BIOACTIVITY AND PHYTOCHEMICAL ANALYSIS OF *HYDNORA AFRICANA* ON SOME SELECTED BACTERIAL PATHOGENS.

By

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DECLARATION

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

Signature: ……

Date: 2011

DEDICATION

This work is dedicated to my late grandfather, Mr Gangashe Muvhi phillimon for his love, encouragement, support and concern before he passed on. May his soul rest in peace.

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My special thanks to God Almighty for the strength and knowledge granted to me during this project, and also for His grace upon my life, without him none of this would be possible.

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Abstract

Medicinal plants have been for long remedies for human diseases because they contain components of therapeutic value. The growing problem of antibiotic resistance by organisms demands the search for novel compounds from plant based sources. The present study was aimed at evaluating the bioactivity and phytochemical analysis of *Hydnora africana* on clinical and standard strains of *Helicobacter pylori* (PE 252C and ATCC 43526), *Aeromonas hydrophila* ATCC 35654, and *Staphylococcus aureus* NCT 6571 in an effort to identify potential sources of cheap starting materials for the synthesis of new drugs against these strains. Ethyl acetate, acetone, ethanol, methanol, and water crude extracts of *H. africana* were screened for activity against the test organisms using the agar well diffusion assay. The Minimum Inhibitory Concentration (MIC_{50}) and Minimum Bactericidal Concentration (MBC) of the most potent extracts were determined by the microdilution method, followed by qualitative phytochemical analysis. Results were analyzed statistically by ANOVA one - way test. Different concentrations (200,100, 50mg/mL) of the methanol, acetone, ethanol and ethyl acetate extracts showed activity against *S. aureus* and *A. hydrophila* while for *H. pylori*, only methanol and ethyl acetate extracts were active; water showed no activity for all studied bacterial pathogens. Mean zone diameter of inhibition which ranged from 0-22mm were observed for all test bacterial pathogens and 14-17mm for ciprofloxacin. The activity of methanol and ethyl acetate extracts were statistically significant ($P< 0.05$) compared to all the other extracts. MIC_{50} and MBC ranged from 0.078 – 2.5mg/mL, 0.78-25mg/mL respectively for all tested bacterial pathogens. For ciprofloxacin, the MIC_{50} and MBC ranged from 0.00976 – 0.078mg/mL and 0.098– 0.78mg/mL respectively. There was no statistically significant difference between extracts (methanol, acetone, ethanol, ethyl acetate) and the control antibiotic (ciprofloxacin) ($P> 0.05$). Qualitative phytochemical analysis confirmed the presence of alkaloids, saponins, steroids, tannins and flavonoids in the methanol, acetone,

ethanol and ethyl acetate extracts. The results demonstrate that *H. africana* may contain compounds with therapeutic potentials which can be lead molecules for semi-synthesis of new drugs.

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CHAPTER ONE

1.1 INTRODUCTION

Medicinal plants have long been recognised as remedies for human diseases because they contain components of therapeutic value (Nostro *et al*., 2000). The study of medicinal plants used in folklore remedies has attracted enormous scientific attention in finding solutions to the problems of multiple resistances to the existing synthetic antibiotics.

It is estimated that plant materials are present in or have provided the models for about 50% of Western drugs and herbal remedies continue to play a role in the cure of diseases (Tabuti *et al*., 2003). In developing countries particularly in South Africa, low income families, especially those of small native communities use folk medicine for the treatment of common infections. These plants are ingested as decoctions, teas and juice preparations to treat respiratory infections (Gonzalez, 1980). They are also made into a poultice and applied directly on the infected wounds or burns by traditional healers (Cowan and Steel, 2004). However, these healers claim that their medicine is cheaper and more effective than modern medicine.

They also claim that the medicinal plant *Hydnora africana* is more efficient to treat infectious diseases than synthetic antibiotics. It is therefore necessary to evaluate, scientifically the potential use of this plant for the treatment of infectious diseases caused by common bacterial pathogens. It can be a possible source for new potent antibiotics to which pathogen strains are not resistant (Fabricant and Farnsworth, 2001). The plant *H. africana* belongs to the family *Hydnoracea*. It is a parasitic plant found in the dry and semi-arid parts of the Succulent Karoo, Little Karoo, Eastern Cape Karoo, and the dry coastal thickets between the Eastern

Cape and KwaZulu-Natal (Asfaw *et al.*, 1999). Infusions and decoctions of the plant are used in folklore remedies for the treatment of ailments such as diarrhoea, dysentery, kidney and bladder complaints. Infusions are used as face wash to treat acne by the Xhosa people (Van wyk and Gericke, 2000).

The choice therefore of *H. africana* is based on ethnobotanical information and preliminary data obtained in our laboratory, to determine its bioactivity against both Gram negative and positive microorganisms, notably: *Helicobacter pylori* ATCC 43526, *Aeromonas hydrophila* ATCC 35654, *Staphylococcus aureus* NCT 6571 and local isolate of *H. pylori* PE 252C.

1.1.1 ***Helicobacter pylori*** is a Gram-negative, microaerophilic bacterium that causes chronic inflammation of the inner lining of the stomach (gastritis) in humans. This bacterium is also the most common cause of ulcers worldwide. They are also associated with stomach cancer and a rare type of lymphocytic tumor of the stomach called MALT lymphoma (Vaira, 2001). *H. pylori* infection is most likely acquired by ingesting contaminated food and water and through person- to- person contact (Chan *et al*., 2008).

The infection is more common in crowded living conditions with poor sanitation (Ndip *et al*., 2004; Dube *et al*., 2009). Once *H. pylori* is detected in patients with peptic ulcer, the normal procedure is to eradicate it and allow the ulcer to heal. The standard first-line therapy is a one week triple therapy consisting of the antibiotics amoxicillin and clarithromycin, and a proton pump inhibitor such as omeprazole (Mirbagheri *et al.*, 2006).

Eradication of the organism has been shown to result in ulcer healing, prevention of peptic ulcer recurrence and may also reduce the prevalence of gastric cancer in high-risk populations (Sepulveda and Coelho, 2002; Ndip *et al*., 2008; Tanih *et al*., 2010). An increasing number of infected individuals are found to harbour antibiotic-resistant strains (Ndip *et al*., 2008; Tanih *et al*., 2010). This results in initial treatment failure and requires

additional rounds of antibiotic therapy or alternative strategies such as a quadruple therapy, which adds a bismuth colloid (Vaira, 2001; Lwai-Lume *et al*., 2005). The emerging resistance to antibiotics, especially metronidazole and amoxicillin limits their use in the treatment of infections (O'Gara *et al*., 2000; Smith *et al*., 2001; Sherif *et al*., 2004).

1.1.2 ***Aeromonas hydrophila*** is a heterotrophic, Gram-negative, rod shaped bacterium, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water. It can survive in aerobic and anaerobic environments (Villari *et al*., 2003).

When it enters the body of its victim, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage (Ormen and Ostensvik, 2001). *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* are all considered to be "opportunistic pathogens," meaning they only infect hosts with weakened immune responses.

A. hydrophila infections occur most during environmental changes, stressors, change in temperature, in contaminated environments, and when an organism is already infected with a virus or another bacterium (Borchardt *et al.*, 2003). It can also be ingested through food products that have already been contaminated with the bacterium (Chauret *et al.*, 2001; El-Taweel and Shaban, 2001). It causes gastroenteritis, cellulitis, myonecrosis and eczema in humans. These diseases can affect anyone, but it occurs most in young children and people who have compromised immune systems or growth problems (Sautour *et al.*, 2003). It can be eliminated using one percent sodium hypochlorite solution and two percent calcium hypochlorite solution. Antibiotic agents such as chloramphenicol, florenicol, tetracycline, sulfonamide, nitrofuran derivatives, and pyrodinecarboxylic acids are used to eliminate and control infection (Gavriel *et al.*, 1998; Chauret *et al.*, 2001; WHO, 2002).

1.1.3 ***Staphylococcus aureus*** is a facultatively anaerobic, Gram-positive coccus and is the most common cause of *staphylococcal* infections. It is a spherical bacterium, frequently part of the skin flora found in the nose, and on skin (Cosgrove *et al*., 2009). It can cause a range of illnesses from minor skin infections, such as pimples, impetigo, boils, cellulitis, folliculitis, carbuncles, scalded skin syndrome and abscesses, to life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome (TSS), bacteremia and sepsis (Kluytmans *et al.*, 1997). Its incidence is from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections. It is still one of the five most common causes of nosocomial infections, often causing postsurgical wound infections.

The treatment of choice for *S. aureus* infection is penicillin; but in most countries, penicillin-resistance is extremely common and first-line therapy is most commonly penicillinase-resistant penicillin (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections like endocarditis (Korzeniowski and Sande, 1982; Bayer *et al.*, 1998) but its use is controversial because of the high risk of damage to the kidneys (Cosgrove *et al.*, 2009). The duration of treatment depends on the site of infection and on severity (Neely and Maley, 2000).

1.2 STATEMENT OF THE PROBLEM

Infectious diseases are the most common cause of morbidity, transience globally and are continually being observed to be a danger to the community. Microorganisms have gained resistance against antibiotics that were before used to treat infectious diseases. This drug resistance phenomenon is troublesome and merits attention. Recently, there has been great interest in controlling the growth of microorganisms by using natural antimicrobials.

Medicinal plants are used as natural antimicrobials to treat bacterial pathogens (Ndip *et al*., 2008) and have been shown to be effective against clinical isolates that have been studied so far (Samie *et al*., 2007). We have preliminary data on the methanol extracts of *H. africana* with anti *H. pylori* activity. However, to the best of our knowledge, *H. africana* has not been evaluated for its antimicrobial activity against *A. hydrophila* and *S. aureus*. There is therefore need to evaluate the potential of this plant in a bid to search for new lead molecules with antimicrobial activity against these pathogens.

1.3 HYPOTHESIS

H. africana can provide potent and cheap leads with antimicrobial activity against *H. pylori*, *A. hydrophila* and *S. aureus*.

1.4 OVERALL OBJECTIVE

The present study is aimed at evaluating the antimicrobial potential of the flower of *H. africana* on some selected bacterial pathogens.

1.4.1 Specific objectives

The specific objectives of this study are to:

1. Screen the extracts of *H. africana* for bioactivity against *H. pylori*, *A. hydrophila* and *S. aureus*.
2. Determine the minimum inhibitory concentration (MIC)
3. Determine the minimum bactericidal concentration (MBC)
4. Identify the active compounds responsible for the antimicrobial properties of the extracts.

CHAPTER TWO

LITERATURE REVIEW

2.1 *HELICOBACTER PYLORI*

2.1.1 HISTORY AND MORPHOLOGY

It has been known for more than a century that bacteria are present in the human stomach (Bizzozero, 1893). These bacteria were thought to be contaminants from digested food rather than true gastric colonizers. About 20 years ago, Barry Marshall and Robin Warren described the successful isolation and culture of a spiral bacterial species, later known as *Helicobacter pylori* (Warren and Marshall, 1983), from the human stomach. Self-ingestion experiments by Marshall (Marshall *et al.*, 1985) and Morris (Morris and Nicholson, 1987) and later experiments with volunteers (Morris *et al.*, 1991) demonstrated that these bacteria can colonize the human stomach, thereby inducing inflammation of the gastric mucosa.

H. pylori is a gram-negative bacterium, measuring 2 to 4 μm in length and 0.5 to 1 μm in width. Although usually spiral-shaped, the bacterium can appear rod shaped (Kusters *et al*., 2006). The organism has 2 to 6 unipolar, sheathed flagella of approximately 3 μm in length, which often carry a distinctive bulb at the end (O`toole *et al.*, 2000). The flagella confer motility and allow rapid movement in viscous solutions such as the mucus layer overlying the gastric epithelial cells (O`toole *et al.*, 2000; Kusters *et al.*, 2006).

2.1.2 PATHOGENESIS AND CLINICAL MANIFESTATION

To colonize the stomach, *H. pylori* must survive the acidic pH of the lumen and burrow into the mucus to reach its niche, close to the stomach's epithelial cell layer. The bacterium has flagella and moves through the stomach lumen and drills into the mucoid lining of the stomach (Ottemann and Lowenthal, 2002). Many bacteria can be found deep in the mucus, which is continuously secreted by mucous cells and removed on the luminal side. To avoid being carried into the lumen, *H. pylori* senses the pH gradient within the mucus layer by chemotaxis and swims away from the acidic contents of the lumen towards the more neutral pH environment of the epithelial cell surface (Schreiber *et al.*, 2004).

This bacterium is also found on the inner surface of the stomach epithelial cells and occasionally inside epithelial cells (Petersen and Krogfelt, 2003). It produces adhesins which bind to membrane-associated lipids and carbohydrates and help it adhere to epithelial cells. It produces large amounts of the enzyme urease, molecules of which are localized inside and outside of the bacterium. Urease breaks down urea (which is normally secreted into the stomach) to carbon dioxide and ammonia which is converted into ammonium ion by taking hydrogen from water upon its breakdown into hydrogen and hydroxyl ions. Hydroxyl ions then react with carbon dioxide, producing bicarbonate which neutralizes gastric acid. The survival of *H. pylori* in the acidic stomach is dependent on urease. The ammonia that is produced is toxic to the epithelial cells, and, along with the other products of *H. pylori* including protease, vacuolating cytotoxin A (VacA), and certain phospholipases damages those cells (Smoot, 1997).

