ANTI-*Escherichia coli* O157: H7 AND ANTI-OXIDANT ACTIVITIES OF ACETONE EXTRACT OF *Piper betle* L. AND ITS FRACTIONS

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by

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LIST OF ABBREVIATIONS AND SYMBOLS

µm	micrometer
µg	microgram
AOA	Anti-oxidant activity
ATCC	American Type Culture Collection
CFU	Colony Forming Unit
DMSO	Dimethyl Sulfoxide
DPPH	Diphenylpicryl-hydrazyl
EHEC	Enterohaemorrhagic *E. coli*
EM	Electron microscope
FRAP	Ferric Reducing
FFNSC	Flavour & Fragrance Natural & Synthetic Compounds
GAE	Gallic acid equivalents
GC-MS	Gas Chromatography Mass Spectrometer
HUS	Haemolytic Uremic Syndrome
INT	2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
LC_{50}	Lethal concentration of 50%
MBC	Minimum Bactericidal Concentration
MHA	Mueller Hinton Agar
MIC	Minimum Inhibition Concentration
mg	miligram
ml	mililiter
NA	Nutrient agar
NB	Nutrient broth
NIST	National Institute of Standards & Technology

R_f	Relative frequency
SD	Standard Deviation
SEM	Scanning Electron Microscope
SMAC	Sorbitol Mac Conkey Agar
STEC	Shiga-toxin producing *E. coli*
Stx	Shiga toxin
TE	Trolox equivalent
TEM	Transmission Electron Microscope
TLC	Thin Layer Chromatography
TPC	Total Phenolic Content
TSB	Trypticase Soy broth
UV	Ultraviolet
Viz.	Namely

AKTIVITI ANTI- *Escherichia coli* O157:H7 DAN ANTI-OKSIDAN EKSTRAK ASETON *Piper betle* L. DAN FRAKSINYA

ABSTRAK

E. coli 0157:H7 merupakan patogen baru yang menyebabkan diarea berdarah sehingga mengakibatkan kekejangan abdomen yang teruk, sindrom uremik berdarah (HUS), trombositopenik purpura trombotik (TTP) dan juga kematian. Terdapat laporan menyatakan bahawa penggunaan antibiotik dalam rawatan boleh meningkatkan risiko pesakit mengalami HUS yang boleh membawa maut. Oleh sebab itu, hasil tumbuhan boleh dijadikan sumber alternatif dalam pencegahan transmisi dan infeksi patogen tersebut di kalangan populasi umum. Melalui asai pembauran cakera, daripada dua puluh empat ekstrak yang diperolehi daripada lapan tumbuhan Malaysia, ekstrak aseton *P. betle* memberikan zon perencatan yang terbesar pada 20.56 ± 0.19 mm. Ekstrak ini juga berhubung kait dengan kandungan jumlah fenolik (TPC) di mana ia menunjukkan jumlah yang tertinggi di antara ekstrak yang diuji pada 430.69 ± 47.50 µg GAE/ mg ekstrak seperti yang telah ditentukan oleh kaedah Folin-Ciocalteu. Melalui asai pencairan mikro, ekstrak aseton *P. betle* memberi nilai MIC terendah iaitu pada 500 µg/ ml dan MBC pada 2000 µg/ ml terhadap *E. coli* O157:H7. Fraksinasi berpandu bio-asai telah dibuat dan sistem larutan klorofom: methanol pada nisbah 19:1 dipilih untuk fraksinasi ekstrak aseton. Fraksinasi dijalankan menggunakan penyediaan kromatografi lapisan nipis (TLC) dan kesemua fraksi yang terkumpul diuji untuk aktiviti antibakteria, TPC dan aktiviti antioksidan. Fraksi 5 memberi nilai MIC paling rendah pada 500 µg/ ml, kandungan fenolik yang tinggi (398.25 ± 7.74 GAE/ mg ekstrak) dan aktiviti anti-oksidan yang tinggi melalui asai DPPH (80.43 ± 0.71%). Aktiviti antibakteria fraksi 5 menurun semasa penyimpanan menyebabkan ia dicampur dengan fraksi 6 dan 7 (dikenali

dengan fraksi A) untuk menjadi lebih stabil. Nilai MIC fraksi A terhadap *E. coli* O157: H7 adalah lebih rendah pada 250 µg/ ml tetapi nilai MBC meningkat kepada > 2000 µg/ ml. Ekstrak aseton dan fraksi A diuji untuk induksi morfologi dan perubahan ultrastruktur *E. coli* O157:H7 pada fasa eksponen di dalam kultur kelompok. Berdasarkan pemerhatian melalui mikroskop elektron imbasan dan transmisi, kerosakan sel oleh fraksi A adalah kurang dibandingkan kerosakan sel yang disebabkan oleh ekstrak aseton pada 2000 µg/ ml. Asai toksisiti menggunakan anak udang (*Artemia salina*) menunjukkan nilai LC_{50} bagi ekstrak adalah 500 µg/ ml dan fraksi A adalah >1000 µg/ ml. Kromatografi gas- spektrometri jisim (GC-MS) untuk fraksi A menunjukkan tiga kompaun fenolik utama, Chavicol, Hydroxychavicol and Chavibetol yang mungkin menyumbang kepada aktiviti antibakteria. Daripada kajian ini, boleh dirumuskan bahawa fraksi A pada 250 µg/ ml ke 2000 µg/ ml dan ekstrak aseton *P. betle* sehingga 500 µg/ ml hanya bersifat bakteriostatik terhadap *E. coli* O157:H7 tetapi ekstrak aseton pada 2000 µg/ ml bersifat bakterisidal. Ekstrak aseton adalah toksik pada kepekatan bakterisidal, sementara fraksi A walaupun hanya bersifat bakteriostatik tetapi ia tidak toksik.

