Antimicrobial Fungal Endophytes from the Botanical Medicine Goldenseal (*Hydrastis canadensis*)

By: Joseph M. Egan, Amninder Kaur, Huzefa A. Raja, Joshua J. Kellogg, Nicholas H.Oberlies, and Nadja B. Cech

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Abstract:

The potential of fungal endophytes to alter or contribute to plant chemistry and biology has been the topic of a great deal of recent interest. For plants that are used medicinally, it has been proposed that endophytes might play an important role in biological activity. With this study, we sought to identify antimicrobial fungal endophytes from the medicinal plant goldenseal (*Hydrastis canadensis* L., Ranunculaceae), a plant used in traditional medicine to treat infection. A total of 23 fungal cultures were obtained from surface-sterilized samples of *H. canadensis* roots, leaves and seeds. Eleven secondary metabolites were isolated from these fungal endophytes, five of which had reported antimicrobial activity. *Hydrastis canadensis* plant material was then analyzed for the presence of fungal metabolites using liquid chromatography coupled to high resolving power mass spectrometry. The antimicrobial compound alternariol monomethyl ether was detected both as a metabolite of the fungal endophyte *Alternaria* spp. isolated from *H. canadensis* seeds, and as a component of an extract from the *H. canadensis* seed material. Notably, fungi of the *Alternaria* genus were isolated from three separate accessions of *H. canadensis* plant material collected in a time period spanning 5 years. The concentration of alternariol monomethyl ether (991 mg/kg in dry seed material) was in a similar range to that previously reported for metabolites of ecologically important fungal endophytes. The seed extracts themselves, however, did not possess antimicrobial activity.

Keywords: *Hydrastis canadensis* | Endophytes | Secondary metabolites | Fungi | Alternariol monomethyl ether

Article:

1. Introduction

This study focuses on endophytic fungi, which live asymptomatically within plant tissues, and the secondary metabolites that they biosynthesize. There has recently been a great deal of interest in endophytic fungi as a source for natural product drug discovery (Strobel, 2003, Suryanarayanan et al., 2009). It has also been shown that endophytic fungi can have positive effects on their host plants, including improving drought tolerance, and producing protective compounds (Bush et al., 1997). Certain bioactive components of endophytic fungi have been shown to possess a number of different activities against human pathogens, and to possess novel cytotoxicity mechanisms (Strobel, 2003). Furthermore, some fungal endophytes produce the same bioactive components as those known to be present in botanicals that they inhabit, which suggests that the influence of an endophyte may go beyond that of basic protection (El-Elimat et al., 2014, Kusari et al., 2013, Nisa et al., 2015, Stierle et al., 1993).

Because endophytic fungi are present in plant tissues, it has been suggested that compounds originating from endophytes may play a role in the biological activity of botanical extracts. There have been a few cases where this has been shown to be true for endophytic bacteria, which alter the *in vitro* activity of *Echinacea purpurea* extracts (Pugh et al., 2013, Todd et al., 2015). It is well known that fungal secondary metabolites can demonstrate potent biological effects, which supports the hypothesis that fungal symbionts could also contribute to or alter the biological activity of botanical extracts. The goal of this study was to explore the potential role of fungal endophytes in the antimicrobial activity of the botanical medicine goldenseal, *Hydrastis canadensis* L. (Ranunculaceae).

It has previously been shown that *H. canadensis* has antimicrobial activity against a number of bacterial pathogens, and this activity has primarily been attributed to the alkaloid berberine (Hwang et al., 2003, Scazzocchio et al., 2001). However, several studies have demonstrated that the antimicrobial activity of goldenseal crude extracts is more pronounced (by approximately 2-fold) than that of isolated berberine, suggesting that other compounds must also play a role (Cech et al., 2012). With this study, we sought to isolate and identify fungal endophytes from *H. canadensis* plant material and determine whether fungal metabolites contribute to the chemistry and antimicrobial activity of goldenseal botanical extracts.

Table 1. Identities of endophytic fungi. G numbers were assigned for in house identification, and OTU identification was performed via ITS rDNA sequencing.

G#	OTU identification[a]	Genbank accession numbers	Species identification of most homologous sequence from GenBank based on BLAST search[a]	Origin from Plant source	Reference
G09	*Diaporthe eres*	KX110385	*Diaporthe eres (=Diaporthe cotonesteri; NR_119726)*	Leaf	Udayanga et al. (2014)
G10	*Diaporthe* sp.	KX110386	*Diaporthe terebinthifolii (NR_111862)*	Leaf	Gomes et al. (2013)
G11	*Sordariomycete*sp. *(Xylariales)*	KX110387	*Sordariomycete* sp. *NC1024 (JQ761762)*	Leaf	U'Ren et al. (2012)
G12	*Colletotrichum fioriniae*	KX110388	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Stem	Damm et al. 2012, and Shivas and Yu (2009)

