



Faculty of Resource Science and Technology

**A Study of Susceptibilities of *Leptospira* Strain Towards Antibiotics and
Indigenous Herbs**

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A Study of Susceptibilities of *Leptospira* Strain Towards Antibiotics and
Indigenous Herbs

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

Leptospirosis is a zoonosis caused by pathogenic *Leptospira* spp.. As for leptospirosis cases has increased over the last decade, thus it's critical to assess the effectiveness of popular antibiotics and medicinal herbs in controlling this disease. Nonetheless, there is minimal evidence on antimicrobial susceptibility of *Leptospira* isolates and medicinal herbs against Leptospirosis in Malaysia. Hence, the objective of this study is to determine antibiotic susceptibility toward *Leptospira* spp. in Sarawak. In addition, this study investigates the antimicrobial activity of *H. formicarum* Jack and *B. stenophylla* against *Leptospira* spp. Then to investigate chemical components containing inside indigenous herbs using Column Chromatography and Gas Chromatography-Mass Spectroscopy (GC-MS) methods. Two herbs in Sarawak that are currently tested are *Hydnophytum formicarum* Jack (family of *Rubiaceae*), and *Boesenbergia stenophylla* (family of *Zingiberaceae*). Twelve isolated local *Leptospira* strains tested were *L. noguchii* strain LT796, and *L. Borgpetersenii* serovar mini (pathogenic *Leptospira*); and *L. wolffii* serovar Khorat strain Khorat-H2, and *L. inadai* serovar Aguaruna strain MW4 (saprophytic *Leptospira* spp.) isolated from the environment were tested against seven antibiotics (penicillin G, ampicillin, amoxicillin, doxycycline, cefotaxime, chloramphenicol and streptomycin) and crude extract of *B. stenophylla* and *H. formicarum* Jack from four different solvents (methanol, ethyl acetate, hexane and chloroform) by using the broth microdilution method. Broth Microdilution method is performed in this study to determine the minimum inhibitory concentrations (MICs) for antimicrobial susceptibilities testing of these selected antibiotics and indigenous herbs against isolated *Leptospira* spp.. As a result, all *Leptospira* spp. showed more susceptible toward cefotaxime and erythromycin with MIC₉₀ 0.2 µg/mL, as compared to amoxicillin and ampicillin with MIC 0.39 µg/mL. Meanwhile, doxycycline exhibited the

highest MIC₉₀ value of 5.938 µg/mL. In the other hand, the present study showed the lowest MIC (125 µg/mL) for all crude extracts has been shown to have higher than the standard antibiotic. However, all crude extract of *B. stenophylla* and *H. formicarum* demonstrated good antimicrobial activity against *Leptospira* spp.. Ethyl acetate is the effective extractant for both *H. formicarum* Jack and *B. stenophylla* extractant against both pathogenic and intermediate *Leptospira* spp. Tuber extract of *H. formicarum* Jack showed better antimicrobial activity against both pathogenic and intermediate *Leptospira* spp.. with lowest MIC values of 125 µg/mL and 62.5 µg/mL. In the other hand, the leave extract of *B. stenophylla* using ethyl acetate of indicate better inhibition against pathogenic *Leptospira* spp.. compare to rhizome extract with the lowest MIC value of 62.5 µg/mL. Besides, this study deals with the identification of phytochemical constituents from *B. stenophylla* and *H. formicarum* Jack by GC-MS analysis. GC-MS confirmed the occurrence of a total of 37 and 39 phytochemical constituents in the different solvent extract of *B. stenophylla* and *H. formicarum* Jack, respectively. The results of the study could be utilised as a future reference for the selection of antibiotics and assist the medical practitioner in the treatment of leptospirosis.

Keywords: *Leptospira* spp., broth microdilution, antibiotics, *Boesenbergia stenophylla*, *Hydnophytum formicarum* Jack

Kajian tentang Kerentanan Strain *Leptospira* terhadap Antibiotik dan Herba Asli

ABSTRAK

Leptospirosis adalah zoonosis yang disebabkan oleh patogenik *Leptospira* spp. Bagi kes *leptospirosis* telah meningkat sejak sedekad yang lalu, maka adalah penting untuk menilai keberkesanan antibiotik dan herba ubatan yang popular dalam mengawal penyakit ini. Namun begitu, terdapat bukti minima tentang kerentanan antimikrob terhadap pencilan *Leptospira* dan herba ubatan terhadap *Leptospirosis* di Malaysia. Oleh itu, objektif kajian ini adalah untuk menentukan kerentanan antibiotik terhadap *Leptospira* spp. di Sarawak. Selain itu, kajian ini menyiasat aktiviti antimikrob *H. formicarum* Jack dan *B. stenophylla* terhadap *Leptospira* spp. Kemudian untuk menyiasat komponen kimia yang mengandungi dalam herba asli menggunakan kaedah Column Chromatography dan Gas Chromatography-Mass Spectroscopy (GC-MS). Dua herba di Sarawak yang sedang diuji ialah *Hydnophytum formicarum* Jack (keluarga Rubiaceae), dan *Boesenbergia stenophylla* (keluarga Zingiberaceae). Dua belas strain *Leptospira* tempatan terpencil yang diuji ialah strain *L. noguchii* LT796, dan *L. Borgpetersenii* serovar mini (*Leptospira* patogenik); dan *L. wolffii* serovar Khorat strain Khorat-H2, dan *L. inadai* serovar Aguaruna strain MW4 (saprophytic *Leptospira* spp.) yang diasingkan daripada alam sekitar telah diuji terhadap tujuh antibiotik (penicillin G, ampicillin, amoxicillin, doxycycline, cefotaxime, chloramphenicol) dan streptomycin dan ekstrak mentah *B. stenophylla* dan *H. formicarum* Jack daripada empat pelarut berbeza (metanol, etil asetat, heksana dan kloroform) dengan menggunakan kaedah pencairan mikro sup. Kaedah Microdilution Broth dilakukan dalam kajian ini untuk menentukan kepekatan perencatan minimum (MIC) untuk ujian kerentanan antimikrob terhadap antibiotik terpilih dan herba asli terhadap *Leptospira* spp terpencil. Akibatnya, semua *Leptospira* spp. menunjukkan lebih mudah terdedah kepada cefotaxime dan erythromycin dengan MIC₉₀ 0.2 µg/mL, berbanding dengan amoksisilin dan ampisilin

dengan MIC 0.39 $\mu\text{g/mL}$. Sementara itu, doxycycline mempamerkan nilai MIC₉₀ tertinggi iaitu 5.938 $\mu\text{g/mL}$. Sebaliknya, kajian ini menunjukkan MIC terendah (125 $\mu\text{g/mL}$) untuk semua ekstrak mentah telah terbukti mempunyai lebih tinggi daripada antibiotik standard. Walau bagaimanapun, semua ekstrak mentah *B. stenophylla* dan *H. formicarum* menunjukkan aktiviti antimikrob yang baik terhadap *Leptospira* spp. Etil asetat adalah pengekstrak berkesan untuk kedua-dua pengekstrak *H. formicarum* Jack dan *B. stenophylla* terhadap kedua-dua patogenik dan perantaraan *Leptospira* spp. Ekstrak ubi *H. formicarum* Jack menunjukkan aktiviti antimikrob yang lebih baik terhadap kedua-dua patogenik dan perantaraan *Leptospira* spp.. dengan nilai MIC terendah iaitu 125 $\mu\text{g/mL}$ dan 62.5 $\mu\text{g/mL}$. Sebaliknya, ekstrak daun *B. stenophylla* menggunakan etil asetat menunjukkan perencatan yang lebih baik terhadap patogen *Leptospira* spp.. berbanding dengan ekstrak rizom dengan nilai MIC terendah iaitu 62.5 $\mu\text{g/mL}$. Selain itu, kajian ini berkaitan dengan pengenalpastian juzuk fitokimia daripada *B. stenophylla* dan *H. formicarum* Jack melalui analisis GC-MS. GC-MS mengesahkan kejadian sejumlah 37 dan 39 juzuk fitokimia dalam ekstrak pelarut berbeza *B. stenophylla* dan *H. formicarum* Jack, masing-masing. Hasil kajian boleh digunakan sebagai rujukan masa depan untuk pemilihan antibiotik dan membantu pengamal perubatan dalam rawatan leptospirosis.

Kata kunci: *Leptospira*, pencairan mikro sup, antibiotik, *Boesenbergia stenophylla*, *Hydnophytum formicarum* Jack

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LIST OF ABBREVIATIONS

<i>B. stenophylla</i>	Boesenbergia stenophylla
CAAT	Cross-Agglutinin Absorption Test
GC-MS	Gas Chromatography-Mass Spectroscopy
CFU/mL	Colonies Forming Unit per Milliliter
EMJH	Ellinghausen-McCullough-Johnson-Harris
<i>H. formicarum</i> Jack	Hydnophytum formicarum Jack
<i>L.</i>	<i>Leptospira</i>
µg/ml	Microgram per Millilitre
MIC	Minimal Inhibitory Concentration
MOH	Ministry of Health
WHO	World Health Organisation
g	Gram
spp	Species

CHAPTER 1

INTRODUCTION

1.1 Study Background

Leptospirosis is an emerging zoonotic disease caused by pathogenic spirochetes of the genus *Leptospira* with global distribution (Bharti et al., 2003; Haake & Levett, 2015). The WHO estimates the global burden of leptospirosis at approximately one million serious human cases each year, with more nations reporting outbreaks (Hartskeerl et al., 2011). Earlier studies have recorded that the disorder is accountable for nearly one million cases and almost sixty thousand deaths annually (Costa et al., 2016). Leptospirosis has been acknowledged as an environmental infection before its etiological agent could be recognized (Tarantola, & Goarant, 2018). This disease affects communities in rural and urban areas living in tropical and temperate climates. Many regions have recorded leptospirosis outbreaks, however human infection is concentrated in tropical and subtropical areas, with Southeast Asia having the highest prevalence (Hinjoy, 2016).

Leptospirosis is a globally important bacterial zoonosis caused by infection with the pathogenic species of *Leptospira*: helical motile spirochetes of the leptospiraceae family. *Leptospira* spp. have categorized as human-infectious and non-infectious environmental saprophytes. Infectious *Leptospira* spp. then further classified as pathogenic and intermediate pathogens based on biological characteristics and molecular phylogeny. More than two hundred and sixty serovars from twenty-four serogroups and about seventeen species belonged to Leptospires. Nevertheless, with members causing lethal human disease, the mechanisms underlying the pathogenesis remain unclear. Infection occurs when pathogenic *Leptospira* spp. is transmitted directly through abraded skin or mucosal membranes, or

indirectly through polluted water bodies or the surroundings. Mostly asymptomatic or briefly present as a flu-like febrile disease, this bacterial infection makes clinical diagnosis difficult. Serious illness such as Weil's disease can be encountered by sufferers, such as bleeding, jaundice, and acute renal failure, and perhaps even serious pulmonary haemorrhage (Haake & Levett, 2015). Rodents are the main sources of contamination, however harmful germs can be carried by many domestic and wild animals (Lau et al., 2010). Despite its obvious impact on human and animal health, this disease is one of the most neglected tropical diseases (Dupouey et al., 2014). Infectious disorders like bacterial and viral hepatitis, influenza, and malaria often mirror leptospirosis, leading to under-reporting and misdiagnosis (WHO, 2011).

Leptospirosis is endemic in Malaysia and has been declared a notifiable illness since December 2010 (Ministry of Health Malaysia, 2011). The illness has continued to be a burden on the Malaysian economy and citizens' lives due to poor livestock productivity, employment losses due to reduced tourism to selected hotspots, and prevention and control costs (Garba et al., 2018). Cases have increased in the last decade, with epidemics linked to recreation and natural calamities (Sapian et al., 2012).

1.2 Problem Statement

Leptospirosis is an emerging zoonotic disease with worldwide distribution caused by pathogenic spirochetes of the genus *Leptospira* (Bharti et al., 2003). Leptospirosis has long been recognized as an environmental infection, before its etiological agent could be identified (Tarantola & Goarant, 2018). This disease affects peoples living in temperate and tropical climates in rural and urban areas. Earlier studies have estimated that the disease is responsible for nearly 1 million cases and almost 60,000 deaths annually (Costa et al., 2015).

This bacterial infection is mostly asymptomatic or briefly present as a flu-like febrile illness, rendering clinical diagnosis difficult. Sufferers may experience serious illnesses such as Weil's disease (jaundice, bleeding and acute renal failure) and maybe even serious pulmonary haemorrhage (Haake & Levett, 2015). However, the clinical presentation of leptospirosis is not clear and sometimes leads to misdiagnosis (Bruce et al., 2005). Misdiagnosis of this infection has become a serious problem in Malaysia whereby dengue, malaria and other infectious diseases with overlapping clinical presentations are endemic. Due to the endemicity of leptospirosis in Malaysia (Benacer et al., 2013) and the significant increase in cases reported over the last decade, it is important to evaluate the effectiveness of common antibiotics and medicinal plants in controlling this species. Nevertheless, there is a few information available on the antimicrobial susceptibility of Malaysian *Leptospira* isolates. In Malaysia, the clinical manifestations of leptospirosis, however, it is not clear and often leads to misdiagnosis (Bruce et al., 2005) and become a significant problem other infectious disease including dengue and malaria are prevalent with overlying clinical symptoms. According to Sarawak Epid News, leptospirosis cases increased from 49 in 2010 to 616 in 2014, including 53 deaths (Sarawak Health Department, 2012). Due to the significant rise in reported cases over the past decade and the endemicity of leptospirosis in Malaysia (Benacer et al., 2013), it is critical to evaluate the efficiency of common antibiotics in monitoring this species. Additionally, there are limited studies in Malaysia on antibiotic susceptibility testing for *Leptospira* locally isolated in Sarawak. Hence, the primary objective of this research is to monitor and analyze the susceptibility of recent local *Leptospira* isolates from Sarawak towards conventional antimicrobial compounds and indigenous herbs for the treatment of leptospirosis.

1.3 Research Objectives

The objectives of this research were:

- i. To determine antibiotic susceptibility toward *Leptospira* spp. in Sarawak by using broth microdilution techniques.
- ii. To investigate the antimicrobial activity of *H. formicarum* Jack and *B. stenophylla* against local *Leptospira* spp. in Sarawak by using broth microdilution techniques.
- iii. To determine the chemical compound in *H. formicarum* Jack and *B. stenophylla* by using Gas Chromatography-Mass Spectroscopy.

CHAPTER 2

LITERATURE REVIEW

2.1 *Leptospira* spp.

Leptospira is a spiral-shaped Gram-negative spirochete that is approximately 0.1 μm in diameter and 6–20 μm in length and belong to the family *Leptospiraceae* and order Spirochaetales (Johnson, 1996; Faine et al., 1999). *Leptospira* features unique hooked ends and two periplasmic flagella that are responsible for locomotion (Levett, 2001; Adler & de la Peña Moctezuma, 2010; Mohammed et al., 2011). The ideal temperature for *Leptospira* growth is between 28 and 30°C (Evangelista & Coburn, 2011). *Leptospira* are obligate aerobic bacteria that thrive best at a temperature of 28-30 °C in laboratory circumstances.

Leptospire are categorised into three classes based on their pathogenicity: pathogenic, intermediate, and saprophytic. Pathogenic *Leptospira* can infect practically any mammal, that leading to leptospirosis. Intermediates can cause infections but have reduced virulence, and saprophytes are typically found in freshwater environments (Goarant, 2016). Pathogenic strains double in about 6-8 hours and reach maximum growth in around 4-7 days. Meanwhile, non-pathogenic leptospire have a doubling time of 3.5 - 4.5 hours and reach maximum growth after a doubling time of 3.5 - 4.5 hours within 2-3 days (Faine et al., 1994).

Adler and de la Peña Moctezuma (2010) reported thirteen pathogenic *Leptospira* species that can cause the disease in animals and humans, which is known as leptospirosis. There are around 260 serovars of pathogenic *Leptospira* species, including *L. alexanderi*, *L. alstonii*, *L. borgpetersenii*, *L. inadai*, *L. interrogans*, *L. fainei*, *L. kirschneri*, *L. licerasiae*, *L. noguchi*, *L. santarosai*, *L. terpstrae*, *L. weilii*, *L. wolffii*, and more.

