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Metabolomics of Endophytic Fungi Producing Associated Plant Secondary Metabolites: Progress, Challenges and Opportunities

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1. Introduction

Microorganisms are indispensable for every aspect of human life, in fact all life on earth, although they cannot be seen by the naked eye. Since time immemorial, every process in the biosphere has been affected by the apparently unending ability of microbes to renovate the world around them. More recently, many discoveries have been made in isolating a special class of microorganisms, mainly fungi but also bacteria, commonly called endophytes, which have been shown to have the natural potential for accumulation of various bioactive metabolites which may directly or indirectly be used as therapeutic agents against a plethora of maladies (Kusari & Spiteller, 2010, 2011). Bioprospecting endophytes have led to exciting possibilities to explore and utilize their potential. Several bioprospecting strategies might be employed in order to discover potent endophytes with desirable traits (Figure 1). These include randomly sampling different plants from any population to isolate the associated endophytes, or first performing a detailed study of an ecosystem in order to determine its features with regard to its natural population of plant species, their relationship with the environment, soil composition, and biogeochemical cycles. Another approach is to evaluate the evolutionary relatedness among groups of plants at a particular sampling site, correlating to species, genus, and populations, through morphological data matrices and molecular sequencing, followed by isolation of endophytes from the desired plants. Traditional medicinal plants are also bioprospected for endophytes, especially for the ones capable of producing one or more of the bioactive secondary metabolites present in the host plants. Finally, the valuable information obtained using the different bioprospecting schemes can be pooled together, comparatively evaluated, and stored for further use applying suitable data mining approaches.

2. What is an endophytic fungus?

Endophytes are microorganisms that internally infect living plant tissues without causing any visible manifestation of disease, and live in mutualistic association with plants for at least a part of their life cycle (Bacon & White, 2000). The term 'endophyte' (Gr. *endon*, within; *phyton*, plant) was first contrived by de Bary (1866). All types of microorganisms (fungi,

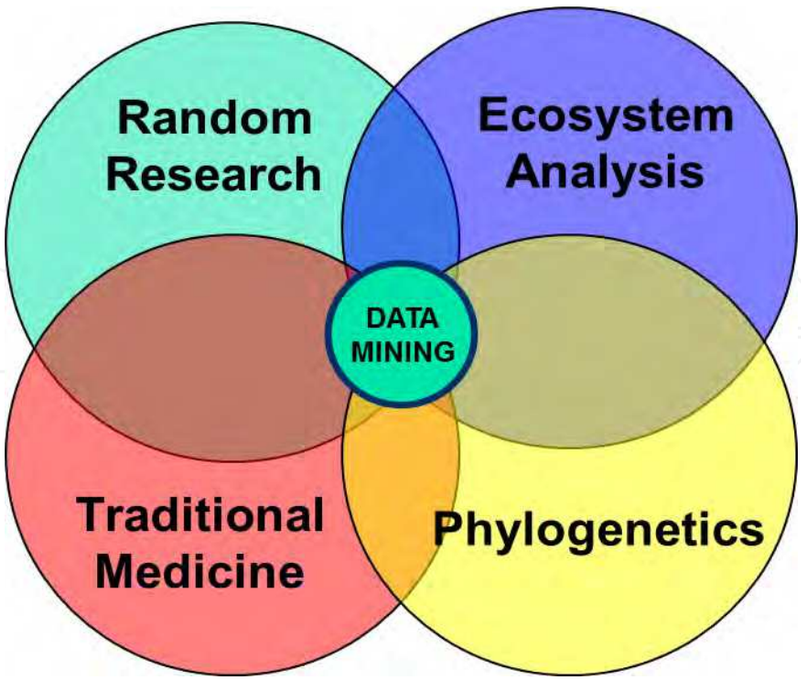


Fig. 1. Different bioprospecting strategies that might be utilized in order to discover novel or competent endophytes with desirable features.

bacteria, and actinomycetes) have been discovered as endophytes. The most frequently encountered endophytes are fungi (Staniek et al., 2008). Fungal endophytes constitute an inexplicably diverse group of polyphyletic fungi ubiquitous in plants, and maintain an indiscernible dynamic relationship with their hosts for at least a part of their life cycle (Figure 2a,b). The existence of fungi inside the tissues of asymptomatic plants has been known since the end of the nineteenth century (Guerin, 1898). Evidence of plant-associated microorganisms found in the fossilized tissues of stems and leaves has revealed that endophyte-plant associations may have evolved from the time higher plants first appeared on the earth (Redecker et al., 2000). However, except for some infrequent studies, it was not until the end of the twentieth century that fungal endophytes began to receive more attention from scientists. Since endophytes were first described in the Darnel (Freeman, 1904), various investigators have isolated endophytes from different plant species. These discoveries led to a worldwide search for novel endophytes for the better understanding and applicability of such a promising group of microorganisms. On the one hand, the ecological aspects of endophytic fungi such as host range, evolutionary relatedness, infection, colonization, transmission patterns, tissue specificity, and mutualistic fitness benefits have been investigated relating to a plethora of plants (Arnold et al., 2003, 2007, Arnold, 2005, 2007; Stone et al., 2004; Schulz &Boyle, 2005; Rodriguez et al., 2009) (Figure 2c). On the other hand, many discoveries have been made in isolating endophytic fungi, which have been shown to have the potential for *de novo* synthesis of various bioactive metabolites that may directly or indirectly be used as therapeutic agents against numerous ailments (Strobel and Daisy, 2003; Strobel et al., 2004; Zhang et al., 2006; Gunatilaka, 2006; Staniek et al., 2008; Suryanarayanana et al., 2009; Aly et al., 2010; Kharwar et al., 2011; Kusari & Spiteller, 2010, 2011).

