ANTIMICROBIAL ACTIVITY OF AQUEOUS *Quercus infectoria* GALL EXTRACT AGAINST PATHOGENIC *Leptospira*

HUSNA BINTI MUSTAFA

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%	Percentage
-	To
/	per
>	Greater than
≤	Less than and equal to
ND	Not done
°C	Degree Celsius
G	Gram
Mg	Milligram
Kg	Kilogram
µm	Micrometer
Mg	Microgram
mL	Milliliter
Cm	Centimeter
Cfu	Colony forming unit
Rpm	Revolution per minute
kV	Kilovolt
sp.	Species
MAT	Microscopic agglutination
HPLC	High performance liquid chromatography
MDR	Multi-drug resistant
MIC	Minimum inhibition concentration
MBC	Minimum bactericidal concentration

MSCoNS	Methicillin resistant coagulase negative *Staphylococcus*
MRSA	Methicillin resistant *Staphylococcus aureus*
SEM	Scanning electron microscope
TEM	Transmission electron microscope
MOF	Multi-organ failure
IV	Intra venous
LPS	Lipopolysaccharide
T2SS	Type two secretion system
GrsD	A type of secretin gene
IM	Inner membrane
OM	Outer membrane
PF	Periplasm flagellar
PG	Peptidoglycan
SP	Signal peptidase
PBPs	Penicillin binding proteins
EF	Endoflagella
OmpL1	Outer membrane protein L1
ImpL63	Inner membrane protein L63
GroEL	Heat-shock protein
OH	Hydroxyl group
EMJH	Ellinghause, McCullough, Johnson and Harris
DFM	Dark field microscope

UV-A	Ultra violet A
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
PBS	Phosphate buffer saline
HDMS	Hexamethyldisilazane
TEMA	Tetrazolium microplate assay

ABSTRAK

AKTIVITI ANTI-MIKROB OLEH EKSTRAK AKUEUS GAL *Quercus infectoria* TERHADAP *Leptospira* PATOGENIK

Leptospirosis adalah penyakit berjangkit zoonosis bakteria yang berlaku di seluruh dunia disebabkan oleh jangkitan *Leptospira* dan boleh menjangkiti manusia melalui pendedahan secara langsung atau tidak langsung dengan air kencing haiwan yang dijangkiti. Ekstrak gal *Quercus infectoria* telah dikenalpasti memiliki aktiviti anti-mikrob spektrum luas secara *in-vitro*. Kajian ini dilakukan untuk menentukan aktiviti anti-mikrob oleh ekstrak akueus gal *Q. infectoria* terhadap *Leptospira* patogenik dan menilai perubahan pada morfologi sel yang dirawat ekstrak menggunakan mikroskop elektron pengimbas (SEM). Ujian siri pencairan kaldu mikro dua kali ganda telah digunakan untuk menentukan kepekatan perencatan minimum (MIC) ekstrak akueus gal *Q. infectoria* terhadap *L. interrogans* serovar Javanica dan *L. interrogans* serovar Icterohaemorhagiae iaitu masing-masing dalam julat kepekatan 4.00 mg/mL hingga 0.0078 mg/mL. Kepekatan bakterisidal minimum (MBC) telah ditentukan melalui subkultur kaldu daripada telaga plat mikro yang tidak menunjukkan pertumbuhan ketara atau kekeruhan ke dalam media EMJH yang baru dan kultur tersebut diperhatikan di bawah mikroskop medan gelap selepas 3 minggu pengeraman untuk mengesan pertumbuhan. Morfologi sel *L. interrogans* serovar Icterohaemorhagiae yang dirawat dan yang tidak dirawat menggunakan ekstrak dianalisis dengan menggunakan SEM. Keputusan ujian pencairan kaldu mikro menunjukkan bahawa ekstrak akueus gal *Q. infectoria* memiliki aktiviti anti-mikrob terhadap kedua-dua serovar *L. interrogans* dengan nilai-nilai MIC iaitu 0.125 mg/mL. Manakala nilai MBC untuk *L.*

interrogans serovar Javanica dan *L. interrogans* serovar Icterohaemorhagiae masing-masing adalah 0.125 mg/mL dan 0.25 mg/mL. Mikrograf daripada analisis SEM terhadap perubahan morfologi *L. interrogans* serovar Icterrohaemorrhagiae menunjukkan perubahan saiz dan bentuk pada sel yang dirawat menggunakan ekstrak (8x MIC) berbanding sel yang tidak dirawat. Kesimpulannya, ekstrak akueus gal *Q. infectoria* mempunyai potensi untuk diterokai keberkesanannya dan penggunaannya dalam rawatan leptospirosis.

ABSTRACT

ANTIMICROBIAL ACTIVITY OF AQUEOUS *Quercus infectoria* GALL EXTRACT AGAINST PATHOGENIC *Leptospira*

Leptospirosis is a bacterial zoonotic infectious disease that occurs worldwide caused by *Leptospira* and transmitted to human through direct or indirect contact with the urine of infected animal. *Quercus infectoria* gall extract is known to have a broad spectrum anti-microbial activity *in-vitro*. This study was conducted to determine the antimicrobial activity of *Q. infectoria* gall extract against pathogenic *Leptospira* and to evaluate the morphological changes of extract-treated cells using scanning electron microscope (SEM). Two-fold serial microdilution broth assay was used to determine the minimum inhibitory concentration (MIC) of aqueous *Q. infectoria* gall extract against *L. interrogans* serovar Javanica dan *L. interrogans* serovar Icterohaemorhagiae at concentration ranging from 4.00 mg/mL to 0.0078 mg/mL. Minimum bactericidal concentration (MBC) was determined by sub-culturing the broth from microtitre plate well showing no apparent growth or turbidity into the freshly prepared EMJH media and observed under dark field microscope after 3 weeks incubation for growth detection. The cell morphology of extract-treated and untreated *L. interrogans* serovar Icterohaemorhagiae were analyzed using SEM. The results of broth microdilution assay demonstrated that aqueous *Q. infectoria* gall extract possessed antimicrobial activity against both *L. interrogans* serovar with MIC values of 0.125 mg/mL. The MBC values for *L. interrogans* serovar Javanica dan *L. interrogans* serovar Icterohaemorhagiae were 0.125 mg/mL and 0.25 mg/mL respectively. The SEM micrograph showed changes of

