ORIGANUM MINUTIFLORUM AS A POTENTIAL THERAPY FOR THE ERADICATION OF *HELICOBACTER PYLORI*

by
Fatma ÖZEN

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ORIGANUM MINUTIFLORUM AS A POTENTIAL THERAPY FOR THE ERADICATION OF *HELICOBACTER PYLORI*

APPROVED BY:

Asst. Prof. May Korachi
(Supervisor)

Assoc. Prof. F. Yeşim Ekinci

Asst. Prof. Mayur Tamhane

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ABSTRACT

ORIGANUM MINUTIFLORUM AS A POTENTIAL THERAPY FOR THE ERADICATION OF *HELICOBACTER PYLORI*

Helicobacter pylori is a gram negative, spiral shaped, microaerophilic bacterium which colonizes the human stomach of more than half of the world's population. *H. pylori* infection plays a crucial role in the pathogenesis of peptic ulcer, gastritis, and gastric cancer. Infection of *H. pylori* treatment consists of a proton pump inhibitor (PPI) with a combination of antibiotics. This study aims to investigate the potential use of commercial extracts of Siberian ginseng (*Eleutherococcus senticosus)*, Black cohosh (*Cimicifuga racemosa*), Red clover (*Trifolium pratense*), Milk thistle (*Silybum marianum)* and various extracts (methanol, ethanol, aqueous, essential oil) of a Turkish endemic plant *Origanum minutiflorum* for the eradication of *H. pylori*. Methanol, ethanol, aqueous and essential oil (EO) extracts of *O. minutiflorum* were collected via soxhlet extractor and clevenger apparatus. The anti-bacterial activity (agar well diffusion method) and the minimum bactericidal concentration (MBC) of all extracts were determined according to CLSI protocols. The chemical composition of the extract showing the strongest antimicrobial activity was determined via gas chromotography mass spectrom (GC-MS). The cell viability effects of the chosen extract were determined on AGS (human adenocarcinoma) cell line by WST-1 assay for 24, 48, and 72 hours. *H. pylori* adhesion and invasion of cultured cells was carried out using a standard gentamicin assay at 1/20,000- 1/40,000 (v/v) dilutions of *O. minutiflorum* EO. Examination of effect of *O. minutiflorum* EO on *H. pylori* and *H. pylori* infected cells were done by scanning electron microscopy (SEM). The results of *in vitro* studies indicated that the EO extract of *O. minutiflorum* (92 µg/ml) displayed the strongest antimicrobial effect against *H. pylori* (ZI= ≥90) in comparison to all other plant extracts. The major compound of the EO was determined as carvacrol (29.22 %). The MBC was observed at a concentration of 46 µg/ml (equal to 1/20,000 (v/v) dilution) EO. The MBC value of EO did not show any cytotoxic effect against AGS cells and exhibited a reduction of more than 80 % *H. pylori* adhered or invaded AGS cells.

The findings of this study show the potential future use of *O. minutiflorum* EO as an alternative therapy to antibiotics for treatment of *H. pylori* infections.

ÖZET

*HELICOBACTER PYLORI'*NİN YOK EDİLMESİNDE POTANSİYEL TERAPİ OLARAK *Origanum minutiflorum*

Helicobacter pylori dünya nüfusunun yarısından fazlasının midesinde kolonize olmuş olan gram negatif, spiral şekilli, mikroarofilik bir bakteridir. *H. pylori* enfeksiyonu peptik ülser, gastrit ve mide kanseri patogenezinde önemli rol oynar. *H. pylori* enfeksiyonunun tedavisinde proton pompası inhibitörü ve antibiyotik kombinasyonundan oluşan tedavi uygulanır. Bu çalışmada Sibirya ginsengi (*Eleutherococcus senticosus*), Karayılan otu (*Cimicifuga racemosa*), Kırmızı yonca (*Trifolium pratense*), Süt devedikeni (*Silybum marianum*) ve Türk endemik bitkisi *Origanum minutiflorum*'un çeşitli ekstratları (metanol, etanol, su, esansiyel yağlar) *H. pylori'*nin yok edilmesi için kullanılmıştır. *Origanum minutiflorum'*un metanol, etanol, su ve esansiyel yağ ekstraktları sokslet ve klevenger cihazları ile elde edilmiştir. Antimikrobiyal aktivite ve minimum öldürücü konsantrasyonu (MBC) CLSI protokolüne göre belirlenmiştir. Güçlü antimikrobiyal aktive gösteren bitki ekstralarının kimyasal kompozisyonu GC-MS ile analiz edilmiştir. Seçilen ektraktların hücre canlılığına olan etkisi AGS hücre hattı üzerinde 24, 48 ve 72 saatte WST-1 ile test edilmiştir. *O. minutiflorum* esansiyel yağının (1/20,000 ve 1/40,000 dilüsyonlarında), *H. pylori*'nin kültür hücrelerine adhezyonu ve invazyonuna etkisi standart gentamisin deneyi kullanılarak tespit edilmiştir. *O. minutiflorum* esansiyel yağının *H. pylori* ve *H. pylori* ile enfekte olmuş hücrelere olan etkisi SEM (taramalı elektron mikroskobu) ile gözlemlenmiştir. Deney ortamında yapılan çalışmaların sonuçları, diğer bitki ektraktları ile karşılaştırıldığında *H. pylori*'ye karşı en güçlü antimikrobiyal etkiye *O. minutiflorum* esansiyel yağının sahip olduğunu göstermiştir (ZI=≥90mm). Esansiyal yağın başlıca bileşeni karvakrol (29.22 %) olarak tespit edilmiştir. Esansiyel yağın MBC konsantrasyonu 46 µg/ml (1/20,000 (v/v) dilusyona eşit) olarak tespit edilmiştir. MBC değeri AGS hücreleri üzerinde herhangi bir sitotoksik etki göstermemiş ve *H. pylori*'nin AGS hücrelerine adhezyonunda ve invezyonunda % 80'den fazla azalmaya sebep olmuştur.

Bu çalışmanın bulguları *H. pylori* enfeksiyonunun tedavisinde *O. minutiflorum* esansiyel yağının antibiyotik tedavisine alternatif olarak gelecekte kullanılabilineceğini göstermektedir.

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LIST OF SYMBOLS / ABBREVIATIONS

AMX	Amoxicillin
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
Cag A	Cytotoxin Associated Antigen
CFU/ml	Colony forming units per mililiter
CLR	Claritromicin
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
DMSO	Dimethyl Sulfoxide
dupA	Duodenal ulcer promoting gene
EO	Essential oil
FBS	Fetal Bovine Serum
GC-MS	Gas Chromatography equipped with Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LPS	Lipopolysaccharides
MALT	Gastric Mucosa Associated Lymphoid Tissue
MBC	Minimum Bactericidal Concentration
MET	Metrodinazole
OD	Optical Density
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute medium -
SEM	Scanning Electron Microscopy
Vac A	Vacuolating Cytotoxin A
WHO	World Health Organization

1. INTRODUCTION

1.1. *Helicobacter pylori*

Helicobacter pylori is a gram negative, spiral shaped, microaerophilic bacterium, measuring 2 to 4 µm in length and 0.5 to 1 µm in width. Although it is usually spiral-shaped, the bacterium can appear as a rod shape, while coccoid shapes appear after prolonged in vitro culture or antibiotic treatment [1]. Although coccoid forms of the bacteria may represent a viable but nonculturable state, these forms cannot be cultured in vitro and are thought to represent dead cells [1, 2]. *H. pylori* has 2 to 6 unipolar, flagella filaments of approximately 3 µm in length, which provides motility and allows rapid movement [3] (Figure 1.1).

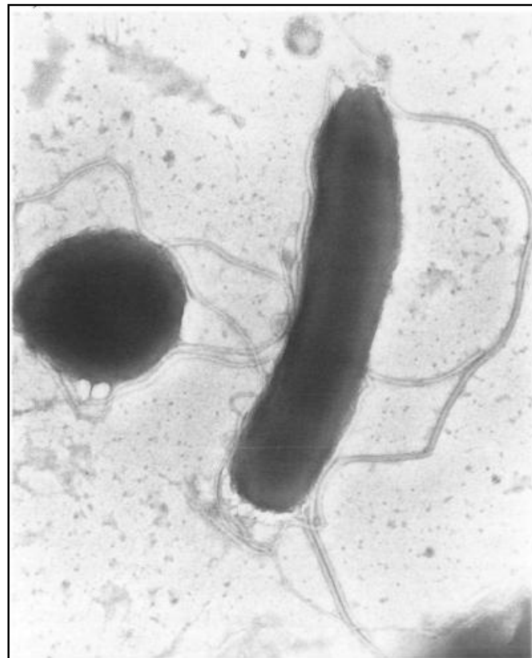

Figure 1.1. Electron micrograph, rod and coccoid forms of *Helicobacter pylori* with associated multiple polar sheathed flagella [5].

H. pylori grows in microaerophilic conditions, with optimal growth at 85% N_2, 5% O_2 and 10% CO_2 and high humidity with an optimum temperature of 37 ^oC and neutral pH [4].

Colonies of the bacteria are cultured on supplemented blood agar and translucent colonies are observed within 3-5 days [5].

1.1.1. History of *Helicobacter pylori*

Helicobacter pylori was discovered by Robin Warren and Barry Marshall in 1983 which was cultivated from gastric biopsy samples [6]. They found that the organism was present in almost all patients with gastric inflammation, duodenal ulcers or gastric ulcers [6]. And, in 1994, *H. pylori* was classified as a group I or definite carcinogen (the only bacterium to be thus classified) by the World Health Organization's (WHO) International Agency for Research on Cancer [7]. This important discovery was rewarded in the 2005with the Nobel Prize for physiology or medicine.

The first *H. pylori* genome sequence was published in 1997 by Tomb et al. [8]. Since then more than 7 full genome sequences have been made available in the public domain [8, 9]. The *H. pylori* genomic comprises of about 1.6 megabases, encoding approximately 1500 predicted open reading frames (ORF). Because of the high spontaneous mutation rate and relatively high recombination frequency, about 20-30 % of the genome is variable between different strains [10].

1.1.2. Taxonomy of *Helicobacter pylori*

The taxonomy of *Helicobacter pylori* has changed over decades. It was first classified by Marshall in 1982, as *Campylobacter pyloridis* and then corrected to *C. pylori* [6, 11]. *C. pylori* was sometimes referred to as Gastric *Camyplobacter* Like Organism (GCLO) [12]. Although *C. pylori* resembles *Campylobacter* in many aspects, it differs in important features such as its flagellum morphology, fatty acid contents, and 16S rRNA sequence. Because of these differences, in 1989 *C. pylori* was transferred to a new genus, *Helicobacter*, and named *Helicobacter pylori* [13].

Helicobacter species can be subdivided into two major lineages which are the gastric *Helicobacter* species and the enterohepatic (nongastric) *Helicobacter* species. The difference between these two groups is high level organ specificity which means that

gastric helicobacters are not able to colonize the intestine or liver [4]. Although all known gastric *Helicobacter* species are urease positive, some enterohepatic *Helicabacter* species do not have urease virulence factor [14].

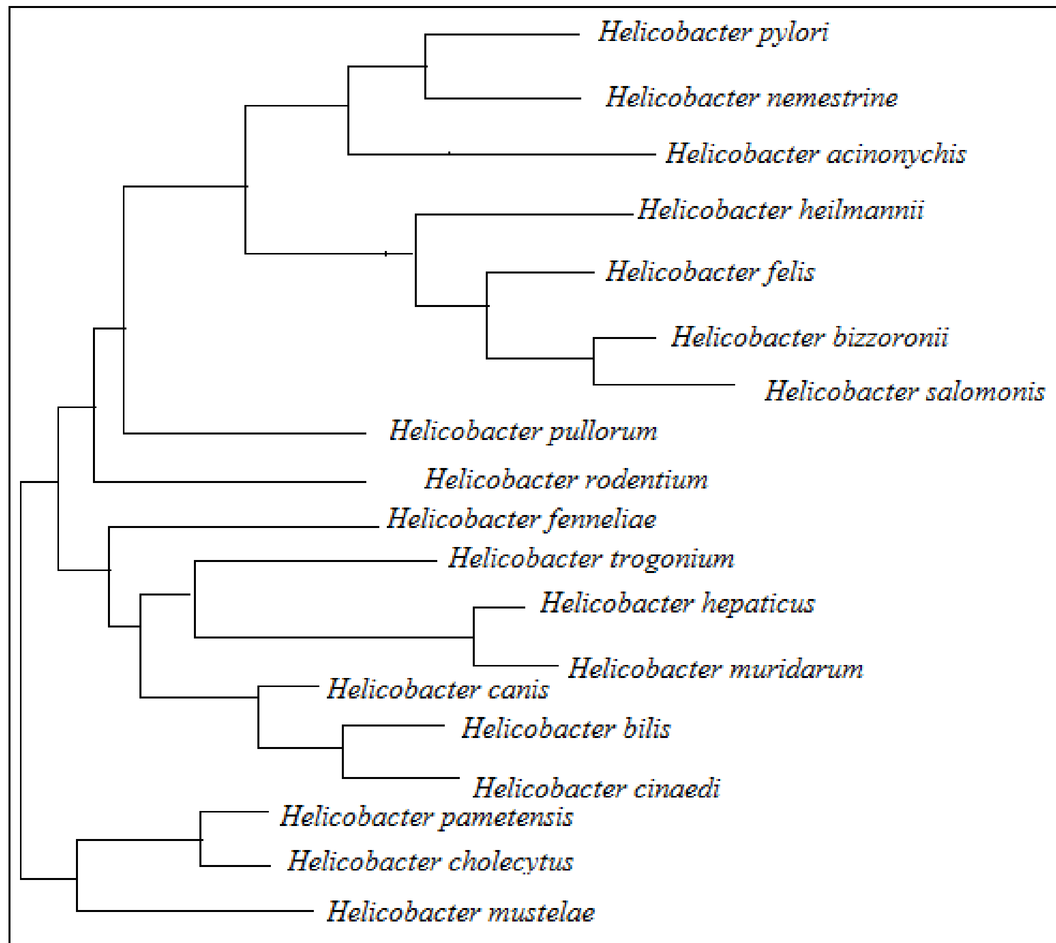

Figure 1.2. Phylogenetic tree of *Helicobacter* species based on the similarities of 16S rRNA sequences [15].

1.1.3. Incidence of *Helicobacter pylori* Infection

H. pylori colonizes the human stomach of more than half of the world's population [16]. *H. pylori* is the main cause of gastritis, peptic ulcer, and gastric cancer which is developed by the direct action of its virulence factors and indirectly by initiation and maintenance of chronic inflammation in the gastric mucosa. Therefore, approximately 70% of all gastric cancer patients are related to prior *H. pylori* infection [17].

The incidence of *H. pylori* infection is related to environmental factors such as age, socioeconomic status, ethnicity, gender and diet [18, 19]. Prevalence of *H. pylori* infection in developed countries is lower than that in developing countries (Figure 1.3). This situation has been associated with a higher incidence in the past due to poor living conditions, and sanitation [20, 21]. Also, in prevalence of infection has been attributed to the rate of acquisition of *H. pylori* in childhood. Moreover, a positive correlation has reported between age and prevalence of *H. pylori* in both developed and developing countries which was found to be higher in adults than in children [22, 23, 24].

Figure 1.3. Incidence of *H. pylori* in the world [25]

Differences in *H. pylori* prevalence among various ethnic and racial groups has been reported [26]. For instance, *H. pylori* infection is an increased risk as an inherent ethnic genetic predisposition for Malaysian, Chinese and Indian populations [26]. Gender is also another important factor that is associated with *H. pylori* prevalence. According to Woodward et al. (2000) [27], there is a higher prevalence of *H. pylori* infection in men than in women.

Although transmission of *H. pylori* is not clear, it is believed to propagate via human-to-human through oral-oral, faecal- oral transmission and/or both routes. Therefore, mother to child transmission is the most common cause of interfamilial spreading of *H. pylori* infection [28]. The number of children per house is also a positive relative risk factor of *H. pylori* infection incidence [29]. For instance, infection rates among children were seen to be significantly higher when their mothers were infected, and a strong link of *H. pylori* infection between children with infected siblings close in age [30, 31].

Diet is also another risk factor that has been related to pathogenicity of *H. pylori*. For example, high salt consumption upregulates several *H. pylori* virulence genes *in vitro* which leads to changes in gastric epithelial cell morphology and function [32]. Contaminated food, water and daily consumables (such as raw milk and vegetables) under unhygienic conditions are also risk factors for transmission of the bacteria [33, 34].

Consequently, the incidence of *H. pylori* infection is related to multiple factors, including host-genetic factors (such as cytokine polymorphisms), *H. pylori* strain heterogeneity and environmental factors.

1.1.4. Virulence Factors *Helicobacter pylori*

Virulence factors enable *H .pylori* to colonize and cause a chronic infection in a niche that cannot normally be colonized by other bacteria. A number of virulence factors have been well-recognised and characterized in disease associated *H. pylori* strain including genes (VacA, CagA), flagella, enzymes (urease), lipopolysaccharides (LPS), and membrane proteins (BabA, OipA).

