Australian *Ganoderma*: Identification, Growth & Antibacterial Properties

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Abstract

Ganoderma species are one of the most widely researched fungi because of their reported potent bioactive properties. Although there is much information related to American, European and Asian isolates, little research has been conducted on Australian *Ganoderma* isolates. *Ganoderma* may only be imported into Australia under strict quarantine conditions, therefore, the isolation of a native strain that possesses bioactivity may be industrially and commercially significant. Three Australian species of this wood-decomposing fungus were isolated in northern Queensland. In this study, they have been identified as three separate species. Further, they have been studied to determine their optimal growth conditions in liquid culture and assessed for their antibacterial properties.

Phylogeny inferred from the Internal Transcribed Spacer Regions (ITS) from the DNA sequences resolved the three Australian *Ganoderma* species into separate clades. Two isolates were identified to be isolates of *Ganoderma cupreum* (H1) and *Ganoderma weberianum* (H2). The third isolate could only be identified to the genus level, *Ganoderma* species, due to the lack of informative data that could be used for comparison.

The effects of short term and long term storage on the viability of the fungi were investigated on agar plates, agar slants and balsa wood at varying temperatures ranging from 10 to 45°C. The most appropriate storage conditions were determined to be -80°C on balsa wood chips for periods of up to 2 years without subculture, and on agar slants at 4°C for up to a maximum of eight weeks. Light was observed to be detrimental to the survival of *Ganoderma* H1 and *Ganoderma* H2 during storage. Growth trials using potato dextrose agar plates determined the optimal temperature and pH for mycelial growth to be 30°C and a pH of 6, for all isolates. Subsequent growth trials in liquid media found that glucose, as the carbohydrate source, supported the greatest mycelial growth of *Ganoderma* H1 and *Ganoderma* H2 and that galactose and fructose supported the greatest growth of *Ganoderma* H3.

Aqueous (hot water) and organic (hexane (HEX), dichloromethane (DCM), ethyl acetate (EtOAc), methanol (MeOH)) extracts from the liquid cultivated mycelium were assessed for their antibacterial activity using disc diffusion assays. Extracts from the mycelium of *Ganoderma* H1 exhibited activity against a greater number of Gram positive bacteria than those from *Ganoderma* H2 and H3. Subsequent studies on the DCM and EtOAc extracts from *Ganoderma* H1 determined the MIC and MBC against a number of Gram positive bacteria, including *Bacillus cereus, B. subtilis, Enterococcus faecalis, Streptococcus pyogenes, Staphylococcus aureus, S. epidermidis* and *Listeria monocytogenes*, as well as *Clostridium* species, including *Clostridium perfringens, C. sporogenes* and *C. difficile*, and some methicillin resistant *Staphylococcus aureus* (MRSA) strains. Time course growth assays confirmed that the DCM and EtOAc extracts predominantly exhibited bactericidal activity. Finally, the active compounds were determined to be terpenoid in structure with some phenolic groups attached.

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Declaration

I hereby declare, that to the best of my knowledge, this thesis contains neither material which has been accepted for the award to the candidate of any other degree or diploma, or any material previously published or written by another person, except where due reference is made in the text of the thesis, and; Where the work is based on joint research or publications, the thesis discloses the relative contributions of the respective workers or authors; I also declare that this thesis has been professionally edited, however, the extent of the editing addressed only the style and grammar of the thesis, and not its substantive content.

Lyndal M. Roberts

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Annotation

Standard chemical symbols and SI units are used without definition.

°C	Degrees Celsius
Amp ^r	Ampicillin resistant
AR	Analytical reagent
AS	Anisaldehyde-sulphuric acid
ATCC	American Type Culture Collection
Basal-G	Basal glucose medium
BB	Berlin blue
BHI	Brain heart infusion
BHI-Cdiff	Brain heart infusion for <i>Clostridium difficile</i>
bp	Base pair
BSA	Bovine serum albumin
cfu	Colony forming units
CNI	Close neighbourhood interchange
dsDNA	Double strand DNA
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
DTT	DL-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtOAc	Ethyl acetate
FBS	Fast blue salt
FTG	Fluid thioglycollate medium
hr	Hour
H1	Ganoderma cupreum (isolate H1)
H2	Ganoderma weberianum (isolate H2)
H3	Ganoderma species (isolate H3)
HEX	Hexane
IGS	Intergenic spacer
IPTG	Isopropyl-β-D-thiogalactopyranoside
ITS	Internal transcribed spacer
LB	Luria-Bertani medium
LBr	Leibermann Burchard reagent
MBC	Minimum bactericidal concentration
ME	Malt extract
MEA	Malt extract agar
MEB	Malt extract broth
MEGA	Molecular Evolutionary Genetics software
MEv	Minimum Evolution
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MeOH	Methanol
MIC	Minimum inhibitory concentration

MIC ₁₀₀	100 % inhibition
MIC ₉₀	90 % inhibition
MIC ₅₀	50 % inhibition
min	Minute/s
MIRD	Monash Institute of Reproduction and Development
MP	Maximum parsimony
MRSA	Methicillin resistant Staphylococcus aureus
MTT	Thiazolyl blue tetrazolium bromide
NA	Nutrient Agar
NB	Nutrient broth
NaCl	Sodium chloride
nd	not done
NP-PEG	Natural products - polyethylene glycol
nt	nucleotide
OD ₅₉₅	Optical density at 595 nm
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
rDNA	Ribosomal DNA
RE	Restriction enzyme
$R_{\rm f}$	Retention factor
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolutions per minute
S	Second/s
SDS	Sodium dodecyl sulphate
TAE	Tris acetate buffer
TBE	Tris borate buffer
TLC	Thin layer chromatography
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultra-Violet
VIS	Visible
VRE	Vancomycin resistant enterococci
VS	Vanillin sulphuric acid
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YE	Yeast extract

Chapter One

Introduction

1.1 Natural Products Industry

The natural products and herbal medicine industry has become increasingly popular over the past three decades (Hamburger & Hostettmann, 1991; Shu, 1998). The recognition of the value of traditional medical systems, particularly of Asian origin, and the identification of indigenous medicinal plants that have shown to have healing power (Elvin-Lewis, 2001), are factors that have had significant influence in the expansion of the natural products industry. Furthermore, there is a constant search for new and effective drugs, which has been driven by the number of pathogenic organisms reported to have multi-resistance against many of the therapeutic products that are currently available on the market (Lipsitch & Samore, 2002; McGowan, 2001; Swartz, 2000; van der Waaij & Nord, 2000).

Many clinically useful drugs have been obtained through the screening of natural products (Shu, 1998). It is reported on average that two or three antibiotics derived from microorganisms are launched every year (Clark, 1996), and over 60% of antitumour and antiinfective agents that have been approved or are in late stages of clinical trials, are of natural product origin (Cragg *et al.*, 1997). Eight of the world's top selling drugs are either natural products or derived from natural products, including: simvastatin, enalapril, pravastatin, the combination of amoxicillin and calvulanate, lovastatin, cyclosporin, clarithomycin and captopril (Quinn, 1999).

Plants have been a major focus of investigations for novel biologically active compounds from natural resources and in recent years pharmaceutical companies have spent a lot of time developing these natural products to produce more affordable and cost effective remedies (Farnsworth, 1994). However, due to exploitation, some traditionally used plants are disappearing and the sustainable usage of natural resources is currently questioned by ecologists (Cragg *et al.*, 1993; Nigg & Seigler, 1992).

Filamentous fungi have been the producers of some of the most powerful secondary metabolites, and these metabolites have been researched and developed into therapeutic agents. Two such major classes of compounds from filamentous fungi that have been developed into drugs are the β -lactam antibiotics and statin drugs.

 β -lactam antibiotics have stemmed from the discovery of the first antimicrobial agent from a species of *Penicillium* by Sir Alexander Fleming in the 1920s (Fleming, 1929). This led to the research and development of the antibiotic (penicillin) by Florey and his team (Florey *et al.*, 1949) and opened the way for the development of other antibiotics and therapeutic compounds. Penicillin still remains among the most active and least toxic antibiotics (Demain & Elander, 1999).

Statins, another example of natural products successfully developed as drugs, are currently used in reducing the risks for hypercholesterolaemia and coronary heart disease (Quinn, 1999; Shu, 1998). The first statin, mevastatin (compactin), was discovered from a species of fungus *Penicillium* (Endo *et al.*, 1976a, 1976b). Since then, other statin inhibitors have been isolated from different sources, such as lovastatin from *Aspergillus terrus* (Alberts *et al.*, 1980), or by the conversion of statins into more active analogues, such as pravastatin (Haruyama *et al.*, 1986) and simvastatin (Hoffman *et al.*, 1986).

1.2 Kingdom Fungi

In 1969, fungi were classified into their own separate kingdom from plants (Whittaker, 1969). This was established on the basis that not one fungus is photosynthetic, fungi must absorb nutrients produced by other organisms and they differ from plants in their cell wall composition, in their body structure and in their modes of reproduction (Whittaker, 1969). The kingdom of Fungi was further classified into four different divisions: 1) Deuteromycota (imperfect fungi), 2) Basidiomycota (club fungi), 3) Ascomycota (sac fungi), and 4) Zygomycota. Since the discovery of Penicillin from the fungus Penicillium (Fleming, 1929), which belongs to the imperfect fungi, there has been much focus on the production of antibacterial agents from the filamentous fungi within this division (al-Hilli & Smith, 1992; Fischer et al., 2000; Florianowicz, 1998; Larena & Melgarejo, 1996; Mayordomo et al., 2000; Rodrigues et al., 2000). Although there is early reference to the antibacterial activities exhibited by fungi that belong to the division, Basidiomycota (Brian, 1951; Robbins et al., 1947; Takeuchi, 1969), it is only within the last ten years that a broader range of genera, species and isolates from within this division has been explored in more detail for antibiotic properties (Anke et al., 1980; Colleto & Mondino, 1991; Lorenzen & Anke, 1998; Rosecke & Konig, 2000;

Wasser & Weis, 1999c; Wasser, 2002). Fungi from the latter two divisions, Ascomycota and Zygomycota, are not mentioned further in this study, and from this point there is little reference to the division of Deuteromycota. The focus of this investigation was on fungi from the genus *Ganoderma*, which belongs to the division of Basidiomycota.

1.3 Basidiomycetes as Sources of Bioactive Substances

Fungi from the division Basidiomycota have been of interest recently due to the number of biological active compounds that have been isolated from them (Eo *et al.*, 1999b; Gan *et al.*, 1998a; Haak-Frendscho *et al.*, 1993; Hatvani, 2001). Fungal fruiting bodies, fungal mycelium or the culture fluid in which the mycelium has been cultivated may all be explored for biological activity. Some advantages of using filamentous fungi over plants as sources of bioactive compounds are that often the fruiting body can be produced in much less time, the mycelium may also be rapidly produced in liquid culture and the culture medium can be manipulated to produce optimal quantities of active products.

When considering natural substances for investigation of medicinal properties, Baker *et al.* (1995) suggested that one should consider the evidence suggesting the traditional usage of the substance by indigenous populations, the abundance of the species in nature and the sustainable utilisation of the species. These criteria were suggested for the discovery of natural products from the Plant Kingdom. However, fungi (a separate Kingdom from plants as mentioned earlier), also fulfil these criteria and most importantly, the sustainability of fungi can be achieved by using artificial cultivation techniques. Another extremely important criterion when searching for novel bioactive compounds is the uniqueness of the organism and its potential to produce secondary metabolites (Donadio *et al.*, 2002b). Many cellular biological responses of natural products are generally associated with inherent properties of secondary metabolites (Shu, 1998) and therefore, represent a large source of compounds that may have potent biological activities.

One of the major rationales for the for antimicrobial compounds from basidiomycetes is that humans (and animals) share common microbial pathogens with fungi, such as *Escherichia coli* and *Staphylococcus aureus*, and therefore we can benefit from defence strategies used by fungi against microorganisms (Zjawiony, 2004).

1.3.1 Medicinal Basidiomycetous Fungi

Basidiomycetous fungi (mushrooms) can be defined as "macrofungi" with distinctive fruiting bodies that are large enough to be seen by the naked eye and to be picked by hand (Chang & Miles, 1992). It is estimated that there are approximately 1.5 million species of mushrooms in the world of which approximately 70,000 species are described (Hawksworth, 1991). About 10,000 of the known species belong to the macro fungi (Kendrick, 1985), of which about 5,000 species are edible and over 1,800 species are considered to have medicinal properties (Chang, 1995). The macro fungi can be divided into four groups: (1) edible flesh, e.g. Agaricus; (2) medicinal, e.g. Ganoderma; (3) poisonous, e.g. Amanita; and (4) miscellaneous, where the properties are less well defined (Chang, 1995). Of edible mushroom species, Agaricus represents the leader in world production and in non-edible medicinal species, Ganoderma, which belongs to the polypores (mushrooms that contain pores that hold reproductive spores, rather than gills) is the leader in terms of production (Chang, 1995). In recent years, more varieties of mushrooms have been isolated and identified, and the number of mushrooms being cultivated for food or medicinal purposes has been increasing rapidly (Chang, 1995). Several mushroom species belonging to the Polyporaceae family are now being regarded as the next candidate producers of valuable medicines (Mizuno et al., 1995a).

Mushroom "nutriceuticals" are bioactive compounds that are extractable from mushrooms, and they have nutritional and medicinal features that may be used in the prevention and treatment of diseases (Chang & Buswell, 1996). Several nutriceutical products have been isolated from medicinal mushrooms and three of these, which are carcinostatic polysaccharide drugs, have been developed from mushrooms in Japan. These are "Krestin" (PSK), from the cultured mycelium of Kawaratake (*Trametes versicolor*), "Lentinan" from the fruiting bodies of Shiitake (*Lentinus edodes*) and "Schizophyllan" (Sonifilan) from the culture fluid of Suehirotake (*Schizophyllum commune*) (Mizuno, 1993). Lentinan and schizophyllan are pure β -glucans whereas PSK is a protein bound polysaccharide (Ooi, 2000). These type compounds are

discussed in more detail in Chapter 2. The biological activity of these three products is related to their immunomodulating properties, which enhance the host's defence against various forms of infectious disease. These immunopotentiators, or immunoinitiators, are also referred to as "biological response modifiers" (BRM).

There have been a number of reviews published on the bioactive substances found in mushrooms, and their medicinal properties (Borchers *et al.*, 1999; Jong *et al.*, 1991; Lorenzen & Anke, 1998; McAfee & Taylor, 1999; Mizuno, 1995; Mizuno *et al.*, 1995c; Ooi & Liu, 1999; Wasser & Weis, 1999b, 1999c). With particular focus on *Ganoderma* species, it is apparent that most of the available data on active extracts and compounds relates to the pharmacological effects on tumour cells, which appear to be based on the enhancement of the host's immune system. A further observation is that there seems to be much less research being performed on the antibacterial action of *Ganoderma* extracts (Mothana *et al.*, 2000; Smania *et al.*, 1999).

1.3.2 Australian Ganoderma

The majority of medicinal investigations on *Ganoderma* species have been performed on species that have been isolated from other parts of the world and there have been few reports on Australian *Ganoderma*. Australian Quarantine Inspection Service (AQIS) have prohibited the importation of the fungus into the country because some species are known to be pathogenic to native trees by causing basal-stem rot (Miller *et al.*, 1999). However, there are numerous *Ganoderma* species that are native to Australia and if these species are examined, quarantine laws are not an issue. Since species within the Ganodermataceae family all evolved from one species, it is then most likely that Australian *Ganoderma* contain similar compounds to *Ganoderma* that have been isolated elsewhere.

Fungi within the same family can produce a different variety of secondary metabolites as a result of stress (Pointing & Hyde, 2001) or from exposure to different environmental conditions (Kim *et al.*, 2002b, 2003). Therefore, the different environmental conditions that Australia has to offer, as well as the different native trees on which the fungi grow, might potentially produce secondary metabolites with interesting biological activities. In addition, there have been reports of different *Ganoderma* species possessing different biologically active compounds (Gao *et al.*, 2000a; Mothana *et al.*, 2003; Smania *et al.*, 2003). Since the majority of medicinal research within the Ganodermataceae has been performed on *G. lucidum*, and it has been reported that this species does not exist in Australia (Moncalvo *et al.*, 1995a), then the investigation of local species may yield compounds with novel medicinal qualities.

An important aspect of the search for natural products with medicinal properties is that the correct identification of the source species is carried out. With the introduction of molecular tools, it has become apparent that the classification of Ganodermataceae is in a state of taxonomic chaos (Ryvarden, 1991; Smith & Sivasithamparam, 2003). This is partially because there has never been a standard nomenclatural system established when using the traditional morphological studies to identify them. As a result, past investigators have unknowingly reported medicinal properties from wrongly identified isolates. In addition, reports that G. lucidum is not conspecific to the Australasian pacific region (Moncalvo et al., 1995a, 1995b) and the number of investigations that have reported medicinal activities from Asian G. lucidum isolates, confuses the matter further. Thus, the correct identification of new species or isolates will help in clarifying some of the taxonomic confusion that exists for Ganoderma. Smith & Sivasithamparam (2000a, 2000b) have addressed the identification of a number of Australian Ganoderma species using molecular techniques. However, there is limited sequence data available for Australian species such that additional data would assist in the identification of new species, as well as confirming the classification of existing ones.

1.4 Aims of This Investigation

There were four main aims of this investigation. The first was to classify three Australian *Ganoderma* isolates that had been isolated from the Cairns State Forest (Chapter 4) using modern molecular techniques rather than traditional morphological taxonomic techniques. Although a number of *Ganoderma* sequences were available from the GenBank database, the data generated in this study would increase the number of available sequences from Australian *Ganoderma* species.

The second aim was to determine suitable storage and growth conditions of the three isolates (Chapter 5). The importance of suitable short term and long term storage of the

fungi was to ensure the fungi remained viable throughout this investigation, as well as for future reference and use. It was also desirable to obtain maximum growth of the fungal mycelium in a liquid cultivation medium so that sufficient material was available to pursue further studies on its biological activity. Therefore, optimal growth of the mycelium was investigated by variation of the environmental parameters, pH and temperature, as well as the nutrient medium and sugar source.

The third aim of this investigation was to investigate the hot water and organic extracts from the three Australian isolates for their antibacterial activity (Chapter 6). Preliminary investigations were performed to assess the three isolates for their antibacterial activity, and then additional studies of the most active isolate could be performed. The chosen isolate was investigated further for its antibacterial effect on a number of bacteria, including some clinically important *Clostridium* strains.

The fourth and final aim of this investigation was to identify, to a certain class of compound, the components within the mycelial extracts that exhibited antibacterial activity (Chapter 7). Partial purification using a bioassay guided fractionation process was performed so that the active components could then be assessed for the class of compound to which they belonged. The identification, as well as the process which identified suitable solvents systems, provided a framework for further studies on the exact identification of the active compounds.

The following review (Chapter 2) focuses on medicinal compounds that have been isolated from mushrooms, and in turn, the therapeutic effects that many of these compounds have shown to possess. Since *Ganoderma* are the fungi under investigation in this study, there is a strong emphasis on the research of fungi from within the family, Ganodermataceae. However, fungi from other families are included for comparison where required, as well as the occasional comparison to biologically active compounds extracted from plants. The classification of *Ganoderma* species is also reviewed, as well as current cultivation techniques using both solid and liquid growth media, with particular focus on the latter.

Chapter Two

Literature Review

2.1 Ganodermataceae

2.1.1 Classification of *Ganoderma*

Ganoderma species belong to the kingdom of Fungi, the division of Basidiomycota, the class of Homobasidiomycetes, the order of Aphyllophorales, the family of Polyporaceae (Ganodermataceae) and the genus of *Ganoderma* (Chang, 1995; Wasser & Weis, 1999a). Fungi from the family of Polyporaceae are classified as such as they have many tiny holes on the underside of the fruiting body, which are pores that contain the reproductive spores. They have a woody or leathery feel and the presence of these pores are obvious characteristics that distinguish polypores from other common types of mushrooms. Polypores, like other fungi, grow on wood as an expansive network of microscopic tubes known as mycelium. They degrade the wood over time and produce a fruiting body (or conk) on the surface of the wood. *Ganoderma* species are among those fungi that can thrive under hot and humid conditions and are usually found in subtropical and tropical regions (Moncalvo & Ryvarden, 1998).

Ganoderma species are not classified as edible mushrooms, as the fruiting bodies are always thick, corky and tough and do not have the fleshy texture characteristic of true edible mushrooms such as the common white button mushroom, *Agaricus bisporus*. Although they are not classified as edible, several types of *Ganoderma* products are available on the market including; ground fruiting bodies or mycelium processed into capsule or tablet form; extracts from the fruiting body or mycelium dried and processed into capsule or tablet form or tea, *Ganoderma* beer and *Ganoderma* hair tonics (Jong & Birmingham, 1992).

Within the genus *Ganoderma*, over 250 taxonomic names have been reported worldwide (Moncalvo *et al.*, 1994; Ryvarden, 1994) including: *G. adspersum*, *G. applanatum*, *G. australe*, *G. boninense*, *G. cupreum*, *G. incrassatum*, *G. lipsiense*, *G. lobatum*, *G. lucidum*, *G. oerstedii*, *G. oregonense*, *G. pfeifferi G. platense*, *G. resinaceum*, *G. sessile*, *G. sinense*, *G. tornatum*, *G. tsugae* and *G. weberianum*, to name a few. However, the majority of reports in the literature appear to be on the one species, *G. lucidum*.

2.1.2 History of *Ganoderma*

Ganoderma lucidum has been treasured in China and Japan for many thousands of years (Willard, 1990). In Chinese, the mushroom is called "Ling Zhi", in Japanese "Reishi, Mannentake or Sachitake", and "Youngzhi" in Korean. Chinese tradition proclaims that *Ganoderma* is also called "miraculous zhi", or "auspicious herb" and is usually considered to "symbolise happy augury, and to bespeak good fortune, good health and longevity, even immortality" (Wasson, 1968).

As early as 800 years ago in the Yuan Dynasty (A.D., 1280 – 1368) *G. lucidum* has been represented in paintings, carvings, furniture, carpet design, jewellery, perfume bottles and many more creative artworks (Wasser & Weis, 1999a). According to the two famous Chinese herb medical books, *Shen Nong Ben Cao Jing* (25-220 A.D., Eastern Han Dynasty) and *Ben Cao Gang Mu* (1590 A.D., Ming Dynasty), there were six know species of *Ganoderma* (Ling Zhi) in China at that time, whereas now more than 250 species have been described (Moncalvo *et al.*, 1994; Ryvarden, 1994).

2.1.3 Medicinal Ganoderma

G. lucidum (Ling Zhi) was the most sought after species within the Ganodermataceae family, as it was believed to be the only mushroom to contain therapeutic properties (Willard, 1990). In the literature today there is much confusion as to which is the true *Ganoderma* species. The Japanese believed that the true *Ganoderma* was red and that a *Ganoderma* species with a different colour was a red *Ganoderma* that had become discoloured due changes in environmental conditions such as temperature, humidity and light (Mayzumi *et al.*, 1997). In China, they believed that the true *Ganoderma* was black as there were reports of a black *Ganoderma* that had unusual medicinal benefits not produced by the red mushroom (Mayzumi *et al.*, 1997).

Chang (1995) suggested that *Ganoderma* (Ling Zhi) encompassed several *Ganoderma* species, although most investigations and therapeutic practices refer to the species, *G. lucidum*. More recently, other species, e.g.: *G. tsugae, G. boninense, G. capense, G. sinense, G. japonicum, G. applanatum, G. tropicum, G. tenue* and *G. luteum*, have become increasingly popular for the investigation of medicinal properties. A number of reviews have described the bioactive substances, medicinal effects and health benefits

of *Ganoderma* species (Chang, 1995; Chang & Buswell, 1999; Chen & Miles, 1996a; Jong & Birmingham, 1992; Mizuno *et al.*, 1995b). It is also noted that the majority of the studies concerning the Ganodermataceae family relate to the antitumour and antiviral effects, while the antioxidant properties associated with this fungus have only recently become apparent (Mau *et al.*, 2002; Yen & Wu, 1999; Zhu *et al.*, 1999). There appears to be limited information available that reports the antimicrobial properties of *Ganoderma* species.

2.2 Systematics of *Ganoderma*

The genus *Ganoderma* was established by Karsten in 1881. Correct citation of the type species is written as *G. lucidum* (Curt.: Fr.) P. Karst. This genus was later divided into two distinct groups, the laccate (cutex layer on the outer surface of the fruiting body that rendered it waxy/shiny) (*G. lucidum* complex) and the non-laccate (*G. applanatum* complex) species, which refer to the subgenera *Ganoderma* and *Elfvingia* respectively. Since then, over 290 taxonomic names in the genus of *Ganoderma* (Ryvarden, 2000) have been published, indicating that this genus is morphologically complex.

The taxonomy of *Ganoderma* species is not clear and it has been noted that the genus is in a state of taxonomic crisis (Ryvarden, 1991). Traditional identification of *Ganoderma* species has been based on morphological features, physiological and developmental characters, and chemical components such as secondary metabolites (Takamatsu, 1998). As a result, the concept of species in this genus is not well established nor universally accepted (Gottlieb *et al.*, 2000).

Over the last few decades, it has been shown that the morphology and culture characteristics of species from the same genus can be greatly affected by growth conditions. This signifies that a large number of synonyms may exist due to the number of species that have been identified based on morphology (Moncalvo, 2000). The shape of the basidiocarp (fruiting body) has been demonstrated to be greatly influenced by the environment (Chen, 1993), the basidiospores by latitude and altitude (Steyaert, 1975) and in some species, the context colour was darker in collections from more southern latitudes on the European continent (Steyaert, 1972). More recently, the age and environment have been shown to have a marked effect on the colour, size and

brightness of the fruiting body, and the presence, absence or length of the stipe (stem) (Moncalvo, 2000). Identification of *Ganoderma* based on these characteristics have contributed greatly to the confusion of the naming of species within this genus, and have resulted in traditional taxonomic methods being inconclusive for establishing a stable classification system for *Ganoderma* species (Hong *et al.*, 2002; Hseu *et al.*, 1996).

Traditional identification parameters are rapidly becoming outdated and new identification methods are being investigated. Alternative approaches that have been used to identify *Ganoderma* species include restriction fragment length polymorphism (RFLP) (Miller *et al.*, 1999), sequence analysis (Hong *et al.*, 2002; Moncalvo *et al.*, 1995a, 1995c; Smith & Sivasithamparam, 2000a) and isoenzyme electrophoresis (Gottlieb *et al.*, 1998; Gottlieb & Wright, 1999b; Smith & Sivasithamparam, 2000b). It is the phylogenetic analysis of amino acid or DNA sequences that is known to have the highest resolving power (Bruns *et al.*, 1991). These modern techniques have helped to clarify the distribution of the different species complexes in the genus *Ganoderma* and in some cases, it has become apparent that species have been misidentified (Gottlieb *et al.*, 1998; Moncalvo *et al.*, 1995c). Taxonomic confusion between *G. lucidum* and *G. tsugae* appears to have resulted in one of the greatest misidentification of species within the genus *Ganoderma* (Hseu & Wang, 1991).

Classification of *Ganoderma* using molecular methods has shown that *G. lucidum* is most likely confined to Europe and does not actually exist in the Australasia Pacific region (Moncalvo *et al.*, 1995a, 1995c). These findings appear to contradict much of the published work in Asia on medicinal *G. lucidum*. In addition, this suggests that documentation of *G. lucidum* in some of the early Chinese and Japanese books, such as *Shen Nong Ben Cao Jing* (25-220 A.D., Eastern Han Dynasty) and *Ben Cao Gang Mu* (1590 A.D., Ming Dynasty), has been cited incorrectly. It appears that this confusion exists because the first botanical specimen was described from Europe in the late eighteenth century (Karsten, 1881) and not from an Asian specimen. This type *G. lucidum* species has been the basis for identification of new species and with the introduction of molecular taxonomy, is observed to be different at the molecular level to

those species reported in Asian countries. This appears to be one of the major reasons why the taxonomy of this species is in a state of crisis.

2.2.1 Molecular Systematics of *Ganoderma*

Nuclear-encoded ribosomal RNA genes (rDNA) have been the primary focus of investigation for new taxonomic approaches in fungal molecular systematics. These genes (rDNA) are arranged in tandemly repeated units (Figure 2.1) with each unit containing the genes for the small subunit (18S), 5.8S, and large subunit (25-28S). Each unit is separated by one or more intergenic spacer (IGS) regions and these IGS regions may contain a separately transcribed coding region for 5S RNA.



Figure 2.1. A ribosomal DNA repeat unit showing the internal transcribed spacer regions and intergenic spacer regions. The diagram shows the location of the ribosomal subunits within the repeat unit. The small subunit (18S RNA) to the large subunit (25-28S RNA) consists of one major rRNA transcript (Bruns *et al.*, 1991).

The coding regions of the 18S, 5.8S and 28S nuclear rDNA genes are highly conserved among fungi and they show little sequence divergence between closely related species and are useful for phylogenetic studies among distantly related organisms (Berbee & Taylor, 1992; Binder & Hibbett, 2001; Swann & Taylor, 1993, 1995). Within each repeat unit, the conserved regions are separated by two internal transcribed spacers, ITS I and ITS II, which show higher rates of divergence (Moncalvo *et al.*, 1995c; Perlin & Park, 2001). It is these ITS regions that are now the most widely sequenced DNA regions in fungi. Variable sequence regions in both the small (18S) and large (25S)

subunits of rDNA genes have also led to numerous molecular approaches that provide rapid identification of fungal species (Perlin & Park, 2001).

Molecular taxonomy of the Ganodermataceae family was first performed by Moncalvo *et al.* (1995a, 1995c). They believed that most of the controversy which has been associated with *Ganoderma* systematics in the past might be resolved with the use of molecular techniques to generate novel taxonomic characters and with the use of phylogenetically based classification methods.

2.2.1.1 Internal Transcribed Spacer (ITS) Regions

The ITS regions are probably the most important regions in fungi for molecular systematics within a genus. The highly conserved ribosomal genes, which flank the ITS regions, are ideal for universal primer targeting and as a result the ITS regions can be amplified by polymerase chain reaction (PCR), the sequences analysed and compared, and evolutionary trees produced. The ITS regions in fungi are highly variable and for this reason are useful in distinguishing between *Ganoderma* species (Moncalvo *et al.*, 1995a, 1995c). In contrast, the nucleotide sequence data from nuclear and mitochondrial rDNA coding regions do not offer enough variation to infer phylogenetic relationships between *Ganoderma* species (Moncalvo, 2000) and therefore are only useful at the genus level. There have been many reports published on the analysis of the ITS regions to establish taxonomic relationships within the *Ganoderma* species (Gottlieb *et al.*, 2000; Moncalvo *et al.*, 1995a, 1995c; Smith & Sivasithamparam, 2000a).

There are specific segments in the ITS regions which have greater variability than other segments. Moncalvo *et al.* (1995a) observed that the frequency of nucleotide substitutions was similar in both ITS regions but found that variations were mostly located in the central region of ITS I and close to the termini in ITS II. They also reported that nucleotide divergence between recently diverged taxa was usually in the ITS II region. This was also observed by Gottlieb *et al.* (2000), who reported that a lower level of resolution of internal phylogenetic branches was obtained from the ITS I data set.

2.2.1.2 Endonuclease Restriction Digestions

Two important techniques that involve digestion of DNA with restriction endonucleases are restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD). While restriction enzyme treatment of the PCR amplified ITS regions can discriminate between most *Ganoderma* species (Park *et al.*, 1996), RAPDs can be used to differentiate between isolates having identical ITS sequences (Hseu *et al.*, 1996).

PCR coupled with RFLP has been a useful tool in phylogenetic studies and is now widely used for fungal phylogeny and taxonomy (Hughes *et al.*, 1998; Magee *et al.*, 1987; Miller *et al.*, 1999). PCR-RFLP was one of the first molecular approaches to approximate the differences in species. This technique, which cleaves chromosomal DNA using restriction endonucleases followed by separation of the fragments by gel electrophoresis, provides a measure of the genetic difference or relatedness between organisms (Perlin & Park, 2001).

RAPD is also another useful tool in molecular biology. Park *et al.* (1996) analysed twenty eight isolates of *Ganoderma* by PCR-RFLP and RAPD and found that the resulting phenograms of genetic relatedness showed similar patterns by the two different methods, although slightly different bands were observed within the *G. lucidum* group by RAPD. Hseu *et al.* (1996) further attempted to differentiate isolates of the *G. lucidum* complex using RAPD analysis and found that groupings based on this technique did not distinguish the same clades as ITS data and they suggested that RAPD might be helpful for systematics at the lower taxonomic level that are unresolved by ITS sequence data.

Single stranded conformational polymorphism (SSCP) is another technique that has been used for taxonomic analysis of *Ganoderma* species. It is advantageous over restriction analysis because it is less time consuming and more economical (Gottlieb *et al.*, 2000). In spite of being useful in determining variability, taxonomic names determined using SSCP does not correspond with the current taxonomic status of some taxa, particularly within the subgenus *Elfvingia*. These groupings were found to be almost the same as those obtained with isoenzyme data (Gottlieb *et al.*, 1998).

2.2.1.3 Isoenzymes

Isoenzymes have been investigated as a means of taxonomic identification of *Ganoderma* species, although this method is not as popular as DNA based techniques because of the need to use comparatively large amounts of fresh material (Smith & Sivasithamparam, 2000b).

Gottlieb *et al.* (1999a, 1999b) used isoenzymic data to distinguish between groups of *Ganoderma* from the subgenus *Ganoderma* and *Elfvingia*, from South America. They observed that many isolates belonging to the same taxa failed to cluster together in the gene trees and in general, correlation between morphological features and isoenzymic patterns could not be established, and therefore the groupings determined by both sets was different (Gottlieb & Wright, 1999a). They concluded that some *Ganoderma* species were misidentified (Gottlieb *et al.*, 1998). Interestingly, groupings obtained by phylogenetic analyses of the ITS regions by Gottlieb *et al.*, 1998).

Smith & Sivasithamparam (2000b) also used phenetic analysis of isoenzymes to examine population and species relationships of *Ganoderma* species in Australia. Their results confirmed the same taxonomic conclusion derived upon analysis of the ITS region (Smith & Sivasithamparam, 2000a), although, two species could not be distinguished from each other highlighting the limitations of using limited numbers of enzyme systems to distinguish taxa (Smith & Sivasithamparam, 2000b).

2.2.2 Phylogenetic Analysis

Taxonomy aims to reflect a natural classification of taxa, and molecular data offer a set of objective characters on which to base taxonomic decisions (Buchanan, 2001). The use of phylogenetic programs to analyse such molecular data has rapidly become popular and it is the resulting phylograms (genetic evolutionary trees) that display monophyletic groups, the members of which share a common ancestor (Buchanan, 2001). The practice of phylogenetic analysis should be conceived as a search for a correct model, as much as a search for the correct tree (Brinkman & Leipe, 2001). The method of phylogenetic inference currently can be classified into three major groups: distance methods, maximum likelihood methods and parsimony methods (Nei, 1996). In distance methods, an evolutionary distance is computed from all pairs of sequences, and a phylogenetic tree (phylogram) is constructed from the pairwise distances (Nei, 1996). In maximum likelihood methods, maximisation of the likelihood is performed for each topology separately and the topology with the highest likelihood is chosen as an estimate of the true tree topology (Nei, 1996). In maximum parsimony methods, a given set of nucleotide sequences are considered, and the nucleotides of ancestral sequences for a hypothetical topology are inferred under the assumption that mutational changes occur in all directions among the four different nucleotides. The smallest number of nucleotide substitutions that explain the entire evolutionary process for the given topology is then computed. The computation is done for all topologies, and the topology that requires the smallest number of substitutions is chosen to be the best tree (Nei, 1996).

There are reviews available on the statistical methods developed for different models and how these models may affect data sets (Brinkman & Leipe, 2001; Nei, 1996). Although numerous phylogenetic algorithms, procedures and computer programs have been devised, their reliability and practicality are, in all cases, dependent on the structure and size of the data (Brinkman & Leipe, 2001). The field of phylogenetic analysis is controversial. Some of the debates have been summarised in reviews (Saitou, 1996; Swafford *et al.*, 1996). Of particular importance is the issue of phylogenetic analysis of large molecular data sets (Graybeal, 1998; Hillis, 1996; Poe, 1998), and the suggestions that statistical reliability is sensitive to sample size (Bremer, 1994; Farris *et al.*, 1996; Felsenstein, 1985; Sanderson & Donoghue, 1989). In contrast, evidence from various studies suggests that increasing sample size generally increases phylogenetic accuracy (Hillis, 1996; Moncalvo *et al.*, 2000).

A matter of concern in the classification of *Ganoderma* is that, frequently, evolution at the morphological and molecular levels is uncoupled and as a consequence studies reveal discordant patterns between them (Gottlieb & Wright, 1999b). In addition, the genus is young in terms of evolution and the speciation process is complex (Moncalvo *et al.*, 1995c; Ryvarden, 1991) and some recently diverged monophyletic groups may
not align with one or more morphologically distinguished taxa and thus, may challenge existing classifications based on features such as fruiting body size and form, cutis microstructure, spore dimensions, host and geographical distribution (Buchanan, 2001).

Knowledge of the physiology and extent of DNA variation of *Ganoderma* species is still only partial (Gottlieb *et al.*, 2000) and the current sequence database for *Ganoderma* species includes about 300 taxa (Moncalvo, 2000). It is clear that a greater sequence database is required to support preliminary research findings and validate phylogenetic hypotheses.

2.3 Preservation and Maintenance of Fungal Cultures

In any study of fungi, it is important to preserve and maintain the fungus throughout the period of investigation. In addition, it is important to retain some form of the reference material as a permanent record and to have stable starter cultures to obtain repeatable results. Cells in fungi can degenerate due to lack of nutrients or oxygen, infections (viruses), a change in substrate pH or the accumulation of unfavourable metabolites (Oei, 1996). If a strain is cultivated for some time it may eventually lose some of the genetic characteristics that it possessed originally (Oei, 1996). Often, the optimal storage conditions are similar for fungi within the same genus. However, there have been cases reported where storage conditions are unique for different fungal strains within the same species (Mayzumi *et al.*, 1997).

There are several techniques used for long term preservation of fungi, each with specific advantages and disadvantages. Such techniques include agar to agar transfer, mineral oil storage on an agar slant, deep freeze, lyophilisation and cryogenic freezing. The agar to agar method is used for 'short term' storage and in laboratories that have simple facilities. It is a relatively cheap method of storage, with little time required for the production of new mycelial growth. Most fungal cultures can be maintained on agar by subculturing at two to six month intervals (Ando, 1997), although, some fungi have the requirements to be sub-cultured as frequently as every two to four weeks (Onions, 1983). A disadvantage of this technique is that it can be unreliable, as storage of the mycelium under refrigeration may result in degeneration of the mycelium, since the fungus can still be growing under these low temperature conditions (Oei, 1996). The

subculturing process of transferring from agar to agar is also thought to be unsatisfactory in terms of 'long term' preservation due to possible contamination, or the possibility of alteration of the fungal characteristics due to, for example, spore production, productivity, pathogenicity and metabolite production (Ando, 1997).

Cultures grown on agar slants and stored under mineral oil, or medicinal paraffin oil, can be stored for periods ranging from one and twenty five years (Onions, 1983). An advantage of this method is that the cultures can be kept at either room temperature or under refrigeration at 4°C (Oei, 1996). A disadvantage of this method is that the depth of the oil layer applied is critical. Too shallow a layer may leave hyphae exposed and these can act as wicks to dry the culture. Too deep a layer restricts the diffusion of oxygen and carbon dioxide (Onions, 1983). In addition, the revival of cultures after extended storage can be a slow process.

Lyophilization and liquid drying methods are difficult to use for the preservation of fungal mycelium (Ando, 1997) and are predominantly used for the preservation of spores that can remain viable for up to 20 years (Oei, 1996). The preferred methods for the preservation of fungal mycelia are to freeze and store the mycelium at liquid nitrogen temperature or, to freeze the mycelium at -80°C in a deep freezer. The most suitable technique is cryogenic freezing because growth is retarded completely, resulting in a stable mother culture (Croan *et al.*, 1999; Oei, 1996). Although cryogenic freezing is thought to be the most effective method for preservation, it has been shown that a successful cryogenic preservation may be difficult due to the large taxonomic variety of fungi and the variability of each new strain should be thoroughly investigated first. Since cryogenic freezing is expensive and requires constant surveillance (Ando, 1997), freezing at low temperatures is the preferred method for preservation in a small scale laboratory.

