Faculty of Resource Science and Technology

Antimicrobial Activity of Red Jerangau (*Boesenbergia stenophylla*) Extract Against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes*

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Bachelor of Science with Honours
(Resource Biotechnology)
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Antimicrobial Activity of Red Jerangau (*Boesenbergia stenophylla*) Extract Against *Staphylococcus aureus, Salmonella typhimurium,* and *Listeria monocytogenes*

Vallirie Easter anak Johnson

A Final Year Project Report Submitted in Partial Fulfilment of Requirement for the Degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr Samuel Lihan

Resource Biotechnology

Faculty Resource Science and Technology

Universiti Malaysia Sarawak

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Final Year Project Report ☑

Masters ☐

PhD ☐

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ABSTRACT

Red jerangau (*Boesenbergia stenophylla*), found mainly in the Borneo highland, is a rare species of ginger used in folklore remedies to treat food poisoning, alcohol intoxication, and stomach aches. In this study, extracts of *B. stenophylla* were tested for antimicrobial properties against *Staphylococcus aureus*, *Salmonella typhimurium*, and *Listeria monocytogenes*, all of which may cause food poisoning. The leaf, rhizome, and stem part of the plant were extracted using hexane, ethyl acetate, and water. These extracts were then screened for their bioactivity using agar-well diffusion method and were further evaluated for their minimum inhibitory concentration (MIC). The zones of inhibition ranged between 0 – 10 mm, while its MIC ranged from >360 – 180 μg/μL. Stem hexane and stem ethyl acetate showed good antimicrobial activity against *S. aureus* and *S. typhimurium*, however the extracts showed to be the least effective against *L. monocytogenes*. Compounds of the two extracts were separated using thin-layer chromatography (TLC) with a solvent system of hexane: ethyl acetate 9:1. The TLC managed to separate a compound with an R_f value of 0.382. Further research on its active compounds should be done to justify the traditional use of the plant and the potential use of it as an antimicrobial agent.

Keywords: Red jerangau, antimicrobial, well diffusion, minimum inhibitory concentration

ABSTRAK

Jerangau merah (Boesenbergia stenophylla), yang ditemui terutamanya di tanah tinggi Borneo, adalah spesis halia yang jarang ditemui dan dijadikan sebagai ubat tradisional bagi merawat keracunan makanan, keracunan alkohol, dan sakit perut. Dalam kajian ini, sifat antimikrobial ekstrak B. stenophylla diuji terhadap Staphylococcus aureus, Salmonella typhimurium, dan Listeria monocytogenes, semuanya boleh menyebabkan keracunan makanan. Bahagian daun, rizom, dan batang tumbuhan diekstrak menggunakan heksana, etil asetat, dan air. Ekstrak ini kemudiannya disaring untuk bioaktiviti mereka dengan menggunakan kaedah penyerapan perigi agar dan selanjutnya dinilai untuk kepekatan penghalang minimum (MIC). Zon inhibisi antara 0-10 mm, manakala MICnya berkisar dari >360-180 μg/μL. Ekstrak batang heksana dan batang etil asetat menunjukkan aktiviti antimikrob yang baik terhadap S. aureus dan S. typhimurium manakala ekstrak itu tidak menunjukkan sebarang keberkesanan terhadap L. monocytogenes. Sebatian dua ekstrak telah dipisahkan menggunakan kromatografi lapisan nipis (TLC) menggunakan system sebatian hexane: etil asetat 9:1. TLC dapat memisahkan kompoun yang mempunyai R_f value sebanyak 0.382. Penyelidikan lanjut mengenai sebatian aktifnya harus bagi membenarkan penggunaan tradisional tumbuhan dan penggunaannya sebagai agen antimikrobial.