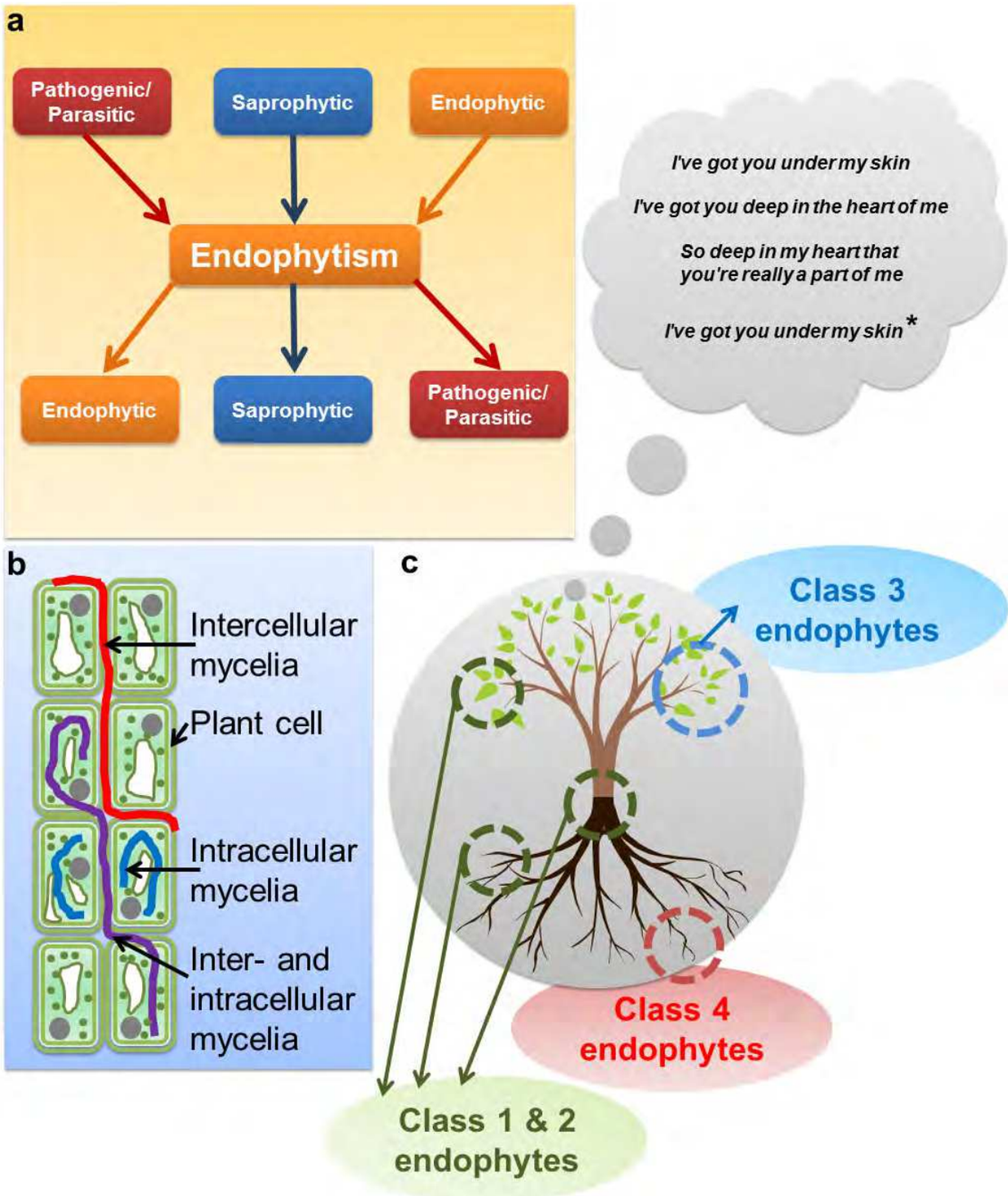


Fig. 2. A diagrammatic representation of endophytic fungal association with their host plants. (a) Possible life histories of endophytic fungi pre- and post-'endophytism'. (b) Different localization patterns of fungal endophytes within plant tissues. (c) Location of the different classes of endophytes (according to Rodriguez et al., 2009). *An artistic rendition of the possible thoughts of a plant about its endophytic counterparts, presented in memory of Cole Albert Porter (1891-1964) and Frank Sinatra (1915-1998).

3. Endophytic fungi producing host plant secondary metabolites

The possibility that endophytes biosynthesize associated plant compounds was first comprehended and published by Stierle et al. (1993), following the highly heralded discovery of endophytic *Taxomyces andreanae* that produces the multi-billion dollar anticancer compound Taxol® (generic name: paclitaxel), which was isolated from the Pacific yew tree *Taxus brevifolia*. Inspired by this discovery, numerous efforts have been made to identify endophytes as sources of associated plant natural products. Endophytes producing antineoplastic camptothecin (CPT) and its structural analogs (Puri et al., 2005; Kusari et al., 2009b, 2011b; Shweta et al., 2010), anticancer pro-drugs podophyllotoxin (Eyberger et al., 2006; Puri et al., 2006) and deoxypodophyllotoxin (Kusari et al., 2009a), antidepressants hypericin and emodin (Kusari et al., 2008, 2009c), and natural insecticides azadirachtin A and B (Kusari et al., 2011c) are some of the interesting discoveries that followed. Novel fungal endophytes capable of producing other associated plant secondary metabolites of therapeutic importance, such as artemisinin, morphine, cannabinoids, and many more, remain to be discovered and characterized.

4. Rationale for plant selection to provide the best opportunities for isolating endophytic fungi producing associated plant natural products

Considering the enormous numbers and the diversity of plants, ingenious strategies should be utilized to narrow the search for endophytes producing plant compounds. A specific rationale for the collection of each plant for endophyte isolation could be proposed to maximize possibility of discovering endophytes equipped with the capacity to produce associated plant natural products. Several hypotheses governing this plant selection strategy might be exploited.

4.1 Plants from inimitable ecological niche, especially those with an uncommon morphology and possessing unusual strategies for subsistence

4.1.1 Case study: *Hypericum perforatum*

Plants from a distinct ecological niche or with unusual biology might also harbor potent endophytes. A fine example of such a plant is *Hypericum perforatum*, which is commonly called St. John's wort (Wichtl, 1986) (Figure 3a). This plant is a pseudogamous, facultatively apomictic, perennial medicinal plant that is native to Europe, West and South Asia, North Africa, North America, and Australia (Hickey and King, 1981; Wichtl, 1986). In general, *Hypericum* has always been a very important medicinal plant occupying a significant place in ancient history. Pedanius Dioscorides, the foremost ancient Greek herbalist, mentioned four species of *Hypericum* - *uperikon*, *askuron*, *androsaimon*, and *koris*, which he recommended for sciatica, "when drunk with 2 heim of hydromel (honey water)" (Gunther, 1959). *H. perforatum* has also been in use, at least from the time of ancient Greece (Tammaro and Xepapadakis, 1986), as an antidepressant, in healing of wounds and menstrual disorders, due to the presence of the-then unknown bioactive compounds in the plant. This plant has also found historical use in India, China, Egypt and many countries of Europe, where the tribal peoples have been burning this plant to represent sun, light, vitality and strength (Hickey & King, 1981). We know now that this plant produces the widely used antidepressant compound hypericin (Brockmann

et al., 1950). Hypericin is a photodynamic compound (Kubin et al., 2005) which is localized and probably also synthesized in the 'dark glands' (Onelli et al., 2002), which are small specialized glandular structures dispersed over all above-ground parts of the plant (flowers, capsules, leaves, stems) but not in the roots (Hölzl et al., 2003) (Figure 3b,c). Therefore, using the rationale that a plant with such an uncommon biology (dark glands) for protecting itself from the photodynamic effects of its own metabolite might also contain endophytes that have been evolutionarily co-adapted to accumulate the same or similar molecules, we undertook bioprospecting endophytic fungi from *H. perforatum* sampled from various populations across Europe and the Himalayan region. This led to the discovery of an endophytic fungus associated with this plant capable of producing hypericin and emodin in axenic cultures (Kusari et al., 2008, 2009c).

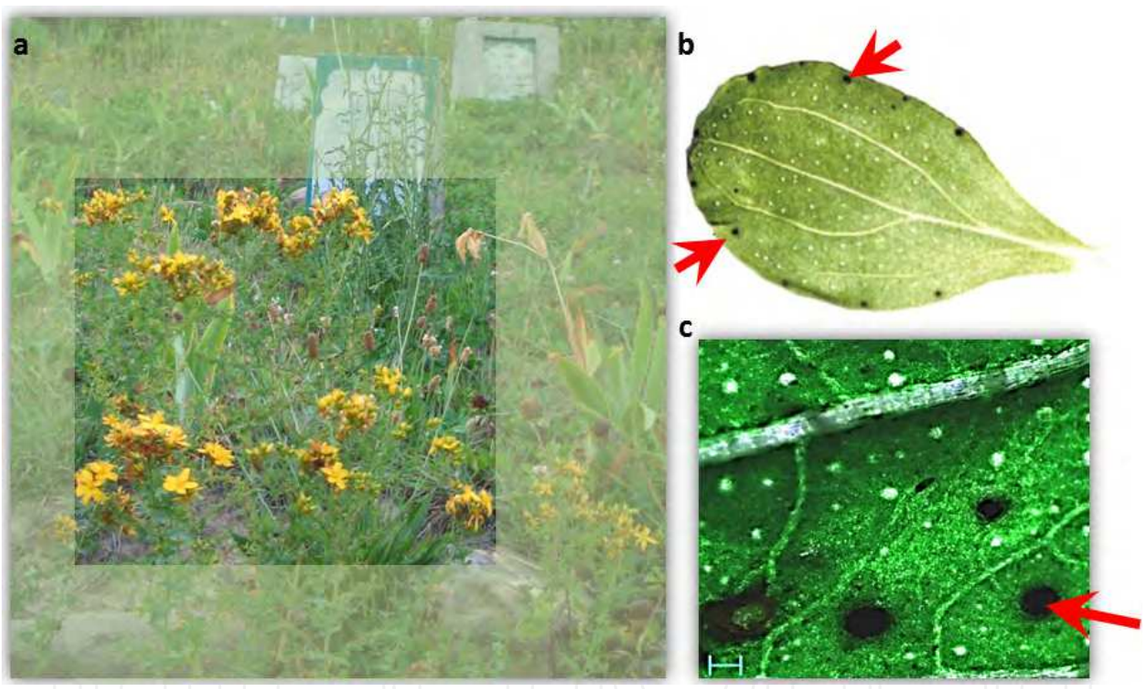


Fig. 3. *Hypericum perforatum* as a suitable example of bioprospecting plants with an unusual morphology, possessing unusual strategies for existence, and from unique environments. (a) Wild *H. perforatum* growing at the Himalayan environments (Harwan, Jammu and Kashmir, India) from where the hypericin- and emodin-producing endophyte was isolated. (Photograph courtesy of M. Spiteller). (b) A representative leaf of *H. perforatum* where the dark glands (arrows) can be seen. (c) A brightfield microscopic image of the leaf adaxial surface where the dark glands (arrow) can be seen as black spots (using Leica S8 APO Greenough stereo microscope, Leica Microsystems GmbH, Wetzlar, Germany; scale = 1 mm. The image was captured using Leica EC3 digital camera and processed using the Leica Application Suite LAS EZ ver. 1.6.0).