2.4 Cultivation of *Ganoderma* Species

2.4.1 Cultivation of *Ganoderma* to Produce Fruiting Bodies

Artificial cultivation of *Ganoderma* fruiting bodies on solid substrates was successfully achieved in mass production in the 1970's by Y. Naoi (Mizuno, 1997) and the process

has been developing rapidly ever since. Solid artificial cultivation is widely adopted by many growers for the commercial production of mushrooms, which is commonly carried out on wood logs and tree stumps (Mayzumi *et al.*, 1997; Naoi, 1997) and in sawdust bags and bottles (Chang & Miles, 1989a; Quimio, 1986; Tong & Chen, 1990). An advantage of using the latter two techniques is that the production time is shortened and higher mushroom yields are achieved (Chang & Miles, 1989a). In addition to this, the substrate may be modified. Triratana *et al.* (1991) investigated the growth using agricultural waste products, such as sugar beet and corncobs, as alternate substrates. *Ganoderma* has also been cultivated for its aesthetic beauty to produce *Ganoderma* bonsai (Chen & Miles, 1996b).

Methods of artificial cultivation typically involve five stages: (1) Spawn run, (2) Primordia ("Antler") formation, (3) Primordia ("Young conk") formation, (4) Fruiting body development, and (5) Cropping Cycle (Chen, 1999; Stamets, 1993). Briefly, spawn is distributed onto a sterile solid substrate, which frequently consists of a mixture of hardwood sawdust and wood chip, and is incubated until colonisation of the substrate is achieved. After this, incubation at decreased temperatures and high carbon dioxide levels is carried out to develop the stalk or antler. Once the desired stalk length is achieved, the conditions are again altered to aid in the fruiting body development. The entire growth cycle from spawn run to cropping in artificial cultivation takes on average approximately 90 to 120 days (Stamets, 1993), but this also depends highly on the method of cultivation used.

2.4.2 Cultivation on a Solid Agar Medium

To examine the preliminary growth patterns of fungi, many researchers have investigated the growth of fungi on various agar media. Bilay *et al.* (2000) investigated the growth rate of thirty mushroom cultures on a variety of media preparations and their pH-modified varieties. They found that the commercial malt extract agar (MEA) and potato dextrose agar (PDA) provided the maximum growth rate for *G. lucidum* and *Agaricus maskae* respectively, whilst an experimental medium, that contained glucose, potassium phosphate and corn steep liquor, provided maximum growth for fifteen cultures. This highlighted the fact that different fungal species have different nutrient requirements.

The diameter of the colony as a reliable measure of growth has been a topic of controversy amongst investigators (Brancato & Golding, 1953; Worley, 1939). It has been noted that under uniform environmental conditions, there is a high degree of radial symmetry in fungal colonies, with all portions of the mycelial front extending at the same rate (Edelstein & Segel, 1983) with no acceleration of growth over time (Brancato & Golding, 1953). Hence, it can be assumed that the growth rate of fungi is constant (Trinci, 1971). As a result, the rate and extent of growth (radii or diameter) has been a useful criterion for comparison of fungi (Lonergan *et al.*, 1993). The ratio of the area of growth over time has also been used as a measure of fungal growth. Worley (1939) suggested a third measurement, the sector area method, which takes into consideration: (1) growth in both radial direction and tangential direction, (2) the number of units contributing to growth, (3) the relationship between initial and total growth, and (4) the relative importance of radial and tangential growth quantities depending on previous conditions.

2.4.3 Cultivation of *Ganoderma* in Liquid Medium

Propagation of mushroom mycelium in submerged culture was initially developed during the 1950's. This method was very successful in growing lower fungi (fungi that do not form basidiocarps) in fermenters for economical production of various natural products (Yang & Liau, 1998). A review by Fang & Zhong (2002c) indicated that there were few investigations into the development of higher fungi (basidiomycetes) bioprocesses.

Since it generally takes several months to cultivate the fruiting body of the fungus and it is also difficult to control the quality of the product during cultivation, researchers are turning to the liquid cultivation of mycelium to obtain useful cellular material or to produce effective active substances. Liquid or submerged culture has the potential advantage over solid substrate cultivation because it requires minimal space, there is less chance of contamination (Bae *et al.*, 2000), the mycelium can be dispersed within the substrate more uniformly, the media parameters can be easily manipulated and the time taken to produce the first crop may be shortened.

2.4.3.1 Investigation of Growth Parameters in Liquid Culture

Different growth factors can influence the quality and quantity of mycelial production. Two important factors that should be considered when cultivating in liquid culture are the components of the culture medium (e.g. nitrogen, carbon and inorganic salts) and the environment (e.g. temperature, pH, aeration and stirring, illumination and inoculum amount). These factors have minimum and maximum enhancing effects and quantification of these limits is necessary in order to model and predict effects on growth of the fungus (Knudsen & Stack, 1991). The quantity, quality, morphology and the nutrient consumption of the mycelium are considered important criteria for determining the optimal conditions for fungal growth (Yang & Liau, 1998). However, in the case where the accumulation and synthesis of an effective component is of interest, these criteria may alter, as some biological components may not exhibit a parallel relationship with the optimal growth parameters.

Although many workers have attempted to grow *G. lucidum* in submerged culture to produce mycelium, only few have investigated the influence of environmental factors (Lin *et al.*, 1973; Yang & Liau, 1998; Zhong *et al.*, 2002) and the effects of the culture medium (Kim *et al.*, 2002a; Lee *et al.*, 1999; Lin *et al.*, 1973; Tseng *et al.*, 1984).

2.4.3.2 Effect of Culture Medium on Mycelial Biomass

The culture medium is extremely important as it provides the nutrients for growth of the mycelium. There have been few investigations reporting the effect that different carbohydrates have on the growth of the mycelium (Lin *et al.*, 1973; Tseng *et al.*, 1984). Lin *et al.* (1973) reported that there was no difference in the production of biomass when glucose or sucrose was used, while Sone *et al.* (1985) who examined the growth of *G. lucidum* in a suite of different carbohydrates, including galactose, glucose, lactose, maltose, mannose, sucrose and xylose, found that lactose produced the highest dry weight of mycelium after seven days, and glucose and glucitol produced the least. In addition, they observed that the mass of mycelium produced in the lactose medium was approximately 1.5 times greater than that in the glucose medium.

The presence of nitrogen in the culture medium is another important factor and it has been observed that no mycelial growth occurs in its absence (Lin *et al.*, 1973). On

review of the literature there appears to be little information on the investigation of different nitrogen sources and how they affect the mycelial growth and formation of *Ganoderma* species.

Culture media buffered with inorganic salts (Lin *et al.*, 1973) and with low concentrations of ammonium phosphate (Lee *et al.*, 1999) have also been reported to result in good mycelial growth.

More recently, researchers have been investigating alternate materials which could increase biomass production including fatty acids (Yang *et al.*, 2000) and plant oils (Schisler & Volkoff, 1977), which have both been found to have a stimulatory effect on the mycelial growth of some mushrooms.

2.4.3.3 Effect of the Environment on Mycelial Biomass

Temperature is one of the most important environmental factors in mycelial growth requiring careful control (Brancato & Golding, 1953). *G. lucidum* isolated from different regions generally has an optimal growth temperature of $30 \pm 2^{\circ}$ C (Lin *et al.*, 1973; Mayzumi *et al.*, 1997; Yang & Liau, 1998), which explains why *Ganoderma* species are often found in hot climates and tropical regions. *G. japonicum* has been observed to grow at an optimal temperature of 25° C (Mayzumi *et al.*, 1997).

The initial pH of the growth medium is another important factor that may affect cell membrane function, cell morphology and structure, the uptake of various nutrients and product biosynthesis (Fang & Zhong, 2002b). It is well known that fungi generally metabolise acids to decrease the pH (Lin *et al.*, 1973) and that during the cultivation of mycelia, the media becomes acidic and when it reaches this acidic level (pH 3 to 4) the growth of the mycelia is retarded (Lin *et al.*, 1973). In contrast to this, Kim *et al.* (2002a) found that the pH of the liquid medium in which *Ganoderma* was grown increased, while the other fungi tested caused a decrease in the medium pH.

It has been demonstrated that the mycelium from a number of fungal species can grow over a wide range of pH values (Lonergan *et al.*, 1993; Yang & Liau, 1998). Yang & Liau (1998) observed that for *G. lucidum* grown in a glucose ammonium chloride

medium the optimal pH for mycelial growth was 4, however, when it was grown in a glucose malt extract medium they observed that the optimal pH for mycelial growth was 5. They concluded that the optimal initial pH for the growth of the mycelium could change depending on the cultivation medium used. Fang & Zhong (2002b) also observed that the initial pH of the cultivation medium affected the production of mycelial biomass. They reported that as the initial pH of the medium decreased from 6.5 to 3.5, the production of mycelial biomass decreased. Earlier, Lin *et al.* (1973) noted the morphology and size of the mycelial pellet varied as the pH of the culture medium changed for a range of *Ganoderma* species. At acidic pH (4), colonies of *Ganoderma* species were covered with 'feather like hairs', whereas at pH 7, the colonies appeared small, with or without short hair like structures.

Shear stress as a result of agitation can affect the morphology of the mycelium, which can directly reduce the cell growth and bioactive compound production (Pace, 1980). Too low an agitation speed may limit the oxygen transfer in the medium, reducing mycelial biomass production, whilst too high an agitation speed has been shown to increase the sheer stress on the mycelium, which in turn, also decreases biomass production (Yang & Liau, 1998). It has been reported that a favourable agitation speed for *Ganoderma* is approximately 100 rpm (Lin *et al.*, 1973; Yang & Liau, 1998). Zhong *et al.* (2002) investigated the production of mycelial biomass with respect to available oxygen. They observed that a high initial oxygen supply increased mycelial biomass production.

Inoculation density has been shown to be an important factor for many cell culture processes (Johansen *et al.*, 1998; Wang *et al.*, 1997a) including the submerged culture process of *Ganoderma* (Fang *et al.*, 2002; Yang & Liau, 1998). Fang *et al.* (2002) observed that a large inoculation density led to a small pellet size while a low inoculation density led to a larger pellet size. The mycelial pellet size has been reported to be another factor of importance in mycelial cultivation (Fang & Zhong, 2002a). Lin *et al.* (1973) suggested that it was best to select a three to four day old culture, when liquid culture is used as the inoculum for mycelial growth, as the fungal mycelium has just started to enter the logarithmic growth phase.

Light is not necessary for the growth of mycelia in liquid cultures or on an agar medium. Submerged cultivation experiments with *Ganoderma* species and many other fungi show that strong light can inhibit the mycelial growth (Lin *et al.*, 1973).

2.4.4 Investigation of Growth Parameters for Bioactive Compounds

More recently, environmental and culture media have been investigated with regards to the effect they have on the extracellular or intracellular bioactive components found in the mycelium of *G. lucidum* (Fang *et al.*, 2002; Fang & Zhong, 2002a, 2002b, 2002c; Kim *et al.*, 2002a; Lee *et al.*, 1999; Tang & Zhong, 2002; Yang *et al.*, 2000; Zhong *et al.*, 2002). Since many metabolites are produced only in minute amounts, it is often an advantage if the medium can be manipulated to ensure that the highest quantity and quality of bioactive components are produced.

The majority of biologically active compounds reported from *Ganoderma* species can be attributed to two major groups of metabolites, the polysaccharides or ganoderic acids (lanostane type compounds, triterpenes). The activity of compounds from within these two groups and their therapeutic effects are discussed in the latter part of this review (Sections 2.5.1 and 2.5.2, respectively). These two classes of metabolites have been used frequently in investigations as markers to determine the effect that different environmental and nutrient conditions can have on the production of valuable metabolites.

It has been observed that good mycelial growth does not ensure that a high production of polysaccharides is achieved (Sone *et al.*, 1985; Tang & Zhong, 2002). The carbon source, in the growth medium, was observed to play an important role in polysaccharide production (Kim *et al.*, 2002a). Tang & Zhong (2002) noted that, while sucrose was suitable for extracellular polysaccharide production but not for cell growth, lactose was a more suitable carbon source for cell growth and intracellular polysaccharide production. Park *et al.* (1994) reported that high levels of mycelial growth were closely related to high production levels of a protein-bound polysaccharide from the mushroom *C. versicolor.* It has also been reported that extracellular polysaccharide production is affected by the pH of the culture medium (Wang & McNeil, 1995) and that lowering the

pH of the medium increases the extracellular and intracellular polysaccharide production in submerged culture (Fang & Zhong, 2002b).

Inoculum size has also been shown to have a marked effect on polysaccharide and ganoderic acid formation (an important secondary metabolite in *Ganoderma* species) in submerged cultures of G. *lucidum* (Fang *et al.*, 2002). A large inoculation density (670 mg L^{-1} dry weight) was found to lead to a small pellet size and high production of extracellular and intracellular polysaccharides, whilst a low inoculation density (70 mg L^{-1}) led to a relatively large pellet size and high accumulation of ganoderic acid. Oxygen supply within the medium has also been shown to have a marked effect on polysaccharide and ganoderic acid production (Zhong *et al.*, 2002).

2.4.5 Effect of Species and Strain on *Ganoderma* Growth

It is recognised by researchers that the growth and dispersal of fungi in natural environments varies among fungal genera, species and strains (Knudsen & Stack, 1991). It is also well known that different strains can produce different compounds (Nishitoba *et al.*, 1986). Consequently, the methods employed in mushroom cultivation, whether it be solid substrate or liquid cultivation, may require modification from one geographical region to another due to different environmental conditions and the presence of different species of microorganisms (Chang, 1999).

The quantity of mycelial biomass produced varies widely depending on the mushroom, substrate and conditions used for cultivation (Yang & Liau, 1998). Kim *et al.* (2002a) studied a number of fungi, which included two *Ganoderma* species, in liquid culture and observed that the rate of mycelial growth was different between genera and some species. It was concluded that two *Ganoderma* isolates, initially thought to be the same species, were actually unique. Not only do cultivation conditions differ between species and strains but storage conditions may vary too. The black *Ganoderma* found in China have been reported to have an extreme intolerance to low temperatures. Mayzumi *et al.* (1997) observed that cultures from the black *Ganoderma* died when frozen for two days or stored at 20°C for twenty days. They also reported the red *Ganoderma* from Japan to be cold tolerant (temperature not reported) (Mayzumi *et al.*, 1997).

Ganoderma species isolated from Asian, European and Northern and Southern American countries have been investigated for their optimal environmental and culture medium conditions. However, there appears to be little information of this type that has been published in peer reviewed journals. There is evidence to suggest that growth data is dependant on the species and the region from which the fungus was originally isolated (Moncalvo *et al.*, 1995a). As a result, a local cultivator would need to investigate the optimal growth conditions for each species and strain they are working with, rather than simply use the conditions reported as optimal for a different environmental region.

2.5 Bioactive Substances in *Ganoderma* Species

Many bioactive compounds have been found in mushrooms, some of which inhibit the growth of cancer cells *in vitro*, have antiviral activity *in vitro*, or have other health benefits *in vivo*. These substances may be useful as starting materials for the development of chemical therapeutic agents in cancer treatment and for other ailments (Mizuno, 1995). Many investigations are currently underway to maximise the production and the utilisation of these functional molecules (Fang & Zhong, 2002b; Kim *et al.*, 2003; Lee *et al.*, 1999; Wang & McNeil, 1995). In addition, the modes of action of these compounds are being investigated (Eo *et al.*, 2000; Gao *et al.*, 2002b; Lei & Lin, 1992; Zhang *et al.*, 2002a).

Polysaccharides, triterpenes, sterols, lectins and proteins are some of the major active constituents that have been isolated from *G. lucidum* and its closely related species, with the first two compounds being the most extensively investigated. A list of the important bioactive components and their biological functions found in *Ganoderma* species is given in Table 2.1.

Table 2.1

Bioactive Constituent	Effects (in vitro)	Source	
Polysaccharides ^a	Immunomodulatory - antitumour	(Gao <i>et al.</i> , 2000a, 2000b; Lei & Lin, 1992; Li <i>et al.</i> , 2000; Li & Zhang, 2000; Lieu <i>et al.</i> , 1992; Ooi <i>et al.</i> , 2002; Sasaki <i>et al.</i> , 1971; Sone <i>et al.</i> , 1985; Wang <i>et al.</i> , 1997b; Zhang & Lin, 1999)	
	Hepatoprotective	(Zhang et al., 2002a)	
	Hypoglycaemic	(Hikino <i>et al.</i> , 19985, 1989; Hikino & Mizuno, 1989; Tomoda <i>et al.</i> , 1986; Zhang & Lin, 2004)	
	Antifibriotic	(Park et al., 1997)	
	Antiinflammatory	(Ukai et al., 1983)	
	Radiation protective and DNA damage, antioxidant, DNA strand breakage	(Kim & Kim, 1999b; Lee et al., 2001; Liu et al., 1997)	
	Antiherpetic	(Eo <i>et al.</i> , 1999a, 2000; Kim <i>et al.</i> , 2000; Oh <i>et al.</i> , 2000)	
Terpenoids and related compounds ^b	Cytotoxicity	(Gan <i>et al.</i> , 1998a; Gao <i>et al.</i> , 2002a; Gonzalez <i>et al.</i> , 2002; Kimura <i>et al.</i> , 2002; Lin <i>et al.</i> , 1991, 2003; Min <i>et al.</i> , 2000; Su <i>et al.</i> , 2000; Wu <i>et al.</i> , 2001)	
	Antiviral	(El-Mekkawy et al., 1998; Min et al., 1998; Mothana et al., 2003)	
	Anticomplement activity	(Min et al., 2001)	
	Hypolipidemic (cholesterol inhibitors)	(Komoda et al., 1989; Shiao, 1992)	
	Hypotensive (ACE)	(Morigiwa et al., 1986)	
	Antiplatelet aggregation activity	(Shiao, 1992; Wang et al., 1991)	
	Hepatoprotective	(Chen & Yu, 1999; Kim et al., 1999)	
	Antioxidant	(Zhu et al., 1999)	
	Antiinflammatory	(Giner-Larza et al., 2000)	
	Enzyme inhibitors	(Lee et al., 1998)	
	Antibacterial	(Smania et al., 1999)	
LZ-8 ^c	Immunomodulatory	(Haak-Frendscho et al., 1993; Miyasaka et al., 1992; van der Hem et al., 1995)	
	Immunosuppressive (antibody production)	(Kino <i>et al.</i> , 1989, 1990, 1991; van der Hem <i>et al.</i> , 1995)	
Lectins ^d	Unknown	(Kawagishi et al., 1997)	
	Mitogenic activity	(Ngai & Ng, 2004)	
Adenosine	Antiplatelet aggregation activity	(Kawagishi <i>et al.</i> , 1993; Shimizu <i>et al.</i> , 1985)	

Major bioactive constituents in Ganoderma species and their function

^a Polysaccharides, one of the main bioactive constituents isolated from *Ganoderma*, discussed in detail in Section 2.5.1; ^b Terpenoids and related compounds, discussed in Section 2.5.2; ^c LZ-8, a protein with biological activity, discussed in Section 2.5.3; ^d Lectins, carbohydrate type proteins, discussed in Section 2.5.4.

There are many biologically active metabolites present in the mycelium of mushrooms grown in submerged cultures (Lorenzen & Anke, 1998), however, it is apparent that many researchers have a principal interest in the fruiting body in the search for bioactive substances. Lorenzen & Anke (1998) noted that the metabolites of fruiting bodies often are not similar to those of mycelia from submerged cultures, which may be influenced by the different stages of development. Therefore, it is useful to investigate all stages of growth of the mushroom for the presence of bioactive molecules. Researchers investigating *Ganoderma* for bioactivity have found active compounds in the liquid cultivated mycelium (Chen *et al.*, 2000; Gao *et al.*, 2000b; Lin & Shiao, 1988; Sone *et al.*, 1985) and in the culture medium (Song *et al.*, 1998; Tasaka *et al.*, 1988a). The spores have also been found to be a valuable source of bioactive compounds (Min *et al.*, 2001; Zhu *et al.*, 2000).

2.5.1 Polysaccharides from *Ganoderma*

Attention has recently focused on the development of immunotherapeutic compounds that can identify and eliminate cancer cells as foreign matter (non-self), or be able to act on substances such as immunopotentiators, immunoinitiators and biological response modifiers (BRM) (Zhou & Gao, 2002). It is generally accepted that mushroom polysaccharides have a mode of action that enhances the body's immune response, rather than having direct cytotoxicity towards the tumour cells. Therefore, they are known to be BRM and it is these polysaccharides that have the potential to be developed into medicines that are not only carcinostatic, but also have antiinflammatory, antiviral, hypoglycaemic, hypotensive, and antithrombotic properties (Mizuno *et al.*, 1995c). The development in research on antitumour polysaccharides in fungi has been extensively reviewed (Mizuno, 1996, 1999; Ooi & Liu, 1999; Wasser, 2002).

2.5.1.1 Structure of Polysaccharides

The major bioactive polysaccharides isolated from *Ganoderma* species are glucans, β -1-3 and β -1-6 D-glucan. The basic structure is β -1-3 D-glucopyronan with 1 to 15 units of β -1-6 monoglucosyl side chains (Mizuno, 1991). Other antitumour polysaccharides of *G. lucidum* are heteropolysaccharides, glycoproteins (polysaccharides connected to proteins), or a group of polysaccharides known as ganoderans A, B and C (Lindequist,

1995). The antitumour polysaccharides differ greatly in their sugar composition and consequently in chemical structure, but one common feature is their relatively high molecular weight (Kim *et al.*, 1993). It has been reported that polyglucans with a higher molecular weight (10^{4-6} Daltons) tend to have greater water solubilities and therefore have a more effective antitumour activity (Mizuno, 1991), although some water insoluble polysaccharides in *Ganoderma* species, known as diet fibres, also have displayed antitumour activity (Wang *et al.*, 1993). Antitumour activity *in vitro* has also been linked to the frequency of polysaccharide branching, which changes with each stage of mycelial growth (Chen & Miles, 1996a).

2.5.1.2 Isolation of Mushroom Polysaccharides

Bioactive polyglycans (polysaccharides) are found in all parts of the mushroom, including the mycelium, or they may be secreted into the growth medium and become extracellular. Bioactive polysaccharides undergo structural changes at different growth stages of the mushroom (Chen & Miles, 1996a). It is also known that different polysaccharides can be extracted from the fruiting bodies and mycelia (Mizuno, 1999), nevertheless, antitumour polysaccharides appear to exist in both the fruiting bodies and the mycelium of Ganoderma species. Of the polysaccharides characterised from Ganoderma species, most have been isolated from the fruiting body of the mushroom (Bao et al., 2002; Wang et al., 1996; Zhang & Lin, 1999a, 1999b, 2004) and from the mycelial biomass cultivated in liquid culture (Chen et al., 2000; Kim et al., 1993; Zhang et al., 1994; Zhao & He, 2002), and only few from the culture medium (Kim et al., 2003; Sone et al., 1985). Polysaccharides that have been characterised from Ganoderma species include: β-D-glucans (Sasaki et al., 1971; Sone et al., 1985; Usui et al., 1983), glycans (Hikino et al., 1985; Tomoda et al., 1986), protein bound polysaccharides (Cheong et al., 1999; Eo et al., 1999a; Kim et al., 1993) and heterogalactans (Miyazaki & Nishijima, 1982; Usui et al., 1981).

According to folklore, *G. lucidum* is the type species that possess the most potent therapeutic properties (Willard, 1990). Hence, only few investigations have been performed on polysaccharides from *G. applanatum* (Nakashima *et al.*, 1979; Sasaki *et al.*, 1971; Usui *et al.*, 1981, 1983), *G. japonicum* (Ukai *et al.*, 1983), *G. tsugae* (Gao *et al.*, 2000a, 2000b; Wang *et al.*, 1993; Zhang *et al.*, 1994) and other *Ganoderma* species.

2.5.1.3 Polysaccharide Mode of Action

It has been widely reported that antitumour and anticancer effects of polysaccharides arise from the enhancement of the host's immune system rather than direct cytocidal effects (Gao *et al.*, 2000a, 2000b; Lieu *et al.*, 1992; Wang *et al.*, 1997b). The mechanisms by which antitumour activity is achieved still remain unclear, and it has only been in the last five years that there have there been reports investigating their mode of action (Cao & Lin, 2003; Eo *et al.*, 2000; Gao *et al.*, 2002b; Ooi *et al.*, 2002; Zhang & Lin, 1999a, 2004; Zhang *et al.*, 2002b). It should also be noted that the effect of cytotoxicity might be dependent on the extract concentration. Cao *et al.* (1993) reported that low concentrations (0.1 or 0.5 mg per mouse) of a polysaccharide from *G. lucidum* had immunostimulating activities but higher concentrations (2 or 4 mg per mouse) were found to be immunosuppressive.

2.5.2 Triterpenoids, Sterols and Related Compounds

Terpenes or terpenoids are amongst the compounds responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants (Dorman & Deans, 2000). Terpenoids can be classified into four main groups, 1) the volatile mono- and sesquiterpenes (C10 and C15), which are commonly known as essential oils; 2) the less volatile diterpenes, (C20); 3) the involatile triterpenoids and sterols (C30); and, 4) the carotenoid pigments (C40) (Harbourne, 1973). The majority of investigations reporting terpenoids from *Ganoderma* have been on the less volatile triterpenoid (triterpene) and sterol type compounds, thus, this review focuses only on compounds from within these classes.

An extensive review by Mahato *et al.* (1992) described the isolation, characterisation and biological activity of triterpenoids found in fungi and plants. Within the kingdom Fungi, most of the triterpenoids and steroids that have been isolated are from *G. lucidum*, although in recent years, other *Ganoderma* species have become a source of interest due to the bioactive constituents they have been found to possess. Triterpenes are thought to be potential anticancer agents due to the biological activity they have exhibited against actively growing tumours *in vitro* (Lin *et al.*, 2003).

2.5.2.1 Structure and Naming of Triterpenoids

There is some confusion over the designated names of *Ganoderma* triterpenoids. Lindequist (1995) suggested that when studying the different triterpenes, one should include the systematic IUC name that indicates the chemical structure of the compound. A summary of most of the triterpenoid components of *G. lucidum* according to their structural relationship has been reported (Kim & Kim, 1999a), but there is still a need for a thorough investigation and coordination of the designated names of triterpenoid constituents within the *Ganoderma* family.

The triterpene chemical structure is based on the ground structure of lanosterol (Figure 2.2), which is an important intermediate in the biosynthetic pathway for steroids and triterpenes in microorganisms and animals (Chang & Buswell, 1999). Stereochemical variation of the structure produces the diversity of triterpenes.



Figure 2.2. Structure of lanosterol (C₃₀H₅₀O) (Moss, 2004).

2.5.2.2 Isolation of Triterpenes

Strigina *et al.* (1971) and Protiva *et al.* (1979) were the first to report the isolation of triterpenes and steroids from *G. applanatum* and two of the most widely investigated triterpenes, ganodermic acid A and B from *G. lucidum*, were first isolated by Kubota *et al.* (1982). Since then, the physiochemical properties of over 100 lanostane-type triterpenoids have been described (Kim & Kim, 1999a).

Later in 1986, Nishitoba *et al.* suggested that triterpenes are specific to *G. lucidum*. Since then, there have been reports of triterpenes isolated from other *Ganoderma* species, including *G. colossum* (Kleinwachter *et al.*, 2001), *G. applanatum* (Chairul & Hayashi, 1994; Chairul *et al.*, 1991; Gan *et al.*, 1998b), *Ganoderma* sp. (Chairul *et al.*, 1990), *G. tsugae* (Chen & Chen, 2003; Gan *et al.*, 1998a; Su *et al.*, 2000), *G. concinna* (Gonzalez *et al.*, 2002), *G. tropicum* (Aryantha *et al.*, 2002) and *G. pfeifferi* (Mothana *et al.*, 2003).

Over the last two decades, the isolation of triterpenes from the fruiting body of Ganoderma species has been well documented (Chairul & Hayashi, 1994; Chairul et al., 1991; Gonzalez et al., 1999, 2002; Kikuchi et al., 1986a, 1986b, 1986c; Luo & Lin, 2001; Luo et al., 2002; Ma et al., 2002; Protiva et al., 1979; Rosecke & Konig, 2000). Many of these isolated triterpenes have shown biological activity (El-Mekkawy et al., 1998; Gao et al., 2002a; Kimura et al., 2002) and therefore these compounds represent promising candidates as new antitumour and antiviral agents. Investigators have also become interested in isolating triterpenes from the spores of Ganoderma (Chen & Yu, 1991, 1999). These compounds have been shown to possess strong antitumour and antiviral activity (Min et al., 1988, 2000, 2001) and have been shown to have higher concentration of ganoderic alcohol and acid content than the fruiting bodies of G. lucidum (Min et al., 1998). It was suggested that there is little triterpene formed in the mycelial pellets of liquid shaking cultures (Su, 1991), however, there have been other reports indicating the isolation of triterpenes from the liquid cultivated mycelial biomass of Ganoderma (Hirotani et al., 1986; Lin & Shiao, 1988; Shiao et al., 1987, 1988a, 1988b). More recently, there have been investigations into the molecular mechanism of antitumour triterpenes from the mycelium of G. lucidum (Lin et al., 2003).

An HPLC procedure has been developed for the isolation and purification of oxygenated triterpenoids from crude extracts (Lin & Shiao, 1987) and this procedure has proved to be an invaluable tool in investigating the triterpenoid patterns within *Ganoderma* species. Using HPLC, Nishitoba *et al.* (1986) investigated the strain specific terpenoid patterns of *G. lucidum* and found that there was a clear difference in terpenoid patterns between the strains, while Hirotani *et al.* (1993) examined the terpenoid patterns of three strains of *G. lucidum* and found that two were similar. Chen

et al. (1999) showed that HPLC analysis of the triterpenoids of different *G. lucidum* strains showed different HPLC patterns, indicating the presence of different triterpenoids, while different *G. tsugae* strains showed similar patterns. They concluded that the spectral patterns between the two mushroom species were different. In addition to this, it has been suggested that HPLC analysis of *Ganoderma* triterpenoids is a simple and easy way to differentiate among different species of the genus (Su *et al.*, 2001).

2.5.2.3 Triterpenoid Mode of Action

Recently, the mode of action of triterpenes has been of interest to many researchers (Gonzalez *et al.*, 2002; Kimura *et al.*, 2002; Lin *et al.*, 2003). The mode of action of triterpenes is different to the mode of action of polysaccharides. Rather than enhancing the immune system, as polysaccharides do, triterpenes have been shown to have direct cytotoxicity against tumour cells (Gonzalez *et al.*, 2002). An investigation by Gonzalez *et al.* (2002) found that three triterpenes induced cell death by apoptosis in human promyelocytic leukaemia HL-60 cells, *in vitro*, while Kimura *et al.* (2002) suggested that the antitumour and antimetatstatic activities of the triterpeneid fraction of *G. lucidum* might be due to the inhibition of tumour-induced angiogenesis.

2.5.2.4 Bitter Triterpenoids

The fruiting bodies of *G. lucidum* contain some intensely bitter compounds (Lindequist, 1995) and this characteristic bitterness of *Ganoderma* is not found in any other mushroom (Mizuno *et al.*, 1995b). It has been suggested that the fruiting body (and not the mycelium) of *G. lucidum* is the only species to contain bitter triterpenoids (Mizuno, 1995). It has also been suggested that the bioactivity is related to the bitterness (i.e. the more bitter, the greater the bioactivity), although the relationship is not fully understood (Mizuno, 1997). Nishitoba *et al.* (1988) investigated the relationship between the chemical structures and bitterness and concluded that the spatial relationship of the hydrophobic methyl groups to the three functional oxygen atoms plays a significant role in generating bitterness.

2.5.2.5 Other Steroidal Constituents

Sterols, compounds closely related to triterpenoids, are also found in *Ganoderma* (Yokokawa & Mitsuhashi, 1981). They have been isolated from the fruiting body and

mycelium (Chen & Yu, 1991; Hirotani *et al.*, 1987; Protiva *et al.*, 1979; Strigina *et al.*, 1971) and have also been shown to exhibit potent cytotoxic activity (Lin *et al.*, 1991). A specific sterol, ergosterolperoxide, was isolated from *G. lucidum* (Arisawa *et al.*, 1986) and has been shown to enhance the inhibitory effect of linoleic acid on the inhibition of mammalian DNA polymerase β (Mizushina *et al.*, 1998b).

2.5.3 Proteins

One of the most important proteins isolated from the mycelium of *G. lucidum* is Ling Zhi-8 (LZ-8) (Kino *et al.*, 1989). LZ-8 is a polypeptide consisting of 110 amino acid residues with an acetylated amino terminus and has a molecular mass of 12 kDaltons (Tanaka *et al.*, 1989). The native form of LZ-8, with a molecular mass of 24 kDaltons is a homodimer of the LZ-8 polypeptide (Tanaka *et al.*, 1989). This protein has been shown to have mitogenic activity *in vitro* and immunomodulating activity *in vivo* (Haak-Frendscho *et al.*, 1993; Kino *et al.*, 1989; van der Hem *et al.*, 1995).

2.5.4 Lectins

Lectins are found in plants, animals, microorganisms and fungi. They are carbohydrateproteins of non-immune origin, which agglutinate cells or precipitate polysaccharides or glycoconjugates (Goldstein *et al.*, 1980). Reviews by Wang *et al.* (1998) and Kawagishi (1995) highlight that there have been relatively few studies conducted on mushroom lectins. The protein LZ-8 displays many biological characteristics of lectins including mitogenic and haemagglutinating activities, although it is not claimed as a lectin *per se* (Wang *et al.*, 1998). Kawagishi *et al.* (1997) isolated a lectin from the mycelium and fruiting body of *G. lucidum* that was distinctly different from the LZ-8 protein isolated by Tanaka *et al.* (1989) and Wang *et al.* (1998). Of the lectins that have been isolated from mushrooms, many have yet to be assessed for their biological activity.

More recently, Ngai & Ng (2004) isolated a lectin from *G. capense* that exhibited potent mitogenic activity toward mouse splenocytes and antiproliferative activity toward leukaemia cells and hepatoma cells *in vitro*. They also observed the lectin to exhibit more potent mitogenic activity than that of concanavalin A (Con A), which is a lectin

extracted from the jack bean (Oxford, 1997) and one of the most widely used and well characterised lectins (Haufe *et al.*, 2004; Martinez-Cruz *et al.*, 1999).

2.6 **Biomedical Applications**

There have been a number of reviews published on the biomedical applications of mushrooms in general (Borchers *et al.*, 1999; Mizuno, 1995; Mizuno *et al.*, 1995a, 1995c; Wasser & Weis, 1999b, 1999c) and *Ganoderma* species in particular (Mizuno *et al.*, 1995b; Su, 1991). Recently, a review was published on the immunomodulating effects of *G. lucidum*, which outlined the mode of actions of a number of biologically extracted compounds (Zhou & Gao, 2002). *Ganoderma* is best known for its immunostimulatory effects in aiding cancer treatment and for its anti-HIV activity. Over the last decade, there has been an increasing amount of research to investigate *Ganoderma* species for new biomedical applications. Some of the applications that the mushroom extracts and constituents have been found to play important roles in include: hepatoprotective activity, hypoglycaemic activity, hypolipidimic activity, antibistamine release, antiinflammatory properties, antiplatelet aggregation activity, anticomplement activity, enzyme inhibition and in the healing of open skin wounds.

2.6.1 Cytotoxic Antitumour Activity

Fractions from *G. lucidum* have been shown to inhibit tumour growth through activation of host-mediated immune responses by stimulating the production of inducing cytokines (or cytotoxic T lymphocytes) by mononuclear leukocytes (Lieu *et al.*, 1992) and promoting the production of interleukin 2 (Lei & Lin, 1992; Ooi *et al.*, 2002). The activity and mechanism of these polysaccharides is still a major area of research. Zhang & Lin (1999) suggested that the antitumour activity of the polysaccharide extract from *G. lucidum* was related to apoptosis induced by TNF- α release from macrophages and IFN- γ release from T lymphocytes. Wang *et al.* (1997b) supported this conclusion and also found that the two cytokines acted synergistically on the inhibition of leukemic cell growth. It has also been observed that the host mediated antitumour effect is increased by the attachment of polyol groups to glucans (Sone *et al.*, 1985).

Antitumour activity against transplanted sarcoma 180 in mice has been exhibited by the β -D-glucan polysaccharides from the fruiting body of *G. applanatum* (Sasaki *et al.*, 1971), *G. lucidum* (Sone *et al.*, 1985) and the culture fluid of *G. lucidum* (Sone *et al.*,

1985). Ooi *et al.* (2002) also observed the suppression of growth of Sarcoma 180 solid tumour in mice and also observed a marked increase in the expression levels of seven immunomodulatory cytokines when in the presence of a hot water extracted *G. lucidum* polysaccharide. In contrast to this, there was no cytotoxic effect observed for a water-insoluble glucan from *G. japonicum* against sarcoma 180 in mice (Ukai *et al.*, 1983) or for the mycelial extract from *G. lucidum* on oral tumour cells *in vitro* (Chen *et al.*, 1991).

Triterpenoids from the fruiting bodies of *G. lucidum* and *G. applanatum*, and malonate half-esters from the fruiting body of *Ganoderma* sp., have shown biological activity as inhibitors against tumour promotion (Chairul & Hayashi, 1994; Chairul *et al.*, 1991; Lin *et al.*, 1991), however, some esters showed toxicity at high concentrations (Chairul *et al.*, 1990). Su *et al.* (2000) examined the cytotoxic activity of lanostanoids from *G. tsugae in vitro* and found them to exhibit significant activity against T-24, HT-3, and CaSKi cells (three cancer cell lines). Further to this, Gan *et al.* (1998a) isolated a lanostanoid and sterol from the same fungus, which caused cell death by apoptosis and suggested that the sterol possessed the activity of cell cycle inhibition. Gonzalez *et al.* (2002) also observed apoptosis in human promyelocytic leukaemia HL-60 cells exposed to three lanostanoids isolated from *G. concinna*.

2.6.2 Hypoglycaemic Activity

Zhang & Lin (2004) observed a polysaccharide isolated from the fruiting body of *G. lucidum* to possess a hypoglycaemic effect in mice, by its insulin-releasing activity due to facilitation of Ca^{2+} inflow to the pancreatic β cells. Earlier, Hikino *et al.* (1985) observed two different glycans from the same species to also have significant hypoglycaemic activity in mice (Hikino *et al.*, 1985). The mechanisms of the glycans were further investigated by Hikino *et al.* (1989) and Hikino & Mizuno (1989). Further studies by Tomoda *et al.* (1986) found that they were not glycans but were peptidoglycans.

2.6.3 Inhibition of DNA Polymerases and Other Enzymes

Polysaccharides have been shown to enhance the activity of DNA polymerases (Lei & Lin, 1993). Lei and Lin (1993) observed the activity of DNA polymerase α (a DNA

polymerase required for nuclear DNA replication) to increase in the splenocytes of 24month-old mice, after they had administered Ganoderma polysaccharides (GL-B) intraperitoneally, once a day for 4 days. In contrast, three triterpenoids isolated from the same fungal species were reported to inhibit the activity of calf DNA polymerase α , as well as rat DNA polymerase β (a DNA polymerase implicated to be involved in DNA repair, recombination and DNA replication) (Mizushina et al., 1999). Mizushina et al. (1999) also observed that two of the terpenes, lucidenic acid O and lucidenic lactone, inhibited the human immunodeficiency virus type 1 (HIV-1) reverse transcriptase. Earlier, two cerebroside compounds were also isolated from G. lucidum that selectively inhibited the activities of replicative DNA polymerases in mammals (Mizushina et al., 1998a). These cerebrosides were observed to have little effect on the activities of eukaryotic DNA polymerase β , prokaryotic DNA polymerases, terminal transferase, HIV deoxynucleotidyl reverse transcriptase, RNA polymerase, deoxyribonuclease I and ATPase.

Morigawa *et* al. (1986) observed that the 70% methanol extract from the fruiting body of *G. lucidum* inhibited angiotensin converting enzyme (ACE) *in vitro*, which is an enzyme involved in the control of hypertension. From this extract they identified ten lanostane triterpenes, of which eight exhibited activity.

