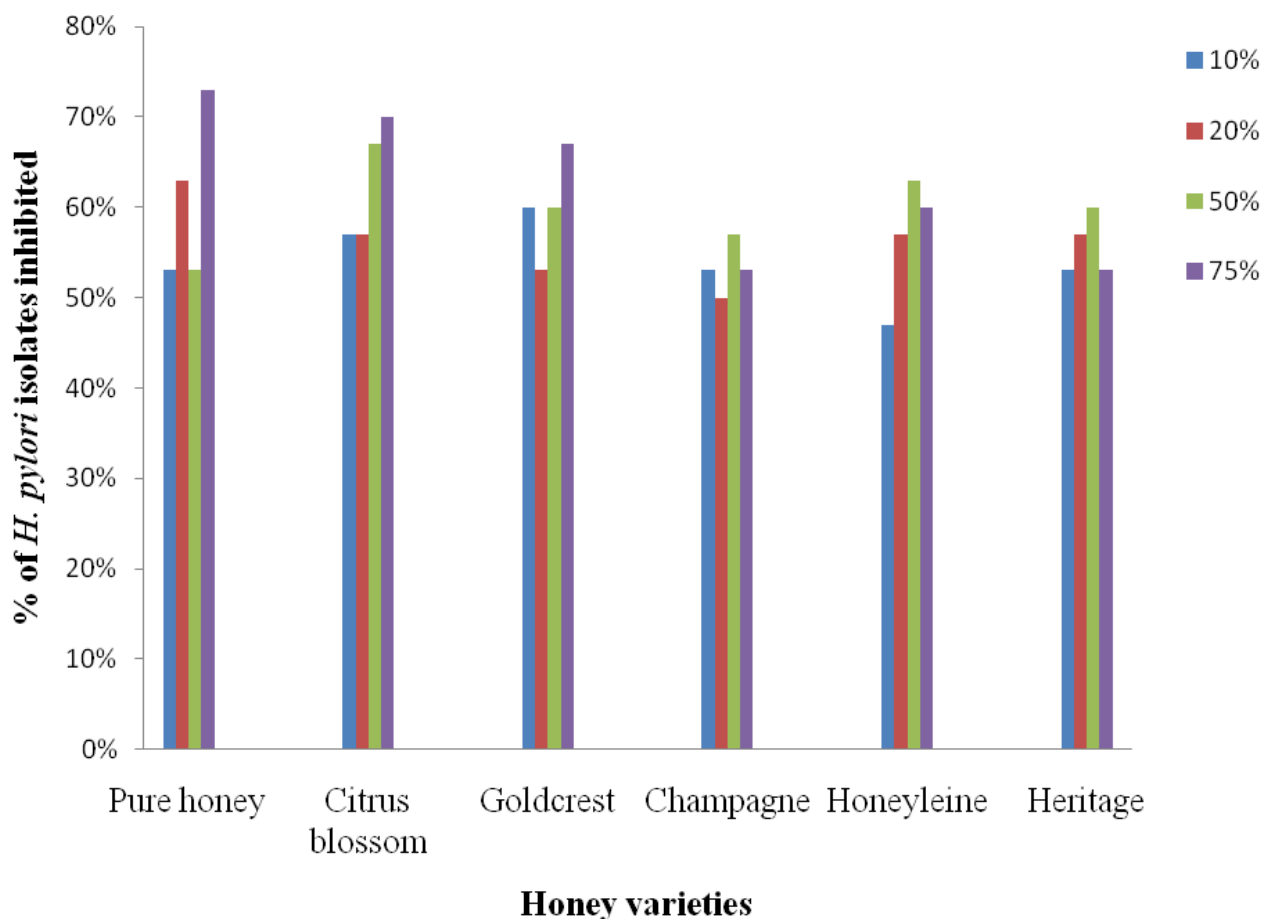


Fig.3.2: Antimicrobial activity of honey varieties at 10% v/v, 20% v/v, 50% v/v, 75% v/v against *H. pylori* isolates. Zone diameters of sensitive isolates were ≥ 14 .

3.3.2 Antimicrobial susceptibility testing of honey solvent extracts

All the solvent extracts of the honeys demonstrated anti-*H. pylori* activity with mean zone diameter of inhibition and percentage susceptibility of isolates in the ranges 14.5- 22.2 mm and 53.3-93.35% respectively (Table 3.3). Based on the percentage susceptibility and mean zone diameter of inhibition, diethyl ether extract of CRT honey was the most active (22.2 ± 6.1 mm; 28/30, 93.3%) while the least antibacterial activity was noted for n-hexane extract of PH honey

(15.8±7.9mm; 16/30, 53.3%). However, no statistically significant difference ($P > 0.05$) was reached comparing the mean zone diameters of solvent extracts to clarithromycin.

Table 3.3: Analysis of the anti-*Helicobacter pylori* activity of solvent extracts by agar well diffusion assay

Honey types	Zones of inhibition (mm) ^a and Percentage susceptibility (%)			
	Solvent extracts of honey varieties			
	n-Hexane	Diethyl ether	Chloroform	Ethyl acetate
Pure honey(PH)	15.8 ± 7.9	18.8 ± 8.3	16.9 ± 6.6	16.3 ± 7.6
	16/30 (53.3)	21/30 (70)	19/30 (63.3)	18/30 (60.0)
Goldcrest (GC)	17.9 ± 8.7	19.9 ± 10.1	15.2 ± 8.7	16.7 ± 9.3
	19/30 (63.3)	22/30 (73.3)	17/30 (56.7)	18/30 (60.0)
Champagne royal train (CRT)	18.5 ± 6.6	22.2 ± 6.1	21.2 ± 6.4	16.2 ± 8.6
	25/30 (83.3)	28/30 (93.3)	27/30 (90.0)	23/30 (76.7)
Honeyleine (HL)	14.5 ± 9.3	17.9 ± 11.0	15.9 ± 9.1	17.5 ± 8.9
	20/30 (66.7)	20/30 (66.7)	19/30 (63.3)	22/30 (73.3)
Clarithromycin		18.0 ± 7.3		
(CRT);0.05µg/mL		23/30(76.7)		

^a mean of triplicate assay ± standard deviation; Zone of sensitive isolate ≥ 14.

3.3.3 Determination of the minimum inhibitory concentration (MIC₅₀)

Inhibitory-zone testing is the primary method for evaluation of the susceptibility of the test isolates to honey solvent extracts. Given the relatively low sensitivity of this method, MIC testing was used as complementary method to evaluate the antibacterial activity of the solvent extracts. The MIC₅₀ values of all the extracts ranged from 0.01-10% v/v concentration (Table 3.4). The two most active extracts were the chloroform extract of PH and CRT honeys with MIC₅₀ ranges of 0.01-10% v/v and 0.625-10% v/v respectively; since there was no statistically significant difference ($P > 0.05$) recorded when their MIC₅₀ values were compared to amoxicillin (0.001-1.25mg/mL), the most sensitive antibiotic in the local treatment regimen. The ethyl acetate extract of HL and n-hexane extract of GC honeys recorded MIC₅₀ values of 0.156-10% v/v and 0.039-10%v/v respectively.

Table 3.4: MICs of the two most active extracts of each honey and reference antimicrobials at 50% bacterial growth inhibition

<i>H. pylori</i> isolates	MIC ₅₀ values in different concentrations									
	Concentrations of solvent extracts (%v/v)								Antibiotics (mg/mL)	
	Pure Honey(PH)		Champagne (CRT)		Honeyleine (HL)		Goldcrest (GC)		Amoxicillin	Metronidazole
	CHLOE	DEE	CHLOE	DEE	EAE	DEE	n-HEX	DEE	AMOX	MET
PE11A	-	-	-	-	-	-	-	-	0.039	5
PE11C	-	-	-	-	-	-	5	5	0.078	5
PE26A	-	-	1.25	-	-	-	-	-	0.02	5
PE76A	1.25	10	10	-	-	-	-	-	0.039	5
PE84C	-	-	-	10	1.25	-	10	-	0.01	2.5
PE93A	1.25	1.25	0.625	0.625	-	0.625	5	5	0.625	5
PE102C	10	-	-	-	10	-	-	10	0.039	5
PE115A	10	-	-	-	10	-	10	-	0.02	5
PE162C	-	-	-	-	5	-	5	5	0.001	5
PE219C	10	1.25	0.625	0.625	2.5	1.25	-	-	0.625	5
PE252C	1.25	-	10	10	2.5	-	2.5	1.25	0.039	5
PE258C	1.25	2.5	2.5	2.5	-	1.25	-	-	0.313	10
PE308C	1.25	10	-	10	2.5	-	2.5	1.25	0.078	5

PE369A	1.25	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE369C	-	-	-	10	0.625	-	10	0.625	0.02	5
PE397C	1.25	-	-	-	1.25	10	5	5	0.625	5
PE406C	2.5	10	-	10	10	-	5	1.25	0.156	5
PE407C	-	10	-	10	-	-	-	-	0.313	5
PE411C	-	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE430A	10	-	-	10	-	-	2.5	-	0.01	5
PE430C	-	10	-	10	0.156	-	-	0.078	0.039	5
PE435A	2.5	1.25	1.25	1.25	-	1.25	-	-	0.039	5
PE436A	-	0.625	0.625	0.625	2.5	1.25	1.25	1.25	0.625	5
PE462A	-	-	-	-	-	-	0.039	-	0.625	5
PE462C	-	-	0.625	-	-	10	-	-	1.25	2.5
PE464A	-	10	-	10	10	-	-	-	0.078	5
PE464C	1.25	-	-	-	2.5	10	1.25	1.25	0.002	2.5
PE466A	10	-	-	-	10	-	10	10	0.01	5
PE466C	0.01	-	-	-	-	-	-	-	0.625	5
PE473A	2.5	-	-	10	2.5	-	2.5	2.5	0.156	5

MIC₅₀ after triplicate assay; -, value not within susceptible range; CHLOE, chloroform; DEE, diethyl ether; HEX, hexane; EAE, ethyl acetate; AMOX, amoxicillin; MET, metronidazole.

3.4 DISCUSSION

The indiscriminate use of antibiotics has developed many resistant micro-organisms creating immense clinical problems in the treatment of infectious diseases such as those caused by *H. pylori*. Therefore, there is a need to develop alternative antimicrobial agents for the treatment of these infectious diseases. A non-antibiotic approach to the treatment and prevention of these infections includes the application of honey. Honey is produced from many different floral sources and its antimicrobial activity varies with origin and processing (Weston, 2000). Considering the enormous potential using honey in a clinical setting, it is important that research continue not only using honeys that are commercially available but also those of local origin with a dearth of information on their antimicrobial potential.

Therefore, in the preliminary screening of PH, CB, GC, CRT, HL and HH honeys at various concentrations (10, 20, 50 and 75% v/v), the data showed that these honeys possess anti-*H. pylori* activity exhibited by zones of inhibition that ranged from 11.0-16.0 mm. This inhibition gives credence to the fact that these honeys could be used as antibacterial agents in the treatment of ailments caused by *H. pylori*.

Furthermore, this result is congruent with the work of Saraf and colleagues (2009) that evaluated the antibacterial activity of local Fijian honeys on both gram- positive and gram-negative bacteria at varying concentrations. With the exception of the other three honeys, the anti-*H. pylori* activity of PH, CB and GC honeys was concentration dependent since they were most active at 75% v/v concentration (Fig.3.2). In addition, there was no statistically significant difference ($P > 0.05$) recorded when the mean zone diameters of these local honeys at their most active concentrations were compared to that of clarithromycin. This may

advocate that these honeys at their specified concentrations could elicit antibacterial potential seeming that of clarithromycin, a key antibiotic in *H. pylori* treatment regimen (Megraud and Lehours, 2007).