Colonization of the stomach by *H. pylori* results in chronic gastritis, an inflammation of the stomach lining (Shiotani and Graham, 2002). Duodenal and stomach ulcers result when the consequences of inflammation allow the acid and pepsin in the stomach lumen to overwhelm

the mechanisms that protect the stomach and duodenal mucosa from these caustic substances. The type of ulcer that develops depends on the location of chronic gastritis, which occurs at the site of *H. pylori* colonization (Dixon, 2000). The acidity within the stomach lumen affects the colonization pattern of *H. pylori* and therefore ultimately determines whether a duodenal or gastric ulcer will form. In people producing large amounts of acid, *H. pylori* colonizes the antrum of the stomach to avoid the acid-secreting parietal cells located in the corpus of the stomach (Kusters *et al*., 2006).

The inflammatory response to the bacteria induces G cells in the antrum to secrete the hormone gastrin, which travels through the bloodstream to the corpus (Blaser and Atherton, 2004). Gastrin stimulates the parietal cells in the corpus to secrete even more acid into the stomach lumen. Chronically increased gastrin levels eventually cause the number of parietal cells to also increase, further escalating the amount of acid secreted (Schubert and Peura, 2008). The increased acid load damages the duodenum, and ulceration may eventually result. In contrast, gastric ulcers are often associated with normal or reduced gastric acid production, suggesting that the mechanisms that protect the gastric mucosa are defective (Schubert and Peura, 2008). *H. pylori* can also colonize the corpus of the stomach, where the acid-secreting parietal cells are located. However, chronic inflammation induced by the bacteria causes further reduction of acid production and, eventually, atrophy of the stomach lining, which may lead to gastric ulcer and increases the risk for stomach cancer (Suerbaum and Michetti, 2002).

2.1.2.1 Gastritis and gastric cancer

Once infected with *H. pylori*, most persons remain asymptomatic. Some infected persons may even clear the infection, with seroreversion rates commonly reported to be in the range of 5% to 10%. It is not known if this seroreversion is spontaneous or results from elimination

of the organism by antibiotic agents used to treat other conditions (Everhart, 2000). However, the typical course of disease in infected patients begins with chronic superficial gastritis, eventually progressing to atrophic gastritis. This progression appears to be a key event in the cellular cascade that results in the development of gastric carcinoma (Morgner *et al.*, 2000).

Although *H. pylori* is associated with the development of adenocarcinoma of the antrum and body of the stomach, it is also clearly linked with gastric mucosa–associated lymphoid tissue (MALT) lymphomas (Zucca *et al.*, 1998). *H. pylori* stimulates lymphocytic infiltration of the mucosal stroma; this infiltration may act as a focus for cellular alteration and proliferation, ultimately resulting in neoplastic transformation to lymphoma (Zucca *et al*., 1998). It appears that *H. pylori* also produces proteins that stimulate growth of lymphocytes in the early stages of neoplasia (Morgner *et al.*, 2000).

2.1.2.2 Peptic ulcer disease

The relationship between *H. pylori* infection and peptic ulcer disease has been studied exhaustively, and it is now accepted that the organism is the major cause of peptic ulcer disease worldwide. Eradicating the infection can alter the natural course of peptic ulcer disease by dramatically reducing its recurrence rate in treated patients, compared with untreated patient. This reduction occurs in patients with duodenal and gastric ulcers who have no history of nonsteroidal anti-inflammatory drug use (Cohen, 2000).

2.1.2.3 Nonulcer dyspepsia

Nonulcer dyspepsia comprises a constellation of varied symptoms, including dysmotility-like, ulcer-like, and reflux-like symptoms. Many possible causes have been suggested for nonulcer dyspepsia, including lifestyle factors, stress, altered visceral sensation, increased

serotonin sensitivity, alterations in gastric acid secretion and gastric emptying, and *H. pylori* infection (Olden and Drossman, 2000).

2.1.2.4 Gastroesophageal reflux disease

Much attention has been focused on the possible relationship between infection with *H. pylori* and gastroesophageal reflux disease (GERD) in its various manifestations (eg, esophagitis, Barrett's esophagus). Some investigators have suggested a link between the presence of *H. pylori* and a decreased risk for developing esophagitis and Barrett's esophagus (Loffeld *et al.*, 2000). Studies have also indicated that certain strains of *H. pylori*, notably the CagA positive strains, may be protective against the development of Barrett's esophagus (Vaezi *et al.*, 2000).

2.1.3 LABORATORY DIAGNOSIS

Currently, there are several methods for detecting the presence of *H. pylori* infection, each having its own advantages, disadvantages, and limitations. Basically, the tests available for diagnosis can be separated according to whether or not endoscopic biopsy is necessary. Histologic evaluation, culture, polymerase chain reaction (PCR), and rapid urease tests are typically performed on tissue obtained at endoscopy (invasive tests) (Stenstorm *et al*., 2008). Alternatively, simple breath tests, serology, and stool assays are sometimes used, and trials investigating PCR amplification of saliva, feces, and dental plaque to detect the presence of *H. pylori* have been described (non-invasive tests) (Bravos and Gilman, 2000).

2.1.3.1 Histology

Histologic evaluation has traditionally been the gold standard method for diagnosing *H. pylori* infection. The disadvantage of this technique is the need for endoscopy to obtain tissue. Limitations also arise at times because of an inadequate number of biopsy specimens

obtained or failure to obtain specimens from different areas of the stomach (Gatta *et al*., 2003). In some cases, different staining techniques may be necessary, which can involve longer processing times and higher costs. However, histologic sampling does allow for definitive diagnosis of infection, as well as of the degree of inflammation or metaplasia and the presence/absence of MALT lymphoma or other gastric cancers in high-risk patients.

2.1.3.2 Culture

Because *H. pylori* is difficult to grow on culture media, the role of culture in diagnosis of the infection is limited mostly to research and epidemiologic considerations. In growing this organism, the media components should include an agar base, growth supplements e.g., sheep and horse blood or serum, and selective supplements containing antimicrobial compounds e.g., vancomycin or teicoplanin to inhibit gram-positive cocci; polymyxin, nalidixic acid, colistin, trimethoprim, or cefsulodin to inhibit gram-negative rods; and nystatin or amphotericin B to inhibit fungi (Ndip *et al.*, 2004; Mégraud and Lehours, 2007). Although costly, time-consuming, and labor intensive, culture does have a role in antibiotic susceptibility studies and studies of growth factors and metabolism (Perez-Perez, 2000; Tanih *et al*., 2010).

2.1.3.3 Polymerase chain reaction

With the advent of PCR, many exciting possibilities emerged for diagnosing and classifying *H. pylori* infection. PCR allows identification of the organism in small samples with few bacteria present and entails no special requirements in processing and transport. Moreover, PCR can be performed rapidly and cost- effectively, and it can be used to identify different strains of *H. pylori* for pathogenic and epidemiologic studies. PCR has also been used in identifying *H. pylori* in samples of dental plaque, saliva, and other easily sampled tissues

(Smith *et al*., 2002; Samie *et al*., 2007). In addition, PCR can detect segments of *H. pylori* DNA in the gastric mucosa of previously treated patients.

2.1.3.4 Rapid urease testing

Rapid urease testing takes advantage of the fact that *H. pylori* is a urease-producing organism. Samples obtained on endoscopy are placed in urea-containing medium; if urease is present, the urea will be broken down to carbon dioxide and ammonia, with a resultant increase in the pH of the medium and a subsequent color change in the pH-dependent indicator. This test has the advantages of being inexpensive, fast, and widely available (Kaklikka *et al*., 2006).

2.1.3.5 Urea breath test

A urea breath test similarly relies on the urease activity of *H. pylori* to detect the presence of active infection. In this test, a patient with suspected infection ingests either ^{14}C- labeled or ^{13}C- labeled urea; ^{13}C- labeled urea has the advantage of being nonradioactive and thus safer for children and women of childbearing age. Urease, if present, splits the urea into ammonia and isotope-labeled carbon dioxide; the carbon dioxide is absorbed and eventually expired in the breath, where it is detected. Besides being excellent for documenting active infection, this test is also valuable for establishing the absence of infection after treatment, an important consideration in patients with a history of complicated ulcer disease with bleeding or perforation (Oderda *et al*., 2001).

2.1.3.6 Serologic tests

In response to *H. pylori* infection, the immune system typically mounts a response through production of immunoglobulins to organism-specific antigens. These antibodies can be detected in serum or whole-blood samples. The presence of IgG antibodies to *H. pylori* can

be detected by use of a biochemical assay. Serologic tests offer a fast, easy, and relatively inexpensive means of identifying patients who have been infected with the organism (Kaklikka *et al*., 2006). This method is also useful in identifying certain strains of more virulent *H. pylori* by detecting antibodies to virulence factors associated with more severe disease and complicated ulcers, gastric cancer, and lymphoma.

2.1.3.7. Stool antigen testing

Stool antigen testing is a methodology that uses an enzyme immunoassay to detect the presence of *H. pylori* antigen in stool specimens. A cost effective and reliable means of diagnosing active infection and confirming cure, such testing has a sensitivity and specificity comparable to those of other noninvasive tests (Ndip *et al*., 2004; Ricci *et al*., 2007).

2.1.4 EPIDEMIOLOGY

The prevalence of *H. pylori* shows large geographical variations. In various developing countries, more than 80% of the population is *H. pylori* positive, even at young ages (Perez-Perez *et. al*, 2004; Ndip *et al*., 2004; Ndip *et al*., 2008). The prevalence of *H. pylori* in industrialized countries generally remains under 40% and is considerably lower in children and adolescents than in adults and elderly people (Ahmed *et al*., 2007). Within geographical areas, prevalence inversely correlates with socioeconomic status, in particular in relation to living conditions during childhood (Kuster *et al*., 2006). In Western countries, the prevalence of this bacterium is often considerably higher among first- and second-generation immigrants from the developing world (Tummuru *et al.*, 1993; Perez-Perez *et al.*, 2005). While the prevalence of *H. pylori* infection in developing countries remains relatively constant, it is rapidly declining in the industrialized world (Genta, 2002). The latter is thought to be caused by the reduced chances of childhood infection due to improved hygiene and sanitation and the active elimination of carriership via antimicrobial treatment. In developing countries, *H.*

pylori infection rates rise rapidly in the first 5 years of life and remain constantly high thereafter, indicating that the bacterium is acquired early in childhood (Fiedorek *et al.*, 1991; Ndip *et al*., 2004). However, in industrialized countries the prevalence of infection is low early in childhood and slowly rises with increasing age. This increase results only to a small extent from *H. pylori* acquisition at later age. The incidence of new infections among adults in the Western world is less than 0.5% per year; the higher prevalence of infection among the elderly thus reflects a birth cohort effect with higher infection rates in the past (Genta, 2002; Asrat *et al*., 2004). The active elimination of *H. pylori* from the population and improved hygiene and housing conditions have resulted in a lower infection rate in children, which is reflected in the age distribution of this lifelong-colonizing bacterium (Roose Ndaal *et al*., 1997; Rehnberg-Laiho *et al.*, 2001). Overall, new infection more commonly occurs in childhood and lasts for life unless specifically treated.

2.1.4.1 Transmission and sources of infection

The exact mechanisms whereby *H. pylori* is acquired are largely unknown. The organism has a narrow host range and is found almost exclusively in humans and some nonhuman primates. It has on rare occasions been isolated from pet animals; thus, the presence of pets may be a risk factor for infection (Dore *et al.*, 2001; Herbarth *et al.*, 2001; Brown *et al.*, 2002). New infections are thought to occur as a consequence of direct human-to-human transmission, via either an oral-oral or fecal-oral route or both. *H. pylori* has been detected in saliva, vomitus, gastric refluxate, and feces (Ferguson *et al.*, 1993; Ferguson *et al.*, 1999; Leung *et al.*, 1999; Parsonnet *et al*, 1999; Allaker *et al.*, 2002; Kabir, 2004; Sinha *et al.*, 2004), but there is no conclusive evidence for predominant transmission via any of these products.

Studies have reported that there was no clear increased risk for being a carrier of *H. pylori* among dentists, gastroenterologists, nurses, partners of an *H. pylori*-positive spouse, or visitors to a clinic for sexually transmitted diseases (Aoki *et al*., 2004). As a result of these and other investigations, it is generally believed that acquisition mostly occurs in early childhood, most likely from close family members (Kivi *et al.*, 2003; Raymond *et al.*, 2004; Konno *et al.*, 2005; Rowland *et al.*, 2006). Premastication of food by the parent is an uncertain risk factor for transmission (Delport *et al*., 2007). Childhood crowding in and outside the family are all positively associated with *H. pylori* prevalence (Goodman and Correa, 2000), whereas among adults crowding appears less important, with the exception of certain circumstances, such as among army recruits (Kyriazanos *et al.*, 2001; Rowland *et al*., 2006). Several studies have reported the presence of *H. pylori* DNA in environmental water sources (Sakamoto *et al.*, 1989; Enroth and Engstrand, 1995; Hegarty *et al.*, 1999; Dube *et al*., 2009), but this probably reflects contamination with either naked DNA or dead *H. pylori* organisms. There is only a single report indicating that H. *pylori* has been successfully cultured from water, but this involved wastewater and as such may well represent fecal contamination of the water source (Momba *et al.*, 2005). Spread via fecal contaminants is supported by the occurrence of *H. pylori* infections among institutionalized young people during outbreaks of gastroenteritis (Laporte *et al.*, 2004). Other possible sources include contaminated food, as *H. pylori* may survive briefly on refrigerated food (Perry *et al*., 2006); direct person-to-person transmission remains the most likely transmission route.

