

BIOACTIVITIES AND MAJOR CONSTITUENTS OF METABOLITES PRODUCED BY ENDOPHYTIC FUNGI FROM *NERIUM OLEANDER*

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Diverse endophytes exist within plant aerial tissues, which constitute a rich bioresource for exploring new natural products. Here we investigate fungal endophytes from a medicinal plant, Nerium oleander L. for antioxidant capacity, xanthine oxidase inhibition, antimicrobial activity, and total phenolic content (TPC). The total antioxidant capacities and TPC of the fungal cultures ranged from 9.59 to 150.8 μmol trolox/100 ml culture, and from 0.52 to 13.95 mg gallic acid/100 ml culture, respectively. A fungal strain Chaetomium sp. showed the strongest antioxidant capacity, contained the highest level of phenolics, and inhibited xanthine oxidase activity. A positive correlation was found between antioxidant capacity and TPC in the tested samples. The major bioactive constituents of the fungal cultures were preliminarily identified as phenolics (e.g. phenolic acids and their derivatives, flavonoids) and volatile and aliphatic compounds by LC-ESI-MS and GC-MS. This study shows that the endophytic fungi associated with N. oleander are a potential bioactive resource.

Key Words: antioxidant activity; antimicrobial activity; endophytic fungi; *Nerium oleander*; total phenolic content; xanthine oxidase inhibition.

Recent studies have shown that fungal endophytes are ubiquitous in plant species (e.g., Petrini et al. 1982; Clay 1988; Espinosa-Garcia and Langenheim 1990; Schulz et al. 1993; Fisher 1996; Faeth and Hammon 1997; Huang et al. n.d.). Endophytic fungi infect and inhabit primarily the aerial tissues of the host plant without causing detectable symptoms. The relationships between endophytes and their host plant are thought to be symbiotic, such as that endophytes obtain nutrients and protection from the host but contribute to effective host defense against pathogens, herbivores or abiotic stress (Saikkonen et al. 1998; Clay and Holah 1999; Redman et al. 2002; Arnold et al. 2003). Globally, there are at least one million species of endophytic fungi in all plants (Dreyfuss and Chapela 1994; Ganley et al. 2004), which can potentially provide a wide variety of structurally unique, bioactive natural products such as alkaloids, benzopyranones, chinones, flavonoids, phenols, steroids, terpenoids, tetralones, xanthenes, and others (Tan and Zou 2001).

As part of our ongoing efforts towards finding novel antioxidant and antimicrobial agents, and other bioactive chemicals from natural resources, we investigated the secondary metabolites of endophytes and their host plants. *Nerium oleander* L. (Apocynaceae), a traditional Chinese medicinal plant, is a small evergreen tree of 2-5 m in height with a wide geographical and ecological distribution (Fu et al. 2005). This plant possesses cardiogenic, antibacterial, antileprotic, anti-inflammatory, anticancer, and antiplatelet aggregation activities, insecticidal activity, mammalian cytotoxicity, and depressants of the central nerve system (Begum et al. 1999; Huq et al. 1999; EI-Shazly et al. 2000; Fu et al. 2005). Various compounds have been reported in connection with these biological activities, such as cardenolides (oleanderin, neriantin, adynerin, deacetyloleanerin, neriifolin), triterpenoidal saponins, oleanderol, rutin, dambonitol in leaves; odorosides (A, B, D, F, G, H, K) in barks; triterpene, steroidal cardenolide, volatile oil, stearic acid, oleic acid in roots; and gitoxigenin, uzarigenin, strosposide, odoroside H in flowers (Siddiqui et al. 1997; Huq et al. 1999; Ji 1999; EI-Shazly et al. 2000; Fu et al. 2005). However, the endophytes of this plant and their medicinal values have not been investigated.

In this study, we isolated a total of 42 endophytic fungi from healthy leaves and stems of *N. oleander*, evaluated the metabolites of 16 selected fungal strains for their antioxidant and antimicrobial activities, and compared these activities with the methanolic extract of the host plant, using the improved ABTS and classical Folin-Ciocalteu methods (Re et al. 1999; Cai et al. 2004). Furthermore, we estimated antimicrobial activities of the fungal metabolites and host plant extract using six test microbes and investigated the xanthine oxidase inhibition of

the host plant and a selected fungal strain *Chaetomium* sp. NoS3 with strongest antioxidant activity. In addition, we identified major bioactive principles in the samples by LC-MS and GC-MS. The research will be helpful for searching new effective antioxidants and antibiotics from endophytic fungi associated with this medicinal plant.

MATERIAL AND METHODS

COLLECTION OF PLANT MATERIAL

Fresh mature leaves and stems of *N. oleander* were collected from a healthy plant grown in Kadoorie Farm and Botanic Garden, Hong Kong, in March 2005. The fresh samples were taken to the laboratory and treated within 8 hr.

