In-vitro antibacterial activity and cytotoxicity of selected medicinal plant extracts from Penang Island Malaysia on metronidazole-resistant-*Helicobacter pylori* and some pathogenic bacteria.

Abdul M. Uyub, Ikenna N. Nwachukwu, Ahmad A. Azlan and Shaida S. Fariza

Research

Abstract

Helicobacter pylori is the major agent in the etiology of chronic active gastritis, duodenal and gastric ulcers and has been linked to gastric carcinoma. The increasing antibiotic resistance of this organism and other bacteria has led to increased treatment failures, especially in therapies involving commonly used antibiotics. Malaysia has an unexplored potential of medicinal plants. This study evaluates the *in-vitro* antimicrobial activity of 32 selected medicinal plants used in Malaysian traditional medicine for gastrointestinal disorders and wounds. Extracts were tested against *H. pylori* by disc diffusion and agar dilution methods. Toxicity of the most potent extracts was also investigated. Organic solvent extracts of *Derris trifoliata* Lour. selectively inhibited *H. pylori*, and thus was further studied. Petroleum ether (PE), chloroform (CHL) and methanol (MET) extracts were tested against 13 other bacterial isolates representing eight genera. Petroleum ether and chloroform extracts showed strong activity against metronidazole-resistant clinical isolates of *H. pylori* with MIC_{90} and MIC_{50} ranging from 1- 4mg/l. All extracts of *D. trifoliata* produced higher inhibition zone diameters against *H. pylori* than for other bacteria. Toxicity tests showed Lc_{50} of PE, CHL and MET extracts on *Artemia salina* Leach as 1.14, 1.1, and 54.9mg/l respectively. Chemical analysis revealed the presence of fatty acids, steroids, triterpenoids, alkaloids, phenols, and phenyl propanoids, tannin, and mucilage in the extracts. *Derris trifoliata* shows potential as a source of selective anti-*H. pylori* agent.

Introduction

Isolation of *Helicobacter pylori* in Australia represented a breakthrough in the scientific understanding of peptic ulcer disease (Warren & Marshall 1983). A Center for Disease Research report shows that two thirds of the world's populations harbor this bacterium, with infection rates much higher in developing countries (NCI 2006, Rahman *et al*. 2009). This infection leads to frequent chronic gastritis world wide (Correa & Piazuelo 2008). *Helicobacter pylori* infection in developing countries is characterized by rapid rate of acquisition, approximately 80% of the population is infected at the age of 20 (Malaty 2007), because the disease is acquired at child hood (Malaty *et al.* 2002). Moujaber *et al*. (2008) reported lower prevalence rates in Australia (15.4%), than (Everhart *et al*. 2000) in the U.S.A. (32.5%). However, there are indications of declining prevalence of *H. pylori* in various parts of the world, resulting in overlapping prevalence rates in the East and West (Leong 2009, Malaty 2007). Correlations have been made between the presence of *H. pylori* and the development of several disease conditions such as gastric adenocarcinoma and gastric lymphoma (Rahman *et al*. 2009), pancreatic adenocarcinoma, ischaemic

Correspondence

Abdul M. Uyub, Ikenna, N. Nwachukwu, Ahmad A. Azlan and Shaida S. Fariza, School of Biological Science, Universiti Sains Malaysia, 11800, Penang, MALAYSIA.
uyub@usm.my; ikennatex@yahoo.com

Ethnobotany Research & Applications 8:095-106 (2010)

Published: May 20, 2010

www.ethnobotanyjournal.org/vol8/i1547-3465-08-095.pdf

heart and cerebrovascular disease, artherosclerosis and skin diseases (Gasbarrini *et al*. 1997, Pakodi *et al.* 2000). Recently also the relationship between *H. pylori* and gastric cancer has been proven (Correa & Piazuelo 2008, Fukase *et al*. 2008). Liou *et al*. (2008) however reported that only 10-15% of infected persons will develop peptic ulcer disease while only about 1% of patients will have gastric cancer or mucosa associated lymphoid tissue lymphoma.

Despite the high incidence of this infection, the reservoirs and transmission pathways to humans are still unclear (Quaglia *et al*. 2008). Some of the most recent literature however suggest that transmission of *H. pylori* occurs from person to person, both via the oral-oral and faecal-oral routes, while others suggest that human infections may occur by contaminated foods and water, zoonotically, and iatrogenically (Cellini *et al*. 2001, Delport & Merwe 2007, Gomes & DeMartinis 2004).

The conventional eradication therapies combine two antibiotics and a proton pump inhibitor (Castillo-Juárez *et al*. 2009). Treatment regimens containing metronidazole, clarithromycin and a proton pump inhibitor are amongst the most efficacious (De Francesco *et al*. 2009, Osato *et al*. 1999). The success rate following this therapy is constantly decreasing, mainly due to antibiotic resistance (De Boer & Tytgat 2000, Kwon *et al*. 2001, Wolle & Malfertheiner 2007).

Resistance of *H. pylori* to antibiotics is a growing world concern and needs urgent public health attention (Jenks & Edwards 2002). The resistance especially to metronidazole and amoxicillin limit their use in the treatment of *H. pylori* infections. Reported frequencies of resistance to antibiotics used in treatment/eradication of *H. pylori* vary widely with different antibiotics and between geographical regions, and even amongst subgroups within a study population. Values vary from about 37.4% for metronidazole in U.S.A. (Osato *et al*. 1999) to about 80-90% in developing countries (Mégraud 2001). A recent study in Egypt (Ragab *et al*. 2009), reported resistance of *H. pylori* to metronidazole (90%), amoxicillin (88%), clarithromycin (20%), tetracycline (11.4%), and ciprofloxacin (7%). A recent research (Salasawati & Ramela 2009), found a high level resistance (minimum effective concentration, MIC >256mg/l) in 24.5% of clinical isolates of *H. pylori* to metronidazole in Kualar Lumpur, Malaysia. This problem is encountered more in Africa (Ndip *et al*. 2007), and other developing countries. Poor patient compliance, due to undesirable side effects of drugs and the significant cost of combination therapy clearly reveals the need to seek alternative available means of eradicating or preventing infections (O'Gara *et al*. 2000).

Currently there has been a resurgence of scientific interest in the use of medicinal plants for the development of new pharmaceutical agents. It is estimated that plant materials as present has provided the models for about 50% of western drugs (Harborne 1998) Studies regarding anti-*H. pylori* activity of medicinal plants have also increased considerably in recent times. Phytochemicals may indeed serve as an alternative source of materials for *H. pylori* eradication because they constitute a rich source of biochemicals. Many natural substances and plants such as ecabet sodium, tea catechins, garlic extracts, honey, *Tephrosia purpurea* (L.) Pers., *Tabebuia impetiginosa* (Mart. ex DC.) Standl. (e.g., Castillo-Juárez *et al*. 2009, Chinniah *et al*. 2009, Gadhi *et al*. 2001, Li *et al*. 2005, Ndip *et al*. 2007, Park *et al*. 2006), have been shown to possess anti-*H. pylori* activities. However, very high MIC and toxicity values have been the major setback confronting the exploitation of these findings.

