EFFECT OF *Centella asiatica* AGAINST GRAM NEGATIVE BACTERIA

by

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CERTIFICATE

This is to certify that the dissertation entitled "Effects of *C. asiatica* against Gram Negative Bacteria" is the bonafide record of research work done by Ms. Mimi Azuana Binti Wagimon during the period from July 2009 to October 2009 under my supervision.

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LIST OF ABBREVIATIONS

CagA	Cytotoxin associated gene A
CFU	Colony Forming Unit
CO_2	Carbon Dioxide
CNS	Central Nervous System
DMSO	Dimetyl Sulfoxide
DNA	Deoxyribonucleic Acid
EPIYA	Membrane-targeting signal of *H. pylori* virulent factor cagA
ExPEC	Extraintestinal pathogen *Escherichia coli*
kg	Kilogram
LB	Luria Bertani Broth
MIC	Minimum Inhibitory Concentration
µL	Microgram
mg	Miligram
ml	Milliliter
nm	Nanometer
N_2	Nitrogen
O_2	Oxygen
OD	Optical Density
pH	Negative logarithm of the activity of dissolved hydrogen ions
PCR	Polymerase Chain Reaction

PPI	Proton Pump Inhibitor
SHP-2	Src homology 2-containing protein-tyrosine phosphatase-2
VagA	Vacuolating cytotoxin A
TSB	Tryptic Soy Broth
WHO	World Health Organization

ABSTRACT

Centella asiatica (*C. asiatica*) is a well-known herb used in traditional medicine for treatment of a wide range of diseases. In this research, crude extract from different parts of *C. asiatica* extracted with two different solvents; methanol and water, were assessed for antibacterial activity against Gram negative bacteria, *Helicobacter pylori* (*H. pylori*) and *Escherichia coli* (*E. coli*). The determination of minimum inhibitory concentration (MIC) was performed using microbroth dilution method. The MICs values for *H. pylori* were 0.04 mg/ml and 0.16 mg/ml for methanolic and water leaves crude extracts, respectively, 0.16 mg/ml and 0.04 mg/ml for methanolic and water stem extracts, respectively and 0.04 mg/ml for both methanolic and water root extracts. The MICs values for *E. coli* were 0.01 mg/ml and 0.02 mg/ml for methanolic and water leaves extracts, 0.01 mg/ml and 1.25 mg/ml for methanolic and water stem extracts while 0.63 mg/ml and 1.25 mg/ml for methanolic and water root extract respectively. The MICs values for positive controls, clarithromycin and ampicillin, were 0.16 mg/ml and 0.01 mg/ml, respectively. Generally, all the *C. asiatica* extracts had a promising antibacterial effect against these bacteria.

ABSTRAK

Centella asiatica (*C. asiatica*) merupakan herba yang sering digunakan dalam perubatan tradisional bagi mengubati pelbagai penyakit. Dalam kajian ini, ekstrak kasar daripada bahagian *C. asiatica* yang berbeza telah diekstrak menggunakan dua jenis pelarut iaitu metanol dan air dan telah diuji aktiviti anti-bakterianya terhadap bakteria Gram negatif, *Helicobacter pylori* (*H. pylori*) dan *Escherichia coli* (*E. coli*). Penentuan kepekatan perencatan minimum (MIC) telah dilakukan menggunakan kaedah pencairan kaldu mikro. Nilai kepekatan perencatan minimum untuk *H. pylori* adalah 0.04 mg/ml dan 0.16 mg/ml, masing-masing untuk ekstrak kasar daripada daun yang diekstrak menggunakan metanol dan air, 0.16 mg/ml dan 0.04 mg/ml, masing-masing untuk ekstrak kasar batang yang diekstrak menggunakan metanol dan air dan 0.04 mg/ml untuk kedua-dua ekstrak kasar akar yang diekstrak menggunakan metanol dan air. Nilai kepekatan perencatan minimum untuk *E. coli* pula adalah 0.01 mg/ml dan 0.02 mg/ml, masing-masing mewakili ekstrak kasar daun yang diekstrak menggunakan metanol dan air. Nilai 0.01 mg/ml dan 1.25 mg/ml pula adalah nilai kepekatan perencatan minimum untuk ekstrak kasar batang yang diekstrak menggunakan metanol dan air manakala 0.63 mg/ml dan 1.25 mg/ml adalah nilai kepekatan perencatan minimum untuk ekstrak kasar akar yang diekstrak menggunakan metanol dan air. Nilai kepekatan perencatan minimum untuk kawalan positif iaitu clarithromycin dan ampicilin pula adalah masing-masing 0.16 mg/ml dan 0.01 mg/ml. Secara amnya, kesemua ekstrak kasar *C. asiatica* menunjukkan kesan anti-bakteria terhadap kedua-dua bakteria ini.