2.1.5 TREATMENT, RESISTANCE MECHANISMS, PREVENTION AND CONTROL

2.1.5.1 Treatment

Once *H. pylori* is detected in patients with peptic ulcer, the normal procedure is to eradicate it and allow the ulcer to heal. The standard first-line therapy is a one week triple therapy consisting of a proton pump inhibitor such as omeprazole and the antibiotics clarithromycin and amoxicillin (Mirbagheri *et al*., 2006). Variations of the triple therapy have been developed over the years, such as using a different proton pump inhibitor, as with pantoprazole or rabeprazole, or replacing amoxicillin with metronidazole for people who are allergic to penicillin (Malfertheiner *et al*., 2007). Such a therapy has revolutionized the treatment of peptic ulcers and has made a cure to the disease possible; previously the only option was symptom control using antacids, H2-antagonists or proton pump inhibitors alone (Rauws and Tytagt, 1990; Graham *et al*., 1991).

An increasing number of infected individuals are found to harbour antibiotic-resistant strains. This results in initial treatment failure and requires additional rounds of antibiotic therapy or alternative strategies such as a quadruple therapy, which adds a bismuth colloid (Fischbach and Evans, 2007; Stenstrom *et al.*, 2008; Graham and Shiotoni, 2008). For the treatment of clarithromycin-resistant strains the use of levofloxacin as part of the therapy has been suggested (Perna *et al*., 2007; Hsu *et al*., 2008).

2.1.5.2 Resistance mechanisms to antibiotics.

H. pylori acquires resistance to all the antibiotics used in the treatment regimens by mutation (Me´graud and Lehours, 2007). The mechanism does not involve plasmids which could be transmitted horizontally but point mutations (nonsense, missense and silent mutations) which

are transmitted vertically; however, transformation may be possible if two strains are present simultaneously in the stomach. The consequence is a progressive increase in the resistance rate due to the selection pressure. As in many bacteria, drug efflux proteins can contribute to natural insensitivity to antibiotics and to emerging antibiotic resistance.

Resistance to macrolides: Macrolides act by binding to ribosomes at the level of the peptidyl transferase loop of the 23S rRNA gene. Resistance of *H. pylori* to macrolides is a major cause of failure of eradication therapies. *H. pylori* resistance is the consequence of point mutations at two nucleotide positions, 2142 (A2142G and A2142C) and 2143 (A2143G), which lead to a conformational change and a decrease in macrolide binding (Occhialini *et al*., 1997; Li *et al*., 2007).

Resistance to amoxicillin: Amoxicillin acts by interfering with peptidoglycan synthesis, especially by blocking transporters named penicillin binding proteins (PBP). The rare amoxicillin-resistant *H. pylori* strains harbour mutations on the *pbp-1a* gene. Amino acid substitution Ser-414_Arg appears to be involved, leading to a blockage of penicillin transport (Van-Zwet *et al*., 1999).

Resistance to fluoroquinolones: Fluoroquinolones inhibit the A subunit of the DNA gyrase, encoded by the *gyrA* gene. Mutations in the quinolone resistance-determining region of *gyrA* are found in *H. pylori* as well as in other bacteria (Tonkic *et al*., 2005; Bogaerts *et al*. 2006).

Resistance to nitroimidazoles: 5-Nitroimidazoles have to be reduced in the cell to alter bacterial DNA. An important gene *rdxA*, an oxygen-insensitive nitroreductase. Mutations in *rdxA* can render the protein ineffective (Hoffman *et al*., 1996). However, it has not been possible to identify a clear panel of point mutations with the *rdxA* gene to explain the phenomenon of resistance (Mégraud, 2004). It is believed that other genes such as *frxA* may also be involved in the reduction process. MTZ-resistance reduces the efficacy of MTZ-

containing regimens but does not make them completely ineffective. There is a discrepancy between *in vitro* MTZ-resistance and treatment outcome which may partially be explained by changes in oxygen pressure in the gastric environment as MTZ-resistant *H. pylori* isolates become MTZ-sensitive under low oxygen conditions *in vitro* (Gerrits *et al*., 2004).

2.1.5.3 Prevention and control

Eradication of the infection in individuals will improve symptoms including dyspepsia, gastritis and peptic ulcers, and may prevent gastric cancer. Rising antimicrobial resistance increases the need for a prevention strategy for the bacteria (Selgrad and Malfertheiner, 2008). There have been extensive vaccine studies in mouse models, which have shown promising results (Hoffelner *et al*., 2008). Researchers are studying different adjuvants, antigens, and routes of immunization to ascertain the most appropriate system of immune protection, with most of the research only recently moving from animal to human trials (Kabir, 2007). An intramuscular vaccine against *H. pylori* infection is undergoing Phase I clinical trials and has shown an antibody response against the bacterium. Its clinical usefulness requires further study (Malfertheiner *et al*., 2008).

A Japanese study found that eating as little as 2.5 ounces of broccoli sprouts daily for two months reduces the number of colonies of *H. pylori* bacteria in the stomach by 40% in mice and humans (Lu *et al*., 2002). This treatment also seems to help by enhancing the protection of the gastric mucosa against *H. pylori*, but is relatively ineffective on related gastric cancers. The previous infection returned within two months after broccoli sprouts were removed from the diet, so an ongoing inclusion in the diet is best for continued protection from *H. pylori* (Yanaka, 2009).

2.2 *STAPHTLOCOCCUS AUREUS*

2.2.1 HISTORY AND MORPHOLOGY

Staphylococcus aureus was discovered in Aberdeen, Scotland in 1880 by the surgeon, Sir Alexander Ogston in pus from surgical abscesses (Dagan, 2000). *S. aureus* is a Gram-positive coccus, non-sporing, non-motile, usually non-capsulate, aerobic and normally facultative anaerobic cocci (1micrometer in diameter) arranged in grape-like clusters when viewed through a microscope (Zhu *et al*., 2008). The cell wall contains peptidoglycan and teichoic acid. The organisms are resistant to temperatures as high as 50°C, to high salt concentrations, and to drying. Colonies are usually large (6-8 mm in diameter), smooth, and translucent. The colonies of most strains are pigmented, ranging from cream-yellow to orange (Liu *et al*., 2008).

2.2.2 VIRULENCE FACTORS

2.2.2.1 Toxins

Depending on the strain, *S. aureus* is capable of secreting several toxins, which can be categorized into three groups. Many of these toxins are associated with specific diseases.

Superantigens: (PTSAgs) have superantigen activities that induce toxic shock syndrome (TSS). This group includes the toxin TSST-1, which causes TSS associated with tampon use. The staphylococcal enterotoxins, which cause a form of food poisoning, are also included in this group (Cosgrove *et al*., 2009).

Exfoliative toxins: EF toxins are implicated in the disease staphylococcal scalded-skin syndrome (SSSS). It also may occur as epidemics in hospital nurseries. The protease activity of the exfoliative toxins causes peeling of the skin observed with SSSS (Neely and Maley, 2000).

Other toxins: Staphylococcal toxins that act on cell membranes include alpha-toxin, beta-toxin, delta-toxin, and several bicomponent toxins. These toxins are associated with folliculitis, furuncle, carbuncle, endocarditis, thromblophlebitis and deep tissue abscess. The bicomponent toxin Panton-Valentine leukocidin (PVL) is associated with severe necrotizing pneumonia in children. The genes encoding the components of PVL are encoded on a bacteriophage found in community-associated methicillin-resistant *S. aureus* (MRSA) strains (Whitt *et al*., 2002).

2.2.2.2 Protein A

Protein A is a protein that is anchored to staphylococcal peptidoglycan pentaglycine bridges by the transpeptidase Sortase A (Schneewind *et al*., 1995). Protein A is an IgG-binding protein that binds to the Fc region of an antibody. In fact, studies involving mutation of genes coding for Protein A resulted in a lowered virulence of *S. aureus* as measured by survival in blood, which has led to speculation that Protein A contributed virulence requires binding of antibody Fc regions(Dagan, 2000). Protein A in various recombinant forms has been used for decades to bind and purify a wide range of antibodies by immunoaffinity chromatography. Transpeptidases such as the sortases that are responsible for anchoring factors like Protein A to the staphylococcal peptidoglycan are being studied in hopes of developing new antibiotics to target MRSA infections (Zhu *et al*., 2008).

2.2.2.3 Role of pigment in virulence

Some strains of *S. aureus* are capable of producing *staphyloxanthin* - a carotenoid pigment that acts as a virulence factor. It has an antioxidant action that helps the microbe evade death by reactive oxygen species used by the host immune system. Staphyloxanthin is responsible for *S. aureus* characteristic golden colour (Cenci- Goga *et al*., 2003). When comparing a

normal strain of *S. aureus* with a strain modified to lack staphyloxanthin, the wildtype pigmented strain was more likely to survive incubation with an oxidizing chemical such as hydrogen peroxide than the mutant strain (Mackay, 2007).

Staphyloxanthin may be key to the ability of *S. aureus* to survive immune system attacks. Drugs designed to inhibit the bacterium's production of the staphyloxanthin may weaken it and renew its susceptibility to antibiotics. In fact, because of similarities in the pathways for biosynthesis of staphyloxanthin and human cholesterol, a drug developed in the context of cholesterol-lowering therapy was shown to block *S. aureus* pigmentation and disease progression in a mouse infection model (Dagan, 2000).

2.2.4 LABORATORY DIAGNOSIS

2.2.4.1 Culture

Depending upon the type of infection present, an appropriate specimen is obtained accordingly and sent to the laboratory for definitive identification by using biochemical or enzyme-based tests. A Gram stain is first performed to guide the way, which should show typical gram-positive bacteria, cocci, in clusters (Dagan *et al*., 2000). Second, the isolate is cultured on mannitol salt agar, which is a selective medium with 7–9% NaCl that allows *S. aureus* to grow, producing yellow-colored colonies as a result of mannitol fermentation and subsequent drop in the medium's pH (Pericone *et al*., 2000); often with hemolysis when grown on blood agar plates (Lysenko *et al*., 2005).

2.2.4.2 Biochemical tests

S. aureus is catalase-positive (meaning that it can produce the enzyme catalase) and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful

to distinguish staphylococci from enterococci and streptococci (Kaplan *et al*., 2004). A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test: *S. aureus* is primarily coagulase-positive that causes clot formation, whereas most other *Staphylococcus* species are coagulase-negative (Grau *et al*., 2008). For staphylococcal food poisoning, phage typing can be performed to determine if the staphylococci is recovered from the food to determine the source of infection (Dagan, 2000).

2.2.4.3 Rapid diagnosis

Diagnostic microbiology laboratories and reference laboratories are key for identifying outbreaks and new strains of *S. aureus*. Recent genetic advances have enabled reliable and rapid techniques for the identification and characterization of clinical isolates of *S. aureus* in real-time. These tools support infection control strategies to limit bacterial spread and ensure the appropriate use of antibiotics. These techniques include real-time PCR and quantitative PCR and are increasingly being employed in clinical laboratories (Borchadt *et al*., 2003).

2.2.5 TRANSMISSION, SOURCES OF INFECTION, TREATMENT AND RESISTANE MECHANISMS.

Spread of *S. aureus* (including MRSA) is through human-to-human contact, although recently some veterinarians have discovered that the infection can be spread through pets, with environmental contamination thought to play a relatively unimportant part (Liu *et al*., 2008).

S. aureus colonizes mainly the nasal passages, but it may be found regularly in most other anatomical locales, including the skin, oral cavity and gastrointestinal tract. About 20% of the human population are long-term carriers of *S. aureus* (Chambers, 2001).

The treatment of choice for *S. aureus* infection is penicillin; but in most countries, penicillin-resistance is extremely common and first-line therapy is most commonly a penicillinase-resistant penicillin (for example, oxacillin or flucloxacillin). Combination therapy with gentamicin may be used to treat serious infections like endocarditis, but its use is controversial because of the high risk of damage to the kidneys. The duration of treatment depends on the site of infection and on severity (Neely and Maley, 2000).

2.2.5.1 Resistance mechanisms to antibiotics.

Alterations in target enzymes, membrane permeability, and efflux mechanisms cause drug resistance in methicillin-sensitive and methicillin-resistant *Staphylococcus aureus* (MRSA). A common mechanism of resistance for *S. aureus* is alterations in type II topoisomerases. Subunits of topoisomerase IV are encoded by genes *grlA* and *grlB.* These genes are analogous to *parC* and *parE,* respectively (O`Donnell *et al*., 2000). Mutations in topoisomerase IV precede alterations in DNA gyrase when exposed to most fluoroquinolones. Studies suggest that topoisomerase IV is the major target of quinolone activity against *S. aureus* (Heaton *et al*., 2000). Moxifloxacin have equivalent activity against *S. aureus*. High MICs are found in *S. aureus* strains that have changes in both target enzymes. Combinations of topoisomerase mutations prevail in quinolone-resistant clinical isolates that are also methicillin resistant (Heaton *et al*., 2000).