In spite of the fact that all the honeys demonstrated antibacterial activity against the test isolates, there was variation in the percentage susceptibilities of the isolates to the different honey types (Fig.3.2). This discrepancy in the observed antibacterial activity could be due to several reasons. One possibility might be related to the differences in susceptibility of each isolate to the honey used, since the pathogen is said to exhibit considerable genetic heterogeneity, therefore no two strains are identical (Logan and Walker, 2002).

Moreover, the variable results observed between honeys has purportedly been attributed to different floral sources and plant species utilized by the bees and geographical factors like temperature, humidity where the honeys were produced (Nzeako and Hamdi, 2000; Ndip *et al.*, 2007). Apparently, the environmental conditions would influence the chemical composition of the honeys and as a consequence their biological properties/functions would differ. This may hold true for this study since the honeys were obtained from different floral sources and localities in South Africa.

Other possible explanation for these observations could be the differences in putative antibacterial agent(s) present in these honeys (Tumin *et al.*, 2005). These agents may utilize hydrogen peroxide and non-peroxide antioxidant component. As reported by others (Melissa *et al.*, 2004; Ndip *et al.*, 2007) dilution of honey enhances hydrogen peroxide mediated antibacterial activity which may explain the discrepancies observed with the antibacterial activity of these honeys because its concentration is determined by the relative levels of

glucose oxidase synthesized by the bee and catalase obtained from plant pollen (Weston, 2000).

The non-peroxide factors of honeys include volatile compounds, phenolic acids and flavonoids (phytochemicals). The accumulation of these phytochemicals depends on climatic conditions (sunlight, moisture), soil characteristics and other factors; therefore it is logical to have disparity in the antibacterial activity between the honeys types due to differences in the composition of pollen or nectar, which have greatest influence on the chemical composition of these honeys (Kaškonienė and Venskutonis, 2010).

In the past, several studies have reported the antimicrobial activity of only the aqueous solution of honey (Taormina *et al.*, 2001; French *et al.*, 2005; Ndip *et al.*, 2007). It is obvious that honey possesses antimicrobial potential but it is not clear whether it is the mass of the honey or some fraction of it. In view of the fact that there might be some specific components (phytochemicals or non-peroxide factors) such as flavonoids, volatile compounds and phenolic acids which may be contributing to the antimicrobial activity, four organic solvents viz n-hexane, diethyl ether, chloroform and ethyl acetate that have been previously employed by Zagloul *et al.* (2001) to possibly extract the afore mentioned phytoconstituents were used.

Accordingly, PH, GC, CRT and HL honeys were employed in the extraction process except HH and CB honeys that were obtained from the same locality as CRT honey. The physical properties (particularly color) credited the continued usage of CRT honey in the subsequent bioassays. The color of honey is closely related to its chemical composition, primarily to the presence of carotenoids, flavonoids and derivatives of tannins and polyphenols (Kaškonienė *et al.*, 2009). Moreover, Yao *et al.* (2003) reported that the antimicrobial potential of honey

could be ascribed to its chemical composition. The solvent extracts of the honeys were evaluated for anti-*H. pylori* activity. The resulting data showed that the most potent antibacterial activity against the test isolates was demonstrated by the diethyl ether extract of CRT honey (22.2 ± 6.1 mm; 28/30, 93.3%) unlike the n-hexane extract of PH honey (15.8 ± 7.9 mm; 16/30, 53.3%) that presented the least antibacterial activity. This was based on mean \pm S.D of zone diameter of inhibition observed with each solvent extract as well as the percentage susceptibilities of the test isolates to each extract (Table 3.3).

Notwithstanding, all the solvent extracts presented good anti-*H. pylori* activity implying that these honeys may contain a diverse range of bioactive components which were extractable into the various solvent extracts. This corroborates the finding of Chauhan *et al.* (2010) that equally reported significant antibacterial activity of solvent extracts of honey against gram negative micro-organisms. In addition, there was no statistically significant difference ($P > 0.05$) recorded when the mean \pm S.D of diameters of zones of inhibition were compared to the positive control (clarithromycin); suggesting that the specific solvent extracts may contain putative antimicrobial compounds whose therapeutic potential are highly comparable to clarithromycin.

Furthermore, we realized an increase in the antimicrobial activity of the solvent extracts of these honeys. Based on mean zone diameter and percentage susceptibilities of test isolates, there was an increase from 16.0mm (crude) to 22.2mm (extracts) and 73.3% (crude) to 93.3% (extract) respectively. It is noteworthy that the diameter of zone of inhibition was considered as a measure of antibacterial activity. Therefore, the larger the zone of inhibition, the more active or the greater the activity of the product (Manyi-Loh *et al.*, 2011). These results

suggest that there was an increase in antibacterial activity of honey after extraction, insinuating the putative antibacterial agents might have been isolated from the bulk of honey.

In the assay to determine MIC₅₀ of the two most active extracts of each honey, amoxicillin and metronidazole were employed as reference antimicrobials. The assay indicated that the MIC₅₀ values varied with extracts and the isolates (Aljadi and Yusoff, 2003). This variation could be attributed to putative components present in the different extracts. The lowest MIC₅₀ range (best antibacterial activity) of 0.01-10% was obtained for the chloroform extract of PH honey.

Both the ethyl acetate extract of HL and n-hexane extract of GC honeys recorded MIC₅₀ values of 0.156-10% v/v and 0.039-10% v/v respectively. In addition, no statistically significant difference ($P > 0.05$) was recorded when MIC₅₀ values of the chloroform extracts of PH (0.01-10% v/v) and CRT (0.625-10% v/v) honeys were compared to amoxicillin (0.001-1.25mg/mL). This may suggest that of the four solvents used for extraction in this study, chloroform extracts demonstrated the highest relative antibacterial activity; advocating that chloroform solvent might have had higher solubility for antimicrobial phytoconstituents because it has been reported that different solvents have the capacity to extract different phytoconstituents based on their solubility or polarity (Doughari, 2006). This may further indicate that these chloroform extracts would contain most active substances (Yeşilada *et al.*, 1999).

3.5 CONCLUSIONS

The results of this study unequivocally shows that PH, GC, CRT and HL honeys (bulk components) as well as the necessary antibacterial components extracted in n-hexane, diethyl ether, chloroform and ethyl acetate solvents from these honeys could become potential candidates to be exploited for further investigation in the discovery of novel natural anti-*H. pylori* compounds, which would be useful on a sustainable basis.

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CHAPTER FOUR

BACTERICIDAL ACTIVITY OF SOLVENT EXTRACTS OF SELECTED SOUTH AFRICAN HONEYS AGAINST CLINICAL ISOLATES OF *HELICOBACTER PYLORI*

ABSTRACT

The growing resistance of *Helicobacter pylori* (*H. pylori*) to antibiotics used in its treatment as well as other inherent limitations of the triple therapy has brought forth the quest for alternative treatment from natural sources. Apitherapy has shown promising effects as an alternative source of *H. pylori* treatment. In this light, selected South African honeys and their solvent extracts have demonstrated considerable anti-*H. pylori* activity. Therefore, in this study the most active solvent extracts of each honey; chloroform extracts of Pure Honey (PH) and Champagne Royal Train (CRT) as well as the n-hexane and ethyl acetate extracts of Goldcrest (GC) and Honeyleine (HL) honeys respectively were evaluated for their bactericidal activity against *H. pylori* strains. The time kill assay of the most active extracts was determined by viability studies over a period of 72hrs. The most potent bactericidal effect against the test isolates was obtained with 5% v/v (1/2 MIC) concentration of chloroform extract of PH from 36-72hrs. Notwithstanding, the other three solvent extracts were most bactericidal at 40% v/v (4MIC) at different time intervals. In conclusion, these honeys and their extracts may contain compounds with anti-*H. pylori* activity and therefore calls for more elaborate phytochemical studies to isolate and characterize the compounds.

4.1 INTRODUCTION

Helicobacter pylori (*H. pylori*), has probably been part of the human gastric biota since time immemorial (Blaser, 1997). It is a human pathogen, which is directly associated with many diseases of the upper gastrointestinal tract including acute and chronic gastritis, non-ulcer dyspepsia, peptic ulcer disease (gastric and duodenal ulcers), and gastric cancers (Williamson, 2001). It is a major public health concern, since these diseases are major causes of death worldwide. Eradication of this pathogen is now a major step in the therapeutic management of the above mentioned diseases (Beasles, 2001).

Conventional treatment of *H. pylori* infection is principally based on the use of triple therapies consisting of a combination of two antibiotics (clarithromycin, amoxicillin, and/or metronidazole) with a proton pump inhibitor (Mégraud and Lehours, 2007). Unfortunately, *H. pylori* is capable to develop drug resistance after a period of repeated use of these drugs especially at suboptimal levels (Matsumoto *et al.*, 1997). Such resistant strains are becoming problematic worldwide (Ochi *et al.*, 2005) and may lead to high failure rates of treatment regimens. In addition, non-compliance of patients to the treatment regimen as well as side effects of the drugs may also result in eradication failure. These necessitate the search for new chemotherapeutic agents with excellent activity, which hopefully can eradicate the pathogen.

Honey is among the attractive sources that has received attention as an alternative treatment for *H. pylori* infections. Apitherapy or therapy with bee products is an age-old therapeutic practice as recorded by several ancient civilizations. Honey is a natural sweet substance,

produced by bees from plant nectars, plant secretions and excretion of plant sucking insects (Williams *et al.*, 2009). It is a supersaturated sugar solution containing vitamins, minerals, proteins, amino acids and nutrients. The largest portion of it as dry matter consists of sugars (79%), which are responsible for much of its physical nature, viscosity, hygroscopicity and energy content (Ansari and Alexander, 2009). The therapeutic effects of honey have been ascribed to its antimicrobial, anti-inflammatory and anti-oxidant properties.

Honey has shown powerful antibacterial effects against pathogenic and non-pathogenic micro-organisms, yeast and fungi even against those that developed resistance to many antibiotics (Molan and Cooper, 2000; Manyi-Loh *et al.*, 2010). Its sugars exert strong osmotic potential attracting water molecules and as such inhibit the growth of bacteria and fungi. In addition, it contains the enzyme glucose oxidase, which acts on glucose in the presence of water, producing hydrogen peroxide and gluconic acid. Hydrogen peroxide is the major contributor to the antimicrobial activity of honey, and the different concentrations of this compound in different honeys result in their varying antimicrobial effects (Molan, 1992).

Furthermore, antioxidants and flavonoids that may function as antibacterial agents are also present. The pH of honey is low and ranges from 3.2 to 4.5 with the most predominant proton donor being gluconic acid. In light of modern science, several important therapeutic effects of honey have been elucidated (Cooper *et al.*, 2002) and these vary with the quality of the honey produced. The type of honey produced is dependent on the flowers blooming in different seasons, in different regions and countries (Ndip *et al.*, 2007). Consequently, there is variation in the chemical composition as well as the physical properties of honey. As a result,

micro-organisms differ in their sensitivity to honeys collected from different sources, regions and countries.

