Fractionations And Analysis of Trunk Exudates From Pistacia Genus In Relation to Antimicrobial Activity

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MChem

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Seek knowledge from the cradle to the grave
To my loving and caring wife Raheleh Shebly
I wish to dedicate this to you
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CERTIFICATE OF ORIGINALITY

I declare that this thesis is my own work and to the best of my knowledge it does not contain any previously published or written by another person and that has not been accepted for the award of any degree or diploma in UWS or any other educational institution, except whereas acknowledgments is made in this thesis.

Signed

Mohammad Sharif Sharifi


ABSTRACT

*H. pylori* is one of the most significant discoveries in gastroenterology in the past century. It is associated with a wide range of gastroduodenal pathologies and gastric cancer. Antibiotic resistance in *H. pylori* has emerged as a significant clinical problem. Furthermore, contemporary therapy is expensive and complex, this presents problems particularly for third world countries. The body of work contained within this dissertation was carried out to investigate an alternative therapy based on observations of the traditional therapy for gastric disease in the Middle East. One of these traditional therapies centres on plants belonging to the *Pistacia* genus. However, the findings went beyond *H. pylori*, leading to the characterisation of what appears to be new classes of antibiotics.

This study represents the first reported investigation into the composition and biological activity of the trunk bark exudates of *Pistacia atlantica Kurdica* (*P. a. Kurdica*), *Pistacia atlantica Mutica* (*P. a. Mutica*) and *Pistacia atlantica Cabolica* (*P. a. Cabolica*), resinous gums that have been termed here ‘Kurdica Gum’, ‘Mutica Gum’ and ‘Cabolica Gum’ respectively.

The antimicrobial screening of the trunk exudates of the genus *Pistacia* led to the characterisation of the most active fraction of the Kurdica gum. This fraction was subsequently subjected to sub-fractionation leading to the discovery of fundamentally new information that went beyond *H. pylori*, expanding the original parameters of the project. The extent of these findings suggests that new classes of antibiotics might have been discovered. Primary studies on their structure and potential mechanism of action has been undertaken.

Thirteen novel antimicrobial agents were identified. Based on the characteristics of these isolated fractions, 50 new compounds were modelled; of which 30 hypothetically have an MIC consistent with contemporary antibiotics and could represent viable lead compounds for commercial development.
LIST OF ABREVIATIONS

ANOVA  Analysis of Variance
APCI-MS  Atmospheric PressureChemical Ionisation-Mass
Spectrometry
ATP  Adenosine 5-triphosphate
BHI  Brain Heart Infusion
BSA  Bovine Serum Albumin
CA  Carbonic Anhydrase
cagA  cytotoxin-associated gene A
CAG  cholesteryl-6-O-tetradecanoyl-a-D-glucopyranoside ()
CagPAI  Cag pathogenicity island
Cfu  colony forming units
CGs  `cholesteryl glucosides
CGL  Cholesteryl-a-D-glucopyranoside
CI  Chemical Ionisation
CPG  cholesteryl-6-O-phosphatidyl-a-D-glucopyranoside
CPVPB  co-poly (vinyl-p-benzoate)
CSA  Campylobacter selective agar
Da  Dalton
DEPC  diethyl pyrocarbonate
DNA  Deoxyribonucleic acid
DNP  2,4-dinitrophenylhydrazine (2,4-DTCM
Dialysis Tubing Cellulose Membrane
EF-G  Elongation Factor G
EI  electron-impact
EOC  Essential Oils of Cabolica
EOK  Essential oils of Kurdica
EOM  Essential Oils of Mutica
GC  Gas Chromatography
GC-FID  Gas Chromatography-Flame Ionisation Detector
GCLO Gastric Campylobacter Like Organism
GC-MS  Gas Chromatography-Mass Spectrometry
GERD  gastroesophageal reflux disease
GPC  Gel Permission Gas Chromatography
H  hour
H2RAs histamine type 2 receptor antagonists
HPLC  High Performance Liquid Chromatography
L  Litre
MALT Mucosa Associated Lymphoid Tissue
MBC  Minimum Bacterial Concentration
mg  milligrams
MIC  Minimum Inhibitory
min  minutes
mL  millilitre
Mp  Melting Point
mRNA Messenger ribonucleic acid
MS  Mass Spectrometry Concentration
nm  nano-meter
NMR  Nuclear Magnetic Resonance
NSAIDs Non-steroidal Anti-Inflammatory Drugs
OD600 Optical density measured at 600 nm.
PBS Phosphate Buffered Saline
PID photoionization detector (PPI proton pump inhibitor
pmol  Pico mols
pM  Pico mols per litre
RI  retention indices
RNA  Ribonucleic acid
SDS  Sodium dodecyl sulfate
TEM transmission electron Microscopy
TCD  thermal conductivity detector
TLC Thin Layer Chromatography
UNSW University of New South Wales
UOW University of Wollongong
UWS University of Western Sydney
VacA vacuolating toxin A
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Chapter 1
Literature review

1.1 Introduction

The discovery of spiral-shaped bacteria in both mucosa and gastric contents of patients goes back to as early as 1889. In 1938 Doenges suggested a causative role of this bacterium in gastric disease (Kidd & Modlin, 1998). Doenges studied both human and *Macacus rhesus* monkeys and observed the organism to invade the gastric glands of 100% of the monkeys that he studied and that the microorganism was present in 43% of human gastric autopsy specimens. He reported that the organism was restricted to the gastric mucosa and it was not observed in the intestinal mucosa. Freedberg and Barron in 1941 (Kidd & Modlin, 1998) were inspired by Doenges’ report and investigated the presence of spirochetes in the gastric tissues of the patients biopsies specimens.

Despite Freedberg and Barron expertise in identifying spirochetes in dogs, unfortunately, they were not easily able to identify the organism, although they demonstrated the frequent presence of spirochetes in ulcerating stomach in comparison to nonulcerated stomachs (53% vs. 14%). However, they concluded that no absolute aetiological role for these organisms could be predicted. Dr. Frank Gorham a specialist of Internal Medicine, of St. Louis, Missouri, in the discussion of this paper noted:

‘I believe that a further search should be made for an organism thriving in hydrochloric acid medium (and variations of hydrochloric acid are normal in all stomachs) as a possible factor of chronicity, if not an etiologic factor, in peptic ulcer.’ (Kidd & Modlin, 1998)
Dr. Gorham used bismuth subnitrate – an antimicrobial agent at the time used to treat syphilis - to treat peptic ulcer over 10 years with some success. Doenges’ suggestion and Dr. Gorham’s practice and note were not accepted by researchers and medical doctors and was buried by the observations of Palmer in 1954 who studied over 1000 gastric biopsies claiming that the observed bacteria were contamination originating from oral microbial flora (Kidd & Modlin, 1998). This conclusion delayed the discovery of the true nature of gastric bacterial infections. The breakthrough came some three decades later by Dr. Robin Warren, an Australian histopathologist at the Royal Perth Hospital.

In 1979 Dr. J. Robin Warren, made an observation that was at first puzzling. As he examined tissue specimens from patients who had undergone an endoscopic examination, where stomach biopsies had been taken, he noticed that upon histological examination several samples contained large numbers of curved and spiral-shaped bacteria (Marshall & Warren, 1983). Warren noted that there appeared to be a correlation between the presence of these bacteria and inflammation of the stomach (gastritis). These observations failed to excite many of Warren’s colleagues and it took the partnership between Warren and a young registrar, Dr Barry Marshall, to progress the work.

One of the early objectives of the collaboration between Warren and Marshall was to attempt to grow the unknown bacterium in culture. Noting the morphological similarities between these gastric bacteria and species of the genus *Campylobacter*, methods used for the cultivation of *Campylobacter jejuni* were employed. After several months of trying to cultivate the organism, success was finally achieved when the cultures were incubated over 4 days during Easter 1982. Once the bacterium was able to be cultured, biochemical and other studies could be undertaken (Marshall & Warren, 1983), that were able to demonstrate that this bacterium was a “new” species. Based on a
number of characteristics, it was initially thought that the gastric bacterium belonged to the *Campylobacter* genus. The early literature (Mobley *et al*, 2001) referred to the bacterium as a Gastric Campylobacter Like Organism (GCLO) that was later named *Campylobacter pyloridis*, changed for grammatical reasons to *Campylobacter pylori* and later validly published as a new genus and species *Helicobacter pylori* (Mobley *et al*, 2001). *Helicobacter pylori* became the type species of a genus which is now known to contain in excess of 20 species infecting the gastrointestinal tract and liver of many animal species in addition to humans.

In 1982 peptic ulcer disease was being managed adequately through the use of acid suppressive drugs. The dictum “no acid, no ulcer” (Lee, 1996) had been the primary rationale for the management of peptic ulcer disease. Although it was known that the mechanism of pathogenesis was not fully understood, the suggestion of Warren and Marshall that a spiral-shaped bacterium was involved in gastric pathology and might even play a causal role in peptic ulcer disease was met with almost universal scepticism. Marshall and Warren gathered evidence to support their hypothesis that *Helicobacter pylori* played a causal role in the development of gastritis and was a factor contributing to the development of peptic ulcer disease. The hypothesis that a bacterium caused inflammation and ulceration was dismissed by most, yet there was sufficient interest generated to drive individual research efforts leading to discoveries that have opened the way for scientist to establish a causal relationship between infection and gastric disease and to discover ways to eradicate peptic ulcer disease and possibly gastric cancer from high percentage of the world’s population (Martin, 1996).

*Helicobacter pylori* is one of the most significant discoveries in gastroenterology in the past century and has earned for Warren and Marshall the Nobel Prize for Medicine. It is associated with a wide range of
gastroduodenal pathologies including peptic ulcer disease and various manifestations of gastric cancer. In the past decade many of the issues concerning \textit{H. pylori} have been clarified. New associations have been suggested from epidemiological studies and further insights into the pathogenesis have been gained by investigating the virulence of the bacteria and the host response to infection (Forman, 1996). In addition to the advances in understanding of pathogenesis, there have been significant improvements in treatment regimes (Forman, 1996).

While it was known that gastric ulcers could result from medication such as Non-steroidal Anti-Inflammatory Drugs (NSAIDs), it is evident that \textit{H. pylori} causes almost all cases of peptic ulcer disease that are not related to the ingestion of NSAIDs. Indeed this infection may cause 95\% of all duodenal ulcer disease (Forman, 1996).

In addition to the link to peptic ulcer disease, in June 1994 the International Agency for Research in Cancer, an arm of the World Health Organization, declared that \textit{H. pylori} to be a Class-1 carcinogen, the highest risk category for cancer-causing agents (Axon & Moayyedi, 1996). The most common form of gastric cancer is gastric adenocarcinoma. A less common cancer of the stomach, called gastric Mucosa Associated Lymphoid Tissue (MALT) lymphoma, also appears to be largely caused by \textit{H. pylori} (Rauws & Tytgat, 1990). Recent evidence suggests that anti-microbial treatment to cure \textit{H. pylori} infection may bring about regression in this subset of tumour (Rauws & Tytgat, 1990). This is an exciting development in both clinical medicine and cancer biology (Aronson, 1998).
1.2 Bacterial Morphology

*H. pylori* is a short curved, Gram-negative bacterium 2.5-5µm long and 0.5-1µm (Figure 1-1), that possesses flagella and requires a microaerobic environment for growth. This organism resides within the gastric mucus layer above the gastric epithelium and colonizes the human gastric epithelium causing chronic gastritis and peptic ulcer (Shimomura *et al* 2004). The bacterium has one to three spirals, giving the organism a characteristic name. The flagella and spiral shape enable the bacterium to propel through the lumen and break into the gastric mucus layer. The flagella also enable the bacterium to survive in the gastric tract as they are sheathed by a membrane structure that protects the filaments against enzymatic and acid damage (Hazell *et al.*, 1986). The spiral shape helps the bacterium to swim through the gastric mucus (Ferrero and Lee, 1988; Hazell *et al.*, 1986).

The bacterium when cultured may appear more rod like. Older cultures may lose their shape developing a coccoid morphology (Hazell *et al.*, 1986). Some researchers have suggested that coccoid forms may be a part of the life cycle of the bacterium, although coccoid forms may simply represent an artefact of aging when grown on artificial media (O’Rourke & Bode, 2001). The morphologic change of the bacterium from a spiral to a coccoid form can be
response to the environmental stresses such as excessive oxygen, alkaline pH, and long term culture (Catrenich & Makin, 1991; Donelli et al, 1998). Coccoid forms of the bacterium conserve the structure of DNA and 16S rRNA and maintain DNA and ATP synthesis (Bode et al, 1993).

Time-dependent alteration in bacterial protein and lipids in association with the conversion from spiral to coccoid has also been studied. *H. pylori* is known to have three cholesteryl glucosides (CGs) in the cell membrane (Hirai et al, 1995). These CGs are: cholesteryl-6-O-tetradecanoyl-a-D-glucopyranoside (CAG), Cholesteryl-a-D-glucopyranoside (CGL), and cholesteryl-6-O-phosphatidyl-a-D-glucopyranoside (CPG) (Fig. 1-2 a-c respectively). The CGs are very rare in bacteria and CPG has been only found in *Helicobacter spp.* (Haque et al, 1996; Haque et al, 1995). However, the CGs are common constituents in plants (Bolt & Clarke, 1970). This study has shown a drastic alteration in the profiles of CGs and other membrane lipids during conversion of spiral to coccoid and that may have been responsible for the morphological change (Shimomura et al, 2004).

![Chemical structure of a; cholesteryl-6-O-tetradecanoyl-a-D-glucopyranoside (CAG), b; Cholesteryl-a-D-glucopyranoside (CGL), and c; cholesteryl-6-O-phosphatidyl-a-D-glucopyranoside (CPG) (Shimomura et al, 2004).](image-url)
1.3 Physiology

To grow *H. pylori*, the use of commercially prepared culture plates is not recommended as often these plates are dry and not suitable for this bacterium. Apart from keeping the culture media moist, the selective antibiotics also are supplied with the blood agar base (described in Chapter 2) to prevent overgrowth of transient gastric microbiota (Hazell, 1993). It is also requiring a special environment consist of 5% oxygen, high (10%) CO$_2$ and humidity that is available in special gas mixture generating kits that are available for microaerophilic bacteria. Cultures should be incubated at least for 2-3 days and often 5-7 days for primary culture from biopsy (Hazell, 1993).

It was hypothesised earlier that *H. pylori* high CO$_2$ acquisition was very important for Carbonic Anhydrase (CA) $\alpha$ and $\beta$ which are the zinc-containing enzyme, catalysing the reversible hydration of CO$_2$ and plays a major role in bacterial physiology. However, later work showed that the expression of CA in three different strains of the bacteria, 26695, J99 and 17.1 was independent of CO$_2$ concentration in the investigated range (0.1-10%) (Chirica et al, 2002).

*H. pylori* metabolism has been studied and it appears that the main source of energy is obtained from the breakdown of proteins and lipids. However, Nuclear Magnetic Resonance (NMR) studies have also shown the catabolism of glucose through a range of biochemical pathways (Mendz & Hazell, 1993).

There are some other important enzymes such as catalase, superoxide dismutase, oxidase, phospholipases, proteases and urease. Urease has been the subject of numerous studies including being used as the basis of the
diagnostic tests for the bacterium. This enzyme has an important role in colonisation and direct damage of gastric mucosa (Mobley et al, 1991).

A lot of bacteria produce urease, including some of the normal flora and non-pathogens; however, urease is a potent virulence factor for some species such as Proteus miabilis, Staphylococcus saprophyticus and it is central to H. pylori metabolism and virulence. Urease is essential for colonisation of the gastric mucosa by bacterium. This enzyme is a vaccine candidate and used for diagnosis and follow-up after treatment (Mobley, 2001). In another study this enzyme has been chosen as a model for chemical cross-linking combined with mass spectrometry analysis to reveal protein topology and identify contact sites between the peptide surfaces that are very important and gives structural details that are useful in vaccine design (Carlsohn et al, 2004).

The enzyme consists of two subunits of 30 and 60 kDa. While it has been reported on the surface of the bacterium, concentrations of the enzyme are found in the periplasm and cytosol. It is very active the kinetics that allow the bacterium to optimize its activity at physiological concentrations of urea in the stomach and gastric mucosa (Mobley et al, 1991).

Urease catalyses the hydrolysis of urea to yield one molecule of ammonia and carbamate. Carbamate spontaneously decomposes to another molecule of ammonia and carbonic acid

\[
\begin{align*}
H_2N-CO-H_2N + H_2O & \rightarrow NH_3 + H_2N-C(O)OH \\
H_2N-C(O)OH + 2H_2O & \rightarrow NH_3 + H_2CO_3
\end{align*}
\]

In aqueous solution, the protonated and de-protonated ammonia and carbonic acid are in equilibrium which leads to an increase in pH that is essential for bacterial colonisation (Mobley, 2001).
Chapter One

*H. pylori* colonises the interface between mucosa and lumen. It possesses features that are necessary for colonisation of the juxtamucosal mucus environment. This pathogen once attached to the gastric epithelial cells, incites an immune response resulting to an active gastritis, which is histologically a characteristic of bacterial infection. It also incites an immunological response marked by the presence of specific IgG. Attachment of tissue antigens (e.g., Lewis B) vacuolating toxin (VacA) (assist the free passage of urea through epithelial cells) and a cytotoxin (CagA) (injected into epithelial cells via a type IV secretion system) ensure the persistence of infection (Marshall, 2002).

1.4 Epidemiology

In this section an overview will be given of the prevalence, mode of transmission, environmental and genetic roles of *H. pylori*. The bacterium causes one of the most common bacterial infection in humans, and is the cause of the majority of upper gastroduodenal disease cases. Epidemiologic studies have linked the infection to peptic ulcer disease, gastric carcinoma, and B-cell mucosa-associated lymphoid tissue (MALT) Lymphoma to *H. pylori* infection (Graham *et al.*, 1992; Marshall *et al.*, 1985; Parsonnet *et al.*, 1994). However, despite of connection between *H. pylori* prevalence and gastric cancer occurrence, studies providing direct evidence supporting a causal link to cancer are not yet available (Hunt, 2004).

Comparison between the prevalence of *H. pylori* in high and low risk (of developing gastric cancer) areas of the Shandong Province in China have produced results which support the hypothesis that *H. pylori*, particularly the CagA positive strains, is the cause of gastric cancer. The prevalence of *H. pylori* in areas which were known to have a high rate of gastric cancer was
much more significant (three times the rate of low risk areas) than the prevalence of *H. pylori* in low risk areas (You *et al.*, 2001).

The prevalence and survival of gastrointestinal cancer patients within different socio-economic groups has been studied to determine whether socio-economic deprivation is related to gastrointestinal prevalence and survival rates. Data from different socio-economic classes were recorded from the population of West Midlands, England from 1986-2000. The results demonstrated a rise of gastrointestinal cancer cases (127%) while there was a steady decrease in cancer prevalence in the socio-economically deprived class (-57%), presumably owing to improved hygiene and as a result less *H. pylori* transmission and cancer cases. Despite the decreasing rates of cancer in deprived classes, it was also demonstrated that the more affluent had a higher chance of surviving gastrointestinal cancer than the socio-economically deprived classes (Roy *et al.*, 2005).

Association between herpes simplex virus type 1 (HSV-1) and *H. pylori* has also been suggested. An epidemiological study was conducted to test this hypothesis. Data from 1090 participants aged 12-19 years were collected and it was discovered that overall, HSV-1 seropositivity is associated with a higher *H. pylori* seroprevalence. The negligible association found in some strata suggests that environmental factors or routes of transmission rather than biological reasons may be primarily responsible for this association (Baccaglini *et al.*, 2006; Baccaglini *et al.*, 2004).

*Helicobacter pylori* is not a gender specific, it can occur worldwide; however, there is a significant differences in prevalence when comparing developed countries and undeveloped countries. Prevalence of *H. pylori* in undeveloped countries is much higher than we would find in developed countries (Goh, 1997; Rothenbacher and Brenner, 2003). For an example, a study was conducted in southern China, this data was then compared to
results obtained from data collected in Australia. It was found that *H. pylori* infection in Chinese subjects was much higher in comparison to those in Australia (44.2% compared to 21%) (Mitchell *et al.*, 1992). So the prevalence of *H. pylori* can be dictated by factors such as, cultural background, occupation, density of living, education. There have been epidemiological studies that have attempted to link *H. pylori* infection to smoking and alcohol consumption. 10537 subjects all infected with *H. pylori* provided data on smoking, usual weekly consumption of alcohol and daily intake of coffee. The study showed that smoking and coffee consumption were not related to active *H. pylori* infection; however, moderate alcohol consumption (of beer and wine) results in lowering the chance of infection by 11% (Murray *et al.*, 2002).

The rate of infection of *H. pylori* can be attributed to living conditions during childhood and the majority of infections are contracted during childhood (Malaty and Graham, 1994). A study was carried out to determine whether the eradication in a population would affect the overall prevalence of *H. pylori* in symptomatic children. 159 patients underwent gastroscopy (after the prevalence of *H. pylori* in their population was determined) and after a set period of time (1997-2004) the prevalence of *H. pylori* was again recorded in their population. The results showed that the overall prevalence had not changed significantly (33.3% in 1997, 27.7% in 2004). It was concluded that eradication schemes are unsuccessful in the reduction of prevalence (Wong *et al.*, 2005). These results draw attention to the prevalence of *H. pylori* in families. There is significant increase in family members of children infected with *H. pylori* going on to infect parents and siblings (Mitchell, 2001). It has also been reported that infected mothers are 8 times more likely to pass the bacterium onto a child than mothers who are not infected while fathers are approximately four times greater (Rothenbacher *et al.*, 1999) than those who are not infected. In these studies it was found that identical strains of *H. pylori* found within the family unit which leads to the idea that interfamilial
transmission has a big impact on the transmission of the bacterium (Mitchell, 2001). However, in saying this, a case-controlled study was conducted in Bangladeshi families and the data suggested that the prevalence of infection in parents of \textit{H. pylori} positive children was the same as that in \textit{H. pylori} negative children. This data may suggest that in certain countries infection of \textit{H. pylori} may occur outside the family home (Sarker \textit{et al}, 1995). It could be hypothesised that un-infected children in the family unit socializing with children outside the family are at greater risk of infection. The number of siblings in families have also been shown to influence the transmission of the bacterium, children who are closer in age are more likely to contract \textit{H. pylori} and it would appear that it is the eldest infecting the youngest (Goodman & Correa, 2000).

The transmission of \textit{H. pylori} from animals to humans has long been of concern. As \textit{H. pylori} is usually contracted in childhood, studies of Polish Shepherd children have been carried out to determine the prevalence of \textit{H. pylori} in children who have contact with sheep. The prevalence of \textit{H. pylori} in children who had contact with sheep was significantly higher (58\%) than those who did not (21.6\%) and also the urban control (26\%) (Plonka \textit{et al}, 2006). There were a number of confounding factors in this study and the consensus within the scientific community is that \textit{H. pylori} infection is not a zoonotic.

The impact of breastfeeding on transmission and/or protection from infection has also been studied. Epidemiological studies provide strong evidence that the breastfeeding of children does not protect them against \textit{H. pylori} infection. In studies carried out in a poor urban community in northeastern Brazil, the prevalence of \textit{H. pylori} infection in breastfed children and non-breastfed children did not differ significantly (55\% for non-breastfed children and 52\% breastfed children). Furthermore, the prevalence of infection was much higher in children whose mothers were infected with \textit{H.
pylori. This study suggests that breastfeeding does not protect children from H. pylori, and that mothers who are infected with H. pylori risk transmitting the infection to their children (Rodrigues et al, 2006).

There have been a number of studies into the proposed acquisition of H. pylori through contaminated water. However, these studies failed to support the belief that water plays a major role in transmission of the bacterium. The obvious conclusion would suggest that direct person-to-person contact is the most important mode of transmission. From studies conducted involving institutionalized subjects, it is clear that this view of close personal contact is needed to necessitate the spread of H. pylori (Mitchell, 2001). There are limited data with regard to transmission of H. pylori between spouses; however, there have been studies suggesting that the risk of infection increases with the number of years of co-habitation but that such transmission is not common (Brenner et al, 1999).

Clustering of H. pylori as an indicator of interfamilial transmission has long been of concern. Some small-scale studies have reported clustering of H. pylori infections as a possible result of intrafamilial transmission. A study was contacted in a large community from Germany that included both high-prevalence and low-prevalence population subgroups. Prevalence of infection among women was higher (34.9%) when their partners were infected with H. pylori and lower (14.5%) if their partners were not infected. Stratification by nationality has shown a strong association of infection for partners of non-German nationality. The results suggest that intrafamilial transmission of infection caused by H. pylori is unlikely to be of relevance in low prevalence population groups, while intrafamilial transmission of H. pylori has been suggested by epidemiological studies in high prevalence population groups (Brenner et al 2006).

The prevalence of H. pylori infection varies widely in different geographical
locations, even within developed countries (Banatvala et al, 1993). In a study conducted in the USA on asymptomatic people residing in the Houston Metropolitan area, it was found that the prevalence of the bacterium increased with age at a rate of 1% per year and was twice as frequent in Blacks American (66%) and Hispanics (65%) compared with White American (26%) (Malaty and Graham, 1994). The prevalence of *H. pylori* in Indigenous Australian is also two to three times higher than in the non-Indigenous Australian population. The prevalence in remote rural communities is much higher 91% in comparison to urban community 60% (Windsor et al, 2005).

There are no specific data indicating the a person's ethnic origins influence susceptibility to *H. pylori*, more appropriately the prevalence of infection appears to be determined factors related to living conditions in childhood. Thus that there appears to be two processes for the acquisition of *H. pylori*, the first is the most common and is the acquisition of the bacterium in early childhood, and the second, more sporadic, is acquisition that can occur throughout adult life.

### 1.5 Disease Profile and Specific Isolates

The world’s population is ageing. Epidemiological studies have shown that aging imparts a variety of physiological changes in the oropharynx, esophagus and stomach the increase for esophageal and gastrointestinal disorders. Older individuals also tend to have a higher prevalence of co-morbidity factors such as *H. pylori* infections. Acid related disorders such as gastroesophageal reflux disease GERD and peptic ulcer disease have unique features when occurring in elderly patients. Therefore, physicians must take the factor of age into consideration when attempting to diagnose or implement treatment for elderly patients. The prevalence of *H. pylori* in the aging population is higher when compared with younger generations; this is the Cohort effect (Greenwald, 2004).
Helicobacter pylori is the most important aetiologic agent of gastritis Fig. 1-3), gastric and duodenal ulcer (peptic ulcer disease) (Fig. 1-4, b and c) and is associated with gastric cancer (Alkopyanz et al., 1998). About 50% of world’s population is infected with H. pylori of which 10% of infected develops disease during their lifetime (Alkopyanz et al., 1998).

Figure 1-3 Chronic gastritis, taken at the time of endoscopy (Martin, 2005)

Figure 1-4 a; normal duodenal, b; duodenal ulcer, c; bleeding duodenal ulcer, at the time of endoscopy (Gastrolab, 2006)
It has been suggested that the spectrum of gastroduodenal disease may be associated with different strains of *H. pylori* (Marshall, 1991). It has also been suggested that strains that are more virulent or have a higher virulence potential may be associated with more serious disease. Certain strains of *H. pylori* are capable of inducing a cytopathic effect on mammalian cells *in vitro* by the formation of intracytoplasmic vacuoles, the product of the vacuolating cytotoxin, *vacA* (Marshall, 1991). The intracytoplasmic vacuoles have an acidic pH and their formation can be potentiated by weak bases such as ammonia. This toxin is believed to form a complex of approximately 1080 kDa in native form but in denatured form has a mass of approximately 90 kDa. Toxin activity is present in almost half of *H. pylori* strains a characteristic of the so called type 1 strains (Ilver et al., 2001).

When first identified, cytotoxicity was strongly associated with the presence of an immunogenic high molecular weight protein of 120-130 kDa in mass. This protein has no cytotoxic activity but the gene coding for it, the cytotoxin associated gene A (*cagA*) is nearly always absent from non-cytotoxic strains (Xiang et al., 1995).

*Helicobacter pylori* strains, isolated from patients with peptic ulcers, produce vacuolating toxin more frequently than isolates from patients without ulcer disease. Mucosal recognition of the *H. pylori* 120kDa protein (CagA) is also positively associated with the activity of gastritis and the extent of surface degeneration. These findings suggest that CagA-positive strains of *H. pylori* have pathogenic factors associated with gastritis and peptic ulceration (Atherton et al., 1995; Van Doorn et al., 1998). The functions that are encoded by a 40-kb chromosomal region are called cag Pathogenicity Island (*cagPAI*) (Alkopyanz et al., 1998). The Pathogenicity Island (*cagPAI*), *H. pylori* cytotoxin-associated gene A (*cagA*), vacuolating toxin A (*VacA*), and factors involved in adherence of *H pylori* to gastric epithelial cells, have been linked to enhanced pathogenicity of the bacterium. The immunoreactive protein
CagA is found in 60-70% of *H. pylori* in the industrialised world (Blaser, 2001; Suerbaum & Michetti, 2002). As the nomenclature suggests, *cagA* maps to the *cagPAI*, a large stretch of contiguous DNA, a cluster of 29 genes that encodes elements of virulence, (Ilver *et al.*, 2001; O'Rourke & Bode, 2001) in which some of them encode a type IV bacterial secretion apparatus, which can translocate CagA into host target cells. Phosphorylation of CagA may occur and activate host signalling-pathways that in turn influence host cellular release, and cell motility (Ilver *et al.*, 2001; O'Rourke & Bode, 2001).

In addition, several other *cagPAI* genes, such as *cagG*, *cagH*, *cagL*, and *cagM*, have been linked to particular epithelial cell responses relevant to *H pylori* pathogenicity (Hsu *et al.*, 2002; Mizushima *et al.*, 2002).

While there are data indicating an association between specific strains and more serious disease, the presence or absence of identified virulence factors are not predictive of disease type or severity although they may have a bearing on inflammatory processes (Blaser & Berg, 2001; Suerbaum & Michetti, 2002). If there are no distinct ulcerogenic strains of *H. pylori*, host and environmental factors must play a role in determining the consequences of *H. pylori* infection (Testerman *et al.*, 2001). The host epithelium provides a protective barrier but colonization by *H. pylori* induces an inflammatory response to the infection, which may be deleterious to the host in that it provides the necessary prerequisite for more serious disease.

*Helicobacter pylori* raises plasma gastrin levels, but it is unclear whether this directly stimulates increased acid secretion. However, in infected individuals with raised acid secretion, one year after clearing the infection, acid secretion returns to normal levels. Therefore *H. pylori* infection in certain individuals promotes an exaggerated acid response, and based on the dictum “no acid, no ulcer”, contributes to the development of duodenal
ulceration. The host factors that determine this response have not been elucidated (Graham & Qureshi, 2001).

1.5.1 Gastric Cancer

There is considerable epidemiological evidence linking *H. pylori* infection to gastric cancer (Fig. 1-5) (Forman, 1996). Correa has proposed a human model of gastric cancer based on histological and clinical evidence. He proposed that gastric cancer develops through a sequence of events from normal gastric mucosa to superficial gastritis, atrophic gastritis, and intestinal metaplasia to gastric carcinoma (Forman, 1996). Prospective cohort studies have investigated whether *H. pylori* infection increases the risk of subsequently developing gastric carcinoma. These studies assessed *H. pylori* seroprevalence in patients who subsequently developed gastric cancer, compared with age and sex matched controls. Such studies have shown that gastric cancer subjects had a higher prevalence of prior infection with *H. pylori* than controls (Huang *et al*, 2003).

Figure 1-5 Gastric cancer, taken at the time of endoscopy (Gastrolab, 2006)
There are some reports suggesting that serology may underestimate the prevalence of *H. pylori* infection in gastric cancer patients. Studies have shown many patients (90%) with gastric cancer who were seronegative had a strong mucosal response to *H. pylori* (Hansson *et al.*, 1995; Hichcock *et al.*, 1995). In a study of patients who had two gastric biopsies taken with a time interval of less than two years and in those who had biopsies taken at an interval of greater than two years, all the patients who had an endoscopic examination with biopsies taken in the interval of less than two years, who had a normal mucosal biopsy at the second examination, had *H. pylori* eradicated. A number of patients who had biopsies taken at an interval greater than two years who had persisting *H. pylori* gastritis had developed mucosal atrophy, while several had progressed to metaplasia and one had developed early gastric cancer (Hansson *et al.*, 1995; Hichcock *et al.*, 1995).

The effects of *H. pylori* infection on gastric epithelial cell kinetics has been investigated using the bromodeoxyuridine (BRDU) test. BRDU is an analogue of thymidine and is taken up by cells in the S phase of cell division. Increased epithelial cell proliferation is an early indicator of a risk of developing carcinoma. Patients who have gastritis induced by *H. pylori* had an increased cell turnover compared to patients who had “gastritis” (not strictly the typical inflammatory response seen in *H. pylori* infected individuals) induced by non-steroidal anti-inflammatory drugs. In addition, the increased cell proliferation returns to normal after clearance of the infection. The increased cell proliferation has also been noted in association with atrophic gastritis, intestinal metaplasia and gastric cancer. This is further evidence of continual change from gastritis to cancer in subjects at risk of developing cancer (Carrillo *et al.*, 2004; Hichcock *et al.*, 1995).

Based on the available evidence, the World Health Organisation in 1994 classified *H. pylori* as a Class 1 (definite) carcinogen (Axon & Moayyedi, 1996). While viruses and parasites have been linked to cancer, this declaration by the WHO was the first definitive citation of a bacterium.
1.5.2 Peptic Ulcer

Peptic ulcer is a break in mucosal layer of the stomach or duodenum, referred to as gastric or duodenal ulcers respectively. About 5% of gastric ulcers are caused by malignant tumor; however, duodenal ulcers are mainly associated with *H. pylori* infection. Peptic ulcers can also be caused by NSAIDs (Quan and Talley, 2002).

