ANTIBACTERIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY STUDIES OF *Lygodium microphyllum*

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ANTIBACTERIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY STUDIES OF *Lygodium microphyllum*

by

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THIS THESIS IS DEDICATED

TO

MY FATHER AHMAD BIN MUSTAFAR (LATE)

&

MY MOTHER ZABIDAH BINTI IBRAHIM

MY HUSBAND REZZA HAZRIQ BIN M. N. KAMAL

MY SON RAYYAN ZAFRAN BIN REZZA HAZRIQ

I OWE SPECIAL THANKS TO ALL OF THEM, ALL MY SIBLINGS & MY BELOVED BIG FAMILY

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LIST OF ABBREVIATIONS

°C	Degree celcius
µg/mL	Microgram per milliliter
Abs	Absorbance
ANOVA	Analysis of variance
ATCC	American type culture collection
BHT	Tert-butylated hydroxytoulene
CO_2	Carbon dioxide
COX	Cyclo-oxygenase
DMSO	Dimethyl Sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Median effective concentration
$FeCl_3$	Ferric chloride
g	Gram
GAE	Gallic acid equivalent
h	Hour(s)
IC_{50}	Inhibitory concentration of 50 %
IL-1	Interleukin-1
L	Litre
MBC	Minimum bactericidal concentration
MeOH	Methyl alcohol
mg	Milligram
mg/ mL	Milligram per mililitre
MIC	Minimum inhibitory concentration
min	Minute(s)

mL	Mililitre
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
$NaHCO_2$	Sodium carbonate
$NaNO_2$	Sodium nitrate
NaOH	Sodium hydroxide
nm	nanometer
NSAID	Non-steroidal anti-inflammatory
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SEM	Standard error of the means
TFC	Total flavonoids content
TLRs	Toll like reseptor
TNF-α	Tumor necrosis factor alpha
TPC	Total phenolic content
WHO	World Health Organization

KAJIAN ANTIBAKTERIA, ANTIOKSIDAN DAN ANTI-INFLAMASI TERHADAP *Lygodium microphyllum*

ABSTRAK

Lygodium microphyllum merupakan paku pakis yang digunakan secara tradisi untuk mengawal cirit-birit berdarah, merawat bengkak dan penyakit kulit, meredakan sedu serta mengawal demam panas dan demam kepialu. Tujuan kajian ini adalah untuk menilai aktiviti antibakteria, antioksidan dan anti-inflamasi lima fraksi pelarut yang diperoleh daripada ekstrak mentah metanol daun paku pakis *L. microphyllum* secara pengekstrakan cecair-cecair dengan heksana, kloroform, etil asetat, n-butanol dan air. Analisis fitokimia dengan menggunakan kaedah "Folin-Ciocalteu" dan kaedah kolorimetri aluminium klorida menunjukkan bahawa fraksi etil asetat mengandungi jumlah kandungan fenolik yang paling tinggi iaitu 11.59 ± 1.02 mg bersamaan sampel asid Gallic (GAE)/g, sementara itu fraksi heksana memaparkan jumlah kandungan flavonoid yang paling tinggi iaitu 60.27 ± 3.53 mg bersamaan sampel quercetin (QE)/g. Keputusan ini juga memperlihatkan bahawa sebatian fitokimia yang lain seperti alkaloid, glikosida, flavonoid, quinone, saponin dan steroid ditemui dalam fraksi heksana dan etil asetat, manakala tanin hanya terdapat dalam fraksi etil asetat. Kajian antibakteria yang dinilai melalui ujian *in vitro* antibakteria menggunakan kaedah mikropencairan medium broth menunjukkan bahawa fraksi etil asetat dan heksana mempamerkan aktiviti antibakteria yang tinggi terhadap semua bakteria enterik klinikal rintang yang diuji, iaitu, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Salmonella typhi* dan *Shigella dysenteriae* serta strain kawalan *Escherichia coli* (ATCC 25922), dengan masing-masing

mempunyai nilai kepekatan perencatan minimum (MIC) dan kepekatan bakterisidal minimum (MBC) iaitu sebanyak 6.25 mg/mL dan 12.5 mg/mL. Aktiviti antioksidan yang ditentukan dengan menggunakan asai pemerangkapan radikal bebas DPPH, asai kuasa penurunan ferricyanide dan asai pelunturan β-karotena/asid linolik menunjukkan bahawa fraksi etil asetat mempunyai aktiviti tertinggi dalam kesemua asai. Sementara itu, kajian anti-inflamasi yang telah dijalankan menggunakan model inflamasi akut, iaitu, model tapak kaki belakang tikus aruhan karaginan menunjukkan bahawa fraksi heksana mempamerkan fraksi yang paling aktif dalam menghalang pembentukan edema. Kesimpulannya, keputusan keseluruhan menunjukkan bahawa paku pakis *L. microphyllum* adalah calon yang berpotensi untuk dikembangkan sebagai agen antibakteria, antioksidan dan anti-inflamasi untuk merawat jangkitan yang disebabkan oleh bakteria enterik, mengurangkan tekanan oksidatif di dalam badan manusia serta sebagai rawatan gangguan inflamasi akut.