Pure honey has demonstrated bactericidal activity against many enteropathogenic organisms, including those of the *Salmonella* and *Shigella* species, and enteropathogenic *E. coli* (Molan, 2001). *In vitro* studies suggested that honey possesses bactericidal activity against *H. pylori* (Al-Somal *et al.*, 1994; Ndip *et al.*, 2007). Even isolates that exhibited resistance to other antimicrobial agents were susceptible to honey (Ali *et al.*, 1999). The antibacterial action of honey could be bacteriostatic (inhibitory) or bactericidal (killing) (Osman *et al.*, 2003).

Based on the promising or considerable activity (inhibitory) presented by the solvent extracts in the previous study, it is therefore necessary to evaluate the bactericidal activity of the most active solvent extract of each honey type (PH, CRT, GC& HL). Consequently, this study was designed to ascertain the rate of kill of *H. pylori* strains by the chloroform extracts of PH and CRT, n-hexane extract of GC as well as ethyl acetate extract of HL honeys.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains

H. pylori strains used were cultured from gastric biopsy specimen obtained from patients with gastroduodenal pathologies attending the endoscopic unit of Livingstone Hospital, Port Elizabeth, South Africa. This was done after informed consent was obtained as per previously reported schemes (Ndip *et al.*, 2008; Tanih *et al.*, 2010). Confirmed isolates were stored in Brain Heart Infusion broth plus 20% glycerol at -80°C for subsequent bioassays. *H. pylori* ATCC (American Type Culture Collection) 43526 was used as the control.

4.2.2 Preparation of honey solvent extracts

Based on the antibacterial activity (MIC₅₀) of the solvent extracts; the chloroform extracts of PH & CRT, n-hexane extract of GC and ethyl acetate extract of HL were the most active solvent extracts of the corresponding honey type. Therefore, each honey was extracted with the stated solvent (i.e. PH & CRT were extracted with chloroform; GC and HL were extracted with n-hexane and ethyl acetate respectively). This was done according to the method of Manyi-Loh *et al.* (2010). Briefly, 100 grams of crude honey was placed in a 500 mL separating funnel, diluted with 150 mL of sterile distilled water and extracted with 150 mL of the stated solvent for each honey type. This was performed as three successive extractions using 50 mL of solvent each time.

The shaking time for each extraction process was 15 min, after which the mixture was allowed to stand to permit the solvent layer to separate. The three layers were collected, mixed and concentrated by evaporating the extract under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Padua, Italy) at 40°C for n-hexane and 50°C for

chloroform and 60 °C for ethyl acetate respectively. Water contaminating extracts was removed by filtration over anhydrous sodium sulphate.

4.2.3 Time-kill assay of solvent extracts

Assay for the rate of kill of *H. pylori* isolates by the most active extract of each honey was determined in accordance with the method of Akinpelu *et al.* (2008) with modifications. Each isolate was subcultured on CBA (Oxoid, England) plates and incubated at 37°C under microaerophilic conditions for 2-3 days. Growth of each isolate was transferred into BHI broth (Oxoid, England) and incubated overnight under the same growth conditions. The turbidity of an 18 h old broth culture of the test isolate was standardized to contain approximately 1.8×10^8 cfu/mL.

A 0.5mL volume of the standardized suspension was added to 4.5 mL of different concentrations of the extracts (1/2MIC, MIC, 2xMIC and 4xMIC). These were incubated at 37°C under microaerophilic condition in an orbital shaker at 120rpm and the killing rate was determined over a period of 72hrs. Exactly 0.5mL volume of each suspension was withdrawn at 6h intervals and transferred to 4.5mL of BHI broth recovery medium containing 3% "Tween 80" to neutralize the effects of the antimicrobial compound carry-overs from the test isolates.

The suspension was serially diluted and 100µL plated out for viable counts. The plates were later incubated at 37 °C for 72hrs. The control plates contained the bacterial cells without the

extract. The emergent bacterial colonies were counted and compared to the counts of the culture control. Time-kill assays were carried out in duplicate.

4.3 RESULTS

4.3.1 Time kill assay of solvent extracts

The most active extract of each honey (i.e. chloroform extract of PH and CRT honeys, n-hexane extract of GC honey as well as ethyl acetate extract of HL honey) was employed in this assay to ascertain the rate of kill of the test isolates. The results of the bactericidal activity of these solvent extracts over the period of 72hrs are presented on figs.4.1- 4.5. With the exception of chloroform extract of PH, the most potent bactericidal effect was established at 4xMIC (40% v/v) against the test isolates by all the honey solvent extracts at different time interval. Interestingly, at 1/2 MIC (5% v/v) the chloroform extract of PH honey demonstrated a profound bactericidal activity over a time interval of 36-72hrs.

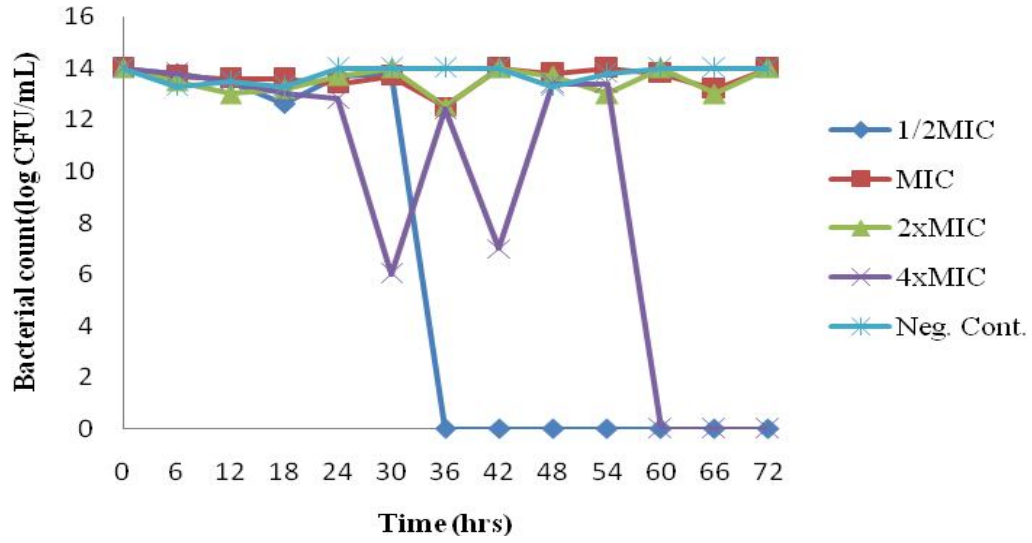


Fig. 4.1: Bactericidal activity of chloroform extract of PH honey against PE 252C

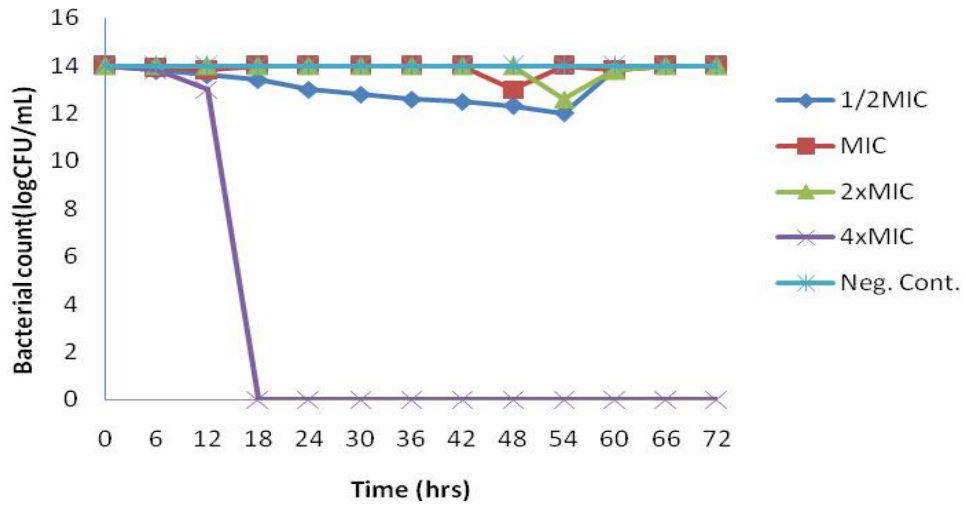


Fig.4.2: Bactericidal activity of chloroform extract of CRT honey against PE 252C

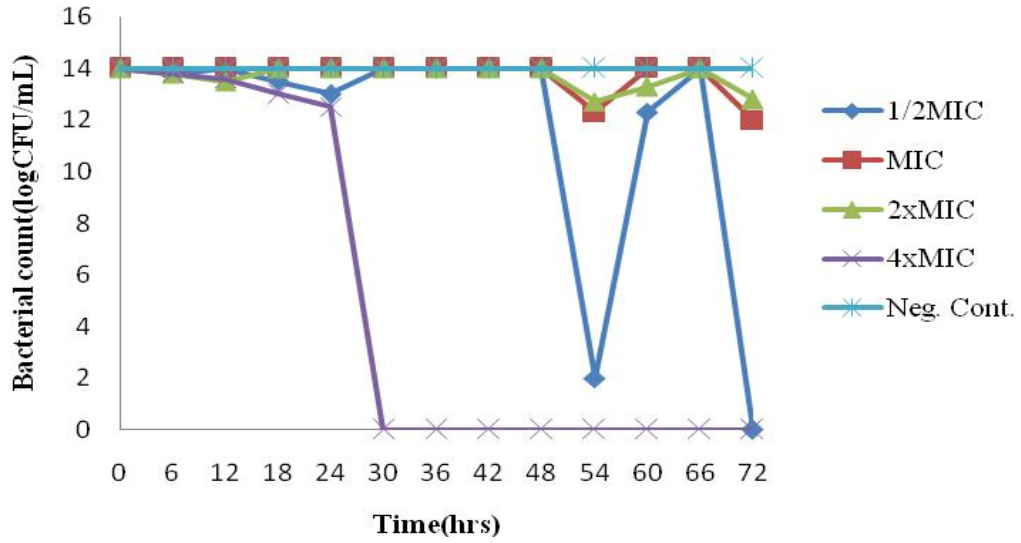


Fig. 4.3: Bactericidal activity of ethyl acetate extract of HL honey against PE 252C

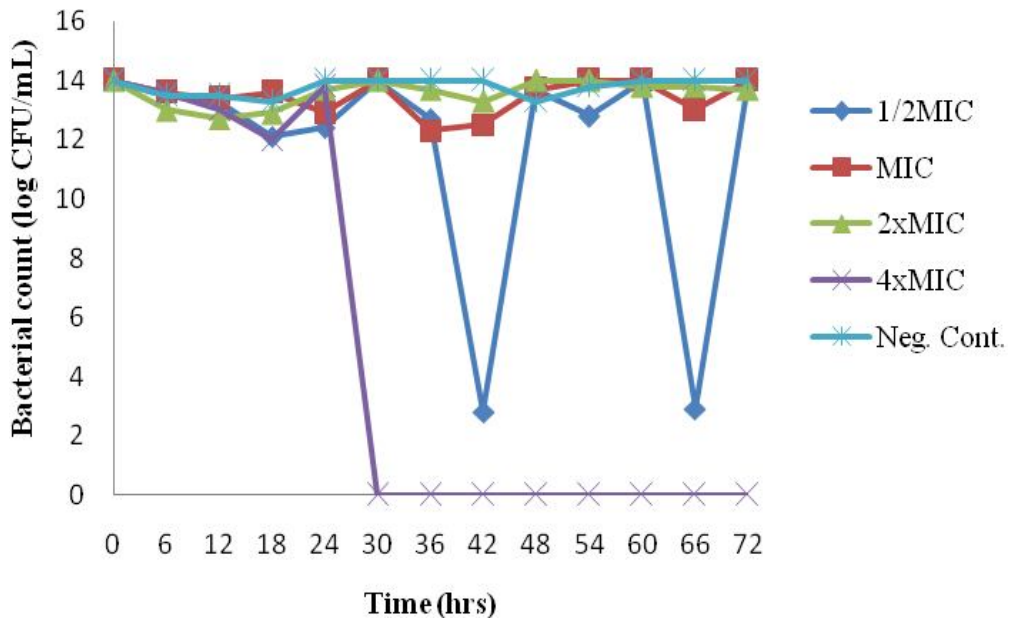


Fig. 4.3: Bactericidal activity of n-hexane extract of GC honey against PE 252C

4.4 DISCUSSION

Antimicrobial chemotherapy of *H. pylori* infections using the triple therapy is fraught with many inherent limitations such as cost, undesirable side effects, resistance and patient non-compliance. The current rising prevalence of antibiotic-resistant *H. pylori* strains has led to a re-evaluation of the therapeutic use of natural products which are perceived as pure, and without side effects (Montbriand, 2004). Several studies have reported the susceptibility of *H. pylori* isolates to various brands of honeys both locally and commercially produced (Ali *et al.*, 1999; Ndip *et al.*, 2007).