CHEMICALS AND REAGENTS

2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), xanthine, and xanthine oxidase were purchased from Sigma/Aldrich (St. Louis, MO), Folin-Ciocalteu reagent from BDH (Dorset, England), and Trolox (6-hydroxy-2,5,7,8-tetramethylchromate-2-carboxylic acid) from Fluka Chemie AG (Buchs, Switzerland). Authentic standards, antibiotics, and other chemicals and reagents used in this study were obtained from Sigma/Aldrich. All the chemicals and reagents were of the analytical grade.

ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI

A total of 20 samples of both leaves and stems from *N. oleander* plants were first washed in running water. The leaves were cut into segments (5×5 mm), and stems were cut into pieces (10 mm in length). Surface sterilization and isolation of endophytic fungi followed a modified method of [Schulz et al. \(1993\)](#), and the details of the procedure were given in a previous study ([Huang et al. n.d.](#)). Identification of fungal strains was based on colony or hyphal morphology of the fungal culture, characteristics of the spores, and reproductive structures if these features were discernible ([Wei 1979](#); [Carmichael et al. 1980](#); [Barnett and Hunter 1998](#)).

CULTIVATION OF ENDOPHYTIC FUNGI

The fresh mycelia (grown on PDA plates at 28°C for 3-6 days) of 16 selected endophytic fungi with different morphology were inoculated in 100 ml flasks containing 50 ml of the

broth (sucrose, 30 g; NaNO₃, 3 g; K₂HPO₄, 1 g; yeast extract, 1 g; KCl, 0.5 g; MgSO₄·7H₂O, 0.5 g; FeSO₄, 0.01 g; H₂O, 1,000 ml), followed by incubation with a shaking incubator (Daihan Labtech CO., LTD) at 140 rpm for 15 days at 28°C. The culture broth of each endophyte was centrifuged at 1,670 g for 10 min and filtered using a Millipore filter with a 0.22-µm nylon membrane under vacuum at ~23°C to remove mycelium. The filtrate sample was stored at 4°C until use within 24 hr.

EXTRACTION OF HOST PLANT SAMPLE

The leaves and stems of *N. oleander* were air-dried in a ventilated oven at 40°C to constant weight and ground into fine powder using a Kenwood Multi-Mill (Kenwood Ltd., UK) and passed through a 24-mesh sieve. The sample (2 g) was extracted with 50 ml of 80% methanol at room temperature for 24 hr in a water bath shaker (Shaking Bath 5B-16) (Techne, Ltd., UK). The extract was filtered using a Millipore filter with a 0.22-µm nylon membrane under vacuum at room temperature and stored at 4°C (Cai et al. 2004).

TOTAL ANTIOXIDANT CAPACITY ASSAY

Total antioxidant capacity was assayed with a Spectronic Genesys 5 spectrophotometer (Milton Roy, NY) using the improved ABTS method (Cai et al. 2004). The results were expressed in terms of trolox equivalent antioxidant capacity (TEAC), i.e., µmol trolox/100 ml culture of endophytic fungus or mmol trolox/100 g dry weight (DW) of plant. All tests were performed in triplicate.

XANTHINE OXIDASE INHIBITION ASSAY

The xanthine oxidase activity with xanthine as the substrate was measured using the method given in Noro et al. (1983) with some modification (Kweon et al. 2001). The fungal strain *Chaetomium* sp. NoS3 and the host plant *N. oleander* were both investigated for xanthine oxidase inhibition with allopurinol, rutin, and chlorogenic acid co-tested as positive control. Xanthine oxidase inhibitory activity was expressed as the percentage of xanthine oxidase inhibition in the above assay system, calculated as $(1 - B/A) \times 100$, where A and B were the activity of the enzyme without and with the test material, respectively. The IC₅₀ value, 50% inhibitory concentration of test sample, was calculated by linear regression analysis, and expressed as µg dry powder weight of plant tissue for *N. oleander* or freeze-

dried metabolite powder weight for endophyte *Chaetomium* sp. NoS3 culture ($\mu\text{g/ml}$). All determinations were conducted in duplicate.

ANTIMICROBIAL ASSAY

Antimicrobial activities were assessed using five bacteria (*Escherichia coli*, *Bacillus cereus*, *Salmonella annatum*, *Staphylococcus aureus*, and *Listeria monocytogenes*), and one fungus (*Candida krusei*). These test microbes were obtained from the Department of Microbiology of the University of Hong Kong. The minimum inhibitory concentration (MIC) of the samples for each test organism was determined using a modified liquid dilution method performed in 96 well micro-trays (NCCLS 1993; Dharmaratne et al. 1999; Mosaddik et al. 2004). The freeze-dried powder of plant extract or fungal culture was dissolved in the phosphate buffer saline (PBS; pH 7.0-7.2) and sterilized by filtration through 0.22 μm sterilizing Millipore express filter (Millex-GP, Bedford). The same amount of test organisms in Mueller Hinton Broth ($\sim 10^6$ colony forming unit (CFU)/ml) was added to a two-fold serial dilutions of the reconstituted extract (50 μl) of each sample to give a final volume of 100 μl . Gentamicin and ketoconazole were co-assayed as positive control for antibacterial and antifungal assays, respectively, and PBS was used as negative control. After incubation at 37°C for 18–24 h (bacteria) or 28°C for 24-48 h (fungus), the plates were examined for growth of the organisms. The lowest concentration of a tested sample or compound was designated as MIC, at which no visual turbidity due to microbial growth was shown. All tests were performed in duplicate.