ANTI-*Escherichia coli* O157:H7 AND ANTI-OXIDANT ACTIVITIES OF ACETONE EXTRACT OF *Piper betle* L. AND ITS FRACTIONS

ABSTRACT

E. coli 0157:H7 is a newly emerging pathogen that cause bloody diarrhoea to severe abdominal cramps, haemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura and even death. There are reports that antibiotic treatment increases the risk of patients developing fatal HUS. Therefore plant products could be an alternative source for prevention of transmission and hence infection of the pathogen among the general populations. By disc diffusion assay, out of twenty four extracts from eight Malaysian plants, acetone extract of *P. betle* inhibited *E. coli* 0157:H7 strongly giving the largest inhibition zone at 20.56 ± 0.19 mm. This correlates strongly with its total phenolic content (TPC) which was the highest among those tested at 430.69 ± 47.50 µg GAE/ mg as determined by Folin-Ciocalteu method. By microdilution assay, acetone extract of *P. betle* gave the lowest MIC at 500 µg/ ml and MBC at 2000 µg/ ml against *E. coli* O157:H7. Bio-assay guided fractionation of acetone extract was done using a solvent system of chloroform: methanol at a ratio of 19:1. Fractionation was done by preparative TLC and the fractions collected were all assessed for antibacterial activity, TPC and AOA. Fraction 5 gave the lowest MIC against *E. coli* 0157:H7 at 500 µg/ ml, high TPC (398.25 ± 7.74) as well as high anti-oxidant activity as assessed by DPPH (80.43 ± 0. 71 %). Upon storage, the antibacterial activity of fraction 5 was reduced. It was subsequently mixed with fraction 6 and 7 (now known as Fraction A) which was more stable. The MIC for fraction A against *E. coli* O157:H7 was much lower at 250 µg/ml but its MBC increased to >2000 µg/ ml. Acetone extract of *P. betle* and fraction A were also tested for induction of morphological and ultrastructural

changes of *E. coli* O157:H7 at exponential phase in a batch culture. Based on observation by SEM and TEM, cellular damage by fraction A was not as severe as that given by acetone extract, both at 2000 µg/ml. Toxicity assay using brine shrimp (*Artemia salina*) showed LC_{50} of acetone extract was 500 µg/ ml and fraction A was >1000 µg/ ml. Gas chromatography- mass spectrometry (GC-MS) of the fraction revealed three major phenolic compounds, Chavicol, Hydroxychavicol and Chavibetol which may contribute to the antibacterial activity. From this study, it can be concluded that fraction A at 250 µg/ ml to 2000 µg/ ml and acetone extract up to 500 µg/ ml were only bacteriostatic towards *E. coli* O157:H7 meanwhile acetone extract was bactericidal at 2000 µg/ ml. Acetone extract was toxic at bactericidal concentration, while fraction A though bacteriostatic was non-toxic.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Escherichia coli O157:H7 is an emerging pathogen that cause significant important effects to human health. Most of *E. coli* is a commensal found in intestinal tracts but the strain used in the study was a subset of the pathogenic *E. coli* which has virulence factors that differentiate it from the normal *E. coli*. *E. coli* O157:H7 was known as enterohaemorrhagic *E. coli* (EHEC) and Shiga toxin producing *E. coli* (STEC) (Centre for Food Security and Public Health, 2009). It was identified as an etiological agent for diarrhoea (Aboaba *et al.,* 2006) and has been the cause of many outbreak related to food contamination. Although there is no outbreak by this pathogen occurred in Malaysia, the prevalence assessed by some researchers showed the existence of it in Malaysian food products such as beef (Frederick, 2011). However, information regarding *E. coli* O157:H7 in Malaysia is scanty thus leading to the use of *E. coli* O157:H7 in this study.

Persons infected with *E. coli* O157:H7 showed the effects from asymptomatic to lethal. The most prominent clinical features of *E. coli* O157:H7 infections are bloody diarrhoea and haemolytic uremic syndrome (HUS) (Keene *et al.,* 1994). The symptom of bloody diarrhoea usually disappear in most patients but the elderly or younger patient infected tend to progress to HUS (Nataro and Kaper, 1998). The illness likely to become severe in patients who is very young or very old.

The treatment of EHEC infection is limited to supportive therapy as there is no specific antibiotic or vaccines available treating the EHEC infection. The usage of antibiotic in patient with this infection showed no alteration of the disease outcome

(Nataro and Kaper, 1998). Nevertheless, some studies showed the usage of antibiotic is necessary depending on the patient's presentation (Collins and Green, 2010). Although it is susceptible to many antibiotics, some strain also showed resistant toward some of the antibiotic used (Frederick, 2011).

