METABOLITES PRODUCED BY HEAT-TOLERANT ENDOPHYTIC FUNGI ASSOCIATED WITH *NEPENTHES AMPULLARIA*

By

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Abstract

Nepenthes species, the tropical carnivorous plant which is commonly known as pitcher plant. This plant can be found in the islands of Borneo, whereby there were several species that were endemic to the state of Sarawak. *Nepenthes* can be divided between two groups, highland and lowland species. Currently, threats such as loss of habitats and over-collection are affecting *Nepenthes*, but climate changes are also threatening the *Nepenthes*, especially the highland species. It is currently still unknown why the highland species are more affected by the increase in global temperature as compared to the lowland species. One of the possibilities for the adaptation of the lowland species to the warmer environment could possibly be due to the presence of fungal endophytes. Besides that, the fungal endophyte also has the ability in producing metabolites which were either same or similar to the host plant, and as well as phytohormones, for instance, gibberellin and indole acetic acid which plays a role the growth and development of their host. Hence, in this study, the endophytic fungi were (i) isolated from two different age group of lowland species, *Nepenthes ampullaria* and (ii) the metabolite produces by the endophytes at different temperature were assessed. Five out of a total of seventy-three isolated endophytic fungi were considered as heat-tolerant endophytes. The identities of these heat-tolerant endophtyes were grouped into fungal class *Dothideomycetes*, *Talaromyces pinophilus* and *Coprinopsis cinerea*. The metabolites produced by these five endophytic fungi were then assessed with liquid chromatography tandem-mass spectrometry (LC-MS/MS). The results from this study showed that the fungal endophytes associated with *Nepenthes ampullaria* can produce metabolites which have the potential of helping the plant to tolerate heat-stress. This study on the endophytic fungi associated with *Nepenthes ampullaria* can serve as a baseline study on the relationship between the fungal endophytes and *Nepenthes*.

Acknowledgement

Declaration

I, Fiona Chung Yi Li hereby declare that this research study entitled "Metabolites produced by heat-tolerant endophyte associated with *Nepenthes ampullaria*" is original and does not contain any material which has been accepted for the award of any other degree or diploma, except where due reference is made in the text of the examinable outcome. To the best of my knowledge, this thesis does not contain any material previously published or written by another person except where due reference is made in the text of the examinable outcome; and where the work is based on joint research or publications, disclosed relative contributions of the respective workers or authors.

Fiona Chung Yi Li

Conference presentation

Poster presenter, Role of heat-resistant fungal endophytes in the survival of lowland *Nepenthes* species, 10th *WESTPAC International Scientific Conference*, 17-20 April 2017, Qingdao, China.

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Abbreviations

°C	Degree Celsius
BLAST	Basic Local Alignment Search Tool
Bt	Beta-tubulin
CAL	Calmodulin
cm	centimetre
csv	Comma separated values
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FDA	Food Drug Administration
g	Gram
GC-MS	Gas Chromatography-Mass Spectrometry
GPS	Global Positioning System
HCOOH	Formic acid
ITS	Internal Transcribed Spacer
IUCN	International Union for Conservative of Nature and Natural Resources
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-QTOF	Liquid Chromatography-quadrupole time-of-flight
m/z	Mass-to-charge
MEGA	Molecular Evolutionary Genetic Analysis
NCBI	National Center for Biotechnology Information
NH_4OAc	Ammonium acetate
NMR	Nuclear Magnetic Resonance spectroscopy
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PLS	Partial Least Square
PLS-DA	Partial Least Square-Discriminant Analysis
rDNA	Ribosomal Deoxyribonucleic acid
ROS	Reactive Oxygen Species
TAE buffer	Tris-acetate-EDTA buffer

TE buffer   Tris-EDTA buffer

Tris-HCl   Tris-hydrochloride

YGCA   Yeast Glucose Chloramphenicol Agar

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Chapter 1 General Introduction

The carnivorous plant, *Nepenthes*, commonly known as pitcher plant can be found in Sarawak state. In fact, there were few *Nepenthes* species that were endemic to Sarawak. *Nepenthes faizaliana*, *Nepenthes glandulifera*, *Nepenthes muluensis* and *Nepenthes murudensis* were some of the examples of endemic pitcher plant in Sarawak (Clarke & Lee 2004). Generally, the pitcher plants can be divided into two groups based on their growing altitude. The two groups were highland species which mainly live at high altitude such as mountain summit, while the other group, lowland species are mainly found living in heath forest (Adam & Hamid 2006; Gray et al. 2017).

In the International Union for Conservative of Nature and Natural Resources (IUCN) Red List, 2/3 of 103 of the listed *Nepenthes* species were categorized as either vulnerable, endangered or critically endangered (Schwallier et al. 2016).

The main anthropogenic threats to carnivorous plant are losing their natural habitat due to agriculture, and as well as over-collection of the carnivorous plants (Jennings & Rohr 2011). However, there were also few studies which show that climate change is also affecting the *Nepenthes* (Gray et al. 2017; Maycock et al. 2011; Schwallier et al. 2016). For example, in a study conducted by Maycock et al. (2011), where two highland species, *Nepenthes macrophylla* (an endemic species to Mount Trus Madi, Sabah) and *Nepenthes lowii* (an endemic species in Borneo) were compared to two dipterocarps species (*Shorea monticola* and *Hopea montana*). They found that the *Nepenthes* species are more susceptible to climate change as compared to the dipterocarps species. This is due to the increase in the mean annual temperature in the higher altitude area as compared to the lower altitude area. Besides that, in another study by Gray et al. (2017), also shows that the highland *Nepenthes* species are more affected by the changes in the climate as compared to the lowland *Nepenthes* species.

As for the endophytes which are a group of microorganisms which is found living in the tissues of the host plant, are widely known for their symbiotic relationship with their host plant (Lata et al. 2018; Rodriguez & Redman 2008; Rodriguez et al. 2009; Rodriguez et al. 2008). It is believed that nearly 300,000 existing plant species is a host for more than one endophytes (Strobel & Daisy 2003). One of the benefits of the endophytes to the plants is their capability of helping the plants in adapting abiotic stress, for examples, tolerating to high temperature and high salinity and increasing resistance to water and drought stress. Besides that, it is also suggested that the endophyte acts as a biological trigger which activates the host defence systems when they are exposed to stress (Aly, Debbab & Proksch 2011).

Besides that, the endophytic fungi also have the capability of producing metabolites with a various biological activity which may help the plants in tolerating stress. The biological activity of the metabolites produce by these endophytes are such as antimicrobial, anticancer and antioxidant (Ludwig-Müller 2015; Pimentel et al. 2011). For example, the metabolites with antimicrobial activity might be involved with protecting the plant against plant pathogenic microorganisms (Gunatilaka 2006). The production and accumulation of the reactive oxygen species (ROS) is a common process by plants when they are exposed to the adverse environment (Singh, Gill & Tuteja 2011). However, when an over-accumulation of ROS occurred, it will cause damages to the proteins, lipids and DNA, thus, making it extremely toxic to the plant cells. As endophytes have the capability in producing metabolites with antioxidant activities which can help in scavenging the accumulated ROS, thus, lowering the ROS in plants and enhancing their tolerance to oxidative stress (Aly, Debbab & Proksch 2011; Hamilton et al. 2012; Huang et al. 2007; White & Torres 2010).

For example, the symbiotic relationship between the fungal endophyte, *Exophiala* species, which is isolated from *Cucumis sativus* (cucumber)'s root. The endophyte increased the host tolerance to heat stress, as better plant growth is observed in the plants associated with *Exophiala* species than the non-inoculated plants. The tolerance to heat stress confers by the endophytes involved in the activities of catalase and peroxidase, whereby the activities of the two enzymes increases when the plant is subjected to heat stress. Besides that, the endophyte also produced more flavonoids metabolites (daidzein and genistein) in the inoculated plant (Khan et al. 2012c). As flavonoids metabolite can

be an antioxidant metabolite, both daidzein and genistein are also involved in helping the host in tolerating heat stress (Khan et al. 2012c; Subramanian, Stacey & Yu 2006).

Therefore, it is hypothesized that the presence of endophytic fungi within the lowland *Nepenthes* species, and as well as the metabolites produced by these endophytes may play a role in the adaptation and survival of the plant in a warmer environment compared to the environment where the highland *Nepenthes* species live in.

Thus, the aim of conducting this research study is to assess the potential role of the heat tolerant endophytic fungi isolated from *Nepenthes ampullaria*.

The objectives of the study mentioned below:

i) Isolation of endophytic fungi from *Nepenthes ampullaria* at different temperatures and identification of the most heat-tolerant endophytic fungi.

ii) Assessment of metabolites produced by the most heat-tolerant endophytic fungi when cultured under different temperatures using LC-MS.

In the following chapter, chapter 2 will be covering a more in-depth detail on the responses of plants when experiencing heat stress, as well as the relationship between the plant and endophyte. This chapter will also explain more on *Nepenthes* plants. Besides that, details on metabolomics (a technique used in either identifying or quantifying metabolites produces by microorganisms), and also the liquid chromatography-mass spectrometry (a common platform use in metabolomics) will also be covered in this chapter too.

The methods that were carried out in the study will be covered in chapter 3. Chapter 4 will be covering on the findings from the study, such as the identity of the isolated endophytes from *Nepenthes ampullaria*, as well as the identity of the metabolites produced by these endophytes when cultured at a different temperature.

The discussion of the results will be covered in chapter 5. In this chapter, the identity of the isolated heat-tolerant endophytic will be discussed. Besides that, the biological activity of the metabolites identified at different temperature will also be discussed in this chapter as well.

Lastly, the conclusion, as well as the future work will be covered in chapter 6.

Chapter 2 Literature Review

2.1   Heat stress

As plant are immobile, they could neither escape nor avoid when they are subjected to any environmental stresses (Hasanuzzaman et al. 2013). Abiotic stresses, for examples, heat, drought, heavy metal and salinity can cause changes in either morphological, physiological, biochemical and/or molecular of the plant. Thus, the changes in any of these will affect both the development and as well as the growth of the plant (Bita & Gerats 2013; Lata et al. 2018).

Due to the constant increase of the temperature, heat stress becomes one of the major abiotic stresses to be experienced by plants (Bita & Gerats 2013; Hasanuzzaman et al. 2013; Lata et al. 2018). This is because it is predicted that there will be an increase in temperature by 1.8 – 4.0°C higher than the current temperature by the year 2100. This is due to the increase of 0.2°C per decade in the global air temperature (Solomon et al. 2007). Besides that, when there's an increase of 5°C to 7°C in the plants' optimal growth temperature, the plants will experience heat stress (Bita & Gerats 2013; Pareek, Sopory & Bohnert 2009).

2.1.1   Plant response to heat stress

Every response by plants to heat stress is different according to their species, their optimal growth temperature, and the duration of been exposed to a high-temperature environment (Hasanuzzaman et al. 2013).

The major impacts caused by this stress are such as a decrease in the rate of germination, production of abnormal seedlings, and poor vigour of seedling (Hasanuzzaman et al. 2013; Kumar et al. 2011; Piramila, Prabha & Nandagopalan 2017; Toh et al. 2008). Besides that, leaf abscission and senescence, scorching of the leaves and stems, growth of roots and shoots are inhibited, discolouration as well as damages on the fruit, are some of the examples of the plants' physiological injuries that can be observed when the plant is under heat stress (Bita & Gerats 2013; Hasanuzzaman et al. 2013; Pareek, Sopory & Bohnert 2009).

Besides that, reactive oxygen species (ROS) are also produced by the plant in response to heat stress (Hasanuzzaman et al. 2013; Rodriguez et al. 2008; Singh, Gill & Tuteja 2011). Even though ROS plays a role as the signalling molecules for the growth, development and defence of the plant, an over-accumulation of ROS, for examples, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), and hydrogen peroxide (H_2O_2) will be extremely toxic to the plant cell. This is because excess ROS will cause oxidative damages to the proteins, lipids, and DNA (Ara et al. 2013; Hasanuzzaman et al. 2013). Hence, in order to survive in a high-temperature environment, the plant will either decrease the production of normal proteins while increasing the synthesis of heat shock proteins (HSPs). Besides that, they will also produce either phytohormone and/or antioxidant metabolites for survival (Bita & Gerats 2013; Rani et al. 2013). Other examples of affected plant stages by heat stress were as listed in Table 2.1.

Table 2.1: The affected plant species and stages, and the effects of heat stress.

Plant	Affected stage	Heat stress effects	References
Capsicum annuum	Reproductive, maturity and harvesting stage	Reduction in fruit width and weight	(Yun-Ying et al. 2009)
Oryza sativa	Reproductive stage	Decreased in grain length, width and weight	(Suwa et al. 2010)
Triticum aestivum	Grain filling and maturity stage	Decreased in kernel weight and yield	(Rahman et al. 2009)
Nicotiana tabacum	Early growth stage	Reduction of antioxidant enzymes activity.	(Gunawardhana & De Silva 2012)
Triticum aestivum	From 60 days after sowing to maturity stage	Reduction in leaf size and smaller grain size produced.	(Djanaguiraman, Prasad & Seppanen 2010)

2.2 The relationship between Plant and Endophyte

The relationship between endophyte and plant may begin hundreds million years ago; where a fossilized tissue of stem and leaves are discovered which act as a proof of evidence on the association of plant and microbes (Firáková, Šturdíková & Múčková 2007; Krings et al. 2007). Three stages as listed below are involved in a successful plant-fungi symbiosis.

(1)   The invasion of the fungi into the tissues of the plants.

(2)   The colonized of plant tissues by the invaded fungi.

(3)   The symbiotic lifestyle expressed by the plant-fungi (Singh, Gill & Tuteja 2011).

The symbiotic relationship between the endophytes and the host plants is vary depending on the host genotypes and the environmental factors (Rodriguez, Redman & Henson 2005). This symbiotic relationship between the plant-endophyte can either be positive (mutualism), neutral (neutralism), or negative (competition) (Rodriguez et al. 2008).

The common microbes that exist as endophytes can either be bacteria or fungi, with fungi being the majority when isolating endophytes from plants (Hardoim et al. 2015).

2.2.1 Endophytic fungi

In the opinion of Rodriguez et al. (2009), all plants that exist in the ecosystem have a symbiotic relationship with fungal endophytes. The fungi that are found living in the plant tissues are known as endophytic fungi. This group of fungi plays significant roles in the plant communities, where they help to defence the plants against pathogens and herbivore, produce phytohormones which supports the growth of the plant and increased in the plant resistance to various abiotic stress, for instance, salinity, temperature and heavy metal toxicity (Lee, Tan & Ting 2014). The fungal endophytes will then gain nutrients from the host plant in return (Tadych & White 2009).

The stress tolerance conferred by the endophytic fungi involve two different mechanisms; which are (a) activation of host plant stress response system when they are exposed to stress (Redman, Ranson & Rodriguez 1999); and (b) production of the anti-stress biochemical by endophytic fungi itself or through the stimulation by the fungal endophytes (Bacon & Hill 1996).

Endophytes can be characterized into two major groups, which are clavicipitaceous endophytes (C-endophytes) and non-clavicipitaceous endophytes (NC-endophytes). The endophytes that infect the grasses belong to the group of C-endophytes. They normally increase the plant biomass, confer drought tolerance, and produce chemicals which are toxic to animals. NC-endophytes are those that are found present in asymptomatic tissues of non-vascular plants, ferns and angiosperms (Rodriguez et al. 2009).

For the second group of endophytes, NC-endophytes, it is further separated into three subclasses, class 2, class 3, and class 4. According to Rodriguez et al. (2008), endophytes belonging to class 2 of NC-endophytes, are made up of both *Ascomycota* and *Basidiomycota*. The endophytes belonging to this class are unique compared to the others because they provide habitat-specific stress tolerance, for examples, drought, disease, heat and salinity, to the host plant. Therefore, it plays a significant role in helping their host to survive in a harsh environment. *Dichanthelium lanuginosum* that are found growing at Yellowstone National Park and Lassen Volcanic National Park in the USA for example. This plant is colonized by *Curvularia protuberata*. These fungal endophytes are able to tolerate high temperature and thus, it helps the host plants to survive in a hot environment (Gond et al. 2010; Márquez et al. 2007; Redman et al. 2002).

Although, the capability of *C.protuberata* in helping its host in tolerating high temperature was found to be linked with a mycovirus, *Curvularia* thermal tolerance virus (CThTV) (Redman et al. 2002). Besides that, *Fusarium culmorum* which colonized *Leymus mollis* that helps the host plants to tolerate seawater salinity (Rodriguez et al. 2008). However, when growing the host plants and the endophytes separately, neither one is capable of tolerating temperature and salinity stress (Márquez et al. 2007; Rodriguez et al. 2009).
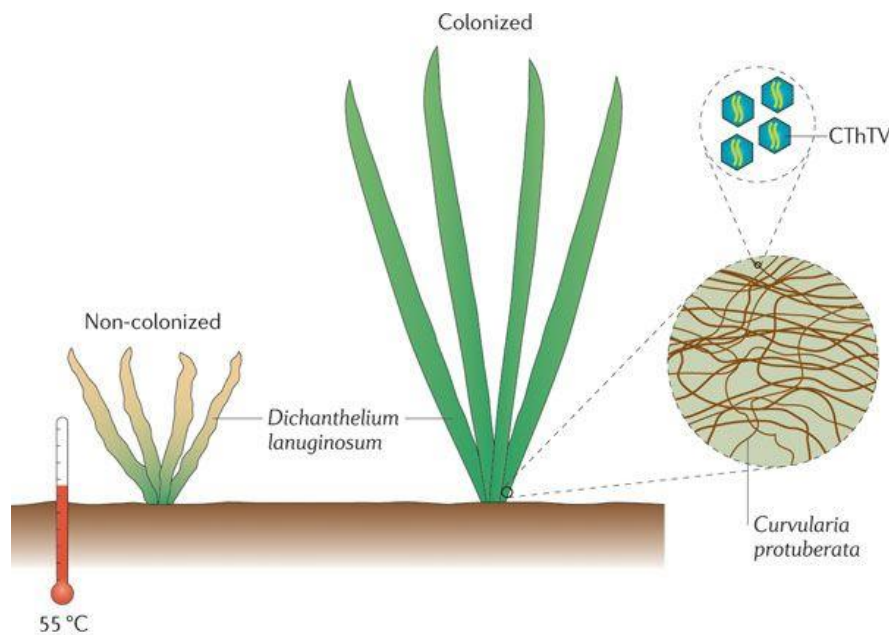
Figure 2.1: The symbiotic relationship between *Dichanthelium lanuginosum* and its endophytic fungi, *Curvularia protuberata*. The thermotolerance conferred by the endophytic fungi were associated with the presence of *Curvularia* thermal tolerance virus (CThTV) (Roossinck 2011).

