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Assessment of anti-urease and consequential inhibitory potential of South African honey extracts on the multiplication of drug-resistant, *vac*A and *cag*A positive *Helicobacter pylori* strains under acidic conditions

by

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Declaration

I, Callote Dube, student number 200803278, hereby declare that this thesis submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture is my original work and has never been presented for a degree in any other university, and that all sources of material used for this thesis have been duly acknowledged.

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Dedication

Family, relatives and friends

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List of abbreviations

Ag^+	Silver ion
***Alp*A**	Adherence-associated lipoprotein
Am	Amoxicillin
***Bab*A**	Blood group antigen binding adhesin
***Cag*A PAI**	Cytotoxin associated gene pathogenicity island
CJBU	Commercial Jack bean urease
Cl	Clarithromycin
CO_2	Carbon dioxide
Cu2+	Copper
DNA	Deoxyribonucleic acid
***Dup*A**	Duodenal ulcer promoting gene
GC-MS	Gas chromatograph – mass spectrometry
GDH	Glutamate dehydrogenase
H_2O_2	Hydrogen peroxide
Hg^+	Mercury ion
Hop	*Helicobacter* outer membrane
HPU1	Urease extracted from *H. pylori* susceptible to drugs
HPU2	Urease extracted from *H. pylori* resistant to clarithromycin

HPU3	Urease extracted from multidrug resistant *H. pylori*
HSP	Heat shock protein
***Ice*A**	Induced by contact to epithelium
IL	Interleukin
KDa	Kilo Dalton
LC-MS	Liquid chromatography – mass spectrometry
MALT	Mucosa associated lymphoid tissue
MBC	Minimum bactericidal concentration
Met	Metronidazole
MHA	Muller Hinton Agar
MIC	Minimum inhibitory concentration
Na_2SO_4	Anhydrous disodium sulphate powder.
NADH	Nicotinamide Adenine dinucleotide
NF-KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NH_3	Ammonia
Ni^{2+}	Nickel
OD	Optimum density
***Oip*A**	Outer membrane inflammatory protein
OMP	Outer membrane protein

PCR	Polymerase chain reaction
PPI	Proton pump inhibitor
PUD	Peptic ulcer disease
RFLP	Restriction fragment length polymorphism
***Sab*A**	Sialic acid binding adhesin
Tet	Tetracycline
***Vac*A**	Vacuolating cytotoxin A
WHO	World Health Organisation

General abstract

Helicobacter pylori, a neutralophile chronically infects the gastric stomach of more than half of the world's population. Infection with the organism is associated with acute or chronic duodenal/gastric ulcer disease, gastritis, gastric adenocarcinoma, mucosa-associated tissue lymphoma (MALT) and primary B-cell gastric lymphoma and has been grouped as a class one carcinogen by the World Health Organisation (WHO). Prevalence of this organism is very high in developing countries especially in Africa, including South Africa. *H. pylori* treatment using the common first and second line regimens, triple therapy with two antibiotics and a proton pump inhibitor (PPI) is showing inefficiency due to increasing drug resistance. However, newly developed treatment regimens seem to be more expensive and are accompanied by more side effects. Honey contains phytochemicals which are a wealthy source of biologically active compounds some of which have been put into good use in the pharmaceutical industry. Pathogenesis of *H. pylori* infection in the human stomach relies on several virulence factors which include the urease enzyme, *cag*A and *vac*A. The urease enzyme actively hydrolyses urea to produce ammonia an important by-product involved in pH regulation favouring the survival of the organism in the acidic human stomach. This study therefore focuses on screening for anti-urease solvent extracts of South African honey, and evaluate whether inhibition of urease offsets the growth of *H. pylori* under acidic conditions.

Locally produced natural honeys; Bush honey, Raw honey, Gold Crest honey, Q Bee honey, Little Bee honey, Fleures honey-radurised, Siyakholwa pure honey and Manuka honey; an import from New Zealand were purchased and the method by Syazana *et al*. (2010) was used for the extraction of compounds in honey. A standard strain ATCC 43526 (American Type Culture Collection, Manassas, VA, USA) and 48 pure cultures obtained from clinical isolates cultured from gastric corpus biopsy specimen of patients with gastric morbidities who were

visiting the endoscopy unit in Livingstone Hospital, Port Elizabeth between June 2008 to December 2008 were initially used as source of urease enzyme as per extraction method done by Amin *et al*. (2013), but with modifications. Prior to urease extraction, *H. pylori* strains were identified by biochemical tests (urease, catalase, oxidase, Gram stain), confirmed by PCR targeting the *glm*M gene (140 bp) and drug resistance profiling was done on all the 48 strains according to Seanego *et al*. (2012). The screening for anti-urease active compounds was done according to Kaltwasser *et al*. (1966), a method relying on the reduction of NADH in a coupled urease dehydrogenase (GDH) system. Acetohydroxamic acid was used as a standard inhibitor.

Prevalence of *cytotoxin-associated* gene A (*cag*A) gene and *vacuolating cytotoxin* gene A (*vac*A) gene was determined among all 48 clinical samples. The standard strains of *H. pylori*, X47 (*cag*A positive), J99 (*vac*A s1m1) and Tx30a (*s*2*m*2) were used as positive controls. *H. pylori*'s growth was then monitored under acidic pH in a cocktail spiked with anti-urease compounds (test samples) and in a cocktail without anti-urease compounds (negative control). Acetohydroxamic acid was used as a standard urease inhibitor. *H. pylori* multiplication was monitored in Brain Heart Infusion Broth (BHIB) adjusted to pH of 2, 3, 4, 5, 6 and 7. The strain MP01 was used as a standard urease negative strain while X47 and J99 were used as positive standards for *cag*A and *vac*A *s*1*m*1 respectively. The compounds that had anti-urease activity and were successful towards suppressing the multiplication of *H. pylori* under acidic environment, all other factors optimised, were subjected to gas chromatograph mass spectrometry (GC-MS) and liquid chromatograph spectrometry (LC-MS) to determine volatile compounds and drugs in honey extracts respectively.

The findings of this study revealed that at a concentration of 50 mg/mL, urease inhibition by petroleum ether extracts of Gold Crest and Fleures honey, hexane extracts of Little Bee and Manuka honey, and chloroform extracts of Bush honey and Q Bee honey had a range above or equal to 50% and there was no significance difference in urease inhibition percentage (I%) of urease from different sources including that extracted from drug resistant *H. pylori* ($p > 0.05$). Virulence factors are important for the pathogenesis of *H. pylori*. All the 48 clinical isolates were *glm*M (140 bp) positive and *cag*A was detected in 97.9% of the test isolates. The *vac*A gene was detected in all isolates but with different subtypes. The *vac*A allelic combination *s*1*m*1 was detected in 75% of the test isolates and *s*1*m*2 allelic combination was detected in 16.7% of the test isolates while the combination *s*2*m*2 was detected in 8.3% of the test isolates. None of the test isolates possessed the allelic combination *s*2*m*1. When *H. pylori* multiplication was monitored under acidic conditions in the presence of anti-urease active compounds, it was revealed that anti-urease active compounds in honey are capable of inhibiting the normal multiplication of *H. pylori* strains that are *cag*A positive, *vac*A positive and drug resistant.

The GC-MS analysis showed that Fleures honey (urease I% = 67.8 – 68.5%) and Gold Crest honey (urease I% = 50.9% – 53.3%), all petroleum ether extracts had 27 and 26 volatile compounds. The hexane extract of Manuka honey (urease I% = 50.0 – 53.2) had 43 compounds detected. The chloroform extract of Q Bee (urease I% = 64.2 – 66.2%) had 13 volatile compounds detected. All the volatile compounds considered as representative samples of GC-MS analysis had a spectral matching ≥ 90% with the NIST11 library. However, the majority of compounds that were detected by LC-MS in representative honey extracts include vardenafil, urapidil, hydrocortisone, e.t.c which are drugs commonly used in the treatment of different ailments or infections that affect human beings. In addition, two

drugs, sulfaquinoxaline and hydroxyquinoline which are used in veterinary medicine and antiseptic, disinfectant and pesticide applications in agricultural activities were detected in Little Bee honey. We therefore conclude that inhibition of urease has a bactericidal effect on drug resistant, *cag*A positive and *vac*A positive *H. pylori* strains growing under acidic environment.

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CHAPTER ONE

1.1 GENERAL INTRODUCTION

Helicobacter pylori is a Gram-negative, microaerophilic, spiral rod-shaped bacillus that is 1–2 µm long, and 1 µm wide displaying 2–6 flagella for mobility (Lertsethtakarn *et al*., 2011; Gu *et al*., 2017). Since it was discovered in 1983 (Marshal and Warren, 1984), it has been associated with the development of several diseases of the human digestive system (Tanih and Ndip, 2012; Seck *et al*., 2013; Chey *et al*., 2017). This bacterium chronically infects the gastric mucosa of more than half of the world's population and is recognized as the main cause of gastritis (Marshal and Warren, 1984; Wu *et al*., 2012).

Infection with *H. pylori* potentially induces chronic gastritis, peptic ulceration, adenocarcinoma of the distal stomach, mucosa associated lymphoid tissue lymphoma (MALT) and primary gastric non-Hodgkin's lymphoma (Permin and Anderson, 2005; Ahmed *et al*., 2007b). *H. pylori* is one of the causative agents of gastric cancer and it's infection of the human host is ranked as a group 1 carcinogen (Chey *et al.*, 2007). The infection with this organism can commence early in life (Dube *et al*., 2009; Khedmat *et al*., 2013) and persist for life if not treated. *H. pylori* infection in paediatrics is associated with pernicious anaemia, growth faltering and susceptibility to water and food borne infections (Thomas *et al*., 2004).

H. pylori infects approximately above 50% of the world's population (Tanih *et al*., 2010; Goh *et al*., 2011; Awuku *et al*., 2017). Of concern is the fact that drug resistance against commonly used drugs of *H. pylori* treatment is on the rise (Seck *et al*., 2009; Tanih *et al.*,

2010; Fathi *et al.*, 2013), thereby increasing the risk of serious health consequences due to prolonged infection. Some studies that have shown that urease negative mutant strains of *H. pylori* fail to effectively inhabit the acidic stomach of host and cause gastritis (Tsuda *et al*., 1994; Amin *et al*., 2010). In addition urease inhibition has been reported in many studies (Krajewska, 1991; Pervez *et al*., 2008; Kumar *et al*., 2009; Du *et al*., 2012). Due to drug resistance, other therapeutic sources have been used in the management of the infection. These include natural products such as honey known to possess a multifaceted medicinal value due to abundance of phytochemicals in its complex that are dependent on geographical location and nectar source (Ndip *et al*., 2007; Mandal *et al*., 2011; Silver *et al*., 2013). Different honey types were chosen as a template of anti-urease compounds. The experimental approach of the study was based on screening for anti-urease active compounds in honey and determining the effectiveness of identified compounds towards suppression of *H. pylori* growth under acidic conditions. This study therefore attempts to explore new ways of treating *H. pylori* infections by targeting the inhibition of the urease enzyme which is a key virulence factor required by this organism to infect the acidic environment of the human stomach.

1.2 RATIONALE

It is a concern that the prevalence and incidences of *H. pylori* infections worldwide has reached alarming rates. Several studies have highlighted high occurrences of *H. pylori* infections in the developing world (Samie *et al.*, 2007; Dube *et al*., 2009; Tanih *et al*., 2010). In South Africa, Samie *et al.* (2007) recorded 84% prevalence in a study conducted in Pretoria from asymptomatic individuals while Dube *et al*. (2009), recorded a prevalence of 86.8% in an asymptomatic population in the Eastern Cape Province. Prevalence studies have been done in other countries including Nigeria with 64% (Jemilohun *et al*., 2011), Cameroon with 78.7% (Ebule *et al*., 2017) and Egypt with 79.7% (Hanafi *et al*., 2017), all cases with

notably high occurrences of *H. pylori* infections. In addition, drug resistance is gradually gaining momentum against currently used treatment regimens, unavoidably making this bug a severe health concern worldwide.

H. pylori infection of the human gastric mucosa is an important risk factor for the development of gastric ulcer, duodenal ulcer, and development of upper gastrointestinal tumor, adenocarcinoma and mucosa associated lymphoid tissue-MALT (Cogo *et al*., 2011; Miernyk *et al*., 2011; Zaki *et al*., 2016a). It has been suggested that up to 95% of duodenal and 70% of gastric ulcers are attributable to *H. pylori* infection and most cases occur in middle aged subjects (Rothenbacher, 2007).

The organism is equipped with several virulence factors which include the vacuolating cytotoxin gene A (*vac*A), cytotoxin associated gene A (*cag*A) and the urease enzyme which all contribute positively to the colonization and pathogenicity of the organism in the human stomach. The *vac*A gene encodes for the production of a vacuolating cytotoxin which influences cytoskeleton changes, vacuolation and apoptosis of host cells (Pacheco *et al*., 2008). A model for vacuolation suggests that anion-selective channels facilitate the transport of chloride ions into host cells resulting in intra-lumenal chloride concentration which eventually leads to diffusion of weak bases into endocytic compartments of cells causing osmotic swelling and vacuolation (Cover and Blanke, 2005). The vacuolating activity of the *vac*A gene in *H. pylori* is associated with presence of four regions, the signal (*s*)-, intermediate (*i*)-, middle (*m*)-, and deletion (*d*)- regions found as either type 1 or 2 (Bakhti *et al*., 2015 ; Hashinaga *et al*., 2016; Trang *et al*., 2016). The allelic combination of *s* region and the *m* region determines cytotoxin production and severity of virulence of the bacterium

(Atherton *et al*., 1995; Palframan *et al*., 2012; Harrison *et al*., 2017). The *vac*A allelic combination *s*1*m*1 is associated with high levels of vacuolating cytotoxin severity as compared to moderate level of vacuolating cytotoxin severity displayed by *s*1*m*2 allelic combination (Cogo *et al*., 2011; Harrison *et al*., 2017). The s2*m*2 allelic combination shows little or no production of vacuolating cytotoxin (Miernyk *et al*., 2011). The *cag*A gene, which codes for a 125–145 kDa *cag*A protein is a marker for the presence of the cytotoxin associated gene pathogenicity island (*cag* PAI). Presence of *cag*A PAI seems to be influential in inflammatory response and having a link with a higher risk of developing peptic ulcer disease (PUD) or gastric cancer in comparison to persons infected by *H. pylori* strains without the *cag* PAI (Parsonnet *et al*., 1997; Zaki *et al*., 2016a).

The ability of *H. pylori* to produce urease enzyme is another important virulence factor that modulates survival of the organism in the hostile acidic environment of the human stomach (Amin *et al*., 2010; 2013). Urease is a multimeric, nickel-containing enzyme which consists of six *ure*A and six *ure*B subunits (Dunn *et al*., 1990; Hu and Mobley, 1990). This enzyme converts urea, into ammonia and bicarbonate. The ammonia is responsible for protecting *H. pylori* against the acidic environment of the human stomach through pH elevation within the microenvironment of the organism (Rektorschek *et al*., 2000; Weeks *et al*., 2000). The bicarbonate protects the organism against the bactericidal activity of peroxynitrite, a nitric oxide metabolite (Kuwahara *et al*., 2000). A study positively linked production of urease by *H. pylori* and colonisation of the acidic environment when it was demonstrated that a urease-negative mutant does not cause gastritis due to difficulties in colonization (Amin *et al*., 2010).

As a class 1 carcinogen (Aguemon *et al*., 2005), high incidences of drug resistance of *H. pylori* has been noted worldwide. Clarithromycin and/or metronidazole drug resistance, the main components of triple therapy remedies is on the rise worldwide (Kadayifci *et al*., 2006; Seck *et al*., 2009; Fathi *et al*., 2013; Tanih *et al*., 2013; Fasciana *et al*., 2015). A systematic review of *H. pylori* resistance to clarithromycin worldwide revealed a 17.2% resistance (Francesco *et al*., 2010). Strains of *H. pylori* obtained from a population of Sicily in Italy had a representation of 25% resistance to clarithromycin (Fasciana *et al*., 2015). A study done in Senegal revealed 90% resistance to metronidazole (Seck *et al*., 2009). In Egypt, another study revealed 100% resistance to metronidazole using an E-test (Fathi *et al*., 2013). Also in a South African population, 20% resistance to clarithromycin and 95.5% resistance to metronidazole was recorded (Tanih *et al*., 2010). The triple therapy consisting of proton pump inhibitor (PPI), clarithromycin and amoxicillin or metronidazole is recommended where clarithromycin resistance is < 15%. Also, in areas with less than 40% metronidazole resistance, PPI with clarithromycin and metronidazole is preferable (Chey *et al*., 2017). The failure of the first line treatment regimens calls for second line or even concomitants or rescue therapies. The advanced therapies which offer better results might utilise the bismuth salt. However, this product is not available in all countries, in addition, such rescue therapies require good infrastructure for the program to be successful. Moreover, some of the drugs suggested could have more side-effects thereby posing new challenges which might eventually lead to failure of the suggested regimens.

Phytochemical research is gaining recognition, many studies have been conducted to evaluate the significance of phytochemicals in the treatment of diseases caused by some notorious pathogens including *H. pylori* (Sibanda and Okoh, 2008; Manyi-Loh *et al.*, 2010; Njume *et al*., 2011; Seanego *et al*., 2012). Honey is a natural product that has been shown to supress

the growth of micro-organisms including *H. pylori* (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010; Mandal *et al*., 2011).

In spite of the demonstrated need of urease activity during colonisation of the harsh acidic environment of the human stomach, not much work has focussed on urease inhibition as a way of treating *H. pylori* infection. Acknowledging that honey has the potential to suppress *H. pylori* growth *in-vitro* (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010; Mandal *et al*., 2011), it becomes necessary to screen for anti-urease active compounds in honey and assess their potential of inhibiting the growth and multiplication of *H. pylori* cells under acidic environments, a mimicry of the human stomach *in-vitro*. The success of this study could help in identifying new lead molecules, which could be used in the development of new and affordable drugs against the pathogen to circumvent the problem of increasing drug resistance.

1.3 HYPOTHESIS

The following null hypotheses were formulated:

1.3.1 There are no active compounds in honey that can suppress the activity of urease and *H. pylori* that is *cag*A positive, *vac*A positive and drug resistant at a neutral pH.

1.3.2 There are no active compounds in honey that inhibit multiplication of *cag*A positive, *vac*A positive and drug resistant *H. pylori* grown under acidic environments.

1.4 OBJECTIVES

1.4.1 Overall objective

The overall objective of this study was to identify honey extracts with anti-urease properties *in-vitro* and test their inhibitory potential on *cag*A positive, *vac*A positive and drug resistant *H. pylori* cultured under acidic environments, a mimicry of the human stomach.

1.4.2 Specific objectives

The specific objectives of this study are:

i. Identify *H. pylori* isolates by biochemical tests (Gram stain, catalase test, oxidase test and urease test) and comfirm by PCR targeting the *glm*M gene (140 bp).

ii. Determine drug resistance pattern of clinical isolates of *H. pylori*.

iii. To extract and purify urease enzyme from *H. pylori* isolates.

iv. To use organic solvents to extract honey compounds.

v. To screen for honey solvent extracts with the most anti-urease activity.

vi. To detect *cag*A gene and *vac*A gene among clinical isolates of *H. pylori*.

vii. To determine the critical pH from which urease activity is a necessity for *H. pylori* growth in an acidic environment.

viii. To determine if not whether kinetic inhibition of urease by anti-urease active compounds in honey can suppress the growth of drug resistant, *cag*A positive and *vac*A positive *H. pylori* strains under acidic environments.

ix. Characterise chemical composition of honey extracts capable of inhibiting urease and hence *H. pylori* growth under acidic environments.

CHAPTER TWO

LITERATURE REVIEW

2.1 DISCOVERY OF *HELICOBACTER PYLORI*

Two Australian researchers, Barry J. Marshall and Robin Warren were awarded a Nobel Prize in Physiology in 2005 after they made a striking discovery of a Gram negative spiral shaped bacilli as the causal agent of gastritis and ulcers (Marshall and Warren, 1983). Evidence was provided when Marshall initially underwent gastric biopsy to prove that he was not infected with the Gram negative bacterium. He then infected himself with the bacterium resulting in a histologically proven mild gastritis over two weeks (Marshall *et al*., 1985). In September 1983, classification of this bug generated a lot of interest at the Second International Workshop on *Campylobacter* Infections held in Brussels, Belgium (Pearson *et al*., 1983). The resemblance of the newly discovered organism to *Campylobacter* included requirement of a rich growth media under microaerophilic conditions, curved morphology and failure to ferment glucose led to its naming as *Campylobacter pyloridis* in 1985 and later to *Campylobacter pylori* in 1987 (Marshall and Goodwin, 1987). However, differences observed between *Campylobacter* species and *Campylobacter pylori* were reasonable for renaming this organism as *Helicobacter pylori* which became the first member of the genus *Helicobacter* (Goodwin *et al*., 1989). Since the discovery of this pathogen, a lot of studies have been done as an attempt to fully understand its biology.

2.2 INFECTION OF THE HUMAN STOMACH AND DISEASE MANIFESTATION

H. pylori infect the stomach of about half of the world's population. The risk of getting the bacterium is more pronounced in developing nations due to precarious hygiene conditions,

overcrowding, contaminated environmental and water sources (Ndip *et al.*, 2003; Dube *et al*., 2009). Modes of transmission of this organism are not clear, but there are acceptable suggestions raised through research which include oral-oral, faecal-oral and person-to-person transmission (Stone, 1999; Ndip *et al*., 2003; Perry *et al*., 2006). Infections with the bacterium can be life long without effective treatment either in an asymptomatic or symptomatic mode. Many studies have linked *H. pylori* infection as the cause of many gastroduodenal complaints such as dyspepsia, gastritis, peptic ulcers and stomach cancer (Ahmed *et al*., 2007b; Rothenbacher, 2007; Tanih *et al*., 2010; Kuo *et al*., 2014).

2.2.1 Gastritis

Gastritis is the inflammation of the stomach and at advanced stages is accompanied by the erosion of the lining of the stomach, and it can either be acute or chronic (Kusters *et al*., 2006). Animal studies revealed a positive correlation of *H. pylori* infection and development of stomach gastritis (Wirth *et al*., 1998; Zhao *et al*., 2015). Serum levels of IL-17 were suggested to serve as a potential biomarker for the diagnosis and prediction of gastritis caused by *H. pylori* (Zhao *et al*., 2015). It is described as a sudden and short-lived inflammation of the gastrointestinal tract that could provoke vomiting and in serious situations can lead to bleeding and hematemesis. Acute gastritis is associated with local irritants such as bacterial endotoxins, caffeine, alcohol, and aspirin. Acute gastritis usually lasts for a few days with complete regeneration and healing occurring promptly (Porth, 2002). On the other hand, chronic gastritis on the other hand is as a result of persistent stomach colonisation by the agent provoking gastritis. There are four major types of chronic gastritis: autoimmune gastritis, multifocal atrophic gastritis, chemical gastritis and *Helicobacter pylori* gastritis (Porth, 2002; Furuta and Delchier, 2009). Prolonged colonisation of the human stomach by

H. pylori has been linked to chronic gastritis. If *H. pylori* infection is not treated successfully, chronic gastritis may last for years or even a life time.

2.2.2 Peptic ulcer disease (PUD)

PUD is a term used to refer to both stomach ulcers and/or duodenal ulcers (Kuster *et al*., 2006; Lanas and Chan, 2017). Stomach or gastric ulcers are open sores that develop along the lining of the stomach, while duodenal ulcers develop along the intestine just beyond the pyloric sphincter. Indigestion, heartburn, burning or gnawing sensation, loss of appetite and weight loss represent the common symptoms of ulcers (Ahmed *et al*., 2007a; Rothenbacher, 2007). Internal bleeding can happen due to ulcers especially if the spot of ulcer development coincides with a blood vessel location. The complications of peptic ulcers include hemorrhage which is caused by bleeding (Figure 2.1).

Figure 2.1: Picture showing ulcer spurting blood

Source: *www.crystalgraphics.com/power**pictures**/images.**photos**.asp?ss*

2.2.3 Gastric cancer/malignant tumor

Cancer is a disease that involves abnormal and uncontrolled cell growth with the potential to invade or spread to other parts of the body. Epidemiological studies have shown that *H. pylori* is a class 1 carcinogen that causes gastric adenocarcinoma, the most common cancers worldwide

(Chey *et al*., 2017). Gastric cancer is the second leading cause of deaths associated with cancer illnesses. A study by Lima *et al*. (2011) revealed that *vac*A *s*1*m*1 and *cag*-PAI genes, *cag*A, *cag*E and *vir*B11 are important *H. pylori* markers for the development of gastric cancer. Tumours are adenocarcinomas developing from mucus-secreting cells in the base of the gastric crypts. About 50% and 60% of gastric cancers occur in the pyloric region or adjacent to the antrum (Furuta and Delchier, 2009) (Figure 2.2).

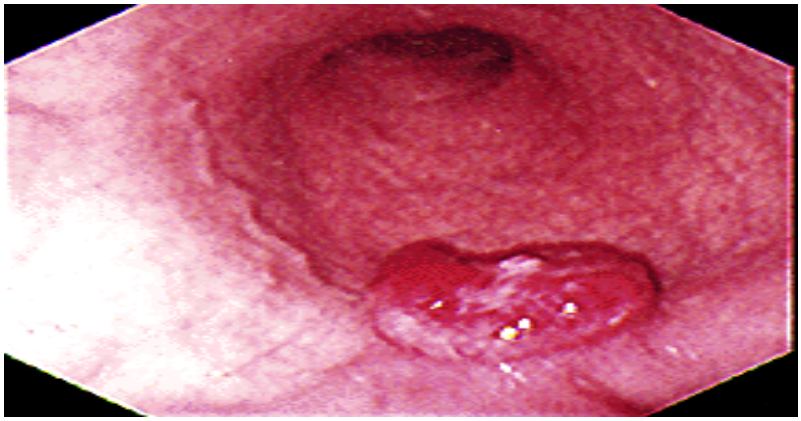


Figure 2.2: Picture showing gastric cancer development

Source: (*www.crystalgraphics.com/powerpictures/images.photos.asp?ss*)

2.2.4 Benign tumor

Benign tumors are non-cancerous cells represented as a mass of cells (tumor) lacking the ability to invade neighboring tissues. These tumors are normally surrounded by an outer fibrous sheath of connective tissue or remain within the epithelium region. The mass effect of benign tumors is a challenge even if these cells do not metastasize. Schwannomas are an example of slow growing benign tumors that originate from Schwann cells of the neutral sheath. In a recent study, a 64 year old woman having Schwannomas had chronic inflammation linked to a microorganism resembling *H. pylori* morphological (Lavy *et al*., 2016).

2.3 *H. pylori* PREVALENCE AND INCIDENCE

The prevalence and incidence of *H. pylori* differ by geographical area and race but the prevalence of *H. pylori* is notably higher in poor developing nations as compared to wealthy developed nations (Ndip *et al*., 2007; Tanih *et al.,* 2010; Watanabe *et al*., 2015). Incidence of *H. pylori* infection has been used to classify the world into two groups i.e. group one having children who become infected at childhood with chronic infection continuing into adulthood while group two comprises of developed nations whereby the minority of children are infected at childhood followed by an increase in prevalence with age (Queiroz *et al*., 2013). There is an *H. pylori* incidence record of 3-10% yearly in developing nations in comparison to 0.5% in developed nations (Rosenberg, 2010). Studies done in countries such as Egypt, India, Nigeria, Cameroon and South Africa show a considerably high prevalence of *H. pylori* infection exceeding 50%, a notable trend associated with developing nations (Dube *et al*., 2009; Eshraghian, 2014). There are studies that have highlighted an increased infection at childhood reaching 50% and above at the age of 5 (Bassily *et al*., 1999; Ndip *et al*., 2004; Dube *at al*., 2009; Awuku *et al*., 2017). Infections have been thought to arise through consumption of contaminated food including vegetables, contaminated water sources, contacts with animals that are *H. pylori* reservoirs as well as consumption of animal derived products such as sheep and cow milk (Khalifa *et al*., 2010). The suggested routes of transmission include direct infections through oral-oral, gastro-oral and faecal-oral routes.

2.4 THE HUMAN STOMACH AS A PREDOMINANT SITE FOR COLONISING *H. pylori* STRAINS

Helicobacter pylori colonises the human stomach and causes a chronic infection. The human stomach lies between the oesophagus and the duodenum of the digestive tract. The lower oesophageal sphincter and the pyloric sphincter help to control movement of the contents of

the stomach in and out of the stomach. The stomach has a mixture of gastric juices responsible for digestion. Also the stomach has a mucus lining along the walls of its inner layer which for protection against self-digestion, a common cause of stomach ulcers. Also this superficial glycoprotein-rich mucosal lining is a defensive mechanism for survival of *H. pylori* in the human stomach. The organism is known to swim through the protective gastric mucus in the stomach and attach to the epithelial cells of the stomach resulting in different disease outcomes over the duration of the infection (Peek, 2005; Celli *et al*., 2009). Pepsin and hydrochloric acid are active ingredients secreted into the stomach by cells lining the stomach. The acid is required for the activation of the enzymes responsible for protein digestion and the human stomach pH can tolerate low pH level up to pH 1.5 (Dressman *et al*., 1990; Russel *et al*., 1993).

The low pH of the human stomach may unavoidably eliminate a wide range of microbial pathogens, but it is a different story with *H. pylori*. This organism actively produces urease enzymes which are either surface localised or cytoplasmic (Krishnamurthy *et al*., 1998). *H. pylori* with cytoplasmic urease (Krishnamurthy *et al*., 1998) and urease negative *H. pylori* (Amin *et al*., 2010) have failed to survive an acidic environment, thereby reinforcing the belief that urease enzyme is required for *H. pylori* to colonise the human stomach. The urease enzyme is highly conserved in *Helicobacter* species and two major subunits of this enzyme have been identified which are *ure*A and *ure*B (Hu *et al*., 1992).

The abundance of the urease produced by *H. pylori* allows rapid hydrolysis of urea producing ammonia and bicarbonate which help neutralise the gastric pH for optimum survival of the organism. Nitrogen metabolism is a requirement for normal health and it is an essential

element in all amino acids derived through dietary protein intake. Under normal conditions, renal nitrogen (urea and ammonia) excretion is equal to nitrogen intake (Weiner *et al*., 2014). The ammonia generated by urease activity in the human stomach might be used in the metabolism of the bacterium for the synthesis of amino acids. Normally, proteins in food are hydrolysed into 9 essential and 11 nonessential amino acids (Weiner *et al*., 2014).

2.5 VIRULENCE FACTORS OF *H. pylori*

Research has gradually focused on pathogenicity makers in an attempt to link virulence markers and disease manifestations. Virulence factors are molecules produced by pathogens to enable colonization of a niche in the host by evading the host's immune response and successfully causing a disease (Backert *et al*., 2011; Cogo *et al*., 2011; Kalali *et al*., 2014). Several virulence factors of *H. pylori* have been documented and these include, the cytotoxin-associated gene A (*cag*A gene), vacuolating cytotoxin gene A (*vac*A) gene, induced by contact with epithelium A gene (*ice*A gene), duodenal ulcer promoting A gene (*dup*A) gene, urease enzyme, adhesins and the flagella (Backert *et al*., 2011; Cogo *et al*., 2011; Pereira *et al*., 2014 Huang *et al*., 2016).

2.5.1 The induced by contact with epithelium gene A (*ice*A)

There are at least two alleles of *ice*A; *ice*A1 and *ice*A2 *(*Arevalo-Galvis *et al*., 2012; Rizzato *et al*., 2012; Huang *et al*., 2016*).* Infection with the *iceA1*-positive *H. pylori* seems to correlate with increased risk for peptic ulcer disease (Huang *et al*., 2016), enhanced mucosal interleukin (IL)-8 and acute antral inflammation (Arevalo-Galvis *et al*., 2012). However, infection with *H. pylori* strains having *ice*A2 gene status had no significant correlation with disease manifestation (Huang *et al*., 2016). The expression of *ice*A1 is instigated by contact

between *H. pylori* and human epithelial cells (Arevalo-Galvis *et al*., 2012). However, heat shock protein 90 (HSP90) is a crucial regulator in *H. pylori* induced IL-8 production (Yeo *et al*., 2004). HSP90 has been revealed to be critical for intracellular signaling that participates in inflammatory response as well as carcinogenesis (Yeo *et al*., 2004).

2.5.2 Duodenal ulcer promoting gene A (*dup*A)

This virulence factor is a *virb*B4 homologue (Lu *et al*., 2005) associated with duodenal ulcer development and reduced risk of gastric carcinoma in some populations (Hussein *et al*., 2010). The *dup*A is located in the plasticity region of *H. pylori* genome. The most common form of *dup*A (*dup*A1) encompasses parts of the jhp0917 and jhp0918 ORF which form a single continuous open reading frame by the insertion of a base C or T after position 1385 in the jhp0913 3' region (Queiroz *et al*., 2011; Pereira *et al*., 2014). The second common form *dup*A2 contains 2 ORFs (Hussein *et al*., 2010).

2.5.3 Vacuolating cytotoxin gene A (*vac*A)

The *vac*A gene encodes for the production of *vac*A protein, a complex of 500 to 600 kDa, consisting of 87 kDa subunits (Haas, 2002). The *vac*A induces the formation of intracellular vacuoles in epithelial cell lines (Atherton *et al*., 1995; Faundez *et al*., 2002). The *vac*A gene is present in almost all *H. pylori* strains and not all are highly toxigenic and pathogenic (Trang *et al*., 2016). The *vac*A gene possess four regions closely associated with the vacuolating activity of *H. pylori*, that is the signal (s)-, intermediate (i)-, middle (m)-, and deletion (d)-regions which are classified as either type 1 or 2 (Hashinaga *et al*., 2016). The *s* region exists as *s*1 or *s*2 and in addition, has subtypes *s*1a, *s*1b and *s*1c (Faundez *et al*., 2002). The *m* region occurs as *m*1 or *m*2. Although all strains of *H. pylori* have a *vac*A gene, there is

variation in the amount of vacuolating activity due to heterogeneity within the *vac*A gene at the 5' end *s* region and the *m* region. The allelic combination *s*1*m*1 and *s*1*m*2 strains produce high and moderate levels of toxin respectively while the *s*2*m*2 allelic combination has little or no toxin and final *s*2*m*1 combination is rare and non-vacuolating (Cogo *et al*., 2011; Pereira *et al*., 2014). All s1m1i1 and s1m2i1 strains are vacuolating and more virulent in comparison to s1m2i2 and s2m2i2 strains (Trang *et al*., 2016). In addition, d1/c1 strains are associated with the production of vacuolating cytotoxin genes (s1m1i1) contrary to d2c2 association with non-vacuolating types s2m2i2 (Trang *et al*., 2016). Studies have found correlation in the presence of the *vac*A *s*1*m*1 genotype and the presence of *cag* PAI in PUD and gastric cancer, while the *vac*A *s*2*m*2 genotype is linked with the absence of the *cag* PAI (Atherton *et al*., 1995; Miernyk *et al*., 2011).

2.5.4 The cytotoxin-associated gene A (*cag*A)

The *cag*A gene encodes a protein that is associated with increased intensity of the gastric inflammation and, subsequently severe clinical outcomes (Miernyk *et al*., 2011). Virulence strains of *H. pylori* possess a 40 kb *cag* PAI containing 31 potential coding regions involved in different type IV secretion system (T4SS) encoding (Jones *et al*., 2010; Jiménez-Soto and Haas, 2016; Zaki *et al*., 2016a). During *H. pylori* infection, adhesins create close bacterial contact with host target cells followed by delivery of effector proteins into host target cells by the T4SS, a needle-like pilus device (Jones *et al*., 2010). There are studies that have confirmed that once injected into the host cell, *cag*A can directly activate NF-κB and induce the release of IL-8 which plays a big role in the development of gatric cancer (Kang *et al*., 2013; Lee *et al*., 2013; Papadakos *et al*., 2013). *Cag*A is classified as an oncogene due to its association with gastric cancer development (Pereira *et al*., 2014).

2.5.5 Adhesins

H. pylori is highly adapted to survival in the gastric environment of the human stomach and it has been shown to encode a varied set of adhesins. More than 30 genes encoding outer membrane proteins (OMPs) have been grouped into *Hop* (*Helicobacter* outer membrane porins) and *Hor* (*Hop* related) subgroups. The adhesins that belong to the *Hop* family are linked with more virulent strains and they include the *bab*A, *sab*A, *alp*A/B, *Hop*Z and *oip*A (Backert *et al*., 2011). More focus has been on BabA and SabA adhesins, however not all *H. pylori* strains express functional BabA or SabA adhesins (Lopez-Bolanos *et al*., 2009) probably explaining the fact that in the human stomach *H. pylori* is found being highly motile in the mucus or attached to the epithelial cells (Oleastro and Ménard, 2013). Adherence to the gastric epithelium allows the bacterium to have persistent colonization without danger of being automatically flushed out by stomach peristalsis. Evasion of the host defense system and efficient delivery of proteins such as *vac*A and *cag*A, into gastric cells are some of the benefits of adherence (Oleastro and Ménard, 2013).

2.5.6 Flagella

H. pylori is equipped with two to six flagella that are in a lophotrich position. Possessing flagella seems to play an important role in colonisation of the host by *H. pylori*. A flagellated strain has been shown to be successful in colonising the stomach of experimental animals while the strain devoid of flagellates colonises the stomach of experimental animals less frequent (Eaton *et al*., 1992). The flagella are important for gastric colonisation through locomotion and counteraction of peristalsis. The flagella enables the organism to achieve different types of movements which include swimming, spreading and swarming motility, attained in liquid media, soft media and semi-solid media respectively (Gu, 2017). Each flagellum has four components, the basal body, the filament, sheath and the hook

(Lertsethtakarn *et al*., 2011). The basal body consist of the c-ring, ms-ring and the type III secretion system. The c-ring (*fli*M, *fli*N, *fli*Y, *fli*G) transfers proteins as well as co-ordinating protein secretion and regulating motor rotation (Lowenthal *et al*., 2009; Tsang *et al*., 2013). The ms-ring (*fli*F) of the basal body is involved in the synthesis of *fl*A, *fla*B and *flg*E (Allan *et al*., 2000). The type III secretion system (*flh*A, *fli*O, *flh*B, *fli*P, *fli*Q, *fli*R) is responsible for the transportation of flagellar proteins towards the end of the flagellar structure (Smith *et al*., 2009; Ibuki *et al*., 2013). The flagellar filament has proteins *fla*A, *fla*B and *fli*D with *fla*A and *fla*B playing an important role in bacterial motility (Josenhans *et al*., 1995). The *fli*D is a filament capping protein of the flagellar assembly (Kim *et al*., 1999). The proteins *hpa*A and *faa*A of the flagellar sheath protect the flagellar subunits from depolymerisation at low pH (Carlsohn *et al*., 2006; Radin *et al*., 2013).

The hook of the flagellar found in *H. pylori* consist of the flagellum export chaperone (*fli*S) and the flagellar hook proteins (*flg*E and *flg*K). The *fli*S chaperone help to prevent premature polymerization of the flagellin and it also participates in flagellum assembly (Lam *et al*., 2010). The *flg*E is responsible for connecting the basal body and the flagellar filament which is also linked to the powerful driving force in a viscous environment (O'Toole *et al*., 1994; Ryan *et al*., 2005). The protein *flg*K of the flagellar hook is responsible for controlling the length of the flagellar hook during flagellum assembly (Douillard *et al*., 2009).

2.6 UREASE ENZYME

Urease enzyme plays a crucial role in *H. pylori* pathogenesis. *H. pylori*, a neutralophile is able to colonise the acidic gastric mucosa of the human stomach by producing a large amount of urease enzymes (Celli *et al*., 2009; Amin *et al*., 2010). The urease enzyme is responsible

for the hydrolysis of urea to ammonia and bicarbonate. The by-products of urea hydrolysis result in elevated pH surrounding *H. pylori* cells from a pH 2 to almost neutral (Allen and Flemstrom, 2005). The pH regulation by urease enzyme has been shown to be crucial during locomotion. The mucus lining of the stomach has been shown to undergo reversible pH dependent sol-gel transition from viscous polymer solution to a soft gel as pH is lowered below 4 and vice versa when pH is raised (Celli *et al*., 2009). The gastric mucin, the glycoprotein content of the mucus is responsible for the pH dependent change of viscosity. The property of the glycoprotein was observed to be important among urease producing strains of *H. pylori* since elevated pH causes alteration of rheological properties of the mucus environment to a much softer form thereby allowing easier propelling during movement (Celli *et al*., 2009).

The crystal structure of *H. pylori* urease shows that it has two subunits (*ure*A and *ure*B) and its catalytic substance contains a dinuclear nickel active site (Lam *et al*., 2010). However, enzymes from other bacteria including *Klebsiella aerogenes* have *ure*ABC structures (Pearson *et al*., 1997; Benini *et al*., 1999). Colonisation of the gastric mucosa of the human stomach requires among virulence factors, production of an active metalloenzyme urease that requires nickel as a cofactor.

It has been demonstrated that urease negative strains or strains of *H. pylori* with *ure*B disruption fail to cause gastritis in mice due to colonisation difficulties (Tsuda *et al*., 1994; Amin *et al*., 2010). Urease enzyme neutralises the gastric acid through production of ammonia (NH_3) and carbon dioxide (CO_2). The NH_3 produced may cause the formation of

NH_3-derived compounds such as monochloramine with cytotoxic effects on host cells (Suzuki *et al*., 1992).

2.6.1 Production and activation of urease in *H. pylori*

The operon of *H. pylori* urease has seven open reading frames (ORFs) *ureABIEFGH* found in a single 6.13 kb cluster of chromosomes (Cussac *et al*., 1992; Fong *et al*., 2013). The *ure*A and the *ure*B are responsible for encoding the urease structural proteins while the remaining five are accessary genes responsible for the synthesis of the catalytically active enzymes (Hu and Mobley, 1993). The *ure*I encodes cytoplasmic membrane protein responsible for the formation of a urea-specific pore. The urea-specific pore channels urea to the urease enzyme located in the cytoplasm of the bacteria (Rectorschek *et al*., 2000). Work on transposon mutagenesis revealed that ure*A*, *ure*B, *ure*F, *ure*G and *ure*H of *H. pylori* are required for the production of functional urease (Cussac *et al*., 1992; Fong *et al*., 2013).

The comparison of urease accessory proteins from different sources including bacteria revealed that ureases contain six amino acids constituting a ligand for the Ni^{2+} metallocenter (Farrugia *et al*., 2013; Fong *et al*., 2013). Metalloenzymes are synthesised as precursors devoid of metalloenzyme active site. Metalloenzymes need an elaborate metallocenter assembly system to have a functional active site (Farrugia *et al*., 2013). The Ni^{2+} metallocenter that activates the enzyme is generated through incorporation of nickel ions to the apoprotein (Fong *et al.*, 2013). The nickel is inserted into the urease active site in a GTP-dependent process with the influence of accessory proteins *ure*D/*ure*H, *ure*E, *ure*F, and *ure*G. The metallocenters are important as they serve crucial biological functions which include transferring electrons, stabilizing biomolecules, binding substrates, and catalysing desirable

reactions and in addition, strict control is needed during metallocenter synthesis to influence specificity of the active site (Farrugia *et al*., 2013; Fong *et al*., 2013; Sujoy and Aparna, 2013).

Maturation of urease requires proper insertion of two nickel ions to the active site in addition to accessory proteins (Carter *et al*., 2009). The *ure*E and *ure*G proteins are soluble and have been researched widely in comparison to *ure*D and *ure*F proteins which are insoluble proteins (Lam *et al*., 2010). *Ure*E seems to be responsible as a structural scaffold for recruitment of GTP to *ure*G i.e. *ure*E serves as a medium to grasp Ni^{2+} from *hyp*A and hence donate it to *ure*G (Yang *et al*., 2015). The accessory protein *ure*D has been renamed *ure*H in *Helicobacter* species (Cussac *et al*., 1992) and has been proposed to be a scaffold towards recruitment of other accessory proteins as well as a facilitator of activation.

*Ure*F is a protein forming a dimer with a novel all-helical topology probably showing a possible binding surface for interaction with other urease components or for urease-related protein-protein interactions (Lam *et al*., 2010a). The *ure*G is soluble and has been highly characterised as reported by different studies (Cussac *et al*., 1992; Lam *et al*., 2010a). It forms sequential complexes with the urease apoprotein. The nucleotide-binding motif (P-loop) in the *ure*G sequence and the resultant seizure of urease activity corresponding to mutation of this site and *in-vitro* GTP-dependent *ure*DFG-urease apoprotein complex activation suggest that *ure*G protein is a GTPase for urease activation (Lam *et al*., 2010a; Farrugia *et al*., 2013).

2.6.2 Urease and inflammation of the human stomach

Urease has been shown to induce primary mucosal macrophages to produce interleukin (IL)-1 beta, IL-6 and tumour necrosis factor (TNF)-alpha (Harris *et al*., 1998). Once in the gastric mucosa, *H. pylori* bind to CD74 on gastric epithelial cells. The urease subunit B-CD74 interaction was shown to induce NF-κB activation and interleukin-8 (IL-8) production (Beswick *et al*., 2006). High expression of IL-8 in gastric mucosa has been linked with risk of gastric cancer (Yamada *et al*., 2013). In addition, heat shock protein (HSP) 90 has been revealed to be critical for intracellular signalling that participates in inflammatory response as well as carcinogenesis (Yeo *et al*., 2004). *H. pylori* also produces [Ni, Fe]-hydrogenase enzymes which oxidise molecular hydrogen to produce enough energy to compensate for the high energy used during early adaptation to survive in acidic environment of the human stomach (Yang *et al.*, 2015).

2.6.3 Urease inhibitors

Urease inhibition has been extensively studied considering potential benefits which include treatment of urease dependent microbial infections and control of pH variations in soil (Upadhyay, 2012; Amin *et al*., 2013). Several classes of urease inhibitors have been discovered worldwide (Upadhyay, 2012; Amin *et al*., 2013; Sujoy and Aparna, 2013; Tan *et al*., 2013; Grant, 2014; Modolo *et al*., 2015). These include inhibitors substrate analogues, substituted urea/thiourea, hydroxyurea, hydroxamic acids, phosphoroamides, and thioles. Varying methods of urease inhibition have been recorded, heavy metal ions seem to react with the active site sulfhydryl group in an analogous way to the formation of metal sulphide (Krajewska, 1991). The phosphoroamide compounds and hydroxamic acids inhibit urease by creating a tetrahedral intermediate with a structure similar to the tetrahedral intermediate postulated to occur during urea hydrolysis (Dixon *et al*., 1980). The boric and boronic acids

seem to form a complex with nickel ions (Breitenbach and Hausinger, 1988). Competitive inhibition of urease has been noted with thiol compounds.

2.6.3.1 Heavy metals as urease inhibitors

Urease activity has been shown to succumb due to presence of trace elements of heavy metals. The heavy metal ions including Ag^{+}, Hg^{2+} and Cu^{2+}, have been shown to inhibit urease activity with silver showing the highest inhibition effect. Time-dependent inhibition studies revealed a biphasic kinetics with all heavy metals ions (Prakash and Vishwakarma, 2001; Kumar *et al*., 2009). The urease enzyme has been shown to quickly bind to Hg^{2+} and then attain a state of slow reversible course to inactivation (Du *et al*., 2012). Prakash and Vishwakarma (2001), in a study showed that urease from the seeds of water melon was inhibited by heavy metal ions including copper, lead, nickel and cobalt and the effectiveness of non-competitive urease inhibition by these heavy metal ions was $Cu^{2+} > Pb^{2+} > Ni^{2+} > Co^{2+}$.

2.6.3.2 Hydroximate and its derivatives as urease inhibitors

Although hydroxamic acids have previously been recorded as potent, specific and non-competitive inhibitors of urease, these inhibitors were later reported to be reversible and slow binding (Mishra *et al*., 2002). A study by Kobash *et al*. (1975) revealed methionine-hydroxamic acid as the best inhibitor of ureases among nineteen alpha-aminoacyl hydroxamic acids which included phenylalanine, serine, alanine, glycine, histidine, threonine, leucine, and arginine-hydroxamic acids in order of decreasing inhibitory ability. Of interest is that the pH optimum required for inhibition shifted to lower pH in the presence of a carboxyl group, and to higher pH in the presence of an amino group. Hydroxamic acid seems to inhibit

urease activity with strict specificity with the exception of aspartic-β-hydroxamic acid, which inhibited asparaginase competitively among studied inhibitors (Kobash *et al*., 1975).

2.6.3.3 Thioles as urease inhibitors

Thiol compounds having a positively charged beta-amino group are potent inhibitors while thiol compounds having an anionic carboxyl group show less inhibitory power towards urease activity (Todd *et al*., 1989). Also their inhibitory interaction is non-specific. Disulfiram, a thiol reagent used in humans approved by FDA for the treatment of chronic alcoholism has been recently shown to inhibit urease activity (Díaz-Sánchez *et al*., 2016).

2.6.3.4 Boric acid as urease inhibitors

Competitive inhibition of Jack bean urease by boric and boronic acids has been reported (Upadhyay, 2012). However phosphate buffering seems to influence inhibition by boric acid in a pH dependent manner with pH of 5 linked to maximal inhibition while the pH of 10 seem to be linked with minimal inhibition of urease (Reddy and Kayastha, 2006). Studies have revealed a lot more compounds showing inhibitory power against urease activity (Reddy and Kayastha., 2006; Amin *et al*., 2013; Tan *et al*., 2013; Díaz-Sánchez *et al*., 2016). Hydroxyurea, sulfur compounds, alpha-hydroxyketones, alpha diketones, triketone oximes, phosphates, biscoumarin and some organic solvents are known to be urease inhibitors (Upadhyay, 2012). Phytochemicals as well have been recommended for their urease inhibition properties (Amin *et al*., 2010; 2013).

2.7 UREASE ASSAYS

Several reliable techniques have been successfully used to confirm and study the chemistry of ureases. Some of them are the Berthlot test/ indophenol assay, pH indicator assay, potentiometric assays coupled urease-glutamate dehydrogenase (GDH) assay and coupled urease-horseradish peroxidase assay.

2.7.1 Berthlot test/ indophenol assay

This test is used to determine ammonia production or presence in a sample. The Berthelot's reagent is an alkaline solution of phenol and hypochlorite. Reaction of Berthelot and ammonia results in a colorimetric blue product which determines the presence of ammonia. Another application of this method is to determine the activity of urease in the presence of urea which eventually leads to the production of ammonia and carbon dioxide. The urease activity assay kit has been used for this test. The kit provides a direct procedure for measuring urease activity in biological and environmental samples. Produced ammonia through hydrolysis of urea by urease, is determined by the Berthelot method which result in a colorimetric product that is measured at 670 nm giving a reading that is proportionate to the urease activity present in the sample.

2.7.2 The pH indicator assays

Urease is active in the pH range of 3.5 to pH 8. In acidic environments, the urease enzymes help to elevate the pH through the hydrolysis of urea to form ammonia and carbon dioxide. The rate of pH change can be monitored spectrophotometrically in the presence of a pH indicator such as phenol red (Hamilton-Miller and Gargan, 1979). This is a convenient and suitable test for routine urease tests but it is not ideal for detailed kinetic analysis.

2.7.3 Coupled urease-glutamate dehydrogenase (GDH) assay/Coupled urease-horseradish peroxidase assay

This method is based on the fact that ammonia produced by urease can stimulate the oxidation of NADH in a coupled enzyme assay and such a reaction can be monitored by measuring reduction of NADH at 340 nm (Kaltwasser *et al*., 1966). The coupled urease-horseradish peroxidase assay utilises the findings that ammonia can stimulate peroxidase activity on o-dianisidine resulting in an increase in absorbance at 460 nm (Stutts and Fridovich, 1964).

2.8 DIAGNOSIS OF *H. pylori* INFECTION

Diagnosis of an infection is crucial for proper management of the disease. There are a several methods that have been used to examine gastric colonization of *H. pylori.* Successes of tests currently used rely on representative samples which must be obtained from infected individuals. Biopsies, blood serum, exhaled air and stool samples have been widely used as *H. pylori* infection test samples (Dube *et al*., 2009; Tanih *et al*., 2010; Harrison *et al*., 2017). Biopsy related methods, and serum based methods are the most common invasive methods that have been widely used to detect and confirm an active *H. pylori* infection (Tanih *et al*., 2010; Kadi *et al*., 2014; Saidu *et al*., 2015; Zaki *et al*., 2016a; Hanafi *et al*., 2017; Harrison *et al*., 2017). Non-invasive tests which include urease breath test and stool antigen test have been successfully used to detect *H. pylori* infection (Dube *et al*., 2009; Harrison *et al*., 2017).

2.8.1 Direct diagnosis tests

Endoscopy is a procedure that has been used to view the inside of the stomach in patients with gastroduodenal complaints. The magnifying narrow-band imaging endoscopy

technology allows clear visualization of the gastric epithelial lining of the stomach (Tahara *et al*., 2009). This visualisation allows gastroenterologist to assess signs of gastritis, ulceration and cancerous cells development in the human stomach (Uemure *et al*., 2001; Tahara *et al*., 2009); hence the representative biopsy samples are collected near or at the area surrounding a suspected disease outcome as observed under endoscopy. Considering any *H. pylori* infection, the gastric mucosa is divided into three states, normal mucosa without a history of *H. pylori* infection (non-gastritis), current *H. pylori* infection (active gastritis), and past history of *H. pylori* infection (inactive gastritis). This requires specialisation and experience for accuracy and persistence with success of endoscopy examination (Watanabe *et al*., 2013).

Culturing of *H. pylori* allows a lot of studies to be conducted in an effort to understand the microbiology, prevalence, virulence and drug resistance patterns of strains circulating in a population. However, to obtain live cultures from clinical specimen can be a challenge because of the fastidious nature of *H. pylori*. Obtaining viable gastric biopsy is one of the methods for diagnosing a current infection. Therefore, samples obtained by endoscopy require specialised transport media so as to maintain viability of *H. pylori* cells. Vials containing approximately 1 mL of cysteine transport medium with 20% glycerol have been successfully used as transport media (Tanih *et al*., 2010). Inoculation of culturing plates soon after a biopsy sample has been collected and sealing cultured plates in Biobags for transportation has been reported to increase chances of recovering the organism. Portagerm pylori agar is another commercial transport media with high recovery rate of *H. pylori* from biopsy samples (Grove *et al*., 2001). A semi-solid *Helicobacter pylori* transport media GESA (publication no. WO/2014/019696, patent pending no. PCT/EP2013/002292; Liofilchems.r.l., Rosetodegli Abruzzi, Teramo, Italy) has also been developed (Cellini *et al*., 2014).

2.8.2 Histopathology and immunohistochemistry (IHC)

Histopathology is a process of examining biopsy or surgical specimen. Biopsy specimens need to be fixed using 10% formaldehyde soon after collection (Mégraud and Lehours, 2007; Jemilohun *et al*., 2010). The fixation of 10% formaldehyde maintains the morphology of the bacteria and also allows different staining techniques to be used, but prolonged storage of the fixed samples lose their features making analysis difficult. *H. pylori* silver stain, modified Giemsa, modified Genta, acridine orange, cresyl violet, Gimenez, Half Gram, Ziehl-Neelsen, and Warthin-Starry are some of the stains that have been used for the histological detection of *H. pylori* in gastric biopsies with better visualization of the organism (Mégraud *et al*., 2007). IHC is a process of selectively imaging antibodies in cells of a tissue section through antibody binding principle to antigens.

2.8.3 Culturing of *H. pylori*

Culturing is the gold standard method of positively identifying an infecting organism and allows direct observation of *H. pylori* growth from biopsy samples. This organism is fastidious and requires a limited and optimised condition for growth. Several studies have highlighted the suitable microaerophilic growth environment consisting of 2% – 6% O_2, 10% CO_2, 80% – 85% N_2 (Kusters *et al*., 2006) made possible by commercial microaerophilic sachets inserted in gas jars together with culture plates, or specialised incubators capable of adjusting atmosphere to set conditions. *H. pylori* is also considered a neutralophile even though it is found inhabiting the acidic environment of the human stomach. Urease producing *H. pylori* have been found to tolerate pH range of 3.5 to 8 (Rektorschek *et al*., 1998; Scott *et al*., 1998; Brown, 2000). The pH of 7 has been shown to provide optimal growth of the organism while the organism's growth is totally inhibited at pH below 3.5 (Scott *et al*., 1998).

The urease activity is over a narrower pH range of pH 6.0 – 8.0 (Scott *et al.*, 1998; Brown, 2000).

Culturing of *H. pylori* also requires a complex source of nutrients almost similar to the nutrients in the human stomach. The generally used solid media for culture of *H. pylori* is Columbia or Brucella agar and Brucella broth supplemented with horse or sheep blood or, faetal calf serum at 5–7% v/v concentration, the *Helicobacter pylori* selective supplements are also included a in the growth media. The Oxoid™ *Helicobacter pylori* selective supplements (Dent) contain vancomycin, trimethoprim, cefsoludin, and amphotericin B. Another type of supplement being used is Skirrow's supplements consisting of vancomycin, trimethoprim, polymyxin B, and amphotericin B (Ndip *et al*., 2003). Brain heart infusion growth media, Brucella growth media and the Muller Hinton growth media are the liquid formulations that are used for the growth of *H. pylori*. The optimum temperature for *H. pylori* growth is ranges from 35^{o}C to 37^{o}C (Grove *et al*., 2001; Tanih *et al*., 2010).

2.8.4 Indirect diagnosis

Indirect tests fall into two categories, the active tests which are capable of detecting an active infection and the passive tests which only reveal the presence of a marker of an organism whether it is a previous exposure or a current infection. The urea breath test and the stool antigen tests are indirect tests that have been used successfully to detect active infections (Dube *et al*., 2009; Harrison *et al*., 2017). Antibody-based tests (serology) also offer reliable results when determining *H. pylori* infections. However, the challenge is that serological tests do not separate between active and non-active infection. Indirect tests are less likely affected

by drugs in use hence they can easily be used as an efficient diagnostic method (Burocua *et al*., 2013).

2.8.5 Molecular techniques

Molecular techniques have been used widely as gold standard techniques in the study of old and newly discovered micro-organisms. The complete genome of most micro-organisms including *H. pylori* and some multicellular organisms has been extensively studied through the use of molecular techniques. The analysis of nucleic acids has been done through polymerase chain reaction (PCR), gel electrophoresis and blotting techniques (Northern, Southern). The analysis of gene expression has been done using real-time PCR and microarrays (DNA chips). Also recombinant DNA technology, Sanger sequencing and next generation sequencing are some of the common techniques that have made research in molecular biology a success.

Molecular techniques have been used for the detection housekeeping genes of *H. pylori* from water, gastric biopsies, stools, luminal aspirates, vomitus, saliva, dental plaque and other oral cavities that can provide a prolonged habitat for the organism as it gains it's way to infect the host (Burocua *et al*., 1999; Ndip *et al*., 2003; Tanih *et al*., 2010; Abu-Sbeih *et al*., 2014; Miftahussurur *et al*., 2016; Harrison *et al*., 2017). Many studies have been published using PCR or PCR/RFLP techniques to study *H. pylori*- related issues from modes of transmission, drug resistance, pathogenesis and many more.

Several genes specific for *H. pylori* have been targeted which include 16S rRNA gene, 23S rRNA and *ure*C/*glm*M (Atherton *et al*., 1995; Burucoa *et al*., 1999; Al-Thwai and Ali, 2013; Nevoa *et al*., 2017). The *ure*C gene is not related to urease production, but encodes for a phosphoglucosamine mutase hence it was renamed *glm*M. It is considered a "housekeeping" gene, which participates directly in cell wall synthesis (Espinoza *et al*., 2011). Other PCR based techniques that have been utilised for *H. pylori* studies include reverse transcriptase PCR (RT-PCR), conventional PCR, real time- PCR, multiplex PCR and nested PCR (Wong *et al*., 2001; Bolek *et al*., 2007; Abu-Almaali *et al*., 2012).

PCR has allowed more research studies to be done on virulence factors of *H. pylori* by targeting virulence genes which include *cag*A , *vac*A , *ice*A , *dup*A , *ure*A , *ure*B genes and adhesins (Essawi *et al*., 2013). In addition, PCR has been used to determine resistance to some of the commonly used drugs in the treatment of *H. pylori* infection. Molecular studies have revealed that clarithromycin resistance by *H. pylori* is due to point mutations in the peptidyl-transferase region encoded in domain V of 23S rRNA for example mutations as a result of the transitions in A2143C and A2142C positions of rRNA and other mutations such as A2143G, A2142G, A2142C and A2144G (Tanih *et al*., 2013; Xiong *et al*., 2013).

2.9 TREATMENT OF *H. pylori* INFECTION

2.9.1 First Line therapies

European and American guidelines recommend primary therapies for *H. pylori* infection to include a PPI, clarithromycin, and amoxicillin or metronidazole (clarithromycin-based triple therapy) twice a day for 7 or 7–14 days or a PPI or (Ranitidine) H_2RA, bismuth subsalicylate,

metronidazole, and tetracycline (bismuth quadruple therapy) twice a day for at least 10 days or 14 days if bismuth is unavailable, this is with regard to populations with less than 20% clarithromycin resistance (Chey *et al*., 2007; Malfertheiner *et al*., 2007). However, in areas with less than 40% metronidazole resistance, PPI with clarithromycin and metronidazole is preferable. Furthermore, a 10-day sequential therapy seems to offer better outcomes. This therapy consists of a dual administration of PPI and amoxicillin followed by triple therapy consisting of PPI, clarithromycin and tinidazole (Zullo *et al*., 2000; Seo *et al*., 2014). In a paediatric study conducted by Arenz *et al*. (2006), the esomeprazole-based one-week triple therapy that was directed by susceptibility testing of *H. pylori* was shown to be effective in eradicating the organism. However, the limitation of this method is that it is effective when there are no double-resistant strains to drugs (clarithromycin or metronidazole). Its efficacy is moreover affected by resistance patterns of amoxicillin and clarithromycin. Recently, a new sequential concomitant hybrid therapy reported a high rate (99%) of treatment success (Hsu *et al*., 2011). The quadruple therapy consisting of PPI, bismuth and two antibiotics (amoxicillin + clarithromycin, or metronidazole + tetracycline) offer superior eradication rates.

The increasing resistance to clarithromycin has led to investigations of fluoroquinolones such as levofloxacin as a surrogate drug for this clarithromycin. A triple therapy with PPI, amoxicillin and levofloxacin has been suggested earlier as an alternative first-line therapy (Katelaris, 2009). Resistance to fluoroquinolones is generally very low (< 10%) worldwide (Boyanova and Mitov, 2010). Recent studies have revealed a low primary and secondary resistance ranging from 6.8% to 25% for floroqunoline (ciprofloxacin, levofloxacin, moxifloxacin and gemifloxacin), which is showing uncertaint on the longevity of the used of regimens that rely on floroquinoline (Teh *et al*., 2014; Zaki *et al*., 2016b).

The meta-analysis of Peedikayil *et al*. (2014) revealed equality in terms of safety and efficacy of both levofloxacin-based first line therapy and the 7 days standard first-line therapy, but of concern is that levofloxacin-based first line therapy do not yield consistant results (Hung *et al*., 2009; Lim *et al.*, 2017). However, the recommendations of Chey *et al*. (2017) are not different from those suggested by European and American guidelines in 2007, but of note is an emphasis on drug selection combination i.e. before a patient is administered with treatment, information on previous infections and drug use needs to be obtained (Chuak *et al*., 2016).

