Screening and purification of antimicrobial compounds from Australian freshwater fungi

by

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LIST OF ABBREVIATIONS

AMA	Antibiotic Medium Agar
ARGF	Australian Genome Research Facility
BLAST	Basic Local Alignment Search Tool
CFU	Colony-forming units
CMA	Cornmeal Agar
DMSO	Dimethyl sulphoxide.
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DOI	Digital Object Identifier
ESI	Electrospray ionization
EtOAct	Ethyl acetate
FTIR	Fourier-transform Infrared spectroscopy
h (unit)	hour(s)
IBM	International Business Machines
ITS	Internal transcribed spacer
LSU	Large sub-unit of ribosome
MEA	Malt extract Agar
M-H	Mueller Hinton medium
MS	Mass spectroscopy
NCBI	National Center for Biotechnology Information
NMR	Nuclear Magnetic Resonance
OD_{600}	Optical density at 600 nm wavelength

PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PYGA	Peptone Yeast extract Glucose Agar
PYGB	Peptone Yeast extract Glucose Broth
rcf	Relative centrifugal force
R_f	Retention factor
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
rT	Room temperature
SPSS	Statistical Package for the Social Sciences
TLC	Thin-layer chromatography
TOF-MS	Time-of-flight mass spectrometry
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
U.S.	United States of America
v/v	Volume per volume
w/v	Weight per volume
XDR	Extensive drug-resistant
ZOI	Zone of inhibition

SUMMARY

Antibiotics are essential for the prevention and treatment of infectious diseases. However, years of misuse of antibiotics have led to the emergence of antibiotic resistance. Therefore, there is an urgent need to find and introduce new antibiotics. Fungi are an important source of antibiotics, yet only a minuscule fraction has been recorded and studied for novel compounds. The freshwater fungi, especially, are understudied compared to other fungal groups. Therefore, this study aims to discover novel antimicrobial compounds from Australian freshwater fungi.

Thirty-eight freshwater fungi strains were previously isolated from submerged wood from three locations in Australia. After screening them on agar and evaluating their ability to produce the antibiotic compounds in solid-state fermentation, and liquid fermentation, six strains with significant activity were selected, including *Penicillium polonicum* MR153A, *Mariannaea* sp. MR33A, *Quintaria submersa* MR119A, *Lentistoma bipolare* CT98B, *Coniochaeta velutina* SCC31A, and *Cosmospora* sp. SCC45A. The extract of MR153A was active against a range of Gram-negative and Gram-positive bacteria, while the extracts of the other five were active against Gram-positive bacteria. The extracts also had weak activity against *Candida* and *Saccharomyces*. Notably, all six strains were active against MRSA.

The crude extracts of MR153A, MR33A, and MR119A were separated with silica-gel column chromatography, and four antimicrobial compounds were purified, one each from the first two fungi and two from the latter. Their minimal inhibition concentration and minimal bactericidal concentration against nine pathogenic bacteria and yeasts were determined. The most active compounds were from MR119A with an MIC of 2 µg/mL against *Staphylococcus aureus*, *Enterococcus faecalis*, and MRSA. One compound has been elucidated: **3-Isopropenyl-z-butenedioic acid monomethyl ester** from strain **MR153A**.

DECLARATION

I certify that this thesis:

- Does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university.
- To the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Student signature

Phung Hien Le

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CHAPTER 1. INTRODUCTION

1.1. Antibiotics - the wonder drugs

1.1.1. A brief history of antibiotics

Antibiotics are medicine used for the prevention and treatment of bacterial infections. They are substances produced naturally by microorganisms or via chemical synthesis that, even at low concentrations, can kill bacteria or suppress their growth.

The term 'antibiotic' was first used in a publication in 1941 by Selman Waksman to describe small molecules produced by a microorganism that can inhibit other microorganisms (Waksman & Woodruff 1941). However, the use of antibiotics can be dated back to the early 20th century, when Paul Ehrlich synthesized arsphenamine, a chemotherapeutic compound that can selectively target syphilis-causing *Treponema pallidum* (Ehrlich & Hafa 1910). The compound was marketed under the name Salvarsan and is considered the first modern antibiotic.

September 1928 marks the beginning of the antibiotic era when Alexander Fleming found that a strain of *Penicillium* can produce a substance that inhibits the growth of various bacterial while being non-toxic to animals in large doses. This substance was named 'penicillin' in the publication in the following year (Fleming, A 1929). The method to produce and purify penicillin on a large scale was published in 1943 (Clifton 1943). Penicillin was used intensively from 1944 to treat infections in World War II, in which it was dubbed a 'wonder drug'.

Roughly half of the antibiotics commonly used today were discovered and introduced between 1945 and 1965, the golden age of antibiotic discovery (Davies 2006; Hutchings, Truman & Wilkinson 2019). The antibiotic discovery rate gradually decreased from the late 1960s. Pharmaceutical companies have since paused the development and commercialization of new antibiotics since they are often not profitable (Plackett 2020). The last class of antibiotics successfully applied in treatment, Diarylquinolines,

was discovered in 2004; and since then, no new class of antibiotics has been introduced (Hutchings, Truman & Wilkinson 2019).

1.1.2. Classification of antibiotics on the basis of mechanisms of action

Antibiotics target specific elements in cell structures and molecular processes of bacteria. The most common modes of action are: Cell wall synthesis inhibition, cell membrane disruption, protein synthesis inhibition, and nucleic acid synthesis inhibition (Reygaert 2018).

Antibiotics can also be categorized into broad-spectrum and narrow-spectrum. Board-spectrum antibiotics such as carbapenem are effective against Gram-positive and Gram-negative bacteria (Papp-Wallace et al. 2011). Meanwhile, narrow-spectrum antibiotics such as colistin and fidaxomicin are only effective against selected groups of bacteria (Poirel, Jayol & Nordmann 2017; Sears et al. 2013)).

1.1.3. Production of antibiotics

A majority of introduced antibiotics are derived from natural sources, specifically microorganisms. Actinobacteria (e.g., *Streptomyces*) is the producer of most classes of antibiotics. Some fungi (e.g., *Penicillium*, *Acremonium*) and other bacteria (e.g., *Bacillus*) are also known for producing antibiotics (Hutchings, Truman & Wilkinson 2019). These antibiotics are produced industrially by fermentation in aerated bioreactors containing liquid medium. The compounds are then extracted, purified, and crystallized into final products. To improve their production tier, antibiotic-producing microbes can be screened via selection, after mutagenesis, or genetic engineering (Smith 1986). Some compounds can be chemically modified to form semi-synthetic derivatives, which have better stability or activity. Many of the currently used antibiotics are analogs of penicillin and cephalosporin. Without the introduction of any new class of antibiotics, most antibiotics marketed in the near future are likely to be semi-synthetic (Coates, Halls & Hu 2011; Volpato, Rodrigues & Fernandez-Lafuente 2010).

1.1.4. Significance of the antibiotic revolution

Prior to the 20th century, infectious diseases such as cholera, diphtheria, pneumonia, and tuberculosis were often fatal, especially for children. For example, in the 1900s, 30% of all death occurred among children less than five years old, and life expectancy was only 47 years. Pneumonia, tuberculosis, and diarrhea enteritis were the three leading causes that accounted for nearly a third of all deaths (*Achievements in Public Health, 1900-1999: Control of Infectious Diseases* 1999). Until World War I, treatment for wound infections was often amputation.

The discovery of penicillin and, later, other antibiotics have revolutionized the treatment of infectious diseases. Penicillin alone has saved approximately 200 million lives since its first use in the 1940s. Infectious disease control has increased the average life expectancy by 29.2 years (*Achievements in Public Health, 1900-1999: Control of Infectious Diseases* 1999).

However, the miracle achievement of the antibiotic revolution is currently being threatened by an emerging crisis, which is antibiotic resistance.

1.2. The antibiotic resistance crisis

1.2.1. Definition and mechanisms of antibiotic resistance

Antibiotic resistance is the ability of bacteria to withstand the effect of an antibiotic that they used to be susceptible to. In the presence of that antibiotic, the resistant bacteria survive or reproduce faster than the susceptible bacteria, therefore increasing the proportion of resistant bacteria in the population. The resistance trait is determined by one or more resistance genes, which bacteria acquire through genetic mutation or horizontal transfer (von Wintersdorff et al. 2016). The antibiotic resistance mechanisms can be: Alterations or destruction of the antibiotic molecule, decrease the permeability of antibiotics through the cell wall and membranes, extrusion of antibiotic molecules out of the cell using efflux pumps, or modification or protection of the target sites (Munita &

Arias 2016). Gram-negative bacteria are more intrinsically resistant to antibacterial agents due to their outer membrane that is impermeable to various solutes (Breijyeh, Jubeh & Karaman 2020).

Bacteria can have resistance to more than one antibiotic and evolve into multi-drug resistant forms.

1.2.2. The history of antibiotic resistance

Just one year after the first administration of penicillin to humans in 1941, four strains of *Staphylococcus aureus* isolated from hospitalized patients were found resistant to this antibiotic (Rammelkamp & Maxon 1942).

One of the first people to warn about the risk of antibiotic resistance was Alexander Fleming in 1945, after winning the Nobel Prize in Physiology or Medicine for his discovery of penicillin. In his Nobel Lecture, he wrote: "The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant" (Fleming, SA 1945).

Fleming's prediction gradually became a reality. After the introduction of penicillin, penicillin-resistance staphylococci had disseminated rapidly. By the late 1960s, more than 80% of *Staphylococcus* isolated from hospitals and communities were penicillin-resistant (Lobanovska & Pilla 2017). In response, many other β-Lactams antibiotics were discovered and introduced, including the semi-synthetic methicillin in 1959. However, the first cases of methicillin-resistant *Staphylococcus aureus* (MRSA) infection were reported in the United Kingdom just two years after the introduction of methicillin and later in many other countries (Enright et al. 2002). This phenomenon eventually occurred to virtually all clinically used antibiotics. Resistant strains emerge only 2 to 15 years after the introduction of an antibiotic (**Figure 1.1**).

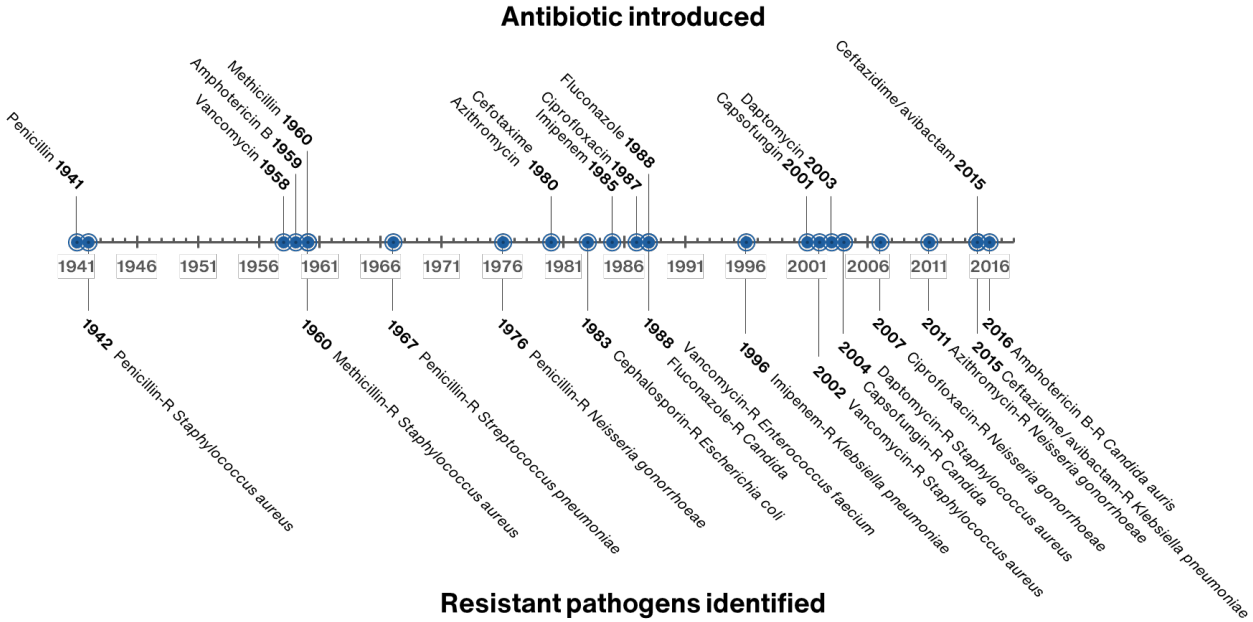

Figure 1.1. *Timeline showing the key dates relating to the antibiotic introduction and antibiotic resistance, recreated based on data from (About Antimicrobial Resistance 2020)*

In the 21st century, along with the drying pipeline of new antibiotics is the emergence of extensive drug-resistant (XDR) bacteria, such as XDR tuberculosis, carbapenem-resistant *Acinetobacter,* that can tolerate a wide range of antibiotics. In 2018, Lee et al. (2018) reported the spread of a near pan-drug-resistant strain of *Staphylococcus epidermidis* that is not susceptible to nearly all antibiotics, including the last-resort vancomycin. A century after the discovery of the first antibiotic, infectious diseases are once again a major threat to global health.

1.2.3. Causes and consequences of antibiotic resistance

Even before the introduction of antibiotics, resistance genes exist naturally in soil and environmental bacteria. The origin and reservoirs for these genes might include the self-protection machinery of antibiotic-producing organisms and intrinsic resistance mechanisms in other bacteria (D'Costa et al. 2011; Peterson & Kaur 2018). The abundance of resistance determinants in the environment creates opportunities for pathogenic bacteria to acquire the trait.

The use of antibiotics, even in an appropriate and regulated manner, still creates selection pressure that benefits resistant strains. There are, however, other artificial factors that accelerate the emergence and dissemination of antibiotic resistance: Over prescription, overuse, and misuse of antibiotics in healthcare and agriculture; poor hygiene and sanitation; inadequate infection prevention and control; and increasing global mobility (World Health Organization 2020).

Antibiotic resistance makes infectious diseases more challenging to treat. More than 35,000 people die each year in the U.S. due to infection by resistant microbes (*About Antimicrobial Resistance* 2020). The safety and efficiency of surgery and chemotherapy are also threatened; up to 50.9% of surgical site pathogens and 26.8% of post-chemotherapy show resistance to standard antibiotics in the U.S. (Teillant et al. 2015). Without effective solutions, humanity will approach the post-antibiotic era where antibiotics are no longer effective.

In the face of the antibiotic resistance crisis, besides implementing more stringent regulations on antibiotics, it is crucial to continue the discovery and introduction of new antibiotics. One potential direction is to investigate the inexhaustible source of bioactive compounds from the microbial world.

1.3. Fungi are a significant source of natural compounds

1.3.1. A brief introduction to fungi

Fungi is a kingdom of heterotrophic eukaryotes that feed by absorption; they get nutrients from a living host (biotrophs) or nonliving organic substrates (saprotrophs). Due to the loss of phagotrophic capabilities, they need to digest extracellularly by secreting lytic enzymes to their substrates. Fungi can grow as unicellular organisms (yeast) or complex mycelium structures composed of filamentous vegetative cells called hyphae. They are capable of reproducing sexually or asexually by budding and sporulation (Reece et al. 2019).

Fungi are ubiquitous in nature and can be found in virtually every habitat, including soil, freshwater, marine, and other organisms. The majority of fungi are mesophiles that grow at temperatures between 5 - 35°C. However, many species are extremotolerant (Dix & Webster 1995; Gostinčar et al. 2009).

Fungi have essential roles in nutrient cycles and ecological interactions. They share the same importance as bacteria in decomposing organic material, especially plant-based materials, and release carbon, nitrogen, and other elements in the form of organic nutrients. Some fungi have a tight mutualistic association with green algae or cyanobacteria to form lichen, the important pioneers on cleared rock and soil surface. They also form mutualistic relationships with apparently all species of plant as endophytes and mycorrhizal fungi. Roughly 30% of known fungi are parasites or pathogens, mostly in plants, but also in other life forms, including animals, microalgae, protists, and other fungi (Reece et al. 2019).

1.3.2. Classification of fungi

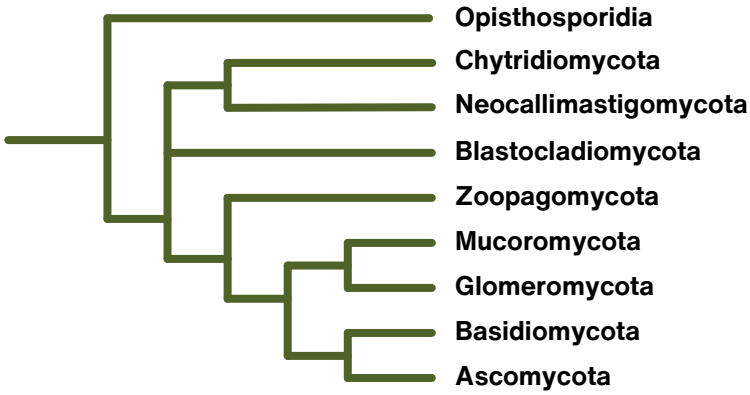

Figure 1.2. *The simplified phylogeny of kingdom Fungi with recognized phyla (Naranjo-Ortiz & Gabaldón 2019)*

Fungi belong to their kingdom of the same name, which belongs to the supergroup Opisthokonta together with animals and other small amoebic relatives (Adl et al. 2012). Approximately 144,000 species of fungi have been described to date (Cannon et al. 2018). However, the newest prediction put the total number of fungal species between 2.2 to 3.8 million (Hawksworth & Lücking 2017). With new species and even taxa being discovered continually, the taxonomy of fungi has undergone various modifications throughout its history. Currently, there are nine phylum-level clades of fungi recognized (**Figure 1.2**).

Traditionally, fungal species were identified based on morphology and phenotypes. Unlike many eukaryotes, the non-reproductive body of fungi typically consists of hyphae or yeast-like cells that offer few distinguishing features. In those cases, classification can be done on characteristics of the cultivated fungal cultures such as the morphology of the colony, colour changes in agar, or the presence of enzymes (Kurtzman et al. 2011; Nobles 2011). The morphology of fungal spores and spore-producing structures, conversely, can provide more diagnostic resolution for the taxonomy; some are distinctive enough to classify the fungus to species level. Until the last decade, the identification of fungi still relied mainly on microscopic observation of these reproductive structures (Kinsey, Paterson & Kelley 2003).

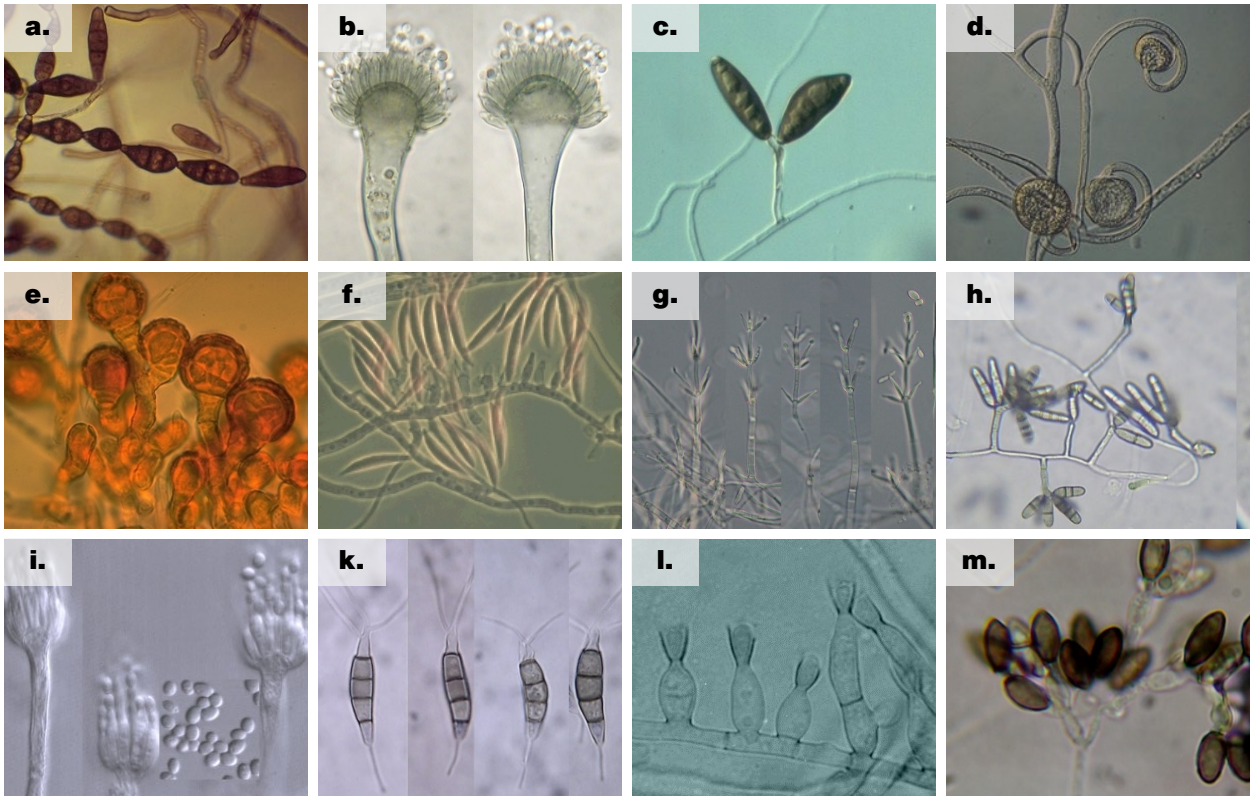

Figure 1.3. *Morphology of spores and conidiophores of several fungi, reproduced with permission from (Mallock n.d.). (a) Alternaria, (b) Aspergillus, (c) Bipolaris, (d) Circinella, (e) Epicoccum, (f) Fusarium, (g) Mariannaea, (h) Paradendryphiella, (i) Penicillium, (k) Pestalotiopsis, (l) Phialophora, (m) Wardomyces.*

Since the 2000s, sequencing technology has provided a powerful tool for the classification of fungi. However, no genetic marker has been universally accepted for this kingdom. In the meantime, the rRNA internal transcribed spacer (*ITS*) region is the

most widely accepted marker since it exhibited a higher correct identification rate than other DNA regions (Schoch et al. 2012). However, the existence of intraspecific and intragenomic heterogeneity of *ITS* in some groups can complicate the sequencing and classification (Dizkirici & Kalmer 2019; Nilsson et al. 2008). In those cases, additional DNA markers such as the rRNA large subunit region (*LSU*) or the RNA polymerase subunit II (*RPB2*) are used to increase reliability (Dizkirici & Kalmer 2019).

1.3.3. Fungi as the source of active compounds

Fungi have many applications in food and beverage, agriculture, bioremediation, and healthcare (Hyde et al. 2019). The many properties of fungi can be traced back to their diverse secondary metabolites. Approximately 60,000 to 80,000 microbial metabolites have been discovered until 2010, more than 40% of which are bioactive compounds. In comparison, less than 10% of known plant metabolites and 33% of known animal metabolites are bioactive (Bérdy 2012).

Among microbes, fungi and actinobacteria are significant sources of metabolites; each accounted for 47% and 41% of microbial metabolites, respectively. Before 1980, most of these compounds were discovered from actinobacteria, especially the genus *Streptomyces*. Since then, the ratio has gradually shifted toward fungi, and they are currently the most significant metabolites producers (Bérdy 2012). Bioactive compounds from fungi were utilized as anticancer agents, anti-angiogenesis agents, immunosuppressants, hypocholesterolemic agents, or antioxidative agents (**Figure 1.4** and **Table 1.1**).

It is worth mentioning that the discovery of metabolites among different groups of fungi is significantly unbalanced. Nearly 99% of fungal metabolites are found from filamentous fungi, while other types of fungi, including yeast and zoosporic fungi, are deemed poor producers (Bérdy 2012). The potential of enzymes from zoosporic fungi in biomass-degradation was suggested in a few studies (Lange et al. 2019), but further research is required.

