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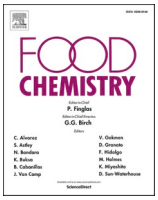

Target directed identification of natural bioactive compounds from filamentous fungi

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ABSTRACT

Some of the most powerful natural antimicrobial compounds originate from filamentous fungi. However, due to the diversity of compounds from plant and fungal origin, separation, isolation, and identification of bioactive constituents can be a long and tedious process. This study explores the effectiveness of thin layer chromatography (TLC) in combination with bioautography in the separation and identification of bioactive compounds from several filamentous fungi. Ultra-performance liquid chromatography coupled to photodiode array detector (UPLC-DAD) was employed to quantitatively identify phenolic composition. The total phenolic content of the selected filamentous fungi ranged from 31.85 mg g^{-1} to 101.77 mg g^{-1}. Additionally, liquid chromatography mass spectrometry (LC/MS) determined the most abundant fatty acids were linoleic, palmitic, oleic, and stearic acid. Submerged cultivation of *Grifola frondosa*, *Monascus purpureus*, *Lentinula edodes*, *Trametes versicolor* and *Pleurotus ostreatus* proved to be an effective method to produce natural antimicrobial compounds.

1. Introduction

In recent years, target-based antimicrobial screening has not yielded new antibiotic discovery, prompting a return to investigation of natural products (Alksne & Dunman, 2008; Brown & Wright, 2016). There is now a renewed drive for natural product solutions with fungi representing a huge area of opportunity for discovery, as they are one of the most diverse groups of organisms on the planet (Hyde, 2022). Current identification of compounds of biological significance are usually directed for discovery from medicinal plants, vegetables, and fruits. However, fungi have long been recognized as functional foods and are widely accepted as a valuable source for development of medicines and nutraceuticals. The harvesting and purification of natural bioactive compounds from fungi has significant pharmacological potential. Different species, their preparation and choice of extraction procedure are known to have substantial influence on both the concentration of metabolites and their bioactivity. In the past, experimentation into the antibacterial activity of fungi has focused mostly on the isolation of compounds from the fruiting body, with a small proportion focusing on liquid cultivated mycelium. Submerged liquid fermentation is of great potential commercially with both the cultured mycelium and culture fluid found to possess antibacterial activity (Hatvani, 2001). The high density of molecular constituents which are present in fungal mycelia or mushroom fruiting bodies means that extraction and separation techniques for the isolation of pure, pharmacologically-active constituents can be a difficult (Sticher, 2008). The content, concentration of metabolites and bioactivity of these compounds is heavily dependent on their preparation and choice of extraction procedure. Consequently, suitable extraction and purification steps as well as, downstream processing post-fermentation are extremely significant. These processes allow for selective removal of interfering compounds and/or concentration of the desired active constituents.

Biological assays and chemical screening methods are important tools for the separation of biologically active compounds from natural sources. Planar chromatographic analysis coupled with biological detection, a method referred to as bioautography; is a simple, inexpensive, and convenient method of allowing target-directed isolation of biologically active compounds of natural origin (Choma & Grzelak, 2011). This technique has proven extremely useful for isolation of compounds prior to quantification using more sophisticated methods of identification. Modern bioautography methodology has re-established standard thin-layer chromatography (TLC) as a screening model for separation of potentially important target compounds, allowing bioactive screening, parallel comparison between samples and semi-

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quantitative analysis. This technique, one of the simplest methods for this purpose, can generate reproducible results requiring little equipment (Marston & Hostettmann, 1999). In addition, the relative simplicity of bioautography is that each stage of the extraction, fractionation and separation procedures may be monitored as the active material is purified. TLC with an *in-situ* bioassay and comparison of R_f values with reference chromatograms, allows for localisation of biologically active constituents within the complex matrix (Choma & Grzelak, 2011). The localisation of constituents with biological activity using various chemical screening methods can eliminate unnecessary separation procedures, which would have downstream expenditure implications (Marston & Hostettmann, 1999).

The aim of this research was to explore the effectiveness of bioautography in the identification of active constituents from the selected filamentous fungi, and in essence from any material of natural origin, such as, plants, vegetables, and fruits, with nutraceutical potential. Quantitative identification of phenolic and fatty acid content of mycelial extracts was then explored using more sensitive and sophisticated techniques.

2. Material and methods

2.1. Preparation of fungal extracts

Pleurotus ostreatus 32783 was supplied by ATCC, American Type Culture Collection, U.S.A. *Monascus purpureus* 1604, *Pleurotus ostreatus* 1833, *Pleurotus citrinopileatus* 5341, *Pleurotus eryngii* 9619, *Pleurotus salmoneo-stramineus* 5338 and *Trametes versicolor* 3086 were supplied by DSMZ GmbH, Braunschweig, Germany. *Grifola frondosa*, *Pleurotus ostreatus* and *Lentinula edodes* fungi were grown and maintained in-house (Alltech, Meath, Ireland). Fungal mycelium per strain was grown by submerged liquid fermentation (SLF) in 500 mL Erlenmeyer flasks (n = 3). Mycelial biomass was collected and freeze-dried using an Alpha 1–4 LD plus freeze drying unit (Christ, Osterode am Harz, Germany). A fine powder was collected (1.0 mm (no. 10) mesh) and the dried extract was further extracted using methanol extraction processes. Freeze-dried mycelial biomass (5 g) was accurately weighed into 100 mL sterilins and shaken overnight (18 – 24 h) with 50 mL of methanol at room temperature. Extracts were then filtered through Whatman paper No. 1 (x 2) by vacuum filtration. Residues were resuspended in another 50 mL of methanol. The process was repeated twice. The combined methanol filtrates were transferred to pre-weighted sterilins, and solvent was evaporated by applying a constant flow of air. Residual methanol was removed by vacuum pressure at 50 °C overnight in an oven and the dry weights recorded.