2.9.2 Second Line therapies

The second line treatment regimens remain the alternative treatment in the event of a rise in clarithromycin and/or metronidazole resistant *H. pylori* strains. In areas with exceedingly high occurrences of clarithromycin and metronidazole resistance, bismuth-based quadruple therapy is used as the first-line treatment (Chey *et al*., 2007; Malfertheiner *et al*., 2007). Otherwise bismuth-based quadruple therapy is the preferred second line regimen that has yielded good eradication results (Melgraud *et al*., 2004; Kadayifci *et al*., 2006; Malfertheiner *et al*., 2007). The second-line regimen currently recommended is a quadruple therapy comprising of PPI, a bismuth salt, tetracycline and metronidazole for 10–14 days (Malfertheiner *et al*., 2007; Davis, 2014). A challenge of this second line treatment regimen is that bismuth salts are not found in all countries, hence an alternative regimen consisting of PPI, doxycycline, metronidazole and amoxicillin is recommended.

Levofloxacin, a fluoroquinolone has a broad spectrum of activity against Gram positive and Gram negative bacteria (Seck *et al*., 2009; Bago *et al*., 2010). Several studies have been conducted to evaluate new fluoroquinolone based regimens as standard second-line therapies

and in comparison to clarithromycin and metronidazole, the new fluoroquinolones seem to be an alternative to the standard second line regimen (Gloker *et al*., 2007; Teh *et al*., 2014; Zaki *et al*., 2016b). Rates of resistance of *H. pylori* to fluoroquinolones is low in different geographical locations, unlike for metronidazole and clarithromycin (Gatta *et al*., 2005; Boyanova and Mitov, 2010; Karczewska *et al*., 2011; Teh *et al*., 2014).

2.9.3 Third-line therapies/ Rescue therapy

European guidelines recommend microbial antibiotic sensitivity testing as a procedure of determining the therapy to be used. Quinolones are ready candidates for third-line therapies and have been observed to possess a wide spectrum activity against both Gram negative and Gram positive bacteria (Boyanova and Mitov, 2010). High doses of PPI plus amoxicillin and levofloxacin or rifabutin regimens have shown higher success rates (Chey *et al*., 2017).

2.10 CHALLENGES IN *H. PYLORI* TREATMENT

Even though various therapeutic regimens have been suggested against *H. pylori*, a multi-purpose regimen against *H. pylori* is still a dream. Over the years, the effectiveness of standard PPI triple therapies has shown a gradually deterioration mainly as a result of increasing drug resistance to key PPI combination with antibiotics (clarithromycin and metronidazole) (Kadayifci *et al*., 2006). Triple therapy consisting of a PPI (Rabeprazole, Dexlansoprazole, Esomeprazole, Lansoprazole, Omeprazole, Pantoprazole), clarithromycin, and amoxicillin or metronidazole was the initially planned regimen for *Helicobacter pylori* eradication. A five year study conducted in Turkey found a decreasing trend in eradication success with triple therapy consisting of a PPI, clarithromycin, and amoxicillin, the PPI-based triple regimens recorded an 84% eradication success in 1997 and decreased to 55.3% in 2004 (Kadayifci *et al*., 2006).

Several proficiently conducted studies globally, have highlighted *H. pylori* resistance against clarithromycin and amoxicillin or metronidazole which appears to vary among different countries. Using melting curve analysis, a study in Turkey revealed a 30% resistance to clarithromycin, also 79.2% of patients who had previously used clarithromycin were resistant to the drug (Kaya *et al*., 2007). In a separate study, Aydin *et al*. (2006), revealed a 58.8% ($p > 0.05$) resistant to clarithromycin when 500mg was administered twice daily for two weeks. A study conducted in South Africa using the GenoType Helico DR recorded 15.38% *H. pylori* resistance to clarithromycin (Tanih *et al*., 2012). Multiple drug resistance is a major problem worldwide. A systematic review of *H. pylori* resistance to clarithromycin worldwide revealed a 17.2% resistance (Francesco *et al*., 2010). Strains of *H. pylori* obtained from a population of Sicily in Italy had a representation of 25% resistance to clarithromycin (Fasciana *et al*., 2015), while a South African population revealed infection with *H. pylori* strains having a 15.38% resistance to clarithromycin (Tanih *et al*., 2013). A study done in Senegal revealed 90% resistance to metronidazole (Seck *et al*., 2009). In Egypt, another study revealed 100% resistance to metronidazole using an E-test (Fathi *et al*., 2013). A study done in Iran, revealed a double resistance to clarithromycin and metronidazole and triple resistance to clarithromycin (17.01%), metronidazole (78.86%) and amoxicillin (27.64%), this study also revealed a high rate of resistance (78.86%) to metronidazole (Ghotaslou *et al*., 2013). Resistant patterns of *H. pylori* against amoxicillin and clarithromycin, however raises doubts regarding the effectiveness of the sequential therapy that consist of a dual therapy of PPI and amoxicillin followed by triple therapy consisting of PPI, clarithromycin and tinidazole. However, cases of amoxicillin resistance are uncommon, with statistics as low as 1.9% resistance recorded among Brazilians (Picoli *et al*., 2014). This therefore calls for more studies on amoxicillin resistant patterns.

Clarithromycin remains one of the most powerful antibiotics against *H. pylori* with minimal inhibitory concentrations (MICs) being acceptably low when compared with other molecules available. However, *H. pylori* resistance to clarithromycin is dampening success rates of *H.*

pylori eradication regimens incorporating clarithromycin. Clarithromycin resistant by *H. pylori* is instigated by point mutations of the 23S RNA gene, the main component of the 50S ribosomal subunit, mostly at positions 2142 and 2143 (2142A~G, 2142A~C, 2142A~T; 2143A~G, 2143A~C) in the peptidyl-transferase region of the variable domain (Versalovic *et al*., 1996; De Francesco *et al*., 2011; Tanih *et al*., 2013; Teh *et al*., 2014). These point mutations prevent the drug from binding with *H. pylori. H. pylori* contain two 23S rRNA genes and mutations usually occur in both copies. Homogenic isolates seem to be more resistant to clarithromycin. The mutation in one copy of the 23S rRNA may be easily transferred to the other 23S rRNA gene by efficient homologous DNA recombination under selective pressure. The increasing resistance to clarithromycin has led to the empiric use of levofloxacin as a surrogate drug for this antimicrobial. However, research has found that the outcome of clarythromicin based therapy in comparison to levofloxin use on eradication rates of *H. pylori* infection and ulcer recurrence are similar (Gopal *et al.*, 2013).

Metronidazole, a core-drug in *H. pylori* treatment, is one of the cornerstones of many triple-therapy formulations for the eradication of *H. pylori*. In *H. pylori* the *rdx*A and *frx*A genes encode reductases that are essential for the activation of metronidazole implying that their inactivation results in metronidazole resistance (Gerrits *et al*., 2004). Different mutations involving the *rdx*A gene which encodes for an oxygen insensitive NADPH nitro-reductase have been identified in metronidazole resistant strains. Presence of truncated *rdx*A, nucleotides insertion/deletion and frame-shift mutation at the C-terminal and missing stop codon at the end of *rdx*A have been observed (Gerrits *et al*., 2004; Teh *et al*., 2014). These mutations are recognized as the main explanation behind metronidazole resistance in *H. pylori*. There are also cases of metronidazole resistant strains involving mutation of gene *frx*A

even though such mutations do not result in a high MIC level of metronidazole resistance (Yang *et al*., 2004b).

Currently, there is no definite standard third-line therapy for treating *H. pylori*. The European guidelines recommend culture method in determining a third-line treatment according to microbial sensitivity to antibiotics (Gisbert, 2012). Efficacy of this method, relies on the availability of well-established laboratories, this however present a challenges to many communities especial in underdeveloped and developing nations.

The multi-use of quinolones due to their broad-spectrum activity against Gram-positive and Gram-negative bacteria could be the explanation for the rising resistance against these drugs by *H. pylori*. In *H. pylori*, single point mutations in the *gyr*A gene, the quinolone resistance-determining region, cause resistance to this class of antibiotics (Tankovic *et al*., 2003). A study conducted in Germany showed that resistance to quinolones and triple resistance to metronidazole, clarithromycin, and ciprofloxacin in *H. pylori* have reached extremely high levels calling for susceptibility testing before administering quinolones especially with patients with records of failed eradication therapy before (Glocker *et al*., 2007). Nevertheless, there is scarcity of information that reveals an effective vaccine that is quite preventive against *H. pylori* infection in various ethnic populations.

All regimens that have been suggested for first line treatment of *H. pylori* (Chey *et al*., 2017) contain PPIs. The PPIs help to reduce the amount of hydrochloric acid released into the stomach and this unavoidably leads to increased stomach pH (Sugimoto *et al*., 2014). Insufficient acid inhibition has been linked with treatment failure during *H. pylori* eradication (Sugimoto *et al*., 2014). Also ingestion of PPIs has been linked to false-negative results of *H.*

pylori by diagnostics tests such as biopsy culture and rapid urease test (Saniee *et al*., 2015). In addition, PPIs have been linked to induction of severe hypomagnesemia, community-acquired pneumonia, iron and vitamin B_{12} deficiency, interstitial nephritis, hip fracture, microscopic colitis and *Clostridium difficile*-associated diarrhoea (Keszthelyi and Masclee, 2012). Acid tolerance of macrolides used to treat *H. pylori* infections is another factor that is important towards success of a treatment regimen. However, with the exception of levoflaxacin triple therapy and fluoroquinolone sequential therapy, all first line suggested regimens seem to contain clarithromycin.

The increasing resistance to clarithromycin has led to investigations of fluoroquinolones such as levofloxacin as a surrogate drug for this clarithromycin. A triple therapy with PPI, amoxicillin and levofloxacin has been suggested earlier as an alternative first-line therapy (Katelaris, 2009). Resistance to fluoroquinolones is generally very low (< 10%) worldwide (Boyanova and Mitov, 2010; Ahmed *et al*., 2012). A recent study, revealed a low primary and secondary resistance of 6.8% and 25% for floroqunoline (ciprofloxacin, levofloxacin, moxifloxacin and gemifloxacin) in comparison to clarithromycin of which *H. pylori* had 6.8% primary and 87.5% secondary resistance to the drug (Teh *et al*., 2014).

In areas with less than 40% metronidazole resistance, PPI with clarithromycin and metronidazole is preferable. Also, a 10-day sequential therapy seems to offer better outcomes. The therapy consists of a dual therapy of PPI and amoxicillin followed by triple therapy consisting of PPI, clarithromycin and tinidazole (Zullo *et al*., 2000). In a pediatric study conducted by Arenz *et al*. (2006), the esomeprazole-based 1-week triple therapy that was directed by susceptibility testing of *H. pylori* was shown to be effective in eradicating the organism. However the limitations of this method are that it is effective when there are no

double-resistant strains to clarithromycin or metronidazole. However, its efficacy is moreover affected by resistance patterns of amoxicillin and clarithromycin. Recently, a new sequential concomitant hybrid therapy reported a high rate (99.1%) of treatment success (Hsu *et al*., 2011). The quadruple therapy mainly consisting of PPI, bismuth and two antibiotics (amoxicillin + clarithromycin, or metronidazole + tetracycline) offer superior eradication rates.

Amoxicillin and tetracycline resistance are still relatively low, rarely exceeding 1% resistance (Megraud, 2004). Amoxicillin resistance in *H. pylori* is believed to be linked with amino acid substitutions in the penicillin binding proteins causing a structural alteration of the protein (Deloney and Schiller, 2000). Amoxicillin-resistant *H. pylori* strains seem to harbour mutations on the *pbp*-1a gene with amino acid substitution Ser-414→Arg being involved resulting in the blockage of penicillin transport (Van-Zwet *et al*., 1999). Tetracycline, a major component of quadruple therapy inhibits bacterial growth by disrupting codon-anticodon interactions during protein synthesis by binding to the 30S subunit hence disturbing the attachment of aminoacyl tRNA to the acceptor site, in addition, mutations of the 16SrRNA-encoding genes seem to affect the binding site of tetracycline (Wu *et al*., 2005). However, horizontal gene exchange involving foreign DNA acquisition by plasmids is common in *H. pylori* (Dharmalingam *et al*., 2003).

2.10.2 NATURE OF ANTIBACTERIAL SUBSTANCES IN HONEY

Honey is slightly acidic, with a pH between 3.2 and 4.5. The low pH is inhibitory to many pathogenic bacteria (Molan, 1995). When honey is diluted, glucose oxidase secreted by bees is activated and converts glucose to gluconic acid and hydrogen peroxide and at this point,

antibacterial activity of honey changes from osmotic to pH and peroxide motion (Molan, 1995; Mandal and Mandal, 2011). However, the nonperoxide and other constituents of honey have been shown to play an important role in the antibacterial property of honey (Zainol *et al.*, 2013).

2.10.2.1 Hydrogen peroxide in honey (H_2O_2)

Hydrogen peroxide (H_2O_2) is a by-product of glucose oxidation catalysed by the enzyme glucose oxidase synthesized by the bees. Dilution of honey has been observed to activate the enzyme glucose oxidase that oxidizes glucose to H_2O_2 and gluconic acid, resulting in the antimicrobial activity of honey (Bang *et al*., 2003; Brudzynski, 2006; Mandal *et al*., 2011). The amount of hydrogen peroxide available as a honey constituency depends on the amount of glucose oxidase produced by the bees and the amount of catalase (flower origin) which actively degrade hydrogen peroxide (Brudzynski, 2007; Mandal *et al*., 2011). The discovery that H_2O_2 is a core compound in honey led to assumptions that it plays a big role in the antimicrobial activity of honey, however, further research has shown that in most honeys, the antibacterial activity is strongly related to H_2O_2 (Brudzynski, 2006; Irish *et al*., 2011). In contrast, Manuka honey showed antibacterial activities after the removal of H_2O_2 (Molan and Russell, 1998).

2.10.2.2 Non-peroxide constituency of honey

The non-peroxide constituency of honey includes sugars together with small amounts of organic acids, phenolic acids, flavonoids, proteins, minerals, vitamins, enzymes and other phytochemicals (Bogdanov, 1997; Kwakman and Zaat., 2012). Honey devoid of hydrogen peroxide show significant antibacterial activity (Mandal and Mandal, 2011). There are two

proteins that contribute to biological activities of honey, the glucose oxidase which originate from bees and catalase which is of floral origin (Zainol *et al*., 2013).

2.10.2.3 Phenolic compounds

Polyphenols are a common and important class of secondary metabolites in honey. Majority of polyphenols arise from the amino acids phenylalanine or tyrosine which when deaminated to cinnamic acids and p-coumaric acids respectively, enter the phenylpropanoid pathway (Kokotkiewicz and Luczkiewicz, (2009). These molecules bear antimutagenic, antibacterial (bacteriostatic, bactericidal), antiviral, antifungul, insecticidal, keratolytic, estrogenic and antioxidant properties (Jaganathan and Mandal. 2009). There are studies that have revealed that the composition of the phenolic compounds in honey depends on the floral source where the bees collect nectar, seasonal and environmental factors, geographic origin and storage conditions (Aljadi *et al*., 2002; Lachman *et al*., 2010; Ramanauskiene *et al*., 2012). Phenolic compounds in honey have been classified into three groups namely flavonoids, cinnamic acids and benzoic acids. Commonly detected phenolic compounds in honey include caffeic, chlorogenic, coumaric, ellagic, ferulic, gallic, homogentisic, phenyllactic, protocatecuic, syringic and vanillic acids, while detected flavonoids include apigenin, chrysin, galangin, hesperetin, kaempferol, luteolin, myricetin, pinobanksin, pinocembrin, quercetin and tricetin (Aljadi *et al*., 2002; Kassim *et al*., 2010; Biesaga and Pyrzyn´ska K, 2013; Campone *et al*., 2014; Moniruzzaman *et al*., 2014).

Studies have revealed that plant derived phenolic compounds can inhibit enzymes (Lin *et al*., 2005; Pereanez *et al*., 2010; Ranilla *et al*., 2010; Al Shukor *et al*., 2013). The study on angiotensin-converting enzyme showed that tannic acid had the highest activity and the

number of hydroxyl groups it has on the benzene ring plays a big role in the activity of phenolic compounds (Al Shukor *et al*., 2013). Also Ranilla *et al*. (2010) observed that even though Chanca Piedra leaves had less phenolic compounds as compared to matico leaves, extracts from Chancapiedra exhibited higher antioxidant activity probably due to free radical scavenging properties of phenolic compounds. Gallic acid, ferulic acid, caffeic acid, propylgallate and epigallocatechingallate were shown to inhibit the enzymatic activity of a phospholipase A_2 (Pereanez *et al*., 2010). Combined extracts from different sources have also proven to offer more inhibitory potential when compared to plant extracts from a single source (Lin *et al*., 2005).

Organisms have a way of balancing the production and removal of free radicals, known as oxidative balance. When oxygen is metabolized, by-products called free radicals are formed by cells. Free radicals move through the cell causing some disruption resulting in cellular damage. . At times there are offsets of this balance resulting in increased levels of reactive oxygen species. This condition is called oxidative stress and indicates an imbalance between the production of free radicals and the antioxidant defence system resulting in damage of all components of the cell, including proteins, lipids and DNA (Wells *et al*., 2009). Oxidative stress plays a significant role in the onset of numerous pathological conditions, thus, foods containing significant levels of antioxidants which can inhibit or delay oxidation of a substrate represent a healthy and logical diet choice. Antioxidants that occur naturally in the body or are consumed through the diet may block damage to cells. Honey intake increases blood vitamin C, beta-carotene and glutathione reductase, and improves antioxidant activity in human plasma (Ali-Waili, 2004; Khalil *et al*., 2010). Antioxidants protect key cell components from damage by neutralizing the free radicals

There is growing evidence that honey exhibit antioxidant activity (Aljadi *et al*., 2002; Piljac-Žegarac *et al*., 2009). Honey antioxidant activity appears to be a result of the combined effect of a range of compounds. Phenolic compounds (flavonoids and phenolic acids), as well as non-phenolic compounds (ascorbic acid, carotenoid-like substances, organic and amino acids, and proteins including certain enzymes such as glucose oxidase and catalase) can contribute to honey antioxidant activity. Different honey types seem not to possess the same therapeutic advantages. Antioxidant potential of honey is directly related to its plant source. Considerable differences in both composition and content of phenolic compounds have been found in different uni-floral honeys. Honeys derived from several floral sources (heterofloral) seem to possess better antioxidant potency assays in comparison to monofloral source (Piljac-Žegarac *et al*., 2009).

2.11 HONEYS AS ALTERNATIVE TO TREAT *H. pylori* INFECTIONS

Phytochemicals are naturally occurring compounds in plants with high biological significance. The underlying fact determining the value of these products is their chemical profile which puts a selection pressure on the use of such product in the treatment of various ailments including life threatening diseases caused by both Gram positive and Gram negative bacteria (Sibanda *et al*., 2008; Njume *et al*., 2011; Nkomo *et al*., 2011). Dating back to ancient times, such biological significance attracted the use of certain plants and natural products in the traditional medicine system e.g. *Aspalathus linearis* is used in the production of herbal tea, called rooibos known to relieve hypertension and easing of severe stomach cramps (Street and Prinsloo, 2013) and *Emblica officinalis* is highly valued for its fruits which enhance digestion, reduce fever and blood purification when eaten (Franklin *et al*., 2012). However, several studies have shown that there are many natural products with anti-*H.*

pylori compounds (Njume *et al*., 2009; Amin *et al*., 2010; Manyi-Loh *et al*., 2010; 2012; 2013; Nkomo *et al*., 2011; Franklin *et al*., 2012; Goswami *et al*., 2012; Awwad *et al*., 2013). Honey, a complex natural sweet substance produced by bees through a process of regurgitation, is one of the natural products that has been shown to possess a broad-spectrum of antibacterial activity against pathogenic micro-organisms (Jedder *et al*., 1985; Ndip *et al*., 2007; Manyi-Loh *et al*., 2010; Mandal *et al*., 2011) and has been used widely as a traditional remedy for the treatment of various ailments including wounds and alleviation from dyspepsia (Tonks *et al*., 2007; Kwakman and Zaat, 2011). The linkage of *H. Pylori* as a causative agent to gastric related illnesses sparked the possibility that honey possess antibacterial properties. In the past decades, honey has been subjected to various laboratory and clinical investigations (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010; 2013; Mandal *et al*., 2011; Silva *et al*., 2013). The antimicrobial properties of honey have been attributed to both the hydrogen peroxide as well as non-peroxide components (Silva *et al*., 2013).

The complex natural sweet substance is also well known for its positive actions within a wound environment including those that are nonresponsive to conventional therapies (Bang *et al*., 2003; Eddy and Gideonsen, 2005; Van den Berg *et al*., 2008; Mandal *et al*., 2011). Honey is known to maintain a moist wound environment that promotes healing, and its high viscosity helps to provide a protective barrier to prevent infection. It is commonly used by many people with belief that it has antibacterial properties. Honey is relatively cheap and is easily available.

2.11.1 Origin of antibacterial substances in honey

It takes a collection of several natural products for bees to construct their hive and produce honey. This collection includes bees-wax, flower volatiles, pollen, nectar, propolis and honey itself. Honey has been reported as a complex mixture of sugars which possess antibacterial activity due to the osmotic effect, together with small amounts of organic acids, phenolic acids, flavonoids, proteins, minerals, vitamins, enzymes and other phytochemicals (Al *et al*., 2009; Kassim *et al*., 2010; Silva *et al*., 2013). Honey containing pollen mainly collected from a single species is classified as mono-floral, while multi-floral honey contains pollen from lots of different species (Ampuero *et al*., 2004). The antimicrobial properties of honey have been credited to both the hydrogen peroxide as well as non-peroxide components (Snow and Manley-Harris, 2004). The non-peroxide factors correlating with antibacterial properties and antioxidant activity of honey include phenolic acids, flavonoids and lysozyme (Arreazroman and Gomezcaravaca, 2006; Silva *et al*., 2013). In addition, low pH and high osmolarity are contributory factors in honey's antimicrobial activity. However, H_2O_2, methylglyoxal, and bee defensing-1combined presence influence the broad-spectrum activity of honey against bacteria (Kwakman *et al*., 2010).

Honey has a wide range of phytochemicals including phenolic compounds which are known to be natural antioxidants (Ramanauskiene *et al*., 2012). Polyphenols and phenolic acids found in the honey vary according to the source of nectar, geographical and climatic conditions (Ramanauskiene *et al*., 2012; Silver *et al*., 2013; Agggad and Guemour, 2014). Hence they can be used as indicators in the studies of floral and geographical origin of honey and propolis (Alzahrani *et al*., 2012; Ramanauskiene *et al*., 2012). Some of them were reported as a specific marker for the botanical origin of the honey. Phenolic compounds

occurring in honey have been classified into three groups, the flavonoids, cinnamic acids and benzoic acids (Valls *et al*., 2009).

Melissopalynological analyses that consist of the qualitative and quantitative microscopic examination of honey pollen grains have been used as a traditional method of determining the geographical origin of honey (Alissandrakis *et al*., 2003; Ruoff *et al*., 2006). In their work (Ruoff *et al*., 2006) validated another technique called the Front-Face Fluorescence Spectroscopy, which was directly applied on honey samples to authenticate 11 uni-floral and poly-floral honey types that have been already categorized using traditional methods of pollen analysis. Due to ever improving technology, a fast and reliable method has been developed based on fingerprinting and barcoding of proteins in honey by use of matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI TOF MS) and MALDI Biotyper 1.1 software, respectively (Wang *et al*., 2009). Regardless of improvement in research techniques, origin of pollen found in honey does not always correlate with the source of the nectar contributed. This poses difficulty in determining the physical, chemical, and pollen analytical characteristics of poly-floral honeys as compared to uni-floral honeys.

Research has also revealed that the geographical location plays an important role in determining the bactericidal properties of different honey types (Alzahrani *et al*., 2012). It was demonstrated in Cameroon that four honey varieties from different geographical locations displayed antibacterial activity against *H. pylori* (Ndip *et al*., 2007). The strongest inhibitory activity (82.22%) was demonstrated by Mountain honey at 75%v/v, followed by Capillano® and Manuka™ honeys (75.56%), and Eco-honey (73.36%) using equal concentrations. The MIC and MBC concentrations of Mountain honey were in the range 0.117 – 0.938µg/mL and 0.366 – 2.965µg/mL respectively (Ndip *et al.*, 2007). In addition,

Manuka (New Zealand) and Medihoney (Australia) honeys made from *Leptospermum* species are accepted as one of the most therapeutically useful honeys. The Manuka honey has unusual gel-like consistency (thixotropy) enhanced by its protein content and the "unique manuka factor" (Mollan, 1995; Mollan and Russel, 1998). In a study done in South Africa, a report revealed an inhibitory effect of honey on uncharacterised clinical *H. pylori* strains, it was observed that inhibitory effect of clarithromycin (zone of inhibition: 18.0 $\pm$ 7.4 mm) was not significantly different ($p > 0.05$) from honeys at a concentration of 75% v/v (Manyi-Loh *et al*., 2010).

2.12 METHODS OF CHARACTERIZING HONEY COMPOUNDS

Harvested honey often requires an effective separation technique to enable the identification of its diverse complex constituents. Solid-phase extraction (SPME) or liquid/liquid extraction followed by the use of Capillary electrophoresis–mass spectrometry (CE-MS), High-performance liquid chromatography (HPLC), Gas Chromatography Mass Spectrometry (GC-MS) and Liquid chromatography mass spectrometry (LC-MS) have been successfully used for the analysis of compounds in honey (Arr´aez-Rom´an *et al*., 2006; Campone *et al*., 2014). These methods offer a simple and cost effective analytical technique of characterizing compounds (Campone *et al*., 2014).

SPME technique has been considered to be a rapid and solvent free method for the extraction of both volatile and nonvolatile organic compounds in honey. It was invented by Pawliszyn in 1989 (Vas and Ve´key, 2004). The SPME improves quality of sample obtained within a short time as well as reduction of costs involved with extraction methods that require solvents. SPME in combination with GC-MS has been exploited well and showed high

sensitivity when a total of 86 compounds in the headspace of 4 types of honey revealed marked different compositions (Wolski *et al*., 2006). Another study demonstrated further the sensitivity of this technique when traces of *p*-dichlorobenzene and/or naphthalene (chemicals used to control wax moth; *Galleria mellonella*) residues in honey were found to exceed the maximum residue concentration (Harizanis *et al*., 2008).

Solid-phase extraction (SP-ME) or liquid/liquid extraction followed by the use of Capillary electrophoresis–mass spectrometry (CE-MS) has also yielded good outcomes in the scientific experimental world. The combination of CE and MS provide high separation efficiency and molecular mass information in a single analysis. CE coupled with electrospray ionization time-of-flight-mass spectrometry (CE-ESI-TOF-MS) has been used also (Kamakshi *et al*., 2013), and it requires small amount of sample, CE offers high resolution and the TOF undeniably is sensitive, highly selective and offers accurate mass and true isotopic patterns of compounds.

High-performance liquid chromatography (HP-LC), a form of improved column chromatography is one of the most powerful tools used for the analysis of compounds in chemistry. However, Liquid Chromatography Mass Spectrometry (LC-MS) which combines liquid chromatography and mass spectroscopy and GC-MS are highly sensitive, accurate and a specific quantitative analysis instruments in comparison to HP-LC (Syazana *et al*., 2013; Moniruzzaman *et al*., 2014).

CHAPTER THREE

In-vitro study on kinetic inhibition of urease enzymes: a focus on urease isolated from drug resistant *H. pylori*

3.1 ABSTRACT

The aim of the study was to evaluate inhibitory potential of South African honey extracts on urease activity. Urease enzyme is produced by pathogenic *H. pylori* so as to evade the acidic defensive mechanism of the human stomach. Commercial Jack bean urease (CJBU), Urease extracted from *H. pylori* susceptible to four drugs - clarithromycin, amoxicillin, metronidazole and tetracycline (HPU1), urease extracted from *H. pylori* resistant to clarithromycin (HPU2) and urease extracted from multidrug resistant *H. pylori* (HPU3) were all exposed in a cocktail assay based on the reduction of NADH in a coupled urease-glutamate dehydrogenase (GDH) system. NADH consumption in the presence of honey extracts (test compounds) and acetohydroxamic acids (standard inhibitor) was monitored using a SynergyMx microplate reader at 340nm. At a concentration of 50mg/ml, urease inhibition by petroleum ether extract of Gold Crest and of Fleures Honey, hexane extract of Little Bee and Manuka Honey and chloroform extract of Bush Honey and of Q had a range above or equal to 50%. The One way ANOVA Test showed no significant difference in urease enzyme inhibitory activity from different sources including urease from drug resistant *H. pylori* strains ($p > 0.05$).

3.2 INTRODUCTION

Nature provides an unlimited source of natural products that are of biological significance (Awaad *et al*., 2013). Honey is a complex product with different constituents dependent on factors such as bee species, foraging behaviour of bees, geographic area, season and mode of storage (Alzahrani *et al*., 2012; Modolo *et al*., 2015). Honey is one of the natural products that have been shown to possess a broad-spectrum of antibacterial activity against pathogenic micro-organisms including *H. pylori* (Jeddar *et al*., 1985; Mandal *et al*., 2011; Alzahrani *et al*., 2012; Nayik and Nanda, 2015). Research has also shown that the geographical location from which honey is harvested plays an important role in determining the bactericidal properties exhibited by different honey types (Alzahrani *et al*., 2012). Apitherapy is an ancient traditional practice that has been used to treat microbial infections as well as application of honey for wound healing (Mandal and Mandal, 2011).

The occurrences of *H. pylori* related infections and incidences are on the rise worldwide especially in developing nations. Infection with *H. pylori* can be life long without an effective treatment (Kadayifci *et al*., 2006; Nayik and Nanda, 2015). It is now widely accepted that *H. pylori* infections are associated with acute or chronic duodenal/gastric ulcer disease, gastritis, gastric adenocarcinoma, mucosa-associated tissue lymphoma (MALT), primary B-cell gastric lymphoma and acid gastric reflux (Ahmed *et al*., 2007b; Seanego and Ndip, 2012; Tanih *et al*., 2013; Abebaw *et al*., 2014).

In the human stomach, urease is produced in enormous quantities by *H. pylori*. The multimeric nickel-containing urease enzyme is a vital virulence factor produced by pathogenic *H. pylori* so as to evade the defence system of the human stomach (Kuwahara *et*

al., 2000; Amin *et al*., 2010; 2013). Urease converts urea into ammonia and bicarbonate. Ammonia causes an increase in pH within the microenvironment surrounding profusely growing *H. pylori* cells in the human stomach thereby protecting the bug from the acid environment of the human stomach. The bicarbonate protects *H. pylori* against bactericidal action of peroxynitrite, a metabolite of nitric oxide (Kuwahara *et al.*, 2000). Urease has been confirmed as a colonization factor of *H. pylori*. In the late 90s, an assessment of importance of cytoplasmic and surface localized urease on *H. pylori* survival in acidic environment revealed that *H. pylori* greatly requires surface localized urease to flourish in an acidic environment (Krishnamurthy *et al*., 1998). Another study revealed that urease-negative mutant strain of *H. pylori* does not cause gastritis due to challenges with colonization (Amin *et al*., 2010). The urease from different sources (plants, bacteria, invertebrates and fungi) have similar structures such as basic trimeric array possessing 1, 2 or 3 subunits that can join to form a hexameric or dodecameric structure (Seck *et al*., 2013). There are studies that have reported that phytochemicals have the potential to inhibit urease activity (Ndip *et al*., 2007; Amin *et al*., 2010).

Antimicrobial agents are important as control effectors of most infectious diseases. *H. pylori* infection is normally treated using a regimen comprising of a PPI, clarithromycin, and amoxicillin or metronidazole (clarithromycin-based triple therapy) given twice a day for 7–14 days or a PPI or (Ranitidine) H_2RA, bismuth subsalicylate, metronidazole, and tetracycline (bismuth quadruple therapy) given twice a day for at least 10 days or 14 days if bismuth is unavailable, in populations with less than 20% clarithromycin resistance (Chey *et al*., 2007; Mandal *et al*., 2011). A second-line regimen called a quadruple therapy comprising of PPI, a bismuth salt, tetracycline and metronidazole for 10–14 days has also been recommended (Gisbert., 2009; Manyi-Loh *et al*., 2010; Mandal *et al*., 2011). The effect of the introduction

of PPIs into the human stomach is an increase of the stomach pH causing digestive disturbances (Sugimoto *et al*., 2014). Also there are studies that have linked the PPIs with the induction of severe hypomagnesemia, community-acquired pneumonia, iron and vitamin B_{12} deficiency, interstitial nephritis, hip fracture, microscopic colitis and *Clostridium difficile*-associated diarrhoea (Keszthelyi *et al*., 2012). In addition, drug resistance is threating successful treatment of *H. pylori* infections (Upadhyay, 2012; Wu *et al*., 2012; Fathi *et al*., 2013; Syazana *et al*., 2013; Gosciniak *et al*., 2014). Proposed rescue therapies seem to be related with unwelcomed side-effects which are associated with defaulting and further development of drug resistance. The European guidelines recommend culture method in determining a third-line treatment according to microbial sensitivity to antibiotics (Gisbert, 2009), however the availability of well-established laboratories determine the success of this approach. This therefore entails a need to explore new approaches that are less harmful while attaining satisfactory control strategies of *H. pylori* infections.

As urease enzyme plays a crucial role in the establishment of an effective infection by *H. pylori*, urease inhibitors may be targeted in search of new leads towards treatment of infections caused by organisms that are urease-dependent. There are studies that have shown that phytochemicals have anti-urease active compounds (Amin *et al*., 2010; Amin *et al*., 2011; Amin *et al*., 2013). Heavy metals, hydroximate and its derivatives, fluoride, thiols, phosphoramides compounds, hydroxyurea, ∝ -hydroxyketones and ∝-diketones, triketone oximes, phosphates, boric acid, sulfur compounds, biscoumarin have been shown to be potential inhibitors of urease (Tanih *et al*., 2010; Upadhyay, 2011). However, the commercially available urease inhibitors such as hydroxamic acid derivatives are toxic, hence inapplicable for clinical use (Megraud, 2012). Continued search for low toxic urease inhibitors is ideal towards targeting urease inhibition to control diseases linked to infection by

urease dependent micro-organisms. Since, honey has been reported as a pool of phytochemicals with a broad-spectrum antibacterial activity depending on various factors such as geographical location and bee foraging behaviour, they has been no report of microorganisms gaining resistance to honey. This study therefore focuses on screening for anti-urease solvent extracts from several South African honey types in search of anti-urease active compounds that could act as a template for cheaper and easily available compounds to control *H. pylori* and other urease dependent micro-organisms.

3.3 MATERIALS AND METHODS

3.3.1 Confirming *H. pylori* isolates and standard strain

A standard strain, ATCC 43526 (American Type Culture Collection, Manassas, VA, USA) and clinical isolates from gastric corpus biopsy specimen of patients with gastro-duodenal disease attending the endoscopy unit at Livingstone hospital, Port Elizabeth, Eastern Cape Province from a previous study done in the MPMERG Laboratory (University of Fort Hare) by Tanih *et al*. (2010) were used for this study. *H. pylori* strains were resuscitated on Columbia Blood Agar supplemented with Skirrow's antibiotics and fetal bovine serum 5% (v/v). The plates were incubated microaerophilically (85% N_2, 10% CO_2 and 5% O_2) using Oxoid^{TM} AnaeroGen^{TM} gas-generating envelopes (Thermo Fisher Scientific) at 37^{o}C for 5 days. Preliminary identification of all *H. pylori* strains was done by colony morphology, Gram stain, catalase test, oxidase test, urease test followed by confirmation by PCR targeting the *glm*M gene of *H. pylori*.

3.3.2 Polymerase Chain Reaction (PCR)

DNA was isolated from presumptive pure colonies by boiling method. Briefly, colonies were suspended in 200 µL of sterile distilled water. Cells were lysed by boiling in a digital Accu dri-block (Labnet International, Woodbridge, United Kingdom) at 100^{o}C for 15 minutes. Cell debris was removed by centrifugation at 13,000 rpm for 5 minutes and 5 µL of supernatant was used as a template in each polymerase chain reaction (PCR) assay. The *glm*M gene was targeted using the primer pair; Forward 5'- GAT AAG CTT TTA GGG GTG TTA GG GG-3', Reverse 5'-GCA TTC ACA AAC TTA TCC CCA ATC-3' as earlier reported (Abu-ALmaali *et al*., 2012). Amplification conditions included an initial denaturation of target DNA at 95°C for 5 min, 40 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. Final elongation was done at 72°C for 5 min. A 25 µL PCR reaction was set up comprising of 12.5 µL of 2X master mix (New England Biolabs, Ipswich, United Kingdom), 6.5 µL nuclease free water (New England Biolabs), 0.5 µL of each primer (Inqaba Biotec, Pretoria, South Africa), and 5 µL of template DNA. Amplification of the 140bp *glm*M gene was carried out using a Bio-Rad thermal cycler (C1000 Touch^{TM} Thermal Cycler). Products were resolved in a 1.5% (wt/vol) agarose gel containing 5 µL of ethidium bromide (Sigma-Aldrich, St Louis, MO). Electrophoresis was carried out in a 0.5X TAE buffer (40 mM Tris-HCL, 20 mM Na-acetate, 1 mM EDTA, pH 8) at 100 volts for 40 min and the gels were visualised under a UV transilluminator (Alliance 4.7 XD-79, Uvitec, Cambridge, United Kingdom).

3.3.3 Drug resistance profiling

This was done according to Seanego *et al*. (2012). In brief, 0.5 McFarland bacterial suspensions was evenly spread using sterile swabs on Muller Hinton agar (MHA) supplemented with 5% foetal bovine serum and Skirrow's antibiotics. Commercial discs impregnated with standard drugs (clarithromycin, amoxicillin, metronidazole and

tetracycline) were placed on agar followed by a 5 day incubation period at 37^{o}C under microaerophilic conditions. Categorizing of resistance was done as recommended by McNulty *et al*., (2002).

- Zone of inhibition < 16mm was read as resistant,
- 16mm ≤ Zone of inhibition < 21mm was read as intermediate
- Zones of inhibition ≥ to 21mm were read as susceptible.

3.3.4 Intracellular crude urease extraction

This was done as previously described by Amin *et al*. (2013) but with modifications. Briefly, 72 hours old broth cultures (50 mL, 0.5 McFarland standard) were centrifuged at 5,000 x *g* at 4^{o}C for 20 minutes and the bacterial mass that settled at the bottom of the tube was washed twice using phosphate-buffered saline (pH 7.4). *H. pylori* cells were suspended in buffer supplemented with complete Mini EDTA-free protease inhibitors (Roche Diagnostics S.A). An Omni Sonic Ruptor, ultrasonic homogenizer was used to disrupt cells using a setting of 40 watts for 60 seconds in ice-bath. Centrifugation at 15,000 X g, 4°C for 30 minutes was done to remove cell debris. The resultant supernatant with crude urease was precipitated in saturated ammonium sulfate solution and then tightly sealed into semipermeable snake skin dialysis tubing and then dialyzed for 24 hours. Urease activity assay kit (SIGMA) was used to determine solutions with active urease which were further eluted through HiPrep 16/60 Sephacryl S-200 High Resolution column (GE Healthcare). The resultant urease solution was stored at -80^{o}C in aliquots until use.

3.3.5 Urease detection

This was done using Urease Activity Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. Using a 96 well microtiter plate the urease enzyme was spiked into standard cocktail to catalyse the hydrolysis of urea resulting in the production of ammonia. The ammonia was determined by the Berthelot method whereby the ammonia produced reacts with the Berthelot reagents (phenol and hypochlorite) to form a blue colorimetric product that was measured at 670 nm using SynergyMx Microplate reader.

3.3.6 Extraction of honey compounds

Seven locally produced natural honeys in South Africa, Bush honey, Raw honey, Gold Crest honey, Q Bee honey, Little Bee honey, Fleures honey-radurised, Siyakholwa pure honey and one imported honey (Manuka honey) from New Zealand were used in this study. All honey products were subjected to a test for the presence of microbial growth by sub-culturing on Colombia blood agar supplemented with Skirrow's antibiotics and 5% horse serum and incubated for 5 days at 37^{o}C under aerobic and anaerobic conditions.

Extraction of volatile compounds was done according to the method described by Syazana *et al.* (2013) but with modifications. Three different organic solvents with increasing polarity were used; petroleum ether, hexane and chloroform. Initially, honey was initially weighed (0.5 g) and diluted with 0.5 mL sterile distilled water (w/v). Petroleum spirit (2 mL) was added to the capped glass tube containing the diluted honey. Then, the mixture was vortexed at 1500 rpm for 3 min before being centrifuged at 2500 rpm for another 5 min to separate the organic layer from the aqueous layer. The top layer containing the organic solvent was transferred to new capped glass tubes while the bottom layer was kept for subsequent

extraction processing using different organic solvents. Approximately 1.5 g of anhydrous disodium sulfate powder ($Na_2SO_{4)}$ was added to the top layer to ensure that residual water is removed from the solvent. The sample was further vortexed for 3 minutes and for GC-MS analysis, the clear top layer containing the organic extract was filtered (using Whatman™ 0.45 µm syringe filter) into a 1 mL autos-ampler vial before GC-MS injection. In preparation for urease/H. pylori inhibition studies, the extracts in capped tubes loosely capped were then placed in a heating block set at 28^{o}C for 7 days to allow drying. The different concentrations of extract solutions were then prepared from dry honey solvent extracts. For the next extraction, 2 mL of hexane was added to the bottom layer from the prior extraction. The above steps were repeated. A similar process was carried out with chloroform followed by ethyl acetate and finally methanol.

3.3.7 Dilution of dried honey extracts to working concentrations (mg/ml)

Totally dried honey extracts were weighed and a 400mg/ml honey/water solution was prepared and kept for 24 hours before it vortexed at 1500 rpm for 3 minutes and filtered through a 0.45 Whatman™ syringe filter from which serial dilutions were done to produce subsequent concentrations i.e. 200 mg/ml, 100mg/ml, 50mg/ml, 25mg/ml and 12.5mg/ml.

3.3.8 Screening for anti-urease active compounds

This was done according to Kaltwasser *et al.* (1966). The method is based on the reduction of NADH in a coupled urease-glutamate dehydrogenase (GDH). Commercial Jack bean urease was used as standard urease; acetohydroxamic acid was used as standard inhibitor, while honey extracts were used as test compounds. Reduction of NADH was monitored using a SynergyMx microplate reader at 340 nm. Inhibition percentage was calculated using the following formula:

$$\textbf{\% Inhibition} = \frac{\textbf{A0} - \textbf{A1}}{\textbf{A0}} \textbf{ X } \frac{\textbf{100}}{\textbf{1}}$$

Where:

- A_0 is NADH decrease in absorbance rate due to urease activity (no inhibitor).
- A_1 is NADH decrease in absorbance rate due to urease (with honey extracts / acetohydroxamic acid)

The assay cocktail was prepared by mixing 240 µL of Tris-HCL buffer [50mM, pH8], 10 µL of NADH [8.5mM] and 10 µL of ά-ketoglutaric acid [25mM pH 5] and this was injected into a 96 well micrititer plate. This was followed by equilibration at 37^{o}C for 10 minutes. Ten microliters (10 µL) of urease [Jack bean urease-10U/mL] and 10 µL of GLDH [250U/mL] in phosphate buffer were then added after which test compounds were added [all 50mg/ml]/acetohydroxamic acid [all 14.3mg/ml or adjusted to 100% inhibition percentage]. Absorbance was read at 340 nm using the SynergyMx microplate reader until reading stability was obtained. This was followed by the addition of 10 µL of 0.3 M urea and kinetic monitoring was done by reading absorbance after 30 seconds for 30 minutes. Three replications were done for each experiment, mean ($\bar{x}$) was calculated and data was then presented in a linear model graph showing rate of NADH reduction as a result of urease activity.

3.3.9 Statistical analysis

The Independent-Sample Kruskal-Wallis Test with the aid of the IBM SPSS statistics version 24 Package was used for statistical analysis with $P < 0.05$ considered statistically significant.

3.4 RESULTS

3.4.1 Confirmation of *H. pylori*

Forty eight clinical samples of *H. pylori* were successfully recovered from a pool of 250 samples that were stored at -80^{o}C. Preliminary identification of the isolates was done by colony morphology, biochemical tests (Gram stain, oxidase test, urease test and catalase tests) and the isolates were confirmed by PCR targeting the *glm*M gene of *H. pylori* (Figure. 3.1).

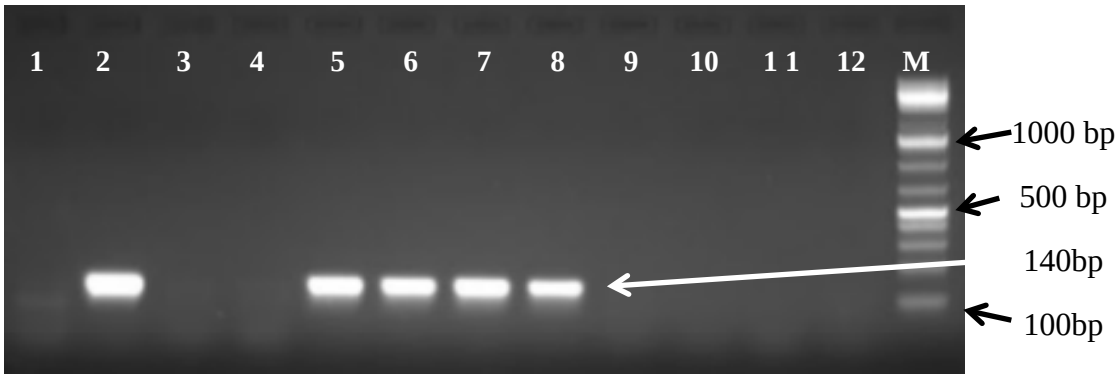


Figure 3. 1: Representative gel picture of PCR product of the *glm*M (140bp) gene of positive isolates. Lanes 1: negative control; Lane 2: positive control; Lanes 2-3, 9-12: negative isolates; Lanes 5-8: positive isolates; Lane M: 100bp ladder

3.4.2 Drug profiling of *H. pylori* strains

Of the 48 samples used in this study, 5/48 (10.4%) were susceptible to clarithromycin, amoxicillin, metronidazole and tetracycline and 3/48 (6.3%) were resistant to all the four drugs (Table 3.1). Resistance was 16.7%, 10.4%, 89.6% and 14.6% for clarithromycin, amoxicillin, metronidazole and tetracycline respectively (Figure 3.2).

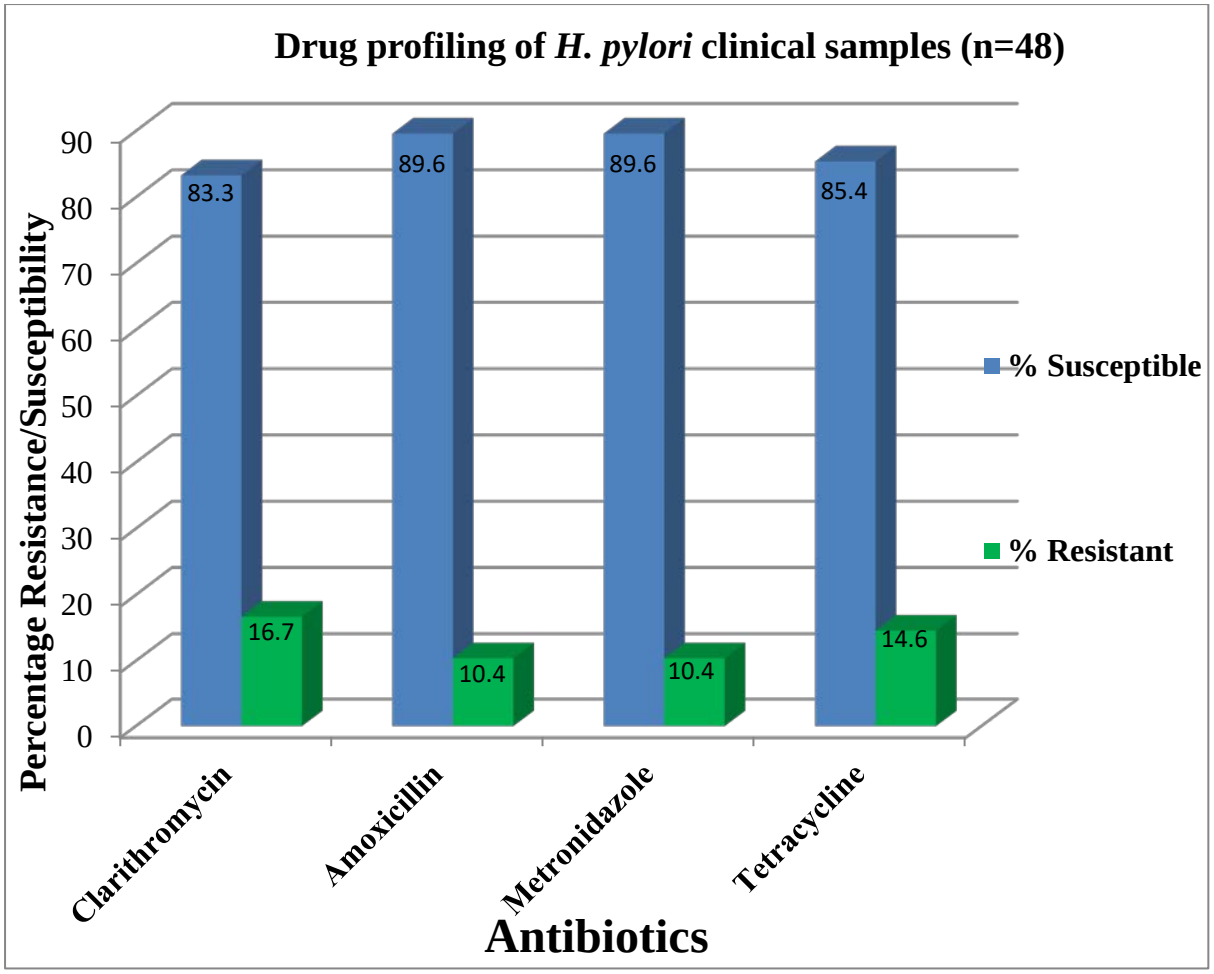


Figure 3. 2: Antibiotic susceptibility testing

Table 3. 1: Resistance of clinical *H. pylori* samples (n=48)

***H. PYLORI* STRAIN**		**DRUG RESISTANCE**				**Strain susceptible to 4 drugs**	**Strain resistant to 4 drugs**
		Cl	**Am**	**Met**	**Tet**		
1	**10 A**	NR	NR	R	R		
2	**467A**	R	NR	R	NR		
3	**56 A**	NR	R	R	NR		
4	**430 A**	NR	NR	R	NR		
5	**96 A**	NR	NR	R	R		
6	**155 A**	R	NR	R	NR		
7	**169 A**	NR	NR	NR	NR	✓	
8	**11 A**	NR	NR	R	R		
9	**402 A**	NR	NR	R	NR		
10	**469 A**	R	NR	R	NR		
11	**265 A**	NR	R	R	NR		
12	**63 A**	NR	NR	R	NR		
13	**87 A**	NR	NR	R	NR		
14	**436 A**	NR	NR	R	NR		
15	**369 A**	R	R	R	R		✓
16	**464 A**	NR	NR	R	NR		
17	**25 A**	NR	NR	NR	NR	✓	
18	**47 A**	NR	NR	R	NR		
19	**93 C**	NR	NR	R	NR		
20	**219 C**	NR	NR	NR	NR	✓	
21	**406 C**	NR	NR	R	NR		
22	**411 C**	NR	NR	R	NR		
23	**467 C**	NR	NR	R	NR		
24	**13 C**	R	NR	R	NR		
25	**32 C**	NR	NR	R	NR		
26	**105 C**	NR	NR	R	NR		
27	**212 C**	NR	R	R	NR		
28	**92 C**	R	R	R	R		✓
29	**300 C**	NR	NR	R	NR		
30	**264 C**	NR	NR	R	NR		
31	**41 C**	NR	NR	R	NR		
32	**405 C**	NR	NR	R	R		
33	**10 C**	NR	NR	R	NR		
34	**464 C**	NR	NR	R	NR		
35	**213 C**	R	R	R	R		✓
36	**350 C**	NR	NR	NR	NR	✓	
37	**98 C**	NR	NR	R	NR		
38	**111 C**	NR	NR	R	NR		
39	**404 C**	NR	NR	R	NR		
40	**53 C**	NR	NR	R	NR		
41	**77 C**	NR	NR	R	NR		
42	**317 C**	NR	NR	NR	NR	✓	
43	**299 C**	NR	NR	R	NR		
44	**162 C**	NR	NR	R	NR		
45	**430 C**	R	NR	R	NR		
46	**14 C**	NR	NR	R	NR		
47	**188 C**	NR	NR	R	NR		
48	**401 C**	NR	NR	R	NR		
Total		**R=8**	**R=6**	**R=43**	**R=8**	**5**	**3**

3.4.3 Kinetic inhibition of ureases by different honey extracts

The results of anti-urease activity of honey extracts isolated using petroleum ether, hexane and chloroform against optimised urease activity cocktail revealed that honey possess compounds of significance importance and the presence of these compounds in honey seem to be influenced by honey type hence floral origin. With the exception of petroleum extract of Siyakholwa pure honey and chloroform extract of Little Bee honey, all the honey extracts under study revealed a level of urease activity inhibition ranging from 0.8 % to a potent level of 67.9 %. At a concentration of 50mg/ml, petroleum ether extract of Gold Crest had inhibition percentage (I%) between 50.9% – 53.3% and that of Fleures Honey was between 67.8%–68.5%, hexane extract of Little Bee recorded an inhibition percentage (I%) between 51.4% – 52.7% and that of Manuka Honey was between 50.0% – 53.2%, lastly chloroform extract of Bush Honey recorded an inhibition percentage (I%) between 63.8% – 66.1% and inhibition percentage of Q Bee was between 64.2%–66.2%, all showing an inhibition percentage range ≥ 50%. (Table 3.2–3.4).

CJBU, HPU1, HPU2 and HPU3 were factors introduced so as to determine whether the source of urease plays a crucial role in urease inhibition by honey extracts. The findings showed that source of urease did not play a crucial role in influencing urease inhibition by anti-urease active compounds ($P > 0.05$). CJBU, HPU1, HPU2 and HPU3, all presented a similar inhibition trend when exposed to similar inhibitory conditions e.g. considering Table (3.2), CJBU, HPU1, HPU2 and HPU3 recorded an inhibition percentage of 20.8%, 22.1%, 21.8% and 22.3% respectively when exposed to the urease kinetic cocktail spiced with petroleum ether extract of Bush Honey.

Table 3. 2: Mean ($\bar{x}$) inhibition percentage (I%) of urease by petroleum ether extracts (50mg/mL) of different honey types

	Bush Honey	**Raw Honey**	**Gold Crest Honey**	**Q Bee honey**	**Little Bee Honey**	**Fleures Pure Honey**	**Siyakholwa Pure honey**	**Manauka Honey**	**Standard inhibitor**	**P value**
CJBU	20.8 %	20.8 %	50.9 %	1.9 %	15.1 %	67.9 %	0.0 %	22.6 %	98.2 %	**0.033**
HPU1	22.1 %	18.1 %	53.3 %	0.8 %	16.2 %	68.5 %	0.0 %	23.3 %	100%	**0.017**
HPU2	21.8 %	23.4 %	51.7 %	1.5 %	15.8 %	68.3 %	0.0 %	22.8 %	99.8 %	**0.041**
HPU3	22.3 %	20.9 %	51.7 %	1.5 %	15.5 %	67.8 %	0.0 %	22.7 %	97 %	**0.029**
P value	**0.562**	**0.467**	**0.165**	**0.739**	**0.216**	**0.625**		**0.391**	**0.106**	

Key: **CJBU** - Commercial Jack bean urease

HPU1 - Urease extracted from *H. pylori* susceptible to four drugs (clarithromycin, amoxicillin, metronidazole and tetracycline)

HPU2 - Urease extracted from *H. pylori* resistant to clarithromycin

HPU3 - Urease extracted from multidrug resistant *H. pylori*

Table 3. 3: Mean ($\bar{x}$) inhibition percentage (%) of urease by Hexane extracts (50mg/mL) of different honey types

	Bush Honey	**Raw Honey**	**Gold Crest Honey**	**Q Bee honey**	**Little Bee Honey**	**Fleures Pure Honey**	**Siyakholwa Pure honey**	**Manauka Honey**	**Standard inhibitor**	**P value**
CJBU	34.5 %	20.8 %	25.9 %	39.7 %	51.7 %	25.9 %	10.3 %	50.0 %	100 %	**0.022**
HPU1	37.2 %	18.1 %	22.6 %	36.9 %	51.4 %	27.2 %	11.2 %	53.2 %	98.6 %	**0.019**
HPU2	37.6 %	23.4 %	25.1 %	40.1 %	52.7 %	25.8 %	10.5 %	51.0 %	96 %	**0.031**
HPU3	36.4 %	20.9 %	24.5 %	39.4 %	51.6 %	26.0 %	10.7 %	50.6 %	99.3 %	**0.041**
P value	**0.239**	**0.518**	**0.753**	**0.491**	**0.217**	**0.164**	**0.092**	**0.152**	**0.155**	

Key: **CJBU** - Commercial Jack bean urease

HPU1 - Urease extracted from *H. pylori* susceptible to four drugs (clarithromycin, amoxicillin, metronidazole and tetracycline)

HPU2 - Urease extracted from *H. pylori* resistant to clarithromycin

HPU3 - Urease extracted from multidrug resistant *H. pylori*

Table 3. 4: Mean ($\bar{x}$) inhibition percentage (%) of urease by Chloroform extracts (50mg/ml) of different honey types

	Bush Honey	**Raw Honey**	**Gold Crest Honey**	**Q Bee honey**	**Little Bee Honey**	**Fleures Pure Honey**	**Siyakholwa Pure honey**	**Manauka Honey**	**Standard inhibitor**	**P value**
CJBU	64.2 %	9.4 %	37.7 %	64.2 %	0.0 %	13.2%	5.6 %	15.1 %	98 %	**0.029**
HPU1	63.8 %	11.4 %	38.2 %	65.7 %	0.0 %	14.0 %	5.3 %	16.0 %	99 %	**0.013**
HPU2	66.1 %	8.5 %	35.8 %	64.9 %	0.0 %	13.8 %	6.1 %	16.3 %	98 %	**0.044**
HPU3	64.5 %	9.1 %	36.3 %	66.2 %	0.0 %	13.5 %	5.8 %	16.5 %	100 %	**0.037**
P value	**0.208**	**0.271**	**0.386**	**0.537**		**0.211**	**0.097**	**0.385**	**0.140**	

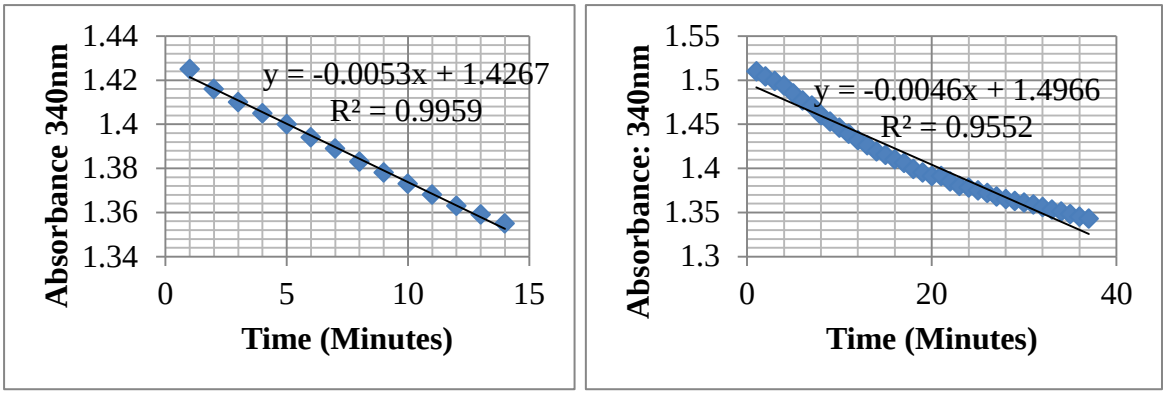
Key: **CJBU** - Commercial Jack bean urease

HPU1 - Urease extracted from *H. pylori* susceptible to four drugs (clarithromycin, amoxicillin, metronidazole and tetracycline)

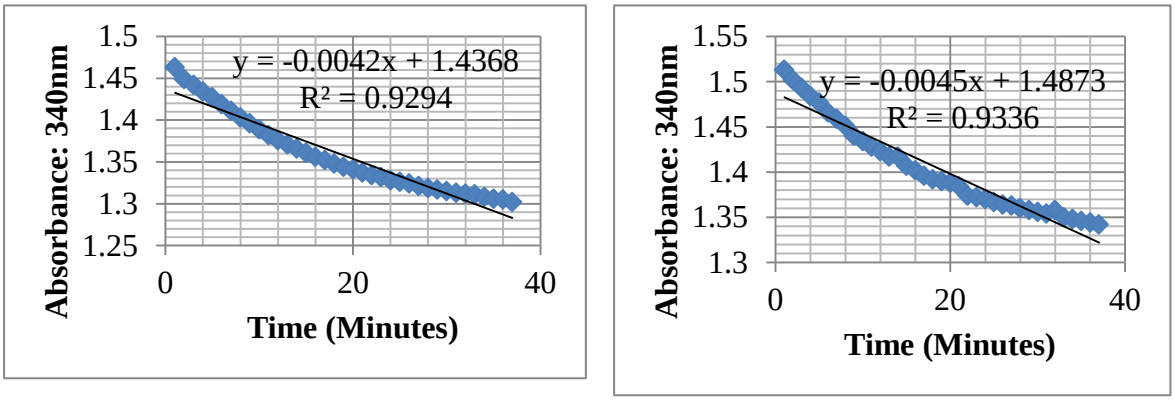
HPU2 - Urease extracted from *H. pylori* resistant to clarithromycin

HPU3 - Urease extracted from multidrug resistant *H. pylori*

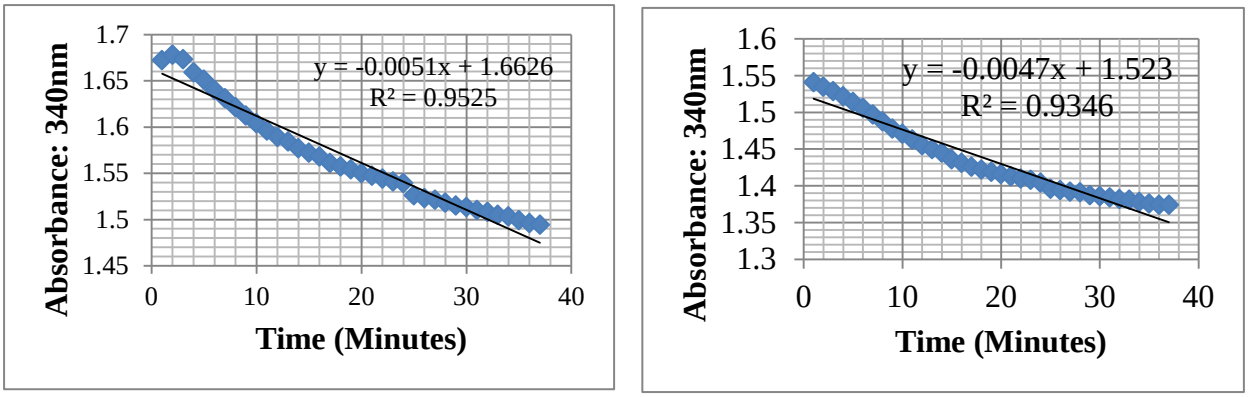
The change in absorbance at 340nm with respect to time was linear for the assay control of this experiment with rate of change being -0.0053 (Figure 3.3 (a)). After 14 minutes of the kinetic study of urease reaction, NADH reduction absorbance was between 1.34 and 1.36. However it took between 35 minutes and 40 minutes for the urease enzyme to reduce NADH to a value closer to the one recorded in the assay control when the reaction cocktail was spiked with honey extracts (Figure 3.3 (a) – (f)). The effects of spiking honey extracts into the assay cocktail seem to follow a similar trend, at 10 minutes of kinetic monitoring, the gradient of the line of best fit and NADH reduction pattern seem to be having same gradient. After 10 minutes of reaction monitoring, the actual NADH reaction continues at a faster rate and then slows down. At around 30 minutes after the commencement of the reaction, the actual NADH reduction and the line of best fit become equal with the line of best fit after which the actual rate of change slows down to almost zero as it gets nearer to 40 minutes.