4.2 Plants that have an ethno-botanical history that is associated with the specific practices or applications of interest

4.2.1 Case study: *Juniperus* species

Juniperus plants (Figure 4) serve an excellent example to describe this rationale, which contain the therapeutically important anticancer lignans podophyllotoxin and deoxypodophyllotoxin (Hartwell et al., 1953). This species was in use as early as in the first century A.D., when Gaius Plinius Secundus mentioned that the smaller species of *Juniperus* could be used, among other things, to stop tumors or swelling (Imbert, 1998). The use of the oil of *Juniperus* species (*J. sabina*, *J. phoenicea* and *J. communis*) for the treatment of ulcers, carbuncles and leprosy was also mentioned by Dioscorides (Gunther, 1959). Generally, the dried needles, called *savin*, or the derived oil was used. In 47 A.D., Scribonius Largus wrote that *savin* oil was used to soften "hard female genital parts" (Sconocchia, 1983). *Savin* was later also used to treat uterine carcinoma, venereal warts and polyps. Based on such historical use by indigenous people, we recently isolated and characterized endophytic fungi harbored in *Juniperus* plants sampled from the natural populations in Dortmund and Haltern, Germany, and Jammu and Kashmir, India. This resulted in the discovery of a deoxypodophyllotoxin-producing endophytic fungus harbored in *J. communis* (Kusari et al., 2009a).

Fig. 4. *Juniperus* as an example of bioprospecting plants having an ethnobotanical history associated with the specific practices or applications of interest. Some *Juniperus* species growing at Rombergpark, Dortmund, Germany are shown. (Photographs courtesy of S. Kusari and M. Spiteller).

4.3 Endemic or endangered plants

4.3.1 Case study I: *Camptotheca acuminata*

Endemic plants are frequently consorted with ecological peculiarities or typical locations that are geographically distinct. Many of these plants are getting vulnerable owing to their therapeutic, agricultural, environmental, and commercial value. These distinct plants might also harbor a plethora of unique endophytes. One out of many examples is the plant *Camptotheca acuminata* (Figure 5a). This plant grows in mainland China, and is commonly called the 'happy tree', which is a direct translation of the Chinese word '*Xi Shu*'. *Camptotheca* was first recorded in 1848 and scientifically described and named by Decaisne (1873). The genus name *Camptotheca* is from the Greek *Campto* (meaning, bent or curved) and *theca* (meaning, a case) referring to the anthers, which are bent inward. The species name *acuminata* is derived from *acuminate*, which refers to the tips of leaves. This

plant has been in use as traditional medicine in China for treatment of psoriasis, liver and stomach ailments and the common cold (Sung et al., 1998). The present application of this plant is on account of the fact that it contains substantial quantities of an important antineoplastic drug, namely camptothecin (CPT). This plant is uprooted and harvested by various sectors, including medical groups, pharmaceutical companies and scientists from around the world, to isolate CPT for numerous purposes (Lorence & Nessler, 2004; Sankar-Thomas, 2010). In addition to the difficulties of the practical total synthesis of this natural compound, the unpredictable problems of nature such as erratic weather and pests (Kusari et al., 2011d) have rendered this plant species vulnerable to extinction. As such, in 2000 and again in 2006, *C. acuminata* was proposed for protection in the CITES (Convention for International Trade in Endangered Species), World Conservation Monitoring Centre, appendix II (Anonymous, 2000, 2006). This appendix lists species that are not necessarily now threatened with extinction but that may become so unless trade is closely controlled. There are, of course, some nurseries growing *C. acuminata* for commercial purposes. These few nurseries, however, cannot meet the demand for CPT production (Sankar-Thomas, 2010). Furthermore, the yields of CPT from field trees vary widely and depend on factors that are difficult to control. For instance, plant diseases such as leaf spot and root rot are some of the major fungal diseases that can limit the cultivation of *Camptotheca* plants (Li et al., 2005) and diminish the production of CPT. Cultivation of *Camptotheca* plants is limited to subtropical climates and it takes about ten years for plants to produce a stable fruit yield (Li et al., 2005; Sankar-Thomas, 2010). The combination of a high demand for CPT and its scarcity from natural plant sources has, therefore, led to a different strategy of bioprospecting the endophytic fungi associated with the *C. acuminata* as alternate sources of CPT and related metabolites (Kusari et al., 2009b, 2011b).

Fig. 5. *Camptotheca acuminata* and *Nothapodytes nimmoniana* as fitting examples of bioprospecting endemic or endangered plants. (a) *C. acuminata* growing at the Southwest Forestry University campus, Kunming, Yunnan Province, China. (b) *N. nimmoniana* growing at the Western Ghats, India. (Photographs courtesy of M. Spiteller).

4.3.2 Case study II: *Nothapodytes nimmoniana*

Another plant containing CPT, *Nothapodytes nimmoniana* of Indian origin (Figure 5b) has also been subjected to extensive harvesting resulting in vulnerability of the plant. Unlike *C. acuminata*, there are no major commercial plantations of *N. nimmoniana* (Shaanker et al., 2008). This has led to harvesting of the plants sampled directly from the wild populations in India to meet the present and projected demands of CPT. In fact, it is estimated that in the last decade alone, there has been at least 20% decline in the population of *N. nimmoniana*, leading to the red-listing of this species (Kumar & Ved, 2000; Hombe Gowda et al., 2002). Therefore, a need for the preservation of this endangered plant species as well as to ensure a continuous supply of CPT has been felt. Endophyte research on this plant has yielded notable results (Puri et al., 2005).

4.4 Plants growing in areas of abundant biodiversity

4.4.1 Case study: *Azadirachta indica*

Plants growing in areas where a multitude of biotic and abiotic factors play essential roles and generating bioactive natural products might harbor diverse endophytic population. *Azadirachta indica* A. Juss. (synonym *Melia azadirachta*), commonly known as the Indian neem or Indian lilac (Butterworth, 1968) is well known in India and its neighboring countries for more than 2000 years as one of the most versatile medicinal plants growing abundantly in regions having high biodiversity of plants (Biswas et al., 2002). Traditionally, neem-based formulations have been used to cure a variety of ailments such as fever, pain, leprosy, and malaria in *Ayurvedic* and *Unani* medical treatments, but the most striking property of neem tree reported to date is its insect-repellent property (Veitch et al., 2008). It is now established that neem plants contain the natural product azadirachtin (i.e., azadirachtin A and structural analogues) that distresses insects as an antifeedent and insect growth regulator (Lay et al., 1993). Numerous natural bioactive compounds have been isolated and characterized from different parts of the neem tree, and new natural products are being discovered every year. Thus, it would seem that *A. indica* is a potential natural resource sheltering unique and competent endophytes having a multitude of desirable traits. As such, neem plants have not only been studied for their endophytic microflora concerning the composition, diversity, and distribution of endophytic microbes within the plants (Rajagopal & Suryanarayanan, 2000; Mahesh et al., 2005; Verma et al., 2007, 2009), but also for endophytes producing bioactive natural products (Li et al., 2007; Kharwar et al., 2009; Wu et al., 2008, 2009). Recently, we isolated an endophytic fungus from this plant that is capable of producing azadirachtin A and B under *in vitro* axenic conditions (Kusari et al., 2011c).

5. Metabolomics of endophytic fungi producing associated plant natural products

5.1 Anticancer compounds

5.1.1 Case study I: Camptothecin (CPT) and structural analogues

Camptothecin (CPT), a pentacyclic quinoline alkaloid, is a potent antineoplastic agent, which was first isolated from the wood of *Camptotheca acuminata* Decaisne (Nyssaceae), a plant native to mainland China (Wall et al., 1966). CPT and its structural analogues (Figure

6) have emerged as one of the most promising agents for cancer treatment owing to the typical action mechanism involving DNA-Topoisomerase I, i.e., they cause DNA damage by stabilizing a normally transient covalent complex between Topoisomerase I (Topo 1) and DNA (Hsiang et al., 1985; Kusari et al., 2011a,d). CPT interacts with the Topo 1-DNA complex, thereby forming a ternary complex that stabilizes the trans-esterification intermediate (Hertzberg et al., 1990; Pommier et al., 1995). Thus, by stabilizing the cleavable complex, CPT transforms the normally useful enzyme Topo 1 into an intracellular, cytotoxic poison, and hence, CPT and structural analogues are called 'topoisomerase poisons' or 'topoisomerase inhibitors' (Lorence & Nessler, 2004).

Endophytic *Entrophospora infrequens* (Puri et al., 2005; Amna et al., 2006) and *Neurospora crassa* (Rehman et al., 2008) isolated from *N. nimmoniana* were initially reported to produce CPT. However, in both cases, there have been no further studies on how the fungi are able to produce CPT and prevent self-toxicity from the intracellular accumulated CPT. Further, no follow-up work on up-scaling the production of CPT has been performed, and there is no published breakthrough in the commercial exploitation of these endophytic fungi as a source of CPT.