Mucosal ulceration may be influenced by endogenous factors, such as gastric acidity and acid-dependent pepsin or exogenous factors such as NSAIDs. *H. pylori* infection increases the levels of gastrin and pepsinogen and reduces the levels of somatostatin subsequently exposure of duodenum to acid increases.

Peptic ulcer disease is one of the most common conditions in clinical practice (Graham, 1991). The natural history of peptic ulcer disease is of remission and relapse. The advent of histamine type 2 receptor antagonists (H\(^2\)RAs) revolutionised the treatment of peptic ulcer disease. These agents are effective in healing ulcers and resulted in a marked decrease in the incidence of complications. However H\(^2\)RAs are effective only in the short term as patients invariably relapse. This resulted in the introduction of long-term maintenance treatment with H\(^2\)RAs for patients with peptic ulcer disease. Maintenance treatment; however, is expensive and the underlying condition is not cured.
1.5.3 *Helicobacter pylori* and Ulcer Relapse

Studies involving over 223 patients confirmed the observation that eradication of *H. pylori* results in a permanent cure of peptic ulcer disease (Arkkila *et al*, 2003). Significantly no credible study has contradicted these finding. It is now broadly accepted by gastroenterologists worldwide that *H. pylori* infection induces peptic ulcer disease (Arkkila *et al*, 2003). Prevention of ulcer relapse by successful therapy is the most important evidence that *H. pylori* plays a major role in gastroduodenal disease. Large, multicentric studies have demonstrated that eradication of *H. pylori* dramatically reduces peptic ulcer relapse rates. In addition, it has been demonstrated that patients presenting with hematemesis secondary to peptic ulcer do not re-bleed following successful eradication of *H. pylori* (Arkkila *et al*, 2003).

The National Institutes of Health in the United States now recommend that all patients who present with gastric or duodenal ulcers and have *H. pylori* infection should receive treatment to cure their *H. pylori* infection (Arkkila *et al*, 2003).

1.5.4 Non-Ulcer Dyspepsia

Non-ulcer dyspepsia is used to describe patients with epigastric discomfort for over three months, who have normal endoscopy (absence of ulcers or tumour), normal biochemistry and normal gallbladder function. The *H. pylori* infection and non-ulcer dyspepsia both are common and frequently one is found with the other (Goddard *et al*, 1999). In a major study, one hundred and twenty dyspeptic *H. pylori* positive patients with endoscopically defined peptic ulcer or non-ulcer dyspepsia were randomly selected to receive a one week course of antibiotics (Goddard *et al*, 1999). *Helicobacter pylori* was successfully eradicated in 96% of the patients with
endoscopically defined peptic ulcer. The eradication results in long-term relief of dyspeptic symptoms and the consumption of antisecretory drug were dramatically reduced in patients with peptic ulcer and not in those with non-ulcer dyspepsia. Non-ulcer dyspepsia is not only common but expensive, as patients require consultation time as well as empirical treatment (Goddard et al, 1999). This finding is similar to that of several other studies.

Despite the lack of evidence of a specific relationship between *H. pylori* infection and non-ulcer dyspepsia, the treatment of such patients appears to be common practice. Effective treatment of *H. pylori* infection has proven difficult with the poor efficacy of single agents necessitating the use of combination therapies (Fallone et al, 2000).

Population screening and treatment of *H. pylori* has been advocated as a means of reducing mortality from gastric cancer as well as dyspepsia and dyspepsia related resource use. In a study aimed at determining the effect of screening for *H. pylori* on dyspepsia and dyspepsia related disorders over 10 years, *H. pylori* positive individuals aged 40-49 years enrolled in a community screening program and were randomised to eradication therapy or placebo. Ten years later they were sent a questionnaire and primary care records were re-examined. Consultation, referral, prescribing and investigation data related to dyspepsia were extracted. The results showed that there was a mean saving in total dyspepsia related costs of US$117 per person. It was concluded there were significant reductions in total dyspepsia related health care costs. The savings made were greater than the initial cost of *H. pylori* screening and treatment (Ford et al, 2005).

Costs benefit analysis of the value of screening and treatment of *H. pylori* patients immediately after they test positive for infection as appears cost effective as compared to the treatment of patients once symptoms appear.
Comparisons of the two alternatives in America have shown that the earlier treatment has a saving of $26 per case, thus proving significantly cost effective (Leivo et al, 2004).

1.6 Contemporary Therapy

In 1994, a National Institute of Health (USA) Consensus Development Conference concluded that there was an association between H. pylori infection and peptic ulcer disease and recommended that ulcer patients with H. pylori be treated with antibiotics. These guidelines suggest that persons with active ulcers or a documented history of ulcer disease should be tested for H. pylori, and if found to be infected must be treated with antibiotics (Martin, 1996).

One of the most widely used regimes to treat H. pylori infection following the acceptance of a role of infection in serious gastric disease consisted of colloidal bismuth subcitrate (DeNol) in combination with two antibiotics, metronidazole and tetracycline or ampicillin/amoxicillin. This triple therapy is complicated as the patient has to take up to 18 capsules a day for the first week and many experienced mild side-effects, including nausea and a metallic taste on the tongue, caused by the 5-nitroimidazole (Van Der Hulst et al, 1996). The bismuth based triple therapy became more effective when combined with acid suppressive proton pump inhibitors (PPI), such as Omeprazole (AstraZeneca). The effectiveness of bismuth based triple therapy ranged from 87.9% and 89.2% for one week and two weeks therapy respectively, with increased efficacy when used in combination with a PPI, with claims of good success rates (94.8%) (Van Der Hulst et al, 1996). The PPI acts by increasing gastric pH, thereby allows the antibiotics to be more active, probably due to bringing the gastric acidity into the range of optimal drug activity.
While classical triple therapy has fallen from favour, new treatment regimens using acid suppressive therapy, particularly proton pump inhibitors, to enhance antimicrobial drugs efficacy have emerged as the preferred therapy for treatment of *H. pylori* infection. These regimens include 1-2 weeks therapy with a proton pump inhibitor ‘e.g., omeprazole twice a day (b.i.d.) 20 mg’ together with two appropriate antibiotics, such as a macrolide (e.g., clarithromycin 500 mg b.i.d.) and amoxicillin (1000 mg b.i.d.), or amoxicillin (500 mg b.i.d.) and a nitroimidazole (e.g., metronidazole 400 mg b.i.d.), or a macrolide (e.g., clarithromycin 250mg b.i.d.) and a nitroimidazole (e.g., metronidazole 400 mg b.i.d.) (Armstrong *et al.*, 1987; Berstad *et al.*, 1996).

There is also evidence that a one week, low-dose regime consisting of omeprazole (20 mgs daily), clarithromycin (250 mgs, b.i.d.) and metronidazole (400 mgs b.i.d.) will eradicate *H. pylori* in 90 - 100% of patients (Alamri *et al.*, 1997). The performance of these various combinations varies depending up the study design, the analysis performed (per protocol or intention to treat), the experience of the investigator and the level of patient education or supervision.

The eradication efficacy with a PPI such as omeprazole or lansoprazole in combination with clarithromycin and amoxicillin or metronidazole is between 79–96% (Hazell *et al.*, 1997; Lind *et al.*, 1996). In practice, it is rare to see successful treatment in greater than 90% of patients.

The successful treatment of *H. pylori* infection results in the eradication of the bacterium, and also cures and prevents the development of the associated diseases (Riberio *et al.*, 2004). However, the development of the resistance by the bacterium to the antibiotics that are currently used is the major impediment for these therapeutic regimens (Hazell *et al.*, 1999; Hyde *et al.*, 1997; Jorgensen *et al.*, 1996).
When treatment with amoxicillin, clarithromycin and/or metronidazole fails then patients will be treated with tetracycline-based combination regimens (Riberio et al, 2004). Also treatment with tetracycline-based regimens is an alternative therapy to reduce the costs of treatment (Goodwin, 1997).

Tetracycline is widely available and cheap and until recently the resistance against this antimicrobial was rare in \textit{H. pylori}. As this antibiotic is cheap and can be obtained in some countries without prescription, the incidence of tetracycline resistance is increasing, particularly in those countries where antibiotic use is less regulated (Hazell et al, 1986; Megraud, 1993; O’Rourke & Bode, 2001). The emergence of tetracycline resistance of \textit{H. pylori} is a serious problem as it has a negative impact on the tetracycline-containing regimens (Riberio et al, 2004).

The challenge now is to find a shorter course of treatment and more patient friendly by considering the antibiotics to one capsule which will be effective, efficacious, have fewer side effects and make \textit{H. pylori} eradication less expensive (Goodwin, 1997). Regrettably, in recent time the pharmaceutical industry appears to have lost interest in the development of specific agents to treat \textit{H. pylori} infection.

A study was conducted to assess the efficacy of triple and quadruple therapy in patients who had failed one or more eradication regimens. The resistance to metronidazole, clarithromycin, and amoxicillin were determined by E-test (a strip impregnated with antibiotics across a gradient within the strip). Patients were randomly divided into two groups: The first group was treated with triple therapy receiving; the PPI pantoprazole 40 mg, amoxicillin 1 g, levofloxacin 250 mg, all twice daily for 10 days. The second group was treated with quadruple therapy receiving; omeprazole 20 mg twice daily, tetracycline 250 mg 4 times daily, metronidazole 500 mg twice daily, and bismuth subcitrate 240 mg. This study showed that the levofloxacin-based
triple therapy was more successful (70% eradication) in comparison to quadruple therapy (37% eradication) (Bilardi et al, 2004).

So far it has been no report of resistance to any bismuth compound that has been used in classical triple therapy (e.g. a nitroimidazole plus a bismuth compound, together with either amoxicillin or tetracycline). This therapy was reported to affect a cure in more than 80% of patients. Unfortunately, problems with poor patient compliance and the ability of \textit{H. pylori} to develop resistance to nitroimidazole, has resulted in recurrent reports of treatment failure (Bateson, 2000).

1.7 Treatment Failure

The antimicrobial resistance patterns should differ among countries and regions as the basis of therapies are different and antimicrobial use is also variable. However, the occurrence of resistance is worldwide. Evidence from a number of countries support the former assertion.

\textit{Helicobacter pylori} resistance to antimicrobial was tested in Poland by \textit{in vitro} susceptibility of 337 isolate from children (N=179) and adult (N=158) that were cultured from various regions of the country between January 2001 and December 2004. All the isolates were susceptible to amoxicillin and tetracycline, 28% were resistant to clarithromycin, 46% were resistant to metronidazole and 20% of isolates were simultaneously resistant to clarithromycin and metronidazole (Dzierżanowska-Fangrat et al, 2005).

A retrospective analysis in Saudi Arabia revealed 35.2% of isolates were resistant to Metronidazole in patients receiving first line therapy and 78.5% in patients receiving second line therapies. In this study only one strain was found to be resistant to tetracycline (Al-Qurashi et al, 2001). The high
resistance to Metronidazole may be a product of the availability and the use of this antibiotic in this region for the treatment of other infections.

In a Korean study, two hundred and twenty patients who failed first line treatment underwent an endoscopic examination and had biopsies taken. Overall 50% of these patients were infected with antibiotic resistant *H. pylori*. The isolates were obtained from both the antrum and corpus. They were tested against various antibiotics and their resistance profile was found to be: amoxicillin (0.5%), clarithromycin (5.9%), furazolidone (1.4%), metronidazole (45.5%), nitrofurantoin (1.4%), and tetracycline (6.8%). Heteroresistance were also present in 38% of the patients. DNA fingerprinting genotype analysis on the 41 pairs of isolates showed identical or similar patterns which in turn suggest the antibiotic-resistant *H. pylori* develop from pre-existing susceptible strain rather than co-infection with a different strain (Kim I et al, 2003). These results could also suggest the resistance may have developed due to the use of low doses of antibiotics during the initial treatment (Kim et at, 2003). These finding suggest that care is needed to ensure appropriate dose and duration of therapy and the importance of patient compliance.

After 25 years since the discovery of *H. pylori* no ideal treatment regimen has been found. Even with the most effective triple or quadruple therapy, 20% of the patients develop resistance to the antibiotics (Gisbert & Pajares, 2002). Even the so called rescue therapies (such as a quadruple combination of PPI, bismuth, tetracycline and metronidazole or levofloxacin-based triple/quadruple therapies) fail to eradicate all *H. pylori* with the best data suggesting success in only 80-90% of patents (Gisbert et al, 2004; Zullo et al, 2003).

Due to the emergence of antibiotic resistance, the effective life span of any given antibiotic against any pathogen can be limited. Worldwide spending on the discovery of new anti-infective agents (including vaccines) is expected
to increase 60% from the spending levels in 1993. New lead compounds, including plant sources are also being investigated (Alper, 1988).

In recent years attempt has been made to screen some natural products including Mastic gum for their anti-microbial activity against *H. pylori*.
1.8 A Suggested Alternative Therapy

Antibiotic resistance in *H. pylori* has emerged as a significant clinical problem. Further, contemporary therapy is expensive and complex, particularly for third world countries. The body of work contained within this dissertation was carried out to investigate an alternative therapy based on observations of the traditional therapy for gastric disease in the Middle East. One of these traditional therapies centres on plants of the genus *Pistacia*.

The resin or gum from *P. lentiscus* (Mastic) has been used by traditional healers for the relief of upper abdominal discomfort, gastralgia, dyspepsia and peptic ulcer. It has also been used as a Masticatory and by dentists for filling carious teeth. It has also been reported to possess stimulant and diuretic properties (Al-Said *et al*, 1986).

In 1984, a double-blind trial of Mastic and placebo in treatment of duodenal ulcer it was claimed that it produced resulted in ulcer healing in 70% of patients as compared to 22% of the placebo group (Al-Habal *et al*, 1984).

However, nothing has been reported in the composition and biological property of its relative *P. atlantica* and its subspecies; *Kurdica, Mutica and Cabolica*. Further, the mechanism of the action the antimicrobial action by the gum of *P. lentiscus* is not understood.
1.9 Genus Pistacia

Phylogenic studies of the genus *Pistacia* have been based on the morphological characterization. Plants of the genus *Pistacia* belongs to the family *Anacardiaceae*, a small family with about 400 species of mainly tropical trees and shrubs some of the well known of these species are:

*Anacardium occidentale* (Cashew, Pajuil, Maranon), *Cotinus coggygria* (Smoke Tree, Venetian Sumach), *Cyrtocarpa edulis*, *Harpephyllum caffrum* (Kaffir Date, Kaffir Plum, Wild Plum), *Malosma laurina* (Laurel Sumac), *Mangifera indica* (Mango), *Operculicarya decaryi* (Jably), *Operculicarya pachypus*, *Pachycormus discolor* (Elephant Tree, Copalquin, Torote Blanco) (Faucon, 1998).

Leaves are alternate and pinnate without stipules in all the European species. Flowers are small with sepals and 3-7 petals in some of the species and without petals in some other. All the members of this family produce a resinous sap. The most well known species of this family are *Anacardium occidentale* and *Pistacia vera*, trees that are cultivated for their nuts (Cashew and Pistachio) (De Rougemont, 1989).

Within the genus *Pistacia* are found eleven species of trees and shrubs. These species are found in some Mediterranean countries, Central Asia and in Southern and Central America (Thomas *et al*, 2000). The most well known of these species are: *Pistacia atlantica* (Mount Atlas Pistache, Mount Atlas Mastic tree), *Pistacia chinensis* (Chinese Pistache), *Pistacia lentiscus* (Mastic Tree, Evergreen Pistache), *Pistacia terebinthus* (Terebinth), *Pistacia vera* (Pistachio) (Faucon, 1998).
Some studies have been undertaken regarding the chemical composition and biological activity of *Pistacia vera* (Fig. 1-6). *Pistacia vera*’s oleoresin has shown anti-inflammatory and antinociceptive activities *in vivo* against carrageenan-induced hind paw edema model in mice without inducing any gastric damage at both 250 and 500 mg/kg. Further fractionation led to identification of α-Pinene which showed a moderate anti-inflammatory effect at a dose of 500 mg/kg (Orhan *et al.*, 2005).

Various parts of *Pistacia vera*’s extract have also been screened for activity against four parasitic protozoa, *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. The cytotoxicity of the extracts on rat skeletal myoblast (L6) cells has also been tested. The branch extract of *Pistacia vera* significantly inhibited (77.3%) the growth of *L. donovani*, and the dry leaf extract was active against *P. falciparum* (60.6% inhibition). These extracts exhibited no cytotoxicity on mammalian cells (Orhan *et al.*, 2005).
In another study lipophytic extracts obtained from different parts of the plant (leaf, branch, stem, kernel, shell skins, seeds) were screened for antibacterial, and antifungal activities against *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans* and *C. parapsilosis*. These extracts were also screened for antiviral activities against Herpes simplex (DNA virus) and Parainfluenza viruses (RNA virus). These extracts demonstrated a moderate antimicrobial activities ranging from 128–256 µg/ml and noticeable antifungal activities. Kernel and seed extracts showed noteworthy antiviral activities (Özçelik *et al*., 2005).

Substantial studies have also been undertaken in regard to chemical compositions of various part of *Pistacia lentiscus*, an evergreen shrub that has been known for centuries because of the production of a resin, so-called “Mastic gum”. The first modern study was carried out in 1956 by Barton and Seaone (Barton & Seaone, 1956). They isolated a new triterpenoid acid “Masticadienonic acid” from the trunk exudate, “Mastic gum”. The essential oils of this gum and also essential oils from leaves of this plant have been analysed. (Castola *et al*., 2000; Duru *et al*., 2003; Papageorgiou *et al*., 1981; Mans, 1991; Mgiatis *et al*., 1999 Vasslios *et al*., 1991) The essential oils and whole gum have been screened for antimicrobial activities (Marone *et al*., 2001; Tassou & Nychas, 1995). However, little work has been undertaken on the composition and biological activity of resin from *Pistacia atlantica* (*P. atlantica*) (Fig. 1-7) and nothing on the subspecies *P. atlantica Kurdica* (*P. a. Kurdica*) (Fig. 1-8), *Pistacia atlantica Mutica* (*P. a. Mutica*) and *Pistacia atlantica Cabolica* (*P. a. Cabolica*).
Figure 1-7 *Pistacia atlantica* (Sharifi, 2001a)

Figure 1-8 *Pistacia atlantica* Kurdica
**Pistacia a. Kurdica** is found widely in the region of the Zagros Mountains, particularly in Western and Northern Iran, Eastern and Northern Iraq, Southern Turkey and Northern Syria so-called Kurdistan (Fig. 1-9).

![Figure 1-9 Distribution of Pistacia atlantica Kurdica](image)

*Figure 1-9 Distribution of Pistacia atlantica Kurdica (Sharifi, 2001a)*

**Pistacia a. Kurdica** shows a discontinuous pattern of distribution over this region and is an important constituent of the natural vegetation in this area. **Pistacia a. Mutica** is found widely in Centre and Eastern Iran (Fig. 1.10).

![Figure 1-10 Distribution of Pistacia atlantica Mutica](image)

*Figure 1-10 Distribution of Pistacia atlantica Mutica (Sharifi, 2001a)*
Pistacia a. Cabolica is found widely around Eastern Iran and Western and Central Afghanistan (Fig. 1-11). These sub-species of P. atlantica are the major source of the gum that is well known in the region, if not more widely, and hereafter will be referred to as Kurdica, Mutica and Cabolica Gum.

Figure 1-11 Distribution of Pistacia atlantica Cabolica (Sharifi, 2001a)

P. a. Kurdica is a native plant of Kurdistan and was classified as a sub-species of P. atlantica because of the presence of leaf rachis wing that are narrower than the type of P. atlantica (Figures 1-12 and 1-13) (Zohary, 1952). However, Yaltirik (1967), classified this plant as a different species Pistacia khinjuk (Yaltirik, 1967), because the leaves are light green on both sides (instead of being dark green above and pale below as in P. atlantica). In addition the nuts of Pistacia khinjuk/P. a. Kurdica are much smaller than P. atlantica’s fruit.
Figure 1-12 *Pistacia atlantica* leaves and flower

(Flores de Almácigo, 2006)

Figure 1-13 *Pistacia atlantica Kurdica* leaves and fruits (It was taken by author)
The body of work presented in this dissertation has as the primary goal the characterization of the chemical composition of Kurdica, Mutica and Cabolica gum with particular reference to their biological activity, specifically their antibiotic activity by reference to the human pathogenic bacterium *H. pylori* and some other Gram-negative and Gram-positive bacteria. This study represents the first reported investigation into the composition and biological activity of the trunk bark exudates of *P. Kurdica*, *P. a. Mutica* and *P. a. Cabolica* a resinous gum that have been termed here ‘Kurdica Gum’, ‘Mutica Gum’ and ‘Cabolica Gum’.

Kurdica gum, known locally in Kurdistan as “Van”, is an exudate of *P. a. Kurdica* that is extracted by injuring the trunk and thick branches of the tree with a specialized tool. The gum slowly exudes from the trunk and is collected by sticking a clay bowl to the trunk (Fig. 1-14). The resinous exudates are collected three times over the summer.
Historically, the resinous exudates of various species of the genus *Pistacia* have been used for the treatment of stomach upsets, a practice spanning many centuries and generally involving chewing the resin of the gum (gum without essential oils) (Avicenna, 1015; Ibn Al-Baytar, 1248).

Archaeologists in 1982 found a late Bronze Age shipwreck with 100 jars filled with Mastic gum, the exudates obtained from the species *Pistacia lentiscus* that had been used by the Egyptians for medicinal purposes (Hairfield & Hairfield, 1990). With the exception of Mastic gum, there have been few reported studies concerning the composition or biological activity of these exudates.

Several studies have been carried out on the chemical composition of the essential oils of *P. lentiscus* and of its bark exudates, Mastic gum. The main constituents of the oils are monoterpenes (Castola *et al*., 2000; Duru *et al*., 2003; Mans, 1991; Mgiatis *et al*., 1999; Papageorgiou *et al*., 1981; Vasslios, 1991). Barton and Seoane (1956) fractionated Mastic gum into two major fractions, an acidic and a neutral fraction (Barton & Seaone, 1956) and Van den Berg *et al.* (1998) identified an unusual polymer constituent, 1,4-poly-β-myrcene.

The aqueous extract of *P. lentiscus* has been investigated for antifungal activity against *Microsporum canis, Trichophyton mentagrophytes* and *Trichophyton violaceum*, with the extract reducing the growth of colonies by 36-100% (Ali-Shtayeh & Abu Ghdeib, 1999). A double blind controlled clinical trial showed Mastic gum to have a significant effect in relieving the symptoms and in healing of duodenal ulcers (Al-Habal *et al*., 1984; Huwez & Al-Habbal, 1986). The antimicrobial activity of the gum has also been evaluated against clinical isolates of *H. pylori* and shown to have a minimal inhibitory concentration (MIC) of 125µg/mL (Marone *et al*., 2001). In addition, components of the essential oils of Mastic gum have been shown to exhibit antimicrobial activity.
against a number of microorganisms in vitro (Mgiatis et al., 1999; Tassou & Nychas, 1995). Although anti-
H. pylori activity has not been reported, the primary components have been reported as being mainly monoterpenes with some level of monoterpenoids (Castola et al., 2000; Duru et al., 2003; Mans, 1991; Mgiatis et al., 1999; Papageorgiou et al., 1981; Vassios, 1991). However, with the exception of a report indicating that the neutral fraction of Mastic gum significantly inhibited the growth of Rhisoctonia solani (Duru et al., 2003), only the whole Mastic gum has been used in these studies.

As peptic ulcer, dyspepsia and some other H. pylori infection associated diseases need multi-therapy and proton pump inhibitor to heal the ulcer and eradicate H. pylori infections this project was outlined to investigate the secret of Pistacia’s gum healing power through physiopathogenicity of H. pylori and natural use of the gum.

1.10 Objectives

The objectives of the work outlined were as follows:

1. To identify and characterize any antimicrobial fractions/components of the Cabolica, Kurdica, Mastic and Mutica gums.

2. To establish a basis for understanding of antimicrobial activity of these fractions/components of the gums.

3. To determine if a relationship exists between the molecular characteristics of the gums and antimicrobial activity, and if present, characterise the relationship.
4. To investigate/identify antimicrobial activity of the individual components with different modes of the action as an alternative for multiple therapy.

5. To investigate if any component/s Cabolica, Kurdica, Mastic and Mutica gums could replace conventional therapy and aid in healing of peptic ulcers caused by *H. pylori*.

6. To investigate these natural remedies as replacements for conventional therapy.

7. To establish relationship between antimicrobial activity of the individual components

8. To investigate the mode of the action/s and potential to enhance the antimicrobial activity.

As a consequence of literature review and above objectives regarding the nature of gastritis and *H. pylori* associated diseases, a number of hypotheses have been formulated and tested. These hypotheses are as follows:

1. The components/some components of the Cabolica, Kurdica, Mastic and Mutica gums have antimicrobial activity

2. The antimicrobial components within the Cabolica, Kurdica, Mastic and Mutica gums might have different mode of the action.
To test the above hypotheses, the gums were fractionated. The fractions were screened for antimicrobial activities. The active fractions were sub-fractionated to individual components. The individual components were screened for antimicrobial activities. The active components were characterised. The mode of the active components was investigated. The final hypothesis was formulated for these natural remedies as replacements for conventional therapy.
Chapter 2
Material and Methods

2.1 Raw Material

2.2 Source of Raw Product used in Studies

2.2.1 Mastic Gum

Mastic gum with the registry No. [61789-92-2] was purchased from Sigma Aldrich Pty Ltd. Australia.

2.2.2 Kurdica Gum

Kurdica gum from the *Pistacia atlantica Kurdica* was collected from the area around the Zagros Mountains in Kurdistan province, Iran over the period June to August 2000 and provided by Kurdistan Saghez Sazi Co., Sanandaj, Iran.

2.2.3 Mutica Gum

Several samples of Mutica gum from the tree *Pistacia atlantica Mutica* were collected from the centre of Iran, Fars province over the period June to August 2000 by the author.
2.2.4 Cabolica Gum

Cabolica gum from the tree of *Pistacia atlantica Cabolica* was collected from Khorasan province in North East of Iran over the period June to August 2000 by the author.

2.3 Steam Distillation

The isolation of the oils from the raw gum was achieved by steam distillation of the gum in a Dean and Stark apparatus, which was modified to give lower phase return of the water. Steam distillation was carried out over 5 hours. Approximately 5ml of pentane >99% spectrophotometric grade (Sigma Aldrich) [109-66-0] was added to the oils to increase their volume. The pentane and oil solution was dried using anhydrous sodium sulfate >99% (Sigma Aldrich) [7757-82-6]. After filtering the solution was transferred to another container and the pentane was allowed to evaporate overnight. The essential oils from the gums were collected for further analysis and stored at -20°C prior to analysis.
2.4 Fractionations

2.4.1 Polymer Fraction

The finely powdered crude resin fraction (40.00g) was dissolved in dichloromethane (500 ml) and precipitated with methanol. The precipitant that had formed was isolated after decanting and classified as ‘High Molecular Weight’ Fraction (HMWF) (Barton & Seaone, 1956; Seoane, 1956). The molecular weight distribution of this fraction was subsequently determined by Gel Permeation Chromatography (GPC).

2.4.2 Acidic and Neutral Fractions

The remaining solution after extracting essential oils and the polymer fraction was evaporated under vacuum (33.10 g) then dissolved in anhydrous diethyl ether (50 ml), diluted with anhydrous methanol (350 ml), and allowed to stand in a stoppered flask overnight. After decanting from any insoluble residue (0.05 g), the solution was evaporated under vacuum. The resulting solid was dissolved in anhydrous diethyl ether (50 ml), diluted with anhydrous methanol (350 ml), allowed to stand, and then decanted from any insoluble residue (1.79 g). After evaporating the supernatant solution under vacuum, the solid product was allowed to dry in air overnight. The dry residue was accurately weighed (31.26 g), dissolved in anhydrous diethyl ether (50 ml), diluted with anhydrous methanol (350 ml) and then this solution was evaporated under vacuum.

The resulting solution was extracted with sodium carbonate solution (5% w/v, 100 ml). The sodium carbonate extract was acidified and then extracted with fresh anhydrous diethyl ether (100 ml). This etherial solution was dried
over anhydrous sodium sulphate, evaporated under vacuum, and so was obtained a sodium carbonate acidic fraction (11.70 g), ‘fraction A’.

The remaining ethereal solution was then extracted with 0.50N sodium hydroxide (70 ml), producing a viscous liquid above the aqueous layer, a light yellow aqueous solution, and an oily precipitate. The oily precipitate was collected in a stoppered bottle for further analysis. The viscous liquid and light yellow solutions were individually acidified, extracted into fresh ether and the ethereal extracts dried over anhydrous sodium sulphate, filtered and evaporated under vacuum. There were thus obtained a sodium hydroxide-(in) soluble acid fraction from the viscous liquid, ‘fraction B’ (5.00 g), a second sodium hydroxide-soluble acid fraction, ‘fraction C’ (2.60 g). The original ethereal layer was then dried over anhydrous sodium sulphate, filtered and evaporated under vacuum, giving the ‘neutral fraction’ (12.30 g).
2.5 Chemical Analysis

2.5.1 Thin-Layer Chromatography (TLC)

Thin-layer Chromatography (TLC), silica gel/aluminum oxide was used to perform simple analysis of the mixtures for tentative comparison. Methylene chloride containing 5% acetone was used as mobile phase, the chromatogram was then visualized with antimony pentachloride (1M in chloroform).

2.5.2 Column Chromatography

A vertical glass column was used to separate the most active fractions of the gum (acidic fraction a). The acidic fraction A (10g) was dissolved in anhydrous ether and added to the top of the column packed with silica gel (SiO2) (20-40 mesh). Elution with benzene and light petroleum 1:3 afforded 48 fractions. TLC used to determine this system for the column chromatography separation and the mobile phase. The mobile phase was added to the top and flows down through the column by gravity. Column chromatography was used as a purification technique to isolates the components of the mixture for further analysis. After testing all the fractions with Mass Spectrometry, the pure fractions were isolated for further analysis.

2.5.3 Gas Chromatography

A gas chromatograph consists of a flowing mobile phase that is a gas, an injection port, a separation column that contains stationary phase and a detector. The technique of gas chromatography can be used to separate volatile organic compounds. An organic compound (analyte) is separated in the column due to differences in its partitioning behavior between the mobile
and stationary phase. The analyte then is detected by the appropriate detector in the detection port. A variety of detectors are used in gas chromatography the flame ionization detector (FID) is the most common detector that is used then thermal conductivity detector (TCD or hot wire detector), electron capture detector (ECD), photoionization detector (PID), flame photometric detector (FPD). thermionic detector. FID was used in all analysis that was performed in University of Wollongong.

2.5.4 Mass Spectrometry

Mass Spectrometry in the commonly used electron-impact (EI) separate molecule/atom according to their mass. In this method of mass spectrometry an electron beam removes a single electron from the molecule in the gas phase to form a molecular ion that is a radical cation (M⁺). Mass spectrometry analysis was performed using EI and also Chemical Ionisation (CI) methods in which the molecular weight of mixtures were determined by CI for comparison between different sub-species’s crude product. The chemical structures were determined by EI and also the high resolution Mass Spectrometry was performed to determined molecular formula of the compounds.

2.6 Analysis of Essential oils

Gas Chromatography-Flame Ionisation Detector (GC-FID) analysis was performed on a Varian 3700 gas chromatograph coupled to a Shimadzu C-R3A integrator and fitted with a fused silica capillary column (25QC/BP5) obtained from SGE, Australia (25m x 0.25mm i.d., 0.25 µm film thickness). The analytical conditions were: carrier gas hydrogen (ca. 1mL/min) injector temperature 260° C, detector (FID) temperature 280° C, oven temperature 40°
C (2 min. hold) to 280°C (5 min. hold) at 4°C/min. Programmed-temperature Kovats retention indices (RI) were obtained by GC-FID analysis of an aliquot of the essential oil spiked with n-alkane mixture containing each homologue from n-C8 to n-C30.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed in electron impact mode (EI, 70 eV) using a VG AutoSpec system interfaced with a Hewlett Packard 5890 Series II gas chromatograph, fitted with a capillary column (BPX5) supplied by SGE, Australia (25m x 0.25mm i.d., 0.25 µm film thickness). Oven temperature settings were the same as those described for GC-FID analysis, with He (ca. 1.4 mL/min) used as carrier gas.

2.7 Chemical Analysis of Acidic and Neutral Fractions

2.7.1 Methylation

The acidic fractions ‘A’ and ‘B’ were combined. One gram of these combined fractions was dissolved in acetonitrile 11mL. To this solution 1.2g potassium carbonate was added. To the stirred solution Methyl Iodide (1.1g) was added and refluxed in 60°C. The reaction then was followed by Thin Layer Chromatography (TLC). After 3.5 hours the solution was cooled and filtered to remove the solid potassium carbonate. The solution then was evaporated under vacuum and solid acidic fractions were analysed by GC-MS.

2.7.2 GC-MS Conditions

The methylated acidic fractions of Kurdica, Mutica, Cabolica and Mastic gum obtained by the above described method were analysed by GC-MS in split
mode (20:1) 0.5 µm injection volume in a Shimadzu QP-5000 GC-MS System using a 30m BP-5 fused silica capillary column of 0.25 mm I.D. and 0.11µm film thickness. The operation conditions were as follows; injector port temperature, 280°C; GC column temperature, 180°C for 0.5min. then at 5°C/min. to 240°C then at 2 °C/min. to 300°C for 15min. and MS interpace at 300°.