(a) Control: No inhibitor (b) Petroleum extract of Gold Crest



(c) Petroleum ether extract of Fleures honey. (d) Chloroform extract of Little bee honey



(e) Hexane extract of Manuka honey (f) Chloroform extract of Bush honey extract.

Figure 3. 3: Reduction of NADH with time due to urease activity in the presence of honey solvent extracts

3.5 DISCUSSION

H. pylori is recognised as a class 1 carcinogen infecting close to half of the world's population (Goh *et al*., 2011; Awuku *et al*., 2017). Several therapies have been formulated worldwide in an effort to carb it's spread and chronic infection. As *H. pylori* lacking urease fail to inhabit the acidic human stomach (Amin *et al*., 2010), it is ideal to focus on urease inhibitors as a control strategy for *H. pylori* infections. Overall, the current study is revealing that honey possess phytochemicals with anti-urease properties. Eight honey types (Bush honey, Raw honey-unheated, Gold Crest honey-pure, Q Bee honey, Little Bee honey, Fleures-radurised honey, Siyakholwa honey and Manuka honey) were used as source of extracts for the urease kinetics inhibition study. While Manuka honey from New Zealand has been well documented for its highly rated bioactive compounds, South African produced honey has challenged that aspect, with five solvent extracts of South African honey origin attaining an inhibition percentage (I%) greater or equal to 50% which is not significantly different to that obtained by Manuka honey, i.e. inhibition percentage (I%) of 50% ($p > 0.05$). The antibacterial activity of honey has been reported as both physical and chemical aspects of its nature i.e. acidity, osmolality, beeswax, nectar, pollen, propolis, volatiles, hydrogen peroxide and proteins (Weston *et al*., 2000; Mandal and Mandal, 2011).

The effect of origin of urease was also studied as a factor that could determine inhibition resistance of urease to honey active compounds. Commercial Jack bean urease (CJBU), *H. pylori* susceptible to four drugs urease (clarithromycin, amoxicillin, metronidazole and tetracycline-HPU1), *H. pylori* resistant to clarithromycin urease (HPU2) and multidrug resistant *H. pylori* urease (HPU3) were extracted from respective strains. On all the experiments performed, there was a similar trend on the inhibition of the different ureases. Inhibition percentage of the control (CJBU) was not significant different from those of other

ureases ($p > 0.05$). The findings suggest that the honey active compounds from the solvent extracts had a similar inhibitory effect on urease irrespective of the source of origin, supporting a statement by Sirko and Brodzik (2000) that ureases from different sources (plants, bacteria, invertebrates and fungi) have similar structures an indication that they might be inhibited by similar compounds.

Nowadays, drug resistance is becoming a big problem to commonly used treatment regimens, therefore the fact that irrespective of source of urease, including urease extracted from drug resistant *H. pylori*, the trend on urease inhibition seemed to be similar. This finding marks an important aspect of the study in the sense that it brings a new inference about targeting urease inhibition as a way to treat infections caused by urease-depended micro-organisms such as *H. pylori*. These data suggest additional research is needed to target the urease enzyme in an effort to offset colonisation of the human stomach by urease-depended *H. pylori*. Such a discovery could probably help surpass drug resistance treatment challenges being observed with current treatment regimes.

3.6 CONCLUSIONS

The findings if this study revealed that:

1. Geographical location from which honey is obtained seems to influence the inhibitory potential of honey against urease activity.
2. The petroleum ether extract of Fleures honey and Gold Crest honey, and the chloroform extract Q Bee honey, all South African honey types are potential candidates of being new source of urease inhibitors.

3. Irrespective of source of urease, urease extracted from drug susceptible *H. pylori* strains and drug resistant *H. pylori* strains seem to be inhibited by same compounds.

CHAPTER FOUR

Detection of cytotoxin-associated gene a (*cag*A) and vacuolating cytotoxin a (*vac*A) gene among 48 clinical isolates of *H. pylori*

4.1 ABSTRACT

Helicobacter pylori (*H. pylori*) infection is associated with various upper gastrointestinal tract (GIT) disorders. Various virulent factors are associated with the successful colonisation of the GIT by this pathogen. The aim of this study was to screen for *cag*A and *vac*A genes among 48 clinical samples of *H. pylori* previously isolated from gastric biopsy specimen of patients with upper gastrointestinal problems. Polymerase chain reaction (PCR) was carried out with specific primers to confirm isolates by targeting the *glm*M gene, and detect the presence of *cag*A gene and *vac*A gene t in *H. pylori* strains. All the isolates were confirmed as *H. pylori* and *cag*A was detected in 97.9% of the test isolates. The *vac*A allelic combination *s*1*m*1 was detected in 75% of the test isolates and *s*1*m*2 allelic combination was in 16.7% of the test isolates while *s*2*m*2 was in 8.3% of the test isolates. The *vac*A *s*2*m*1 allelic combination was not detected in any of the test isolates. In conclusion, the test isolates of *H. pylori* under study possess genes associated with upper gastrointestinal problems.

4.2 INTRODUCTION

Helicobacter pylori (*H. pylori*) is a medically important bacterium that expresses resistance to antibiotics in addition to a variety of virulence factors (Tanih *et al*., 2010; Cogo *et al*., 2011; Harrison *et al*., 2017), this eventually leads to therapy failure and serious disease manifestations due to infection. As a class-1 carcinogen, this organism infects the gastric mucosa of more than half of the world's adult population (Ndip *et al*., 2004; Ahmed *et al*., 2006). There is strong evidence that infection with the organism is the main cause of the peptic ulcers, gastritis and the development of adenocarcinomas of the stomach and primary gastric mucosa-associated lymphoid tissue lymphoma (Warren and Marshal, 1983; Oleastro *et al*., 2006; Braganca, 2007; Chey *et al*., 2017), thus being known as the main instrumental factor resulting in gastric cancer (Al-Marhoon *et al*., 2004). Animal studies have demonstrated that *cag*A plays an important role in disease progression in a Mongolian gerbil model where gastric cancer develops within 12 weeks (Peek Jr. *et al*., 2000; Franco *et al*., 2008).

H. pylori genome was fully sequenced in 1997 (Tomb *et al*., 1997; Alm *et al*., 1999), resulting in the follow up of more studies on the biology, pathology, and immunology of *H. pylori* infection. The clinical outcome of long-term infection is subjective and is depended on the infecting bacterial strains' virulence factors (Wang *et al*., 2003; Jones *et al*., 2010; Miernyk *et al*., 2011) and host genotype. *H. pylori* secrete many proteinaceous factors that help the bug during initial colonization and successive persistence which can be life-long without effective treatment (Aguemon *et al*., 2005; Dube *et al*., 2009). However, there are two putative bacterial markers for virulence associated with the pathogenesis of *H. pylori* infection, the *cag* A gene and the *vac* A gene (Achtman *et al*., 1999; Palframan *et al*., 2012;

Zaki *et al*., 2016a). These two virulence factors are polymorphic and affect different host cellular pathways.

The *cag*A gene, encodes a 125 to 145 kDa protein (*cag*A), a marker for the presence of the cytotoxin-associated gene pathogenicity island (*cag*PAI) that is associated with an increase in intensity of gastric inflammation. *Cag*A, an oncoprotein is an abundant protein produced by *H. pylori* (Jimenez-Soto and Haas, 2016; Zaki *et al*., 2016a). The presence of the *cag*A gene is an indicator for the presence of all the genes found in the *cag*PAI including *cag*T, *cag*M and *cag*E. The *cag*PAI family gene is responsible for the bacterial type IV secretion system, which injects *cag*A into the cytosol of gastric epithelial cells of host (Jones *et al*., 2010; Jimenez-Soto and Haas, 2016). The bacterial type IV secretion system, translocate DNA and protein substrates to other target cells by a mechanism that depends on direct cell-cell contact. The subfamily of the effector translocators, are used by Gram negative bacteria (*H. pylori*) to deliver virulence proteins to host cells for modulation of different physiological processes during infection (Christie *et al*., 2013). In an unphosphorylated state, once in host cell, *cag*A can directly influence cellular tight junction, cellular polarity, cell proliferation and differentiation, cell scattering, induction of inflammatory response as well as cell elongation (Jones *et al*., 2010). In addition, in the host cell *cag*A localizes to the plasma membrane where it can be phosphorylated by either Abl kinase or Src family kinases.

The *vac*A gene encodes the production of the vacuolating cytotoxin. The cytotoxin has been shown to cause vacuolation of epithelial cells *in vitro* and promote epithelial cell damage and mucosal ulceration in mice (Telford *et al*., 1994; Leunk *et al*., 1999; Miernyk *et al*., 2011). The *vac*A is one of the proteins secreted from the bacteria through the type V auto-transport

secretion system. The V-secretion system is important for the Gram-negative bacteria either for the secretion of virulent factors for pathogens or by secreting factors contributing to the survival of non-invasive environmental micro-organisms (van Ulsen *et al*., 2013). *Vac*A is an 88kDa toxin comprising of p33 and p55 subunits. The *vac*A toxin is believed to bind to host cells and internalised causing severe vacuolation characterized by the accumulation of large vesicles that possess hallmarks of both late endosomes and early lysosomes. The anion-selective channels seem to facilitate the transport of chloride ions resulting in increased intra-lumenal chloride concentrations (Cover and Blanke, 2005). Apart from vacuolating effects of *vac*A, it has been hypothesized that *vac*A can form membrane-embedded pores at the inner-mitochondrial membrane resulting in the degeneracy of the mitochondrial electrochemical membrane potential (Palframan *et al*., 2012).

The *vac*A gene is found in almost all *H. pylori* strains, however not all infections of *H. pylori* result in cell vacuolation *in-vitro,* earmarking sequence heterogeneity of this gene (Cogo *et al*., 2011; Palframan *et al*., 2012). This gene has two variable regions, the *s* region and the *m* region. The s region encodes the signal peptide which exists as two alleles, the *s*1 or the *s*2. The *s*1 type can be re-subtyped into *s*1a, *s*1b, and *s*1c (Miernyk *et al*., 2011). The *m* region is located in the middle region of the gene and consists of the *m*1 or *m*2 alleles. The *m*1 type can be further subtyped into *m*1a and *m*1b (Miernyk *et al*., 2011). Bacterial strains possessing allelic combination s1*m*1 produce high levels of vacuolating cytotoxins while those with allelic combination s1*m*2 produce moderate levels. Interestingly, the *s*2 type encodes a *vac*A protein with an N-terminal hydrophilic amino acid extension which does not influence cell vacuolation (Letley *et al*., 2003; Miernyk *et al*., 2011), hence allelic combination *s*2*m*2 has little or no vacuolating cytotoxin effect on host cells (Miernyk *et al*., 2011; Arevalo-Galvis *et al*., 2012). *Cag*A, an oncoprotein, closely associated with presence of *s*1 (Atherton *et al*.,

1995). Recently, virulent strains of *H. pylori* that express *cag*A, have been shown to be significantly associated with coronary artery disease (Pietroiusti *et al*., 2002; Kowalski *et al*., 2006).

4.3 MATERIALS AND METHODS

4.3.1 Standard strains

Standard strains of *H. pylori* including X47 (*cag*A positive), J99 (*vac*A *s*1*m*1 positive) and Tx30a (*s*2*m*2) were supplied by Professor Rainer Haas (Ludwig-Maximilians-Universit*ä*t M*ü*nchen) in Munich, Germany.

4.3.2 DNA isolation

DNA was isolated from the clinical isolates as well as the standard strains by the boiling method as previously described in section 3.3.2.

4.3.2 Polymerase Chain Reaction (PCR)

PCR reaction was performed directly on DNA obtained from presumptive pure colonies by boiling method as explained in Chapter 3. The extracted DNA was used as a template in each polymerase chain reaction (PCR) to target the *glm*M, *cag*A, *vac*A *s1*, *vac*A *s2*, *vac*A *m1* and *vac*A *m2* using a set of primers supplied by Inqaba Biotec Industries (Pty) Ltd. A 25µL PCR reaction was set up comprising of 12.5 µL of 2X matermix (New England Biolabs, Ipswich, United Kingdom), 6.6 mL nuclease free water (New England Biolabs), 0.5 µL of each primer (Inqaba Biotec, Pretoria, South Africa), and 5 µL of template DNA. Amplification was carried out using a Bio-Rad thermal cycler (C1000 TouchTM Cycler). The following cycling

parameters were used: initial denaturation at 95˚C for 5 min, followed by 40 cycles of 95˚C for 1 min, 54˚C for 1 min and 72˚C according to amplicon length (1 min per 1000 bp). This was followed by final elongation for 5 min. The PCR products were resolved in a 1.5% (wt/vol) agarose gel containing 5 µL of ethidium bromide (Sigma-Aldrich, St Louis, MO). Electrophoresis was done in a 0.5X TAE buffer (40 mM Tris-HCL, 20 Mm Na-acetate, 1 mM EDTA, pH 8) for 40 min at 100 volts. Visualisation was done under a UV transilluminator (Alliance 4.7 XD-79, Uvitec, Cambridge, United Kingdom) in parallel with molecular size makers, GeneRulerTM 100bp DNA Ladder (Fementas).

Figure 4. 1: List of target genes, primers and amplicon size

Target gene	**Forward primer**	**Reverse primer**	**Amplicon size**	**Reference**
*glm*M	GATAAGCTTTTAGGGGTGTTAGGGG	GCATTCACAAACTTATCCCCAATC	140 bp	Abu-ALmaali *et al*., 2012
*Cag*A	ACCGCTCGAGAACCCTAGTCGGTAATGGG	CAGGTACCGCGGCCGCTTAAGATTTTT	981 bp	Smith *et al*., 2004
*Vac*A *s*1	CTGCTTGAATGCGCCAAAC	ATGGAAATACAACAAACACAC	259 bp	Atherton *et al*., 1995
*Vac*A *s*2	CTGCTTGAATGCGCCAAAC	ATGGAAATACAACAAACAC	286 bp	
*Vac*A *m*1	GGTCAAAATGCGGTCATGG	CCATTGGTACCTGTAGAAAC	290 bp	
*Vac*A *m*2	CATAACTAGCGCCTTGCAC	GGAGCCCCAGGAAACATT	352 bp	

4.3 RESULTS

4.3.1 Prevalence of *cag*A and *vac*A

Of the 48 clinical samples used in this study, 47 samples (97.9%) were *cag*A positive. The *vac*A gene was also detected, 36 of the 48 isolates (75%) possessed *s*1*m*1 allelic combination, 8 of the 48 clinical samples (16.7%) possessed *s*1*m*2 allelic combination, no clinical sample possessed *s*2*m*1 allelic combination and 4 of the 48 clinical samples (8.3%) had *s*2*m*2 allelic combination (Table 4.3).

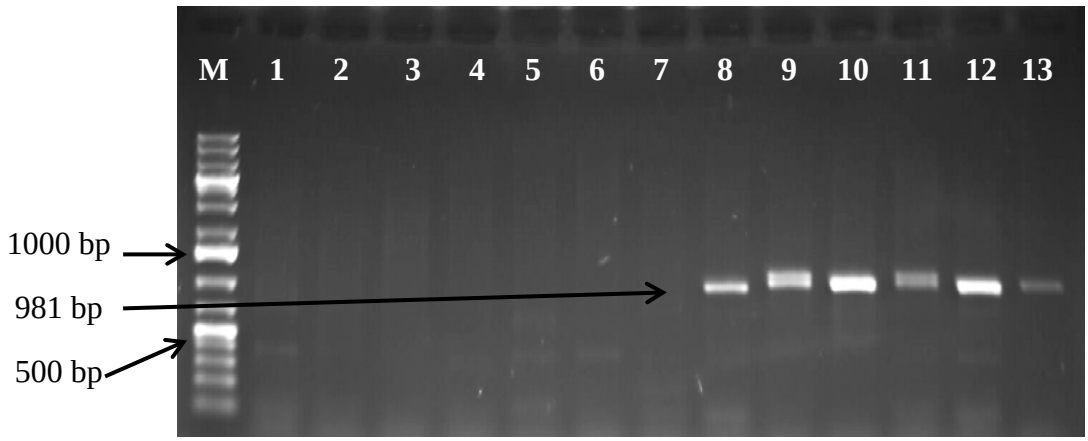


Figure 4. 2: Agarose gel electrophoresis photograph showing specific detection of 981 bp *cag*A gene. M-100 bp molecular weight marker, Lane 1: negative control; Lanes 2- 7: negative isolates; Lanes 8-12 positive isolates; Lane 13: positive control.

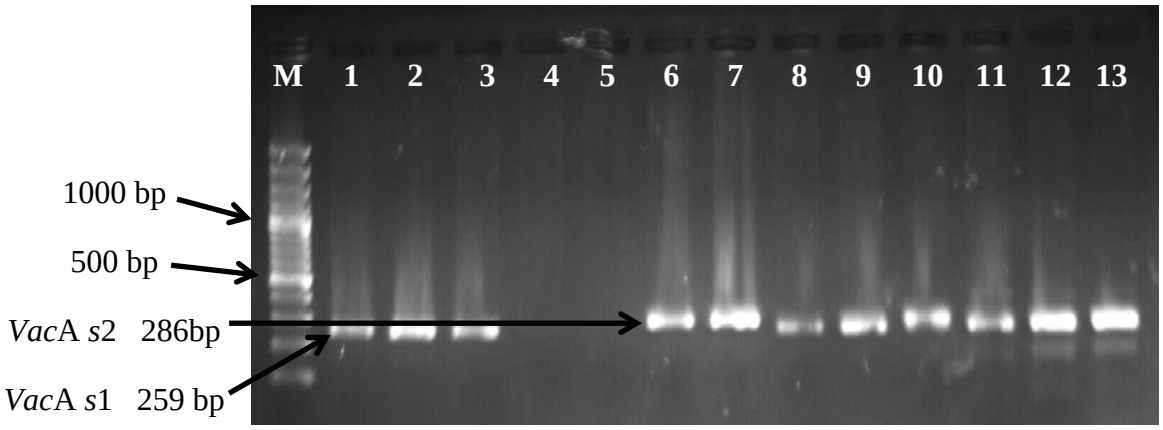


Figure 4. 3: Agarose gel electrophoresis photograph showing specific detection of 259bp *vac*A *s*1 and 286 bp *vac*A *s*2 genes. M- 100bp molecular weight marker, Lanes 1-3; 8-12: *s*1 positive isolates; Lane 6-7; *vac*A *s*2 positive isolates; Lane 4: negative control *vac*A *s*1

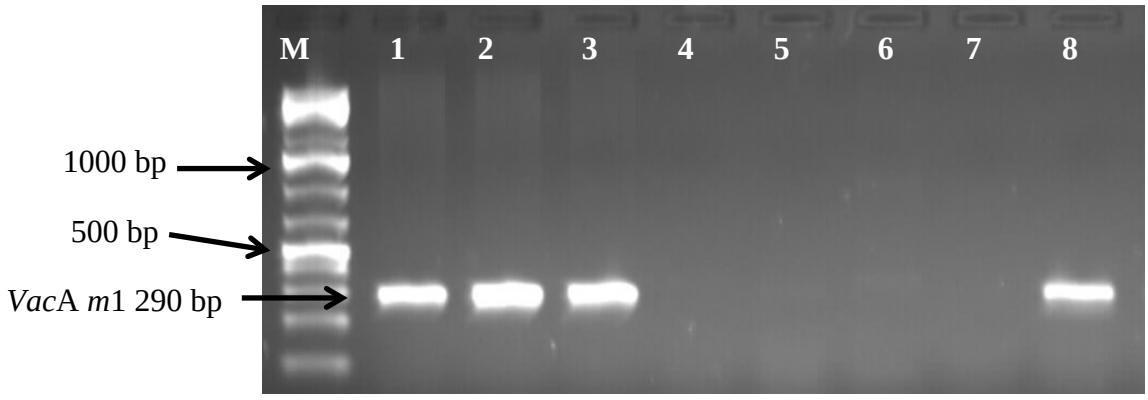


Figure 4. 4: Agarose gel electrophoresis photograph showing specific detection of 290 bp *vac*A m1 gene. M- 100 bp molecular weight marker, Lane 1-3: positive isolates; Lane 4-7: negative isolates; Lane 8: positive control

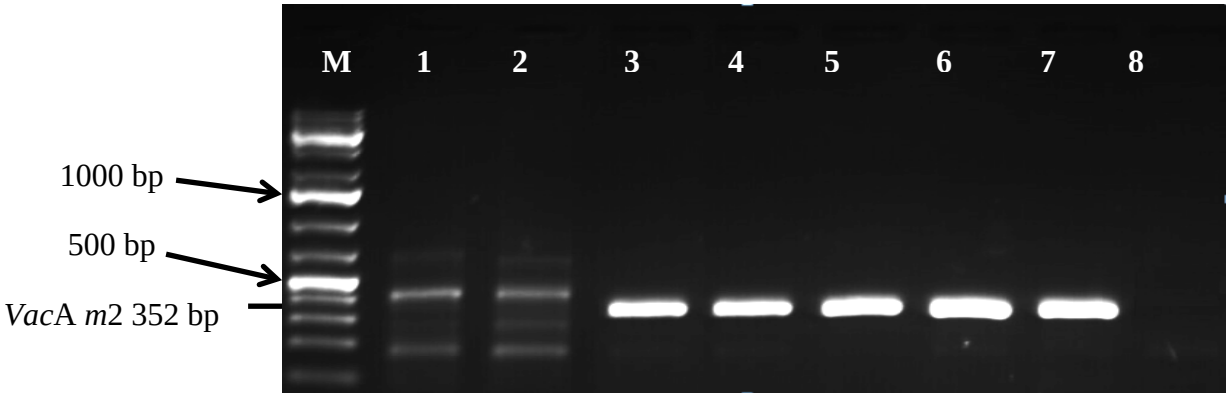


Figure 4. 5: Agarose gel electrophoresis photograph showing specific detection of 352 bp *vac*A *m*2 gene. M- 100 bp molecular weight marker, Lane 1-2: unspecified amplicons, Lanes 3-6: positive isolates; Lane 7: Positive control; Lane 8: negative control

Table 4. 1: Presence of *cag*A and allelic variants of *vac*A of *H. pylori* strains obtained from patients with upper gastrointestinal problems

			***Vac*A**				***Vac*A allelic combinations**			
Strain		***Cag*A**	**s1**	**s2**	**m1**	**m2**	**s1m1**	**s1m2**	**s2m1**	**s2m2**
1	10 A	+	+	-	+	-	✓			
2	467A	+	+	-	+	-	✓			
3	56 A	+	+	-	+	-	✓			
4	430 A	+	+	-	+	-	✓			
5	96 A	+	+	-	-	+		✓		
6	155 A	+	+	-	+	-	✓			
7	169 A	+	-	+	-	+				✓
8	11 A	+	+	-	-	+		✓		
9	402 A	+	+	-	+	-	✓			
10	469 A	+	+	-	+	-	✓			
11	265 A	+	+	-	+	-	✓			
12	63 A	+	+	-	+	-	✓			
13	87 A	+	+	-	+	-	✓			
14	436 A	+	+	-	+	-	✓			
15	369 A	+	+	-	-	+		✓		
16	464 A	+	+	-	+	-	✓			
17	25 A	+	+	-	+	-	✓			
18	47 A	+	+	-	+	-	✓			
19	93 C	+	+	-	+	-	✓			
20	219 C	+	+	-	+	-	✓			
21	406 C	+	+	-	+	-	✓			
22	411 C	-	+	-	-	+		✓		
23	467 C	+	+	-	+	-	✓			
24	13 C	+	-	+	-	+				✓
25	32 C	+	+	-	+	-	✓			
26	105 C	+	+	-	+	-	✓			
27	212 C	+	+	-	+	-	✓			
28	92 C	+	+	-	+	-	✓			
29	300 C	+	+	-	+	-	✓			
30	264 C	+	-	+	-	+				✓
31	41 C	+	+	-	-	+		✓		
32	405 C	+	+	-	+	-	✓			
33	10 C	+	+	-	+	-	✓			
34	464 C	+	+	-	+	-	✓			
35	213 C	+	+	-	-	+		✓		
36	350 C	+	+	-	+	-	✓			
37	98 C	+	+	-	+	-	✓			
38	111 C	+	+	-	+	-	✓			
39	404 C	+	+	-	-	+		✓		
40	53 C	+	+	-	+	-	✓			
41	77 C	+	+	-	+	-	✓			
42	317 C	+	+	-	+	-	✓			
43	299 C	+	+	-	+	-	✓			
44	162 C	+	-	+	-	+				✓
45	430 C	+	+	-	-	+		✓		
46	14 C	+	+	-	+	-	✓			
47	188 C	+	+	-	+	-	✓			
48	401 C	+	+	-	+	-	✓			
	X47	+								
	J99		+		+					
	Tx30a			+		+				
Total Positive		**47**	**44**	**4**	**36**	**12**	**36**	**8**	**0**	**4**
% Total		**97.9%**	**91.7%**	**8.3%**	**75%**	**25%**	**75%**	**16.7%**	**0%**	**8.3%**

4.4 DISCUSSION

H. pylori infection is common in South Africa (Letley *et al*., 1999; Samie *et al*., 2007; Dube *et al*., 2009; Tanih *et al*., 2010). Drug resistant strains, *cag*A positive strains and *vac*A positive strains among those equipped with the most virulence genes have been reported among *H. pylori* strains circulating in South Africa (Letley *et al*., 1999; Tanih *et al*., 2013). This poses a serious health concern if attention is not given to drug resistant pattern and virulence genes of *H. pylori* that are circulating in South Africa.

There are studies that have associated the risk of *cag*A as an oncogen and *vac*A as a vacuolating cytotoxin (Cogo *et al*., 2011; Miernyk *et al*., 2011; Harrison *et al*., 2017). This study shows that *cag*A genes (97.9%) and *vac*A *s*1*m*1 allelic combinations (75%) were frequent among strains of *H. pylori* affecting the study population. This is not surprising since the study population was entirely patients who were undergoing endoscopic examination for upper gastrointestinal problems. *Vac*A allelic combination *s*2*m*1 was not detected in the *H. pylori* strains circulating in the study population however even if *s*2*m*2 was detected, it is less common with 8.3% prevalence. The pattern of existence of these virulence genes seem to be influenced by the study population. Since, this study was done after years of sample collection, it was difficult to obtain all the patient information such as disease manifestations, hence limitations of the study to link virulence genes detected with disease manifestations. However, this study was a success in regard to the study focus of the research. We managed to obtain the drug profile of the resuscitated strains of *H. pylori* as well as the virulence genes possessed by *H. pylori* strains, the necessary findings of the next phase of the study.

4.4 CONCLUSION

*Cag*A and *vac*A (*s*1*m*1) genes are highly frequent among *H. pylori* strains infecting patients with gastrointestinal problems. However, further studies are needed to link virulent genes of *H. pylori* including subtypes, to the disease manifestations in study population due to specific *H. pylori* strain infection. In addition, the correlation between infecting bacterial genotypes in terms of *cag*A and *vac*A in relation to patients' gastrointestinal problems contributes to earlier findings in the understanding of disease manifestations associated with *H. pylori* infections.

CHAPTER FIVE

Assessment of urease inhibition effect on survival of drug-resistant, *vacuolating cytotoxin* gene-A and *cytotoxin-associated* gene-A positive *H. pylori* strains under acidic conditions.

5.1 ABSTRACT

H. pylori produce large quantities of urease to neutralize the acidic environment of the human stomach during colonization. *H. pylori* lacking urease has been shown to fail to cause gastritis in animals. Highly pathogenic organisms possess *cag*A, an oncoprotein and *vac*A genes. Drug resistance is now common against *H. pylori* regimens currently used, and this is becoming a health concern especially in communities associated with the prevalence of highly pathogenic strains of *H. pylori*. Information gathered in ealier chapters of this work was used to categorise clinical isolates as drug-resistant, *cag*A positive and *vac*A positive. Agar well diffusion was used to determine zone of inhibition of honey extracts with anti-urease activity against *H. pylori* growth at pH of 7 and clarithromycin (0.5mg/L) was used as a standard drug. Urease inhibition studies were done by culturing *H. pylori* strains in Brain Heart Infusion Broth (BHIB) adjusted to pH of 2, 3, 4, 5, 6 and7. Strain MP01 was used as a standard urease negative strain while X47 and J99 were used as positive standards for *cag*A and *vac*A *s*1*m*1 respectively. The data generated from this study reveal that anti-urease active compounds in honey have the potential to offset the normal growth of *H. pylori* strains that are *cag*A positive, *vac*A positive and drug resistant under acidic conditions.

5.2 INTRODUCTION

Helicobacter pylori (*H. pylori*) is a spiral shaped, microaerophilic and Gram-negative microorganism that colonizes the highly acidic human stomach (Allen., 2000; Lu *et al*., 2005; Tanih *et al*., 2010), an environment unsuitable for the survival of most micro-organisms. Since the discovery of *H. pylori* in the human stomach (Marshall and Warren, 1984), many studies have linked this organism as a causative agent of benign and malignant gastrointestinal diseases (Seck *et al*., 2009; Singh *et al*., 2009; Tanih *et al*., 2010; Harrison *et al*., 2017).

Pathogenicity of *H. pylori* is associated with the display of *cag*A gene and *vac*A genes (Faundez *et al*., 2002; Jones *et al*., 2010; Zabaleta, 2012; Zaki *et al*., 2016a). The *cag*A gene is a marker for the presence of the *cag*PAI (Covacci *et al*., 1993; Backert *et al*., 2010). The *cag*PAI family gene is responsible for the type IV secretion system, which transports *cag*A positive strains into the cytosol of gastric epithelial cells (Viala *et al*., 2004; Jones *et al*., 2010). In an un-phosphorylated state, once *cag*A is inside host cells, it influences the induction of inflammatory response (Brandt *et al*., 2005), cell proliferation and differentiation (Murata-Kamiya *et al*., 2007; Lee *et al*., 2010), cell scattering, cellular tight junction (Oliveira *et al*., 2009), and cellular polarity (Zeaiter *et al*., 2008). In addition, *cag*A has been shown to be localized in the plasma membrane where it is phosphorylated by Src or Abl kinases (Selbach *et al*., 2002; Tammer *et al*., 2007). *Cag*A, an oncoprotein is closely associated with the presence of *s*1 allele of the *vac*A gene (Artherton *et al*., 1995). Presence of this gene therefore promotes intensified gastric inflammation with serious clinical outcomes which can result in severe damage to the gastric mucosa.

*Vac*A, is a toxin that induces vacuoles in gastric epithelial cells, as well as other systemic changes such as induction of apoptosis in gastric epithelial cells (Cover *et al*., 2003) and inhibition of proliferation and IL-2 secretion by T cells (Gebert *et al*., 2003). The *vac*A gene is present in all *H. pylori* strains (Pereira *et al*., 2014), The four regions of *vac*A, the signal (*s*)-, intermediate (*i*)-, middle (*m*)-, and deletion (*d*)- regions found as either type 1 or 2 (Palframan *et al*., 2012; Bakhti *et al*., 2015 ; Hashinaga *et al*., 2016; Trang *et al*., 2016). Studies have shown that the mosaic combination of the alleles from both regions contribute to variations in the vacuolating activity of different *H. pylori* strains with strains possessing *s*1/*m*1 combination being the most cytotoxic followed by the *s*1/*m*2 strains and the *s*2/*m*2 (Atherton *et al*., 1995, Van Doorn *et al*., 1998; Cogo *et al*., 2011; Miernyk *et al*., 2011; Palframan *et al*., 2012). In addition, all strains possessing s1m1i1 and s1m2i1 combinations are vacuolating and more virulent as compared to the strains possessing the combinations s1m2i2 and s2m2i2 (Trang *et al*., 2016). Also, the strains with d1/c1 combination are linked with the production of vacuolating cytotoxin genes (s1m1i1) while the combination d2c2 is linked to failure to non-vacuolating types *s*2*m*2*i*2 (Trang *et al*., 2016).

However, current drug resistant patterns on *H. pylori* infection are worrying. The ideal antimicrobial therapy should have an eradication success of at least 90% in conjunction with tolerable levels of side effects. Several studies have highlighted high levels of drug resistant to commonly used drugs in the treatment of *H. pylori* (Seck *et al*., 2009; Singh *et al*., 2009; Tanih *et al*., 2010; Fathi *et al*., 2013). The study by Singh *et al*., (2009), involving test samples from patients with signs and symptoms of duodenal or gastric ulcer/gastritis/gastric adenocarcinoma/non ulcer dyspepsia based on endoscopic findings; found that all test isolates were resistant to metronidazole, while 65% were resistant to amoxicillin and 4.7% were resistant to clarithromycin. Another study involving patients visiting endoscopy unit by Tanih

et al., (2010), found that 20% of the test isolates were resistant to clarithromycin and 95.5% of the test isolates were resistant to metronidazole. In Senegal 90% resistance to metronidazole were reported (Seck *et al*., 2009), this was not significantly different to 100% resistance to metronidazole recorded in Egypt (Fathi *et al*., 2013).

H. pylori infect and occupy the acidic human stomach. It has been demonstrated that *H. pylori* releases urease in enormous quantities as a tool to strive in the acidic environment of the human stomach (Amin *et al*., 2010; Amin *et al*., 2013; Modolo *et al.*, 2015). Urease is a nickel-containing metalloenzyme of high molecular weight; the enzyme catalyses the hydrolysis of urea resulting in the formation of ammonia and carbon dioxide. However, there has been a demonstration that urease-negative *H. pylori* mutant strains fail to colonize the host resulting in the development of gastritis (Amin *et al*., 2010). Research has shown that in prolonged unfavourable conditions, *H. pylori* are capable of attaining different morphologies in a non-culturable coccoid state (Atherton., 1997; Fujimura *et al*., 2004).

High prevalence of *H. pylori* infection in developing nations (Samie *et al*., 2007; Dube *et al*., 2009; Tanih *et al*., 2010) in conjunction with increasing drug resistance (Singh et al., 2009; Gościniak *et al*., 2014) and virulence genes *cag*A and *vac*A put the whole world at risk in regard to diseases that occur as a result of *H. pylori* infection. Hence a search for alternative forms of *H. pylori* treatment to current treatment regimens will be of importance. In this study, we therefore focused on inhibition of urease using pre-selected honey extracts that have been demonstrated in an earlier chapter to possess anti-urease activity. Also an acidic growth environment was introduced so as to mimic the acidic human stomach when

determining the consequential inhibition of the growth of *H. pylori* when the urease enzyme is inhibited during culturing of *H. pylori.*

5.3 MATERIALS AND METHODS

5.3.1 *H. pylori* strains

Table 5. 1: List of *H. pylori* strains

Strain	Description	Source	Storage
Clinical isolate 369A	Resistant to clarithromycin, amoxicillin, metronidazole and tetracycline	MPMERG UFH	Stored at -80 ^{o}C
Clinical isolate 219C	Susceptible to clarithromycin, amoxicillin, metronidazole and tetracycline	MPMERG UFH	Stored at -80 ^{o}C
X47	*Cag*A +ve	LMU Munich	Stored at -80 ^{o}C
J99	*Vac*A *s*1*m*1	LMU Munich	Stored at -80 ^{o}C
MP01	Urease –ve	LMU Munich	Stored at -80 ^{o}C

5.3.2 Antimicrobial susceptibility testing of honey extracts at neutral pH

This was done according to Seanego *et al*. (2012), with modifications. In brief, chloroform extract of Bush honey (Inhibition percentage range = 63.8-66.1%) and the chloroform extract of Q bee (Inhibition percentage range = 64.2-66.2%) were selected for the study based on urease inhibition percentage ≥ 50% observed in the previous study (Chapter 3). A colony

from 5 day cultures of strains 369A (Resistant to the four drugs tested), 219C (susceptible to the four drugs tested), X47, J99 and MP01 were taken to represent each bacterial sample.

The bacterial isolates were grown on Muller Hinton Agar (MHA) supplemented with Skirrow's antibiotics and 5% v/v fetal bovine serum with expected pH of 7.3 ± 0.1 at 25^{o}C. Plates were swabbed with sterile swab sticks impregnated with a 0.5 McFarland standard bacterial inoculum. Five wells were bored into the agar medium using sterile 6 mm cork-borer. The first three wells were filled with honey extracts at 50% v/v in respect to the diluent Phosphate buffered saline (PBS) at pH 6. The other two wells were filled with a positive control, clarithromycin (CLR) at 0.5mg/L and a negative control, PBS at pH 6. The plates were kept for 20 minutes prior to incubation to allow diffusion of the solution into the medium. The plates were incubated at 37°C for 5 days and the zones of inhibition were measured for all test organisms. The experiment was replicated three times and zones of inhibition reported as mean ± SD.

5.3.3 Assessment of *H. pylori* growth under acidic conditions

H. pylori strains were initially grown on Columbia Blood Agar supplemented with Skirrow's antibiotics and fetal bovine serum 5% (v/v) at 37^{o}C under micro-aerophilic conditions (85% N_2, 10% CO_2 and 5% O_2 - Helico-Campy Pack gas-generating envelopes). Urease, catalase, oxidase and PCR targeting the *glm*M gene of *H. pylori* were done to confirm presence of the organism.

Fifty milliliters (50mL) of Brain Heart Infusion Broth (BHIB) was prepared in five 100mL bottles; pH was adjusted to 2, 3, 4, 5 and 6, while normal prepared BHIB represented media

with neutral pH. The BHIB was autoclaved, allowed to cool and Skirrow's supplements and 5% fetal bovine serum added to the broth under sterile conditions and stored at 4^{o}C until use.

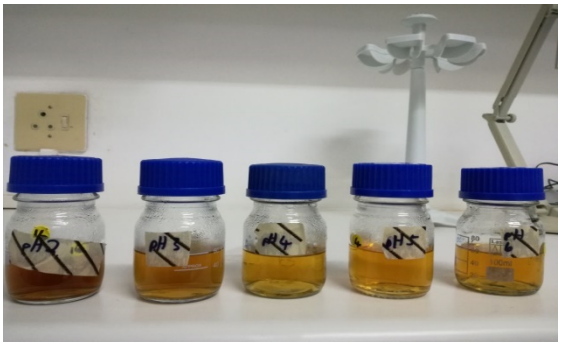


Figure 5. 1 : Broth adjusted to pH 2; 3; 4; 5 and 6

5.3.3.1 Critical pH for urease activation

H. pylori strains, J99 (*s*1*m*1 positive), MP01 (Urease negative mutant strain) and one clinical isolate (369A) where cultured in BHIB supplemented with Skirrows antibiotics and 5% fetal bovine serum with pH ranging from 2-7. Incubation was done at 37^{o}C under micro-aerophilic conditions (85% N_2, 10% CO_2 and 5% O_2 - Helico-Campy Pack gas-generating envelopes). The test medium cocktail consisted of 260µL of BHIB, 10µL of 0.3M urea, 5µL of 0.5 McFarland standard bacterial suspensions in BHIB with respective pH. All the experiments were replicated three times. The blank used was the absobance read at 600nm before incubation which was subtracted from the final absorbance read after an incubation of 3-5 days under micro-aerophilic conditions giving the actual difference in absorbance due to pH change.

5.3.3.2 Inhibition of *H. pylori* under acidic environment

One hundred and forty microliters (140µL) of broth was dispensed into all the wells of the microtiter plate. One hundred and forty microliters of 2X extract solution was pipetted into column 1. Using a multi-pipeter set at 120µL, contents of column 1 were mixed. One hundred and forty microliters of solution from column 1 was withdrawn and pipetted into column 2, mixed and 140µL of solution withdrawn and pipetted into column 3. The procedure was repeated up to column 10 and 140µL of solution from column 10 was discarded. Five microliters of 0.5 McFarland standard bacterial suspensions was pipetted into each well. In addition, 30µL of 0.3M of urea was pipetted into all wells of two different micro-titre plates. After mixing, the micro-titre plates were incubated for 5 days under microaerophilic conditions at 37^{o}C. Optimum density (OD) was read at 600nm. All the experiments were replicated three times. The blank used was the absobance read before incubation which was subtracted from the final absorbance read after an incubation of 5 days under micro-aerophilic conditions giving the actual difference in absorbance due to pH acidity exposure.

Table 5. 2: Micro-titre plate experimental lay-out

	pH2	BL	pH3	BL	pH4	BL	pH5	BL	pH6	BL	pH7	pH8	Strains
	1	2	3	4	5	6	7	8	9	10	11	12	
A													X47
B													
C													J99
D													
E													MP01
F													
G													369A
H													Negative control

5.3.3.3 Ammonium test

The Urease Activity Assay Kit (Sigma Aldrich) was used to determine urease activity. In this assay, the urease enzyme catalyses the hydrolysis of urea resulting in the production of ammonia. The ammonia produced was determined by the Berthelot method resulting in colorimetric product measure at 670 nm, proportionate to the urease activity present I the sample. The estimation of ammonia production/concentration was done using the table below adapted from Urease Activity Assay Kit (Sigma Aldrich) according to the manufacturer's instructions. All the experiments were replicated three times. Actual ammonium produced was determined by subtracting OD_{670} value before incubation (Blank) from OD_{670} value after incubation.

Table 5. 3 : The standards used for estimating ammonia concentration at OD670

	Standard OD_{670}	Ammonia concentration (µM)
A	1.252	500 µM
B	1.109	400 µM
C	0.912	300 µM
D	0.734	200 µM
E	0.536	150 µM
F	0.388	100 µM
G	0.233	50 µM
H	0.056	0 µM

5.3.3.4 Re-culturing of *H. pylori* exposed to acidic and toxic environment

Samples previously cultured for 5 days in growth media with anti-urease compounds were sub-cultured for 5 days to determine biostatic/bactericidal potential of anti-urease active honey extracts. In brief, maintaining steril environment, sterile loops were used to spread bacterial inoculum on Columbia Blood Agar supplemented with Skirrow's antibiotics and fetal bovine serum 5% (v/v) and incubation was done at $37^{o}C$ under micro-aerophilic conditions (85% N_2, 10% CO_2 and 5% O_2 – Oxoid^{TM} AnaeroGen^{TM} gas-generating envelopes, Thermo Fisher Scientific) in gas jars. After 5 days of culturing, observation on *H. pylori* growth was done. Three replications were done for this experiment.

5.4 RESULTS

5.4.1 Antimicrobial susceptibility testing of honey extracts

Table 5. 4 : Zone of inhibition ± SD (mm) of honey extracts and clarithromycin (0.05µg/mL) at pH 7.3 ± 0.1

Honey extracts	Strain	Zone of inhibition (mm) at different concentrations (mg/mL) at pH 7.3 ± 0.1			
		50	100	200	Clarithromycin (0.5mg/L)
Bush honey	X 47	08 ± 0.2	09 ± 0.5	11 ± 0.3	22 ± 0.7
	J99	09 ± 0.7	10 ± 0.2	10 ± 0.5	23 ± 1.3
	MP01	07 ± 0.3	08 ± 0.3	11 ± 0.7	21 ± 1.1
	369A	08 ± 1.2	11 ± 0.6	11 ± 0.8	10 ± 2.6
	219C	09 ± 0.5	09 ± 2.1	10 ± 0.9	22 ± 0.9
Q Bee	X 47	09 ± 0.4	08 ± 2.6	11 ± 0.5	22 ± 0.7
	J99	10 ± 1.9	10 ± 0.4	11 ± 2.2	23 ± 1.3
	MP01	08 ± 0.7	09 ± 0.9	11 ± 1.6	21 ± 1.1
	369A	08 ± 1.8	08 ± 2.7	10 ± 2.4	10 ± 2.6
	219C	08 ± 0.6	09 ± 1.8	11 ± 0.9	22 ± 0.9

The results of susceptibility testing of honey extracts with anti-urease activity (Table 5.4) on growth of *H. pylori* strains indicate that the anti-urease activity of the selected extracts do not effectively inhibit growth of *H. pylori* at neutral pH, the highest inhibition zone recorded was 11 ± 2.2 at 200 mg/L for strain J99. At pH of 7.3, the growth of strain MP01 (mutant devoid of urease) was normal and not different from other strains that are capable of producing urease. The strain 369A is the only one which showed resistance to clarithromycin while strains X47, J99, MP01 and 219C were susceptible to clarithromycin.

5.4.2 Inhibition of *H. pylori* growth under acidic environments

Table 5. 5 : Inhibition of *H. pylori* growth by anti-urease honey extracts at 200mg/mL concentration

	X47 (*Cag*A, Urease +ve)				**J99** (*Vac*A *s*1*m*1, urease +ve)				**MP01**				**CLR RES (369A)**				**B.I.T**	
	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	**N.E.T**	**W.E.T**
pH 7	0.543	**0.267**	0.841	**0.143**	0.577	**0.101**	0.847	**0.149**	0.495	**0.183**	0.811	**0.113**	0.562	**0.286**	0.857	**0.159**	**0.276**	**0.698**
pH 6	0.429	**0.258**	0.748	**0.098**	0.432	**0.061**	0.754	**0.104**	0.238	**0.067**	0.690	**0.040**	0.441	**0.27**	0.739	**0.089**	**0.171**	**0.650**
pH 5	0.283	**0.09**	0.744	**NON**	0.281	**0.088**	0.769	**NON**	0.200	**NON**	0.596	**NON**	0.280	**0.087**	0.726	**NON**	**0.193**	**0.778**
pH 4	0.217	**0.023**	0.711	**NON**	0.210	**0.016**	0.710	**NON**	0.203	**NON**	0.674	**NON**	0.224	**NON**	0.714	**NON**	**0.194**	**0.716**
pH 3	0.178	**NON**	0.700	**NON**	0.183	**NON**	0.704	**NON**	0.256	**NON**	0.691	**NON**	0.183	**NON**	0.703	**NON**	**0.247**	**0.692**
pH 2	0.224	**NON**	0.643	**NON**	0.234	**NON**	0.651	**NON**	0.218	**NON**	0.658	**NON**	0.227	**NON**	0.642	**NON**	**0.225**	**0.647**

KEY: N.E.T: No extract turbidity; W.E.T: With extract turbidity; P.T: Projected turbidity; B.I.T: Before incubation turbidity

Table 5. 6: Inhibition of *H. pylori* growth by anti-urease honey extracts at 100mg/mL concentration

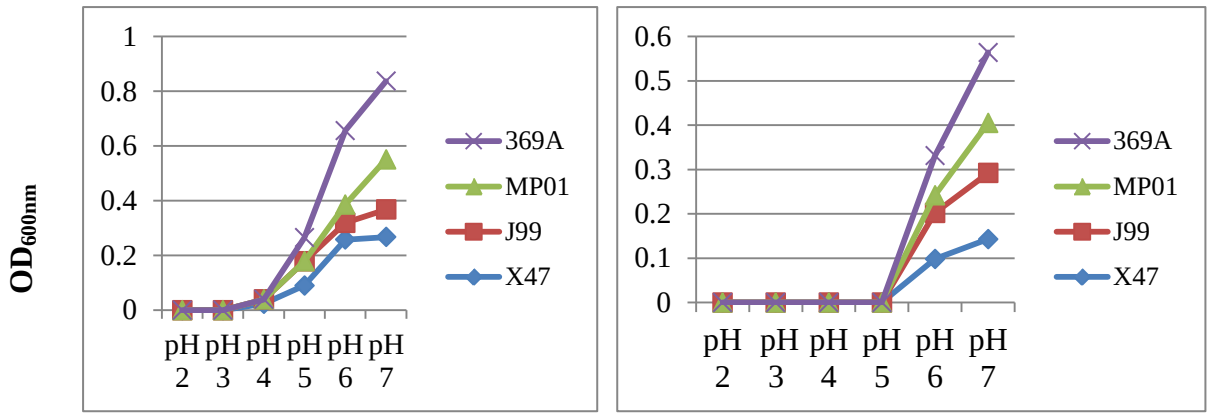
	X47 (*Cag*A, Urease +ve)				**J99 (*Vac*A *s*1*m*1, urease +ve)**				**MP01**				**CLR RES (369A)**				**B.I.T**	
	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	P.T	W.E.T	P.T	N.E.T	W.E.T
pH 7	0.481	**0.177**	0.543	0.103	0.392	**0.088**	0.489	**0.049**	0.554	**0.25**	0.666	**0.226**	0.395	**0.091**	0.479	**0.039**	0.304	0.440
pH 6	0.317	**0.099**	0.439	0.004	0.216	**0.011**	0.439	**0.004**	0.250	**0.032**	0.432	**NON**	0.220	**0.004**	0.441	**0.006**	0.218	0.435
pH 5	0.248	**0.110**	0.449	NON	0.146	**0.008**	0.451	**0.001**	0.172	**NON**	0.441	**NON**	0.241	**0.003**	0.450	**0.000**	0.238	0.450
pH 4	0.230	**NON**	0.407	NON	0.222	**0.002**	0.409	**NON**	0.221	**NON**	0.419	**NON**	0.221	**0.001**	0.413	**NON**	0.220	0.414
pH 3	0.244	**NON**	0.394	NON	0.247	**NON**	0.407	**NON**	0.219	**NON**	0.398	**NON**	0.243	**NON**	0.399	**NON**	0.237	0.403
pH 2	0.254	**NON**	0.406	NON	0.259	**NON**	0.420	**NON**	0.207	**NON**	0.406	**NON**	0.254	**NON**	0.411	**NON**	0.247	0.413

KEY: N.E: No extract turbidity; W.E: With extract turbidity; P.T: Projected turbidity; B.I.T: Before incubation turbidity

Table 5. 7: Inhibition of *H. pylori* growth by anti-urease honey extracts at 50 mg/mL concentration

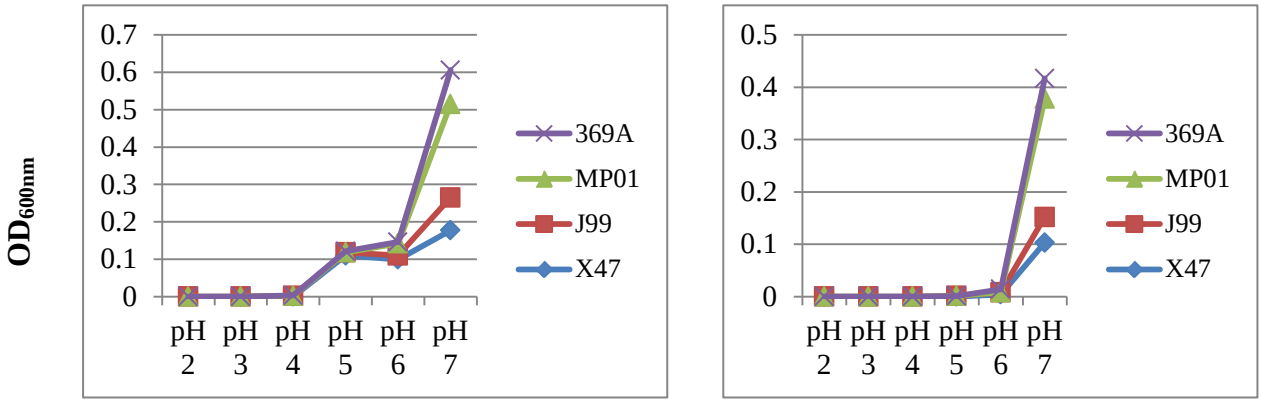
	X47 (*Cag*A, Urease +ve)				**J99 (*Vac*A *s*1*m*1, urease +ve)**				**MP01**				**CL RS (369A)**				**B.I.T**	
	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	**P.T**	W.E.T	**P.T**	N.E.T	W.E.T
pH 7	0.413	**0.134**	0.400	**0.108**	0.421	**0.129**	0.371	**0.079**	0.631	**0.352**	0.598	**0.306**	0.508	**0.216**	0.416	**0.124**	0.279	0.292
pH 6	0.341	**0.118**	0.226	**0.005**	0.239	**0.018**	0.254	**0.033**	0.268	**0.045**	0.225	**0.004**	0.228	**0.007**	0.278	**0.057**	0.223	0.221
pH 5	0.290	**0.054**	0.132	**NON**	0.006	**0.016**	0.235	**NON**	0.223	**NON**	0.164	**NON**	0.161	**0.022**	0.147	**0.008**	0.236	0.139
pH 4	0.200	**00**	0.195	**NON**	0.217	**0.017**	0.192	**NON**	0.211	**NON**	0.195	**NON**	0.208	**0.008**	0.195	**NON**	0.201	0.200
pH 3	0.193	**NON**	0.222	**NON**	0.213	**NON**	0.209	**NON**	0.217	**NON**	0.199	**NON**	0.223	**NON**	0.217	**NON**	0.214	0.214
pH 2	0.209	NON	0.196	NON	0.290	**NON**	0.216	**NON**	0.302	**NON**	0.219	**NON**	0.297	NON	0.205	NON	0.286	0.212

KEY: N.E: No extract turbidity; W.E: With extract turbidity; P.T: Projected turbidity; B.I.T: Before incubation turbidity



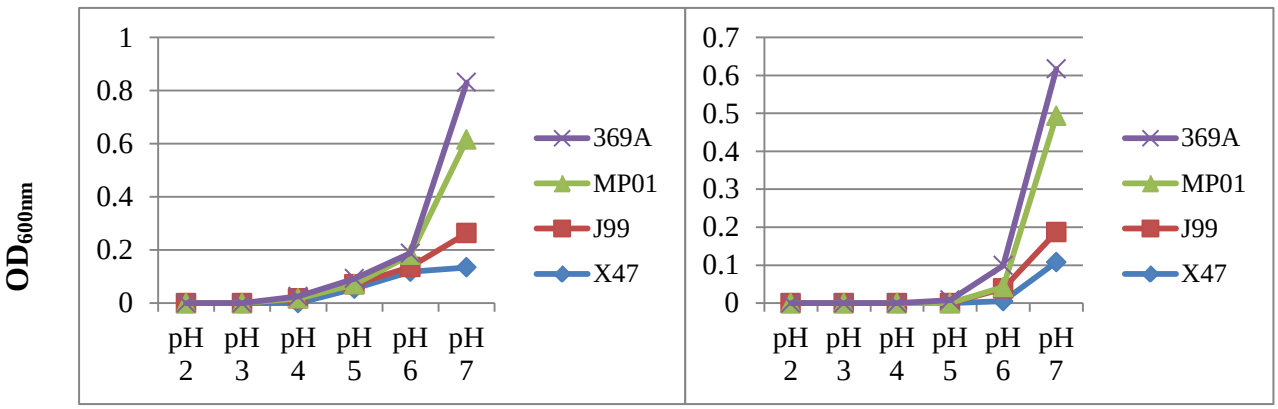
(a) No extract/inhibitor (b) With extract/inhibitor

Figure 5. 2 (a-b): Inhibition of *H. pylori* growth by anti-urease honey extracts at 200mg/L concentration at different pH levels



(a) No extract/inhibitor (b) With extract/inhibitor

Figure 5. 3 (a-b): Inhibition of *H. pylori* growth by anti-urease honey extracts at 100mg/L concentration at different pH levels



(b) No extract/inhibitor (b) With extract/inhibitor

Figure 5. 4 (a-b): Inhibition of *H. pylori* growth by anti-urease honey extracts at 50 mg/L concentration at different pH levels

The urease negative mutant (MP01) recorded a good growth at pH of 7, but there was a sharp decline of viability of this strain when it was cultured at pH 6, eventually losing viability at pH 5 and downwards (Table 5.5─5.7).

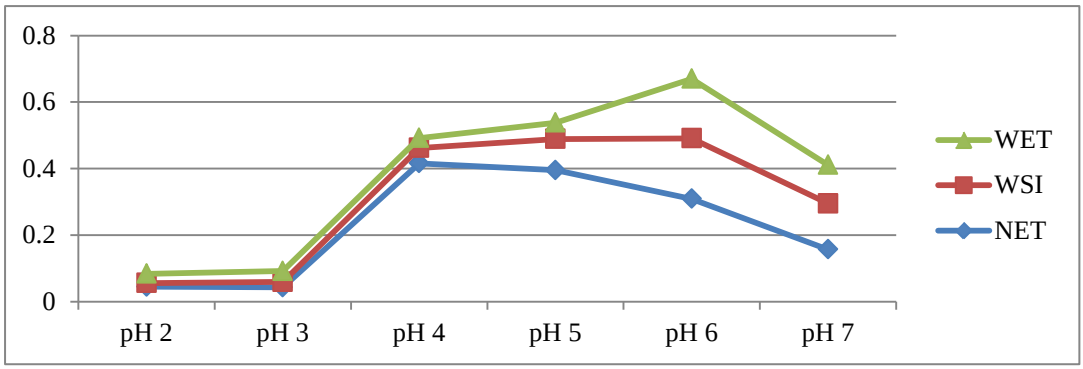
The exposure of X47, J99 and 369A to acidic growth media revealed sustainability of viability up to pH 4 (Figure 5.2-5.4). The total loss of viability or no-growth was recorded at pH 3 and 2 implying that viability might have been lost between pH 4 and pH 3. Introduction of anti-urease honey extracts into the growth media seemed to offset growth of *H. pylori* under acidic conditions. At a concentration of 200mg/mL viability of *H. pylori* strains X47, J99 and 369A was lost between pH 6 and pH 5 (Table 5.5). At a concentration of 100mg/L, viability of strains X47 and J99 was lost between pH 6 and pH 5 while the viability of strain 369A was lost between pH 5 and pH 4 (Table 5.6). Also growth of the organism was observed in growth media spiked with anti-urease honey extracts at a concentration of 50mg/L (Table 5.7). The findings reveal that strains X47 and J99 lost viability between pH 6 and pH 5, the trend notably similar to growth pattern observed when growth was monitored in growth media spiked with 100mg/L (Table 5.6). However, strain 369A lost viability between pH 5 and pH4 which is the similar trend that was noticed when growth was monitored in media spiked with 100mg/L.

5.4.3 Determining urease activity by ammonia test

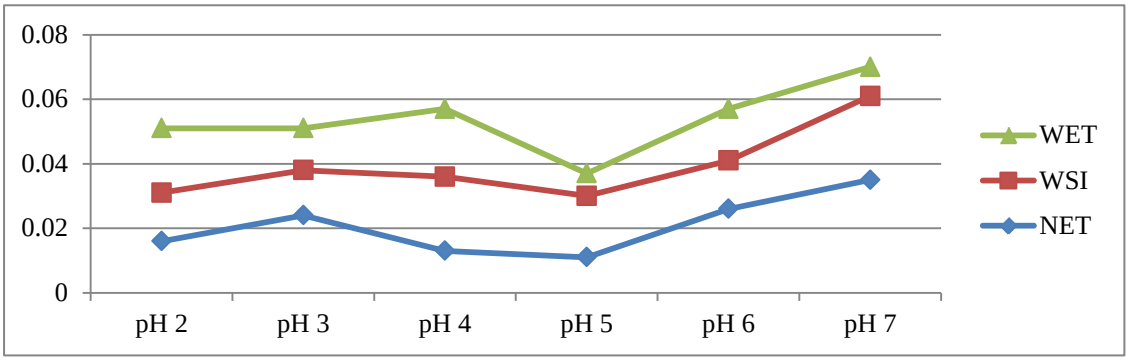
Table 5.8: Ammonia concentration (µM) after 5 days of *H. pylori* growth in media spiked with chloroform extract of Bush honey at 50mg/L concentration and acetohydroxamic acid as a standard inhibitor (14.3 mg/mL)

	X47 (*Cag*A, Urease +ve)			**J99** (*Vac*A *s*1*m*1, urease +ve)		**MP01** (Urease negative)			**369A** Clarithromycin resistant		
	N.E.T	**W.S.I**	**W.E.T**	**N.E.T**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**
pH 7	0.157	0.138	0.116	0.172	0.127	0.035	0.026	0.009	0.183	0.129	0.121
pH 6	0.309	0.182	0.179	0.299	0.154	0.026	0.015	0.016	0.311	0.188	0.183
pH 5	0.395	0.094	0.049	0.374	0.033	0.011	0.019	0.007	0.386	0.072	0.039
pH 4	0.416	0.046	0.030	0.421	0.035	0.013	0.023	0.021	0.437	0.059	0.028
pH 3	0.043	0.016	0.033	0.040	0.026	0.024	0.014	0.013	0.037	0.024	0.031
pH 2	0.045	0.011	0.028	0.037	0.030	0.016	0.015	0.020	0.032	0.017	0.023

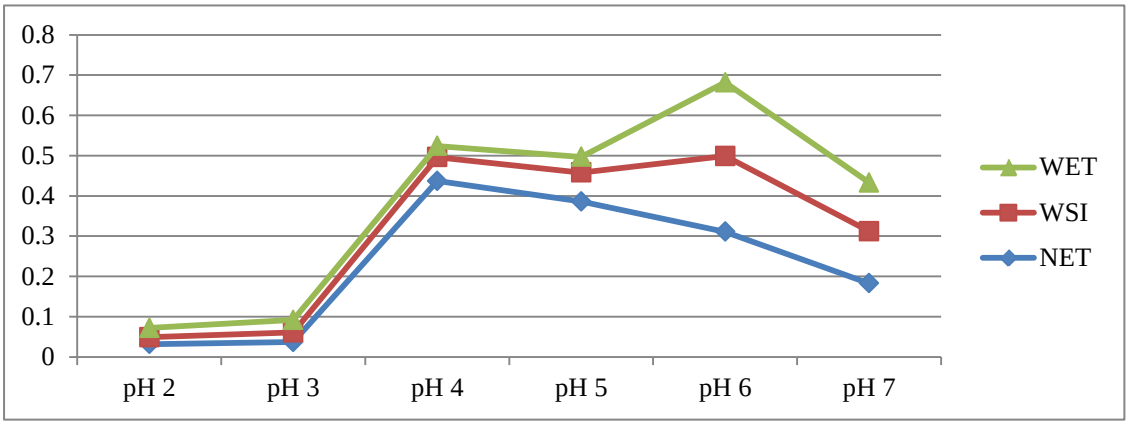
KEY: N.E.T.: No extract turbidity; W.S.I: With standard inhibitor turbidity; W.E.T: With extract turbidity



(a) Strain X47



(b) Strain MP01 (Urease negative)



(c) Strain 369A

Figure 5. 5 (a-c): Ammonia concentration (µM) after 5 days of *H. pylori* growth in media spiked with chloroform extract of Bush honey at 50mg/L concentration and acetohydroxamic acid as a standard inhibitor (14.3 mg/mL)

The Urease Activity Assay Kit (Sigma Aldrich) was used to determine ammonia production in samples of *H. pylori* cultured for 5 days under microaerophilic conditions with/without anti-urease active compounds (Figure 5.5). Ammonia was not detected in media suspension spiked with strain MP01 (urease negative). However, media spiked with strains X47, J99 and 369A (clinical isolate-clarithromycin resistant) had traces of ammonia being detected using the Urease Activity Assay Kit. Ammonia detection seemed to be dependent to pH level, all culturing media with *H. pylori* strains X47, J99 and 369A had traces of ammonia ranging between 0 μM and 50 μM at pH of 7. At pH of 6, culturing media without urease inhibitors but spiked with strains X47, J99 and 369A had ammonia at a concentration between 50 μM and 100 μM. At a pH of 6 as well, culturing media spiked with urease inhibitors and strains X47, J99 and 369A had an ammonia concentration between 0 μM and 50 μM. At pH level of 5, media without urease inhibition factors recorded an ammonia concentration ranging from 50 μM to 150 μM for strains X47, J99 and 369A. The same strains were also cultured at pH of 5 with media supplemented with urease inhibitors, the resultant ammonia concentration ranged from 0 μM to 50 μM. The media with urease negative mutant strain (MP01) recorded an insignificant level of ammonia concentration regardless of presence/non-presence of urease inhibitors in the culturing medium. The pH level of 4 resulted in the highest ammonia concentration detected using Urease Activity Assay Kit-spectrophotometer assay. At this pH, the concentration of ammonia ranged from 100 μM to 150 μM for media spiked with *H. pylori* strains X47, J99 and 369A but without urease inhibiting factors. Introduction of urease inhibitors to media at pH level of 4, led to sharp decrease in the production of ammonia in samples linked to the presence of strains X47, J99 and 369A, equating to ammonia concentration (between 0 μM and 50 μM) detected at the same pH of 4 when MP01 strain was cultured in the presence/absence of urease inhibitors. The pH level of 3 and 2 resulted in none/little production of ammonia with regard to strains X47, J99, 369A and MP01.