The efflux mechanism responsible for quinolone resistance in *S. aureus* is mediated by the production of the NorA protein. This cytoplasmic protein is an efflux transporter of quinolones and may be an inducible method of resistance. NorA-mediated resistance exists in isolates with and without accompanying topoisomerase mutations. NorA appears to have greater affinity for pumping hydrophilic quinolones (norfloxacin, enoxacin) (O`Donnell *et al*., 2000).

2.2.6 PREVENTION

Emphasis on basic hand washing techniques is effective in preventing the transmission of *S. aureus* (Bayer *et al*., 1998). No vaccine is generally available that stimulates active immunity against staphylococcal infections in humans. A vaccine based on fibronectin binding protein induces protective immunity against mastitis in cattle and might also be used as a vaccine in humans. However, vaccine therapies represent a new and innovative approach in broadening the available clinical tools against the global health problem of community and healthcare-associated *S. aureus* bacterial infections (Cosgrove *et al*., 2009).

Hyperimmune serum or monoclonal antibodies directed towards surface components (e.g., capsular polysaccharide or surface protein adhesions) could theoretically prevent bacterial adherence and promote phagocytosis by opsonization of bacterial cells. Also, human hyperimmune serum could be given to hospital patients before surgery as a form of passive immunization. When the precise molecular basis of the interactions between *staphylococcal* adhesins and host tissue receptors is known, it might be possible to design compounds that block the interactions and thus prevent bacterial colonization. These could be administered systemically or topically (Dagan *et al*., 2000).

The pharmaceutical company, Nabi, has developed a trivalent staphylococcal polysaccharide conjugate vaccine called TriStaph. It contains the two main capsular types, 5 and 8, found in the outer coating of more than 80% of *S. aureus* strains, conjugated to nontoxic recombinant *Pseudomonas* exotoxin A (Zhu *et al*., 2008). To enhance the efficacy of this vaccine, a surface polysaccharide, 336, is added. *S. aureus* Type 336 accounts for approximately 20% of *S. aureus* infections that do not form a polysaccharide capsule in the human bloodstream. The 336 conjugate vaccine, has been shown to be safe and generate antibodies in humans that are specific and mediate protection against 336-positive strains of *S. aureus* (Zhu *et al*., 2008).

Together, these polysaccharide conjugates can cover all clinically-significant serological types of *S. aureus*.

2.3 *AEROMONAS HYDROPHILA*

2.3.1 MORPHOLOGY

Aeromonas hydrophila is a heterotrophic Gram-negative, non-spore-forming, rod-shaped, oxidase-positive, facultative anaerobic bacilli belonging to the family Aeromonadaceae and it is the only one of six *Aeromonas* species that is known to be pathogenic in humans (Villari *et al.*, 2003). It is usually from 0.3 to 1 micrometer in width, and 1 to 3 micrometers in length. *Aeromonas hydrophila* does not form endospores, and can grow in temperatures as low as four degrees celsius. These bacteria are motile by polar flagella (Havelaar *et al.*, 1992; Janda and Abbott, 1998; Villari *et al.*, 2003).

2.3.2 PATHOGENESIS AND CLINICAL MANIFESTATIONS

It was believed that the pathogenicity of *Aeromonas* species is mediated by a number of extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins and enterotoxins (Albert, 2000). The type III secretion system (TTSS) mediated pathogenic mechanism has been proven to play a pivotal role in *Aeromonas* pathogenesis. The TTSS is specialized protein secretion machinery that export virulence factors delivered directly to host cells. These factors subvert normal host cell functions in ways that are beneficial to invading bacteria. In contrast to the general secretory pathway, type III secretion system is triggered when a pathogen comes in contact with host cells. ADP-ribosylation toxin is one of the effector molecules secreted by several pathogenic bacteria and translocated through TTSS and delivered into the host cytoplasm leads to interruption of NF-κB pathway, cytoskeletal damage and apoptosis. This toxin has been characterized in *Aeromonas hydrophila* (human

diarrhoeal isolate), *Aeromonas salmonicida* (fish pathogen) and *Aeromonas jandaei* GV17, a pathogenic strain which can cause disease both in human and fish (Chopra *et al*., 2000).

2.3.2.1 Clinical manifestation

One of the diseases it can cause in humans is gastroenteritis. This bacterium has been known to cause a generalized infection and spread throughout the body in persons with weak or defective immune systems, malignancies and other preexisting diseases. In such individuals, a generalized infection can be life-threatening. This bacterium is linked to two types of gastroenteritis. The first type is a disease similar to cholera, which causes rice-water diarrhea. The other is dysenteric gastroenteritis, which causes loose stools filled with blood and mucus. Dysenteric gastroenteritis is the most severe of the two types, and can last for multiple weeks (Sautour *et al*., 2003).The organism is also associated with cellulitis, an infection that causes inflammation in the skin tissue (Gavriel *et al*., 1998). It also causes diseases such as myonecrosis and eczema in people with compromised immune systems (Chauret *et al*., 2001).

2.3.3 LABORATORY DIAGNOSIS

2.3.3.1 Culture

Depending upon the type of infection present, an appropriate specimen is obtained accordingly and sent to the laboratory for definitive identification by using biochemical tests. A Gram stain is first performed, which should show typical rod shaped, gram-negative bacteria (Dagan *et al*., 2004). *A. hydrophila* can easily grow on culture media e.g., nutrient or Muller- Hilton agar, the role of culture in diagnosis of the infection is limited mostly to research and epidemiologic considerations but does have a role in antibiotic susceptibility studies and studies of growth factors and metabolism (Perez-Perez, 2000).

2.3.3.2 Polymerase chain reaction

PCR is a powerful tool that is used effectively in the identification and detection of *A. hydrophila* infection. PCR allows identification of the organism in small samples with few bacteria present and entails no special requirements in processing and transport. Moreover, PCR can be performed rapidly and it can be used to identify different strains for pathogenic and epidemiologic studies (Bravos and Gilman, 2000).

2.3.4 TRANSMISSION, SOURCES OF INFECTION AND TREATMENT

The common routes of infection suggested for *Aeromonas* are the ingestion of contaminated water or food or contact of the organism with a break in the skin (Schubert, 1991). No person-to-person transmission has been reported (Havelaar *et al.*, 1992; Moyer *et al.*, 1992; Hänninen and Siitonen, 1995; WHO, 2002; Borchardt *et al.*, 2003). The growth of *A. hydrophila* is temperature dependent. Therefore, the risk of infection occur most during environmental changes, stressors, change in temperature; is highest in the summer months, when these microorganisms are multiplying more rapidly (Holmes and Nicolls, 1995).

A. hydrophila is a bacterium that is commonly found in freshwater environments and in brackish water (lakes, rivers, marine waters, sewage effluents, and drinking waters) (Allen *et al.*, 1983; Nakano *et al.*, 1990; Poffe and Op de Beeck, 1991; Payment *et al.*, 1993; Ashbolt *et al.*, 1995; Bernagozzi *et al.*, 1995). They can survive in aerobic and anaerobic environments.

The organism can be eliminated using one percent sodium hypochlorite solution and two percent calcium hypochlorite solution. Antibiotic agents such as chloramphenicol, florenicol, tetracycline, sulfonamide, nitrofuran derivatives, and pyrodinecarboxylic acids are used to eliminate and control the infection (Chauret *et al.*, 2001; El-Taweel and Shaban, 2001).

2.4 MEDICINAL PLANTS AND SOLVENTS EMPLOYED IN THE STUDY OF PLANT ANTIMICROBIALS.

Medicinal plants are sources of alternative and complementary medicine. Ethnobotanical studies are often significant in revealing locally important plant species especially for the discovery of drugs. From its beginning, the documentation of traditional knowledge, especially on the medicinal uses of plants, has provided many important drugs of modern day (Adebolu, 2005). Traditional medicine still remains the main resource for a large majority of the people in Africa for treating health problems (Abebe and Hagos, 1991; Addis *et al.*, 2001).

Medicinal plants signify a rich source from which antimicrobial agents may be obtained. Studies revealed that natural antimicrobials can act as resistant microbial inhibitors (Adebolu, 2005). There is an increased need for the isolation and identification of new antimicrobials that are capable of inhibiting and treating a wide range of microorganisms including multi-drug resistant strains. Plants have been documented to have these compounds that contribute to their antimicrobial activity including phytochemicals such as flavonoids, phenolics and propolis which are not fully characterized but posses antimicrobial activity against bacterial pathogens (Cushnie and Lamb, 2005).

The type of solvent used may have an effect on the nature of the compounds extracted and the resulting bioactivity of the extract (Eloff, 1998b; Eloff *et al*., 2008). To ascertain the value of each extractant therefore, several parameters, including the rate of extraction, the quantity extracted, the diversity of compounds extracted, the diversity of inhibitory compounds extracted, the ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and the potential health hazard of the extractants have to be evaluated. The efficiency of extraction has to be optimized to ensure that as many of the potentially active constituents as possible are extracted. A series of solvents of varying polarity (hexane,

carbon tetrachloride, di-isopropyl ether, ethyl ether, methylene dichloride, tetrahydrofuran, chloroform, acetone, ethanol, ethyl acetate, methanol, water or mixtures of different solvents) are used on the plant material (Eloff *et al*., 2008). In enormous reports, methanol or ethanol are used for alkaloid extraction; acetone for flavonoids and steroids, hexane, diethyl ether and chloroform for fat soluble oils, wax, lipids and esters; dichloromethane for terpenoids, ethyl acetate for esters, ethanol may also be used for sterols, polyphenols, tannins and water for the water soluble components like glycosides, polysaccharides, polypeptides and lectins, which are very effective against pathogens probably because of their ability to intercalate with DNA and/or cell membranes (Büssing, 1996). The crude extracts or mixtures of compound-rich residues are used for the initial screening of plants for anti-microbial activities. Thin Layer Chromatography (TLC), other chromatography separations and several solvent systems are used for the elution of many water and organic solvent soluble anti-microbial compounds (Eloff, 1998b; Eloff *et al*., 2008).

2.4.1 *Hydnora africana*

2.4.1.1 Description, Distribution and habitat.

Hydnora africana is a parasitic plant of the genus *Euphorbia.* It has such an unusual physical appearance that one would never say it is a plant. It looks astonishingly similar to fungi and is only distinguishable from fungi when the flower has opened. *Hydnora africana* is specifically associated with species of *Euphorbia*, commonly *E. mauretanica* and *E. tirucalli,* found in the dry and semi-arid parts of the Succulent Karoo, Little Karoo, Eastern Cape Karoo, and the dry coastal thickets between the Eastern Cape and KwaZulu-Natal province of South Africa. It grows very close to its host plant but may not be seen in the drier parts of the year. It occurs in both winter and summer rainfall areas with the most common vegetation being the Succulent Karoo, and Eastern Cape Karoo. It is found from the western coastal areas of

Namibia, southwards to the Cape and then northwards throughout Swaziland, Botswana, KwaZulu-Natal and as far as Ethiopia (Asfaw *et al.*, 1999).

2.4.1.2 Uses and cultural aspects

The fruit of *Hydnora africana* is said to be a traditional Khoi food, but there are no recorded details to confirm this. The fruit is delicious when baked and has a sweetish taste. Jackal food is used in a series of Cape dishes as recorded in the recipe book of Betsie Rood, *Kos uit die veldkombuis* (Rood, 1994). One of the recipes describes how the fruit pulp can be mixed with cream to make a delicious dessert. The fruit is extremely astringent and has been used for tanning and preserving fishing nets. Diarrhoea, dysentery, kidney and bladder complaints are all treated with infusions and decoctions of the plant. Infusions used as a face wash also treat acne (Van wyk and Gericke, 2000).

Despite the documented uses of this plant in traditional medicine and the growing resistance of common bacterial pathogens, especially those under investigation in this study, we are not aware of any study that has investigated the antimicrobial potential of this plant against these pathogens.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Bacterial strains

The following standard strains of bacteria *Aeromonas hydrophila* ATCC 35654, *Staphylococcus aureus* NCT 6571, *Helicobacter pylori* ATCC 43526 and local clinical strain of *Helicobacter pylori* PE 252C isolated in our laboratory (Tanih *et al*., 2010) were used. Cultures of confirmed organisms were maintained on nutrient agar slants at 4°C (Cheesbrough, 1982; Cowan and Steel, 2004). *H. pylori* was suspended in 20% glycerol and stored at -80°C.

3.2 Preparation of plants extracts

H. africana was selected based on ethnobotanical information and preliminary data obtained in our laboratory. It was identified in collaboration with botanists of the University of Venda, where voucher specimens have been deposited.