Meanwhile, saprophytic *Leptospira* spp., which include *L. biflexa*, *L. meyeri*, *L. yanagawae*, *Leptospira kmetyi*, *L. vanthielii* and *L. wolbachii*, and more than 60 serovars (Adler & de la Peña Moctezuma, 2010), are bacterium that are not harmful and do not cause disease (Picardeau *et al.*, 2008).

Intermediate strains of *Leptospira* spp. have an unknown pathogenicity, causing mild to severe sickness in infected people (Perolat *et al.*, 1998). According to Chiriboga and colleagues (2015), the average proportion of intermediate strains identified in the human body is 96 percent, whereas pathogenic strains account for only 4 percent. As a result, this challenges the conventional public health assumption and knowledge that pathogenic strains of *Leptospira* are the only clusters that cause leptospirosis. As a result of this study, it is probable that intermediate *Leptospira* can also cause leptospirosis. Table 1.1 lists the *Leptospira* species that are pathogenic, intermediate, and saprophytic.

Table 1.1: List of *Leptospira* species (Adler & de la Peña Moctezuma, 2010; Picardeau, 2017).

Pathogenic <i>Leptospira</i>	Intermediate <i>Leptospira</i>	Saprophytic <i>Leptospira</i>
<i>Leptospira alexanderi</i>	<i>Leptospira fainei</i>	<i>Leptospira biflexa</i>
<i>Leptospira alstonii</i>	<i>Leptospira broomii</i>	<i>Leptospira idonii</i>
<i>Leptospira borgpetersenii</i> ,	<i>Leptospira licerasiae</i>	<i>Leptospira meyeri</i>
<i>Leptospira inadai</i>	<i>Leptospira wolffii</i>	<i>Leptospira wolbachii</i>
<i>Leptospira interrogans</i>		<i>Leptospira vanthielii</i>
<i>Leptospira fainei</i>		<i>Leptospira terpstrae</i>
<i>Leptospira kirschneri</i>		<i>Leptospira yanagawae</i>
<i>Leptospira licerasiae</i>		
<i>Leptospira Noguchi</i>		
<i>Leptospira santarosai</i> ,		
<i>Leptospira terpstrae</i> ,		
<i>Leptospira weilii</i>		
<i>Leptospira wolffii</i> ,		

The survival and permanence of *Leptospira* in the environment are determined by the interaction of several factors, including abiotic soil and water conditions, as they can survive in soil with a moisture content greater than 20%, water and soil with pHs ranging

5.5 to 7.6, and temperatures ranging from 4 to 40 °C (Barragan et al., 2017). As a result, they may thrive in alkaline soil, muck, marshes, streams, rivers, and tissues and organs of both living and dead animals (Mohammed et al., 2011). Saprophytic species grow at temperatures ranging from 5 to 10 °C (Mohammed et al., 2010), are more common in the aquatic environment, and are native to freshwater surfaces than pathogenic *Leptospira*.

Leptospirosis is caused by pathogenic *Leptospira*. This bacterium is commonly found in the kidneys of wild mammals such as rats, swine, dogs, cats, raccoons, cattles, mongooses, and bandicoots. The most prevalent reservoir host animal for *Leptospira* species is rats. Between 1970 and 1986, nine serovars of leptospirosis were described in Malaysia, including *Pyrogenes*, *Autumnalis*, *Canicola*, *Hebdomadis*, *Icterohemorrhagiae*, *Pomona*, *Grippotyphosa*, *Celledoni*, and *Sejroe* (Benacer et al., 2013). Thayaparan et al. reported the presence of *Leptospira* in wildlife around tourism zones in Sarawak (2013). Meanwhile, Su'ut et al. (2011) emphasised the distribution of *Leptospira* in the Rejang Basin (2011). According to Pui et al. (2015), the major intermediate *Leptospira* found circulating in Tanjung Datu National Park and Bako National Park in Sarawak is *L. wolffii* serovar Khorat. Pathogenic strains are typically responsible for disease transmission. However, the intermediate strain, *L. wolffii* serovar Khorat strain Khorat H-2, was recovered from the urine of a patient with probable leptospirosis in Thailand and has the potential to cause severe illness (Slack et al., 2008).

2.2 Leptospirosis

Leptospirosis is a significant zoonotic illness spread by the spirochete *Leptospira*. *Leptospira* is more resistant to infection in humid and warm conditions, and is therefore more prevalent in tropical regions than in temperate countries (Zavitsanou & Babatsikou,

2008). This disease is seen worldwide in rural areas as well as urban areas in both developed and developing countries (Bharti et al., 2003). Flood and strong rains also the causes of leptospirosis occurrence (Haake and Levett, 2015). Farmers and urban dwellers are particularly susceptible to poor sanitation, occupation, and substandard housing which is lead to leptospirosis infection.

Leptospirosis is an infection that is spread through the environment and can affect both humans and animals (Maciel et al., 2008). Common animal reservoirs include rodents and domestic animals. Leptospirosis is transmitted to humans either directly or indirectly through contact with the urine of animal reservoirs, moist soil, or water contaminated with animal reservoirs' urine (Levett, 2001). The extent to which leptospirosis is transmitted is determined by factors such as the degree of contact with infected hosts, the climate, and population density.

Leptospirosis typically manifests as a non-specific acute febrile disease characterised by headache, myalgia, and fever. According to Haake & Levett (2015), the majority of human leptospiral infections in endemic locations are moderate or asymptomatic. Three factors have a role in the progression of increasingly severe infections: host vulnerability, pathogen virulence, and epidemiological circumstances.

2.3 Pathogenesis of *Leptospira*

The species that can cause leptospirosis in humans include pathogenic *Leptospira* spp. The pathogenicity of the bacterium is currently unknown, but some ongoing studies suggest that the LipL32 lipoprotein is responsible for the species' virulence (Evangelista & Coburn, 2010). According to some studies, the attachment of extracellular matrix (ECM)

components to pathogen outer surface proteins enhances the virulence of pathogenic *Leptospira* spp. (Ghazaei, 2018).

After invasion, *Leptospira* will spread and survive in mammalian host tissue, causing acute sickness symptoms. According to Evangelista and Coburn (2010), the lipoprotein Loa22 may be a potential candidate for pathogenic strain pathogenicity. During an acute *Leptospira* infection, Loa22 is elevated. Even when a mutant Loa22 is created using transposon mutagenesis, *Leptospira* remains avirulent when injected into a host. Only after the mutant's complementation does the virulence return. Essentially, some proteins, such as outer membrane proteins and lipoproteins, may be implicated in pathogen strain pathogenesis, but in an overlapping functional process (Ghazaei, 2018).

The first phase in the pathogenesis of leptospirosis is the penetration of tissue barriers in order to obtain access to the hosts' bodies. Pathogenic *Leptospira* species can infect humans by direct or indirect contact with polluted urine from diseased animals. *Leptospira* can enter the body through the mucous membranes of the mouth cavity and conjunctiva, as well as through wounded skin caused by cuts or abrasions on the skin, which also allows *Leptospira* entrance (Marinho & Cardoso, 2014; Haake & Levett, 2015). Water contaminated by the urine of reservoir host animals can also transmit leptospirosis. The oral cavity is a possible entrance point, as studies have shown that drinking contaminated water while swimming is a potential risk for leptospirosis, as proven by the 2005 outbreak of leptospirosis among Adventure Race participants in Florida (Stern et al., 2010).

The following stage is hematogenous dispersion, during which the pathogenic *Leptospira* penetrates and stays in the host bloodstream (Haake & Levett, 2015). As a result, *Leptospira's* persistence in the host's bloodstream can lead to bacterial pneumonia, shock,

sepsis, organ dysfunction, and other serious consequences, or even death. Evangelista & Coburn (2011) also reported that hemolytic enzymes, lipopolysaccharides, proteins on the outside of the cell's membrane, adhesion molecules, and other surface proteins are possible virulence factors that could play a role in leptospiral pathogenesis.

Leptospirosis can develop in 1 day to 4 weeks after exposure and persist for months (Evangelista & Coburn, 2011), while human infection conditions may differ based on the infection serotype of *Leptospira*, the patient's age, health, and immunological competency. The disease's symptoms can range from minor to severe depending on the stage of infection. Kidney failure, liver failure, chronic lung haemorrhage, and even mortality are all symptoms of asymptomatic leptospirosis (Pui et al., 2017a). Hemolysis of erythrocytes can also occur as a result of *Leptospira's* synthesis of hemolysins, resulting in hemoglobinuria (Narayanavari et al., 2015). Unfortunately, the mechanisms of leptospirosis remain unknown at the moment (Marinho & Cardoso, 2014).

2.4 Antibiotics

Antibiotics are antimicrobial medications used to treat and prevent microbial infections. Alexander Fleming discovered penicillin in 1927 as the first antibiotic. Antibiotics can be created naturally by fungi or bacteria that are capable of inhibiting the pathogens that cause disease, or they can be produced synthetically (Ventola, 2015). Antibiotics are drugs that are used to treat infectious diseases as well as to prevent the spread of pathogenic germs. Antibiotics are also known to induce a variety of adverse effects, including nausea, indigestion, diarrhoea, and hypersensitivity.

Antibiotics are roughly classified into six classes: penicillins, tetracyclines, cephalosporins, fluoroquinolones, macrolides, and aminoglycosides, each with a unique

mode of action. Both penicillins and cephalosporins are bactericidal antibiotics used to block cell wall formation, and bacteriostatic antibiotics like aminoglycosides and tetracyclines are bactericidal antibiotics that utilized to inhibit protein synthesis, while the bactericidal antibiotics fluoroquinolones inhibit DNA synthesis (National Health Service, 2016). Each sort of antibiotic is only effective against a certain type of infectious disease as it based on their mechanism.

Only three medications, penicillin G, doxycycline, and ceftriaxone, were evaluated and found to be effective against leptospirosis, according to Murray and Hospenthal (2004a). Recently, penicillin, doxycycline, ceftriaxone, and ampicillin are commonly used antibiotics to treat leptospirosis (Lim et al., 2011) and the current options for leptospirosis treatment are also cefotaxime and azithromycin (Devishree, 2015). These three antibiotics belong to various antibiotic classes, including beta-lactam for penicillin and doxycycline, and tetracycline for ceftriaxone. Intravenous penicillin is the first-line antibiotic therapy for severe leptospirosis, whereas doxycycline is used to treat mild leptospirosis (Charan et al., 2013). Most symptoms of leptospirosis, such as fever, are reduced when antibiotics are used (Lim et al., 2011).

Antibiotic susceptibility testing in laboratories is required for the selection of antibiotics for the treatment of infectious diseases (Jenkins & Schuetz, 2012). Since there is a scarcity of evidence from existing clinical investigations, the appropriate treatment of leptospirosis remains a significant clinical challenge (Raptis, Pappas & Akritidis, 2006).

2.5 Antibiotic Susceptibility Testing

Both according to Fluit et al. (2001), detecting antibiotic susceptibility is required to generate optimal antimicrobial therapy and restrict the spread of antibiotic-resistant

bacteria or antibiotic resistance genes in the population. On the other hand, the purpose of antibiotic susceptibility testing is to validate the bacteria's susceptibility to the antibiotics of choice as well as to discover resistance (Reller et al., 2009), besides help physicians decide on the best treatment for conditions based on the bacteria discovered. Bacterial antibiotic susceptibilities can be classified into three categories: sensitive, intermediate, and resistant. Furthermore, antimicrobial susceptibility testing information is useful in keeping track of the antibiotic resistance levels of bacteria on a worldwide scale, which serves as surveillance. This reveals that antibiotic resistance levels in bacteria have considerably increased in recent decades, with multi-resistant infections identified (Kerr, 2005).

Antibiotic resistance develops because of the massive use of antibiotics for clinical purposes. Although *Leptospira* species are still susceptible to routinely used medications antibiotic resistance may have emerged or evolved. According to Benacer et al. (2017), there is a shortage of antibiotic susceptibility data among Malaysian *Leptospira* isolates. Hence, antibiotic susceptibility testing is used to track the evolution of antibiotic resistance to prevent the establishment of multidrug-resistant strains of *Leptospira* spp. that are still treatable with those antimicrobial drugs (Perreten et al., 2005).

According to prior research, broth microdilution testing is one of the most frequent used tests to determine the antibiotic susceptibility of *Leptospira* spp. (Reller et al., 2009). This test is carried out in 96-well round-bottom microtiter plates and is then analysed using Alamar Blue, a growth detector. When the colour of Alamar Blue changes from dark blue to brilliant pink, it signifies that bacterial organism are growing and developing. The quantitative findings of broth microdilution testing can be generated and reported as the Minimal Inhibitory Concentration (MIC). MICs are the lowest antibiotic concentrations

employed that do not cause colour changes in alamar Blue. The MIC also shows the lowest dose of the tested substance that inhibits growth completely, with an Optical Density (OD) OD reading equal to the OD of the blank (negative control) (Kheadr et al., 2007). The most often used method for assessing *Leptospira* antibiotic susceptibility is broth microdilution, which yields quantitative findings in g/mL.

2.6 Medicinal Plants

Prior to the invention of the internet, technology was not as highly developed or as advanced as it is now. Thus, there was no artificial medicine, vaccine, or antimicrobial agents to cure any illness or condition that existed at the time of writing. Throughout history, humans have relied on nature as a source of medicine to treat and cure a variety of ailments. A medicinal plant was traditionally used to treat people, and it is reported that roughly 80 percent of the population still relies on this method of therapy today. Up to 90% of the population relies on frequent prescriptions, most of which are grown locally (Duke, 1985). Herbal medicine is a well-known medical system worldwide.

As per Amenu (2014), most traditional remedies and therapies are derived from natural products such as plant extracts. Natural products, such as plant extracts, are still considered an important source of information in studies or research to produce pharmaceuticals today (Cos et al., 2006). Besides, natural products of medicinal plants have been shown to include an extensive supply of biologically active chemicals, which can be found in large quantities in their natural state. It appears to be a much healthier choice when compared to manufactured pharmaceuticals because it has fewer adverse effects. Phytochemicals found in natural products have a variety of health-promoting effects, including antidiarrheal, antibacterial, anticancer, and analgesic properties (Sasidharan et al.,

2011). These insights have resulted in a greater understanding of the subject and serve as a foundation for therapeutic development. Unfortunately, only a fraction of the 500,000 plant species has their antibacterial activity examined to date (Palombo, 2011).

In the latter part of the twentieth century, medicinal plants gained wide acceptance around the world because of the rise in antibiotic-resistant bacterial strains, widespread recognition of the importance of traditional remedies, and the incorporation of derivatives from medicinal plants into pharmaceutical formulations. There are several types of medications have their origins in traditional medical folklore. These include ephedrine, reserpine, digitoxin, and tubocurarine, to name a few. Malaysia is renowned for its vast tropical forest. Many plant species have been identified in Malaysia as medicinal herbs, representing a diverse range of uses. According to Khalilur and Raden Supathan (1981), traditional medicine has been used in Malaysian multi-ethnic community for decades. More than 1300 medicinal plant species have been identified in Peninsular Malaysia alone, according to official records. Table 1.2 below lists some examples of medicinal plants found in Malaysia, as well as their therapeutic potential.

Table 1.2: List of medicinal use of folklore medicines in Malaysia (Alsarhan et al., 2014)

Family	Scientific name	Local name	Medicinal use
<i>Verbenaceae</i>	<i>Callicarpa arborea</i> Roxb.	Tambang besi	Cuts, gastric, rheumatism
			

Table 1.2 continued

<i>Caricaceae</i>	<i>Carica papaya</i> L.	Betik	Asthma, fever, gastric
			
<i>Myrtaceae</i>	<i>Psidium guajava</i> L.	Jambu batu	Diarrhea, stomachache
			
<i>Zingiberaceae</i>	<i>Curcuma domestica</i> Valetton	Kunyit	Bloating, coughs, gastric
			
	<i>Zingiber officinale</i> Rose.	Halia	Flatulence, gastric
			

2.7 *Boesenbergia stenophylla*

In the botanical world, the Zingiberaceae family, usually referred to as the ginger family, is a group of flowering plants that includes approximately 1600 recognised species. It is most common to find members of the Zingiberaceae family prospering in moist parts of the tropics and subtropics, which may include locations that are dry seasonally, and in the subtropics. The Zingiberaceae family of ginger plants, which are members of the ginger family, can be found in abundance throughout Southeast Asia. It is widely used in a variety of applications, including medicine, food, spices, colours, and perfume. The members of the ginger family generally have underground stems or fleshy rhizomes that grow from the ground. Numerous genera are economically valuable as spices, attractive plants, or traditional remedies, among other things.