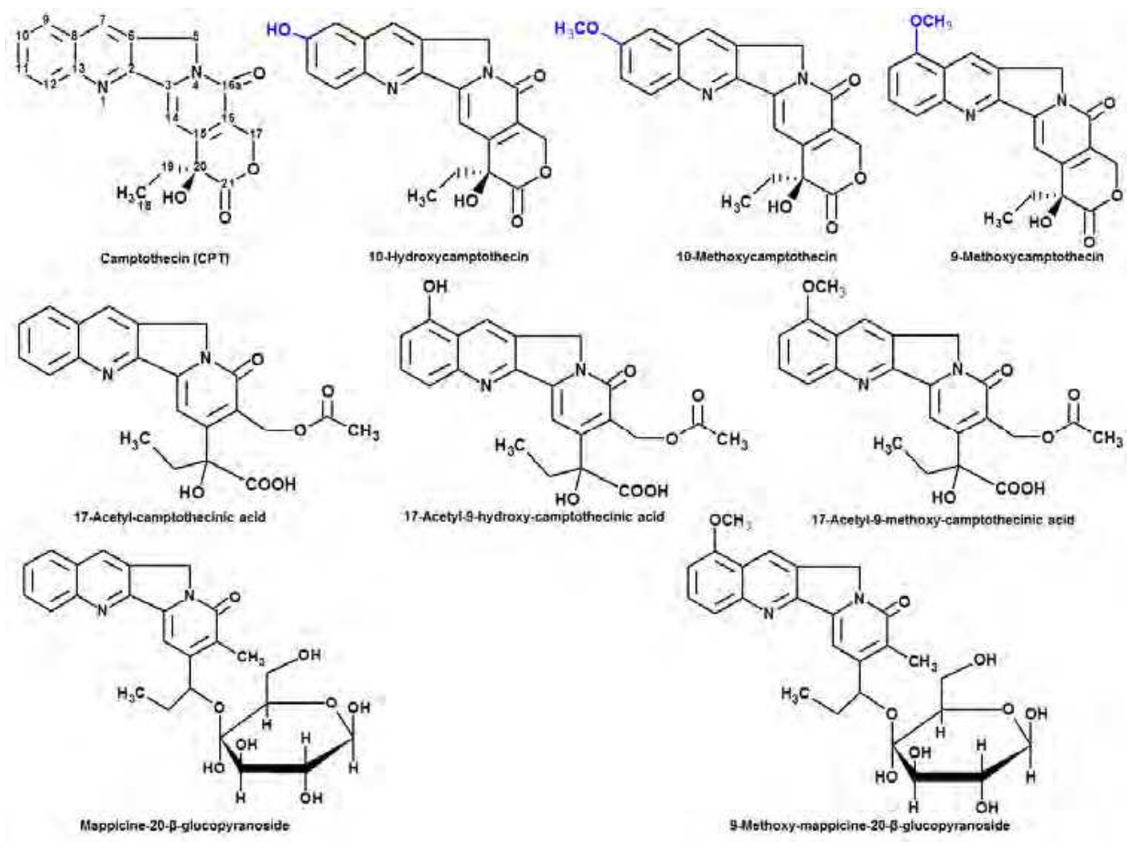


Fig. 6. Camptothecin (CPT) and some important natural analogues found in plants.

Recently, we isolated an endophytic fungus, *Fusarium solani*, from the inner bark of *Camptotheca acuminata* Decaisne, obtained from the Southwest Forestry University (SWFU) campus, Kunming (Yunnan Province), People's Republic of China (Figure 7a). This

endophyte is capable of indigenously producing CPT, 9-methoxycamptothecin (9-MeO-CPT), and 10-hydroxycamptothecin (10-OH-CPT) in submerged *in vitro* axenic culture (Kusari et al., 2009b). We further investigated how this endophytic fungus, capable of producing CPT, ensures self-resistance before being incapacitated by its own and the associated plant's CPT biosyntheses. We discovered its survival strategy by examining the fungal *Top1* (Topo 1) structure with emphasis on the CPT-binding and catalytic domains (Kusari et al., 2011a). The typical amino acid residues Asn352, Glu356, Arg488, Gly503, and Gly717 (numbered according to human *Top1*) were identified that ensure fungal resistance towards intracellular CPT. The substitution Met370Thr is identical to that found in CPT-resistant human cancer cells, but different from the host *C. acuminata*. This work denoted the significance of resistance mechanisms employed by endophytes to bear toxic host metabolites, and provided a deeper understanding of plant-microbe coevolution.

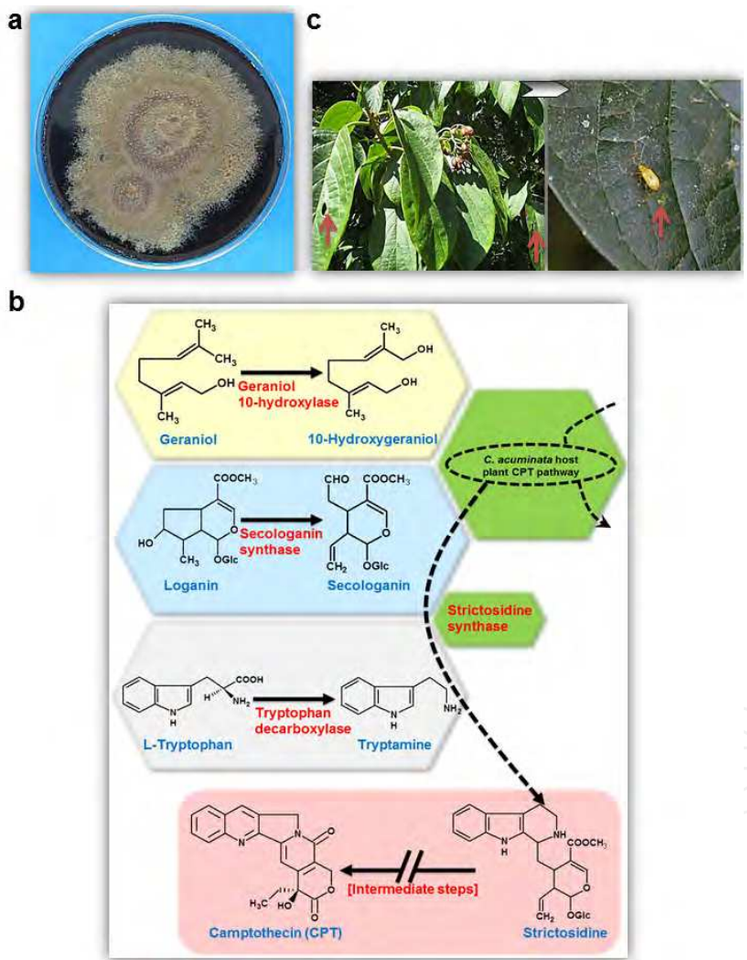


Fig. 7. (a) Endophytic *Fusarium solani* that produces CPT, 9-MeO-CPT and 10-OH-CPT. (b) A cross-species CPT biosynthetic pathway where the endophyte utilizes indigenous enzymes to biosynthesize CPT precursors (10-hydroxygeraniol, secologanin, and tryptamine), but requires the host strictosidine synthase to complete the pathway. (c) Perforations on the leaves *N. nimmoniana* plant (Western Ghats, India) (red arrows) are caused by feeding of Chrysomelid leaf beetles. (Photographs courtesy of M. Spiteller).