Prevention and treatment without major side effect can be done as long as suitable substances were found. With the emergence of new resistant strains of the bacteria and treatment failure of some modern drugs, alternative approaches were done to search for a new plant-based medicine. Complication of the antibiotics used for treating EHEC infection lead to this study to find an alternative to prevent its transmission.

Plants have been used for treating many diseases and infections by many traditional practices. They have ability to synthesize aromatic substances including antimicrobial phytochemical such as phenolic and polyphenols (Cowan, 1999). Phenolic compounds apart from being identified as having the antibacterial property also act as an antioxidant which is important in preventing the oxidative stress known to be related with the aetiology of human disease (Rathee *et al.,* 2006). This substances were used and exploited by pharmaceutical industries as the source of their drugs (Aboaba *et al.,* 2006). With the basis of plant has been used as medicine since ancient time, the study was aimed to search for potential medicinal plant found widely in Malaysia. Jamal *et al.* (2010) showed that, Malaysian plants contain valuable compounds such as phenol and thus it has potential to be used as alternative treatment. In this study, several Malaysian plants will be screened for their anti- *E. coli* O157:H7 activity and the selected plant extract will be assessed in several assays to support the fact that it has the potential as a new alternative source to treat and prevent the transmission of *E. coli* O157:H7.

1.2 Problem statement

There is no specific treatment for *E. coli* O157:H7 as the antibiotics used to treat patients infected with the pathogen increased the risk of patient developing HUS. The study was carried out to provide new information on the phenolic content, antioxidant activity and antibacterial activity against *E. coli* O157:H7 of selected Malaysian plants. The usage of plant as alternative treatment for *E. coli* O157:H7 is suitable because of the fewer side effects and availability. Besides treatment, the plant can be used to prevent the transmission of *E. coli* O157:H7 by external usage.

1.3 Research Objectives

The objectives of this study were:

1. To determine anti-*Escherichia coli* O157:H7 activity of twenty four extracts derived from eight selected Malaysian plants
2. To determine and compare the total phenolic content (TPC) and anti-oxidant activities (AOA) of the plant extracts that exhibited anti-*E. coli* O157:H7 activity.
3. To assess the induction of morphological and ultra-structural changes by potential fraction on *E. coli* O157:H7 in a batch culture under SEM and TEM.
4. To determine the toxic activity of selected fraction and extract by brine shrimp toxicity assay
5. To determine the compounds present in the active fraction using Gas Chromatography- Mass Spectrophotometry.

CHAPTER 2

LITERATURE REVIEW

2.1 Medicinal plants

Medicinal plants refer to plants which exhibit medicinal potential. In Malaysia, the diverse flora was diverse making it excellent to screen for different biological activity attributed by their secondary metabolites (Jamal *et al.*, 2010). Information on their medicinal uses usually derived from various knowledge of traditional medicine and this forms the basis for most of drug discovery (Fabricant and Farnsworth, 2001).

It is estimated that 80% of the population in developing countries depend on medicinal plants for their health requirements (Milow *et al.*, 2011; Patra *et al.*, 2009). The knowledge of the medicinal plant has played a role in a new drug discovery. These were shown in a report by Saklani and Kutty (2008) that twenty six plant based drugs were approved or launched between 2000 to 2006, including a drug named polyphenon E (Veregen) in a form of ointment. The main compound is polyphenol (catechin) extracted from green tea and used to treat genital and perianal warts.

In this study, several plants were selected for screening of anti-bacterial and anti-oxidant activity. The plants were used as traditional medicine and known locally. Table 3.1 showed the list of the plants and its traditional used. From the list, some plants were used for other purposes apart from being used as medicine. For examples, *Samanea saman*, which is known as rain tree, have been used as source of

timber and its wood have been crafted for sale to the tourists (Staples and Elevitch, 2006) and *Pelthopharum pterocarpum* known to be ornamental plant along the road side and park. The bark of the plant has been utilized for making furniture and source of fire wood (Ong, H.C., 2004). In this study, *Piper betle* have been the most potential plant for having the anti-*E. c*oli O157:H7 activity thus more review of the plant in section **2.7.**

2.2 Antibacterial agent

An antibacterial agent is a compound that is natural, synthetic or semi synthetic and active against bacteria (Vasanthakumari, 2007). Antibiotic which was used frequently for treating the bacterial infection was a natural product or a derivative from it. The actions of the antibiotic towards the bacteria are listed as below:

(a) Inhibit cell wall synthesis by preventing the synthesis of peptidoglycan which is a major component of Gram positive bacterial cell wall, e.g., Penicillin.

(b) Inhibit protein synthesis by inhibiting the process of transcription and translation, e.g., Chloramphenicol.

(c) Inhibit nucleic acid synthesis by inhibiting the DNA replication and translation in the microorganisms, e.g., Metronidazole.

(d) Damage the cell membrane by attaching to the membranes phospholipid causing problem of membrane permeability, e.g., Polimyxine B.

(e) Inhibit specific enzyme systems by resembling the metabolite such as para-amino-benzoic acid (PABA) which is attached to the enzyme responsible for bacterial growth, e.g., Trimethoprime.