Over 80% of all ginger species may be found in India, which has a total of 20 taxa and approximately 200 species. Each genus has a distinguishing characteristic that allows it to be distinguished from the others. Rhizomes of distinct species are coloured in a variety of ways, including pink, pastel yellow, greenish blue, and other hues. Additionally, some species have scale leaves that protect their rhizome, while others have no or only a few leafy shoots, such as the *Curcuma* spp., which have a pseudostem with clasping leaf sheaths, and the *Kaempferia* spp., which have a small true stem, among other differences. There are several genera that have been economically used for medicinal purposes, including *Alpina*, *Hedychium*, *Amomum*, *Elettaria*, *Kaempferia*, *Curcuma*, and *Zingiber* (Kumar et al., 2013). Several species, including *Alpina galangal*, *Boesenbergia pandurata*, *Curcuma amada*, *Curcuma longa*, *Curcuma zedoaria*, *Kaempferia galangal*, *Zingiber officinale*, and *Zingiber zerumbet*, are commonly used to provide benefits to humans, according to Voravuthikunchai (2007).

Boesenbergia spp., which are members of the Zingiberaceae family, are endemic to tropical areas such as India and Southeast Asia. Baker used to classify ginger as belonging to the *Kaempferia* genus, which was later reclassified as belonging to the *Boesenbergia* genus. In Borneo, there are thought to be 25 species of *Boesenbergia* spp., all of which are endemic to the country (Larsen, 2003). It is most found in shaded and moist areas, such as those near streams or other bodies of water. *Boesenbergia* spp. plants have rhizomes, which are parts of the plants that can be used for medicinal purposes (Ling et al., 2010).

B. stenophylla, also referred as Jerangau merah in Malay, is an extremely unique plant that is only found on the island of Borneo. *B. stenophylla* is a member of the genus *Boesenbergia*. It can only be found at high altitudes (about 3000 ft) since it demands a cool environment to develop at its optimal level of efficiency. The medical use of *B. stenophylla* is beneficial in the treatment of stomach discomfort, food poisoning, and alcohol intoxication, among other things. It has been suggested that the plant's continued existence may be threatened by factors such as harvesting the plants and climate change, among others (Toyat et al., 2015).

2.8 *Hydnophytum formicarum* Jack

H. formicarum Jack, is an epiphytic plant belonging to the *Rubiaceae* family that has been designated as such because of its relationship with ants, in which the ants' caudex is used for protection and shelter (Huxley, 1978). Borneo and South-East Asia are the most common locations for this species (Prachayasittikul et al. 2008; Hamsar and colleagues 2012; Gay and colleagues 2018). This plant is known by several names in Malaysia, including Sarang Semut, Kepala Beruk, and Raja Satong. As a traditional medicine for the treatment of diabetes, as an anti-inflammatory and neurotonic cure, it has

been used for thousands of years (Prommee, 1988; Prachayasittikul et al., 2008). This species has also been demonstrated to be beneficial in the treatment of headaches, rheumatism, and diarrhoea, due to the presence of a bioactive component in its leaves (Prachayasittikul et al., 2008).

A small number of studies have been undertaken on the evaluation of the medicinal qualities of *H. formicarum* Jack, with a special emphasis on the plant's potential as an alternative cancer treatment (Darwis et al., 2014; Senawong et al., 2013). The tubers of *H. formicarum* Jack. have so far yielded eight known chemicals, including sitosterol (steroid), stigmasterol (steroids), protocathechualdehyde, isoliquiritigenin, butein (phenolic compounds), butein, sinapinic acid, and 7,3',5'-trihydroxyflavanone (flavonoid). In addition, the anti-proliferative, anti-inflammatory, anti-parasitic, and anti-oxidative effects of plant extracts and isolated chemicals have been demonstrated in animal studies to be highly effective in humans (Abdullah et al., 2010; Darwis et al., 2014). It is the scarcity of information and previous studies on this species' antileptospiral activity that has prompted this investigation into the species' activity by ants. In exchange, the plants offer food for the ants in the form of nectar or other protein-rich sources, which is why *H. formicarum* Jack. is commonly referred to as the ant plant.

2.9 Maceration

Maceration is an extraction technique in which coarsely powdered drug material (either leaves, stem bark, or root bark) is placed within a container and the menstruum is poured on top until the drug material is entirely coated by the menstruum. It is described as follows: The container is then sealed and kept for a minimum of three days after that (Ingle et al. 2017). Stirring the contents on a regular basis is recommended. If the contents are

placed in a bottle, shaking the bottle from time to time is also recommended. The micelle is removed from the marc at the end of the extraction process using filtration or decantation. The micelle is then separated from the menstruum by evaporation in an oven or on top of a water bath, which is followed by filtration (Ingle et al. 2017). This approach is simple to use and is particularly well suited for thermolabile plant material.

2.10 Determination of chemical compounds present in natural product by using GC-MS

Compounds present in plant samples can be determined and identified using gas chromatography and mass spectrometry (GC-MS), which is a combined analytical approach (Uma & Balasubramaniam, 2012). GC-MS is critical in the phytochemical analysis and chemotaxonomic investigations of medicinal plants that contain biologically active constituents (Hethelyi et al., 1987), as well as in the identification of new medicinal plants. The components were identified using their retention indices, and the mass spectrum was interpreted using the National Institute of Standards and Technology database (NIST). The database contains around 62,000 recognised compound patterns. The acquired spectra of the unknown components of the Hibiscus asper fraction were compared to standard mass spectra of known components contained in the NIST collection (NISTII).

CHAPTER 3

MATERIALS AND METHODOLOGIES

3.1 Bacterial Strain Preparation and Growth Conditions

A total of twelve *Leptospira* strains isolates from soil (n = 9) and water (n = 3) were tested against seven selected antibiotics (penicillin G, ampicillin, amoxicillin, doxycycline, cefotaxime, chloramphenicol and streptomycin). These environmental isolates were previously isolated between April 2014 to February 2015 from different locations in Sarawak, Malaysia (Pui et al., 2017b). The cultures were obtained from *Leptospira* culture collection at Microbiology Laboratory, Faculty of Resources Science and Technology, Universiti Malaysia Sarawak and the bacterial strains were maintained in *Leptospira* Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco BD, USA). Table 3.1 and Table 3.2 listed tested pathogenic and intermediate *Leptospira* strains, respectively. Each sample were suspended in 10 mL of EMJH liquid medium to produce the pure culture of each sample. These cultures were resuscitated by growing in EMJH which contain 0.1 g of 5-fluorouracil and enrichment media at room temperature. All cultures were incubated at 30 °C for 30 days. Table 3.1 shows the selected saprophytic *Leptospira* strains that were used in this study. Final concentration of the culture is 10^6 to 10^8 CFU/mL as mentioned by Ristow *et al.* (2008).

Table 3.1: List of pathogenic *Leptospira* spp.

ID	Species	Locality	Source
CFP16	<i>L. noguchii</i> strain LT796	Sungai Rait Camp Miri	Soil
CFP18	<i>L. noguchii</i> strain LT796	Kubah National Park	Soil
CFP21	<i>L. noguchii</i> strain LT796	Samunsam Wildlife Sanctuary	Soil
CFP3	<i>L. Borgpetersenii</i> serovar mini	Bako National Park	Soil
CFP34	<i>L. Borgpetersenii</i> serovar mini	Sungai Rait Camp Miri	Soil
CFP35	<i>L. Borgpetersenii</i> serovar mini	Kubah National Park	Soil

Table 3.2: Tested intermediate *Leptospira* spp.

ID	Species	Locality	Source
CFG4	<i>L. wolffii</i> serovar Khorat strain Khorat-H2	Bako National Park	Soil
CFG12	<i>L. wolffii</i> serovar Khorat strain Khorat-H2	Sungai Rait Camp Miri	Soil
CFG14	<i>L. wolffii</i> serovar Khorat strain Khorat-H2	Kubah National Park	Soil
CFG3	<i>L. inadai</i> serovar Aguaruna strain MW4	Tanjung Datu National Park	Soil
CFG15	<i>L. inadai</i> serovar Aguaruna strain MW4	Kubah National Park	Soil
CFG18	<i>L. inadai</i> serovar Aguaruna strain MW4	Kampung Barieng, Bau	Soil

3.2 Antimicrobial Agents Preparation

Seven antibiotics: penicillin G (Fisher Bioreagents, USA), doxycycline (Acros

Organics, USA), ampicillin (Fisher Bioreagents, USA), amoxicillin (Fisher Bioreagents, USA), cefotaxime (Acros Organics, USA), chloramphenicol (Acros Organics, USA), and erythromycin (Fisher Bioreagents, USA) were purchased from the respective companies. The antimicrobial stock solutions were prepared from reagent grade powders using solvents and diluents to produce 1 mg/ml solutions as suggested by the Clinical and Laboratory Standards Institute document M100-S22. The antibiotic stock solutions were then stored at -20 °C in divided one-time use aliquots.

3.3 Antibiotic Susceptibility Testing

The Broth microdilution testing was performed as suggested by Murray and Hoshpenthal (2004a), and Chakraborty et al (2010). 96-well, flat-bottom microtiter plates were used to perform this testing. Each plate included positive control (*Leptospira* only), negative controls (EMJH broth only), and serial twofold dilutions of each of the seven antibiotics, all in EMJH broth. The final concentrations of each antibiotic in antibiotic-containing well, ranging from 0.20 – 50 µg/mL (units/ml for penicillin). 7-day-old cultures grown in liquid EMJH medium at 30 °C were prepared for *Leptospira* inoculum used for testing. A 100 µL of *Leptospira* inoculum of 2×10^6 CFU/mL was added into the antibiotic-containing and positive control wells. The final volume of each well was 200 µL, which was then incubated at 30 °C. After three days of incubation, 20 µL of 10X AlamarBlue was added to each well. AlamarBlue is a cell viability indicator dye that turns from dark blue to bright pink when growth bacteria are existing. On the fifth day of incubation, the colour of each well was observed. The MICs were noted visually as the lowest concentration of antibiotics in the wells without colour change. AlamarBlue is an oxidation-reduction indicator that changes color from dark blue to bright pink in response to chemical reduction of the growth medium resulting from cell growth.

3.4 Plant Crude Extraction

Tubers of *H. formicarum* Jack were purchased from the Lachau local market, Sarawak and *B. stenophylla* were collected from Bario, Sarawak. Maceration extraction was performed to obtain crude extract from tubers of *H. formicarum* Jack and leaves and rhizomes of *B. stenophylla* (Prachayasittikul et al., 2008). The extraction was carried out using chloroform, ethyl acetate, hexane, and methanol to give the corresponding chloroform, ethyl acetate and methanol extracts. (Prachayasittikul et al., 2008). 20 g of crude extract powder were macerated in 200 ml extractant.

3.5 Antimicrobial Activity of *H. formicarum* Jack. and *B. stenophylla* Crude Extract

Broth microdilution testing was performed as reported previously (Murray et al. 2004; Ressler et al. 2008). Each 96-well flat-bottom plate included serial twofold dilutions of the crude extracts, positive controls (bacteria without a crude extract), and negative controls (medium only), all in EMJH medium. The inoculum of *Leptospira* used for testing was prepared from 7-day-old cultures grown in EMJH medium at 30 °C. A *Leptospira* inoculum of 2×10^6 leptospiral organisms/ml was added, and the plates were incubated at 30°C with a final volume of 200 µL in each well. After 3 days of incubation, 20 µL of 10 Alamar Blue was added to each well. The color of each well was documented on the fifth day of incubation, and the MICs were recorded as the concentration in the well containing the lowest concentration without a blue to pink color change.

3.6 GC-MS Analysis

GC-MS profiling of *H. formicarum* jack and *B. stenophylla* crude extract were analysed using a Shimadzu 2010 Plus GC-MS instrument. The carrier gas was helium and

the capillary column was BPX-5 (30.0 m x 0.25 mm). The diluted sample was then injected into a split less injector. For 2 minutes, the column will be heated to 60 °C. The temperature was then increased to 300 °C at 10°C/min for 5 minutes. Injector and detector temperatures will be 250 °C and 300 °C, respectively. Finally, the substance was identified by matching their mass spectrum to the NIST library (Wang et al., 2009).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Antibiotic Susceptibility Testing towards Pathogenic and Intermediate *Leptospira* spp.

Table 4.1 and Table 4.2 shows the MIC values of seven antibiotics for 12 locally isolates of *Leptospira* spp. All isolates MIC range of 0.2 to 6.25 µg/mL toward all tested antibiotics. In this study, doxycycline exhibited the highest MIC value of 6.25 µg/mL, whereas penicillin G and chloramphenicol showed the same MIC value of 3.13 µg/mL. Overall, cefotaxime and erythromycin presented good inhibition activity against all tested environmental isolates with MIC 0.2 µg/mL. The isolates CFP3, CFP34, CFP35, CFG3, CHG15, and CFP18 were susceptible to amoxicillin, ampicillin, cefotaxime and erythromycin with lowest MICs value of 0.2 µg/mL whereas doxycycline had MICs value ranged from 0.78 to 12.5 µg/mL where CFP18 and CFP21 showed higher MICs value of 6.25 µg/mL and 12.5 µg/mL, respectively. Overall, all tested isolates *Leptospira* spp. more susceptible toward cefotaxime and erythromycin with MIC 0.2 µg/mL, as compared to amoxicillin and ampicillin with MIC 0.39 µg/mL.

Table 4.1: Antibiotic Susceptibility Testing of Pathogenic *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)						
	Pen G ^b	Amo	Amp	Doxy	Cefo	Chlor	Em
CFP 3	0.39	0.20	0.2	0.78	0.20	1.56	0.20
CFP 16	0.78	0.39	0.39	1.56	0.20	1.56	0.20
CFP 18	3.13	0.39	0.39	6.25	0.20	3.13	0.20
CFP 21	3.13	0.39	0.39	12.5	0.20	3.13	0.20
CFP 34	0.39	0.20	0.20	0.78	0.20	1.56	0.20
CFP 35	0.39	0.20	0.20	0.78	0.20	1.56	0.20

^a Minimum inhibitory concentration

^b Values for Penicillin are in units/mL; all others are in µg/mL.

Pen G: Penicillin G, Amo: Amoxicillin, Amp: Ampicillin, Doxy: Doxycycline, Cefo: Cefotaxime, Chlor: Chloramphenicol, Em: Erythromycin.

Table 4.2: Antibiotic Susceptibility Testing of Intermediate *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)						
	Pen G ^b	Amo	Amp	Doxy	Cefo	Chlor	Em
CFG 3	0.39	0.20	0.20	0.78	0.20	0.78	0.20
CFG 4	0.78	0.39	0.39	1.56	0.20	1.56	0.20
CFG 12	0.78	0.39	0.39	1.56	0.20	1.56	0.20
CFG 14	3.13	0.39	0.39	3.13	0.20	3.13	0.20
CFG 15	0.39	0.20	0.20	0.78	0.20	1.56	0.20
CFG 18	0.39	0.20	0.20	0.78	0.20	0.78	0.20

^a Minimum inhibitory concentration

^b Values for Penicillin are in units/mL; all others are in µg/mL.

Pen G: Penicillin G, Amo: Amoxicillin, Amp: Ampicillin, Doxy: Doxycycline, Cefo: Cefotaxime, Chlor: Chloramphenicol, Em: Erythromycin.

The present study documented the inhibition of penicillin, amoxicillin, ampicillin, doxycycline, erythromycin, cefotaxime, and chloramphenicol against *Leptospira* spp. by using broth microdilution method. The MICs were documented visually as the lowest concentration of antibiotics in the wells without blue-to-pink colour change and MIC represents the MIC value at which $\geq 90\%$ the *Leptospira* spp. are inhibited.

In this study, cefotaxime and erythromycin displayed the lowest MIC (0.2 µg/mL) against all tested isolates, which agrees with the findings of Murray and Hospenthal (2004b). Erythromycin and cefotaxime have been revealed to have the highest efficacy against all isolates *Leptospira* sp. studied and the MICs of these two antibiotics have been consistently lower than the current leptospirosis drugs of choice, penicillin G and doxycycline (Murray & Hospenthal, 2004b).