The discovery of this endophytic fungus that is capable of producing CPT led us to envisage the possibility of using this organism to produce CPT under controlled fermentation conditions in an economical, environment-friendly, and reproducible manner amenable to industrial scale-up. Unfortunately, it was observed that a substantial decrease occurred in the production of CPT and 9-MeO-CPT by this *in vitro*-cultured endophyte following repeated subculturing (i.e., in successive subculture generations) (Kusari et al., 2009b). Optimized fermentation conditions and the addition of precursors as well as various host plant tissue extracts did not restore the production. We then deciphered the chemical ecology of the endophyte-host interaction, where the fungal endophyte utilizes indigenous *G10H* (geraniol 10-hydroxylase), *SLS* (secologanin synthase), and *TDC* (tryptophan decarboxylase) to biosynthesize CPT precursors. However, to complete the cross-species CPT biosynthetic pathway, the endophyte requires the host *STR* (strictosidine synthase) (Kusari et al., 2011b) (Figure 7b). The fungal CPT biosynthetic genes destabilized *ex planta* over successive subculture generations. The seventh subculture predicted proteins exhibited reduced homologies to the original enzymes proving that such genomic instability leads to dysfunction at the amino acid level. The endophyte with an impaired CPT biosynthetic capability was artificially inoculated into the living host plants and then recovered after colonization. CPT biosynthesis could still not be restored. This demonstrated that the observed phenomenon of genomic instability was possibly irreversible (Kusari et al., 2011b). Following our discovery of the endophytic fungus *F. solani*, another endophytic fungus has been isolated from *Apodytes dimidiata* capable of producing the same compounds (Shweta et al., 2010). Furthermore, an endophytic *Xylaria sp.* has recently been isolated from *C. acuminata* capable of producing only 10-OH-CPT, and remarkably, not the parent compound CPT (Liu et al., 2010). In both these cases, no further follow-up studies have been reported so far. Recently, it was reported that chrysomelid beetles (*Kanarella unicolor* Jacobby) feeds on the leaves of *N. nimmoniana* without any apparent adverse effect (Ramesha et al., 2011) (Figure 7c). Interestingly, most of the CPT in the insect body was found in the parental form without any major metabolized products.

5.1.2 Metabolomics resources used for CPT and structural analogues

For analyzing both the host plants and the isolated endophytes, we employed a number of state-of-the-art analytical tools and methodologies (Kusari et al., 2009b, 2011b,d). CPT, 9-MeO-CPT, and 10-OH-CPT were identified by high-performance (pressure) liquid chromatography (HPLC) coupled to multicomponent high-resolution tandem mass spectrometry (LC-HRMS and LC-HRMS^n fragment spectra) using a LTQ-Orbitrap spectrometer, Thermo Scientific. The compounds were quantified using TSQ Quantum Ultra AM mass spectrometer (Thermo Finnigan, U.S.A.) equipped with an ESI ion source (Ion Max). The mass spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of pump, flow manager, and autosampler (injection volume 0.6 µL). Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. The separations were performed by using a Phenomenex Gemini C_{18} column (3 µm, 0.3 × 150 mm) (Torrance, CA) with a H_2O (+ 0.1% HCOOH) (A)/acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 4 µL min^{-1}). Samples were analyzed by using a gradient program as follows: 95% A isocratic for 5 min, linear gradient to 60% A within 12 min, and to 100% B in 29 min. After 100% B isocratic for 5 min, the system returned to its initial condition (95% A) within 1 min and was equilibrated for 7 min. The spectrometer was operated in positive

mode (1 spectrum s^{-1}; mass range: 200-800) with nominal mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz, with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass; *m/z* 391.284286; bis-(2-ethylhexyl)-phthalate. MS^2 led to the corresponding CO_2 loss of the precursor (CID of 45). The final MS^3 measurement was performed under CID of 45 and resulted in characteristic fragments of the compounds. The compounds were additionally confirmed using ^1H NMR, performed at 298 K with a Bruker DRX-400 spectrometer using 5 mm tubes with $CDCl_3$ (Merck, Darmstadt, Germany) as solvent.

For the host *C. acuminata* plants, the LC-MS/MS data were subjected to a number of different chemometric evaluations for metabolite profiling and correlating the phytochemical loads among the various *Camptotheca* plants (infraspecific), among the organic and aqueous phases, and among the different aerial tissues (dry and fresh in parallel) to reflect the metabolome profiles of the studied plants (Kusari et al., 2011d). The analyses included multivariate analysis (MVA), Kruskal's multidimensional scaling (MDS), principal component analysis (PCA), linear discriminant analysis (LDA), and hierarchical agglomerative cluster analysis (HACA). All analyses were performed using the statistical software XLSTAT-Pro (Addinsoft, NY, U.S.A.), except for MVA which was performed using the statistical software QI Macros (KnowWare International Inc., CO, U.S.A.). Both the statistical software packages were used in combination with Microsoft Excel (part of Microsoft Office Professional, Microsoft Corporation, U.S.A.).

Furthermore, we used the high-precision isotope-ratio mass spectrometry (HP-IRMS) by compound-specific carbon isotope (CSCI) and compound-specific nitrogen isotope (CSNI) modules, to confirm that the endophytic fungus actually utilizes host strictosidine synthase, as detailed above (Kusari et al., 2011b). The CPT produced by the cultured endophyte (first generation) outside the host plant in a nitrogen-free media was compared to CPT from the tissue (not containing the same *F. solani*) of original *C. acuminata* host (from SWFU) to check both the $\delta^{13}C/^{12}C$ (by CSCI) and the $\delta^{15}N/^{14}N$ (by CSNI). It was possible to trace the exact pattern of the accumulation of both 'carbons' and 'nitrogens' with the source of the enzyme(s) (fungal or plant) concerned up to and including the formation of CPT in the endophytic fungus and in the host plant. Briefly, the samples were readied for HP-IRMS in each case by placing 0.5 mg CPT in 3.5 × 5 mm tin capsules (HEKAtech GmbH, Germany), lyophilizing completely and finally rolling the capsules into small spheres. The HP-IRMS measurements were performed in compound-specific carbon isotope (CSCI) and compound-specific nitrogen isotope (CSNI) modules, using a FlashEA 1112 elemental analyzer (Thermo Fisher, Italy) coupled to a DELTA V Plus isotope-ratio mass spectrometer (Thermo Fisher, Bremen, Germany) interfaced through a ConFlo IV universal continuous flow interface (Thermo Fisher, Bremen, Germany) (Kusari et al., 2011b). The combustion furnace (oxidation reactor) was maintained at 1020°C, and flash combustion was initiated by injecting a pulse of O_2 at the time of sample drop. Helium was used as the carrier with a flow rate of 120 mL min^{-1}. NO_x species were reduced to N_2 in a reduction furnace at 680°C. Water was removed by phosphorus pentoxide in a water trap and CO_2 was separated from N_2 using a Porapak-packed N_2/CO_2-separation column (3 m × 6.5 mm, Thermo Electron S. p. A.) operated isothermally at 85°C. Each sample was analyzed in quadruplet. Acetanilide (Fisons Instruments) was used as the reference standard.

5.1.3 Case study II: Podophyllotoxin and deoxypodophyllotoxin

The first literature report on the extraction of 'Podophyllum' was that of King, who called the resin he obtained from alcohol extraction as 'podophyllin' (King, 1857). 'Podophyllum' is the dried roots and rhizomes of species of *Podophyllum* (Figure 8), which was described and its first modern botanical name given by Linnaeus (1753). The first successful chemical investigation was later carried out by Podwyssotzki (1881, 1882, 1884). The correct empirical formula for podophyllotoxin was first advanced by Borsche and Niemann (1932), and later confirmed (Gensler et al., 1954; Gensler and Wang, 1954; Petcher et al., 1973).

Strikingly, the first documented proof of the discovery of deoxypodophyllotoxin was not from *Podophyllum*. The Leech book of Bald, 900-950 A.D., an early English medicinal book, has reported on the use of root of *Anthriscus sylvestris* (Imbert, 1998). These roots were reported contain lignans such as deoxypodophyllotoxin and were used in ointments prepared from a large number of plants and plant extracts like *savin* to cure cancer (Cockayne, 1961). Podophyllotoxin and deoxypodophyllotoxin share the same action mechanism based on the core structure of deoxypodophyllotoxin as evidenced by the SAR studies. They inhibit the formation of the microtubules, i.e., inhibit the formation of the mitotic spindle, resulting in an arrest of the cell division process in metaphase and clumping of the chromosomes (Imbert, 1998; Canel et al., 2000; Liu et al., 2007). Under *in vitro* conditions, they bind to tubulin dimers giving lignan-tubulin complexes. This stops further formation of the microtubules at one end but does not stop the disassembly at the other end leading to the degradation of the microtubules. This mode of action is comparable to the alkaloid colchicin, and for their mode of action these compounds are called 'spindle poisons' (Liu et al., 2007).