H.pylori is genotypically and phenotypically a heterogeneous bacteria that has special features to overcome hosts defense mechanisms. For instance, the stomach has an acidic environment, so *H. pylori* have to survive in the acidic gastric juice for at least a short period of time. Moreover, the bacteria need to enter the highly viscous gastric mucus layer to reach and adhere to epithelial gastric cells, obtain nutrients and to avoid the host immune response. The bacterial virulence factors are related with the survival mechanisms

of *H. pylori.* Strains of *H. pylori* have been grouped into two families referred to as type I and type II based on whether they produce VacA and the CagA antigen [35].

1.1.4.1. The Cytotoxin Associated Antigen (Cag A) Pathogenicity Island

Cytotoxin associated gene (Cag) pathogenicity island (a large DNA fragment in the genome of a pathogen that contains virulence genes) (Cag PAI), encoded T4SS is a 40 kilobase segment of DNA, containing 31 genes, many of which encode components of a type 4 bacterial secretion system [36, 37]. The secretion system acts as a molecular syringe for delivery of bacterial products (CagA and peptidoglycan component) into eukaryotic cells [38]. The cag PAI plays a crucial role during pathogenesis of *H. pylori*, most strains of *H. pylori* carry the cag pathogenicity island which the presence this region of the genome are less likely to be related with gastric cancer [39, 40]. CagA is a variable molecular mass 121–145 kDa protein, encoded by one of the genes (CagA) within the cag PAI [41]. Translocation of CagA into the host cell is recognized as a signaling molecule and activated by phosphorylation on tyrosine residues in EPIYA motifs (its five amino-acid Glu-Pro-Ile-Tyr-Ala) [42, 43] by host Src family kinases [44, 45]. Phosphorylated CagA interacts with a range of host signaling molecules, such as tyrosine phosphatase SPH-2 [46] which induces MAP kinase [47] resulting in morphological changes (hummingbird phenotype), abnormal proliferation and movement of host gastric cells [48] (Figure 1.4).

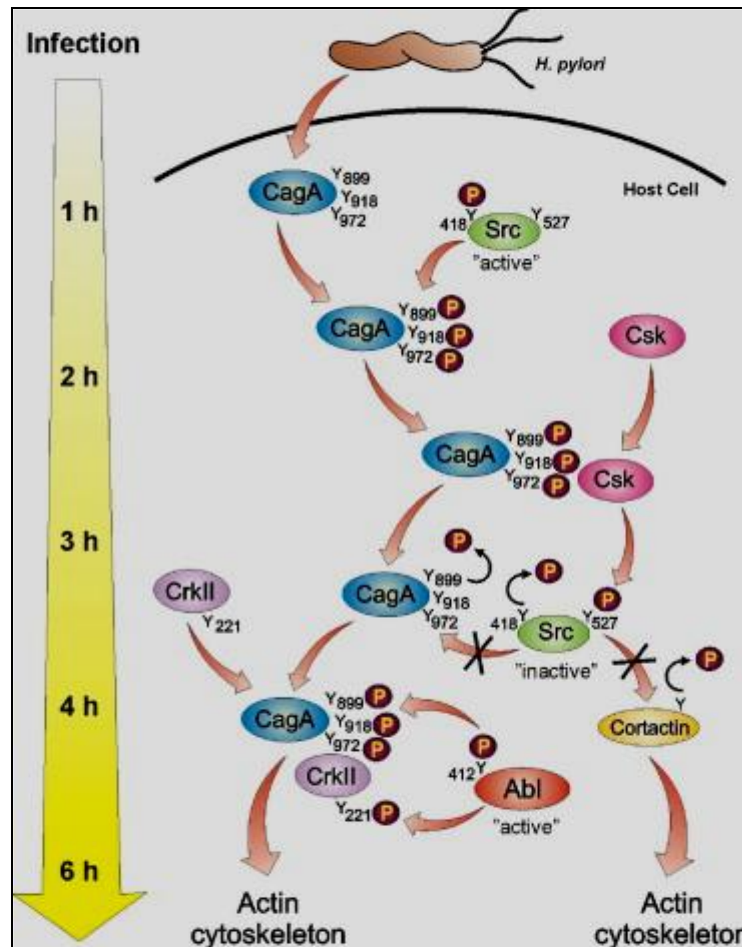

Figure 1.4. Model for CagA phosphorylation and CagA-induced signaling leading to the cytoskeletal rearrangements of infected cells [49].

However, non-phosphorylated CagA interacts with certain host cell proteins; the epithelial tight junction-scaffolding protein zonulin (ZO-1), the cell adhesion protein E-cadherin, the hepatocyte growth factor receptor c-Met, the cadherin-associated protein b-catenin, the adaptor protein GRB-2 and the kinase PAR1 [50, 51, 52, 53, 54, 55]. These non-phosphorilated CagA–host protein interactions disrupt tight and adherent junctions, leading to a loss of cell polarity, and inducing pro-inflammatory and mitogenic response-effects that may be important in the development of gastric carcinoma.

CagA also has the ability to activate nuclear factor κB (NF-κB) (regulator of many cellular processes including the control of the immune response and inflammation) and induces IL-8 (proinflammatory cytokine) secretion [56, 57].

1.1.4.1. The Vacuolating Cytotoxin (Vac A)

Another major *H. pylori* virulence factor is the vacuolating cytotoxin (VacA), which induces cytoplasmic vacuolation in host epithelial cells [58]. Vacuolating exotoxin (*VacA*) also exhibits high levels of genetic diversity amongst the *H. pylori* genome [59] which has the importance of forming intracellular vacuoles in gastric and other epithelial cells. All *H. pylori* strains contain a copy of the gene. VacA is a moleculer mass 140 kDa protoxin protein. VacA has been reported to produce multiple structural and functional alterations in epithelial cells; disrupts endosomal maturation resulting in vacuolation, selectively increase the permeability of polarized epithelial cell monolayers leading to barrier dysfunction at tight junctions and also induces mitochondrial damage, cytochrome c release and gastric epithelial cell apoptosis [60, 61, 62, 63, 64] (Figure 1.5.).

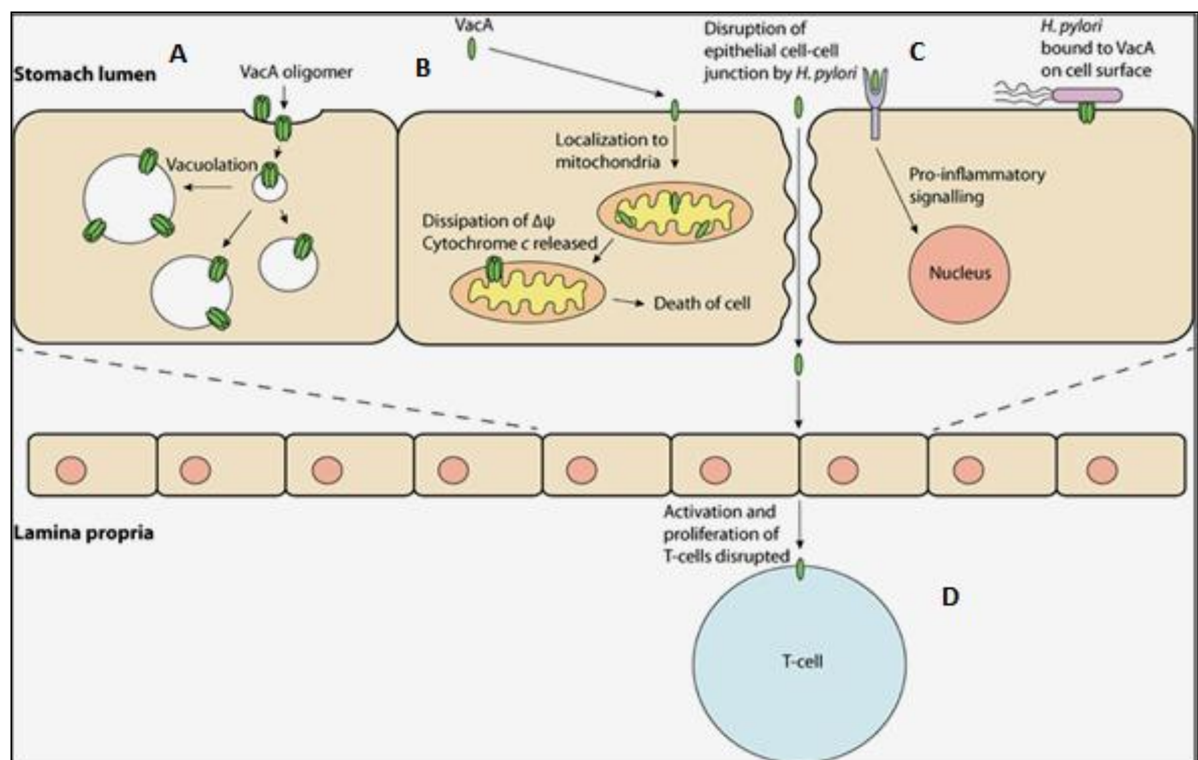

Figure 1.5. Schematic view of *H. pylori* virulence factor VacA. A: VacA be taken up by the cell and produce vacuoles. B: Localize to the mitochondria and induce apoptosis. C: Bind to a protein on the cell membrane and induce inflammation. D: Pass through the tight junction and obstruct T-cell activation and proliferation [65].

1.1.4.2. Adhesions and Outer Membrane Proteins

H. pylori can adhere tightly to human gastric epithelial cells which is relevant to its colonization and the persistence of infection. Although more than ten different binding specificities have been reported for glycoproteins, carbohydrates and phospholipids [66], two types of adhesins have been well characterized by molecular cloning and the construction of isogenic mutants; Blood Group Antigen Binding Adhesion A (BabA), Outer Inflammatory Protein A (Oip A) (HopH), and SabA (Figure 1.6).

BabA is a 78-kDa outer membrane protein, encoded by the babA2 gene, it binds to Lewis b antigens and ABO antigen on the human host cells [67]. Adherence is an important virulence factor for *H. pylori*. Functional receptors for *H. pylori* adherence include fucosylated ABO blood group Lewis b antigen and sialyl-Lewis x/a antigens. Adhesion avoids bacterial shedding and enables *H. pylori* to efficiently use its secretion systems for delivery of their effector molecules to modulate the host [68].

The outer membrane protein A (OipA) is a member of the Hop protein family. The gene encoding the OipA protein, a 34 kDa outer membrane protein, is present in all *H. pylori* strains. OipA has been significantly associated with duodenal ulceration [69, 70] and gastric cancer [70].

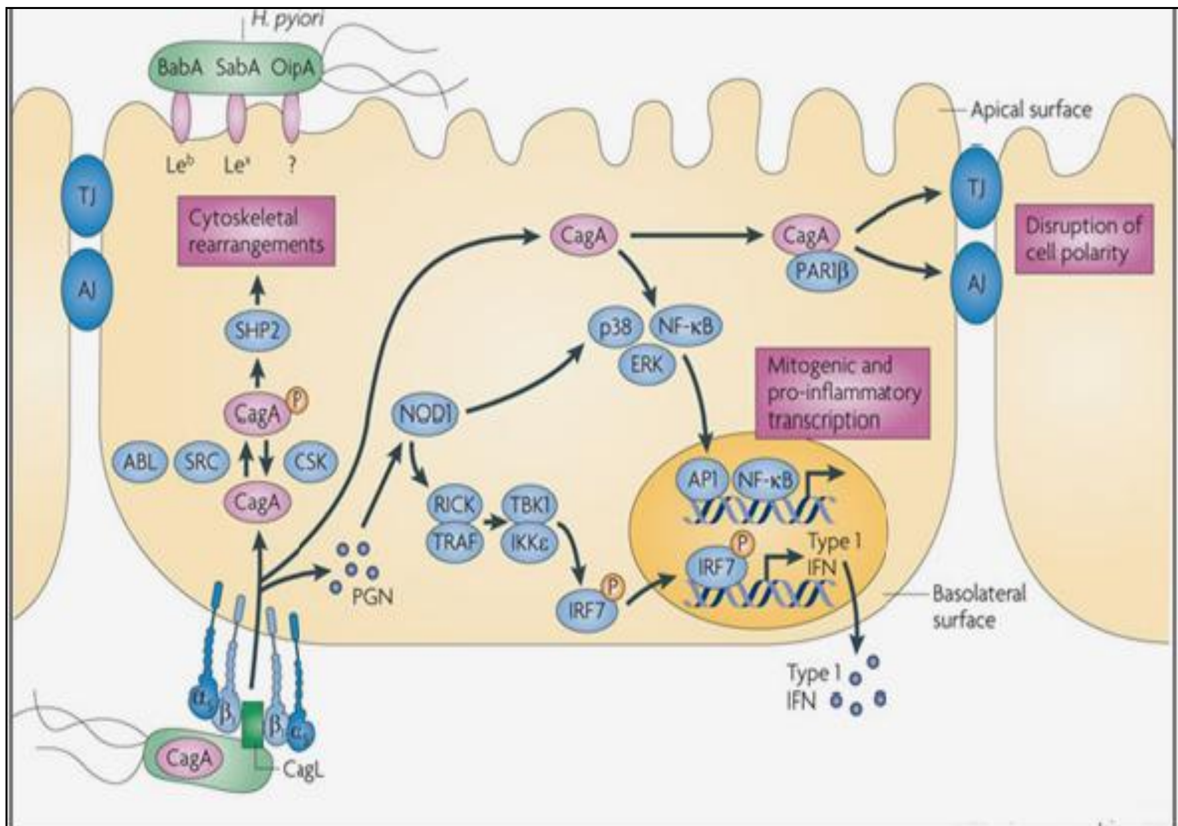

Figure 1.6. Schematic view of *Helicobacter pylori* adhesions and outer membrane proteins; BabA, OipA, and Sab A virulence factors [71].

1.1.4.4. Duodenal ulcer promoting gene (dupA)

A duodenal ulcer promoting gene (dupA), located in the ‘‘plasticity region” of the *H. pylori* genome, is a novel virulence marker. The infection with dupA-positive strains was found significantly associated with duodenal ulceration but negatively associated with gastric cancer [68]. However, *H. pylori* virulence genes have been associated with both ulcer disease and gastric cancer, whereas patients with duodenal ulcers are paradoxically at a decreased risk of gastric cancer [72].

1.1.4.5. Urease Enzyme

Although the gastric mucosa is well protected against bacterial infections, *H. pylori* is highly adapted to its ecological niche using a bundle of polar flagella, a potent urease and additional features that allow its oriented swimming and multiplication in the mucus,

attachment to epithelial cells, evasion of the immune response, and persistent colonization and transmission.

H. pylori produces large amounts of urease which is a virulence factor of *H. pylori* [73, 74]. The absence of urease enzymatic activity renders the bacterium unable to colonize the gastric mucosa, so it is the major factor in the resistance to gastric acidity. *H. pylori* is not an acidophilic bacterium, however it can survive the acidic gastric environment in a pH of 2-3. The reaction catalyzed by urease produces 2 mole ammonia and 1 mole carbon dioxide for every mole of urea hydrolyzed (Figure 1.7.). The ammonia produced by this reaction increases the pH of the environment [75]. Urease activity is present in all *H. pylori* strain and urease is located on the cell surface, from where it can create a cloud of ammonia around the cells [75]. It is assumed that one role of this enzyme is the regulation of pH during the time that *H. pylori* is present in the stomach [73].

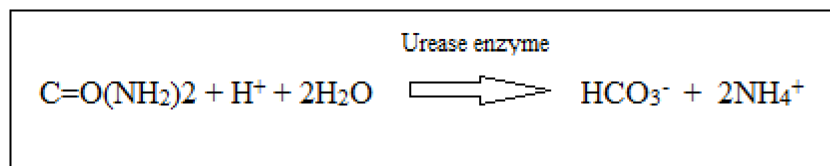

Figure 1.7. Urease enzyme reaction.

1.1.5. Diagnosis of *H. pylori*

Helicobacter pylori can be diagnosed by invasive tests based on gastric samples and non-invasive tests based on clinical specimens, such as blood, breath, faeces, urine, or saliva. These samples can be screened by serology, urea test, polymerase chain reaction (PCR), or enzyme like immunosorbent assay (ELISA) (anti *H. pylori* IgG antigen test) [76]. The choice of a specific test for a patient depends on several factors, including gastric complaints, age, local experience, costs, and clinical information. When the alarm symptoms such as weight loss, anaemia, and dyspepsia are absent, endoscopic examination is usually not required [77]. Diagnosis of *H. pylori* is limited by non-invasive methods. For example, serology is limited to the confirmation of infection by presence because of anti *H. pylori* serum can be detected several months after treatment [78].