2.7.3 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) was used to separate, identify, purify, and quantify compounds. As the fractions were needed as they are without any changes such as methylation. Water HPLC Micromass ZQ-2000 equipped with Mass Lynx software and Phenomenex Column C18 (100 x 4.6 mm) was used in Atmospheric Pressure Chemical Ionisation (APCI) mode to collect different components of the acidic fractions for chemical structure determination and further analysis by Nuclear Magnetic Resonance NMR spectroscopy/Crystallography and biological assays of individual components. This method is used to separate the polar compounds that are not too labile.
2.7.4 HPLC Conditions

Instrument Parameters

Polarity ESCi+

<table>
<thead>
<tr>
<th>Calibration</th>
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<tr>
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<tr>
<td>Corona (uA)</td>
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<td>APCI Cone (V)</td>
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<td>Extractor (V)</td>
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<td>Cone Gas Flow (L/Hr)</td>
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<tr>
<td>Desolvation Gas Flow (L/Hr)</td>
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<td>360</td>
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<tr>
<td>LM 1 Resolution</td>
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<td></td>
</tr>
<tr>
<td>HM 1 Resolution</td>
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<td></td>
</tr>
<tr>
<td>Ion Energy 1</td>
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<tr>
<td>Multiplier (V)</td>
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</tr>
</tbody>
</table>

Experimental Record

------------------------------------------- Run method parameters -------------------------------------------

Waters 600 HPLC Pump Initial Conditions

| A%   | 28   | (H₂O) |
| B%   | 0    | (CH₃CN) |
| C%   | 70   | (MeOH) |
| D%   | 2    | (0.14576100mic) |
| Flow (ml/min) | 1.000 |
| Stop Time (mins) | 25.0 |
High Pressure (psi) 2500
Low Pressure (psi) 0
Set Temperature (° C) 0
Temperature Limit (° C) 25

Waters600 HPLC Pump Gradient Timetable

The gradient Timetable contains 2 eateries which are:

<table>
<thead>
<tr>
<th>Time</th>
<th>A%</th>
<th>B%</th>
<th>C%</th>
<th>D%</th>
<th>Curve</th>
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</thead>
<tbody>
<tr>
<td>0.00</td>
<td>28</td>
<td>0</td>
<td>70</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>10.00</td>
<td>0</td>
<td>0</td>
<td>98</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

Waters600 HPLC Pump External Event Timetable
No Entries in the Pump External Event Timetable.
2.7.5 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is the study of the interaction of electromagnetic radiation with matter. This phenomenon is used to determine the chemical structure of the compounds. NMR spectroscopy one/two-dimensional techniques were used to determine chemical structures of polymeric and acidic fractions.

2.8 Mastication

A 2.00 g sample of the resin fraction of gums was Masticated for 4 h, then washed with distilled water and dried in a desiccator (2.34 g). One portion (50 mg) of the dried Masticated resin was then dissolved in methanol (2 ml) and a level of oxidation confirmed by adding a few drops of the solution to 1 ml of 2,4-dinitrophenylhydrazine (2,4-DNP) reagent. A similar test was carried out on unMasticated resin.

2.9 Oxidation of High Molecular Weight Fraction (HMWF) (Polymeric Fraction)

A sample of the HMWF sub-fraction (50 mg) was dissolved in dichloromethane (2 ml) and tested for the presence of carbonyl groups by adding a few drops of the solution to 1 ml of 2,4-DNP reagent. Finely powdered HMWF (1.00 g) was suspended in 50 ml of distilled water and analytical grade air bubbled through the suspension for 24 h. The solid was filtered off and allowed to air dry (1.11 g). The aerated, dried HMWF (50 mg) was then dissolved in dichloromethane (2 ml) and oxidation confirmed...
by adding a few drops of the solution to 1 ml of 2,4-DNP reagent. Same procedure was carried out to oxidise synthetic poly myrcene.

2.10 Purification of the synthetic polymer (Dialysis)

Dialysis Tubing Cellulose Membrane (DTCM) (10 mm) D9277 was purchased from Sigma-Aldrich Sydney Australia. DTCM D9277 retains >90% of polymer (M.W. 12,400) in solution over a 10-hour period.

Glycerol that was included as a humectant was removed by washing the tube in running water for 4 hours then it was placed in a 0.3% (w/v) solution of sodium sulfide at 80ºC for 1 minute to remove sulfur compounds. The tube was then washed for 2 min in hot water 60º C, followed by acidification with a 0.2% (v/v) solution of sulfuric acid. It was then rinsed with hot water to remove the acid. This tube retains most polymer of molecular weight 12,000 or greater.

2.11 Bacteria and Culturing Conditions

2.11.1 Helicobacter pylori

The following strains of Helicobacter pylori (H. pylori) were obtained from the culture collection of the University of New South Wales.
Table 2-1 The strains of \textit{H. pylori} obtained from University of New South Wales (UNSW)

<table>
<thead>
<tr>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Helicobacter pylori} 26695</td>
</tr>
<tr>
<td>\textit{H. pylori} J99 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} RSB6 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} P10 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} SS1 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} SS2000 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} N6 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} NCTC11637 37° C in CO2 incubator</td>
</tr>
<tr>
<td>\textit{H. pylori} RU1 37° C in CO2 incubator</td>
</tr>
</tbody>
</table>

2.11.2 Agar Plates

\textit{H. pylori} were cultured routinely on \textit{Campylobacter} selective agar (CSA) (A2.4.1) 36-48 h in a Stericult incubator (Forma Scientific, USA) at 37°C with 95% relative humidity and 10% CO₂ to reduce oxygen and provide microaerophillic environment. All the strains were cultured from Cryo-preserved stocks and did not passage a maximum 5 times to avoid any major genetic mutations resulted from excessive passaging. Cultures purity was confirmed by phase contrast microscopy and additions of urease reagent (A2.5.9) and hydrogen peroxide for rapid positive urease and catalase activities respectively.

2.11.3 Liquid Cultures

The strains of \textit{H. pylori} were cultured in Brain Heart Infusion broth, supplemented with horse serum (A2.4.5) for 36-48 h in 3 L anaerobic jar with constant shaking of 70/min. All inoculations were carried out aseptically in
a laminar flow cabinet to avoid cross contaminations. Cultures purity was confirmed once again by phase contrast microscopy, urease, hydrogen peroxide test and Gram staining.

2.11.4 Gram-negative and Gram-positive bacteria

Table 2-2 Gram-positive and Gram-negative bacteria obtained from UNSW

<table>
<thead>
<tr>
<th>Gram-negative bacteria</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> type 1</td>
<td>UNSW 048200, 37°C</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>UNSW 086300, 37°C</td>
</tr>
<tr>
<td><em>Serratia marscens</em></td>
<td>UNSW 052001, 37°C</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>UNSW 029101, 37°C</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>UNSW 034000, 37°C</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>UNSW 045800, 37°C</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>UNSW 036800, 30°C</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>UNSW 027800, 37°C</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>UNSW 048200, 37°C</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gram-positive bacteria</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>UNSW 052300, 37°C</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>UNSW 056201, 37°C</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>UNSW 055440, 37°C</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>UNSW 001402, 37°C</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>UNSW 030702, 30°C</td>
</tr>
<tr>
<td><em>Corynebacterium sp.</em></td>
<td>UNSW 000400, 37°C</td>
</tr>
</tbody>
</table>
2.11.5 Agar Plates

All other Gram-negative and Gram-positive bacteria were grown on Luria Bertani (A2.4.4) medium. Plates were incubated at appropriate temperature for 12-18 h in an aerobic/anaerobic environment. Agar plates were used for maintaining cultures and also as a source of inoculum for liquid cultures.

2.11.6 Liquid Culture

LB broth (A2.4.3) was used to grow all Gram-positive and Gram-negative that is listed in section 2.7.4. The cultures were grown for 12-18 h in aerobic/anaerobic environment. Liquid cultures were used to determine Minimum Inhibitory Concentration (MIC) and kill kinetics.

2.12 Cryopreservation of Bacteria

2.12.1 Helicobacter pylori

Preserved cryovial of the strains of H. pylori was prepared by harvesting the culture of bacteria on CSA plates. The bacteria was aseptically harvested with Brian Heart Infusion (BHI) (A2.4.5) containing 20% (v/v) glycerol. The harvested cultures were stored in -80°C freezer.

2.12.2 Gram-negative and Gram-positive bacteria

Two methods were used to preserve all other gram negative and gram positive bacteria. For long term preservation, an overnight culture grown in LB broth (800 µL) was mixed with 200 µL of glycerol. They then preserved at -80°C. For short term preservations (maximum of 4 weeks), bacteria were grown on LB agar plates wrapped in parafilm and stored at 4°C. In both kind of preservation, bacteria recovered overnight.
2.13 Minimum Bacterial Concentration (MBC) and Minimum Inhibitory Concentration (MIC)

2.13.1 Essential Oils

Isosensitest (Oxoid Basenstoke UK) broth was prepared containing 0.5% Tween 80 and 16% Essential Oil of Kurdica (EOK). Serial dilutions were made until the EOK concentration of 0.0625% was achieved. Dilution tubes containing 1ml of mixture were stored at 4°C (6).

The inoculum was prepared with Isosensitest broth from 36 h cultures grown on CSA, using *H. pylori* strains 26695, J99, RSB6, P10, SS1, SS2000, N6, NCTC11637 and RU1 diluted to an optical density (OD) of 0.1 (0.5 McFaellan standard) at $A_{600}$ with Isosensitest broth containing 0.5% Tween-80. Each culture was incubated for 2 h to allow recovery of the bacteria. The serial dilutions of EOK were then inoculated with 1ml of diluted culture and incubated using tissue culture flask at 37°C in a CO$_2$ incubator for 48h. CSA plates were then inoculated with samples from the dilutions using a spiral platter and incubated in an atmosphere of 10% CO$_2$ in air, 95% relative humidity at 37°C to determine colony forming units (cfu).

Control cultures were prepared in the same manner without adding EOK. The MBC was defined as the concentration of EOK that killed the entire inoculum and was equivalent to the MIC.

The same procedure was carried out to determine the MIC of the Essential Oil of Mutica (EOM) and the Essential Oil of Cabolica (EOC).
2.13.2 The crude products and all other fractions and isolates

The MIC and MBC were determined using a broth micro-dilution method. The strains of *H. pylori* (Table 2.1) and all other Gram-positive and Gram-negative bacteria listed (Table 2.2) were used to determine the MBC and MIC values for the crude gums and all other fractions and isolate compounds. Cells were harvested from CSA blood agar (Section) and adjusted to approximately 6 to 7 log CFU/mL in Isosensitest broth with 5% horse serum. Prior to inoculating the cultures, the various test compounds were diluted into sterile culture broth. The diluted test compounds then serially diluted in microtitre wells, giving a final volume of 50 µL in each well. All the test compounds were performed in quadruplicate. The microtitre plates containing the diluted test compounds were preheated for 1 h at 37°C before inoculating 50 µL of the previously prepared cultures into each well, giving a final volume of 100 µL. After incubating the plates in a microaerobic atmosphere for 36 h, two small portions of (~5 µL) were taken from each well; one portion was dispensed onto CSA blood agar to determine the MIC. The other portion was diluted with sterile broth culture to neutralise the effect of the test compounds followed by dispensing onto CSA blood agar for MBC determination. The agar plates were again incubated for up to 72 h. Controls supplemented only with appropriate solvents without test compounds were also performed.

2.14 Kill Kinetics

2.14.1 *H. pylory*, strain 26695

Time-kill kinetic experiments were performed with static liquid cultures containing Isosensitest broth (Oxoid) supplemented with 5 % horse serum (Oxoid). The inoculum was harvested with Isosensitest broth from 36 h
cultures grown on Campylobacter Selective Agar (CSA). To ensure that the strain was at the same phase of growth, the OD600 of the inoculum was adjusted to 0.1 and diluted in the culture medium to give a starting concentration of $1.00 \times 1.00 \times 10^7$ at 600 nm. The H. pylori strain 26695 was chosen for all kill kinetics study. Each culture was incubated for 2 h to allow recovery of the bacteria before the gums and their fractions were added at their respective MIC and 5X MIC concentrations. Control cultures at MIC and 5XMIC containing ethanol/sodium carbonate solution (5%) or sodium hydroxide 0.5 N were also performed. Samples were taken from 0 to 24 h at 5 or 30 min intervals depending on the fraction/s, diluted with 0.9 % saline, plated on CSA using a spiral platter and incubated at 37°C in a CO₂ incubator for 72 h to determine viable colonies.

2.14.2 Gram-negative and Gram-positive bacteria

A 100 ml Isosensitest broth (Oxoid) culture was inoculated with a 10% inoculum from an 18 h overnight E. coli type 1 culture. The culture was allowed to grow to stationary phase. The inoculums were then adjusted with the culture medium to give a starting concentration of $1.00 \times 1.00 \times 10^7$. It was then divided into twenty equal 5 ml portions (10 control and 10 test) and the appropriate dilutions were made to determine total cell No. To each test tube Kurdica gum or one of its fraction (table 4-5) was added to a final concentration equal to MIC or 5 X MIC. To the control tubes ethanol, sodium carbonate solution 5% or sodium hydroxide 0.5 N were added depending on the fraction. All the tubes were incubated at 37°C with shaking 150 rev/min and at intervals, dilutions were made with 0.9 % saline, plated on Luria Broth (Oxoid UK) (LB) agar plates using a spiral platter and incubated at 37°C in an incubator for 48 h to determine viable colonies for both controls and tests.

For all other gram negative and positive bacteria same procedure was repeated and incubated at appropriate temperature listed in table 4-2.
2.14.3 Analysis of Variance (ANOVA)

Statistic analysis was performed on data as appropriate. Students t-test and Analysis of Variance (ANOVA) were used as appropriate. The methods used were adapted (Hibbert & Gooding, 2006) to statically analyse the behavior of the synthetic and natural antimicrobial substances in kill kinetics.
2.15 Transmission Electron Microscopy

Micrographs were taken by Transmission Electron Microscopy (TEM) at the Electron Microscope Unit at the University of New South Wales (UNSW). Samples were taken from kill kinetic in 5 min interval to visualize changes in cell morphology after exposure to the fractions and isolated components.

Bacterial culture aliquots (1 mL) were taken and centrifuged at 16000 x g for 1 min at room temperature. They were then re-suspended in PBS and washed 3 times with centrifugation to pellet the cells. After the final wash the pellet were fixed in 100 µL of fixing solution [2.5% (w/v) glutaraldehyde, 0.1 M sodium cacodylate-HCl solution pH 7.4] for 3 h.

The samples were centrifuged again and the pellet were washed several times with 100 µL of cacodylate buffer (1 M sodium cacodylate-HCl) until glutaraldehyde was totally removed from the cell pellet. The pellets were incubated for 1 h with 50 µL of osmium fixation solution (2% osmium tetroxide in 0.1 M cacodylate buffer solution) and then were left overnight in cacodylate buffer.

The fixed pellets were centrifuged and the osmium fixative was removed. They were then rinsed several times with 100 µL of 2% (w/v) sodium acetate to reduce the precipitating of the buffer by the post fixing process with uranyl acetate. The sodium acetate solution was then removed and the pellet incubated for 1 h in 2% uranyl acetate.

To remove water, the pellet were washed with a series of 15 min ethanol washes as follows; 50% ethanol plus 0.1% NaCl, 70% ethanol, 95% ethanol
and 100% ethanol then 30 min wash with 100% ethanol, they were then placed in dry acetone twice, each time 15 min.

After removing the acetone from bacterial pellet, they were infiltrated in epoxy resin using the following procedure; 1:1 (v/v) acetone/resin mix for 1 h then 1:9 (v/v) acetones/resin mix overnight, in both cases, they were kept in room temperature. It was then incubated for 30 min at 60° C, the resin mix was then removed and fresh resin was added to the bacterial cell, it was then cured at 60° C for 48 h in polypropylene tubes.

After 48 h the embedded samples were taken out from their polypropylene tube and were shaped manually using magnetic glass and scalpel. They were then cut using Reichert Ultracut E Ultramicrotome with a glass knife to an approximately 100 nm thicknesses slices. The sliced samples were flattened with chloroform, and they were placed on a copper grid (micron 200) and dried on a filter paper.

The samples were stained using the following procedure; 2% uranyl acetate in 50% ethanol was added to the sliced sample that was on the cooper grid for 10 min, then 0.4% (w/v) lead citrate plus 1 M NaOH solution were added to them for 2 min. They were dried on the filter paper prior to examination by TEM. The samples were examined and photographed using the Hitachi 7000 TEM with charged coupled device digital imaging.

### 2.16 Ribosome Purification

Ribosome extraction is a meticulous process and ribosomes are very susceptible to nucleases. High levels of cleanliness was necessary to preserve the material and gloves were always worn while handling ribosomes and during the entire preparation procedure as contamination from the skin
is a common problem in extracting ribosomes. All of the glassware and tubes were sterile including centrifugation tubes. The glassware was never been touched with bare hands. As RNases (as opposed to DNases) are very robust, and can survive autoclaving, so it was extremely important to avoid contamination. All the buffers were sterile, and DEPC-treated water was used to inactivate nucleases.

The ribosome were first purified to test the compounds as it would be difficult to tell from SDS-PAGE whether the EF-G band is derived from ribosome-bound or free EF-G.

To wash any bound factors off the ribosomes (including EF-G) that may mask the effect of the compounds 1 molar ammonium chloride was used in the sucrose cushion.

Also to see the amounts of EF-G that was pelleted with the ribosomes (and is therefore presumably ribosome-bound) the pellet was re-suspended and run on a gel.

One portion of S30 extract (the supernatant from step 6 of the protocol, which contains ribosomes and EF-G) was incubated with the compounds for some time prior to making S100 extract and one portion was incubated with Fusidic acid with the same manner.

This allowed the Fusidic acid to stabilise EF-G on the ribosome and the test compounds (if this was in fact what it does with these compounds).

The S30 extract was then centrifuged to make S100 (the first part of step 7 of the protocol). The ribosome pellet was then re-suspended and run on a gel to observe differences in the amount of the EF-G in the pellet between
extracts that was incubated with the test compounds versus extracts that were not exposed to the test compounds. Same procedure was performed for Fusidic acid as a reference.

2.16.1 Preparation of Ribosome from *Escherichia coli*

1. Using a single colony of *E. coli*, a 20-ml overnight culture of pre-warmed LB medium in a 125-ml flask was inoculated. It was then incubated overnight at 37°C.

2. 2 x 1l of pre-warmed LB medium were inoculated with 10 ml of overnight culture and incubated with shaking at 37°C to an OD$_{600}$ = 0.6-0.8.

3. They were chilled on ice for 10 min, and then harvested by centrifuging in a GS3 rotor at 5000 rpm for 10 min at 4°C.

4. The cell pellet was re-suspended in a total of 250 ml H$^{10}$M$^{10}$A$^{1000}$β$^{5}$ [A7]. It was then pelleted in GS3 rotor at 5000 rpm for 10 min, re-suspending in 50 ml H$^{10}$M$^{10}$A$^{50}$β$^{5}$[A7] followed by pelleting in SS-34 rotor, 13000 rpm 10 min 4°C. The yield was around 2.0 g of cells.

5. The cell pellet were re-suspended in 5 ml H$^{10}$M$^{10}$A$^{50}$β$^{5}$[A7]. 25 µl of 1 mg/ml DNase I was added and pressed with one pass on high pressure through the French Press at 24 000 psi [1260 on gauge].

6. The cell debris was pelleted in 50.2 Ti rotor at 30 000 rpm, 15 min, 4°C.
7. The supernatant (S30) was layered over sucrose cushion (10 ml of 
H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{1000}β\textsuperscript{5} + 20% sucrose) [A7] It was then centrifuged in 50.2 Ti 
rotor at 45 000 rpm, 4 h, 4°C. The ribosome pellet was then rinsed 
with 1 mL H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{50}β\textsuperscript{5} [A7], removing brown membranous 
material, it was then re-suspended in 1-2 ml of H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{50}β\textsuperscript{5} [A7] and 
 microfuged for 2 min. to remove any debris.

8. The supernatant was layered onto 20 ml H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{50}β\textsuperscript{5} + 40% sucrose 
[A7] and centrifuged in 50.2 Ti rotor at 30000 rpm, 14 h, 4°C (or 
equivalent \(\omega^2t\)).

9. The ribosome pellets was re-suspended in 250 µl H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{50}β\textsuperscript{5} [A7]. 
The debris was pelleted in microfuge, A\textsubscript{260} was taken (a dilution of 
1:1000 was made for measuring the A\textsubscript{260}), it was then aliquot and 
frozen. 
A\textsubscript{260} reading was multiplied by dilution factor and then divided by 
14.5 to give µg/µl, and then divided by 2.7 to give pmol/µl.
2.17 SDS-PAGE

Detecting complex mixture of Elongation Factor G (EF-G)~GDP~ribosome~Fusidic acid/isolated compounds was carried out using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE). Precast Polyacrylamide Mini Gels (12%) were purchased from iGELS. Sample buffer (A7) was added to incubated extract with the test compounds/Fusidic acid and heated in boiling water for 5 min to denature proteins. The SDS in the sample buffer gave the denatured protein a net negative charge allowing the proteins move downward where the positive electrode was situated. The denatured proteins were loaded into the wells of the stacking gels. A 20 mA Direct Current (DC) was passed through the gels stacked then the amperage was increased to 40 mA till the dye front migrated a short distance from the bottom of the gel toward the positive electrode.

2.17.1 Staining and Destaining of SDS-PAGE Gels

The gels were then placed in Coomassie brilliant solution (A2.5.7) over night with agitation. They were then destained in “destain” (A2.5.8) with agitation and a 30 min frequent changes, followed by washing in Milli-Q water. The gels were then placed in clear plastic bag and they were sealed and photographed.
Chapter 3
GC/MS Analysis and Antimicrobial activity of the Essential Oils

3.1 Introduction

Essential oil is an aromatic volatile substance derived from The French word of essence. Essential oil is extracted from plant by conventional method of steam distillation, however, some other methods may be employed such as cold press expression and solvent extraction, in which the latest one mainly is used in laboratory. Essential oils are used as a flavor and fragrance in food, detergents, toiletry, and perfumes (Brophy & Doran, 1996).

Essential oil is obtained from flowers such as rose and jasmine, from leaves such as eucalyptus, fruits, bark exudates, wood and root. Essential oils are widely used in food, including confectionary, edible oils, backed products, meat, pickle and caned vegetable (Brophy & Doran, 1996). They are used as antioxidant in edible oils.

Essential oils of *Pistacia lentiscus* (*P. lentiscus*), *Commiphora myrrh* (*C. myrrh*), and *Boswellia serrata* (*B. serrata*) resins have shown strong antioxidant activity in sunflower oil. *Pistacia lentiscus* and *B. serrata* showed good antioxidant activity in lard, and *P. lentiscus* resin and its essential oil have demonstrated high antioxidant activity in virgin olive oil (Assimopoulou *et al.*, 2005).

The essential oils of oregano, rosemary and garlic were incorporated with whey protein isolate films 1.0-4.0% (wt/vol) and used as an antimicrobial food packaging. Their antimicrobial activities evaluated against *Escherichia coli* O157:H7 (ATCC 35218), *Staphylococcus aureus* (ATCC 43300), *Salmonella enteritidis* (ATCC 13076), *Listeria monocytogenes* (NCTC 2167) and *Lactobacillus*
plantarum (DSM 20174). The film containing oregano essential oil demonstrated more activity than those containing garlic and rosemary (Seydim & Sarikus, 2006).

The essential oils of the aerial parts of two species of genus Pimpinella; Pimpinella anisatum, Pimpinella flabellifolia were analysed by GC and GC/MS. The major components of P. anisatum were (E)-anethole (82.8%) and methyl chavicol (14.5%), and the major constituents of P. flabellifolia were limonene (47.0%), (E)-anethole (37.9%) and α-pinene (6.0%). They were screened for antioxidant activity. P. anisatum demonstrated greater antioxidant activity than that of P. flabellifolia oil. They were also screened for antimicrobial and antifungal activities. These oils showed moderate activities against all microorganisms tested (Tepe et al, 2006).

The essential oils of Satureja subspicata was collected by hydrodistillation and analyzed by GC-MS in Dalmatia (Croatia). The oil constitutes carvacrol (16.76%), α-pinene (13.58), p-cymene (10.76%), [gamma]-terpinene (9.54%) and thymol methyl ether (8.83%) as the main components. The oils also contained myrcene, linalool, β-caryophyllene, limonene, geranyl acetate, 1-Octen-3-ol, nerol, thymol and borneol. The oils were screened against 13 bacteria and 9 fungal strains and showed that the oils had a great antimicrobial and antifungal activity against all the microorganisms ranging from 0.09 to 6.25 µL/mL (Skocibusic et al, 2006).

Essential oils of Thymbra capitata (Thymus capitatus) collected from Southern Apulia (Italy) were analysed by GC and GC-MS. This oil constitutes of 75 components in which the major components were thymol and carvacrol, constituted more than 50% of the oils, as well as [gamma]-terpinene, borneol and p-cymene (Miceli et al, 2006).
The essential oils of *Cinnamomum cassia*, *Origanum compactum*, *Origanum heracleoticum*, *Satureja hortensis*, *Satureja montana*, *Thymus vulgaris carvacroliferum*, and *Thymus vulgaris thymoliferum* demonstrated strong antimicrobial activity against *Pseudomonas putida* a strain of meat origin, associated with meat spoilage with MIC of 0.05 (wt/vol).

Ten other essential oils of *Cinnamomum verum* (from leaf and bark), *Eugenia caryophyllus*, *Cymbopogon martini* var. *motia*, *Cymbopogon nardus*, *Melaleuca linariifolia*, *Origanum majorana*, *Pimenta dioica*, *Thymus satureoides*, and *Thymus serpyllum* were screened against the same microorganism and showed high antimicrobial activities with MIC ranging from 0.1% to 0.4% (Oussalah *et al*., 2006).

The essential oils of *Thymus eriocalyx* and *Thymus x-porlock* were analyzed by GC and GC/MS and studied for their antifungal activities with special reference to the mechanism of inhibition of *Aspergillus niger* growth at ultrastructural level. In this study the MIC and Minimal Fungicidal Concentrations (MFC), and fungicidal kinetics of these oils were determined. Transmission electron microscopy (TEM) of *A. niger* that was exposed to MIC levels showed that the essential oils damaged the cell wall, cell membrane and cellular organelles (Rasooli *et al*., 2006).

The essential oils are used as pesticides as well. Nineteen essential oils, from different plants belonging to the family *Labiatae* of Moroccan origin, were screened for their insecticidal activity against Hessian fly (*Cecidomyiidae*) adults and eggs that is the major pest of wheat in Morocco. The essential oils from species *Mentha pulegium*, *Origanum compactum*, and *Origanum majorana* were mostly active against adults, and the oils from species *Ammi visnaga*, *Pistacia lentiscus*, *O. compactum*, and *M. pulegium* were more active against eggs (Lamiri *et al*, 2001).

They are also used as pharmaceutical ingredients, dental products, and in aromatherapy and phytotherapy (Brophy & Doran, 1996).

Chemical analysis of the essential oils from different parts of the plant *Pistacia* genus has been carried out by a number of researchers. The essential oils of leaf, branch and fruit of *Pistacia lentiscus* from Tuscany (Italy), was analysed and identified by GC and GC-MS. The major components of the oils from the leaf were α-pinene (16.1-25.3%), limonene (6.6-12.3%), terpinen-4-ol (7.6-12.7%) and germacrene (9.6-14.3%). The components branch oil were; α-pinene (34.4-46.2%), myrcene (6.3-11.6%) and limonene (8.1-13.0%), while the essential oils of the fruit contained α-pinene (7.5-11.2%), myrcene (68.2-71.0%) and limonene (9.6-19.7%) (Veriano *et al.*, 2004).

Essential oils, particularly essential oil of tea tree oil have been used for topological infection (external use) and also as antiseptic and surface disinfectant. Tea tree oil has been screened against a wide range of medically important micro-organism (Belaiche *et al.*, 1996; Carson & Riely, 1993; Carson *et al.*, 1995)
The chemical composition of the essential oil obtained by hydrodistillation of *Pistacia vera* L. gum was analyzed using Gas Chromatography-Mass Spectrometry (GC-MS), α-Pinene, β-pinene, and α-thujene were found to be the major constituents. This oil has shown bacteriostatic activity against 12 clinical isolates of *H. pylori*. The MIC was determined to be 1.55 mg/ml for all isolates (Ramezani *et al.*, 2004).

The essential oils of Mastic gum has demonstrated activity against Gram-positive and Gram-negative bacteria (Tassou & Nychas, 1995).

Lipophilic monoterpenes such as α-pinene, β-pinene, limonene and β-myrcene are the major constituents of the essential oil of Mastic gum. As the major components of Kurdica, Mutica and Cabolica are also lipophilic such as α-pinene, β-pinene, they are expected to have similar effect on microorganisms. Therefore it was essential to isolate the essential oils of Mastic, Kurdica, Mutica and Cabolica gum and to determine the activity of the other fractions of the gum and whether they are also participative in antimicrobial activities.

Chemical analysis of the essential oils was carried out by GC/MS as it has been described (Papageorgiou *et al.*, 1981) to allow the comparison between essential oils of Mastic gum and Kurdica, Cabolica and Mutica gum. These analyses led to further work on α-pinene, the major constituent of the Kurdica, Mutica and Cabolica gum.

The objective of this project was not that to determine the antimicrobial activity of the essential oils of these gums but to screen all the fractions of the gums including essential oils and its oxidised form against bacteria particularly *H. pylori* listed in Table 2.1.
3.2 Experimental

3.2.1 Steam Distillation

The isolation of the oils from the raw gum was achieved by hydro distillation in a Dean and Stark apparatus, which was modified to give lower phase return of the water. The collected essential oils from the gums were stored at -20˚C prior to analysis as described in details (2.3).

3.2.2 Analysis of Essential oils

Gas Chromatography-Flame Ionisation Detector (GC-FID) analysis was performed on a Varian 3700 gas chromatograph coupled to a Shimadzu C-R3A integrator and fitted with a fused silica capillary column (25QC/BP5) obtained from SGE, Australia (25m x 0.25mm i.d., 0.25 μm film thickness) as described in details (2.6).

3.2.3 Oxidation of Pure α-pinene

Pure α-pinene (20 mL) (Appendix A3) was exposed to areal oxidation for 24 h. This procedure was carried out using analytical grade oxygen that was bubbled through α-pinene. Oxidation was confirmed by comparing IR spectra of α-pinene and its oxidised after areal oxidation (Spectra 3.6-3.7).
3.2.4 Antimicrobial Screening of Essential Oils

3.2.4.1 Minimum Bacterial Concentration (MBC), Minimum Inhibitory Concentration (MIC) and Kill kinetics

Isosensitest (Oxoid Basenstoke UK) broth (Appendix A3) was prepared containing 0.5% Tween 80 and 16% Essential oils of Kurdica (EOK), Essential Oils of Mutica (EOM) and Essential Oils of Cabolica (EOC). Appropriate serial dilutions were made until a concentration of 0.0625% of the essential oils was achieved. Dilution tubes containing 1ml of mixture were stored at 4°C as described (2.13.1). *H. pylori* strains listed in Table 2.1 Chapter 2 were grown on *Campylobacter* Selective Agar (CSA) (Appendix A3) used. Then the MIC and the kill kinetics of these essential oils were determined to obtain more data and to investigate the antibacterial activity of these gums particularly against *H. pylori*.

3.3 Results

3.3.1 Chemical Identification

Identification of the oil components was carried out by a comparison of mass spectra with literature data (NIST, NISTREP) and also by a comparison of their retention indices (RI) with those of authentic compounds or with those in the literature (Adams, 1995). Table 3.1-3.3 show the identified compounds of the EOK, EOK and EOC respectively in the order of elution time on the capillary column used for the GC-MS analysis, as previously described (2.6)
Table 3-1: The composition of essential oil of Kurdica gum in the order of elution time

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compounds</th>
<th>RA(^a) (%)</th>
<th>RI(^b) (Exp)</th>
<th>RI(^c) (lit)</th>
<th>MWt</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Thujene</td>
<td>0.07</td>
<td>920</td>
<td>931</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>α-Pinene</td>
<td>97.18</td>
<td>935</td>
<td>939</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Camphene</td>
<td>0.41</td>
<td>946</td>
<td>953</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Sabinene</td>
<td>0.16</td>
<td>972</td>
<td>976</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>5</td>
<td>β-Pinene</td>
<td>1.26</td>
<td>975</td>
<td>980</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>Δ3-Carene</td>
<td>0.11</td>
<td>1010</td>
<td>1011</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>7</td>
<td>Limonene</td>
<td>0.06</td>
<td>1089</td>
<td>1088</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td></td>
<td></td>
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</table>

Table 3-2: The composition of essential oil of Mutica gum in the order of elution time

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Compounds</th>
<th>RA(^a) (%)</th>
<th>RI(^b) (Exp)</th>
<th>RI(^c) (lit)</th>
<th>MWt</th>
<th>Identification</th>
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<tbody>
<tr>
<td>1</td>
<td>α-Thujene</td>
<td>3.64</td>
<td>920</td>
<td>931</td>
<td>136</td>
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<td>2</td>
<td>α-Pinene</td>
<td>85.74</td>
<td>935</td>
<td>939</td>
<td>136</td>
<td>1,2</td>
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<tr>
<td>3</td>
<td>Camphene</td>
<td>0.67</td>
<td>946</td>
<td>953</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>β-Phellandrene</td>
<td>1.86</td>
<td>972</td>
<td>976</td>
<td>136</td>
<td>1,2</td>
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<tr>
<td>5</td>
<td>β-Pinene</td>
<td>4.83</td>
<td>975</td>
<td>980</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>β-Myrcene</td>
<td>0.83</td>
<td>991</td>
<td>991</td>
<td>136</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Δ3-Carene</td>
<td>0.40</td>
<td>1010</td>
<td>1011</td>
<td>136</td>
<td>1,2</td>
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<td>8</td>
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<td></td>
<td>136.1</td>
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<td>9</td>
<td>(+)-2-Carene</td>
<td>1.02</td>
<td></td>
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<td>136</td>
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<td>10</td>
<td>1,2,8-p-Menthatriene</td>
<td>0.73</td>
<td></td>
<td></td>
<td>134</td>
<td>1,2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
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</tbody>
</table>
Table 3-3 The composition of essential oil of Cabolica gum in the order of elution time

<table>
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<tr>
<th>Peak No.</th>
<th>Compounds</th>
<th>RA(^a) (%)</th>
<th>RI(^b) (Exp)</th>
<th>RI(^c) (lit)</th>
<th>MWt</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-Thujene</td>
<td>3.46</td>
<td>920</td>
<td>931</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>α-Pinene</td>
<td>87.1</td>
<td>935</td>
<td>939</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>3</td>
<td>Camphene</td>
<td>0.79</td>
<td>946</td>
<td>953</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Sabinene</td>
<td>1.59</td>
<td>972</td>
<td>976</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>5</td>
<td>β-Pinene</td>
<td>4.66</td>
<td>975</td>
<td>980</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>β-Myrcene</td>
<td>0.77</td>
<td>991</td>
<td>991</td>
<td>136</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>Δ3-Carene</td>
<td>0.32</td>
<td>1010</td>
<td>1011</td>
<td>136</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>Limonene</td>
<td>0.90</td>
<td>1028</td>
<td>1031</td>
<td>136</td>
<td>1,2</td>
</tr>
<tr>
<td>9</td>
<td>Terpinolene</td>
<td>0.42</td>
<td>1089</td>
<td>1088</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) RA; relative area (raw peak area relative to total peak area).