The usage of antibacterial agents however is based on a number of factors including pathogen identity, infection site, the interaction of the agent and the side effect it cause to the patient, pharmacokinetics and pharmacodynamics of the agent, cost and its administration (Hessen and Kaye, 2004). Current therapy used the antibacterial agents by single or in combination to demonstrate greater activity. Although it is widely used in current medical practice, there are still issues arising from the usage. The side effects such as renal impairment may occur as a result of the drug accumulation which then relates to its toxicity (Hessen and Kaye, 2004). Alternatively, the plants have been used widely in traditional medicine thus offering an option to aid in the treatment of diseases cause by pathogens.

2.3 Antibacterial activity of medicinal plants

Medicinal plants have been known to exhibit a lot of pharmacological activity. The natural mechanism of plants against microorganism, insect and other organisms and their ability to produce limitless substances has given the researchers the opportunity to explore their use as anti-microbial agents. In addition, the side effects related to the use of synthetic drugs (Jamal *et al.*, 2010) and the availability of few pharmaceutical dispenses use for antimicrobial activity (Cowan, 1999) also stimulate many researchers searching for new anti-infections agent from natural resources (Rios and Recio, 2005). The activity exhibited by plants was contributed by the compound they synthesized. Table 2.1 showed some medicinal plants that exhibited the antibacterial activity and table 2.2 shows the phytochemical compounds from plants with its action as antimicrobial.

Table 2.1: Examples of medicinal plants with antibacterial activity.

Plants	**Bacterial Species**	**References**
Vaccinium macrocarpon	*Listeria monocytogenes, Salmonella typhimurium, Staphylococcus aureus*	Wu *et al*. (2008)
Samanea saman	*S. aureus and Escherichia coli*	Jagessar *et al.* (2011)
Litsea glutinosa, Vitex peduncularis and *Elephantopus scaber*	*S. aureus, Pseudomonas aeruginosa, Proteus mirabilis, Enterococcus faecalis* and *E. coli*	Prusti *et al.* (2008)
Curcuma manga	*P. aeruginosa, Bacillus subtilis* and *S. aureus*	Philip *et al.* (2009)
Ficus racemosa, Vitex negundo, Ocimum basilicum and *Etlingera elatior*	*Klebsiella pneumonia, S. aureus, Salmonella typhi, Proteus vulgaris* and *P. aeruginosa*	Renisheya *et al.* (2011)
Derris trifoliata	*Helicobacter pylori*	Uyub *et al.* (2010)
Bupleurum falcatu and *Stachys pubescens*	*S. aureus, L. monocytogenes, Streptococcus pneumonia, P. aeruginosa, K. pneumonia, E. coli* and *S. typhi*	Nazari *et al.* (2012)

Cont. Table 2.1

Plants	**Bacterial Species**	**References**
Zingiber zerumbet	*Vibrio parahemolyticus*	Kader *et al.* (2011)
Coleus amboinicus	Methicillin-Resistant *S. aureus* (MRSA)	Sahgal *et al*. (2009)
Marrubium vulgare	*Mycobacterium tuberculosis*	Buzayan and El-Garbulli, (2012)
Phyllanthus amarus	*S. aureus, Serratia marcescens, E. coli, Enterobacter* sp., *Streptococcus fecalis, K. pneumonia, P. mirabilis* and *P. aeruginosa*	Saranraj and Sivasakthivelan, (2012)

Table 2.2: Major classes of antimicrobial compounds from plants (Cowan, 1999).

Class	**Subclass**	**Example (s)**	**Mechanism**
Phenolics	Simple phenols	Catechols	Substrate deprivation
		Epicatechin	Membrane disruption
	Quinones	Hypericin	Inactivate enzymes
	Flavonoids	Chrysin	Bind to adhesins
	Flavones	Abyssinone	Inactivate ezymes
	Tannins	Ellagitannin	Membrane disruption Bind to protein, adhesins
	Coumarins	Warfarin	Interact with eukaryotic DNA
Terpenoids		Capsaicin	Membrane disruption
Alkaloids		Berberine Piperine	Intercalate into cell wall / DNA
Lectins and polypeptides		Mannose-specific agglutinin	Block viral fusion or adsorption
		Fabatin	Form disulphide bridges

2.4 *Escherichia coli* O157:H7

2.4.1 *E. coli* O157:H7 discovery

The first isolate of this serotype was from a sporadic case of haemorrhagic colitis in 1975 known as non-invasive or non-toxigenic by a standard test conducted (Riley *et al*., 1983). It was not recognized as etiologic of foodborne illness just until the report of two outbreaks of gastrointestinal illness which occurred in 1982 associated with this pathogen (Riley *et al.,* 1983). Since then, it caused many major outbreaks related food-borne infections and caused a serious concern in public health.