1.1.6. Treatment of *H. pylori*

Helicobacter pylori is susceptible to most antibiotics *in vitro*, however only a few antibiotics have shown a good effect in infected people [79]. *In vivo* limitations of antibiotics are due to the inactivation of drugs at low pH's (1.5 to 3.5), the slow growth rate of *H. pylori,* and inability of drugs to reach appropriate levels in the gastric mucus layer [80, 81]. Metronidazol, clarithromycin, amoxicillin, tetracycline and bismuth are the most commonly used antibiotics for treatment. For the first line therapy, two antibiotics and one proton pump inhibitor (PPI) or ranitidine bismuth citrate (RBC) are used for *H. pylori* infection for 7-14 days. Although high success rates have been seen in clinical practice, in general 20 % of first line therapies fail, therefore patients are usually re-treated with a second line regimen by another combination of antibiotics. The recommended second-line therapy is the quadruple regimen which includes; tetracycline, metronidazole, bismuth salts and a PPI. However, the efficacy of this therapy is also limited due to its side effects, number of tablets per day, and long duration. Moreover, bismuth and metronidazole are not available especially in developing countries. As an alternative to this quadruple second line regimen, a longer-lasting (i.e. 10 to 14 days) PPI or RBC triple therapy with two alternative antibiotics are commonly used. The choice of antibiotics for second line therapy depends on the first line regimen. For example, if clarithromycin was used as an antibiotic in first line therapy, as a second line regimen a different antibiotic should be suggested such as; metronidazole, amoxicillin, and tetracycline. Unfortunately, in case of second-line treatment failure, there is a limited choice of antibiotics and no standard third-line therapy [82] (Figure 1.8.). Antibiotic susceptibility can be detected using biopsies and/or faecal samples by culture (e.g. agar dilution, E-test, broth dilution, and disc diffusion) and nucleic acid-based (e.g. PCR, immunoassays, and FISH) techniques in *H. pylori*. Because of several factors including antibiotic resistance, patient related factors, treatment related factors, failure of *H. pylori* treatment could occur [83].

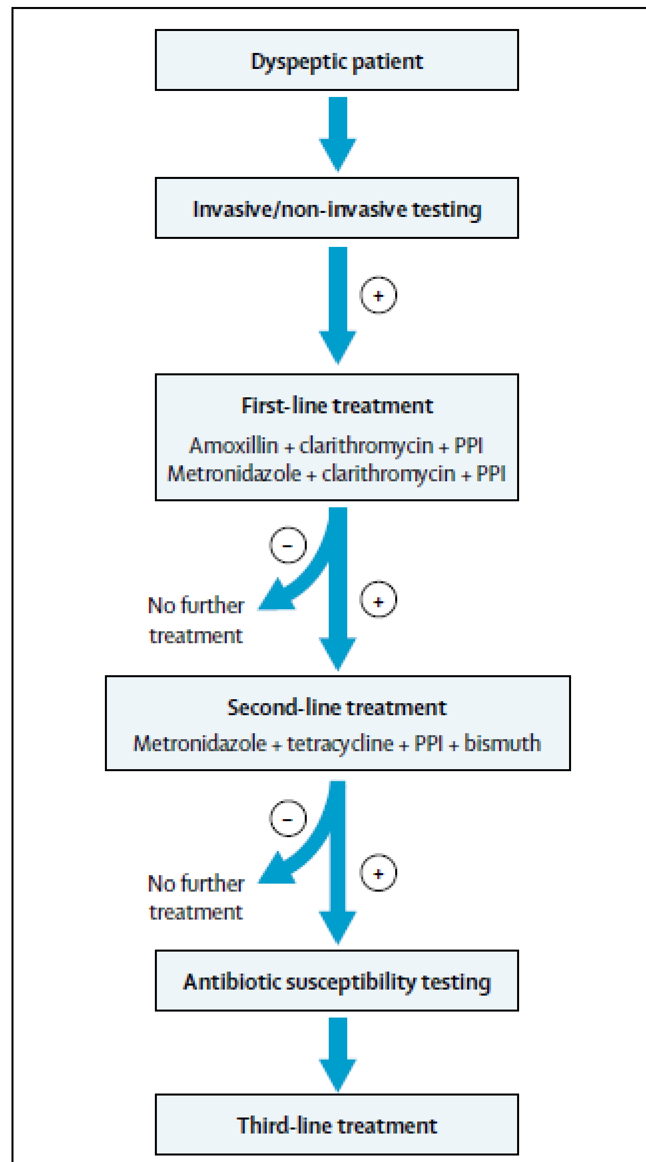

Figure 1.8. *H. pylori* treatment regimen [82].

1.1.6.1. Antibiotic Resistance

H. pylori is predominantly extracellular, but is occasionally described in an intracellular location within gastric epithelial cells. The intracellular location of *H.* pylori may facilitate resistance to antibiotics, especially in gastric cancers [8].

The available antimicrobial therapies for *H. pylori* have many shortcomings because of antimicrobial resistance that is a major problem during treatment. While the incidence of *H. pylori* resistance to metronidazole ranges from 15 to 40% in Europe and USA and from 9 to 12% in Japan [84, 85], it is much higher in developing countries, ranging from 50 to

80% (Figure 1.9) [86, 87]. The prevalence of *H. pylori* resistance to clarithromycin range is 1.7 to 25% in the world (Figure 1.10.).

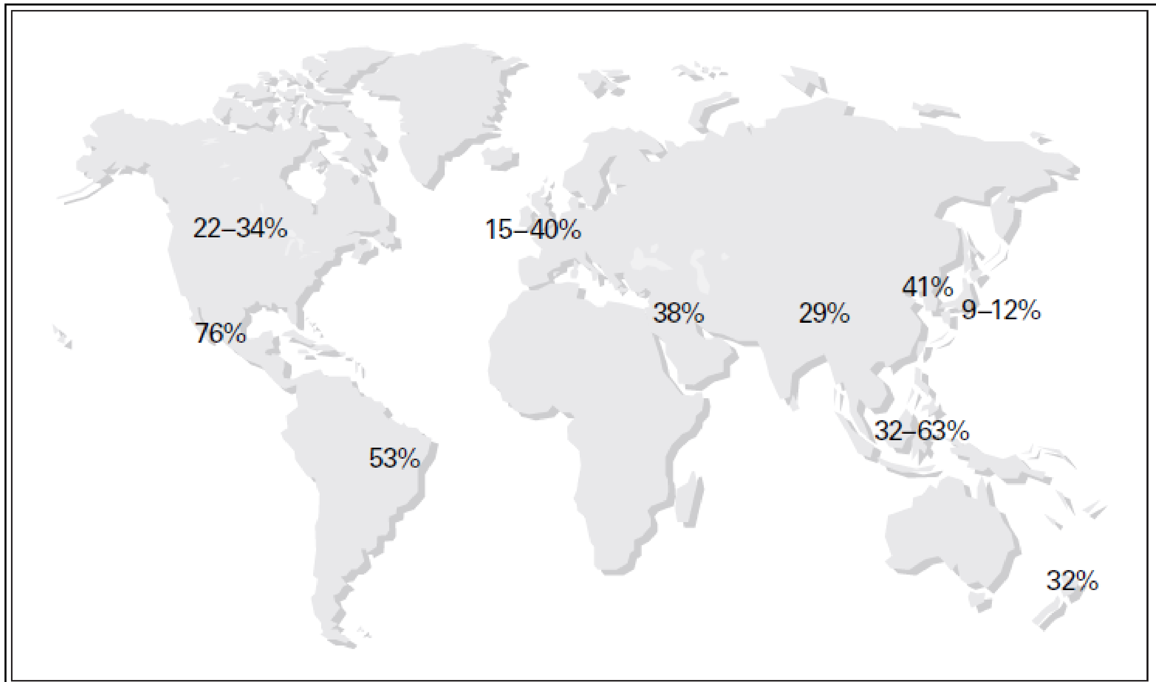

Figure 1.9. Resistance of *H. pylori* to metronidazole in worldwide [88].

These patients are usually retreated with an adapted second-line regimen because of the first line therapy failure, by another combination of antibiotics. The reason for choosing different antibiotic combinations during second line therapy is developed of secondary resistance to the prescribed antibiotics [89]. For instance, according to Buckly *et al.*, after failure of first line therapy, the clarithromycin resistance rate increased from 3.4 to 58% in Ireland [90].

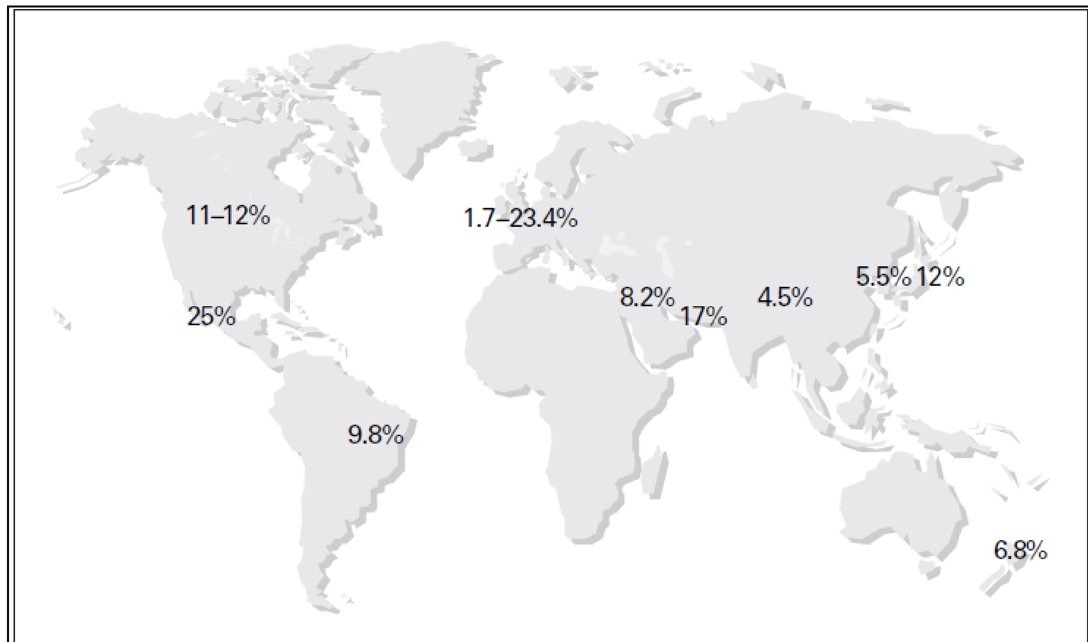

Figure 1.10. Resistance of *H. pylori* to clarithromycin in worldwide [88].

Overall, the components, dosage and duration of treatment play a crucial role during *H. pylori* eradication. To select the right antibiotic treatment during first line therapy is important to prevent secondary antimicrobial resistance. For this reason, geographical prevalence of antimicrobial resistance should be considered during first line therapy. However, to date there are not enough studies available to make an accurate estimate of the effect of these resistances on treatment success.

1.2. *Helicobacter pylori* RELATED DISEASES

H. pylori infects more than half of the world's population [16] and also remains one of most common bacterial infections in the world. Although *H. pylori* colonization is not a disease in itself, it affects the relative risk of developing disorders of the gastrointestinal and extra-gastroduodenum. In addition, *Helicobacter pylori* is directly associated with many gastrointestinal diseases (Figure 1.11) including acute and chronic gastritis, peptic ulceration, MALT lymphoma, gastric cancer, and gastroesophageal reflux disease (GERD), it may be also a risk factor for pancreatic cancer and some extra-gastric diseases, including several autoimmune diseases [4].

Colonization of the gastric mucosa by *H. pylori* results in the development of acute and chronic gastritis which in turn modifies the composition of the gastric juice, either making it more or less acidic in virtually all infected individuals [91]. Gastric or duodenal ulcers (commonly referred to as peptic ulcers) are described as mucosal defects with a diameter of at least 0.5 cm penetrating through the muscularis mucosa. Over 80 % of peptic ulcers show the presence of *H. pylori* infection [92]. Bleeding is the most common complication of peptic ulcer disease and is estimated to occur in 15 to 20% of ulcers.

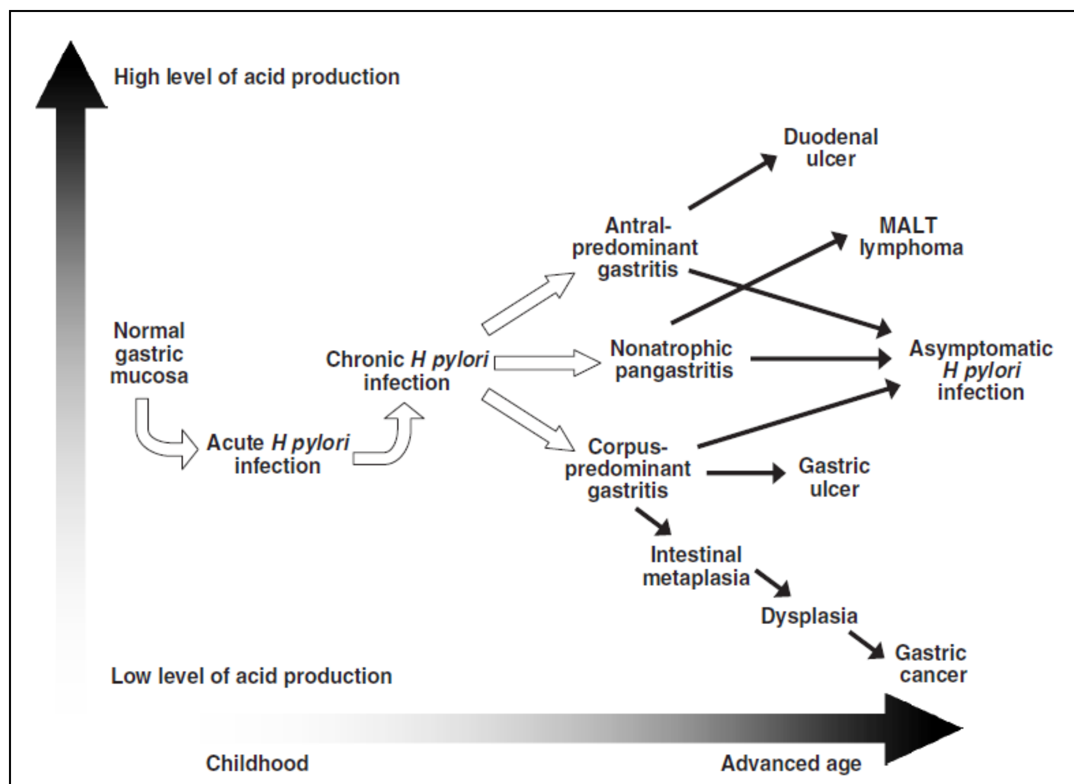

Figure 1.11. Clinical outcomes of *H. pylori* infection [93].

Gastric mucosa associated lymphoid tissue (MALT) lymphoma is a form of lymphoma (a cancer of the lymphatic system) involving the mucosa-associated lymphoid tissue; however the gastric mucosa does not normally contain lymphoid tissue. Although MALT lymphoma is a unique and distinct form of marginal zone B-cell- non-Hodgkin's lymphoma, according to various studies *H. pylori* was found strongly associated with the development of MALT lymphoma, the most common gastric and possibly gastrointestinal lymphoma [94, 95, 96]. Almost all MALT lymphoma patients were *H. pylori* positive, and

H. pylori-positive subjects showed a significant increased risk for the development of gastric MALT lymphoma [97, 98]. However, the exact incidence in *H. pylori* positive patients is unknown, the reason being the diagnostic controversies and the relative rarity of this disorder. It has been shown that approximately 80% of patients with low grade gastric MALT lymphoma have been treated after the eradication of *H. pylori* within 12 months [95, 96, 99, 100].

1.2.1. Gastric Cancer

Gastric cancer is the second most common cause of death in the world reaching approximately 700.000 patients each year [16]. Incidence of the gastric cancer is higher in developing countries than developed countries [101]. Although gastric cancer incidence and mortality rates have slowly been decreasing in developed countries over the last five decades, it is the fifth most common type of cancer and the fourth leading cancer related to disease in Europe [102, 103].

The clinical history of tumor-related symptoms includes; weight loss, decreasing appetite, anemia, nausea, and vomiting. However, about 40% of patients never report these symptoms within their recent or remote medical history [104]. The 1st year and 5th year survival percentages are low with only 42% and 24%, respectively [105].

Development of gastric cancer is much higher in *H. pylori*-infected populations than in uninfected populations. Therefore, *H. pylori was* classified as a group I or definite carcinogen (the only bacterium to be thus classified) by the World Health Organization's (WHO) International Agency for Research on Cancer in 1994 [7].

Although there is a close association between *H. pylori* infection and gastric cancer, most infected persons do not develop the disease**.** For example, some large populations with high prevalence of *H. pylori* infection have low gastric cancer rates [106], showing that beside environmental factors such as smoking [108] and dietary salt intake [108], genetic diversity of both the bacteria and the host seem to play an important role in determining disease risk. A single gene mutation is also responsible the development of hereditary gastric cancer with familial association. For instance, polymorphisms were determined as

relevant factors in the human interleukin- beta (IL-β) gene and in the interleukin-1 (IL-1) receptor antagonist [109].

H. pylori plays a crucial rule at the beginning of a sequential paradigm of gastric cancer that is known Correa's cascade [110] (Figure 1. 12). Correa' cascade is explained that development of gastric cancer after initiation by *H. pylori* infection and continuation by environmental and host factors [110]. Histologic steps of gastric cancer development is initiated by the transition from normal mucosa to chronic superficial gastritis, which then leads to atrophic gastritis and intestinal metaplasia, and finally to dysplasia and adenocarcinoma [111].