2.2. Inoculum preparation and storage

Escherichia coli 10778, *Staphylococcus aureus* 1104, *Staphylococcus epidermidis* 1798, were supplied by DSMZ GmbH, Braunschweig, Germany. *Salmonella enterica* 15,480 was supplied by ATCC, American Type Culture Collection, Virginia, U.S. *E. coli* 8879 was supplied by NCIMB, UK. Frozen stocks of bacteria (5 % (v/v) in 70 % glycerol) were stored at −70 °C with the acquired medium. A single colony was isolated and transferred to approximately 25 mL of broth and grown overnight. OD at λ_{595nm} was adjusted accordingly using a spectrophotometer (Shizmadzu Corporation, Kyoto, Japan). Working plates were prepared by transferring a single colony using spread plate technique to fresh agar and incubating overnight. Streaked plates were stored at 4 °C for up to six months.

2.3. Thin layer chromatography

Thin layer chromatography (TLC) was performed on silica gel, 60 F254, Alu backed plates, 2 mm (10 cm × 20 cm) (Merck). They were carefully scored using a scalpel and cut to the appropriate size. Volumes, in μL of the extract to be examined, were spotted onto the TLC plate approximately 2 cm from the bottom. The spots were dried under a warm current of air and the plate was developed in a solvent saturated glass TLC tank using an appropriate solvent system. Optimisation of the solvent system for different fractions was performed. When the mobile phase reached approximately 1 cm from the top of the plate, the plate was removed from the TLC tank and allowed to dry. Plates were examined under visible light, UV-λ_{254nm} and UV-λ_{365nm}, with or without gentle heating. Compounds with native fluorescence were viewed as bright zones on a dark background under UV light. Absorbing compounds (fluorescence quenching) were detected as dark violet spots on a bright green background. The retention factor (R_f) was recorded for each compound in each methanol extract following TLC development, to allow direct comparison between the different fungi. The $R_f \times 100$ values (%) were calculated using the following equation:

$$\left[R_f(b)/R_f(a)(mm)\right] \times 100$$

where, the R_f value for each isolate was the distance it had travelled from the origin (b) divided by the distance travelled by the solvent mobile phase (a).

2.4. TLC detection reagents

Fast blue salt (FBS) reagent was used for the detection of flavonoids and phenolic compounds. A TLC plate was sprayed with 6 – 8 mL of a 0.5 % (w/v) aqueous FBS solution, dried and then observed under visible light for the presence of red to brown zones, with or without warming.

Aluminium chloride (AlCl3) solution (1 %, v/v) in ethanol was used to confirm the presence of flavonoids, which was indicated by yellow fluorescence. The appearance of blue spots revealed phenolic acids. For the detection of anthraquinones (red) and coumarins (blue) the TLC plate was sprayed with 10 mL of freshly prepared KOH ethanolic solution (10 % (w/v), dried and then viewed under UV-λ_{365nm} or visible light, with or without warming. For the detection of lipids, a plate was dipped in 10 % (v/v) sulphuric acid solution and placed in an oven (105 °C) for 30 min. This resulted in the charring of compounds which contained mainly lipids and allowed visualisation of compounds under normal light. However, this process is destructive to the constituents on the plate and therefore cannot be used for further processing. Anisaldehyde-sulphuric acid (AS) reagent was used for the nonspecific detection of terpenes. The reagent consisted of anisaldehyde (0.5 mL) mixed with 10 mL glacial acetic acid, followed by methanol (85 mL) and concentrated sulphuric acid (5 mL), in that order. The plate was sprayed with approximately 10 mL, warmed at 100 °C for 5 – 10 min and evaluated under visible light. Iodine vapour was used for the detection of compounds with conjugated double bonds. A TLC plate was placed into the TLC tank, which had been previously saturated with iodine vapour by the addition of iodine crystals. The plate was removed and evaluated in visible light.

2.5. Bioautography

A single colony of bacteria was isolated and transferred to approximately 25 mL of broth and grown overnight. Optical density (OD) at λ_{595nm} was measured by spectrophotometer (Shizmadzu Corporation, Kyoto, Japan). Developed plates were cut to size and placed inside sterile petri dishes (150 × 15 mm). Agar was inoculated with an aliquot of the overnight bacterial culture, mixed thoroughly by pipetting to avoid formation of bubbles, and then poured into the petri dish immersing the TLC plate in agar. The agar was spread uniformly over the plate to give an agar overlay thickness of approximately 1 mm. The plate was rotated slowly to remove any air bubbles from the surface. After 30 min, the plates were inverted and incubated overnight at 37 °C.

