IDENTIFICATION OF AN ENDOPHYTIC FUNGUS, *ASPERGILLUS TERREUS* MP15 ISOLATED FROM *SWIETENIA MACROPHYLLA* LEAVES WITH ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES

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By

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LIST OF ABBREVIATIONS

BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CREA	Creatine sucrose agar
CYA	Czapek yeast extract sucrose agar
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenylpicrylhydrazyl
EC_{50}	Half maximal effective concentration
EPS	Extracellular polysaccharides
GAE	Gallic acid equivalents
GC-MS	Gas chromatography-mass spectrometry
GMB	Methylene blue dye
GRAS	Generally recognized safe
HGT	Horizontal gene transfer
IBRL	Industrial Biotechnology Research Laboratory
IC_{50}	50% inhibitory concentration
INT	p-iodonitrotetrazolium violet salt
LC_{50}	50% lethal concentration
MBC	Minimum bactericidal concentration
MEA	Malt extract agar
MHA	Muller Hinton agar
MHB	Muller Hinton broth
MIC	Minimum inhibitory concentration
MOH	Ministry of Health
NA	Nutrient agar
PDA	Potato dextrose agar
rDNA	Ribosomal Deoxyribonucleic acid
ROS	Reactive oxygen species
SDB	Sabouraud dextrose broth
SEM	Scanning electron microscope
TLC	Thin layer chromatography
TPC	Total phenolic content
WHO	World Health Organization
YES	Yeast extract sucrose

PENGECAMAN KULAT ENDOFIT, *ASPERGILLUS TERREUS* MP15 YANG DIPENCILKAN DARIPADA DAUN *SWIETENIA MACROPHYLLA* DENGAN AKTIVITI ANTIBAKTERIA DAN ANTIOKSIDAN

ABSTRAK

Penyelidikan ini telah dijalankan untuk mengkaji aktiviti antimikrob kulat endofit yang telah dipencilkan daripada daun *Swietenia macrophylla* King. Dalam penyaringan pertama, satu daripada sembilan kulat endofit mempamerkan aktiviti antimikrob terhadap sekurang-kurangnya satu daripada mikroorganisma ujian. Kulat endofit ini telah dipilihkan untuk pemeriksaan selanjutnya serta dikenalpastikan sebagai *Aspergillus terreus* berdasarkan ciri-ciri morfologi dan juzukan molekul. Berdasarkan keputusan yang diperolehi daripada kaedah peresapan cakera, ekstrak etil asetat (ekstrasel) kulat ini menunjukkan aktiviti antibakteria yang signifikan dan stabil terhadap semua kultur bakteria Gram positif yang diuji. Zon perencatan yang telah direkodkan adalah dalam julat 10.3 ± 0.4 hingga 13.7 ± 0.6 mm, manakala kepekatan perencatan minimum adalah dalam julat 0.75 hingga 2.00 mg/mL serta kepekatan bakterisid minimum adalah dalam julat 2.00 hingga 8.00 mg/mL. Ekstrak ini adalah bakterisid terhadap *Bacillus subtilis*, *Bacillus spizizenii*, *Staphylococcus aureus*, manakala bakteriostat terhadap *Bacillus cereus*. Selain itu, penambahan ekstrak tumbuhan perumahnya ke dalam kaldu fermentasi tidak mempengaruhi aktiviti antibakteria kulat endofit dengan signifikan, menunjukkan kulat endofit ini mungkin mempunyai laluan biosintesis untuk menghasilkan sebatian bioaktif yang tersendiri. Tambahan pula, corak pertumbuhan dalam ujian bakteria telah menunjukkan bahawa aktiviti antibakteria ekstrak etil asetat ini terhadap *S. aureus* adalah bergantung kepada kepekatan ekstrak. Selain itu, bermula daripada jam

keempat untuk proses rawatan, semua sel bakteria telah dibunuh dengan kepekatan perencatan minimum tanpa hidup semula dalam jam-jam seterusnya. Kesan bakterisid ekstrak yang signifikan terhadap *S. aureus* juga telah dibuktikan apabila diperhatikan di bawah mikroskop elektron imbasan. Sel-sel bakteria menjadi hancur, perubahan bentuk asal yang bukan lagi kokus, dan pembentukan kaviti pada permukaan sel. Selain daripada itu, ekstrak kulat *A. terreus* MP15 ini menunjukkan aktiviti antioksidan yang berpotensi dan juga mengandungi jumlah kandungan fenolik yang tinggi. Tambahan pula, dalam asai bioautograf, keputusan telah menunjukkan bahawa satu titik berwarna kuning dengan nilai relatif pengerakan, R*f* 0.52 daripada ekstrak ini telah menyumbangkan kepada aktiviti antibakterianya. Kemudian, fraksi 2a berwarna kuning yang telah diperoleh daripada penyisihan separa ekstrak menunjukkan aktiviti antibakteria yang lebih berkesan kerana nilai-nilai kepekatan perencatan minimum dan kepekatan bakterisid minimum adalah dalam julat 0.25 hingga 0.50 mg/mL. Selain itu, ujian kematian anak udang telah menunjukkan fraksi tersebut tidak menyebabkan ketoksikan pada tahap akut dan kronik. Analisa gas kromatografi spektrometer jisim ke atas fraksi kuning ini juga telah menunjukkan sebatian utama ialah di-n-octyl phthalate, yang berkemungkinan menyumbang kepada aktiviti-aktiviti antibakteria dan antioksidan ekstrak ini. Kesimpulannya, ekstrak daripada *A. terreus* MP15 ini adalah sumber berpotensi untuk menjadi pengawet makanan yang bersifat antibakteria dan antioksidan, serta sebagai pewarna kuning.