CHAPTER 1

INTRODUCTION

1.1 Plant Material (*Centella asiatica*)

1.1.1 Plant Description

Centella asiatica (*C. asiatica*) is a plant that belongs to the family Umbelliferae. It has been found in many Asian countries such as in parts of India, Sri Lanka, China, Indonesia, Malaysia, Australia and Southern and Central Afrika (Verma *et al.*, 1999), thus has different names. *C. asiatica* or locally called 'Pegaga' has been used as traditional medical herbs, as in India, it is used in Ayurveda medicine and known as 'Mandukaparni' (Zainol *et al.*, 2008). In China, it has given names like 'Luci Gong Gen' and 'Tung Chain' (Saniah, 2005) while in Sri Lanka and Indonesia it is known as 'Thankuni Sak' (Jayashree, 2003).

C. asiatica (Figure 1.3) is an easy growing plant that being found in moist places and can grow in different soil composition (Devkota and Jha, 2009). This plant is a polymorphous, creeping, rooting at nodes, with sometimes significant tap root, cylindrical and glabrous stems.

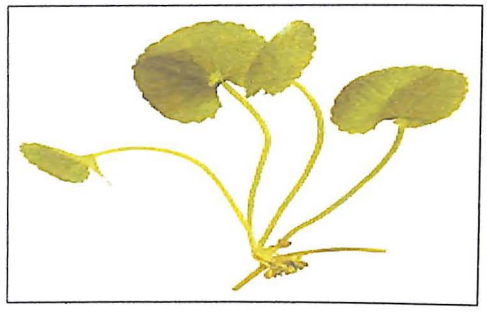

Figure 1.1: *C. asiatica* (source: http://www.ramuan.com/pages/herbs.htm)

1.1.2 Medicinal Applications

C. asiatica, known for its role in folk medicines has many applications in human health. It has been used years ago to heal wound. Study performed by Biswas and Mukherjee (2003) on rat dermal has shown that an alcoholic extract of *C. asiatica* has an effect on wound healing following oral and topical administration. To assess the effectiveness on wound healing, an increased in DNA, protein and collagen content of granulation tissues are proves that cellular proliferation and synthesis occur at the site of wound.

It has been used in the Ayurvedic medicine to treat depression and stress. Thus, in India, it is regarded as the most spiritual of all herbs. Furthermore, *C. asiatica* also been used by yogis to improve meditation. Besides, it also used as an anti-tumor. A study by Babu *et al.* (1995) showed that methanolic extract of *C. asiatica* has 100% cytotoxicity in two cell lines after 3 hours incubation at 37°C.

C. asiatica has been proved to have antibacterial activity. The essential oil of *C. asiatica* showed a broad spectrum of antibacterial activity against Gram positive and Gram negative bacteria (Oyedeji and Afolayan, 2005). It was found that the antibacterial activity was greater in Gram positive bacteria compared to Gram negative bacteria. This may due to the component structures of Gram negative bacteria that have a unique outer membrane, composed of lipopolysaccharides (LPS), porin channels and murein lipoprotein, which Gram positive bacteria lack. Gram negative bacteria are resistant to lysozyme and penicillin attack and LPS block antibiotics, dye and detergents, protecting the sensitive inner membrane and cell wall.

In addition, the hydroalcoholic extract of *C. asiatica* leaves possesses potential anticonvulsant, antioxidant and central nervous system (CNS) depression actions (Ganachari *et al.*, 2004).

1.1.3 Bioactive Constituents

The medicinal properties of *C. asiatica* has attributed by its bioactive constituents. *C. asiatica* contains pentacyclic triterpenes, mainly known as asiaticoside, madecassosoide, siatic acid and madecossic acid (Inamdar, 1996). *Centella* triterpenoids can be regarded as phytoanticipins due to their antimicrobial activities and protective role against attempted pathogen infections. Among pentacyclic triterpenoid saponin component, asiaticoside has identified as the major component claimed for main therapeutic interest (Zainol *et al.*, 2008). Table 1.1 shows extracts of *C. asiatica* with the composition and applications.