In Malaysia, plant derived medicines have been part of the traditional health care for ages, especially against stomachic complaints and infection related aliments. Given the alarming resistance in a bacterium proven to be the etiologic agent of active chronic gastritis, peptic ulcer disease and gastric cancer amongst other diseases, (Uemura *et al*. 2001) the search for cheaper, safer and more effective anti *H. pylori* agents is indeed necessary.

This paper reports anti-*H. pylori* activity of 32 Malaysian plants used in Malaysian ethnomedicine for treatment of stomach complaints, giving special attention to the organic solvent extracts of *Derris trifoliata* Lour. with regard to its activity against metronidazole resistant clinical isolates of *H. pylori*, and some common pathogenic and opportunistic pathogenic bacteria. Toxicity and preliminary phytochemical analysis of the extracts are also reported.

Materials and Methods

Plant selection and preparation of extracts

Thirty-two plants were selected based on ethnobotanical information obtained from traditional medicine practitioners in Malaysia. The plants are used for treatment of stomachic complaints and cure of wounds or external tissue injury. All the plants are commonly found and were collected around Penang Island, Malaysia. Plant vouchers were kindly identified and stored by the Botanical Garden, Universiti Sains Malaysia, Pulau Pinang Malaysia. Either parts or whole plants were used for extraction (Table 1).

Material of each plant was dried at room temperature (28 ±3^{o}C) by exposure to atmosphere. The dried materials were pulverized into very fine powder. Ten grams of each sample was soxhlet extracted sequentially using Petroleum Ether (PE), Chloroform (CHL) (Uemura *et al*. 2001), Methanol (MET) at 60^{o}C, and water at boiling temperature. The crude extract was vacuum dried, redissolved in Dimethyl Sulfoxide (DMSO, Fluka, Swizerland) and finally sterilized by filtration (Whatman 0.2µm).

Table 1. Plants screened for anti-*Helicobacter pylori* activity. Plants selected based on ethnobotanical information obtained from traditional medicine practitioners in Malaysia.

Plant species	Parts used						
	Flowers	Leaves	Rhizomes	Roots	Seeds	Stems	Whole plants
Calotropis gigantea (L.) W.T. Aiton		X					
Centella asiatica (L.) Urb.							X
Chromolaena odorata (L.) R.M. King & H. Rob.		X					
Colubrina asiatica (L.) Brongn.		X					
Cosmos caudatus Kunth		X					
Cymbopogon citratus (DC.) Stapf						X	
Desmos cochinchinensis Lour.		X					
Derris trifoliata Lour.						X	
Ficus deltoidea Jack		X					
Hibiscus rosa-sinensis L. (white variety)		X				X	
Jatropha podagrica Hook.		X		X		X	
Kaempferia galanga L.		X	X				
Labisia pumila Benth. & Hook. f				X			
Languas galanga (L.) Stuntz			X				
Limnocharis flava (L.) Buchenau		X					
Melastoma malabathricum L. (blue variety)		X				X	
Mimosa pudica L.							X
Mitrasacme alsinoides R. Br.		X					
Neptunia oleracea Lour.		X					
Orthosiphon stamineus Benth.		X				X	
Parkia speciosa Hassk.					X		
Pereskia sacharosa Griseb.		X					
Phaeomeria imperialis (Roscoe) Lindl.	X						
Phyllanthus niruri L.							X
Piper betle L.		X					
Pluchea indica (L.) Less.		X					
Polygonum minus Huds.		X					
Psidium guajava L.		X					
Sesbania grandiflora (L.) Pers.		X					
Solarnum torvum Sw.					X		
Tinospora cordifolia (Willd.) Miers						X	
Zingiber officinale Roscoe			X				

Chemical analysis of extracts

Phytochemical analysis of the extracts was carried out according to the methods of the National Pharmaceutical Control Bureau, Malaysia. PE extract was tested for the presence of fat, fatty acids, steroids, terpenoids and carotenoids, while the CHL extract was tested for alkaloids, phenolics, flavonoids, and anthraquinones. MET extract was tested for alkaloids, glycosides, tannin and mucilage.

Bacterial cultures

H. pylori were isolated from antral biopsies of patients undergoing routine gastroduodenoscopy for stomach complaints at a hospital in northern peninsular Malaysia. Biopsies were transported in Stuart transport medium (Oxoid, U.K.), inoculated onto Eugon agar (BBL, U.S.A.) plus 10% (v/v) human blood. Incubation was at 37°C under CO_2 + air atmosphere for 4 days (Uyub & Sasidharan 2000). Presence of *H. pylori* was confirmed by rapid urease test and microscopy. *H. pylori* SJ47 isolated from a 60 year old woman was used as a working culture for screening of anti-*H. pylori* activity of plants.

Other pathogenic bacteria such as *Enterobacter* sp., *Escherichia coli* ATTC 25922, *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Salmonella* sp., *Salmonella paratyphi* A, *Shigella boydii*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Vibrio cholerae*, and *Vibrio parahaemolyticus* were obtained from Departmental stock cultures, School of Biological Sciences, Universiti Sains Malaysia. They were grown aerobically on Mueller- Hinton agar (BBL, USA) at 37°C. All bacteria were preserved in tryptic soy broth (BBL, U.S.A.) + 10% (v/v) glycerol at -80°C prior to revival. In each case they were revived shortly before antimicrobial assay.

Screening of plants with anti H. pylori activity

A disc diffusion assay was initially used to screen plants for anti-*H. pylori* activity. Cells were harvested from growth medium and turbidity was adjusted to a scale of 0.5 on the MacFarland standard (Biomerieux, France). A sterile wire swab was immersed into the suspension and rotated firmly against the inner wall of the tube to squelch excess liquid. The agar surface was then swabbed three times, rotating the plates 60 degrees between swabbing to ensure even distribution of cells. Disks (AA, 6mm, Whatman U.K.) which had been impregnated with 20µl of each extract was deposited on the agar and gently pressed with sterile forceps to ensure complete contact with the agar surface. Six disks, two as negative controls (each impregnated with water and DMSO), and the remaining 4, each impregnated with PE, CHL, MET, and water extracts were used per plate. They were deposited with their centers at least 25mm apart.