In addition to the capability in conferring heat and salinity tolerance to their host plants, the endophyte fungi can also help the plants to tolerate drought, where the mechanism of in conferring drought tolerance involve the adjustment of the osmolyte concentration or stomatal activity of the plant (Malinowski & Belesky 2000). For examples, both *Colletotrichum protuberata* and *Fusarium culmorum* were able to help both their own hosts and unrelated plants, for examples, tomato and rice in tolerating similar levels of drought tolerance (Rodriguez et al. 2008). Another example, the pathogenic *Colletotrichum* also confer drought tolerance to cucurbits, pepper and tomato when they undergo non-pathogenic lifestyles (Redman, Dunigan & Rodriguez 2001; Rodriguez, Redman & Henson 2004).

In some recent studies, endophytic fungi were also reported to be able to tolerate heavy metals (Ban et al. 2012; El-Gendy et al. 2011; Khan & Lee 2013). For examples, the isolated fungal endophytes from the roots of soybean plants, *Metarhizium anisopliae* and *Penicillium funiculosum* has the capability of tolerating copper (Khan & Lee 2013). Besides that, tolerance against copper and cadmium were also demonstrated by the fungal endophytes *Rhizopus oryzea*, *Aspergillus luchuensis*, *Monacrosporium elegans*, *Penicillium lilacinum*, *Curvularia lunata*, *Drechslera hawaiiensis*, *Verticillium fungicola*, and *Pestalotiopisis clavispora* (El-Gendy et al. 2011).

The previously reported tolerance conferred by endophytic fungi to respective abiotic stress were summarised in Table 2.2.

Table 2.2: The stress tolerance conferred by the endophytic fungi for their respective host plant.

Abiotic stress	Endophytic fungi	Host plant	Reference
Heat	*Fusarium* species	*Lycopersicon esculentum*	(Rodriguez & Redman 2008)
	Curvularia protuberata	*Lycopersicon esculentum*	(Rodriguez et al. 2008)
	Curvularia protuberata	*Dichanthelium lanuginosum*	(Redman et al. 2002)
	Curvularia species	*Lycopersicon esculentum*	(Rodriguez & Redman 2008)
	Alternaria species	*Lycopersicon esculentum*	(Rodriguez & Redman 2008)
Drought	*Phialophora* species	*Festuca pratensis*	(Malinowski et al. 1997)
	Colletotrichum gloeosporioides	*Capsicum annuum*	(Redman, Dunigan & Rodriguez 2001)
	Neotyphodium sp.	*Festuca arizonica*	(Morse, Day & Faeth 2002)
	Piriformospora indica	*Arabidopsis* species	(Sherameti et al. 2008)
	Fusarium species	*Lycopersicon esculentum*	(Rodriguez & Redman 2008)
	Trichoderma harzianum TH-56	*Oryza sativa*	(Pandey et al. 2016)

Salinity	*Piriformospora indica*	*Oryza sativa*	(Jogawat et al. 2016)
	Fusarium culmorum	*Leymus mollis*	(Rodriguez et al. 2008)
Heavy metal	*Neotyphodium coenophialum* and *Neotyphodium uncinatum*	*Festuca arundinacea and Festuca pratensis*	(Soleimani et al. 2010)
	Neotyphodium lolii	*Lolium perenne*	(Monnet et al. 2001)
	Neotyphodium uncinatum	*Lolium perenne*	(Ren et al. 2006)

2.2.2 Bioactive compounds from endophyte

Within the last decade, great attention is paid to endophytes due to their potential of synthesizing bioactive secondary metabolite (Ludwig-Müller 2015). In the symbiotic relationship between plant and fungal endophytes, where the nutrients and shelter are provided by the plants for the endophytes, while the endophyte produces secondary metabolite which helps the plants to tolerate abiotic stresses (Gunatilaka 2006). Besides that, there were also some fungal endophytes which have the ability in producing either same or similar metabolite as the ones produced by the host plant (Zhao et al. 2011).

The metabolites produced by these fungal endophytes also possess different biological activities, for examples, anticancer, antimicrobial, antioxidant and others (Pimentel et al. 2011). For instance, alkaloids, lignans, steroids, xanthones, phenols, terpenoids, isocoumarins, and flavonoids are some of the examples of the metabolites that are produced by the endophytes (Kaul et al. 2012; Ludwig-Müller 2015; Singh et al. 2017).

2.2.2.1 Antioxidant compound

It is found that all endophytic fungi of several plant species can produce antioxidant metabolites; which are the substances that capable and effective in protecting the cells from damages caused by the ROS (Huang et al. 2007). For examples, the antioxidant metabolites, pestacin and isopestacin, produced by fungal endophyte, *Pestalotiopsis microspora* isolated from *Terminalia merobensis* (Firáková, Šturdíková & Múčková 2007; Kaul et al. 2012). The pestacin have at least ten times higher in its antioxidant activity compared to Trolox, through the cleavage of unusually reactive C-H bond and through O-H abstraction at a lesser degree (Harper et al. 2003). Due to having a similar structure to flavonoids, isopestacin are capable of scavenging superoxide and hydroxyl free radicals (Strobel et al. 2002). The chemical structure of pestacin and isopestacin are shown in Figure 2.2 below.
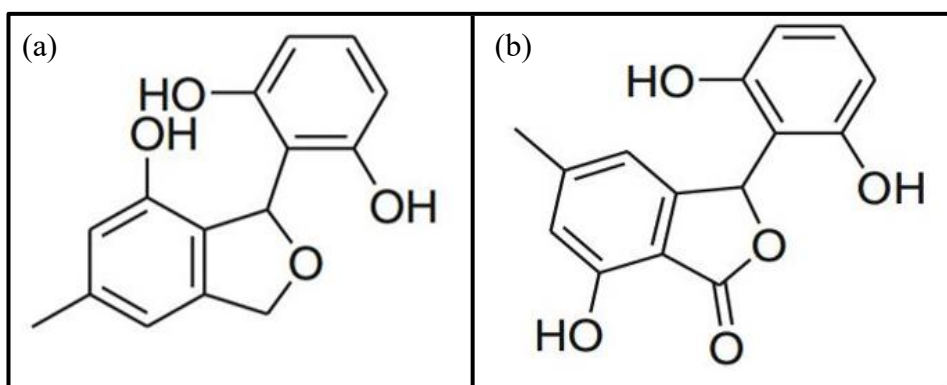
Figure 2.2: The chemical structures of (a) pestacin and (b) isopestacin, antioxidant metabolites from *Pestalotiopsis microspore*; (Kaul et al. 2012).

Besides of scavenging the ROS, the various antioxidant metabolites produced are also involved in the enhancement of the host tolerance to stresses (Herrera-Carillo et al. 2009; Malinowski, Belesky & Lewis 2005; Schulz et al. 2002; Yuan, Zhang & Lin 2010). This is observed in multiple studies, where the production of antioxidant metabolites in symbiotic plants increases when the plants are exposed to stresses (Harper et al. 2003; Huang et al. 2007; Richardson et al. 1992). For instance, the concentration for both mannitol and other potential metabolites with antioxidant properties in *Festuca arundinacea* Schreb., which is colonized by the endophytic fungi, *Acremonium coenophialum* is higher compared to the non-colonized plants when both of the colonized and non-colonized plants experience drought stress (Richardson et al. 1992).

2.2.2.2 Anticancer compound

Besides antioxidant metabolites, endophytic fungi are also shown to produces metabolite with anticancer properties. For example, taxol or also can be known as paclitaxel which is probably one of the popular anticancer metabolites to be discovered from endophytic fungi, *Taxomyces andreanae*. This diterpenoid metabolite was first isolated and commonly found from the plant, *Taxus brevifolia* (Stierle, Strobel & Stierle 1993). As approved by the Food and Drug Administration (FDA), taxol has been used for treating lung, breast, prostate and ovarian cancer (Pimentel et al. 2011; Zhao et al. 2011). Besides *Taxomyces andreanae*, taxol also has been found in other species of endophytic fungi such as *Wollemia nobilis* (Strobel et al. 1997), *Pestalotiopsis terminaliae* (Gangadevi & Muthumary 2009), *Phyllosticta spinarum* (Senthil Kumaran, Muthumary & Hur 2008), and *Taxodium distichum* (Li et al. 1996). Please refer to Figure 2.3 below for the chemical structure of taxol.
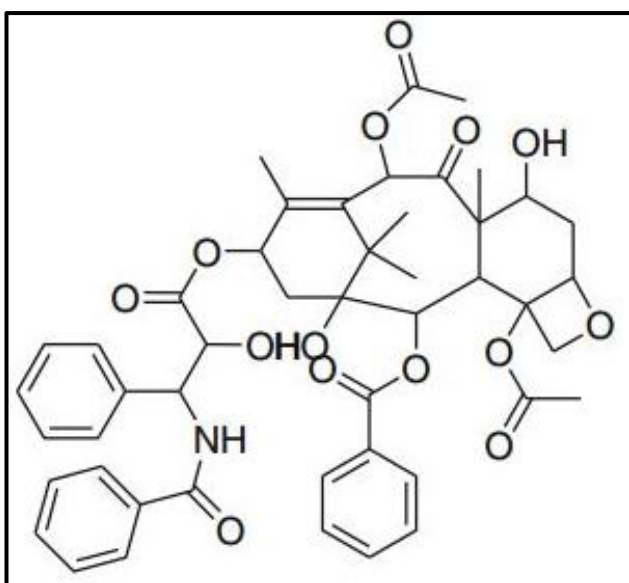
Figure 2.3: The chemical structures of taxol, an anticancer metabolite isolated from *Taxomyces andreanae* **(Kaul et al. 2012).**

2.2.2.3 Antimicrobial compound

Other than antioxidant and anticancer metabolites, antimicrobial metabolites are also produced by the fungal endophytes. The antimicrobial metabolites are the metabolites that have a low molecular weight which produced by endophytes against other competing microbes. The antimicrobial metabolites are shown to be active against the competing microbes at lower concentrations (Guo et al. 2008). These antimicrobial metabolites produced by endophytes are classified into different classes, for examples, peptides, alkaloids steroids, phenols, terpenoids, flavonoids, and quinines (Guo et al. 2008; Pimentel et al. 2011). For examples, pyrrocidines A and pyrrocidines B are two metabolites that produce by the fungal endophyte, *Acremonium zeae* which can be found in maize plants. These two metabolites not only possess antifungal properties which work against *Aspergillus flavus* and *Fusarium verticollioides*, but it also was shown to have antibacterial activities that can be used against Gram-positive bacteria (Donald et al. 2005). Another example of the antimicrobial metabolites from endophytic fungi is cryptocandin (isolated from *Cryptosporiopsis* cf. *quercina*) which have antifungal activity. This metabolite can be used against *Candida albicans*, *Trichophyton rubrum, Sclerotinia sclerotiorum and Botrytis cinerea* (Strobel et al. 1999). Please refer to Figure 2.4 for the structures of pyrrocidines A and B, and also the structure of cryptocandin.
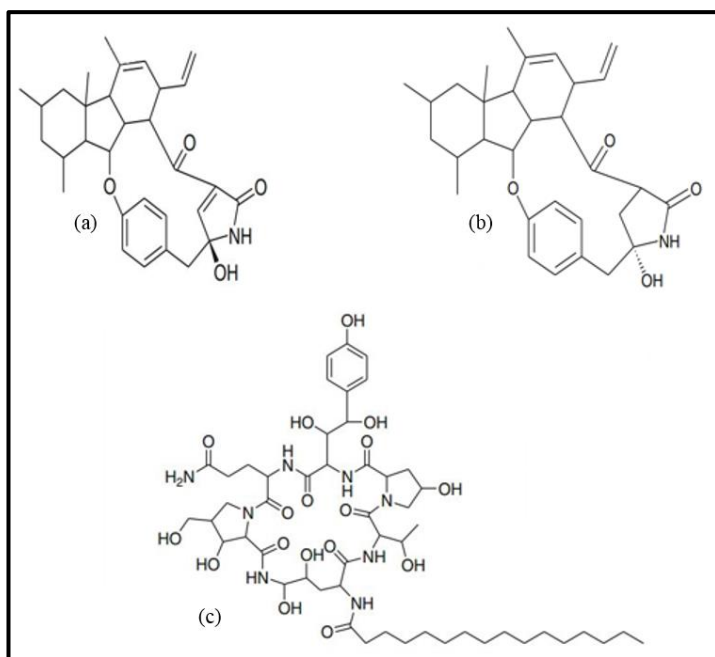
Figure 2.4: The structures of antimicrobial metabolites: (a) pyrrocidines A, (b) pyrrocidines B, and (c) cryptocandin (Kaul et al. 2012).

Please refer to Table 2.3 below for other examples of metabolites produced by fungal endophytes and the biological activities for respective metabolites.

Table 2.3: The biological activities of metabolites produced by various endophytic fungi.

Endophyte	Host plant	Metabolites	Biological activity	References
Penicillium pinophilum	*Allium schoenoprasum*	Skyrin Dicatenarin	Anticancer	(Koul et al. 2016)
Curvularia lunata	*Niphates olemda*	Lunatin	Antimicrobial	(Jadulco et al. 2002)
Cephalosporium sp. IFB-E001	*Trachelospermum jasminoides*	Graphislactone A	Antioxidant	(Song et al. 2005)
Pestalotiopsis microspora	*Terminalia morobensis*	Isopestacin Pestacin	Antimicrobial and antioxidant	(Harper et al. 2003; Strobel et al. 2002)
Pestalotiopsis microspore and *Monochaetia* sp	*Torreya taxifolia*	Ambuic acid	Antimicrobial	(Li et al. 2001)

Phomopsis sp.	*Erythrina cristagalli*	Phomol	Weak cytotoxic activity, antimicrobial, and anti-inflammatory	(Weber et al. 2004a)
Phoma medicaginis	*Medicago sativa* and *Medicago lupulina*	Brefeldine A	Initiate apoptosis in a cancer cell, and antimicrobial	(Weber et al. 2004b)
Microsphaeropsis olivacea	*Pilgerodendron uviferum*	Graphislactone A	Antioxidant	(Hormazabal et al. 2005)
Fusarium sp.	*Cajanus cajan*	Cajaninstilbene acid	Antioxidant	(Zhao et al. 2012)
Cephalotheca faveolata	*Eugenia jambolana*	Sclerotiorin	Anticancer	(Giridharan et al. 2012)

2.3 Nepenthes species

Nepenthaceae, the carnivorous plant family which contained only a single genus *Nepenthes*. It is commonly known as pitcher plant or periuk kera (monkey cup) by the local. This plant species have a diverse geographical distribution as they can be found in Madagascar, Australia, New Caledonia and India. However, they are more commonly found on the islands of Borneo, Philippines and Sumatra (Figure 2.5) (Adam & Hamid 2006; Maycock et al. 2011). There are few endemic *Nepenthes* species can be found in the state of Sarawak, such as *Nepenthes faizaliana*, *Nepenthes murudensis*, *Nepenthes muluensis*, and *Nepenthes glandulifera* (Clarke & Lee 2004).
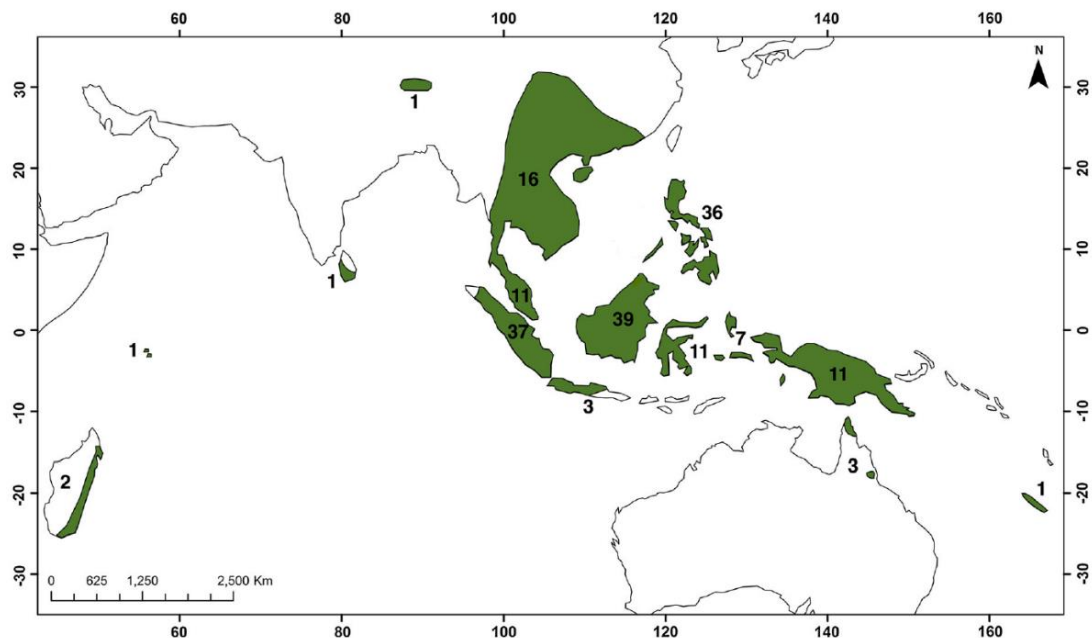
Figure 2.5: The distributions of the *Nepenthes* species are shaded in green, while the number in the map represent the approximate number of known *Nepenthes* in each country, taken from Van der Ent, Sumail and Clarke (2015).

In Sarawak, it is not common to find this plant in the lowland tropical rainforest, as this habitat have a low light level and high nutrient on the forest floor, which makes it unsuitable for the *Nepenthes* to grow (Clarke & Lee 2004). However, they can be commonly found growing in habitats such as heath forest (unusable land for growing rice after clearing the forest), mountain summits, peat swamps forest, and limestone cliffs (Adam & Hamid 2006; Clarke & Lee 2004). The common things between these habitats are that they all have high light levels and low nutrient in the soil, which are suitable for the *Nepenthes* to grow (Clarke & Lee 2004).

Nepenthes are divided into two main groups which based on the altitude where they are found growing. The first group, the lowland species are usually found from 0 m to approximate 1,100 m above sea levels, growing at a temperature of around 27°C (Adam & Hamid 2006; Chua 2013; Gray et al. 2017). The lowland species that can be found in Sarawak are *Nepenthes ampullaria*, *Nepenthes albomarginata*, *Nepenthes mirabilis*, and *Nepenthes rafflesiana* (Clarke & Lee 2004). Those that are found in a cooler and montane habitat falls under the highland species group. The highland species are usually found above 1,100 m above sea level with temperatures of around 25°C during the day, and temperature of as low as 12°C during the night (Adam & Hamid 2006; Chua 2013; Gray et al. 2017). Examples of highland species found in Sarawak are *Nepenthes fusca*, *Nepenthes muluensis*, and *Nepenthes platychila* (Clarke & Lee 2004). Figure 2.6 showed the *Nepenthes* species which were found in Sarawak.