Table 1.1: Product range of extracts from *C. asiatica* indicating the specific chemical composition and treatment (adapted from James and Dubery, 2009)

Extract	**Chemical composition**	**Applications**
Asiatic acid	>95% Asiatic acid	Anti-ageing cosmetics, application after laser therapy, cosmeceutics
Titrated Extract of *Centella Asiatica* (TECA)	55-66% Genins 34-44% Asiaticoside	Anti-cellulite, slimming products, breast creams, stretch marks, scarred skin, anti-ageing cosmetics, moisturizing care
TECA cosmetics	>40% Genins > 36% Asiaticoside	Anti-cellulite, slimming products, breast creams, stretch marks, scarred skin, anti-ageing

		cosmetics, moisturizing care
Heteroside	>55% Madecassoside >14% Asiaticoside	Slow release effect, anti-ageing cosmetics, for moisturizing nightcreams
Asiaticoside	>95% Asiaticoside	Anti-inflammatory, against irritated and reddened skin, antiallergic
Genins	>25% Asiatic acid >60% Madecassic acid	Natural antibiotic, antibacterial properties, for anti-acne products, intimate hygiene

1.2 *Helicobacter pylori*

1.2.1 History

Before *Helicobacter pylori* (*H. pylori*) was discovered by Barry Marshall and James Robyn Warren in 1982 (Marshall and Warren, 1984), actually there was another investigator who observed spiral organisms in the stomach of dogs for the first time in 1892. This *Helicobacter* pioneer was the Italian Giulio Bizzozzero. There were also claims from many other researchers from France, Poland, Germany, the United Kingdom, the United States, Greece, China and Soviet Union on the discovery of bacteria in gastric mucosa. However, these researchers's work has not been well known in the scientific field (Ciacci and Mazzacca, 2006).

This bacterium was originally classified into *Campylobacter pyloridis*, and then the name was changed to *Campylobacter pylori* (*C. pylori*). Nevertheless, some features of *C. pylori* seem very different from all other *Campylobacter* and it has been suggested that *C. pylori* belong to another genus and should not be included in the genus *Campylobacter*. *C. pylori* seems more closely related to genus *Wolinella succinogenes* but there are inconsistencies in five major groups of taxonomic features of these bacteria that caused *C. pylori* should be excluded from genus *Wolinella* and thus, brings into establishment of new genus known as *pylori* (Goodwin *et al.*, 1989).

1.2.2 Microbiology Characteristics

H. pylori (Figure 1.1) is Gram-negative, rod shaped and microaerophilic organism. It has rapid and darting motility by means of multiple unipolar or bipolar-sheathed flagella (Feirtag and Velázquez, 1999; Goodwin *et al.*, 1989). It also has been described as spiral-shaped (Clyne and Drumm, 1993) or straight unbranched cells, 2.5 to 5 µm long and 0.5 to 1 µm wide (Goodwin *et al.*, 1989).

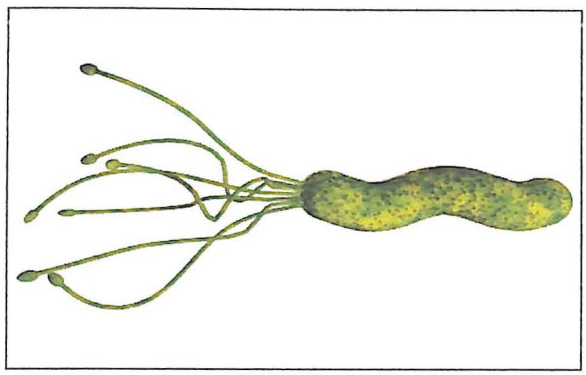

Figure 1.2: *H.pylori* (source: www.medgadget.com/archives/img/hpylori_lg.jpg)

This bacterium is a fastidious microorganism that requires optimal growth at 37°C and sometimes growth at 30°C but not at 25°C while the growth is variable at 42°C (Feirtag and Velázquez, 1999). Since it is a microaerophilic organism, *H. pylori* needs a low concentration of oxygen supply. The required atmosphere for optimal growth included 10% CO_2, 5% O_2 and the rest is N_2 (Busolo *et al.*, 1998).