shape and size of the *L. interrogans* serovar Icterohaemorrhagiae extract-treated cells (at 8x MIC) as compared to the untreated cells. In conclusion, aqueous *Q. infectoria* gall extract has the potential to be explored for its efficacy and use in the treatment of leptospirosis.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Leptospirosis is a worldwide infectious disease affecting animals and humans and is caused by the infection with spiral-shaped bacteria known as *Leptospira* species (Tilahun *et al.*, 2013). It is also known as a zoonotic disease as the disease can be directly or indirectly transmitted from infected animal to human usually through contaminated food and water (Wang *et al.*, 2007). People may acquire the disease when they come into contact with infected animals or contaminated the environment with *Leptospira* (Chadsuthi *et al.,* 2012*).* Rat and other rodents are primary hosts for *Leptospira* while other mammals like pig, cattle, sheep and dog can also carry and transmit the disease but they act as secondary hosts (Hui Yi *et al.*, 2011).

The spirochete genus of *Leptospira* comprises of both saprophytic and pathogenic species (Evangelista and Coburn, 2010). Saprophytic species are naturally present in environmental water and soil and usually do not cause disease (Mohammed *et al.*, 2012; Benacer *et al.*, 2013). However, several pathogenic strains such as *L. interrogans* serovars can survive in low-nutrient environments like moist soil and fresh water for long periods of time especially when the pH is slightly alkaline (Trueba *et al.*, 2004; Evangelista and Coburn, 2010). In *in vitro* cultivation, *Leptospira* is observed to have optimal growth at the temperature between 28°C and 30°C in Ellinghausen-McCullough/Johnson-Harris (EMJH) medium that contains oleic acid, bovine serum albumin and polysorbat (Evangelista and Coburn, 2010). In addition, a

strain of serovar Javanica was reported to survive in distilled water with pH 7.8 for 152 days (Trueba *et al.*, 2004)

Leptospirosis has been reported to cause more than 500 000 cases per year worldwide (Chadsuthi *et al.*, 2012). Tropical and subtropical countries are ideal for *Leptospira* to survive due to its high humidity and warm temperature (Benacer *et al.*, 2013). The incidence in the tropics area is approximately 10 times higher than in temperate regions (Tilahun *et al.*, 2013). In Malaysia, the incidence of leptospirosis was not well documented previously as it was not included in the list of notifiable diseases. Currently, available leptospirosis country data for Malaysia is based on the Report of Morbidity and Mortality for Ministry of Health Hospitals. The cases of leptospirosis in Malaysia increased from 236 in 2004 to 5370 in 2015 (Zainudin, 2015). The first fatal case of leptospirosis reported in Malaysia was in 1925 due to infection with serovar Icterohaemorrhagiae as reported by Fletcher in 1928 (El Jalii and Bahaman, 2004). *L. borgpetersenii* serovar Javanica and *L. interrogans* serovar Bataviae were considered as the major pathogenic *Leptospira* circulating among the urban rat population in Kuala Lumpur (Benacer *et al.*, 2013).

Leptospirosis in human displays wide array of clinical presentations that commonly present in other well-known infectious diseases such as meningitis, pneumonitis, hepatitis, nephritis, pancreatitis, erythema nodosum and death (Wang *et al.*, 2007). In animal, leptospirosis will result in kidney disease, liver disease or reproductive dysfunction (Spickler and Leedom Larson, 2013)

Patients with leptospirosis usually response to the antibiotics such as penicillin and tetracycline at sufficient doses in the early phase of the infection (Chandan *et al.*, 2012). Despite of using modern drug which prone to produce unwanted adverse effects,

traditional medicine has become an alternative for the treatment of infectious diseases like leptospirosis. Traditional medicine refers to herbal-based therapeutic agent or the use of natural sources such as herbs, fruits and vegetable for treatment (Chandan *et al.*, 2012). There are many plant species that are being reported to be effective against various pathogens *in vitro* and it is able to provide a valuable lead in the discovery of new and more efficacious drugs (Chander *et al.*, 2015).

Quercus infectoria gall is a part of *Q. infectoria* plant known to be used as one of traditional medicines for centuries. The gall has been documented to possess various pharmacological effects including astringent, antidiabetic, local anaesthetic and anti-inflammatory effects as well as anti-microbial activity (Umachigi *et al.*, 2008). There are several reports on the anti-microbial activity of *Q. infectoria* extracts against bacteria and yeast (Wan Nor Amilah *et al.*, 2014; Baharuddin *et al.*, 2015). *Q. infectoria* gall extract can also be a source of antibacterial agent especially against multi drug resistance (MDR) Gram-positive bacteria (Wan Nor Amilah *et al.*, 2014).

The natural products derived from medicinal plants are known to produce biologically active compounds which contribute to pharmacological action of the plant. The chemical constituents of *Q. infectoria* gall extract are composed of tannic acid (50-70%), gallic acid (2-4%), ellagic acid, starch and sugar (Basri *et al.*, 2011). There was a study reported that tannic acid plays an important role in resulting the antimicrobial effect of *Q. infectoria* gall extract by which it prevents the adherence of bacteria to cell surface receptors that caused the inability of bacteria to multiply within the cell (Vasconcelos *et al.*, 2006).