Figure 2.6: Examples of highland and lowland *Nepenthes* species that can be found in Sarawak; (from left to right) *Nepenthes faizaliana*, *Nepenthes glandulifera*, *Nepenthes ampullaria*, and *Nepenthes gracilis*, image taken from Clarke and Lee (2004).

Nepenthes species produced leaves which are formed into a jug or cup or pitcher shape, which is their most fascinating and unique aspect. Not only does the pitcher are in bright colour, but they also emit scents and provide nectar which attracts preys, especially insects (Adam & Hamid 2006; Clarke & Lee 2004). Please refer to Figure 2.7 for the functional zone of the pitchers. The pitchers can be categorized into three functional zones (refer to Figure 2.7) with the details for each zone as mentioned in the following:

(1)   The peristome (attraction zone), in which the lid and the peristome are involved to attract and trap insect preys.

(2)   The slippery and waxy inner of the pitcher (conductive zone) which cause the insect prey to lost their footing, and thus, trapping them, due to microscopic wax plates that covered the pitcher.

(3)   The inner bottom part of the pitcher (absorption zone) which contains digestive fluid for retention and digestion of the insect preys (Mithöfer 2011; Pavlovič, Slováková & Šantrůček 2011).
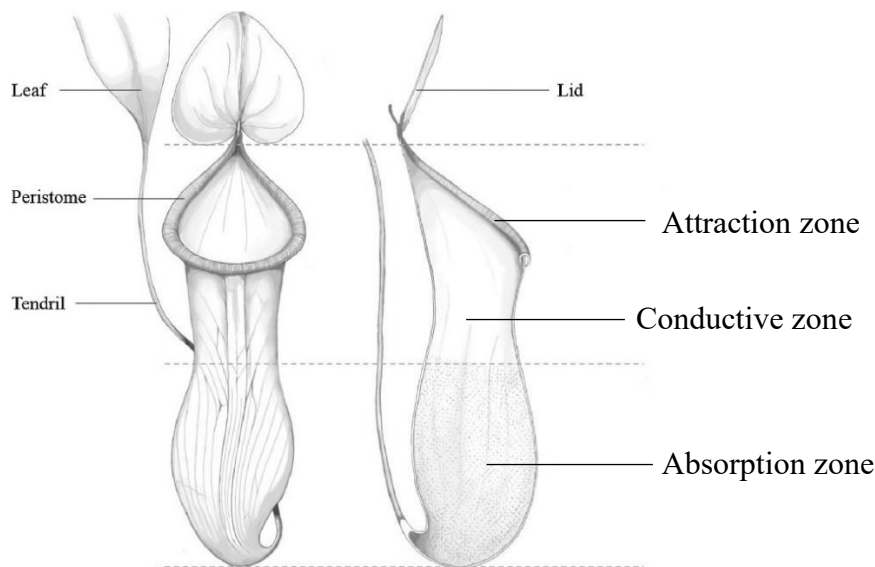
Figure 2.7: The front and side view of the *Nepenthes* pitcher, and the three functional zones of the pitcher (Sissi, Alain & Frédéric 2018).

2.3.1   Threats to *Nepenthes*

In the International Union for Conservative of Nature and Natural Resources (IUCN) red list, about 70% listed *Nepenthes* in the IUCN red list were categorized either as vulnerable, endangered or critically endangered (Schwallier et al. 2016). For example, an endemic *Nepenthes* species in Sarawak, *Nepenthes murudensis*, is currently categorized as an endangered species in the IUCN red list (IUCN 2017).

Loss of the natural habitats and over-collection of the carnivorous plants were the two main threats facing by *Nepenthes* species (Jennings & Rohr 2011). Besides that, in recent studies, climate change was also reported as one of the threats that are currently facing by all *Nepenthes*, especially the highland *Nepenthes* species. For example, in the study conducted by Maycock et al. (2011) at the Trus Madi Forest Reserve, where they compared the effects of climate change between two highland *Nepenthes* species (*Nepenthes macrophylla* and *Nepenthes lowii*) and two dipterocarps species (*Shorea monticola* and *Hopea montana*). They found that the *Nepenthes* species were more susceptible to the changes in the climate compared to the two dipterocarps species, which is due to the increase in the mean annual temperature at a higher altitude area compared with the lower altitude area, where the dipterocarps are found (Maycock et al. 2011).

Besides that, studies involving the comparison of highland *Nepenthes* species and lowland *Nepenthes* species also showed that the highland species will lose most of their suitable habitats based on the tested models (Gray et al. 2017; Schwallier et al. 2016). The reason why such occurrence occurs is because of the inabilities of the highland *Nepenthes* species adjusting themselves to the changes in the environment due to the less exposure in varies temperature, which cause them to has a low thermal-tolerance (Addo-Bediako, Chown & Gaston 2000; Araújo et al. 2013; Ghalambor et al. 2006).

Hence, the endophytic fungi from the lowland *Nepenthes* species were selected for this study. This is due to the ability of endophytes in helping their host in adapting and tolerating abiotic stresses (Lee, Tan & Ting 2014).

2.4   Identification of fungi

With the estimation of 1.5 to 5.1 million species, the second largest group of eukaryotes exists on the planet Earth is represented by fungi (Blackwell 2011; Hawksworth 1991; O'Brien et al. 2005). Hence, they are ecologically, metabolically, morphologically and phylogenetically diverse. They are also known to produce various bioactive molecules, therefore, making them very useful for discovering new bioactive compounds (Raja et al. 2017). Thus, the scientific names of the fungi are important for the identification of other closely related species, for better prediction of the evolution of chemical-gene clusters, and as well as prioritizing taxonomically related strains (Schmitt & Barker 2009).

2.4.1   Morphological identification of fungi

Identifying fungi based on their morphology such as the spore-producing structure due to either asexual or sexual reproduction is a traditionally used method which is still been used in current time (Hyde, Abd-Elsalam & Cai 2011). This method is important as it uses to understand the morphological characteristic evolution of a fungi species (Raja et al. 2017).

However, fungi can only be identified up till family-level only by using morphological identification (Wang et al. 2016). This is because some of the morphology of the fungi can be misleading either due to hybridization (Hughes et al. 2013; Olson & Stenlid 2002), cryptic speciation (Foltz, Perez & Volk 2013; Giraud et al. 2008; Kohn 2005; Lücking et al. 2014) and as well as convergent evolution (Brun & Silar 2010). Besides, it can be quite a challenge to identify fungi based on their morphology only, as the morphological characters that can be used to identify fungi are quite limited (Raja et al. 2017). For example, endophytic fungi which is not unusual for them to not sporulate in culture. When this happened, the fungi could not be identified based on their morphology as there's no phenotypic characteristic to be used for morphological identification (Ko et al. 2011).

2.4.2 Molecular identification of fungi

The used of a molecular technique for fungi identification began approximately over two decades ago by White et al. (1990), which described the fungal nuclear ribosomal operon primers. The generated fungal DNA sequences by using the primers for the large subunit (nrLSU-26S or 28S), small subunit (nrSSU-18S), and also the entire internal transcribed spacer region (ITS1, 5.8S, ITS2) are what started the molecular identification of fungi (Seifert, Wingfield & Wingfield 1995; T D Bruns, T J White & Taylor 1991).

All these three separate regions have different rates of evolution, and hence, have a variety of genetic levels. Among these three separate regions, SSU is the slowest to evolve, hence it has the lowest variation, whereas the region that exhibits the highest variation is the ITS region which evolve the fastest (Mitchell & Zuccaro 2006; T D Bruns, T J White & Taylor 1991).

Not only does the ITS region is the fastest to evolve, but it also has the highest probability of successful identification for a broad range of fungi. Besides that, this region can be amplified easily, widespread use, and as well as having an appropriate large barcode gap. Hence, the ITS region is chosen as the official DNA barcode for fungi identification (Schoch et al. 2012).

However, the ITS region may not be effective in amplifying fungi with highly speciose genera, for examples *Penicillium* (Seifert et al. 2007), *Aspergillus* (Samson et al. 2014), and *Fusarium* (Al-Hatmi et al. 2016), despite being the official region for fungi identification. This is due to these fungi either have a narrow or there is no gap in their ITS regions (Schoch et al. 2012). Hence, protein-coding genes are used together with ITS for better precision in identifying fungi at the species-level (Raja et al. 2017). This is due to the intron presence in the gene which sometimes evolves at a faster rate than the ITS region. These genes are also believed to occur as a single copy in fungi, less variable in length, and fewer mutations in their exon, and thus, allowing an easier recognition of homology and convergence (Berbee & Taylor 2001; Einax & Voigt 2003). The translation elongation factor 1-alpha (Rehner & Buckley 2005), the largest and second largest subunits of RNA polymerase (Liu & Hall 2004; Matheny et al. 2002; Reeb, Lutzoni & Roux 2004; Stiller & Hall 1997), and beta-tubulin (Glass & Donaldson 1995) are some examples of the commonly use protein-coding genes for fungi identification.

2.5   Metabolomics

Metabolomics is carried out to measure (quantify and/or identify) the metabolites (small molecules) present within a biological sample (Bartel, Krumsiek & Theis 2013; Wang et al. 2017). Examples of metabolites that can be found present in a biological sample are such as organic acids, vitamins, alkaloids, amino acids, peptides, and polyphenols (Zhang et al. 2012).

Besides, metabolomics methods were used by several studies in identifying the metabolites produced by different species of fungal endophytes. For example, hypericin and emodin were identified from the endophytic fungi from *Hypericum perforatum* (Kusari et al. 2008). Furthermore, camptothecin, 10-hydroxycamptothecin and 9-methoxycamptothecin were also found to be produced by *Fusarium solani* (endophytic fungi from *Apodytes dimidiata*) (Shweta et al. 2010). Besides that, *Fusarium* oxysporum, *Fusarium solani* and *Fusarium proliferatum*, endophytic fungi of *Cajanus cajan* L. Millsp., are found to be able in producing an antioxidant metabolite, cajanistillbene acid (Zhao et al. 2012). In addition, in a study conducted by Dastogeer et al. (2017), the metabolite produced by *Nicotiana benthamiana* endophytes are shown to cause an increase of different metabolites such as cytosine, glycerol, oleic acid, and succinate when it is exposed to water-stress.

The three most commonly used analytical platforms in metabolomics studies are as follows:

(1)   Nuclear magnetic resonance spectroscopy (NMR)
(2)   Gas chromatography-mass spectrometry (GC-MS)
(3)   Liquid chromatography-mass spectrometry (LC-MS) (Wishart 2016; Zhang et al. 2012).

Each of the respective platforms each has their own advantages and disadvantages. For instance, in GC-MS platforms, the samples need to be derivatives first, whereas, it is not required in the LC-MS platforms (Wishart 2016). Please refer to Table 2.4 below for other advantages and disadvantages for the three commonly used analytical platforms.

Table 2.4: The advantages and disadvantages of the three commonly used platforms in metabolomics (Gromski et al. 2015; Wishart 2016).

Platforms	Advantages	Disadvantages
Nuclear magnetic resonance spectroscopy (NMR)	• Non-destructive • Provide specific metabolite structural information • Robust • Do not require derivation and separation	• Costly • Low sensitivity compared to mass-spectrometry • Time-consuming in interpreting spectral
Gas chromatography-mass spectrometry (GC-MS)	• Sensitive • Organic and inorganic molecules can be detected • Reproducible	• Requires derivation • Generally cannot analyse heat sensitive metabolites
Liquid chromatography-mass spectrometry (LC-MS)	• Sensitive • Derivation of the metabolite is not required • Used for complex mixture, polar and non-polar metabolites • Less expensive than NMR	• Requires separation • Matrix effects

The analysis of the metabolites can be grouped in two methods; targeted metabolomics and untargeted metabolomics (Bartel, Krumsiek & Theis 2013). Targeted metabolomics is a method which focuses on quantifying and identifying of selected and/or known metabolites. The advantages in using targeted metabolomic methods are no carried over of analytical artefacts to downstream analysis, and the study only involved a selected group of metabolites. Untargeted metabolomics is the method that quantified and identified all metabolites which are present in the biological system or sample. Besides that, the advantage of using this method is it could analyse a larger number of metabolites compared to the targeted metabolomics method (Bartel, Krumsiek & Theis 2013; Gorrochategui et al. 2016).

As metabolomics usually resulted in a large quantity of data, it can be divided into two statistical analyses (univariate and multivariate analysis) to gain more relevant and significant information from the data. Univariate analysis is carried out when only one variable is measured, hence, the interpretation of the data can be reduced to only metabolites that show the strongest responses. When comparing with other samples sets, univariate analysis will include t-test and analysis of variance (ANOVA). Whereas, for multivariate analysis, the sample data with two or more variables are measured in to observe the metabolite changes between different samples groups. The most common test that is carried in multivariate analysis is principal component analysis (PCA) and partial least squares regression (PLS) (Bartel, Krumsiek & Theis 2013; Saccenti et al. 2014).

2.5.1 Liquid Chromatography-Mass Spectrometry

Liquid chromatography-mass spectrometry (LC-MS) is the one of the most commonly use hyphenated platforms or technique. This is because LC-MS has higher sensitivity, high-throughput and also the possibility of confirming the identity of the metabolites produced by the endophytic fungi (Songsermsakul & Razzazi-Fazeli 2008; Villas-Bôas et al. 2005).

In this platform, the components present in a mixture are separated between the stationary and mobile phase via the liquid chromatography. This separation depends on the molecules relative affinity to these phases. Examples of liquid chromatography that was commonly used can be either high-performance liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC) (Malysheva 2013).

The principle behind the mass spectrometry which was coupled to LC is based on the ionization and separation of the molecules according to the mass-to-charge (m/z) ratio. The molecules respective m/z and abundance will be detected by the platform.

Generally, the mass spectrometry that was coupled to the LC was made up of three major parts, which were:

(1)   Conversion of the molecules presents in a sample into ions by the ion source.
(2)   Resolving the converted ions either by an electromagnetic field or time-of-flight tube by the mass analyser.
(3)   Detection of the resolved ions by the detector (Zhou et al. 2012).

Electrospray ionization (ESI), atmospheric pressure photoionization (APPI), and atmospheric pressure chemical ionization (APCI) are some examples of ion sources instruments. For example, ESI is the most common used ionization instruments. This is due to its capability in ionizing the molecules in the liquid phase. Besides, it can also be used for ionizing both small and large molecules. Furthermore, it does not cause significant fragmentation on the molecular ions due to its soft ionization capability (Gowda & Djukovic 2014; Zhou et al. 2012).

The most common mass analyser that used, are for examples, ion trap, quadrupole, and time-of-flight (TOF). The main goal in the mass analyser is to achieve high resolution and high sensitivity. It is also common to use a combination of two or more analysers (which also can be known as tandem mass spectrometry, MS/MS). Quadrupole-TOF (Q-TOF), triple quadrupole (TQ), triple-quadrupole ion trap (QTrap) and linear-quadrupole ion trap-Orbitrap (LTQ-Orbitrap) are some examples of mass analyser that can be used in MS/MS. The commonly use mass analyser for targeted metabolomics are TQ and QTrap, whereas, for untargeted metabolomics, Q-TOF and LTQ-Orbitrap are the commonly use mass analyser because of their higher resolving mass capability (Gowda & Djukovic 2014).

Chapter 3 Methodology

3.1 Sampling Site

The collection of the plant sample, *Nepenthes ampullaria* was carried out at Bau area, Kuching, Sarawak, Malaysia with the Global Positioning System (GPS) coordinates of 1°29'23.4"N,110°12'41.9"E as shown in Figure 3.1. Two different age group (old and young) *Nepenthes ampullaria* (Figure 3.2) were collected. The young *Nepenthes ampullaria* had smaller pitcher size (approximately 2 cm) as compared to the old *Nepenthes ampullaria* pitcher which was approximate 6 cm. The plants which were exposed to direct sunlight were selected. The plants were collected by carefully digging out the whole plant without damaging the roots. The collected *Nepenthes ampullaria* were then transported back to the laboratory, and the isolation of endophytic fungi was carried out within 12 hours of collection.
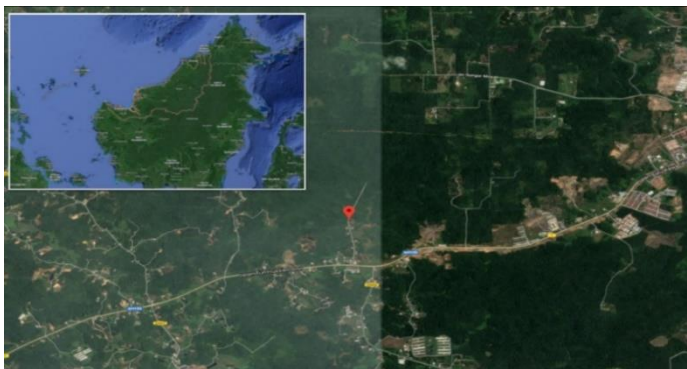
Figure 3.1: The location indicated by a red point was the location where the plant samples, *Nepenthes ampullaria* were collected. Inlet (top left) shows the state of Sarawak (area within the red line) in the Borneo Island (Source: Google Map).

Figure 3.2: The collected *Nepenthes ampullaria*; (left) old *Nepenthes ampullaria*, (right) young *Nepenthes ampullaria*.

3.2 Isolation of Endophytic Fungi from *Nepenthes ampullaria*

3.2.1 Isolation of Endophytic Fungi

When isolating endophytes, it is important to surface sterilized the plant samples. This is because this step will remove or kills all the microorganisms that live on the surfaces of the plant tissues (Greenfield et al. 2015). Calcium hypochlorite, ethanol and sodium hypochlorite are the commonly used chemicals in sterilizing the surface of plant samples (Barampuram, Allen & Krasnyanski 2014). Different endophytic fungi can be isolated when different sterilization methods with different concentration of chemical and different sterilization timing are carried out (Schulz et al. 1993).

The fungal endophytes were isolated based on the method from Lee, Tan and Ting (2014) with slight modifications. The collected *Nepenthes ampullaria* were cleaned thoroughly with running tap water to remove all the soil and sediments on the plant samples. Then, the endophytes were isolated from the three different tissues, namely pitcher, leaf and root of the old and young *Nepenthes ampullaria* respectively. The pitcher, leaf and root parts were selected as these three different parts can be used as a baseline for a whole *Nepenthes ampullaria*.