Activity of D. trifoliata against H. pylori and other pathogenic bacteria

In subsequent experiments, when only extracts from *D. trifoliata* were studied, extract amounts were standardized at 240µg per disk. The extracts were tested against *H. pylori* SJ47 and 13 other bacteria. Only 4 disks were used impregnated with one each of DMSO (as negative control), PE, CHL, and MET extracts were deposited per plate. Their centers were at least 30mm apart. This test was carried out to determine whether the extracts could also inhibit other bacteria that could inhabit the gastrointestinal tract, whether as pathogens or normal flora. Plates inoculated with *H. pylori* were incubated microaerophilically as described, while plates inoculated with other bacteria were incubated aerobically at 37°C.

***Toxicity assay using brine shrimps, Artemia salina* Leach**

Cytotoxicity of extracts from *D. trifoliata* was evaluated in triplicate using *Artemia salina* Leach. Brine shrimp eggs (Focus, U.S.A.) were hatched in artificial sea water (Kester *et al*. 1967). After 48 hours the phototrophic nauplii were collected and a suspension of about 30–50 nauplii in 100 µl were placed into bijoux bottles containing 900 µl of extract in artificial sea water. Eight final concentrations of the extract ranging from 1-128 µg/ml, at two fold serial increments were used in the assay. The control bijoux bottle contained the nauplii in 1000 µl artificial sea water but devoid of the extract. After 24 hours of incubation, the dead nauplii were counted under a dissecting microscope. Subsequently 200 µl of methanol was added to each bottle and 60 minutes later the total number of nauplii were counted. Cytotoxicity, expressed as LC_{50} was calculated using the formula:

Log LC_{50} – Log Concentration A / Log Concentration B – Log Concentration A = 50% - M / N - M

Where concentration A is the concentration before LC_{50}, concentration B is the concentration after LC_{50}, M is the % killed before LC_{50}, and N is the % killed after LC_{50}.

Determination of Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentrations (MICs) were determined by an agar dilution method with final concentrations ranging from 0.025 to 32mg/l for metronidazole (Sigma, U.S.A.), 1 to 128mg/l PE extract, and 1 to 1024mg/l for CHL extract, all tested at two-fold serial increments. *Helicobacter pylori* (84h agar culture) was suspended in sterile distilled water and after adjusting turbidity to a scale of 0.5 on the MacFarland standard (Biomerieux, France), 20 µl was spotted onto growth medium (control) and respectively onto metronidazole PE extract, CHL extract containing growth media. Media used for this test was Eugon agar (BBL, U.S.A.) plus 10% (v/v) human blood. Incubation was at 37°C under CO_2 + air atmosphere. MIC was defined as the lowest concentration of the antibiotic or extract tested that showed no observable bacterial growth.

Results

Chemical constituents of D. trifoliata extracts

Chemical analysis revealed that PE extract contained fatty acids, steroids and triterpenoids, CHL extract contained

alkaloids, phenols and phenyl propanoids, while MET extract contained phenyl propanoids, tannin and mucilage.

Sensitivity of H. pylori towards the extracts

All the plants tested possessed anti *H. pylori* activity. Ninety-eight percent of the organic solvent extracts (108/110) inhibited *H. pylori* as compared to only slightly more than 10% (4/38) of water extracts. The 4 water extracts were prepared from *Desmos cochinchinensis* Lour. leaf, *Orthosiphon stamineus* Benth. leaf, and stem, and *Psidium guajava* L. leaf. Two plants *D. cochinchinensis*, and *Labisia pumila* Benth. & Hook.f. yielded only MET extract as the only organic extract. These are shown on Table 2.

The amount of extract per disc was not standardized in the initial screening for anti-*H. pylori* activity. Therefore, for some extracts, a wide inhibition zone was only indicative of potential anti-*H. pylori* activity. This does not necessarily mean stronger antibacterial activity compared to other extracts that exhibited smaller inhibition zones. Thus the ratio of inhibition zone diameter to amount of extract per disk was used to indicate differential strength of anti-*H. pylori* activity (Table 2, shown in parenthesis). The two

Table 2. Inhibition of *Helicobacter pylori* by traditional medicinal plants from Malaysia.

Plants	Part used	Inhibition zone diameter (mm) by respective extract			
		Petroleum ether	Chloroform	Methanol	Water
Calotropis gigantea (L.) W.T. Aiton	Leaf	13.2 ±0.8 (11.8)	14.0 ±0. 9 (12.5)	9.8 ±1.2 (1.4)	NZ
Centella asiatica (L.) Urb.	Whole	8.5 ±0.6 (7.1)	8.2 ±0.4 (14.6	13.0 +0.9 (0.9)	NZ
Chromolaena odorata (L.) R.M. King & H. Rob.	Leaf	20.3 ±1.4 (8.8)	25.7 ±1.0 (29.2)	25.3 ±1.6 (2.8)	NZ
Colubrina asiatica (L.) Brongn.	Leaf	11.0 ±0.9 (0.4)	10.0 ±0.9 (8.3)	16.3 ±2.1 (0.7)	NZ
Cosmos caudatus Kunth	Leaf	16.0 ±0.6 (8.3)	11.7 ±0.5 (29.2)	23.0 ±0.9 (2.1)	NZ
Cymbopogon citratus (DC.) Stapf	Stem	29.5 ±1.5 (16.0)	18.0 ±1.4 (75.0)	28.5 ±1.5 (1.5)	NZ
Desmos cochinchinensis Lour.	Leaf	NE	NE	30.0 ±2.1 (1.4)	10.0 ±0. 6 (3.6)
Derris trifoliata Lour.	Stem	42.0 ±0.9 (87.5)	47.0 ±1.7 (117.5)	47.0 ±0.9 (5.0)	NZ
Ficus deltoidea Jack	Leaf	8.0 ±0.1 (7.7)	10.0 ±0.6 (17.9)	12.0 ±0.6 (2.4)	NZ
Hibiscus rosa-sinensis L. (white variety)	Leaf	11.5 ±1.1 (24.0)	11.2 ±1.2 (46.5)	14.3 ±1.0 (2.1)	NZ
	Stem	13.2 ±0.8 (5.7)	9.6 ±0.6 (10.9)	13.7 ±1.2 (2.7)	NZ
Jatropha podagrica Hook.	Leaf	13.0 ±1.1 (54.2)	10.0 ±0.5 (62.5)	8.0 ±0.5 (6.3)	NZ
	Stem	15.5 ±1.4 (38.8)	14.0 ±0.9 (25.0)	9.2 ±0.8 (0.8)	NZ
	Root	47.3 ±3.0 (10.4)	42.0 ±0.5 (27.6)	34.0 ±2.5 (2.2)	NZ
Kaempferia galanga L.	Leaf	62.0 ±0.1 (19.9)	66.0 ±0.1 (63.5)	46.0 ±0.1 (7.6)	NZ
	Tuber	18.3 ±1.0 (17.6)	18.3 ±1.0 (13.5)	11.0 ±0.6 (1.6)	NZ
Labisia pumila Benth. & Hook.f.	Root	NE	NE	8.0 ±0.5 (3.7)	NZ
Languas galanga (L.) Stuntz	Tuber	39.3 ±2.1 (14.1)	24.2 ±1.6 (18.9)	21.5 ±1.9 (2.3)	NZ
Limnocharis flava (L.) Buchenau	Leaf	24.0 ±0.6 (5.5)	14.0 ±0.6 (2.2)	11.0 ±1.1 (0.5)	NZ
Melastoma malabathricum L. (blue variety)	Leaf	14.0 ±2.3 (6.3)	22.2 ±1.3 (4.5)	25.7 ±0.8 (2.0)	NZ
	Stem	10.5 ±0.8 (16.4)	7.2 ±0.4 (29.9)	18.0 ±0.6 (1.6)	NZ
Mimosa pudica L.	Whole	8.5 ±0.6 (26.6)	8.8 ±1.6 (0.4)	14.2 ±1.9 (2.8)	NZ
Mitrasacme alsinoides R. Br.	Leaf	11.0 ±0.6 (2.2)	9.5 ±1.1 (5.4)	13.3 ±2.3 (1.0)	NZ
Neptunia oleracea Lour.	Leaf	10.5 ±0.8 (8.2)	10.7 ±2.0 (1.5)	28.3 ±4.1 (1.1)	NZ
Orthosiphon stamineus Benth.	Leaf	17.7 ±2.8 (8.5)	18.3 ±2.2 (11.5)	22.0 ±2.4 (4.4)	9.0 ±1.3 (3.2)
	Stem	12.7 ±0.5 (79.2)	11.3 ±1.0 (47.2)	16.0 ±0.9 (4.2)	8.0 ±0.1 (2.9)
Parkia speciosa Hassk.	Seed	10.5 ±0.8 (2.6)	26.0 ±0.6 (25.0)	18.0 ±0.1 (1.6)	NZ