\(^b\) RI (Exp); programmed temperature retention indices as determined on BP-5 column using a homologous series of \(n\)-alkanes (Cs-C\(_{30}\)) as internal standard and H\(_2\) as carrier gas.

\(^c\) RI (lit); values from literature data using He as carrier gas, with T denoting programmed temperature values.

MWt: molecular weight; values with subscript “d” confirmed from GC-MS (Cl) data.

\(^e\) 1; based on retention index, 2; based on comparison of mass spectra with literature data (NIST, NISTREP) or authentic sample, 3; retention time identical to authentic compound.

Typical chromatograms of the essential oils of *P. a. Kurdica, P. a. Mutica* and *P. a. Cabolica* are shown in (Spectra 3-1-3-3). The prominent component 97.2% is α-pinene in Kurdica, 85.74% in Mutica and 87.1% in Cabolica that show a unique characteristic for sub-species of *P. atlantica*
Spectrum 3-1 Typical chromatogram of the essential oil of Kurdica gum

Spectrum 3-2 Typical chromatogram of the essential oil of Mutica gum
Spectrum 3-3 Typical chromatogram of the essential oil of caboilca gum
3.3.2 Oxidation of Pure $\alpha$-pinene

Spectra 3.4 and 3.5 showed the IR spectra that were taken before and after areal oxidation of $\alpha$-pinene.

Spectrum 3-4 IR Spectrum of alpha pinene

Spectrum 3-5 IR Spectrum of oxidised alpha pinene
3.3.3 MIC and Kill Kinetics

The MIC values obtained were dependent upon the *H. pylori* strain used. Table 3-1 shows the MIC results for EOK, EOM and EOC.

EOK killed all *H. pylori* strains within 8-9.5 hours depending on the strain at their respective MIC concentration while the 5XMIC killed all the *H. pylori* strains in 2 hours at their 5XMIC concentration. EOM killed the entire strains within 9.5-10 hours depends on the strains in their respective MIC and finally EOC killed all *H. pylori* strains within 7.5-8 hours in their respective MIC. As observed with EOK, all *H. pylori* strains were killed by EOM and EOC at their respective 5XMIC concentration.

The pure α-pinene completely killed *H. pylori* 26695 at its respective MIC of 1000µg/ml within 12h while the oxidised α-pinene killed all *H. pylori* within 3h. (Fig. 3-1).
<table>
<thead>
<tr>
<th>No.</th>
<th>H. pylori strains</th>
<th>MIC EOK µg/ml</th>
<th>MIC EOM µg/ml</th>
<th>MIC EOC µg/ml</th>
<th>MIC EOMa µg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>26695</td>
<td>1000</td>
<td>1000</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>2</td>
<td>J99</td>
<td>1000</td>
<td>1000</td>
<td>500</td>
<td>500</td>
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<tr>
<td>3</td>
<td>RSB6</td>
<td>1000</td>
<td>1000</td>
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<td>500</td>
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<td>7</td>
<td>N6</td>
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<td>1000</td>
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<td>8</td>
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</tr>
</tbody>
</table>
Figure - 3-1 Kill Kinetics of alpha-pinene and its oxidised against *H. pylori* 26695

![Graph showing kill kinetics](image)

H. pylori 26695

---

**Control** - **Alpha-pinene MIC** - **Oxidised alpha-pinene MIC**

---

Figure -3-1 Kill Kinetics of alpha-pinene and its oxidised against *H. pylori* 26695
3.4 Discussion

Table 3-4 (A, B and C) shows the composition of the essential oils of *Pistacia lentiscus* from the literature (Adams, 1995; Castola *et al*., 2000; Duru *et al*., 2003; Mgiatis *et al*., 1999; Papageorgiou *et al*., 1981; Vassios, 1991) in comparison to the results of this study with *P. a. Kurdica*, *P. a. Mutica* and *P. a. Cabolica*.

The major constituent of the species of *P. lentiscus* and *P. atlantica*, is α-pinene ranging from 21.70 to 78.90 for *P. lentiscus* and 85.74-97.20 for *P. a. Kurdica*, *P. a. Mutica* and *P. a. Cabolica* that gives a unique characteristic to this species. β-Pinene also has been reported in all analytical data from the literature and from these results, ranging 1.26-38.70. β-Myrcene is present in all reported data and this data ranging from 0.10-12.27 but Kurdica and Turkish sub-species of *atlantica* (Duru *et al*., 2003). Limonene is reported in literature for *P. lentiscus*, ranging from 0.95-11.52; however, has not been found in this analysis for *P. a. Mutica*. Some of the reported data also are not reliable as in some report (Adams, 1995; Castola *et al*., 2000; Duru *et al*., 2003; Mgiatis *et al*., 1999; Papageorgiou *et al*., 1981; Vassios, 1991) 1.7-10% of the total composition of the essential oils is not known.

EOK, EOM and EOC have some antimicrobial activity against Gram-positive and Gram-negative bacteria. The highest level of activity was demonstrated by EOC then EOK. Considering the composition of both EOC and EOK having oxygenated monoterpenes in comparison to EOM that has no oxygenated monoterpenes at all suggests that oxidation or oxygenated components plays a big role in antimicrobial activity.

As the major components of EOC, EOK and EOM are α-pinene so pure α-pinene and oxidised α-pinene was chosen to determine MIC and kill kinetics of *H. pylori* 26695 for comparison. The MIC value of oxidised α-pinene (500µg/ml) was the half of the MIC of pure α-pinene (1000µg/ml). The pure α-pinene killed the entire of *H. pylori* 26695 at its respective MIC of 1000µg/ml within 12h while the oxidised α-pinene completely killed *H. pylori* within 3h. (Fig. 3-1).
The IR spectra 3.5 show the presence of oxygen in comparison to spectra 3.4.

Thus the concept of a naturally occurring monoterpenes becoming more active through a process of oxidation generating reactive aldehyde or keton groups, including processes involving bacterial metabolism, is consistent with our understanding of the mechanism of action of essential oils (Mucciarelli et al, 2001; Rasooli et al, 2006).

The MIC data of these essential oils were not consistent with contemporary antibiotics and were more consistent with a topical “disinfectant” like compound and this finding is consistent with literature and it was expected (Baylac & Racine, 2003 Sokmen et al, 2004; Tepe et al, 2004). Thus while this work has provided an insight into “natural” mechanisms activating components of essential oils in Pistacia sp. Increasing antimicrobial activity, as suggested by the oxidation of $\alpha$-pinene, these compounds are more appropriately used as feed supplements to replace more potent antibiotics.

The purpose of isolating and screening of these oils in this work was to investigate the antimicrobial activity of gum extracted from Pistacia sp., with the view to seeking to identify lead compounds for novel antibiotics that may act systemically against H. pylori and other bacterial pathogens. While further work could be undertaken to further characterise the mode of the actions of the individual components of the essential oils and enhancement of their “disinfectant” properties, this would be of limited value as it was apparent that the observations were convergent with already developed fields and therefore it was decided to finalise the studies of these essential oils and their antimicrobial activities at this point and to explore other fraction/s that potentially would be more productive avenues.
Table 3-5 The composition of the essential oils of Kurdica, Mutica and Cabolica in compare to Mastic gum from literature

(A)

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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</thead>
<tbody>
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<td>Undecan-2-one</td>
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<td>0.14</td>
<td></td>
<td>0.13</td>
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<td></td>
<td>3.64</td>
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<td>0.14</td>
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<td>1.16</td>
<td>1.59</td>
</tr>
<tr>
<td>α-Thujene</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>t</td>
<td>3.64</td>
<td>3.46</td>
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<td>0.40</td>
<td>0.41</td>
<td>0.67</td>
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Chapter 4
4.1 Introductions

A common historical practice for treating stomach ulcers has been the chewing of crude Mastic gum. A practice which has spanned many centuries (Al-Habal et al., 1984; Al-Said et al., 1986; Huwez & Al-Habbal, 1986; Mills & White, 1989; Stern et al. 2003). In 1982 a late Bronze Age shipwreck was discovered by archaeologists containing 100 jars filled with Mastic gum, the exudates having been obtained from the species *Pistachia lentiscus*, that had been used by the Egyptians for medicinal purposes (Hairfield & Hairfield, 1990; Mills & White, 1989). Despite these findings, and with the exception of Mastic gum, there have been few reported studies concerning the composition or biological activity of these exudates.

Mediterranean countries and Southern and Central America are the homes of the genus *Pistacia* from the *Anacardiaceae* family which consists of eleven species of trees and includes the two species *Pistacia lentiscus* and *Pistacia atlantica* (Yaltirik et al., 1967). *Pistacia atlantica Kurdica*, one of the sub-species, is a tree that grows up to 15 meters high and is widely spread around the Zagros Mountains and particularly in Western and Northern Iran, and in Eastern and Northern Iraq, an area formerly referred to as Kurdistan. The tree is an important constituent of the natural vegetation in the area and shows a discontinuous pattern of distribution over this region (Zohary, 1952).
The investigation, in this thesis, into the fractions or biological activity of the trunk bark exudate of *Pistacia atlantica* Kurdica, *Pistacia atlantica* Mutica, and *Pistacia atlantica* Cabolica (*P. a. Kurdica, P. a. Mutica* and *P. a. Cabolica*) is the first reported investigation of its kind. These resinous gums have been termed here ‘Kurdica Gum’, ‘Mutica Gum’ and ‘Cabolica Gum’.

The chemical composition of the essential oils of *P. lentiscus* and its bark exudate, Mastic gum has been the subject of several studies. The main constituents of the oils are monoterpenes (Chrysoula *et al*, 1995; Duru *et al*, 2003; Lamiri *et al*, 2001; Mans, 1991; Mgiatis *et al*, 1999; Tassou & Nylchas, 1995; Veriano *et al*, 2004). In 1956 Barton and Seoane, fractionated Mastic gum into two major fractions, an acidic and a neutral fraction (Barton & Seoane, 1956; Seoane, 1956) and an unusual polymer constituent, 1,4-poly-β-myrcene, was identified in 1998 (Van den Berg *et al*, 1998).

Antifungal activity of the aqueous extract of *P. lentiscus* has been investigated (Duru, 2003). Mastic gum has been shown to have a significant effect in relieving the symptoms and in healing of duodenal ulcers, as demonstrated by a double blind controlled clinical trial. (Al-Habal, 1984). The gum has also been evaluated for antimicrobial activity against clinical isolates of *H. pylori* and shown to have an MIC value of 125µg/ml (Marone *et al*, 2001). However, with the exception of a report indicating that the neutral fraction of Mastic gum significantly inhibited the growth of *Rhisoctonia solani* (Duru *et al*, 2003), only the whole Mastic gum has been used in these reported studies.

This chapter examines anti-*H. pylori* activity of the individual fractions of the Kurdica gum, together with that of the whole gum, against nine strains of *H. pylori* and a range of Gram-positive and Gram-negative bacteria. The resin fraction obtained from the gum before and after Mastication has been also investigated for any activity, and details of possible mode of the actions will be discussed in next Chapters.
4.2 Experimental

Mastic gum was purchased from Sigma-Aldrich (2.2.1) Kurdica (2.2.2), Mutica (2.2.3) and Cabolica (2.2.4) gums, were collected from different parts of Iran over the period of June to August 2000 by author, as described (2.2). Composite samples of the collected gums were then used in this study. The methods used to fractionate the gum have followed equivalent reported methods relating to the fractionation of Mastic gum (Barton & Seaone, 1956) in order to allow a direct comparison of the composition and activity of the four exudates gums. After a primarily MIC determination of the fractions, Thin Layer Chromatography, Mass Spectrometry and GC-Mass Spectrometry were conducted to identify and compare the active fraction/s with non-active fraction/s and to isolate the most active component/s of the active fraction/s by Column Chromatography or Liquid Chromatography (LC).

Isolation, analysis, and identification of the far most active component/s and their antimicrobial activities are discussed in Chapter 6.

The Highest Molecular Weight Fraction (HMWF), acidic fractions ‘A’ and ‘B’, neutral fraction, fraction ‘C’ and ‘D’, whole gum, Masticated, oxidised and methylated acidic fractions were screened against 9 strains of *H. pylori* and Gram-positive and Gram-negative bacteria listed in Table 2.1 and 2.2.

4.2.1 Steam Distillation

The residue of the gum (Resin) was isolated from the essential oils by steam distillation in a Dean and Stark apparatus, which was modified to give lower phase return of the water. The time of steam distillation was carried out over 5 hours (2.3)
4.2.2 Fractionations

4.2.2.1 Polymer Fraction

The finely powdered crude resin fraction (40.00 g), resulting from distillation, was dissolved in dichloromethane (500 ml) and precipitated with methanol. The precipitant that had formed was isolated after decanting and classified as ‘High Molecular Weight’ Fraction (HMWF) and characterised by Gel Permeation Chromatography (GPC) as it described (2.4.1).

4.2.2.2 Acidic and Neutral Fractions

The resulting solution after extracting essential oils and the polymer fraction was evaporated under vacuum (33.10 g) then dissolved in anhydrous diethyl ether (50 ml), diluted with anhydrous methanol (350 ml), and allowed to stand in a stoppered flask overnight. The acidic fractions of this solution were then extracted with sodium carbonate and sodium hydroxide from neutral fractions. The details of these methods have been described in Chapter 2 (2.4.2)
4.2.3 Antimicrobial Activity

4.2.3.1 Minimum Inhibitory Concentration (MIC) and Kill Kinetics

The MIC and MBC values were determined for the crude gums and all other fractions and isolated compounds listed in (Table 4-1) against 9 strains of *H. pylori* (Table 2-1) and all other Gram-positive and Gram-negative bacteria listed in table 2-2 using a broth micro-dilution method as described in Chapter 2.

The strain 26695 *H. pylori* was chosen for time-kill kinetic experiments with static liquid cultures as described in Chapter 2.
4.3 Results

4.3.1 Fractionations

The yield of essential oils obtained were 20.00 % (w/w) of the Kurdica gum, 15.00% (w/w) of Mutica gum, 13.00% (w/w) of Cabolica gum and 1.80% of Mastic gum. The yield of strongest acid fractions, from the sodium carbonate extract (fraction ‘A’), were 23.4 % (w/w) of the Kurdica gum, 18.80% (w/w) of Mutica gum, 19.20% (w/w) of Cabolica gum and 12.20% of Mastic gum. The weaker acidic fractions ‘B’ from the sodium hydroxide extract were 10.00 % (w/w) of the Kurdica gum, 12.60% (w/w) of Mutica gum, 13.80% (w/w) of Cabolica gum and 7.10% of Mastic gum. There were thus obtained a sodium hydroxide-(in) soluble acid fraction from the viscous liquid, ‘fraction B’ a second sodium hydroxide-soluble acid fraction, ‘fraction ‘C’. This fraction was 5.20 % (w/w) of the Kurdica gum, 6.60% (w/w) of Mutica gum, 7.60% (w/w) of Cabolica gum and 5.10% of Mastic gum (Table 4.1).

The oily fraction (tri-glycerides, possibly contamination during collection process) was just present in Kurdica gum and it was 1.80% (w/w). The neutral fraction represented a yield of 24.0 % (w/w) of the Kurdica gum, 25.60% (w/w) of Mutica gum, 24.60% (w/w) of Cabolica gum and 36.30% of Mastic gum. The high molecular weight fraction (HMWF) represented 13.80 % (w/w) of the Kurdica gum, 19.40% (w/w) of Mutica gum, 20.00% (w/w) of Cabolica gum and 35.20% of Mastic gum (Table 4.1).

The HMWF of the gums were analysed by Gel Permeation Chromatography (GPC) and Nuclear Magnetic Resonance (NMR) to determine tentatively the number average molecular weight and possible structure of this fraction. The obtained data was compared with literature and will be discussed in a separate chapter. However, the antimicrobial activity of this fraction is reported in this chapter.
In addition the mode of the action of HMWF, the method that enhanced the activity of this fraction, synthesising a polymer with a similar active site and their MIC and Kill kinetics will be discussed in a separate chapter.

4.3.2 Antimicrobial Activities

4.3.2.1 Minimum Inhibitory Concentration

The MIC results have been tabulated in tables 4-2-4-13 for all the fractions of the Mastic, Kurdica, Mutica and Cabolica gums, against the strains of *H. pylori* listed in table 2-1 and all other Gram-positive and Gram-negative bacteria listed in table 2-2.

4.3.2.2 Kill Kinetics

The data collected from kill kinetics were recorded and plotted out using Microsoft Excel. The analysed graphs showed biocidal activities for all the fractions but fraction ‘C’ that was biostatic.
Table 4-1 The composition of Kurdica, Mutica, Cabolica and Mastic gum

<table>
<thead>
<tr>
<th></th>
<th>Essential Oils %</th>
<th>Resin %</th>
<th>Acidic Fraction (A) %</th>
<th>Acidic Fraction (B) %</th>
<th>Acidic Fraction (C) %</th>
<th>Acidic Fraction (D) %</th>
<th>Neuteral Fraction %</th>
<th>High Molecular Weight Fraction (HMWF) %</th>
<th>Insoluble Residue %</th>
<th>Whole Gum (Total weight) %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. a. Kurdica</em></td>
<td>20.00</td>
<td>80.00</td>
<td>23.40</td>
<td>10.00</td>
<td>5.20</td>
<td>1.80</td>
<td>24.00</td>
<td>13.80</td>
<td>1.80</td>
<td>100</td>
</tr>
<tr>
<td>(Kurdica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. a. Mutica</em></td>
<td>15.00</td>
<td>85.00</td>
<td>18.80</td>
<td>12.60</td>
<td>6.60</td>
<td>0.00</td>
<td>25.60</td>
<td>19.40</td>
<td>2.00</td>
<td>100</td>
</tr>
<tr>
<td>(Mutica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. a. Cabolica</em></td>
<td>13.00</td>
<td>87.00</td>
<td>19.20</td>
<td>13.80</td>
<td>7.60</td>
<td>0.00</td>
<td>24.60</td>
<td>20.00</td>
<td>1.80</td>
<td>100</td>
</tr>
<tr>
<td>(Cabolica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. lentiscus</em></td>
<td>1.80</td>
<td>98.20</td>
<td>12.20</td>
<td>7.10</td>
<td>5.10</td>
<td>0.00</td>
<td>36.30</td>
<td>35.20</td>
<td>2.3</td>
<td>100</td>
</tr>
<tr>
<td>(Mastic gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4-2 The MIC values of Kurdica gum and its fractions against the strains of *H. pylori* (µg/mL)

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Whole Kurdica Gum</th>
<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
<th>Masticated Resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>26695</td>
<td>150</td>
<td>1000</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
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<td>250</td>
<td>50</td>
</tr>
<tr>
<td>J99</td>
<td>150</td>
<td>1000</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>125</td>
<td>50</td>
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<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
<td>P10</td>
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<td>1000</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
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<td>500</td>
<td>100</td>
<td>50</td>
<td>750</td>
<td>750</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>200</td>
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<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
<td>NCTC11637</td>
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<td>1000</td>
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<td>50</td>
<td>1000</td>
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<td>500</td>
<td>250</td>
<td>50</td>
</tr>
<tr>
<td>RU1</td>
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<td>1000</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
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</tr>
<tr>
<td>Weight (%)</td>
<td>100.00</td>
<td>20.00</td>
<td>80.00</td>
<td>23.40</td>
<td>10.00</td>
<td>5.200</td>
<td>1.80</td>
<td>24.00</td>
<td>13.80</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N/A: Non Applicable
NA: No Activity
Table 4-3 The MIC values of Kurdica gum and its fractions against Gram-negative bacteria (µg/mL)

<table>
<thead>
<tr>
<th>Gram Negative Bacteria</th>
<th>Whole Kurdic Gum</th>
<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
<th>Masticated Resin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> type 1</td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>400</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>150</td>
<td>500</td>
<td>100</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>400</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>150</td>
<td>500</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
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<td>500</td>
<td>100</td>
<td>20</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>300</td>
<td>100</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
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<td>500</td>
<td>100</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
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<td>100</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
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<td>500</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>125</td>
<td>100</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>150</td>
<td>500</td>
<td>100</td>
<td>50</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
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<td>Weight (%)</td>
<td>100.00</td>
<td>20.00</td>
<td>80.00</td>
<td>23.40</td>
<td>10.00</td>
<td>5.20</td>
<td>1.80</td>
<td>24.00</td>
<td>13.80</td>
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</table>

N/A: Non Applicable
NA: No Activity
Table 4-4 The MIC values of Kurdica gum and its fractions against Gram-positive bacteria (µg/mL)

<table>
<thead>
<tr>
<th>Gram Positive Bacteria</th>
<th>Whole Kurdica Gum</th>
<th>Essential Oils</th>
<th>Resin Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
<th>Masticated Resin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacillus cereus</td>
<td>200</td>
<td>400</td>
<td>200</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
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<td>250</td>
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<td>Staphylococcus aureus</td>
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<td>200</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>300</td>
<td>500</td>
<td>200</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
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</tr>
<tr>
<td>Staphylococcus epidermidis</td>
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<td>400</td>
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<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>500</td>
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</tr>
<tr>
<td>Bacillus subtilis</td>
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<td>500</td>
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<td>100</td>
<td>1000</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Corynebacterium sp.</td>
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<td>1000</td>
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<td>Weight (%)</td>
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<td>20.0</td>
<td>80.00</td>
<td>23.40</td>
<td>10.0</td>
<td>5.20</td>
<td>1.8</td>
<td>24.00</td>
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<td>-</td>
</tr>
</tbody>
</table>

N/A: Non Applicable
NA: No Activity
### Table 4-5 The MIC values of Mutica gum and its fractions against the strains of *H. pylori* (µg/mL)

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Whole Mutica Gum</th>
<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
</tr>
</thead>
<tbody>
<tr>
<td>26695</td>
<td>150</td>
<td>1000</td>
<td>150</td>
<td>150</td>
<td>1000</td>
<td>1000</td>
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</tr>
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<td>J99</td>
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<td>1000</td>
<td>150</td>
<td>100</td>
<td>1000</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>125</td>
</tr>
<tr>
<td>RSB6</td>
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<td>1000</td>
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<td>150</td>
<td>1000</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>P10</td>
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<td>1000</td>
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<td>1000</td>
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<td>NA</td>
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<td>250</td>
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<td>500</td>
<td>150</td>
<td>100</td>
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<td>500</td>
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NA: No Activity
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<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
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NA: No Activity
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<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
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N/A: Non Applicable
NA: No Activity
Table 4-8 The MIC values of Cabolica gum and its fractions against the strains of *H. pylori* (µg/mL)

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<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
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N/A: Non Applicable
NA: No Activity
Table 4-9 The MIC values of Cabolica gum and its fractions against Gram-negative bacteria (µg/mL)

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<th>Gram Negative Bacteria</th>
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<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
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<th>HMWF</th>
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<td>NA</td>
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N/A: Non Applicable
NA: No Activity
Table 4-10 The MIC values of Cabolica gum and its fractions against Gram-positive bacteria (µg/mL)

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N/A: Non Applicable
NA: No Activity
Table 4-11 The MIC values of Mastic gum and its fractions against the strains of *H. pylori* (µg/mL)

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</tbody>
</table>

N/A: Non Applicable  
NA: No Activity
Table 4-12 The MIC values of Mastic gum and its fractions against Gram-negative bacteria (µg/mL)

<table>
<thead>
<tr>
<th>Gram Negative Bacteria</th>
<th>Whole Mastic Gum</th>
<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF</th>
<th>Oxidised HMWF</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> type 1</td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>500</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Serratia marcescens</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>1000</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Alcaligenes faecalis</em></td>
<td>200</td>
<td>500</td>
<td>200</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Enterobacter aerogenes</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>500</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudomonas fluorescens</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>500</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>500</td>
<td>1000</td>
<td>NA</td>
<td>NA</td>
<td>250</td>
<td>100</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>200</td>
<td>500</td>
<td>150</td>
<td>150</td>
<td>200</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>100.0</td>
<td>20.0</td>
<td>85.0</td>
<td>23.4</td>
<td>10.0</td>
<td>4.0</td>
<td>1.8</td>
<td>25.0</td>
<td>4.2</td>
<td>-</td>
</tr>
</tbody>
</table>

N/A: Non Applicable
NA: No Activity
<table>
<thead>
<tr>
<th>Gram Positive Bacteria</th>
<th>Whole Mastic Gum</th>
<th>Essential Oils</th>
<th>Resin</th>
<th>Acidic Fraction (A)</th>
<th>Acidic Fraction (B)</th>
<th>Acidic Fraction (C)</th>
<th>Acidic Fraction (D)</th>
<th>Neutral Fraction</th>
<th>HMWF Oxidised HMWF</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus cereus</em></td>
<td>200</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>200</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td><em>Streptococcus faecalis</em></td>
<td>200</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>250</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>250</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td><em>Corynebacterium sp.</em></td>
<td>250</td>
<td>400</td>
<td>150</td>
<td>150</td>
<td>750</td>
<td>1000</td>
<td>N/A</td>
<td>NA</td>
<td>1000</td>
</tr>
<tr>
<td>Weight (%)</td>
<td>100.0</td>
<td>20.0</td>
<td>85.0</td>
<td>23.4</td>
<td>10.0</td>
<td>4.0</td>
<td>1.8</td>
<td>25.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>

N/A: Non Applicable  
NA: No Activity
4.4 Discussion

The resinous trunk bark exudates from species of the *Pistacia* genus have a history of informal use for gastrointestinal disorders over a period spanning at least ten centuries (Avicenna, 1015; Hairfield & Hairfield, 1990; Ibn Al-Baytar, 1248). More contemporary informal practice has almost exclusively involved Mastic gum, the exudate of the *P. lentiscus*, and reported studies concerning the composition or biological activity of such exudates have correspondingly concentrated upon this gum.

Whole Mastic gum has been shown to have *in vitro* antimicrobial activity against *H. pylori* (Marone *et al.*, 2001) and to be effective in clinical trials against duodenal ulcers (Al-Habbal *et al.*, 1984; Al-Said *et al.*, 1986; Huwez & Al-Habbal, 1986). Also, antimicrobial activity against a number of microorganisms has been demonstrated *in vitro* by various components of the essential oils of Mastic gum (Duru *et al.*, 2003; Mgiatis *et al.*, 1999; Tassou & Nychas, 1995) (although anti-*H. pylori* activity has not been reported and that has been reported in Chapter 3), these components being mainly monoterpenes with some level of monoterpenoids (Duru *et al.*, 2003; Vasslios *et al.*, 1991). Despite the fact that a number of other plants incorporate these components at various levels in their essential oils, comparable historical use or advocacy of these plants in anti-ulcer therapy has not however been reported. The question arises therefore as to why *Pistacia* trunk bark exudates, might seemingly be so singularly and historically advocated for this purpose.

There are three aspects by which these gums might differ from other plants in this regard. It has been shown to incorporate.
• An unusually high molecular weight polymer, polymyrcene (Van den Berg et al, 1998).

• A range of characteristic di- and tri-terpenes and –terpenoids (Barton & Seaone, 1956; Seoane, 1956) as a part of its components.

• They have been Masticated in use (particularly Kurdica gum), a practice consistent with its physical form and properties.

This chapter has therefore sought to

• Investigate the extent to which the trunk bark exudate of Pistacia atlantica Kurdica (P. a. Kurdica), Pistacia atlantica Mutica (P. a. Mutica) and Pistacia atlantica Cabolica (P. a. Cabolica) might demonstrate antimicrobial activity against H. pylori

• Fractionate the gums in a manner similar to that reported for Mastic gum, such that a direct comparison with the four gums might be made, and to investigate any anti-H. pylori activity of its various major fractions

• Isolate the highest molecular weight fraction of the gums that contributes to Masticating property of the gum and investigate the activity of these fractions and investigate the possibility of a potential increase in activity of the gum as a consequence of its Mastication, a process which, amongst other things, would result in exposure of the gum to the possibility of areal oxidation for a sustained period

• To compare components of Kurdica, Mutica, Cabolica gum (the sub-species of Pistacia atlantica) and Mastic gum in chemical composition (fractions) (Table 4-1) and antimicrobial activities Table (4-2 to 4-13).
It is reported here anti-\textit{H. pylori} activity demonstrated by Kurdica, Mutica, Cabolica and Mastic gum against nine strains of \textit{H. pylori} (Tables 4-2, 4-5, 4-8 and 4-11). Activity was demonstrated by the essential oils fraction of the gum, and by the acidic fractions of the gum. It has been also demonstrated the highest anti-\textit{H. pylori} activity coming from the acidic fraction ‘A’ and that the methylated of this fraction and other acidic fractions have no activity at all. It is also reported here activity of the highest molecular weight fraction (HMWF) and enhancement of the activity of this fraction upon its simple aerial oxidation. It have been also demonstrated both an oxidised resin from the gum (the remainder of the gum once the essential oils have been removed) and Masticated (as a consequence of Mastication), and an associated enhancement of the anti-\textit{H. pylori} activity of this resin that will be discussed in a separate chapter. Also the antimicrobial activities of the whole above mentioned fractions have been reported in Tables 4-2 to 4-13.

The highest activity is shown by those components of the resin, which demonstrate strong acid properties particularly the sodium carbonate soluble fraction of the Kurdica gum ‘fraction A’. Lower levels of activity are demonstrated by the remaining weak (sodium hydroxide soluble) acid fractions, essential oils and HMWF of Cabolica. Whilst no activity was detected at the concentration levels tested for the neutral fraction, it is interesting to note in this regard that antifungal activity has been reported for the neutral fraction of Mastic gum, but no significant activity was reported for the acid fraction (Duru \textit{et al}, 2003).

Mastication of the resin was shown to result in the introduction of aldehyde and/or ketone structures within it, consistent with an oxidation process having taken place. It also resulted in a significant increase in its anti-\textit{H. pylori} activity (Tables 4-2-4-13) Similarly, exposure of the HMWF to oxygen resulted in both
the incorporation of aldehyde/ketone structures and a significant increase in activity that will be discussed in a separate chapter.

These results are consistent with the concept that the anti-microbial activity of the components of the gum increases as their polarity increases, and in particular as a consequence of the presence of aldehyde and/or carboxylic acid groups within their structures, the level of these groups potentially increasing as a consequence of oxidation \textit{in situ}. 
Chapter 5
Polymeric Fractions of the Gums, Their antimicrobial activity and Mechanism of action

5.1 Introduction

The use of natural and synthetic organic polymers has become the mainstay of many familiar products due to their excellent electrical, mechanical, optical and thermal properties. The natural and synthetic polymers dominate the large and important industries such as rubber, plastics, packaging, specialist coatings, adhesives, medical devices and pharmaceuticals. In pharmaceuticals polymers are of interest as a means of drug deliver or as a drug itself, e.g., antimicrobials (Batz et al, 1974; Donaruma, 1974).

The emergence of antibiotic resistance, where bacteria exhibit reduced susceptibility to antimicrobials by mechanisms such as altered drug uptake, drug target alternation and/or drug inactivation has become a major problem, particularly in hospitals (Cloete, 2003; Smith, 2005). These mechanisms undoubtedly make a major contribution to antibiotic failure in the clinic.

A particular problem is where medical devices are contaminated with bacteria growing as adherent biofilms; particularly device-related infections associated with artificial joints, and venous catheters. Biofilms are also associated with some other chronic infections, such as those occurring in the respiratory tract (Smith, 2005). To overcome these problems, the process of biofilm formation has been studied with a view to preventing or treating infections (Kerr et al, 2001). Biofilms are targets for “anti-fouling” systems and novel drug delivery
technologies (Kerr et al, 2001). These include surface modification of devices to reduce bacterial attachment and incorporation of antimicrobials (anti-microbial co-polymers) to prevent bacteria colonisation (Kalyon & Olgun, 2001). Electronic instruments have also been used either to release antimicrobials from device surfaces or to drive antimicrobials through the biofilm (Smith, 2005). Other technologies include aerosolized delivery of antibiotics to the lung and formulation into liposome and polymer-based vehicles (Smith, 2005). Many biodegradable polymer-based carrier systems have also been used, such as poly(lactide-co-glycolide) and thermoreversible hydrogels (Smith, 2005).

Intravascular devices used for vascular access are also associated with biofilm formation and the risk of device-related bloodstream infection, which causes an increase of morbidity and mortality, prolonged hospitalisation and as a result a considerable increase of medical costs (Piozzi et al, 2004). To prevent bacterial colonization, two antibiotics, rifampin and amoxicillin, have been adsorbed on polyurethanes exhibiting acidic or basic properties (Piozzi et al, 2004). This type of antibiotic–polymer interaction has shown increases activity both with the introduction in the polymer side-chain of functional groups and with the matrix hydrophilicity (Piozzi et al, 2004).