2.6.4 Influence on Aggregation of Platelets

Blood platelets play an important role in thrombosis and haemostasis and the aggregation of platelets occurs when the cells respond to agonist stimulation. Ganodermic acid S, from *G. lucidum*, has been shown to induce platelet aggregation by stimulating the hydrolysis of phosphatidylinositol 4,5-bisphosphate (Shiao, 1992). Wen *et al.* (1997) found that *G. japonicum* exhibited an antithrombotic effect and the inhibition of blood coagulation and platelet activation. Tao & Feng (1990) showed that a Chinese herbal medicine made from *G. lucidum* inhibited platelet aggregation when given to patients with atherosclerotic diseases. Adenosine has also been shown to inhibit platelet aggregation (Shimizu *et al.*, 1985). In contrast to this, Gau *et al.* (1990) found that the administration of crude *Ganoderma* extracts, known to have a high content of adenosine, had no effect on platelet aggregation in haemophiliac patients who

were HIV positive. An epimer of 5'-deoxy-5'-methylsulphinyladenosine was found to inhibit platelet aggregation *in vitro* (Kawagishi *et al.*, 1993).

2.6.5 Hypolipidimic Activity

Elevation of cholesterol level in plasma is a main risk factor for the development of atherosclerosis and heart disease. Therefore, agents with hypolipidemic activity, for instance inhibitors of cholesterol biosynthesis and of cholesterol absorption, are of great therapeutical value. The powdered fruiting bodies of *G. lucidum* were reported to lower plasma cholesterol levels in spontaneously hypertensive rats (Kabir *et al.*, 1988). In particular, sterols (Komoda *et al.*, 1989) and oxygenated triterpenes (Shiao, 1992) have been shown to inhibit cholesterol synthesis *in vitro*. In contrast, a polysaccharide, ganoderan B, was shown to have no inhibitory effect (Hikino *et al.*, 1989).

2.6.6 Inhibition of Histamine Release

Histamine release from mast cells is an important step in the pathogenesis of inflammation, allergies and anaphylactic shock. An ethanolic extract from the mycelium of *G. lucidum* has been shown to exhibit significant antiinflammatory effects (Kendrick, 1985). In addition, the compounds cyclooctasulfur (Tasaka *et al.*, 1988b) and oleic acid (Tasaka *et al.*, 1988a), isolated from the culture broth of *G. lucidum*, were both found to inhibit histamine release from rat peritoneal mast cells and impede calcium uptake into these cells without affecting the cyclic AMP content.

2.6.7 Hepatoprotective

Polysaccharides from *G. lucidum* have been reported to have potent hepatoprotective activity (Kim *et al.*, 1999; Zhang *et al.*, 2002a). Song *et al.* (1998) isolated an extracellular polymer from the culture fluid of *G. lucidum* that exhibited hepatoprotective properties by lowering the glutamic pyruvic transaminase (GPT) activity in the serum of intoxicated rats. This mechanism was also observed by Chen & Yu (1999) who studied the effects of ganosporeric acid A, a triterpene from the spores of *G. lucidum*, in mice with liver injury. Shieh *et al.* (2001) concluded that the hepatic and renal protective mechanism of *G. lucidum* might be due at least in part to its prominent superoxide scavenging effect, because the *Ganoderma* extract could protect the liver and kidney from superoxide induced hepatic and renal damages. In addition,

Lin *et al.* (1995) observed that the greatest antihepatotoxic activity also exhibited the greatest free radical scavenging activity.

2.6.8 Antioxidants

Reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals, can induce carcinogenesis and other pathological diseases by acting as initiating or promoting agents (Lakshmi *et al.*, 2003; Lee *et al.*, 2001). Therefore, the inactivation or elimination of ROS may be critical in preventing these diseases.

Protein bound polysaccharides from *G. lucidum* (Lee *et al.*, 2001; Liu *et al.*, 1997; You & Lin, 2002), methanolic extracts from *G. tsugae* (Yen & Wu, 1999) and ethanolic extracts from *G. lucidum* (Lakshmi *et al.*, 2003) have exhibited superoxide and hydroxyl radical scavenging activity. A hot water extract of *G. lucidum* has exhibited antioxidative effect on mouse liver and kidney lipid peroxidation (Shieh *et al.*, 2001) and extracts have also been found to reduce strand breakage in DNA caused by UV induced photolysis of hydrogen peroxide (Kim & Kim, 1999b; Lee *et al.*, 2001). Liu *et al.* (1997) concluded that the lower the polysaccharide/protein ratio in protein bound polysaccharides, the greater the activity. Phenols have also been identified as a major constituent in methanolic extracts of mushrooms as naturally occurring antioxidants (Mau *et al.*, 2002). *Ganoderma* species were also found to possess higher antioxidant activity, reducing power, and scavenging and chelating abilities than the other mushrooms studied. There have been few investigations of the antioxidant activities of triterpenes from *Ganoderma* species (Zhu *et al.*, 1999).

2.6.9 Wound Management

Fungi are an attractive source of chitin for medical application because the chitin is produced in a filamentous form and it can be manufactured under standard conditions (Hamlyn & Schmidt, 1994). The use of chitin to promote wound healing has been reported (Balassa & Prudden, 1978) and it has been suggested that chitin accelerates the healing process (Olsen *et al.*, 1989). A chitin membrane, known as "sacchachitin", was prepared and developed from the residue of the fruiting body of *G. tsugae* (Su & Hsu, 1995). The membrane was shown to enhance the proliferation and migration of fibroblasts around wounds when applied as skin substitutes (Su *et al.*, 1996, 1997,

1999). Further, it was reported by Hung *et al.* (2001) that sacchachitin showed no cytotoxicity and its action in promoting wound healing may have been due to its chemotactic effect for inflammatory cells, which in turn may facilitate subsequent angiogenesis, granulation tissue formation, and faster new tissue formation, leading to faster wound healing. Chemical analysis of the sacchachitin membrane revealed that the treated residue was a copolymer of beta-1,3-glucan (ca 60%) and N-acetylglucosamine (ca 40%) (Su *et al.*, 1997).

2.7 Antimicrobial Compounds

In recent years, there have been a significant number of human pathogenic bacteria becoming resistant to antimicrobial drugs (Davis, 1994; Donadio *et al.*, 2002a; Lorenzen & Anke, 1998; Mulligen *et al.*, 1993) and this is in part due to the misuse and overuse of current antibiotics (Monroe & Polk, 2000). Antimicrobial drug resistance is of major economic concern having an impact on physicians, patients, health care administrators, pharmaceutical producers and the public (McGowan, 2001). In addition, bacteria and fungal pathogens have complicated the treatment of infectious diseases (Baratta *et al.*, 1998; Diamond, 1993). Given the increase in multiple drug resistance of human pathogenic microorganisms, it is imperative that new and effective therapeutic agents are developed.

Traditional tribal communities, indigenous people and the eastern world have been using plants, spices and fungi for thousands of years as therapeutic agents. For the past two decades attention has turned to the extracts and biologically active compounds used in traditional herbal medicine to uncover the scientific basis of their remedial effects and to seek new lead compounds for development into therapeutic drugs (Cragg *et al.*, 1997).

Plant extracts as antimicrobial agents have been investigated (Dorman & Deans, 2000; Palombo & Sempe, 2001, 2002; Vlietinck *et al.*, 1995) and have been found to contain many biologically active components (Cowan, 1999). An extensive review by Cowan (1999) reported the major groups of antimicrobial compounds found in plants and the important experimental methods required in determining new active compounds.

In addition to plants extracts as sources of antimicrobial agents, research is being performed on fungi for their ability to mobilize the body's humoral immunity and in turn prevent bacterial, viral, or fungal pathogens that are resistant to current therapeutic agents (Wasser & Weis, 1999c). Fungi are well known for the production of important antibiotic compounds such as penicillin, however, the occurrence of antibiotics in the class of fungi known as Basidiomycetes (the mushrooms) is less well documented (Miles & Chang, 1997) and there are only few reviews that summarise the antibacterial activity from these type mushrooms (Gao *et al.*, 2003; Wasser & Weis, 1999b, 1999c; Zjawiony, 2004). The following section (Section 2.7.1) reports the antibacterial activity of fungi from the division Basidiomycota, with particular focus on the antibacterial activity of *Ganoderma* extracts. Other antibiotic and drug producing filamentous fungi, such as *Penicillium* and *Aspergillus* species, are not covered in this review as they are from a different division to that of *Ganoderma*.

2.7.1 Antibacterial Activity

2.7.1.1 Activity of Mushroom Extracts

There appear to be an increasing number of reports on Gram-positive bacteria developing resistance to virtually every clinically available drug (Donadio *et al.*, 2002a), and basidiomycetous mushrooms have been shown to possess antibacterial activity against this group of bacteria. The mycelium of *Flammulina velutipes* was shown to have bacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* (Ishikawa *et al.*, 2001). In addition to these two bacteria, the fruiting body extract from *Armillariella mellea* also exhibited activity against *B. cereus*, *in vitro* (Obuchi *et al.*, 1990). The antibiotics, Psalliotin and Coriolin, from *Agaricus xanthoderma* and *Trametes* sp., respectively, have also been shown to inhibit Grampositive bacteria (Dornberger *et al.*, 1986; Takeuchi, 1969).

Early studies proposed that most antibacterial components from basidiomycetous fungi were potent against Gram-positive bacteria only (Lee *et al.*, 1982; Robbins *et al.*, 1947) but more recent work showed that extracts were also active against Gram-negative organisms, *Proteus vulgaris* and *Escherichia coli*, *in vitro* (Yoon *et al.*, 1994). Some *Agaricus* species (*A. campestis*, *A. bisporus* and *A. arvensis*) have been shown to produce compounds active against both Gram-positive and Gram-negative bacteria (Wasser & Weis, 1999b) and, extracts from *Lentinus edodes* have also been shown to be active against both types of bacteria (Hirasawa *et al.*, 1999). Overall, extracts from mushrooms are observed to be more active against Gram-positive bacteria than Gramnegative bacteria (Smania *et al.*, 1999).

The cell walls and inner cell membrane of Gram-positive bacteria are thick and consist primarily of peptidoglycan, while the cell walls of Gram-negative bacteria consists of an inner cell membrane, a peptidoglycan layer and a thick outer layer of a lipid-polysaccharide complex (Villee *et al.*, 1989). The greater resistance of Gram-negative bacteria is most likely to be the result the Gram-negative bacteria having this outer membrane acting as a barrier to many environmental substances, including antibiotics (Tortora *et al.*, 2001).

An aqueous extract of *Dendropolyporus umbellatus* demonstrated antibiotic action *in vitro* against *S. aureus* and *E. coli* (Chang & But, 1986). A polysaccharide Schizophyllan from *Schizophyllum commune* exhibited protective effects against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* infections in mice (Komatsu *et al.*, 1973) and against an infection in yellowtail fish caused by a *Streptococcus* species (Matsuyama *et al.*, 1992). Other polysaccharides that have exhibited antibacterial activity are the commercial products, Lentinan and Krestin, from the cultivated fruiting body of *L. edodes* (Jong & Birmingham, 1993) and the cultured mycelium of *Trametes versicolor*, respectively (Sakagami *et al.*, 1991; Tsukagoshi *et al.*, 1984).

The less polar organic extracts from mushrooms have also been shown to exhibit strong antibacterial activity (Beltran-Garcia *et al.*, 1997; Hirasawa *et al.*, 1999; Mothana *et al.*, 2000) and most of these have been extracted with a chlorinated organic solvent. In addition, the dichloromethane (DCM) extract from the fungus *Formitopsis pinicola* (Swartz ex Fr.) Karst. exhibited antibacterial activity against the Gram-positive bacterium, *B. subtilis* (Keller *et al.*, 1996).

There have been reports showing that triterpenes have a great antibacterial effect (Pinducciu *et al.*, 1995; Wilkens *et al.*, 2002) and it is well documented that triterpenes

are one of the major constituents isolated from *Ganoderma*. Armillaric acid, isolated from *A. mellea*, is one such terpenoid that inhibits Gram-positive bacteria (Obuchi *et al.*, 1990). Sesquiterpenes have also been shown to be one of the antibacterial agents in mushrooms (Donnelly *et al.*, 1985; Ishikawa *et al.*, 2001). Interestingly, Anke & Sterner (1991) observed that derivatisation of sesquiterpenes to less polar compounds increased the antimicrobial and cytotoxic affects.

Many researchers investigating the antibacterial activity of mushrooms focus on the fruiting bodies and only a few have investigated the liquid cultivated mycelium for activity. Donnelly *et al.* (1985), Donnelly & Hutchinson (1990) and Ishikawa *et al.* (2001) investigated the mycelial extracts from the fungi, *A. mellea* and *F. velutipes*, and found the extracts to possess antibacterial activity against Gram-positive bacteria. In addition, Hatvani (2001) found the culture fluid of *L. edodes* to possess antibacterial activity.

2.7.1.2 Antibacterial Extracts from Ganoderma

Ganoderma species have been widely investigated for their therapeutic properties as antitumour and antiviral agents but have been far less investigated as a source of new antibacterial agents. A review by Gao *et al.* (2003) on the antibacterial and antiviral value of *Ganoderma* species supported this observation, as there were few citings on research in this area.

Antibacterial activity has been observed against Gram-positive bacteria from the fruiting body extracts of *G. lucidum* (Kim *et al.*, 1993) and *G. orgonense* (Brian, 1951). Furthermore, Sudirman & Mujiyati (1997) observed that seven Indonesian *Ganoderma* species inhibited the growth of *B. subtilis*. Yoon *et al.* (1994) investigated the additive effect on the activity of an aqueous extract of *G. lucidum* with four known antibiotics and observed that the antibacterial activity increased. It is interesting to note that the majority of antibacterial investigations on *Ganoderma* species have been performed on the fruiting body and there are relatively few on extracts from the liquid cultivated mycelium. Coletto & Mondino (1991) observed that methanolic extracts of the mycelia and culture extracts of *G. recinaceum* and *G. lucidum* inhibited *B. subtilis* and *G. recinaceum* also inhibited *Staphylococcus aureus*.

Compounds shown to have an antibacterial effect include steroidal compounds from the fruiting body of *G. applanatum*, which were found to have a broad spectrum antibacterial activity with bactericidal effects (Smania *et al.*, 1999), as well as two ganomycins, namely A and B, from the fruiting body of *G. pfeifferi*, which exhibited antibacterial activity against both Gram-negative and Gram-positive bacteria (Mothana *et al.*, 2000).

2.7.2 Antifungal Activity

Antifungal drugs available today treating are not always successful in immunocompromised patients due to the ineffectiveness or toxicity that many of them have on the host (Seltrennikoff, 1995), and hence, there is a need for the identification of novel antifungal agents. Plant extracts have been under investigation for their antifungal properties and many have been found to possess activity (Binns et al., 2000; Diallo et al., 2001; Feresin et al., 2001). Since basidiomycetes belong to the kingdom of Fungi, they are thought to have weak antifungal activities (Mizuno, 1995) and therefore have rarely been investigated for their bioactivity as antifungal agents. It is only recently that they have become of interest due to their secondary metabolites exhibiting a wide range of antimicrobial activities.

Ishikawa *et al.* (2001) isolated two cuparene-type sesquiterpenes from the culture medium of *F. velutipes*, and observed both to have antifungal activity against the fungus *Cladosporium herbarum*. Hatvani (2001) tested the culture fluid of the mushroom *L. edodes* and found that it exhibited poor activity against the yeast *Candida albicans*. Earlier, Anke *et al.* (1980) isolated an antibiotic from the submerged culture of the mushroom *Marasmius scorodonius* (Fr.) Fr., which exhibited activity against bacteria, yeasts and filamentous fungi. Antifungal compounds have also been found in the extracts of *L. edodes* (Takazawa *et al.*, 1982).

2.7.3 Antiviral Activity

The propagation of viruses depends on the metabolic activity of the host cells (Eo *et al.*, 1999b). The goal of antiviral chemotherapy is the discovery of antiviral agents that are specific for the inhibition of viral multiplication without affecting normal cell

metabolism (Eo *et al.*, 1999b). Herpes simplex virus (HSV) and human immunodeficiency virus (HIV) are important viruses being investigated. Recently, a review by Gao *et al.* (2003) was published on the antiviral value of the genus *Ganoderma*, which summarised the major biologically active constituents and their effect or mode of action toward a number of viruses.

Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) are responsible for a broad range of human infectious diseases. HSV-2 is also known as an oncogenic virus that has the ability to convert cells into tumour cells. In addition, HSV infections are recognised as risk factors for the human immunodeficiency virus (HIV) infection (Hook III *et al.*, 1992). Polysaccharide fractions from *G. lucidum* exhibited activity against HSV-1 and HSV-2 (Eo *et al.*, 1999b, 2000; Kim *et al.*, 2000; Oh *et al.*, 2000). Lanostane type triterpenes from *G. pfeifferi* have also been shown to exhibit activity against HSV-1, as well as inhibit the influenza A virus (Mothana *et al.*, 2003).

There have been reports of low molecular weight aqueous fractions strongly inhibiting multiplication of HIV-1 (Kim *et al.*, 1997) and, of triterpenes inhibiting both HIV-1 protease (Min *et al.*, 1998) and HIV-2 protease (El-Mekkawy *et al.*, 1998).

2.8 Overview

This review summarised important areas of investigation being performed on *Ganoderma* species around the world, with particular emphasis in areas of research that have been focused on this investigation.

Firstly, this review highlighted the state of taxonomic confusion that the Ganodermataceae family is currently in, thus, emphasising the importance of correctly identifying new species. With the establishment of a modern classification system using molecular tools and evolutionary studies, misidentification of species should become a thing of the past.

In the search for active compounds from *Ganoderma* species, the majority of research has been performed on extracts from the fruiting body and there has been far less studies on extracts from the liquid cultivated mycelium. It appears that there are a

number of biologically active compounds to be found in the mycelium and the benefits of liquid cultivation over solid cultivation include: the ability to manipulate the cultivation medium to optimise mycelial growth; a shorter cultivation time; less contamination; and, less space is required. This review also brought attention to the fact that the manipulation of environmental or nutrient variables during the liquid cultivation process can greatly affect the production of mycelial biomass or biologically active constituents, or both.

In addition to the focus of research that has been put on the fruiting body of *Ganoderma*, it is also apparent that most of the research has been performed on the type species, *G. lucidum*. However, other species, such as *G. tsugae* and *G. applanatum* have also shown to possess biologically active components, some of which are similar to those found in *G. lucidum*.

The medicinal activities of a number of major classes of compounds that have been extracted from *Ganoderma*, namely triterpenoids and polysaccharides, were reviewed in detail. Other major compounds that have been shown to have pharmacological properties, such as lectins and proteins, were also reviewed. However, these have been far less investigated than terpenoids and polysaccharides. The review also demonstrated that only recently has the mode of action of these compounds been of interest, and that further studies need to be performed to establish mechanisms.

Lastly, this review reported the antimicrobial activities exhibited by *Ganoderma* extracts. It highlighted that there are a number of investigations into the antiviral activities of *Ganoderma*, however, there appears to be only few studies that have reported the antibacterial and antifungal activity of *Ganoderma* extracts. Since preliminary studies on some *Ganoderma* extracts have shown antibacterial activity, this appears to be a promising area of research.

Chapter Three

Materials and Methods

3.1.1 Chemicals

All chemicals used in this investigation were of analytical reagent (AR) grade and were purchased from Sigma Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise specified. Components of fungal and bacterial growth media were purchased from Oxoid (Basingstoke, Hampshire, UK) or Difco (Becton Dickinson, Maryland, USA). Analytical grade agarose was purchased from Promega Corporation (Madison, USA). All water used was deionised, unless otherwise stated.

3.1.2 Enzymes

All enzymes used in the manipulation of DNA were from Promega Corporation (Madison, USA) or Novagen (Madison, USA).

3.1.3 Buffers

Buffers used in the extraction of DNA (AP1, AP2, AP3/E, AW and AE) were supplied with the DNeasy® Plant Mini Kit (QIAGEN, Germany). Buffers used in the manipulation of DNA (*Taq* DNA polymerase 10X reaction buffer without MgCl₂, Buffer B, Buffer C and MulticoreTM) were purchased from Promega Corporation (Madison, USA). The 2X rapid ligation buffer containing T4 DNA ligase was supplied with the pGEM[®]-T Easy Vector System (Promega Corporation, Madison, USA). Methods used to prepare reagent buffers used throughout this investigation are described in Table 3.1.

Table 3.1

Buffer reagents used in this investigation

Buffer	Preparation
10 X Tris Borate (TBE)	108 g of tris base, 55 g of boric acid and 20 mL of 0.5 M EDTA (pH 8) were made up to 1 L with water.
10 X Tris Acetate (TAE)	48.4 g of tris base, 11.42 mL of glacial acetic acid and 20 mL of 0.5 M EDTA (pH 8) were made up to 1 L with water.

3.2 Sterilisation

All glassware and equipment used for the handling of fungal and bacterial cultures were sterilised prior to use. Unless otherwise specified, all sterilisation procedures were performed by autoclaving at 121°C (101 kPa) for 15 minutes.

3.3 Freeze Drying

Dehydration of organisms and mycelial biomass was performed under reduced pressure at -50°C in a Dynavac Freeze Drying Unit (Dynavac, Burwood, Australia).

3.4 pH Determination and Adjustment

The pH of all solutions was measured using an Activon ACE69 model pH meter, which had been calibrated prior to use using a two point buffering system at pH 4 and 7. Adjustment of the pH of solutions was achieved using sterile HCl (1 M or 6 M) (Section 3.2), sterile NaOH (1 M or 6 M) (Section 3.2), or filter sterilised 10% (w/v) tartaric acid solution. Media required to be at pH levels of 5, 6 or 7, were adjusted prior to sterilisation. Media required to be at pH levels of 3, 4 8 or 9, were adjusted after sterilisation due to hydrolysis of the agar at low pH values and a change in pH at high pH values during the autoclaving process.

3.5 Organisms

3.5.1 Basidiomycetous Fungi

A range of fungi was used throughout this study. Three wood rotting fungal species of *Ganoderma* were isolated in the Cairns State Forest, Northern Queensland, Australia. These have been designated *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 throughout this investigation. The other fungal organisms were used to allow a direct comparison of growth trends for white-rot fungi are listed in Table 3.2.

3.5.2 Storage of Basidiomycetous Fungi

Frozen stocks of fungi were stored on balsa wood at -70° C. Sub-sampling was performed at appropriate time intervals (described in Chapter 5.3.1) to ensure that the organisms remained viable. Frozen stocks were thawed, aseptically transferred to fresh

agar medium and then incubated at the appropriate temperature until confluent growth was achieved. Mycelial plugs were then used for subculture.

Fungi	Source ^a /Strain No.
Ganoderma sp.	SUT / H1
<i>Ganoderma</i> sp.	SUT / H2
Ganoderma sp.	SUT / H3
Ganoderma lucidum	ATCC / 46755
Ganoderma applanatum	ITBB / app
Pycnoporus cinnabarinus	CBS / 101046

 Table 3.2

 Basidiomycetous fungi used in this investigation

^a SUT, Swinburne University of Technology, Hawthorn, Australia; ATCC, American Type Culture Collection, Manassas, VA, USA; ITBB, Institut Teknologi Bandung, Bandung, Indonesia; CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands.

3.5.3 Test Organisms

3.5.3.1 Bacteria and Culture Conditions

Table 3.3 lists the range of bacterial organisms used throughout this study. These were used for the investigation of antibacterial activity of fungal extracts. The bacteria were propagated using the conditions described in Table 3.3 according to the recommendations of the supplier. All bacteria were grown aerobically except *Clostridium*, which was grown anaerobically using a BBL GasPak (Becton Dickinson, Cockeysville, Maryland, USA) in airtight anaerobic jars with anaerobic indicators (Oxoid, Hampshire, UK).

3.5.3.2 Storage of Bacterial Organisms

'Long term' frozen stocks of bacteria were stored at -70° C in a bead storage system in nutrient medium (Technical Service Consultants Ltd., Lancashire, UK). 'Short term' stocks were prepared by transferring previously isolated single colonies to fresh agar slants and then incubating at the appropriate temperature (Table 3.3). These slants were stored at 4°C for up to six months.

Table 3.3

Bacteria used	in	this	investigation	
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Bacterial species	Liquid Medium ^a	Solid Medium ^b	Temp (°C)	Source ^c /Strain No.
Bacillus cereus	NB	NA	37	ACMO / 2997
B. subtilis	NB	NA	37	ACMO / 40
Clostridium butyricum	FTG/BHI	CMG	37 An ^d	ACMO /2646
C. difficile	FTG/BHIm	CMG	37 An	MU / JIR1166
C. difficile	FTG/BHIm	CMG	37 An	$RCH / cd1^{g}$
C. difficile	FTG/BHIm	CMG	37 An	RCH / cd8 ^g
C. difficile	FTG/BHIm	CMG	37 An	RCH / cd14 ^h
C. perfringens	FTG/BHI	CMG	37 An	ACMO / 3902
C. perfringens	FTG/BHI	CMG	37 An	MU / JIR248
C. perfringens	FTG/BHI	CMG	37 An	MU / JIR235 ^e
C. sporogenes	FTG/BHI	CMG	37 An	ACMO / 354
Enterococcus faecalis	BHI	HBA	37	ATCC / 19433
E. faecalis [#]	BHI	HBA	37	MU / M259849
E. faecalis [#]	BHI	HBA	37	MU / M233165
E. faecalis [#]	BHI	HBA	37	MU / M252807
E. faecalis [#]	BHI	HBA	37	MU / M193292
E. faecalis [#]	BHI	HBA	37	MU / M169600
E. faecalis [#]	BHI	HBA	37	MU / M255048
Escherichia coli K12	NB	NA	37	ACMO / 2641
Klebsiella pneumoniae	NB	NA	37	ACMO / 9
Listeria monocytogenes 4B	BHI	HBA	37	ACMO / 98
Micrococcus luteus	NB	NA	28	ACMO / 975
Proteus vulgaris	NB	NA	37	SUT / pv1
Pseudomonas aeruginosa	NB	NA	37	ACMO / 495
Salmonella typhimurium	NB	NA	37	SUT/ st1
Shigella sonnei	NB	NA	37	SUT / ss7
Staphylococcus aureus	NB	NA	37	SUT / sa8
S. aureus	NB	NA	37	ATCC / 25923
S. aureus *	NB	NA	37	MU / M67638
S. aureus *	NB	NA	37	MU / M67783
S. aureus *	NB	NA	37	MU / M99320
S. aureus *	NB	NA	37	MU / M173525
S. aureus *	NB	NA	37	MU / M180920
S. aureus *	NB	NA	37	MU / M183909
S. epidermidis	NB	NA	37	SUT / S10
Streptococcus pyogenes	BHI	HBA	37	ACMO / 178

^a NB, nutrient broth; FTG, fluid thioglycollate medium; BHI, brain heart infusion; BHIm, modified brain heart infusion; ^b NA, nutrient agar; CMG, cooked meat glucose; HBA, horse blood agar; ^c ACMO, Australian Collection of Microorganisms, Department of Microbiology, University of Queensland; MU, Department of Microbiology, Monash University, Melbourne; RCH, Royal Childrens Hospital, Parkville, Melbourne; SUT, Environment and Biotechnology Centre, Swinburne University of Technology, Melbourne; ATCC, American Type Culture Collection, Manassas, VA, USA; ^d An, anaerobic growth; ^e Plasmid carrying strain, Tc^RCm^REm^R; ^g *C. difficile* clinical isolate resistant to 32 μg mL⁻¹ tetracycline; ^h *C. difficile* clinical isolate with no resistant genes; * Methicillin resistant *Staphylococcus aureus*; [#] Vancomycin resistant *Enterococcus faecalis*.

3.5.3.3 Test Fungi

Table 3.4 lists the range of test fungi, including some yeast strains, used throughout this study. They were used for the investigation of antifungal activity of the *Ganoderma* extracts. All yeast and test fungi were grown on MEA (Table 3.4) at 37°C. Yeast were grown for a period of 24 hours and fungi were grown for either a period of 24 hours to produce mycelia, or a period of 72 hours to produce spores.

	Organism	Source ^a /Strain No.
Yeast	<i>Candida</i> sp.	SUT / cs1
	Saccharomyces cerevisiae	SUT / sc
	Yeast	SUT/ 2906
	Yeast	SUT / 6034
	Yeast	SUT / 2119
	Rhizopus sp.	SUT / rs1
	Aspergillus niger	ATCC / 16404
F	Mucor sp. +	SUT / ms+
Fungi	<i>Mucor</i> sp	SUT / ms-
	Penicillium sp.	SUT / ps1
	Fusarium oxysporium	SUT / fo1

 Table 3.4

 Yeast and test fungi used in this investigation

^a ATCC, American Type Culture Collection, Marysville, USA; SUT, Swinburne University of Technology, Hawthorn, Australia.

3.6 Nutrient Media

All solid and liquid culture media were sterilised after preparation according to the method described in Section 3.2, unless stated otherwise. Nutrient media were prepared to the manufacturer specifications and are listed in Table 3.5, on the following page.
Medium	Mass (g) per 1 L of water	Natural pH
Brain Heart Infusion (BHI)	37.0	7.4 ± 0.2
Malt Extract Agar (MEA) ^a	50.0	5.4 ± 0.2
Malt Extract Broth (MEB) ^a	20.0	5.4 ± 0.2
Mueller Hinton Agar (MHA)	38.0	7.3 ± 0.1
Mueller Hinton Broth (MHB)	21.0	7.4 ± 0.2
Nutrient Agar (NA)	28.0	7.4 ± 0.2
Nutrient Broth (NB)	13.0	7.4 ± 0.2
Potato Dextrose Agar (PDA)	39.0	5.6 ± 0.2
Potato Dextrose Broth (PDB)	24.0	5.1 ± 0.2
Fluid Thioglycollate Medium (FTG)	29.5	7.1 ± 0.2
Tryptic Soy broth (TSB)	30.0	7.3 ± 0.2

 Table 3.5

Nutrient media prepared to manufacturer specifications

^a Sterilisation by autoclaving at 115°C (101kP) for 10 min, as per the manufacturer specification.

3.6.1 Fungal Growth Media

Media prepared to the manufacturer specifications are listed in Table 3.5. To investigate the effect of carbohydrate source on fungal growth, media were prepared according to the method described by Kawagashi *et al.* (1997) with minor modifications (Table 3.6). Modification was by addition of different carbohydrate sources to the base medium. This medium has been designated "Basal" or "Basal-G", (where the "G" denotes glucose) throughout this investigation.

 Table 3.6

 Basal medium for the investigation of carbohydrate source

Medium	Amount
Carbohydrate source ^a	50.0 g
Malt extract (ME)	10.0 g
Yeast extract (YE)	2.0 g
Bacto-peptone	2.0 g
KH ₂ PO ₄	5.0 g
MgSO ₄	2.5 g
Water	to 1000 mL

^a Carbohydrate source was: glucose, lactose, sucrose, fructose, xylose, galactose, mannose or cellobiose; The natural pH of all media was 5.1 ± 0.1 , except galactose (4.8 ± 0.7) and fructose (5.2 ± 0.1).

3.6.2 Bacterial Growth Media

Media prepared to manufacturer's specifications are listed in Table 3.5. BHIm for *C*. *difficile* was prepared by adding 18.5 g of BHI, 2.5 g of yeast extract (YE) in 500 mL of water. The mixture was shaken and sterilised by autoclaving at 116°C for 15 minutes. The medium was left to cool in a 55°C water bath and then 5 mL of filter sterilised 10% (w/v) L-cysteine, 7.5 mL of filter sterilised 25% (w/v) glucose and a few grains of FeSO₄·H₂O were added. BHIm agar medium for *C. difficile* was prepared in the same way, except 7.5 g of bacteriological agar was added to the mixture prior to autoclaving. FTG medium for *C. perfringens* was prepared by the addition of 14.5 g of FTG medium and 2.5 g of K₂HPO₄ to 500 mL of water. The medium was sterilised by autoclaving at 121°C for 20 minutes.

3.6.3 Media used to Propagate *E. coli* Carrying Recombinant Plasmids

Luria-Bertani (LB) broth was prepared according to Sambrook & Russell (2001). Briefly, 2.5 g of NaCl, 5.0 g of tryptone and 2.5 g of yeast extract were dissolved in 500 mL of water. The mixture was sterilised by autoclaving at 121°C for 15 minutes. LB ampicillin (LB^{Amp}) agar was prepared by cooling 500 mL of LB broth to 50°C after sterilisation and then adding 750 μ L of filter sterile ampicillin (40 mg mL⁻¹).

SOC medium was prepared according to Sambrook & Russell (2001). Briefly, 20 g of tryptone, 5 g of yeast extract (YE) and 0.5 g of NaCl were added to 950 mL of deionized water. The solution was shaken until all solutes dissolved and then 10 mL of 250 mM KCl was added. The pH was adjusted to 7 using 5 M NaOH (~ 0.2 mL) and then made up to 1 L with water. The solution was then sterilised by autoclaving at 121°C for 20 minutes. Immediately before use, 5 mL of sterile 2 M MgCl₂ and 20 mL of sterile 1 M glucose were added.

3.7 Fungal Maintenance

3.7.1 Storage on Slants

Fungal samples were inoculated onto MEA or PDA slants in clear glass McCartney bottles (or Universal bottles) and incubated for 5 to 6 days, in darkness, at the desired

temperature. After incubation, the bottles were wrapped in aluminium foil to protect the samples from light and then stored at 4°C.

3.7.2 Storage under Mineral Oil

Fungal samples were inoculated onto agar slants in clear glass containers and incubated for 5 to 6 days at the desired temperature. Sterilised medicinal paraffin oil (specific gravity 0.865 - 0.890) was then poured over the mycelium on the agar slants to a depth of no more than 1 cm from the top of the slant. The samples were stored at 4°C.

3.7.3 Storage in Water

Fungal samples were inoculated into 4 mL of sterile water and stored at 4°C or at room temperature.

3.7.4 Storage on Wood

Fungal samples were inoculated onto sterile balsa wood (5mm x 5mm x 1mm) and incubated for 5 to 6 days at the desired temperature. The samples were then stored at room temperature, 4° C or at -80° C.

3.8 Incubation of Fungal Cultures

3.8.1 Static Cultures

Culture plates were placed on large, plastic trays. To maintain a humid atmosphere, a small jar of water was placed onto the tray and the trays were then wrapped in plastic film. The trays were subsequently placed in incubators at the desired temperature.

3.8.2 Shaker Flasks Cultures

Polycarbonate shaker flasks that contained four baffles were securely tightened in a Bioline shaker incubator. They were shaken at 30°C and at 100 rpm for 30 days, unless otherwise stated.

3.9.1 Slant Preparation

Agar slants were prepared by dispensing 10 mL aliquots of molten medium into 30 mL McCartney bottles and sterilising as described in Section 3.2. The bottles were then laid on a 30° angle and allowed to set.

3.9.2 Plate Preparation

Using sterile technique, 20 mL aliquots of sterile molten medium were transferred to gamma sterilised Petri dishes, 90 mm x 15 mm (Biotech) with an automatic liquid dispenser. After solidifying, the plates were dried in a 40°C oven for 15 minutes and subsequently subjected to UV radiation for 20 minutes.

3.9.3 Sub Culturing

Sub cultures were prepared by excising small pieces of mycelium (1 cm x 1 cm) from culture slants and placing them on freshly prepared agar plates. These were incubated at the desired temperature until the mycelium was approximately 10 mm from the outer edge of the plate. These have been designated as "working cultures" throughout this investigation. A second sub-culture step was performed by cutting 6 mm round plugs from the growing mycelial edge of the working cultures using a sterile plug borer and transferring them onto the centre of the experimental agar plates. These have been designated the "experimental cultures" throughout this investigation.

3.9.4 Radial Growth Zone Measurements

Radial growth measurements were performed following the method of Lonergan *et al.* (1993). Briefly, radial zone measurements were performed in triplicate on the different fungal colonies grown on PDA. PDA was used in preference to MEA because of its translucent nature, which enabled the growing edge to be seen and measured clearly. Growth assays were performed on all the plates by measuring the mycelial radius (minus the plug radius) of the colony in mm. The measurements were taken from four different points and the average radius was recorded. Measurements were taken at 24 hours intervals over a period of 6 days. A light box fitted with a magnifying glass was used to facilitate viewing and enabled accurate measurement of the fungi.

3.9.5 Mycelial Growth Rate

The rate of mycelial growth was performed subsequently to the completion of the radial growth zone measurements (Section 3.9.4). The average rate of mycelial growth (mm day⁻¹) was determined over a four day period. To determine this, the following calculation was employed:

$$\{[G(d6) - G(d5)] + [G(d5) - G(d4)] + [G(d4) - G(d3)] + [G(d3) - G(d2)]\} \div 4$$

Where: G(d6) is equal to the average mycelial growth (in mm) on day 6 and G(d5) is equal to the average mycelial growth (in mm) on day 5.

If the mycelial growth had reached the perimeter of the plate before day 6, then day five was used as the starting point for the calculation.

3.10 Broth Cultivation Techniques

3.10.1 Shaker Flask Preparation

Plastic polypropylene shaker flasks containing four baffles were used. Liquid medium, 125 mL, 250 mL or 1000 mL, was prepared into 250 mL, 500 mL or 2000 mL shaker flasks, respectively. The flasks were plugged with non-absorbent cotton wool (Smith and Nephew, Australia) and then sterilised as described in Section 3.2.

3.10.2 Mycelial Mat and Fragment Inoculation

A conical flask containing 50 mL of MEB was prepared, plugged with non-absorbent cotton wool and sterilised. Once the medium had cooled to room temperature, two 6 mm plugs were taken from the growing edge of a 5 day culture and transferred to the culture medium. The flask was then incubated without shaking for a period of 7 days. The mycelial mat culture was homogenised using an Ystral GmbH homogeniser with a sterile blade (2 mm) for 40 seconds. A 1, 2.5 or 10 mL aliquot of the homogenised sample was then transferred to a 250, 500 or 1000 mL shaker flask, respectively, containing the experimental medium.

3.10.3 Separation of Biomass from the Culture Medium

The mycelial biomass was separated from the culture medium by vacuum filtration through No. 54 type Whatman filter paper. The mycelial biomass was then rinsed with water until the water ran clear (approx. 3 volumes) and the resulting biomass freeze dried as described in Section 3.3.

3.11 Liquid - Solid Extractions

3.11.1 Hot Water Aqueous Extraction

Four to 5 g of freeze dried mycelial biomass, which had been ground to a fine powder using a mortar and pestle, was accurately weighed into a 250 mL beaker containing a magnetic stirrer. After addition of 100 mL of water, the mixture was heated at 95 to 100°C for 2 hours, whilst stirring. After this time, the hot water extract (filtrate) was separated from the mycelial biomass by vacuum filtration using No. 54 type Whatman filter paper. The biomass was rinsed with water until the water ran clear (approx. 3 volumes). The biomass was placed into a fresh 250 mL beaker and another 100 mL volume of water was added. The process of heating, stirring and filtration was repeated. The combined filtrates were collected and evaporated to dryness by freeze drying (Section 3.3). The mass of extract per gram of mycelium was determined.

3.11.2 Organic Extraction

Four to 5 g of freeze dried mycelial biomass, which had been ground to a fine powder using a mortar and pestle, was accurately weighed into a 20 x 80 mm cellulose extraction thimble (Whatman, Maidstone, UK). The extraction solvent (100 mL) was added to a 500 mL round bottom flask. Soxhlet extraction was carried out in a hot water bath for a period of 6 hours. Consecutive organic solvents used in the extraction process, boiling points and the bath temperatures are listed in Table 3.7. After the extraction process was complete, the solvent was evaporated to dryness under reduced pressure by rotary evaporation. The mass of the extract was calculated per gram of mycelium.

Table	3.7
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Organic Solvent	Boiling Point (°C) ^a	Bath Temperature (°C)
Hexane	68.5	90
Dichloromethane	42.0	60
Ethyl Acetate	76-77	95
Methanol	64.5	90

Organic solvents used in this investigation

^a Boiling points taken from Vogel (1948).

3.12 Chromatography

3.12.1 Gravity Column Chromatography

Gravity column chromatography was performed using silica gel 60 Å, 70 to 230 mesh. The column had an internal diameter of 15 mm and a length of 260 mm. The column was packed using the slurry method and equilibrated with CHCl₃ for eight column volumes. A gradient step elution was required to elute all compounds from the column. Twenty mL volumes of each eluant starting with CHCl₃:MeOH (99:1) and ending with CHCl₃:MeOH (2:8) was added to the column. Fractions were collected in 5 mL volumes and the flow rate was 1 mL min⁻¹. Approximately 10 µL of each fraction was spotted onto TLC plates and developed in an appropriate solvent system (Section 3.12.2). Like fractions were combined and then subjected to further analysis.