Plants	Part used	Inhibition zone diameter (mm) by respective extract			
		Petroleum ether	Chloroform	Methanol	Water
Pereskia sacharosa Griseb.	Leaf	13.3 ±0.5 (83.3)	NZ	NZ	NZ
Phaeomeria imperialis (Roscoe) Lindl.	Flower	18.0 ±1.1 (7.3)	14.0 ±0.6 (10.9)	16.3 ±1.4 (4.5)	NZ
Phyllanthus niruri L.	Whole	14.0 ±1.6 (8.0)	9.8 ±0.8 (11.2)	29.7 ±1.4 (3.6)	NZ
Piper betle L.	Leaf	54.2 ±0.8 (6.3)	25.8 ±0.8 (4.9)	23.5 ±0.8 (3.9)	NZ
Pluchea indica (L.) Less.	Leaf	13.7 ±1.9 (5.9)	11.0 ±0.6 (6.9)	23.0 ±1.3(13.7)	NZ
Polygonum minus Huds.	Leaf	15.5 ±0.6 (12.1)	12.3 ±0.8 (22.0)	15.5 ±1.1 (1.5)	NZ
Psidium guajava L.	Leaf	8.5 ±0.8 (5.1)	10.0 ±0.6 (3.6)	33.0 ±2.3 (1.3)	7.0 ±0.5 (2.5)
Sesbania grandiflora (L.) Pers.	Leaf	10.8 ±1.0 (2.4)	8.8 ±1.3 (3.9)	17.3 ±1.6 (0.4)	NZ
Solarnum torvum Sw.	Seed	11.0 ±0.9 (17.2)	8.7 ±0.8 (18.1)	12.3 ±0.8 (2.2)	NZ
Tinospora cordifolia (Willd.) Miers	Stem	10.7 ± 0.8 (11.1)	19.2 ± 5.0 (10.9)	13.7 ± 2.7 (1.7)	NZ
Zingiber officinale Roscoe	Tuber	33.3 ± 1.6 (10.2)	41.5 ± 7.0 (7.2)	19.7 ± 1.5 (1.9)	NZ

Number in parenthesis indicates ratio of inhibition zone diameter: mg extract per disc. Negative control (20 µl DMSO incorporated per disc) gave no inhibition zone against *H. pylori*. NZ= not inhibited. NE=No extract produced.

highest values recorded were 117.5 and 87.5 exhibited by CHL and PE extracts of *D. trifoliata* respectively. The lowest value (0.4) was exhibited by MET extract of *Sesbania grandiflora* (L.) Pers., PE extract of *Centella asiatica* (L.) Urb. and CHL extract of *Mimosa pudica* L.

Sensitivity of other bacteria towards PE, CHL, and MET extracts

In view of their strong activity against the focal bacteria *H. pylori*, both PE and CHL extracts were further evaluated for their activity against thirteen other pathogenic and non pathogenic bacteria, representing eight genera. MET extract was also evaluated for comparison.

Table 3. Inhibitory activity of *Derris trifoliata* Lour. against *Helicobacter pylori* and other common pathogenic and non pathogenic bacteria by disc diffusion assay.

Bacteria tested	Diameter* of zone of inhibition (mm) produced		
	PE extract	CHL extract	MET extract
Enterobacter sp.	NZ	NZ	8.0±0.1
Escherichia coli ATCC 25922	NZ	NZ	15.0±1.0
Escherichia coli	NZ	NZ	8.0±0.1
Proteus vulgaris	NZ	NZ	12.7±0.6
Pseudomonas aeruginosa	7.3±0.6	14.0±1.0	11.3±1.0
Pseudomonas stutzeri	NZ	7.0±0.1	NZ
Salmonella paratyphi A	NZ	7.0±0.5	NZ
Salmonella sp.	NZ	NI	13±1.0
Shigella boydii	NZ	7.0±0.1	NZ
Shigella dysenteriae	NZ	7.0±0.1	NZ
Staphylococcus aureus	NZ	NZ	7.0±0.1
Vibrio cholerae	6.5±0.1	6.5±0.1	NZ
Vibrio parahaemolyticus	NZ	7.0±0.1	NZ
Helicobacter pylori SJ47	42.0±1.0	38.0±1.0	8.5±1.0

*mean of three values; NZ= not inhibited.