The bactericidal activity of the chloroform extracts of PH and CRT honeys, n-hexane extract of GC honey as well as ethyl acetate extract of HL honey was determined using viability studies. At different time intervals during cultivation, broth constituted with different concentrations of the solvent extracts were sampled, diluted and the total colony counts on the plates were enumerated in order to determine the relationship between the concentration of the solvent extracts, treatment time and bactericidal activity (Wang and Huang, 2005). Actually, the isolates were initially cultivated in the presence of the solvent extracts contained in the broth and subsequently, sampled, diluted and re-cultivated in the absence of the solvent extracts. If no colony formation was observed at that point, the extract had bactericidal activity (Coudon and Stratton, 1998).

In this assay, it was noticed that at the time interval of 36-72hrs the test isolate was killed at a concentration as low as 5% v/v (1/2xMIC) by the chloroform extract of PH honey, thus it was the most effective bactericidal extract (Fig.4.1). At the highest concentration of 40% v/v (4x MIC), chloroform extract of CRT, ethyl acetate extract of HL, and n-hexane extract of GC presented potent bactericidal activity over the time interval of 18-72hrs and 30-72hrs

respectively (Fig.4.2- 4.5). This may suggest that with a further increase in extract concentration better results would be obtained. In addition, there was growth of bacterial cells at 10% v/v (MIC), showing they were inhibited as expected.

4.5 CONCLUSION

Chemical antibiotics have become obsolete within a short period of time due to multidrug resistance presented by micro-organisms of medical importance particularly *H. pylori*. The need for an alternative antibacterial substance derived from natural products on a sustainable manner has become a subject of interest to scientists' the worldover. In this study, all the solvent extracts of the different honeys demonstrated bactericidal activity against the test strains at varying time intervals.

Furthermore, due to the good inhibitory and bactericidal activity elicited by the chloroform extracts of PH and CRT, it is therefore needed to isolate and characterize their bioactive constituents in a bid to have a clear understanding on the antibacterial activity of these honey types.

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CHAPTER FIVE

ISOLATION AND IDENTIFICATION OF VOLATILE BIOACTIVE COMPOUNDS

WITH ANTI- *HELICOBACTER PYLORI* ACTIVITY FROM HONEY

CHLOROFORM EXTRACTS

ABSTRACT

The chloroform extracts of Pure Honey (PH) and Champagne Royal Train (CRT) were fractionated with n-hexane, chloroform and subsequently with the solvent systems, n-hexane: ethyl acetate: acetic acid (S1) or methanol: acetic acid: water (S2) respectively, and obtained several fractions that were evaluated for anti-*Helicobacter pylori* activity. All the fractions obtained with S1 solvent system demonstrated potent anti-*H. pylori* activity with PHF5 being the most active with a mean MIC₅₀ value of 1.25mg/mL that was not significantly different ($P > 0.05$) from amoxicillin. Chemical analysis of the fractions led to the identification of 24 volatile compounds belonging to known chemical families present in honey. From these, the pure compound, linalool demonstrated potent antibacterial activity at a concentration of 0.002 and 0.0313mg/mL with inhibitory activity of 95% growth inhibition. The minimum bactericidal concentration (MBC) was in the range 0.0039-0.313mg/mL. Thiophene and N-methyl-D3-aziridine were novel compounds identified in this study. They are small heterocyclic compounds which serve as essential intermediates in the synthesis of natural products and pharmaceuticals endowed with immense biomedical activities. Thus, the solvent extracts and fractions of these honeys could serve as potential anti-*Helicobacter pylori* regimens with therapeutic potential.

5.1 INTRODUCTION

Antibiotics provide an invaluable tool for the control of microbial infections, however, microorganisms are gaining multi-resistance to these antibiotics after repeated treatment and this is a growing problem worldwide. In a bid not to select for further resistant strains, there has been a lot of interest in the investigation of natural products as sources of new antibacterial agents. Natural products and their derivatives (including antibiotics) represent more than 50% of all drugs in clinical use in the world (Mohapatra *et al.*, 2010). Honey is a natural product with several known biological activities and has been used in folkloric medicine in different cultures for a long time. It may be used alone or in combination with other substances and has been administered both orally and topically.

It is well established that honey inhibits a broad spectrum of bacterial species including aerobes and anaerobes, Gram positives, and Gram negatives. Its antimicrobial action could be bacteriostatic as well as bactericidal and may be principally useful against bacteria, which have developed resistance to antibiotics (Patton *et al.*, 2006). The antimicrobial activity in honey has been attributed to its high sugar content, low water content, acidity and hydrogen peroxide.

Hydrogen peroxide is known as the major contributor to the antibacterial activity, however, it could be destroyed when honey is diluted in the presence of catalase; as a consequence the antibacterial activity is reduced or eliminated. It appears that there are some honeys that retain their antibacterial activity even in the presence of catalase or when heated. These honeys therefore rely on components other than hydrogen peroxide for their potency with their antimicrobial compounds being postulated to be phytochemicals (Gheldof *et al.*, 2002).

Besides its antimicrobial activity, honey can clear infections in a number of ways, including boosting the immune system, having anti-inflammatory and antioxidant activities, and via stimulation of cell growth (Al-jabri, 2005; Manyi-Loh *et al.*, 2011).

Despite the beneficial effects reported by researchers, there are some unpleasant attributes. Honey may be contaminated by bacterial spores (*Bacillus* and *Clostridium* genus), by yeast (*Saccharomyces*, *Schizosaccharomyces* and *Torula* strains) and by fungi (*Penicillium* and *Mucor* strains). Furthermore, altered mental status could be caused by grayanotoxin contaminated in honey (Gunduz *et al.*, 2008).

Depending on the sources of nectar, the composition of honey can be variable and diverse between bee species, locations or seasons due to foraging from different plant sources. This is directly related to the fact that the properties of honey depend on various factors, including the plant sources, climate, environment and bee species (Chanchao, 2009). Honey contains about 181 substances, including sugars, proteins, moisture, vitamins, minerals, hydroxymethylfurfural (HMF), enzymes, flavonoids, phenolic acids, volatile compounds *etc.* (Ayoub *et al.*, 2009). Some of these substances could be extracted with organic solvents and have been reported to exhibit antimicrobial, antioxidant and other health beneficial properties.

Flavonoids and phenolic molecules have been demonstrated to have direct antimicrobial activity against important clinical isolates including *E. coli*, *Salmonella sp*, *Klebsiella sp*, *Enterobacter sp*, *Pseudomonas aeruginosa*, *Helicobacter pylori* (*H. pylori*), methicillin-resistant *Staphylococcus aureus* (MRSA) and pathogenic fungi by inhibiting nucleic acid

synthesis, energy metabolism or by disrupting cell membrane function (El-Gendy *et al.*, 2008).

H. pylori is a pathogenic bacterium that persistently colonizes the gastric mucosa of human stomach unless treated. Currently, it is estimated that half of the world's population harbors this bacterium which may occur as normal flora in some people but causes inflammation of gastric mucosa (gastritis) as well as duodenitis and peptic ulcers in others. If not completely eradicated, superficial gastric inflammation can drive a cascade of histological changes, resulting in an increased risk of gastric malignancies (Parsonnet, 2005).

The treatment of choice for these infections entails a combination of two antibiotics, amoxicillin or clarithromycin and metronidazole with a proton pump inhibitor such as omeprazole. Eradication of *H. pylori* is still a challenge because of the rapidly increasing prevalence of multi-drug resistant strains worldwide (Fuccio *et al.*, 2008). Tanih *et al.* (2010) in their study reported multi-drug resistance of isolates in the locality. It is therefore of great significance to search for alternative and complementary treatment to eradicate this pathogen and as well as adverse side effects encountered in the conventional treatment regimen.

The anti-*H. pylori* activity of natural products have been documented in previous studies (Ndip *et al.*, 2007; Njume *et al.*, 2011a; Manyi-Loh *et al.*, 2010). Recently, Manyi-Loh *et al.* (2010) reported the susceptibility of *H. pylori* isolates to selected South African honeys and their solvent extracts; the chloroform extracts of Pure honey and Champagne royal train exhibited bacteriostatic and bactericidal activities. As a follow up to this study, the present study sought to isolate, partially purify and identify the phytocomponents with anti-*H. pylori*

activity present in the chloroform extracts of Pure honey (PH) and Champagne Royal Train (CRT).

5.2 MATERIALS AND METHODS

5.2.1 Solvents and reagents

Distilled water, vanillin reagent (Sigma-Aldrich), n-hexane, ethyl acetate, chloroform, concentrated sulphuric acid, acetone, dimethyl sulphoxide (DMSO), methanol and acetic acid used were obtained from Merck (South Africa) and linalool compound of highest purity (98.7%), from Dr. Ehrenstorfer GmbH, Augsburg, Germany.

5.2.2 Bacterial isolates

Seven local strains of *H. pylori* isolated in the laboratory (Tanih *et al.*, 2010) and a reference strain ATCC (American Type Culture Collection) 43526 were used. These strains have been characterized as clarithromycin and metronidazole resistant (Tanih *et al.*, 2010). Prior to sensitivity testing, all *H. pylori* strains were revived on Columbia blood agar plates enriched with Skirrow's antibiotics and 7% laked horse blood; subsequently, their identification was re-evaluated by morphological and biochemical characterization. The bacteria were harvested in Brain Heart Infusion broth supplemented with Skirrow's antibiotics and 5% horse serum and incubated in a microaerophilic atmosphere (CampyGen BR0056A, Oxoid) at 37°C for 18h.

5.2.3 Preparation of honey solvent extract

Floral sources of PH were *Citrus limon* and *Citrus sinensis* while that of CRT was the vineyards. Crude honeys (PH and CRT) were each extracted with chloroform solvent

according to the procedures that have been previously reported by Manyi-Loh *et al.* (2010). Briefly, 100 grams of crude honey was placed in a 500 mL separating funnel, diluted with 150 mL of sterile distilled water and extracted with 150 mL of chloroform solvent. This was performed as three successive extractions using 50 mL of solvent each time.