IDENTIFICATION OF AN ENDOPHYTIC FUNGUS, *ASPERGILLUS TERREUS* MP15 ISOLATED FROM *SWIETENIA MACROPHYLLA* LEAVES WITH ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES

ABSTRACT

This research was aimed to study the antimicrobial activity of the endophytic fungi previously isolated from the leaves of *Swietenia macrophylla* King. In the primary screening, one out of nine endophytic fungal isolates exhibited antimicrobial activity against at least one of the tested microorganisms. This fungal isolate was selected for further tests and was then identified as *Aspergillus terreus* based on its cultural features and molecular sequences. Based on the result obtained from the disc diffusion assay, the ethyl acetate extract (extracellular) of this fungal isolate showed promising and stable antibacterial activity against all the tested Gram positive bacteria. The zones of inhibition recorded were ranged from 10.3 ± 0.4 to 13.7 ± 0.6 mm, whilst minimal inhibitory concentration (MIC) ranged from 0.75 to 2.00 mg/mL and minimal bactericidal concentration (MBC) ranged from 2.00 to 8.00 mg/mL. The extract was bactericidal against *Bacillus subtilis*, *Bacillus spizizenii* and *Staphylococcus aureus*, while bacteriostatic against *Bacillus cereus*. Furthermore, the addition of host plant extract in the fermentation broth had no significant effect on the antibacterial activities of the endophytic fungal isolate, suggesting that it may have its own biosynthetic pathway to produce bioactive compounds. Besides, time kill study revealed that the antibacterial activity of the ethyl acetate extract was concentration dependant towards *S. aureus*. Moreover, starting at the 4th hour of treatment, the bacterial cells were completely killed with MIC without any regrowth

in subsequent hours. The significant bactericidal effect of extract against *S. aureus* was also evidenced when viewed under scanning electron microscope. The bacterial cells were collapsed, no longer in coccal shape and there were formation of cavities on the cell surfaces. On the other hand, this *A. terreus* MP15 extract exhibited potential antioxidant activity and also consisted of high total phenolic content. Furthermore, on bioautography assay, it revealed that a yellow spot with retention factor, R*f* value 0.52 from this extract contributed to the antibacterial activity. Subsequently, the yellow fraction labeled as 2a of the extract exhibited more potent antibacterial activity as the MIC and MBC values were lowered, both ranged from 0.25 to 0.50 mg/mL. Besides, brine shrimp toxicity test also demonstrated that the fraction was non-toxic. The gas chromatography-mass spectrometry analysis of this yellow fraction revealed that the major compound was di-n-octyl phthalate, which may contribute to the antibacterial and antioxidant activities of the extract. Therefore, the extract of *A. terreus* MP15 can be regarded as a safer and potential source as antibacterial and antioxidant food preservatives, as well as yellow colorant.

CHAPTER 1.0 INTRODUCTION

1.1 Problem statements

Food safety is a significant and increasing public health issue (Rocourt *et al.*, 2003). Despite effort is continually done to ensure food safety, foodborne diseases remain the serious concern in both developed and developing countries. Moreover, approximately 1.8 million lives around the world are taken in addition to diarrheal diseases annually (WHO, 2006). In Malaysia itself, food poisoning cases are increasing as 62.47 cases per 100,000 populations were reported in 2008, while 36.17 in 2009 (Sharifa Ezat *et al*., 2013). However, the number of cases reported may only represent the tip of actual cases in addition to complex chain of reporting and monitoring processes (Soon *et al*., 2011).

Foodborne pathogens are causative agents of foodborne illnesses. Therefore, eradication of foodborne pathogens should be done at ease as Sharifa Ezat *et al.* (2013) reported that foodborne illnesses can cause significant impact on economic and trade in Malaysia besides mortality. However, the eradication of foodborne is complicated in addition to emergence of antimicrobial resistance in foodborne related microorganisms. Lim (2002) reported *Salmonella typhi* resistance in Malaysian government hospital, whereas Kadariya *et al.* (2014) and Shaheen (2009) reported resistance of *S. aureus* and *B. cereus*, respectively. Therefore, food spoilage microbes should be eradicated beforehand with application of antimicrobial preservatives during food processing to ensure food safety. However, currently available antimicrobial preservatives had been growingly associated with allergic reactions, cancer, birth defects and other health problems (Jeon, 2011). In addition to that, naturally occurring antimicrobial compounds should be discovered for more choices and effective food preservatives.

On the other hand, oxidative process and accumulation of reactive oxygen species also impacted on food and human health, respectively. Lipid peroxidation causes rancidity and hence food spoilage. Meanwhile, accumulation of free radicals causing oxidative stress in human body may also result in significant cell damages. Moreover, it leads to various diseases such as cancer, stroke and heart diseases (Wiseman and Halliwell, 1996). In addition to combat the accumulation or to scavenge free radicals, natural antioxidants are suitable candidates. They are effective free radical scavengers that can initiate chain breaking of reactive species to form stable compounds (El-Diwani *et al*., 2009).

1.2 Rationale of the study

In addition to the urgent need to discover new and more effective natural products for food preservation, endophytes are considered as the potential sources of novel bioactive compounds. It remains as an untapped source as approximately 300,000 plant species which exist on earth, each of them harbors more than one endophyte (Strobel and Daisy, 2003).

Endophytes are microorganisms which colonize the living internal tissues of various host plants and establish mutual relationship without any symptom of diseases (Zhang *et al*., 2006). Upon colonizing the host plant, endophytes synthesize secondary metabolites to aid in survival and stress tolerance of host plant; and in turn the host plants supply nutrients and as habitat for the endophytes (Firakova *et al*., 2007). Therefore, in addition to the interaction of endophytes with the host plant, the endophytes acquired the ability to produce similar or more pronounced bioactive compounds form the host plant (Zhao *et al*., 2010a). More specifically, endophytic fungi are known as outstanding sources of bioactive compounds (Kaul *et al*., 2013; Strobel and Daisy, 2003). Strobel (2003) also reported that endophytic fungi are able to produce antibacterial, antioxidant, antiviral and anticancer compounds with pharmaceutical importance.

Since there are vast numbers of plant species in the world, plant selection for the study of the residing endophytes is essential in order to narrow the discovery of potential endophytes (Strobel, 2003). Plant that has ethnobotanical history associated with specific uses or interest can be a criterion for plant selection (Strobel and Daisy, 2003). In addition to search for new antimicrobial and antioxidant compounds, *Swietenia macrophylla* King commonly known as “Pokok Tunjuk Langit” in Malay or big leaf mahogany was used in this study. It is a medicinal plant used to treat diabetes, epilepsy, hypertension, diarrheal and malaria traditionally (Pullaiah, 2006). Furthermore, it has been commonly used in Asia and other countries to treat wide range of diseases in addition to its antimicrobial, anti-inflammatory, antioxidant, anticancer and anti-diarrheal activities (Eid *et al*., 2013; Moghadamtousi *et al*., 2013). Therefore, in addition to its medicinal value, it is believed to harbor potential endophytes which can produce novel bioactive compounds with diverse structure (Strobel, 2003). Moreover, Darah *et al*. (2014a) reported that the leaves of *S. macrophylla* are the valuable sources for discovery of endophytic fungi with bioactive properties.