Petroleum ether extract inhibited *Pseudomonas aeruginosa* and *Vibrio cholerae* but inhibitory effect based on inhibition diameter was significantly smaller (6.5 – 7.3mm) compared to *H. pylori* (42mm)(Table 3). CHL inhibited a wider range of bacteria; *Vibrio parahaemolyticus*, *V. cholerae*, *S. paratyphi* A, *S. boydii*, *S. dysenteriae*, and *P. stutzer*. It is worthy of note that the inhibitory activity was also smaller (6.5-7.0mm) compared to that for *H. pylori* (38mm). MET inhibited a wide range of bacteria but it was relatively inactive against *H. pylori* exhibiting only 8.5mm inhibition zone diameter compared to 11 to 15mm against *E. coli* ATTC 25922, *Salmonella* sp and *Pseudomonas* sp. Of the three extracts tested, MET extract was least active against *H. pylori*.

Toxicity of the extracts on Artemia salina

Toxicity tests were carried out in triplicates using *A. salina*. Using the formula given in section 2.6, PE extracts proved to be the most toxic of the *D. trifoliata* extracts tested LC_{50} = 1.4 µg/ml, followed by CHL extract (1.1 µg/ml) and MET extract (54.9 µg/ml). These are shown on Table 4.

Table 4. Toxicity of extracts of *Derris trifoliata* Lour. against *Artemia salina* Leach.

Log conc. of extract (µg/ml)	PE extract			CHL extract			MET extract		
	Total nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed	Total nauplii	Total nauplii killed	% killed
2.107	32	32	100	29	29	100	32	26	81.25
1.806	36	36	100	34	34	100	31	19	61.29
1.505	32	32	100	40	40	100	30	3	10.00
1.204	30	30	100	45	45	100	32	1	3.13
0.903	38	34	89.47	41	41	100	32	0	0
0.602	33	23	69.70	51	46	90.20	34	0	0
0.301	31	19	61.29	48	32	66.67	34	0	0
0.001	40	16	40.00	54	26	48.15	31	0	0

LC_{50} *D. trifoliata* PE extract = 1.4 µg/ml; LC_{50} *D. trifoliata* CHL extract = 1.1 µg/ml; LC_{50} *D. trifoliata* MET extract = 54.9 µg/ml.

MIC of PE and CHL extracts on H. pylori

Considering their narrow spectrum of activity, PE and CHL extracts of *D. trifoliata* were selected for MIC determination. Agar dilution method was used to determine the sensitivity of the 47 clinical isolates of *H. pylori* to Metronidazole, PE and CHL extracts of *D. trifoliata*. Table 5 shows the sources of and clinical isolates used for the determination of MIC. Only SJ 47 and SJ 779 were metronidazole susceptible (MIC = 0.5 and 8.0 mg/l respectively). The other isolates had MIC values >8mg/l and were considered as metronidazole-resistant (Goh *et al*. 2000).

Fifteen clinical isolates (SJ 1, 2, 6-13, 17-19, 26 and 32) were used for the determination of MIC against PE extract. SJ 95 and SJ 155 were subsequently lost and therefore only the remaining 45 isolates were used for MIC determination against CHL extract.

Table 5. Source of, and minimum inhibitory concentration (MIC) of metronidazole (Metron), Petroleum Ether (PE), and Chloroform (CHL) extracts of *Derris trifoliata* Lour. against clinical isolates of *Helicobacter pylori*. NR = no report, ND = not done, DN = duodenitis, PPU = prepyloric ulcer, PG = pyloric gastritis, DU=duodenal ulcer, NUD = non-ulcer dyspepsia, AG= antral gastritis, M=Malay, C = Chinese, I = Indian, ML = male, F = female

No.	Isolate	Ethnic group	Gender	Age	Endoscopic findings	MIC (µg/ml)		
						Metron	PE	CHL
1.	SJ 46	M	ML	50	AG	>32	2	2
2.	SJ 47	I	F	60	AG	0.5	2	2
3.	SJ 65	C	F	46	AG	>32	ND	2
4.	SJ 69	C	F	70	NUD	>32	ND	2
5.	SJ 70	C	F	63	NUD	>32	ND	2
6.	SJ 77	M	ML	24	AG	>32	1	1

No.	Isolate	Ethnic group	Gender	Age	Endoscopic findings	MIC (µg/ml)		
						Metron	PE	CHL
7.	SJ 95	C	F	68	AG	>32	1	ND
8.	SJ 98	C	ML	69	DU	>32	1	1
9.	SJ 109	I	ML	47	AG	>32	2	2
10.	SJ 115	C	ML	26	AG	>32	2	2
11.	SJ 136	I	ML	37	DU	>32	4	4
12.	SJ 137	I	ML	33	AG	>32	4	4
13.	SJ 143	C	ML	45	AG	>32	1	2
14.	SJ 147	I	ML	40	NUD	>32	ND	2
15.	SJ 148	I	ML	45	AG	>32	ND	4
16.	SJ 152	I	ML	56	PPU	>32	ND	4
17.	SJ 153	C	ML	67	PPU	32	1	1
18.	SJ 154	C	ML	42	PPU	>32	1	1
19.	SJ 155	M	ML	34	DU	>32	1	ND
20.	SJ 157	C	ML	70	AG	32	ND	4
21.	SJ 160	I	ML	60	AG	>32	ND	2
22.	SJ 164	C	ML	51	AG	>32	ND	2
23.	SJ 175	B	ML	30	AG	>32	ND	8
24.	SJ 179	I	ML	30	AG	>32	ND	4
25.	SJ 184	I	ML	41	PG	>32	ND	4
26.	SJ 185	I	ML	46	AG	>32	1	1
27.	SJ 195	C	ML	67	NR	>32	ND	4
28.	SJ 198	C	ML	37	AG	>32	ND	2
29.	SJ 205	I	F	55	AG	>32	ND	2
30.	SJ 248	B	ML	47	NUD	>32	ND	2
31.	SJ 251	M	ML	45	AG	>32	ND	2
32.	SJ 255	I	ML	60	AG	>32	1	1
33.	SJ 270	I	ML	42	AG	>32	ND	1
34.	SJ 295	B	ML	25	NUD	>32	ND	1
35.	SJ 300	C	F	55	AG	>32	ND	1
36.	SJ 334	M	ML	38	AG	>32	ND	1
37.	SJ 371	C	ML	45	DU	>32	ND	4
38.	SJ 381	M	ML	45	AG	32	ND	1
39.	SJ 392	I	ML	48	PPU	>32	ND	8
40.	SJ 393	I	F	45	NUD	>32	ND	4
41.	SJ 422	I	F	48	AG	>32	ND	1
42.	SJ 443	I	ML	60	AG	>32	ND	1
43.	SJ 452	B	ML	32	AG	>32	ND	4
44.	SJ 779	B	ML	43	DN	8	ND	4
45.	SJ 808	C	ML	62	DU	>32	ND	4
46.	SJ 815	M	ML	70	DN	32	ND	1
47.	SJ 885	I	ML	56	DN	>32	ND	4

Using PE extract, 60% 0f the isolates were inhibited by 1mg/l, 87% by 2mg/l and 100% by 4mg/l. The isolate SJ 47 which was sensitive to metronidazole was inhibited by PE extract at 2mg/l. MIC_{50} and MIC_{90} was about 1mg/l and 2mg/l respectively.