3.12.2 Thin Layer Chromatography (TLC)

TLC was performed on silica gel, 60 F_{254} , aluminium backed plates (Darmstadt, Germany). They were cut to the appropriate size using a sharp scalpel. Volumes, in μ L of the extract to be examined, were spotted onto the TLC plate approximately 1 cm from the bottom of the plate. The spots were dried with a warm current of air and then the plate was developed in a saturated glass TLC tank using the appropriate solvent system. The solvent systems differed for the different extracts and extract fractions. Optimisation of the solvent system for the different fractions from the dichloromethane (DCM) extract was investigated in Chapter 7. When the mobile phase had travelled the required distance, the plates were removed from the TLC tank and allowed to dry. In some cases, the developing procedure was repeated in the same mobile phase. Compounds were viewed under Visible and UV light (254 and 365 nm), or were

subjected to further chemical analysis (as described in Section 3.14) for the identification of classes of compounds.

3.12.3 Preparative TLC

Preparative TLC plates were prepared as follows. A slurry was made using 60 g of silica G60 F_{254} in 150 mL of distilled water. The slurry was allowed to sit for 1 hour and then poured onto glass plates in a TLC plate maker. The plates were made to 1 mm thickness and allowed to dry for 2 days. Before use, impurities were removed from plates by running the plates in a slightly more polar phase than the anticipated mobile phase. The plates were activated at 120°C for 2 hours and then used as described in Section 3.12.2. Spots of interest were scraped from the plate and like spots combined. An appropriate solvent was used to redissolve the compound and the silica gel removed by centrifugation. The silica gel was washed with the solvent three times to ensure maximum removal of the compound of interest.

3.13 Antibacterial Assays

3.13.1 Disc Diffusion Assay

A 4 mL volume of the propagation broth was inoculated with bacteria from a stock culture and incubated overnight at the appropriate temperature. The overnight broth culture was then adjusted to a 0.5 McFarland standard in 3 mL of a 0.85% (w/v) saline solution. Four mL of molten MHA kept at 50°C in a water bath was inoculated with 200 μ L of the bacterial saline suspension, mixed and poured evenly into a Petri dish containing a bottom layer of solid MHA (16 mL). Blank sterile discs (6 mm) (Oxoid, Hampshire, UK) were impregnated with a known amount of extract and placed gently onto the agar overlay. The plates were incubated at 37°C overnight or 48 hours, or 28°C for 2 days, depending on the growth requirements of the bacterium. Negative controls of the solvent only and positive controls of antibiotics (e.g. tetracycline, ampicillin) were also prepared. The antibacterial activity was recorded by measuring any zones of growth inhibition around the disc.

3.13.2 Minimum Inhibitory Concentration (MIC)

MIC determinations were performed in gamma sterilised 96 well microtitre plates (Cellstar, Greiner Bio-One). A two-fold serial dilution containing the growth medium

and extract was prepared to a volume of 100 μ L per well. To this, a 10 μ L aliquot of the test organism (adjusted to a 0.5 McFarland standard in 0.85% (w/v) saline solution) was added to each well. A second two-fold serial dilution containing the growth medium and extract was also prepared. To this, a 10 μ L aliquot of 0.85% (w/v) saline solution was added. This second serial dilution served as a negative turbidity control. Positive controls were also prepared. All two-fold dilutions and controls were prepared in triplicate. The plates were incubated under aerobic or anaerobic conditions for 24 or 48 hours, depending on the bacterium used. After the appropriate incubation time, the wells were mixed thoroughly and the optical density measured at 595 nm (OD_{595}), in triplicate, using an Emax Precision Microplate Reader (Molecular Devices). The OD₅₉₅ due to the growth of the test organism was calculated by subtracting the OD₅₉₅ of the turbidity control solutions (Second two-fold serial dilutions) from the corresponding OD₅₉₅ of the sample solutions (First two-fold serial dilution). The MIC was then determined to be at the concentration where there was no increase in the OD_{595} , or at the concentration where the OD_{595} was zero. The experiment was repeated in triplicate to check for reproducibility.

3.13.3 Minimum Bactericidal Concentration (MBC)

The MBC was determined for samples that previously exhibited an MIC. Directly after the MIC was determined (Section 3.13.2), a sample was taken from each well that exhibited no increase in OD_{595} (no bacterial growth) and was streaked onto freshly prepared agar plates. The fresh agar plates were then incubated at the appropriate growth temperature for either 24 or 48 hours. After this time, the plates were inspected for growth of the bacteria. The MBC was determined to be the concentration at which no bacterial growth was observed.

3.13.4 Time Kill Assay

Time course growth assays were determined at the MBC for the extract against the test bacteria. If no MBC was previously detected then the MIC was used instead. For each bacterium, the extract was prepared in a total volume of 2 mL in the appropriate growth medium to give a final concentration equal to that of the MBC. At time zero, a 100 μ L aliquot of the test bacteria (which had been previously adjusted to 0.5 McFarland standard in 0.85% (w/v) saline solution) was added to the sample and incubated

aerobically or anaerobically, depending on the bacterium, for up to 48 hours. At time 0, 1, 2 or 3 and 4 hours, a 100 μ L aliquot was taken from the sample and a ten-fold dilution series prepared. A sample from each dilution (100 μ L) was spread onto the surface of fresh agar plates. The plates were allowed to dry and then incubated under the appropriate conditions, depending on the bacterium used. The plates were then inspected for growth and the number of colony forming units (cfu) per mL determined. A control assay was also performed for comparison of growth. The growth was also checked at 24 or 48 hours, for some organisms.

3.13.5 Bioautography Agar-Overlay

Bioautography was performed using TLC plates (Section 3.12.2) that contained spots of interest. The TLC plates were cut to size and placed into square Petri dishes (10 x 10 cm). A volume of 12 mL of molten agar, kept at 50°C, was inoculated with 200 μ L of an overnight bacterial culture, mixed thoroughly, and then poured over the TLC plate in the Petri dish. The agar was spread uniformly over the plate to give an agar overlay thickness of approximately 1 mm. The surface of the agar was flamed briefly to remove any air bubbles. Once the agar was set, the plates were inverted and incubated overnight at 37°C. The plates were sprayed with MTT (thiazolyl blue tetrazolium bromide) (2 mg mL⁻¹) and reincubated for 30 minutes at 37°C. The plates were then observed for spots that exhibited clear zones of growth inhibition against a purple background of live bacteria.

3.14 Phytochemistry

Photochemistry was performed on TLC plates that had been spotted with extract and allowed to develop (Section 3.12.2). For each detection reagent, two identical plates were prepared along side each other. The first plate was used as the reference whilst the second plate was subjected to spray reagents and detection. Methods for detection were performed as described in Wagner *et al.* (1984) and Krebs *et al.* (1969). Detection of compounds was by: 1) visualisation under visible or UV light (254 or 395 nm), 2) gently heating the TLC plate and then visualisation of spots under visible or UV light (254 or 395 nm), or 3) lightly spraying (using a Preval Sprayer purchased from Sigma-Aldrich) the TLC plates with detection reagents and observing under visible or UV light.

3.14.1 Detection Reagents

3.14.1.1 Anisaldehyde – Sulphuric Acid (AS)

AS reagent was used for the unspecific detection of steroids, terpenes, essential oils, bitter principles and saponin drugs. The reagent was prepared by mixing 0.5 mL of anisaldehyde with 10 mL of acetic acid, followed by 85 mL of MeOH and 5 mL of concentrated H₂SO₄. A TLC plate was then sprayed with 10 mL of AS reagent, warmed at 100°C for 5 to 10 minutes and evaluated under UV (365 nm) or visible light.

3.14.1.2 Vanillin Sulphuric Acid (VS)

VS reagent was used for the unspecific detection of essential oils, bitter and pungent principles, and saponin drugs. This reagent consisted of two parts. a) A 5% (w/v) ethanolic H_2SO_4 solution, and b) a 1% (w/v) ethanolic vanillin solution. The TLC plate was sprayed with 10 mL of (a) followed by 5 to 10 mL of (b), warmed at 100°C for 5 to 10 minutes and evaluated under visible light.

3.14.1.3 Liebermann – Burchard Reagent (LBr)

Freshly prepared LBr reagent was used for the detection of triterpenes, steroids (saponins and bitter principles) and sterols (cholesterol and esters). The reagent was prepared by carefully adding 5 mL of acetic anhydride and 5 mL of conc. H_2SO_4 to 50 mL of absolute ethanol on ice. The TLC plate was sprayed with 5 to 10 mL of LBr reagent, warmed at 100°C for 5 to 10 minutes and evaluated under visible or UV (365 nm) light.

3.14.1.4 Potassium Hydroxide (KOH)

Freshly prepared KOH reagent was used for the detection of anthraquinones, anthrones and coumarins and arbutin drugs. The TLC plate was sprayed with 10 mL of a 10% (w/v) ethanolic KOH solution, dried and then observed under UV (365 nm) or visible light, with or without warming.

3.14.1.5 Kedde Reagent

Freshly prepared Kedde reagent was used for the detection of cardiac glycoside drugs. The reagent was prepared by mixing 5 mL of a 3% (w/v) ethanolic 3,5-dinitrobenzoic acid solution with 5 mL of 2 M NaOH. A volume of 5 to 8 mL of the reagent was

sprayed onto the TLC plate and then observed under visible light for the presence of pink zones.

3.14.1.6 Natural Products - Polyethylene Glycol (NP-PEG)

NP-PEG was used for the detection of anthracene derivatives, coumarins, arbutin drugs, bitter principle and flavonoids. Ten mL of NP (1% (w/v) methanolic diphenylboryloxyethylamine) followed by 8 mL of PEG (5% (w/v) ethanolic polyethyleneglycol-4000) was sprayed onto the TLC plate. The plate was then observed under UV light at 365 nm.

3.14.1.7 Fast Blue Salt (FBS)

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

3.14.1.8 Berlin Blue Reagent (BB)

Freshly prepared Berlin blue reagent was used for the detection of arbutin drugs. The reagent was prepared by adding 10 g of FeCl₃ and 0.5 g of $K_3[Fe(CN)_6]$ in 100 mL of H₂O. The TLC plate was then sprayed with 5 to 8 mL of BB and evaluated in visible light for the presence of blue zones.

3.14.1.9 Fluorescein

Fluorescein was used for the detection of lipids. A TLC plate was lightly sprayed with a 0.01% (w/v) ethanolic solution of fluorescein and dried in warm air. The plate was then lightly sprayed with water and evaluated in visible light for the presence of yellow zones.

3.14.1.10 Iodine

Iodine vapour was used for the detection of compounds with conjugated double bonds. A TLC plate was placed into a TLC tank, which had been previously saturated with iodine vapour by the addition of iodine crystals. The TLC plate was removed and evaluated in visible light.

3.14.1.11 Folin-Ciocalteu Reagent

Folin-Ciocalteu reagent was used for the detection of phenolic compounds and was purchased ready made from Merck (Darmstadt, Germany). The TLC plate was sprayed with 5 to 10 mL and then evaluated in visible light for the presence of blue zones.

3.14.1.12 Aluminium Chloride

AlCl₃ was used for the detection of flavonoids. A TLC plate was sprayed with 5 to 10 mL of a 1% (w/v) ethanolic AlCl₃ solution and evaluated under UV (365 nm) light.

3.14.1.13 Dragendorff Reagent

Freshly prepared Dragendorff reagent was used for the detection of alkaloids. The reagent was prepared by dissolving 8 g of KI in 20 mL of H_2O . This solution was then added to a second solution containing 0.85 g of basic bismuth nitrate in 40 mL of H_2O and 10 mL of acetic acid. The TLC plate was sprayed with 10 mL and observed under visible light for the presence of yellow zones.

3.15 Extraction of DNA from Fungi

The extraction of DNA from fungal matter was performed using a DNeasy® Plant Mini Kit (QIAGEN, Germany). Briefly, 20 mg of freeze dried mycelium was ground to a fine powder under liquid nitrogen. The mycelium was transferred to a 1.5 mL microtube and 400 µL of buffer AP1 and 4 µL of RNase were added and the mixture and vortexed. The sample was then incubated for 10 minutes at 65°C, mixing three to four times during the incubation. Buffer AP2 (130 µL) was added and the sample was vortexed then incubated on ice for 5 minutes. The sample was centrifuged for 5 minutes at 11,000 rpm in a M-24 centrifuge (Boec, Germany) to separate the lysate from the cell debris. The lysate was then applied to the QIAshredder spin column sitting in a 2 mL collection tube and centrifuged for 2 minutes at 11,000 rpm. The flow through fraction was transferred to a new 1.5 mL microtube. A volume of buffer AP3/E, one and a half times greater than the lysate, was then added and mixed by pipetting. The lysate mixture was then applied to the DNeasy spin column sitting in a 2 mL collection tube and was centrifuged for 1 minute at 10,000 rpm. The DNeasy column was then placed into a new 2 mL collection tube and 500 µL of buffer AW was added, mixed and centrifuged for 1 minute at a speed greater than 8,000 rpm. Another 500 µL of buffer

AW was added to the DNeasy column, but this time it was subjected to centrifugation for 2 minutes at 11,000 rpm to dry the membrane. The DNeasy column was then removed from the collection tube and transferred to a new sterile 1.5 mL microtube and 100 μ L of buffer AE (at 65°C) was pipetted directly onto the DNeasy membrane. The column was incubated at room temperature for 5 minutes and then centrifuged for 1 minute at a speed greater than 8,000 rpm to elute the DNA. The DNA eluent was put aside. Again, 100 μ L of buffer AE was added to the same column and centrifuged for 1 minute at a speed greater than 8,000 rpm. The two DNA eluants were designated DNA extract 1 and 2.

3.16 Oligonucleotide Primers for PCR and Sequencing

Oligonucleotides used in this investigation (Table 3.8) were either purchased from GeneWorks (Adelaide, SA, Australia) or provided by Monash Institute of Reproduction and Development (MIRD). Oligonucleotides were provided desalted and lyophilised. The oligonucleotides were resuspended in water to obtain a concentration of 1 μ g μ L⁻¹ and stored at –20°C. The location and orientation of oligonucleotides used in the amplification the ITS I and ITS II region is shown in Figure 3.1.

Name	Sequence (5' - 3')	Reference
BMB-CR^a	GTACACACCGCCCGTCG	(Gottlieb et al., 2000)
LRO ^a	GCTTAAGTTCAGCGGGT	(Gottlieb et al., 2000)
T7 promoter ^b	TAATACGACTCACTATAGGG	(Anonymous, 1999)
SP6 ^b	TTCTATAGTGTCACCTAAAT	(Anonymous, 1999)

Table 3.8Oligonucleotides used in this investigation

^a Primers used for the amplification of the entire ITS I to ITS II region. ^b Primers used for sequencing of the recombinant plasmids.



Figure 3.1. One major rRNA transcript showing the ITS I and ITS II regions. The ITS regions are the most widely sequenced region in fungal DNA. The diagram displays the orientation and location of oligonucleotides primers used during the PCR amplification in this investigation (Bruns *et al.*, 1991).

3.17 Electrophoresis of DNA

3.17.1 Agarose Gel Electrophoresis

Electrophoresis of DNA through agarose was performed as described in Sambrook & Russell (2001). Agarose (0.7 to 2.0% (w/v)) was prepared in 1 X TBE or 1 X TAE buffer (Section 3.1.3) and electrophoresis was performed in the same buffer at 100 V. Samples containing a tracking dye (ratio 5:1) were loaded into the wells of the agarose gel. A sample of DNA standards (100 bp ladder) was also loaded to allow comparison of DNA band size. The agarose gel contained 1 μ L of aqueous ethidium bromide (10 mg mL⁻¹) and the DNA was visualised by UV trans-illumination.

3.17.2 Polyacrylamide Gel Electrophoresis (PAGE)

Electrophoresis of DNA through polyacrylamide was performed as described by Sambrook and Russell (2001) with some minor modifications. A polyacrylamide gel (8% stacking gel and 10% resolving gel) was prepared in a 1 X TBE buffer solution. Samples containing a tracking dye (ratio 5:1) were loaded into the wells. A sample of DNA standards (50 bp ladder) was also loaded. Electrophoresis was carried out at 25 mA until the tracking dye reached the bottom of the gel. The gel was removed, carefully rinsed with water and then soaked in an ethidium bromide solution (1 μ g per 100 mL⁻¹ water). The DNA fragments were visualised by UV trans-illumination.

3.18 Polymerase Chain Reaction (PCR) of Fungal DNA Regions

PCR was performed using standard procedures as described in Sambrook and Russell (2001) with minor modifications. Each 25 μ L reaction contained final concentration of

4 μ M deoxy nucleotide tri-phosphates (dNTP's, Promega), 1.5 mM MgCl₂, 1 X reaction buffer (Promega, final concentrations in buffer 10 mM Tris-HCl (pH 9), 50 mM KCl, and 1% Triton[®]X-1000), 2.5 units of *Taq* DNA polymerase (Promega), 1 μ M of each oligonucleotide template and approximately 0.2 ng of DNA template. Control reactions without DNA template were also prepared. The amplification of the ITS I and ITS II regions of the fungal DNA was performed using a PTC-150 MiniCycler thermocycler with a heated lid under the conditions shown in Table 3.9.

Table 3.9

Thermo-eyening	conditions for the an	ipinication of the 1151 a	nd 115 11 regions
Step	No. of cycles	Temperature (°C)	Time (min)
Denaturation	1	95	2.0
Amplification	30	50	0.5
		72	2.0
		95	1.0
Extension	1	72	7.0

4

 ∞

Thermo-cycling conditions for the amplification of the ITS I and ITS II regions

3.19 Purification of DNA

Stop

3.19.1 Purification of DNA from Agarose Gels

1

DNA to be purified was electrophoresed through 1% (w/v) agarose in 1 X TAE buffer containing ethidium bromide. DNA was visualised by UV trans-illumination and the expected size DNA band was excised from the gel using a sterile scalpel and placed into a 1.5 mL microtube. The DNA was then purified using an UltraCleanTM DNA Purification Kit (Mo Bio laboratories, Inc., CA, USA) according to the manufacturer's specifications. Briefly, the mass of the gel slice was calculated and three volumes of ULTRA SALT were added. The microtube was suspended in a water bath at 65°C until the gel was completely dissolved. For the recovery of 1 µg of DNA, 6 µL of ULTRA BIND was added. The sample was incubated at room temperature for 5 min, mixing several times. The sample was then centrifuged for 5 seconds and the supernatant removed and discarded. The pellet was resuspended in 1 mL of ULTRA WASH, mixed thoroughly and centrifuged for 5 seconds. The supernatant was removed. The sample was again centrifuged and carefully aspirated to remove any left over traces of supernatant. The DNA pellet was resuspended in 12 µL of TE, mixed by pipetting and

incubated for 5 minutes at room temperature. The sample was then centrifuged for 1 minute and the supernatant carefully removed, without disturbing the pellet, and transferred to a new microtube. The purified DNA was stored at -20° C.

3.19.2 Purification of Recombinant DNA from *E. coli*

Recombinant plasmid DNA was purified from a 2 mL overnight E. coli culture using the alkaline lysis method (Sambrook & Russell, 2001). A 1.5 mL sample of the overnight culture was transferred to a sterile microtube and centrifuged at 11,000 rpm. The pellet was resuspended in 200 μ L of TE plus RNase (100 μ g mL⁻¹) and mixed by pipetting. Subsequently, 100 µL of ice cold Solution I (50 mM glucose, 25 mM Tris-Cl (pH 8), 10 mM EDTA (pH 8)) was added. An aliquot (200 µL) of freshly prepared Solution II (0.2 N NaOH, 1% SDS) was then pipetted with force into the microtube. The mixture was incubated for 5 minutes at room temperature and then a 200 µL aliquot of Solution III (60 mL of 5 M CH₃COOK, 11.5 mL of glacial CH₃COOH and 28.5 mL of H₂O) was gently added to the side of the sample tube. The microtube was inverted three times and centrifuged for 8 minutes at 11,000 rpm. The supernatant (600 μ L) was carefully transferred to a new tube and the pellet discarded. A 150 µL sample of phenol:chloroform (50:50) was added to the tube, the mixture vortexed for 5 seconds and then centrifuged for 3 minutes at 11,000 rpm. The top layer (approx. 500 µL) was carefully transferred to a new tube. One mL of 100% EtOH was then added, mixed and allowed to incubate for 5 minutes at room temperature. The mixture was then centrifuged at 11,000 rpm for 10 minutes to obtain a white pellet. The EtOH was aspirated from the microtube and the pellet carefully washed twice in 70% EtOH. The ethanol was removed and the microtubes inverted to dry. The recombinant plasmid DNA was then redissolved in 40 μ L of TE (pH 8) and stored at -20°C.

3.20 Enzymatic Modifications of DNA

3.20.1 Restriction Digest of Recombinant DNA

Digestion of the purified recombinant DNA plasmid by endonucleases was performed by adding 4.4 μ L of sterile water, 1 μ L of 10X MulticoreTM buffer (Promega, 1X final concentration was 25mM tris acetate pH 7.8 (25°C), 100 mM potassium acetate, 10 mM DL-dithiothreitol (DTT)), 0.1 μ L of Bovine serum albumin (BSA) (10 mg mL⁻¹) and 4 μ L of the purified recombinant DNA into a 0.6 mL microtube. A 0.5 μ L sample of the appropriate restriction enzyme was added to the microtube and mixed by pipetting. Two enzyme systems were used. The first system used two enzymes, *SacI* (10 units μ L⁻¹) and *ApaI* (10 units μ L⁻¹), and the second only one enzyme, *Eco*RI (12 units μ L⁻¹). A negative control was also prepared in the same manner omitting the restriction enzyme. The samples were incubated for 1 hour at 37°C. The resulting digests were analysed by agarose gel electrophoresis (Section 3.17.1) on a 1% (w/v) agarose gel.

3.20.2 Restriction Enzyme Digestion of PCR DNA

Restriction endonuclease digestion of the purified PCR DNA was performed in 0.6 mL PCR tubes. Separate digestions were prepared using the restrictions enzymes, *Apa*I, *Hae*III, *Hha*I and *Hin*dIII. Each 20 μ L reaction contained a final concentration of 20 μ g BSA (10mg mL⁻¹), 1 X reaction Buffer B (*Alu*I and *Hin*dIII) or C (*Hae*III and *Hha*I) (Promega) (1X final concentrations in: Buffer B, 6 mM tris-HCl, 6 mM MgCl₂, 50 μ M NaCl, 1 mM DTT, pH 7.5 at 37°C; Buffer C, 10 mM tris-HCl, 10 mM MgCl₂, 50 mM NaCl, 1 mM DTT, pH 7.9 at 37°C), 5 units of restriction enzyme (Promega) and approximately 750 ng of DNA template. Samples were incubated for 6 hours at 37°. The resulting restriction digests were analysed by PAGE (Section 3.17.2).

3.21 Cloning of the Fungal ITS I and ITS II Region

3.21.1 Plasmids used for Cloning

The pT7Blue-3 Perfectly Blunt Vector (Novagen, Madison, WI, USA) was used for blunt end cloning and the pGEM[®]-T Easy Vector system (Promega Corporation, Madison, WI, USA) was used for cohesive end cloning in this investigation.

3.21.2 Cloning into pT7Blue-3

The cloning of purified PCR DNA into the pT7Blue-3 vector was performed using a Perfectly Blunt Cloning Kit (Promega Corporation, Madison, U.S.A.). Briefly, end conversion of the DNA was performed by mixing 0.2 μ L of the purified PCR product, 5.0 μ L of the end conversion mix and 0.3 μ L of nuclease free water in a 0.6 mL microtube. The solutions were mixed and incubated for 15 minutes at 22°C. The sample was then heated at 75°C for 5 minutes and then cooled on ice for 2 minutes.

The ligation proceeded by adding 1 μ L of Blunt Vector and 1 μ L of T4 DNA ligase to the end conversion mix. The solutions were mixed and incubated at 22°C overnight. Transformation of the ligation mix was performed by the addition of 1 to 12 μ L of the ligation mix to a microtube containing 50 μ L NovaBlue singles competent *E. coli* cells, which were supplied with the cloning kit. The solution was mixed gently and kept on ice for 5 to 30 minutes. A heat shock was performed by incubation for 30 seconds in a 42°C water bath. The microtube was again placed on ice for 2 minutes and 250 μ L of SOC medium was added.

3.21.3 Cloning into pGEM[®]-T Easy

Cloning of purified PCR DNA into the pGEM[®]-T Easy vector was performed using a cloning kit obtained from Promega Corporation (Madison, U.S.A.). A ligation reaction was prepared by adding 2 μ L of deionised water, 5 μ L of 2X rapid ligation buffer, 1 μ L of the vector (50 ng), 1 μ L of the purified PCR product (36.5 ng) and 1 μ L of T4 DNA ligase into a 0.6 mL microtube. The sample was mixed and incubated at 4°C overnight. The microtubes were then centrifuged briefly and 2 μ L of the sample was transferred to a new 1.5 mL microtube on ice. The transformation mix was prepared by transferring 50 μ L of *E. coli* JM109 competent cells into the ligation mix and gently mixing. The microtube was left on ice for 20 min, heat shocked for 45 to 50 seconds in a water bath at 42°C and then returned to the ice for 2 minutes. A 950 μ L sample of SOC medium was then added to the transformation mix and left to incubate at 37°C for 1.5 hours, with shaking at a speed of 150 rpm.

3.21.4 Blue / White Screening for Recombinant Plasmids

Using a sterile glass spreader, the surface of freshly prepared LB agar plates was spread with 35 μ L of 50 mg mL⁻¹ X-gal in dimethylformamide and 20 μ L of 100 mM IPTG in water. Once the plates were dry, the transformation mix was spread over the surface of the plates. The plates were incubated overnight at 37°C and then inspected for growth. White colonies were indicative of the bacteria carrying the recombinant plasmid, whilst blue colonies were indicative of the bacteria carrying the vector that contained no insert. To produce large amounts of recombinant plasmid for further purification, single white colonies were selected and grown in 2 mL of LB broth at 37°C overnight.

3.22 DNA Sequencing

Recombinant DNA was sequenced at the Wellcome Trust Sequencing Centre, MIRD. Sequences were determined by the chain termination method with the use of the DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, UK), using an AVI377 automated DNA sequencer. A typical reaction consisted of 400 ng of plasmid DNA together with 3.2 pmol of primer. Primers used for sequencing are listed in Table 3.8. For each sample under investigation, three recombinant clones were chosen and sequenced in the forward and reverse direction. DNA sequence data were compiled and analysed using the program Sequencher version 3.0 (Genecodes, Ann Arbor, MI, U.S.A.).

3.23 Sequence Data Analysis

Sequence data was compared to some Australian isolates (Smith & Sivasithamparam, 2000a) and some isolates used by Moncalvo et al. (1995a) and are listed in Table 3.10. DNA Sequences (ITS I, ITS II and ITS I + ITS II) were aligned using ClustalW program in Biology Workbench version 3.2 (Subramaniam, 1999). Final alignments were visually examined and adjusted manually. Phylogenetic analysis was conducted using the program MEGA version 2.1 (Kumar *et al.*, 2001). Maximum parsimony (MP) analysis was performed on separate ITS I, ITS II data sets and on the combined data set. Missing data/gaps were treated as pairwise deletions. A heuristic search was performed using close neighbourhood interchange (CNI) branch swapping on starting trees generated with twenty random addition sequences. A strict consensus tree was derived The robustness of the internal branches was evaluated by 100 from the results. bootstrap replications using a heuristic search starting with twenty random addition sequences. Also evaluated were MP trees set to complete deletion (all gaps/missing data were removed from the analysis) and Minimum Evolution Trees (MEv). Pairwise nucleotide differences were calculated using Pairwise distance options in MEGA.

Table 3.10

Group ^a	Ganoderma Species ^b	Collection No. ^c	Isolation ^d	ITSI/ITSII Accession No. ^e
-	G. cupreum (H1)	SUT H1	QLD	AY569450
-	G. weberianum (H2)	SUT H2	QLD	AY569451
-	Ganoderma sp. (H3)	SUT H3	QLD	AY569452
-	Ganoderma sp.	SE1 BS	?	-
5.1	G. weberianum	CBS 219.36	Philippines	X78734/X78755
5.1	G. weberianum	CCRC 37081	Taiwan	Z37064/Z37086
5.1	G. weberianum*	RSH 0821	Taiwan	X78751/X78772
5.2	G. weberianum**	DFP4483	QLD	-
5.2	G. weberianum	DFP 8401	NSW	-
5.2	G. weberianum**	DFP 8405	NSW	-
6.3	Grp. 6.3 ^{###}	UWA 8	WA	-
6.3	Grp. 6.3 ^{###}	QFRI 8147.1	QLD	-
6.3	Grp. 6.3 ^{###}	QFRI 8647.1	QLD	-
6.3	Grp. 6.3 ^{###}	DAR 73779	WA	-
7	Grp. 7 ⁺⁺	RSH 1109	Japan	X78747/x78768
7	Grp. 7 ⁺⁺	RSH BLC	Taiwan	Z37097/z37078
7	Grp. 7 ⁺⁺	RSH J2	Japan	X78746/x78767
7	Grp. 7**	HMAS 60537	China	Z37050/Z37074
8.1	G. sinense	ZHANG 1734	China	Z37066/Z37103
8.1	G. sinense [#]	RSH 0109	Taiwan	X78752/X78773
8.2	G. cupreum	DFP 3896	QLD	-
8.2	G. cupreum	DFP 4336	NSW	-
8.2	G. cupreum***	QFRI 8678.1	QLD	-
11.3	G. australe	DAR 73781	WA	-
11.3	<i>G. australe</i> ^{##}	DFP 3386	TAS	-
11.3	G. australe	UWA 92	WA	-
11.3	G. australe	UWA 108	QLD	-
10	G. incrassatum	DAR 73783	NT	-
10	G. incrassatum	SE3	Thailand	-
11.2	G. adspersum	CBS 351.74	Belgium	X78742/X78763
11.2	G. adspersum ^{##}	IMI157816	UK	_
-	G. lucidum	ATCC 46755	USA	Z37052/Z37076
-	Fomitopsis cf. rosea ^f	JMM T92-10	Taiwan	X78754/X78775

Ganoderma isolates used for the phylogenetic analysis in this investigation

Shaded rows are *Ganoderma* species determined in this investigation; ^a Classification from Smith & Sivasithamparam (2000a); ^b Identification at time of isolation: **G. microsporum*, ***G. lucidum*, ****G. chalceum*, [#]*G. Formosan*, ^{##}*G. applanatum*, ^{###} *Ganoderma* sp. aff. *lucidum*, ⁺⁺*G. tsugae*, and were renamed by Smith & Sivasithamparam (2000a); ^c ATCC, American Type Culture Collection, Maryland, USA; CBS, Centraalbureau voor Schimmelcultures, Baarn, The Netherlands; CCRC, Culture Collection and Research Centre of Hsinchu, Taiwan; CSIRO, Melbourne, Australia; IMI, International Mycological Institute, Egham, Surrey, UK; DAR, Orange Agricultural Institute, Orange, Australia; DFP, Division of Forest Products; HMAS, Herbarium Mycologicum Academiae Sinicae, Institute of Microbiology, Academia Sinica, Beijing, China; QFRI, Queensland Forest Research Institute, Department of primary industries, forestry, Indooropilly, Queensland; RSH, Collection of J. M. Moncalvo, Duke University, Durham, NC, USA; SUT, Swinburne University of Technology, Hawthorn , Australia; UWA, personal collection of B. J. Smith; ZHANG, X. Q. Zhang, Beijing, China; ^d Australian state or territory abbreviations, NSW, New South Wales; NT, Northern Territory; QLD, Queensland; TAS, Tasmania; VIC, Victoria; WA, Western Australia; ^e Accession numbers obtained from the National Centre for

Biotechnology Information (NCBI) database, all other sequence data was obtained from Smith & Sivasithamparam (2000a). F *Fomitopsis* cf. *rosea* (not a *Ganoderma* species) was used as an outgroup in the phylogenetic analysis.

Chapter Four

Molecular and Phylogenetic Analysis

4.1 Introduction

The taxonomic identification of an organism is an essential part of the investigation of a new species or isolate of a known species. It is imperative that the organism is correctly identified so that when reference is made to published material, the experimental data becomes valid and may be used and compared by other investigators. Classical identification techniques used in the identification of fungi include the macroscopic and microscopic comparison of morphological features such as the fruiting body colour, spore size, pore size and cutex. With the progress in molecular genetics, these identification techniques are now being replaced by more modern methods that analyse and compare the ribosomal RNA (rRNA) genes from different fungal isolates. Such molecular techniques used for fungal identification to date include PCR coupled with RFLP (Miller et al., 1999), isozyme analysis (Gottlieb et al., 1998; Gottlieb & Wright, 1999b; Smith & Sivasithamparam, 2000b) and direct sequencing of the rRNA genes (Hong et al., 2002; Moncalvo et al., 1995a, 1995c; Smith & Sivasithamparam, 2000a). In addition, developments in the field of bioinformatics have enabled the interpretation of this sequence information to yield information about the evolutionary relationships between new and existing fungal species. This chapter reports the identification of three Australian Ganoderma isolates using molecular techniques and bioinformatic tools.

4.2 Overview

The identification of the three Australian *Ganoderma* isolates using molecular techniques and tools was performed. DNA was extracted from the fungal mycelium of the three isolates. Specific ITS regions were amplified using PCR and analysed by RFLP. The specific regions were also cloned into the pGEM[®] -T Easy vector to produce recombinant DNA. Sequence analysis of the recombinant plasmids afforded sequence data, which in turn, were examined to produce consensus sequences for each isolate. Consensus sequences were then compared against sequence data from other known *Ganoderma* species using sequence analysis software.

4.3 **Results and Discussion**

4.3.1 Isolation of Three Australian *Ganoderma* Species

Three *Ganoderma* species were isolated from the Cairns State Forest in Queensland, Australia, by a local mushroom cultivator, Mr. Henk Voogt. At the time of isolation, the three fungi were identified to be from the Ganodermataceae family because of typical characteristics they had in common with other members of this family. For this reason, the three isolates have been designated *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 in this investigation. Both *Ganoderma* H1 and H3 were isolated from the trunks of dead trees, whilst *Ganoderma* H2 was isolated from the soil. *Ganoderma* H2 appeared to be growing on the matter in the soil, such as rotting branches and roots. The exact coordinates from where they were isolated are 16.59'51"S, 145.40'01"E (H1), 16.48'06"S, 145.36'59" (H2) and 16.56'04", 145.36'56" (H3). Figure 4.1 shows photos taken at the time of isolation of the three living fungal isolates.



Figure 4.1. Representative photos of the three Australian *Ganoderma* species isolated from the Cairns State Forest. (a) *Ganoderma* H1, (b) *Ganoderma* H2, and, (c) *Ganoderma* H3. All three isolates were living at the time of photography and can be seen growing on either wood or organic matter.

Attempting to identify new fungi based on traditional classification methods could possibly lead to misidentification, especially if one is not a mycological taxonomist. Ultimately, this would contribute to the taxonomic crisis in the classification of *Ganoderma* species (Ryvarden, 1991). Therefore, traditional identification techniques were not employed and only the molecular identification was considered in this investigation.

4.3.2 Molecular Analysis

Molecular techniques are becoming increasingly popular in the identification of new fungal species. In this section the identification of the *Ganoderma* isolates was performed using modern molecular techniques including PCR, RFLP and the cloning and sequencing of DNA fragments. DNA was extracted from the mycelium of the three isolates and the ITS I and ITS II regions of the rRNA genes amplified using specific primers. This specific region, which contains fungal evolutionary information, was then subjected to analysis by RFLP. A type *G. lucidum* specimen was also included for comparison against the three isolates. The specific ITS regions were also cloned, subjected to sequence analysis and in turn, the sequence data analysed and compared against other *Ganoderma* isolates using appropriate software.

4.3.2.1 DNA Extraction and Amplification by Polymerase Chain Reaction (PCR)

DNA was extracted from the dried fungal mycelium of *Ganoderma* H1, H2, H3 and *G. lucidum*, which had been cultivated in MEB in shaker flasks (Section 3.10.1). The extraction process employed a commercial extraction kit and is described in Section 3.15. The resulting DNA was electrophoresed through a 1% (w/v) agar gel (Section 3.17.1) and it was observed that good yields of fungal DNA were obtained (results not shown).

PCR of *Ganoderma* H1, H2, H3 and *G. lucidum* (Section 3.18) DNA, using the primer pairs BMB-CR/LR0, amplified the internal transcribed spacer (ITS I and ITS II) regions, the 5.8S region and a small portion of the 18S and 25S rDNA genes (Figure 3.1). All four isolates yielded an amplified product of approximately 750 to 800 bp, as well as some impurities (Figure 4.2a). These impurities were removed upon gel

purification (Section 3.19.1) and again electrophoresed through a 1% (w/v) agarose gel. Figure 4.2 depicts two typical agarose gels showing the PCR product obtained from each isolate before (Figure 4.2a) and after purification (Figure 4.2b).



Figure 4.2. PCR and purification of DNA from four *Ganoderma* isolates. (a) PCR DNA before purification and (b) purified PCR DNA. Lanes 1, 2, 3 and 4 contain DNA from *Ganoderma* H1, *Ganoderma* H2, *Ganoderma* H3 and *G. lucidum*, respectively. Lane 5 in (a) is a blank, and lane M contains a standard DNA ladder consisting of fragments ranging from 100 to 1500 bp;

The purified PCR product, for all four isolates, corresponds to the single DNA fragment that can be seen at approximately 800 bp (Figure 4.2b), indicating that the size of the combined ITS I and ITS II region was close to that observed by Moncalvo *et al.* (1995c) and Gottlieb *et al.* (2000). The exact size of the PCR fragments that had been generated with the primer pair LRO/BMB-CR was determined after sequence analysis and is described later in Section 4.3.2.5. This was found to be 774 bp, 770 bp and 758 bp for *Ganoderma* H1, H2 and H3 respectively. The exact size of the *G. lucidum* fragment was not determined in this investigation.

4.3.2.2 Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) analysis was performed on the *Ganoderma* isolates to establish that they were different species, as different *Ganoderma* species generally display different restriction patterns (Gottlieb *et al.*, 2000). One *G. lucidum* isolate was also included in the RFLP analysis for comparison against a type species. The single restriction digestions were performed on the purified

PCR product of each isolate as described in Section 3.20.2. The digests were then analysed by polyacrylamide gel electrophoresis (PAGE) using an 8% stacking and 10% resolving gel (Section 3.17.2). The restriction patterns seen on the polyacrylamide gel are shown in Figure 4.3. To enable accurate estimation of fragments, a theoretical restriction digest was performed on the sequence data obtained from sequence analysis of the three Australian isolates determined later in this Chapter (Section 4.3.2.5). This was used for comparison against the experimental data. No theoretical digest was possible on *G. lucidum*, as GenBank only held sequence data for the separate ITS regions and not the entire ITS I to ITS II region, which incorporates the 5.8S rRNA. A restriction map showing the location of sites on the sequences of the three Australian *Ganoderma* isolates is also presented in Figure 4.4. This map gives a clear indication of where each fragment came from within the sequence.

Suitable enzymes for the RFLP analysis were ones that recognised many restriction sites on the sequences to be analysed. Six restriction enzymes were tested in a preliminary study (results not shown) and the four that gave the best restriction patterns were selected for comparative analysis. The patterns observed for the four restriction enzymes, *AluI*, *HaeIII*, *HhaI* (*CfoI*) and *HindIII*, were also compared to patterns that have been published for some known *Ganoderma* species (Gottlieb *et al.*, 2000).

Digestion using the enzyme *Alu*I gave different restriction patterns for the three Australian *Ganoderma* isolates and *G. lucidum* (Figure 4.3a). A fragment of approximately 350 bp was seen for all four isolates. This corresponded to the theoretical fragment of 336/335 bp for *Ganoderma* H2 and *Ganoderma* H3 (Table 4.1). However, this fragment was not expected for *Ganoderma* H1 and was thought to be a result of some incomplete digestion at the site located at nucleotide (nt) 551 (Figure 4.4). Subsequent digestions of *Ganoderma* H1 using *Alu*I confirmed this (results not shown). *Ganoderma* H1 also produced a fragment of 260 bp, which was not observed for any of the other *Ganoderma* isolates (Figure 4.3a). The fragment seen at 195 bp for the three Australian *Ganoderma* H1). This fragment was the result of a digestion at the site at nt 195 (or nt 196 for *Ganoderma* H1) for each of the sequences (Figure 4.4).



