CHAPTER 1: GENERAL INTRODUCTION

Catharanthus roseus commonly called Madagascar periwinkle is a medicinal plant belonging to the family Apocynaceae (Gajalakshmi et al., 2013). Even though the plant is native to Madagascar; it also could be found in Malaysia and has been called as Kemunting Cina (Ayob & Simarani, 2016) where it is popularly employed in landscaping or gardening due to its colourful flowers. This ornamental plant is also reported to be used as anticancer (Balaabirami & Patharajan, 2012) since it could produce a lot of important compounds, especially Vinca alkaloids such as vinblastine and vincristine (Krishnan, 1995; Manganey et al., 1979). Besides, this plant produces vindoline and catharanthine, which are the major monomer alkaloids as well as a biosynthetic precursor for vinblastine and vincristine (Noble, 1990).

In view of the importance of these alkaloids in the medical applications, several studies have been made to discover the potential source for these metabolites. The plant's associated microorganisms are believed to be able to produce similar metabolites as their host plant. Associated microorganism could be as symbiosis endophytes, are microbes that refer to microorganisms that live inside the tissues of plants without causing any apparent harm or diseases to the host plant (Petrini, 1991; Strobel, 2002). In fact, they promote the host plant's growth and the formation of secondary metabolites related to the plant defence (Petrini, 1991; Chandra et al., 2010).

The microorganisms could produce valuable bioactive compounds with a varied application in both research and applied fields (Ravindra et al., 2014). Endophytes also recognized as rich sources of secondary metabolites of multifold importance (Tan & Zou, 2001) include enzymes and plant growth hormones (Carol, 1988). Some of these metabolites are bioactive compounds that demonstrated potent anticancer, antibacterial and antiarthritis activity.

Fungi are one of the endophytic microorganisms that could be found in a plant. By having a wide spectacular array of shapes, sizes and colours, this species diversity actually remains unexplored (Srinivasam & Muthumary, 2007). Endophytic fungi spend the whole part of their life cycle living symbiotically within the healthy tissues of the host plant (Tan & Zou, 2001; Ravindra et al., 2014). It also has been recognized as one of the important and novel resources of natural bioactive products (Strobel et al., 2004) since most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defence against pathogens (Guo et al., 2008). Some of these compounds have proven useful for discovering a novel drug (Yan et al., 2011). There are many reports that endophytic fungi isolated from a medicinal plant produce a new drug or compound that similar to the host plants. All these findings will help to fill the demands of the drugs. In fact, the manufacturing cost of the drugs from endophytic fungi is cheaper than the production of the plants since it takes a shorter period to produce it.

Breast cancer is a first killer for women in the world followed by cervical and ovarian cancer. World Health Organization (W.H.O) has estimated that by the year 2030, 12 million people will be diagnosed with the breast cancer. Unfortunately, some people refused to get a cancer treatment since they afraid that they could not afford to pay for the high cost. Due to very expensive vinblastine and vincristine used in chemotherapy process for certain types of cancer including breast cancer. Naturally, these alkaloids were produced by the *Catharanthus roseus*. However, this plant takes about one year before it is ready to be harvested and lead the high cost of production. Balandrin and Klocke (1988) reported it needs 500 kg of *Catharanthus roseus*'s leaves to produce 1 g of purified vinblastine and vincristine that cost ranging from 1 mil USD to 3.5 mil USD/kg (Chandra, 2012).

In 1960, vinblastine was introduced to treat certain types of cancer, including breast cancer, testicular cancer and Hodgkin's disease (Armstrong et al., 1964). While in 1963, vincristine was introduced through oxidization of vinblastine to treat leukaemia (Evans et al., 1963). Thus, the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA) as a symbol of giving a hope for cancer patients (Appendix A).

Currently, there are only three reports on these alkaloids produced by the endophytic fungi which were *Alternaria* sp., *Fusarium oxysporum* and unidentified fungi from *Catharanthus roseus* (Guo et al., 1998; Zhang et al., 2000; Yang et al., 2004). An alternative producer of these alkaloids in the short period is required where endophytic fungi could produce vinblastine and vincristine within a month. Thus, this research was carried out in order to find a new endophytic fungus that could be other source of anticancer compounds from the host plant *Catharanthus roseus*.

The objectives of the study are:

1. To isolate and identify the endophytic fungi from *Catharanthus roseus*.
2. To determine the metabolites properties; antimicrobial and hydrolytic enzymes produced by four identified strains of endophytic fungi.
3. To study the phytochemical analysis and antioxidant properties of the endophytic fungi isolated from *Catharanthus roseus*.
4. To compare the effectiveness of anti-carcinogenic alkaloids; vinblastine and vincristine produce by *Catharanthus roseus* and endophytic fungi on breast cell line cancer (MDA-DB 231) through MTT and apotopsis assays.

CHAPTER 2: LITERATURE REVIEW

2.1 Medicinal plant

Medicinal plants were used in many regions, especially in traditional Chinese and Indian medicine for years ago. It is still being used till now for many treatments all over the world. However, researchers always try to find an alternative source that can replace plants since it will become good practices and environmentally-friendly. Song et al. (2005) stated that medicinal plant provides a special environment for endophytes and there are a lot of endophytic fungi with novel bioactive natural products that can be obtained from the medicinal plant (Gomes et al., 2007). Thus, it is known as a good storage for endophytic fungi (Krishnamuthy et al., 2008) which is becoming an important source of various secondary metabolites and bioactive compounds valuable for the pharmaceutical industries (Khan et al., 2010).

Agarwood (*Aquilaria sinensis*) is a fragrant wood that has been used in a traditional Chinese medicine and fragrance additive for over 2000 years (He et al., 2005). It was followed by a few reports on this medicinal plant that has a significant activity of anti-cancer (Gunasekera et al., 1981), antidepressant activities (Okugawa et al., 1996), anti-flammatory activities (Zhou et al., 2008), antimicrobial and antitumor (Cui et al., 2011). All these activities (antimicrobial, anticancer and antimalarial) also found in endophytic fungi from 81 types of Thailand medicinal plants (Wiyakrutta et al., 2004).

2.1.1 *Catharanthus roseus*

Catharanthus roseus (L) G. Don is a herbaceous subshrub also known as Vinca rosea (Akhtar et al., 2007) from a family Apocynaceae and native to Madagascar. According to Nayak and Percira (2006), this plant is native to the Caribbean Basin and has historically been used to treat a wide assortment of disease. However, it is grown

commercially in certain regions, especially Australia, Africa, India and southern Europe for its medicinal uses (Kumar et al., 2013).

C. roseus is observed as a rich source of pharmaceutically important terpenoid indole alkaloids (TIA) where it produced about 130 TIA (Wang et al., 2011) and more than 200 important compounds (Krishnan, 1995). Catharanthine and vindoline are major monomer alkaloids and biosynthetic precursors to the dimeric alkaloids; vinblastine and vincristine (Figure 2.1). These two well-known anticancer drugs used in the treatment of acute leukaemia, Hodgkin's disease (Noble, 1990) and extensively used in modern medicine as potential anticancer compounds (Krishnan, 1995; Jaleel et al., 2008) as an anticancer chemotherapeutic (Pasquier & Kavallaris, 2008).

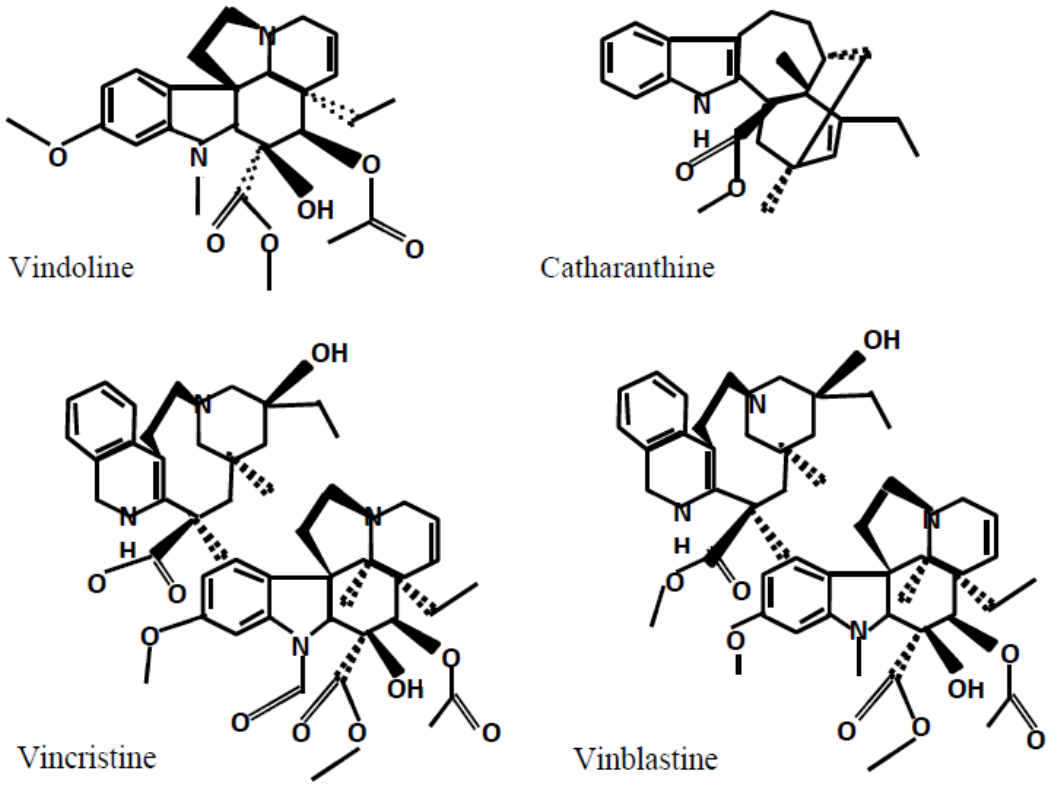


Figure 2.1: The chemical structures of main Vinca alkaloids vinblastine, vincristine and their monomeric precursors; vindoline, catharanthine from *C. roseus* (Mu et al., 2012).

Other alkaloids produced by this plant and commercially been used in the pharmaceutical industry are ajmalicine and serpentine which are known as hypertension and anxiety drugs respectively (Hedhili et al., 2007) (Figure 2.2). The difference between these alkaloids and main alkaloids (vindoline, catharanthine, vinblastine and vincristine) is the source of plant materials. It can be extracted from the roots of *Catharanthus roseus*, instead of leaves part (Iwase et al., 2005).

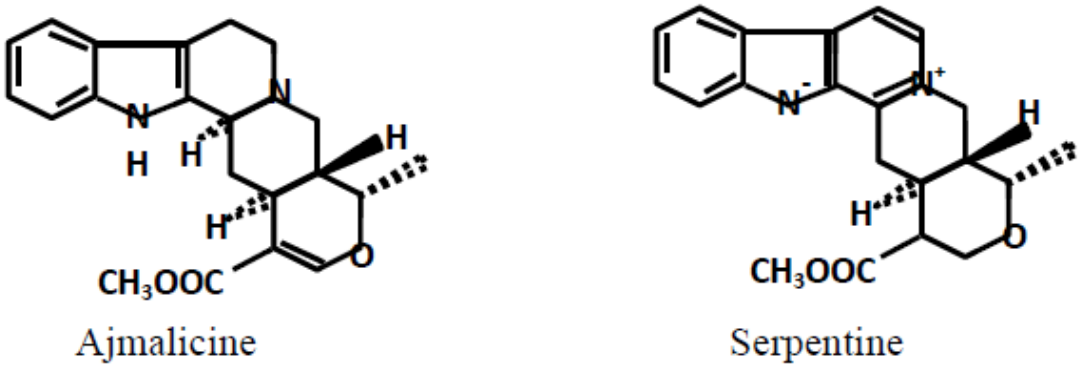


Figure 2.2: The chemical structures of ajmalicine and its oxidized form, serpentine from *C. roseus* (Hirata et al., 1987).

Even though there are more than 2000 TIA synthesized by different families of plant species (Gerasimenko et al., 2002; Barleben et al., 2007; Guirimand et al., 2010), there are no other plant species that can synthesize vinblastine and vincristine since they need a monomer alkaloids as a biosynthetic precursor to the dimeric alkaloids (Van der Hajden et al., 2004) and lead the price of these alkaloids become expensive.

2.2 Endophytes

Endophyte is defined as an organism that colonized intra and intercellular internal plant tissues (Fouda et al., 2015). It refers to microorganisms; fungi, yeast and bacteria. They reside or invade the tissues of living plants without causing any disease or injury to them (Chandra et al., 2010), and have proven to be rich sources of bioactive natural products (Li et al., 2006; Tan & Zou, 2001). It is because they develop a special

mechanism to be inherent in the host plant by penetrating tissues and forms a close association. Kogel et al. (2006) reported this mutualism interaction between endophytes and host plants may result in fitness benefits for both partners. The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine (Strobel & Daisy, 2003). Chandra (2012) also found out that the metabolic interactions between endophytes and its host plant may favour the synthesis of some similar secondary metabolites such as enzyme and antimicrobial properties.

Hence, the study of endophytes including their distributions, biodiversity, biochemical characteristics, secondary metabolites, agents of biological control and other useful characteristics would be found by further exploration of endophytes (Selvanathan et al., 2011) since it is relatively unstudied and potential sources of novel natural products for exploitation in medicine, agriculture and industry (Strobel & Daisy, 2003). According to Guo et al. (2008) and Yan et al. (2011), most endophytes are capable of synthesizing bioactive compounds that might be used as a defence against pathogens and some of the compounds have been proven useful for novel drug discovery.

In fact, endophytes are likely to be a rich and reliable source of genetic diversity and biological novelty (Bacon & White, 2000) and previous research has proven that novel endophytic microbes usually produce novel natural products (Strobel, 2002). It happened when endophytes are able to develop special metabolites to penetrate inside the host tissue, residing in mutualistic association and later their biotransformation abilities open a new platform for the synthesis of novel secondary metabolites (Chandra, 2012). They produce metabolites to compete with the epiphytes and also with

the plant pathogens toward maintaining the critical balance between fungal virulence and plant defence (Chandra, 2012).

2.3 Endophytic fungi

Plant endophytic fungi are defined as the fungi which spend the whole or part of their life cycle by colonizing inter and/or intracellularly inside the healthy tissues of the host plants and causing no apparent harm to the host (Zhang et al., 2006). There are approximately 30,0000 known plant species may host at least one endophytic fungi (Strobel & Daisy, 2003).

According to Dreyfuss and Chapela (1994), there are two basic groups of endophytic fungi; i) Generalist, which is found in high abundance among different plant species. ii) Singleton which is found in low abundance and in specific host plants. This relationship may have begun from the time that higher plants first appeared hundreds of millions of years ago where an evidence of plant-associated fungi has been discovered in fossilized tissues of stems and leaves (Taylor et al., 1999) where it has been reported to be associated with plants for over 400 million years (Krings et al., 2007). It has estimated that there are about 1.3 million species of endophytic fungi all over the world (Dreyfuss & Chapela, 1994). However, it is estimated that there are over 1 million of them are wasting in nature (Petrini, 1991).

Therefore, a lot of research has been done on endophytic fungi in order to keep it useful to human life and nature. Until now, plant endophytic fungi have been recognized as important and novel resources of natural bioactive products with a potential application in agriculture, medicine and food industry (Strobel et al., 2004; Gunatilaka, 2006; Verma et al., 2009) and a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity (Giridharan et al., 2012). In fact, due to the chemical constituents of medicinal plants were complex, more

and more endophytic fungi with novel metabolites of pharmaceutical importance were isolated from medicinal plants and a series of new and useful compounds were obtained (Huang et al., 2007; Guo et al., 2008; Kusari et al., 2009). They also play important physiological (Malinowski et al., 2004) and ecological (Malinowski & Belesky, 2006) roles in plant symbiosis, which protect their hosts from infectious agents and stressful environment by secreting bioactive secondary metabolites (Sun et al., 2014) and a number of bioactive constituents for helping the host plants to resist external biotic and abiotic stresses. On the other hand, the host plants can supply plenteous nutrient and easeful habitation for the survival of endophytic fungi (Rodriguez et al., 2009; Silvia et al., 2007).

Thus, there is no doubt that exploring a variety of new natural products from endophytic fungi of medicinal plants have become a hot spot of new drug research (Cui et al., 2011). Stierle et al. (1993) has become the founder that discovered first bioactive compounds paclitaxel (Taxol) from the endophytic fungi *Taxomyces andreanae* in 1993. Since some endophytic fungi have developed the ability to produce the same or similar bioactive substances as they originated from their host plants (Zhang et al., 2006), it leads the researchers become more interested studying endophytic fungi as a potential producer of a novel and biologically active compounds with antimicrobial, insecticidal, cytotoxic and anticancer activities (Table 2.1). These bioactive compounds could be classified as terpenoids, alkaloids, steroids, phenols, Quinones, lignans, and lactones (Zhang et al., 2006; Xu et al., 2008). However, sometimes endophytic fungi can be passive residents or act as an assemblage of latent pathogens in their host (Ganley et al., 2004).

Table 2.1: Bioactive compounds produced by endophytic fungi isolated from medicinal plants.

Host plants	Endophytic fungi	Bioactive compounds	References
Taxus brevifolia	*Taxomyces andreanae*	Taxol	Stierle et al., 1993
Taxus wallichiana	*Pestalotiopsis microspora*	Paclitaxol	Strobel et al., 1996
Torreya taxifolia	*Pestalotiopsis microspora*	Torreyanic acid	Lee et al., 1996
Taxus brevifolia	*Taxomyces andreanae*	Paclitaxol	Strobel, 2002
Artemisia annua	*Colletotrichum gloeosporioides*	Artimisinin (antimalarial)	Wang et al., 2002
Justicia gendarussa	*Colletotrichum gloeosporioides*	Taxol	Gangadevi & Muthumary, 2008
Nothapodytes nimmoniana	*Neurospora crassa*	Camptotechin	Kusari et al., 2009
Tamarindus indica	*Fusarium solani*	Taxol	Merlin et al., 2012
Eugenia jambolana	*Cephalotheca faveolata*	Sclerotiorin	Giridharan et al., 2012
Phyllanthus amarus	*Trichothecium* sp.	Trichothecinol-A	Tawaare et al., 2014

Chandra et al. (2010) reported that endophytic fungi also promote the growth of the host plants and the formation of secondary metabolites related to plant defence. As a significant of these long-term associations, some of these endophytic fungi may have developed genetic systems that allow the exchange of information between themselves and the higher plants. The exchange would allow the endophytic fungi to be more efficiently coping with the environmental conditions and perhaps increase compatibility with the host plants (Petrini et al., 1992; Strobel & Daisy, 2003).

2.3.1 Metabolites from endophytic fungi

Plants have been used in the traditional medicine of many cultures for years and has been extensively documented (Kapoor, 1990) and it has been estimated about 25,000 species of plants in the world and probably 10 % of them have been tested for some type of biological activities (Verpoorte et al., 1997). However, researchers always try to find alternative resources of bioactive compounds that have a potential to be used in various industries especially in the pharmaceutical industry. Furthermore, by discovering of endophytes as untapped resource of bioactive natural products, it means that all the damage for which plants were earlier being exploited can be synthesized from endophytic fungi associated with the plants, consequently reducing the requirement of any other part of the plant and sparing them from extinction in most places as well as the drugs could be available at low costs (Huang et al., 2007; Kusari et al., 2009). It's supported by Buss and Hayes (2000) where they reported the Mayans already used fungi were grown on roasted green corn to treat intestinal ailments nearly 3000 years ago.

As known, plants and endophytic fungi produce some similar metabolites through mutualism motion (Preeti et al., 2009). Baker et al. (2000) reported that natural products are naturally derived metabolites by-products from organisms, plants or

animals. It's supported by Tan and Zou (2001) where they are found to be a rich source of functional metabolites and fungi have been known to be a major source of active compounds used in medicine (Wiyakrutta et al., 2004). Hence, they represent a relatively unexplored ecological source, and their secondary metabolites are particularly active because of their interactions with their hosts (Krohn et al., 2007; Kharwar et al., 2011).

According to Chandra (2012), endophytes synthesis metabolites to compete with epiphytes with pathogens to colonize the host and also to regulate host metabolism in balanced association by possessing exoenzymes necessary to colonize their host and grow well in the Apo plastic washing fluid of the host. Once the roots are colonized, the association with the host becomes mutualistic. These allow growth of the host and stock the endophytes with enough nourishment to extensively colonize the host's roots (Chandra, 2012). On the other hand, endophytes can produce bioactive metabolites to enhance the growth and competitiveness of the host besides to protect it from herbivores and plant pathogen (Guo et al., 2008; Yan et al., 2011).

First secondary metabolite discovered by the researcher is Taxol produced by endophytic fungus *Taxomyces andreanae* which was isolated from *Taxus brevifolia* (Stierle et al., 1993) known as the pacific yew tree (George et al., 1994). This multi-million drug is proved as an important antimitotic agent who is active against lung cancer, ovarian cancer, breast cancer, head-neck cancer and advanced forms of Kaposi's sarcoma (Merlin et al., 2012) by inhibiting cell proliferation through binding to the β-subunit of the tubulin heterodimers and promoting its polymerization (Kovacs et al., 2007).

This finding also proves endophytic fungi that experience a long-term coexistence in direct contact with the host have exchanged genetic material to share the

bioactive compound with their host (Wang & Dai, 2011; Nadeem et al., 2012). Traditionally, this bioactive compound is produced by a yew tree, but it required 10,000 kg of the bark of this plant to obtain 1 kg of Taxol which is equivalent to 3,000 trees. In addition, each patient needs about 2 g of Taxol to complete course of treatment administered several times over many months (Dewick, 2009). Thus, this finding of secondary metabolites from endophytic fungi has given hope to the pharmaceutical industry.

2.3.2 Antimicrobial

Antimicrobial agents are undeniable as one of the most important therapeutic discovery of the 20th century. However, Peterson and Dalhoff (2004) have found that by way of the 'antibiotic era' which is barely five decades old, mankind is now faced with the global problem of emerging resistance in virtually all pathogens. It's supported by Eloff (2000) where his surveys have revealed that almost no group of antibiotics has been introduced in which resistance had not been observed based on the report by Murray and Lopez (1997). In their report, out of the 39.5 million of death in the developing world, 9.2 million were estimated to have been caused by infectious and parasitic diseases, and that 98 % of death in children in developing countries resulted mostly from infectious diseases.

Thus, there is a need to search for new antimicrobial agents because infections disease is still a global problem due to the development and spread of drug-resistant pathogens (Read et al., 2011). Antibiotics are defined as low molecular weight organic, natural products made by microorganisms (Demain, 1981). The antibiotic resistance of bacterial pathogens has become a serious health concern and encourages the search for novel and efficient antimicrobial metabolites (Liang et al., 2012). Therefore, nowadays, an exploitation of novel classes of antimicrobial metabolites is increasingly noticeable

by investigating the diversity, ecological role, secondary metabolites and bioactivity of the endophytic fungi isolated from various medicinal plants (Vaz et al., 2009). Endophytes are a common source of the antibiotics where the natural products or bioactive compounds from these microbes have been observed to inhibit or kill a wide variety of harmful disease – causing agents, but not limited to phytopathogens as well as bacterial, fungi, virus and protozoa that affect humans and animals (Strobel & Daisy, 2003). Thus, it has been the tremendous increase in concern in screening endophytes for their antimicrobial activities (Liang et al., 2012). Furthermore, antimicrobial metabolites produced by the fermentation of endophytic fungi have many advantages, including sustainable use, no destruction of resources, easy large-scale industrial production and quality control (Liang et al., 2012).

2.3.3 Enzyme

Endophytic fungi yield several metabolites including enzymes with a potential to hydrolyse several plant-derived macromolecules (Zaferanloo et al., 2013). Enzymes are biocatalysts protein in nature which catalyse the biochemical reaction taking place in the living cell without any overall change (Jain & Jain, 2006) where they are obtained from plants, animals and microorganisms (Boboye & Ajay, 2011). Bacteria and fungi are mostly used organism for enzyme production (Maheswari & Sherley, 2012) where it involved in the metabolism of carbohydrate including the trehalose metabolism in microbes either in association with another organism such as plants or when they are unaccompanied (Boboye, 2004). Plus, enzymes produced by microorganisms are so great coupled with their fast growth rates and the ease of enzyme recovery (Boboye & Ajay, 2011).

According to Ajayi and Boboye (2012), microbes have some advance over plants and animals as sources of enzymes, especially endophytic fungi which have the

ability to produce abundant extracellular enzyme such as pectins, cellulase, lipase, amylase, laccase and protease (Sunitha et al., 2013). These fungal enzymes play the key role in the biodiversity and hydrolysis against pathogenic infection and to obtain their nutritional need from the host plants (Sunitha et al., 2013). However, Valipour et al. (2014) reported that enzyme production by bacteria is greater since the growth rate of bacteria is usually higher than fungi.

Different fungal strains produce a diverse enzyme with natural properties suited to the environmental conditions in which they must act (Webster & Weber, 2007). It is hypothesized that their metabolic compatibility has been strongly influenced by natural selection, such as it thrives in a competitive environment (Glover, 1995). Thus, by screening fungal resource for novel metabolites of enzymes and their application, it becomes major goals of current research to accomplish environmentally friendly technological development. (Poosarla et al., 2012). However, bioactive product discovery are depending on the knowledge of habitats when fungi are abundant and the strength of culture collection (Hyde, 2001) as reported earlier by Biabani et al. (1998) that fungi have proven themselves invaluable sources of natural products for agriculture as well as biomedical development for over a half century.

2.3.3.1 Cellulase

Cellulase is a group of hydrolytic (Bhavsar & Bhalerao, 2012) and synergistic enzyme that is used to break up the cellulose into glucose or other oligosaccharide compounds (Chellapandi & Jani, 2008) (Figure 2.3). This enzyme is capable of degrading lignocellulose materials and has a wide range of applications (Bhavsar & Bhalerao, 2012). According to Enari (1983), cellulose the most abundant renewable material in the world and is an important raw material in sugars, biofuels and other industries. Thus, there is a considerable economic interest to develop processes for the

effective treatment and utilization of cellulosic waste as an inexpensive carbon source. Therefore, cellulase provides a key opportunity for exploring the tremendous benefits of biomass utilization (Lynd et al., 2002).

Valipour et al. (2014) reported, it is estimated that approximately 20 % of a billion USD of the world's sale of industrial enzyme consist of cellulase, hemicellulase and pectinase where these enzymes are used in textile, food (Balnave & Graham, 1995), brewery and wine as well as in pulp and paper industries (Sadler, 1993). The most endophytic fungi used as a cellulase producing fungi are *Aspergillus, Rhizopus* and *Trichoderma* sp. (Saito et al., 2003). Generally, the cellulase system in fungi is considered to comprise three hydrolytic enzymes i) endo-(1,4) -D-glucanase (endoglucanase, endocellulase, CMCase) which clears linkage at random commonly in the amorphous parts of cellulose. ii) exo – (1,4)- D-glucanase (cellobiohydrolase, exocellulase, microcrystalline cellulase) which release cellobiose from non-reducing or reducing and generally from the crystalline parts of cellulose. iii) Glucosidase (cellobiose) which relates glucose from cellobiose and short chain cello oligosaccharides (Bhat & Bhat, 1997) (Figure 2.4).