The isolation of the endophytic fungi from each part of *Nepenthes ampullaria* was done in duplicates, whereby a total of six tissues samples (excluding the negative controls) used for isolating the endophytic fungi associated with *Nepenthes ampullaria* from the two different age group respectively.

First, the sample segments were surface sterilized with 95% ethanol for 60 seconds. Then, it was rinsed thrice with sterilized distilled water. Next, the sterilized plant tissue was blotted dried with sterilized tissue paper and cut into smaller segments before placing them onto Yeast Glucose Chloramphenicol Agar (YGCA) plates, as this media contained chloramphenicol which can suppress bacteria growth. A control plate was made by swabbing the surface of the wet sterilized plant's tissue. This is to confirm that the isolated fungi were endophytes and are not others microbes which survive the surface sterilisation steps. The plates were then sealed with parafilm, labelled and cultured at different temperatures (25°C, 30°C, 35°C, 40°C, and 45°C). Please refer to Figure 2.2 for the overview method in isolating the endophytes from the host plant, *Nepenthes ampullaria*.

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┌─────────────────────────────────┐
│   Collection of plant samples.  │
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│ Separate plant samples to 3 segments; pitcher, leaf   │
│ and root.                                              │
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│ Surface sterilization with 95% ethanol for 60 seconds.│
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│ Rinse 3 times with sterilized distilled water.        │
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│ Blot dried plant tissue surface with sterilized tissue.│
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│ Cut into smaller segments.                            │
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│ Placed segments onto yeast extract glucose            │
│ chloramphenicol agar (YGCA) plates.                   │
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│ Incubated plates at different temperatures (25°C, 30°C,│
│ 35°C, 40°C, and 45°C).                                │
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Figure 3.3: A schematic flowchart in isolating the endophytic fungi from *Nepenthes ampulllaria*.

3.2.2 Purification of Isolated Endophytic Fungi

The fungal endophytes growing out from either side of the sterilized plant tissues (as shown in Figure 3.4) were re-isolated and was placed onto the Potato Dextrose Agar (PDA) plate by using a sterilized plastic straw. Then, the isolates were then further sub-cultured on PDA plate to obtain a pure fungal strain (Figure 3.5).
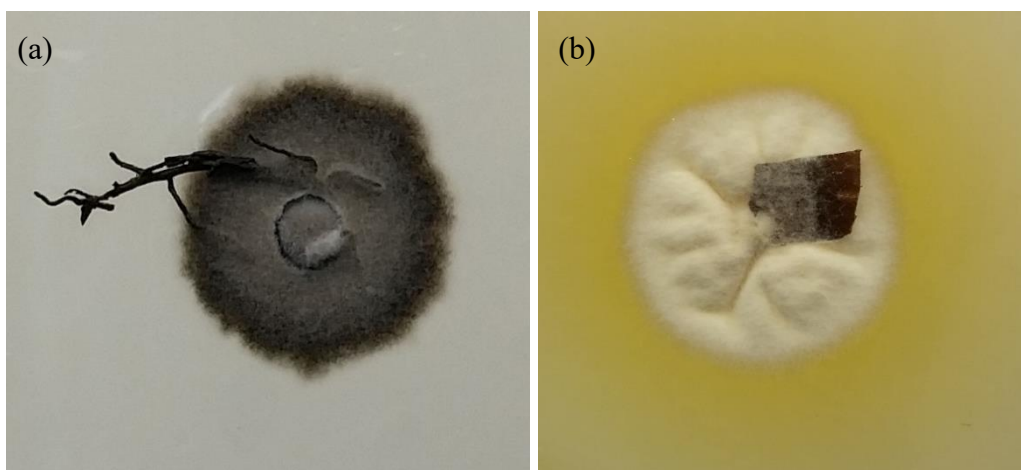
Figure 3.4: Fungal endophytes were observed growing out from either side of the surface sterilized plant tissue on YGCA plates; (a) root and (b) pitcher.
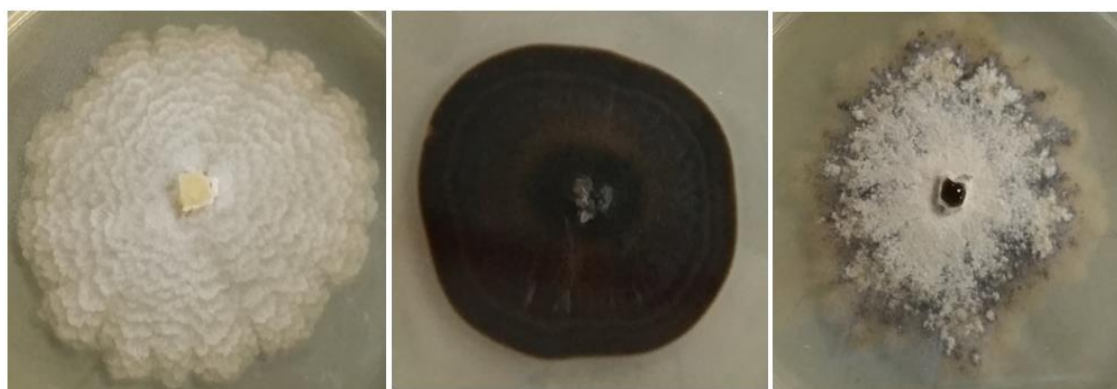
Figure 3.5: Purified isolated endophytic fungi on the PDA plates.

3.3 Molecular Identification of Selected Endophytic Fungi

The traditional method for identifying endophytic fungi is based on its morphological characteristics. However, this method not only requires extensive taxonomical knowledge on fungi (Gherbawy & Voigt 2010) but it also relies on the production of reproductive structure and sporulation of the fungi where most endophytes do not produce (Jones & Pang 2012). Hence, the fungi Internal Transcribed Spacer (ITS) regions are commonly used in identifying fungi through molecular technique (Arnold 2007).

The fungal endophytes isolated at 40°C and 45°C (total number of five endophytes; four from 40°C and one from 45°C) were selected for molecular identification. These five endophytes were cultured on PDA plates for approximate 7 days.

The DNA extraction protocol for identifying the selected fungal endophytes is modified based on the paper from Yeates et al. (1998). A volume about 0.5 mL of 1.0 mm glass beads (BioSpec Products, Inc.) was added to 2 mL microcentrifuge tube. Then, 1 ml of sterile 1X TE buffer (1 M Tris-HCl [pH 8], 0.5 M EDTA [pH8]) were added to the tube. The mycelia of the fungi were scrapped from the plate and transferred to the tube. The tubes were then vortex for 15 minutes, and stored at -20°C for later use.

Three loci of the endophytes were selected to be amplified for identification. The three loci were rDNA ITS, beta-tubulin (β-tubulin), and calmodulin. The official DNA barcode marker for fungi identification at species-level, the rDNA ITS were selected due to its high probability for correct fungi identification (Raja et al. 2017; Schoch et al. 2012). However, fungi identification with using only ITS region may not be enough for some fungi species, thus, protein-coding genes can be used for more precise fungi identification (Raja et al. 2017). β-tubulin and calmodulin were among the two useful protein-coding gene regions for identifying fungi at species-level (Samson et al. 2014; Visagie et al. 2014).

Each of the three loci was amplified using ITS5/ITS4 primer for rDNA ITS, Bt2a/Bt2b for β-tubulin, and CAL223F/CAL737R for calmodulin. The sequence for each primer was listed in Table 3.1 below.

The total volume of 25 µL per reaction of Polymerase Chain Reaction (PCR) master mix containing the following; 12.5 µL of MyTaq Mix (Bioline), 1 µL of respective primer, 1 µL of the crude DNA extract and sterilized distilled water, was prepared. All three of the different primer pairs used the same amplification PCR conditions as shown in Table 3.2. The amplified PCR products were then loaded into 1% agarose gel stained with 3 µL of RedSafe in TAE buffer and visualized under UV light in GelDoc image system (Bio-Rad).

Table 3.1: The sequence for the primers used in the PCR.

Primer name	Primer sequence
ITS 4	5'-TCCTCCGCTTATTGATATGC-3'
ITS 5	5'-GGAAGTAAAAGTCGTAACAAGG-3'
Bt2a	5'-GGTAACCAAATCGGTGCTGCTTTC-3'
Bt2b	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'
CAL223F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'
CAL737R	5'-CATCTTCTGGCCATCATGG-3'

Table 3.2: The PCR conditions for the three primer sets.

Initial denaturation	95°C for 5 minutes	
Denaturation	95°C for 30 seconds	
Annealing	55°C for 30 seconds	35 cycles
Extension	72°C for 30 seconds	
Final extension	72°C for 7 minutes	

The amplified PCR products together with the primers used were then send to First BASE Laboratories Sdn Bhd for sequencing. The obtained sequences from First BASE Laboratories Sdn Bhd were then analysed and identified by matching it against the sequences from the National Center for Biotechnology Information (NCBI) database, using the Basic Local Alignment Search Tool (BLAST).

Molecular Evolutionary Genetics Analysis Version 6.0 (MEGA 6) were used in constructing the phylogenetic tree of the endophytes for each primer sets. The calculated genetic distance and bootstrap replication values of 500 using MEGA 6 were used in constructing the phylogenetic tree. The algorithm used in constructing the phylogenetic tree was based on the Tamura-Nei model (Tamura et al. 2013). Please refer to Figure 3.6 for the summarise method in extracting the DNA from the selected endophytic fungi.

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│ Culture the selected 5 endophytic fungi on PDA for 7 days. │
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│ Scrapped and transferred fungal mycelia into 1 mL sterile 1X │
│ TE buffer in 2 mL tube containing 1.0 mm glass beads.     │
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        │ Vortex tube for 15 minutes.       │
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│ 1 µL of crude DNA extract was transferred into the PCR master mix. │
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│ Amplification program for PCR:                            │
│ Initial denaturation: 95°C, 5 minutes                     │
│ Denaturation: 95°C, 30 seconds    ┐                       │
│                                   ├ 35 cycles             │
│ Annealing: 55°C, 30 seconds       │                       │
│ Extension: 72°C, 30 seconds     ┘                         │
│ Final extension: 72°C, 7 minutes                          │
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        ┌───────────────────────────────────┐
        │ Visualize on 1% agarose gel.      │
        └───────────────────────────────────┘
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                            ▼
        ┌───────────────────────────────────┐
        │ Send to First BASE for sequencing. │
        └───────────────────────────────────┘
                            │
                            ▼
┌─────────────────────────────────────────────────────────┐
│ Analysed obtained sequences against BLAST program in NCBI. │
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                            │
                            ▼
        ┌───────────────────────────────────┐
        │ Construct phylogenetic tree using MEGA 6. │
        └───────────────────────────────────┘
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Figure 3.6: A schematic flowchart of the method of identifying the selected endophytes with molecular technique.

3.4   Preparation of Fungal Extract

The endophytes were inoculated onto sterilized barley in a 250 mL conical flask. The flask was incubated for 30 days at different temperatures; ranging from 25°C, 30°C, 35°C, 40°C, and 45°C. For each temperature will have three replicates for each endophyte and one negative control (containing only sterilized barley). The metabolite was extracted with ethyl acetate acting as the solvent. In the extraction process, 150 mL ethyl acetate was added to the conical flasks with the cultured endophytes. Then, the flasks were stirred at 25°C, 150 rpm for overnight. The fungal extracts were then filtered through filter paper. Next, the filtrate (Figure 3.7) were concentrated with the rotary vacuum evaporator. Later, the concentrated extract was then transferred into the universal bottle and air dried inside a fume hood. The extracts were then stored at -20°C. Figure 3.8 shows the summarise method in preparing the crude fungal extract with ethyl acetate as the solvent.
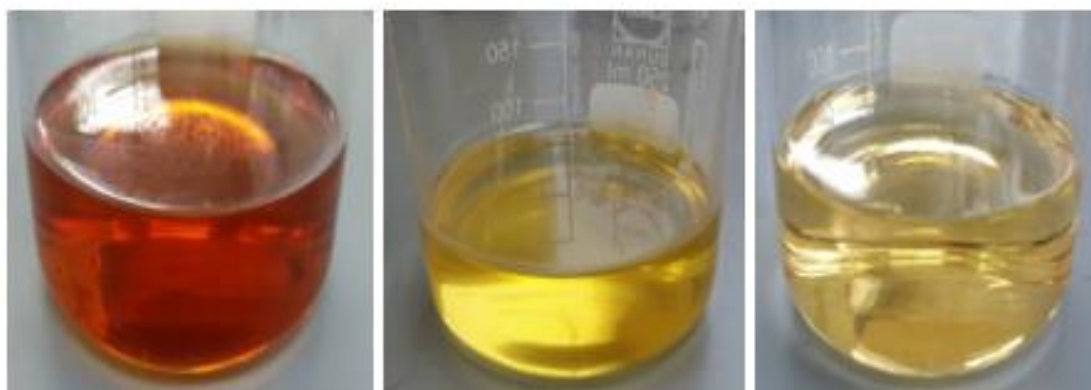
Figure 3.7: Filtered fungal extracts; (left) OR fungal extract at 30°C; (centre) OL fungal extract at 40°C; and (right) OR fungal extract at 30°C.

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│  Inoculate selected endophytes onto sterilized barley.  │
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        │  Incubate for 30 days at 25°C, 30°C,       │
        │  35°C, 40°C, and 45°C                      │
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        │  Metabolite was extracted with ethyl acetate. │
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        │  Stirred flasks at 25°C, 150 rpm for overnight. │
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            │  Filter filtrate with filter paper. │
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    │  Concentrate crude extract with rotary vacuum evaporator. │
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        │  Dried under fume hood and storage at -20°C for │
        │  further use.                              │
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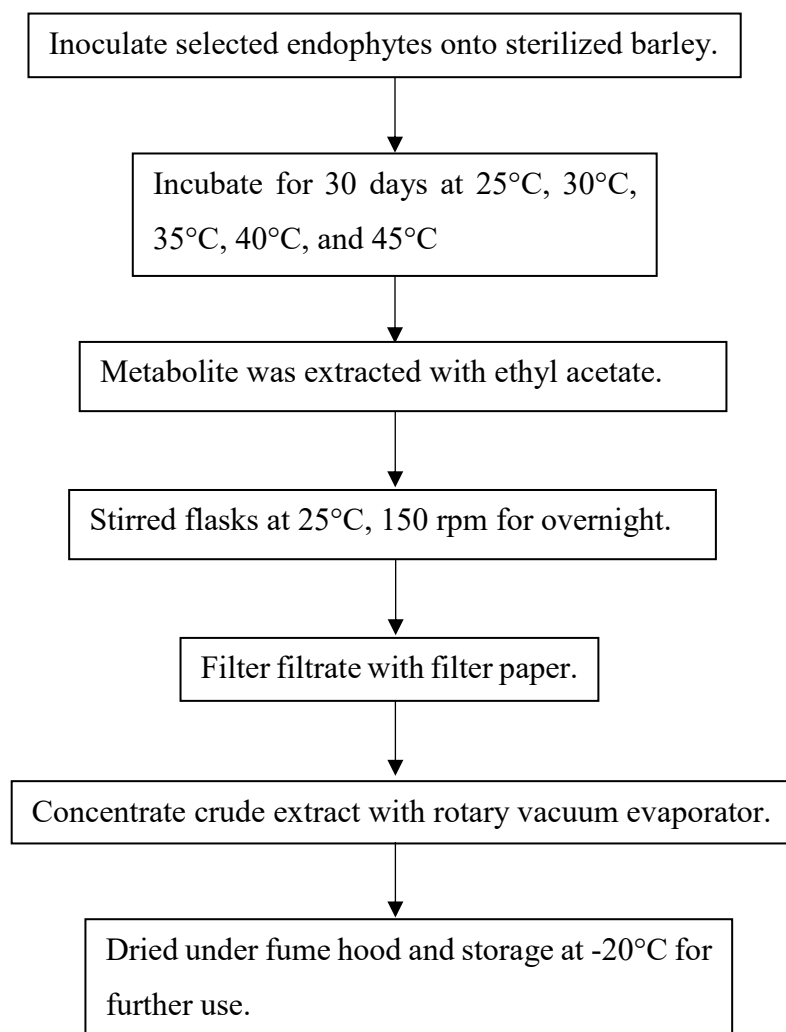
Figure 3.8: A schematic view of preparing the fungal extract with using ethyl acetate as solvent.

3.5 Metabolomic Analysis of Selected Endophytic Fungi Cultured at Different Temperature

From the three commonly used platforms in metabolomics, liquid chromatography-mass spectrometry(LC-MS) was used as the analytical platform for this study (Wishart 2016; Zhang et al. 2012). LC-MS was selected due to its high sensitivity and throughput, and as well as the possibility in confirming the identity of the metabolites present in the fungal extracts (Songsermsakul & Razzazi-Fazeli 2008; Villas-Bôas et al. 2005).

In this study, the metabolites are analysed using untargeted metabolomics method, and multivariate statistical analysis will be carried out as well. The analysis of the variability among the samples was carried out using multivariate analysis which involved the principal component analysis (PCA) and partial least square analysis discriminant analysis (PLS-DA) (Bartel, Krumsiek & Theis 2013; Khoomrung et al. 2017; Saccenti et al. 2014).

3.5.1 Sample Preparation

Eleven fungal extracts (3 biological replicates for each sample) which were completely dried (Figure 3.9) were selected and brought over to University Malaysia Sabah (Bartel, Krumsiek & Theis). Approximate 1 mg of crude fungal extract was dissolved with 400 µL of methanol:chloroform:water with 1% sodium chloride (ratio of 1:1:1 v/v). The mixture was then vortexed until the extract was fully dissolved. Then, the mixture was centrifuged at 9,520 g for 30 minutes at 4°C. Prior to air-dry the supernatant, it was transferred to another new tube. The dried extracts were re-dissolved with methanol first, before filtering and transferring the samples extracts to LC-MS vials. The vials were then stored at -80°C before subjecting it to the LC-MS platform on the next day.
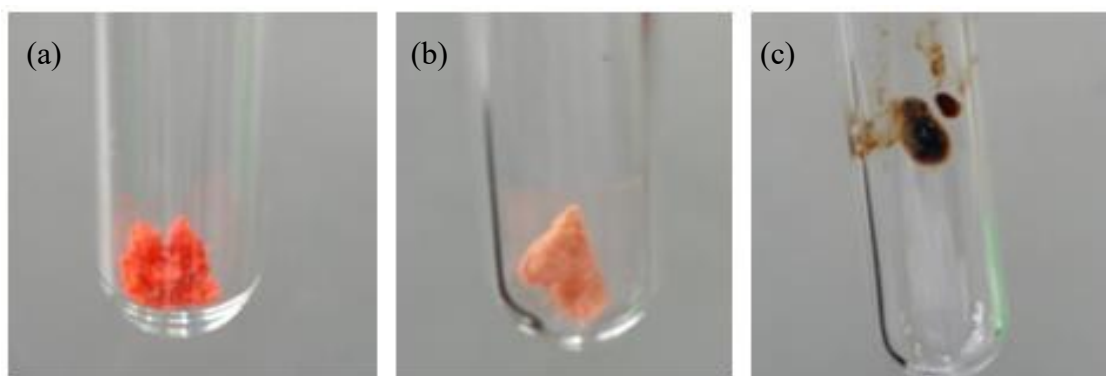
Figure 3.9: Dried crude fungal extracts that were brought over to University Malaysia Sabah (Bartel, Krumsiek & Theis). (a) Dried crude fungal extract of sample YL cultured at 25°C; (b) dried fungal extract of sample YL cultured at 30°C; and (c) dried crude extract of sample OR cultured at 30°C.

