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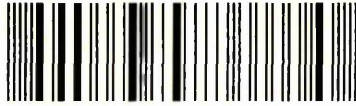
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**An Antimicrobial Agent from Celery Seed  
Active against *H. pylori***

**Yong Zhou**

**A thesis submitted in partial fulfillment of the requirements of  
Sheffield Hallam University  
for the degree of Doctor of Philosophy**

**September 2008**





## **Abstract**

As well as peptic ulcers, *Helicobacter pylori* is associated with the development of gastritis, gastric adenocarcinoma and lymphoma, and has been classified as a class I carcinogen in humans (International Agency for Research on Cancer Working Group, 1994). Although the bacteria can be eradicated in up to 90% of patients, side effects, poor compliance and the resistance of the bacteria to antibiotics are common causes of frequent treatment failure. Celery seed extracts (CSE) from a unique source in India has been used as herbal medicine since antiquity and found to have anti-inflammatory and gastroprotective properties (Butters *et al.*, 2004; Whitehouse *et al.*, 2001). This study followed on observations that crude extracts exhibited anti-helicobacter activity (Rainsford & Liu, 2006).

CSE was selectively fractionated followed by HPLC. Fractions were collected and bio-assayed against different strains of *H. pylori* using conventional culture methods. The most potent component that was obtained from HPLC and purified was designated celery seed with anti-Helicobacter activity (CAH). This component has strong bactericidal effects against *H. pylori*; the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were  $3.15 \mu\text{g ml}^{-1}$  and  $6.25 - 12.5 \mu\text{g ml}^{-1}$ , respectively. This compares favourably with the MIC and MBC of tetracycline, which are in the region of  $3.15 \mu\text{g ml}^{-1}$ . The isolated compound has highly specific inhibitory effect on *H. pylori*, since no inhibitory activity was detected against *Campylobacter jejuni* or *Escherichia coli* at these levels. The molecular ion of CAH was measured as 384.23 by mass spectrometry, giving the empirical formula as  $\text{C}_{24}\text{H}_{32}\text{O}_4$ . The MS and NMR data strongly suggest this compound is a phthalide dimer. From radioactive bioassay, CAH inhibits RNA synthesis by 50% of that seen in a negative control in 3 days, while DNA and protein synthesis were unchanged.

These suggested that the new compound may be suitable for further investigation as an agent for treating *H. pylori* infections.

# CONTENTS

<u>Abstract</u>	i
<u>Contents</u>	iii
<u>Acknowledgements</u>	vii
<u>List of Figures</u>	viii
<u>List of Tables</u>	x

## Chapter I

### Introduction and Survey of Literature

<u>1. 1 <i>Helicobacter pylori</i> and associated diseases</u>	2
1.1.1 Introduction	2
1.1.2 Discovery of <i>Helicobacter pylori</i>	3
1.1.3 Microbiology	6
1.1.3.1 Genus description	6
1.1.3.2 Morphology and general properties	7
1.1.3.3 Genome structure	10
1.1.3.4 Proteome characterization	14
1.1.3.5 Metabolism	15
1.1.3.6 Growth requirements	28
1.1.4 Pathogenesis	29
1.1.4.1 Animal models	29
1.1.4.2 Virulence factors	33
1.1.4.3 Immune response	39
1.1.4.4 Cytokines	41
1.1.4.5 Infection processing	42
1.1.5 Epidemiology	44
1.1.5.1 Prevalence and geographical distribution	44
1.1.5.2 transmission	47
1.1.6 Important <i>H. pylori</i> -associated diseases	48
1.1.6.1 Acute and chronic gastritis	48
1.1.6.2 Peptic Ulcer disease (PUD)	49
1.1.6.3 Gastric cancer	52
1.1.6.4 NSAIDs	54
1.1.7 Diagnosis	55
1.1.8 Current treatment and their limitations	57
1.1.8.1 Current therapies for PUD and control of <i>H. pylori</i>	57
1.1.8.2 Vaccination	60
1.1.8.3 Limitations of current therapies	63

1.1.9	Conclusion and relevance of this project	66
-------	--	----

## **1.2 Celery seed in medicine**

1.2.1	History	69
1.2.2	Character	69
1.2.3	Extraction and isolation	70
1.2.4	Composition	73
1.2.5	Pharmacognosy of celery seed extract	74
1.2.6	Relevant claims of CSE	81
1.2.7	Available celery seed extracts	82

1.3	<b><u>Hypothesis for the present investigations</u></b>	85
-----	---	----

	<b><u>Aims and objectives</u></b>	87
--	-----------------------------------	----

## **Chapter II**

### **Effects of various extracts of celery seed on the growth and viability of *H. pylori***

2.1	Introduction	89
2.2	Methods	91
2.2.1	Bacterial strains and culture condition	91
2.2.2	Identification of <i>H. pylori</i>	92
2.2.2.1	Urease test	92
2.2.2.2	16S rRNA gene sequence	93
2.2.3	CSE sources	93
2.2.4	Antimicrobial activity test	94
2.3	Results	96
2.3.1	Initial cultivation of microorganisms	96
2.3.2	Urease test (CLO)	96
2.3.3	16S rRNA gene sequence identification	100
2.3.4	Inhibitory effects of A-CSE against different strains of <i>H. pylori</i>	100
2.3.5	Positive control	106
2.3.6	Inhibitory effects of related solvents	106
2.3.7	Inhibitory effects of various CSE against <i>H. pylori</i>	112
2.4	Discussion	115

## **Chapter III**

### **Isolation, chemical characterization and identification of the principal active component**

3.1	Introduction	121
3.2	Methods	122
3.2.1	Bacteria strains, culture condition and CSE source	122
3.2.2	Antimicrobial activity test	123

3.2.3	Organic extraction and separation	123
3.2.4	Spectroscopic methods	125
3.3	Results	126
3.3.1	Antibacterial activity of CSE and their purification	126
3.3.2	Effects of CAH against other bacteria	147
3.3.3	Bioactivity of a known chemical in celery seed, sedanolide	147
3.3.4	Chemical characterization of CAH	151
3.4	Discussion	159

#### Chapter IV

#### Mechanism of action of celery seed extracts, fractions and principally active component on *H. pylori*

4.1	Introduction	164
4.2	Materials and methods	166
4.2.1	Bacteria, culture conditions and preparation of CSE component	166
4.2.2	Bactericidal and bacterostatic assays	167
4.2.2.1	Microplate assay	167
4.2.2.2	Trypan blue exclusion	167
4.2.3	Electron microscopy	168
4.2.4	Effect of CSE component on <i>H. pylori</i> membrane potential	168
4.2.5	Effect of CSE component on protein and nucleic acid synthesis	170
4.2.6	1-D and 2-D Electrophoresis and protein identification	171
4.2.6.1	1-D Electrophoresis	171
4.2.6.2	2-D Electrophoresis and protein identification	172
4.3	Results	174
4.3.1	Bactericidal and bacteriostatic testing	174
4.3.2	Transmission and scanning electron micrographs	175
4.3.3	1-D and 2-D Electrophoresis and protein identification	181
4.3.3.1	1-D gel	181
4.3.3.2	2-D gels	185
4.3.4	Membrane potential measurement	185
4.3.5	Effects CSE component on protein, DNA and RNA synthesis	190
4.4	Discussion	197
4.4.1	CAH has a bactericidal effect on <i>H. pylori</i>	197
4.4.2	Treatment with CAH induces a significant morphological change..	198
4.4.3	CAH does not compromise <i>H. pylori</i> membrane potential	199
4.4.4	CAH significantly inhibits total RNA synthesis in <i>H. pylori</i>	200
4.4.5	Effect of CAH on protein expression profiles	200
4.5	Conclusions	202

#### Chapter V

#### Overall discussion and conclusions

5.1	Constituents of celery seed and bioactivity	204
5.2	Activity of CSE toward and specificity for <i>H. pylori</i>	206
5.3	Novel mechanisms	208
5.3.1	Bactericidal effect	210
5.3.2	CAH does not cause disruption of the cell membranes	210
5.3.3	Selective effect of CAH on protein expression profiles	211
5.3.4	CAH significantly inhibits overall RNA synthesis in <i>H. pylori</i>	212
5.3.5	CAH in the context of antibiotic resistance	212
5.4	Economics of CAH for pharmaceutical exploitation	213
	<b><u>Suggested Future Investigations</u></b>	<b>215</b>
	<b><u>References</u></b>	<b>217</b>
	<b><u>Publication and conferences relevant to the thesis</u></b>	<b>267</b>

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## List of Figures

Fig. 1-1 Morphology of <i>H. pylori</i> strain ATCC 43504	9
Fig. 1-2 Circular representation of the <i>H. pylori</i> 26695 chromosome	12
Fig. 1-3 The relationships of acid resistance and metabolisms of <i>H. pylori</i>	16
Fig. 1-4 Glucose Metabolism	18
Fig. 1-5 The noncyclic Krebs cycle	20
Fig. 1-6 Lipid metabolism	22
Fig. 1-7 The respiratory electron transport chain of <i>H. pylori</i>	25
Fig. 1-8 Cag type IV secretion system	35
Fig. 1-9 The roles of Vac A in pathogenesis of <i>H. pylori</i>	37
Fig. 1-10 Prevalence and geographical distribution of <i>H. pylori</i> infection	46
Fig. 1-11 Age and sex-related incidence of peptic ulcer	50
Fig. 1-12 Summary of recommended treatment strategy for eradication of <i>H. pylori</i>	62
Fig. 1-13 Primary resistance of <i>H. pylori</i> to the common antibiotics	65
Fig. 1-14 The appearance of the dry celery seed	71
Fig. 1-15 The known components of celery seeds	75
Fig. 2-1 Culture <i>H. pylori</i> on chocolate agar plate	97
Fig. 2-2 Comparison of growth of <i>H. pylori</i> strains 3339 and 26695	98
Fig. 2-3 Results of urease test	99
Fig. 2-4 PCR results of <i>H. pylori</i> strain 3339 and 26695	101
Fig. 2-5 16S rRNA sequence of <i>H. pylori</i> strains 3339	102
Fig. 2-6 Dye termination sequencing of the PCR product	103
Fig. 2-7 Inhibitory effect of A-CSE against <i>H. pylori</i>	104
Fig. 2-8 Inhibitory effect of tetracycline against <i>H. pylori</i>	107
Fig. 2-9 Inhibitory effect of DMSO solvent against <i>H. pylori</i> strain 3339	108
Fig. 2-10 Inhibitory effect of acetonitrile solution against <i>H. pylori</i> strain 3339	109
Fig. 2-11 Inhibitory effect of methanol solution against <i>H. pylori</i> strain 3339	110
Fig. 2-12 Inhibitory effect of iso-propanol against <i>H. pylori</i> strain 3339 at 5 days	111

Fig. 2-13 Inhibitory effect of various CSE against <i>H. pylori</i> 3339	113
Fig. 2-14 Inhibitory effect of various CSE extracts against <i>H. pylori</i> 26695	116
Fig. 3-1 Fractionation of A-CSE	127
Fig. 3-2 Inhibitory effect of fractions of CSE against <i>H. pylori</i> 3339	128
Fig. 3-3 Examination of TLC on sub-fractions of petroleum ether phase	131
Fig. 3-4 Effects of subfractions of petroleum ether phase against <i>H. pylori</i>	132
Fig. 3-5 Examination of TLC on the 12 <sup>th</sup> sub-fractions	138
Fig. 3-6 Inhibitory effects of subfractions of SF12 against <i>H. pylori</i>	139
Fig. 3-7. Absorbance trace of sample SF12-I in SCN HPLC system	140
Fig. 3-8 Inhibitory effects of SFa,b,c,r against <i>H. pylori</i>	142
Fig. 3-9 Absorbance trace of SFc in RP-HPLC system	143
Fig. 3-10 Absorbance trace of SF12-i in RP-HPLC system	145
Fig. 3-11 Inhibitory effects of SFoa, SFob and SFoc against <i>H. pylori</i>	146
Fig. 3-12 Inhibitory effect of CAH against other bacteria	148
Fig. 3-13 Inhibitory effect of sedanolide against <i>H. pylori</i> 3339	152
Fig. 3-14 Mass spectrum of CAH recorded on the Q-star mass spectrometer	153
Fig. 3-15 MALDI-Tof MS analysis of CAH	154
Fig. 3-16 NMR analysis of CAH	156
Fig. 4-1 Bactericidal effects of CAH against <i>H. pylori</i> strain 26695	176
Fig. 4-2 Trypan blue exclusion	177
Fig. 4-3 Scanning electron micrographs	179
Fig. 4-4 Transmission electron micrographs	182
Fig. 4-5 <u>1-D gels</u> showing the effects of CAH on protein expression profiles	184
Fig. 4-6 <u>2-D gels</u> showing the effects of CAH on protein expression profiles	186
Fig. 4-7 Fluorescent microscopic versions of <i>H. pylori</i> following Rh123 staining	191
Fig. 4-8 Effects of CAH on protein and nucleic acid synthesis	195



## **List of Tables**

<b>Table 1-1 Pathology of <i>H. pylori</i> associated gastritis</b>	<b>30</b>
<b>Table 1-2 Diagnosis of <i>H. pylori</i></b>	<b>56</b>
<b>Table 1-3 Agents for eradication of <i>H. pylori</i> and active mechanisms</b>	<b>59</b>
<b>Table 1-4 Regimens for treatment of <i>H. pylori</i> infection</b>	<b>61</b>
<b>Table 1-5 Comparative costs of eradication therapy and other treatment</b>	<b>67</b>
<b>Table 3-1 Summary of MICs of subfraction 1-16</b>	<b>136</b>
<b>Table 4-1 Tentative identification of protein spots</b>	<b>188</b>
<b>Table 4-2 Flow cytometric analysis of <i>H. pylori</i></b>	<b>189</b>

## ABBREVIATIONS

A-CSE	ethanolic extract of celery seed
ACP	acyl carrier protein
BB	brucella broth
Cag A	cytotoxin-associated gene A
CAH	celery seed anti- <i>Helicobacter</i>
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
C-CSE	CO <sub>2</sub> supercritical extract
CHAPS	3-[3-(Cholamidopropyl)dimethylammonio]-1-proanesulfonate
CLO test	<i>Campylobacter</i> like organism test
COX	cyclooxygenase
1 and 2DE	1 and 2 dimensional electrophoresis
DMSO	dimethyl sulfoxide
DPPH	2, 2- diphenyl-1-picrylhydrazyl
DTT	dithiothreitol
EGTA	ethylene glycol tetraacetic acid
FAK	focal adhesion kinase
FBS	fetal bovine serum
GC	gas chromatograph
GERD	gastroesophageal reflux disease
γ-GT	γ-glutamyl transeptidase
HPLC	high performance liquid chromatography
IL-1β	interleukin-1β
INF-γ	interferon-gamma
LDS	lithium dodecyl sulfate
Le <sup>x</sup> and Le <sup>y</sup>	lewis X and Y
LPS	lipopolysaccharide
LPx	liposomal peroxidation X

MALT	mucosa associated lymphoid tissue
MALDI	matrix-assisted laser desorption ionization
ME-CSE	methyl ethyl ketone extracts
MBC	minimum bactericidal concentration
MIC	minimal inhibitory concentration
NADPH	nicotinamide adenine dinucleotide phosphate
NSAID	nonsteroidal antiinflammatory drug
PAI	pathogenicity island
PBP	penicillin-binding protein
PBS	phosphate buffered saline
PE	phosphatidylethanolamine
PGs	prostaglandins
PGE <sub>2</sub>	prostaglandin E2
POR	pyruvate:acceptor oxidoreductase
PPI	proton pump inhibitor
PUD	peptic ulcer disease
RANTES	regulated on activation normal T expressed and secreted
ROI	region of interest
RP-HPLC	reversed phase HPLC
SEM	scanning electron micrographs
SGC column	system change number column
SFE	supercritical fluid extraction
TCA	trichloroacetic acid
TEM	transmission electron microscopy
Th1 and Th2	T-helper type phenotype cell 1 and 2
TLRs	toll-like receptors
TLC	thin-layer chromatography
TNF- $\alpha$	tumor necrosis factor alpha
Vac A	vacuolating toxin A

## **Chapter I**

### **Introduction and Survey of Literature**

## **1. 1 *Helicobacter pylori* and Associated Diseases**

### **1.1.1 Introduction**

About 20 years ago, Drs, Barry Marshall and Robin Warren first reported the successful isolation and culture of a spiral bacterial species from human stomach, later known as *Helicobacter pylori* (Warren and Marshall, 1983). Self experiments by Marshall and later experiments with volunteers demonstrated that the bacteria could colonize the human stomach, thereby inducing inflammation of the gastric mucosa (Marshall *et al.*, 1985; Mori and Nicholson, 1987; Morris *et al.*, 1991). These initial studies stimulated further research, which showed that gastric colonization with *H. pylori* can lead to variety of upper gastrointestinal disorders and diseases. These resulted in insights into the pathogenesis of chronic gastric diseases and in a major clinical impact with regard to the management of the relevant diseases. These pioneering results culminated in the award of the 2005 Nobel Prize in Physiology and Medicine to Professors Barry Marshall and Robin Warren for their “discovery of bacterium *Helicobacter pylori* and its roles in gastritis and peptic ulcer disease” (Press release, 2005).

*Helicobacter pylori* is recognized as one of the most common bacterial pathogens worldwide. It infects more than 50% of the human population and >80% of populations from developing countries (Gatta *et al.*, 2003), and is

most frequently acquired during childhood (Oezcay *et al*, 2004). The prevalence of *H. pylori* in gastric ulcer disease is >90% and cure of the infection results in cure of gastric ulcer disease (Nilsson and Utt, 2002). In the last 5 years, there have been approximately 1500 peer-reviewed publications per year (Kusters *et al.*, 2006) on *Helicobacter*, which involved all aspects of the important issues such as gene, transmission route, prevalence, pathogenesis and treatment. Infection with *H. pylori* can be diagnosed by a variety of tests and often be successfully treated with antibiotics. Unfortunately, the increasing frequency of antibiotic resistance and associated costs affects the efficacy of treatment, and effective vaccination strategies still do not exist.

#### **1.1.2 Discovery of *Helicobacter pylori***

Peptic ulcer disease is a general public health problem throughout the world. Statistics from available sources since the end of the 1970s indicate that 10% or more of Western populations may be afflicted by the disease at some time in their lives and 10% of all adult admissions to general medical and surgical hospitals accounts for the disease (Langman, 1979). Before the involvement of a bacterial infection in causing peptic ulcer was realized, age and sex were recognized as influential factors in causing the disease, others have been recognized patient factors are geographical and environmental factors, smoking and alcohol habits, non-steroidal anti-inflammatory drugs (NSAIDs) and corticosteroid drugs, and genetic and psychological factors (Langman,

1979). Also, the decline in the prevalence of *H. pylori* infection in recent decades is probably one major factor leading to a reduction in peptic ulcer mortality and hospital admissions for peptic ulcer among young people (Kang *et al.*, 2002).

For many years, the environment of the stomach has been considered in a sterile site, and because of its acidity too extreme to support microbial life (Abigail and Dixie, 2002). After examination of 1140 gastric suction biopsy specimens, Palmer concluded that no living bacteria could be found in human stomach, except the oral contaminations (Marshall and Warren, 1984). The discovery that *H. pylori* could colonize the human broke the rule of the bacteria-free stomach. In 1983, Marshall and Warren first identified curved bacilli adjacent to the gastric epithelium of patients with chronic gastritis (Marshall and Warren, 1984). Also, an association between these microorganisms and gastroduodenal diseases was established.

Warren (then at the University of Adelaide, Department of Pathology) observed small curved and S-shaped bacilli in 135 gastric biopsy specimens in the early 80's (Marshall and Warren, 1984). The bacteria were closely associated with the surface epithelium, both within and between the gastric pits. These bacteria were at that time almost unknown to clinicians and pathologists (Marshall and Warren, 1984). Marshall (then at the University of Western

Australia) confirmed that Warren's bacteria had only one or two spirals per cell and resembled *Campylobacter* rather than spirochaetes. They also found that *Campylobacter* isolation techniques were effective in isolating them. These bacteria did not fit any known species either morphologically or biochemical (Marshall and Warren, 1984).

Support for a pathogenic role for these microorganisms in humans initially came from independent studies by Marshall and Morris who established that ingestion of large inoculums of the cells resulted in infection (Marshall *et al*, 1985; Morris *et al*, 1987). These investigators satisfied Koch's postulates by establishing histological confirmed gastritis following the ingestion of viable organisms and resultant recovery of the ingested bacteria (Samra *et al*, 2004). During this study, a volunteer (Dr. Marshall) who had histological normal gastric mucosa ingested the bacteria by mouth, and this resulted in development of a histological proven gastritis on the 14<sup>th</sup> day. It was proposed that this disorder may then progress to a chronic infection, which was further key to predispose to the individual to peptic ulceration (Marshall *et al.*, 1985).

The morphology of these organisms resembles the *Campylobacter* species and consequently they were initially called *Campylobacter*-like organisms or *Campylobacter pylori*. However, morphologic, biochemical and genetic differences were soon identified and *Campylobacter pylori* was designated a



new genus and species name, *Helicobacter pylori* (Blanchard *et al*, 2004). In biochemical markers, *H. pylori* is positive for urease activity. It is susceptible to the antibiotic cephalothin and resistant to nalidixic acid. Compared to *Campylobacter* species, *H. pylori*, a more specialized pathogen, is restricted to the human or animal's stomach, with a unique combination of virulence factors including urease, the *cagA* pathogenicity island and VacA. It has an incomplete citric acid cycle, a simple respiratory chain and few regulatory systems. Also *H. pylori* and *C. jejuni* respond differently to oxygen and oxidative stress (Kelly, 2001). Since the publication of the genome sequence of *C. jejuni* in 2000, and those of *H. pylori* strains 26695 and J99 in 1997, 1999 respectively, the direct comparison of their genome structures became possible (Tomb *et al.*, 1997; Alm *et al.*, 1999 and Parkhill *et al.*, 2000).

### **1.1.3 Microbiology**

#### **1.1.3.1 Genus description**

The genus *Helicobacter* belongs to the subdivision of the *Proteobacteria*, order *Campylobacterales*, family *Helicobacteraceae*. Members of the genus *Helicobacter* are all microaerophilic organisms and are catalase and oxidase positive in most cases; many are also urease positive (Gillespie and Bamford, 2000).

*Helicobacter* species can be sub-divided into the gastric *Helicobacter* specie

(*H. pylori*) and the enterohepatic *Helicobacter* species. *H. pylori* in general can colonize the mammal stomach and is unable to colonize the intestine and liver. It is genetically heterogeneous, suggesting a lack of clonality. This results in every *H. pylori*-positive subject carrying a distinct strain (Kansau *et al.*, 1996). Their heterogeneity can be seen at DNA level (genetic heterogeneity) and the nucleotide level (transcriptional and translation variation and mutation) (Achtman and Suerbaum, 2000).

Strains of *H. pylori* are grouped into two broad families, type I and type II, on the basis of whether they express a biologically active *cagA* antigen and/or *Vac A* (Veronique *et al*, 2000). The type I strains contain one or more copies of the pathogenicity island (PAI) gene and/or *Vac A*. The cytotoxin-associated gene protein (*cagA*) was served as a marker for a *cag* PAI presented as *cagA* gene (Hassan *et al*, 2003). At least 60% of *H. pylori* strains in the United States possess this pathogenicity island, and 90% of strains are *cag* PAI positive in other countries (Blaser *et al.*, 1995). The type I strains are found more frequent than type II in people who develop gastric cancer (Peek *et al.*, 1997).

#### **1.1.3.2 Morphology and general properties**

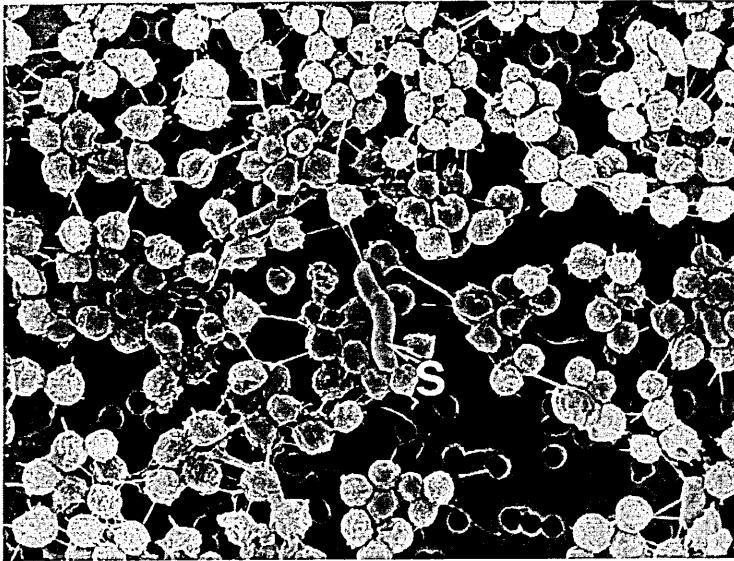
The Gram-negative, curved or spiral bacterium *H. pylori* live in the mucous layer above the gastric epithelium. The *H. pylori* cell is about 3 micrometers long with a diameter of about 0.5 micrometer (Fig.1-1A). As a Gram-negative

bacterium has an integument consisting of an outer membrane, a periplasmic space containing the peptidoglycan, and an inner (cytoplasmic) membrane (Scott, *et al.*, 1998). The cells have five or six flagella at one pole (Fig.1-1B) (Mégraud, 1995). They are nutritionally fastidious bacteria and microaerophilic, and they grow best in an atmosphere of reduced oxygen (about 5%) and elevated CO<sub>2</sub> (about 10%). *H. pylori* produces abundant urease activity, which catalyzes the hydrolysis of urea into ammonia and carbon dioxide (Schaechter *et al.*, 1998).

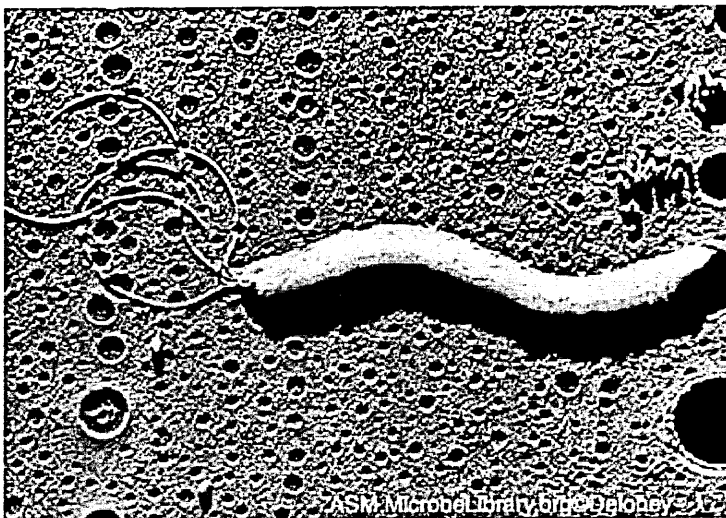
The outer membrane of the cell wall of *H. pylori* consists of phospholipids and lipopolysaccharide (LPS). The phospholipid moiety contains cholesterol glucoside, which is very rare in bacteria (Haque *et al.*, 1996). The LPS moiety consists of lipid A, core oligosaccharide, and an O side chain. Lipid A has low biological activity compared to that from other bacteria (Muotiala *et al.*, 1992). The O side chain of *H. pylori* can mimic Lewis blood group antigens (Lewis x [Le<sup>x</sup>] and Le<sup>y</sup>) and aid molecular mimicry of host antigens. It was also associated immune evasion (Appelmek *et al.*, 1997). LPS phase variation via length variation of poly(C) sequences in the genes encoding  $\alpha$ -1,3-fucosyltransferases (Appelmek *et al.*, 1999) and a poly(C) and poly (TAA) repeats in the gene encoding the  $\alpha$ -1,2-fucosyltransferase (Wang *et al.*, 1999), contributes to population heterogeneity and may allow adaptation of *H.*

**Fig.1-1 Morphology of *H. pylori* strain ATCC 43504**

A



B



A: Coexistence of spiral form (S) and coccoid forms.

B: The cells have five or six flagella at one pole

Reproduced from Mizoguchi *et al.* (1998)

*pylori* to changing conditions in the gastric mucosa (Moran *et al.*, 2002).

Like most flagella, the *H. pylori* flagellum has a hook structure that connects the flagella motor in the cytoplasmic membrane with the part of flagellum that protrudes from the cell surface. Also, the flagella of *H. pylori* are different from usual structures. They are composed of two protein subunits, FlaA and FlaB. These genes of flaA and flaB are not only located in different parts of the chromosome but are under different control. Also the protein shaft of the flagellum is encased in a membranous sheath (Salyers and Whitt, 2002).

Morphologically, *H. pylori* is divided into a spiral (Fig.1-1B) and a coccoid forms. In general, the spiral *H. pylori* transforms to the coccoid form under stressful environmental conditions (Saito *et al.*, 2003). These conditions include high O<sub>2</sub> concentration, alkaline pH, high temperature, extended incubation, or treatment with antibiotics (Mizoguchi *et al.*, 1998; Bode *et al.*, 1993). Several investigators have suggested that the coccoidal form represents a degenerative form with no infectious capability (Hua *et al.*, 1996), or that it has only weak metabolic activity (Vijaykumari *et al.*, 1995).

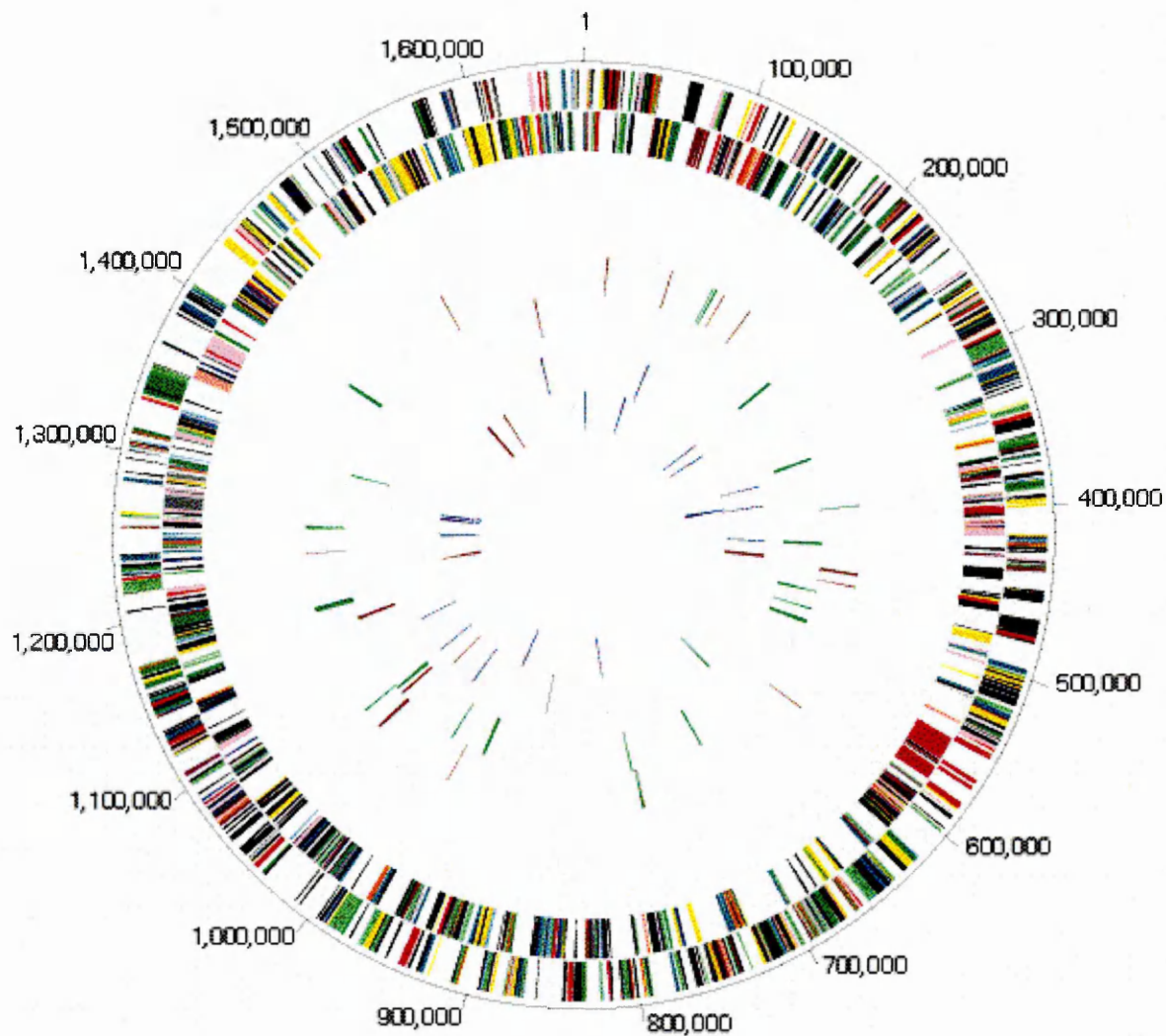
#### **1.1.3.3 Genome structure**

There are now 2 genomic sequences for *H. pylori* in the public domain ([www.genomesonline.org](http://www.genomesonline.org)). In addition, the genome size of *H. pylori* has

recently been determined for 30 isolates by pulsed-field gel electrophoresis after digestion with *NotI* and *NruI*. The range of genome size is 1.60-1.72Mb (average 1.67Mb) (Mégraud, 1995). As shown in Fig. 1-2, the genome of *H. pylori* strain 26695 consists of a circular chromosome with a size of 1,667,867 base pairs (bp) (1,643,831 bp in J99) and average G+C content of 39%. Five regions in strain 26695 (nine in strain J99) within the genome have a significantly different G+C composition (3,280,  $P < 0.01$ ). Region 2 (35% G+C) of strain 26695 is the *cag* pathogenicity island associated with production of the *CagA* antigen and upregulation of interleukin 8, which is flanked by 31-bp direct repeats, and appears to be the product of lateral gene transfer. Regions 1 and 3 (33% G+C) contain one or more copies of the insertion sequence *IS605* at one end, 55 rRNA genes and a 521 bp repeat near the other. Also, region 1 contains the *virB4* genes, which encodes a protein similar to proteins involved in the transfer of the T-DNA from *Agrobacterium tumefaciens* to plant cells and in the secretion of the *Bordetella pertussis* toxin. Region 4 (43% G+C) contains fused *rpoB* and *rpoC* genes, and *fusA* gene. Region 5 (33% G+C) contains two restriction/modification systems (Tomb *et al.*, 1997; Marais *et al.*, 1999).

A total of 1590 open reading frames (ORFs) have been identified representing 91% of chromosome in strain 26695 and in strain J99 1495 identified ORFs represent 90.8% of chromosome (Tomb *et al.*, 1997; Alm *et al.*, 1999). The

**Fig. 1- 2. Circular representation of the *H. pylori* 26695 chromosome.**



Outer concentric circle: predicted coding regions on the plus strand classified as to role. Second concentric circle: predicted coding regions on the minus strand. Third and fourth concentric circles: IS elements (red) and other repeats (green) on the plus and minus strand, respectively. Fifth and sixth concentric circles: tRNAs (blue), rRNAs (red), and sRNAs (green) on the plus and minus strand, respectively,

Reproduced from Tomb *et al.* (1997).

function of 58% of the open reading frames can be predicted, 18.5% have orthologues of unknown function in other species, and 23.5% are *H. pylori* specific (Peter *et al.*, 2001).

*H. pylori* have relatively few regulatory genes. There are 62 genes in the pathogenesis category in the genome. Both strains sequenced (26695 and J99) have an approximately 40 kb long *Cag* pathogenicity island that contains over 40 genes known or suspected to be involved in pathogenicity (Salyer and Whitt, 2002)

Comparison of the two available genome sequences confirms that *H. pylori* is genetically diverse. About 7% of genes in each of the genomes are unique, and about half of these unique genes are clustered together on the chromosome and may thus have come into the strain from an outside source (Salyer and Whitt, 2002). This genetic heterogeneity is possible and an adaptation of *H. pylori* to the gastric conditions of its host, as well as to the distinct patterns of the host-mediated immune response to *H. pylori* infection (Kuipers *et al.*, 2000). It is thought to occur via several methods of DNA rearrangement and the introduction and deletion of foreign sequences (Achtman and Suerbaum, 2000). Diversity at the nucleotide level arises via several mechanisms, including transcriptional and translation phase variation and mutation (Ge *et al.*, 1999). Phase variation often occurs with a single



mutation via causing a shift in translation of an affected gene due to reversible slipped-strand mispairing in hemopolymeric G or C tracts (Achtman and Suerbaum, 2000).

#### **1.1.3.4 Proteome characterization**

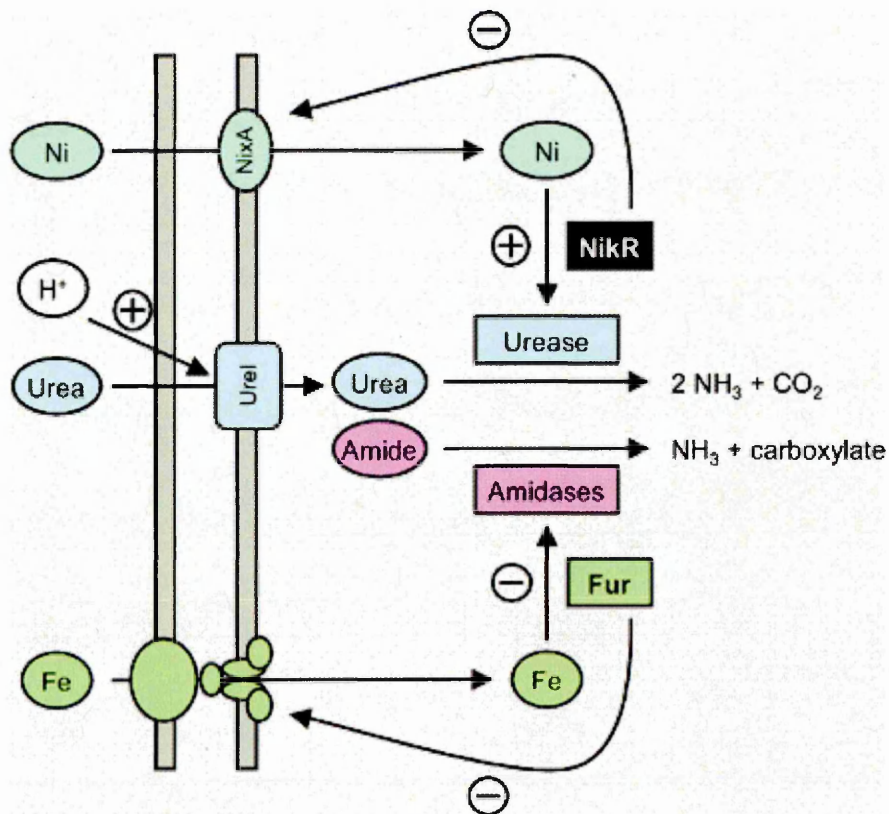
Since the first complete bacterial genome (*Haemophilus influenzae*) was reported in 1995 (Fleishmann *et al.*, 1995; Tomb *et al.*, 1997; Marais *et al.*, 1999), protein identification has changed remarkably. Two-dimensional gel electrophoresis of proteins allows the separation of up to 10,000 protein species in one iso-electrophoretic focusing run (Klose and Kobalz, 1995). This resolution clearly exceeds the number of genes predicted for establish of the two *H. pylori* strains sequenced and is sufficient for proteome analysis of this microorganism. Studies on *H. pylori* proteins have suggested that the organism may be very conservative at the protein level. Many proteins in *H. pylori* are subjected to high degree of post-translational modification (Robert *et al.*, 2001). For *H. pylori* strains 26695 and J99, comparison of their genomic nucleotide sequences suggests that nucleotide variation does not translate into highly divergent proteomes. There are only eight genes with 98% or greater nucleotide identity between the two strains, but 310 proteins with 98% or greater amino acid conservation, including 41 with perfect identity (Robert *et al.*, 2001).

A number of the unique proteins produced by *Helicobacter pylori* are now known to be involved in host colonization and transmission of this pathogen. The growing list of those identified for *H. pylori* includes: (1) proteins involved in adhesion, such as BabA, which mediated binding to Lewis<sup>b</sup> histo-blood group antigen associated with the presence of *cag* pathogenicity island (Ilver *et al.*, 1998), and AlpA and AlpB, which are members of a large family of related outer membrane proteins (Tomb *et al.*, 1997); or (2) proteins required for motility, such as flagellins; or (3) factors involved in acid neutralization, especially urease; or (4) proteins involved in iron uptake and storage, such as a lactoferrin-binding protein (Doig *et al.*, 1993); or (5) proteins involved in pathogenicity such as the Cag pathogenicity island encoded proteins and the vacuolating toxin VacA (Jungblut *et al.*, 2000); or (6) heat shock proteins (Hsps), which are highly conserved immunogenic molecules. *H. pylori* produce at least two Hsp's, the HspA (13000-Mr) sharing homology at amino acid level with the GroES family and HspB (54000-Mr) identified to be a homologue of the class belonging to the GroEL family (Nilsson and Utt, 2002); or (7) neutrophil activating protein (HP-NAP): *H. pylori* infection induces an inflammatory cell response, and the severity of mucosal injury appears to be directly correlated with the extent of neutrophil infiltration (Craig *et al.*, 1992).

#### **1.1.3.5 Metabolism**

*H. pylori* seems to have a “stripped-down” metabolism (**Fig. 1-3**), which has

**Fig. 1-3 Schematic representation of the relationships of acid resistance and metabolisms**



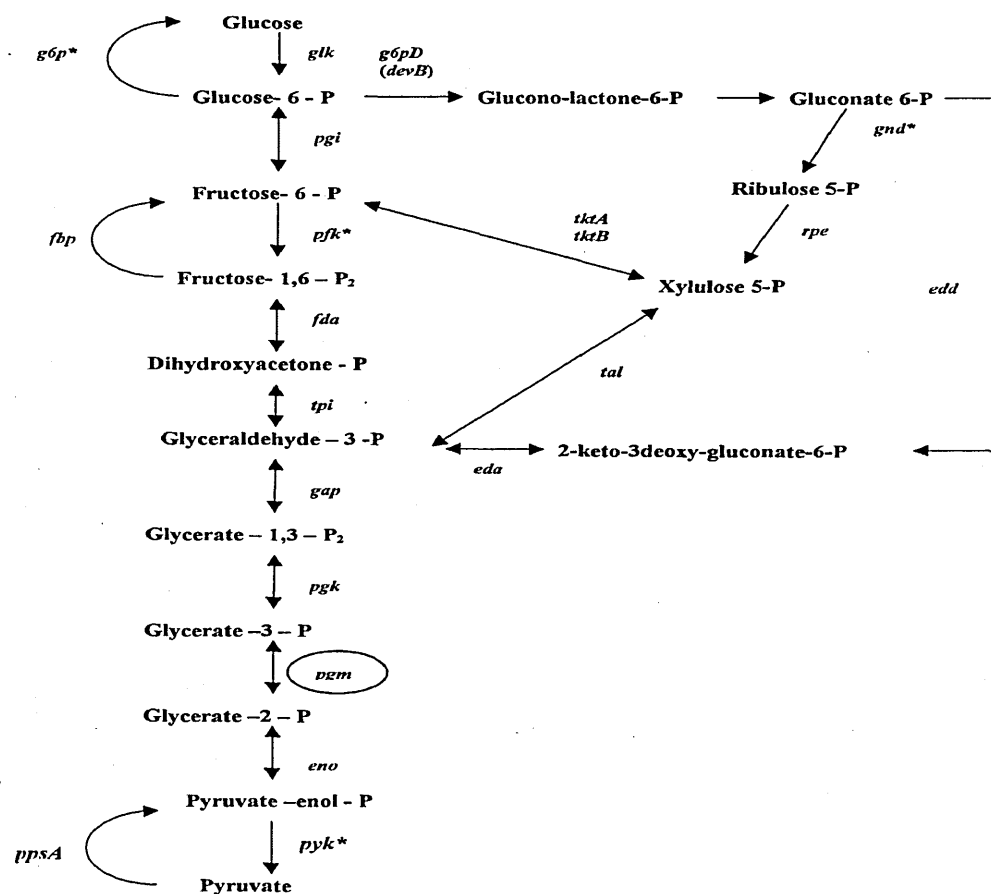
Relationships of acid resistance (urease activity and urea transport), nitrogen metabolism (ammonia production), metal metabolism (iron uptake and nickel uptake), and gene regulation (Fur and NikR) in *H. pylori*. (Fe: ferrous iron. Fur: ferric uptake regulator homolog. Ni: Nickel)

From Kusters *et al.*, 2006

very few and single biosynthetic pathways for some amino acids (Nedenskov, 1994). This limited metabolic flexibility may be related to its narrow host and target organ range, but is clearly highly effective in human, because its infection usually contains for the rest of the host life. As stated previously, *H. pylori* is urease, catalase, and oxidase positive characteristics, which are often used in its identification. The availability of two complete genome sequences of *H. pylori* and microbiology combines with biochemical evidence to give an understanding of its metabolism (Kusters *et al.*, 2006).

**Glucose Metabolism and Pyruvate Metabolism:** *H. pylori* can metabolize glucose by both oxidative and fermentative pathways (Burns *et al.*, 1993) and glucose appears to be the only carbohydrate utilized by the bacterium (Mendz *et al.*, 1993). Glucose is imported into the cells by a permease, which is specific for D-glucose and galactose. This transporter is unaffected by the known glucose permeases inhibitors of other bacteria (Burns *et al.*, 1993). Intracellular phosphorylation of glucose is performed by a glucokinase (Mendz *et al.*, 1993). Mendz and Hazell (1991) revealed the presence of enzymatic activities, which are part of the oxidative and nonoxidative steps of the pentose-phosphate pathway in *H. pylori* (**Fig. 1-4**). This pathway is an efficient mechanism to provide NADPH and NADH for reductive biosynthesis and C<sub>5</sub> phosphorylated carbohydrates essential for nucleotide synthesis. Alternatively, glucose- 6-phosphate can be utilized by the Entner-Doudoroff pathway, which

**Fig. 1-4 Glucose Metabolism in *H. pylori***



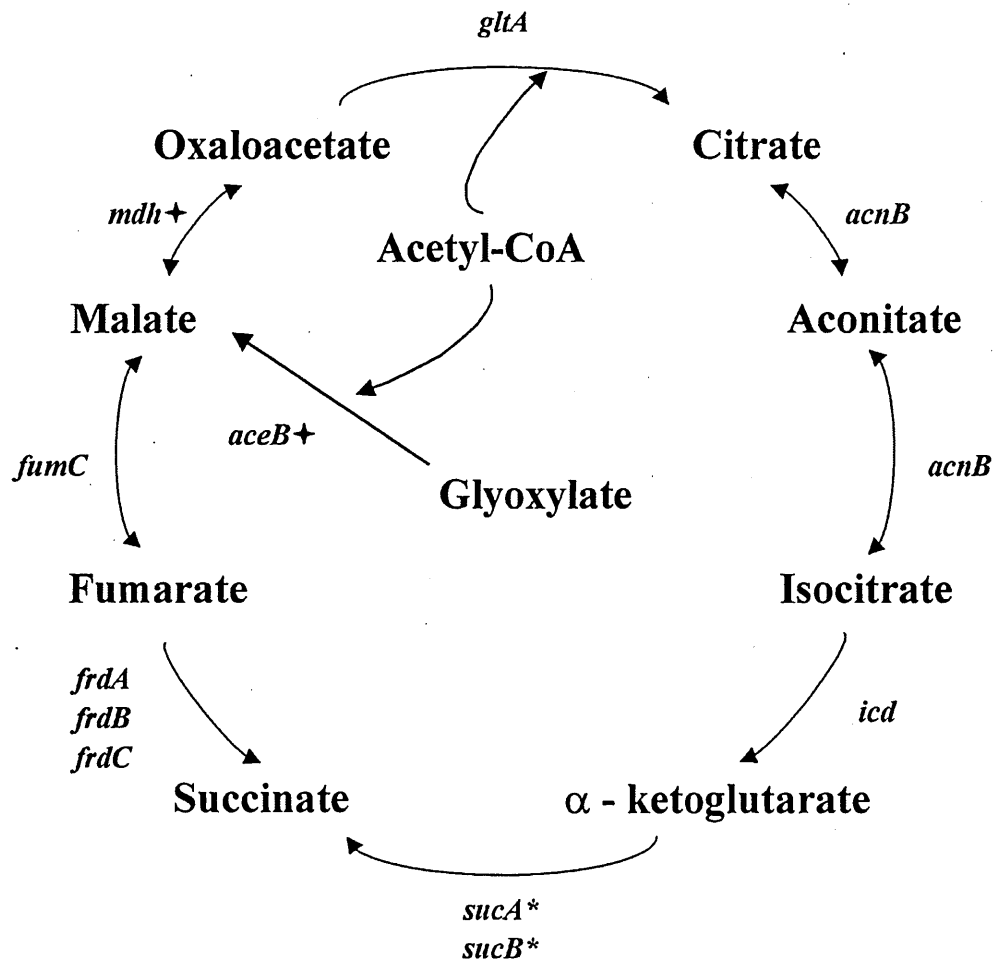
Glycolysis, gluconeogenesis, pentose phosphate, and Entner-Doudoroff pathways. Glycolysis: *glk*, glucokinase; *pgi*, phosphoglucose isomerase; *pfk*, phosphofructokinase; *fda*, fructose-1,6-bisphosphate aldolase; *tpi*, triose-phosphate isomerase; *gap*, glyceraldehyde-3-phosphate dehydrogenase; *pgk*, phosphoglycerate kinase; *pgm*, phosphoglycerate mutase; *eno*, enolase; *pyk*, pyruvate kinase. Gluconeogenesis: the same enzymes as in glycolysis but with unidirectional steps, i.e., *ppsA*, phosphoenol pyruvate synthase; *fbp*, fructose-1,6 bisphosphatase; and *g6p*, glucose-6 phosphatase. Pentose phosphate: *g6pD* (*devB*), glucose-6-phosphate dehydrogenase; lactonase; *gnd*, 6-phosphogluconate dehydrogenase; *rpe*, D-ribulose-5-phosphate 3 epimerase; *tal*, transaldolase; *tkt*, transketolase. Entner-Doudoroff: *edd*, 6-phosphogluconate dehydratase; *eda*, 2-keto-3-deoxy-6-phosphogluconate aldolase. Asterisks denote enzymes for which no gene was identified in the *H. pylori* sequence. The circle denotes an enzyme whose enzymatic activity has not been observed but whose corresponding gene was identified (from Marais *et al.*, 1999).

consists of two reactions including a dehydratase-catalyzed formation of 2-keto-3-deoxygluconate- 6-phosphate from gluconate-6-phosphate and an aldolase-catalyzed production of pyruvate and glyceraldehyde-3-phosphate (**Fig. 1-4**) (Chalk *et al.*, 1994).

Pyruvate is an end product of both the glycolytic and Entner-Doudoroff pathways. To enter the Krebs cycle, pyruvate must be converted to acetyl-CoA (oxidative decarboxylation of pyruvate) carried out in *H. pylori* by a pyruvate:acceptor oxidoreductase (POR) associated with mixed-acid fermentation (Hughes *et al.*, 1998). Flavodoxin is an electron acceptor for POR. The pyruvate-flavodoxin oxidoreductase of *H. pylori* is composed of four subunits previously detected only in hyperthermophilic organisms (Hughes *et al.*, 1998). The genome analysis of *H. pylori* supports these experimental data (Tomb *et al.*, 1997).

**The Krebs Cycle and Related Enzymes:** In *H. pylori*, the Krebs cycle (tricarboxylic acid cycle) likes to be a branched noncyclic pathway. As shown in **Fig.1-5**, one way of dicarboxylic acid proceeds reductively from oxaloacetate through malate and fumarate to succinate, and other tricarboxylic acid starts oxidatively from oxaloacetate through citrate and isocitrate and to  $\alpha$ -ketoglutarate to succinate finally (Hazell *et al.*, 1997).

**Fig. 1-5 Dicarboxylic and tricarboxylic acid branches of the noncyclic Krebs pathway in *H. pylori***



*gltA*, citrate synthase; *acnB*, aconitase; *icd*, isocitrate dehydrogenase; *sucAB*,  $\alpha$ -ketoglutarate dehydrogenase; *frdABC*, fumarate reductase; *fumC*, fumarase; *mdh*, malate dehydrogenase; *aceB*, malate synthase. Asterisks denote enzymes for which no genes were identified in the *H. pylori* sequence; crosses denote enzymes whose enzymatic activities were observed but the corresponding genes were not identified. Reprinted from reference 237 with permission of the publisher (Marais *et al.*, 1999).

**Amino Acid Metabolism:** Amino acids are potential sources of carbon, nitrogen, and energy. All the strains of *H. pylori* tested required arginine, histidine, isoleucine, leucine, methionine, phenylalanine, and valine. Some of them also required alanine (8 of 10 strains), and serine was also needed for 5 of them (Nedenskov, 1994).

**Lipid Metabolism:** Metabolism of lipid is similar to that in most aerobic bacteria (Fig. 1-6), with acetyl-CoA being initially catalyzed to malonyl-CoA by acetyl carboxylase. This enzyme consists of a complex of three individual proteins: biotin carboxyl carrier protein, biotin carboxylase, and carboxyltransferase ( $\alpha$  and  $\beta$  subunits), encoded by the genes, HP0370 (*accC*), HP0371 (*fabE*), HP0557 (*accA*), and HP0950 (*accD*), respectively (Burns *et al.* 1995). The next steps in the initiation of fatty acid biosynthesis involve the attachment of the acyl carrier protein (ACP) to the acetyl and malonyl moieties, which encoded by the genes, HP0559, HP0962 (*acpP*), and HP0808 (*acpS*), respectively. The transfer reactions of CoA-bearing acyl chains to ACP are catalyzed by acetyl-CoA:ACP transacylase and malonyl-CoA:ACP transacylase (Fig. 1-6). This function could be performed by a thiolase product of HP0690 (*fadA*). Malonyl-CoA is converted to malonyl-CoA:ACP by the malonyl-CoA:ACP transacylase encoded by HP0090 (*fabD*) (Fig. 1-6).





The most abundant phospholipids of the lipid composition of *H. pylori* are phosphatidylethanolamine (PE), cardiolipin, and phosphatidylglycerol (PG) (Hirai *et al.*, 1995). The biosynthesis pathway for phospholipids utilizes *sn*-glycerol-3-phosphate (Glp). Glp is acylated first by the glycerol-3-phosphate acyltransferase, using the acyl-ACP products of fatty acid synthesis, and then a second fatty acid is added by a 1-acyl-Glp acyltransferase to form phosphatidic acid, encoded by genes HP0201 (*p/sX*) and HP1348 (*p/sC*), respectively (Rock and Cronan, 1982). Phosphatidic acid is catalyzed to CDP-diglycerol by CDP-diglycerol synthase. CDP-diglycerol reacts with serine to form phosphatidylserine (PS) or with Glp to form phosphatidylglycerolphosphate (PGP). The decarboxylation of PS by PS decarboxylase yields PE, and the dephosphorylation of PGP by PGP phosphatase yields PG. Three ORFs, HP0215, HP1016, and HP1357 involved in this reaction (Ge *et al.*, 1997).

**Lipopolysaccharide (LPS) metabolism:** The surface-exposed lipopolysaccharide (LPS) molecules are important immunomodulators and potent stimulators of the immune system of hosts (Moran, 1995). The lipid A portion, one of three components of LPS, is responsible for the immunological and endotoxic properties (Moran *et al.*, 1997). The *H. pylori* LPS is remarkable in its low toxicity, contained Lewis<sup>x</sup> and Lewis<sup>y</sup> antigenic motifs that mimic Lewis antigens present on parietal cells of the human gastric mucosa (Aspinall

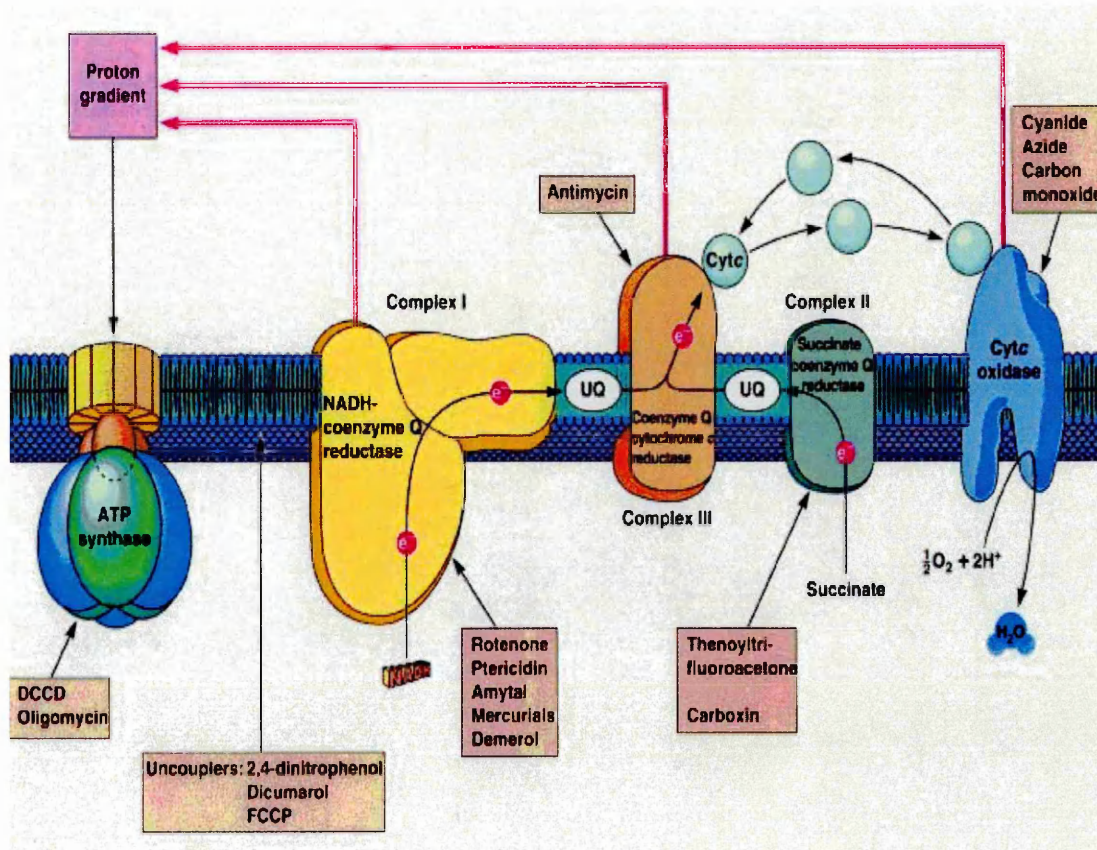
*et al.*, 1996). At least 27 genes found in *H. pylori* are likely to be involved in LPS biosynthesis (Tomb *et al.*, 1997).