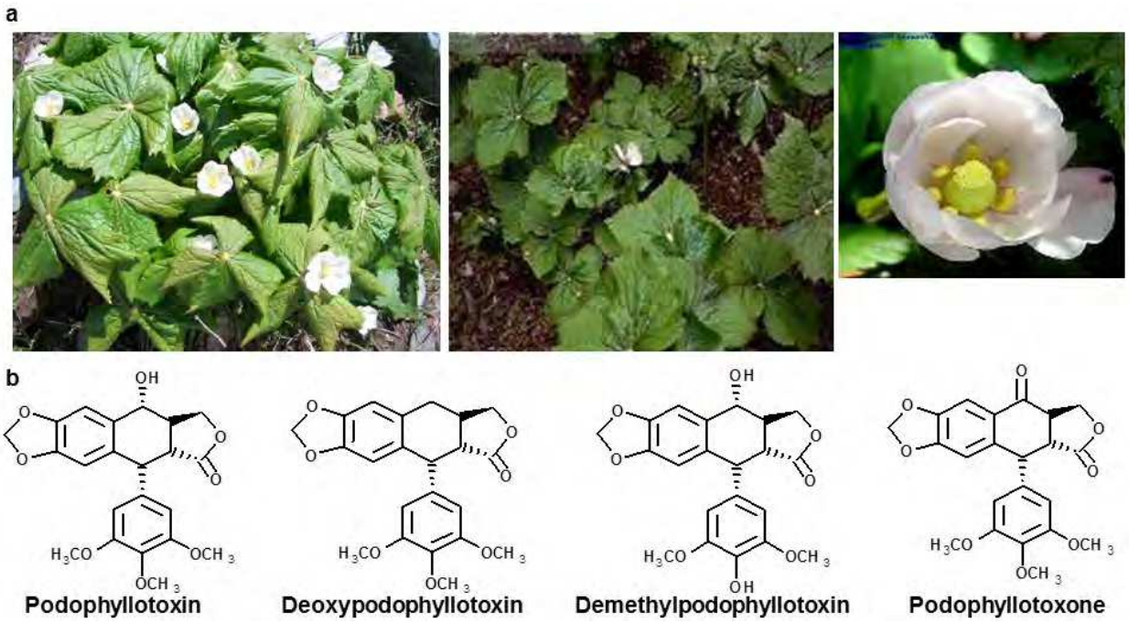


Fig. 8. (a) *Podophyllum hexandrum* Royale. (Photographs courtesy of M. Spiteller). (b) Podophyllotoxin and some important structural analogues found in plants.

Discoveries of podophyllotoxin-producing endophytic fungi include *Phialocephala fortinii* isolated from *P. peltatum* (Eyberger et al., 2006), *Trametes hirsuta* isolated from *P. hexandrum* (Puri et al., 2006), and *Fusarium oxysporum* isolated from *Juniperus recurva* (Kour et al., 2008). Unfortunately, in all the above cases, there has been no follow-up work on scale-up, and there is no published breakthrough in the commercial exploitation of these endophytic fungi as a source of podophyllotoxin. We recently isolated an endophytic fungus, *Aspergillus fumigatus* Fresenius, from *Juniperus communis* sampled from the Rombergpark botanical gardens, Dortmund, Germany, which produced deoxypodophyllotoxin under *in vitro* axenic conditions (Kusari et al., 2009a). The growth and production kinetics showed the potential of the endophyte in indigenous production of deoxypodophyllotoxin, but *in vitro* subculturing showed no production from the third subculture generation.

5.1.4 Metabolomics resources used for podophyllotoxin and structural analogues

The *Juniperus* and *Podophyllum* plants sampled by us from diverse populations were extracted and subjected to metabolomics analyses (Kusari et al., 2011e). HPLC analysis of the extracts was performed using a Surveyor HPLC system. Compounds were separated on a Hydro-RP column (150 × 2 mm, 4 µm particle size) from Phenomenex (Torrance, CA). The mobile phase consisted of 10 mM ammonium acetate in distilled water (A) and acetonitrile with 0.1% formic acid (B). Gradient elution was performed using the following solvent gradient: from 85A/15B (held for 3 min) in 16 min to 17A/83B, then in 1 min to 0A/100B and after 7 min, back to the initial conditions (85A/15B); each run was followed by an equilibration period of 8 min. The flow rate was 0.22 mL min^{-1} and the injection volume was 5 µL. All separations were performed at 30°C. Mass spectra were obtained using a TSQ Quantum Ultra AM mass spectrometer equipped with an ESI ion source (Ion Max) operating in positive mode (Kusari et al., 2011e). Nitrogen was employed as both the drying and nebulizer gas (40 AU). Capillary temperature was 200°C and capillary voltage was 3.5 kV. The calibration curves of the available reference compounds podophyllotoxin and demethylpodophyllotoxin were constructed by dilution of external standards with methanol to give the desired concentrations. The concentrations of standard solutions were 0.1, 0.5, 1, 5, 10, 50, 80, 120, and 160 µg mL^{-1}. Correlation coefficient for the linear calibration curve was >0.99 for both podophyllotoxin and demethylpodophyllotoxin. All procedures were carried out under light protection. Concentrations of the commercially unavailable compounds deoxypodophyllotoxin and podophyllotoxone were calculated with the assumption of similar precursor ion response like that of podophyllotoxin. The LOQs were 0.05 µg mL^{-1} (demethylpodophyllotoxin) and 0.2 µg mL^{-1} (podophyllotoxin, deoxypodophyllotoxin, and podophyllotoxone), respectively. The LOD (3 times noise intensities) and LOQ (10 times noise intensities) were calculated/estimated from signal to noise ratio using signal intensities of the analytes and the noise near the retention time of the analytes. Estimation was necessary for the derivatives (deoxypodophyllotoxin and podophyllotoxone) due to unavailability of reference standards.

All the above secondary metabolites were re-verified using the highly selective and sensitive LC-ESI-$HRMS^n$. HPLC analysis of the extracts was performed using an Agilent (Santa Clara, U.S.A.) 1200 HPLC system consisting of LC-pump, PDA detector (λ = 254 nm), autosampler (injection volume 10 µL) and column oven (30°C). Compounds were separated using a Synergi Fusion RP80 column (150 x 3 mm, 4 µm particle size) from Phenomenex (Torrance,

CA) with a H_2O (+ 0.1% HCOOH, + 10 mM ammonium acetate) (A)/acetonitrile (+ 0.1% HCOOH) (B) gradient (flow rate 400 µL min^{-1}). Samples were analyzed by using gradient program: 95% A isocratic for 3 min, linear gradient to 100% B over 20 min, after 100% B isocratic for 10 min, the system returned to its initial condition (95% A) within 1 min, and was equilibrated for 5 min. The FT-full scan and MS/MS spectra were obtained with an LTQ-Orbitrap XL spectrometer (Thermo Fisher, U.S.A.) equipped with H-ESI-II source. The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 250-1000) with nominal mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using an internal standard; bis(2-ethylhexyl)phthalate: *m/z* 391.284286. MS/MS experiments were performed in HCD (higher-energy C-trap dissociation, 35 eV) mode. The following parameters were used for experiments: spray voltage 5 kV, capillary temperature 260°C, and tube lens 70 V. Nitrogen was used both as sheath gas (45 AU) and auxiliary gas (10 AU). Helium served as the collision gas.

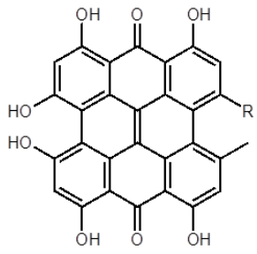
The LC-MS/MS data were subjected to a number of different chemometric evaluations for metabolite profiling and correlating the phytochemical loads among the various plants of the studied *Juniperus* and *Podophyllum* species (infraspecific), between the organic and aqueous extracts, among populations of the same species from different locations, among populations of different species from the same location, among populations of different species from different locations, as well as among populations of different genera (infrageneric) from the same and different locations (Kusari et al., 2011e). The chemometric algorithms and methodologies were used similar to those used for the *C. acuminata* plants (*vide supra*).

The extracts of the isolated endophytic fungi, both from *Juniperus* and *Podophyllum* plants, were evaluated with an LTQ-Orbitrap spectrometer (Kusari et al., 2009a). The spectrometer was operated in positive mode (1 spectrum s^{-1}; mass range: 50–1000) with nominal mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high accuracy mass within 1 ppm deviation using one internal lock mass, polydimethylcyclosiloxane – $[(CH_3)_2SiO]_6$: *m/z* 445.120025. The spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of pump, flow manager and autosampler (injection volume 0.5 µL). Nitrogen was used as sheath gas (5 AU) and helium served as the collision gas. The separations were performed by using a Phenomenex Gemini C_{18} column (3 µm, 0.3 × 150 mm) (Torrance, CA, U.S.A.) with a H_2O (+0.1% HCOOH) (A) / acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 4 µL min^{-1}). Samples were analyzed by using a gradient program as follows: 90% A isocratic for 2 min, linear gradient to 100% B over 8 min, after 100% B isocratic for 10 min, the system was returned to its initial condition (90% A) within 1 min, and was equilibrated for 9 min. Furthermore, for the deoxypodophyllotoxin-producing endophyte, quantitation of the compound was achieved by accurate mass (maximum deviation 1 ppm) single ion monitoring (SIM) of the $[M+H]^+$ ion of deoxypodophyllotoxin (Kusari et al., 2009a). Since deoxypodophyllotoxin is unavailable from commercial sources, the calibration was performed using podophyllotoxin as standard and detector response was assumed to be in the same range. The calibration graph was linear from 50 ng mL^{-1} up to 10000 ng mL^{-1}. Furthermore, a high-resolution full scan run was performed in order to check for the accumulation of structural analogues of podophyllotoxin and deoxypodophyllotoxin by that particular endophyte in axenic culture.

