CHAPTER II

LITERATURE REVIEW

2.1 General consideration of endophytic fungi

The term endophyte was originally defined by De Bary in 1866. At the most basic level, it simply refers to the organism: "endo" is the Greek word meaning within and "phyte" is the Greek word for plant, so an endophyte is a microorganisms (mostly fungi and bacteria) which lives inside a plant. Endophytes, in contrast to epiphytes, are contained entirely within the substrate plant (Figure 2.1) and may be either parasitic or symbiotic. Endophytic fungi are asymptomatic and may be described as mutualistic (Clay, 1991). Endophytic fungi were found in a wide range of plant groups including mosses, ferns, lichens, orchids, grasses and trees (Marchisio *et al.*, 1985; Clay, Hardy, and Hammond, 1988; Petrini, Hake, and Dreyfus, 1990; Petrini, Fisher, and Petrini, 1992; Weber, 1995; Kowalski and Rolf, 1996), are extremely abundant and are often very diverse (Stone and Petrini, 1997; Schulthess and Faeth, 1998). The major features of mutualistic symbioses include the lack of destruction of most cells or tissues, nutrient or chemical cycling between the fungus and host, enhanced longevity and photosynthetic capacity of cells and tissues under the influence of infection, enhanced survival of the fungus, and a tendency toward greater host specificity than seen in nectrophic infections (Lewis, 1973).

Members of the Ascomycotina, Basidiomycotina, Deuteromycotina, and some Oomycetes have been isolated as endophytes. Endophytic fungi have been isolated from phanerogams in alpine, temperate and tropical regions, although the plants of the Coniferae, Ericaceae and Gramineae have been most intensively sampled (Clay, 1991; Petrini, 1986; Siegal *et al.*, 1987).

Some fungal endophyte-grass associations, such as that between the perennial rye grass *Lolium perenne* and the fungus *Epichloe* (anamorph *Acremonium*), are common and widespread in natural populations. The association has become very close and the endophyte invades the flowers, is incorporated in the seeds, and passed to the next generation of host grass. The life cycles of endophytic fungi in grasses are shown in the Figure 2.2 (Carlile *et al.*, 2001).

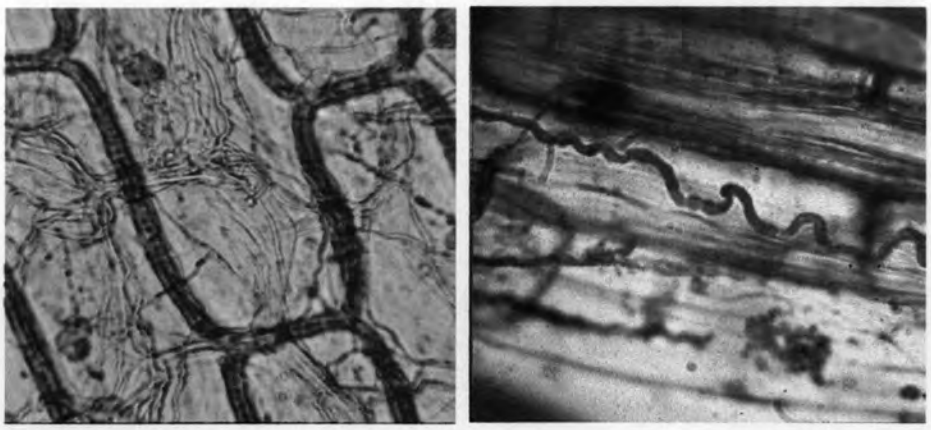

Figure 2.1 Vegetative growths in endophytic fungi of grasses. (A) A hi-power shot of the fungal endophytes within the leaf sheaths of *Arizona fescue* (B) Mycelium of endophytic fungal (*Neothyphodium coenophialum*) in tall festue leaf sheath.