3.5.2   LC-QTOF Acquisition

Extracted samples in the LC-MS vials were subjected to Vanquish™ Horizon UHPLC system (Thermo Fisher Scientific, USA) coupled with electrospray ionization Impact II QTOF-mass spectrometry system (Bruker Daltonic, Germany). 10 µL of the samples were then injected into Kinetex F5 (2.1 × 100 mm, 2.6 µm; Phenomenex, Torrance, CA, USA). During analysis, the Kinetex F5 was maintained at 40°C with 600 µl/min flow rate. The mobile phase was composed of 2 solvents; solvent A (H_2O - 0.1% HCOOH - 1% 10 mM NH_4OAc) and solvent B (acetonitrile/methanol [6:4 v/v] - 1% of 0.1% HCOOH - 1% 10 mM NH_4OAc).

The gradient elution program was initiated from 1% to 40% solvent B in 5 min, followed by 100% solvent B from 5.1 to 8 min and maintained for 2 min. Before injecting the next sample, the initial gradient was used in conditioning the column for 3 minutes.

The m/z between 50 and 1,500 was set for data acquisition. The positive heated electrospray ionization (ESI) were deployed at 4,200 V for positive. The following was set for the ion source conditions: 300°C for gas temperature, 12 l/min for drying gas flow, and 5.0 bar for nebulizer flow. 10 mM sodium formate as mass calibration standard was introduced post-column through a 6-port valve at 0.1 – 0.3 minutes. The *m/z* values of acquired data were calibrated against the introduced sodium formate, and then, subsequently converted into a mzXML file format.

3.5.3   Pre-processing of Data and Multivariate Data Analysis

The obtained raw data for the samples were pre-processed using MZmine in order to compensate for the variations in the retention times and m/z values for each samples analysis. The peak list tables of the pre-processed data were then saved in a comma separated values (*.csv) file format. In the table, the rows represent the samples while the columns represent the integrated and normalized peak areas.

The .csv file format of the data for the isolated endophytes was then subjected and analysed in MetaboAnalyst, an integrated software tool used for statistical analysis. Prior to statistical analysing the data, the data were log transformed and Pareto scaled. Statistical analysis involving principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were carried out on the data that had been transformed and scaled. The variables that its position are the furthest away from the centre of the loading plot were selected for identification.

3.5.4   Identification of Metabolite

The precursor ions $[M-H]^+$ mass-to-charge (m/z) ratio of the selected variables were searched and matched with the online available databases, MetFrag and METLIN for the identification of the metabolites. MetFrag is an in silico fragmentation software tool which is able to screen the metabolite candidates retrieved from databases including the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Wolf et al. 2010). The METLIN database is a database that focused on the LC-MS mass spectral, whereby the identification of the metabolites based on the accurate mass data (Sana et al. 2008; Smith et al. 2005).

Please refer to Figure 3.10 for the summarize overview of the methods in analysing the metabolites produced by the selected endophytic fungi.

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┌─────────────────────────────────────────────────┐
│ Dissolve dried crude extract with 400 µL of     │
│ MeOH:CHCL₃:H₂O+1% NaCl (1:1:1 v/v).             │
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│ Centrifuge at 9,520 g, 30 minutes at 4°C.       │
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│ Transferred of supernatant to another tube and  │
│ air-dried.                                       │
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│ Re-dissolved extract with methanol. Filtered    │
│ and transfered extract to LC-MS vials.          │
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│ Subject vials to Vanquish™ Horizon UHPLC        │
│ system (Thermo Fisher Scientific, USA) coupled  │
│ with electrospray ionization Impact II QTOF-mass│
│ spectrometry system (Bruker Daltonic, Germany). │
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│ Pre-processing of data using MZmine.            │
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┌─────────────────────────────────────────────────┐
│ Statistical analysis with MetaboAnalyst.        │
└─────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────┐
│ Metabolite identification with online database. │
└─────────────────────────────────────────────────┘
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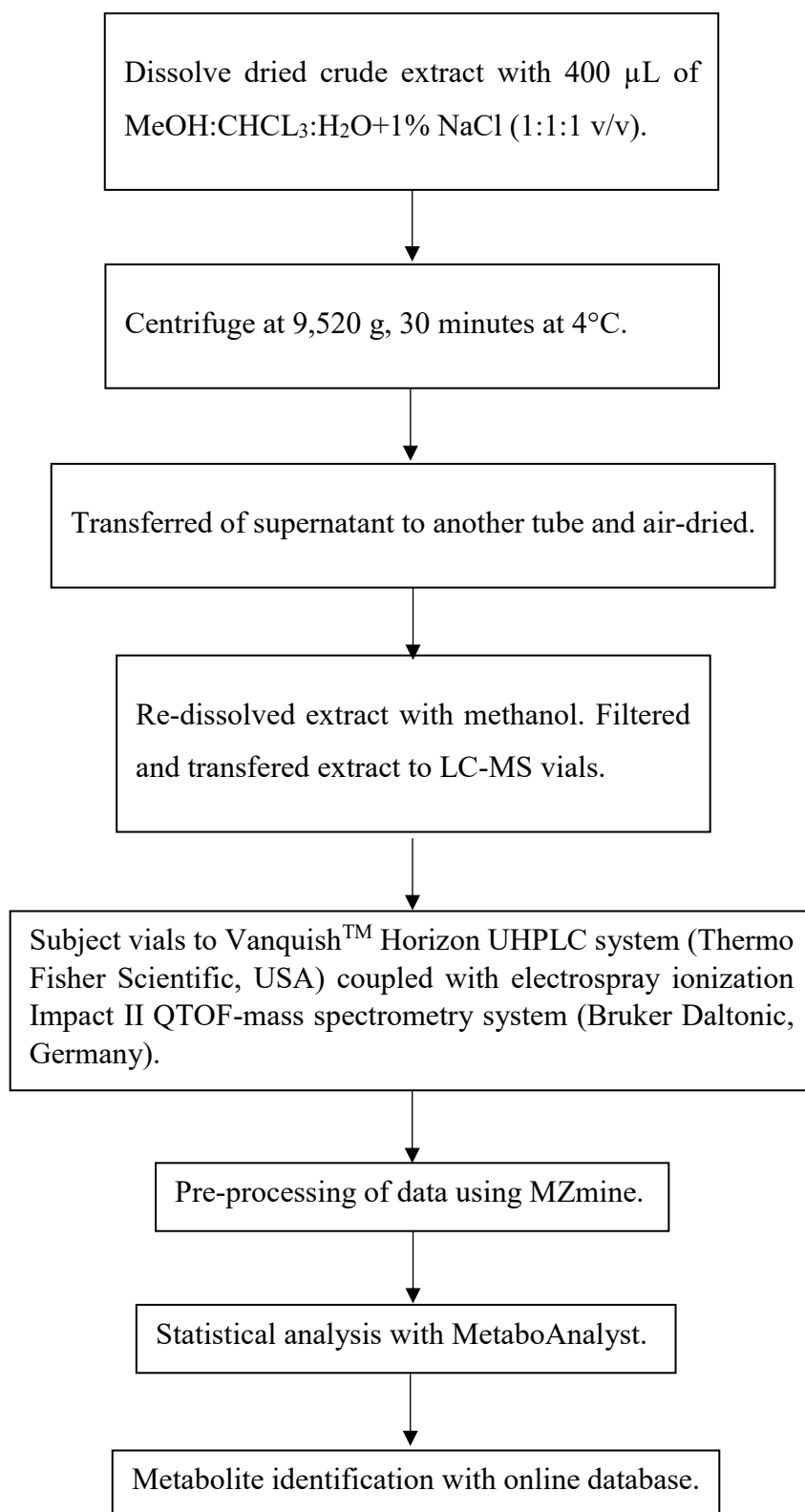
Figure 3.10: A schematic overview of the overall of LC-MS methodology.

Chapter 4 Result

4.1   Isolation of Endophytes from *Nepenthes ampullaria*

The endophytic samples were labelled based on the age group and parts of the *Nepenthes ampullaria*, for example, OP referred to old *Nepenthes ampullaria*'s pitcher. The labelling used for each isolated fungal endophyte were recorded in Table 4.1.

Table 4.1: Definition of labels used in labelling the isolated endophytic fungi.

Labels	Definition of the label
YP	Young *Nepenthes ampullaria* pitcher
YL	Young *Nepenthes ampullaria* leaf
YR	Young *Nepenthes ampullaria* root
OP	Old *Nepenthes ampullaria* pitcher
OL	Old *Nepenthes ampullaria* leaf
OR	Old *Nepenthes ampullaria* root

The negative control plates which were swabbed with the wet surfaces of the sterilized plant tissues remained clean (as shown in Figure 4.1) indicated that the fungi growing from the plant tissue were endophytes. A sum of seventy-three fungal endophytes was successfully isolated from both young and old *Nepenthes ampullaria*. Based on Figure 4.2. 35°C has the highest number of endophytes isolates (24 isolates) from both the young and old *Nepenthes ampullaria*, followed by room temperature (23 isolates), 30°C (21 isolates), 40°C (4 isolates), and lastly at 45°C, where only one endophyte was isolated. However, out of the total of seventy-three isolated endophytes, only five were considered heat-tolerant endophyte based on the temperature where they were isolated from. This is because heat-tolerant fungi or also known as thermotolerant fungi has an optimal growth temperature range of 40°C to 50°C (Mouchacca 2000). From the five heat-tolerant endophytes, four of them were isolated at 40°C, while only one was isolated at 45°C.
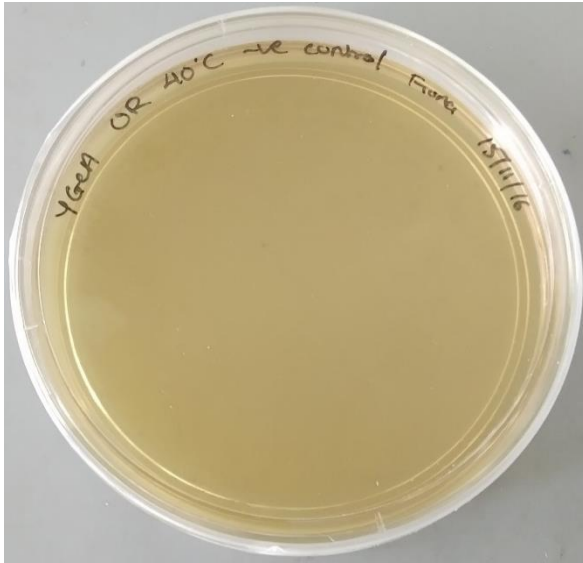
Figure 4.1: The negative control plate which was swabbed with the sterilized wet roots of old *Nepenthes ampullaria*.
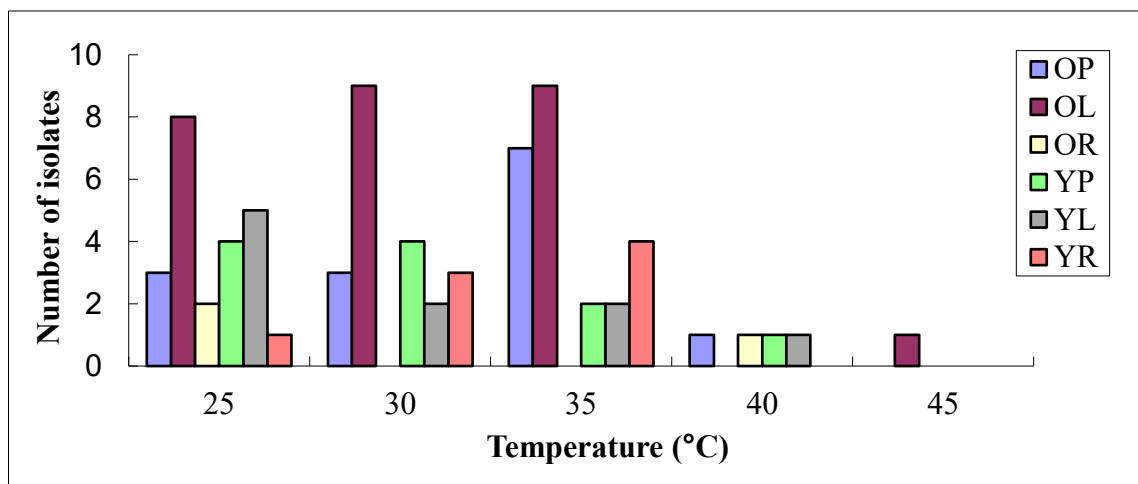
Figure 4.2: The number of isolated fungal endophytes from three different parts of both old and young *Nepenthes ampullaria* at a different temperature.

The four endophytes that were isolated at 40°C was from the leaf and pitcher of the young *Nepenthes ampullaria*, and from the old *Nepenthes ampullaria*, the endophytes were isolated from the roots and pitcher. For the one and only endophyte isolated at 45°C was from the leaf of the old *Nepenthes ampullaria*. Figure 4.3 shows the morphology of the five heat-tolerant endophytes cultured on PDA.
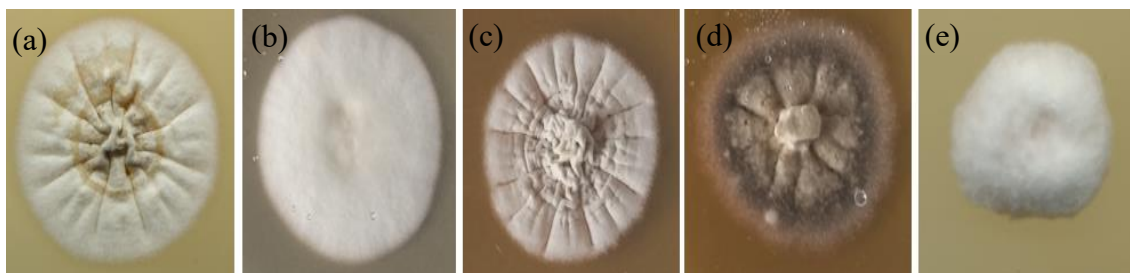
Figure 4.3: The endophytes isolated from (a) pitcher of old *Nepenthes ampullaria* (OP); (b) root of old *Nepenthes ampullaria* (OR); (c) pitcher of young *Nepenthes ampullaria* (YP); (d) leaf of young *Nepenthes ampullaria* (YL); and (e) leaf of old *Nepenthes ampullaria* (OL). From (a) to (d) were the endophytes isolated at 40°C; while (e) was from 45°C.

4.2 Identification of Isolated Endophytic Fungi

The five heat-tolerant endophytic fungi were selected for identification with molecular technique. Multi-locus phylogeny was used to identify the endophytic fungi, with the usage of three different sets of primers, ITS5/ITS4, Bt2a/Bt2b, and CAL228F/CAL737R. The ITS5/ITS4 primer set is the universal primer pair used in amplifying the ITS region. The primer sets, Bt2a/Bt2b (universal primer for fungal β-tubulin) (Zampieri et al. 2009) and CAL228F/CAL737R (primer used in amplifying calmodulin region) (Carbone & Kohn 1999) were used for further fungi identification.

Figure 4.4 below shows all the successfully amplified PCR products with the three different primers. For the ITS primers, the fungal DNA band size is approximately 600 bp to 700bp. Band size for the PCR product by Bt primers are approximate 500 bp, whereas approximately 300 bp to 500 bp band size were observed for CAL primers products. The Bt primers successfully in amplifying the DNA from sample OP, YP, YL and OR (loaded in the sequence from lane 8 to lane 11). There's only two DNA successfully amplified with using CAL primers, which were OP and OL (loaded in lane 12 and lane 13).
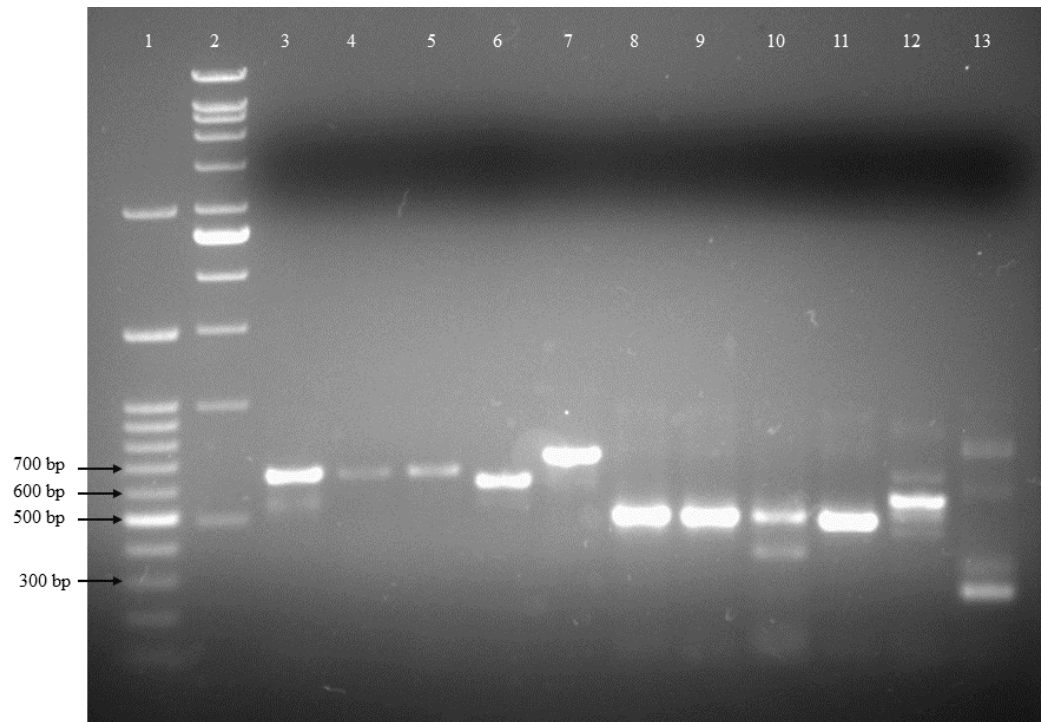
Figure 4.4: The PCR products of the extracted DNA from the five selected fungal endophytes using ITS, Bt and CAL primers. Lane 1 was 100 bp ladder; lane 2 was 1 kb ladder. From lane 3 to lane 7 were samples amplified with ITS primer, starting with OP, YP, YL, OR and lastly OL in lane 7. Lane 8 to lane 11 were loaded with samples amplified with Bt primer pair, OP, YP, YL and OR. For the last two lanes, lane 12 and lane 13 were the CAL PCR products, OP and OL.