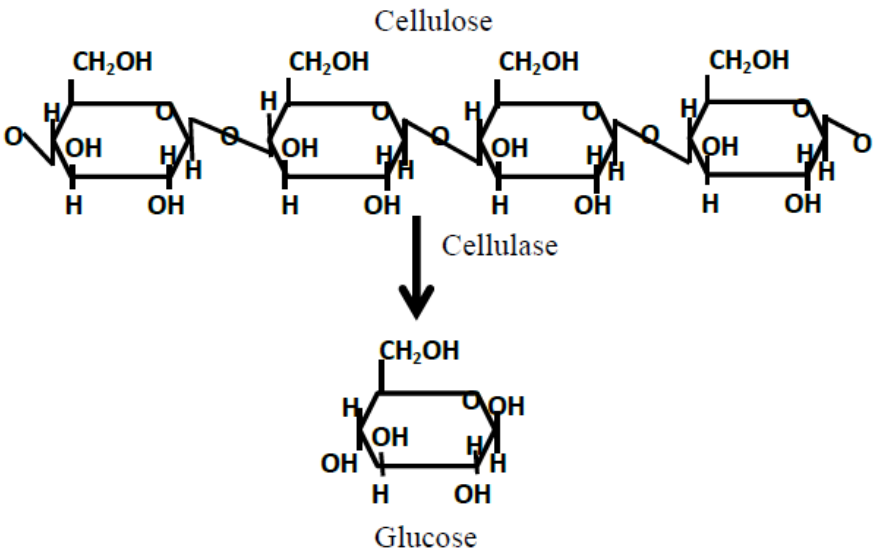


Figure 2.3: The reaction of cellulase enzyme (Held, 2012).

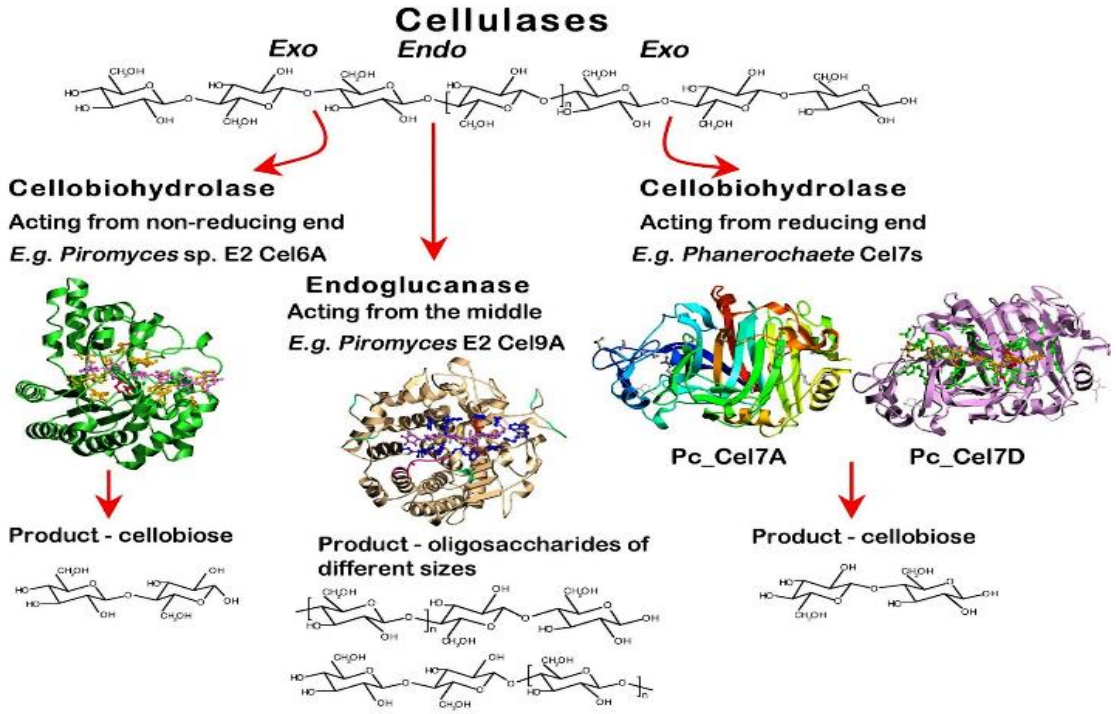


Figure 2.4: The cellulase system in fungi which is divided into three groups (www.emeraldbiology.com).

2.3.3.2 Amylase

Amylase is universal enzyme being widespread in animals, fungi, plants, unicellular eukaryotes and prokaryotes, which is the most important enzymes used in biotechnology (Burhan et al., 2003). It is estimated that approximately 30 % of world enzymes production are amylase (Sivaramakrishnan et al., 2006) and followed by report from Maheswari and Sherley (2012) that this enzyme leading the world's market with a 65 % of enzymes production due to the high demand in various sectors such as food, pharmaceutical, textiles and detergents (Zaferanloo et al., 2013). However, this enzyme is widely used in the industry for starch hydrolysis (Maheswari & Sherley, 2012) (Figure 2.5).

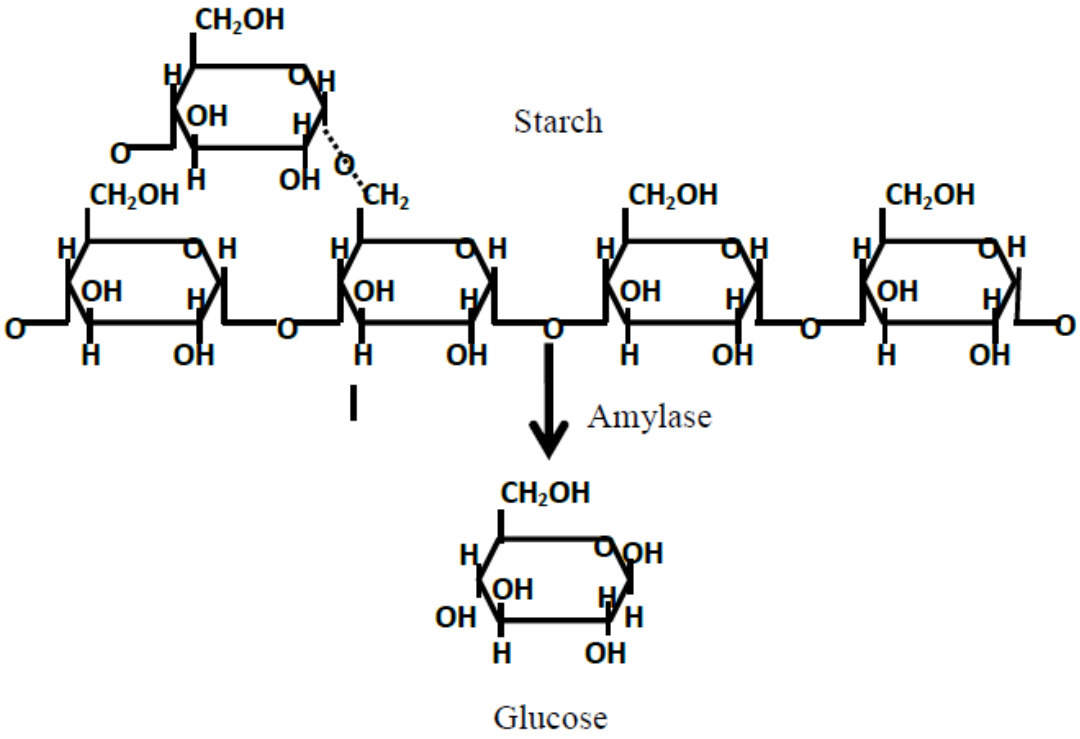


Figure 2.5: The structure of starch polysaccharide and its digestion of glucose by using an amylase enzyme (Held, 2012).

According to Maheswari and Sherley (2012) on their report, this enzyme is used commercially for the production of sugar syrups which is hydrolyzed from the starch which consists of glucose, maltose and higher oligosaccharide. Besides, it also catalyses the endo-cleavage of the α-1,4 glycoside linkages and release of short oligosaccharide with a limitation of dextrin where the most important amylase for industrial and biotechnology applications are glucoamylases and α-amylases (Sivaramakrishnan et al., 2006) (Figure 2.6). In order to meet the demands of this enzyme, low cost medium is required for the production of amylase.

Therefore, this demand has driven the exploitation of endophytes as enzyme sources for promising industrial applications in agriculture, medicine and food industry (Laird et al., 2006). Plant endophytic fungi spend the whole or part of their lifecycle colonizing the inside of healthy tissues of the host plants either inter/ and or intracellular

(Zhao et al., 2010) where fungal sources of amylase are mostly terrestrial isolating such as *Aspergillus* sp. (Zaferanloo et al., 2013).

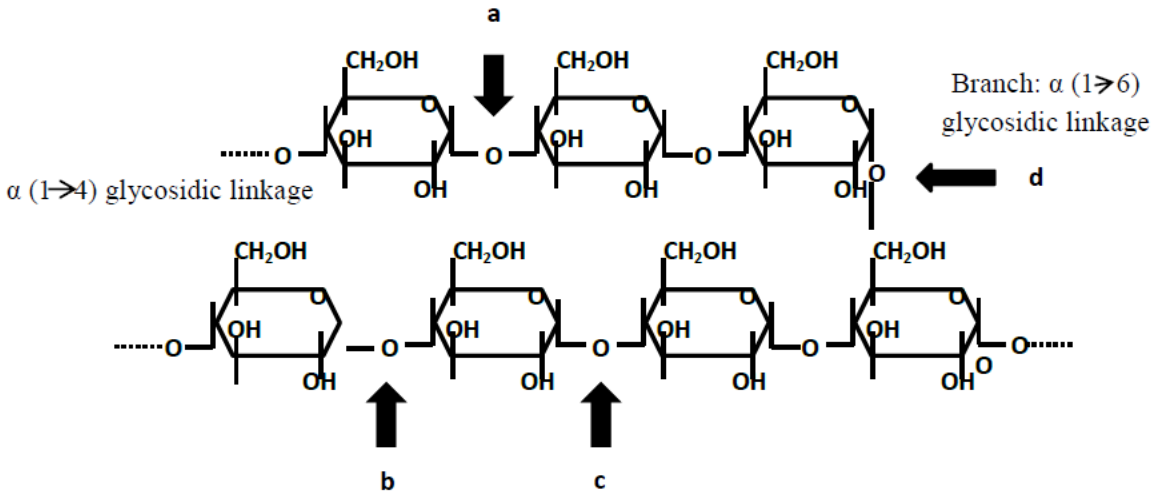


Figure 2.6: The structure of amylopectine with amylase action; a: α-amylase, b: amyloglucosidases, c: β-amylase and d: isoamylase and pullulanase (Miguel et al., 2013).

2.3.3.3 Protease

Protease constitutes one of the most significant groups of industrial enzyme that are now used in a widespread range of industrial enzymes such as detergent, food, pharmaceutical, leather and silk industries (Adinarayana et al., 2003) due to the its physiological functions ranging from generalized protein digestion to the more specific regulated processes (Mussarat et al., 2000). This enzyme hydrolyzes peptide bonds of proteins and breaks them down into polypeptides or free amino acids (Figure 2.7). Thus, it differs in its substrate specificity, catalytic mechanism and active site (Sumantha et al., 2006). However, bacterial protease is the most substantial compared with plant, animal and fungal protease since they possess almost all characteristics desired for their biotechnological application (Rao et al., 1998).

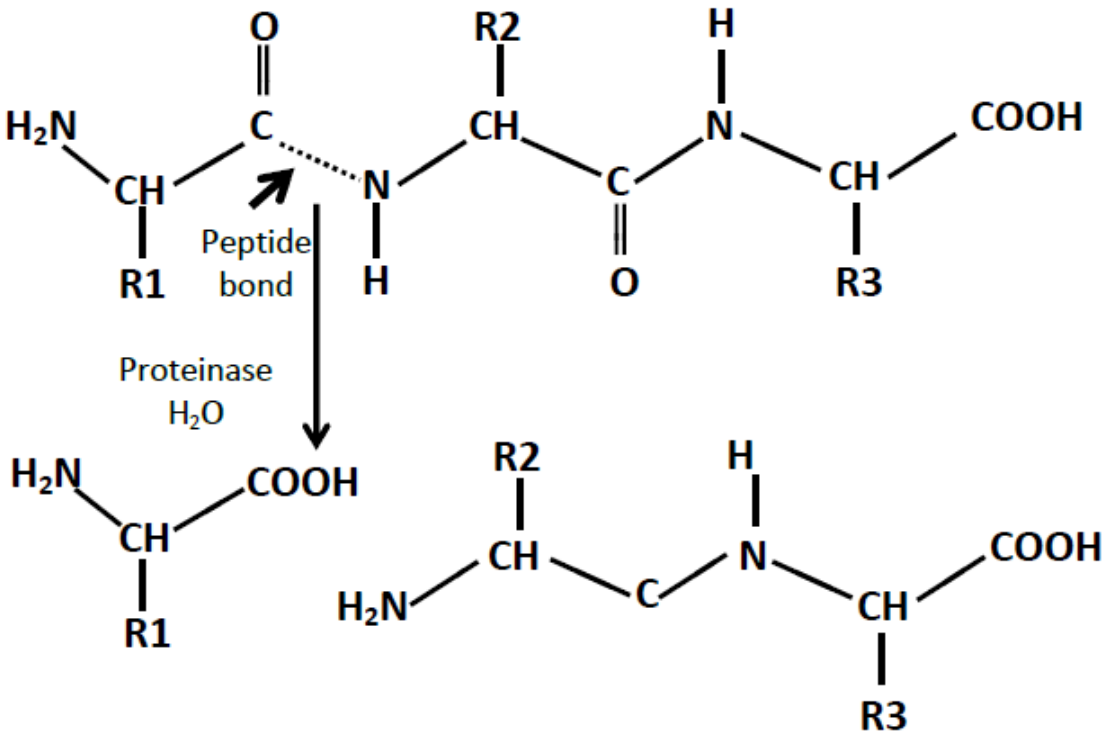


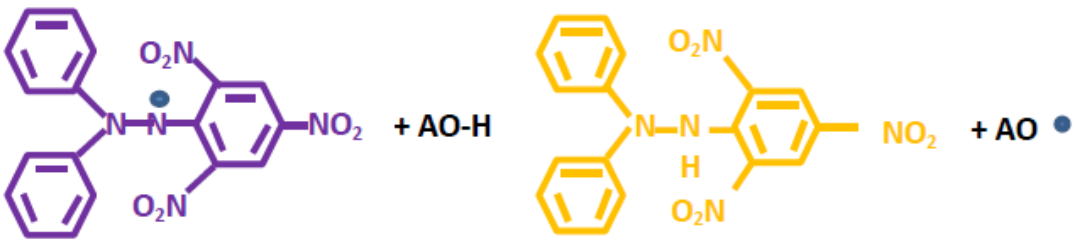
Figure 2.7: The basic chemical structure of a protein's amino acid, which are connected by a peptide bond and the presences of protease to cut the proteins into two fragments (Donohue and Osna, 2004).

2.3.4 Antioxidant

Antioxidant is defined as a compound that can delay, inhibit or prevent the antioxidant of oxidisable materials through scavenging free radicals and diminishing oxidative stress (Dai & Mumper, 2010). This is supported by Halliwell et al. (1995) from their report that antioxidant is any substances that present anti-tumour at low concentration compared to those of an oxidizable substrate significantly delays or prevents oxidation of that substrate. The mechanism of this antioxidant compound is by associating with decreased DNA damage; diminished lipid peroxidation maintained immune functions and inhibited malignant transformation of cells (Gropper et al., 2009). Antioxidant properties can be determined using few methods such as DPPH scavenging activities, ferric reducing antioxidant power and ferrous chelating activities.

However, DPPH scavenging activity is the most popular method to determine the antioxidant properties since it is a simple method (Huang et al., 2005).

DPPH is stable and nitrogen – centred free radical which produces violet colour in ethanol solution (Pushpalatha et al., 2011) with a chemical compound of $C_{18}H_{12}N_5O_6$. According to Huang et al. (2005), by being a stable free radical, the DPPH assay is used to evaluate the ability of antioxidant to scavenge free radicals. It gives reliable evidence concerning the antioxidant ability of the tested compounds to act as free radical scavengers or hydrogen donors. It is supported by Yamagushi et al. (1998) when DPPH free radicals become paired with hydrogen from a free radical scavenging antioxidant, its purple colour fades rapidly to yellow to form reduced DPPH-H (Figure 2.8). Hence, it was reduced to a yellow coloured produced with the addition of the fractions in a concentration (Pushpalatha et al., 2011).



DPPH DPPH-H

Figure 2.8: The mechanism of DPPH reaction on radical scavenging activity where the DPPH compound in purple colour changed into yellow colour as a reaction receiving the hydrogen from the other compound (Perez and Aguilar, 2013).

Therefore, the studies on antioxidant activities are important for pharmaceutical industries. Part of that, the major groups of phytochemicals that have been suggested as a natural source of antioxidant may contribute to the total antioxidant activity of plant material, including polyphenols, carotenoid and traditional antioxidant vitamins such as vitamin C and E (Maisarah et al., 2013). Phenolic is a compound possessing one or more aromatic rings with one or more hydroxyl groups (Dai & Mumper, 2010) and has

been considered as a powerful antioxidant in vitro and proved to be more potent antioxidant than vitamin C, vitamin E and carotenoids (Rice-Evans et al., 1996) where natural phenolic can affect basic cell functions that related cancer development by many different mechanisms. Generally, the mechanisms of phenolic compounds for antioxidant activities are neutralizing lipid free radicals and preventing decompositions of hydro peroxides into free radicals (Li et al., 2006) due to the presence of hydroxyl groups (Pushpalatha et al., 2011).

According to Hodek et al. (2002), first of all in the initiation stage, phenolic may inhibit the activation of pro-carcinogen by inhibiting phase 1 metabolizing enzyme such as cytochrome. Then, it may inhibit the formation and growth of tumours by induction of cell cycle arrest and apoptosis. Malignant cells are characterized by excessive proliferation, inability to terminally differentiate or perform apoptosis under normal conditions and an extended or immortalized life span. The regulation of cell cycle is altered in these cells. Consequently, any perturbation of cell cycle specific proteins by phenolic can potentially affect and / or block the continuous proliferation of these tumorgenic cells (Dai & Mumper, 2010).

2.3.5 Anticancer

Cancer is a main killer disease for all women over the world and more than six million new cases reported every year (Chandra, 2012) (Figure 2.9) where it is a multi-step disease, incorporating environmental, chemical, physical, metabolic and genetic factors which play a direct and/ or indirect role in the induction and deterioration of cancers (Lv et al., 2010). It occurs when cells become abnormal and keep dividing and forming new cells without any control (Chandra, 2012) (Figure 2.10). Nowadays, breast cancer becomes serious threat to women's health in the world (Zhang et al., 2013)

where almost 30 % of early stage breast cancer patients eventually develop recurrence and metaphases (Lv et al., 2010).

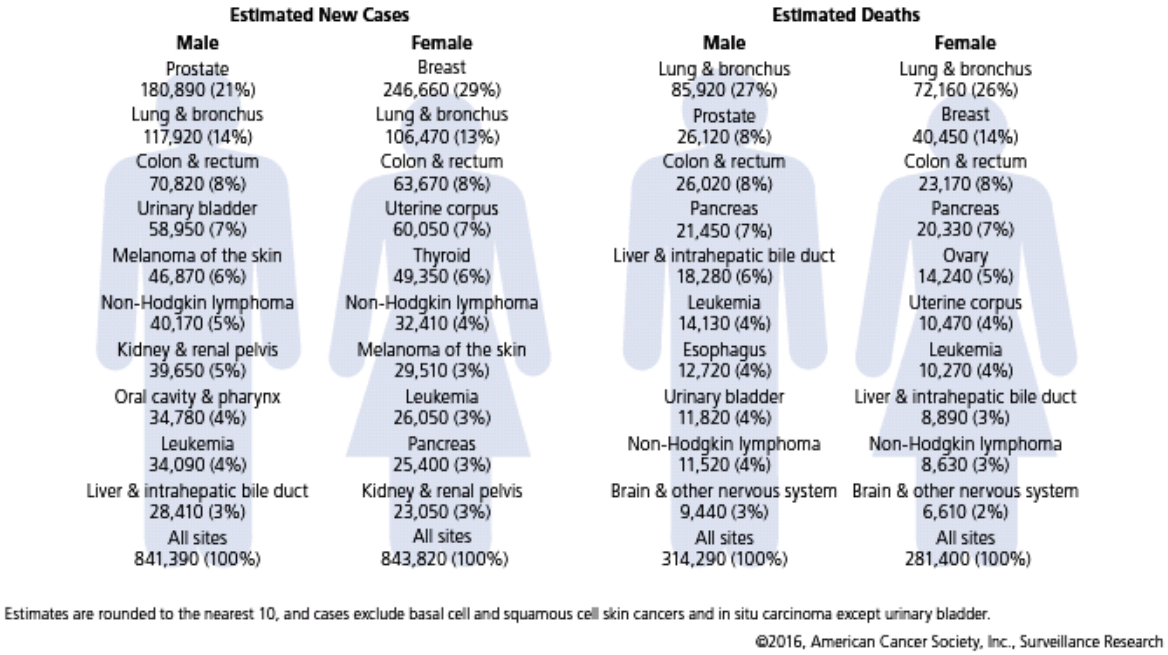


Figure 2.9: The estimated new cases and deaths in America (American Cancer Society-www.m.cancer.org, 2016).

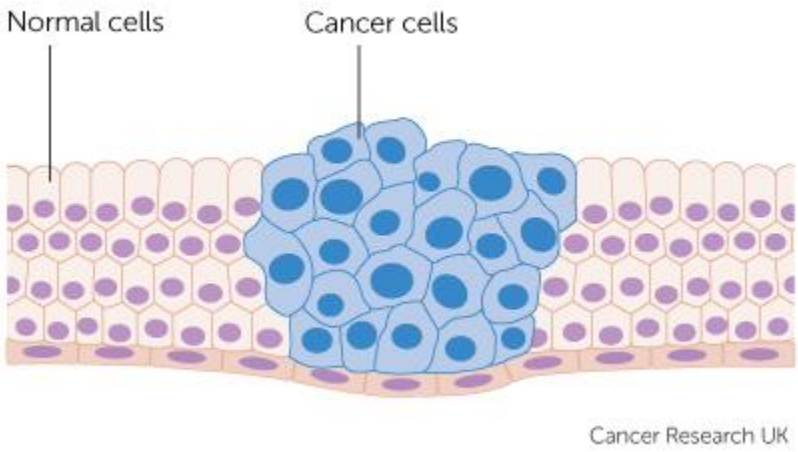


Figure 2.10: The development of cancer cells (Cancer Research UK, 2016).

Thus, the chemotherapeutic drugs are used as a treatment for cancer, which is usually DNA damaging agents and these drugs invariably result in the DNA strand breakage, chromosome breaks and / or gain chromosomes. These DNA damaging

agents has been reported to cause phenotypic abnormalities like spontaneous abortion, congenital malformations and malignant transformations (Sandberg, 1980).

Reasearch showed that the use of these regimens has resulted in the increased survival of patients receiving the treatments. However, chemotherapeutic agents and radiation cause DNA harm not only to the malignant cells but the normal cells also suffer damage to their genome. This damage could be boosted when both cytotoxic drugs and radiation are combined for therapeutic purposes. This may result in the reduction of the latency period of neoplastic transformation and the assessment of chromosomal damage helps to predict the mutagenicity and carcinogenicity of these agents (Devi et al., 2012).

2.3.5.1 Anti-carcinogenic vinblastine and vincristine

According to Tikhomiroff and Jolicoeur (2002), the production of secondary metabolites by plant cells and tissues has become and active field of study because of its potential as a source of valuable pharmaceutical compounds. *C. roseus* (Apocynacea) is found to contain alkaloids about 100 which have been isolated so far (Hughes & Shanks, 2002). *C. roseus* has historically been used to treat a wide assortment of disease since it contains over 130 compounds and many of which have cytotoxicity (Noble, 1990; Zu et al., 2006). Sertel et al. (2011) reported that the attention in this species arises from its therapeutic role as the source of the anticancer alkaloids; vinblastine and vincristine. These Vinca alkaloids are prescribed for a wide variety of cancers including non-small cell lung cancer, breast cancer, bladder cancer, lymphomas and leukaemia (Damen et al., 2010). Barnett et al. (1978) also reported earlier that vinblastine and vincristine are used as major drugs in the treatment of lymphoma and leukaemia since they are potent mitotic inhibitors that have been used clinically in the treatment of a variety of neoplasms (Fergus en et al., 1984) due to the their ability to lower the number of white blood cells (Chandra, 2012).

Thus, the secondary metabolite pathways of these Vinca alkaloids have already been documented (Morgan & Shanks, 1999; De Luca & Laflamme, 2001) and are quite complex (Tikhomiroff & Jolicoeur, 2002). Figure 2.11 showed the pathway of these alkaloids where it starts with a tryptophan and converted to tryptamine. Tryptamine is considered with secologanine to produce strictocidine, the common precursor of all indole alkaloids and divided into three branches (Tikhomiroff & Jolicoeur, 2002).

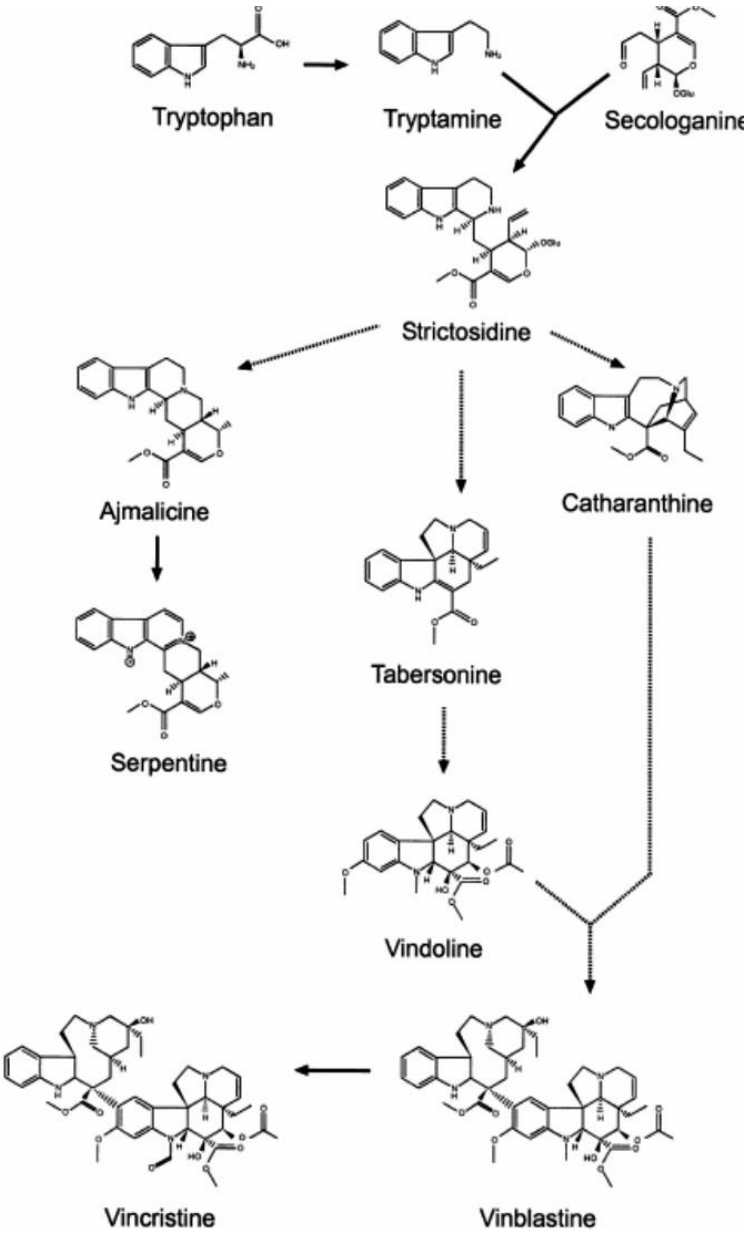


Figure 2.11: The pathway of Vinca alkaloids produced by *C. roseus* plant (Tikhomiroff & Jolicoeur, 2002).

Vinblastine and vincristine are bisindole antitumour alkaloids which are synthesized from the leaves and flowers of this plant and accumulate in very low concentration (Singh et al., 2008). Both of these terpenoid indole alkaloids are two well-known anticancer agents which are derived from the coupling of vindoline and catharanthine monomers (Perez et al., 2002). Report from Potier (1980) and Tam et al. (2010) showed that these alkaloids are commercially produced semi-synthetically by dimerization of their natural precursor's vindoline and catharanthine.