Using the CHL extract, 64% of the isolates were inhibited by 2mg/l, 95.6% by 4mg/l and 100% by 8mg/l. The isolates SJ 47 and SJ 779 were killed by complete inhibition by I and 2mg/l respectively. MIC_{50} and MIC_{90} of CHL extract was about 2 and 4mg/l respectively.

Discussion and conclusion

H. pylori resistance to antibiotics is a world wide problem. Our primary objective was to evaluate *in-vitro* activity of 32 ethnobotanical Malaysian plants used for the treatment of abdominal ailments, especially *D. trifoliata* for anti-clinical *H. pylori* activities.

Successful treatment of *H. pylori* infections is dependent on the antibacterial susceptibility pattern of the organism and patient compliance (Osato *et al*. 1999). The increasing resistance of *H. pylori* strains to metronidazole, one of the primary drugs of choice (used in combination) for the treatment of *H. pylori* infection is of great concern especially in tropical and developing countries. Reduced success rates of current therapies have been reported and a correlation made between metronidazole and treatment failure (Jenks & Edwards 2002).

The prevalence of *H. pylori* resistant to metronidazole reported in this study (95.7%) is alarmingly high. This value is comparatively higher than values obtained in other developing countries (Ani *et al.*1999, Mégraud 2001, Osato *et al*. 1999, Ragab *et al*. 2009, Rahman *et al*. 2009).This might be as a result of the use of this agent for the treatment of genitourinary infections and other parasitic infections. It could also have arisen as a result of abuse of this drug owing to illegal practices and poor compliance with pharmaceutical regulations. This study also revealed a lower occurrence of metronidazole resistance in strains of *H. pylori* isolated from females (88.89%) compared with those isolated from males (97.39%). Other studies have often showed higher frequencies of metronidazole resistance from females, (Osato *et al*. 1999). Our data however may have been skewed owing to the small number of females available for analysis.

Although all the plants tested in this study possessed anti-*H. pylori* activities, the index of their activities varied. One limitation was that the tests were performed on crude extracts without isolating and testing the pure bioactive compounds. As such, it may not be known if the biological action (inhibitory and (or) toxic) is due to one or several compounds. Lai *et al*. (2008) agreed that the isolation of pure compounds does not necessarily lead to an increase in *H. pylori* inhibition as expected, with *Phyllanthus urinaria* L. Given that limitation, the PE extract of *D. trifoliata* contained fatty acids, and tripenoids, a class of compounds that has previously been reported to be inhibitory to *H. pylori* (Murphy Cowan 1999, Gadh*i et al*. 2001, Petschow *et al*. 1996). The CHL extract of that same plant contained alkaloids, another group of compounds known to have members specifically inhibitory to *H. pylori* (Hamasaki *et al*. 2000, Klausmeyer *et al*. 2004). The fact that PE and CHL extracts of *D. trifoliata* exhibited strong *in-vitro* anti-*H. pylori* activity and relatively ineffective against other common bacteria tested suggest the presence of compounds possessing more selective and specific action against *H. pylori*. Therefore, given the age long traditional use of this plant in Malaysia, further investigation of these two extracts is indicated.

MIC values in this study were determined for PE and CHL extracts. These were favored because of their narrower antibacterial spectrum of activity. Our findings show that MIC values for the extracts ranged from 1-4 µg/ml. These values are higher than therapeutically acceptable for many antibiotics used in *H. pylori* eradication. However, they are lower than values suggested for metronidazole, tetracycline, rifampin and tinidazole. (McDermott *et al*. 2009). McDermott *et al*. (2009) in their review suggested interpretative breakpoints for the most common antibiotics used in the treatment of *H. pylori* induced gastritis. Their suggested susceptible MIC values for therapy with amoxicillin, ciprofloxacin, clarithromycin, levofloxacin, and mixifloxacin is <1µg/ml, for furazolidone, nitrofurantoin is <2µg/ml, for rifampin is 4µg/ml, for tinidazole, metronidazole is <8µg/ml, and tetracycline is <16µg/ml. These values corroborate therapeutically effective susceptibility values reported for eradication of *H. pylori* (Okabe *et al*. 1997). It is worthy of note that MIC of PE extract reported in this work is at least 4 times lower, compared to MIC values found in literature for several anti *H. pylori* extracts from plant sources (Castillo-Juárez *et al*. 2009, Cellini *et al*. 2001, Chinniah *et al*. 2009, Fabry *et al*. 1996, Ingolfsdottir *et al*. 1997, Jones *et al*. 1997, Li *et al*. 2005, Mabe *et al*. 1999, Tabak *et al*. 1999). This makes this plant eligible for further studies. Though the extracts were effective at low concentrations, LC_{50} at 1.4µg/ml indicated toxicity at the concentration inhibitory to most *H. pylori* tested. This may undermine its usefulness. Toxicity of samples has been shown to be dependent on the concentration of specific compound(s) in the sample (Svensson *et al*. 2005). They, also showed that in some impure complicated samples, the data obtained by a biologist may be more diffuse. The level of toxicity observed in this study may have resulted from presence of multiple compounds in the crude extracts, acting in synergy or independently. Thus, isolation and testing of the pure anti-*H. pylori* component, may produce lower levels of toxicity.

Among the extracts tested, PE extract showed the widest diameter zone of inhibition (42mm). This extract also

interestingly possessed activity against *P. aeruginosa* and *V. cholerae* which are gastro intestinal pathogens, but showed no activity against *E. coli* and other normal microflora of the gastro intestinal tract. This finding raises hope for the possibility of developing an antibacterial agent from this extract that can exhibit selective and specific inhibitory action against *V. cholerae* and *H. pylori* without affecting mutualistic organisms that inhabit the gastrointestinal tract.

Acknowledgment

This study received financial support from The Malaysian Government under IRPA programme number 0102051084EA001 and FRGS grant account number 203/P.Biologi/671036. Authors thank Dr Pandyraj and Dr Ballan Kannan, Department of Surgery, Hospital Seberang Jaya, Penang, Malaysia for the endoscopic biopsies.