5.4.4 Re-culturing of *H. pylori* after acid exposure

At pH 7, strains X47, J99, MP01 and a clinical isolate (369A- clarithromycin resistant) showed good growth regardless of addition of urease inhibitors in the culturing medium during the 3─5 days culturing period. The good growth of strain MP01 (urease negative) at pH of 7 implies that *H. pylori*'s growth is not urease dependent at that pH, which explains the ineffectiveness of urease inhibitors to offset *H. pylori* growth of urease producing strains at pH of 7.

Table 5. 8: Re-growth of *H. pylori* after 3─5 day's acid exposure in growth media spiked with honey extracts at 200mg/L concentration

	X47 (*Cag*A, Urease +ve)			**J99** (*Vac*A *s*1*m*1, urease +ve)		**MP01**			**369 A CLR RES**		
	N.E.T	**W.S.I**	**W.E.T**	**N.E.T**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**
pH 7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pH 6	✓	✓	✓	✓	✓	X	X	X	✓	✓	✓
pH 5	✓	X	X	✓	X	X	X	X	✓	X	X
pH 4	✓	X	X	✓	X	X	X	X	X	X	X
pH 3	X	X	X	X	X	X	X	X	X	X	X
pH 2	X	X	X	X	X	X	X	X	X	X	X

Key: Growth ✓ No growth ☒ N.E.T: No extract turbidity W.E.T: With extract turbidity

W.S.I.: With standard inhibitor

Table 5. 9: Re-growth of *H. pylori* after 3-5 days acid exposure in growth media spiked with honey extracts at 100mg/mL concentration

	X47 (*Cag*A, Urease +ve)			**J99** (*Vac*A *s*1*m*1, urease +ve)		**MP01**			**369A CLR RES**		
	N.E.T	**W.S.I**	**W.E.T**	**N.E.T**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**
pH 7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pH 6	✓	✓	✓	✓	✓	X	X	X	✓	✓	✓
pH 5	✓	X	X	✓	**X**	X	X	X	✓	✓	✓
pH 4	✓	X	X	✓	X	X	X	X	✓	X	X
pH 3	X	X	X	X	X	X	X	X	X	X	X
pH 2	X	X	X	X	X	X	X	X	X	X	X

Key: Growth ✓ No growth ☒ N.E.T: No extract turbidity W.E.T: With extract turbidity

Table 5. 10: Re-growth of *H. pylori* after 3─5 day's acid exposure in growth media spiked with honey extracts at 50mg/L concentration.

	X47 (*Cag*A, Urease +ve)			**J99** (*Vac*A s1m1, urease +ve)		**MP01**			**369 A CLR RES**		
	N.E.T	**W.S.I**	**W.E.T**	**N.E.T**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**	**N.E.T**	**W.S.I**	**W.E.T**
pH 7	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
pH 6	✓	✓	✓	✓	✓	X	X	X	✓	✓	✓
pH 5	✓	X	X	✓	✓	X	X	X	✓	✓	✓
pH 4	X	X	X	✓	X	X	X	X	✓	X	X
pH 3	X	X	X	X	X	X	X	X	X	X	X
pH 2	X	X	X	X	X	X	X	X	X	X	X

Key: Growth ✓ No growth ☒ N.E.T: No extract turbidity W.E.T: With extract turbidity

The urease negative strain MP01 succumbed and lost viability when exposed to pH of 2, 3, 4, 5 and 6. Strains X47, J99 and 369A (clinical isolate) maintained viability in media without urease inhibiting compounds when exposed to pH 6, 5 and 4, at pH 3 and pH 2, none of the strains were successfully re-cultured after 5 days of exposure to the stated pH values. Strains X47, J99 and 369A could not be re-cultured from 5 day old cultures spiked with urease inhibiting compounds at pH levels 2, 3, 4, 5 and 6. X47 lost viability between pH 5 and 6 regardless of the concentration of urease inhibiting compounds. J99 and 369A lost viability between pH 5 and 6 at a concentration of 200mg/mL (Table 5.8) of urease inhibiting compounds while viability was lost between pH 4 and 5 at a concentration of 100mg/ml of urease inhibiting compounds (Table 5.9). At 50mg/ml, viability of strain J99 was lost between pH 5 and 6 while the viability of strain 369A was lost between pH 4 and 5 (Table 5.10).

5.5 DISCUSSION

H. pylori release urease in the human stomach. The urease enzyme is released in abundance by *H. pylori* and plays an important role in pH regulation by splitting urea into ammonia and carbon dioxide. Ammonia eventually leads to elevation of the pH in the human stomach. In this controlled experiment, we targeted inhibition of urease so as to observe the growth of *H. pylori* under acidic environments.

The organism is also equipped with several virulence factors, which include the *cag*A, an oncoprotein and the *vac*A genes. The allelic combination of these two genes influences risk of disease outcome due to *H. pylori* infection. The organism is recognised as a class 1 carcinogen by WHO, and many eradication protocols have been suggested due to drug

resistance development to currently approved therapies. In the present experiment, honey extracts with anti-urease activity were selected as test compounds. The anti-urease extracts were used to eliminate activity of urease enzyme among *H. pylori* strains grown under acidic environments, a mimicry of the human stomach so as to determine the value of targeting urease inhibition as a treatment strategy of *H. pylori* infections.

Our findings support previous studies that urease dependent micro-organisms could be controlled by application of urease inhibition techniques (Zaborska *et al*., 2001; Zhang *et al*., 2006; Amin *et al*., 2012; Amin *et al*., 2013). The strain MP01 is a mutant devoid of urease enzyme. There was an important observation when this mutant strain was cultured in media with different pH values. Strain MP01 succumbed to a dramatic drop in the growth between pH 7 and 6 when grown in media without urease inhibitors, probably because the change of pH from 7 to 6 signalled more production of urease enzyme so as to stabilise pH at a neutral level. Since this is a mutant strain that is unable to produce urease, the acidic environment led to poor growth implying that the critical pH for urease activation lies between pH 7 and pH 6. Also this observation implies that the optimum pH for growth of *H. pylori* is pH 7. The current findings support earlier reports that surface localisation of urease help to maintain a neutral pH around the organism (Phadnis *et al*., 1996).

There are other studies that have shown that at pH 7, honey has a greater inhibitory effect on Gram negative bacteria (Ali-Naama, 2009; Manyi-Loh *et al*., 2010; Alzahran *et al*., 2012). In this study, at the pH of 7 (a presumptive pH for commercial media for the growth of *H. pylori*) the honey extracts with urease inhibition compounds did not show much inhibitory effect on the growth of *H. pylori* probably because of the screening of honey extracts to only

those with high concentration of urease inhibitors. The findings of this study suggest that at a neutral pH, urease inhibition does not suppress the growth of *H. pylori*.

Contrary, important observations were made when strains of *H. pylori* (X47, J99 and 369A) growth was monitored in the absence/presence of urease inhibitors in acidic growth media (pH adjusted from pH 7 to pH 2). In comparison to the mutant strain, these strains (X47, J99 and 369A) showed a reasonable decline in growth rate when cultured in media without urease inhibitors when subjected to decreasing pH up to pH of 4 after which no growth was observed. However, spiking of anti-urease active extracts of honey (200mg/L) led to a sharp decline in growth of *H. pylori* with growth undetectable between pH 5 and pH 6 for strains X47, J99 and 369A. The 100mg/L and 50mg/L anti-urease extracts of honey resulted in no growth of strain X47 and J99 between a pH of 5 and 6 while strain 369A showed no growth between pH 4 and 5. In comparison to cultures grown without anti-urease active compounds, where growth was lost between the pH of 3 and 4 or pH4 and 5, urease inhibiting compounds in honey seem to be playing an active role which causes *H. pylori* to lose its multiplication capabilities. Surface localised urease enzyme is known to maintain an optimum pH for the growth of the organism in an acidic environment (Phadnis *et al*., 1996; Amin *et al*., 2010). The observed results therefore suggest that urease inhibitors in honey weaken the protective effect of the urease enzyme causing the organism to succumb to the acidic pH. The current observations demonstrate that the urease enzyme is an important virulent factor required by *H. pylori* to colonise the acidic environment such as the human stomach.

This study revealed that the concentration of urease inhibiting compounds in culturing media did not affect re-growth *of H. pylori* after exposure to culturing media with pH of 7 (Tables

5.9–5.10). However, it was observed that all the strains showed no growth between pH 3 and pH 4 and re-culturing of these strains was not successful after 5 days of incubation at 37^{o}C under miroaerophilic conditions. This could suggest that urease activity was totally stalled between pH 3 and pH 4, probably marking bactericidal pH range of honey extracts, or could be that because of prolonged unfavourable growth conditions, *H. pylori* cells changed to its coccoid state which is viable but non-culturable.

The standard strain X47 is *cag*A positive and the *cag*A gene encodes a protein that is linked with increased intensity of gastric inflammation. J99 is *vac*A *s*1*m*1 positive while strain 369A is a clinical isolate that is multidrug resistant. The findings of this study therefore may imply that the selected anti-urease honey extracts have the potential to eradicate the growth of *cag*A positive, *vac*A positive and drug resistant strains exposed to acidic environment.

5.6 CONCLUSION

H. pylori devoid of urease (MP01) were shown to have difficulties in surviving the acidic environment while urease producing *H. pylori* tolerated acid exposure with success. This leads to a conclusion that the urease enzyme is an important virulence factor for the organism's survival under acidic conditions. The data generated in this study also demonstrate that anti-urease compounds in honey extracts are bioactive against urease depended *H. pylori* under acidic conditions and have the potential to offset the growth of *H. pylori* strains that are *cag*A positive, *vac*A positive and drug resistant under acidic conditions. In addition, anti-urease active compounds have been shown to be antibacterial against urease producing *H. pylori* under acidic conditions.

CHAPTER SIX

Characterisation of bioactive compounds in honey extracts by GC-MS and LC-MS

6.1 ABSTRACT

Gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are sensitive techniques frequently used in the detection of volatile and non-volatile compounds respectively. Extracts for analysis were chosen on the bases of known urease inhibition potential of the extracts as determined in chapter 3. Volatile compounds in honey were detected using the GC-MS technique while commonly used drugs/pesticides/insecticides applied during agricultural activities were screened using LC-MS run on a positive mode. Fleures honey (I% = 67.8–68.5%), Gold Crest honey (I% = 50.9%–53.3%), Raw honey (I%=8.1–23.4%) and Siyakholwa honey (I% = 0), all petroleum ether extracts recorded 27, 26, 22, and 29 compounds respectively that were positively identified by spectral matching ≥ 90% with the NIST11 library during GC-MS analysis. Manuka honey (I% = 50.0 – 53.2%) and Siyakholwa honey (I% = 10.3% – 11.2%), all hexane extracts recorded 43 and 23 compounds respectively that were positively identified by spectral matching ≥ 90% with the NIST11 library during GC-MS analysis. Regarding chloroform extracts, Raw honey (I% = 8.5 – 11.4%), Q Bee (I% = 64.2 – 66.2%) and Siyakholwa honey (I% = 5.3% – 6.1%), revealed 19, 13 and 12 compounds respectively with spectral matching ≥ 90% with the NIST11 library during GC-MS analysis. There were six compounds that were identified by LC-MS to be constituents of both Q Bee honey and Little Bee honey hexane extracts. The majority of compounds detected in Little Bee honey are drugs commonly used in the treatment of a variety of ailments affecting human beings. Two drugs, sulfaquinoxaline and hydroxyquinoline used in veterinary medicine and, antiseptic,

disinfectant and pesticide applications in agricultural produce were also detected in Little Bee honey. To conclude, geographical location influences honey's value as a rich source of urease inhibiting compounds.

6.2 INTRODUCTION

Honey is a natural sweet product commonly used for food or food additive and traditional medicinal applications (Selcuk and Nevin, 2002). This natural sweet product has been used in ethno-medicine dating back to ancient times. Treatment of wounds, burns, skin ulcer and gastro-duodenal disorders are some of the complications that have been alleviated by use of honey (Selcuk and Nevin, 2002; Orhan *et al*., 2003).

The geographical location and floral origin affect the physiochemical composition, antioxidant activities and phenolic components of honey (Eleazu *et al*., 2013). Several studies have documented different classes of chemical compounds in honey including acids, aldehydes, alcohol, ketones, terpenes, hydrocarbons, furans (Sirko and Brodzik., 2000; Barra *et al*., 2010; Manyi-Loh *et al*., 2010; Modolo *et al*., 2015). While it is acceptable that geographical location from which bees forage play an important role in determining the medicinal value of honey, veterinary medicines and insecticides used in agriculture always get incorporated into the honey passively by foraging bees (Hoopingarner and Nelson, 1998; Kuhn and Wittrig, 2007).

Different techniques are being used for determining chemical components in honey. Capillary electrophoresis–mass spectrometry (CE-MS), High-performance liquid chromatography (HPLC), Gas Chromatography Mass Spectrometry (GC-MS) and Liquid chromatography mass spectrometry (LC-MS) are the most commonly used techniques (Arr´aez-Rom´an *et al*., 2006; Campone *et al*., 2014).

GC-MS has been widely used in the analysis of chemical components of honey with much success. Liquid Chromatography Mass Spectrometry (LC-MS) which combines liquid chromatography and mass spectroscopy is a highly sensitive, accurate and specific quantitative analysis method. The GC-MS is suitable for the analysis of volatile chemicals, mostly non-polar or chemicals amenable to changes to enhance volatility (Wolski *et al*., 2006; Syazana *et al*., 2013). The LC-MS is highly recommended for more polar chemicals (Campone *et al*., 2014). The purpose of this study was therefore to assess the composition of volatile and non-volatile compounds in South African honey that have anti-urease potential as well as determining whether there are any likelihood of insecticide or chemical traces in honey that have been incorporated by bees after foraging on plants or pollen sprayed using pesticides.

6.3 MATERIALS AND METHODS

6.3.1 Honey preparation for GC-MS and LC-MS

Extraction of honey compounds for GC-MS and LC-MS analysis was done according to the method described by Syazana *et al.* (2013) as already explained in chapter 3. However the anhydrous disodium sulfate powder ($Na_2SO_{4)}$ was not added to honey extracts prepared for LC-MS analysis.

6.3.2 GC-MS analysis

Analysis of compounds was done at the Mass Spectrometry Laboratory, School of Chemistry; University of KwaZulu Natal-Pietermaritzburg, South Africa. GC-MS analysis was performed using a Shimadzu GC-MS-QP2020 mass spectrometer. The column was an SH-RtxTM-VMS (30m X 0.25mmID, 1.40 µm) column, and helium, a carrier gas was kept at a constant linear velocity of 34cm/sec at 40^{o}C. One microliter volumes were injected using a splited mode at an injector temperature of 200^{o}C. The oven temperature was ramped from 40 to 220^{o}C (4 minute hold) at a rate of 16^{o}C/min, then at 32^{o}C/min (hold 5 minutes). The oven temperature was held at 150oC for 6 minutes following each analysis. The total run time for each sample was almost 90 minutes. The GC-MS interface temperature was set to 150oC. Mass spectrometry mode was used during analytical scanning from 35-300 atomic mass unit (amu). The ion source temperature was set to 200^{o}C. The blank was first injected, and was followed by the sample injection. The chromatograms obtained from the total ion count (TIC) were integrated without any correction for co-eluting peaks and the results were expressed as total abundance. All the peaks were identified based on mass spectral matching (≥ 90%) from both the NIST and Wiley libraries. Only compounds with 90% or greater spectral matching accuracy are reported. Qualitative analysis report obtained from the total ion count (TIC) was

summarised without any alteration for co-elution peaks. All the peaks were identified based on mass spectral matching greater or equal to 90% (≥ 90%) with the NIST11 library. Only compounds with 90% or greater spectral matching accuracy were reported.

6.3.3 Liquid Chromatography-Mass Spectrometry (LC-MS)

Hexane extracts of Raw Honey, Goldcrest Honey, Q Bee Honey, Fleures Honey and Little Bee Honey were subjected to Liquid Chromatography Mass Spectroscopy (LC-MS) so as to identify possible compounds responsible for urease inhibition. LC-MS analysis was done using Triple TOF^{TM} 5600 System Technology and Duospray^{TM} Ion Source on a positive electrospray ionisation mode according to Andrews *et al*., 2017 but with modifications. The following conditions for MS were used: ion spray voltage, 4.5KV-4.5KV; ion source temperature, 600^{o}C; curtain gas (GS 1) at 30 psi; nebulizer gas at 50 psi; heater gas (GS 2) at 50 psi; declustering potential at 80 V. The following mass ranges were used, m/z 100-600 for TOF MS scan, 50-600 for TOF MS/MS experiments. In the TOF MS/MS experiments, the most intensive 8 ions were selected for MS/MS fragmentation. The dynamic background subtraction was done to match the information dependent acquisition (IDA) criteria. The collision energy (CE) was set at 30eV-40eV and the collision energy was at 10eV for MS/MS experiments. The data was analysed using PeakView Software^{TM} and the Metabolite Pilot Software on Drug Discovery was used to determine compounds in honey extracts and the potential compounds were selected based on being positive in mass error, isotope and library hit scores.

6.4 RESULTS

6.4.1 Volatile compounds in honey detected by GC-MS

6.4.1.1 Petroleum ether extracts and urease inhibition

There were 27, 22 and 26 compounds that were detected in Fleures honey, Raw Honey and Gold Crest honey respectively by GC-MS by spectral matching greater than 90% with the NIST11 library (Figure 6.1). The compounds in Fleures honey petroleum ether extract compared very well with compounds detected in Gold crest honey with a spectral matching greater or equal to 90%. Tetradecane, heneicosane, nonadecane and octadecanoic acid were potential compounds found in Gold crest honey with spectral matching ≥ 90% and inhibition percentage (%) of 50.9% but not in Fleures honey that had an inhibition percentage of 67.9%.

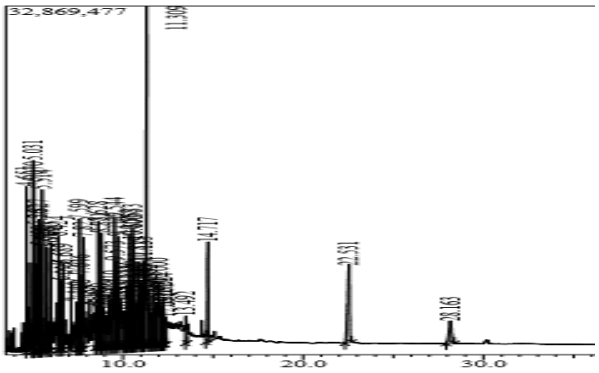
6.4.1.2 Hexane extracts and urease inhibition

All the peaks were identified based on mass spectral matching ≥ 90% from both the NIST11. Two hexane extracts were analysed with GC-MS (Figure 6.1). The hexane extract of Manuka honey had forty three of the eighty four (51.2%) compounds with 90 % or greater spectral matching with NIST11 library (Table 6.2). Siyakholwa honey recorded a lower number of compounds with a 90% or greater spectral matching compared to Manuka honey, twenty three out of eighty two (28%) of the compounds revealed a 90% or greater spectral matching (Table 6.2). Comparing volatile compounds with spectral matching from 90% and above in both extracts, it was observed that twenty two (88%) out of twenty five compounds identified in Siyakholwa honey hexane extract were also found in the Manuka honey hexane extract. Hexadecane, phenol and tetradecanamide were the three compounds that were not positively identified in Manuka honey by spectral matching (≥ 90%) even though they were identified

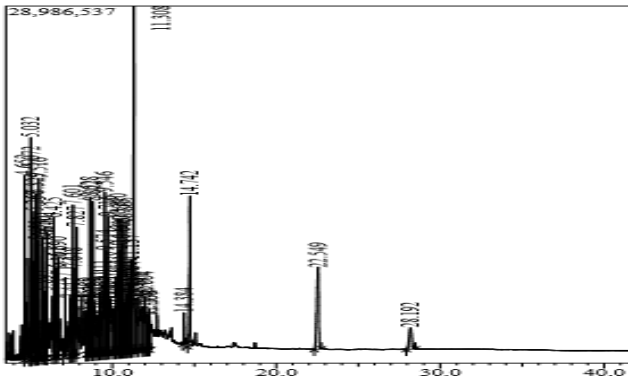
by spectral matching (≥ 90%) in Siyakholwa honey hexane extract. Manuka honey exhibited an inhibition percentage ranging from 50% to 53.2% on urease activity under observation. Siyakholwa honey hexane extract performed poorly on urease inhibition with an inhibition percentage ranging from 10.3% to 11.2% on the ureases selected for this study.

6.4.1.3 Chloroform extract and urease inhibition

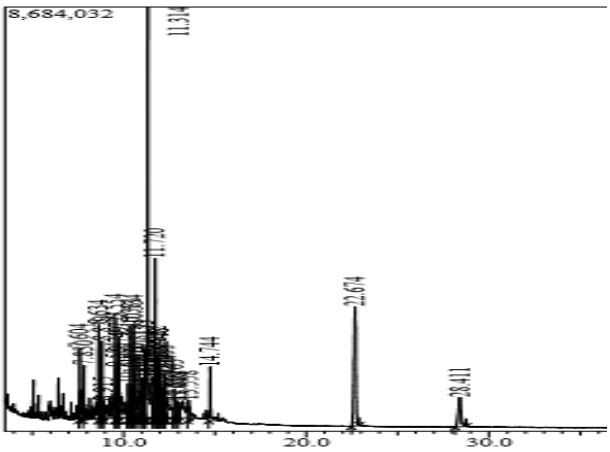
Chloroform was also used as an extraction solvent for honey compounds. Three honey extracts were analysed using GC-MS (Figure 6.1). Few compounds reached a 90% library hit in comparison to petroleum extracts and hexane extracts. Raw honey had nineteen out of sixty seven (28.4%) compounds with spectral matching of 90% and above in the NIST11 library (Table 6.3). Q Bee honey recorded thirteen of the thirty five (37.1%) compounds reaching a spectral matching of 90% and above in the NIST11 library (Table 6.3). Siyakholwa honey extract had twelve out of thirty eight (31.6%) of the compounds reaching 90% and above spectral matching with the NIST11 library (Table 6.3). Of the three, Siyakholwa honey extracts gave the lowest urease inhibition percentage (%) ranging from 5.3% to 6.1% which did not differ much from a range of 8.5% to 11.4% attained by Raw honey extracts under similar experimental environments.



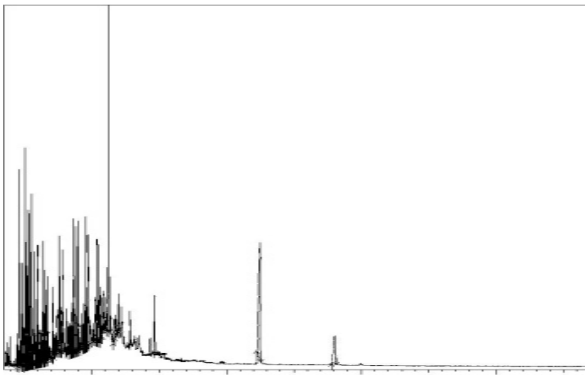
(a) Petroleum ether chromatogram of Fleures honey



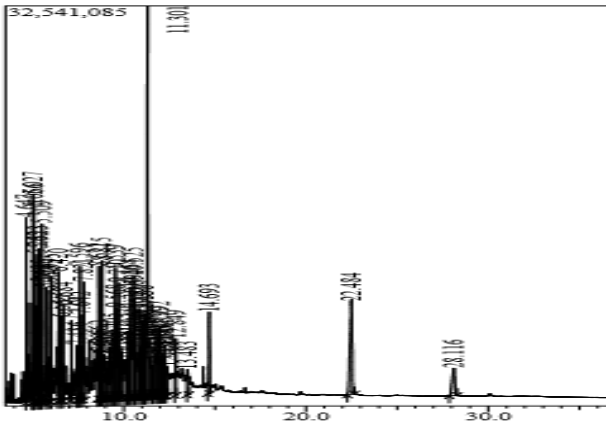
(b) Petroleum ether chromatogram of Gold Crest honey



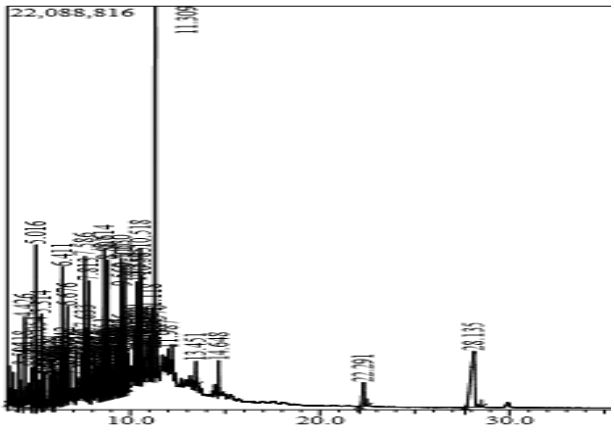
(c) Hexane chromatogram of Little Bee honey



(d) Hexane chromatogram of Manuka honey



(e) Chloroform chromatogram of Bush honey



(f) Chloroform chromatogram of Q Bee honey

Figure 6. 1 (a-f): GC-MS chromatograms of honey extracts

Table 6. 1: GC-MS analysis of petroleum extracts in reference to inhibition percentage

	Compound	Fleures honey I%=67.8-68.5%		Gold Crest I% = 50.9-53.3%		Raw Honey I% = 8.1-23.4%		Siyakholwa I% = 0.0%	
		Relative %	**✓/*X***	**Relative %**	**✓/*X***	**Relative %**	**✓/*X***	**Relative %**	**✓/*X***
1	Benzene	0.87	✓	0.84	✓	0.54	✓	1.00	✓
2	Undecane	0.91	✓	0.92	✓	0.54	✓	1.05	✓
3	Octane	0.91	✓	0.92	✓		***X***	1.05	✓
4	Decane	0.91	✓	0.92	✓	0.54	✓	1.05	✓
5	Dodecane	0.91	✓	0.75	✓	0.47	✓	1.05	✓
6	Tetradecane	0.91	✓	0.75	✓	0.47	✓	1.05	✓
7	Ethanol	0.63	✓	0.65	✓	1.89	✓		***X***
8	Tridecane	0.79	✓	0.75	✓	0.54	✓	0.90	✓
9	Pentadecane	0.91	✓	0.83	✓	0.54	✓	0.90	✓
10	Nonane	1.00	✓	1.02	✓	0.48	✓	1.18	✓
11	Hexadecane	0.75	✓	1.02	✓	0.48	✓	1.04	✓
12	Heptadecane	0.59	✓	1.02	✓	0.73	✓	0.76	✓
13	Eicosane	0.80	✓	0.83	✓	0.73	✓	0.93	✓
14	Heneicosane	1.52	✓	1.62	✓	0.73	✓	1.70	✓
15	Tetracosane	0.58	✓		***X***	0.90	✓	0.84	✓
16	Tetradecane		***X***		***X***	0.47	✓	1.05	✓
17	Tetratetracontane	1.91	✓	0.57	✓	0.90	✓	2.13	✓
18	Hexatriacontane	1.02	✓	1.43	✓		***X***	0.85	✓
19	Hexadecanamide	1.11	✓	0.65	✓		***X***	1.10	✓
20	Dodecanamide	1.11	✓	0.65	✓		***X***	1.10	✓
21	Octadecanamide	1.11	✓	0.65	✓		***X***	1.10	✓
22	Decanamide	1.11	✓	0.65	✓		***X***	1.10	✓
23	Tetradecanamide	1.11	✓	0.65	✓		***X***	1.10	✓
24	Tetratriacontane	1.25	✓	1.28	✓		***X***	1.21	✓
25	2-methyloctacosane	1.25	✓	0.57	✓		***X***	0.75	✓
26	9-Octadecenamide	5.88	✓	4.76	✓	2.22	✓	5.93	✓
27	Cholesta-3,5-diene	1.86	✓	2.53	✓		***X***	1.42	✓
28	Cholesterol	1.86	✓	2.53	✓		***X***	1.42	✓
29	Heneicosane		***X***		***X***	0.73	✓	1.70	✓
30	9,12-Octadecadienoic acid		***X***		***X***	1.89	✓		***X***
31	Cis-13,16-Docasadienoic acid		***X***		***X***	1.89	✓		***X***
32	Oxacycloheptadec		***X***		***X***	1.89	✓		***X***
33	Phthalic acid		***X***		***X***		✓		***X***
34	Diisooctyl phthalate		***X***		***X***		***X***	1.26	✓
Total compounds identified out of all peaks		**27/70**		**26/84**		**22/86**		**29/72**	
%total compounds identified out of all peaks		**38.5%**		**31%**		**25.6%**		**40.3%**	

Key: ✓ - detected, ***X*** - not detected

Table 6. 2: GC-MS analysis of hexane extracts in reference to inhibition percentage

	Compound	Manuka Honey I% = 50-53.2%		Siyakholwa I%=10.3-11.2%	
		Relative %	**✓/*X***	**Relative %**	**✓/*X***
1	n-Butyl ether	0.44	✓		***X***
2	Isobutyl ether	0.44	✓		***X***
3	Oxalic acid	0.44	✓		***X***
4	Malonic acid	0.44	✓		***X***
5	Nonane	0.51	✓	0.82	✓
6	Heptane	0.51	✓		***X***
7	Decane	0.51	✓		***X***
8	Dodecane	0.51	✓	0.82	✓
9	D-Limonele	0.46	✓		***X***
10	Cyclobutane	0.46	✓		***X***
11	Cyclohexene	0.46	✓		***X***
12	Octane	1.72	✓		***X***
13	Undecane	1.72	✓		***X***
14	Nonane	1.72	✓		***X***
15	Benzene	0.45	✓		***X***
16	Ethanol	0.79	✓		***X***
17	Naphthalene	0.51	✓		***X***
18	Azulene	0.51	✓		***X***
19	2,4-Dimethyldodecane	0.82	✓		***X***
20	Tridecane	0.82	✓		***X***
21	Eicosane	0.61	✓		***X***
22	Hexadecane	0.53	✓	0.79	✓
23	Tetradecane	0.53	✓	0.79	✓
24	Heptadecane	0.53	✓	2.20	✓
25	3-Ethyl-3-methylheptane	0.52	✓		***X***
26	Pentadecane	1.00	✓		***X***
27	Sulfurous acid	0.51	✓		***X***
28	Heneicosane	0.45	✓	2.20	✓
29	Nonadecane	0.76	✓	0.79	✓
30	Tetratetracontane	0.76	✓		***X***
31	2-Methyloctacosane	0.51	✓	0.90	✓
32	Tetracosane	2.74	✓		***X***
33	Hexatriacontane	0.68	✓	1.85	✓
34	2-Methylhexacosane	1.48	✓	2.82	✓
35	Hexadecanamide	1.38	✓	1.84	✓
36	Octadecanamide	1.38	✓	1.84	✓
37	Tetradecanamide	1.38	✓	1.84	✓
38	Dodecanamide	1.38	✓	1.84	✓
39	Decanamide	1.38	✓	1.84	✓
40	Tetratriacontane	1.38	✓	1.85	✓
41	Tetracosane	1.38	✓	1.12	✓
42	9-Octadecenamide	10.32	✓	10.63	✓
43	Cholesta-3,5-diene	1.00	✓	1.25	✓
44	Eicosane		***X***	0.79	✓
45	Phenol		***X***	0.90	✓
46	Tetratetracontane		***X***	3.27	✓
47	Pentadecane		***X***	1.06	✓
Total compounds identified out of all peaks		**43/84**		**23/82**	
% total compounds identified out of all peaks		**51.2%**		**28%**	

Key: ✓ - detected, ***X*** - not detected

Table 6. 3: GC-MS analysis of hexane extracts in reference to inhibition percentage

	Compound	Raw honey I% = 8.5-11.4%		Q Bee honey I%= 64.2-66.2%		Siyakholwa 5.3-6.1%	
		Relative %	✓/*X*	Relative %	✓/*X*	Relative %	✓/*X*
1	Ethanol	0.81	✓		***X***		***X***
2	Dodecane	1.29	✓		***X***	1.46	✓
3	Nonane	1.29	✓		***X***		***X***
4	Hexadecane	1.29	✓	2.09	✓	1.46	✓
5	Tetradecane	1.29	✓		***X***	1.46	✓
6	Decane	0.91	✓		***X***		***X***
7	Pentadecane	0.91	✓		***X***	1.46	✓
8	Heptadecane	0.83	✓	2.09	✓	1.46	✓
9	Heneicosane	0.83	✓	2.21	✓	2.12	✓
10	Eicosane	2.43	✓	2.09	✓	2.12	✓
11	Phenol	0.97	✓		***X***		***X***
12	Tetratetracontane	2.75	✓	2.21	✓	2.66	✓
13	2-methyloctacosane	2.75	✓	2.21	✓	2.66	✓
14	Tetracosane	0.72	✓	1.99	✓	1.78	✓
15	Tetratriacontane	1.70	✓	1.88	✓		***X***
16	Hexatriacontane	1.50	✓	2.06	✓	1.96	✓
17	2-methylhexacosane	0.75	✓	2.06	✓		***X***
18	Cholesta-3,5-diene	2.24	✓	2.66	✓		***X***
19	Cholesteryl benzoate	2.24	✓	2.66	✓		***X***
20	Cholesterol		***X***	2.66	✓		***X***
21	Benzoic acid		***X***		***X***	1.20	✓
Total compounds identified out of all peaks		**19 / 67**		**13 / 35**		**12 / 82**	
% total compounds identified out of all peaks		**28.4%**		**37.1%**		**14.6%**	

Key: ✓ - detected, ***X*** - not detected

6.4.2 Volatile compounds in honey and urease inhibition

The solvent extracts that resulted in urease kinetic inhibition of 50% and more were selected for this analysis. Two extracts of petroleum ether (Gold Crest honey and Fleures Honey), one hexane extract of Manuka honey and one chloroform extract of Q bee honey were selected to link compounds in honey with inhibitory power on kinetic activity of the urease enzyme. The area percentage was the only variable considered for this analysis.

Thirty seven compounds were detected by GC-MS in the petroleum ether extract of Gold Crest honey with eicosane having the highest area percentage of 26.11% followed by cholester-5-en-3-ol (3.beta) with 13.72% (Table 6.4). The petroleum ether of Gold Crest honey had an inhibition percentage of urease activity ranging from 50.9% to 53.3%. The urease percentage inhibition of Fleures honey ranged from 67.8% to 68.5%.

The petroleum ether extract of Fleures honey had 35 compounds detected by GC-MS. The cholesteryl valerate had the highest area percentage of 20.94% of the compounds followed by eicosane with 16.79%. Cholesterol- pentafluoropropionate had 9.35% area representation while 9-octadecanamide recorded 6.56% area representation (Table 6.5). The hexane extract of Manuka honey had an inhibition percentage of urease activity ranging from 50% to 53.2%. GC-MS analysis revealed that 38.42% of the area % of compounds was represented by eicosane followed by 10.32% of 9-octadecenamide and 8.62% of 1,4-epoxynaphthalene-1(2H)-methanol (Table 6.6). The chloroform extracts were represented by Q Bee honey which had urease inhibition percentage ranging from 64.2% to 66.2%. Also eicosane had the largest share of area percentage (23.19%) followed by 9- octadecanamide (16.65%) and silane, diethylheptyloxyoctadecyloxy with 12.29% (Table 6.7).

Table 6. 4: Petroleum ether extracts of Gold Crest honey showing area %

No	Volatile compounds	RT (min)	Area %
1	Mesitylene	4.655	0.85
2	Nonane, 5-(2-methylpropyl	5.035	1.36
3	Spiro [3.5]nona-5,7-dien-1-one, 5,9,9-trimethy	5.075	1.16
4	Benzene, 1-ethyl-3,5-dimethyl	5.272	0.89
5	Undecane	5.332	1.07
6	Benzene, 1,2,3,5-tetramethyl-	5.494	3.00
7	Ethanol, 1-(2-butoxyethoxyl)	5.896	0.77
8	Dodecane, 4,6-dimethyl, 4-methyl	5.969	3.34
9	Azulene	6.004	0.93
10	Tetradecane, 5-methyl	6.346	1.44
11	Tridecane, 6-methyl	7.185	0.53
12	Heptadecane	7.376	3.43
13	Hexadecane,1-iodo	7.445	4,51
14	Eicosane	7.605	26.11
15	Pentadecane	7.651	0.91
16	Phenol, 2,4-bis(1,1-dimethylethyl)	7.712	0.87
17	Octacosyl triflouroacetate	8.764	0.72
18	Heneicosane	9.233	3.84
19	Silane, trichlorooctadecyl	9.579	1.01
20	Benzenepropanoic acid, 3,5-bis (1,1-dimethyle	9.645	0.84
21	Hexatriacontyl trifluoroacetate	9.675	0.70
22	11-Bromoundecanoic acid	9.747	0.90
23	9-Octadecanamide. (Z)	9.817	5.66
24	Cis-13,16-Docasadienoic acid	10.449	1.56
25	Hexadecanamide	10.612	0.81
26	Cholest-5-en-3-ol (3.beta)-,	10.712	13.72
27	Octadecanoic acid, 2-hydroxyethyl ester	10.805	2.85
28	Tetratetracontane	10.860	2.69
29	Bis(dodecanamido) methane	11.388	0.62
30	Cholesta-3,5-diene	11.544	3.19
31	Hexatriacontane	11.857	0.98
32	Bis(tridecyl) phthalate	12.006	1.59
33	2-methylhexacosane	12.183	0.64
34	Silane, diethylheptyloxyoctadecyloxy	22.594	3.47
35	1.4-Epoxynaphthalene-1(2H)-methanol, 4,5,7	28.243	1.16
36	Propanoic acid, 3.3’-thiobis-, didodecyl ester	30.274	0.23
37	Longifolenaldehyde	87.317	0.71
Total			**100%**

Table 6. 5: Petroleum ether extracts of Fleures honey showing area %

No	Volatile compounds	RT (min)	Area %
1	Mesitylene	4.651	0.86
2	Pentadecane	5.031	1.22
3	Spiro[3.5]nona-5,7-dien-1-one, 5,9,9-trimethy	5.070	1.06
4	Benzene	5.267	3.54
5	Undecane	5.327	0.91
6	Dodecane, 4,6-dimethyl	5.965	2.67
7	Naphthalene	6.000	0.86
8	Tetradecane	6.559	1.34
9	Eicosane	7.440	16.79
10	Pentadecane	7.646	0.68
11	Phenol, 2.4-bis(1,1-dimethylethyl)	7.708	0.70
12	Heneicosane	8.487	0.58
13	Docosyl pentafluoropropionate	8.760	0.60
14	Nonane, 5-methyl-5-propyl	8.905	0.91
15	Heptadecane	9.054	0.64
16	Silane, trichlorooctadecyl	9.573	0.99
17	Hexadecane, 1-iodo	10.078	2.06
18	Octadecane, 1-chloro	10.152	0.72
19	E,E,Z,1,3,12-Nonadecatriene-5,14-diol	10.443	0.66
20	Bis(dodecanamido)methane	10.533	2.45
21	Hexadecanamide	10.600	1.11
22	Cholesta-3,5-diene	10.727	3.32
23	Octadecanoic acid, 2 hydroxyethyl ester	10.819	3.74
24	Tetratetracontane	10.997	2.84
25	Cholest-5-en-3-ol (3.beta)	11.163	1.7
26	9-Octadecanamide	11.309	6.56
27	Bis(tridecyl) phthalate	12.000	1.97
28	Hexatriacontane	12.261	0.78
29	5.5-Diethylheptadecane	12.391	0.68
30	13-Docosenamide, (Z)	13.492	0.74
31	Silane, diethylheptyloxyoctadecyloxy	22.531	3.43
32	1.4-Epoxynaphthalene-1(2H)- methanol, 4,5,7	28.163	1.26
33	Cholesteryl valerate	85.148	20.94
34	3Beta-methoxy-5-cholesten-19-oic acid	85.345	1.34
35	Cholesterol, pentafluoropropionate	86.493	9.35
Total			**100%**

Table 6. 6: Hexane extracts of Manuka honey showing area %

No	Volatile compounds	RT (min)	Area %
1	n-Butyl ether	3.829	0.44
2	4-Butoxy-2-butanone	4.118	0.72
3	Oxalic acid, cyclohexyl propyl ester	4.428	1.44
4	Nonane, 2,6-dimethyl, 5-butyl	4.786	4.91
5	D-Limonene	4.880	0.46
6	Octane, 5-ethyl-2-methyl	5.015	3.6
7	Decane, 3,7-dimethyl, 2,3,7-trimethyl	5.350	1.3
8	Benzene, 1,2,4,5-teteamethyl	5.498	1.05
9	Ethanol, 1-(2-butoxyethoxy)	5.880	0.79
10	Tetradecane	5.952	0.78
11	Naphthalene	5.994	0.51
12	Undecane, 2,5-dimethyl	6.030	1.1
13	Dodecane, 4,6-dimethyl	6.275	3.72
14	Tetradecane	7.107	0.68
15	Hexadecane	7.168	1.43
16	Eicosane	7.428	38.42
17	Pentadecane, 2,6,10,14-tetramethyl	7.632	1.71
18	Phenol, 2,4-bis (1,1-dimethylethyl)	7.693	1.04
19	1-Dodecanol, 2-hexyl	7.729	0.48
20	Sulfurus acid, pentadecyl 2-propyl ester	8.157	0.51
21	1-Heptanol, 2,4-diethyl	8.745	0.97
22	Heptadecane	9.040	0.74
23	Phthalic acid, butyl tetradecyl ester	9.340	0.47
24	n-Tetradecyltrichlorosilane	9.559	1.58
25	Benzenepropanoic acid, 3,5-bis(1,1-dimethyl	9.625	0.79
26	Tetracontyl	9.655	2.31
27	Ethanone, 1-phenyl	10.290	0.56
28	Hexadecanamide	10.583	1.38
29	Octadecanoic acid, 2 hydroxylethyl ester	10.808	1.17
30	9-Octadecenamide, (Z)	11.293	10.32
31	Bis(dodecanamido)methane	11.375	0.82
32	Tetratetracontane	11.984	0.43
33	5,5-Diethylpentadecane	12.198	0.57
34	Cholesta-3,5-diene	14.644	1.00
35	Silane, diethylheptyloxyoctadecyloxy	22.296	2.51
36	1,4-Epoxynaphthalene-1(2H)-methanol	28.051	8.62
Total			**100%**

Table 6. 7: Chloroform extracts of Q Bee honey showing area %

No	Volatile compounds	RT (min)	Area %
1	Eicosane	7.604	23.19
2	Nonane, 5-methyl-5-propyl	18.915	1.03
3	Silane, trichlorooctadecyl	9.584	1.21
4	Pentadecanoic acid	9.749	1.73
5	9-Octadecanamide, (Z)	10.620	16.65
6	Octadecanoic acid, 2-hydroxyethyl ester	10.813	3.91
7	Hexatriacontane	11.019	5.41
8	1,3,5-Trisilacyclohexane	11.720	6.52
9	1H-Indene, 1-hexadecyl-2,3-dihydro	11.822	3.21
10	Squalane	11.873	4.54
11	Hexadecanoic acid, 2-hydroxy-	11.948	2.73
12	5,5-Diethylpentadecane	12.210	5.06
13	1,3,5-Trisilacyclohexane	12.763	2.41
14	Oxalic acid, 3,5-difluorophenyl tetradecyl ester	12.891	1.05
15	2-Methylhexacosane	13.558	2.19
16	Cholesta-3,5-diene	14.744	2.66
17	Silane, diethylheptyloxyoctadecyloxy	22.674	12.29
18	1,4-Epoxynaphthalene-1(2H)-methanol,4,5,7	28.411	4.20
Total			**100%**

6.4.3 LC-MS analysis

Six hexane extracts of different honeys were analysed using LC-MS (Appendix C). The honey extracts showed differences in compound composition. The hexane extract of Little Bee honey which scored an inhibition percentage (I%) ≥ 50% during urease inhibition assays in the previous chapter has 16 compounds positively identified according to mass error, isotope and library of Metabolite Pilot Software on Drug Discovery Software installed in the Triple TOF^{TM} 5600 System Technology and Duospray^{TM} Ion Source system. The hexane extract of Little Bee honey (I% = 51.6% – 52.7%) and hexane extract of Q Bee honey (I% = 36.9% – 40.1%) both had 6 similar compounds in their matrix namely vardenafil, caproylresorcinol, hydrocortisone, altretamine, urapidil and oxeladin (Table 6.8). The 10 compounds that were found in hexane extract of Little Bee honey but not in other hexane

extracts of other honeys are clotiapine, sulfaquinoxaline, Lisinopril, raubasine, hydroxyquinoline, pilocarpine, levocabastine, aceclidine, diazoxide and benzylpenicillin (Table 6.8). There were 13, 5, 18, 14 and 16 compounds that were positively identified in hexane etracts of Raw honey, Gold Crest honey, Q Bee honey, Fleures honey and Siyakholwa honey respectively.

Table 6. 8: Compounds detected by positive mode of LC-MS in hexane extract of South African honey according to mass error, isotope and library scores

Raw Honey)	**Goldcrest)**	**Q Bee**	**(Fleures)**	**Siyakholwa)**	**DHE (Little Bee)**
ALL POSITIVE (Mass error, isotope and Library)	ALL POSITIVE (Mass error, isotope and Library)	ALL POSITIVE (Mass error, isotope and Library)	ALL POSITIVE (Mass error, isotope and Library)	ALL POSITIVE (Mass error, isotope and Library)	ALL POSITIVE (Mass error, isotope and Library)
Vardenafil Vincamine Yohimbine Tamoxifen Urapidil Dihydralazine Theophylline Sulfalene Sulfamethoxypyrida Acepromet azine Hydrocortisone 8-Hydroxyquinoline Azapropazone	Trapidil Urapidil Pergolide Oxprenolol Benzoctamine	Vardenafil Caproylresorcinol Alprenolol Moxisylyte Amisulpride Quinine Acetylsalicylamid Chinine Viquidil Hydrocortisone Altretamine Urapidil Cyclovalone Oxeladin Theophylline Theobromine 7-Aminodesmethylflu Buprofezin	Vincamine Yohimbine Caproylresorcinol Grepafloxacin Ropinirole Oxymetazoline Pilocarpine Urapidil Benzylpenicillin Moxisylyte Floctafenine Levobunolol Etiroxate Quinidine	Vardenafil Alprenolol Nandrolone Urapidil Diltiazem Adenine 7-aminodesmethylflu Vincamine Yohimbine Tamoxifen Salsalate Hydrocortisone Oxprenolol Caproylresorcinol Flupentixol	Vardenafil Clotiapine Sulfaquinoxaline Lisinopril Raubasine Caproylresorcinol Urapidil 8-Hydroxyquinoline Pilocarpine Hydrocortisone 21-ac Altretamine Levocabastine Aceclidine Diazoxide Benzylpenicillin Oxeladin
Total = 13	Total = 5	Total = 18	Total = 14	Total = 16	Total = 16

6.5 DISCUSSION

Acids, aldehydes, alcohol, ketones, terpenes, hydrocarbons, furans are chemical compounds that have been observed as components of honey in previous studies (Awaad *et al*., 2010; Alzahrani *et al*., 2012; Seanego *et al*., 2012). This current study, as well confirmed the presence of the named chemical compounds in honey extracts of South African honey. A compilation based on a spectral matching (≥ 90%) from NIST11 library was made on four petroleum extracts, two hexane extracts and three chloroform extracts. With reference to petroleum extract GC-MS analysis, Fleures honey, Gold Crest honey, Raw honey and Siyakholwa honey recorded 38.5%, 31%, 25.6% and 40.3% respectively, compounds that had 90% or greater spectral matching with the NIST11 library. Fleures honey and Gold Crest honey had an inhibition percentage above 50 % while Raw honey and Siyakholwa honey recorded an inhibition percentage (I%) that is less than 10%. However, the major difference in chemical composition was with Raw honey versus Fleures honey and Gold Crest but not Siyakholwa honey. Octane, hexatriacontane, hexadecanamide, dodecanamide, octadecanamide, decanamide, tetradecanamide, tetratriacontane, 2-methyloctacosane, cholesta-3,5-diene and cholesterol are compounds that were detected in Fleures honey, Gold Crest and Siyakholwa honey but not in Raw honey. If these compounds mattered the most towards urease inhibition, this could imply that an inhibition percentage of zero (0%) obtained by Siyakholwa extract of petroleum ether could be attributed to experimental error.

GC-MS analysis of Manuka honey and Siyakholwa honey extracts of hexane revealed a 51.2% and 28% respectively of NIST11.l library matching greater or equal to 90%. Furthermore, Manuka honey had a urease inhibition percentage (I%) of 50–53.2% compared to 10.3–11.2% scored by Siyakholwa honey. Octane, hexatriacontane, dodecanamide, octadecanamide, decanamide, tetradecanamide, tetratriacontane, 2-methyloctacosane,

cholesta-3, 5-diene and with the exception of hexadecanamide and cholesterol are the compounds with spectral matching of 90% or greater with the NIST11 library in the hexane extract of Manuka honey which scored an inhibition percentage (I%) that is greater than 50%. However, the Siyakholwa honey extract of hexane possessed most of these compounds. Chloroform extracts were also analysed by GC-MS technique, 28.4 % of compounds in Raw honey chloroform extract had a 90% or greater spectral matching with the NIST11 library while 14.6% of the Siyakholwa honey chloroform extracts revealed a 90% or greater spectral matching with the NIST11 library.The chemical compounds that were recorded had a 90% or greater spectral matching with the NIST11 library which leaves out unknown compounds which might also have contributed to high urease inhibition percentage.

The honey extracts that exhibited 50% or more inhibition percentage (%) of urease kinetic activity had an enormous area percentage represented by eicosane, cholest-5-en-3-ol, 9-octadecanamide (Z), cholesteryl valerate, silane, diethylheptyloxyoctadecyloxy, hexatriacontane and 1,4-epoxynaphthalene- 1(2H)-methanol. Therefore, these named compounds seem to be potential candidates responsible for urease inhibition. However, this does not rule out the other compounds as potential candidates for urease inhibition. Nevertheless, the findings of this study suggest that honey possesses a pool of valuable compounds for the medical fraternity that aims to utilise urease inhibitors to control/treat infections caused by urease depended organism.

LC-MS is a sensitive instrument in detecting molecules (Kamakshi *et al*., 2013). Several compounds were detected in South African honey based on mass error, isotope and library scores. The number of compounds detected by LC-MS per honey extract was dependent on the type of honey supporting the idea that compounds in honey depend on honey source or

geographical area from which the honey is harvested. However, the LC-MS analysis of drugs present in these honeys showed that agricultural activities such as pesticides application did not influence the medicinal value of the different types of honeys under study. This might be so because the majority of the compounds detected are not recommended as agricultural chemicals. Compounds that were detected include vardenafil, an oral drug used to treat impotence. This drug inhibits phosphodiesterase type 5 (PDE_5) resulting in an increased arterial blood leading to enlargement of corpus cavernosum (Ormrod *et al*., 2012). Also vincamine was detected and is a peripheral vasodilator leading to increase blood flow to the brain, mainly used to boost brainpower, memory and focus, this drug is a monoterpenoid indole alkaloid found in the leaves of Apocynaceae family (Na *et al*., 2016). Tamoxifen was also detected in Raw honey. This drug is used in the treatment of oestrogen receptor-positive breast cancer (Cuzick *et al*., 2015). Urapidil is an α-1-adrenoreceptor antagonist drug, important in lowering blood pressure (Grimm and Flack, 2011). In addition, dihydralazine can induce peripheral vasodilation resulting in lowered peripheral vascular resistance which leads to a drop in blood pressure (Yang *et al*., 2004). Hydrocortisone is a steroid hormone produced by the adrenal gland at a rate of 1mg/kg body mass per day under non-stressful conditions (Goding, 2009) and atretamine is a drug approved for minimising the symptoms of ovarian cancer (Kwon *et al*., 2009). Final, oxeladin also a common compound that was found in both Q Bee and Little Bee hexane extracts is a cough suppressant use to treat different cough etiologies (Zayed and Issa, 2013).

However, the detected drug such as sulfaquinoxaline, might have been incorporated into honey matrix through contaminated environment and water commonly visited by foraging bees. Sulfaquinoxaline is a sulphonamide antibiotic used in veterinary medicine, meat and poultry industries to treat coccidiosis a common protozoan disease (Adewole, 2012).

Administration of sulfaquinoxaline can be through drinking water or feed. Hydroxyquinoline has antiseptic, disinfectant and pesticide properties (Mandewale *et al*., 2015). Due to such properties, hydroxyquinoline has been shown to be active in preserving longevity of cut Gerbera flowers (Banaee *et al*., 2013).

Nevertheless, the identified compounds may act as bases for further studies in search of answers that will elucidate the anti-urease activity of honey extracts. While it remains difficult to pin point which compounds carry inhibitory activity towards urease, the identified compounds can be isolated and inhibition tests run on pure compounds so as to ascertain the effectiveness of individual compounds and combined effect of these compounds towards urease inhibition.

6.6 CONCLUSION

1. South African honey serves as a rich source of natural compounds whose importance calls for further studies, in addition the compounds in South African honeys compared very well with Manuka honey which is well known for its high level of medicinal value.
2. The anti-urease activity of honey seems to be influenced by geographical area being foraged by bees and agriculturally used chemicals did not influence the urease inhibitory potential of honey extracts.

CHAPTER 7

7.1 GENERAL DISCUSSION

Research on the treatment success of *H. pylori* infections has been on-going for years, however the challenges surpass discovery of correct antibiotic combinations including the high cost of combination therapy to counteract drug resistance, therapy failures and adverse side effects. Also rescue drugs, such as tetracycline, quinolones, rifambutin or bismuth salts, are either contraindicated or not approved for children. However, there is a growing trend in research towards the use of new therapeutic approaches such as traditional medicines because of proven effectiveness. Several studies have shown that there are natural products with anti-*H. pylori* compounds (Manyi-Loh *et al*., 2010; Manyi-Loh *et al.*, 2013; Nkomo *et al*., 2011; Franklin *et al*., 2012; Goswami *et al*., 2012) and honey being one of the readily available natural products that has been shown to possess bactericidal activity on different species of pathogenic micro-organisms (Ndip *et al*., 2007; Manyi-Loh *et al*., 2010). Of interest, the prolonged use of honey seems not to result in eradication resistant patterns as its action is not mediated via a single mechanism (Mohammad and Mohammad, 2007).

While Warren and Marshal., (1983) were the first to discover *H. pylori* in the human stomach, and the first as well to show that infection of the human stomach by *H. pylori* causes gastritis, there are many studies that have looked into the pathogenesis of *H. pylori*. Several virulence factors have been outlined which include the cytoxin-associated gene A (*cag*A), vacuolating cytotoxin gene A (*vac*A) and the urease enzyme. The presence of the *cag*A gene has been shown to be associated with increased intensity of gastric inflammation leading to serious clinical outcomes (Miernyk *et al*., 2011; Papadakos *et al*., 2013). Also *cag*A is associated with development of gastric cancer, hence it is referred to as an oncogene (Pereira *et al*., 2014). The *vac*A gene is present in all strains and has two variable regions, the *s* and *m*

regions. There is variation of vacuolating rate due to heterogeneity within the *vac*A gene. The strains possessing allelic combinations *s*1/*m*1 and *s*1/*m*2 produce high and moderate toxin levels while *s*2/*m*2 has little or no toxin (Cogo *et al*., 2011; Pereira *et al*., 2014).

The urease enzyme is produced by *H. pylori* in enormous quantities and is known to catalyse conversion of urea to ammonia and carbon dioxide, resulting in neutralisation of the pH in the human stomach. There are studies that have reported that *H. pylori* devoid of urease have difficulties in colonising the acidic environment (Tsuda *et al*., 1994; Amin *et al*., 2010). The strain MP01 is a mutant devoid of urease enzyme. Its failure to withstand a small deviation of pH 7 to pH 6 which led to significant loss of viability of the strain supports earlier findings by Amin *et al*. (2010) where they reported that urease-negative mutant strain of *H. pylori* does not enhance gastritis due to challenges with colonisation. The urease enzyme was extracted from different strains, including a commercial Jack bean urease, of interest is that honey active compounds affected these ureases in a similar fashion, this support earlier reports that ureases from different sources possess similar structures (Seck *et al*., 2013). The mucus lining of the human stomach undergoes reversible pH dependent solution-gel transition from viscous polymer solution to a soft gel at low pH (Celli *et al*., 2009). This implies that if urease inhibitors are to be a target towards *H. pylori* infection management, incidences of *H. pylori* infections might drop dramatically because infecting cells will fail to move through the mucus layer and attach to the epithelial lay of the human stomach where *H. pylori* cells normally attach with the help of adhesins. Attachment by adhesins is important in helping the organism to dodge being automatically flushed out by the continual peristaltic movement of the digestive tract.

Different honey types seem to have different therapeutic advantages; otherwise antioxidant potential of honey seems to be directly related to floral source. Therefore, to obtain much more positive results pertaining antimicrobial activity of honey, floral source of both mono-floral and hetero-floral honey needs consideration when searching and screening for the most active components of honey. Of interest, the prolonged use of honey seems not to result in eradication resistant patterns as its action is not mediated via a single mechanism (Mohammad and Mohammad, 2007). Therefore, this factor makes honey an important source of anti-*H. pylori* compounds if its mechanism of antibacterial action is understood.

The overall objective of the study was to identify honey extracts with anti-urease properties *in-vitro* and test their inhibitory potential on *cag*A positive, *vac*A positive and drug resistant *H. pylori* cultured under acidic environments, a mimicry of the human stomach. This study revealed that geographical location plays an important role in determining the medicinal value of honey. In South Africa, there are honey types that have urease inhibiting compounds i.e. petroleum ether extracts of Fleures honey and Gold Crest honey; the hexane extract of Little Bee honey; and the chloroform extract of Bush honey and Q Bee honey. Of importance is that the named South African honey extracts, compared very well with Manuka honey, a New Zealand product that has been well documented for its antibacterial activity.

The honey extracts with urease inhibiting activities were also used to determine the effect of inhibiting urease activity of *H. pylori* grown in acidic media. Strains of *H. pylori* possessing *cag*A, *vac*A and multidrug resistance properties were grown under controlled environments for 5 days. The observations made revealed that urease inhibitors can offset growth of *H. pylori* under acidic environments. The strains possessing *cag*A, *vac*A and drug resistance properties were affected in a similar manner which implies that targeting urease enzyme with

urease inhibitors as a way of treating *H. pylori* infection might be a success. Of importance is that the *in-vitro* analysis on urease inhibition is showing that current drug resistance patterns noticed with clarithromycin, amoxicillin, metronidazole and tetracycline is totally eliminated when using an approach that targets urease inhibition by solvent honey extracts to offset growth of *H. pylori* in acidic environments.

7.2 CONCLUSION

In line with the current study, the following can be drawn:

1. Treatment success with commonly used regimens for the treatment of *H. pylori* infections is under threat due to drug resistance.
2. *H. pylori* rely on the urease enzyme to survive the acidic gastric niche of the human host.
3. South African honeys are a source of valuable medicinal compounds with chemical constituents influenced by geographical location from which bees forage.
4. Inhibition of urease unavoidably impact negatively on the growth of *H. pylori* under acidic conditions.
5. GC-MS and LC-MS are useful techniques of identifying compounds in fractions of solvent extracts.

7.3 RECOMMENDATIONS

1. Further studies involving more advanced separation techniques are required to purify the anti-urease active compounds in honey so as to identify the exact compounds responsible for urease inhibition.

2. Melissopalynogy studies need to be done to determine the original source of compounds.

3. More studies are needed to elucidate the mechanism of action by which honey extracts inhibit urease activities.

3. Toxicology experiments to evaluate suitability of the honey extracts on safety measures prior to clinical trials.

4. Also animal studies to determine the therapeutic potential of the identified compounds would be of importance prior to subsequent clinical evaluation.

7.4 REFERENCES

Abebaw W., Kibret M. and Abera B. (2014). Prevalence and risk factors of *H. pylori* from dyspeptic patients in Northwest Ethiopia: A hospital based cross-sectional study. *Asian Pacific Journal of Cancer Prevention*. 15(11):4459–4463. doi:10.7314/APJCP.2014.15.11.4459.

Abu-Almaali H.M., Al-Khatabi H.A., Nasr-Allah H.A. and Al-Khafaji Z.M. (2012). Duplex PCR primers for detection of *Helicobacter pylori* DNA directly from gastric biopsy samples. *Kerbala Journal of Pharmaceutical Sciences*. (3):201–212.

Abu-Sbeih R.S., Hawari A.D., Hassawi D.S. and Al-Daghistani H.I. (2014). Isolation and detection of *Helicobacter pylori* from patients suffering from peptic ulcer using biochemical tests and molecular techniques. *American Journal of Biochemistry and Biotechnology*. 10(1):58–68. doi:10.3844/ajbbsp.2014.58.68.

Achtman, M., Azuma, T., Berg, D.E., Ito. Y., Morelli, G., Pan, Z-J., Suerbaum. S., Thompson, S.A., van der Ende, A. and van Doorn, L.J. (1999). Recombination and clonal groupings within *Helicobacter pylori* from different geographical regions. *Molecular Microbiology*. 32: 459–470.

Adeniyi B.A and Anyiam F.M. (2004). *In vitro* Anti-*Helicobacter pylori* potential of methanol extract of *Allium ascalonicum* Linn. (Liliaceae) leaf: Susceptibility and effect on urease activity. *Journal of Phototherapy Research*. 18:358–361.

Adewole, S.O. (2012). The efficacy of drugs in the treatment of coccidiosis in chicken in selected poultries. *Academic Research International*. 2(1):20–24.

Aggad H. and Guemour D. (2014). Honey antibacterial activity. *Medicinal and Aromatic Plants*. 3:2. http://dx.doi.org/10.4172/2167-0412.1000152

Aguemon B.D., Struelens M.J., Massougbodji A. and Ouendo E.M. (2005). Prevalence and risk-factors for *Helicobacter pylori* infection in urban and rural Beninese populations. *Clinical Microbiology and Infectious Diseases*. 11(8):611–617.

Ahmed K.S., Ghebremedhin A.A., Khan A.A., Tiwari S.K., Ahi J.D. and Ahmed I. (2012). Determination of antibiotic sensitivity pattern of *Helicobacter pylori* isolates from South India population by epsilometer test (E-Test). *Advances in Microbiology*. 2:263–267.

Ahmed K.S., Khan A.A., Ahmed I., Tiwari S.K., Habeeb A., Ahi J.D., Abid Z., Ahmed N. and Hahibullah C.M. (2007a). Impact of household hygiene and water source on the prevalence and transmission of *H. pylori*: A South Indian perspective. *Singapore Medical Journal.* 48(6):543–549.

Ahmed K.S., Khan A.A., Ahmed I., Tiwari S.K., Habeeb M.A., Ali S.M., Ahi J.D., Abid Z., Alvi A., Hussain M.A., Ahmed N. and Habibullah C.M. (2006). Prevalence study to elucidate the transmission pathways of *Helicobacter pylori* at oral and gastroduodenal sites of a South Indian population. *Singapore Medical Journal*. 47(4):291–296.

Ahmed, K., Farzana, R., Walter, M., Godfrey, L. and Martin, H. (2007b). Histopathological profile of gastritis in adult patients seen at a referral hospital in Kenya. *World Journal of Gastroenterology*. 14:4117–4121.