***Table 1.1**. Examples of bioactive compounds discovered from fungi and their application*

Compound	Originated fungus	Application	Reference
Taxol (paclitaxel)	*Ascochyta medicaginicola*	Anticancer agent	Zaiyou, Li and Xiqiao (2017)
Camptothecin	*Trichoderma atroviridi*		Pu et al. (2013)
Fumagillin	*Aspergillus fumigatus*	Anti-angiogenesis	Guruceaga et al. (2019)
Cyclosporine	*Tolypocladium inflatum*	Immunosuppressant used in organ transplants	Kobel and Traber (1982); Colombo and Ammirati (2011)
Lovastatin	*Aspergillus terreus*	Both are statin, an important class of hypocholesterolemic agents	Manzoni and Rollini (2002)
Mevastatin	*Penicillium citrinum*		
Astaxanthin	*Xanthophyllomyces dendrorhous*	Bioactive pigment, its antioxidative activity is ten times stronger than β-carotene	Donoso et al. (2021); Visser, van Ooyen and Verdoes (2003)
Compounds from applied technology	*Aspergillus niger*	Producer of many food enzymes	Li et al. (2020)
	Saccharomyces cerevisiae	Producer of many biopharmaceuticals and protein with human-like glycosylation	Nielsen, J (2013); Karbalaei, Rezaee and Farsiani (2020); Manfrão-Netto, Gomes and Parachin (2019); Hamilton and Zha (2015)
	Pichia pastoris		
	Hansenula polymorpha		

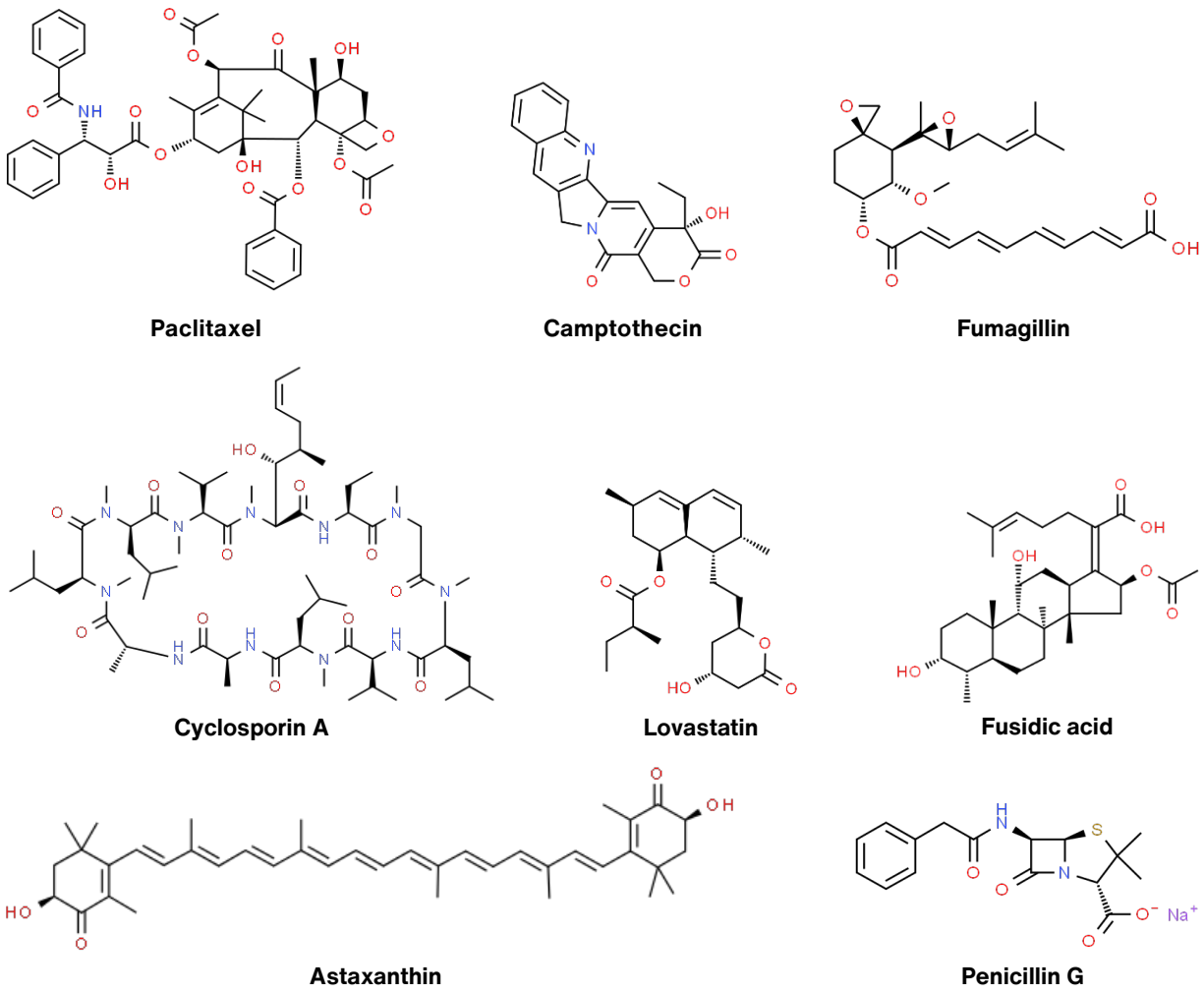

Figure 1.4. *The molecular structure of some bioactive compounds discovered from fungi listed in* ***Table 1.1****. Images were obtained from ChemSpider (http://www.chemspider.com/).*

1.3.3.1. Fungi are an important source of antibiotics

Much like other bioactive compounds, the major sources of antibiotics are actinobacteria and fungi. Fungi produce approximately 20% of clinically significant classes of antibiotics, which is three times lower than the number of classes of antibiotics discovered from actinobacteria (Hutchings, Truman & Wilkinson 2019). Fungi produce 22% of antibiotic compounds known in 1955, which numbered nearly 12,000 (Demain & Martens 2017). This percentage is now expected to be higher due to the increased significance of fungi as metabolite producers (Bérdy 2012).

Although fewer in number, many antibiotics from fungi are among the most important in terms of use, i.e., penicillins and cephalosporins. These two families of antibiotics were initially discovered from *Penicillium* and *Acremonium* species, respectively, and both belong to the β-lactam class, together with carbapenem (Jakubczyk & Dussart 2020). They have been some of the most prescribed antibiotics for over 70 years (Mitić et al. 2014) and still constitute a significant portion of the antibiotic market. In 2006, the market for penicillins and cephalosporins reached $6.7 billion and $9.4 billion, respectively (Demain & Martens 2017).

Despite being exploited for antibiotics discovery for almost a century, fungi are still considered a potential source of new antibiotics. For one reason, within the documented fungal species, 90% have not been studied for antimicrobial activities or valuable compounds (Volk 2013). Nielsen et al. (2017) studied 24 genome sequences from the genus Penicillium, the producer of the first antibiotic penicillin, and identified more than 1,300 biosynthetic gene clusters, as well as a previously undescribed compound of yanuthone class.

The abundance of some antibiotic gene clusters across microbial producers is exceptionally low. In actinobacteria, it was estimated that 22.5% and 1.25% of screened species could produce streptothricin and streptomycin, respectively. Meanwhile, the probability that vancomycin, erythromycin, and daptomycin can be found in screened species is $1/10^5$, $1/10^6$, and $1/10^7$, respectively (Clardy, Fischbach & Currie 2009). A similar pattern can be expected in fungi. With the vast majority of the Fungi kingdom, approximately 95% of species, remaining unexplored (Cannon et al. 2018; Hawksworth & Lücking 2017), the undiscovered fungi potentially possess new metabolite pathways and novel antibiotics.

1.4. Freshwater fungi are an understudied group with potential chemical diversity

1.4.1. The diversity of freshwater fungi

Freshwater fungi are a polyphyletic group of fungi consisting of species from different lineages. Their defining characteristic is the capability to accomplish their entire life cycle underwater, including the sporulation (El-Elimat et al. 2021; Ittner, Junghans & Werner 2018; Krauss et al. 2011). Most of the documented freshwater fungal species are Ascomycota and Chytridiomycota (Shearer et al. 2007). As of May 2021, 738 ascomycetes have been reported in freshwater habitats (Shearer & Raja HA n.d.). While there have not been any studies on the number of chytridiomycetes species in freshwater, they are considered a significant component of the habitat (Gleason et al. 2008). Otherwise, only 115 freshwater basidiomycetes have been recorded; most are yeast-like species (Jones et al. 2014*)*. Only about 40 filamentous basidiomycetes species have been documented (Jones et al. 2014); the most noticeable is *Psathyrella aquatica*, the only aquatic gilled mushroom (Frank, Coffan & Southworth 2010).

The number of described freshwater fungi represents less than 1% of the approximately 144,000 documented fungal species and is a minuscule fraction of the estimated millions of existing species. This data suggests that this group of fungi has not received adequate attention compared to their terrestrial relatives. However, there is increasing evidence indicating that the diversity of freshwater fungi is significant. Previous studies suggest that fungi can make up more than 50% of all eukaryotic sequences in freshwater bodies (Grossart et al. 2019) and may represent 17% of eukaryotic diversity in these habitats (Debroas et al. 2017). Particularly in the initial decomposition of leaves, their biomass can reach 90 – 95% of all microbial biomass (Grossart et al. 2019). Molecular data also show that Basidiomycota may have high diversity in freshwater habitats, comparable to Ascomycota in terms of active operational

taxonomical units (Lepère et al. 2019), in contrast to the number of recorded freshwater species from these two phyla.

1.4.2. Distinctive features of freshwater fungi

Zoosporic fungi such as chytrids are most probably native to these environments since their primitive flagellated motile spores usually require free water to disperse. On the other hand, freshwater filamentous fungi most likely originated from terrestrial ancestors but evolved morphological adaptations to colonize the water environments. Most of them produce bulky spores with multiradiate, sigmoid, or branched conformation and sticky sheaths or appendages, which help the spore attach to new submerged substrates (El-Elimat et al. 2021; Grossart et al. 2019; Krauss et al. 2011) (**Figure 1.5**). Some freshwater ascomycetes developed deliquescent asci or asci with specialized discharge mechanisms adapted for dispersal in water (Wong et al. 1998).

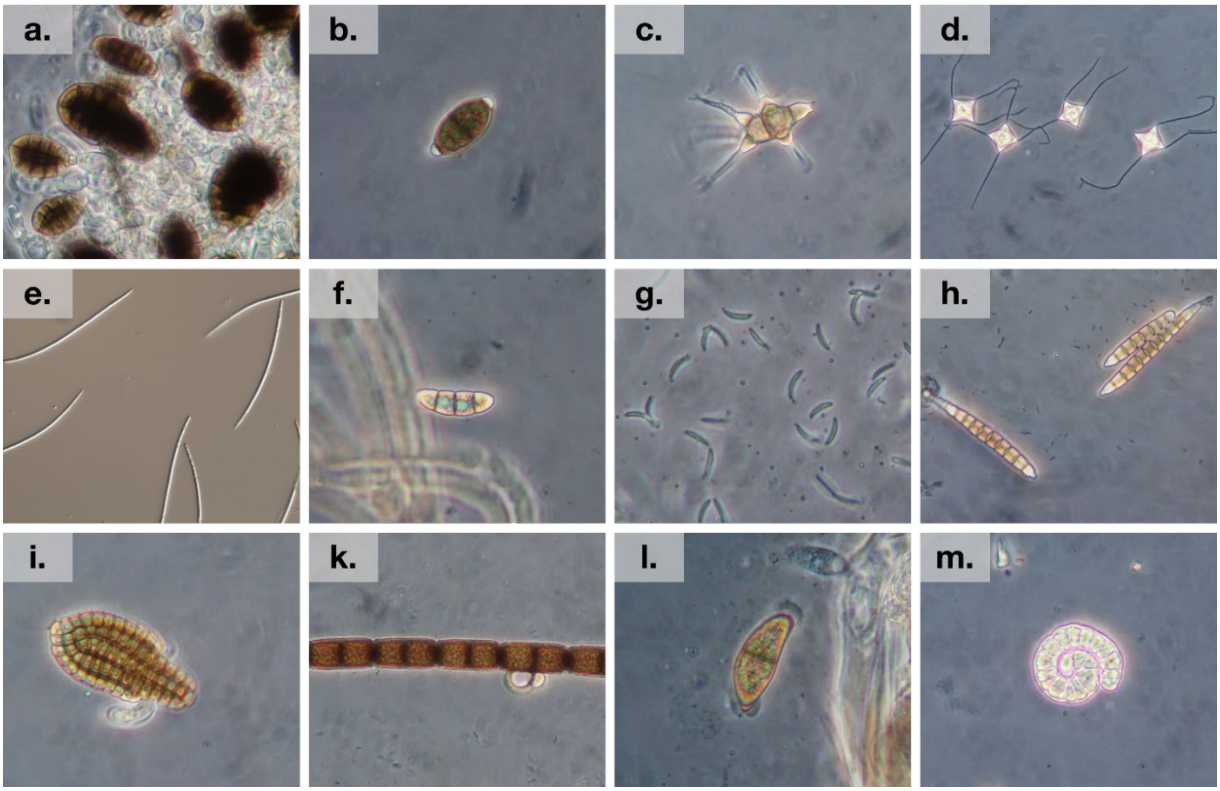

Figure 1.5. *Morphology of ascospores and conidia of several freshwater fungi, images kindly provided by Dr. Sally Fryar. (a) Canalisporium sp., (b) Savoryella sp., (c) Arachnophora longa, (d) Nawawia filiformis, (e) Ophioceras commune, (f) Annulusmagnus triseptatus, (g) Vermiculariopsiella sp., (h) Bactrodesmium longisporum, (i) Dictyosporium bulbosum, (k) Sporoschisma parcicuneatum, (l) Jahnula seychellensis, (m) Helicoma sp.*

Freshwater hyphomycetes are important organisms associated with the decaying of allochthonous plant material. They are, in particular, dominant decomposers of leaves in streams (Ittner, Junghans & Werner 2018; Wong et al. 1998). Chytrids, another significant component in freshwater habitats, might contribute to decomposing particulate organic matter and converting inorganic to organic compounds (Gleason et al. 2008). Conversely, yeasts are not well-studied in freshwater habitats, and their ecological roles in these environments remain elusive (Ittner, Junghans & Werner 2018).

1.4.3. Bioactive compounds from freshwater fungi.

Since 1992, only about 280 metabolites have been published from freshwater fungi. Of these, 80 compounds were published prior to 2010 (El-Elimat et al. 2021), a minuscule number compared to roughly 15,000 known fungal active compounds at that time (Bérdy 2012).

Most freshwater fungal metabolites recorded from 1992 to 2010 were published because of their antimicrobial properties (e.g., Kirschsteinin from *Kirschsteiniothelia* sp., Oxasetin from *Vaginatispora aquatica*) or nematocidal properties (e.g., Caryospomycins from *Caryospora callicarpa*, Pseudohalonectrin from *Pseudohalonectria adversaria*) (Hernández-Carlos & Gamboa-Angulo 2011). Among them, the most significant one is Pneumocandin B_0, an antifungal compound from a fungus isolated from pond water filtrate, *Glarea lozoyensis*. Pneumocandin B_0 is used in the semi-synthesis of caspofungin acetate. FDA approved the latter in 2001 to treat fungal infections (Heinz, Buchheidt & Ullmann 2016).

The majority of published compounds from freshwater fungi were from the 2010s, including many polyketides, phenylpropanoids, terpenes, alkaloids, and peptides. Some of them exhibit antibacterial (e.g., Clearanol C from *Paraphoma radicina*), antifungal (e.g., Clavariopsins from *Clavariopsis aquatica*), and anticancer activity (e.g., Greensporone A from *Halenospora* sp.) (El-Elimat et al. 2021).

Since the diversity of freshwater fungi, and fungi in general, has only been poorly explored, the few published metabolites certainly do not represent all the fungal chemistry from this ecological group. However, it shows the potential of freshwater fungi in making clinically relevant compounds such as infection treatment drugs. Most freshwater habitats around the world have not been studied for freshwater fungal metabolites, especially South America, Africa, and Australia (El-Elimat et al. 2021), and they may very well hide many novel antibiotics waiting to be discovered.

1.5. Research plan

1.5.1. Hypothesis

This study hypothesizes that freshwater fungi are potential sources of novel antimicrobial compounds.

1.5.2. Aims of the project

The overall aim is to discover novel antimicrobial compounds from the provided freshwater fungi. The specific objectives of this project are:

- Screening antimicrobial compounds from freshwater fungi.
- Scaling up the production of the antimicrobial compounds from the selected fungi with solid-state and liquid fermentation.
- Purification of antimicrobial compounds from freshwater fungi for MIC and MBC determination and elucidation of their structure.
- Molecular identification of the selected fungi.

CHAPTER 2. MATERIALS AND METHODS

2.1. Freshwater fungal species

A total of 38 strains of freshwater fungi were used in this study; they were kindly provided by Dr. Sally Fryar, College of Science and Engineering, Flinders University (**Table 2.1** and **Figure 2.1**). These fungi were isolated from decaying wood in freshwater streams in three regions of Australia: Mulgrave River (MR) in April 2015, Daintree Rainforest (CT) in October 2015, and Scott Creek Conservation Park (SCC) in August 2020. Samples from the Daintree Rainforest were collected under the permit WITK15834715. They were preliminarily identified by Dr. Sally Fryar based on morphological features.

Table 2.1. *The list of 38 strains of freshwater fungi isolated from Mulgrave Rive, Daintree Rainforest, and Scott Creek Conservation Park by Dr. Sally Fryar*

Collection sites	Strain code	Classification based on morphology
Woobadda Creek (15°58'03.7"S, 145°22'34.0"E)	**CT98B**	*Lophiostoma bipolare* complex
Daintree Cascades (16°10'14.8"S, 145°24'19.0"E)	**CT154A**	
	CT134B	*Heliomyces* sp. or *Neoheliomyces* sp.
	CT147B	*Tiarosporella* sp.
Oliver Creek (16°08'14.6"S, 145°26'24.3"E)	**CT164A**	*Dictyosporium bulbosum*
Mulgrave River - Site 3 (17°10'38.1"S 145°43'25.5"E)	**MR33A**	*Mariannaea aquaticola*
	MR119A	*Quintaria submersa*
	MR144B	Not yet identified
Mulgrave River - Site 4 (17°08'30.8"S 145°45'43.7"E)	**MR153A**	Not yet identified
	SCC5A	*Mariannaea* sp.
	SCC6A	
	SCC9A	
	SCC10A	*Hongkongomyces* sp.

Scott Creek Conservation Park (35°05'32.2"S 138°40'59.2"E)	**SCC54E**	
	SCC31A	*Coniochaeta* sp.
	SCC31C	*Pleurothecium* sp.
	SCC31D	*Dictyochaeta* sp.
	SCC75A	
	SCC76A	
	SCC31E	*Pleurothecium recuvatum*
	SCC31G	Coelomycete sp.
	SCC32A	*Phaoisaria clematidis*
	SCC34A	*Beverwykella pulmonaria*
	SCC45D	
	SCC44A	*Cosmospora* sp.
	SCC45A	
	SCC52A	*Helicodendron* sp.
	SCC57B	Not yet identified
	SCC57C	*Pleurotheciella* sp.
	SCC65A	*Sporoschisma* sp.
	SCC73A	Not yet identified
	SCC73B	Not yet identified
	SCC75B	*Dactylaria* sp.
	SCC75C	*Sporidesmium* sp.
	SCC81A	Hyphomycete sp.
	SCC82A	*Annulatascus* sp.
	SCC88A	Not yet identified
	SCC89B	*Candelabrum* sp.


CT134B
CT154A*
CT164A
MR33A
MR119A*
MR144B
MR153A
SCC5A
SCC6A
SCC9A
SCC10A
SCC31A
SCC31C*
SCC31D*
SCC31E*
SCC31G*
SCC32A*
SCC34A
SCC44A
SCC45A
SCC45D
SCC52A
SCC54E*
SCC57B*
SCC57C*
SCC65A
SCC73A
SCC73B
SCC75A*
SCC75B

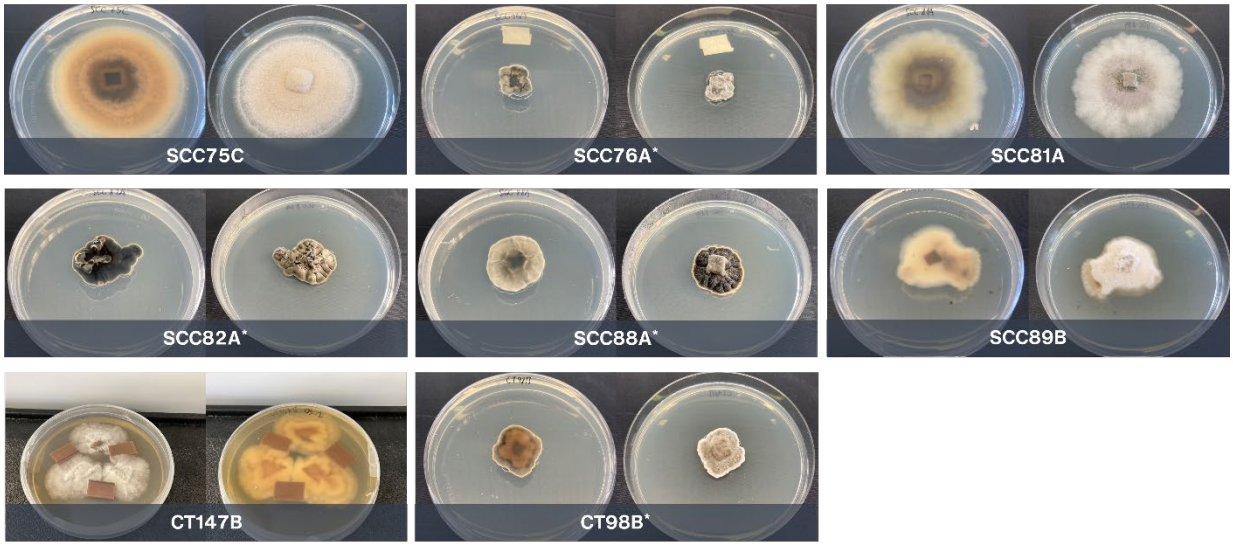

Figure 2.1. *Photos of 38 freshwater fungi used in this study. The fungi were cultured on PDA medium in 9-cm plates. The cultures marked with asterisk (*) were slow-growing strains, the photos were taken on day 30 after the inoculation. Other cultures were medium and fast growing strains, the photos were taken on day 14 after the inoculation.*

2.1.1. Maintenance of freshwater fungi

The freshwater fungi were maintained on Potato Dextrose Agar (PDA) (**Table 2.2**) with a sterile slab of birch wood to facilitate hyphae development. The culture was incubated at 26 °C for 14 days or up to 30 days for slow-growing cultures. Grown cultures were stored at 4 °C for up to 6 months.

2.2. General material and methods

2.2.1. Frequently used media

The list of frequently used media in this study is shown in **Table 2.2**. All media and its container were sterilized by autoclaving at 122 °C for 30 minutes.