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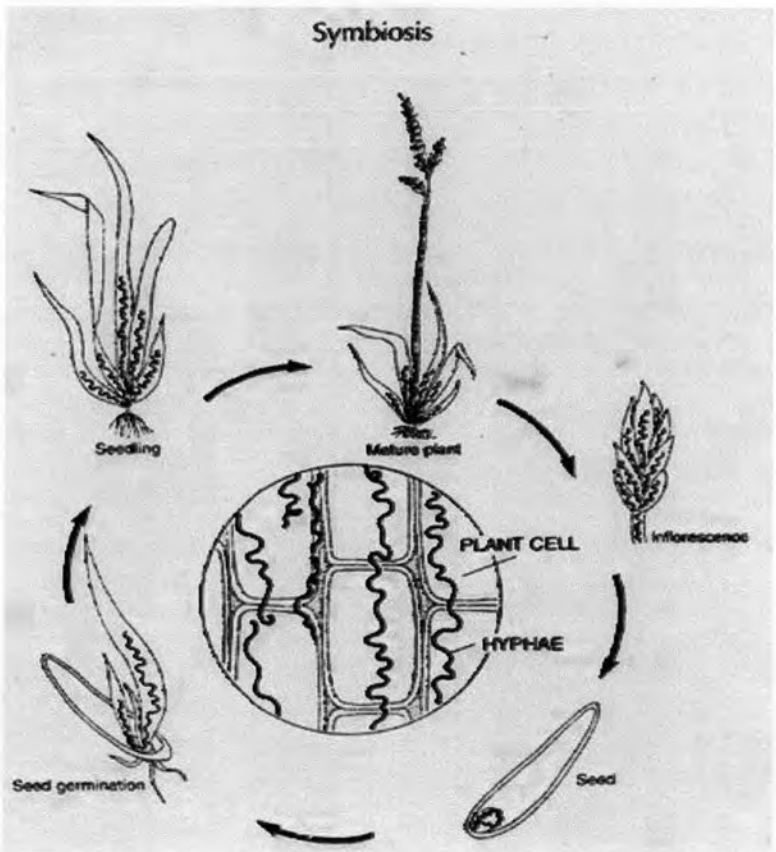


Figure 2.2 Life cycle of *Acremoium coneophiaum*, an endophytic fungus of grass. The fungus only produces hyphae that grow between plant cells (Paracer and Ahmadjian, 2000).

2.2 Endophytic symbioses

Modern usage of the term endophytic in mycology refers to those fungi which live almost entirely within the leaves and stems of apparently healthy host plants, doing so asymptomatically, causing no visible signs of infection. The term endophyte was originally defined by De Bary (1866) to distinguish those species which invade and reside within host tissues or cell from epiphytes, those fungi living on the outer surfaces of host plants. Parasitic antagonistic symbionts which cause visible disease symptoms are more usually referred to as pathogens (e.g. rusts and mildews), even though these may live almost entirely within host tissues (Petrini, 1986). Additionally, although mycorrhizal fungi live both in and on host tissues such associations are not usually included within the endophyte category.

A great deal of interest in endophytes has emerged recently, with the suggestion that such species may be useful as biocontrol agents against insect pests of grasses and with the discovery that such species produce toxins in host plant tissues (Clay, 1986, 1989). There is presently a great deal of discussion concerning the extent to which endophytes are in fact latent pathogens or examples of fungi co-evolving with plants from parasitism to mutualism (Clay, 1988). However, in many cases mutualistic relationships between host plants and fungal endophytes are suspected and in many instances such associations have been identified.

2.3 Host specificity

The degree of host specificity which operates in endophytic fungi is not yet clear. Some species are commonly occurring and may be isolated from various host plant species and from different locations with differing environmental conditions. In general terms, the geographical occurrence of endophytes is related to the distribution of host species. In some cases almost all individuals in a plant population may be infected by endophytes, which are common such as *Cladosporium* spp., *Nodulisporium* spp. and *Pleospora* spp. However, some endophytes do not show such a wide species range and are often isolated from plants of the same family or closely related families. Other species are only rarely detected (Isaac, 1992).

The degree to which endophytes are tissue or organ specific is also not yet clear. Some species are most commonly isolated from similar tissues, particularly the endophytes of conifer needles (Carroll and Petrini, 1983). In other cases the occurrence is less distinct. However, only limited surveys have been carried out to date (Isaac, 1992).

2.4 Natural products from endophytic fungi

The following section shows some examples of natural products obtained from endophytic fungi and their potential in the pharmaceutical and agrochemical arenas.

2.4.1 Anticancer compounds

Paclitaxel (Taxol) and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes (Figure 2.3). Taxol, a highly functionalized diterpenoid, is found in each of the world's yew (*Taxus*) species, but was originally isolated from *Taxus brevifolia* (Wani *et al.*, 1971; Suffness, 1995). By the early 1990s, however, no endophytic fungi had been isolated from any of the world's representative yew species. After several years of effort, a novel paclitaxel-producing endophytic fungus, *Taxomyces andreanae*, was discovered in *T. brevifolia* (Strobel *et al.*, 1993). This compound interacts with tubulin during the mitotic phase of the cell cycle, and thus prevents the disassembly of the microtubules and thereby interrupts the cell division. The original target diseases for this compound were ovarian and breast cancers, but now it is used to treat a number of other human tissue-proliferating diseases as well.