**Nucleotide metabolism:** *H. pylori* appears to be able to synthesize pyrimiding nucleotides *de novo*, many of the pyrimidine nucleotides, and to have a limited utilization of the pyrimidine salvage pathways. The bacterium shows a greater capacity to salvage preformed purines, but at the same time it is able to grow and proliferate, synthesizing *de novo* purine nucleotides.

**Respiratory Chains:** *H. pylori* is a microaerophilic bacterium that does not tolerate high oxygen conditions, but it requires at least 2% O<sub>2</sub>, since *H. pylori* uses oxygen as a terminal electron acceptor (**Fig. 1-7**) (Mendz *et al.*, 1997). The terminal respiratory acceptor can be oxygen (aerobic respiration) or other substrates (anaerobic respiration). *H. pylori* has genes coding for proteins involved in both types of respiration (Tomb *et al.*, 1997). The apparent paradox in having an operative aerobic respiratory chain and anaerobic respiration at the low oxygen tensions in *H. pylori*, has not been resolved (Marais *et al.*, 1999).

**Superoxide defence:** There are three principal mechanisms which enable *H. pylori* to resist oxidative damage, and they are catalyzed by the enzymes superoxide dismutase, catalase, and alkylhydroperoxide reductase (Ahp)

**Fig. 1-7 The respiratory electron transport chain of bacteria of *H. pylori***



light brown boxes show specific inhibitors that act at each stage of respiration.

From Saunders (1995)

(Hazell *et al.*, 1991; Spiegelhalder *et al.*, 1993). Inflammation within the gastric mucosa leads to an increase in toxic oxygen metabolites (Nalini *et al.*, 1992). The superoxide anion is a highly reactive oxygen species formed as part of the oxidative burst of polymorphonuclear leukocytes, and is dismutated to H<sub>2</sub>O<sub>2</sub> by superoxide dismutase (Moran, 1996). Hydrogen peroxide is in turn converted to oxygen and water by catalase.

**Nitrogen metabolism** The main route of ammonia production in *H. pylori* is through the highly active urease enzyme (Bauerfeind *et al.*, 1997). Urease is a nickel-containing enzyme that consists of 12 UreA and 12 UreB subunits (Ha *et al.*, 2001), encoded by *ureA* and *ureB* genes (Labigne *et al.*, 1991). Urea transport into the cell is controlled via the H<sup>+</sup>-gated urea channel UreI (Bury-Mone *et al.*, 2001), resulting in increased urea transport in acidic conditions. For facilitating survival and growth in acidic conditions, the ammonia produced via enzymatic degradation of urea is used for amino acid biosynthesis. The importance of ammonia in *H. pylori* metabolism and virulence is underlined by the presence of several alternative routes for ammonia production, via enzymatic degradation of diverse amides as well as amino acids (Bury-Mone *et al.*, 2003).

**Metal metabolism:** *H. pylori*, like other bacteria, requires iron-scavenging systems to survive in its natural environment (Earhart, 1996). Fe(III) is the

oxidation state under aerobic conditions, which at neutral pH forms insoluble hydrated oxide polymers. Fe(II) is the predominant iron form under anaerobic conditions. Thus, in an oxidizing atmosphere, organisms have to develop efficient systems for iron assimilation (Earhart, 1996).

**Urease uptake and pH Regulation:** *H. pylori* produces high levels of urease, up about 6 -10% of the total bacterial protein (Labigne and Reuse, 1996). This enzyme breaks down urea into ammonia and carbon dioxide, providing an acid-neutralizing cloud of ammonia that could protect the bacterium from gastric acidity (Marshall *et al.*, 1990). The function of urease plays an important role in the physiology of *H. pylori* (Hazell and Mendz, 1997) and in bacterial colonization (Labigne and Reuse, 1996). *H. pylori* urease has ultimate characteristics, compared to those of other bacterial species. First, the enzyme is found in the cytoplasm as well as on the bacterial surface (Dunn *et al.*, 1997; Phadnis *et al.*, 1996). Second, it has two optimal pHs, one of which is acidic (Ferrero and Lee, 1991), and it displays higher substrate affinity than other bacterial ureases (Moblely and Foxall, 1994). Third, it is composed of only two subunits, UreA and UreB (Clayton *et al.*, 1990), whereas other bacterial ureases contain three subunits (Moblely *et al.*, 1995).

The ability of *H. pylori* to survive exposure to low pH is likely to depend on a combination of cytoplasmic and surface-associated urease activities

(Krishnamurthy *et al.*, 1998). Glucose metabolism can take place in *H. pylori* at environmental pHs between 3.5 and 8.6, and cytoplasmic urease activity allows metabolism in the pH range from 2.5 to 4.0 by maintaining the periplasmic pH at 6.2 (Rektorschek *et al.*, 1998). Also surface-associated urease decreases *H. pylori* survival at neutral pH (Meyer-Rosberg *et al.*, 1996). *H. pylori* has adapted itself to the acidic environment of the stomach and can be classified as an acid-tolerant neutrophile (Bauerfeind *et al.*, 1997).

#### **1.1.3.6 Growth requirements**

*H. pylori* strains are homogeneous with regard to their culture requirement: media, atmosphere and temperature (Mégraud, 1995). Growth of *H. pylori* has been demonstrated in nutrient-rich media such as Brucella broth. Typical supplements added to these growth media include whole blood, serum, lysed erythrocytes, hemin, yeast extract and peptone (Kitsos and Stadtlander, 1998). These supplements may act as additional sources of nutrients and possibly also protect against the toxic effects of long-chain fatty acids. The bacterium only optimally grows at the narrow pH range of 5.5 – 8.0 (Scott *et al.*, 2002). Normally, the microaerophilic conditions used in the laboratory are at 10% CO<sub>2</sub>, 3% O<sub>2</sub> (2% -5%) and 87% N<sub>2</sub> at 37 °C (34 – 40 °C) (Momin and Nair, 2001). The conditions for optimal growth are very important for this organism, since it does not tolerate prolonged exposure to air (Park, 2002). *H. pylori* can be

stored for the long term (1-2 years) at  $-80^{\circ}\text{C}$  in liquid media with either 15 – 20% glycerol or 10% dimethyl sulfoxide. The optimal viability requires the use of cultures less than 48 hour, with more than 90% spiral-shaped cells (Kusters *et al.*, 2006).

#### **1.1.4 Pathogenesis**

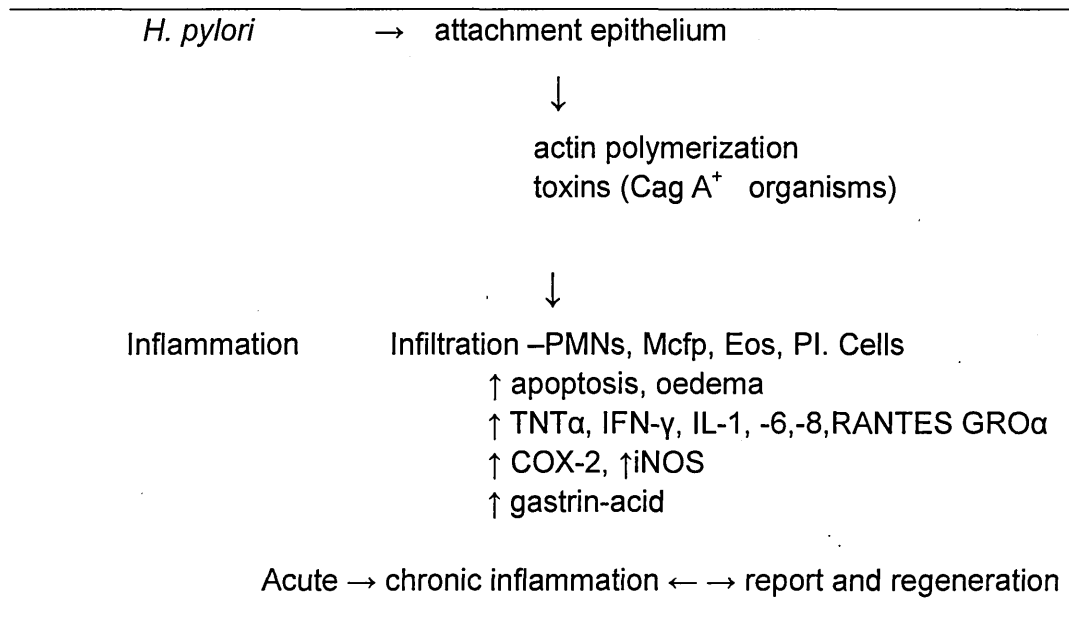
The pathogenesis of *H. pylori* can be described in three steps: (a) gain of entry and colonization of the unique niche of the human gastric mucosa; (b) avoidance, subversion, or exploitation of the nonspecific and specific human immune systems; and (c) multiplication, tissue damage, and transmission to a new susceptible host or spread to adjacent tissue (McGee and Mobley, 1999). Pathology of *H. pylori* associated gastritis and biochemical changes were shown in **Table 1-1** (Rainsford, 2001). This pathogenesis processing depends on a variety of factors, including characteristics of the colonizing strain, host genetics and immune response, diet, and the level of acid production (Kusters *et al.*, 2006).

##### **1.1.4.1 Animal models**

Animal models have been very helpful in understanding of the mechanisms inducing disease development and the pathogenic properties of *H. pylori*, as well as testing the effects of treatment and vaccination on the pathogenesis.



**Table 1-1 Pathology of *H. pylori* associated gastritis**



Repeated cycles of cell injury. Oxyntic gland “replacement”: Irreversible; Fibrosis: Intestinalized epithelium; Gross loss of glands in Lamina propria; ↓acid production; adenocarcinoma (Rainsford, 2001)

The choice of the most appropriate model is dependent on the hypothesis that is being tested.

**Mouse:** The mouse is the most widely used animal host for investigations of the pathogenicity of *H. pylori* and other bacteria, since mice are readily available and their housing is relatively cheap. Also genetic variants are available that are useful in defining host resistance and host susceptibility. *H. pylori* infection of many commonly used mouse strains results likely in lymphocytic gastritis without progression to *H. pylori*-associated pathology, such as peptic ulcers or gastric cancer (Kodama *et al.*, 2004; O'Rourke *et al.*, 2003). Also, the model of the murine stomach is different from that of the human stomach with lacks components involved in the development of gastric pathology. Finally, the murine stomach is not sterile except when stimulated with acid secretory agents and thus other bacteria may influence the outcome of *H. pylori* infection, compared to the healthy human stomach (O'Rourke *et al.*, 2003, Pritchard *et al.*, 2004). Therefore, the use of the mouse model is mostly restricted to testing the colonization properties of distinct *H. pylori* mutants.

**Mongolian gerbil:** This animal model has advantages similar to the mouse model in being easy to rise and with size. After long-term colonization by *H. pylori*, the gastric pathology of gerbils is similar to that in human subjects, for

instance in the development of peptic ulcers, intestinal metaplasia (Hirayama *et al.*, 1996, Honda *et al.*, 1998) and gastric adenocarcinoma (Franco *et al.*, 2005). The Mongolian gerbil model was mostly helpful to test the colonization abilities of defined *H. pylori* mutants (Wirth *et al.*, 1998), identify virulence factors, and determine the efficacy of treatment and vaccination (Jeremy *et al.*, 2005; Kavermann *et al.*, 2003).

**Guinea pig:** The guinea pig model is also similar to the mouse model. In addition, the guinea pig stomach has several features in common with the human stomach, such as sterility, the production of IL-8, the lack of a nonglandular region, and the presence of a cylindrical epithelium (Durrani *et al.*, 2003; Keenan *et al.*, 2003). Furthermore, like humans, the guinea pig has a nutritional requirement for exogenous vitamin C. The lowered vitamin C levels in humans are related to *H. pylori* infection (Sobala *et al.*, 1991).

**Gnotobiotic piglets:** One of the first animal models for *H. pylori* infection was based on the gnotobiotic piglet. The pig is a monogastric mammal with dietary habits similar to those of humans, and it has a stomach with similar anatomical and physiological characteristics (Krakowka *et al.*, 1987). Colonization of gnotobiotic piglets with *H. pylori* results in gastritis (Eaton and Krakowka, 1992),

gastric ulcers and MALT lymphoma (Green *et al.*, 1997). Many early studies of the role of virulence factors of *H. pylori* infection were performed with this model.

**Nonhuman primates:** A natural choice for an animal model of *H. pylori* infection would be a nonhuman primate such as the rhesus monkey model, as these are genetically closely related to humans (Dubois *et al.*, 1996). The model has been used for testing the efficacy of therapeutic intervention by antimicrobials (Dubois *et al.*, 1996), anti-adhesion compounds (Mysore *et al.*, 1999), and vaccines (Dubois *et al.*, 1998), and it has permitted study of the role of mucins and Lewis antigens in adhesion of *H. pylori* (Linden *et al.*, 2004).

#### **1.1.4.2 Virulence factors**

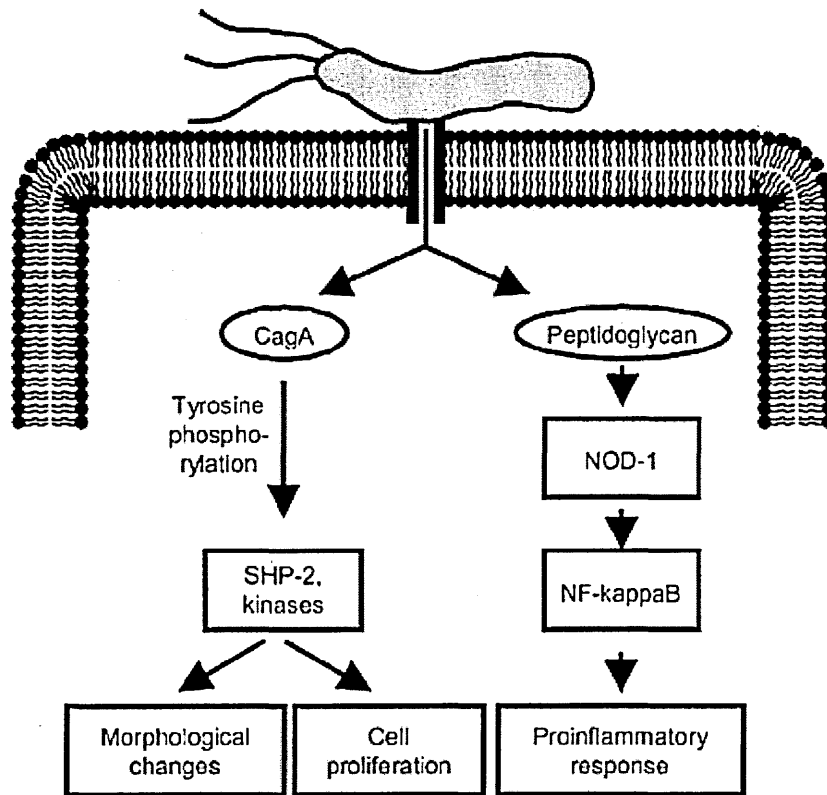
Several virulence factors of *H. pylori* have now been suggested to play a direct role in the ability of the bacteria to colonize the gastric mucosa and/or to contribute to the pathogenesis of disease (Blanchard *et al.*, 2004).

**Urease:** The enzyme urease, which by hydrolyzing urea into CO<sub>2</sub> and ammonia, allows *H. pylori* to survive in the highly acidic environment, was one of the first virulence factors described (Hu *et al.*, 1995).

**cag PAI:** The *cag* pathogenicity-island (*cag* PAI) and cytotoxin-associated gene (*CagA*) protein, which serves as a marker for a *cag* PAI, are the most notable among virulence factors (Kausar *et al*, 2005). Recent studies have provided a molecular basis for the pathological actions of *CagA* on gastric epithelial cells. After attachment of *cagA*-positive *H. pylori* to gastric epithelial cells, *CagA* is directly injected from the bacteria into the cells via the bacterial type IV secretion system and undergoes tyrosine phosphorylation in the host cells (Yamazaki *et al*, 2005). Tyrosine-phosphorylated *Cag A* then binds specifically to SHP-2 tyrosine phosphatase and deregulates activity. *Cag A*-activated SHP-2 dephosphorylates focal adhesion kinase (FAK) and inhibits kinase activity, and causes sustained Erk MAP kinase activation (Masanori *et al.*, 2006). The roles of *Cag A* and PAI are summarized in **Fig. 1-8**.

**VacA:** The vacuolating cytotoxin antigen (*VacA*), a 95-kDa protein of *H. pylori* is another virulence factor that is considered to constitute an increased risk for development of peptic ulcers and gastric cancer, and an important antigen in the human immune response to *H. pylori* (Montecucco and Rappuoli 2001; Salama *et al*, 2001). *VacA* induces the formation of large cytoplasmic vacuoles in culture cells. *VacA*-induced vacuolization has several consequences for cellular physiology that might contribute to pathogenesis and to *H. pylori* survival. It causes a marked decrease of the proteolytic activity in the endocytic pathway. Consequently, *VacA* inhibits the stimulation of T-cell clones, specific

**Fig. 1-8 Schematic representation of the different roles of the Cag type IV secretion system in immune modulation, cell proliferation, and morphological changes.**



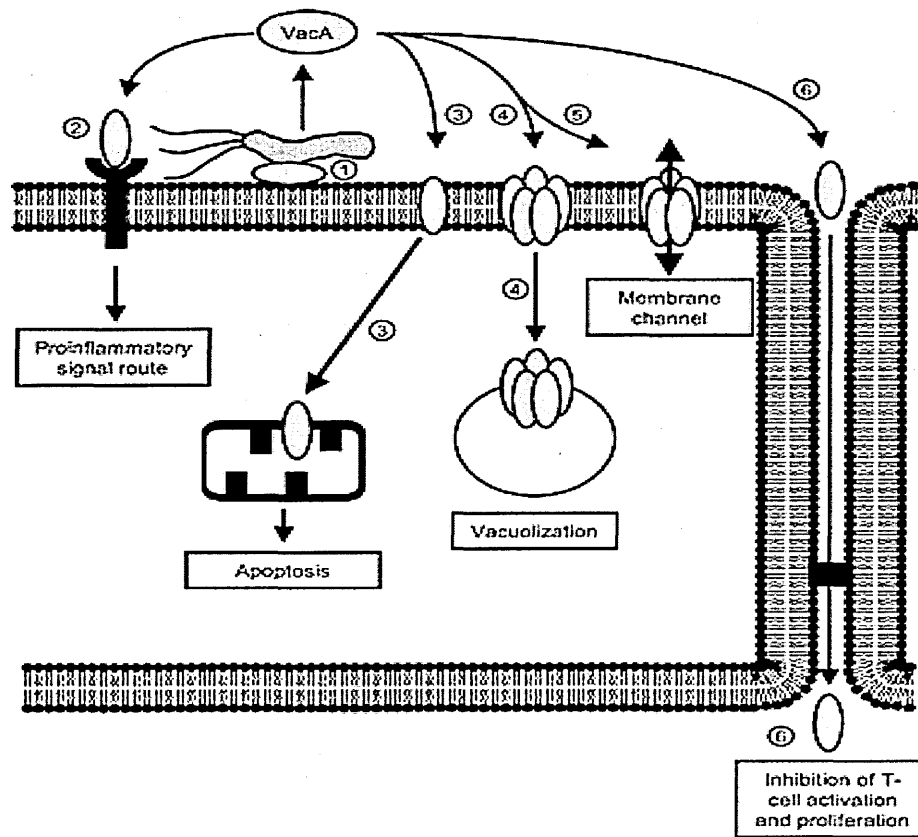
Reproduced from Kusters *et al.* (2006).

for epitopes generated in the antigen-processing compartment (Molinari et al 1998). In artificial membranes, VacA forms anion-selective channels, indicating that the vacuolating activity derives from osmotic imbalance of intracellular acidic components (Montecucco and Bernard, 2003). In mouse bone marrow derived mast cells, VacA has chemotactic activity, and induces production of pro-inflammatory cytokines (Gebert, 2003). The roles of VacA are summarized in Fig. 1-9.

**LPS:** The endotoxin, lipopolysaccharide (LPS) is released from the outer cell membrane of the bacteria. LPS from *H. pylori* is shown to impair the mucosal integrity due to the binding to laminin, one of the important components of extracellular matrix (Moran, 1996; Piotrowski et al., 1991). Moreover, the bacterial LPS induces the septic shock and the multiorgan dysfunctions due to the activation of mediators of tissue injury such as macrophage-derived cytokines and free oxidant species (Kusters et al., 2006). Also, LPS causes inhibition of gastric mucus secretion to interfere with somatostatin receptor and to enhance pepsinogen secretion (Brzozowski et al., 1997)

**Adhesion associated proteins:** (1) BabA (HopS) is a 78 kDa protein, encoded by the *babA* gene. BabA mediates binding to fucosylated Lewis b

**Fig. 1-9 The roles of Vac A in pathogenesis of *H. pylori***



The VacA protein influences cellular processes via different routes, thus assisting in chronic colonization of the gastric mucosa by *H. pylori*. (1) Surface-bound VacA may be directly delivered to the cell membrane. Secreted VacA may either (2) bind to a cell membrane receptor and initiate a proinflammatory response, (3) be taken up directly by the cell and be trafficked to the mitochondria and induce apoptosis, (4) be taken up by pinocytosis and induce vacuolization, (5) form a membrane channel, resulting in leakage of nutrients to the extracellular space, or (6) pass through the tight junctions and inhibit T-cell activation and proliferation (Kusters *et al.*, 2006).



(Le<sup>b</sup>) blood group antigens on the human host cells (Boren *et al.*, 1993). There are two distinct *babA* alleles, *babA1* and *babA2*, but only *babA2* can encode a full-sized (active) bacterial adhesion protein. BabA-mediated adhesion is relevant for the colonization and pathogenesis of *H. pylori* (Guruge *et al.*, 1998). The adhesion is predominantly toward MUC5AC-specific ligands. The Le<sup>b</sup> antigen is an important factor in this site-specific colonization (Van de Bovenkamp *et al.*, 2003). BabA is suggested to play a role in the virulence of *H. pylori*, as the *babA2* allele is strongly associated with peptic ulcer disease and gastric adenocarcinoma (Gerhard *et al.*, 1999). (2) OipA (HopH), a 34-kDa OipA protein may play an adhesin role (Yamaoka *et al.*, 2000). Expression of OipA is strongly associated with increased IL-8 expression (Yamaoka *et al.*, 2002). (3) SabA (HopP) mediates binding to sialic acid-containing glycoconjugates (Lu *et al.*, 2002). *H. pylori*-induced gastric inflammation and gastric carcinoma are associated with the replacement of nonsialylated Lewis antigens by sialylated Le<sup>x</sup> and sialylated Le<sup>a</sup> (Mahdavi *et al.*, 2002). Human granulocytes also carry sialylated carbohydrates on their surface, and consequently these cells are specifically recognized by SabA. *In vitro*, binding granulocytes by *H. pylori* results in the nonopsonic activation of these cells (Unemo *et al.*, 2005), potentially allowing the bacterium to control these cells. SabA is also involved in the binding of the extracellular matrix protein laminin to damage the host immune surveillance and thus allows the bacterium to control

the immune response through direct transfer of CagA, VacA, and other virulence factors (Walz *et al.*, 2005).

*H. pylori* also produces several other factors that are believed to be important in its pathogenesis, including a protease, lipase, catalase, phospholipase and oxidase (Thompson and Taylor, 2000).

#### **1.1.4.3 Immune response**

**Activation of the immune response:** Toll-like receptors (TLRs) on epithelial cells recognize and react to *H. pylori* products such as flagella (TLR5), peptidoglycan (TLR2), CpG motifs (TLR9), and LPS (TLR4) (Hornef *et al.*, 2005). TLR2 and TLR5, rather than TLR4, are likely to be the predominant receptors for *H. pylori* antigen-induced NF- $\kappa$ B activation and chemokine expression in the gastric mucosa cells (Ding *et al.*, 2005). TLR-independent mechanisms seem to predominate in the activation of the innate response against *H. pylori* (Gobert *et al.*, 2004). The intracellular peptidoglycan, transferred from bacterium into the cytoplasm of the epithelial cells by *cag* PAI-mediated contact may play a major activator of the innate response against *H. pylori* (Viala *et al.*, 2004). This peptidoglycan from *H. pylori* is recognized by Nod1 (Viala *et al.*, 2004), and then Nod proteins act as intracellular (Nod1) and extracellular (Nod2) receptors in epithelial cells (Chamaillard *et al.*, 2003).

**Immune modulation:** *H. pylori* downregulates inflammation and control the host's immune response through a wide range of virulence factors that are involved in both provoking and maintaining a proinflammatory immune response. *H. pylori*-induced pathology seems to be predominantly a T-cell-mediated disease. This adaptive immune response is initiated and maintained by monocytes and Th1 lymphocytes. The immunoregulatory and proinflammatory cytokines e.g. interleukin-2 (IL-2) and INF- $\gamma$  induced in mononuclear cells by *H. pylori*, influence the natural T-cell to divide helper T (Th) cells to Th1 and Th2. Th1 promotes cell-mediated immunity by producing interleukin-2 (IL-2) (Lindholm *et al.*, 1998). Also, *H. pylori* infection results in upregulation of MIP-3 $\alpha$  gene expression in gastric epithelial cells, thus inducing an influx of monocytic cells into the lamina propria of the gastric mucosa (Nishi *et al.*, 2003). These cells may be functionally impaired, as *H. pylori* is capable of inhibiting phagocytosis by macrophages (Allen, 2001). This not only results in reduced anti-*H. pylori* activity of the macrophages but more importantly results in decreased and altered processing of *H. pylori* antigens by activated macrophages (dendritic cells). Since the activation of B and T cells are dependent on the presentation of *H. pylori* antigens by dendritic cells, this is of crucial importance for the outcome of the immune response. IL-10-producing T cells seem to be crucial in the control of *H. pylori*-induced inflammation and enable the bacterium to persist in the gastric mucosa

(Stromberg et al., 2003). Therefore, much of the pathology associated with *H. pylori* infection results from the activities of the host immune system rather than from direct bacterial activity.

Although it is now generally accepted that the development of *H. pylori*-induced gastritis and/or pathology depends predominantly on Th1 cells and Th1 cytokines antibodies can effectively prevent infection and reduce colonization in animal models (Marnila et al., 2003).

**1.1.4.4 Cytokines:** The characterization of local inflammation in *H. pylori* infection is the increased production of several cytokines (Moss et al., 1994).

(1) INF- $\gamma$ : The increased levels of INF- $\gamma$  might contribute to gastric inflammation by activating mononuclear phagocytes and neutrophils, up-regulating the expression of major histocompatibility complex type II molecules on epithelial cells, and decreasing the epithelial barrier function (Lindholm et al., 1998). (2) IL-1 $\beta$ : IL-1 $\beta$  is suggested to inhibit gastric acid secretion (Muller and Hunt, 1993). IL-1 $\beta$ , a subset of the IL-1 cytokine is a potent proinflammatory cytokine and the most potent known inhibitor of acid secretion (Calam, 1999). This subsequently leads to reduced acid output, which is associated with corpus colonization by *H. pylori*, resulting in pangastritis, formation of atrophic gastritis, and increased risk of gastric cancer (El-Omar, 2001). (3) TNF- $\alpha$  (tumor necrosis factor alpha) is a proinflammatory

cytokine, and influences gastrin production and thus increased acid production by gastric parietal cells (Suzuki *et al.*, 2001). (4) Cytokine, IL-10, is associated with an inflammatory response (El-Omar *et al.*, 2003). (5) *H. pylori* increased IL-8 and COX-2 in the antral mucosa, but did not influence COX-2 and local cytokines in gastric ulcer (Wu *et al.*, 2006). (6) The mucosal levels of cytokines IL-1, IL-6, and TNF- $\alpha$  and of chemokines IL-8, GRO- $\alpha$ , and RANTES (regulated on activation normal T expressed and secreted) were all significantly decreased after the treatment of *H. pylori* (Hahm *et al.*, 1998). (7) The apoptotic index and iNOS score were significantly reduced after the eradication of *H. pylori* (Hahm *et al.*, 1998). (8) Cytokine interleukin-18 induces production of interferon-  $\gamma$  by activated T lymphocytes and promotes a Th1 profile. It can enhance host chemokine response to *H. pylori* infection (Andrew *et al.*, 2004).

#### **1.1.4.5 Infection process**

*H. pylori* has special features to overcome host defence mechanisms and be successful as a pathogen. Urease and motility are essential for the ability of *H. pylori* to colonize the gastric mucus layer. The bacteria adhere to the gastric epithelium via host cell glycan and glycoprotein receptors and the bacteria proteins, BabA and the adhesion-associated lipoproteins (AlpA and AlpB), and trigger cellular signaling events that lead to cytoskeletal rearrangements and pedestal formation. VacA toxin and urease cause cytotoxic damage to the

gastric epithelium, and the bacteria stimulate nuclear factor kb (NFkB) activation and the release of inflammatory mediators (Sebastian and Christine, 1999). *H. pylori* infection damages the mucosa through an inhibition of mucus-bicarbonate secretion and gastric blood flow, breaking the mucosal barrier and promoting acid back-diffusion with consecutive mucosal inflammation and cell injury (Konturek *et al.*, 1999). By stimulating specific local T and B cell response and a systemic antibody response, *H. pylori* infection induces a local pro-inflammatory cytokine response. Interleukin-8 (IL-8), which is expressed and secreted by gastric epithelial cells, may be an important host mediator inducing neutrophil migration and activation (Crabtree, 1996).

Tissue invasion and the elaboration of chemical mediators provoke an intense polymorph response. The epithelium responds to infection by marked degenerative changes including mucin depletion, cellular exfoliation and syncytial regenerative changes. This histological picture is termed as acute neutrophilic gastritis (Dixon, 1993).

The major features of *H. pylori*-associated chronic gastritis are the filtrate of lymphocytes and plasma cells as mediators of the mucosal immune response, surface epithelial degeneration indicating direct cytotoxic effects by bacterial products, and continuing polymorph activity provoked by bacterial products,

complement activation or cytokine release. Long-standing chronic gastritis is characterized by the development of glandular atrophy and intestinal metaplasia (Dixon, 1993).

*H. pylori* infection frequently causes gastritis, and this is an important condition for initiating gastric ulcer and duodenal ulcer. The genetics of the host plays a role in the development of human ulcer disease. A greater-than-normal ability to secrete acid may be one of the genetic host factors that may underlie duodenal ulcer disease. Any factors that induce the duodenal acid overload (e.g. smoking, which inhibits pancreatic bicarbonate secretion) could enhance the ability of *H. pylori* to grow in the duodenal bulb (Graham *et al.*, 1996).

### **1.1.5 Epidemiology**

#### **1.1.5.1 Prevalence and geographical distribution**

*H. pylori* has infected 50% of the world's population and the prevalence of *H. pylori* infection varies widely by geographic area, age, race, and socioeconomic status (Brown, 2000).

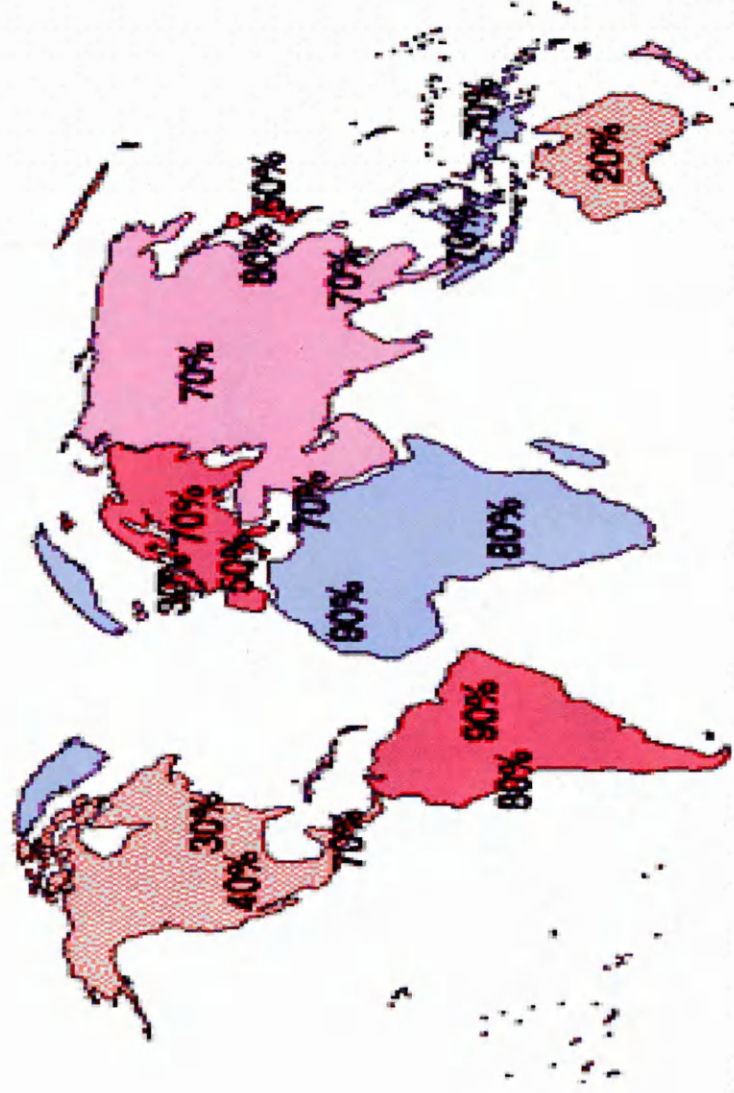
Rates appear to be higher in developing than in developed countries and they seem to be decreasing with improvements in hygiene practices. Poor hygiene and crowded conditions may facilitate transmission of infection among family

members and cause institutional clustering of infections (Brown, 2000). The infection rate of *H. pylori* in Latin America is more than 90%, Eastern Europeans > 50%, African Americans about 40-50%, UK >30%, Austria around 20%, and Asia about 70-80% (**Fig. 1-10**) (Fennerty, 2005). In Western countries, the epidemiology of *H. pylori* shows a consistent pattern. This infection is uncommon in persons less than the age of 40 years old, but its prevalence is rising to reach approximately 50% in elderly persons. Also the prevalence of *H. pylori* has been decreasing with a 50% reduction in the past 25 years. This can all be explained by the concept of birth cohorts (Marshall, 1993).

Epidemiological studies for *H. pylori* showed acquisition in early childhood. In the United States, *H. pylori* is present in 10% to 15% of children under the age of 12 years compared with 50% to 60% of persons greater than 60 years of age (Richard and Peek, 2004). In developing countries, most of the population is infected by the age of 10 years. Intra-familial transmission in young children and *H. pylori* prevalence in the mothers is a crucial determinant for the child's risk of children being infected. In developed countries, the age-dependent increase of seropositivity is mainly due to the decreasing rate of childhood infection (Apostolopoulos *et al.*, 2002; Selimoğlu *et al.*, 2002; Malaty and Nyrent, 2003).



**Fig. 1-10 Prevalence and geographical distribution of *H. pylori* infection**



The frequency of infection is in order: Latino America > 90%, Eastern Europeans > 50% African Americans 40-50%, UK >30% (Fennerty, 2005).

### 1.1.5.2 Transmission

The specific mode of transmission of *H. pylori* is not known. There has been numerous suggestions as to its mechanisms among these. (1) Human to human transmission: The most likely mode of transmission is from person to person. Both faecal-oral and oral-oral routes have been proposed (Allaker *et al.*, 2002). Persons who have greater contact with human faeces and secretions have high infection rates (Parsonnet *et al.*, 1999). *H. pylori* has been detected in saliva, vomitus, gastric refluxate, and faeces (Ferguson *et al.*, 1999), (2) By family members: It is generally believed that acquisition mostly occurs in early childhood, most likely from close family members (Kivi *et al.*, 2003). Feeding by the parents is an uncertain risk factor for transmission of *H. pylori* (Kurosawa *et al.*, 2000). (3) In clinical trials: This organism has been found from dental plaque (Song *et al.*, 1999). Data obtained using techniques for detection of *H. pylori* DNA indicate that both faeces and saliva/dental plaque contain specific *H. pylori* gene fragments, which indicate the presence of *H. pylori* cells or cell debris (Hazell, 1993). It appeared that there was no clear increased risk for being a carrier of *H. pylori* among dentists, gastroenterologists, nurses, partners of an *H. pylori*-positive spouse, or visitors to a clinic for sexually transmitted diseases (Mégraud, 1995). (4) By environment: Waterborne transmission, probably due to the faecal contamination, may be an important source of infection, especially in areas where used untreated water is drunk. Recent studies in the UK have linked

clinical *H. pylori* infection with the cells in contaminated well water (Enroth and Engstrand, 1995). (5) By animals: *H. pylori* has rare occasions been isolated from pet animals. Transmission via animals has also been suggested for this infection, for instance via sheep and housefly (Selimoglu *et al.*, 2002). The presence of pets may be a risk factor for this infection.

### **1.1.6 Important *H. pylori* -associated diseases**

#### **1.1.6.1 Acute and chronic gastritis**

**Acute gastritis.** Acute infection of stomach has been found in animal models, when *H. pylori* was infected intentionally (Graham *et al.*, 1988). Similarly, in humans a challenge model for *H. pylori* infection resulted in acute gastritis was observed (Graham *et al.*, 2004). This infection is often associated with colonization of *H. pylori* and low stomach acid level in both the proximal and distal stomach mucosa, which can last for months. The patients with acute *H. pylori* infection always suffer from nonspecific dyspeptic symptoms, such as fullness, nausea, and vomiting (Granstrom *et al.*, 1997). It is unclear whether this initial colonization can be followed by spontaneous clearance and resolution of gastritis (Perez-Perez *et al.*, 2003).

**Chronic gastritis.** When colonization does become persistent, the development of gastritis relates to the bacterial colonization and the level of acid secretion. The colonization is associated with an antrum-predominant

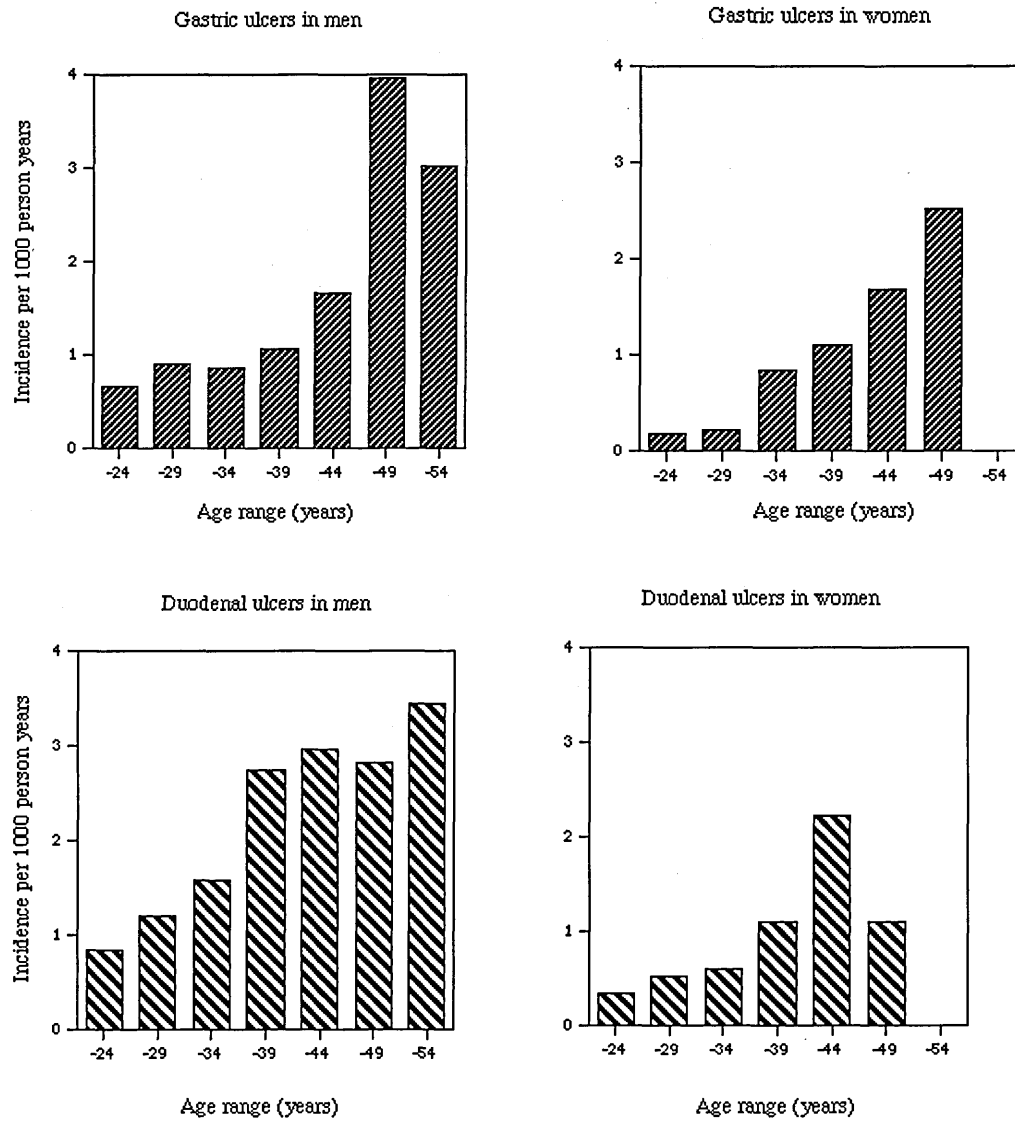
gastritis and a corpus-predominant pangastritis (Kuipers *et al.*, 1995). Impairment of acid secretion improved this colonization to induce gastritis. The reduction in acid secretion can be due to a loss of parietal cells as a result of atrophic gastritis. Also, the local inflammatory factors such as cytokines, including interleukin-1 $\beta$  (IL-1 $\beta$ ), have a strong suppressive effect on parietal cell function (El-Omar *et al.*, 1997).

#### **1.1.6.2 Peptic ulcer disease (PUD)**

Data on PUD indicate that 0.1 – 0.2% of the world population will develop an ulcer annually, roughly 2-5% will have an ulcer at some time in their life and up to 0.002% of them will die each year from perforation or bleeding (Kurata and Haile, 1984; Shearman, 1989). In the UK, the prevalence of PUD on the population is of 6-13% for men and 2-5% for women between the ages of 15 and 64 years. The mean annual incidence per 1000 inhabitants aged over 15 years for duodenal ulcer is 2.2‰ for men and 0.6‰ for women; for gastric ulcer, the figures are 0.5‰ and 0.3‰ respectively. The overall average incidence is 1.8 per 1000 inhabitants per year.

A study of 21,440 persons in the municipality of Tromsø in Norway (Johnsen *et al.*, 1992) gives detailed sex and age-related incidences over the age range 20-49 years. The overall incidence rates are similar, if slightly higher than those above, and those data are summarized in **Fig. 1-11**.

**Fig. 1-11 Age and sex-related incidence of peptic ulcer**



Data are taken from Johnsen *et al.* (1992), describing the age and sex specific incidences of duodenal and gastric ulcers censored for death and migration in a population of 21,440 persons aged between 20 and 54 years in the municipality of Tromsø. Insufficient data were available for women aged over 49 years.

PUD is considered as one of the gastro-duodenal diseases most strongly linked to *H. pylori* infection (Parsonnet, 1998). In general, the prevalence of *H. pylori* infection has been found in range from 80% to 100% in patients with duodenal ulcers, and from 50% to 100% in gastric ulcers (Sugiyama et al., 2001). In the United States, the percentage is 75% for both ulcers (Fennerty, 2005). In a report from China, 87.2% of PUD patients had histological evidence of *H. pylori* infection, and after excluding the patients with anti-*H. pylori* treatment, 98.9% of duodenal ulcers, 100% of gastric ulcers and 100% of the coexistent ulcers had evidence of this infection (Hu et al., 1995). Kuipers (2006) from the Netherlands reported that an individual infected with *H. pylori* has an estimated lifetime risk of 10 - 20% for the development of PUD, and this infection can be diagnosed in 90 –100% of duodenal and in 60 – 100% of gastric ulcer patients. In North-West India, *H. pylori* presents in 76.09% of duodenal ulcer and 50% of gastric ulcer (Romshoo et al., 1999). In the UK, the prevalence of PUD in patients with *H. pylori* infection was seven times greater than that of those without this infection (Varia et al., 1994; Sobala et al., 1991).

*H. pylori* plays an important role in the pathogenesis of PUD. The following hypotheses have been proposed to explain the association between the bacteria and PUD: (1) Leaking roof hypothesis: *H. pylori* though a non-invasive organism damages the gastric mucosa by secreting various toxins and enzymes, which cause local mucosal damage (Goodwin 1988); (2) Gastrin link

hypothesis: the cells destruct antral D cells which are sources of somatostatin (inhibitor for gastrin release). Inhibition of somatostatin causes increase in gastrin release and hence increases gastric acidity, thereby leading to gastroduodenal injury (Levis and Haddad, 1989); (3) Bacterial virulence factor hypothesis: Specific bacterial virulence factors are considered to be important in the development of peptic ulcer diseases. *H. pylori* colonizes gastric metaplastic epithelium, leading to local inflammation and mucosal injury and eventually to the ulcer formation (Konturek *et al.*, 1999). Those bacterial substances include urease, catalase, mucinase, lipase, hemolysins, phospholipase A, leukotriene-B<sub>4</sub>, interleukin-1, 4, and 6 (Bruce, 1993). *H. pylori* type A strains have been postulated to be more ulcerogenic. (4) The host inflammatory response may be important for clarification of the pathogenesis of *H. pylori* – positive PUD. Also Tatsuji *et al.* (2000) suggested that mucosal  $\alpha$  - and  $\beta$ -chemokines may be important to ulcerogenesis in PUD.

#### **1.1.6.3 Gastric cancer**

According to the International Agency for Research on Cancer, there are 900,000 people annually afflicted by gastric cancer worldwide and most of whom will die of this disease. More than a decade ago the link between *H. pylori* and gastric cancer was recognized, but recently this association has been strengthened. There is sufficient evidence in humans for the carcinogenicity of infection with *H. pylori*. Thus, infection with *H. pylori* is

defined to be carcinogenic to humans (Group 1) (IARC, 1994). In Japan, *H. pylori* is associated with a twofold to threefold higher rate of gastric cancer among males (Yammagata *et al.*, 2000). The most common type of cancer linked to *H. pylori* infection is gastric adenocarcinoma, which is the second most common cause of cancer-related mortality worldwide and the 14<sup>th</sup> overall cause of death (Parkin *et al.*, 1988).

The evidence to support *H. pylori* as a causal factor in gastric cancer is as follows. (1) Epidemiological data indicate a geographic association between prevalence of *H. pylori* and prevalence of gastric cancer. Epidemiological trend show declining incidence of gastric cancer in countries with falling rates of *H. pylori* infection. The most significant evidence to date is the EUROGAST study (1993). The conclusion was that a 100% infection rate with *H. pylori* conferred about a six-fold risk of gastric cancer compared to a similar population with no infection after examination of a total of 17 populations in 13 countries; (2) Histopathological examination of gastric biopsy specimens showed that *H. pylori* infection was more common in patients with gastric cancer than in those with no pathology symptoms. The Forman *et al* (1994) study indicated that there was a higher rate of seropositivity and higher antibody concentrations in cancers than in controls subjects (69% vs. 47 %; 90 µg/ml vs. 3.6 µg/ml); (3) Inoculation of *H. pylori* into two humans resulted in chronic active gastritis, an early precursor lesion for gastric cancer; (4) There is evidence that *H. pylori*



infection in animal models results in progression to atrophy and metaplasia, which are accepted as preneoplastic lesions; (5) Animal models, including ferrets, gerbils, and mice, show that gastric *H. pylori* infection can lead to experimentally induced gastric cancer; (6) Eradication of *H. pylori* in animal models and humans appears to lower the risk of developing gastric cancer (Jeanmariie and Timothy, 2005).

At the molecular level, this cancer risk has been attributed to mutations associated with chronic inflammation, imbalance of epithelial proliferation and apoptosis, and growth of bacteria producing carcinogenic nitrogen metabolites (Mahmood *et al.*, 2001).

There is considerable controversy on the role of the bacterium in dyspepsia and gastroesophageal reflux disease (GERD), and the effects of irradiation of *H. pylori* infection on GERD symptoms (Sanders and Peura, 2002).

#### **1.1.6.4 *H. pylori* and NSAIDs**

*H. pylori* and NSAIDs are the major causes of gastroduodenal ulcer disease. The relationship between *H. pylori* and NSAIDs in peptic ulcerogenesis is complex. *H. pylori* and NSAIDs are independent risk factors for peptic ulcer and peptic ulcer bleeding (Malfertheiner *et al.*, 2002). Eradication of *H. pylori* in chronic NSAID users decreases the incidence of ulcer disease (Huang *et al.*,

2002). A report from Hong Kong showed that eradication of *H. pylori* infection significantly reduced the risk of ulcers for patients starting long-term NSAID treatment, but is insufficient to prevent ulcers and ulcer complications as a gastroprotective measure in high-risk cases (Chan *et al.*, 2002). In other studies, *H. pylori* eradication by omeprazole treatment is useful for prevention of recurrent NSAID-associated ulcer bleeding and healing of NSAID-associated ulcers (Chan *et al.*, 2001; Hawkey *et al.*, 1998).

Conversely, other studies reported that NSAIDs have some partially protective effects on the inflammation induced by *H. pylori* and *H. pylori* may overcome the ulcerogenic effects of NSAIDs by stimulating prostaglandins (Rainsford, 2001). The two agents may undertake different mechanisms in mucosal injury. Rainsford (2001) concluded the difference of pathways: (1) Producing persistent ulcers and leukocyte infiltration with chemokine productions in *H. pylori* infection; (2) predominant ischaemia-oxyradical mechanism by NSAIDs; (3) Physico-chemical changes by NSAIDs in the impairment of mucosal defences and cellular necrotic reactions.

#### **1.1.7 Diagnosis**

The available tests are generally divided into invasive tests and noninvasive tests, which have specific advantages and disadvantages (**Table 1-2**)

**Table 1-2 Diagnosis of *H. pylori***

Diagnosis methods	Sensitivity	Advantages and disadvantages
<b>Invasive methods</b>		
Histology	> 90%	Gold standard. Requires expert pathologist.
Culture biopsy	> 95%	Gold standard. Requires microbiologist.
Rapid urease test	> 90%	Rapid and cheap. Requires add confirmation.
<b>Noninvasive methods</b>		
Urea breath test	> 95%	Gold standard. Requires expensive equipment.
Fecal antigen test	> 90%	Simple. No reliable
Serology	80-90%	Used for epidemiological study. No reliable for ongoing infection.

From Logan (1998).

(Logan, 1998). The invasive tests are based on gastric specimens for histology, culture, or other methods. The noninvasive tests based on peripheral samples, such as blood, breath samples, stools, urine, or saliva for detection of antibodies, bacterial antigens, or urease activity. The choice of a specific test for an individual patient depends on local experience and the clinical setting. Breath tests and serology are commonly used, due to them being quick and simple. In research protocols, a combination of two methods is often applied. For routine diagnostic purposes, histology, urea breath testing, and culture are currently most often used, whereas the use of serology is most appropriate for large epidemiological studies. In hospital-based care, many patients undergo endoscopy, which is then combined with an invasive test for *H. pylori*. Faecal antigen tests are always used in children without the need for endoscopy or vena puncture (Vaira *et al.*, 1999; Zagari *et al.*, 1999).

### **1.1.8 Current treatment and their limitations**

#### **1.1.8.1 Current therapies for eradication of *H. pylori***

*H. pylori* infection is strongly associated with the diseases detailed above, and appear to play an important role in their pathogenesis. Therefore, it is reasonable to suggest that eradication of *H. pylori* infection may benefit patients with those diseases and prevention of its infection may benefit people's health in many cases.

A number of agents with activity against *H. pylori* have been used, which include bismuth compounds, macrolides (clarithromycin), nitromidazoles (metronidazole), penicillins (amoxicillin), tetracyclines (tetracycline), fluoroquinolones (ciprofloxacin), rifamycins (rifabutin), nitrofurans, and proton pump inhibitors (omeprazole). Amoxicillin, clarithromycin and metronidazole are the most commonly utilized antibiotics for the pharmacological treatment of *H. pylori* infection (Gerrits *et al.*, 2006). Their mechanisms of action against *H. pylori* are shown in **Table 1-3**.

Regimens to eradicate *H. pylori* have been developed over the past 20 years, initially starting with monotherapy and then dual therapy. None of the antibiotics in monotherapy constitutes an effective treatment (Tytgat and Noach, 2003). Dual treatments combining a proton pump inhibitor with amoxicillin / clarithromycin were popular two decades ago, as amoxicillin and clarithromycin are acid-sensitive, and therefore gastric acid secretion must be potentially inhibited by use of PPI during the eradication therapy for these antibiotics to be more stable and bio-available in the stomach (Mitsushige *et al.*, 2006). Their actions were readily explained and they were well tolerated. But these combinations are now rarely used because of recognition of a lack of efficacy long term and because there are more efficacious therapies available (Boer and Tytgat, 2000).

**Table 1-3 Agents for eradication of *H. pylori* and active mechanisms**

Antimicrobial	commonly used compound	Mechanisms
Penicillins	amoxicillin	Binding of $\beta$ -lactam antibiotic to penicillin-binding protein (PBP) inhibits cell division
Marcrolides	clarithromycin erythromycin	Binds 23S rRNA ribosomal subunit, results in inhibition of protein synthesis.
Nitromidazoles	metronidazole tinidazole	Reduction of prodrug by nitroreductases leads to formation of nitro-anion radical and imidazole intermediates and subsequent DNA damage
Tetracyclines	tetracycline	Binding to ribosomal prevents association with aminoacyl-tRNA and subsequent protein synthesis.
Fluoroquinolones	ciprofloxacin	Inhibition of DNA gyrase and topoisomerases, interfering with DNA replication.
Rifamycins	rifabutin	Binding to RNA polymerase, resulting in transcription inhibition.
Nitrofurans	furazolidone	Reduction of prodrug by nitroreductases leads to formation of nitro anion radicals and subsequent DNA damage.
Proton pump inhibitor	omeprazole	Inhibits the proton motive force of the bacterium, and destabilises its site of colonization in the stomach.
Bismuth	bismuth subcitrate	Inhibits protein, ATP, and cell membrane synthesis.

The current most frequent recommendations are triple or quadruple therapies, since they are effective in 80-90% of cases (Blanchard *et al.*, 2004). Clinical trials have shown that the antibiotic combination of amoxicillin/clarithromycin and amoxicillin /metronidazole together with a proton pump inhibitor (PPI) are both effective in eradicating *H. pylori* infection. The specific dose regimens used in many countries are shown in **Table 1-4**. Treatment should be a package of first – and (if necessary) second – line eradication therapies together. If there are failures in second line therapy, patients are referred to a specialist (**Fig. 1-12**) (Malfertheiner *et al.*, 2002).

