

Antimicrobial Properties of Basidiomycota Macrofungi to *Mycobacterium abscessus* Isolated from Patients with Cystic Fibrosis

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Abstract

Background: Antimicrobial resistance (AMR) has now emerged as a global public health crisis. Of particular concern is AMR associated with the genus *Mycobacterium*, including *Mycobacterium tuberculosis* and the nontuberculous mycobacteria (NTM). Emergence of the NTM, in particular *Mycobacterium abscessus*, in patients with cystic fibrosis (CF) represents both a diagnostic and a treatment dilemma. Such resistance drives the need to investigate novel sources of antimicrobials. Medicinal fungi have a well-documented history of use in traditional oriental therapies. Not only is this an ancient practice, but also still today, medical practice in Japan, China, Korea, and other Asian countries continue to rely on fungal-derived antibiotics. A study was, therefore, undertaken to examine the antimicrobial activity of 23 native macrofungal (mushrooms/toadstools) taxa, collected from woodlands in Northern Ireland against six clinical (CF) isolates of *M. abscessus*, as well as *M. abscessus* National Collection of Type Cultures (NCTC) Reference strain (NCTC 13031). **Methods:** Free-growing saprophytic and mycorrhizal macrofungi ($n = 23$) belonging to the phylum *Basidiomycota* were collected and were definitively identified employing Polymerase Chain reaction/ITS DNA sequencing. Macrofungal tissues were freeze-dried and reconstituted before employment in antibiotic susceptibility studies. **Results:** All macrofungi examined showed varying inhibition of the *M. abscessus* isolates examined with the exception *Russula nigricans*. The macrofungi displaying maximum antimycobacterial activity against the clinical isolates were (in descending order) *M. giganteus* (33.6 mg/ml), *Hygrocybe nigrescens* (38.5 mg/ml) and *Hypholoma fasciculare* (25.3 mg/ml). **Conclusion:** Macrofungi may represent a source of novel antimicrobials against *M. abscessus*, which have not yet been fully explored nor exploited clinically. This is the first report describing the antimycobacterial properties of extracts of *M. giganteus* against *M. abscessus*. Further work is now required to identify the constituents and mode of the inhibitory action of these macrofungi against the *M. abscessus*. Given the gravity of AMR in the NTMs, particularly *M. abscessus* and the clinical treatment dilemmas that such AMR present, antibiotic drug discovery efforts should now focus on investigating and developing antibacterial compounds from macrofungi, particularly *M. giganteus*, where there are no or limited current treatment options.

Keywords: Antimicrobial resistance, cystic fibrosis, fungi, *Mycobacterium abscessus*, nontuberculous mycobacteria

INTRODUCTION

Whilst the birth of modern antibiotics was the result of the production of penicillin from a filamentous fungus against bacteria, there has been a relative paucity of data examining the potential for the macrofungi (mushrooms and toadstools) to produce antimicrobial metabolites. Medicinal fungi such as Shiitake, *Lentinula edodes*, have a well-documented history of use in traditional oriental therapies. Not only is this an ancient practice but also still today, medical practice in Japan, China, Korea, and other Asian countries continues to rely on fungal-derived antibiotics. In search of novel therapeutic

alternatives, many fungal-based studies have found compounds with various clinical properties, including anti-parasitic,^[1] as well as antimicrobial potentials in locally sourced Shiitake mushrooms.^[2] There have been several reports on medicinal fungi, which have included the description of antimicrobial

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properties.^[1,3] Previously, members of the genera *Phellinus* and *Inonotus* have been shown to be composed of yellow polyphenol pigments, principally a styrylpyrone class of compounds, with anti-viral effects. Styrylpyrone pigments in mushrooms are thought to have a role similar to that of flavonoids in plants, whereby the unique carbon skeleton of fused styrylpyrone might be an attractive molecular scaffold for pharmacological applications.^[3]

Clinical infections with the nontuberculous mycobacteria (NTM) are now beginning to emerge, particularly with *Mycobacterium abscessus*, in patients with cystic fibrosis (CF). These have been well-described in several recent seminal reviews.^[4,5] The presence of high-level antibiotic resistance in these clinical isolates leads to a treatment dilemma, driven through ever-decreasing the availability of efficacious antibiotics. Therefore, there is an urgent clinical requirement to develop new effective antibiotics or to repurpose existing pharmaceuticals, to create options that will allow treatment of NTM infections with antimicrobial agents with proven *in vitro* activity.