Biodegradable macroporous polymer–ceramic composites have become widely used to treat bone defects in clinical practice. Composite poly(-caprolactone)-iodine/calcium hydroxide (PCLI2/Ca(OH) has been developed to a macroporous sponge-like structure that has shown excellent antimicrobial activities against Escherichia coli and Staphylococcus aureus (Almeida et al, 2004). The implanted composite has also had a perfect adhesion in the root to the implant site (Almeida et al, 2004). Anti-microbial bioabsorbable polymers are used to protect autogenous cancellous bone grafts that are placed into the peri-implant bone defect and release doxycycline slowly (Buchter, et al 2004).
The antimicrobial activity of a variety of proanthocyanidin polymer fractions with different structures and functional groups from the leaves of the forage legume *Dorycnium rectum* have been evaluated against *Clostridium aminophilum* and *Butyrivibrio fibrisolvens*. Sensitivity of *Cl. proteoclasticum* was significantly dependent upon the polymer structure. However, *Ruminococcus albus* and *Peptostreptococcus anaerobius* were sensitive to all proanthocyanidin structures (Sivakumaran *et al*, 2004).

Chitin is a glucose-based polysaccharide widely distributed in nature as the principal component of exoskeletons of crustaceans and insects (Ikinci *et al*, 2002). These compounds can be extracted from the waste products of the seafood industry in the form of shells of shellfish. Chitin can also be found in the cell wall of some bacteria and fungi (Lucero *et al*, 2002). Chitosan is a deacetylated polymer obtained from the alkaline deacetylation of chitin. Chitosan has a number of biological applications, particularly as a biomaterial in pharmaceutical and medical fields (Valenta *et al*, 1998). It is also used for systematic delivery of drugs and vaccines (Kelly *et al*, 2004). Chitosan has also been used as a coating to further retard drug release in the treatment of periodontitis (Kelly *et al*, 2004).

This polymer has a number of distinct advantages, including availability, biocompatibility, biodegradability, non-toxicity, antimicrobial properties, heavy metal ion chelation, gel forming properties, ease of chemical modification and high affinity for proteins (Krajewska, 2005). Chitosan is predicted to be a dominant material in the pharmaceutical industry (Ikinci *et al*, 2002).

To prevent catheter-related infection, 10¹²–¹³ activated silver nanoparticles per gram in polyurethane and silicone was evenly distributed that resulted in an
excellent antimicrobial activity against a broad spectrum of organisms \textit{in vitro} (Samuel & Guggenbichler, 2004).

Mastic gum contains an unusually high molecular weight polymer (Cis-1,4-poly-\(\beta\)-myrcene) (Van den Berg \textit{et al}, 1998) that has not been examined for its antimicrobial activity. As a part of the investigation into the anti-microbial activity of the whole gums and their fractions, the polymers were isolated and the molecular weights of these fractions determined by Gel Permission Chromatography as previously described (2.4.1). The antimicrobial activities of these fractions have been reported briefly in Chapter 4, and they will be discussed along with their kill kinetics in this chapter.

In this chapter, the isolation of the High Molecular Weight Fractions (polymer fractions) of the gums, their characteristics, their molecular weight and functional groups in relation to their anti-microbial activity will be discussed. The mode of the action by which the polymeric fractions of the gums may kill bacteria will also be considered.

5.2 Objectives

The objectives of the work outlined in this chapter were as follows:

1. To identify and characterize the polymeric fraction of the gums.

2. To establish a basis for understanding the antimicrobial activity of the polymeric fractions of the gum.
3. To establish relationship between molecular characteristics and antimicrobial activity.

4. To establish the relationship between antimicrobial activity of the polymer and its monomer.

5. To investigate the mode of the action/s and potential to enhance the antimicrobial activity.

As a consequence of the information presented and the data outlined in Chapter 4, regarding antimicrobial activity of the polymer and the minimum inhibitory concentration (MIC) values, in this chapter a number of hypotheses have been formulated and tested. These hypotheses are as follows:

1. The antimicrobial activity of the polymer increases with the molecular mass.

2. The antimicrobial behaviour of the polymer is distinct from that of the monomer subunits forming the polymer.

3. The antimicrobial activity of the polymer depends on its functional groups.

4. The water solubility of the polymer is important in its antimicrobial activity.

To test the above hypotheses, pure Cis-1,4-poly-β-myrcene was synthesised, its functional group was identified and its mode of the action was investigated and
compared with its monomer. To understand the potential for oxidized forms to enhance antimicrobial activity, the impact of oxidation was tested.

A novel water soluble polymer “co-poly (vinyl-p-benzoate)” (CPVPB) with different molecular mass were also synthesised to investigate the impact that molecular mass and functional groups exposed on the polymer have on antimicrobial activity and also the effect of solubility on the Gram-positive and Gram-negative bacteria.
5.3 Experimental Design

The polymeric fractions of the gums were isolated from the resin by dissolving the resins with dichloromethane (Van den Berg et al, 1998) or anhydrous diethyl ether as it has been described in Chapters 2 and 4. The collected polymer was then analysed by NMR to confirm the structure and by GPC to determine their MW.

5.3.1 Synthesis of the Polymyrcene

5.3.1.1 Free Radical Polymerisation

Four important processes are used to conduct free radical polymerisation. These are: bulk, solution, suspension, and emulsion polymerisation. In bulk polymerisation pure monomer is polymerised using a suitable initiator.

Bulk polymerisation method was used to synthesise Cis-1,4-poly-β-myrcene (Fig. 5-1).

\[
\begin{align*}
\text{Figure 5-1 Cis-1,4-poly-β-myrcene}
\end{align*}
\]
Free radical polymerisation is a chain reaction involving three steps: initiation, propagation and termination. The tree steps of polymerisation are as follows:

Initiation:

Two reactions are involved in initiations. The first reaction is the dissociation (here $k_d$ is the dissociation rate constant) of an initiator (I) to form a pair of radicals $R^*$.

$$
I \xrightarrow{k_d} 2R^*
$$

The second reaction in the initiation step is a chain initiation that involves the addition of the monomer (M) to the active species $R^*$. Further monomer is added to the activated molecule $RM^*$ (propagation) until the monomer is exhausted (living polymers) or the activated end of the growing chain is destroyed (termination).

These steps can be summarised as follows:

\[\begin{align*}
I & \rightarrow R^* \\
R^* + M & \rightarrow RM^* \\
RM^* + M & \rightarrow R(M)_n M^* \\
R(M)_n M^* & \rightarrow \text{Inactive polymer}
\end{align*}\]
In a 50 mL beaker containing 10 mL of β-myrcene, 0.5 mL of hydrogen peroxide 35% was added. The beaker was covered with aluminium foil and incubated at 60°C overnight. The polymerised β-myrcene in the beaker was collected for MW determination by GPC, NMR analysis and antimicrobial activity.

5.3.2 Synthesise of co-poly (vinyl-p-benzoate) (CPVPB) - A Novel Anti-microbial Polymer

Four polyvinyl alcohols (Fig. 5-2) with molecular weights ranging from 13,000-146,000 were chosen to synthesis novel soluble anti-microbial polymers to test the hypothesis of the relationship between molecular weight of the polymer and anti-microbial activity. Methyl-p-hydroxybenzoate (Fig. 5-3) used in food preservatives was chosen to incorporate into the structure of the polymer.

In order to synthesis CPVPB, two methods of direct esterification and transesterification were employed in which both resulted in the incorporation of a p-hydroxybenzoate structure into polyvinyl alcohol. The methods, together with the level of incorporation influenced the structure of the polymer product which in turn may influence the antimicrobial activity against a range of Gram-positive and Gram-negative bacteria. As the level of incorporation of p-hydroxybenzoate using transesterification was relatively higher than direct esterification, the product of transesterification was chosen for detailed studies.
5.3.2.1 Transesterification:

12g of polyvinyl alcohol was suspended in a solution of 400 mL dry dimethylformamide (DMF), 100 mL of dry benzene (used to azeotrope out any small quantities of water), 15 mL triethanolamine (catalyst) and 2g of methyl p-hydroxybenzoate. The mixture was heated and refluxed using a Dean and Stark apparatus, removing 125 mL of distillate. The polymer was then dissolved in DMF. A further 10g of methyl p-hydroxybenzoate was dissolved in 25 mL of dry DMF and added down the condenser and the reflux was continued. Approximately 150 mL of distillate was removed slowly over a period of 6 h and then the reflux was continued for a further 15 h. The resulting solution was allowed to cool to room temperature. A semi-transparent gel was the product.
The gel was broken up and suspended in 1.25 L of absolute alcohol. The mixture was stirred for 2 h until the DMF, triethanolamine and un-reacted methyl p-hydroxybenzoate had been leached from the product. The resulting polymer was filtered through Whatman grade 40/8µm (Sigma-Aldrich) and dissolved in 300 mL of milli-Q water, then was precipitated into 1.5 L of acetone and stirred to remove remaining contaminants. Any large polymers “lumps” were broken up. As a result a white and stringy product was obtained. The solid product was filtered off through Whatman grade 40/8µm and solid product was then suspended in 400 mL of absolute ethanol and stirred for 1h. This solid polymer was filtered off through Whatman grade 40/8µm and then dissolved in 300 mL of distilled water.

The process of dissolution (water), precipitation (acetone), suspension (ethanol) with stirring, was repeated until no detectable Ultra Violet (UV) absorption characteristic of the p-hydroxybenzoate structure was detected in the supernatant ethanol.

The solid white polymer product CPVPB (Fig. 5-4) was then filtered off through Whatman grade 40/8µm and air dried. These transesterification conditions were expected to result in a low level of incorporation of the p-hydroxybenzoate into the polymer and the polymer being completely insoluble in the acetone. Thus the yield was expected to be approximately the same weight as the original polymer (12g).
5.3.2.2 Purification of the polymer

Any remaining low molecular weight impurities under MW 124,000 (including those from the solvents) were removed from the polymer by dialysis against water using high molecular weight cut-off dialysis tubing consisting of a cellulose membrane (DTCM), prior to antimicrobial trials. This was to ensure that any positive result is indeed due to activity of the polymer and not the product of any low molecular weight contaminants.

Some DTCM was softened in distilled water over night. One end of the tube was well sealed, product added and the other end sealed. The tube containing product was then placed in a large measuring cylinder filled with distilled water, which was slowly stirred by a magnetic stirrer. Every half hour small aliquots of the water was taken from the cylinder and UV absorption was measured.

No rapid increase in the UV absorbance was observed in the water soon after placing the tube into the cylinder. This was indicative of both the integrity of...
the tubing and the absence of a high level of contamination from low molecular weight compounds.

The dialysis water was changed several times until no UV absorption could be detected. The solution within the dialysis tube was removed and then dried for determining the level of incorporation of p-hydroxybenzoate by UV followed by antimicrobial trials. The above procedure was repeated for all four different molecular weight compounds.

5.3.3 Determination of the Level of Incorporation by UV

Methyl-p-hydroxybenzoate absorbs at 255 nm at neutral pH and shifts to 285 nm in basic conditions (Spectra 5-11 and 5-12). The polymer-ester exhibited maximum absorbance at 257 nm in neutral aqueous conditions and 299.9 nm under basic conditions (Spectra 5-13 and 5-14); slightly different from the methyl ester in neutral condition and markedly different under basic condition.

As the molar absorption coefficient of this ester structure was not known, the level of p-hydroxybenzoate incorporation was determined by hydrolysing the p-hydroxybenzoate off the polymer in alkaline conditions and determining the quantity of p-hydroxybenzoic acid (di-salt) in the hydrolysate (maximum absorption at 249 nm in neutral conditions and 280 nm under alkaline conditions) (Spectra 5-15 and 5-16).

The polymer (product) was accurately weighed (0.31g) and then was dissolved in 1L of water. The UV spectrum of the solution was taken and the solution was diluted to determine the position of any maxima (257 nm).
The solution was diluted with a known quantity of cold alkali solution (0.1 M NaOH) and immediately the spectrum was run again. The position of the maximum of the phenolic salt of the polymer-ester was 299.9 nm (theoretically absorbance maximum at 300.5 nm) (Spectrum5-14).

To liberate p-hydroxybenzoic acid (as its di-sodium salt), the alkaline solution was warmed to 70°C for 5 min and was then cooled to 5°C. The UV spectrum of the solution was taken, the hydrolysis was quantitative and the maximum absorbance at 280 nm was measured (Spectrum 5-16).

The hydrolysate (alkaline solution) was acidified with sufficient acid solution (0.2 M HCl) and its UV spectrum was taken at 249 nm (Spectra 5-15) to quantitatively determine the concentration of p-hydroxybenzoic acid present in its protonated form. The p-hydroxybenzoic acid used as a standard and its absorption characteristics under similar alkaline and acid conditions was determined, thus allowing a comparison and to determine the quantity of this structure incorporated into the polymer.

The molar extinction coefficient ($E$) of the p-hydroxybenzoic acid was calculated from the absorbance ($A$) (Spectrum 5-15) using Lambert-Beer law. Using the calculated molar extinction coefficient, the concentration of the 4-p-hydroxybenzoic acid incorporated into the polymer was calculated (Spectrum 5-17).
5.3.4 Anti-microbial activity of the natural and synthetic polymers

5.3.5 Minimum Inhibitory Concentration

The MIC and MBC values were determined for HMWF of the gums as it has been reported in Chapter 4 and is reported again in this chapter for comparison. The MIC and MBC values were also determined for synthetic poly-myrcene and CPVPB against 9 strains of *H. pylori* (Table 2-1) and all other Gram-positive and Gram-negative bacteria listed in Table 2-2 using a broth micro-dilution method as previously described (2.13).

The strain 26695 *H. pylori* was chosen for time-kill kinetic experiments with static liquid cultures as described (2.14.1).

5.3.5.1 *H. pylori*, strain 26695

Time-kill kinetic were performed with static liquid cultures containing Isosensitest broth (Oxoid) supplemented with 5 % horse serum (Oxoid). The inoculum was harvested with Isosensitest broth from 36 h cultures grown on *Campylobacter* Selective Agar (CSA) as previously described in detail (2.11.5).

5.3.5.2 Gram-negative and Gram-positive bacteria

A 100 mL Iso-sensitest broth (Oxoid) culture was inoculated with a 10% inoculum from an 18 h overnight Gram-positive or Gram-negative bacterial culture. The culture was allowed to grow to stationary phase and that was determined by taking the Optical Density 600 (OD600) of the culture. The inoculums were then adjusted with the culture medium to give a starting
concentration of 1.00 x 1.00 E + 08. The details have been described in Chapter 2.

Each culture was incubated for 2 h to allow recovery of the bacteria before the HMWF, cis-1,4-\(\beta\)-polymyrcene and their oxidised form, 4-hydroxybenzoic acid and CPVPB were added at their respective MIC and 5 X MIC concentrations. Control cultures at MIC and 5 X MIC containing ethanol/water were also performed.

5.4 Results

The high molecular weight fraction (HMWF) represented 13.80 % (w/w) of the Kurdica gum, 19.40% (w/w) of Mutica gum, 20.00% (w/w) of Cabolica gum and 35.20% of Mastic gum (Table 5-1). The number average molecular weight, weight average molecular weight and the distribution of molecular weight of these fractions as well as synthetic Cis-1,4-poly-\(\beta\)-myrcene are shown in Table 5-1, which was obtained by GPC (Spectra 5-1-5-5).
Table 5-1 Polymeric fraction of the gums and synthetic polymyrcene

<table>
<thead>
<tr>
<th></th>
<th>High Molecular Weight Fraction (HMWF) %</th>
<th>Whole Gum (Total weight) %</th>
<th>Number average molecular weight (Mn)</th>
<th>Weight average molecular weight (Mw)</th>
<th>Distribution of molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. a. Kurdica</em></td>
<td>13.80</td>
<td>100</td>
<td>1023</td>
<td>11876</td>
<td>250-55000</td>
</tr>
<tr>
<td>(Kurdica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. a. Mutica</em></td>
<td>19.40</td>
<td>100</td>
<td>906</td>
<td>3584</td>
<td>250-50000</td>
</tr>
<tr>
<td>(Mutica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. a. Cabolica</em></td>
<td>20.00</td>
<td>100</td>
<td>682</td>
<td>3611</td>
<td>250-30000</td>
</tr>
<tr>
<td>(Cabolica gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. lentiscus</em></td>
<td>35.20</td>
<td>100</td>
<td>20000</td>
<td>80000</td>
<td>50000-130000</td>
</tr>
<tr>
<td>(Mastic gum)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis-1,4-poly-β-myrcene</td>
<td>N/A</td>
<td>N/A</td>
<td>221823</td>
<td>405703</td>
<td>50000-500000</td>
</tr>
</tbody>
</table>
Spectrum 5-1 Molecular weight distribution within HMWF of "Kurdica gum" (Mn 1023, Mw 11876,)

Spectrum 5-2 Molecular weight distribution within the HMWF of "Mutica gum" (Mn 906, Mw 3584)
Spectrum 5-3 Molecular weight distribution within the HMWF of "Cabolica gum" (Mn 682, Mw 3611)

Spectrum 5-4 Molecular weight distribution within the HMWF of "Mastic gum" (Mn 80,000, Mw above 120,000)
Spectrum 5-5 Molecular weight distribution within synthetic polymyrcene (Mn 221823, Mw 405703)
5.4.1 NMR Analysis

The Nuclear Magnetic Resonance (NMR) spectra of HMWF ($^{13}$C) showed some similarity in constituents of *P. lentiscus* (Spectrum 5-6) with *P. a. Kurdica* (Spectrum 5-7) and *P. a. Mutica* (Spectrum 5-8). However, *P. a. Cabolica*’s NMR spectrum (Spectrum 5-9) is totally different and it is indicating different structure and also has much lower antimicrobial activity than HMWFs of other gums. The NMR spectrum of Mastic gum is consistent with literature (Van den Berg *et al.*, 1998) and it has been proven to be Cis-1,4-poly-β-myrcene (Fig. 5-1). NMR spectrum of synthetic Cis-1,4-poly-β-myrcene (Spectrum 5-10) is consistent with NMR spectra of Mastic gum and literature (Newmark & Majumdar, 1988).
Spectrum 5-\textsuperscript{6}\textsuperscript{13}C NMR (C\textsubscript{2}D\textsubscript{2}Cl\textsubscript{4}) of the HMWF of Mastic gum

Spectrum 5-\textsuperscript{7}\textsuperscript{13}C NMR spectrum (C\textsubscript{2}D\textsubscript{2}Cl\textsubscript{4}) of the HMWF of Kurdica gum
Spectrum 5-13C NMR spectrum (C\textsubscript{2}D\textsubscript{2}Cl\textsubscript{4}) of the HMWF of Mutica gum

Spectrum 5-913C NMR spectrum (C\textsubscript{2}D\textsubscript{2}Cl\textsubscript{4}) of the Cabolica gum
Spectrum 5-10 NMR spectrum ($\text{C}_2\text{D}_2\text{Cl}_4$) of the synthetic cis-1,4-β-polymyrcene
5.4.2 Aerial Oxidation and Mastication

No change was observed when the HMWF was treated with 2, 4-DNP, while a yellow precipitate formed with the aerated sample, confirming that HMWF was susceptible to oxidation. A yellow precipitate was also formed when a sample of Masticated Kurdica gum resin (the only gum that is suitable for Mastication) was treated with 2, 4-DNP, but no change was observed when un-Masticated resin was treated with 2,4-DNP, indicating that the Mastication process resulted in some level of oxidation. No change was observed when synthetic polymer “cis-1,4-poly-β-myrcene” was treated with 2, 4-DNP, while a yellow precipitate similar to the aerated HMWF was formed with aerated polymer, indicating oxidation.

The MIC of highest molecular weight fraction HMWF ranged from 250-500µg/mL. Upon oxidation of this fraction, its MICs shifted to a range of 125 to 250µg/mL.

5.4.3 UV Spectroscopy

The UV Spectra 5-11-5-17 showed the level of incorporation Methyl-p-hydroxybenzoate (Fig. 5-3) into the structure of the polymer. The incorporation percentages have been tabulated in Table 5-2.
Spectrum 5-11 Methyl-p-hydroxybenzoate at neutral pH

Peak Table
Peak Style          Peaks
Peak Threshold      0.0100
Range              400.1nm to 200.0nm

Wavelength (nm)   Abs
255.0             0.253

Spectrum 5-12 Methyl-p-hydroxybenzoate at basic pH

Peak Table
Peak Style          Peaks
Peak Threshold      0.0100
Range              400.1nm to 200.0nm

Wavelength (nm)   Abs
285.0             0.1415
205.0             2.8833
Spectrum 5-13 Poly-ester at neutral pH

Peak Table
Peak Style: Peaks
Peak Threshold: 0.0100
Range: 400.1nm to 200.0nm

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>257.0</td>
<td>0.273</td>
</tr>
</tbody>
</table>

Spectrum 5-14 Poly-ester at basic pH

Peak Table
Peak Style: Peaks
Peak Threshold: 0.0100
Range: 400.1nm to 200.0nm

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>299.9</td>
<td>0.2845</td>
</tr>
</tbody>
</table>
Spectrum 5-15 p-hydroxybenzoic acid at neutral pH

Peak Table
Peak Style                        Peaks
Peak Threshold                    0.0100
Range                             400.1nm to 200.0nm

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>249.9</td>
<td>0.0916</td>
</tr>
</tbody>
</table>

Spectrum 5-16 p-hydroxybenzoic acid at basic pH

Peak Table
Peak Style                        Peaks
Peak Threshold                    0.0100
Range                             400.1nm to 200.0nm

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>280.0</td>
<td>0.3765</td>
</tr>
<tr>
<td>205.0</td>
<td>2.8265</td>
</tr>
</tbody>
</table>
Spectrum 5-17 p-Hydroxybenzoic acid incorporated into polyvinyl alcohol MW 13000-23000

<table>
<thead>
<tr>
<th>Wavelength (nm)</th>
<th>Abs</th>
</tr>
</thead>
<tbody>
<tr>
<td>249.0</td>
<td>0.891</td>
</tr>
</tbody>
</table>

The molar extinction coefficient was calculated as follows:

\[ A = Ecb \]

Where  \( A = \) absorbance (optical density) = (MW 13000-23000 = 0.891), (MW 31000-50000 = 0.801), (MW 50000-85000 = 0.750) and (MW 85000-146000 = 0.690)

\( c = \) concentration of solute in mol per liter = unknown

\( b = \) path length through the sample in centimeter = 1

\( E = \) the molar extinction coefficient = 25,230
The incorporated percentage of p-hydroxybenzoic acid into co-poly (vinyl-p-benzoate) for MW of 13000-23000, 31000-50000, 50000-85000 and 85000-146000 were calculated by the above equation are shown in Table 5-2.

Table 5-2 Percentage of p-hydroxybenzoic acid incorporated into the polymer

<table>
<thead>
<tr>
<th>Molecular weight</th>
<th>Incorporated p-hydroxybenzoate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13000-23000</td>
<td>0.15</td>
</tr>
<tr>
<td>31000-50000</td>
<td>0.14</td>
</tr>
<tr>
<td>50000-85000</td>
<td>0.13</td>
</tr>
<tr>
<td>85000-146000</td>
<td>0.12</td>
</tr>
</tbody>
</table>
5.4.4 MIC and Kill kinetics

The MIC results have been tabulated in Tables 5-7, 5-8 and 5-9 for HMWF of the gums, synthetic cis-1,4-poly-β-myrcene and all other co-poly(vinyl-p-benzoate) and 4-hydroxybenzoic acid against the strains of *H. pylori* listed in Table 2-1 and all other Gram-positive and Gram-negative bacteria listed in Table 2-2.

The MIC values for HMWF ranged from 200-1000 µg/mL against the strains of *H. pylori* and all other Gram-negative bacteria (Table 5-7 and 5-8) and ranged from 500-1000 µg/mL against Gram-positive bacteria (Table 5-9).

The MIC values for Cis-1,4,-β-polymyrcene was 100 µg/mL against the strains of *H. pylori* and all other Gram-negative bacteria (Table 5-7 and 5-8) and 1000 µg/mL against Gram-positive bacteria (Table 5-9). The MICs of oxidised Cis-1,4,-β-polymyrcene was 50 µg/mL against the strains of *H. pylori*, 75 µg/mL for all other Gram-negative bacteria and 1000 µg/mL against Gram-positive bacteria.

The MIC values for 4-hydroxybenzoic acid CPVPB against the strains of *H. pylori*, all other Gram-negative and Gram-positive bacteria was >2000 µg/mL.

5.4.4.1 Statistical Analysis

The data collected from kill kinetics and MIC values were recorded on Microsoft Excel and analysed using Microsoft Excel, Sigma plot and MATLAB. Multi-way ANOVA was performed in all cases. Statistically significant results were determined by a P value of less than or equal to 0.05.
Table 5-3 shows the five-way ANOVA for the first set of 5x3x2x2x2.

Table 5-3 Five-way ANOVA

<table>
<thead>
<tr>
<th>Sum of Squares</th>
<th>degree of freedom</th>
<th>Mean Squares</th>
<th>F</th>
<th>Prob&gt;F or α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
<td>9.44E+07</td>
<td>4</td>
<td>2.36E+07</td>
<td>4.0245</td>
</tr>
<tr>
<td>Bacteria</td>
<td>1.21E+08</td>
<td>2</td>
<td>6.06E+07</td>
<td>10.3309</td>
</tr>
<tr>
<td>Polymerization state</td>
<td>3.11E+03</td>
<td>1</td>
<td>3.11E+03</td>
<td>5.30E-04</td>
</tr>
<tr>
<td>Oxidation state</td>
<td>5.69E+06</td>
<td>1</td>
<td>5.69E+06</td>
<td>0.97</td>
</tr>
<tr>
<td>Concentration</td>
<td>1.99E+07</td>
<td>1</td>
<td>1.99E+07</td>
<td>3.3896</td>
</tr>
<tr>
<td>Error</td>
<td>6.45E+08</td>
<td>110</td>
<td>5.87E+06</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.87E+08</td>
<td>119</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-4 shows the five-way ANOVA in which *S. aureus* is excluded - Gram-negative only-
Table 5-4 Five-way ANOVA areas from kill kinetics

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>degree of freedom</th>
<th>Mean Squares</th>
<th>F</th>
<th>Prob&gt;F or α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
<td>1.84E+07</td>
<td>4</td>
<td>4.60E+06</td>
<td>15.5136</td>
<td>3.64E-09</td>
</tr>
<tr>
<td>Bacteria</td>
<td>1.06E+04</td>
<td>1</td>
<td>1.06E+04</td>
<td>0.0357</td>
<td>0.8506</td>
</tr>
<tr>
<td>Polymerization state</td>
<td>3.86E+07</td>
<td>1</td>
<td>3.86E+07</td>
<td>130.4236</td>
<td>0</td>
</tr>
<tr>
<td>Oxidation state</td>
<td>1.22E+06</td>
<td>1</td>
<td>1.22E+06</td>
<td>4.1159</td>
<td>0.0462</td>
</tr>
<tr>
<td>Concentration</td>
<td>8.16E+06</td>
<td>1</td>
<td>8.16E+06</td>
<td>27.5604</td>
<td>1.52E-06</td>
</tr>
<tr>
<td>Error</td>
<td>2.10E+07</td>
<td>71</td>
<td>2.96E+05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.74E+07</td>
<td>79</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5.5 shows a five-way ANOVA, including all data, without oxidation state.

Table 5-5 Four-way ANOVA; all data without oxidation

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>degree of freedom</th>
<th>Mean Squares</th>
<th>F</th>
<th>Prob&gt;F or α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
<td>1.06E+09</td>
<td>5</td>
<td>2.12E+08</td>
<td>31.15</td>
<td>9.99E-16</td>
</tr>
<tr>
<td>Bacteria</td>
<td>6.77E+07</td>
<td>2</td>
<td>3.38E+07</td>
<td>4.9766</td>
<td>0.0099</td>
</tr>
<tr>
<td>Polymerization state</td>
<td>2.91E+06</td>
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<td>2.91E+06</td>
<td>0.428</td>
<td>0.5154</td>
</tr>
<tr>
<td>Concentration</td>
<td>1.34E+07</td>
<td>1</td>
<td>1.34E+07</td>
<td>1.9733</td>
<td>0.1651</td>
</tr>
<tr>
<td>Error</td>
<td>4.22E+08</td>
<td>62</td>
<td>6.80E+06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1.56E+09</td>
<td>71</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 5-6 shows the impact of polymerisation state without *S. aureus*.
Table 5-6 Four-way ANOVA; summarized of results

<table>
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<th>Sum of Squares</th>
<th>degree of freedom</th>
<th>Mean Squares</th>
<th>F</th>
<th>Prob&gt;F or α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substance</td>
<td>7.78E+08</td>
<td>5</td>
<td>1.56E+08</td>
<td>147.0414</td>
<td>0</td>
</tr>
<tr>
<td>Bacteria</td>
<td>1.82E+06</td>
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<td>0.1975</td>
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<tr>
<td>Polymerization state</td>
<td>8.66E+06</td>
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<td>8.66E+06</td>
<td>8.1856</td>
<td>0.0068</td>
</tr>
<tr>
<td>Concentration</td>
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<td>6.35E+06</td>
<td>6.0018</td>
<td>0.0189</td>
</tr>
<tr>
<td>Error</td>
<td>4.13E+07</td>
<td>39</td>
<td>1.06E+06</td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>8.36E+08</td>
<td>47</td>
<td>1.56E+08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial Strains</td>
<td>β-myrcene/Oxidised</td>
<td>Mastic/HMWF</td>
<td>Kurdica/HMWF</td>
<td>Mutica/HMWF</td>
<td>Cabolica/HMWF</td>
</tr>
<tr>
<td>------------------</td>
<td>-------------------</td>
<td>-------------</td>
<td>--------------</td>
<td>-------------</td>
<td>--------------</td>
</tr>
<tr>
<td>26695</td>
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<td>500/250</td>
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<td>250/125</td>
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<td>1000/500</td>
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<td>250/100</td>
<td>500/250</td>
<td>250/125</td>
<td>1000/1000</td>
</tr>
</tbody>
</table>

Table 5-7 MIC values of polymeric fractions of the gums and their oxidised, cis-1,4-β-myrcene and CPVPB against the strains of *H. pylori* (µg/mL)
Table 5-8 The MIC values of polymeric fractions of the gums and their oxidised, cis-1,4-β-polymyrccene and CPVPB against gram negative bacteria (µg/mL)

<table>
<thead>
<tr>
<th>Gram negative bacteria</th>
<th>β-myrcene/HMWF</th>
<th>Mastic Oxidised HMWF</th>
<th>Kurdica Oxidised HMWF</th>
<th>Mutica Oxidised HMWF</th>
<th>Cabolica Oxidised HMWF</th>
<th>Polymyrccene Oxidised Polymyrccene</th>
<th>4-hydroxybenzoic acid CPVPB MW</th>
<th>CPVPB MW</th>
<th>CPVPB MW</th>
<th>CPVPB MW</th>
<th>CPVPB MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Escherichia coli type 1</td>
<td>1000/1000</td>
<td>250/100</td>
<td>400/250</td>
<td>500/250</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Salmonella typhimurium</td>
<td>1000/1000</td>
<td>250/100</td>
<td>500/200</td>
<td>250/125</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>1000/1000</td>
<td>250/100</td>
<td>500/400</td>
<td>1000/500</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
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<tr>
<td>Pseudomonas aeruginosa</td>
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<td>500/250</td>
<td>500/250</td>
<td>1000/1000</td>
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<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
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<td>Alcaligenes faecalis</td>
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<td>250/100</td>
<td>500/300</td>
<td>1000/500</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Enterobacter aerogenes</td>
<td>1000/1000</td>
<td>250/100</td>
<td>500/200</td>
<td>1000/500</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>1000/1000</td>
<td>250/100</td>
<td>250/125</td>
<td>250/125</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Proteus vulgaris</td>
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<td>250/100</td>
<td>250/125</td>
<td>250/125</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
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<td>500</td>
</tr>
<tr>
<td>Porphyromonas gingivalis</td>
<td>1000/1000</td>
<td>200/100</td>
<td>200/125</td>
<td>250/125</td>
<td>1000/1000</td>
<td>100/75</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
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</tr>
</tbody>
</table>
Table 5-9 The MIC values of polymeric fractions of the gums and their oxidised, cis-1,4-β-myrcene and CPVPB against gram positive bacteria (µg/mL)

<table>
<thead>
<tr>
<th>Gram positive bacteria</th>
<th>β-myrcene/ Oxidised</th>
<th>Mastic HMWF/ Oxidised</th>
<th>Kurdica HMWF/ Oxidised</th>
<th>Mutica HMWF</th>
<th>Cabolica HMWF/ Oxidised</th>
<th>Polymyr-cene/ Oxidised</th>
<th>4-hydroxybenzoic acid MW</th>
<th>CPVPB 13000-</th>
<th>CPVPB 23000-</th>
<th>CPVPB 50000-</th>
<th>CPVPB 85000-</th>
<th>CPVPB 146000</th>
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</thead>
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<tr>
<td>Bacillus cereus</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 250</td>
<td>500/250</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 250</td>
<td>500/250</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>Streptococcus faecalis</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 200</td>
<td>500/200</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
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<td>500</td>
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<tr>
<td>Staphylococcus epidermidis</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 200</td>
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<td>1000/ 1000</td>
<td>1000/ 1000</td>
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<td>1000</td>
<td>750</td>
<td>500</td>
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<td>500</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 200</td>
<td>500/200</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
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<td>1000</td>
<td>750</td>
<td>500</td>
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<td>500</td>
</tr>
<tr>
<td>Corynebacterium sp.</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>500/ 200</td>
<td>500/200</td>
<td>1000/ 1000</td>
<td>1000/ 1000</td>
<td>2000</td>
<td>1000</td>
<td>750</td>
<td>500</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>
5.5 Discussion

The percentages of the HMWFs within the gums and their average molecular weight (Mn)s showed differences ranging from 13.8-35.2% and 682-221823 respectively (Table 5-1). NMR Spectra (5-6-5-10) are indicative of different physical and biological properties with respect to natural and artificial polymers. Apart from Cabolica gum, the rest of the polymeric fractions of the gums were assigned to be cis-1,4-β-poly-myrccene based on the similarity of NMR spectra of the gums when compared with synthesised cis-1,4-β-poly-myrccene (Spectra 5-6-5-10) together with NMR spectra from the literature (Newmark & Majumdar, 1988; Van den Berg, 1998).