#### **1.1.8.2 Vaccination**

During the past decade, many studies have focused on the development of alternative treatments for *H. pylori*, in particular, vaccination. A study during the early 1990s provided evidence of vaccination (contained bacterial antigens) against *H. pylori* infection. The main mechanism of protective immunity against the bacteria occurred via stimulation of T-helper type phenotype cells, which are induced by the production of interleukins 4 and 10, and not by antibody production (Sutton, 2001). Another study reported that mucosal immunization with a variety of antigens in combination with mucosal adjuvants such as cholera toxin (AB5 toxin, CT), the heat-labile toxin of *Escherichia coli*, or Freund adjuvants, which induce a Th2 response, prevents or cures an infection by *Helicobacter* spp., while Th1 response-inducing adjuvants enhance

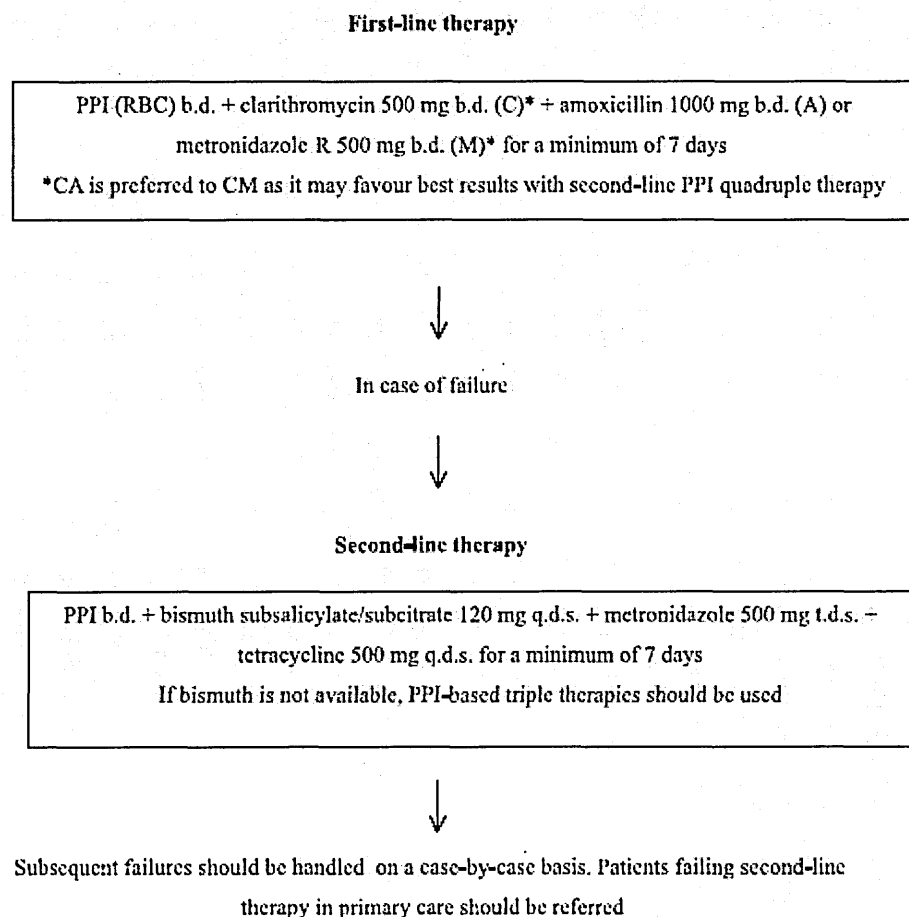
**Table 1-4 Regimens for treatment of *H. pylori* infection**

Abbr	Drug	Dosage	Efficacy	Recommended Duration
<b>OC</b>				
	Omeprazole	40 mg	60-80%	14 days
	Clarithromycin	500 mg t.i.d.		
<b>RBC+C</b>				
	Ranitidine bismuth citrate	400 mg b.i.d.	70-85%	14 days
	Clarithromycin	500 mg t.i.d.		
<b>BTM + H<sub>2</sub> antagonist</b>				
	Bismuth subsalicylate*	2 tabs q.i.d.	80-95%	14 days
	Metronidazole	250 mg q.i.d.		
	Tetracycline HCl	500 mg q.i.d.		
<b>PPIAC</b>				
	PPI	bid	80-95%	10-14 days
	Amoxicillin	1 gm b.i.d.		
	Clarithromycin	500 mg b.i.d.		
<b>PPIMC</b>				
	PPI	bid	80-95%	10-14 days
	Metronidazole	500 mg b.i.d.		
	Clarithromycin	500 mg b.i.d.		
<b>BMT quadruple therapy</b>				
	Bismuth	2 tablets q.i.d.	90-99%	14 days
	Metronidazole	500 mg t.i.d.		
	Tetracycline	500 mg q.i.d.		
	PPI	b.i.d.		
<b>Furazolidone quadruple salvage therapy</b>				
	Bismuth	2 tablets q.i.d.	90-99%	14 days
	Furazolidone	100 mg t.i.d.		
	Tetracycline	500 mg q.i.d.		
	PPI	b.i.d.		

From Qureshi and Graham (2000).



**Fig. 1-12 Summary of recommended treatment strategy for eradication of *H. pylori***



The management of *H. pylori* infection- The Maastricht 2 – 2000 consensus report. PPI: proton pump inhibitor; RBC: ranitidine bismuth citrate; R: metronidazole 400 mg. From Malfertheiner *et al.* (2002)

inflammation rather than eliminating it (Ferrero *et al.*, 1995). So, the alternative treatment or prevention strategies such as vaccines may attract attention in the future.

#### **1.1.8.3 Limitations of current therapies**

Although several meta-analyses of studies of *H. pylori* eradication regimens have been published recently, the eradication rates vary in the different regions and countries (Gisber *et al.*, 2000). Such therapeutic regimens may fail because of increasing antimicrobial resistance of *H. pylori* and side-effects of the therapies, poor compliance and high costs (Ann, 2003). Also individual factors affect antimicrobial agents, which are the physical environment of the stomach with its low pH, active secretion, and the thick mucus layer. Antimicrobials may not penetrate this barrier, as well as frequent emptying of stomach contents thus limiting the time for agents to act locally (Graham, 1993).

Antibiotic resistance is one of the most common reasons for treatment failure and a growing problem in the world (Qureshi and Graham, 2000). Either application of one-drug regimens or the widespread application of antimicrobial regimens to treat *H. pylori* infection may magnify the problem of drug resistance (Sharara *et al.*, 2002; Branca *et al.*, 2004; Ruggiero *et al.*, 2004). Many studies have been performed to determine the prevalence of *H. pylori* to

antibiotics. Resistance is described as either “primary inherent resistance” or “acquired developed resistance”. Mégraud (2004) concluded that worldwide frequencies of primary resistance ranged from 1.7 to 25% to clarithromycin, from 9 to 62.7% to metronidazole, up to 0.5% to tetracycline and up to 0.9% to amoxicillin (**Fig. 1-13**).

Percentage indicates proportion of isolates that were resistant to the antibiotic. Failure of treatment is also related to an organism having acquired resistance during treatment. Resistance to commonly used antibiotics is significantly more frequent in patients where a previous eradication treatment has failed. Miyaji *et al* (1997) reported that metronidazole resistance was detected in 28% of patients before eradication treatment and 66.7% after unsuccessful treatment with this drug. Clarithromycin resistance increased from 32% before treatment to 70.6% after unsuccessful treatment.

In *H. pylori*, antibiotic resistance mechanisms are mainly based on point mutations located on the bacterial chromosome. Those are to reduce or abolish activity of electron transport proteins (e. g. RdxA, FrxA, or FdxB), to alternate in the penicillin-binding proteins PBP-D and PBP1A, to mutate in the rRNA genes for 16S and 23S rRNA and to decrease outer membrane permeability (Monique *et al.*, 2006).

**Fig. 1-13 Primary resistance of *H. pylori* to the common antibiotics in different parts of the world**

Country	years studied	No. of strains tested	clarithromycin prevalence (%)	metronidazole prevalence (%)	tetracycline prevalence (%)	amoxicillin prevalence(%)
Europe						
Bulgaria	96-98	103	8.7	Not determined ( ND)	ND	ND
Croatia	2001	196	8	33	ND	0
France	96-99	659	1.5	31.5	ND	0
Germany	95-00	1644	2.2	26.2	0	0
Germany	95-96	188	4	32	ND	ND
Italy	98-02	406	23.4	36.7	ND	0
Italy	99	167	1.8	14.9	ND	0
Netherlands	97-98	231	1.7	21.2	0	0
Portugal	90-99	132	22	34.1	0	0
Spain	95-98	235	12.9	23.5	0.7	0
Sweden	97-98	203	2.9	26.1	ND	0
UK	94-99	1064	4.4	40.3	0.5	0
UK	95-98	843	3.9	36	ND	0.4
North America						
Mexico	95-97	144	25	76.3	ND	0
USA	93-99	3439	10.6	21.6	ND	0.08
USA	98-99	422	12	ND	ND	0
USA	00-01	106	12.2	33.9	ND	ND
South America						
Brazil	96-00	203	9.8	53	ND	ND
Middle East						
Iran	02	120	17	ND	ND	ND
Israel	00-01	110	8.2	38.2	0	0.9
Far East						
Hong Kong	97-01	991	4.5	29	0.5	0.3
Japan	95-00	593	11	9	ND	0.3
Korea	94-99	456	5.9	40.6	5.3	0
Korea	96-00	224	5.4	41.9	ND	ND
Singapore	93-96	459	ND	62.7	ND	ND
Singapore	02	120	ND	31.7	ND	ND
New Zealand	93-98	225	6.8	32	ND	ND

Data from Mégraud (2004).

Also, the increasing cost for these regimens is other problem on current therapies. Bulletin (1994) has investigated the comparative costs of different treatment in peptic ulcer disease (Moore, 1995). The cost of antibiotic treatment with acid suppressing drugs is more expensive than general eradication therapy. The maintenance therapy is the most expensive (Table 1-5). For the cost of prescriptions in England in 1991 –1993, spending on histamine antagonists has remained static at about £ 180 million, while spending on omeprazole and other proton pump inhibitors has increased from £20 million to £94 million in two years (Moore, 1995).

For the vaccination treatment or prevention strategies, recent research has been disappointing and at present there is no vaccine to prevent infection (Li et al., 2003). Several issues remain in regard to development of a safe and effective vaccine against *H. pylori* infection. Firstly, a safe mucosal adjuvant or vector to stimulate an immune response must be identified. Secondly, the optimal route of administration needs to be defined. Finally, different regimens need to be developed to ensure complete sterilization of the gastric mucosa (Hardin and Wright, 2002).

#### **1.1.9 Conclusion and relevance of this project**

For more than 20 years, *H. pylori*, a spiral microaerophilic Gram-negative bacterium, has been known to colonize the human gastric mucosa. The

**Table 1-5 Comparative costs of eradication therapy and other treatment**

<b>Treatment</b>	<b>Cost (£)</b>
<b>Eradication therapy</b>	
Standard triple therapy	22
Triple therapy with ranitidine, 4 weeks	47
Omeprazole & amoxycillin	40
Omeprazole & clarithromycin	101
<b>Acid suppressing healing</b>	
Cimetidine, 8 week course	16
Ranitidine, 8 week course	51
Omeprazole, 4 week course	36
<b>Maintenance therapy with acid suppressants</b>	
Cimetidine, 400mg od, 1 year	50
Ranitidine, 150mg od, 1 year	170
Ranitidine, 150mg bd, 1 year	340
Omeprazole, 20mg od, 1 year	474

From Moore, 1995.

persistence of the colonization leads to the development of gastric diseases, especially in PUD and gastric cancer. *H. pylori* has been classified as Class I carcinogen. Thus, the eradication of *H. pylori* is critical to reduce the risk of gastric diseases. A triple therapy was recommended as a first-line choice for its eradication, based on a combination treatment with anti-secretory drugs or bismuth and two antibiotics for 1-2 weeks.

Despite this careful approach, the treatment regimens have serious drawbacks. The high cost, length of treatment, harmful side effects and increasing drug resistance frequently contribute to treatment failure. To overcome these problems, efforts are being invested in the development of new medicines to combat *H. pylori* infection. There have been a number of reports that natural plants and foods such as garlic, honey, and capsicum can inhibit *H. pylori in vitro* and each report has suggested that such ingredients could be used for treatment of the infection (Graham *et al.*, 1999). This study concentrated on celery seed extracts, which were previously shown to have *anti-H. pylori* activity (Rainsford and Liu, 2006), as a source of novel *anti-H. pylori* agents.

## **1.2 Celery seed in medicine**

### **1.2.1 History**

Celery has been cultivated for the last 3000 years, notably in pharaonic Egypt, and was known in China in the fifth century BC (Chevalier, 1998). Celery seed (the seed of *Apium graveolens*) has been used as herbal medicine for various conditions for thousands of years in the world (Usher, 1974). In the Middle Ages, it was discovered that cultivation produced a much superior plant (Idrisi, 2005). At that time people began to use it more widely as a vegetable. It was not until the 19<sup>th</sup> century that the seeds were used in recipes, appearing first in pickling recipes (Icbs-online, 2004). Medicinal preparations began to emerge in the late 19<sup>th</sup> century and these generally contained the juice of crushed celery seed. It was recommended as a cure for rheumatism, a tonic for asthma and herbalists used to treat liver diseases such as hepatitis, bronchitis, fever and flatulence. During ancient times, India's Ayurvedic physicians (vaidvas) prescribed celery seed as a diuretic and as a treatment for colds, flu, water retention, indigestion, various types of rheumatism, and ailments of the liver and spleen (Mdidea, 2006; Usher, 1974).

### **1.2.2 Character**

The celery seed preparation is the dried seed of *Apium graveolens*, a biennial of the parsley family. This is the same genus and species used for growing table celery. It is very small, about 1.6 mm, an ovate, brown cremocarp (**Fig**



1-14) and has a characteristic odor and a warm aromatic taste (Papamichail *et al.*, 2000; Usher, 1974). The seed is collected when ripe in September.

### 1.2.3 Extraction and isolation

Grinding had a dramatic effect on the efficiency of release of solvent extractable components from celery seed. The total weight of extract obtained from ground seed is in the order of six times the yield from whole seed (Falzari and Menary, 2005).

Many studies have been done on the extraction of the volatile and water-soluble constituents of celery and its seed over the past decades (Brown *et al.*, 1988). There are three major extraction techniques, which are supercritical fluid extraction (SFE), steam distillation and organic solvent extraction (Catchpole and Grey, 1996).

Steam distillation is the most widely practiced and cheapest, but is limited to essential oil production only and can sometimes cause chemical change (Catchpole and Grey, 1996).

Organic solvent extraction is of relatively low costs in terms of capital and operating cost (Catchpole and Grey, 1996). The two extracting solvents compared are the polar solvent, chloroform and the non-polar solvent, hexane.

**Fig. 1-14 The appearance of the dry celery seed**



Mdidea-online. (2006) Products. [online]. Last accessed on 17<sup>th</sup> Nov. 2006 at URL: [http:// www.mdidea. com/products/](http://www.mdidea.com/products/)

The proportion of phthalides extracted with chloroform is greater than in the hexane extracts. Although statistically different, the increased yield using chloroform as opposed to hexane may not be enough to be economically worth the extract complications of using a polar solvent. The costs are associated with extract clean-up and solvent recovery (Falzari and Menary, 2005).

As the use of non-toxic and volatile solvents, such as CO<sub>2</sub>, SFE protects from thermal degradation and solvent contamination (Papamichail *et al.*, 2000). In 1963, Gold and Wilson first reported the existence of four unique trace branched-chain alkylidenephthalides of the volatile components of celery by organic solvent extraction (Gold and Wilson, 1963).

In the early studies, some components of celery seed could not be successfully separated by gas chromatography (GC) due to their instabilities in GC columns (Kurobayashi *et al.*, 2006). In 1987, Uhlig successfully separated 3-n-butylphthalide, 3-n-butyl-4,5-dihydrophthalide (sedanenolide) and 3-n-butyl-tetrahydro- phthalide (sedanolide) from celery by HPLC (Uhlig *et al.*, 1987). In 2002, several phthalide and derivatives with antioxidant, cyclooxygenase and topoisomerase inhibitory effects have been purified from celery seed by using RP-HPLC, eluting with methanol and water (Momin and Nail, 2002). Momin also reported that anti-mosquito and antibacterial components were isolated and purified from the hexane extract of celery seed

by using normal phase HPLC, eluting with hexane (Momin *et al.*, 2000). From the water-soluble portion of the methanol extract of celery seed, five sesquiterpenoid glucosides, three phthalide glycosides, six aromatic compound glycosides, two norcarotenoid glucosides and a lignan glucoside were isolated by using general chromatography and HPLC (Kitajima *et al.*, 2003).

#### **1.2.4 Composition**

To summarize the literature sources, the principal constituents of celery seed are 1.5-3% volatile oil; coumarins (seselin, osthenol, apigravin, celerin, umbelliferone); furanocoumarins (including bergapten); flavonoids (apigenin, apiin); phenolic compounds, choline, ascorbate, involatile oil, fatty acids and unidentified alkaloids (Holistic-online, 2004). Major components are limonene, selinene, phthalide, furocoumarins, and glucosides of furocoumarins, and flavonid (Kitajima *et al.*, 2003). A high proportion of the essential oil consists of monoterpene hydrocarbons (46%) and phthalides (42.3%) (MacLeod and Ames, 1989).

In several literature reports, there are the identified chemical structures of the phthalides and derivatives from celery seeds, including sedanolide (3-butyltetrahydrophthalide), sedanenolide(3-n-butyl 4,5-dihydrophthalide), senkyunolide-N, senkyunolide-J, 3-butylphthalide(3-n-butylphthalide), and

3-hydroxymethyl-6-methoxy-2,3-dihydro-1H-indol-2-ol, 7-[3-(3,4-dihydroxy-4-hydroxymethyl-tetrahydro-furan-2-yloxy)-4,5-dihydroxy-6-hydroxymethyl-tetrahydro-pyran-2-yloxy]-5-hydroxy-2-(4-hydroxy-3-methoxy-phenyl)-chromen-4-one (**Fig. 1-15**) (Kitajima *et al.*, 2003; Momin and Nair 2002; Macleod *et al.*, 1988).

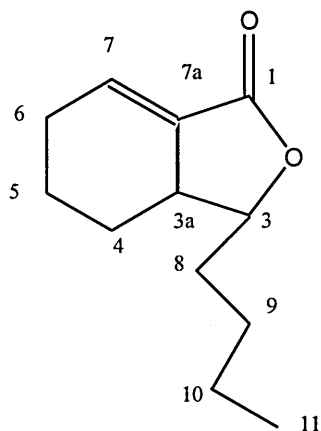
The ISO standard for celery seed oil defines the levels of the following five components:  $\beta$ -pinene 0.5-2%, myrcene 0.3-1.4%, limonene 58-79%,  $\beta$ -selinene 5-20% and sedanenolide 1.5-11% (Falzari and Menary, 2005).

#### **1.2.5 Pharmacognosy of celery seeds**

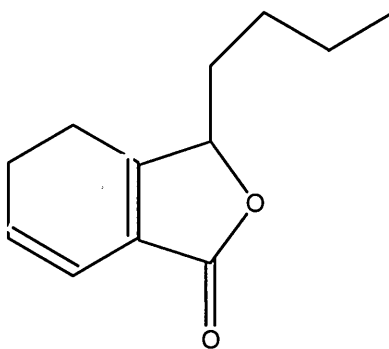
Celery seed is a mild diuretic and urinary antiseptic, and has been used in the treatment of urinary calculi (Oiye and Muroki, 2002). It has a calming effect on the gut, and can be used in the relief of flatulence and griping pains (Raffa, 2005). It can reduce visceral spasm, stimulate the smooth muscle of the womb and bring on delayed menstruation. After childbirth it helps the uterus readjust and encourages the flow of breast milk (Riddle, 2004). The phthalides are the constituents that are responsible for the antispasmodic, sedative and diuretic properties

Celery seed is beneficial in any oedematous condition that accompanies arthritis, because of its direct action on the kidneys, increasing the elimination

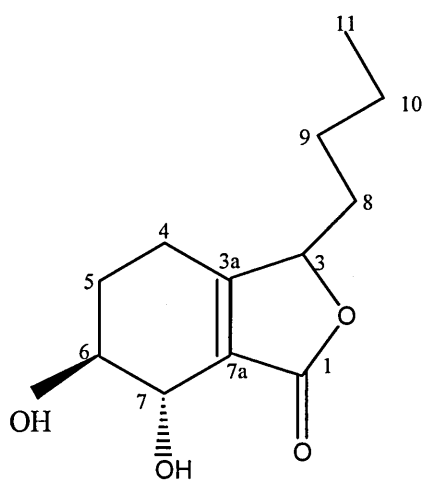
**Fig. 1-15 Some of the known chemical components of celery seed**



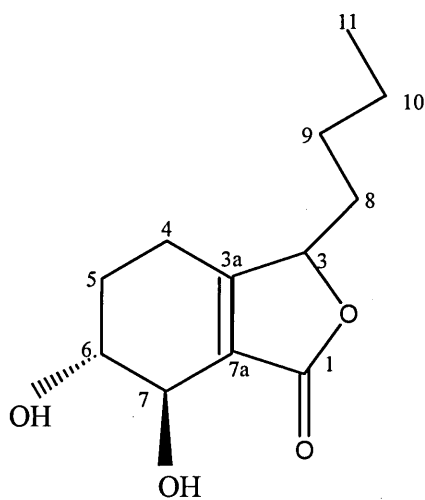
1. Sedanolid



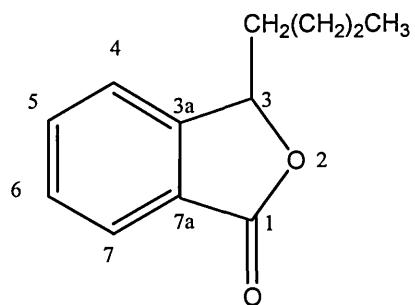
2. Sedanenolide (V)



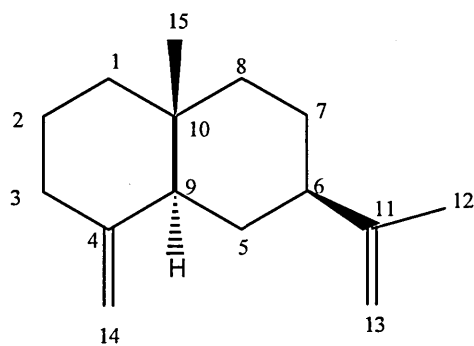
3. Senkyunolide-N



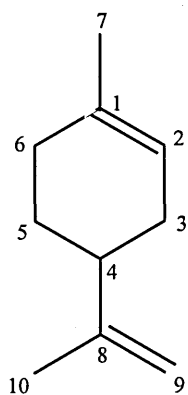
4. Senkyunolide-J



5. 3-n-Butylphthalide



6. B-Selinene



7. d-Limonene

of water and speeding up the clearance of accumulated toxins from the joints. It is often administered with Dandelion (*Taraxacum radix*) to increase the efficiency of elimination by both the kidneys and liver (Cousin and Hartvig, 2004). *A. graviolens* also has the effect of reducing blood glucose concentration combat therefore to be helpful in diabetes, in which it involves a direct action by stimulating the pancreas to insulin secretion (Carper, 1993). Clinical studies in China have revealed an anti-hypertensive action for tincture of celery seed, accompanied by increased urine output (Tierra, 1998). The flavonoid, apigenin, which is a component of celery seed, exhibits significant anti-platelet activity *in vitro* (Mdides, 2006).

Researchers at the University of Chicago have discovered that a chemical in celery called 3-butylphthalide reduces blood pressure in laboratory rats by relaxing the smooth muscle lining of blood vessels. Once relaxed, the vessels dilate, allowing the blood to flow more freely throughout the body. When fed a dose of phthalide equivalent to what is found in four stalks of celery a day, the rats experienced a 13 percent drop in blood pressure and a 7 percent drop in cholesterol (Mindell, 1994). Phthalides from celery seed appear to help lower blood pressure by acting as a diuretic and vasodilator through impacting the production of prostaglandins, acting in a similar manner as calcium – channel blockers lowering blood cholesterol levels, reducing the formation of arterial plaque, and effecting on areas and systems of the brain that control vascular



resistance (Tsi and Tan, 1997; Le and Elliot, 1991, Mimura et al., 1995).

Celery seed extracts show anti-inflammatory activity *in vivo* and effects on components of inflammation *in vitro* (Lewis *et al.*, 1985; Hammer *et al.*, 1999). Celery contains a phytosterol with some anti-inflammatory activity but the major anti-inflammatory effect is due to unidentified polar substances. Mannitol from celery seed shows anti-inflammatory action in an adjuvant arthritic rat model, but it does not reduce carrageenan-induced oedema in the rat (Lewis *et al.*, 1985). In xylene-induced ear oedema in mice (which is a model of acute, exudative phase, inflammation) and in the cotton pellet granuloma tests in rats (which is a model of chronic, glaucomatous, inflammation), ethanolic extract of celery seed showed an anti-inflammatory effect only against the chronic inflammation model, indicating an anti-proliferative effect (Atta and Alkofahi, 1998; Butters *et al.*, US Patent 6,352,728, 2002; Butters *et al.*, US Patent 6,761,913, 2004). In rats, the extracts are powerful nutraceuticals that amplify the potency of salicylates and prednisone for treating pre-established chronic inflammation (arthritis, fibrosis) and reduce the steroid's gastrototoxic and lymphopenic side effects (Whitehouse and Butters, 2003). Popovic *et al.* (2006) reported that *in vitro* the extracts of celery seed were good scavengers of deoxyribose (OH<sup>•</sup>) and 2, 2- diphenyl-1-picrylhydrazyl (DPPH<sup>•</sup>) radicals and reduce liposomal peroxidation (LPx) intensity in liposome, which points to their protective (antioxidant) activity. Also, *in vivo* the examined extracts showed a

certain protective effect on antioxidant systems (activities of glutathione peroxidation, glutathione reductase, peroxidation, catalase, xanthine oxidase, reduced glutathione content and intensity of LPx) in liver homogenate and blood of mice. In other study, polyacetylenes from celery seed were proved as a pronounced toxicity against acute lymphoblastic leukaemia cell line (Zidorn *et al.*, 2005).

Momin (2001) reported the mosquitocidal, nematocidal and antifungal activities of compounds of the methanolic extract of celery seed *in vitro*. Sendanolide, senkyunolide-N and senkyunolide-J were extracted from celery seed, which produced 100% mortality at 25, 100, and 1000 µg/ml, respectively, on the nematode, *Panagrellus redivivus*. Also, sendanolide gave 100% mortality at 50µg/ml on nematode, *Caenorhabditis elegans* and fourth-instar mosquito larvae, and inhibited the growth of the fungal pathogens *Candida parapsilasis* and *Candida albicans*. The five-membered lactone ring along with the butyl side chain in phthalides from those extracts above may be important for the observed biological activities. Another study suggested that celery seed extracts were active against many bacteria including *Staphylococcus aureus*, *Staphylococcus albus*, *Shigella dysenteriae*, *Salmonella typhi*, *Streptococcus faecalis*, *Streptococcus pyogenes* and *Pseudomonas solanacearum* (Popovic *et al.*, 2006). Friedman (2002) showed that the extracts had bactericidal activities against *Campylobacter jejuni*, *Escherichia coli*, *Listeria*

*monocytogenes* and *Salmonella enterica*.

Celery seed extracts have been identified by the US National Cancer Institute as possessing cancer-preventive properties (Caragay, 1992). There are cancer chemoprotective phytochemicals in celery seed, including coumarins, phthalides, polyacetylenes and terpenoids (Robbers *et al.*, 1994). These beneficial substances act as antioxidants and electrophilic scavengers, stimulate the immune system, inhibit nitrosation and the formation of DNA adducts with carcinogens, inhibit hormonal actions and metabolic pathways associated with development of cancer, and induce phase I or II detoxification enzymes (Craig, 1999). Phthalides from celery seed inhibit tumor formation by stimulating the protective phase II enzyme, glutathione transferase. Terpenoids (Limonene) from celery seed increase tumor latency and decrease tumor multiplicity (Sauberlich *et al.*, 1994). Also, the similar chemopreventive activity of methanolic extract of celery seed has been found in the Solt Farber protocol of induced hepatocarcinogenesis, as well as in oxidative stress and induction of positive foci of r-GT in the liver of Wistar rats (Sultana, *et al.*, 2005).

A significant hepatoprotective activity of the methanolic extract of the celery seeds was observed in paracetamol and thioacetamide intoxications in rats.

Stimulation of hepatic regeneration could be the explanation for the hepatoprotective effect of celery seed extract (Singh and Handa, 1995). Also, celery seed extract is potent against experimentally induced hepatocarcinogenesis in Wistar rats. Prophylactic treatment with this extract protected against diethylnitrosoamine (DEN) -, 2-acetylaminofluorine (AAF)- and partial hepatectomy (PH) - induced hepatocarcinogenesis and other related events such as induction of  $\gamma$ -glutamyl transepeptidase ( $\gamma$ -GT) positive foci, in a dose-dependent manner *in vivo* (Sultana *et al.*, 2005; Anubha and Handa, 1995).

The essential oil of celery has the cercaricidal effect against cercariae *Schistosoma mansoni*, one of the stages in the life cycles of this parasite, which causes schistosomiasis (Saleh *et al.*, 1985).

#### **1.2.6 Relevant patents of CSE**

Celery seed extracts have been reported in patents (US Patent 6,761,913 and 6,352,728,) in anti-inflammatory function and treatment of gastrointestinal irritation. These patents revealed that biologically active extracts of celery seed were produced by various chemical methods and possessed activity for the treatment and prevention of acute and chronic pain, inflammation and gastrointestinal irritation in animal models. Celery seed extracts were found to have marked inhibitory effects on PGE<sub>2</sub> production by porcine gastric mucosal

explants in organ culture (Butters et al., 2004). The gastroprotective effects are probably mediated through non-prostaglandin mechanisms (Whitehouse *et al.*, 2001).

Tani (2005) claimed that celery seed extracts provided an anti-inflammatory agent exhibiting an anti-inflammatory function. This natural herb or combination of other herbs is effective to relax and suppress symptoms of allergy, particularly holistic symptoms of allergic rhinitis, without causing any substantive adverse effects. Also, inclusion of the agent into foods and drinks is useful (Tani, 2005, US Patent 5916565).

Rainsford and Liu (2001; US Patent 2006001396) suggested that celery seed extracts had shown interesting antimicrobial activity against *H. pylori*.

Japanese patents JP57056416 and 587058327 refer to mouthwash compositions containing an alcoholic extract of celery.

#### **1.2.7 Available celery seed extracts**

Celery seed oil is traded with increasing demand on world markets. The phthalide components and selinene are the critical components imparting both the typical celery flavor and fragrance and the therapeutic value. The market for medical herbs and related commodities is growing. The 1999 market for herbs in the US exceeded \$ 2 billion (Craig, 1999). In 1984, an annual celery

seed oil production was 25 tons and a celery herb oil production was 0.5 tones (Lawrence, 1987). One current estimate of the annual consumption of celery seed oil is 40 tones / annum, with some 50% of this produced in India (Falzari and Menary, 2005).

Celery seed is available in the following forms: fresh or dried seeds, tablets, encapsulated oil or extracts to make tea, by pouring boiling water over crushed seeds. Among the treatments recommended from Health stories are celery seed oil capsules or tablets: One to two capsules or tablets three times a day, as directed by your health care provider. Celery seed extract: 1/4 to 1/2 tsp three times a day, or as directed by your health care provider. Whole celery seeds: Prepare a tea by pouring boiling water over one teaspoon (1 to 3 g) of freshly crushed seeds. Let it steep for 10 to 20 minutes before drinking. Drink three times a day. Of the combinations, Apium combines well with menyanthes and/or guaiacum in rheumatic disease. The therapeutic action of Apium is potentiated by taraxacum. High blood pressure: either consume 8 ribs of celery per day or take a celery extract standardized to contain 85% 3-n butyl phthalide and other celery phthalides at a dosage of 75 to 150 mg twice daily. For the relief of join and muscle pain (including pain due to osteoarthritis, rheumatoid arthritis, and fibromyalgia): one tablet two to three times daily. For gout: two tablets twice daily.

In Australia, more than 100,000 people have used the celery seed extract standardized at 85% phthalides without any reports of side effects. The recommendations for joint and muscle diseases in the clinical experience are to use a celery seed extract with standardized containing 85% 3n-butyl and other phthalides (Aura hitech & health center – online, 2007).

### **1.3 Relevant background and hypothesis for the present investigations**

Overall pre-clinical assessment of over the counter medications, including herbal medicines, the treatments of infection of *H. pylori* still have many drawbacks (detailed in section 1 - 2 of chapter 1). The current standard treatment of *H. pylori* infections is antimicrobial schemes, Nevertheless, antimicrobial eradication treatment success of *H. pylori* infections is jeopardized by a couple of interfering factors, such as patients' compliance, the clinical course *H. pylori*-related diseases or microbial virulence factors involved in the degree of inflammation and by host characteristics including gastric pH, diabetes or smoking. Resistance to antimicrobials in use is generally accepted as the most important jeopardizing factor (Wolle, 2007; Byun et al., 2006). In the vaccine development, the fact that the organism is prevalent worldwide, is responsible for significant morbidity and mortality, and is difficult and expensive to eradicate makes it a prime target for vaccine therapy (Hardine and Wright, 2002). As emerging drug resistance continues to plague efforts to eradicate *H. pylori* infection and developing vaccine therapy in the future, new therapeutic regimens incorporating existing antibiotic agents and newly developed compounds are essential.

A satisfactory solution would be a cheaper effective therapy, potential for safer use especially where infections persist and therapies with special mechanisms for attach on the bacteria as well as with avoidance of use of antibiotics that



may cause alterations in GI flora and contradicts resistance.

Plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years (Craig, 1999). The World Health Organization estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components. They are always cheap, and may be effective with few side effects (Tripathi et al., 2007). In view of the variety of the effects exhibited by active components from celery seed, it was of interest to examine whether they might have an anti-*H. pylori* effect. Earlier studies by Rainsford and Liu (2001; US Patent 2006001396) suggested that celery seed extracts had shown interesting antimicrobial activity against *H. pylori*. Five compounds have been purified which are partly responsible for the antimicrobial properties. Those compounds had been confirmed the chemical structures and quantified the inhibitory concentration against *H. pylori*. But they are not responsible for the most anti-*H. pylori* activity, the constituent(s) maybe reside in other fractions and sub-fractions. Also the antibacterial mechanisms need to be investigated.

Hypothesis: Based on the above observations and review of current literature, it is postulated in this thesis that " Component(s) of celery seed may inhibit the growth and viability of *H. pylori* by novel mechanisms of action".

# Aims and Objectives

## Aims

1. To characterize the effects of celery seed extracts against *Helicobacter pylori*.
2. To identify the active components from celery seed extracts.
3. To investigate the mechanism of action of celery seed extracts against *H. pylori*.
4. To establish from these studies the potential of CSE to be a natural treatment for clinical practice to act as a prophylactic or eradicate *Helicobacter pylori*.

## Objectives

- To determine the effects of the CSE components following their isolation and chemically characterization on the growth and viability of *H. pylori* and to define the mechanisms of their inhibitory effects.
- The component fractions were obtained from 3 sources, i.e.
  - a. The raw ethanolic extract obtained from Chennai, India (A-CSE).
  - b. The CO<sub>2</sub> critical fluid extract applied to the ethanolic extract above (C-CSE).
  - c. Methyl ethyl ketone extracts of the abovementioned ethanolic (ME-CSE) extract.
- As a known component of CSE fractions (sedanolide) was obtained

commercially, it was investigated for potential anti-*Helicobacter* activity and compared with that of the extracts some of which may contain these components.

- The mechanisms of inhibitory effects on the growth and viability of *H. pylori* will be determined by initially examining the effects on protein and nucleic acid synthesis and of viability using dye exclusion methods. Further exploration of these effects was undertaken following on the outcome from these initial studies and employs molecular biological techniques.

## **Chapter II**

### **Effects of Various Extracts of Celery Seed on the Growth and Viability of *H. pylori***

## 2.1 Introduction

In the search for a new agent to combat *H. pylori* infection, celery seed has attracted much attention with regard to its anti-microbial activity (Popovic *et al.*, 2006; Friedmen, 2002, Rainsford and Liu 2006; US Patent 2006001396). Celery seed (the seed of *Apium graveolens*) has been used as a herbal medicine for thousands of years in India. In pharmacognosy, celery seed has been reported to be useful for the treatment of urinary calculi, gut diseases, the relief of flatulence and griping pains, reduction of visceral spasm, and stimulation of the smooth muscle of womb. It also promotes hypoglycemia to reduce blood glucose and may thus be useful in treating diabetes mellitus (Mddides-online, 2006). Momin (2001) reported the antifungal, mosquitocidal and nematicidal activities of compounds from the methanolic extract of celery seed. Moreover, Friedmen (2002) showed that celery seed extracts have bactericidal activities against *Campylobacter jejuni*, *Esherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*. Ethanolic extracts have also been found to have anti-inflammatory and anti-ulcer activity in rodent models (Butters *et al.*, 2004).

The extracts of celery seed that have been investigated here are the raw ethanolic extract of celery seed (A-CSE), CO<sub>2</sub> supercritical extract (C-CSE) and methyl ethyl ketone extracts (ME-CSE). The microplate assay is basic and effective to evaluate the bactericidal and bacteriostatic activity levels. Liquid inhibitory assays in BB media were chosen. (Feresin *et al*, 2003). The extracts

have been tested to identify their effects on the growth and viability of *H. pylori*. Also, the bactericidal versus bacteristatic activity was distinguished by using the same assay.

Both *H. pylori* strains were cultured with and without those known CSEs for 5 – 7 days. Then the optical density was measured to compare the growth of *H. pylori* between untreated and treated samples. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration were derived. Therefore, the effects of various CSEs against *H. pylori* were characterized.

## **2.2 Methods**

### **2.2.1 Bacterial strains and culture condition**

The strain 3339 of *H. pylori* employed in the present investigations was isolated from a British patient with a peptic ulcer (duodenal ulcer or gastritis). Another strain 26695 was obtained from Prof. David Kelly (University of Sheffield), and is one of the strains for which a full genome sequence is available (Tomb *et al.*, 1997). Their identities were confirmed by Gram stain, urease reaction and 16S RNA gene sequence (see the identification section of result part).

The bacteria were store at –80°C in aliquots of 1 ml of brucella broth (BBL, USA) containing 15% [v/v] glycerol (Kitsos and Stadtlander, 1998) and cultured

on chocolate agar plates (Biomérieux, France) for 5 days under microaerophilic conditions (10% CO<sub>2</sub>, 3% O<sub>2</sub> and 87% N<sub>2</sub>, at 37 °C). A liquid culture of *H. pylori* was grown in brucella broth. For liquid culture media, Brucella broth (BB) (28g) (Oxoid) was added to 1L of distilled water. The medium was sterilized autoclaving at 120 °C for 15 min and allowed to cool. Fetal bovine serum (FBS) (Invitrogen, UK) was added to 5% [v/v] immediately before use (Morgan *et al.*, 1987).

## **2.2.2 Identification of *H. pylori***

### **2.2.2.1 Urease test**

The CLO test (*Campylobacter* Like Organism) was the first commercial test available developed by Prof. Barry Marshall (Marshall and Warren, 1984). This test is performed in liquid media with urea and phenol red presence, a dye that turns pink at a pH of 6.0 or greater. In the presence of *H. pylori*, the *Campylobacter*-like organism, which converts urea to ammonia by way of its urease activity, the pH will rise above 6.0 and the phenol red in the medium changes from yellow to magenta. Here, the urease test was performed by using suspended cells. A solution of urea (Sigma) (10%) and a solution of phenol red (1%) were prepared. For the working solution, two drops of phenol red solution were mixed in 1 ml of the urea solution. The reagent is stable for 2 weeks at 4-8 °C. Each sample was added to 0.2 ml of the reagent and incubated at room temperature (24 °C) for one minute. For positive result, the

reagents changed from yellow to magenta (Finegold and Baron 1986).

#### **2.2.2.2 16S rRNA gene sequence**

For identification of *H. pylori* at the genome level, the QIAGEN genomic kits (Qiagen, West Sussex, UK) were chosen for direct isolation of *H. pylori* DNA.

An internal fragment of the 16S rRNA gene was amplified using the following primers; 5'-AGAGTTTGATCMTGGCTCAG-3' (16S-1, 20-mer) and 5'-TACGGYTACCTTGTTACGACTT-3' (16S2, 22-mer) (Song *et al.*, 1999).

PCR amplification was carried out through 30 cycles consisting of a denaturation step of 95 °C for 1 min, a primer-annealing step of 55 °C for 1 min and an extension step at 72 °C for 2 min, with a single final extension step of 72 °C for 10 mins (Clayton *et al.*, 1992). The 1466 bp PCR product was purified with the Wizard SV Gel and PCR Clean-up System. The purified product DNA was sent to MWG-Biotech (London) for dye termination sequencing, using same primers as well used for PCR.

#### **2.2.3 CSE sources**

CSE for antimicrobial tests was obtained from 3 sources. The raw ethanolic extract of celery seed (A-CSE) was obtained from Chennai, India; the other two sources of celery seed extracts were obtained from Prof. MW. Whitehouse, Griffith University and University of Queensland, Brisbane, Queensland, Australia, which were the CO<sub>2</sub> super critical fluid extract (C-CSE) applied to the



ethanolic extract above and methyl ethyl ketone extracts (ME-CSE) of the same original ethanolic extract. All the CSE extracts were dark green highly viscous liquids that were dissolved in dimethylsulphoxide (DMSO) to prepare stock solutions.

#### **2.2.4 Antimicrobial activity test**

The liquid inhibitory method using BB broth plus FBS was performed using 24-well plates, including an uninhibited control, culture extract controls and positive control samples (Xia *et al.*, 1999).

For using in the testing, colonies of the bacteria were inoculated into the Brucella broth (BB) plus FBS and adjusted to turbidity equivalent to a No.2 McFarland standard for broth dilution method (McFarland No. 2 Standard =  $1 \times 10^7$  ---  $1 \times 10^8$  CFU/ml,  $0.3 \text{ abs@}560\text{nm} = 2.4 \times 10^8$  CFU/ml) (Burger *et al.*, 2000; Haim *et al.*, 2004).

Each assay was performed in multiple wells and contained 0.1ml sample plus 1.0ml of early log phase *H. pylori* culture in Brucella broth plus fetal bovine serum. Samples comprising 1mg of various CSEs were dissolved in 10  $\mu$ l of solvent (DMSO) to get 100 mg/ml, and then diluted 100 fold into brucella broth plus FBS to get 1mg/ml. Control assays using up to 1% [v/v] of solvent concentration used in the experimental inoculation showed that the solvents

themselves did not have significant antimicrobial activity at the concentration used. The serial dilutions of components of CSE were added to the column well of the each plate. Next 0.1 ml of cell suspension was inoculated into each well in each row.

After the plates were inoculated at 37°C under microaerophilic conditions, bacterial growth was monitored by method of turbidometry, using a microplate reader at 550nm (Wallac 1420, Perkinelmer, Finland). Turbidometry is the process of measuring the amount of light that a solution absorbs. A photoelectric cell collects the selected light which passes through the cuvette. A measurement is then given for the amount of absorbed light to find the number of cells in a solution (Fiach and Dmitri, 2006). MICs were determined by using a two fold dilution series of the samples in BB + FBS.

Bactericidal activity was determined as follows. *H. pylori* was cultured in the liquid media with serial dilution of the extracts as detailed above in 24-well plates under the same microaerophilic conditions for 5 days, including a negative control. Then the aqueous cultures were centrifuged at 16,000 x g for 5 min. After discarding the supernatants, the pellets were re-suspended in fresh medium and inoculated for 3 days under the same conditions. Bactericidal activity was concluded if the culture failed to re-grow in the fresh

medium.

## **2.3 Results**

### **2.3.1 Initial cultivation of microorganisms**

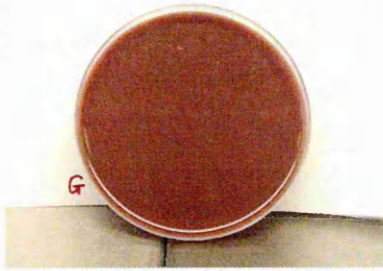
In **Fig. 2-1**, the growth of the *H. pylori* strain 26695 and 3339 were investigated on chocolate agar plates. During the period of culture all the colonies had similar morphology, which is consistent with the absence of contaminating microorganisms.

Also the two strains of *H. pylori* were cultured in BB liquid media plus fetal bovine serum. Both of strains grew very well in this medium. The growth of strain 3339 (from 0.05 to 0.342 @ OD<sub>550</sub> in 3 days) was different from that of strain 26695 (from 0.049 to 0.088) under same conditions (**Fig. 2-2**). The growth rate of strain 3339 was markedly greater than that of strain 26695.

### **2.3.2 Urease test (CLO)**

In the urease test (**Fig.2-3**), in the presence of both of *H. pylori* strain 3339 and strain 26695 the reagents changed from yellow to magenta, meanwhile the colour did not change when the reagents were added into *E-coli* or the water control. The presence of *H. pylori* converts urea to ammonia by way of its urease activity, the pH rose above 6.0 and the phenol red in the medium changed from yellow to magenta.

**Fig. 2-1 Culture *H. pylori* on chocolate agar plate**



Day 0



Day 1



Day 2



Day 3



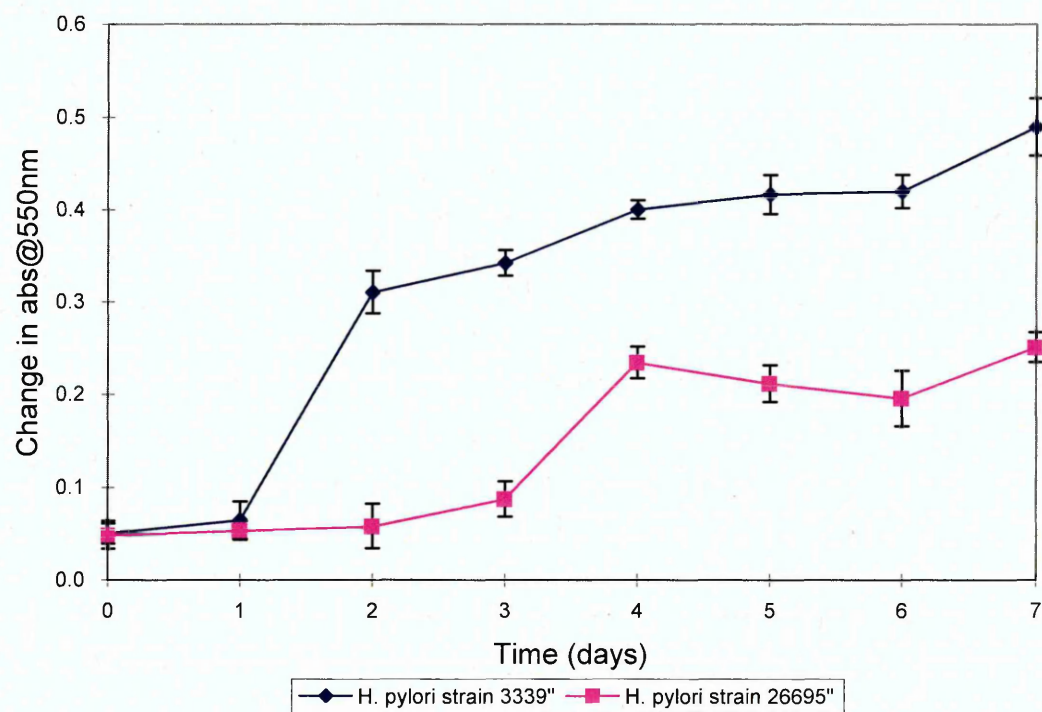
Day 4



Day 5

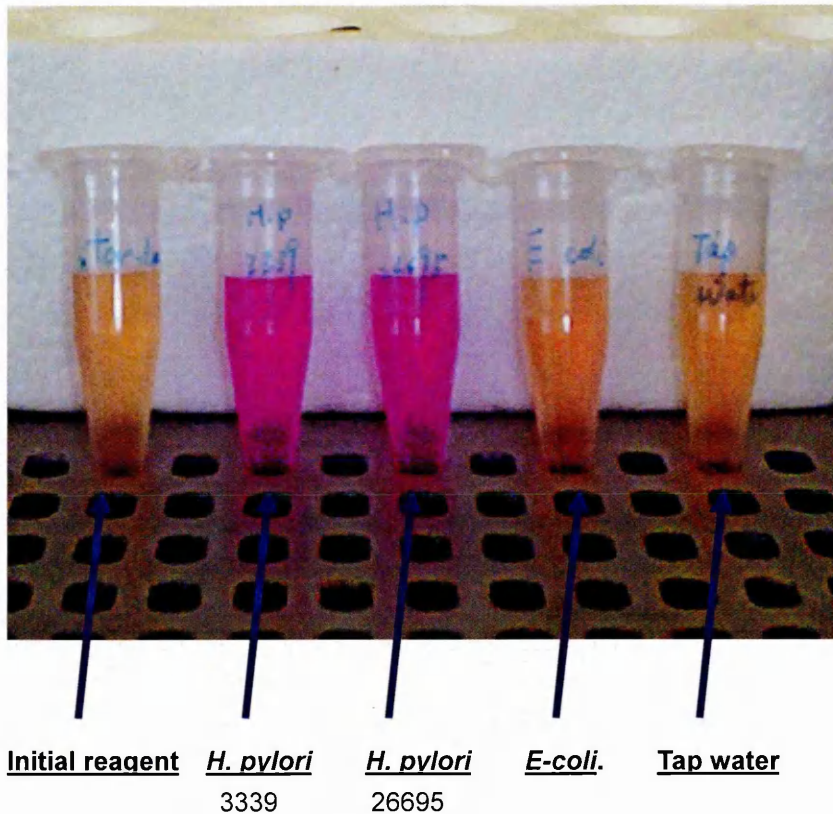
*H. pylori* strain 3339 was streaked on chocolate agar and cultured for 5 days under microaerophilic conditions. There were colonies of *H. pylori* to display after 1day and the most amounts of colonies could be found after 3 days. All colonies were similar size and color.

**Fig. 2-2 Comparison of growth of *H. pylori* strains 3339 and 26695**



Both strains of *H. pylori* were cultured in BB media plus FBS to observe their growth ratio. These results have been repeated (n = 5) under same conditions.

**Fig. 2-3 Results of urease test**



All the test tubes contained 1 m<sup>98</sup>l of fresh urea/phenol red reagent prepared. The initial reagent is yellow shown in the first left below. The colour change was observed in 10 mins after adding samples. For adding *H. pylori*, the colour changed from yellow to magenta. There was not change in tubes with *E coli* and tap water.

### 2.3.3 16S rRNA gene sequence identification

Strain 26695, for which there is a completed genome sequence in the public domain, was obtained from Professor David Kelly at University of Sheffield.

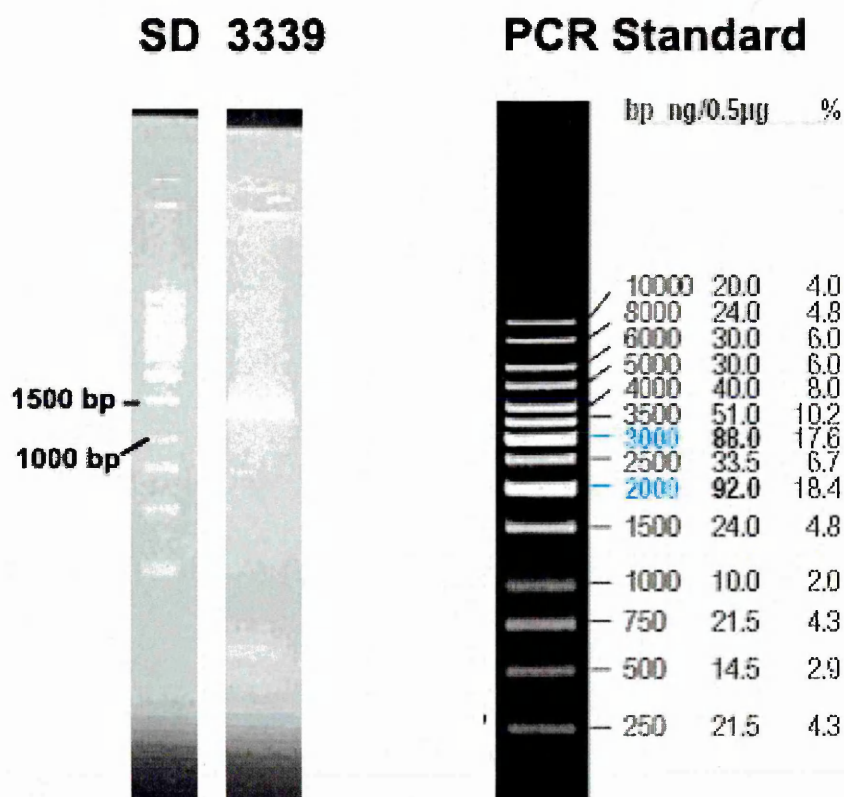
Strain 3339 is a less well characterized clinical isolate and so its identity as a strain of *H. pylori* was confirmed by sequences of the 16S rRNA gene. The 16S rRNA gene was amplified by using PCR from the universal eubacterial primers 5'-AGAGTTTGATCMTGGCTCAG-3' and 5'-TACGGYTACCTTGTTACGACTT-3'. Sequencing of the 1466 bp PCR product (**Fig. 2-4**). Both PCR primers gave 99.2% identity to the 16S rRNA gene of *H. pylori* over 238 aligned bases (**Fig. 2-5, 2-6**). Sequencing of the PCR product of strain 3339 from primers was performed by MWG Biotech (Ebersberg, Germany).

### 2.3.4 Inhibitory effects of A-CSE against different strains of *H. pylori*

In the assays for inhibitory activity of A-CSE against *H. pylori* strain 3339 and 26695, A-CSE exhibited variable antimicrobial activity to the different strains (**Fig. 2-7**). The concentrations of A-CSE were varied between 6.25 µg /ml and 500 µg /ml and the data were recorded each 24 hours over 7 days. The minimum inhibitory concentration (MIC) of A-CSE against *H. pylori* strain 3339 was at 200 µg /ml, meanwhile it was at 300 µg /ml for strain 26695 at day 5 (**Fig.2-7a**). At 7 days, the values of MIC were 400 µg /ml for strain 3339 and >



**Fig. 2-4 PCR results of *H. pylori* strain 3339 and 26695**



The PCR product represented the 16S ribosomal subunit gene of *H. pylori* strain 3339. The mobility of the product band was consistent with the expected size of 1466 bp.

The standards on the right are copied from the manufacturer (Qiagen, UK) and motilities of DNA bands are not comparable with the gels on the left.