Figure 4.3. RFLP-PAGE of *Ganoderma* H1, *Ganoderma* H2, *Ganoderma* H3 and *G. lucidum* using the enzymes: (a) *AluI*, (b) *HaeIII*, (c) *HindIII* and, (d) *HhaI*. Lanes 1 to 4 contain *Ganoderma* H1, H2, H3 and *G.* lucidum, respectively. Lane M contains the DNA standards. Fragment sizes are shown for the DNA standard. The inset of (c) shows the results of a subsequent digestion of *Ganoderma* H3 using the restriction enzyme *HindIII*.

The four smallest fragments observed in the *Alu*I restriction digest of *Ganoderma* H1 (72, 75, 76 and 95 bp) and three smallest fragments seen for *Ganoderma* H2 (69, 75 and 97 bp) corresponded with those for the theoretical digest (Table 4.1). Two of the similar sized fragments (75 and 76 bp) for *Ganoderma* H1 were just resolved (Figure 4.3a). The theoretical digest of *Ganoderma* H3 displayed three small fragments, one of 70 bp and two of 79 bp, whilst the experimental digest only displayed two small fragments at 70 and 80 bp (Figure 4.3a). This was a result of the two fragments of equal size (79 bp) migrating through the gel as the same fragment. On close inspection of the gel it could be seen that the fragment at 80 bp was thicker than the other fragments, indicating a doublet band (Figure 4.3a). *G. lucidum* only showed two smaller sized fragments at 70 and 50 bp. The digestion pattern exhibited by *G. lucidum* using *Alu*I

was the same as those observed by Gottlieb *et al.* (2000) for some different *G. lucidum* isolates.

Table 4.1

Comparison of the RFLP fragments of three Australian Ganoderma isolates and an American type Ganoderma lucidum

Ganoderma	Restriction Enzyme Fragment ^a				
Isolate	AluI	HaeIII	HhaI (CfoI)	HindIII	
H1	400* 260 200 95 80 73	600 200	300 290 195	$770^{*}590$ 220	
H1 ^b	260 196 95 76 75 72	574 200	302 282 190	549, 225	
H2	350 200 95 77 70	400 200 180	500 300	770	
H2 ^b	334 195 97 75 69	393 199 178	492 278	770	
Н3	350 195 (80) 70	500 200 185	(300) 190	550 220	
H3 ^b	335 195 79 79 70	377 199 182	287 281 190	528 230	
G. lucidum	350 195 70 50	400 200 180	310 290 190	550 220	

^a The RFLP of the PCR fragments from *Ganoderma* H1 (777 bp), H2 (773 bp) and H3 (760 bp), and *G. lucidum*. The exact size of the PCR fragment for *G. lucidum* was not determined; Restriction digestion was performed on the entire ITS I to ITS II region. Each number indicates the size of the fragment observed in bp; ^b The theoretical RFLP fragment sizes. This restriction enzyme products were determined from the sequence data; ^{*} Incomplete digestion; numbers in brackets () indicate bands with double intensity, i.e.: doublet bands; number in **bold** typeface indicates an unexpected result.

When locations of the *Alu*I restriction sites on the sequences of the three Australian *Ganoderma* isolates were compared, a distinct difference was observed (Figure 4.4). The locations of *Alu*I on the sequences of *Ganoderma* H1 and H2 were similar (nt 196, 291, 551, 626 and 698 and nt 195, 292, 626 and 695, respectively), with a discrepancy at nt 551 (Figure 4.4). The sequence of *Ganoderma* H3 exhibited slightly different restriction sites, with the exception of the first site, at nucleotide positions 195, 530, 609 and 679 (Figure 4.4). On general observation and comparison of the three restriction maps, the cut sites appear to align (Figure 4.4). However, it was observed that the restriction sites at the 3' end on the *Ganoderma* H3 sequence were always approximately twenty nucleotides distal to those sites on the *Ganoderma* H1 and *Ganoderma* H2 sequences. This was due to approximately twenty to thirty missing nucleotides in the middle of the ITS I region of *Ganoderma* H1 (Appendix 1A). Comparing the three isolates, it was clear that two nucleotide positions for *Alu*I were different (nt 551/530 and nt 292/291) (Figure 4.4).



Figure 4.4. Comparison of the restriction enzyme sites on the sequences of: (a) *Ganoderma* H1 (774 bp), (b) *Ganoderma* H2 (770 bp) and (c) *Ganoderma* H3 (758 bp); The restriction digestion was performed on the combined ITS I to ITS II regions, which incorporated the 5.8S region; The four enzymes used were *AluI*, *HaeIII*, *HindIII* and *HhaI*. The location of the restriction site is the number of nucleotides along the sequence in the direction 5' to 3'.

Ganoderma H1 had only one restriction site for the enzyme *Hae*III at nt 199, whilst both *Ganoderma* H2 and *Ganoderma* H3 had two restriction sites at nt 199 and 592 and nt 199 and 576, respectively (Figure 4.4). As previously observed, the restriction site at the 3' end of the *Ganoderma* H3 sequence was approximately twenty nucleotides distal to the corresponding position on the *Ganoderma* H2 sequence. Overall, restriction digestion with *Hae*III resulted in two fragments for *Ganoderma* H1 (600 and 200 bp)

and three fragments for *Ganoderma* H2, *Ganoderma* H3 and *G. lucidum* (400, 200 and 180 bp; 500, 200 and 185 bp; 400, 200 and 180, respectively) (Figure 4.3b). The sizes of fragments in the experimental digests correlated with those in the theoretical digest, with the exception of one fragment from *Ganoderma* H3, which was observed to be 500 bp instead of 377 bp (Table 4.1). This was not expected and could not be explained, as the size of the *Ganoderma* H3 DNA analysed was 760 bp (Figure 4.3b) and not 885 bp, as indicated by the results (Figure 4.3b and Table 4.1).

There were no *Hin*dIII restriction sites present in the sequence of *Ganoderma* H2, resulting in only one fragment of 730 bp (the original fragment) on the polyacrylamide gel (Figure 4.3c). Initial digestion with *Hin*dIII also did not cut the *Ganoderma* H3 sequences, however, subsequent digests yielded two fragments (inset of Figure 4.3c). There was little difference between the restriction patterns of *Ganoderma* H1, H3 and *G. lucidum*, as *Hin*dIII cut these isolates at one site of similar location resulting in two fragments (Figure 4.3c and Figure 4.4). In contrast, the *G. lucidum* isolates investigated by Gottlieb *et al.* (2000) did not contain any restriction sites for *Hin*dIII. In addition, they found only one isolate within the subgenus *Ganoderma*, *G. tuberculosum*, was cut with *Hin*dIII. *Ganoderma* H1 also displayed an additional band at 770 bp, which was the undigested original fragment (Figure 4.3c). Overall the experimental digestions corresponded with the theoretical digestions resulting in similar fragment sizes (Table 4.1).

The *Hha*I restriction enzyme cut at two sites of similar positions for both *Ganoderma* H1 and H3 (nt 302 and 492 and nt 281 and 471, respectively), taking into consideration the twenty nucleotide difference for *Ganoderma* H3 (Figure 4.1). This resulted in similar restriction patterns with two fragments of slightly different sizes (300, 290 and 195 bp for *Ganoderma* H1, and 300, 300 and 190 bp for *Ganoderma* H3) (Figure 4.3d). The 300 bp and 290 bp fragments of *Ganoderma* H1 were only just resolved on close inspection of the polyacrylamide gel, whilst the two fragments at 300 bp for *Ganoderma* H3 were unresolved, but the band was wide and therefore, indicative of a doublet (Figure 4.3d). All fragments observed in the experimental digests corresponded to those of the theoretical digest (Table 4.1). *Hha*I cut *Ganoderma* H2 at one position, nt 492

(Figure 4.4), giving two fragments sized 492 and 281 bp, which also corresponded to the theoretical digest of this isolate (Table 4.1).

Overall, the sum of fragments for the different RFLPs was similar in size to the undigested PCR product. Some discrepancies were observed, such as the extra bands observed in the digest of *Ganoderma* H1 with *Alu*I and *Hin*dIII. On supplementary digestions, these bands were confirmed to be products of incomplete digestion (results not shown).

Considering the four RFLPs together for each Australian isolate, no two groups of restriction patterns were the same, which can be clearly seen on the restriction map of the sequences (Figure 4.4). This strongly suggested that the three Australian isolates belonged to different species. Restriction patterns were also different to those seen by Gottlieb *et al.* (2000). This suggested that the Australian isolates investigated were not a type species of *G. platense*, *G. praelongum*, *G. sessile*, *G. sessiliforme*, *G. tuberculosum*, *G. zonatum*, *G. lipsiense*, *G. lobatum* or *G. tornatum*. It has been reported that RFLP results can be ambiguous and that RFLPs may in fact produce different patterns within the same species (Gottlieb *et al.*, 2000). This was the case for the RFLP pattern for *G. lucidum* in this investigation in that its restriction pattern was not similar to any of the patterns reported by Gottlieb *et al.* (2000) for the same species. This demonstrates the sensitivity of using DNA in taxonomic studies and highlights the need to use more than one tool in the identification process.

4.3.2.3 Sequencing of the Purified PCR Fragment

An attempt was made to directly sequence the purified DNA fragments. After repeated attempts, sequence data could not be obtained from the DNA fragments. Direct sequencing of the purified PCR product from *Ganoderma* species has been successfully performed by (Moncalvo *et al.*, 1995a, 1995c). The inability to obtain sequence data from the purified PCR fragment could not be explained, although, it was thought that the most likely problem was due to the low melting temperatures (T_M) of the primers (LRO $T_M = 47^{\circ}$ C, BMB-CR $T_M = 54^{\circ}$ C) used in the sequencing process. Primers with low T_M have been shown to inhibit the sequencing process (personal communication, Dr. Gerard Gibbs, MIRD, Clayton, VIC, Australia). The inability to obtain sequence

data directly from the PCR fragment prompted the decision to utilise alternative methods such as the production of recombinant DNA (Section 4.3.2.4).

4.3.2.4 Cloning of the Purified PCR Fragment

The purified PCR fragments were cloned into two commonly used vectors to produce recombinant plasmids. This enabled sequencing of the plasmids using universal primers. Initially, the PCR product was cloned into the pT7Blue-3 blunt end vector. Screening for transformants gave a low yield of colonies on a number of attempts (approximately three white colonies were obtained on each attempt). Low yields of white colonies were also observed after altering variables such as ligation reaction volume and time, as well as transformation time. In addition, analysis of these white colonies indicated that there were no PCR inserts of the expected size between 750 and 800 bp (results not shown). Therefore, a different vector system was employed, pGEM[®]-T Easy.

4.3.2.4.1. Cloning into pGEM[®]-T Easy Vector System

The purified PCR fragment of *Ganoderma* H1, H2 and H3 was successfully cloned into the pGEM[®]-T Easy vector (Figure 4.5). Preparation, ligation and transformation of the vector and DNA insert was performed as described in Section 3.21.3. A recombinant vector of *G. lucidum* was not prepared, as sequence data for the ITS I and ITS II regions were available in GenBank. Blue/white screening for recombinants gave a ratio of 40:60 (blue: white) (Section 3.21.4). White colonies were picked from the plate and plasmid purification was performed as described in Section 3.19.2. The recombinant plasmids carrying the *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 PCR products were designated pGEM-H1, pGEM-H2 and pGEM-H3, respectively.

4.3.2.4.2. Restriction Digest Analysis of the Recombinant Plasmids

The screening of the recombinant plasmids led to some valuable information about restriction sites in the *Ganoderma* H1, H2 and H3 DNA fragments. A number of purified plasmids were chosen and analysed further for the expected 750 to 800 bp insert by restriction digestion (Section 3.20.1). The resulting digests were electrophoresed through 1% (w/v) agarose gels (Section 3.17.1). Two different digest systems were employed for the screening of the recombinant plasmids. The first system

used the restriction enzymes *Apa*I and *Sac*I, which cut at nt 15 and 110, respectively, in the plasmid vector sequence. The second system used the restriction enzyme *Eco*RI. There were two restriction sites in the plasmid vector for this enzyme at nt 53 and 71 in the vector.



Figure 4.5. A vector map of pGEM[®] -T Easy showing: (a) the original pGEM[®]-T Easy vector (3015 bp) (b) the vector with the *Ganoderma* H1 PCR insert, pGEM-H1 plasmid (3789 bp); The diagrams show the β -lactamase coding region, Amp^r (aqua) and the *lacZ* region (green), which is split when the PCR product is inserted into the multiple cloning region (white).

The PCR products for *Ganoderma* H1, H2 and H3 were inserted into the region between nucleotides 60 and 61 in the pGEM[®]-T Easy vector (Figure 4.5b). Therefore, each plasmid increased in size by the insert PCR piece (i.e. vector (3015 bp) plus *Ganoderma* H1 (774 bp) or *Ganoderma* H2 (770 bp) or *Ganoderma* H3 (758 bp)). Figure 4.5b shows a representative plasmid with the *Ganoderma* H1 insert (774 bp), which increases the plasmid to 3789 bp. Figure 4.5 also shows the location of the restriction sites in the plasmid. This demonstrates that when the plasmid was cut with the enzymes *Apa*I and *Sac*I, the size of the insert fragment became slightly larger (869 bp) than the original insert (774 bp). This larger sized fragment was seen in the following digests using the enzymes *Apa*I and *Sac*I (Figures 4.6a, 4.7a, 4.8a). *Eco*RI did not increase the size of the insert fragment, as the restriction sites for this enzyme closely flanked the region of where the PCR product was inserted into the vector (Figure 4.5b).

The screening process was primarily performed to select three recombinant plasmids to be sequenced (Section 4.3.2.5), for each of the isolates. During PCR of the DNA fragments, there is a small chance that the enzyme, *taq* DNA polymerase, may introduce a base error into the sequence. For this reason, deriving a consensus sequence from three clones reduces the chances of obtaining the DNA sequence with an incorrect base.



Figure 4.6. Restriction digests of a number of recombinant plasmids, pGEM-H1, using (a) *ApaI* and *SacI*, and (b) *EcoRI*. Lane M contains the 100 bp DNA marker, lanes 1 to 6 contain the purified recombinant plasmid, lane 7 has purified PCR DNA and lane 8 has the plasmid vector pGEM®-T Easy.
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isolates of pGEM-H1, using the restriction enzymes (a) ApaI and SacI, and (b) EcoRI. All recombinant plasmids in lanes 1 to 6, when cut with ApaI and SacI (Figure 4.6a) produced a large plasmid fragment (3000 bp) and a smaller fragment. The expected fragment size, providing the insert did not contain the restriction sites for the two enzymes, was approximately 900 bp when taking into account the location of the restriction sites on the vector (Figure 4.5). Lanes 1, 2 and 6 showed fragments approximately 700 bp in size, whilst lanes 3, 4 and 5 showed the expected fragment size (900 bp). When *Eco*RI was used as the restriction enzyme, the expected fragment size of 800 bp was seen in lanes 1, 2, 5 and 6 (Figure 4.6b). Although the correct insert fragment size was seen in lane 1 and 2 when cut with *Eco*RI (Figure 4.6b), the previous digest using ApaI and SacI resulted in a fragment size that did not correspond with the insert (Figure 4.6a). Therefore, plasmids in lanes 1 and 2 were identified as not containing the correct insert. The digests in lanes 3 and 4 showed one large fragment (3000 bp), which corresponded to the vector DNA, and two smaller fragments at 500 and 350 bp. This demonstrated that the Ganoderma H1 insert had an EcoRI site, as indicated by the digestion of the pure DNA in lane 7. One of the fragments in lane 3 looked slightly smaller than that in lane 4, which suggested that the inserts were in opposite directions in the two recombinant plasmids. From the results obtained, recombinant plasmid in lanes 3, 4 and 5 for Ganoderma H1 contained the desired insert and were chosen for further analysis.



Figure 4.7. Restriction digests of a number of recombinant plasmid, pGEM-H2, using (a) *Apa*I and *Sac*I, and (b) *Eco*RI. Lane A contains the 100 bp DNA marker, lanes 1 to 6 contain the purified recombinant plasmid, lane 7 has purified PCR DNA and lane 8 has the plasmid vector pGEM[®]-T Easy.

The restriction digests of the pGEM-H2 isolates using the enzymes *Apa*I and *Sac*I (Figure 4.7a) resulted in two fragments in all lanes. The first fragment at 3000 bp corresponded to the vector DNA and the second fragment at approximately 900 bp corresponded to the *Ganoderma* H2 PCR insert. This second fragment suggested that all recombinant plasmids contained the insert. The correct insert in all recombinant plasmids was confirmed by the digestion with *Eco*RI (Figure 4.7b). All digests (lanes 1 to 6) yielded a plasmid fragment at 3000 bp and two smaller fragments at approximately 500 bp and 350 bp, which indicated that the *Ganoderma* H2 insert also had an *Eco*RI site, as indicated in lane 7. *Ganoderma* H2 recombinant plasmids in lanes 3, 4 and 5 were chosen for further analysis.



Figure 4.8. Restriction digestion of a number of recombinant plasmids, pGEM-H3, using (a) *Apa*I and *Sac*I, and (b) *Eco*RI. Lane A contains the 100 bp DNA marker, lanes 1 to 9 contain the purified recombinant plasmid, lane 10 has purified PCR DNA and lane 11 has the plasmid vector pGEM[®]-T Easy.

Restriction digestion of pGEM-H3 using the enzymes *Apa*I and *Sac*I (Figure 4.8a) resulted in an assortment of fragment sizes in lanes 1 to 9. All lanes contained the vector fragment at 3000 bp. Lanes 3, 4, 5, 6 and 9 displayed an insert fragment of the expected size at approximately 900 bp. Lanes 1, 2 and 8 showed a fragment slightly larger than 900 bp, but it was thought to be too large to be the correct insert. The recombinant plasmid in lane 7 did not contain the correct insert, as the fragment size of 800 bp was too small. An *Eco*RI digest of the pGEM-H3 recombinant plasmids (Figure 4.8b) confirmed the correct insert in lanes 3, 4, 5 and 9. Lane 10 shows the digestion of the purified PCR product, which exhibits two fragments of the sizes 450 and 350 bp. Restriction digestion of the pGEM-H3 plasmids in lanes 3, 4, 5 and 9 yielded two

fragments of 450 and 350 bp, indicating that they were all the correct insert (Figure 4.8b). All other recombinant plasmids were determined to not contain the correct plasmid. *Ganoderma* H3 recombinant plasmids in lanes 3, 4 and 5 were chosen for further analysis.

Overall, the restriction analysis showed that the insert for each *Ganoderma* isolate, which encompassed the ITS I, ITS II and 5.8S regions, contained one restriction site for *Eco*RI, but no restriction sites for either *Apa*I or *Sac*I. The sizes of the resulting two *Eco*RI fragments suggested that this restriction site was located in the 5.8S region. This is in agreement with published data (Vilgalys *et al.*, 1994).

4.3.2.5 Sequencing of the Recombinant Vectors

Since the insert DNA was derived by PCR, it is possible that *in vitro* mutations may be present as a result of the error prone nature of *Taq* polymerase (Sambrook & Russell, 2001). Therefore, three isolates of each recombinant plasmid, pGEM-H1, pGEM-H2 and pGEM-H3, were characterised by double strand DNA (dsDNA) sequencing (Section 3.22). A representative chromatogram showing the sequence output is shown in Figure 4.9. The set of sequence data for each isolate was aligned, compared and constructed into a consensus sequence using the program Sequencher version 3.0 (Genecodes, Ann Arbor, MI, U.S.A.). The consensus sequences obtained for *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 are displayed in Figures 4.10a,b,c, respectively. The consensus sequences yielded the exact size of the combined ITS I to ITS II region, which were determined to be 774 bp for *Ganoderma* H1, 770 bp for *Ganoderma* H2 and 758 bp for *Ganoderma* H3.



Figure 4.9. Typical dsDNA sequence data obtained from sequence analysis of recombinant plasmid.

a.

b.

c.

TGTACACCGCCCGTCGCTACTACCGATTGAATGGCTTAGTGAGGTCTTGGGATTGGCTTCGGGGAGCCGGCAACGGCACCCTGTCG CTGAGAACTTGATCAAACTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGGAAGGATCATTATCG AGTTTTGACTGGGTTGTAGCTGGCCTTCCGAGGCATCGTGCACGCCTGCTCATCCACTCTACACCTGTGCACTTACTGTGGGTTATG GATTGCGTGTAAAAGCGCGTCCGTGCCTGCGTCTTACCACAAACACTATAAAAGTATCAGAATGTGTATTGCGATGTAACGCATCTAT ATACAACTTTCAGCAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAG TGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGAAGTGATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATCTTCAACCTACA AGCTTTTGAAATAATGGCTTGTTAGGCTTGGACTTGGAGGCTTGTCGGCCGTTACCGGCGCTCCTCTCAAACGCATTAGCTTGGTT CCTTTGCGGATCGGCTTGTCGGTGTGATAATGTCTACGCCGCGGACCGTGACGCGTTTGGCGAGCTTCTAATCGTCTCCGCTTTTGGGA CAACTTTTATGACCTCTGACCTCAAATCAGGTAGGACTACCCGCTGAACTTAAGCA

Figure 4.10. The consensus sequence for: (a) *Ganoderma* H1 (774 bp), (b) *Ganoderma* H2 (770 bp) and, (c) *Ganoderma* H3 (758 bp). Each consensus sequence starts at the ITS I region and finishes at the end of the ITS II region and are written in the 5' to 3' direction. Highlighted sections indicate sequences complementary to universal primers in fungal analysis in red (<u>BMB-CR</u>) and blue (<u>LRO</u>).

The amplification of the ITS I and ITS II regions has been performed separately using the primer pairs BMB-CR/5.8S and 5.8SR/LRO (Gottlieb *et al.*, 2000). In this investigation, the primers BMB-CR/LRO were used to amplify the entire ITS I to ITS II region. Therefore, sequence data included the ITS I, ITS II and 5.8S region. Organisation of the sequence data into separate regions was performed manually by aligning against already published sequences and removing the non aligned regions.

Nucleotide sequences were deposited into GenBank and given accession numbers, which are listed in Table 3.10 in Materials and Methods, Chapter 3.

4.3.3 Phylogenetic Analysis

To determine the evolutionary relationships of the three Australian *Ganoderma* isolates with each other and other *Ganoderma* species at the molecular level, and in turn identify the species, a phylogenetic study was performed. Sequence data from the ITS I, ITS II and combined ITS I + ITS II regions of the three Australian isolates (Section 4.3.2.5) was aligned against published sequence data using the data analysis programs available from Biology Workbench version 3.2 (Subramaniam, 1999). The aligned sequences were then analysed for evolutionary relationships using MEGA version 2.1 (Kumar *et al.*, 2001). They were also subjected to pairwise distance analysis in MEGA.

As the outcome of an evolutionary study can vary depending on the input data (Brinkman & Leipe, 2001; Nei, 1996), careful consideration was taken in choosing appropriate taxa to include in the study. Initial analyses were performed on a large number of closely related taxa (> 45) (results not shown). This gave an estimate of the evolutionary relationships between *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 and a wide range of *Ganoderma* species. From these evolutionary relationships a smaller number of taxa were selected to be included in the final analyses. It has been shown that for highly variable regions such as the ITS I and ITS II region, the more distant the species, the poorer the statistical support will be (Moncalvo *et al.*, 2000). Hence, it was important to select different species that were closely related so conclusions would be reliable and statistically robust.

A selection of Australian and Asian *Ganoderma* isolates that had been previously analysed in a larger study by Smith & Sivasithamparam (2000a) and grouped according to Moncalvo *et al.* (1995b) were included in the phylogenetic analysis. The groups and subgroups previously assigned (Moncalvo *et al.*, 1995b; Smith & Sivasithamparam, 2000a), as well as an additional group 12, have also been used in this investigation to aid in discussion. Since the primary aim of this investigation was to identify the three Australian *Ganoderma* isolates, the relationship between many of the species was not of interest and therefore has not been discussed in depth. Overall, thirty three taxa were included in the phylogenetic analysis. They have been listed in Table 3.10 in Materials and Methods, Chapter 3.

4.3.3.1 Sequence Alignment

Sequence alignment of the thirty three taxa was carried out as described in Section 3.23. The sequence alignment included the out group, *Fomitopsis rosea* JMM T92-10, which was used to root the tree in the phylogenetic study (Section 4.3.3.2). Gaps were also introduced into the sequences to increase their alignment similarity. Alignments of the ITS I region and ITS II region have been included in Appendix 1 (1A and 1B).

Nucleotide sequences in the ITS I region aligned in 222 sites, with 85 (38.3%) variable sites and 53 (23.9%) parsimony informative sites. The ITS II region aligned in 224 sites, with 98 (44.1%) variable sites and 70 (31.3%) parsimony informative sites. The frequency of variable sites was similar in the two ITS regions, but variations were mostly located in the central region of the ITS I alignment and the termini of the ITS II alignment. This was also noted by Moncalvo *et al.* (1995c).

4.3.3.2 Evolutionary Trees

The data was analysed using maximum parsimony as described in Section 3.23. Maximum parsimony only considers cladistically informative characters, which are sites in the alignment with at least two different kinds of nucleotides each represented twice (Kumar *et al.*, 2001). Therefore, 23.9% of the ITS I region and 31.3% of the ITS II region were used in the analysis.

Maximum parsimony using both complete deletion and pairwise deletion was investigated, as insertions and deletions can sometimes give important information about the data set (Nei, 1996). The consensus trees produced by both models had the same topology and only differed in the statistical support of internal branches (results not shown). The strict consensus trees derived using pairwise deletion gave better statistical support than complete deletion. Therefore, pairwise deletion phylogenies are presented in this investigation. The evolutionary trees produced from the separate datasets, ITS I and ITS II, and the combined dataset ITS I + ITS II, are shown in Figure 4.11, Figure 4.12 and Figure 4.13, respectively.

A number of analyses using different tree making and distance models were also considered. These models included distance methods such as UPMGA, and NJ methods and the tree building method maximum likelihood. Preliminary analyses using these models produced topologically similar trees that varied slightly in statistical support (data not shown). This indicated that the topology of the tree was close to the true evolutionary tree (Brinkman & Leipe, 2001).

For the ITS I dataset, an heuristic search based on close neighbourhood interchange (CNI) branch swapping starting with 20 random addition sequences produced 446 equally parsimonious trees, of 146 steps, with a consistency index of 0.7534 and a retention index of 0.9043. The strict consensus tree for the ITS I dataset is shown in Figure 4.11. *Ganoderma* H1 formed a statistically well supported clade (96% bootstrap value) with *G. cupreum* (group 8.2). *Ganoderma* H2 clustered with the Australian *G. weberianum* (subgroup 5.2), but the statistical support was poor (56% bootstrap value). When the clade incorporated both the Australian and Asian *G. weberianum* isolates (group 5) the statistical support of the clade was robust (98% bootstrap value). *Ganoderma* H3 did not form a clade with any of the other isolates, although it was grouped with the next closest isolate *Ganoderma* sp. SE 1. This other isolate (*Ganoderma* sp. SE 1) had not yet been taxonomically identified but was thought to be *G. boninense* (personal communication, Dr. Brendan J. Smith, CSIRO, Canberra, ACT, Australia).



Figure 4.11. Phylogenetic relationship of *Ganoderma* species inferred from nucleotide sequences of the internal transcribed spacer region, ITS I. The tree depicted is a strict consensus tree of 446 equally parsimonious trees obtained by an heuristic search in MEGA using CNI branch swapping and 20 random addition sequences (Kumar *et al.*, 2001). Out group for the analysis was *Fomitopsis* cf. *rosea* JMM T92.10. Groups and subgroups are according to Smith & Sivasithamparam (2000a). Values displayed below branches are confidence levels after 100 bootstrap replications. Consistency Index = 0.75342; retention index = 0.904255; tree length = 146.



Figure 4.12. Phylogenetic relationship of *Ganoderma* species inferred from nucleotide sequences of the internal transcribed spacer region, ITS II. The tree depicted is a strict consensus tree of 71 equally parsimonious trees obtained by an heuristic search in MEGA using CNI branch swapping and 20 random addition sequences (Kumar *et al.*, 2001); Out group for the analysis was *Fomitopsis* cf. *rosea* JMM T92.10. Groups and subgroups are according to Smith & Sivasithamparam (2000a); Values displayed below branches are confidence levels after 100 bootstrap replications; Consistency index = 0.668394; Retention index = 0.849765; tree length = 193.



Figure 4.13. Phylogenetic relationship of *Ganoderma* species inferred from nucleotide sequences of the internal transcribed spacer regions, ITS I and ITS II regions combined. The tree depicted is a strict consensus tree of 293 equally parsimonious trees obtained by an heuristic search in MEGA using CNI branch swapping and 20 random addition sequences (Kumar *et al.*, 2001); Out group for the analysis was *Fomitopsis* cf. *rosea* JMM T92.10. Groups and subgroups are according to Smith & Sivasithamparam (2000a); Values displayed at divergent branches are confidence levels after 100 bootstrap replications; Consistency index = 0.690751; retention index = 0.866584; tree length = 346.

For the ITS II dataset, an heuristic search based on CNI branch swapping starting with 20 random addition sequences produced 71 equally parsimonious trees, of 193 steps, with a consistency index of 0.6684 and a retention index of 0.8498. The strict consensus tree for the ITS II dataset is shown in Figure 4.12. The phylogram for the ITS II dataset (Figure 4.12) was of similar topology to the ITS I data set (Figure 4.11), with a few minor differences. *Ganoderma* H2 clustered with the Asian *G. weberianum* isolates (subgroup 5.1) with statistical support of 82%, as opposed to the ITS I clade with the Australian isolates (subgroup 5.2) which had lower statistical support (56%) (Figure 4.11). Statistical support for the larger clade that incorporated both Asian and Australian *G. weberianum* isolates was slightly better (87%) than the subgroup 5.1 (82%) (Figure 4.12), indicating that ITS II phylogeny supported only one species of *G. weberianum*. *Ganoderma* H3 formed a clade with *Ganoderma* sp. SE 1, although statistical support of that branch was moderate (76%).

Maximum parsimony analysis of the combined data sets (ITS I + ITS II) is depicted in Figure 4.13. An heuristic search based on CNI branch swapping starting with 20 random addition sequences produced 293 equally parsimonious trees, of 346 steps, with a consistency index of 0.6908 and a retention index of 0.8666. The internal branches of the combined datasets had greater statistical bootstrap support (Figure 4.13) than the single data sets (Figure 4.11 and Figure 4.12). Like the ITS II phylogeny, *Ganoderma* H2 clustered with group 5.1 (Asian *G. weberianum* isolates). However, the statistical support of this branch was lower in the combined dataset (58%) than in the ITS II phylogeny (82%). Overall, the larger clade (group 5), which included the two subsets (5.1 and 5.2), had strong statistical support (99%), again supporting this clade as the one species, and not separate species of *G. weberianum*. *Ganoderma* H3 formed a clade with *Ganoderma* sp. SE 1, but again, statistical support was only moderate (80%).

Bootstrap analysis of the different phylogenies (ITS I, ITS II and ITS I + ITS II datasets) gave a pattern of strong statistical support for: 1) *Ganoderma* H1 clustering with *G. cupreum* (group 8.2) with 96%, 98% and 100% bootstrap values in the ITS I, ITS II and ITS I + ITS II phylograms, respectively; and 2) *Ganoderma* H2 clustering with *G. weberianum* (group 5) with 98%, 87% and 99% bootstrap values in the ITS I, ITS II and ITS I + ITS II phylograms, respectively. Although *Ganoderma* H2 had the

strongest statistical support with group 5 (that incorporated both Asian and Australian subgroups), in this report it was grouped within subgroup 5.2 (Australian *G. weberianum* isolates), as it was also an Australia isolate.

An important observation was the low statistical support of branches in this analysis between the less closely related species. Terminal taxa resolved by Smith & Sivasithamparam (2000a) was similar in our analysis, but they did not perform bootstrap analysis so no comparisons could be made. Moncalvo *et al.* (1995c) also observed low statistical support for the less closely related species and suggested that the ITS I region is rapidly evolving. Therefore, to overcome low statistics in evolutionary models one would need to use a number of closely related species. This was a problem in the analysis of the Australian species, as there is only limited sequence data available. Hence, this highlights the requirement for a larger number of Australian and Asian *Ganoderma* sequences so that reliable evolutionary trees can be determined

4.3.3.3 Intraspecies Sequence Divergence

The percentage nucleotide divergence between taxa was calculated from the pairwise distance data computed in MEGA (Kumar *et al.*, 2001). Table 4.2 shows the nucleotide divergence in the ITS I region (lower triangle) and the ITS II region (upper triangle). A 1% nucleotide divergence corresponds to two nucleotide substitutions between two taxa, and the boxes indicate a group or clade as determined in the phylogenetic analysis (Section 4.3.3.2). Only taxa or groups closely related to the three Australian *Ganoderma* isolates are included in Table 4.2.

As determined in the phylogenetic analysis, *Ganoderma* H1 clustered with group 8.2, *G. cupreum* (Section 4.3.3.2). The average nucleotide divergence of *Ganoderma* sp. H1 with the isolates within this group was 0.5% nucleotide divergence in the ITS I region and 1.0% in the ITS II region. The average nucleotide variation (0.75% for combined ITS regions) was within the less than the 2% intraspecific ITS variation allowed for *Ganoderma* species (Moncalvo *et al.*, 1995b, 1995c). This, combined with the strong statistical support (> 95% bootstrap values) in the ITS I phylogram (Figure 4.11), ITS II phylogram (Figure 4.12) and combined dataset phylogram (Figure 4.13) supported the classification of *Ganoderma* H1 as a *G. cupreum* isolate.

Table 4.2

Percentage of nucleotide difference between different Ganoderma isolates

	Perce	ntage	of Nu	cleotic	le Sub	ostitut	ion B	etweer	n Tax	a ^a											
	Grp 5	.1		Group	o 5.2		Grou	p 6.3		Grou	p 8.1		Grou	p 8.2		Grou	p 10		Grouj	p 12	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1 G. weberianum CBS 219.3		1.6	0.0	3.2	3.2	3.2	1.1	12.0	10.9	11.5	12.6	15.1	16.1	16.1	16.1	16.7	16.1	16.0	16.0	15.6	16.9
2 G. weberianum RSH 0821	0.0		1.6	4.3	4.3	4.3	2.1	11.5	10.4	11.0	12.1	16.2	17.3	16.8	16.8	17.3	16.8	16.1	16.1	15.6	17.0
3 G. weberianum CCRC 37081	0.0	0.0		3.7	3.8	3.7	1.6	12.5	11.4	12.0	13.0	15.0	16.0	16.0	16.0	16.7	16.0	16.0	16.0	15.5	17.4
4 G. weberianum DFP 8401	2.0	2.0	2.0		0.0	0.0	2.1	9.8	8.7	9.2	10.3	15.1	16.1	15.1	15.1	15.1	15.1	13.9	13.9	14.4	15.3
5 G. weberianum DFP 4483	2.0	2.0	2.0	1.0		0.0	2.2	9.9	8.8	9.3	10.4	15.2	16.3	14.6	14.6	14.7	14.6	14.0	14.0	14.5	15.4
6 G. weberianum DFP 8405	1.5	1.5	1.5	1.5	1.5		2.1	9.8	8.7	9.3	10.4	15.1	16.1	15.1	15.1	15.1	15.1	13.9	13.9	14.4	15.3
7 Ganoderma sp. SUT H2	1.5	1.5	1.5	1.5	1.5	0.0		10.9	9.8	10.3	11.4	14.9	16.0	14.9	14.9	15.0	14.9	15.3	15.3	14.8	15.7
8 Ganoderma sp. QFRI 8147.1	8.0	8.0	8.0	9.0	9.1	7.5	7.5		1.1	0.5	1.6	13.7	14.8	13.0	13.0	13.1	13.1	13.0	13.0	15.0	13.8
9 Ganoderma sp. QFRI 8647.1	8.0	8.0	8.0	9.0	9.1	7.5	7.5	0.0		0.5	1.6	13.7	14.8	13.0	13.0	13.1	13.1	13.0	13.0	13.9	12.7
10 Ganoderma sp. DAR 73779	7.5	7.5	7.5	8.5	8.6	7.0	7.0	0.5	0.5		1.1	13.1	14.2	12.5	12.5	12.6	12.6	12.4	12.4	14.4	13.3
11 Ganoderma sp. UWA 8	8.0	8.0	8.0	9.0	9.1	7.5	7.5	1.5	1.5	2.0		14.2	15.3	12.5	13.6	13.7	13.7	12.4	12.4	15.6	14.4
12 G. sinense ZHANG 1734	10.2	10.2	10.2	11.2	10.8	10.7	10.7	11.7	11.7	12.2	12.8		0.5	9.9	9.9	10.5	9.8	13.5	13.5	12.3	11.6
13 G. sinense RSH 0109	9.7	9.7	9.7	10.7	10.3	10.2	10.2	11.3	11.3	11.8	12.4	0.0		11.0	11.0	11.6	10.9	14.6	14.6	13.4	12.6
14 G. cupreum DFP 4336	11.1	11.1	11.1	11.1	10.7	10.6	10.6	12.1	12.1	11.6	13.7	5.1	5.1		1.0	1.6	1.6	10.4	10.4	11.3	11.2
15 G. cupreum QFRI 8678.1	11.1	11.1	11.1	11.1	10.7	10.6	10.6	12.1	12.1	11.6	13.7	5.1	5.1	0.0		0.5	0.5	10.4	10.4	11.3	10.6
16 G. cupreum DFP 3896	11.1	11.1	11.1	11.1	10.7	10.6	10.6	12.1	12.1	11.6	13.7	5.1	5.1	0.0	0.0		1.0	11.0	11.0	11.9	10.7
17 Ganoderma sp. SUT H1	11.6	11.6	11.6	11.6	11.2	11.1	11.1	12.6	12.6	12.1	14.2	5.6	5.6	0.5	0.5	0.5		10.9	10.9	11.2	10.5
18 G. incrassatum SE 3	12.5	12.5	12.5	13.0	13.1	11.5	11.5	11.0	11.0	10.5	12.6	10.7	10.3	9.5	9.5	9.5	10.1		0.0	12.2	14.1
19 G. incrassatum DAR 73783	12.0	12.0	12.0	12.5	12.6	11.0	11.0	10.5	10.5	10.0	12.1	10.2	9.7	9.5	9.5	9.5	10.1	1.5		12.2	14.1
20 Ganoderma sp. SE1 BS	11.4	11.4	11.4	13.4	13.5	12.9	12.9	12.0	12.0	11.5	13.6	8.5	8.6	8.0	8.0	8.0	8.5	11.9	11.4		9.8
21 Ganoderma sp. SUT H3	8.9	8.9	8.9	10.1	9.6	8.4	8.4	8.5	8.5	8.5	9.6	7.8	7.8	7.2	7.2	7.2	6.7	7.9	7.3	6.1	

^a Pairwise distance (MEGA) from the nucleotide sequences alignment between taxa, using pairwise deletion; 0.5 % of divergence between two taxa corresponds to approximately one nucleotide substitution; Lower and upper triangles correspond to the values obtained for the ITS 1 and ITS II regions, respectively.

The next closest group to subgroup 8.2 (*G. cupreum*) was subgroup 8.1 (*G. sinense*), which had a nucleotide divergence of 5.1% in the ITS I region and 10.1% in the ITS II region (Table 4.2). These were clearly separate groups as the average nucleotide difference (7.6% for the combined ITS regions) was greater than 6% allowed for interspecific variation for *Ganoderma* species (Moncalvo *et al.*, 1995c). In addition, the ITS variation between *Ganoderma* H1 and subgroup 8.1 was 8.0% for the combined ITS regions (Table 4.2), which confirmed that it was not an isolate of *G. sinense*.