ANTIBACTERIAL, ANTIOXIDANT AND ANTI-INFLAMMATORY STUDIES OF *Lygodium microphyllum*

ABSTRACT

Lygodium microphyllum is a fern which has been used traditionally for controlling dysentery, treating swellings and skin diseases, curing hiccups as well as controlling high fever and typhoid fever. The aim of the study was to evaluate the antibacterial, antioxidant and anti-inflammatory activities of five solvent fractions obtained from crude methanol extract of *L. microphyllum* leaves by liquid-liquid extraction of hexane, chloroform, ethyl acetate, n-butanol and water. Phytochemical analysis via Folin-Ciocalteu's method and aluminium chloride colorimetric method showed that ethyl acetate fraction obtained the highest total phenolic content of 11.59 ± 1.02 mg gallic acid equivalents (GAE)/g sample, whereas hexane fraction displayed the highest total flavonoid content of 60.27 ± 3.53 mg quercetin equivalents (QE)/g sample. The results also revealed that other phytochemical compounds such as alkaloid, glycosides, flavonoids, quinones, saponin and steroid were found in hexane and ethyl acetate fractions, whilst tannins were found only in the ethyl acetate fraction. The antibacterial study assessed via *in vitro* antibacterial test using broth microdilution method showed that ethyl acetate and hexane fractions displayed potent antibacterial activity against all tested clinically resistant enteric bacteria, namely, *Escherichia coli, Klebsiella pneumonia*, *Proteus mirabilis*, *Salmonella typhi and Shigella dysenteriae* as well as the control strain isolate *Escherichia coli* (ATCC 25922) with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 6.25 mg/mL and 12.5 mg/mL,

respectively. The antioxidant activity determined via DPPH free radical scavenging assay, ferricyanide reducing power assay and β-carotene/linoleic acid bleaching assay showed that ethyl acetate fraction had the highest activity in all assays. Meanwhile, in the anti-inflammatory study, which was carried out using acute inflammatory model, i.e., carrageenan-induced rat hind paw oedema model showed that hexane fraction exhibited the most active fraction in inhibiting the oedema formation. In conclusion, the results indicated that *L. microphyllum* is a potential candidate to be developed as an antibacterial, antioxidant and anti-inflammatory agent for the treatment of enteric infection, minimizing the oxidative stress in human body as well as treatment of acute inflammatory disorders.

CHAPTER 1

INTRODUCTION

In recent years, interests on the screening and development of new drugs for pharmacological effects including anti-malarial, anti-parasitic, anti-angiogenic, anti-bacterial, anti-inflammatory, anti-diabetic and anti-cancer from plant-based products especially medicinal plants have been studied worldwide. Bioactive compounds that exhibit pharmacological properties from plants have been extracted, separated, isolated and identified continuously today in myriad ways (Sasidharan *et al*., 2011; Azmir *et al.*, 2013). The isolated bioactive compounds for therapeutic purposes are known as plant-derived drugs and have been used for millennia to cure various human diseases including infectious diseases.

Infectious diseases involve two factors which are pathogen and human host. Pathogen agent refers to microorganisms and mostly pathogenic to vulnerable host like human. People around the globe are suffering from infectious diseases caused by a wide range of pathogens, including bacteria (tuberculosis, leprosy, and diarrhea), virus (Human immunodeficiency virus (HIV) and Zika virus) and parasite (malaria). Reactive oxygen species (ROS) is generated from either endogenous (infections and various inflammatory processes) or exogenous source (produce in response to ultraviolet (UV) radiation due to global warming) which contributed in elevating levels of ROS. Disruption of normal cellular homeostasis by redox signaling contributes to disease in virtually every organ including the development of cancer. Factors likes global warming and unpredictable climate changes across the century play an important role in causing the emergence and resurgence of infectious

diseases worldwide. Consequently, the changes of the survival, reproduction and life cycle of microorganisms particularly bacteria through environmental stress have occurred. Therefore, not surprisingly, the impact of extreme climate changes like El Nino, La Nina, flood and drought were being followed by the outbreak of various infectious diseases around the world (Wu *et al.*, 2016). The uncontrolled outbreaks of many infectious diseases in developing countries are due to financial restraint and shortage of medical resources, and have become worst after the emergence of drug-resistant particularly in the 21st century.

Morbidity and mortality amongst humans due to infectious diseases caused by pathogenic microorganisms such as bacterial infection particularly gram-negative bacteria have increased. Since the discovery of penicillin and its beneficial healing effect in multiple infectious diseases, mankind is fully depended upon it and have extensively used the antibiotic as treatment against all kinds of bacterial infections. On the contrary, bacteria has increased their ability to prevent the antibiotic binding to their targets by means of activating the efflux pumps, enzymatic inactivation of antibiotic, and altering of their targets site. Subsequently, the bacteria has managed to develop resistance to antibiotics, thereby declining the effectiveness of available antibiotics against the targeted bacteria and resulting in the human life-threatening infections worldwide (Bhatt *et al.,* 2015).

Enteric bacteria are representative of gram-negative bacteria and known as bacterial gastroenteritis from family Enterobacteriaceae. Habitually, enteric bacteria are capable of triggering infection of the intestinal tract causing diarrhea and dehydration mostly in young children (Petri *et al.*, 2008). Enteric bacteria like *Escherichia coli*, *Shigella* sp. and *Salmonella* sp. are able to colonize and disrupt intestinal function and often lead to diarrhea disease; additionally, enteric bacteria have significant

clinical concerns on their resistance to antibacterial agents especially *E. coli*, in which this enteric infection will end up in treatment failure. No new relevant compounds of drugs have been developed mainly to combat the emergence of antibiotic-resistance in bacteria particularly the gram-negative enteric bacteria (Zabawa *et al.*, 2016). Therefore, the development of new antibiotics able to treat infections caused by enteric bacteria, which cover a broad range of antibiotics inhibiting the target of these bacteria is seriously needed.