The MIC values and the kill kinetics data of the HMWFs (naturally occurred Cis-1,4-β-poly-myrccene -tentatively-), β-myrccene, and synthetic cis-1,4-β-poly-myrccene, 4-hydrobenzoic acid and CPVPB and their antimicrobial relationship were statistically interpreted as follows:

Multi-way ANOVA as described (2.14.3) was applied to the kill kinetic data (area under the curve) from the effect of the substances 4-Hydroxy benzoic acid, β-myrccene, HMWFs of Cabolica, Kurdica, Mastic and Mutica (polymers and their monomer), two oxidation forms (non-oxidized and oxidized). These were tested on three species of bacteria, namely *E. coli*, *H. pylori* and *S. aureus* in two concentrations (1 and 5 X MIC). The factors and number of levels in each factor are summarized in Table 5.10. There are (six levels in the first factor: 4-Hydroxy benzoic acid, β-myrccene, HMWFs of Cabolica, Kurdica, Mastic and Mutica) for the substance, there are three levels in the second factor (*E. coli, H. pylori* and *S. aureus*), two levels in the third factor (polymer and monomer), two levels in the fourth factor and two levels in the fifth factor.
If all the possible combination of factors and levels were assessed, there would be 144 experiments needed to be carried out in a full factorial design experiment; however, as oxidation does not apply to 4-Hydroxy benzoic acid and its polymer CPVPB, the oxidation state experiments were redundant. Consequently 132 kill kinetic curves were recorded for this study and the area under each curve was used as an estimate of the effectiveness of substance in killing bacteria.

A kill kinetic curve represents the bacterial counts versus time, and thus the smaller area under the curve, the more effective the substance is in terms of the bactericidal activity. The area data then were analysed by multi-way ANOVA with four separate multi-way ANOVA being employed in this study.

To evaluate the effect of all variables (including oxidation state) a five-way ANOVA was established using only five levels in the first factor (substance)
excluding 4-hydroxy benzoic acid. The resultant design had a dimension of 5 x 3 x 2 x 2 x 2. The outcome of the five-way ANOVA for the first set is given in table 5.3.

As Table 5-3 shows, the effect of substance and bacteria are both highly significant ($\alpha < 0.05$). This indicates that at least two or more substances are significantly different with respect to all others. These data indicate that there are significant differences in the killing potential of the substances tested. This result is as expected. In the same way, at least one of the bacterial species was significantly different from the others. This is not unexpected, given the different bacteria employed in this study.

The key information of interest here is the impact of the polymerization state. Some what surprisingly the analysis (ANOVA) shows a very high $\alpha$ (0.9817) which could be interpreted as indicating that substances in polymer form do not kill the bacteria better than when they are in the monomer form. This is a general statement interpreted from ANOVA, meaning that the polymerization state as a whole is insignificant. This would be reasonable if all three bacterial species exhibit similar behaviour and the starting materials were essentially equivalent; however, this is not the case.

To further explore that data, a second five-way layout was set up in which the S. aureus data were excluded (only Gram-negative bacteria considered). Again the areas from the kill kinetic curves were analysed using a five-way ANOVA and the results were summarized in Table 5-4.

As Table 5-4 suggests, excluding the S. aureus data from the results makes a difference. Similar to Table 5.3, the substance effect is significant in Table 5.4, but the effect of bacteria becomes insignificant ($\alpha=0.8506 >>0.05$), i.e. E. coli and H. pylori are affected in a similar fashion. This is consistent with intrinsic Gram-negative potency. Further, it indicates that from the perspective of
antimicrobial activity against Gram-negative bacteria, *H. pylori* is no different to *E. coli*.

More interestingly, where polymerization is concerned, the results indicate that with respect to Gram-negative bacteria (Table 5-4) there is a highly significant effect. This is an indication of higher activity of polymers in killing Gram-negative bacteria. Oxidation state shows the similar pattern and may be interpreted to indicate that oxidation of selected polymers increases the killing effect.

As it was mentioned above, 4-Hydroxybenzoic acid and its polymer CPVPB were excluded from some analyses, as oxidation state did not apply to them. To examine the whole data including 4-Hydroxy benzoic acid, a third multi-way ANOVA was undertaken including all data, without that of the oxidation state (Table 5-5).

Table 5-5 shows similar results to Table 5-3; that is significant effect for bacteria and insignificant effects for polymerization state and blank concentration. These results could be an indication of the same effect of *S. aureus*, as explained earlier. Thus again to investigate the impact of the polymerization state the analysis was performed without *S. aureus* (Table 5-6).

By removing the *S. aureus* data, the effect of bacteria (Gram-negative) is insignificant while the polymerization state and concentrations (MIC and 5MIC) are significant. It implies that *S. aureus* is affected differently from the other two bacterial species. Thus is may be concluded that the effect of polymers on *E. coli* and *H. pylori* are similar and these effects are different to those observed for *S. aureus*.
Table 5-6 also supports the effectiveness of polymer against Gram-negative bacteria, which was predictable as these compounds are hydrophobic. The antimicrobial activities of the polymeric fractions of the gum suggest $\beta$-myrcene, the monomer of these fractions, as being important with respect to antimicrobial activity as seen in Table 5-7-5-9. However, while knowing the structure, the question arises as to what are the active sites of these polymeric fractions and why they are more active against Gram-negative than Gram-positive bacteria, despite of the fact that the monomer has the same MIC values for Gram-positive and Gram-negative bacteria Table 5-7-5-9.

One factor is the effect of oxidation. As a result of oxidation or Mastication, a yellow precipitate formed. When oxidised polymers were treated with 2, 4-DNP the data were consistent with the formation of aldehydes or ketones (Fig. 5-5), consistent with a polymeric backbone carrying reactive aldehyde groups able to react at the bacterial surface.

![Figure 5-5 Oxidation and Mastication of polymeric fraction](image)

As illustrated in the above reaction, the double bond between carbon 7 and 8 can be broken resulting in an aldehyde on the polymeric backbone and releasing the ketone. The antimicrobial activities of aldehyde and ketones are well known (Melrose et al, 1988). The broad-based antimicrobial properties of polymer having the repeating polymeric unit of an aldehyde (Fig. 5-6) has been patented by Chemeq Limited (Melrose et al, 2000). Interestingly, in 2000 Chemeq announced that results from a preliminary
study conducted by Murdoch University demonstrated antimicrobial activity of the following structure (Fig. 5-6) against *H. pylori* (Chemeq, 2000).

![Figure 5-6 Active functional group](image)

Thus the concept of a naturally occurring polymer being “activated” through a process of oxidation generating reactive aldehyde groups, including processes involving Mastication, is consistent with our understanding of the mechanism of action of synthetic antimicrobial polymers.

The MIC data of these polymeric compounds were not consistent with contemporary antibiotics and were more consistent with a topical “disinfectant” like compound. Thus while this work has provided an insight into “natural” mechanisms activating components of polymers found in *Pistacia* sp. increasing antimicrobial activity, as suggested by the work of Chemeq Ltd., these compounds are more appropriately used as feed supplements to replace more potent antibiotics.

The purpose of the work presented in this thesis was to investigate the antimicrobial activity of gum extracted from *Pistacia* sp., with the view to seeking to identify lead compounds for novel antibiotics that may act systemically against *H. pylori* and other bacterial pathogens. While further work could be undertaken to further characterise the polymers and their “disinfectant” properties, this would be of limited value as it was apparent that the observations were convergent with already developed fields and therefore it was decided to finalise the studies of these polymers at this point and to explore other potentially more productive avenues.
Analysis, Identifications and Antimicrobial Activity of the Isolated Components of the Acidic Fractions

6.1 Introduction

The chemical composition of Mastic gum has been studied by a number of researchers. The first attempt to characterise the chemical composition of Mastic gum was made in 1904 by Tschirsh and Reutter followed by Casparis and Naef 1934 (Barton & Seaone, 1956). However, they failed to demonstrate any of those components that are known to us today. The first published account detailing elements of the chemical composition of Mastic gum was by Barton and Seaone (1956). They isolated and identified three crystalline compounds from the “acidic fraction” (Masticadienonic, isoMasticadienonic and oleanonic acids) and one compound from the “neutral fraction” of Mastic gum (tirucallol) (Barton & Seaone, 1956; Seoane, 1956). In 1973 nine esters were isolated from “the acidic fraction” of the galls of *Pistacia lentiscus* (*P. lentiscus*) by chromatography after methylation with diazomethane (Monaco *et al*, 1973). They also isolated eight triterpenes from the neutral fraction of the galls produced by *Aploneura lentisci* (Monaco *et al*, 1973). These data were used as a source for authentic measurements of melting point (m. p.) and optical rotations.

Isolated triterpenes from the bled resin of *Pistacia vera* have been documented to show some similarity with those that had been isolated from *P. lentiscus* (Caputo *et al*, 1978). As Mastic had been used as a protective layer for artistic works including painting, there was an interest in understanding the characteristics of the gum and so it was subjected to pyrolysis gas chromatography-mass spectrometry (Chiavari *et al*, 1995) for chemical identification. Similar works have been undertaken by other researchers looking for varnishes that had been
used for art and paintings (Rene de la Rie, 1989; Van der Doelen, 1998; Van der Doelen & Boon, 2000; Zumbuhl et al, 1998). Eight components were identified by other workers with High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) using atmospheric pressure Chemical Ionisation (APCI) (Van der Doelen et al, 1998).

Much of the work that had been undertaken prior to 1987 has been summarized (Mills & White, 1989). Interestingly, they have also identified components of Mastic resin together with some other resins from wrecked ships of the late Bronze Age (Mills & White, 1989). While good work has been undertaken, some attempts at characterization have not been reliable as they used Thin Layer Chromatography (TLC) and others are perhaps trivial or irrelevant.

TLC has been used for preliminary comparisons (Hairfield & Hairfield, 1990). Some of the previously identified components of Mastic identified by less reliable techniques have been confirmed using different methods. For example, Papageorgiou and associates have identified ten triterpenoid acids from acidic fraction of Mastic gum by Gas Chromatography-Mass Spectrometry (GC-MS) (Papageorgiou et al, 1997). Also, the chemical composition of the resins extracted from insect galls found on the plant species of *Pistacia* but not *atlantica* has been analysed by a number of researchers (Caputo et al, 1978).

The identified triterpenes and triterpenoids from acidic fractions of Mastic gum had structures that mimic those of steroidal compounds. Therefore action was taken to search the literature with respect to possible antimicrobial activity that triterpenes and steroids may exhibit. In China ten triterpenic acids and two steroids had been isolated from the root of *Rubus innominatus* from the *Rosaceae* family in which some of these components were shown to exhibit antibacterial activity (Mingkui et al, 2003). Steroid compounds have also been isolated from
the sponge *Erylus lendenfeldi* (Geodiidae) collected in the Red Sea with demonstrated antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Al-Trabeen *et al*, 2004). They have also shown antifungal activity against *Candida albicans* (Al-Trabeen *et al*, 2004). Cholic acid also exhibits a structure similar to some of the acidic fractions of Mastic gum (Bangwei *et al*, 2004). Some novel cationic steroid antibiotics have been synthesized by conjugating tripeptides to a triamino analog of cholic acid. These compounds have demonstrated activity against Gram-negative and Gram-positive bacteria (Bangwei *et al*, 2004).

Preliminary analysis of extracts from *Bryophyllum Pinnatum* (Lam) Oken have shown the presence of steroids, flavanoids, saponins, tannins, glycosides and acids. Such extracts have shown antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* (Akpuaka *et al*, 2003).

Two novel steroidal phenols have been synthesized and screened against strains of multiresistant *Staphylococcus aureus*, a vancomycin resistant *Enterococcus faecalis* and fast growing mycobacteria (Lange *et al*, 2004). Their antibacterial activity was dependant on the length of alkyl chain (Lange *et al*, 2004). Similarly, stigmasterol and β-stigmasterol glycoside were isolated and identified by 2D NMR; these compounds demonstrated significant antimicrobial activity (Nacef *et al*, 2003).

A tetraoxane derivative of steroid: \( I \ [R = \text{H, ethanoyl, propanoyl, benzoyl}; R1 = \text{H, Me, Et, isopropyl}; R2 = \text{H, Me, Et}; R3 = \text{H, Me, Et}; R4 = \text{H, Me, Et, tert-Bu, aryl, ester, etc.}; X = \text{alkoxy, amino, N-alkylamino, N-arylamino}; n = 0-3] \), and all other possible stereoisomers has demonstrated high antimicrobial activity against the malarial parasite *Plasmodium falciparum* chloroquine-susceptible
strain D6, and the chloroquine-resistant strain W2 respectively (Solaja et al., 2003) (Fig. 6-1).

The chemical compositions of gum extracted from the *atlantica* species and its sub-species are not known but are expected to be similar to that of Mastic. The acidic fractions of the sub-species of *atlantica*, particularly *Kurdica* have been demonstrated to contain compounds exhibiting anti-microbial activity as previously described; therefore work was undertaken to identify the compositions of extracted fractions and compare the spectra with the published spectra in literature. In this chapter, the analysis of acidic fractions of Mastic gum as a reference and Kurdica, Mutica and Cabolica gums are reported. The identification of the GC-MS peaks was performed using published mass spectra and retention characteristics of Mastic gum (Papageorgiou et al., 1997). The retention time and characteristics of Mastic gum that was obtained by GC-MS was first verified with published data, and then the verified obtained data was used as a criterion for identification of sub-species of *atlantica.*

Figure 6-1 Tetraoxane derivative of steroid
The acidic fractions of Mastic (Sigma Aldridge), Kurdica, Mutica and Cabolica gum (Kurdistan Saghez Sazi and Surij from Iran) were analysed using GC-MS (CI and EI as described 2.5.3, 2.5.4 and 2.7). Thirteen triterpenoid acids were identified by retention characteristics as their methyl esters (i.e., Moronic acid, Oleanonic acid, Ursonic acid, Oleanolic acid, IsoMasticadienonic acid, 3-epi-isoMasticadienolic acid, Masticadienonic acid, DihydroMasticadienonic acid, 3-O-acetyl-3epi(iso)Masticadienolic acid, Masticadienolic acid, DihydroMasticadienonic acid, 3-acetoxy-3-epi(iso)Masticadienolic acid, 3-acetoxy-3-epiMasticadienolic acid) in Mastic gum. Their structures were identified by analysis of their spectral data, optical rotation and melting point and also by comparing with authentic reported data and co-injection with authentic samples (Chiavari et al, 1995; Papageorgiou et al, 1997; Van der Doelen & Boon, 2000).

This data was used to identify the chemical composition of Kurdica, Mutica and Cabolica gums and to compare the composition by reference to their biological activity. The objectives of the work outlined in this chapter were as follows:

6.2 Objectives:

1. To identify the individual components of Mastic gum in relation to their antimicrobial activities.

2. To use Mastic gum data (GC-MS) for identification of Kurdica, Mutica and Cabolica gums components.
3. To screen for compounds in gum extracts that have antimicrobial activity, seeking to identify and isolate specific compounds with substantial antimicrobial activity.

4. To investigate any differences between the active components identified within the fractions.

5. To perform kill kinetics on individual chemical isolates seeking to identify bactericidal or bacteriostatic activities.

6. To observe any morphological changes in bacteria that has been exposed to the active components.

7. To identify any common structure/s of the active component/s.
6.3 Experimental

6.3.1 GC-MS Analysis of Acidic Fractions

The acidic fractions ‘A’ and ‘B’ were combined. The combined fractions were dissolved in acetonitrile 11mL. This solution was then methylated as previously described. The methylated solution was evaporated under vacuum and solids were analysed by GC-MS. The methylated acidic fractions of Kurdica, Mutica, Cabolica and Mastic gum were analysed by GC-MS in split mode (20:1) 0.5 µm injection volume in a Shimadzu QP-5000 GC-MS System with a 30m BP-5 fused silica capillary column of 0.25 mm I.D. and 0.11µm film thickness as described previously (2.7.2). High Resolution Mass Spectrometry was used to determine the molecular formula. The obtained spectrum of Mastic gum was used in order to obtain a retention time for the ten previously identified methylated triterpenoid.

6.3.2 Column Chromatography

A vertical glass column was used to separate and collect the most active fractions of the gum (acidic fraction ‘A’ and ‘B’). In this technique, the mixture to be analysed is placed on the top of the column and flows down through the column (by either gravity or external pressure). This process of fractionation was performed parallel to GC-MS for further validations and also to collect the fractions for antimicrobial assay in the existing form whereas in GC-MS these fractions were methylated. While the intention was to repeat the method reported in the literature (Barton & Seaone, 1956), allowing the comparison with authentic data, some modification in the mobile phase was necessary to
optimize the separation. This optimization was obtained by TLC and has been described in Chapter 2. For each gum 48 fractions were collected. Purity of the fractions was tested by Chemical Ionisation (CI) Mass Spectrometry, followed by identification by EI Mass- Spectrometry. These spectra were then correlated with authentic spectra in the literature (Chiavari et al, 1995; Monaco et al, 1973; Papageorgiou et al, 1997; Van der Doelen & Boon, 2000).

The impure sub-fractions were discarded and the pure components were then crystallized from ether/benzene or methanol. Rotations were determined in CHCl₃, at the concentration of 0.2%, UV absorptions were taken by Varian UV spectrophotometer equipped with Carry 50 software. The molecular weight, m/z fragments, peak intensities and [α]D are tabulated in Table 6-1. The pure components were then kept for further analysis and screening for any antimicrobial activities against the strains of *H. pylori* and Gram-positive and Gram-negative bacteria listed in Table 2-1 and 2-2 of Chapter 2.

6.3.3 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is a method of analysis that is not limited by the volatility or stability of the sample compound. HPLC is used to separate, identify, purify and quantify various compounds. Atmospheric Pressure Chemical Ionisation-Mass Spectrometry (APCI-MS) was used to avoid any changes in chemical structure of triterpenoids components of the acidic fractions (Van der Doelen et al, 1998) and also to validate the identification made as a result of data obtained by GC-Mass and collected following column chromatography. The identified collected fractions were kept for antimicrobial screening against the strains of *H. pylori* and Gram-positive
and Gram-negative bacteria (Table 2-1 and 2-2) and also to investigate the mode of the action reported in Chapter 7.
6.3.4 Antimicrobial Activity of the Isolated Components of the Acidic Fractions

6.3.4.1 Minimum Inhibitory Concentration (MIC)

The MIC and MBC values were determined for all the sub-fractions of acidic fractions ‘a’ and ‘b’ and all other derivatives of the sub-fractions that are listed in table 6.1 against 9 strains of *H. pylori* (Table 2.1) and all other Gram-positive and Gram-negative bacteria listed (Table 2.2) using the broth micro-dilution method as described in Chapter 2.

6.3.4.2 Time-kill kinetic

The 26695 strain of *H. pylori*, *Escherichia coli type 1* and *Staphylococcus aureus* were chosen for time-kill kinetic experiments with static liquid cultures. The cultures were allowed to grow to stationary phase and that was determined by taking the Optical Density 600 (OD600) of the cultures. The inoculums were then adjusted with the culture medium to give a starting concentration of 1.00 x 1.00 E + 08. Each culture was incubated for 2 h to allow recovery of the bacteria before the isolated components were added at their respective MIC and 5 X MIC concentrations. Control cultures mimicking the active tests at MIC and 5 X MIC using appropriate concentrations of ethanol/water were also performed.

The data collected from kill kinetics with MIC and 5 X MIC for individual components were recorded on Microsoft Excel and analysed using Microsoft Excel, Sigma plot and MATLAB. Multi-way ANOVA was performed in all cases and statistically significant results were determined by a P value of less than or equal to 0.05. (2.14.3)
6.3.4.3 *Helicobacter pylori* strain 26695

Time-kill kinetics were determined using static liquid cultures containing Isosensitest broth (Oxoid) supplemented with 5% horse serum (Oxoid). The inoculum was harvested with Isosensitester broth from 36 h cultures grown on *Campylobacter* Selective Agar (CSA) (2.4.1).

6.3.4.4 *Escherichia coli* type 1 and *Staphylococcus aureus*

A 100 mL Isosensitest broth (Oxoid) culture was inoculated with a 10% inoculum from an 18 h overnight *E. coli* type 1 or *S. aureus* culture. The cultures were then allowed to grow to the stationary phase as described above.

6.3.5 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was performed on *H. pylori* strain 26695 in parallel with kill kinetics as described in Chapter 2 (2.15)
6.4 Results

6.4.1 Column Chromatography, GC-MS Spectrometry, High Resolution Mass Spectrometry, HPLC-APCI

Spectrum 6-1 shows the spectra from literature (Papageorgiou et al, 1997) in comparison with Spectrum 6-2 which was obtained by the described method in (2.5.3 and 2.5.4).

As can be seen the Spectra 6-1 and 6-2 show a strong correlation. Samples were run to obtain good resolution in order to use the retention time and mass spectrometry data of the Mastic gum components as a criterion to identify some of the atlantica gum components and compare the similarities.

Spectra 6-3, 6-4 and 6-5 obtained by GC-MS are from acidic fractions of Kurdica, Cabolica and Mutica gums respectively. Spectra 6-6-6-13 GC-MS, EI fragments that have been identified and listed in Table 6-1. Spectra 6-15 obtained by HPLC-APCI for further validation, their fragmentation spectra are listed in Appendix A6-1. The chemical compositions of the acidic fractions ‘A’ and ‘B’, their retention time, molecular weight, rotation and fragments intensity (in GC-MS) of Mastic, Kurdica, Mutica and Cabolica gums have been tabulated in Table 6-1.
Spectrum 6-1 GC-MS analysis of Mastic gum from literature (Papageorgiou et al., 1997)

Spectrum 6-2 GC-MS analysis of Mastic gum
Spectrum 6-3 GC-MS analysis of Kurdica gum

Spectrum 6-4 GC-MS analysis of Cabolica gum
Spectrum 6-5 GC-MS analysis of Mutica gum
Chapter Six

Spectrum 6-6 Mass spectrum (EI) of moronic acid (methyl ester)

Spectrum 6-7 Mass spectrum (EI) of oleanonic acid (methyl ester)

Spectrum 6-8 Mass spectrum (EI) of ursonic acid (methyl ester)
Spectrum 6-9 Mass spectrum (EI) of ursonic acid (methyl ester)

Spectrum 6-10 Mass spectrum (EI) of (iso)Masticadienonic acid (methyl ester)

Spectrum 6-11 Mass spectrum (EI) of 3-epiisomasicadienolic and Masticadienolic acids (methyl esters)
Spectrum 6-12 Mass spectrum (EI) of 3-O-Acetyl-3-epi(iso)Masticadienolic acid (methyl ester)

Spectrum 6-13 Mass spectrum (EI) of dihydroMasticadienonic acid (methyl ester)

Spectrum 6-14 Mass spectrum (EI) of dihydroMasticadienonic acid (methyl ester)
Table 6-1 Chemical composition of acidic fractions ‘a’ and ‘b’ of Mastic, Kurdica, Cabolica and Mutica gum

<table>
<thead>
<tr>
<th>No.</th>
<th>I.Time - F.Time</th>
<th>Mastic</th>
<th>Kurdica</th>
<th>Cabolica</th>
<th>Mutica</th>
<th>Common name/Systematic name</th>
<th>mp</th>
<th>$[\alpha]_D$</th>
<th>M</th>
<th>m/z characteristic ions of methylated compounds</th>
</tr>
</thead>
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<tr>
<td>1</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>CHCl3 468 (99), 243 (65), 189 (30)</td>
</tr>
<tr>
<td>2</td>
<td>34.267– 34.600</td>
<td>0.71</td>
<td>0.39</td>
<td>0.53</td>
<td></td>
<td></td>
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<td>CHCl3 468 (61), 249 (55), 189 (100)</td>
</tr>
<tr>
<td>3</td>
<td>36.517– 36.783</td>
<td>0.85</td>
<td></td>
<td>0.23</td>
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<td>CHCl3 468 (82), 242 (53), 189 (100)</td>
</tr>
<tr>
<td>4</td>
<td>37.983– 38.350</td>
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<td>11.97</td>
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</table>
Figure 6-2 Methyl moronate

Figure 6-3 Methyl oleanonate

Figure 6-4 Ursonic acid (Methyl ester)

Figure 6-5 Methyl oleanolate
Figure 6-7 (R=O), Figure 6-7 (R=α-OH, H) and Figure 6-10 (α-CH₃COO)

Figure 6-6 (R=O), Figure 6-9 (R=β-OH, H) and Figure 6-11 (α-CH₃COO)
6.4.2 HPLC –Atmospheric Pressure Chemical Ionisation (HPLC-APCI)

The components that were listed in Table 6.1 isolated by HPLC-APCI as well (2.7.3) for further validation (Spectra 6-15), the individual spectra are included in Appendix A6.1. The spectra are correlating with those published in the literature (Van der Doelen et al, 1998).

Spectrum 6-15 HPLC-APLC analysis of Acidic Fraction 'A' of Kurdica gum
6.4.3 Minimum Inhibitory Concentration

The MIC results have been tabulated in Tables 6-2-6-4 for all the components of acidic fractions of the Mastic, Kurdica, Mutica and Cabolica gums, against the strains of *H. pylori* listed in Table 2.1 and all other Gram-positive and Gram-negative bacteria listed in Table 2.2.

The MIC values for the components listed in Table 6-1 ranged from 0.1-50 µg/mL against the strains of *H. pylori* and all other Gram-negative bacteria (Table 6-2 and 6-3) and ranged from 2-100 µg/mL against Gram-positive bacteria (Table 6-4).

6.4.4 Kill Kinetics

The rate of killing for all the components was almost constant (P < 0.05) in their respective MIC and 5MIC and no statistically significant differences were observed (2.14.3).

![Diagram of kill kinetics](image)

Figure 6-12 The typical kill kinetics graph of 3-O-acetoxy-3-epiisoMasticadienolic acid against *H. pylori* 26695 has shown in Figure 6-12.
6.4.5 Transmission Electron Microscopy

Analysis of the strain 26695, treated with the components listed in Table 6-2 (1 X MIC), using TEM showed progressive changes in the morphology of this strain. As the change of cell morphology in the cells treated with all the components were similar, only the one that was treated with Masticadienonic acid is shown here.

As it is shown in Figure 6-12 initially the cells are bacilli shaped with an electron dense cytoplasm with inclusion bodies present in majority of them with outer cell membrane closely fit with cell wall. As the exposure time elapsed, a growing number of cells becoming coccoid increased and the background electron density of the cytoplasm reduced. At 12 h, electron dense particles were present within the cytoplasm and periplasm and within lining of the outer membrane and blebbing in outer membrane.

Unlike the isolated components treated culture, the control cultures all remained viable for the 12 h duration of the experiment, displaying bacilli shape, an electron dense cytoplasm with some inclusion bodies.
Figure 6-13 The effect of Masticadienonic acid on cellular structure (H. pylori 26695); a: at time zero, b: at 5 h, c: at 12 h, no viable bacteria was cultured at this point. 1: inclusion bodies, 2: electron dense cytoplasm, 3: loss of electron dense cytoplasm, 4: electron dense particles.
Table 6-2 The MIC values of the isolated components of the acidic fractions of the gums against the strains of *H. pylori* (µg/mL)

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<th>Sub-fractions of acidic fractions of the gums</th>
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<th>RSB6</th>
<th>P10</th>
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Table 6-3 The MIC values of the isolated components of the acidic fractions of the gums against the Gram-negative bacteria (µg/mL)

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<th>Salmonella typhimurium</th>
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Table 6-4 The MIC values of the isolated components of the acidic fractions of the gums against the Gram-positive bacteria (µg/mL)

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<tr>
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</table>
6.5 Discussion

The chemical characteristics of the specific components of Mastic gum, galls from *P. lentiscus* and *P. vera* are well known. Some of these characteristics were used as authentic data (Monaco *et al.*, 1973).

The characteristics of isolated components of acidic fractions of Mastic, Kurdica, Mutica and Cabolina gums such as molecular weight, GC-MS data, melting point and optical rotations listed in Table 6.1 correlate with literature (Barton & Seaone, 1956; Papageorgiou *et al.*, 1997; Seoane, 1956).

When this study began in January 2000, no published data were available on antimicrobial activity of the Mastic gum and other related gums and their fractions. The first abstract from this work was published in June 2001 (Sharifi *et al.*, 2001). Soon after that abstract was published, a short paper was published reporting on the antimicrobial activity of whole Mastic gum (not defined fractions) against *H. pylori* (Marone *et al.*, 2001). In 2003 a patent was published reporting the antimicrobial activity of the Mastic as a whole together with a mixture of some of the fractions, that is not pure chemical entities (Fotinos *et al.*, 2003).

In this discussion the data obtained from this study are discussed making reference to relevant published data by way of comparison.

Compound No. 4 in Table 6.1 constitutes 11.20%, 11.97%, 7.38% and 8.20% of the acidic fractions of Mastic, Kurdica, Cabolina and Mutica gums respectively. The retention time of this compound was slightly different from a compound designated by a number of researchers to be methyl moronate (Papageorgiou, Bakola-Christianopoulou *et al.* 1997). The observed differences were not considered to be material as the data
generated here employed a different instrument and columns and based on appropriate analysis were statistically insignificant (P > 0.01), (Spectra 6-1 and 6.2). Thus compound No. 4 was assigned as methyl moronate (Spectrum 6-6) with oleanane base, having the observed mass spectra (GC-MS) peaks at m/z 468, 409, 249 and 189 (Spectrum 6-6). The molecular ion was observed at m/z 468, the peak m/z 409 indicating the loss of carbomethoxy substituent at C-17. This assumption is confirmed by peak m/z 249 and the loss of carbomethoxy group, resulting in the most intense peak (100) observed at m/z 189 (Fig. 6-14).

![Mass spectra fragments of methyl moronate](image)

The Melting Point (MP) of this methyl ester was 145-147º and the MP of its corresponding acid collected from column chromatography was 218-220º, which being further confirmation of this structure. The stereochemistry of this structure was then confirmed by polarimeter giving [$\alpha$]$_D$ +59 (CHCl$_3$, c 0.2 at 20º) for methyl ester and [$\alpha$]$_D$ +60 for its corresponding acid, which correlates with data in the literature (Gonzalez, Amaro et al. 1983).
Compound No. 5 in table 6.1 constitutes 7.87%, 4.93%, 4.80% and 5.20% of the acidic fractions of Mastic, Kurdica, Cabolica and Mutica gums respectively. This compound has previously been identified and isolated from Mastic gum by a number of researchers with its retention time correlating to methyl oleanonate (Ahsan et al., 1995; Gonzalez et al., 1983; Pozzo-Balbi et al., 1978; Stern, 2003).

The molecular ion of methyl oleanonate (Spectrum 6-7) was observed at m/z 468, indicative an oleanane skeleton base. The peak at m/z 408 is indicative of the loss of carbomethoxy group. The fragments m/z 408 (A – COOMe), 262 (B) and the most intense peak at m/z 203 (Fragment E) indicate a double bond at C-12, a carbomethoxy substituent at C-17 and an oxo at C-3 (Fig. 6-15).

The MP of oleanonate was 180-182º, it rotated polarized light $\left[\alpha\right]_D +85^\circ$ (CHCl$_3$, c 0.2 at 20º) for methyl ester and +75 for its corresponding acid, which correlates with the literature (Gonzalez, Amaro et al. 1983).

Figure 6-15 Mass spectra fragments of methyl oleanonate
Compound No. 6 has a molecular ion of 468 (Spectra 6-8). It correlates to the spectra of ursonic acid (methyl ester) as reported in the literature (Van der Doelen et al, 1998). Compound No. 6 has not previously been reported as a constituent of Mastic. Compound No. 6 constitutes 0.76%, and 0.31%, in Kurdica and Mutica gums respectively but was not detected in Cabolica. This compound is found in Dammar resin (Brewis & Halsall, 1961) and the resin from the Daemonorops draco (Nasini & Piozzi, 1981). The molecular ion of ursonic acid (Spectrum 6-7) was observed at m/z 468, Figure 6-16 (A) indicative an oleanane/ursen skeleton base, m/z 409 and (B) indicative of the loss of carbomethoxy group, pointing to a carboxylic acid substitution. The most intense fragments was observed at m/z 262 (C), this fragment and the signal at m/z 203 (D) indicates a double bond at C-12, a carbomethoxy substituent at C-17 and an oxo at C-3 (Fig. 6.4). Its mp was 174-176º, and it rotated polarized light [α]D +84º (CHCl₃, c 0.2 at 20ºC).

Figure 6-16 Mass spectra fragments of ursonic acid methyl ester
Compound No. 10 has a molecular ion of 470 (Spectra 6-9) and correlates with the spectra of oleanolate acid (methyl ester) as described in the literature (Papageorgiou et al, 1997). It constitutes 0.53%, 3.39%, 1.02% and 2.80% of the acidic fractions of Mastic, Kurdica, Cabolica and Mutica gums respectively. Compound 10 has been previously identified and isolated from Mastic gum by Papageorgiou et al (1997). The molecular ion of oleanolic acid (Spectrum 6-9) was observed at m/z 470 followed by m/z 409, Figure 6-17 (A and B) indicative an oleanane skeleton base, the fragment m/z 409 (B) is indicative of the loss of carbomethoxy group and hydrogen of hydroxyl group on C-3, pointing to a carboxylic acid and a hydroxyl group substitute. The m/z 262 (C), and the most intensive peak m/z 203 (Fig. 6-5 C) indicate a double bond at C-12, a carbomethoxy substitute at C-17 (Fig. 6-5 E). Its MP was 196-198° with the rotation of [α]D +84° (CHCl₃, c 0.2 at 20°C).

Figure 6-17 Mass spectra fragments of oleanolic acid methyl ester
Compounds No. 11, 12, 16, 19, 20 and 24 are triterpenoids with many structural characteristics similar to steroids. However, despite the similarities they are not steroids as they lack all the characteristics of true steroids. That is, the formula of steroids, C_{27}H_{46}O, is not observed in the terpenoid. Notwithstanding, a 30-carbon triterpene is a precursor that has been converted by an enzyme-catalysed reaction into cholesterol. The structural analysis of triterpenoids fragments in this study was mainly based on a comparison with the published data of triterpenes and the recorded spectra of analogue steroids.