Nucleotide variation between the *Ganoderma* H2 isolate and all other isolates in group 5 (G. weberianum) was observed to be 0 to 1.5% in the ITS I region and 1.1 to 2.1% in the ITS II region (Table 4.2). The average nucleotide intraspecific variation between Ganoderma H2 and the other isolates within group 5 (1.6% for the combined ITS regions) was also within less than the allowable 2% intraspecific ITS variation in Ganoderma species (Moncalvo et al., 1995b, 1995c). There were nucleotide differences observed between *Ganoderma* H2 and the separate subgroups 5.1 and 5.2. The average nucleotide variation in the ITS I region for subgroup 5.2 (1%) was less than that for subgroup 5.1 (1.5%). Conversely, the average nucleotide variation in the ITS II region for subgroup 5.2 (2.1%) was greater than that of subgroup 5.1 (1.6%). This was reflected in the ITS I (Figure 4.11) and ITS II (Figure 4.12) phylogenies in that Ganoderma sp. H2 clustered with the different subgroups in each phylogram. However, bootstrap analysis of the phylograms (> 87%) supported a single species of G. weberianum. This was in agreement with Smith & Sivasithamparam (2000a) who analysed a larger number of Ganoderma species and suggested that the two clades (subgroups 5.1 and 5.2) were allotropic forms of the single species G. weberianum.

The next closest group to *G. weberianum* (group 5) was group 6.3, which had been designated *Ganoderma* sp. after being incorrectly identified as *G. lucidum* (Smith & Sivasithamparam, 2000a). The sequence variation between group 6.3 and subgroup 5.1 (9.3% combined ITS regions) and subgroup 5.2 (8.6% combined ITS regions) (Table 4.3) was greater than the 6% level for interspecific variation of the ITS regions for *Ganoderma* species (Moncalvo *et al.*, 1995c).

Ganoderma H3 and the unidentified Australian isolate, *Ganoderma* sp. SE 1, that was thought to be the same species (group 12) had nucleotide variation of 6% and 9% in the ITS I and ITS II regions, respectively (Table 4.2). The nucleotide difference between these two isolates was strong evidence that they were separate species, as the combined ITS regions (7.5%) was greater than the allowable 2% for intraspecific ITS variation in *Ganoderma* species (Moncalvo *et al.*, 1995c). In addition, the statistical support in the phylogenies was not robust (< 80%) (Figures 4.11, 4.12 and 4.13). The next closest groups to *Ganoderma* H3 were group 8.2, *G. cupreum* (8.9% nucleotide difference), and group 10, *G. incrassatum* (10.9% ITS nucleotide difference). Both groups had an interspecific ITS variation greater than 6% with *Ganoderma* H3, which demonstrated that *Ganoderma* H3 was neither of these species. Since the classification of *Ganoderma* H3 could not be certain, it has been designated *Ganoderma* sp. H3 until further identification can be performed using a larger number of sequences.

Table 4.3

Nucleotide difference between groups of Ganoderma taxa

		Percei	itage of	f nucleo	tide su	bstituti	on betw	veen gro	oups ^a		
		1	2	3	4	5	6	7	8	9	10
1	Grp 5.1		3.00	10.75	9.25	14.83	15.29	15.00	13.00	13.13	14.83
2	Grp 5.2	1.75		9.00	7.25	14.50	13.88	13.38	13.50	10.75	13.63
3	Grp 6.3	7.88	8.13		7.22	13.00	11.94	11.75	11.50	10.38	12.75
4	Grp 7	10.13	10.88	8.25		12.31	10.50	9.38	10.44	7.88	11.06
5	Grp 8.1	9.75	10.38	11.63	14.88		10.13	13.50	11.00	11.38	11.75
6	Grp 8.2	11.13	10.75	12.38	14.25	5.13		10.25	9.75	11.88	10.38
7	Grp 10	12.25	12.00	11.00	13.38	10.00	9.63		10.00	9.38	12.50
8	Grp 11.2	13.00	13.25	12.25	13.63	11.25	10.63	5.50		7.88	12.75
9	Grp 11.3	12.63	12.38	12.13	13.50	8.88	9.75	3.88	4.38		14.38
10	Grp 12	9.75	10.69	10.00	11.88	7.75	7.25	9.25	11.13	9.88	

^a Values determined by Pairwise distance in MEGA from the nucleotide sequences alignment between taxa, using pairwise deletion; 0.5% of divergence between two taxa corresponds to approximately one nucleotide substitution; Lower triangle corresponds to values obtained for ITS I; Upper triangle corresponds to values obtained for ITS II.

4.3.3.4 Taxonomic Conclusion

Smith & Sivasithamparam (2000a) considered the phylogenetic classification inferred from sequence data to be superior over the use of sequence variation statistics for identifying taxa. In this study, both sequence variation statistics and phylogenies were

in agreement on the classification of two of the unidentified Australian isolates, *Ganoderma* H1 and *Ganoderma* H2. Phylogenetic analysis of the third isolate, *Ganoderma* H3, grouped it with another Australian isolate (although bootstrap confidence was moderate (76 to 80%)), but the results from the sequence variation analysis conflicted with this grouping and indicated that they were distinctly different species. It is likely that if there were a greater number of sequences available, then results closer to the true tree and hence, true species, could have been achieved for this particular taxon.

There was a higher nucleotide divergence observed in the ITS II region than in the ITS I region (Table 4.2). This was also observed by Moncalvo *et al.* (1995a, 1995c) and Gottlieb *et al.* (2000). In addition, a lower level of resolution of internal branches for the ITS I phylogeny was seen by Gottlieb *et al.* (2000), as was the case in this investigation.

Overall, the evolutionary trees and nucleotide sequence variation data supported the molecular identification of *Ganoderma* H1 to be *G. cupreum* and *Ganoderma* H2 to be *G. weberianum*. *Ganoderma* H3 was not identified to be an isolate of any defined species used in the molecular identification and therefore was designated *Ganoderma* sp. H3.

4.4 Summary

In this Chapter, three new *Ganoderma* species were isolated from the Cairns state forest and sent to Swinburne University of Technology to be analysed using modern molecular techniques. They were tentatively named *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3.

Ribosomal DNA was extracted from the fungal mycelium that had been grown in liquid culture. The ITS I to ITS II region, was amplified using PCR and specific primers. The PCR products were purified and estimated to be approximately 750 to 800 bp in size, for all three isolates (Figure 4.2). The size of the entire ITS region was similar to that reported by Moncalvo *et al.* (1995c).

The purified product of the three isolates was analysed by RFLP-PAGE, using the enzymes *AluI*, *Hin*dIII, *HhaI* (*CfoI*) and *Hae*III (Section 4.3.2.2). Restriction patterns were similar for some enzymes and *Ganoderma* isolates, but the overall restriction pattern discriminated between the three different patterns (Table 4.1). This strongly indicated that the three Australian isolates were in fact three different species. The restriction patterns were also compared against those previously reported by Gottlieb *et al.* (2000), of which none were similar.

Several attempts were made to directly sequence the purified PCR product of which none were successful. The PCR product was then cloned into a vector to produce recombinant DNA. The recombinant DNA was successfully sequenced and the sequence data analysed to produce a consensus sequence for each Australian isolate. The entire ITS I to ITS II region was determined to be 774, 770 and 758 bp for *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3, respectively (Figure 4.10). The actual size of the entire ITS region correlated well with the estimated size of the purified PCR product.

To study the evolutionary relationship of the three Australian *Ganoderma* isolates, a phylogenetic study was carried out using the molecular evolutionary program MEGA (Kumar *et al.*, 2001). Preliminary studies on a large number of taxa identified closely related species in the geographic regions of Australia and Asia. A selection of the closest related species and isolates were chosen for further analysis.

Maximum parsimony analysis was the model chosen to analyse the data as this has been used frequently in the analysis of *Ganoderma* (Gottlieb *et al.*, 2000; Smith & Sivasithamparam, 2000a). An heuristic search using CNI branch swapping starting with 20 random addition trees was performed on the separate ITS I and ITS II region, as well as the combined ITS I + ITS II region. The three phylograms produced from the analysis had similar topology, which indicated good representation of the evolutionary relationships (Figures 4.11, 4.12 and 4.13). Bootstrap analysis was also performed to evaluate the robustness of the internal branches. *Ganoderma* H1 clustered with *G. cupreum* in the three phylograms and had strong statistical support (> 95%). *Ganoderma* H2 also had strong statistical support when it formed a clade with *G.*

weberianum in the three phylograms (> 87%). *Ganoderma* H3 formed a clade with another unknown *Ganoderma* sp. SE 1, thought to be *G. boninense*, in both the ITS II and ITS I + ITS II phylograms, but the statistical support was only moderate (76 to 80%).

Nucleotide variation data was also resolved and used to support the results obtained from the evolutionary trees. Moncalvo *et al.* (1995c) showed that intraspecific variation in the combined ITS regions was < 2%, while interspecific variation was usually > 6%. Less than 2% ITS variation was observed for *Ganoderma* H1 in the *G. cupreum clade* (group 8.2) and *Ganoderma* H2 in the *G. weberianum* clade (group 5) (Table 4.2). *Ganoderma* H3 and the unknown *Ganoderma* sp. SE 1, had nucleotide variation of 7.5% (Table 4.2). This, combined with the low statistical support of the clade in the phylograms (Figure 4.11 and 4.12) suggested that it was not the same species as the *Ganoderma* sp. SE 1.

In conclusion, *Ganoderma* H1 was identified as an isolate of *G. cupreum* and *Ganoderma* H2 as an isolate of *G. weberianum*, while the species classification of *Ganoderma* H3 was unable to be determined.

Chapter Five

Preservation and Mycelial Growth Optimisation

5.1 Introduction

The type species, Ganoderma lucidum, has been the primary focus of investigation for approximately the last twenty years and there has been relatively little attention paid to the other species within this genus. This has led to large amounts of information being produced on the growth, cultivation and medicinal effects of the type species, G. lucidum. Due to this wealth of information, conditions employed for the growth and preservation of G. lucidum have also been adopted for the growth of other species within this genus. There is evidence, however, to suggest that closely related species within the same genus might require different growth conditions and nutrient sources (Mayzumi et al., 1997). Fungi isolated from various regions around the world may adapt to different environmental factors and in turn require alternative growth conditions (Bilay et al., 2000; Kim et al., 2002a). When investigating a new fungal species or isolate, it is essential to understand the environment under which the fungus can be stored, the environmental and nutritional factors necessary for development, and factors that may affect the growth rate of the fungus. This Chapter reports the storage and growth requirements of the three Australian Ganoderma isolates, G. cupreum (H1), G. weberianum (H2) and Ganoderma sp. (H3), used in this study.

5.2 Overview

The three Australian *Ganoderma* isolates, H1 (*G. cupreum*), H2 (*G. weberianum*) and H3 (*G.* species), were investigated for their ability to survive in storage using a variety of preservation techniques. These included slant and plate storage, mineral oil storage and storage at -80° C. This viability investigation was performed to establish the appropriate long term preservation required to maintain a stable starter culture and short term preservation for a stable working culture. The environmental factors, temperature and pH, were also investigated to determine optimal working conditions for growth of the mycelium. The three isolates were then cultivated in shaker flask cultures. A variety of growth media was subsequently investigated for the production of mycelial biomass and in turn, various carbohydrate sources were examined to observe which source promoted the maximum production of mycelial biomass.

5.3 **Results and Discussion**

5.3.1 Preservation and Viability of *Ganoderma* H1, H2 and H3

The three *Ganoderma* isolates, H1, H2 and H3, which had been identified as three different species (Chapter 4), were investigated for their ability to be stored on agar slants under mineral oil and at -80°C, for long term preservation. The three isolates were also examined for short term preservation on agar slants and plates and on agar slants under mineral oil. In addition a study was performed to assess the effect that light had on the storage of the fungi. The ability of the fungi to produce rapid subcultures after storage in these conditions was investigated, since this has a major impact on the time required for this study to take place.

5.3.1.1 Long Term Preservation

The isolates were examined for their ability to be preserved on agar slants under mineral oil stored at both 4°C and room temperature (Section 3.7.2), and on balsa wood at –80°C (Section 3.7.4). These two methods were chosen for long term preservation of the fungal cultures because they were inexpensive and readily accessible at Swinburne University of Technology.

Mineral oil storage of the isolates was performed as described in Section 3.7.2. PDA and MEA slants were inoculated and incubated until there was confluent fungal growth on the slant. The slant was then covered with mineral oil, to a depth of 1 cm from the top of the slant. The slants were stored at room temperature and in the refrigerator at 4°C. After repeated attempts with different samples, *Ganoderma* H1 and H3 could not be recovered after being stored under mineral oil, both at room temperature and at 4°C (results not shown). However, the mycelium of *Ganoderma* H2 could be successively recovered at monthly intervals for up to 1 year when stored in the same way (results not shown). *Ganoderma* H1 and H3 exhibited either poor growth or did not survive when stored in water at room temperature, 4°C and -80°C, and on balsa chips at 4°C (results not shown). Due to the non recovery of *Ganoderma* H1 and H3, the above methods of storage were not used and the option of long term storage on balsa wood at -80°C was investigated.

Storage on balsa wood at -80° C was performed as described in Section 3.7.4. Mycelium was grown on balsa wood and then frozen at -80° C. When preserving fungal cultures at -80° C in vials, it is convenient in terms of space to store many balsa wood chips in a few vials, rather than having one balsa chip per vial. When the mycelium was required to be subcultured, a process of thawing the stored vial and then refreezing the unused balsa chips was performed. The thawing and refreezing of mycelium in this way has been known to create problems in the viability of fungi (Ando, 1997). Therefore, this investigation studied the survival rate of the three *Ganoderma* isolates after repeated thawing and refreezing at -80° C. Three tubes of fifty balsa chips were prepared. After the tubes were stored at -80° C. The remaining chips in the tubes were replaced, to be stored again at -80° C. This process was repeated at monthly intervals (starting at day zero) up until the sixth month, and then every 6 months for up to 2 years.

Table 5.1

The effect of thawing and refreezing at -80°C on the viability of Australian Ganoderma isolates

	Prese	Preservation Period (months) ^a										
Fungi	0	1	2	3	4	5	6	12	12 ^b	18	24	24 ^b
Ganoderma H1	5/5 [°]	5/5	4/5	4/5	3/5	1/5	0/5	0/5	5/5	0/5	0/5	5/5
Ganoderma H2	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5
Ganoderma H3	5/5	5/5	5/5	2/5	0/5	0/5	0/5	0/5	4/5	0/5	0/5	4/5

The fungi were stored on balsa wood chips (50 per vial) at -80° C and checked for viability at monthly intervals for up to two years; ^a The number of thawing and refreezing cycles. Up to 6 months, 1 month equals one thawing/freezing cycle, after this, 6 months equals one thawing/freezing cycle; ^b Control maintained at -80° C without thawing; ^c The numerator shows the number of chips from which viable fungi were recovered (the growth was checked on the tenth day) and the denominator shows the number of balsa chips tested.

Table 5.1 shows the survival rates of *Ganoderma* H1, H2 and H3, after repeated thawing and refreezing. The isolate, *Ganoderma* H2, showed the greatest viability with repeated thawing and refreezing in that it had a 100% survival rate (5/5) after 24 months. Ando (1997), who tested the viability of nine strains from two *Ganoderma* species (species were not noted), that were stored at –80°C for 1 year, found similar results in that all nine strains had a 100% survival rate. In contrast, there was no viability of *Ganoderma* H1 observed after month 6 and no viability of *Ganoderma* H3

observed after month 4 (Table 5.1). These two isolates showed a similar viability pattern in that the viability decreased with repeated thawing and refreezing. Ando (1997) also found that the survival rates of repeated thawing and refreezing could decrease the viability of some fungal cultures, although this was not observed of *Ganoderma* species. Even though the *Ganoderma* isolates H1 and H3 could not withstand continued thawing and refreezing, the results showed that they were still viable after long periods of time when they were not subjected to thawing (Table 5.1). The viability of the chips from the tubes that were opened after 1 and 2 years (with no thawing and refreezing) was good, yielding a 100% (5/5) and 80% (4/5) viable chips for *Ganoderma* H1 and H3, respectively.

Overall, the thawing and freezing of the cultures had a negative effect on the viability of *Ganoderma* H1 and H3, and no effect on the viability of *Ganoderma* H2. Furthermore, the storage of all three isolates, when no thawing and refreezing occurred, was good. Based on these results, the long term preservation of the three Australian *Ganoderma* isolates was achieved by preparing a minimum of 6 vials, each with 10 balsa chips, to avoid the reduction of viability after repeated thawing and refreezing.

The growth of the mycelium after storage on the balsa chips at -80° C was also investigated. Samples were taken from fresh separate vials (which had not been thawed and refrozen) so that the effect that the low temperature had on the mycelial growth could be observed. The radial diameter of the mycelium was measured after thawing and subculturing onto fresh agar plates, at 6 month intervals for up to 2 years.

Table 5.2 shows the mycelial radial diameter of the *Ganoderma* isolates after storage on balsa chips after 6, 12, 18 and 24 months. It can be seen that on regrowth of the isolates H1 and H3, the radial growth became slower after longer periods of storage (from an initial radial diameter of 30 mm to 7 mm after 24 months for *Ganoderma* H1 and from an initial radial diameter of 38 mm to 14 mm after 24 months for *Ganoderma* H3). The storage at –80°C appeared to have no effect on the mycelial growth of isolate H2, as the initial radial diameter of 36 mm was comparable to that obtained after 24 months, 35 mm. There is no literature available that shows the radial growth of *Ganoderma* after long periods of storage, so comparisons cannot be made and therefore, this method was

adopted for the storage of the three *Ganoderma* isolates for up to 2 years. After this time, transfer of the cultures to a fresh medium would need to be carried out. Due to slower growth of *Ganoderma* H1 and H3 after longer terms of storage, further investigations for short term storage were performed so that rapid growing working cultures could be produced at short notice.

Table 5.2

The effect of long term preservation at -80°C on the mycelial growth of Australian Ganoderma isolates

	Preserva	tion Perio	d (months)	a	
Fungal Organism	0	6	12	18	24
Ganoderma H1	38 ± 1	21 ± 1	9 ± 2	9 ± 1	7 ± 0
Ganoderma H2	36 ± 1	36 ± 1	35 ± 3	35 ± 2	35 ± 2
Ganoderma H3	38 ± 0	29 ± 1	18 ± 1	15 ± 1	14 ± 2

The fungi were grown on sterile moist balsa wood for 6 days and then stored in a deep freezer at -80° C for up to two years. The mycelial diameter was calculated on the fifth day of growth; ^a The number of months the culture was stored at -80° C; Growth of subcultures was performed in triplicate.

5.3.1.2 Short Term Preservation

The three Australian *Ganoderma* isolates were investigated for their ability to survive in storage at 4°C, both on slant and plate cultures, which were sealed to prevent drying. The effect of light on the storage cultures was also examined. The growth media, MEA and PDA, were chosen for investigation because they are commonly used for the growth and storage of fungal species (Chen, 1999; Stamets, 1993). The viability was determined by subculture of the stored fungi, at weekly intervals, onto fresh media and measuring the radial growth on the fifth day. The storage viability at 4°C was evaluated for up to 12 weeks and the maximum radial growth was taken to be 40 mm.

The results of the storage viability for the *Ganoderma* isolates when stored on different media at 4°C are presented in Figure 5.1. They give a representation of the ability of the fungi to be able to withstand low temperature, as well as a comparison of the differences between the three isolates. Firstly, it must be noted that *Ganoderma* H2 had the greatest viability when stored at 4°C, and although not identical, *Ganoderma* H1 and H3 had similar viability profiles. The similar profile was also observed in the examination of the viability after thawing and refreezing (Table 5.1) and was in agreement with the

phylogenetic analysis presented in Section 4.3.3, which showed that *Ganoderma* H1 and H3 were more closely related to each other than to *Ganoderma* H2.

An interesting observation of isolate H2 was when it was stored on slants, the fungus survived for the 12 week period with little to no decrease in the subculture growth rates (Figure 5.1h,k). In contrast, when *Ganoderma* H2 was stored on plates, the cultures survived for only 8 and 9 weeks on MEA and PDA plates, respectively (Figure 5.1b,e). This suggested that the available nutrients in the plates were depleted more quickly than those in the slants, enabling the longer survival of *Ganoderma* H2 on the slants. Slants, rather than plates, have been a form of storage for a number of years (Oei, 1996; Onions, 1971). This nutrient depletion effect was observed in both the slants and plates for isolates H1 (Figure 5.1a,d,g,j) and H3 (Figure 5.1c,f,i,l), giving a similar subculture growth pattern for both storage on slants and plates.

The overall survival of the three isolates when stored on plates at 4°C declined over time and resulted in the non-survival for *Ganoderma* H2 and H3 on both MEA and PDA plates, respectively, and *Ganoderma* H1 on MEA plates (Figure 5.1a-f). The exception was that H1 on the PDA plate was still viable after storage for 12 weeks (Figure 5.1d). It was apparent that *Ganoderma* H3 had the lowest tolerance when stored at 4°C on the different media, surviving only for a maximum of 9 weeks in storage, when stored on MEA slants (Figure 5.1c). Croan *et al.* (1999) also reported that most tropical isolates when stored on MEA plates have not maintained viability after storage for two months at 4°C.

The results obtained for the three isolates when stored on slants exposed to light show that there was a distinct negative effect on the viability of both H1 and H3 (Figure 5.1m,o,p,r). Both these cultures died after week 1 and week 5, respectively, indicating that they were sensitive to light. It has been previously reported that strong light can inhibit the mycelial growth of *Ganoderma* species (Lin *et al.*, 1973). There was no detrimental effect by light on the viability of *Ganoderma* H2.



Figure 5.1. Short term viability of *Ganoderma* H1, H2, H2, and H3 on different media. *Ganoderma* was stored on MEA and PDA plates, MEA and PDA slants and MEA and PDA slants exposed to light, at 4°C. Graphs are labelled (a) to (r) for ease of discussion. Subcultures were taken at weekly intervals and the radial growth measurements were recorded on the fifth day of mycelial growth.

Another observation, when comparing the more similar species, H1 and H3, was that they appeared to grow to some extent differently on the two storage media used. Isolate H1 had a slightly better storage survival rate when PDA was the nutrient medium (Figure 5.1d,j), while in contrast, isolate H3 survived for a longer period when MEA was the nutrient medium (Figure 5.1c,i). This is indicative of the different nutrient requirements of different fungi.

Overall, the information gained from the storage survival rates is informative in terms of storage profile, temperature capabilities and light sensitivities for each of the three isolates. It is clear that *Ganoderma* H1 and H3 are similar in their basic survival requirements, whilst *Ganoderma* H2 appears to be more robust under the conditions examined. This also demonstrates that the different fungal species from the same genus can require different storage conditions as was demonstrated in the long term preservation studies (Section 5.3.1.1).

For short term storage and rapid subculture growth it was determined that the three *Ganoderma* isolates could be successively stored on plates for no longer than 4 weeks and on slants for no longer than 6 weeks, away from direct light. Either PDA agar or MEA was deemed suitable for storage for these time periods.

5.3.2 Examination of Standard Growing Conditions

One aim of this investigation was to understand the growth conditions required by the Australian isolates. This was performed with a view to obtaining large quantities of mycelial biomass, which could then be investigated for any biological activity. Growth requirements for other *Ganoderma* species, predominately *G. lucidum*, have been published in the literature (Tseng *et al.*, 1984; Yang & Liau, 1998). However, there is no evidence to suggest that different species from the same genus require the same growth and nutritional factors. In addition, isolates from a different region may not necessarily grow under the same conditions, as illustrated by the variety in growth conditions published for *Ganoderma* species around the world (Chen, 1999; Chen & Miles, 1996b; Mayzumi *et al.*, 1997; Stamets, 1993; Tong & Chen, 1990). The effect of the environmental factors, pH and temperature, were examined and the optimal

conditions determined. These optimal conditions were then used to examine other variables, such as the mycelial growth rate, growth medium and nutrient source.

5.3.2.1 Effect of pH and Temperature on the Radial Growth of Fungal Mycelia

The three Australian *Ganoderma* isolates, H1, H2 and H3, were investigated for the effect that pH and temperature had on the radial growth of the mycelium grown on agar plates, as described in Section 3.9.4. The fungi were incubated at different temperatures on PDA plates that had been altered to varying pH values, ranging from pH 3 to 9 (Section 3.4). The radial growth of the mycelium was measured on day 5 and the results plotted to produce radial growth curves. Radial growth curves of an American *G. lucidum* isolate and an Indonesian *G. applanatum* isolate were also investigated and used for comparison against the three radial growth curves of *Ganoderma* H1, H2 and H3.

It can be seen from Figure 5.2 that a wide variation in growth rate, as well as pH and temperature tolerance, exists for the different species studied. From the range of temperatures examined, the optimal growth temperature for the three Australian *Ganoderma* isolates, H1, H2 and H3, was determined to be 30°C (Figure 5.2a,b,c). This temperature has also been shown to be optimal for other *Ganoderma* species (Lin *et al.*, 1973; Yang & Liau, 1998) and is frequently used for the cultivation of *Ganoderma* in liquid culture (Fang & Zhong, 2002b; Lee *et al.*, 1999; Yang *et al.*, 2000). The Indonesian isolate was also observed to grow best at 30°C, but also grew well at 37°C (Figure 5.2e). The American *Ganoderma* isolate investigated in this study had a lower optimal temperature at 25°C (Figure 5.2d). Taking into consideration the region that the different species were isolated from (*Ganoderma* H1, H2 and H3 isolated from a tropical region of Australia whilst *G. lucidum* was isolated from a temperate region in America) a relationship may exist between the geographical origin and the optimal temperature. This region/temperature relationship has also been shown for other fungi (Vidal *et al.*, 1997).



Figure 5.2. The pH and temperature growth profiles of (a) *Ganoderma* H1, (b) *Ganoderma* H2, (c) *Ganoderma* H3, (d) *Ganoderma lucidum* and, (e) *Ganoderma applanatum*, all grown on PDA; The mycelial radial growth was measured in mm on the fifth day of growth; The inset of (d) displays the legend for the pH values. Growth was performed on triplicate plates but for ease of representation error bars are not shown. Errors calculated as standard deviations are included in Appendix 2.

The pH/temperature profiles were observed to be a similar shape for the four isolates *Ganoderma* H1, *Ganoderma* H2, *Ganoderma* H3 and *G. lucidum* (Figure 5.2a,b,c,d), showing a range of temperatures in which growth was supported. The three Australian isolates, H1, H2 and H3, that were all isolated from the same tropical region, exhibited different growth rates at different temperatures (Figure 5.2a,b,c). *Ganoderma* H1 and H2 had a higher growth rate between 25 to 37°C, whilst *Ganoderma* H3 had a higher growth rate between the lower range of 20 to 30°C. This suggested that *Ganoderma* H1 and H2 had a greater tolerance for higher temperatures than *Ganoderma* H3. It was expected that the growth profiles of *Ganoderma* H1 and H3 would be more similar, as

The optimal growth temperature for *G. applanatum*, which was observed to be over the range 30 to 37°C, was higher than that of the three Australian isolates or that of the *G. lucidum*. The growth profile of *G. applanatum* was not like any of the *Ganoderma* isolates examined. The profile was more like that of an Australian white rot fungus, *Pycnoporus cinnabarinus*, which is included for comparison in Figure 5.3. In addition, orange mycelia rings were observed for the growth of *G. applanatum*, which is a typical characteristic of the mycelium from *Pycnoporus* species (personal communication, Prof. Greg Lonergan, SUT, Hawthorn, VIC, Australia). This demonstrates how easily species can be misidentified when using traditional taxonomic methods and hence, the significance of establishing a universal taxonomic system that can be employed worldwide.

they were found to be more closely related in the phylogenetic studies in Chapter 4

(Section 4.3.3) and the viability studies earlier in this Chapter (Section 5.3.1).

All the fungal isolates examined exhibited no growth at 10°C (Figure 5.2). Little or slow growth of fungi at temperatures of 10°C or less is a common observation (Lonergan *et al.*, 1993; Vidal *et al.*, 1997). It is this retardation of growth that is the major factor in the choice of low temperatures for the storage of many fungal cultures. The cultures that did not grow at low temperatures were not further investigated to see if their growth was renewed at optimal temperatures, as the effect of low temperature on *Ganoderma* H1 and H3 was previously found to be detrimental (Section 5.3.1.2). With the exception of *G. applanatum*, none of the *Ganoderma* H3 and *G. lucidum* at 37°C. A

narrow optimal growth temperature range (30 to 35°C) for submerged culture of *G. lucidum* has been reported (Yang & Liau, 1998). Reports have shown that after fungal cultures have been subjected to high temperatures (lethal temperature) there is no renewal of growth at the optimal temperature (Grigansky *et al.*, 1999). Therefore, the renewal of cultures that had been subjected to temperatures greater than 37°C was not investigated.



Figure 5.3. The pH and temperature growth profiles of (a) *Ganoderma applanatum* and (b) *Pycnoporus cinnabarinus*, grown on PDA. The mycelial radial diameter was measured in mm on the fifth day of growth. The inset of (b) displays the legend for the pH values. Growth was performed on triplicate plates but for ease of representation error bars are not shown on the graphs. Errors calculated as standard deviations are included in Appendix 2.

The optimal pH range of the fungal isolates is also represented in Figure 5.2. It has been noted that most fungi will grow over a wide range of pH values (Yang & Liau, 1998). This was observed for the *Ganoderma* isolates examined in this study, which grew well over the pH range of 4 to 8 (Figure 5.2). The pH at which the Australian and American isolates grew best was different. The Australian *Ganoderma* isolates H1, H2 and H3 were observed to grow best at pH 6 (Figure 5.2a,b,c) while *G. lucidum* was observed to grow best on media with a pH of 4 (Figure 5.2d). These initial pH values compare with those in the literature, which report that favourable initial pH for the growth of *Ganoderma* is between 4 and 6 (Chen, 1999; Lin *et al.*, 1973; Yang & Liau,

1998) and optimal pH for some other basidiomycetous fungi has been reported around pH 6 (Grigansky *et al.*, 1999; Kim *et al.*, 2002b).

The pH of the medium that supported the least growth for all isolates was found to be pH 3 and 9 (Figure 5.2). *Ganoderma* H1 and H2 were observed to have the slowest growth at pH 3, while in contrast, *Ganoderma* H3 had the slowest growth at pH 9. Lin (1973) found that there was slow growth when the pH was 7 to 8. This was not observed for the Australian *Ganoderma* isolates. In fact, growth at these two pH values was comparable to the growth at the optimal pH determined for all three isolates.

5.3.2.2 Growth Rate of Fungal Mycelia on PDA Plates

The growth rate of the *Ganoderma* isolates, H1, H2 and H3, was investigated at the optimal pH and at two favourable temperature values. The average rate of mycelial growth was determined as described in Section 3.9.5. The fungi were grown on PDA plates and incubated at 25 and 30°C for up to 7 days. The average mycelial growth (mm day⁻¹) was then determined over a 4 day period (day 2 to day 6 of growth). The growth rate of *G. lucidum* was also determined for comparison. The two incubation temperatures, 25 and 30°C, were chosen because they covered the optimal growth temperatures for both the American isolate and the three Australian isolates, (Figure 5.2).

Mycelial growth rate has been shown to be a good means of comparing the growth of different fungal species under different environmental conditions (Bilay *et al.*, 2000; Vidal *et al.*, 1997). In this investigation, linear growth rates were observed for *Ganoderma* H1, H2 and H3, and *G. lucidum*, at the different pH and temperatures examined (results not shown). The growth rates (mm day⁻¹) for each isolate grown on PDA at either 25 or 30°C are presented in Table 5.3.

	Mycelial Rad	ial Growth Rate at th	e Optimal pH ^a
Fungal Organism	Optimal pH	Growth/day @ 25°C (mm)	Growth/day @ 30°C (mm)
Ganoderma H1	6 ^b , 8	4.7 ± 1.3	8.2 ± 1.6
Ganoderma H2	6	9.6 ± 0.1	10.8 ± 0.5
Ganoderma H3	6	8.2 ± 0.6	9.1 ± 1.6
G. lucidum	4	4.3 ± 0.6	1.3 ± 0.3

Table 5.3

	Mycelial Rad	ial Growth Rate at th	e Optimal pH ^a
Fungal Organism	Optimal pH	Growth/day @ 25°C (mm)	Growth/day @ 30°C (mm)
Ganoderma H1	6 ^b , 8	4.7 ± 1.3	8.2 ± 1.6
Ganoderma H2	6	9.6 ± 0.1	10.8 ± 0.5
Ganoderma H3	6	8.2 ± 0.6	9.1 ± 1.6
G. lucidum	4	4.3 ± 0.6	1.3 ± 0.3

Growth Rates of Ganoderma species at 25 and 30°C

^a The average radial growth rate was calculated by taking the average radial growth (mm) on day 2 to 6 of growth. PDA plates, which had been adjusted to the optimal pH, were employed as the growth medium and rates were measured at the temperatures of 25°C and 30°C for comparison; ^b indicates the pH at which growth rates were calculated.

There was a wide range of mycelial growth rates for the four isolates examined ranging from 1.3 ± 0.3 to 10.8 ± 0.5 mm day⁻¹ at 30°C and 4.3 ± 0.6 to 9.6 ± 0.1 mm day⁻¹ at 25°C. Relative growth rates varied with temperature (Figure 5.2) and showed that the Australian isolates had a faster growth rate than the American isolate (Table 5.3). This is also evident in the growth profiles presented in Figure 5.2, which displayed smaller growth curves for G. lucidum. Overall Ganoderma H1, H2 and H3, and G. lucidum had a mycelial growth rate of 8.2 ± 1.6 , 10.8 ± 0.5 , 9.1 ± 1.6 and 4.3 ± 0.6 mm day⁻¹, respectively, when grown at their optimal pH and temperature (Table 5.3). Bilay et al. (2000), who investigated the growth of one G. lucidum strain amongst other species, observed a slow growth rate of 2.17 mm day⁻¹ on the same PDA medium at a temperature of 27°C. This growth rate lies within the range of 4.3 ± 0.6 (at 25°C) and 1.3 ± 0.3 (at 30°C) mm day⁻¹, which was also observed for G. lucidum in this study (Table 5.3). Besides this comparison of growth rates, there is little information in the literature on the growth rates of *Ganoderma* species grown on nutrient plates.

5.3.2.3 Effect of Growth Media on Fungal Biomass in Shaker Flask Cultures

The three Australian Ganoderma isolates were investigated for the effect that cultivation media had on the production of mycelial biomass and final pH in shaker flask cultures (Sections 3.10.1 and 3.8.2). Three cultivation media were chosen for initial experimentation, which included the two undefined media, PDA and MEA, and a medium used by Kawagishi (1995), which is referred to as Basal-G in this investigation.

All media were left at their natural pH, as this was close to that determined as the optimal pH for growth of *Ganoderma* H1, H2 and H3 (Section 5.3.2.1).

The mycelial biomass for *Ganoderma* H1, H2 and H3, cultivated in MEB, PDB and Basal-G medium, over a period of 10, 20 and 30 days is shown in Figure 5.4. The maximum amount of mycelial biomass produced by *Ganoderma* H1, H2 and H3 was when cultivation occurred in Basal-G medium for 30 days. The biomass was 1.15 ± 0.13 , 1.90 ± 0.05 and 1.10 ± 0.15 g, respectively, in 125 mL of cultivation medium. The final pH of the culture medium was also recorded and is presented in Table 5.4.

When PDB was used as the cultivation medium, the amount of biomass increased up to day 20 for all three isolates and then did not increase further (Figure 5.4). It was observed that the fungal growth in PDB was slow and this resulted in a small amount of mycelial biomass being produced. It was also noted that the amount of biomass at each sampling point (10, 20 and 30 days) was lower than that produced in MEB and Basal-G medium. A similar observation was also seen by Bilay *et al.* (2000), who found the growth rate of *G. lucidum* on PDA (2.17 mm day⁻¹) was less than the growth rate on MEB (3.44 mm day⁻¹).

		Final pH of Cultivation Medium ^a							
Days	<i>Ganoderma</i> sp.	MEB ^b	PDB ^c	Basal-G ^d					
10	H1	3.7 ± 0.0	3.7 ± 0.1	3.4 ± 0.0					
	H2	4.1 ± 0.1	4.2 ± 0.1	4.0 ± 0.0					
	Н3	3.6 ± 0.0	4.4 ± 0.2	4.4 ± 0.1					
20	H1	3.5 ± 0.1	3.9 ± 0.1	3.4 ± 0.0					
	H2	4.2 ± 0.1	4.7 ± 0.2	4.1 ± 0.0					
	Н3	5.8 ± 0.1	3.4 ± 0.2	4.0 ± 0.1					
30	H1	7.1 ± 0.8	4.2 ± 0.1	3.5 ± 0.1					
	H2	7.6 ± 0.0	5.4 ± 0.2	4.0 ± 0.0					
	Н3	7.8 ± 0.5	4.5 ± 0.6	3.6 ± 0.2					

Table 5.4
Final pH of culture medium after 10, 20 and 30 days cultivation

^a Final pH was calculated after growth of the mycelium in 125 mL of the culture medium in 250 mL shaker flasks. Growth was performed in triplicate shaker flasks and the average and STD calculated; ^b Initial pH of MEB is 5.4 ± 0.4 ; ^c Initial pH of PDB is 5.6 ± 0.2 ; ^d Initial pH of Basal-G medium is 5.1 ± 0.1 .



Figure 5.4. Mycelial biomass of (a) *Ganoderma* H1, (b) *Ganoderma* H2 and, (c) *Ganoderma* H3, cultivated in MEB, PDB and Basal-G growth media; Biomass was measured after 10, 20 and 30 days; Mycelium was grown in 125 mL of growth medium in a 250 mL shaker flask for the required number of days. Flasks were incubated at 30°C and shaken at 100 rpm; All cultures were prepared in triplicate.

It is generally known that the by-products of fungal metabolic processes are acids, which decrease the pH of the culture medium (Fang & Zhong, 2002b; Lin *et al.*, 1973). In addition, the utilization of some compounds in the medium may result in adverse changes in pH (Griffin, 1994). The final pH of the media that was used for the cultivation of the three *Ganoderma* isolates was found to have decreased to an acidic level at day 10 (3.4 ± 0.0 to 4.4 ± 0.2) (Table 5.4). Over the course of the cultivation period, the pH of the media was seen to remain around this acidic level with the exception of the culture medium MEB, which increased to approximately pH 7. MEB contains no buffering salts and it has been suggested that a buffer is required to control the pH of the medium (Lin *et al.*, 1973). PDB contains no buffers either, but there was no increase in pH observed when the fungi were grown in this medium (Table 5.4). This observation was thought to have been a function of the low yield of biomass produced in PDB, which in turn resulted in slow consumption and hence, no depletion of the nutrients in the medium.

It has been reported that when the pH of the culture medium reaches a low level (~ 3.8), the growth of the mycelium ceases (Lin *et al.*, 1973). This was not observed for any of the isolates examined in the different media (Figure 5.4) and this is an observation consistent with that of Kim et al. (2002a). When the pH of all media reached acidic levels and remained at this level over a period of 30 days, the growth of the mycelium actually increased over the course of the cultivation period (Figure 5.4 and Table 5.4). This suggested that there were nutrients (energy sources) still available in the culture medium that the fungi could metabolise. In fact, the opposite effect occurred, whereby, when the pH of the culture medium increased, the mycelial growth either stopped or decreased in mass, as was the case for cultivation in PDB and MEB after day 20 (Table 5.4 and Figure 5.4). This suggested that if the final pH of the cultivation medium was low, then mycelial growth in that particular medium would be high. This observation was similar to that of Yang and Liau (1998) who also observed that at lower pH levels better growth of G. lucidum mycelia was achieved. Lin et al. (1973) reported that discontinuation of mycelial growth took place after 7 days of cultivation at low pH. However, longer incubation times were not investigated.
Overall there appeared to be a general trend of increased biomass production over time when the isolates were cultivated in both the Basal-G medium and PDB (Figure 5.4). This was not the case when they were cultivated in MEB. When MEB was used as the cultivation medium, a decline in the biomass for all isolates was observed between day 20 and day 30 of cultivation. As the biomass decreased between day 20 and 30 (Figure 5.4), there was a significant increase in the pH of the medium, changing to a slightly alkaline pH ranging between 7.1 ± 0.8 to 7.8 ± 0.5 (Table 5.4). Generally, the utilisation and exhaustion of compounds within the growth medium can lead to changes in the pH and prevention of growth (Chang & Miles, 1989b; Griffin, 1994). Fang & Zhong (2002b) reported that when the residual glucose was exhausted in the cultivation medium of *G. lucidum*, an increase in pH (to a pH of 7) was observed. They determined that the glucose consumption was directly related to cell growth.