**Fig. 2-5 16S rRNA sequence of *H. pylori* strains 3339**

```

16S_H.pylori    ACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGATGAAGCTTCTAGCTTGCTAGAAT 240
3339-seq_rxn    -----CTAACTTGCTAGAAT 15
                                     *** *****

16S_H.pylori    GCTGATTAGTGGCGCACGGGTGAGTAACGCATAGGTTATGTGCCTCTTAGTTGGGATAG 300
3339-seq_rxn    GCTGATTAGTGGCGCACGGGTGAGTAACGCATAGGTCATGTGCCTCTTAGTTGGGATAG 75
*****

16S_H.pylori    CCATTGGAAACGATGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGCTAAGA 360
3339-seq_rxn    CCATTGGAAACGATGATTAATACCAGATACTCCCTACGGGGGAAAGATTTATCGCTAAGA 135
*****

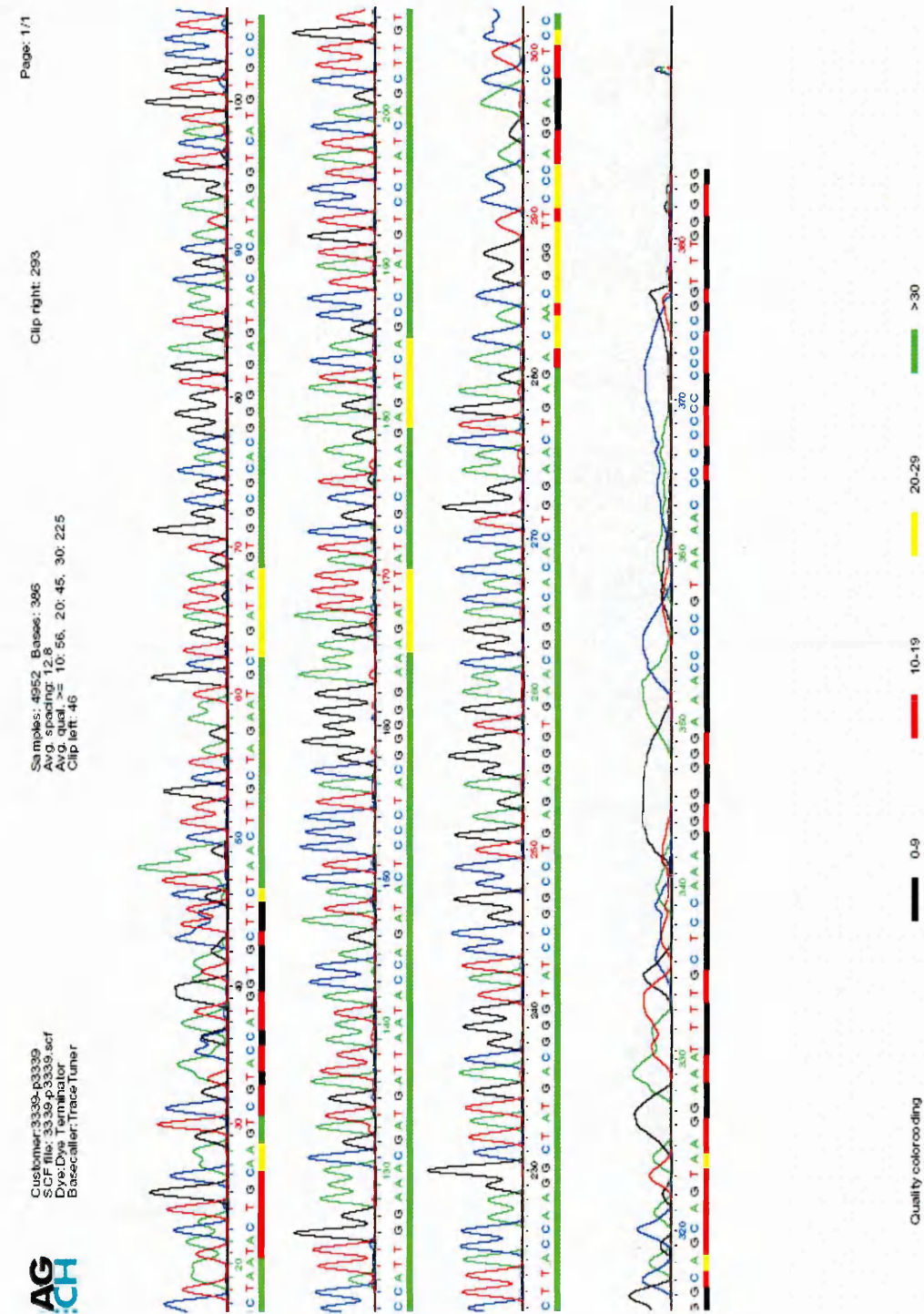
16S_H.pylori    GATCAGCCTATGTCCTATCAGCTTGTGGTAAGGTAATGGCTTACCAAGGCTATGACGGG 420
3339-seq_rxn    GATCAGCCTATGTCCTATCAGCTTGTGGTAAGGTAATGGCTTACCAAGGCTATGACGGG 195
*****

16S_H.pylori    TATCCGGCCTGAGAGGGTGAACGGACACACTGGAAGTGAAGACACGGTCCAGACTCCTACG 480
3339-seq_rxn    TATCCGGCCTGAGAGGGTGAACGGACACACTGGAAGTGAAGACAA----CGGGTTCC---- 247
*****          * * ***

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The alignment to *H. pylori* 16S rRNA sequence from database and sequences were 99.2% identical.

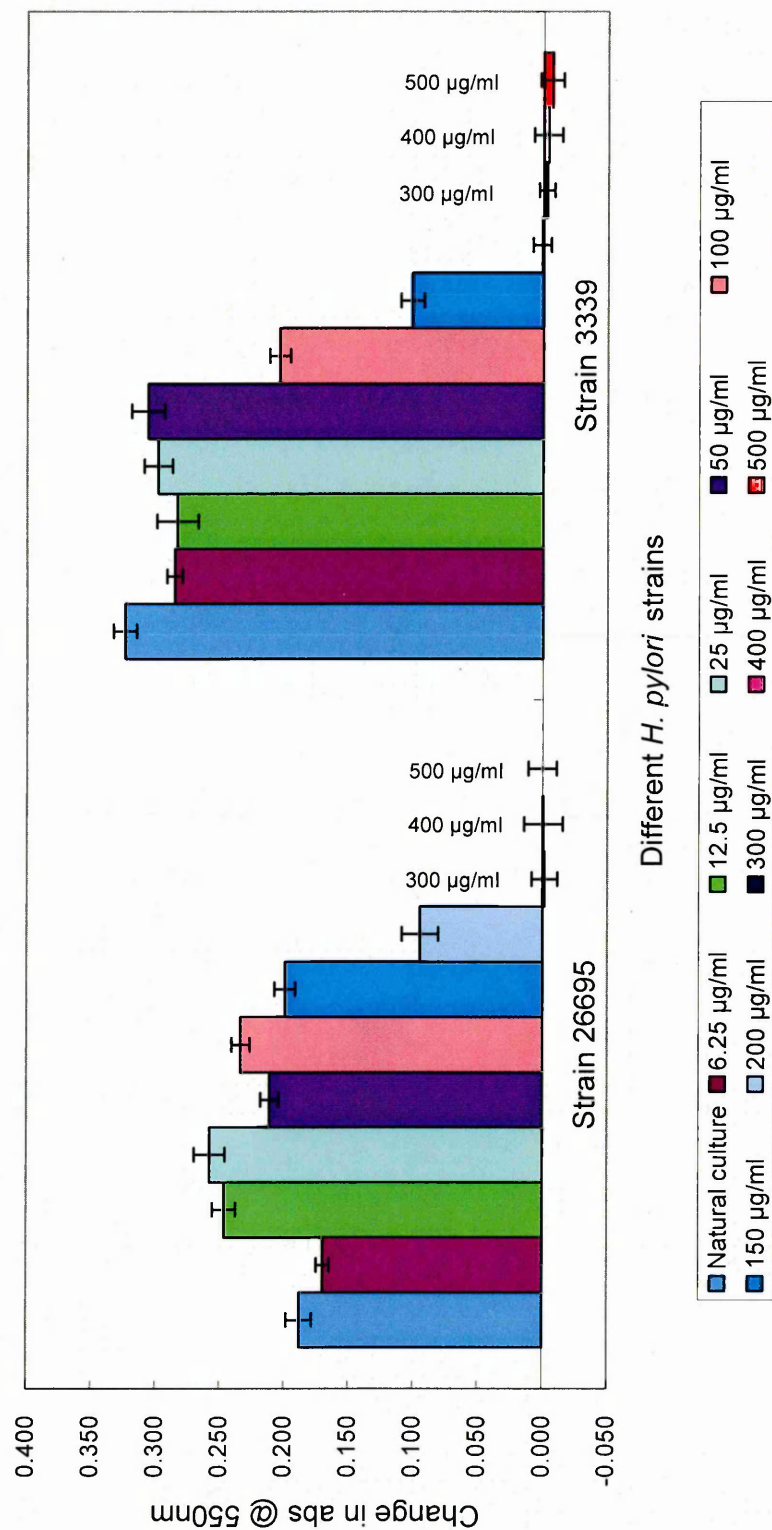
**Fig. 2-6 Dye termination sequencing of the PCR product from strain 3339**



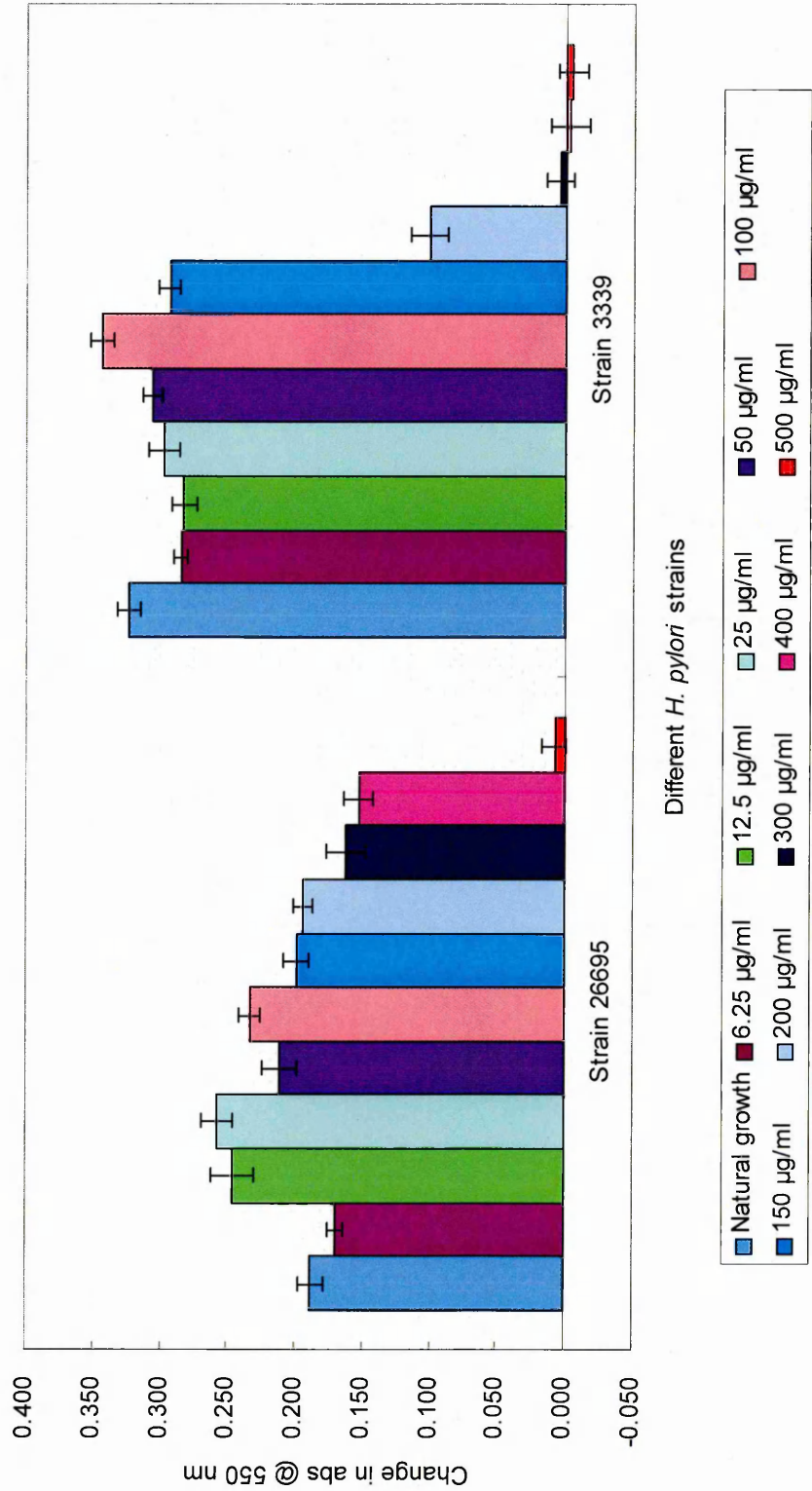
Automated sequencer output that was used to determine the sequence of the 16S rRNA gene from *H. pylori* strain 3339.

**Fig. 2-7 Inhibitory effect of A-CSE against *H. pylori* strain 26695 and strain 3339**

**A. Inhibitory effect after 5 days**



**B. Inhibitory effect after 7 days**



The growth of various *H. pylori* treated with different concentrations of A-CSE resulted in a significant decrease ( $P \leq 0.01$ ) from pre-treatment levels in  $OD_{550}$ . Results are the means of 5 independent replications; error bars show SD. Natural culture is without added antimicrobial agent.

500 µg/ml for strain 26695 (**Fig.2-7b**).

### 2.3.5 Positive control

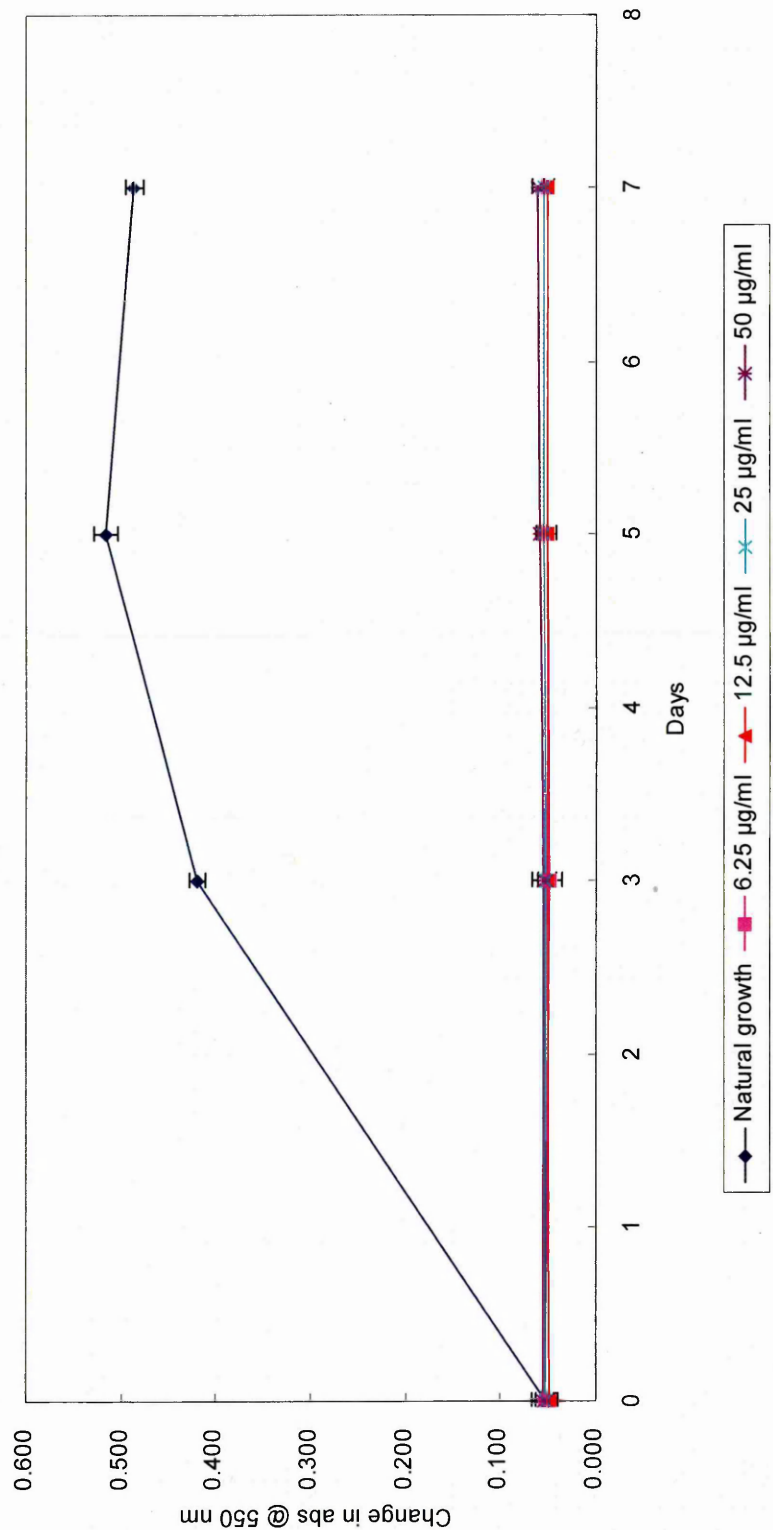
Tetracycline was chosen as the positive control. MIC of tetracycline against *H. pylori* strain 3339 was consistently observed to be 6.25 µg/ml when measured using an incubator the period of 7 days and there was a strong inhibitory effect against the bacteria (**Fig.2-8**).

### 2.3.6 Inhibitory effects of related solvents

DMSO, acetonitrile, iso-propanol and methanol can be employed to dissolve CSE and its fractions and these were used in different experiments. It was, therefore, important to assess the inhibitory effects of those solvents against *H. pylori*. As shown in **Fig.2-9**, concentrations of DMSO up to 1% (v/v) did not inhibit the growth of the bacteria at day 3, 4 and 5, compared with its growth in the medium without added organic solvents. At most concentrations tested, the DMSO slightly stimulated growth and so any inhibition observed in the presence of CSE fractions made up in DMSO must be due to the CSE derived materials.

Also, comparable results were observed in growth in BB plus FBS with acetonitrile (**Fig.2-10**), methanol (**Fig.2-11**) or iso-propanol (**Fig.2-12**). Therefore, it can be concluded that concentrations of these solvents up to 1%

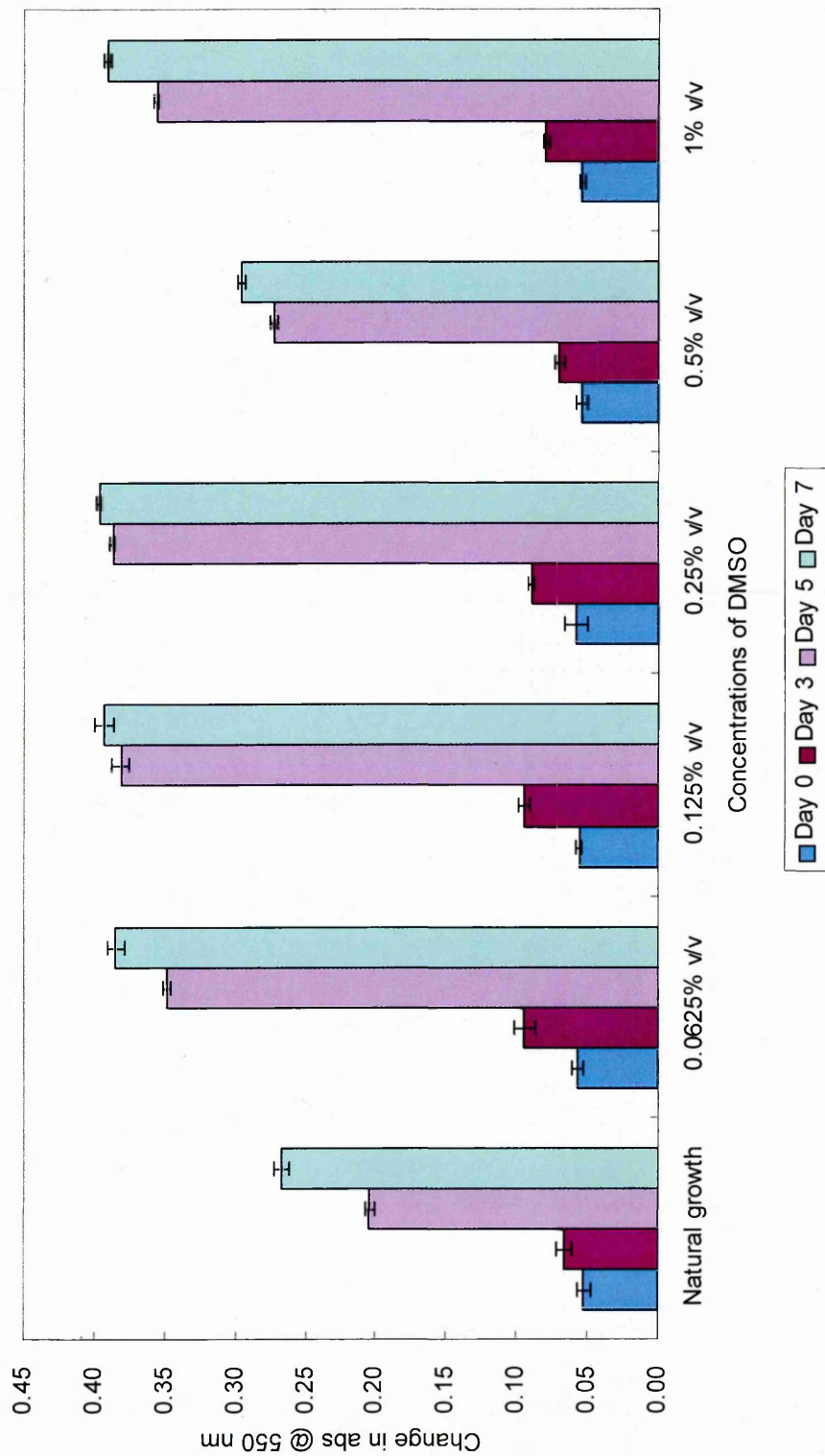
**Fig. 2-8 Inhibitory effect of tetracycline against *H. pylori***



*H. pylori* without treatment (natural growth) grew regularly. The growth of *H. pylori* treated with more than 6.25 µg/ml of concentration of tetracycline resulted in a significant inhibition ( $P \leq 0.01$ ) from pre-treatment levels in OD<sub>550</sub>. Results are the means of 5 independent replications; error bars show SD.

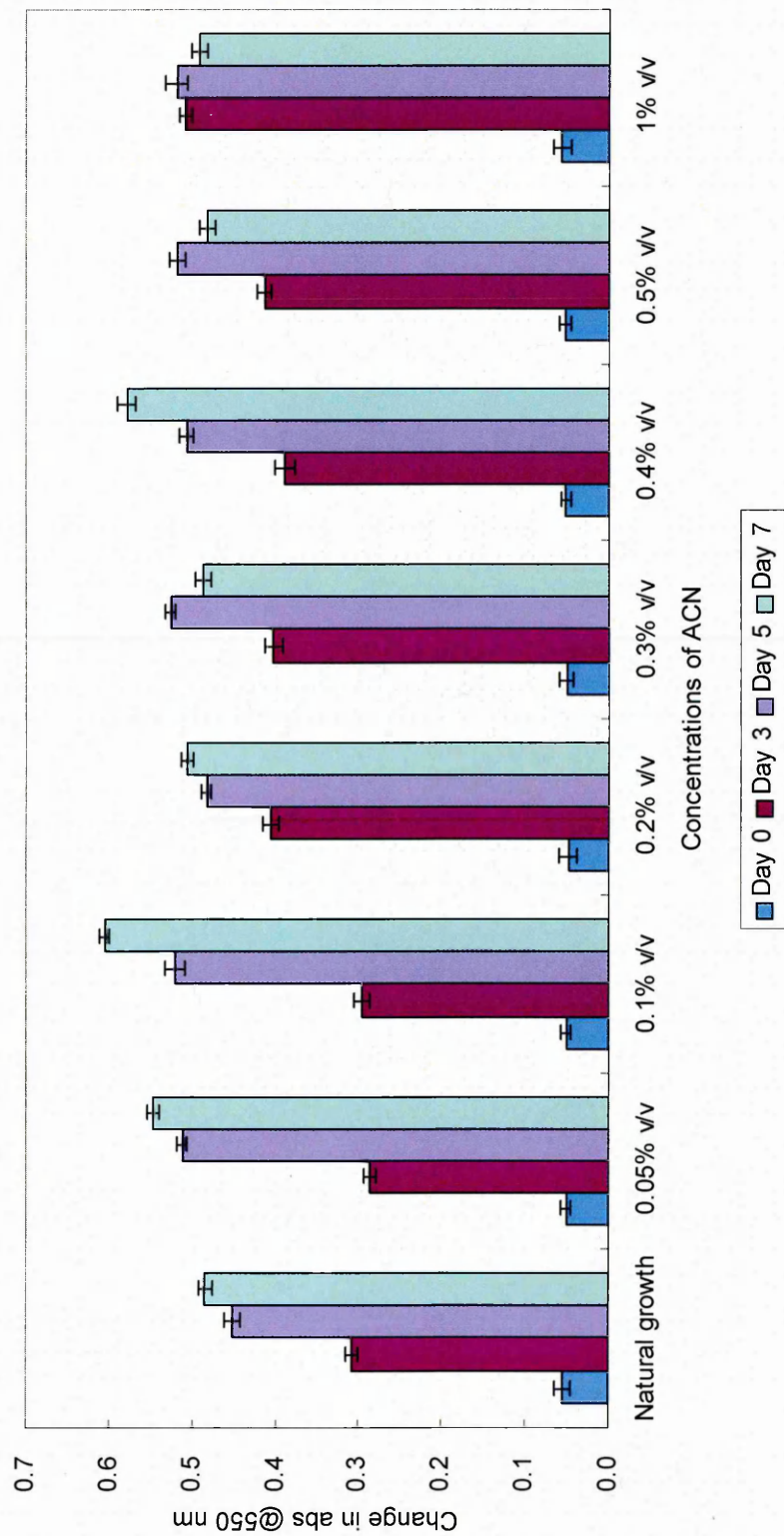


**Fig. 2-9 Effect of DMSO solvent against *H. pylori* strain 3339**



The effect of DMSO solvent against *H. pylori* 3339 is shown above. DMSO (up to 1% v/v of concentration) had no significant inhibitory effect on growth of the cells by liquid media culture. Results are the means of 5 independent replications; error bars show SD. Natural culture is without added any solvent.

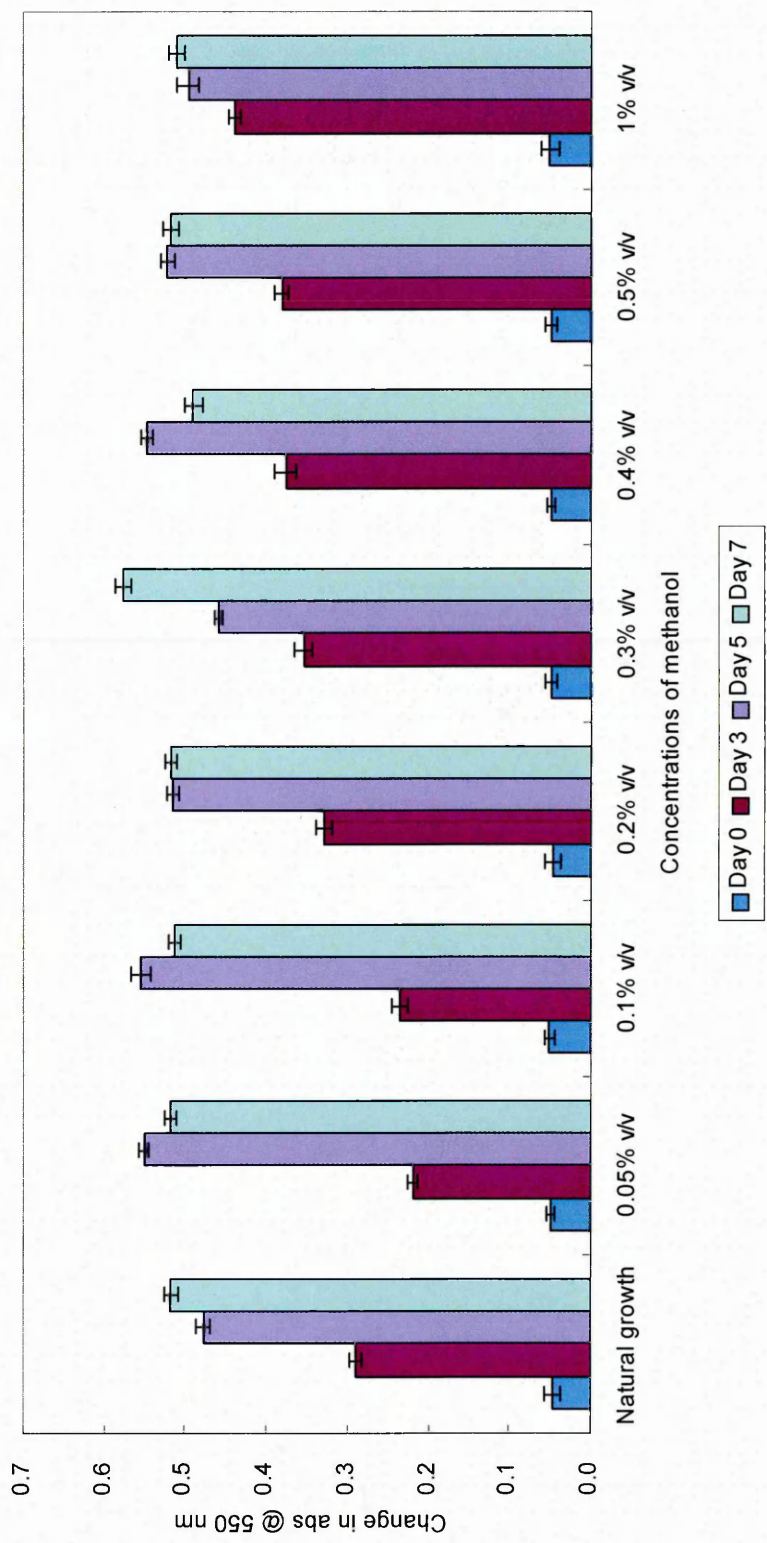
**Fig. 2-10 Effect of acetonitrile solution against *H. pylori* strain 3339**



The effect of ACN solvent against *H. pylori* 3339 is shown above. ACN (up to 1 v/v% of concentration) had no significant inhibitory effect on growth of the cells by liquid media culture. Results are the means of 5 independent replications; error bars show SD. Natural culture is without added any solvent.

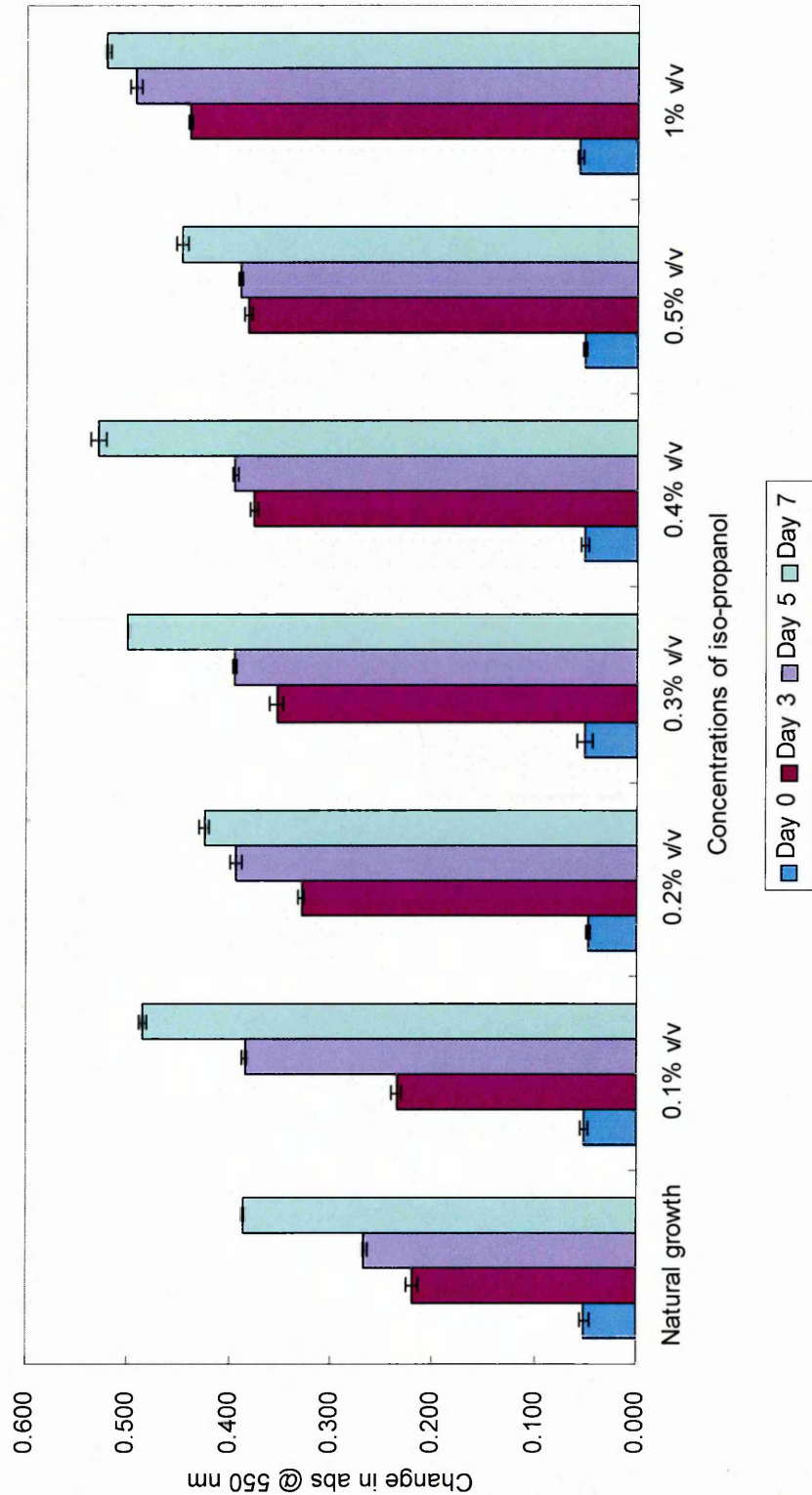


**Fig. 2-11 Effect of methanol solution against *H. pylori* strain 3339**



The effect of methanol solvent against *H. pylori* 3339 is shown above. ACN (up to 1 v/v% of concentration) had no significant effect on growth of the cells by liquid media culture. Results are the means of 5 independent replications; error bars show SD. Natural culture is without added any solvent.

**Fig. 2-12 Effect of iso-propanol against *H. pylori* strain 3339 at 5 days**



The effect of iso-propanol solvent against *H. pylori* 3339 is shown above. Iso-propanol (up to 1 v/v%) had no significant inhibitory effect on growth of the cells by liquid media culture. Results are the means of 5 independent replications; error bars show SD. Natural culture is without added any solvent.

(v/v) did not mask substantial antimicrobial effects of the solute against *H. pylori* strain 3339. So, in all of the subsequent experiments, the concentrations of DMSO, acetonitrile, methanol and propanol for dissolving samples was kept less than 1% (v/v).

### **2.3.7 Inhibitory effects of various CSE against *H. pylori***

All of CSE (A-CSE, C-CSE and ME-CSE) sources were tested for their inhibitory effects against *H. pylori*. This experiment has been replicated at least 5 times. The student's test was used to compare the means, with significance assessed at the 95% confidence level (the student's t-test).

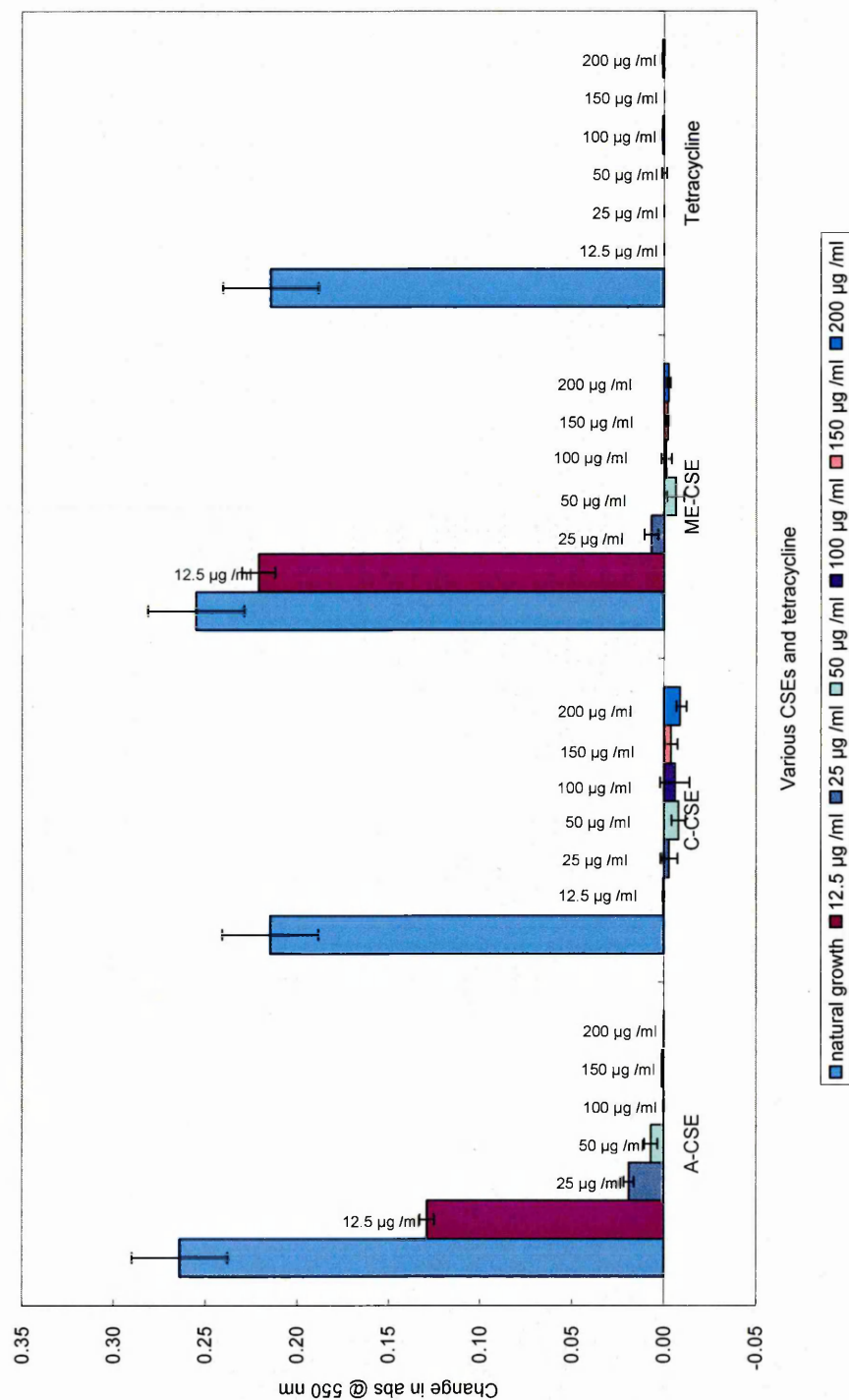
In **Fig.2-13a**, A-CSE exhibited an antimicrobial activity with 200 µg/ml of MIC against *H. pylori* strain 3339 at Day 5. The value for the MIC of C-CSE was 25 µg/ml and that of ME-CSE was 50 µg/ml. Also, similar effects were found at 7 days. The value of MIC being 25 µg/ml for C-CSE and 50 µg/ml for ME-CSE, but for A-CSE had increased to 300 µg/ml (**Fig.2-13b**).

In the testing of C-CSE and ME-CSE against *H. pylori* 3339 at concentration greater than the MIC, the optical density actually fell during the experiment (**Fig. 2-13**). It was likely that C-CSE and ME-CSE induced the cell lysis under these conditions.

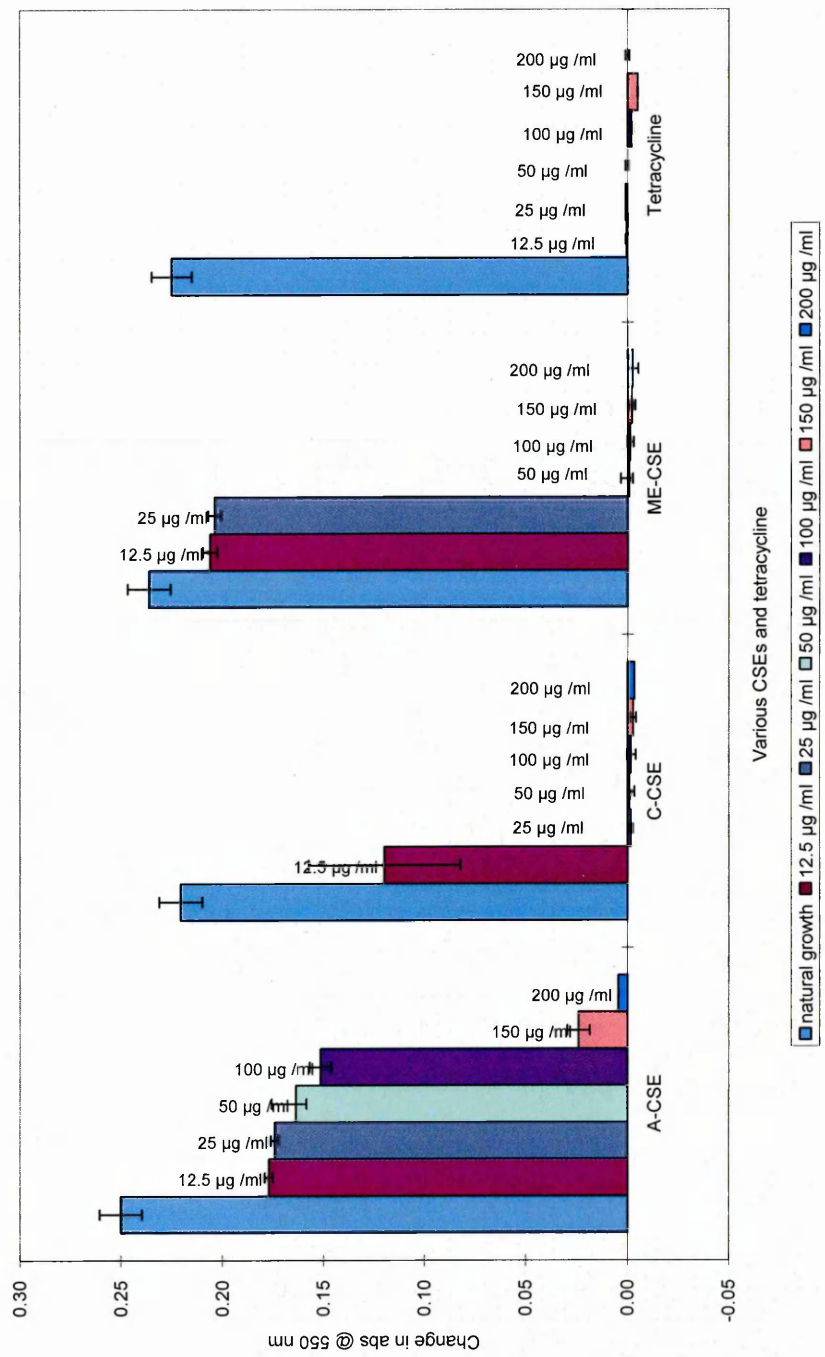
In the experiments with *H. pylori* 26695, similar results were obtained to those

**Fig. 2-13 Inhibitory effect of various CSE against *H. pylori* 3339**

**A. Inhibitory effects after 5 days**



**B. Inhibitory effects after 7 days**



The inhibitory effects of various against *H. pylori* 3339 after (a) 5 days and (b) 7 days are shown below (mean  $\pm$  SD) (n=5). The growth of *H. pylori* treated with different CSE resulted in a significant decrease ( $P \leq 0.01$ ) from pre-treatment levels in OD<sub>550</sub>. Also, for comparison, the growth of the cell treated with positive control (tetracycline) resulted in a significant decrease ( $P \leq 0.01$ ).

above for 3339. Thus, the MICs were 25 µg/ml, 50 µg/ml and 300 µg/ml of MICs for C-CSE, ME-CSE and A-CSE respectively at day 5 (**Fig.2-14a**). With increase in the incubation time to 7 days, the MICs of C-CSE and ME-CSE were still same as observed at earlier times, but that of A-CSE had increased to 400 µg/ml (**Fig. 2-14b**).

## 2.4 Discussion

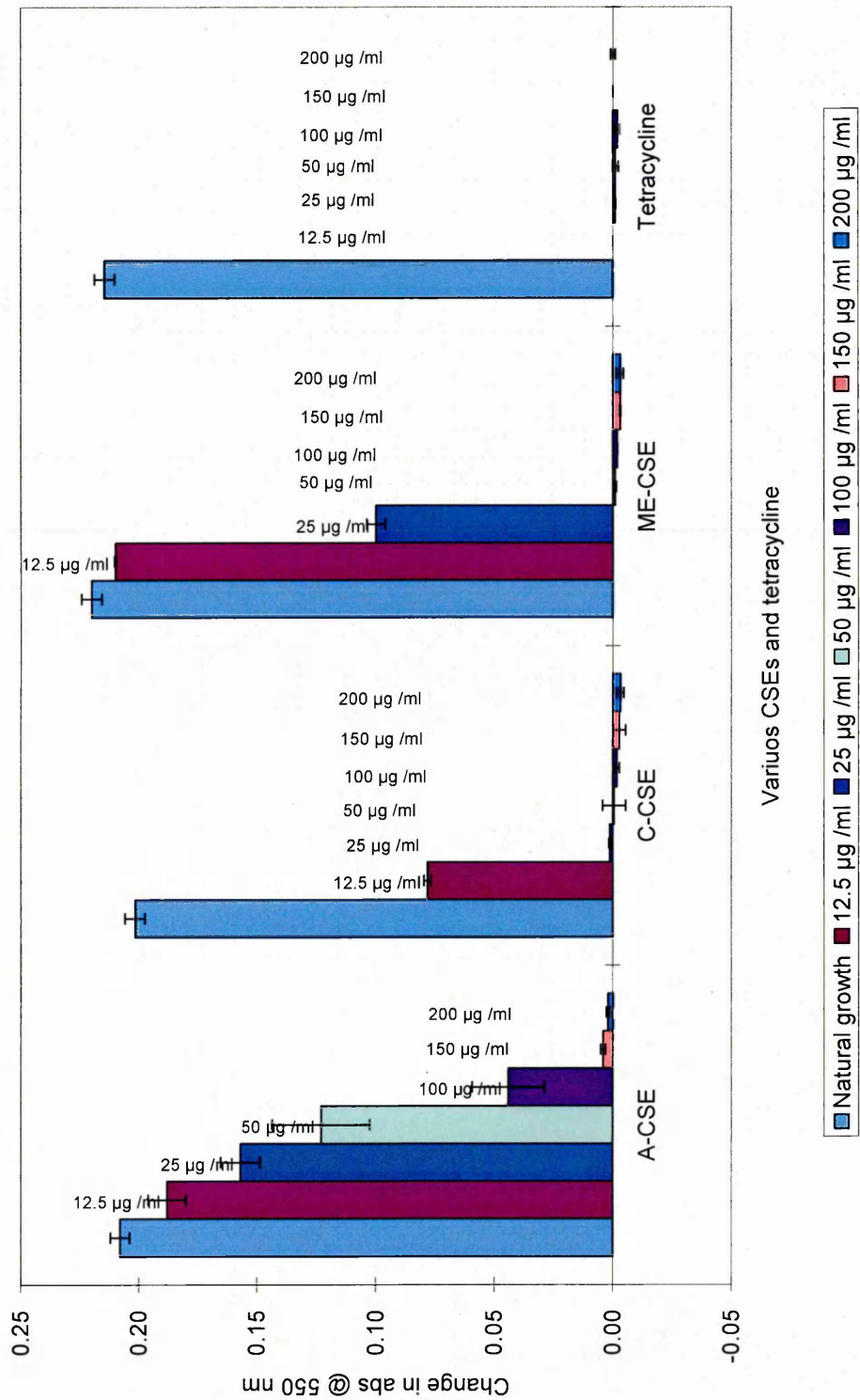
Two strains of *H. pylori* were used during this work, strain 26695 (for which a genomic sequence is available) and the clinical isolate 3339. The strains had slightly different colony morphologies and strain 3339 grew slightly more quickly than strain 26695 under the conditions employed. As shown in the results (section 3 of this chapter), the properties of both strains are consistent with them being *H. pylori*, since they were found to be strongly urease positive and both grew on chocolate agar in an atmosphere containing 3% O<sub>2</sub> and 10% CO<sub>2</sub> but not on the same medium in unmodified air. Moreover, 16S rRNA gene sequencing showed that strain 3339 had identical 16S rRNA genes to *H. pylori* from database and therefore the strains used are genuine *H. pylori* strains.

As detailed in the Introduction to this chapter, Celery seed extracts have been known for their bactericidal, anti-inflammatory, diuretic effects, and as an inhibitor of adjuvant in arthritic disease in rats and in rheumatic conditions (Whitehouse *et al*, 2001; Kitajima *et al*, 2003) and applied as an antimicrobial

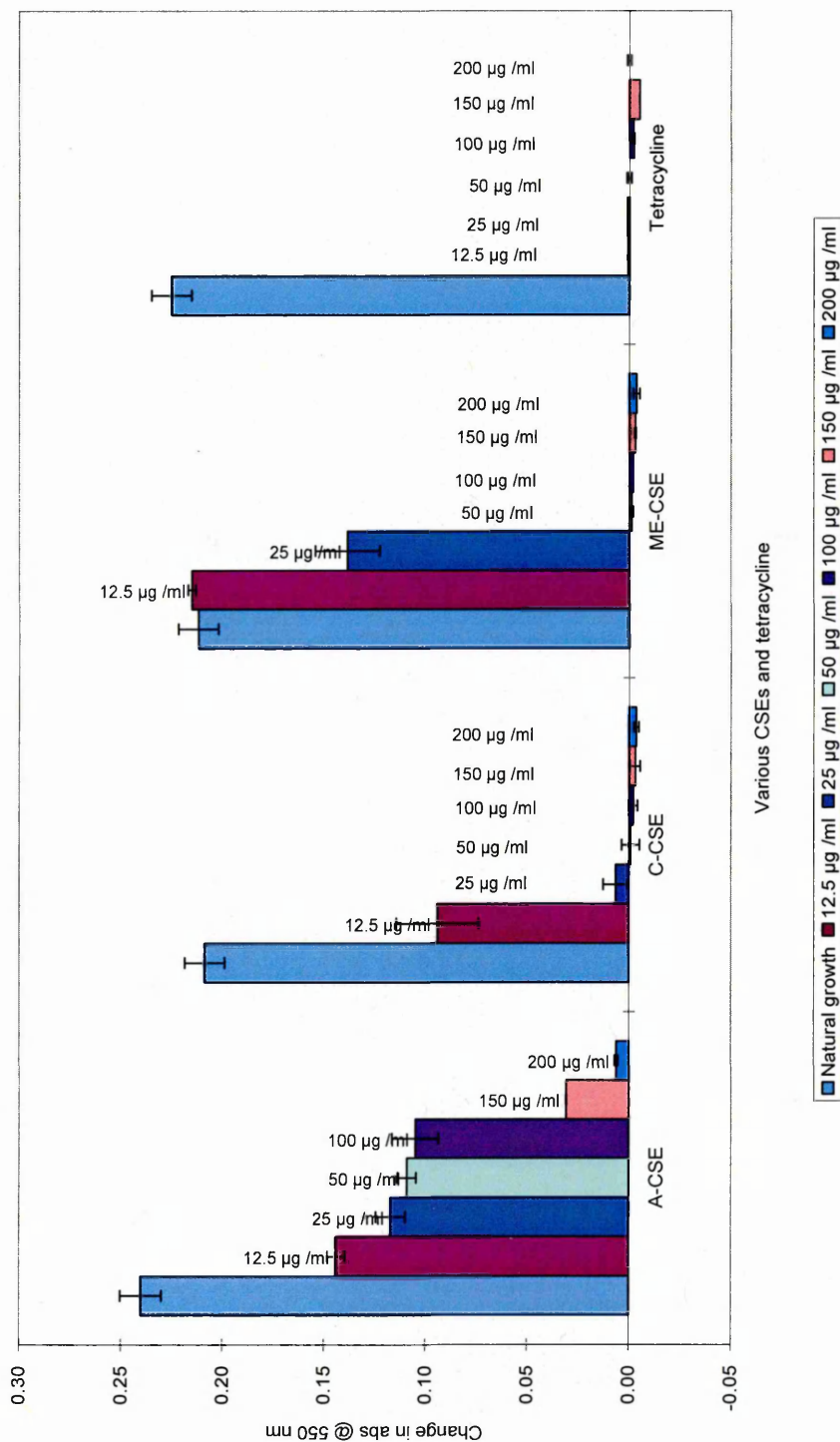


**Fig. 2-14 Inhibitory effect of various CSE extracts against *H. pylori* 26695**

**A. Inhibitory effects after 5 days**



## B. Inhibitory effect after 7 days



The inhibitory effects of various CSE against *H. pylori* 26695 after (a) 5 days and (b) 7 days are shown below (mean  $\pm$  SD) (n=5). The growth of *H. pylori* treated with different CSE resulted in a significant decrease ( $P \leq 0.01$ ) from pre-treatment levels in OD<sub>550</sub>. Also, for comparison, the growth of the cell treated with positive control (tetracycline) resulted in a significant decrease ( $P \leq 0.01$ ).



against *H. pylori* suggested by researchers in Rainsford's group. The data presented here show that A-CSE exhibits an antimicrobial activity with the MIC of 200 - 300 µg/ml against *H. pylori* strains 3339 and 26695. Thus it is clear that there are the antibiotic agents in CSE that are active against the important human pathogen of *H. pylori*.

Interestingly, the data of inhibitory effects of the various CSE sources show that C-CSE and ME-CSE exhibit more potent inhibitory effects with MIC of 25 µg/ml and 50 µg/ml against *H. pylori* strains, respectively, compared to an MIC of 200 - 300 µg/ml obtained with A-CSE. It is likely that the critical compounds with this effect are resident in the low polarity fractions of CSE, for instance essential oils, because supercritical CO<sub>2</sub> extraction and methyl ethyl ketone extraction are generally accepted as the standard extraction techniques for obtaining essential oils from herbs (Catchpole and Grey, 1996). In the experiments described above, C-CSE and ME-CSE are the extracts of CSE that were produced by using the techniques above, so their main constituents are likely to be essential oils. Also, among the known constituents of CSE, the essential oils have attracted attention in the past with regarded to bactericidal activities and are used in the treatment of inflammatory conditions (Woods et al, 2001 and Friedman *et al.*, 2002).

In the present studies, the determination of MIC in liquid samples against *H.*

*pylori* has enabled comparison of the effectiveness of different extracts to be clearly differentiated. It has been shown that low concentrations (<1%) of various solvents did not significantly inhibit the growth of the cultures.

## **Chapter III**

### **Isolation, Chemical Characterization and Identification of the Principal Active Component**

### 3.1 Introduction

From the data and observations in the Chapter 2, it was found that the fractions of CSE termed A-CSE, C-CSE and ME-CSE exhibited potent antimicrobial activity against *H. pylori* strains 26695 and 3339; this not being due to the solvents used in solubilizing these fractions. These experiments revealed that the inhibitory effects of C-CSE and ME-CSE against *H. pylori* were greater than those of A-CSE. The critical fluid extraction (or CO<sub>2</sub> extraction) and methyl ethyl ketone extraction techniques used to prepare C-CSE and ME-CSE respectively are conventional procedures used for obtaining essential oils from plant sources (Catchpole and Grey, 1996). These active constituents may thus be components of the essential oil fractions (details in section 2 of chapter 1). In this Chapter, the investigations were aimed at isolating and identifying the components of these celery seed extracts that have antimicrobial effect against *Helicobacter pylori*.

A-CSE was available in substantially larger quantities than the other extracts and so A-CSE was used as starting materials for purifying the active component. To purify the active component, A-CSE was successively fractionated by means of selective solvent extractions and followed by column and liquid chromatography steps, with individual fractions being assayed for inhibitory activity against *H. pylori* at each stage. Analytical thin-layer chromatography (TLC) was used for routine analysis of the purified fractions.

Bacterial strains and culture conditions, and microplate antimicrobial activity assay were employed as described in Chapter 2.

The principal active component was analyzed for its chemical structure using both (a) mass spectrometry (MS) and accurate Q-star MS analysis to determine the molecular weight, molecular formula and main fragment ions, and (b)  $^{13}\text{C}$  and  $^1\text{H}$  NMR.

## 3.2 Methods

### 3.2.1 Bacteria strains, culture condition and CSE source

The strains 3339 and 26695 of *H. pylori* used were same as in Chapter 2, and were grown and showed in the same manner as described above. Briefly, the bacteria were store at  $-80^\circ\text{C}$  in BB plus 5% FBS and cultured on chocolate agar plates and BB plus 5% FBS under microaerobic conditions (10%  $\text{CO}_2$ , 3%  $\text{O}_2$  and 87%  $\text{N}_2$ , at  $37^\circ\text{C}$ ). For *Campylobacter jejuni*, all the culture and testing conditions were same as for *H. pylori*. *Escherichia coli* and *Staphylococcus aureus* (SH1000) were cultivated under aerobic condition at  $37^\circ\text{C}$  in LB broth.

The crude ethanolic extract of celery seed (A-CSE) used was the same as in chapter 2. The known celery seed constituent, sedanolide, was obtained from Sigma, UK.

### 3.2.2 Antimicrobial activity test

Microplate antimicrobial activity assays were used to evaluate the bactericidal activity levels using a 24-well plate growth inhibition assay as described in Chapter 2. The only difference in the conditions that were employed is that iso-propanol was added as the solvent to dissolve the fractions.

### 3.2.3 Organic extraction and separation

A-CSE samples (100g) were partitioned sequentially with organic solvents, comprising 2 L of petroleum ether (Sigma, UK) followed by extraction with 500 ml of water to yield the petroleum ether phase and aqueous phases, respectively. The first aqueous phase (500 ml) was extracted with 500 ml of diethyl ether to yield the diethyl ether phase and final water phase. Assay of the three phases revealed that petroleum ether phase contained the major inhibitory activity against *Helicobacter* (Rainsford and Liu, 2006).

This petroleum ether phase was fractionated using Silica Gel Chromatography (SGC) on a column comprising of 200g (dry weight) of Silica gel 60 (VWR, England), which was loaded into a 5 cm diameter glass column; the column was eluted sequentially with hexane/ethyl acetate (95:5; 7:3 v/v), ethyl acetate (AR) and finally methanol (AR) (Dey and Harborne, 1991). Fractions, 15 ml, were collected. Changes in colour of the fraction were used as a guide to

separation of the A-CSE into its constituent parts. A total of 16 fractions were collected. Analytical thin-layer chromatography (TLC) was then used to determine the purity of the fractions and was performed on precoated fluorescence indication silica gel plates (Plastic backed GF<sub>254</sub>, E. Merck, Damstadt, Germany), using hexane-ethyl acetate (7:3 v/v) as the mobile phase. Spots were located by their absorption under ultraviolet (UV) light (at 254 and 366 nm wavelengths). Following bioassay of these fractions, the 12<sup>th</sup> fraction (SF12) was found to exhibit the most activity.

Further separation on SF12 was performed by using SGC exactly as described as above, except that the column was eluted with hexane/diethyl ether (70:30 v/v). Fractions with spots of the same R<sub>f</sub> values on TLC were combined to give 10 pooled fractions (A-J). The anti-*Helicobacter* assay of these pooled fractions revealed that one of the fractions, hereinafter termed SF12-I, exhibited the most antibacterial activity.

SF12-I was further purified by employing one of two different HPLC methods. In preparative RP-HPLC (Phenomenex, C18, particle diameter 5 µm, column dimensions 250x10 mm), acetonitrile/water (70:30 v/v) was used as the mobile phase, with the flow rate being 5 ml/min. In the analytical HPLC (Phenomenex, SCN, particle diameter 5 µm, column dimensions 250 x 4.6 mm), a mixture of hexane and diethyl ether (70:30 v/v) was used as the mobile phase, being

eluted at a flow rate of 1 ml/min. For these HPLC separations, a Waters 510 system (Waters, Milford, MA) was used and the LC-UV traces were recorded online with a Waters 486 photodiode-array detector and Sp4290 integrator with detection at 212 nm for RP-HPLC and 240 nm for normal phase HPLC.

### 3.2.4 Spectroscopic methods

1-D nuclear magnetic resonance (NMR) spectra were recorded in  $\text{CDCl}_3$  at room temperature on a Bruker Unity Ac 250MHz NMR spectrometer ( $^1\text{H}$ , 250MHz;  $^{13}\text{C}$ , 62.9 MHz) in the BMRC of Sheffield Hallam University (by Mrs. J Hague). 2-D NMR spectra were recorded in  $\text{CDCl}_3$  at RT on a Bruker Unity instrument (Ac 500 MHz) ( $^1\text{H}$ , 500MHz;  $^{13}\text{C}$ , 125.8 MHz) in the Department of Chemistry at Sheffield University (by Dr. B. Taylor).

Mass spectra of the final purified fraction (later referred to as celery seed anti-Helicobacter, CAH) were obtained on several instruments. Mass spectra were determined using a VG 70/70 Sector Mass Spectrometer (Micromass, Manchester, UK) in the BMRC at Sheffield Hallam University.

Accurate mass measurements to determine the molecular formula of CAH were made for electrospray mass spectrum by using an API Q-star mass spectrometer (Applied Biosystems, Foster, USA). A LCQ ion trap (Thermo Scientific, Waltham, USA) was made for tandem MS studies by using APCI in



the BMRC as well. A Kratos Concept ISQ for electron ionization spectrum was performed by Dr. Noel Davies at the University of Tasmania. HPLC-UV data were obtained on a Waters Alliance 2690 with 996 diode array detector.

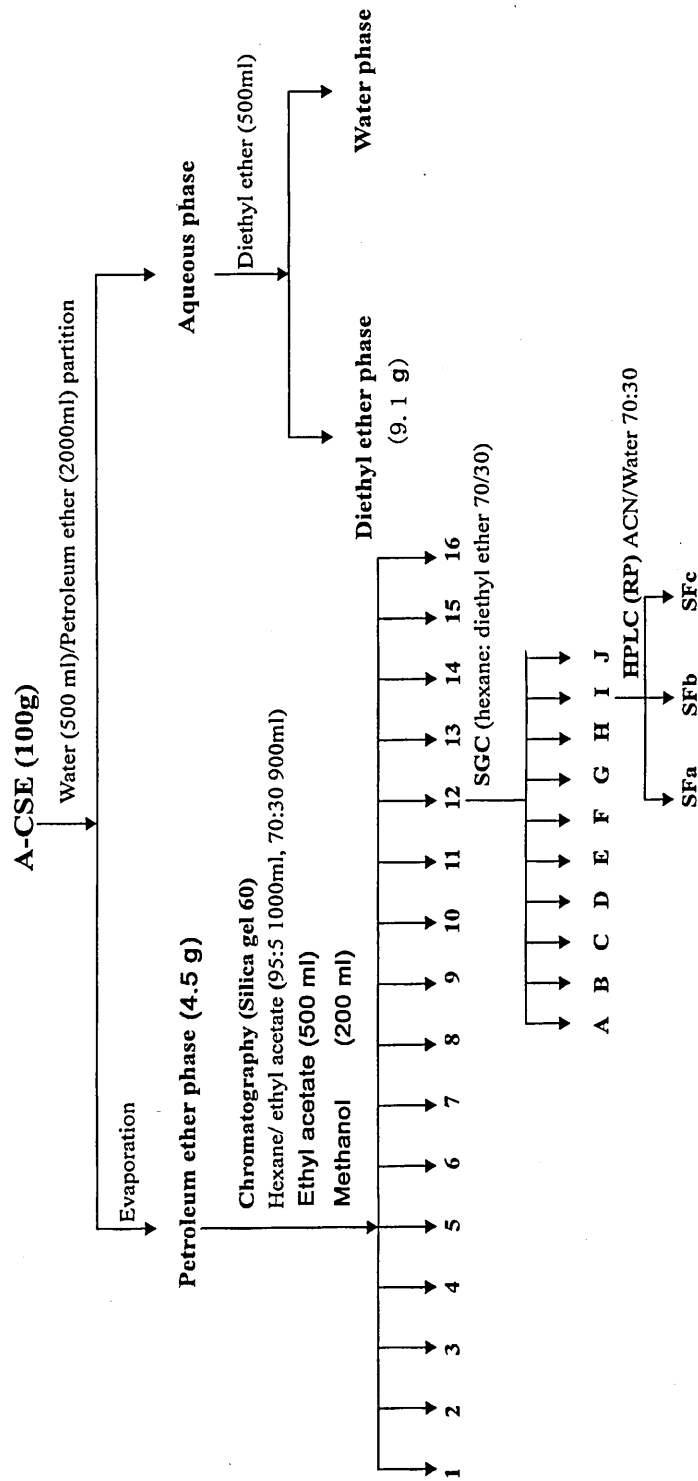
### 3.3 Results

#### 3.3.1 Antibacterial activity of celery seed extracts and purification of the active components

As outlined in the scheme shown in **Fig. 3 –1**, A-CSE (100 g) was mixed in 500 ml of water and partitioned sequentially with petroleum ether and diethyl ether as described in the method section. After evaporation of the solvent, the petroleum ether fraction was obtained, which gave the appearance of a pale yellow oil with a distinct celery odour. In contrast, the diethyl ether fraction had a dark green appearance, which was a viscous liquid having a light celery odour. The amounts of the evaporated petroleum ether fraction, diethyl ether fraction and water fraction were 4.5 g, 9.1 g and 86 g, respectively. All fractions (petroleum ether fraction, diethyl ether fraction and water fraction) were tested for antibacterial activity against *H. pylori* strain 3339 and compared to the activity of the original A-CSE.

As shown in **Fig.3 – 2a**, the inhibitory activity appeared predominantly in petroleum ether fraction (MIC=25 µg/ml) as compared to the diethyl ether fraction (MIC=50 µg/ml) and aqueous fraction (MIC>500 µg/ml) at 5 days. The

**Fig. 3-1 Fractionation of A-CSE**

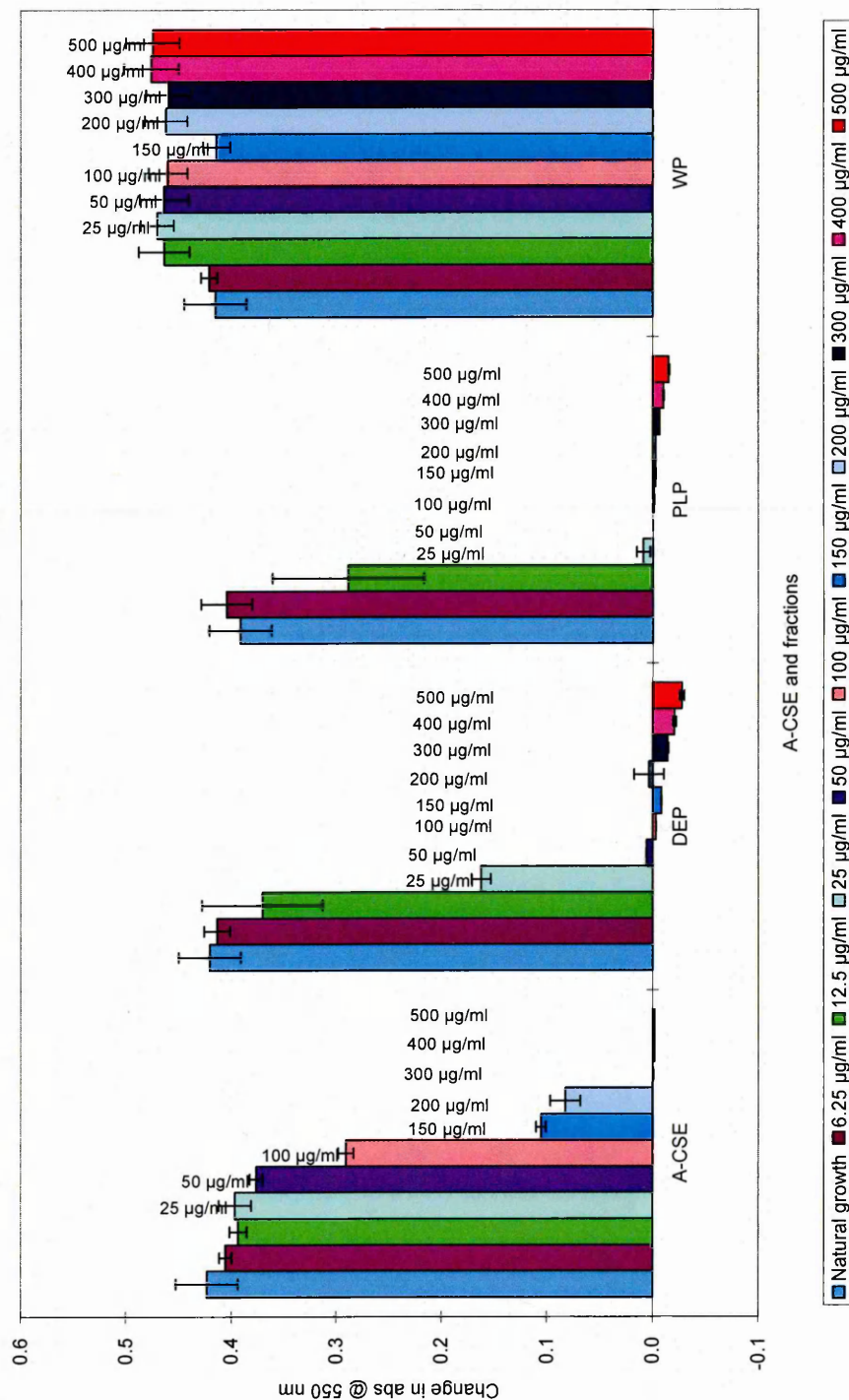


MIC: SF6: 25 µg/ml, SF12:12.5 µg/ml; SF12-I: 6.25 µg/ml; SFc: 3.15 µg/ml

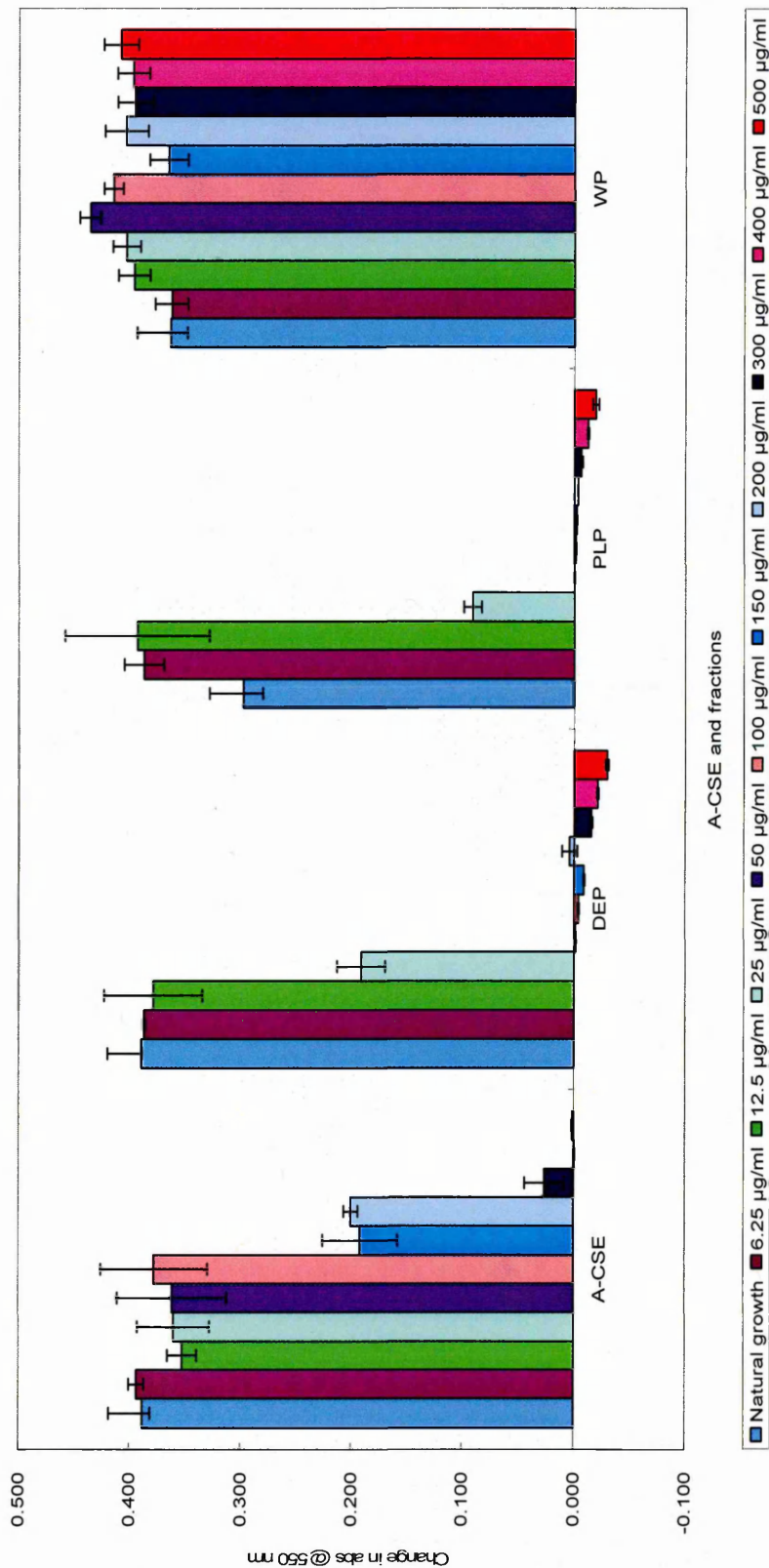
Among the constituents of A-CSE, the petroleum ether phase exhibited the anti-*H. pylori* activity. Among the subfractions obtained by chromatography of the petroleum ether phase, the subfraction 12 has the most activity. After further separation of SF12, the most activity was observed in SF12-i. SF12-i was further purified to obtain 3 new subfractions (SFa, SFb and SFc). SFc was a pure component and has the most inhibitory effect against *H. pylori*.

**Fig. 3-2 Inhibitory effect of fractions of CSE against *H. pylori* 3339**

**A. Inhibitory effect after 5 days**



## B Inhibitory effect after 7 days



The growth of *H. pylori* treated with CSE or petroleum ether phase (PEP) or diethyl ether phase (DEP) resulted in a significant decrease ( $P \leq 0.01$ ) from pre-treatment levels in  $OD_{550}$  after (a) 5 days and (b) 7 days. PEP pronounced the most antimicrobial active. The water phase (WP) had no significant effect on the growth of *H. pylori* at up to 500 µg/ml of concentration. Results are the means of 5 independent replications; error bars show SD.

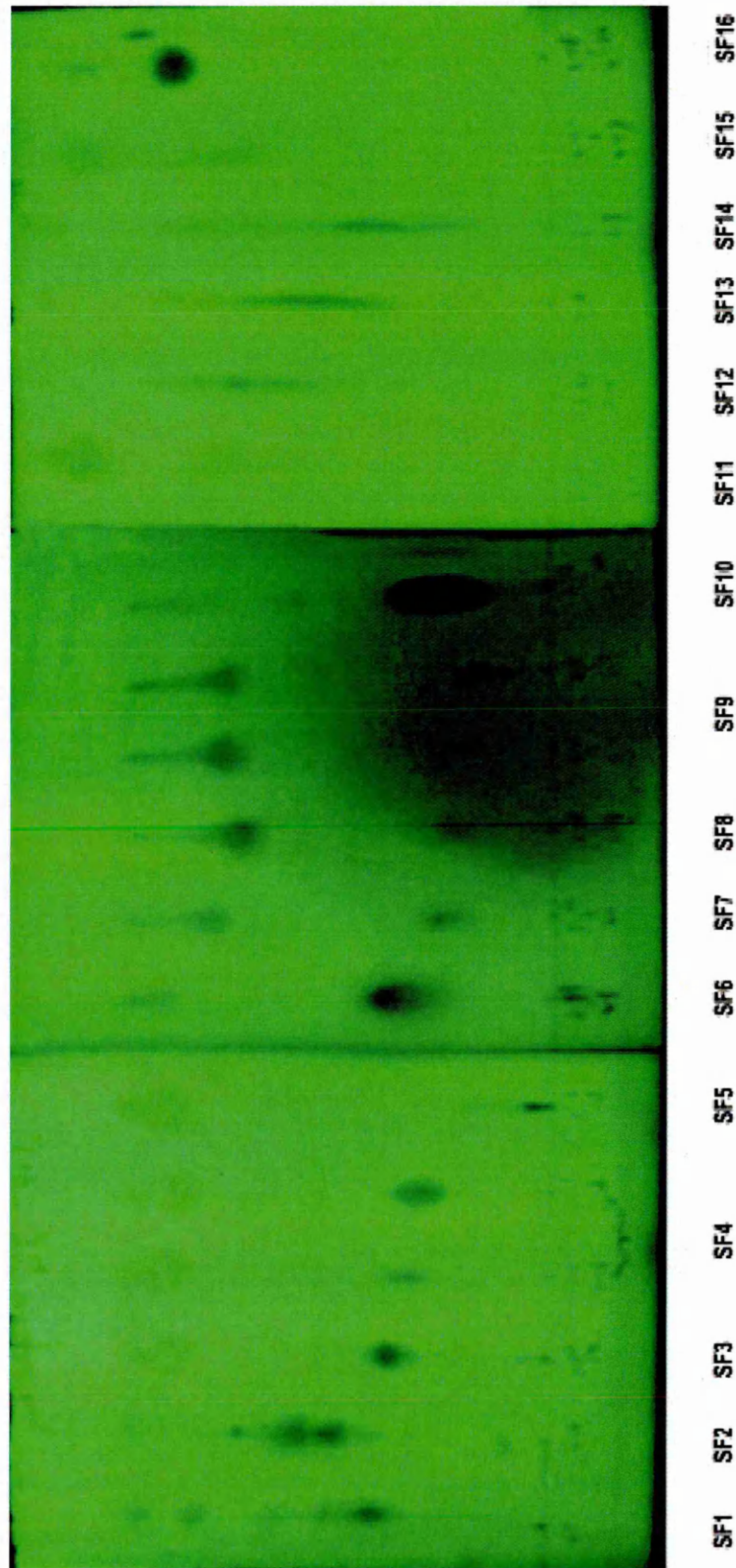
MIC of the petroleum ether fraction and the diethyl ether fraction after 7 days incubation was unchanged compared with that at day 5 (**Fig.3 – 2b**). As observed previously (Chapter 2), the MIC of A-CSE increased from 200 µg/ml at day 5 to 400 µg/ml at day 7 (**Fig.3 – 2b**). Therefore, among these fractions above, the most active against *H. pylori* was the petroleum ether fraction (MIC = 25 µg/ml).

This petroleum ether phase containing greater inhibitory effects was then subjected to further fractionation via column chromatography on a Silica gel 60 (SGC), eluted with hexane-ethyl acetate (95:5, 1000ml), hexane-ether acetate (70:30, 900ml), pure ether acetate (500ml) and finally pure methanol (200ml) as the mobile phases (Fig. 3-1). Fractions with spots with the same R<sub>F</sub>s on TLC analysis were combined to yield 16 major subfractions 1-16 (SF1-16) (**Fig. 3-3**).

Each subfraction was tested for effects against *H. pylori* strain 3339 by antimicrobial activity assay and the MICs were determined (**Fig. 3-4**). The MIC values of the different subfractions are summarized in **Table 3-1**. The most pronounced antimicrobial activity resided in the SF12 fraction (MIC=12.5 µg/ml). There were minor peaks of antimicrobial activity in the SF6 and SF11 fraction (MIC=25 µg/ml). Of the remaining subfractions, the values of their MIC were ≥ 50 µg/ml. The fraction, SF12 had the appearance of a green oil with a

**Fig. 3-3 Examination of TLC on sub-fractions of petroleum ether phase**

TLC performed on  $10 \times 10 \text{ cm}^2$  of plates, using hexane and diethyl ether (95:5) as the TLC solvent. All collections were combined to give 16 subfractions with spots of RF in each pooled fraction.

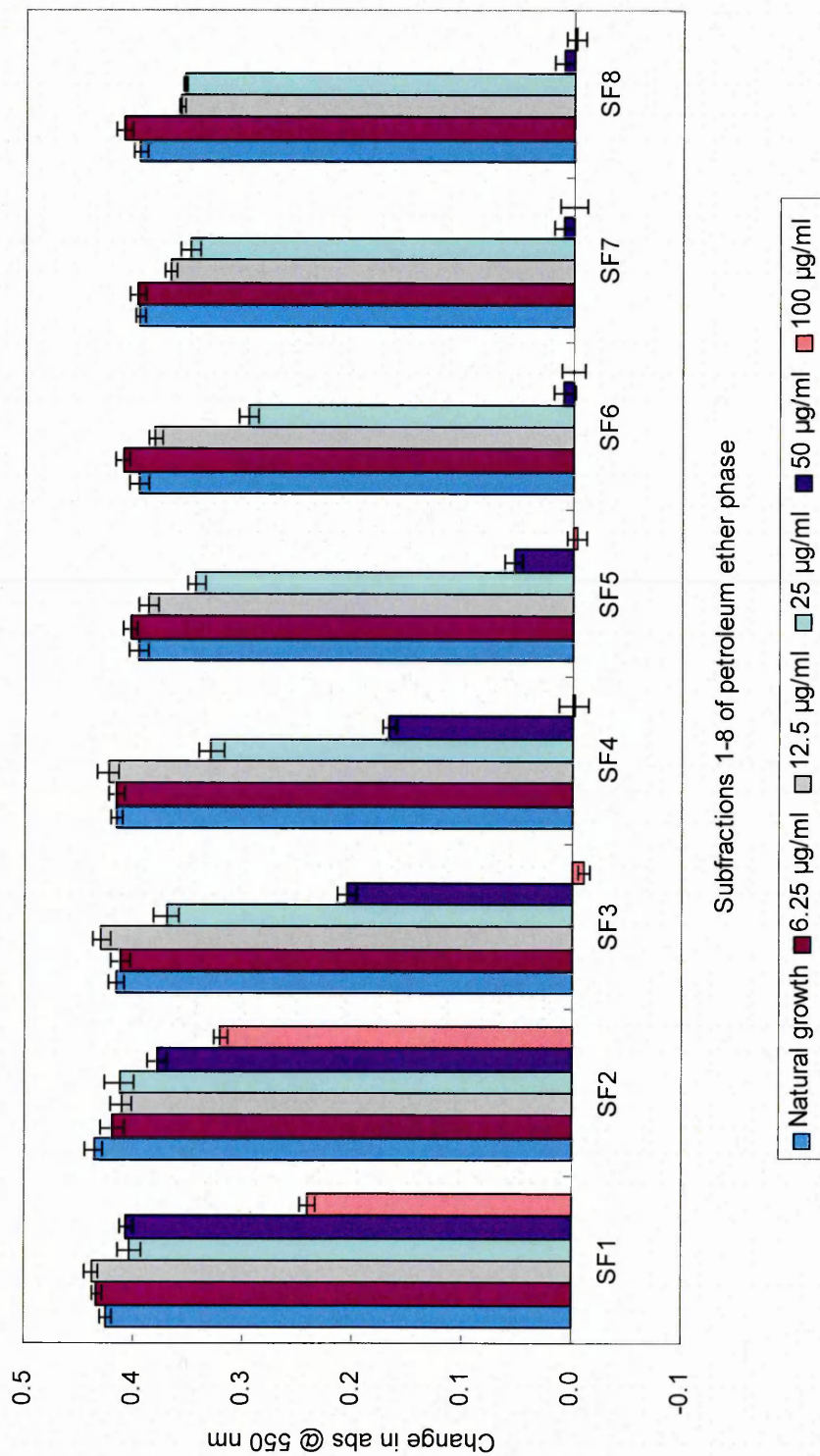




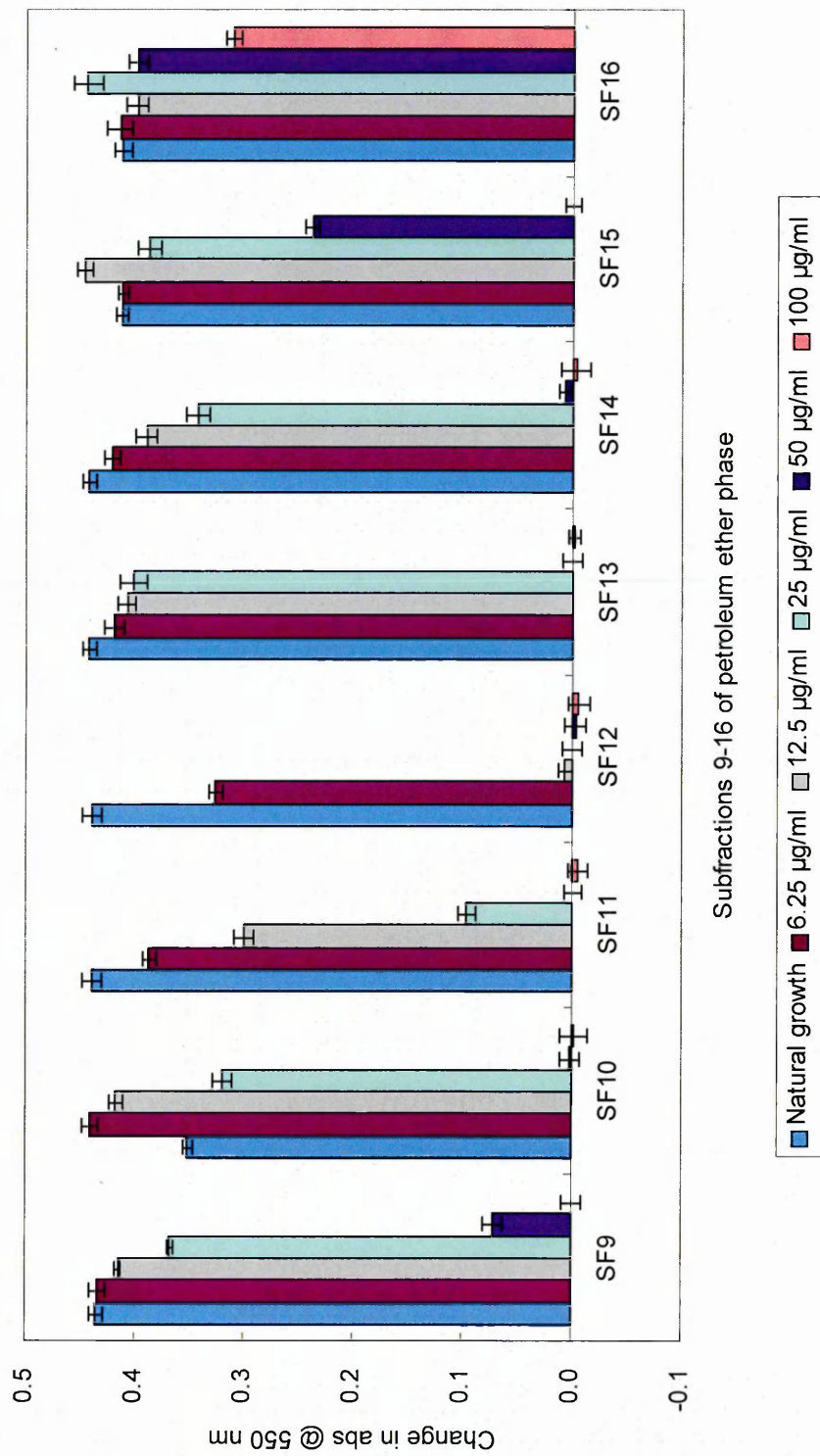
**Fig. 3-4 Inhibitory effects of subfractions of petroleum ether phase against *H. pylori* 3339**

The inhibitory effects against *H. pylori* 3339 are shown below as the mean of 5 independent replications after (a) 5 days and (b) 7 days.

**A. After 5 days**

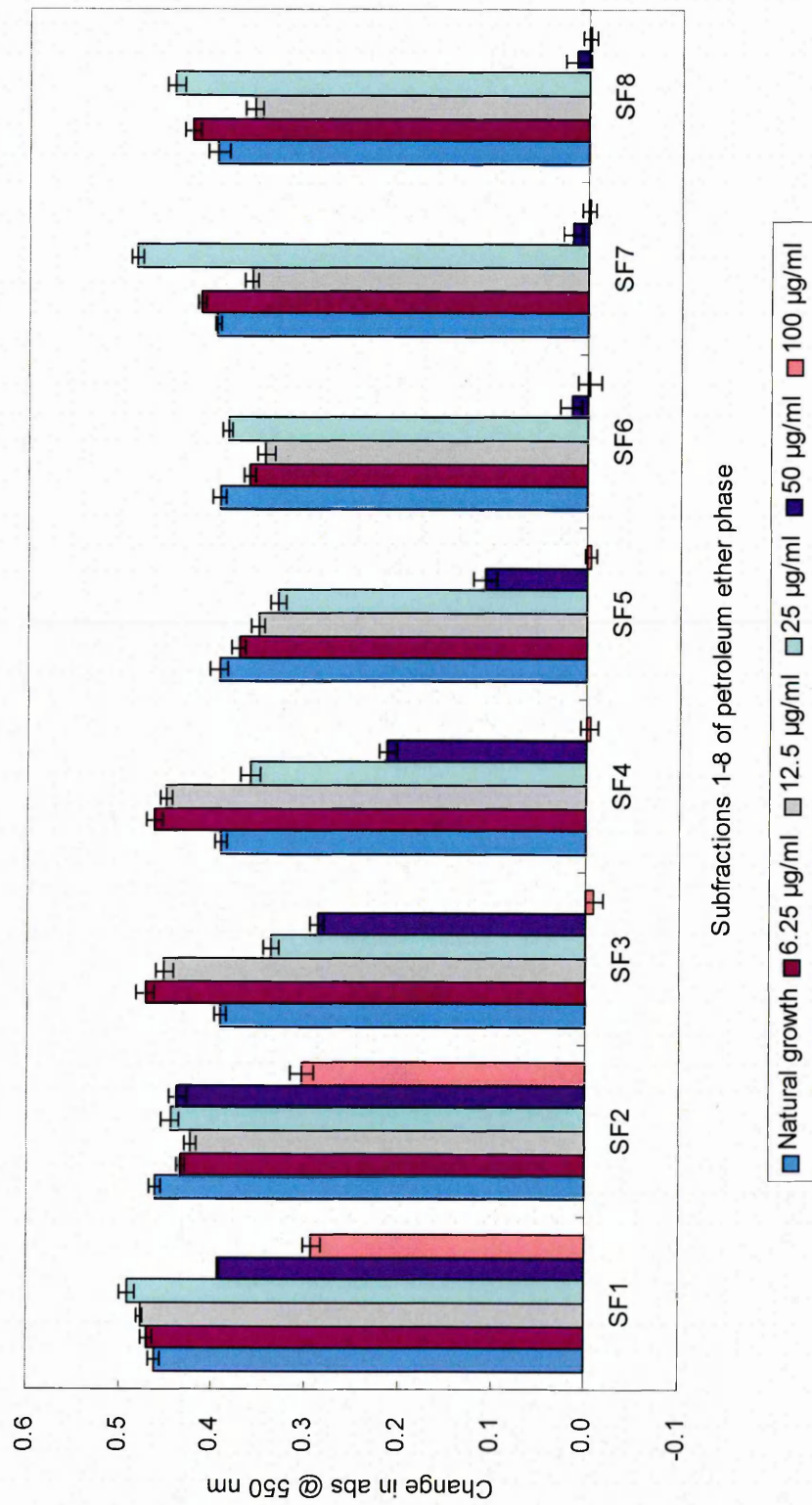


Continued

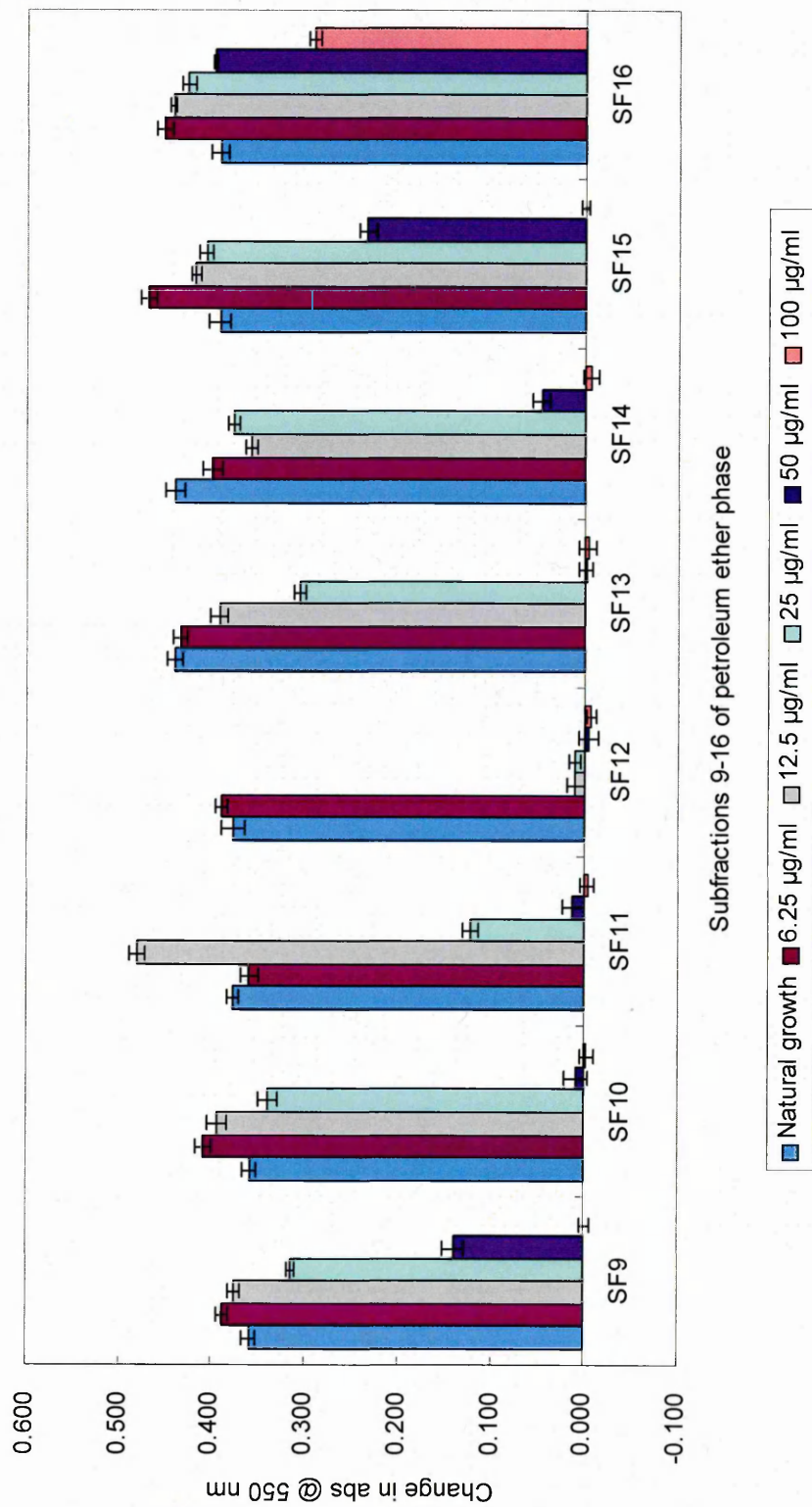




# **B. After 7 days**



Continued



**Table 3-1 Summary of MICs of subfraction 1-16**

<b>Subfractions</b>	<b>MIC (µg/ml)</b>	<b>Subfractions</b>	<b>MIC (µg/ml)</b>
<b>SF1</b>	<b>≥100</b>	<b>SF9</b>	<b>≥100</b>
<b>SF2</b>	<b>≥100</b>	<b>SF10</b>	<b>≥100</b>
<b>SF3</b>	<b>≥100</b>	<b>SF11</b>	<b>25</b>
<b>SF4</b>	<b>≥100</b>	<b>SF12</b>	<b>12.5</b>