Al M.L., Daniel D., Moise A., Bobis O., Laslo L. and Bogdanov S. (2009). Physico-chemical and bioactive properties of different floral origin honeys from Romania. *Food Chemistry*. 112:863–867

Al M.L., Daniel D., Moise A., Bobis O., Laslo L. and Bogdanov S. (2009). Physico-chemical and bioactive properties of different floral origin honeys from Romania. *Food Chemistry*. 112:863–867.

Al Shukor N., Van Camp J., Gonzales G.B., Staljanssens D., Struijs K., Zotti M.J., Raes K. and Smagghe G. (2013). Angiotensin-converting enzyme inhibitory effects by plant phenolic compounds: a study of structure activity relationships. *Journal of Agricultural and Food Chemistry*. 61(48):11832–9.

Alissandrakis E., Daferera D., Tarantilis P.A., Polissiou m, and Harizanis P.C. (2003). "Ultrasound-assisted extraction of volatile compounds from citrus flowers and citrus honey," *Food Chemistry*. 82(4):575–582.

Ali-Waili N.S. (2004). Effects of Daily Consumption of Honey Solution on Hematological Indices and Blood Levels of Minerals and Enzymes in Normal Individuals. *Journal of Medicinal Food*. 6(2): 135–140. https://doi.org/10.1089/109662003322233549

Aljadi A.M.and Yusoff K.M. (2002). Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turkey Journal of Medical Science*. 33:229–236.

Allan E., Dorrell N., Foynes S., Anyim M. and Wren B.W. (2000) Mutational analysis of genes encoding the early flagellar components of *Helicobacter pylori*: evidence for transcriptional regulation of flagellin: A biosynthesis. *Journal of Bacteriology*. 182(18):5274–5277.

Allen A. and Flemstrom G. (2005). Gastroduodenal mucus bicarbonate barrier: Protection against acid and pepsin. *American Journal of Physiology*. 288:C1–19

Allen, L-A. H. (2000). Modulating phagocyte activation: the pros and cons of *Helicobacter pylori* virulence factors. *The Journal of Experimental Medicine*. 191, 1451–1454.

Alm, R.A., Ling, L. and Moiretal, D.T. (1999). "Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*". *Nature.* 397(6715): 176–180.

Al-Marhoon, M.S., Nunn, S. and Soames R.W. (2004). The Association Between *cag*A+ *H. pylori* Infection and Distal Gastric Cancer: A Proposed Model. *Digestive Diseases and Sciences.* Vol. 49, Nos. 7(8):1116–1122.

Al-Naama R.T. (2009). Evaluation of *in-vitro* inhibitory effect of honey on some microbial isolate. *Journal of Bacteriology Research*. 1(6):064-067.

Al-Thwai A.N. and Ali S.F. (2013). Detection of *Helicobacter Pylori* in saliva and biopsy specimens of some Iraqi patients using PCR technique. *International Journal of Advanced Biological Research*. 3(4):593–598.

Alzahrani H.A., Alsabehi R., Boukraâ L., Abdellah F., Bellik Y. and Bakhotmah B.A. (2012). Antibacterial and antioxidant potency of floral honeys from different botanical and geographical origins. *Molecules*. (17):10540–10549. doi:10.3390/molecules170910540.

Amin M., Anwar F., Naz F., Mehmood T. and Saari N. (2013). Anti-*Helicobacter pylori* and Urease Inhibition Activities of Some Traditional Medicinal Plants. *Molecules.* (18):2135–2149. doi:10.3390/molecules18022135.

Amin M., Iqbal M.S., Hughes R.W., Khan S.A., Reynolds P.A., Enne V.I., Rahman S. and Mirza A.S. (2010). Mechanochemical synthesis and in vitro anti-*Helicobacter pylori*

and urease inhibitory activities of novel zinc (II)-famotidine complex. *Journal of Enzyme Inhibition and Medicinal Chemistry*. 25:383–3890.

Amin, M., Anwar, F., Janjua, M.R.S.A., Iqbal, M.A. and Rashid, U. (2012). Green Synthesis of Silver Nanoparticles through Reduction with Solanum xanthocarpum L. Berry Extract: Characterization, antimicrobial and urease inhibitory activities against *Helicobacter pylori*. *International Journal of Molecular Science*. 13:9923–9941.

Ampuero S., Bogdanov S, and Bosset J.O. (2004). "Classification of unifloral honeys with an MS-based electronic nose using different sampling modes: SHS, SPME and INDEX," *European Food Research and Technology*. 218(2):198–207.

Andrews G.L., Simons B.L., Young J.B., Hawkridge A.M. and Muddiman D.C. (2011). Performance characteristics of a new hybrid Triple Quadrupole Time of Flight Tandem Mass Spectrometer. *Analytical Chemistry*. 83(13):5442-5446.

Anukam, K.C., Osazuwa, E., Osemene, G.I., Ehigiagbe, F., Bruce, A.W. and Reid, G. (2006). Clinical study comparing probiotic *Lactobacillus* GR-1 and RC-14 with metronidazole vaginal gel to treat symptomatic bacterial vaginosis. *Microbes and infection*. 8(12-13):2772–2776.

Arenz T., Antos D., Ru¨ssmann H., Alberer M., Buderus S., Kappler M. and Koletzko S. (2006). Esomeprazole-based 1-week triple therapy directed by susceptibility testing for eradication of *Helicobacter pylori* infection in children. *Journal of Pediatric Gastroenterology and Nutrition.* 43:180–184.

Arevalo-Galvis A., Trespalacios-Rangel A.A., Otero W., Mercado-Reyes M.M. and Poutou-Pinales R.A. (2012). Prevalence of *cag*A, *vac*A, *bab*A2 and *ice*A genes in *H. pylori*

strains isolated from Colombian patients with functional dyspepsia. *Polish Journal of Microbiology.* 61(1):33-40.

Arevalo-Galvis A., Trespalacios-Rangell A.A., Otero W., Mercado-Reyes M.M. and Poutou-Pinales R.A. (2012). Prevalence of *cag*A, *vac*A, *bab*A and *ice*A genes in *H. pylori* strains isolated from Colombian patients with functional dyspepsia. *Polish Journal of Microbiology*. 61:33–40.

Arr´aez-Rom´an D., G´omez-Caravaca A.M., G´omez-Romero M., Segura-Carretero A. and Fern´andez-Guti´errez A. (2006). Identification of phenolic compounds in rosemary honey using solid-phase extraction by capillary electrophoresis–electrospray ionization-mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis*. 41:1648–1656.

Atherton J.C. (1997). The clinical relevance of strain types of *Helicobacter pylori*. *Gut.* 40:701-703

Atherton J.C., Cao P., Peek Jr R.M., Tummuru M.K., Blaser M.J. and Cover, T.L. (1995). Mosaicism in vacuolating cytotoxin alleles of *Helicobacter pylori*. Association of specific *vac*A types with cytotoxin production and peptic ulceration. *Journal of Biological Chemistry*. 270:17771–17777.

Awaad S.A., El-Meligy M.R., and Soliman A.G. (2013). Natural products in treatment of ulcerative colitis and peptic ulcer. *Journal of Saudi Chemical Society*. 17:101–124

Awuku Y.A., Simpong D.L., Alhassan I.K., Tuoyire D.A., Afaa T. and Adu P. (2017). Prevalence *of Helicobacter pylori* infection among children living in a rural setting in Sub-Saharan Africa. *BCM Public Health*. 17:360. DOI 10.1186/s12889-017-4274-z

Awuku Y.A., Simpong D.L., Alhassan I.K., Tuoyire D.A., Afaa T. and Adu P. (2017). Prevalence of *Helicobacter pylori* infection among children living in a rural setting in Sub-Saharan Africa. *BMC Public Health*. 17:360. DOI 10.1186/s12889–017–4274-z

Aydin A., Onder G., Akarca U., Tekin F., Tuncyurek M. and Ilter T. (2006). Comparison of 1- and 2-week pantoprazole-based triple therapies in clarithromycin-sensitive and resistant cases. *European Journal of Internal Medicine*. 18:496–500.

Backert S., Clyne M. and Tegtmeyer N. (2011). Molecular mechanisms of gastric epithelial cell adhesion and injection of *cag*A by *Helicobacter pylori. Cell Communication and Signaling*. 9:28. http://www.biosignaling.com/content/9/1/28

Backert S., Tegtmeyer N. and Selbach M. (2010). The versatility of *Helicobacter pylori cag*A effector protein functions: the master key hypothesis. *Helicobacter* 15:163–176.

Bago J., Majstorović K., Belošić-Halle Z., Kućišec N., Bakula V., Tomić M., Bago P. and Troskot R. (2010). Microbial resistance of *H. pylori* to the outcome of 10-days vs. 7-days Moxifloxacin based therapy for the eradication: a randomized controlled trial. *Annals of Clinical Microbiology and Antimicrobials*. 9(13):1–6.

Bakhti S.Z., Latifi-Navid S., Mohammadi S., Zahri S., Bakhti F.S., Feizi F., Yazdanbod A and Siavoshi F. (2015). Relevance of *Helicobacter pylori vac*A 3’ – end region polymorphism to gastric cancer. *Wiley Online Library*. 21(4): 305-316. https://doi.org/10.1111/hel.12284

Banaee S., Hadavi E. and Moradi P. (2013). Effect of Ascorbic Acid, 8-Hydroxyquinoline Sulfate and Sucrose on the Longevity and Anthocyanin Content of Cut Gerbera Flowers. Current Agriculture Research Journal. 1(1):29–33.

Bang L.M., Buntting C. and Molan P. (2003). The Effect of Dilution on the Rate of Hydrogen Peroxide Production in Honey and Its Implications for Wound Healing. *The Journal of Alternative and Complementary Medicine*. 9(2):267–273.

Barra M.P.G., Ponce-Díaz M.C., Venegas-Gallegos C. (2010). Volatile compounds in honey produced in the central valley of Ñuble Province, Chile. *Chilean Journal of Agricultural Research.* 70(1):75–84.

Barra M.P.G., Ponce-Díaz M.C., Venegas-Gallegos C. (2010). Volatile compounds in honey produced in the central valley of Ñuble Province, Chile. *Chilean Journal of Agricultural Research.* 70(1):75–84.

Bassily S., Frenck W.R., Mohareb W.E., Wierzba T., Savarino S., Hall E., Kotkat A., Naficy A., Hyams C.K. and Clemens J. (1999). Seroprevalence of *Helicobacter pylori* among Egyptian newborns and their mothers: A preliminary report. *American Journal of Tropical Medicine and Hygiene*, 61(1):37–40.

Benini S., Rypniewski W.R., Wilson K.S., Miletti S., Ciurli S., Mangani S. (1999). A new proposal for urease mechanism based on the crystal structures of the native and inhibited enzyme from *Bacillus pasteurii*: Why urea hydrolysis costs two nickels. *Structure*. 7:205–216.

Beswick E.J., Pinchuk I.V., Minch K., Suarez G., Sierra J.C., Yamaoka Y. and Reyes V.E. (2006). The *Helicobacter pylori* Urease B Subunit Binds to CD74 on Gastric

Epithelial Cells and Induces NF-κB Activation and Interleukin-8 Production. *Infection and Immunity*. 74(2):1148–1155.

Biesaga M. and Pyrzyn´ska K. (2013). Stability of bioactive polyphenols from honey during different extraction methods. *Food Chemistry*. 136:46–54.

Biesaga M. and Pyrzyn´ska K. (2013). Stability of bioactive polyphenols from honey during different extraction methods. *Food Chemistry*. 136:46–54.

Bolek, B. K., Salih, B. A. and Sander, E. (2007). Genotyping of *Helicobacter pylori* strains from gastric biopsies by multiplex polymerase chain reaction. How advantageous is it? *Diagnostic Microbiology and Infectious Disease*. 58:67–70.

Bolek, B. K., Salih, B. A. and Sander, E. (2007). Genotyping of *Helicobacter pylori* strains from gastric biopsies by multiplex polymerase chain reaction. How advantageous is it? *Diagnostic Microbiology and Infectious Disease*. 58:67–70.

Boyanova L. and Mitov I. (2010). Geographic map and evolution of primary *Helicobacter pylori* resistance to antibacterial agents. *Expert Review of Anti Infective Therapy.* 8:59–70.

Braganca S.M., Azevedo N.F., Simoes L.C., Keevil C.W. and Vieira M.J. (2007). Use of fluorescent in situ hybridization for the visualization of Helicobacter pylori in real drinking water biofilms. *Water Science and Technology*. 55(89):387-393.

Brandt, S., Kwok, T., Hartig, R., Konig, W., and Backert, S. (2005). NF-kappaB activation and potentiation of pro-inflammatory responses by the *Helicobacter pylori cag*A protein. *Proceedings of the National Academy of Sciences of the United States of America*. 102:9300–9305.

Breitenbach JM, Hausinger RP. (1988). *Proteus mirabilis* urease: Partial purification and inhibition by boric acid and boronic acids. *Biochemical Journal*. 250:917–920.

Brown L.M. (2000). *Helicobacter pylori*: epidemiology and routes of transmission. *Epidemiology Reviews*. 22:283–297.

Brudzynski K. (2006). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Canadian Journal of Microbiology*. 52:1228–1237.

Brudzynski K. (2007). Effect of hydrogen peroxide on antibacterial activities of Canadian honeys. *Canadian Journal of Microbiology*. 52(12):1228–1237. DOI10.1139/w06-086

Burocua C., Delchier J.C., Courillon-Mallet A. and Fauchere J.L. (2013). "Comparative evaluation of 29 commercial *Helicobacter pylori* serological kits," *Helicobacter*. 18(3):169–179.

Burucoa C., Lhomme V. and Fauchere J. L. (1999). Performance criteria of DNA fingerprinting methods for typing of Helicobacter pylori isolates: Experimental Results and Meta-Analysis. *Journal of Clinical Microbiology*. 37(12):4071–4080.

Cambau. E., Allerheiligen V., Coulon C., Corbel C., Lascols C., Deforges L., Soussy C.J., Delchier J.C.and Megraud F. (2009). Evaluation of a New Test, GenoType HelicoDR, for Molecular Detection of Antibiotic Resistance in *Helicobacter pylori*. *Journal of Clinical Microbiology*. 47(11):3600–3007.

Campone L., Piccinelli A.L., Pagano I., Carabetta S., Di Sanzo R., Russo M and Rastrelli L. (2014). Determination of phenolic compounds in honey using dispersive liquid–liquid microextraction. *Journal of Chromatography*. 1334:9–15.

Carlsohn E., Nystrom J., Bolin I., Nilsson C.L. and Svennerholm A.M. (2006). HpaA is essential for *Helicobacter pylori* colonization in mice. *Infection and Immunity*. 74(2):920–926.

Carter E.L., Flugga N., Boer J.L., Mulrooney S.B., Hausinger R.P. (2009). Interplay of metal ions and urease. *Metallomics.* 1:207–221.

Celli J.P., Turnerb B.S., Afdhal N.H., Keates S., Ghiran I., Kelly C.P., Ewoldt R.H., McKinley G.H., Sod P., Erramillia S. and Bansila R. (2009). *Helicobacter pylori* moves through mucus by reducing mucin viscoelasticity. *Proceedings of National Academy of Sciences.* 106(34):14321–14326.

Cellini L., Di Campli E., Di Bartolomeo S., Bessa L.J., Baffoni M. and Di Giulioa M. (2014). New Transport Medium for Cultural Recovery of *Helicobacter pylori*. *Journal of Clinical Microbiology*. 52(12): 4325–4329.

Chey W.D. and Wong B.C. (2007). American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *The American Journal of Gastroenterology.* 102:1808–1825 [PMID: 17608775 DOI: 10.1111/j.1572-0241.2007.01393.x]

Chey W.D., Leontiadis G.I., Howden C.W. and Moss S.F. (2017). ACG Clinical Guideline: Treatment of *Helicobacter pylori* Infection. *American Journal of Gastroenterology*. 112:212–238; doi: 10.1038/ajg.2016.563

Christie P.J., Whitaker N. and Gonzalez-Rivera C. (2013). Mechanism and structure of the bacterial type IV secretion systems. *Biochimica et Biophysica Acta*. 1843:1578–1591.

Chuah S.K., Liang C.M., Lee C.H., Chiou S.S., Chiu Y.C., Hu M.L., Lu L.S., Chou Y.P., Chang K.C., Kuo C.H., Kuo C.M., Hu T.H. and Tai W.C. (2016). A randomized control trial comparing 2 levofloxacin-containing second line therapies for *Helicobacter pylori* eradication. *Medicine*. (19):e3586. Doi:10.1097/MD.000000000003586.

Cogo L.L., Monteiro C.L.B., Nogueira K.S., Palmeiro J.K., Ribeiro M.L., Camargo E.R., Neves D.L., Nascimento A.J. and Costa L.M.D. (2011). Characterization of virulence genes *cag*A and *vac*A in *Helicobacter pylori* and their prevalence in gastrointestinal disorders. *Brazilian Journal of Microbiology*. 42: 1289–1295 ISSN 1517–8382

Covacci A., Censini S., Bugnoli M., Petracca R., Burroni D., Macchia G., Massone A., Papini E., Xiang Z. and Figura N. (1993). Molecular characterization of the 128- kDa immunodominant antigen of *Helicobacter pylori* associated with cytotoxicity and duodenal ulcer. *Proceedings of National Academy of Sciences of the United States of America.* 90:5791–5795.

Cover T. L. and Blanke S. R. (2005). *Helicobacter pylori vac*A, aparadigm for toxin multi-functionality. *Nature Reviews Microbiology*. 3:320–332.

Cover T.L., Krishna U.S., Israel D.A. and Peek R.M. Jr. (2003). Induction of gastric epithelial cell apoptosis by *Helicobacter pylori* vacuolating cytotoxin. *Cancer Research*. 63(5):951–957. PMID: 12615708

Cussac V., Ferrero R.I. and Labigne A. (1992). Expression of *Helicobacter pylori* urease genes in *Escherichia coli* grown under nitrogen limiting conditions. *Journal of Bacteriology*. 174:2466–2473.

Cuzick J., sestak I., Cawthorn S., Hamed H., Holli K., Howell A., Forbes J.F. (2015). Tamoxifen for prevention of breast cancer: extended long-term follow-up of IBIS-I breast cancer prevention trial. *The Lancet Oncology*. 16(1):67–75

De Francesco V., Zullo A., Hassan C., Giorgio F., Rosania R. and Ierardi E. (2011). "Mechanisms of *Helicobacter pylori* antibiotic resistance: an updated appraisal." *World Journal of Gastrointestinal Pathophysiology*. 2(3):35–41.

Deloney C. R. and Schiller N. L. (2000). Characterization of an in-vitro selected amoxicillin resistant strain of *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy*. 44:3363–3373.

Dharmalingam S., Rao U. A., Jayaraman G. and Thyagarajan S. P. (2003). Relationship of plasmid profile with the antibiotic sensitivity pattern of *Helicobacter pylori* isolates from peptic ulcer disease patients in Chennai. *Indian Journal of Medical Microbiology*. 21(4):257–261.

Díaz-Sánchez A.G., Alvarez-Parrilla E., Martínez-Martínez A., Aguirre-Reyes L., Orozpe-Olvera J.A., Ramos-Soto M.A., Núñez-Gastélum J.A., Alvarado-Tenorio B. and de la Rosa L.A. (2016). Inhibition of Urease by Disulfiram, an FDA-Approved Thiol Reagent Used in Humans. *Molecules*. 21:1628. doi:10.3390/molecules21121628

Dixon N.E., Hinds J.A., Fihelly A.K., Gazolla C., Winzor D.J. and Blakeley R.L. (1980). Jack bean urease (EC 3.5.1.5): the molecular size and the mechanism of inhibition by hydroxamic acids. Spectrophotometric titration by enzymes with reversible inhibitors. *Canadian Journal of Biochemistry*. 58:1323

Douillard F.P., Ryan K.A., Hinds J. and O'Toole P.W. (2009). Effect of FliK mutation on the transcriptional activity of the σ54 sigma factor RpoN in *Helicobacter pylori*. *Microbiology*. 155(Pt 6):1901–1911.

Dressman J. B., Berardi R. R., Dermentzoglou L. C., Russell T.L., Schmaltz S.P, Barnett J. L. (1990). Upper gastrointestinal (GI) pH in young, healthy men and women. *Pharmaceutical Research*. 7:756–761. PMID: 2395805

Du N., Chen M., Liu Z., Sheng L., Xu H. and Chen S. (2012). Kinetics and mechanism of jack bean urease inhibition by Hg^{2+}.*Chemistry Central Journal*. 6:154. http://journal.chemistrycentral.com/content/6/1/154.

Dube C., Nkosi T.C., Clarke A.M., Mkwetshana N., Green E. and Ndip R.N. (2009). *Helicobacter pylori* antigenemia in an asymptomatic population of Eastern Cape Province, South Africa: public health implications. *Reviews on Environmental Health*. 24(3):249–255.

Dunn B. E., Campbell G.P., Perez-Perez G.I., and Blaser M.J. (1990). Purification and characterization of urease from *Helicobacter pylori*. *Journal of Biological Chemistry*. 265:9464–9469.

Eaton, K. A., Morgan D.R. and Krakowka S. (1992). Motility as a factor in the colonisation of gnotobiotic piglets by *Helicobacter pylori*. *Journal of Medical Microbiology*. 37:123–127.

Ebule I.A., Djune F.A. K., Sitedjeya M.I. L., Tanni B., Heugueu C., Longdoh A.N., Noah N.D., Okomo A.M.C., Paloheimo L., Njoya O. and Syrjanen, K. (2017). Prevalence of *H. pylori* Infection and Atrophic Gastritis among dyspeptic subjects in Cameroon

using a Panel of Serum Biomarkers (PGI, PGII, G-17, HpIgG). *Scholars Journal of Applied Medical Sciences*. 5(4A):1230–1239

Eddy J.J. and Gideonsen M.D. (2005). Topical honey for diabetic foot ulcers. *Journal of Family Practice*. 54:1–6.

Eisig J.N., Silva F.M., Barbuti R.C., Navarro-Rodriguez T., Moraes-Filho J.P.P. and Pedrazzoli J. (2011). *Helicobacter pylori* antibiotic resistance in Brazil: clarithromycin is still a good option. *Arquivos De Gastroenterologia*. 48(4):261–264.

Eleazu C.O., Iroaganachi M.A., Eleazu K.C. and Okoronkwo J.O. (2013). Determination of physico-chemical composition, microbial quality and free radical scavenging activities of some commercially sold honey samples in Aba, Nigeria: 'The effect of colours'. *International Journal of Biomedical Research*. Doi:10.7439/ijbr

Eshraghian A. (2014). Epidemiology of Helicobacter pylori infection among the healthy population in Iran and countries of the Eastern Mediterranean Region: A systematic review of prevalence and risk factors. *World Journal of Gastroenterology*. 20(46):17618–17625. doi: 10.3748/wjg.v20.i46.17618

Espinoza, C., Vazquez, G., Mendez, M., Vargas, R. and Cerezo, G. J. (2011) *Journal of Clinical Microbiology*. 49 (4):1650.

Essawi T., Hammoudec W., Sabri I., Sweiden W. and Farraji M.A. (2013). Determination of *Helicobacter pylori* virulence genes in gastric biopsies by PCR. *ISRN Gastroenterology*. http://dx.doi.org/10.1155/2013/606258.

Farrugia M.A., Macomber L. and Hausinger R.P. (2013). Biosynthesis of the urease metallocenter. *Journal of the Biological Chemistry*. 288:13178–13185. doi: 10.1074/jbc.R112.446526.

Fasciana T., Calà C., Bonura C., Carlo E.D., Matranga D., Scarpulla G., Manganaro M., Camilleri S. and Giammanco A. (2015. Resistance to clarithromycin and genotypes in *Helicobacter pylori* strains isolated in Sicily. *Journal of Medical Microbiology*. 64(11):1408-1414. DOI:10.1099/jmm.0.000163

Fathi M.S., EL-Folly R.F., Hassan R.A. and El-Arab M.E. (2013). Genotypic and phenotypic patterns of antimicrobial susceptibility of *Helicobacter pylori* strains among Egyptian patients. *The Egyptian Journal of Medical Human Genetics*. 14:235–246.

Faundez G., Troncoso M. and Figueroa G. (2002). *Cag*A and *vac*A in strains of *Helicobacter pylori* from ulcer and non-ulcerative dyspepsia patients. *BCM Gastroenterology*. 2:20. http://www.biomedcentral.com/1471-230X/2/20

Fong Y.H., Wong H.C., Yuen M.H., LauP.H., Chen Y.W. and Wong K. (2013). Structure of ureG/ureF/ureH complex reveals how urease accessory proteins facilitate maturation of *Helicobacter pylori* urease. *PLOS Biology*. https://doi.org/10.1371/journal.pbio.1001678

Franco A. T., Johnston E., Krishna U., Yamaoka Y., Israel D. A., Nagy T. A., Wroblewski L. E., Piazuelo M. B., Correa P. and Peek R. M., Jr. (2008). Regulation of gastric carcinogenesis by *Helicobacter pylori* virulence factors. *American Association for Cancer Research*. 68:379–387.

Franklin A., Rao U.A., Vijayakumar A. and Srikumar R. (2012). *In-vitro* Anti-*Helicobacter Pylori* Activity of *Emblica officinalis. International Journal of Microbiological Research*. 3 (3): 216–220.

Furuta, T. and Delchier, J. C. (2009). *Helicobacter pylori* and non-malignant diseases. *Helicobacter*. 14:29-35.

Gardner T.B. and Hill D.R. (2001). Treatment of giardiasis. *Clinical microbiology reviews.* 14(1):114–28. https://doi.org/10.1128/CMR.14.1.114-128.2001 PMID: 11148005.

Gatta L., Zullo A. and Perna F. (2005). A 10-day levofloxacinbased triple therapy in patients who have failed two eradication courses. *Alimentary Pharmacology and Therapeutics*. 22(1):45–49.

Gebert B., Fischer W., Weiss E., Hoffmann R. and Haas R. (2003). *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science*. 301(5636):1099–102. https://doi.org/10.1126/science.1086871 PMID: 12934009

Gerrits M.M., van der Wouden E.J., Bax D.A., van Zwet A.A., van Vliet A.H.M., de Jong A., Kusters J.G., Thijs J.C. and Kuipers E.J. (2004). Role of the rdxA and frxA genes in oxygen-dependent metronidazole resistance of *Helicobacter pylori. Journal of Medical Microbiology*. 53:1123–1128.

Ghotaslou R., Milani M., Akhi M.T., Hejazi M.S., Nahaei M.R., Hasani A. and Sharifi Y. (2013). Relationship between Drug Resistance and *cag*A Gene in *Helicobacter pylori*. *Jundishapur Journal of Microbiology*. 6(10): e8480.

Gisbert J.P. (2012). Rescue Therapy for *Helicobacter pylori* Infection. *Gastroenterology Research and Practice.* 10.1155/2012/974594.

Gisbert, J.P. (2009). Second-line rescue therapy of *Helicobacter pylori* infection. *Therapeutic Advances in Gastroenterology*. 2(6):331–356. doi: 10.1177/1756283X09347109.

Glocker E., Stueger H.P. and Kist M. (2007). "Quinolone resistance in *Helicobacter pylori* isolates in Germany," *Antimicrobial Agents and Chemotherapy*. 51(1):346–349.

Goding A.M. (2009). The effect of hydrocortisone on angiogenesis. *Journal of the British Institute of Organ Studies* 80(2):59–65. https://doi.org/10.1893/011.080.0202

Goodwin C. S., Armstrong J. A., Chilvers T., Peters M., Collins M. D., Sly L., McConnel W. and Harper W. E. S. (1989). Transfer of *Campylobacter pylori* and *Campylobacter mustelae* to *Helicobacter* gen. nov. as *Helicobacter pylori* comb. nov. and *Helicobacter mustelae* comb. nov., respectively. *International Journal of Systematic and Evolutionary Bacteriology*. 39:397–405.

Gopal R., Elamurugan T.P., Kate V., Jagdish S. and Basu D. (2013). Standard triple versus levofloxacin based regimen for eradication of *Helicobacter pylori*. *World Journal of Gastrointestinal Pharmacology and Therapeutics*. 4(2):23–27. doi:10.4292/wjgpt.v4.i2.23

Gościniak G., Biernat M., Grabińska J., Bińkowska A., Poniewierka E. and Iwańczak B. (2014). The Antimicrobial Susceptibility of *Helicobacter pylori* Strains Isolated from Children and Adults with Primary Infection in the Lower Silesia Region, Poland. *Polish Journal of Microbiology*. 63(1):57-61.

Gościniak G., Biernat M., Grabińska J., Bińkowska A., Poniewierka E. and Iwańczak B. (2014). The Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from

children and adults with primary infection in the lower Silesia region, Poland. *Polish Journal of Microbiology*. 63(1):57–61.

Goswami S., Bhakuni S.R., Chinniah A., Pal A., Kar K.S. and Dasa K.P. (2012). Anti-*Helicobacter pylori* Potential of Artemisinin and Its Derivatives. *Journal of Antimicrobial Agents and Chemotherapy*. 56(9):4594–4607.

Graham D.Y. and Shiotani A. (2008). New concepts of resistance in the treatment of *Helicobacter pylori* infections. *Nature Clinical Practice Gastroenterology and Hepatology*. 5: 321–331.

Grant C.A. (2014). Use of NBPT and ammonium thiosulphate as urease inhibitors with varying surface placement of urea and urea ammonium nitrate in production of hard red spring wheat under reduced tillage management. *Canadian Journal of Plant Science*. 94:329–335. doi:10.4141/CJPS2013-289.

Grimm R.H. and Flack J.M. (2011). Alpha 1 adrenoreceptor antagonists. *The Journal of Clinical Hypertension*. 13(9):654-657.

Grove D.I., McLeay R.A.B., Byron K.E. and Koutsouridis G. (2001). Isolation of *Helicobacter pylori* after transport from a regional laboratory of gastric biopsy specimens in saline, Portagerm pylori or cultured on chocolate agar. *Pathology*. 33:362–364.

Gu H. (2017). Role of Flagella in the Pathogenesis of *Helicobacter pylori*. *Current Microbiology*. 74:863–869.

Haas, R. (2002). *Helicobacter pylori*. In: Molecular infection biology: interaction between microorganisms and cells. Edited by Hacker, J. and Heesemann, J. A. John Wiley &

Sons, Inc. and Specktrum Akademischer Verlag co-publication, Heidelberg – Berlin, pp 256–258.

Hamilton-Miller JMT and Gargan RA. (1979). Rapid screening for urease inhibitors. *Journal of Investigative and Clinical Urology*. 16:327–328.

Hanafi N.F., Mikhael I.L. and Younan D.Y. (2017). Prevalence of *Helicobacter pylori* Antibodies in Egyptians with Idiopathic Thrombocytopenic purpura and in the General Egyptian Population: A Comparative Study. *International Journal of Current Microbiology and Applied Sciences.* ISSN: 2319-7706, 6(5):2482–2492.

Harizanis P.C., Alissandrakis E., Tarantilis P.A. and Polissiou M. (2008). Solid-phase microextraction/gas-chromatographic/mass spectrometric analysis of *p* - dichlorobenzene and naphthalene in honey. *Taylor and Francis online*. 25(10): 1272-1277

Harris P.R., Ernst P.B., Kawabata S., Kiyono H., Graham M.F. and Smith P.D. (1998). Recombinant *Helicobacter pylori* urease activates primary mucosal macrophages. *Journal of Infectious Diseases*. 178(5):1516–1520.

Harrison U., Fowora M.A., Seriki A.T., Loell E., Mueller S., Ugo-Ijeh M., Onyekwere C.A., Lesi O.A., Otegbayo J.A., Akere A., Ndububa D.A, Adekanle O., Anomneze E., Abdulkareem F.B., Adeleye I.A., Crispin A., Rieder G., Fischer W., Smith S.I. and Haas R. (2017). *Helicobacter pylori* strains from a Nigerian cohort show divergent antibiotic resistance rates and a uniform pathogenicity profile. *Plos One*. https://doi.org /10.1371/ journal.pone. 0176454

Hashinaga M., Suzuki R., Akada J., Matsumoto T., Kido Y., Okimoto T., Kodama M., Murakami K. and Yamaoka Y. (2016). Differences in amino acid frequency in *cag*A

and *vac*A sequences of *Helicobacter pylori* distinguish gastric cancer from gastric MALT lymphoma. *Gut Pathogens*. 8:54. DOI 10.1186/s13099-016-0137-x

Hoopingarner R. and Nelson K. (1998). American foulbrood clean-up rate using three terramycin treatments. *American Bee Journal*. 128:120–121.

Hsu P.I., Wu D.C., Wu J.Y. and D. Y. Graham D.Y. (2011). Modified sequential *Helicobacter pylori* therapy: proton pump inhibitor and amoxicillin for 14 days with clarithromycin and metronidazole added as a quadruple (hybrid) therapy for the final 7 days. *Helicobacter*. 16(2):139–145.

Hu L., Foxall P.A., Russell R. and Mobley H.L.T. (1992). Purification of Recombinant *Helicobacter pylori* Urease Apoenzyme Encoded by *ure*A and *ure*B. *Infection and Immunity*. 60(7):2657–2666.

Hu L.T. and Mobley H.L. (1993). Expression of catalytically active recombinant *Helicobacter pylori* urease at wild-type levels in Escherichia coli. *Infection and Immunity*. 61:2563–2569.

Hu, L. T., and H. L. Mobley. 1990. Purification and N-terminal analysis of urease from *Helicobacter pylori. Infection and Immunity*. 58:992–998.

Huang X.J., Deng Z.M., Zhang Q., Li W.Y., Wang B.N. and Li M.Y. (2016). Relationship between the *iceA* gene of *Helicobacter pylori* and clinical outcomes. *Therapeutics and Clinical Risk Management*. 12:1085–1092.

Hussein N.R., Argent R.H., Marx C.K., Patel S.R., Robinson K. and Atherton J.C. (2010). *Helicobacter pylori* dupA is polymorphic, and its active form induces pro-inflammatory cytokine secretion by mono nuclear cells. *The journal of Infectious Diseases*. 202(2):261–269.

Ibuki T., Uchida Y., Hironaka Y., Namba K., Imada K. and Minamino T. (2013). Interaction between FliJ and FlhA, components of the bacterial flagellar type III export apparatus. *Journal of Bacteriology*. 195(3):466–473.

Irish J., Blair S. and Carter D.A. (2011). The Antibacterial Activity of Honey Derived from Australian Flora. *Antibacterial Honey from Australian Plants*. 6(3):e18229.

Jaganathan S.K. and Mandal M. (2009). Antiproliferative effects of honey and of its polyphenols: A review. *Journal of Biomedicine and Biotechnology*. doi:10.1155/2009/830616

Jeddar A., Kharsany A., Ramsaroop U.G., Bhamjee A., Hafejee I.E., Moosa A. (1985). The antibacterial action of honey. An *in vitro* study. *South African Medical Journal*. 67:257–258.

Jemilohun A.C., Otegbayo J.A., Ola S.O., Oluwasola O.A. and Akere A. (2011). Prevalence of *Helicobacter pylori* among Nigerian patients with dyspepsia in Ibadan. *Pan African Medical Journal*. http://www.panafrican-med-journal.com/content/article/6/18/full/

Jemilohun A.C., Otegbayo J.A., Ola S.O., Oluwasola O.A. and Akere A. (2010). Prevalence of *Helicobacter pylori* among Nigerian patients with dyspepsia in Ibadan. *Pan African Medical Journal*. http://www.panafrican-med-journal.com/content/article/6/18/full

Jiménez-Soto L.F. and Haas R. (2016). The *cag*A toxin of *Helicobacter pylori*: abundant production but relatively low amount translocated. *Scientific Reports*. 6:23227, DOI: 10.1038/srep23227

Jones K.R., Whitmire J.M. and Merrell D.S. (2010). A tale of two toxins: *Helicobacter pylori cag*A and *vac*A modulate host pathways that impact disease. *Frontiers in Microbiology*. doi: 10.3389/fmicb.2010.00115

Josenhans C., Labigne A. and Suerbaum S. (1995) Comparative ultrastructural and functional studies of *Helicobacter pylori* and *Helicobacter mustelae* flagellin mutants: both flagellin subunits, *Fla*A and *Fla*B, are necessary for full motility in *Helicobacter species*. *Journal of Bacteriology.* 177(11):3010–3020.

K. Goh., W. Chan, S. Shiota and Y. Yamaoka. (2011). Epidemiology of *Helicobacter pylori* infection and public health implications. *National Institute of Health*. 16(01):1-9. Doi:10.1111/j.1523-5378.2011.00874.x.

Kadayifci A., Buyukhatipoglu H., Savas M.C. (2006). Simsek I. Eradication of *Helicobacter pylori* with Triple Therapy: An Epidemiologic Analysis of Trends in Turkey Over 10 Years. *Journal of Clinical Therapeutics.* 20(11):960–1966.

Kadi R.H., Halawani E.M. and Abdelkader H.S. (2014). Prevalence of *H. pylori* strains harbouring *cag*A and iceA virulence genes in Saudi patients with gastritis and peptic ulcer disease. *Microbiology Discovery*. http://www.hoajonline.com/journals/pdf/2052-6180-2-2.pdf doi:10.7243/2052–6180–2–2

Kalali B., Mejias-Luque R., Javaheri A. and Gerhard M. (2014). *H. pylori* virulence factors: Influence on immune system and pathology. *Mediators of Inflammation*. http://dx.doi.org/10.1155/2014/426309

Kaltwasser H. and Schlegel H.G. (1966). NADH-Dependent coupled enzyme assay for urease and other ammonia-producing systems. *Analytical Biochemistry*. 1966; 16(1):132–138.

Kamakshi V.G., Bhat S.S. and Bhargava H.R. (2013). Antioxidative property and LC-MS analysis of Apis honey samples collected from different geographical locations of India. *World Applied Sciences Journal.* 25(6):850–853.

Kang D.W., Hwang W.C. and Park M.H. (2013). "Rebamipide abolishes *Helicobacter pylori cag*A-induced phospholipase D1 expression via inhibition of NfkappaB and suppresses invasion of gastric cancer cells". *Oncogene*. 32(30):3531–3542.

Karczewska E., Wojtas-Bonior I., Sito E., Zwoli˜nska-Wcisło M. and Budak A. (2011). Primary and secondary clarithromycin, metronidazole, amoxicillin and levofloxacin resistance to *Helicobacter pylori* in Southern Poland. *Pharmacological Reports*. 63(3):799–807.

Kassim M., Achoui M., Mustafa M.R., Mohd M.A. and Yusoff K.M. (2010). Ellagic acid, phenolic acids, and flavonoids in Malaysian honey extracts demonstrate in vitro anti-inflammatory activity. *Nutrition Research*. 30:650–659.

Katelaris PH. (2009). *Helicobacter pylori*: antibiotic resistance and treatment options. *Journal of Gastroenterology and Hepatology*. 24:1155–1157

Kaya A.D., OztOrk C.E., Akcan Y., Behcet M., Esra Karakoc E., Yucel M., Mislrlloglu M. and Tuncer S. (2007). Prevalence of *Helicobacter pylori* in symptomatic patients and detection of clarithromycin resistance using melting curve analysis. *Current Therapeutic Research.* 68(3):151–160.

Keszthelyi D. and Masclee A.D. (2012). Effects of proton pump inhibitor therapy in the distal gut: Putting the pieces together. *Digestive Diseases and Sciences*. 57:2487–2489. DOI:10.1007/s10620–012–2339–5.

Khalil M.I., Sulaiman S.A. and Boukraa L. (2010). Antioxidant properties of honey and its role in preventing health disorder. *The Open Nutraceuticals Journal*. 3:6–16

Khedmat H., Karbasi-Afshar R., Agah S. and Taheri S. (2013). *Helicobacter pylori* infection in the general population: A Middle East perspective. *Caspian Journal of Internal Medicine*. 4(4):745–753.

Kim J.M., Kim J.S. and Kim N. (2008). "Gene mutations of 23SrRNA associated with clarithromycin resistance in *Helicobacter pylori* strains isolated from Korean patients". *Journal of Microbiology and Biotechnology*. 18(9):1584–1589.

Kim J.S., Chang J.H., Chung S.I., Yum J.S. (1999). Molecular cloning and characterization of the *Helicobacter pylori fli*D gene, an essential factor in flagellar structure and motility. *Journal of Bacteriology*. 181(22):6969–6976.

Kobashi K., Takebe S., Terashima N. and Hase J. (1975). Inhibition of urease activity by hydroxamic acid derivatives of amino acids. *Journal of Biochemistry*. 77(4):837–843.

Koelz H. (1992). Gastric acid in vertebrates. *Scandinavian Journal of Gastroenterology*. 193:2–6.

Kokotkiewicz A. and Luczkiewicz M. (2009). Honeybush (Cyclopia sp) – A rich source of compounds with high antimutagenic properties. *Elsevier*. 80(1): 3–11

Kowalski M., Pawlik M., Konturek J.W. and Konturek S.J. (2006). *Helicobacter pylori* infection in coronary artery disease. *Journal of Physiology and Pharmacology*, 57:101–111.

Krajewska B. (1991). Urease immobilized on chitosan membrane. Inactivation by heavy metal ions. *Journal of Chemical Technology and Biotechnology*. 52:157–162.

Krishnamurthy P., Parlow M., Zitzer J.B., Vakil N.B., Mobley H.L.T., Levy M., Phadnis S.H. and Dunn B.E. (1998). *Helicobacter pylori* containing only cytoplasmic urease is susceptible to acid. *Infection and immunity*. 66(11):5060–5066.

Kuhn E. and Wittrig B. (2007). Analysis of nitrofurans in honey using LC/MS?mS and an ultra C18 column. *Food, Flavors and Fragrances*. 3:11

Kumar S. and Kayastha A.M. (2009). Inhibition studies of soybean (*Glycine max*) urease with heavy metals, sodium salts of mineral acids, boric acid, and boronic acids. *Journal of Enzyme Inhibition and Medicinal Chemistry*. 25(5):646–652. https://doi.org/10.3109/14756360903468155.

Kusters J.G., van Vliet A.H.M. and Kuipers E.J. (2006). "Pathogenesis of *Helicobacter pylori* infection," *Clinical Microbiology Reviews*. 19(3):449–490.

Kuwahara H., Miyamoto Y., Akaike T., Kubota T., Sawa T., Okamoto S. and Maeda H. (2000). *Helicobacter pylori* urease suppresses bactericidal activity of peroxynitrite via carbon dioxide production. *Infection and Immunity*. 68(8):4378–4383.

Kwakman P.H.S. and Zaat S.A.J. (2011). Antibacterial components of honey. 64(1):48-55. Doi:10.1002/iub.578

Kwakman P.H.S., te Velde A.A., de Boer L., Speijer D., Vandenbroucke-Grauls C.M.J.E. and Zaat S.A.J. (2010). How honey kills bacteria. *The Federation of American Societies for Experimental Biology Journal*. 24:2576–2582.

Kwok C.S., Nijjar R.S. and Loke Y.K. (2011). Effects of proton pump inhibitors on adverse gastrointestinal events in patients receiving clopidogrel: systematic review and meta-analysis. *Drug Safety*. 34:47–57.

Kwon Y.S., Nam J., Kim D., Suh D., Kim J., Kim Y.M. and Kim Y.T. (2009). Hexamethylmelamine as Consolidation Treatment for Patients with Advanced Epithelial Ovarian Cancer in Complete Response after First-Line Chemotherapy. *Journal of Korean Medical Sciences*. 24: 679–683. DOI: 10.3346/jkms.2009.24.4.679.

Lachman J., 1, Hejtmánková A., Sýkora J., Jindřich Karban J., Orsák M. and Rygerová B. (2010). Contents of major phenolic and flavonoid antioxidants in selected Czech honey. *Czech Journal of Food Science*. 28(5):412–426.

Lam R., Romanov V., Johns K., Battaile K.P., Wu-Brown J., Guthrie J.L., Hausinger R.P., Pai E.F. and Chirgadze N.Y. (2010). Crystal structure of a truncated urease accessary protein *ure*F from *Helicobacter pylori*. *Proteins.* 78(13):2839–2848.

Lam W.W., Woo E.J., Kotaka M., Tam W.K., Leung Y.C., Ling T.K. and Au S.W. (2010) Molecular interaction of flagellar export chaperone FliS and cochaperone HP1076 in *Helicobacter pylori*. *Federation of American Societies of Experimental Biology Journal*. 24(10):4020–4032.

Lanas A and Chan F.K.L. (2017). Peptic ulcer disease. *Lancet*. 5;390(10094):613-624. Doi:10.1016/S0140-6736(16)32404-7

Lavy D.S., Paulin E.T., Parker M.I., Zhang B., Parker G.S. and Schwartz M.R. (2016). *H. Pylori* in a gastric schwannoma: a case report. *Annals of Translation Medicine*. 4(7):137

Lee I. O., Kim J. H., Choi Y. J., Pillinger M. H., Kim S. Y., Blaser M. J., and Lee Y. C. (2010). *Helicobacter pylori cag*A phosphorylation status determines the gp130-activated SHP2/ERK and JAK/ STAT signal transduction pathways in gastric epithelial cells. *Journal of Biological Chemistry*. 285:16042–16050.

Lee K.E., Khoi P.N., Xia Y., Park J.S., Joo Y.E., Kim K.K., Choi S.Y. and Jung Y.D. (2013). *Helicobacter pylori* and interleukin-8 in gastric cancer. *World Journal of Gastroenterology*. 19(45):8192-8202. Doi:10.3748/wjg.v19.i45.8192

Lertsethtakarn P., Ottemann K.M. and Hendrixson D.R. (2011). Motility and chemotaxis in *Campylobacter* and *Helicobacter*. *Annual Review of Microbiology.* 65:389–410.

Letley D. P., Lastovica A., Louw J. A., Hawkey C. J. and Atherton J. C. (1999). Allelic diversity of the *Helicobacter pylori* vacuolating cytotoxin gene in South Africa: rarity of the *vac*A s1a genotype and natural occurrence of an *s*2/*m*1 allele. *Journal of Clinical Microbiology*. 37(4):1203–1205.

Leunk R. D., Johnson P. T., David B. C., Kraft W. G. and Morgan, D. R. (1988). Cytotoxic activity in broth-culture filtrates of *Campylobacter pylori*. *International Journal of Medical Microbiology*. 26:93–99.

Lim J.H., Kim S.G., Song J.H., Hwang J.J., Lee D.H., Han J.P., Hong .J., Kim J.H., Jeon S.W., Kim G.H., Shim K.N., Shin W.G., Kim T.H., Kim S.M., Chung I.K., Kim H.S., Kim H.U., Lee J. and Kim J.G. (2017). Efficancy of levofloxacin-based third line

therapy for the eradication of *Helicobacter pylori* in peptic ulcer disease. 11(2):226-231. Doi:10.5009/gnl16099.

Lima V.P., Silver-Fernandes J.L., Alves M.K.S and Rabenhorst S.H.B. (2011). Prevalence of *Helicobacter pylori* genotypes (*vac*A, *cag*A, *cag*E and *vir*B11) in gastric cancer in Brazilian's patients: An association with histopathological parameters. *Cancer Epidemiology*. 35:e32–e37.

Lin Y.T., Kwon Y.I., Labbe R.G. and Shetty K. (2005). Inhibition of *Helicobacter pylori* and Associated Urease by Oregano and Cranberry Phytochemical Synergies. *Applied and Environmental Microbiology*. 71(12): 8558–8564.

Lopez-Bolanos CC, Guzman-Murillo MA, Ruiz-Bustos E, Ascencio F. (2009). The role of heparan sulfate on adhesion of 47 and 51 kDa outer membrane proteins of *Helicobacter pylori* to gastric cancer cells. *Canadian Journal of Microbiology*. 55: 450–456.

Lowenthal A.C., Hill M., Sycuro L.K., Mehmood K., Salama N.R. and Ottemann K.M. (2009) Functional analysis of the *Helicobacter pylori* flagellar switch proteins. *Journal of Bacteriology*. 191(23):7147–7156.

Lu C.Y., Kuo C.H., Lo Y.C., Chuang H.Y., Yang Y.C., Wu I.C., Yu F.J., Lee Y.C., Jan C.M., Wang W.M. and Wu D.C. (2005). The best method of detecting prior *Helicobacter pylori* infection. *World Journal of Gastroenterology*. 11(36):5672–5676.

Lu H., Hsu P.I., Graham D.Y., Yamaoka Y. (2005). Duodenal ulcer promoting gene of *Helicobacter pylori*. *Gastroenterology*. 128(4):833–848.

Malfertheiner P., Megraud F., O'Morain C., Bazzoli F., El-Omar E. and Graham D. (2007). The European *Helicobacter* Study Group (EHSG). Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III. Consensus Report. *Gut*.56:772–781.

Mandal M.D. and Mandal S. (2011). Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine.* 1(2):154–160.

Mandal M.D. and Mandal S. (2011). Honey: its medicinal property and antibacterial activity. *Asian Pacific Journal of Tropical Biomedicine.* 1(2):154–160.

Mandewale M.C., Thorat B.R., Shelke D. and Yamgar R. (2015). Fluorescence and molecular docking studies of some new Schiff bases of 6-chloro-2- hydroxyquinoline-3-carbaldehyde. *Journal of Chemical and Pharmaceutical Research*. 7(6):900–909.

Manyi-Loh C.E., Clarke A.M. and Ndip R.N. (2011). Identification of volatile compounds in solvent extracts of honeys produced in South Africa. *African Journal of Agricultural Research.* 6(18):4327–4334, DOI: 10.5897/AJAR11.754.

Manyi-Loh C.E., Clarke A.M. and Ndip R.N. (2012). Detection of phytoconstituents in column fractions of n-hexane extract of Goldcrest honey exhibiting anti-*Helicobacter pylori* activity. *Archives of Medical Research Journal.* 43:197–204.

Manyi-Loh C.E., Clarke A.M., Green E. and Ndip R N. (2013). Inhibitory and bactericidal activity of selected South African honeys and their solvent extracts against clinical isolates of *Helicobacter pylori. Pakistan Journal of Pharmaceutical Sciences*. 26(5):897–906.

Manyi-Loh C.E., Clarke A.M., Munzhelele T. and Ndip R.N. (2010). Selected South African honeys and their extracts possess in vitro anti-*Helicobacter pylori* activity. *Archives of Medical Research*. 41(5):324–331. DOI: 10.1016/j.arcmed.2010.08.002

Marshall B. J., Goodwin C. S. (1987). Revised nomenclature of *C. pyloridis*. *International Journal of Systematic and Evolutionary Bacteriology*. 37:68

Marshall B.J. and Warren J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; 1: 1311–1315

Marshall B.J. and Warren J.R. (1984). Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. *Lancet* 1984; 1: 1311–1315

Marshall BJ, Armstrong JA, McGechie DB, Glancy RJ. (1985). Attempt to fulfill Koch's postulates for pyloric campylobacter. *Medical Journal of Australia*. 142:436–439.

Marshall, M. J. and Warren, R. J. (1983). Unidentified curved bacilli on gastric epithelium active chronic gastritis. *Lancet.* 1273–1275.

McClelland M., Sanderson K.E., Clifton SW., Latreille P., Porwollik S., Sabo A., Meyer R. and Bieri, T. (2004). Comparison of genome degradation in Paratyphi A and Typhi, human restricted serovars of *Salmonella enterica* that causes typhoid. *Nature Genetics*. 36: 1268–1274.

McClelland, M., Sanderson, K.E., Clifton, S.W., Latreille, P., Porwollik, S., Sabo, A., Meyer, R. and Bieri, T. (2004). Comparison of genome degradation in Paratyphi A and Typhi, human restricted serovars of *Salmonella enterica* that causes typhoid. *Nature Genetics*. 36:1268–1274.

Mégraud F. (2012). The challenge of *Helicobacter pylori* resistance to antibiotics: The comeback of bismuth-based quadruple therapy. *Therapeutic Advances in Gastroenterology*. 5(2):103–109.

Mégraud F. and Lehours P. (2007). *Helicobacter pylori* Detection and Antimicrobial Susceptibility Testing. *Clinical Microbiology Reviews*. 20(2):280–322.

Mégraud, F. (2004). *Helicobacter pylori* antibiotic resistance: prevalence, importance and advances in testing. *Gut*. 53:1374–1384

Miernyk K., Morris J., Bruden D., McMahon B., Hurlburt D., Sacco F., Parkinson A., Hennessy T. and Bruce M. (2011). Characterization of *Helicobacter pylori cag*A and *vac*A genotypes among Alaskans and their correlation with clinical disease. *Journal of Clinical Microbiology*. 49(9):3114-3121. Doi:10.1128/JCM.00469-11

Miernyk K., Morris J., Bruden D., McMahon B., Hurlburt D., Sacco F., Parkinson A., Hennessy T. and Bruce M. (2011). Characterization of *Helicobacter pylori cag*A and *vac*A Genotypes among Alaskans and Their Correlation with Clinical Disease. *Journal of Clinical Microbiology*. 49(9):3114–3121.

Miftahussurur M. and Yamaoka Y. (2016). Diagnostic Methods of *Helicobacter pylori* Infection for Epidemiological Studies: Critical Importance of Indirect Test Validation. *Bio Med Research International*. http://dx.doi.org/10.1155/2016/4819423

Mishra H., Parril A.L. and Williamson J.S. (2002). Three dimensional quantitative structure-activity relationship and comparative molecular field analysis of dipeptide hydroxamic acid *Helicobacter pylori* urease inhibitors. *Antimicrobial Agents and Chemotherapy*. 46:2613–2618.

Modolo L.V., de Souza A.X., Horta L.P., Araujo D.P. and de F*á*tima Â. (2015). An overview on the potential of natural products as urease inhibitors: A review. *Journal of Advanced Research*. 6:35–44.

Modolo L.V., de Souza A.X., Horta L.P., Araujo D.P. and de Fatima A. (2015). An overview on the potential of natural products as ureases inhibitors: A review. *Journal of Advanced Research*. 6:35-44.

Mohammad F. and Mohammad H. P. (2007). A study on the healing effect of honey on infected open wounds. *Pakistan Journal of Medical Sciences*. 23:327–329.

Molan P.C. (1995). The antibacterial properties of honey. *Chemistry in New Zealand*. July:10–14.

Molan P.C. and Rusell K.M. (1998). Non-peroxide antibacterial activity of some New Zealand honeys. *Journal of Apicultural Research*. 27:62–67.

Moniruzzaman M., An C.Y., Rao P.V., Hawlader. M.N.I., Azlan S.A.B.M., Sulaiman S.A. and Gan S.H. (2014). Identification of Phenolic Acids and Flavonoids in Monofloral Honey from Bangladesh by High Performance Liquid Chromatography: Determination of Antioxidant Capacity. *BioMed Research International*. Article ID 737490.

Murata-Kamiya N., Kurashima Y., Teishikata Y., Yamahashi Y., Saito Y., Higashi H., Aburatani H., Akiyama T., Peek R. M. Jr., Azuma T. and Hatakeyama M. (2007). *Helicobacter pylori cag*A interacts with E-cadherin and deregulates the beta-catenin signal that promotes intestinal trans-differentiation in gastric epithelial cells. *Oncogene*. 26:4617–4626.

Na R., Jiajia L., Dongliang Y., Yingzi P., Juan H., Xiong L., Nana Z., Jing Z. and Yitian L. (2016). Identification of vincamine indole alkaloids producing endophytic fungi isolated from *Nerium indicum*, Apocynaceae. *Microbiological Research*. 192:114–121.

Nakamura H., Yoshiyama H., Akeuchi H., Mizote T., Okita K. and Nakazawa T. (1998). Urease plays an important role in the chemotactic motility of *Helicobacter pylori* in a viscous environment. *Infection and Immunity*. 66:4832–4837.

Nayik G.A. and Nanda V. (2015). Characterization of the volatile profile of uni-floral honey from Kashmir Valley of India by using solid-phase micro-extraction and gas chromatography–mass spectrometry. *European Food Research and Technology*. doi:10.1007/s00217-015–2413-2.

Ndip N.R., Malange E.A., Akoachere T.F.J., MacKay G.W., Titanji K.P.V. and Weaver T.L. (2004). *Helicobacter pylori antigens* in the faeces of asymptomatic children in the Buea and Limbe health districts of Cameroon: A pilot study. *Tropical Medicine and International Health.* 9(9):1036–1040.

Ndip R.N., MacKayW.G., Farthing M.J.G. and Weaver L.T. (2003). Culturing *Helicobacter pylori* from clinical specimens: review of microbiologic methods. *Journal of Pediatric Gastroenterology and Nutrition*. 36:616-622.

Ndip R.N., Malange T.A.E., Echakachi C.M., Malongue A., Akoachere J.F.T.K., Ndip L.M. and Luma H.N. (2007). *In-vitro* antimicrobial activity of selected honeys on clinical isolates of *Helicobacter pylori*. *African Health Sciences.* 7(4):228–231.

Nevoa J.C., Rodrigues R.L., Menezes G.L., Lopes A.R., Nascimento H.F., Santiago S.B., Morelli M.L. and Barbosa M.S. (2017). Molecular technique for detection and

identification of *Helicobacter pylori* in clinical specimens: a comparison with the classical diagnostic method. *Jornal Brasileiro de Patologia e Medicina Laboratorial.* 53(1):13–19.

Ngan L.T.M., Moon J.K., Shibamoto T., Ahn Y.J. (2012). Growth-inhibiting, bactericidal, and urease inhibitory effects of Paeonia lactiflora root constituents and related compounds on antibiotic-susceptible and resistant strains of *Helicobacter pylori*. *Journal of Agricultural and Food Chemistry*. 60:9062–9073.

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

Njume, C., Afolayan, A. J. and Ndip, R. N. (2009). An overview of antimicrobial resistance and the future of medicinal plants in the treatment of *Helicobacter pylori* infections. *African Journal of Pharmacy and Pharmacology*. 3(13):685-699.

Nkomo L.P, Green E and Ndip R.N. (2011). Preliminary phytochemical screening and *in vitro* anti-*Helicobacter pylori* activity of extracts of the leaves of *Lippia javanica*. *African Journal of Pharmacy and Pharmacology*. 5(20):2184–2192.

O'Toole P.W., Kostrzynska M. and Trust T.J. (1994). Non-motile mutants of *Helicobacter pylori* and *Helicobacter mustelae* defective in flagellar hook production. *Molecular Microbiology*. 14(4):691–703

Occhialini A., Urdaci M., Doucet-Populaire F., B´eb´ear C.M., Lamouliatte H. and M´egraud F. (1997). "Macrolide resistance in *Helicobacter pylori*: rapid detection of

point mutations and assays of macrolide binding to ribosomes". *Antimicrobial Agents and Chemotherapy*. 41(12):2724–2728.

Oleastro M. and Ménard A. (2013). The Role of *Helicobacter pylori* Outer Membrane Proteins in Adherence and Pathogenesis. *Biology*. *2*(3):1110–1134. doi:10.3390/biology2031110

Oliveira M. J., Costa A. M., Costa A. C., Ferreira R. M., Sampaio P., Machado J. C., Seruca R., Mareel M. and Figueiredo C. (2009). *Cag*A associates with c-Met, E-cadherin, and p120catenin in a multiproteic complex that suppresses *Helicobacter pylori*-induced cell-invasive phenotype. *Journal of Infectious Diseases*. 200:745–755.

Orhan F., Sekerel B.E., Kocabas C.N., Sackesen C., Adalioglu G and Tuncer A. (2003). Complementary and alternative medicine in children with asthma. Annals of Allergy, Asthma and Immunology. 90:611–615.

Ormrod D., Easthope S.E. and Figgitt D.P. (2012). Drugs aging. Springer International Publishing. 19:217. https://doi.org/10.2165/00002512-200219030-00005

Owen J.R. (1998). *Helicobacter* - Species classification and identification. *British Medical Bulletin.* 54(1):17-30.

Pacheco J.B.P. and Anunciação C.E. (2008). Diagnóstico molecular por PCR dos genes de virulência *cag*A e *vac*A de *Helicobacter pylori* diretamente de biópsia gástrica humana e correlação com gastrite e úlcera. Ensaios e Ciência: C. Biológicas, Agráriase da Saúde. 12:49–62.

Palframan S.L., Kwok T. and Gabriel K. (2012). Vacuolating cytotoxin A (*vac*A), a key toxin for *Helicobacter pylori* pathogenesis. *Frontiers in Cellular and Infection Microbiology*. doi: 10.3389/fcimb.2012.00092

Papadakos K.S., Sougleri I.S., A.F. Mentis A.F., Hatziloukas E. and Sgouras D.N. (2013). "Presence of terminal EPIYA phosphorylation motifs in *Helicobacter pylori cag*A contributes to IL-8 secretion, irrespective of the number of repeats." *PLoS One.* 8(2): e56291.

Papamichael K. and Mantzaris G.J. (2012). Pathogenesis of *Helicobacter pylori* infection: Colonization, virulence factors of the bacterium and immune and non-immune host response. *Hospital Chronicles*. 7(1): 32–37.

Parsonnet J., Friedman G.D., Orentreich N. and Vogelman H. (1997). Risk for gastric cancer in people with *cag*A positive or *cag*A negative *Helicobacter pylori* infection. *Gut.* 40:297–301.

Paul R., Postius S., Melchers K. and Schafer K.P. (2001). Mutations of the *Helicobacter pylori* genes *rdx*A and pbp1 cause resistance against MTZ and amoxicillin. *Antimicrobial Agents and Chemotherapy*. 45:962–965.

Pearson M.A., Michel L.O., Hausinger R.P. and Karplus P.A. (1997). Structure of Cys319 variants and acetohydroxamate-inhibited *Klebsiella aerogenes* urease. *Biochemistry*. 36:8164–8172.

Pearson, A. D., and Great Britain Public Health Laboratory Service. (1983). *Campylobacter II: Proceedings of the Second International Workshop on Campylobacter Infections*, Brussels, 6–9 September 1983. Public Health Laboratory Service, London, United Kingdom

Peedikayi M.C., AlSohaibani F.I. and Alkhenizan A.H. (2014). Levofloxacin-Based First-Line Therapy versus Standard First-Line Therapy for *Helicobacter pylori*

Eradication: Meta-Analysis of Randomized Controlled Trials. *PLoS ONE*. 9(1): e85620. doi:10.1371/journal.pone.0085620

Peek M.R. (2005). Events at the host-microbial interface of the gastrointestinal tract IV. The pathogenesis of *Helicobacter pylori* persistence. *American Journal of Physiology,Gastrointestinal and Liver Physiology*. 289:G8–G12.

Peek R. M. Jr., Wirth H. P., Moss S. F., Yang M., Abdalla A. M., Tham K. T., Zhang T., Tang L. H., Modlin I. M. and Blaser M. J. (2000). *Helicobacter pylori* alters gastric epithelial cell cycle events and gastrin secretion in Mongolian gerbils. *Gastroenterology*. 118:48–59.

Pereañez J.A., Núñez V., Patiño A.C., Londoño M. and Quintana J.C. (2010). Inhibitory effects of plant phenolic compounds on enzymatic and cytotoxic activities induced by a snake Venom phospholipase A_2. *Journal of Pharmacology and Toxicology*. 18(3):295–304.

Pereira W.N., Ferraz M.A., Zabaglia L.M., de Labio R.W., Orcini W.A., Ximenez J.P.B., Neto A.C., Payao S.L.M. and Rasmussen L.T. (2014). Association among *H. pylori* virulence markers *dup*A, *cag*A and *vac*A in Brazilian patients. *Journal of Venomous Animals and Toxins including Tropical Diseases.* 20:1. http://www.jvat.org/content/20/1/1

Permin, H. and Anderson, P. L. (2005). Inflammation, immunity and vaccines for *Helicobacter* infection. *Helicobacter*. 10:21–30.

Perry S., Sanchez M.L., Yang S., Haggerty T.D., Hurst P., Perez-Perez G. and Parsonnet J. (2006). Gastroenteritis and Transmission of *Helicobacter pylori* Infection in Households. *Emerging Infectious Diseases*. 12(11):1701–1708.