Glucose, the sugar source in MEB, is the carbon source that provides for both the structural and energy requirements of the fungal cell (Chang & Miles, 1989b). The depletion of this source generally leads to an adverse affect on cell growth. When the isolates were grown in MEB medium, the decrease in biomass and increase in pH (Figure 5.4) may have been due to the depletion of the carbon source within the medium before the completion of the 30 day incubation cycle. There are other nutrients present in the medium, such as nitrogen, that if depleted, may also lead to an adverse effect on the growth of the cell (Chang & Miles, 1989b; Griffin, 1994). However, this was not investigated further as the aim of this investigation was to produce large enough quantities of mycelial biomass for secondary metabolite investigation (antibacterial activity) and not to investigate, in depth, the different nutrient requirements required by the three isolates. In addition, it has been suggested that many secondary metabolite pathways are negatively affected by nitrogen sources favourable for growth (Adrio & Demain, 2003).

Due to the maximum amount of biomass being produced when all three isolates were grown in the Basal-G medium (Figure 5.4), this medium was chosen for further nutritional study in the following section.

5.3.2.4 Effect of Carbohydrate Source on Mycelial Biomass Production

The three Australian *Ganoderma* isolates were investigated for the effect that different carbohydrate sources had on the production of the mycelial biomass in shaker flask cultures (Sections 3.10.1 and 3.8.2). The fungi were grown in Basal-G growth medium (Section 3.6.1), substituting glucose with different carbohydrate sources for the comparison of the mycelial growth. The carbohydrate sources investigated were the monosaccharides; glucose, galactose, fructose, mannose and xylose, and the disaccharides; lactose, sucrose and cellobiose. Shaker flasks were prepared in triplicate. The mycelial biomass and final pH were determined after 30 days of growth at the optimal growth temperature, 30°C.

Figure 5.5 shows the effects of different carbohydrate sources on cell growth and the final culture medium pH of *Ganoderma* H1, H2 and H3. Comparing the profiles of the three isolates, it is evident that these fungi utilise different carbohydrate sources in different ways, demonstrating that related species can have different growth needs.

When *Ganoderma* H1 was grown in a glucose supplemented medium (Figure 5.5a) the maximum amount of mycelium was obtained $(1.23 \pm 0.10 \text{ g})$. Glucose has been also observed to be the best energy source for biomass production for another medicinally important fungus, *Lentinus edodes* (Song & Cho, 1987). A glucose supplemented medium was also highly favourable to the growth of *Ganoderma* H2 (Figure 5.5b). However, it was also noted that there was no difference between the amount of biomass produced when glucose $(1.91 \pm 0.15 \text{ g})$, xylose $(1.88 \pm 0.21 \text{ g})$ and lactose $(1.84 \pm 0.09 \text{ g})$ were the carbohydrate source for *Ganoderma* H2. A similar observation reported by Tang & Zhong (2002), also found that glucose and lactose (as well as maltose) were comparable in the production of the mycelial biomass of *G. lucidum*, after 10 days of cultivation.



Figure 5.5. The effect of carbohydrate source on mycelial biomass production and culture medium pH of (a) *Ganoderma* H1, (b) *Ganoderma* H2 and, (c) *Ganoderma* H3. Carbohydrate source was in the form of monosaccharides: glucose, galactose, fructose, mannose and xylose; and disaccharides: lactose, sucrose and cellobiose. The scale on the left of the graph corresponds to the biomass in grams (bars). The right scale, final pH of the cultivation medium is represented on the graph by the scatter points. Cultures were grown in 125 mL of medium in 250 mL shaker flasks for a period of 30 days.

The general concept that glucose, among all the hexoses, is biologically the most effective energy source (Cochrane, 1958) was supported by the high levels of mycelial growth of both *Ganoderma* H1 and H2 in media containing glucose. However, glucose did not support the best mycelial growth for *Ganoderma* H3 (Figure 5.5c), and the more complex sugars, galactose and fructose, resulted in larger amounts of mycelium being produced. The amount of biomass produced when galactose (2.05 ± 0.30 g) and fructose (2.02 ± 0.19 g) were the carbohydrate source was double that when glucose (0.99 ± 0.24 g) was the carbohydrate source in the culture medium.

When cellobiose was used as the carbohydrate source, the least amount of biomass was produced for all three isolates (Figure 5.5). This carbohydrate source gave a yield of 0.48 ± 0.11 , 1.08 ± 0.10 and 0.70 ± 0.10 g of mycelium in 125 mL of culture medium. Bae *et al.* (2000) reported that cellobiose was one of the most favourable carbohydrate sources for producing high biomass production of another higher fungus, *Paecilomyces japonica*.

Glucose is the most widely utilised carbon source by fungi, most probably due to it being the most commonly available sugar, found in cellulose, starches, and other carbohydrates (Griffin, 1994). Fructose and mannose are the next most commonly utilised sugars by fungi, followed by galactose (Griffin, 1994). Reports have shown that different types of carbohydrate sources can aid the growth of *Ganoderma* species. Sone et al. (1985), who studied the growth of G. lucidum, found lactose to support the greatest amount of mycelial growth after 7 days of cultivation. The presence of lactose was also favourable to the growth of the three Australian isolates, although other sugars supported better growth (Figure 5.5). It must be noted that the Australian isolates were cultivated for a longer period (30 days), which may largely affect the mycelial growth, as was the case when the three isolates were cultivated in MEB (Figure 5.4). Comparative studies by Lin (1973) on a different *Ganoderma* sp. showed that there was no significant difference in biomass production when either glucose or sucrose was the carbohydrate source in the culture medium. This was not found in the studies of the three Australian isolates, as the growth of mycelium in a sucrose supplemented medium was less than that in a glucose supplemented medium for all three isolates. Again, the

cultivation time used by Lin (1973) was only 7 days while in the case of the three Australian isolates, the cultivation time was 30 days.

It was shown previously that *Ganoderma* H2 had a faster growth rate than *Ganoderma* H1 and H3 (Table 5.3). The ability of *Ganoderma* H2 to grow rapidly was also apparent when comparing the three carbohydrate source graphs in Figure 5.5. They showed that the overall biomass of *Ganoderma* H2 (Figure 5.5b) was much greater than the overall biomass of *Ganoderma* H1 (Figure 5.5a) and *Ganoderma* H3 (Figure 5.5c) when taking into account the growth in all of the cultivation media with different carbohydrate sources. Overall, *Ganoderma* H1 produced the least amount of biomass (Figure 5.5a), again demonstrating that *Ganoderma* H1 was the slowest growing isolate out of the three isolates examined. The slow growth of *Ganoderma* H1 was also seen in the comparison of growth rates in Section 5.3.2.2 (Table 5.3).

The final pH of the culture medium was another factor of interest since there have been reports that suggest that the lower the final pH, the greater the mycelial growth obtained (Yang & Liau, 1998). The final pH of the culture medium for the three Australian isolates is also presented in Figure 5.5 and is represented by the points on the graphs. The final pH of all the media investigated ranged from 3.5 to 4.8 for the three isolates, showing that there was a drop in pH from the initial pH of 5.1 ± 0.1 (Table 3.6), which is commonly observed when cultivating fungi in liquid culture (Chen, 1999; Fang & Zhong, 2002b). Ganoderma H1 produced the least amount of biomass overall as mentioned earlier (Figure 5.5). It also decreased the final pH of seven media out of eight, to a pH below 4, (pH 3.46 ± 0.22 to 3.85 ± 0.13) (Figure 5.5a). In comparison, Ganoderma H2 reduced four media out of eight to just below a pH of 4 (pH 3.86 ± 0.05 to 3.93 ± 0.01) (Figure 5.5b) and *Ganoderma* H3 decreased the pH of only one medium (fructose) to a pH of 3.88 (Figure 5.5c) after 30 days of cultivation. The idea that a low final culture medium pH gives a high yield of biomass does not hold up when comparing different isolates against each other. However, there appeared to be a general trend of a low final culture medium pH being proportional to a high biomass yield, when comparing the growth of the same isolate in different media. In the case of both Ganoderma H1 and H2, when biomass production was large, the final pH was

observed to be lower than that when the biomass production was low (Figure 5.5a,b). This pattern was not observed for *Ganoderma* H3 (Figure 5.5c).

Further investigations of the optimal carbohydrate concentration in the liquid medium were not performed. Fang & Zhong (2002c) reported that the highest levels of biomass, as well as intracellular polysaccharides and ganoderic acids, were obtained at an initial glucose concentration of 50 g L⁻¹. This concentration was used in the cultivation medium in this investigation. In addition, nutrients such as nitrogen and inorganic salts were not examined in this study, as at this point in the investigation, the production of biomass was deemed sufficient to proceed with the examination of the biological activity of the three isolates. In addition, growth factors such as yeast extract, peptone and phosphate ions, which have been reported to promote good mycelial growth (Bae *et al.*, 2000; Fang & Zhong, 2002c; Song & Cho, 1987), were already incorporated into the cultivation medium. For future studies, it would be recommended to examine all nutrient sources and the effect they had on the biological activity (which has been determined in the following Chapter) of the fungus.

Overall, glucose was determined to be the best carbohydrate source for *Ganoderma* H1, glucose, xylose and lactose for *Ganoderma* H2, and both galactose and fructose for *Ganoderma* H3. Fang and Zhong (2002c) observed that high biomass produced when glucose was the carbohydrate source also produced a high ganoderic acid concentration in the mycelium. This relationship of high ganoderic acids and high biomass production was also seen by Tang & Zhong (2002), and Fang & Zhong (2002b). Because the biological activity of the cultivated mycelium from the three Australian *Ganoderma* isolates was of interest, in particular the organic extracts from the mycelium as discussed in the following Chapter, the high biomass production using glucose as the primary carbohydrate source appeared promising in the production of biologically active compounds.

5.4 Summary

In this Chapter, the three Australian *Ganoderma* isolates, H1, H2 and H3, were investigated for their viability and their growth behaviour in selected environmental and nutritional growth conditions. These factors were investigated to determine the optimal

growth requirements of the fungi and to assess whether they were comparable to other *Ganoderma* species that have been isolated from other parts of the world. In addition, optimal growth conditions were established so that the rapid production of good quality mycelium could be produced.

The viability of the three isolates was determined for both long and short term storage (Section 5.3.1). It was determined that the best method for long term storage for the three isolates was on balsa wood chips, stored at -80°C for periods of up to 2 years without subculture. Viability studies were not performed on the fungi after this time. Long term storage resulted in the decrease of subculture growth for both *Ganoderma* H1 and H3. This prompted further investigation on the short term storage of the fungi, so that rapid subculture of the mycelium could be performed. It was determined that all three isolates were to be stored on either, PDA or MEA slants at 4°C for up to periods of 8 weeks before transferring to fresh slants. It was also important to store the slants away from the direct light, in particular *Ganoderma* H1 and H3, as the light had a detrimental effect on the viability of the mycelium.

The two important environmental factors, pH and temperature, were investigated (Section 5.3.2.1). The isolates were found to grow best at a temperature and pH similar to what has been reported for other *Ganoderma* species (Yang & Liau, 1998). From the range of temperatures studied, it was determined that 30°C was optimal for all three isolates, although growth at 37°C for *Ganoderma* H1 and H2, and growth at 25°C for *Ganoderma* H3 was also highly favourable. Under the growth conditions tested, an initial cultivation medium of pH 6 was determined to be optimal for all three isolates, although it must be noted that they grew well in the pH range of 4 to 8. The growth rates of the three fungi were also studied (Section 5.3.2.2) and it was observed that at the optimal pH and temperature, *Ganoderma* H2 had the fastest growth rate whilst *Ganoderma* H1 had the slowest.

The type of cultivation medium and the carbohydrate source in the medium were examined for the ability to provide the greatest amount of mycelial growth (Section 5.3.2.3 and Section 5.3.2.4). A defined growth medium, which contained phosphates, organic nitrates and minerals, was found to support the best mycelial growth of the three

Australian isolates. The growth of the fungi, in defined medium supplemented with different carbohydrate sources, was explored and it was found that glucose supported the greatest growth of both *Ganoderma* H1 and H2, and galactose and fructose supported the greatest growth of *Ganoderma* H3.

Overall, the viability and growth studies examined in this Chapter gave some valuable information about the preservation, growth and cultivation of the three Australian *Ganoderma* isolates. Of particular interest was the observation that although *Ganoderma* H1 and H3 had similar profiles in the viability studies (Section 5.3.1) and were more closely related in the phylogenetic studies (Chapter 4, Section 4.3.3), there appeared to be more similarities between *Ganoderma* H1 and H2 when some growth requirements were investigated. This highlights the significance that closely related species or strains may require different growth conditions and that it is important that these optimal growth conditions be identified for each new isolate.

Chapter Six

Mycelial Extraction and Antibacterial Activity

6.1 Introduction

The extraction process is an important step in the investigation of biologically active compounds. When extracting compounds from fungi, or any living source, the type of solvent used, the extraction process employed and the age, part or type of cultivation of the living tissue, can all have a marked effect on the type of compound that can be extracted. Aqueous extractions using hot water have been performed on the fruiting bodies and mycelium of *Ganoderma* species and have resulted in the extraction of many proteins, lectins and polysaccharides (Bao *et al.*, 2002; Cheong *et al.*, 1999; Kawagishi *et al.*, 1997). When the extraction process employs organic solvents such as chloroform or methanol, which are non-polar and highly polar, respectively, different compounds such as flavonoids, alkaloids, coumarins, fatty acids and triterpenes are commonly extracted (Cowan, 1999; Lin & Shiao, 1988; Shiao *et al.*, 1988b).

Many of the compounds extracted from both the mycelium and fruiting body of *Ganoderma* have been found to exhibit biological activity towards tumours and virus infected cells (Gao *et al.*, 2000a; Kim *et al.*, 2000; Kimura *et al.*, 2002; Lin *et al.*, 2003; Mothana *et al.*, 2003; Oh *et al.*, 2000; Zhang & Lin, 1999a, 199b). In addition, compounds from *Ganoderma* have also been reported to have hepatoprotective roles against liver injury (Kim *et al.*, 1999; Zhang *et al.*, 2002a) and protective effects towards organelles that are affected by reactive oxygen species (Lee *et al.*, 2001; You & Lin, 2002). On review of the literature, there appears to be fewer studies performed on the antimicrobial activity of the aqueous or organic extracts from *Ganoderma* species. This Chapter reports the extraction of compounds and their antibacterial activities from the liquid cultivated mycelium of the three Australian *Ganoderma* isolates.

6.2 Overview

Both aqueous and organic extracts from the liquid cultivated mycelium of the three Australian *Ganoderma* isolates were investigated for antibacterial activity. Aqueous extractions employed hot water. Organic extractions involved Soxhlet extraction using four organic solvents ranging in polarity. Initial extractions were performed on the mycelial biomass that had been cultivated in a commercially available nutrient medium, MEB, for 30 days. The inhibitory activity of all crude aqueous and organic extracts was investigated against a range of Gram positive and Gram negative bacteria. Preliminary

results indicated that both the aqueous and organic extracts from *Ganoderma* H1 possessed activity against a greater range of bacteria than the other *Ganoderma* isolates and hence, this species was used for a more in-depth investigation. Crude organic extracts from the mycelium of *Ganoderma* H1, which had been cultivated in three different media, were examined for their activity against a range of aerobic and anaerobic bacteria, including some clinically important pathogenic species. Effective concentrations of the most active organic extracts were investigated by means of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays. Finally, time course growth assays were studied to investigate the effect that the extracts had on the different test bacteria.

6.3 Results & Discussion

6.3.1 Preliminary Antibacterial Investigations of *Ganoderma* H1, H2 & H3

Preliminary antibacterial screening was performed early in this investigation to determine whether the extracts possessed any biological activity. Both the aqueous (Section 6.3.1.2) and organic extracts (Section 6.3.1.4) from the three isolates, H1, H2 and H3 were investigated for their antibacterial activity. At this stage, the biomass productivity of the three isolates cultivated in different media, which was discussed in the previous Chapter (Sections 5.3.2.3 and 5.3.2.4), had not yet been explored. It is therefore important to note that the growth medium MEB, which was used for the cultivation of the three isolates, was initially selected purely as a means of producing mycelial biomass. Once activity was confirmed, further analysis was performed in an alternative cultivation media and is discussed in the latter part of this Chapter. In addition, antibacterial assays were initially performed to identify which isolate, if any, contained the greatest antibacterial activity so a more in-depth study of only one isolate could be performed. The Australian Ganoderma isolate H1, which was found to possess the greatest activity under the initial growth conditions, is further explored and discussed in the latter part of this Chapter and in Chapter 7.

6.3.1.1 Aqueous Extraction of the Mycelium from *Ganoderma* H1, H2 & H3

Aqueous extractions on the mycelial biomass of the three *Ganoderma* isolates H1, H2 and H3, were performed as described in Section 3.11.1. The mycelium was cultivated

in MEB for 30 days (Sections 3.10.1 and 3.8.2), freeze dried (Section 3.3) and then subjected to aqueous extraction by boiling for 2 hours at 100°C. Aqueous extracts were then evaporated to dryness. The mass and appearance/consistency of the aqueous extracts are presented in Table 6.1.

Table 6.1

	Hot Water Extract ^b				
<i>Ganoderma</i> isolate ^a	mass (mg g ⁻¹)	Appearance; Consistency			
H1	15 ± 3	Dark brown; Powder			
H2	22 ± 2	Dark orange/brown; Powder			
Н3	15 ± 3	Dark brown; Powder			

Hot water extraction yield of three Australian Ganoderma isolates H1, H2 and H3

^a Ganoderma isolates were cultivated in MEB for 30 days in shaker flasks; ^b Hot water extract yield was calculated in mg of extract per gram of mycelium and is a result of triplicate extractions.



Figure 6.1. Aqueous extracts from the mycelial biomass of *Ganoderma* H1, H2 and H3 that had been cultivated in MEB for a period of 30 days. (a) Dried and ground extract. (b) Extracts (10% w/v) resuspended in water.

The yield of aqueous extract for both *Ganoderma* H1 and H3 resulted in the same mass $(15 \pm 3 \text{ mg g}^{-1})$ after triplicate extractions (Table 6.1). *Ganoderma* H2 provided a larger extract yield $(22 \pm 2 \text{ mg g}^{-1})$. The appearance/consistency of the three extracts was

similar for both *Ganoderma* H1 and H3 (dark brown powder), however for *Ganoderma* H2, the extract was a dark brown to orange powder. The dried and ground extracts, and the 10% (w/v) solutions resuspended in water, are shown in Figure 6.1a,b. Yoon *et al.* (1994), who investigated the aqueous extraction of the fruiting bodies of *G. lucidum* also observed a similar colour.

6.3.1.2 Antibacterial Activity Screening of the Aqueous Extracts from *Ganoderma* H1, H2 and H3

Preliminary screening for antibacterial activity was performed using a disc diffusion assay (Section 3.13.1) and was performed for all aqueous extracts reported in Section 6.2.1.1. The extracts from *Ganoderma* H1, H2 and H3 were applied to separate discs and the inhibitory effect was measured against a number of Gram positive and Gram negative bacteria. The final amount of extract applied to each disc was 6000 μ g. The results of the preliminary investigation are presented in Table 6.2.

The data in Table 6.2 show that both *Ganoderma* H1 and H2 exhibited incomplete inhibition against the Gram positive bacterium, B. cereus. Ganoderma H1 also exhibited activity against B. subtilis. There was no activity observed for any of the extracts against any of the Gram negative bacteria tested. It is difficult to compare these results against literature values, as there appears to be little information available on the antibacterial activity of aqueous extracts from the mycelium of *Ganoderma*. Yoon *et* al. (1994) who investigated the bioactivity of aqueous extracts from the fruiting body of G. lucidum found that the extracts also exhibited inhibitory activity towards the Bacillus species. In addition, they found their aqueous extracts to have strong activity against some other Gram positive and Gram negative bacteria. It has been suggested that there can be variation in the bioactive component of fungal mycelium compared to the fruiting body (Lindequist, 1995; Lorenzen & Anke, 1998) and that during the different stages of growth, there can be structural change of the bioactive components (Chen & Miles, 1996a). The aqueous extracts obtained by Yoon et al. (1994), from the fruiting body of *Ganoderma*, were likely to have contained different active compounds to those in the aqueous extracts from Ganoderma H1, H2 and H3. Therefore, the smaller range of activity exhibited in this investigation (Table 6.2) could be attributed to the fact that the extracts were from the mycelium and not the fruiting bodies of the fungi.

Table 6.2

		Antibacterial Activity of Crude Aqueous Extracts					
	Test Bacteria ^a	H1	H2	Н3			
Gram	Bacillus cereus	[7]	[6.5]	-			
positive	Bacillus subtilis	[7]	-	-			
	Enterococcus faecalis	-	-	-			
	Listeria monocytogenes	-	-	-			
	Micrococcus luteus	-	-	-			
	Streptococcus pyogenes	10 (19)*	- (17)*	7 (14)*			
	Staphylococcus aureus (SUT)	-	-	-			
	Staphylococcus epidermidis	-	-	-			
Gram	Escherichia coli	-	-	-			
negative	Klebsiella pneumoniae	-	-	-			
	Pseudomonas aeruginosa	-	-	-			
	Proteus vulgaris	-	-	-			
	Salmonella typhimurium	-	_	_			
	Shigella sonnei	-	-	-			

Preliminary antibacterial screening of the aqueous extracts from the liquid cultivated mycelium of *Ganoderma* H1, H2 and H3.

^a Cultures were incubated at 37°C for 24 hours, except *Micrococcus luteus*, which was incubated at 30°C for 48 hours. Culture conditions and growth media are listed in Table 3.3 of Chapter 3; ^b Activity was determined by disc diffusion assays on lawn cultures. A zone of inhibition > 6mm was considered positive. [], incomplete inhibition was observed (i.e.; reduced growth, but not complete elimination of growth). -, no inhibition was observed. * Two zones were observed, the first complete zone of inhibition was observed prior to chemical treatment and after spraying with MTT, a larger second zone was observed which is shown in the brackets (); Results are the average of triplicate experiments; Blanks of solvent only (processed in the same way) were also tested for antibacterial activity, and gave no inhibition; Crude extracts were from fungi grown in MEB for 30 days. H1, H2 and H3 represent *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3 respectively.

Ganoderma H1 and H3 exhibited the strongest (and only complete) zone of inhibition against *S. pyogenes* (Table 6.2 and Figure 6.2). There appears to be no literature available that reports the sensitivity of *S. pyogenes* towards aqueous extracts from *Ganoderma* species, although, Smania *et al.* (1999) found that organic extracts displayed inhibitory activity towards this bacterium. To aid in the visual observation of the zones of inhibition were observed for *Ganoderma* H1 and H3 (giving a second zone of inhibition) and a large zone of inhibition was also observed for *Ganoderma* H2 (Figure 6.2b). MTT is reduced by the metabolically active cells (i.e. live bacterial cells) and results in an intracellular purple formazan product that accumulates in the cells (Hostettmann, 1999). Hence, non-viable cells (or dead cells) do not reduce MTT and will not produce a purple product. As a result, if a plate is sprayed with MTT, there will

be a non-coloured (clear) zone of inhibition (bacterial cells that have been killed by the extract) clearly visible against the darker purple coloured viable cells. A representative diagram of this process is shown in Figure 6.3. However, on close inspection of this new larger zone of inhibition, there was a definite area where bacterial growth could be seen. The bacteria within this area did not reduce the MTT to form a purple complex indicating that the bacteria within this larger zone were not living (Figure 6.3) (and was confirmed by the inability of the subcultured bacteria to grow during subsequent incubation). One possibility of this observation is that *S. pyogenes* had time to grow within this region before the extract fully diffused through the agar medium. It would then appear that the extract continued to move through the agar medium and kill the bacteria after growth occurred.



Figure 6.2. Representative photos of the disc diffusion assay showing complete inhibition of *Streptococcus pyogenes* by the aqueous extract of *Ganoderma* H1 after a 24 h incubation period. (a) Depicts the zone of inhibition (dark area) around the disc that contains the extract. The lighter area outside the zone of inhibition is the growth of the organism that is visible to the eye. (b) Depicts the larger zone of inhibition (clear zone) around the same disc with extract, after spraying with MTT. The area outside the zone of inhibition is a purple formazan complex formed from the reduction of MTT by the live bacteria.

There is much debate regarding the use of the diameter of zone of inhibition as a reliable measure of antibacterial activity, as the size of the zone of inhibition is affected by the rate of diffusion of the compound through the agar gel, and different compounds diffuse at different rates (Barry & Thornsberry, 1985). The observation of the larger zone highlighted this issue and illustrated that inhibition of each bacterium depends largely on the ability of the extract to diffuse through the medium.



Figure 6.3. Representative diagram of the reduction of MTT by living bacteria after 24 hours incubation. The plate is sprayed with MTT, which is reduced to form a purple complex by the living bacteria. Dead bacteria do not reduce MTT and therefore, leave a zone of inhibition. Bacteria that do not reduce MTT can be confirmed dead by performing a subculture.

Overall, the aqueous extracts from the mycelium of the three Australian *Ganoderma* isolates exhibited a small range of antibacterial activity against some Gram positive bacteria. This activity appeared to be much lower than that reported in the literature. However, it must be noted that the aqueous extraction process employed in this investigation (near boiling for 2 hours, Section 3.11.1) may inactivate some types of potential antibacterial compounds, such as proteins. Nevertheless, this is the first account of aqueous extracts from the mycelium of *Ganoderma* exhibiting antibacterial activity. The small amount of activity exhibited by the aqueous extracts, combined with the fact that the organic extracts exhibited antibacterial activity against a greater range of bacteria (which is discussed in the following Sections in this Chapter), led to the decision to not pursue the investigation of aqueous extracts any further.

6.3.1.3 Organic Extraction of the Mycelium from *Ganoderma* H1, H2 & H3

Organic extractions were performed on the mycelial biomass of *Ganoderma* H1, H2 and H3 as described in Section 3.11.2. The fungal isolates were cultivated in MEB and harvested on day 30 (Sections 3.8.2 and 3.10.3). The mycelial biomass was freeze dried and then subjected to successive Soxhlet extractions using a range of organic solvents. The solvents used were in the order of increasing polarity starting with hexane (HEX),

dichloromethane (DCM), ethyl acetate (EtOAc) and then methanol (MeOH). The resulting solvent obtained from each extraction was evaporated to dryness under reduced pressure and the mass (Table 6.3) and appearance/consistency (Table 6.4) of the crude extracts noted.

Solvents of increasing polarity were used in the extraction process to try and extract components of varying polarity into the different solvents. Generally, extraction using a less polar solvent (e.g. hexane) will extract compounds of a similar polarity, and extraction using a more polar compound (e.g. methanol) will then extract compounds that have a greater polarity. Ideally, different compounds will be extracted into the solvents at different phases in the Soxhlet process. However, one rarely achieves complete separation of constituents and the same compound may be recovered (in varying proportions) in several fractions (Harbourne, 1973). Nevertheless, using solvents of increasing polarity in a Soxhlet apparatus is a classical chemical procedure for obtaining chemical constituents from dried material.

The yield of the extracts for the three *Ganoderma* isolates grown in MEB are given in Table 6.3. When comparing the mass of extracts between different successive solvents within the same isolate, there was a wide range of extract masses obtained ranging from 13 ± 3 to 197 ± 54 mg g⁻¹, 5 ± 2 to 69 ± 17 mg g⁻¹ and 2 ± 1 to 119 ± 18 mg g⁻¹ for H1, H2 and H3, respectively. A wide range of extract mass was also observed between the different isolates extracted using the same solvent. For example, the MeOH extract yielded 197 ± 54 mg g⁻¹, 69 ± 17 mg g⁻¹ and 119 ± 18 mg g⁻¹ for H1, H2 and H3, respectively (Table 6.3). This large range of masses indicated that there were differences in the mycelium of the three *Ganoderma* isolates. Rosecke & Konig (2000) and Yen & Wu (1999) also observed extract yields ranging from 2 to 25 mg g⁻¹ (HEX extract) and 40 to 103 mg g⁻¹ (MeOH extract), from the fruiting bodies of different *Ganoderma* isolates.

Extraction	Organic Extracts from	Organic Extracts from Australian <i>Ganoderma</i> Isolates ^a						
Solvent	H1 (mg g^{-1})	H2 (mg g^{-1})	H3 (mg g^{-1})					
HEX	$54 \pm 9^{\mathbf{b}}$	16 ± 8	12 ± 6					
DCM	45 ± 3	7 ± 2	4 ± 1					
EtOAc	13 ± 3	5 ± 2	2 ± 1					
МеОН	197 ± 54	69 ± 17	119 ± 18					
Total crude extract ^c	309 ± 54	96 ± 17	137 ± 18					

 Table 6.3

 Organic extraction yield of *Ganoderma* isolates H1, H2 and H3

^a *Ganoderma* isolates were cultivated in MEB for 30 days in 1 L shaker flasks; ^b Crude extract yield was calculated in mg of extract per gram of mycelium and is a result of triplicate extractions; ^c The total crude extract was calculated by addition of all successive extracts (HEX, DCM, EtOAc, MeOH) from the same isolate.

The extracts obtained from each species by the same solvent exhibited similar colours and textures (Table 6.4). All HEX extracts were observed to be an orange oily consistency. This observation was similar to that obtained by Rosecke and Konig (2000), who noted their HEX extracts to be either a yellow or orange oily substance. The DCM extract was observed to be an orange solid, EtOAc to be a yellow/orange/brown solid and the MeOH to be a chunky brown to dark red solid (Table 6.4). Again, the observation for the crude extracts were similar those obtained by Rosecke and Konig (2000), who observed their MeOH and DCM extracts to consist of a dark red solid and orange solid respectively.

Table 6.4

Extraction	Organic Extracts from Australian <i>Ganoderma</i> Isolates ^a						
Solvent	H1	H2	Н3				
	Appearance; Consistency	Appearance; Consistency	Appearance; Consistency				
HEX	Orange; Oily	Orange; Oily	Orange; Oily				
DCM	Dark orange; Solid	Dark orange; Solid	Dark orange; Solid				
EtOAc	Yellow/orange; Solid	Orange/brown; Solid	Orange/brown; Solid				
MeOH	Brown/red; Solid	Brown/red; Solid	Brown/red; Solid				

The appearance and consistency of the organic extracts from Ganoderma H1, H2 and H3

^a Ganoderma isolates were cultivated in MEB for 30 days in 1 L shaker flasks.

6.3.1.4 Antibacterial Activity Screening of the Organic Extracts from *Ganoderma* H1, H2 and H3

Preliminary screening for antibacterial activity was performed using the disc diffusion assay (Section 3.13.1) and was carried out on all crude organic extracts. Initial bioactivity experiments were performed on crude extracts from different Soxhlet batches. Once bioactivity was confirmed, the Soxhlet batches were pooled to obtain large amounts of crude extract for further analysis. As mentioned earlier, preliminary screening was performed early in this investigation to assess the crude extracts for any biological activity present. Therefore, preliminary extractions, and in turn antibacterial assays, were performed on the mycelium that had been cultivated in a MEB medium.

Preliminary antibacterial testing of the crude organic extracts from the three Australian *Ganoderma* isolates H1, H2 and H3 are shown in Table 6.5. Note that antibacterial activity in Table 6.5 is reported as either a positive or negative result, and not as the diameter of the zone of inhibition, as was reported for the aqueous extracts in the previous section (Section 6.3.1.2, Table 6.2). Further antibacterial assays were performed on the organic extracts (Section 6.3.2.1), thus, zones of inhibition are reported and discussed later in this chapter. This section on the preliminary testing of the organic extracts is primarily included to report the process of selection, of one *Ganoderma* isolate, for further antibacterial analysis.

Table 6.5 shows that the crude HEX, DCM and EtOAc extracts of *Ganoderma* H1 exhibited activity against the Gram positive bacteria, *B. cereus*, *B. subtilis*, *S. pyogenes*, *S. aureus*, *S. epidermidis* and *L. monocytogenes*. There was no inhibition observed for the organic extracts from *Ganoderma* H2 or H3 against any of the bacteria, with the exception of incomplete inhibition of *B. cereus* and complete inhibition of *S. pyogenes*, exhibited by the DCM extract from *Ganoderma* H2.

Table 6.5

		Antibacterial Activity of Crude Organic Extracts ^b											
			HEX			DCM		EtOAc		МеОН			
	Test Bacteria ^a	H1	H2	Н3	H1	H2	Н3	H1	H2	H3	H1	H2	H3
	Bacillus cereus	[+]	-	-	+	[+]	-	+	-	-	-	-	-
	Bacillus subtilis	+	-	-	+	-	-	+	-	-	-	-	-
e	Enterococcus faecalis	-	-	-	-	-	-	-	-	-	-	-	-
itiv	Streptococcus pyogenes	+	-	-	+	+	-	+	-	-	-	-	-
sod mi	<i>Staphylococcus aureus</i> (SUT)	[+]	-	-	+	-	-	[+]	-	-	-	-	-
Gra	Streptococcus epidermidis	-	-	-	[+]	-	-	-	-	-	-	-	-
	Listeria monocytogenes	[+]	NT	NT	[+]	NT	NT	[+]	NT	NT	-	NT	NT
	Micrococcus luteus	-	-	-	_	-	-	_	-	-	_	-	-
	Escherichia coli	-	-	-	-	-	-	-	-	-	-	-	-
ive	Klebsiella pneumoniae	-	-	-	-	-	-	-	-	-	-	-	-
negat	Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-
am	Proteus vulgaris	-	-	-	-	-	-	-	-	-	-	-	-
Ģ	Salmonella shigella	-	-	-	-	-	-	-	-	-	-	-	-
	Shigella sonnei	-	-	-	-	-	-	-	-	-	-	-	-

Preliminary antibacterial screening of the organic extracts from the liquid cultivated mycelium of *Ganoderma* H1, H2 and H3

^a Cultures were incubated at 37°C for 24 hours, except *M. luteus*, which was incubated at 30°C for 48 hours. Culture conditions and growth media are listed in Table 3.3 of Chapter 3; ^b Activity was determined by disc diffusion assays. A zone of inhibition > 6mm was considered positive. + a zone of inhibition was observed; - no inhibition was observed; [+] incomplete inhibition was observed (i.e.; reduced growth, but not complete elimination of growth); NT, not tested; Results are the average of triplicate experiments; Blanks, consisting of the solvent without extract (which had been processed in the same way) were also tested for antibacterial activity and gave no zones of inhibition; Crude extracts are from fungi grown in MEB for 30 days. H1, H2 and H3 represent *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3, respectively.

Ganoderma H1 was observed to possess activity against the greatest range of bacteria (Table 6.5). The DCM extract from *Ganoderma* H1 exhibited activity against six bacteria, whilst the DCM extract from *Ganoderma* H2 and H3 exhibited activity against two and zero bacteria, respectively. The yield of DCM extract for the three isolates H1, H2 and H3 was also different (Table 6.3). The DCM extract from *Ganoderma* H1 (45 \pm 3 mg g⁻¹) was as much as six to fourteen times greater than the DCM extract obtained from *Ganoderma* H2 (7 \pm 2 mg g⁻¹) and H3 (4 \pm 1 mg g⁻¹) respectively. Since the mass of extract (6000 µg) assessed for activity was the same for all extracts, this suggested that the active compound/s was/were not in the DCM extract of *Ganoderma* H3, or

was/were not in a concentration high enough to cause an antibacterial effect. A similar case was also observed for the HEX and EtOAc extracts.

Like the aqueous extracts (Table 6.2), the organic extracts had no inhibitory effect towards any of the Gram negative bacteria tested (Table 6.5). Other reports have shown organic extracts from *Ganoderma* to have antibacterial activity against some selected Gram negative bacteria (Mothana *et al.*, 2003; Smania *et al.*, 1999). It must be noted however, this literature reports bioactivity of the organic extracts of the fruiting body of the mushroom and not the liquid cultivated mycelium, as is the case in this investigation. This again suggests that different compounds may be produced in the liquid cultivated mycelium to that of the fruiting body.

All the MeOH extracts obtained from the three Australian isolates displayed no inhibitory effects against all the bacteria tested. This was not expected as previous studies have shown that the MeOH extract from a *Ganoderma* species has inhibited the growth of Gram positive bacteria (Sudirman & Mujiyati, 1997). Again, these inhibitory effects have been observed from extracts of the fruiting bodies of *Ganoderma* and not the liquid cultivated mycelium. However, there appeared to have been no investigations on MeOH extracts from the liquid cultivated mycelium, therefore warranting the research of these extracts.

There appears to be no evidence in the literature of the antibacterial effect of organic extracts of the liquid cultivated mycelium of *Ganoderma* species, with the exception of one such paper, which explores the activity of ganoderic acids (extracted using orgnics) against some bacteria (Li *et al.*, 2000b). Therefore, this is one of the first account of antibacterial activity from *Ganoderma* mycelial extracts. Since the mycelium of *Ganoderma* H1 produced the greatest yield of crude extract for each solvent (Table 6.3) and these organic extracts displayed antibacterial activity against the greatest number of bacteria (Table 6.5), it was decided that only the organic extracts from this isolate would be further studied. This does not suggest that *Ganoderma* H2 and H3 did not possess biological activity, however, time restrictions and the large number of variables that could be explored on three isolates meant that this investigation would be limited to only one Australian *Ganoderma* isolate from this point forward.

6.3.2 Investigation of the Antibacterial Activity of *Ganoderma* H1 Organic Extracts

The Australian *Ganoderma* isolate H1 was further cultivated in different nutrient media. Successive Soxhlet extractions were performed and the extracts were examined for antibacterial activity against a range of bacteria. The nutrient media chosen for the cultivation of *Ganoderma* H1 were two commercial based products, PDB and MEB, and a defined medium, Basal-G. The investigation of mycelial growth in the three media is reported in Chapter 5, and it was found that Basal-G medium supported the maximum production of mycelial biomass for *Ganoderma* H1 (Section 5.3.2.3). It has been reported that a linear relationship does not necessarily exist between maximum biomass production and maximum biological activity (Lee *et al.*, 1999). Therefore, although the Basal-G medium resulted in the greatest growth of mycelial biomass (Figures 5.4 and 5.5) it may not necessarily provide the greatest production of bioactive compounds. For this reason, extracts from the mycelial biomass grown in different cultivation media were investigated for antibacterial activity. For ease of discussion, a crude organic extract sample that has been extracted using "solvent X" from the fungus cultivated in "medium Y" has been denoted as "X(Y)".

6.3.2.1 Organic Extraction of the Mycelium Cultivated in Three Different Growth Media

The Australian *Ganoderma* isolate H1 was cultivated for 30 days in MEB, PDB and Basal-G growth media (Sections 3.8.2 and 3.10.1). The mycelial biomass was harvested (Section 3.10.3) and freeze dried (Section 3.3). The biomass was then subjected to successive Soxhlet extractions (Section 3.11.2) using organic solvents in the order of HEX, DCM, EtOAc and MeOH. The resulting solvent obtained from each extraction was evaporated to dryness and the mass and appearance/consistency of the crude extract noted. Representative extracts from the Basal-G medium are presented in Figure 6.4, which show the difference in extract colour.

During the preparation of extracts, an insoluble layer formed at the bottom of the HEX, EtOAc and MeOH extract, and as a top layer on the DCM extract (Figure 6.4a). These layers were removed, prepared as separate extracts and tested against a suite of bacteria for antibacterial activity. No antibacterial activity was observed (results not shown).

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The soluble fraction, presented in Figure 6.4b as a 20% (v/v) solution, is the crude extract referred to throughout this investigation.

b.

Figure 6.4. *Ganoderma* H1 crude organic extracts, extracted from mycelium that had been cultivated in Basal-G growth medium for a period of 30 days. For each photo the extracts are from left to right: HEX, DCM, EtOAc and MeOH. (a) The extracts were prepared as a 20% (w/v) solution, into the same solvent used for the extraction process. A precipitate was observed for all extracts at the bottom of the vial, except for the DCM extract, which gave an oily/milky top layer on resuspension. (b) The soluble fractions of the samples from (a) were separated from the precipitate, concentrated to a solid and then prepared in the same solvent, 20% (w/v).