1.3 Objectives of study

The aims of the present study are as follows:

1. To screen, select and identify endophytic fungus with promising antimicrobial activity from *Swietenia macrophylla* leaves.
2. To evaluate the antimicrobial and antioxidant activities of the crude extract of *Aspergillus terreus* MP15.
3. To partially purify the bioactive fraction of the *A. terreus* MP15 extract and to identify the possible compounds with antibacterial and toxicity activities.

CHAPTER 2.0 LITERATURE REVIEW

2.1 The need to discover new antimicrobial agents for food preservation

2.1.1 Foodborne diseases as a threat to food safety

Foodborne diseases are illnesses which onset in addition to ingestion of food contaminated with microorganisms or other agents (Giuseppe *et al.*, 2010). The contaminants can be classified into three main categories namely biological, chemical and physical agents. In addition to that, according to Centers for Disease Control and Prevention (CDC), 90% of foodborne diseases are caused by biological agents such as bacteria, virus, fungi and parasites (Simonne, 2010). Microbes are prone to contaminate food supply at any point during production, processing, packing, transportation and even preparation prior to consumption (Unnevehr, 2003). Following that, Table 2.1 is showing the list of examples of microorganisms which are causative agents in foodborne illnesses. In addition to foodborne illnesses, the common symptoms are vomiting, diarrhea, abdominal pain and fever. Some diseases can also lead to more serious complications and even fatal (Scallan *et al.*, 2011), including cholera, salmonellosis, typhoid fever and *E. coli* gastroenteritis (Kaferstein, 2003). Foodborne diseases impose heavy toll in human life particularly the infants, children, the elderly and immunocompromised group (Van de Venter, 2000).

Furthermore, in addition to interconnected global food chains and trade, it escalates international incidents involving contaminated food sources (WHO, 2015). Foodborne diseases now represent a serious and emerging public health concern in both developed and undeveloped countries (Giuseppe *et al.*, 2010). In each year, millions of citizens are infected with foodborne illnesses and even fatal upon consumption of unsafe food. WHO (2015) reported that diarrheal diseases alone kill an approximately 1.5 million children worldwide annually. In the United States,

Table 2.1 The types of microorganisms which cause foodborne illnesses. (CDC, 2001)

Microorganisms	Incubation period	Signs and symptoms	Duration of illness	Associated food
Bacillus cereus	1-6 hrs	Sudden onset of nausea and vomiting. Diarrhea may be present.	24 hrs	Improperly refrigerated cooked rice, meat.
Campylobacter jejuni	2-5 days	Diarrhea, cramps, fever and vomiting.	2-20 days	Meat, stews.
Escherichia coli	1-8 days	Severe diarrhea with blood present, abdominal pain and vomiting.	5-10 days	Undercooked beef, unpasteurized milk and raw fruits and vegetables.
Salmonella spp.	1-3 days	Diarrhea, fever and vomiting.	4-7 days	Contaminated eggs, poultry and unpasteurized milk.
Shigella spp.	24-48 hrs	Abdominal cramps, fever and diarrhea.	4-7 days	Food or water contaminated with fecal matters. Fecal-oral transmission
Staphylococcus aureus	1-6 hrs	Sudden onset of severe nausea and vomiting. Diarrhea and fever	24-48 hrs	Unrefrigerated meat, potato, egg salads.
Vibro cholerae	24-72 hrs	Profuse watery diarrhea and vomiting, which can lead to severe dehydration and death.	3-7 days	Contaminated water, fish and shellfish.
Yersinia enterocolitica	24-48 hrs	Diarrhea, vomiting, fever and abdominal pain.	1-3 weeks	Undercooked pork, unpasteurized milk

there were approximately 76 million illnesses with 325,000 hospitalisations and 5000 deaths reported each year (Lim, 2002). On the other hand, in Malaysia itself, there was a doubling in incidence rate of food poisoning cases from 2008 to 2009, which was 36.17 cases per 100,000 population to 62.47 (MOH, 2010). Despite that, the officially reported cases may only represent the tip of the actual incidence of foodborne cases in Malaysia. There may be a huge number of cases left undiagnosed or officially unreported (Lim, 2002).

Once foodborne diseases are not eradicated, this issue significantly affects food safety as it relates to both human health and economical trade development (Noraini, 2007). In terms of the effects on human health, it includes the cost of mortality, morbidity and quality of life. On the other hand, food safety problems also cause high losses in income for producers and as well as reduced food supply for trading (Bhat and Vasanthi, 2003). In United States, foodborne disease is estimated to cost at $6.5 billion to $34.9 billion annually. Besides that, in Australia, foodborne is estimated for costing A$1.2 billion every year. However, in Malaysia, cost study analysis was not done to evaluate the cost or losses for foodborne diseases (Sharifa Ezat *et al*., 2013).

Therefore, eradicate foodborne diseases and hence improve food safety is vital to ensure healthy and sufficient food supply in both developing and developed countries (Unnevehr, 2003). It is essential as WHO (2015) reported that approximately 70% of the new infectious diseases emerged over the decade, were spread by bacteria, viruses and other microbes in animals and animal products. Therefore, any food product and livestock should be prevented from infected or contaminated with biological hazard even at the farm level. Thus far, naturally occurring antimicrobial agents as natural food preservatives should be utilised to eradicate spoilage microbes and hence to ensure food safety.

2.1.2 Antibiotics resistance in foodborne microorganisms

Antibiotics have long been used in food industry to enhance food safety. They have been applied to eliminate pathogens or food spoilage microorganisms and hence to enhance the shelf life of food product (Crozier-Dodson *et al*., 2005). Besides that, since the 20th century, antimicrobial agents are given to livestock and poultry for the prevention, control and treatment of infections. Thus far, the advent of antibiotics to treat infectious diseases significantly promoted the health and life expectancy of human and also health and welfare of animals (Verraes *et al*., 2013).