Table 2.2. *Frequently used media in this study and their composition. Inside the round bracket is the abbreviation name of the medium and the manufacture of the medium/ingredient*

Medium	Composition
Tryptic Soy Broth (TSB) (Oxoid – Thermo Fisher)	Tryptone 17 g/L; Enzymatic digest of soya bean 3 g/L; NaCl 5 g/L; K_2HPO_4 2.5 g/L; Glucose 2.5 g/L in demineralized water
Tryptic Soy Agar (TSA) (Oxoid – Thermo Fisher)	Tryptone 15 g/L; Enzymatic digest of soya bean 5 g/L; NaCl 5 g/L; Agar 15 g/L in demineralized water

Antibiotic Medium (AMA) (Oxoid – Thermo Fisher)	Peptone 6 g/L; Tryptone 6 g/L; Yeast extract 3 g/L; 'Lab-Lemco' powder 1.5 g/L; Glucose 1 g/L; Agar 11.5 g/L in demineralized water
Cornmeal Agar (CMA) (Oxoid – Thermo Fisher)	Corn Meal Extract 2 g/L; Agar 15 g/L in tap water
Malt Extract Agar (MEA) (Oxoid – Thermo Fisher)	Malt extract 30 g/L; Mycological peptone 5 g/L; Agar 15 g/L in tap water
Mueller Hinton Broth (MH) (Difco – BD)	Beef extract 3 g/L; Acid Hydrolysate of Casein 17.5 g/L; Starch 1.5 g/L; 20 mg/L Ca^{2+} as $CaCl_2$; 10 mg/L Mg^{2+} as $MgCl_2$ in demineralized water
Potato Dextrose Broth (PDB) (Difco – BD)	Potato extract 4 g/L, Dextrose 20 g/L in tap water
Potato Dextrose Agar (PDA) (Difco – BD)	PDB medium supplied with agar 15 g/L
Peptone Yeast Extract Glucose Broth (PYGB)	Yeast extract 5 g/L (Sigma); Peptone 10 g/L (Sigma); Glucose 20 g/L (Sigma) in tap water
Peptone Yeast Extract Glucose Agar (PYGA)	PYGB medium supplied with agar 15 g/L

2.2.2. Test organisms

Nine test organisms used in this study are shown in **Table 2.3**. They were maintained in Tryptic Soy Agar (TSA) medium using the streak plate method.

Table 2.3. *The list of test organisms used in this study*

Classification	Test organism
Yeast	*Candida albicans*
	Saccharomyces cerevisiae
Gram-negative bacteria	*Escherichia coli*
	Klebsiella pneumoniae
	Pseudomonas aeruginosa
	Serratia marcescens

Gram-positive bacteria	*Enterococcus faecalis*
	Staphylococcus aureus
Antibiotic-resistant Gram-positive bacterium	Methicillin-resistant *Staphylococcus aureus* (MRSA)

2.2.3. Evaluating antimicrobial activity

In this study, the antimicrobial activity of an extract or solution was evaluated using the agar well diffusion method (Perez, Pauli & Bazerque 1990) with some modifications. The test organism was inoculated in 10 mL of Tryptic Soy Broth (TSB) medium and incubated at 37 °C for 24 hours. The inoculum was diluted in sterile 0.9% (w/v) NaCl to an optical density at 600 nm wavelength (OD_{600}) of 0.20. Freshly autoclaved Antibiotic Medium (AMA) was seeded with the diluted inoculum. The inoculum percentage was 4% (v/v) for *S. aureus*, *E. faecalis*, *C. albicans*, *S. cerevisiae,* and 2% (v/v) for other organisms. The seeded medium was poured into sterile 9-cm Petri plates at 25 mL per plate and was left until solidified. Then, wells were punched into the agar using a sterile 5-cm diameter cork borer. Each well was filled with 50 µL of the test solution. Colistin (400 µg/mL), vancomycin (400 µg/mL), and amphotericin B (400 µg/mL) were used as the positive controls for Gram-negative, Gram-positive bacteria, and yeast, respectively. Any solvent present in the extract was tested as negative control. The plates were incubated overnight at 37 °C. After that, the diameter of the zone of inhibition (ZOI) was measured.

An example of zones of inhibition is shown in **Appendix 2.** Example of a zone of inhibition in antimicrobial activity tests.

2.2.4. Preparation of the standard curve of test organism' cell count versus optical density

The test organism was inoculated in a 20 mL Mc Cartney bottle containing 10 mL of Mueller-Hinton (M-H) medium and incubated at 37 °C for 24 hours. The broth was

diluted with fresh M-H medium to OD_{600} of 0.10, 0.20, and 0.40, and viable cell count (CFU/mL) was determined using the Miles and Misra drop plating method (Miles, Misra & Irwin 1938). The OD_{600} and cell count values were plotted into a linear standard curve, which was used to estimate the cell density of a test organism culture, given the OD_{600} value of the culture broth. The standard curves were used in this study to prepare a $5x10^5$ CFU/mL cell culture for the MIC and MBC determination in **Subsection 2.9**.

2.2.5. Bioautography analysis

Contact bioautography (Narasimhachari & Ramachandran 1967) was used to visualize the antimicrobial compounds among the separated compounds in the TLC plate. Visible bands of compounds in the developed TLC plate were marked with a pencil. The TLC plate was then placed face down onto the pathogen-seeded agar surface for 1 hour for the compounds to diffuse from the silica gel into the agar. The position of bands of compounds was marked on the back of the agar plate. The TLC plate was then removed, and the agar plate was incubated overnight at 37 °C. Growth inhibition zones would be observed in the bioautogram corresponding to the location of the antimicrobial compounds in the TLC plate.

2.3. Primary screening of the freshwater fungi

In the primary screening, the antimicrobial production ability of the freshwater fungi was detected by testing the methanol extract of fungal cultures grown on four different agars. The procedure for each fungal strain is as follow:

Four 0.5 x 0.5 cm pieces of agar were cut from a 14-day-old fungal culture, or 30-days old for slow-growing strains, and placed upside-down in the center of a 6-cm Petri dish containing 10 mL of either PDA, MEA, PYGA, or CMA medium. The plates were incubated for 14 days at 26 °C. After that, a 2 x 2 cm piece of agar was cut from the center of the culture plate and transferred into a 15-mL centrifuge tube with 2 mL of methanol. For slow-growing strain, a 1 x 1 cm piece of agar was cut from the center of

the culture plate and transferred into a 15-mL centrifuge tube with 0.5 mL of methanol. The tube was agitated in an orbital shaker at 100 rpm for 1 hour and then stored at 4 °C for 24 hours to diffuse the compounds into methanol. The methanol extract was tested for antimicrobial activity against *E. coli* and *S. aureus* following **Subsection 2.2.3**.

2.4. Secondary screening of the freshwater fungi

The fungi selected from the primary screening were evaluated for antimicrobial productivity in solid-state fermentation using four substrates: basmati rice, jasmine rice, brown rice, and mungbean (purchased from Coles, Australia). The procedure for each fungal strain is as follows:

Fifty milliliters of PYGB supplemented with 2% (w/v) dissolved agar were prepared in a 250 mL conical flask. It was inoculated with a 1 x 1 cm piece of a 14-day-old fungal agar culture, or 30-days old for slow-growing strains. The flask was incubated in an orbital shaker incubator at 100 rpm, 26 °C, for six days. The flask was then used as a seed culture.

Forty grams of a substrate was spread evenly in a 19 cm x 11 cm x 4.5 cm aluminum foil tray (purchased from Woolworths, Australia). The tray was sprayed lightly with tap water and covered with double-layer foil. It was then autoclaved twice with a 24-hour interval between each time. After that, 80 mL tap water containing 0.2 g yeast extract was added to the tray. The tray was autoclaved one more time and was allowed to cool before inoculation. Ten milliliters of a six-day-old seed culture were pipetted and spread into the tray. The tray was incubated at 26 °C for 12 days.

Each fungal strain was grown in twelve trays, with three trays for each of the four substrates. On days 7, 10, and 12, a 2 x 1 cm piece of the solid substrate was cut from the middle region of the tray culture and transferred into a 15-mL centrifuge tube with 2.5 mL of methanol. The tube was agitated in an orbital shaker at 100 rpm for 1 hour and then stored at 4 °C for 24 hours. The methanol extract was tested for antimicrobial

activity against *E. coli* and *S. aureus* following **Subsection 2.2.3**. After the sampling on day 12, all culture trays were stored at 4 °C to halt the growth.

The fungal strains which produced extract that exhibited significant antimicrobial activity were selected for further experiments. For these strains, the remaining content of each culture tray was collected into a 500-mL conical flask and extracted with 150 mL of methanol. The flasks were agitated in an orbital shaker at 100 rpm for 1 hour and then stored at 4 °C for 24 hours. The methanol extract was collected and stored at 4 °C for further experiments. Due to the high moisture content of the solid-state cultures, the methanol extracts are expected to have a water content of 20% to 30%.

The old culture trays were discarded if the result showed a temporal decrease in antimicrobial activity for any selected fungal strain. New tray cultures were inoculated and incubated at 26 °C for a shorter period, and the contents were extracted and stored in the same manner.

2.5. Investigating the production of the antimicrobial compound productivity from the selected fungi in liquid cultures

For each selected fungus, a seed culture was prepared following **Subsection 2.4**. Three liquid media were tested in this experiment: PDB medium, PDB medium supplemented with 2% (w/v) dissolved agar (PDB-A medium), and PDB medium in perlite substrate (PDB-P medium).

Fifty milliliters of PDB and PDB-A medium were prepared in 250-mL flasks. PDB-P flasks were prepared by pouring 50 ml of autoclaved PDB-A medium into 250-mL flasks containing 100 mL of sterile perlite (super coarse grade). Each flask was inoculated with 5 mL of the well-grown seed culture. Each fungal strain would be grown in nine flasks, three for each medium. The flask was incubated in an orbital shaker incubator at 100 rpm, 26 °C, for ten days.

On days 4, 7, and 10, 1 mL of culture from each flask was collected into a 1-mL microcentrifuge tube and centrifuged at 10 000 rcf for 10 minutes. The supernatant was collected into a separate tube, and the pellet was mixed with 1 mL of methanol. The tubes were stored at 4 °C for 24 hours to allow diffusion. The supernatant and pellet extract were tested for antimicrobial activity against *E. coli* and *S. aureus* following **Subsection 2.2.3**. The cultures that exhibited significant antimicrobial activity were selected for further experiments.

2.6. Preliminary investigation of characteristics of the antimicrobial compounds in the selected extracts

2.6.1. Thermostability of the antimicrobial compounds

One milliliter of each extract (from the solid-state fermentation) or supernatant (from the liquid culture) was transferred into a 2.5-mL microcentrifuge tube. The tubes were incubated on a dry block heater at each of the following conditions: 40 °C for 1 hour and 3 hours; 60 °C for 1 hour and 3 hours; 22 °C for 8 hours and 24 hours. The stability was evaluated by testing the samples for antimicrobial activity against *E. coli* and *S. aureus*.

2.6.2. Assessing the partition coefficient of the antimicrobial compounds in different solvents

One milliliter of each extract/supernatant was transferred into a 1-mL microcentrifuge tube. Three tubes of each extract were evaporated to dryness using a centrifuge evaporator (Labconco, U.S.); two tubes were reconstituted in 1 mL of Milli-Q water each, the remaining tube was reconstituted in 1 mL of methanol. All samples were homogenized in an ultrasonic bath for 30 minutes. For EtOAct/Water extraction, 1 mL of ethyl acetate was added to one water-reconstituted tube; the tube was agitated using a vortex mixer for 1 minute and was left until the layers separated. The extracts from the methanol tube, the water tube, and both phases of the EtOAct/Water tube were tested for activity against *E. coli* and *S. aureus*.

As discussed in **Subsection 3.4.2**, the result indicates that the antimicrobial compounds in the extracts are separatable to varying degrees with EtOAct/water extraction. Therefore, ethyl acetate was used for the extraction and separation of the compounds in **Subsection 2.7**.

2.6.3. The stability/solubility of the antimicrobial compounds at different pH

One milliliter of each extract was transferred into a 2.5-mL microcentrifuge tube. The extract was evaporated to dryness using a centrifuge evaporator (Buchi, Switzerland). The dried extract was reconstituted in 1 mL of methanol and homogenized in an ultrasonic bath for 30 minutes. The pH of the reconstituted extracts was adjusted to 4, 7, and 10 using NaOH 2M or HCl 2M solution. The pH of a solution was determined using a universal pH indicator strip (Macherey-Negel, Germany). The extracts were incubated at room temperature for 1 hour, then were adjusted to pH 7. The pH-treated samples and controls were tested for antimicrobial activity against *E. coli* and *S. aureus*.

2.6.4. Separation of the extracts and supernatants using thin-layer chromatography

Thin-layer chromatography (TLC) was used in this study to detect compounds in extract solutions and the separation of compounds in different eluent systems.

TLC silica gel 60 F_{254} plates (Supelco Cat. No. 105554), 10 cm in height, were used as the stationary phase. Twenty microlitres of each extract were loaded onto the plate 1-cm from the bottom edge of the plate. The plate was developed in a closed chamber saturated with an appropriate mobile phase. The TLC plate was removed from the chamber when the solvent front was 5 mm to the top edge. The separated compounds were observed and photographed under 254 nm and 365 nm wavelengths. The retention factor of each compound was calculated using the following equation:

$$R_f = \frac{Distance\ traveled\ by\ the\ compound}{Distance\ traveled\ by\ slovent}$$

Initially, three solvent systems were used as the mobile phase, including Chloroform:Methanol:Water (65:35:1) lower phase, EtOAct:Methanol (9:1), and Chloroform:Methanol (9:1). The separated compounds were observed under 254 mn and 365 nm wavelengths. The spots corresponding to the antimicrobial compounds would be visualized using bioautography against *E. coli* and *S. aureus*.

2.6.5. Antimicrobial activity of the extracts against additional pathogens

The selected extracts were tested against all nine pathogens listed in **Table 2.3** using the method in **Subsection 2.2.3**.

2.7. Purification of the antimicrobial compounds

2.7.1. Acid-base extraction of extracts

Some extracts were partially purified using the acid-base extraction method. In this study, only the acid-first approach was employed. The procedure for each extract is as follow:

A volume of methanol extract (from **Subsection 2.4**) was condensed using a rotary evaporator to remove methanol, leaving only the aqueous residue. This step could be skipped for water-reconstituted extracts and liquid culture broth (from **Subsection 2.5**). The pH of the aqueous solution was adjusted to 3; the pH-3 aqueous was transferred into a separation funnel with an equal volume of ethyl acetate. The funnel was agitated and lay still to allow separation. Both phases were collected; the solvent and aqueous phases were called phase E and phase A, respectively. The E phase is transferred into a separation funnel with an equal volume of pH-10 Milli-Q water. The funnel was agitated and lay still to allow separation. Both phases were collected; the solvent and aqueous phases were called phase E.E and phase E.A, respectively. The pH was adjusted using 2M NaOH and 2M HCl. The samples from all steps were collected and separated in TLC plates and analyzed using bioautography to evaluate the presence and purity of the antimicrobial compounds.

2.7.2. Column chromatography purification of the compounds

A volume of methanol extract (from **Subsection 2.4**) was condensed using a rotary evaporator to remove methanol, leaving only the water residue. The aqueous solutions were extracted twice with an equal volume of ethyl acetate using a separation funnel. For more polar compounds, the residual water phase was extracted again with an equal volume of n-butanol. The ethyl acetate extract was evaporated to dryness using a rotary evaporator.

To pack the column, a slurry of 100 g of silica gel in 200 mL of chloroform was poured into a fritted glass column, 4 cm in diameter, and was allowed to settle for 1 hour to form a 20 cm tall silica gel bed. Subsequently, between 0.5 to 1 g of the evaporated ethyl acetate extract was dissolved in a minimum volume of chloroform and loaded onto the silica gel column. The sample was eluted with a solvent system. The composition of the solvent system for each run was determined based on the results of **Subsection 2.6.4**. The eluant was collected into 50 mL fractions. Fractions were separated on TLC plates and analyzed using bioautography to evaluate the presence and purity of the antimicrobial compounds.

Pure antimicrobial compounds were evaporated to dryness using a rotary evaporator and stored at -20 °C for further experiment.

2.8. Elucidation of the molecular structure of the antimicrobial compounds

The purified antimicrobial compounds were sent to Assoc. Prof. Martin Johnston for structure elucidation. The structure of the compounds was determined with NMR spectroscopy, mass spectroscopy, and FTIR spectroscopy.

NMR spectra were recorded on a Bruker 600 MHz Avance III NMR spectrometer at 600 MHz and 150 MHz for ^{1}H and ^{13}C, respectively. All spectra were obtained at 298 K and referenced to the residual solvent signal. Resonances were assigned with the aid of

2D homonuclear (^{1}H-^{1}H) correlation spectroscopy (COSY) and heteronuclear (^{1}H-^{13}C) correlation spectroscopy (HSQC, HMBC).

Mass spectroscopy analysis was carried out by Flinders Chemical Analysis Services. Spectra were run using a Perkin Elmer, AxION 2 TOF, DSA-ToF in Positive ion mode using a mass range of 120 to 2200. Alternatively, a Waters Synapt HDMS was utilized in positive ESI mode. Key instrument settings: capillary voltage 3.0 kV, sample cone 20 V, extraction cone 4, source temp 80 °C, desolvation gas flow rate 500 L/hr, desolvation temp 300 °C, trap CE 6 V, transfer CE 2 V.

FT-IR spectra were recorded using a PerkinElmer Frontier spectrometer with an attenuated total reflectance (ATR) diamond crystal attachment and are reported in wavenumbers (cm^{-1}).

2.9. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antimicrobial compounds

The MIC and MBC of the compounds were determined using the broth microdilution method. For each compound, only the susceptible test organisms as determined in **Subsection 2.6.5** were used for this test. The procedure for each compound–test organism combination was as follow:

The test organism was inoculated in a 20-mL Mc Cartney bottle containing 10 mL of M-H medium and incubated at 37 °C for 24 hours. The broth was diluted with fresh M-H medium to get a seed culture with a cell density of 5×10^{6} CFU/mL. The cell density was determined from the calibration line from **Subsection 2.2.4**.

5.12 mg of the purified compound was dissolved in 1 mL of M-H medium and filtered through a 0.22 µm sterile membrane to yield a 5.12 mg/mL stock solution. If the compound has poor solubility in water, 5.12 mg of the purified compound was dissolved in 50 µL of DMSO and diluted with 950 µL of M-H medium for a stock solution of the same concentration. 500 µL of the stock solution were diluted in 500 µL of M-H medium

to yield a 2.56 mg/mL solution. This two-fold serial dilution was repeated ten times, with the final solution having a concentration of 2.5 µg/mL.

In a sterile 96-well microplate, 160 µL of M-H medium was pipetted into each well. Next, 20 µL of the compound solution was added to each well of the same row in decreasing order of concentration. Then, 20 µL of the seed culture was added to each well. The final test volume in each well was 200 µL, with a cell density of 5×10^5 CFU/mL and compound concentration ranged from 512 µg/mL to 0.25 µg/mL. To test the compound at 1.024 mg/mL concentration, 40 uL of the 5.12 mg/mL stock solution were added into the well with 140 µL of M-H medium and 20 µL of the seed culture. The plate was sealed with sterile sealing film and incubated at 37 °C for 24 hours. The lowest concentration of compound from which no visual growth was observed was the MIC of the compound against that organism.

Subsequently, 10 µL of broth from the MIC well and two subsequent higher concentration wells were dropped on a TSA plate. The plate was incubated at 37 °C for 24 hours. The lowest concentration of compounds from which no visual colony was formed was the MBC of the compound against that organism.

2.10. Species identification of the selected fungal strain using the ITS and LSU DNA sequence

Approximately 50 mg of mycelium was collected from a 14-day old agar culture. The sample was dried under a vacuum and disrupted with a bead beater. Total genomic DNA was extracted from the disrupted sample using the DNeasy Plant Mini Kit (QIAGEN CAT. No. 69104) following the provided instructions.

Primers ITS1 (5' – TCCGTAGGTGAACCTGCGG – 3') and ITS4 (5' – TCCTC CGCTTATTGATATGC – 3') (White et al. 1990) were used to amplify the ITS sequence using polymerase chain reaction (PCR). The LSU sequence was amplified using primers

LR0R (5' – ACCCGCTGAACTTAAGC – 3') (Rehner & Samuels 1995) and LR5 (5' – TCCTGAGGGAAACTTCG – 3') (Vilgalys & Hester 1990).

For each sequence, the PCR was carried out in a 50-µL reaction mixture containing 5 µL of Standard *Taq* Buffer, 1 µL of 10 mM dNTP, 0.25 µL Hot Start *Taq* DNA Polymerase (New England BioLab, U.K.), 1 µL of 10 µM of each primer, 2 µL of the total DNA as a template, and 39.75 µL of RNAase-free water. The amplification was performed in a 2720 Thermal Cycler (Applied Biosystems, U.S.) with an initial denaturation at 95 °C for 3 minutes, followed by 30 cycles of 95 °C for 1 minute, 52 °C for 50 seconds, and 72 °C for 1 minute, and a final elongation step at 72 °C for 10 minutes. The PCR products were cleaned up using a QIAquick PCR Purification Kit (QIAGEN CAT. No. 28104) following the provided instruction.

The purity of the PCR products and the purified DNA were evaluated using agarose gel electrophoresis. The purified DNA samples were sent to ARGF (Australia) for Sanger sequencing. The sequencing results were assembled and trimmed with Geneious Prime 2021.2.2 (https://www.geneious.com) to generate the final sequences. Each sequence was analyzed with NCBI BLAST for related sequences. The LSU and ITS sequence of each strain were aligned with similar sequences from the BLAST results and concatenated using Geneious Prime 2021.2.2. A Bayesian phylogenetic tree was constructed with the concatenated LSU and ITS sequence using MrBayes plugin on Geneious Prime 2021.2.2. Classification of the strain was determined based on the phylogenetic tree.

2.11. Statistical analysis

Data were analyzed using IBM SPSS Statistics 27. Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey's test, in which a *p*-value smaller than 0.05 was considered to be statistically significant.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Primary screening of the freshwater fungi

The aim of this study was to evaluate Australian freshwater fungi as a potential source of novel antibiotics. A random screening approach was employed, in which the fungi were grown in several media and were screened for antimicrobial activity. This approach was chosen for the primary screening due to its relative simplicity and the fact that this approach ensures that some forms of activity could be discovered regardless of the modes of action.

The results of the primary screening of 38 strains of freshwater fungi on four types of agar against *E. coli* and *S. aureus* are shown in **Table 3.1**, with the antimicrobial activity of the active strains shown in **Figure 3.1**.

Table 3.1. *The list of 38 strains of freshwater fungi screened, 17 strains exhibited antimicrobial activity were marked black while 21 strains exhibited no activity are in gray*

CT98B	CT134B	CT147B	CT154A	CT164A	**MR119A**	MR144B	**MR153A**
MR33A	SCC5A	SCC6A	**SCC09A**	SCC10A	**SCC31A**	SCC31C	SCC31D
SCC31E	**SCC31G**	SCC32A	SCC34A	**SCC44A**	**SCC45A**	**SCC45D**	**SCC52A**
SCC54E	SCC57B	SCC57C	SCC65A	**SCC73A**	**SCC73B**	**SCC75A**	SCC75B
SCC75C	**SCC76A**	**SCC81A**	SCC82A	SCC88A	SCC89B		

The activity against *S. aureus* of these fungi could be grouped into **(a)** high activity with the zone of inhibition (ZOI) diameters of 17-19 mm observed in CT98B and MR119A; **(b)** intermediate activity with ZOI diameters of 13-15 mm for MR33A, SCC31A, SCC44A, and SCC45A; **(c)** low activity with the ZOI diameters of 8-11 mm for MR153A, SCC9A; **(d)** SCC81A, SCC31G, SCC45D, SCC52A, SCC73A, SCC73B, SCC75A, SCC75C, and SCC76A with weak activity with ZOI diameters smaller than 8 mm (**Figure 3.1**). A total of 17 strains, which represented 44% of the tested fungi, exhibited antimicrobial activity against *S. aureus*, with no apparent activity against *E. coli* observed. This is a lower

proportion than in a similar study (Wang et al. 2008), where 77% of the 30 freshwater fungi isolated from lakes in Yunnan, China, exhibited antimicrobial activity.