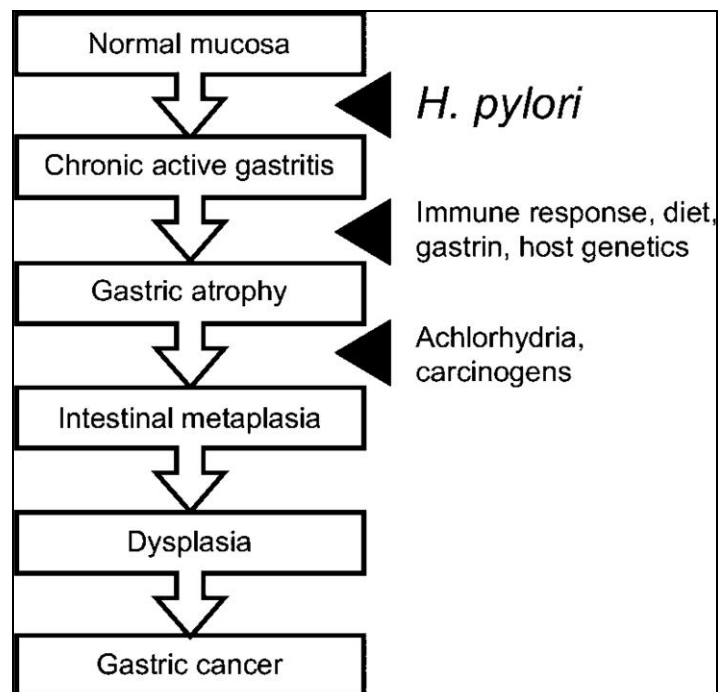

Figure 1.12. Histological steps of gastric cancer development. Correa's cascade [110].

Previous studies have shown that *H. pylori* infection of Mongolian gerbils can lead to gastric cancer and are used as animal models [112]. Mongolian gerbils represent tractable systems that allow understand to insights into the effects of the host, pathogen, and environmental factors development of gastric cancer [113]. A study presented that antimicrobial therapy induces apoptosis and inhibits proliferation of gastric cells in *H. pylori* infected gerbil [114]. Animal studies have suggested that primary prevention of

gastric cancer by *H. pylori* eradication regimen is more effective. Although, the effects in human of this type of prevention have not supported sufficiently with large scale randomized controlled studies in China, non-randomized studies have suggested that H pylori eradication therapy prevents the development of gastric cancer [115, 116, 117].

1.2.2. *H. pylori* Related Extra-Gastric Diseases

Since the discovery of *Helicobacter pylori,* colonization has been hypothetically associated with a variety of extra-gastric disorders; coronary heart disease [118], neurodegenerative diseases (such as Parkinson disease) [119], dermatological disorders (such as rosacea) [120], autoimmune thyroid disease [121], thrombocytopenic purpura [122], iron deficiency anemia [123], Raynaud's phenomenon [124, 125], glaucoma, other ocular diseases [126] and many autoimmune diseases [127]. Although many studies have published a positive relationship between *H. pylori* and various extra-gastric diseases, the association between *H. pylori* infection and other extra-gastric diseases remains controversial and unclear. A positive relationship is being mostly supported by case studies, small pilot studies or *in vitro* data. However the hypothesis of an etiological role has not yet been fully investigated.

1.3. PHYTOTHERAPY

Phytotherapy is a form of medical treatment which relies on the use of whole or in the form of prepared extracts of plants. Herbal medicine is a kind of phytotherapy described by The World Health Organization (WHO) as: "a plant-derived material or preparation with therapeutic or other human health benefits which contains either raw or processed ingredients from one or other plant materials, or combinations." Herbal medicines include 1) herbs (crude plant material such as leaves, flowers, fruit, seed, stems, wood, bark, roots, rhizomes or other plant parts, which may be entire, fragmented or powdered), 2) herbal materials (fresh juices, gums, fixed oils, essential oils, resins and dry powders of herbs), 3) herbal preparations, and 4) finished herbal products that contain parts of plants [128].

1.3.1. Phytotherapy in the Global Market

Worldwide consumption of phytotherapeutic agents has markedly increased over the past years. Therefore, clinical and pharmacologic interest in the efficacy and safety of herbal supplements and medicines has increased. For example, although global sales of herbal products were estimated to US $60 billion in 2000, in 2008 it reached approximately US $83 billion. It is forecast to reach $107 billion by the year 2017 [129]. In developing countries, especially some Asian and African countries, 80% of the population uses traditional medicine for primary health care. In addition, 70% to 80% of the population in many developed countries uses traditional medicines in some form of alternative or complementary medicine (e.g. acupuncture) [128].

1.3.2. Advantages and Disadvantages of Phytotherapy

Recent publications have highlighted the severe consequences (dangerous and lethal side effects) from side effects from certain herbal products [130]. These side effects may occur through several different mechanisms, including potential toxic effects of the herbal plants, effects of contaminants, and interactions of herbal plants with drugs or other herbs. Furthermore, the use of fake or different herbs, to the ones shared has contributed to the negative effects about the safety and efficacy of herbal products [131]. However, recent advances in analytical chemistry (e.g. gas chromatography (GC), high performance liquid chromatography (HPLC), ultra-violet spectroscopy (UV), infra-red spectroscopy (IR), raman spectroscopy, nuclear magnetic resonance spectroscopy (NMR), mass spectroscopy (MS), X-ray diffraction, gas chromatography-mass spectrometry (GC–MS) and HPLC–MS) and related disciplines have helped to elucidate the complex chemical compositions (eg. toxic compounds) of herbal remedies. Side effects may also occur due to contaminants in herbal products, as heavy metals, including lead, mercury, or arsenic [132].

1.3.3. Plant Extraction Techniques

Extraction of plant materials can be done by various extraction procedures; conventional and non-conventional methods. Non-conventional methods have many advantages including, more environmental friendly due to decreased use of synthetic and organic

chemicals, reduced operational time, and better yield and quality of extract. Ultrasound [133], pulsed electric field [134], enzyme digestion [135], extrusion [136], microwave heating [137], supercritical fluids [138] are examples of non-conventional methods. Bioactive compounds from plant materials can be extracted by classical extraction methods including, soxhlet extraction, maceration and hydrodistillation [139]. These conventional methods are based on the extracting power of different solvents in use and the application of heat and/or mixing. The Soxhlet extraction is not limited to the extraction of lipids but also many desired compounds can be extracted by the soxhlet extraction depending on the choice of solvents [140]. The polarity of the targeted compound is the most important factor for solvent choice. Maceration is also a popular and inexpensive way to get essential oils and bioactive compounds. Hydrodistillation is a traditional method for extraction of bioactive compounds and essential oils from plant materials. During hydrodistillation process, organic solvents are not involved and it can be performed before dehydration of plant materials.

1.3.4. Alternative Therapies for Eradication of *H. pylori*

In the literature, many studies are related to gastro-protective effects of plant extracts including; Green tea (Camellia sinensis L.), Encholirium spectabile Mart. (Bromeliaceae), and Zingiber Officinale Roscoe (Zingiberaceae) [141, 142, 143].

1.3.4.1. Eleutherococcus senticosus (Siberian ginseng)

Ginseng is widely used as a herbal product in China, and many other asian countries such as Korea, Japan, Malay, Europe, North Africa, and in the United States. The most common type of ginseng is Asian ginseng, often called Panax, Chinese, or Korean ginseng. The other commonly used type of ginseng is Siberian ginseng, which is derived from the roots and rhizome of *Eleutherococcus senticosus* (Figure 1.13). It is called ‘‘Ciwujia’’ in Chinese and ‘‘Shigoka’’ or ‘‘Ezo-ukogi’’ in Japanese.

Figure 1.13. Image of *Eleutherococcus senticosus* (Siberian ginseng) [144].

Many products come in the form of capsule, powder, or tea bags, with the name of ‘‘Siberian ginseng’’ and ‘‘Eleuthero’’ and are widely available in the health food markets. For thousands of years, ginseng has been traditionally used as a tonic to build up nonspecific resistance against various ailments such as depression, fatigue, diabetes, damaged immune functions, etc. In addition the major constituents such as eleutheroside B, eleutheroside E, and isofraxidin of aqueous and alcohol extracts of Siberian ginseng have been reported to have anti-fatigue, anti-stress, antioxidant effects, immune-enhancing, anticancer, inhibition of gastric ulcer and antidepressant effects [145, 146, 147, 148, 149].

1.3.4.2. Cimicifuga racemosa (Black cohosh)

Black cohosh (Actaea racemosa L.), a member of the Ranunculaceae family, is an herbaceous plant native to the eastern United States (Figure 1.14). It has been traditionally used for centuries for a variety of health benefits. Black cohosh has been found as an anti-inflammatory, antipyretic, analgesic agent to treat infectious diseases [150, 151]. Moreover, the black cohosh extracts has been used to relieve symptoms associated with female medical conditions, especially menopause [152, 153].

Figure 1.14. Image of *Cimicifuga racemosa* (Black cohosh) [154]

Phytochemical studies have identified more than 50 secondary metabolites in black cohosh, including flavonoids, alkaloids, and polyphenolic fukiic acid esters [153, 154]. The major two bioactive compouns of black cohosh are triterpene glycosides and phenylpropanoids. Although, recent studies show that the extracts of blach cohosh enriched triterpene glycosides and specific triterpene glycosides have anticancer activity, the mechanisms and nature of these active compounds are not clear [155, 156].

1.3.4.3. Trifolium pratense (Red clover)

Trifolium pratense (Red clover) is a species of clover, an herbaceous plant native to Europe and Western Asia (Figure 1.15). It is the most common herb used for relief of menopausal symptoms. It has been used for over 100 years in Europe and America to treat whooping cough, pain, as a sedative and expectorant. Although there is not much scientific data about the effect mechanism, studies about the antitumoral, anti-inflamatory and oestrogenic effects of the plant have been confirmed [157]. The compounds of the plant (e.g. isoflavones biochanin A and genistein) present oestrogenic activity [158. Also its isoflavones may play a role in reducing the risk of cancer, including breast cancer [159].

Figure 1.15. Image of *Trifolium pratense* (Red clover) [160].

1.3.4.4. Silybum marianum (Milk thistle)

Milk thistle (*Silybum marianum* L.) is a medicinal plant widely used traditionally in European medicine for over 2000 years [161, 162]; it is indigenous to Kashmir, and was once grown in Europe as a vegetable. The major source of milk thistle is silymarin which is a flavonoid complex. Silymarin is used for the therapy of liver disease and also has been used to treat patients with alcoholic cirrhosis [163, 164]. In addition the antifibrotic, anti-inflammatory, and immunomodulating activities of silymarin have been reported [165, 166]. Although it is a popular herbal plant in the market, there is no detailed information on its safety or interaction with other drugs.

Figure 1.16. Image of *Silybum marianum* (Milk thistle) [167].

1.3.4.5. Origanum minutiflorum (Turkish oregano)

Turkey is regarded as an important centre for the family Lamiceae which is represented throughout the world by 50 species and in Turkey by 22 species or 32 taxa [168]. Origanum mostly grow in the mountainous areas of the Mediterranean region. Moreover, Turkey exports 5.000-7.500 tons of oregano for a return 13-15 million US dollars, of which *Origanum minutiflorum* represents the majority of Turkey's oregano exports. *Origanum minutiflorum* is an endemic plant of Isparta, in Turkey which is locally called "Sütçüler Kekiği" [169], and /or "Toka kekiği" [170]. It is not only used as a spice and herbal tea but also as a traditional medical herb to cure stomach-aches and respiratory colds in Anatolia. It is also be added to all kinds of food products as seasoning. The positive effects of oregano extracts on human health have been attributed to its antioxidant activity [170].

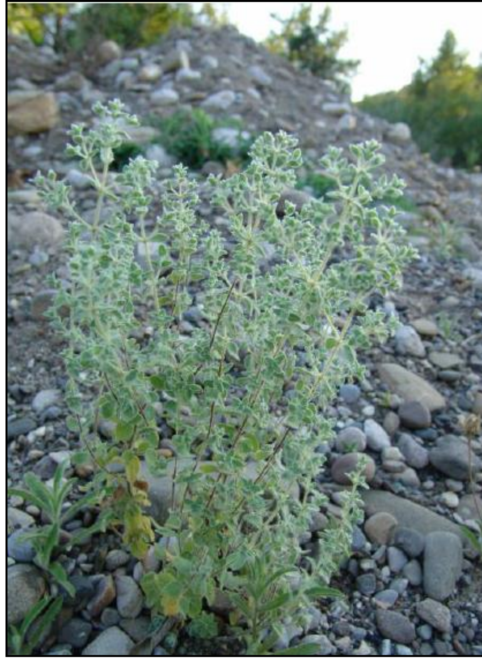

Figure 1.17. Image of *Origanum minutiflorum* (Turkish oregano) [171].

Extracts of *O. minutiflorum* have been previously tested for their antioxidant, phenolic, and antimicrobial activity on food pathogens [169, 172, 173, 174, 175]. For instance, methanol extracts of the plant have shown to contain high amounts of antimicrobial chemicals such as rosmarinic acids, carvacrol, and erodictil [169]. Furthermore, the composition of *Origanum minutiflorum* essential oil have been seen to include thymol, carvacrol, pinene, terpinene, and cymene which are known to have antimicrobial, antifungal and antioxidant properties [172, 174, 176, 177]. In addition, the literature has shown *O. minutiflorum* extracts to have a wide range of antimicrobial activity against many gram negative and gram positive bacteria including *E.coli O157:H7*, *L. monocytogenes*, *S. typhimurium*, *S. aureus*, and *C. jejuni* etc. [173, 174, 175].

2. MATERIALS

2.1. PLANT SAMPLES

- Turkish oregano (*Origanum minutiflorum*) (Sutculer, Isparta, Turkey)
- Siberian ginseng (*Eleutherococcus senticosus)* (Herbal Authority, UK)
- Black cohosh (*Cimicifuga racemosa*) (Herbal Authority, UK)
- Red clover (*Trifolium pratense*) (Herbal Authority, UK)
- Milk thistle (*Silybum marianum)* (Herbal Authority, UK)

2.2. BACTERIAL STRAIN

- *Helicobacter pylori* J99 (ATCC 700824) (Rocville, MD, USA)

2.3. TISSUE CULTURE

- Human gastric cancer AGS cells (gastric adenocarcinoma, ATCC CRL 1739) (Rocville, MD, USA)

2.4. LABORATORY EQUIPMENT

The laboratory equipments used during this study were;

- Laminar flow cabinet (ESCO Labculture Class II Biohazard Safety Cabinet 2A, Singapore)
- Gas chromatography mass spectrometry (Thermo Fisher Scientific, USA)
- Spectrophotometer ((Thermo Scientific Labsystems, Massachusetts, USA)
- ELISA plate reader (Bio-Tek EL x 800, USA)
- Water bath (Wisebath, Korea)
- CO_2 incubator (Nuaire NU5510/E/G, USA)
- Inverted microscope (Nikon, Japon)
- Liquid nitrogen tank (Arpege 140, France)

- -20 °C freezer (Arçelik, Turkey)
- -80ºC freezer (Thermo Forma -86 C ULT Freezer, USA)
- Freeze-drier (Christ A 2-4 LD, UK)
- Incubator (Binder, USA)
- Autoclave (Hirayama, Japon)
- Rotary evaporator (Buchi, Italy)
- Gas Chromatography/ Mass Spectrometry (GC/MS) (Thermo Scientific, USA)
- Anaerobic workstation (Don Whithley Scientific, UK),
- Shaker (BS-T, Sartorious, Aubagne, France),
- Centrifuge (Hettich EBA 200, Germany and Eppendorf 5810 R, USA)
- Vortex (Stuart SA8, UK)
- Chemical hood
- Micropipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Soxhlet and Clevenger apparatuses (Çalışkan Cam, Turkey)
- Sputter coater (Baltec SCD 005, USA)
- Scanning electron microscopy (Evo-40, Carl Zeiss AG, Germany)

2.5. CHEMICALS

- Brucella Broth (Remel, USA)
- RPMI (Roswell Park Memorial Institute medium)-1640 medium (pH 7.4; Gibco, USA)
- Fetal Bovine Serum (FBS) (Gibco, USA)
- Trypsin-EDTA (Sigma, USA)
- Dimethyl sulfoxide (Santa Cruz sc-202581, USA)
- Absolute Ethanol (≥99.9 %) (AppliChem, Germany)
- Methanol (≥99.9 %) (Sigma- Aldrich, USA)
- Sodium sulphate (≥99,8, Sigma, Germany)
- Hydrogen peroxide (% 50)(Sigma, USA)
- Urea Agar (Salubris, Massachusetts, USA)
- Oxidase Ready Test (Remel, USA)
- Metrodinazole (50 mg) (Oxoid, UK)

- Claritromicin (15 mg) (Oxoid, UK)
- Amoxicillin (10 mg) (Oxoid, UK)
- Gentamicin (Sigma, USA)
- Sodium cacodylate trihydrate powder (Sigma, Germany)
- Glutaraldehyde (Sigma, Germany)
- Hexamethyldisilazane (HMDS)
- WST1 cell proliferation kit (Roche, USA)
- Glycerol (Sigma, USA)
- Microaerophilic gas pack (Oxoid, UK)

2.6. LABORATORY CONSUMABLES

- Serological pipettes 25, 10, 5, 2 ml (Grenier-Bio or Axygen, USA)
- Micro pipettes 1000, 200, 100, 10, 2.5 µl (Thermo Scientific, USA)
- Polypropylene centrifuge tubes, 50 ml, 15 ml, 2 ml, 1 ml, 0.5 ml (Isolab, Germany)
- Cell culture flasks, T-25, T-75, T-150 and cell culture plates, 6-well, 96-well, (TPP Switzerland or Grenier-Bio, Germany)
- Cryovials (TPP, Switzerland)
- Parafilm
- Napkins
- Scissors
- Standard ruler
- Scrappers (Isolab, Germany)
- Loops (Isolab, Germany)
- Gloves
- Glass tubes
- Anaerobic jar (Oxoid, UK)

3. METHODS

3.1. PLANT SAMPLES

Turkish oregano (*Origanum minutiflorum*) was collected from the district of Sutculer, Isparta, Turkey at the end of the summer in August 2012 which included leaves, flowers, and roots together. *O. minutiflorum* was stored at room temperature in a dark place in a plastic container. Commercial extracts of Siberian ginseng (*Eleutherococcus senticosus)*, Black cohosh (*Cimicifuga racemosa*), Red clover (*Trifolium pratense*) and Milk thistle (*Silybum marianum)* were obtained from Herbal Authority (UK) and kept at 4°C.