However, in addition to misuse and overuse of antibiotics, antimicrobial resistance is now recognized as the top health challenge nowadays (Marshall and Levy, 2011). Antimicrobial resistance is the ability of microorganism to resist the effect of antimicrobial agent beyond its normal susceptibility (Acar and Rostel, 2001). Moreover, several mechanisms were postulated to involve in conferring resistance in bacteria, namely disruption of bacterial cell wall permeability, enzymatic degradation and target modification of antimicrobial compounds, as well as deviation from normal physiological pathways to escape from antimicrobial action (Verraes *et al*., 2013). Besides, Furuya and Lowy (2006) also reported that antimicrobial resistance is acquired possibly when a susceptible strain obtained specific resistance gene from a resistant bacteria via horizontal gene transfer (HGT). Following that, the three mechanisms of HGT namely conjugation, transformation and transduction are illustrated in Figure 2.1.

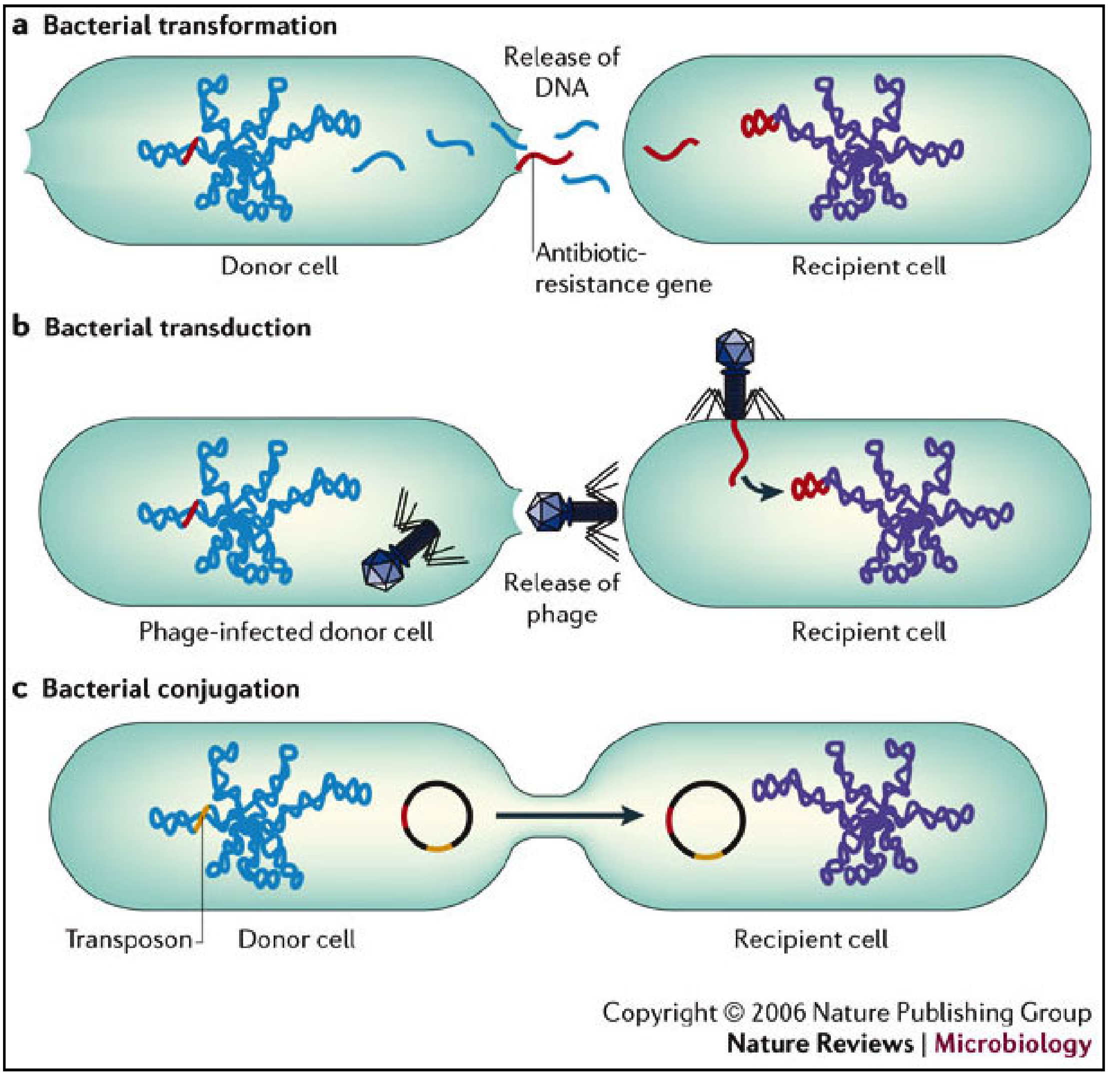

Figure 2.1 Possible three mechanisms of HGT for acquisition of resistance gene from resistant bacteria. (Furuya and Lowy, 2006)

Since the first use of antibiotics treatment for foodborne illnesses, there is ascending number of resistant bacteria over the decades. *E. coli*, *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., *S. aureus* are among the commonly reported resistant foodborne bacteria (Angulo *et al*., 2004). In addition to that, *E. coli* O157:H7 was firstly reported by Ratnam *et al*. (1988) to resistant against several commonly used antimicrobial agents. Unfortunately, resistance in *E. coli* is emerging as Horii *et al*. (1999) reported that it was resistant to other six antibiotics namely ampicillin, streptomycin, tetracycline, ticarcillin, kanamycin and sulfasoxazole.

Besides, *Salmonella* species especially multi-drug resistant *Salmonella typhimurium* DT104 has been reported as emerging causative agent for human infections (Threlfall, 2000). Furthermore, in 1972, *Yersinia enterocolitica* was reported to resistant against several antibiotics namely carbenicillin, ticarcillin and cephalothin (Preston *et al*., 1994), yet there are increasing reports on its emergence of antibiotic resistance (White *et al*., 2002). Moreover, another rapidly emerging foodborne pathogen, methicillin-resistant *S. aureus* (MRSA) was also detected in year 2005 as reported by Armand-Lefevre *et al*. (2005). On the other hand, *Bacillus cereus* as one of the common foodborne pathogens was also reported to resistant against several antibiotics namely ampicillin, ampiclox, cotrimazole and cloxacillin (Umar *et al*., 2006).