Dr. Ikenna Nwachukwu holds Academy of Science for Developing World (TWAS) - Universiti Sains Malaysia Postdoctoral fellowship. He is from The Federal University of Technology Owerri, Nigeria.

Literature Cited

Ani, A.E., A.O. Malu, J.A. Onah, D.M.M. Queiroz, G. Kirschner & G.R. Rocha. 1999. Antimicrobial susceptibility test of *Helicobacter pylori* isolated from Jos, Nigeria. *Transactions of The Royal Society of Tropical Medicine and Hygiene* 93:659-661.

Castillo-Juárez, I., V. González, H. Jaime-Aguilar, G. Martínez, E. Linares, R. Bye & I. Romero. 2009. Anti- *Helicobacter pylori* activity of plants used in Mexican traditional medicine for gastrointestinal disorders. *Journal of Ethnopharmacology* 122:402-405.

Cellini, L., L. Marzio, G. Ferreo, A. Del Vino, E. Di Campli, L. Grossi, S. Toracchio & L. Artese. 2001. Transmission of *Helicobacter pylori* in an animal model. *Digestive Diseases and Sciences* 46:62-68.

Chinniah, A., S. Mohapatra, S. Gowami, A. Mahapatra, S.K. Kar, U.V. Mallavadhani & P.K. Das. 2009. On the potential of Tephrosia purpurea as anti *Helicobacter pylori* agent. *Journal of Ethnopharmacology* 24:642-645.

Correa, P. & M.B. Piazuelo. 2008. Natural history of *Helicobacter pylori* infection. *Digestive and Liver Disease* 40:490-496.

De Boer, W.A. & G.N. Tytgat. 2000. Treatment of *Helicobacter pylori* infection. *British Medical Journal* 320:321-334.

De Francesco, V., A. Zullo, E. Lerardi, & D. Vaira. 2009. Minimal Inhibitory Concentration (MIC) values and different point mutations in the 23s rRNA gene for clarithromycin resistance in *Helicobacter pylori*. *Digestive and Liver Disease* 41:610-11.

Delport, W. & S. W. van der Merwe. 2007. The transmission of *Helicobacter pylori*: The effects of analysis of method and study population on inference. *Best Practice and Research Clinical Gastroenterology* 21:215-236.

Everhart, J.E., D. Kruszon-Moran, G.I. Perez-Perez, T.S. Tralka & G. McQuillan. 2000. Seroprevalence and ethnic differences in *Helicobacter pylori* infection among adults in the United States. *Journal of Infectious Diseases* 181(4):1359-1363.

Fabry, W., P. Okemo & R. Ansorg. 1996. Activity of. East African medicinal plants against *Helicobacter pylori*. *Chemotherapy* 42:315-317.

Fukase, K., M. Kato, S. Kikuchi, K. Inoue, N. Uemura, S. Okamoto, S. Terao, K. Amagai, S. Hayashi, M. Asaka and for the Japan Gast Study Group. 2008. Effect of eradication of Halicobacteria pylori on incidence of metachronous gastric carcinoma after endoscopic resection of early gastric cancer: An open-label randomised controlled trial. *The Lancet* 372(9636):392-397.

Gadhi, C.A., A. Benharref, M. Jana & A. Lozniewski. 2001. Anti *Helicobacter* activity of *Aristolochia paucinervis* Pomel extracts. *Journal of Ethnopharmacology* 75:203-205.

Gasbarrini, A., F. Franceschi, I.G. Gasbarrin & P. Pola. 1997. Extraintestinal pathology associated with *Helicobacter* infection. *European Journal of Gastroenteronology and Hepathology* 9:231-233.

Goh, K.I., N. Parasakthi, P.I. Cheah, C.K. Ranjeev, I.M. Rosmawat, Y.-M. Tan & S.-C. Chins. 2000. *Helicobacter pylori* infection and gastric mucosal lesion.Efficacy of a 1-week pantoprazole triple therapy in eradicating *Helicobacter pylori* in Asian patients. *Journal of Gastroenterology and Hepatology* 15:910-914.

Gomes, B.C. & E.C.P. DeMartinis. 2004. The significance of *H. pylori* in water, food and environmental samples. *Food Control* 15:397-403.

Harborne, J.B. 1998. *Phytochemical Methods. A guide to modern techniques of plant analysis*. 3rd edition. Chapman and Hall, London.

Ingolfsdottir, K., M.A. Hjalmarsdottir, A. Sigurdsson, G.A. Gudjonsdottir, A. Brynjolfsdottir & O. Steingrimsson. 1997. In vitro susceptibility of *Helicobacter pylori* to protolichesterinic acid from the lichen *Cetraria islandica*. *Antimicrobial Agents and Chemtherapy* 41:215-217.

Hamasaki, N., E. Ishii, K. Tominaga, Y. Tezuka, T. Nagaoka, S. Kadota, T. Kuroki & I. Yano. 2000. Highly selective antibacterial activity of novel alkyl quinolone alkaloids from a Chinese herbal medicine, Gosyuyu (Wu-Chu-Yu), against *Helicobacter pylori* In Vitro. *Microbiology and Immunology* 44:9-15.

Jenks, P.J. & D.I. Edwards. 2002. Metronidazole resistance in *Helicobacter pylori*. *International Journal of Antimicrobial Agents* 19:1-7.

Jones, N.L, S. Shabib & P.M Sherman. 1997. Capsaicin as an inhibitor of the growth of the gastric pathogen *Helicobacter pylori*. *Federation of European Microbiology Societies Microbiology Letters* 146:223-227.

Kester, D.R., I.W. Duedall, D.N. Connors & R.M. Pytkowicz. 1967. Preparation of artificial seawater. *Limnology and Oceanography* 12:176-179.

Klausmeyer, P., G.N. Chmurny, T.G. McCloud, K.D. Tucker & R.H. Shoemaker. 2004. A novel antimicrobial indolizinium alkaloid from *Aniba panurensis*. *Journal of Natural Products* 67:1732-35.

Kwon, D.H., K. Hulten, M. Kato, J.J. Kim, M. Lee, F.A.K. El-Zaatari, M.S. Osato & G.Y. Graham. 2001. DNA sequence analysis of *rdxA* and *frxA* from 12 pairs of metronidazole-sensitive and -resistant clinical *Helicobacter pylori* isolates. *Antimicrobial Agents and Chemotherapy* 45:2609-2615.

Lai, C.-H., S.-H. Fang, Y.K. Rao, M. Geethangili, C.-H. Tang, Y.J. Lin, C.-H. Hung, W.-C. Wang & Y.-M. Tzeng. 2008. Inhibition of *Helicobacter pylori* induced inflammation in human gastric epithelial AGS cells by *Phyllanthus urinaria* extracts. *Journal of Ethnopharmacology* 118:522-526.