Natural products have been recognized as source of therapeutic agents against bacterial infectious diseases. Natural sources from plants are attracting attention globally as potential sources of antibacterial agents (Taylor, 2013). Nowadays, the discovery and development of compounds from the plant kingdom which possess activity against pathogenic bacteria have been explored extensively. Therefore, plant-based products are candidates to be studied to defeat drug-resistance enteric bacteria.

Human diseases caused by adverse effects of oxidative stress have become a serious problem nowadays. Oxidative stress is the situation when there is an imbalance of number between the reactive oxygen species (ROS) and antioxidant available in the body. Normally, the amount of ROS surpasses the normal level, disturb and damage the cells, finally give problems to our health. This imbalance creates an antioxidant deficiency environment and may lead to various diseases, namely, Alzheimer's disease, Parkinson's disease, rheumatoid disease, heart disease, eye disease, cancer and inflammatory disorders. Natural product, namely, plant-based product can play a crucial role to overcome diseases caused by ROS. According to Krishnaiah *et al.* (2011), increasing the level of antioxidant in the body by consuming supplement diet from antioxidant compounds found in plant products is the best solution to prevent oxidative damage from occurring. Antioxidant compounds are able to reduce the

reaction of ROS in damaged tissues by terminating the radical chain reactions, thereby decreasing the incidence of human diseases. Therefore, in this study the antioxidant activity of *Lygodium microphyllum* extracts was explored.

The emergence of various diseases nowadays are caused by the infection of pathogen and the effects of oxidative stress resulting from the reaction of ROS, whereby these factors have a strong correlation with inflammation. Inflammation is believed to be a protective mechanism of the body to eradicate the hazardous stimuli such as morbific pathogens, damaged tissues caused by ROS and also irritants as well as promoting the healing process of the affected tissues by restoring back their normal function and structure. The eradication failure of the hazardous stimuli by immune system can lead to chronic inflammation. Autoimmune diseases such as rheumatoid arthritis, Crohn's disease, lupus, Type 1 diabetes and Addison's disease are examples of chronic inflammations due to persistent attack of leukocytes to healthy and unaffected tissues (Ehlers & Kaufmann, 2010; Franceschi & Campisi, 2014). Therefore, it is important to protect our tissues from damage and gain back the normal tissue functions, and at the same time reduces the potency of the illness getting worse.

Steroids and non-steroidal anti-inflammatory drugs (NSAIDs) are the common drugs used worldwide for the treatment of inflammation. Unfortunately, these type of drugs possess side effects for example corticosteroids causes inherent insufficiency of adrenal (Alves *et al.,* 2008), and aspirin contributes to gastrointestinal problems (Laine, 2003). Due to the side effects; there is now an urgent need to develop new anti-inflammatory agents from natural plant-based which are believed to have less side effects to substitute conventional drugs (Mahesh *et al*., 2011).

Almost all classes of plants play an important role in the development of potent therapeutic agents. Pteridophytes, the non-flowering primitive vascular plants, have also been found to be utilized as traditional medicines of the Chinese (Cao *et al.,* 2015), Indian (Talukdar & Talukdar, 2013) and Native American (Karadeniz *et al.,* 2015). Secondary metabolites are produced by all species of plants including fern for the purpose of defending themselves against environmental stress in order to adapt to their environment (Mazid *et al.*, 2011). Ferns existing on earth for millions of years have survived and successfully adapted along with the environmental stressess due to climate changes. Therefore, there is a belief that ferns consist more functional bioactive compounds that exhibit various pharmacological effects compared to flowering vascular plants (Lee & Shin, 2011).

Lygodium microphyllum (Schizaeaceae) is one of the ferns which has important contributions for prevention and treatment of various human ailments. Studies of the fern leaf decoction have revealed its effects on the control of dysentery, as poultices for skin diseases and swellings, and curing of hiccups (Benniamin, 2011). The Malay old folks have been using these fern leaves to control high fever and typhoid fever. Unfortunately, to date, no comparative studies have been reported on the pharmacological effects of this fern, primarily in Malaysia.

Hence, based on the abundance of *L. microphyllum* in Malaysian ecosystem, and its potential as therapeutic agents, the current research was undertaken to explore the potential pharmacological activities of *L. microphyllum* leaves extracts as an antibacterial, antioxidant and anti-inflammatory agent. In addition to these, the bioactive activities of the crude methanol *L. microphyllum* extract and its fractions may give us ideas for better understanding the correlation of the chemical compounds in exhibiting their pharmacological effects.

1.1 Objectives

The current study was conducted with the following objectives:

i. To evaluate the Malaysian fern crude methanol *L. microphyllum* extract and its fractions as potential anti-bacterial against enteric bacteria and perform the phytochemical analysis.

ii. To identify the antioxidant activities of the crude methanol *L. microphyllum* extract and its fractions.

iii. To investigate the effectiveness of crude methanol *L. microphyllum* extract and its fractions as anti-inflammatory agent.

CHAPTER 2

LITERATURE REVIEW

2.1 Overviews of infectious diseases

Relationships between host and pathogenic bacteria are complex and dynamic. The host such as humans are infected when bacteria are invade, growing and multiplying within the human body. Additionally, many pathogenic bacteria have specialized strategy, i.e., entry into human cells and tissues for survival and multiplication. The nature of an infection is varied and based on the severity, location (appropriate environment within the host, e.g., nutrient, temperature, and pH) and the number of pathogens invading and multiplying to produce an infection. Examples of various types of infection associated with pathogenic bacteria are bacteremia (a condition of viable bacteria present in the blood), septicemia (blood poisoning associated with persistence of bacteria and their toxin in the blood), localized (the infections are restricted to a limited or certain anatomical regions) and abscess (a localized infection with a collection pus surrounded by inflamed area) (Prescott *et al.*, 2008).