Resistant bacteria can be exposed or transmitted to humans through many ways as they can be found in soil, water, human or animal feces. They can contaminate poultry during slaughter, while may also affect plant during production possibly being exposed to contaminated water or sewage discharge (Schluter *et al*., 2007). Furthermore, after food processing, there is a high risk of post contamination and as well as during food handling by consumers. In addition to that, possible occurrence of resistant bacteria transmission to human significantly increase the risk of treatment failures and severity of infection, prolonged duration of sickness, as well as increase in hospitalisation and mortality (Newell *et al*., 2010). Therefore, eradication of resistant microbes is at ease as it is affecting human health and welfare, yet many of the currently available antimicrobial agents for food preservation are reported hazardous for human and animal. To date, the safety of many food preservatives is the subject of discussion or debate among the researchers and authorities major in food science, toxicity study and biology fields (Abdulmumeen *et al*., 2012).

2.1.3 Health considerations for currently used antimicrobial preservatives

Food preservatives are substances which are added in food directly or indirectly to affect the characteristics of any food (FDA, 2014). More specifically, antimicrobial preservatives are one of the types of preservatives which can inhibit or kill bacteria or fungi, including mold to extend shelf life. Common antimicrobial preservatives used are including sodium nitrate, calcium propionate, sulphites and so on (Abdulmumeen *et al*., 2012). Since the year 1858, boric acid had been firstly used as an antimicrobial food preservative. In the past, people assumed that small volume of a substance sufficient to act as preservative may scarcely harmful (Luck and Jager, 1995). Thereafter, benzoic acid and ascorbic acid were introduced in food preservation to prevent microbial growth (Dziezak, 1986). Following that, acetic acid, fumaric acid, lactic acid and pumaric acid are also applied to prevent growth of foodborne bacteria sequentially (Podolak *et al*., 1996).

However, in the nineteen-fifties, in addition to advent of toxicology study, the safety of preservatives was re-evaluated (Luck and Jager, 1995). Preservatives are subjected to various tests to ensure that they are safe for human consumption. Nowadays, animals and human volunteers are utilised for advanced stages of trials. Besides that, preservatives are now assessed in stepwise basis based on following criteria: a) biochemical aspects, b) acute toxicity, c) short and long term of toxicity, d) long term studies of toxicity and carcinogenicity, e) genotoxicity and f) reproductive toxicity (WHO, 2010).

After tests and toxicological evaluation, many antimicrobial preservatives have been growingly associated with allergic reactions, carcinogenic, birth defects and other health problems (Jeon, 2011). In addition to that, boric acid, a commonly used preservative was banned in the year 1950s due to its toxicity. Furthermore, benzoates which are widely used as antimicrobial preservatives also reported to cause worsening asthma, allergic rhinitis and chronic urticaria. Another type which is sorbates also tested to cause urticaria and contact dermatitis (Kinderlerer and Hatton, 1990). Furthermore, Table 2.2 is showing other examples of antimicrobial preservatives used in different food products and their side effects. Thereafter, the number of banned preservatives is ascending. Therefore, new and more effective naturally occurring antimicrobial compounds should be discovered to replace the harmful food preservatives.

Table 2.2 Examples of antimicrobial preservatives and their side effects (Pankey and Upadhyay, 2012).

Name	Types of food	Possible negative effects
Sodium benzoate	Carbonated drinks, pickles, sauces.	Carcinogenic, neurotoxic, and may cause fetal abnormalities.
Sulphur dioxide	Carbonated drinks, dried fruits, juices, potatoes	Induce gastric irritation, nausea, causes fetal abnormalities.
Sodium metabisulphite	Potato chips, raisins and apples	May cause severe asthma
Potassium nitrate	Canned meat products	Lower oxygen carrying capacity of blood, carcinogenic, affect adrenal gland
Calcium benzoate	Cereals and meat products	May inhibit digestive enzyme function
Calcium sulphite	Biscuits and burgers	May cause bronchial problems, low blood pressure, and anaphylactic shock.
Benzoic acid	Drinks, cereals and meat products	May inhibit digestive enzyme function

2.2 Antibiotics

2.2.1 Definitions and sources

The term ‘antibiotic’ was a Greek derivation which literally means *against life* and was firstly reported or used in literatures by Waksman and Woodruff (1942). It was also defined as substance of microbial origin which selectively inhibits the growth of other microorganisms. Meanwhile, following the production of antibiotics from microbial sources, the discovery of antibiotics now have been extended to plants, chemically modified natural antibiotics and synthetic compounds. Therefore, Keyes *et al*. (2008) now defined antibiotics as low molecular weight compounds, whether of microbial origin, metabolites of organisms or synthetic compound, which can kill or inhibit the growth of other microorganisms at low concentrations.

Flashback to the history of antibiotics, the introduction of the first antibiotic, Penicillin from microbial origin by Sir Alexander Fleming in 1929 had been regarded as one of the noble contributions in addition to its ability to selectively treat staphylococcal infections (Bisht *et al*., 2009). Following the production of penicillin, other antibiotics namely streptomycin, chloramphenicol and tetracycline were introduced (Clardy *et al*., 2009). Besides that, since the advent of antibiotics, it increased the average lifespan of population and controlled some of the infectious diseases (Berdy, 2012). However, the report on antibiotic resistance in the 1940s had rendered the efficacy of natural-derived antibiotics low. Following that, semi synthetic antibiotics which were modified compounds from natural products were introduced to circumvent antibiotics resistance. Sulfonamides were the first semi-synthetic antibiotics introduced in year 1937 (Davies and Davies, 2010). Despite the introduction of semi-synthetic drug, the search of antibiotics from natural sources is an ongoing trend (Newman and Cragg, 2007). In the pipeline of antibiotics production, natural-derived antibiotics are remained dominants. Among 40 antibacterial compounds undergoing clinical trials, 20 are natural-derived, 18 are synthetic and 2 are from other sources (Butler and Cooper, 2011). Importantly, the natural-derived compounds dominated the phase III of clinical trials while only few synthetic ones were approved in phase III. Therefore, in addition to disappointing results obtained in developing synthetic compounds, it has prompted the continuous effort on discovery of antibiotics from natural products.