2.6. UPLC-DAD analysis of phenolic constitution

Solid phase extraction was performed according to the method of Dopico-García et al. (2007) with slight modifications. Freeze-dried mycelial biomass (0.5 g) was accurately weighed and mixed with 5 % methanol, pH 2. The mixture (25 mL) was placed in an ultrasonic bath at 40 °C for 20 min. The extracts were filtered and collected. This process was repeated 5 times per extract. The solution was passed through an Agilent Bond Elut-C18 column (Element, Kildare, Ireland) previously conditioned with 30 mL of methanol and 70 mL acid water (pH 2 with HCL). Methanol (50 mL) was used as the elution solvent to recover phenolic compounds. The extracts were concentrated to dryness under reduced pressure (45 °C) using a concentrator plus/Vacufuge® plus (Eppendorf AG, Hamburg, Germany) and redissolved in 30 % methanol (1 mL).

The phenolic extracts (10 μL) were analysed using an Agilent 1290 Infinity Ultra Pure Liquid Chromatography (UPLC) system, coupled a to photodiode array detector (UPLC-DAD) (Agilent, California, U.S.). Separation was achieved on an Agilent Poroshell 120 EC-C18 column (3.0 × 50.0 mm, 2.7 μm) thermostatted at 28 °C. The sample volume injection was 10 μL. A solvent system consisting of 0.2 % formic acid (solvent A) and methanol (solvent B) was used with the following gradients; 0 – 1 min isocratic at 100 % A, following a linear gradient for the subsequent conditions outlined in Table S1. The linear gradient was applied at 23 – 25 min from 0 to 100 % A (Table S1). At 25.5 min the sample loop was rinsed and the gradient repeated. The flow rate was kept constant at 1 mL min^{-1}. Phenolic compounds were identified by comparison of retention times of standard materials and quantification was achieved by the absorbance recorded in the chromatograms relative to eighteen external standards, including; 4-hydroxybenzoic acid, caffeic acid, catechin, cyanocinnamic acid, ferulic acid, flavone, homogentisic acid, kaempferol, myricetin, naringenin, *ρ*-coumaric acid, *ρ*-hydroxybiphenyl acid, protocatechuic acid, pyrogallol, rutin, salicylic acid, vanillic acid and vanillin. Detection was performed with a multiple wavelength UV detector, set at the following wavelengths: λ_{254nm}, λ_{265nm}, λ_{280nm} and λ_{320nm}. Chromatogram quantification was acheived using a calibration plot of external standards.

2.7. Fatty acid analysis using liquid chromatography mass spectrometry (LC/MS)

Fatty acid composition analysis of the whole methanol extract and extracted TLC fractions were performed on an Agilent 6530 accurate-mass quadrupole time-of-flight (Q-ToF) LC/MS (Agilent Technologies, U.S.). The mobile phase used was 2 mM ammonium acetate (solvent A) and acetonitrile with 2 mM ammonium acetate (solvent B), with an injection volume of 10 μL. Separation was achieved on an Agilent Eclipse Plus C18 column (2.1 × 150 mm, 3.5 μm), thermostatted at room temperature. A slight stepwise gradient from 60 to 100 % solvent B was applied at a flow rate of 0.4 mL min^{-1} for 15 min. Electrospray mass spectra data were recorded on a negative ionisation mode for a mass range *m/z* 100 to *m/z* 1000. Identification was performed by comparing retention times and their masses with those of 38 authentic standards (Table S2). Fatty acid analysis of separated components was performed according to relative R_f value (Results of which can be found in the Supplementary Information (Table S8 – S12)). Fractions were subjected to two successive overnight elution processes, firstly with diethyl ether (1 mL) at 500 rpm. Following centrifugation (14,000 rpm for 10 min) the supernatants were collected individually and dried at 45 °C for approximately 2 h using an Eppendorf concentrator plus/Vacufuge® plus (Eppendorf AG, Hamburg, Germany). The pellet was resuspended in methanol and the process was repeated. The fractionated components of the extracted isolates were redissolved in methanol and analysed by LC/MS.

3. Results and discussion

3.1. Target directed identification of active compounds

Lyophilised biomass was subjected to methanol extraction. The quantity of recovered dry extract (yield (%)) following the methanol extraction process from crude biomass is outlined in Table 1. The extraction yield (%) recovered from filtrate of the selected species ranged from 597 ± 219 mg (*Monascus purpureus*) to 3393 ± 210 mg (*Pleurotus ostreatus 1833*) (Table 1).

The extract recovery from *M. purpureus* (an Ascomycete) was substantially less compared to the recoveries noted from the Basidiomycetes due to the characteristic morphological differences between them. Preliminary separation and identification of bioactive compounds within the lyophilised biomass of the methanol extract was performed using TLC and examined by exposure to UV light allowing characterisation of individual compounds within the sample. The mobile phase; hexane:ethyl acetate (1:1, v/v) resulted in effective separation of at least fifteen components in the methanol extract of *M. purpureus* (Fig. 1a-c). Fewer components were visible in *Pleurotus* spp., *L. edodes*, *G. frondosa* and *T. versicolor*, making *M. purpureus* the most diverse species of the selected fungi. Due to the varying intensities of the different separated regions observed under UV light, it was evident that the methanol extract from each species contained diverse components of varying concentration. Of the other species examined; according to UV detection, twelve compounds were separated from *P. citrinopileatus*, ten compounds from *Pleurotus ostreatus*, *P. 1833*, *P. 32783* and *P. salmoneo-stramineus*, seven compounds from *G. frondosa*, eight compounds from *L. edodes* and eight compounds from *T. versicolor* (Tables S3 – S7). Comparison of relative retention times is the most reliable index for compound identification (Banwart et al., 1985). R_f values combined with universal and specific TLC detection reagents, determined the class of compounds within each separated isolate, indicating them to be made up of mostly phenolic or lipid constituents (Tables S3 – S7). Reactivity to detection reagents as well as intensity of reaction demonstrated the natural variation in concentration of phenolic or lipid components between the various species of fungi and further indicated that some compounds may be present in fungi of the same genus at different concentrations. The agar over-lay method in conjunction with tetrazolium salts (MTT) helped to localise and measure zones of bacterial inhibition (Fig. 1d,e).