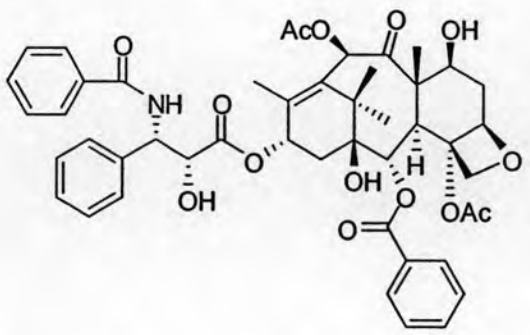


Figure 2.3 The structure of taxol, the world's billion dollar antitumor agent.

An examination of the endophytes of *Taxus wallichiana* yielded *Pestalotiopsis microspora*, and a preliminary monoclonal antibody test indicated that it might produce taxol. Furthermore, other *P. microspora* isolates were obtained from a bald cypress tree in South Carolina and also were shown to produce taxol (Li *et al.*, 1996).

Torreyanic acid, a selectively cytotoxic quinone dimer and potential anticancer agent, was isolated from a *P. microspora* strain (Figure 2.4). This strain was originally obtained as an endophyte associated with the endangered tree *Torreya taxifolia* (Florida torreya) (Lee *et al.*, 1996). Torreyanic acid was tested in several cancer cell lines, and it demonstrated 5-10 times more potent cytotoxicity in those cell lines that are sensitive to protein kinase C agonists and causes cell death by apoptosis.

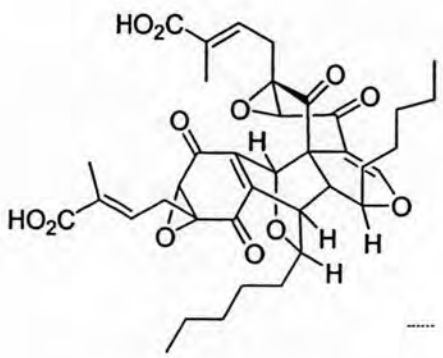


Figure 2.4 Structure of torreyanic acid.

Alkaloids are also commonly found in endophytic fungi. Such fungal genera as xylaria, phoma, hypoxylon, and chalara are representative producers of a relatively large group of substances known as the cytochalasins (Figure 2.5), of which over 20 are now known (Wagenaar *et al.*, 2000). Many of these compounds possess antitumor and antibiotic activities, but because of their cellular toxicity, they have not been developed into pharmaceuticals. Three novel cytochalasins have recently been reported from *Rhinocladiella* sp., an endophyte on *Tripterygium wilfordii.* These compounds have antitumor activity and have been identified as 22-oxa-[12]-cytochalasins (Wagenaar *et al.*, 2000). Thus, it is not uncommon to find one or more cytochalasins in endophytic fungi, and this provides an example of the fact that redundancy in discovery does occur, making dereplication an issue even for these under-investigated sources.

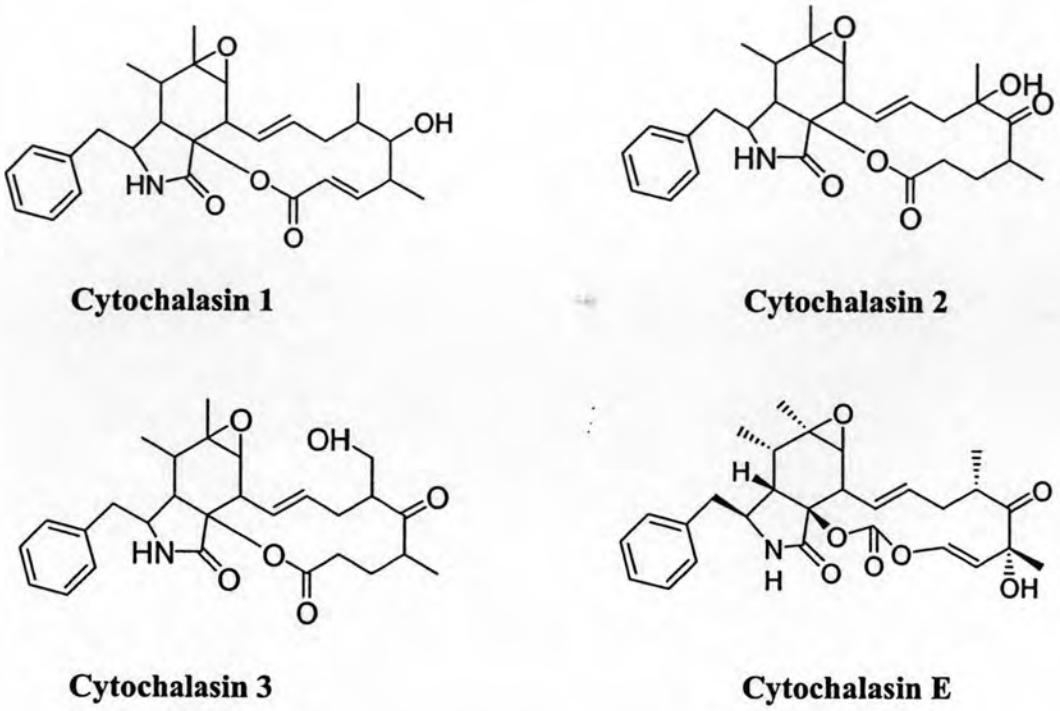


Figure 2.5 The structure of cytochalasins 1, 2, 3, and E.