2.4.2 Characteristics

*E. coli O157:*H7, similar to other *E. coli*, is a Gram negative and rod-shape bacterium but differentiated from other *E. coli* strains by its serogroups, pathogenicity and virulence (Nataro and Kaper, 1998). The pathogen expresses somatic (O) antigen 157 and flagella (H) antigen 7 and cannot produce β-glucoronidase (Lim *et al*., 2010). The biochemical reactions of *E. coli* O157:H7 are the same as typical *E. coli* except for it does not ferment sorbitol thus yielding colourless colonies on the Sorbitol MacConkey agar (SMAC) (March and Ratnam, 1986). The strain also shows no haemolytic reaction on sheep and rabbit blood agar (Wells *et al*., 1983).

2.4.3 Pathogenicity

E. coli O157:H7 cause bloody diarrhoea (haemorrhagic) to severe abdominal cramps, HUS, thrombotic thrombocytopenic purpura and even death (Griffin *et al.*, 1988; Keene *et al.*, 1994). This pathogen produces several virulent factors. The most

important virulent factors of this pathogen causing death and other symptoms in patients infected are Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) (Baker *et al*., 2007). The toxin produced was identical at the genetic and protein levels to the Stx produced by *Shigella dysentariae* hence the alternative name for the pathogen is STEC (Shiga toxin-producing *E. coli*) (Nataro and Kaper, 1998). The ability to produce Stxs makes them important as the toxins have been linked to the HUS that can lead to renal failure (Mohawk and O'Brien, 2011). Beside STEC, the strain was known to cause hemorrhagic colitis which led them to be named as enterohaemorrhagic *E. coli* or EHEC (Centre for Food Security and Public Health, 2009).

The pathogenesis of *E. coli* O157:H7 was established in a model of *E. coli* O157:H7 oral infection of BALB/c mice (Mohawk *et al.*, 2010) whereby after the pathogen was ingested, it will colonize the cecum and allowing the Stx2 to enter the blood stream through the colonization site in the gastrointestinal tract. The toxins travel to the kidney and altered the kidney function hence causing HUS. Cattle which are major reservoirs of *E. coli* O157:H7 show no symptom although they are infected. Cattles are not sensitive to shiga toxins and have different site for colonization of the pathogens compared to the human thus making them tolerant hosts with continuous transmission of the pathogen (Nguyen and Sperandio, 2012).

Apart from the two major virulence factors (Stx1 and Stx 2 toxins), four other factors may contribute to the virulence of this pathogen. First, enterohemolysin which cause the erythrocyte to hemolyse releasing the source of iron needed for bacterial growth. Second, the intestinal adherence factor known as intimin which help the pathogen to attach to the intestinal wall causing lesion. Third, an iron transport system which is a special system possessed by *E. coli* O157:H7 that helps

them using the product of haemolysed erythrocyte as the source of iron and lastly, the O157 lipopolysaccharide (O157 LPS) which help to boost the cytotoxicity of the Stx toxins on human vascular endothelial cells (Nataro and Kaper, 1998).

2.4.4 Epidemiology and Transmission

Since the outbreak of *E. coli* O157:H7 in 1982 (Riley *et al.,* 1983), several other outbreaks have been reported till that recently occurred in USA related to organic spinach and spring mix blend in December 2012 (Centre for Disease Control and Prevention, 2013). Along with the outbreak and surveillance study conducted, the transmissions of infection were identified and established.

Report by World Health organization in 1997, since 1992 until 1997, 42 cases of outbreak were reported out of which 25 cases were foodborne, 10 cases by person to person contact and 2 cases by contact with animals. The transmission of 90 outbreaks occurred in UK, Denmark, Norway, Finland, USA, Canada and Japan between 1982 and 2006 confirmed that the source was from food (42.2%), dairy products (12.2%), animal contacts (7.8%), water (6.7%), environmental (2.2%) and unknown (28.9%) (Pennington, 2010). Thirteen outbreaks reported by CDC (2013) within the period of 2006 until 2012 were linked to transmission by fresh spinach in 2006, frozen beef ground patties and pizza in 2007, ground beef in 2008, pre-packaged cookie dough and beef in 2009, beef and cheese in 2010, Romaine lettuce, Lebanon bologna and in shell hazelnuts in 2011 and by organic spinach in 2012. All of the cases occurred in the USA.

In Asia, *E. coli* O157:H7 have been isolated in Japan, India and China (Radu *et al*., 1998) but the major outbreaks only occurred in Japan with 9,451 cases and

twelve deaths. The transmission was caused by food including white radish sprouts, salad, seafood sauce and raw beef (WHO, 1997). Another outbreak with death occurred in Jiangsu Province, China in 1999 where ninety five patients infected with *E. coli* O157:H7 developed acute renal failure and 87% fatality rate (Centre for Health Protection, 2010).

In Malaysia, most of the foodborne outbreaks are caused by *Salmonella* sp., *Shigella* sp., *Salmonella typhi* and *Vibrio* sp. (FAO/WHO, 2004). None of the outbreak was due to *E. coli* O157:H7 but, the pathogen have been isolated from beef (Radu *et al.*, 1998; Sahilah *et al.*, 2010), organic vegetables and chicken marketed in Malaysia (Chang *et al.*, 2013). Although a large number of foodborne disease incidence have been reported in Malaysia, there is still less effort to show the impact and its importance, therefore the true incidence of foodborne outbreaks occurred is unknown (Lim, 2002).