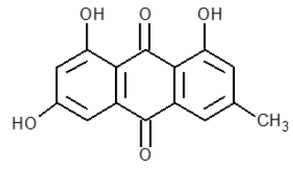
5.2 Antidepressants and photodynamic compounds

5.2.1 Case study: Hypericin and emodin

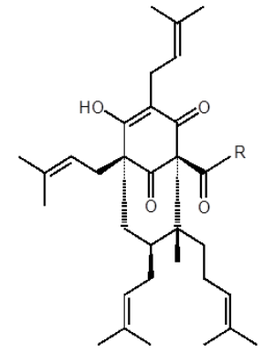
Hypericin (2,2'-dimethyl-4,4',5,5',7,7'-hexahydroxy-mesonaphtodianthrone) (Figure 9), a naphthodianthrone derivative, is a plant derived compound of high medicinal value. It is one of the main constituents of *Hypericum* species. The first detailed report of the isolation of hypericin was from the medicinal herb *Hypericum perforatum* L., published by Brockmann et al. (1939). The molecular formula of hypericin was first reported in 1942 by the same author as $C_{30}H_{16}O_8$ (Brockmann et al., 1942) and eight years later the correct structure was published (Brockmann et al., 1950). Various species of the genus *Hypericum* have long been used as medicinal plants in various parts of the world due to their therapeutic efficacy (Yazaki and Okada, 1994). Their main constituents are napthodianthrones, primarily represented by hypericin, pseudohypericin, protohypericin, protopseudophypericin (Brockmann et al., 1939, 1942, 1957), the anthraquinone emodin, and derivatives occurring in very low concentrations such as isohypericin, demethyl-pseudohypericin, hyperico-dehydro-dianthrone, pseudo-hyperico-dehydro-dianthrone (Brockmann et al., 1957), and cyclopseudohypericin (Häberlein et al., 1992). Hypericin has long been in use, at least from the time of ancient Greece (Tammaro and Xepapadakis, 1986), as an antidepressant due to its monoamine oxidase (MAO) inhibiting capacity, having effects similar to bupropion (Nahrstedt and Butterweck, 1997) and imipramine (Raffa, 1998). Potential uses of hypericin extend to improved wound healing, anti-inflammatory effects (Zaichikova et al., 1985), antimicrobial and antioxidant activity (Radulovic et al., 2007), sinusitis relief (Razinkov et al., 1989), and seasonal affective disorder (SAD) relief (Martinez et al., 1993). Hypericin also has remarkable antiviral activity against a number of viruses (Kusari et al., 2008). Several recent *in vitro* studies have revealed the multifaceted cytotoxic activity of hypericin as a result of its photodynamic activity (Kubin et al., 2005).



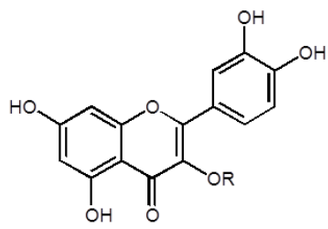
Hypericin: R = CH_3
Antidepressive, antimicrobial, wound healing, anticancer, antiviral (esp. enveloped viruses like HIV-1, HSV, etc.), antiSAD, photodynamic
Pseudohypericin: R = CH_2OH



Emodin
Laxative, purgative, ion transporter (Cl^- channel), antimicrobial, antioxidant anti-inflammatory, antitumor, photodynamic



Hyperforin: R = $CH(CH_3)$
Adhyperforin: R = $CH(CH_3)CH_3CH_3$



Quercetin: R = H
Quercitrin: R = Rhamnose
Hyperoside: R = Galactose
Rutin: R = Glucose-Rhamnose

Fig. 9. Hypericin and related compounds found in plants.

Using the rationale that the plants containing hypericin may also contain endophytic fungi that are able to accumulate the same or similar molecules, a selective search for fungal endophytes was pursued. A number of endophytic fungi were isolated from various organs of the *Hypericum* plants, which were morphologically different from the strains isolated from unsterilized explants (surface-contaminating fungi). Only one endophytic fungus was able to produce hypericin and emodin under axenic submerged shake-flask fermentation (Kusari et al., 2008). The fungus was identified as *Thielavia subthermophila* by its morphology and authenticated by 28S rDNA and ITS-5.8S rDNA analyses. The growth of the endophyte and production of hypericin remained independent of the illumination conditions and media spiking with emodin. Protohypericin could not be detected, irrespective of either spiking or illumination conditions. The *hyp-1* gene, suggested to encode for the Hyp-1 phenolic coupling protein in plant cell cultures, was absent in the genome of the endophyte. Thus, it was proposed that emodin anthrone is the common precursor of both hypericin and emodin in the fungal endophyte, which is governed by a different molecular mechanism than the host plant or host cell suspension cultures (Kusari et al., 2009c).

5.2.2 Metabolomics resources used for hypericin and related compounds

The *Hypericum* plants sampled from diverse populations in Europe and the Himalayan region were extracted and subjected to a metabolic profiling (Kusari et al., 2009d). The compounds were separated on a Luna C_{18} 100 Å column (3 µm, 250 mm; Phenomenex, Torrance, CA) at 30°C. Chromatographic conditions were optimized for the separation of hypericin, pseudohypericin, hyperforin and emodin (gradient 1), and for the separation of flavonoids (gradient 2) at the Surveyor HPLC system (Thermo Finnigan, U.S.A.). The mobile phase consisted of 10 mM ammonium acetate buffer adjusted to pH 5.0 with glacial acetic acid (A) and a 9:1 mixture of acetonitrile and methanol (B). First gradient elution was performed using the following solvent gradient: from 55A/45B held for 2 min to 0A/100B in 8 min, thereafter holding for 13 min; each run was followed by an equilibration period of 6 min. The flow rate was 0.3 mL min^{-1} and injection volume was 3 µL. Second gradient elution was performed using the following solvent gradient: start for 2 min at 95A/5B, in 6 min to 75A/25B, then in 2 min to 50A/50B and in another 2 min to 100B. After holding for 13 min returned to initial conditions (95A/5B) within 1 min and held for 8 min. The eluent flow rate was 0.25 mL min^{-1} and the injection volume was 5 µL. Highly selective and sensitive selected reaction monitoring (SRM) was performed using a TSQ Quantum Ultra AM mass spectrometer (Thermo Finnigan, U.S.A.) equipped with an ESI ion source (Ion Max) operating in negative mode (Kusari et al., 2009d). Nitrogen was employed as both the drying and nebulizer gas. The capillary voltage was 5 kV and capillary temperature was set at 200°C. Sheath gas (nitrogen) was set at 45 arbitrary units and collision gas pressure was 1.5 mTorr. Each mass transition was monitored at a peak width of 0.5 and dwell time of 0.3 s. All the secondary metabolites were re-verified using the highly selective and sensitive LC-ESI-$HRMS^n$ (LTQ-Orbitrap spectrometer). External calibration was performed in the range 0.01-10 µg mL^{-1} for emodin, 0.05-50 µg mL^{-1} for hyperforin, pseudohypericin, and hypericin, as well as 0.5-100 µg mL^{-1} for hyperoside, rutin, quercetin, and quercitrin. Correlation coefficient for the calibration curves were >0.99 for all analytes. The relative standard deviation (RSD) of the analytical method was determined by eight injections of an extract and was below 6% for all compounds. The LOD (limit of detection) and LOQ were

determined by minimum signal to noise ratio of 3 and 9, respectively. Instrumental LOQ of the compounds varied between 0.003 µg mL^{-1} (emodin) and 2 µg mL^{-1} (quercitrin).

Similar to the *C. acuminata* plants, the LC–MS/MS data for these plants were also subjected to a number of different statistical evaluations for metabolite profiling and correlating the phytochemical loads among the various parts of the plants of the studied *Hypericum* samples (same and different species), between the organic and aqueous extracts, among the different species, among populations of the same species from different locations, among populations of different species from the same locations, as well as among populations of different species from different locations (Kusari et al., 2009d).