DETERMINATION OF TOTAL PHENOLIC CONTENT

Total phenolic content (TPC) was estimated using the Folin-Ciocalteu colorimetric method as described by Cai et al. (2004) with minor modification. The appropriate dilutions of the samples (0.2 ml) were oxidized with 0.5 N Folin-Ciocalteu reagent for 4 min at room temperature. Then the reaction was neutralized with saturated sodium carbonate (75 g/l). The absorbance of the resulting blue color was measured at 760 nm with spectrophotometer after incubation in dark for 2 hr at room temperature. Quantification was done on the basis of the standard curve of gallic acid. The results were expressed as gallic acid equivalent (GAE), i.e., mg gallic acid/100 ml culture or g gallic acid/100 g DW. All tests were performed in triplicate.

LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS)

The LC-MS-2010EV system used in this study consisted of a LC-20AD binary pump, a SIL-20AC auto-sampler, a photodiode-array detector, a central controller, and a single quadrupole MS detector with electrospray ionization (ESI) interface (Shimadzu, Japan). The analytical column used was VP-ODS C₁₈ column (250 × 2.0 mm, 4.6 μm) (Shimadzu, Japan). LC conditions were as follows: solvent A, 0.1% formic acid, and solvent B, MeOH with 0.1% formic acid. A gradient elution used was 0-5 min, 5% B; 5-15 min, 5-30% B; 15-40 min, 30-40% B; 40-60 min, 40-50% B; 60-65 min, 50-55% B; 65-90 min, 55-100% B; 90-95 min, 100% B; 95-96 min, 100-5% B; 96-100 min, 5% B. The flow rate was 0.2 ml/min, injection volume was 5-10 μl, and detection was at 280 nm. The LC elute was introduced directly into the ESI interface without flow splitting. The scan range of ESI-MS was *m/z* 160-800. The ESI voltage was 4.5 kV in positive ion mode and 3.5 kV in negative ion mode. A nebulizing gas of 1.5 l/min and a drying gas of 10 l/min were applied for ionization using nitrogen in both cases.

GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS)

A GCMS-QP2010 system (Shimadzu, Japan) was employed for analysis of volatile and aliphatic compounds. A CP-WAX 52 CB column (50 m × 0.20 mm, ø with 0.2 μm film thickness) was used with helium as a carrier gas at a flow rate of 1 ml/min and injection volume was 1 μl. The GC oven temperature was kept at 40°C for 0.5 min and programmed to 150°C at a rate of 6°C/min, to 250°C at a rate of 8°C/min, and kept constant at 250°C for 8 min. Splitless injections were done with both headspace and liquid injection methods. The MS was operated in electron ionization mode (70 eV) with a scan range of *m/z* 40-600. The interface and ion source temperatures were 260°C and 200°C, respectively. Library search was carried out using NIST and SZTERP libraries.

STATISTICAL ANALYSIS

The results of TEAC and TPC in [Table 2](#) were calculated as mean ± standard deviation (SD) in this study. Differences between mean values were compared using the least significant difference (*LSD*) calculated with the Statistical Analysis System (SAS Institute, Inc, Cary, NC). Coefficients of determination (*R*²) were calculated using Microsoft Excel 2000.

RESULTS

ISOLATION AND IDENTIFICATION OF ENDOPHYTIC FUNGI

A total of 42 endophytic fungal strains were isolated from *N. oleander* and classified into 14 different taxa. Twenty-two of the strains isolated from leaf segments belong to seven different taxa, and 20 strains isolated from stem pieces belong to nine different taxa (two of them were the same as those in the leaves) (Table 1). Hyphomycete sp. and *Torula* sp. colonized both leaves and stems of *N. oleander*. *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., and *Phoma* spp. were found in the stems, and Ascomycete sp. was only found in the leaves. Half of the fungal stains (50% relative frequency) lacking sporulating structures were grouped into mycelia sterilia spp. Both leaves and stems were colonized by mycelia sterilia: they showed different cultural characters and were grouped into six morphosepcies.