An unhealthy state is a condition of any part of the human body that are not capable of doing their normal functions due to the presence of bacteria or its products, is described as a phenomena of infectious disease. Generally, the end result of the relationship between host and bacteria is dependent on the degree of pathogenicity or virulence of the bacteria, the number of invading bacteria to the host as well as the degree of host's defenses (Wilson *et al.*, 2002).

Pathogenic bacteria has at least one reservoir which is a place to live and multiply. Human, animal and the environments are the common reservoirs for pathogenic

bacteria. Initial vehicle of the pathogenic bacteria to the reservoir (or host) such as human is a vital feature in the development of infectious diseases. Bacteria can be transmitted via direct contact which is from human to human by sneezing and coughing; besides, bacteria is also able to transmit indirectly by various ways. For examples, infected humans spread bacteria into their surroundings, and then the bacteria deposited on various surfaces which indirectly transmitted to other humans or resuspended into the air. In addition, soil, water and food also play a role in transmitting the bacteria to humans indirectly (Girard *et al*., 2006).

After being transmitted to the human, bacteria must be able to adhere to and colonize cells or tissues. Pathogenic bacteria can penetrate epithelial surface via passive mechanisms such as ulcers or injury in a mucous membrane, wounds or burns on the skin's surface, tissue damage as well as from the cellular internalization pathways of human which are phagocytosis and endocytosis (Prescott *et al.*, 2008; Kim *et al*., 2012). Generally, pathogenic bacteria attached to the epithelial surface, and then penetrate the human's mucous membranes and epithelium. Bacterial pathogen likely to penetrate deeper into the tissues and spread to all organs and tissues through the production of specific virulence factors which consist of enzymes or bacterial's product assist the dissemination and subsequently entering the small terminal lymphatic capillaries that surrounded the epithelial cells. Consequently, the bacteria successfully reached the whole system and damage the circulatory system; thus, the final determinant of a successful bacterial pathogen is its capability of leaving the human and invading other human or reservoirs (Prescott *et al.,* 2008). Typically, the pathogenic bacteria leave human via urine, feces, droplets, or saliva, and then transmitted to other reservoirs.

Bacterial pathogen can contaminate food and water, and thus causing human infections such as acute gastroenteritis, which is an infection that involved in inflammation of the stomach and intestinal lining. Gastroenteritis can occur in two ways. The first way, the pathogenic bacteria colonized gastrointestinal and multiply within it, and then invades human tissues or release exotoxins. The second way, the exotoxin released by the pathogenic bacteria may contaminate food, that consumed by humans. Food contaminated with bacterial pathogen may produce a food-borne infection and the condition often referred as food poisoning. Meanwhile, food contaminated by bacterial's products is referred to food intoxication and the condition referred to as enterotoxin poisoning. Enterotoxin is released by several bacterial pathogens and the targets are intestines. Toxin secreted by the bacterial pathogen impeded the function of the intestinal mucosa by altering permeability apical membrane of the mucosa cells of the intestinal wall and then kills the cells, thus consequently leads to show symptoms of gastrointestinal infection, viz., vomiting, nausea, abdominal pain, and diarrhea.

2.2 Diarrheal disease

The major health problem nowadays is diarrheal disease. Diarrhea is one of the symptoms and indicative of gastrointestinal infections, which can be caused by various bacterial pathogens such as enteric bacteria (Godana, 2013; Karambu *et al.*, 2013). Additionally, diarrheal disease lead morbidity and mortality particularly among young children around the world (WHO/UNICEF, 2013). Diarrheal infection disseminated through various ways, either from contaminated food or water; or spread from human to human due to poor hygiene.

According to Godana (2013), clinical types of diarrhea can be divided into three, i.e., acute watery diarrhea, acute bloody diarrhea (dysentery) and persistent/chronic diarrhea. However, duration and severity of diarrhea are different between each type of diarrhea, of which the sign and symptom of acute diarrhea are watery diarrhea and bloody diarrhea lasts no longer than two weeks; meanwhiles, the episode of diarrhea of persistent/chronic diarrhea lasts longer than two weeks. In addition, the clinical types of diarrhea also lean on its particular causes and the sources. Therefore, following patient's historical descriptions such as the presence of associated enteric symptoms; history of food ingestion, camping, and travelling; as well as animal exposure may help to recognize the causes or sources of the patient's diarrhea.

Normally, mild watery diarrhea, a condition of not showing the symptom of fever or bloody feces is associated with non-invasive enteropathogens; whereas, severe watery diarrhea, which shows enteric symptoms often linked to the presence of enteric bacterial pathogens, i.e., enterotoxigenic *E. coli (*ETEC) (Kumar *et al.*, 2015). Meanwhile, bloody diarrhea (dysentery) which showed enteric symptoms, bloody feces and the microscopic presence of red and white blood cells in the stool sample is caused by enteropathogens such as bacterial pathogens from the genera *Salmonella*, *Shigella*, *Yersinia* and *Campylobacter* that attack intestinal tract (Chowdhury *et al.*, 2010).

Dehydration is the most severe threat during diarrhea episode. Dehydration occurs when water as well as electrolytes such as potassium, chloride and sodium, which lost via liquid stools, sweat, urine and vomit are not replaced. Severe dehydration may occur if the water and electrolytes are not replenished; thus, severe dehydration often followed by death. Zinc supplements are proven life-saving of young children less than five years of age by reducing the duration of diarrhea; since, diarrhea

problem of young children always associated with zinc deficiency and during diarrhea, zinc also loses through dehydration process (Sazawal *et al.*, 1995; Shimelis *et al.,* 2008). Therefore, use of zinc supplements with oral rehydration salts (ORS) reduces morbidity and mortality of diarrhea disease particularly infants and younger age children (WHO/UNICEF, 2013).