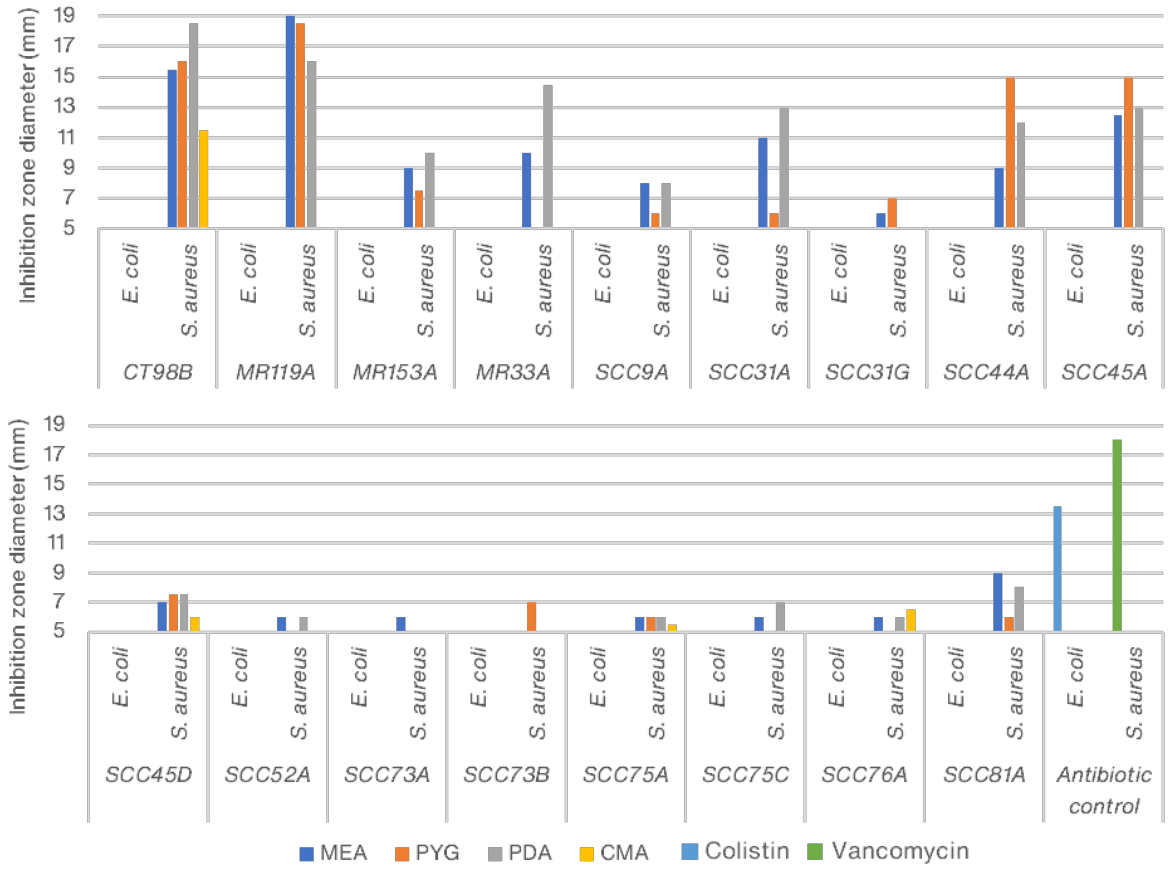

Figure 3.1. *The antimicrobial activity against E. coli and S. aureus of the fungal strains grown on four types of agar media. Colistin and vancomycin (400 µg/mL) was used as controls*

It is evident that the antimicrobial activity is not the same for different strains of the same species. For example, the highly active strain CT98B and the inactive strain MR154A are both identified as *Lophiostoma bipolare*; and the active strain SCC9A is the same species, *Marianaea* sp., as strains SCC5A and SCC6A, which were inactive. This indicates that the specific biosynthetic pathways the produce antimicrobial metabolites are strain-specific rather than genus- or species-specific. A similar phenomenon was recorded in other studies of fungi isolated from water (Wang et al. 2008), soil (Vizcaino et al. 2005), and plants (Phongpaichit et al. 2006; Santos et al. 2015). For example, (Wang et al. 2008) reported strains of *Pseudohalonectria lignicola* and *Dictyosporium heptasporum* with activities against *Bacillus aureus, E. coli, and S. aureus*, while other strains of the same species did not produce any activity.

The difference in the antimicrobial activity for the same culture grown on different agar was also observed, which results from the difference in the nutritional profile of the media. Sixteen out of seventeen active strains exhibited antimicrobial properties after growing on PDA and MEA. These are two commonly used media in mycology with high sugar content and low levels of nitrogen. Meanwhile, PYGA is very nutrient-rich with high sugar and peptide content, which promote biomass accumulation and antimicrobial compound production by some fungi. Metabolic pathways are often regulated by nutrients such as carbon sources (Ruiz-Villafán et al. 2021), nitrogen sources (Tudzynski 2014); the overabundance of certain nutrients could suppress some secondary metabolic pathways (Baral, Akhgari & Metsä-Ketelä 2018). Strains like SCC44A and SCC45A produce the highest activity on PYGA, while strains MR153A, MR33A, SCC9A, or SCC31A respond poorly to this medium. Differently, only strain CT98B exhibits high antimicrobial activity in CMA, a nutritionally impoverished medium (**Figure 3.1**).

Many active strains in the primary screening belong to genera from which antimicrobial compounds have been isolated, including terperstacin and marianins from *Mariannaea* spp. (the same genus as strains MR33A and SCC9A) (Botta et al. 2020; Fukuda et al. 2011), heliconols from *Helicodendron giganteum* (the same genus as strain SCC52A) (Mudur et al. 2006), circinophoric acid from *Sporidesmium circinophorum* (the same genus as strain SCC75C) (Buttachon et al. 2016), and coniosetin from *Coniochaeta ellipsoidea* (the same genus as strain SCC31A) (Segeth et al. 2003).

Even though antimicrobial compound has been discovered in *Lophiostoma bipolare* strain BCC25910 (Intaraudom et al. 2015), the same group as CT98B; *L. bipolare* was taxonomically revised in (Hashimoto et al. 2018) to be a complex of 11 species. Based on the phylogenetic tree in **Figure 3.16**, the strain BCC25910 is more closely related to the genus *Vaginatispora*. Differently, CT98B belongs to *Lentistoma,* a genus with no antimicrobial documented.

Antimicrobial activities have also been reported from the genus *Dictyochaeta* (strains SCC75A and SCC76A) (Wu et al. 2012), and *Quintaria* (strains MR119A) (Zainuddin et al. 2010), although no specific compounds were reported. This study is the first to report the antimicrobial activity of fungi from the genera *Cosmospora* (strains SCC44A and SCC45A) and *Beverwykella* (strain SCC45D). It is expected that these less common and unexplored genera could possess distinct metabolite pathways and novel compounds.

Eleven out of seventeen strains were selected for the secondary screening, including CT98B, MR119A, MR153A, MR33A, SCC9A, SCC31A, SCC45A, SCC52A, SCC73A, SCC75C, and SCC81A. The first seven in the list were the strains of interest because of their notable activity against *S. aureus*.

3.2. Secondary screening of the freshwater fungi

Solid-state fermentation was used in the secondary screening since the production of compounds using this method can be scaled up to produce sufficient compounds for structure elucidation and other assays and potentially applicable for scale-up of production (Lizardi-Jiménez & Hernández-Martínez 2017).

Eight out of eleven strains exhibited antimicrobial activity, which is shown in **Figure 3.2**. No activity was observed with strains SCC73A, SCC75C, and SCC81A. All eight active strains could inhibit *S. aureus*, with MR119A having the highest activity against *S. aureus* with a ZOI diameter up to 20 mm. This activity is comparable to that of the PDA culture in the primary screening.

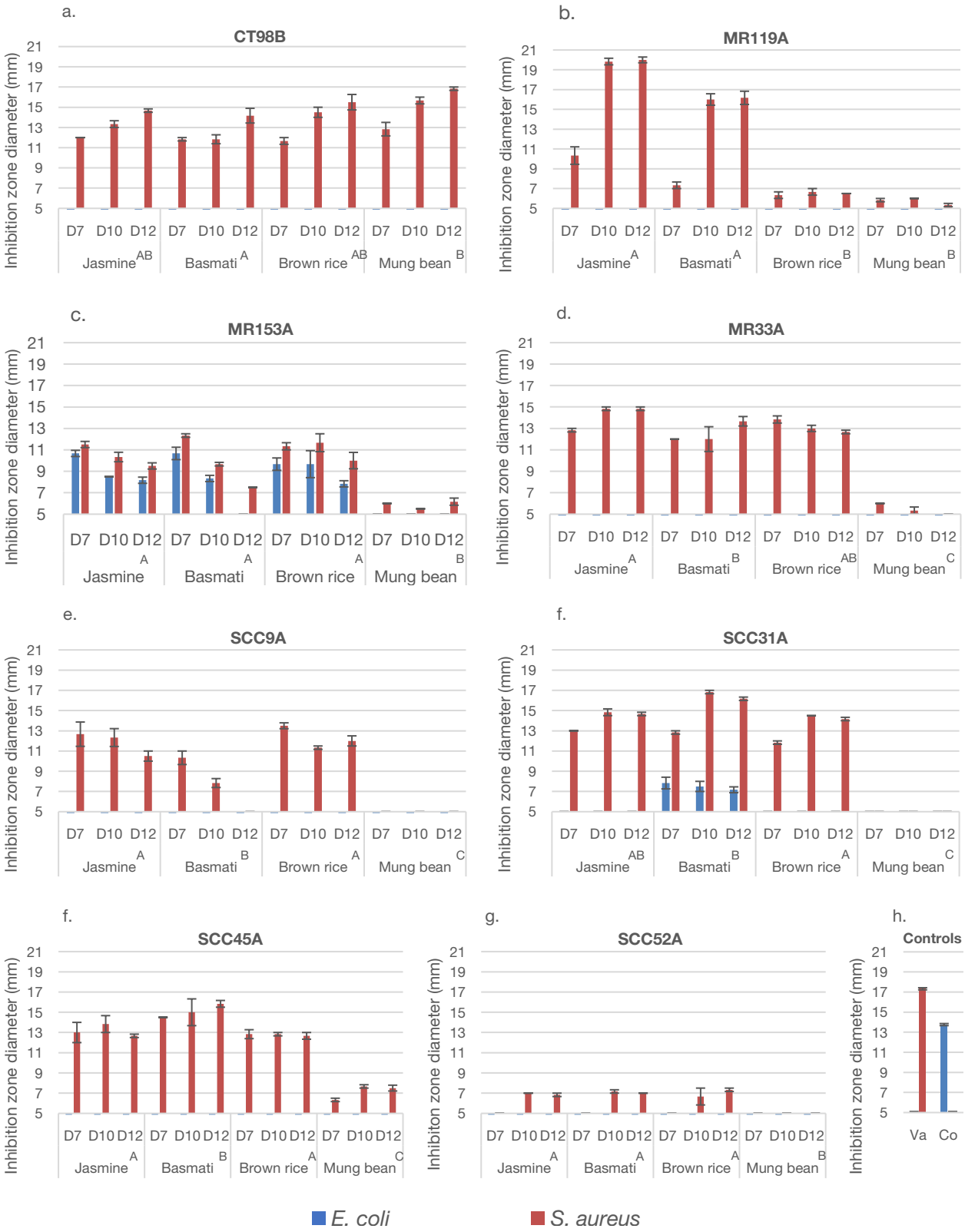

***Figure 3.2**: The antimicrobial activity against E. coli and S. aureus of the fungal strains grown on four types of solid substrate in the secondary screening. Charts (a) to (h) display the activity of strain CT98B, MR119A, MR153A, MR33A, SCC9A, SCC31A, SCC45A, and SCC52A, and the antibiotic control, respectively. The error bars represent the standard error of the mean. The same letter (A, B, C) indicates no significant difference ($p > 0.05$) between inhibition diameter values from each fungal strain (same chart) cultivated in different substrates. (D7, D10, D12: Day 7, Day 10, Day 12).*

The solid-state fermentation of strain CT98B, SCC31A, and SCC45A produced activity against *S. aureus* with a ZOI of approximately 16 – 17 mm in diameter (**Figure 3.2**). The results of SCC31A and SCC45A are higher compared to those in the primary screening. Meanwhile, the activity against *S. aureus* of CT98B in the secondary screening is lower than that in the primary screening, in which the activity reached 18.5 mm with PDA cultures. However, there was an increasing trend in the ZOI diameter on all solid-state cultures of strain CT98B from day 7 to day 12, which indicates that the activity may increase after day 12.

The solid-state fermentation of MR33A produced a ZOI against *S. aureus* of 14 mm in diameter, similar to the results on PDA. In the secondary screening, the highest activity against *S. aureus* of MR153A and SCC9A was slightly higher than in the primary screening. The poor production by SCC52A was noted in both the primary and secondary screening.

Notably, two out of the eight active strains, MR153A and SCC31A, exhibited activities against *E. coli* in the secondary screening, while no such activity was observed in the primary screening. The highest activity of MR153A against *E. coli* is 11 mm, while SCC31A could weakly inhibit this pathogen with ZOIs ranging from 7 mm to 8 mm.

Of the four substrates used, overall, the mung bean cultures of all strains, except for CT98B, produced low or no activity, even though the fungal growth was similar to the rice cultures. Among the three types of rice, jasmine supported the most consistent antimicrobial producing ability among all fungal strains, while basmati and brown rice has low antimicrobial productivity for strain SCC9A and MR119A. Rice grains have been utilized as a fungal-fermentation substrate in previous studies to produce various compounds (El-Elimat et al. 2014; Shang et al. 2012) and was deemed a good substrate for fungal metabolite production (VanderMolen et al. 2013); for example, El-Elimat et al. (2017) isolated 18 compounds from *Clohesyomyces* grown in rice. However, no other study has compared fungal metabolite production with different varieties of rice as a

substrate. This study demonstrated that the type of rice does affect the production of antimicrobial metabolites of fungi.

This study suggests that mung bean is unsuitable for the production of antimicrobial metabolites for freshwater fungi, though the sample size is very small. The differences in nutritional profile between mung bean and rice might repress some fungal metabolic pathways and cause the low observed activity, though the exact factor is unknown. Strain CT98B is the only fungus that produces significant antimicrobial activity when grown on mung beans. It was noted in the primary screening that this strain exhibits activity on all four types of agar media.

Six strains were chosen for further experiments from the eight active fungal strains, including CT98B, MR119A, MR33A, SCC31A, SCC45A for their high activity against *S. aureus*, and MR153A for its activity against *E. coli*. MR119A and SCC45A, in particular, belong to genera from which no antimicrobial compounds had been reported and have a higher chance of possessing novel compounds.

For the first five strains, all solid-state fermentation trays were harvested, each tray was extracted with 150 mL of methanol. The extracts of the following cultures were used for preliminary investigation and purification of the antimicrobial compounds: the mung bean cultures of CT98B, the basmati rice culture of MR33A, SCC31A, SCC45A, and the jasmine rice culture of MR119A. For strain MR153A, there were temporal decreases in the activity of the rice cultures; therefore, new rice cultures were inoculated and incubated for nine days before the methanol extraction. The extract of the brown rice cultures of MR153A was used for preliminary investigation and purification of the antimicrobial compounds.

3.3. Antimicrobial compound production by six selected fungi in liquid culture

The six selected fungal strains were grown in three liquid media. As shown in **Figure 3.3**, five strains exhibited antimicrobial activity, including MR153A, MR119A, CT98B, SCC31A, and SCC45A. No activity was observed with strain MR33A.

MR119A had the highest activity against *S. aureus* compared to other strains with a ZOI diameter of more than 19 mm, achieved in all three media. The activity of SCC31A against *S. aureus* was also notable, with the highest ZOI diameter of 16.5 mm on PDB and PDB-A medium and low activity on PDB-P. In comparison, MR153A and CT98B had an intermediate activity against *S. aureus* with a ZOI diameter that peaked at 12 – 12.5 mm. That activity was observed in all three media for MR153A, but only in PBD-A media for CT98B. SCC45A exhibit low activity against *S. aureus* with a ZOI of only 7 mm on PDB media. Activity against *E. coli* was observed only in MR153A cultures in PDB and PDB-A media, with the largest ZOI diameter of 8.2 mm.

For MR153A, MR119A, CT98B, and SCC31A, antimicrobial activity was detected in both pellet and supernatant, which means that both components could be used to extract the antimicrobial compounds.

For strain MR153A, MR119A, and SCC31A, there was no significant difference in the activity against *S. aureus* between the liquid media and the solid-state media screening. However, the activity of MR153A against *E. coli* was lower in liquid media, and the activity of SCC31A against *E. coli* was not observed. In contrast, CT98B decreased significantly in activity, and SCC45A and MR33A had very low to no antimicrobial activity in liquid media. Previous studies indicated that fungi, including freshwater fungi, generally produce more complex metabolites profiles in solid-state fermentation than in liquid fermentation, even though they can grow well in both cultures (Bills, Dombrowski & Goetz 2012; El-Elimat et al. 2021); for example, VanderMolen et al.

(2013) used aurofusarin and PC3 as marker compounds to track the potential metabolite production, and found that rice and grits produced higher quantities while liquid media such as MEB and PDB produced low levels of these compounds.

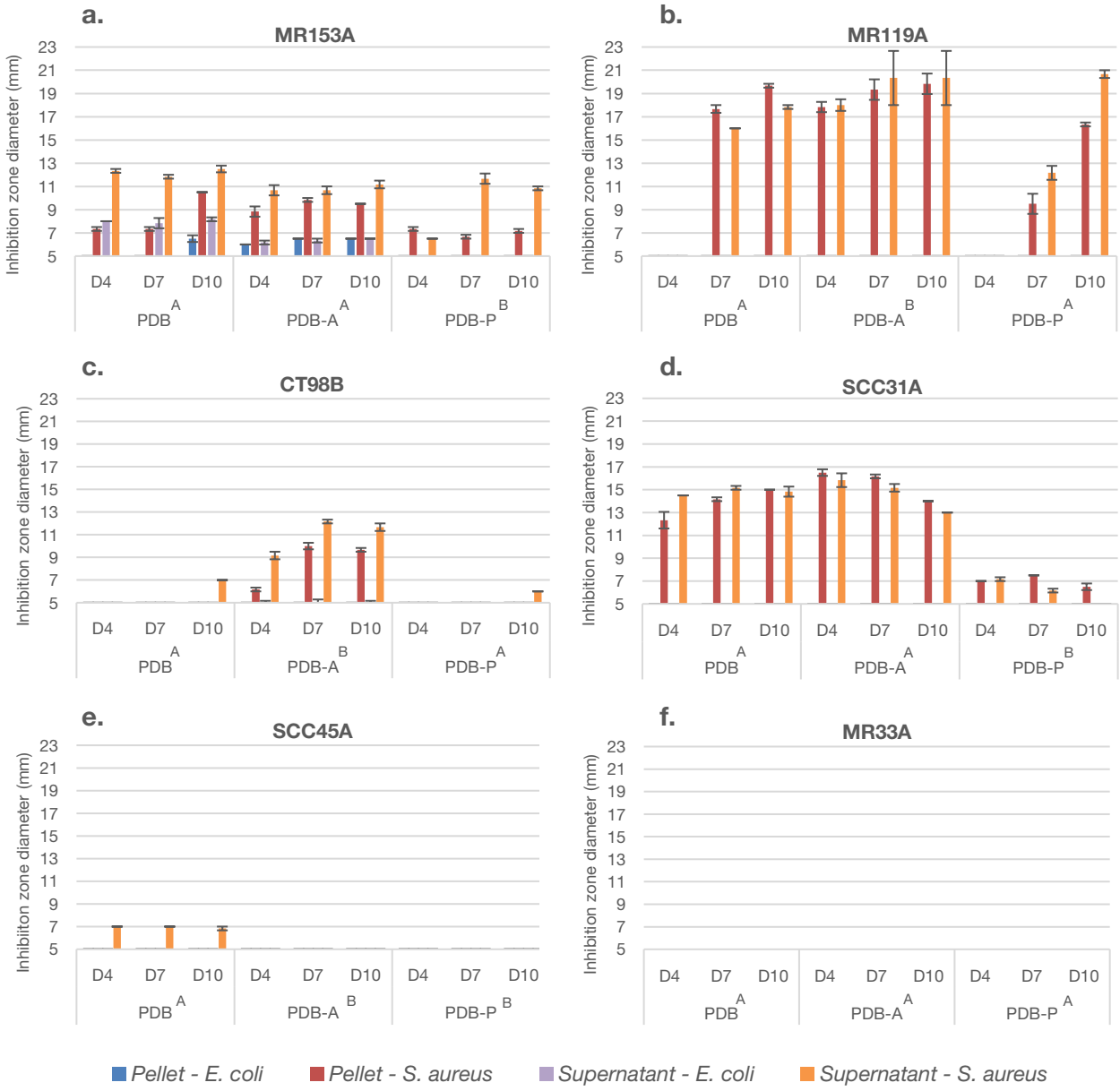

***Figure 3.3**: The antimicrobial activity against E. coli and S. aureus of the pellet and supernatant of six fungal strains grown in three liquid cultures. Charts (a) to (f) display the activity of strain MR153A, MR119A, CT98B, SCC31A, SCC45A, and MR33A, respectively. The error bars represent the standard error of the mean. The same superscript letter (A, B, C) indicates no significant difference ($p > 0.05$) between inhibition diameter from different liquid cultures of each fungal strain (same chart). (D7, D10, D12: Day 7, Day 10, Day 12).*

Despite the forementioned drawback of liquid culture, it was still investigated in this study since this method is more scalable than solid-state fermentation for the industrial production of compounds. Also, if the fungi still retain the antimicrobial productivity in liquid media, as in the case of MR119A and SCC31A, a less complex metabolite profile would make purification of the antimicrobial compounds easier.

In liquid culture, fungi either proliferate as spherical pellets of various sizes or as dispersed hyphae. The pellet formation can positively or negatively impact metabolite productivity; however, the dense mycelium can hinder chemical exchanges of inner hyphae and thus decrease biomass density (Veiter, Rajamanickam & Herwig 2018). For that reason, besides conventional PDB medium, two modified PDB media with the addition of agar and perlite were tested in this study. Agar increases the viscosity of the medium and can reduce pellet formation (Veiter, Rajamanickam & Herwig 2018). Perlite is a porous inorganic material that serves as an inert substrate matrix for the mycelium. A fungal fermentation method, FERMEX, that utilize vermiculite, a similar inert mineral, and broth in rolling bottles were previously described (Bills, Dombrowski & Goetz 2012).

As shown in **Table 3.2**, PDB medium with added agar (PDB-A) reduced pellet formation in MR119A, CT98B, and SCC31A cultures. This observation coincided with a higher activity of these strains in this medium compared to the PDB medium, especially on day 4, as shown in **Figure 3.3**. There were no notable differences in the morphology of strain MR153A, as well as its activity, between PDB and PDB-A medium. On the other hand, the activity of all strains decreased significantly in the PDB with perlite (PDB-P) medium. This result might be because the PDB-P culture was incubated on an orbital shaker instead of a roller, as described in the FERMEX method. The mechanical force from the shaking broke perlite into micro-particles, which might, in turn, damage the fungal cells and cause a reduction in activity.

The pellet and supernatant of PDB and PDB-A cultures of MR119A and SCC31A were collected for further experiments because of their high activity.