TOTAL ANTIOXIDANT CAPACITY AND PHENOLIC CONTENT

The improved ABTS method has been widely used to assess total antioxidant capacity of crude extracts in both hydrophilic and lipophilic systems *in vitro* (Re et al. 1999; Cai et al. 2004; Surveswaran et al. n.d.). Therefore, this method was also employed in the present study to assay total antioxidant capacity of the methanolic extract from leaves and stems of *N. oleander* and metabolites of sixteen endophytic fungal cultures (Table 2). The host plant *N. oleander* showed medium antioxidant activity with the value of 17.89 mmol trolox/100 g DW. The TEAC values of the 16 endophytic fungi cultures exhibited a wide variation ranging from 9.59 to 150.8 μmol trolox/100 ml culture. The mean value of all the tested endophytic fungi was 36.55 μmol trolox/100 ml culture. Most of the fungal strains (75%) showed moderate antioxidant capacities (20 to 50 μmol trolox/100 ml culture). The strain NoS3 (*Chaetomium* sp.) possessed the highest antioxidant capacity (150.8 μmol trolox/100 ml culture). In contrast, the control broth without endophytic fungi showed nearly no activity (1.28 μmol trolox/100 ml).

Total phenolic contents of the metabolites of 16 endophytic fungal strains and their host plant *N. oleander* are shown in Table 2. The host plant contained 3.07 g gallic acid/100 g DW, and the 16 fungal strains showed different total phenolic contents ranging from 0.52 to 13.95 mg gallic acid/100 ml culture (mean = 2.64 mg/100 ml). Most endophytic fungi had medium TPC values (1.0-4.0 mg gallic acid/100 ml). The strain NoS3 had the highest TPC value

(13.95 mg gallic acid/100 ml culture). The TPC value of control broth without endophytic fungi was close to zero (0.02 mg/100 ml).

A highly positive linear correlation ($R^2 = 0.9406$) was found between the total antioxidant capacity (y) and total phenolic content (x) in the 16 fungal strains ($y = 10.078x + 9.9918$). This indicated that the phenolic compounds in the endophytic fungi significantly contributed to their antioxidant activity. Previous studies also revealed that phenolic compounds are major antioxidant constituents in medicinal plants, vegetables, fruits, and spices (Zheng and Wang 2001; Cai et al. 2004; Shan et al. 2005; Surveswaran et al. n.d.). Our investigation of endophytic fungi revealed the same relationship as found in plants.

XANTHINE OXIDASE INHIBITION ACTIVITY

Xanthine oxidase plays an important role in the metabolism of xanthines and it is closely related to hyperuricemia, therefore inhibition of xanthine oxidase is an effective therapeutic approach for treating gout, kidney stones and myocardial ischemia (Li et al. 1999; Kong et al. 2000; Kweon et al. 2001). The fungal strain *Chaetomium* sp. NoS3 and its host *N. oleander* were both tested for their xanthine oxidase inhibition activities. The methanolic crude extract of *N. oleander* was inhibitory against xanthine oxidase with an IC_{50} value of 218 $\mu\text{g/ml}$, while the crude culture of *Chaetomium* sp. NoS3 inhibited the enzyme with an IC_{50} value of 109.8 $\mu\text{g/ml}$. The IC_{50} values of three pure standards (allopurinol, rutin, and chlorogenic acid) co-assayed in this study as positive control were shown to be 3.1 $\mu\text{g/ml}$, 30.1 $\mu\text{g/ml}$, 27.2 $\mu\text{g/ml}$, respectively.

ANTIMICROBIAL ASSAY

The host plant *N. oleander* and the 16 endophytic fungal metabolites were tested for *in vitro* antimicrobial actions against five pathogenic bacteria and one pathogenic fungus (Table 3). The host plant was moderately inhibitory to *C. krusei*, *S. aureus*, *E. coli*, *L. monocytogenes*, *B. cereus*, and *S. annatum* with MICs of 1.56, 6.25, 12.5, 25, 25, and 25mg/ml, respectively. Most of the tested fungi isolated from *N. oleander* possessed better antibacterial and antifungal activities than the host plant. Six of the fungal strains (NoL3, NoL14, NoS3, NoS7, NoS10, and NoS16) inhibited the growth of all six test microbes (MICs ≤ 10 mg/ml). Of the six endophytes, NoS16 exhibited strongest antibacterial activity against *L. monocytogenes* with an MIC at 0.08 mg/ml, followed by NoS12 and NoS7 with MICs at 0.16 and 0.31 mg/ml, respectively. Furthermore, 11 of the 16 endophytes also possessed good

antifungal activities against *C. krusei* (MICs \leq 10 mg/ml), and four strains (NoL12, NoS3, NoS15, and NoS16) exhibited the strongest antifungal activity (MIC = 1.25 mg/ml). Positive control co-assayed gentamicin was antibacterial against *L. monocytogenes*, *B. cereus*, *S. aureus*, *S. annatum*, and *E. coli* with MICs of 1.22, 2.44, 9.75, 9.75, and 9.75 μ g/ml, respectively, and ketoconazole was antifungal against *C. krusei* at 7.81 μ g/ml.