Kata kunci: *Jerangau merah, antimikrobial, penyerapan perigi agar, kepekatan penghalang minimum*

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List of Abbreviations

%	Percentage
°C	Degree Celcius
ELISA	Enzyme-linked immunosorbent assay
g	Gram
hVISA	Heterogenous vancomycin-intermediate *S. aureus*
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
mrMRSA	Multi-resistant MRSA
MRSA	Methicilllin-resistant *S. aureus*
MSSA	Methicillin-susceptible *S. aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mg	Milligram
MIC	Minimum inhibitory concentration
mL	Millilitre
mm	Millimetre
OD_{600}	Optical density measured at 600 nm
R_f	Retention factor
rpm	Rotation per minute
Spp.	Species
TLC	Thin-layer chromatography
UV	Ultraviolet
µg	Microgram
µL	Microlitre

CHAPTER 1

INTRODUCTION

Boesenbergia stenophylla or its common name, red jerangau is classified under the family Zingiberaceae. This plant is considered to be highly endemic to Borneo highland and is mainly found at an altitude of above 3,000 ft. in the Tropical Heat Forest of Sarawak. It is used by the locals to cure food poisoning, stomach aches and alcohol intoxication (Toyat *et al.*, 2015).

Staphylococcus aureus and *Listeria monocytogenes* are both gram-positive bacteria while *Salmonella typhimurium* is a gram-negative bacterium which causes food poisoning. Infections caused by these bacteria are usually treated with antibiotics. However, the problem of antibiotic resistance, especially towards commonly used antibiotics for treatment has prompted further research on natural antimicrobial compounds contained within medicinal plants in search for new alternatives for the treatment of drug-resistant infections (Sibanda *et al.*, 2008).

Throughout human history, plants have been used to cure diseases and enhance human health worldwide. Extensive research has been carried out to evaluate its medicinal value and it has been proven to be a useful drug. Several studies on chemotherapeutic potentials of medicinal plants have demonstrated that they can be used as a valuable source for antimicrobial compounds. Protein-like compounds, phenolics compound, tannins and flavonoids, for instance, which are found in medicinal plants plays a crucial role as antimicrobial agents (Ahmad *et al.*, 1998).

The antimicrobial activities of several *Boesenbergia* spp. such as *B. rotunda* and *B. pandurata* have been researched (Pattaratanawadee *et al.*, 2006; Voravuthikunchai *et al.*, 2006), However, there are no published studies in regards of the antimicrobial activities of *B.*

stenophylla.

Therefore, the objectives of this study are:

1. To screen the plant extracts of the leaf, rhizome and stem parts for any antimicrobial activity

2. To determine the antimicrobial minimum inhibitory concentration (MIC) of *B. stenophylla* extracts

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal plants

Throughout human history, people have been utilizing nature as a source of medicine to cure diseases. Towards the late part of 20th century, medicinal plants have become mainstream worldwide due to the increased antibiotic resistant bacterial strains, widespread acceptance of the importance of traditional remedies, as well as the integration of derivatives from medicinal plants into pharmaceutical products. Several types of drugs that originated from medicinal folklore include ephedrine, reserpine, digitoxin, and tubocurarine. In Malaysia, an extensive variety of plant species has been recorded as a medicinal plant species. In Peninsular Malaysia alone, there are recorded to be more than 1300 medicinal plant species. Table 1 below shows the examples of medicinal plants in Malaysia and its medicinal use.

Table 1. Medicinal use of folklore medicines in Malaysia

Family	Scientific name	Local name	Medicinal use
Verbenaceae	*Callicarpa arborea* Roxb.	Tambang besi	Cuts, gastric, rheumatism
Caricaceae	*Carica papaya* L.	Betik	Asthma, fever, gastric
Myrtaceae	*Psidium guajava* L.	Jambu batu	Diarrhea, stomach ache
Zingiberaceae	*Curcuma domestica* Valeton	Kunyit	Bloating, coughs, gastric
	Zingiber officinale Rosc.	Halla	Flatulence, gastric

Note. Adapted from "Review on Some Malaysian Traditional Medicinal Plants with Therapeutic Properties," by A. Alsarhan *et al.*, 2014, *Journal of Basic & Applied Sciences, 10*, p. 160. Copyright 2014 by Lifescience Global.

2.2 Zingiberaceae family

Zingiberaceae family, also known as the ginger family is a family of flowering plants consisting of about 1600 known species. Members of the Zingiberaceae family are typically found to be thriving in moist areas of the tropics and substropics, which include regions that

are dry seasonably. The members of the ginger family often have underground stems or fleshy rhizomes. Many species are economically important as spices, ornamental plants or traditional medicines. Examples of the species are as shown in Table 1 as well as *B. stenophylla*, which is the plant used in this study.