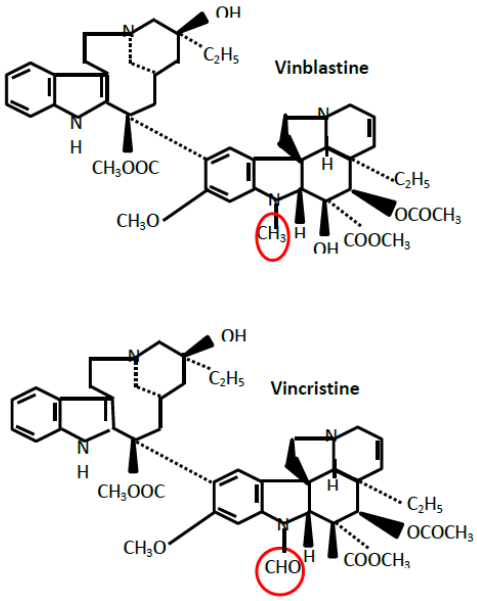


Figure 2.12: The chemical structures of vinblastine and vincristine (www.chm.bris.ac.uk).

Vinblastine has introduced in 1960 and used to treat specific types of cancer, including Hodgkin's disease, breast cancer, testicular cancer and non- small cell living cancer while vincristine is an oxide form of vinblastine and was introduced in 1963 used in the treatment of acute lymphoblastic leukaemia (Armstrong, 1964). Even though both of these alkaloids differ in their molecular structure by only one carbonyl group (Figure 2.12), vinblastine and vincristine exhibit quite different spectra of activity in both their targets of antitumor activity and their site of dose limiting toxicity (Fergusen et al., 1984; Mu et al., 2012).

The anticancer activity of these alkaloids has prevented mitosis in metaphase and they bind to tubulin thus prevent the cell from making the spindles it needs to divide. Their intracellular binding to tubulin with the subsequent dissolution of microtubules and arrest of cells in mitosis is considered necessary to mediate their cytotoxic action (Creasey, 1979). Hence, it is attributed to their ability to disrupt microtubules causing the dissolution of mitotic spindles and metaphase arrest in dividing cells (Jordan & Wilson, 2004; Zu et al., 2004) (Figure 2.13).

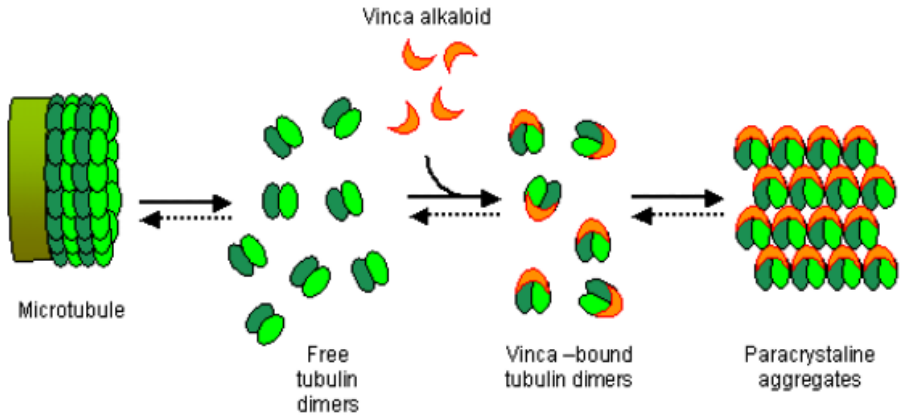


Figure 2.13: The mechanism of destabilization of microtubules by Vinca alkaloids (Turin University-www.flipper.diff.org).

Although these alkaloids have only minor structural differences and behave in the same way at the level of drug tubulin interaction (Himes et al., 1976; Owellen et al., 1977) their toxicity and spectrum of clinical activity differ considerably (Chandra, 2012). Other reports showed that the primary action mechanisms of vincristine are via interference with microtubule formation and mitotic spindle dynamics, disruption of intracellular transport and decreasing tumour blood flow with the latter probably as a consequence of anti- angiogenesis (Damen et al., 2010; Moore et al., 2009; Kavallaris et al., 2008 and Perez et al., 2002)

Even though vinblastine and vincristine have important pharmacological activities, both of them are synthetically challenging (Demain & Vaishnar, 2011)

because these alkaloids are species specific (Van der Hajden et al., 2004) which is only could be produced by *C. roseus* plant only. Thus, alternative sources of these alkaloids always being searched. Guo et al., (1998) first reported endophytic fungi *Alternaria* sp. isolated from the phloem of *C. roseus* that had the ability to produce vinblastine. Later, Zhang et al. (2000) discovered an endophytic fungi *Fusarium oxysporum* from the phloem of *C. roseus* that could produce vincristine and followed by reports from Yang et al. (2004) also found an unidentified vincristine producing fungi from the leaves of *C. roseus*. Plus, Joel and Bhimba (2012) discovered crude extracts of 84 endophytic fungi had anticancer activities against A375 (human malignant melanoma), SW620 (human colorectal adenocarcinoma) Kato III (human gastric carcinoma), HepG2 (human liver hepatoblastoma) and Jurkat (human acute T cell leukaemia).

CHAPTER 3: ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FILAMENTOUS FUNGI FROM *Catharanthus roseus*

3.1 Introduction

Catharanthus roseus commonly called Madagascar periwinkle is herbaceous sub - shrub of latex producing plants belonging to the family *Apocynaceae* (Gajalakshmi, 2013). This plant is a native to Madagascar but also found in Malaysia, where it is called Kemunting Cina and is popularly employed in landscaping or gardening due to its colourful flowers. This ornamental plant is also reported to be used as anticancer where it produced the alkaloids called vincristine and vinblastine (Balaabirami & Patharajan, 2012). The report on the medicinal efficacy of this plant incurs the current surge in its global market, thus the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA). However, this plant takes about one year to harvest and lead to the high cost of production. Alternative producer of these alkaloids in the short period is required.

In view of the importance of these alkaloids in the medical applications, several studies have been made to discover the potential source for this metabolite. The plants associated microorganisms are believed to be able to produce similar metabolites as their host plant. Endophytes are microbes that colonize healthy tissues of the plant for at least part of their life cycle without causing any apparent disease symptoms in their host (Petrini, 1991). These endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan & Zou, 2001) including enzymes and plant growth hormones (Carol, 1988). Some of these metabolites are bioactive compounds that demonstrated potent anticancer, antibacterial and antiarthritis activity.

Endophyte microorganisms that could be found in plants including fungi. By having a wide spectacular array of shapes, sizes and colours, this species diversity actually remains unexplored (Srinivasam & Muthumary, 2007). The capability of these fungi to grow and produce their metabolites within 2 - 4 weeks become more economic. Thus, the objective of this study is to identify fungi strains isolated from a medicinal plant, *Catharanthus roseus* through a molecular technique and observation of morphological characteristics.

3.2 Materials and methods

3.2.1 Plant sample collection, preparation and fungal isolation

The root and leaves of two types of wild grown *Catharanthus roseus* (purple and white) were taken from a peat soil in Alor Setar, Kedah (6.1167 °N, 100.3667 °E) which is the northern part of Peninsular Malaysia. The fungi strains were isolated according to Robert and Terry (1978). The root samples were cleaned under running tap water to remove the soil particles. The cleaned samples of roots and leaves were cut into 1 cm size and surface sterilized was done by immersion in 10 % (v/v) bleach for 15 min. Then, it was rinsed using distilled water for 5 min and blotted dry with a sterile paper. The piece of samples was placed on Malt Extract Agar (MEA: OXOID). Plates were incubated at 25 °C and observed daily (Appendix B). The seven days old of fungal culture were cut into small plug of mycelium using sterile borer and transferred to a new plate of Malt Extract Agar (MEA). Sub culturing was repeated several times in order to get a pure culture. The pure culture was maintained on Potato Dextrose Agar (PDA) slant agar at 4 °C.

3.2.2 Identification of fungi

3.2.2.1 Molecular identification

a. DNA extraction and PCR amplification

Deoxyribonucleic Acid (DNA) was isolated from fresh mycelia taken from the surface plate of each endophytic fungus. The DNA was recovered from scrapped mycelia using i-Genomic BYF DNA Extraction Mini Kit (Intron Biotechnology, Inc.) according to manufacturer's instructions. The quality of genomic DNA was evaluated using a Nanodrops spectrophotometer (Thermo Scientific) and followed by 1 % (v/v) agarose gel electrophoresis.

The Polymerase Chain Reaction (PCR) amplification was conducted using universal primer Internal Transcribed Spacer Ribosomal DNA (ITS rDNA). ITS 1F (forward primer 5' CTT GGT CAT TTA GAG GAA GTA A 3') and ITS 4 (reverse primer 5' TCC TCC GCT TAT TGA TAT GC 3') (White et al., 1990). The 20 µL PCR reaction mixture contained 4 µL of buffer, 1.2 µL of $MgCl_2$, 2 µL of dNTP, 0.6 µL of primer F, 0.6 µL of primer R, 10.4 µL of distilled water and 1 µL of DNA. The PCR was performed using 2720 PCR Thermo Cycler followed the standard procedure: initial denaturation at 94 °C for 1 min followed by 35 cycles for each denaturation (94 °C for 1 min), annealing (51 °C for 1 min) and extension (72 °C for 1 min). The last stage was the final extension at 72 °C for 8 min and cooling to 10 °C. Then, these PCR products were analysed on electrophoresis using 1 % (v/v) agarose gel that was run at 100 v, 400 mA for 30 min. The gel was stained with ethidium bromide and a band was photographed on a UV light transilluminator.

The amplicons were cleaned after ascertaining their integrity before automated sequencing directions. The sequences were then aligned using the data from a GenBank at the NCBI (http://www.ncbi.nlm.nih.gov) and the most identical sequences of fungi were identified.

3.2.2.2 Morphological identification

The isolated fungi were identified based on the morphology and cultural characteristics. It included the colours of spores and growth profiles of the colonies. Meanwhile, the microstructures of each fungus were observed under scanning electron microscope (SEM).

a. Physical assessment

A 1 cm diameter agar disk from the margin of seven days old growing colony of each isolate grown at 25 °C was used to centrally inoculate each replicate. The plates were incubated at the same condition above and the experiment consisted of three replicates (n=3). Assessment of growth was made daily during the seven days incubation period. For each colony, the mean radial mycelia growth was calculated by measuring two different colony radii on each of the three plates. The growth was corrected by subtracting the 1 cm diameter of the original plug of inoculum and plotted against time.

b. Microstructure analysis

The microstructures of spore and hyphae configuration of these fungi were investigated from seven days old fungal culture. The specimen was cut into a small size (3 mm) and soaked in 8 % (v/v) of Glutaraldehyde and Sorenson's Phosphate Buffer (1:1) for 1 h. Then, the samples were washed using distilled water and Sorenson's Phosphate Buffer (1:1). After that the samples were fixed into 4 % (v/v) of osmium and distilled water (1:3) for 14 h. Next, all the samples were dehydrated in an ascending series of ethanol from 10 % to 90 % (v/v) for 15 min each. Then the samples were soaked twice in 100 % (v/v) of ethanol (15 min each) before transferring into intermediate fluids of ethanol – acetone mixture with a ratio of 3:1, 1:1, and 1:3 for 20 min each. Finally the samples were soaked into 100 % acetone four times (20 min each) before proceeding to a Critical Point Drying (CPD) phase. The samples were coated

with gold in an ion-coating and viewed under a Scanning Electron Microscope (JEOL JSM-6400) (Appendix C).

3.3 Results and discussion

3.3.1 Molecular identification

A total of five strains of filamentous fungi were isolated from leaves and roots of both varieties of *Catharanthus roseus.* These endophytic fungi were identified by using molecular technique. The high quality DNA extracted from these fungi was used for PCR by using ITS1 and ITS4 as a primer and a marker (100 bp). The PCR amplification of ITS regions successfully amplified the genomic DNA of the fungi (Figure 3.1). These DNA nucleotide sequences have been identified and deposited in European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena/) with the accession number (Table 3.1). The result showed four different species of fungi were successfully identified and was confirmed through neighbour-joining tree using Molecular Evolutionary Genetics Analysis (MEGA) (Figure 3.2).

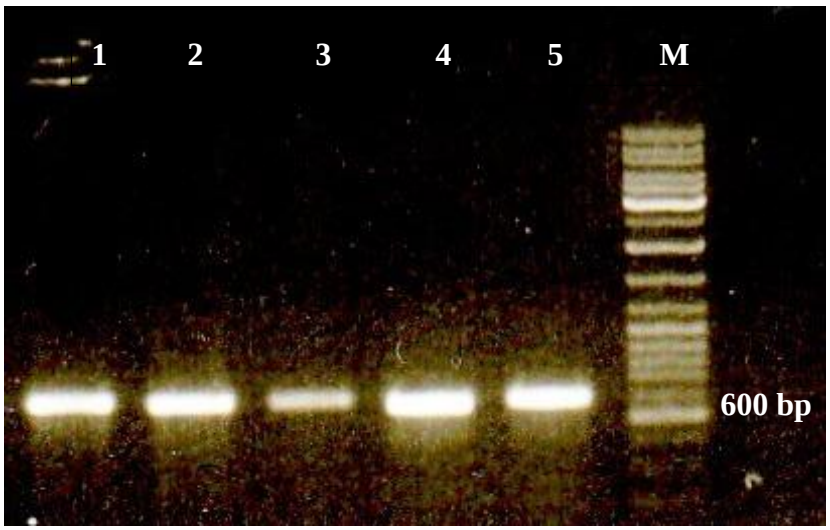


Figure 3.1: The amplification of DNA from pure culture of fungi isolated from *C. roseus* using the primer set ITS1/ITS4. 1 and 3: *Colletotrichum gloeosporioides*, 2: *Macrophomina phaseolina*, 4: *Nigrospora sphaerica*, 5: *Fusarium solani* and M: 100 bp DNA marker.

Table 3.1 Fungal species isolated from different varieties and tissues of *C. roseus* and respective accession number of their ITS sequences.

Strain	Host Plant	Tissues	Species	Molecular size (bp)	Similarity (%)	Accession No. GenBank	Accession No. ENA
1 & 3	*C. roseus* (purple & white)	Leaf	*Colletotrichum gloeosporioides*	530	100	KT 968450.1	HG 938365
2	*C. roseus* (purple)	Root	*Macrophomina phaseolina*	521	100	KC 822431.1	HG 938366
4	*C. roseus* (white)	Leaf	*Nigrospora sphaerica*	240	99	KC 505176.1	HG 938367
5	*C. roseus* (white)	Root	*Fusarium solani*	541	100	JQ 277276.1	HG 938368

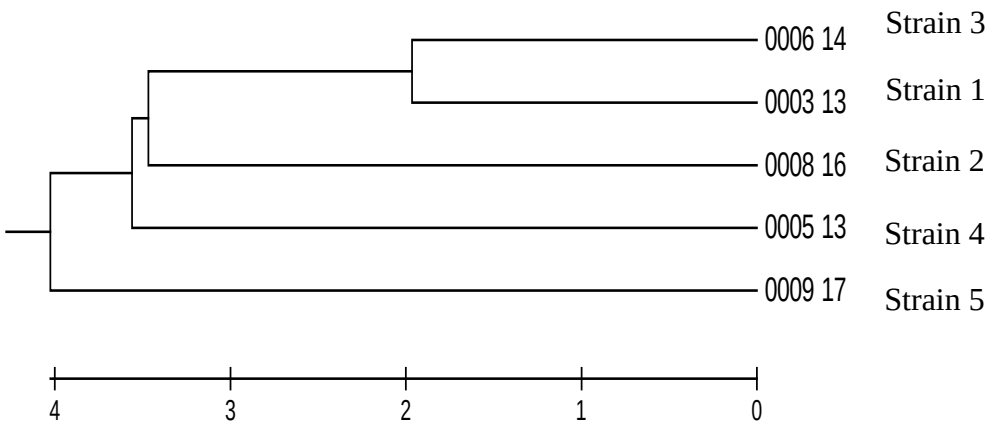


Figure 3.2: Neighbour-joining tree of all endophytic fungi isolated from *C. roseus,* which were produced in MEGA 5. Strain 1 and 3 were *C. gloeosporioides,* and strains 2, 4 and 5 were *M. phaseolina*, *N. sphaerica* and *F. solani* respectively.

3.3.2 Morphological identification

Morphological characteristics had been studied to confirm the identification of these endophytic fungi. Table 3.2 and Figure 3.3 showed the morphological appearances of these endophytic fungi.

Table 3.2: The morphological appearance of endophytic fungi isolated from different tissues and colours of *C. roseus* after a week incubated on Malt Extract Agar (MEA) at 25 °C.

Endophytic Fungi	Host Plant	Tissue	Morphological Appearance
Colletotrichum gloeosporioides	*C. roseus* (purple)	Leaf	White coloured
Macrophomina phaseolina	*C. roseus* (purple)	Root	Light black coloured
Nigropsora sphaerica	*C. roseus* (white)	Leaf	Red to green coloured (turn the media into red coloured)
Fusarium solani	*C. roseus* (white)	Root	White and yellowish coloured

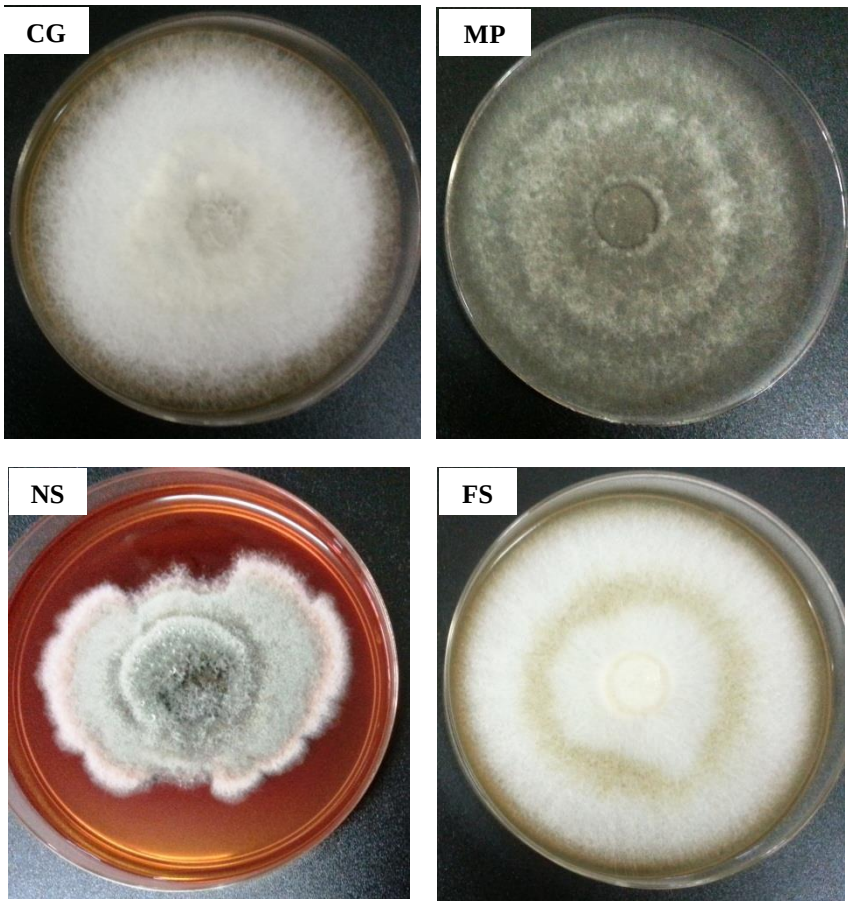


Figure 3.3: Morphological appearances of endophytic fungi isolated from *C. roseus.* CG: *C. gloeosporioides,* MP: *M. phaseolina,* NS: *N. sphaerica* and FS: *F. solani*.

A study on the fungal growth on the MEA plate showed the rapid growth after 3 days of cultivation. The observation was made only for seven days since the mycelia of most strains was compact and covered the entire Petri dish within a week except *Nigropsora sphaerica*. The mycelia growth curves of all strains are shown in Figure 3.4. The microstructures of fungi were observed under scanning electron microscope (SEM) to get the real shape and size of spore and hyphae for each fungus for more identification. The round shape of spores was observed from endophytic fungi *Colletotrichum gloeosporioides, Macrophomina phaseolina* and *Nigrospora sphaerica* while the spores of strain 2 were alike a raspberry shape. Meanwhile, only *Fusarium solani* showed a new moon shape of the spore. All the fungi had a septate hyphae range from 12 µm to 25.6 µm (Figure 3.5).

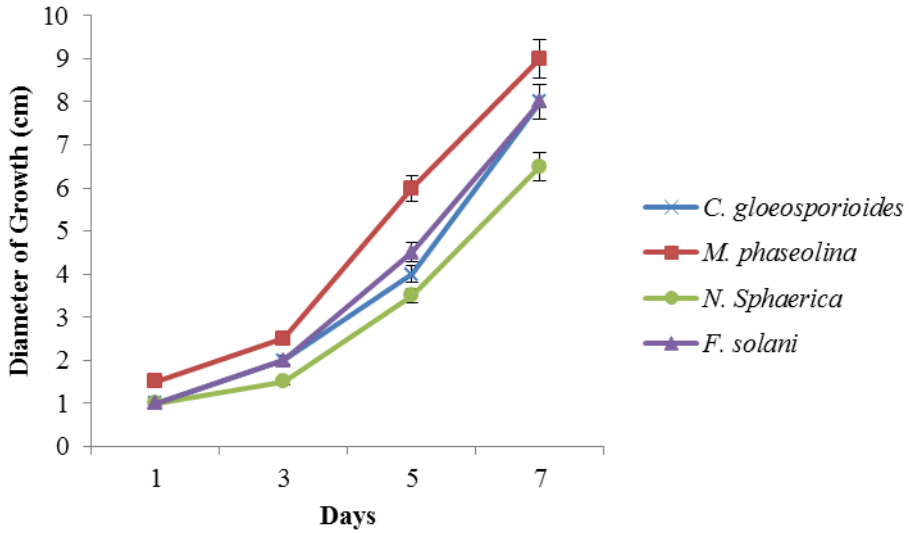


Figure 3.4: The growth profiles of fungi strain isolated from *C. roseus*. All endophytic fungi were incubated for seven days on MEA at 25 °C. Values presented are the means ± SD, (n = 3).

Colletotrichum gloeosporioides has been isolated from both host plant's leaf. Previously, this whitey coloured fungus was found by Sharma et al. (2013) and Koelsch et al. (1995) but as a plant pathogen to *Catharanthus roseus* plant. This species was rarely been found in *Catharanthus roseus* compared to its genus *Colletotrichum*. Xuan et al. (2012) had found *Colletotrichum* sp. in *Cephalotaxus hainanensis;* the Chinese medicinal plant. Both of *C. roseus* and *C. hainanensis* plants produced alkaloids that had been used for anticancer and this fungus is expected could generate constituents which are similar to the host plants (Stierle et al., 1993). *Macrophomina phaseolina* also known as a soilborne fungus (Endraki & Banihashemi, 2010; Arias et al., 2011) was isolated from the root of *C. roseus* (purple). *Nigrospora sphaerica* is the imperfect fungus that was found in *C. roseus* (white) leaf. It has a unique appearance by changing the colour from white to dark green. It is also able to secrete the metabolites that change the plate culture medium from brownish to red colour. This fungus was reported earlier as an endophytic or pathogenic fungus (Zhang et al., 2009) and only a few literatures on this fungus. Meanwhile, *Fusarium solani* with a new moon shape of spore was isolated from a white *Catharanthus roseus'* root.

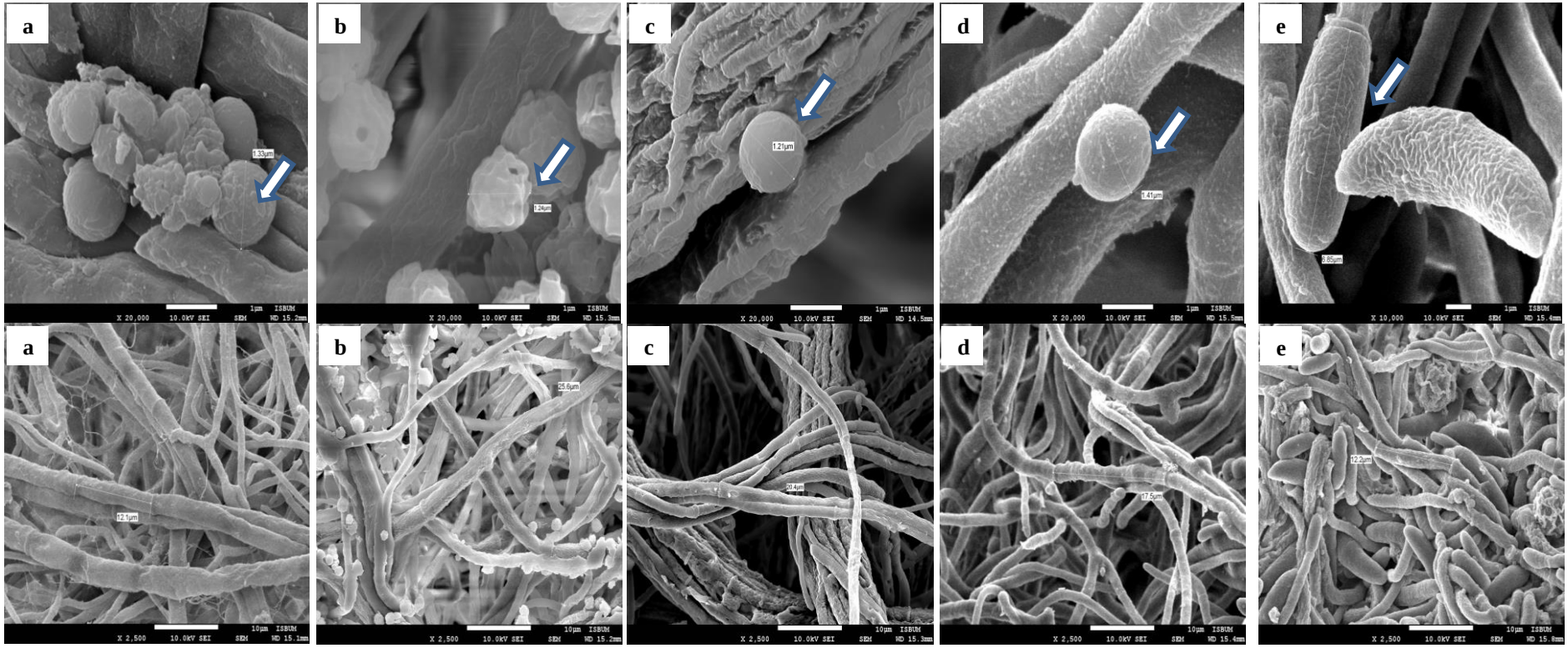


Figure 3.5: The scanning electron micrographs of endophytic fungi (a) strain 1, (b) strain 2, (c) strain 3, (d) strain 4 and (e) strain 5 showed a various shapes of spores (arrow) and hyphae. The spores and hyphae were observed under 20,000 x and 2500 x .

M. phaseolina, *N. sphaerica* and *F. solani* were reported as pathogens of many agricultural crops and plants (Anandi et al., 2005; Siavosh & Seyed, 2012). This *M. phaseolina* causes seedling blight, root rot, and charcoal rot of more than 500 crops and non-crop species. It has a very wide distribution covering most of the tropics and subtropics, where high temperatures and water stress occur during the growing season. Initial infections occur at seedling stage, but remain latent until the plant approaches maturity. Plants may wilt and die. Even though a lot of research were reported on endophytic fungi from *C. roseus* plant, these three strains; *C. gloeosporioides, M. phaseolina* and *N. sphaerica* have become new findings for this host plant (Table 3.3).