Table 3.2. *The morphology of six fungal strains in three liquid media.*

Fungal strain	PDB medium	PDB-A medium	PDB-P medium
MR153A	Small pellet	Small pellet	Dispersed hyphae
MR119A	Small pellet	Dispersed hyphae	Dispersed hyphae
CT98B	Large pellet	Small pellet	Dispersed hyphae
SCC31A	Small pellet	Dispersed hyphae	Dispersed hyphae
SCC45A	Dispersed hyphae	Dispersed hyphae	Dispersed hyphae
MR33A	Dispersed hyphae	Dispersed hyphae	Dispersed hyphae

3.4. Preliminary investigation of characteristics of the antimicrobial compounds in the selected extracts

This experiment used methanol extracts of six strains from the selected solid-state fermentation cultures (MR153A, MR33A, MR119A, CT98B, SCC31A, and SCC45A) and two supernatants of two strains from the liquid cultures (MR119A and SCC31A). For convenience, the solid-state fermentation methanol extracts and liquid culture supernatants will be referred to as 'extracts'. The solid-state fermentation methanol extracts and culture supernatant of strain SCC31A were labeled 'SCC31A.S' and 'SCC31A.L', respectively, and likewise for other extracts.

3.4.1. Thermostability of the antimicrobial compounds

As shown in **Figure 3.4**, all eight extracts were thermostable, with no difference in antimicrobial activity observed between the heat-treated and non-treated samples. The stability of compounds allows them to be stored at 4°C for a long period of time. Furthermore, based on these results, the extracts and compounds could be concentrated using a rotary evaporator with a heated water bath (up to 60°C) in subsequent experiments.

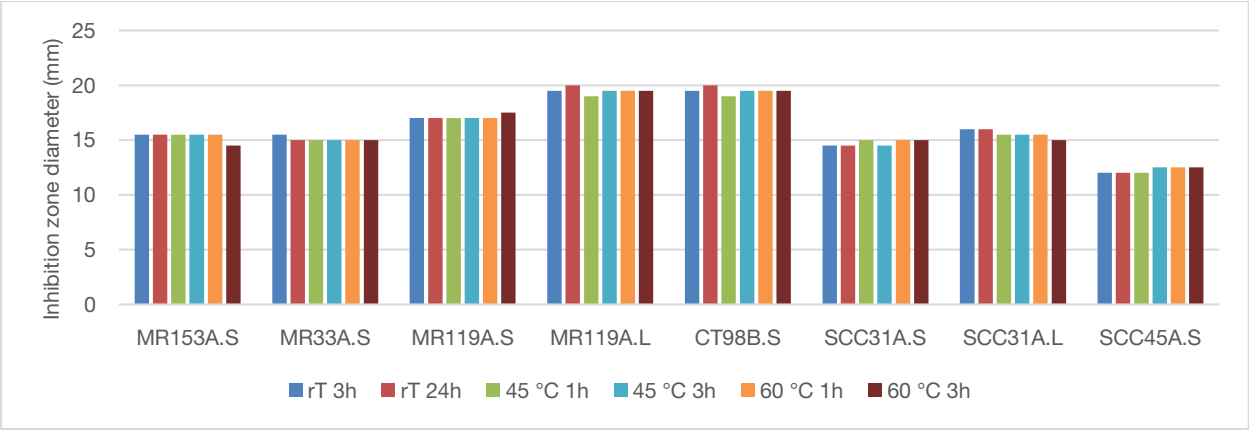

Figure 3.4. *The antimicrobial activity of eight extracts against S. aureus after treatment with different temperature conditions (rT: room temperature)*

3.4.2. The partition coefficient of the antimicrobial compounds in different solvents

The activity of the reconstituted extracts corresponded to the solubility of the antimicrobial compounds in different solvents. As shown in **Figure 3.5**, the activity of all methanol-reconstituted solutions was comparable to the crude extracts; therefore, the corresponding antimicrobial compounds were soluble in methanol.

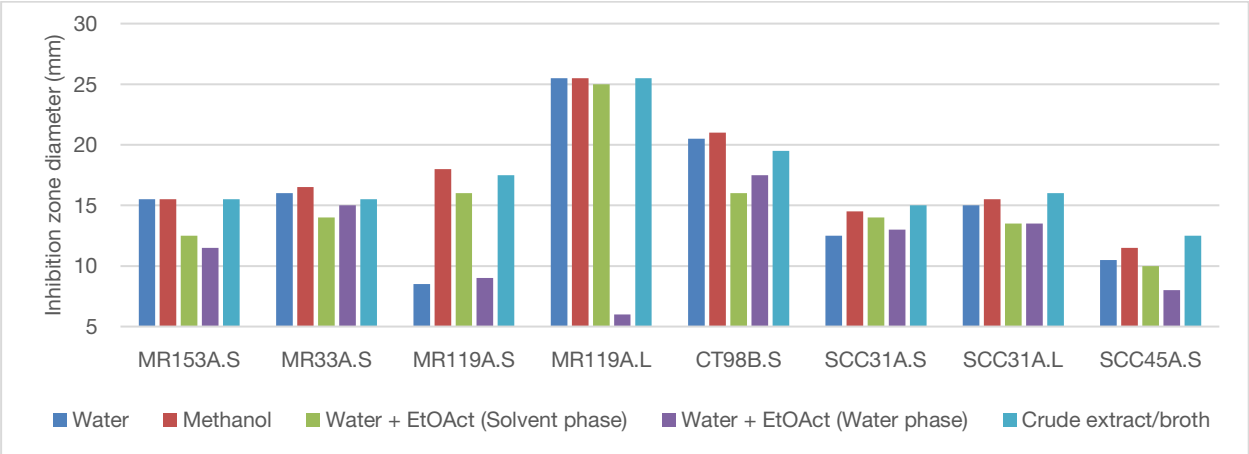

Figure 3.5*. The activity against S. aureus of eight evaporated extracts, reconstituted in water, methanol, or separated with Water/EtOAct extraction*

MR119A.S had poor solubility in water, suggesting the lowest polarity of its compound. The compounds of other extracts had higher polarity as they could dissolve in water. However, all compounds were considered nonpolar since they are extractable with a

water-immiscible solvent such as EtOAct. This result determined how the water-reconstituted extracts and culture supernatants were extracted in **Subsection 3.5**.

3.4.3. The solubility/stability of the antimicrobial compounds in different pH

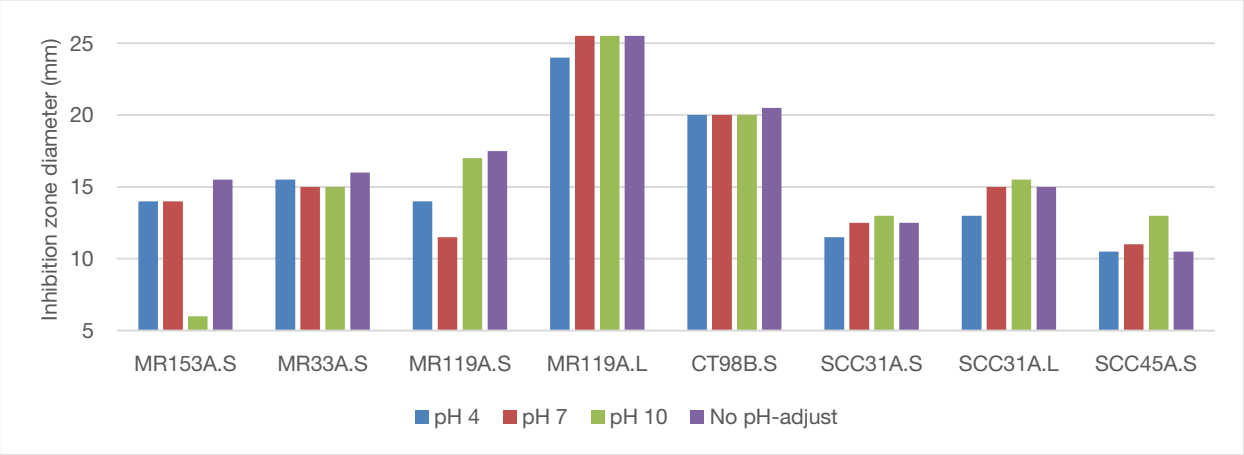

***Figure 3.6**. The activity against S. aureus of eight evaporated extracts reconstituted in water and adjusted to pH 4, 7, and 10*

A decrease in antimicrobial activity could result from the decrease of either solubility or stability of the antimicrobial compounds. As shown in **Figure 3.6**, the solubility/stability of antimicrobial compounds in MR33A.S and CT98B.S was unaffected by different pH levels. The solubility/stability of compounds from MR153A.S decreased at basic conditions. The compounds in MR119A.S were likely more soluble under acidic or basic conditions. Differently, the solubility/stability of compounds in MR119A.L, SCC31A.S, SCC31A.L, and SCC45A.S increase at higher pH levels.

None of the antimicrobial compounds in all eight extracts were unstable/insoluble at low pH levels. Therefore, these compounds could be separated with silica gel, a weakly acidic stationary phase.

3.4.4. Separation of the extracts using thin-layer chromatography

Thin-layer chromatography reveals a complex compounds profile in all extracts, as shown in **Figure 3.7** and **Figure 3.8**. More than one antimicrobial compound was detected in all six extracts, especially MR119A.S with six compounds and MR33A.S with more than ten (**Table 3.3**). In the scope of this study, only the most prominent compound

from each extract was focused on. The prominent antimicrobial compound of MR153A.S was denoted MR153A.S.Cp, and likewise for the compound of other extracts.

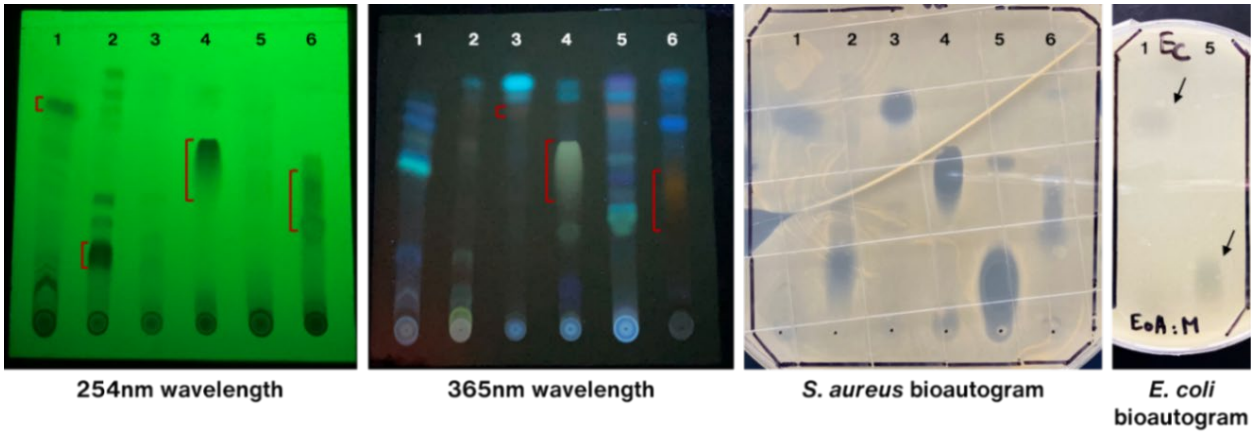

***Figure 3.7**. Separation of six SSF extracts in silica gel TLC plate with the EtOAct:Methanol (9:1) solvent system. Plates were visualized under UV light, and the antimicrobial compounds were visualized in bioautograms. Brackets indicate antimicrobial compounds visible under UV light. Lane 1 to 6 were the SSF extracts of MR153A, MR33A, MR119A, CT98B, SCC31A, SCC45A, respectively.*

Band of the prominent compound from all extract except for SCC31A.S were visualized under UV light. In the case of SCC31A.S.Cp, it may not have an absorbance spectrum that can be visualized on TLC. Except for SCC31A.S.Cp and SCC31A.L.Cp, the R_f value of prominent compounds was different, suggesting that they are different compounds.

Based on the bioautograms in **Figure 3.7**, the active spot of MR153A.S against *S. aureus* and *E. coli* both overlapped with the band of MR153A.S.Cp. The same observation was found with SCC31A.S and SCC31A.S.Cp. The prominent compounds of MR153A.S and SCC31A.S were responsible for the activity of these extracts against both *S. aureus* and *E. coli*. Therefore, in further experiments, only antimicrobial tests against *S. aureus* were used when evaluating the presence of these compounds.

For strains MR119A and SCC31A, the SSF extract was higher than the LF extract in quantity and fluorescence intensity of compound bands, as shown in **Figure 3.8**. This result also agrees that freshwater fungi can produce a more complex metabolite on solid-state fermentation than liquid fermentation (El-Elimat et al. 2021). MR119A.S.Cp was distinct from MR119A.L.Cp, with the former being more polar and producing a

higher antimicrobial activity than the latter. On the other hand, based on the shape and R_f of the active band, SSC31A.S.Cp and SSC31A.L.Cp were likely the same or very similar compounds. Also considering the complexity of the two SCC31A extracts, only SSC31.L were used in further experiments.

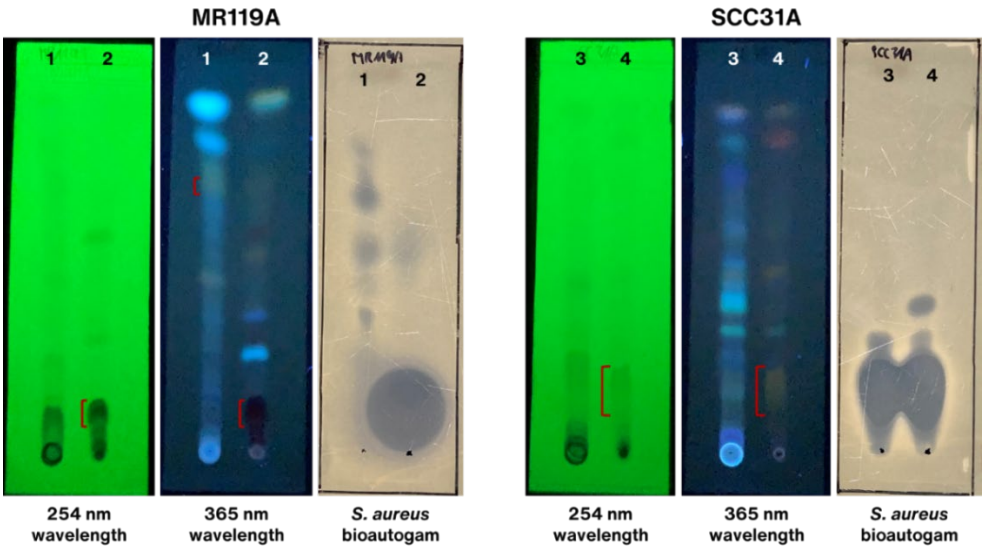

***Figure 3.8**. Comparison of the TLC images and bioautograms of the SSF extract and the LF supernatant of strains MR119A and SCC31A in Chloroform:Methanol (9:1) solvent system. Lane 1 to 4 were MR119A SSF, MR119A LF, SCC31A SSF, and SCC31A LF, respectively. Brackets indicate antimicrobial compounds under UV light.*

On silica gel TLC, a higher R_f value corresponds to a lower polarity of the compound. With the three solvent systems used, all the compounds were found relatively non-polar; however, the compound differed in R_f values, which indicated differences in polarity. The range of R_f values observed with all prominent antimicrobial compounds indicates that it is feasible to separate, purify, elute these compounds with silica gel chromatography and different combinations of the solvent system.

***Table 3.3**. Antimicrobial compounds from eight extracts and supernatants separated in silica gel TLC plate with three solvent systems. (C: Chloroform, M: Methanol, W: Water, E: Ethyl acetate, AMCP: Antimicrobial compound. Only the lower phase of the C:M:W system was used.)*

Extract/ supernatant	Number of AMCPs	R_f value of the most prominent AMCP		
		C:M:W 65:35:10	C:M 9:1	E:M 9:1
MR153A.S	2	0.97	0.60	0.74
MR33A.S	>10	0.99	0.34	0.24
MR119A.S	6	0.99	0.68	0.73
MR119A.L	3	Not tested	0.12	0.28
CT98B.S	2	0.97	0.26	0.60
SSC31A.S	2	0.99	0.16	0.15
SCC31A.L	3	Not tested	0.16	0.15
SCC45A.S	3	0.99	0.06	0.38

3.4.5. Antimicrobial activity of the extracts against additional pathogens

As shown in **Figure 3.9**, all seven extracts exhibited antimicrobial activity against *S. aureus* and *E. faecalis*, both of which are Gram-positive bacteria. The activity of extracts against *S. aureus* was consistent with the result from the screening, which is comparable to the activity of those against *E. faecalis*. The extracts were also active against MRSA, which is an antibiotic-resistant strain. In contrast, only MR153A.S was active against Gram-negative bacteria. The extract was more active against *E. coli* and *K. pneumoniae* with ZOI diameters of 13 mm and less active against *S. marcescens* and *P. aeruginosa* with ZOI diameters of 10 to 11 mm. Except for SCC31A.L, other extracts exhibited weak antifungal activity. MR33A.S was only active against *S. cerevisiae,* while the rest were active against both *S. cerevisiae* and *C. albicans*.

Only one out of seven extracts were found active against Gram-negative bacteria. It is harder to find compounds that are active against Gram-negative bacteria since they are more intrinsically resistant to antibacterial agents (Breijyeh, Jubeh & Karaman 2020).

MR153A.S.Cp might be a broad-spectrum antibacterial agent that is active against both Gram-positive and Gram-negative bacteria.

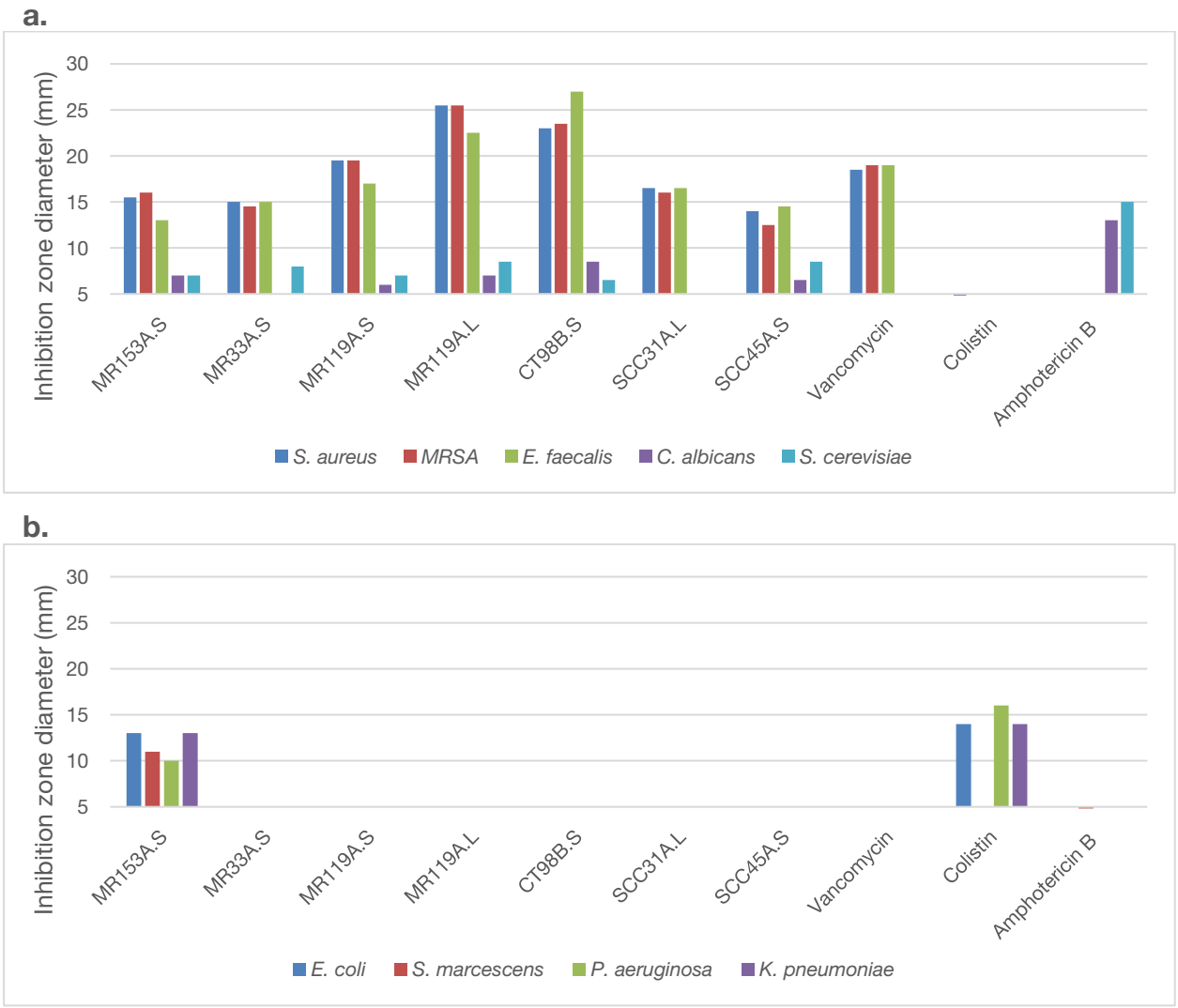

Figure 3.9. *Antimicrobial activity of seven extracts against Gram-positive bacteria and yeast (a) and Gram-negative bacteria (b). Vancomycin, colistin, amphotericin B were positive controls.*

3.5. Purification of the antimicrobial compounds

Due to the limited time of this project, it was decided to purify compounds that showed high activity and were single components. Three extracts were selected for the separation and purification step. MR153A.S was chosen because of the broad-spectrum activity; MR119A.L and CT98B.S were chosen because of their high activity and less complex compounds profile.

3.5.1. Acid-base extraction of MR119A.L

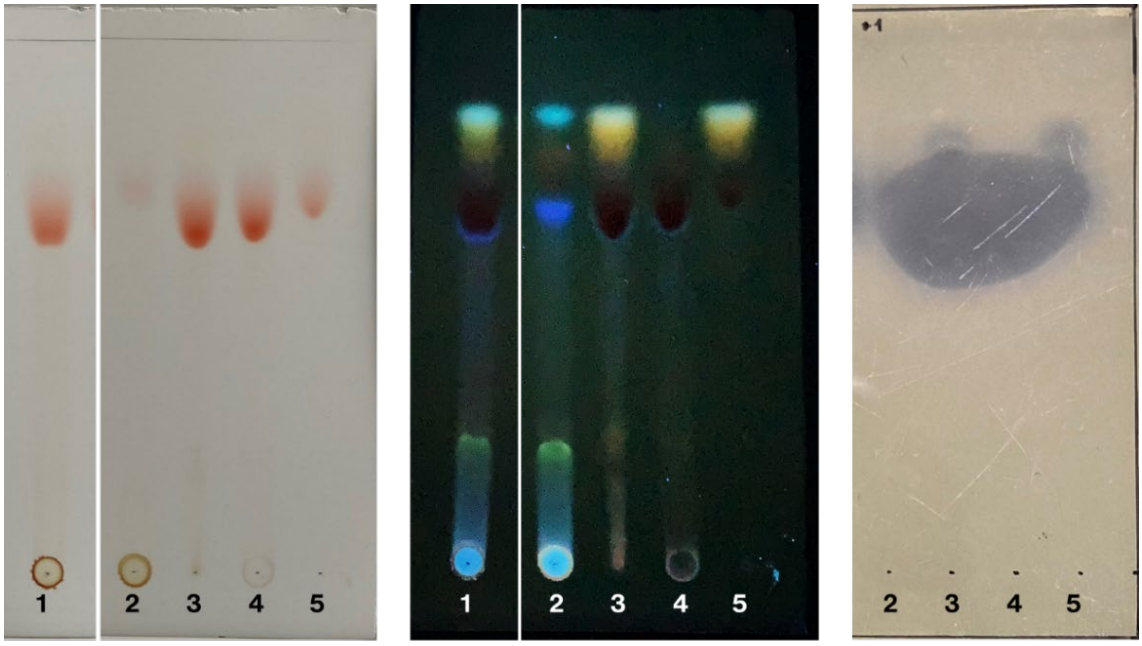

***Figure 3.10**. TLC separation in EtOAct:Methanol (8:2) and bioautogram of phases of MR119A.L after acid-base extraction. Lane 1 was the crude extract, lanes 2 to 5 were phases A, E, E.A, and E.E, respectively, as called in **Subsection 2.7.1**. The compound MR119A.L.Cp was red in color.*

MR119A.L was found to be halochromic, as shown in **Appendix 4**. The compound was yellow in low pH and red in high pH, suggesting that it might be protonated (deprotonated) by acids (bases). Therefore, this compound might be separable with acid-base extraction. The result in **Subsection 3.4.3** indicated that the compound in MR119A.S is less soluble in water at low pH; therefore, the acid-first approach was used.