2.4.2 Antibacterial and antifungal compounds

Cryptocandin was isolated and characterized from *Cryptosporiopsis* cf. *quercina*. This compound contains a number of peculiar hydroxylated amino acids and a novel amino acid: 3-hydroxy-4-hydroxymethylproline (Figure 2.6). *Cryptosporiopsis* cf. *quercina* is the imperfect stage of *Pezicula cinnamomea*, a fungus commonly associated with hardwood species in Europe. It was isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia. On petri plates, *C. quercina* demonstrated excellent antifungal activity against some important human fungal pathogens including *Candida albicans* and *Trichophyton* spp. (Strobel *et al.,* 1999). The bioactive compound is related to the known antimycotics, the echinocandins and the pneumocandins (Walsh, 1992). Cryptocandin is also active against a number of plant pathogenic fungi including *Sclerotinia sclerotiorum* and *Botrytis cinerea.* Cryptocandin and its related compounds are currently being considered for use against a number of fungal-causing diseases of the skin and nails.

Cryptocin, a unique tetramic acid, is also produced by *C. quercina* (Figure 2.7). This unusual compound possesses potent activity against *Pyricularia oryzae* as well as a number of other plant pathogenic fungi (Li *et al.,* 2000). The compound was generally ineffective against a general array of human pathogenic fungi.

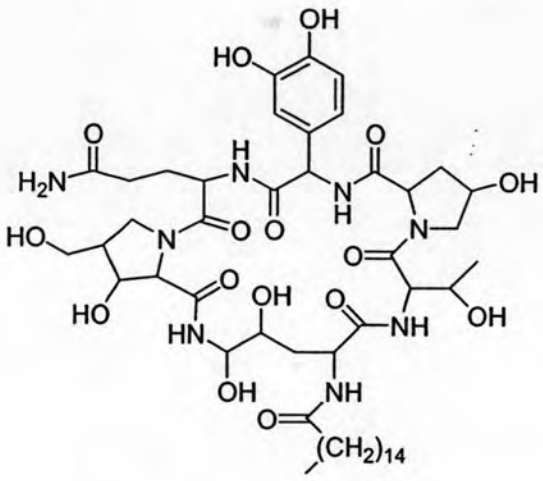


Figure 2.6 The structure of cryptocandin.

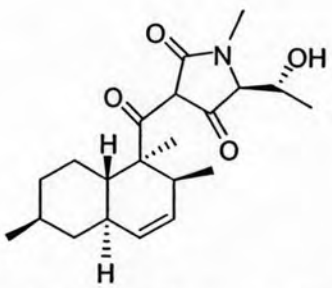


Figure 2.7 The structure of cryptocin.

Ambuic acid, an antifungal agent, which has been recently described from several isolates of *P. microspora* found as representative isolates in many of the world's rainforests (Figure 2.8) (Li *et al.,* 2001). This compound as well as another endophyte products, therein, have been used as models to develop new solid-state NMR tensor methods to assist in the characterization of molecular stereochemistry of organic molecules.

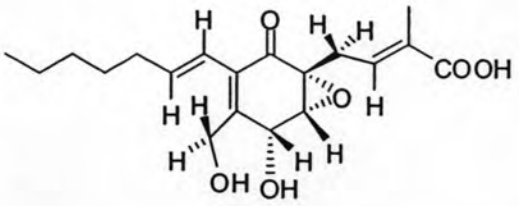


Figure 2.8 Structure of ambuic acid.

In addition, several compounds including pestaloside, an aromatic *β*-glucoside (Figure 2.9), pestalopyrone and hydroxypestalopyrone (Lee *et al.*, 1995), was also isolated from a strain of *P. microspora* which was found in the endangered tree *Torreya taxifolia*. These products also possess phytotoxic properties. Other newly isolated secondary products obtained from *P. microspora* (endophytic on *Taxus brevifolia*) include two new caryophyllene sesquiterpenes, pestalotiopsins A and B (Pulici *et al.*, 1996a).