PRIMARY IDENTIFICATION OF BIOACTIVE CONSTITUENTS FROM ENDOPHYTIC METABOLITES AND HOST PLANT EXTRACT

Preliminary identification of major types of phenolic compounds by LC-ESI-MS and other bioactive principles (volatile and aliphatic constituents) by GC-MS showed that major types of bioactive compounds in the host plant and endophytic fungal metabolites included phenolic acids and their derivatives, flavonoids, phenolic terpenoids, volatile constituents, and aliphatic compounds. Some representative compounds identified in this study are given in [Table 2](#). Chlorogenic acid (5-*O*-caffeoylquinic acid), di-*O*-caffeoylquinic acid, and rutin were identified to be dominant phenolic compounds in *N. oleander*, and phenolic acid derivatives and terpenoids were detected in its endophytic fungal cultures NoL10, NoL11, NoL13, NoS13, and NoS16 ([Fig. 1 and Table 2](#)). Aliphatic compounds (9-octadecenoic acid and 9,12-octadecadienoic acid) and terpenoids were also found in the crude extract of *N. oleander*. Many aliphatic compounds (e.g., hexadecanoic acid methyl ester, octadecanoic acid methyl esters, octadecadienoic acid methyl esters) and volatile fermented constituents (e.g., acetol, 2,3-butanediol) were also detected in some crude cultures of the endophytic fungi ([Fig. 2 and Table 2](#)).

DISCUSSION

Endophytes have been found in most taxa in the plant kingdom including algae, mosses, ferns and vascular plants and are potential sources of novel natural products for exploitation for medicinal, agricultural and industrial use ([Strobel and Long 1998](#); [Arnold et al. 2001](#); [Tan and Zou 2001](#); [Schulz et al. 2002](#)). In this study, 42 endophytic fungal strains isolated from *N. oleander* were grouped into 14 different taxa, including Ascomycete sp., *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., Hyphomycete sp., mycelia sterilia spp. (6 species), *Phoma* spp. (2 species), and *Torula* sp. Most of the taxa were common endophytic fungi ([Photita et al. 2005](#); [Zou and Tan 2000](#)). Cultures lacking reproductive structures or distinctive features were grouped into morphospecies based on similar cultural characters. Morphospecies cannot be identified to species or genus level without molecular analysis,

which is a common problem concerning the identification of endophytes (Arnold et al. 2000; Gamboa and Bayman 2001; Wiyakrutta et al. 2004; Wang et al. 2005).

Antioxidants are thought to be highly profitable in the management of reactive oxygen species-mediated tissue impairments. It has been reported that many antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Halliwell 1994; Mitscher et al. 1996; Owen et al. 2000; Sala et al. 2002; Cozma 2004). Some antioxidant compounds isolated from endophytic fungi and their antioxidant activities have also been reported (Harper et al. 2003; Song et al. 2005). The improved ABTS method (Re et al. 1999; Cai et al. 2004; Shan et al. 2005), a rapid and reliable test of total antioxidant capacity *in vitro* for crude extracts of plants, was successfully used in the present study to evaluate endophytic fungal cultures as well as the host plant. The results showed that most endophytic fungi isolated from *N. oleander* exhibited antioxidant capacity to some extent. The fungal strain *Chaetomium* sp. NoS3 with the strongest antioxidant activity was further screened for bioactive compounds.

The phenolic compounds (e.g., phenolic acids and their derivatives, flavonoids, and phenolic terpenoids) and certain volatile and aliphatic constituents identified in the present study might be responsible to some extent for the total antioxidant capacity of the host plant and the isolated endophytic fungi. Chlorogenic acid, phenolic acid derivatives, and rutin possess a wide range of biological activities, such as antioxidant, antimutagenic, immunomodulatory, and antiviral activity (Li et al. 2005; dos Santos et al. 2005; Yu et al. 2005). Biogenic volatile organic compounds are involved in multiple inter-organism interactions and are also important in the flavor and fragrance, pesticide, and perfumery industries (Zini et al. 2003), such as acetol (1-hydroxy-2-propanone) which has applications as a pharmaceutical and dye intermediate (Cameron and Cooney 1986) and recently has been reported as a potent inhibitor of urease (Tanaka et al. 2004). Aliphatic constituents are frequently encountered as structural subunits in many natural products and some also have shown to be highly toxic towards fungi, bacteria, and mammalian cells, and to display neurotoxic, anti-inflammatory, nematocidal activities and anti-platelet-aggregatory effects (Nogueira et al. 1996; Christensen and Brandt 2006). Hexadecanoic acid methyl ester, an aggregation pheromone was reported attractive to both males and virgin females (Takacs et al. 2001). Some of these bioactive compounds were detected in the host plant *N. oleander* and/or its endophytic fungi.

Many antibiotics have encountered drug resistance or cause severe adverse drug reactions, and there is an urgent need to search for new antibiotics (Rouveix 2003). Several recent studies (Ezra et al. 2004; Shiono et al. 2005; Shiono 2006) reported that novel compounds with antimicrobial activity were isolated from the cultured endophytic fungi. Wiyakrutta et al. (2004) also reported that endophytic fungi with anti-microbial, anti-cancer and anti-malarial activities were isolated from Thai medicinal plants. The present study shows that endophytic fungi isolated from *N. oleander* also have a wide range of antimicrobial activities.