As shown in **Figure 3.10**, MR119A.L could be partially purified with acid-base extraction. The aqueous phase E.A was the final product of the extraction, and it was reasonably cleaner than the crude extracts. Therefore, 900 mL of MR119A.L was adjusted to pH 3 and extracted with 900 mL of ethyl acetate. The solvent phase was collected and extracted with 900 mL of pH 10 Milli-Q water. From this, 870 mL of the aqueous phase was collected.

3.5.2. Column chromatography purification of the compounds

3.5.2.1. *Purification of MR119A.L.Cp*

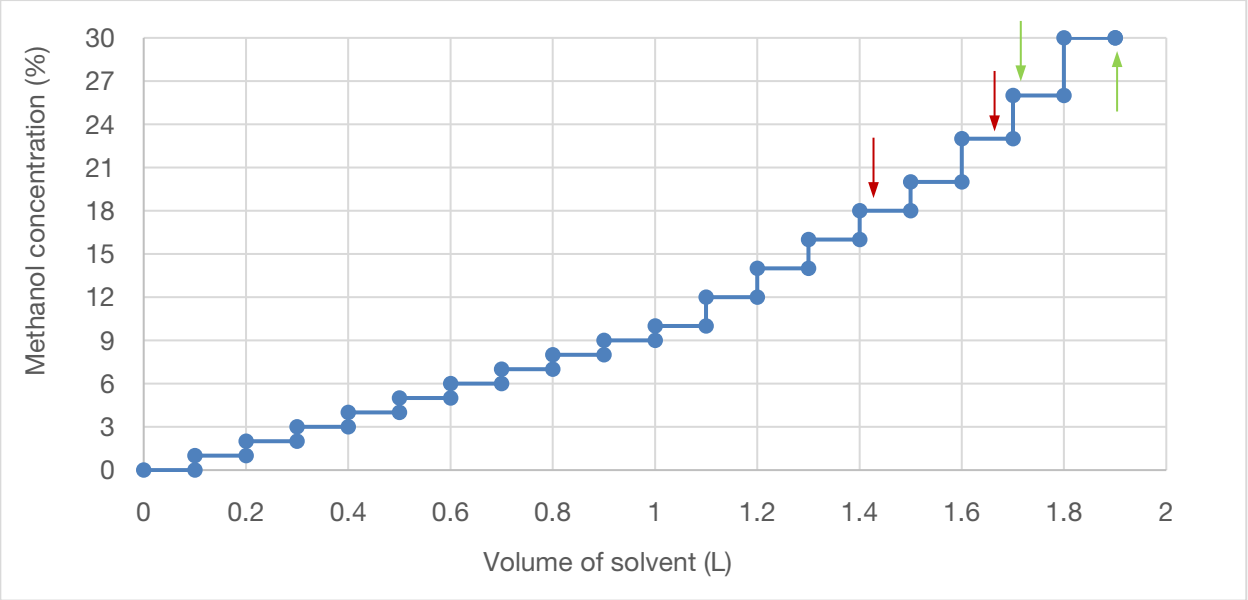

Figure 3.11. *Stepwise gradient of methanol in chloroform used to purify MR119A.L.Cp1 and MR119A.L.Cp2. Red arrows indicate the start and end of MR119a.L.Cp1 elution, and green arrow for MR119a.L.Cp2.*

400 mL of the acid-base extracted aqueous of MR119A.L was extracted again with 200 mL of ethyl acetate and condensed into a solid sample. The sample was added to a silica gel column and separated with a stepwise concentration gradient of methanol in chloroform, from 0% to 8% (v/v). Two antimicrobial compounds were eluted, MR119A.L.Cp1 was eluted between 18% and 23% methanol concentration, and MR119A.L.Cp2 between 23% and 26%. These compounds showed up as the same spot in TLC, suggesting their structures are similar. The extraction yielded 26.1 mg of MR119A.L.Cp1 and 19.6 mg of MR119A.L.Cp2, and both are red in color.

3.5.2.2. *Purification of CT98B.S.Cp*

Ninety milliliters of CT98B.S extract were condensed with an evaporator, extracted with 20 mL of ethyl acetate, and condensed again into a solid sample. The result in **Subsection 3.4.4** indicates that MR153A.S.Cp could be separated with a chloroform:methanol solvent system. Therefore, the solid sample was added to a silica

gel column and separated with a stepwise concentration gradient of methanol in chloroform, started at 0%, and finished at 20% (v/v). Compound CT98B.S.Cp was eluted between 8% and 12% methanol concentration and concentrated into 129.2 mg of light yellow powder.

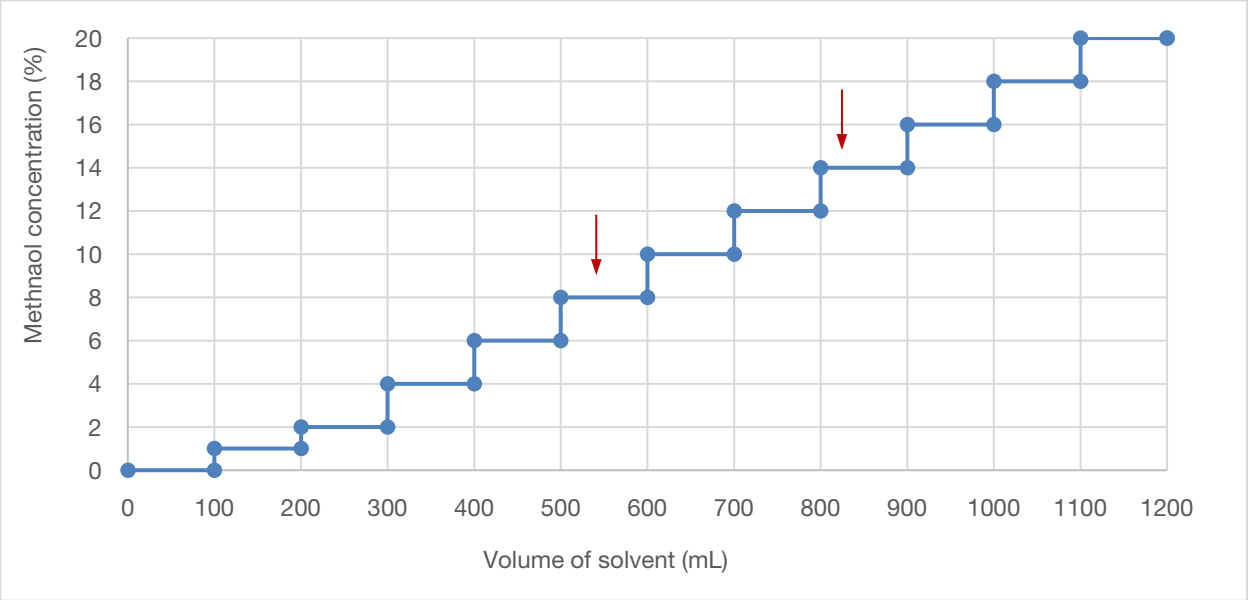

Figure 3.12. *Stepwise gradient of methanol in chloroform used to purify CT98B.S.Cp. Arrows indicate the start and end of CT98B.S.Cp elution.*

3.5.2.3. *Purification of MR153A.S.Cp*

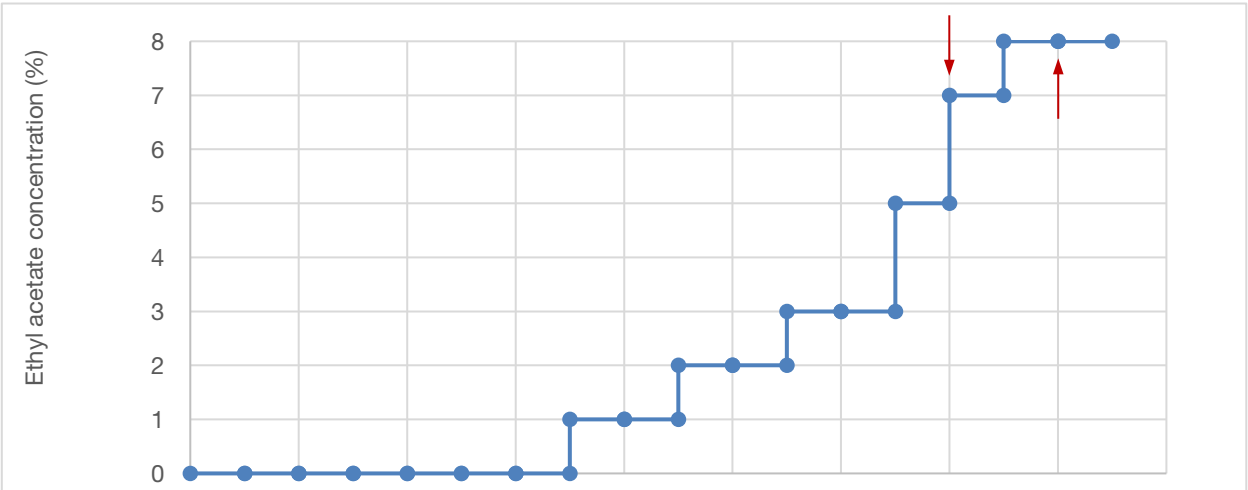

Figure 3.13. *Stepwise gradient of ethyl acetate in chloroform used to purify MR153A.S.Cp. Arrows indicate the start and end of MR153A.S.Cp elution.*

133 mL of MR153A.S extract was condensed with an evaporator, extracted with 100 mL of ethyl acetate, and condensed again into a solid sample. Since MR153A.S.Cp

was highly nonpolar and had many adjacent compounds, ethyl acetate was used instead of methanol for more delicate control of the polarity of the eluent. The sample was added to a silica gel column and separated with a stepwise concentration gradient of ethyl acetate in chloroform, from 0% to 8% (v/v). Compound MR153A.S.Cp was eluted between 7% and 8% ethyl acetate concentration and concentrated to 10.7 mg of orange powder.

Photos on the separation of the four antimicrobial compounds are provided in **Appendix 3**.

3.6. Elucidation of the chemical structure of the antimicrobial compounds

The four purified compounds were sent to Assoc. Prof. Martin Johnston for structure elucidation. As of the submission date of this thesis, only the structure of MR153A.S.Cp was determined while the three other compounds were still being elucidated. Information on the structure elucidation is provided in **Appendix 8**.

3.6.1. Compound MR153A.S.Cp: 3-Isopropenyl-z-butenedioic acid monomethyl ester

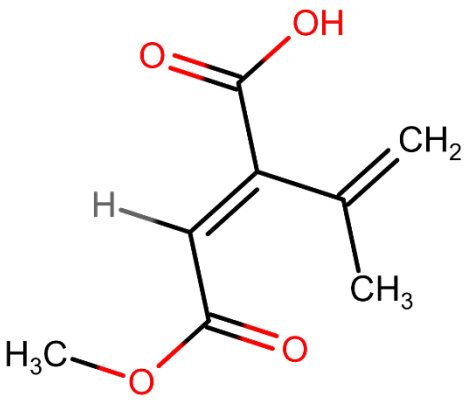

Figure 3.14. *The molecular structure of MR153A.S.Cp - 3-Isopropenyl-z-butenedioic acid monomethyl ester*

MR153A.S.Cp was determined to be 3-Isopropenyl-z-butenedioic acid monomethyl ester with the molecular structure shown in **Figure 3.14**.

This compound was isolated from an endophytic fungi *Verticillium* sp. (Peng et al. 2013) and a *Penicillium* sp. (Ye, Ai & Liu 2021). Peng et al. (2013) reported significant cytotoxicity activity of this compound against the HL-60 cancer cell line (IC_{50} 0.83 µg/mL). A patent has been granted for the use of this compound as a pesticide against

Xanthomonas oryzae; the compound was produced by *Aspergillus sclerotiorum* in liquid PDB (JIANG et al. 2018). In this study, the compound was isolated from *Penicillium polonicum* (see **Subsection 3.8** for classification).

The finding of a previously documented compound was not unexpected since this study utilized a random screening approach where the only criterion for selection is antibiotic activity, and analytical techniques of dereplication were not employed,

A thorough search of relevant literatures found no antimicrobial compounds with similar structures. The antimicrobial mechanism of this compound has also not been investigated. This study reports for the first time the antimicrobial activity of 3-Isopropenyl-z-butenedioic acid monomethyl ester against a wide range of bacteria and yeast other than *Xanthomonas oryzae*.

3.7. The MIC and MBC of the antimicrobial compounds

As shown in **Table 3.4**, MR153A.S.Cp (3-Isopropenyl-z-butenedioic acid monomethyl ester) is active against *S. aureus* and *E. faecalis*, with an 8 – 16 µg/mL MIC and an MBC of 16 – 32 µg/mL. It is also as active against MRSA as *S. aureus*. The compound is active against Gram-negative bacteria, with an MIC and MBC against *E. coli* and *K. pneumoniae* of 16 µg/mL and 32 µg/mL. The compound is less active against *S. marcescens,* with an MIC and MBC of 128 µg/mL.

CT98B.S.Cp is only active against Gram-positive bacteria, with an MIC of 8 – 32 µg/mL and MBC of 32 – 64 µg/mL.

MR119A.L.Cp1 and MR119A.L.Cp2 have similar activity against Gram-positive bacteria with impressive MIC of 2 µg/mL and MBC of 2 – 4 µg/mL. All compounds have weak activity against yeast, with the MIC and MBC greater than 512 µg/mL.

***Table 3.4**. The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of four compounds against nine test organisms (N/A: Not applicable, the compound was not tested against the strain.)*

Pathogen	MR153A.S.Cp		CT98B.S.Cp		MR119A.L.Cp1		MR119A.L.Cp2	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	8	32	16 - 32	32 - 64	2	2	2	4
MRSA	8	16 - 32	32	32 - 64	2	2	2	4
E. faecalis	8	16	8 - 16	32	2	4	2	4
E. coli	16	32	N/A	N/A	N/A	N/A	N/A	N/A
S. marcescens	128	128	N/A	N/A	N/A	N/A	N/A	N/A
P. aeruginosa	32	128	N/A	N/A	N/A	N/A	N/A	N/A
K. pneumoniae	16	32 - 64	N/A	N/A	N/A	N/A	N/A	N/A
C. albicans	1024	>1024	>1024	>1024	1024	>1024	1024	>1024
S. cerevisiae	1024	>1024	>1024	>1024	512	512 - 1024	512	512 - 1024
The MIC and MBC value was measured as µg/mL								

All four compounds inhibit *S. aureus* at about the same strength compared to MRSA, which suggests that MRSA is susceptible to these compounds.

The activity of MR119A.L.Cp1 and MR119A.L.Cp2 against *S. aureus* and MRSA were remarkable. The recorded MIC and MBC values were comparable to many clinically available antibiotics such as erythromycin, gentamicin, ciprofloxacin, and linezolid (O'Riordan et al. 2015).

Strain MR119A and CT98B belong to genus *Quintaria* and *Lentistoma*, respectively. No antimicrobial compounds from these genera have been recorded. Therefore, the

chance of CT98B.S.Cp, MR119A.L.Cp1, and MR119A.L.Cp2 being novel compounds were high.

3.8. Species identification of the selected fungal strain using the ITS and LSU DNA sequences

The ITS and LSU sequences of six selected fungal strains, as well as the sequences used for constructing phylogenetic trees, are provided in **Appendix 6** and **Appendix 7**.

Strain MR153A was classified as *Penicillium polonicum* based on the phylogenetic analysis of the ITS and LSU sequence (**Figure 3.16**). However, strain MR153A did not sporulate in agar media, which is different from typical *Penicillium polonicum* and other *Penicillium* species. This feature complicates the morphological classification of this strain.

Phylogenetic analysis of ITS and LSU sequences indicated that strain MR33A is closely related to *Mariannaea cinerea* (**Figure 3.16**). However, the morphology of this strain is unlike that of *M. cinerea* and is highly similar to *M. aquaticola*. Therefore, MR33A may represent a new species of *Mariannaea*.

Strain MR119A and SCC31A were classified as *Quintaria submersa* and *Coniochaeta velutina*, respectively, based on the phylogenetic analysis of the LSU sequence (**Figure 3.15**). This result also matched the morphology-based classification. ITS sequence was not used to classify these fungi since there is a lack of reliable ITS sequence of *Coniochaeta* spp. on NCBI GenBank.

Strain CT98B was morphologically classified as *Lophiostoma bipolare*, a complex of morphologically similar species in family Lophiostomataceae (Hashimoto et al. 2018). However, phylogenetic analysis of both ITS and LSU sequences indicates that strain CT98B is a sister taxon to other strains of *Lentistoma bipolare*, a species in the *Lophiostoma bipolare* complex (**Figure 3.16**). Strain CT98B may belong to *Lentistoma*

bipolare or may be a novel species of *Lentistoma.* Further research is needed to confirm the classification.

Phylogenetic analysis of ITS and LSU sequences placed strain SCC45A in the genus *Cosmospora* as a sister taxon of both *C. stilbosporae* and *C. stegonsporii* (**Figure 3.16**). The morphology of strain SCC45A is also distinct from these two species. It is highly probable that strain SCC45A represents a new species of *Cosmospora*.

Table 3.5*. Classification of the six freshwater fungi based on morphology and DNA sequences.*

Strain	**Classification based on morphology (Previously done by Dr. Sally Fryar)**	**Classification based on DNA sequences**
MR153A	No ID	*Penicillium polonicum*
MR33A	*Mariannaea aquaticola*	Possibly new species of *Mariannaea*
MR119A	*Quintaria submersa*	*Quintaria submersa*
CT98B	*Lophiostoma bipolare* complex	*Lentistoma bipolare*
SCC31A	*Coniochaeta* sp.	*Coniochaeta velutina*
SCC45A	*Cosmospora* sp.	New species of *Cosmospora*

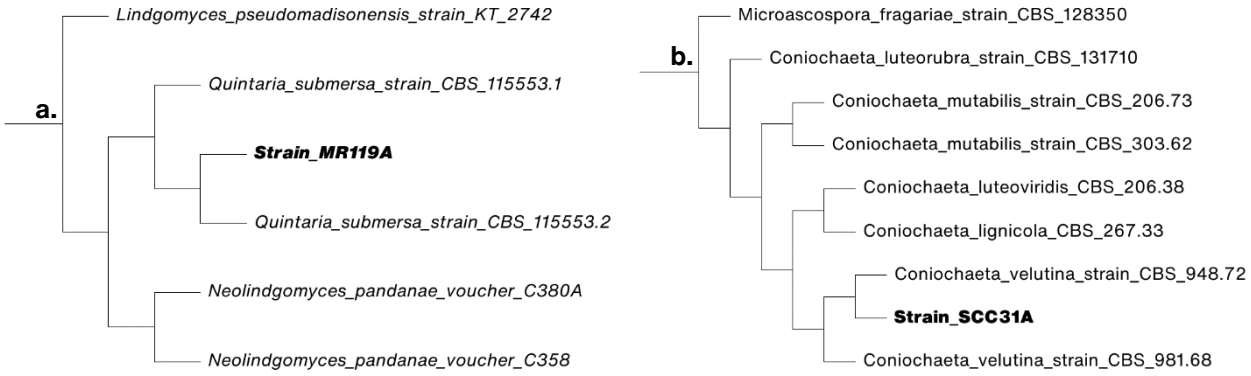

Figure 3.15. *Phylogenetic tree of strains MR119A (a) and SCC31A (b). The trees were constructed from the LSU sequence using Bayesian analysis.*

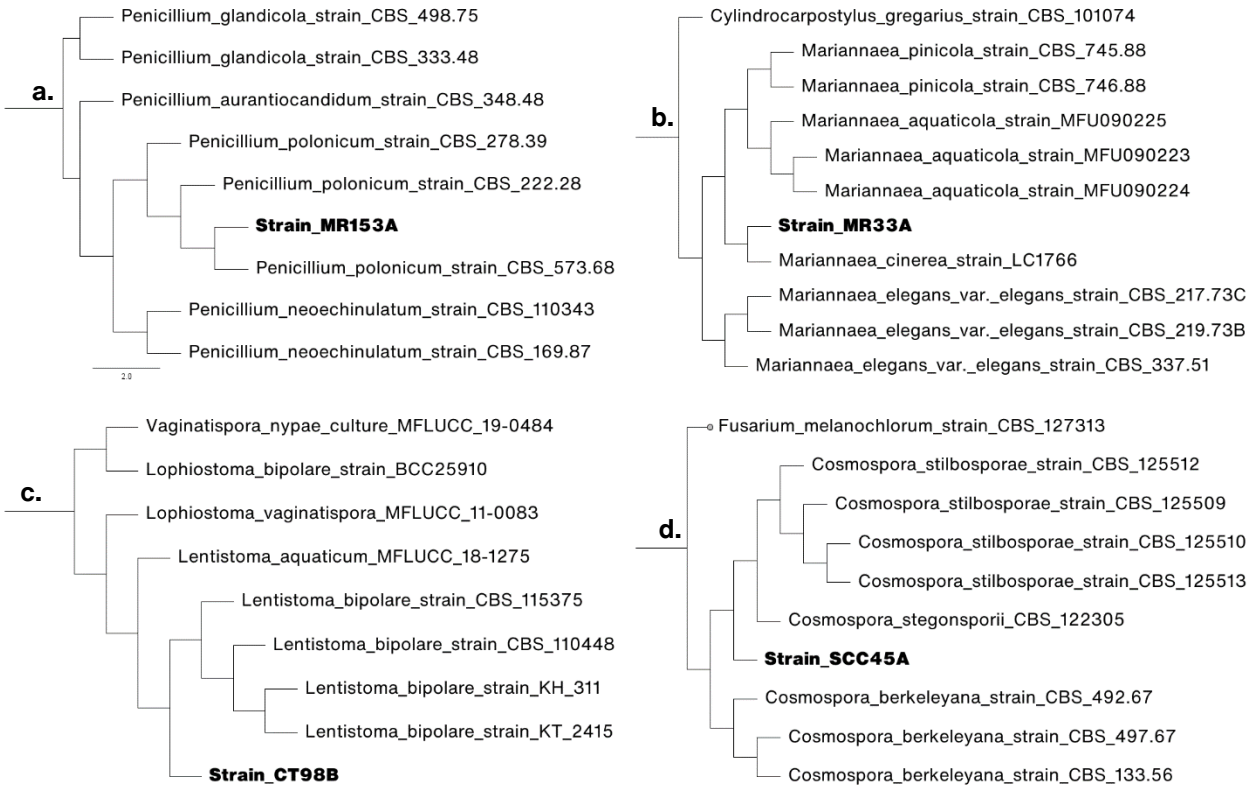

Figure 3.16*. Phylogenetic tree of strains MR153A (a), MR33A (b), CT98B (c), and SCC45A (d). The trees were constructed from concatenated ITS and LSU sequences using Bayesian analysis.*

CHAPTER 4. CONCLUSION AND FUTURE DIRECTION

4.1. Summary of the key finding of this study

This study hypothesized that freshwater fungi are potential sources of novel antimicrobial compounds as they constitute a new source of fungi that are among the least investigated in terms of secondary metabolite production. To test this hypothesis, a general screening approach was utilized, in which thirty-eight fungi were grown on multiple types of media and tested for antimicrobial activity. In fact, 44% of the fungal strains in this study exhibit some level some activity. To test for novelty within the timeframe of the project, it was decided to purify compounds that showed high activity and were single components. Four compounds were purified from three fungal strains. All three strains are novel and previously untested, two of which are from genera with no documented antimicrobial compound. To date, one of the compounds has been identified as 3-Isopropenyl-z-butenedioic acid monomethyl ester. This compound has been previously reported to be produced by three different genera, which indicates that some metabolite pathways are common to many fungi, including freshwater fungi. The issue of dereplication is not uncommon in these random screening approaches as the only criterion for selection is antibiotic activity. Therefore, we have to assess the hypothesis when the structures of the three other compounds are elucidated. If at least one of the compounds is found to be novel, it will indicate that this screening approach is still valid and shows the potential of freshwater fungi as a source of new antimicrobial compounds. Otherwise, it might be worth implementing more specialized approaches such as specific mode-of-action screening or genome mining for a higher chance of finding new antimicrobial compounds.