Table 2 summarizes the active areas of interest from each genus of fungi according to their R_f values, as determined using bioautography agar over-lay. A total of fourteen antimicrobial compounds were separated from *M. purpureus*, ten from the *Pleurotus* species, seven from *G. frondosa*, eight from *L. edodes* and six from *T. versicolor*. In each of the various species of fungi tested, differing intensities of antibacterial activity were observed in each separated region, indicative of varying

Table 1
Extraction recovery from dry filtrate (%).

Fungi	% Recovery[a]
	Methanol extract
Grifola frondosa	3061 ± 289
Lentinula edodes	3301 ± 488
Monascus purpureus	597 ± 219
Pleurotus ostreatus	2357 ± 339
Pleurotus ostreatus 1833	3393 ± 210
Pleurotus ostreatus 32783	2749 ± 805
Pleurotus citrinopileatus	2057 ± 1120
Pleurotus eryngii	3315 ± 231
Pleurotus salmoneo-stramineus	1981 ± 611
Trametes versicolor	3060 ± 279

Data represents the mean ± standard deviation (n = 3) of recovered dry extract (mg) following methanol extraction from crude mycelial biomass (5 %, w/v). [a]Total (%) dry weight recovered following extraction.

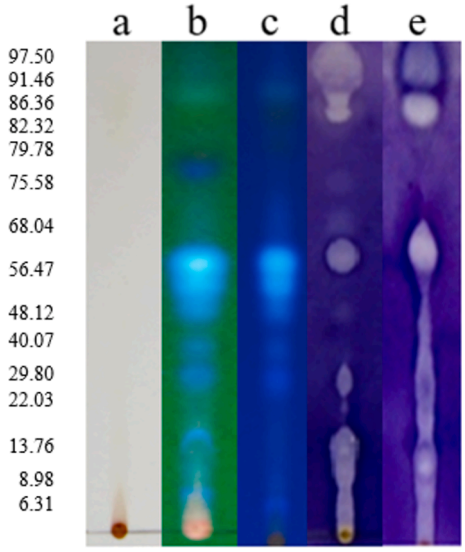

Fig. 1. Separation of bioactive compounds of *Monascus purpureus* using TLC and bioautography. TLC plates visualised under (a) visible light, (b) λ_{254nm} and (c) λ_{365nm}. Antimicrobial activity of extracts using bioautography tested against (d) *E. coli* 10,778 and (e) *S. epidermidis*. R_f values (R_f x100) are displayed to the left. The methanol extract (1 μL) of *M. purpureus* was suspended in $CHCl_3$:acetone:MeOH:H_2O (20:20:10:1, v/v/v/v) (100 mg mL^{-1}). The plate was developed in hexane:ethyl acetate (1:1, v/v). Clear zones of microbial growth inhibition are demonstrated against a purple background of viable bacteria grown on solidified nutrient agar. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

concentrations of an active agent or the presence of multiple antibacterial elements. For each fungus, the most polar bioactive (R_f value; 97.50) was noted to contain different constituents as this active region reacted positively with most detection reagents, suggesting a combination of polar lipid, terpenoid or phenolic constituents (Table S3-S7). Phenolic acids, flavonoids and coumarins from edible fungi are known to exhibit antibacterial activity, with studies showing that action against gram-positive and gram-negative bacteria correlates with the extent of phenols, flavonoids, ascorbic acid, β-carotene, and lycopene present at various stages of the developing mushroom fruiting body, including development of mycelium (Barros et al., 2007). Altogether, bioautographic analysis of the components determined that antibacterial activity was due to the presence of both polar and non-polar compounds within each fungal extract. Bioautography also indicated the potential presence of different compounds within each separated spot on the TLC plate. The observed differences in antimicrobial activity between species was related to the solvent solubility of the active compound(s) and the concentration of the active component particular to the species of fungi. A full summary of bioactivity of each of the separated regions of the selected fungal species toward *E. coli* 8879, *E. coli* 10778, *Staphylococcus aureus* 1604, *Staphylococcus epidermis* 1798 and *Salmonella enterica* 15480, combined with each region's reaction to specific detection reagents is available in the Supplementary Information (Table S3 – S7). Extracts from each species were noted to have a broad spectrum of antibacterial activity.

Use of universal detection reagents indicated a strong presence of fatty acids as well as, phenolic or flavonoid constituents. Naturally derived antioxidants such as polyphenols (phenols, quinines, flavonoids, tannins and coumarins) from fungi have been suggested as alternatives to antimicrobial agents (Kao et al., 2010; Smith et al., 2015). Additionally, certain fatty acids such as lauric, linoleic, oleic, and linolenic

Table 2
Antimicrobial analysis of individual separated regions established by agar over-lay bioautography.