Most of the outbreaks of *E. coli* O157: H7 infections occurred were linked with the consumption of contaminated food by the pathogen. Several factors associated with the infection include dose of infection, source of food, food processing and probably the environment that supports the survival of the pathogen. Beef was associated with most of the contaminated food occurred in the outbreak and source of isolates. The animals, especially ruminants are reservoir hosts for *E. coli* O157:H7.

Cornick and Helgerson (2004) reported high dose of bacterial inoculum (10^5 to 10^7 CFU) were required to colonize the ruminants such as mature cattle and sheep. Along with the dose of infection, the susceptibility of the animals toward the pathogen was also taken into consideration so that it will sustain the population. The

animal infected with the pathogen can transmit the pathogen by shedding it in their faeces (Cornick and Helgerson, 2004). Human could be infected by the pathogen by the consumption of the food contaminated directly from the source or by contaminated surface. High infectious dose is required to infect the animals, while a lower dose is required to cause infection in humans. It was reported that the infectious dose for human can be as low as 50 to 700 organisms (Tilden *et al*., 1996, Tuttle *et al*., 1999).

2.4.5 Treatment and prevention

Treatment of *E. coli* O157: H7 infection usually involves the patient's appearance and the severity of the infection. In less severe cases, the management include resting and replacement of fluid and electrolyte where else surgery may be considered in critically ill patients with abdominal pain (Collins and Green, 2010). Antibiotics are used in septic patients but for *E. coli* O157:H7 infection, the use of antibiotics is not established as no clinical studies have proven that it is effective in reducing the infection and duration of bloody diarrhoea (Collins and Green, 2010). Several mechanisms have been proposed by Kimmit *et al.* (2000) explaining why the use of antibiotics in treating *E. coli* O157:H7 infection was not effective. The antibiotics may kill the intestinal normal flora leading to the overgrowth of *E. coli* if it was resistant to the antibiotics. Another reason is that antibiotics may cause the *E. coli* O157:H7 to lyse and release the Shiga toxins or induce the expression of Stx genes.

Although the issue regarding the use of antibiotic in *E. coli* O157:H7 treatment is controversial, Safdar *et al.* (2002) using meta-analysis studies showed no association between the usage of antibiotic with the risk of developing fatal HUS. It

is contrary with another cohort study by Wong *et al.* (2000) which reported that the antibiotic treatment in children infected by the pathogen increase the risk of HUS. The antibiotic frequently used for the treatment of *E. coli* O157:H7 infection is Trimethoprim-sulfamethoxazole and Fosfomycin (Safdar *et al*., 2002).

Beside treatment, prevention was done to eradicate the *E. coli* O157:H7 infection. Most of the practices have been applied and some are still in research or have not been established. For example, microbial intervention procedure was done at slaughter houses such as hot water washes and steam pasteurization (Berry and Wells, 2010), actually helps in reducing the number of bacteria from the cattle carcasses. Disinfection to kill or inactivate the organism can be done using several disinfectants such as 70% ethanol, glutaraldehyde, and iodine-based disinfectants, moist heat (121^{o}C, 15 minutes), dry heat (160-170^{o}C, 60 minutes) and for food, it is safe to cook them at a minimum temperature of 71^{o}C (CFSPH, 2009). Another recommendation to reduce the infection is by drinking the pasteurized milk and juice, wash fruits and vegetable before eating, wash hands after touching animals and avoid swimming in lakes which are also used by cattles (Buchanan and Doyle, 1997).

2.4.6 Anti- *E. coli* O157:H7 from plant extract.

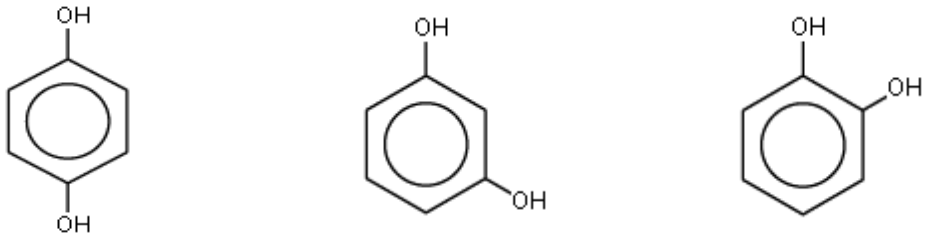
It is known that plants have been widely used in traditional medicine. The plants can be consumed in a large quantity by human with fewer side effects compared to the existing chemical anti-bacterial agents. Some plants are used as anti-bacterial agent to treat the infection and this lead some researcher to conduct a study of anti-bacterial activity of plant. Table 2.3 shows some plants exhibiting anti-*E. coli* O157:H7 activity.