Secondary metabolism in nature evolved in response to stress and needs of the natural environment. It was believed that over the years, the producers of secondary metabolites such as bacteria and fungi undergo evolutionary processes to naturally select the biosynthetic genes of potential compounds. Those genes are retained and even further modified for improvement (Demain, 2009). Furthermore, secondary metabolites produced aid in survival of interspecies competition and confer defensive mechanism for its producer (Vaishnav and Demain, 2011). Therefore, the metabolites produced are in diverse array of reactive functional groups and are possible novel antibiotics (Verdine, 1996). In addition to that, 100,000 metabolites are derived from plants, whereas 200,000 microbial compounds are isolated annually (Knight *et al.*, 2003). However, the number of antibiotics discovered is descending over the decades. Therefore, more effort should be put on to isolate potential and novel antimicrobial compounds from the natural sources.

2.2.2 Mode of action

2.2.2.1 Antibacterial agent

Antibiotics are classified based on its main group of target microorganisms, namely antibacterial, antifungal, anti-yeast and antiviral. The inhibitory or killing effect of antibiotics is a complex process which firstly involves the physical interaction of the drug and specific target in microorganisms, and followed by the alterations at the biochemical, molecular or structural levels (Kohanski *et al*., 2010). In addition to explanations on antibacterial agents, they are substances which are involved in killing or inhibit the growth of bacteria. They are of different types of classes with distinct in modes of action, specificity and range (Zaffiri *et al*., 2012). They can be from classes such as quinolones, rifamycins, β-lactams, macrolides and so on. Furthermore, it was postulated that they act on bacteria via inhibition of DNA, RNA, cell wall and protein synthesis (Lewis, 2013).

In a replicating bacterial cell, the DNA synthesis, mRNA transcription and cell division all require the DNA-topoisomerase complexes. With that, the quinolones class of antibiotics target and interferes with the production machinery of these complexes. They will trap the enzymes topoisomerase II and IV during DNA cleavage and then halt the joining of DNA strands (Drlica *et al*., 2008). In addition to disruption in DNA replication process, it has lethal effect on the nucleic acid metabolism and hence resulting bacterial cell death.

Besides that, the inhibition of RNA synthesis is also another mode of action for rifamycins group of antibiotics. Rifamycins are microbial antibiotics which originally isolated from *Amycolatopsis mediterranei* in the 1950s (Sensi, 1983). It inhibits DNA replication through binding to the transcribing RNA polymerase, hence preventing the synthesis of RNA strand (Floss and Yu, 2005).

On the other hand, another mode of action of antibiotics to treat on bacterial cells is through disruption of cell wall synthesis. The bacterial cell wall is bounded by layers of peptidoglycan which are essential to retain its mechanical strength for survival (Cabeen and Jacobs-Wagner, 2005). In addition to that, classes of antibiotics such as β-lactams and glycoproteins kill the bacteria through interference of cell wall biosynthesis. In response to that, it will cause changes in cell shape and size, activation of cell stress responses and finally cell death (Tomasz, 1979).

Furthermore, inhibition of protein synthesis is also one of the modes of action for antibiotics from classes such as macrolides, lincosamides, amphenicols and oxazolidones (Katz and Ashley, 2005). They are involved in blocking the protein translation or translocation of tRNAs, which are crucial for the elongation of peptide chains (Kohanski *et al*., 2010). In addition to disruption in peptide elongation, the formation of protein cannot be preceded and hence inhibit or kill the bacterial cells.

2.2.2.2 Antifungal agent

On the other hand, antifungal agent is defined as drug which eradicates fungal infection selectively with minimal toxicity to the host (Dixon and Walsh, 1996). There are three main categories of antifungal drugs in clinical use which are azoles, polyenes and allylamine. Majority classes of antifungal drugs target ergosterol or 1,3-β-D-glucan, which are major components in the fungal cell wall (Shapiro *et al*., 2011). They are components involved in regulation of membrane fluidity and also integrity of fungal membrane (White *et al*., 1998). Thus far, antifungal agent acts on pathogenic fungi through direct interaction with ergosterol and their synthesis processes (Parks and Casey, 1996).

Azole-based antifungal drug exert activity through inhibition of cytochrome P_{450} dependent enzymes, which involved in the biosynthesis of ergosterol. Following that, the depletion of ergosterol leads to accumulation of sterol precursors, hence resulting in the alteration of fungal membrane structure and function (Ghannoum and Rice, 1999). This group of antifungal agents includes ketoconazole, fluconazole and itraconazole. On the other hand, allylamines-based antifungal agent such as terbinafine and naftifine exert antifungal action through inhibition of ergosterol synthesis. Furthermore, another group of antifungal agent is polyenes. Amphotericin, nystatin and primaricin are examples of this type of antibiotics. These polyenes-based antibiotics exert antifungal action through formation of pores and cause leakage of cellular matters to the extracellular environment (Dixon and Walsh, 1996). Despite amphotericin is an effective drug, it has narrow spectrum of antifungal activity and thus limiting its activity. Therefore, new choices of antifungal drug should be discovered from the natural sources to inhibit or kill the mold or fungi for food preservation.

2.2.3 Antibiotics as food preservatives

In addition to provide safer food supply for human consumption and hence reduces the occurrence of foodborne illnesses, antimicrobial food preservatives are utilised in food to control the growth of harmful microorganisms. They are substances added in food to prevent deterioration by killing or inhibiting the growth of microorganisms (Juneja *et al.*, 2012). Generally, they have been applied in the product formulation step as direct food additives for reduction or elimination of spoilage organisms and in the food production process as secondary food additives (Crozier-Dodson *et al.*, 2005).

In addition to that, food preservatives can be grouped into three categories including processing aids, secondary direct food additives and direct food additives (FDA, 2014). For processing aid, it is added to food during processing and following that, it will be removed or converted into normal food constituents which have insignificant non-functional residuals. Besides that, for direct additives, it will be added and are functional for final food product. On the other hand, secondary direct food additives are applied during manufacture but are removed from the food product lastly (Crozier-Dodson *et al.*, 2005).

Furthermore, an antimicrobial preservative should posses the following characteristics: a) wide spectrum of antimicrobial activity, b) non-toxic, c) will not encourage development of resistant strain and d) will not affect the flavor, taste and aroma of the food (Gupta, 2007). Besides that, the antimicrobial preservatives which are safe for consumption are labelled as generally recognized safe (GRAS). In addition to possible adverse effect of synthetic or chemical preservatives imposed on human, natural additives are in great interest for discovery of the researchers. They are known to be more effective and safer (Lucera *et al*., 2012; Sharma and Fumio, 2004). Besides that, they can be found available from plants and microorganisms (Brul and Coote, 1999). Following that, Table 2.3 is showing the list of examples of antimicrobial preservatives isolated from natural sources.