Polarity scale	Rf × 100	*Grifola frondosa*	*Lentinula edodes*	*Pleurotus ostreatus*	*Monascus purpureus*	*Pleurotus 1833*	*Pleurotus 32783*	*Pleurotus citrino.*	*Pleurotus eryngii*	*Pleurotus salmoneo stramineus*	*Trametes versicolor*
1	6.31 ± 0.96	c,d	c	a,b,d,e	b,c,d,e	a,b,d,e	a,b,d,e	a,b,d,e	a,b,d,e	a,b,d,e	d
2	8.98 ± 0.71				b,c,d,e						
3	13.76 ± 1.99	b,d,e	d	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	b,c,d,e
4	22.03 ± 2.16			b,d	b,c,d	b,d	b,d	b,d	b,d	b,d	
5	29.80 ± 1.67		e	b	b,d,e	b	b	b	b	b	
6	40.07 ± 6.89				d,e						
7	48.12 ± 0.75				a,c,d,e						
8	56.47 ± 2.89	c		a,b,c,d,e	b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	d
9	68.04 ± 2.12			a,c		a,c	a,c	a,c	a,c	a,c	
10	75.58 ± 1.84	c,d,e	c,d,e	c,e	a,b,c,e	c,e	c,e	c,e	c,e	c,e	a,b,c,d,e
11	79.78 ± 1.17			a,e	e	a,e	a,e	a,e	a,e	a,e	
12	82.32 ± 0.83				a,b,c						
13	86.74 ± 0.68		d		b,c,d						
14	91.46 ± 2.93	b,c,e	b,c,e	b,c,e	a,b,c,e	b,c,e	b,c,e	b,c,e	b,c,e	b,c,e	
15	97.50 ± 0.74	b,c,d,e		a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	a,b,c,d,e	e

Data is representative of antimicrobial activity of seperated compounds against (a) *E.coli* 8879; (b) *E.coli* 10778; (c) *S. aureus*; (d) *S. epidermidis* and (e) *S. enterica*. Separation of each fungal methanol extract (100 mg mL^{-1}) was performed using mobile phase hexane:ethyl acetate (1:1, v/v) in triplicate. Polarity scale representative of least polar (1) to most polar (15) compound. Abbreviations: *Pleurotus citrino.,Pleurotus citrinopileautus*.

acid, amongst others, including their derivatives are known to have strong bacteriostatic activity against both gram-positive and gram-negative bacteria (Bergsson et al., 2011; Kabara et al., 1972). Based on the intensity of a reaction with UV light and detection reagents, combined with bioautography, biological activity of fungal mycelial extracts was most likely due to a combination of multiple constituents, with the main active agent to be of phenolic and/or lipid structure.

3.2. UPLC-DAD analysis of phenolic compounds

To quantitatively identify natural phenolic compounds from the selected filamentous fungi, ultra-performance liquid chromatography coupled to photodiode array detector (UPLC-DAD) was performed. Mixtures of phenolic acids may contain considerable flavonoid components as the two are often found in a common matrix, therefore a solvent gradient was applied to allow effective separation (Banwart et al., 1985). In addition, for effective separation of compounds that may have similar retention times under specific gradient analysis (co-elution), measurement of the absorbance simultaneously at different wavelengths was applied (Banwart et al., 1985). Prior to analysis by UPLC, solid phase extraction (SPE) was used to selectively remove interfering compounds and concentrate the compounds of interest allowing efficient extraction and purification of phenolic compounds. The total phenol profile of the selected filamentous fungal species was carried out by comparing retention times and absorption spectra of peaks with those of eighteen relevant authentic standards (Table 3). The data revealed that some compounds vary in absorption at specific wavelengths, which confirms that measuring the absorbance of the sample simultaneously at different wavelengths increases the accuracy of identification.

Analysis confirmed that the sum of the different constituents made up the total concentration of phenolics in each whole extract. Different species contained different phenolic compounds, ranging from fifteen to the maximum eighteen analysed (Table 3). The phenolic composition of *Pleurotus* spp. is comparable to those in the literature (Kim et al., 2008; Palacios et al., 2011), however; the specific and characteristic composition of each species is likely to be associated with the environmental factors of their growth conditions. The total phenolic content in each of the filamentous fungi ranged from 31.85 mg g^{-1} (*M. purpureus*) to 101.77 mg g^{-1} (*P. citrinopileatus*). Nine phenolic compounds, namely, myricetin, rutin, protocatechuic, vanillic, caffeic, ferulic, ρ-coumaric, α-cyano-4-hydroxycinnamic and homogentisic acids were dominant among all ten fungal species (Table 3). *P. 32783* and *P. eryngii* did not show evidence for the presence of catechin. With this exception, every phenolic compound tested was detected in each *Pleurotus* species. The benzoic acids, salicylic acid and 4-hydroxybenzoic acid, in addition to the flavonoid naringenin, were not detected in *M. purpureus*. Vanillin and ρ-hydroxybiphenyl were not detected in *L. edodes*, and *G. frondosa* did not contain flavone, kaempferol or ρ-hydroxybiphenyl (Table 3). Similar to Kim et al. (2008), homogentisic, protocatechuic, catechin, myricetin and certain cinnamic acids were detected in extracts of *Pleurotus ostreatus* and *P. eryngii*; however, much greater concentrations were detected from the extracts in the present study (Table 3). The difference most likely is due to levels detected were extracted from the fruiting body and not mycelium, as was examined in the present study. *G. frondosa* and *M. purpureus* contained fifteen phenolic compounds, while *Pleurotus ostreatus*, *P. 1833*, *P. citrinopileatus*, *P. salmoneo-stramineus* and *T. versicolor* contained all eighteen (Table 3). *P. citrinopileatus* had the largest concentration of phenolic compounds (101.77 mg g^{-1}) detected by UPLC, while *M. purpureus* contained the least (31.85 mg g^{-1}) (Table 3). There are few studies concerning the individual profiles of phenolic compounds in therapeutic and edible fungi (Palacios et al., 2011). The results show that production of individual phenolics can depend on growth conditions, stage of growth and is largely species specific.