3.1.1. Preparation of *Origanum minutiflorum* Plant Extracts

Turkish oregano (*O. minutiflorum)* was collected, and air dried at room temperature for 10 days. Dried *O. minutiflorum* was crushed by pestle and mortar.

Since it is known that different extraction procedures and solvents produce different bioavailable components of a plant, the first part of the project was to perform on plant extraction, using various solvents methanol, ethanol and water.

3.1.1.1. Methanol and Ethanol Extraction

Thirty grams of crushed *O. minutiflorum* was transferred into separate cone-shaped filter papers (Whatman No: 1, UK) which were then placed into soxhlet extractor (Çalışkan Cam, Turkey) (Fig 3.1).

Plant materials were extracted with 400 ml methanol (≥ 99.8%) (Sigma, USA), or ethanol (≥ 99.8%) (Sigma, USA) separately in round bottom flasks. The soxhlet extraction process was carried out for 6 h. Following extraction, the solvent (methanol or ethanol) was evaporated off by use a rotary evaporator (Burchi, Italy) under reduced pressure at 40 °C until 20 ml of solvent remained. The extracts were incubated at -80 °C (Thermo Forma, USA) for 30 min. Following incubation, the extracts were freeze-dried (Christ, UK) in a freeze drier for 4 days and stored at 4 °C until used.

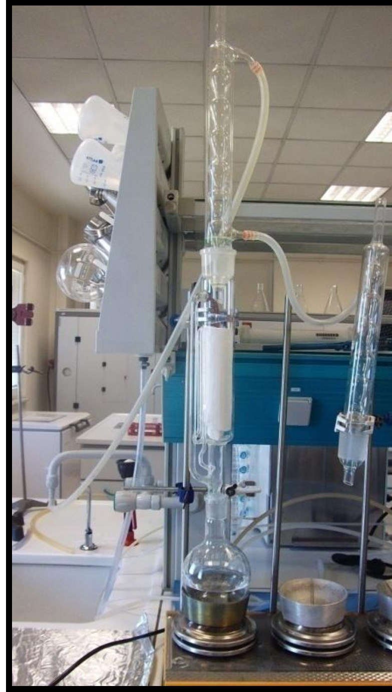

Figure 3.1. Soxhlet extractor and heater

3.1.1.2. Aqueous Extraction

Thirty grams of dried *O. minutiflorum* was incubated for 10 min in 1 L hot water (80 ^oC) in a 2 L closed glass bottle (Isolab, Germany) to eliminate evaporation of essential oil compounds and stirred occasionally. Following incubation, the infusion was centrifuged (Eppendorf, USA) at 3200xg for 10 min. The extract was then filtered with Whatman No. 1 filter paper (Whatman, UK), cooled at 4 ^oC in a freezer, and freeze-dried (Christ, UK) for 4 days, then stored at 4 ^oC until used.

3.1.1.3. Essential Oil Extraction

The essential oil (EO) of *O. minutiflorum* was isolated by the hydro-distillation method using a clevenger apparatus (Çalışkan Cam, Turkey). Thirty grams of dried plant and 400 ml of distilled water were added to a 1000 ml round bottom flask which was placed on a heating device (Isolab, Germany) and a clevenger apparatus was connected to the flask as shown in figure 3.2. Hydro-distillation was carried out for 3 h. The collected EOs was dried over anhydrous sodium sulphate (≥99,8) (Sigma, Germany) in order to absorb the residual water from EO. EOs was put into a sterile glass vial (Interlab, Turkey) and sealed

with aluminum foil and stored at -20 °C until used. This procedure was repeated until enough EO was extracted. All extracts were mixed together.

Figure 3.2. Essential oil extraction system composed of Clevenger apparatus and heater.

3.2. CALCULATION OF EXTRACT YIELDS

Extraction yields were calculated as the weight of the crude extract to that of raw material (30g) as follows:

$$\text{Extraction yield (\%)} = \frac{\text{weight of the freezed dried extract (mg)}}{\text{weight of original sample (mg)}} \text{ x } 100 \quad (3.1)$$

The relative density of the EO was determined as follows: mass of sterile empty glass vial (Interlab, Turkey) was determined (m_1). One ml essential oil was added into glass vial. Mass of glass vial and essential oil was also determined (m_2). The relative density of the essential oil was then calculated according to the equation:

$$d = m_2 - m_1 \quad (3.2)$$

Table 3.1. Explanation of equation for calculation of essential oil density

d	Relative density of essential oil
m_1	Mass of empty glass vial
m_2	Mass of 1 ml essential oil and empty glass vial

3.3. GROWTH AND IDENTIFICATION OF BACTERIA

Helicobacter pylori J99 (ATCC 700824) was obtained as a -80 ^{o}C stock culture from American Tissue Type Culture Collection (ATCC) (Rocville, MD, USA).

-80 °C stock bacteria were cultivated in Brucella broth (Remel, USA) with 10 % fetal bovine serum (GIBCO, USA), and Brain Heart Infusion (BHI) Agar with 5% sheep blood plates (Salubris, Massachusetts, USA) under aseptic conditions. Bacteria were then incubated at 37 °C for 48 h in an anaerobic workstation (Don Whitley Scientific, UK) containing 85 % N_2, 5 % O_2 and 10 % CO_2 gas mixture (Habaş, Turkey) or anaerobic jar with microaerophilic gas pack (Oxoid, UK) under 95 % humidity.

The strain was identified by gram staining, morphology and biochemical testing (catalase, urease, and oxidase positive).

3.3.1. Catalase Test

A colony of pure *H. pylori* culture was transferred by a sterile loop onto a slide and 20 µl of 50% H_2O_2 (Sigma-Aldrich, USA) was dropped onto the colony. The evolution of gas was examined, which indicates catalase activity.

3.3.2. Oxidase Test

Oxidase activity was tested by the oxidase reagent test (Remel, USA). One colony of *H. pylori* and one drop of oxidase solution was transferred to a filter paper (Whatman, UK). A positive result was determined with the appearance of a blue color within 10 sec.

3.3.3. Urease Test

Urease activity was tested by urea agar (Salubris, Massachusetts, USA). A full lawn of growth of *H. pylori* was cultured onto urea agar. The urea agar was incubated at 37 °C for 6 h under microaerophilic conditions. Urease production was indicated by a pink color while a yellowish color displayed urease negative.

3.3.4. Growth Curve

One ml Brucella broth with 10% FBS was placed on to the center of a 48 h growth *H. pylori* culture plate. *H. pylori* was harvested from the plate using a sterile swab (Isolab, Germany), which was used as a stock culture. The stock bacterial culture was inoculated to 10 ml of Brucella broth (Remel, USA) with 10 % FBS medium in a 25 cm^2 tissue culture flask (TTP, Germany) at 450 nm (≤0.05 OD) and incubated under microaerophilic condition in an anaerobic workstation (Don Whitley Scientific, UK) (85 % N_2, 5 % O_2 and 10 % CO_2 gas mixture) under 95 % humidity at 37°C while shaking at 150 rpm with a shaker (Thermo Scientific, USA). The optical density of bacterial culture was determined by measuring the absorbance (optical density, OD) every hour at 450 nm in a spectrophotometer (Thermo Scientific Labsystems, USA). Ten fold serial dilutions were prepared at OD_{450} of 0.1 and 10 µL of each dilution were cultured onto BHI with 5 % sheep blood agar and plates were incubated for 5 days under microaerophilic conditions at 37 ^{o}C. Following incubation, the number of colony forming unit (CFU) per ml were determined. The bacteria in logarithmic phase were used in the experiments.

3.3.5. Screening of Antimicrobial Effect of Plant Extracts

The in vitro anti-bacterial activities of commercial and prepared extracts were determined by the agar well diffusion method [179]. Briefly; methanol, ethanol and aqueous extracts of *Origanum minutiflorum* were dissolved in 0.5% (v/v) dimethylsulfoxide (DMSO) (Sigma, USA) with a concentration 2000 µg/ml (100 % concentration) according to previous studies [172]. EOs were diluted at a concentrations of 92 mg/ml and 9.2 mg/ml (1/10 and 1/100 times diluted concentrations) with 0.5% DMSO.

A total volume of 100 µl of *H. pylori* suspension (1×10^7 CFU/ml) was cultured onto BHI agar plates containing 5 % sheep blood. The wells (7.0 mm in diameter) on the plate were made by sterile glass pasteur pipette and 70 µl of commercial and prepared extracts (methanol, ethanol, aqueous and EOs) were added to the wells. 0.5 % DMSO was used as a negative control and antibiotics amoxicillin (AMX, 0.05 mg/ml) (Sigma, USA), clarithromycin (CLR, 0.05 mg/ml) (Oxoid, UK), and metronidazole (MTZ, 0.8mg/ml) (Sigma, USA) were used as positive controls. Following incubation for 72 h at 37 ^{o}C under microaerophilic condition, the zone of inhibition (ZI) was measured. The antibacterial activity was classified according to Lee et al. (2004) [180], as follows: very strong response, zone diameter ≥30mm; strong response, zone diameter 21–29mm; moderate response, zone diameter 16–20mm; weak response, zone diameter 11–15mm; and little or no response, zone diameter ≤10mm. All tests were replicated three times.

The type of extract that displayed the highest zone of inhibition and thus the strongest antimicrobial property was used for the reminder of the study.

3.3.6. Determination of Minimum Bactericidal Concentrations (MBCs)

The MBC were determined according to CLSI [181] with some modifications. Serial dilutions of EOs (1/2 to 1/512, 1/1000 to 1/64000 and 1/10000 to 1/80000) (v/v), (or antibiotics as positive control) were prepared. *H. pylori* was suspended in Brucella broth containing 10 % FBS and diluted to reach a final dilution of 1×10^6 CFU/ml. 500 µl of inoculums and 500 µl of each EO concentration were mix in 2 ml sterile tubes. The tubes were incubated under microaerophilic conditions at 37°C for 48h while shaking at 150 rpm with a shaker (Thermo Scientific, USA). Following 48 h incubation, 100 µl aliquots of the test tubes were plated onto BHI containing 5 % sheep blood agar plates to determine the viable CFUs. The plates were incubated for 5 days. The MBC was defined as the lowest concentration of the tested sample that completely inhibited visible bacterial growth on BHI with 5 % sheep blood agar plate. All inhibition tests were replicated three times.

3.4. ANALYSIS OF CHEMICAL COMPOSITION OF EXTRACTS

Components of the extracts were analyzed by gas chromatography mass spectrometry (GC-MS) (Thermo Fisher Scientific, USA). In order to dilute the extracts, 1µl of extract was dissolved in 999 µl n-hexane (≥85 %) (Sigma, USA). Analysis of extracts were performed with TRACE ISQ (Thermo Scientific, USA) GC-MS system with TR WAX MS column (30mm x 0.25mm 0.25 µm film thickness) series gas chromatography with helium as carrier gas at flow rate of 10 ml/min; split ratio 10 ml/min. Injector and detector temperatures were set at 250 ^{o}C. The oven temperature was kept at 50 ^{o}C for 5 min, then programmed to 200 ^{o}C at rate of 5 ^{o}C / min for 5 min, and held at 250 ^{o}C for 10 min. Diluted samples (1/1000 in hexane, v/v) of 1 µl was injected manually. The identification of the chemical constituents were achieved from TIC chromatogram, the mass spectra of the extracts components were automatically searched using in-house Wiley7, Nist, and Tutor libraries of the extracts constituents of the equipment by comparison of the date base including mass spectra of the fragment of those chemicals [178].

3.5. CELL CULTURE STUDIES

Human gastric cancer AGS cells (gastric adenocarcinoma, ATCC CRL 1739) were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA) and cultured in RPMI-1640 medium (pH 7.4; GIBCO, USA), supplemented with 10 % FBS (GIBCO, USA) at 37 ^{o}C in a humidified atmosphere of 5 % CO_2 incubator (Binder, USA).

3.5.1. Cytotoxic Effect of *O. minutiflorum* EOs on AGS Cell Line

Cells were grown in medium supplemented with 10 % FBS, in a CO_2 incubator at 37 ^{o}C. Cells were diluted to $1x10^5$ cells/ml and 100µl added to each well except the last two columns on a 96-well plate. Control wells were seeded in another 96 well plate to eliminate the effect of volatile compounds on surrounding wells. Plates were incubated at 37 ^{o}C in a 5% CO_2 incubator overnight. Following incubation, cells were treated with various concentrations of EO (serial dilutions from 1/8000 to 1/80000) (v/v) for 24, 48 and 72 hours. Three replicate wells per concentration were used and repeated in triplicate for all time intervals. Untreated medium and 0.5 % DMSO were used as control. Following the

incubation, cells were washed with PBS three times. WST-1 (Roche, USA) assay was carried out according to manufacturer's recommendations. WST-1 proliferation reagent (5µl WST1 and 45µl medium) were added to each well and incubated for 2 hours at 37°C (plate is covered with aluminum foil). Following incubation, the OD's were read by ELISA plate reader at 490nm (Bio-Tek ELx800, USA).

$$\text{Percent (\%) of viability} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of untreated cells}} x\ 100 \quad (3.3)$$

According to the cell cytotoxicity assay, the concentration which did not show any considerable growth inhibition on AGS cell line following 72 h incubation was chosen.

3.5.2. Bacterial Adhesion and Invasion Assay

H. pylori adhesion and invasion of cultured cells was carried out using a standard gentamicin assay as previously described [182]. $1x10^5$ cell /well were cultured in 24-well plates (TTP, Germany) and incubated at 37 °C in a 5% CO_2 incubator overnight. Following incubation, *H. pylori* (10^7) at a multiplicity of infection (MOI) of 100 in log-phase was added directly on the cell and plates were incubated for 1.5 h at 37 °C in a 5% CO_2 incubator. Following incubation, the infected cells were washed with PBS three times. The extracts were diluted with concentrations of 46 µg/ml and 23 µg/ml (1/20000 and 1/40000 (v/v)) in RPMI cell culture medium with 0.05% DMSO and added directly on the cell for 1.5 h. Inoculation of wells with RPMI medium with 0.05 % DMSO used as positive control. The plate was incubated at 37 °C for 1.5 h under a 5 % CO_2 humidified incubator. Following incubation, to determine the number of cell-adhesion and invasion bacteria, infected cells were washed three times to remove unattached bacteria and then lysed with distilled water for 10 min. Lysates were diluted in PBS, plated onto BHI agar with 5 % sheep blood agar plates and cultured for 4–5 days, after which the CFUs were determined.

3.6. SCANNING ELECTRON MICROSCOPY (SEM)

Examination of effect of *O. minutiflorum* EO on *H. pylori* and *H. pylori* infected cells were done by scanning electron microscopy (SEM). The MBC concentration of EO was chosen for SEM analysis.

Sterile coverslips were placed in each well of a polystyrene 6-well tissue culture plate (TPP, Switzerland). The AGS cells (2×10^5 cells/ well) were cultured on glass coverslips and incubated at 37 ^oC in a 5% CO_2 incubator overnight. Following incubation, *H. pylori* (10^7) at a multiplicity of infection (MOI) of 100 was added directly on the cell and plates were incubated for 1.5 h at 37 ^oC in a 5% CO_2 incubator. Following incubation, the infected cells were washed with PBS three times. The extract was diluted (1/20000) in RPMI cell culture medium and added directly on the cell for 1.5 h. Following incubation, the cells on coverslips in the absence (negative control well) or presence (test well) of the extract and/or *H. pylori* were prepared for SEM.