2.2.1 *Boesenbergia stenophylla*

Boesenbergia stenophylla, or commonly known as jerangau merah by the Malays and kaburo adak by the Kelabit and Lun Bawang communities, is considered as a rare species under the family Zingiberaceae. It is highly endemic to the Borneo highland, found to be mainly thriving at an altitude of 3,000 ft. in the Tropical Heat Forest of Sarawak. They prefer to grow on slopes of nearby streams under heavily shaded forest floors. Local communities utilize these rhizomes as a mean to treat alcohol intoxication, food poisoning and stomach aches (Toyat *et al.*, 2015). As of now, there are not much research studies on this plant. Figure 1 shows the red jerangau plant with its leaf, stem, and rhizome labeled.

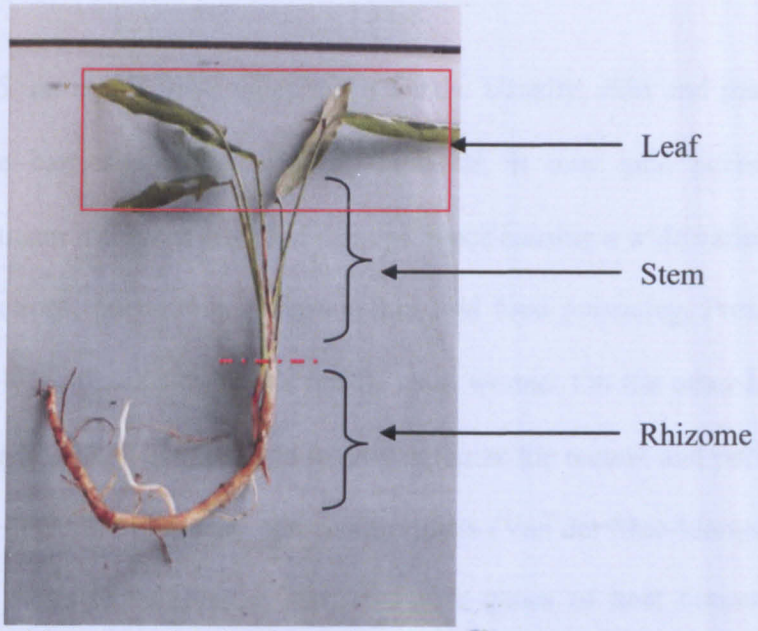

Figure 1. Red jerangau showing its leaf, rhizome and stem.

2.3 *Staphylococcus aureus*

Staphylococcus aureus is a gram-positive, facultatively anaerobic bacteria belonging to the *Staphylococcaceae* family. It is known to affect all known mammalian species, hence it can be transmitted from one species to another. Non-pathogenic *S. aureus* are typically found existing as a commensal, living as a member of the body's normal flora (on the skin, nose), whilst pathogenic strains can cause boils, cellulitis, pneumonia and food poisoning.

2.3.1 Morphology

S. aureus, is a gram-positive coccus with a diameter of 0.5 – 1.5 μm. *S. aureus* are characterized by their individual cocci, dividing itself in more than one plane, which appears as grape-like clusters when observed under the microscope. When grown on agar plates, it will appear as smooth, round, slightly raised, large golden to yellow colonies (Ryan *et al.*, 2004).

2.3.2 Pathogenesis

Often, the transmission of *S. aureus* is via contaminated hands. Usually, skin and mucous membranes as an effective barrier against infection, however, it may gain access to underlying tissues or bloodstream if there is any skin damage hence causing a wide variety of diseases including skin infections, pneumonia, osteomyelitis, and food poisoning. Portal of entry of *S. aureus* are usually a minute surgical or needle stick wound. On the other hand, methicillin-resistant *S. aureus* (MRSA) are found to mainly colonize the rectum and perineal area, as well as the vascular devices, pulmonary and urinary tracts (Van der Mee-Marquet *et al.*, 2004). Once *S. aureus* starts to colonize a host, virulence genes of host tissues are triggered. *S. aureus* show many virulence factors (surface proteins) which promote host

tissues colonization, invasins (kinases, hyaluronidase and leukocidin) that promotes spread of bacteria, and surface factors (protein A and capsule) that inhibit engulfment by phagocytes (Todar, 2004). Microbial Surface Components Recognizing Adhesive Matrix Molecules (MSCRAMM) are a variety of molecules used by the *S. aureus* to adhere and invade host epithelial cells. These molecules, with the aid of the bacterial products, play an important role in the attachment and adhesion to nasal epithelial cells (Weidenmaier *et al.,* 2004).