The method described by Ndip *et al*. (2008) to prepare extracts was employed with modifications. The plant was harvested, air dried for 2 weeks and ground to fine powder using a blender (ATO MSE mix, 702732, England). Organic solvents including methanol, ethanol, acetone, ethyl acetate (100%) and water were used for extraction. The dried plant material, 2.5-2.8 kg, was macerated in five fold excess of the solvent in extraction pots such that the level of the solvents was above that of the plant material. The slurry was put in a shaker incubator (Edison, N.J., USA) regulated at room temperature (RT) for 48 hours then centrifuged at 300 rpm for 5 mins (Model TJ-6 Beckman, USA) and filtered using filter papers of pore size 60^{Å}.The process was repeated twice for a total of three extractions. The combined extracts was concentrated in a rotavapor (BUCHI R461, Switzerland) and

transferred to appropriately labelled vials and allowed to stand at room temperature to permit evaporation of residual solvents. A 3 gram sample of each plant extract was used for the preliminary bioassay, and 3 g kept in the extract bank for subsequent use. Stock solutions were prepared by dissolving the extracts in 10% Dimethyl Sulphoxide (DMSO).

3.3 Antibacterial susceptibility test

The agar well diffusion technique was employed as previously described by Dastouri *et al*. (2008). For *H. pylori*, Columbia base agar was prepared following the manufacturer's instructions, supplemented with 7% defibrinated horse blood and skirrow's antibiotics while for *A. Hydrophila* and *S. aureus*, Muller-Hilton agar was prepared following the manufacturer`s instructions. A 0.5 McFarland standard was prepared by the method of Koneman *et al*. (1992), and 5mL put into a sterile test tube. An inoculum of each microorganism was prepared from subculture of bacterial suspension. With a sterile wire loop, four to five colonies of the same morphological type were picked and emulsified in 0.9% physiological saline. The turbidity of the suspension was adjusted to correspond to 0.5 MacFarland standard. An inoculum with the required turbidity was estimated to contain 10^8 colony forming unit and used to evenly inoculate specific agar plates depending on the microorganisms. Five wells were cut in each agar plate with a cooled, flamed cork borer of 6mm diameter, and the agar plugs removed with a sterile needle. About 100µL of the different concentrations (200, 100, 50 mg/mL) of the extract were put separately into each well, in each plate. Ciprofloxacin (0.0125mg/mL) was used as positive control. The plates were incubated at 37ºC for 24 hours for *A. hydrophila* and *S. aureus* and 3-5 days for *H. pylori* under microaerobic conditions and the diameter of the zone of inhibition measured and recorded in millimeters. The experiment was repeated 2x for each strain.

3.4 Determination of minimum inhibitory concentration (MIC_{50})

MIC_{50} was carried out as described by (Banfi *et al*., 2003, Njume *et al*., 2010) with modifications. The microdilution test was performed in 96-well plates. Two-fold dilutions of the most potent extracts and antibiotic (ciprofloxacin) were prepared in the test wells in complete Brian Heart Infusion (BHI) broth, the final extracts and antibiotic concentrations ranged from 0.0024 –5mg/mL. Twenty microlitres of each bacterial suspension was added to 180 µL of extract -containing culture medium. Control wells were prepared with culture medium and bacterial suspension only. Also included was culture medium and extract only at different concentrations. An automatic ELISA micro plate reader (Model 680, Bio-Rad, Japan) adjusted to 620nm was used to measure the absorbance of the plates before and after 24 hours incubation. The absorbencies were compared to detect an increase or decrease in bacterial growth. The lowest concentration of the test extract resulting in inhibition of 50% of bacterial growth was recorded as the MIC.

3.5 Determination of minimum bactericidal concentration (MBC)

To determine the MBC, 0.2mL of the contents of the MIC was serially diluted tenfold in 0.9% physiological saline (Ndip *et al.*, 2007). A loop full was taken from each tube and inoculated onto BHI agar plates. The MBC was recorded as the lowest concentration of the extract that gave complete inhibition of colony formation of the test bacteria at the latter cultivation.

3.6 Phytochemical screening of the extracts

A small portion of the dry extract was subjected to the phytochemical test using previously established methods (Akinpelu *et al.,* 2008) to test for alkaloids, tannins, flavonoids, steroids and saponins.

3.6.1 Test for alkaloids

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

3.6.2 Test for tannins

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

3.6.3 Test for flavonoids

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

3.6.4 Test for saponins

Two grams of the extract was boiled in 20mL of distilled water in a water bath and filtered (Acrodisc syringe filter pall, USA). Aprroximately 10mL of the filtrate was mixed with 5mL of distilled water and shaken vigorously for a stable persistent froth. The frosting was mixed

with 3 drops of olive oil and shaken vigorously, then observed for the formation of an emulsion

3.6.5 Test for steroids

About 0.5 g of the extract was dissolved in 3 mL of $CHCl_3$ and filtered. Concentrated H_2SO_4 was added to the filtrate to form a lower layer. A reddish brown colour was taken as positive for steroid ring.

3.7 Statistical analysis

Analysis was performed using the SPSS Version 17.0 (Illinois USA, 2009). The one way ANOVA test was used to determine if there was any statistically significant difference in the diameter of zones of inhibition of the different solvents extract of *H. africana* ; the MIC_{50} of the extracts and the control antibiotic (ciproxacillin). P-values <0.05 were considered significant**.**

CHAPTER FOUR

RESULTS

4.1 Extract yield

Different solvents including ethyl acetate, acetone, ethanol, methanol and water were used for extraction because the type of solvent used may have an effect on the nature of the compounds extracted, the quantity extracted and the resulting bioactivity of the extract. Water extracted the highest quantity followed by methanol, ethanol, acetone and ethyl acetate (11.2, 9.9, 5.6, 3 and1.5g) respectively (fig 1).

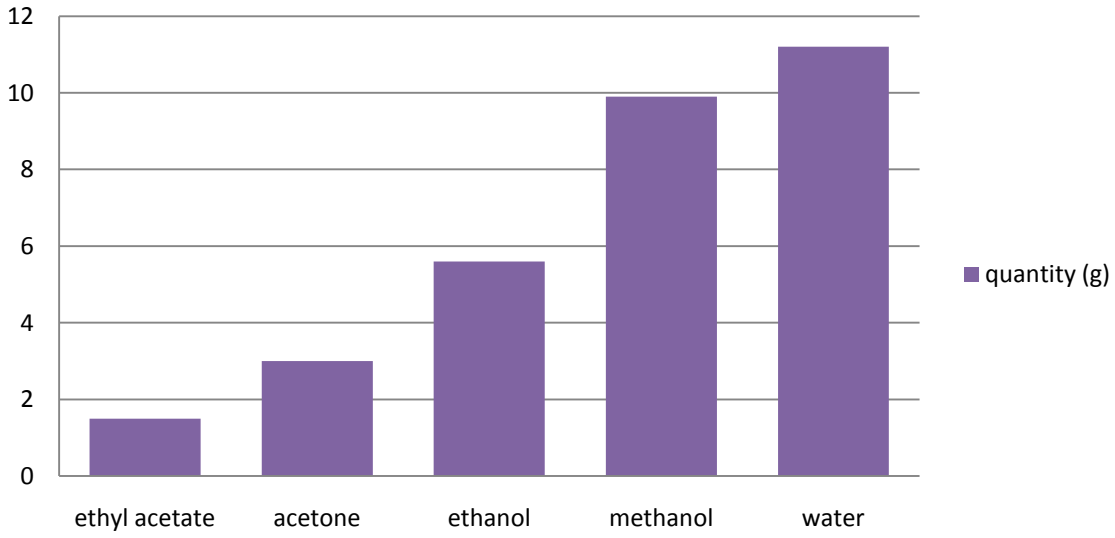

Fig 1: Quantity (grams) of *H.africana* flower extracted with different solvents. X-axis shows the different solvents used for extraction and Y-axis shows quantity extracted in grams.

4.2 Antimicrobial Susceptibility testing

The plant extracts showed *in vitro* activity against all the bacterial pathogens used *(S. aureus, A. hydrophila, H. pylori* 43526 and clinical isolate of *H. pylori* P.E 252C) with the exception of water extracts. The mean zone diameter of inhibition ranged from 0mm to 22mm (Table 1). Acetone, methanol and ethyl acetate were the most active extracts against *S. aureus, A. hydrophila* with mean zone diameter of inhibition ranging from 13-22mm, while for *H. pylori*, methanol and ethyl acetate extracts showed activity with mean zone diameter ranging from 14-21mm. The most active crude extracts (methanol and ethyl acetate) against all test microorganisms were statistically significant ($P < 0.05$) compared to all other extracts. DMSO used as negative control, showed no activity. Ciprofloxacilin (0.0125mg/mL) was used as a positive control, with mean zone diameter ranging from 14-17mm. An inhibition zone of ≥ 6mm was chosen as representative of bacterial susceptibility to the extracts. The breakpoint of ciprofloxacin (0.05mg/mL) is 21mm (CLSI, 2008).

Table 1: Antibacterial activity of extracts of *H.africana* against selected bacterial pathogens

	Zone diameter at different concentration (mm)*															
	Methanol			water			Acetone			Ethyl acetate			Ethanol			Cipro
	mg/mL															
SBP	200	100	50	200	100	50	200	100	50	200	100	50	200	100	50	0.025
S.a	17±2.1	21±2.1	22±2.1	0	0	0	20±0.7	19±1.4	22±3.5	16±0.7	18±0.7	19±0.7	14±1.4	16±1.4	17±1.4	17±0.7
A.h	17±0.7	16±0.7	15±1.4	0	0	0	17±2.1	17±1.4	18±1.4	14±1.4	13±1.4	15±0	13±0.7	16±1.4	14±0.7	17±1.4
H.p1	20±0.7	16±1.4	15±1.4	0	0	0	0	0	0	15±2.8	17±1.4	14±1.4	0	0	0	14±1.4
H.p 2	17±2.1	21±2.1	18±0.7	0	0	0	0	0	0	16±0.7	19±1.4	17±0.7	0	0	0	15±0.7

SBP, selected bacterial pathogens; *S.a*, *S.aureus*; *A.h*, A.*hydrophila*; *H.P*1, *H.pylori* 43526; *H.p* 2, *H.pylori* PE 252C; Cipro, ciprofloxacin;
*, experiment was repeated twice and zone of inhibition recorded as mean zone diameter ±SD. Sensitivity zone ≥ 6mm

4.3 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) determination

The active extracts were further assayed to determine their MIC_{50} and MBC against the bacterial pathogens. Although only methanol and ethyl acetate extracts showed activity against *H. pylori*; methanol, acetone, ethanol and ethyl acetate extracts were active against *S.aureus* and *A. hydrophila*. Subsequently methanol and ethyl acetate extracts were used for the determination of MIC_{50} and MBC for *H. pylori* (ATCC 43526 and PE 252C) and methanol, acetone, ethanol and ethyl acetate extracts were used to determine MIC_{50} and MBC for *S.aureus* and *A. hydrophila*. The MIC_{50} and MBC ranged from 0.078 – 2.5 mg/mL and 0.78 – 25mg/mL respectively for all studied microorganisms (fig 2-5), (Table 2). MIC_{50} and MBC of ciprofloxacin ranged from 0.00976 – 0.078mg/mL and 0.098– 0.78mg/mL respectively (fig 6), (Table 2). This drug served as the positive control.

The MIC_{50} was 0.078mg/mL, 0.15625mg/mL, 0.15625mg/mL and 0.625mg/mL for ethyl acetate, acetone, ethanol and methanol extracts against *S. aureus* respectively (fig 2). Furthermore, the MIC_{50} of *A. hydrophila* was 0.078mg/mL, 0.15625mg/mL and 0.3125mg/mL for ethyl acetate, acetone and methanol extracts in that order; ethanol showed no activity at MIC_{50} (fig 3). For ethyl acetate and methanol extracts, the MIC_{50} was 1.25mg/mL, 2.5mg/mL against *H. pylori* 43526 respectively (fig 4). Finally, the MIC_{50} was observed to be 2.5mg/mL for the ethyl acetate extract against *H. pylori* PE 252C*;* however the methanol extract showed no inhibition at MIC_{50} against *H. pylori* PE 252C (fig 5). The MIC_{50} of the antibiotic ciprofloxacin was 0.00976mg/mL, 0.00976mg/mL, 0.078mg/mL, 0.078mg/mL; while the MBC were 0.098mg/mL, 0.098mg/mL, 0.78mg/mL and 0.78mg/mL for *S. aureus, A. hydrophila, H. pylori* 43526 and *H. pylori* PE 252C respectively (fig 6), (Table 2).

Gram positive bacteria (*S aureus*) was most susceptible to *H. africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). However, there was no statistically significant difference (P>0.05) between the MIC_{50} and MBC of different solvents against Gram negative and Gram positive organisms. Also, there was no statistically significant difference in activity between the extracts (methanol, acetone, ethanol, ethyl acetate) and the control antibiotic (ciprofloxacin).