1.2 Rationale of the study

The study on anti-leptospiral activity of *Q. infectoria* gall extract is still limited as there was no published report on the effect of *Q. infectoria* gall or its extract against pathogenic *Leptospira*. On the other hand, there was a study conducted on *Phyllunthus amarus* plants extract which contains tannic acid as one of their active compounds and the extract was tested positive for the anti-leptospiral acitivity (Verma *et al.*, 2014). Phytochemical test using HPLC of previous study found that *Q. infectoria* gall extract contain large amount of tannic acid which considered as the bioactive compound that lead to antimicrobial activity (Nur Syukriah *et al.*, 2014). Hence, there is a possibility that the *Q. infectoria* gall extract can exhibit anti-leptospiral activity against pathogenic *Leptospira*. Besides that, the inhibition or killing mechanism of *Leptospira* spp. by medicinal plant is still poorly understood. Thus, the information on the morphological changes that occurs prior to and after the treatment can provide some clue on killing mechanisms of *Leptospira* by the medicinal plant like *Q. infectoria* gall.

Emergence of antibiotic resistance has becomes a serious threat to global public health and has necessitated the need to search for new antibacterial agents, thus research and development to find effective and safe alternative agents are necessary to overcome the problems. Herbal alternatives to antibiotics in treating infectious diseases are becoming more popular in our current culture and their promising treatment outcomes can be identified in years to come. In addition, the effective treatment for leptospirosis remains a problem as it is difficult to diagnose and the disease usually become recognized at the severe stage. This is because at the early stage the broad spectrum symptoms of leptospirosis can be confused with other common bacterial infection leading to a progressive and severe infection (Hartskeerl *et al.*, 2011). Leptospirosis

usually response to various antibiotics if the treatment is given at the early stage of infection or the administration of antibiotics is in sufficient doses. Nevertheless, injudicious antibiotic use for the empirical treatment of infectious diseases can lead to widespread cross-resistance of the bacterial agent. In order to minimize this problem, medicinal plant including *Q. infectoria* can be used as an alternative for the treatment of leptospirosis as the previous studies reported on its broad spectrum anti-microbial activity.

1.3 Objectives of the study

1.3.1 General objective

- To study the antimicrobial activity of aqueous *Q. infectoria* gall extract against *L. interrogans* serovars.

1.3.2 Specific objectives

- To determine the minimum inhibition concentration (MIC) of aqueous *Q. infectoria* gall extract against *L. interrogans* serovars.
- To determine the minimum bactericidal concentration (MBC) of aqueous *Q. infectoria* gall extract against *L. interrogans* serovars.
- To analyse the morphological changes of *Leptospira* cells treated with the aqueous *Q. infectoria* gall extract using scanning electron microscope (SEM).