2.3.3 Clinical manifestations

According to the Minnesota Department of Health (2017), infections caused by *S. aureus* are skin and soft tissue infections such as cellulitis or boils. Cellulitis is an infection of the underlying skin layers. It is usually a result of a cut or scrape in the skin, which in turn, results in the entrance of the said bacteria. Cellulitis commonly occurs on the arms or legs, however, it may occur anywhere else on the body. Symptoms of cellulitis include swelling, redness as well as pain at infection site. Boils or abscesses, are the forming of a warm, painful pus-filled pocket of infection at the site of injury. Surrounding area of the boil is usually red and swollen. Osteomyelitis causes the skin and soft tissues over the infected bone to become red and swollen. Symptoms exhibited include fever, chills and bone pain. *S. aureus* can also cause infection of the lungs (pneumonia) which causes breathing difficulties, fever, malaise, or chills. Asides from that, *S. aureus* is a pathogen that causes food poisoning. Upon ingestion of contaminated chicken and other poultry products, patients may exhibit symptoms of infection by having diarrhea and vomiting after two to six hours.

2.3.4 Treatment

In some cases, oral antibiotics are prescribed for skin infections. However, antibiotic ointments such as non-prescription triple antibiotic mixture are usually used to treat minor

skin infections. If boils or abcesses are present, they are surgically drained. Severe infections involve hospitalization and are treated with intravenous antibiotics. Different types of antibiotics are given by the doctors to treat *S. aureus* infection, depending on the drug-resistance pattern of the bacteria and the severity of the infection. Most *S. aureus* are now resistant to penicillin and methicilllin-resistant *S. aureus* are a common occurrence in the hospital. Nevertheless, some of the main choices for managing serious methicillin-susceptible *S. aureus* (MSSA) infections are penicillinase-resistant penicillins such as dicloxacillin and flucloxallin. As for less serious MSSA infections such as soft tissue and skin infections, first generation cephalosporins (cephalothin, cephalexin, cefazolin), clindamycin, erythromycin and lincomycin plays an important role. Parenteral vancomycin are used to treat all serious MRSA infections. Teicoplanin is used as an alternative if the patient is allergic to vancomycin. The combination of two oral antimicrobials, typically fusidic acid and rifampicin, are used to treat multi-resistant MRSA (mrMRSA) strains. They are used in combination as resistance are rapidly developed if a single agent is used. Quinupristin and linezolid are new antibiotics with good antistaphylococcal activity. However, they are very expensive, hence they are usually reserved for patients who are failed to be treated via conventional therapy or those infected with highly resistant strains such as heterogenous vancomycin-intermediate *S. aureus* (hVISA) (Rayner & Munckhof, 2005).

2.3.5 Prevention and control

According to the Centers for Disease Control and Prevention (CDC) (2016a), it is important to emphasize on a good hand and body hygiene. Hands should be washed often, body cleaned regularly, especially after exercising. Any wounds, cuts or scrapes should be cleaned and covered until it is fully healed. Personal items like razors or towels should not be shared. In order to avoid food poisoning caused by *S. aureus*, it is also advisable to wear disposable

gloves if there are any wounds or infections present on the hands or wrist, although it is better to not prepare food at all with this condition. Food should also be stored in a wide and shallow container. Then, it must be refrigerated as soon as possible (CDC, 2016b).

2.4 *Salmonella typhimurium*

Salmonella typhimurium is a pathogenic gram-negative, flagellated facultative anaerobe belonging to the family of *Enterobacteriaceae*. Mainly, it is found in the gastrointestinal tract of mammals. It is also a non-typhoidal serotype of *Salmonella* which induces clinical manifestations such as bacteraemia (blood stream infections) and diarrhea.