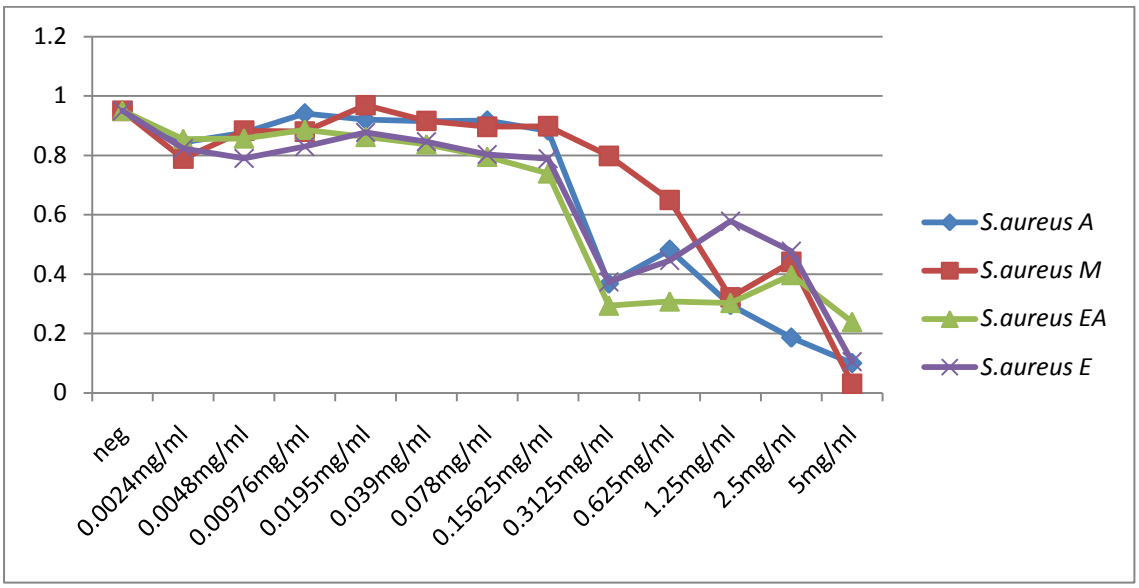

Fig 2: MIC_{50} of different solvent extracts against *S. aureus*. X-axis shows concentration of extracts and Y-axis viability of *S. aureus.* neg, broth and isolate only; 0.0024mg/mL-5mg/mL, different concentration of crude extracts of *H. africana;* A, acetone; M, methanol; EA, ethyl acetate; E, ethanol.

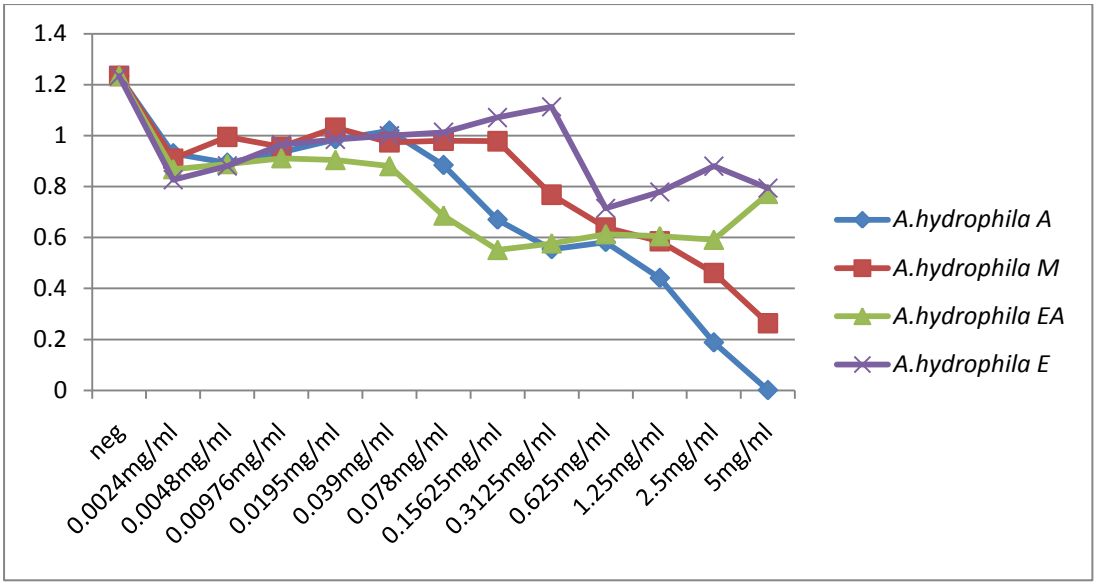

Fig 3: MIC_{50} of different solvent extracts against *A. hydrophila*. X-axis shows concentration of extracts and Y-axis viability of *A. hydrophila*. neg, broth and isolate only; 0.0024mg/mL-5mg/mL, different concentration of crude extracts of *H. africana*. A, acetone; M, methanol; EA, ethyl acetate; E, ethanol.

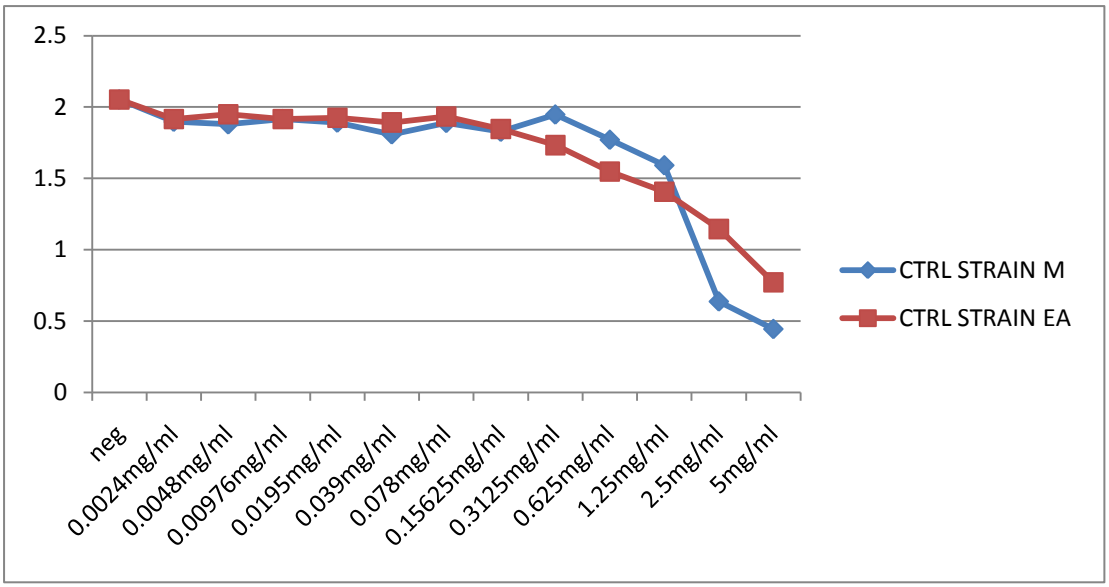

Fig 4: MIC_{50} of different solvent extracts against *H. pylori* 43526 (CTRL strain). X-axis shows concentration of extracts and Y-axis viability of *H. pylori* 43526. neg, broth and isolate only; 0.0024mg/mL- 5mg/mL, different concentration of crude extracts of *H. africana*. A, acetone; M, methanol; EA, ethyl acetate; E, ethanol.

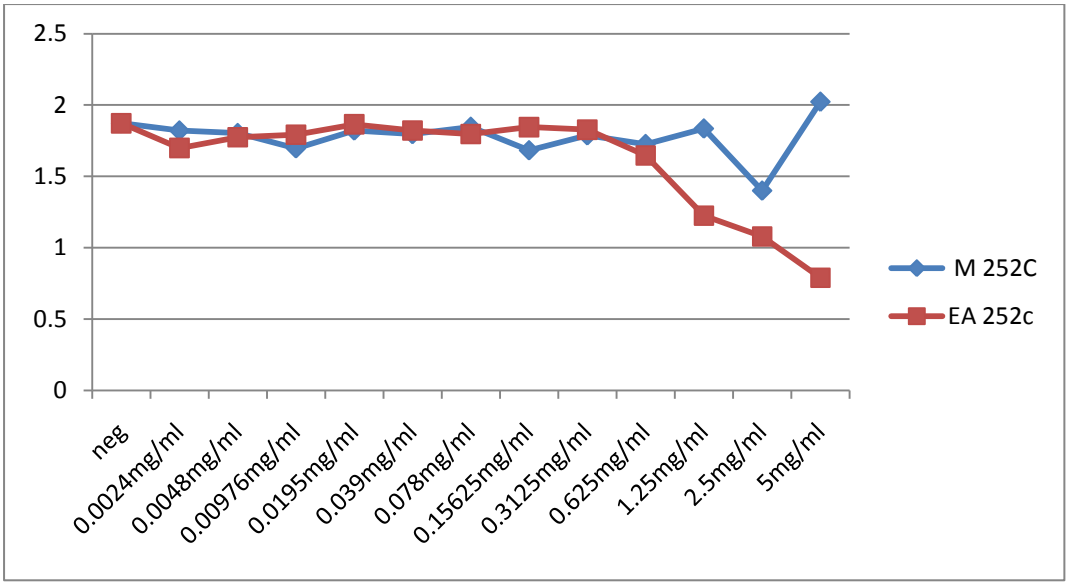

Fig 5: MIC_{50} of different solvent extracts against *H. pylori* PE 252C. X-axis shows concentration of extracts and Y-axis viability of *H. pylori*. neg, broth and isolate only; 0.0024mg/mL- 5mg/mL, different concentration of crude extracts of *H. africana*. A, acetone; M, methanol; EA, ethyl acetate; E, ethanol.

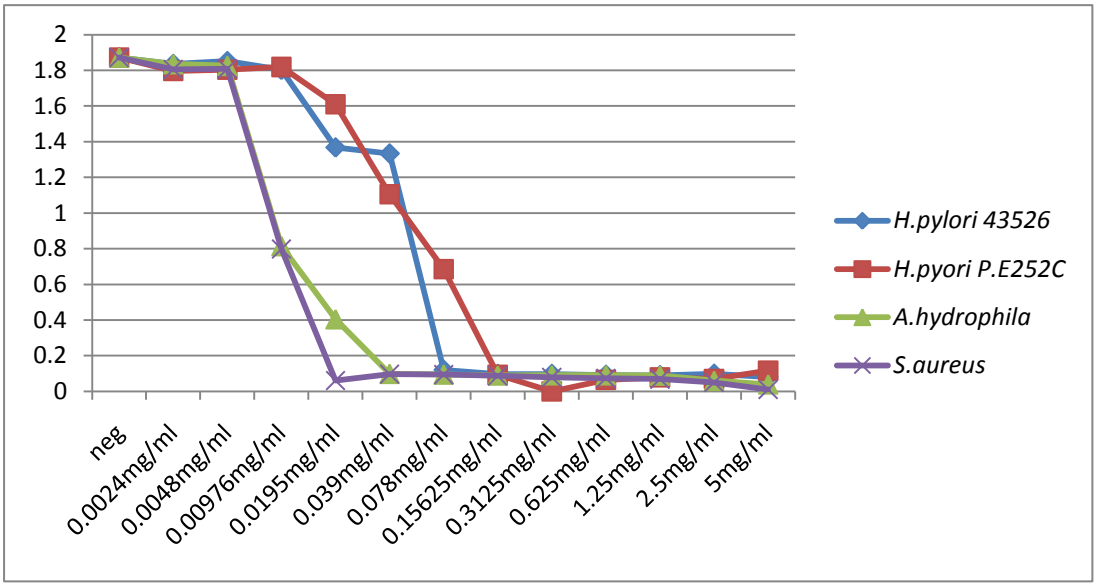

Fig 6: MIC_{50} of antibiotic (ciprofloxacin) against selected bacterial pathogens. X-axis shows concentration of antibiotic and Y-axis viability of *H. pylori*. neg, broth and isolate only; 0.0024mg/mL- 5mg/mL, different concentration of ciproxacillin.

Table 2: MBC (mg/mL) of different solvent extracts of *H. africana* and antibiotic against selected bacterial pathogens

	Extracts/ Antibiotic (mg/mL)				
SBP	Methanol	Acetone	Ethanol	Ethyl acetate	Ciprofloxacin
S.aureus	6.25	1.56	1.56	0.78	0.098
A.hydrophila	3.125	1.56	_	0.78	0.098
H.pylori43526	25	ND	ND	12.5	0.78
*H.pylori*252C	_	ND	ND	_	0.078

SBP, selected bacterial pathogens; –, MBC values not within susceptible range; ND, not determined.

4.5 Phytochemical analysis

Phytochemical analysis of four extracts (methanol, acetone, ethanol and ethyl acetate) of *H. africana* is summarized in Table 3. The results revealed the presence of the following secondary metabolites: alkaloids, saponins, tannins, steroids and flavoinds, based on colour, heamolysis, turbidity, layers, emulsification and precipitation following the reactions.

Table 3: Phytochemical constituents of different solvent extracts of *H.africana*

Phytochemicals	Solvent extracts			
	Methanol	Acetone	Ethanol	Ethyl acetate
Alkaloids	+++	++	+++	++
Saponins	+++	++	++	+++
Tannins	+++	+++	+++	+++
Flavonoids	+++	+++	+++	+++
Steroids	+++	+++	+++	+++

+++, Present in large quantity; ++, Present in moderate quantity.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS.

5.1 Discussion

The phytoconstituents of various plants have longed been known and their antimicrobial properties have been widely reported (Nostro *et al*., 2000; Roy *et al.*, 2006). The antimicrobial activities of plant extracts have been linked to the presence of some bioactive compounds. These secondary metabolites also serve to protect the plants themselves against bacterial, fungal and viral infections (De and Ijeoma, 2002; El-Mahmood and Amey, 2007). These bioactive compounds are known to work synergistically to produce various effects on the human and animal subjects (Amagase, 2006). However, most reports on *Hydnora africana* have focused mainly on the morphology of the plant because it has a bizarre shape, while information on its activity against hospital based pathogens is scanty (Bolin *et al*., 2009).