Leong , R.W. 2009. Differences in peptic ulcer between the east and the west. *Gastroenterology Clinics of North America* 38:363-379.

Li, Y., C. Xu, Q. Zhang, J.Y. Liu & X. Tan. 2005. Invitro anti-*Helicobacter pylori* action of 30 Chinese medicines used to treat ulcer diseases. *Journal of Ethnopharmacology* 98:329-333.

Liou, J.-M., J.-T. Lin, Y-C. Lee, C.-Y. Wu & M.-S. Wu. 2008. *Helicobacter pylori* infection in the elderly. *International Journal of Gerontology* 2:145-152.

Mabe, K., M. Yamada, I. Ogun & T. Takahashi. 1999. In-vitro and in-vivo activities of tea catechins against *Helicobacter pylori*. *Antimicrobial Agents and Chemtherapy* 43:1788-1791.

Malaty, H. 2007. Epidemeology of *Helicobacter pylori* infection. *Best Practice and Research. Clinical Gastroenterology* 21:205-214.

Malaty, H.M., A.B. El-Kasbany, D.Y. Graham, C.C. Miller, S.G. Reddy, S.R. Srinivasan, Y. Yamaoka & G.S. Berenson. 2002. Age at acquisition of *Helicobacter pylori* infection: A follow up study from infancy to adulthood. *Lancet* 359:931-935.

McDermott, P.F., J.L. Simala-Grant & D.E. Taylor. 2009. Anitimicrobial resistance in *Helicobacter* and Campylobacter. Pp. 847-864 in *Antimicrobial Drug Resistence, Mechanisms of Drug Resistence*. Volume 1. Edited by D.L. Mayers. Humana Press, Totowa, New Jersey.

Mégraud, F. 2001. Global spectrum of *Helicobacter pylori* Antibiotic Resistance. *World Journal of Gastroenterology* 6:19-20.

Moujaber, T., C. Maclntyre, J. Backhouse, H. Gidding, H. Quinn & T.G. Gilbert. 2008. The seroepidermeology of *Helicobacter pylori* infection in Australia. *International Journal of Infectious Diseases* 12:500-504.

Murphy Cowan, M. 1999. Plant products as antimicrobial agents. *Clinical Microbiology Reviews* 12:564-582.

NCI. 2006. *H. pylori* and cancer: Fact sheet. www.cancer.gov/cancertopics/factsheet/risk/h-pylori-cancer

Ndip, R.N., A.E. Malange Tarkang, S.M. Mbullah, H.N. Luma, A. Malongue, L.M. Ndip, K. Nyongbela, C. Wirmum, S.M.N. Efange. 2007. *In vitro* anti-*Helicobacter pylori* activity of extracts of selected medicinal plants from North West Cameroon. *Journal of Ethnopharmacology* 114:452-457.

O'Gara, E.A., D.J. Hill & D.J. Maslin. 2000. Activities of garlic oil, garlic powder, and their diallyl constituents against *Helicobacter pylori*. *Applied and Environmental Microbiology* 66:2269-2273.

Okabe, N., T. Hooya, T. Takahashi & F. Matsumoto. 1997. Incidence of *Helicobacter pylori* infections and therapy for anastomotic ulcers and residual gastritis in patients with residual stomachs. *Journal of Infection and Chemotherapy* 3:205-208.

Osato, M., R. Reddy & D. Graham. 1999. Metronidazole and clarithromycin resistance amongst *Helicobacter pylori* isolates from a large metropolitan hospital in the United States. *International Journal of Antimicrobial Agents* 12:341-347.

Pakodi, F., O.M.E. Abdel-salam, A. Debreceni & G. Mozsik. 2000. *Helicobacter pylori*; One bacterium and broad

spectrum of human disease: An Overview. *The Journal of Physiology-Paris* 94:139-152.

Park, B., H. Lee, S. Lee, X. Piao, G.R. Takeoka, R.Y. Womg, Y. Ahn & J. Kim. 2006. Antibacterial activity of *Tabebuia impetiginosa* Martius ex DC (Taheebo) against *Helicobacter pylori*. *Journal of Ethnopharmacology* 105:255-262.

Petschow, B.W., R.P. Batema & L.L. Ford. 1996. Susceptibility of *Helicobacter pylori* to bactericidal properties of medium-chain monoglycerides and free fatty acids. *Antimicrob Agents and Chemotherapy* 40:302-306.

Quaglia, N.C., A. Dambrosio, G. Normanno, A. Parisi, R. Patrono, G. Ranier, A. Rella & G. Celano. 2008. High occurrence of *Helicobacter pylori* in raw goat, sheep, and cow milk inferred by glm M gene: A risk of food borne infection. *International Journal of Food Microbiology* 124:43-47.

Ragab, Y., A. Al-hejin & F. Amer. 2009. Resistance of *Helicobacter pylori* to antibiotics and associated risk factors. *International Journal of Antimicrobial Agents* 34 (Suppl.):566-569.

Rahman, Z., A.M. Rhaman, M.S. Arfin, M.M. Alam, I.M. Bhuiyan, N. Ahmed & S.N. Hassan. 2009. *Helicobacter pylori* infection and strain types in adult dyspeptic patients undergoing endoscopy in a specialized hospital of Dhaka City. *Bangladesh Journal of Medical Microbiology* 30(1):4-9.

Salasawati, H. & M. Ramela. 2008. Antibiotic resistance patterns and genotypes of *Helicobacter pylori* isolated from ethnically different dyspeptic patients in Kualar Lumpur Malaysia. *International Journal of Infectious Diseases* 12(supp):e124.

Svensson, B.M., L. Mathiasson, L. Martensson & Y. Bergstoms. 2005. *Artemia salina* as test organism for assessment of acute toxicity of leachate water from land fills. *Environmental Monitoring and Assesment* 102:309-321.

Tabak, M., R. Armon & I. Neeman. 1999. Cinnamon extracts' inhibitory effect on *Helicobacter pylori*. *Journal of Ethnopharmacology* 67:269-277.

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

Uyub, A.M. & S. Sasidharan. 2000. *Development and Evaluation of Storage Medium for Helicobacter pylori.* Paper presented at symposium of The Malaysian Society for Microbiology.

Warren, J.R. & B. (1983).. Marshall. 1983. Unidentified curved bacilli on gastric epitelium in active chronic gastritis. *Lancet* (letters). 1:1273-1275.

Wolle, K. & P. Malfertheiner. 2007. Treatment of *Helicobacter pylori*. *Best Practice and Research Gastroenterology* 21:315-324.