For the isolated endophytic fungi, quantitation of the hypericin and emodin was performed by using a Thermo Finnigan Surveyor HPLC system consisting of Surveyor MS-pump and Surveyor Autosampler-Plus (injection volume 5 µL) (Kusari et al., 2008, 2009c). The compounds were separated on a Luna C_{18} (50 × 3 mm, 3 µm particle size) column from Phenomenex (Torrance, CA). The mobile phase consisted of water containing 10 mM ammonium acetate (pH 5.0) (A) and acetonitrile-methanol, 9:1 (B). Samples were separated using a gradient program as follows: (flow rate of 250 µL min^{-1}) 55% A isocratic for 2 min, linear gradient to 100% B over 6 min (flow rate 300 µL min^{-1}). After 100% B isocratic for 7 min, the system was returned to its initial conditions (55% A) within 1 min and was equilibrated for 4 min before the next run was started. MS detection (multiple reaction monitoring mode) was performed by using a TSQ Quantum Ultra AM spectrometer equipped with an ESI ion source (Ion Max) operating in negative mode. Nitrogen was employed as both the sheath (50 arbitrary units) and auxiliary (8 arbitrary units) gas, and argon served as the collision gas with a pressure of 1.5 mTorr. The capillary temperature was set at 250°C. External calibration was performed in the range 0.01-10.0 µg mL^{-1} for hypericin and 0.005-10.0 µg mL^{-1} for emodin. Correlation coefficients for the linear calibration curves were >0.995 for both compounds.

Hypericin and emodin were identified by HRMS fragment spectra (LTQ-Orbitrap spectrometer), which were consistent with authentic standards (Kusari et al., 2008, 2009c). The spectrometer was equipped with a Dionex HPLC system Ultimate 3000 consisting of pump, flow manager, and autosampler (injection volume 1 µL). Nitrogen was used as sheath gas (6 arbitrary units), and helium served as the collision gas. The separations were performed by using a Phenomenex Gemini C_{18} column (3 µm, 0.3 × 150 mm) (Torrance, CA) with a H_2O (+0.1% HCOOH, +1 mM ammonium acetate) (A)/acetonitrile (+0.1% HCOOH) (B) gradient (flow rate 4 µL min^{-1}). Samples were analyzed by using a gradient program as follows: 30% A isocratic for 1 min, linear gradient to 100% B over 10 min; after 100% B isocratic for 60 min, the system was returned to its initial condition (30% A) within 1 min and was equilibrated for 9 min. The spectrometer was operated in negative mode (1 spectrum s^{-1}; mass range 50–1000) with nominal mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation using one internal lock mass (*m/z* 386.7149314; CsI_2^-).

Additional screening for emodin anthrone and protohypericin was also performed in full scan negative mode (Kusari et al., 2009c). For that, the spectrometer was equipped with a Thermo Surveyor system consisting of a LC-pump and autosampler (injection volume 5 µL). N_2 was used as sheath gas (5 arbitrary units), and He served as the collision gas. The

separations were performed by using a Phenomenex Synergi Fusion RP column (4 µm, 2 × 150 mm) with a H_2O (+0.1% HCOOH, +10 mM NH_4OAc) (A)/MeCN (+0.1% HCOOH) (B) gradient (flow rate 0.25 mL min^{-1}). Samples were analyzed by using a gradient program as follows: 50% A isocratic for 2 min, linear gradient to 100% B over 8 min; after 100% B isocratic for 48 min, the system was returned to its initial condition (50% A) within 1 min and was equilibrated for 6 min. The spectrometer was operated in negative mode (1 spectrum s^{-1}; mass range 200-1000) with mass resolving power of 60000 at *m/z* 400 with a scan rate of 1 Hz with automatic gain control to provide high-accuracy mass measurements within 2 ppm deviation.

6. Current progress and future challenges

The production of bioactive compounds by endophytes, especially those exclusive to their host plants, is significant both from the molecular and biochemical perspective, and the ecological viewpoint. The production of beneficial secondary metabolites (including those produced by plants) by endophytes nurtures expectations of utilizing them as alternative and sustainable sources of these compounds. However, the commercial implication of production of desirable compounds by endophytic fungi still remains a future goal (Kusari & Spiteller, 2011). A major obstacle preventing the biotechnological application of endophytes is the perplexing problem of reduction of secondary metabolite production on repeated subculturing under axenic monoculture conditions. In addition to a constant pursuit of discovering competent endophytes with potential for pharmaceutical use, it is essential to follow-up these discoveries with advanced research to establish, restore and sustain the *in vitro* biosynthetic capability of endophytes. This can be achieved by a multifaceted approach involving complete elucidation of the dynamic endophyte-endophyte interactions pertaining to their biological, biochemical and genetic frameworks. Considering the fact that endophytes reside within plants and are constantly communicating with their hosts, it is compelling that plants would have a substantial influence on the *in planta* metabolic processes of the endophytes. Moreover, recent whole-genome sequencing strategies have shown that the known secondary metabolites of various bacteria and fungi are largely outnumbered by the number of genes encoding the biosynthetic enzymes in these microorganisms (Winter et al., 2011). This is accentuated by the fact that endophytic fungi always remain in versatile interactions with the host plant and other endophytes, and even slight variation in the *in vitro* cultivation conditions can impact the kind and range of secondary metabolites they produce (Scherlach & Hertweck, 2009). Further research to systematically understand the endophyte–endophyte and endophyte-host interspecies crosstalk is desirable for sustainable production of compounds using endophytes (Kusari & Spiteller, 2011).

7. Overcoming the obstacles

The potential of novel fungal endophytes capable of biosynthesizing plant metabolites has undoubtedly been recognized. However, there is still no known breakthrough in the biotechnological production of these bioactive secondary metabolites using endophytes. It is important to elucidate the metabolome in endophytes correlating to their associated plants on a case-by-case basis to understand how the biogenetic gene clusters are regulated and their expression is affected *in planta* and *ex planta* (by environmental changes and axenic

culture conditions). Only a deeper understanding of the host-endophyte relationship at the molecular and genetic levels might help to induce and optimize secondary metabolite production under laboratory conditions to yield plant metabolites in a sustained manner using endophytes. The biosynthesis of plant metabolites in endophytes could further be manipulated to yield new lead structures which could act as pro-drugs. In addition to identifying new natural products, genome mining, metabolic engineering and metagenomics would certainly have an impact on the understanding and manipulation of secondary metabolite production by endophytic fungi. Likewise, it is well-known that an unidentified proportion of endophytic fungi are uncultivable *in vitro* under axenic conditions. For such unculturable species, environmental PCR strategies might be employed for recovery, suitably coupled with a culture-independent metagenomic approach or compound structure-based gene targeting to study their desirable biosynthetic gene clusters (Kusari & Spiteller, 2011). Further research along these directions is highly desirable in order to elucidate comprehensively the endophytic fungal biosyntheses since this knowledge can then be utilized for heterologous expression of the preferred final products in large quantities using suitable model organisms like *Saccharomyces cerevisiae* or *Escherichia coli*. Another advantage of endophytic fungi over plants is that the biosynthetic gene modules of a natural product produced by a cascade of biosynthetic steps might be arranged as an operon in highly contiguous clusters in the fungal genome. This could allow swift *in silico* detection of signature genes or gene domains that are pathway-specific followed by the possibility of expressing them in heterologous organisms. We stand at the cross-roads of time when the world's biodiversity is declining at an alarming rate. Many endemic, endangered and medicinally valuable plants are on the verge of extinction. Along with these plants, the endophytes harbored in them are also threatened. Further fundamental research must be addressed to ensure a continuous and sustained supply of bioactive pro-drugs against the present and emerging diseases.

8. Acknowledgements

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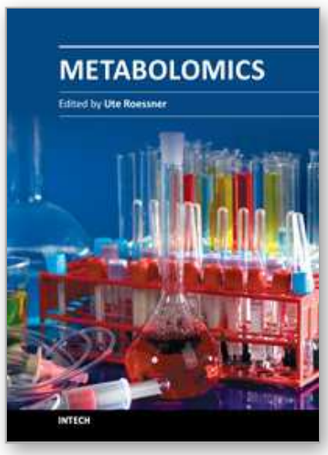

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Metabolomics is a rapidly emerging field in life sciences, which aims to identify and quantify metabolites in a biological system. Analytical chemistry is combined with sophisticated informatics and statistics tools to determine and understand metabolic changes upon genetic or environmental perturbations. Together with other 'omics analyses, such as genomics and proteomics, metabolomics plays an important role in functional genomics and systems biology studies in any biological science. This book will provide the reader with summaries of the state-of-the-art of technologies and methodologies, especially in the data analysis and interpretation approaches, as well as give insights into exciting applications of metabolomics in human health studies, safety assessments, and plant and microbial research.

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