The media that support the growth of *H. pylori* is the one that rich in nutrient. Media are usually supplemented with blood (Feirtag and Velázquez, 1999) and the incubation period varies from 3 days (Wang and Huang, 2006) to 5-7 days (Voravuthikunchai and Mitchell, 2008), depends on the strains. As the culture aged, *H. pylori* might change from spiral rod

shaped to coccoid forms. This change is influenced by many factors such as nutritional deprivation, increased oxygen tension, alkaline pH, increased temperature, exposure to different antibiotics and aging (Dubini *et al.*, 1999).

Besides the morphological on Gram smear, *H. pylori* also can be identified based on the biochemical characteristics such as urease, catalase and oxidase (Busolo *et al.*, 1998). It has been reported as urease, catalase and oxidase positive (Windsor and O'Rourke, 2000). The confirmation of these characteristics provides sufficient information in the identification of *H. pylori* in patient.

1.2.3 Virulence Factors

H. pylori is a human pathogen that colonizes the area of stomach and thus, has an ability for the occurrence of gastritis, gastric ulcer as well as gastric carcinoma (Bhattacharjee *et al.*, 2002). Other bacteria normally seem not to be colonized in a niche that known for the extremity of pH which is approximately 2, but, *H. pylori* make it possible by having some unique characteristics that enable it to colonizes the host and cause pathogenesis to the host.

The ability of *H. pylori* to adapt well within its ecological niche is due to some features that enable the colonization and ultimately responsible for a chronic infection to the niche. There are some virulence factors that have been identified such as adherence, urease enzyme, vacA and also cagA and the cag pathogenicity island (Suerbaum and Josenhans, 1999).

H. pylori have a unique mechanism that allowed it to adhere to the human gastric epithelial cells. The capability of attachment ease the colonization of *H. pylori* and therefore exhibit chronic infection. The adherence to host cells usually facilitates the bacteria to obtain nutrients from the host and avoid the shedding of the mucous gel layer. *H. pylori* bind to host cell receptors of adhesins. Some types of adhesins that have been identified including AlpA, AlpB, HopZ (Israel and Peek Jr., 2006) and BabA2 (Suerbaum and Josenhans, 1999).

Since human stomach release acidic gastric juice and this condition were totally not suitable for growth of other bacteria, *H. pylori* had encountered this problem by releasing urease enzyme. This enzyme will neutralized the acidic environment by hydrolyzes urea to yield ammonia, which is a strong base and carbon dioxide. But, another effect appears upon the production of ammonia. Ammonia known for its toxic properties that caused direct toxic damage to epithelial cells, activation of the oxidative burst in phagocytes and leading to apoptosis (Suerbaum and Josenhans, 1999).

Other specific virulence factor is vacA. It is a protein cytotoxin that stimulates injuries in epithelial cells by inducing toxic vacuolization in certain cell lines. VacA is present in all *H. pylori* strains but only approximately 50% of *H. pylori* strains expressed this protein cytotoxin. This is depended on the two types of vacA allelic variable parts named s-region and m-region. The s-region can be divided into s1 and s2 while the m-region exists as m1 and m2. It has been identified that type s1/m1 strains secrete high amount of toxin whereas type s1/m2 strains produce moderate levels. While s2/m2 type of strains show little or no vacuolating cytotoxin (Van Doorn *et al.*, 1999).

If vacA present in all *H. pylori* strains, another virulence factor that believed to cause pathogenesis, cagA, is only exist in 60-80% of *H. pylori* strains (Pakodi *et al.*, 2000). Suerbaum and Josenhans (1999) have been reported that more than 90% of strains from ulcer patients are $cagA^+$. CagA is a part of pathogenicity island (cag-PAI) and strains $cagA^+$ are those that consist of cag-PAI. The difference between $cagA^+$ and $cagA^-$ is the ability to generate virulent responses in host cells. $cagA^+$ is more virulent strains that stimulate the emission of interleukin-8 (IL-8). This cytokine is responsible for the pathogenesis of *H. pylori* gastritis.

1.2.4 Epidemiology

1.2.4.1 Prevalence of Infection

Kaya *et al.* (2007) had claimed that *H. pylori* as a frequent cause of chronic infection worldwide. Approximately, 50% or at least half of the world's population has been infected by *H. pylori* (Frenck and Clemens, 2003; Sasidharan and Uyub, 2009). But, the trend of infection is differing from one country to another. Geographic area, age, race, ethnicity and sosio-economic status are some factors that influenced the prevalence of *H. pylori* infection (Sasidharan and Uyub, 2009).