The obtained sequences from FIRST Base were analysed against the GenBank database in NCBI with using BLAST search program. The closest matching species from the search for the isolated endophytes were listed in Table 4.2. The closest match species for the endophytes were selected based on the species with the highest similarity percentages.

Table 4.2: The closest matching species for the isolated endophytic fungi for the three different primer sets.

Primer used	Fungal isolate	Closest match (Accession number)	Similarities (base pairs, percentages)	Phylogenetic Division
ITS	YP	Fungal sp. PDB99 (KP236115)	552/553 (99%)	*Dothideomycetes*
	YL	Fungal sp. PDB99 (KP236115)	562/563 (99%)	*Dothideomycetes*
	OP	Fungal sp. PDB99 (KP236115)	568/569 (99%)	*Dothideomycetes*
	OR	*Talaromyces pinophilus* clone OTU49 (KY965441)	522/522 (100%)	*Talaromyces*
	OL	*Coprinopsis* sp. BAB-4086 (KJ670305)	631/631 (100%)	*Coprinopsis*
Bt	YP	*Dothideomycetes* sp. UFMGCB 9905 (KX024589)	340/379 (89%)	*Dothideomycetes*
	YL	*Dothideomycetes* sp. UFMGCB 9905 (KX024589)	259/291 (89%)	*Dothideomycetes*

Bt	OP	*Dothideomycetes* sp. UFMGCB 9905 (KX024589)	357/405 (88%)	*Dothideomycetes*
	OR	*Talaromyces pinophilus* strain 1-95 (KP342453)	363/363 (100%)	*Talaromyces*
CAL	OP	*Macrophomina phaseolina* strain CPC 21534 (KF951982)	423/464 (91%)	*Macrophomina*
	OL	*Coprinopsis cinerea* okayama7#130 (AB764051)	180/181 (99%)	*Coprinopsis*

The three primer sets each has a phylogenetic tree constructed (Figure 4.5 to Figure 4.7) by comparing the isolated endophytic fungi sequences with the closest matches sequences of known fungi from NCBI database. The diagram which displays the lines of the evolutionary origin of different organisms that shares a common ancestry is known as a phylogenetic tree (Baum 2008). The phylogenetic tree for the three different primer sets was constructed with the maximum likelihood method which based on the Tamura-Nei model, with 500 bootstrap values and cut-off values of 50%, which was calculated with Molecular Evolutionary Genetic Analysis version 6 (MEGA 6) software.
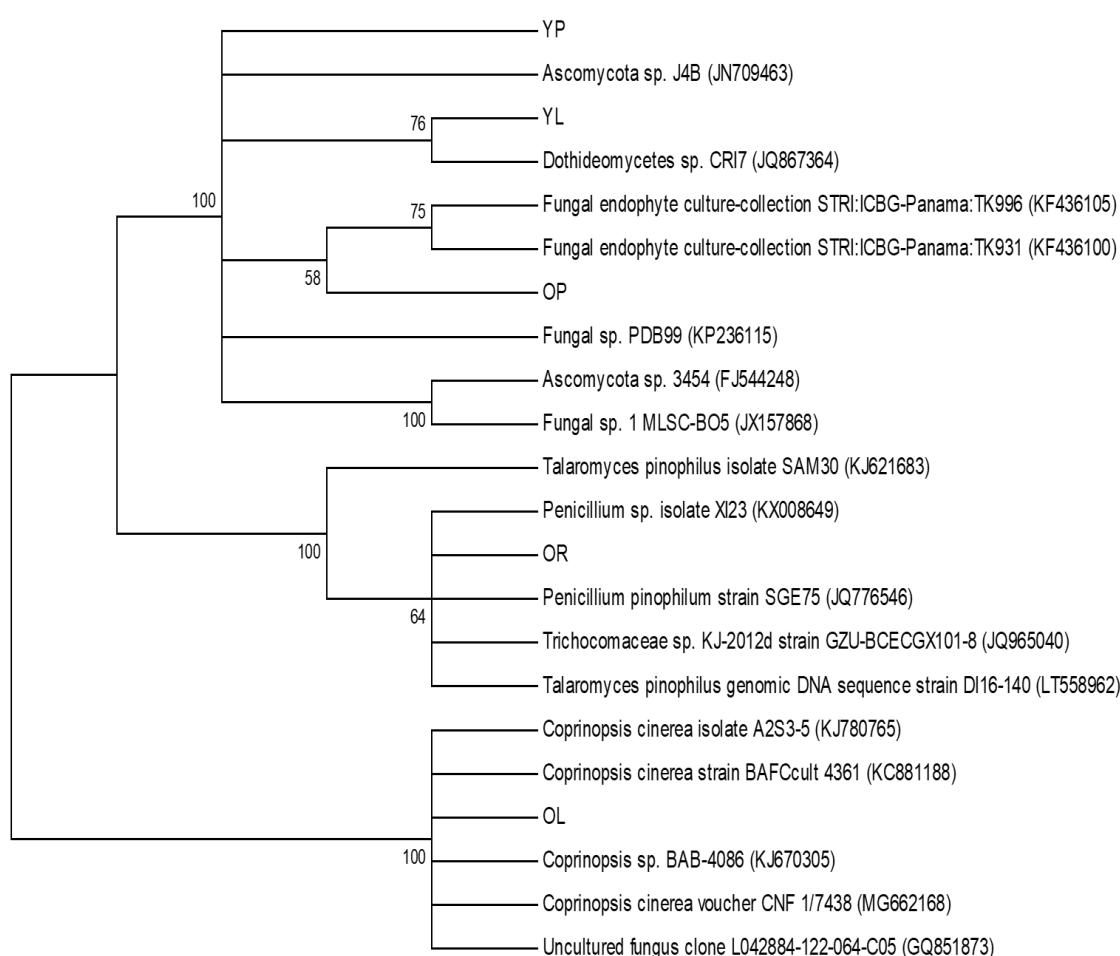
Figure 4.5: Phylogeny tree based on the ITS sequences of the five selected endophytic fungi was constructed using the Tamura-Nei model with bootstrap replication at 500 with 50% cut-off value.
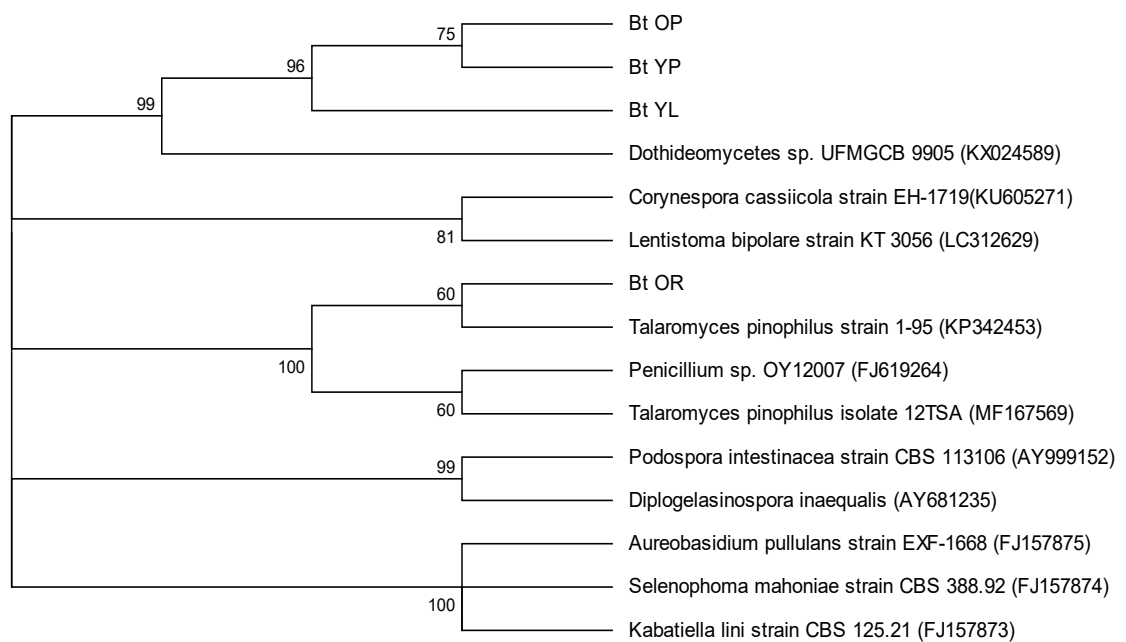
Figure 4.6: Phylogeny tree constructed for the endophytes amplified with Bt primer sets which were based on the Tamamura-Nei model with bootstrap replication at 500 and 50% cut-off value.
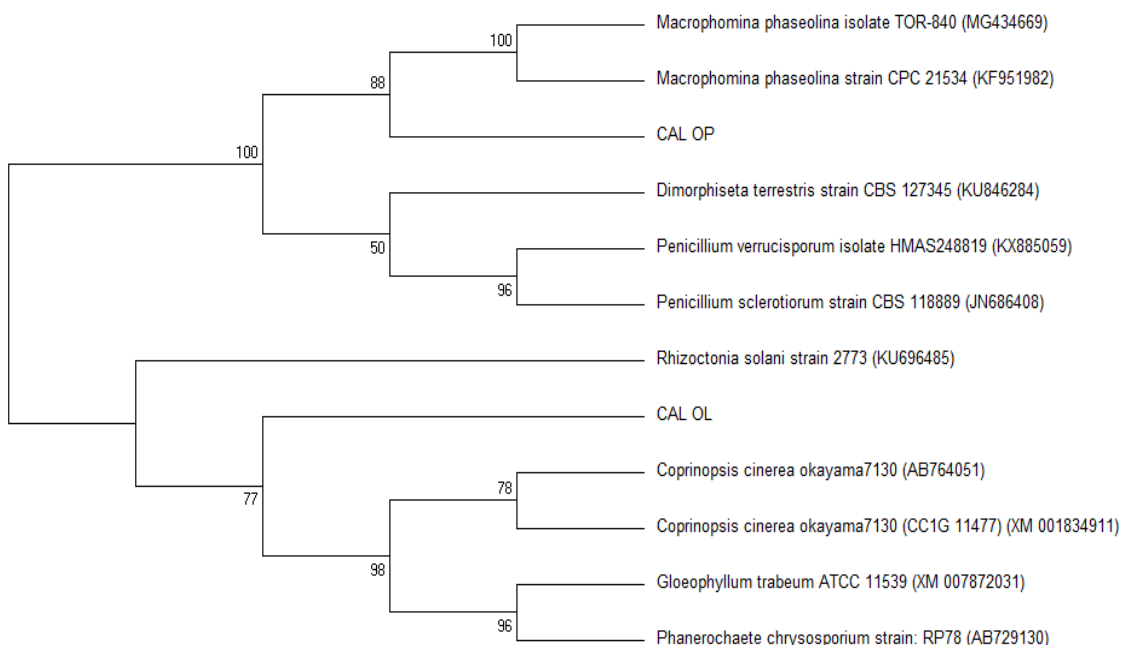
Figure 4.7: Phylogeny tree of OP and OL amplified using CAL primer, which was constructed based on Tamamura-Nei model with the same bootstrap replication and cut-off values as Figure 4.5 and Figure 4.6.

From the five isolated endophytes, three of the endophytes were identified up till the class level only, which is the fungal class *Dothideomycetes*, while the other two are identified as *Talaromyces pinophilus* and *Coprimopsis cinerea* as seen in Table 4.2.

The identities of the five endophytes which were amplified using ITS primers were grouped into fungal class *Dothideomycetes*, *Talaromyces pinophilus*, and *Coprinopsis cinerea* as seen in Table 4.1. The BLAST search results for YP, YL, and OP showed a high similarity to fungal species PDB99 (accession KP236115), which was a fungus belonging to the fungal class *Dothideomycetes*. Besides that, these three endophytes also share a common ancestry between them to other fungi belonging to class *Dothideomycetes* and phylum *Ascomycota*. For fungal endophyte OR, it was identified as *Talaromyces pinophilus*, while the fungal endophyte OL was identified as *Coprinopsis cinerea*.

The beta-tubulin (β-tubulin) loci were also amplified to identify the endophyte as well. The endophytes which were identified using Bt primers were grouped into two groups, which were fungal class *Dothideomycetes*, and *Talaromyces pinophilus* (referred to Table 4.2 for the closest matching species and Figure 4.6 for the phylogenetic tree for sequences amplified with Bt primers).

For calmodulin (CAL), only endophyte OP and OL were successfully amplified. The closest matching for OP was identified as *Macrophomina phaseolina*, while *Coprinopsis cinerea* was identified as the closest matching species for OL. *Macrophomina phaseolina* is a fungus that falls under *Dothideomycetes* fungal class, thus, this further confirmed the identity of OP as a fungal class *Dothideomycetes*.

4.3   Screening of Metabolites from the Endophytes

The crude extracts were separated into two groups; polar and non-polar, when it was dissolved with a mixture of methanol:chloroform:water with 1% sodium chloride. The polar metabolite was extracted by the methanol, while the non-polar was extracted by chloroform as shown in Figure 4.9 below.
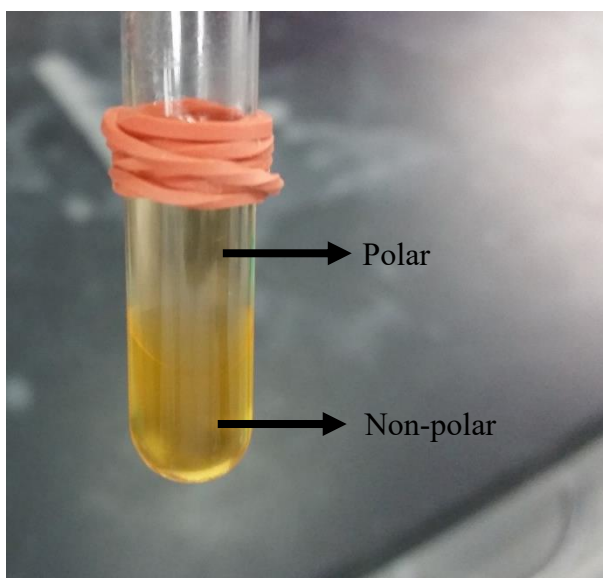
Figure 4.8: The separation of the polar and non-polar metabolite of the crude fungal extracts after dissolving it in a mixture of methanol:chloroform:water with 1% sodium chloride.

Prior to identifying the metabolites produced by the endophytic fungi, the obtained raw data were pre-processed with MZmine for compensating the variations in the retention times and m/z values. Then, a multivariate statistical analysis using the principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA) were carried out on the pre-processed data. Both PCA and PLS-DA were conducted using MetaboAnalyst version 4.0, a web-based tool for analysing metabolomics data (Chong et al. 2018).

Only four of the five endophytes metabolites based on two temperatures, 30°C and 40°C, were analysed due to limited sample amount at University Malaysia Sabah (Bartel, Krumsiek & Theis) for LC-MS analysis.

Good separation was observed in the PCA between the endophytes in Figure 4.9, where the separation is suggested to be due to the endophyte identity, for examples, the OL sample extracts only clustered among themselves and was separated from YP, OP and OR. In Figure 4.9 (a), the OR sample was further separated between the two cultured temperature, which was 30°C and 40°C. However, the metabolites by YL and OP were overlapping with each other as shown in Figure 4.9 (a) and Figure 4.9 (b).
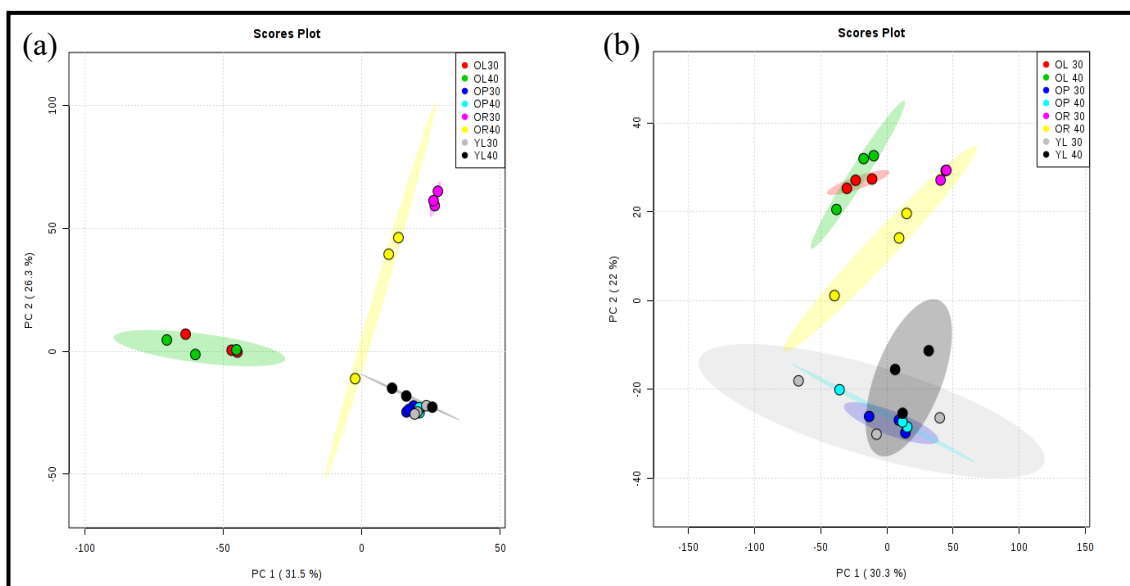
Figure 4.9: The PCA score plot of both (a) polar and (b) non-polar metabolites produces by the endophytes. Each samples extracts is represented by different coloured dots and represents data from three biological replicates.