According to Dreyfuss and Chapela (1994), there are two basic groups of endophytic fungi; i) Generalist, which is found in high abundance among different plant species. ii) Singleton which is found in low abundance and in a specific host plant. Endophytic fungi often are symptoms symbionts living within the above ground tissues of their angiosperm hosts and are not affected by surface sterilization techniques (Selvanathan et al., 2011). However, in this study, these three fungi may play an important role as endophytes. It is probably due to the flexibility of *C. roseus* in producing antimicrobial activity. Balaabirami and Patharajan (2012) reported that the leaf extract of *C. roseus* gave a positive result for antimicrobial test against *Asperigillus niger*. It was indicated that these fungi helped to synthesize bioactive agent that could be used by plants for defence against other pathogens (Selim et al., 2012).

Thus, when these fungi became endophytes, it is believed to carry out a resistance mechanism in order to overcome a pathogenic invasion by producing secondary metabolites bearing antimicrobial activity (Selim et al., 2012).

Table 3.3: The endophytic fungi isolated from a *C. roseus* plant

Endophytic fungi	References
Fusarium oxysporum	Kumar & Ahmad, 2013
Alternia sp.	Sreekanth et al., 2017
Taldromyces radicus	Palem et al., 2015
Curvularia sp.	Pandey et al., 2016
Chaonephora infundibulifera	Pandey et al., 2016
Pestalotiopsis sp.	Srivinasan & Muthusamy, 2009

3.4 Conclusion

In summary, we successfully identified the putative endophytic fungi isolated from different tissues of wildly grown *Catharanthus roseus* (purple and white) using a morphology and a molecular technique. The ITS 28s ribosomal RNA gene showed that the species were *Colletotrichum gloeosporioides, Macrophomina phaseolina, Nigrospora sphaerica* and *Fusarium solani* based on the sequences from a GenBank of BLAST system. Thus, these endophytic fungi will further be studied to find out the secondary metabolites containing natural products and bioactive compounds that could be produced by these endophytic fungi since they have been recognized as important and novel resources of natural bioactive products with a potential application in agriculture, medicine and food industry (Strobel et al., 2004; Gunatilaka, 2006; Verma et al., 2009) and a rich source of novel organic compounds with interesting biological activities and a high level of biodiversity (Giridharan et al., 2012).

CHAPTER 4: ANTIMICROBIAL AND HYDROLYTIC ENZYME PROPERTIES OF ENDOPHYTIC FUNGI FROM *Catharanthus roseus*

4.1 Introduction

The antibiotic resistance of pathogens has become a serious concern around the world (Aksoy & Unal, 2008) due to the evolution of resistance by existing pathogenic bacteria and fungi to commercial drugs, which is a relevant problem faced by health services (Costelloe et al., 2010). Resistance to drugs used in the treatment of many infectious diseases which caused by bacterial and fungal or parasitic is increasing, further complicating on rendering treatment of critical infectious illness (Ramos et al., 2010). This phenomenon encourages the search for novel and effective antimicrobial metabolites (Liang et al., 2012). According to Levy (2005), increasing prevalence of multi-resistant bacteria has turned the search for new antimicrobial agents an important strategy for alternative therapies useful in the handling of difficult infections.

Natural products remain to be an important source of new pharmaceutical products (Newman & Cragg, 2007). Since some of them are produced by the organisms as a result of selection in favor of improved defense against competing deleterious microorganisms (Ramos et al., 2010). The production of defensive secondary metabolites by them is important due to their original natural function in response to environmental challenges (Lu & Shen, 2004).

Metabolites isolated from the fungal endophytes are good sources of novel secondary metabolic products having diverse structural groups and showing antibacterial, antifungal, anticancer, antiviral, antioxidant, insecticide, antidiabetic and immunosuppressive activities (Demain, 1999; Tan & Zou, 2001). Therefore, tremendous increase in interest on screening endophytes for their antimicrobial

activities (Liang et al., 2012). Furthermore, antimicrobial metabolites produced by endophytic fungi have many advantages, including sustainable use, no destruction of resources, easy large-scale industrial production and quality control (Liang et al., 2012). (Table 4.1).

Other than that, endophytic fungi also yield enzyme; a biocatalyst protein in nature which catalyse the biochemical reaction taking place in the living cell without any overall change (Jain & Jain, 2006) with a potential to hydrolyse several plant-derived macromolecules (Zaferanloo et al., 2013). These enzymes are obtained from plants, animals and microorganisms (Boboye & Ajay, 2011). However, bacteria and fungi are mostly used microorganism for enzyme production (Maheswari & Sherley, 2012) due to their fast growth rates and the ease of enzyme recovery (Boboye & Ajay, 2011). They also involved in the metabolism of carbohydrate including the trials metabolism in microbes either in association with another organism such as plants or when they are unaccompanied (Boboye, 2004). Therefore, the objective of this research is to determine the antimicrobial properties of these endophytic fungi isolated from *Catharanthus roseus* plant and its hydrolytic enzymes produced that functionalize as degradation materials to penetrate plant tissues as endophytes.

Table 4.1: The secondary metabolites (antibiotic) produced by endophytic fungi from medicinal plants.

Host plants	Endophytic fungi	Bioactive compounds	References
Gingko biloba	*Chaetomium globosum*	Chaetoglobosins	Zhang et al., 2013
Cinnamomum leanehirae	*Fusarium oxysporum*	Beauvericin	Wang et al., 2011
Kennedia nigricans	*Streptomyces* sp.	Manumycin	Castillo et al., 2002
Artemisia mongolica	*Colletotrichum gloeosporioides*	Colletotric acid	Zou et al., 2000
Artemisia annua	*Colletotrichum* sp.	Artemisinin	Lu et al., 2000
Tripterygium wilfordii	*Cryptosporiopsis quercina*	Cryptoxin	Strobel et al., 1999
Taxus brevifolia	*Pestalotiopsis microspora*	Pestalotiopsins	Pulici et al., 1996
Tripterygium wilfordii	*Fusarium* sp.	Subglutinol A & B	Lee et al., 1995

4.2 Materials and methods

4.2.1 Cultivation and extraction

The preparation of crude fungal extract (CFE) was carried out following Wiyakrutta et al. (2004) with a minor modification where two types of extracts have been prepared; crude supernatant extract (CSE) and crude mycelia extract (CME) (Figure 4.1). Briefly, six pieces of the plug (1 cm) of each seven days grown fungal culture were inoculated into yeast extract sucrose broth (YESB) medium and incubated for 21 days at 25 °C under static condition. Then, the grown culture was harvested using filter separation methods. In order to prepare crude supernatant extract, the cell free

filtrate was extracted thrice with a dichloromethane (200 mL) and evaporated to dryness using a rotary evaporator. Meanwhile, for the preparation of crude mycelia extract, the mycelia biomass were freeze-dried and extracted twice with a mixture of dichloromethane: methanol (1:1, v/v) for 1 h. The extracted mycelia mixtures were then air dried and weighed before kept in the air tight container until further used. Both of the samples, crude mussel extract and crude supernatant extract were then dissolved separately in dimethylsulphoxide (DMSO) to obtain 1 g/mL of concentration.

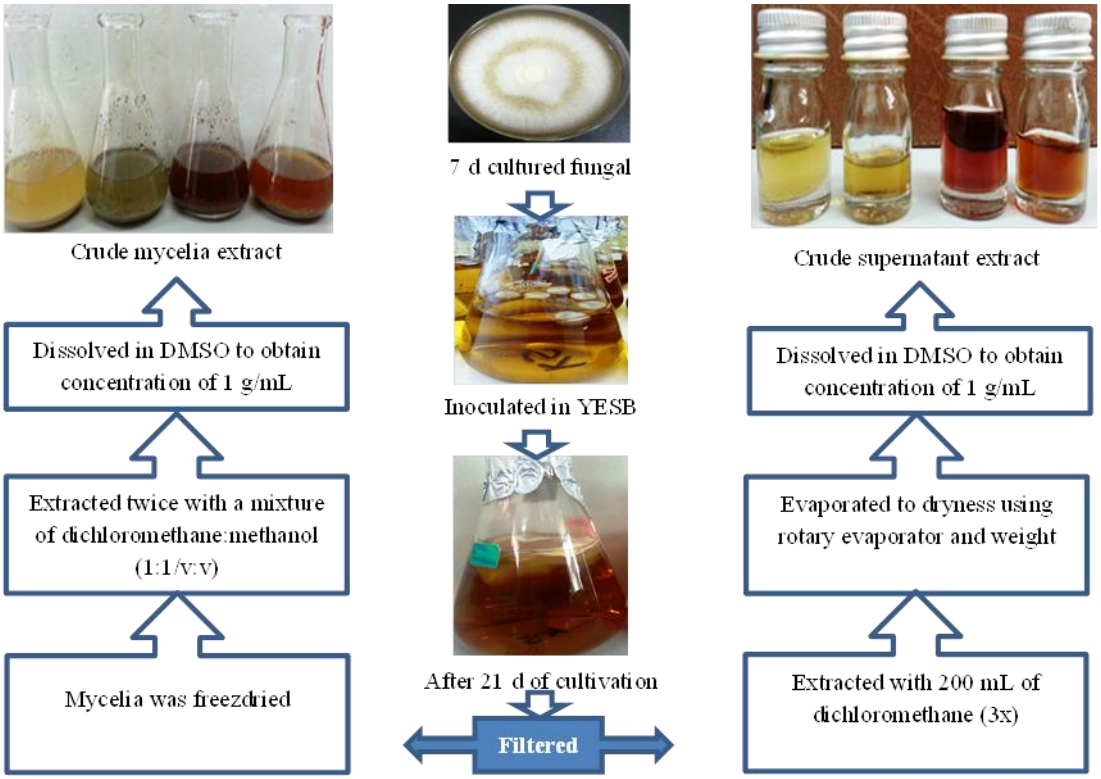


Figure 4.1: Schematic digram of he preparation of crude fungal extract (crude mycelia and crude supernatant extract).

4.2.2 Preparation of microorganisms

All pathogens for antimicrobial test were supplied by Department of Microbiology, Institute of Biological Sciences, Faculty of Malaya, Kuala Lumpur, Malaysia.

3.5 Fungal strain

Aspergillus niger (plant pathogen) was cultured in a Petri dish contained Malt Extract Agar (MEA: OXOID) and incubated at 25 °C for 7 d. Meanwhile, *Candida albicans* (human pathogen) was cultured on Yeast Extract Peptone Dextrose agar (YEPD; 3 g of yeast extract, 10 g of peptone, 20 g of dextrose, 15 g of agar, and 1 L distilled water) and incubated at 25 °C for 7 d. After the incubation period, plates were stored in the 4 °C cool room until further use.

4.2.2.1 Bacterial strain

Staphylococcus aureus (Gram-positive) and *Escherichia coli* (Gram-negative) were cultured on Nutrient Agar (NA: OXOID) and incubated at 37 °C for 24 h. After incubation, these bacteria were stored in the 4 °C cool room until further use.

4.2.3 Determination of antibacterial properties

Four fungi strains had been identified earlier (*C. gloeosporioides*, *M. phaseolina*, *N. sphaerica* and *F. solani*) were tested for the antibacterial properties against *S. aureus* and *E. coli*. For this test, each fungal strain was prepared in three different samples; i) fungal plug, ii) crude mycelia extract and iii) crude supernatant extract. Then all these samples were cultured in different NA plate which has been lawn with *S. aureus* and *E. coli* separately.

For the test using fungal plug samples, 1 cm diameter of the plug was taken from a seven days of culture. While above test for both samples of crude mycelia and crude supernatant extracts was conducted using the Kirby-Bauer disk diffusion technique (Bauer et al., 1966). It was prepared by using sterile disc that eventually had been added 20 µL of extract with different concentrations (1, 5 and 10 mg/mL). Erythromycin (15 µg) disc was used as a positive control while distilled water as a negative control (Figure 4.2). All plates were incubated at 37 °C for 24-48 h. After an

incubation period, plates were observed for zones of inhibition (ZoI) by using Equation 4.1.

$$ZoI = (D - d) \quad \text{(Equation 4.1)}$$

Where,

D= diameter of colony + clear zone

d= diameter of colony

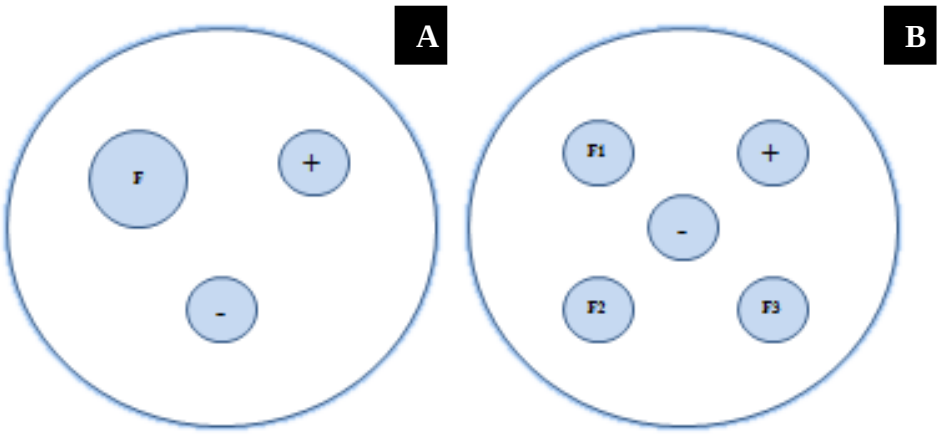


Figure 4.2: The design of plates for antibacterial test against *S. aureus* and *E. coli* using fungal plug samples (A) and crude fungal extract (mycelia and supernatant) with different concentrations (B). All plates were incubated at 37 °C for 24-48 h. (F) Fungal plug; (F1, F2 and F3) disc with extract samples 1, 5 and 10mg/mL, respectively; (+) erythromycin disc as a positive control and (-) distilled water disc as a negative control.

4.2.4 Determination of antifungal properties

For antifungal properties, all fungi strains were tested against *Aspergillus niger*; a filamentous fungus (multicellular) and *Candida albicans*; a yeast (unicellular) using a confrontation culture method (Dennis and Webster, 1971). A one cm of the fungal plug of both sample and pathogenic fungal was placed on the same plate contained MEA and YEPD agar medium for *A. niger* and *C. albicans* respectively (Figure 4.3). The plates were incubated at 25 °C. After 7 days, the plates were observed for the antagonistic on

the inhibition of mycelia growth. The percentage of inhibition (POI) of mycelia growth was calculated according to Fokkema (1973):

$$POI = \frac{(R_1 - R_2) \times 100}{R1} \quad \text{(Equation 4.2)}$$

Where,

R_1 = radius of the pathogen away from the antagonist

R_2 = radius of the pathogen towards the antagonist

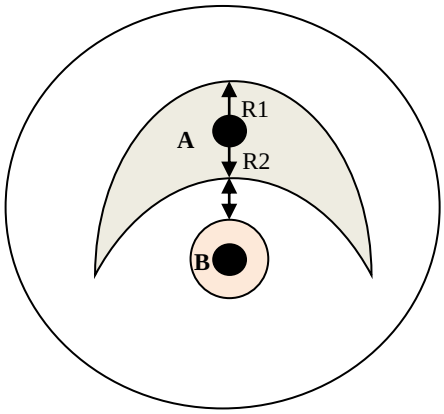


Figure 4.3: The design of plates for antifungal test against *A. niger* and *C. albicans* using confrontation culture method. (A) pathogenic fungal plug samples and (B) antagonist; tested fungal plug sample (B). All plates were incubated at 25 °C for 7 d. R1 and R2 are radius of pathogen.

4.2.5 Hydrolytic enzymes test

The endophytic fungi isolated from a *Catharanthus roseus* were qualitatively tested in the presence of its hydrolytic enzymes; cellulase, amylase and protease. The enzyme productivity of these fungi was expressed as the diameter of clear zone performed from the colony:

$$EA = (D - d) \quad \text{(Equation 4.3)}$$

Where,

D= diameter of colony + clear zone

d= diameter of colony

4.2.5.1 Cellulase

The qualitative test of cellulase for endophytic fungi isolated from a *C. roseus* was carried out according to Stewart et al. (1982). The media were prepared by using CMC medium (10 g of carboxymethyl cellulose, 6.5 g of sodium nitrate, 6.5 g of dipotassium phosphate, 0.3 g of yeast extract, 6.5 g of potassium chloride, 3 g of magnesium sulphate heptahydrate, 17.5 g of agar and 1 L of pure water; pH 6). The fungal plug with one cm sized was inoculated onto this medium for 5 d at 28 °C. To test the presence of enzyme, the plate was stained by using 1 % (w/v) of Congo red for one hour. Then, 1 M sodium chloride was introduced on the plate for 20 min for washing the previous staining. The enzyme prodictivity was determined when the yellow colour appeared around the fungal colonies on the plates and indicate the cellulase activity.

4.2.5.2 Amylase

The qualitative test of amylase for endophytic fungi isolated from a *C. roseus* was carried out according to Namasivayam (2009). Briefly, 1 cm of the fungal plug was inoculated onto starch agar medium (3 g of beef extract, 10 g of soluble starch, 12 g of agar and 1 L of purified water; pH 6). After 4 d incubated at 32 °C, the plate was tested for the presence of amylase by flooding 1 % (w/v) of iodine solution onto the fungal growth. The positive result was indicated by the formation of a clear zone on the plates.

4.2.5.3 Protease

The qualitative test of protease for endophytic fungi isolated from a *C. roseus* was carried out according to Hasan et al. (2013). A plug of fungus (1 cm) was placed onto a plated contained 5 g of peptone, 3 g of beef extract, 5 g sodium chloride, 15 g of agar and 1 L of distilled water with a pH 6. The plate was then incubated at 28 °C for 7 d before checking with a mercuric chloride reagent and the clear zone appeared showed a positive result for this enzyme productivity.

4.2.6 Statistical analysis

All data were analysed using IBM SPSS Statistics, Version 20 (IBM Corp., Armonk, New York).

4.3 Results and discussion

4.3.1 Antimicrobial properties

Four endophytic fungi associated with a *C. roseus* plant were tested for antimicrobial properties; antibacterial and antifungal activities. Figure 4.5 showed the overall results of the antibacterial properties of three different sample preparations of these endophytic fungi. Eventhough previous research showed a positive result for these fungal strains (Table 4.2), but through this test, it can be seen that only a plug of *N. sphaerica* and CSE of *F. solani* were positive against *S. aureus* with the MIC of 1 mg/mL with the inhibition zone of 0.1±0.05 cm (Figure 4.4). However, none of these endophytic fungi were significantly positive against *E. coli* (Table 4.3).

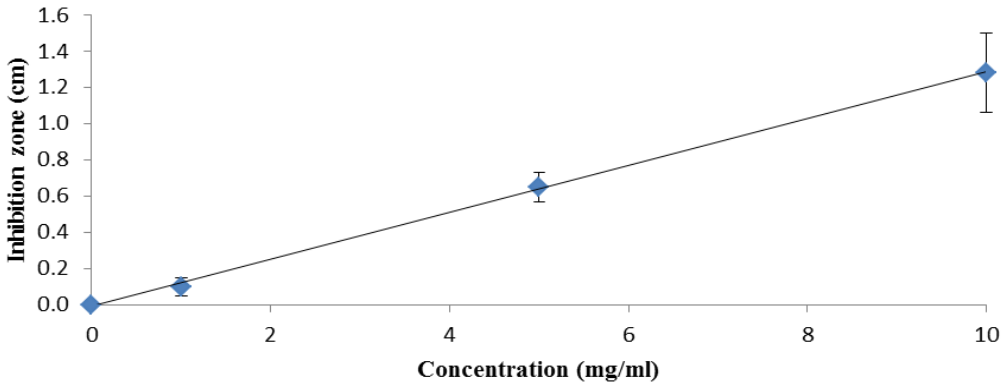


Figure 4.4 The minimum inhibitory concentration (MIC) of a crude supernatant extract *F. solani* isolated from a *C. roseus* against *S. aureus*.

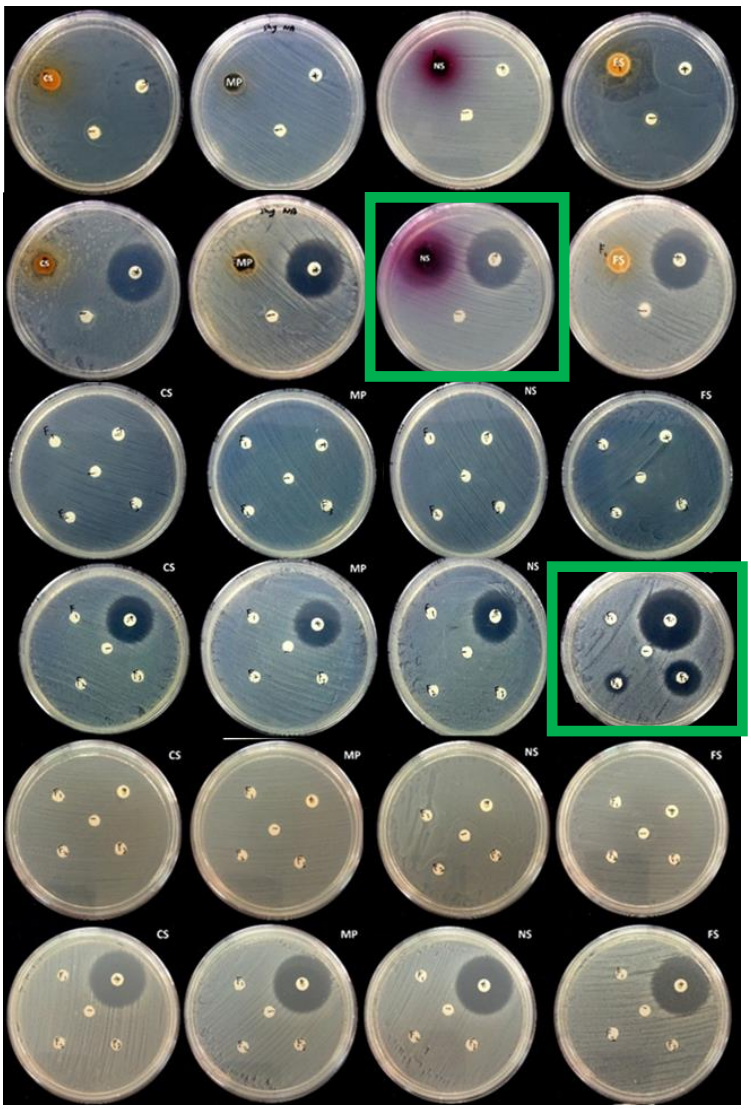


Figure 4.5: The antibacterial properties of endophytic fungi isolated from a *C. roseus*. All fungi were tested against pathogenic bacteria *E. coli* (row 1) and *S. aureus* (row 2) with a different samples; fungal plug (row A), crude supernatant extract (row B) and crude mycelia extract (row C). All plates were incubated for 48 h at 37 °C. The green boxes were the only plates that positive for antibacterial test against *S. aureus* which were *N. sphaerica* and *F. solani*. Positive control was erythromycin (+) and control distilled water (-). From the left; CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

Table 4.2: Previous studies on antibacterial properties of endophytic fungi from plants that positively against pathogens.

Endophytic fungi	Host plant	Pathogens	References
C. gloeosporioides	*Madhuca longifolia*	*S. aureus* *E. coli* *C. albicans*	Denise et al., 2008
	Cinnamomum malabatrum	*S. aureus* *E. coli* *C. albicans*	Packiaraj et al., 2016
	Lannea corammendalica	*S. aureus* *E. coli*	Premjanu & Jaynthy, 2015
	Indigofera suffruticosa	*S. aureus* *E. coli*	Santos et al., 2015
N. sphaerica	*Indigofera suffruticosa*	*S. aureus* *E. coli*	Santos et al., 2015
	Swietenia macrophylla	*S. aureus*	Ibrahim et al., 2015
	Bauhinia racemosa	*E. coli*	Kandasamy, 2015
F. solani	*Taxus baccata*	*S. aureus* *E. coli* *C. albicans*	Tayung et al., 2011
	Solanum lycopersicum	*S. aureus* *E. coli*	Hateet et al., 2014
	Calotropis procera	*S. aureus* *E. coli*	Rani et al., 2017
	Withania somnifera	*S. aureus* *E. coli*	Salini et al., 2014

It was reported that Gram-negative bacteria were more resistant compared to the Gram-positive bacteria (Cosa et al., 2006) which was commonly more sensitive to the tested extracts (Shan et al., 2007). This happened because both of bacteria have different samples of biochemical composition and structures of cell wall (Goyal et al., 2008). Gram-negative bacteria possess an outer membrane and a unique periplasmic

space which is not found in Gram-positive bacteria (Duffy & Power, 2001). This membrane contained lipopolysaccharide and protein layer that surrounding the peptidoglycan layer of the cell wall. Thus, it can prevent the bacteria from being attacked.

Meanwhile, *S. aureus* was the most virulent species of *Staphylococci* which causes a variety of superficial and deep pyogenic infections such as skin infection, folliculitis, boils and food poisoning (Priya et al., 2012). Plus, it was a frequent and important human pathogen which is also found as non-pathogenic microorganisms in human samples (Creech et al., 2005) where almost a third of human were colonized with *S. aureus* (Grundmann et al., 2006). This species was the most virulent species and resistant to various antibiotics (Forbes et al., 1998). However, through this research, two fungal strains (*N. sphaerica* and *F. solani*) have the ability to resist the growth of *S. aureus* by inhibiting the protein, which cross-links peptidoglycans in the cell wall.

Table 4.3: The antibacterial properties of endophytic fungi isolated from a *C. roseus* against pathogenic bacteria *E. coli* and *S. aureus* with a different samples; fungal plug, crude supernatant extract and crude mycelia extract. plates were incubated for 48 h at 37 °C. Positive control was erythromycin (+) and negative control was distilled water (-).

Samples	Fungi	Pathogenic bacteria									
		S. aureus					*E. coli*				
		Diameter of inhibition zone (cm)									
			F		(+)	(-)		F		(+)	(-)
Fungal plug	CG		-		2.20±0.06	-		-		-	-
	MP		-		2.20±0.05	-		-		-	-
	NS		1.68±0.08		2.30±0.05	-		-		-	-
	FS		-		2.27±0.05	-		-		-	-
		1 mg/mL	5 mg/mL	10 mg/mL	(+)	(-)	1 mg/mL	5 mg/mL	10 mg/mL	(+)	(-)
Crude supernatant extract	CG	-	-	-	2.42±0.04	-	-	-	-	-	-
	MP	-	-	-	2.45±0.05	-	-	-	-	-	-
	NS	-	-	-	2.45±0.05	-	-	-	-	-	-
	FS	0.1±0.05[a]	0.65±0.08[b]	1.28±0.22[c]	2.43±0.05	-	-	-	-	-	-
Crude mycelia extract	CG	-	-	-	2.46±0.05	-	-	-	-	-	-
	MP	-	-	-	2.45±0.05	-	-	-	-	-	-
	NS	-	-	-	2.48±0.04	-	-	-	-	-	-
	FS	-	-	-	2.42±0.04	-	-	-	-	-	-

Values presented are the means ± SD, (n = 3). Values within the same row and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

The results on the antifungal properties determined by using an antagonistic method were shown in Figure 4.6. Among these endophytic fungi, *F. solani* and *C. gloeosporioides* gave the highest result on the antagonistic against *A. niger* and *C. albicans*, which were 80 % and 65 %, respectively (Table 4.4).

Figure 4.6: The antifungal properties of endophytic fungi isolated from a *C. roseus*. These endophytic fungi were positively tested against pathogenic fungi *C. albicans* (row A) and *A. niger* (row B). All plates were incubated for 7 d at 25 °C. From the left; CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

Table 4.4: The percentage of inhibition on endophytic fungi isolated from a *C. roseus* against pathogenic fungi *A. niger* and *C. albicans*.