2.4.1 Morphology

S. typhimurium is a rod-shaped bacterium. It forms spherical smooth colonies of a diameter of about two to four mm when grown on ordinary agar. It will form bluish-green colonies with black centres indicating, no fermentation of lactose by the species when grown on Hektoen enteric agar (Pier *et al.*, 2004).

2.4.2 Pathogenesis

Most of *Salmonella* spp. serotypes are able to adapt to a variety of animal hosts, including humans. One of the most frequently isolated foodborne pathogens are of the *Salmonella* species. They are usually found in poultry, eggs and dairy products as well as fresh fruits and vegetables (Pui *et al.*, 2011). *S. typhimurium* is commonly transmitted via food, including via eggs and any egg products or processed foods such as jalapeño peppers and chocolate (Feasey *et al.*, 2012; Pande *et al.*, 2016). Infection of *S. typhimurium* occurs when the cells enter the epithelial cells of the intestinal lining. This causes ruffling of host cells, damaging the microvilli on the cell surface temporarily. White blood cells then rushes into the mucosa,

fluoroquinolone and cephalosporins (Feasey *et al.*, 2012).

2.4.5 Prevention and control

According to Eng *et al.* (2015), an effective measure to eliminate the possible transmission of *S. typhimurium* is by ensuring the safety of the water consumed. Asides from that, as the *Salmonella* spp. can be in a wide variety of food, it is encouraged to handle and cook the food properly to prevent contamination of food. Another way to prevent contamination of *S. typhimurium* is by restricting the use of antibiotics inappropriately in food animals and their food.

2.5 *Listeria monocytogenes*

Listeria monocytogenes is a gram-positive, non-spore forming bacterium belonging to the *Listeriaceae* family. It can be resistant towards the effects of heat, drying and freezing hence, allowing it to grow on refrigerated foods (Sallami *et al.*, 2006).

2.5.1 Morphology

L. monocytogenes is a rod-shaped bacterium. When grown on a haemolytic ceftazidime lithium chloride agar with horse blood incorporated in the agar overlay, *L. monocytogenes* will form blue-gray colonies that are surrounded by narrow zones of β-hemolysis (Poysky *et al.*, 1993).

2.5.2 Pathogenesis

Infection of *L. monocytogenes* causes listeriosis. It is typically associated with the ingestion of raw and contaminated foods, such as raw vegetable, raw poultry, unpasteurized milk, and

smoked fish (Rouquette & Berche, 1996; Dykes, 2003). The bacteria infect the organism by invading the gastrointestinal epithelium, then entering the macrophages, monocytes, or polymorphonuclear leukocytes of the host. Once it is blood-borne, the *L. monocytogenes* multiplies inside the host's body. This causes it to have access to the transplacental migration to the fetus in pregnant women and the brain (Kazmierczak *et al.*, 2005).

2.5.3 Clinical manifestations

Listeriosis is a severe foodborne disease that can cause a variety of symptoms. Some causes diarrhea and fever. Infection of elderly patients, immunosuppressed individuals, and pregnant women may exhibit a more severe clinical manifestations including bacteraemia. Compared to other common foodborne pathogens (*Camplylobacter* spp. *Shigella* spp., *Salmonella* spp.), the incidence of listeriosis is rather low. However, the symptoms are more severe and often times, are fatal. One of the clinical manifestations of listeriosis is gastroenteritis (Schuppler & Loessner, 2010). Another one is neonatal listeriosis. This occurs when a pregnant woman contracts listeriosis. Typically, they experience mild fever and other flu-like symptoms such as muscle aches and fatigue. Rarely do they contract a full blown case of listeriosis which manifests as a respiratory distress syndrome, pneumonia, shortness of breath, rash and either hyperthermia or hypothermia. Infections during pregnancy could lead to miscarriage, premature delivery, stillbirth or life-threatening infection of the newborn. In most cases, neonatal deaths from congenital listeriosis are due to respiratory failure and pneumonia (Farber & Peterkin, 1991).