2.3 Enteric bacteria (*Enterobacteriacae*)

Enteric bacteria are bacteria in the family *Enterobacteriaceae,* a large family of gram-negative bacteria and consist of 44 genera which constituent the genera that can be characterized by recognizing the metabolic properties of this family. The morphology of *Enterobacteriaceae* is rod-shaped bacterial with simple nutritional requirement, peritrichously flagellated or nonmotile, non spore-forming and have 0.3 - 1.0 X 1.0 - 1.06 µm of cell dimensions. Most members of *Enterobacteriaceae* are facultative anaerobes and chemoorganotrophs, which exhibit both respiratory and fermentative metabolism. The representative genera of this family are *Escherichia, Salmonella, Shigella, Citrobacter, Klebsiella, Proteus, Enterobacter, Erwinia, Serratia and Yersinia.* Several genera of this family possess vital human pathogens responsible for causing various diseases.

Members of *Enterobacteriaceae* are so common and often called as enteric bacteria which reside normally in the intestines of humans including animals. Based on their ability to cause diarrheal disease of humans, *Enterobacteriaceae* can be divided into a group of pathogens and non-pathogens. However, the most pathogenic genera of this family are *Salmonella* and *Shigella* as well as some strains of *Escherichia*. In contrast, members of *Enterobacteriaceae* which possessed minimum pathogenicity are known as classic opportunists which able to cause disease when the bacteria

strains have gained access to the other tissues sites or when the host, such as human is immunocompromised. Examples of opportunistic infections caused by opportunistic *Enterobacteriaceae* are respiratory tract infections, wound infections and urinary tract infections. Normally, the genus of *Klebsiella, Proteus, Citrobacter, Enterobacter, Serratia, Morganella* and *Providencia* are referred to as opportunistic *Enterobacteriaceae* (Prescott *et al.*, 2008).

2.3.1 *Escherichia coli* infections

The large intestine has the highest microbial population in the body and *Escherichia coli* is a common member regularly found in the large intestine. Eventhough, majority of *E. coli* strains are non-pathogenic members of the normal intestinal flora, but some strains may cause diarrheal disease by several mechanisms thus, *E. coli* is often known as an essential food-borne pathogen. The strains of *E. coli* which associated with gastrointestinal disease, including diarrheal disease are classified into common strains, i.e., consist of Enteropathogenic *Escherichia coli* (EPEC), Enterotoxigenic *Escherichia coli* (ETEC) and Enterohaemorrhagic *Escherichia coli* (EHEC); and rare strains of pathogenic *E. coli* which was isolated from young children with diarrhea, i.e., Enteroaggregative *Escherichia coli* (EAggEC) and Enteroinvasive *Escherichia coli* (EIEC).

EPEC is an important cause of diarrhea in children residing in developing countries. This strain attaches to the epithelial cells, multiplying and causing cell damage called effacing lesions, thereby causing vomiting, fever and prolonged diarrhea mainly in infants less than 5 years. ETEC is often referred to as traveler's diarrhea, which is responsible for watery diarrhea due to production of plasmid-mediated toxins, namely heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT) in infant and

adults. EHEC causes life-threatening haemorrhagic colitis with severe abdominal pain and cramps followed by bloody diarrhea in all ages and frequently without fever. This infection occurs by ingesting contaminated meat and dairy products, as well as unpasteurized milk. *E .coli* 0157: H7, which carry the genes for Shiga-like toxin is a major form of EHEC. Shiga-like toxin (Stx-1 and STX-2 proteins) is able to kill vascular endothelial cells, which are detected by the bacteriophage-encoded genetic of this bacterial strain, have also been involved in the extra-intestinal disease, which is haemolytic uraemic syndrome that leads to renal failure (Nguyen & Sperandio, 2012).

EAggEC causes chronic watery diarrhea and vomiting, mainly in children. This strain adheres to epithelial cells in localized regions, forming clumps of bacteria with the appearance of brick-like (aggregates). Meanwhile, EIEC strains causes dysentery, fever and colitis, with mucus, blood and present of many pus cells in feces. The ability of this strain to invade intestinal epithelial cells by penetrating and multiplying in cells is associated with the presence of a large plasmid. It is believed that this strain may also produce an enterotoxin and cytotoxin (Prescott *et al.*, 2008)

2.3.2 Salmonellosis

Salmonellosis is referred to as *Salmonella* gastroenteritis can be caused by many *Salmonella* serovar (serological variation or strains). Typically, in developing countries, Typhimurium and Enteritidis are frequently isolated serovars from human. Salmonellae are aerobes and facultative anaerobes; besides, they are gram negative, motile, and non-sporing rods. Human acquired the infection by ingesting Salmonellae, from contaminated food directly or indirectly from the intestinal tracts

of animals and human sources. Common sources of infection are beef and beef products, poultry, egg and egg products and water.

The incubation time of Salmonellae in the body is around 8 to 48 hours. Once the Salmonellae is in the body, they multiplied and invaded the intestinal mucosa which subsequently produced enterotoxin and cytotoxin to destroy the epithelial cells. Thus, symptoms such as diarrhea, fever, vomiting, abdominal pain and cramps as well as nausea may occur within 12 to 36 hours after ingesting infected food. Normally, these prominent symptoms persist up to 5 days, but able to last for several weeks. In acute infection diarrhea diseases, blood and mucus appear in feces as well as abundant Salmonella shed in the feces. Usually, patients like infants, elderly and immunosuppressed persons are at higher risk of loss of fluids in the body due to diarrhea, thereby increasing the rate of mortality especially among infants. Besides diarrheal disease, food-poisoning *Salmonella* strains can also cause bacteraemia, septicaemia and inflammation of the gall bladder (Sánchez-Vargas *et al.*, 2011).