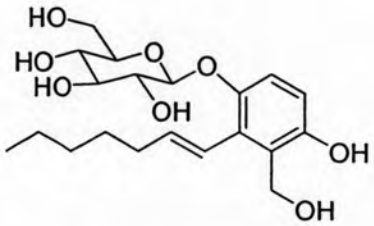


Figure 2.9 Structure of pestaloside.

P. jesteri is a newly described endophytic fungal species from the Sepik river area of Papua New Guinea, and it produces jesterone (Figure 2.10) and hydroxyjesterone, which exhibit antifungal activity against a variety of plant pathogenic fungi (Li and Strobel, 2001). These compounds as a cyclohexenone epoxide, are highly functionalized cyclohexenone epoxides. Jesterone, subsequently, has been prepared by organic synthesis with complete retention of biological activity (Hu *et al.*, 2001).

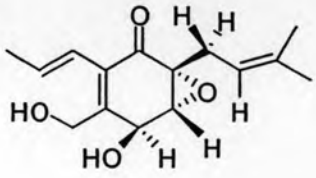


Figure 2.10 Structure of jesterone.

Phomopsichalasin, a metabolite from an endophytic *Phomopsis* sp. (Figure 2.11), represents the first cytochalasin-type compound with a three-ring system replacing the cytochalasin macrolide ring. This metabolite exhibits antibacterial activity in disk diffusion assays (at a concentration of 4 μg/disk) against *Bacillus subtilis, Salmonella gallinarum,* and *Staphylococcus aureus.* It also displays a moderate activity against the yeast *Candida tropicalis* (Horn *et al.*, 1995).

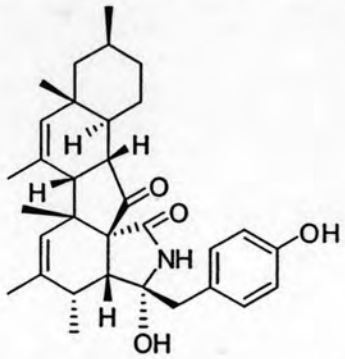


Figure 2.11 Structure of phomopsichalasin.

An endophytic fungi (*Fusarium* sp.), from the interior of a surface-sterilized piece of *Selaginella pallescens* stem tissue, collected in the Guanacaste Conservation Area of Costa Rica, were screened for antifungal activity. CR377, a new pentaketide antifungal agent (Figure 2.12), was isolated from the culture broth of fungi, that showed potent activity against *Candida albicans* in agar diffusion assays (Brady and Clardy, 2000).

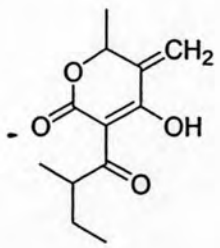


Figure 2.12 Structure of CR377.

Colletotric acid (Figure 2.13), a metabolite of *Colletotrichum gloeosporioides*, an endophytic fungus isolated from *Artemisia mongolica*, displays antibacterial activity against bacteria as well as against the fungus *Helminthsporium sativum* (Zou *et al.*, 2000).

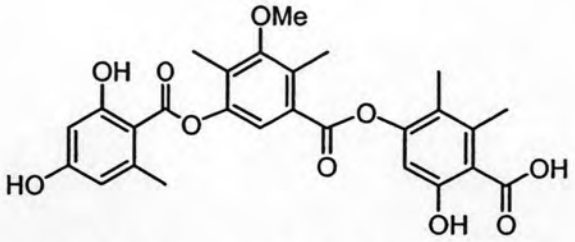


Figure 2.13 Structure of colletotric acid.

The *Colletotrichum* sp. found in *Artemisia annua* which showed antimicrobial activity, produced a new indole derivative 6-isoprenylindole-3-acetic acid (Figure 2.14), not only metabolite with activity against human pathogenic fungi and bacteria but also metabolite that was fungistatic to plant pathogenic fungi (Lu *et al.*, 2000).

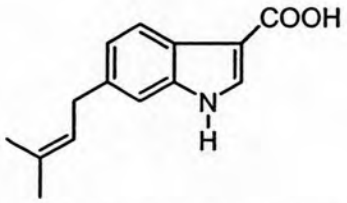
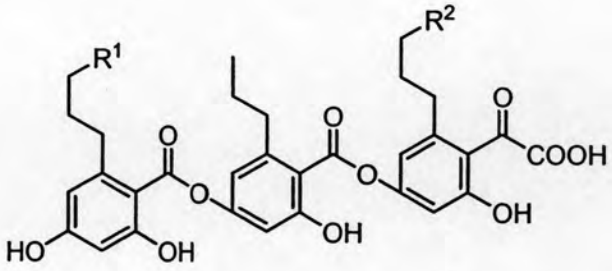


Figure 2.14 Structure of 6-isoprenylindole-3-acetic acid.