All coverslips were washed once with 1X PBS solution (pH 7.4) and the culture medium in the wells discarded. Glutaraldehyde solution (2.5 %) prepared in 0.15 M cacodylate buffer was added into the wells to fix the cells /bacteria on coverslips which were then incubated for 2 h at room temperature.

Following incubation, glutaraldehyde was removed from the wells and coverslips were rinsed three times, 10 min each, with 0.15 M cacodylate buffer. Samples were dehydrated by exposing the coverslips to ascending ethanol series (30 % → 50 % → 70 % → 80 % → 90 % → 95 % → 100 %) for 10 min each. Final drying step of coverslips was done with hexamethyldisilazane (≥ 99 per cent, Sigma, Germany) by three rinses (10 min each) of coverslips. In the final rinse, hexamethyldisilazane was not discarded but was allowed to evaporate slowly at room temperature.

Following evaporatoin, coverslips were coated with gold for 40 s with sputter coater (Baltec SCD 005, USA) and the cells on coverslips in the absence/presence of essential oils/ bacteria were examined with scanning electron microscopy (Evo-40, Carl Zeiss AG,

Germany). Images were taken at different magnifications ranging from 500X-10000X at 10 kv.

3.7. STATISTICAL ANALYSIS

Minitab software version 16 (Minitab, Inc., State College, PA) was used for data analysis. The results were presented as mean ± standard deviation. The correlation of antibacterial adhesion, invasion activity of treated AGS epithelial cells relative to DMSO control wells were determined by Student's t-test. Cytotoxicity data was evaluated using one-way ANOVA (Analysis of Variance) followed by the Tukey test. A value of $p < 0.05$ was considered significant.

4. RESULTS AND DISCUSSION

In this study the potential use of *Origanum minutiflorum*'s extract(s) and four different commercially available methanol extracts of plants were evaluated for their ability to eradicate *Helicobacter pylori.* Furthermore, the effects of the plant extracts on the inhibition of *H. pylori* adhesion and invasion to human gastric cell lines (AGS) were examined.

4.1. PLANT EXTRACTION OF *Origanum minutiflorum*

The aerial parts of *O. minutiflorum* were subjected to hydro-distillation for 3 h, using a Clevenger-type apparatus to yield 2.4 % (w/w) of yellow (in colour) oil. The relative density of the *O. minutiflorum* essential oil was calculated and found as 920 mg/ml at room temperature. The extraction yield of *O. minutiflorum* extracts are shown in Table 4.1.

Table 4.1. Yield of *O. minutiflorum* plant extract

	Percent (%) (w/w)
Methanol Extract	10.63
Ethanol Extract	6.77
Aqueous Extract	10.4

The average extraction yield of dried *O. minutiflorum* using aqueous, methanol and ethanol extraction were 10.4 % (w/w), 10.63 % (w/w), and 6.77 % (w/w), respectively. Differences in the extraction yield can be explained by the fact that, different extraction solvents can yield the bioavailability of different components in a plants chemical composition. This has been observed in a previous study by Oke et al., where differences in the yield of *O. minutiflorum* extracts were obtained by the use of different solvents including; acetone, ethanol, methanol, n-hexane, and water [172]. The highest yield was detected in water (27.4 % w/w) followed by ethanol (20.7% w/w) and methanol (20.3% w/w) while n-hexane (4.3 % w/w) showed the least extraction yield for *O. minutiflorum* [172]. Alternative techniques are being studied in order to enhance the overall yield of bioactive

components from plant materials with non-conventional methods including; ultrasound [133], pulsed electric field [134], enzyme digestion [135], extrusion [136], microwave heating [137] and supercritical fluids [138].

4.2. GROWTH AND IDENTIFICATION OF *Helicobacter pylori*

A stock bacterium stored at -80 °C was cultivated in Brucella broth with 10 % FBS, and BHI with 5% sheep blood agar under aseptic conditions. Upon close scrutinization of colonies following incubation at 37 °C for 48 h, under microaerophilic conditions, small, translucent colonies were observed (Figure 4.1.).

Figure 4.1. Growth of *H. pylori* on BHI agar with 5 % sheep blood at 37 °C for 48 h in an anaerobic workstation.

The identity of *H. pylori* J99 ATCC 700824 strain was confirmed by morphological and biochemical tests (Figure 4.2.). *H. pylori* J99 strain displayed a catalase positive result following the observation of gas formation (Figure 4.2 A). An oxidase positive result was determined by the appearance of a blue colour (Figure 4.2 B). Urease production was determined by a pink color indicating a urease positive result (Figure 4. 2 C). Finally, gram negative, rod shaped *H. pylori* was observed under a light microscope (1000 X magnification) with immersion oil following gram staining (Figure 4.2 D).

In general all *H. pylori* strains are gram negative and they have the same biochemical characteristics including catalase, oxidase and urease positive activity [4]. These properties are often used for identification of *H. pylori. H. pylori* is a spiral (S) shaped bacteria measuring 2 to 4 µm in length and 0.5 to 1 µm in width. The bacterium can also appear as rod shaped, which are known to convert to a coccoidal shape following prolonged in vitro culturing or antibiotic treatment [1]. Coccoid forms of the bacteria may also represent a viable but non-culturable state. These forms cannot be cultured in vitro and are thought to represent dead cells [1, 2]. Moreover, a coccoidal formation of *H. pylori* has been observed in starvation conditions including acid stress or lack of glucose [183].

Figure 4.2. Identification of *H. pylori* by biochemical testing
A) Catalase, B) Urease C) Oxidase test and D) Gram staining

4.3. GROWTH CURVE

The growth curve of *H. pylori* J99 was determined by an indirect spectrophotometric method over a period of 24 h (Figure 4.3). *H. pylori* strains often form aggregates in liquid culture, which impairs the reproducible quantification of bacterial cells under microaerophilic conditions at 37 ^{o}C [184]. It is for this reason that liquid cultures of bacteria incubated under microaerophilic conditions in this study were shaken at 150 rpm during growth curve experiments.

The lag phase of *H. pylori* was observed between 0 -9 h of cultivation in Brucella broth with 10 % FBS. The number of CFU's per ml was detected and an OD_{450} of 0.1 was found equal to $1x10^{8}$ bacteria/ ml. In this study, the logarithmic phase of *H. pylori* J99 was detected after 9h. Doubling time of *H. pylori* J99 was determined at approximately 4h. Similar results have been observed in previous studies where the doubling time of *H. pylori* J99 was seen at 3.7 h [185]. This long doubling time is due to its slow growth as a microaerophilic microorganism. Furthermore, since its only carbon source is glucose and the bacterium is constantly motile it therefore requires higher energy consumption. These factors all contribute to an increase in the doubling time [185]. According to a report on a different strain of *H. pylori* 26695 (ATCC), the logarithmic phase was observed at 10h and stationary phase started after 30 h [184]. These findings are similar to our results with *H. pylori* J99.

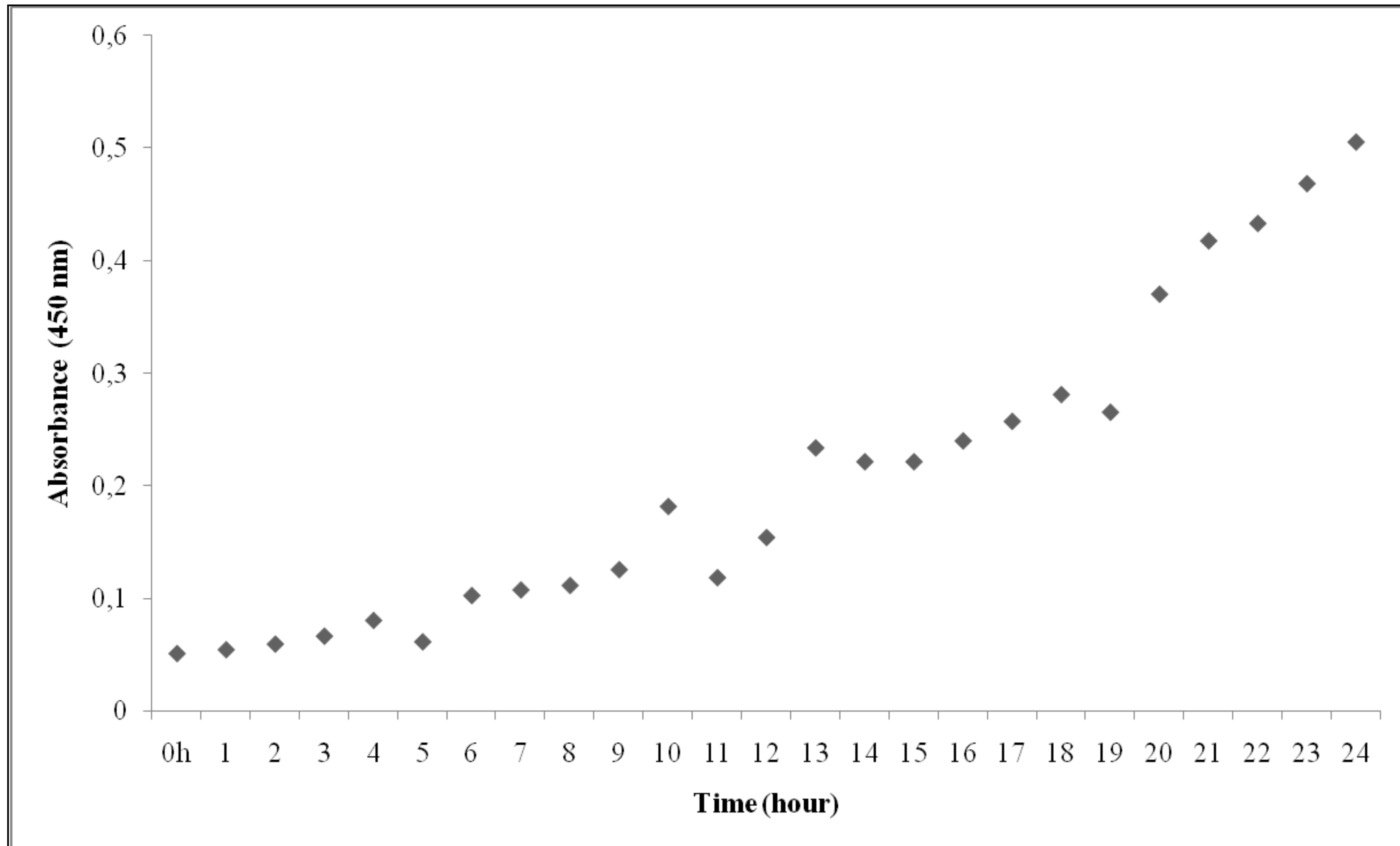

Figure 4.3. Growth curve of *H. pylori* J99 in Brucella broth with 10 % FBS .

4.4. ANTIMICROBIAL EFFICACY OF PLANT EXTRACTS

The antimicrobial activity of commercially available methanol extracts of Siberian ginseng (*E. senticosus)*, Black cohosh (*C. racemosa*), Red clover (*T, pratense*), Milk thistle (*S. marianum)* and methanol, ethanol, aqueous and EO extracts of *O. minutiflorum* were tested by the well diffusion assay against *H. pylori* J99 reference strain. According to our knowledge this the first study to test these extracts for anti-*Helicobacter* effects. Results of the antimicrobial efficacy of these plant extracts are shown in Table 4.2.

The antibacterial activity was classified as follows: very strong response, zone diameter ≥30mm; strong response, zone diameter 21–29mm; moderate response, zone diameter 16–20mm; weak response, zone diameter 11–15mm; and little or no response, zone diameter ≤10mm [180].

Commercially available methanol extracts Siberian ginseng (500 mg/ml), Black cohosh (500 mg/ml), Red clover (500 mg/ml) and Milk thistle (500 mg/ml) all showed moderate inhibitory effects (ZI: 11 mm, 17.25 mm, 20.0 mm and 22.0 mm, respectively). These extracts showed significantly weaker antimicrobial activity than positive controls (AMX, 0.05 mg/ml; MTZ, 0.08 mg/ml, and CLR 0.05 mg/ml) ($p< 0.05$).

In comparison between methanol and ethanol extracts no significant difference was observed with regards to their antimicrobial effects against *H. pylori* J99 ($p>0.05$). Methanol and ethanol extracts showed weak inhibitory effects ZI 16 mm and 14.5 mm at a concentration of 2 mg/ml, respectively. Moreover, aqueous extracts of *O. minutiflorum* did not show any antimicrobial activity against *H. pylori* J99 at a concentration of 2 mg/ml. The EO extracts of *O. minutiflorum* at two different concentrations 920 mg/ml (100 %) and 92 mg/ml (10%), showed the same potent anti-*H. pylori* effect with a ZI ≥90 mm. This value was greater than the ZI observed for the control antibiotics (AMX (0.05 mg /ml), MTZ (0.08 mg/ml) and CLR (0.05 mg/ml) (ZI: 66 mm, 70 mm, 61 mm, respectively)) ($p<0.05$).

Table 4.2. Inhibitory effects of plant extracts against *Helicobacter pylori* determined by well diffusion assay.

Tested Sample	Inhibition zone (mm)[a]
Black Cohosh (500 mg/ml)	17.25± 2.22
Milk Thistle (500 mg/ml)	22.0±2
Syberian Ginseng (500 mg/ml)	11.0±0
Red Clover (500 mg/ml)	20.0±2.45
Ethanol Extract of *O. minutiflorum* (2 mg/ml)	16.0±4.24
Methanol Extract of *O. minutiflorum* (2 mg/ml)	14.5±4.95
Aqueous Extract of O. *minutiflorum* (2 mg/ml)	0
EO (92 mg/ml)	≥ 90 mm
EO (9.2 mg/ml)	32.5±3.54
CLR (0.05 mg/ml)	61.67±7.64
MTZ (0.08 mg/ml)	70.0±0
AMX (0.05 mg/ml)	66.67±7.64
DMSO	0

DMSO (0.05 %) was used as negative control.

[a] Results are presented as mean ± standard deviations.

To the best our knowledge, no study to date has investigated the antimicrobial effects of Siberian ginseng and Black cohosh on any microorganisms. However a study on the compounds of Siberian ginseng has found the presence of caffeic acid which is a known antimicrobial agent [186]. Only one study has investigated the antimicrobial effects of a methanol extract of Red clover against *Clostridium sticklandii* a ruminal hyper ammonia-producing bacterium [187]. In a study by Lee et al, the antimicrobial activity of milk thistle was tested against gram negative bacteria *Escherichia coli,* and *Proteus vulgaris*; gram positive *Bacillus subtilis* and *Staphylococcus epidermidis*; yeasts, *Trichosporon beigelli, Saccharomyces cerevisiae,* and *Candida albicans.* An antimicrobial effect was only determined against gram positive bacteria [188].

A previous study comparing the antimicrobial effect of methanol and ethanol extracts of *O. minutiflorum* on *E. coli O157:H7*, *Shigella sonnei* RSKK 878, *S. aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, showed a

moderate antimicrobial activity on these microorganisms [172]. Whereas, the aqueous extracts of *O. minutiflorum* did not have any antimicrobial effects against any of these microorganisms [172]. These differences in activity can be explained by the difference in solvent extracts used, the compounds being extracted by each solvent, the polarity of the solvents, and their intrinsic bioactivity [189].

Previous studies have shown growth inhibitory activity of *O. minutiflorum* EOs against the growth of many bacteria [173, 175]. A study on ciprofloxacin resistant *Campylobacter spp.* showed a inhibitory effect ranging between 10-28 mm (1/10 diluted in ethanol). In comparison with the results of this study an approximate three fold higher antimicrobial effect was observed at the same dilution on *H. pylori* [173].

Another study which investigated the inhibitory effects of EO of *O. minutiflorum* on a wide range of microorganisms (10^6-10^7cfu/ml) including; *Bacillus brevis, Bacillus cereus, E. coli, Listeria monocytogenes*, *Micrococcus luteus, Yersinia enterocolitica, Staphylococcus aureus, Proteus vulgaris, Bacillus amyloliquefaciens, Enterococcus faecalis, Klebsiella pneumoniae, Aeromonas hydrophila, Corynebacterium xerosis* and *Bacillus subtilis* found moderate to strong antimicrobial activity of the EO. The best antimicrobial effect was observed against *Proteus vulgaris* (ZI= 51 mm) at a concentration of 1/50 v/v. Our results showed a lower ZI (32 mm) however the concentration was more diluted (1/100). At a similar concentration (1/100) the best antimicrobial effects were observed against *C. xerosis* (ZI= 38.5 mm) a slightly higher inhibition than that determined for *H.pylori* (ZI = 32 mm). Inhibitory effects at this concentration were also observed for *E. faecalis* (ZI=36mm), *B. amyloliquefaciens* (ZI= 35.5mm), *Aeromonas hydrophila* (ZI=34 mm), *Proteus vulgaris*, (ZI=33.5) and *Micrococcus luteus* (ZI=31.5 mm). All these values were similar to those observed for *H.pylori* in this study. No antimicrobial activity was observed for the other test bacteria studied at this concentration of EO [175].