<b>SF5</b>	<b>≥100</b>	<b>SF13</b>	<b>50</b>
<b>SF6</b>	<b>25</b>	<b>SF14</b>	<b>≥100</b>
<b>SF7</b>	<b>50</b>	<b>SF15</b>	<b>≥100</b>
<b>SF8</b>	<b>≥100</b>	<b>SF16</b>	<b>≥100</b>

slight celery odor, compared to SF6, which was a light yellow oil with a distinct celery odor.

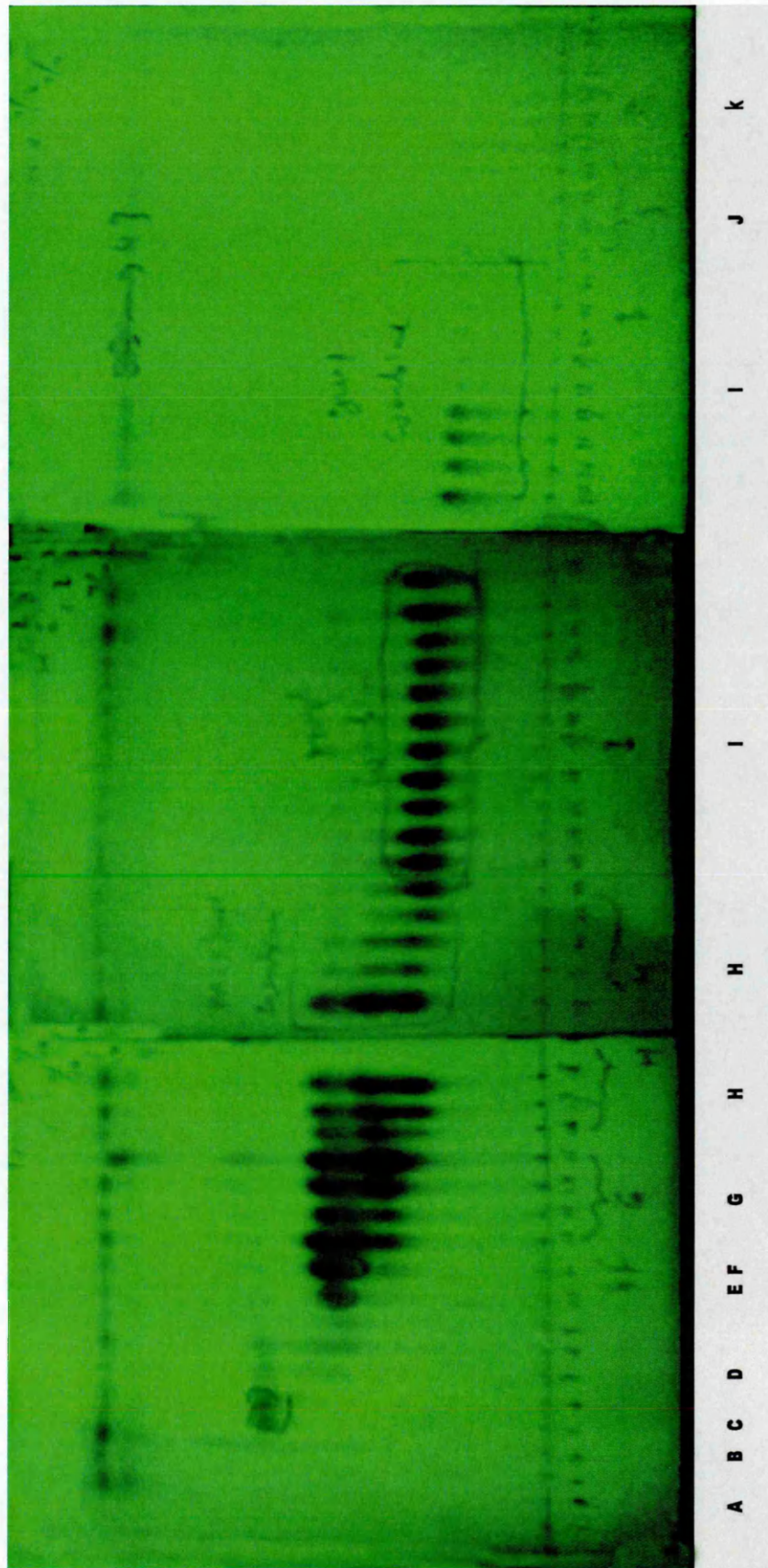
SF12 was then subjected to column chromatography on a silica gel 60 column as above, using hexane: diethyl ether (70:30, 800ml) as the mobile phase. Fractions with the same TLC patterns were combined to yield 10 major subfractions A-J (SF12a-j) (**Fig. 3-5**). After antimicrobial assays of these fractions, the most pronounced inhibitory activity resided in the SF12-i (MIC=6.25 µg/ml) (**Fig. 3-6**). In appearance, SF12-i was a green viscous oil with a celery odor, similar to the original SF12.

The nonvolatile material in SF12-i was dissolved in propan-2-ol or DMSO to a final concentration at 100 mg/ml and then further purified via two separated methods, analytical SCN HPLC and semi-preparative RP-HPLC, as described in the above methods section.

In the analytical SCN HPLC, SF12-i was dissolved in DMSO and eluted with hexane and diethyl ether (70:30) as mobile phase at a flow rate of 1 ml / min. As shown in **Fig. 3-7**, the first main peak of absorbance, referred to as SFa, appeared at 13.19 min, and was a single unresolved peak. The second peak of absorbance, termed as SFb, appeared at 14-16 min and consisted of the two admixed peaks. The last main peak, SFc, appeared at 17.81 min and was a

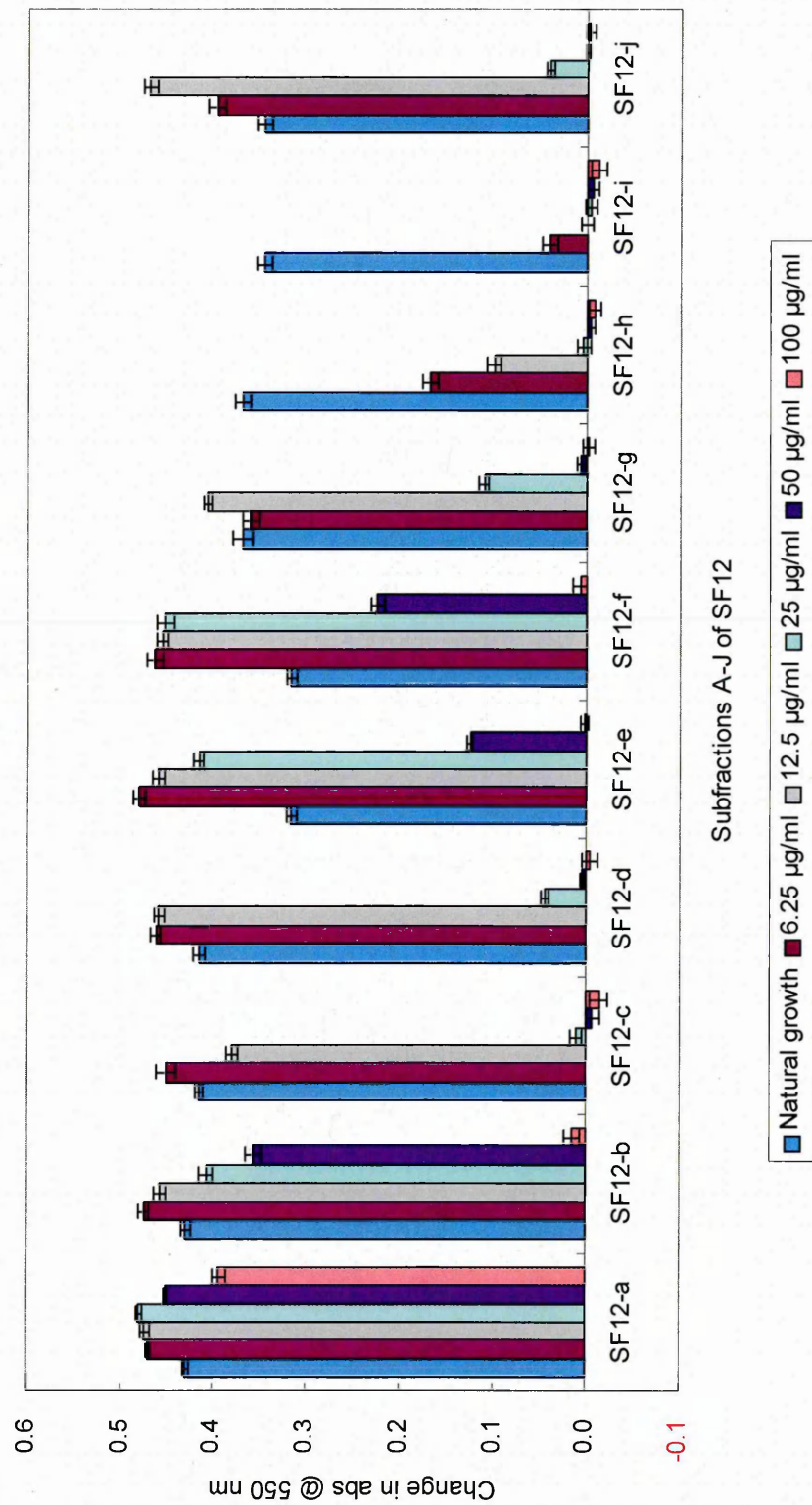
**Fig. 3-5 Examination of TLC on the 12<sup>th</sup> sub-fractions**

TLC performed on  $10 \times 10 \text{ cm}^2$  of plates, using hexane: diethyl ether (95:5) as the TLC solvent. All collections were combined to give 10 subfractions with spots of the same RF in each pooled fraction.



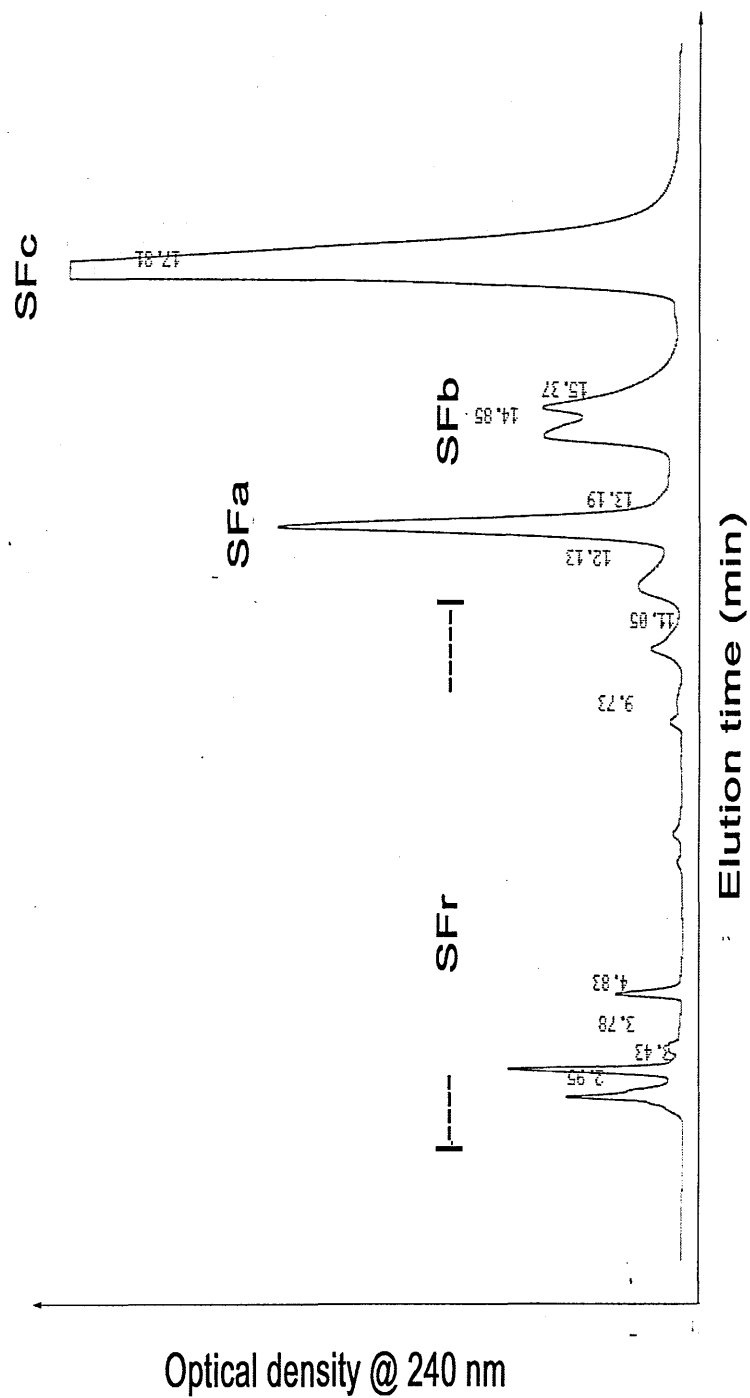


**Fig. 3-6 Inhibitory effects of subfractions of SF12 against *H. pylori***



The inhibitory effects of subfractions of SF12 against *H. pylori* are shown above after 5 days incubation. Results are the means of 5 independent replications; error bars show SD.

**Fig. 3-7. Absorbance trace of sample SF12-I in SCN HPLC system.**



The four major fractions collected are indicated. System conditions: SCN column. Mobile phase hexane/diethyl ether. Flow rate: 1ml/min.

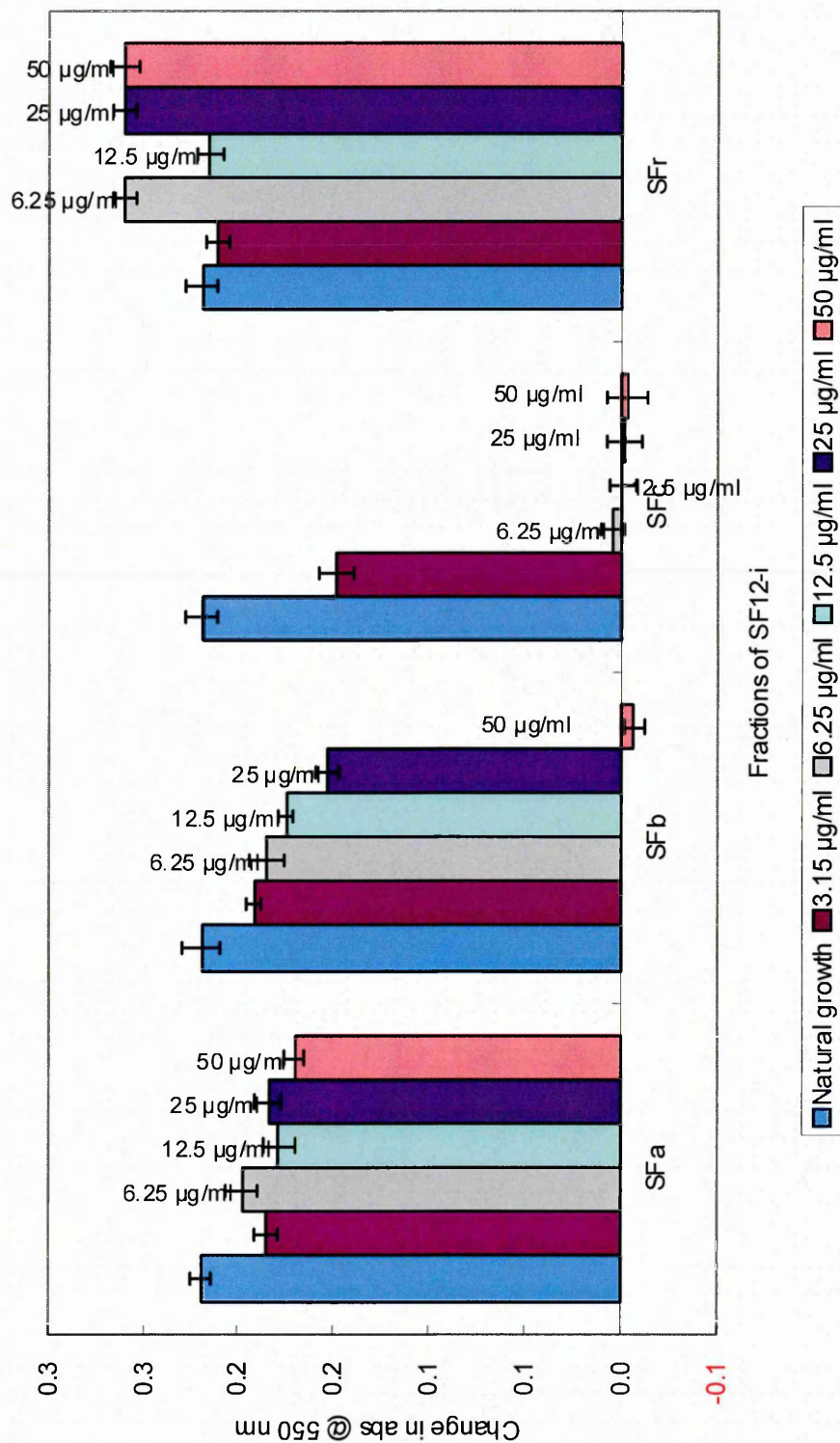
single high peak. To avoid missing any of the components, the large numbers of small peaks that appeared between 2 min and 13 min were collected, and pooled to give a fraction termed SFr. After evaporation of the solvent, each of the four SCN HPLC fractions was evaluated for antibiotic activity against *H. pylori*. The results showed that the MICs of SFa, SFb, SFc and SFr were >50, 25, 3.15 and >50 µg/ml, respectively (**Fig. 3-8**). SFc thus contained the highest concentration of bioactivity.

In order to obtain a suitable large quantity of the purified bioactive component subfraction C (SFc), a semi-preparative RP-HPLC system was used for subsequent isolation, and was eluted with acetonitrile/water (75:25) as the mobile phase. Firstly, SFc from SCN column was loaded onto the RP-HPLC column to estimate its elution time and purity. The result of the RP-HPLC UV recording indicated that SFc still appeared as a single high peak at about 22.92 minutes of elution time under these conditions (**Fig. 3-9**).

A new aliquot, SF12-i from the second SGC (**Fig. 3-1**) was then separated on the same RP-HPLC system using the same conditions, except that the flow rate was decreased to 6.0 ml/min to improve the separation. The HPLC profile and anti-*H. pylori* activity of the fractions were used to determinate the position of the active fraction SFc under these new chromatographic conditions. In the RP-HPLC trace of SF12-i, there were three main single peaks that appeared at

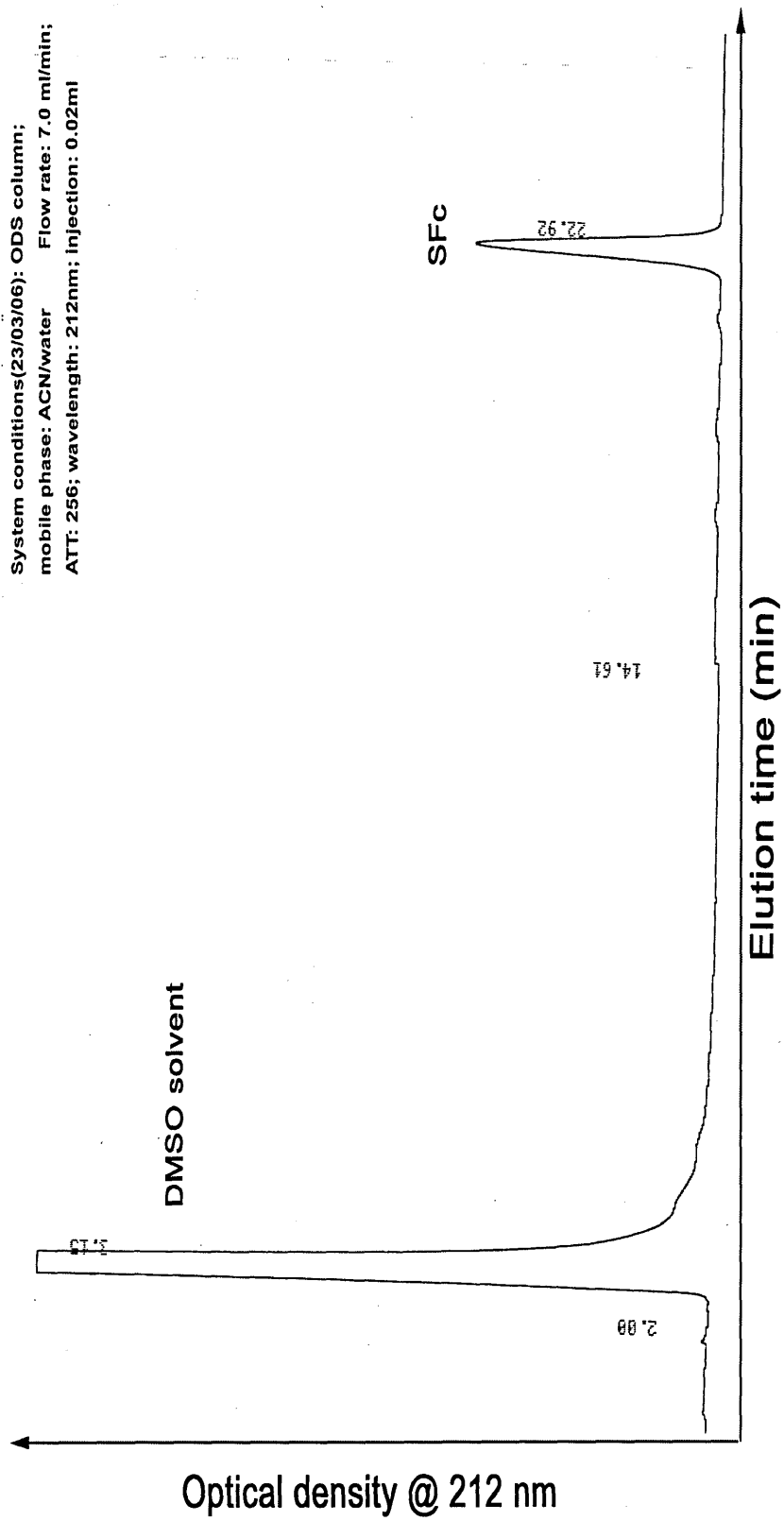


**Fig. 3-8 Inhibitory effects of SFa,b,c,r against *H. pylori***



The inhibitory effects against *H. pylori* are shown above after 5 days incubation. Results are the means of 5 independent replications; error bars show SD.

Fig. 3-9 Absorbance trace of SFc in RP-HPLC system



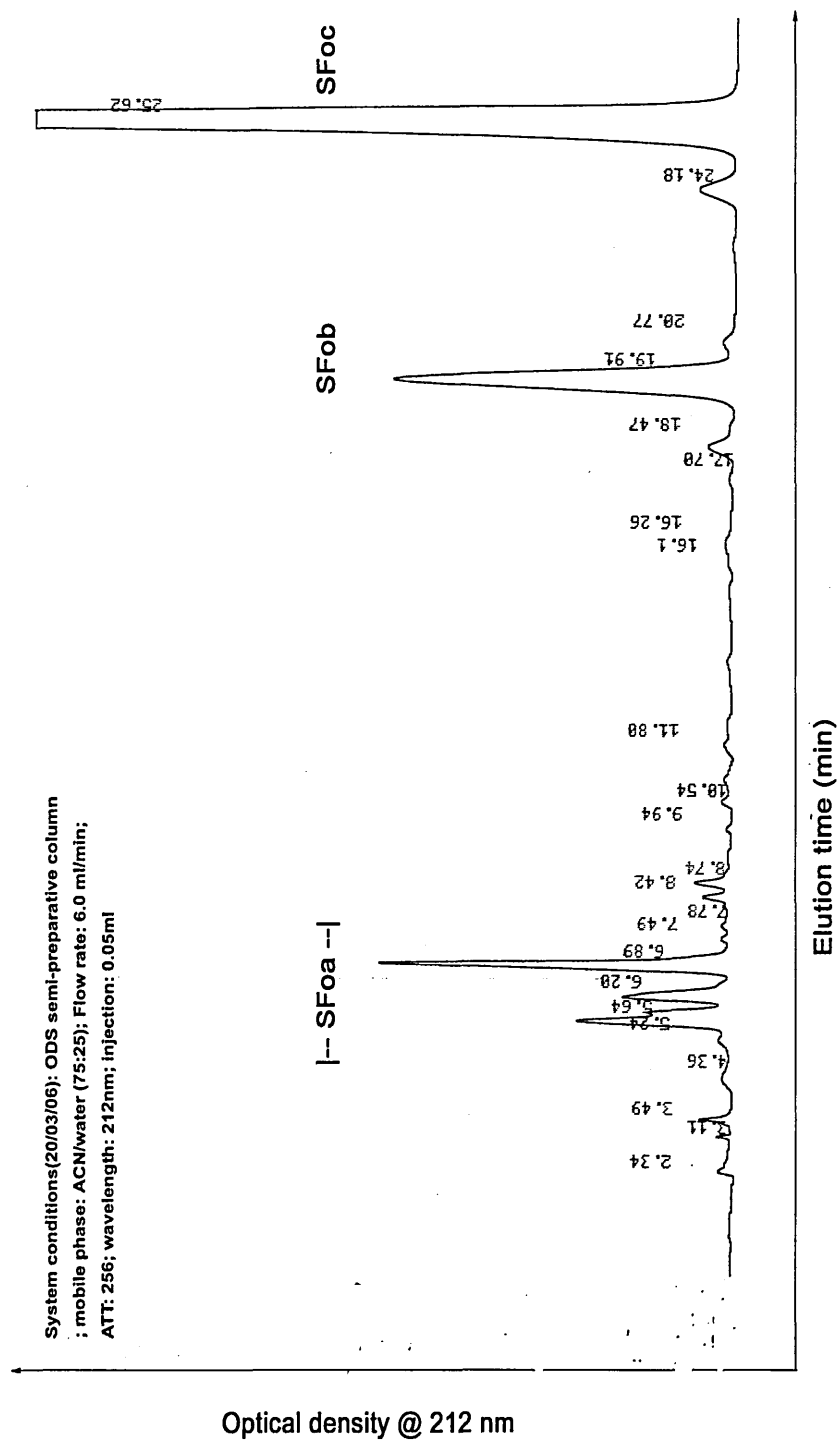
SFc was dissolved into DMSO for injection. The major peaks and system conditions are indicated

6.98, 19.91 and 23.62 min, respectively, which were designated as SFoa; SFob and SFoc as obtained from ODS column (different from the previous SCN column) in **Fig. 3-10**. Compared with the elution time of SFc in the same system (ET=22.92 min) (**Fig. 3-9**), the last single high peak (SFoc in **Fig. 3-10**) at 23.62 min is the most likely to have same component as SFc obtained previously, taking into account the slight decrease in flow rate. Also, both SFc and SFoc gave single spots with the same RF on TLC analysis, using hexane: diethyl ether (70:30) as the TLC solvent (data not shown).

For comparison of bioactivity, the eluted material was collected as three fractions from ODS column: SFoa was a pool of at least 3 unresolved peaks, whereas fractions SFob and SFoc were each well resolved peaks (**Fig. 3-10**). Each of the three RP-HPLC fractions was evaluated for antibiotic activity against *H. pylori*. The results showed that the MIC of SFoa, SFob and SFoc were 50, 12.5 and 3.15 µg/ml, respectively (**Fig. 3-11**). The iso-propanol solvent employed in solubilizing these fractions did not affect the viability of the bacterial cells. Therefore, the inhibitory effect of SFoc against *H. pylori* is probably identical to that of SFc and they contain same components as shown by TLC and HPLC traces.

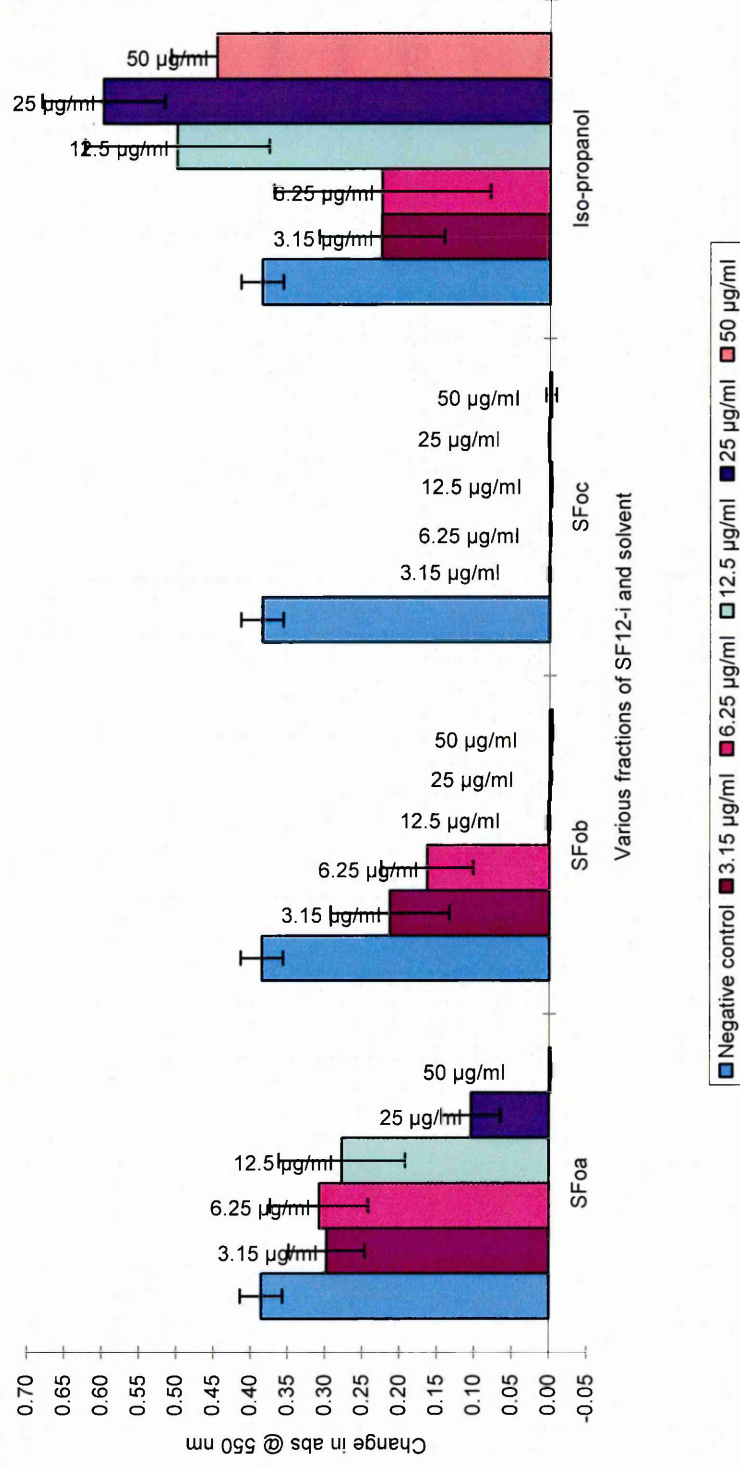
The fraction with the most pronounced antimicrobial activity was SFoc or SFc, which was a colorless and almost odourless oil, and its pure active component

**Fig. 3-10 Absorbance trace of SF12-i in RP-HPLC system.**



SF12-i was dissolved into iso-propanol for injection. The major peaks and system conditions are indicated.

**Fig. 3-11 Inhibitory effects of SFoa, SFob and SFoc against *H. pylori***



The inhibitory effects of SFoa, SFob and SFoc against *H. pylori* after five days' incubation. Results are the means of 5 independent replications; error bars show SD. Natural growth is the culture without adding antimicrobial agent.

was formed CAH (for celery seed anti-*Helicobacter* fraction). It was noticeable that the MIC of this most active fraction compared favourably to that of the known antibiotic, tetracycline, which was found to have inhibitory effects at 3-5 µg/ml in parallel experiments (Chapter 2, result section).

### 3.3.2 Effects of CAH against other bacteria

In antimicrobial assay against *Campylobacter jejuni*, there was no significant inhibitory effect of CAH after 48 hr, at concentration up to 50 µg/ml. In contrast, the parallel MIC of positive control, tetracycline was 3.15 µg/ml against *C. jejuni* (Fig. 3-12).

The antimicrobial assays of CAH with *Escherichia coli* and *Staphylococcus aureus* were performed under aerobic conditions. As shown in Fig. 3-12, MICs of CAH against both bacteria were more than 50 µg/ml. In contrast, both positive controls showed strong inhibitory effects (MIC of ampicillin against *S. aureus* = 3.15 µg/ml, MIC of tetracycline against *E. coli* = 12.5 µg/ml).

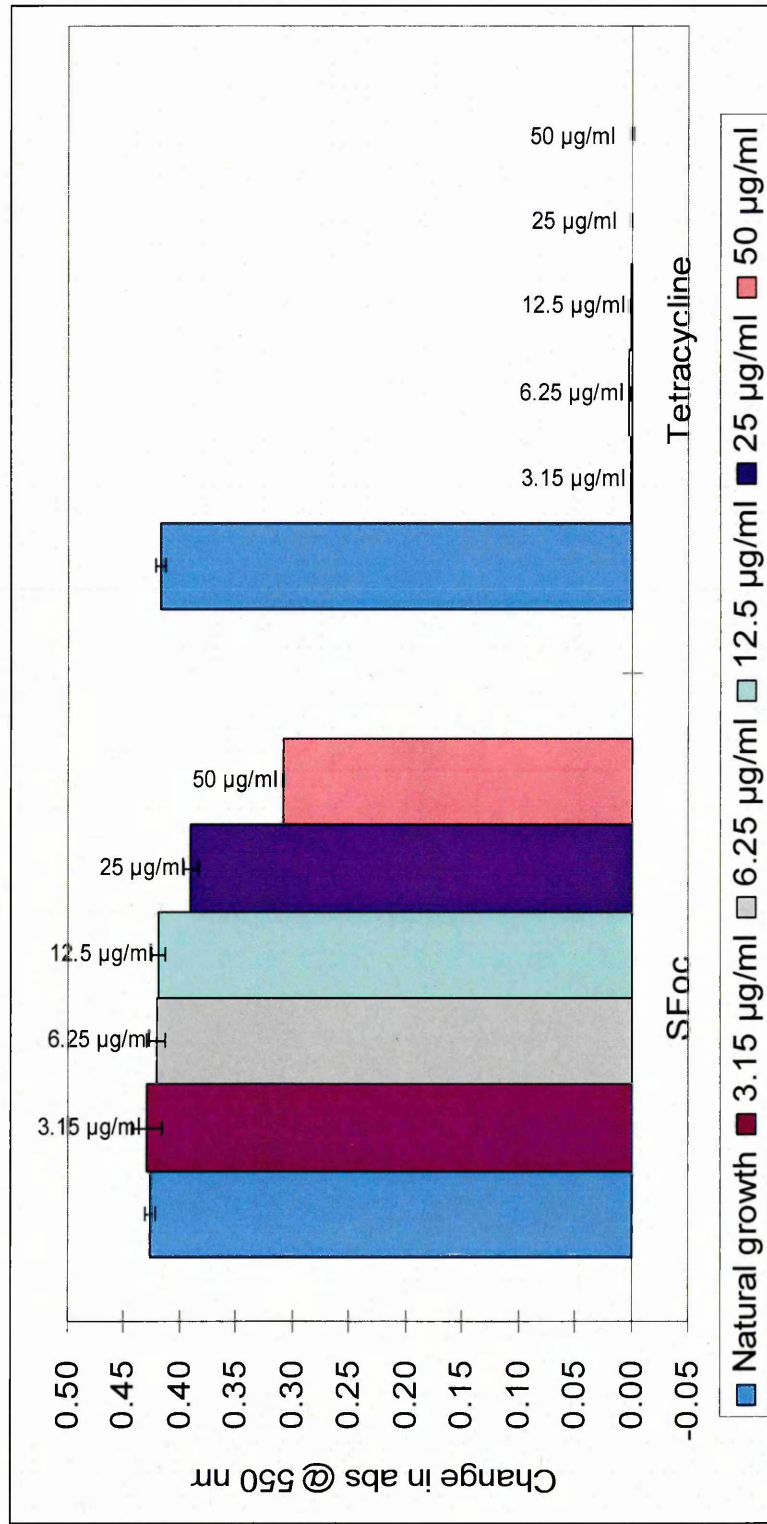
### 3.3.3 Bioactivity of a known chemical in celery seed, sedanolide

Well known component of celery seed, sedanolide, was tested for inhibitory effect against *H. pylori* strain 3339. The results indicated that MIC of sedanolide was 50 µg/ml during a 5 day assay; the MIC of tetracycline in a parallel positive control was < 6.25 µg/ml (i.e. same as in previous experiments

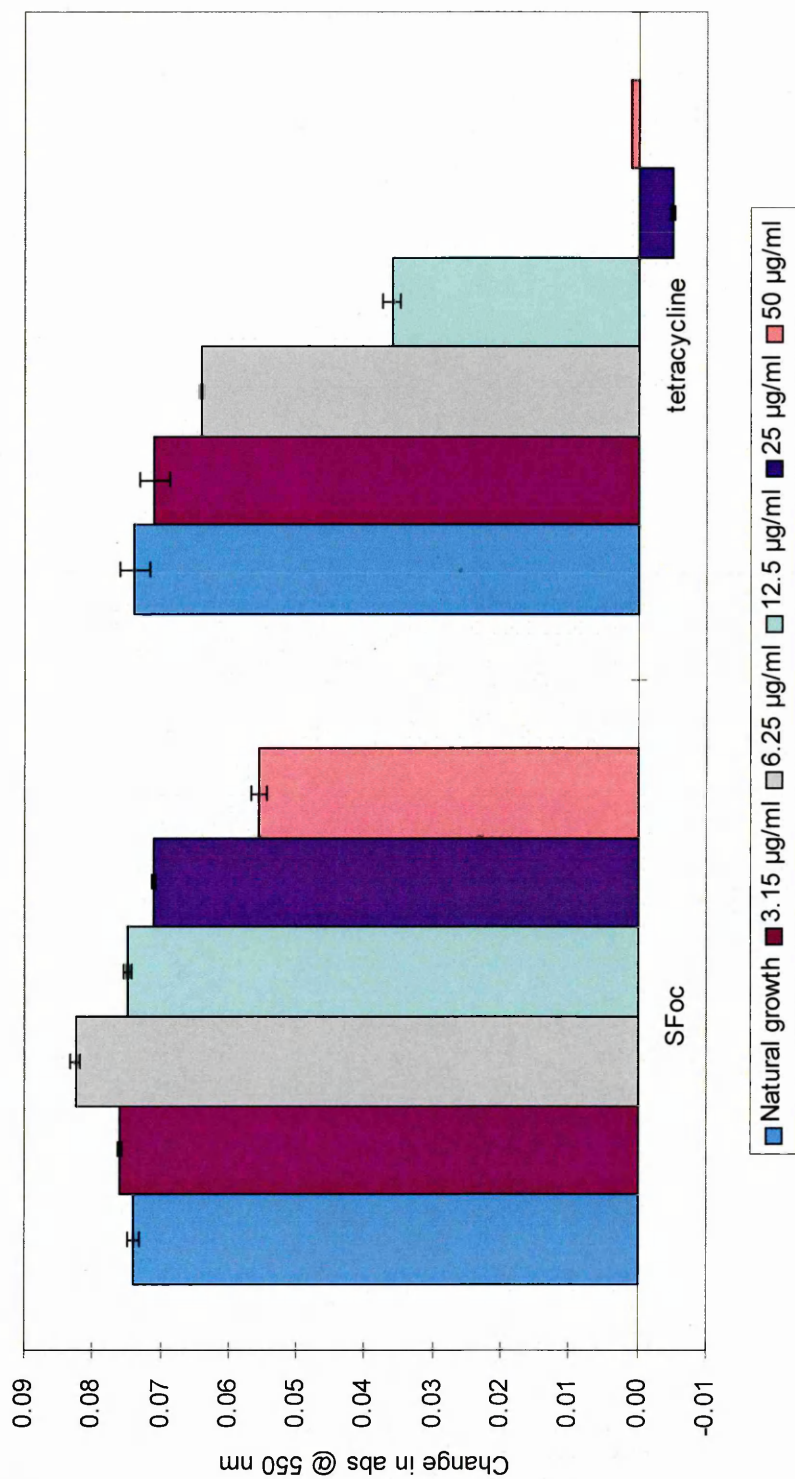
**Fig. 3-12 Effects of CAH against other bacteria**

The inhibitory effects are shown below in mean of 5 independent replications (mean  $\pm$  SD). A: Effect against *Campylobacter jejuni* after 48 hr; B: Effect against *Escherichia coli* after 2 hr; C: Effect against *Staphylococcus aureus* after 24 hr.

A: Effect against *Campylobacter jejuni*

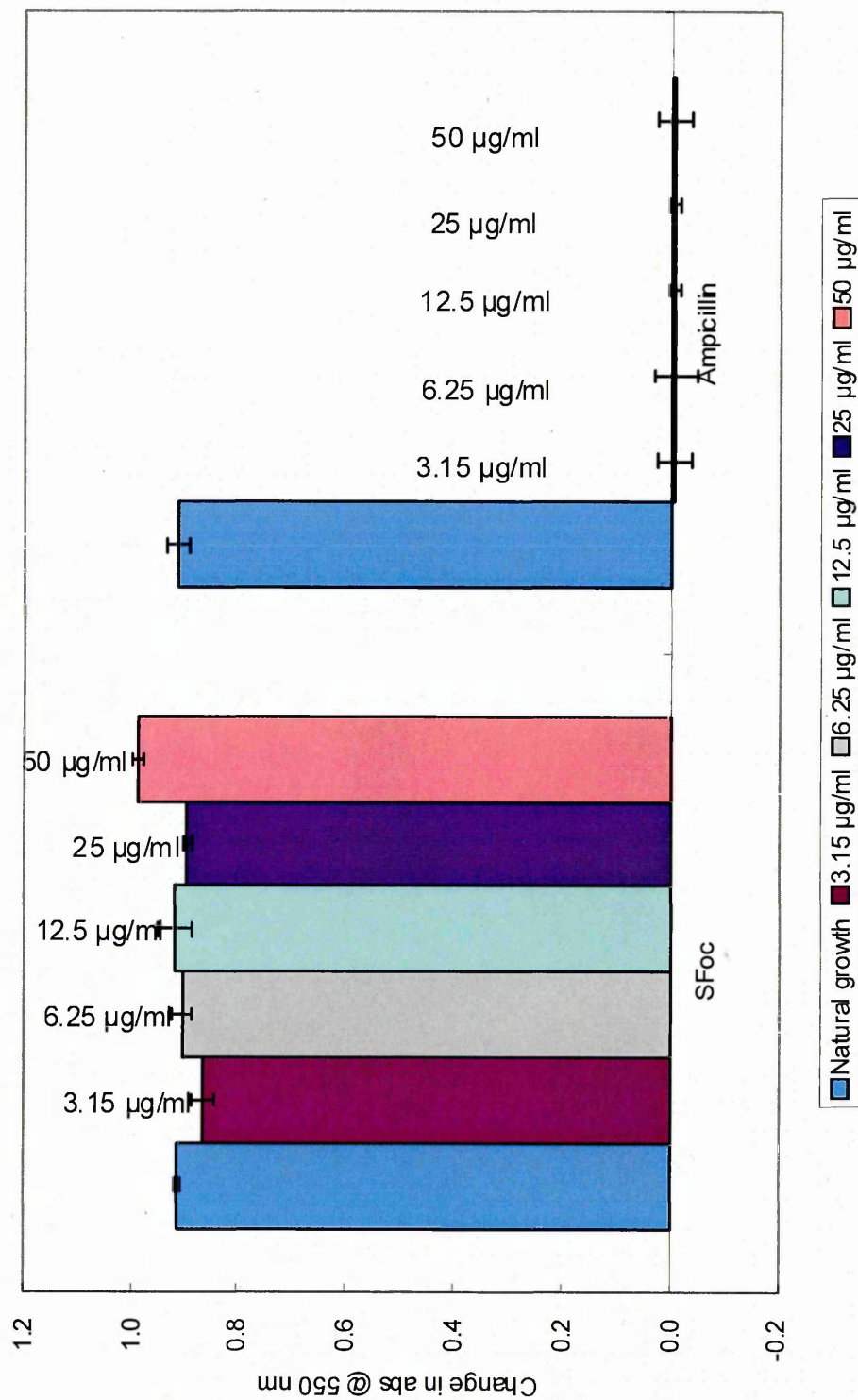


B: Effect against *Escherichia coli* (continued)





C: Effect against *Staphylococcus aureus* (continued)



) (Fig. 3-13). Hence, sedanolide had a significant inhibitory effect against *H. pylori*, but was not active enough to account for the activity of CAH (Rainsford and Liu, 2006).

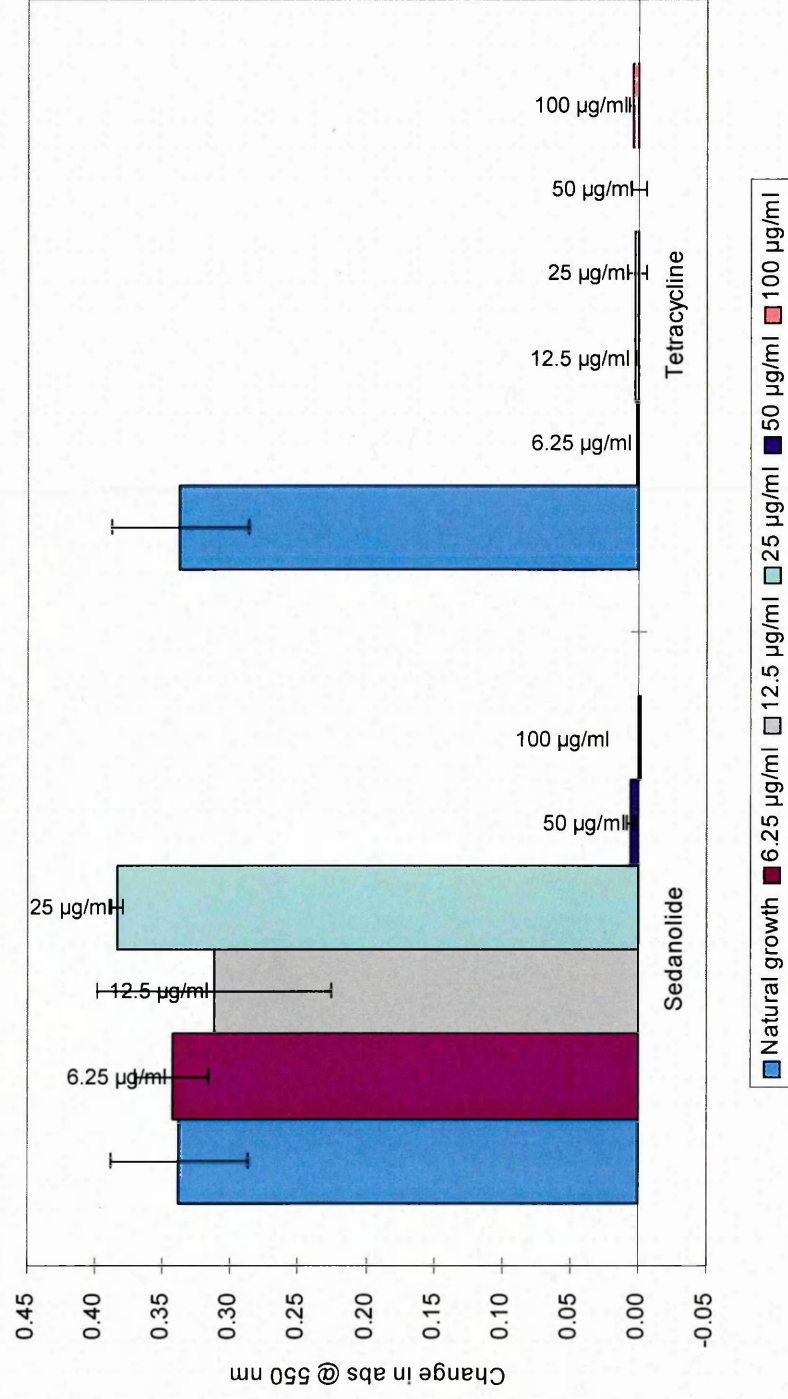
#### 3.3.4 Chemical characterization of the highly anti- *H. pylori* major fraction CAH

The electrospray mass spectrum of CAH showed ions at  $m/z$  385.23, 407.23 and 423.20 (Fig. 3-14). These ions represent  $[M+H]^+$ ,  $[M+Na]^+$  and  $[M+K]^+$ , respectively for a compound of molecular weight 384. This was subsequently confirmed by electron ionisation which showed a weak  $M^+$  ion at  $m/z$  384 (1%) and prominent fragment ions at  $m/z$  193 (100%), 192 (80%) and 107 (55%) (Fig. 3-15). The molecular ion was measured as 384.22943 by peak matching, giving the empirical formula as  $C_{24}H_{32}O_4$  (within 1.6ppm of the calculated value). Tandem MS studies on an ion trap mass spectrometer showed the prominent product ion from the  $[M+H]^+$  ion at 385 to be a water loss to  $m/z$  367, and the major products of this ion were at  $m/z$  349, 321, 193, 175 and 147. The UV absorbance maximum of the active component was at 220nm in 80:20 methanol/water, as determined by Noel Davies and co-worker (University of Tasmania, Australia)

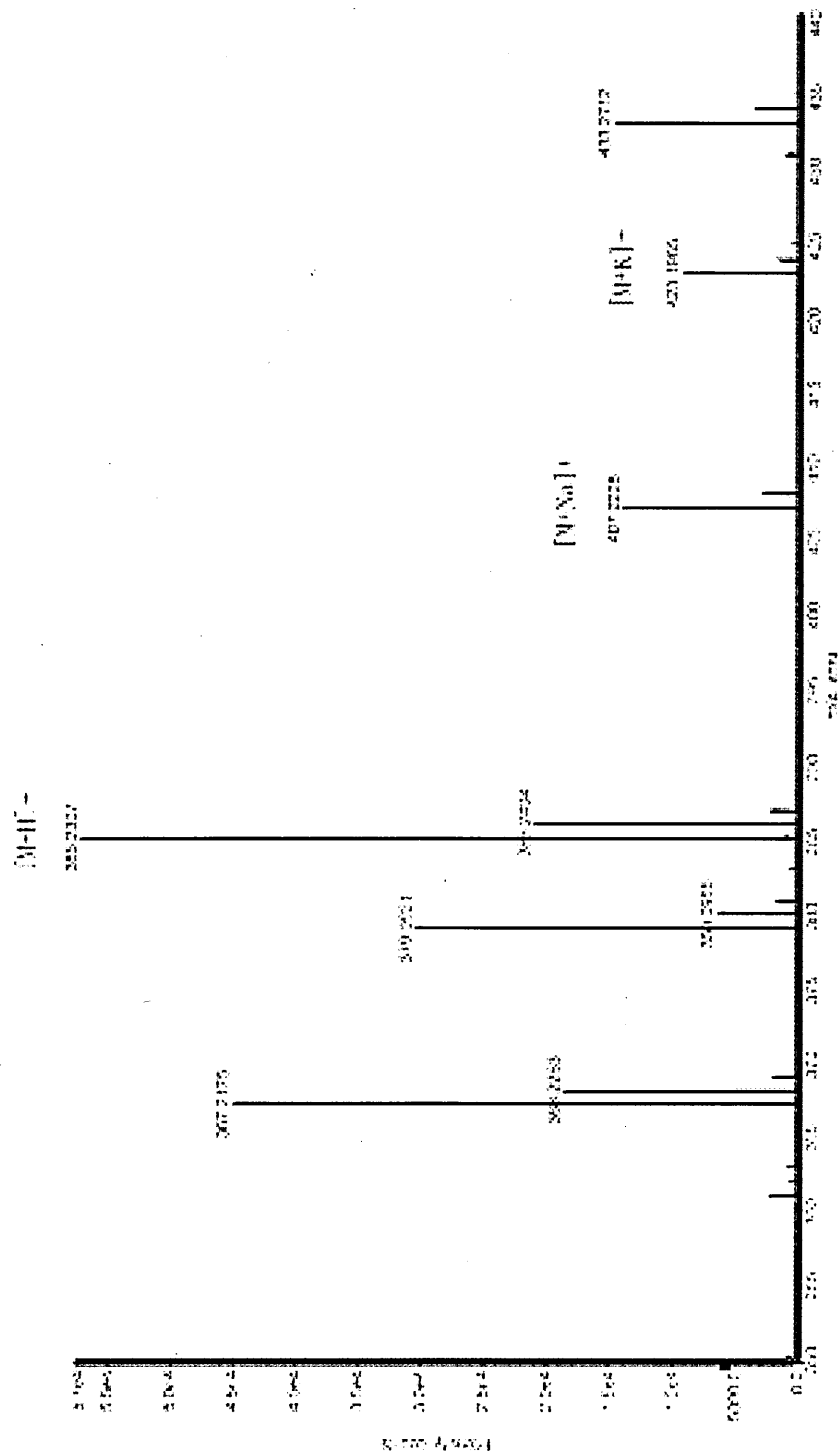
The  $^{13}C$  and  $^1H$ - $^{13}C$  correlation spectra indicate that there are 24 carbon signal

**Fig. 3-13 Inhibitory effect of sedanolide against *H. pylori* 3339**

The inhibitory effects are shown below in means ( $\pm$  SD) of 5 independent replicates after 5 days incubation.

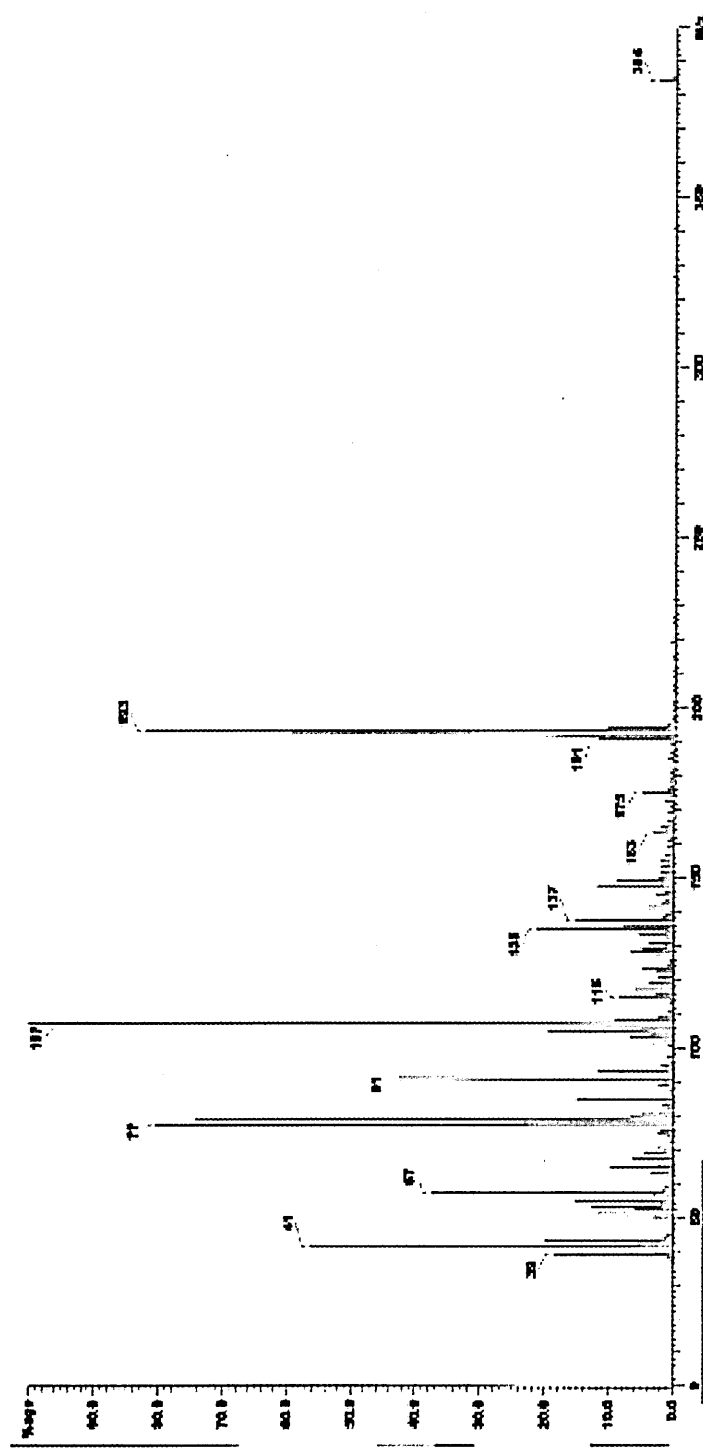


**Fig. 3-14 Mass spectrum of CAH recorded on the Q-star mass spectrometer**



Mr. J. Hague measured this data.

**Fig. 3-15 MALDI-Tof MS analysis of CAH**



Mr. J. Hague measured this data.

, including 2 of carbonyls (C=O), 1 of  $sp^2$  CH, 1 of  $sp^2$  C, 7 as CH and 12 as  $CH_2$  groups. Also two of the CH groups have quite a low chemical shift that could indicate nearby oxygen and there are 2 terminal  $-CH_3$  groups, one on short chain, the other probably on a long chain (**Fig. 3-16**).

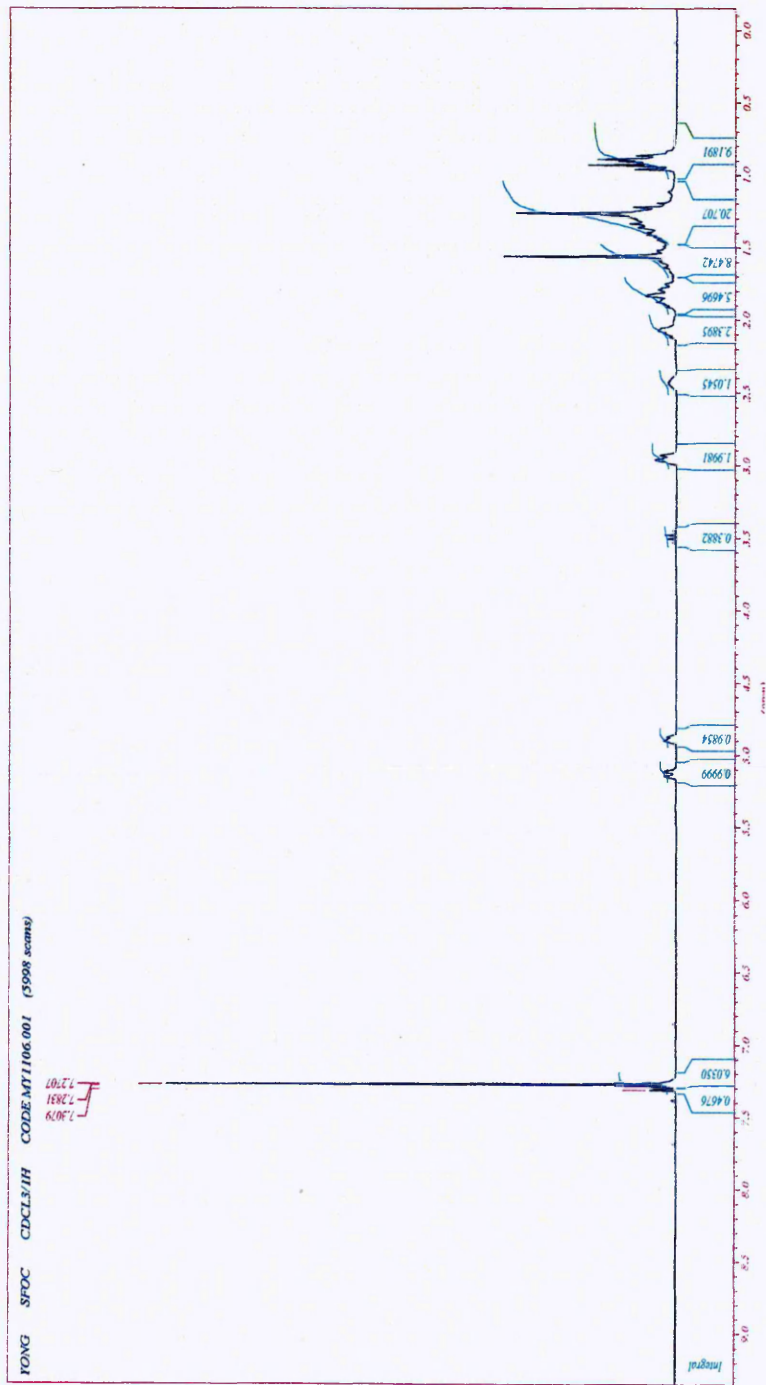
These data are entirely consistent with a phthalide dimer. The strong ions at  $m/z$  192 and 193 in the EI mass spectrum (**Fig. 3-15**) and intense  $MS^3$  product ion at  $m/z$  193 are consistent with bond cleavage with and without hydrogen transfer to monomeric subunits. Sedanenolide (**Fig. 1-15**),  $C_{12}H_{16}O_2$ , (molecular weight 192) is a significant phthalide monomer in celery seed oil (Bjeldanes and Kim, 1977), and a dimer of this would have the required formula. n-Butylphthalide (**Fig. 1-15**) (molecular weight 190) and sedanolide (**Fig. 1-15**), (molecular weight 194) are also present as monomers in celery seed oil. A heterodimer of (2) and (3) would also produce the correct formula for CAH. However the intense product ions at  $m/z$  192/193 by different MS techniques argues strongly for CAH being a heterodimer.

A comprehensive literature search showed the only reports of phthalide dimers of this formula related to partial hydrogenation products prepared in the characterisation of the more unsaturated natural phthalide dimers angeolide and Z-6,6',7,3a'-diligustilide found in other members of the Apiaceae family (Banerjee *et al*, 1982, Delgado *et al*, 1988). The genus *Angelica* (Apiaceae)

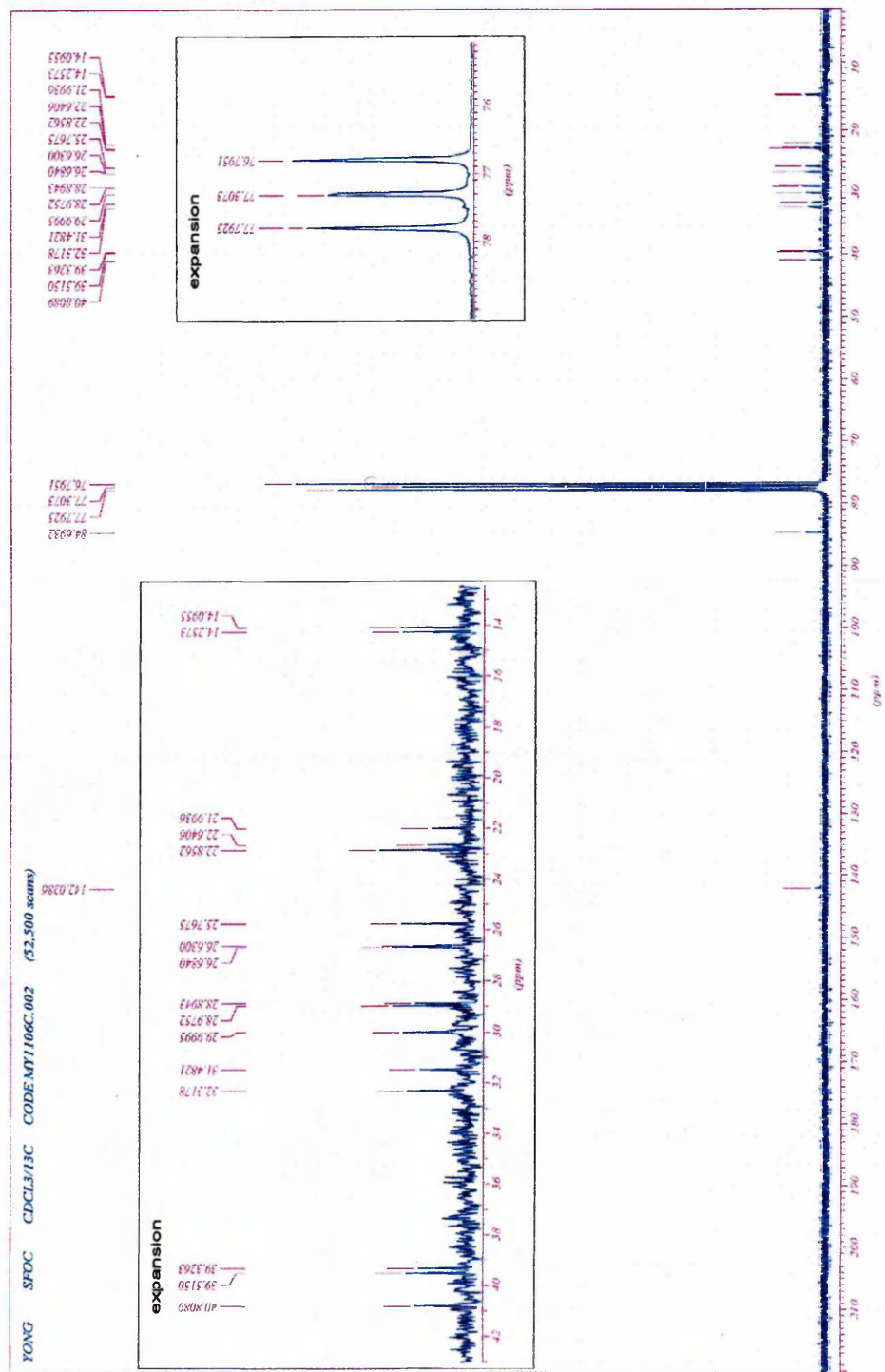
**Fig. 3-16 NMR analysis of CAH**

Dr. B. Taylor (Sheffield University) measured the data below.

A:  $^1\text{H}$  – NMR spectrum

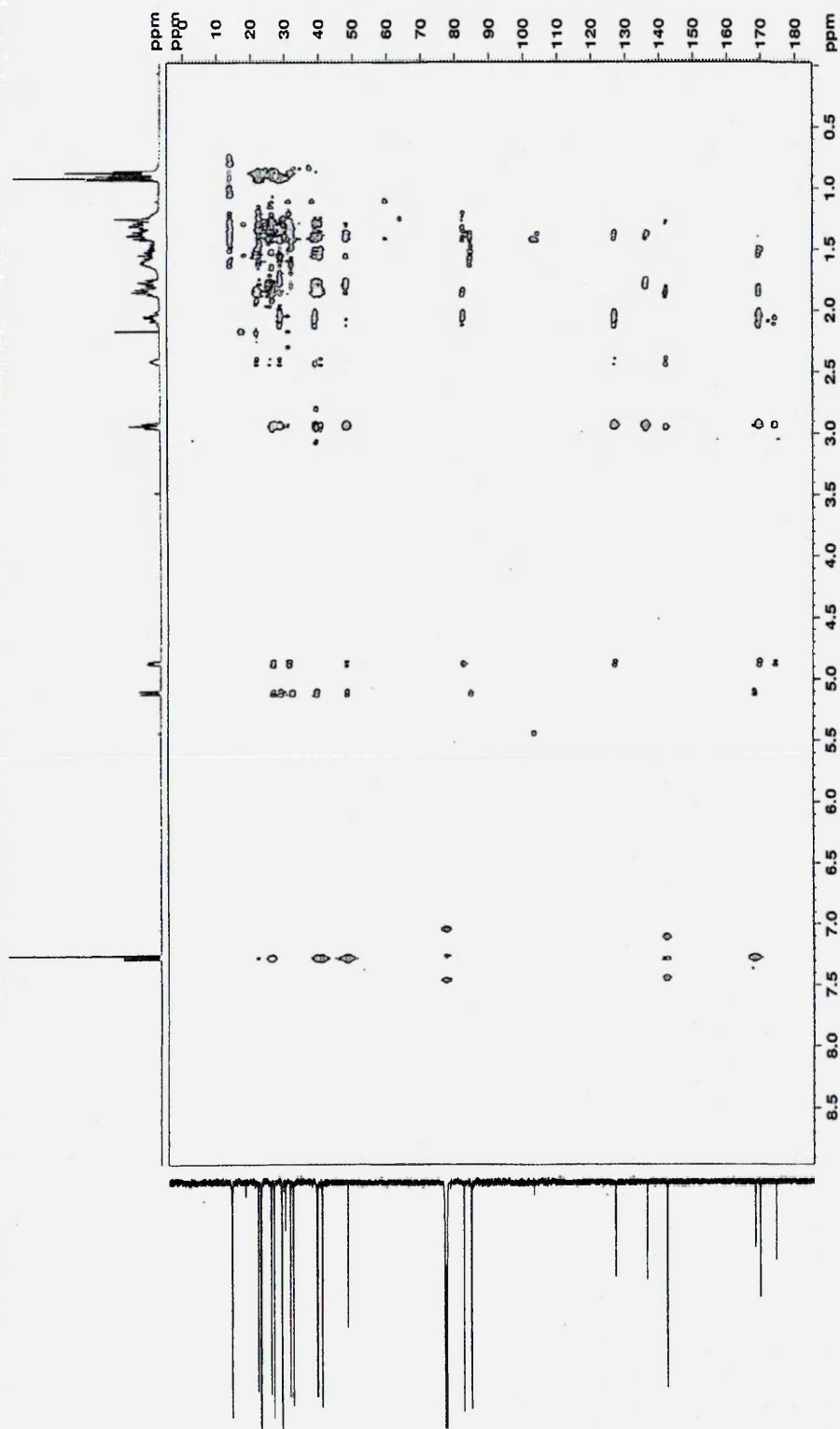


# B: $^{13}\text{C}$ -NMR spectrum





C:  $^1\text{H}$  –  $^{13}\text{C}$  correlation NMR spectrum



is a particularly rich known source of phthalides and phthalide dimers, with other examples of the latter including riligustilide (Meng *et al*, 1983), tokinolides A and B (Tsuchida *et al*, 1987), and gelispirolide (Deng *et al*, 2006). There are many theoretically possible phthalide dimers with the formula  $C_{24}H_{32}O_4$ , as dimerisation of phthalides can occur across any pair of double bonds, and also in the case of sedanenolide via Diels-Alder addition.

### 3.4 Discussion

As shown in Chapter 2, CSE exhibited a pronounced inhibitory effect on the growth of *H. pylori* and the critical compounds with this effect are resident in the low polarity fractions of CSE, which include the essential oils. The conclusion that the active component was of low polarity was initially derived from the observation that it partitioned into the most hydrophilic petroleum ether phase, upon initial fractionation of the A-CSE. The literature on volatile compounds of CSE also suggested they have been credited with many medicine properties, for instance bactericidal activity (Oiye and Muroki, 2002; Raffa, 2005; Riddle, 2004. See also introduction).

Since concern of low polarity compounds with activity of CSE above, the isolation of CSE was starting to purify the low polarity fractions. Many studies have been done on the volatile constituents of celery and its seed over the past decades. In the early reported studies, some components of celery seed (i.e.

phthalides) could not be successfully separated by gas chromatography (GC) due to their instabilities in GC columns (Kurobayashi *et al.*, 2006). In 1987, Uhlig successfully separated 3-n-butylphthalide, 3-n-butyl-4,5-dihydrophthalide (sedanenolide) and 3-n-butyl-tetrahydrophthalide (sedanolide) from celery by HPLC (Uhlig *et al.*, 1987). In 2002, several phthalide and derivatives with antioxidant, cyclooxygenase and topoisomerase inhibitory activities have been described, which were purified from celery seed by using RP-HPLC, eluted with methanol and water (Momin and Nail, 2002). Similarly, anti-mosquito and antibacterial components were isolated and purified from the hexane extract of celery seed by using normal phase HPLC, eluting with hexane, the methods employed in this project (Momin *et al.*, 2000). In this project, CAH the most bioactive component, was isolated and purified from both normal phase HPLC and RP-HPLC, respectively.

Mass and NMR spectral investigations were employed to as ascertain the SF12-i chemical structure. These methods have been successfully used to determine the constituents of celery seed (Momin and Nair, 2002). Most significantly, antibiotic phthalides from fungal sources and some synthetic analogues have already been shown to be highly effective or specific against *H pylori* (Dekker *et al.*, 1997, Radcliff *et al.*, 2008), and the presence of a spiroketal ring was shown to greatly enhance this activity. While several known phthalide dimers from the Apiaceae do contain a sprioketal ring, this

occurs through a dimerisation process involving cyclization of a butylidene phthalide side-chain which is not present in monomeric sedanenolide, sedanolide or n-butylphthalide which all have a saturated n-butyl side-chain. The exact chemical structure of CAH remains to be determined.

Also, some dimeric phthalides extracted from other medicinal plants have been reported with bioactivity such as a preventive and therapeutic agents against atherosclerosis and a potent and specific activator of the progesterone receptor (Zhang *et al.* 2003; Lim *et al.* 2006; Lin *et al.*, 1998). From the present study, CAH, a dimeric phthalide from celery seed, is the first reported component to have selective bactericidal activity against *H. pylori in vitro*. The MIC of tetracycline, which is one of currently confined antibiotics for effective *H. pylori* treatment (Zullo *et al.*, 2003), was 3.15 µg ml<sup>-1</sup> in the present study and tetracycline served as the positive control in each set of anti-*Helicobacter* data presented here.

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The results in this study indicate the inhibitory activity of CAH against *H. pylori* was comparable with tetracycline *in vitro*. Moreover, this inhibitory bioactivity of CAH is specific for *H. pylori*, since there was no effect on growth of other bacteria, *Campylobacter jejuni*, *Escherichia coli* and *Staphylococcus aureus*. This contrasts with observation that some celery seed extracts or compounds had been reported to have inhibitory effects on a wide range of bacteria as well

as having mosquitocidal, nematocidal and antifungal activities (Popovic *et al.*, 2006; Friedman, 2002; Momin *et al.*, 2001). Interestingly, the monomer phthalide, sedanolide (Sigma) from celery seed was not found to exhibit the inhibitory effect against *H. pylori* at the low concentrations at which CAH was active.

## **Chapter IV**

**Mechanism of Action of Celery Seed Extracts,  
Fractions and Principally Active Component on  
*H. pylori*.**

## 4.1 Introduction

The most common mechanisms by which antibiotics inhibit growth of bacterial cells are in order (a) inhibition of cell wall synthesis (most common), (b) inhibition of protein synthesis (translation) (second largest class), (c) alteration of cell membranes, (d) inhibition of nucleic acid synthesis, and (e) anti-metabolite activity (Hancock, 2005). In order to investigate mechanisms of inhibitory effects of the main active constituent of CSE, CAH was investigated to see whether its effect was bactericidal or bacterostatic. The effect of CAH on membrane potential, cell morphology, protein expression profiles, and bulk protein and nucleic synthesis in *H. pylori* were investigated as well as follows.

1. The influence on membrane integrity and membrane potential of *H. pylori* was evaluated by using a fluorescent dye method. The lipophilic dye rhodamine123 was used to identify cells with an active membrane potential since it accumulates within bacterial cells in an energy-dependent fashion, with this accumulation being reversible by treatment of cells with compounds that uncouple the membrane potential (Watson *et al.*, 1998). Analysis was performed via epifluorescence microscopy and flow cytometry.

2. The morphological changes of cells were observed when bacterial growth was inhibited by CAH. Samples of *H. pylori* obtained at various stages after addition of CAH were analyzed to detect any significant morphological

changes by using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Kuster *et al.*, 1997). Electron microscopy was performed in the Sorby Centre at the University of Sheffield.

3. To determine whether the CSE fractions were bactericidal or bacteriostatic, experiments were undertaken to assess cell viability count assays (MBC measurement and trypan blue exclusion), CAH was distinguished with bactericidal activity against *H. pylori*. Minimum bactericidal concentration (MBC) is defined as the lowest concentration at which the tested compound completely kills the cell (Wang *et al.*, 2005). MBC assays were performed on multiple-well microplates. Trypan blue exclusion was used microscopically to determine cell viability.

4. The effect of CAH on protein expression profiles was also investigated by performing one and two dimensional (1-D and 2-D) electrophoresis. A generally accepted approach for proteomic analysis employs solubilization of proteins and their separation by 2-D electrophoresis followed by identification using a range of mass-spectrometry methods (Govorun *et al.*, 2003). After treatment with CAH, there were some protein spots that disappeared from the treated gel and the 5 main spots of this type were identified from the control gel.



5. The interference of CAH with protein, DNA and RNA syntheses was investigated by a pulse-labeling technique (Tateda *et al.*, 1996). CSE samples were incubated with individual  $^3\text{H}$ -labelled precursors, involved in the synthesis of DNA, RNA and protein. After washing to remove the unincorporated radioactively labeled precursors, the radioactivity in the isolated macromolecules was determined to quantify the extent of inhibitory effects (Pollard, 2006).

## **4.2 Materials and methods**

### **4.2.1 Bacteria, culture conditions and preparation of CAH**

As in the earlier studies, the strain 26695 of *H. pylori* was used. As previously, the bacteria were stored at  $-80^{\circ}\text{C}$  in BB with 5% FBS, and cultured in chocolate agar plates or liquid media (BB plus 5% FBS) for up to 7 days under microaerophilic conditions (10%  $\text{CO}_2$ , 3%  $\text{O}_2$  and 87%  $\text{N}_2$ , at  $37^{\circ}\text{C}$ ).

CAH was isolated as described in Chapter 3 using the purification protocol that included RP-HPLC. The purity of the resulting material was assessed by using analytical thin-layer chromatography (TLC) and the CAH was found to be pure. CAH (1 mg) was dissolved in 10  $\mu\text{l}$  of solvent (iso-propanol) to obtain a concentration of 100 mg/ml, and then diluted 100 fold into *Brucella* broth plus fetal bovine serum (5%) to give on 1 mg/ml of CAH. From this stock solution, CAH was further diluted in BB plus FBS as required at the concentrations

detailed below.

#### **4.2.2 Bactericidal and bacterostatic assays**

**4.2.2.1 Microplate assay:** *H. pylori* was cultured in the liquid media with two-fold serial of concentrations of CAH in 24-well plates under the same microaerophilic conditions for 5 days as employed above, together with an identical bacterial culture without any added fractions which served as the negative control. The cultures were centrifuged at 16,000 x g for 5 min. After discarding the supernatants, the pellets were re-suspended in BB plus FBS with no added CSE fractions and cultured under microaerobic conditions again for 3 days. The optical density of the samples was measured daily to monitor the growth of the cells.

**4.2.2.2 Trypan blue exclusion:** *H. pylori* strain 26695 was cultured with 5 µg/ml of CAH and without any treatment under microaerobic conditions for 3 days. To 100 µl of each cell suspension was added and an equal volume of 0.4% trypan blue (Life Technologies, Inc.) and the suspension gently mixed. The suspension was incubated for 5 minutes at room temperature. An aliquot (10 µl) of the stained cell suspension was placed in a haemocytometer and the number of viable (unstained) and dead (blue stained) cells were counted. The average number of unstained or stained cells in each quadrant (1:1 mm) was multiplied by  $2 \times 10^4$  to determine the number of stained/unstained cells/ml.

#### 4.2.3 Electron microscopy

*H. pylori* strain 26695 was cultured in BB plus 5% FBS with 5 µg ml<sup>-1</sup> of CAH under microaerobic conditions for 3 days as above. Two negative controls were performed, one where *H. pylori* 26695 was added with no additions to the BB + FBS media, and another where iso-propanol (the solvent used for CAH) was added to the same concentration (0.05%) as in the CAH containing culture. All samples were centrifuged (16,000 × g; 5 min; 4 °C), the pellets collected and the supernatants were discarded. For examination under the scanning electron microscope at 20 kV (Phillips 500 SEM, University of Sheffield), the pellets were fixed in 2% (v/v) freshly prepared paraformaldehyde and 2.5% (v/v) glutaraldehyde in 4% PSB (pH 7.2), followed by 2% (w/v) osmium tetroxide. After washing free of fixative the pellets were then dehydrated by passage through a graded series of ethanol treatments and air dried. Mean cell diameters were calculated from measurements of 50 randomly selected cells from each micrograph (Tang *et al.*, 2004). For examination with transmission electron microscopy (using an FEI Tecnai 20 TEM, University of Sheffield), a drop of bacterial samples was applied to the electron microscope grid, and then negatively stained with 1% (w/v) phosphotungstic acid (pH 7.2) for 20 s (Tang *et al.*, 2004).