In contrast to cefotaxime and erythromycin, the local isolates exhibited high MIC toward doxycycline. CFP21 showed the highest MIC (12.5 µg/mL) with doxycycline as compared to other isolates. Nevertheless, doxycycline was still effective as the MIC against all tested isolated *Leptospira* spp. were 5.938 µg/mL. For minor leptospirosis treatment, doxycycline has been commonly prescribed and used (Suputtamongkol et al., 2004). Athletes who had intake doxycycline before the challenge, they were secure from infection during the outbreak of leptospirosis that happened in an eco-challenge multisport race in Sabah, Malaysia (Sejvar et al., 2003).

Unlike doxycycline, penicillin group demonstrated better inhibition against *Leptospira* spp. The MIC for ampicillin, amoxicillin, and penicillin G ranged between 0.39 and 3.13 µg/mL. However, the MIC results of penicillin G were 87.54% higher than amoxicillin and ampicillin. Nevertheless, penicillin G is commonly prescribed for treating serious leptospirosis is due to the low level of toxicity and the ability to deliver the drug intravenously at high dosages at the initial stage of infection (Watt et al., 1988). In contrast, ampicillin and amoxicillin have lower MIC than penicillin. This finding agrees with previous studies where Ampicillin and amoxicillin had lower MICs than penicillin or doxycycline for almost all tested strains (Hospenthal & Murray, 2003). Previous studies have reported the use of ampicillin as a possible alternative for *in vivo* and *in vitro* treatment of leptospirosis, but the ampicillin reaction against leptospirosis is limited and cannot be spread to all organ tissues, for instance, heart and kidneys, causing it ineffective in the clearance of protected site leptospirae (Truccolo et al. 2002). Chloramphenicol has the same MIC (3.13 µg/mL) as penicillin G.

4.2 Antimicrobial Activity of Crude Extract *H. formicarum* Jack

Table 4.3 showed antimicrobial activity of crude extract of *H. formicarum* Jack against pathogenic *Leptospira* spp.. Ethyl acetate crude extract showed 66.67% potential in inhibit tested pathogenic *Leptospira* strains (CFP16, CFP3, CFP34 and CFP35) with values of 125 µg/mL. Methanol crude extract of *H. formicarum* Jack also shown same MIC values as Ethyl acetate crude extract (125 µg/mL) against CFP16 whereas another isolate with 250 µg/mL. Hexane crude extract showed inhibition at range 250 µg/mL (66.67%) to 500 µg/mL (33.33%). Chloroform crude extract shows highest MIC value which were 500 µg/mL.

Table 4.3: Antiantimicrobial Activity of Crude Extract of *H. formicarum* Jack against Pathogenic *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	ME	HE	CE
CFP16	125	125	250	500
CFP18	250	250	500	500
CFP21	250	250	500	500
CFP3	125	250	250	500
CFP34	125	250	250	500
CFP35	125	250	250	500

a Minimum inhibitory concentration

*EA_E= Ethyl Acetate Extract; ME= Methanol Extract; HE= Hexane Extract; CE= Chloroform Extract

Table 4.4 showed antimicrobial activity of crude extract of *H. formicarum* Jack against intermediate *Leptospira* strains. Ethyl acetate crude extract showed 50% potential in inhibit tested intermediate *Leptospira* strains (CFG3, CFG15 and CGFG 18) with values of 62.5 µg/mL. Methanol crude extract of *H. formicarum* Jack showed 50% inhibition against tested intermediate *Leptospira* strains with 125 µg/mL (CFG4, CFG15, and CFG18) and 250

µg/mL (CFG12, CFG14, and CFG3). Meanwhile, hexane crude extract of *H. formicarum* Jack inhibit 83.33% of tested intermediate *Leptospira* spp. with 125 µg/mL while 250 µg/mL against CFG3. On the other hand, chloroform crude extract of *H. formicarum* Jack against intermediate *Leptospira* spp. appeared better inhibition than pathogenic *Leptospira* spp. with MIC values of 250 µg/mL (83.33%). Figure 4.1 and Figure 4.2 shows antimicrobial activity of crude extract of *H. formicarum* Jack. against pathogenic and intermediate *Leptospira* spp.

Table 4.4: Antimicrobial Activity of Crude Extract of *H. formicarum* Jack against Intermediate *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	M _E	H _E	C _E
CFG4	125	125	125	250
CFG12	125	250	125	250
CFG14	250	250	125	500
CFG3	62.5	250	250	250
CFG15	62.5	125	125	250
CFG18	62.5	125	125	250

^a Minimum inhibitory concentration

* EA_E = Ethyl Acetate Extract; M_E = Methanol Extract; H_E = Hexane Extract; C_E = Chloroform Extract

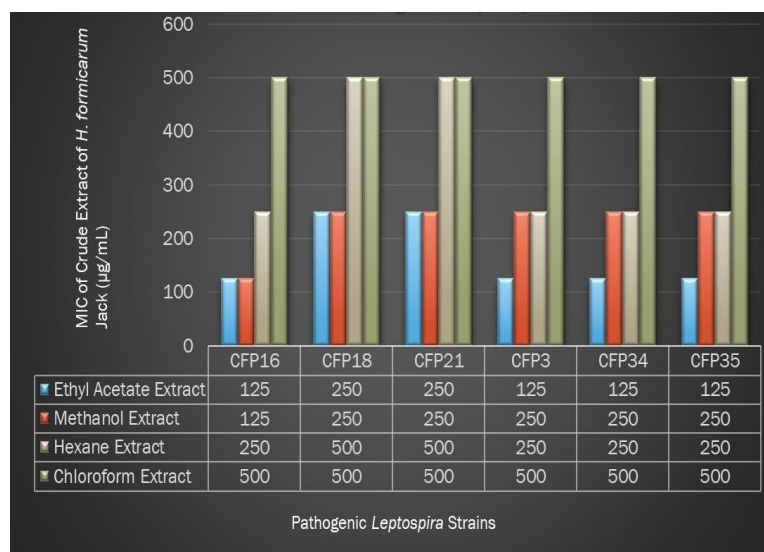


Figure 4.1: Antimicrobial activity of crude extract of *H. formicarum* Jack against pathogenic *Leptospira* spp.

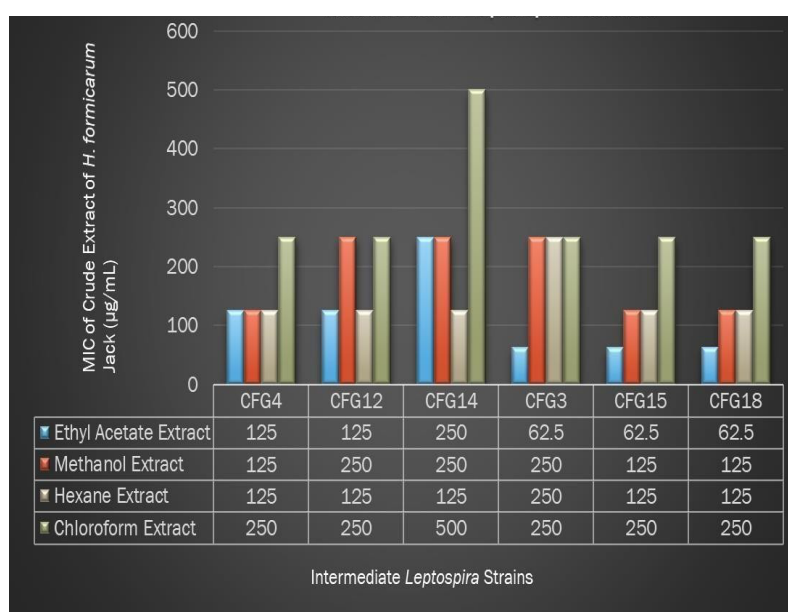


Figure 4.2: Antimicrobial activity of crude extract of *H. formicarum* Jack against intermediate *Leptospira* spp.

4.3 Antimicrobial Activity of Crude Extract of *B. stenophylla*.

Table 4.5 showed antimicrobial activity of leaves crude extract of *B. stenophylla* against pathogenic *Leptospira* spp. Ethyl acetate leaves crude extract of *B. stenophylla* showed inhibition of 66.67% against tested pathogenic *Leptospira* spp. (CFGP 16, CFP 3, CFP 34, and CFP 35) with MIC values of 62.5 µg/mL. Both methanol extract and hexane extract give lowest MIC values of 62.5 µg/mL against CFP 34. Meanwhile, chloroform extract gives highest MIC values of 500 µg/mL (33.3%) against both CFP18 and CFP21.

Table 4.5: Antimicrobial Activity of Leave Crude Extract of *B. stenophylla* against Pathogenic *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	M _E	H _E	C _E
CFP 16	62.5	125	125	250
CFP 18	125	250	250	500
CFP 21	125	250	250	500
CFP 3	62.5	125	125	250
CFP 34	62.5	62.5	62.5	125
CFP 35	62.5	125	125	250

^a Minimum inhibitory concentration

* EA_E = Ethyl Acetate Extract; M_E = Methanol Extract; H_E = Hexane Extract; C_E = Chloroform Extract

Table 4.6 showed antimicrobial activity of leaves crude extract of *B. stenophylla* against intermediate *Leptospira* spp. The highest MIC values of 500 µg/mL shown by chloroform leave crude extract against CGG 14. However, three crude extracts give same MIC values against all intermediate *Leptospira* spp. Ethyl acetate, methanol and hexane extract leave crude extract of *B. stenophylla* inhibit with MIC value of 62.5 µg/mL (CFG 3, CFG 15 and CGFG 18), 125 µg/mL (CFG 4 and CGFG 12) and 250 µg/mL (CFG 14).

Table 4.6: Antimicrobial Activity of Leave Crude Extract of *B. stenophylla* against Intermediate *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	M _E	H _E	C _E
CFG 4	125	125	125	250
CFG 12	125	125	125	250
CFG 14	250	250	250	500
CFG 3	62.5	62.5	62.5	125
CFG 15	62.5	62.5	62.5	125
CFG 18	62.5	62.5	62.5	125

^a Minimum inhibitory concentration

EA_E = Ethyl Acetate Extract; M_E = Methanol Extract; H_E = Hexane Extract; C_E = Chloroform Extract

Table 4.7 antimicrobial activity of leaves crude extract of *B. stenophylla* against pathogenic *Leptospira* spp. The lowest MIC values poses by *B. stenophylla* rhizome crude extract against tested pathogenic *Leptospira* spp. was 125 µg/mL. Ethyl acetate, methanol and hexane give same MIC value of 125 µg/mL against CFP34, whereas ethyl acetate crude extract inhibit CFP16 with same MIC values.

Table 4.7: Antimicrobial Activity of Rhizome Crude Extract of *B. stenophylla* against Pathogenic *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	M _E	H _E	C _E
CFP 16	125	250	250	500
CFP 18	250	500	500	>500
CFP 21	250	500	500	>500
CFP 3	250	250	250	500
CFP 34	125	125	125	250
CFP 35	250	250	250	500

^a Minimum inhibitory concentration

* EA_E = Ethyl Acetate Extract; M_E = Methanol Extract; H_E = Hexane Extract; C_E = Chloroform Extract

Table 4.8 showed antimicrobial activity of leaves crude extract of *B. stenophylla* against intermediate *Leptospira* spp. Ethyl acetate, methanol and hexane crude extract give same inhibition activity against CFG3, CFG 15 and CFG18 with MIC values of 62.5 µg/mL, respectively. Chloroform crude extract also give the same MIC value of 125 µg/mL on CFG3, CFG 15 and CFG18. Figure 4.3, Figure 4.4, Figure 4.5 and Figure 4.6 shows antimicrobial activity of crude extract of *B. stenophylla* Jack against pathogenic and intermediate *Leptospira* spp.

Table 4.8: Antimicrobial Activity of Rhizhome Crude Extract of *B. stenophylla* against Intermediate *Leptospira* spp.

Strain ID	MIC ^a (µg/mL)			
	EA _E	M _E	H _E	C _E
CFG 4	125	250	250	250
CFG 12	125	250	250	250
CFG 14	250	250	250	500
CFG 3	62.5	62.5	62.5	125
CFG 15	62.5	62.5	62.5	125
CFG 18	62.5	62.5	62.5	125

^a Minimum inhibitory concentration

* EA_E = Ethyl Acetate Extract; M_E = Methanol Extract; H_E = Hexane Extract; C_E = Chloroform Extract

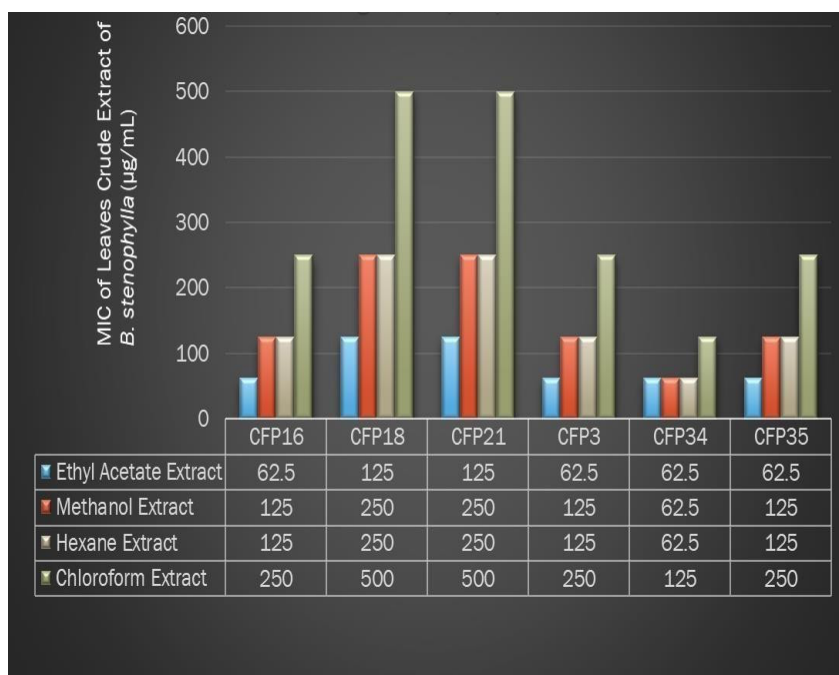


Figure 4.3: Antimicrobial activity of leaves crude extract of *B. stenophylla* against pathogenic *Leptospira* spp.

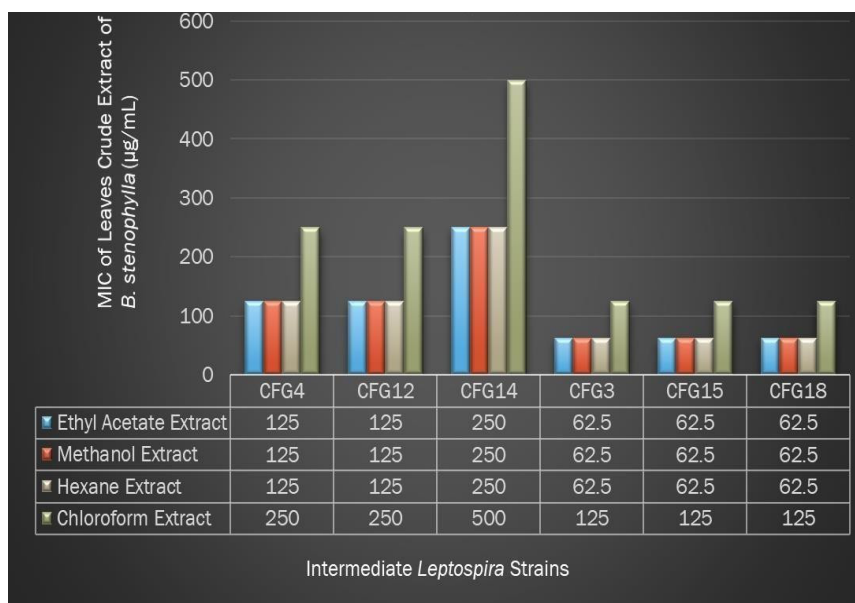


Figure 4.4: Antimicrobial activity of leaves crude extract of *B. stenophylla* against intermediate *Leptospira* spp..