There is a difference in the prevalence of *H. pylori* infection between developed and developing country. Most of the reports showed that the developing country has higher prevalence rates compared to developed country (Alborzi *et al.*, 2006; Frenck and Clemens, 2003; Raj *et al.*, 2008; Sasidharan *et al.*, 2008). At present, the infection of *H. pylori* in children in many developing countries is up to 50% before reaching 10 years old and 80%

to 90% become infected in adulthood while the prevalence in children of developed countries is near 10% (Bosques-Padilla *et al.*, 2003).

Many developing countries have lower socioeconomic status, contrasted with the developed countries. The lower socioeconomic status leads to a poor living standard with lower hygiene level, lower levels of education, poor sanitation and contaminated environment. All these factors caused the infection rates of *H. pylori* is higher in developing countries (Pillay *et al.*, 2007).

In Malaysia, a multi-racial country, the prevalence rates among the three major races also different although they are living in the same geographical area. Studies done by Goh (1997); Raj *et al.* (2001); Raj *et al.* (2008) and Sasidharan *et al.* (2008) have shown that Malay populations has the lowest prevalence than Indian and Chinese populations. This differences might correlated with factor genetic and socio-cultural practices (Raj *et al.*, 2001), socioeconomic and dietary (Sasidharan *et al.*, 2008).

Considering that the Chinese and Indians are immigrant from China and India respectively, the original immigrant passed the high infection rates to their descendent born in Malaysia. The high prevalence rates in their origin countries have reflect the prevalence in Malaysia. Besides, Raj *et al.* (2008) suggested that genetic or other host factors inherent to Malay community protect them from *H. pylori* infection. Malay populations also have been reported to consume a lot of herbs as this provides protection to them from being infected by *H. pylori* (Sasidharan *et al.*, 2008).

1.2.4.2 Source of Infection

According to Frenck and Clemens (2003), water and food sources that become polluted could be highly risky for being infected with *H. pylori*. Contaminated water resource may be one of the factors for *H. pylori* infection since there was a research done before that successfully isolates *H. pylori* from the untreated wastewater (Lu *et al.*, 2002). Besides, Klein *et al.* (1991) had studies the possibility of water source as a risk factor for infection of *H. pylori* in Peruvian children. The result showed that the risk is high in children who used the untreated water. All these studies support the theory that water could possibly be one of the sources of infection especially in the developing countries where the water treatments were limited.

1.2.4.3 Routes of Transmission

Although the transmission of *H. pylori* is still unclear, Delport and van der Merwe (2007) suggested that it would be transmitted through oral-oral and fecal-oral routes. This idea has been supported by van Duynhoven and Jonge (2001) that person-to-person contact is mostly considered for transmission route. The first possible route is iatrogenic which the most frequent mode of transmission. *H. pylori* can be transmitted when tubes or endoscopes of infected person are used for another patient.

Another potential route is the fecal-oral pathway. This route could be possible since there were attempts of isolation of *H. pylori* in feces of infected children (Dunn *et al.*, 1997) but the isolation process is actually very difficult and required sophisticated and expensive techniques. *H. pylori* from samples other than gastric tissues usually tend to change in the morphology, metabolism and growth patterns (Bode *et al.*, 1993). Here, a development of

viable but non-culturable (VNC) coccoid form occurred. This type of cells is metabolically active but do not go through cellular division. That's why there is no colonies developed in the culture plate.

The third promising route is oral-oral. Even though samples from mouth have rarely been cultured, but the presence of *H. pylori* can be detected by molecular biology technique using PCR amplification of DNA fragments (Parsonnet *et al.*, 1999). The assuming of oral-oral as a route of transmission has been supported by several studies. A study done by Clemens *et al.* (1996) in *H. pylori* infection among Bangladeshi children claimed that behavior of the mothers that coat the nipples with saliva before breast-feeding had showed high prevalence of infection. It become worst when the hygienic aspect does not practiced by the mother who did not washing her hands and nipples prior to breast-feeding (Frenck and Clemens, 2003).

Furthermore, there was a study by Chow *et al.* (1995) among Melbourne Chinese immigrants to look for evidence that usage of chopsticks could be one of the methods of transmission through oral-oral pathway. Reuse of the chopsticks by other person might increase the risk for that person to be infected by *H. pylori*. In addition, the pre-mastication of food by mother before feeding their child also linked the association of oral-oral route with the high prevalence rates. This situation can be seen in African cultures (Megraud, 1995), Bangladesh (Clemens *et al.*, 1996) and in Ethiopia (Lindkvist *et al.*, 1998).