The shaking time for each extraction process was 15 min, after which the mixture was allowed to stand to permit the solvent layer to separate. The three layers were collected; mixed and concentrated by evaporating the extract under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Padua, Italy) at 50°C. Water contaminating extracts was removed by filtration over anhydrous sodium sulphate.

5.2.4 Separation of chemical components of chloroform extracts

Thin-layer chromatographic (TLC) technique using Silica gel plates was employed as per the method of Magano *et al.* (2008) to separate and determine the chemical compounds in the extracts. A concentration of 50% v/v of each extract was prepared in chloroform solvent from which 10µL was spotted on to TLC plates (Silica gel 60F₂₅₄, Kieselgel, 20x20cm, Darmstadt, Germany), 1 cm from the bottom and labeled according to the honey type (i.e. PH and CRT). The spotted plates were air-dried and introduced into a TLC tank containing the developing solvent system. The tank was closed with a glass lid so as to have the chamber completely filled with the solvent vapors. Different solvent systems were tested for effective separation of compounds in these extracts.

The two most suitable solvent systems standardized based on better separation of compounds were; methanol: acetic acid: water (MAAW) (10:7:3) and n-hexane: ethyl acetate: acetic acid (HEA) (7.8:3.5:1.25). The plates were run until the mobile phase was about 1 cm from the

top; subsequently they were removed from the tank and air-dried. Separated components were visualized under UV light at a wavelength of 254nm. To improve detection of compounds, the TLC chromatograms were sprayed with a mixture of vanillin-sulphuric acid (0.1g vanillin: 28mL methanol: 1mL concentrated sulphuric acid). The plates were heated at 100°C for 4 min for optimal color development. R_f values of the compounds separated on the TLC plates were determined using the formula: ratio of the distance substance travels to the distance solvent travels up the plate.

5.2.5 Fractionation of bioactive compounds and purification by silica gel Column Chromatography

Separation and purification of compounds in the chloroform extracts by column chromatography was done according to the methods of Hassan *et al.* (2007) and Shrivastav *et al.* (2009). Slurry of Silica gel (MN Kieselgel 60, 0.063-0.2mm, Darmstadt, Germany) was prepared in n-hexane solvent and was used to pack two separate columns of dimension 40 x 2.5cm (125mL); one for PH extract and the other for CRT extract. The columns were gently tapped to ensure uniform packing of the particles of the stationary phase as well as to eliminate air bubbles. They were equilibrated with n-hexane and a small quantity of the solvent was allowed to remain at the top of each column (about 4 cm). Each extract (6g) was mixed separately with Silica gel (12g) in n-hexane; they were gently mashed until the extract was adsorbed on the Silica gel and was allowed to dry.

Each mixture in a powder form was loaded to the top of its corresponding column and eluted at a flow rate of 41drops /min (3mL/min), with the solvent system, n-hexane: ethyl acetate: acetic acid (7.8:3.5:1.25) referred to as S1, which gave a common separation of compounds in both honey solvent extracts in the TLC analysis. Fractions were collected separately from

both columns based on the polarity of the solvent system; however, 100% n-hexane was used for elution of columns before employing the solvent system. Twelve (12) fractions were obtained from the column loaded with extract of PH (PHHex, PH F1-F11) and 9 fractions from the other (CRTHex, CRT F1-F8).

Furthermore, a third column of the same dimension was packed with slurry of silica gel prepared in chloroform solvent. All the other subsequent procedures mentioned previously were followed strictly. The column was loaded with the chloroform extract of PH honey prepared in chloroform and elution was carried out firstly with chloroform solvent that was used for equilibration (i.e. PHCL) and later with the solvent system, methanol: acetic: water (10:7:3) referred to as S2, that gave the best separation of compounds in the TLC analysis of this extract. Four fractions were obtained (i.e. PH F1, PH F2, PH F3 &PH F4).

All column fractions were concentrated by evaporation under reduced pressure using a rotary evaporator (Steroglass, Strike 202, Padua, Italy). The residues obtained were weighed and their masses were recorded as shown in Table 5.1.

Table 5.1: Residual weights (grams) of column fractions after evaporation at the rotary evaporator

Column fractions	Masses (grams)		
	Solvent system used as mobile phase		
	S1		S2
	PH	CRT	PH
Hexane	0.0018	0.0029	NA
Chloroform	NA	NA	0.0464
F1	0.012	0.012	0.002
F2	0.015	0.023	1.063
F3	0.015	0.030	2.017
F4	0.01	0.016	0.555
F5	0.0227	0.030	NA
F6	0.0257	0.046	NA
F7	0.0132	0.050	NA
F8	0.0273	0.093	NA
F9	0.0356	NA	NA
F10	0.059	NA	NA
F11	0.102	NA	NA

NA, Not applicable; S1, n-hexane: ethyl acetate: acetic acid; S2, methanol: acetic acid: water

These fractions (excluding the ones with negligible masses) were tested for antibacterial activity by broth micro dilution method as well as for purity by TLC technique.

5.2.6 Anti-*Helicobacter pylori* activity of column fractions and linalool

The minimum inhibitory concentrations (MIC₅₀) of these fractions against a local *H. pylori* clarithromycin/metronidazole resistant strain (PE93A) and *H. pylori* reference strain (ATCC 43526), were evaluated following the method of Njume *et al.* (2011b) using broth micro dilution test. The column fractions obtained while eluting with S1 solvent system were each dissolved in 80% acetone and those obtained while eluting with S2 solvent system were dissolved in 10% DMSO. A stock of 20mg/mL was prepared for each fraction. Two-fold dilutions were prepared in 96-well-round-bottom microtitre plates (Greiner Bio-One, Frickenhausen, Neuburg, Germany) in BHI broth (Oxoid, England); the final concentration of

each fraction was 0.01-10mg/mL. Similarly, amoxicillin (0.0012 - 1.25 mg/mL), was two-fold diluted and tested on the same plate with the column fractions as reference antimicrobial. Control wells were also prepared with culture medium only, culture medium with fraction and culture medium with bacterial isolate only.

The inoculum of each strain was diluted tenfold in sterile normal saline. Twenty μL of the bacterial suspension (10^8 CFU/mL) was aliquoted into each well. The final volume in each well of BHI broth, fraction, and inoculum was 120 μL . The absorbencies were read using an ELISA microtitre plate reader (Model 680, S/N 19138, Biorad, Japan) adjusted at 620nm. The micro plates were sealed and incubated at 37°C under microaerophilic conditions for 2-3days, agitated and the absorbencies were again read at the same wavelength. The absorbencies were compared to the values obtained before incubation to detect any increase or decrease in bacterial growth. The lowest concentration of the extract resulting in inhibition of bacterial growth by 50% was taken as MIC₅₀.

Subsequently, the very active fractions were subjected to GC-MS analysis to determine their chemical composition. In order to trace and identify the active ingredient(s), the effect of linalool was tested (found in the most active fraction, PH F5) against an increased number of *H. pylori* drug resistant strains (Bergonzelli *et al.*, 2003). The MIC assay was carried out as previously described above and a stock concentration of linalool, 1mg/mL prepared in 80% acetone or 100% ethanol (EtOH) was employed. Stock concentrations of amoxicillin, 0.0000019-0.002mg/mL were equally used as the positive control. MIC was the lowest concentration of linalool or amoxicillin that resulted in inhibition of bacterial growth by 95%.

The minimum bactericidal concentration (MBC) was evaluated by the method of Vila *et al.* (2010). Following MIC, 2 μ L of each sample was spread on Columbia blood agar plates and incubated at 37°C under microaerophilic conditions (CampyGen, Oxoid, England) for 72hrs. Plates were checked for bacterial growth and MBC was recorded as the lowest concentration at which no colony of *H. pylori* was formed on the plates. Each experiment was done in triplicate and amoxicillin was used as the positive control.

5.2.7 Test for purity of column fractions

To test for purity of these fractions (i.e. to check if the fractions were composed of a single compound), a secondary TLC analysis was carried out employing the solvent systems, n-hexane; ethyl acetate: acetic acid (7.8: 3.5: 1.25) or methanol: acetic acid: water (10:7:3) depending on the fraction. The developed chromatograms were viewed under UV light at 254nm before and after staining with vanillin-sulphuric acid (0.1g vanillin: 28mL methanol: 1mL concentrated sulphuric acid). R_f values of the spots were calculated.

5.2.8 Analysis of the chemical components of honey solvent extracts by Gas chromatography-Mass spectrometry (GC-MS)

Considering the mean MIC₅₀ values of the fractions, PHF5, PHF6, PHF10, PHF11, CRTF2, CRTF3, CRTF5 obtained with S1 solvent system and PHF2, PHF3 and PHF4 obtained with S2 solvent system were subjected to GC-MS analysis to determine their volatile constituents. The GC-MS analyses of the volatiles were carried out using Hewlett-Packard HP 5973 mass spectrometer interfaced with an HP-6890 gas chromatograph with an HP5 column (Wiley, New York, USA). The following conditions were used: initial temperature 70 °C, maximum temperature 325 °C, equilibration time 3 min, ramp 4 °C/min, final temperature 240 °C; inlet:

split less, initial temperature 220 °C, pressure 8.27 psi, purge flow 30 ml/min, purge time 0.20 min, gas type helium; column: capillary, 30 m × 0.25 mm i.d., film thickness 0.25 µm, initial flow 0.7 ml/min, average velocity 32 cm/s; MS: EI method at 70 eV. The components of the oils 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 (Joulain *et al.*, 2001; Abd El-Moaty, 2010). The yield of each component was calculated per kg of the honey material, while the percentage composition was calculated from the summation of the peak areas of the total oil composition.

5.2.9 Statistical analysis

ANOVAs –one-way test was carried out while employing MINITAB (version 14.0 for Windows) to determine if there was any statistically significant difference in comparing the MIC₅₀ values of the column fractions to each other as well as to the positive control, amoxicillin. Equally, the MIC₉₅ and MBC values of linalool were compared to amoxicillin.

5.3 RESULTS

5.3.1 Thin layer chromatography analysis of chloroform extracts

Of all the solvent systems employed as mobile phases, the best separation of compounds (8 bands) was obtained from the chloroform extract of PH honey with the solvent system, methanol: acetic acid: water (10:7:3) while for CRT there was smearing throughout the plate (no distinct bands). Furthermore, a common separation of three bands was achieved with the solvent system, n-hexane: ethyl acetate: acetic acid (7.8:3.5:1.25) for both chloroform extracts. Calculated R_f values for the separated bands are shown in Table 5.2.

Table 5.2: R_f values of separated bands presented by the different honey chloroform extracts resolved by thin layer chromatography (TLC) with different solvent systems

Solvent systems used as mobile phase	R_f values	
	Chloroform extract of PH	Chloroform extract of CRT
10-methanol	0.81	Smear
7-acetic acid	0.69	
	0.67	
	0.64	
	0.48	
	0.35	
	0.31	
	0.16	
7.8-n-hexane	0.83	0.83
3.5-ethyl acetate	0.7	0.68
1.25-acetic acid	0.6	0.53

R_f , retention factor

5.3.2 Evaluation of MIC₅₀ of column fractions

The anti-*Helicobacter pylori* effect of the column fractions in the MIC₅₀ assay is shown in table 5.3. All the fractions (PHF1-F11, CRTF1-F8 and PHCL, PHF2-F4) demonstrated antibacterial activity with MIC₅₀ values that ranged from 1.25 - 10mg/mL. Considering the different eluting solvent system, PH F5 and PH F11 were the most potent of all the fractions, although there was no significant difference ($P > 0.05$) in the activity of all fractions (except PHF1 & CRTF1) compared to the positive control, amoxicillin (MIC₅₀ range 0.625-1.25mg/mL). Of the four fractions obtained with the solvent system S2, PH F3 presented the best activity with MIC₅₀ values in the range 2.5-5mg/mL even though there was significant difference in activity of all the fractions ($P < 0.05$) compared to amoxicillin.