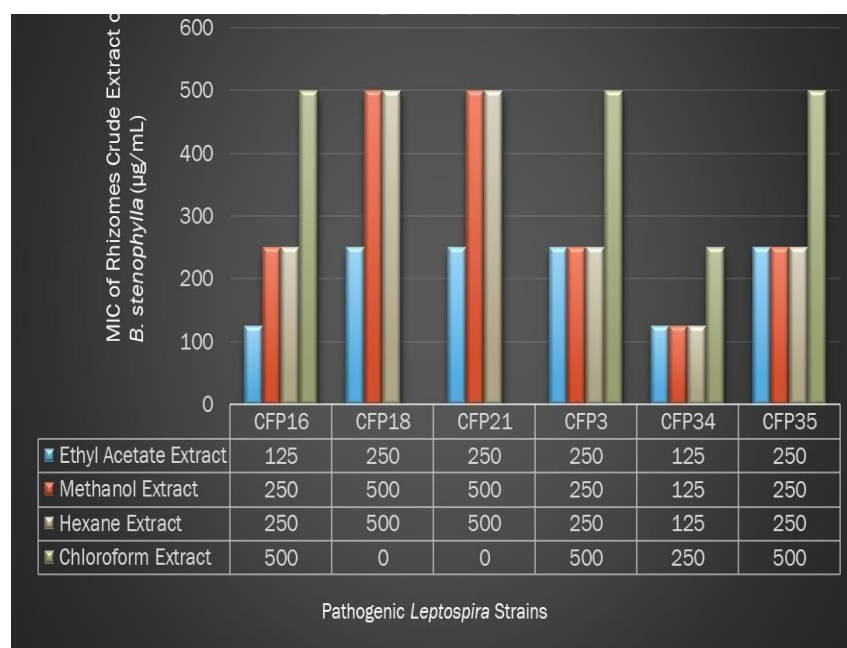


Figure 4.5: Antimicrobial activity of rhizomes crude extract of *B. stenophylla* against pathogenic *Leptospira* spp.

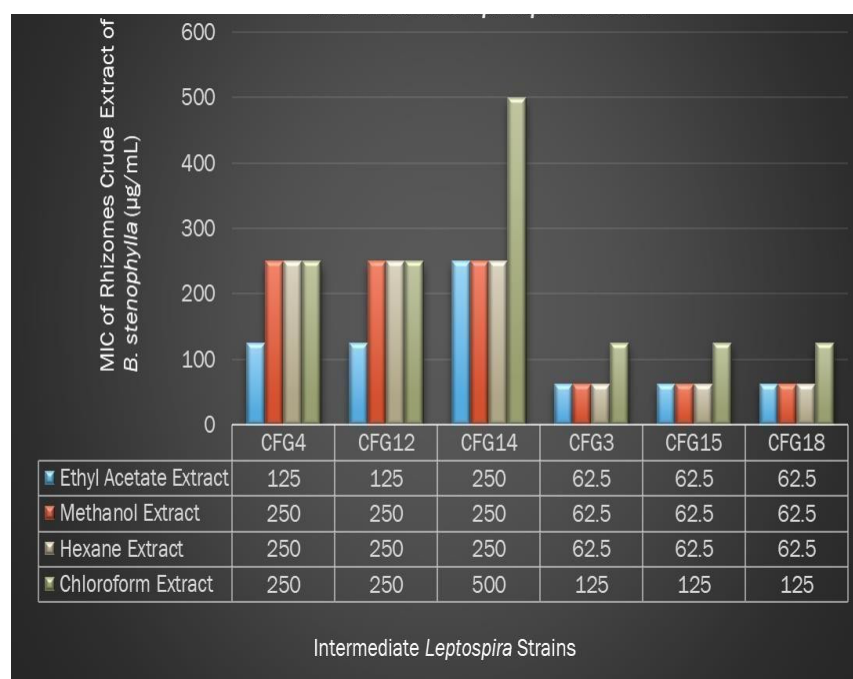


Figure 4.6: Antimicrobial activity of rhizomes crude extract of *B. stenophylla* against intermediate *Leptospira* spp.

According to Ventola (2015), antibiotic resistant bacteria (ARB) are emerging due to overuse, misuse, and a lack of novel medicines from the pharmaceutical sector. ARB strains can cause illnesses like gastritis, urinary tract infections (UTIS), encephalitis, and food poisoning (Lee & O'Rourke, 1993; Mailles et al., 2009). ARB strains are currently deemed a major public health issue by the WHO (2014), because conventional antibiotics may not cure ARB infections. Antibiotic development has slowed dramatically in recent decades, with only two approved between 2008 and 2012 (Li & Webster, 2018). Hence, it is vital to examine for new alternatives, such as those derived from medicinal plants as medicinal plants have useful phytochemicals that can be utilised as bactericides (Chakraborty et al., 2014) and can be successful in the defence against infectious diseases like leptospirosis while causing only minor side effects.

Hence, the purpose of this study was to determine the antimicrobial activity of *H. formicarum* Jack and *B. stenophylla* extract against locally isolated pathogenic *Leptospira* and intermediate *Leptospira* spp. in Sarawak. Antimicrobial activities were determined by using broth microdilution method and the crude extract MIC were determined using a 96- well broth microdilution method based on colour change of alamar Blue. Alamar Blue is a reagent based on resazurin that produced a rapid and easily discernible colour change, allowing for visual measurements. Previous study uses alamar Blue as indicator to read visual MIC values (Lall et al., 2013). There is no published anti-leptospiral study of *H. formicarum* Jack and *B. stenophylla* against local isolated *Leptospira* strain in Sarawak.

In this study, the findings shows that both *H. formicarum* Jack and *B. stenophylla* had potent anti-leptospiral activity. Although there is no previous study for *H. formicarum* Jack and *B. stenophylla* against *Leptospira* spp, hence no comparable previous study to current study, yet this finding will give preliminary anti-leptospiral activity information or trend of *H. formicarum* Jack and *B. stenophylla*.

A prior analysis revealed that ant plant ethanolic tuber extract had low antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Prajubjinda et al., 2012). This has been ascribed to differences in antibacterial efficiency reported between diverse areas for ant plant growth (Andriani et al., 2017) but seasonal fluctuations and plant variety are other potential factors influencing extract quality. In this research, *H. formicarum* Jack and *B. stenophylla* material was obtained from Sarawak. Intermediate *Leptospira* was inhibited more than pathogenic *Leptospira* by crude extract *H. formicarum* Jack and *B. stenophylla*. This could be owing to variations in the structure of the bacterial cell envelope between pathogenic and intermediate *Leptospira* spp. which alter permeabilization and membrane disruption after antibacterial drug exposure (Silhavy et al., 2010).

4.4 GC-MS Analysis

In this study, the chemical constituent in crude extract of *H. formicarum* Jack and *B. stenophylla* for four different solvent which is methanol, ethyl acetate, chloroform and hexane were analysed using GC-MS. Table 4.9, Table 4.10, Table 4.11, and Table 4.12 showed chemical compositions crude extract of *H. formicarum* Jack, whereas Table 4.13, Table 4.14, Table 4.15 and Table 4.16 showed chemical compositions crude extract of *B. stenophylla* I, respectively.

Table 4.9: Chemical compositions Methanol Extract of *H. formicarum* Jack

Rt	Name of compound	Peak Area %
16.641	Eugenol	3.50
17.372	3-Phenyl-2-Propenoate	12.03
17.472	1-ethenyl-1-methyl-2,4-bis (1-methylethenylcyclohexane)	1.32
18.113	α -Santalene	1.10
19.971	2,4a,5,6,7,8,9,9a-octahydro-3,5,5-1H-Benzocycloheptene	1.10
20.260	γ -Muurolene	2.16
22.232	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo [9.1.0] d	1.27
22.540	Isospathulenol	8.33
28.325	l-(+)-Ascorbic acid 2,6-dihexadecanoate	1.06
34.485	(E)-4-Methoxy-6-styryl-2H-pyran-2-one	23.80

Table 4.10: Chemical compositions Ethyl Acetate Extract of *H. formicarum* Jack

Rt	Name of compound	Peak Area %
17.376	1-Tetradecene	0.82
20.000	2,4-Di-tert-butylphenol	4.30
21.595	1-Heptadecene	2.59
24.910	Tetradecanoic acid	0.74
25.389	1-Nonadecene	3.74
26.254	6,10,14-trimethyl-2-Pentadecanone	0.88
28.560	n-Hexadecanoic acid	22.11
28.832	Behenic alcohol	5.43
30.012	Heptadecanoic acid	1.03
31.199	Oleic Acid	0.72
31.659	Octadecanoic acid	11.86
31.963	Docosyl heptafluorobutyrate	3.69
34.766	Bis(2-ethylhexyl) adipate	0.82
34.838	Docosyl heptafluorobutyrate	2.08
35.772	1,3,5-triphenyl-Cyclohexane	6.02
36.363	n-Hexacosane	1.26
36.956	Bis(2-ethylhexyl) phthalate	1.51

Table 4.11: Chemical compositions Hexane Extract of *Hydnophytum formicarum* Jack

Rt	Name of compound	Peak Area %
26.257	6,10,14-trimethyl-2-Pentadecanone	9.32
27.677	Hexadecanoic acid, methyl ester	1.48
28.257	Dibutyl phthalate	1.10
28.363	n-Hexadecanoic acid	2.69
28.799	Hexadecanoic acid, ethyl ester	3.11
30.508	n-Tetracosane	0.78
32.031	Pentacosane	0.75
32.286	Decanedioic acid, bis(2-ethylhexyl) ester	11.26
33.422	Octacosanol	0.62
33.496	n-Tricosane	3.62
34.262	4,8,12,16-Tetramethylheptadecan-4-olide	1.37
34.897	n-Tetracosane	2.26
36.384	Tetracontane	39.36
36.957	Bis(2-ethylhexyl) phthalate	11.94
37.994	1-Hexacosanol	2.40
38.076	n-Hexacosane	2.71

Table 4.12: Chemical compositions Chloroform Extract of *H. formicarum* Jack

Rt	Name of compound	Peak Area %
26.255	6,10,14-trimethyl-2-Pentadecanone	1.92
27.695	Hexadecanoic acid, methyl ester	15.48
28.801	Hexadecanoic acid, ethyl ester	4.31
29.337	Heptadecanoic acid, methyl ester	1.37
30.346	Hexadecanoic acid, propyl ester	1.25
30.526	(E)-9-Octadecenoic acid, methyl ester	4.58
30.928	Methyl stearate	5.92
31.439	(Z)-18-Octadec-9-enolide	1.82
31.537	(E)-9-Octadecenoic acid ethyl ester	1.33
33.896	methyl-Eicosanoate	2.34
36.368	Tetracontane	12.08
36.565	Dotriacontane	6.82
36.944	Bis(2-ethylhexyl) phthalate	3.11

Table 4.13: Chemical compositions of methanol crude extract of *B. stenophylla*

Rt	Name of compound	Peak Area %
16.235	δ-Elemene	1.27
16.663	Eugenol	3.81
17.402	3-Phenyl-2-Propenoic acid methyl ester	10.16
17.488	1-Ethenyl-1-methyl-2,4-bis(1-methylethenyl) cyclohexane	1.54
18.127	α-Santalene	1.95
18.357	1-ethenyl-1-methyl-2-(1-methyl Cyclohexane)	0.80
19.011	α-Humulene	0.97
19.542	Germacrene D	1.80
19.742	decahydro-4a-methyl-1-methyle Naphthalene	0.69
19.982	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octahydro	1.41
22.247	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabicyclo[9.1.0]dodeca-3,7-diene	1.29
22.566	Isospathulenol	7.49
23.202	Neointermedeol	1.23
30.154	Kaurene	14.36
34.549	(E)-4-Methoxy-6-styryl-2H-pyran-2-one	17.88
36.504	Tetracontane	0.79
36.870	Triphenylphosphine oxide	3.16

Table 4.14: Chemical compositions of ethyl acetate crude extract of *B. stenophylla*

Rt	Name of compound	Peak Area %
16.644	Eugenol	2.70
17.375	2-Propenoic acid, 3-phenyl-methyl ester	7.39
19.989	2,4-Di-tert-butylphenol	8.95
21.582	Octadec-1-ene	1.44
22.543	Isospathulenol	1.92
25.375	Octadec-1-ene	1.41
28.338	l-(+)-Ascorbic acid 2,6-dihexadecanoate	1.75
28.812	1-Nonadecene	1.08
31.946	Eicosyl trifluoroacetate	0.72
34.508	(E)-4-Methoxy-6-styryl-2H-pyran-2-one	46.27

Table 4.15: Chemical compositions of Chloroform crude extract of *B. stenophylla*

Rt	Name of compound	Peak Area %
23.412	1,6-dimethyl-4-(1-methylethyl)- Naphthalene	0.95
24.803	6,10,14-trimethyl-2-Pentadecanone	1.10
27.386	Di-3,7-dimethyl-1-octyl phthalate	2.18
28.243	Dibutyl phthalate	1.25
35.598	1,3,5-triphenyl- Cyclohexane	1.73
36.854	Phthalic acid, bis(2-ethylhexyl) ester (6CI,8CI	4.90
36.923	Bis(2-ethylhexyl) phthalate	5.23

Table 4.16: Chemical compositions of Hexane crude extract of *Boesenbergia stenophylla*

Rt	Name of compound	Peak Area %
14.689	Octadecane <n->	0.86
17.211	Copaene <alpha->	0.86
17.484	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-me	2.01
18.126	Santalene <alpha->	3.78
18.231	Bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-	1.03
19.433	Dotriacontane <n->	3.53
19.733	Naphthalene, decahydro-4a-methyl-1-methyle	1.27
20.378	Heneicosane	1.79
23.639	Heptatriacontane <n->	3.25
24.455	Eicosane	1.30
27.399	Dotriacontane	3.26
28.125	Dotriacontane	1.72
30.188	Kaur-16-ene	62.90
30.799	Dotriacontane	2.92

The crude methanol extract of *H. formicarum* showed that (E)-4-Methoxy-6-styryl-2H-pyran-2-one is the major compound with 23.80%, followed by 3-Phenyl-2-Propenoate (12.03%), Isospathulenol (8.33%) and other 7 chemical compounds with composition between 1.06% to 3.50%. As stated in Table 4.10, 17 compounds were identified in crude

ethyl acetate extract of *H. formicarum*. It comprised of n-Hexadecanoic acid (22.11%), followed by Octadecanoic acid (11.86%) and 1,3,5-triphenyl-Cyclohexane (6.02%) and other chemical composition (29.61). Sixteen compounds listed in Table 4.11, where the major compound identified in hexane crude extract for *H. formicarum* were tetracontane with 39.36%. whereas major chemical composition for chloroform extract was Hexadecanoic acid methyl ester with 15.48%. Both methanol and ethyl acetate crude extract *B. stenophylla* yield in same major compound which is (E)-4-Methoxy-6-styryl-2H-pyran- 2-one with 17.88% and 46.27%, respectively. Bis(2-ethylhexyl) 1,2-benzenedicarboxylate with 4.90% have been identified as major compound in chloroform extract. Last but not least, Kaur-16-ene was major compound in hexane crude extract in *B. stenophylla* with composition of 62.90%.

As whole, the major phytochemical constituents in *B. stenophylla* included 16-kaurene from hexane (62.9%) and chloroform (32.07%) extract, and (E)-4-methoxy-6-styryl-2H-pyran-2-one from ethyl acetate (46.27%) and methanol (17.88%) extract. In the other hand, tetracontane (39.36%), n-hexadecanoic acid (22.11%), hexadecanoic acid methyl ester (15.48%) and, 3-phenyl-2-propenoic acid methyl ester (12.03%) were major phytochemical constituents found in *H. formicarum* Jack from chloroform, ethyl acetate, hexane and methanol extracts, respectively. Most of the identified compounds are reported showed their antimicrobial, antifungal, antioxidant, anti-cancer, and anti-inflammatory properties. Inclusive, all tested antibiotics and crude extracts employed in this study showing their effectiveness against isolated *Leptospira sp.* and the results obtained from the study could be used as a future reference for the selection of antibiotics and assist the medical practitioner in the treatment of leptospirosis. Figure 4.7, Figure 4.8, Figure 4.9 and Figure 4.10 are chromatogram produce from crude extract *H. formicarum* Jack, meanwhile, Figure

4.11, Figure 4.12, Figure 4.13 and Figure 4.14 are chromatogram produce from crude extract *B. stenophylla*.