2.4.3 Antiviral compounds

Two novel human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B (Figure 2.15), have been isolated from solid-state fermentation of the endophytic fungus *Cytonaema* sp. (Guo *et al*, 2000). It is apparent that the potential for the discovery of compounds having antiviral activity from endophytes is in its infancy. The fact, however, that some compounds have been found already is promising.



cytonic acid A R^1 = Et, R^2 = H

cytonic acid B R^1 = H, R^2 = Et

Figure 2.15 Structure of cytonic acids A and B.

2.4.4 Volatile antibiotics from endophytic fungi

Muscodor albus is a newly described endophytic fungus obtained from small limbs of *Cinnamomum zeylanicum* (cinnamon tree) (Worapong *et al.*, 2001). This xylariaceaous (non-spore producing) fungus effectively inhibits and kills certain other fungi and bacteria by producing a mixture of volatile compounds. The majority of these compounds have been identified by GC/MS, synthesized or acquired, and then ultimately formulated into an artificial mixture. This mixture not only mimicked the antibiotic effects of the volatile compounds produced by the fungus but also was used to confirm the identity of the majority of the volatiles emitted by this organism (Strobel *et al.*, 2001). Each of the five classes of volatile compounds produced by the fungus had some microbial effects against the test fungi and bacteria, but none was lethal. However, collectively they acted synergistically to cause death in a broad range of plant and human pathogenic fungi and bacteria. The most effective class of inhibitory compounds was the esters, of which isoamyl acetate was the most biologically active. The composition of the medium on which *M. albus* grows dramatically influences the kind of volatile compounds that are produced (Ezra and Strobel, 2003). The ecological implications and potential practical benefits of the "mycofumigation" effects of *M. albus* are very promising given the fact that soil fumigation utilizing methyl bromide will soon be illegal in the United States. The potential use of mycofumigation to treat soil, seeds, and plants may soon be a reality. The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits, and other plant parts in storage and while being transported.

Using *M. albus* as a screening tool, it has now been possible to isolate other endophytic fungi producing volatile antibiotics. The newly described *M. roseus* was twice obtained from tree species growing in the Northern Territory of Australia. This fungus is just as effective in causing inhibition and death of test microbes in the laboratory as *M. albus* (Woropong *et al.*, 2002). In addition, for the first time, a non-muscodor species (*Gliocladium* sp.) was discovered as a volatile antibiotic producer. The volatile components of this organism are totally different than those of either *M. albus* or *M. roseus*. In fact, the most abundant volatile inhibitor is [8]-annulene, formerly used as a rocket fuel and discovered for the first time as a natural product. However, the bioactivity of the volatiles of this *Gliocladium* sp. is not as good or comprehensive as that of the *Muscodor* spp. (Stinson, Ezra, and Strobel, 2003).

2.4.5 Products of endophytic fungi with antioxidant activity

Two compounds, pestacin and isopestacin, have been obtained from culture fluids of *Pestalotiopsis microspora*, an endophyte isolated from a combretaceaous plant, *Terminalia morobensis*, growing in the Sepik River drainage system of Papua New Guinea (Strobel *et al.*, 2002; Harper *et al.*, 2003a). Both pestacin and isopestacin display antimicrobial as well as antioxidant activity. Isopestacin was attributed with antioxidant activity based on its structural similarity to the flavonoids (Figure 2.16). Electron spin resonance spectroscopy measurements confirmed this antioxidant activity; the compound is able to scavenge superoxide and hydroxyl free radicals in solution (Strobel *et al.*, 2002). Pestacin (Figure 2.17) was later described from the same culture fluid, occurring naturally as a racemic mixture and also possessing potent antioxidant activity (Harper *et al.*, 2003). Proposed antioxidant activity of pestacin arises primarily via cleavage of an unusually reactive C-H bond and, to a lesser extent, through O-H abstraction. The antioxidant activity of pestacin is at least 1 order of magnitude more potent than that of trolox, a vitamin E derivative (Harper *et al.*, 2003).

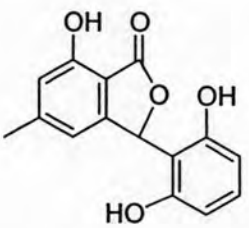


Figure 2.16 Structure of isopestacin.

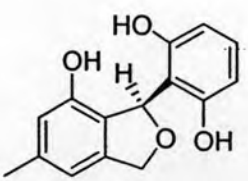
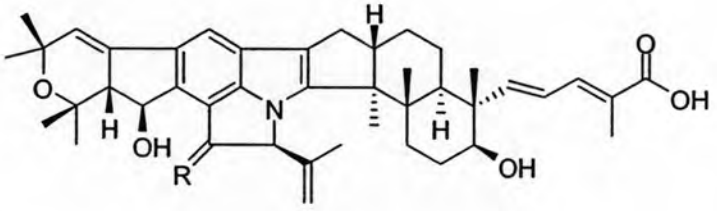


Figure 2.17 Structure of pestacin.