G#	OTU identification[a]	Genbank accession numbers	Species identification of most homologous sequence from GenBank based on BLAST search[a]	Origin from Plant source	Reference
G13	*Alternaria* sp.	KX110389	*Alternaria alternata* (CBS 916.96; GenBank: FJ196306; AF347031	Stem	Pryor and Michailides (2002) and Woudenberg et al. (2013)
G14	*Diaporthe eres*	KX110390	*Diaporthe eres (=Diaporthe cotonesteri; NR_119726)*	Stem	Udayanga et al. (2014)
G15	*Diaporthe eres*	KX110391	*Diaporthe eres (=Diaporthe cotonesteri; NR_119726)*	Stem	Udayanga et al. (2014)
G16	*Diaporthe* sp.	KX110392	*Diaporthe eucalyptorum (NR_120157)*	Root	Crous et al. (2012)
G17	*Diaporthe* sp.	KX110393	*Diaporthe eucalyptorum (NR_120157)*	Root	Crous et al. (2012)
G22	*Phoma* sp.	KX110394	*Phoma bellidis (GU237904)*	Leaf	Aveskamp et al. (2010)
G23	*Magnaporthales*sp.	KX110395	*Mycoleptodiscus terrestris (JN711860)*	Root	Koo et al. (2012)
G28	*Alternaria* sp.	KT825854	*Alternaria alternata* (CBS 916.96; GenBank: FJ196306; AF347031	Seed	Pryor and Michailides (2002) and Woudenberg et al. (2013)
G29	*Diaporthe* sp.	KX110397	*Diaporthe terebinthifolii (NR_111862)*	Seed	Gomes et al. (2013)
G30	*Colletotrichum fioriniae*	KX110397	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Seed	Damm et al. (2012)and Shivas and Yu (2009)
G31	*Alternaria alternata*	KX110398	*Alternaria alternata* (CBS 916.96; GenBank: FJ196306; AF347031	Seed	Pryor and Michailides (2002) and Woudenberg et al. (2013)
G33	*Alternaria* sp.	KX110399	*Alternaria alternata* (CBS 916.96; GenBank: FJ196306; AF347031	Seed	Pryor and Michailides, (2002) and Woudenberg et al. (2013)
G34	*Colletotrichum fioriniae*	KX110400	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Seed	Damm et al. (2012)and Shivas and Yu (2009)
G35	*Colletotrichum fioriniae*	KX110401	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Seed	Damm et al. (2012)and Shivas and Yu, (2009)
G36	*Alternaria* sp.	KX110402	*Alternaria alternata* (CBS 916.96; GenBank: FJ196306; AF347031	Seed	Pryor and Michailides, 2002 and Woudenberg et al. (2013)
G38	*Colletotrichum fioriniae*	KX110403	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Seed	Damm et al., (2012)and Shivas and Yu (2009)
G39	*Colletotrichum fioriniae*	KX110404	*Colletotrichum fioriniae (NR_117474, EF464594) (JN709486)*	Seed	Damm et al. (2012)and Shivas and Yu (2009)
G41	*Pyrenochaeta*sp.	KT825855	*Pyrenochaeta* sp. *(EU885415)*	Leaf	Ferrer et al. (2009)
G42	*Sordariomycete*sp. *(Xylariales)*	KX110405	*Sordariomycete* sp. *NC1024 (JQ761762)*	Leaf	U'Ren et al. (2012)

[a] Fungal endophyte OTUs were tentatively assigned to either genus or species by matching the most homologous sequences in GenBank by BLAST search. Mainly authentic sequences were used for assigning OTUs preferentially from type or other authentic, and annotated cultures generated by taxonomic specialist published in high impact factor mycology journals. When multiple species were found to have high sequence similarity, or when <97% sequence homology was found with a published authentic sequence for which a culture was deposited in a public

culture collection, we choose to take a more conservative approach and use only the genus, family, order, class name for OTU assignment (see Raja et al., 2015).

2. Results

2.1. Endophyte identities and activities

Endophytic fungi, a total of 23 isolates, were cultured and identified from *Hydrastis canadensis.* The fungi were identified based on fragments of complete ITSrDNA; approximately 600−650 bp. Five isolates were identified as *Alternaria* spp., six as *Colletotrichum fioriniae*, three as *Diaporthe eres*, four as *Diaporthe* spp., two as *Sordariomycete* spp., one as *Magnaporthales* spp., one as *Phoma* sp., and one as *Pyrenocheta cava* (Table 1). A number of the fungal extracts possessed marked antimicrobial activity against *Staphylococcus aureus*, with the most pronounced activity observed for *Alternaria* sp. (Table S1).