2.3.3 Typhoid fever

Typhoid fever, is the most serious form of disease, caused by *Salmonella* enterica serovar Typhi (*Salmonella* Typhi) and is acquired by ingestion of water contaminated with feces of infected human. Meanwhile, paratyphoid fever, a milder form of the disease, is caused by *Salmonella enterica* subspecies *enterica* serovars Paratyphi A, B and C (*Salmonella* Paratyphi A, B and C) and is acquired by ingestion of food contaminated with these strains. Infection caused by *S. typhi* among children and young adults (less than 19 years) is a major public health concern in developing countries. Furthermore, untreated typhoid fever may result in death (Stoesser *et al.*, 2013; Bula-Rudas *et al.*, 2015) .

Generally, the incubation period of the bacteria after entering the small intestine is about 10 to 14 days. The bacteria will colonize the small intestine, then invade the epithelium and spread to the blood, gall bladder, liver and lymphoid tissue. The noticeable symptoms of this infection including headache, fever, abdominal pain, anorexia and malaise, which persist up to several weeks. However, once the bacteria reinfected the gastrointestinal tract, the prominent symptoms, i.e., diarrhea and rash (rose spot) appeared (Sánchez-Vargas *et al.*, 2011). Paratyphoid fever caused by *S.* paratyphi A, B and C has symptoms similar to typhoid fever, however, the infection of this strains tends to be milder and has lower mortality rate (Girard *et al.*, 2006).

2.4 Antibacterial activity

2.4.1 Antibacterial agent

In the 21st century, modern medicine is dependent on chemical agents or chemotherapeutic agents that are used to treat many infectious diseases in order to destroy pathogenic organisms or inhibit their growth. Pathogenic organisms residing in the human bodies can cause disease, thus the control and immediate destruction of the pathogenic agents is important. Mostly, infectious diseases caused by pathogenic agents are treated with antibiotics which is chemotherapeutic agents at the minimum concentration that enough to treat infections without harming the host.

Drugs, which kill or inhibit microbial pathogen are variable according to their range of effectiveness. Broad-spectrum drugs that effective for many types of pathogens (such as aerobic and anaerobic organisms; gram-negative and gram-positive bacteria). Meanwhile, narrow-spectrum drugs are effective only against specified pathogens. Besides, drugs may also be divided into several microbial groups, i.e.,

antibacterial, antifungal, antiprotozoal and antiviral based on their actions against bacterial, fungi, protozoa and virus, respectively. Generally, the term of antibiotic is used to describe antimicrobial agents, but usually refers to antibiotic agents.

Antibiotic are classified into natural (produced and isolated from bacterial and mold), semi-synthetic (modified chemically from natural antibiotics) and synthetic (manufactured by chemical procedures without microbial activity) drugs that can destroy susceptible pathogens or inhibit their growth. Most semi-synthetic drugs have a broad-spectrum of antibacterial activity compared to their parent constituent. Antibiotics can be either bactericidal or bacteriostatic. Those that kill the target bacteria are bactericidal agents, however, their activity depends on the concentration that may be at low concentration and exhibit bacteriostatic action. Whereas, those reversibly inhibit growth are referred as bacteriostatic agents. Nevertheless, if the agents is removed, the bacteria will grow again (Prescott et al., 2008).

Antibiotics can be grouped according to their mechanism of action, e.g., cell wall synthesis inhibition, protein synthesis inhibition and nucleic acid synthesis inhibition. Tetracycline is a family of antibiotics with a common four-ring structure of which a variety of side chains are attached. This antibiotic is related to antibacterial agent that inhibit bacterial protein synthesis by binding to the bacterial ribosome, and combined with 30 S (small) subunit of the ribosome. Thus, inhibits the binding of aminoacyl-tRNA molecules to the ‘A’ site of the ribosomal acceptor. The effectiveness of treatment using this antibiotic depends on active host resistance to the pathogen since their action is bacteriostatic. In addition, tetracyclines are broad-spectrum antibiotic that are used actively against gram-negative and gram-positive bacteria. Common side effects of tetracyclines include gastrointestinal disturbance, renal injury and teeth discoloration with presence of no major adverse side effects, thereby making

them attractive for use in the treatment of bacterial infections (Chopra and Roberts, 2001; Eliopoulos et al., 2003).

2.4.2 Antibacterial resistance

In the 21st century, the spread of drug-resistant bacteria is a growing worldwide problem. Infectious diseases are difficult to treat because of the antibacterial resistance due to intensive use and misuse of available antibiotics. Common mechanisms of resistant bacteria to survive doses of antibiotics that would previously exhibit bactericidal effects are due to the production of enzymes which inactivate or modify antibiotic, modify the target site and changes in the bacterial cell membrane, thereby preventing the uptake of antibiotic. Resistant bacteria undergo genetic change, which may occur by mutation process or by the acquisition of new genetic material (Wilson *et al.,* 2002).