1.4 Flowchart of the study

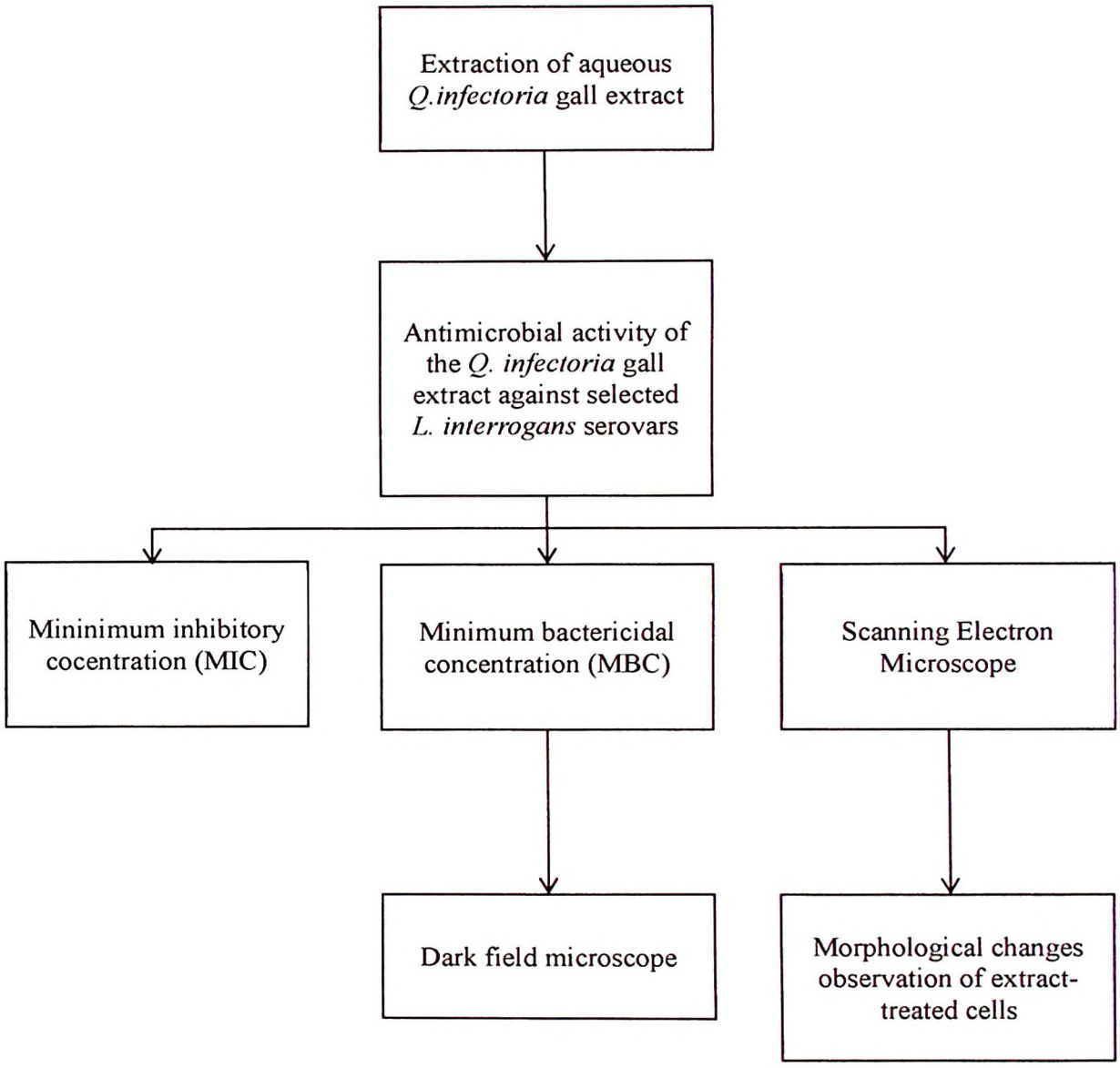

Figure 1.1: Flow chart of the study

CHAPTER 2

LITERATURE REVIEW

2.1 Leptospirosis

Leptospirosis is a bacterial zoonotic disease that human can be infected through the exposure or contact directly or indirectly with the animal especially rodent like rat and mice. Zoonotic disease is a disease that can be passed between animal to human which can be caused by microorganisms like viruses, bacteria, parasites and fungi. Leptospirosis is a result of the infection with spirochete bacterium namely *Leptospira* species especially the pathogenic *Leptospira* .This infections could result in morbidity and mortality of both human and also animals (Thayaparan *et al.*, 2013).

Leptospirosis had different alternate name at different regions. The alternate names of leptospirosis include Weil's syndrome, swamp fever, mud fever, autumn fever, swineherd's disease, rice-filed fever, hemorrhagic jaundice, Stuttgart disease, Conicula fever and redwater of calves (Spickler and Leedom Larson, 2013). Leptospirosis can also be considered as a seasoning infectious disease as the cases of leptospirosis commonly reported during the summer and fall season (Chadsuthi *et al.*, 2012). The disease occurs worldwide but most common in the temperate regions in the late summer and early fall and usually occur during rainy season in the tropical region (Tilahun *et al.*, 2013). Besides that, the cases also frequently reported after flooding and natural disasters (Hui Yi *et al.*, 2011).

2.1.1 Epidemiology of the disease

Leptospirosis outbreak accounting for thousands of deaths worldwide is considered as re-emerging disease but severely neglected infectious disease (Utzinger *et al.*, 2012). Leptospirosis was particularly endemic in tropical and subtropical regions with the incidences of the disease reported between 0.1 to 1 case and more than 10 cases per population per year in both regions respectively. The incidences of leptospirosis were only based on severe leptospirosis in which 70% to 90% of the cases required hospitalization (Hartskeerl *et al.*, 2011). There was also a study done on the annual incidences of leptospirosis worldwide, the researcher found that there were 28 countries from all over the world that has highest incidence of leptospirosis where Sri Lanka, Thailand and Philippines were also listed in that rank. The annual incidence in Sri Lanka, Thailand and Philippines were 54%, 48.9% and 4.8% per million populations respectively. Globally, the endemicity of leptospirosis was mainly located in the Caribbean and Central, South America, as well as in Southeast Asia and Oceania (Pappas *et al.*, 2008).

In Malaysia, the incidence of leptospirosis is not well documented previously as it is not included in the notifiable diseases list. Currently available leptospirosis data in Malaysia is based on the Report of Morbidity and Mortality for Ministry of Health Hospitals. The cases of leptospirosis in Malaysia increased yearly from 263 in 2004 to 5370 in 2015. The mortality rate of leptospiral infection also increased where in 2004 and 2005 the mortality were 20 cases but in 2006 and 2007 become increased to 22 deaths. In 2008 and 2009 the mortality rate of leptospirosis infection dramatically increased to 47 and 62 cases respectively. In 2014, the number of death increased to 92 cases (Zainudin, 2015). Human leptospirosis case in Malaysia was first discovered by

Fletcher in 1925 and many investigations on human leptospirosis cases in Malaysia disclosed a high prevalence of infection. These finding indicates that human leptospirosis was endemic in this country (El Jalii and Bahaman, 2004).

2.1.2 Pathogenesis

Leptospirosis can be transmitted to human through direct or indirect contact with urine infected animal such as rodent which is a major reservoir for a pathogenic *Leptospira* (Tilahun *et al.*, 2013). Besides that, *Leptospira* can also be transmitted through handling infected animal tissues and ingestion of contaminated food and water which also possible to cause leptospirosis in human (WHO, n.d). Humans are considered as accidental hosts as they will become infected with the *Leptospira* when they come into direct contact with urine of infected animals or through indirect contact with soil, water or plant that have been contaminated by urine of infected animals. The infection cause by *Leptospira* will begin when spirochete *Leptospira* entering the human body through cut or skin damage, mucous membrane or conjunctivae, aerosol inhalation of microscopic droplets and oral mucosa. The oral mucosa is more important route of entry after ingestion as compared to the intestinal tract (Levett and Haake, 2010). Once it passes into human body, the bacteria will enter blood stream to spread themselves throughout the human body which will result in bacterial infection that can lead to severe complication in the human body (Tilahun *et al.*, 2013). In the blood stream, *Leptospira* will last up to 7 days and this phase was known as bacteremic phase. In this phase, the number of *Leptospira* in blood and tissues will reach a critical level. During this phase, the endothelium of small blood vessels will be damaged leading to

localized ischemia in organs, renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis and placentitis. However, the specific action of *Leptospira* to produce the lesion is undefined. Moreover, there will be circulating antibodies in the blood stream and tissue to fight the infection by removing *Leptospira* through opsonophagocytosis (Adler and de la Peña Moctezuma, 2010). This phase known as immune phase that will occur during second week of infection in which antibodies production occur and *Leptospira* can be found in the urine of infected individual (Levett, 2001).