Table 5.3: Anti-*Helicobacter pylori* effect of column fractions recorded as MIC₅₀

<i>H. pylori</i> isolates	MIC ₅₀ values of all the chromatographic fractions and positive control																							
	Solvent system used as mobile phase																							AMOX
	S1												S2											
	Chloroform extract of PH											Chloroform extract of CRT								Chloroform extract of PH				
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F1	F2	F3	F4	F5	F6	F7	F8	PHCL	F2	F3	F4	
PE 93A	5	5	5	5	1.25	1.25	2.5	5	5	5	1.25	5	5	5	ND	2.5	ND	ND	10	ND	5	2.5	5	
ATCC43526	10	5	2.5	2.5	1.25	5	10	2.5	2.5	1.25	2.5	10	2.5	5	5	5	2.5	2.5	2.5	10	5	5	5	1.25
Mean	7.5	5	3.75	3.75	1.25	3.13	6.25	3.75	3.75	3.13	1.88	7.5	3.75	5	ND	3.75	ND	ND	6.25	ND	5	3.75	5	0.94

ND, Not determined.

5.3.3 Test for purity of fractions

The secondary TLC analysis of all the fractions obtained with S1 solvent system showed that they were partially purified since the chromatograms presented more than one band (Figs.5.1a-c). The chromatogram of the four fractions obtained with the S2 solvent system presented a single broad black band in each fraction. However, the volatile constituents in the fractions were identified by GC-MS analysis.

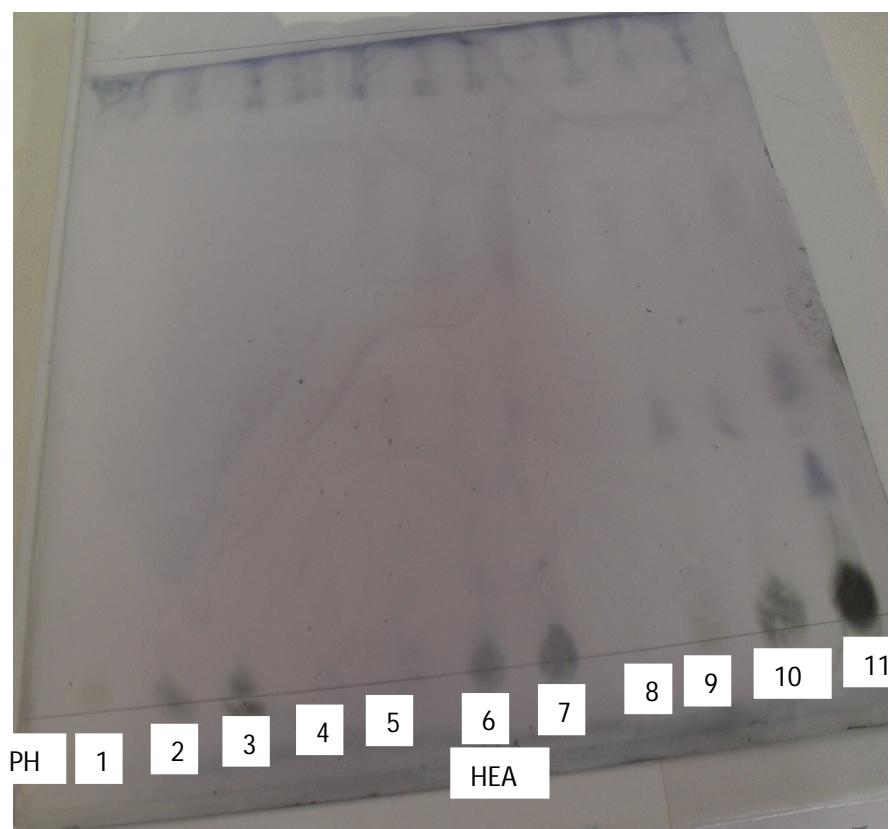


Fig.5.1a: TLC chromatograms of PH fractions employing S1 solvent system (HEA)

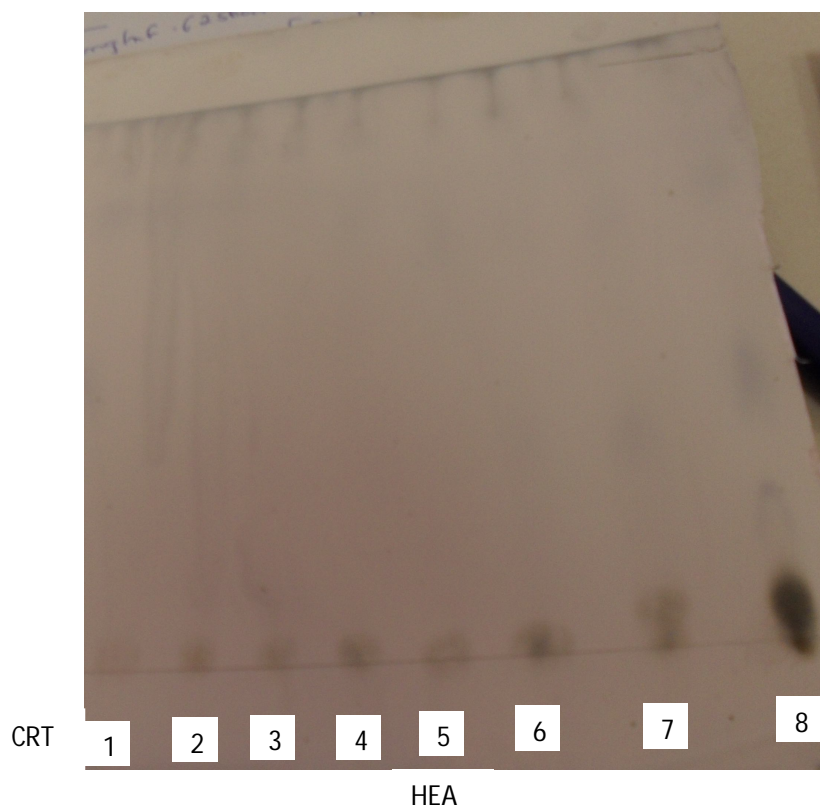


Fig. 5.1b: TLC chromatograms of CRT fractions employing S1 solvent system (HEA)

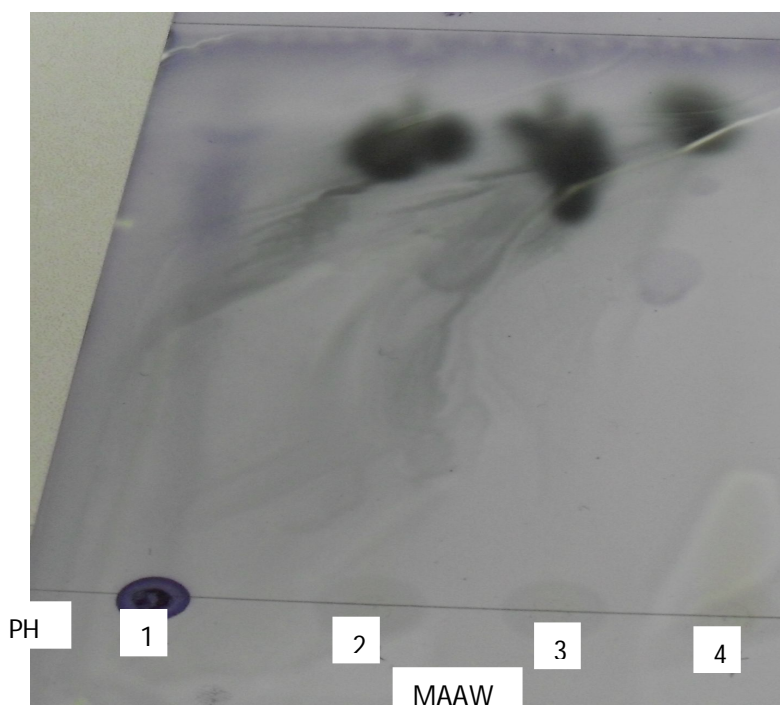


Fig. 5.1c: TLC chromatograms of PH fractions employing S2 solvent system (MAAW)

5.3.4 Analysis of the chemical components of honey solvent extracts by Gas chromatography-Mass spectrometry (GC-MS).

The volatile compounds which are part of the chloroform extract were analyzed by GC-MS, and the data are reported in table 5.4. A total of 24 volatile compounds were identified in all the column fractions. However, 15 volatile compounds were obtained with the S1 solvent system and 12 with the S2 solvent system. The PH F5 fraction contained the highest number of volatiles.

Compounds identified included hydrocarbons, alcohol, aldehydes, ketones, acids, terpenes and its derivatives, benzene derivatives, furan and pyran derivatives, thiophene, N-methyl-D3 aziridine and propanenitrile.

Table 5.4: Volatile compounds identified in column fractions of chloroform extracts of PH and CRT honeys

Compounds	Area percentage (%) of volatile compounds in each column fraction									
	Solvent system used as mobile phase									
	S1					S2				
	PH5	PH6	PH10	PH11	CRT2	CRT3	CRT5	PH2	PH3	PH4
Hexane	89.4	96.8	-	7.75	54.83	17.86	4.56	-	-	19.98
Octane	0.37	-	-	-	-	-	-	-	-	-
Cyclohexane	5.97	-	-	-	-	-	-	-	-	-
Acetic acid	-	-	29.26	11.12	-	-	-	80.63	70.57	54.73
Hexadecanoic acid	0.45	-	-	-	-	-	-	-	-	-
Butanal	-	-	-	-	-	-	5.55	-	-	23.66
Succinaldehyde	-	-	-	-	-	-	-	-	0.62	-
3-cyclohexene-1-acetaldehyde	0.15	-	-	-	-	-	-	-	-	-
2-propanone	-	-	-	-	-	-	-	6.47	10.06	1.63
2,5-furandione	-	-	-	-	-	-	-	1.72	2.21	-
Toluene	1.52	1.69	-	-	-	-	-	-	-	-
1,2-dimethyl benzene	0.26	0.23	-	-	-	-	-	-	-	-
1,3-dimethyl benzene	1.36	1.29	-	-	-	-	-	-	-	-
1,3-benzenediamine	-	-	-	-	-	-	-	-	0.95	-
Epoxy linalool	0.20	-	-	-	-	-	-	-	-	-
Linalool	0.17	-	-	-	-	-	-	-	-	-
1-octen-7-methylocta-1,3(z),5(E)-triene	0.13	-	-	-	-	-	-	-	-	-
Furfuryl alcohol	-	-	-	-	-	-	-	3.02	4.22	-
1-Butanol	-	-	-	-	-	-	-	1.42	-	-
Methanol	-	-	-	-	45.17	-	-	-	-	-
2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-	-	-	-	-	-	-	6.75	9.2	-
N-methyl-D3-Arizidine	-	-	-	-	-	-	-	-	0.43	-
Thiophene	-	-	-	-	-	-	-	-	1.74	-
Methanamine hydrochloride	-	-	70.74	81.13	-	82.14	89.89	-	-	-

-,Not Found; S1, n-hexane; ethyl acetate; acetic acid (7.8:3.5:1.25); S2, methanol; acetic acid: water (10:7:3)

5.3.5 Evaluation of the anti-*Helicobacter pylori* activity of linalool pure compound (Standard)

MIC₉₅ and MBC values of linalool compound in the different diluents against the test isolates were as observed in table 5.5. The compound demonstrated inhibitory activity at a concentration ranging from 0.002 - 0.0313mg/mL while the bactericidal activity against the isolates was in the range 0.0039 - 0.0313m/mL. Amoxicillin recorded MIC₉₅ and MBC values that ranged from 0.00000195 - 0.002mg/mL.