There are several studies highlighting the anti-*H. pylori* activity of other plant extracts. Lai *et al*. used methanol (ZI= 19mm) and chloroform extracts (ZI= 21mm) of *Phyllanthus urinaria* against *H. pylori* strain 26695. The tested extracts at a concentration of 100 mg/ml showed similar effects to standard antibiotic treatments CLR, 0.05mg/ml ZI= 22mm; MTZ, 0.08 mg/ml ZI=8mm; AMX, 0.05 mg/ml ZI= 15mm [190]. This plant showed less

potent antimicrobial effects than *O. minutiflorum* (92 mg/ml ZI≥ 90) in this study. Another study examined 60 essential oils for their antimicrobial potential against *H. pylori* P1. ZI's ranged from 7-63 mm [191]. In this study Cinnamon bark (*Cinnamomum zeylanicum*) at a concentration of 100 mg/ml showed the highest antimicrobial activity (ZI=63 mm).. Furthermore, in the same study *Oreganum vulgaris* (100 mg/ml) showed antimicrobial activity of ZI= 19 mm. However, in our study *O. minutiflorum* EO at concentration of 92 mg/ml showed a higher antimicrobial property ZI ≥ 90 mm against *H. pylori* J99. The different results could also be due to the use of different extraction solvents, different plant compositions and variety of *H. pylori* strain tested.

4.5. MBC OF *Origanum minutiflorum* ESSENTIAL OIL

Since EO of *O. minutiflorum* was found to have the strongest efficacy, only this plant extract was used for the determination of MBC against *H. pylori* J99 strain. The MBC value is defined as the lowest concentration of the test agent that completely inhibits visible (*H.* pylori) growth on BHI with 5 % sheep blood agar [190].

This study determined the MBC at a concentration of 46 µg/ml (equal to 1/20000 (v/v) dilution). Although, there is no study to date on the inhibitory effects or MBC of *O. minutiflorum* extracts against *H. pylori*, many studies have reported the various effects of other plant extracts on *H. pylori. Oregano vulgaris*, a member of the Lamicea family, has also been tested against *H. pylori*. The EO showed a similar, MBC value of 40µg/ml with *O. minutiflorum* EO (MBC 46 µg/ml) in this study [191]. Moreover, *Phyllanthus urinaria* pure components isolated with HPLC and crude extracts (chloroform and methanol extracts) were tested against *H. pylori*. The MBC value of both crude extracts (97.7 µg/ml) was found to be lower than those for the pure isolates (MBC 15.6-67.5 µM) [190].

4.6. CHEMICAL COMPOSITION OF *Origanum minutiflorum* ESSENTIAL OIL

Components of the *O. minutiflorum* EO were analyzed by GC-MS GC-MS data (Figure 4.4), shows the identification of the components listed according to their elution on the TR

WAX MS column. In total, 40 components were identified (Table 4.3). GC-MS analysis revealed that the major compound was carvacrol (29.22 %). The other most abundant components were borneol (7.68 %), o-cymene (5.92 %), β-caryophyllene (4.96 %), β-mycrene (4.12 %), 4-terpineol (3.59 %), terpineol (3.54 %), and camphene (3.17 %).

The compounds identified from *O. minutiflorum* EO have previously been found in a variety of other plant extracts [191, 174] and have shown to inhibit the growth of a wide range of microorganisms [174, 178].

Previous studies on the chemical composition of *O. minutiflorum* EO by GC-MS have also shown carvacrol to be the most abundant component. However; the % of this component was much higher in all studies, this could be due to the fact that only a few components in total were identified compared to the larger number (40) identified in this study [173, 174, 175]. Aslim et al. found twenty nine components and detected 73.0 % presence of carvacrol [173]. Dadaloglu et al. found seventeen components of *O. minutiflorum* EO 68.23% presence of carvacrol [174]. In another study, only eight major constituents were determined by GC-MS, with carvacrol present at 84.6 % [175]. These differences in components and percent of major constituents could be due to several factors including; location, geography, genotype, seasonal changes, harvest time and experimental conditions [173]. Such effects are confirmed in a study on *Origanum vulgare,* where *O. vulgare* from the inland contained a higher amount of terpenoid alcohols, whereas *O. vulgare* grown in a Mediterranean climate contained higher amount of phenols [192].

Table 4.3. Relative Chemical Composition of *O. minutiflorum* Essential Oil

		RT	% Area
1	α- thujene	4.01	0.03
2	furan	4.32	0.34
3	camphene	4.81	3.17 ǂ
4	β-pinene	5.87	0.90
5	δ-3- Carene	7.10	0.24
6	l-Phellandrene	7.60	0.44
7	β-Myrcene	7.72	4.12 ǂ
8	α-Humulene	8.06	2.58 ǂ ф
9	dl-Limonene	8.61	0.78
10	1,8-Cineole	8.87	2.34
11	2- Hexenal	9.25	0.32
12	Trans-Ocimene	9.89	0.07
13	ɽ-Terpenine	10.11	6.84 ǂ
14	ocimene	10.38	0.11
15	o-Cymene	10.85	5.92 ǂ
16	α-Terpinolene	11.17	0.49
17	3-Hexen-1-ol	14.15	0.10
18	3-Octanol	14.52	0.21
19	1-Octen-3-ol	16.03	0.99
20	terpineol	16.35	3.54 ǂ
21	linalool oxide	16.50	0.09
22	sabinene hydrate	18.45	2.00
23	L-LINALOOL	18.55	1.97
24	endobornyl acetate	19.23	0.08
25	β-Caryophyllene	19.60	4.96 ǂ ф
26	4- Terpineol	19.76	3.59 ǂ
27	aromadendrene	19.82	0.80
28	dihydrocarvone	20.28	0.73
29	verbenol	21.53	0.09

Table 4.3. Relative Chemical Composition of *O. minutiflorum* Essential Oil [continued]

30	ledene	21.91	0.52
31	Borneol *	22.06	7.68 ǂ
32	myrcenol	22.81	0.10
33	germacrne-D *	23.34	0.12
34	ρ-cymen-8-ol *	25.33	0.25
35	(-)-Caryophyllene oxide	28.20	4.00 ǂ Φ
36	ledol	30.03	0.19
37	(+) spathulenol	30.94	2.21
38	eugenol*	31.58	0.23
39	thymol *	31.97	1.46
40	carvacrol *	32.61	29.22 ǂ

RT: Retention time (Compounds listed in order of elution from column).

ǂ indicates major components.

* indicates compounds with known antimicrobial properties.

Φ indicates components with known cytotoxic activity.

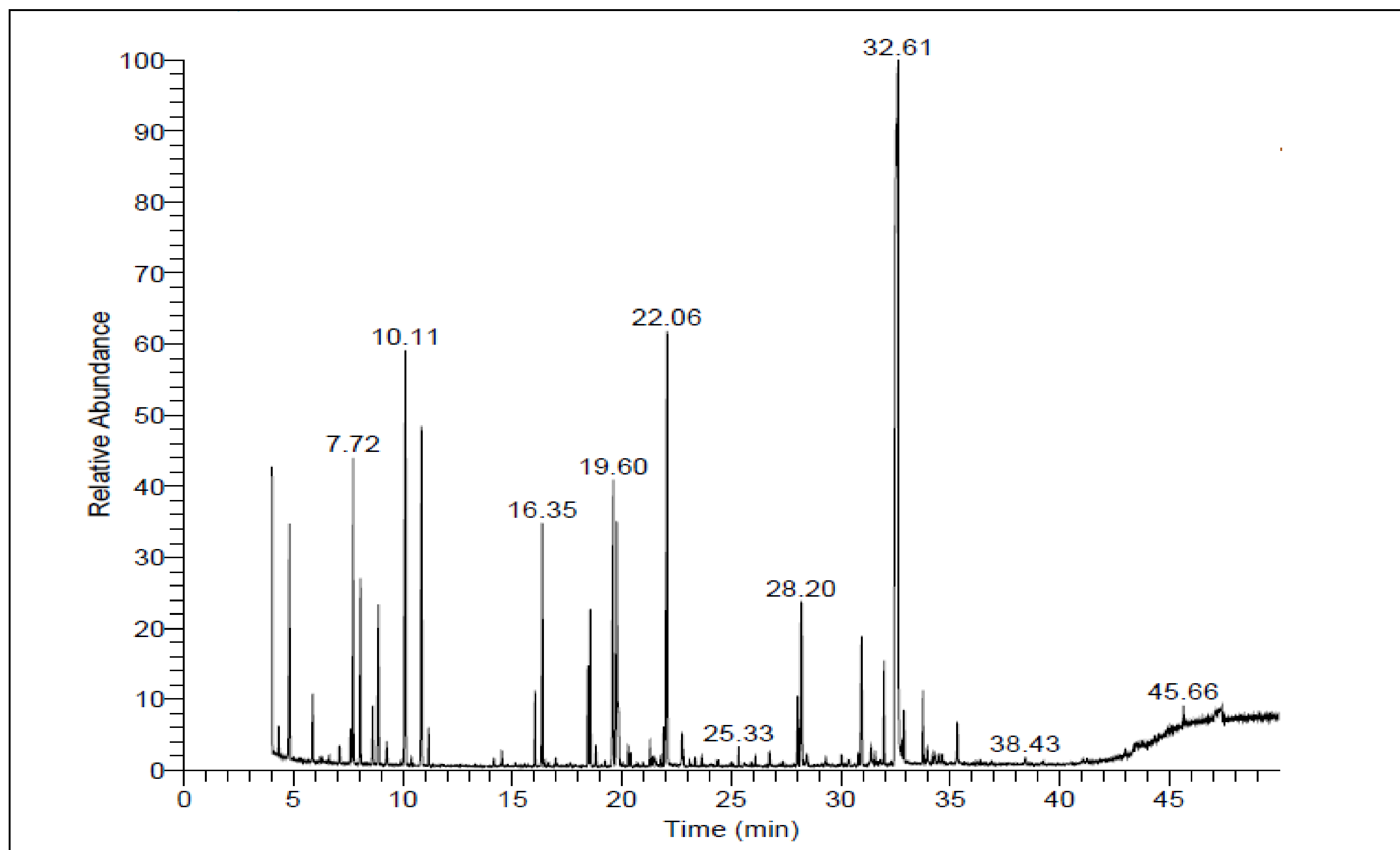

Figure 4.4. GC-MS chromatogram of essential oil extract of *O. minutiflorum*

4.7. CYTOTOXIC EFFECT OF ESSENTIAL OIL ON AGS CELL LINE

The cytotoxicity assay was carried out in order to determine the concentrations at which the EO did not affect the cell viability.

Dilutions of (1/2 to 1/80,000 v/v) EO were tested for cytotoxic effects on AGS cell lines. Dilutions between (1/2- 1/4,000 showed a total (100%) cytotoxic effect against AGS cell line (data not shown) (Figure 4.5). Dilutions of 1/8,000 v/v and 1/10,000 v/v EO following 24, 48 and 72 h incubation, displayed a statistically significant decrease in cell proliferation ($p < 0.05$) with cell viabilities of 25%, 11% and 32% respectively at 1/8,000 v/v dilution and 39%, 74% and 84% at 1/10,000 v/v respectively. However, 1/20,000 v/v, 1/40,000 v/v and 1/80,000 v/v dilutions did not significantly affect cell viability ($p > 0.05$) even after 72h incubation

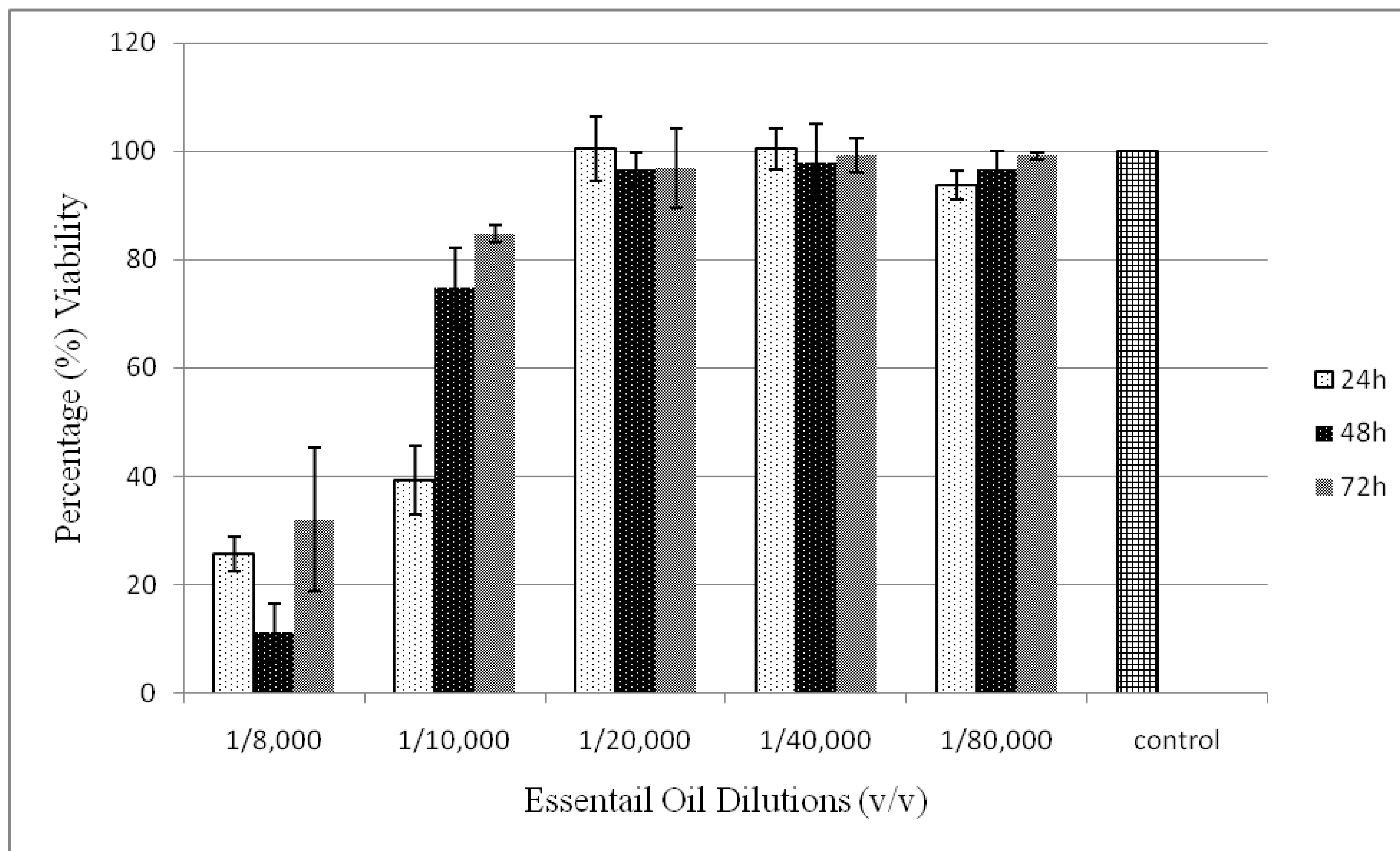

Figure 4.5. Percentage viability of AGS cells in the presence of *O. minutiflorum* EO.

Control: 0.05% DMSO. Values given as mean ±standard deviation of triplicate independent experiments.

In this study, the MBC concentration of 1/20,000 v/v EO for *H. pylori*, was observed as not toxic to AGS cells. Although antimicrobial and antioxidant activities of *O. minutiflorum* extracts have been previously studied and reported, no previous study has been done on the cytotoxic activity of *O. minutiflorum EO* on any cell line [170, 172, 173, 174, 176]. However, studies investigating the cytotoxic potential of certain available components of *O. minutiflorum* EO, β-caryophyllene (4.96 %), α-humulene (2.58 %), and caryophyllene oxide (4.00 %) have shown these components to be cytotoxic to cells [193].

In another study, the cytotoxic activity of methanol and aqueous extracts of *O. minutiflorum* (10–100 μg/ml) were tested on baby hamster kidney fibroblast cell line, which did not show any cytotoxic effect at all test concentrations [172]. However, the antimicrobial effects of the aqueous extracts (2 mg/ml) when tested on various microorganisms also did not show any inhibitory effects. Furthermore, while the methanol extracts showed a good antimicrobial effect against several microorganisms the ZI were lower than those observed for *O. minutiflorum EO*. Moreover, cytotoxic effects of different extracts of Origanum species were evaluated on various cell lines [193, 194]. An example of such a study is by Al-Kalaldeh *et al.* who did not find any cytotoxic effect of *Oreganum vulgare* extracts on MCF7 cell line (breast cancer cell line) at an IC_{50} of 64.09 μg/ml [193]. However, other studies on AGS cell lines have shown that at a concentration of (0.2 μM), *Antrodia camphorate* isolates and *Anisomeles indica* (60 μM), had no significant cytotoxic effect [195, 196].

4.8. ADHESION AND INVASION ASSAY

Since *O. minutiflorum* EO extract was not only determined to inhibit *H. pylori* growth at a minimum concentration of 46 μg/ml (1/20000 v/v dilution) but also to be non-toxic against AGS cell, this concentration (46 μg/ml) and lower concentrations of 23 μg/ml (1/20000 (v/v) and 1/40000 (v/v) dilutions) were used in the adhesion and invasion assay. The EO extract at these concentrations exhibited a marked reduction of more than 80 % *H. pylori* adhered or invaded AGS cells ($P<0.01$) (Figure 4.6). Statistical comparison of these two concentrations showed no significant difference in the % reduction of *H. pylori* adhered or invaded bacteria ($p>0.05$).