Leptospirosis also commonly happen in the animal as *Leptospira* can infect all mammals. Furthermore, there are many wild and domestic animals species have been identified as a host of infecting leptospiral organisms (Desvars *et al.*, 2013). In the animal, tissue damage can also recognised and severe damage of the tissue is reversible and completely repair although the damage is long lasting damage. The recovery of damage is recognised in the kidneys of pigs and dogs by forming scar and observe as "white spots" macroscopically (Adler and de la Peña Moctezuma, 2010).

2.1.3 Clinical Manifestation

Clinically, the syndrome of leptospirosis can be recognized in two form of clinical syndromes include anicteric and icteric leptospirosis. Anicteric is a self-limited and mild flu-like illness while icteric refers to severe illness characterized by the involvement of organ dysfunction or failure which is also known as Weil's disease (Gompf and Green-McKenzie, 2016). Leptospirosis is also known as biphasic illness which involves two distinct phases including a primary acute or septicemia phase and a

secondary immune phase (Hui Yi *et al.*, 2011). Both phases can be observed in mild form of leptospirosis or during anicteric leptospirosis. In icteric leptospirosis, these two phases of illness are common and often indistinguishable (Gompf and Green-McKenzie, 2016).

The anicteric leptospirosis can last for about a week. In this stage, the patients suffer from very mild symptoms and present with febrile illness of sudden onset. The fever can be biphasic and remission occurs after 3 to 4 days. The other signs and symptoms that can be recognized during this stage include chills, headache, myalgia, abdominal pain, conjunctiva suffusion and skin rash. However, the symptom of skin rash is less common and transient which last less than 24 hours. Patient with leptospirosis may present with severe headache, retro-orbital pain and photophobia (Levett , 2001).

Icteric leptospirosis contributes to high mortality rate as it is much more severe than the anicteric leptospirosis as it is a rapidly progressive form of leptospirosis. Icteric leptospirosis usually could lead to hepatic dysfunction, renal insufficiency, haemorrhage and multi-organ failure (MOF). Haemorrhage appears as petechiae, purpura, conjunctival haemorrhage and gastrointestinal haemorrhage. The infection with *L.* icterohaemorrhagiae commonly could lead to icteric leptospirosis (Weil's syndrome) (Dutta and Christopher, 2005).

2.1.4 Laboratory diagnosis of leptospirosis

Diagnosis of leptospirosis in the laboratory was classified into direct evidence and indirect evidence detections of *Leptospira*. Direct evidence involves the isolation of organism or demonstration of *Leptospira* under dark field microscope or amplification of specific fragment of leptospiral DNA. Indirect evidence refers to the detection of antibodies to *Leptospira*. Furthermore, the methods of detection of *Leptospira* in laboratory were categorized into bacteriological, microscopic, serological and molecular (Vijayachari *et al.*, 2008). Usually, leptospirosis was diagnosed in laboratory through serological technique based on detection of antibodies against *Leptospira* organism in the samples like blood, urine or tissue (WHO, n.d.). Antibodies of *Leptospira* appear within few days of onset of illness and persist for weeks or months or even years in some cases.

Microscopic agglutination (MAT) was a gold standard serological test with high specificity toward the detections of antibodies. MAT involve the use of a paired of sera collected during acute and convalescent phases of the disease to established rises in antibody titres (Vijayachari *et al.*, 2008). The sensitivity antibody detection of by MAT during first week of illness was 41% and rises to 82% during second to fourth week and beyond the fourth week of illness, 96% was detected (Seghal *et al.*, 1999; Vijayachari *et al.*, 2008). MAT was considered as an alternative confirmatory diagnosis for leptospirosis besides the isolation of the organisms from the clinical samples (Vijayachari *et al.*, 2008). Other antibody detection methods include macroagglutination, latex agglutination, lateral flow assays and IgM dipstick (Adler *et al.*, 2010).

2.1.5 Treatment of leptospirosis

Commonly used antibiotic like penicillin and doxycycline could be beneficial in the treatment of mild leptospirosis (Hospenthal and Murray, 2003). In severe leptospirosis, hospitalization is required and the antibiotics would be injected directly into the blood stream (Nhs.uk, 2014). The recommended treatment for leptospirosis was doxycycline at dose of 5 mg/kg through intravenous (IV) administration twice daily for 14 days which is thought to clear the bacteria from the blood stream and also from the kidney (Cordner, 2012). Ampicillin and amoxicillin are also recommended in mild disease, whereas penicillin G and ampicillin are indicated for severe disease (Bharti *et al.*, 2003).

The susceptibility of antibiotics include ampicillin, doxycycline, and ofloxacin with different dose of each drug include 10 mg/kg, 100 mg/kg and 30 mg/kg respectively was assessed against *L.interrogans* serovar Icterohaemorrhagiae strain *Verdun* in a Syrian hamster model. From the finding, doxycycline was able to clear the *Leptospira* from blood and all tissues in 2 days, except for liver, which required 3 days. Ampicillin cleared *Leptospira* from the host, except kidneys and heart, which still had 102 leptospires/g at day 6 and ofloxacin was unable to clear bacteria from blood or kidneys (Truccolo *et al.*, 2002; Bharti *et al.*, 2003).

2.2 Microorganism

2.2.1 Cell biology of *Leptospira*

The genus *Leptospira* belongs to the phylum *Spirochetes* and includes both pathogenic and saprophytic species (Paster *et al.*, 1991; Raddi *et al.*, 2012). *Leptospira* are obligate aerobic spirochetes that share the features of both gram-negative and Gram-positive bacteria as shown in Figure 2.1. The double-membrane (inner membrane and outer membrane) and the presence of lipopolysaccharide (LPS) are characteristic of Gram-negative bacteria, while the close association of the cytoplasmic membrane with murein cell wall is reminiscent of Gram-positive envelope architecture (Evangelista and Coburn, 2010). Besides that, the attachment of peptidoglycan to the inner membrane also resembles to the nature of gram positive bacteria (Vijayachari *et al.*, 2008). LPS and integral membrane proteins such as porin OmpL1 and type two secretion system (T2SS) secretin GspD located in the outer membrane constitute to be antigenic for *Leptospira* (Adler and de la Peña Moctezuma, 2010).