Table 5.5: Antibacterial activity of Linalool (mg/mL), a volatile compound characterized from the chloroform extract of Pure honey

<i>H. pylori</i> isolates	Minimum Inhibitory Concentration (MIC)		Minimum Bactericidal Concentration (MBC)	
	Linalool	Amoxicillin	Linalool	Amoxicillin
PE 93A	0.0156	0.001	0.0156	0.001
PE 219C	0.002	0.00000195	0.0039	0.00000195
PE252C	0.0156	0.002	0.0156	0.002
PE 308C	0.0156	0.002	0.0156	0.0005
PE369A	0.0078	0.000125	0.0313	0.000125
PE435A	0.0156	0.0005	0.0156	0.0005
PE436A	0.0313	0.0005	0.0313	0.0005
ATCC 43526	0.0156	0.0005	0.0156	0.0005
Mean	0.0149		0.0117	

5.4 DISCUSSION

Investigations into honey varieties as alternative sources of antimicrobials have become more common over the past few years, due to the increased rate of development of antibiotic resistant organisms. Honey has been recognized for their many pharmacological activities such as its anti-oxidant, antimicrobial, anti-inflammatory, anticancer and immune modulating properties (Manyi-Loh *et al.*, 2011).

In the TLC analysis of chloroform extracts of PH and CRT honeys, the main aim was to ascertain the number and chromatographic behavior of the compounds present in each extract. Of all the solvent systems employed MAAW (10:7:3) resolved the mixture of chloroform extract of PH into eight distinct bands while a common separation of three bands was obtained for both extracts with HEA (7.8:3.5:1.25); thus suggesting the presence of compounds since an earlier report by Stalikas (2007) stated that non-polar compounds such as hydrocarbons, waxes, fatty acids, alkaloids and essential oils present in natural products could be extracted with chloroform.

The fractions obtained from column chromatographic separation of both extracts were subjected to MIC assay to determine their anti-*H. pylori* effect. All the fractions demonstrated anti-*H. pylori* activity with MIC₅₀ values that ranged from 1.25 - 10mg/mL and was dependent on the fractions, test strain and the solvent system. This inhibitory activity may be an indication that different compounds with antibacterial activity might have been among the phytochemical constituents of the chloroform extracts of these honeys. This result is in conformity with the

work of Nwodo *et al.* (2010) that equally reported good antibacterial activity of their column fractions.

Although, there was no significant difference ($P>0.05$) in activity between fractions obtained with the S1 solvent system compared to the positive control, amoxicillin, fractions PHF5 (1.25mg/mL) and PHF11 (1.88mg/mL) were potent in activity and close to the control. This may indicate that the compounds in these fractions might demonstrate antibacterial activity seeming that of amoxicillin.

On the other hand, there was a significant difference ($P<0.05$) in activity of all the fractions obtained with the S2 solvent system compared to the positive control, amoxicillin. PHF3 (3.75mg/mL) in this category demonstrated the best antibacterial activity. This may imply that the compounds present in PHF3 could be of better antimicrobial potential than those in the other fractions. Taking into consideration the MIC₅₀ values of PH obtained with the different solvent system, it is important to note that better antimicrobial activity was generally elicited by fractions obtained with the S1 solvent system. This may suggest that the solubility or polarity of this solvent system allowed many bioactive compounds to dissolve in it and thus became eluted (Doughari, 2006).

Volatile compounds in honey have been reported to have their origins, in general terms, in different chemical families such as: alcohols, hydrocarbons, ketones, aldehydes, acids, esters, terpenes, norisoprenoids, benzene compounds and their derivatives, furan and pyran derivatives (Cuevas-Glory *et al.*, 2007). In this study, a total of 24 volatile compounds were identified in the

chloroform extracts of two different honeys with different solvent systems. They were classified into the following; hydrocarbons (3), alcohols (2), aldehydes (3), ketones (2), benzene derivatives (4), acids (2), terpenes and its derivatives (3), furan and pyran compounds (2), sulphur compound (1), aziridine derivative (1) and nitrile compound (1).

Our most potent fractions PHF3, PHF5 and PHF11 contained these compounds. Cyclohydrocarbons, benzene derivatives, fatty acids and terpenes have been reported to exhibit antimicrobial activity; due to their lipophilicity they present hydrophobic interaction with lipid bilayer membrane of the organism thus affect the functioning of the membrane and membrane embedded proteins (Trombetta *et al.*, 2005). Consequently, the membrane becomes more permeable, and other compounds present in the fraction could make their way into the bacterium (Nalina and Rahim, 2007).

Meotti *et al.* (2003) in their study reported that furan derivatives are a new important class of pharmacological agents which have been found to show antibacterial, antiviral, antifungal and antioxidant activities. In addition, linalool as well as pyran derivatives obtained by maillard reaction have been reported to possess anti-oxidant activity. The presence of these compounds in this study may suggest that their antioxidant property could be of prime importance during respiratory burst in *H. pylori*-induced mucosal damage, scavenging for free reactive oxygen species (Li *et al.*, 2001).

Furthermore, linalool and epoxy linalool are monoterpenes which are the dominant group of chemical components found in nearly all essential oils of many types of plants and flowers.

However, since there were many compounds in each of these fractions, there may be need to determine the active compound (s). Identification of the active component(s) in the most active fraction should allow better standardization of the antimicrobial activity. In this light, the anti-*H. pylori* effect of linalool compound was tested. Though its relative abundance in the PH fraction is low, it has previously been identified as a characteristic marker of citrus floral source and has a wide range of activity spectrum (Bergonzelli *et al.*, 2003; Castro-Várquez *et al.*, 2007).

Taking into consideration, that essential oil and many of its components have limited solubility in aqueous medium which in turn could affect its activity (Bergonzelli *et al.*, 2003; Kubo *et al.*, 2001); linalool compound was diluted in both 80% acetone and 98 % EtOH and its activity tested against an increased number of *H. pylori* drug resistant strains by broth microdilution. The MIC₉₅ ranges for the compound in 80% acetone and EtOH were 0.002 - 0.0313mg/ml and 0.0078-0.0313mg/mL respectively; which were not significantly different ($P > 0.05$) from the MIC₉₅ values of the positive control (0.00000195 - 0.002mg/mL). This may suggest that the therapeutic potential of linalool compound against *H. pylori* strains is highly comparable to the classical antibiotic, amoxicillin (Manyi-Loh *et al.*, 2010). Consequently, 80% acetone could be used to substitute EtOH that has often been reported as the solvent of choice for diluting most essential oils and their components prior to susceptibility testing (Bergonzelli *et al.*, 2003; Koutsoudaki *et al.*, 2005).

As observed in table 5.5, all the isolates were sensitive to linalool although, the strain PE 219C was the most sensitive been inhibited and killed at concentrations as low as 0.002mg/mL and 0.0039mg/mL respectively. The test compound demonstrated significant anti-*H. pylori* activity

that ranged from 0.002 - 0.313mg/mL (mean MIC 0.0149mg/mL or 14.9µg/mL). This is in contrast with the finding of Vila *et al.* (2010) who reported a MIC value of 62.5µg/mL against their *H. pylori* isolates. The MBC values of the test compound were between 0.0039 and 0.0313mg/mL. With the exception of strains PE 219C and PE369A, MIC₉₅ and MBC values were markedly the same for the other six strains, indicating that the activity of the compound at the said concentrations was bactericidal. This result is in line with the finding of Kubo *et al.* (2001).

The most remarkable finding was the presence of thiophene and aziridine compounds in the chloroform extract of PH. They are heterocyclic compounds whose nuclei represent a very important field in drug discovery, since they serve as precursors for the synthesis of natural/synthetic biological products endowed with cosmic pharmacological activities including antiviral, antibacterial, antifungal, anticonvulsant, antiasthmatic, anti-inflammation and antiprotozoal effects (Ismail *et al.*, 2009; Sivadas *et al.*, 2011). In the continuous venture to develop potent and novel antimicrobial agents, small molecules have traditionally been a reliable source consequently; heterocycles (thiophenes and aziridines) would remain an active area of investigation (Sivadas *et al.*, 2011).

However, only four volatile compounds were identified in the fractions obtained from CRT honey. As shown in table 5.4, we can safely conclude that the non-peroxide anti-*H. pylori* activity of this honey may not be due to volatile compounds but to other phytochemicals as the phytochemicals in honey could be grouped into carbohydrates, phenolic compounds (flavonoids & non-flavonoid phenolic compounds) and volatiles (Kaškonienė and Venskutonis, 2010).

5.5 CONCLUSION

The anti-*H. pylori* activity of chloroform extract of Pure honey could be attributed to a combination of several compounds even though not all have been identified in this study. It would be essential to examine each antibacterial component of the extract separately, and in combination to ascertain whether they act alone or synergistically. Nevertheless, the chloroform extract of PH and CRT could serve as a natural source of lead molecules that could be further harnessed for the development of novel anti-*H. pylori* agents that may greatly contribute to the efficient control of this bacterial pathogen which is ubiquitous and whose mode of transmission is still elusive. Further studies are needed to identify the non-volatile phytochemicals in the extracts with anti-*H. pylori* activity as well as to test for toxicity.

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CHAPTER SIX

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 GENERAL DISCUSSION

The traditional use and anecdotal evidence of “Iqhilika” (Mead) (obtained from fermentation of honey in the presence of yeast and water) as medicine in the treatment of stomach ailments by the Xhosa people in the Eastern Cape Province provided the basis for suggesting that honey and its solvent extracts may be useful for specific medical conditions.

In this study, all the honeys at concentrations between 10% and 75% v/v had inhibitory effect on the growth of *H. pylori* strains. The antibacterial activity was evidenced by the observation of clear zones of inhibition around each well with diameter which ranged from 11.0-16 mm. Based on zone diameter of sensitive strain ≥ 14 mm, the percentage of susceptible strains recorded was between 46.7 and 73.3. These results are consistent with earlier reports on the antibacterial activity of locally produced honeys in different parts of the world (Omojasola, 2002; Wilkinson and Cavanagh, 2005). However, the results were variable; with three honeys (PH, CB and GC) being most active at 75% v/v concentration while the others (CRT, HL and HH) were most active at 50% v/v concentration.

In crude and unprocessed honey, its osmolarity, acidity and hydrogen peroxide content are factors exhibiting antibacterial activity (Mandal and Mandal, 2011). Hydrogen peroxide has been reported as the leading antibacterial factor in honey and its concentration is determined by the relative levels of glucose oxidase, synthesized by the bee and catalase originating from flower

pollen (Weston, 2000). Therefore, there is the possibility that peroxide activity could vary from one local honey sample to another. On dilution of some types of honeys, glucose oxidase generates hydrogen peroxide at levels lethal to the bacteria (Halawani and Shohayeb, 2011). This may suggest that hydrogen peroxide could be responsible for the variation in antibacterial activity of the tested honeys obtained from different floral sources since they were diluted.