Besides that, separation of the extracts based on the identities of the endophytes were also seen in the PLS-DA (showed in Figure 4.10). In Figure 4.10 (a), a further separation was also observed, which is due to the difference in the temperature when culturing the endophytes.
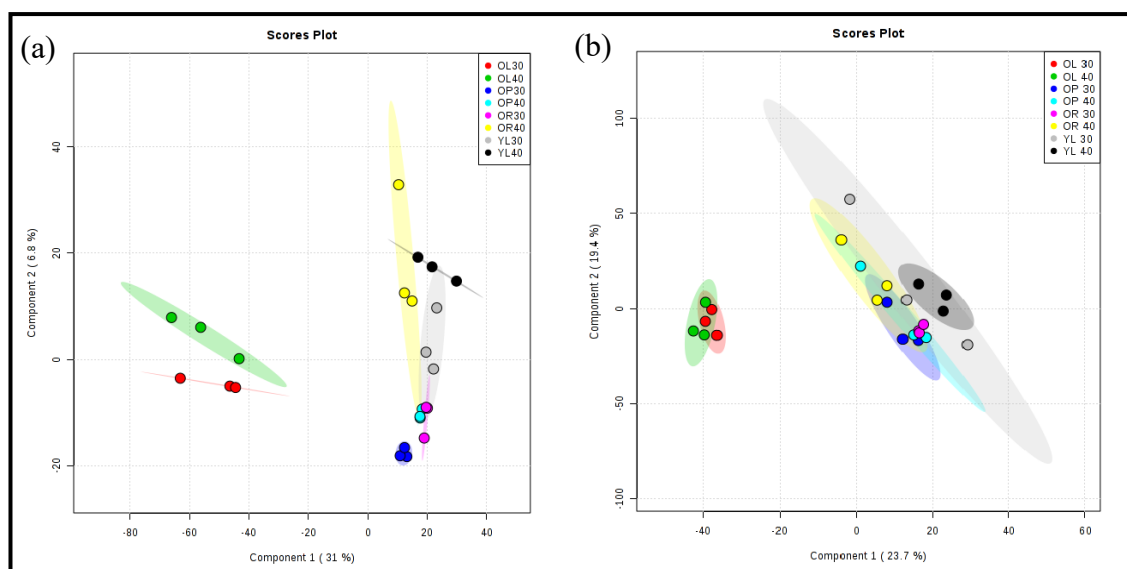
Figure 4.10: PLS-DA score plot for (a) polar and (b) non-polar metabolites from the endophytes cultured at 30°C and 40°C. Each samples extracts were represented by different colour dots, and each of the samples has three biological replicates.

The identification of the metabolites for each sample was selected from their respective loading plot of PCA and PLS-DA, whereby the variables or outlier in the loading plot, which were located away from the centre or cluster were selected for identification. This is because the same or similar metabolite will cluster together, whereas those that were distinct from the others would be observed as outliers (Chanana et al. 2017; Macintyre et al. 2014). For example, as shown in Figure 4.11 were the respective individual loading plot for two different fungal extracts, OP and OL. The selected outliers (the dots within the red circles) were selected due to the metabolites being distinctive to those which were clustered together when comparing between the same extracts from two temperatures.

The metabolites were identified by matching the selected variables precursor ions mass-to-charge (m/z) with the mass-to-charge (m/z) of known metabolites in the available database Metfrag which includes that Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Besides that, METLIN database was also used in identifying the selected variables. The identified metabolites from respective endophytic fungi extracts using the two mentioned databases (Metfrag and METLIN) were listed in Table 4.3.
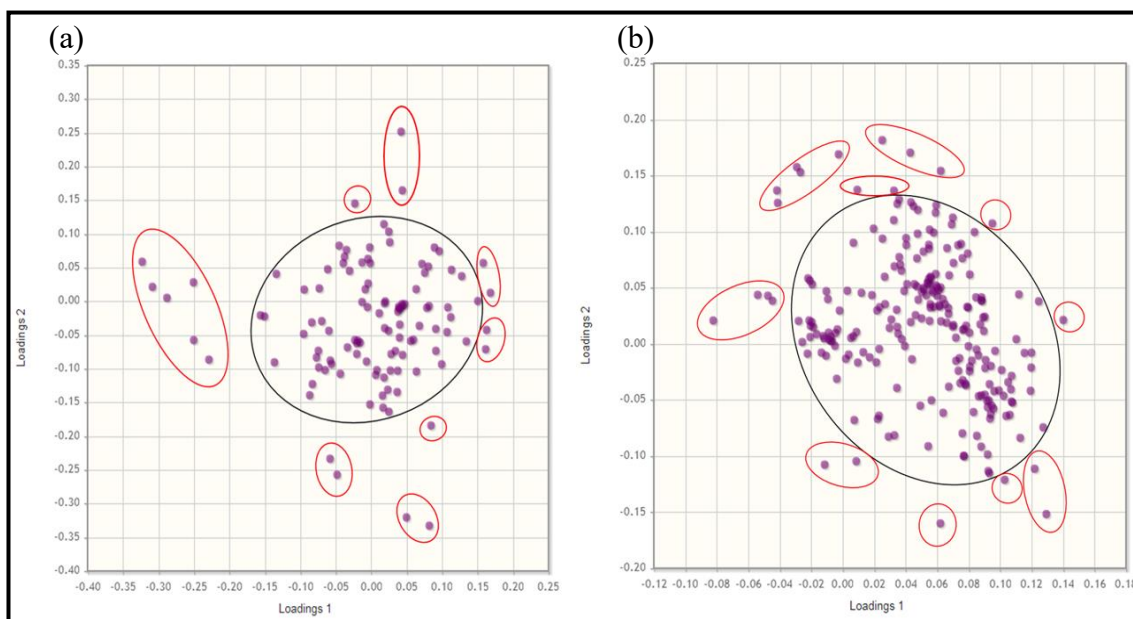
Figure 4.11: The PCA loading plot for the polar extract of (a) OP sample and (b) OL sample. The purple dots within the red circles were selected for metabolite identifications.

Table 4.3: Identified metabolites produced by the selected endophytes at different temperatures.

Endophyte	Identified metabolites	Temperature (°C)		Biological activities	References
		30	**40**		
YL	Matairesinol	✓	✓	Antimicrobial, antioxidant, anticancer	(Csapi et al. 2010; Donoso-Fierro et al. 2009; Kumarasamy et al. 2003)
	5,6,7-trimethoxycoumarin	✓	✓	Antimalaria	(Baxter, Harborne & Moss 1998)
	Deltaline	✓		Analgesic	(Wang et al. 2011)
	Coniferyl aldehyde		✓	Antimicrobial, potential heat shock factor 1 (HSF1) inducer	(Choi et al. 2017; Shreaz et al. 2013)
	13-docosenamide	✓	✓	Antimicrobial, anticancer	(Prasher & Dhanda 2017; Xu et al. 2009)
OP	Pinolidoxin	✓		Phytotoxic	(Stergiopoulos et al. 2012)

	Matairesinol	✓	✓	Antimicrobial, antioxidant, anticancer	(Csapi et al. 2010; Donoso-Fierro et al. 2009; Kumarasamy et al. 2003)
	5,6,7-trimethoxycoumarin	✓	✓	Antimalaria	(Baxter, Harborne & Moss 1998)
	Lariciresinol		✓	Antifungal, antioxidant	(Bajpai et al. 2017; Hwang et al. 2011)
	Deltaline	✓		Analgesic	(Wang et al. 2011)

OR	Matairesinol	✓	✓	Antimicrobial, antioxidant, anticancer	(Csapi et al. 2010; Donoso-Fierro et al. 2009; Kumarasamy et al. 2003)
	Adenine	✓		Promoting the plants' growth	(Hardoim et al. 2015; Pirttilä et al. 2004)
	Gibberellin A8-catabolite	✓		Phytohormone	(Khan et al. 2008)
	Magnoshinin	✓		Anti-inflammation	(Kadota et al. 1987; Kimura et al. 1985)
	13-docosenamide	✓	✓	Antimicrobial, anticancer	(Prasher & Dhanda 2017; Xu et al. 2009)
	Gardenin B	✓	✓	Antioxidant, anticancer	(Charoensinphon et al. 2013; Wang et al. 2018)

OL	Phenylacetaldehyde	✓	✓	Antimicrobial, antioxidant	(Al-Fatimi, Wurster & Lindequist 2016; Cecotti et al. 2013)
	Pentalenolactone F		✓	Antibiotic	(Tetzlaff et al. 2006)
	(9Z,12Z,15Z)-octadecatrienoic acid	✓		Anti-inflammatory, anti-arthritic, insecticide	(Kumar, Kumaravel & Lalitha 2010; Kumaradevan et al. 2015)

Majority of the metabolites produced by the endophytes were detected at 30°C (a total of 11 different metabolites) as compared to 40°C (total of 8 different metabolites). As shown in Table 4.3, there were also some metabolites such as gardenin B were detected at both temperatures. Besides that, matairesinol was seemed to be produced by three different endophytic fungi, YL, OP and OR.

Chapter 5 Discussion

5.1 Isolation and Identification of Endophytes from *Nepenthes ampullaria*

A total of seventy-three endophytic fungi were successfully isolated from three different parts of both young and old *Nepenthes ampullaria*. However, only five out of the seventy-three were considered as heat tolerant endophytes. as these five endophytes were isolated at a temperature of 40°C and 45°C. This is because thermotolerant fungi are fungi with optimum growth temperature which falls in the range of 40°C to 50°C (Mouchacca 2000).

Two out of the five heat-tolerant endophytes were isolated from the young *Nepenthes ampullaria*, and the remaining three endophytes were isolated from the old *Nepenthes ampullaria*. This is due to the distribution and population of the endophytes that can be found within a plant are affected by the age of their host (Jia et al. 2016). Hence, this explains why the only endophyte that was isolated at 45°C were from the old *Nepenthes ampullaria*, as the plant-endophyte had already adapted to the warmer environment as compared to young *Nepenthes ampullaria.*

The five fungal endophytes were then identified via molecular technique. Based on Table 4.2, the identities of the five endophytes can be grouped into three groups, fungal class Dothideomycetes, *Talaromyces pinophilus* and *Coprinopsis cinerea*. These three groups of fungi had been successfully isolated and reported as endophytic fungi of other plant species.

The endophyte YP, YL and OP were identified as fungi belonging to *Dothideomycetes* class based on their rDNA ITS region (99%), beta tubulin loci (89%) and calmodulin loci (91%). Although endophyte OP was identified as *Macrophomina phaseolina* (89% similarity), which belongs to fungal class *Dothideomycetes* with CAL primers set, different morphology was observed between endophyte OP and *M.phaseolina* (shown in Figure 5.1).

Figure 5.1: The comparison of the morphology of (a) *Macrophomina phaesolina* **(Trakunsukharat 2011) and (b) endophyte OP; with both of the fungi cultured on potato dextrose agar (PDA).**

Dothideomycetes fungal class is the largest and most diverse class of fungi under the Ascomycota division (Schoch et al. 2009). They can be found present in every continent and many are found to be tolerant against the extreme environment as they can also be found in Antartica (Ohm et al. 2012). Fungi belonging to this class can be found existed as a pathogen, endophytes, epiphytes and as well as saprobe (Schoch et al. 2006). Examples of fungi belonging to *Dothideomycetes* class that were found as endophytic fungi were such as *Pleosporales* species and *Paraconiothyrium* species in *Sarracenia*, another carnivorous plant species (Glenn & Bodri 2012). The endophytic fungi, *Lasiodiplodia* species (belonging to *Dothideomycetes* fungal class was also isolated from the pitcher of *Nepenthes ampullaria* (Lee, Tan & Ting 2014). Other examples of endophytic fungi belonging to this class were *Paecilomyces formosus* (Khan et al. 2012a), *Alternaria alternate*, *Phoma sorgina*, *Epicocum nigrum* (Szilagyi-Zecchin et al. 2016), *Cladosporium cladosporioides* (Paul & Yu 2008; Xing & Guo 2011), and *Phomopsis* species (Ferreira et al. 2015; Kumaran & Hur 2009; Yang et al. 2015).

Besides that, the endophyte OR was identified as *Talaromyces pinophilus* with 100% similarity based on its ITS region and beta-tubulin loci. *Talaromyces pinophilus*, or also can be known as *Penicillium pinophilum*, which is the anamorph stage (Abdel-Rahim & Abo-Elyousr 2018). This fungus is widely used to degrades cellulose (Pol, Laxman & Rao 2012) and also as the renewable pigments and colourants sources (Caro et al. 2015). The endophyte, *Talaromyces pinophilus* were also reported to be isolated as a fungal endophyte from the roots of *Drosera rotundifolia* (commonly known as sundews which is also a carnivorous plant) which was collected in the summer (Quilliam & Jones 2010). Besides that, it is also isolated from the roots of *Ceriops tagal* (Xing & Guo 2011). Others previously reported host plant of *Talaromyces pinophilus* or *Penicillium pinophilum* were *Curcuma amada* (Krishnapura & Belur 2016), *Allium schoenoprasum* (Koul et al. 2016), *Sophora tonkinensis* (Yao et al. 2017), *Arbutus unedo* (Vinale et al. 2017) and *Limonium tetragonum* (Khalmuratova et al. 2015).

Endophyte OL which was identified as *Coprinopsis cinerea* (100% similarity) was the only endophyte that was isolated at 45°C. *Coprinopsis cinerea* which was previously known as *Coprinus cinereus,* and classified as *Psanthyrellaceae* family, is an edible mushroom (Srivilai & Loutchanwoot 2009). *Coprinopsis cinerea* or also commonly known as ink cap mushroom is considered unique organism due to the development of blank ink when it's fruiting body matured, whereas other mushroom species scatter their spores either through air or raindrops (Kim et al. 2017). Due to its well defined and short life cycle (development of mature fruiting bodies in 2 weeks), and easy cultivation in laboratory conditions, *Coprinopsis cinerea* is commonly use as model for studies such as reproduction and development of basidiomycetes (Burns et al. 2010; Kim et al. 2017; Srivilai & Loutchanwoot 2009). Besides that, *Coprinopsis cinerea* was also previously isolated as an endophyte from *Eugenia jambolana* (Yadav et al. 2014), *Panax ginseng* (Park et al. 2012), and *Zea mays* (Renuka & Ramanujam 2016).

5.2   Screening of Metabolites from the Endophytes

Based on the result in Table 4.2, the majority of the identified metabolites produced by the endophytes associated with *Nepenthes ampullaria* was at 30°C compared to 40°C. The identified metabolites can be then categorized into five groups based on their previously reported biological activities. Majority of the identified metabolites were reported to possess antimicrobial activity, followed by antioxidant, anticancer and lastly anti-inflammatory activity.

Matairesinol, a lignan which was previously reported to be isolated from the seeds of *Centaurea scabiosa* (Kumarasamy et al. 2003), and *Centaurea* arenaria (Csapi et al. 2010). Besides that, this lignan is also produced by the fungus of *Monascus kaoling*-fermented rice (Cheng et al. 2010). Matairesinol was reported to have antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Serratia marcescens* (Kumarasamy et al. 2003). This metabolite also has antioxidant activity as it shows prominent scavenging activity in 2,2-Diphenyl-1-picrylhydrazyl (DPPH) antioxidant assay. For example, in the DPPH antioxidant assay carried out in a study by Donoso-Fierro et al. (2009), showed that the antioxidant activity of matairesinol is greater than 60%. In the same study, it was revealed that the antioxidant capability of the metabolite depends on the number and position of the hydroxyl group in the matairesinol, and also the methyl group in the phenolic ring which also influenced the phenolic activity of the metabolite. Not only does matairesinol has the antimicrobial and antioxidant activity, but it also has anti-cancer properties too, where it was tested with MTT assay against skin epidermoid carcinoma (A431), cervix adenocarcinoma (Hamilton et al.), and breast adenocarcinoma (MCF7) cells. It showed significant inhibition on the proliferation for the three cell lines, with IC_{50} values (inhibitory concentration of 50% on the cell proliferation) of 7.51 µM (Hamilton et al.), 19.92 µM (A431), and 36.23 µM (MCF7) (Csapi et al. 2010).

Another lignan metabolite, lariciresinol, which was only identified in the extract from endophyte OP. Lariciresinol was isolated from *Ephedra viridis* (Pullela et al. 2005), *Rubia phillipinensis* (Bajpai et al. 2017), and *Sambucus williamsii* (Hwang et al. 2011). It's reported that this lignan metabolite has antifungal and antioxidant properties. It displayed its antifungal properties against *Candida albicans*, T*richosporan beigelii*, and *Malassezia furfur* (Hwang et al. 2011). Furthermore, the antioxidant activity of lariciresinol was speculated to have strong capacity in scavenging free radicals via either the transfer of the hydrogen atom or the donation of the electron (Bajpai et al. 2017).

Although there's no reported matairesinol and lariciresinol isolated from endophytic fungi, the precursors for the two lignans, pinoresinol have been isolated and identified from *Eucommia ulmoide* Oliv. and its endophytic fungi, *Phomopsis* species (Gao et al. 2018). The pinoresinol can be converted to lariciresinol and secoisolaricirecinol by pinoresinol-lariciresinol reductase (PLR). Then, from secoisolariciresinol to matairesinol by secoisolariciresinol dehydrogenase (SIRD) (as shown in Figure 5.2) (Gao et al. 2018; Teponno, Kusari & Spiteller 2016).
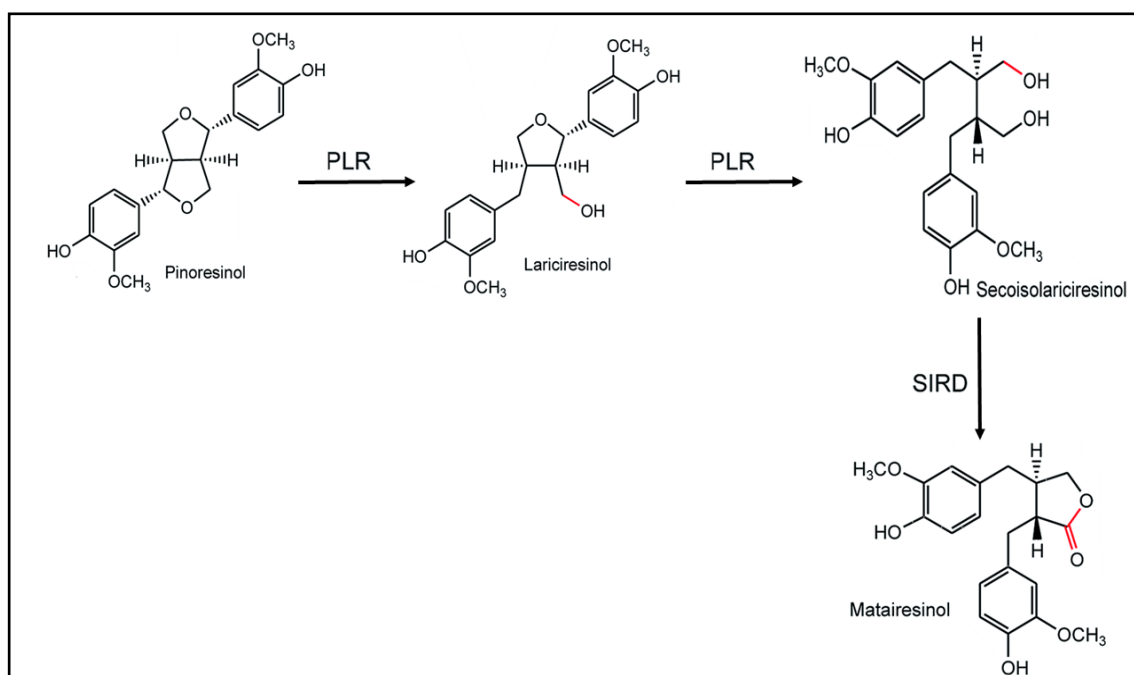

Figure 5.2: Biosynthesis pathway of conversion of pinoresinol to matairesinol.