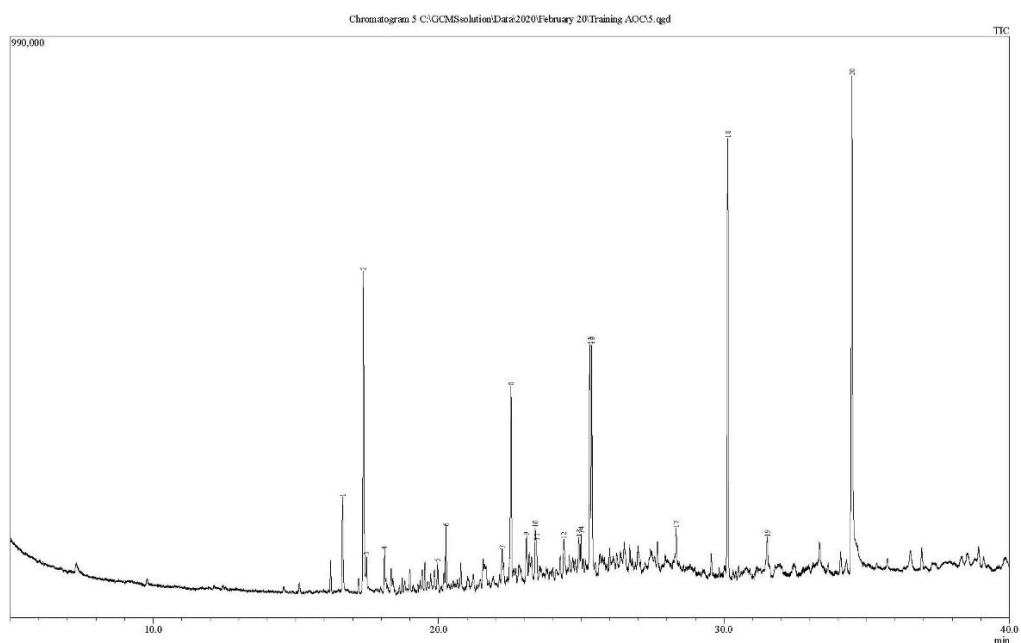


Figure 4.7: Chromatogram of methanol extract of *H. formicarum* Jack

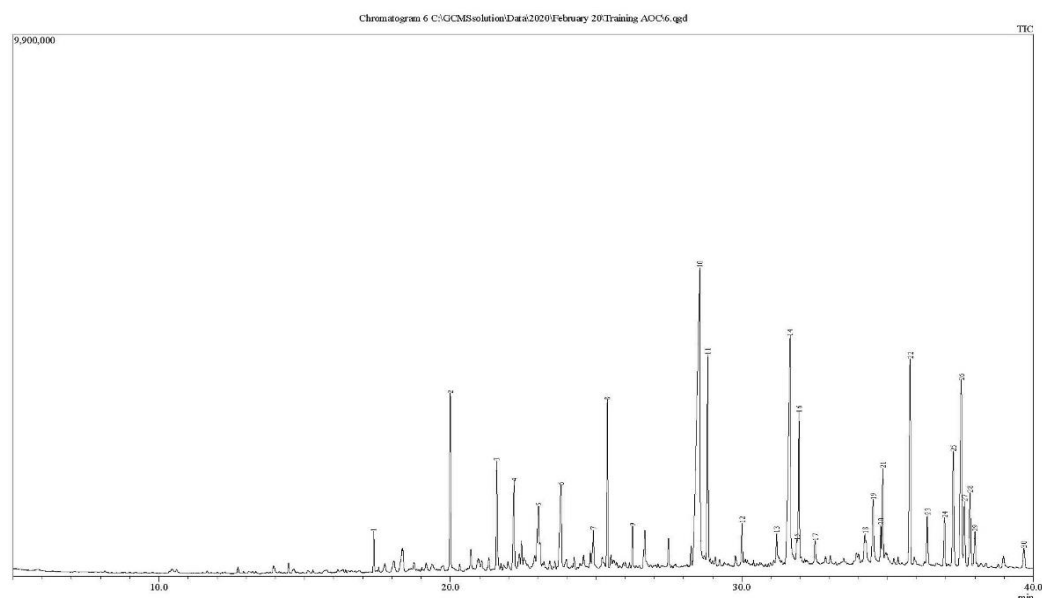


Figure 4.8: Chromatogram of ethyl acetate extract of *H. formicarum* Jack

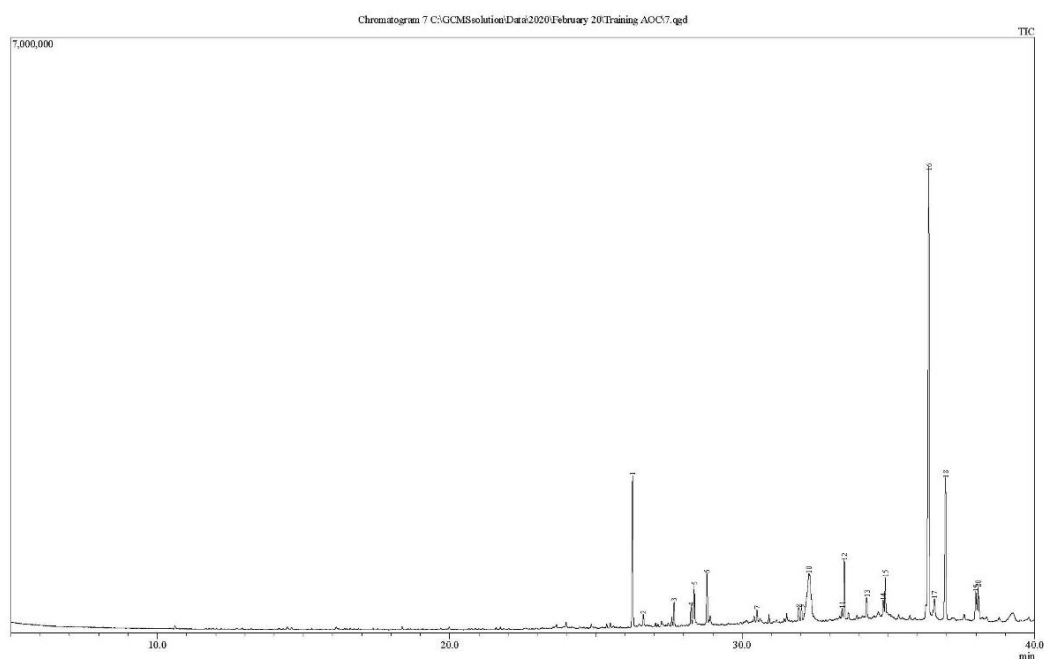


Figure 4.9: Chromatogram of chloroform extract of *H. formicarum* Jack

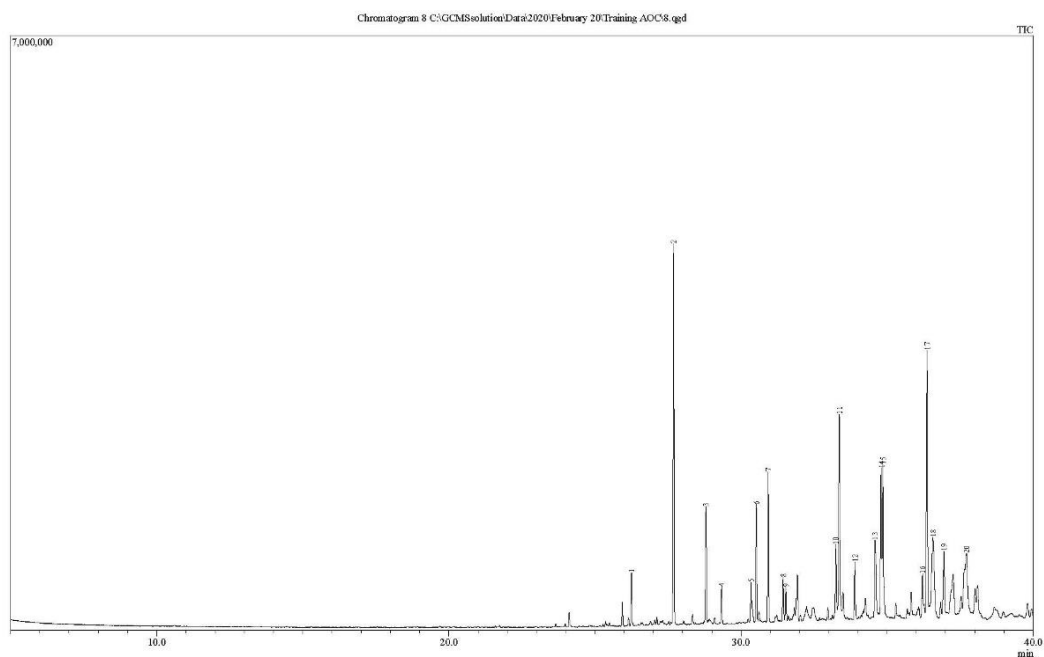


Figure 4.10: Chromatogram of hexane extract of *H. formicarum* Jack

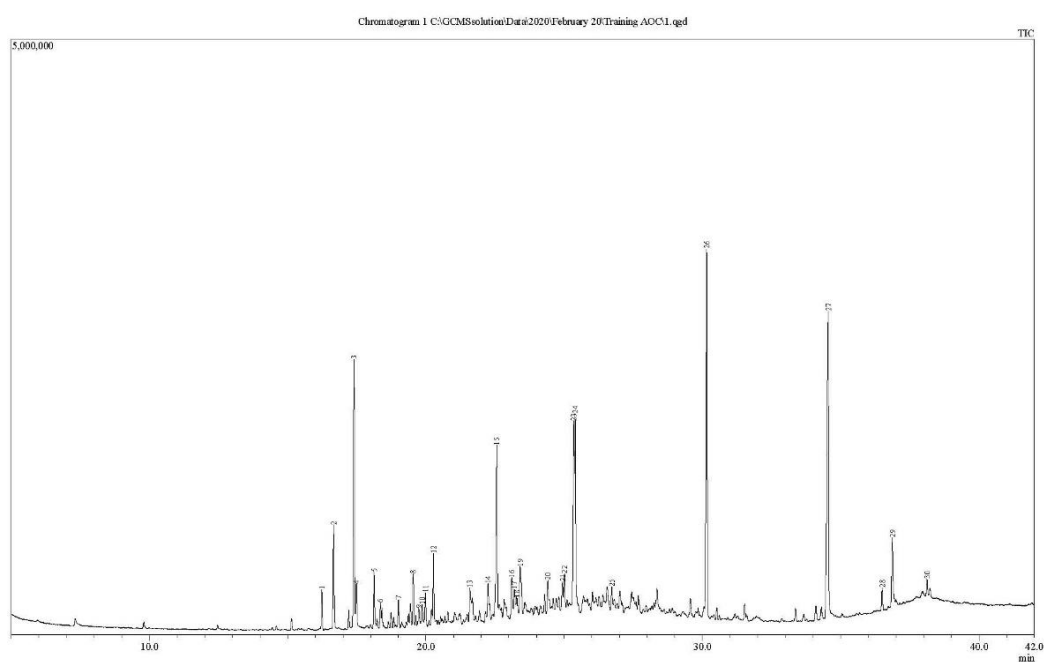


Figure 4.11: Chromatogram of methanol crude extract of *B. stenophylla*

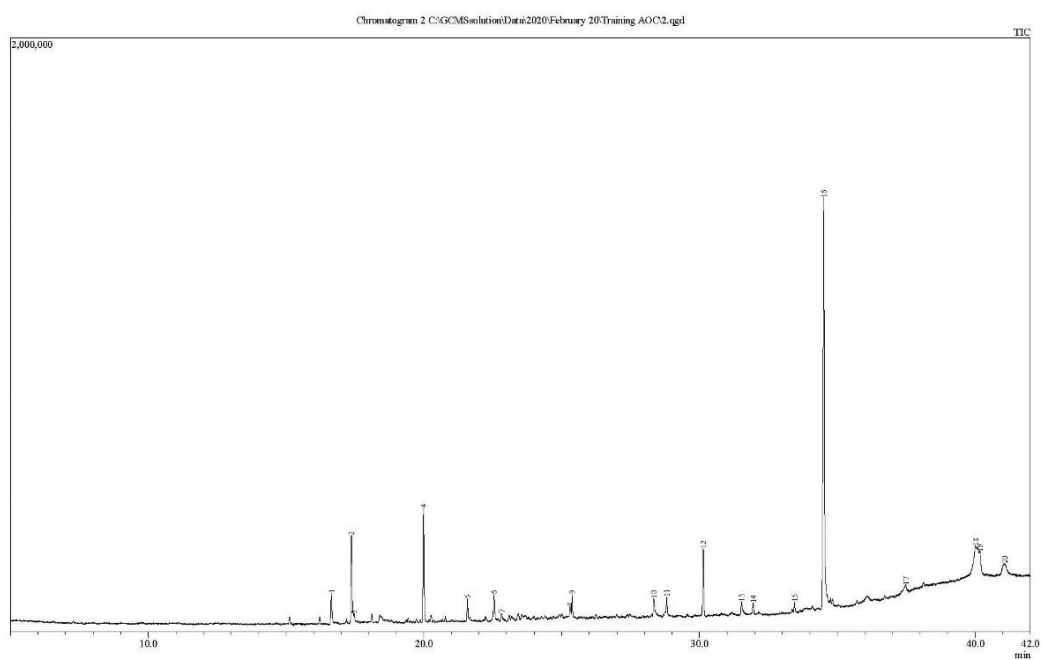


Figure 4.12: Chromatogram of ethyl acetate crude extract of *B. stenophylla*

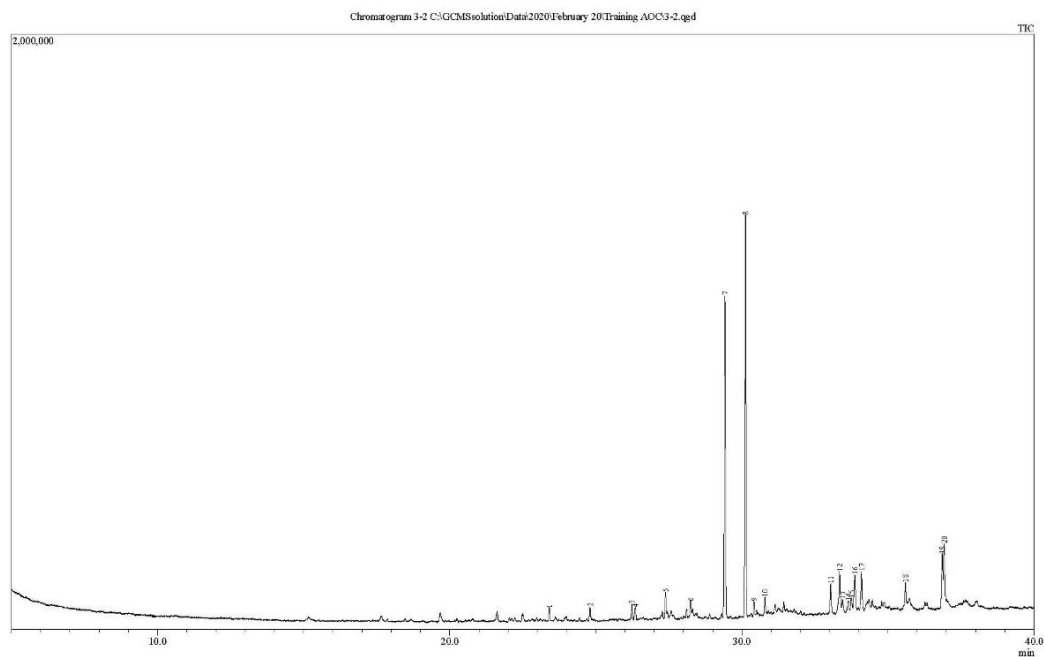


Figure 4.13: Chromatogram of chloroform crude extract of *B. stenophylla*

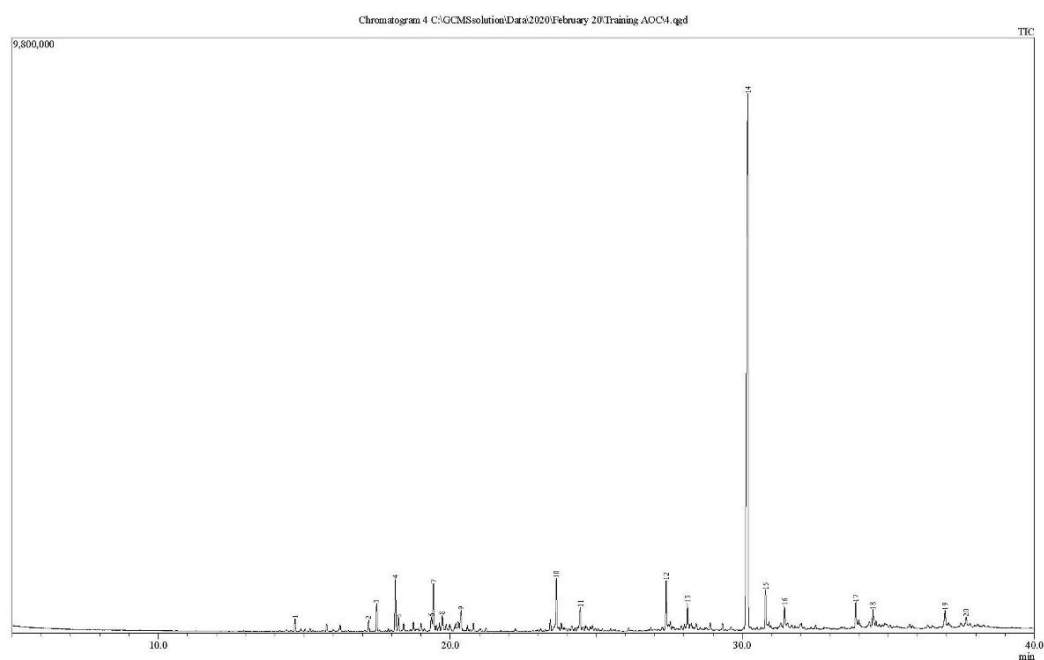


Figure 4.14: Chromatogram of hexane crude extract of *B. stenophylla*

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Overall, all tested antibiotics employed in this study showing their effectiveness against isolated *Leptospira* spp. in Sarawak. In this study, the broth microdilution method of antimicrobial susceptibility testing has been successfully conducted to assess the minimum inhibitory concentration of antibiotics and anti-leptospiral activity of two indigenous herbs against locally isolated pathogenic and intermediate *Leptospira* spp. in Sarawak. Overall, locally isolated *Leptospira* spp. susceptible against tested antibiotic and two indigenous herbs show antimicrobial activity against *Leptospira* spp..