#### 4.2.4 Effect of CAH on *H. pylori* membrane potential

Aliquots (1 ml) from 3-day liquid cultures of *H. pylori* strain 26695 were

centrifuged at 16,000 x g for 5 min. The supernatant was discarded and the cell pellets were washed by re-suspension in PBS (Invitrogen) and centrifuged under the same conditions. Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (200 µl, 0.1 mM, Sigma) was added to re-suspend each pellet and the suspensions were incubated for 5 min to permeabilize the outer membrane. The cells were centrifuged at 20 °C (16,000 x g), and the pellets re-suspended in 200µl of rhodamine123 in PBS solution (5 µg/ml, Sigma) and incubated in the dark for 30 min under the microaerophilic conditions as above. The tubes were then centrifuged under the same conditions as above and the supernatant discarded. The cell pellet was then resuspended in 200 µl of CAH (25 µg/ml in iso-propanol). Three controls were also performed in parallel using cells prepared in an identical way and then, in place of CAH, treated with an equal volume of the following (1) PBS, (2) carbonyl cyanide 3-chlorophenylhydrazone (CCCP; 15 µM) in PBS and (3) PBS plus the same amount of iso-propanol used in the experimental reaction (Diaper and Edwards, 1994; Mason *et al.*, 1995; Comas and Vives-Rego, 1997 and Watson *et al.*, 1998). After 4 h, the samples were analysed by flow cytometry (Becton Dickinson FACScan), with side scatter thresholds set for the analysis of the bacteria, in which fluorescence emission was acquired using a 4 decade log amplifier calculated on an arbitrary linearised scale (1-10,000) in the fluorescence 1 channel (630 nm). The excitation and emission wavelengths were set at 600 nm and 665 nm,

respectively. After selecting a well defined region of interest (ROI) on forward versus side scatter, average fluorescence of the bacteria falling within this ROI was measured (Logan *et al.*, 1998). The extracellular probe is not fluorescent and, therefore, does not affect the reading. Bacteria were distinguished from debris and background noise on the basis of their forward- and side-scatter characteristics. The yellow fluorescence of 10,000 bacteria was analyzed by LYSIS software (Becton Dickinson, UK). The mean fluorescence in the defined range of fluorescence intensity represents the metabolic activity of the cells, as indicated by the presence of the membrane potential (Kuster *et al.*, 1997).

To validate the FACS analysis of fluorescent labeling of *H. pylori*, microscopic observations were also performed at 1000 x under oil immersion using an Olympus BX60 fluorescence microscope (Olympus Optical Co., Germany) (Logan *et al.*, 1998). Slides were prepared with 5 µl aliquot of each tube and viewed using the fluorescence microscope. Specifications for this were PanFluor Ph2 (Olympus). Digital images were recorded using a cool SNAP-PRO camera (Sony) and viewed using Labworks (Perkin-Elmer, USA) computer program. Cell counts were taken from at least 2 randomly selected fields and data analyzed using the  $\chi^2$  test for significance.

#### **4.2.5 Effect of CAH on gross protein and nucleic acid synthesis**

The methods used for incorporation of  $^3\text{H}$ -labelled precursors (Amersham

Biosciences, Amersham, UK) to investigate the effect of celery seed extracts on synthesis of protein, RNA and DNA in *H. pylori* 26695, were those described by Onishi *et al.* (1996). Thus, bacteria were grown to early log phase in liquid culture as described above. Aliquots of culture were transferred to sterile tubes containing various concentrations of CAH, together with radioactive precursors as follows: (1)  $^3\text{H}$ -leucine at  $2.5\ \mu\text{Ci/ml}$  to monitor protein synthesis; (2)  $^3\text{H}$ -uracil at  $0.3\ \mu\text{Ci ml}^{-1}$  to monitor RNA synthesis or (3)  $^3\text{H}$ -thymidine at  $0.75\ \mu\text{Ci ml}^{-1}$  plus uridine ( $50\ \mu\text{g ml}^{-1}$ ) to determine DNA synthesis (Onishi *et al.*, 1996). After 24 h, 48 h or 72 h (as stated for each experiment), the reactions were stopped by adding trichloroacetic acid (TCA) to a final concentration of 10 % (w/v). The precipitates were collected with a cell harvester and washed five times with 10% (w/v) TCA. The filters carrying the washed precipitates were placed into scintillation tubes with 2 ml of scintillation cocktail solvent, comprising 7 g of PPO (2,5-diphenyloxazole), 0.6 g of dimethyl-POPOP (1,4-bis{4-methy-5-phenyl-2-oxazdyl}benzene), and 100 ml of Bio-Solv BBS-3 (Yoshiyuki and Kunihiro, 1972). Radioactivity was then quantified by using a Packard 3320 scintillation counter (Minnesota, USA).

#### **4.2.6 1-D and 2-D Electrophoresis and protein identification**

##### **4.2.6.1 1-D Electrophoresis**

*H. pylori* strain 26695 was cultured 3-5 days in BB plus FBS (5%) with half of MIC of the CSE and/or other fractions or solvents (added to produce the same

concentrations as in CSE samples). The OD<sub>600nm</sub> was measured and the culture was diluted with fresh medium to give OD<sub>600nm</sub>=1.0. Samples tested in this way included A-CSE (100 µg/ml), petroleum ether phase (25 µg/ml), SF12 (12.5 µg/ml) and CAH (1.8 µg/ml). Control cultures were inoculated into BB plus FBS (5%) alone under the same conditions. Aliquots (1 ml) of the samples were taken and cells were harvested by means of centrifugation (16,000 x g, 5 min), washed with sterile water and then re-centrifuged. The cell pellets were then re-suspended in 150 µl of SDS loading buffer (Invitrogen, Paisley, UK) and then heated at 85 °C for 3 min to lyse them. Aliquots (25 µl) of each sample were loaded onto a 1-D mini-gel (Novex, 4-20% polyacrylamide, Tris-Glycine Gels, 1.0mm thick, 10 wells, Invitrogen). After loading, gel electrophoresis was performed by using SDS running buffer (Invitrogen) at 125 V for about 90 min, until the dye front reached the bottom of gel, according to recommendations of the manufacturer. After removing the gel, Coomassie blue R250 stain (Eckerskon *et al.*, 1988) was added to visualize the protein spots.

#### **4.2.6.2 2-D Electrophoresis and protein identification**

*H. pylori* 26695 obtained from liquid cultures (5 ml) with CAH at half of MIC (1.8 µg/ml) under microaerobic conditions for 3 days, were harvested by centrifugation (16,000 x g, 5 min), washed with sterile water and then re-centrifuged. The cell pellets were resuspended in Sample Rehydration buffer (Invitrogen, Paisley, UK) containing 8M urea, 0.5% CHAPS and 200 mM

DTT (dithiothreitol) to obtain total protein extracts. After sonication for 30 min (Branson 1210 sonicator, 47 kHz, 80W), the total protein concentration was determined using the Bradford assay (Bradford, 1976). Aliquots diluted to about 300 µg of total protein per sample were obtained. Isoelectric focusing was performed using 7-cm IPG strips, pH 3-10L (Invitrogen) according to recommendations of the manufacturer. After loading the protein sample (10 µg) isoelectric focusing was performed at 200 V for 20 min, 450 V for 15 min, 700 V for 15 min and finally 2,000 V for 3.5 h. The strips were then incubated in lithium dodecyl sulfate (LDS) sample buffer with reducing reagent (Invitrogen) for 15 min and subsequently placed in LDS sample with idoacetamide (Invitrogen) for 15 min. The second dimension separation was then performed on 1-mm-thick polyacrylamide mini-gels (gradient 4-12%, Invitrogen) in MES running buffer at 200 V for 45 min. Silver stain (Invitrogen) was used to visualize the protein spots. Gel images were analyzed by using 2-D Gel Analysis Software Version (Phoretix 2-D Expression v2004). Gels from CAH treated samples were compared with control gels from samples treated with the solvent in place of CAH.

Silver-stained spots were excised from 2-DE gels and transferred into Proteome System<sup>®</sup> (Shiamdzu-Biotech, Milton Keynes, UK) and processed for protein digestion according to the manufacturer's instructions. In-gel digestion of the excised protein spots was performed using trypsin (1µg/ml) and the



sample was then mixed (1:1) with a saturated  $\alpha$ -cyano-4-hydroxycinnamic acid solution in 50% acetonitrile – 0.3% trifluoroacetic acid, and 2  $\mu$ l was applied to the sample template of a matrix-assisted laser desorption ionization (MALDI) time of flight (ToF) mass spectrometer instrument (Voyager Elite, PerSeptive Biosystems Inc., Framingham, MA, USA). The instrument was operated at an accelerating voltage of 20 KV and a grid voltage as 90% of the accelerating voltage. The time of flight was measured by a 500 MHz transient digitizer board in the computer, and the data were analyzed using GRAMS/386 software (Galactis Industries Corp., Salem, NH, USA). The resulting peptide fingerprint data were analyzed by using Mascot software (<http://www.matrixscience.com/>) or MS-Fit (UCSF spectrometry Facility; <http://propspector.ucsf.edu>), and the search employed protein database of the National Center for Biotechnological Information (<http://www.ncbi.nlm.nih.gov>), of the complete genome of *H. pylori* 26695 (Pleißner *et al.*, 2004). The accuracy of  $MH^+$  mass determination was  $\leq 0.01\%$  and possible modification of cysteine residues by acrylamide and methionine oxidation were taken into consideration.

## **4.3 Results**

### **4.3.1 Bactericidal and bacteriostatic testing**

To determine whether CAH had bactericidal as well as bacteriostatic activity, bacteria were initially cultivated in the presence of the compound and

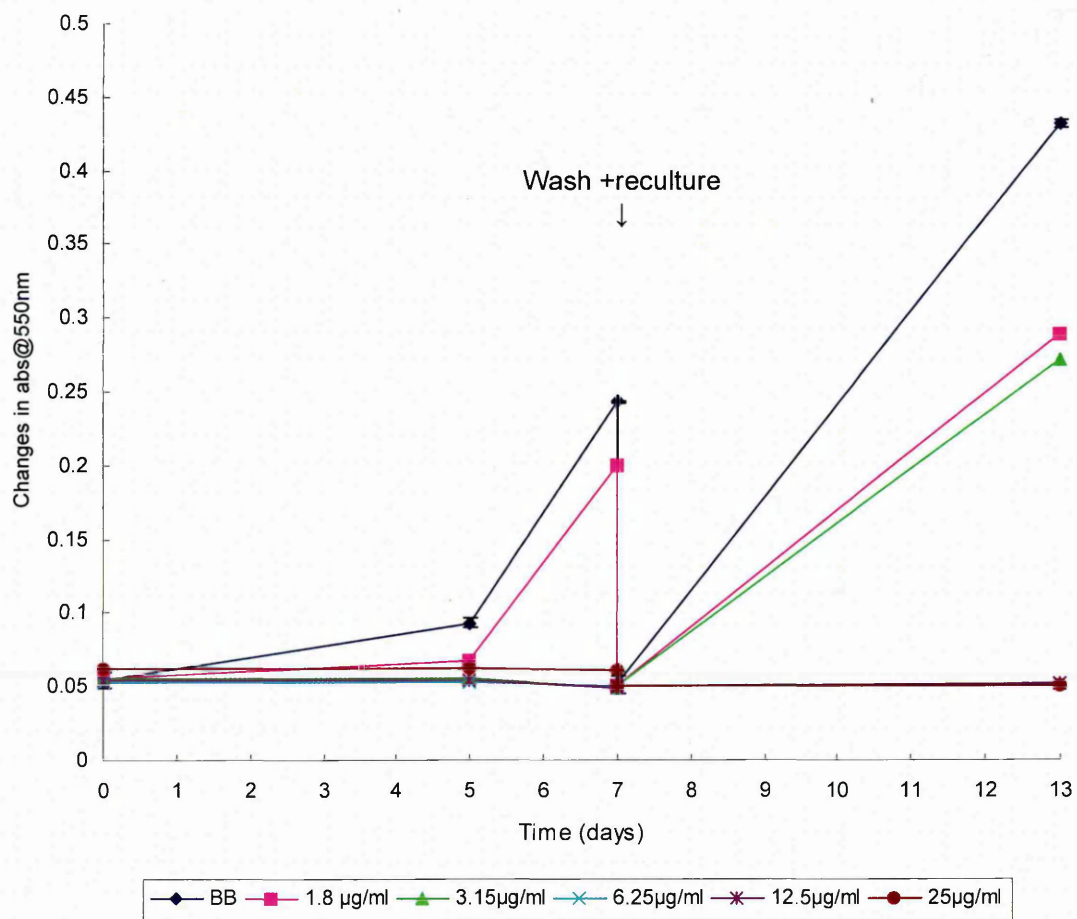
subsequently re-cultured in its absence (Wang *et al.*, 2005). The minimum bactericidal concentration (MBC) is defined as the lowest concentration at which a compound gives complete killing. As showing in **Fig 4-1**, the growth of cells has been inhibited at more than 3.15  $\mu\text{g ml}^{-1}$  of concentration of CAH, and the cells could not be re-grown at 6.25  $\mu\text{g ml}^{-1}$  of concentration of CAH or more. Therefore, MBC of CAH was 6.25  $\mu\text{g ml}^{-1}$  and approximately twofold higher than the MIC. The results indicated that CAH has bacteriostatic effect against *H. pylori* at concentration up to 3.15  $\mu\text{g ml}^{-1}$  and with bactericidal effect as concentration more than 6.25  $\mu\text{g /ml}$  (2 x MIC).

Also, the bactericidal effect of CAH against *H. pylori* was confirmed by the trypan blue exclusion experiments. 3 days after incubating with CAH in or iso-propanol alone, the proportion of the viable cells observed were 2% and 94 %, respectively. In the negative control (no addition of CAH or solvents to the culture), 98% of the cells were viable after 3 days (**Fig. 4-2**). The cell viability in the CAH-treated samples did differ from negative control and solvent treated sample ( $X^2$ ,  $P<0.01$ ). The proportion treated with solvent did not differ from control. The results indicated that CAH enhanced *H. pylori* viability *in vitro*.

#### **4.3.2 Transmission and scanning electron micrographs**

**Fig. 4-3** shows scanning electron micrographs (SEM) of *H. pylori* strain 26695 were cultured in the presence of CAH at more than MIC (5  $\mu\text{g ml}^{-1}$ ) for 72 hrs.

**Fig. 4-1 Bactericidal effects of CAH against *H. pylori* strain 26695**

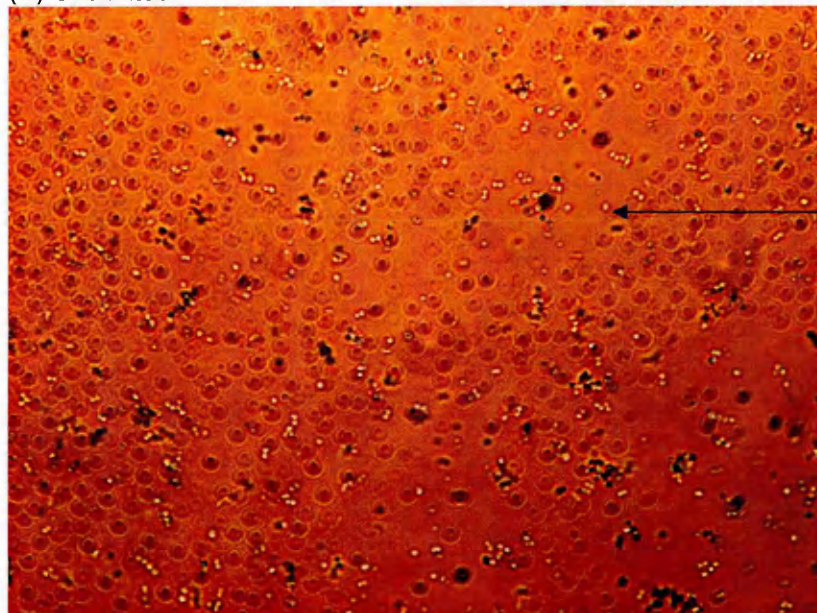


*H. pylori* 26695 were cultured with a range of concentrations of CAH for 7 days. After centrifuging, pellets were re-cultured in BB plus FBS without any treatment for 5 days. The effects are shown below as mean of 5 independent replications. Below 3.15 µg/ml of CAH, the inhibition was reversible. Above 6.25 µg/ml (the MBC), growth was irreversibly inhibited.

#### **Fig. 4-2 Trypan blue exclusion**

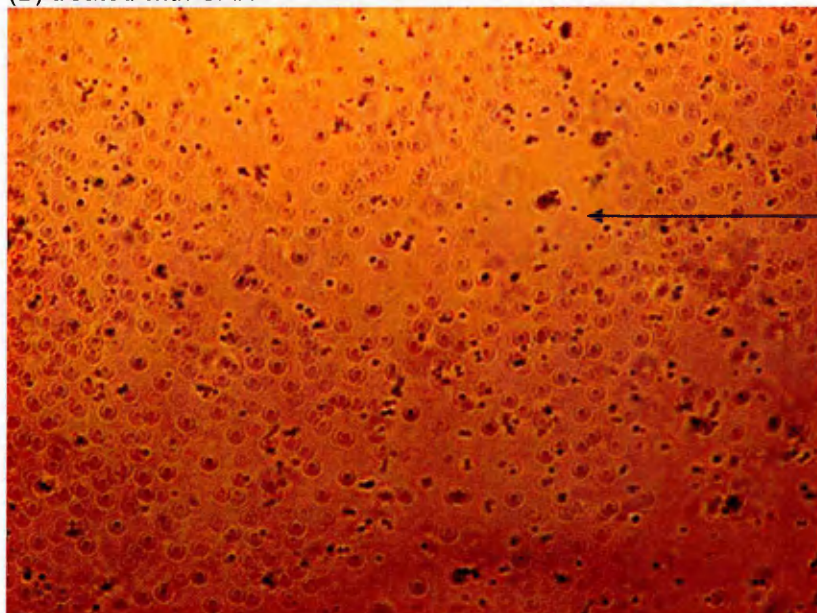
*H. pylori* strain 26695 was observed under an Olympus microscope (1000 x under oil immersion), which stained with 0.4 % trypan blue for 5 minutes after treated with CAH (B) and with the same amount of iso-propanol (C) as was present as solvent in (B). (A) is the negative control without any treatment. This experiment has been repeated for 3 times and the very similar results were observed.

(A) Untreated



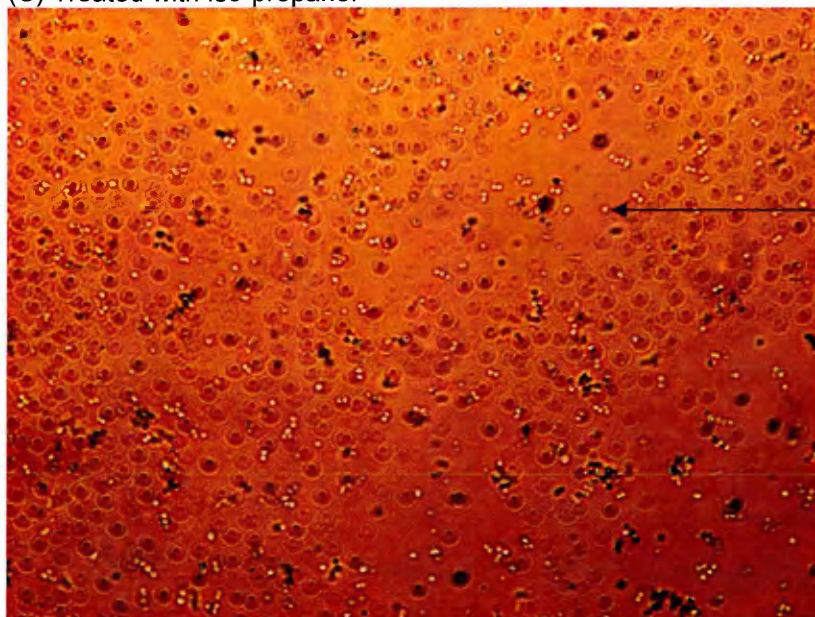
Viable cell without staining.

(B) treated with CAH



Dead cell with blue staining.

(C) Treated with iso-propanol



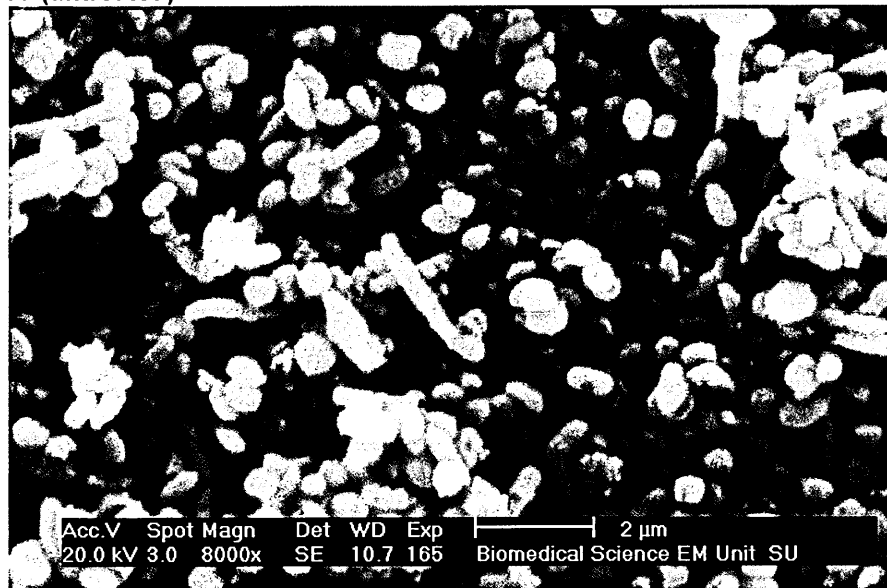
→ Viable cell without staining.



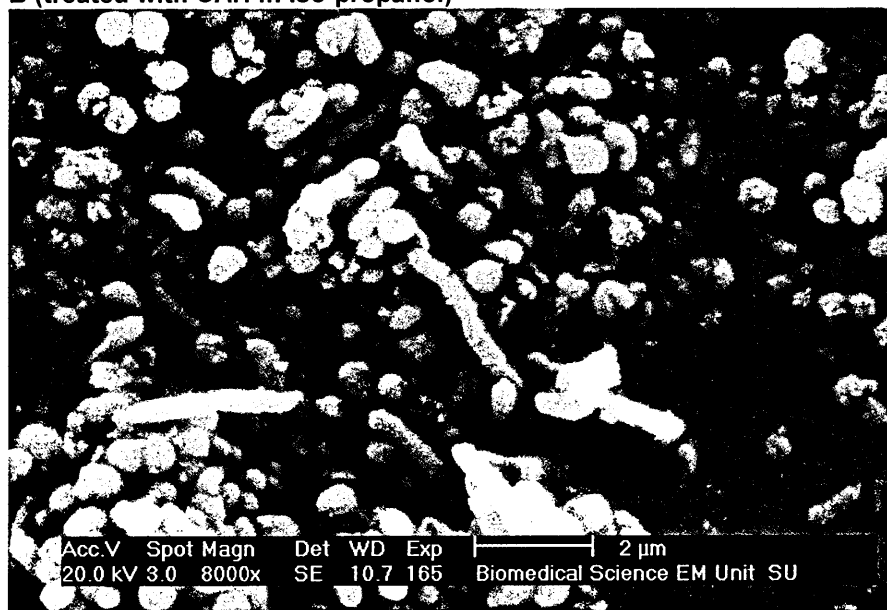
### **Fig. 4-3 Scanning electron micrographs**

*H. pylori* 26695 was incubated under microaerobic conditions for 72 h, (A) without treatment, (B) treated with 5 mg ml<sup>-1</sup> of CAH, added as a stock solution in iso-propanol, and (C) treated with the same amount of iso-propanol as was present as the solvent in (B).

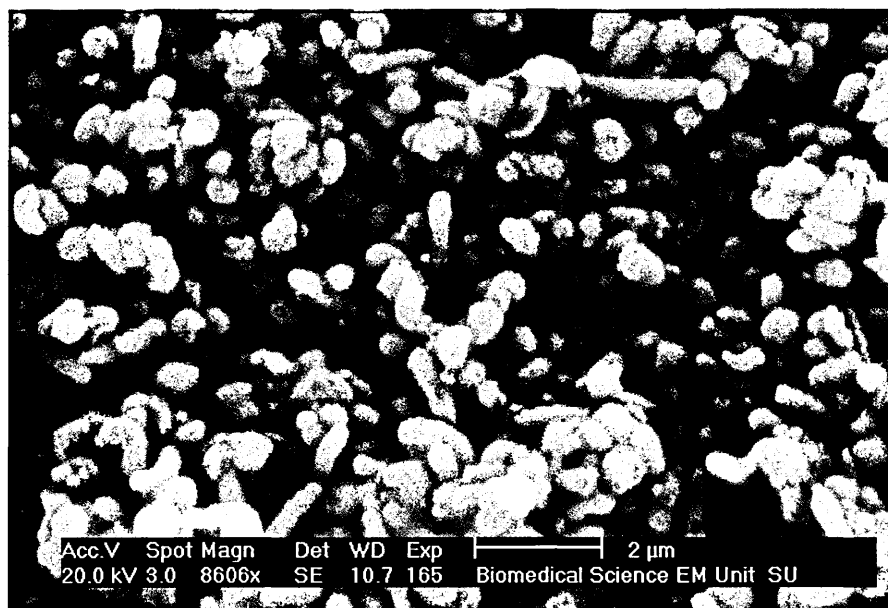
**A (untreated)**



**B (treated with CAH in iso-propanol)**



C (treated with 0.05 % (v/v) of iso-propanol solvent)



In the CAH-treated sample there were 19.7 % in the rod form (226/1141) and 80.3% in the coccoid form (917/1141) by counting all sight versions. In the untreated sample, 29.8% of the bacterial cells were in the rod form (206/692) and 71.2% in the coccoid form (486/692). This observed increase in the proportion of coccoid cells is significant at the 5 % level according to the Chi-square statistical test ( $\chi^2$ ,  $P < 0.05$ ).

The average sizes ( $1.5 \pm 0.3 \mu\text{m} \times 0.15 \mu\text{m}$ ) of the cells treated with CAH were smaller than that of untreated samples ( $3.0 \pm 0.6 \mu\text{m} \times 0.2 \mu\text{m}$ ). These changes are significant change using to the Chi-square statistical test ( $\chi^2$ ,  $P < 0.05$ ). Inoue *et al.* (2005) also reported a similar morphological change in *H. pylori* ATCC43504 in the presence of bactericidal agents.

From TEM results, there was a loss of regions of low electron dense materials in the cells after treatment with CAH (**Fig. 4-4**).

#### **4.3.3 1-D and 2-D Electrophoresis and protein identification**

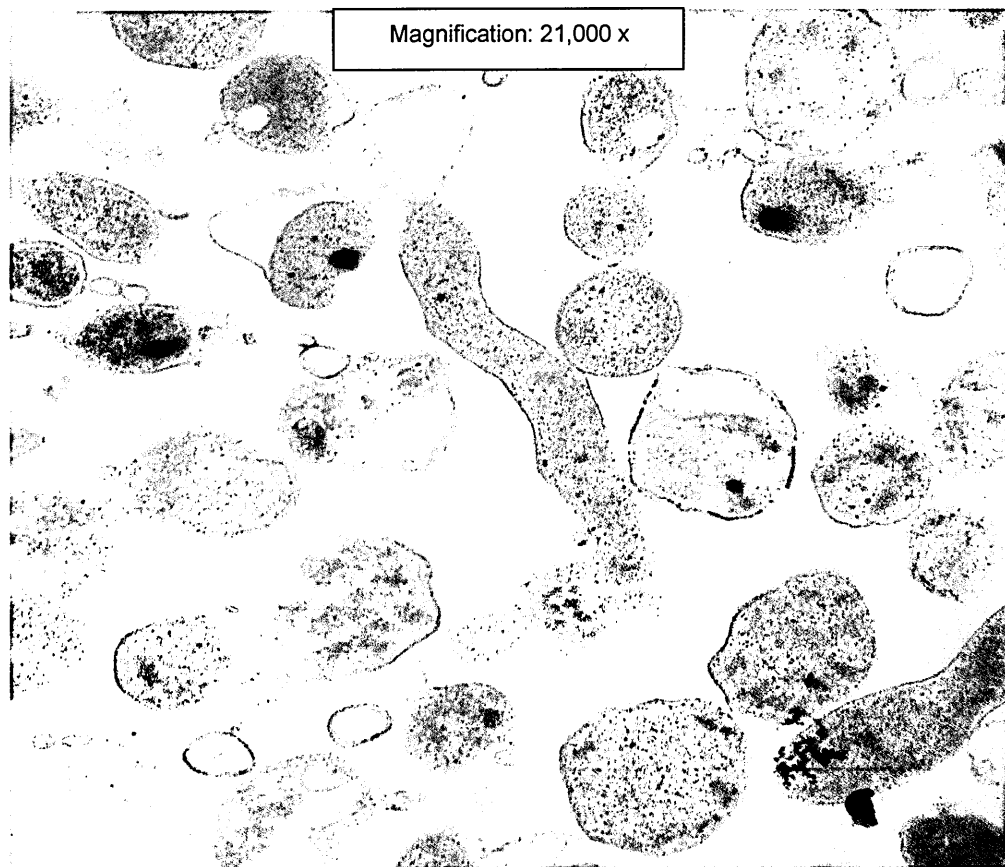
**4.3.3.1 1-D gel:** all samples showed a large expression of a doublet of proteins at about 60 kDa. At least one of those was probably the heat-shock protein Gro EL, which is expressed in response to the general stress of exposure (Ford *et al.*, 2005). No major changes in protein profile due to addition of the celery seed fraction could be discerned from 1-D gel (**Fig. 4-5**).



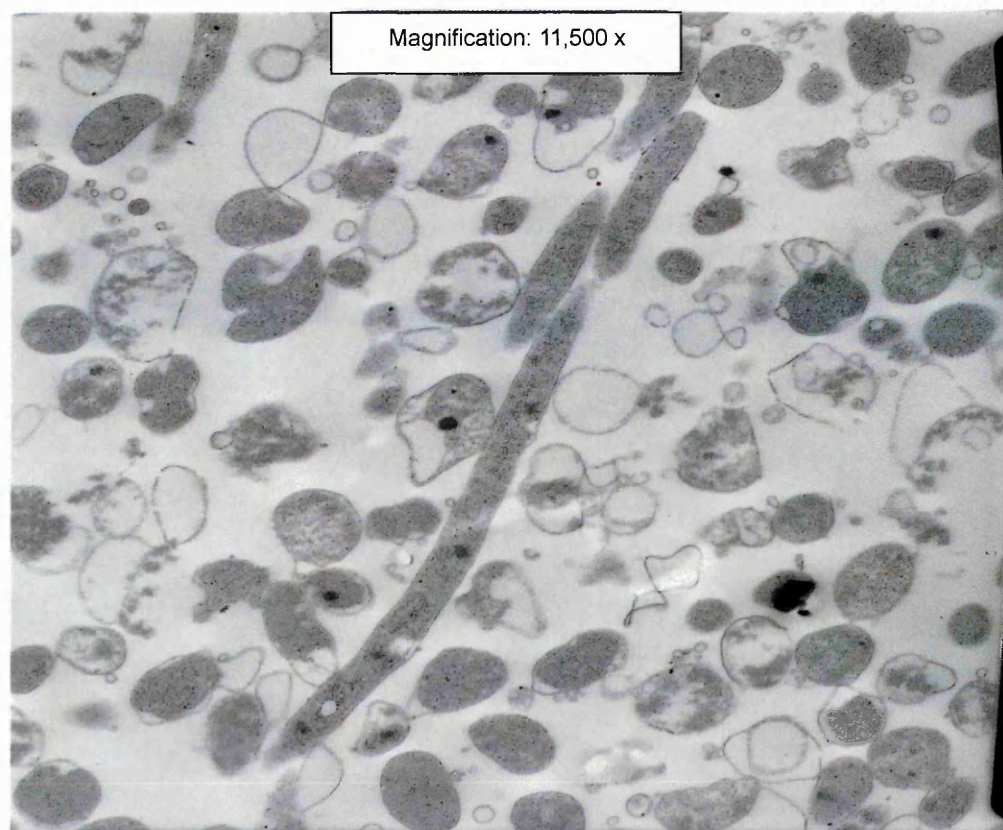
**Fig. 4-4 Transmission electron micrographs**

*H. pylori* 26695 incubated under same condition as in SEM, (A) without treatment, (B) treated with 5 µg/ml of CAH.

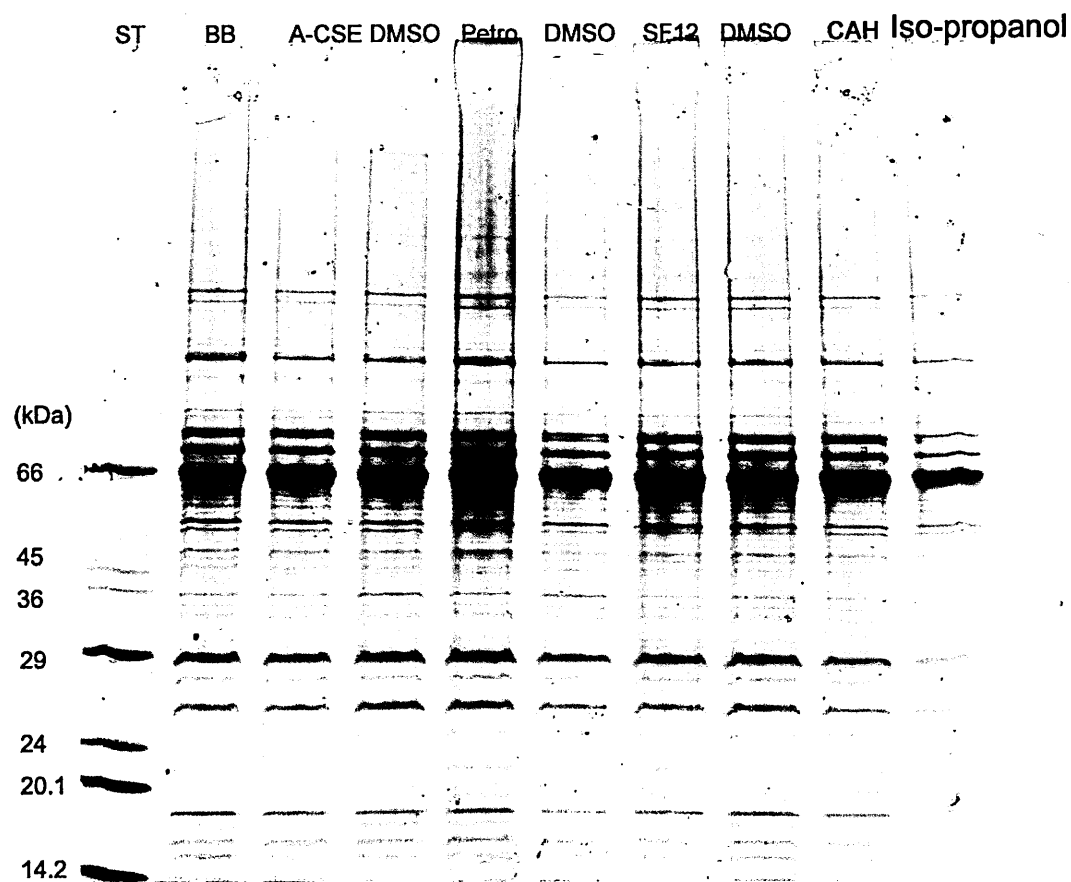
(A) Without treatment



(B) Treated with CAH



**Fig. 4-5 One dimensional gels showing the effects of CAH on protein expression profiles of *H. pylori* 26695**



One dimensional gel showing the effects of CSE and fractions on protein expression profiles of *H. pylori* 26695 incubated under microaerobic conditions for 72 h. These strips are from reactive incubated with CSE and fractions included A-CSE (100 µg/ml), petroleum ether phase (25 µg/ml), SF12 (12.5 µg/ml) and CAH (1.8 µg/ml). The solvent-treated sample (DMSO and iso-propanol) next to CSE and fractions were from incubates performed under the same conditions as in CSE and fractions treated samples.

**4.3.3.2 2-D gels:** Global protein-expression profiles of *H. pylori* strain 26695 treated with CAH and untreated were resolved on 2-D gels (**Fig. 4-6**). In both treated and untreated samples, the proteins spots were spread over the whole the *pI* range of 3-10 and the *Mr* range 5-150 kDa. These results agree with the comparative proteome analysis of *H. pylori* reported by Jungbult *et al.* (2000). By using 2-D Gel Analysis Software Version, there were 338 spots detected from the untreated cell gel and 326 spots from treated cell gel. From the same software, 289 spots were found in the same position in the same intensity for both gels. Gel digestion and mass spectrometry allowed identification of the five most prominent protein spots from the untreated gel that decreases in intensity upon treatment with CAH. These proteins are initial on **Fig. 4-6** and listed in **Table 4-1**.

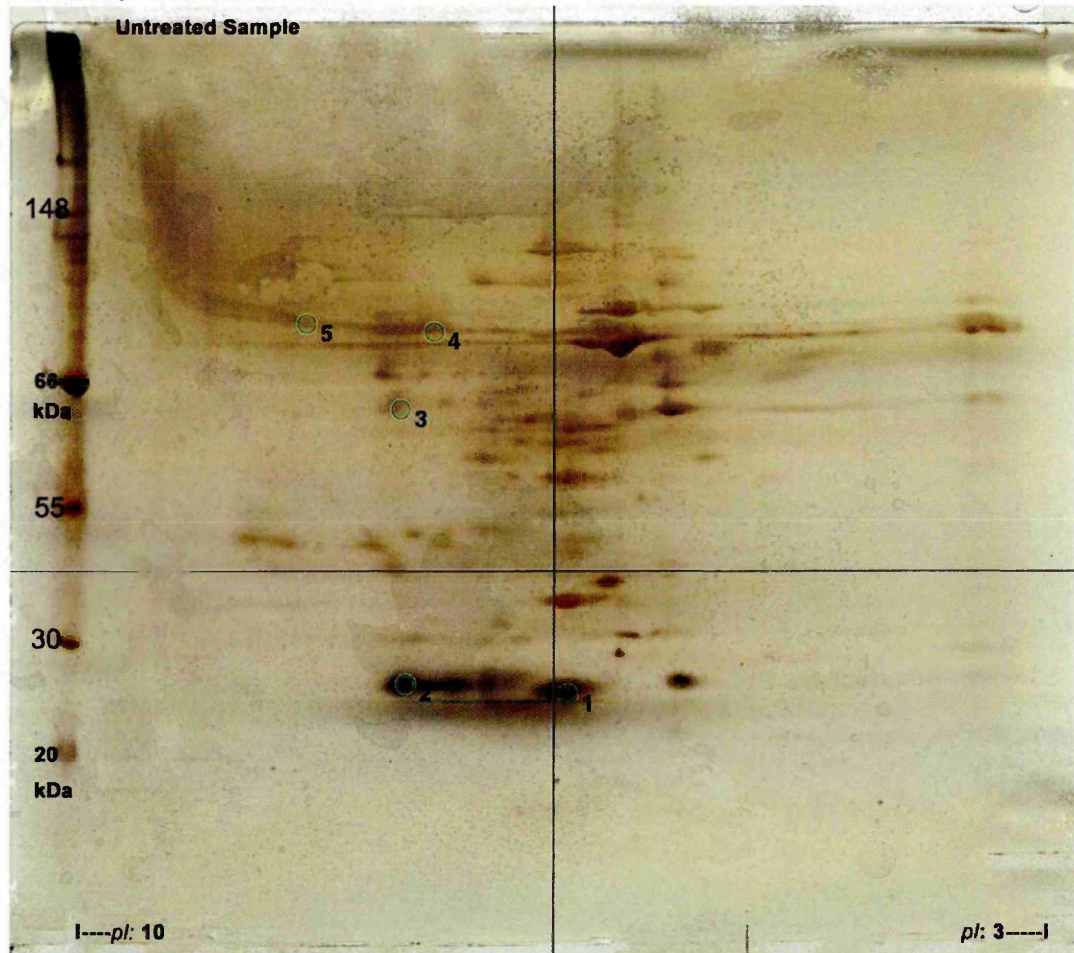
#### **4.3.4. Membrane potential measurement**

Rh123 staining was used to discriminate between viable and dead cells in cultures treated with CAH (25 µg/ml). As shown in **Table 4-2**, flow cytometric analysis revealed that the average fluorescence of *H. pylori* treated with CAH, as well as that of cells treated with solvent alone, was slightly greater than the florescence due to untreated cells (fluorescence 1 channel, 630 nm). However, these changes were not significant when analyzed with the Chi-squared test. For confirmation of the inhibitory effects, analysis of cells treated with the membrane potential uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydra-

**Fig. 4-6 2-D dimensional gels showing the effects of CAH on protein expression profiles of *H. pylori* 26695**

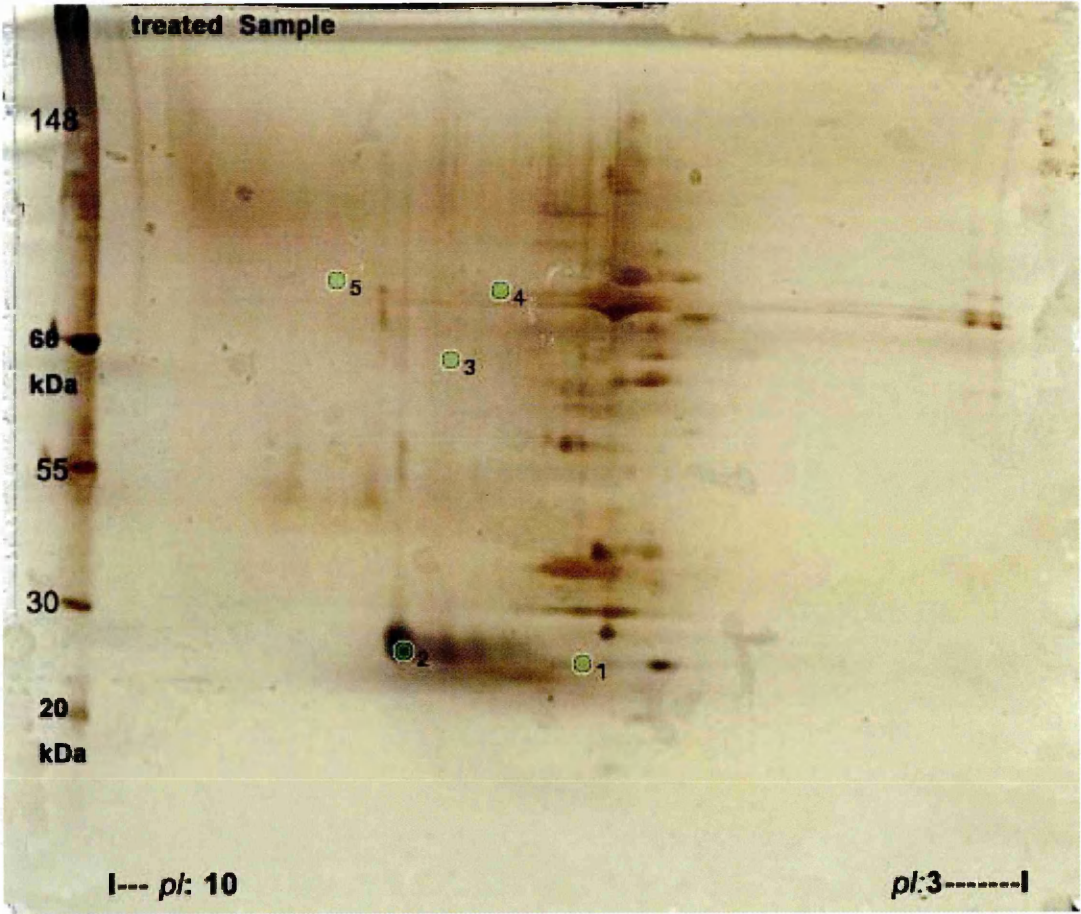
Two dimensional gels showing the effects of CAH on protein expression profiles of *H. pylori* 26695 incubated under microaerobic conditions for 72 h, (A) without and (B) with half of MIC of CAH. The five protein spots marked with number showed clearly different intensities at the same positions (markedly lower intensity in gel treated with CAH) and were chosen for identification via MS.

**A: Without treatment**





B: Treated with CAH



**Table 4-1 Tentative identification of protein spots that decreased upon treatment with CAH**

Spot No.	Hp No.	Gene	Protein identified	MP	<i>T<sub>p</sub></i>	<i>T<sub>M</sub></i> (Da)
1	HP-0389	SodB	Superoxide Dismutase	12	6.0	24518
2	HP-1240		Maf-like protein	6	6.8	21159
3	HP-0239	hemA	Glutaryl-tRNA reductase	5	8.4	51903
4	HP-0705	uvrA	UvrABC system protein A	29	7.3	104062
5	HP-1072	CopA	Copper-transporting ATPase	9	8.3	81853

Spot no. refers to the numbers assigned in Fig 4-6; Hp no. refers to the 26695 genome annotation ([www.tigr.org](http://www.tigr.org)); Matching peptides (MP) refer to the number of matching tryptic digest fragments; *T<sub>p</sub>* and *T<sub>M</sub>*: theoretical isoelectric point and molecular mass.

**Table 4-2 Flow cytometric analysis of *H. pylori* following Rh123 staining**

Samples	fluorescence intensity of <i>H. pylori</i> (Mean $\pm$ SD)
Negative control	120.2 $\pm$ 4.7
+ CAH	133.12 $\pm$ 5.7
+ Solvent	133.6 $\pm$ 9.9
+ CCCP	90.86 $\pm$ 4.0

The ROI (Region of interest) was defined as including 95% of *H. pylori* with fluorescence emission of the treatment with EGTA and Rh123 alone in total 10,000 of cells at fluorescence 1 channel (630 nm). Values expressed are the means  $\pm$  SD of four independent experiments. Rh123 was used as a fluorescent probe for membrane potential, and CCCP was used as an uncoupling agent. The samples treated with CAH or solvent showed no significant difference from the negative control. In contrast, the samples treated with CCCP were significantly decreased ( $P < 0.01$ ).



-zone ) was performed. As a result of treatment with CCCP, the average fluorescence was significantly decreased ( $90.86 \pm 4.0$ )( $\chi^2$ -test,  $P < 0.01$ ), compared to untreated cells.

Rhodamine123 labelled cells were also analyzed by means of fluorescence microscopy. The proportion of the control cells not cultured with CAH that showed fluorescence indicating the presence of membrane potential was  $4.6 \pm 0.3\%$ . After treatment with CCCP, this decreased to  $0.8 \pm 0.1\%$ , which was a significant change ( $P < 0.01$ ,  $\chi$ -test). By contrast, the proportion of fluorescent cells in samples treated with CAH and solvent were  $5.7 \pm 0.2\%$  and  $5.9 \pm 0.3\%$ , respectively (**Fig. 4-7**). These changes were not significant with respect to the control sample as assessed by using the Chi-square test. This observation is in agreement with the results of cytometric analysis shown in **Table 4-2**.

#### **4.3.5 Effects on protein, DNA and RNA synthesis**

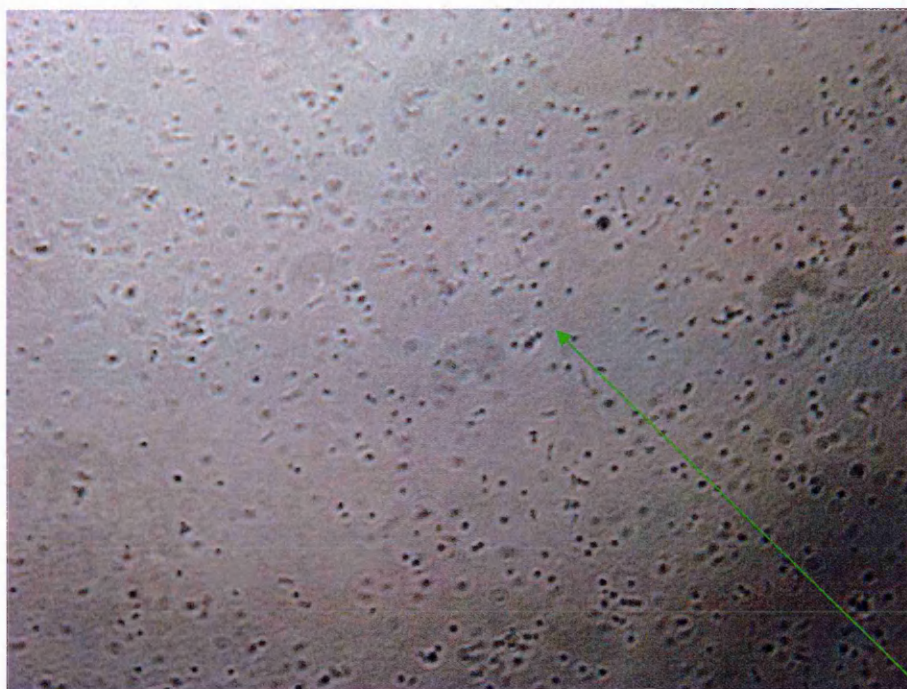
To determine if CAH inhibits the overall synthesis of proteins, DNA or RNA, the cells were incubated with the specific radiolabelled precursors for these three types of macromolecule. After treating with CAH ( $3.15 \mu\text{g/ml}$ ) in the presence of radiolabelled precursors, the radioactivity in the TCA-insoluble materials were analyzed in different days of inoculation by scintillation counting. These results were shown in **Fig. 4-9**. Cells of the CAH-treated samples did not show any change in  $\text{OD}_{595}$ , which indicates CAH inhibited the growth of cells under

**Fig. 4-7 Fluorescent microscopic versions of *H. pylori* following Rh123 staining**

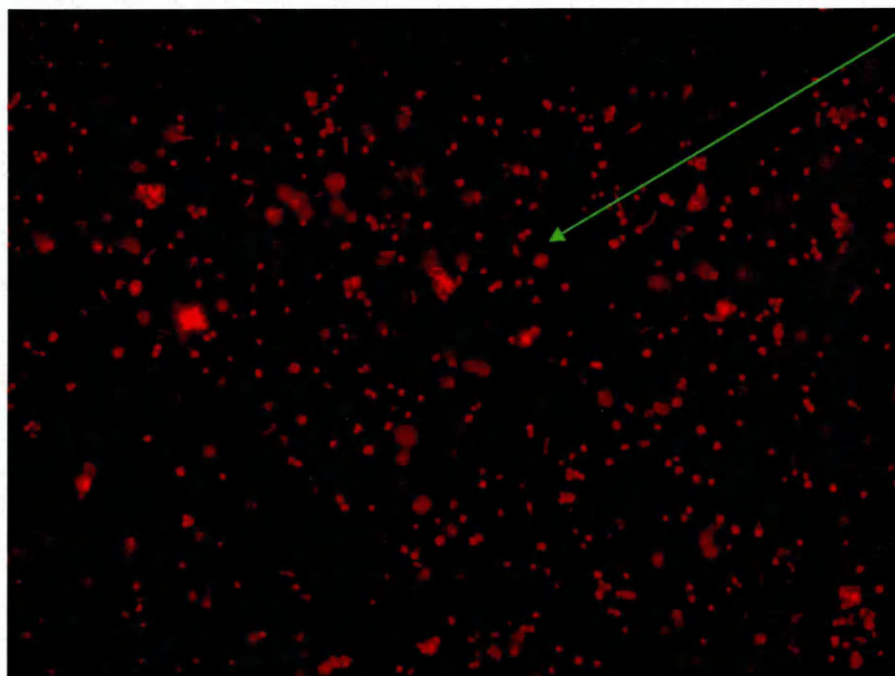
Phase-contrast microscopy was performed at x 1000 under oil immersion. The excitation and emission wavelength of the fluorescence microscopy filters were 455nm and 490nm, respectively.

**(A) Negative control**

**Phase-contrast microscopy**



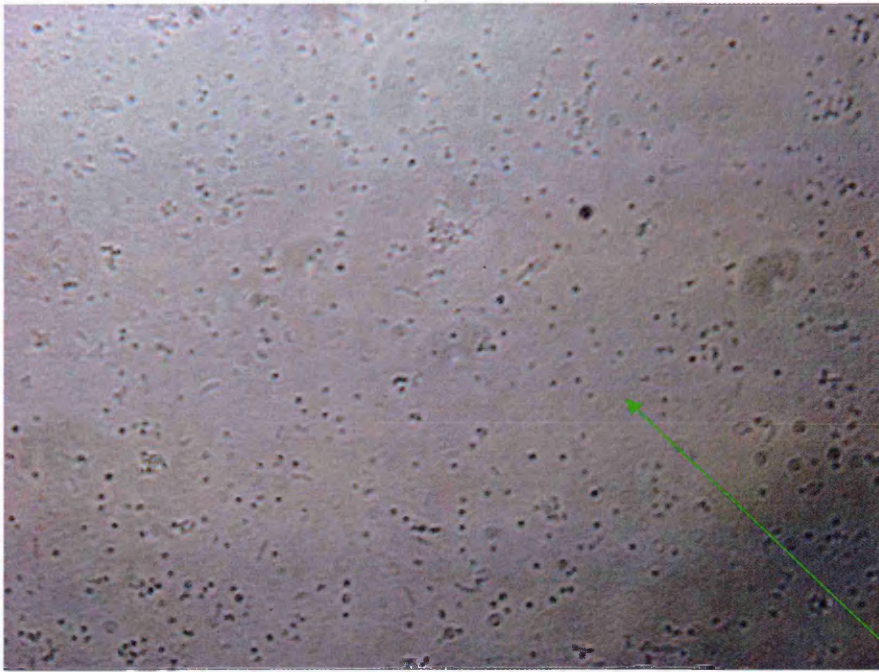
**Fluorescence microscopy**



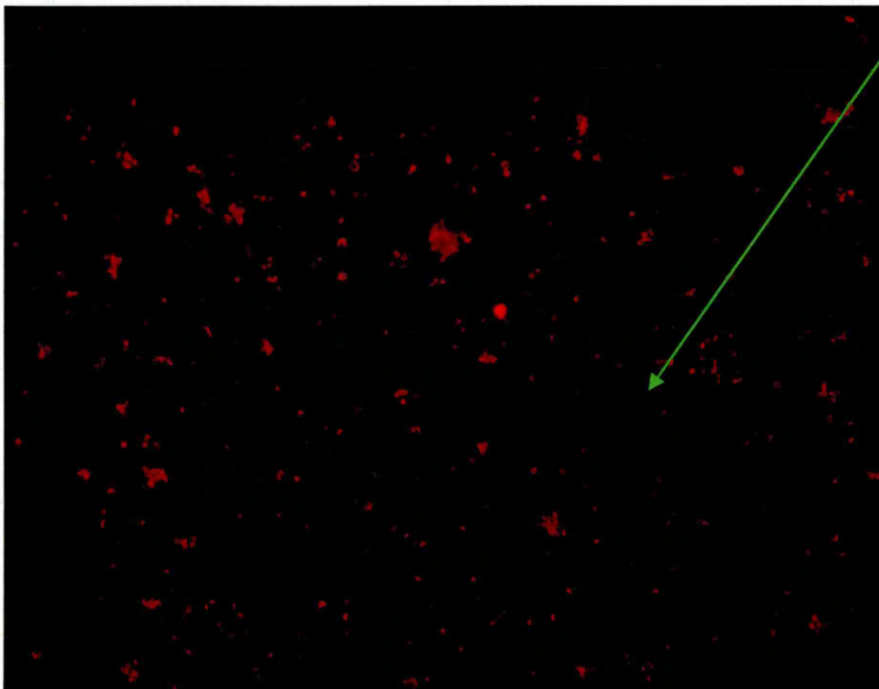
Natural growth  
with fluorescence

**(B) Treated with CCCP**

**Phase-contrast microscopy**



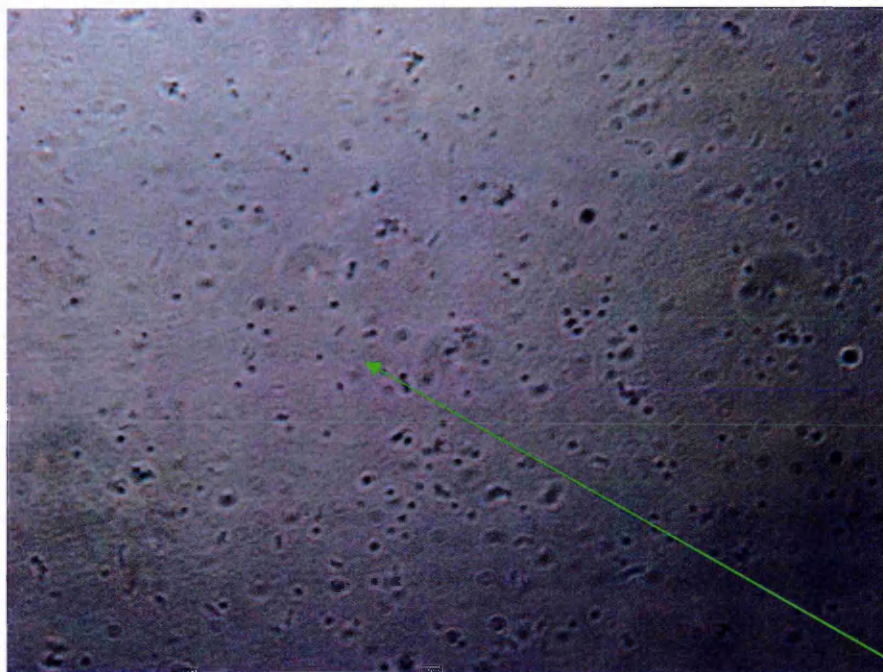
**Fluorescence microscopy**



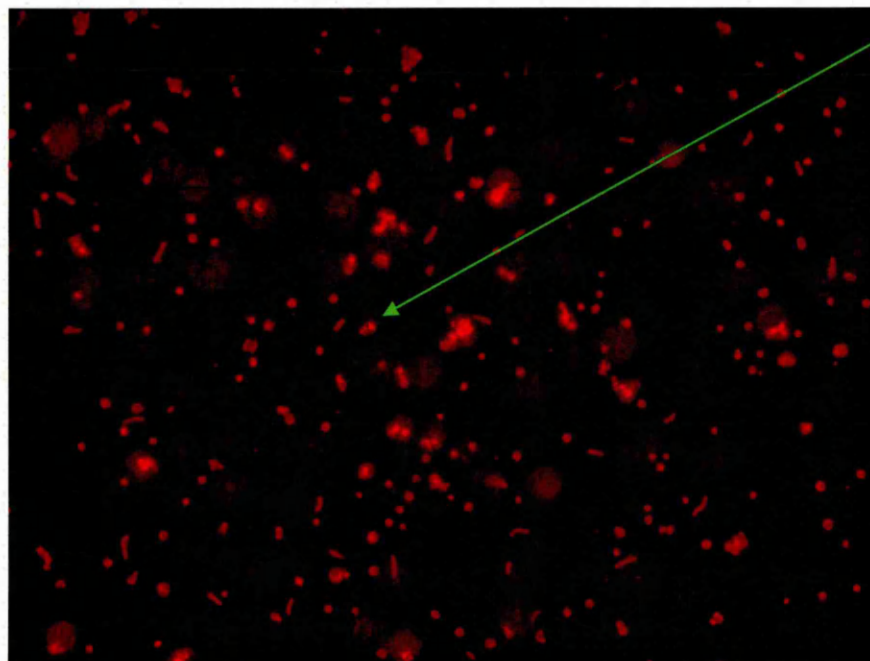
CCCP reduces  
fluorescence

(C) Treated with CAH

Phase-contrast microscopy



Fluorescence microscopy

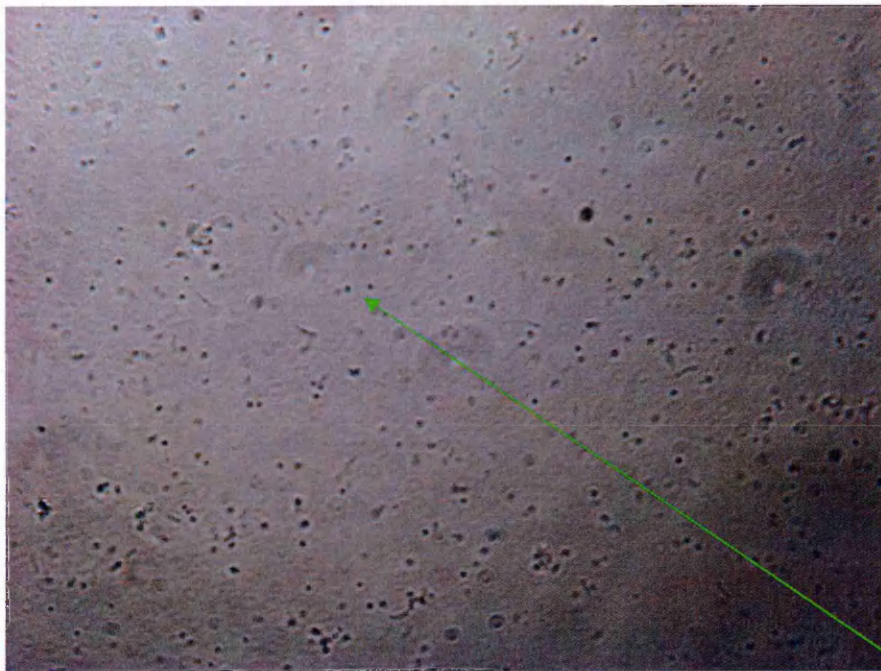


No effect on the  
fluorescence

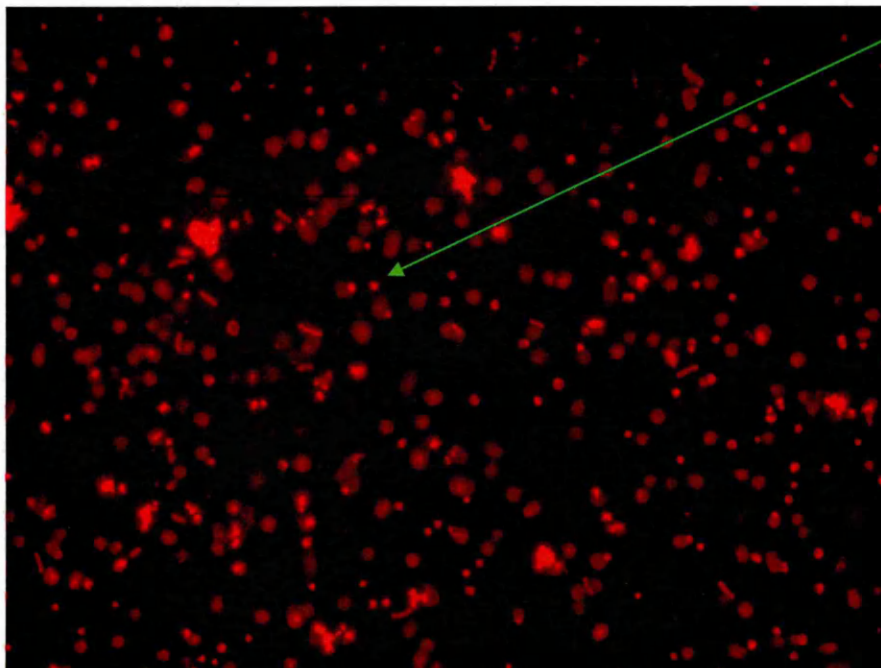


(D) Treated with solvent

Phase-contrast microscopy



Fluorescence microscopy

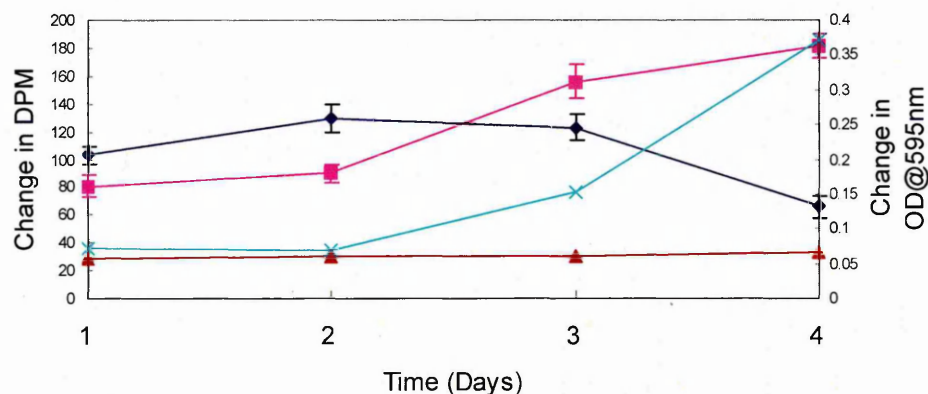


No effect on the  
fluorescence

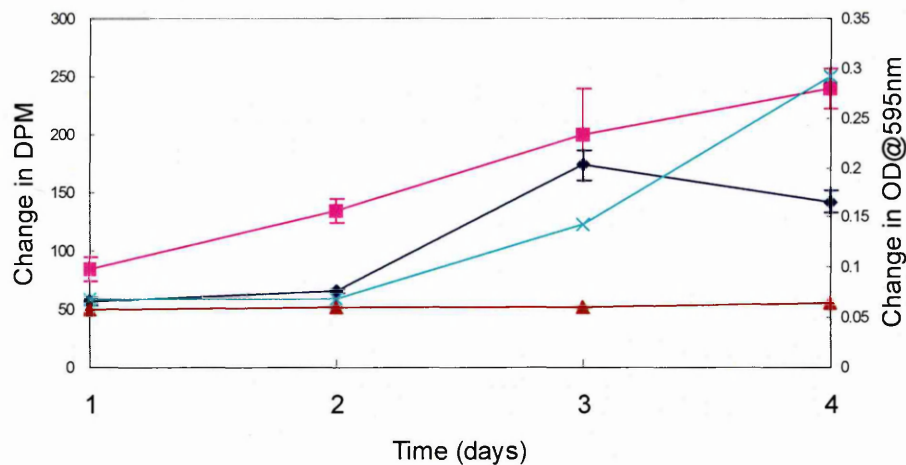
#### Fig. 4-8 Effects of CAH on protein and nucleic acid synthesis

Samples were treated with CAH at the MIC (3.15  $\mu\text{g/ml}$ ), added as a solvent in iso-propanol. Controls contained the same amount of solvent without CAH. Changes in culture OD were measured at the same time as the amount of radioactive label in disintegration per minute (DPM), associated with the macromolecular (TCA-precipitated) fraction. —□—: Change in DPM of CAH-treated experiment; —■—: change in DPM of control; —▲—: change in OD of CAH-treated culture; —x—: Change in OD of control culture. Values of DPM are expressed as means  $\pm$  SD of three independent experiments.

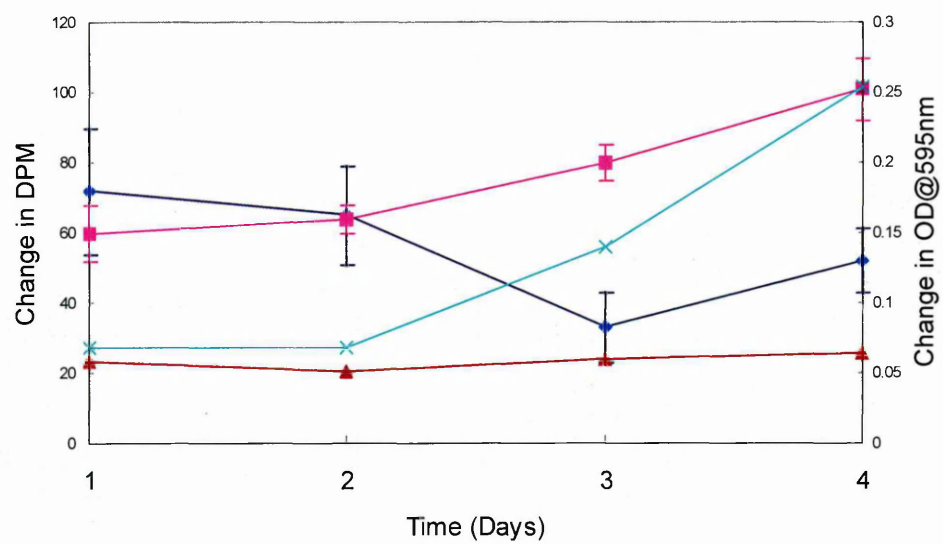
Effect of CAH on protein synthesis (3H leucine label)



Effects OF CAH on DNA synthesis (3H thymidine label)