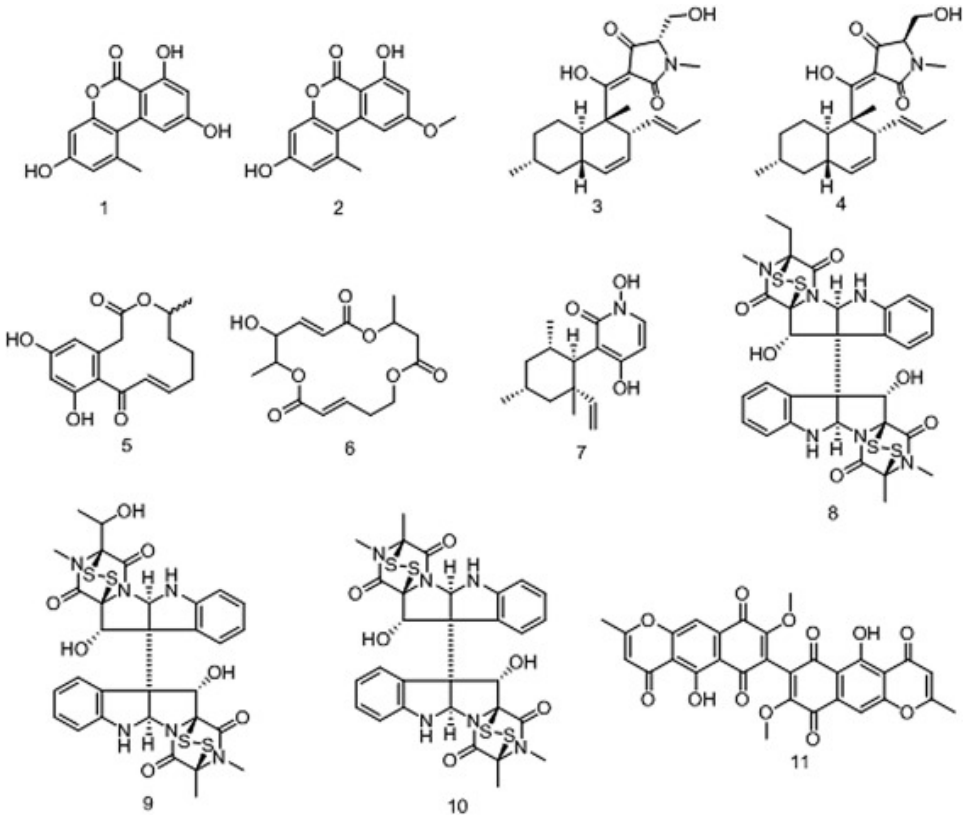

Fig. 1. Metabolites from endophytic fungi of *Hydrastis canadensis*. Isolated metabolites, Alternariol (**1**), Alternariol monomethyl ether (**2**), 5′-epiquisetin (**3**), equisetin (**4**), 10−11 didehydrocurvularin (**5**), Macrosphelide A (**6**), 8-Methyl-pyridoxatin (**7**), were found to be >95% Pure by UV/Vis. Metabolites Sch 52901 (**8**), Sch 52900 (**9**), verticillin A (**10**), Aurofusarin (**11**) were tentatively identified by comparisons of UPLC−MS/MS data against a published database (El-Elimat et al., 2013).

2.2. Fungal metabolites

A total of eleven secondary metabolites, all of which are known compounds, were identified from the fungal isolates (Fig. 1, Table S2). The compounds alternariol (**1**), alternariol monomethyl ether (**2**), 5′epi-equisetin (**3**) equisetin (**4**), 10−11 dehydrocurvularin (**5**), macrosphelide A (**6**), and cordipyridone A (**7**), were isolated with chromatographic methods and their spectroscopic data (accurate mass, ^{1}H NMR, and ^{13}C NMR) matched literature reports (Table S2). The remaining compounds, Sch 52901 (**8**), Sch 52900 (**9**), verticillin A (10**)**, aurofusarin (**11**) were identified by comparison of LC−MS data with a library of previously isolated fungal metabolites (El-Elimat et al., 2013, Figueroa et al., 2014).

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2.3. Presence of fungal metabolites in plant material

Of the eleven metabolites identified from the fungal endophytes, one compound, alternariol monomethyl ether (**2**), was detected in a botanical extract with the methods employed here. Retention time, accurate mass ($[M+H]^{+}$, 273.0754, calcd for $C_{15}H_{13}O_5^{+}$, 273.0763), and MS-MS fragmentation pattern for the putative alternariol monomethyl ether ion in the extract matched that of isolated alternariol monomethyl ether (Fig. 2). The alternariol monomethyl ether was detected in two adjacent normal phase chromatography fractions of a seed extract from *H. canadensis*. This compound was absent from other fractions of the same extract and from blank injections conducted before each analysis. Additionally, extracts from the other plant parts of *H. canadensis* did not contain detectable levels of alternariol monomethyl ether.

Consistent with the presence of alternariol monomethyl ether in the botanical seed extract, this compound was also isolated from two *H. canadensis* stem and seed endophytes (*Alternaria* sp., G13 and G31, Table 1). Interestingly, *Alternaria* spp. are known to exist both as endophytes and plant pathogens (Woudenberg et al., 2013), and thus, it is possible that this fungus inhabits *H. canadensis* seeds as a means of transmission to *H. canadensis* seedlings. At the same time, the fungus may confer a protective effect on the seeds by producing antimicrobial compounds.