Table 2.3: Plants with anti- *E. coli* O157:H7 activity

Plant	**Local name**	**References**
Punica granatum,	Delima	Voravuthikunchai *et al*. (2006)
Peltopharum pterocarpum	Jemerlang Laut	
Eugenia caryophyllata,	Cengkih	Stonsaovapak *et al.* (2000)
Cinamonum zeylanicum	Kayu manis	
Vaccinium macrocarpon	Kranberi	Wu *et al.* (2008)
Brassica oleracea.	Kailan	Hafidh *et al.* (2011)
Ascophyllum nodosum	Rumpai laut	Wang *et al*. (2009)
Quercus infectoria	Manjakani	Suwalak and Voruthikunchai, (2009)
Cinnamon zeylanicum	Kayu manis	Senhaji *et al.* (2007)

2.5 Phenolic compound

Phenolic compound is a substance containing one or more aromatic rings bearing one or more hydroxyl groups (Harborne, 1973). The simple phenols or polyphenols are grouped based on the phenol unit present in the molecule (Khoddami *et al*., 2013) which means a simple phenol is a phenol with a substitution pattern of the benzene ring and the functional group is hydroxyl while polyphenols refer to more than one phenolic hydroxyl group attached to one or more benzene rings (Vermerris and Nicholson, 2006). Figure 2.1 shows some molecular structure of the phenols. Harborne and Simmonds (1964) classified the phenolic compounds into groups based on the number of carbons in the molecule (Table 2.4).



Hydroquinone Resorcinol Catechol

Figure 2.1: Molecular structure of phenols.

Table 2.4: Classification of phenolic compounds.

Structure	**Class**
C_6	Simple phenolics
C_6-C_1	Phenolic acids and related compounds
C_6- C_2	Acetophenones and phenylacetics acids
C_6- C_3	Cinnamic acids, Cinnamyl aldehydes, Cinnamyl alcohols
C_6-C_3	Coumarins, isocoumarins and chromones
C_{15}	Chalcones, aurones, dihydrochalcones
C_{15}	Flavans
C_{15}	Flavones
C_{15}	Flavanones
C_{15}	Flavanonols
C_{15}	Anthocyanidins
C_{15}	Anthocyanins
C_{30}	Biflavonyls
$C_6 – C_1$- C_6, C_6,- C_2- C_6	Benzophenones,Xanthones, Stilbenes
C_6, C_{10}, C_{14}	Quinones
C_{18}	Betacyanins
Lignans, neolignans	Dimers or Oligomers
Lignin	Polymers
Tannins	Olygomers or polymers
Phlobaphenes	Polymers

Phenolic compounds are essential metabolites for plant growth, reproduction and serve in plant defence mechanisms against pathogens (Mujica *et al.,* 2009). Harborne (1973) reported phenolic compounds were one of the compounds included as phytoalexins (higher plant product of metabolism in response to microbial attack) and preinfective substances (secondary constituents occurred naturally which is important in imparting disease resistance to plants).

They also contribute to the properties of food mainly vegetal origin in term of the quality, nutritional value, colour, taste, aroma and flavour (Mujica *et al.,* 2009; Memmune *et al.*, 2009). Plant phenolics, in general, are highly effective free radical scavengers and antioxidants (Tung *et al.,* 2007). Their structure determines antioxidant activity and this was referred to as structure-activity relationship (SAR) (Balasundram *et al.,* 2006). The fact was supported by studies showing good correlation between the phenolic content of plants and their antioxidant activity (Dasgupta and De, 2004; Cai *et al.,* 2004).

Phenolic compounds also exhibit anti-bacterial, anti-inflammatory, anti-mutagenic and anti-nociceptive activities (Thèriault *et al.,* 2006; Güvenç *et al.,* 2010; Maddox *et al.,* 2010; Muthuraman *et al.,* 2011).

2.6 Anti-oxidant activity (AOA)

Oxidative stress occurs in response to the free radical which try to take electron from the body causing damage to the human cells related to human diseases such as chronic inflammation, atherosclerosis, degenerative disease and certain type of cancer (Karadag *et al*., 2009; Kumar, 2012). Anti-oxidant activity indicates the capability of the anti-oxidants to counteract the oxidative stress as they intercept and

react with the free radicals and inhibit the oxidation process (Wright *et al*., 2001; Tirzitis and Bartosz, 2010). A number of mechanisms can be served by anti-oxidants as preventive agents. These include acting as physical barriers by preventing the generation and access of reactive oxygen species (ROS) to the crucial biological site, trapping the energy and electrons, neutralizing or diverting ROS by acting as catalytic system, inactivating or binding thus preventing the generation of ROS and acting as chain-breaking anti-oxidants which scavenge and destroy ROS (Wright *et al.,* 2001; Karadag *et al.,* 2009).

Anti-oxidants present in cells are known as endogenous anti-oxidants and function to neutralize the oxidative stress occurred in the body. Anti-oxidants from the dietary intake are needed to assisst the endogenous antioxidants for acting their role efficiently (Pham-Huy *et al.,* 2008). Anti-oxidants are abundant in fruits and vegetables, tea, cereal, aromatic plants and others existing as natural anti-oxidants with some of the compounds with a well-known source such as tocopherols and ascorbic acids and some are too complex such as plant phenols (Dimitrios, 2006; Hamid *et al.,* 2010). It is very difficult to classify the anti-oxidants as their molecular constituents are heteregenous. Table 2.5 shows classes of the anti-oxidants and examples based on Vertuani *et al.* (2004).

Table 2.5: Various classes of anti-oxidants.