Leptospira are thin, helically coiled and motile spirochetes usually 6 to 20 μm in length. The helical amplitude is approximately 0.1 to 0.15 μm and the wavelength is about 0.5 μm (Levett, 2001). Motility of *Leptospira* is provided by the periplasmic flagella (PF) that are located between inner membrane (IM) and outer membrane (OM) as shown in the Figure 2.2. The short extend of PF from each pole in *Leptospira* result in the characteristic hook-shaped formation at the end of the cells (Goldstein and Charon, 1988; Raddi *et al.*, 2012). PF in *Leptospira* is attached at each end of protoplasmic cell cylinder. They reside inside the cell within periplasmic space which differ from other bacteria and the researchers believe that motility is likely to play an important role in the disease process (Li *et al.*, 2000).

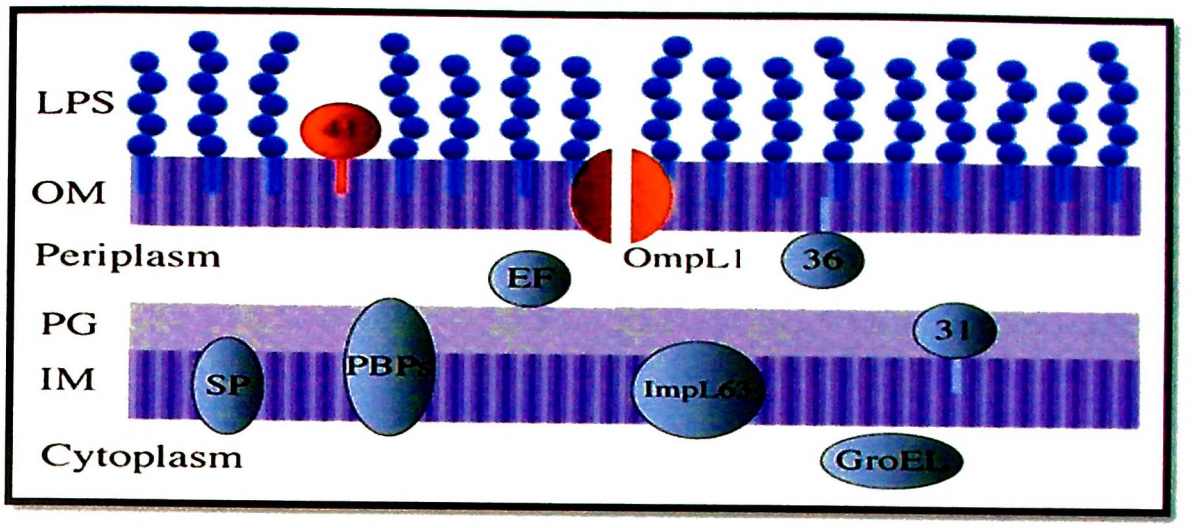

Figure 2.1: Membrane protein of *Leptospira* [adapted from UCLA/VA Leptospirosis Research Laboratory, (2002)]. LPS: lipopolysaccharide; OM:outer membrane; PG: Peptidoglycan; IM: inner membrane; SP: single peptidase PBP: penicillin binding proteins; EF: endoflagella; OmpL1: outer membrane protein L1; ImpL63: inner membrane protein L63; GroEL: heat-shock protein

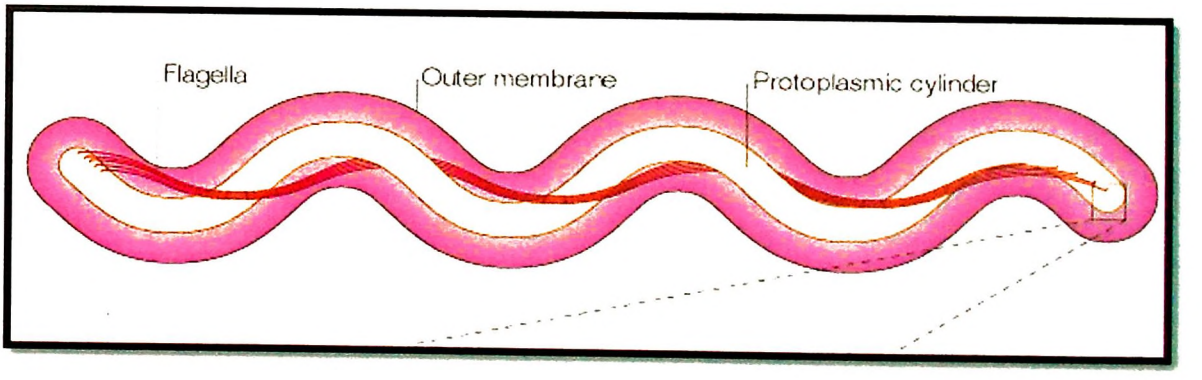

Figure 2.2.: Endoflagella (periplasmic flagella) of spirochetes bacteria [adapted from Rosa *et al.* (2005)]

2.2.2 Classification of *Leptospira*

Serovar is the basic unit of *Leptospira* taxonomy and consists of closely related isolates based on serological reactions to the lipopolysaccharide of *Leptospira* (Spickler and Leedom Larson, 2013). Each serovar has a characteristic antigenic make-up and serovars that have antigenic similarities are formed into serogroups. Nowadays, over 200 pathogenic serovars which are divided into 25 serogroups. For non-pathogenic serovars, at least 50 serovars have been identified (Spickler and Leedom Larson, 2013).