2.4.6 Products of endophytic fungi with insecticidal activity

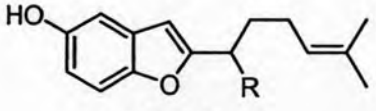
Nodulisporic acids (Figure 2.18), novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels. The first nodulisporic acids were isolated from an endophyte, a *Nodulisporium* sp., from the plant *Bontia daphnoides*. This discovery has since resulted in an intensive search for additional *Nodulisporium* spp. or other producers of more potent nodulisporic acid analogues (Bills *et al.*, 2002). Insect toxins have also been isolated from an unidentified endophytic fungus from wintergreen (*Gaultheria procumbens*). The two new compounds, 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran (Figure 2.19), both show toxicity to spruce budworm, and the latter is also toxic to the larvae of spruce budworm (Findlay *et al.*, 1997).



R = O, Nodulisporic acid A

R = H_2, Nodulisporic acid B

Figure 2.18 Structure of nodulisporic acids A and B.



1 R = O
2 R = OH

Figure 2.19 The structure of 5-hydroxy-2-(1'-hydroxy-5'-methyl-4'-hexenyl) benzofuran and 5-hydroxy-2-(1'-oxo-5'-methyl-4'-hexenyl) benzofuran.

2.4.7 Products of endophytic fungi with antidiabetic activity

A nonpeptidal fungal metabolite (L-783, 281) was isolated from an endophytic fungus (*Pseudomassaria* sp.) collected from an African rainforest near Kinshasa in the Democratic Republic of the Congo (Zhang *et al.*, 1999). This compound acts as an insulin mimetic but, unlike insulin, is not destroyed in the digestive tract and may be given orally. Oral administration of L-783, 281 in two mouse models of diabetes resulted in significant lowering in blood glucose levels. These results may lead to new therapies for diabetes (Zhang *et al.*, 1999).

2.4.8 Products of endophytic fungi with immunosuppressive activity

The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinols A and B (Figure 2.20) (Lee *et al.*, 1995a). Subglutinols A and B are equipotent in the mixed lymphocyte reaction (MLR) and thymocyte proliferation (TP) assays with an IC_{50} of 0.1 μM. In the same assay systems, the famous immunosuppressant drug cyclosporine A, also a fungal metabolite, was roughly as potent in the MLR assay and 10^4 more potent in the TP assay. Still, the lack of toxicity associated with subglutinols A and B suggests that they should be explored in greater detail as potential immunosuppressants (Lee *et al.*, 1995a).

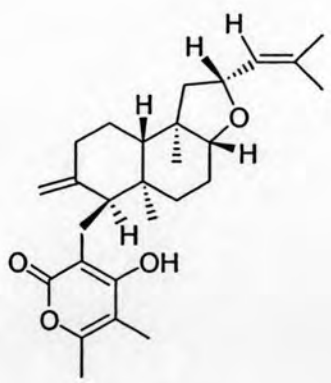


Figure 2.20 Structure of subglutinol A.

2.5 Plant sample

Hydnocarpus anthelminthicus Pierre ex Laness., known in Thai as "Kra-Boa-Yai" (กระเบาใหญ่), belongs to the family Flacourtiaceae which have many species widely distributed in many countries, including China, India, Malasia, Vietnam and Thailand. It is a medium-sized woody plant, frequently grows along streams in evergreen forests at an altitude of 100-500 m. It can be cultivated for ornamental and shading purposes in parks and along roads, as in sunny places it gives more branches and forms beautiful shape. Plenty of flowers and fruits are produced every year (Hai *et al*, 1999).

2.5.1 Botanical description

The followings are the descriptions for *H. anthelminthicus*: Tall tree, 10 m high or more. Stems erect; bark whitish-grey. Leaves alternate, oblong-lanceolate, coriaceous, shining green, base rounded, apex attenuate-obtuse, 10-30 cm long, 3-7 cm wide, veins conspicuous forming a dense net, especially in the lower surface; young leaves entirely pinkish (Figure 2.21).

Inflorescence in axillary raceme, few-flowered; flowers unisexual or polygamous, rose; both sexes on the same plant; sepals 5, villous; petals 5 to which linear scales opposite; stamens 5; ovary superior.