2.5.4 Treatment

Listeriosis typically clears away spontaneously within seven days. However, individuals at increased risk, such as an immunocompromised patient, may require IV antibiotic treatment

to prevent a more severe disease. It is important to administer early effective antibiotic treatment for pregnant women. Currently, ampicillin, either alone or in combination of gentamicin is the main choice to treat infection of *L. monocytogenes*. Some individuals may need to acquire alternative antibiotic treatments due to allergies, for example. Alternatives include the usage of vancomycin, erythromycin and fluoroquinolones (Temple & Nahata, 2000).

2.5.5 Prevention and control

Some of the recommendations to prevent listeriosis by CDC (2016c) are to consume only pasteurized milk and milk products, keep milk and its products refrigerated at minimum a temperature of 5 ^{0}C, store opened hot dog packages no longer than one week and sealed packages no longer than two weeks in the refrigerator. Also, melons are recommended to be consumed or refrigerated (5 ^{0}C) right after it was cut. Melons that are left at room temperature for more than four hours should be thrown away.

2.6 Plant extraction techniques

2.6.1 Soxhlet extraction

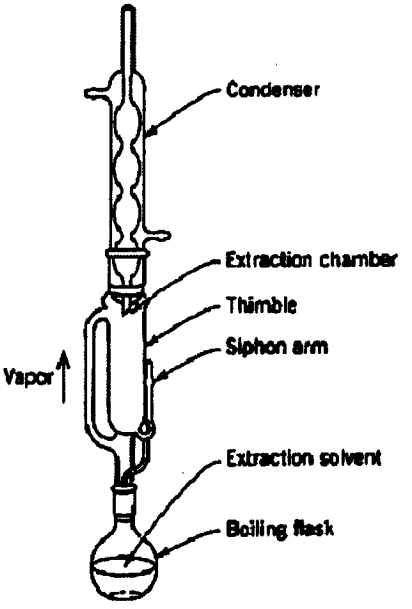

Figure 2. Soxhlet apparatus. Adapted from "Oil extraction from *Calophyllum inophyllum* L. via soxhlet extraction: Optimization using response surface methodology (RSM)," by N. M. Shamsuddin et al., 2015, *10th Asian Control Conference (ASCC)*. Copyright 2015 by IEEE.

In this method, finely ground sample is placed at the thimble chamber. The extraction solvents are heated at the bottom flask of the Soxhlet apparatus, which vaporizes into the sample thimble, then condenses in the condenser and finally dripping back. Once the liquid contents reach the siphon arm, the liquid content is emptied into the bottom flask. This method is not environmentally friendly and solvents used needs to be of high-purity (Azwanida, 2015). Extraction to extract *Azadirachta indica* (Neem) leaf powder in methanol using the Soxhlet extraction method shows numerous phytochemicals were able to be retrieved (Amid *et al.*, 2010).

2.6.2 Maceration

The maceration procedure includes the soaking of plant materials, either powdered or coarse, in a stoppered container containing a solvent. Then, it is allowed to stand at room temperature with frequent agitation for a minimum time period of 3 days. After, the mixture will be strained via filtration. It is the easiest extraction technique. However, due to the usage of a large volume of solvent, organic waste becomes an issue. Hence, a proper waste management is required (Azwanida, 2015). In a study conducted by Arya *et al.* (2012), maceration is used to extract *Psidium guajava* L. leaves. It was found that compared to chloroform, water and petroleum ether extracts, hydroalcohol and ethanol extracts resulted in the highest extraction yield with the highest amount of phytoconstituents.

2.7 Identification and characterization techniques

2.7.1 Chromatographic techniques

Thin layer chromatography (TLC) is a quick and simple method used to determine the number of components in a mixture. It also supports the identity of a compound by comparing the R_f of that compound to the R_f of a known compound. Additional tests using TLC includes the spraying of phytochemical screening reagents that leads to colour changes depending on the phytochemicals present in the plant extracts; or via viewing of TLC plate under the UV lights. Another chromatographic technique is bio-autography. It is used to determine bioactive compound with antimicrobial activity. Bio-autographic technique uses the microorganisms' growth inhibition to detect the antimicrobial components of the plant extracts that are chromatographed on the TLC layer. Clear zones produced on the TLC plates will be used to visualize the position of the bioactive compound with antimicrobial activity by referencing to its R_f values (Sasidharan *et al.*, 2011). On the other hand, gas chromatography–mass spectrometry (GC-MS) is method which combines both the features of