Xanthine oxidase is a key enzyme that catalyzes the oxidation of hypoxanthine or xanthine to uric acid. Therefore, inhibition of xanthine oxidase is an effective therapeutic approach for treating hyperuricemia (Li et al. 1999; Kweon et al. 2001). As the current clinical drug used to inhibit xanthine oxidase causes severe adverse effects, it is necessary to search for new inhibitors of the enzyme (Kong et al. 2000). Some flavonoids have been shown to be inhibitory against it (Nagao et al. 1999; Li et al. 1999), and endophytic naphthopyrone metabolites were obtained as the enzyme inhibitor by Song et al. (2004). In this study, we showed that the crude extract of *N. oleander* and the culture of *Chaetomium* sp. NoS3 could both inhibit the enzyme, indicating that they contain certain xanthine oxidase inhibitors. High levels of rutin, chlorogenic acid and its derivatives which have good inhibition of the enzyme activity were indeed detected in the crude extract of *N. oleander* (Fig. 1B). However, the related bioactive constituents in the crude culture of *Chaetomium* sp. NoS3 could not be identified by LC-ESI-MS and GC-MS. This could be due to their low quantity in the fungal culture, or other types of inhibitors of xanthine oxidase exist that are yet to be identified.

Different bioactive compounds including phenolics normally possess specific chromatographic behavior and UV-vis spectral characteristics (Sakakibara et al. 2003; Santos-Buelga and Williamson 2003; Cai et al. 2004; Shan et al. 2005). In this study, only the major phenolic compounds from *N. oleander* and the 16 fungal metabolites were analyzed using LC-ESI-MS and compared with authentic phenolic standards and related literature data (Tan and Zou 2001; Sakakibara et al. 2003; Cai et al. 2004). Because of the diversity and complexity of the natural mixtures of bioactive compounds in the crude plant extract and fungal cultures, it is rather difficult to characterize every compound present and elucidate its structure in a single study. Further investigation is still needed to discover the unidentified/unknown bioactive constituents in the endophytic fungal isolates.

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FIGURE LEGENDS

Fig. 1. HPLC chromatograms (280 nm) and ESI-MS data of phenolics from **(A)** a typical endophytic fungus (NoL11) and **(B)** its host plant (*N. oleander*). Tentative identification of major peaks: (1), phenolic acid derivatives; (2) and (3), phenolic terpenoid (MW 310) and its isomer (MW 310); (4), unknown terpenoid (262); (5) and (6), chlorogenic acid (5-*O*-caffeoylquinic acid) (MW 354) and its isomer (MW 354); (7), rutin (MW 610); (8), di-caffeoylquinic acid (MW 516); (9), phenolic terpenoid (MW 534).

Fig. 2. GC chromatograms of volatile and aliphatic compounds from two typical endophytic fungi **(A)** NoS16 and **(B)** NoL11, and **(C)** their host plant (*N. oleander*). Molecular weight and formula are noted for most peaks in the figure. Tentative identification of major peaks: (1), acetol (1-hydroxy-2-propanone); (2), hexadecanoic acid methyl ester; (3), 9-octadecadienoic acid methyl ester; (4), 9,12-octadecadienoic acid methyl ester; (5) and (6), 2,3-butanediol and its isomer; (7), 9-octadecenoic acid; (8), 9,12-octadecadienoic acid.

TABLE 1. FUNGAL TAXA AND THE NUMBER OF ISOLATED ENDOPHYTES FROM *N. OLEANDER*.

Taxa	Isolates in leaves	Isolates in stems	Relative frequency of isolation (%) ^a
Ascomycete sp.	1		2.38
<i>Chaetomium</i> sp.		1	2.38
<i>Cladosporium</i> sp.		2	4.76
<i>Colletotrichum</i> sp.		1	2.38
Hyphomycete sp.	2	3	11.9
<i>Mycelia sterilia</i> spp.	(14)	(7)	(50.0)
<i>Mycelia sterilia</i> sp. 1	9		21.4
<i>Mycelia sterilia</i> sp. 2	3		7.14
<i>Mycelia sterilia</i> sp. 3	1		2.38
<i>Mycelia sterilia</i> sp. 4	1		2.38
<i>Mycelia sterilia</i> sp. 5		5	11.9
<i>Mycelia sterilia</i> sp. 6		2	4.76
<i>Phoma</i> spp.		(4)	(9.52)
<i>Phoma</i> sp. 1		2	4.76
<i>Phoma</i> sp. 2		2	4.76
<i>Torula</i> sp.	5	2	16.7
Total	22	20	100

^a Relative frequency of isolation used for indicating species abundance was calculated as the number of identified fungal isolates of a taxon divided by the total number of endophytic fungal isolates (22 + 20 = 42) from the leaf segments and stem pieces (Photita et al., 2001).