Fungi	Percentage of inhibition (%)	
	A. niger	***C. albicans***
CG	75.71±0.06[a]	65.00±0.00[a]
MP	48.50±0.00[b]	23.08±0.06[b]
NS	57.06±0.10[c]	41.18±0.06[c]
FS	79.75±0.06[d]	62.96±0.06[d]

Values presented are the means ± SD, (n=3). Values within the same column and having different superscript letters are significantly different ($p < 0.05$). ND: Not detected, CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

The antimicrobial properties of four fungal strains were ranged between 49 % to 80 % and 23 % to 65 % of inhibition against *A. niger* and *C. albicans* respectively (Figure 4.7). Previous research reported by Cui et al. (2011) was also found that *F.*

solani isolated from the medicinal plant *Aquilaria sinensis* gave a positive effect on the pathogenic microbes; *C. albicans, E. coli* and *S. aureus*. A similar observation was reported against *C. albicans* and *S. aureus* which were isolated from a different medicinal plant; *S. pallescens* and *C. leanehirae* respectively (Sean & Jon, 2000; Wang et al., 2011). The highest antimicrobial properties from *Fusarium* sp. might be due to production of mycotoxins; the toxic secondary metabolites formed under appropriate environmental conditions by filamentous fungi (Bennett & Klich, 2003).

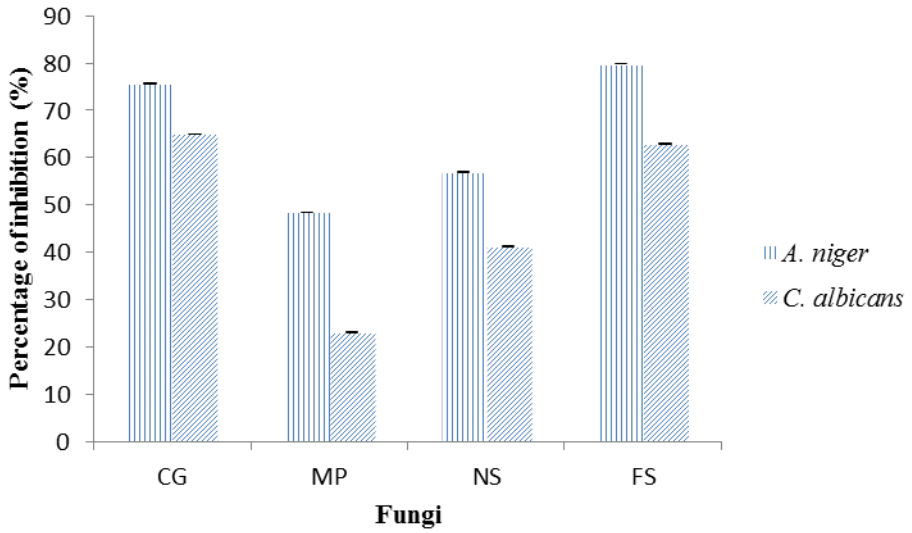


Figure 4.7: The percentage of inhibition on the endophytic fungi isolated from a *C. roseus* against pathogenic fungi *A. niger* and *C. albicans*. CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

4.3.2 Hydrolytic enzyme

Endophytic fungi are known to have a huge proficiency in producing hydrolytic enzymes that act as a mechanism to protect itself against pathogenic invasion and to gain nutrition from the host such as cellulases (Caldwell, 2000), amylase (Choi *et al*., 2005) and proteases (Petrini *et al.,* 1992). Cellulase and protease play and important role in degradation of plant tissue. This caused the enzyme producing microbes penetrate the root and leaves of plants. Four endophytic fungi isolated from a *C. roseus* plant showed a positive result (Figure 4.8) on cellulase enzyme. *N. sphaerica* has the highest enzyme activity, according to the clear zone produced after 5 days of incubation time (Table 4.5). This hydrolytic (Bhavsar & Bhalerao, 2012) and synergistic enzyme that used to break up the cellulose or lignocellulose into glucose or other oligosaccharide compounds (Chellapandi & Jani, 2008) for a wide range of applications (Bhavsar & Bhalerao, 2012).

However, only three of these endophytic fungi were positively present for amylase and protease endophytes except *M. phaseolina*. Meanwhile, *F. solani* was a great producer of both enzymes with a diameter of clear zone 5.78±0.05 cm and 5.65±0.06 cm respectively (Table 4.4). Amylase is universal enzyme being widespread in animals, fungi, plants, unicellular eukaryotes and prokaryotes, which is the most important enzymes used in biotechnology (Burhan et al., 2003). It is estimated that approximately 30 % of world enzymes production are amylase (Sivaramakrishnan et al., 2006) and followed by report from Maheswari and Sherley (2012) that this enzyme leading the world's market with a 65 % of enzymes production due to the high demand in various sectors such as food, pharmaceutical, textiles and detergents (Zaferanloo et al., 2013). However, this enzyme is widely used in the industry for starch hydrolysis (Maheswari & Sherley, 2012).

According to Maheswari and Sherley (2012) on their report, this enzyme is used commercially for the production of sugar syrups which is hydrolyzed from the starch which consists of glucose, maltose and higher oligosaccharide. Besides, it also catalyses the endo-cleavage of the α-1,4 glycoside linkages and release of short oligosaccharide with a limitation of dextrin where the most important amylase for industrial and biotechnology applications are glucoamylases and α-amylases (Sivaramakrishnan et al., 2006). In order to meet the demands of this enzyme, low cost medium is required for the production of amylase. Therefore, this demand has driven the exploitation of endophytes as enzyme sources for promising industrial applications in agriculture, medicine and food industry (Laird et al., 2006). Plant endophytic fungi spend the whole or part of their lifecycle colonizing the inside of healthy tissues of the host plants either inter/ and or intracellular (Zhao et al., 2010) where fungal sources of amylase are mostly terrestrial isolating such as *Aspergillus* sp. (Zaferanloo et al., 2013).

Meanwhile, protease constitutes one of the most significant groups of industrial enzyme that are now used in a widespread range of industrial enzymes such as detergent, food, pharmaceutical, leather and silk industries (Adinarayana et al., 2003) due to the its physiological functions ranging from generalized protein digestion to the more specific regulated processes (Mussarat et al., 2000). This enzyme hydrolyzes peptide bonds of proteins and breaks them down into polypeptides or free amino acids. Thus, it differs in its substrate specificity, catalytic mechanism and active site (Sumantha *et al*., 2006).

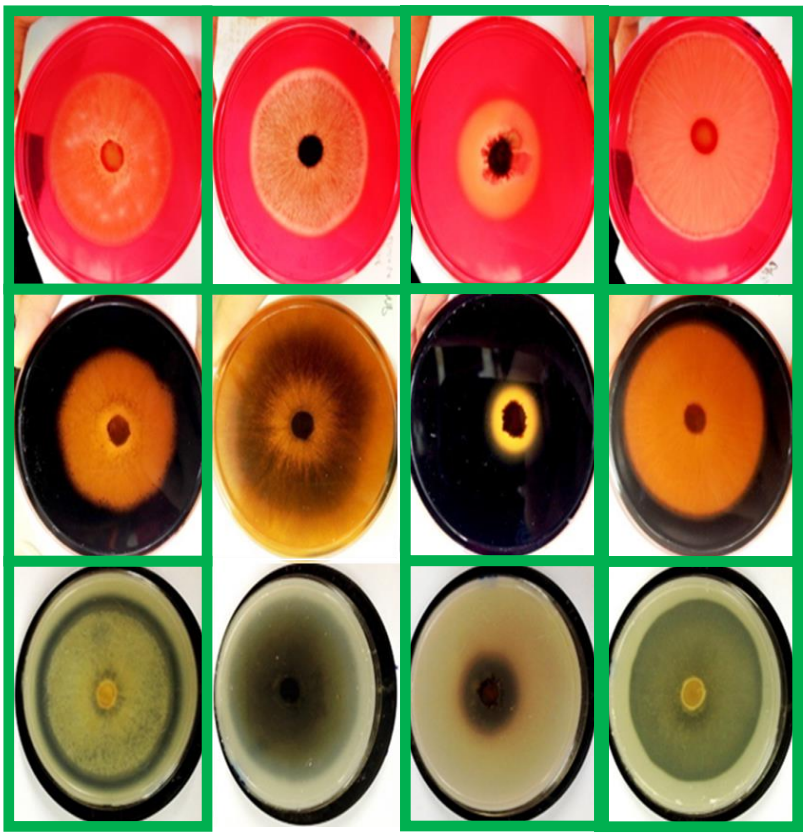


Figure 4.8: The qualitative enzymes test of endophytic fungi isolated from a *C. roseus*. The green boxes were the only plates that positive for the qualitative enzymes test of cellulase (A), amylase (B) and protease (C). All strains were positive for the three enzymes except *M. phaseolina* which was only positive for cellulase and protease. From the left; CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

Table 4.5: Enzyme productivity of the endophytic fungi isolated from a *C. roseus* plant.

Fungi	Diameter of clear zone (cm)		
	Cellulase	**Amylase**	**Protease**
CG	0.10 ± 0.00^{a}	4.20 ± 0.00^{a}	0.45 ± 0.06^{a}
MP	0.15 ± 0.06^{a}	ND	ND
NS	2.80 ± 0.00^{b}	1.00 ± 0.00^{b}	2.30 ± 0.00^{b}
FS	0.20 ± 0.00^{c}	5.78 ± 0.05^{c}	5.65 ± 0.06^{c}

Values presented are the means ± SD, (n = 3). Values within the same column and having same superscript letters are not significantly different ($p < 0.05$). ND: Not detected, CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica* and FS: *F. solani*.

4.4 Conclusions

The antibacterial properties of the endophytic fungi *C. gloeosporioides*, *M. phaseolina*, *N. sphaerica* and *F. solani* were tested against *S. aureus* and *E. coli*. *N. sphaerica* showed a positive result against *S. aureus* (1.68 ± 0.08 cm) for plug sample. While crude supernatant extract (CSE) of *F. solani* showed a positive result against *S. aureus* (0.1 ± 0.05 cm, 0.65 ± 0.08 cm and 1.28 ± 0.22 cm) of concentrations (1 g/mL, 5 g/mL and 10 g/mL) respectively. Meanwhile, the antifungal test showed that all these endophytic fungi were positive against *A. niger* and *C. albicans*.

These endophytic fungi also showed a presence of cellulase enzyme through the qualitative enzymes test where *N. sphaerica* gave a highest diameter of clear zone 2.80±0.00 cm. However, only *C. gloeosporioides*, *N. sphaerica* and *F. solani* were positively present for amylase and protease where *F. solani* was a great producer of both enzymes with an diameter of clear zone 5.78±0.05 cm and 5.65±0.06 cm respectively. The results indicate these fungi were endophytes due to the capability to penetrate the plant cell by producing those enzymes.

CHAPTER 5: ANTIOXIDANTS AND PHYTOCHEMICAL ANALYSIS OF ENDOPHYTIC FUNGI FROM *Catharanthus roseus*

5.1 Introduction

Antioxidant is a stable molecule which donates an electron to a rampaging free radical and terminates the chain reaction before vital molecules are damaged where it delays or inhibits cellular damage (Halliwell, 1995). The contribution of this free radicals in the pathogenesis of a large number of diseases is well acknowledged where a potent scavenger of free radicals may serve as a possible preventive intervention for the diseases (Gyamfi et al., 1999). A great variety of plants have been studied for a new source of natural antioxidants, especially phenolic and flavanoid compounds. These compouds derived from plants were proved to be potent antioxidant and free radical scavengers (Silva et al., 2005). Furthermore, plants also in particular have historically been viewed as the main source of novel bioactive compounds. However, plant availability is a limiting factor in the commercial success of some natural products due to a large quantity of plant is required to produce sufficient amounts of the bioactive compounds for clinical use. Thus, it has raised concerns like environmental degradation, loss of biodiversity and threat to endangered species (Alvin et al., 2014).

Endophytes are the symbiotic microbes that inhabitat into the internal plant tissues without causing any apparent harm to their host and these endosymbiotic fungi found to synthesis bioactive secondary metabolites (Schulz et al., 2002; Strobel, 2003) like flavonoids, phenols, saponins, steroids, tannins and terpenoids. All these metabolites were used to protect the host from infectious diseases, parasitic infections and herbivore tissue invading pathogens (Abirami & Boominathan, 2016). However, they were poorly investigated group among other microorganisms since they were hidden within healthy host plant. (Srinivasan et al., 2010). In fact, they represent an

abundant and dependable source of novel bioactive compounds with huge potential for exploitations in a wide variety of medicinal, agricultural and industrial areas (Tan & Zou, 2001). The research on naturally occurring antioxidants had increased greatly and endophytic fungi are one of the potential source producing effective secondary metabolites in recent decades. Thus, the objective of this study is to determine the antioxidant properties of endophytic fungi from *C. roseus* plant and phytochemical analysis of these endophytic fungi.

5.2 Materials and methods

5.2.1 Determination of polyphenolics content in the endophytic fungi

5.2.1.1 Total phenolic contents

Total phenol content (TPC) was measured by the Folin-Ciocalteu method (Muller et al., 2010). Briefly, 20 µL of crude fungal extract (CFE) was mixed with 100 µL of Folin-Ciocalteu reagent (diluted 10-fold with distilled water) in a 96-well microplate and incubated for 5 min before added with 75 µL of sodium carbonate solution (75 g/L). After 2 h incubated in darkness at room temperature, the absorbance was measured at 740 nm with a microplate reader (Tecan Sunrise, Austria). Gallic acid (100 – 1000 µM) was used as a standard for calibration and construction of a linear regression line and water was used as a blank. The total phenolic content was estimated as mg gallic acid equivalent (mg GAE)/g of dry extract.

5.2.1.2 Total flavonoid contents

Total flavonoid content (TFC) was measured according to Sasipriya and Siddhuraju (2012) and Abdulwali et al. (2014). Briefly, 50 µL of crude fungal extract (CFE) were added with 70 µL of distilled water and 15 µL of sodium nitrite solution (5 %) in a 96-well microplate. The solutions were well mixed and incubated at room temperature for 5 min. Then, 15 µL of aluminium chloride solution (10 %) was added

into the mixture and incubated for 6 min. Then, 100 µL of 1 M sodium hydroxide solution was added and the absorbance was measured at 510 nm with a microplate reader (Tecan Sunrise, Austria). The total flavonoid content (TFC) was estimated from quercetin (200 – 1000 µM) standard curve and the results were expressed as mg quercetin equivalent (mg QE)/g of dry extract.

5.2.2 Antioxidant activity assays

5.2.2.1 DPPH radical – scavenging activity

The free radical scavenging activity of crude fungal extract (CFE) was measured in terms of hydrogen donating ability by using DPPH radical according to Margithas et al. (2009) and Ablat et al. (2014). Briefly, 40 µL of crude fungal extracts with different concentrations (0.05 – 2 mg/mL) were mixed with 200 µL of 50 µM DPPH solution in methanol. Then, the mixture was immediately shaken before incubated in the dark at room temperature for 15 min. The absorbance was measured at 517 nm with a micro plate reader (Tecan Sunrise, Austria). Butylated hydroxyanisole (BHA) with concentrations of 5 – 80 µg/mL was used as a standard while the control was ethanol. The percentage of inhibition activity of the extracts was calculated according to the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_1 - A_2) \times 100}{A_1}$$

(Equation 5.1)

Where ,

A_1 = Absorbance of control

A_2 = Absorbance of sample

The DPPH radical scavenging activity was estimated from the graph plotted against the percentage inhibition and compared with the standard in order to find the Half Maximal Inhibitory Concentration (IC_{50}).

5.2.2.2 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) activities of crude fungal extracts were measured according to Muller et al. (2010). Briefly, 20 µL of crude fungal extract (CFE) in methanol were mixed with 200 µL of daily prepared ferric reducing antioxidant power (FRAP) reagent contained 5 mL of 10 mM tripyridyl triazine (TPTZ) in 40 mM hydrogen chloride (HCl), 5 mL of 20 mM iron (III) chloride ($FeCl_3$), and 50 mL of 0.3 M acetate buffer; pH 3.6 in 96-well microplate. The formation of the TPTZ-Fe^{2+} complex in the presence of antioxidant compounds in the extract was measured at 595 nm with a microplate reader (Tecan Sunrise, Austria) after incubating for 8 min. Ferrous sulphate (FeSO4) solution (0.2 – 1 mm) was used for a standard calibration curve while the methanol was used as blank. The ferric reducing antioxidant power (FRAP) value was evaluated according to the linear regression between standard solutions and absorbance at 595 nm and the results were estimated as mmol Fe^{2+}/g of dry extract from triplicated tests.

5.2.2.3 Metal chelating activity

Metal chelating activity or ferrous ion chelating activity (FCA) of the CFE was determined by measuring the formation of the ferrozine (Fe^{2+}) complex, according to the procedure described by Decker and Welch (1990). 10 µL of extracts with different concentrations (50 – 800 µg/mL) were mixed with 120 µL of distilled water and 10 µL of 2 mM iron (II) chloride ($FeCl_2$) in a 96-well microplate. 20 µL of 5 mM ferrozine was added to the mixture in order to initiate the reaction. The reaction mixture was incubated for 20 min at room temperature and was measured at 562 nm along with

EDTA-Na_2 (5 – 80 µg/mL) as a standard metal chelator. 20 µL of distilled water was used as a blank while 100 µL of methanol was used as a control. The percentage of inhibition of ferrozine (Fe^{2+}) complex formation was calculated according to the following formula:

$$\text{Ferrous ion chelating activity (\%)} = \frac{(A_1 - A_2) \times 100}{A_1}$$

(Equation 5.2)

Where,

A_1 = Absorbance of control

A_2 = Absorbance of sample

The metal chelating activity was estimated from the graph plotted against the percentage inhibition and compared with the standard in order to find the Half Maximal Inhibitory Concentration (IC_{50}).

5.2.3 Statistical analysis

All data were analysed by using analysis of variance (IBM SPSS Statistics, Version 20; IBM Corp., Armonk, New York) to determine the significant studies on antioxidant and polyphenolics compounds. The regression among variables was showed in surface plot graph.

5.3 Results and discussion

Total phenolic contents (TPC) of the crude fungal extract (CFE) were tested using Folin-Ciocalteu's reagent and expressed as Gallic acid equivalent ($y = 0.0042x + 0.0196$, $r^2 = 0.9996$) as in Appendix D. While total flavonoid contents (TFC) were measured based on the equivalent of quercetin ($y = 0.0676x + 0.0142$, $r^2 = 0.9997$) as in Appendix E. The results showed that crude supernatant extract (CSE) of *N. spherica* had a higher value of both total phenolic contents (TPC) and total flavonoid contents

(TFC), which were 0.030 mg GAE/g and 0.038 ± 0.001 QE/g respectively (Table 5.1). The lowest total phenolic contents (TPC) and total flavonoid contents (TFC) were crude mycelia extract (CME) of *N. sphaerica* (0.023 mg GAE/g) and CFE of *C. gloeosporioides* (0.022 ± 0.001 QE/g) respectively.

Table 5.1: The polyphenolics content of crude fungal extracts

Samples	TPC (mg GAE/g)	TFC (mg QE/g)
CG (CME)	0.024 ± 0.001^{f}	0.022 ± 0.002^{f}
CG (CSE)	0.028 ± 0.002^{c}	0.022 ± 0.001^{f}
MP (CME)	0.024 ± 0.001^{f}	0.034 ± 0.001^{b}
MP (CSE)	0.027 ± 0.001^{d}	0.023 ± 0.001^{e}
NS (CME)	0.023 ± 0.000^{g}	0.025 ± 0.002^{d}
NS (CSE)	0.030 ± 0.000^{a}	0.038 ± 0.001^{a}
FS (CME)	0.025 ± 0.001^{e}	0.029 ± 0.003^{c}
FS (CSE)	0.029 ± 0.001^{b}	0.023 ± 0.001^{e}

Values presented are the means ± SD, (n = 3). Values within the same column and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, TPC: total phenolic contents, TFC: total flavonoid contents CME: crude mycelia extract and CSE: crude supernatant extract.

According to Huang et al. (2010), main natural products of secondary metabolites in plant and fungi were phenolic compounds where phenol and flavonoid compounds have been reported to possess different bioactivities. The antioxidant activity of crude fungal extracts was determined by using a methanol solution of DPPH reagent, ferric reducing antioxidant power (FRAP) and metal chelating activity (FCA). The DPPH radical scavenging activity of crude fungal extracts was tested at different concentrations (50 - 250 µg/mL) against the BHA as a standard (Appendix F). The percentage of DPPH radical scavenging activity ranged from 9.03 % to 17.15 % and 0.26 % to 9.03 % of mycelia and supernatant samples respectively (Table 5.2). However, there was no data for half maximal inhibitory concentration (IC_{50}) due to low scavenging activity.

Table 5.2: The DPPH radical- scavenging activity of crude fungal extracts

Concentration (µg/mL)	DPPH radical – scavenging activity (%)										
	C. gloeosporioides		*M. phaseolina*		*N. sphaerica*		*F. solani*		VB	VC	BHA
	CME	CSE	CME	CSE	CME	CSE	CME	CSE			
50	20.73 ±0.15[b]	8.94 ±0.77[f]	26.49 ±0.15[a]	3.97 ±0.26[b]	9.52 ±0.69[e]	0.26 ±0.02[h]	10.75 ±0.90[d]	4.46 ±0.38[g]	17.57 ±0.83[c]	10.17 ±0.86[d]	0.32 ±0.01[a]
100	4.99 ±0.42[d]	9.37 ±0.80[b]	16.98 ±0.74[a]	9.44 ±0.74[b]	9.03 ±0.65[b]	0.26 ±0.02[e]	9.86 ±0.83[b]	8.42 ±0.72[c]	9.73 ±0.78[b]	8.47 ±0.71[c]	0.64 ±0.01[b]
150	9.19 ±0.75[f]	7.76 ±0.67[h]	20.82 ±0.20[b]	10.12 ±0.79[e]	16.80 ±0.64[d]	9.03 ±0.40[f]	10.14 ±0.85[e]	8.29 ±0.71[g]	24.89 ±0.39[a]	18.76 ±0.81[c]	0.96 ±0.26[c]
200	15.75 ±0.67[e]	7.84 ±0.67[h]	19.22 ±0.84[c]	8.91 ±0.69[g]	17.15 ±0.64[d]	0.26 ±0.02[i]	9.73 ±0.81[f]	7.68 ±0.66[h]	29.05 ±0.23[a]	24.06 ±0.42[b]	1.28 ±0.05[d]
250	8.83 ±0.72[g]	13.39 ±0.59[d]	25.53 ±0.08[a]	11.21 ±0.45[e]	10.29 ±0.76[f]	0.26 ±0.02[h]	15.85 ±0.71[g]	8.16 ±0.70[h]	24.98 ±0.36[b]	24.22 ±0.44[b]	1.60 ±0.34[e]

Values presented are the means ± SD, (n = 3). Values within the same row and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS*: N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract, CSE: crude supernatant extract and BHA:Butylatedhydroxyanisole.

The ferric reducing antioxidant power assay was measured based on the reaction between Fe^{3+}-TPTZ and antioxidant potentials to form Fe^{2+}-TPTZ in the fungus extract. The highest results showed that crude supernatant extract (CSE) of *N. sphaerica* produced 0.477±0.11 mmol Fe^{2+}/g of extract while the lowest result was crude mycelia extract (CME) of *C. gloeosporioides* produced 0.336±0.01 mmol Fe^{2+}/g of extract (Table 5.3).

Table 5.3: Ferric reducing antioxidant power (FRAP) of crude fungal extracts

Samples	Ferric reducing antioxidant power (mmol Fe^{2+}/g extract)
CG (CME)	0.336 ± 0.01^{f}
CG (CSE)	0.400 ± 0.04^{d}
MP (CME)	0.357 ± 0.01^{e}
MP (CSE)	0.423 ± 0.04^{c}
NS (CME)	0.347 ± 0.01^{d}
NS (CSE)	0.477 ± 0.11^{b}
FS (CME)	0.340 ± 0.01^{f}
FS (CSE)	0.437 ± 0.11^{c}
VB	0.103 ± 0.01^{g}
VC	0.097 ± 0.01^{g}
$FeSO_4$	0.595 ± 0.02^{a}

Values presented are the means ± SD, (n = 3). Values within the same raw and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract, CSE: crude supernatant extract and $FeSO_4$: Ferrous sulphate.

The metal chelating (FCA) activity was measured based on the chelation of ferrous ion with ferrozine and formed ferrous-ferrozine complex. The fungus extract was prepared in different concentrations (50-800 µg/mL) with data ranged from 12.33 % to 39.72 % and 15.78 % to 39.16 % for crude mycelia extract (CME) and crude supernatant extract (CSE) respectively (Table 5.4 and Figure 5.1).

Table 5.4: Metal chelating activity (FCA) of crude fungal extracts.

Samples	Metal chelating activity (%)				
	Concentration (µg/mL)				
	50	100	200	400	800
CS (CME)	20.48±0.87^{i}	17.92±0.63^{h}	27.55±0.78^{g}	18.01±0.64^{h}	16.53±0.50^{i}
CS (CSE)	38.86±0.05^{e}	37.85±0.14^{d}	39.16±0.01^{c}	37.07±0.06^{d}	37.78±0.13^{d}
MP (CME)	20.76±0.50^{i}	18.65±0.73^{g}	12.33±0.13^{j}	18.47±0.72^{h}	21.51±0.55^{h}
MP (CSE)	24.44±0.59^{h}	18.96±0.70^{g}	18.33±0.64^{h}	18.67±0.67^{h}	23.61±0.58^{g}
NS (CME)	37.25±0.10^{f}	30.80±0.44^{e}	32.16±0.21^{e}	28.87±0.63^{f}	27.08±0.75^{e}
NS (CSE)	34.42±0.22^{g}	23.34±0.66^{f}	30.64±0.36^{f}	29.58±0.29^{e}	26.66±0.78^{f}
FS (CME)	39.72±0.15^{d}	30.59±0.75^{e}	38.31±0.12^{d}	29.06±0.88^{e}	32.44±0.50^{e}
FS (CSE)	24.41±0.66^{h}	19.06±0.79^{f}	15.78±0.28^{i}	25.79±0.49^{g}	26.26±0.47^{f}
VB	95.97±0.03^{c}	94.38±0.03^{b}	93.18±0.05^{a}	91.02±0.04^{b}	89.55±0.02^{b}
VC	97.50±0.03^{b}	95.27±0.00^{a}	93.91±0.01^{a}	92.52±0.05^{a}	92.41±0.05^{a}
EDTA-Na_2	98.29±0.11^{a}	93.71±0.05^{c}	88.11±0.15^{b}	83.31±0.09^{c}	77.59±0.02^{c}

Values presented are the means ± SD, (n = 3). Values within the same row and having different superscript letters are significantly different ($p < 0.05$). CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract.

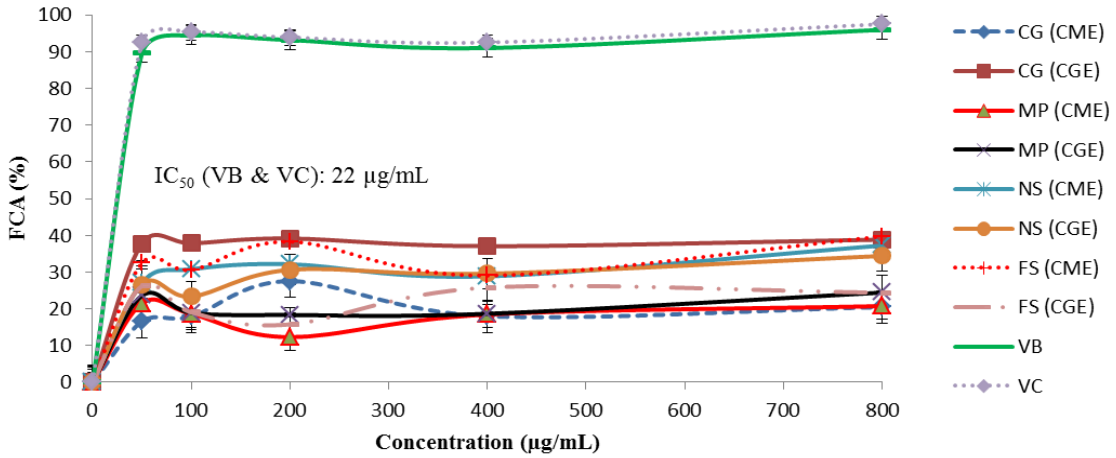


Figure 5.1: The antioxidant assay of crude fungal extracts from *C. roseus* plant by using ferrous ion chelating activity (FCA) at 562 nm. CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract.