The extraction of active compounds from plant material depends on the type of solvent used in the extraction process (Parekh *et al*., 2005; Majhenic *et al*., 2007). In this study, it was observed that plant extractions with organic solvents provided stronger antibacterial activity than extraction with water. This study confirms the results of previous studies, which reported that water is not a suitable solvent for extraction of antibacterial compounds from medicinal plants compared to organic solvents, such as methanol, acetone and ethyl acetate (Karaman *et al*, 2003; Moniharapon and Hashinaga, 2004; Parekh *et al*, 2005; Majhenic *et al*, 2007). This finding is also correlated with the medicinal preparations that use rum and liquor to extract the active plant components (Jhon *et al*., 2006). Extract yields of water, methanol,

ethanol, acetone and ethyl acetate were 11.2g, 9.9g, 5.6g, 3g and 1.5g respectively after one extraction.

H. africana exhibited a stronger antibacterial activity against *H. pylori* than previously reported plants including *Eryngium foetidium, Euphorbia hirta* and *Tapienachilus ananassae* (Ndip *et al*., 2007). Their mean zone diameter of inhibition ranged from 0–18mm (Ndip *et al*., 2008) while in the present study the mean zone diameter ranged from 0–21mm (Table 1). In line with the findings of this study, another study had demonstrated very potent antibacterial activity of *Hydnora abyssinica*. In their study, Saadabi and Ayoub *et al.* (2009) screened crude extracts of the family *Hydnoracea* (*H. africana* also belong in this family), and reported potent antibacterial activity against common pathogenic gram-negative and positive bacteria including *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis* and *Staphylococcus aureus*.

In the present study MIC_{50} and MBC recorded for *H. africana* against all studied microorganisms ranged from 0.078–2.5mg/mL and 0.78–25mg/mL respectively. However, the methanol extract showed no inhibition at MIC_{50} against *H.pylori* PE 252C. The MIC results confirm earlier findings by Nariman *et al*. (2004) who documented MIC ranges of 0.0037–2 mg/mL. Also, MIC values of 0.0625–0.5 mg/mL have been documented for East African medicinal plants against similar bacteria pathogens (Fabry *et al*., 1996).

Moreover in line with our finding, another study had demonstrated very potent antibacterial activity of *Afzelia Africana*. In their study, Akinpelu *et al*. (2008) screened crude extracts of *A. africana* commonly used to treat bacterial infections. They tested this plant on common pathogenic gram-negative and positive bacteria including *Staphylococcus aureus* amongst other microorganisms. Their lowest MIC recorded was 1.56 mg/mL and the lowest MBC was 3.13 mg/mL. MIC_{50} and MBC of the antibiotic (ciprofloxacin) ranged from 0.00976-0.078mg/mL; 0.098– 0.78mg/mL for all tested bacterial pathogens respectively and was not

statistically significant in activity (P>0.05) compared to the extracts. Such results provide evidence that some medicinal plants might be potential sources of new antibacterial agents even against some resistant strains.

Gram positive bacteria (*S. aureus*) was most susceptible to *H.africana* compared to the Gram negative bacteria (*A. hydrophila and H. pylori*). Most plants extracts are most active against Gram positive bacteria; this has been attributed to the fact that the cell wall of Gram positive bacteria is easier to penetrate than the Gram negative bacteria which contains outer membrane with a lipopolysacharide layer which is impermeable to certain antibiotics and antibacterial compounds (Nikaido, 1996; Fennell *et al*., 2004).

Phytochemical analysis of the extracts of *H. africana* revealed the presence of alkaloids, saponins, tannins, flavonoids and steroids (Table 3). These phytochemical compounds are known to be biologically active and thus aid the antimicrobial activities of plants. Alkaloids was one of the phytochemical compounds identified in this study. They have been allied with medicinal uses for centuries. Most common biological properties of alkaloids are their toxicity against cells of foreign organisms, antiinflammatory, anti-asthmatic, and anti-anaphylactic properties (Gopalakrishnan *et al.,* 1979; Ganguly and Sainis, 2001; Staerk *et al.,* 2002) and may be responsible for the observed activity.

The presence of flavonoids in crude extract of *H. africana* is important since they have been reported to exhibit antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic, antioxidant, antitrypanosomal and antileishmanial properties (Hodek *et al*., 2002). Flavonoids exhibit a wide range of biological activities such as the ability of scavenging hydroxyl radicals, superoxide anion radicals and lipid peroxyradicals. These radicals are important for prevention of diseases associated with oxidative damage of membrane, proteins and DNA (Ferguson, 2001). Flavonoids in human diet may reduce the risk of various cancers,

coronary heart diseases as well as preventing menopausal symptoms (Xu *et al*., 2000; Hodek *et al*., 2002; Tasdemir *et al*., 2006).

Saponins and tannins were also reported in this study. Saponins are responsible for numerous pharmacological properties and are known to produce inhibitory effects on inflammation (Just *et al*., 1998; Estrada *et al*., 2000). Tannins exert antimicrobial activities by iron deprivation, hydrogen bonding or specific interactions with vital proteins such as enzymes in microbial cells (Njume *et al*., 2009). Herbs that have tannins are astringent in nature and are used for treating intestinal disorders such as diarrhoea and dysentery (Dharmananda, 2003). Motar *et al*. (1985) revealed the importance of tannins for the treatment of inflamed or ulcerated tissues. Tannins were observed to have remarkable activity in cancer prevention (Li *et al.*, 2003), this is important noting that *H. africana* could have potentials as a source of important bioactive molecules for the treatment of cancer (Trease and Evans, 1983). Lastly steroidal compounds were also present in the crude extracts of *H. africana;* they have drawn much interest in pharmacy due to their relationship with such compounds as sex hormones (Okwu, 2001).

5.2 Conclusion

From the results obtained, the following conclusions can be drawn:

1. The study demonstrated the *in vitro* activities of the crude extracts of *H.africana* and provides preliminary evidence for the use of this plant in traditional medicine.
2. The MIC_{50} and MBC of the crude extracts ranged from 0.078–2.5mg/mL, 0.78-25mg/mL respectively.
3. Alkaloids, saponnins, tannins, flavonoids and steroids were identified in the extracts of the plant.

5.3 Recommendations

1. Bioassay-guided fractionation should be conducted to determine the active compounds in *H. africana.*
2. The toxicity of the compounds should be determined.
3. *In vivo* animal model studies should be conducted to ascertain their healing potential.

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APPENDICES

Appendix 1

Representative photographs of sites of infection and plant under study

Fig 1: H.africana **(flower)**

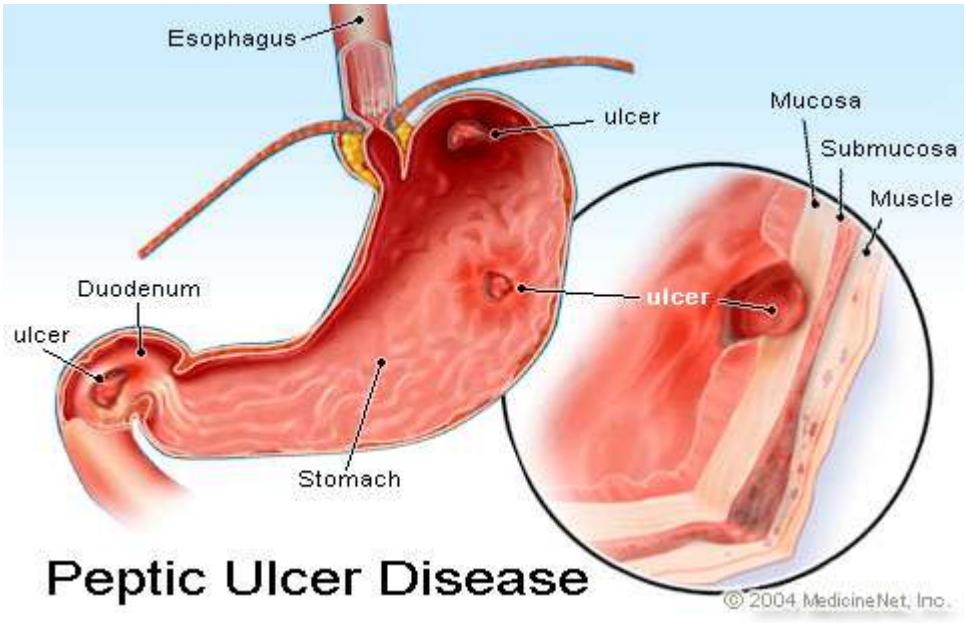

Fig 2: Stomach ulcers caused by *H.pylori*

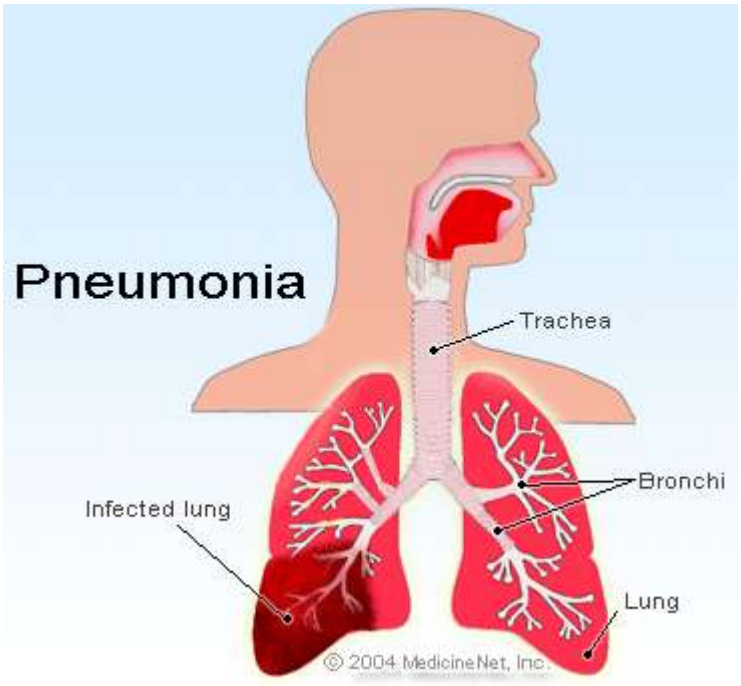

Fig 3: Pneumonia caused by *Staphylococcus aureus*

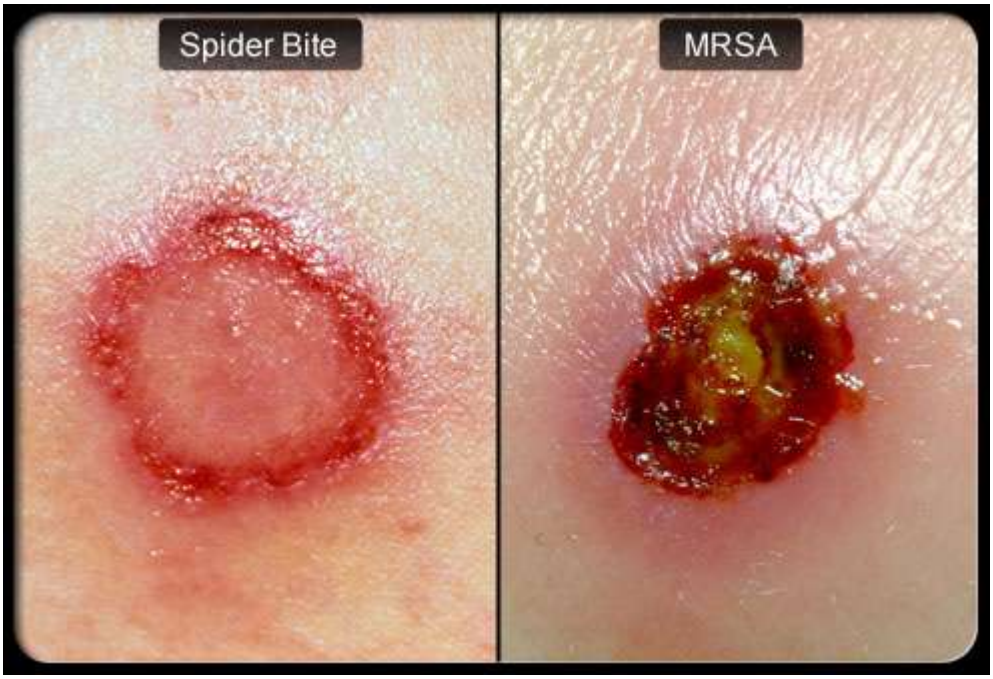

Fig 4: Wound caused by *Staphylococcus aureus*

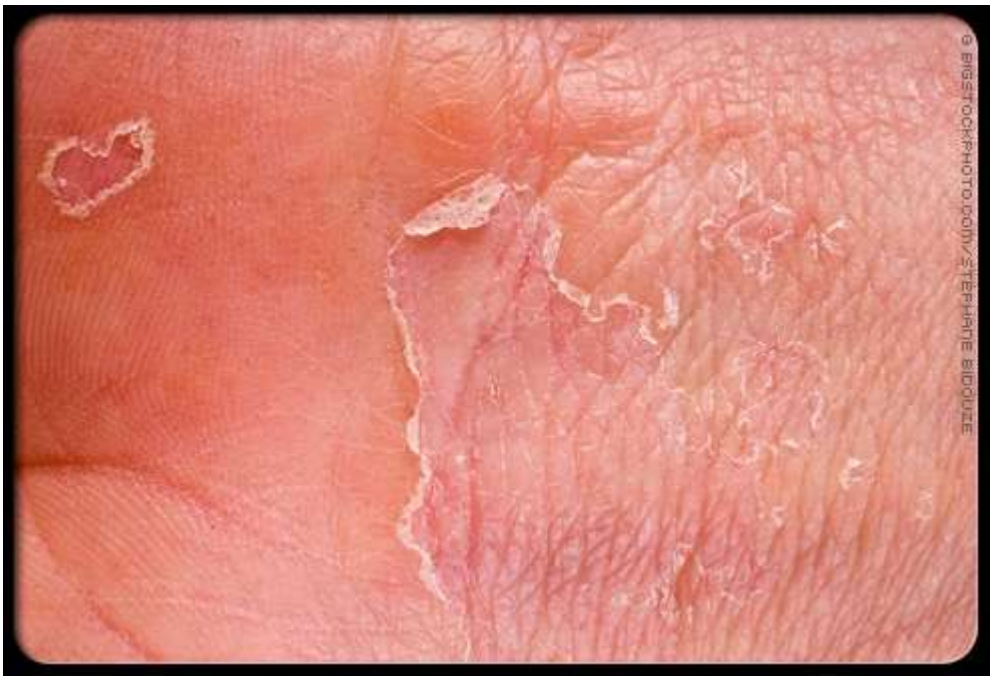

Fig 5: Eczema caused by *Aeromonas hydrophila*

Appendix 2

Media used in this study and preparation.