There are two different classifications of *Leptospira* which include serological method and genetic technique (Levett, 2001). In the serological method, the organisms are divided into serogroups while the genetic technique classifies the organism into species. In the serological classification, the genus of *Leptospira* is divided into *L. interrogans*, which comprise of all pathogenic strains and *L. biflexa*, the environmental saprophytic strain (Thayaparan *et al.*, 2013).

In genetic technique, *Leptospira* are divided into 21 species and the species that have been detected in the clinical cases include *L. interrogans, L. borgpetersenii. L. alexanderi, L. alstonii, L. inadai, L. fainei, L. kirschneri, L. licerasiae, L. noguchi, L. santarosai, L. terpstrae, L. weilii* and *L. wolffii* (Spickler and Leedom Larson, 2013). In addition, the genus of *Leptospira* had been recognised recently which consist of 20 species including 9 pathogenic, 5 intermediate and 6 saprophytic species. There are three major species of pathogenic *Leptospira* with a global distribution include *L. interrogans, L. borgpetersenii,* and *L. kirschner* (Spickler and Leedom Larson, 2013).

In traditional classification through serological method, all pathogenic *Leptospira* are grouped under *L. interrogans* serovars (Plank and Dean, 2000;

Evangelista and Coburn, 2010). However, there were seven *Leptospira* species that had been discovered to be the main agent for leptospirosis includes *L. interrogans, L. borgpetersenii, L. santarosai, L. noguchii, L. weilli, L.kirschneri* and *L. alexanderi.* These identification of seven species was identified through genotypic method (Ahmed *et al.*, 2006; Evangelista and Coburn, 2010).

L. interrogans is a pathogenic *Leptospira* and it is known as primary agent to cause leptospirosis (Raddi *et al.*, 2012). Pathogenic *Leptospira* live in the proximal renal tubules of the kidneys of reservoir host like rodent, although other tissues and organs may also serve as a source of infection (Adler and de la Peña Moctezuma, 2010). Besides that, *L. interrogans* can also survive in low-nutrient environments, such as moist soil and fresh water for long periods, with salt concentration, pH and viscosity as a critical factors (Trueba *et al.*, 2004; Evangelista and Coburn, 2010).

L. interrogans generally shares the similar cell features with other spirochetes (Raddi *et al.*, 2012). However, recent study on genomic structure of *L. interrogans* and *L. biflexa* had discovered the unique characteristics in *L. interrogans* that might be potentially associated with virulence and pathogenesis of *L. interrogans* (Nascimento *et al.*, 2004; Picardeau *et al.*, 2008; Raddi *et al.*, 2012). The study represents data on ultrastructure of *L. interrogans* and *L. biflexa*. From the finding, a major structural difference between *L. interrogans* and *L. biflexa* was the third layer of the OM, which appears more pronounced in *L. interrogans* than in *L. biflexa* (Raddi *et al.*, 2012). Furthermore, LPS appears more abundant and longer (9.2 nm) in *L. interrogans* than that in *L. biflexa* which supports the finding of the model that LPS was highly variable on cell surfaces of pathogenic and saprophytic species and plays an essential role in *Leptospiral* virulence (Nahori *et al.*, 2005; Raddi *et al.*, 2012).

Furthermore, most of severe leptospirosis in human being principally caused by *L. interrogans* serovar Icterohaemorrhagiae (Merien *et al.*, 2000). This is because the primary reservoir host for *Leptospira* was rodent like rat and it was the typical reservoir for *L. interrogans* serovar Icterohaemorrhagiae (Bharti *et al.*, 2003). In Malaysia, the recent study revealed two predominant pathogenic *Leptospira* serovars from two species that include *L. borgepetersenii* serovars Javanica and *L. interrogans* serovars Bataviae being isolated from rats in Kuala Lumpur (Benacer *et al.*, 2016).

2.2.3 Media for cultivation of *Leptospira* sp.

Leptospira are slow growing organisms having a generation time of approximately 24 hours at 30°C and the requirement for a rich medium at neutral pH predisposes the cultivation of those microorganisms sometimes troublesome, particularly from natural sources. All *Leptospira* spp. prefer the narrow pH window of 7.2 to 7.6, and usually fail to proliferate in acidic condition (Miraglia *et al.*, 2009).

The nutritional requirements of *Leptospira* are unique although simple. Vitamin B1, B 12 and long-chain fatty acids are the only organic compounds that are known to be required. Fatty acids are their source of energy and carbon. It is also required as a source of cellular lipids since *Leptospira* cannot synthesize fatty acids from de novo reaction. Furthermore, the non-essential nutrient such as pyruvate, enhances the initiation of growth of the pathogenic *Leptospira* (Cameron, 2015).

A variety of media can be used for the cultivation of *Leptospira* in which Ellinghause, McCullough, Johnson and Harris (EMJH) medium is the most commonly used. EMJH medium composed of ammonium chloride, a nitrogen source, and

thiamine, a growth factor. The pH of this media is buffered by sodium phosphate and potassium phosphate while sodium chloride maintains the osmotic balance of this medium. In modified EMJH medium, polysorbate 80-albumin *Leptospira* enrichment is used in replacing rabbit serum that commonly used in traditional culture medium of *Leptospira*. Besides that, the addition of 5-fluorouracil is lethal to various microorganisms, but not to *Leptospira*, in concentrations of 200-400 μg/mL. This substance is widely used as an additional medium to obtain pure primary culture of isolated *Leptospira* (Miraglia *et al.*, 2009).