4.2. Overcoming the challenge

This study involved screening a group of relatively slow-growing fungi, with some strains taking up to one month to cover a third of a 6-cm Petri disk. Furthermore, the

antimicrobial-production media for this group of fungal strains had not been extensively studied. Due to these factors, it was a challenge to produce and purify antimicrobial compounds within a 9-month time limit. The structure elucidation also requires further resources and time. Despite these limitations, the project successfully purified four compounds, with one having its chemical structure elucidated.

4.3. Future research directions

The structure elucidation of the three remaining antimicrobial compounds is still ongoing. If at least one of those compounds were novel, they need to be evaluated as potential drugs. A continuation of this study will be on optimizing and scaling up the production of the compound, testing the efficacy and toxicity *in vitro* and *in vivo*, and determination of the mechanism of action of the compound. In light of the antimicrotic resistance crisis, it is necessary to test the compound against a wider spectrum of bacteria, including multi-drug resistant strains.

Given the high percentage of obtaining novel compounds, another direction is to purify the antimicrobial compounds from all seventeen active fungal strains. Additionally, from the thirty-eight freshwater fungi, it will be worthwhile to screen for other valuable bioactivities where specialized screening systems are available.

It is also recommended that further research should screen more freshwater fungi from a number of geographic locations for potentially novel compounds to combat antibiotic resistance.

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APPENDICES

Appendix 1. Example photos of fungi on solid-state fermentation

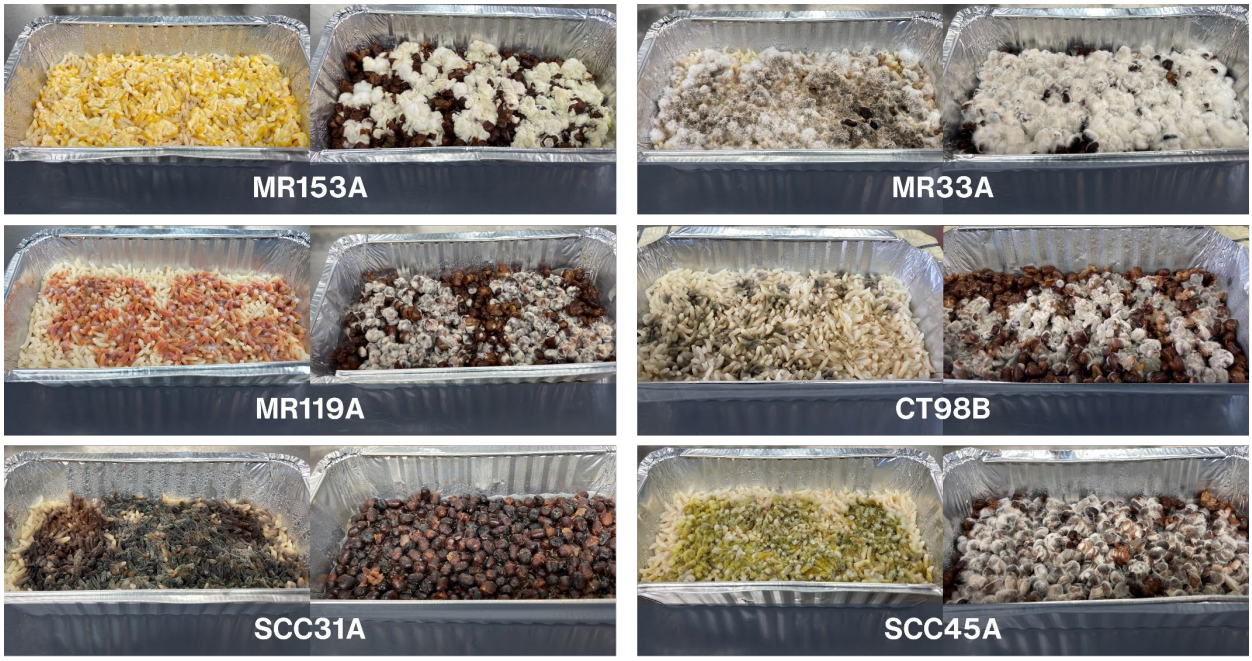

Figure A.1. *Photos of day-7 cultures of six fungal strains on the solid-state substrate. For each strain, the photo on the left is the jasmine culture and on the right is the mung bean culture.*

Appendix 2. Example of a zone of inhibition in antimicrobial activity tests

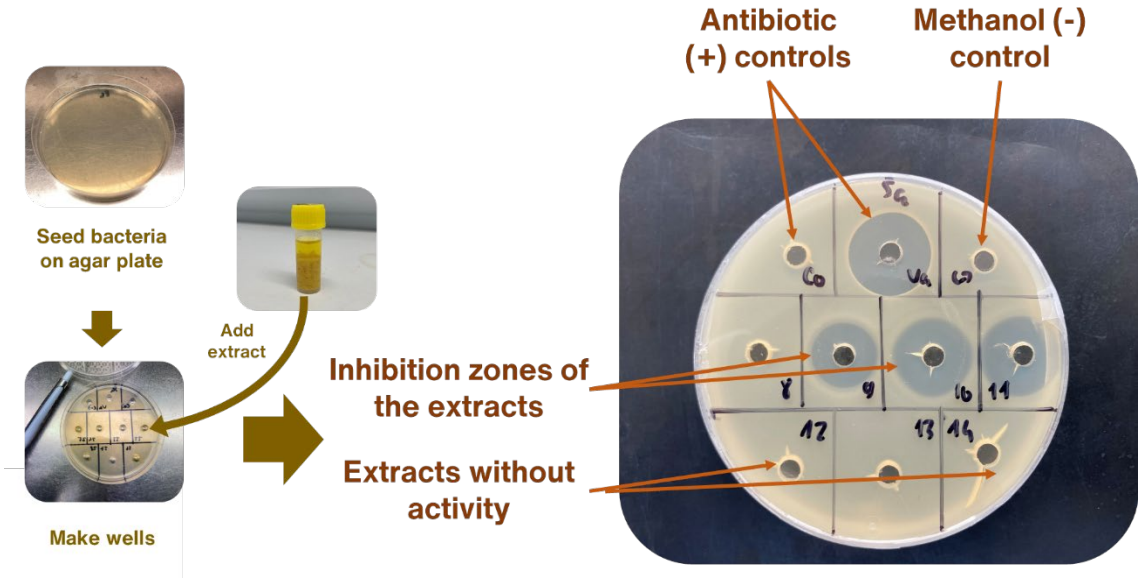

Figure A.2. *The process of evaluating the antimicrobial activity of extracts and the example of inhibition zones.*

Appendix 3. Separation of four antimicrobial compounds

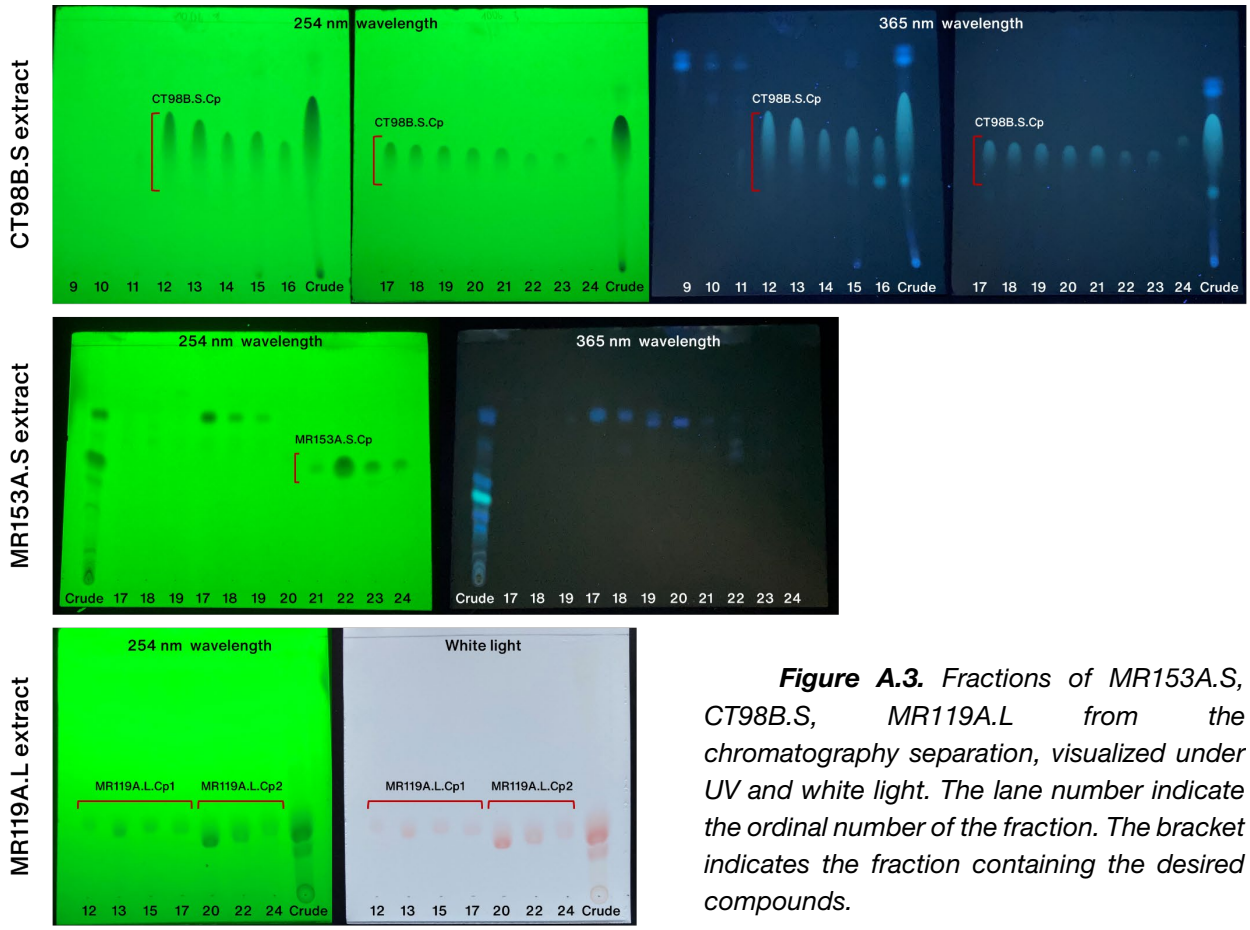

Figure A.3. *Fractions of MR153A.S, CT98B.S, MR119A.L from the chromatography separation, visualized under UV and white light. The lane number indicate the ordinal number of the fraction. The bracket indicates the fraction containing the desired compounds.*

Appendix 4. Halochromic properties of compounds in MR119A.L

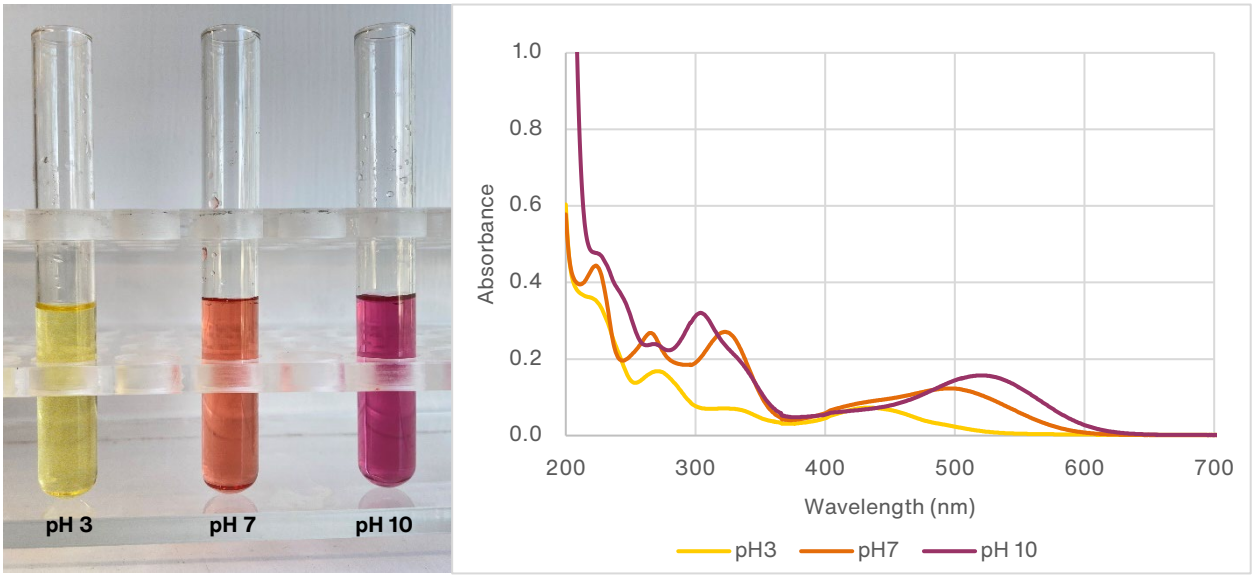

Figure A.4. *The shift in color and absorbance spectrum of MR119A.L extract from pH3 to pH 10, showing the halochromic properties of the antimicrobial compounds*

Appendix 5. Example of the standard curves for from OD_{600} to cell density

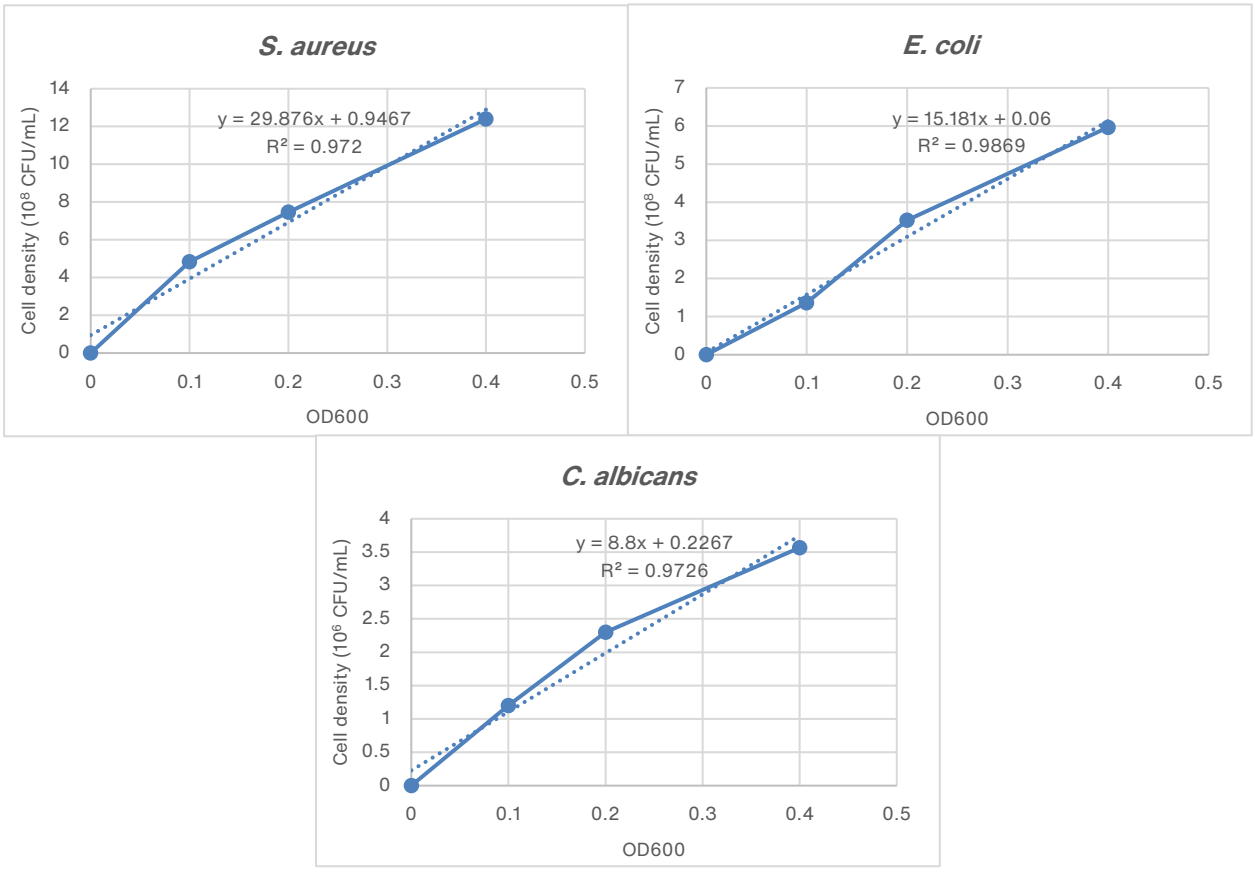

Figure A.5. *Optical density versus cell density standard curves for S. aureus, E. coli, and C. albicans*

Appendix 6. ITS and LSU DNA sequences of six fungi MR153A, MR33A, MR119A, CT98B, SCC31A, and SCC45A

Strain	ITS sequence	LSU sequence
MR153A	TTCCGTAGGTGAACCTGCGGAAGG ATCATTACCGAGTGAGGGCCCTTT GGGTCCAACCTCCCACCCGTGTTT ATTTTACCTTGTTGCTTCGGCGGG CCCGCCTTTACTGGCCGCCGGGGG GCTCACGCCCCCGGGCCCGCGCCC GCCGAAGACACCCCCGAACTCTGT CTGAAGATTGAAGTCTGAGTGAAA ATATAAATTATTTAAAACTTTCAA CAACGGATCTCTTGGTTCCGGCAT CGATGAAGAACGCAGCGAAATGCG ATACGTAATGTGAATTGCAAATTC AGTGAATCATCGAGTCTTTGAACG CACATTGCGCCCCCTGGTATTCCG GGGGGCATGCCTGTCCGAGCGTCA	GCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGAT TGCCCCAGTAACGGCGAGTGAAGCGGCAAGAGCTCAAA TTTGAAAGCTGGCTCCTTCGGGGTCCGCATTGTAATTT GCAGAGGATGCTTCGGGAGCGGTCCCCATCTAAGTGCC CTGGAACGGGACGTCATAGAGGGTGAGAATCCCGTATG GGATGGGGTGTCCGCGCCCGTGTGAAGCTCCTTCGACG AGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGTGGT AAATTTCATCTAAAGCTAAATATTGGCCGGAGACCGAT AGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTT TGAAAAGAGAGTTAAAAAGCACGTGAAATTGTTGAAAG GGAAGCGCTTGCGACCAGACTCGCTCGCGGGGTTCAGC CGGCATTCGTGCCGGTGTACTTCCCCGCGGGCGGGCCA GCGTCGGTTTGGGCGGTCGGTCAAAGGCCCTCGGAAGG TAACGCCCCTAGGGGCGTCTTATAGCCGAGGGTGCAAT GCGACCTGCCTAGACCGAGGAACGCGCTTCGGCTCGGA

	TTGCTGCCCTCAAGCCCGGCTTGT GTGTTGGGCCCCGTCCTCCGATTC CGGGGGACGGGCCCGAAAGGCAGC GGCGGCACCGCGTCCGGTCCTCGA GCGTATGGGGCTTTGTCACCCGCT CTGTAGGCCCGGCCGGCGCTTGCC GATCAA	CGCTGGCATAATGGTCGTAAGCGACCCGTCTTGAAACA CGGACCAAGGAGTCTAACATCTACGCGAGTGTTCGGGT GTCAAACCCGTGCGCGAAGTGAAAGCGAACGGAGGTGG GAACCCTTACGGGTGCACCATCGACCGATCCTGAAGTC TTCGGATGGATTTGAGTAAGAGCGTAGCTGTTGGGACC CGAAAGATGGTGAACTATGCCTGAATAGGGCGAAGCCA GAGGAAACTCTGGTGGAGGCTCGTAGCGGTTCTGACGT GCAAATCGATCGTCGAATTTGGGTATAGGGGCGAAAGA CTAATCGAACCATCTGGTAGCTGGTTCCTGCC
MR33A	AACCTGCGGAGGGATCATTACCGA GTTTACAACTCCCAAACCCCTGTG AACATACCTGTTTTGTTGCTTCGG CGGTGCCCTCGCTCTCGCGGCGAG GCCCGCCAGAGGACCCAAACAAAC TCTTTGATTTTATCTAGGATTTTA CTTCTGAGTGATATACAAAATAAA TCAAAACTTTCAACAACGGATCTC TTGGTTCTGGCATCGATGAAGAAC GCAGCGAAATGCGATAAGTAATGT GAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGC CCGCCAGTATTCTGGCGGGCATGC CTGTTCGAGCGTCATTTCAACCCT CAAGCTCCGGCTTGGTGTTGGGGA TCGGCAGCCCGGCTTCGCGCCGAC GCCGTCCCCCAAATGCAGTGGCGG TCACGCTGTAGCTTCCTATGCGTA GTAGCACACCTCGCACTGGAAAGC AGCGCGGCCACGCCGTAAAACCCC CTACTTTTTGTGGTTGACCTCGAA TCAGGTAGGACTACCCGCTGAACT TAAGCATATCAATAA	CATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATT GCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAAT TTGAAATCTGGCCCTCGGGTCCGAGTTGTAATTTGTAG AGGATGCTTTTGGTGCGGTGCCTTCCGAGTTCCCTGGA ACGGGACGCCATAGAGGGTGAGAGCCCCGTCTGGTTGG ACGCCAATCCTCTGTAAAGCTCCTTCGACGAGTCGAGT AGTTTGGGAATGCTGCTCTAAATGGGAGGTATATGTCT TCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACA AGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGA GAGTTAAACAGTACGTGAAATTGTTGAAAGGGAAGCGC TTATGACCAGACTTGGGCTTGGTAGATCAACCTGGGTT CTCCCTGGTGCACTCTTCCTCGTCCAGGCCAGCATCAG TTCGCTGCGGGGGATAAAGGCTTCGGGAATGTGGCTCC CTCGGGAGTGTTATAGCCCGTTGCGTAATACCCTGCGG TGGACTGAGGTTCGCGTTCTGCAAGGATGCTGGCGTAA TGGTCATCAGCGACCCGTCTTGAAACACGGACCAAGGA GTCGTCTTCGTATGCGAGTGTTCGGGTGTTAAACCCCT ACGCGTAATGAAAGTGAACGCAGGTGAGAGCTTCGGCG CATCATCGACCGATCCTGATGTTCTCGGATGGATTTGA GTAAGAGCATACGGGGCCGGACCCGAAAGAAGGTGAAC TATGCCTGTATAGGGTGAAGCCAGAGGAAACTCTGGTG GAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCA AATATGGGCATGGGGGCGAAAGACTAATCGAACCTTCT AGTAGCTGGTTTCCGCC
MR119A	GGAAGGATCATTACCGTTCATGGA GAGAGAATTGAGAGAACAGGCACA GGATGTGGTTTTGGGCTGATATGC ACCACATGGAGGTCAGGCACGGTT TCCCGATAGTCCTGCAAAAGCCGC ACCTACACGTCTGTTGGGGTCGGT ACCTCTGGGAGATGCCACACTGGG GTCCAGCAAGTCGATTACGTTTGC CAGCCTAGTGGTGACCCAGCATCT CTGTATGTCGTGTGGTGCGCCCCC CTCAAGCCGCGAAGCTCCCAGCCA CGCTGGGAGCTGGCACCGCATCCT GTCCTGTCGCAATTTACTCCTACC ACCCTTGCCTATCTGTACCTTCTG TTTCCTCGGCAGGCTCGCCTGCCA GTTGAGGACCTTCTCAAACACCTT	AGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGA TTGCCTCAGTAACGGCGAGTGAAGCGGCAACAGCTCAA ATTTGAAATCTGGCCCCTTTGGAGTCCGAGTTGTAATT TGCAGAGGGTGCTTTGGTGTTGGCCCTGGTCTAAGTTC CTTGGAACAGGACGTCGCAGAGGGTGAGAATCCCGTAT GTGGCCGCGGGCCTCCGCCTTGTATAGCCCCTTCGACG AGTCGAGTTGTTTGGGAATGCAGCTCTAAATGGGAGGT AAATTTCTTCTAAAGCTAAATACTGGCCAGAGACCGAT AGCGCACAAGTAGAGTGATCGAAAGATGAAAAGCACTT TGGAAAGAGAGTCAAAAAGCACGTGAAATTGTTGAAAG GGAAGCGCTTGCAGCCAGACTTGCCTGCGGTTGCTCAG CCAGGCTTCTGCCTGGTGCACTCTTCCGCATGCAGGCC AGCATCAGTTCGGGCGGTTGGATAAAGGCCTTGGGAAT GTGGCTCCTCTCGGGGAGTGTTATAGCCCGGGGTGCAA TGCAGCCAGCCTGGACTGAGGTCCGCGCATTTGCTAGG ATGCTGGCGTAATGGCTGTAAGCGGCCCGTCTTGAAAC