TABLE 2. ANTIOXIDANT CAPACITY, TOTAL PHENOLIC CONTENT AND MAJOR CONSTITUENTS OF 16 SELECTED ENDOPHYTIC FUNGAL CULTURES AND THE HOST (*N. OLEANDER*) EXTRACT.

Sample	Taxa	TEAC ($\mu\text{mol trolox}/100$ ml ferment broth)	TPC (mg gallic acid/100 ml ferment broth)	Major types (representative compounds) of phenolics and other constituents
NoL3	Ascomycete sp.	42.73 \pm 0.478	2.92 \pm 0.074	Not identified
NoL6	Mycelia sterilia sp. 1	37.42 \pm 0.907	3.82 \pm 0.110	Terpenoids, volatile compounds (acetol, 2,3-butanediol)
NoL9	Mycelia sterilia sp. 2	10.50 \pm 0.517	0.52 \pm 0.024	Terpenoids, volatile compounds (acetol)
NoL10	Hyphomycete sp.	23.89 \pm 0.639	0.97 \pm 0.028	Phenolic acid derivatives
NoL11	Mycelia sterilia sp. 1	14.68 \pm 0.157	1.46 \pm 0.078	Terpenoids, volatile compounds (acetol, 2,3-butanediol)
NoL12	<i>Torula</i> sp.	38.38 \pm 0.860	2.65 \pm 0.065	Volatile compounds (acetol)
NoL13	Mycelia sterilia sp. 3	17.45 \pm 0.266	2.19 \pm 0.031	Phenolic acid derivatives, volatile compounds (acetol)
NoL14	Mycelia sterilia sp. 4	26.98 \pm 0.841	1.90 \pm 0.069	Aliphatic compounds (hexadecanoic acid methyl ester, 7-octadecenoic acid methyl ester)
NoS3	<i>Chaetomium</i> sp.	150.8 \pm 2.045	13.95 \pm 0.109	Not identified
NoS5	<i>Cladosporium</i> sp.	9.59 \pm 0.121	0.64 \pm 0.063	Volatile compounds (acetol)
NoS7	<i>Phoma</i> sp. 2	26.80 \pm 0.841	1.40 \pm 0.064	Not identified
NoS10	<i>Phoma</i> sp. 1	46.78 \pm 0.429	2.67 \pm 0.028	Aliphatic compounds (hexadecanoic acid methyl ester)
NoS12	<i>Colletotrichum</i> sp.	37.10 \pm 0.788	2.04 \pm 0.072	Not identified
NoS13	Mycelia sterilia sp. 6	42.51 \pm 0.440	1.71 \pm 0.028	Phenolic acid derivatives, volatile compounds (2,3-butanediol, acetol)
NoS15	<i>Torula</i> sp.	30.07 \pm 0.672	1.54 \pm 0.032	Volatile compounds (acetol, 2,3-butanediol)
NoS16	Mycelia sterilia sp. 5	29.07 \pm 0.343	1.78 \pm 0.018	Phenolic acid derivatives, volatile compounds (acetol), aliphatic compounds (hexadecanoic acid methyl ester, 9-octadecadienoic acid methyl ester, 9,12-octadecadienoic acid methyl ester)
Mean		36.55	2.64	
LSD ($p < 0.05$) *		1.247	0.097	
Control broth		1.28	0.02	
<i>N. oleander</i> extract ^a		17.89 \pm 0.010	3.07 \pm 0.008	Phenolic acid (chlorogenic acid, di-caffeoylquinic acid), flavonoids (rutin), terpenoids, volatile compounds, aliphatic compounds (9-octadecenoic acid, 9,12-octadecadienoic acid)

* LSD ($p < 0.05$), least significant difference, was used for comparison among means of various endophytic fungal cultures.

^a mmol trolox /100 DW for TEAC and g gallic acid/100 DW for TPC.

TABLE 3. IN VITRO ANTIMICROBIAL ACTIVITIES OF ENDOPHYTIC FUNGAL METABOLITES AND THEIR HOST PLANT (*N. OLEANDER*) EXTRACT.

Test sample	MIC (mg/ml) of test microbe ^a					
	<i>Listeria monocytogenes</i>	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Salmonella anatum</i>	<i>Escherichia coli</i>	<i>Candida krusei</i>
NoL3	0.63	2.50	10.0	5.00	5.00	2.50
NoL6	>10	2.50	10.0	>10	>10	>10
NoL9	>10	>10	>10	>10	>10	>10
NoL10	10.0	2.50	>10	>10	>10	5.00
NoL11	1.25	2.50	>10	10.0	>10	>10
NoL12	2.50	1.25	>10	10.0	10.0	1.25
NoL13	>10	>10	>10	>10	>10	10.0
NoL14	5.00	5.00	10.0	10.0	10.0	2.50
NoS3	5.00	1.25	10.0	10.0	10.0	1.25
NoS5	>10	10.0	>10	>10	>10	>10
NoS7	0.31	10.0	5.00	10.0	10.0	10.0
NoS10	2.50	2.50	5.00	10.0	5.00	2.50
NoS12	0.16	5.00	10.0	10.0	5.00	>10
NoS13	1.25	>10	>10	>10	>10	2.50
NoS15	0.63	1.25	>10	10.0	10.0	1.25
NoS16	0.08	2.50	10.0	10.0	10.0	1.25
<i>N. oleander</i>	25.0	6.25	25.0	25.0	12.5	1.56
Positive control ^b						
gentamicin	1.22	9.75	2.44	9.75	9.75	n.d. ^c
ketoconazole	n.d.	n.d.	n.d.	n.d.	n.d.	7.81