EMJH medium can be prepared in three forms including solid, semi-solid and liquid. Liquid media is preferable in the standard leptospiral culture. Maximum inoculum concentrations are smaller than for other bacteria, and often reach only 10^7/mL or 10^8/mL where the medium will display an obvious turbidity. In the semi solid media which composed of 1:1000 ratio of agar, the bacteria tend to appear in cluster around particles of the agar. In the solid media with ratio of 1:100 agar, the bacteria grow under the agar surface and the growth is extremely slow which take several weeks to be observed using naked eyes. Thus, liquid or broth EMJH medium is preferred for the growth of *Leptospira* sp. as the bacteria can be freely motile and the growth of *Leptospira* can be observed within a week (WordPress, n.d).

2.3 Microscopic examination of *Leptospira* sp.

2.3.1 Dark field microscopy

Dark field microscopic technique is one of the direct detection methods to confirm the growth of *Leptospira* in the culture media. The technique involves the

visualization of unstained *Leptospira* by enhancing the contrast of the microscope. In dark field microscopy (DFM), the entire field of view appears dark when there is no sample on the microscope stage. However, when a sample is placed on the stage it appears bright against a dark background (Microscopeworld.com, 2016). It is one of the methods that can be used in laboratory for the diagnosis of leptospirosis in the biological sample like blood and urine. DFM has low sensitivity as compared to other methods of leptospirosis diagnosis. Approximately 10^2 to 10^6 leptospires per mL of blood is necessary for visualization by dark field microscope which results in low sensitivity compared to other diagnostic methods. The sensitivity of DFM was shown to be 61% and 93.3% and the specificity was 60% which slightly lower than ELISA method (79%) and other technique available. Besides that, DFM observation can be misinterpreted with the fibrin or other protein threads which result in false positive result. Therefore, DFM method is not recommended for laboratory diagnosis of leptospirosis (Yaakob *et al.*, 2015).

Nevertheless, most of the studies on antimicrobial activity of antimicrobial agents like antibiotic or medicinal plant against *Leptospira* use DFM to observe the inhibition of *Leptsopira* determined based on the motility and the concentration of the organisms (Nelson *et al.*, 2013; Prabhu *et al,* 2014). In addition, various forms of *Leptospira* can be visualized under DFM, such as hooked, straight, spiral, coccal and other forms (Sharma and Kalawat, 2008).

2.3.2 Scanning electron microscopy

Scanning electron microscope (SEM) reveals the three dimensional structure of the specimen which provide information on surface topography of the specimen (Vernon-Parry, 2000). SEM uses a focused beam of high-energy electrons to generate a variety of signals at the surface of solid specimens. The signals that derive from electron-sample interactions reveal information about the sample including external morphology, chemical composition, and crystalline structure and orientation of materials making up the sample (Susan, 2016).

In microbiological field, SEM is a valuable tool that can help in the analysis of the morphological structure of the microorganisms and the understanding their physical characteristics associated with the pathogenesis of the microorganisms towards the particular disease. Previously, there were several studies carried out to observe the morphology of *Leptospira* under SEM including the size of *Leptospira* (Voronina *et al.*, 2014), the spiral direction (Yoshii, 1978), hook formation and so on. A study on the morphology of a *Leptospira* strain demonstrated that the cells were corkscrew-shaped with end hooks with sizes of 0.12 μm in diameter and from 9.44 to 10.14 μm in length (Voronina *et al.*, 2014).

However, the report on the morphological study of treated *Leptospira* with antimicrobial agents observed under SEM is still lacking and not well understood. But, there was a study reported on the effect of UV-A radiation on *L. interrogans* serovar Canicola observed under SEM showing the elongation and deformed in the structure (Chadsuthi *et al.*, 2010).

2.4 *Quercus infectoria* plant

Q. infectoria plants are from the family of Fagaceae and commonly known as gall oak which can be found in Greece, Asia Minor and Iran. Physically, the plant is a small tree or shrub growing up to 4 to 6 feet tall, crooked, with smooth and bright leaves as shown in Figure 2.3. *Q. infectoria* grows well in the partial shade or partial sun to full sun and requires moist soil. However, the plant also can grow in semi-shade or no shade with moist soil (Shrestha *et al.*, 2014).

Q. infectoria plants possess monoecious flower that pollinated by wind. Besides that, there is also gall which arising on branches of the tree. In local India, the gall of *Q. infectoria* is called as " *majupal*" in Sanskrit, "*machakai*" in Kannada, India (Shrestha *et al.*, 2014) and "*manjakani*" in Malaysia. Macroscopically, the galls of *Q. infectoria* are globule in shape with numerous horny appearances on its external surface which give rough touch. The external color of the gall is greyish-brown to brownish-black in color and dark brown buff colored as shown in Figure 2.4. It has unpleasant odor and the size is about 1.4 to 2.3 cm in length and 1 to 1.5 cm in diameter (Shrestha *et al.*, 2014).

Figure 2.3: The tree of *Q. infectoria* [adapted from toxicology centre.com, (n.d.)]

Figure 2.4: The gall of *Q. infectoria* [adapted from Dehlvi Naturals (n.d.)]

2.4.1 Extraction of the plant

Plant extraction is the method that commonly used to isolate or extract the desired chemical compounds from the plant materials for further separation and characterization. It is considered as the crucial steps in the analysis of medicinal plant. The basic operation steps of plant extraction include, pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and also increasing the contact of sample surface with the solvent system (Sasidharan *et al.*, 2011). The extraction steps should be carefully and properly carried out in order to ensure that the potential constituent are not lost during the process.

There are various techniques can be used to extract the medicinal plant such as sonification, heating under reflux, soxhlet extraction, and maceration or percolation. However, these extraction techniques has become the traditional technique of plant extraction as there are several modern technique of plant extraction that had been developed include solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages. The advantages of the modern medicinal plant extraction include elimination of additional sample clean-up and also improve the extraction efficiency, selectivity, and kinetics of the extraction product (Huie, 2002).

Maceration method was the simplest method of crude extraction with the basic principle of grinding the plant materials into small or finer dry or wet materials. This principle will increase the surface area of the plant materials for the extraction process