Through ferric reducing antioxidant assay, it showed that crude supernatant extract (CSE) of *N. sphaerica* has the highest result 0.477±0.11 mmol Fe^{2+}/g of extract while crude mycelia extract (CME) of *C. gloeosporioides* has the lowest result 0.336±0.01 mmol Fe^{2+}/g of extract (Table 5.5). This analysis also revealed that crude

supernatant extract (CSE) of *N. sphaerica* has the highest properties for both antioxidant and polyphenolic; total phenolic content (TPC) and total flavonoid content (TFC). The previous studies also showed that *N. sphaerica* isolated from *Bauhinia racemosa* and *Cresentia cujete* has highest value for both antioxidant and phenolic content (Kandasamy et al., 2015; Prabukumar et al., 2015).

According to Ablat et al. (2014), the extract containing the high value of total phenolic content and total flavonoid content showed the highest reducing power activity. The ferric reducing ability correlated well with total phenolic content (r^2 = 0.9996), total flavonoid content (r^2 = 0.997) contents in the fraction and this was supported by Velioglr et al. (1998) from their report that the antioxidant activity of plant material is very well correlated with the content of phenolic compounds. The most significant studies on antioxidant and polyphenolics compounds were determined using an analysis of variance (ANOVA). Table 5.6 showed the regression of fungi*crude fungal extract*test was significantly different. It was supported by the surface plot where it showed relationships among these variables (Figure 5.2). Besides, the correlation among test was also significant at the 0.01 level.

Table 5.5: Antioxidant and phytochemical analysis of crude fungal extracts.

Samples	DPPH (IC_{50} µg/mL)	FRAP (mmol Fe^{2+}/g extract)	FCA (IC_{50} µg/mL)	TPC (mg GAE/g)	TFC (mg QE/g)
CG (CME)	ND	0.336 ± 0.01^{g}	ND	0.024 ± 0.001^{f}	0.022 ± 0.002^{f}
CG (CSE)	ND	0.400 ± 0.04^{d}	ND	0.028 ± 0.002^{c}	0.022 ± 0.001^{f}
MP (CME)	ND	0.357 ± 0.01^{e}	ND	0.024 ± 0.001^{f}	0.034 ± 0.001^{b}
MP (CSE)	ND	0.423 ± 0.04^{c}	ND	0.027 ± 0.001^{d}	0.023 ± 0.001^{e}
NS (CME)	ND	0.347 ± 0.01^{f}	ND	0.023 ± 0.000^{g}	0.025 ± 0.002^{d}
NS (CSE)	ND	0.477 ± 0.11^{a}	ND	0.030 ± 0.000^{a}	0.038 ± 0.001^{a}
FS (CME)	ND	0.340 ± 0.01^{f}	ND	0.025 ± 0.001^{e}	0.029 ± 0.003^{c}
FS (CSE)	ND	0.437 ± 0.11^{b}	ND	0.029 ± 0.001^{b}	0.023 ± 0.001^{e}
VB	ND	0.103 ± 0.01^{h}	22.00^{a}	-	-
VC	ND	0.097 ± 0.01^{i}	22.00^{a}	-	-

Data are mean ± SD (n = 3). Values within the same row and having different superscript letters are significantly different ($p > 0.05$). DPPH: diphenyl-1-picrylhydrazyl, FRAP: ferric reducing antioxidant power, FCA: ferrous ion chelating activity, TPC: total phenolic content, TFC: total flavonoid content, ND: not detected, CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, VB: Vinblastine, VC: Vincristine, CME: crude mycelia extract and CSE: crude supernatant extract.

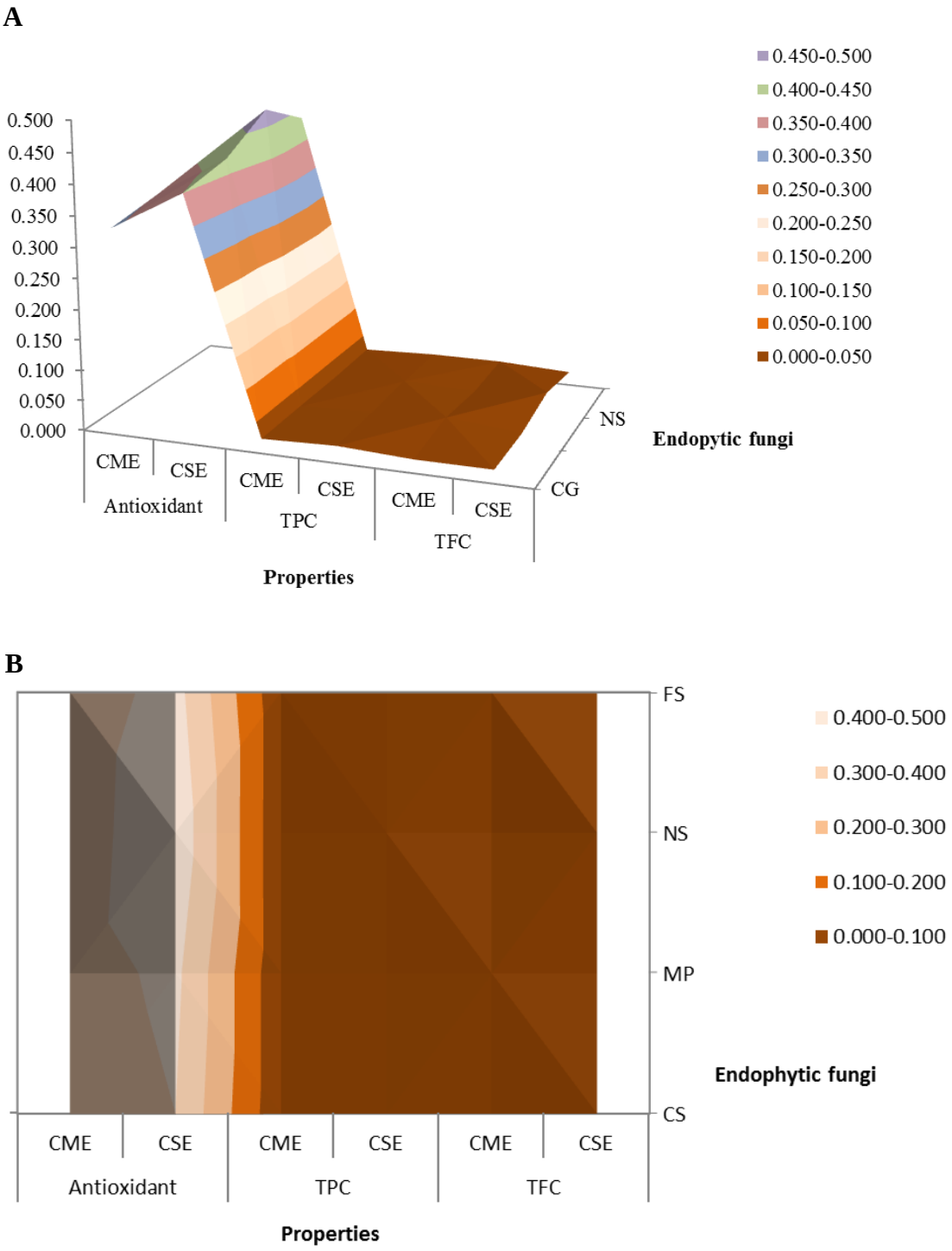


Figure 5.2: The surface plots of fungi properties *vs*. crude fungal extract and endophytic fungi; A: 3D surface plot, B: contour plot, CG: *C. gloeosporioides*, MP: *M. phaseolina*, NS: *N. sphaerica*, FS: *F. solani*, CME: crude mycelia extract, CSE: crude supernatant extract.

Table 5.6: The ANOVA of antioxidant and polyphenolic content on endophytic fungi from *C. roseus***.**

	Regression			
	Sum of Squares	**Mean Square**	**F**	**Sig.**
Fungi	0.000	0.000	0.011	0.919
Fungi*CFE	0.006	0.003	0.089	0.915
Fungi*CFE*Test	0.532	0.177	18.637	0.000
	Correlation			
	Sum of Squares	**Pearson Correlation**	**Sig. (1-tailed)**	**Sig. (2-tailed)**
Fungi	0.102	0.022	0.459	0.919
CFE	0.186	0.089	0.339	0.679
Test	-2.901	-0.853	0.000**	0.000**

Fungi (CG, MP, NS, FS); CFE (CME, CSE); Test (Antioxidant, TPC, TFC). **correlation was significant at the 0.01 levels.

5.4 Conclusion

N. sphaerica isolated from a medicinal plant *C. roseus* has potential as an antioxidant agent due to the significant result on both antioxidant and polyphenolic properties compared to the other species. Since there was no result on IC_{50} of DPPH radical – scavenging activity and IC_{50} of ferrous ion chelating activity, the data on ferric reducing antioxidant power was used as a comparison to a polyphenolic property. From the analysis, all CSE showed the highest result on the antioxidant properties through ferric reducing antioxidant power (FRAP) assay compared to crude mycelia extract (CME) of each endophytic fungi and this also applied to the total phenolic content (TPC) analysis. Thus, as a summary, the crude supernatant extract (CSE) samples were more productive on antioxidant and polyphenolic assays. Meanwhile, *N. sphaerica* has a great potential as the antioxidant agents and also could produce the polyphenolic compounds.

CHAPTER 6: ANTI-CARCINOGENIC ALKALOIDS VINBLASTINE AND VINCRISTINE PRODUCED BY ENDOPHYTIC FUNGI FROM *Catharanthus roseus*

6.1 Introduction

Endophytes are microbes that refer to microorganisms that live inside the tissues of plants without causing any apparent harm or diseases to the host plant (Strobel, 2002). In fact, they promote the host plant's growth and the formation of secondary metabolites related to the plant defence (Chandra et al., 2010; Petrini, 1991). They could produce valuable bioactive compounds with a varied application in both research and applied fields (Ravindra et al., 2014). Endophytic fungi spend the whole part of their life cycle living symbiotically within the healthy tissues of the host plant (Tan & Zou, 2001; Ravindra et al., 2014). It also has been recognized as one of important and novel resources of natural bioactive products (Strobel et al., 2004) since most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defence against pathogens (Guo et al., 2008). Some of these compounds have proven useful for discovering a novel drug (Yan et al., 2011).

There are many reports that endophytic fungi isolated from a medicinal plant produce a new drug or compound that similar to the host plants (Table 6.1). All these findings will help to fill the demands of the drugs. In fact, the manufacturing cost of the drugs from endophytic fungi is cheaper than the production of the plants since it takes a shorter period to produce it.

Table 6.1: List of previous research on endophytic fungi that produces drugs.

Host Plants	Endophytic fungi	Drugs	References
Phyllanthus amarus	*Trichothecium* sp.	Trichothecinol-A	Ravindra *et al*., 2014
Eugenia jambolana	*Cephalotheca faveolata*	Sclerotiorin	Giridharan *et al*., 2012
Camptotheca acuminate	*Fusarium solani*	Camptothecin	Kusari *et al*., 2009
Taxus brevifolia	*Taxomyces andreanale*	Taxol	Strobel, 2002
Tylophora indica	*Fusarium ssolani*	Taxol	Merlin *et al*., 2012

Catharanthus roseus or well known as a Madagascar periwinkle is a medicinal plant belonging to the family Apocynaceae (Gajalakshmi et al., 2013). Even though this plant is native to Madagascar; it also can be found in Malaysia and has been called as Kemunting Cina (Ayob & Simarani, 2016). This plant is also well-known to produce a lot of important compounds, especially Vinca alkaloids vinblastine and vincristine (Krishnan, 1995; Manganey et al., 1979). Besides, this plant produces vindoline and catharanthine as well, which are the major monomer alkaloids as well as a biosynthetic precursor for vinblastine and vincristine (Noble, 1990).

In 1960, vinblastine was introduced to treat certain types of cancer, including breast cancer, testicular cancer and Hodgkin's disease (Armstrong et al., 1964). While in 1963, vincristine was introduced through oxidization of vinblastine to treat leukaemia (Evans et al., 1963). So far, there are only three reports on these alkaloids produced by the endophytic fungi which were *Alternaria* sp., *Fusarium oxysporum* and unidentified fungi from *Catharanthus roseus* (Guo et al., 1998; Zhang et al., 2000; Yang et al., 2004). Thus, the objective of this research is to find a new endophytic fungus that could produce Vinca alkaloids, vinblastine and vincristine from the host plant *C. roseus*. The purified alkaloids will be tested for cytotoxicity test through MTT assay against breast cell line cancer.

6.2 Materials and methods

6.2.1 Preparation of crude fungal extract and crude leaf extract

The preparation of crude fungal extract (CFE) was carried out following Wiyakrutta et al. (2004) with a minor modification. Briefly, six pieces of the plug (1 cm) of fungal growth culture were inoculated into a 250 mL conical flask containing 250 mL of yeast extract sucrose broth (YESB) medium (20 g of yeast extract, 40 g of sucrose and 1L of distilled water with a pH 5.8). The flasks were then incubated at 25 °C under static condition for 21 d. The grown culture was harvested by filter separation methods and used for crude extract preparation.

The crude fungal extract from the cell free filtrate and mycelia biomass were prepared. The cell free filtrate was extracted thrice with a dichloromethane (200 mL) and evaporated to dryness using a rotary evaporator. The sample was weighed to constitute the crude fungal extract (CFE). Meanwhile, the mycelia biomass were freeze-dried and extracted twice with a mixture of dichloromethane: methanol (1: 1, v/v) for 1 h. The mycelia extract was air dried and weighed and kept in the air tight container until further used. Both of crude extract were then dissolved separately in dimethylsulphoxide (DMSO) to obtain 1 g/mL of concentration.

Meanwhile, the crude extracts of *C. roseus* leaves were prepared by soaking 5 g powdered of samples thrice with 90 % (v/v) ethanol (3 x 30 mL) for 12 h each at room temperature. The alcohol extract was filtered and concentrated in vacuo to 10 mL diluted with 10 mL of water, acidified with 10 mL of 3 % (v/v) hydrochloric acid and then washed with hexane (3 x 30 mL). The aqueous portion was basified with ammonia to pH 8.5 and extracted using chloroform (3 x 30 mL). The chloroform extract was washed with pure water, dried over sodium sulphate and concentrated under vacuum before dissolving in 10 mL of methanol (Gupta et al., 2005) (Appendix G).

6.2.2 Determination and purification of vinblastine and vincristine

The presence of Vinca alkaloids in both crude extracts was detected using Liquid Chromatography–Mass Spectrometry (AB Sciex 3200Q Trap LCMS/MS), method Multiple Reaction Monitoring (MRM), equipped with a column Agilent Zorbax XDB C18 (150 mm x 4 mm x 5 µM) and buffered with (A) pure water, ammonium formate and formic acid, (B) acetonitrile, ammonium formate and formic acid. The experiment was run for 10 min and rapidly screened for the peaks because it should have two fragment ions from the same compounds for further confirmation of the peaks.

The fungal crude extracts were then purified using High-Performance Liquid Chromatography (Agilent 1220 Infinity Gradient LC – G4294B) for vinblastine and vincristine. The 10 µL samples were injected in HPLC column C18 Merck (50 mm x 2 mm x 1.5 µM) and isocratic elution was performed using methanol and Nano pure water with 0.01 % acetic acid at a flow rate of 1 mL/min. A dual wavelength 254 nm and 260 nm were used to detect the compounds eluting from the column. The purified vinblastine and vincristine were collected using an Agilent Fraction Collector (G1364C) and tested in a cytotoxicity test.

6.2.3 Cytotoxicity activity (MTT assay)

Cytotoxicity of vinblastine and vincristine produced by both crude extracts were assessed using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma) assay against breast cell line cancer MDA-MB 231. The cancer cell was grown in Dulbecco's Modified Eagle's Medium (DMEM) and washed in phosphate buffer solution (PBS) before 100,000 cells were placed in each well of 96 well plates and treated with fungal extract. After 72 h incubated at 37 °C and 5 % CO_2, the MTT reagent was removed from the plate and replaced with a DMSO and gently shaken for 30 min. The absorption was determined at 570 nm. The percentage of inhibition (POI) was calculated using the formula:

$$POI = \frac{(A_1 - A_2) \times 100}{A_1}$$

(Equation 6.1)

Where,

A_1 = Absorbance of control

A_2 = Absorbance of sample

The Half Maximal Inhibitory Concentration (IC_{50}) was determined from the graph of samples concentration vs. percentage of inhibition.

6.2.4 Determination of apoptosis by Annexin V/PI staining

Experiment was carried out according to the manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit I, BD Biosciences, USA). Cells were seeded in 6-well plates at 1.3 x 10^5 cells/mL, incubated for 24 h and treated with fungal extract. Following 72 h incubation, the floating and adherent cells were collected and centrifuged at 100 x *g* to obtain a pellet. The pellet were washed twice with cold PBS and then resuspended in 1X binding buffer at a concentration of 1 x 10^6 cells/mL. Then, 100 µL of the resuspended pellet (1 x 10^5 cells) was transferred to a 5 mL polystyrene round-bottom tube and stained with 5 µL of Annexin V-fluorescein isothiocyanate (FITC) and 5 µL of propidium iodide (PI). The cells were then gently vortexed and incubated for 15 min at RT (25° C) in the dark. Thereafter, 400 µL of 1X binding buffer was added to each tube. Samples were analysed immediately by CyAN ADP flow cytometer (Beckman Coulter, Brea, CA, USA). For each sample, 10,000 events were collected. The results were analysed by using Summit v4.3 software (Beckman Coulter, Brea, CA, USA).

6.2.5 Statistical analysis

All data were analysed using IBM SPSS StatistiCG, Version 20 (IBM Corp., Armonk, New York).

6.3 Results and discussion

Five endophytic fungi were isolated from the leaf and roots of *C. roseus* plant and have been identified as a *C. gloeosporioides, M. phaseolina, N. sphaerica* and *F.solani.* The crude fungal extract was prepared by using the 21 days of fungal cultured (Figure 6.1) while the crude leaf extract was prepared using a dried leaf of wildly grown *C. roseus* - purple and white. The crude leaf extract of *C. roseus* showed a presence of both vinblastine and vincristine. However, only crude mycelia extract of *N. sphaerica* and *C. gloeosporioides* showed a positive result on vinblastine and vincristine respectively by using Liquid Chromatography–Mass Spectrometry (LCMS). It was determined by the presence of two fragment ions from a peak in the same compounds and indicated a confirmation of the compound detected even though the retention time slightly different among all samples (Figure 6.2 and Figure 6.3). Meanwhile, there was no result on both alkaloids in a crude supernatant fungal extract. This result indicated that the vinblastine and vincristine were intracellularly produced by both fungi.

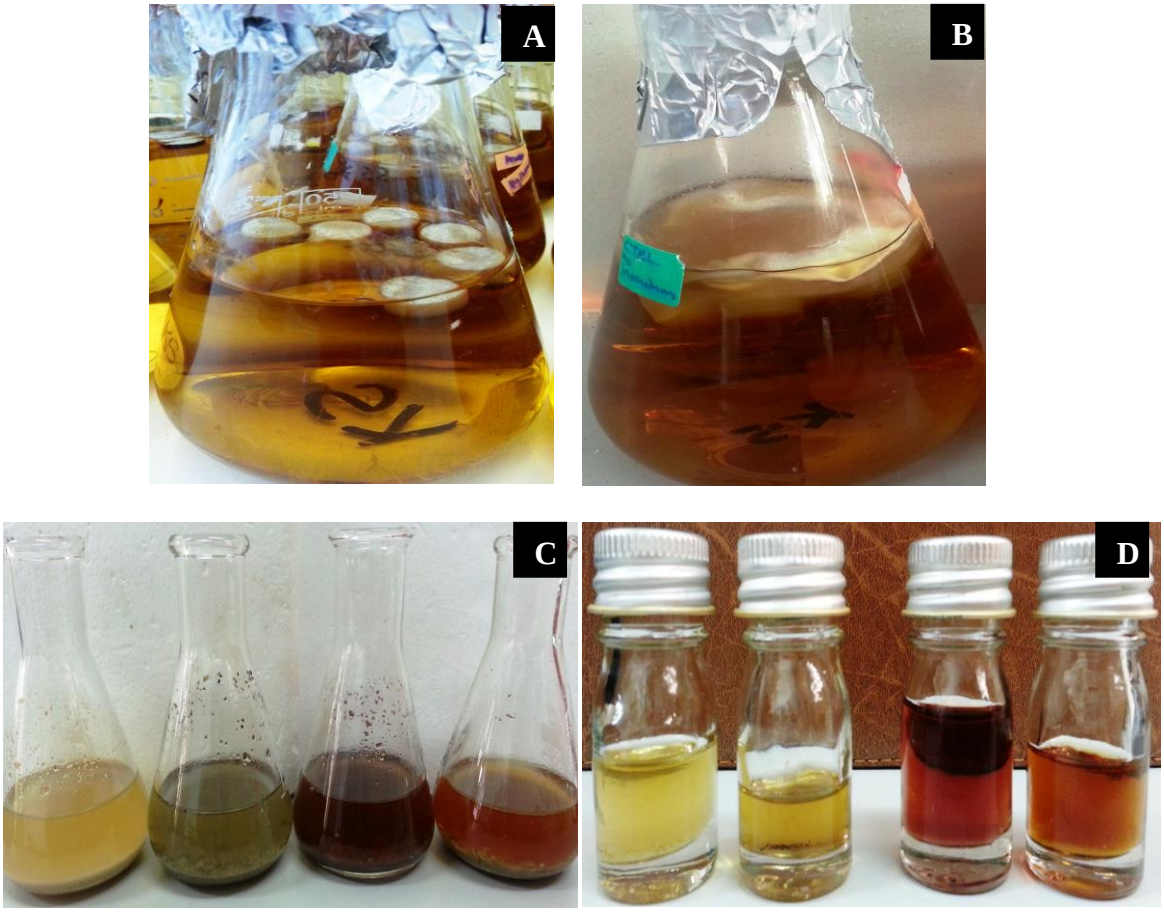


Figure 6.1: The crude fungal extract was prepared by using a broth culture technique where the inoculum was cultured in a yeast extract sucrose broth (YESB) medium and incubated at 25 °C under static condition (A). After 21 days (B), the cultured can be harvested using filter separation methods and the crude mycelia extract (C) and crude supernatant extract (D) had been prepared.

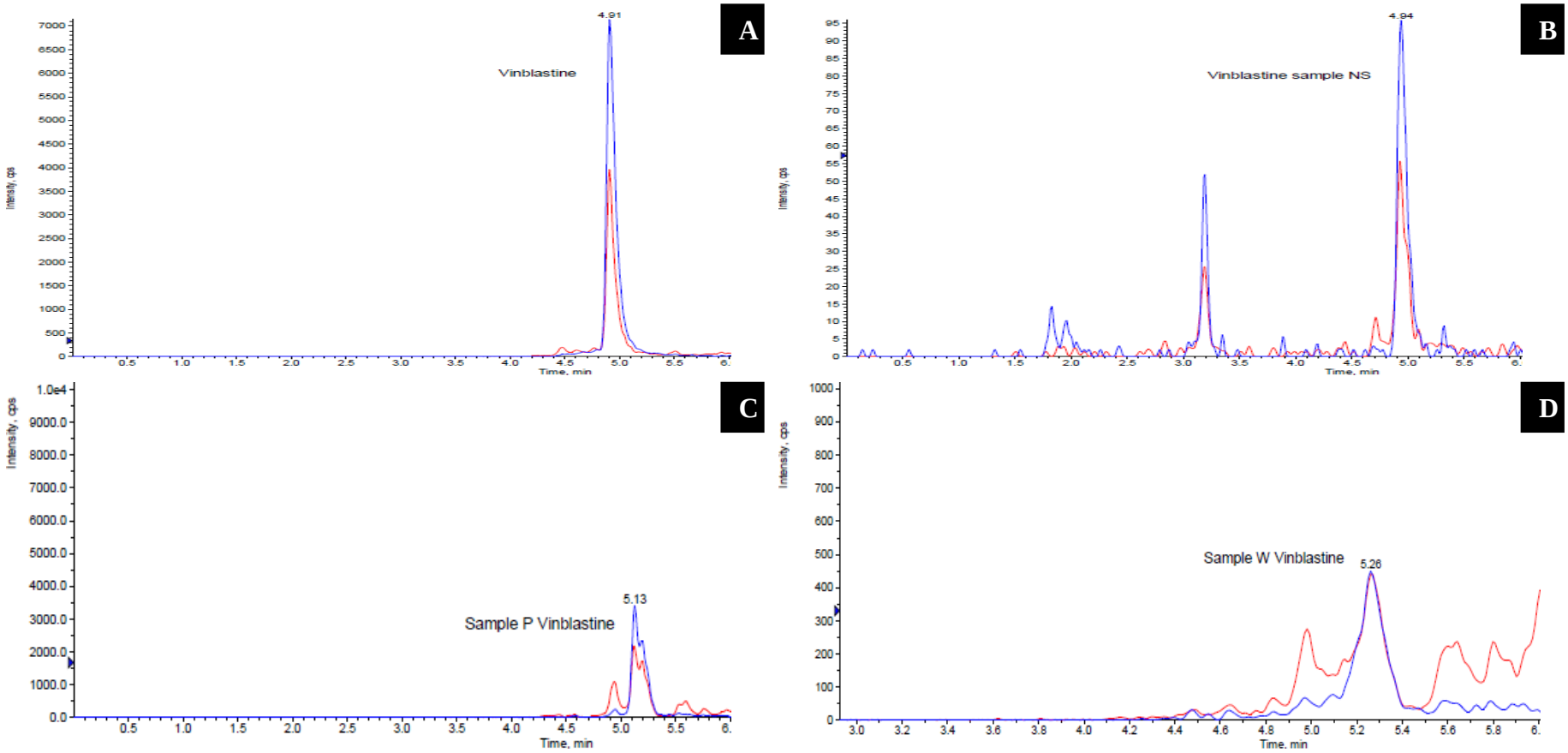


Figure 6.2: The Liquid Chromatography–Mass Spectrometry (LCMS) chromatograph of commercial standard vinblastine (A), crude mycelia extract of *N .sphaerica* isolated from *C. roseus* plant (B), crude leaf extract of *C. roseus* - purple (C) and crude leaf extract of *C. roseus* – white (D) showed a presence of vinblastine at retention time of 4.91 min, 4.94 min, 5.13 min and 5.26 min respectively. Blue line = 1st fragment ion, Red line = 2nd fragment ion.