13-docosenamide or also known as erucamide (Figure 5.3), a metabolite previously reported to be isolated from *Frosskaolea tenacissma* (Kim et al. 2015; Prasher & Dhanda 2017). It was also isolated from endophytic fungi such as *Nigrospora sphaerica* (Prasher & Dhanda 2017) and *Paecilomyces* sp. (Xu et al. 2009). The 13-docosenamide from *Paecilomyces* sp. was reported of showing antifungal activities when tested against *Aspergillus fumigatus*, *Candida albicans*, *Trichophyton rubrum,* and *Cryptococcus neoformans* (Xu et al. 2009). Besides having antifungal properties, this metabolite also has anticancer properties (Xu et al. 2009).

Phenylacetaldehyde (Figure 5.3), an important aldehyde with scents belonging to numerous plants, for examples, roses, petunia, tomato, and grape (Kaminaga et al. 2006). Besides that, this metabolite also has been identified in fungi as volatile oil, for examples, *Ganoderma pfeifferi* (Al-Fatimi, Wurster & Lindequist 2016), *Boletopsis leucomelas* (Nosaka & Miyazawa 2014), and *Pleurotus eryngii* var. *ferulae* (Usami et al. 2014). Furthermore, phenylacetaldehyde is also an essential oil component possessing antimicrobial properties as shown by the volatile oil of *G.pfeifferi* against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* (Al-Fatimi, Wurster & Lindequist 2016; Cecotti et al. 2013). Besides that, phenylacetaldehyde was also considered to be involved in the antioxidant activity of volatile oil of *G.pfeifferi*, which showed strong antioxidant activity (Al-Fatimi, Wurster & Lindequist 2016).

The first isolated sesquiterpenoid antibiotic from *Streptomyces roseogriseus* in 1957 is pentalenolactone F (Figure 5.3) (Koe, Sobin & Celmer 1956). Besides, it also reported being produced by several other *Streptomyces* species such as *S. arenae*, *S. albofaciens*, *S. baarnensis*, *S. omiyaensis*, *S. chromofucus*, *S. viridifaciens*, and *S. griseochromogenes* (Tetzlaff et al. 2006). This metabolite shows to has antibiotic activity against both Gram-positive and Gram-negative bacteria, and also fungi. The inhibition of the enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which caused the irreversible inactivation of GAPDH is the mode of action of pentalenolactone F (Duszenko, Balla & Mecke 1982; Hartmann et al. 1978; Tetzlaff et al. 2006; Zhu et al. 2011).

Coniferyl aldehyde (Figure 5.3), a phenolic metabolite previously isolated from *Eucommia ulmoides*'s bark (Choi et al. 2017). Besides, the coniferyl aldehyde is also known as 4-hydroxy-3-methoxycinnamaldehyde (Kim et al. 2015; Shreaz et al. 2013). This metabolite shows antifungal activity when it was tested against *Candida* species. The results showed the minimum inhibitory concentration in inhibiting 90% growth (MIC_{90}) of the *Candida* sp. for coniferyl aldehyde falls between the range of $100 - 300$ µg/mL (Shreaz et al. 2013). Furthermore, the coniferyl aldehyde is also reported to have the potential in inducing heat shock factor 1 (HSF1), which regulates the response of heat shock. When cells were exposed to stresses such as heat, the HSF1 will be activated, and thus, increasing the heat shock proteins (HSPs) expression. In addition, the HSF1 stability is also regulated by a coniferyl aldehyde, whereby this is mediated by phosphorylation of HSF1. Therefore, it was indicated that coniferyl aldehyde is responsible for increasing the stability of the protein, and also the transcriptional activity by HSF1 (Choi et al. 2017).

Gardenin B (Figure 5.3), or also known as either demethyltangeretin or 5-demethyltangeretin (Kim et al. 2015). It is a polymethoxyflavones metabolite which can be found in citrus peel (Charoensinphon et al. 2013; Wang et al. 2018). Besides, the antioxidant activities of gardenin B were tested by using *Streptomyces cerevisiae* as a model organism when subjecting it to oxidative stress, whereby the tolerance of *S.cerevisiae* toward the stress was improved with the decrease of lipid peroxidation and ROS. It was reported that the antioxidant activity of this metabolite against cadmium sulfate and hydrogen peroxide will require cytosolic catalase and glutathione (Wang et al. 2018). Besides that, it also has anticancer activity, whereby gardenin B has greater growth inhibition effect when tested in human colon cancer as compared with nobiletin and tangeretin (Charoensinphon et al. 2013).
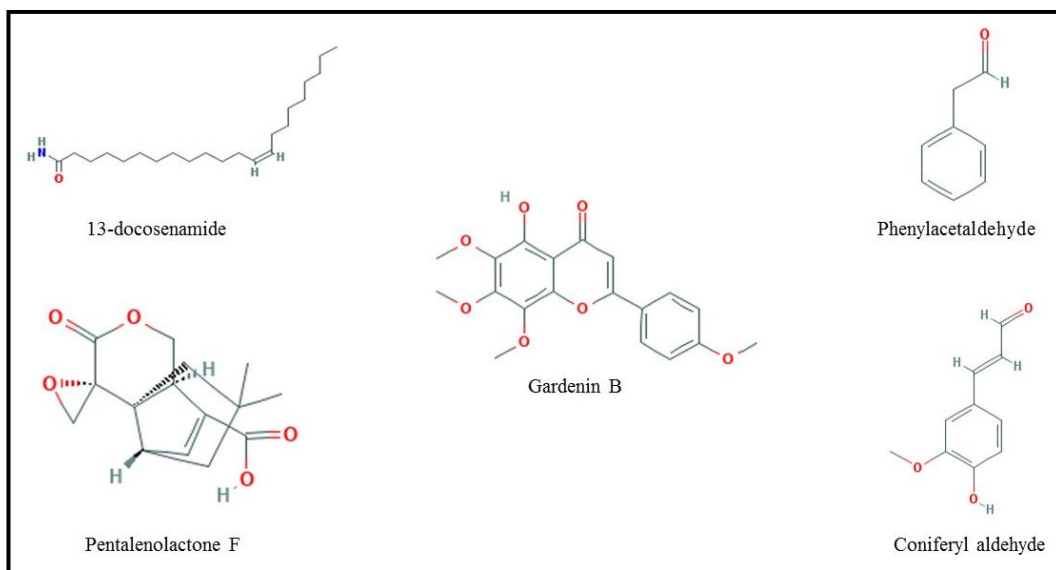
Figure 5.3: The chemical structures for the metabolites with either antimicrobial, antioxidant and/or anticancer activities which were produced by the isolated endophytes (Image is taken from Kim et al. (2015)).

Besides identifying metabolites with antimicrobial, antioxidant and anticancer activity, metabolites with anti-inflammatory activity were also found. For examples, magnoshinin, a neolignan isolated from the buds of *Magnolia salicifolia*, which significantly inhibited the formation of granuloma tissue (Kadota et al. 1987; Kimura et al. 1985). The structure of magnoshinin is as shown in Figure 5.4.

(9Z,12Z,15Z)-octadecatrienoic acid (Figure 5.4), or known as linolenic acid, which was previously identified in *Clerodendrum phlomidis* and the leaf of *Vitex negundo* (Kumar, Kumaravel & Lalitha 2010; Kumaradevan et al. 2015). Besides that, this metabolite was also found in the endophyte, *Aspergillus fumigatus* as volatile constituents (Xu et al. 2014). It was reported that (9Z,12Z,15Z)-octadecatrienoic acid has the properties of anti-inflammatory, anti-arthritic, nematicide, insecticide and also cancer preventive (Kumar, Kumaravel & Lalitha 2010; Kumaradevan et al. 2015).

Figure 5.4: Chemical structure of metabolites with anti-inflammatory activity (Image is taken from Kim et al. (2015)).

Plant growth promoting metabolites were also identified in one of the heat-tolerant endophytic fungi from *Nepenthes ampullaria*. These metabolites were adenine and gibberellin A8-catabolite (please refer to Figure 5.5 for the chemical structure of adenine and gibberellin A8-catabolite).
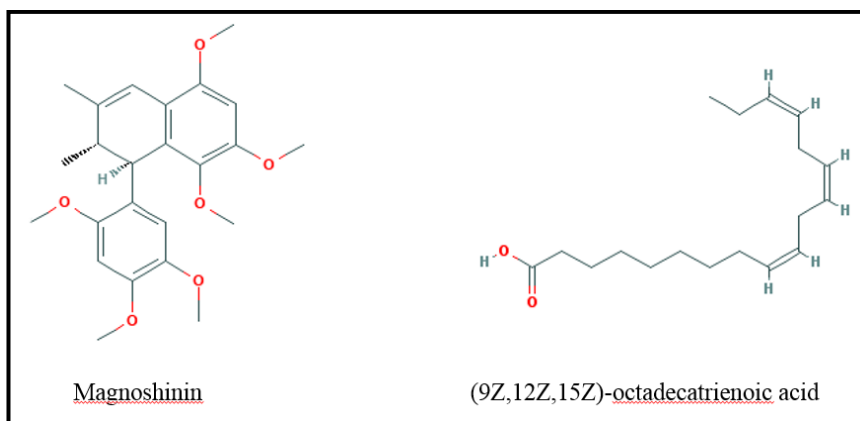
Adenine, which was previously reported to be produced by several endophytic fungi, for examples, *Penicillium* species from *Huperzia serrata* and *Ginkgo biloba* (Ying et al. 2013; Yuan et al. 2014), *Rhodutorula minuta* from the buds of Scots pine (Pirttilä et al. 2004). This metabolite can also be produced by endophytic bacteria such as *Methylobacterium extorquens* and *Pseudomonas synxantha* (Pirttilä et al. 2004). This metabolite has been reported to help in promoting the plant's growth (Hardoim et al. 2015; Pirttilä et al. 2004). This was shown by the mutated *Arabidopsis thaliana* which were able to tolerate oxidative stress due to the mutation in the gene expressing adenine phosphoribosyltransferase (APT1), an enzyme which involves in the conversion of adenine to adenosine monophosphate (AMP). It was shown that the mutation caused a decrease in the expression and enzymatic activity of APT1, but an increase in the level of adenine. Hence, it was suggested that the level of adenine may trigger the responses which can lead to enhanced stress tolerance (Sukrong et al. 2012). Besides that, adenine can also be used to induce growth of plant tissue cultures, however, the mechanism of action of adenine in inducing the growth of the plant is still unknown (George & Sherrington 1984).

Gibberellin A8-catabolite, a gibberellin (GA) is known to be one of the important plant growth hormones from the diterpenoid family. , This hormone is involved in the plant's growth and development, for examples, regulating the gene expression in the germination of seed, stem elongation, development or flower and fruits (Bömke & Tudzynski 2009; Khan et al. 2012a; Olszewski, Sun & Gubler 2002; Pusztahelyi, Holb & Pócsi 2015). Besides that, when plants detected any changes in the environment and to respond to the changes, they alter their physiology and biochemistry rapidly with the presence of gibberellin (Olszewski, Sun & Gubler 2002).

Not only gibberellin were produced by plants, but it can also be produced by fungi along with other important terpenes (Pusztahelyi, Holb & Pócsi 2015). Gibberellin was first identified as secondary metabolites from *Fusarium fujikoroi*, a rice pathogenic fungi that caused the "bakanae" disease (Sawada 1917). Besides that, there were also several species of endophytic fungi that were reported to have the capability of producing gibberellin. *Aspergillus fumigatus* (Khan et al. 2011), *Paecilomyces formosus* (Khan et al. 2012a), *Phoma glomerata* (Waqas et al. 2012), *Chrysosporium pseudomerdarium (Hamayun et al. 2009), Penicillium* species (Leitão & Enguita 2016; Waqas et al. 2012), and *Porostereum spadiceum* (Hamayun et al. 2017) were some examples of endophytic fungi that can produce gibberellin. In addition, *Talaromyces pinophilus*, a fungal endophyte from *Suaeda glauca* Bunge (Khalmuratova et al. 2015), which is the identity for the endophyte OR that was isolated in this study, was reported to be able in producing gibberellin. Furthermore, it was reported that gibberellin produced by *A.fumigatus* when it was exposed to salinity stress act as a similar growth modulator to indole-acetic acid (IAA), abscisic acid (ABA) and cytokinin. Besides that, in the study carried out by Wu et al. (2016), where the effects of high-temperature stress were tested on rice plants. It was reported that the level of cytokinin and ABA increased, while the level of IAA and GA decreases when subjecting the plants to high temperature. As the level of IAA and GA decreases, the fertility of the plant and its grain weight were reduced (Wu et al. 2016).
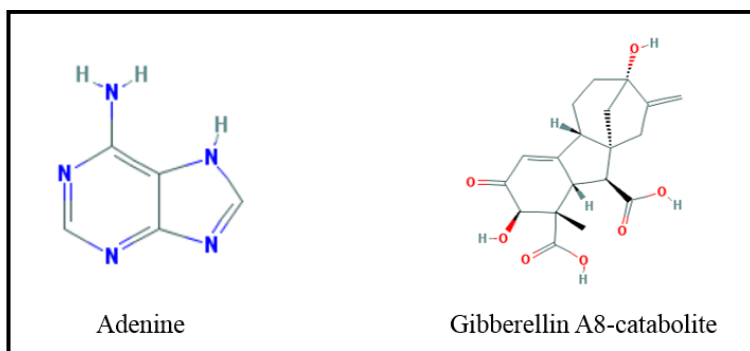
Figure 5.5: Chemical structure of adenine and gibberellin A8-catabolite.

As shown by the results from this study, the metabolites produced by the endophytes associated with *Nepenthes ampullaria* has the potential in helping the plants in tolerating heat stress. For instance, coniferyl aldehyde which was produced by the endophytes isolated from young *Nepenthes ampullaria*. This metabolite is reported to be a potential inducer of heat shock factor 1 (HSF1), which has played the role as the main regulator of the transcription expression of the heat shock proteins (Choi et al. 2017; Dai et al. 2007). The heat shock proteins can be found in all organisms, including fungi. Together with heat shock factor, the heat shock protein plays important role in responding to heat stress (Kotak et al. 2007). For example, *Fusarium oxysporum* produced the heat shock protein within 10 minutes after exposing to heat stress (Freeman, Ginzburg & Katan 1989).

Besides coniferyl aldehyde, there also several of the metabolites with antioxidant activities produced by the selected endophytes. These antioxidant metabolites produced by the fungal endophytes might also play a role in helping to protect the plant cells from oxidative damages due to excess ROS (Huang et al. 2007). The production of ROS is a common process occurred when the plant was experiencing stress such as heat (Hasanuzzaman et al. 2013). In a normal environment, the production of the antioxidant metabolites is sufficient in scavenging the produced ROS. However, the balance between the antioxidant metabolites and ROS are disturbed when the plant is experiencing stress, thus, causing over accumulation of ROS (Hamilton & Bauerle 2012).

Chapter 6 Conclusion and Future Work

This research study has presented (i) the identity of the heat-tolerant endophytic fungi isolated from *Nepenthes ampulllaria*, and (ii) the metabolites produced by the heat-tolerant endophyte under different temperature.

In this study, a total of seventy-three fungal endophytes was successfully isolated from three different parts for both young and old *Nepenthes ampullaria*. The five endophytes isolated from temperature 40°C and 45°C were chosen and identified through molecular technique and they are group under *Dothideomycetes* fungal class, *Talaromyces pinophilus*, and *Coprinopsis cinerea*.

Majority of the identified metabolites from the endophytes were at 30°C compared to 40°C. A total of 14 metabolites were identified produced at both 30°C and 40°C, with the majority of the metabolites having antimicrobial activity, followed by metabolites with antioxidant and anticancer activities. There were also metabolites having more than one biological activity such as matairesinol, which previously reported to have antimicrobial, antioxidant and anticancer activities. Besides that, compound that was reported as a potential inducer for heat shock factor was also identified from the extract of endophyte YL cultured at 40°C. Other than that, two plant growth promoting metabolites, adenine and gibberellin A8-catabolite, were also identified from the endophyte extracts.

To our best knowledge, this is the first study which isolates heat-tolerant endophytic fungi from *Nepenthes ampullaria*, and as well as assessing the metabolites produced by these endophytes at a different temperature. Thus, the *Nepenthes ampullaria* can be used as a model plant for similar studies based on the obtained results from this research study.

As the five endophytes were considered as heat-tolerant fungi, it is possible that these fungi may be responsible for the survival of lowland *Nepenthes* species in a warmer environment. Hence, we would like to suggest to introduce these endophytes to other plant species in order to test out whether they have the capability of conferring tolerance to heat stress. Besides that, determination of the structure of the metabolites by using nuclear magnetic resonance, as well as quantifying the metabolites can be carried out to further confirmed the compounds identity and their concentration at a different temperature.

Furthermore, previous studies showed that inoculating plant species with endophytic fungi can improve the plant's tolerance towards abiotic stress. For examples, when *Cucumis sativus* were grown in high-temperature conditions, better growth qualities were observed in the inoculated plants with the endophyte, *Paecilomyces formosus* LHL10 compared to the wild-type plants (Khan et al. 2012b). In another study, the fungal endophyte, *Chaetomium* species isolated from *Lasiurus scindicus*, is introduced into rice seedlings shows enhancement in the survival percentage of the rice seedlings compared to wild-type rice seedlings (Sangamesh et al. 2018).

Therefore, the heat-tolerant endophytic fungi associated with *Nepenthes ampullaria* could be used as the potential microbes in developing heat-tolerant highland *Nepenthes* species.

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