Ethyl acetate is the effective extractant for both *H. formicarum* Jack and *B. stenophylla* extractant against both pathogenic and intermediate *Leptospira* spp. Tuber extract of *H. formicarum* Jack showed better antimicrobial activity against both pathogenic and intermediate *Leptospira* spp.. with lowest MIC values of 125 µg/mL and 62.5 µg/mL. In the other hand, the leave extract of *B. stenophylla* using ethyl acetate of indicate better inhibition against pathogenic *Leptospira* spp.. compare to rhizome extract with the lowest MIC value of 62.5 µg/mL.

In this study, the possibility of antibiotic resistance among local pathogenic and intermediate *Leptospira* spp. cannot be ruled out. Antibiotic resistance is a worldwide medical concern that can have major medical consequences and make treating individuals with leptospirosis challenging. Hence, it is important to continue find new alternative solution by using natural products. Besides, to determine effective antibiotic for leptospirosis treatment and the sensitivity of local isolates to antibiotics, further study on larger numbers of environmental isolates in Sarawak and an increase in the class of antibiotics is required.

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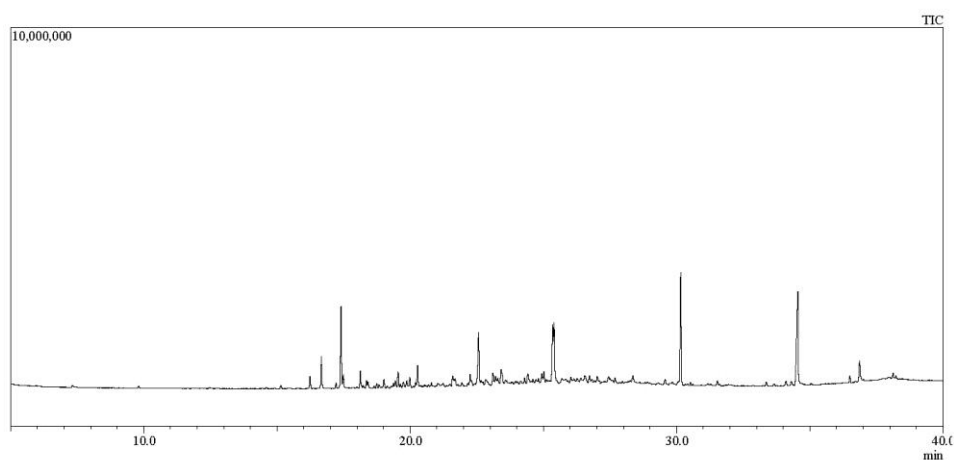
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APPENDICES

Appendix A: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/29/2020 3:31:45 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name :
 Sample ID : 1
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 1
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC1.qgd

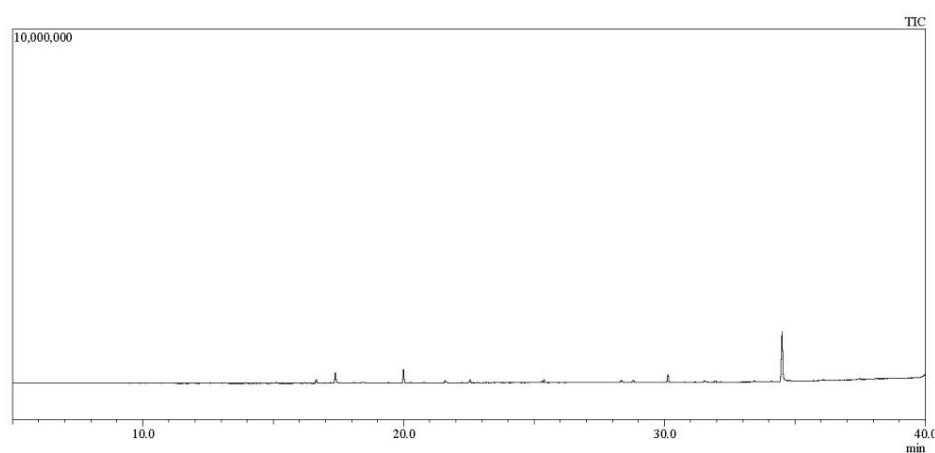


Peak#	R.Time	Area	Area%	Height	Height%	Similarity	Index	Name
1	16.235	752367	1.27	332023	1.68	96		Elemene <delta>
2	16.663	2267515	3.81	861945	4.35	97		Eugenol
3	17.402	6039749	10.16	2239425	11.31	97		2-Propenoic acid, 3-phenyl-, methyl ester
4	17.488	915428	1.54	351879	1.78	93		Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methyl-2-propenyl)-
5	18.127	1159164	1.95	464155	2.34	96		Santalene <alpha>
6	18.357	474098	0.80	205050	1.04	94		Cyclohexane, 1-ethenyl-1-methyl-2-(1-methyl-2-propenyl)-
7	19.011	578434	0.97	230407	1.16	96		Humulene <alpha>
8	19.542	1072120	1.80	430480	2.17	91		Germaacene D
9	19.742	411041	0.69	135965	0.69	93		Naphthalene, decalhydro-4a-methyl-1-methyl-
10	19.865	556622	0.94	173636	0.88	87		Cubebol <epi>
11	19.982	841509	1.41	277586	1.40	90		1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octa-
12	20.274	1398223	2.35	583592	2.95	85		Murolene <gamma>
13	21.589	422187	0.71	182411	0.92	77		cis-1-Chloro-9-octadecene
14	22.247	767471	1.29	286540	1.45	94		(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabi-
15	22.566	4456497	7.49	1399441	7.07	95		Isospathulenol
16	23.106	1107004	1.86	325860	1.65	88		Cadin-4-en-10-ol
17	23.202	732137	1.23	221567	1.12	92		Neointermedeol
18	23.295	597240	1.00	148272	0.75	85		(1R,4S)-4-Isopropyl-1,6-dimethyl-1,2,3,4-tetra-
19	23.409	1717361	2.89	401651	2.03	80		trans-Sesquibutene hydrate
20	24.411	536421	0.90	215966	1.09	77		Allopregnane-7.alpha.,11.alpha.-diol-3,20-diol-
21	24.940	726254	1.22	223781	1.13	76		.alpha.-Kessyl acetate
22	25.015	774098	1.30	287263	1.45	72		5,8,10-Undecatrien-3-ol, 2,3-dimethyl-
23	25.347	4534389	7.62	1537210	7.77	88		Isospathulenol
24	25.397	4259439	7.16	1600751	8.09	80		1,1,4,7-Tetramethyldecalhydro-1H-cyclopropa]
25	26.725	438971	0.74	171509	0.87	74		(1S,3aR,4R,8R,8aS)-1-Isopropyl-3a-methyl-7-
26	30.154	8537765	14.36	3054868	15.43	90		Kaurene
27	34.549	10632020	17.88	2555405	12.91	92		(E)-4-Methoxy-6-styryl-2H-pyran-2-one
28	36.504	472191	0.79	188079	0.95	96		Tetracontane

Appendix B: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/29/2020 4:25:00 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 2
 Sample ID : 2
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 2
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC32.qcd

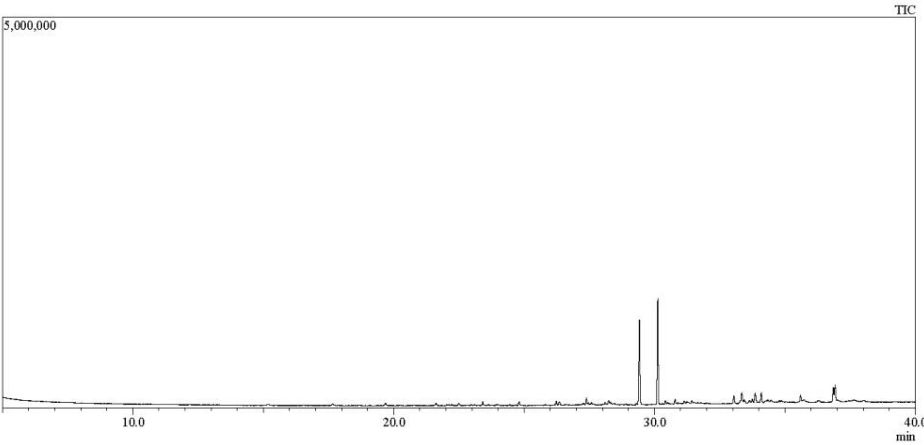


Peak Report TIC						
Peak#	R.Time	Area	Area%	Height	Height%	Similarity Index Name
1	16.644	315444	2.70	105495	3.32	97 Eugenol
2	17.375	862864	7.39	295565	9.29	94 2-Propenoic acid, 3-phenyl-, methyl ester
3	17.472	66960	0.57	26696	0.84	88 Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-me
4	19.989	1045512	8.95	387795	12.19	95 2,4-Di-tert-butylphenol
5	21.582	168109	1.44	71602	2.25	95 Octadec-1-ene
6	22.543	224273	1.92	83140	2.61	94 Isospathulenol
7	22.825	63315	0.54	22083	0.69	74 Benzene, 1-(1-buten-3-yl)-2-vinyl-
8	25.304	86576	0.74	33478	1.05	87 Isospathulenol
9	25.375	165213	1.41	75271	2.37	96 Octadec-1-ene
10	28.338	204311	1.75	60084	1.89	92 4-(+)-Ascorbic acid 2,6-dihexadecanoate
11	28.812	126254	1.08	55117	1.73	95 1-Nonadecene
12	30.132	615966	5.28	226450	7.12	88 Kaurene
13	31.522	118114	1.01	37719	1.19	72 Isopropyl myristate
14	31.946	83596	0.72	35893	1.13	93 Eicosyl trifluoroacetate
15	33.448	75918	0.65	33199	1.04	87 13-Tetradecen-1-ol acetate
16	34.508	5401949	46.27	1393723	43.80	92 (E)-4-Methoxy-6-styryl-2H-pyran-2-one
17	37.483	175585	1.50	25918	0.81	80 Octacosanol
18	40.016	1214359	10.40	99236	3.12	83 gamma-Sitosterol
19	40.178	336820	2.88	80602	2.53	87 Decanedioic acid, bis(2-ethylhexyl) ester
20	41.069	324892	2.78	33276	1.05	82 Stigmasteryl
		11676030	100.00	3182342	100.00	

Appendix C: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
Analyzed : 9/30/2020 8:58:33 AM
Sample Type : Unknown
Level # : 1
Sample Name : 3-2
Sample ID : 3-2
Sample Amount : 1
Dilution Factor : 1
Vial # : 3
Injection Volume : 1.00
Data File : C:\GCMSsolution\Data\2020\February 20\Training AOC3-2.qgd

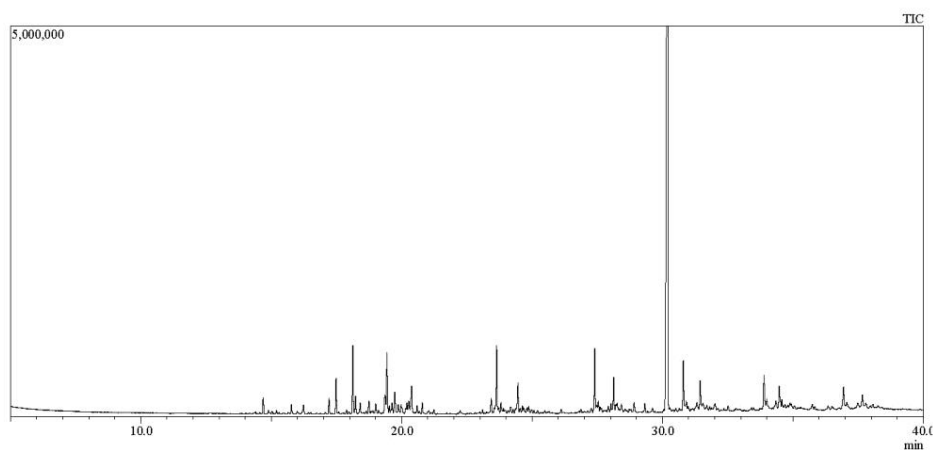


Peak Report TIC							
Peak#	R.Time	Area	Area%	Height	Height%	Similarity	Index Name
1	23.412	103574	0.95	44098	1.13	95	Naphthalene, 1,6-dimethyl-4-(1-methylethyl)-
2	24.803	120057	1.10	45567	1.17	93	2-Pentadecanone, 6,10,14-trimethyl-
3	26.237	110280	1.01	50469	1.29	93	2-Pentadecanone, 6,10,14-trimethyl-
4	26.354	116779	1.07	38870	1.00	89	Hexacosane, 1-iodo-
5	27.386	237505	2.18	88461	2.27	79	Di-3,7-dimethyl-1-octyl phthalate
6	28.243	135578	1.25	52822	1.35	95	Dibutyl phthalate
7	29.420	3209741	29.51	1080172	27.68	88	Kaurene
8	30.125	3488765	32.07	1356564	34.76	89	Kaur-16-ene
9	30.416	115130	1.06	47643	1.22	89	Dotriacontane
10	30.788	127253	1.17	56891	1.46	89	Tetracosane
11	33.042	276332	2.54	97974	2.51	75	1-Naphthalenepropanol, alpha-ethyldecylhydr
12	33.340	423374	3.89	133481	3.42	75	Androstan-6-one, (5.alpha.)-
13	33.435	114396	1.05	42165	1.08	79	13-Tetradecen-1-ol acetate
14	33.646	95889	0.88	29795	0.76	75	(E)-Labda-8(17),12-diene-15,16-dial
15	33.747	129817	1.19	39980	1.02	86	Tetracosane
16	33.862	400483	3.68	119902	3.07	72	1-Heptatriacontanol
17	34.094	382969	3.52	129171	3.31	76	(E)-Labda-8(17),12-diene-15,16-dial
18	35.598	188660	1.73	75041	1.92	90	Cyclohexane, 1,3,5-triphenyl-
19	36.854	532518	4.90	174536	4.47	95	Phthalic acid, bis(2-ethylhexyl) ester (6CI,8CI
20	36.923	568671	5.23	198838	5.10	95	Bis(2-ethylhexyl) phthalate
		10877771	100.00	3902440	100.00		