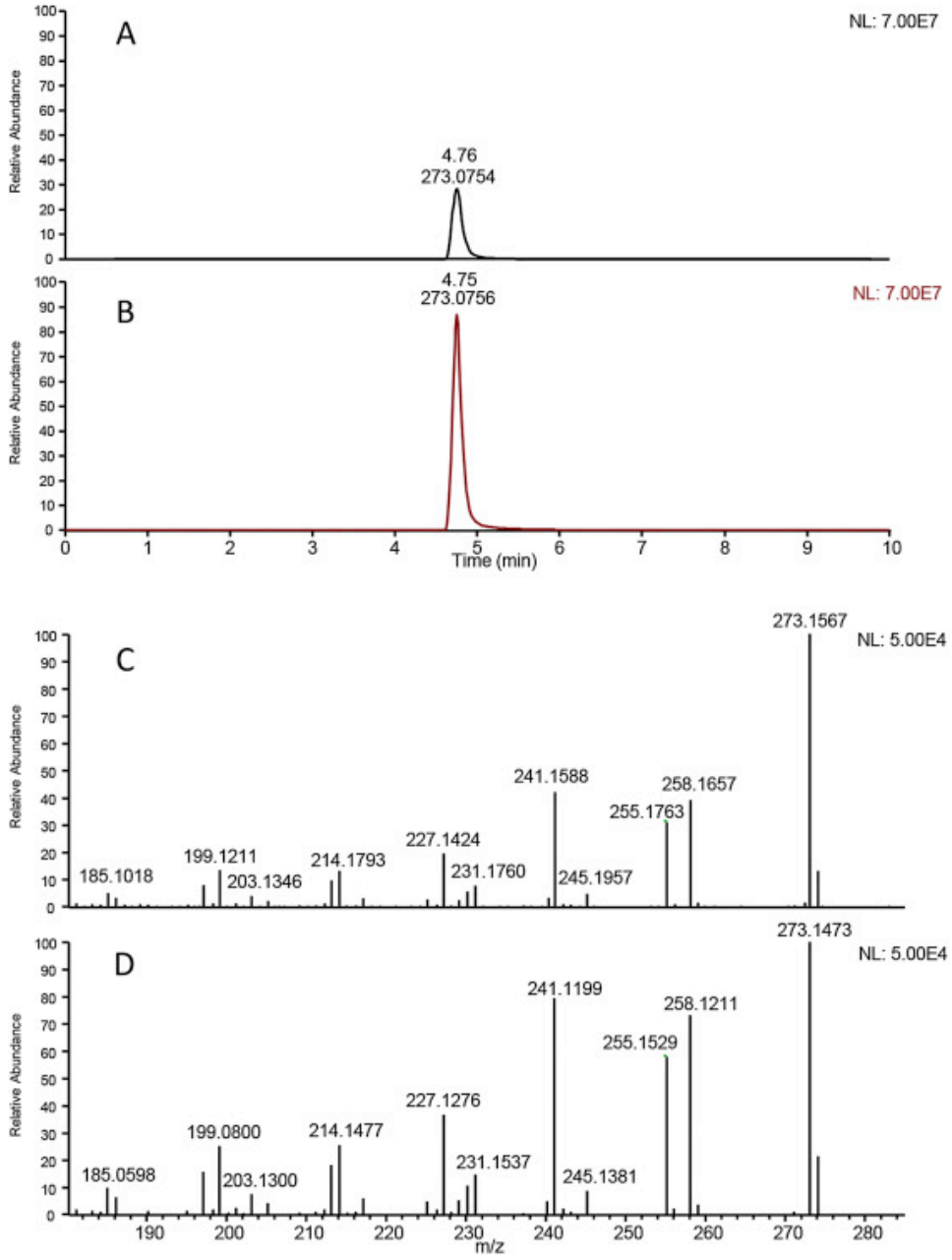

Fig. 2. Selected ion chromatogram for *m*/*z* 273.0755 in a concentrated fraction from an extract of *H. canadensis* seeds (analyzed at 1 mg/mL) (A) and isolated alternariol monomethyl ether from *Alternaria* sp. at a concentration of 1 mg/mL (B). MS-MS fragmentation patterns for the precursor ion at *m*/*z* 273.0754 are in excellent agreement between the compound detected in the plant material (C) and isolated alternariol monomethyl ether (D). NL indicates the level of mass spectrometric signal to which each spectrum or chromatogram is normalized.

The presence of alternariol monomethyl ether in the extract of *H. canadensis* seeds known to be colonized by *Alternaria* sp. is highly suggestive of fungal origin for this compound. The possible explanation that alternariol monomethyl ether is produced by the plant itself cannot, however, be ruled out. Alternariol monomethyl ether has been widely reported in the literature as a fungal metabolite. Aly et al. (2008) also detected this metabolite in the plant *Polygonum senegalense*,

which was colonized with *Alternaria* sp., and attributed its presence to the fungus, not the botanical. Onocha et al. detected alternariol monomethyl ether in a botanical, *Anthocleista djalonensis*. These authors assumed alternariol monomethyl ether to be of botanical origin on the basis of a lack of visible mold on the botanical sample (Onocha et al., 1995). However, another explanation would have been its production by fungal endophytes, which can be present without any visible symptoms of infection.

2.4. Antimicrobial activity of fungal metabolites and botanical extracts

To explore the potential relevance of alternariol monomethyl ether to the biological activity of *H. canadensis*, antimicrobial activity against *Staphylococcus aureus* was measured for all of the botanical extracts and for isolated alternariol monomethyl ether. *S. aureus* was chosen as a test case for this study because it is a common Gram-positive bacterial pathogen (Kaatz and Seo, 1995). As demonstrated in Table 2, the seed extract showed only weak antimicrobial activity (Fig. S1), with minimum inhibitory concentration (MIC) of ≥200 μg/mL. The extract of the *H. canadensis* aerial portion extract was slightly more active, with an MIC of 200 μg/mL (Fig. S2). The antimicrobial activity of this extract is likely due to plant derived alkaloids, which are known to be abundant in *H. canadensis* leaves (Ettefagh et al., 2011 Purified alternariol monomethyl ether was more active as an antimicrobial than the botanical extracts, with an MIC of 75 μg/mL (Fig. S3).