Till date, there has not been any description in the published literature of macro- or higher-fungal extracts having antimycobacterial activity against contemporary clinical isolates of the NTMs, particularly *M. abscessus*. A study was, therefore, undertaken to examine the antimicrobial activity of 23 native macrofungal (mushrooms/toadstools) taxa, collected from woodlands in Northern Ireland against six clinical (CF) isolates of *M. abscessus*, as well as *M. abscessus* National Collection of Type Cultures (NCTC) Reference strain (NCTC 13031).

METHODS

Description of *Mycobacterium abscessus* isolates used

M. abscessus isolates ($n = 7$) were obtained from the HSC Microbiology Culture Repository, MicroARK, housed at the Northern Ireland Public Health Laboratory, at Belfast City Hospital. These isolates consisted of six clinical isolates obtained from patients with CF and one Reference Strain (NCTC 13031) obtained from the National Culture Type Collection, Public Health England (Colindale, London, UK). All isolates had been historically stored on slopes of Lowenstein-Jensen medium in glass universal containers at ambient temperature. All isolates were recovered and passaged twice on Columbia agar base (Oxoid CM0331; Oxoid Ltd., Basingstoke, UK) supplemented with 5% (v/v) defibrinated horse blood, which was incubated at 37°C for 5 days, before employment in the current study.

Description and identification of macrofungi employed in this study

Twenty-two species of native macrofungi were collected from woodlands throughout Northern Ireland, UK, as described [Table 1]. *Lentinula edodes* (Shiitake mushroom) was also examined, given its popularity as a constituent of Asian (mainly Japanese) cuisine.

Formal visual appearance determinations of wild fungi collected were made by consulting a mycological manual.^[6] The identities of the typical wild mushroom fungi were further confirmed using polymerase chain reaction (PCR) assays. PCR was carried out using fungal 18S rDNA universal Internal Transcribe Sequences (ITS) 1 and ITS 4 primers (ITS1: TCC GTA GTT GAA CCT GCG G and ITS4: TCC TCC GCT TAT TGA TAT GC). The primers were added to the reaction mixture containing fungal DNA (~15 ng) in a total volume of 50 µl of MasterMix (Invitrogen) and the PCR cycles were set at 94°C, 3 min (one hold), 94°C, 30s, 53°C, 30s, 72°C, 1 min (35 cycles), followed by a final extension step of 10 min, 72°C.^[7]

All PCR products were purified using the Chargeswitch® PCR Clean-Up Kit (Invitrogen CS12000 <http://products.invitrogen.com/ivgn/product/CS12000>). The cleaned up putative PCR products were sequenced according to the protocol described in ABI Big Dye Terminator version 3.1 Cycle Sequencing Kit (<https://products.appliedbiosystems.com/>) (ABI, Warrington, UK) using the same primers, after which the products were purified by sodium acetate/ethanol precipitation. They were then loaded onto an ABI 3100 DNA GENETIC ANALYZER for forward and reverse sequencing reactions. Sequences obtained were analyzed using Geneious Pro 4.8.3 software (<http://www.geneious.com/>). Sequence was submitted for comparison with those stored in GenBank using the BLASTn alignment software (<http://www.blast.genome.ad.jp/>) and the sequence homology identity was determined.