Preparation of culture media

The culture media was composed of Columbia Blood Agar (CBA), skirrow`s antibiotics and 7% horse blood for *H. pylori*. CBA was prepared using the manufacturer`s instructions which indicated 39g/L of the agar for *H. pylori*; while for *S. aureus* and *A. hydrophila,* Muller-Hilton agar was prepared following manufacturer`s instructions which indicated 38g/L. The mixtures were boiled to dissolve and sterilized in an autoclave at 121°C for 15 minutes. It was allowed to cool then poured in plates.

Preparation of Brain Heart Infusion agar

It was prepared following the manufacturer`s instructions which indicated 47g/L of the broth. The mixture was boiled to dissolve and sterilized in an autoclave at 121°C for 15min. It was allowed to cool (50°C), after which 2ml of skirrow`s antibiotics and 35ml of 7% horse blood were added.

Appendix 3

Statistical observations

Antibacterial activity of different solvents extract of *H.africana* against tested bacterial strains at 200mg/ml

Multiple Comparisons

Zd

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	8.2500	4.1370	.315	-4.525	21.025
	3	10.6250	4.1370	.127	-2.150	23.400
	4	2.2500	4.1370	.981	-10.525	15.025
	5	17.2500*	4.1370	.006	4.475	30.025
2	1	-8.2500	4.1370	.315	-21.025	4.525
	3	2.3750	4.1370	.977	-10.400	15.150
	4	-6.0000	4.1370	.607	-18.775	6.775
	5	9.0000	4.1370	.241	-3.775	21.775
3	1	-10.6250	4.1370	.127	-23.400	2.150
	2	-2.3750	4.1370	.977	-15.150	10.400
	4	-8.3750	4.1370	.301	-21.150	4.400

	5	6.6250	4.1370	.519	-6.150	19.400
4	1	-2.2500	4.1370	.981	-15.025	10.525
	2	6.0000	4.1370	.607	-6.775	18.775
	3	8.3750	4.1370	.301	-4.400	21.150
	5	15.0000*	4.1370	.018	2.225	27.775
5	1	-17.2500*	4.1370	.006	-30.025	-4.475
	2	-9.0000	4.1370	.241	-21.775	3.775
	3	-6.6250	4.1370	.519	-19.400	6.150
	4	-15.0000*	4.1370	.018	-27.775	-2.225

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

1=Methanol, 2=Acetone, 3=Ethanol, 4=Ethyl acetate, 5=water

Antibacterial activity of different solvents extract of *H.africana* against tested bacterial strains at 100mg/ml

Multiple Comparisons

Zd

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	9.5000	4.3853	.244	-4.042	23.042
	3	11.2500	4.3853	.128	-2.292	24.792

	4	1.8750	4.3853	.992	-11.667	15.417
	5	18.5000*	4.3853	.006	4.958	32.042
2	1	-9.5000	4.3853	.244	-23.042	4.042
	3	1.7500	4.3853	.994	-11.792	15.292
	4	-7.6250	4.3853	.442	-21.167	5.917
	5	9.0000	4.3853	.290	-4.542	22.542
3	1	-11.2500	4.3853	.128	-24.792	2.292
	2	-1.7500	4.3853	.994	-15.292	11.792
	4	-9.3750	4.3853	.255	-22.917	4.167
	5	7.2500	4.3853	.489	-6.292	20.792
4	1	-1.8750	4.3853	.992	-15.417	11.667
	2	7.6250	4.3853	.442	-5.917	21.167
	3	9.3750	4.3853	.255	-4.167	22.917
	5	16.6250*	4.3853	.013	3.083	30.167
5	1	-18.5000*	4.3853	.006	-32.042	-4.958
	2	-9.0000	4.3853	.290	-22.542	4.542
	3	-7.2500	4.3853	.489	-20.792	6.292

	4	-16.6250*	4.3853	.013	-30.167	-3.083

*. The mean difference is significant at the 0.05 level. 1=Methanol, 2=Acetone, 3=Ethanol, 4=Ethyl acetate, 5=water

Antibacterial activity of different solvents extract of *H.africana* against tested bacterial strains at 50mg/ml

Multiple Comparisons

Zd

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	7.3750	4.7445	.546	-7.276	22.026
	3	9.6250	4.7445	.300	-5.026	24.276
	4	1.1250	4.7445	.999	-13.526	15.776
	5	17.2500*	4.7445	.018	2.599	31.901
2	1	-7.3750	4.7445	.546	-22.026	7.276
	3	2.2500	4.7445	.989	-12.401	16.901
	4	-6.2500	4.7445	.685	-20.901	8.401
	5	9.8750	4.7445	.278	-4.776	24.526
3	1	-9.6250	4.7445	.300	-24.276	5.026

	2	-2.2500	4.7445	.989	-16.901	12.401
	4	-8.5000	4.7445	.413	-23.151	6.151
	5	7.6250	4.7445	.515	-7.026	22.276
4	1	-1.1250	4.7445	.999	-15.776	13.526
	2	6.2500	4.7445	.685	-8.401	20.901
	3	8.5000	4.7445	.413	-6.151	23.151
	5	16.1250*	4.7445	.028	1.474	30.776
5	1	-17.2500*	4.7445	.018	-31.901	-2.599
	2	-9.8750	4.7445	.278	-24.526	4.776
	3	-7.6250	4.7445	.515	-22.276	7.026
	4	-16.1250*	4.7445	.028	-30.776	-1.474

*. The mean difference is significant at the 0.05 level. 1=Methanol, 2=Acetone, 3=Ethanol, 4=Ethyl acetate, 5=water

MIC*50* of different solvent extracts

Multiple Comparisons

Mic

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	5.0781250000	2.9719521691	.361	-3.745307128	13.901557128
	3	4.9218750000	2.9719521691	.386	-3.901557128	13.745307128

	4	3.8086250000	2.9719521691	.591	-5.014807128	12.632057128
2	1	-5.0781250000	2.9719521691	.361	-13.901557128	3.745307128
	3	-.1562500000	2.9719521691	1.000	-8.979682128	8.667182128
	4	-1.2695000000	2.9719521691	.973	-10.092932128	7.553932128
3	1	-4.9218750000	2.9719521691	.386	-13.745307128	3.901557128
	2	.1562500000	2.9719521691	1.000	-8.667182128	8.979682128
	4	-1.1132500000	2.9719521691	.981	-9.936682128	7.710182128
4	1	-3.8086250000	2.9719521691	.591	-12.632057128	5.014807128
	2	1.2695000000	2.9719521691	.973	-7.553932128	10.092932128
	3	1.1132500000	2.9719521691	.981	-7.710182128	9.936682128

1=Methanol, 2=Acetone, 3=Ethanol, 4=Ethyl acetate

MBC of different solvent extracts

Multiple Comparisons

mbc

Tukey HSD

(I) ext	(J) ext	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	4.3433614000	3.0918916392	.520	-4.836159155	13.522881955
	3	4.3827490000	3.0918916392	.513	-4.796771555	13.562269555
	4	4.3827474250	3.0918916392	.513	-4.796773130	13.562267980
2	1	-4.3433614000	3.0918916392	.520	-13.522881955	4.836159155
	3	.0393876000	3.0918916392	1.000	-9.140132955	9.218908155
	4	.0393860250	3.0918916392	1.000	-9.140134530	9.218906580
3	1	-4.3827490000	3.0918916392	.513	-13.562269555	4.796771555
	2	-.0393876000	3.0918916392	1.000	-9.218908155	9.140132955
	4	-.0000015750	3.0918916392	1.000	-9.179522130	9.179518980
4	1	-4.3827474250	3.0918916392	.513	-13.562267980	4.796773130
	2	-.0393860250	3.0918916392	1.000	-9.218906580	9.140134530

3	.0000015750	3.0918916392	1.000	-9.179518980	9.179522130

1=Methanol, 2=Acetone, 3=Ethanol, 4= Ethyl acetate

Multiple Comparisons FOR MICS of the extracts and antibiotics

MIC

Tukey HSD

(I) EXTS	(J) EXTS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	.96354167	.60662512	.527	-.9266680	2.8537513
	3	.80729167	.60662512	.678	-1.0829180	2.6975013
	4	-.30595833	.60662512	.986	-2.1961680	1.5842513
	5	.95541667	.60662512	.535	-.9347930	2.8456263
2	1	-.96354167	.60662512	.527	-2.8537513	.9266680
	3	-.15625000	.56162573	.999	-1.9062441	1.5937441
	4	-1.26950000	.56162573	.215	-3.0194941	.4804941
	5	-.00812500	.56162573	1.000	-1.7581191	1.7418691
3	1	-.80729167	.60662512	.678	-2.6975013	1.0829180
	2	.15625000	.56162573	.999	-1.5937441	1.9062441
	4	-1.11325000	.56162573	.323	-2.8632441	.6367441
	5	.14812500	.56162573	.999	-1.6018691	1.8981191
4	1	.30595833	.60662512	.986	-1.5842513	2.1961680
	2	1.26950000	.56162573	.215	-.4804941	3.0194941
	3	1.11325000	.56162573	.323	-.6367441	2.8632441
	5	1.26137500	.56162573	.219	-.4886191	3.0113691
5	1	-.95541667	.60662512	.535	-2.8456263	.9347930
	2	.00812500	.56162573	1.000	-1.7418691	1.7581191
	3	-.14812500	.56162573	.999	-1.8981191	1.6018691
	4	-1.26137500	.56162573	.219	-3.0113691	.4886191

1=methanol, 2=acetone, 3=ethanol, 4=ethyl acetate, 5=ciprofloxacin

Multiple Comparisons FOR MBC of the extracts and the antibiotic

MBC

Tukey HSD

(I) EXT	(J) EXT	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	2	-.0290548000	.0286896214	.845	-.118450040	.060340440
	3	.0103328000	.0286896214	.996	-.079062440	.099728040
	4	.0025831750	.0286896214	1.000	-.086812065	.091978415
	5	.0047738000	.0286896214	1.000	-.084621440	.094169040
2	1	.0290548000	.0286896214	.845	-.060340440	.118450040
	3	.0393876000	.0265614282	.589	-.043376310	.122151510
	4	.0316379750	.0265614282	.756	-.051125935	.114401885
	5	.0338286000	.0265614282	.711	-.048935310	.116592510
3	1	-.0103328000	.0286896214	.996	-.099728040	.079062440
	2	-.0393876000	.0265614282	.589	-.122151510	.043376310
	4	-.0077496250	.0265614282	.998	-.090513535	.075014285
	5	-.0055590000	.0265614282	1.000	-.088322910	.077204910
4	1	-.0025831750	.0286896214	1.000	-.091978415	.086812065
	2	-.0316379750	.0265614282	.756	-.114401885	.051125935
	3	.0077496250	.0265614282	.998	-.075014285	.090513535
	5	.0021906250	.0265614282	1.000	-.080573285	.084954535
5	1	-.0047738000	.0286896214	1.000	-.094169040	.084621440
	2	-.0338286000	.0265614282	.711	-.116592510	.048935310
	3	.0055590000	.0265614282	1.000	-.077204910	.088322910
	4	-.0021906250	.0265614282	1.000	-.084954535	.080573285

1=methanol, 2=acetone, 3=ethanol, 4=ethyl acetate, 5=ciprofloxacin

Appendix 4

Manuscripts in preparation

1. Bioactivity and phytochemical analysis of *Hydnora africana* on some selected bacterial pathogens.
2. Bioactivity of *Hydonora africana* on some selected bacterial pathogens: Preliminary phytochemical screening.