Nonetheless, no statistical significant difference was found in the antibacterial activity among the honey varieties; consequently PH, GC, CRT and HL honeys with better antibacterial activity were extracted with four organic solvents viz. n-hexane, diethyl ether, chloroform and ethyl acetate. All the extracts of each honey demonstrated potent antibacterial activity with no significant difference in activity between the extracts even though variations occurred in diameter of zones of inhibition as well as in the percentage of susceptible isolates. This may suggest that honey contains copious amounts of bioactive molecules which could easily solubilise in organic solvents (Maji *et al.*, 2010).

Subsequently, in the assay to determine the MIC at 50% bacterial growth inhibition of the two most active extracts of each honey, it was revealed that the most active extract of each honey were; n-hexane extract of GC, ethyl acetate extract of HL, and chloroform extracts of PH and CRT respectively. These results indicate that the inhibitory effect was dependent on the choice of solvent (Aljadi and Yusoff, 2003) as well as the honey type since phytoconstituents in honey varies with geographical location and climate (Kaškonienė and Venskutonis, 2010).

Furthermore, the above-mentioned active extracts of each honey were subjected to time kill assay in order to determine their bactericidal activity against *H. pylori* over 72 hour's time interval. Interestingly, at 1/2 MIC (5% v/v) the chloroform extract of PH honey demonstrated a profound bactericidal activity over a time interval of 36-72hrs. For the other solvent extracts, the most potent bactericidal effect was established at 4xMIC (40%v/v) against the test isolates at different time interval. This may suggest that with a further increase in extract concentration better results would be obtained. In addition, there was growth of bacterial cells at 10%v/v (MIC), showing they were inhibited as expected.

In the TLC assay to separate the compounds present in the chloroform extracts of PH and CRT, eight bands were found on the TLC plate spotted with extract of PH and developed with the solvent system, methanol: acetic acid: water (MAAW) (10:7:3). A common separation of three bands was observed on the TLC plate spotted with both PH and CRT extracts and developed with the solvent system, n-hexane: ethyl acetate: acetic acid (HEA) (7.8: 3.5:1.25). The presence of these bands creates awareness of the possibility of phytochemical compounds in the extracts. The chemical constituents may be therapeutically active or inactive. The ones which are active are called active constituents and the inactive ones are known as inert chemical constituents (Kamba and Hassan, 2010). Notwithstanding, the active constituents have the advantage of being combined with the inactive compounds whose complementary effect gives honey as a whole safety and efficiency much superior to that of its isolated and pure active compounds (Shariff, 2001).

Upon separation and purification of chloroform extracts with two solvent system; HEA (S1) and MAAW (S2) by column chromatographic technique, numerous fractions were obtained designated PHF1-PHF11 and PHCL, PHF1-PHF4 (S2) for PH extract and CRTF1-CRT F8 for CRT extract and evaluated for antibacterial activity. Overall, the best antibacterial activity was demonstrated by fractions PHF5 and PHF11 with MIC values of 1.25mg/mL and 1.88 mg/mL respectively, which were not significantly different from the positive control, amoxicillin (0.94mg/mL). This may suggest that the therapeutic potential of these fractions could be comparable to that of the classical antibiotic (Ndip *et al.*, 2007). Albeit, fractions PHF6, PHF10, PHF2, PHF3, PHF4 and CRTF2, CRTF3 and CRTF5 demonstrated slight antibacterial activity. It is believed that further fractionation and purification of such fractions might still result in the isolation of useful bioactive substances (Njume *et al.*, 2011).

Accordingly, it is worth significance to identify the chemical constituents present in these active fractions. Knowledge about the chemical constituents may lead to the discovery of the true relevance of the folkloric medicinal use of these honeys in different cultures for the treatment of multiple ailments. The volatile compounds identified in the active fractions of both solvent extracts belonged to known chemical groups; alcohols, acids, ketones, aldehydes, benzene derivatives, terpenes and its derivatives, furan and pyran derivatives previously identified in honey by several other authors (Castro-Várquez *et al.*, 2006; De la Fluente *et al.*, 2007). However, only four volatile compounds, methanol, hexane, butanal and methanamine hydrochloride were identified in the chloroform extract of CRT.

Of novelty was the presence of thiophene and N3-methyl-D3-aziridine compounds, essential precursors used for the synthesis of natural products and pharmaceuticals endowed with vital biomedical properties, in the chloroform extract of PH. However, some volatile compounds might have been lost during solvent evaporation of the extracts and fractions as well as not being detected by the GC column due to masking effect of the solvent (Cuevas-Glory *et al.*, 2007). Furthermore, some of the above mentioned compounds have been reported for their known antimicrobial, anti-inflammatory and anti-oxidant properties against other pathogenic bacteria (Paduch *et al.*, 2007; Manilal *et al.*, 2009).

In order to trace the compound responsible for the antibacterial activity of the most active fractions, the anti-*H. pylori* activity of linalool pure compound was evaluated against an increased number of *H. pylori* strains. Linalool demonstrated potent antibacterial activity with MIC at 95% bacterial growth inhibition that ranged from 0.002 to 0.0313mg/mL and bactericidal activity between 0.0039 and 0.0313mg/mL. These results are in agreement with previous studies on the significant anti-*H. pylori* activity of linalool compound (Koutsoudaki *et al.*, 2004; Vila *et al.*, 2009).

Even though, the other known antimicrobial compounds identified in the solvent extract of PH, have not been evaluated singly for anti-*H. pylori* activity, the possibility of synergistic and or antagonistic actions amongst these compounds should not be overlooked. Therefore, Pure honey could provide a natural, economic and readily available source of bioactive compounds. In

addition, these volatile compounds might not only be employed for the synthesis of novel drugs or therapeutic agents but could also aid in the determination of botanical origin of these honeys as well as the aroma profile (Castro-Várquez *et al.*, 2006).

6.2 GENERAL CONCLUSIONS

From the results of this study, it is worth mentioning that;

- 1) Crude honeys such as PH, CB, GC, CRT, HH &HL obtained in South Africa demonstrated potent anti-*H. pylori* activity at concentrations $\geq 10\%$ v/v.
- 2) Solvent extracts of these honeys (PH, GC, CRT, HL) equally exhibited profound bacteriostatic and bactericidal activities, with the chloroform extracts of PH and CRT being the most efficient; thus presenting chloroform solvent as the best medium for the extraction of active pharmacological agents in honey.
- 3) Upon fractionation and purification of the chemical compounds present in the chloroform extract of PH, numerous volatile compounds were identified belonging to known chemical groups such as alcohols, aldehydes, acids, ketones, terpenes, benzene and their derivatives, furan and pyran derivatives which have been previously identified by several authors (Castro-Várquez *et al.*, 2007; Barra *et al.*, 2010).
- 4) Two novel compounds; thiophene and N-methyl-D3-aziridine were identified in this extract and could be part of the antibacterial components responsible for the potent antibacterial activity revealed by the chloroform extract of PH.

- 5) Linalool compound elicited profound bacteriostatic and bactericidal actions against *H. pylori* isolates thus could be one of those compounds responsible for the antibacterial activity of the chloroform extract of PH.
- 6) Above all, these honeys in their crude form could become part of a dietary-based approach for the management of *H. pylori* infections in asymptomatic individuals, especially since they are of natural origin and pose a low risk for the development of resistance or could be used as adjunct in conventional triple therapy regimen to alleviate the unpleasant side effects of the drugs.

6.3 RECOMMENDATIONS

- 1) Further studies should be done to identify other compounds with anti-*H. pylori* activity among the volatile components identified in the chloroform extract of PH.
- 2) Further purification of the active column fractions obtained from chloroform extract of PH should be conducted in order to obtain pure forms of the compounds.
- 3) Subsequently, toxicological analysis of the compounds becomes necessary.
- 4) The non volatile constituents (flavonoid and non-flavonoid phenolic compounds) in the active column fractions obtained from the chloroform extracts of PH and CRT need to be investigated by High Performance Liquid chromatography and the anti-*H. pylori* activity of the identified compounds evaluated.
- 5) The mechanisms of action of the identified active compounds need to be evaluated *in vivo*.
- 6) The crude honeys and their solvent extracts should be tested against other pathogenic organisms including Gram positive and negative bacterial species as well as fungi species in a bid to evaluate their broad spectrum activity.

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APPENDICES

APPENDIX 1



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



Eastern Cape Department of Health

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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

LIST OF PUBLICATIONS AND MANUSCRIPTS SUBMITTED

LIST OF PUBLICATIONS

Manyi-Loh, C.E., Clarke, A.M., Munzhelele, T., Green, E., Mkwetshana, N.F. and Ndip, R.N. (2010). Selected South African honeys and their extracts possess *in vitro* anti-*Helicobacter pylori* activity. *Archives of Medical Research*. **41**:324-331.

Manyi-Loh, C.E., Clarke, A.M., Mkwetshana, N.F. and Ndip, R.N. (2010). Treatment of *Helicobacter pylori* infections: Mitigating factors and prospective natural remedies. *African Journal of Biotechnology*. **9**:2032-2042.

Manyi-Loh, C.E., Clarke, A.M. and Ndip, R.N. (2011). An overview of honey: Therapeutic properties and contribution in nutrition and human health. *African Journal of Microbiology Research*. **5**: 844-852.

Manyi-Loh, C.E, Clarke, A.M. and Ndip, R.N. (2011). Identification of volatile compounds in solvent extracts of honeys produced in South Africa. *African Journal of Agricultural Research*. **6**:4327-4334.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. (2011). Volatile compounds in honey: a review on their involvement in aroma, botanical origin determination and potential biomedical activities. *International Journal of Molecular Sciences*. **12**: 9514-9532.

MANUSCRIPTS SUBMITTED

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. Goldcrest honey and its solvent extracts: a natural product with anti-*Helicobacter pylori* activity. *African Journal of Pharmacy and Pharmacology*.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. *In vitro* evaluation of the antimicrobial activity of selected South African honeys and their solvent extracts on *Helicobacter pylori* isolates. *Pharmacognosy Magazine*.

Manyi-Loh, C.E., Clarke, A.M., Green, E. and Ndip, R.N. Inhibitory and bactericidal activity of selected South African honeys and their solvent extracts against clinical isolates of *Helicobacter pylori*. *Pakistan Journal of Pharmaceutical Research*.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. Detection of phytoconstituents in column fractions of n-hexane extract of Goldcrest honey exhibiting anti-*Helicobacter pylori* activity. *Archives of Medical Research*.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. Isolation and identification of volatile bioactive compounds with anti-*Helicobacter pylori* activity from honey chloroform extracts. *Molecules*.

APPENDIX 3

CONFERENCE PRESENTATIONS

Manyi-Loh, C.E., Clarke, A.M. and Ndip, R.N. (2010). Goldcrest honey and its solvent extracts: a natural product with anti *H. pylori* activity. Presented at Traditional Medicine conference, 28-30 July, ICC Durban.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. (2010). *In vitro* antimicrobial activity of selected South African honeys and their solvent extracts on *Helicobacter pylori* isolates. Presented at international conference on antimicrobial research. ICAR .Valladolid (Spain), 3-5 November.

Manyi-Loh, C.E., Ndip, R.N. and Clarke, A.M. (2011). Inhibitory activity of selected South African honeys and their solvent extracts on *Helicobacter pylori* isolates. Presented at South African Society of Microbiologists, 6-9 November, Southern Sun Cape Sun Hotel, Cape Town.