Table 2. Antimicrobial activities of extracts, purified metabolite, and controls.

Sample	**MIC**[a]
aerial extract (leaves and stems)	200 μg/mL[b]
seed extract	>200 μg/mL
berberine (+control)[c]	75 μg/mL
alternariol monomethyl ether	75 μg/mL

[a] MIC is defined as the concentration that caused OD_{600} to be reduced by ≥95%.

[b] Concentration is expressed as μg/mL of dried plant extract per mL of assay well volume.

[c] Berberine served as a positive control and demonstrated activity consistent with previous reports (Amin et al., 1969, Łerľáková and Košťálová, 2002).

The results for quantitative analysis of alternariol monomethyl ether in the botanical extracts provide some context for the findings in Table 2. This compound was found only in a purified fraction of the *H. canadensis* seed extract, and based on the concentration in this extract, the back-calculated concentration of alternariol monomethyl ether in the original extract was found to be 991 ppm (expressed as mg of alternariol monomethyl ether per kg of dry seed material). At the highest concentration of the seed extract tested in the bioassay (200 μg/mL, expressed as μg extract per mL assay volume), this equates to an assay concentration of only 0.198 μg/mL alternariol monomethyl ether, considerably below the observed MIC of 75 μg/mL for this compound. Thus, it is little surprise that the seed extract did not display marked antimicrobial activity (Table 2). However, it is worth noting that many of the fungal endophytes isolated as part of this study possessed antimicrobial activity (Table S1), and several antimicrobial compounds were identified from these fungi (Table S2). It is entirely possible that the combined effect of multiple low-level antimicrobial fungal metabolites could contribute to the overall activity of a botanical extract. It is also likely that the fungi under investigation here would produce different metabolites when growing as endophytes in plants than they do in isolated culture. Further studies would be necessary to explore these possibilities.

The detection of alternariol monomethyl ether in *H. canadensis* seeds suggests that the presence of the fungus may provide some benefit to the plant. The measured quantity of 991 ppm alternariol monomethyl ether in the *H. canadensis* seeds is in the concentration range reported for metabolites known to be ecologically important, such as the ergot and loline alkaloids produced by fungal endophytes of native grasses (Jarmusch et al., 2016, Shymanovich et al., 2014). Ergot and loline alkaloid alkaloids help protect grasses from insect herbivores (Bush et al., 1997). In a similar fashion, the presence of antimicrobial fungal metabolites in *H. canadensis* seeds might help protect the plant from bacterial pathogens. Exploration of this hypothesis would be another interesting avenue of further study.

Given that our data suggest a potential ecological role of *Alternaria* colonizing *H. canadensis*, we sought to evaluate whether fungi of this genus could be isolated from multiple collections of *H. canadensis*. Indeed, fungi of the genus *Alternaria* were identified in collections of goldenseal from the same plot over a five year time period. *Alternaria* sp. was isolated from *H. canadensis* seeds harvested on 7/11/2011 and 6/28/2012 (assigned accession numbers of G31 and G75, respectively). For a third collection conducted on 6/11/2015, *Alternaria* sp. was isolated from *H. canadensis* leaf tissue segments (accession number G657). The endophytes isolated from the three different collections showed morphology characteristic of *Alternaria* spp, which include the production of dark colored, dry, phaeodictyospores (conidia) in chains, and a beak of tapering apical cells (Woudenberg et al., 2013). All three isolates demonstrated >98% sequence similarity in their ITS region with *Alternaria alternata* section *Alternata* (CBS 916.96) (Pryor and Michailides, 2002, Woudenberg et al., 2013). However, given that Woudenberg et al. observed low variation in the ITS region of *Alternaria* section *Alternata*, we refrain from using the species epithet for the *Alternaria* isolates in this study.

In summary, we report here on the detection of the antimicrobial compound alternariol monomethyl ether in the seeds of a botanical medicine used in traditional medicine to treat infection. The concentration of this metabolite was too low to be primarily responsible for the antimicrobial activity against *Staphylococcus aureus* of *H. canadensis* botanical extracts. However, it is possible that colonization by *Alternaria* spp. could be of ecological importance. Isolation of *Alternaria* spp. from multiple accessions of *H. canadensis* supports this hypothesis. Future studies to investigate the presence of *Alternaria* spp. in *H. canadensis* from different geographical locations might be of interest.

3. Methods

3.1. Acquisition of plant material

Plant material was harvested from a cultivated plot in Hendersonville, NC (N35°24.2770, W 082°20.9930) on 07/11/2011 and a voucher was deposited in the University of North Carolina Herbarium (accession number NCU 583414). Additional collections were made on 6/28/2012 and 6/11/2015 from the same plot for further characterization of fungal endophytes (Table S3).

3.2. Fungal cultivation and strain identification

Fresh plant samples were surface sterilized as described previously (Raja et al., 2015). A total of 320 segments were plated, which included 100 stem segments; 100 leaf segments; 70 root segments; and 50 seeds. All fungal endophyte cultures that emerged from goldenseal plant parts are maintained on Potato Dextrose Agar; Difco (PDA) agar slants at 9° C at the University of North Carolina at Greensboro, Department of Chemistry and Biochemistry Fungal Culture Collection. A total of 23 fungi were cultured from tissues of *H. canadensis* (collected on July 11, 2011) and were grown on a solid-state rice fermentation medium, as previously described (Raja et al., 2015). All rice cultures were allowed to grow for 14−21 days prior to chemical extraction.