	GTGCAGTACCCAGTGGTTGTCGGC AGCAATAACAATCGAAATCAAAAC TTTCAACAACGGATCTCTTGGTTC TGGCATCGATGAAGAACGCAGCGA AATGCGATAAGTAGTGTGAATTGC AGAATTCCGTGAATCATCGAATCT TTGAACGCACATTGCGCCCTTTGG TATTCCACTGGGCATGCCTGTTCG AGCGTCGTTGAGAACCTTCAAGCC TAGCTTGGTGTTGGGCGCCTGTCC CGCCTCGTGTGCGGGGACTCGCCT TAAATCCATTGGCAGCGGGCAGGG CAGCTGACTGCGCAGTAGACTTC	ACGGACCAAGGAGTCTAACATCTATGCGAGTGTTTGGG TGTTAAGCCCGGGCGCGCAATGAAAGTGAACGGAGGTG GGAACCCGCAAGGGCGCACCATCGACCGATCCTGATGT CTTCGGATGGATTTGAGTAAGAGCATAGCTGTTGGGAC CCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCC AGAGGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACG TGCAAATCGATCGTCAAATTTGGGCATAGGGGCGAAAG ACTAATCGAACTATCTAGTAGCTGGTTCCTGCC
CT98B	ACCTGCGGAAGGATCATTATTGTG AGGGCTTCGGCCCATTGCATAATG CAAACCCTTGCCTTTTTAGTAGTA TCTCTGTTTCCTCGGCAGCTTAGT GCTAACAGCTTTGCCTGTCAAAGG ACCCCTAAAACAAAACCTTTGTAA TCTCGATCATGTTCAGAAAAACTC AATTTTTACAACTTTCAACAATGG ATCTCTTGGCTCTGGCATCGATGA AGAACGCAGCGAAATGCGATAAGT AGTGTGAATTGCAGAATTCAGTGA ATCATCGAATCTTTGAACGCACAT TGCGCCCTTTGGTATTCCTTAGGG CATGCCTGTTCGAGCGTCATTTAA AACCTCAAGCACGGCTTAGTGGCA GGGACCCTAAGAGGGACCTACCCG TAGCCCAGCTTGGTGATGGGCGCC TGTCCCGCCGTGGTGCGCGGACTC GCCTCGAATGCAGTTGGCAGCCCC AATACCTCCTCAATCGGATCCGAA GTAGAATTCGCAGGCCGTGGGTGG TGGTGGCTCTCCAAAGGTAGGCAA CCCGTAAAGCGTCTCCTCAATCGG ATCCGAAGTAAAATTCGCAGGCCG TGGACGGCGGAGGGGTCCTACGAG CAATTCACCCTGCTCTA	AGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGA TTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAA ATTTGAAATCTGGCCTTCGGGTCCGAGTTGTAATTTGC AGAGGGCGCTTTGGCATCGGCTGTGGTCTAAGTTCCTT GGAACAGGACGTCGCAGAGGGTGAGAATCCCGTACGTG GCCGCCAGCCTTTACCGTGTAAAGCCCCTTCGAAGAGT CGAGTTGTTTGGGAATGCAGCTCTAATTGGGAGGTAAA TTTCTTCTAAAGCTAAATATCTGCCAGAGACCGATAGC GCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGG AAAGAGAGTCAAAAAGCACGTGAAATTGTTGAAAGGGA AGCGCTTGCAGTTAGACGTGCCTTTAGATGCTCAGCCG GGCTTTGGCCCGGTGCACTCTTCTATTGGCAGGCCAGC ATCAGTTTGGGCGGTCGGATAAAGGCCTATTAAACGTG ACTCCCCTCGGGGAGAGCTTATAGGGTAGGCGTCATGC GATCAGCCTGGACTGAGGTCCGCGCATCTGCTAGGATG CTGGCGTAATAGTTGCAAGCGGCCCGTCTTGAAACACG GACCAAGGAGTCTAACATCTATGCGAGTGTTTGGGTGT CAAGCCCGGATGCGTAATGAAAGTGAATGGAGGTGGGA TCCCTTCGGGGGTGCACCATCGACCGATCCTGATGTTTT CGGATGGATTTGAGTAAGAGCATAGCTGTTGGGACCCG AAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGA GGAAACTCTGGTGGAGGCTCGCAGCGGTTCTGACGTGC AAATCGATCGTCAAATTTGGGCATAGGGGCGAAAGACT TAATCGAACTATCTAGTAGCTGGTTCCTGCCGAA
SCC31A	TGCGGAGGGATCATTATTAGAAGC CGAAAGGCTACTTAAAACCATCGC GAACTCGTCCAAGTTGCTTCGGCG GCGCGGCCCCCCTCACGGGGGCGC CGCAGCCCCGCCTCTCCGGAGGTG TGGGGCGCCCGCCGGAGGTACGAA ACTCTGTATTATAGTGGCATCTCT GAGTAAAAAACAAATAAGTTAAAA	GCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGAT TGCCCCAGTAACGGCGAGTGAAGCGGCAACAGCTCAAA TTTGAAATCTGGCTTCGGCCCGAGTTGTAATTTGTAGA GGATGCTTTTGGTGAGGTGCCTTCTGAGTTCCCTGGAA CGGGACGCCAGAGAGGGTGAGAGCCCCGTATAGTCGGC CACCGATCCTCTGTAAAGCTCCTTCGACGAGTCGAGTA GTTTGGGAATGCTGCTCAAAATGGGAGGTATATCTCTT CTAAAGCTAAATATTGGCCAGAGACCGATAGCGCACAA

	CTTTCAACAACGGATCTCTTGGTT CTGGCATCGATGAAGAACGCAGCG AAATGCGATAAGTAATGTGAATTG CAGAATTCAGTGAATCATCGAATC TTTGAACGCACATTGCGCCCGCTA GTACTCTAGCGGGCATGCCTGTTC GAGCGTCATTTCAACCCTCAAGCC CTGCTTGGTGTTGGGGCCCTACGG CTGCCGTAGGCCCTGAAAGGAAGT GGCGGGCTCGCTACAACTCCGAGC GTAGTAATTCATTATCTCGCTAGG GACGTTGCGGCGCGCTCCTGCCGT TAAAGACCATCTTTAACTCAAGGT TGACCTCGGATCAGGTAGGAATAC CCGCTGAACTTAAGCATATCAATA AGCGG	GTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAG GGTTAAATAGCACGTGAAATTGTTGAAAGGGAAGCGCT TGTGACCAGACTTGTGCCGGGCTGATCATCCAGTGTTC TCACTGGTGCACTCGACCCGGCTCAGGCCAGCGTCGGT TCTCGCGGGGGGATAAAAGCTCCGGGAACGTGGCACCT CCGGGTGTGTTATAGCCCGCTGCTTAATACCTTCGCGG GGACCGAGGTTCGCGCTCTGCAAGGACGCTGGCATAAT GGTCATCAGCGACCCGTCTTGAAACACGGACCAAGGAG TCGAGGTTTTGCGCGAGTGTTTGGGTGTAAAACCCGCA CGCGTAATGAAAGTGAACGTAGGTGAGAGCTTCGGCGC ATCATCGACCGATCCTGATGTATTCGGATGGATTTGAG TAAGAGCGTATAGCCTCGGACCCGAAAGATGGTGAACT ATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGG AGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCAA ATTTGGGCATGGGGGCGAAAGACTAATCGAACCATCTA GTAGCTGGTTACCGCCGA
SCC45A	CCGTAGGTGAACCTGCGGAGGGAT CATTACCGAGTTTACAACTCCCAA ACCCCTGTGAACATACCTATGTTG CTTCGGCGGGATCGCCCCGGCGCC TTCGGGCCCGGACCCAGGCGCCCG CCGGAGGACCCAAACTCTTGTCTC CATGAGAATCTTCTGAGTGATACA AGCAAATAAATTAAAACTTTCAAC AACGGATCTCTTGGTTCTGGCATC GATGAAGAACGCAGCGAAATGCGA TAAGTAATGTGAATTGCAGAATTC CGTGAATCATCGAATCTTTGAACG CACATTGCGCCCGCCAGTATTCTG GCGGGCATGCCTGTTCGAGCGTCA TTTCAACCCTCAAGCCCCCGGGCT TGGTGTTGGGGATCGGCCCGCCCT CCGGCGCGCCGGCCCCGAAATCTA GTGGCGGTCTCGCTGTAGCCTCCT CTGCGTAGTAGCACACCTCGCAGC GGAACGCAGCCTGGCCACGCCGTT AAACCCCCCACTTCTGAAGGTTGA CCTCGGATCAGGTAGGAATACCCG CTGAACTTAAGCATATCAATAAGC GGAGG	AGCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGA TTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAA ATTTGAAATCTGGCCCTTGTGGTCCGAGTTGTAATTTG TAGAGGATGCTTTTGGTGAGGTGCCTTCCGAGTTCCCT GGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCTGGT TGGACACCGAGCCTCTGTAAAGCTCCTTCGACGAGTCG AGTAGTTTGGGAATGCTGCTCTAAATGGGAGGTATATG TCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGC ACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAA AGAGGGTTAAATAGTACGTGAAATTGTTGAAAGGGAAG CGCTTGTGACCAGACTTGAGCCCGGTTAATCATCCAGC CTTCTGGCTGGTGCACTTGGCCGGCTCAGGCCAGCATC AGTTCGGCGCGGGGGATAAAGGCTTCGGGAATGTGGCT CCTCCGGGAGTGTTATAGCCCGTTGTGTAATACCCTGT ACTGGACTGAGGTTCGCGCATCTGCAAGGATGCTGGCG TAATGGTCATCAGCGACCCGTCTTGAAACACGGACCAA GGAGTCGTCTTCGTATGCGAGTGTTCGGGTGTCAAACC CCTACGCGTAATGAAAGTGAACGCAGGTGAGAGCTTCG GCGCATCATCGACCGATCCTGATGTTCTCGGACGGATT TGAGTAAGAGCATACGGGGCCGGACCCGAAAGAAGGTG AACTATGCCTGTATAGGGTGAAGCCAGAGGAAACTCTG GTGGAGGCTCGCAGCGGTTCTGACGTGCAAATCGATCG TCAAATATGGGCATGGGGGCGAAAGACTAATCGAACCT TCTAGTAGCTGGTTTCCGCC

Appendix 7. GenBank accession numbers of sequences used in this study to build the phylogeny trees

Phylogeny tree of strain MR153A

- ITS sequences: MH859186.1, MH859186.1, NR_163549.1, MH856013.1, JN942719.1, MH856383.1, MH856371.1, MH860946.1
- LSU sequences: NG_069811.1, MH870903.1, MH866462.1, MH867511.1, JN939298.1, MH867930.1, NG_069770.1, MH867919.1

Phylogeny tree of strain SCC45A

- ITS sequences: MH864521.1, MH863673.1, MH863670.1, MH863671.1, MH863674.1, NR_159868.1, MH859038.1, MH859041.1, MH857543.1
- LSU sequences: MH875956.1, MH875137.1, MH875134.1, MH875135.1, MH875138.1, NG_067453.1, MH870755.1, MH870758.1, MH869080.1

Phylogeny tree of strain SCC31A

- LSU sequences: MH876344.1, MH877339.1, MH878308.1, MH869757.1, NG_067348.1, NG_067344.1, MH878310.1, MH870991.1

Phylogeny tree of strain CT98B

- ITS sequences: MN608549.1, NR_132901.1, NR_171878.1, LC312507.1, LC312511.1, LC312512.1, LC312506.1
- LSU sequences: MN577418.1, NG_060309.1, NG_073805.1, LC312536.1, LC312540.1, LC312541.1, LC312535.1

Phylogeny tree of strain MR119A

- LSU sequences: LC149916.1, GU301866.1, GU479810.1, MK347995.1, MK347985.1

Phylogeny tree of strain MR33A

- ITS sequences: KM231746.1, GQ153838.1, GQ153834.1, GQ153836.1, KM231754.1, MH862153.1, KX986135.1, MH860670.1, MH860673.1, MH856885.1
- LSU sequences: KM231614.1, GQ153837.1, GQ153833.1, GQ153835.1, MH873845.1, MH873846.1, KX986142.1, MH872373.1, MH872376.1, MH868407.1

Appendix 8. Supplemented information on the structure elucidation of four compounds in this study, kindly provided by Prof. Martin Johnston

- Compounds CT98B.S.Cp:

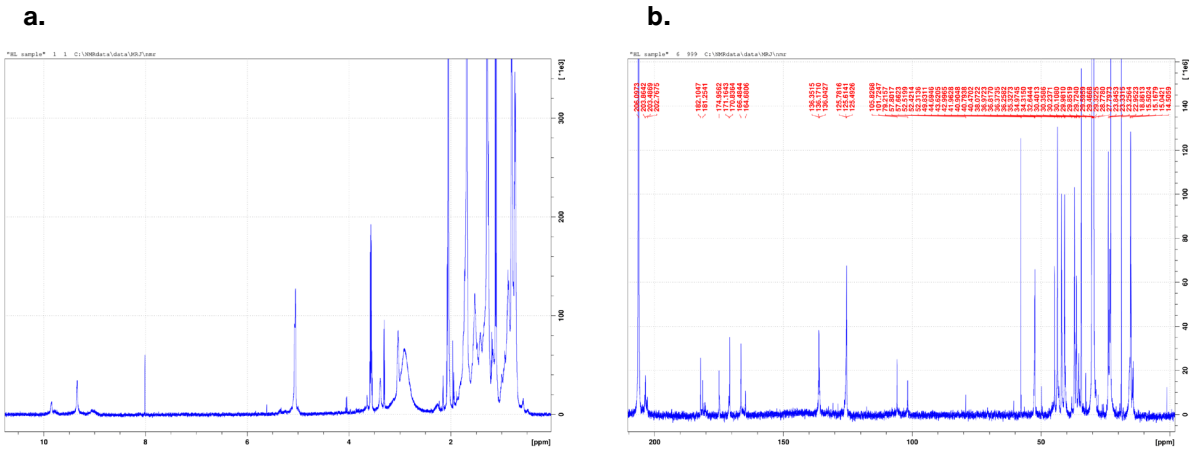

Figure A.6. *Information on the structure elucidation of CT98B.S.Cp: ^{1}H-NMR spectrum in acetone-D6 (a), ^{13}C-NMR spectrum in acetone-D6 (b) of the compound*

- Compounds MR153A.S.Cp:

^{1}H NMR (600MHz, $CDCl_3$, 25 ˚C): d 5.528 (bs, 1H), 5.25 (1H, bs), 5.175 (1H, s), 4.90 (1H, bs), 3.95 (3H, s), 1.81 (3H, s).

^{13}C NMR (150MHz, $CDCl_3$, 25 ˚C): d 179.24, 171.20, 139.57, 115.46, 102.95, 89.36, 59.90, 17.30.

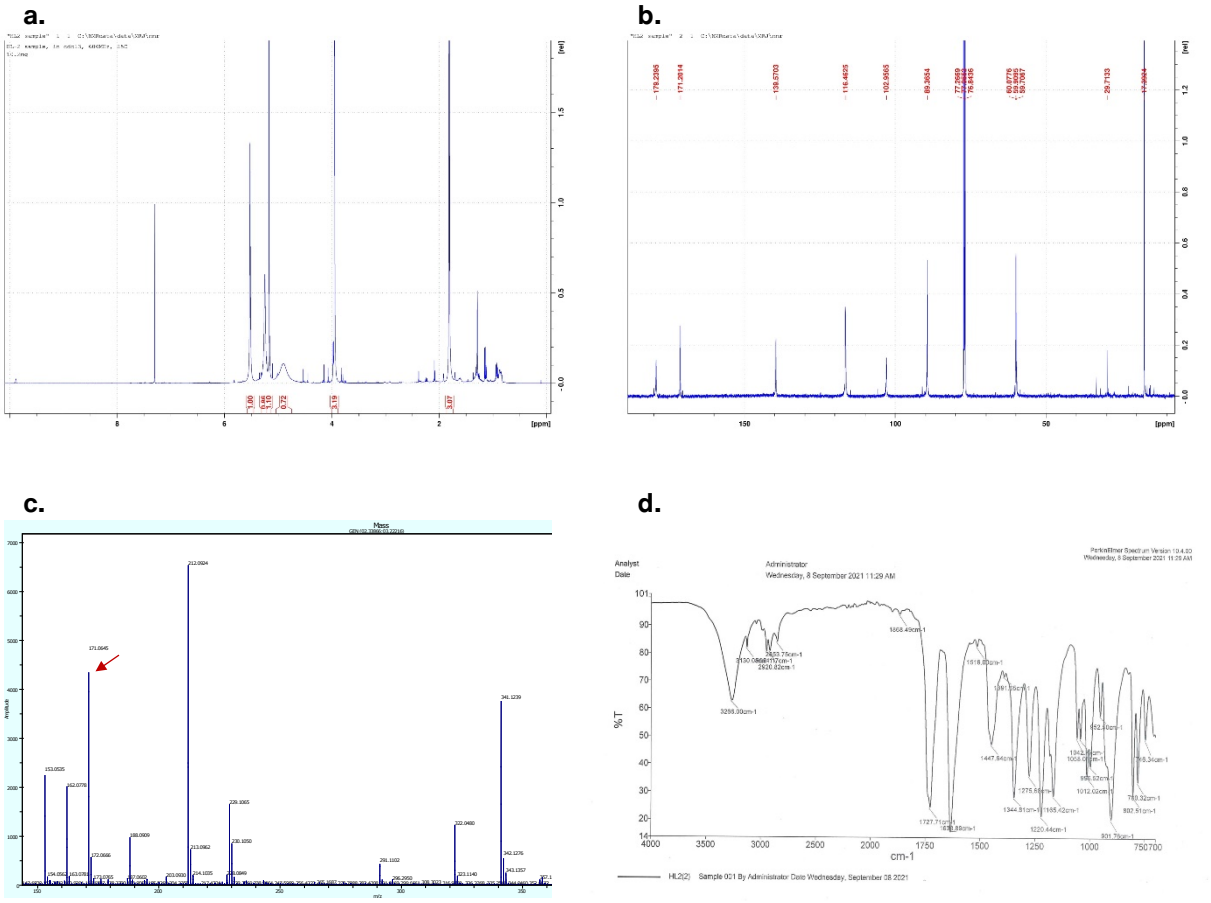

Figure A.7*. Information on the structure elucidation of MR153A.S.Cp (3-Isopropenyl-z-butenedioic acid monomethyl ester): ^{1}H-NMR spectrum in chloroform-D (a), ^{13}C-NMR spectrum in chloroform-D (b), Mass spectrum (c), and FTIR spectrum (d) of the compound.*

- Compounds MR119A.L.Cp1:

a. **b.**

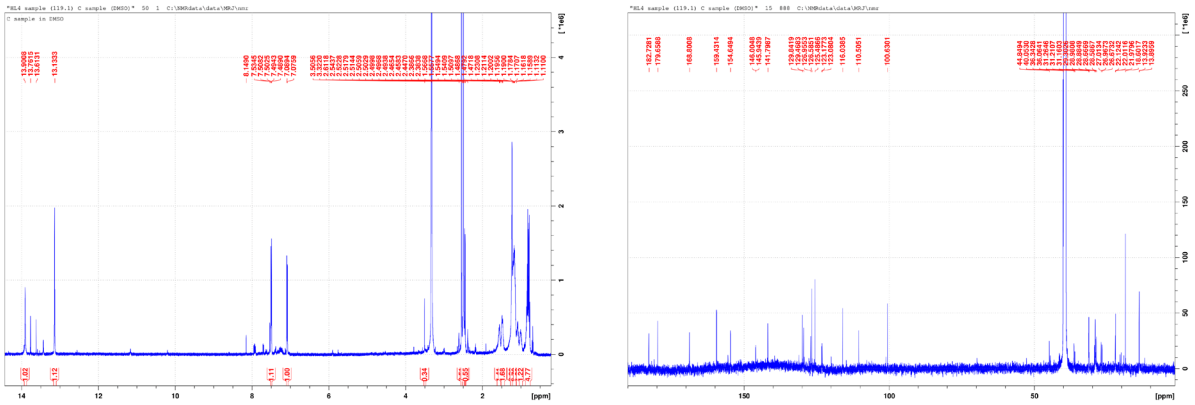

Figure A.8. *Information on the structure elucidation of MR119A.L.Cp1: ^{1}H-NMR spectrum in DMSO-D6 (a), ^{13}C-NMR spectrum in DMSO-D6 (b) of the compound*

- Compounds MR119A.L.Cp2:

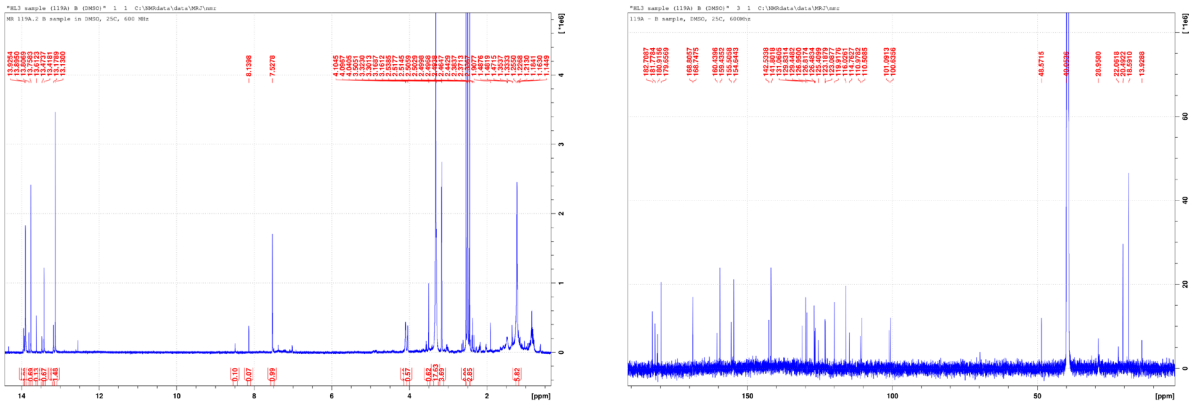

Figure A.9. *Information on the structure elucidation of MR119A.L.Cp2: ^{1}H-NMR spectrum in DMSO-D6 (a), ^{13}C-NMR spectrum in DMSO-D6 (b) of the compound*