Appendix D: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/30/2020 2:13:38 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 4
 Sample ID : 4
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 4
 Injection Volume : 1.00
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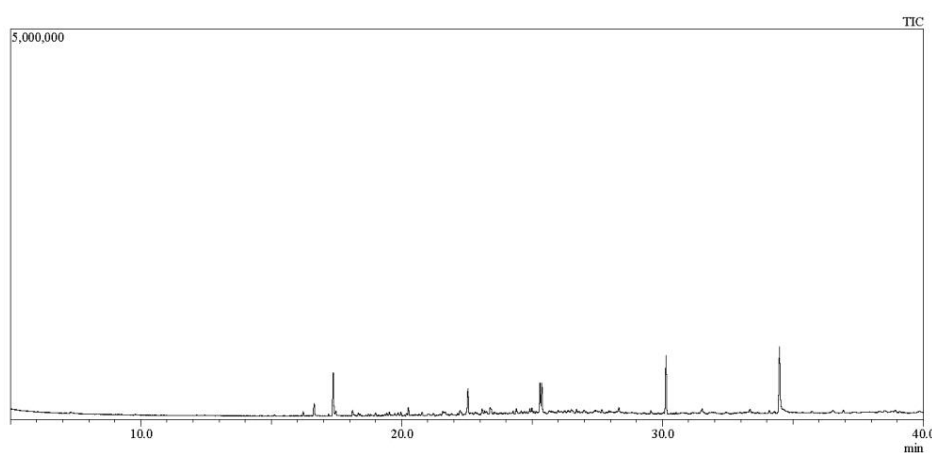


Peak Report TIC						
Peak#	R.Time	Area	Area%	Height	Height%	Similarity
1	14.689	438909	0.86	208795	1.25	93
2	17.211	438625	0.86	196528	1.18	95
3	17.484	1022221	2.01	452517	2.72	94
4	18.126	1923842	3.78	869755	5.22	96
5	18.231	521301	1.03	222521	1.34	95
6	19.360	719911	1.42	224201	1.35	89
7	19.433	1796540	3.53	766816	4.60	91
8	19.733	647842	1.27	253048	1.52	95
9	20.378	912575	1.79	328676	1.97	91
10	23.639	1653228	3.25	800124	4.80	92
11	24.455	661924	1.30	320778	1.93	91
12	27.399	1658870	3.26	775680	4.66	91
13	28.125	872615	1.72	409997	2.46	91
14	30.188	31985564	62.90	8799203	52.81	91
15	30.799	1486828	2.92	608887	3.65	90
16	31.446	891303	1.75	345539	2.07	89
17	33.891	1126625	2.22	409230	2.46	89
18	34.477	886342	1.74	277938	1.67	89
19	36.943	783811	1.54	252348	1.51	86
20	37.664	425979	0.84	140283	0.84	88
		50854855	100.00	16662864	100.00	

Appendix E: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/30/2020 3:00:59 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 5
 Sample ID : 5
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 5
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC\5.qgd

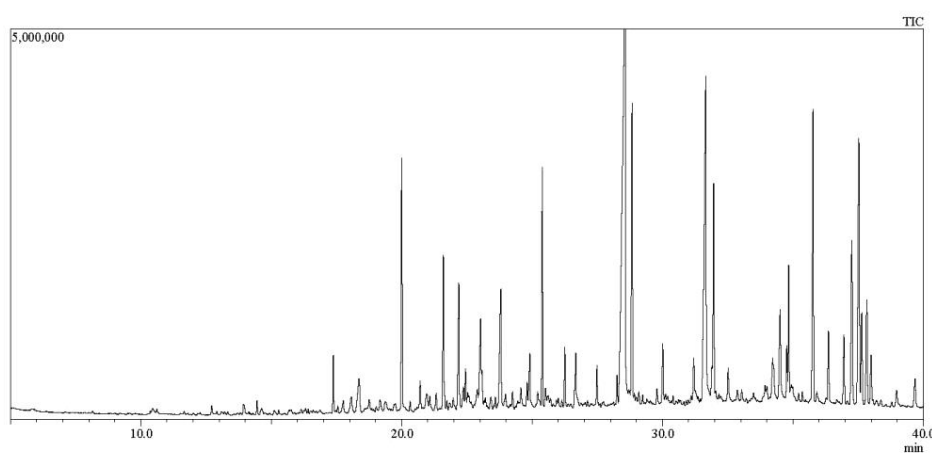


Peak Report TIC							
Peak#	R.Time	Area	Area%	Height	Height%	Similarity	Ref. Index Name
1	16.641	399737	3.50	160593	3.80	97	Eugenol
2	17.372	1376275	12.03	546749	12.93	98	2-Propenoic acid, 3-phenyl-, methyl ester
3	17.472	151379	1.32	57593	1.36	94	Cyclohexane, 1-ethenyl-1-methyl-2,4-bis(1-methyl-2-propenyl)-
4	18.113	126265	1.10	62061	1.47	94	Santalene <alpha>
5	19.971	125333	1.10	48094	1.14	90	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-octa
6	20.260	246988	2.16	107844	2.55	85	gamma-Muurolene
7	22.232	145767	1.27	59046	1.40	92	(1R,3E,7E,11R)-1,5,5,8-Tetramethyl-12-oxabi
8	22.540	952162	8.33	331044	7.83	95	Isospathulenol
9	23.085	206523	1.81	72809	1.72	88	Cadin-4-en-10-ol
10	23.392	231373	2.02	89188	2.11	80	4-(2,2-Dimethyl-6-methylenecyclohexyl)butan
11	23.432	159447	1.39	68623	1.62	63	Cadalene
12	24.395	215340	1.88	62134	1.47	80	Allopregnane-7.alpha.,11.alpha.-diol-3,20-diol
13	24.916	140195	1.23	53987	1.28	75	1,1,4,7-Tetramethyldecalhydro-1H-cyclopropa
14	24.993	153523	1.34	59900	1.42	71	5,8,10-Undecatrien-3-ol, 2,3-dimethyl-
15	25.302	960968	8.40	383392	9.07	87	Isospathulenol
16	25.371	967110	8.46	385202	9.11	80	1,1,4,7-Tetramethyldecalhydro-1H-cyclopropa
17	28.325	121550	1.06	55332	1.31	91	l-(+)-Ascorbic acid 2,6-dihexadecanolate
18	30.134	1914094	16.74	749384	17.72	88	Kaurene
19	31.520	119424	1.04	44483	1.05	62	Tetradecanoic acid
20	34.485	2722228	23.80	831157	19.66	92	(E)-4-Methoxy-6-styryl-2H-pyran-2-one
		11435681	100.00	4228615	100.00		

Appendix F: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/30/2020 3:48:19 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 6
 Sample ID : 6
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 6
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC\6.qgd

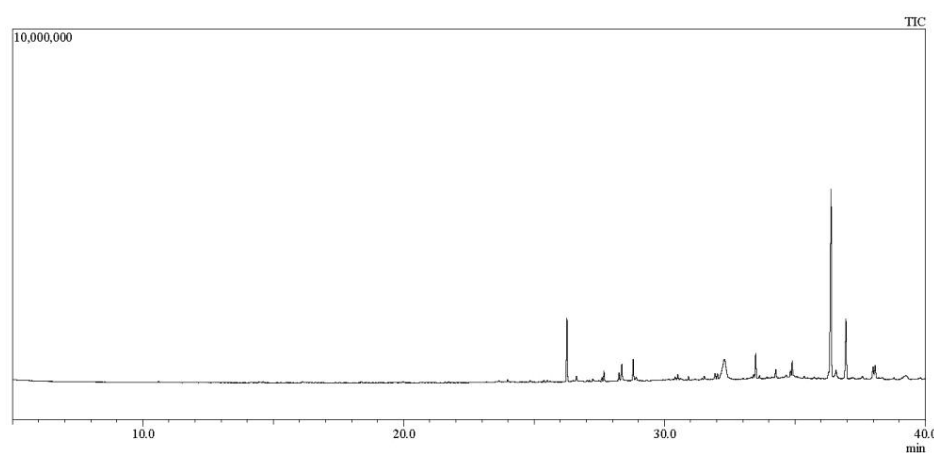


Peak Report TIC							
Peak#	R.Time	Area	Area%	Height	Height%	Similarity	Ref. Index Name
1	17.376	1513109	0.82	730088	1.45	96	1-Tetradecene
2	20.000	7932726	4.30	3199208	6.37	94	2,4-Di-tert-butylphenol
3	21.595	4775555	2.59	1958191	3.90	95	1-Heptadecene
4	22.191	5057807	2.74	1573378	3.13	84	Bicyclo[3.3.1]nonan-2-one
5	23.025	3728954	2.02	1029457	2.05	81	Ethanol, 2-(3,3-dimethylcyclohexylidene), (Z)
6	23.801	5856207	3.17	1450383	2.89	78	Cyclopropanemethanol, 2-methyl-2-(4-methyl-
7	24.910	1359373	0.74	569038	1.13	93	Tetradecanoic acid
8	25.389	6907876	3.74	3043841	6.06	96	1-Nonadecene
9	26.254	1626020	0.88	743298	1.48	93	2-Pentadecanone, 6,10,14-trimethyl-
10	28.560	40813144	22.11	5327110	10.61	96	n-Hexadecanoic acid
11	28.832	10022058	5.43	3700879	7.37	92	Behenic alcohol
12	30.012	1895849	1.03	718912	1.43	92	Heptadecanoic acid
13	31.199	1328347	0.72	474308	0.94	93	Oleic Acid
14	31.659	21886956	11.86	4073296	8.11	93	Octadecanoic acid
15	31.882	1294134	0.70	326290	0.65	81	Octadecanamide
16	31.963	6818331	3.69	2664400	5.31	94	Docosyl heptafluorobutyrate
17	32.516	1128056	0.61	393835	0.78	88	9,12-Octadecadienoic acid (Z,Z)-
18	34.228	2570788	1.39	494367	0.98	79	Oxiraneoctanoic acid, 3-octyl-, cis-
19	34.509	4309492	2.33	1118226	2.23	88	Eicosanoic acid
20	34.766	1511032	0.82	639656	1.27	96	Bis(2-ethylhexyl) adipate
21	34.838	3837796	2.08	1612634	3.21	94	Docosyl heptafluorobutyrate
22	35.772	11119405	6.02	3690300	7.35	92	Cyclohexane, 1,3,5-triphenyl-
23	36.363	2332381	1.26	886784	1.77	96	Hexacosane <n>->
24	36.956	2792329	1.51	883547	1.76	95	Bis(2-ethylhexyl) phthalate
25	37.257	7309873	3.96	2082254	4.15	82	(2,3-Diphenylcyclopropyl)methyl phenyl sulfo
26	37.529	13306164	7.21	3401664	6.78	82	(2,3-Diphenylcyclopropyl)methyl phenyl sulfo
27	37.637	3643564	1.97	1155154	2.30	83	(2,3-Diphenylcyclopropyl)methyl phenyl sulfo
28	37.832	4633288	2.51	1326012	2.64	82	(2,3-Diphenylcyclopropyl)methyl phenyl sulfo

Appendix G: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/30/2020 4:35:38 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 7
 Sample ID : 7
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 7
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC\7.qgd

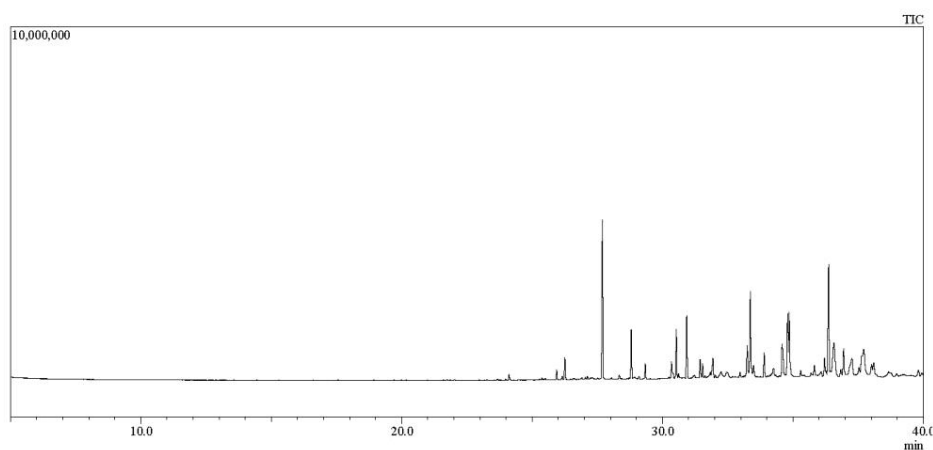


Peak Report TIC							
Peak#	R.Time	Area	Area%	Height	Height%	Similarity	Ref. Index Name
1	26.257	3920421	9.32	1769789	12.74	93	2-Pentadecanone, 6,10,14-trimethyl-
2	26.625	312903	0.74	130019	0.94	86	Phthalic acid, 4,4-dimethylpent-2-yl isobutyl e
3	27.677	622633	1.48	287203	2.07	96	Hexadecanoic acid, methyl ester
4	28.257	461745	1.10	217266	1.56	97	Dibutyl phthalate
5	28.363	1132075	2.69	441359	3.18	94	n-Hexadecanoic acid
6	28.799	1306733	3.11	583945	4.21	95	Hexadecanoic acid, ethyl ester
7	30.508	329497	0.78	137807	0.99	96	Tetracosane <n>
8	31.937	408756	0.97	155517	1.12	89	Stearate <ethyl>
9	32.031	313958	0.75	145248	1.05	96	Pentacosane
10	32.286	4736187	11.26	511698	3.68	96	Decanedioic acid, bis(2-ethylhexyl) ester
11	33.422	260480	0.62	113824	0.82	91	Octacosanol
12	33.496	1520940	3.62	688926	4.96	97	Tricosane <n>
13	34.262	577181	1.37	232800	1.68	94	4,8,12,16-Tetramethylheptadecan-4-olide
14	34.826	448273	1.07	178915	1.29	88	Eicosyl trifluoroacetate
15	34.897	948532	2.26	427833	3.08	96	Tetracosane <n>
16	36.384	16548640	39.36	5289625	38.09	96	Tetracontane
17	36.576	1030277	2.45	227373	1.64	89	2-Pentatriacontanone
18	36.957	5019876	11.94	1662454	11.97	96	Bis(2-ethylhexyl) phthalate
19	37.994	1009341	2.40	318784	2.30	92	1-Hexacosanol
20	38.076	1140484	2.71	366218	2.64	96	Hexacosane <n>
		42048932	100.00	13886603	100.00		

Appendix H: GC-MS Spectrum

GCMS ANALYSIS

Analyzed by : Admin
 Analyzed : 9/30/2020 5:23:11 PM
 Sample Type : Unknown
 Level # : 1
 Sample Name : 8
 Sample ID : 8
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 8
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Data\2020\February 20\Training AOC\8.qcd



Peak Report TIC						
Peak#	R.Time	Area	Area%	Height	Height% Similarity	Index Name
1	26.255	1307328	1.92	613117	2.44	94 2-Pentadecanone, 6,10,14-trimethyl-
2	27.695	10565445	15.48	4457554	17.76	95 Hexadecanoic acid, methyl ester
3	28.801	2945228	4.31	1357721	5.41	95 Hexadecanoic acid, ethyl ester
4	29.337	938268	1.37	429656	1.71	97 Heptadecanoic acid, methyl ester
5	30.346	850370	1.25	388672	1.55	91 Hexadecanoic acid, propyl ester
6	30.526	3124295	4.58	1380323	5.50	95 9-Octadecenoic acid, methyl ester, (E)-
7	30.928	4039424	5.92	1764221	7.03	97 Methyl stearate
8	31.439	1240666	1.82	509974	2.03	97 (Z)-18-Octadec-9-enolide
9	31.537	906071	1.33	388661	1.55	94 (E)-9-Octadecenoic acid ethyl ester
10	33.245	1910233	2.80	812117	3.24	87 Hexadecanoic acid, hexyl ester
11	33.367	5864757	8.59	2312477	9.22	89 Hexadecanoic acid, hexyl ester
12	33.896	1595191	2.34	668670	2.66	97 Eicosanoate <methyl>
13	34.585	3142712	4.60	873401	3.48	84 Hexadecanoic acid, cyclohexyl ester
14	34.795	4579960	6.71	1718192	6.85	87 Hexadecanoic acid, cyclohexyl ester
15	34.855	4529096	6.63	1758398	7.01	86 Hexadecanoic acid, cyclohexyl ester
16	36.213	980763	1.44	421377	1.68	85 Octadecanoic acid, hexyl ester
17	36.368	8243114	12.08	3048721	12.15	96 Tetracontane
18	36.565	4654980	6.82	820580	3.27	96 Dotriacontane
19	36.944	2122358	3.11	725826	2.89	96 Bis(2-ethylhexyl) phthalate
20	37.714	4724768	6.92	642372	2.56	88 15-Methylnonacosane
		68265027	100.00	25092030	100.00	