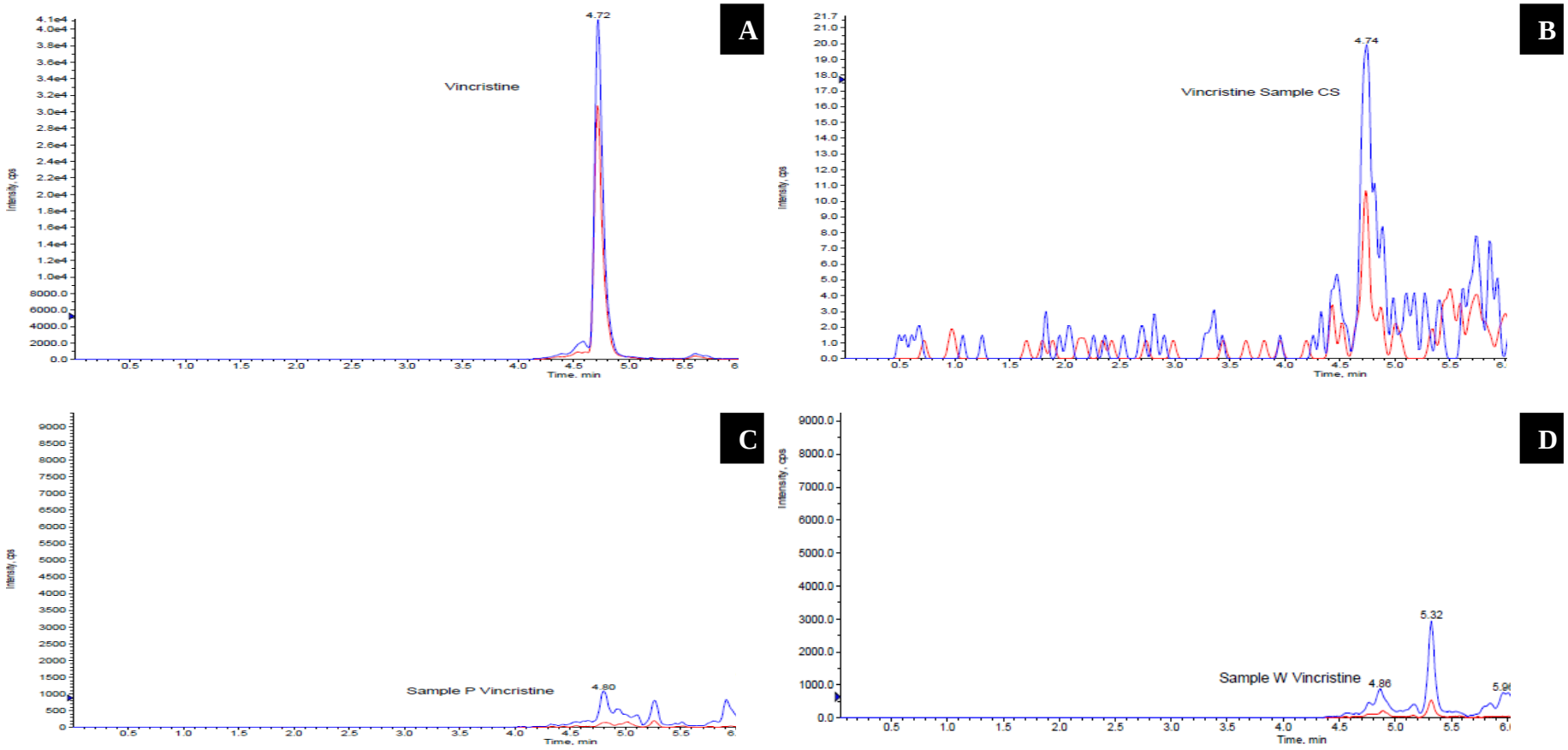


Figure 6.3: The Liquid Chromatography–Mass Spectrometry (LCMS) chromatograph of commercial standard vincristine (A), crude mycelia extract of *C. gloeosporioides* isolated from *C. roseus* plant (B), crude leaf extract of *C. roseus* - purple (C) and crude leaf extract of *C. roseus* – white (D) showed a presence of vincristine at retention time of 4.72 min, 4.74 min, 4.80 min and 4.86 min respectively. Blue line = 1st fragment ion, Red line = 2nd fragment ion.

Purified vinblastine was obtained by using High-Performance Liquid Chromatography (HPLC). The crude mycelia extract of this fungus and crude leaf extract were eluted for five minutes of retention time when loaded on the analytical C18 column with a flow rate of 1 ml/min. The crude mycelia extract of *N. sphaerica* fermented in yeast extract sucrose broth and crude leaf extract of *C. roseus* produced 0.868 µg/mL and 0.666 µg/mL of vinblastine respectively. From the chromatograph of HPLC, the retention time for almost each peak of crude extract, fungal showed a same result with a crude leaf extract since the fungal was isolated from the *C. roseus* (Figure 6.4 and Figure 6.5).

Cytotoxicity of alkaloids produced by endophytic fungus *N. sphaerica* and *C. roseus* were tested against human breast carcinoma MDA-MB 231 cell line cancer with various concentrations (6.35 to 400 µg/mL). The results showed it was significantly different from concentrations of alkaloids, but not from the sources of these alkaloids (Table 6.2). The test showed a positive result with a Half Maximal Inhibitory Concentration (IC_{50}) value of > 32 µg/mL and 350 µg/mL for CFE and CLE respectively (Figure 6.6). This happened when the Vinca alkaloid was binding to beta-tubulin and disruption of microtubule function during mitosis, which turn leads to mitosis arrest and cell death (Damen et al., 2010). Therefore, after treated 72 h with the alkaloids produced by CFE and CLE, some of the breast cancer cells started to die (Figure 6.7). The apotopsis assay was carried out to support the data on MTT assay by using AnnexinV staining assay where the important mechanism of this assay is programmed cell death or apoptosis in the chemotherapeutic technique (Dickson & Schwartz, 2009; Call et al., 2008).

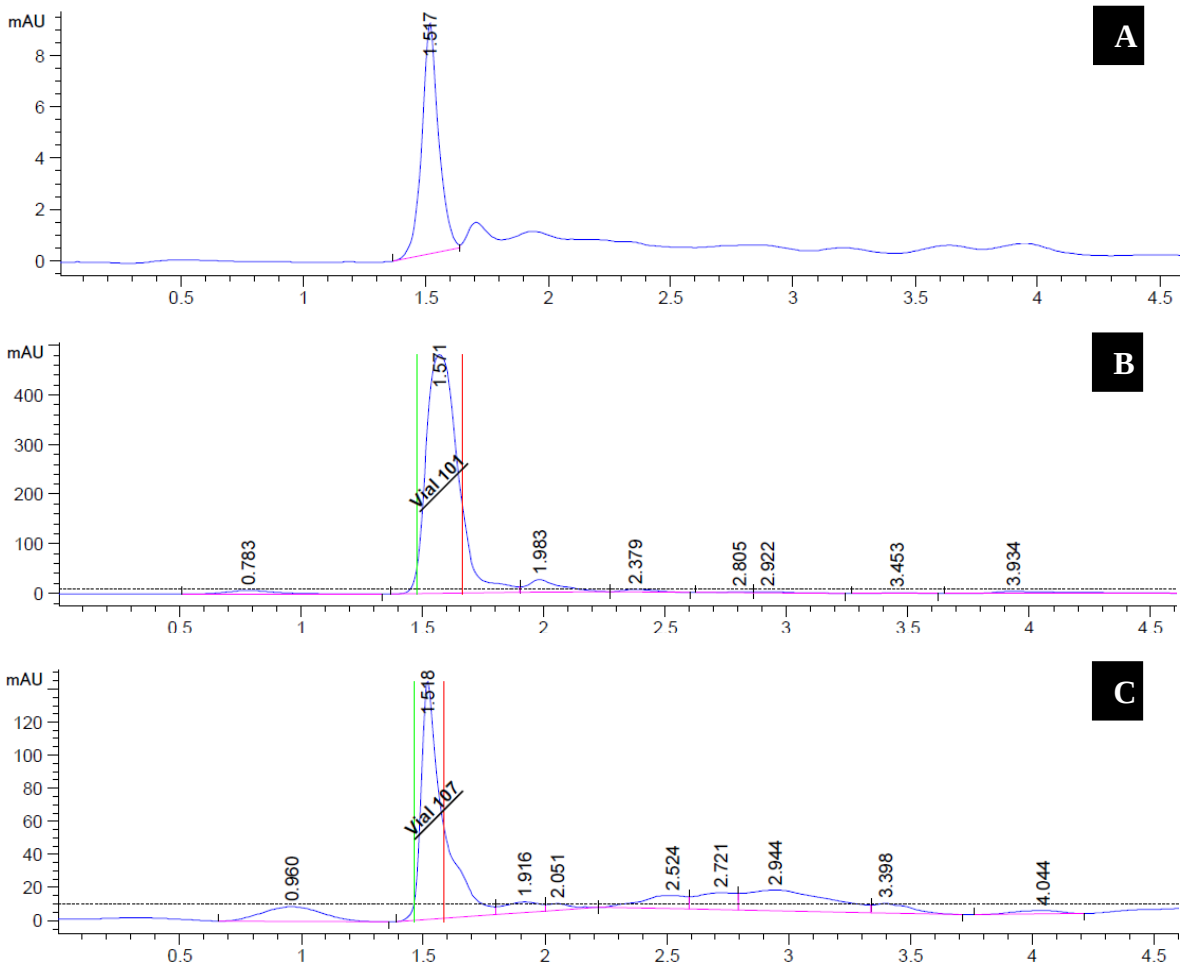


Figure 6.4: The High-Performance Liquid Chromatography (HPLC) chromatograph of commercial standard vinblastine (A), vinblastine produced by the crude mycelia extract of *N.sphaerica* isolated from *C. roseus* – white plant (B) and crude leaf extract of *C. roseus* – white (C) has been purified at retention time of 1.52 to 1.57 min.

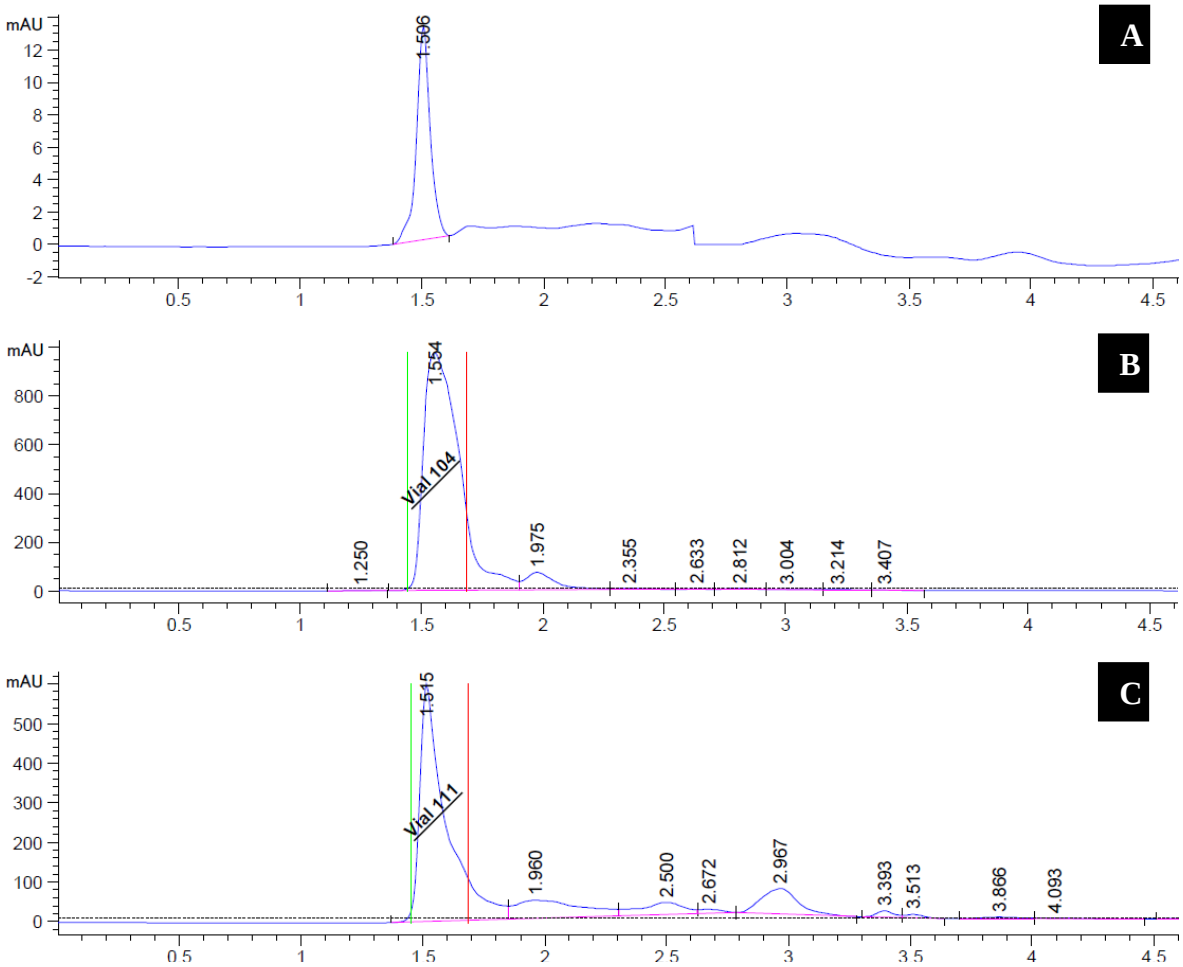


Figure 6.5: The High-Performance Liquid Chromatography (HPLC) chromatograph of commercial standard vincristine (A), vincristine produced by the crude mycelia extract of *C. gloeosporioides* isolated from *C. roseus* – purple plant (B) and crude leaf extract of *C. roseus* – purple (C) has been purified at retention time of 1.51 to 1.56 min.

This assay may distinguish between cell death or late apoptosis, early apoptosis and intact cells (Cheng et al., 2004) by staining with Annexin VFITC and propidium iodide (PI) (Lay et al., 2014). The result showed that breast cell line cancer MDA-MB 231 treated with alkaloids purified from *N. sphaerica, C. gloeosporioides*, *C. roseus* (white and purple) have a percentage of early apoptosis 3.1 %, 1.61 %, 1.20 % and 1.34 % , and a percentage of late apoptosis 4.57 %, 7.82 %, 4.15 % and 3.16 % respectively.These data were compared with the sample treated with commercial standard alkaloid vinblastine and vincristine as where they have a percentage of early apoptosis 6.83 % and 10.73 % and a percentage of late apoptosis 8.04 % and 9.75 % respectively. Meanwhile, the control sample showed a percentage of early and late apoptosis 16.18 % and 7.94 % respectively. All these data were shown in Figure 6.8 and 6.9. The results indicated that the effects of programmed cell death or apoptosis on breast cell line cancer MDA-MB 231 were induced by purified alkaloids (vinblastine and vincristine) from both crude fungal extract and crude leaf extract.

Jordan and Wilson (2004) in their reports also mentioned that the anticancer activity of these alkaloids was attributed to their ability to disturb microtubules metaphase arrest in dividing cell. Before this only one report on vinblastine produced from endophytic fungi *Alternaria* sp. isolated from the *C. roseus* (Gua et al., 1998). This new finding will help to fill the demand of this valuable natural product for cancer treatment. This was supported by Ravindra et al. (2011) that among the metabolites produced by the endophytic fungi attention is attracted to the compound with anti-cancer properties. So far, there were 100 anticancer substances classified in 19 different chemical classes with an activity against 45 different cancer cell lines that have been isolated from 50 fungal species (Abdulmyanova et al., 2015).

Table 6.2: The percentage of inhibition on MTT assay of alkaloids purified from a crude mycelia extract (CME) of *C. gloeosporioides*, *N.sphaerica* isolated from *C. roseus* plant and a crude leaf extract (CLE) of *C. roseus* against breast cell line cancer MDA-DB 231 with various concentrations 6.35 to 400 µg/mL.

	Percentage of inhibition (%)						
	Concentration (µg/mL)						
	6.35	**12.50**	**25.00**	**50.00**	**100.00**	**200.00**	**400.00**
CG	42.36±0.00^{a}	45.14±0.00^{c}	49.31±0.00^{e}	29.17±0.00^{g}	34.72±0.00^{i}	30.56±0.00^{k}	42.46±0.01^{m}
NS	31.17±0.01^{a}	30.52±0.00^{c}	41.56±0.00^{e}	63.64±0.00^{g}	61.04±0.00^{i}	61.67±0.00^{k}	60.39±0.00^{m}
CR(P)	11.11±0.00^{a}	14.14±0.00^{c}	25.02±0.00^{e}	27.27±0.00^{g}	20.20±0.01^{i}	24.24±0.00^{k}	35.35±0.00^{m}
CR(W)	27.89±0.00^{a}	45.26±0.00^{c}	30.53±0.00^{e}	25.79±0.01^{g}	29.47±0.01^{i}	32.63±0.01^{k}	55.26±0.00^{m}
VB	49.57±3.35^{b}	49.52±3.91^{d}	50.69±1.55^{f}	52.83±5.94^{h}	56.24±6.34^{j}	64.54±5.28^{l}	75.88±6.61^{n}
VC	44.22±6.35^{b}	48.10±7.40^{d}	52.06±5.10^{f}	57.19±8.43^{h}	64.58±11.71^{j}	71.25±8.39^{l}	79.50±7.45^{n}

Values presented are the means ± SD, (n = 3). Values within the same column and having same superscript letters are not significantly different ($p < 0.05$). CG: *C. gloeosporioides*, NS: *N. sphaerica*, CR(P): *C. roseus* purple, CR(W): *C. roseus* white, VB:Vinblastine and VC:Vincristine.

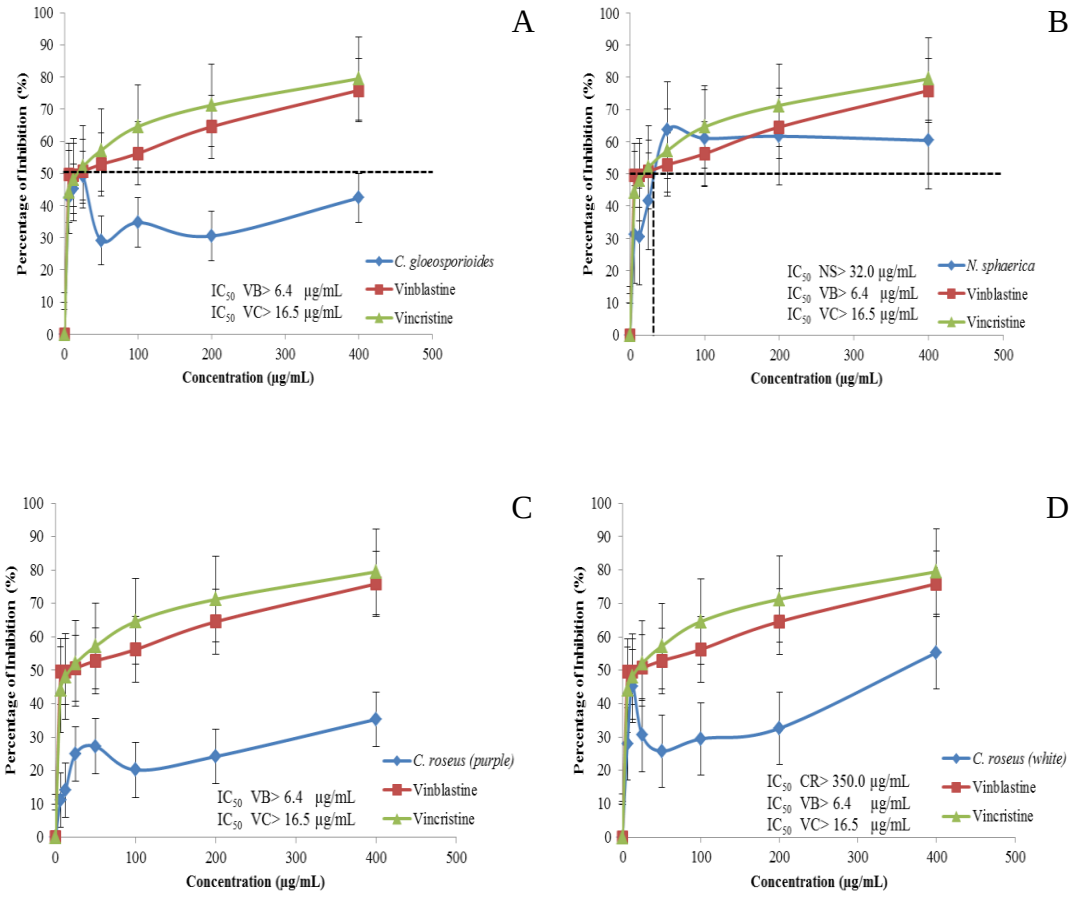


Figure 6.6: The MTT assay of alkaloids purified from a crude mycelia extract (CME) of *C. gloeosporioides* (A), *N. sphaerica* (B) isolated from *C. roseus* plant and a crude leaf extract (CLE) of *C. roseus*-purple (C) and *C. roseus*-white (D) against breast cell line cancer MDA-DB 231 with various concentrations 6.35 to 400 µg/mL.

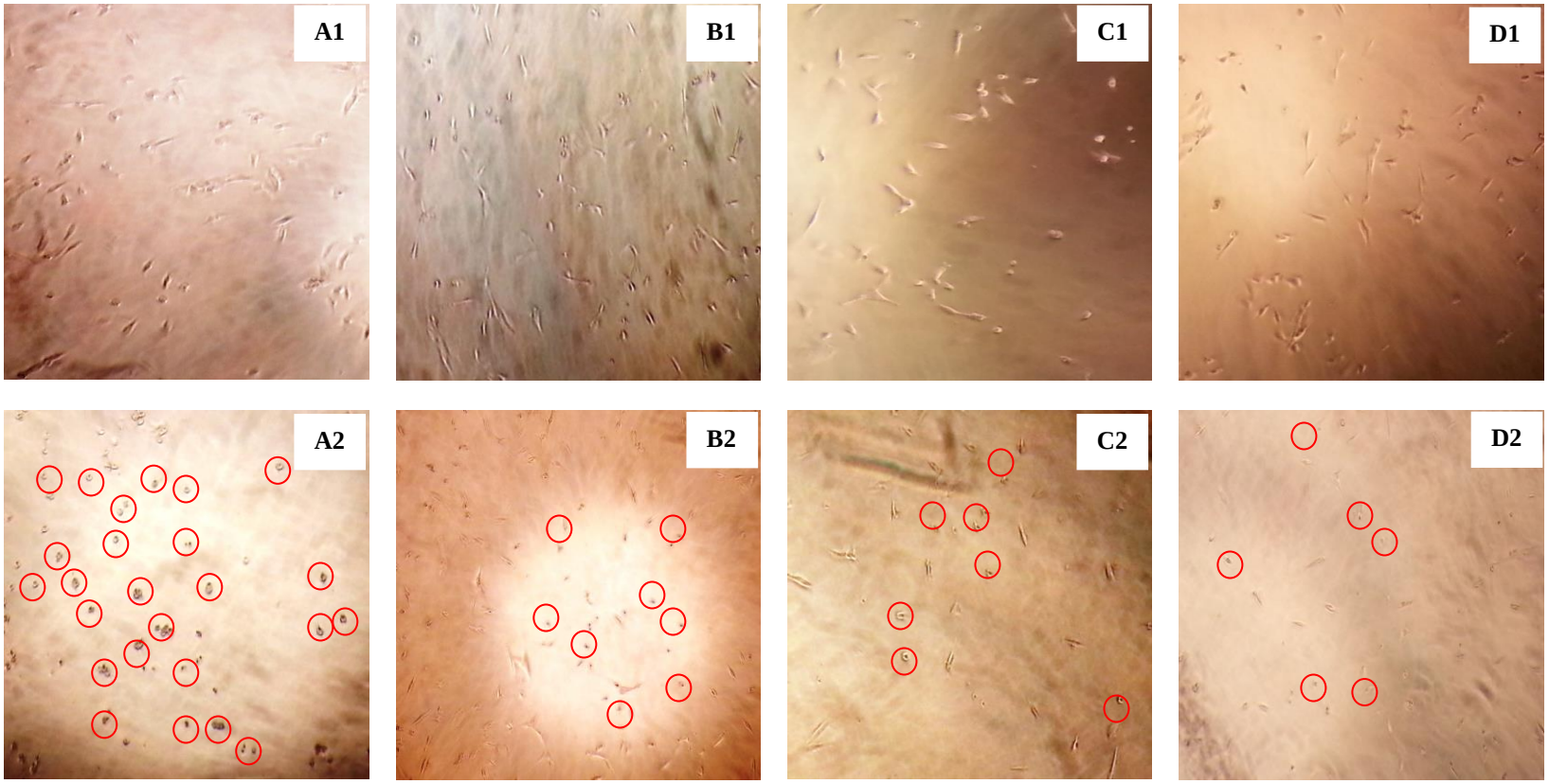


Figure 6.7: The structure of breast cell line cancer MDA-DB 231 (1) before treated with alkaloids purified from a crude mycelia extract of *N. sphaerica* (A1), crude mycelia extract (CME) of *C. gloeosporioides* (B1), crude leaf extract (CLE) of *C. roseus* - white (C1) and crude leaf extract of *C. roseus* – purple (D1). (2) After 72 h treated with different concentration of vinblastine and vincristine produced by all crude extracts, the structure of breast cell lines changed from a rod shape into a round shape (red circle).

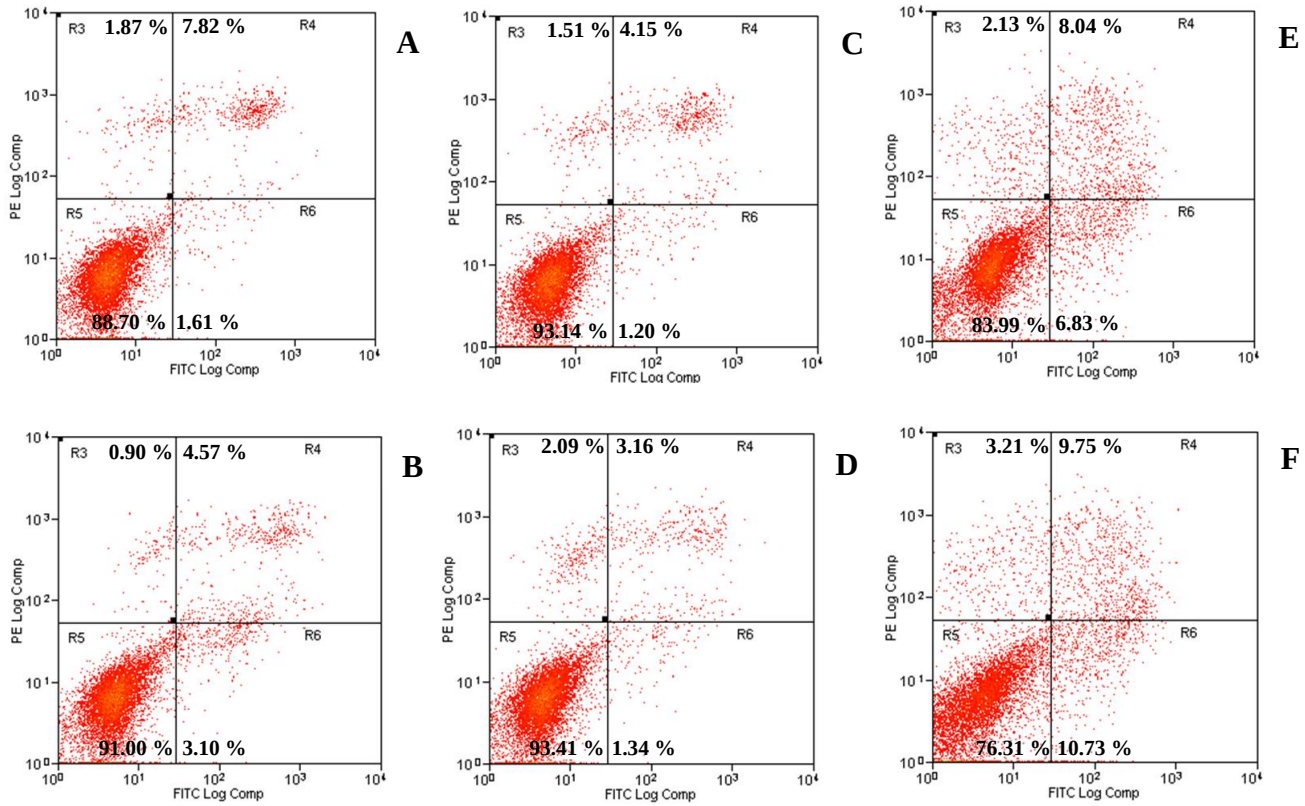


Figure 6.8: The percentage of viable, early apoptotic cells, late apoptotic/early necrotic cells and late necrotic cells of breast cell line cancer MDA-DB 231 after 72 h treated with alkaloids purified from a crude mycelia extract *C. gloeosporioides* (A) and *N. sphaerica* (B), crude leaf extract of *C. roseus* - purple (C) and *C. roseus* – white (D), commercial standard vinblastine (E) and vincristine (F) . The result was determined by flow cytometry analysis using Annexin-V/PI. R5= viable cells, R6= Early apoptosis, R4= Late apoptosis/ early necrosis and R3= Late necrosis.