Pervez H., Iqbal M., Tahir M.Y., Nasim F., Choudhary M.I. and Khan K.M. (2008). In vitro cytotoxic, antibacterial, antifungal and urease inhibitory activities of some N4-substituted isatin-3-thiosemicarbazones. *Journal of Enzyme Inhibition and Medicinal Chemistry*. 23(6):848–854. https://doi.org/10.1080/14756360701746179

Phadnis S.H., Parlow M.H., Levy M., Ilver D., Caulkins C.M., Connors J.B. and Dunn B.E. (1996). Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. 64(3):905–912.

Picoli S.U., Mazzoleni L.E., FernÀndez H., De Bona L.R., Neuhauss E., Longo L. and Prolla J.C. (2014). Resistance to amoxicillin, clarithromycin and ciprofloxacin of *Helicobacter pylori* isolated from Brazil patients. *Rev Inst, Med. Trop. Sao Paulo*. 56(3):197-200. DOI: 10.1590/S0036-46652014000300003

Pietroiusti A., Diomedi M. and Silvestrini M. (2002). Cytotoxin-associated gene A positive *Helicobacter pylori* strains are associated with atherosclerotic stroke. *Circulation Research*. 106:580–584.

Piljac-Žegarac J., Stipčević T. and Belščak A. (2009). Antioxidant properties and phenolic content of different floral origin honeys. *Journal of ApiProduct and ApiMedical Science*. 1(2):43–50.

Porth C. M. (2002). Alterations in gastrointestinal function. In Pathophysiology: concepts of altered health states. Porth, C. M., and Kunert, M. P. eds Lippincott Williams & Wilkins; 6th Ed. 27:831–856.

Prakash O. and Vishwakarma D.K. (2001). Inhibition of urease from seeds of water-melon (*Citrullus vulgaris*) by heavy metal ions. *Journal of Plant Biochemistry and Biotechnology*. 10(2):147–149.

Quastel J.H. (1933). The action of polyhydric phenols on urease; the influence of thiol compounds. *Biochemistry Journal*. (01):1116–1122.

Queiroz D.M. Carneiro J.G. and Braga-Neto M.B. (2012). Natural history of *Helicobacter pylori* infection in childhood: eight-year follow-up cohort study in an urban community in northeast of Brazil. *Helicobacter*. 17*:* 23–*2*9.

Queiroz D.M., Rocha G.A., Rocha A.M., Moura S.B., Saraiva I.E., Gomes L.I., Soares T.F., Melo F.F., Cabral M.M., Oliveira C.A. (2011). *Dup*A polymorphisms and risk of *Helicobacter pylori*-associated diseases. *International Journal of Medical Microbiology*. 301(3):225–228.

Radin J.N., Gaddy J.A., Gonzalez-Rivera C., Loh J.T., Algood H.M. and Cover T.L. (2013) Flagellar localization of a *Helicobacter pylori* autotransporter protein. *MBio* 4(2):e00613–12

Ramanauskiene K., Stelmakiene A., Briedis V., Ivanauskas L., and Jakstas V. (2012). The quantitative analysis of biologically active compounds in Lithuanian honey. *Journal of Food Chemistry*. 132(2012):1544–1548.

Ranilla L.G., Kwon Y., Apostolidis E. and Shetty K. (2010). Phenolic compounds, antioxidant activity and in vitro inhibitory potential against key enzymes relevant for hyperglycemia and hypertension of commonly used medicinal plants, herbs and spices in Latin America. *Bioresource Technology*. 101:4676–4689.

Reddy K.R. and Kayastha A.M. (2006). Boric acid and boronic acid inhibition of pigeonpea urease. *Journal of Enzyme Inhibition and Medicinal Chemistry*. 21:467–470.

Rektorschek M., Buhmann A., Weeks D., Schwan D. and Bensch K.W. (2000). Acid resistance of *Helicobacter pylori* depends on the ureI membrane protein and an inner membrane proton barrier. *Molecular Microbiology*. 36:141–152.

Rektorschek M., Weeks D., Sachs G. and Melchers K. (1998). Influence of pH on metabolism and urease activity of *Helicobacter pylori*. *Gastroenterology*. 115:628-641.

Rimbara E., Sasatsu M., Graham D.Y. (2013) PCR Detection of *Helicobacter pylori* in Clinical Samples. In: Wilks M. (eds) PCR detection of microbial pathogens. Methods in molecular biology (Methods and Protocols), vol 943. *Humana Press*, Totowa, NJ

Rizzato C., Torres J., Plummer M., Munoz N., Franceschi S., CamorlingaPonce M., Fuentes-Panana E.M., Canzian F. and Kato I. (2012). Variations in *Helicobacter pylori* cytotoxin-associated genes and their influence in progression to gastric cancer: implications for prevention. *PLoS One*. 7:e29605.

Rosenberg J.J. (2010). *Helicobacter pylori*. *Paediatrics in Review*. 31(2):85–86.

Rothenbacher D. (2007). Is *Helicobacter pylori* infection a necessary condition for non-cardia gastric cancer? A view from epidemiology. *Arquivos de Medicina.* 21:3–4.

Ruoff K., Luginbu W., Kunzli R., Bogdanov S., Bosset J.O., von der Ohe K., von der OHE W., and Amado R. (2006). Authentication of the Botanical and Geographical Origin of Honey by Front-Face Fluorescence Spectroscopy. *Journal of Agricultural and Food Chemistry*. 54:6858–6866.

Russell T.A., Berardi R.R., Barnett J.L., Dermentzoglou L.C., Jarvenpaa K.M., Schmaltz S.P. (1993). Upper gastrointestinal pH in seventy-nine healthy, elderly, North American men and women. *Pharmaceutical Research*. 10:187–196. PMID:8456064.

Ryan K.A., Karim N., Worku M., Penn C.W. and O'Toole P.W. (2005). *Helicobacter pylori* flagellar hook-filament transition is controlled by a FliK functional homolog encoded by the gene HP0906. *Journal of Bacteriology*. 187(16):5742–5750.

Saidu, A.Y., Munir, G., Salihu, Y., N. M. Sani., Muhammad, Y. and Dodo, A. M. (2015). Seroprevalence of *Helicobacter Pylori* among Adult in Sokoto Metropolis. *IOSR Journal of Nursing and Health Science*. 4(5):64-69. e-ISSN: 2320–1959.p- ISSN: 2320–1940.

Samie A., Obi C.L., Barrett L.J., Powell S.M. and Guerrant R.L. (2007). Prevalence of *Campylobacter* species, *Helicobacter pylori* and *Arcobacter* species in stool samples from the Venda region, Limpopo, South Africa: Studies using molecular diagnostic methods. *Journal of Infection*. 54:558–566.

Saniee P., Shahreza N. and Siavoshi F. (2015). Negative Effect of Proton-pump Inhibitors (PPIs) on *Helicobacter pylori* Growth, Morphology, and Urease Test and Recovery after PPI Removal – An In vitro Study. *Helicobacter* 21(2): doi: 10.1111/hel.12246.

Scott D., Weeks D., Melchers K. and Sachs G. (1998). The life and death of *Helicobacter pylori*. *Gut*. 43(1):S56–S60S56.

Seanego C.T. and Ndip R.N. (2012). Identification and antibacterial evaluation of bioactive compounds from *Garcinia kola* (Heckel) Seeds. *Molecules*. (17):6569–6584. doi:10.3390/molecules17066569.

Seck A., Burucoa C., Dia D., Mbengue M., Onambele M., Raymond J. (2013). Breurec S. Primary antibiotic resistance and associated mechanisms in *Helicobacter pylori* isolates from Senegalese patients. *Annals of Clinical Microbiology and Antimicrobials*. (12):3 https://doi.org/10.1186/1476-0711-12-3.

Seck A., Mbengue M., Gassama-Sow A., Diouf L., Ka M.M. and Boye C.S. (2009). Antibiotic susceptibility of *Helicobacter pylori* isolates in Dakar, Senegal. *Journal of Infection in Developing Countries.* 3(2):137–140.

Selbach M., Moese S., Hauck C. R., Meyer T. F. and Backert S. (2002). *Src* is the kinase of the *Helicobacter pylori cag*A protein *in-vitro* and *in-vivo*. *The Journal of Biological Chemistry*. 277:6775–6778.

Selcuk H. and Nevin K. (2002). Investigation of antimicrobial effect of honey collected from various regions of Turkey. *Pakistan Journal of Biological Sciences*. 5(3):325-328.

Selgrad M. and Malfertheiner P. (2011). Treatment of *Helicobacter pylori. Current Opinion in Gastroentorogy*. 27: 565–570.

Seo J.H., Woo H.O., Youn H.S. and Rhee K.H. (2014). Antibiotics resistance of *Helicobacter pylori* and treatment modalities in children with *H. pylori* infection. *Korean Journal of Pediatrics*. 57(2):67-71. http://dx.doi.org/10.3345/kjp.2014.57.2.67

Sibanda T. and Okoh A.I. (2008). *In-vitro* antimicrobial regimes of crude aqueous and acetone extract of *Garcinia kola* seeds. *Journal of Biological Sciences*. 8:149–154.

Siddaraju M.N. and Dharmesh S.M. (2007). Inhibition of gastric H+,K+-ATPase and *Helicobacter pylori* growth by phenolic antioxidants of *Zingiber officinale*. *Molecular Nutrition and Food Research*. 51:324–332.

Silva T.M.S., Santos F.P., Evangelista-Rodrigues A., Silva E.M.S., Silva G.S., Novais J.S. (2013). Phenolic compounds, melissopalynological, physicochemical analysis and antioxidant activity of jandaı'ra (Melipona subnitida) honey. *Journal of Food Composition and Analysis*. 29:10–18.

Singh V., Mishra S., Maurya P., Rao G.R.K., Jain A.K., Dixit V.K., Gulati A.K. and Nath G. (2009). Drug resistance pattern and clonality in *H. pylori* strains. *Journal of Infection in Developing Countries.* 3(2):130-136.

Singh V., Mishra S., Maurya P., Rao G.R.K., Jain A.K., Dixit V.K., Gulati A.K. and Nath G. (2009). Drug resistance pattern and clonality in *H. pylori* strains. *The Journal of Infection in Developing Countries*. 3(2):130–136. doi:10.3855/jidc.60

Sirko A. and Brodzik R. (2000). Plant ureases: Roles and regulation. *Acta Biochimica Polonica*.4(4):1189–1195.

Smith T.G., Pereira L. and Hoover T.R. (2009) *Helicobacter pylori* FlhB processing-deficient variants affect flagellar assembly but not flagellar gene expression. *Microbiology*. 155(Pt 4):1170–1180.

Snow M.J. and Manley-Harris M. (2004). On the nature of non-peroxide antibacterial activity in New Zealand manuka honey. *Food Chemistry* 84:145–147.

Snow M.J. and Manley-Harris. (2004). On the nature of non-peroxide antibacterial activity in New Zealand Manuka honey. *Food Chemistry* 84:145–147.

Stachniuk A. and Fornal E. (2016). Liquid Chromatography-Mass Spectrometry in the Analysis of Pesticide Residues in Food. Food Anal. Methods. 9:1654–1665.

Stone M.A. (1999). Transmission of *H. pylori*. *Post Graduate Medical Journal*. 75:198–200.

Street R.A. and Prinsloo G. (2013). Commercially important medicinal plants of South Africa: A review. *Journal of Chemistry*. http://dx.doi.org/10.1155/2013/205048.

Stutts P. and Fridovich I. (1964). A continual spectrophotometric determination of ammonia-producing systems. *Analytical Biochemistry*. 8:70–74.

Sugimoto M., Uotani T., Sahara S., Ichikawa H., Yamade M., Sugimoto K. and Furuta T. (2014). Efficacy of tailored *Helicobacter pylori* eradication treatment based on clarithromycin susceptibility and maintenance of acid secretion. *Helicobacter*. 9(4):312–318. doi: 10.1111/hel.12128.

Sujoy B. and Aparna A. (2013). Enzymology, Immobilization and Applications of Urease Enzyme. *International Research Journal of Biological Sciences*. 2(6):51–56. ISSN 2278–3202

Suzuki M., Miura S., Suematsu M., Fukumura D., Kurose I., Suzuki H., Kai A., Kudoh Y., Ohashi M. and Tsuchiya M. (1992). *Helicobacter pylori*-associated ammonia production enhances neutrophil-dependent gastric mucosal cell injury. *American Journal of Physiology*. 263:G719–G725

Syazana N., Gan S.H. and Halim A.S. (2013). Analysis of volatile compounds of Malaysian Tualang (*Koompassia excelsa*) honey using gas chromatography mass spectrometry. *African Journal of Traditional, Complementary, and Alternative Medicines*. 10(2):180–188. http://dx.doi.org/10.4314/ajtcam.v10i2.2.

Tahara T., Shibata T., Nakamura M. (2009). “Gastric mucosal pattern by using magnifying narrow-band imaging endoscopy clearly distinguishes histological and serological severity of chronic gastritis,” *Gastrointestinal Endoscopy*. 70(2):246–253.

Tammer I., Brandt S., Hartig R., Konig W., and Backert S. (2007). Activation of Abl by *Helicobacter pylori*: a novel kinase for *cag*A and crucial mediator of host cell scattering. *Gastroenterology*. 132:1309–1319.

Tanih F.N. and Ndip N.R. (2012). Molecular Detection of Antibiotic Resistance in South African Isolates of *Helicobacter pylori. Gastroenterology Research and Practice*. 10.1155/2013/259457:1–6.

Tanih N.F. and Ndip R.N. (2013). Molecular detection of antibiotic resistance in South African isolates of *Helicobacter pylori*. *Gastroenterology Research and Practice*. ArticleID259457, http://dx.doi.org/10.1155/2013/259457.

Tanih N.F., Okeleye B.I., Ndip L.M., Clarke A.M., Naidoo N., Mkwetshana N., Green E. and Ndip R. N. (2010). *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape province – race and disease status. *South African Medical Journal*. 100(11):734-737.

Tanih N.F., Okeleye B.I., Ndip L.M., Clarke A.M., Naidoo N., Mkwetshana N. and Green E. (2010). Ndip, R.N. *Helicobacter pylori* prevalence in dyspeptic patients in the Eastern Cape Province - Race and disease status. *South African Medical Journal*. 100(11):734–737.

Tankovic J., Lascols C., Sculo Q., Petit J.C. and Soussy C.J. (2003). Single and double mutations in *gyr*A but not in *gyr*B are associated with low- and high-level fluoroquinolone resistance in *Helicobacter pylori*. *Antimicrobial Agents and Chemotherapy*. 47:3942–3944.

Teh X., Khosravi Y., Lee W.C., Leow A.H.R., Loke M.F., Vadivelu J. and Goh K.L. (2014). Functional and Molecular Surveillance of *Helicobacter pylori* Antibiotic

Resistance in Kuala Lumpur. *H. pylori* Antibiotics Resistance in Malaysia. *Plos One*. 9(7):e101481.

Telford J. L., Ghiara P., Dell'Orco M., Comanducci M., Burroni D., Bugnoli M., Tecce, M. F., Censini, S., Covacci, A., Xiang Z., Papini, E., Montecucco, C., Parente, L. and Rappuoli, R. (1994). Gene structure of the *Helicobacter pylori* cytotoxin and evidence of its key role in gastric disease. *Journal of Experimental Medicine*. 179:1653–1658.

Thomas, J. E., Dale, A., Bunn, J. E., Harding, M., Coward, W., Cole, T. J. and Weaver, L. T. (2004). Early *Helicobacter pylori* colonization: the association with growth faltering in The Gambia. *Archives of Diseases in Childhood*. 89(12):1149–1154.

Todd M.J. and Hausinger R.P. (1989). Competitive inhibitors *of Klebsiella aerogenes* urease: Mechanisms of interaction with the nickel active site. *Journal of Biological Chemistry*. 264:15835–15842.

Tomb J. F., White O. and Kerlavage A. R. (1997). "The complete genome sequence of the gastric pathogen *Helicobacter pylori*". *Nature*. 388(6642):539–547.

Tonks A.J., Dudley E., Porter N.G., Parton J., Brazier J., Smith E.L. and Tonks A. (2007). A 5.8-kDa component of manuka honey stimulates immune cells via TLR4. *Journal of Leukocyte Biology*. 82:1147–1155. doi: 10.1189/jlb.1106683

Tsang J., Smith T.G., Pereira L.E. and Hoover T.R. (2013) Insertion mutations in *Helicobacter pylori flh*A reveal strain differences in RpoN-dependent gene expression. *Microbiology*. 159(1):58–67.

Tsuda M., Karita M., Morshed M.G., Okita K. and Nakazawa T. (1994). A urease-negative mutant of *Helicobacter pylori* constructed by allelic exchange mutagenesis lacks the ability to colonize the nude mouse stomach. *Infection and Immunity*. 62:3586–3589.

Uemure N., Okamoto S., Yamamoto S., Matsumura N., Yamaguchi S., Yamakido M., Taniyama K., Sasaki N. and Schlemper R.J. (2001). *Helicobacter pylori* infection and the development of gastric cancer. *The New England Journal of Medicine*. 345(11):784–789.

Upadhyyay L.S.B. (2012). Urease inhibitors: A review. *Indian Journal of Biotechnology*. 11:381–388.

Valls J., Millán S., Martí M.P., Borràs E. and Arola L. (2009). Advanced separation methods of food anthocyanins, isoflavones and flavanols. *Journal of Chromatography A.* 1216:7143–7172.

Van Den Berg A.J., Van Den Worm E., Van Ufford H.C., Jalkes S.B., Hoekstra M.J. and Beukelman C.J. (2008). An in-vitro examination of the antioxidant and anti-inflammatory properties of buukwheat honey. *Journal of Wound Care*. 17(4):172–178.

Van Doorn L.J., Figueiredo C., Sanna R., Plaisier A., Schneeberger P.M., Boer W. and Quint W. (1998). Clinical relevance of the *cag*A, *vac*A, and *ice*A status of *Helicobacter pylori*. *Gastroenterology*. 115:58–66.

Van Ulsen P., ur Rahman S., Jong W.S.P., Daleke-Schermerhorn M.H. and Luirink J. (2013).Type V secretion: From biogenesis to biotechnology. *Biochimica et Biophysica Acta.* 1843:1592–1611, http://dx.doi.org/10.1016/j.bbamcr.2013.11.006

Van-Zwet A. A., Vandenbrouke-Grauls C. M. J. E., Thijs J. C., van der Wouden E. J., Gerrits M. M. and Kusters, J. G. (1999). Stable amoxicillin resistance in *Helicobacter pylori*. *Lancet.* 353:154.

Vas G. and Ve´key K. (2004). Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. *Journal of Mass Spectrometry*. 39: 233–254

Versalovic J., Shortridge D. and Kibler K. (1996). "Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori.*" *Antimicrobial Agents and Chemotherapy*. 40(2):477–480.

Viala J., Chaput C. and Boneca I.G. (2004). Nod1 responds to peptidoglycan delivered by the *Helicobacter pylori* cag pathogenicity island. *Nature Immunology*. 5:1166-1174.

Wang J., Doorn l., Robinson P., Ji X., Wang D., Wang Y., Ge L., Telford J.L. and Crabtree J.E. (2003). Regional variation among *vac*A alleles of *Helicobacter pylori* in China. *Journal of Clinical Microbiology*. 41(5):1942–1945.

Wang J., Kliks M.M., Qu W., Jun S., Shi G,and Li Q.X. (2009). Rapid determination of the geographical origin of honey based on protein fingerprinting and barcoding using MALDI TOF MS. *Journal of Agricultural and Food Chemistry*. 57(21):10081–10088.

Warren J.R. and Marshall B. (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet.* 1(8336):1273–1275.

Watanabe K., Nagata N., Shimbo T., Nakashima R., Furuhata E., Sakurai T., Akazawa N., Yokoi C., Kobayakawa M., Akiyama J., Mizokami M. and Uemura N. (2013). Accuracy of endoscopic diagnosis of *Helicobacter pylori* infection according to

level of endoscopic experience and the effect of training. *BCM Gastroenterology*. 13:128. https://doi.org/10.1186/1471-230X-13-128.

Watanabe M., Ito H., Hosono S., Oze I., Ashida C., Tajima K., Katoh H., Matsuo K. and Tanaka H. (2015). Declining trends in prevalence of *Helicobacter pylori* infection by birth-year in a Japanese population. *Journal of the Japanese Cancer Science.* 106(12):1738–1743. DOI: 10.1111/cas.12821

Weeks D. L., Eskandari S., Scott D.R. and Sachs G. (2000). A H1-gated urea channel: the link between *Helicobacter pylori* urease and gastric colonization. *Science* 287:482–485.

Weiner D., Mitch W.E. and Sands J.M. (2014). Urea and Ammonia Metabolism and the Control of Renal Nitrogen Excretion. *Clinical Journal of the American Society of Nephrology.* doi: 10.2215/CJN.10311013CJASN

Wells P.G., McCallum G.P., Chen C.S., Henderson J.T., Lee C.J., Perstin J., Preston T.J., Wiley M.J. and Wong A.W. (2009). Oxidative stress in developmental origins of disease: Teratogenesis, neurodevelopmental deficits, and cancer. *Toxicological Sciences*. 108:4–18.

Weston R.J. (2000). The contribution of catalase and other natural products to the antibacterial activity of honey: a review. *Food Chemistry*. 71(2000):235-239.

Wirth H., Beins M.H., Yang M., Tham K.T. and Blaser M.J. (1998). Experimental infection of Mongolian gerbils with wild-type and mutant *Helicobacter pylori* strains. *Infection and Immunity*. 66(10):4856–4866.

Wolski T., Tambor K., Rybak - Chmielewska H. , Kêdzia B. (2006). Identification of honey volatile components by solid phase microextraction (SPME) and gas

chromatography/mass spectrometry (GC/MS). *Journal of Apicultural Science*. 50(2):115-126

Wong, B. C. Y., Yin, Y., Berg, D. E., Xia, H. H-X., Zhang, J. Z., Wang, W. H., Wong, W. M., Huang, X. R., Tang, V. S. Y. and Lam, S. K. (2001). Distribution of distinct *vac*A, *cag*A, *ice*A alleles in *Helicobacter pylori* in Hong Kong. *Helicobacter*. 6(4):317–324.

Wu J. Y., Kim J. J., Reddy R., Wang W. M., Graham D. Y. and Kwon D. H. (2005). Tetracycline-resistant clinical *Helicobacter pylori* isolates with and without mutations in 16SrRNA-encoding genes. *Antimicrobial Agents and Chemotherapy*. 49(2):578–583.

Wu W., Yang Y. and Sun G. (2012). Recent insights into antibiotic resistance in *Helicobacter pylori* eradication. *Gastroenterology Research and Practice*. Article ID 723183. doi:10.1155/2012/723183.

Xiong, L. j., Tong, Y., Wang, Z. and Mao, M. (2013). Detection of Clarithromycin-Resistant *Helicobacter pylori* by Stool PCR in Children: A Comprehensive Review of Literature. *Helicobacter*. 18: 89–101. doi: 10.1111/hel.12016

Yamada S., Kato S., Matsuhisa T., Makonkawkeyoon L., Yoshida M., Chakrabandhu T., Lertprasertsuk N., Suttharat P., Chakrabandhu B., Nishiumi S., Chongraksut W. and Azuma T. (2013). Predominant mucosal IL-8 mRNA expression in non-*cag*A Thais is risk for gastric cancer. *World Journal of Gastroenterology*. 19:2941–2949.

Yang X., Li H., Lai T. and Sun H. (2015). UreE-UreG complex facilitates nickel transfer and pre-activates GTPase of ureG in *Helicobater pylori*. *The Journal of Biological Chemistry*. 290(20):12474–12485.

Yang X., Wu D. and Li H. (2004). Flow injection chemiluminescence determination of dihydralazine sulphate based on permanganate oxidation sensitized by rhodamine B. Luminescence. 19:322–327.

Yang Y.J., Wu J.J., Sheu B.S., Kao A.W. and Huang A.H. (2004). The *rdx*A gene plays a more major role than frxA gene mutation in high-level metronidazole resistance of *Helicobacter pylori* in Taiwan. *Helicobacter*. 9(5):400–407.

Yeo M., Park H., Lee K., Lee K.J., Kim J.H., Cho S.W. and Hahm K. (2004). Blockage of HSP 90 modulates *Helicobacter pylori*-induced IL-8 productions through the inactivation of transcriptional factors of AP-1 and NF-*K*B. *Biochemical and Boiphysical Communication*. 320:816–824.

Yoshida, H., Hirota, K., Shiratori, Y., Nihei, T., Amano, S. and Yoshida, A. (1998) Use of a gastric juice-based PCR assay to detect *Helicobacter pylori* infection in culture-negative patients. *Journal of Clinical Microbiology*. 36:317–320.

Zabaleta J.1. (2012). Multifactorial etiology of gastric cancer. *Methods in Molecular Biology*.863:411–435.

Zaborska W., Krajewska B., Kot M. and Karcz W. (2007). Quinone-induced inhibition of urease: Elucidationof its mechanisms by probing thiol groups of the enzyme. *Bioorganic Chemistry*. 35:233–242.

Zaborska W., Krajewska B., Leszko M. and Olech Z. (2001). Inhibition of urease by Ni^{2+} ions: Analysis of reaction progress curves. *Journal of Molecular Catalysis B: Enzymatic.*13:103–108.

Zainol M.I., Yusoff K.M. and Yusof M.Y.M. (2013). Antibacterial activity of selected Malasian honey. *Journal of Complementary and Alternative Medicine.* 13:129.

Zaki M.E.I., Elewa A., Ali M.A. and Shehta A. (2016a). Study of Virulence Genes *cag*A and *vac*A in *Helicobacter pylori* Isolated from Mansoura University Hospital Patients by Multiplex PCR. *International Journal of Current Microbiology and Applied Sciences.*ISSN: 2319-7706, 5(2):154–160.

Zaki M.E.S., Othman W., Ali M.A. and Shehta A. (2016b). Fluoroquinolone-Resistant *Helicobacter pylori* strains Isolated from One Egyptian University Hospital: Molecular Aspects. Journal of Microbiology and Antimicrobial Agents. 2 (1):26–31. ISSN 2396–880X

Zayed S.I.M. and Issac Y.M. (2013). Plastic Membrane, Carbon Paste and Multiwalled Carbon Nanotube Composite Coated Copper Wire Sensors for Determination of Oxeladin Citrate Using Batch and Flow Injection Techniques. Journal of Brazilian Chemical Society. 24(4):585–594.

Zeaiter Z., Cohen D., Musch A., Bagnoli F., Covacci A., and Stein M. (2008). Analysis of detergent-resistant membranes of *Helicobacter pylori* infected gastric adenocarcinoma cells reveals a role for MARK2/Par1b in *cag*A mediated disruption of cellular polarity. *Cell Microbiology Journal*. 10:781–794.

Zhang L., Mulrooney S.B., Leung A.F.K., Zeng Y., Ko B.B.C., Hausinger R.P. and Sun H. (2006). Inhibition of urease by bismuth (III): Implications for the mechanism of action of bismuth drugs. *BioMetals*. 19:503–511. DOI 10.1007/s10534-005-5449-0

Zhao Y., Zhou Y., Lin G., Hu W. and Du J. (2015). Association between IL-17, IL-8 and IL-18 expression in peripheral blood and *Helicobacter pylori* infection in Mongolian gerbils. *Jundishapur Journal of Microbiology*. 8(8):e21503. DOI:10.5812/jjm.21503.

Zullo A., Rinaldi V. and Winn S. (2000). A new highly effective shout term therapy schedule for *Helicobacter pylori* eradication. *Alimentary Pharmacology and Therapeutics*. 14(6):715–718.

APPENDICES

APPENDIX A: BUFFERS

A 1: 36% HCl to 1M HCl

- Add 83.5 mL of 36% HCl acid to 600mL of deionised water in a 1L measuring cylinder and top up with deionised water to 1L.

A 2: 1M NaOH

- Dissolve 40 grams of NaOH in 1L deionised water.

A 3: Phosphate buffered saline (PBS) pH 7.4

NaCl	8g
KCl	0.2g
$NaHPO_4$	1.44g
Kh_2PO_4	0.272g
Deionised water	800ml

- Adjust pH to 7.4 using HCl/NaOH before filling up to 1L with deionised water

A 4: 0.1 M potassium dihydrogen phosphate

- Dissolve 13.61g KH_2PO4 in 1000mL deionised water

A 5: 0.1M Disodium hydrogen phosphate

- Dissolve 17.8g Na_2HPO_4 in 1000mL of deionised water.

A 6: 10x TAE electrophoresis buffer

- Dissolve 48.4g of Tris base [tris(hydroxymethyl)aminomethane], 14.4mL of glacial acetic acid (17.4 M) and 3.7 EDTA, disodium salt in 1L deionised water.

A 7: Preparation of Tris-HCl buffer (50mM, pH 8) from 100X concentrate

-Ratio is 10 mL Tris-HCl buffer concentrate: 990 mL deionised water

A 8: Preparation of NADH (8.5 mM)

- Ratio: add 6.32757g of NADH to 1 L NaOH (0.01M) buffer

A 9: Preparation of α-ketoglutaric acid: 25mM

- Ratio: 3.65275g α-ketoglutaric acid : 1L of deionised water.

A 10: Preparation of 10U/mL of urease

- Weigh 1g of urease and add 10mL of Tris-HCl buffer

A11: Preparation of glutamate dehydrogenase (GLDH), 250u/mL

- Weigh 5.682 mg and add 10mL phosphate buffer

A12: Preparation of 0.3 M urea

- Weigh 18.018g urea and add 1 L of deionised water.

APPENDIX B: GC-MS QUALITATIVE ANALYSIS REPORT OBTAINED FROM THE TOTAL ION COUNT (TIC)

B 1: Petroleum extract of Gold Crest honey

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	4.655	16400976	0.85	16464410	2.35	1.00	Mesitylene
2	5.035	26320774	1.36	21813930	3.11	1.21	Nonane, 5-(2-methylpropyl)-
3	5.075	22380949	1.16	18579715	2.65	1.20	Spiro[3.5]nona-5,7-dien-1-one, 5,9,9-trimethyl
4	5.272	17271472	0.89	13789079	1.97	1.25	Benzene, 1-ethyl-3,5-dimethyl-
5	5.332	20637957	1.07	15112427	2.15	1.37	Undecane
6	5.494	14967390	0.77	11404529	1.63	1.31	Benzene, 1,2,3,5-tetramethyl-
7	5.520	20002441	1.03	17583325	2.51	1.14	Benzene, 1,2,3,5-tetramethyl-
8	5.733	23312308	1.20	12108313	1.73	1.93	Benzene, 1,2,4,5-tetramethyl-
9	5.896	14835622	0.77	7498483	1.07	1.98	Ethanol, 1-(2-butoxyethoxy)-
10	5.969	17879243	0.92	12024439	1.71	1.49	Dodecane
11	6.004	17910191	0.93	12239880	1.74	1.46	Azulene
12	6.346	12716936	0.66	6307082	0.90	2.02	Tetradecane, 5-methyl-
13	6.429	25009414	1.29	14932093	2.13	1.67	Dodecane, 4,6-dimethyl-
14	6.564	18730104	0.97	9639668	1.37	1.94	Tetradecane
15	6.693	21806144	1.13	11230527	1.60	1.94	Dodecane, 4-methyl-
16	7.125	15134614	0.78	9480514	1.35	1.60	Tetradecane
17	7.185	10285063	0.53	3935769	0.56	2.61	Tridecane, 6-methyl-
18	7.376	11576837	0.60	3526697	0.50	3.28	Heptadecane
19	7.445	20035414	1.03	6177599	0.88	3.24	Hexadecane
20	7.605	40102776	2.07	16424426	2.34	2.44	Eicosane
21	7.651	17682964	0.91	10275029	1.46	1.72	Pentadecane
22	7.712	16775828	0.87	7063938	1.01	2.37	Phenol, 2,4-bis(1,1-dimethylethyl)-
23	7.830	34986943	1.81	14106506	2.01	2.48	Eicosane
24	7.986	10497878	0.54	3519689	0.50	2.98	Eicosane
25	8.145	12340014	0.64	7064259	1.01	1.75	Hexadecane
26	8.368	12576900	0.65	3887386	0.55	3.24	Heptadecane
27	8.417	13476016	0.70	4859729	0.69	2.77	Eicosane
28	8.491	14589113	0.75	5765066	0.82	2.53	Heptadecane
29	8.548	10645318	0.55	4402774	0.63	2.42	Heptadecane
30	8.633	46865375	2.42	15695787	2.24	2.99	Eicosane
31	8.671	17574795	0.91	7242594	1.03	2.43	Eicosane
32	8.764	13959555	0.72	6004570	0.86	2.32	Octacosyl trifluoroacetate
33	8.825	34303980	1.77	15948665	2.27	2.15	Eicosane
34	8.926	25173776	1.30	5513184	0.79	4.57	Hexadecane, 1-iodo-
35	9.059	16946545	0.88	4508315	0.64	3.76	Heptadecane
36	9.233	17095657	0.88	6582938	0.94	2.60	Heneicosane
37	9.302	11694965	0.60	4526358	0.65	2.58	Eicosane
38	9.414	18014361	0.93	7454132	1.06	2.42	Heneicosane
39	9.464	14000156	0.72	6931150	0.99	2.02	Eicosane
40	9.549	25320748	1.31	15911486	2.27	1.59	Eicosane
41	9.579	19485276	1.01	11446221	1.63	1.70	Silane, trichlorooctadecyl-
42	9.645	16211319	0.84	8807961	1.26	1.84	Benzenepropanoic acid, 3,5-bis(1,1-dimethylet
43	9.675	13488813	0.70	7166836	1.02	1.88	Hexatriacontyl trifluoroacetate
44	9.720	23526929	1.22	14273879	2.03	1.65	Eicosane
45	9.747	17385580	0.90	10687319	1.52	1.63	11-Bromoundecanoic acid
46	9.787	12434041	0.64	6586489	0.94	1.89	Heneicosane
47	9.817	12777863	0.66	6869209	0.98	1.86	Octadecanamide
48	9.900	22721878	1.17	6141276	0.88	3.70	Eicosane
49	10.020	11306167	0.58	4207446	0.60	2.69	Eicosane
50	10.064	23184722	1.20	4936521	0.70	4.70	Eicosane
51	10.161	18219509	0.94	4875585	0.69	3.74	Hexadecane, 1-iodo-
52	10.241	36907940	1.91	8350229	1.19	4.42	Eicosane
53	10.374	42127753	2.18	12992811	1.85	3.24	Eicosane
54	10.449	30202664	1.56	11574558	1.65	2.61	cis-13,16-Docasadienoic acid
55	10.529	37915236	1.96	13500978	1.92	2.81	Eicosane
56	10.612	15697733	0.81	11411002	1.63	1.38	Hexadecanamide
57	10.635	16158648	0.83	7680535	1.09	2.10	Heneicosane
58	10.712	16777387	0.87	5024821	0.72	3.34	Cholest-5-en-3-ol (3.beta.)-, 3,5-dinitrobenzoa
59	10.775	10482492	0.54	5810194	0.83	1.80	Eicosane
60	10.805	32769513	1.69	14526973	2.07	2.26	Octadecanoic acid, 2-hydroxyethyl ester
61	10.860	13495992	0.70	6281952	0.90	2.15	Tetratetracontane
62	11.003	28088346	1.45	7172022	1.02	3.92	Tetratetracontane
63	11.043	10422857	0.54	5260146	0.75	1.98	Tetratetracontane
64	11.095	16693815	0.86	5584149	0.80	2.99	Eicosane
65	11.141	31666273	1.64	9175927	1.31	3.45	Eicosane
66	11.315	96736032	5.00	30140432	4.30	3.21	9-Octadecenamide, (Z)-
67	11.388	12020644	0.62	5294031	0.75	2.27	Bis(dodecanamido)methane
68	11.544	10425134	0.54	3345373	0.48	3.12	Cholesta-3,5-diene
69	11.605	22515928	1.16	5524752	0.79	4.08	Octadecanoic acid, 2-hydroxyethyl ester
70	11.808	10797201	0.56	3423877	0.49	3.15	Heneicosane
71	11.857	18897517	0.98	3983436	0.57	4.74	Hexatriacontane
72	12.006	30685361	1.59	4710909	0.67	6.51	Bis(tridecyl) phthalate
73	12.183	12340975	0.64	3318037	0.47	3.72	2-methylhexacosane
74	12.268	11703250	0.60	3081905	0.44	3.80	Hexadecane, 1-iodo-
75	12.385	11146850	0.58	2357107	0.34	4.73	Cholest-5-en-3-ol (3.beta.)-, nonanoate
76	14.390	6404605	0.33	2736747	0.39	2.34	Cholesta-3,5-diene
77	14.750	44951463	2.32	13420856	1.91	3.35	Cholesta-3,5-diene
78	22.594	67109710	3.47	7849155	1.12	8.55	Silane, diethylheptyloxyoctadecyloxy-
79	28.243	22453261	1.16	2184559	0.31	10.28	1,4-Epoxynaphthalene-1(2H)-methanol, 4,5,7-
80	30.274	4381239	0.23	446999	0.06	9.80	Propanoic acid, 3,3'-thiobis-, didodecyl ester
81	86.515	237527641	12.27	1315773	0.19	180.52	Cholest-5-en-3-ol (3.beta.)-, 9-octadecenoate,
82	87.317	13832939	0.71	552705	0.08	25.03	Longifolenaldehyde
		1935916008	100.00	701568152	100.00		

B 2: Petroleum extract of Fleures honey

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	4.651	14877252	0.86	15582063	3.28	0.95	Mesitylene
2	5.031	21234601	1.22	18010715	3.79	1.18	Pentadecane
3	5.070	18471921	1.06	15978645	3.37	1.16	Spiro[3.5]nona-5,7-dien-1-one, 5,9,9-trimethyl
4	5.267	15107383	0.87	11871373	2.50	1.27	Benzene, 1-ethyl-3,5-dimethyl-
5	5.327	15856301	0.91	12434156	2.62	1.28	Undecane
6	5.489	12227900	0.70	9907490	2.09	1.23	Benzene, 1,2,3,5-tetramethyl-
7	5.514	15788308	0.91	15211003	3.20	1.04	Benzene, 1,2,3,5-tetramethyl-
8	5.728	18399722	1.06	9752151	2.05	1.89	Benzene, 1,2,4,5-tetramethyl-
9	5.965	13678323	0.79	9687890	2.04	1.41	Dodecane
10	6.000	14879303	0.86	10174171	2.14	1.46	Naphthalene
11	6.424	17291112	1.00	10724896	2.26	1.61	Dodecane, 4,6-dimethyl-
12	6.559	12977785	0.75	6522005	1.37	1.99	Tetradecane
13	6.689	15195754	0.88	8099491	1.71	1.88	Dodecane, 4-methyl-
14	7.120	10267174	0.59	6273682	1.32	1.64	Tetradecane
15	7.440	13824140	0.80	4456858	0.94	3.10	Eicosane
16	7.599	26343003	1.52	12211539	2.57	2.16	Eicosane
17	7.646	11892463	0.68	7001775	1.47	1.70	Pentadecane
18	7.708	12189287	0.70	4611363	0.97	2.64	Phenol, 2,4-bis(1,1-dimethylethyl)-
19	7.825	24677399	1.42	10502377	2.21	2.35	Eicosane
20	8.487	10153813	0.58	4020298	0.85	2.53	Heneicosane
21	8.628	33138368	1.91	11939384	2.52	2.78	Eicosane
22	8.760	10385121	0.60	4187480	0.88	2.48	Docosyl pentafluoropropionate
23	8.821	23776143	1.37	10822822	2.28	2.20	Eicosane
24	8.905	15869668	0.91	3786271	0.80	4.19	Nonane, 5-methyl-5-propyl-
25	9.054	11180279	0.64	3047688	0.64	3.67	Heptadecane
26	9.229	13898349	0.80	4508266	0.95	3.08	Eicosane
27	9.410	11019076	0.63	5322615	1.12	2.07	Eicosane
28	9.544	18802772	1.08	12235134	2.58	1.54	Eicosane
29	9.573	17199634	0.99	8118769	1.71	2.12	Silane, trichlorooctadecyl-
30	9.714	26652049	1.54	10400151	2.19	2.56	Eicosane
31	9.895	17779985	1.02	4157621	0.88	4.28	Eicosane
32	10.078	11739019	0.68	3371933	0.71	3.48	Hexadecane, 1-iodo-
33	10.152	12548231	0.72	3372532	0.71	3.72	Octadecane, 1-chloro-
34	10.236	26448657	1.52	5722874	1.21	4.62	Eicosane
35	10.368	29473716	1.70	10570940	2.23	2.79	Eicosane
36	10.443	11517846	0.66	5301356	1.12	2.17	E,E,Z-1,3,12-Nonadecatriene-5,14-diol
37	10.533	42593305	2.45	11098160	2.34	3.84	Bis(dodecanamido)methane
38	10.600	19342940	1.11	10007004	2.11	1.93	Hexadecanamide
39	10.632	11198451	0.65	5382848	1.13	2.08	Hexadecane, 1-iodo-
40	10.727	12440884	0.72	3397328	0.72	3.66	Cholesta-3,5-diene
41	10.819	34250021	1.97	7944187	1.67	4.31	Octadecanoic acid, 2-hydroxyethyl ester
42	10.997	20660393	1.19	5912932	1.25	3.49	Tetratetracontane
43	11.091	14553744	0.84	4981239	1.05	2.92	Tetratetracontane
44	11.135	14516567	0.84	7981125	1.68	1.82	Eicosane
45	11.163	13212243	0.76	5406010	1.14	2.44	Cholest-5-ene, 3-ethoxy-, (3.beta.)-
46	11.309	102001375	5.88	31978246	6.74	3.19	9-Octadecenamide, (Z)-
47	11.382	11720876	0.68	5266061	1.11	2.23	Octadecanamide
48	11.538	16259910	0.94	3048388	0.64	5.33	Cholest-5-en-3-ol (3.beta.)-, 3,5-dinitrobenzoa
49	11.648	30806333	1.77	4573369	0.96	6.74	Octadecanoic acid, 2-hydroxyethyl ester
50	11.742	12767808	0.74	3225518	0.68	3.96	Cholesta-3,5-diene
51	11.851	12732906	0.73	4022600	0.85	3.17	Hexadecane, 1-iodo-
52	12.000	34126762	1.97	5868492	1.24	5.82	Bis(tridecyl) phthalate
53	12.179	14091257	0.81	3697852	0.78	3.81	Tetratetracontane
54	12.224	11094576	0.64	4422430	0.93	2.51	Eicosane
55	12.261	13547825	0.78	3426431	0.72	3.95	Hexatriacontane
56	12.391	11877170	0.68	2564494	0.54	4.63	5,5-Diethylheptadecane
57	13.492	12800840	0.74	2572728	0.54	4.98	13-Docosenamide, (Z)-
58	14.717	32335258	1.86	9430406	1.99	3.43	Cholesta-3,5-diene
59	22.531	59463291	3.43	7237503	1.52	8.22	Silane, diethylheptyloxyoctadecyloxy-
60	28.163	21925658	1.26	2103157	0.44	10.43	1,4-Epoxynaphthalene-1(2H)-methanol, 4,5,7-
61	85.148	186289946	10.73	2399357	0.51	77.64	Cholesteryl valerate
62	85.345	23242043	1.34	2205971	0.46	10.54	3Beta-methoxy-5-cholesten-19-oic acid
63	86.493	162334008	9.35	2421764	0.51	67.03	Cholesterol, pentafluoropropionate
64	86.870	177177773	10.21	2238954	0.47	79.13	Cholesteryl valerate
		1736126020	100.00	474714331	100.00		

B 3: Hexane extract of Manuka honey

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	3.596	3155427	0.58	2098612	0.85	1.50	
2	4.118	2405530	0.44	2773939	1.12	0.87	
3	4.426	5030864	0.92	4802600	1.94	1.05	
4	4.880	2360391	0.43	2043366	0.83	1.16	
5	5.016	8542635	1.57	8786982	3.55	0.97	
6	5.054	3823808	0.70	3576786	1.45	1.07	
7	5.314	5612964	1.03	4974876	2.01	1.13	
8	5.350	2483237	0.46	2319810	0.94	1.07	
9	5.400	2283859	0.42	1168207	0.47	1.96	
10	5.498	2868613	0.53	1655746	0.67	1.73	
11	5.716	2804503	0.52	1518213	0.61	1.85	
12	5.877	4515788	0.83	2465075	1.00	1.83	
13	5.952	4089032	0.75	2124257	0.86	1.92	
14	5.990	2305535	0.42	1719989	0.70	1.34	
15	6.030	2564217	0.47	1287569	0.52	1.99	
16	6.202	2380622	0.44	1332678	0.54	1.79	
17	6.275	3803217	0.70	1237961	0.50	3.07	
18	6.329	4179176	0.77	2196459	0.89	1.90	
19	6.411	11691825	2.15	7252429	2.93	1.61	
20	6.462	3190348	0.59	2110123	0.85	1.51	
21	6.497	2818667	0.52	1847594	0.75	1.53	
22	6.542	6104181	1.12	2622243	1.06	2.33	
23	6.676	8478054	1.56	4999517	2.02	1.70	
24	6.731	2608037	0.48	1560488	0.63	1.67	
25	6.780	2409752	0.44	1594096	0.64	1.51	
26	7.108	3610021	0.66	2481028	1.00	1.46	
27	7.169	4996751	0.92	1199183	0.49	4.17	
28	7.428	5351795	0.98	2008354	0.81	2.66	
29	7.586	16261512	2.99	7501098	3.03	2.17	
30	7.633	5773812	1.06	3610723	1.46	1.60	
31	7.665	2660307	0.49	1650832	0.67	1.61	
32	7.693	3174452	0.58	2251190	0.91	1.41	
33	7.813	15486192	2.84	6280465	2.54	2.47	
34	7.863	2789438	0.51	1995716	0.81	1.40	
35	7.913	2393473	0.44	1551229	0.63	1.54	
36	7.969	2812019	0.52	1267582	0.51	2.22	
37	8.127	2489860	0.46	2266558	0.92	1.10	
38	8.157	2550614	0.47	1542679	0.62	1.65	
39	8.350	3110348	0.57	1269491	0.51	2.45	
40	8.400	3950378	0.73	1548967	0.63	2.55	
41	8.475	4489489	0.82	2185221	0.88	2.05	
42	8.515	3570001	0.66	1450202	0.59	2.46	
43	8.614	19354591	3.55	7957534	3.22	2.43	
44	8.654	4177492	0.77	2826537	1.14	1.48	
45	8.746	5074309	0.93	2112960	0.85	2.40	
46	8.807	14580979	2.68	7275337	2.94	2.00	
47	8.850	3169317	0.58	2246139	0.91	1.41	
48	8.910	5964696	1.10	1840232	0.74	3.24	
49	8.960	4264366	0.78	2203254	0.89	1.94	
50	9.039	3284638	0.60	1523067	0.62	2.16	
51	9.128	2898214	0.53	920504	0.37	3.15	
52	9.215	3860292	0.71	2223155	0.90	1.74	
53	9.280	2585186	0.47	1204713	0.49	2.15	
54	9.340	2886098	0.53	1526090	0.62	1.89	
55	9.396	4623734	0.85	2618735	1.06	1.77	
56	9.445	5809526	1.07	2151559	0.87	2.70	
57	9.530	12448901	2.29	7189501	2.91	1.73	
58	9.560	8404795	1.54	5650309	2.29	1.49	
59	9.626	4390648	0.81	2828237	1.14	1.55	
60	9.655	4688218	0.86	2262874	0.92	2.07	
61	9.700	13909298	2.55	6636693	2.68	2.10	
62	9.792	7353470	1.35	2055431	0.83	3.58	
63	9.882	5329005	0.98	1931713	0.78	2.76	
64	10.220	10055350	1.85	2437564	0.99	4.13	
65	10.354	8855113	1.63	5287722	2.14	1.67	
66	10.380	3528190	0.65	2149953	0.87	1.64	
67	10.480	6466418	1.19	2362376	0.96	2.74	
68	10.518	13753315	2.53	7136697	2.89	1.93	
69	10.585	8411165	1.54	5638015	2.28	1.49	
70	10.635	3715113	0.68	1709822	0.69	2.17	
71	10.807	11265505	2.07	1922278	0.78	5.86	
72	10.984	4694378	0.86	2102550	0.85	2.23	
73	11.075	4664736	0.86	1357438	0.55	3.44	
74	11.118	5695200	1.05	3412883	1.38	1.67	
75	11.150	2357903	0.43	1537444	0.62	1.53	
76	11.309	67704564	12.43	20178939	8.16	3.36	
77	11.370	4058078	0.75	2270515	0.92	1.79	
78	11.987	2425883	0.45	1055945	0.43	2.30	
79	13.451	3037801	0.56	1238750	0.50	2.45	
80	14.648	3803451	0.70	1772154	0.72	2.15	
81	22.291	9227437	1.69	1292681	0.52	7.14	
82	28.135	43792012	8.04	3064040	1.24	14.29	
		544550129	100.00	247242473	100.00		

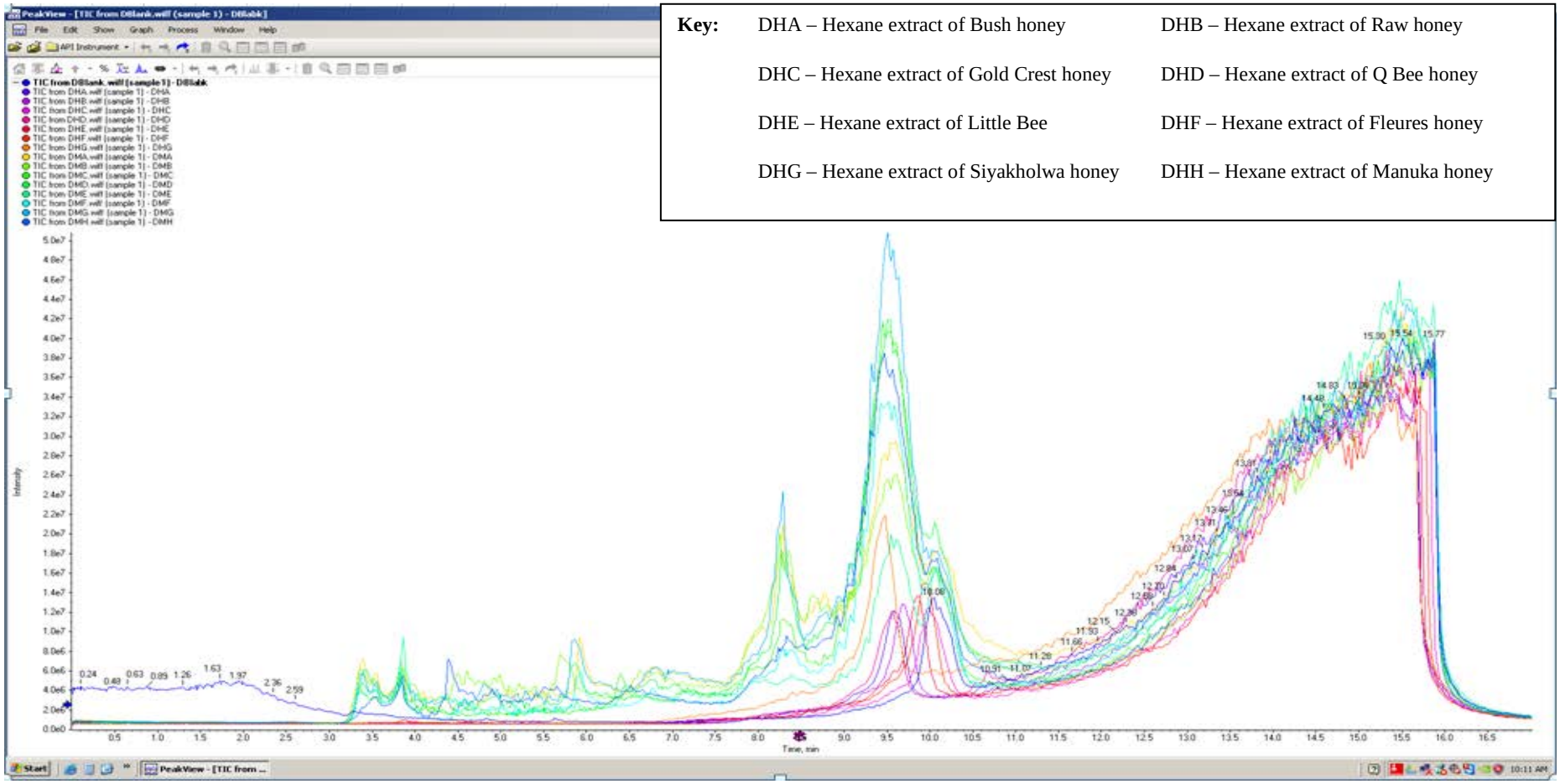
B 4: Chloroform extract of Q Bee honey

Peak Report TIC

Peak#	R.Time	Area	Area%	Height	Height%	A/H	Name
1	7.604	2810308	1.86	1482470	3.04	1.90	Eicosane
2	7.830	2390731	1.58	1131640	2.32	2.11	Eicosane
3	8.634	2991472	1.98	1968532	4.04	1.52	Eicosane
4	8.829	2841721	1.88	1629013	3.34	1.74	Eicosane
5	8.915	1562360	1.03	397199	0.82	3.93	Nonane, 5-methyl-5-propyl-
6	9.554	2700481	1.78	2094341	4.30	1.29	Eicosane
7	9.584	1838091	1.21	1093911	2.25	1.68	Silane, trichlorooctadecyl-
8	9.725	2367689	1.56	1726608	3.54	1.37	Eicosane
9	9.749	2624520	1.73	1415993	2.91	1.85	Pentadecanoic acid
10	10.249	2799896	1.85	760033	1.56	3.68	Eicosane
11	10.381	4059280	2.68	1899454	3.90	2.14	Eicosane
12	10.534	6149473	4.06	1968951	4.04	3.12	Eicosane
13	10.620	2877749	1.90	970550	1.99	2.97	Octadecanamide
14	10.813	2952856	1.95	1353493	2.78	2.18	Octadecanoic acid, 2-hydroxyethyl ester
15	11.019	2460055	1.62	882941	1.81	2.79	Hexatriacontane
16	11.113	1669465	1.10	669652	1.37	2.49	Eicosane
17	11.153	4335929	2.86	1450926	2.98	2.99	Eicosane
18	11.314	22336836	14.75	8465906	17.37	2.64	9-Octadecenamide, (Z)-
19	11.611	2960820	1.96	897028	1.84	3.30	Octadecanoic acid, 2-hydroxyethyl ester
20	11.720	9876224	6.52	3326395	6.83	2.97	1,3,5-Trisilacyclohexane
21	11.822	4864848	3.21	1327537	2.72	3.66	1H-Indene, 1-hexadecyl-2,3-dihydro-
22	11.873	4806114	3.17	1214045	2.49	3.96	Squalane
23	11.948	4129064	2.73	1280246	2.63	3.23	Hexadecanoic acid, 2-hydroxy-1-(hydroxymetl
24	12.039	5736947	3.79	958131	1.97	5.99	Hexatriacontane
25	12.210	2519369	1.66	684321	1.40	3.68	5,5-Diethylpentadecane
26	12.255	2350934	1.55	945177	1.94	2.49	5,5-Diethylpentadecane
27	12.294	2801099	1.85	734412	1.51	3.81	5,5-Diethylpentadecane
28	12.763	3642084	2.41	647344	1.33	5.63	1,3,5-Trisilacyclohexane
29	12.891	1588659	1.05	407568	0.84	3.90	Oxalic acid, 3,5-difluorophenyl tetradecyl este
30	13.018	2077340	1.37	382760	0.79	5.43	Squalane
31	13.558	3314008	2.19	414478	0.85	8.00	2-methylhexacosane
32	14.744	4021092	2.66	1106722	2.27	3.63	Cholesta-3,5-diene
33	22.674	18611831	12.29	2442068	5.01	7.62	Silane, diethylheptyloxyoctadecyloxy-
34	28.411	6354534	4.20	595229	1.22	10.68	1,4-Epoxynaphthalene-1(2H)-methanol, 4,5,7-
		151423879	100.00	48725074	100.00		

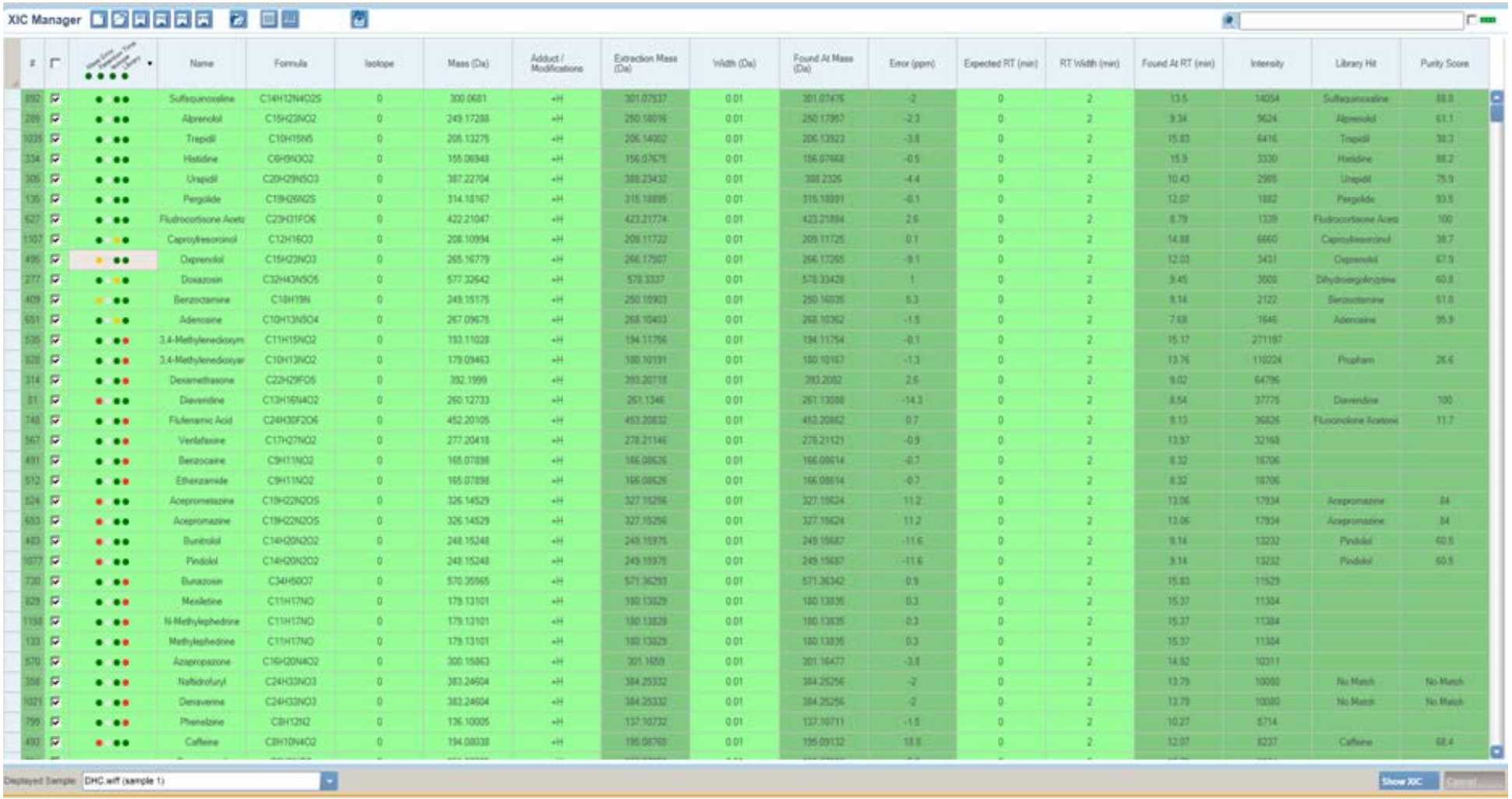
APPENDIX C: LC-MS RESULTS ANALYSIS USING METABOLITE PILOTE SOFTWARE ON DRUG DISCOVERY

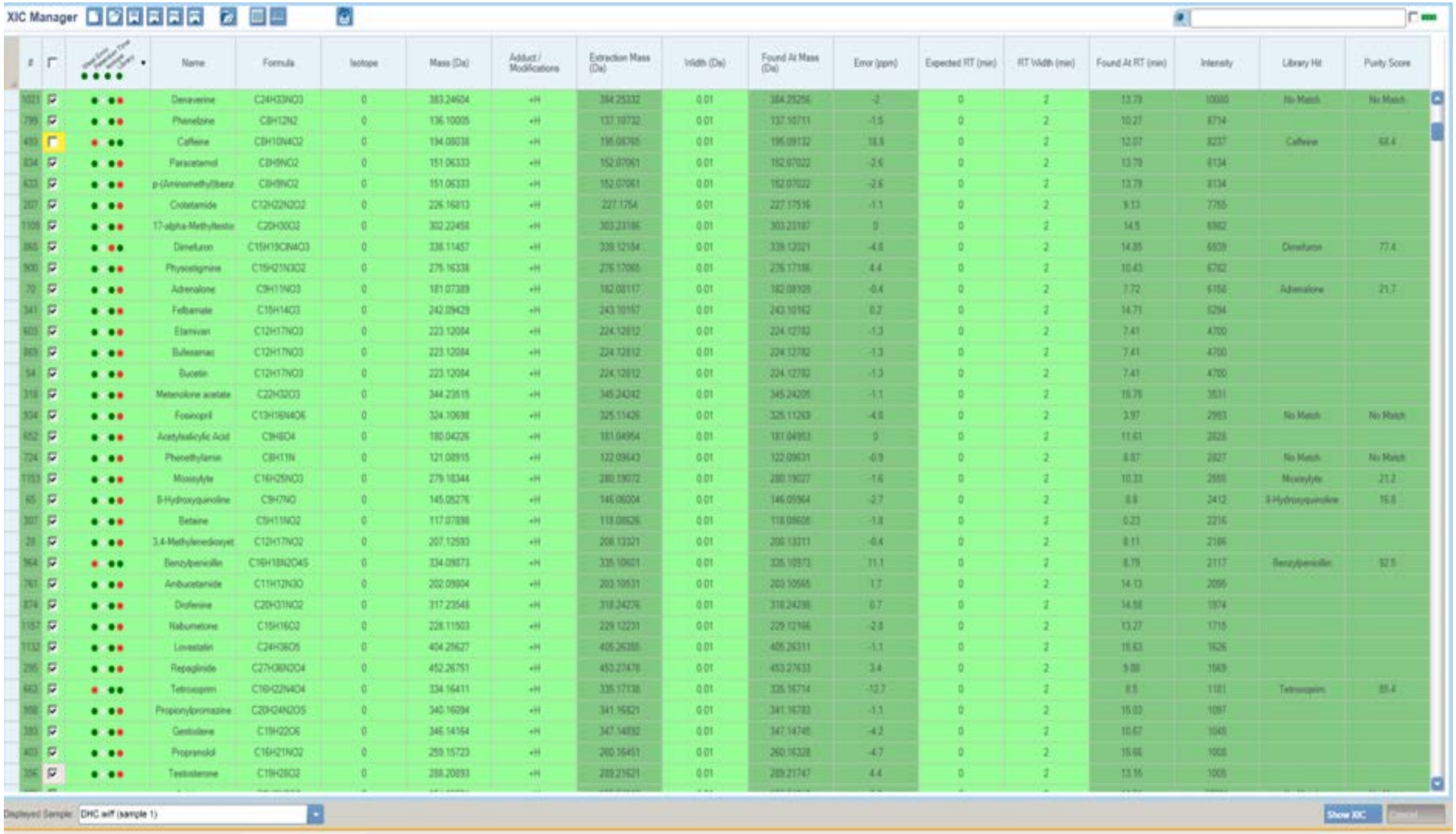
C:1 Peak view TIC –DHA- DHG



Key:	DHA – Hexane extract of Bush honey	DHB – Hexane extract of Raw honey
	DHC – Hexane extract of Gold Crest honey	DHD – Hexane extract of Q Bee honey
	DHE – Hexane extract of Little Bee	DHF – Hexane extract of Fleures honey
	DHG – Hexane extract of Siyakholwa honey	DHH – Hexane extract of Manuka honey