Since the turn of this century, the world is interested in combating multi-drug resistant (MDR) bacteria mainly in the gram-positive bacteria like methicillin-resistant *Staphylococcus* (MRSA) and vancomycin-resistant enterococci (VRE). Many efforts have been done by researchers and drug companies to develop new compounds. Giske *et al.* (2008) reported antibiotics, such as linezolid and daptomycin which are able to fight these bacteria. Unfortunately, during the world fight with infections caused by antibiotic-resistance gram-positive bacteria strain, gram-negative bacteria take placed and become dominant; thus, diseases caused by them become a serious problem nowadays. According to Bhatt *et al.* (2015), previously, drug-resistant gram-negative bacteria was initially threatened as nosocomial bacteria that spread the infections within environment of health-care facilities, but now, their ability to spread the infections to the community have been

reported. Furthermore, no new compounds of drugs are currently developed solely to treat infections caused by antibiotic-resistant gram-negative bacteria.

Antibiotic resistance also increased rapidly in health care facility like hospital due to overuse or misuse of antibiotic. The extensive growth of antimicrobial resistance by pathogens sooner or later will be limiting the life span of common antibiotic. Consequently, new effective antibiotics are urgently needed to combat resistance bacteria. The pharmaceutical drug such as carbapenems and polymyaxin have been used as the last resort against multidrug-resistant gram-negative bacteria, and has become as first-line therapy after all other options of antibiotic proved ineffective (Walsh & Amyes, 2004; de Oliveira *et al.,* 2015). However, the utilization of these drugs has been limited due to appearance of carbapenem-resistant bacteria and the toxicity concerning polymyxin. Antibiotic resistant continuously grow and spread rapidly from time to time exceeding the number of discovery and development of new anti-resistant antibiotics. Therefore, the intensive research and development of new antibacterial drugs from natural sources are urgently needed in the present situation.

2.4.3 Evaluation of antibacterial susceptibility test

In the treatment and control of infectious diseases, susceptibility testing is used to determine the effectiveness of antibacterial against specific pathogens. Besides, susceptibility testing can give an estimation of proper therapeutic dose of antibacterial agents for further treatment. Laboratory antibacterial susceptibility testing can be performed using dilution susceptibility and disk diffusion tests. Dilution susceptibility test is a manual or semi-automated dilution techniques to assess the minimum inhibitory concentration (MIC). This technique can also be used

to determine the minimum bactericidal concentration (MBC) which measures the lowest concentration of antibacterial needed to kill bacteria. A dilution test can be done in both agar and broth media, but broth dilution is the most commonly used method to determine the antibacterial susceptibility (Balouiri *et al.,* 2016) .

A twofold dilution is carried out by diluting the concentrated tested antibacterial stock solution. Then, a standardized inoculum of the test organism is added and incubated overnight. After incubation, the lowest concentration of diluted tested antibacterial required to prevent visible growth, which refer to MIC is reported (CLSI, 2016). The MBC can be assessed if the broth showing no visible growth is subcultured into fresh medium. The lowest antibiotic concentration from which the bacteria do not grow when subcultured to agar medium is the MBC value. Broth microdilution represent the performance of the broth dilution test in microdilution plates with a capacity of less than or equal to 400 µL per well. In addition, microdilution procedure is believed to produce fast and reliable quantitative result of MIC (Nijs *et al.*, 2003; Balouiri *et al.*, 2016)

2.5 Antioxidant activity

2.5.1 Antioxidant agents

Reactive Oxygen Species (ROS) is referred to as reactive molecules and free radicals derived from oxygen. The oxygen based radicals is an unstable molecule, where the unpaired electron can shift or paired with other electrons. Reactive molecules can be termed as active oxygen species, where it is generated after our system in the body utilized the oxygen, and they are more reactive compare to oxygen molecule

(Yoshikawa and Naito, 2002). Examples of active oxygen species are superoxide radical, hydrogen peroxide, hydroxyl radical, hydroxyl ion and nitric oxide.

Respiratory burst, a condition of increased in oxygen consumption due to phagocytes ingest bacteria has occurred in the tissues and resulting in production of ROS such as superoxide and hydrogen peroxide (Rada & Leto, 2008). ROS molecules are antibacterial and toxic to bacteria. The degradation of bacteria via oxygen-dependent degradation depended on the molecules of ROS including hydroxyl radicals and singlet oxygen, which able to kill the bacteria (Robinson, 2008; Petropoulos *et al.*, 2015).

The protective agent to counteract oxidative stress, which is referred to as antioxidant prevents and repairs the damage caused by ROS particularly free radicals, thereby enhancing the immune defense and depleting risk of chronic diseases to human body. ROS at high concentration is harmful to human tissue by subjecting the tissue to the deleterious process and damaging the cell; whereas, ROS at low concentration is beneficial especially in cellular and immune responses (Pham-Huy *et al.*, 2008).

Antioxidants are able to quench the free radicals from the reactive oxygen-based radicals (ROS-based radicals). The endogenous antioxidants present in our body are divided into two functional groups - the enzymatic antioxidants (e.g., primary enzymes: superoxide dismutase, glutathione peroxidase and catalase) and the non-enzymatic antioxidants (e.g., Vitamin A, uric acid, coenzyme Q10, and glutathione), which are responsible to inhibit the production of free radicals or able to neutralize the free radicals of ROS-based radicals (Carocho & Ferreira, 2013). Antioxidant deficiencies in our body can occur when the endogenous antioxidant system is inadequate to eliminate the free radicals; thus, our system needs to uptake exogenous

antioxidant compounds such as Vitamin C, Vitamin E, Vitamin K, phenolic acids, flavonoids, β-carotene and some minerals. These can be taken in naturally particularly from plant-based products, and via diets to balance the concentration of free radical production in our body.