# Effect of SFoc on RNA synthesis (3H uridine label)



these experimental conditions. After  $\leq 3$  days, radioactive counts with  $^3\text{H}$ -uracil were significantly decreased from 72 DPM to 33 DPM (minus background), meanwhile the radioactive counts with  $^3\text{H}$ -leucine and  $^3\text{H}$ -thymidine were increasing as well as in the untreated cells. Between 3 and 4 days, the two latter counts were progressively reduced. The results indicate a substantial initial inhibition of RNA synthesis to 50% of that seen in untreated cells, but that synthesis of DNA and protein is significantly delayed relative to inhibition of RNA synthesis. It is clear that the loss of RNA synthesis up to day 3 is not due to lack of cell growth since DNA and protein were synthesized in the CAH-treated cells during this period.

#### **4.4 Discussion**

##### **4.4.1 CAH has a bactericidal effect on *H. pylori***

The results (Fig. 4-1) show that *H. pylori* cells inoculated at a concentration 6.25  $\mu\text{g/ml}$  of CAH do not resume growth upon transfer to fresh medium that is free from CAH. Hence CAH is bactericidal agent with a minimum bactericidal concentration (MBC) of 6.25  $\mu\text{g/ml}$ . The MBC is approximately twofold higher than the MIC.

Trypan blue selectively gives appearance of a blue colour to dead cells. Since cells are selective in the compounds allowed to pass through the membrane, in a viable cell trypan blue is not taken up. However, it passes through any



damaged membranes in dead cells. Hence, dead cells are shown as a distinctive blue colour under the microscope. From the results in Section 3 above, the proportion of viable cells treated with CAH was significantly decreased in comparison with those in control samples. This result was in agreement with observation following MBC testing, in which CAH was showing to be bactericidal against *H. pylori*. Thus, it is possible to conclude that the treatment with CAH leads to damage to the cell membrane, allowing trypan blue to enter the cells.

#### **4.4.2 Treatment with CAH induces a significant morphological change in *H. pylori***

Changes in cell morphology of bacteria resulting from starvation are well known (Kusters *et al.*, 1997). In the case of *H. pylori*, the cells convert to the coccoid form from the helical form and this conversion is a passive process that results from cell death (Kusters *et al.*, 1997). Inoue *et al.* (2005) also reported that these morphological changes occur in the presence of agents that have bactericidal actions against *H. pylori* ATCC43504.

From the results in section 3 above, the effects of CAH on the morphology of *H. pylori* are modest and comprise changes to the relative abundance of rods and cocci as well as the sizes of cells. Coincident with the bactericidal effects, the bacteria tended to change from the rod to coccoid shapes and shrank in size.

#### **4.4.3 CAH does not compromise *H. pylori* membrane potential**

Disruption of the cell membrane via binding to it or disrupting its integrity is one of the established mechanisms of antibiotic action on bacterial cells (Hancock, 2005). Fluorescence labeling techniques combined with direct optical detection methods can be used for the rapid assessment of bacterial membrane potential to assess the effect of an antimicrobial agent on membrane potential (Bunthof *et al.*, 2001; Ueckert *et al.*, 1995). These methods are highly sensitive and are of relatively high resolution (Breeuwer and Abee, 2000). Analysis can be performed by epifluorescence or confocal microscopy, scanning or image cytometry as well as flow cytometry. In particular, flow cytometry has been shown to be a powerful tool for analyzing populations rapidly on cell-by-cell basis thus allowing single-sample as well as multi-sample processing. Statistical significance of the data can be determined (Davey, 2002; Nebevon-Caron *et al.*, 2000). Also, flow cytometry facilitates near real-time monitoring, as fluorescence labeling of the cells is rapid (Hewitt and Nebe-Von-Caron, 2001).

In the present study, both fluorescence microscopy observations and flow cytometry were performed to investigate the effects of CAH on bacterial membrane potential and no significant such effects due to CAH were observed. Compared with this treatment the known uncoupling agent, CCCP, led to

substantial and significant reduction in the proportion of cells exhibiting a membrane potential, CAH at 8 times the MIC had no observable effect on the membrane potential of the sub-population of cells that took up Rh123.

#### **4.4.4 CAH significantly inhibits total RNA synthesis in *H. pylori***

Inhibition of protein and nucleic acid synthesis are also established mechanisms of antibiotic action against bacterial cells, in the case of natural compounds as well as chemical medicines (Hancock, 2005). From the results in section 3 this chapter, CAH was initially observed to inhibit RNA synthesis, ie, prior to protein synthesis. One possible mechanism of action is therefore that CAH inhibits the action of RNA polymerase-I. CAH inhibits RNA synthesis by 50% compared that seen in the negative control after 3 days, whilst the inhibition of DNA and protein synthesis delayed approximately one day compared to this.

#### **4.4.5 Effect of CAH on protein expression profiles**

One and two dimensional protein expression profiles were performed in order to establish whether CAH had an effect on expression of any specific proteins in *H. pylori*. Based on the complete genome sequences, there are 1590 predicted genes in the genome of *H. pylori* strain 26695 (Tomb et al., 1997) and 1495 predicated genes for strain J99 (Alm et al., 1999). In previous studies, more than 1800 protein species have been resolved by 2-DE and more than

200 of these have been identified (<http://www.mpiibberlin.mpg.de/2D-PAGE>) (Bumann *et al.*, 2001). The total number of protein spots generated by 2-DE is greater than the number of theoretical ORFs (open reading frames), since a number of proteins can be processed by proteolytic enzymes or modified by methylation, glycosylation and phosphorylation (Cho *et al.*, 2002). To begin to address the influence of CAH and its underlying mechanisms, a few of the proteins of *H. pylori* strain 26695 were differentially expressed in the presence of CAH, when compared with expression profiles during normal growth, and these were chosen for identification. Since no spots were clearly observed to increase in intensity upon treatment with CAH, 5 protein spots were chosen that clearly decreased in intensity or disappeared when CAH was added (**Table 4-1**). These five proteins were characterized and presumptively identified using MS corrected with bioinformatics. These identified glutamyl-tRNA reductase and superoxide dismutase (SOD) for which it is the easiest to see how their low of expression may be linked to antimicrobial activity. Glutamyl-tRNA reductase has been demonstrated to be involved the NADPH-dependent reduction of the initial step of tetrapyrrole biosynthesis in *Escherichia coli* (Lüer *et al.*, 2007). Glutamyl-tRNA reductase (HemA protein) reduces glutamate to form glutamate-1-semialdehyde, which is then converted to 5-aminolevulinic acid (ALA) and can be necessary for growth of the nutritionally versatile organisms including *H. pylori*. It has previously been identified as a target for development of another novel antimicrobial agent

(Wang *et al.*, 1999). Superoxide dismutase has been shown to be a virulence factor for this pathogenic microaerophile (Richard *et al.*, 2001). SOD is part of the mechanism that protects *H. pylori* against toxic stress (Chuang *et al.*, 2005) and low SOD upon treatment with CAH is consistent with a breakdown of the cells stress defence mechanism.

#### **4.5 Conclusions**

In these studies, it has been found that CAH is a bactericidal agent. Five proteins specifically reduced in expression in response to CAH have been identified. The effects of the CAH on inhibition of RNA synthesis may give insight into the mechanism of anti-*H. pylori* action but since only 50% inhibition of RNA synthesis was observed, it may be that inhibition of RNA polymerase is not the prime site of action of CAH. Whether CAH has any direct effect on integument integrity remains an open question, because of the contrasting results observed from typan blue exclusion and Rh123 fluorescence.

## **Chapter V**

### **Overall Discussion and Conclusions**

The project is the first comprehensive investigation of the inhibitory (bactericidal) effects of components of celery seed on growth and viability of *H. pylori*. The results of the present study have shown that: (1) CAH is a novel component from celery seed with specific inhibitory effects against *H. pylori*; (2) the actions of CAH are novel and target *H. pylori* growth mechanisms. (3) CAH has been tentatively identified as a dimeric molecule with molecular weight 384 and the molecular formula  $C_{24}H_{32}O_4$ . CAH has potential for safer use and a low cost therapy, and may avoid the alterations in GI flora. This research could provide the pharmaceutical industry with a novel component of celery seed oil in the search for new drugs and medicines for eradication of *H. pylori*.

### **5.1 Constituents of celery seed and bioactivity**

Previous chemical studies had indicated that the principal constituents of celery seed are 1.5-3% volatile oil, coumarins, furanocoumarins, flavonoids, phenolic compounds, choline, ascorbate, involatile oil, fatty acids and unidentified alkaloids (detailed in section 2 of chapter 1). The major components of these essential oils are monoterpene hydrocarbons (46%) and phthalides (42.3%) (MacLeod and Ames, 1989). Previous research in pharmacognosy has attempted to correlate the well characterized bioactive effects of celery seed with the phthalide constituents (Oiyee and Muroki, 2002; Raffa, 2005; Riddle, 2004). Previously described active monomeric phthalide components from celery seed have been characterized at the chemical

structure level, including sedanolide (3-butyltetrahydrophthalide), sedanenolide (3-n-butyl-4,5-dihydrophthalide), 3-n-butylphthalide and N- or J- senkyunolide (**Fig.1-15**) (Kitajima *et al.*, 2003; Momin and Nair, 2002; Macleod *et al.*, 1988). Also, phthalides have been found to exist together with dimeric derivatives in a number of important medicinal herbs of the umbelliferae, including *Ligusticum eallichii*, *L. chuangxiong*, *L.portere*, *Levisticum officinale* and *Cnidium officinal* (Lin *et al.*, 1998). These previously identified dimeric phthalides have been reported as religustilde ( $M_r=380$ ), 3,8-dihydro-diligustilide ( $M_r=382$ ); 4,5-dihydro-diligustilide ( $M_r=378$ ); angelicide ( $M_r=380$ ); levistolide ( $M_r=380$ ) and tokinolide ( $M_r=380$ ) (Lin *et al.*, 1998; Lim *et al.* 2006; Zhang *et al.* 2003). Also, several known phthalide dimers from the Apiaceae do contain a spiroketal ring and this occurs through a dimerisation process involving cyclization of a butylidene phthalide side-chain, which is not present in monomeric sedanenolide, sedanolide or n-butylphthalide which all have a saturated n-butyl side-chain.

From the bioactivity profile of CSE, several investigators have reported that phthalides from CSE are responsible for the antispasmodic, sedative and diuretic properties, and acted to promote anti-hypertension, anti-inflammation and antibacterial effects (detailed in Chapter 1). Most significantly, antimicrobial phthalides have already been shown to be very effective and highly specific against *H pylori*, due to the presence of a spiroketal ring to



greatly enhance this activity (Dekker *et al*, 1997, Radcliff *et al*, 2008). More interestingly, some dimeric phthalides extracted from other medicinal plants have been reported to have bioactivity. The traditional Chinese medicinal herb *chuangxiong*, which is used as a preventive and therapeutic agent against atherosclerosis, may owe its beneficial properties to the presence of dimeric phthalides (Zhang *et al*. 2003). 3,8-Dihydro-diligustilide extracted from *chuangxiong* was found to be a potent and specific activator of the progesterone receptor. Progesterone is a steroid hormone with an essential role in human reproduction (Lim *et al*. 2006). Phthalides and dimeric derivatives from *Danggui* are useful to treat anemia, hypertension, chronic bronchitis, asthma, rheumatism and cardiovascular diseases via tonifying (thinning) the blood and alleviating female irregular menstruation and amenorrhoea (Lin *et al.*, 1998). From the present study, CAH, a dimeric phthalide from celery seed, was found to be bactericidal against *H. pylori* *in vitro* and its chemical structure remains to be determined.

## **5.2 Activity of CSE toward and specificity for *H. pylori***

The activity of an antibiotic against microorganisms is measured in terms of its minimal inhibitory concentration (MIC), which, although not as informative as the minimal bactericidal concentration, is easier to measure (Mégraud, 1998). The microplate assay used in the present study is a basic and effective way to evaluate the antibacterial activity at the MIC level. Currently available

antibiotics for effective *H. pylori* treatment are confined to amoxycillin, clarithromycin, metronidazole and tetracycline. The effectiveness of any new anti-*Helicobacter* agent must therefore be compared to these existing drugs (Zullo *et al.*, 2003). In a previous Korean study in 2003, MICs of clinical *H. pylori* isolates to these established antibiotics are in the ranges of amoxycillin 0.5 – 8 µg/ml; clarithromycin 0.0625 – 64 µg/ml; metronidazole 1-8 µg/ml and tetracycline 3 - 5 µg/ml and is agreement with Kim *et al.* (2004). The MIC of tetracycline was measured as 3.15 µg/ml in the study above, in agreement with the reference above. In the present studies, tetracycline was employed as the positive control. The results of this project indicated the bioactivity of CAH against *H. pylori* was comparable to tetracycline *in vitro*. Also, this bioactivity of CAH is specific for *H. pylori*; as no effect on growth of other bacteria tested (*Campylobacter jejuni* and *Escherichia coli*) was detected.

Changes in the morphology of the bacteria may also give information relevant to the agent's mechanisms of action (Mai *et al.*, 1989). In *E.coli*, the cells generally become smaller and almost spherical during exposure to antibiotics and thus exhibit similar morphological changes to those seen during starvation (Siegele and Kolter, 1992). In *Campylobacter spp.*, a related bacterium to *H. pylori*, the cells change from a bacillary form to a coccoid form under unfavorable conditions. The coccoid form is now considered to be a degenerate morphological phase associated with dead bacteria that does not

pose an infectious risk (Hazeleger et al., 1995). From the present study, the effects of CAH on the morphology of *H. pylori* are modest and comprised changes to the shapes and sizes of rods and coccid, coincident with the bactericidal effects, a significant proportion of the bacteria changed from the rod to coccoid shape and shrank in size (Section 4.3.2 of Chapter 4). Inoue *et al.* (2005) also reported that the same morphological change happened in the presence of bactericidal agents against *H. pylori* ATCC43504. In case of *H. pylori*, Kusters *et al.* (1997) revealed that the conversion of the helical form to a coccoid form is a passive process that results from cell death (Kusters, *et al.*, 1997). This conclusion is consistent with the bactericidal effects of CAH and its effect on trypan blue exclusion.

### **5.3 Novel mechanisms**

In general, the mode of inhibition of bacterial growth by antibiotic operates via one or more of following basic mechanisms:

(1) Inhibition of cell wall synthesis or disruption of the cell membrane, which is by far the most common mechanism. This effect involves inhibition of cell wall transpeptidation, (e.g. by penicillin and cephalosporin), or transport of cell wall components by carriers (e.g. by bacitracin). The disruptive effects on the structure of membrane may occur via binding to the membrane or disrupting its integrity (e.g. by polymyxin B).

(2) Inhibition of protein synthesis (which is the second largest class). These effects include interference with the ribosomal 30S subunit (e.g. by aminoglycosides and tetracycline), 50S subunit (e.g. by chloramphenicol and macrolides) or ribosomal translocation (e.g. fusidic acid).

(3) Inhibition of nucleic acid synthesis. As well as inhibition of DNA polymerase, this effect includes interference with DNA gyrase (e.g. by ciprofloxacin and quinolones) and inhibition of reverse transcriptase including that of retroviruses (e.g. by azithromycin).

(4) Interference with the transcription by RNA polymerase (e.g. by rifampacin).

(5) Action as an inhibitor of metabolic activity, e.g. inhibition of folic acid synthesis (by sulfonamides), blockage of tetrahydrofolate synthesis (e.g. by trimethoprim) or disruption of NAD<sup>+</sup> metabolism (e.g. by isoniazid) (Hancock, 2005; Prescott *et al.*, 2002).

These actions of conventional antibiotics serve as a basis for comparison for understanding the mechanisms of the inhibitory effects of the novel component such as the novel component, CAH, from celery seed.

**5.3.1 Bactericidal effect:** Antibiotics act against bacteria that caused diseases by in one of two ways, which are the bacteriostatic actions (interfering with bacterial growth) and bactericidal actions (killing bacteria directly) (Prescott *et al.*, 2002). Some antibiotics can have both bactericidal and bacteriostatic activities, depending upon the concentration of agent that is present. Bacteriostatic agents interfere with bacterial growth and reproduction by inhibiting essential metabolic processes such as the synthesis of proteins or nucleic acids (see previous section), while bactericidal agents kill or prevent growth of bacteria by their effect on vital bacterial structures and processes. From the present experiments (MBC measurement and trypan blue exclusion), CAH was shown to have bactericidal effect against *H. pylori*. Also, CAH has bacteriostatic effect at concentration  $\geq 3.15 \mu\text{g/ml}$ . This implied the inhibition of essential metabolic processes as a mechanism of action of this agent. These effects have been confirmed in the radiolabelling experiments involving investigations of effects on nucleic acid and protein synthesis.

**5.3.2 CAH does not cause disruption of the cell membranes:** As mentioned in the beginning of this section, disruption of the membrane following binding of drugs to the membranes or disruption of their integrity is one of the most common mechanisms of the antibiotic action on bacterial cells. Like most bacteria, *H. pylori* use an electrochemical gradient of hydrogen ions across its cytoplasmic membrane in order to transduce cellular energy. This potential is

approximately 131 mV in nature at physiological pH values determined by using fluorescent dyes (Scott, *et al.*, 1998). The transmembrane potential reflects the viability of the organism by demonstrating the maintenance of a proton motive force. In this project, both fluorescent microscopic assessment and flow cytometry were performed to investigate the effects of CAH on bacterial membrane potential. The results from both forms of studying the membrane potential indicated that CAH did not significantly compromise the membrane integrity in *H. pylori*. Nonetheless, It is possible that CAH leads to permeabilisation of the integument of *H. pylori*, since it reduced the properties of cells that excluded trypan blue.

**5.3.3 Selective effect of CAH on protein expression profiles:** Various authors have reported significant difference in the 2-DE protein profiles of *H. pylori* under stress conditions, as well as antibiotics. There has been relatively little work to investigate the effect of antibiotics on the proteome of *H. pylori*. Recently, Chuang *et al.* (2005) showed that two major proteins of *H. pylori*, AhpC and UreE were consistently reduced in expression under oxygen stress. They proposed that inhibition of AhpC resulted in the absence of anti-oxidative capacity of the cells and repression of UreE was a critical factor for decline of urease activity. In the present study, a total of more than 300 protein spots were observed in 2-D gels of treated and untreated samples. The five proteins that change most markedly in response to CAH were identified. Among those

proteins that changed most significantly in response to CAH, all of which decreased in abundance when CAH was added. The two largest changes lay in (1) glutamyl-tRNA reductase involved in the NADPH-dependent reduction during the initial step of tetrapyrrole biosynthesis and (2) superoxide dismutase known to be a virulence factor for *H. pylori* (Lüer *et al.*, 2007; Richard *et al.*, 2001). These changes must have resulted from either the direct inhibition of protein/gene expression or at the level of protein degradation and protein processing (Kusters *et al.*, 1997).

#### **5.3.4 CAH significantly inhibits overall RNA synthesis in *H. pylori*:**

Inhibition of RNA synthesis is one of the five basic mechanisms of antibiotic action against bacterial cells due to inhibition of RNA polymerase or inhibition of the synthesis of the precursors of RNA (Hermann and Westhof, 1998). Rifamycins are the common antibiotics that inhibit RNA synthesis. In the case of *H. pylori*, rifamycins bind to rRNA polymerase and topoisomerases to result in transcriptional inhibition (Gerrits *et al.*, 2006). The component from celery seed, CAH inhibits RNA synthesis by 50% compared to that seen in the negative control at 3 days, while DNA and protein synthesis do not change significantly. More work is need to determine the step during RNA synthesis that is (partially) inhibited by CAH.

#### **5.3.5 CAH in the context of antibiotic resistance: Increasing antibiotic**

resistance is one of the most common reasons for treatment failure and a growing problem worldwide (Qureshi and Graham, 2000). In Bulgaria, Boyanova *et al.* (2008) reported that the primary clarithromycin resistance rates in *H. pylori* increased significantly from 1996-1999 (10%) to 2005-2007 (17.9%  $P<0.02$ ) and resistance rates in treated adults were significantly higher than those in untreated adults ( $P<0.01$ ). Increase in clarithromycin resistance has been also detected by Japanese, French, and Italian authors (Masuda, *et al.*, 2004; De Francesco, *et al.*, 2007 and Raymond, *et al.*, 2007). Mégraud (2005) concluded that frequencies of primary resistance of *H. pylori* ranged from 1.7 to 25% to clarithromycin, from 9 to 62.7% to metronidazole, up to 0.5% to tetracycline and up to 0.9% to amoxicillin. Despite improved efforts toward the rational use of existing agents, there remains a pressing need for novel antibiotics (Lira *et al.*, 2007). This new component, CAH from celery seed may become starting material for the synthesis of new drugs. Future work may reveal whether CAH could be used as a therapy against *H. pylori* strains that are resistant to established antibiotics. Also, studies are required to determine how readily *H. pylori* might develop resistance to CAH.

#### **5.4 Economics of CAH for pharmaceutical exploitation**

The increasing cost of antibiotics is one of the chief problems of current anti-*H.pylori* therapies. For the cost of prescriptions in England in 1991 –1993, spending on omeprazole and other proton pump inhibitors has increased from