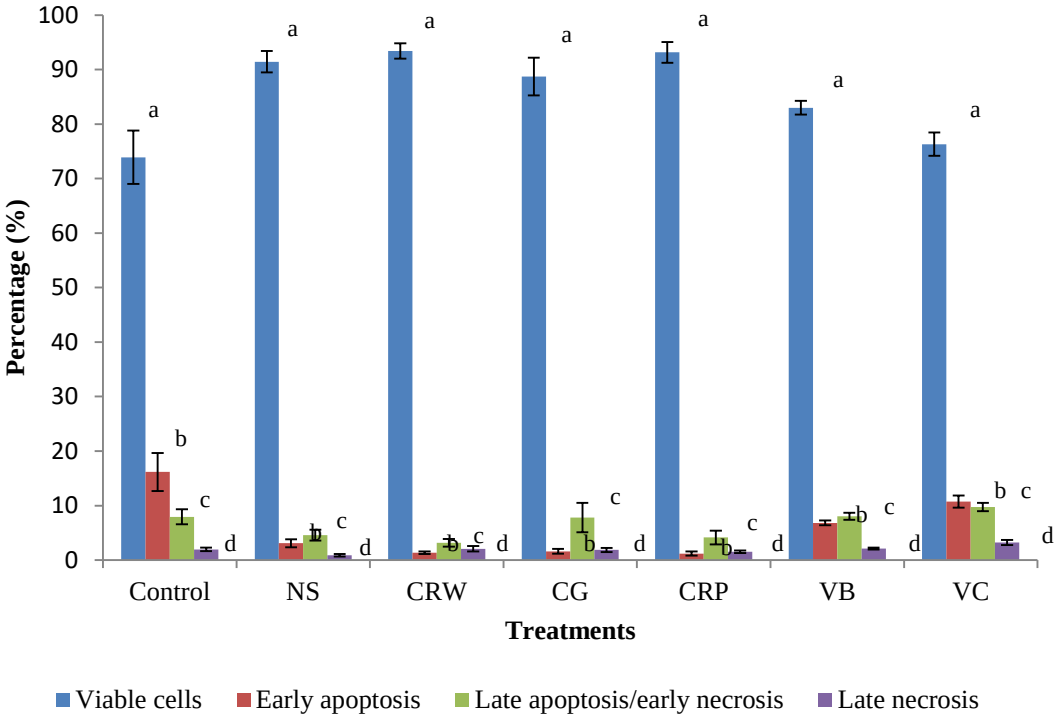


Figure 6.9: The percentage of viable, early apoptotic cells, late apoptotic/ early necrotic cells and late necrotic cells of untreated and alkaloids-treated MDA-MB-231 cells for 72 h as determined by flow cytometry analysis using Annexin-V/PI. Values presented are the means ± SD, (n = 3). Values having the same superscript letters are not significantly different ($p < 0.05$). CG: *C. gloeosporioides*, NS: *N. sphaerica*, CRP: *C. roseus* purple, CRW: *C. roseus* white, VB:Vinblastine and VC:Vincristine.

6.4 Conclusion

This is the first report on *N. sphaerica* and *C. gloeosporioides* isolated from a medicinal plant *C. roseus*. The crude mycelia extract (CME) of *N. sphaerica* was positive produced vinblastine with a concentration of 0.868 µg/mL compared to the crude leaf extract (CLE) which produced a concentration of 0.666 µg/mL. While the crude mycelia extract of *C. gloeosporioides* was positive produced vincristine. However the concentration was very low. The cytotoxicity test using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against breast cell line cancer MDA-MB 231 for both extracts showed that vinblastine produced by crude mycelia extract of *N. sphaerica* has a better half maximal inhibitory concentration (IC_{50}) > 32

µg/mL compared to the vinblastine from a crude leaf extract of *C. roseus* > 350 µg/mL. The programmed cell death or apoptosis assay gave a solid result of anticancer by inducing the purified alkaloids from both crude fungal extract and crude leaf extract to the breast cell line cancer MDA-MB 231. Currently, the commercial vinblastine in the market was produced by *C. roseus*. However, it takes almost one year before it is ready for harvesting. On the other hand, vinblastine produced by *N. sphaerica* only takes only a month (cultivation, extraction and purification) before it is ready to use. As a conclusion, the vincristine and vinblastine produced by crude mycelia extract of *C. gloeosporioides* and *N. sphaerica* respectively could be produced faster with a huge amount instead of current resource of this alkaloid; *C. roseus*.

CHAPTER 7: CONCLUSIONS AND RECOMMENDATIONS

7.1 Conclusions

The main objective of this research was to find a new endophytic fungus that could produce vinca alkaloids, vinblastine and vincristine from the host plant *Catharanthus roseus*. Thus, this chapter will conclude all the findings regarding to all the endophytic fungi associated with a *C. roseus* plant.

1. In summary, we successfully identified the putative endophytic fungi isolated from different tissues of wildly grown *C. roseus* (purple and white) using a morphology and a molecular technique. The ITS 28s ribosomal RNA gene showed that the species were *C. gloeosporioides, M. phaseolina, N. sphaerica* and *F. solani* based on the sequences from a GenBank of BLAST system. Thus, these endophytic fungi were further studied to determine the natural bioactive secondary metabolites.

2. The antibacterial properties of the endophytic fungi *C. gloeosporioides, M. phaseolina, N. sphaerica* and *F. solani* were tested against *S. aureus* and *E. coli*. *N. sphaerica* showed a positive result against *S. aureus* (1.68 ± 0.08 cm) for plug sample. While crude supernatant extract (CSE) of *F. solani* showed a positive result against *S. aureus* (0.1 ± 0.05 cm, 0.65 ± 0.08 cm and 1.28 ± 0.22 cm) of concentrations (1 g/mL, 5 g/mL and 10 g/mL) respectively. Meanwhile, the antifungal test showed that all these endophytic fungi were positive against *Aspergillus niger* and *Candida albicans*. These endophytic fungi also showed a presence of cellulase enzyme through the qualitative enzymes test where *N. sphaerica* has a highest diameter of clear zone 2.80±0.00 cm. However, only three of these endophytic fungi were positively present for amylase and protease endophytes except *M. phaseolina* where *F. solani* was a great producer of both

enzymes with diameter of clear zone 5.78±0.05 cm and 5.65±0.06 cm respectively.

3. *N. sphaerica* isolated from a medicinal plant *C. roseus* has a potential as an antioxidant agent (based on FRAP test result) due to the significant result on both antioxidant and polyphenolic properties. From the analysis, all crude supernatant extract (CSE) showed the highest result on the antioxidant properties through ferric reducing antioxidant power (FRAP) assay compared to crude mycelia extract (CME) of each endophytic fungi and this also applied to the total phenolic content analysis. Thus, as a summary, the crude supernatant extract (CSE) samples were more potent on antioxidant and polyphenolic assays. Meanwhile, *N. sphaerica* has a great potential as the antioxidant agents and also could produce the polyphenolic compounds.

4. The crude mycelia extract (CME) of *N. sphaerica* was positive produced vinblastine with a concentration of 0.868 µg/mL compared to the crude leaf extract (CLE) which produced a concentration of 0.666 µg/mL. While the crude mycelia extract of *C. gloeosporioides* was positive for vincristine. However the concentration was very low. The cytotoxicity test using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay against breast cell line cancer MDA-MB 231 for both extracts showed that vinblastine produced by crude mycelia extract of *N. sphaerica* has a better half maximal inhibitory concentration (IC_{50}) > 32 µg/mL compared to the vinblastine from a crude leaf extract of *C. roseus* > 350 µg/mL. The apoptosis test by staining with Annexin VFITC and propidium iodide showed that breast cell line cancer MDA-MB 231 treated with alkaloids purified from *N. sphaerica, C. gloeosporioides, C. roseus* (white and purple) have a percentage of early apoptosis 3.1 %, 1.61 %, 1.20 % and 1.34 % , and a percentage of late apoptosis 4.57 %, 7.82 %, 4.15 %

and 3.16 % respectively. Currently, the commercial vinblastine in the market was produced by *C. roseus*. However, it takes almost one year before it is ready for harvesting. On the other hand, vinblastine produced by *N. sphaerica* only takes only a month (cultivation, extraction and purification) before it is ready to use. As a conclusion, the vincristine and vinblastine produced by crude mycelia extract of *C. gloeosporioides* and *N. sphaerica* respectively could be produced faster with a huge amount instead of current resource of this alkaloid; *C. roseus*.

7.2 Recommendations for future works

Through this research, it was motivating to find out other secondary metabolites could be produced by endophytic fungi *N. sphaerica* since it showed a great data on all aspects that we studied. The optimization of the anti-carcinogenic alkaloids produced by these endophytic fungi could be studied in order to fulfil the demands of these drugs in pharmaceutical industries.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. Publications

1) Ayob, F.W and Simarani, K. (2016). Endophytic filamentous fungus from a *Catharanthus roseus*: Identification and its hydrolytic enzymes. *Saudi Pharmaceutical Journal 24*; 273-278.

2) Ayob, F.W., Simarani, K., Zainal Abidin, N. and Mohamad, J. (2016). First report on *Nigrospora sphaerica* isolated from *Catharanthus roseus* plant with an anticarcinogenic properties. *Microbial Biotechnology, 10*(4), 926-932.

B. Conferences

1. Ayob, F.W., Simarani, K. and Mohamad, J. (2015a). Anti-carcinogenic natural products vinblastine and vincristine using endophytic fungi isolated from ornamental plant *Catharanthus roseus*. Seminar PhD University of Malaya, Kuala Lumpur, Malaysia.

2. Ayob, F.W., Simarani, K. and Mohamad, J. (2015b). Production of anti-carcinogenic natural products vincristine & vinblastine using endophytic fungi isolated from a local ornamental plant. Tech Plan Grand Prix Malaysia. University of Malaya, Kuala Lumpur, Malaysia

3. Ayob, F.W., Simarani, K. and Mohamad, J. (2015c). Alternative production method of natural products vincristine & vinblastine as anti-carcinogen from endophytic fungi. 3 Minutes Thesis Competition, University of Malaya, Kuala Lumpur, Malaysia.

4. Ayob, F.W., Simarani, K. and Mohamad, J. (2014). Antimicrobial properties of enzyme producing fungi isolated from a medicinal plant (*Catharanthus roseus*). 4th ASEAN Science Congress and Sub Committee Conferences, Bogor, Indonesia.

5. Ayob, F.W., Simarani, K. and Mohamad, J. (2013). The high value medicinal plant (*Catharanthus roseus*) associated with the economic value endophytic fungi. 5th Global Summit on Medicinal and Aromatic Plant, Miri, Sarawak, Malaysia.

microbial biotechnology

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First report on a novel *Nigrospora sphaerica* isolated from *Catharanthus roseus* plant with anticarcinogenic properties

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Summary

This paper reports on the vinca alkaloid produced by a novel *Nigrospora sphaerica* isolated from *Catharanthus roseus*. Through liquid chromatography–mass spectrometry (LCMS), only the crude mycelia extract of this fungus was positive for determination of vinblastine. This vinca alkaloid was then purified by using high-performance liquid chromatography (HPLC) and tested for cytotoxicity activity using MTT assays. The breast cell line cancer (MDA-MB 231) was treated with a purified vinblastine which was intracellulary produced by *N. sphaerica*. The purified vinblastine from extracted leaf of *C. roseus* was used as a standard comparison. A positive result with a value of half maximal inhibitory concentration (IC_{50}) of > 32 μg ml^{-1} was observed compared with standard (IC_{50}) of 350 μg ml^{-1} only. It showed that a vinblastine produced by *N. sphaerica* has a high cytotoxicity activity even though the concentration of vinblastine produced by this endophytic fungus was only 0.868 μg ml^{-1}.

Introduction

Endophytes are microbes that refer to microorganisms that live inside the tissues of plants without causing any apparent harm or diseases to the host plant (Strobel, 2002). In fact, they promote the host plant's growth and the formation of secondary metabolites related to the plant defence (Petrini, 1991 & Chandra *et al.*, 2010). They could produce valuable bioactive compounds with a varied application in both of research and applied fields (Ravindra *et al.*, 2014). Endophytic fungi spend the whole part of their life cycle living symbiotically within the healthy tissues of the host plant (Tan and Zhou, 2001; Ravindra *et al.*, 2014). It also has been recognized as one of important and novel resources of natural bioactive products (Strobel *et al.*, 2004) as most endophytes are capable of synthesizing bioactive compounds that may provide plants with a defence against pathogens (Guo *et al.*, 2008). Some of these compounds have proven useful for discovering a novel drug (Yan *et al.*, 2011). There are many reports that endophytic fungi isolated from a medicinal plant produce a new drug or compound that similar to the host plants (Table 1). All these findings will help to fill the demands of the drugs. In fact, the manufacturing cost of the drugs from endophytic fungi is cheaper than production from the plants as it takes a shorter period to produce it.

Catharanthus roseus or well known as a Madagascar periwinkle is a medicinal plant belonging to the family Apocynaceae (Gajalakshmi *et al.*, 2013). Even though this plant is native to Madagascar, it also can be found in Malaysia. Here, it is called as Kemunting Cina and the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA) (Ayob and Simarani, 2016). This plant also well known to produce a lot of important compounds especially vinca alkaloids vinblastine and vincristine (Manganey *et al.*, 1979; Krishnan, 1995). Besides, this plant also produces vindoline and catharanthine which are the major monomer alkaloids as well as a biosynthetic precursor for vinblastine and vincristine (Noble, 1990). In 1960, vinblastine was introduced to treat certain types of cancer including breast cancer, testicular cancer and Hodgkin's disease (Armstrong *et al.*, 1964), while in 1963, vincristine was introduced through oxidization of vinblastine to treat leukaemia (Evans *et al.*, 1963). So far, there are only three reports on these alkaloids produced by the endophytic fungi, which were *Alternaria* sp., *Fusarium oxysporum* and unidentified fungi from *C. roseus* (Guo *et al.*, 1998; Zhang *et al.*, 2000; Yang *et al.*, 2004). Thus, this research was carried out to find a novel endophytic fungus that could produce vinca alkaloids, vinblastine and vincristine from the host plant *C. roseus*. The purified alkaloids will be tested for cytotoxicity test through MTT assay against breast cell line cancer.

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ORIGINAL ARTICLE

Endophytic filamentous fungi from a *Catharanthus roseus*: Identification and its hydrolytic enzymes

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Available online 29 April 2016

KEYWORDS

Hydrolytic enzyme;
Medicinal plant;
Symbiotic microorganisms,
Molecular and morphological identification

Abstract This paper reported on the various filamentous fungi strains that were isolated from a wild grown *Catharanthus roseus*. Based on the morphological characteristics and molecular technique through a Polymerase Chain Reaction and DNA sequencing method using internal transcribed spacer (ITS), these fungi had been identified as a *Colletotrichum* sp., *Macrophomina phaseolina*, *Nigrospora sphaerica* and *Fusarium solani*. The ultrastructures of spores and hyphae were observed under a Scanning Electron Microscope. The hydrolytic enzyme test showed that all strains were positive in secreting cellulase. *Colletotrichum* sp. and *F. solani* strains also gave a positive result for amylase while only *F. solani* was capable to secrete protease. These fungi were putatively classified as endophytic fungi since they produced extracellular enzymes that allow them to penetrate plant cell walls and colonize with symbiotic properties.

1. Introduction

Catharanthus roseus commonly called Madagascar periwinkle is a herbaceous sub-shrub of latex producing plants belonging to the family *Apocynaceae* (Gajalakshmi et al., 2013). This plant is a native to Madagascar but also found in Malaysia, where it is called Kemunting Cina and is popularly employed in landscaping or gardening due to its colorful flowers. This ornamental plant is also reported to be used as anticancer where it produced the alkaloids called vincristine and vinblastine (Balaabirami and Patharajan, 2012). The report on the medicinal efficacy of this plant incurs the current surge in its global market, and thus the flower of this plant was chosen as a logo for the National Cancer Council Malaysia (MAKNA). However, this plant takes about one year to harvest and leads the high cost of production. Alternative producer of these alkaloids in the short period is required.

In view to the importance of these alkaloids in the medical applications, several studies have been made to discover the potential source for these metabolite. The plant associated microorganisms are believed able to produce similar metabolites as their host plant. Endophytes, microbes that colonize healthy tissues of the plant for at least part of their life cycle without causing any apparent disease symptoms in their host (Petrini, 1991). These endophytes are also recognized as rich sources of secondary metabolites of multifold importance (Tan and Zou, 2001) including enzymes and plant growth hormones (Carol, 1988). Some of these metabolites are bioactive compounds that demonstrated potent anticancer, antibacterial and antiarthritis activities.

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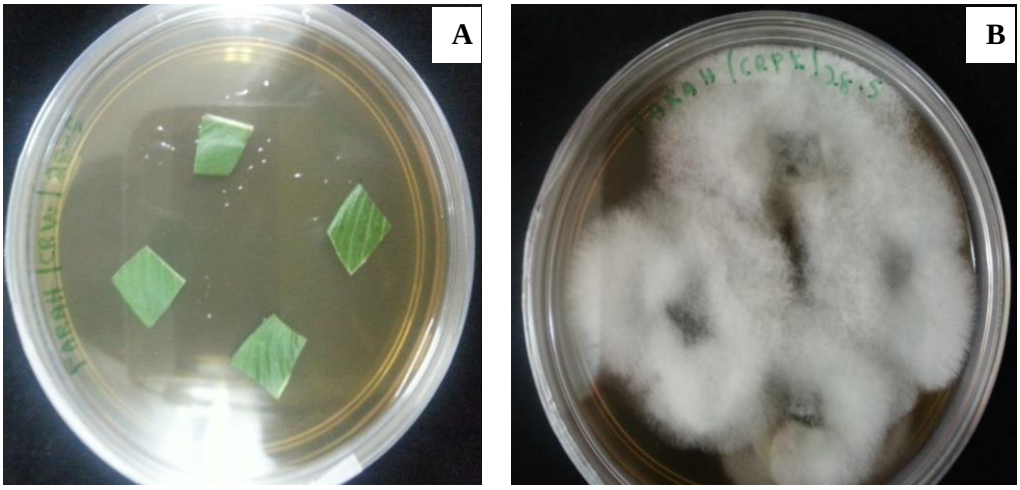
APPENDIX A

MAKNA
Majlis Kanser Nasional
National Cancer Council
A Meaning to Life

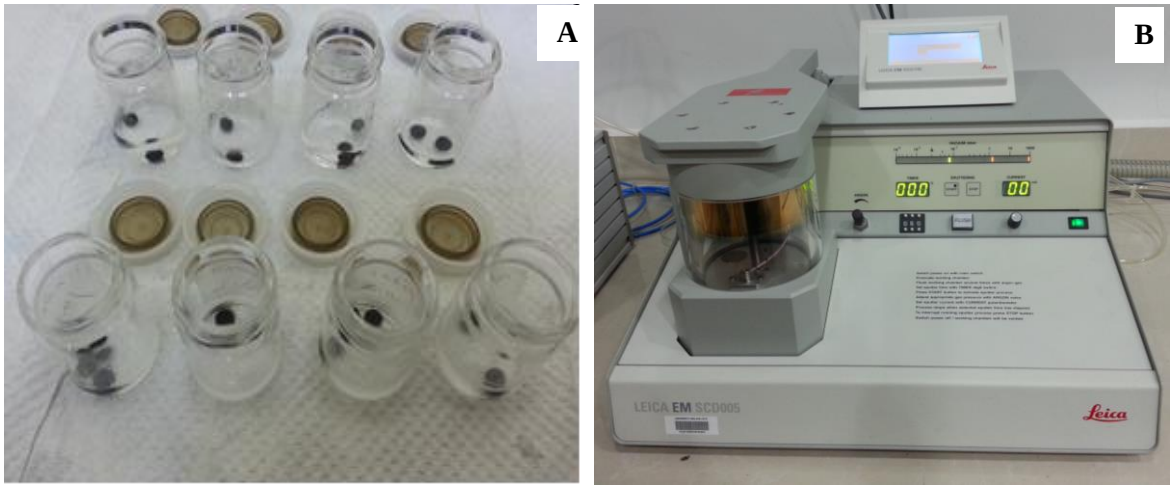
Appendix A : Logo of the National Cancer Council Malaysia (MAKNA) inspired from *C. roseus* plant as a symbol for giving a hope for cancer patients.

APPENDIX B



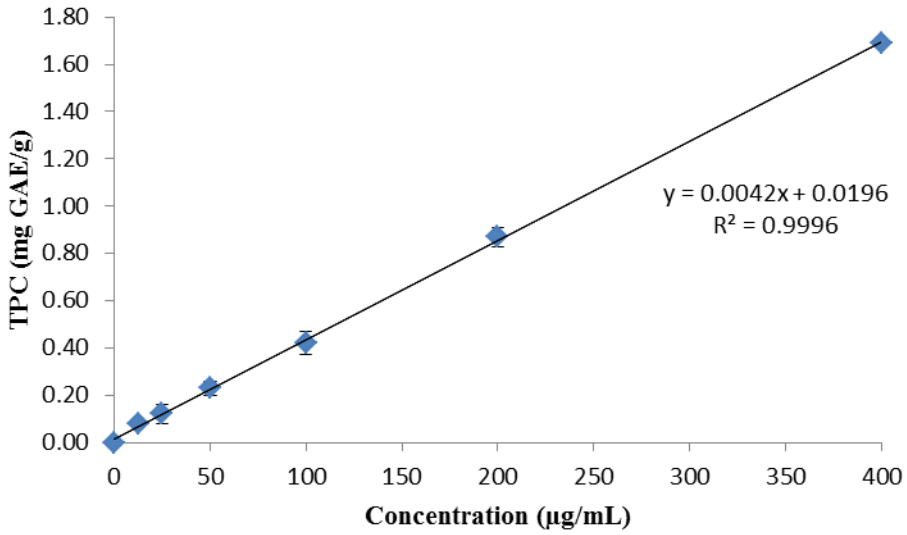
Appendix B: Fungal isolation from a leave of *C. roseus* (A) and the growth of fungus on MEA plate (B).

APPENDIX C



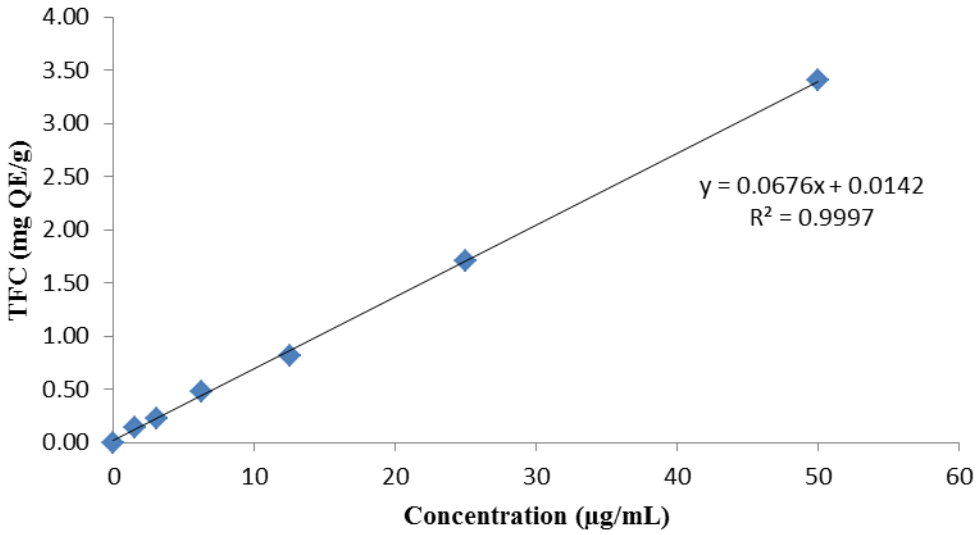
Appendix C: Sample preparation for microstructure identification (A) and coated with gold in an ion-coating (B) before viewing under Scanning Electron Microscope (SEM).

APPENDIX D



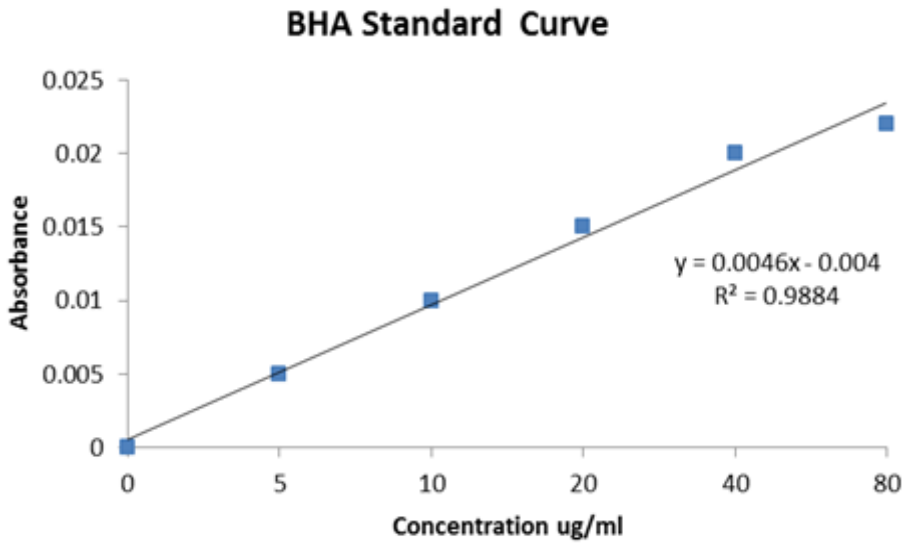
Appendix D: Gallic acid standard curve of total phenolic content.

APPENDIX E



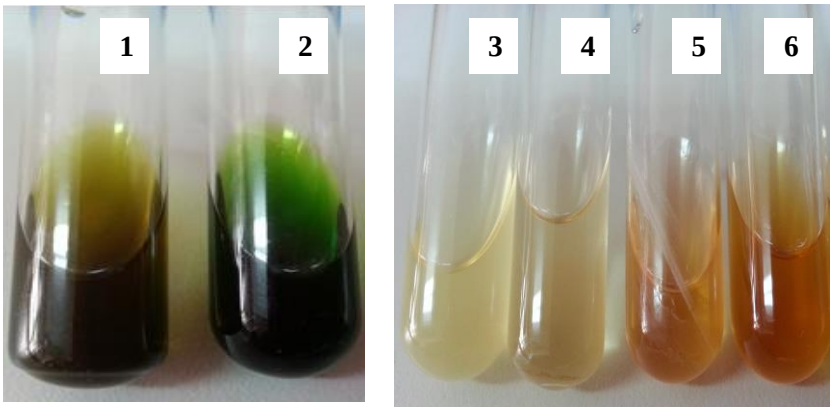
Appendix E: Quercetin standard curve of total flavonoid content.

APPENDIX F



Appendix F: BHA standard curve for DPPH Radical Scavenging Assay.

APPENDIX G



Appendix G: Crude leaf extract (1: *C. roseus*-purple, 2: *C. roseus*-white) and crude fungal extract (3: *C. gloeosporioides*, 4: *M. phaseolina*, 5: *N. sphaerica* and 6: *F. solani*.).