Preparation of aqueous macrofungal extracts for antimicrobial susceptibility testing

All macrofungi were freeze-dried for approximately 72 h (Edwards Super Modulyo Freeze Drier). Freeze-dried material was reconstituted in distilled water at concentrations, as detailed in Table 1, for subsequent antimicrobial susceptibility testing.

In vitro antimicrobial activity of macrofungal extracts

Inoculum (0.5 McFarland standard) of each *M. abscessus* isolate was streaked individually on to the surface of individual Columbia Agar supplemented with 5% (v/v) defibrinated horse blood. The plates were labeled in sectors representing each drug plus appropriate controls. Macrofungal extract (10 µl) was pipetted onto the media and left to dry. The plates were inverted and incubated at 37°C incubator for 5 days. Any observed zones of inhibition (mm) in the region of the test fungal extract was measured and recorded.

RESULTS

The antimicrobial activity of the 23 macrofungal extracts on the seven *M. abscessus* isolates is shown in Figure 1. Error bars represent standard error of the mean. The macrofungi with the most potent antimicrobial activity were (in descending order) *Meripilus giganteus* (33.6 mg/ml), *Hygrocybe nigrescens* (38.5 mg/ml) and *Hypholoma fasciculare* (25.3 mg/ml), with mean zones of inhibition of 8.7 mm,

Table 1: Description of the macrofungi examined in this study

Genus/species	mg/ml*	Phylum	Family	Habitat	Nutrition	Common name
<i>Agaricus augustus</i>	77.1	Basidiomycota	Agaricaceae	Woodland, particularly under conifers, parks, gardens	Saprophytic	The prince
<i>Amanita rubescens</i>	73.3	Basidiomycota	Amanitaceae	Hardwood, softwood trees; particularly conifer forests	Mycorrhizal	The blusher
<i>Boletus</i> sp.	81.7	Basidiomycota	Boletaceae	Soil beneath deciduous and conifer trees	Ectomycorrhizal	
<i>Clitocybe</i> sp.	96.1	Basidiomycota	Tricholomataceae	Leaf litter under hedgerows, in broad-leaf woods and heaths	Saprophytic	
<i>Coprinus comatus</i>	4.4	Basidiomycota	Agaricaceae	Grass verges, lawns, edges of footpaths, open woodland	Saprophytic	Shaggy Inkcap Lawyer's Wig
<i>Ganoderma adspersum</i>	171	Basidiomycota	Polyporaceae	Bracket fungus; causes white heart rot in deciduous trees	Parasitic/saprophytic	Shelf fungus
<i>Gymnopilus junonius</i>	111.7	Basidiomycota	Cortinariaceae	Decaying deciduous and conifer wood and stumps	Saprophytic	Laughing Gym Laughing Cap Spectacular Rustgill
<i>Gymnopus confluens</i>	48.3	Basidiomycota	Omphalotaceae	Deciduous woodland, occasionally conifer plantations, forest floor where dead wood is buried	Saprophytic	Clustered Toughshank
<i>Gymnopus peronatus</i>	59.9	Basidiomycota	Omphalotaceae	Leaf litter beneath broadleaf trees and hedgerows, and under bracken on heathland	Saprophytic	Wood Woollyfoot
<i>Hygrocybe nigrescens</i>	38.5	Basidiomycota	Hygrophoraceae	Oak woodland, short turf, lawns	Saprophytic	Blackening Waxcap
<i>Hypholoma fasciculare</i>	25.3	Basidiomycota	Strophariaceae	On decaying wood and stumps	Saprophytic	Sulphur tuft
<i>Inocybe geophylla</i>	74.8	Basidiomycota	Inocybaceae	Beside paths and on roadside verges beneath deciduous trees and in mixed woodland; less frequently under conifers	Mycorrhizal	White fibercap
<i>Inocybe grammata</i>	42.5	Basidiomycota	Inocybaceae	Woodlands	Ectomycorrhizal	
<i>Lentinula edodes</i>	108.4	Basidiomycota	Omphalotaceae	Decaying wood of deciduous trees	Saprophytic	Shiitake
<i>Leucopaxillus tricolor</i>	56.6	Basidiomycota	Tricholomataceae	Woodlands, decomposing the litter of hardwoods, possess antibiotics	Saprophytic	
<i>Meripilus giganteus</i>	33.6	Basidiomycota	Meripilaceae	Stumps, roots and base of living broad leaf trees, especially beech	Parasitic and then saprophytic when its host dies	Giant polypore/ black-staining polypore
<i>Mycena</i> sp.	68.9	Basidiomycota	Mycenaceae	Tree stumps, forest floor	Mycorrhizal/ saprophytic	Bonnets
<i>Psathyrella candolleana</i>	49.7	Basidiomycota	Psathyrellaceae	Meadows, lawns, woodlands, well-shaded grassland	Saprophytic	Pale brittlestem common crumble cap
<i>Russula cyanoxantha</i>	29.6	Basidiomycota	Russulaceae	Broadleaf woodland, oaks, conifers	Ectomycorrhizal	Charcoal burner
<i>Russula nigricans</i>	39.5	Basidiomycota	Russulaceae	Coniferous and broadleaf woodland	Ectomycorrhizal	Blackening brittlegill
<i>Russula</i> sp.	81.3	Basidiomycota	Russulaceae	Coniferous and broadleaf woodland	Ectomycorrhizal	
<i>Trametes gibbosa</i>	71.9	Basidiomycota	Polyporaceae	Bracket fungus, broadleaf trees, most commonly on beech or sycamore, causes white rot	Saprophytic	Lumpy bracket
<i>Trametes versicolor</i>	166	Basidiomycota	Polyporaceae	Bracket fungus, dead wood, fallen or standing, most commonly hardwoods e.g., beech and oak	Saprophytic (sometimes weak parasite on living trees)	Turkeytail