£20 million to £94 million in two years (Moore, 1995). Due to their low cost and absence of side effects, plants have played a significant role in maintaining human health and improving the quality of human life for thousands of years (Craig, 1999). The World Health Organization estimated that 80% of the earth's inhabitants rely on traditional medicine for their primary health care needs, and most of this therapy involves the use of plant extracts or their active components.

Celery is widely cultivated in the temperate zones as an important food crop and the leaf stalks are relished as a popular vegetable (Momin *et al.*, 2000). In 1984, an annual celery seed oil production was 25 tonnes and a celery herb oil production was 0.5 tonnes (Lawrence, 1987). One current estimate of the annual consumption of celery seed oil is 40 tonnes / annum (Falzari and Menary, 2005). Raw material for producing CAH on the industrial scale is therefore readily available at low cost. Also, if structural information for CAH becomes available in the public domain, potentially low-cost chemical routes to this valuable molecule may be developed.

### **Suggested Future Investigations**

Further *in vitro* studies are needed to define the mechanism of action of CAH. Thus, it would be beneficial to investigating the effects of CAH on intermediary and lipid metabolism of *H. pylori*, for instance glucose metabolism, pyruvate metabolism, lipid metabolism, respiratory chains, and urease and nickel uptake.

This study has reported CAH is defined as an antibiotic agent on *H. pylori*, which is liable to have a novel antibiotic mechanism *in vitro*. For the development of CAH toward a new medicine, the future pharmacological and clinical studies need to be performed to investigate this effect on animal (e.g. mouse) and human (gastric environment) models of infections from *H. pylori*. Also, the possibility of resistance to CAH should be explored by testing it against strains resistant to other antibiotics and performing experiments to see whether resistant mutant or whether resistant mutants can be isolated during laboratory growth.

In this project, CAH purified from celery seed has been isolated and identified as a pure component by thin layer chromatography. For large-scale production, development of a chemical or biocatalytic route to CAH would be a great advantage. Also, availability of chemically synthesized CAH would verify this molecule as the active component in the celery seed derived material, and

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**Publication and Conferences relevant to**  
**the Thesis**

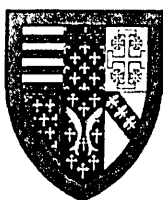
# *INFLAMMOPHARMACOLOGY 2008*

*Queens' College, Cambridge  
September 8<sup>th</sup> to 10<sup>th</sup>, 2008*



*Watson Buchanan 1930 - 2006*

*Programme and Abstracts*



## A NOVEL COMPOUND FROM CELERY SEED WITH A BACTERICIDAL EFFECT AGAINST *HELICOBACTER PYLORI*

Yong Zhou<sup>1</sup>, Brian Taylor<sup>2</sup>, Thomas J Smith<sup>1</sup>, Malcolm Clench<sup>1</sup>, Noel Davies<sup>3</sup> and KD Rainsford<sup>1</sup>

<sup>1</sup> Biomedical Research Centre, Sheffield Hallam University, Sheffield. S1 1WB, UK

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<sup>3</sup> Central Science Laboratory, University of Tasmania, Private Bag 74, Hobart, Tasmania, 7001, Australia

As well as peptic ulcers, *Helicobacter pylori* is associated with the development of gastritis, gastric adenocarcinoma and lymphoma; it being been classified as a class I carcinogen in humans (International Agency for Research on Cancer Working Group, 1994). Although the bacteria can be eradicated in up to 90% of patients, side effects, poor compliance and the resistance of the bacteria to antibiotics are common causes of frequent treatment failure. Celery seed extracts (CSE) from a unique source in India has been used a herbal medicine since antiquity and found to have anti-inflammatory and gastroprotective properties (Butters et al., 2004; Whitehouse et al., 2001). This study followed on observations that crude extracts of CSE exhibited anti-helicobacter activity (Rainsford & Liu, 2003).

CSE was selectively fractionated using organic solvents followed by HPLC. Fractions were collected and bio-assayed against different strains of *H. pylori* using conventional culture methods. The most potent component that was obtained from HPLC and purified was designated CAH. This compound had potent bactericidal effects against *H. pylori*; the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) being  $3.15 \mu\text{g ml}^{-1}$  and  $6.25 - 12.5 \mu\text{g.ml}^{-1}$ , respectively. This compares favorably with the MIC and MBC of tetracycline, which are both  $3.15 \mu\text{g.ml}^{-1}$ . The isolated compound had highly specific inhibitory effects on *H. pylori*, since no inhibitory activity was detected against *Campylobacter jejuni* or *Escherichia coli* at these levels. The molecular ion of CAH was measured as 384.23 by mass spectrometry (using peak matching), giving the empirical formula as  $\text{C}_{24}\text{H}_{32}\text{O}_4$ . The MS and NMR data strongly suggest this compound is a sedanenolide dimer. From radioactive bioassay, CAH inhibits RNA synthesis by 50% of that seen in a negative control in 3 days, while DNA and protein synthesis was unchanged.

In conclusion, (1) CAH is a novel component from celery seed with effective and specific inhibition against *H. pylori*; (2) there are novel anti- *H. pylori* mechanisms and there is no evidence of resistance; (3) it has potential for safer use and a low cost therapy; (4) it may avoid the alterations in GI flora caused by using antibiotics. These results suggest that the new compound may be suitable for further investigation as an

agent for treating *H. pylori* infections.

International Agency for Research on Cancer Working Group. (1994) *IARC Monogr. Eval. Carcinog. Risks Hum.* 61: 1-124

Butters D.E. *et al.* (2004) *US Patent* 6,761,913;

Whitehouse M.W. *et al.* (2001) *Inflammopharmacology*, 9: 201-209

Rainsford K.D. & Liu Z.-P. (2003) *US Patent* 2006001396



# A Novel Compound from Celery Seed with a Bactericidal Effect against *Helicobacter pylori*

Yong Zhou<sup>1</sup>, Brian Taylor<sup>2</sup>, Thomas J Smith<sup>1</sup>, Malcolm Clench<sup>1</sup>, Noel Davies<sup>3</sup> and KD Rainsford<sup>1</sup>

<sup>1</sup> Biomedical Research Centre, Sheffield Hallam University, Sheffield. S1 1WB, UK <sup>2</sup> Department of Chemistry, University of Sheffield, Sheffield, UK

<sup>3</sup> Central Science Laboratory, University of Tasmania, Private Bag 74, Hobart, Tasmania, 7001, Australia

## Introduction

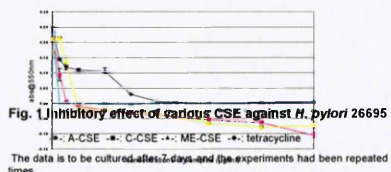
As well as peptic ulcer, *H. pylori* is associated with the development of acute, chronic gastritis, gastric adenocarcinoma and gastric lymphoma (MALT), and has been classified as a class I carcinogen in humans. Although the bacteria can be eradicated in up to 90% of patients by the costly combination of a proton pump inhibitor with multiple antibiotics, side effects, poor compliance and the resistance of the bacteria to the antibiotics are common causes of frequent treatment failure.

Celery seed (*Apium graveolens*) has been used as herbal medicine for thousands of years in Indian. It is useful for the treatment of urinary calculi, gut diseases, the relief of flatulence and griping pains, reduction of visceral spasm, stimulation of the smooth muscle of womb. Momin (2001) reported the antifungal, mosquitocidal and nematocidal activities of the methanolic extract of celery seed. Ethanolic extracts have also been found to have anti-inflammatory and anti-ulcer activity in rodent models (Butters *et al.*, 2004, US Patent: 6,761,913; Michio, 2005, US Patent: 5916565).

In present studies, based on an initial observation of ethanolic extracts of celery seed having anti-*H. pylori* properties by Rainsford and Liu (US Patent: 20060013906), we investigated these effects, identified and characterized the components from the complex mixture of essential oils in celery seed oil, which was found to be active against *H. pylori*.

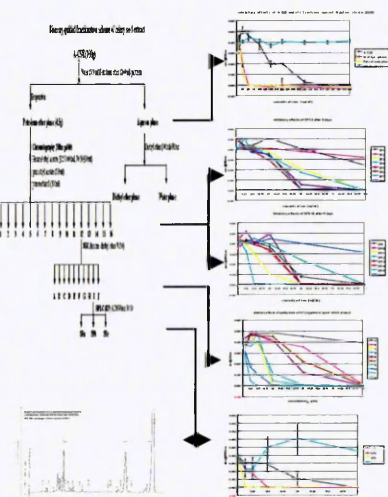
## Effects of various extracts of celery seed on the growth and viability of *H. pylori*

Three celery seed extracts (A-CSE, C-CSE and ME-CSE) were initially tested for their inhibitory effects against *H. pylori* strain 26695 by using microplate antimicrobial activity assays. All three fractions showed activity against *H. pylori* 26695 with MICs being 300 µg/ml (A-CSE), 25 µg/ml (C-CSE) and 50 µg/ml (ME-CSE) respectively, (Fig.1). As the OD<sub>550</sub> of culture treated with C-CSE and ME-CSE decreased, it is likely that these extracts induced lysis of *H. pylori* cells.



## purification of the active components and the antibacterial activity

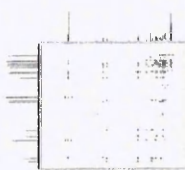
A-CSE was fractionated step by step in the following scheme. All fractions in each step were tested for antibacterial activity against *H. pylori* strain 26695 and the MICs were determined.



## Chemical characteristic of the highly anti- *H. pylori* major fraction CAH

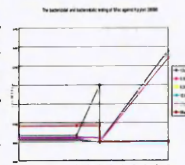
The electrospray mass spectrum of CAH showed ions at  $m/z$  385.23, 407.23 and 423.20. These ions represent  $[M+H]^+$ ,  $[M+Na]^+$  and  $[M+K]^+$ , respectively for a compound of molecular weight 384. This was subsequently confirmed by electron ionisation which showed a weak  $M^+$  ion at  $m/z$  384 (1%) and prominent fragment ions at  $m/z$  193 (100%), 192 (80%) and 107 (55%). The molecular ion was measured as 384.22943 by peak matching, giving the empirical formula as  $C_{24}H_{32}O_4$ .

$^{13}C$  and  $^1H$ - $^{13}C$  correlation spectra: 24 carbon signals, including 2 of carbonyls ( $C=O$ ), 1 of  $sp^2$   $CH$ , 1 of  $sp^2$   $C$ , 7 of  $sp^3$  carbons as  $CH$  and  $CH_2$  groups and 12  $sp^3$  carbons as  $C$  and  $CH_3$  groups.

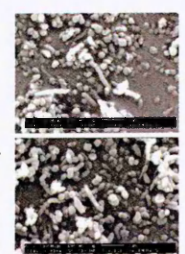


## Studies of anti-*H. pylori* mechanism

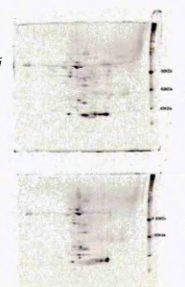
After bacteria were initially cultivated with the compound and re-cultured in its absence, CAH is bactericidal having an MBC of 6.25 µg/ml, which was approximately twofold higher than the MIC.



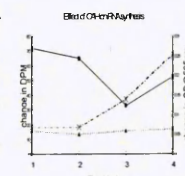
After treating with CAH, there were 19.7 % of *H. pylori* to preserve in the rod form and 80.3% in the coccoid form (Fig.A), compared to 29.8% and 71.2% in untreated sample (Fig.B). The exposure to SFC gave rise to the morphological changes in which the rod forms were converted to coccoid forms.



By using 2-D electrophoresis, both the 2-DE profiles of *H. pylori* grown under normal (A) and treated with CAH (B) exhibited the distinct spots between masses 14 and 100 kDa and  $pI$  3 and 9 range analysis. There were 338 spots found in the untreated gel and there were 189 of spots, which matched those in the treated gel.



From radioactive testing, a substantial inhibition of RNA synthesis by 50% (72 DPM to 33 DPM) of that seen in untreated cells by CAH firstly in 3 days, meanwhile synthesis of DNA and protein is not significantly decreased.



## Conclusion

CAH was the pure component of CSE, as a colorless oil without odor, with 384 of molecular weight and  $C_{24}H_{32}O_4$  molecular formula. It may be a dimerisation of phthalide across any pair of double bonds, and also in the case of sedanolide via Diels-Alder addition.

The present results have shown: (1) CAH is a novel component from celery seed with effective and specific inhibition against *H. pylori*; (2) there are novel anti-*H. pylori* mechanisms and no resistance; (3) it is potential for safer use and a low cost therapy; (4) it may avoid the alterations in GI flora caused by using antibiotics.

## References:

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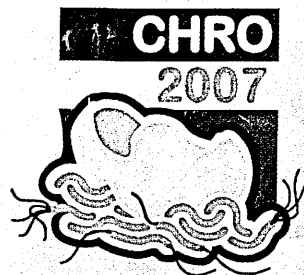
Butters, D.E., Davis, C.H., McGeary, R.P., Pownall, M.C., Rainsford, K.D., Whitmore, M.W. (2004) Extracts of celery seed (Apium graveolens) and their potential as anti-inflammatory and anti-ulcer agents. J. Pharm. Med. 6, 27-37.

VOLUME 54 • SUPPLEMENT 1 • 2007

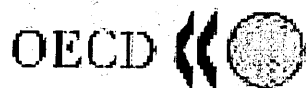
# Zoonoses

## AND PUBLIC HEALTH

EDITOR: MARY TORRENCE



**14th International Workshop on  
Campylobacter, Helicobacter and Related Organisms Rotterdam,  
The Netherlands, 2-5 September 2007**



P157

# Genomic Analysis of *Helicobacter pylori* Strain Isolated from a Patient with Gastric Cancer

T. L. Cover and M. S. McClain  
Vanderbilt University, Nashville, TN, USA

**Introduction:** Genome sequence analysis of two *Helicobacter pylori* strains (26695 and J99) revealed a high level of genetic diversity, and analyses of short segments of DNA in collections of *Helicobacter pylori* strains revealed geographic diversity. In this study, we sought to analyze the genome sequence of an *H. pylori* strain isolated from an East Asian patient with gastric cancer.

**Methods:** The *Helicobacter pylori* strain containing a type *slc vacA* allele and able to translocate CagA into AGS cells was selected for analysis. Chromosomal DNA was isolated and the genome sequence was analyzed by pyrosequencing.

**Results:** Approximately 1.6 MB of sequence data was obtained. The organization of the *cag PAI* in this strain was similar to that in strains 26695 and J99. Blast score ratio analysis of the predicted open reading frames from the three genomes identified 60–100 strain-specific genes in each genome and about 1350 ORFs that were conserved among all three strains.

**Conclusion:** Proteins encoded by strain-specific alleles may contribute to the pathogenesis of gastric cancer.

P158

# Analysis of the *Helicobacter pylori* Proteome by Multidimensional Liquid Chromatography Coupled to Mass Spectrometry (LC/MS/MS)

T. L. Cover, M. S. McClain, L. J. Zimmerman and A. Ham  
Vanderbilt University, Nashville, TN, USA

**Introduction:** *Helicobacter pylori* genomes contain about 1600 ORFs. Previous analyses of *H. pylori* proteomes by 2D gel electrophoresis identified several hundred bacterial proteins expressed during growth *in vitro*. In this study, we investigated whether a more comprehensive analysis of the *H. pylori* proteome could be accomplished by using multi-dimensional liquid chromatography coupled to mass spectrometry (LC/MS/MS).

**Methods:** *Helicobacter pylori* strain 26695 was grown in broth culture, the bacteria were harvested, and bacterial proteins were solubilized with detergent. Proteins were run into an SDS-PAGE gel, and then in-gel digestion and peptide extraction were performed. The resulting peptides were fractionated using strong cation exchange chromatography and analyzed by reversed-phase chromatography coupled to an LTQ ion trap mass spectrometer. Tandem mass spectra were searched against an *H. pylori* strain 26695 database using the QUEST algorithm.

**Results:** A total of 559 *H. pylori* proteins were identified by at least one or more unique peptides, with a false-positive rate of < 2%. Many of these *H. pylori* proteins have not been detected previously by proteomic analyses using 2D gel electrophoresis. Multiple proteins coded by the *cag PAI* and 24 outer membrane proteins (including pQ and two *VacA* paralogues) were identified. In addition, numerous proteins annotated as 'conserved hypothetical proteins' (n = 40) or '*H. pylori*-specific hypothetical proteins' (n = 96) were identified. An analysis of the broth culture supernatant identified *VacA* and 41 other *H. pylori* proteins.

**Conclusion:** The comprehensive analysis of bacterial proteomes using MS/MS-based proteomic platforms may provide new insights into

the expression of bacterial proteins during growth under various conditions.

P159

# The Role of Bacteriophage in the Genome Dynamics of *Campylobacter jejuni*

I. F. Connerton, A. E. Scott, A. R. Timms, P. L. Connerton, C. Loc Carrillo and K. A. Radzum  
University of Nottingham, Nottingham, United Kingdom

Contamination of poultry meat is a common source of *Campylobacter*. *Campylobacter*-specific bacteriophage was reported from broiler chickens and retail poultry (Atterbury et al., 2003 Appl. Environ. Microbiol. 69:4511; 2005 Appl. Environ. Microbiol. 71:4885). Lytic *Campylobacter* phage represent a means by which the *Campylobacter* load in poultry could be controlled through the use of phage therapy (Loc-Carrillo et al., 2005 Appl. Environ. Microbiol. 71:6554; Wagenaar et al., 2005 Vet. Microbiol. 109:275). The emergence of phage resistance has been suggested as a barrier to the use of phage therapy. However, phage resistance often comes at a price to the bacterial host. Here we suggest this is an inability to colonise poultry. Following bacteriophage CP34-treatment of chickens colonised by *Campylobacter jejuni* HPC5, bacteriophage resistant colonies were recovered at a frequency of 4% from the intestinal contents of birds. Although stable *in vitro* these isolates readily reverted to phage sensitivity in chickens, such that they were treatable with the original phage that selected them. The resistant isolates and reversion strains recovered from chickens retained their multi-locus sequence types but showed marked changes in their macro-restriction profiles. Further analyses revealed chromosomal inversions up to 590kb about the origin of replication of *C. jejuni* in response to exposure to lytic bacteriophage. These inversions took place about  $\mu$ -like prophage sequences, and in phage resistant types is associated with the production of functional  $\mu$ -like bacteriophage. These data suggests that bacteriophage therapy of *C. jejuni* would remain a sustainable measure to reduce poultry contamination but demonstrate how bacterial genomes can evolve under the strong and widespread pressure of bacteriophage predation in the environment.

P160

# A Novel Component From Celery Seed With a Bactericidal Effect Against *Helicobacter pylori*

Y. Zhou, T. J. Smith and K. D. Rainsford  
Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom

The seeds of celery (*Apium graveolens*) have been used as an herbal medicine since antiquity and beneficial properties have been reported to include anti-bacterial, anti-inflammatory and diuretic effects. Here, by means of solvent extractions and chromatographic steps, we have fractionated an alcoholic extract of celery seeds and investigated the fractions for their activity against *Helicobacter pylori*. As part of this work, we have been able to purify a component of celery seeds that has strong bactericidal effects against *H. pylori*; the minimum inhibitory concentration (MIC) and minimum bactericidal concentrations are within the range 3–15 mg ml<sup>-1</sup>, respectively. Interestingly, the compound appears very specific to *H. pylori*, since we detected no significant activity against *Campylobacter jejuni* or *Escherichia coli*. These results suggest that the new compound may be suitable for further investigation as an agent for treating *H. pylori* infections.



# A Novel Compound from Celery Seed with a Bactericidal Effect against *Helicobacter pylori*

Yong Zhou, Thomas J Smith, KD Rainsford

Biomedical Research Centre, Sheffield Hallam University, Sheffield, S1 1WB, UK

## Introduction

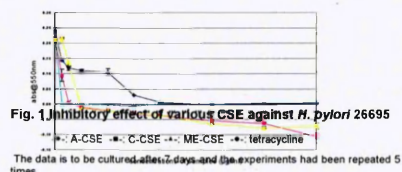
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Celery seed (*Apium graveolens*) has been used as herbal medicine for thousands of years in Indian. It is useful for the treatment of urinary calculi, gut diseases, the relief of flatulence and griping pains, reduction of visceral spasm, stimulation of the smooth muscle of womb. Momin (2001) reported the antifungal, mosquitocidal and nematocidal activities of the methanolic extract of celery seed. Friedman (2002) showed that celery seed extracts (CSE) have bactericidal activities against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*. Ethanolic extracts have also been found to have anti-inflammatory and anti-ulcer activity in rodent models.

In our search for a new agent to combat *H. pylori* infection, we initially observed that ethanolic extracts of celery seed had antimicrobial properties. In present studies, we investigated these effects, identified and characterized the components from the complex mixture of essential oils in celery seed, which was found to be active against *H. pylori*.

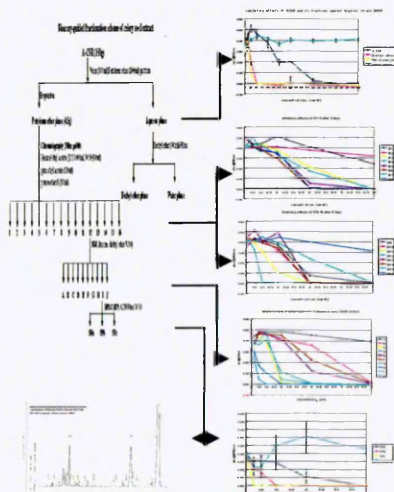
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## Purification of the active components and the antibacterial activity

A-CSE was fractionated step by step in the following scheme. All fractions in each step were tested for antibacterial activity against *H. pylori* strain 26695 and the MICs were determined.

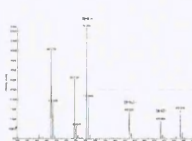


## Chemical characteristic of the highly anti-*H. pylori* major fraction SFC

From Q-Star MS analysis, the molecular weight of the component of SFC is 384 and its molecular formula declared from the exact mass values is  $C_{24}H_{32}O_4$ .

$^{13}C$  and  $^1H$ - $^{13}C$  correlation spectra: 24 carbon signals, including 2 of carbonyls ( $C=O$ ), 1 of  $sp^2$  CH, 1 of  $sp^2$  C, 7 of  $sp^3$  carbons as CH and  $CH_3$  groups and 12  $sp^3$  carbons as C and  $CH_2$  groups.

The MS-MS spectrum of SFC presented the molecular ion at  $m/z$  384 and major fragments at  $m/z$  193, 191, 135, 107 and 91. The ion at  $m/z$  193 and 191 in MS spectrum was observed that the main product ions were identical with sedanolide and ligustilide unit, respectively.



## Studies of anti-*H. pylori* mechanism

To determine bactericidal or bacteriostatic activity, bacteria were initially cultivated with the compound and re-cultured in its absence. The results indicated that SFC is bactericidal having an MBC of 6.25 µg/ml, thus showing that it was approximately twofold higher than the MIC.

After treating with SFC, there were 19.7 % of *H. pylori* to preserve in the rod form and 80.3% in the coccoid form (Fig.A), compared to 29.8% and 71.2% in untreated sample (Fig.B). The exposure to SFC gave rise to the morphological changes in which the rod forms were converted to coccoid forms.

By using 2-D electrophoresis, both the 2-DE profiles of *H. pylori* grown under normal (A) and treated with SFC (B) exhibited the distinct spots between masses 14 and 100 kDa and  $pI$  3 and 9 range analysis. There were 338 spots found in the untreated gel and there were 189 of spots, which matched those in the treated gel.

## Discussion

SFC, a colorless oil without odor was the pure fraction from separation of the nature vegetable, CSE, step by step and has the higher activity against *H. pylori* at 3.15 µg/ml of MIC. It can compare to the tetracycline, which is 3-5 µg/ml of MIC at same condition.

From the chemical structure analysis, the molecular weight of SFC is 384 and its molecular formula is  $C_{24}H_{32}O_4$ . It maybe had a dimmer structure comprised of a sedanolide and ligustilide unit.

This extract must be bactericidal of *H. pylori*, as the concentration is more than 2 fold of MIC. The effects of the morphology are modest and comprised the shapes and sizes of rods and coccid. There were the inhibitory effects on proteins, due to missing spots on 2-D gels after treatment. The radio labeled assay is in process to investigate the effects on protein and nucleic acid syntheses.

## References:

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- Momin, P. S. and Moin, M. S. (2001) Nematocidal, antifungal and antibacterial compounds from *Apium graveolens* L. seeds. J. Pharm. Med. 2001: 1-10.
- Friedman, J. (2002) Medicinal plants: A practical approach. Oxford University Press, Oxford, UK. 1-10.