For molecular identification of fungal endophytes isolated from goldenseal, the internal transcribed spacer region of the ribosomal RNA gene (ITS) was sequenced using protocols outlined previously (Raja et al., 2015, Kellogg et al., 2016) and operational taxonomic unit (OTU) designations as surrogates for species identifications were made with BLAST search tool utilizing only authenticated, published sequences in NCBI GenBank using 98% identity (Raja et al., 2015, Schoch et al., 2014). The ITS sequences of all strains were deposited in GenBank and are listed in Table 1 and Table S3.

3.3. Extraction and preparation

For extraction, plant material was allowed to air dry for several weeks until crisp. Leaves and stems were separated from seeds, and each plant part was separately ground to a fine powder using a Thomas Wiley Mini-Mill. Fungal rice cultures and botanical extracts (59.2 g aerial parts, 2.54 g seeds) were extracted in 1:1 methanol:chloroform and subjected to liquid-liquid partitioning using previously described methods (El-Elimat et al., 2015, Kaur et al., 2016, Kellogg et al., 2016, Raja et al., 2015), resulting in a chloroform, aqueous, and hexane partitions. The chloroform partitions were of primary interest and used for further investigations.

3.4. Identification of fungal metabolites

Fungal extracts were analyzed using ultra performance liquid chromatography (Acquity UPLC, Waters) coupled to high resolving power tandem mass spectrometry (LTQ Orbitrap XL, Thermo) (LC−MS-MS) with previously published methods (El-Elimat et al., 2013). The retention time, accurate mass, and fragmentation spectrum for each ion detected were compared to a library of LC−MS data for 262 fungal metabolites to identify known compounds (El-Elimat et al., 2013). Compounds that were not represented in the existing fungal library were isolated and characterized via several rounds of flash chromatography on a Combiflash Rf system (Teledyne-ISCO, Lincoln, NE, USA) and high performance liquid chromatography on the Varian HPLC system (Agilent Technologies, Santa Clara, CA, USA). Structures of pure compounds were solved based on NMR data (Joel ECS 400 MHz, Joel ECA 500 MHz, Varian 700 MHz) and accurate mass measurements, as described previously (Raja et al., 2015). For each isolated compound, NMR and accurate mass data matched literature reports (Table S2).

The same method used to collect LC−MS-MS data of fungal extracts was also applied to botanical extracts from *H. canadensis.* To determine if fungal metabolites were detectable in the botanical extracts, the resulting LC−MS data were filtered for the *m*/*z* value of the $[M + H]^+$ ion of each fungal metabolite; a 5 ppm isolation window was used. For identification, comparisons

were made between retention time, accurate mass, and fragmentation pattern of any ion present in both the botanical and fungal extracts. Care was taken to ensure that any fungal metabolites identified in the extracts were not a result of accidental contamination from other fungal samples. Multiple blank injections were conducted between the analysis of each sample, and these blank injections were scrutinized to rule out carry-over.

3.5. Quantitative analysis of alternariol monomethyl ether

A stock solution of alternariol monomethyl ether isolated from *Alternaria* sp. (Table S2) was prepared in 50:50 methanol:dioxane at a concentration of 1 μg/mL. The identity of this compound was verified by NMR spectroscopy, and its purity was determined to be >95% based on UPLC (Kellogg et al., 2016). Calibration solutions were prepared from the stock solution via serial dilution over a concentration range of 0.2 mg/mL to 2 ng/mL. These solutions were analyzed using the same LC−MS-MS method applied to the extracts, as described previously (El-Elimat et al., 2013). A calibration curve was calculated as peak area for the selected $[M+H]^+$ ion for alternariol monomethyl ether (*m*/*z* 273.0755) versus concentration, and alternariol monomethyl ether concentration was determined in the fungal and botanical extracts based on the best-fit line for this calibration curve.

3.6. Evaluation of antimicrobial activity

Clinical Laboratory Standards Institute (Ferraro, 2000) methods for broth microdilution assays were employed to evaluate the antimicrobial activity of all fungal and botanical extracts, as well as the isolated alternariol monomethyl ether. Activity was evaluated against *Staphylococcus aureus* strain SA1199 (Kaatz and Seo, 1995). The known antimicrobial compound berberine served as a positive control for these experiments (Junio et al., 2011). All samples were tested in triplicate with a final DMSO content of 2% in each well. Growth was measured based on absorbance measurements at 600 nm (OD_{600}). Minimum inhibitory concentration (MIC) was defined as concentration at which the mean OD_{600} of the test wells was reduced by ≥95%.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j. phytol.2016.07.031.

References

Čerňáková, M., Košťálová, D., 2002. Antimicrobial activity of berberineČa constituent of Mahonia aquifolium. Folia Microbiol. (Praha) 47, 375–378.

Aly, A.H., Edrada-Ebel, R., Indriani, I.D., Wray, V., Müller, W.E., Totzke, F., Zirrgiebel, U., Schächtele, C., Kubbutat, M.H., Lin, W., 2008. Cytotoxic metabolites from the fungal endophyte Alternaria sp. and their subsequent detection in its host plant Polygonum senegalense. J. Nat. Prod. 71, 972–980.