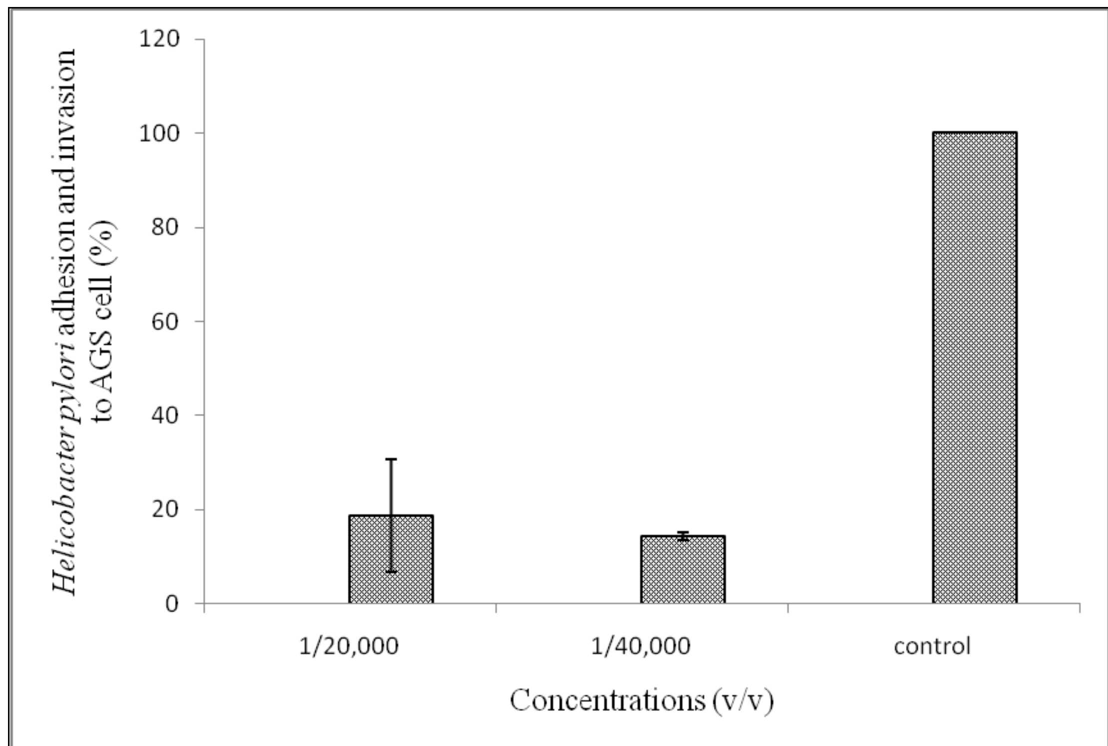

Figure 4.6. Effect of *O. minutiflorum* EO on *H. pylori* adhesion and invasion to AGS cell. Values given as means ±standard deviations of triplicate independent determinations from a typical experiment.

According to the literature, there is no previous study on the effects of any type of EO on the anti-adhesion and invasion of *H. pylori* J99.

A variety of other plant extracts have been reported to inhibit *H. pylori* adhesion and invasion to gastric epithelial cells [190, 195, 196,]. Methanol and chloroform extracts of *Phyllanthus urinaria* were assayed in order to determine their ability to inhibit the adhesion and invasion of *H. pylori* strain 26695 to AGS cells (125 - 500 µg/ml). results showed that a reduction of more than 60% for concentrations between 250 to 500 µg/ml [190]. According to results of this study *O. minutiflorum* EO showed the higher inhibitory effects (46 µg/ml) than those observed for *Phyllanthus urinaria* extracts.

4.9. SCANNING ELECTRON MICROSCOPY (SEM)

To further clarify the effects of *O. minutiflorum* essential oil on *H. pylori* and the adhesion of *H. pylori* to AGS cells, SEM analysis was carried out. The MBC value of essential oil was chosen because at this concentration a bactericidal and non toxic effect on *H. pylori* and AGS cells respectively were observed.

Following 2 days incubation under microaerophilic conditions at 37 ^{o}C rod and coccoid shapes of *H. pylori* were observed (Figure 4.7A). The coccoid form, which is known to appear under stress conditions, was observed in response to the reduction in glucose levels [1]. Furthermore a clear reduction in the number of CFU's in the presence of EO (MBC value 1/20,000) was observed (Figure 4.7B).

Figure 4.8A shows the normal morphology of AGS cells before attachment or invasion of *H. pylori* without EO. SEM results showed that following the inoculation of *H. pylori* to AGS cells (Figure 4.8B), a morphological change to the hummingbird phenotype occurred to AGS cells. This phenotype has typically been observed upon attachment of *H. pylori* to human gastric epithelial cells and is a major step in the initiation of the bacterial infection [196, 197]. This process leads to the colonization and efficient delivery of virulence factors such as cytotoxin-associated antigen A (CagA) into AGS cells, and finally the development of pathogenic diseases. The morphological changes induced by phosphorylation of CagA in the gastric epithelial cell line has been called as the ''hummingbird" phenotype (cellular elongation) and characterized by formation of needle-like cell protrusions [46]. Hummingbird phenotype results in proliferation of epithelial and compensatory cells [48].

Figure 4.9 A shows the hummingbird phenotype following infection of AGS cells. Furthermore, a clear view of the attachment of *H. pylori* to the AGS cell surface can be observed. Following incubation of the infected cells with the MBC value of EO Figure 4.9 B for 1.5 h a clear eradication of attached *H. pylori* cells was achieved. Moreover, the needle like protrusion morphology, seen typically in the infected cells changed. These observations are indicative of a positive anti adherence and anti invasion effect of the EO on AGS cells.

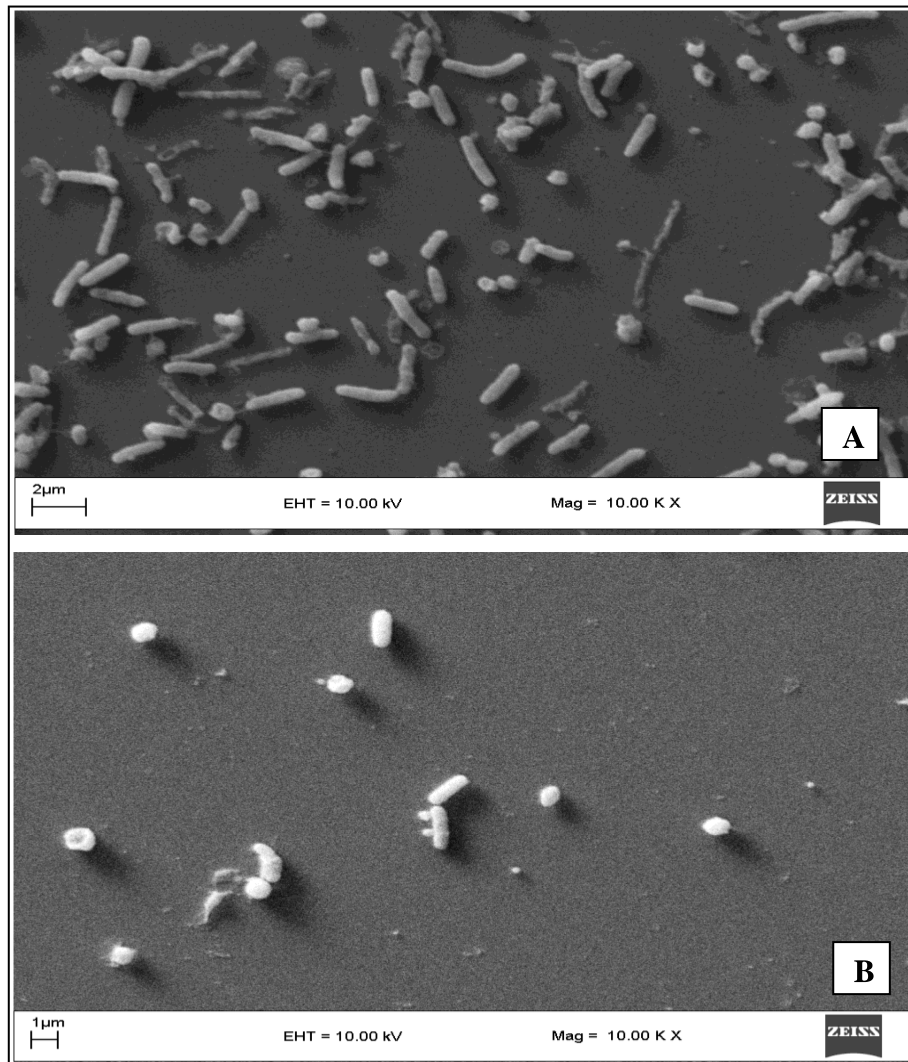

Figure 4.7. Scanning electron microscopy images of a 48-h culture of *H. pylori* (10^7) A) without EO, B) in the presence of 1/20000 (v/v) diluted EO at 10000 X magnification.

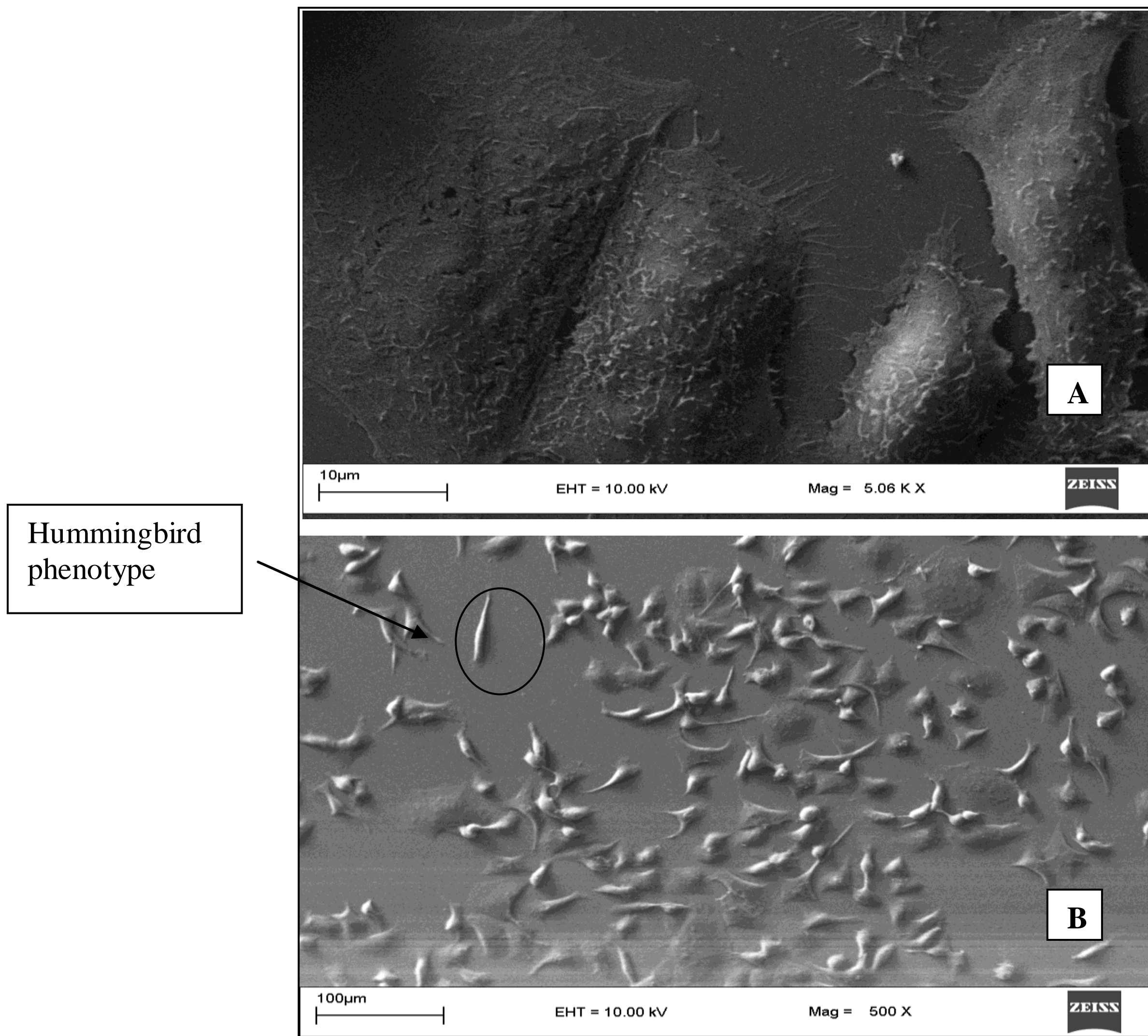

Figure 4.8. Scanning electron microscopy images of AGS cell (A) without *H. pylori* or EO. (5000 X) and B) with *H. pylori* (10^7) at 500 X magnification. The hummingbird phenotype of AGS cells is observed indication infection with *H. pylori*.

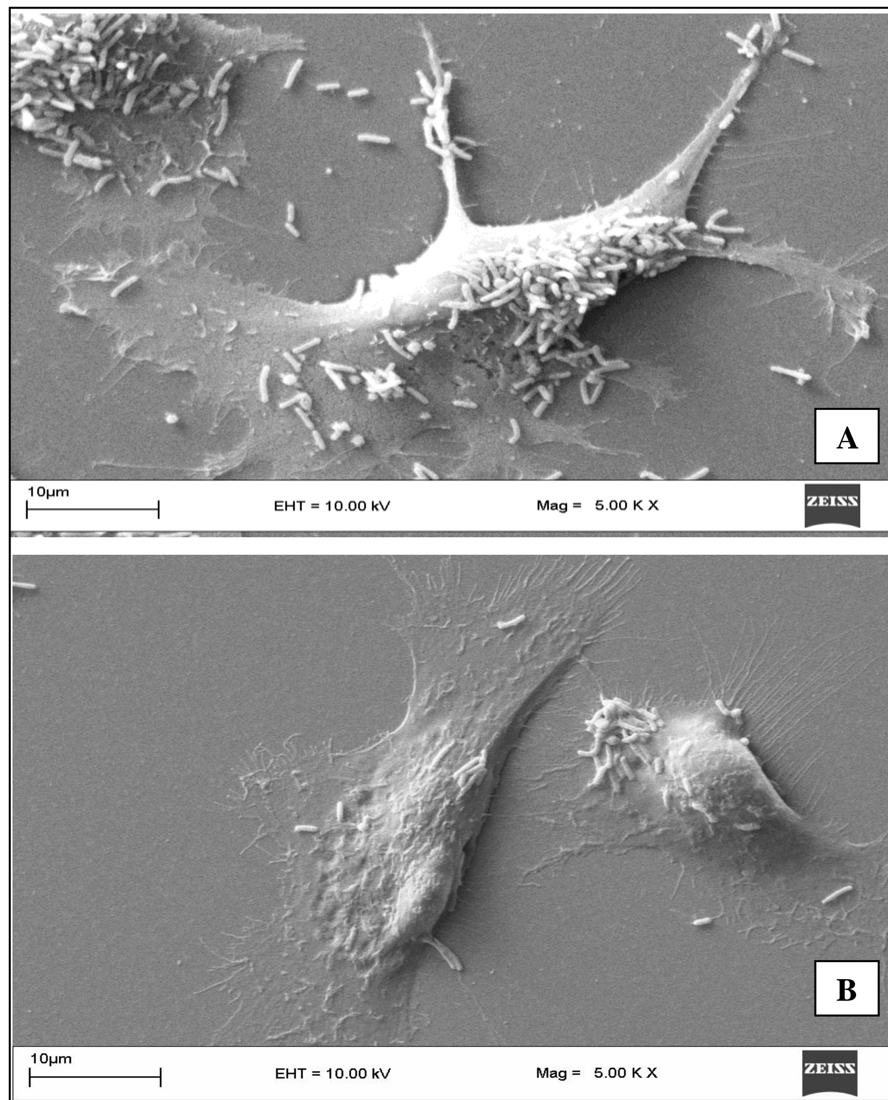

Figure 4.9. Scanning electron microscopy images of AGS cells infected with *H. pylori* (10^7) A) without EO as a control
B) in the presence of 1/20000 (v/v) diluted EO for 1.5 h at 5000 X magnification.

5. CONCLUSION

In this study several herbal extracts were tested for the eradication efficacy of *H. pylori* J99. Although all tested plant extracts showed antimicrobial activity (except aqueous extracts of *O. minutiflorum*), the strongest antimicrobial activity was observed by EO extract of *O. minutiflorum*. The concentration of EO which is not only bactericidal for *H. pylori* but also non-toxic for AGS cells was also determined. The EO of this plant also showed a good potential for inhibiting and eliminating *H. pylori* that had both attached and invaded AGS cells.

The preliminary results from this study show a high potential for the use of *O. minutiflorum* EO as an alternative treatment in the future for the treatment of *H. pylori* infections in gastric cells.

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