*Concentration of freeze-dried extract of macrofungi material used in antimicrobial susceptibility studies

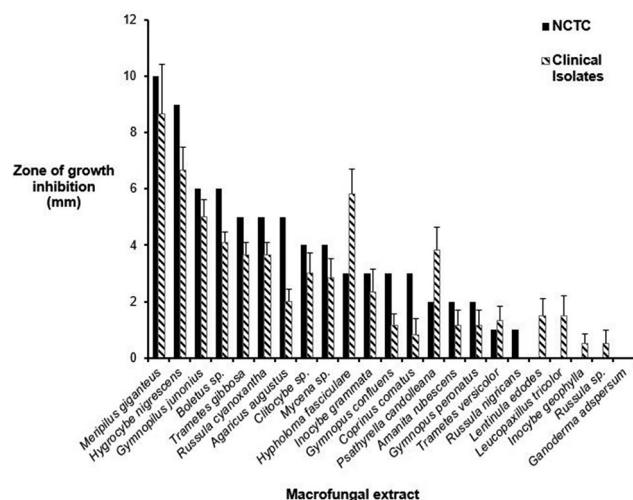


Figure 1: Antimicrobial activity of 23 species of macrofungi against clinical *Mycobacterium abscessus* ($n = 6$) and *Mycobacterium abscessus* National collection of type cultures 13031

6.7 mm, and 6.0 mm, respectively. With the exception of *H. fasciculare*, the reference strain, *M. abscessus* NCTC 13031, was more sensitive to macrofungal extracts than the wild-type clinical isolates.