C: 2 XIC manager (DHC- Hexane extract of Gold Crest wiff sample 1)

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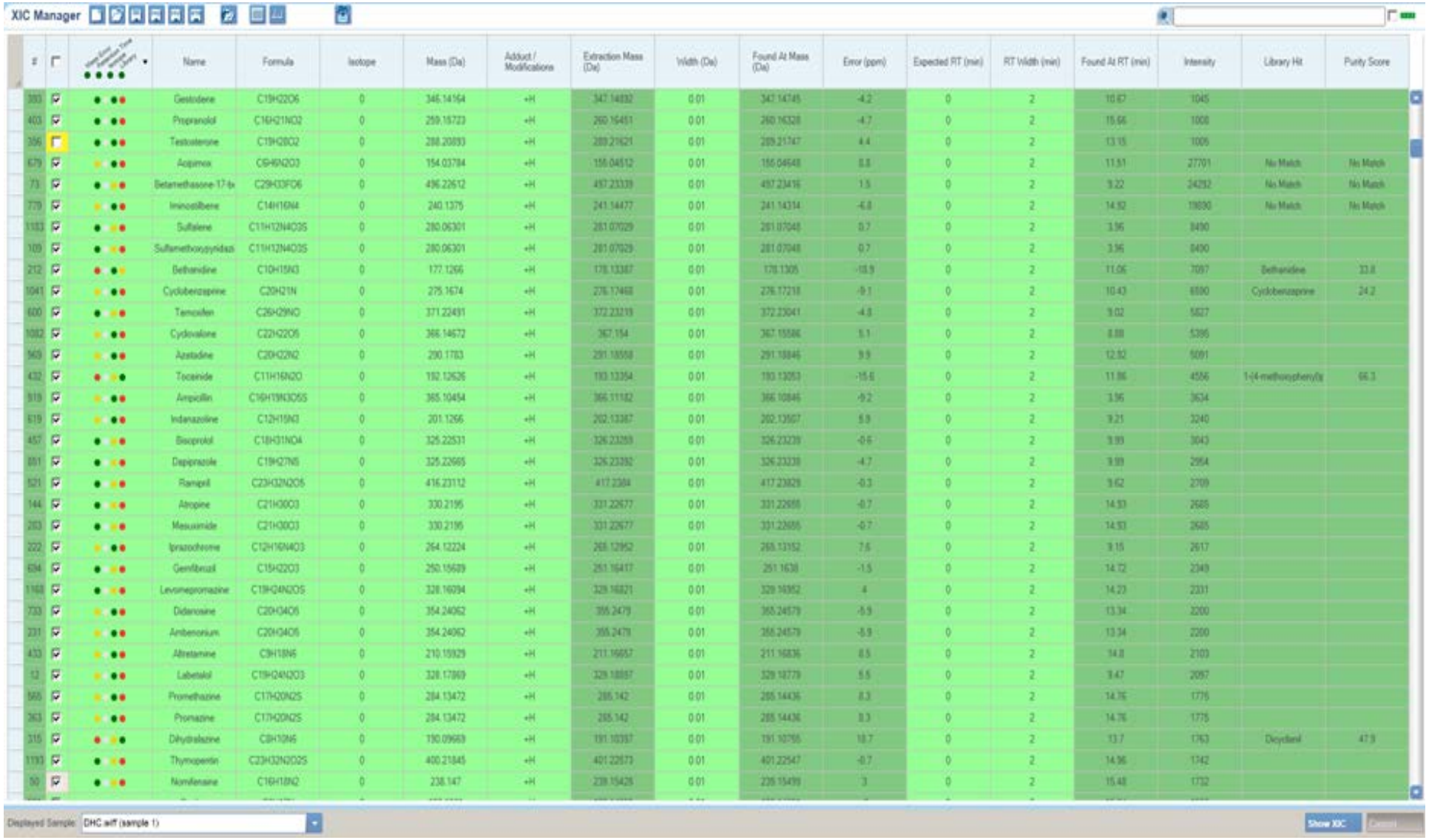
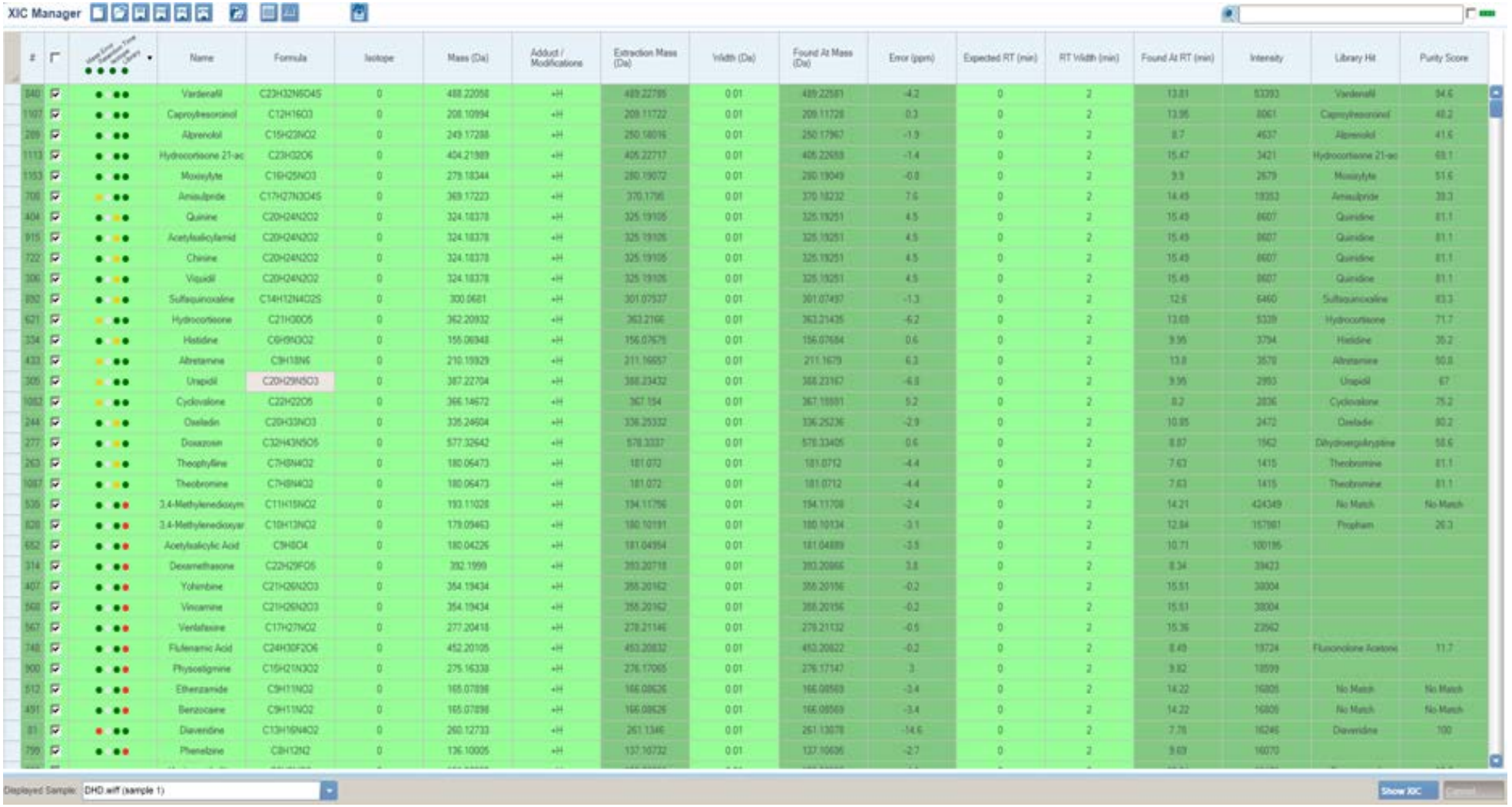
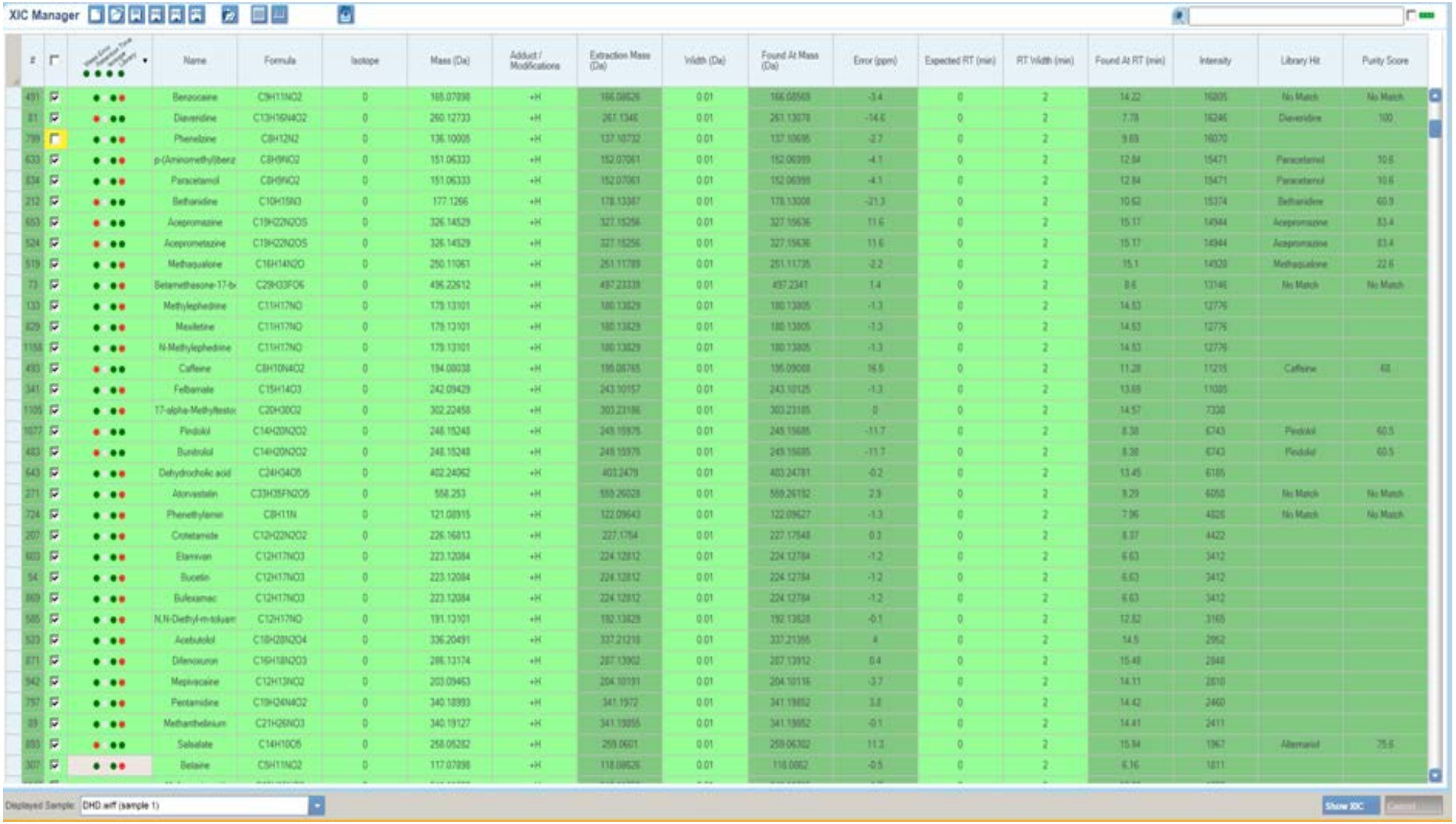
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Displayed Sample:
DHC wiff (sample 1)
Show XIC


XIC Manager
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Gestodene
Propranolol
Testosterone
Acipimox
Betamethasone 17-bu
Imiquimod
Sulfalene
Sulfamethoxypyridazi
Bethanidine
Cyclobenzaprine
Tamoxifen
Cydovalone
Azatadine
Tocainide
Ampicillin
Indanazoline
Bisoprolol
Dapiprazole
Ramipril
Atropine
Mesuximide
Iprazochrome
Gemfibrozil
Levomepromazine
Didanosine
Ambenonium
Altretamine
Labetalol
Promethazine
Pronazine
Dihydralazine
Thymopentin
Nomifensine
Displayed Sample:
DHC wiff (sample 1)
Show XIC

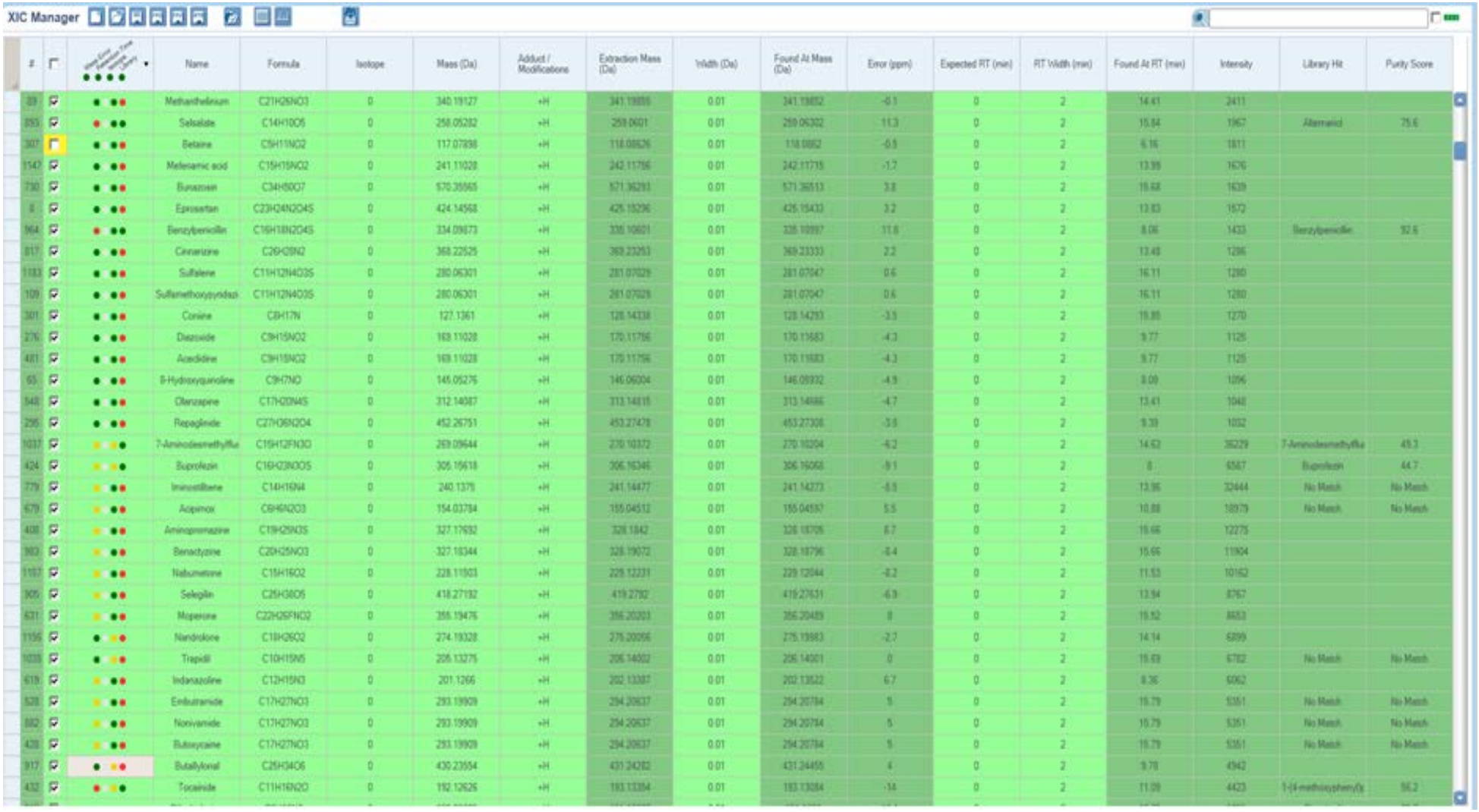
C: 3 XIC manager (DHD- Hexane extract of Q Bee wiff sample 1)



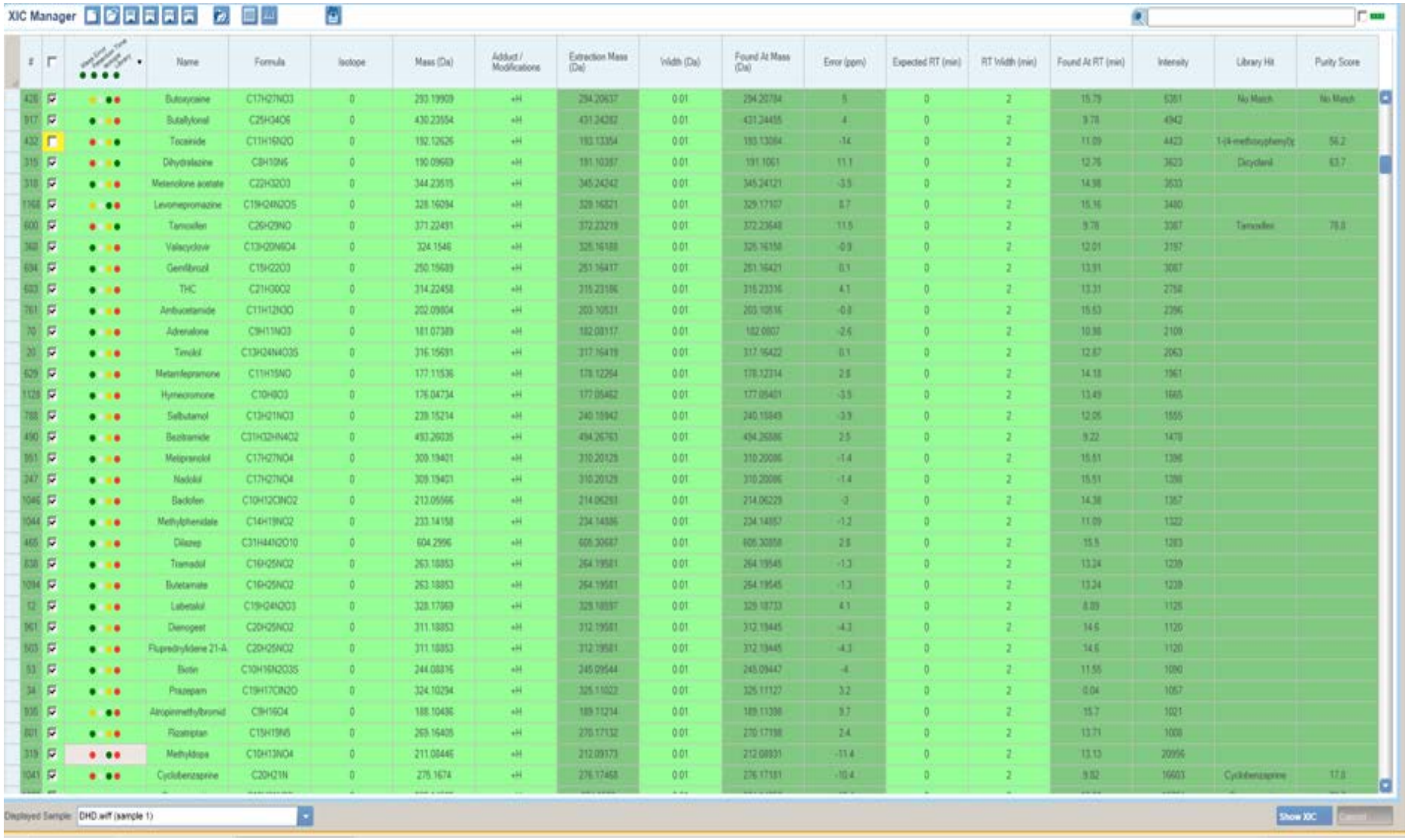
#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
840	Vardenafil	C23H32N6O4S	0	488.22058	+H	489.22785	0.01	489.22581	-4.2	0	2	13.81	53393	Vardenafil	94.6
1107	Caproylresorcinol	C12H16O3	0	208.10994	+H	209.11722	0.01	209.11728	0.3	0	2	13.95	8061	Caproylresorcinol	48.2
289	Alprenolol	C15H23NO2	0	249.17288	+H	250.18016	0.01	250.17967	-1.9	0	2	8.7	4637	Alprenolol	41.6
1113	Hydrocortisone 21-ac	C23H32O6	0	404.21989	+H	405.22717	0.01	405.22658	-1.4	0	2	15.47	3421	Hydrocortisone 21-ac	69.1
1153	Moxisylyte	C19H25NO3	0	279.18344	+H	280.19072	0.01	280.19049	-0.8	0	2	9.9	2679	Moxisylyte	51.6
708	Amisulpride	C17H27N3O4S	0	369.17223	+H	370.1795	0.01	370.18232	7.6	0	2	14.49	18353	Amisulpride	39.3
404	Quinine	C20H24N2O2	0	324.18378	+H	325.19105	0.01	325.19251	4.5	0	2	15.49	8607	Quinidine	81.1
915	Acetylsalicylamid	C20H24N2O2	0	324.18378	+H	325.19105	0.01	325.19251	4.5	0	2	15.49	8607	Quinidine	81.1
722	Chinine	C20H24N2O2	0	324.18378	+H	325.19105	0.01	325.19251	4.5	0	2	15.49	8607	Quinidine	81.1
306	Visodil	C20H24N2O2	0	324.18378	+H	325.19105	0.01	325.19251	4.5	0	2	15.49	8607	Quinidine	81.1
892	Sulfaquinoxaline	C14H12N4O2S	0	300.0681	+H	301.07537	0.01	301.07497	-1.3	0	2	12.6	6460	Sulfaquinoxaline	83.3
621	Hydrocortisone	C21H30O5	0	362.20932	+H	363.2166	0.01	363.21405	-6.2	0	2	13.69	5329	Hydrocortisone	71.7
234	Histidine	C6H9NO2	0	155.06948	+H	156.07676	0.01	156.07684	0.6	0	2	9.95	3794	Histidine	35.2
433	Abrotanine	C9H18N6	0	210.15929	+H	211.16657	0.01	211.1679	6.3	0	2	13.8	3578	Abrotanine	50.8
305	Urapidil	C20H29N5O3	0	387.22704	+H	388.23432	0.01	388.23167	-6.8	0	2	9.95	2993	Urapidil	67
1082	Cyclovalone	C22H22O5	0	366.14672	+H	367.154	0.01	367.15581	5.2	0	2	8.2	2836	Cyclovalone	75.2
244	Oxeladin	C20H33NO3	0	335.24604	+H	336.25332	0.01	336.25236	-2.9	0	2	10.85	2472	Oxeladin	80.2
277	Doxazosin	C23H43N5O5	0	577.32642	+H	578.3337	0.01	578.33405	0.6	0	2	8.87	1562	Dihydroergokryptine	58.6
263	Theophylline	C7H8N4O2	0	180.06473	+H	181.072	0.01	181.0712	-4.4	0	2	7.63	1415	Theobromine	81.1
1087	Theobromine	C7H8N4O2	0	180.06473	+H	181.072	0.01	181.0712	-4.4	0	2	7.63	1415	Theobromine	81.1
535	3,4-Methylenedioxym	C11H15NO2	0	193.11028	+H	194.11756	0.01	194.11708	-2.4	0	2	14.21	424349	No Match	No Match
828	3,4-Methylenedioxyar	C10H13NO2	0	179.09463	+H	180.10191	0.01	180.10134	-3.1	0	2	12.84	157981	Prophen	26.3
652	Acetylsalicylic Acid	C9H8O4	0	180.04226	+H	181.04954	0.01	181.04889	-3.5	0	2	10.71	100196		
314	Dexamethasone	C22H29FO5	0	392.1999	+H	393.20718	0.01	393.20866	3.8	0	2	8.34	39423		
407	Yohimbine	C21H26N2O3	0	354.19434	+H	355.20162	0.01	355.20156	-0.2	0	2	15.51	38004		
568	Vincamine	C21H26N2O3	0	354.19434	+H	355.20162	0.01	355.20156	-0.2	0	2	15.51	38004		
567	Venlafaxine	C17H27NO2	0	277.20418	+H	278.21146	0.01	278.21132	-0.5	0	2	15.36	23562		
748	Flufenamic Acid	C24H30F2O6	0	452.20105	+H	453.20832	0.01	453.20822	-0.2	0	2	8.49	19724	Fluocinolone Acetone	11.7
900	Physostigmine	C15H21N3O2	0	275.16338	+H	276.17065	0.01	276.17147	3	0	2	9.82	18599		
512	Etherzamide	C9H11NO2	0	165.07898	+H	166.08626	0.01	166.08569	-3.4	0	2	14.22	16809	No Match	No Match
491	Benzocaine	C9H11NO2	0	165.07898	+H	166.08626	0.01	166.08569	-3.4	0	2	14.22	16809	No Match	No Match
81	Diavendine	C13H16N4O2	0	260.12733	+H	261.1346	0.01	261.13078	-14.6	0	2	7.78	16246	Diavendine	100
799	Phenebine	C8H12N2	0	136.10005	+H	137.10732	0.01	137.10636	-2.7	0	2	9.69	16070		

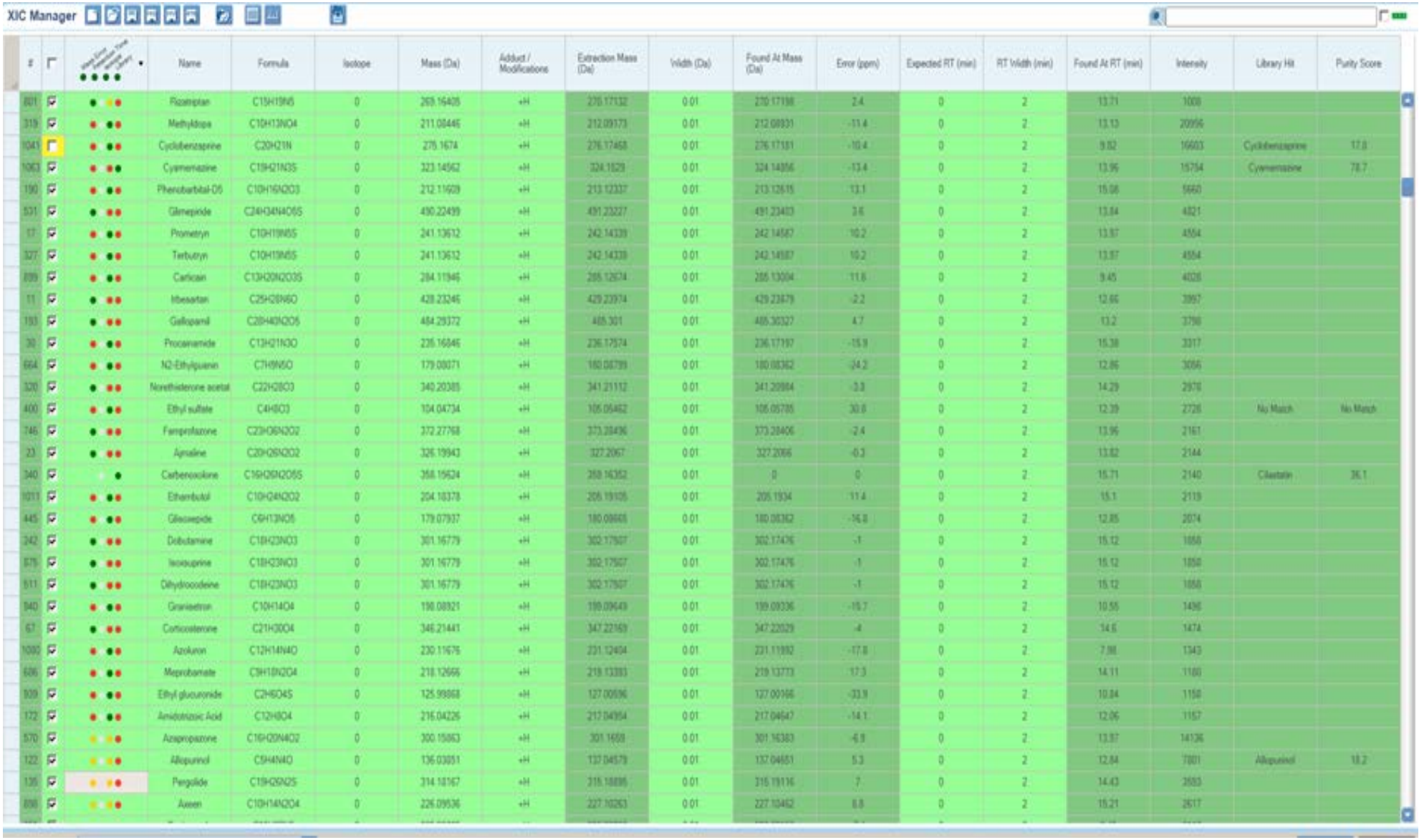


#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
491	Benzocaine	C9H11NO2	0	165.07898	+H	166.08626	0.01	166.08568	-3.4	0	2	14.22	16805	No Match	No Match
81	Diaveridine	C13H16N4O2	0	260.12733	+H	261.1346	0.01	261.13078	-14.6	0	2	7.78	16246	Diaveridine	100
799	Phenelzine	C8H12N2	0	136.10005	+H	137.10732	0.01	137.10695	-2.7	0	2	9.69	16070		
633	p-(Aminomethyl)benz	C8H9NO2	0	151.06333	+H	152.07061	0.01	152.06999	-4.1	0	2	12.84	15471	Paracetamol	10.6
634	Paracetamol	C8H9NO2	0	151.06333	+H	152.07061	0.01	152.06999	-4.1	0	2	12.84	15471	Paracetamol	10.6
212	Bethanidine	C10H15N3	0	177.1266	+H	178.13387	0.01	178.13308	-21.3	0	2	10.62	15374	Bethanidine	60.9
653	Acepromazine	C19H22N2OS	0	326.14529	+H	327.15256	0.01	327.15636	11.6	0	2	15.17	14944	Acepromazine	83.4
524	Aceprometa zine	C19H22N2OS	0	326.14529	+H	327.15256	0.01	327.15636	11.6	0	2	15.17	14944	Acepromazine	83.4
519	Methaqualone	C16H14N2O	0	250.11061	+H	251.11789	0.01	251.11735	-2.2	0	2	15.1	14928	Methaqualone	22.6
73	Betamethasone-17-bu	C28H37FO6	0	496.22612	+H	497.23339	0.01	497.2341	1.4	0	2	8.6	13146	No Match	No Match
133	Methylephedrine	C11H17NO	0	179.13101	+H	180.13829	0.01	180.13805	-1.3	0	2	14.53	12779		
829	Mexiletine	C11H17NO	0	179.13101	+H	180.13829	0.01	180.13805	-1.3	0	2	14.53	12779		
1158	N-Methylephedrine	C11H17NO	0	179.13101	+H	180.13829	0.01	180.13805	-1.3	0	2	14.53	12779		
493	Caffeine	C8H10N4O2	0	194.08038	+H	195.08765	0.01	195.09068	16.5	0	2	11.28	11215	Caffeine	68
341	Felbamate	C11H14O3	0	242.09429	+H	243.10157	0.01	243.10125	-1.3	0	2	13.68	11085		
1105	17-alpha-Methyltestos	C20H30O2	0	302.22458	+H	303.23186	0.01	303.23185	0	0	2	14.57	7338		
1077	Pindolol	C14H20N2O2	0	248.15248	+H	249.15975	0.01	249.15685	-11.7	0	2	8.38	6743	Pindolol	60.5
483	Bunitrolol	C14H20N2O2	0	248.15248	+H	249.15975	0.01	249.15685	-11.7	0	2	8.38	6743	Pindolol	60.5
643	Dehydrocholic acid	C24H34O5	0	402.24062	+H	403.2479	0.01	403.24781	-0.2	0	2	13.45	6185		
271	Atorvastatin	C33H35FN2O5	0	558.253	+H	559.26028	0.01	559.26192	2.9	0	2	9.29	6050	No Match	No Match
724	Phenethylamin	C8H11N	0	121.08915	+H	122.09643	0.01	122.09627	-1.3	0	2	7.96	4826	No Match	No Match
207	Crotetamide	C12H22N2O2	0	226.16813	+H	227.1754	0.01	227.17548	0.3	0	2	8.37	4422		
603	Etamivan	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12784	-1.2	0	2	6.63	3412		
54	Bucetin	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12784	-1.2	0	2	6.63	3412		
869	Bufexamac	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12784	-1.2	0	2	6.63	3412		
585	N,N-Diethyl-m-toluam	C12H17NO	0	191.13101	+H	192.13829	0.01	192.13828	-0.1	0	2	12.82	3165		
523	Acebutolol	C18H28N2O4	0	336.20491	+H	337.21219	0.01	337.21355	4	0	2	14.5	2952		
671	Difenoxuron	C16H18N2O3	0	286.13174	+H	287.13902	0.01	287.13912	0.4	0	2	15.48	2948		
942	Mepivacaine	C12H13NO2	0	203.09463	+H	204.10191	0.01	204.10116	-3.7	0	2	14.11	2610		
797	Pentamidine	C19H24N4O2	0	340.18993	+H	341.1972	0.01	341.19852	3.8	0	2	14.42	2460		
89	Methantheline	C21H26NO3	0	340.19127	+H	341.19855	0.01	341.19852	-0.1	0	2	14.41	2411		
893	Salsalate	C14H10O5	0	258.05282	+H	259.0601	0.01	259.06302	11.3	0	2	15.94	1967	Alternariol	75.6
307	Betaine	C5H11NO2	0	117.07898	+H	118.08626	0.01	118.0862	-0.5	0	2	6.16	1831		



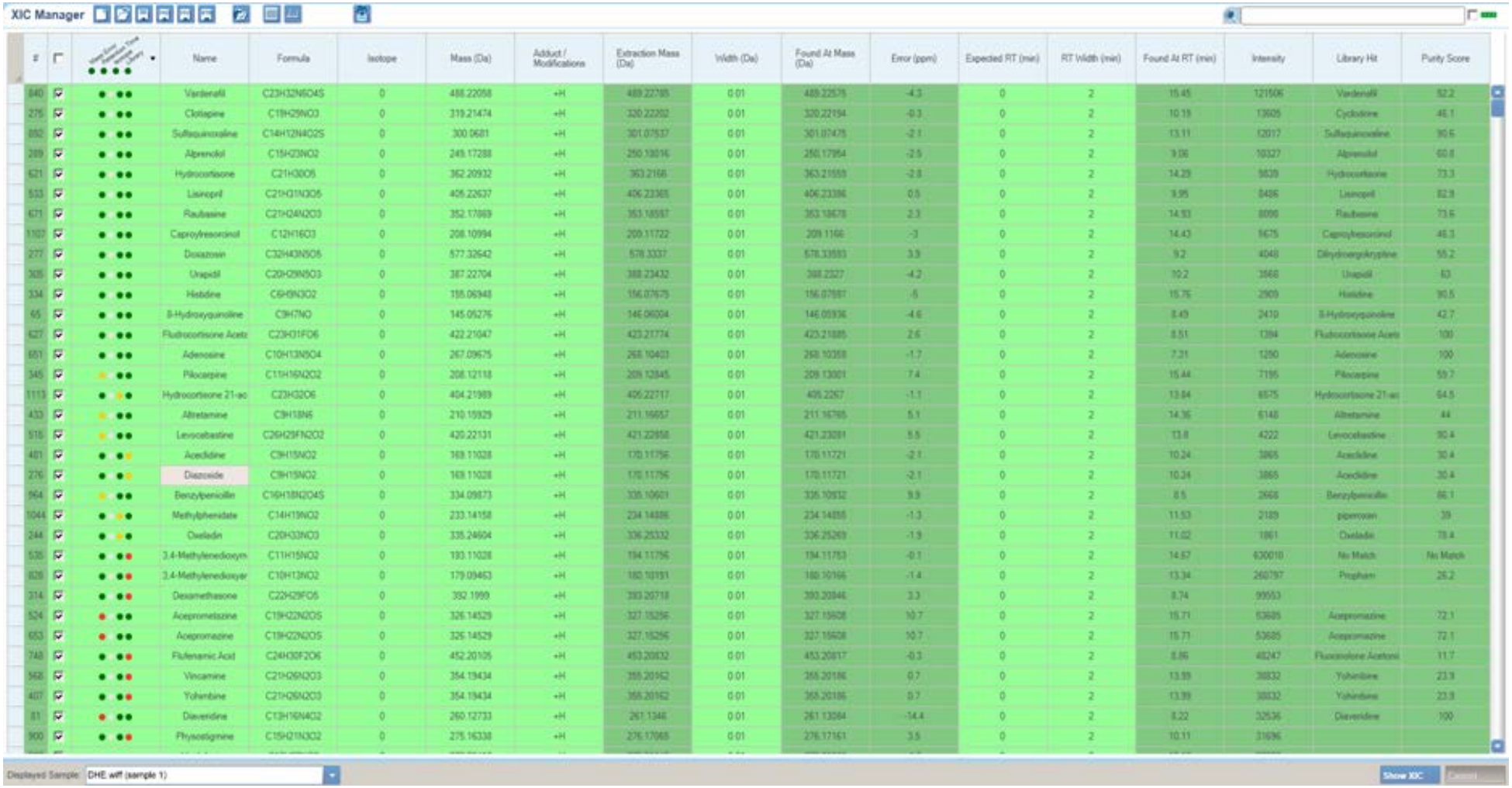
#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
89	Methanthelinium	C21H26NO3	0	340.19127	+H	340.19855	0.01	340.19852	-0.1	0	2	14.41	2411		
893	Selsalate	C14H10O5	0	258.05282	+H	259.0601	0.01	259.06302	11.3	0	2	15.84	1967	Alternaria	75.6
307	Betaine	C5H11NO2	0	117.07898	+H	118.08626	0.01	118.0862	-0.5	0	2	6.16	1811		
1147	Meloxamic acid	C15H19NO2	0	241.11028	+H	242.11756	0.01	242.11715	-1.7	0	2	13.99	1676		
730	Burazocin	C34H50O7	0	570.35565	+H	571.36293	0.01	571.36513	3.8	0	2	15.68	1639		
8	Eprosartan	C23H24N2O4S	0	424.14568	+H	425.15296	0.01	425.15433	3.2	0	2	13.83	1572		
964	Benzylpenicillin	C16H18N2O4S	0	334.09873	+H	335.10601	0.01	335.10897	11.8	0	2	8.06	1433	Benzylpenicillin	92.6
817	Cinnarizine	C26H28N2	0	368.22525	+H	369.23253	0.01	369.23333	2.2	0	2	13.48	1286		
1183	Sulfalene	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07047	0.6	0	2	16.11	1280		
109	Sulfamethoxypyridazi	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07047	0.6	0	2	16.11	1280		
301	Coniine	C8H17N	0	127.1361	+H	128.14338	0.01	128.14293	-3.5	0	2	15.85	1270		
276	Diazoxide	C8H7N3O2	0	169.11028	+H	170.11756	0.01	170.11683	-4.3	0	2	9.77	1125		
481	Azadirone	C8H7N3O2	0	169.11028	+H	170.11756	0.01	170.11683	-4.3	0	2	9.77	1125		
65	8-Hydroxyquinoline	C9H7NO	0	145.05276	+H	146.06004	0.01	146.05932	-4.9	0	2	8.09	1096		
548	Olanzapine	C17H20N4S	0	312.14087	+H	313.14815	0.01	313.14686	-4.1	0	2	13.41	1048		
296	Repaglinide	C27H36N2O4	0	452.26751	+H	453.27478	0.01	453.27308	-3.8	0	2	9.39	1032		
1037	7-Aminodesmethylflu	C15H12F3NO	0	279.09644	+H	280.10372	0.01	280.10204	-6.2	0	2	14.62	35229	7-Aminodesmethylflu	49.3
424	Buprofezin	C16H23N3OS	0	305.15618	+H	306.16346	0.01	306.16068	-9.1	0	2	8	6587	Buprofezin	44.7
779	Iminostilbene	C14H11N	0	240.1375	+H	241.14477	0.01	241.14273	-8.5	0	2	13.96	32444	No Match	No Match
679	Acipimox	C6H6N2O3	0	154.03784	+H	155.04512	0.01	155.04597	5.5	0	2	10.88	18979	No Match	No Match
408	Aminopromazine	C19H25N3S	0	327.17932	+H	328.1842	0.01	328.18705	8.7	0	2	15.66	12275		
983	Benactyzine	C20H25NO3	0	327.18344	+H	328.19072	0.01	328.18796	-8.4	0	2	15.66	11904		
1107	Nabumetone	C15H16O2	0	228.11503	+H	229.12231	0.01	229.12044	-8.2	0	2	11.53	10162		
905	Selegiln	C25H38O5	0	418.27192	+H	419.2792	0.01	419.27631	-6.9	0	2	13.94	8767		
631	Moperone	C22H26FNO2	0	355.19476	+H	356.20203	0.01	356.20489	8	0	2	15.52	8653		
1155	Nandrolone	C18H26O2	0	274.19328	+H	275.20056	0.01	275.19983	-2.7	0	2	14.14	6899		
1033	Trapidil	C10H15N5	0	205.13275	+H	206.14002	0.01	206.14001	0	0	2	15.69	6782	No Match	No Match
618	Indanazoline	C12H15N3	0	201.1266	+H	202.13387	0.01	202.13522	6.7	0	2	8.36	6062		
528	Enbutamide	C17H27NO3	0	293.19909	+H	294.20637	0.01	294.20784	5	0	2	15.79	5351	No Match	No Match
882	Nonivamide	C17H27NO3	0	293.19909	+H	294.20637	0.01	294.20784	5	0	2	15.79	5351	No Match	No Match
428	Butoxycaine	C17H27NO3	0	293.19909	+H	294.20637	0.01	294.20784	5	0	2	15.79	5351	No Match	No Match
917	Butallylonal	C25H34O6	0	430.23554	+H	431.24282	0.01	431.24455	4	0	2	9.78	4942		
432	Tocainide	C11H16N2O	0	192.12626	+H	193.13354	0.01	193.13084	-14	0	2	11.09	4423	1-(4-methoxyphenyl)(	55.2

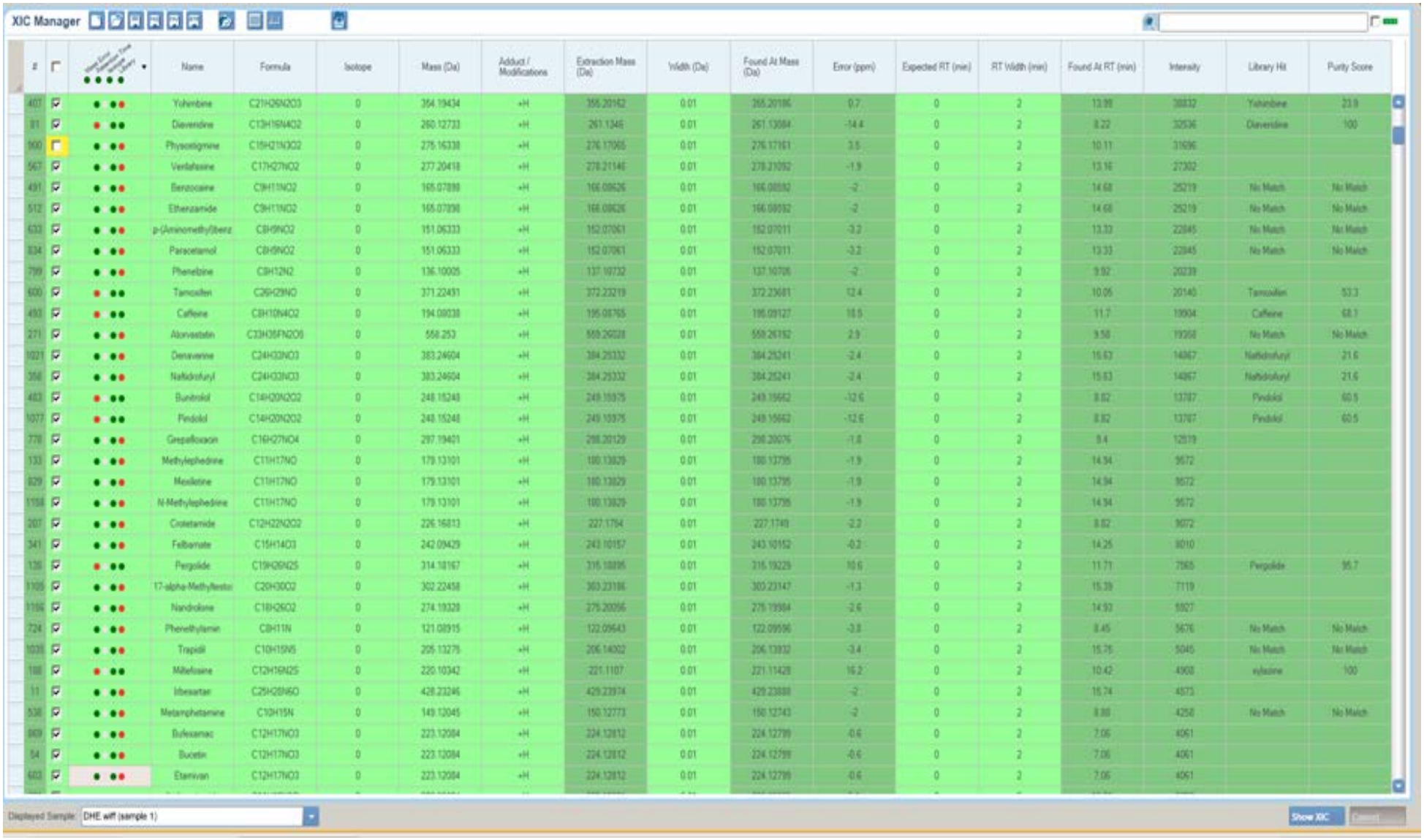

XIC Manager
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Butoxycaine
Butallylonal
Tocainide
Dihydralazine
Meterolone acetate
Levomepromazine
Tamoxifen
Valacyclovir
Gemfibrozil
THC
Ambucetamide
Adrenalone
Timolol
Metamfepramone
Hymecromone
Salbutamol
Bezitramide
Metipranolol
Nadolol
Badofen
Methylphenidate
Diazep
Tramadol
Butetamate
Labetalol
Dienogest
Flupredrylidene 21-A
Biotin
Prazepam
Atropinemethylbromid
Rizatriptan
Methyldopa
Cyclobenzaprine
Displayed Sample:
DHD.wiff (sample 1)
Show XIC



#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
801	Rizatriptan	C15H19N5	0	269.16405	+H	270.17132	0.01	270.17198	2.4	0	2	13.71	1008		
319	Methyldopa	C10H13NO4	0	211.08446	+H	212.09173	0.01	212.08931	-11.4	0	2	13.13	20956		
1041	Cyclobenzaprine	C20H21N	0	275.1674	+H	276.17468	0.01	276.17181	-10.4	0	2	9.82	16603	Cyclobenzaprine	17.8
1063	Cyamemazine	C19H21N3S	0	323.14562	+H	324.1529	0.01	324.14856	-13.4	0	2	13.96	15754	Cyamemazine	78.7
190	Phenobarbital-D5	C10H16N2O3	0	212.11609	+H	213.12337	0.01	213.12615	13.1	0	2	15.08	5660		
531	Glimepiride	C24H34N4O5S	0	490.22499	+H	491.23227	0.01	491.23403	3.6	0	2	13.84	4821		
17	Prometryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14587	10.2	0	2	13.87	4554		
327	Terbutryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14587	10.2	0	2	13.87	4554		
899	Carticain	C13H20N2O3S	0	284.11946	+H	285.12674	0.01	285.13004	11.6	0	2	9.45	4026		
11	Irbesartan	C25H28N6O	0	428.23246	+H	429.23974	0.01	429.23879	-2.2	0	2	12.66	3997		
193	Gallopamil	C28H41NO5	0	471.29372	+H	472.301	0.01	472.30327	4.7	0	2	13.2	3798		
30	Procainamide	C13H21N3O	0	235.16846	+H	236.17574	0.01	236.17797	-15.9	0	2	15.38	3317		
664	N2-Ethylguanin	C7H9N5O	0	179.08071	+H	180.08799	0.01	180.08362	-24.2	0	2	12.86	3056		
320	Norethisterone acetat	C22H28O3	0	340.20385	+H	341.21112	0.01	341.20984	-3.8	0	2	14.29	2976		
400	Ethyl sulfate	C4H6O3	0	104.04734	+H	105.05462	0.01	105.05785	30.8	0	2	12.39	2726	No Match	No Match
746	Fenspiclazone	C23H26N2O2	0	362.27768	+H	373.28496	0.01	373.28406	-2.4	0	2	13.96	2161		
23	Apraline	C20H26N2O2	0	326.19943	+H	327.2067	0.01	327.2066	-0.3	0	2	13.82	2144		
340	Carbenoxolone	C15H26N2O5S	0	358.15624	+H	359.16352	0.01	0	0	0	2	15.71	2140	Cilastatin	36.1
1011	Ethambutol	C10H24N2O2	0	204.18378	+H	205.19105	0.01	205.1934	11.4	0	2	15.1	2119		
445	Gliclazide	C15H13NO5	0	179.07937	+H	180.08665	0.01	180.08362	-16.8	0	2	12.85	2074		
242	Dobutamine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17476	-1	0	2	15.12	1858		
675	Isoxsuprine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17476	-1	0	2	15.12	1858		
511	Dihydrocodeine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17476	-1	0	2	15.12	1858		
940	Granisetron	C10H14O4	0	198.08921	+H	199.09649	0.01	199.09336	-15.7	0	2	10.55	1496		
67	Corticosterone	C21H30O4	0	346.21441	+H	347.22169	0.01	347.22029	-4	0	2	14.6	1474		
1092	Azoluron	C12H14N4O	0	230.11676	+H	231.12404	0.01	231.11892	-17.8	0	2	7.98	1343		
686	Meprobamate	C9H18N2O4	0	218.12666	+H	219.13393	0.01	219.13773	17.3	0	2	14.11	1160		
939	Ethyl glucuronide	C2H6O4S	0	125.99868	+H	127.00596	0.01	127.00166	-33.9	0	2	10.84	1158		
172	Amidotrizoic Acid	C12H9O4	0	215.04226	+H	217.04954	0.01	217.04647	-14.1	0	2	12.06	1157		
570	Azaperpazone	C19H20N4O2	0	300.15863	+H	301.1659	0.01	301.16383	-6.8	0	2	13.87	14136		
122	Allopurinol	C5H4N4O	0	136.03851	+H	137.04579	0.01	137.04651	5.3	0	2	12.84	7001	Allopurinol	18.2
135	Pergolide	C19H26N2S	0	314.18167	+H	315.18895	0.01	315.19116	7	0	2	14.43	2693		
898	Axsen	C10H14N2O4	0	226.09536	+H	227.10263	0.01	227.10462	8.8	0	2	15.21	2617		

C: 4 XIC manager (DHE- Hexane extract of Little Bee wiff sample 1

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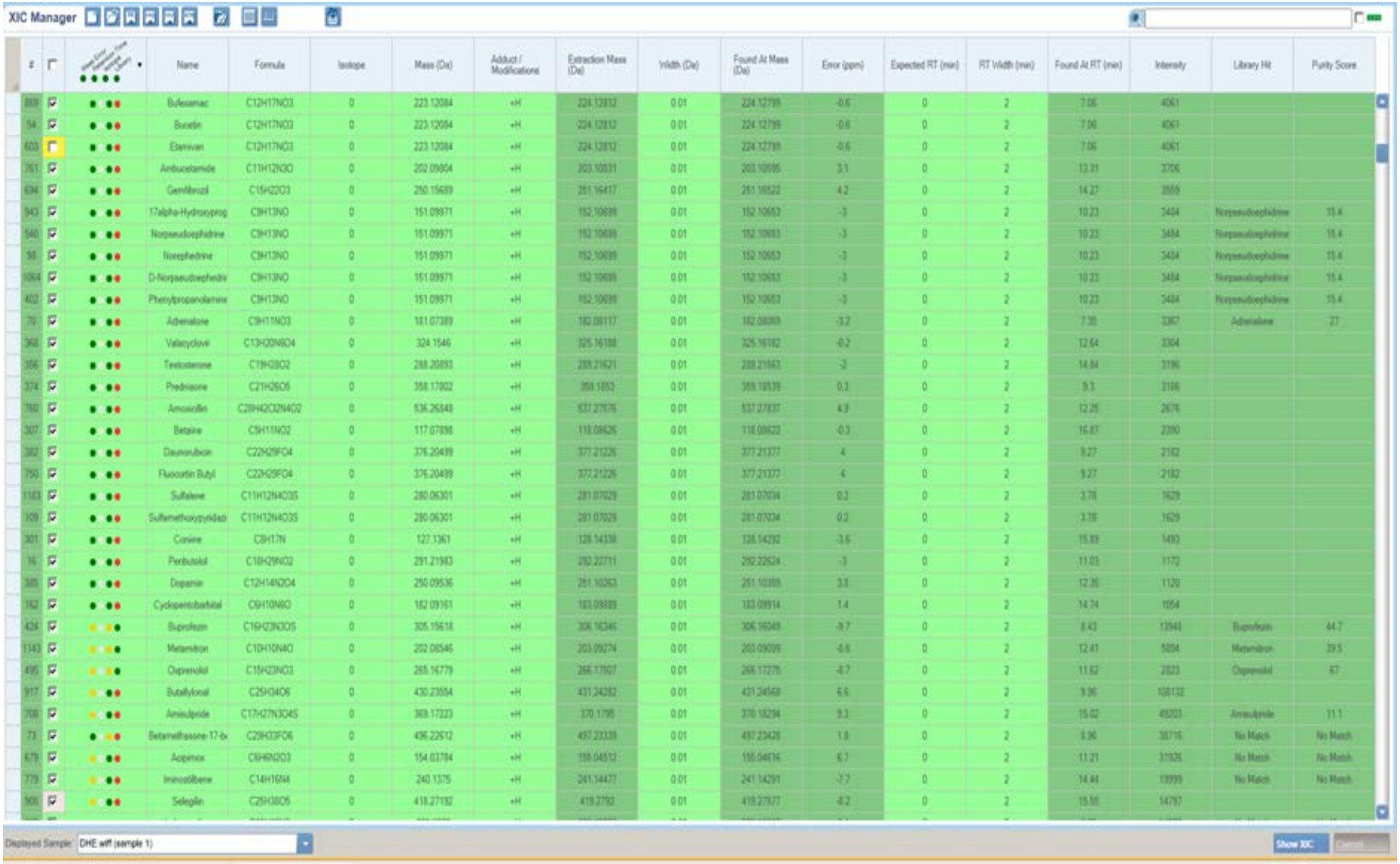
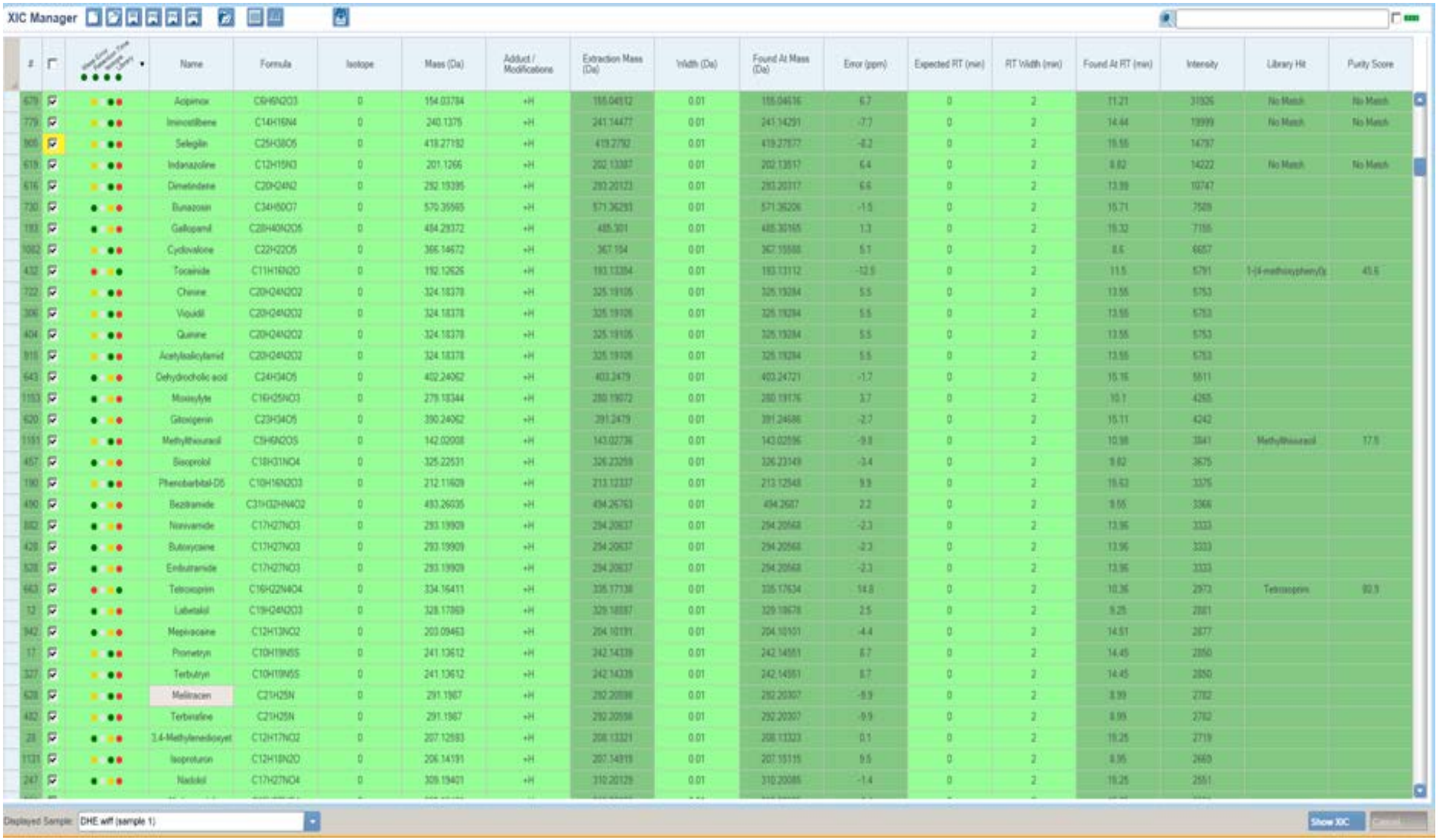
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Yohimbine
Reserpine
Physostigmine
Verbascoside
Benzocaine
Etanercept
Paracetamol
Phenelzine
Tamoxifen
Caffeine
Atorvastatin
Denaverine
Nafidrofuryl
Buntrolol
Pindolol
Gepefloxacin
Methylephedrine
Mesiletine
N-Methylephedrine
Crotetamide
Felbamate
Pergolide
17-alpha-Methylestosterone
Nandrolone
Phenethylamine
Trapidil
Mibefradil
Irbesartan
Metamphetamine
Bufexamac
Bucetin
Etanivan
Displayed Sample:
DHE.wiff (sample 1)
Show XIC



#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
889	Bufexamac	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12799	-0.6	0	2	7.06	4061		
54	Bucetin	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12799	-0.6	0	2	7.06	4061		
603	Etamivan	C12H17NO3	0	223.12084	+H	224.12812	0.01	224.12799	-0.6	0	2	7.06	4061		
761	Ambucetamide	C11H12N2O	0	202.09804	+H	203.10531	0.01	203.10696	3.1	0	2	13.31	3706		
694	Gemfibrozil	C15H22O3	0	250.15689	+H	251.16417	0.01	251.16522	4.2	0	2	14.27	3559		
943	17alpha-Hydroxyprog	C9H13NO	0	151.09971	+H	152.10699	0.01	152.10653	-3	0	2	10.23	3484	Norpseudoephidrine	55.4
540	Norpseudoephidrine	C9H13NO	0	151.09971	+H	152.10699	0.01	152.10653	-3	0	2	10.23	3484	Norpseudoephidrine	55.4
98	Norephedrine	C9H13NO	0	151.09971	+H	152.10699	0.01	152.10653	-3	0	2	10.23	3484	Norpseudoephidrine	55.4
1064	D-Norpseudoephedri	C9H13NO	0	151.09971	+H	152.10699	0.01	152.10653	-3	0	2	10.23	3484	Norpseudoephidrine	55.4
402	Phenylpropanolamine	C9H13NO	0	151.09971	+H	152.10699	0.01	152.10653	-3	0	2	10.23	3484	Norpseudoephidrine	55.4
70	Adrenalone	C9H11NO3	0	181.07389	+H	182.08117	0.01	182.08059	-3.2	0	2	7.35	3367	Adrenalone	27
368	Valacyclovir	C13H20N6O4	0	324.1546	+H	325.16188	0.01	325.16182	-0.2	0	2	12.64	3304		
356	Testosterone	C19H28O2	0	288.20893	+H	289.21621	0.01	289.21663	2	0	2	14.84	3196		
374	Prednisone	C21H26O5	0	358.17802	+H	359.1853	0.01	359.18539	0.3	0	2	9.3	3186		
760	Amoxicillin	C28H42C2N4O2	0	536.26548	+H	537.27276	0.01	537.27837	4.9	0	2	12.25	2676		
307	Betaine	C5H11NO2	0	117.07898	+H	118.08626	0.01	118.08622	-0.3	0	2	16.87	2390		
382	Daunorubicin	C22H29FO4	0	376.20499	+H	377.21228	0.01	377.21377	4	0	2	9.27	2182		
750	Flucortin Butyl	C22H29FO4	0	376.20499	+H	377.21228	0.01	377.21377	4	0	2	9.27	2182		
1183	Sulfalene	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07034	0.2	0	2	3.78	1629		
109	Sulfamethoxypyridazi	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07034	0.2	0	2	3.78	1629		
301	Coniine	C8H17N	0	127.1361	+H	128.14338	0.01	128.14292	-3.6	0	2	15.89	1493		
16	Penbutolol	C18H29NO2	0	291.21983	+H	292.22711	0.01	292.22624	-3	0	2	11.03	1172		
385	Dopamin	C12H14N2O4	0	250.09536	+H	251.10263	0.01	251.10359	3.8	0	2	12.35	1120		
162	Cyclopentobarbital	C9H10N6O	0	182.09161	+H	183.09889	0.01	183.09914	1.4	0	2	14.74	1054		
424	Buprofezin	C16H23N3OS	0	305.15618	+H	306.16346	0.01	306.16049	-9.7	0	2	8.43	13948	Buprofezin	44.7
1143	Metamitron	C10H10N4O	0	202.08546	+H	203.09274	0.01	203.09099	-8.6	0	2	12.41	5804	Metamitron	39.5
495	Ospremidil	C15H23NO3	0	265.16779	+H	266.17507	0.01	266.17275	-8.7	0	2	11.62	2823	Ospremidil	67
917	Bubalbyosal	C25H34O6	0	430.23554	+H	431.24282	0.01	431.24568	6.6	0	2	9.96	108132		
708	Amisulpride	C17H27N3O4S	0	369.17223	+H	370.1795	0.01	370.18234	9.3	0	2	15.02	49203	Amisulpride	11.1
73	Betamethasone-17-b	C29H37FO6	0	496.22612	+H	497.23339	0.01	497.23428	1.8	0	2	8.96	38716	No Match	No Match
679	Aopenox	C9H6N2O3	0	154.03784	+H	155.04512	0.01	155.04616	6.7	0	2	11.21	31926	No Match	No Match
779	Imiconstilbene	C14H16N4	0	240.1375	+H	241.14477	0.01	241.14291	-7.7	0	2	14.44	13999	No Match	No Match
909	Selegilin	C25H38O6	0	418.27192	+H	419.2792	0.01	419.27577	-8.2	0	2	15.55	14797		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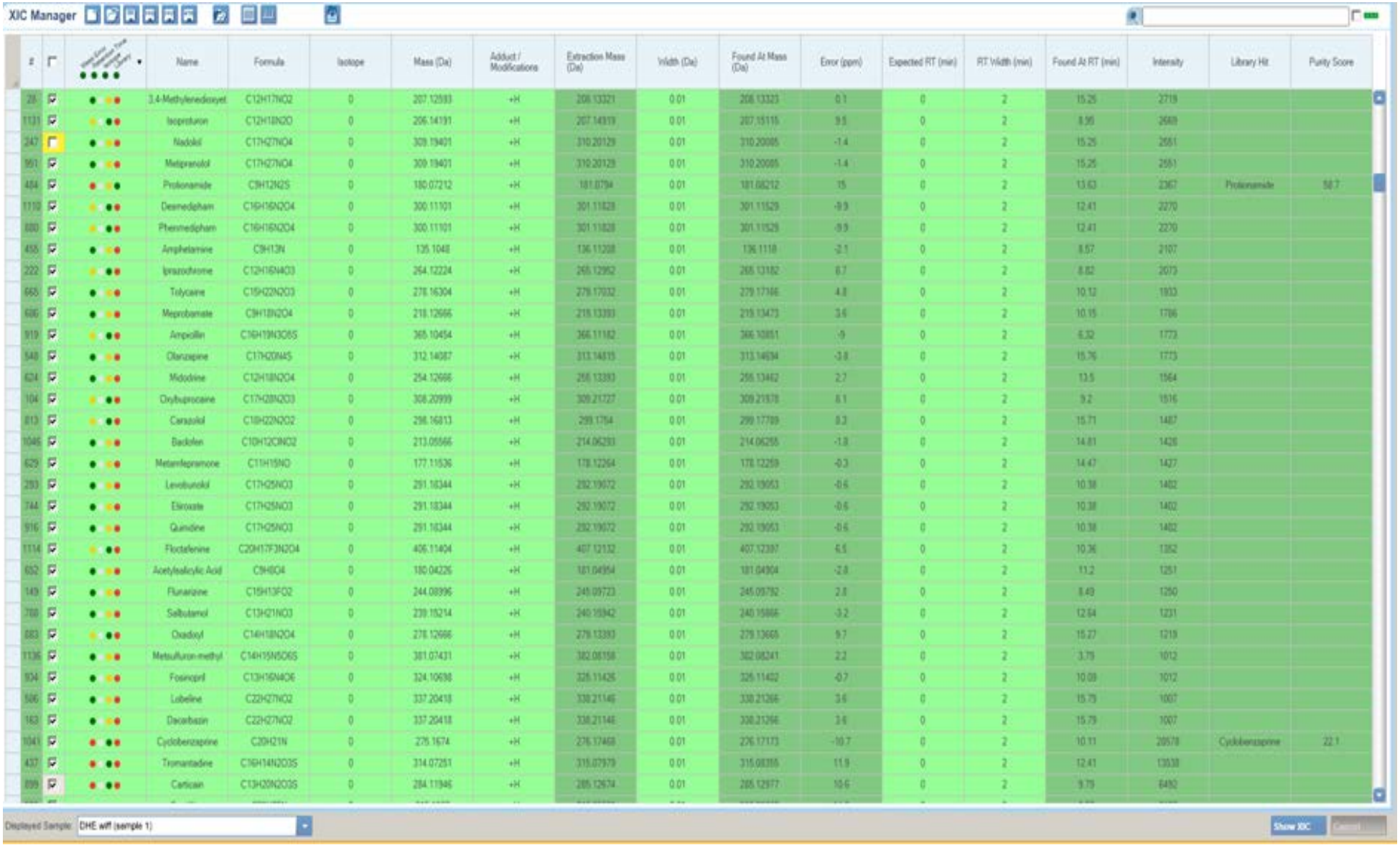
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Displayed Sample:
DHE wiff (sample 1)
Show XIC