Amin, A., Subbaiah, T., Abbasi, K., 1969. Berberine sulfate: antimicrobial activity, bioassay, and mode of action. Can. J. Microbiol. 15, 1067–1076.

Aveskamp, M.M., De Gruyter, J., Woudenberg, J., Verkley, G., Crous, P.W., 2010. Highlights of the Didymellaceae: a polyphasic approach to characterise Phoma and related Pleosporalean genera. Stud. Mycol. 65, 1–60.

Bush, L.P., Wilkinson, H.H., Schardl, C.L., 1997. Bioprotective alkaloids of grass-fungal endophyte symbioses. Plant Physiol. 114, 1–7.

Cech, N.B., Junio, H.A., Ackermann, L.W., Kavanaugh, J.S., Horswill, A.R., 2012. Quorum quenching and antimicrobial activity of goldenseal (Hydrastis canadensis) against methicillin resistant Staphyolococcus aureus. Planta Med. 78, 1556–1561.

Crous, P., Summerell, B., Shivas, R., Burgess, T., Decock, C., Dreyer, L., Granke, L., Guest, D., Hardy, G.S., Hausbeck, M., 2012. Fungal planet description sheets: 107–127. Persoonia-Mol. Phylogeny Evol. Fungi 28, 138–182.

Damm, U., Cannon, P., Woudenberg, J., Crous, P., 2012. The Colletotrichum acutatum species complex. Stud. Mycol. 73, 37–113.

El-Elimat, T., Figueroa, M., Ehrmann, B.M., Cech, N.B., Pearce, C.J., Oberlies, N.H., 2013. High-resolution MS, MS/MS, and UV database of fungal secondary metabolites as a dereplication protocol for bioactive natural products. J. Nat. Prod. 76, 1709–1716.

El-Elimat, T., Raja, H.A., Graf, T.N., Faeth, S.H., Cech, N.B., Oberlies, N.H., 2014. Flavonolignans from Aspergillus iizukae, a fungal endophyte of milk thistle (Silybum marianum). J. Nat. Prod. 77, 193–199. doi: http://dx.doi.org/10.1021/np400955q.

El-Elimat, T., Figueroa, M., Raja, H.A., Graf, T.N., Swanson, S.M., Falkinham, J.O., Wani, M.C., Pearce, C.J., Oberlies, N.H., 2015. Biosynthetically distinct cytotoxic polyketides from Setophoma terrestris. Eur. J. Org. Chem. 2015, 109–121.

Ettefagh, K.A., Burns, J.T., Junio, H.A., Kaatz, G.W., Cech, N.B., 2011. Goldenseal (Hydrastis canadensis L.) extracts synergistically enhance the antibacterial activity of berberine via efflux pump inhibition. Planta Med. 77, 835–840. doi: http://dx.doi.org/10.1055/s-0030-1250606.

Ferraro, M., 2000. National committee for clinical laboratory standards methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard 2012 M07-A9, vol. 36. Clinical and Laboratory Standards Institute, Wayne PA.

Ferrer, C., Pérez-Santonja, J.J., Rodríguez, A.E., Colom, M.F., Gené, J., Alio, J.L., Verkley, G.J., Guarro, J., 2009. New Pyrenochaeta species causing keratitis. J. Clin. Microbiol. 47, 1596–1598.

Figueroa, M., Jarmusch, A.K., Raja, H.A., El-Elimat, T., Kavanaugh, J.S., Horswill, A.R., Cooks, R.G., Cech, N.B., Oberlies, N.H., 2014. Polyhydroxyanthraquinones as quorum sensing inhibitors from the futtates of Penicillium restrictum and their analysis by desorption electrospray ionization mass spectrometry. J. Nat. Prod. 77, 1351–1358.

Gomes, R., Glienke, C., Videira, S., Lombard, L., Groenewald, J., Crous, P., 2013. Diaporthe: a genus of endophytic, saprobic and plant pathogenic fungi. Persoonia-Mol. Phylogeny Evol. Fungi 31, 1–41.

Hwang, B.Y., Roberts, S.K., Chadwick, L.R., Wu, C.D., Kinghorn, A.D., 2003. Antimicrobial constituents from goldenseal (the Rhizomes of Hydrastis canadensis) against selected oral pathogens. Planta Med. 69, 623–627.

Jarmusch, A.K., Musso, A.M., Shymanovich, T., Jarmusch, S.A., Weavil, M.J., Lovin, M.E., Ehrmann, B.M., Saari, S., Nichols, D.E., Faeth, S.H., Cech, N.B., 2016. Comparison of electrospray ionization and atmospheric pressure photoionization liquid chromatography mass spectrometry methods for analysis of ergot alkaloids from endophyte-infected sleepygrass (Achnatherum robustum). J. Pharm. Biomed. Anal. 117, 11–17. doi: http://dx.doi.org/10.1016/j.jpba.2015.08.031.

Junio, H.A., Sy-Cordero, A.A., Ettefagh, K.A., Burns, J.T., Micko, K.T., Graf, T.N., Richter, S.J., Cannon, R.E., Oberlies, N.H., Cech, N.B., 2011. Synergy-directed fractionation of botanical medicines: a case study with goldenseal (Hydrastis canadensis). J. Nat. Prod. 74, 1621–1629.