3.3. Fatty acid analysis

To further examine target directed identification of active compounds, a fatty acid composition profile for each of the selected fungal species was generated using LC/MS. Twenty-two different fatty acids were identified by comparing their retention times and characteristic MS spectral data with those of authentic standards. In consideration of the possible presence of one or more hydroxyl and/or carboxylic acid groups, the tentative mass spectrum for compounds was acquired in negative ionisation mode ($[M-H]^-$). By this technique, the diagnostic fragmentation patterns of the compounds during collision induced dissociation (CID) elucidated structural information of the compounds analysed. The combination of accurate mass measurement to determine the elemental composition and liquid chromatography to separate isomeric compounds provided a powerful tool for identification of lipid diversity in each of the ten species of filamentous fungi. Linoleic, palmitic, oleic, and stearic acid were among the most abundant fatty acids (Table 4).

Although each fungal species in the present investigation is within the same subphylum (Ascomycetes and Basidiomycetes); it was determined that *M. purpureus* had the most diverse fatty acid composition which included, pelargonic, lauric, myristic, pentadecylic, palmitic, palmitoleic/palmitelaidic, heptadecanoic, stearic, oleic/elaidic, linoleic, arachidic, gadoleic, arachidonic/eicosatetraenoic, nonadecylic, dihomolinoleic, EPA, DPA, DHA and tricosylic acids (Table 4). The composition of mycelial fatty acids has been observed to vary greatly depending on growth conditions such as nutritional factors, oxygen, and temperature (Pedneault et al., 2007; Suutari, 1995). In fungi, the major fatty acids that typically occur in membrane phospholipids and storage triacylglycerols are palmitic and stearic acids and their unsaturated derivatives palmitoleic, oleic, linoleic and linolenic acids (Suutari, 1995), most of which demonstrated in each of the fungal extracts in the present investigation (Table 4).

A full fatty acid profile using LC/MS was used to identify the individual separated active constituents of lipid structure by comparing R_f values to relevant standards (Tables S8 – S12). This allowed association of biological activity to a particular class or constituent type based on the R_f value of the TLC spot of interest. Analysis of individual TLC separated isolates indicated that the fatty acid components successfully migrated through the TLC development system, however failed to selectively separate components based on polarity. LC/MS analysis of individual isolates revealed the non-specific nature of lipid detection, using detection reagents in conjunction with TLC analysis. Whilst TLC can identify individual isolates/active components and different derivatives, it can be limited when assessing a complex mixture consisting of homologues with different side chain length and unsaturation, leading to potential underestimation of content. Nonetheless, a growing interest in an effect-directed analysis has led to the success of TLC with microbial detection, and this chromatographic method is more efficient than HPLC, where post column derivatisation is normally more difficult (Fuchs et al., 2011). This study demonstrates that several different constituents made up the total concentration of fatty acids in each isolated region and that several active and possibly non-active constituents make up the separated compounds detected.

Altogether, LC/MS and UPLC-DAD analysis allowed quantification and identification of relevant bioactive fungal compounds in the methanol extract. LC/MS allowed quantitative identification of separated active and non-active regions of interest. However, these techniques also revealed the complexity of the biological mixture and demonstrated the necessity for preliminary extraction procedures prior to target based antimicrobial compound separation. Certain phenols and fatty acids are expected to have similar polarity and as a result this would lead to co-elution by TLC, consequently having similar capacity to cross the cell membrane (Vaquero et al., 2011).

Analysis indicated that several different constituents made up the total concentration of phenolics and other components in each fungus,

Table 3
UPLC-DAD directed identification and quantification of phenolic compounds from filamentous fungi.