XIC Manager
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
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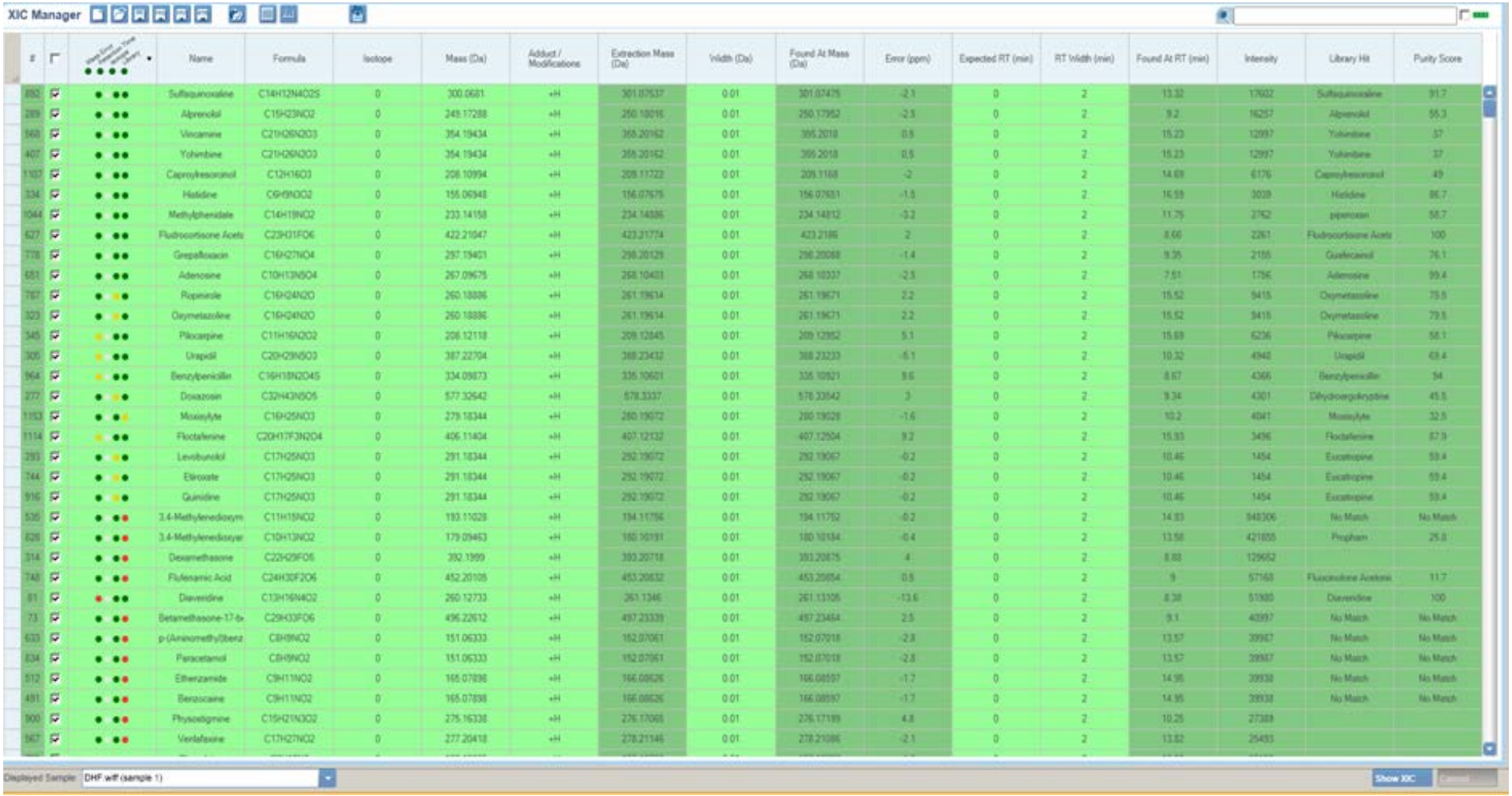
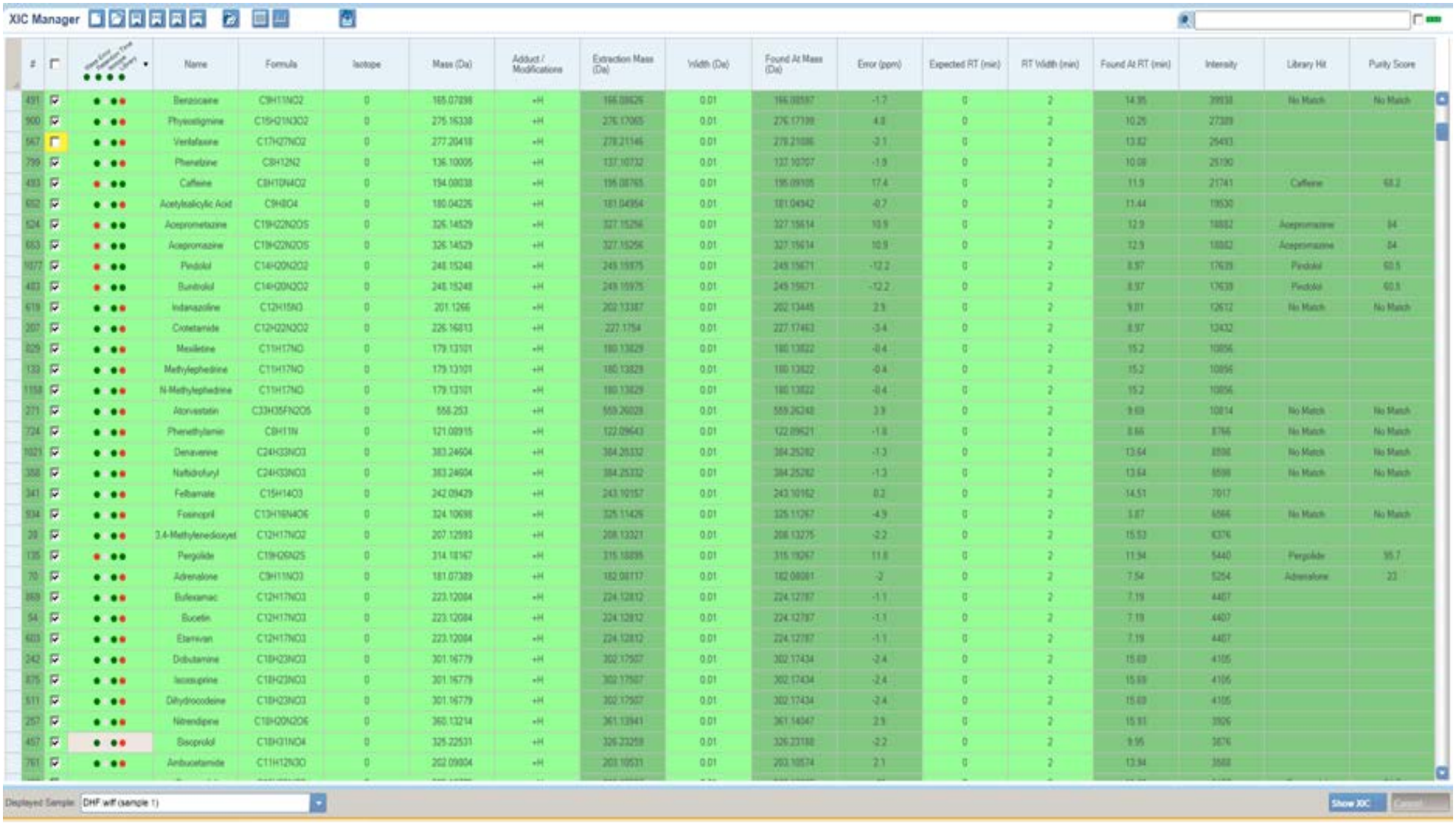
Isoproturon
Nadolol
Metipranolol
Prolonamide
Desmedipham
Phenmedipham
Amphetamine
Iprazochrome
Tolycaine
Meprobamate
Ampicillin
Olanzapine
Midodrine
Oxybuprocaine
Carzolol
Backofen
Metamfepramone
Levobunolol
Eliroxate
Quinidine
Floctafenine
Acetylsalicylic Acid
Flunarizine
Salbutamol
Oxadoyl
Metsulfuron-methyl
Fosinopril
Lobeline
Dacarbazin
Cyclobenzaprine
Tromantadine
Carticain
Prolonamide
Cyclobenzaprine
Displayed Sample:
DHE wiff (sample 1)
Show XIC

C: 5 XIC manager (DHF- Hexane extract of Fleures wiff sample 1)




XIC Manager
Name
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Benzocaine
Physostigmine
Venlafaxine
Phenelzine
Caffeine
Acetylsalicylic Acid
Acepromazine
Pindolol
Buntrolol
Indanazoline
Cretamide
Mexiletine
Methylephedrine
N-Methylephedrine
Atorvastatin
Phenethylamin
Denaverine
Nafidrofuryl
Felbamate
Fosinopril
Pergolide
Adrenalone
Bufexamac
Bucetin
Etamivan
Dobutamine
Isoxsuprine
Dihydrocodeine
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Ambucetamide
Caffeine
Acepromazine
Pindolol
No Match
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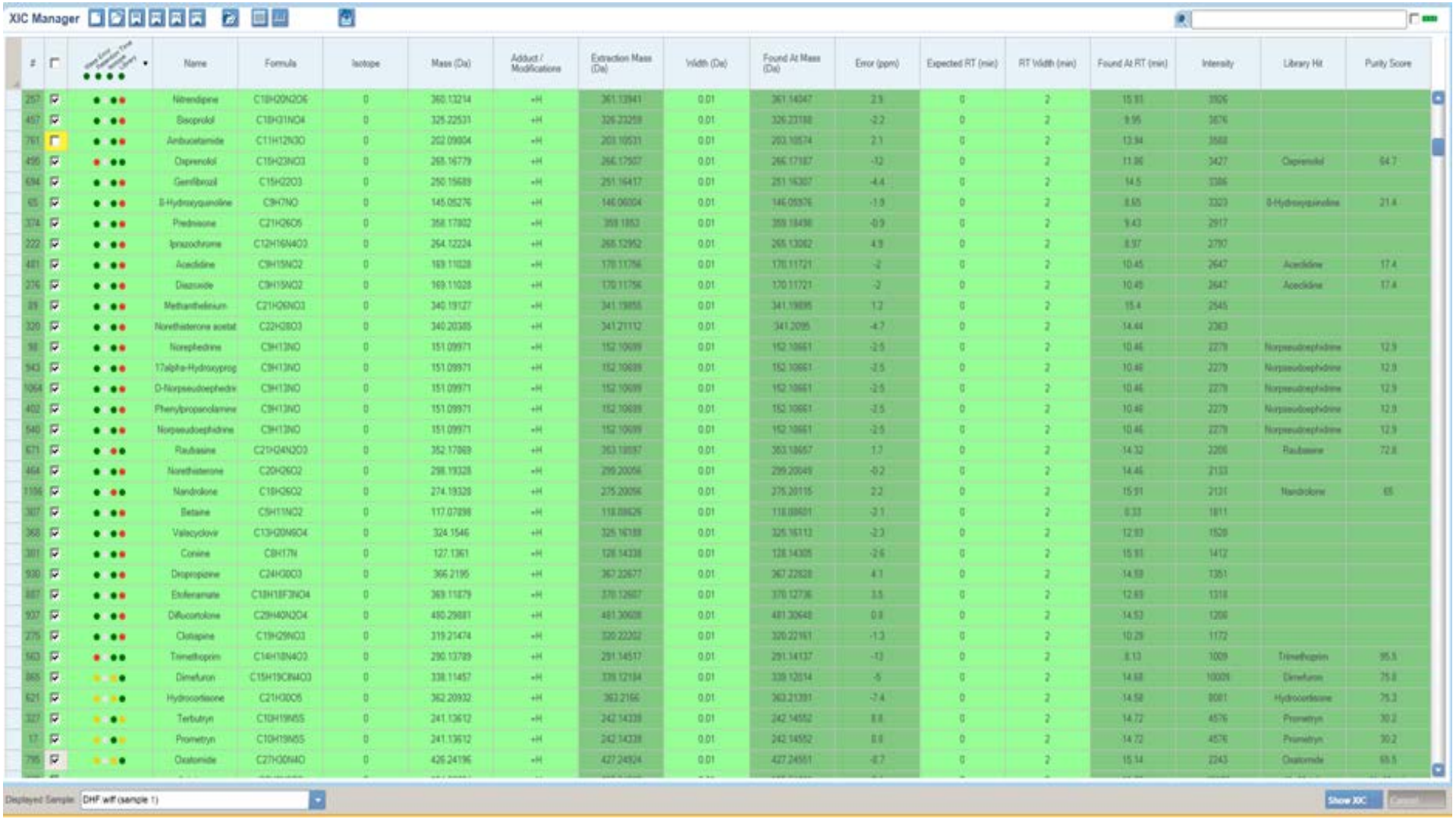
DHF wiff (sample 1)
Show XIC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DHF wiff (sample 1)
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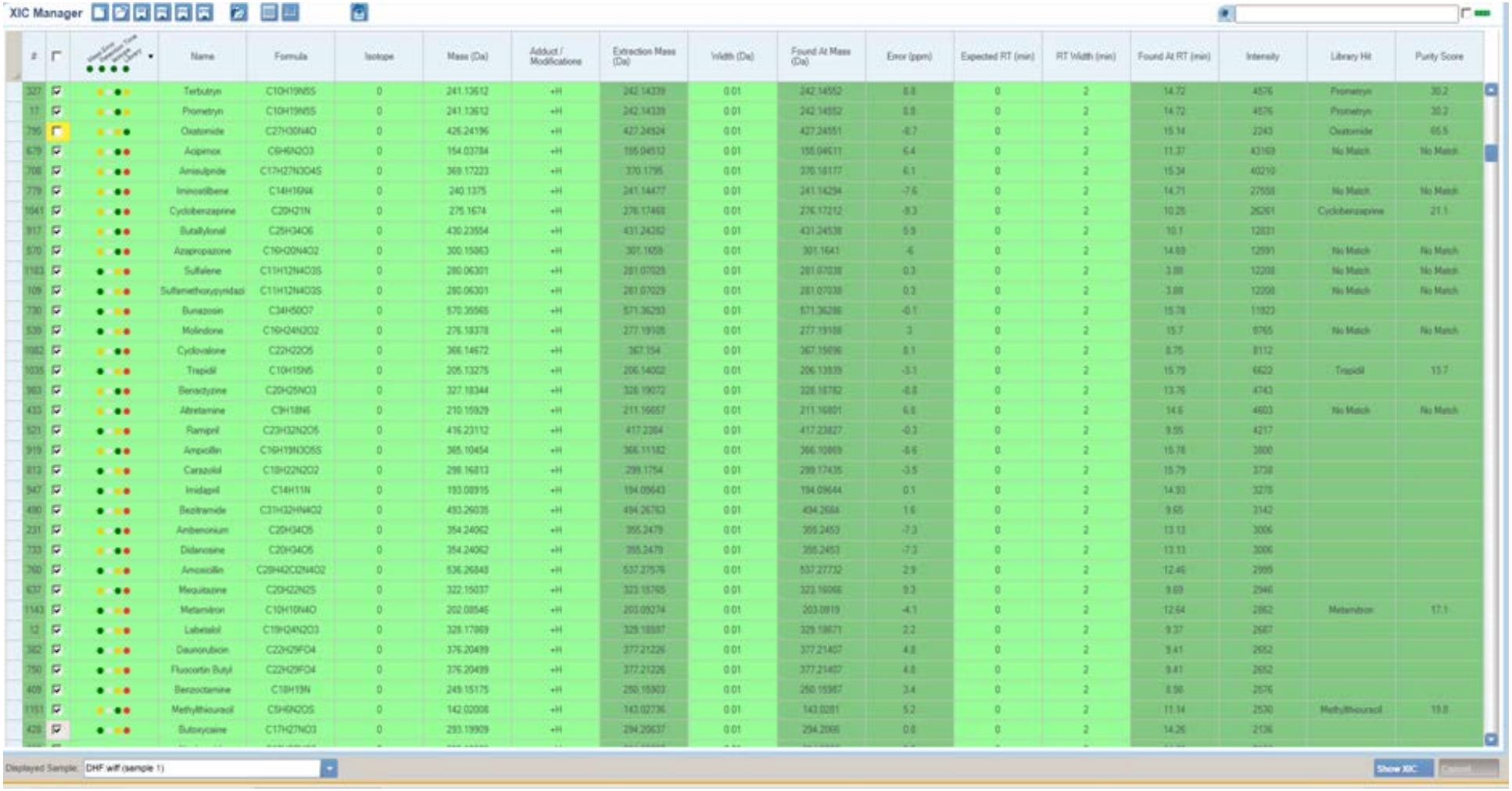
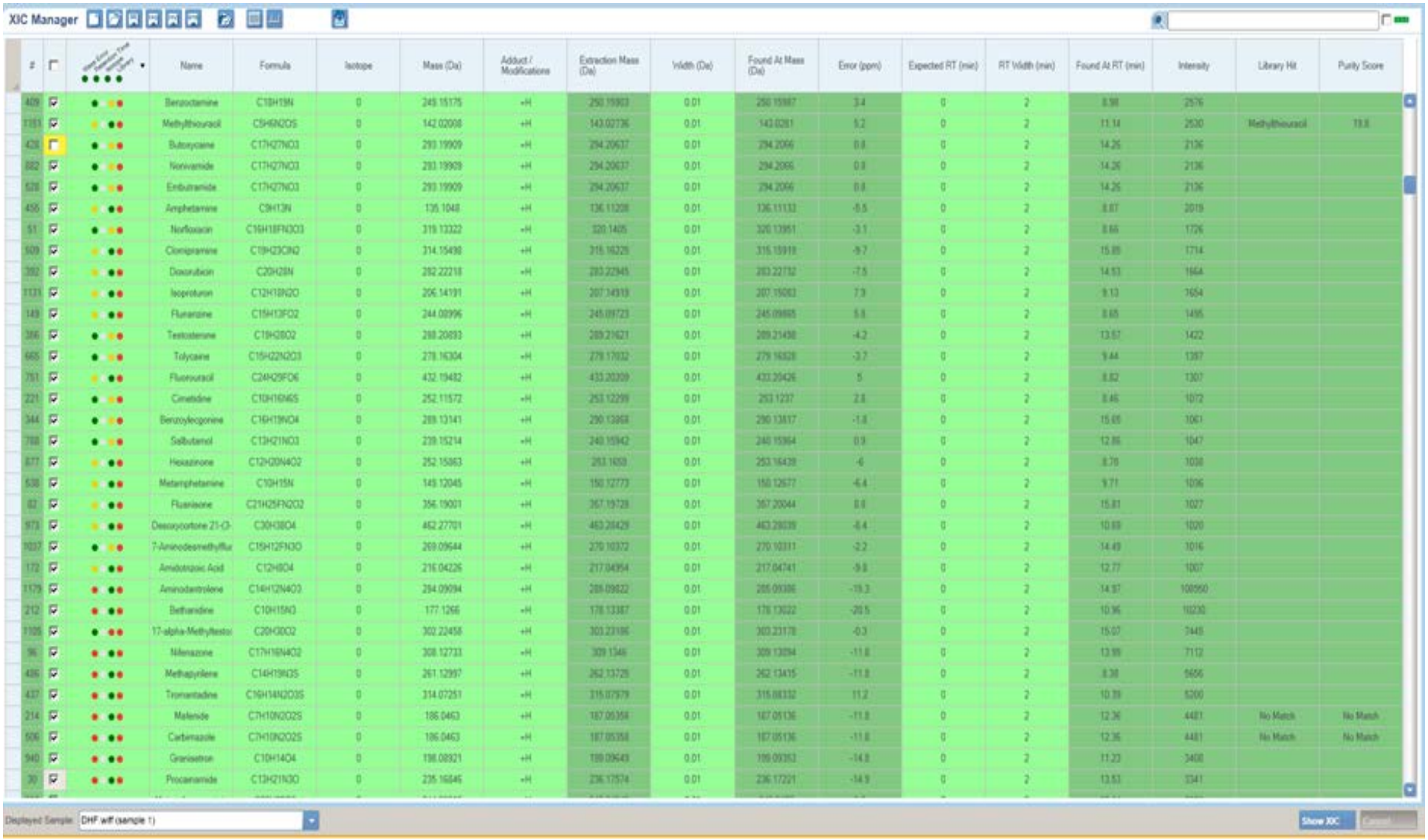


#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
327	Terbutryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14552	8.8	0	2	14.72	4876	Prometryn	30.2
17	Prometryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14552	8.8	0	2	14.72	4876	Prometryn	30.2
790	Oxatomide	C27H30N4O	0	426.24196	+H	427.24924	0.01	427.24551	-8.7	0	2	15.14	2243	Oxatomide	65.5
679	Acipimox	C6H6N2O3	0	154.03784	+H	155.04512	0.01	155.04611	6.4	0	2	11.37	43169	No Match	No Match
708	Amisulpride	C17H27N3O4S	0	369.17223	+H	370.1795	0.01	370.18177	6.1	0	2	15.34	40210		
779	Iminostilbene	C14H11N	0	240.1375	+H	241.14477	0.01	241.14294	-7.6	0	2	14.71	27558	No Match	No Match
1641	Cyclobenzaprine	C20H21N	0	275.1674	+H	276.17468	0.01	276.17212	-9.3	0	2	10.25	26261	Cyclobenzaprine	21.1
917	Butallylonal	C25H34O6	0	430.23554	+H	431.24282	0.01	431.24538	5.9	0	2	10.1	12831		
570	Azapropazone	C16H20N4O2	0	300.15863	+H	301.1659	0.01	301.1641	-6	0	2	14.69	12591	No Match	No Match
1183	Sulfalene	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07038	0.3	0	2	3.88	12208	No Match	No Match
109	Sulfamethoxypyridazi	C11H12N4O3S	0	280.06301	+H	281.07029	0.01	281.07038	0.3	0	2	3.88	12208	No Match	No Match
730	Bunazosin	C34H50O7	0	570.35565	+H	571.36293	0.01	571.36286	-0.1	0	2	15.78	11923		
539	Molindone	C16H24N2O2	0	276.18378	+H	277.19105	0.01	277.19188	3	0	2	15.7	9765	No Match	No Match
1082	Cyclovalone	C22H22O5	0	366.14672	+H	367.154	0.01	367.15696	8.1	0	2	8.75	8112		
1035	Trapidil	C10H15N5	0	205.13275	+H	206.14002	0.01	206.13939	-3.1	0	2	15.79	6622	Trapidil	15.7
983	Benactyzine	C20H25NO3	0	327.18344	+H	328.19072	0.01	328.18782	-8.8	0	2	13.76	4743		
433	Abretamine	C9H18N6	0	210.15929	+H	211.16657	0.01	211.16801	6.8	0	2	14.6	4603	No Match	No Match
521	Ramipril	C23H32N2O5	0	416.23112	+H	417.2384	0.01	417.23827	-0.3	0	2	9.55	4217		
919	Ampicillin	C16H19N3O5S	0	365.10454	+H	366.11182	0.01	366.10869	-8.6	0	2	15.78	3800		
813	Carazolol	C18H22N2O2	0	298.16813	+H	299.1754	0.01	299.17435	-3.5	0	2	15.79	3738		
947	Imidapril	C14H11N	0	193.08915	+H	194.09643	0.01	194.09644	0.1	0	2	14.93	3278		
490	Bezitramide	C31H32N4O2	0	492.25035	+H	494.26763	0.01	494.2684	1.6	0	2	9.65	3142		
231	Ambenonium	C20H34O5	0	354.24062	+H	355.2479	0.01	355.2453	-7.3	0	2	13.13	3006		
733	Didanosine	C20H34O5	0	354.24062	+H	355.2479	0.01	355.2453	-7.3	0	2	13.13	3006		
760	Amoxicillin	C29H42C0N4O2	0	536.26848	+H	537.27576	0.01	537.27732	2.9	0	2	12.46	2999		
637	Mequitazine	C20H22N2S	0	322.15037	+H	323.15765	0.01	323.16066	9.3	0	2	9.69	2946		
1143	Metamizol	C10H10N4O	0	202.08546	+H	203.09274	0.01	203.0919	-4.1	0	2	12.64	2862	Metamizol	17.1
12	Labetalol	C19H24N2O3	0	328.17869	+H	329.18597	0.01	329.18671	2.2	0	2	9.37	2687		
382	Daunorubicin	C22H29FO4	0	376.20499	+H	377.21226	0.01	377.21407	4.8	0	2	9.41	2652		
750	Fluocortin Butyl	C22H29FO4	0	376.20499	+H	377.21226	0.01	377.21407	4.8	0	2	9.41	2652		
409	Benzoctamine	C18H19N	0	249.15175	+H	250.15903	0.01	250.15987	3.4	0	2	8.98	2676		
1151	Methylthiouracil	C5H6N2OS	0	142.02008	+H	143.02736	0.01	143.0281	5.2	0	2	11.14	2530	Methylthiouracil	19.8
428	Butorphanol	C17H27NO3	0	293.19909	+H	294.20637	0.01	294.2066	0.8	0	2	14.26	2136		


XIC Manager
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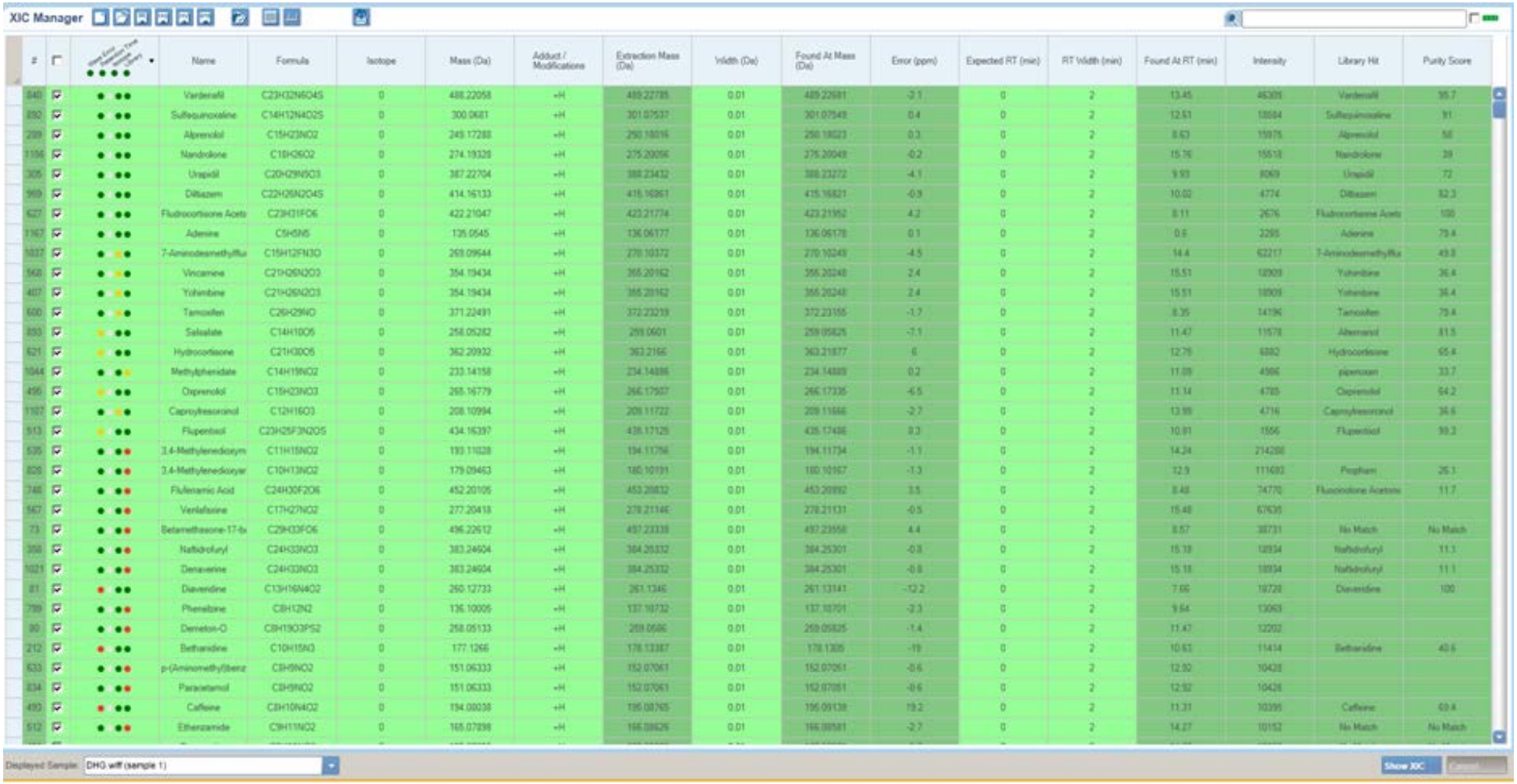
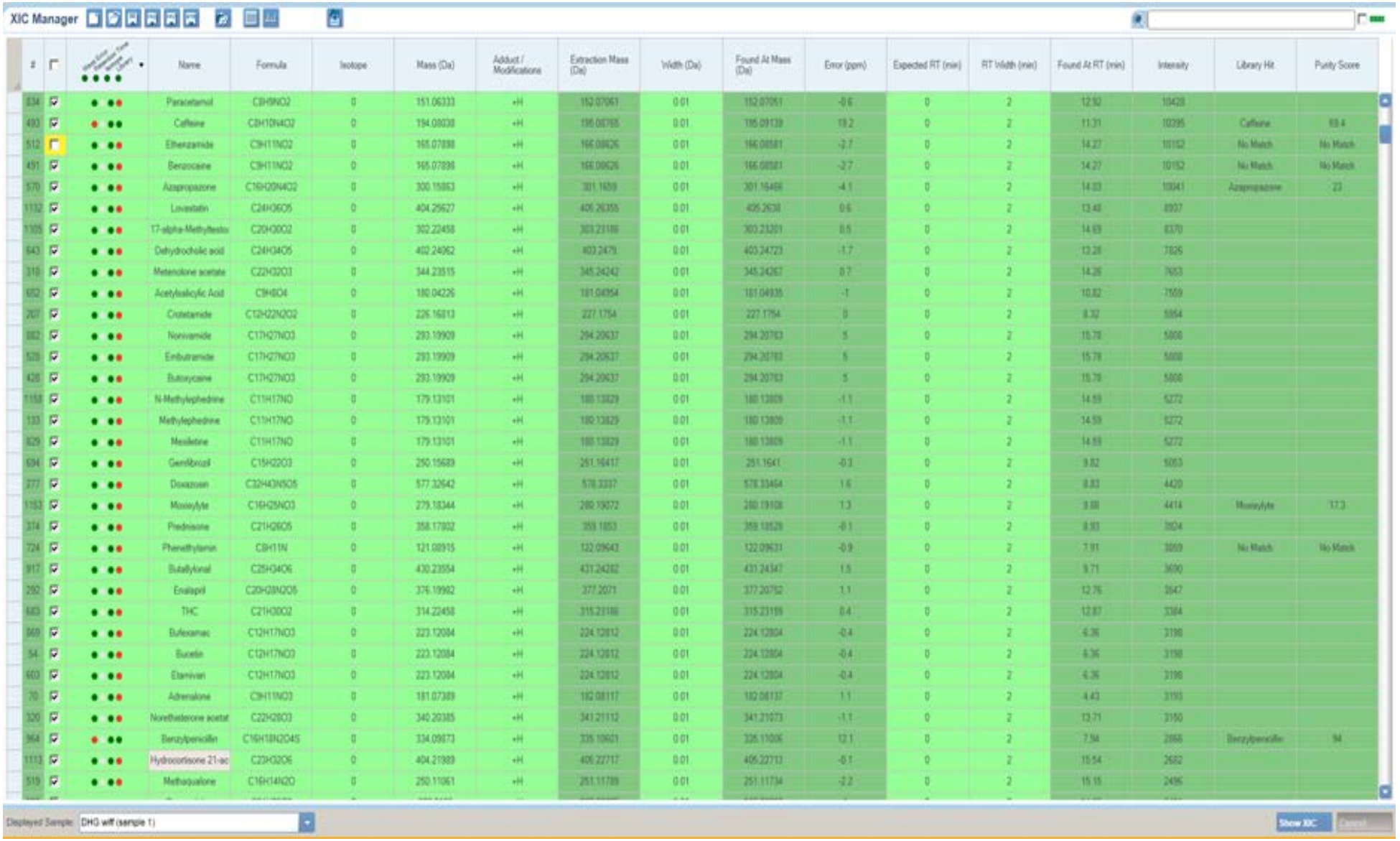
Formula
Isotope
Mass (Da)
Adduct / Modifications
Extraction Mass (Da)
Width (Da)
Found At Mass (Da)
Error (ppm)
Expected RT (min)
RT Width (min)
Found At RT (min)
Intensity
Library Hit
Purity Score
Displayed Sample:
DHF.wiff (sample 1)
Show XIC

C: 6 XIC manager (DHG- Hexane extract of Siyakholwa honey wiff sample 1




XIC Manager
| Name | Formula | Isotope | Mass (Da) | Adduct / Modifications | Extraction Mass (Da) | Width (Da) | Found At Mass (Da) | Error (ppm) | Expected RT (min) | RT Width (min) | Found At RT (min) | Intensity | Library Hit | Purity Score
834 | Paracetamol | C8H9NO2 | 0 | 151.06333 | +H | 152.07061 | 0.01 | 152.07061 | -0.6 | 0 | 2 | 12.92 | 10428
493 | Caffeine | C8H10N4O2 | 0 | 194.08038 | +H | 195.08765 | 0.01 | 195.09139 | 19.2 | 0 | 2 | 11.31 | 10295 | Caffeine | 69.4
512 | Etenzamide | C9H11NO2 | 0 | 165.07898 | +H | 166.08626 | 0.01 | 166.08581 | -2.7 | 0 | 2 | 14.27 | 10152 | No Match | No Match
491 | Benzocaine | C9H11NO2 | 0 | 165.07898 | +H | 166.08626 | 0.01 | 166.08581 | -2.7 | 0 | 2 | 14.27 | 10152 | No Match | No Match
570 | Azapropazone | C16H20N4O2 | 0 | 300.15863 | +H | 301.1659 | 0.01 | 301.16466 | 4.1 | 0 | 2 | 14.03 | 10041 | Azapropazone | 23
1132 | Lovastatin | C24H36O5 | 0 | 404.25627 | +H | 405.26355 | 0.01 | 405.2638 | 0.6 | 0 | 2 | 13.48 | 8937
1105 | 17-alpha-Methyltestos | C20H30O2 | 0 | 302.22458 | +H | 303.23186 | 0.01 | 303.23201 | 0.5 | 0 | 2 | 14.69 | 8370
643 | Dehydrocholic acid | C24H34O5 | 0 | 402.24062 | +H | 403.2479 | 0.01 | 403.24723 | -1.7 | 0 | 2 | 13.28 | 7826
318 | Metenolone acetate | C22H32O3 | 0 | 344.23515 | +H | 345.24242 | 0.01 | 345.24267 | 0.7 | 0 | 2 | 14.26 | 7653
652 | Acetylsalicylic Acid | C9H8O4 | 0 | 180.04226 | +H | 181.04954 | 0.01 | 181.04935 | -1 | 0 | 2 | 10.82 | 7559
207 | Crotetamide | C12H22N2O2 | 0 | 226.16813 | +H | 227.1754 | 0.01 | 227.1756 | 0 | 0 | 2 | 8.32 | 5954
882 | Nonivamide | C17H27NO3 | 0 | 293.19909 | +H | 294.20637 | 0.01 | 294.20783 | 5 | 0 | 2 | 15.78 | 5808
528 | Embutramide | C17H27NO3 | 0 | 293.19909 | +H | 294.20637 | 0.01 | 294.20783 | 5 | 0 | 2 | 15.78 | 5808
426 | Butoxycaine | C17H27NO3 | 0 | 293.19909 | +H | 294.20637 | 0.01 | 294.20783 | 5 | 0 | 2 | 15.78 | 5808
1158 | N-Methylephedrine | C11H17NO | 0 | 179.13101 | +H | 180.13829 | 0.01 | 180.13809 | -1.1 | 0 | 2 | 14.59 | 5272
133 | Methylephedrine | C11H17NO | 0 | 179.13101 | +H | 180.13829 | 0.01 | 180.13809 | -1.1 | 0 | 2 | 14.59 | 5272
829 | Mesildrine | C11H17NO | 0 | 179.13101 | +H | 180.13829 | 0.01 | 180.13809 | -1.1 | 0 | 2 | 14.59 | 5272
694 | Gemfibrozil | C15H22O3 | 0 | 250.15689 | +H | 251.16417 | 0.01 | 251.1641 | -0.3 | 0 | 2 | 9.82 | 5063
277 | Doxazosin | C23H43N5O5 | 0 | 577.32642 | +H | 578.3337 | 0.01 | 578.33464 | 1.6 | 0 | 2 | 8.83 | 4420
1153 | Moxisylyte | C16H25NO3 | 0 | 279.18344 | +H | 280.19072 | 0.01 | 280.19108 | 1.3 | 0 | 2 | 9.88 | 4414 | Moxisylyte | 17.3
374 | Prednisone | C21H26O5 | 0 | 358.17802 | +H | 359.1853 | 0.01 | 359.18528 | -0.1 | 0 | 2 | 8.93 | 3924
724 | Phenethylamin | C8H11N | 0 | 121.08915 | +H | 122.09643 | 0.01 | 122.09631 | -0.9 | 0 | 2 | 7.91 | 3859 | No Match | No Match
917 | Butallylonal | C25H34O6 | 0 | 430.23554 | +H | 431.24282 | 0.01 | 431.24347 | 1.5 | 0 | 2 | 9.71 | 3690
292 | Enalapril | C20H28N2O5 | 0 | 376.19982 | +H | 377.2071 | 0.01 | 377.20752 | 1.1 | 0 | 2 | 12.76 | 3647
683 | THC | C21H30O2 | 0 | 314.22458 | +H | 315.23186 | 0.01 | 315.23199 | 0.4 | 0 | 2 | 12.87 | 3384
869 | Bufexamac | C12H17NO3 | 0 | 223.12084 | +H | 224.12812 | 0.01 | 224.12804 | -0.4 | 0 | 2 | 6.36 | 3198
54 | Bucetin | C12H17NO3 | 0 | 223.12084 | +H | 224.12812 | 0.01 | 224.12804 | -0.4 | 0 | 2 | 6.36 | 3198
603 | Etamivan | C12H17NO3 | 0 | 223.12084 | +H | 224.12812 | 0.01 | 224.12804 | -0.4 | 0 | 2 | 6.36 | 3198
20 | Adrenalone | C9H11NO3 | 0 | 181.07389 | +H | 182.08117 | 0.01 | 182.08137 | 1.1 | 0 | 2 | 4.43 | 3193
320 | Norethisterone acetat | C22H28O3 | 0 | 340.20385 | +H | 341.21112 | 0.01 | 341.21073 | -1.1 | 0 | 2 | 13.71 | 3150
964 | Benzylpenicillin | C16H18N2O4S | 0 | 334.09873 | +H | 335.10601 | 0.01 | 335.11006 | 12.1 | 0 | 2 | 7.94 | 2866 | Benzylpenicillin | 94
1113 | Hydrocortisone 21-ac | C23H32O6 | 0 | 404.21989 | +H | 405.22717 | 0.01 | 405.22713 | -0.1 | 0 | 2 | 15.54 | 2682
519 | Methaqualone | C16H14N2O | 0 | 250.11061 | +H | 251.11789 | 0.01 | 251.11734 | -2.2 | 0 | 2 | 15.15 | 2496
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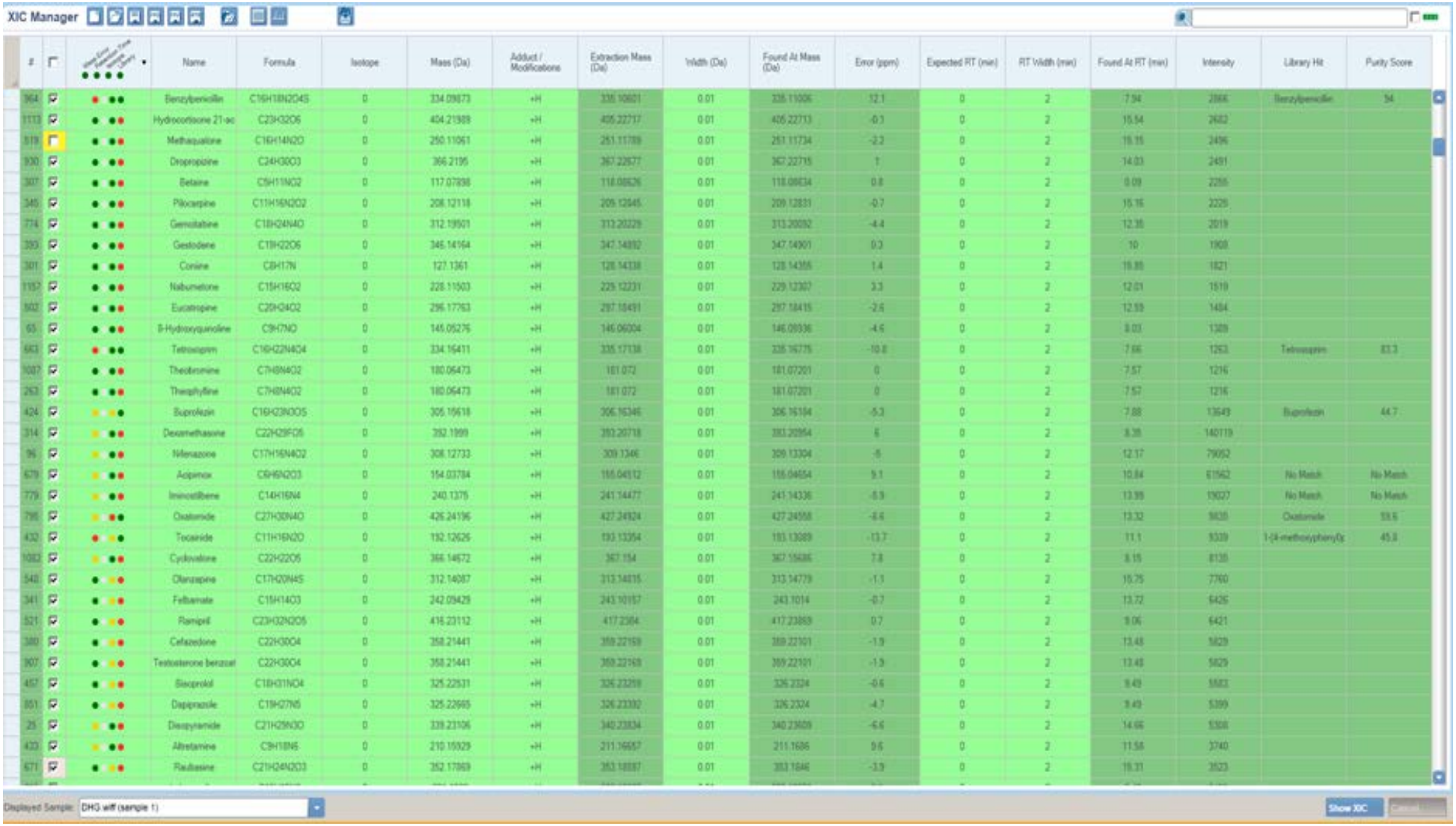
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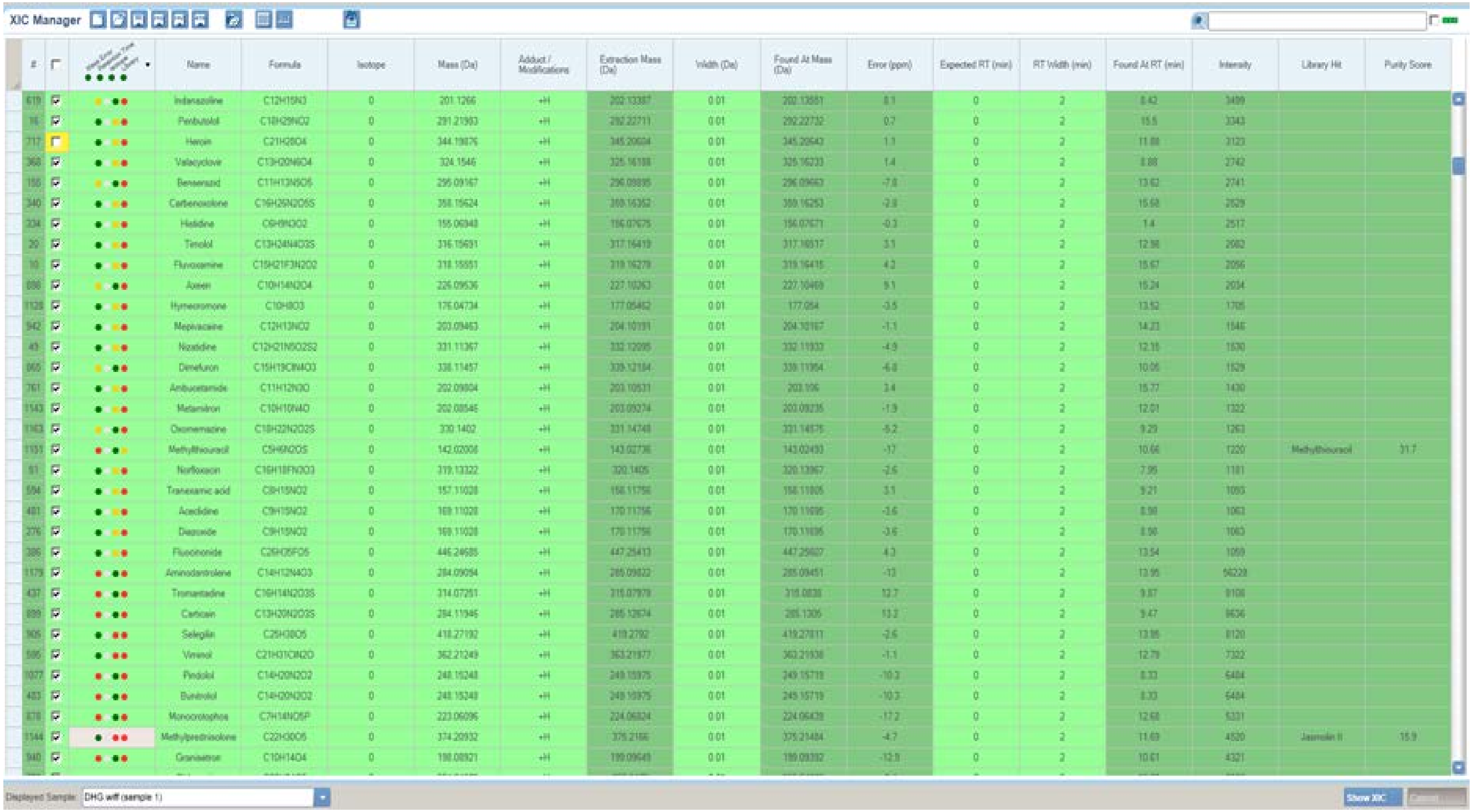

XIC Manager

#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
619	Indanazoline	C12H15N3	0	201.1266	+H	202.13387	0.01	202.13551	8.1	0	2	8.42	3499		
16	Penbutolol	C18H29NO2	0	291.21983	+H	292.22711	0.01	292.22732	0.7	0	2	15.5	3343		
717	Heroin	C21H23NO5	0	344.19876	+H	345.20604	0.01	345.20643	1.1	0	2	11.88	3123		
368	Valacyclovir	C13H20N6O4	0	324.1546	+H	325.16188	0.01	325.16233	1.4	0	2	8.88	2742		
155	Benserazid	C11H13N3O5	0	295.09167	+H	296.09895	0.01	296.09663	-7.8	0	2	13.62	2741		
340	Carbenoxolone	C16H26N2O5S	0	358.15624	+H	359.16352	0.01	359.16253	-2.8	0	2	15.58	2529		
334	Histidine	C6H9N3O2	0	155.06948	+H	156.07675	0.01	156.07671	-0.3	0	2	1.4	2517		
29	Timolol	C13H24N4O3S	0	316.15691	+H	317.16419	0.01	317.16517	3.1	0	2	12.98	2082		
10	Fluvoxamine	C15H21F3N2O2	0	318.15551	+H	319.16279	0.01	319.16415	4.2	0	2	15.67	2056		
898	Azeen	C10H14N2O4	0	226.09536	+H	227.10263	0.01	227.10469	9.1	0	2	15.24	2034		
1128	Hymecromone	C10H8O3	0	176.04734	+H	177.05462	0.01	177.054	-3.5	0	2	13.52	1705		
942	Mepivacaine	C12H13NO2	0	203.09463	+H	204.10191	0.01	204.10167	-1.1	0	2	14.23	1546		
49	Nizatidine	C12H21N5O2S2	0	331.11367	+H	332.12095	0.01	332.11933	-4.9	0	2	12.15	1530		
865	Dimefuron	C15H19ClN4O3	0	338.11457	+H	339.12184	0.01	339.11964	-6.8	0	2	10.05	1529		
761	Ambucetamide	C11H12N3O	0	202.09804	+H	203.10531	0.01	203.106	3.4	0	2	15.77	1430		
1143	Metamitron	C10H10N4O	0	202.08546	+H	203.09274	0.01	203.09235	-1.9	0	2	12.01	1322		
1163	Oxomemazine	C18H22N2O2S	0	330.1402	+H	331.14748	0.01	331.14575	-5.2	0	2	9.29	1263		
1151	Methylthiouracil	C5H6N2OS	0	142.02008	+H	143.02736	0.01	143.02493	-17	0	2	10.66	1220	Methylthiouracil	31.7
51	Norfloxacin	C16H18FN3O3	0	319.13322	+H	320.1405	0.01	320.13967	-2.6	0	2	7.95	1181		
594	Tranexamic acid	C8H15NO2	0	157.11028	+H	158.11756	0.01	158.11805	3.1	0	2	9.21	1093		
481	Acedidine	C9H15NO2	0	169.11028	+H	170.11756	0.01	170.11695	-3.6	0	2	8.98	1063		
276	Diazoxide	C9H15NO2	0	169.11028	+H	170.11756	0.01	170.11695	-3.6	0	2	8.98	1063		
386	Fluocinonide	C26H35FO6	0	446.24685	+H	447.25413	0.01	447.25607	4.3	0	2	13.54	1059		
1179	Aminodantrolene	C14H12N4O3	0	284.09094	+H	285.09822	0.01	285.09451	-13	0	2	13.95	56228		
437	Tromantadine	C16H14N2O3S	0	314.07251	+H	315.07979	0.01	315.0838	12.7	0	2	9.87	9106		
899	Carticain	C13H20N2O3S	0	284.11946	+H	285.12674	0.01	285.1305	13.2	0	2	9.47	9636		
905	Selegilin	C25H38O5	0	418.27192	+H	419.2792	0.01	419.27811	-2.6	0	2	13.98	8120		
595	Viminol	C21H31ClN2O	0	362.21249	+H	363.21977	0.01	363.21938	-1.1	0	2	12.79	7322		
1077	Pindolol	C14H20N2O2	0	248.15248	+H	249.15975	0.01	249.15719	-10.3	0	2	8.33	6484		
403	Bunitrolol	C14H20N2O2	0	248.15248	+H	249.15975	0.01	249.15719	-10.3	0	2	8.33	6484		
878	Monocrotophos	C7H14NO5P	0	223.06096	+H	224.06824	0.01	224.06439	-17.2	0	2	12.68	5331		
1144	Methylprednisolone	C22H30O5	0	374.20932	+H	375.2166	0.01	375.21484	-4.7	0	2	11.69	4520	Jasmolin II	15.9
940	Granisetron	C10H14O4	0	198.08921	+H	199.09649	0.01	199.09392	-12.9	0	2	10.61	4321		

Displayed Sample: DHG.wiff (sample 1)

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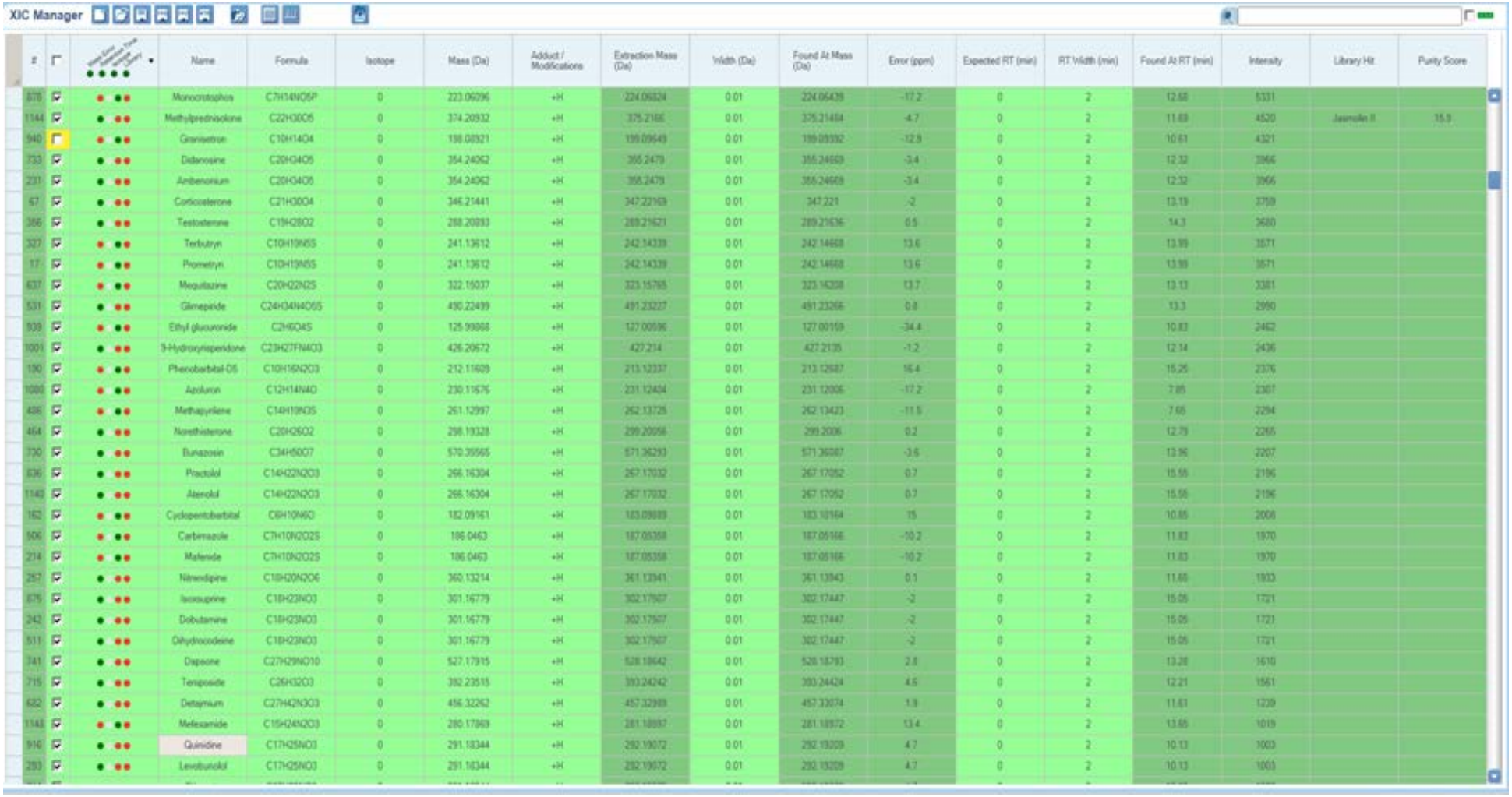

#	Name	Formula	Isotope	Mass (Da)	Adduct / Modifications	Extraction Mass (Da)	Width (Da)	Found At Mass (Da)	Error (ppm)	Expected RT (min)	RT Width (min)	Found At RT (min)	Intensity	Library Hit	Purity Score
878	Monocrotophos	C7H14NO5P	0	223.06096	+H	224.06824	0.01	224.06429	-17.2	0	2	12.68	5331		
1144	Methylprednisolone	C22H30O5	0	374.20932	+H	375.2166	0.01	375.21484	-4.7	0	2	11.69	4520	Jasmolin II	15.9
940	Granisetron	C18H14O4	0	198.08921	+H	199.09649	0.01	199.09302	-12.9	0	2	10.61	4321		
733	Didanosine	C20H24O5	0	354.24062	+H	355.2479	0.01	355.24669	-3.4	0	2	12.32	3966		
231	Ambenonium	C20H24O5	0	354.24062	+H	355.2479	0.01	355.24669	-3.4	0	2	12.32	3966		
67	Corticosterone	C21H30O4	0	346.21441	+H	347.22169	0.01	347.221	-2	0	2	13.19	3759		
366	Testosterone	C19H28O2	0	288.20893	+H	289.21621	0.01	289.21636	0.5	0	2	14.3	3680		
327	Terbutryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14668	13.6	0	2	13.99	3571		
17	Prometryn	C10H19N5S	0	241.13612	+H	242.14339	0.01	242.14668	13.6	0	2	13.99	3571		
637	Mequitazine	C20H22N2S	0	322.15037	+H	323.15765	0.01	323.16208	13.7	0	2	13.13	3381		
531	Glimepiride	C24H34N4O5S	0	490.22499	+H	491.23227	0.01	491.23266	0.8	0	2	13.3	2990		
939	Ethyl glucuronide	C2H6O4S	0	125.99868	+H	127.00596	0.01	127.00159	-34.4	0	2	10.83	2462		
1001	9-Hydroxyrisperidone	C23H27FN4O3	0	426.20672	+H	427.214	0.01	427.2135	-1.2	0	2	12.14	2436		
190	Phenobarbital-D5	C10H16N2O3	0	212.11609	+H	213.12337	0.01	213.12687	16.4	0	2	15.25	2376		
1080	Apolunn	C12H14N4O	0	230.11676	+H	231.12404	0.01	231.12006	-17.2	0	2	7.85	2307		
436	Methapyrilene	C14H19N3S	0	261.12997	+H	262.13725	0.01	262.13423	-11.5	0	2	7.65	2294		
464	Norethisterone	C20H26O2	0	298.19328	+H	299.20056	0.01	299.2006	0.2	0	2	12.79	2265		
730	Bunazosin	C34H50O7	0	570.35565	+H	571.36293	0.01	571.36087	-3.6	0	2	13.96	2207		
636	Practolol	C14H22N2O3	0	266.16304	+H	267.17032	0.01	267.17052	0.7	0	2	15.55	2196		
1140	Atenolol	C14H22N2O3	0	266.16304	+H	267.17032	0.01	267.17052	0.7	0	2	15.55	2196		
162	Cyclopentobarbital	C9H12N2O	0	182.09161	+H	183.09889	0.01	183.10164	15	0	2	10.85	2066		
506	Carbenazole	C7H10N2O2S	0	186.0463	+H	187.05358	0.01	187.05166	-10.2	0	2	11.83	1970		
214	Mafenide	C7H10N2O2S	0	186.0463	+H	187.05358	0.01	187.05166	-10.2	0	2	11.83	1970		
257	Nitrendipine	C18H20N2O6	0	360.13214	+H	361.13941	0.01	361.13843	0.1	0	2	11.68	1933		
675	Isoxsuprine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17447	-2	0	2	15.05	1721		
242	Dobutamine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17447	-2	0	2	15.05	1721		
511	Dihydrocodeine	C18H23NO3	0	301.16779	+H	302.17507	0.01	302.17447	-2	0	2	15.05	1721		
341	Dapsone	C27H29NO10	0	527.17915	+H	528.18642	0.01	528.18793	2.8	0	2	13.28	1610		
715	Tenposide	C26H32O3	0	392.23515	+H	393.24242	0.01	393.24424	4.6	0	2	12.21	1561		
622	Detajmium	C27H42N2O3	0	456.32262	+H	457.32989	0.01	457.33074	1.9	0	2	11.61	1229		
1143	Meliszamide	C15H24N2O3	0	280.17869	+H	281.18597	0.01	281.18372	13.4	0	2	13.65	1019		
916	Quinidine	C17H25NO3	0	291.18344	+H	292.19072	0.01	292.19209	4.7	0	2	10.13	1003		
283	Levobunolol	C17H25NO3	0	291.18344	+H	292.19072	0.01	292.19209	4.7	0	2	10.13	1003		