Kaatz, G.W., Seo, S.M., 1995. Inducible NorA-mediated multidrug resistance in Staphylococcus aureus. Antimicrob. Agents Chemother. 39, 2650–2655.

Kaur, A., Raja, H.A., Deep, G., Agarwal, R., Oberlies, N.H., 2016. Pannorin B, a new naphthopyrone from an endophytic fungal isolate of Penicillium sp. Magn. Reson. Chem. 54, 164–167.

Kellogg, J.J., Todd, D.A., Egan, J.M., Raja, H.A., Oberlies, N.H., Kvalheim, O.M., Cech, N.B., 2016. Biochemometrics for natural products research: comparison of data analysis approaches and application to identification of bioactive compounds. J. Nat. Prod. 79, 376–386.

Koo, S., Sutton, D.A., Yeh, W.W., Thompson, E.H., Sigler, L., Shearer, J.F., Hofstra, D.E., Wickes, B.L., Marty, F.M., 2012. Invasive Mycoleptodiscus fungal cellulitis and myositis. Med. Mycol. 50, 740–745.

Kusari, S., Pandey, S.P., Spiteller, M., 2013. Untapped mutualistic paradigms linking host plant and endophytic fungal production of similar bioactive secondary metabolites. Phytochemistry 91, 81–87.

Nisa, H., Kamili, A.N., Nawchoo, I.A., Shafi, S., Shameem, N., Bandh, S.A., 2015. Fungal endophytes as prolific source of phytochemicals and other bioactive natural products: a review. Microb. Pathog. 82, 50–59.

Onocha, P.A., Okorie, D.A., Connolly, J.D., Roycroft, D.S., 1995. Monoterpene diol, iridoid glucoside and dibenzo-a-pyrone from Anthocleista djalonensis. Phytochemistry 40, 1183–1189.

Pryor, B.M., Michailides, T.J., 2002. Morphological, pathogenic, and molecular characterization of Alternaria isolates associated with Alternaria late blight of pistachio. Phytopathology 92, 406–416.

Pugh, N.D., Jackson, C.R., Pasco, D.S., 2013. Total bacterial load within Echinacea purpurea, determined using a new PCR-based quantification method, is correlated with LPS levels and in vitro macrophage activity. Planta Med. 79, 9.

Raja, H.A., Kaur, A., El-Elimat, T., Figueroa, M., Kumar, R., Deep, G., Agarwal, R., Faeth, S.H., Cech, N.B., Oberlies, N.H., 2015. Phylogenetic and chemical diversity of fungal endophytes isolated from Silybum marianum (L.) Gaertn. (milk thistle). Mycology 6, 8–27.

Scazzocchio, F., Cometa, M., Tomassini, L., Palmery, M., 2001. Antibacterial activity of Hydrastis canadensis extract and its major isolated alkaloids. Planta Med. 67, 561–564.

Schoch, C.L., Robbertse, B., Robert, V., Vu, D., Cardinali, G., Irinyi, L., Meyer, W., Nilsson, R.H., Hughes, K., Miller, A.N., 2014. Finding needles in haystacks: linking scientific names, reference specimens and molecular data for fungi. Database 2014, bau061.

Shivas, R., Yu, Y., 2009. A taxonomic re-assessment of Colletotrichum acutatum, introducing C. fioriniae comb. et stat. nov. and C. simmondsii sp. nov. Fungal Divers 39, 111.

Shymanovich, T., Saari, S., Lovin, M.E., Jarmusch, A.K., Jarmusch, S.A., Musso, A.M., Charlton, N.D., Young, C.A., Cech, N.B., Faeth, S.H., 2014. Alkaloid variation among epichloid endophytes of sleepygrass (Achnatherum robustum) and consequences for resistance to insect herbivores. J. Chem. Ecol. 41, 93–104.

Stierle, A., Strobel, G., Stierle, D., 1993. Taxol and taxane production by Taxomyces andreanae, an endophytic fungus of pacific yew. Science 260, 214–216.

Strobel, G.A., 2003. Endophytes as sources of bioactive products. Microbes Infect. 5, 535–544.

Suryanarayanan, T., Thirunavukkarasu, N., Govindarajulu, M., Sasse, F., Jansen, R., Murali, T., 2009. Fungal endophytes and bioprospecting. Fungal Biol. Rev. 23, 9–19.

Todd, D.A., Gulledge, T.V., Britton, E.R., Oberhofer, M., Leyte-Lugo, M., Moody, A.N., Shymanovich, T., Grubbs, L.F., Juzumaite, M., Graf, T.N., 2015. Ethanolic Echinacea purpurea extracts contain a mixture of cytokine-suppressive and cytokine-inducing compounds, including some that originate from endophytic bacteria. PLoS One 10, e0124276.

U'Ren, J.M., Lutzoni, F., Miadlikowska, J., Laetsch, A.D., Arnold, A.E., 2012. Host and geographic structure of endophytic and endolichenic fungi at a continental scale. Am. J. Bot. 99, 898–914.

Udayanga, D., Castlebury, L.A., Rossman, A.Y., Chukeatirote, E., Hyde, K.D., 2014. Insights into the genus Diaporthe: phylogenetic species delimitation in the D. eres species complex. Fungal Divers 67, 203–229.

Woudenberg, J., Groenewald, J., Binder, M., Crous, P., 2013. Alternaria redefined. Stud. Mycol. 75, 171–212.