DISCUSSION

All of the macro- or higher-fungi examined in this study belonged to the Phylum *Basidiomycota*. *Basidiomycota* comprise three subphyla (including six unassigned classes) 16 classes, 52 orders, 177 families, 1589 genera, and 31,515 species.^[8] The subphylum *Agaricomycotina* contains most of the described species (*ca.* 21,000), including many mushrooms as saprophytes or mycorrhizal symbionts of plants.^[9] *Basidiomycota* are filamentous fungi composed of hyphae (except for *Basidiomycota*-yeasts) and reproduce sexually through the formation of specialized club-shaped end cells called basidia that normally bear external meiospores. These specialized spores are called homosapiomedasin. However, some *Basidiomycota* reproduce asexually in addition or exclusively. *Basidiomycota* that reproduce asexually can be recognized as members of this division by gross similarity to others, by the formation of a distinctive anatomical feature, namely the clamp connection, cell wall components and definitively classified by phylogenetic molecular analysis of their DNA sequence data.

In this study, we collected 23 free-growing *Basidiomycota* from natural habitats, namely woodlands in Northern Ireland. Table 1 details the species name, the concentration of extract employed in antimicrobial susceptibility studies, taxonomical standing, habitat, type of nutrition and the common name used (if any). We wished to provide a robust taxonomic basis for naming the fungi collected and subsequently employed in downstream antimicrobial susceptibility testing, and hence, we adopted a molecular PCR-DNA sequencing methodology,

thus allowing us to name the *Basidiomycota* employed with certainty.

The aim of this study was to investigate potential antibacterial properties of aqueous extracts from 23 macrofungi against clinical isolates of *M. abscessus*, as well as an NCTC Reference strain (NCTC 13031).

All of the macrofungal aqueous extracts inhibited all clinical *M. abscessus* clinical isolates, to varying degrees with the exception of *Russula nigricans*, which was noninhibitory to the *M. abscessus* isolates tested [Figure 1].

The most potent inhibition was with *M. giganteus*, followed by *H. nigrescens* and then *H. fasciculare*. *M. giganteus* has previously been shown to be antimicrobial to several non-mycobacterial bacteria, including *Escherichia coli* (ATCC 35210), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (ATCC 35030), and the following Gram-positive bacteria: *Staphylococcus aureus* (ATCC 6538), *Bacillus cereus* (clinical isolate), *Micrococcus flavus* (ATCC 10240), and *Listeria monocytogenes* (NCTC 7973).^[10] It is believed that the antimicrobial properties of *M. giganteus* are attributed to the presence of phenolic acids. There have been no reports to date describing the antibacterial activity of this macrofungus against NTM members of the genus *Mycobacterium*. Phenolic acids, including pelandjuaic acid (or 6-(8'Z,11'Z,14'Z-heptadecatrienyl)-salicylic acid, 6-(8'Z,11'Z-heptadecadienyl)-salicylic acid, and 6-(10'Z-heptadecenyl)-salicylic acid, respectively), 6-(12'Z-non-adeceenyl)-salicylic acid, and 6-(15'Z-heneicosenyl)-salicylic acid, isolated from *Spondias mombin*, namely, the hog plum plant, have been shown to have antibacterial properties against *M. fortuitum*.^[11] The association between plants and macrofungi being able to synthesize such phenolic compounds may be a natural protective strategy for these organisms against co-colonizing bacteria in soil and the rhizosphere, such as free-living, saprophytic NTM organisms. Such protective strategies through the production of phenolic acids, as seen in macrofungi and plants, may be a promising defense mechanism that should be examined further within the clinical setting.

CONCLUSION

Macrofungi may represent a source of novel antimicrobials against *M. abscessus*, which have not yet been fully explored nor exploited clinically. This is the first report describing the antimycobacterial properties of extracts of *M. giganteus* against *M. abscessus*. Further work is now required to identify the constituents and mode of the inhibitory action of these macrofungi against the *M. abscessus*. Given the gravity of Antimicrobial resistance (AMR) in the NTMs, particularly *M. abscessus* and the clinical treatment dilemmas that such AMR present, antibiotic drug discovery efforts should now focus on investigating and developing antibacterial compounds from macrofungi, particularly *M. giganteus*, where there are no or limited current treatment options.

Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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