Table 2.3 List of antimicrobial preservatives isolated from natural sources.

Sources	Types of antimicrobial preservatives	References
Plants		
Basil	Essential oil	Wan *et al*. (1998)
Solanum spp.	Saponins	Davidson and Naidu (2000)
Cedrus deodara	Flavonoids	Zeng *et al*. (2011)
Fungi		
Ganoderma boninense	Dodecanoic acid	Chong *et al*. (2014)
Xylaria sp. YX-28	7-amino -4-methylcoumarin	Liu *et al*. (2008)
Calcarisporium sp.	Cibaric acid, 1-hydroxy-trans-8-decenoic acid	Ji *et al*. (2004)
Bacteria		
Bacillus amyloliquefaciens ES-2	Fengycin and surfactin	Sun *et al*. (2006)
Bacillus subtilis SC-8	Surfactin	Yeo *et al*. (2012)
Lactobacillus acidophilus	Bacteriocins	Mohankumar and Murugalatha (2011)

2.3 The need to discover antioxidants for food preservation

2.3.1 Rancidity in food and chronic diseases

Lipid peroxidation and followed by rancidity is leading cause of food spoilage. In year 1992, a foodborne outbreak due to rancid biscuits affected 45 children and they suffered from vomiting, diarrhea and abdominal pain (Bhat *et al.*, 1995). Lipid peroxidation happens when atmospheric oxygen reacts with the food components especially fats and oils, resulting in undesirable flavor and odor of the food (Bozin *et al.*, 2007). In detail, it occurs via free radical chain reaction and involved the initiation of reactive oxygen species (ROS). ROS includes hydroxyl radical, superoxide anion, hydrogen peroxide and singlet oxygen (Min and Ahn, 2005). Furthermore, the chain reaction involved three major steps namely initiation, propagation and termination. Initiation takes place when free radical abstracts a hydrogen atom to form radicals. Following that, the radicals formed then tend to abstract hydrogen atom from neighboring lipids to form lipid hydroperoxide. This chain reaction will only be terminated when the radicals react with other molecules to form non-radical products (Min and Ahn, 2005).

Moreover, Coupland and McClements (1996) reported that rancidity will produce potentially toxic products such as hydrocarbons, peroxides, aldehydes and dangerous free radicals in food. Upon consumption of rancid food, it will lead to accumulation of free radicals in the human body and hence cause adverse health effects (Fife, 2004). Free radicals are unstable, highly reactive and upon accumulation, it will lead to oxidative stress. Each of them has an unpaired electron which can attack essential macromolecules such as DNA, proteins, carbohydrates and lipids, leading to cell destruction and homeostatic disruption (Young and Woodside, 2001). Following that, it causes diseases such as cancer, atherosclerosis, stroke, diabetes and many others (Gilbert, 2000). In a study done by Feng *et al.* (2007), they evidenced that consumption of rancid butter and sheep fat significantly associated with nasopharyngeal carcinoma. In addition to that, in order to curb the problem of rancidity, antioxidants are ideal candidates as they can inhibit the formation of free radicals (Dauqan *et al.*, 2011). Therefore, they should be used as food preservatives and as well as to play essential roles in the body defense system against the ROS to maintain human good health (Ou *et al.*, 2002). To date, researchers are gaining interest on discovery of natural antioxidants in addition to their role in human health (Dauqan *et al.*, 2011).

2.4 Antioxidants

2.4.1 Definitions and sources

Antioxidants are defined as compounds which are stable to donate an electron to reactive free radical and neutralize it, hence reducing its damages (Lobo *et al.*, 2010). The reactive free radical is containing unpaired electron and thus antioxidant will bind to this unpaired electron followed by neutralising it. They will terminate the chain reaction of free radicals before any damages on the essential macromolecules occur, as well as delay lipid peroxidation (Dauqan *et al.*, 2011). Early research on antioxidants in biology focused on preventing rancidity, and only after the discovery of vitamin A, C, E as antioxidants, researchers also realized the importance of antioxidants in the biochemistry of living organisms. Despite human body is able to produce some antioxidants such as uric acid, ubiquinol and glutathione, they are only sufficient to cope with physiological rate of free radical generation (Agarwal and Prabakaran, 2005). Some other antioxidants such as vitamin C, E, β-carotene and so on are to be acquired from diet (Levine *et al.*, 1999).

Antioxidants are mainly reducing agents such as ascorbic acid, tocopherols and polyphenols (Sies, 1997). In addition to that, plants and microbes are rich sources of potential natural antioxidants (Gupta *et al*., 2013). Natural antioxidants can provide chain breaking antioxidants which are highly reactive against free radicals for forming stable compounds (El-Diwani *et al*., 2009). Man and Tan (1999) also reported that natural antioxidants from rosemary and sage extracts were comparable to the antioxidant activities of synthetic antioxidants namely butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in preventing lipid peroxidation of potato chips. Furthermore, great interest has been put on natural antioxidants in addition to emerging problem regarding toxicity of synthetic antioxidants (Venskutonis, 2004). In addition to that, blueberry, cranberry, grape fruit, lemon, orange and spinach are plants producing these compounds. Following that, Table 2.4 is showing the examples of naturally occurring classes of antioxidants and the substances.

Table 2.4 The naturally occurring classes of antioxidants and examples of substances (Pokorny, 2007).

Antioxidant class	**Examples of substances**
Flavonoids	Quercetin, catechin, rutin
Anthocyanins	Delphinidin
Tannins	Procyanidin, ellagic acid, tannic acid
Lignans	Sesamol
Stilbenes	Resveratrol
Coumarins	*ortho*-coumarine
Essential oils	S-Carvone

2.4.2 Mechanisms of action of antioxidants

Antioxidants scavenge free radicals by accepting or donating an electron to form non free radical molecule. Different types of antioxidants have distinct mechanisms of action. Moreover, they commonly act by neutralizing free radical molecule to non-free radical molecule (Dauqan *et al*., 2011). Vitamin C or ascorbate is one of the essential dietary constituents for human as it is an effective reducing agent for various types of free radicals (El-Beltagi and Mohamed, 2013). Vitamin C, $AscH^-$ acts as an antioxidant through donating a hydrogen atom to a free radical and hence neutralizing it. Subsequently, it will become an ascorbate, Asc^- as final product, which is a stable free radical (Best, 1990). Following that, Figure 2.2 is showing the reaction involved for free radical scavenging activity of vitamin C.