Compound No. 11 and 16 with a molecular ion of 468, have an identical mass spectra (Spectra 6-10) with different retention time. Their mass spectra and retention times can be correlated to isoMasticadienonic acid and isoMasticadienonic acid (methyl esters) respectively (Papageorgiou et al., 1997). They constitutes 30.74% and 40.13 in acidic fractions of Mastic, 14.79% and 20.06 in Kurdica, 13.16 and 21.11 in Cabolica and 20.50, 32.90 in Mutica respectively.

They have a lanosta skeleton base with the most intense peak (100) at m/z 453 (Fig. 6-18) indicative of the loss of a methyl group followed by the loss of methanol resulting to an observed peak at m/z 421. The MP of the isoMasticadienonic and Masticadienonic acids collected by column chromatography were 178-180° and 166-168° (both from ether-light petroleum) with the optical rotation of [α]_{D} -78° and +35 (CHCl_{3}, c 0.2 at 20°C) respectively.
The structure was further confirmed by MP of their corresponding methyl esters 110-112º and 123-124º with the optical rotations of $[\alpha]_D^\circ +37^\circ$ and $-71^\circ$ respectively. These structures were then validated by HPLC-APCI Appendix A 6.1.

Compounds No. 12 and 20 are distinguished from isoMasticadienonic and Masticadienonic acids by their characteristic peaks at m/z 455 and 437, indicating a hydroxyl substitute on C-3, pointing to the loss of methyl group from m/z 470 followed by the loss of water (Fig. 6-19), resulting the presence of two strong peaks at m/z 455 and 437 (Spectra 6-11). Their GC-MASS spectra are correlating with methyl-3-epiiso-Masticadienolic acid and methyl Masticadienolic acid (methyl esters) with MP 140-142º and 121-122º and rotations of $[\alpha]_D^\circ +12^\circ$ and $-44^\circ$ (CHCl$_3$, c 0.2 at 20ºC) respectively. Compound 12 constitutes 0.87% of the acidic fractions in Mastic, 0.1.16% in Kurdica, 0.43% in Cabolica and 1.10% in Mutica. Compound 20 constitutes 0.79%, 0.62%, 3.45% and 0.71% of acidic fractions of Mastic, Kurdica, Cabolica and Mutica respectively.
Compound No. 18 with a molecular ion of m/z 470 was found only in Kurdica gum (Spectrum 6-12). Its CI mass spectrometry following column chromatography showed one single peak with no fragmentation at all at peak m/z 455. It constituted 0.60% of the acidic fractions of Kurdica gum.

It had an lanosta skeleton base with the most intense peak (100) at m/z 455 (Fig. 6-20) indicative of the loss of a methyl group followed by the loss of methanol resulting to an observed peak at m/z 423. Its MP was 90-92º with the optical rotation of [α]D -75 (CH3OH, c 0.2 at 20ºC) and correlated with dihydroMasticadienonic acid methyl ester (Barton & Seaone, 1956).
Compound No. 23 is distinguished by its characteristic peaks at m/z 472 and 457, 454 and 439 indicating a hydroxyl substitute on C-3, pointing to the loss of methyl group from m/z 472 and the loss of water (Fig. 6-21). This resulted in the presence of two strong peaks at m/z 454 and 439 (spectra 6-13). Its MP of 115-116º and optical rotations [α]D -44º and -44º (CHCl₃, c 0.2 at 20ºC) correlates with dihydroMasticadienolic acid methyl ester (Barton & Seaone, 1956). Compound 23 was only found in Kurdica gum and constitutes 1.06% of the acidic fractions of this gum.
Compounds No. 24 and 25 are correlated in retention time and GC-Mass spectrum with methyl 3-acetoxy-3-epi-isoMasticadienolate and 3-acetoxy-3-epi-Masticadienolate (Spectra 6-12 and 6-13) respectively. The observed peak at m/z 512 in compound No. 24 is the major differences between these two compounds. The observed peak at m/z 497 in both spectra is indicative of the loss of methyl group, and the peaks at m/z 452 and m/z 437 are indicative of the loss of acetoxy substitute attached to C-3 (Fig. 6-22). These compounds (methyl esters) had MP of 118-122° and 100-102° and optical rotations of $[\alpha]_D +22^o$ and -45° (CHCl$_3$, c 0.2 at 20°C) respectively.

The spectrum of compound No. 19 correlates to 3-O-acetyl-3-epi-isoMasticadienolic acids. This compound constitutes 9% of the acidic fractions of the Kurdica gum and was only found in this gum. The presence of this triterpenoid was the major differences in terms of constituents having antimicrobial activity between Kurdica gum and all other gums examined. There were some other constituents that did not appear to have substantial antimicrobial activity, subsequently no attempt was made to characterise these. Compounds No. 19 had an identical GC-MS with compounds 24 and
25; however, it had a different MP of 85-87° and optical rotation of \([\alpha]_D -2^o\) (CHCl₃, c 0.2 at 20°C) and correlated to 3-O-acetyl-3epi-iso-Masticadienolic acid.

Important features of the chemical entities identified and isolated above is their antimicrobial activity. Most of the chemical entities above have not been tested for antibacterial activity previously, particularly with respect to *H. pylori*. Thus it was important to further characterise these compounds with respect to their capacity to inhibit or kill bacteria.

The antimicrobial screening of these sub-fractions led to fundamentally new information that went beyond *H. pylori*, expanding the original parameters of the project. Such was the extent of these findings that a new class of antibiotics has emerged and their structure were characterised. The mechanism of their action and structural related activities will be discussed in next chapter. Furthermore, the potential to enhance the antimicrobial activity of antibiotics has been incremented and as a result the ability to design a new class of antibiotics has become possible.

The MIC values for moronic acid ranged from 5-20 \(\mu g/mL\). The strain SS1 was more sensitive with MIC 5 \(\mu g/mL\) (Table 6-2). MIC values ranged for all other Gram-negative bacteria tested from 10-20 \(\mu g/mL\) (Table 6.3) and for Gram-positive bacteria ranged from 50-100 \(\mu g/mL\) (Table 6-4). Antimicrobial activity of moronic acid isolated from *Ozoroa mucronata* has been previously reported in 1979 (Hostetttmann-Kaldas & Nakanishi, 1979). However, antiviral activity of this substance is well known and it is reported to be active against Herpes (Kurokawa *et al*, 1999). Purified Moronic acid from *Rhus javanica* has shown significant anti-HSV activity *in vitro* and *in vivo* with therapeutic index of (10.3–16.3). The effective concentrations for 50% plaque reduction of moronic acid for wild type HSV type 1 (HSV-1) was 3.9 mg/mL (Kurokawa *et al*, 1999). Moronic acid has also been isolated from
*Myrceugenia euosma* and shown significant anti-HIV activity with therapeutic index of over 186 (Singh *et al.*, 2005). This substance and its derivatives were classed “Structure I” for structural analyses, which will be discussed in Chapter 7 (Table 6-5).

The MIC values for oleanonic acid against the nine strains of *H. pylori* ranged from 5-10 µg/mL. Oleanolic acid and ursonic acid were less active against the 9 strains of *H. pylori* with MIC values ranged from 25-100 µg/mL (Table 6-2), for all other Gram-negative bacteria the MIC was 50 µg/mL and for all other Gram-positive bacteria the MIC was 100 µg/mL (Table 6-3 and 6-4). Antimicrobial activity of oleanonic acid and its derivatives have not been previously reported. Oleanonic and ursonic structures and their derivatives were classed “Structure II and Structure III” respectively (Table 6-5). Lanosta base skeletons were classed Structure IV, V and VI table 6-6.

MIC values for Masticadienonic acid, isoMasticadienonic acid and Masticadienolic acid against 9 strains of *H. pylori* was 5µg/mL. Testing Masticadienonic acid against the strain P10 of *H. pylori* yielded an MIC of 10 µg/mL (Table 6-2). Masticadienonic acid and isoMasticadienonic acid had MIC values of 5 µg/mL and Masticadienolic acid, 2 µg/mL against all other Gram-negative bacteria (Table 6-3). Their MIC values against Gram-positive bacteria ranged from 5-10 µg/mL; (Table 6-4) these three compounds are designated structures IVb, VIb and IVd respectively (Table 6-6).

MIC values for dihydroMasticadienonic acid against all the strains of *H. pylori* and all other Gram-positive and Gram-negative bacteria listed in Table 2-1 and 2-2 ranged from 1 to 5 µg/mL. The MIC values of dihydroMasticadienolic acid exhibited a tight cluster ranging from 0.5-2 µg/mL (Table 6-2). This compound is designated structure Va (Table 6-6). MIC values for 3-epi-isoMasticadienolic acid against *H. pylori* strains ranged 5-10 µg/mL. This MIC was higher than that observed for all other Gram-
negative bacteria at 1 µg/mL and was similar to that observed in Gram-positive bacteria 5 µg/mL (Table 6-2-6-4). No particular antimicrobial pattern with respect to Gram-positive and Gram-negative bacteria was identified. This compound exhibited broad spectrum activity with an MIC ranged of 1-10 µg/mL and is designated structure VIId (Table 6-6).

MIC values for 3-acetoxy-3-epiisoMasticdienolic acid and 3-acetoxy-3-epiMasticdienolic acid ranged from 0.1-0.5 µg/mL against H. pylori, 1 µg/mL against all other Gram-negative bacteria tested with the exception of E. coli which had an MIC of 5 µg/mL. When tested against Gram-positive bacteria these agents exhibited an MIC of 2 µg/mL (Table 6-2-6-4).

The most active chemical isolate was 3-O-acetoxy-3-epiisoMasticdienolic acid with the MIC values ranged from 0.01 to 5 µg/mL against all the strains of H. pylori and all other bacteria listed in Table 2-1 and 2-2. This compound is found only in the acidic fraction of the Kurdica gum (Table 6-1). This compound is designated structure VIj (Table 6-6) and constitutes 9.00% of this fraction. Hypothetically, the higher activity of the acidic fraction of Kurdica gum in comparison to acidic fractions of the other gums may be attributed to this compound. Antimicrobial activities of all the Structure IV, V and VI and their derivatives have not previously been reported (Table 6-6) thus the identification and characterisation of these compounds may represent an important finding that could lead to the development of a novel class of antimicrobial agents that may have application in the treatment of infectious disease.

Antibacterial agents can be bactericidal or bacteriostatic. The difference can be significant clinically. Bactericidal agents may be more effective in the treatment of disease particularly in immuncompromised individuals. The isolated components were all bactericidal, the typical kill kinetics graph of 3-
O-acetoxy-3-epiisoMasticadienolic acid against *H. pylori* 26695 has shown in Figure 6-12.

The isolated components were divided into two major groups; Olean base skeleton, with three sub-group of Structure I, II and III (Table 6-5) and Lanosta Base skeleton with three sub-group of Structure IV, V and VI (Table 6-6). These components mimic steroid compounds, and the known antibiotic Fusidic acid. As a consequence an investigation of the possible mode of the action/s was undertaken which is reported in Chapter 7.

Table 6-5 Chemical structure of isolated components of the gums

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<tr>
<th>Structure I</th>
<th>Structure II</th>
<th>Structure III</th>
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<td>a: R= O, RI= COOMe</td>
<td>a: R= O, RI= COOMe</td>
<td>a: R= H, α-OH, RI= CHO</td>
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<td>Moronate</td>
<td>Oleanonate</td>
<td>Ursonic aldehyde</td>
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<tr>
<td>Moronic acid</td>
<td>Oleanonic acid</td>
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</tr>
<tr>
<td>c: R= O, RI= CHO</td>
<td>c: R= H, β-OH, RI= COOMe</td>
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<tr>
<td>Moronic aldehyde</td>
<td>Oleanolate</td>
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</tr>
<tr>
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<tr>
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<td>Oleanolic aldehyde</td>
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203
Table 6-6 Chemical structure of isolated components of the gums

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<tr>
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<tr>
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<tr>
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<td></td>
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<tr>
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<tr>
<td>Masticadienolic acid</td>
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**Structure V**

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<tr>
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<tr>
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<tr>
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Chapter 7
Structural Analysis of the Isolates from the Acidic Fractions of the Gums and Their Mode of the Action

7.1 Introduction

As discussed in Chapter 6, isolated triterpenoids from acidic fractions of the gums studied here are structurally similar to steroids; however, they are not steroids as their characteristic numbers of carbon atoms are different. The formula of steroids $C_{27}H_{46}O$ does not fit the expected pattern of a triterpenoid. A 30-carbon triterpene is a precursor that can be converted by an enzyme-catalyzed reaction into cholesterol.

The chemical isolates characterized in Chapter 6 can be divided into two major groups based on their skeleton being olean and lanosta. The structural analysis of these triterpenoids in relation to their antimicrobial activities has been discussed in Chapter 6.

As these components mimic steroid compounds, including particularly the antibiotic Fusidic acid, action was taken to investigate the possible mode of action/s of the extracts in comparison to the mode of action of Fusidic acid.

The steroid antibiotic Fusidic acid was first isolated in 1962 from *Fusidium coccineum* (Godtfredsen *et al*, 1962). This antibiotic, chemically has a basic structure of an anti-inflammatory steroid and not other biologic steroids (Crosbie, 1962), so they do not have the physiological action of the steroid hormones (Johnsen, 1962). Fusidic acid is chemically related to Cephalosporin P and Helvelic acid, antibiotics that have not been developed commercially (Decazes & Vodant-Modai, 1988; Mainardi, 1997). Cephalosporin was first isolated in 1951 from the genus *Cephalosporium*
Cephalosporins represent an important class of broad spectrum antibiotics that have undergone continuing evolution. For example, a Cephalosporin has recently shown to be active against Bangladeshi diarrheagenic Hafina alvei-like strain that has been recently classified as a new species *Escrichia albertii* (Stock *et al.*, 2005).

It is known that Fusidane type compounds inhibit protein synthesis in bacterial systems of *E. coli*. Fusidic acid does not cross the outer membrane of Gram-negative bacteria, so the cell lysate and purified ribosome of *E. coli* are used for protein inhibition assays of Fusidic acid (Ovchinnikov *et al.*, 1982). Fusidic acid inhibits bacterial protein synthesis by inhibiting dissociation of Elongation Factor G (EF-G) which is a protein of M 74000, (Tanaka *et al.*, 1968) and the GDP-ribosome in the cyclic reaction and translocation of peptidyl-tRNA from the acceptor site to the donor site on the ribosome. (Bodley *et al.*, 1969; Okura *et al.*, 1970). It was also suggested by Tanaka *et al.*, using puromycin reaction, that these antibiotics inhibit translocation of peptidyl-tRNA on the ribosomes (Tanaka *et al.*, 1968). Fusidane type compounds inhibit GTPase activity that is observed in combination of the ribosomes and EF-G. The localization of Fusidic acid action with respect to the ribosome and EF-G of drug-sensitive *E. coli* cells and those of resistant cells has shown that the Fusidic acid sensitivity is associated with EF-G and not with the ribosome (Kinoshita *et al.*, 1968).

There are three main steps in chain elongation in which one amino acid is added to the nascent polypeptide chain (Liljas, 1990; Spirin, 1985). These steps are:

1. The correct aminoacyl-tRNA (Fig. 7-1) positions in the A site of the ribosome (Zavialov & Ehrenberg, 2003), this step is catalyzed by Elongation Factor Tu (EF-Tu) that is a monomeric protein of M,
43000 that has a binding site for GTP (Nissen et al., 2004). EF-Tu-GTP-aminoacyl-tRNA molecules form a ternary complex that is thermodynamically favoured as all the aminoacyl-tRNA molecule in vivo is found in this ternary complex (Nilsson & Nissen, 2005).

Then ternary complexes bind to the A site if the codon and anticodon matches. Once the correct base pairs are formed between the anticodon of an aminoacyl-tRNA and the codon of the A site, then the ternary complex is repositioned into the P site. These repositioning hydrolyses GTP to GDP and Pi' and the EF-Tu-GDP complex leaves the ribosome (Valle et al., 2003). Another elongation factor called EF-Ts catalyses the phosphorylation of GDP into GTP enabling EF-Tu-
GTP to bind with another aminoacyl-tRNA (Hilgenfeld, 1995; Wurmbach et al, 1979). Basically, GTP is hydrolysed for every aminoacyl-tRNA that docks at the A site successfully by EF-Tu (Fig. 7-2) (Moran et al, 1994; Pingoud et al, 1990).

**EF-Tu:Ts cycle for binding aa-tRNAs**

![Diagram](image)

Figure 7-2 EF-Ts cycle for binding aa-tRNAs (PennState)

2. In the second step, once peptide bond has formed, the newly formed peptidyl-tRNA is situated partially in A and partially in P sites, as shown in Figure 7-3 (Moran et al, 1994; PennState).

3. In the third step the deacylated-tRNA is displaced from the P site to the E site and should be released allowing next codon to be translated (Fig. 7-3) (Moran, 1994 #4804).
As a consequence of the above, the peptidyl-tRNA should move completely from the A site to P site with the movement of the mRNA by one codon. This step is called the translocation (Moran et al., 1994) (Fig. 7-4). Translocation reaction is the specific site of Fusidic acid attack.

Figure 7-3 Three sites of interaction with tRNAs (PennState)

Figure 7-4 Translocation step (PennState)
As it is shown in Figure 7-5 these reactions are catalyzed by EF-G and GTP which forms a labile complex with the ribosome (Misumi & Tanaka, 1980). Fusidic acid forms a stable complex with EF-G, GTP and the ribosome and prevents the release of EF-G from the ribosome that is required to hydrolyse GTP for the next round of translocation (Fig. 7-5) (Beres & Lucas-Lenard, 1973).

![Figure 7-5 The mechanism of Fusidic acid; inhibition of the GTPase reaction(Okura et al, 1971)]
The structure of Fusidic acid was determined by crystallography in 1966, (Cooper, 1966) confirming the structure that was proposed by Godtfredsen et al in 1962. Godtfredsen et al (1962) determined the structure from calculated three dimensional Patterson and electron density distributions and from partly refined by square calculations (Cooper, 1966). Cooper et al (1966) determined the crystal structure and absolute configuration of Fusidic acid methyl ester 3-p-bromobenzoate by X-ray diffraction methodology. The Fusidic acid structure was solved from the 3-dimensional Patterson and Fourier syntheses and was refined by block-diagonal least-squares to a final R factor. For the first time the unusual configuration of the steroid skeleton was revealed. It was known to have the configurations 3α-OH, 4α-CH3, 5α-H, 8α[middle dot]CH3, 9β-H, 10β-CH3, 11α-OH, 13α-H, 14β-CH3, 16β-OCOCH3 with all ring junctions trans fused and rings A and C syn to ring B. Rings A and C had chair conformations while ring B was a flattened boat, giving a unique characteristic structure to this antibiotic, distinguishing from the steroid hormones and known common triterpenoids structures (Cooper & Hodgkin, 1968). However, this structure is still classed as a triterpenoid.

Fusidic acid is slightly soluble in water; however, its sodium salt, Fucidin, is readily soluble in water. Fusidic acid (Fucidin, sodium salt of Fusidic acid) is bacteriostatic in low concentrations and bactericidal in concentrations of 0.12 µg/mL or higher (Coutant et al, 1996; McDonald, 1965; Turnidge, 1999). Fucidin stops the growth of bacteria after approximately 2 minutes after that addition of the antibiotic to a culture of bacteria in vitro. However, it was noted that DNA and RNA synthesis may continue up to two hours following the addition of Fucidin (Jensen & Lassen, 1964).

Most wild strains of Staphylococcus, (Garaud & Vachon, 1985) Streptococcus, Neisseria, Corynebacterium, Clostridium and some mycobacteria are sensitive to Fusidic acid. Fusidic acid has limited activity against Gram-negative bacteria and that is believed to be due to differences in cell wall permeability
This is similar to Penicillin G which is active against Gram-positive bacteria and Neisseria spp. but not other Gram-negative bacteria due to outer membrane permeability.

Fusidic acid is used to treat the primary topical skin infections such as impetigo contagiosa, erythrasma and secondary skin infections (Wheat et al., 1984) such as infected wounds and infected burns caused by sensitive strains of S. aureus, Streptococcus species and C. minutissimum (Collignon & Turnidge, 1999).

Resistance to Fusidic acid has recently increased in hospitals where cross infections are common. The prevalence of resistance seems to be higher in methicillin-resistant strains of S. aureus (Irish et al., 1998; Wolff, 1997). Resistance occurs by a number of mechanisms, which includes alterations in EF-G and drug permeability. However, the frequency of resistance is not high in clinical practice. Nevertheless, evidence suggests that Fusidic acid in combination with other antibiotics results in less resistance emergence (Turnidge & Collignon, 1999).

Fusidic acid has been shown to have the capacity to stabilize the ribosome-translocation factor-GDP complex. It was shown that the presence of a carboxyl group at C-20 and a 17, 20-double bond group appear to be critical in forming the complex. It was also shown that the geometry of the double bond (16, 21-cis) is the most essential feature to this unique activity (Fig. 7-6). It was concluded that the nature and stereochemistry of the other functional groups are important but not essential (Bodley & Godtfredsen, 1972).
The compounds investigated in this study were extracts from plants that exhibited characteristics similar to steroids and Fusidic acid. While not having the structural characteristics of Fusidic acid (Crosbie, 1962; Duvold et al., 2001) there remains a potential that the triterpinoids extracted from Mastic and other gums may act through a mechanism interfering with ribosome function.

The objectives of the work outlined in this chapter were as follows:

7.2 Objectives:

1. To investigate the mode of action/s of the isolated components by comparison to the mode of the action of Fusidic acid.

2. To run protein inhibition assays using Fusidic acid in parallel to isolated components of the gums.

3. To analyse the isolated components structurally in order to find the common structure/s that have/would have same mechanism of action.
7.3 Experimental

7.3.1 Culture of *Escherichia coli*

*E. coli* type 1 (UNSW 048200) was obtained from the culture collection of University of New South Wales (UNSW). The bacteria was recovered overnight and re-cultured on LB plates. Plate was incubated at 37°C in an aerobic environment for 18 hours.

A single colony was used to inoculate with 20 mL of pre-warmed LB medium in a 125 mL flask. The flask was then incubated overnight at 37°C. Then 2 x 1L of pre-warmed LB medium were inoculated with 10 mL of overnight culture and incubated with shaking at 37°C to obtain a culture with an OD$_{600}$ = 0.6-0.8. Following incubation, the resultant cells were chilled on ice for 10 min. and then harvested by centrifuging in a GS3 rotor at 5000 rpm for 10 min at 4°C. The details of this protocol have been described in Chapter 2 (2.16.1).

7.3.2 Ribosome Purification

Critical to the successful isolation of ribosomes from the bacterial cells is the realization that these sub-cellular organelles are quite fragile. Successful isolation depends on appropriate handling and care with the selection of buffers and other reagents. In the protocol used here only those buffers that are listed in the method were used (A2.5.11).

Ribosomes are highly susceptible to nucleases, with ribonuclease contamination from the skin of the experimentalist being a common risk. To mitigate such risk, gloves were worn while handling ribosomes (and during the entire preparation procedure) and the gloves were changed often.
All glassware and tubes were cleaned and sterilised prior to use. As RNAses (as opposed to DNases) are heat stable, and can thus survive autoclaving, all glassware and other materials were DEPC-treated to inactivate nucleases (as described in the protocol). After treatment none of the glassware or tubes were touched with bare hands.

It was very important to purify the ribosome to validate the experiments as it would be difficult to determine from SDS-PAGE alone whether the EF-G band is derived from ribosome-bound or free EF-G. The details of the purification and the protocol that was used have been described in Chapter 2 (2.16.1).

Briefly, one portion of S30 extract (supernatant from step 6 of the protocol, which contains ribosomes and EF-G) was incubated with the compounds under investigation for 20 min at 0° prior to preparing an S100 extract. As a control, one portion was incubated with Fusidic acid using identical methods. This allowed the Fusidic acid to stabilise EF-G on the ribosome and the test compounds; if this was in fact the mechanism by which the test compounds interact with ribosomes.

Following the procedure outlined above, the S30 extract was centrifuged to make the S100 fraction (the first part of step 7 of the protocol). The ribosome pellet was then re-suspended and run on an SDS-PAGE gel to observe differences in the amount of the EF-G in the pellet between extracts that were incubated with the test compounds versus extracts that were not exposed to the test compounds. The same procedure was performed using Fusidic acid as a reference. Control (blank) was incubated under identical conditions in the absence of Fusidic acid or the test compounds.
7.3.3 SDS-PAGE

Detecting complex mixtures of Elongation Factor G (EF-G)~GDP~ribosome~Fusidic acid/test compounds listed in Table 7-1 was carried out using sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) as described in Chapter 2. Precast Polyacrylamide Mini Gels (12%) were purchased from iGELS (iGELS Sydney Australia). Sample buffer (A2.5.9) was added to incubated extract with the test compounds/Fusidic acid and GTP, this was then heated in a boiling water for 5 min to denature proteins (Chapter 2).

7.3.4 Staining and Destaining of SDS-PAGE Gels

Following each run, the gels were stained with Coomassie brilliant blue solution over night. They were then destained (2.17.1) followed by washing in Milli-Q water. The gels were then placed in a clear plastic bag and photographed.
<table>
<thead>
<tr>
<th>Compound’s No. (CN)</th>
<th>Isolated components subjected to SDS-PAGE</th>
<th>Lane No. on the Gel</th>
<th>Incubated at 0° for 20 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank</td>
<td>2</td>
<td>Purified Ribosome (PR) (incubated with buffer + 1mM GTP)</td>
</tr>
<tr>
<td>2</td>
<td>Fusidic acid</td>
<td>4,18-19</td>
<td>FR (incubated with 1mM CN2 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>3</td>
<td>Moronic acid</td>
<td>3</td>
<td>FR (incubated with 1 mM CN3 + 1mM GTP)</td>
</tr>
<tr>
<td>4</td>
<td>Oleanonic acid</td>
<td>11</td>
<td>FR (incubated with 1mM CN4 + 1mM GTP)</td>
</tr>
<tr>
<td>5</td>
<td>Ursonic acid</td>
<td>12</td>
<td>FR (incubated with 1mM CN5 + 1mM GTP)</td>
</tr>
<tr>
<td>6</td>
<td>Oleanolic acid</td>
<td>13</td>
<td>FR (incubated with 1mM CN6 + 1mM GTP)</td>
</tr>
<tr>
<td>7</td>
<td>Acidic Fractions (Kurdica gum)</td>
<td>5</td>
<td>FR (incubated with 1mM CN7 + 1mM GTP)</td>
</tr>
<tr>
<td>8</td>
<td>IsoMasticadienonic acid</td>
<td>6</td>
<td>FR (incubated with 1mM CN8 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>9</td>
<td>3-epi-isoMasticadienolic acid</td>
<td>7</td>
<td>FR (incubated with 1mM CN9 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>10</td>
<td>Masticadienonic acid</td>
<td>8</td>
<td>FR (incubated with 1mM CN10 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>11</td>
<td>DihydroMasticadienonic acid</td>
<td>9</td>
<td>FR (incubated with 1mM CN11 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>12</td>
<td>3-O-acetyl-3-epi(iso)Masticadienolic acid</td>
<td>10</td>
<td>FR (incubated with 1mM CN12 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>13</td>
<td>Masticadienolic acid</td>
<td>14</td>
<td>FR (incubated with 1mM CN13 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>14</td>
<td>DihydroMasticadienolic acid</td>
<td>15</td>
<td>FR (incubated with 1mM CN14 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>15</td>
<td>3-acetoxy-3-epiisoMasticadienolic acid</td>
<td>16</td>
<td>FR (incubated with 1mM CN15 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
<tr>
<td>16</td>
<td>3-acetoxy-3-epiMasticadienolic acid</td>
<td>17</td>
<td>FR (incubated with 1mM CN16 + 1mM GTP) co-sedimentated with EF-G</td>
</tr>
</tbody>
</table>
7.4 Results

Figure 7-6 and 7-7 showed the results obtained from poly-acrylamide gel electrophoresis (SDS-PAGE).

Lane 1 is the molecular mass marker while lanes 3 to 19 are a comparison of Fusidic acid/test compound dependent co-sedimentation with 70s ribosome. Lane No. 2 is a purified ribosome that has not been incubated with Fusidic acid or test compounds but with 1mM buffer H\textsuperscript{10}M\textsuperscript{10}A\textsuperscript{50}\beta\textsuperscript{5} (A2.5.11) instead, followed by 20 min incubation at 0\textdegree.

As it is shown in figure 7.6 no band of 74kDa has been observed in this lane (Lane 2) indicating of non co-sedimentation of EF-G with ribosome 70s due to lack of Fusidic acid.

Clear strong band of 74kDa in lanes 4, 18 and 19 were observed. This is indicative of co-sedimentation of Fusidic acid with the ribosome 70s (Fig. 7-6 and 7-7).

Compounds 3-6 (indicated by CN in the table 7-1) have not been bound with EF-G and ribosome 70s, as no band of 74kDa was observed (Fig. 7-6 and 7-7).

A diffuse but strong band was observed in lane 5 for the acidic fractions of Kurdica gum, which contains a mixture of compounds. Given the mix of compounds it may be expected that the SDS-PAGE would yield such a product.

For the compounds 8-16 a band was present corresponding to \(\sim 74\) kDa, indicating co-sedimentation of these compounds with ribosome 70s and GDP (Fig. 7-6 and 7-7; lanes 6-10 and 14-17).
The results from the gels has been summarized in Table 7-2.

<table>
<thead>
<tr>
<th>Compounds No. (CN)</th>
<th>Isolated components subjected to SDS-PAGE</th>
<th>Lane No. on the Gel</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Blank</td>
<td></td>
<td>2</td>
<td>No band at 74 kDa</td>
</tr>
<tr>
<td>2 Fusidic acid (FA)</td>
<td></td>
<td>4, 18–19</td>
<td>A band at 74 kDa</td>
</tr>
<tr>
<td>3 Moronic acid</td>
<td></td>
<td>3</td>
<td>No band at 74 kDa</td>
</tr>
<tr>
<td>4 Oleanonic acid</td>
<td></td>
<td>11</td>
<td>No band at 74 kDa</td>
</tr>
<tr>
<td>5 Ursonic acid</td>
<td></td>
<td>12</td>
<td>No band at 74 kDa</td>
</tr>
<tr>
<td>6 Oleanolic acid</td>
<td></td>
<td>13</td>
<td>No band at 74 kDa</td>
</tr>
<tr>
<td>7 Acidic Fractions (Mixture)</td>
<td></td>
<td>5</td>
<td>Smear and un-clear in the entire lane</td>
</tr>
<tr>
<td>8 IsoMasticadienonic acid</td>
<td></td>
<td>6</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>9 3-epi-isomasticadienolic acid</td>
<td></td>
<td>7</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>10 Masticadienonic acid</td>
<td></td>
<td>8</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>11 Dihydromasticadienonic acid</td>
<td></td>
<td>9</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>12 3-O-acetyl-3-epi(iso)masticadienolic acid</td>
<td></td>
<td>10</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>13 Masticadienolic acid</td>
<td></td>
<td>14</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>14 Dihydromasticadienolic acid</td>
<td></td>
<td>15</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>15 3-acetoxy-3-epiisoMasticadienolic acid</td>
<td></td>
<td>16</td>
<td>A band at 74 kDa as FA</td>
</tr>
<tr>
<td>16 3-acetoxy-3-epiMasticadienolic acid</td>
<td></td>
<td>17</td>
<td>A band at 74 kDa as FA</td>
</tr>
</tbody>
</table>
Figure 7-7 Comparison of Fusidic acid/isolates-dependent co-sedimentation with 70s ribosome

Figure 7-8 Comparison of Fusidic acid/isolates-dependent co-sedimentation with 70s ribosome
7.5 Discussion

The test compounds that were subjected to SDS-PAGE are listed in Table 7-1 and 7-2. These components were isolated from Kurdica gum. These isolates were divided into two major groups based on their skeleton; being lanosta and olean, each having three sub-groups. These triterpenoids mimic certain steroidal-like compounds, particularly the well known antibiotic Fusidic acid. They were subjected to a protein inhibition assay to investigate their potential mode of action. The results of co-sedimentation of ribosome with Fusidic acid/test compound was interpreted from the SDS-PAGE and tabulated in Table 7-2. These results indicated that there was some similarity in the mechanism of action of the lanosta type skeleton and Fusidic acid. However, the olean type structures did not exhibit the pattern observed with Fusidic acid and the lanosta type compounds. Notwithstanding, the negative results obtained for the olean type structures are very important from the perspective of structure and function analysis, as these compounds could appear to act by a mechanism distinct from steroidal antibiotics such as Fusidic acid and the potential that these compounds represent a new family of antimicrobial compounds should be explored. Further, even though the lanosta type compounds exhibited characteristics similar to Fusidic acid, further work is required to determine if these compounds represent newly described members of an existing family of antibiotics or a new family exhibiting a similar mode of action to Fusidic acid.

Fusidic acid structure-activity relationship has been subjected to analysis by a number of researchers in the past due to similarity of this structure to steroid compounds (Crosbie, 1962) and triterpenoids (Fried et al, 1965). However, only derivatives of this antibiotics and fusidane type antibiotics Cephalosporin and Helvolic acid have been analysed in relationship to their antimicrobial activity (Crosbie, 1962; Duvold et al, 2001). In this chapter
work was undertaken to determine if there were potential structural relationships within the library of isolated triterpenoid acids with respect to their antimicrobial activities and their mode of actions.

The presence of carboxylic acid in all acidic fractions of the gum is necessary for their antimicrobial activities, as their methyl ester had no activity under the conditions tested. The position of carboxyl group in olean base skeleton is located at C28, these group of compounds have much lower antimicrobial activity than lanosta base skeleton, in some cases they are 1000 fold less active.