Fruit large, globose; pericarp woody, blackish-brown; seeds are about 18.14 mm. broad, little longer, resemble a small bulb in shape, weigh barely 2 g (the albumen 0.6 g), are brownish black, rough, hard, and have a large rayed hilum. The integument is 1.5 mm, thick, and has a lighter colored inner layer, 0.15 mm thick, and consisting of tangentially arranged, and relatively little thickened, stone cells; the cells of the middle layer are placed at right angles to the surface; those of the exterior layer are tangentially arranged, and those of the adhering pulp are rather small and frequently interspersed with groups of stone cells. The cells of the endosperm are smaller than in *Gynocardia*, and do not contain the yellow bodies seen in the latter, but besides oil contain numerous colorless roundish albuminoid granules (Hai *et al*, 1999).

Flowering period: April-June. Fruiting period: July-November.

Figure 2.21 Leaves and stems of *Hydnocarpus anthelminthicus* Pierre.

2.5.2 Traditional uses

Seeds of mature fruit, picked when fully ripe. Separated from the fruit, the seeds are washed clean, dried and pressed or extracted with solvents to yield oil. This oil increases body weight, the strength of collagen tissue (Wiart, 2002), and used in treating leprosy rheumatism, sprain, cancer, inflammatory, mycobacteriosis, scabies, impetigo and some other dermatitides (ธงชัย เปาอินทร์, 2544; นันทวัน บุณยะประภัศร, 2539; Hai *et al*, 1999).

2.5.3 Chemical composition

The seed kernels contain 40-50% oil consisting of glycerides of chaulmoogric, hydnocarpic and gorlic acids (Figure 2.22). The oil is brownish yellow and has a special odour. At temperature below 25°C, the oil usually condenses into white soft masses. Some analytical indices of hydnocarpus oil are as follows: d_{25}, 0.94-0.96; n_D30, 1.473; $[\alpha]_D^{15}$, (+47°) - (+54°), mp., 20-25°; saponification value, 201-212; acid value, 8; iodine value, 83-91; unsaponifiable matter, 1%.

The oil dissolved in benzene, chloroform, ether, petroleum ether, slightly in cold alcohol and entirely in boiling alcohol (Hai *et al*, 1999).

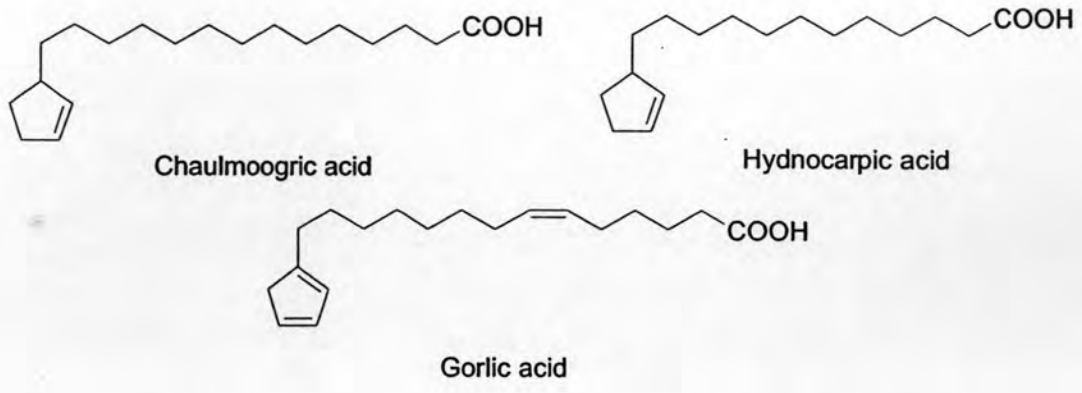


Figure 2.22 Structures of fatty acid from seeds.

2.5.4 Pharmacological actions

The seed oil and its derivatives inhibit acido-resistant bacteria such as *Mycobacterium tuberculosis* and *M. leprae.* It is difficult to penetrate into the bacteria for the oil itself, so its inhibiting action is mild, while its derivativtes are more active. Sodium salts of chaulmoogric and hydnocarpic acids, the main acids existing in seed oil, at the concentration of 1:100,000 still inhibit *M. tuberculosis* and protect guinea-pigs infected with this bacterium (Hai *et al*, 1999).

The *H. anthelminthica* oil is excitative to body tissues. When applied to skin, it causes rubefaction and occasionally blisters. Its oral administration excites the gastro-intestinal tract, produces vomiting and diarrhea (Hai *et al*, 1999).

2.5.5 Therapeutic uses

The seed oil has been employed to cure leprosy since long. At present, it is substituted by other more effective and convenient anti-leprosy drugs. Because of its vomitive action, the oil should be given in gradually increasing doses to reduce excitative effect to the gastro-intestinal tract. Better results are obtained by intramuscular injection, especially in the form of iodinated derivatives. Sodium salt, ethyl and esters of the fatty acids are commonly used in leprosy therapy (Hai *et al*, 1999).