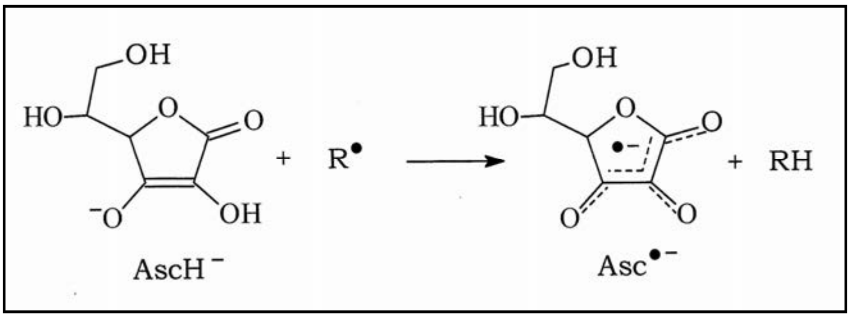

Figure 2.2 The free radical scavenging activity of vitamin C through formation of ascorbate free radical. (Best, 1990)

Another group of antioxidant is known as tocopherols or more specifically, vitamin E. It is a natural, highly tolerable molecule and effective as membrane stabilizing agent (Fryer, 1992). Furthermore, it is known to interfere with the free radical chain reactions by capturing free radicals. It has unpaired hydroxyl group on the aromatic ring which is responsible for its anti-oxidative properties. Moreover, this hydrogen group will interact with the free radical, leading to relatively stable form of vitamin E (Engin, 2009). Following that, Figure 2.3 is showing the antioxidant effect of vitamin E on lipid hydroperoxyl radical (LOO•).

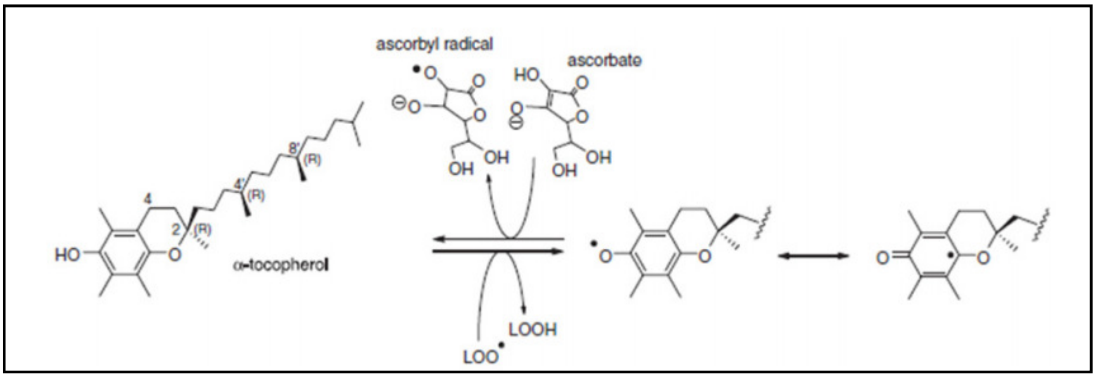

Figure 2.3 Antioxidant effect of vitamin E. It reacts with lipid hydroperoxyl radical and become stabilized radical. (El-Beltagi and Mohamed, 2013)

Furthermore, carotenoid is another type of antioxidant which capable of detoxifying various forms of reactive oxygen species. The examples of carotenoids are such as alphacarotene, beta-carotene, lutein and lycopene (Zed and Mehmood, 2004). In terms of its antioxidant properties, it exerts this ability via several ways. It reacts with the oxidation products to terminate the chain reactions, scavenge singlet oxygen and release the heat energy, and also interact with triplet or excited molecules to prevent formation of singlet oxygen. Moreover, it is an effective antioxidant in addition to the extended system of conjugated double bonds in its structure (El-Beltagi and Mohamed, 2013).

Besides, phenolic compounds are characterized by having more than one aromatic ring bearing at least one hydroxyl group. They exhibit strong antioxidant activity as they are efficient hydrogen and electron donors, and as well as able to form polyphenol-derived radical to neutralize the unpaired electron (Rice-Evans *et al.*, 1997). Another distinctive ability of phenolic in antioxidant ability is to modify the lipid structure and lower membrane fluidity to restrict the diffusion of free radicals into the cells (Arora *et al*., 2000). Therefore, as antioxidants are essential dietary constituent and can prevent rancidity in food, they should be discovered from natural sources such as endophytic fungi. Yadav *et al.* (2014b) reported that endophytic fungi are producing phenols and terpenes, which are the main constituents for antioxidants.

2.4.3 Antioxidants as food preservatives

Antioxidants are one of the types of food preservatives used in processing and storage of food. It is added to reduce oxidation of unsaturated fatty acids, oils and lipids, thus preventing food product to turn rancid (Rahul and Shalinee, 2012). In addition to that, it can promote safety and palatability of food for human consumption. Furthermore, antioxidants can also be added in functional food to enhance human health for protection against free radicals damages (Gupta *et al*., 2013). Natural antioxidants have been utilised in food manufacturing process since 1920s as synthetic antioxidants were not available at that time (Nahas, 2011). However, due to their variability in composition and efficiency, synthetic antioxidants have been invented (Pokorny, 2007).

In addition to that, there are two major groups of antioxidants used in food preservation, which are natural and synthetic. Natural antioxidants include tocopherols, ascorbic acids, polyphenols, while synthetic antioxidants consist of butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA) and proply gallate (PG) (Marmesat *et al*., 2010). They are applied to food to suppress lipid oxidation upon long storage and also to reduce concentration of free radicals upon consumption (Pokorny, 2007). However, there are reports on the undesirable health effects of several synthetic antioxidants. In addition to that, BHT and BHA may be carcinogenic to humans and also cause DNA damages (Pankey and Upadhyay, 2012).