Endogenous and exogenous antioxidants have a synergistic effect to prevent the debut of oxidative stress. Nevertheless, the concentration of exogenous antioxidants should be controlled to prevent them from turning to pro-oxidant agents (Bouayed & Bohn, 2010; Carocho & Ferreira, 2013). Despite its ability to display pro-oxidant activity, the exogenous antioxidants from plant-based products are receiving great attention to overcome diseases caused by ROS. Thus, the demand of using natural antioxidants from plant sources in the pharmaceutical, nutraceutical and cosmetic products has increased worldwide (Singh *et al*., 2013). Furthermore, synthetic antioxidants like propyl gallate, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are used as additives in food industries have controversial issues on their adverse effects on human health due to its carcinogenic potentials (Krishnaiah *et al.,* 2011; Carocho & Ferreira, 2013). Consequently, demand of using of plant-based antioxidant products, particularly in the food industry has increased.

Plants have a self-defense system to protect the cells from damage due to abiotic and biotic stresses. The defense system consists of enzymatic and non-enzymatic antioxidants such as superoxide dismutase, ascorbate peroxidase, phenolic acids, flavonoids, and carotenoid (Hossain *et al.,* 2007; Lee & Shin, 2011). These antioxidant compounds play an important role in human defense system since our body needs these sources of exogenous antioxidants from plant products to help balance the production and neutralization of free radicals in the body.

2.6 Anti-inflammatory activity

2.6.1 Overview of inflammation and anti-inflammatory

Human is continuously exposed to pathogenic bacteria and their metabolic products that can cause diseases. However, human has an immune system that protects them from adverse effects consequences of this exposure. Immune system consists of cells, tissues and organs that recognize the intrusion of foreign materials into the body. Immune system has also the capability to mobilize defense cells and cell products to help neutralize or destroy a particular foreign material speedily and effectively. Immunity is categorized as innate (nonspecific immune response) and adaptive immunity (specific immunity).

Innate immunity is also refers to the body's natural ability to recognize and destroy foreign materials. The components of innate immunity are (a) mechanical methods which prevents the entry of microbes into the body or physically remove them from the body surface, (b) chemical mediator - acts directly against foreign materials or that activates other mechanisms, leading to the destruction of foreign materials, (c) cells - phagocytise the production of chemicals involved in the immune system response and (d) inflammation - mobilises the immune system and isolates foreign materials until they can be destroyed. There are general mechanisms inherited as part of the innate function and acts as a first line defense. Innate immunity is a nonspecific immune response defends against foreign substances equally but has no immunological memory (Serhan, 2008).

Infection, activation of the immune system, inflammation response and ischemia are exemplified of endogenous sources of reactive oxygen species (ROS) including free

radicals and non-radicals (oxidants). The imbalance between production and neutralization of ROS (including nitric oxide) in human tissues leads to oxidative stress, thereby resulting the oxidative damage tissue associated with the occurrence of numerous diseases including inflammatory disorders.

Inflammation is an important nonspecific defense reaction to hazardous stimuli such as pathogenic invader, damaged cells and also irritants. Acute inflammation is the immediate response of the body to hazardous stimuli. Resident cells of the human tissue consist of monocytes, macrophages, dendritic cells and Kupffer cells, which have receptors known as pattern recognition receptors (PRRs), responsible to recognize pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). PAMPs are products derived from pathogens that are able to interact with PRRs; whereas, DAMPs are chemical factors released by damaged or injured tissue that are able to bind to PRRs (Rubartelli & Lotze, 2007; Srikrishna & Freeze, 2009). After the activation of PRRs by binding either to PAMPs or DAMPs, these resident cells (e.g., macrophages) released inflammatory mediators and activate the immune response (Tang *et al.*, 2012).

For example, when there is an invading agent such as pathogenic bacteria invades human tissue, the body tries to destroy that agent by activating a cascade of events including (a) activation of specific immune cells, (b) release of chemical mediators from these active cells, and (c) increased infiltration of phagocytes in response to the chemical mediators, that result in the development of the signs of inflammation which causes pain, redness, sweeling, heat and loss of function in the affected area (Ryan & Majno, 1977; Ward & Lentsch, 1999). The symptom or sign may appear for a few days, then, inflammation has three possibilities whether fully recover, develops into an abscess or getting worse and become a chronic inflammation. Example of

diseases results from acute inflammation, including acute bronchitis, acute appendicitis, acute dermatitis, acute infective meningitis and acute tonsillitis. However, if the inflammation is not controlled, it may lead to tissues damage, fibrosis and necrosis.

In general, eliminating any hazardous stimuli is the main target of the innate immune response, and if necessary, aided by the adaptive immune response. Failure to eliminate the hazardous stimuli or inefficient termination of the inflammatory response, could lead to chronic inflammation. Therefore, inflammatory process should be well regulated by the immune system to avoid tissue damage.

An adequate treatment of acute inflammation becomes compulsory to protect any tissue damage, restore the tissue structure and function, as well as to turn off the inflammatory response after eliminating the hazardous stimuli. This is to prevent overproduction of the pro-inflammatory cytokine [interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and chemokines] and inflammatory mediators (including ROS and NO) that will likely perpetuate immune reaction and tissue damage (Kanterman *et al*., 2012).

Conventional anti-inflammatory drugs, namely steroids and non-steroidal anti-inflammatory drugs (NSAIDs) have been used until now to treat inflammation, especially acute inflammatory disorders; however, currently used steroids and NSAIDs are associated with bad side effects (e.g., diarrhea, kidney and liver failures). The discovery of new anti-inflammatory compounds of plant sources have received great attention from researchers especially in academia and pharmaceutical industry due to few side effects (Fürst & Zündorf, 2014).