Common Name	Concentration (mg/g)* Chemical Name	*Grifola frondosa*	*Lentinula edodes*	*Pleurotus ostreatus*	*Monascus purpureus*	*Pleurotus 1833*	*Pleurotus 32783*	*Pleurotus citrino.*	*Pleurotus eryngii*	*Pleurotus salmoneo stramineus*	*Trametes versicolor*
Benzoic acids											
4-hydroxybenzoic	4-hydroxybenzoic acid	0.05^{c}	0.01^{c}	0.05^{c}	*nd*	0.02^{c}	0.02^{c}	0.08^{c}	0.01^{c}	0.02^{c}	$\leq 0.01^{c}$
Protocatechuic acid	3,4-dihydroxybenzoic acid	0.41^{d}	0.91^{a}	4.87^{a}	0.02^{b}	2.74^{a}	3.37^{d}	12.03^{a}	18.70^{a}	6.90^{a}	1.44^{a}
Salicylic acid	2-hydroxybenzoic acid	1.68^{c}	1.20^{c}	2.33^{c}	*nd*	1.99^{c}	1.93^{c}	1.80^{c}	5.34^{c}	0.53^{c}	0.49^{c}
Vanillic acid	4-hydroxy-3-methoxybenzoic acid	0.86^{b}	1.98^{b}	17.30^{b}	**10.87^{b}**	5.58^{b}	**19.92^{b}**	1.16^{a}	4.61^{b}	**15.19^{b}**	8.39^{b}
Benzaldehydes											
Vanillin	4-hydroxy-3-methoxybenzaldehyde	0.02^{d}	*nd*	0.02^{a}	0.06^{a}	0.02^{b}	0.03^{a}	0.03^{c}	0.02^{a}	0.03^{d}	0.01^{a}
Phenols											
Pyrogallol	1,2,3-benzenetriol	**17.16^{c}**	**17.06^{b}**	11.02^{a}	2.03^{a}	5.63^{c}	3.25^{a}	**20.61^{a}**	4.95^{c}	14.78^{a}	8.11^{c}
ρ-hydroxybiphenyl	4-phenylphenol	*nd*	*nd*	0.30^{c}	0.05^{a}	0.81^{c}	0.31^{c}	1.48^{c}	0.21^{c}	0.37^{c}	**19.98^{c}**
Cinnamic acids											
Caffeic acid	3,4-dihydroxycinnamic acid	0.16^{c}	0.13^{a}	0.25^{a}	0.06^{a}	0.23^{a}	0.08^{b}	0.90^{b}	0.29^{c}	0.28^{a}	0.19^{a}
Ferulic acid	4-hydroxy-3-methoxycinnamic acid	2.09^{b}	1.89^{b}	12.16^{b}	2.52^{b}	4.87^{b}	6.14^{b}	14.26^{b}	0.99^{b}	13.00^{b}	3.69^{b}
ρ-coumaric	4-hydroxycinnamic acid	2.21^{a}	2.75^{a}	3.35^{a}	1.60^{a}	3.87^{a}	3.39^{a}	14.17^{a}	4.42^{a}	4.13^{a}	1.70^{a}
α-cyano-4 -hydroxycinnamic acid	3-phenyl-2-propenoic acid	10.16^{c}	3.06^{a}	**27.83^{a}**	2.62^{a}	**23.42^{c}**	5.99^{c}	12.22^{a}	5.49^{a}	9.77^{a}	1.53^{a}
Flavanones											
Naringenin	4′,5,7-trihydroxyflavanone	0.19^{d}	0.97^{a}	3.45^{a}	*nd*	2.11^{a}	7.38^{a}	12.55^{a}	6.88^{a}	2.75^{b}	3.85^{a}
Catechin	3,3′,4′,5,7-pentahydroxyflavanone	3.53^{a}	5.70^{a}	0.09^{a}	4.17^{a}	0.02^{c}	*nd*	1.05^{a}	*nd*	0.30^{a}	0.04^{a}
Flavones											
Flavone	2-phenyl-4*H*-1-benzopyran-4-one	*nd*	0.44^{b}	0.84^{b}	0.79^{b}	0.63^{b}	0.49^{a}	4.80^{b}	0.85^{c}	0.67^{b}	0.59^{b}
Kaempferol	3,4′,5,7-tetrahydroxyflavone	*nd*	0.12^{c}	0.14^{c}	0.80^{c}	0.13^{a}	0.13^{c}	0.28^{c}	0.16^{a}	0.45^{a}	0.31^{c}
Myricetin	3,3′,4′,5,5′,7-hexahydroxyflavone	0.19^{c}	0.24^{c}	0.15^{c}	0.06^{c}	0.17^{c}	0.09^{c}	0.32^{c}	0.28^{a}	0.12^{c}	0.17^{a}
Flavone glycosides											
Rutin	Quercetin-3-rutinoside	4.21^{c}	10.34^{c}	0.40^{a}	5.97^{c}	0.15^{a}	2.71^{c}	3.54^{c}	**35.73^{c}**	0.14^{a}	1.51^{c}
Other standards:											
Homogentisic acid	2,5-dihydroxyphenylacetic acid	1.76^{a}	0.15^{a}	3.05^{a}	0.23^{a}	3.15^{a}	2.27^{b}	0.49^{a}	0.88^{a}	1.63^{b}	2.04^{a}
Total phenolic content (mg g^{-1})*		44.68	46.95	87.60	31.85	55.54	57.50	101.77	89.81	71.06	54.04

Data is representative of the phenolic compounds present in ten selected fungal species using UPLC-DAD analysis. Data is representative of the total concentration of phenols as detected using authentic standards. Concentration (mg/g dry extract)* is based on maximum UV detection at wavelengths of λ^{a}_{254nm}, λ^{b}_{265nm}, λ^{c}_{280nm}, and λ^{d}_{320nm}. The main phenolic acid of each species is indicated in bold. Abbreviations: *Pleurotus ostreatus**, *Pleurotus ostreatus*; *Pleurotus citrino.*, *Pleurotus citrinopileatus*; *nd*, not detected.

Table 4
LC/MS directed identification of fatty acids in selected filamentous fungi.