Eburicoic acid is a triterpenoid that could convert into an antimicrobial active ring A seco-acid (Fig. 7-9) by microbial enzymatic activity. It is important to know that in the steroid antibiotics, carboxyl group is located at C-21 and it is a part of the side chain, while in olean base structure is located at C-28. However, the antimicrobial activity of the seco-acid –a dicarboxylic acid- is dependant on the presence of carboxyl group at C-3 according Fried et al (Fig. 7-2). In their work the effectiveness of carboxyl group at C-3 has been tested by methylation of its second carboxyl group at C-21. From these data and the literature one could conclude that:

![Diagram of Eburicoic acid conversion](image)

Figure 7-9 Eburicoic acid converted by microbial enzyme to antimicrobial active ring A seco-acid
1. Carboxyl group is essential for antimicrobial activities of steroidal and triterpenoid, as the methylated form of these compounds had no antimicrobial activity.

2. The position of carboxyl group may be located at C-3, C-21 and/or C-28. It also possible that the carboxyl group could be located at any of a number of possible sites on the molecules.

3. In the dicarboxylic acid form that has two anionic sites at distant parts of the molecule (i.e. C-3 and C-21), one group appears detrimental to activity. This may be the carboxylic acid at C-3 as was claimed by Fried et al.

4. In dicarboxylic acid, trans-acids are active and cis-acids are inactive (Fried et al).

5. The presence of an oxygen moiety or a double bond that may take the place of the oxygen moiety appears to be very important. The presence of an oxygen at C-3 in structures Ib, IIb and IIId in comparison to hydroxyl group at C-3 in structures IIIa (Table 7-3) illustrates this point as the first three compounds are 5-10 fold more active than latter structure IIIa, ursonic acid. An extra methyl group also has made this compound more hydrophobic and may be responsible for the apparent decrease in activity against Gram-positive bacteria (Table 7-3, structure IIIa).

6. Masticadienonic acid (compound IVb) has a double bond at C-8 and C-9 and the location of double bond in isoMasticadienonic (compound VIb) is at C-7 and C-8. These two isomers have the same activity (Table 7-4); however, the epi-isomer of Masticadienolic acid is 5 fold
more active in the Gram-positive bacterium *S. aureus* and 10 fold more active against *H. pylori*.

7. The configuration of the side chain of Fusidic acid (Duvold *et al*, 2001) and lanosta skeleton base of isolates is detrimental in antimicrobial activity. The stereoisomers differing in the configuration at C-17 and C-20 have different activities. Such was the differences that some of these stereoisomers were not active at all in the range that was tested.

8. Acetyl and acetoxy groups are increasing the activity of the isolates in some cases by 100 folds, for comparison structures VI* f* and VI*i versus structure IV*d.

9. Saturation of the double bond between C-24 and C-25 in dihydroMasticadienonic acid and dihydroMasticadienolic acid increases the activity by 10 folds against Gram-positive and Gram-negative bacteria, in comparison to Masticadienonic acid, Masticadienolic acid, and compounds V*b*, V*d, IV*b* and IV*d respectively (Table 7.4)

10. Tetracyclic ring system of the isolates chair-chair-chair configuration hypothetically has given this type of compounds broader antimicrobial activity spectrum (against Gram-positive and Gram-negative bacteria) in comparison to Fusidic acid with tetracyclic ring system of –chair-boat-chair configuration (Crosbie, 1962; Duvold *et al*, 2001).

Within the isolates from acidic fraction ‘A’ there are two classes of novel antimicrobial agents in that their MIC are consistent with contemporary antibiotics; one is similar (yet distinct) to Fusidic acid and the other one very different. These were defined as lanosta base skeleton, that mimics the well
know antibiotic, Fusidic acid. This class of compounds appears to interact at
the level of protein synthesis and may have the same mode of the action as
Fusidic acid. The other class of compounds had an olean base skeleton that
did not mimic the action of Fusidic acid and may exert its antimicrobial effect
by a different mechanism that is yet to be determined at the molecular level.
There are also some common themes related to antimicrobial activities of
these isolates

These classes of compounds have not been identified in past. They do have
strong antimicrobial activities more and broader than Fusidane type
antibiotics. The results obtained from this work have shed light on the study
of antimicrobial activities of the steroid and triterpenes and enhancement of
these activities.

As a result of these findings new classes of antibiotics has emerged and their
structure and mechanism of action characterised. Furthermore, the potential
to enhance the antimicrobial activity of extant antibiotics has been increased
and the ability to design new antibiotics has become possible.

The obtained data could also be used to design more novel antibiotics that
will be discussed in Chapter 8.
Table 7-3 Chemical structure of the isolated components of the acidic fractions of the gums

<table>
<thead>
<tr>
<th>Structure</th>
<th>MIC</th>
<th>S. aureus/ H. pylori</th>
<th>26695 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Structure I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a: R = O</td>
<td>RI= COOMe</td>
<td>&gt;100/100</td>
<td></td>
</tr>
<tr>
<td>Moronate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b: R = O</td>
<td>RI= COOH</td>
<td>50/10</td>
<td></td>
</tr>
<tr>
<td>Moronic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Structure II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a: R = O</td>
<td>RI= COOMe</td>
<td>&gt;100/100</td>
<td></td>
</tr>
<tr>
<td>Oleanonate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b: R = O</td>
<td>RI= COOH</td>
<td>50/10</td>
<td></td>
</tr>
<tr>
<td>Oleanonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c: R = H, β-OH</td>
<td>RI= COOMe</td>
<td>&gt;100/100</td>
<td></td>
</tr>
<tr>
<td>Oleanolate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d: R =O</td>
<td>RI= COOH</td>
<td>50/20</td>
<td></td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Structure III</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a: R = H, α-OH</td>
<td>RI= COOH</td>
<td>100/50</td>
<td></td>
</tr>
<tr>
<td>Ursonic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b: R = H, α-OH</td>
<td>RI= COOH</td>
<td>&gt;100/100</td>
<td></td>
</tr>
<tr>
<td>Ursonic acid (methyl ester)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c: R = H, α-OH</td>
<td>RI= COOMe</td>
<td>&gt;100/100</td>
<td></td>
</tr>
<tr>
<td>Oleanolate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d: R =O</td>
<td>RI= COOH</td>
<td>50/20</td>
<td></td>
</tr>
<tr>
<td>Oleanolic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 7-4 Chemical structure of the isolated components of the acidic fractions of the gums

<table>
<thead>
<tr>
<th>Structure IV</th>
<th>MIC</th>
<th>Structure V</th>
<th>MIC</th>
<th>Structure VI</th>
<th>MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure IV" /></td>
<td>S. aureus/H. pylori</td>
<td>26695 µg/mL</td>
<td>S. aureus/H. pylori</td>
<td>26695 µg/mL</td>
<td>S. aureus/H. pylori</td>
</tr>
<tr>
<td>a: R = O, RI = COOMe</td>
<td>&gt;100/100</td>
<td>a: R = O, RI = COOEt</td>
<td>&gt;100/100</td>
<td>a: R = O, RI = COOMe</td>
<td>&gt;100/100</td>
</tr>
<tr>
<td>Masticadienonate</td>
<td>5/5</td>
<td>b: R = O, RI = COOH</td>
<td>5/1</td>
<td>b: R = O, RI = COOH</td>
<td>5/5</td>
</tr>
<tr>
<td>Masticadienonic acid</td>
<td>10/5</td>
<td>c: R =H, β-OH, RI = COOMe</td>
<td>&gt;100/100</td>
<td>c: R =H, α-OH, RI = COOMe</td>
<td>&gt;100/100</td>
</tr>
<tr>
<td>Masticadienolate</td>
<td>&gt;100/100</td>
<td>d: R =H, β-OH, RI = COOMe</td>
<td>2/0.5</td>
<td>d: R =H, α-OH, RI = COOH</td>
<td>2/0.1</td>
</tr>
<tr>
<td>Masticadienolic acid</td>
<td>5/0.5</td>
<td>e: R = α-CH₃COO, RI = COOMe</td>
<td>5/0.5</td>
<td>e: R = α-CH₃COO, RI = COOMe</td>
<td>2/0.1</td>
</tr>
<tr>
<td>3-α-acetoxy-3-epiMasticadienolate</td>
<td>5/5</td>
<td>f: R = α-CH₃COO, β-OH, RI = COOH</td>
<td>0.05/0.01</td>
<td>f: R = α-CH₃COO, β-OH, RI = COOH</td>
<td>0.05/0.01</td>
</tr>
<tr>
<td>3-α-acetoxy-3-epiMasticadienolic acid</td>
<td>0.05/0.01</td>
<td>g: R =H, α-OAc, RI = COOMe</td>
<td>0.05/0.01</td>
<td>g: R =H, α-OAc, RI = COOMe</td>
<td>0.05/0.01</td>
</tr>
<tr>
<td>i: R =H, α-OAc, RI = COOH</td>
<td>0.05/0.01</td>
<td>j: R =H, α-OAc, RI = COOH</td>
<td>0.05/0.01</td>
<td>j: R =H, α-OAc, RI = COOH</td>
<td>0.05/0.01</td>
</tr>
</tbody>
</table>
Chapter 8
Identification and Characterisation of a New Class of Antimicrobial Agents

Natural products have been the source of most of the world’s supply of antimicrobial agents. While the genomics revolution holds the promise of designed antimicrobials, the natural environment can be expected to continue to yield new drugs and drug leads.

The antimicrobial screening of the trunk exudates of the genus \textit{Pistacia} led to the characterization of the most active fraction of the Kurdica gum which was the Acidic fraction ‘A’, (Chapter 4). This acidic fraction was subsequently subjected to sub-fractionation leading to fundamentally new information that went beyond \textit{H. pylori}, expanding the original parameters of the project. Such was the extent of these findings that a new class of antibiotics has emerged and their structure and mechanism of action characterised. Furthermore, the potential to enhance the antimicrobial activity of extant antibiotics has been increased and as a result the ability to design new antibiotics has become possible.

This chapter is a summary of the major findings of this project and in addition sets out a direction for a future research. The conclusions for each area can be found in the discussion section of each chapter. Thus while this chapter repeats some of these, it also links together the major issues that have been addressed in this study.

\textit{Helicobacter pylori} is one of the most significant discoveries in gastroenterology in the past century and has earned for Warren and Marshall the Nobel Prize for Medicine. \textit{H. pylori} is the most important aetiologic agent of gastritis, gastric, duodenal ulcer (peptic ulcer disease) and is causally associated with gastric cancer (Alkopyanz \textit{et al}, 1998). About 50% of
world population is infected with *H. pylori* in of which 10% of infected develops disease during their lifetimes (Alkopyanz *et al.*, 1998).

While it was known that gastric ulcers could result from medication such as Non-steroidal Anti-Inflammatory Drugs (NSAIDs), it is evident that *H. pylori* causes almost all cases of peptic ulcer disease that are not related to the ingestion of NSAIDs. Indeed this infection evidently causes ~95% of all duodenal ulcer disease.

In addition to the link to peptic ulcer disease, in June 1994 the International Agency for Research in Cancer, an arm of the World Health Organization, declared that *H. pylori* to be a Class-1 carcinogen, the highest risk category for cancer-causing agents (Axon & Moayyedi, 1996).

Treatment regimens using acid suppressive therapy, particularly proton pump inhibitors, to enhance antimicrobial drugs efficacy have emerged as the preferred therapy for treatment of *H. pylori* infection. (Armstrong *et al.* 1987; Berstad & Olafsson 1996).

The successful treatment of *H. pylori* infection results in the cure of the infection, and as a consequence also cures and prevents the development of the associated diseases (Riberio *et al.*, 2004). However, the development of resistance by the bacterium to the antibiotics that are currently used is the major impediment for these therapeutic regimens (Hazell *et al.*, 1999; Hyde *et al.*, 1997; Jorgensen *et al.*, 1996).

The challenge now is to find an effective course of treatment that is more “patient friendly”, overcomes issues of resistance, ideally works as a single agent (with or without acid suppressive therapy), have fewer side effects and in the longer term make *H. pylori* eradication less expensive (Goodwin, 1997). While in recent time the pharmaceutical industry appears to have lost
interest in the development of specific agents to treat \textit{H. pylori} infection, basic research in this area may hold the key to new and better therapies.

Antibiotic resistance in \textit{H. pylori} has emerged as a significant clinical problem. Further, contemporary therapy is expensive and complex, particularly for third world countries. The body of work contained within this dissertation was carried out to investigate an alternative therapy based on observations of the traditional therapy for gastric disease in the Middle East. One of these traditional therapies centres on plants of the genus \textit{Pistacia}. However, the findings went beyond \textit{H. pylori}, leading to the characterisation of what appears to be a new class of antibiotics.

The resin or gum from \textit{P. lentiscus} (Mastic) has been used by traditional healers for the relief of upper abdominal discomfort, gastralgia, dyspepsia and peptic ulcer. It has also been used as a Masticatory and by dentists for filling carious teeth. It has also been reported to possess stimulant and diuretic properties (Al-Said \textit{et al}, 1986).

The chemical compositions of Kurdica, Mutica and Cabolica gums were characterised with particular reference to their biological activity, specifically their antibiotic activity by reference to the human pathogenic bacterium \textit{H. pylori} and some other Gram-negative and Gram-positive bacteria.

This study represents the first reported investigation into the composition and biological activity of the trunk bark exudates of \textit{P. Kurdica}, \textit{P. a. Mutica} and \textit{P. a. Cabolica} a resinous gum that have been termed here ‘Kurdica Gum’, ‘Mutica Gum’ and ‘Cabolica Gum’.

The trunk exudates constitutes four major fractions;
1. Essential oils
2. Acidic fraction (‘A’, ‘B’ and ‘C’)
3. Neutral fraction
4. Polymeric fraction

The main constituents of the essential oils of the gums were monoterpenes, with some level of monoterpenoids (Chapter 3). Despite the fact that a number of other plants incorporate these components at various levels in their essential oils, comparable historical use or advocacy of these plants in anti-ulcer therapy has not been reported.

There are three aspects by which these gums might differ from other plants in this regard. It was shown to include:

- An unusual high molecular weight polymer, polymeric fractions

- A range of characteristic di- and tri-terpenes and tri-terpenoids as a part of its components (Acidic and Neutral fractions).

- That they have historically been Masticated in use (particularly Kurdica gum), a practice consistent with its physical form and properties.

After isolation of the resin from essential oils that had a general mild antimicrobial activity, there were three acidic fractions ‘A’, ‘B’ and ‘C’ with a broad antimicrobial activities spectrum in which the activity of the fraction ‘A’ was considered to be consistent with contemporary antibiotics and hence was subjected to sub-fractionation and further analysis. There was also a neutral fraction, which was not active in the range that was tested (Chapter 4), and High Molecular Weight Fraction (HMWF) that was active; however, this activity was not consistent with contemporary antibiotics (Chapter 5).
The neutral fractions are the methyl ester of the fractions ‘A’, ‘B’ and ‘C’ and hypothetically would be subjected to hydrolysis by stomach acid, converting to active acidic fraction ‘A’ and ‘B’.

Within the acidic fraction ‘A’ there are two classes of compounds that had MIC values consistent with contemporary antibiotics. These were defined as lanosta base skeleton, that mimics the well known antibiotic, Fusidic acid. This class of compounds appear to interact at the level of protein synthesis and may have the same mode of the action as Fusidic acid (Chapter 7). The other class of compounds had an olean base skeleton that did not mimic the action of Fusidic acid and may exert its antimicrobial effect by a different mechanism that is yet to be determined at the molecular level.

The HMWF (polymeric fractions) had a mild antimicrobial activity that was increased by oxidation (Chapter 5). The polymer fraction has an MIC more consistent with a disinfectant than a classical antibiotic. However, we may speculate that this polymer may act as an antimicrobial “bandage”, protecting the ulcer from contact with stomach acid while exerting a local antiseptic effect.

Therefore, within these plant extracts that have been used as traditional medicines, there may be multiple classes of antimicrobial agents with different mode of the action. Novel antibiotics within the acidic fraction ‘A’, essential oils with mild antimicrobial activities and an antimicrobial polymer that could hypothetically protect the ulcers from the acid.

These observations could be a base for future work. Until then, it would appear that these natural remedies may be of clinical value when used in its existing form, without modification. However, understanding the characteristics and action of the components of these natural products opens the prospect of new and better therapies.
As it was mentioned above, these findings went beyond *H. pylori*. Some components of the acidic fraction ‘A’ mimic a well known antibiotic, Fusidic acid. Fusidic acid is a narrow spectrum antibiotic, used primarily against Gram-positive bacteria. Most wild-type strains of *Staphylococcus*, (Garaud & Vachon, 1985) *Streptococcus*, *Neisseria*, *Corynebacterium*, *Clostridium* and some mycobacteria are sensitive to Fusidic acid. It is also used to treat topical skin infections and infected wounds and burns caused by sensitive strains of *S. aureus*, *Streptococcus* species and *C. minutissimum* (Collignon & Turnidge, 1999).

Fusidic acid has little or no effect against Gram-negative bacteria and that is believed to be due to differences in cell wall permeability (Collignon & Turnidge, 1999). Disrupting or by-passing the outer membrane of Gram-negative bacteria renders these cells sensitive to these drugs. An important observation from this work is that the lanosta skeleton sub-fractions of acidic fraction ‘A’ is effective in vitro against Gram-negative bacteria as well as Gram-positives. This broader spectrum of antimicrobial activity opens important possibilities for the design of new agents active against Gram-negative bacteria. This could be due to ‘chair-chair-chair’ configurations as it is described in Chapter 7 and warrants further investigation.

Reports of resistance to Fusidic acid has recently increased, mainly in hospitals where cross infections are common. The rate of resistance is higher in methicillin-resistant strains of *S. aureus* (Irish et al, 1998; Wolff, 1997) and it occurred by a number of mechanisms that includes alterations in EF-G and drug permeability (Turnidge & Collignon, 1999). Fusidic acid has shown the capacity to stabilize the ribosome-translocation factor-GDP complex. It was shown that the presence of a carboxyl group at C-20 and a 17, 20-double bond was critical in forming the complex. Since the positions of carboxylic group and the double bond of side chain are different with the lanosta base skeleton, then the Fusidic acid resistance bacteria may not be resistant to this
class of compounds. This hypothesis should be tested in future work as effective treatments for MRSA and Vancomycin resistant S. aureus are critically needed.

Also It has been shown in the studies reported here that the geometry of the double bond (16, 21-cis) appears to be an essential feature to this unique activity (Chapter 7). It was concluded by others that the nature and stereochemistry of the other functional groups were important but not essential (Bodley & Godtfredsen, 1972). This is why attempts have been made to synthesis new derivatives of Fusidic acid by altering the geometry of the double bond and also by adding new functional groups for more solubility (Duvo et al, 2001). The results reported here suggest new approaches to this issue.

Novel analogs of Fusidic acid -with one or more carbohydrate units attached- have been synthesised and patented by University of Michigan. Some of these glycosylated analogs of Fusidic acid have enhanced solubility properties in comparison to unmodified Fusidic acid and some other glycosylated analogs may be employed as chemotherapeutic agents. The glycosylated analogs may have antimicrobial activities as is claimed by the investigators.

The carbohydrate unit that has attached to Fusidic acid may be a monosaccharide or disaccharide selected from the group consisting of the following structures:
Figure 8-1 Monosaccharide and disaccharide that were attached to lanosta/olean base skeleton using ACDLAB computer analysis
The structure-activity relationship of novel analogs of Fusidic acid—with one or more carbohydrate (Koreeda et al., 2000) (Figure 8.1; structure I-X) was analysed using computer assisted analysis (ACDLAB 8.0). In this analysis Fusidic acid was replaced by lanosta base skeleton and olean base skeleton. This modeling work provides a basis for novel structural analogues that may have enhanced clinical utility. Testing of this hypothesis with respect to directives with lanosta/olean base skeleton would be a priority for future work.

Some of the model structures are as follows:

![Figure 8-2 3-(2,3-dideoxy-alpha-D-erythro-hexanopyranoside) Masticadienonic acid](image)
Figure 8-3 3-(4,6-di-O-acetyl-2,3-dideoxy-alpha-D-erythro-hex-2-enopyranoside)-Masticadienonic acid

Figure 8-4 3-[4-O-(2,3,4,6-tetra-O-acetyl-alpha-D-glucopyranosyl)-6-O-acetyl-23-dideoxy-alpha-D-erythro-hex-2-enopyranoside] Masticadienonic acid
Figure 8-5 3-[4-O-(beta-D-glactopyranosyl)-2,3-dideoxy-alpha-D-erythro-hex-2-enopyranoside] Masticadienonic acid

Figure 8-6 3-(4,6-di-O-acetyl-2,3-dideoxy-alpha-D-erythro-hex-2-enopyranoside)-Oleanonic acid

Figure 8-7 3--(4,6-di-O-acetyl-dideoxy-alpha-D-erythro-hex-2-enopyranoside)-Oleanonic acid
Figure 8-8 3-(2,3-dideoxy-alpha-D-erythro-hexanopyranoside) Oleanonic acid

Figure 8-9 3-[4-O-(2,3,4,6-tetra-O-acetyl-alpha-D-glucopyranosyl)-6-O-acetyl-23-dideoxy-alpha-D-erythro-hex-2-enopyranoside] Oleanonic acid

Figure 8-10 3-[3-[4-O-(beta-D-glactopyranosyl)-2,3-dideoxy-alpha-D-erythro-hex-2-enopyranoside] Oleanonic acid
The aforementioned structures could be also synthesised with Moronic acid, Oleanolic acid, Ursonic acid, IsoMasticadienonic acid, Masticadienolic acid, IsoMasticadienolic acid, DihydroMasticadienonic acid and DihydroMasticadienolic acid, bringing the total of the suggested antimicrobial compounds to 50 compounds in which 30 of these compounds have a MIC consistent with contemporary antibiotics and could be commercialised.

The above discussion illustrates a wealth of work that could and should be undertaken to advance our understanding of the nature and mode of action of a range of “natural” antimicrobial compounds. This dissertation sets out the advances that have been made and opens the door to a rich field of study.
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necessary for expression of the vacuolating cytotoxin. Infection and Immunity 63, 94-98.


Appendix A1 Raw Materials

A1.1 Mastic Gum

Registry No., [61789-92-2], was purchased from Sigma Aldrich Pty Ltd. Australia.

A1.2 Kurdica Gum

Kurdica gum from the *Pistacia atlantica Kurdica* was collected from the area around the Zagros Mountains in Kurdistan province, Iran over the period June to August 2000 and provided by Kurdistan Saghez Sazi Co., Sanandaj, Iran.

A1.3 Mutica Gum

Mutica gum; exudates trunk of *Pistacia atlantica Mutica* were collected from the centre of Iran, Fars province over the period June to August 2000 by the author.

A1.4 Cabolica Gum

Cabolica gum; exudates trunk of *Pistacia atlantica Cabolica* was collected from North East of Iran Khorasan province over the period June to August 2000 by the author.
Appendix A2 Chemical and Reagents, Bacteriological Media, Buffers and Solutions

A2.1 Chemical and Reagents

Unless stated otherwise, all reagents were anhydrous AR.

Chloroform, Glacial Acetic Acid, Glycerol, Hydrogen peroxide, Isoamyl Alcohol, Propan-2-ol (isopropanol), Sodium Acetate, Sodium Hydroxide and Sucrose were purchased from AnalR/BDH Chemicals, Victoria Australia.

Ethanol, Methanol, Hydrochloric acid, Polyvinyl alcohol, Methyl-4-hydroxybenzoate, 4-hydroxbenzoic acid, β-myrcene, α-pinene, Dichloromethane, Benzene, Antimony Pentachloride, Mastic Gum, Acetonitrile, Diethyl Ether, 2,4-dinitrophenylhydrazin were purchased from Sigma-Aldrich New South Wales Australia.

β-mercaptoethanol, Coomassie Brilliant Blue R-250, were from BioRad, New South Wales Australia.

A2.2 Bacteriological Media and Supplements

A2.2.1 Bacteriological Media and Supplements

Unless stated, all bacteriological media was made using Milli-Q water and was sterilised followed by autoclaving for 15 min at 121°C.
A2.3 Selective Supplements

A2.3.1 Skirrow’s Selective Supplement

Polymyxin B  161.5 μg/mL (1250u/mL)
Trimethoprim  2.5 mg/mL
Vancomycin  5 mg/mL

Trimethoprim was dissolved in a minimal volume of 95% ethanol. Polymyxin B and vancomycin were added. The solution then was made up with Milli-Q water. The antibiotic solution was filter sterilised and stored at –20°C in 16 mL aliquots (335).

A2.3.2 Fungizone®

Amphotericin B  4.2 mg/mL
Sodium De-oxycholate  3.4 mg/mL
Sodium Phosphate  1.7 mg/mL

Fungizone® contains the anti-fungal agent Amphotericin B. The Fungizone was filter sterilised and stored in dark due to its photosensitivity in 4 mL aliquots at –20°C.
A2.4 Bacteriological Media

A2.4.1 Campylobacter Selective Agar (CSA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Base No. 2</td>
<td>38g/L</td>
</tr>
<tr>
<td>Defibrinated Horse Blood</td>
<td>70 mL/L</td>
</tr>
<tr>
<td>Fungizone®</td>
<td>500 µL/L</td>
</tr>
<tr>
<td>Skirrow’s Selective Supplement</td>
<td>2 mL/L</td>
</tr>
</tbody>
</table>

CSA is used as a media for the culture of *H. pylori*. The agar base was autoclaved for 15 min at 121°C and allowed to cool to 44°C. The horse blood, Skirrow’s and Fungizone® were then added. The CSA plates were poured by a Pourmatic mp1000 (New Brunswick Scientific Edition, N.J. USA).

A2.4.2 Campylobacter Selective Agar + Kanamycin (CSAK)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Agar Base No. 2</td>
<td>38g/L</td>
</tr>
<tr>
<td>Defibrinated Horse Blood</td>
<td>70 mL/L</td>
</tr>
<tr>
<td>Fungizone®</td>
<td>1 mL /L</td>
</tr>
<tr>
<td>Skirrow’s Selective Supplement</td>
<td>2 mL/L</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>250 µL/L</td>
</tr>
</tbody>
</table>

CSAK is designed to select for *H. pylori* transformants – the isogenic mutants. However, CSAK was used here for kill kinetics as the plates were incubated longer in the CO₂ incubator so the quantity of Fungizone in CSAK was doubled. The agar base was autoclaved and allowed to cool to 44°C prior to the addition of the horse blood, Skirrow’s and Fungizone®. The CSAK plates were poured by hand.
A2.4.3 IsoSensitest Broth/Agar

IsoSensitest Agar (or Broth)  34.1 g/L
Horse Serum  50 mL/L
Fungizone®  830 µL/L
Skirrow’s Selective Supplement  2167 µL/L

IsoSensitest is often used in antibiotic sensitivities testing. IsoSensitest broth/Agar was autoclaved for 15 min at 121°C and allowed to cool to 44°C. The horse serum and selective supplements were then added.

A2.4.4 Luria-Bertani Broth/Agar

Agar  15 g/L
Sodium Chloride  5 g/L
Tryptone
Yeast Extract  5 g/L

Luria-Bertani (LB) broth and agar* was used to culture variety of the bacteria listed in table 2.2 particularly to culture \( E. coli \), as described in Chapter 2. The LB was sterilised by autoclaving and was allowed to cool to 44°C prior to the addition (if any) of selective supplements.

A2.4.5 Brain Heart Infusion Broth

Brain Heart Infusion Broth  37 g/L
Horse Serum  50 mL/L
Fungizone®  830 µL/L
Skirrow’s Selective Supplement  2167 µL/L

Brain Heart Infusion was primarily used for liquid cultures of \( H. pylori \).
A2.5 Buffers & Solutions

Unless stated otherwise, all buffers and solutions were made using sterile Milli-Q water.

A2.5.1 M Ethylenediamine tetra-acetic acid (EDTA)

Ethylenediamine tetra-acetic acid (EDTA) (9.3g) was mixed with 40 mL of water to make a solution of 0.5M of EDTA. To dissolve EDTA the pH was adjusted at 8.0 using 0.5M NaOH, then the volume made up to 50 mL., followed by autoclaving. This buffer was then stored at room.

A2.5.2 Magnesium Chloride

The solution of 2M Magnesium Chloride was made by dissolving 19 g of MgCl₂ in 80 mL of water. It was then made up to 100 mL., followed by autoclaving.

A2.5.3 Sodium Acetate

The solution of 3M sodium acetate was prepared by dissolving 40.81g sodium acetate in 80 mL of water. The pH was then adjusted to 5.3 using glacial acetic acid. The volume was made up to 100mL. This solution was kept at room temperature.
A2.5.4 10% Sodium Dodecyl Sulfate (SDS)

Sodium Dodecyl Sulfate (SDS) 100g was dissolved in 700 mL of water. It was then heated to 65°C and volume was made up to 1000mL. The solution was kept at room temperature.

A2.5.5 Sodium Carbonate

A stock solution of 10% sodium carbonate (w/v) was made by dissolving 100g of Sodium carbonate in 700 mL of water made up to 1L. This solution was kept in room temperature.

A2.5.6 Sodium Hydroxide 10 M

A stock solution of 10 M NaOH was made by dissolving 400.00 g of NaOH pellets in 1L of water and kept in room temperature.

A2.5.7 Staining Buffer

Coomassie Brilliant blue was used to stain proteins in Polyacrylamide gels (Chapter 2). Coomassie Brilliant Blue R250 1g was dissolved in 500 mL of methanol and 100 mL of Glacial acetic acid it was then made up to 1L with Milli-Q water.
A2.5.8 Destaining Buffer

Glacial acetic acid (100 mL) was added to the methanol 500 mL., the volume was then made up to 1L with Milli Q water. This buffer was re-used after decolourising with Whatman quantitative filter papers Grade 40/8μm (Sigma-Aldrich, Sydney Australia).

A2.5.9 Urease Reagent

Urease reagent, was used to determine the purity of Helicobacter cultures. This reagent was prepared by dissolving 0.44 g of NaH$_2$PO$_4$.2H$_2$O and 2.58 g of Na$_2$HPO$_4$.12H$_2$O in 900 mL of Milli-Q water. To this solution was then 20g of Urea, 0.5g Phenol Red and 0.2g of Sodium Azide were added and made up to 1L, and the pH was adjusted to 6.5.

A2.5.10 Sucrose Stock Solution (40% w/v)

Stock solutions 40% of sucrose (w/v) were prepared just prior to use. The solutions were made by adding sucrose to hot sterile water followed by filter sterilised using a syringe filter. This solution was used in ribosome purification as a sucrose cushion as described in Chapter 2 and 7.

A2.5.11 Ribosome Purification Buffer

Diethyl Pyrocarbonate (DEPC)-treated water used for preparation of the following buffers except for HEPES stock. DEPC was added to Nanopure water to 0.1% (v/v) and stirred vigorously for 2 h in fume hood. It was then autoclaved and stored in cold room. A solution of 1 M HEPES-KOH pH 7.62
was made by dissolving 2.14 g solid KOH in 100mL. Nanopure water, the solution was then autoclaved.

\[ H^{10}M^{10}A^{1000}\beta^5 \]
10 mM HEPES-KOH pH7.6 1 ml 1 M HEPES-KOH pH 7.6
10 mM MgCl₂ 1 ml 1 M MgCl₂
1 M NH₄Cl 20 ml 5 M NH₄Cl
5 mM β-mercaptoethanol 35 µl β-mercaptoethanol
78 ml H₂O
100 ml

\[ H^{10}M^{10}A^{50}\beta^5 \]
10 mM HEPES-KOH pH7.6 5 ml 1 M HEPES-KOH pH 7.6
10 mM MgCl₂ 5 ml 1 M MgCl₂
50 mM NH₄Cl 5 ml 5 M NH₄Cl
5 mM β-mercaptoethanol 175 µl β-mercaptoethanol
485 ml H₂O
500 ml

\[ H^{10}M^{10}A^{1000}\beta^5 + 20\% \text{ sucrose} \]
10 mM HEPES-KOH pH7.6 1 ml 1 M HEPES-KOH pH 7.6
10 mM MgCl₂ 1 ml 1 M MgCl₂
1 M NH₄Cl 20 ml 5 M NH₄Cl
5 mM β-mercaptoethanol 35 µl β-mercaptoethanol
20 g
H₂O to 100 ml
100 ml

\[ H^{10}M^{10}A^{50}\beta^5 + 40\% \text{ sucrose} \]
10 mM HEPES-KOH pH7.6 2 ml 1 M HEPES-KOH pH 7.6
10 mM MgCl₂ 2 ml 1 M MgCl₂
50 mM NH₄Cl 2 ml 5 M NH₄Cl
5 mM β-mercaptoethanol 70 µl β-mercaptoethanol
80 g
H₂O to 200 ml
200 ml
Appendix A3 Essential oil GC-MS Profile

Spectrum 0-1 GC analysis of kuridca

Spectrum 0-2 GC analysis of Mutica
Spectrum 0-3 GC analysis of Cabolica
Spectrum 0-4 Typical chromatogram and identifications of the components (Kurdica)
Spectrum 0-5 Typical GC-MS analysis (Kurdica)

Spectrum 0-6 Typical GC-MS analysis (Kurdica)
Appendices

Spectrum 0-7 Typical GC-MS analysis (Kurdica)

Spectrum 0-8 Typical GC-MS analysis (Kurdica)
Spectrum 0-9 Typical GC-MS analysis (Kurdica)

Spectrum 0-10 Typical GC-MS analysis (Kurdica)
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Spectrum 0-11 Typical GC-MS analysis (Kurdica)

Spectrum 0-12 Typical GC-MS analysis (Kurdica)
Spectrum 0-13 Typical GC-MS analysis (Kurdica)

Spectrum 0-14 Typical GC-MS analysis (Kurdica)
A6.2 HPLC- APCI-MS Spectra

Spectrum 0-15 APCI-MS Acidic fractions of Kurdica gum

Spectrum 0-16 APCI-MS Acidic fractions of Kurdica gum
Spectrum 0-17 APCI-MS Acidic fractions of Kurdica gum