Class	**Example**
Vitamins	Retinol (Vitamin A), Vitamin E, Vitamin C, Nicotinamide (Vitamin B3), Riboflavin and Niacin
Fats and Lipids	PUFAs (Omega 3 and 6)
Amino acids	Taurine, Glutamine, L-Arginine, Histidine, Glycine, Thiols, N- Acetyl Cysteine,
Peptides	Carnosine, Gamma- glutamyl cysteinyl glycine (GSH)
Proteins	Albumin, Thioredoxin, Lactoferrin, Transferrin, Bilirubin, Ceruloplasmin
Plant derived products	Phenols (Tocopherols and tocotrienols), polyphenols, flavonoids, Carotenoids, Biochanin A, Glucosinolates, Allicin
Minerals	Zinc, copper, iron, Selenium, toxic metals,
Enzymes	Coenzyme Q

2.7 *Piper betle* L. (Sirih)

2.7.1 Taxonomy

Family Piperaceae comprises approximately 5 genera and 1400 species. The most representative are from genera *Piper* and *Peperomia* with 700 and 600 species, respectively (Vaghasiya *et al.*, 2007). The *Piper* species are scandent herbs, shrubs or infrequently trees and have high commercial, economical and medicinal importance (Parmar *et al*., 1997). *Piper betel* Linn are extensively grown in India, Sri Lanka, Malaysia, Thailand, Taiwan and other Southeast Asian countries with its common names known as betel (in English), paan (in Indian), phlu (in Thai) and Sirih (in Bahasa Indonesia) (Suppakul *et al*., 2006; Datta *et al.*, 2011). Taxonomy of *P. betle* is as follows (United States of Agricultural Department [USDA]):

Kingdom:	:	Plantae
Subkingdom:	:	Tracheobionta
Superdivision:	:	Spermatophyta
Division:	:	Magnoliophyta
Class:	:	Magnoliopsida
Subclass:	:	Magnoliidae
Order:	:	Piperales
Family:	:	Piperaceae
Genus:	:	*Piper* L.
Species:	:	*Piper betle* L.

Figure 2.2: *Piper betle* tree

Figure 2.3: *Piper betle* leaves

2.7.2 Usage

In traditional health system, the values of the *P. betle* L. are recognized and it has many uses. Apart from being traditionally used for chewing purposes with other condiments (Arambewela *et al.,* 2005a), betel leaves are also used in poultices to relieve stomach pains, antiseptic, treat ulcers, wounds and burns, detoxication (Joseph *et al.,* 2009; Kumar *et al.,* 2010). Its light yellow aromatic essential oil with sharp burning is valued as being aromatic, antiseptic, treating respiratory or digestive problems and even as aphrodisiac (Joseph *et al.,* 2009; Kumar *et al.,* 2010). The roots are believed to be contraceptive for women and favouritely chewed by singer for voice improvement while the flowers are used as ingredients in the betel quid and fruit for cough (Ghosh and Bhattacharya, 2005; Nagori *et al.,* 2011).

2.7.3 Chemical constituents

A number of studies have reported on the chemical constituents of *P. betle* L. A review paper by Parmar *et al.* (1997) classified nearly 600 chemical constituents belonging to *Piper* species within the period of 1907 to June 1996. Among them, compounds which were detected in the *P. betle* belonging to different classes of bioactive compounds such as alkaloids/amides (Piperine and Piperlonguminine), propenylphenols (Allylpyrocatechol diacetate, Chavibetol, Chavicol, Eugenol, Hydroxychavicol, Isoeugenol and Safrole), terpenes (Camphene, β-Caryophyllene, ρ-Cymene, Limonene, Myrcene, β–Pinene, α-Terpinene and α-Terpineol acetate), steroids (Sitosterol, β-Sitosteryl palmitate, γ-Sitosterol), Most of the compounds identified were found in the leaves of *P. betle*. The leaf of *P. betle* also consist of water, proteins, carbohydrates, minerals, fat, fibre, essential oil, tannin, alkaloid,

vitamin C, vitamin A, Thiamine, Riboflavin, minerals such as calcium, iron, iodine, phosphorus and potassium (Pradhan *et al.*, 2013).

Eugenol, safrole, quarcetin, β-sitosterol, Piperine, Piperlonguminine, Linalool, isoeugenol, flavones, α-pinene and β-caryophyllene are found in the flower, stem and roots of *P. betle* (Ghosh and Bhattacharya, 2005; Nagori *et al.*, 2011). Apart from the plant part, betel oil contains eugenol, caryophyllene, 3-benzodioxole and 5-(2-propenyl) (Caburian and Osi, 2010). Table 2.6 shows some of the activity exhibited by the compounds of *P. betle.*

Table 2.6: Biological activities of some chemical constituents of *P. betle* extracts (Kumar *et al.*, 2010)

Chemical constituent	**Biological activity**
Hydroxychavicol	Antidiabetic, Cytokine production in Th cells, inhibits platelet aggregation, chemoprotective
Allypyrocatechol	Healing gastric ulcer, anti-infalmmatory
Chavibetol	Radioprotective
Piperbetol, Piperol A, Piperol B, Methylpiperbetol	Selectively inhibit platelet aggregation

2.7.4 Pharmacological activities of *Piper betle* L.

The traditional usage and the constituents of *P. betle* have been pointed out previously and its pharmacological activity is listed in table 2.7.