^a *L. monocytogenes*, *S. aureus*, *B. cereus*, *S. annatum*, and *E. coli* are bacteria, and *C. krusei* is a fungus.

^b µg/ml for MIC of positive control.

^c n.d., no determination.

Fig. 1.

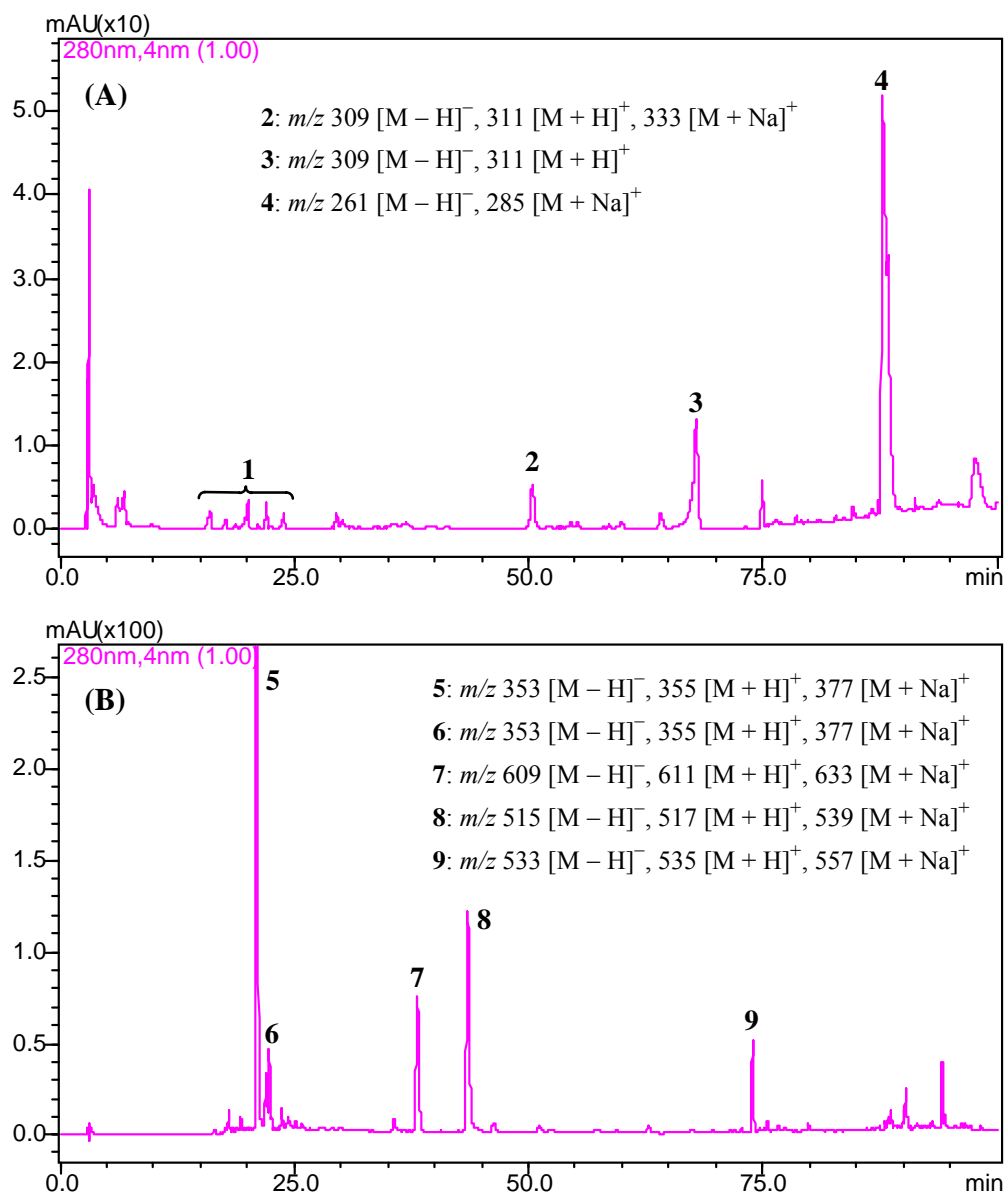


Fig. 2.