Fatty acid	Chemical formula	Exact mass of negative ion [M−H]⁻	Exact mass of neutral ion	Carbon atom: Double bond (C:D)	Strain[a]
Caprylic	$C_8H_{16}O_2$	143.108	144.115	08:0	L, G, P, T
Pelargonic	$C_9H_{16}O_2$	157.123	158.131	09:0	L, G, M, P, T
Undecylic	$C_{11}H_{20}O_2$	185.155	186.162	11:0	L, T
Lauric	$C_{12}H_{20}O_2$	199.170	200.178	12:0	L, G, M
Myristic	$C_{14}H_{28}O_2$	227.202	228.209	14:0	L, G, M, P, T
Pentadecylic	$C_{15}H_{30}O_2$	241.217	242.225	15:0	L, G, M, P, T
Palmitic	$C_{16}H_{32}O_2$	255.233	256.240	16:0	L, G, M, P, T
Palmitoleic/ Palmitelaidic	$C_{16}H_{30}O_2$	253.217	254.225	16:1	L, G, M, P, T
Heptadecanoic	$C_{16}H_{30}O_2$	269.249	270.256	17:0	L, G, M, P, T
Stearic	$C_{17}H_{34}O_2$	283.264	284.272	18:0	L, G, M, P, T
Oleic/Elaidic	$C_{18}H_{36}O_2$	281.249	282.256	18:1	L, G, M, P, T
Linoleic	$C_{18}H_{34}O_2$	279.233	280.241	18:2	L, G, M, P, T
Linolenic (α + γ)	$C_{18}H_{34}O_2$	277.217	278.225	18:3	L, T
Nonadecylic	$C_{18}H_{30}O_2$	297.280	298.287	19:0	M, P
Arachidic	$C_{18}H_{28}O_2$	311.296	312.303	20:0	M
Gadoleic	$C_{19}H_{38}O_2$	309.280	310.287	20:1	M
DihomoLinoleic	$C_{20}H_{38}O_2$	307.264	308.272	20:2	M
Arachidonic/ Eicosatetraenoic	$C_{20}H_{34}O_2$	303.233	304.240	20:4	M
EPA	$C_{20}H_{34}O_2$	301.217	302.247	20:5	M
DPA	$C_{22}H_{40}O_2$	329.249	330.256	22:5	M
DHA	$C_{22}H_{38}O_2$	327.233	328.240	22:6	M
Tricosylic	$C_{22}H_{36}O_2$	353.343	354.350	23:0	M

Summary of peak assignments detected from selected filamentous fungi; [a]M.; *Monascus purpureus*, P.; *Pleurotus ostreatus*, G.; *Grifola frondosa*, L.; *Lentinula edodes*, T.; *Trametes versicolor*. Full list of standards examined are detailed in Table S2. *Pleurotus ostreatus* is representative of combined *P. 1833, P. 32783, P. citrinopileatus, P. eryngii* and *P. salmoneo-stramineus* strains.

indicating that biological activity may be correlated with synergism among the different polyphenols, flavonoids, phenolic acids and/or a range of structurally diverse fatty acids occurring in the various species of fungi. In a less complex mixture, bioautography would be a useful tool for identification and semi-quantitative antimicrobial determination. At present, there is little information available on the synergy of compounds in relation to antibacterial activity (Vaquero et al., 2011).

In general, the detection of active constituents from extracts depends on both the biological activity of the extract and the sensitivity of the techniques used. Further fractionation, purification and biological analysis is required for specific isolation of an active constituent. A number of techniques or a combination thereof, such as ethanol precipitation, fractional precipitation, ion-exchange chromatography, gel filtration, and affinity chromatography are generally used for the intention of purification (Zhang et al., 2007).

4. Conclusion

Filamentous fungi are being increasingly investigated for their biological activity, which has led to different applications in pharmaceuticals, biotechnology, and food preservations. Submerged cultivation of *Grifola frondosa*, *Monascus purpureus*, *Lentinula edodes*, *Trametes versicolor* and *Pleurotus ostreatus* proved to be an effective method to produce natural antimicrobial compounds.

TLC provided a good basis for preliminary identification of compound class. Target-directed antibacterial screening using bioautography resulted in identification and localization of bioactive constituents and allowed for association of activity to compound structure. The data revealed that biological activity was associated with a combined action of multiple active constituents of phenolic and/or lipid structure, which may work synergistically or additively. The detection of linoleic, oleic, stearic, and palmitic acid in the mycelium produced by submerged liquid fermentation and identified in the methanol extract, indicated that this bioprocess did not prevent the production or isolation of these predominant fatty acids as also observed in relevant research (Papaspyridi et al., 2012; Pedneault et al., 2007). Similarly, the solid phase extraction process did not prevent the isolation of bioactive phenolic compounds.

Fungi represent a potentially powerful source of new pharmaceutical agents (Ainsworth et al., 2011; Marx, 2004; Wasser, 2002) and SLF represents a process of commercial significance due to its high production value (Hatvani, 2001). This is the first report of phenolic compositional data from *M. purpureus*, *L. edodes*, *G. frondosa* and *T. versicolor* isolated by methanolic extract from biomass cultivated in submerged culture. This study determined that the selected filamentous fungi produce broad-spectrum antimicrobial compounds. Thereby, these species may not only be an important source of dietary fiber which could be utilized as dietary supplements but could contribute to further work in the investigation of fungal nutraceuticals.

CRediT authorship contribution statement

Helen Smith: Investigation, Writing – original draft. **Sean Doyle:** Supervision. **Richard Murphy:** Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [Authors Helen Smith and Richard Murphy are employed by Alltech Biotechnology which funded this work. Alltech Biotechnology specialise in natural product research with potential to contribute and promote animal health and nutrition.]

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.

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