Table 6.6

Extraction Solvent	Organic Extracts from <i>Ganoderma</i> H1 ^a					
	Basal-G (mg g ⁻¹)	PDB (mg g^{-1})	MEB (mg g^{-1})			
HEX	41 ± 9^{b}	17 ± 1	54 ± 9			
DCM	18 ± 7	25 ± 9	45 ± 3			
EtOAc	20 ± 4	7 ± 1	13 ± 3			
MeOH	264 ± 26	326 ± 17	197 ± 54			
Total crude extract ^c	344 ± 26	375 ± 17	309 ± 54			

Organic extract yield of Ganoderma H1 grown in different cultivation media

^a Ganoderma H1 was cultivated in MEB, PDB and Basal-G at 30°C for 30 days in shaker flasks; ^b The crude extract yield was calculated in mg of extract per gram of mycelium and is an average result of thirty extractions for HEX, DCM and EtOAc and five extractions for MEOH; ^c Total crude extract was calculated by the addition of successive extract masses (HEX, DCM, EtOAc, MeOH), from mycelium that had been cultivated in the same growth medium.

The yield of crude extracts from the mycelium of *Ganoderma* H1 grown in MEB, PDB and Basal-G media are summarised in Table 6.6. The total amount of extractable organic compounds (using the solvents HEX, DCM, EtOAc and MeOH), when grown in PDB (375 mg g⁻¹) and Basal-G medium (344 mg g⁻¹) was found to be higher than that when grown in MEB (308.8 mg g⁻¹). This demonstrated that different growth media could alter the production of extractable metabolites produced by *Ganoderma* H1. This

was a similar observation to that reported in the previous Chapter 6, whereby the different medium affected the production of mycelial biomass (Section 5.3.2.3). A recent report has also shown that by manipulating the cultivation media, production of the bioactive metabolite may be increased or decreased (Fang & Zhong, 2002c).

A range in yield was also observed for extracts from the same solvent, for the different cultivation media (Table 6.6). This again highlighted the effect that different media can have on the production of metabolites, suggesting that different concentrations of metabolites were produced in the mycelium grown in different media. Comparing all extracts, the results show that the MeOH extraction gave the largest yield of extract, ranging from 197 ± 54 to 326 ± 17 mg g⁻¹ for *Ganoderma* H1 cultivated in MEB and PDB respectively (Table 6.6). However, there was little or no antibacterial activity observed for any of the MeOH extracts when both preliminary testing (Table 6.5) and further testing (Table 6.8) was performed. Of interest were the HEX, DCM and EtOAc extracts, which exhibited antibacterial activity against a range of Gram positive indicator organisms (Table 6.5). These extracts gave relatively low yields of extract compared to the MeOH extraction, but were comparable to those who have used a similar extraction system for *Ganoderma* isolates (Mothana *et al.*, 2000; Smania *et al.*, 1999).

The extracts obtained from each medium ranged in colour and texture from a yellow oily substance (HEX) to a dark red solid (MeOH) (Table 6.7). One difference noted was that the HEX extract from the mycelium grown in PDB was much lighter in colour than all the other HEX extracts.

Table 6.7

The appearance and consistency of organic extracts from Ganoderma H1 grown in different media

Extraction	Organic Extracts from <i>Ganoderma</i> H1 ^a						
Solvent	Basal-G	PDB	MEB				
	Appearance; Consistency	Appearance; Consistency	Appearance; Consistency				
HEX	Orange; oily	Yellow; Oily	Orange; Oily				
DCM	Dark orange; Solid	Orange; Solid	Orange: Solid				
EtOAc	Dark orange; Solid	Orange; Solid	Orange; Solid				
МеОН	Dark red; Solid	Dark red; Solid	Brown/red; Solid				

^a Ganoderma isolates were cultivated for 30 days in 1 L shaker flasks.

6.3.2.2 Antibacterial Activity of the Organic Extracts from the Mycelium Grown in Different Cultivation Media

The organic extracts of *Ganoderma* H1 (Table 6.6) were assessed for their antibacterial activity using the disc diffusion assay (Section 3.13.1). Prior to analysis, the amount of extract to be applied to the discs was determined by a small optimisation experiment (results not shown). This was performed to identify a suitable amount of extract that would provide zones of inhibition that could be measured. Figure 6.5 displays two examples of results obtained from the disc diffusion assay. Zones of complete inhibition are exhibited by the EtOAc(PDB) and DCM(PDB) extracts against *S. aureus* and *B. cereus*, respectively, and can be clearly identified surrounding each disc.

Table 6.8 displays the zones of inhibition measured for the different extracts against a range of test bacteria. The HEX, DCM and EtOAc extracts exhibited inhibitory activity against a range of Gram positive bacteria, whilst the MeOH extracts only displayed incomplete inhibition and only slight inhibition against the Gram positive bacteria, *E. faecalis* and *S. pyogenes*, respectively. The antibacterial activity of the HEX, DCM and EtOAc extracts was not extended to activity against *M. luteus*. This result was thought to be unusual, as the bacterium *M. luteus* is a highly sensitive microbe and is among the most susceptible of the Gram positive bacteria (personal communication, Prof. Warren Baker, SUT, Hawthorn, VIC, Australia). This was the case observed by Yoon *et al.* (1994) who found that aqueous extracts from *G. lucidum* exhibited antibacterial activity against a range of Gram positive bacteria and the greatest activity was seen towards *M. luteus*.



Figure 6.5. Representative photos of the disc diffusion assay showing: (a) complete inhibition of *Staphylococcus aureus* by the EtOAc(PDB) extract (8 mm zone), and; (b) complete inhibition of the *Bacillus cereus* by the DCM(PDB) extract (7 mm zone).

Table 6.8

The antibacterial activity of the crude organic extracts from the mycelium of *Ganoderma* H1 against a range of bacteria

		Antibacterial Activity of <i>Ganoderma</i> H1 Organic Extracts ^a					
		HEX			DCM		
	Test Bacteria	Basal-G	MEB	PDB	Basal-G	MEB	PDB
	Bacillus cereus	[8]	7	-	7	$7[8]^{\#}$	7
	Bacillus subtilis	7	7	-	7	7	7
	Enterococcus faecalis	[8]	S	S	7	S	S
ve	Streptococcus pyogenes	8[10]#	8[10]#	8	7	7	7
positiv	<i>Staphylococcus aureus</i> (SUT)	[8]*	[7]*	[7]*	9[10] [#]	[8]	9[10] [#]
Gram	Staphylococcus aureus (25923)	7	-	-	7	-	[7]
-	Staphylococcus epidermidis	-	NT	NT	9[12]	NT	NT
	Listeria monocytogenes	[13]	NT	NT	7[8]	NT	NT
	Micrococcus luteus	-	-	-	-	-	-
n ve	Escherichia coli	-	-	-	-	-	-
jrar oati	Klebsiella pneumoniae	-	-	-	-	-	-
o e	Proteus vulgaris	-	-	-	-	-	-
٩	<i>S. aureus</i> (M67638)	-	NT	NT	[8]	[7]	[15]
SA	<i>S. aureus</i> (M67783)	-	NT	NT	[9]	-	[14]
MR	S. aureus (M99320)	-	NT	NT	[9]	-	[16]
cal	S. aureus (M173525)	-	NT	NT	[8]	-	[12]
lini	S. aureus (M180920)	-	NT	NT	[9]	-	[16]
U	S. aureus (M183909)	-	NT	NT	[16]	-	[18]
	E. faecalis (M259849)	-	-	-	-	-	-
εE.	E. faecalis (M233165)	-	-	-	-	-	-
VR	<i>E. faecalis</i> (M252807)	-	-	-	-	-	-
nical	E. faecalis (M193292)	-	-	-	-	-	-
Clir	E. faecalis (M169600)	-	-	-	-	-	-
	E. faecalis (M255048)	-	-	-	-	-	-

^a Activity was determined by disc diffusion assays. 6000 µg of extract was applied to each disc. A zone of inhibition > 6 mm was considered positive; **s** a very slight zone of inhibition observed; [] incomplete inhibition was observed (i.e.; reduced growth, but not complete elimination of growth); [#] a double zone of inhibition was observed. The larger second zone, was a zone of incomplete inhibition, indicated by the value in parentheses []; - no inhibition was observed; **NT** not tested; Negative solvent blanks were also tested. These showed no inhibition (results not shown); Positive blanks of either, Ampicillin, Tetracycline, Chloramphenicol or Streptomycin, were also tested (results not shown); * blank was recorded to be 6.5 mm; Numbers are the average of triplicate discs and triplicate experiments performed on different days; ^b Clinical MRSA, methicillin resistant *Staphylococcus aureus* isolates; ^c Clinical vancomycin resistant *Enterococcus* isolates; Crude extracts were from fungi grown in Basal-G, MEB and PDB for 30 days.

		Antibacterial Activity of Ganoderma H1 Organic Extracts					
		EtOAc MeOH					
	Test Bacteria	Basal-G	MEB	PDB	Basal-G	MEB	PDB
	Bacillus cereus	7*	6.5*	-	-	-	-
	Bacillus subtilis	6.5	-	-	-	-	-
	Enterococcus faecalis	S	S	-	[9]	[9]	[9]
ve	Streptococcus pyogenes	8	8[10]	[8]	S	S	S
positi	Staphylococcus aureus (SUT)	7[8]	[8]	8	-	-	-
Gram	Staphylococcus aureus (25923)	7	[7]	[7]	-	-	-
	Staphylococcus epidermidis	-	NT	NT	-	NT	NT
	Listeria monocytogenes	[8]	NT	NT	-	NT	NT
	Micrococcus luteus	-	-	-	-	-	-
n ve	Escherichia coli	-	-	-	-	-	-
jrar gati	Klebsiella pneumoniae	-	-	-	-	-	-
) on	Proteus vulgaris	-	-	-	-	-	-
٩	<i>S. aureus</i> (M67638)	[10]	[7]	[7]	NT	NT	NT
SA	S. aureus (M67783)	[9]	-	[7]	NT	NT	NT
MR	S. aureus (M99320)	[10]	[7]	[9]	NT	NT	NT
ical	S. aureus (M173525)	[9]	7	7	NT	NT	NT
Clin	S. aureus (M180920)	[9]	[7]	[7]	NT	NT	NT
Ŭ	S. aureus (M183909)	[9]	[7]	[11]	NT	NT	NT
	<i>E. faecalis</i> (M259849)	-	-	-	-	-	-
E.	E. faecalis (M233165)	-	-	-	-	-	-
I VF	<i>E. faecalis</i> (M252807)	-	-	-	-	-	-
nica	E. faecalis (M193292)	-	-	-	-	-	-
Clir	E. faecalis (M169600)	-	-	-	-	-	-
	<i>E. faecalis</i> (M255048)	_	-	-	_	_	_

Table 6.8... continued

The antibacterial activity of the crude organic extracts from the mycelium of *Ganoderma* H1 against a range of bacteria

^a Activity was determined by disc diffusion assays. 6000 µg of extract was applied to each disc. A zone of inhibition > 6 mm was considered positive; s a very slight zone of inhibition observed; [] incomplete inhibition was observed (i.e.; reduced growth, but not complete elimination of growth); [#] a double zone of inhibition was observed. The larger, second zone was a zone of incomplete inhibition, indicated by the value in parentheses []; - no inhibition was observed; NT not tested; Negative solvent blanks were also tested. These showed no inhibition (results not shown); Positive blanks of either, Ampicillin, Tetracycline, Chloramphenicol or Streptomycin, were also tested (results not shown); * blank was recorded to be 6.5 mm; Numbers are the average of triplicate discs and triplicate experiments performed on different days; ^b Clinical MRSA, methicillin resistant *Staphylococcus aureus* isolates; ^c Clinical vancomycin resistant *Enterococcus* isolates; Crude extracts were from fungi grown in Basal-G, MEB and PDB for 30 days.

All the crude extracts from the mycelium of *Ganoderma* H1, cultivated in MEB, PDB and Basal-G growth medium, displayed no inhibitory activity against the Gram negative bacteria tested (Table 6.8). This was expected because there was no inhibitory activity observed from the organic extracts in the preliminary investigation (Table 6.5). Extracts from *G. applanatum* (Smania *et al.*, 1999) and *G. pfeifferi* (Mothana *et al.*, 2000) have been shown to possess antibacterial activity against the Gram negative bacterial species, *E. coli*. However, the extraction methods were different in either cases and biological compounds found in the species *G. applanatum* and *G. pfeifferi* may not necessarily be the same as those found in *Ganoderma* H1 (i.e. *G. cupreum* as determined in Chapter 4). In addition, the greater resistance of Gram negative bacteria is well documented and similar observations have been reported in analysis of extracts from other fungal species (Beltran-Garcia *et al.*, 1997; Ishikawa *et al.*, 2001; Smania *et al.*, 1999). This greater resistance by the Gram negative bacteria is reported to be because of the characteristics of the outer membrane, which acts as a barrier to environmental substances, including antibiotics (Tortora *et al.*, 2001).

The overall activity of the different extracts (Table 6.8) shows that the DCM extracts displayed antibacterial activity against the greatest range of bacteria. Strong antibacterial activity of DCM extracts from other fungi has been reported. The DCM(MEB), DCM(PDB) and DCM(Basal-G) extracts showed growth inhibition against five or more aerobic bacteria, whilst extracts from within the HEX and EtOAc group, namely HEX(PDB) and EtOAc(PDB), showed a lesser range of antibacterial activity. Chlorinated extracts from different G. lucidum isolates have been investigated and have been shown to possess biologically active compounds that can positively affect the immune system (Koyama et al., 1997; Min et al., 2001). With specific regards to antibacterial activity, there have also been reports of chlorinated extracts from Ganoderma species (Mothana et al., 2000; Smania et al., 1999) and other Basidiomycetous fungi (Beltran-Garcia et al., 1997; Hirasawa et al., 1999) that have been shown to possess antibacterial activity. The antibacterial activity of the organic extracts from the Ganoderma H1 is the first account of an Australian Ganoderma isolate with antibacterial activity. In addition, it is the first report of the antibacterial activity from this particular *Ganoderma* species (G. cupreum).

6.3.2.3 Antibacterial Activity of the Organic Extracts Against Clinical MRSA and VRE Isolates

The HEX, DCM, EtOAc and MeOH extracts that exhibited activity against the growth of the pathogenic organisms *E. faecalis* and *S. aureus* (Table 6.8), were further investigated for their abilities to inhibit clinical isolates of vancomycin resistant enterococci (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA). The results of the assay are also presented in Table 6.8. Two representative photos of the disc diffusion assay showing zones of inhibition, one of complete and the other of incomplete inhibition, are given in Figure 6.6.

Further examination of the organic extracts that inhibited *E. faecalis* against clinical VRE indicated no inhibitory effects (Table 6.8). It was expected that the HEX(Basal-G) and DCM(Basal-G) extracts were to exhibit some antibacterial activity towards the VRE's, as these two extracts exhibited zones of inhibition towards *E. faecalis*, whilst the other extracts only showed slight inhibitory activity. However, there was no activity observed. There have been no reports in the literature on the activity of *Ganoderma* against enterococci, so no comparisons can be made.



Figure 6.6. Representative photos of the disc diffusion assay showing incomplete inhibition and a weak incomplete inhibition towards an MRSA isolate. (a) DCM (PDB) extract exhibiting strong incomplete inhibition against MRSA (M183909), and (b) DCM (Basal-G) extract exhibiting weak incomplete inhibition against MRSA (M183909).

Table 6.8 displays the sizes of the zones of inhibition of the extracts that had activity towards the two strains of *S. aureus* examined. EtOAc(PDB) was the only extract to exhibit a complete zone of inhibition against *S. aureus* (SUT). The other extracts tested were observed to exhibit incomplete inhibition towards the same bacterium, with the

exception of EtOAc(Basal-G), DCM(Basal-G) and DCM(PDB), which exhibited double zones of inhibition. The double zones of inhibition consisted of a smaller zone of complete inhibition and a second, larger zone of incomplete inhibition. The extracts tested from the Basal-G medium (HEX, DCM and EtOAc) all exhibited complete zones of inhibition against a different *Staphylococcus* isolate (*S. aureus* 25923). However, the same solvent extracts from different media (MEB and PDB) did not inhibit this bacterium. Further testing of the MeOH extracts against the MRSA isolates were not performed, as there was no inhibitory activity observed for any of the MeOH extracts towards either of the *S. aureus* strains.

The DCM(Basal-G), DCM(PDB), EtOAc(Basal-G) and EtOAc(PDB) extracts displayed activity toward all six MRSA isolates, whilst the DCM(MEB) and EtOAc(MEB) extracts exhibited activity towards only one isolate and five isolates, respectively. The DCM(PDB) extracts displayed larger zones of incomplete inhibition than those of DCM(Basal-G) extracts against all MRSA strains. This comparison of incomplete inhibition is shown in Figure 6.6b. In contrast, the EtOAc(Basal-G) gave larger zones of incomplete inhibition than the EtOAc(PDB) extracts against all MRSA. Overall, the extracts from the mycelium grown in MEB were observed to be the least effective against the MRSA isolates.

The extracts from the mycelium of *Ganoderma* H1 cultivated in both Basal-G and PDB growth media appeared to have an inhibitory effect against the clinical MRSA isolates, although the incomplete inhibition observed indicated that, at the concentration used the crude extracts were bacteriostatic and not bactericidal against the MRSA isolates. To confirm this, further evaluation via a different method would need to be conducted. Additional testing of the extracts against the MRSA isolates was not performed, as only bacteria that showed strong sensitivity (complete zones of inhibition) were chosen for further analysis. Nevertheless, on review of the literature there appear to be no such investigations reported on the antibacterial effect of *Ganoderma* extracts against MRSA species and therefore, the findings here are the first report of the antibacterial activity of a *Ganoderma* species against clinical MRSA isolates.

6.3.2.4 Comparison of Crude Extracts from the Liquid Cultivated Mycelium of *Ganoderma* H1

To further explore the antibacterial activities of the crude extracts, a sufficient quantity of mycelial biomass was required to enable maximum extraction of the crude extracts. Therefore, a culture medium was required that could produce large amounts of mycelium with bioactivity. Basal-G medium was identified as the medium that could produce the largest amount of mycelial biomass in 30 days (Sections 5.3.2.3 and 5.3.2.4). In addition, Basal-G was also identified as the growth medium that produced mycelium that contained bioactivity towards a number of test organisms (Table 6.8). As a result, Basal-G was chosen as the growth medium for the further cultivation and investigation of the mycelium from *Ganoderma* H1.

As mentioned previously, the DCM extracts exhibited strong antibacterial activity against a range of Gram positive bacteria (Table 6.8) and therefore, the DCM extract was of particular interest for further investigation. Previous observations (Section 6.3.2.1, Table 6.6) showed that the yield of extract for DCM(Basal-G) $(18 \pm 7 \text{ mg g}^{-1})$ was lower than that for the other two extracts, DCM(PDB) $(25 \pm 9 \text{ mg g}^{-1})$ and DCM(MEB) $(45 \pm 3 \text{ mg g}^{-1})$ (Table 6.6). However, when considering the mycelial biomass production of *Ganoderma* H1 in the three media (Section 5.3.2.3, Figure 5.4), the quantity of mycelial biomass produced in PDB or MEB. Therefore, taking into account the amount of DCM extract from the mycelial biomass produced in a 250 mL shaker flask, the overall mass of the DCM(Basal-G) extracts (20.70, 8.5 and 18.90 mg per 250 mL, respectively) (Table 6.9). Thus, Basal-G was chosen as the growth medium for further analysis of *Ganoderma* H1.

Table 6.9 shows the average mass of extract obtainable in a 250 mL shaker flask. Mycelium grown in the Basal-G growth medium enables a larger mass of extract to be obtained for the DCM, EtOAc, and MeOH extracts, than when grown in MEB or PDB. The overall extract mass of the HEX(Basal-G) extract (47.15 mg) was greater than that of the HEX(PDB) (5.78 mg) extract, but not that of the HEX(MEB) (54.42 mg).

However, the HEX extract was not studied further due to its insolubility in aqueous solvents.

	Ganoderma H1 ^ª					
Extraction Solvent ^b	Basal-G ^c (mg)	PDB ^d (mg)	MEB ^e (mg)			
HEX	47.15	5.78	54.42			
DCM	20.70	8.50	18.90			
EtOAc	23.00	2.38	5.46			
МеОН	303.60	110.84	82.74			

 Table 6.9

 Total extract obtainable in 250 mL culture flasks

^a Ganoderma H1 was cultivated for 30 days in 125 mL of culture medium in 250 mL shaker flasks; ^b The total amount of crude extract obtainable in 125 mL of culture medium in a 250 mL shaker flask. Errors are not shown as this mass was calculated by obtaining the total amount of mycelium produced in 125 mL of culture fluid and factoring in the amount of extract in mg extracted from 1 g of mycelium; The total amount of mycelium produced after 30 days of cultivation in 125 mL of ^e Basal-G medium, 1.15 ± 0.13 g; ^d PDB, 0.34 ± 0.02 ; and ^e MEB, 0.42 ± 0.02 g.

The cultivation of *Ganoderma* H1 was scaled up and carried out in 2 L shaker flasks consisting of 1 L of culture medium. The average biomass produced for each of the three media in the larger flasks was found to be proportionally greater than what was produced in 125 mL of culture medium in the 250 mL shaker flasks (results not shown). Therefore, 2 L shaker flasks that contained 1 L of Basal-G growth medium were used for the remainder of this investigation to produce large amounts of mycelium from *Ganoderma* H1.

6.3.2.5 Antibacterial Activity of the Organic Extracts Against *Clostridium* isolates

The activity exhibited by the HEX, DCM and EtOAc extracts against the aerobic spore forming bacilli, *B. cereus* and *B. subtilis* (Table 6.8), led to the investigation of other spore forming anaerobic bacteria. Extracts from the mycelium cultivated in Basal-G growth medium were assessed for their antibacterial activity using the disc diffusion assay (Section 3.13.1) against some pathogenic *Clostridium* species. This included some clinical isolates of *C. perfringens* and *C. difficile*. Although the MeOH extracts did not exhibit any previous activity towards bacteria within this genus (Table 6.8), they were included in the analysis.

Table 6.10 displays the results for the antibacterial activity assay for all extracts against a range of *Clostridium* isolates. The HEX, DCM and EtOAc extracts exhibited an inhibitory effect towards all anaerobic isolates tested, with the exception of EtOAc towards *C. butyricum*, whereby no inhibition was observed. The zones of inhibition observed were larger than what had been previously observed against the spore forming and other bacteria (Table 6.8), indicating that the *Clostridium* isolates were highly sensitive to the crude extracts of *Ganoderma* H1. It is noted that the larger zones may have been due to the slower growth of the anaerobes (48 hours, as opposed to 24 hours for aerobes), which would have allowed more time for the extract to diffuse through the agar medium making these slower growing microorganisms appear more susceptible (Barry & Thornsberry, 1985). However, this appeared to not be the case, as MIC values and time kill assays (reported and discussed later in this Chapter) showed otherwise.

Again, it was apparent that the DCM extract contained components that had the greatest effect towards the test bacteria, as the zones of inhibition for the DCM extracts were larger (10 to 15 mm) than those for the HEX (7 to 12 mm) and EtOAc (8 to 11 mm) extracts. The difference in the zones of inhibition can be clearly seen in the representative photos shown in Figure 6.7. This figure depicts the inhibition of *C. perfringens* by the HEX(Basal-G) and DCM(Basal-G) extracts, and the inhibition of *C. sporogenes* by the DCM(Basal-G) extract.



Figure 6.7. Representative photos of the disc diffusion assay showing *Ganoderma* H1 extracts exhibiting complete inhibition against some *Clostridium* isolates. Complete inhibition of *Clostridium perfringens* (SUT) by **(a)** HEX(Basal-G) extract and **(b)** DCM(Basal-G) extract. **(c)** Complete inhibition of *Clostridium sporogenes* by the DCM(Basal-G) extract.

Table 6.10

	Zones of Inhibition Exhibited by the Crude Extracts from <i>Ganoderma</i> H1 ^a					
Test Bacteria	HEX	DCM	EtOAc	MeOH		
C. butyricum	7	10	-	-		
C. sporogenes	10	13	10*	-		
C. perfringens (SUT)	7	10	8	-		
<i>C. perfringens</i> (cp8) [#]	11	11	11	8		
C. perfringens (cp9) [#]	9	11	10	8		
<i>C. difficile</i> (cd1) [#]	12	15	11	-		
<i>C. difficile</i> (cd8) [#]	12*	15*	11	-		
$C_{\rm difficile} (cd14)^{\#}$	11	14	11	-		

Antibacterial activity of Ganoderma H1 organic extracts against some anaerobic Clostridium bacteria

^a Crude organic extracts from the mycelium of *Ganoderma* H1 that had been cultivated for 30 days in Basal-G growth medium; 6000 μ g of extract was applied to each disc; Assays were performed in triplicate; The diameter of the zone of inhibition was measured in mm; * solvent blank was recorded to have a slight inhibition of 7; [#] clinical isolate; – no inhibition was observed.

The MeOH extracts tested displayed no inhibitory activity towards the *Clostridium* isolates, with the exception of the two clinical anaerobic *C. perfringens* isolates (Table 6.10). This was an unexpected result because in earlier assays (Table 6.8), little or no activity had been observed for the MeOH extracts against the aerobic bacteria tested.

On review of the literature, there appear to be no reports on the inhibitory effects of *Ganoderma* extracts against *Clostridium* isolates and therefore, no comparisons can be made. However, one account of the extracts from the liquid cultivated mycelium from a different basidiomycetous species, *Cordyceps militaris*, has shown to have potent growth inhibitory activities towards a number of *Clostridium* species (Ahn *et al.*, 2000). There have been studies reporting the inhibitory activity of *Ganoderma* extracts against the aerobic spore forming bacilli (Smania *et al.*, 1999), but this is the first account of the organic extracts from a *Ganoderma* species possessing activity toward the anaerobic spore former, *Clostridium*.

6.3.2.6 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the Organic Extracts

Minimum inhibitory concentration (MIC) is defined as "the lowest concentration which results in maintenance or reduction of inoculum viability" over a 24 h period (Carson *et al.*, 1995), and the minimum bactericidal concentration (MBC) is defined as the lowest concentration that kills at least 99.9% of the original inoculum (Schoenknecht *et al.*, 1985). In this investigation, the MIC and MBC were determined for the DCM and EtOAc extracts on a selection of test bacteria. Although the HEX extract displayed activity against a range of bacteria, it was not investigated further for its MIC and MBC. This was due to insolubility of the extract in the propagation media, as well as time limitations, which did not allow for further investigations of this nature. The MeOH extract was not investigated further, as this extract showed little to no inhibitory activity towards the range of bacteria tested.

The MIC and MBC were investigated using a modified micro dilution method as described in Sections 3.13.2 and 3.13.3, respectively. A two-fold serial dilution containing the extract and test organism was prepared and analysed in triplicate at an optical density of 595 nm (OD_{595}). A second two-fold serial dilution containing only the extract served as a negative turbidity control. To determine the OD_{595} due to the growth of the test organism, the OD_{595} readings of the control solutions were subtracted from the corresponding OD_{595} readings of the sample solutions. The MIC was then determined to be at the concentration where there was no increase in the OD_{595} , or at the concentration where the OD_{595} was zero. The MBC was determined by monitoring the viability of bacteria in each well that exhibited no increase in OD_{595} , after a 24 h period. The final extract concentration at which no growth was observed was recorded as the MBC. The MIC and MBC results are presented in Table 6.11.

The DCM extract exhibited MIC values ranging from 750 to 6000 μ g mL⁻¹ against the aerobic bacteria tested. The MBCs for the corresponding bacteria were found to be two-fold higher in concentration than that of the MIC. No MBC was observed for the DCM extract against *L. monocytogenes* and *S. epidermidis*, which correlated with previous observations of incomplete inhibition in the disc diffusion assay (Table 6.8). The MICs of an aqueous *Ganoderma* extract against *B. subtilis* (3500 μ g mL⁻¹), *B.*

anthracis (3500 μ g mL⁻¹) and *B. cereus* (2000 μ g ml⁻¹) (Yoon *et al.*, 1994), were similar to what was seen in this investigation for the DCM against the bacillus isolates (3000 μ g mL⁻¹) (Table 6.11).

	Crude Extract from <i>Ganoderma</i> H1 ^a						
-	DC	CM	EtC	DAc			
Test Bacteria	MIC ^b	MBC ^c	MIC	MBC			
Bacillus cereus	3000	6000	NT	NT			
Bacillus subtilis	3000	6000	NT	NT			
Listeria monocytogenes	6000	-	-	-			
Staphylococcus epidermidis	6000	-	NT	NT			
Streptococcus pyogenes	750	1500	750	750			
Clostridium perfringens (cp8)	6000	6000	-	-			
Clostridium perfringens (cp9)	375	375	1500	1500			
Clostridium perfringens (SUT)	187	187	750	750			
Clostridium butyricum	3000	3000	-	NT			
Clostridium sporogenes	3000	3000	12000	-			
Clostridium difficile (cd1)	375	3000	12000	12000			
Clostridium difficile (cd8)	3000	3000	12000	12000			
Clostridium difficile (cd14)	3000	3000	12000	12000			

 Table 6.11

 The MIC and MBC of the crude organic extracts from *Ganoderma* H1

^a DCM and EtOAc extracts from *Ganoderma* H1 that had been cultivated in Basal-G medium for 30 days; ^b MIC, minimum inhibitory concentration (μ g mL⁻¹); ^c MBC, minimum bactericidal concentration (μ g mL⁻¹); All results recorded after a 24 h period; – the MIC/MBC was not observed at the concentrations examined; **NT**, not tested.

Compared to the other bacteria, *S. pyogenes* exhibited a higher sensitivity toward both the DCM and EtOAc extracts (Table 6.11). The bacterium was approximately three to four times more susceptible to the DCM extract than the other bacteria tested. The sensitivity of this bacterium towards some purified organic extracts from *G. applanatum* has also been reported by (Smania *et al.*, 1999). They found *S. pyogenes* to be four to twenty times more sensitive than the other bacteria tested.

The DCM extract exhibited MIC values ranging from 187 to 6000 μ g mL⁻¹ against the anaerobic bacteria tested (Table 6.11). The same MIC and MBC was obtained for each individual *Clostridium* isolate, with the exception of the DCM extract against *C*. *difficile* (cd1). The MIC endpoint of an agent is generally taken as the lowest concentration of the drug showing no growth visible to the naked eye (Schoenknecht *et*
al., 1985). However, subculture can often show that some bacteria will be recoverable at the MIC and only at a greater concentration will death have occurred (as was the case for the DCM extract against the aerobes). This was not the case for the extract against the *Clostridium* isolates, as at the MIC, 99.9% reduction of the inoculum was observed, which is therefore, recorded as the MBC. This suggested that the extract had a strong bactericidal effect towards these bacteria (at the MIC/MBC). However, at concentrations lower than the MBC, a decreased inhibitory effect of the DCM extract was observed (discussed in the latter part of this section). Overall, the DCM extract was observed to have a stronger inhibitory effect against the anaerobic bacteria, as the MIC and MBC were less than those for the aerobic bacteria. This was also evident in the disc diffusion assays, whereby the largest zones of inhibition were observed against the *Clostridium* isolates (Table 6.11).

The EtOAc extract exhibited MIC values ranging from 750 to 12000 μ g mL⁻¹ against the range of bacteria tested (Table 6.11). For the different concentrations examined, there was no MIC determined for the EtOAc extract against *C. butyricum*, *L. monocytogenes* and *C. perfringens* (cp8) (Table 6.11). Previous assays, using the disc diffusion method, also showed no inhibitory activity towards *C. butyricum* (Table 6.10). However, previous assays showed that the EtOAc extract exhibited incomplete inhibition against *L. monocytogenes* (Table 6.8) and complete inhibition against *C. perfringens* (cp8) (Table 6.10).

Graphical presentation of the MIC assays, which displays the concentration response of the DCM extract and EtOAc extract against the bacteria, are shown in Figure 6.8 ((DCM) Figure 6.8 a,b,c,e,f,h,j,l,n,p,r,t,v and (EtOAc) Figure 6.8 d,g,i,l,k,m,o,q,s,u,w). The graphs show the inhibitory activity of the extracts at concentrations below the MIC. In addition, the graphs demonstrate that an extract may still have an inhibitory effect, even if there was no MIC observed against a bacterium.

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Figure 6.8a-e. MIC concentration response graphs of the organic extracts (DCM and EtOAc) from the mycelium of *Ganoderma* H1. () DCM extract against: (a) *Bacillus cereus*, (b) *Bacillus subtilis*, (c) *Listeria monocytogenes* and (e) *Staphylococcus epidermidis*, and () EtOAc extract against: (d) *Listeria monocytogenes*. Percent inhibition was calculated by comparing OD_{595} of the growth of the test organism with and without extract after a 24, 48 or 72 hour period. The MIC₁₀₀ can be seen at 100% inhibition, and is marked on the graphs. MBCs are not shown, or calculated for the extracts.



Figure 6.8 (f-k). MIC concentration response graphs of the organic extracts (DCM and EtOAc) from the mycelium of *Ganoderma* H1. (DCM extract against: (f) *Streptococcus pyogenes*, (h) *Clostridium perfringens* (cp8), (j) *Clostridium perfringens* (cp9), and (DECM) EtOAc extract against: (g) *Streptococcus pyogenes*, (i) *Clostridium perfringens* (cp8), (k) *Clostridium perfringens* (cp9). Percent inhibition was calculated by comparing OD₅₉₅ of the growth of the test organism with and without extract after a 24, 48 or 72 hour period. The MIC₁₀₀ can be seen at 100% inhibition, and is marked on the graphs. MBCs are not shown, or calculated for the extracts



Figure 6.8 (I-q). MIC concentration response graphs of the organic extracts (DCM and EtOAc) from the mycelium of *Ganoderma* H1. () DCM extract against: () *Clostridium perfringens* (SUT), (n) *Clostridium butyricum*, (p) *Clostridium sporogenes*, and () EtOAc extract against: (m) *Clostridium perfringens* (SUT), (o) *Clostridium butyricum*, (q) *Clostridium* sporogenes. Percent inhibition was calculated by comparing OD₅₉₅ of the growth of the test organism with and without extract after a 24, 48 or 72 hour period. The MIC₁₀₀ can be seen at 100% inhibition, and is marked on the graphs. MBCs are not shown, or calculated for the extracts



Figure 6.8 (r-w). MIC concentration response graphs of the organic extracts (DCM and EtOAc) from the mycelium of *Ganoderma* H1. () DCM extract against: (r) *Clostridium difficile* (cd1), (t) *Clostridium difficile* (cd8), (v) *Clostridium difficile* (cd14), and () EtOAc extract against: (s) *Clostridium difficile* (cd1), (u) *Clostridium difficile* (cd8), (w) *Clostridium difficile* (cd14). The percent inhibition was calculated by comparing OD_{595} of the growth of the test organism with and without extract after a 24, 48 or 72 hour period. The MIC₁₀₀ can be seen at 100% inhibition, and is marked on the graphs. MBCs are not shown, or calculated for the extracts

The concentration response of the EtOAc extract toward *L. monocytogenes* is shown in Figure 6.8d. At the concentrations examined, the MIC cannot be determined (MIC = MIC₁₀₀ = 100% reduction or maintenance of the inoculum). However, the graph displays 20 to 50% inhibitory activity of *L. monocytogenes* at EtOAc extract concentrations ranging from 94 to 12000 μ g mL⁻¹. It is common in the discovery and analysis of new antibiotics to discuss the minimum inhibitory concentrations in terms of the percent inhibition, as concentrations below the MIC can also give important information regarding the effectiveness of the compound at different concentrations. Two inhibitory values that are commonly cited are the MIC₅₀ (50% inhibition) and MIC₉₀ (90% inhibition) (Alhambra *et al.*, 2004; Baykam *et al.*, 2004). Although there was no MIC₁₀₀ (100% inhibition of the growth of the inoculum, MIC₁₀₀) determined for the EtOAc extract towards *L. monocytogenes*, a 40 to 50% inhibitory effect was observed over an EtOAc extract concentration range of 750 to 12000 μ g mL⁻¹. This inhibitory effect was seen in previous results as zones of incomplete inhibition towards *L. monocytogenes* (Table 6.8).

There was also no MIC_{100} observed for the EtOAc extract against *C. perfringens* (cp8) and C. butyricum, however, varying degrees of bacterial inhibition over the range of extract concentrations was exhibited (Figure 6.81,0). At the highest extract concentrations examined (12000 μ g mL⁻¹), an 80 to 90% inhibitory activity against C. perfringens (cp8) was observed (Figure 6.81). Since a low percentage of bacterial growth was determined at the high extract concentrations in the MIC assay, it was hypothesised that the bacterial growth in the disc diffusion assay was not visible to the eye. Therefore, in the disc diffusion assay a clear zone of inhibition was observed against *C. perfringens* (cp8) rather than an incomplete zone of inhibition (Table 6.10). Subculturing the zone of inhibition onto fresh agar would have possibly confirmed the presence of live bacteria, however, this was not performed, as it was considered not necessary at this point in the investigation. The low inhibitory activity of the EtOAc extract toward C. butyricum, no greater than 10% inhibition (Figure 6.80), was seen in the disc diffusion assay as no zones of inhibition (Table 6.10). These concentration response curves illustrate the usefulness of using two different methods to evaluate the antibacterial activity of an extract.

The DCM extract exhibited a MIC₁₀₀, with a concentration dependent inhibitory response below the MIC₁₀₀, toward all the bacteria tested (Figure 6.8). In particular, there was a clear dose dependent response of the extract toward *L. monocytogenes* and *S. pyogenes* (Figure 6.8c,e). The inhibition of *L. monocytogenes* increased from approximately 10% to 85% inhibition, over the extract concentration range of 12 to 3000 μ g mL⁻¹ (Figure 6.8c), and an MIC observed at an extract concentration of 6000 μ g mL⁻¹. This gradual inhibitory activity was similar for the DCM extract against *S. pyogenes* (Figure 6.8e), *C. butyricum* (Figure 6.8n). However, it must be noted that the MIC was not the same and that the extract concentrations exhibited different degrees of inhibition. A decrease in inhibition was also observed for the DCM against other bacteria, *B. cereus*, *B. subtilis*, *S. pyogenes*, *C. perfringens* (cp8), *C. perfringens* (cp9), *C. perfringens* (SUT) and *S. sporogenes*, however, the decrease appeared to be not as proportional (Figure 6.8a,b,f,h,j,l,p).

The DCM extract also showed inhibitory activities at varying degrees toward the *C*. *difficile* isolates below the MIC₁₀₀ values (Figure 6.8r,t.v). The MIC₁₀₀ for DCM against *C. difficile* (cd1), *C. difficile* (cd8) and *C. difficile* (cd14) was 375, 3000 and 3000 μ g mL⁻¹, respectively. At DCM extract concentrations of 375 μ g mL⁻¹, and 750 μ g mL⁻¹, there was at least 80% inhibition of *C. difficile* (cd8) and *C. difficile* (cd14), demonstrating that the extract still had the ability to inhibit the bacteria, however, these *Clostridium* strains appeared to be slightly more resistant to the DCM extract.

The same MIC was observed for the EtOAc toward the three *C. difficile* isolates, at 12000 μ g mL⁻¹ (Table 6.10). At extract concentrations of 3000 μ g mL⁻¹ (*C. difficile* (cd1) and *C. difficile* (cd8)) and 1500 μ g mL⁻¹ (*C. difficile* (cd14)), greater than 90% inhibition of the bacteria was still observed (Figure 6.8s,v,w). T-tests were performed on the results that gave close inhibitory values to that of the MIC (Appendix 3). All T-tests showed that there was a significant difference between the MIC and the mean value obtained for extract inhibitory concentrations below the MICs. Therefore, the MIC was confirmed to be 12000 μ g mL⁻¹ for the EtOAc toward the *C. difficile* isolates.

The EtOAc extract also exhibited a dose dependent response towards *S. pyogenes*, *C. perfringens* (cp9), *C. perfringens* (SUT) and *C. sporogenes* (Figures6.8g,k,m,q). The

EtOAc extract had a smaller range of concentrations than the DCM extract, which had an inhibitory effect on the bacteria. In addition, the concentrations below the MIC that were effective had a decreased inhibitory effect on the same test organisms. An example of this is shown in Figure 6.8v,w, which depicts the inhibitory effect of the EtOAc extract at different concentrations towards *C. difficile* (cd14).

Overall, the dose response was unique for each test organism, which suggested that the inhibitory action by the extracts was concentration dependent and that the concentration would depend on the target organism. This is similar to the observation that the extracts have a different MIC against the different test organisms (Table 6.11). This difference in inhibitory activity and MIC towards different bacteria is reported widely in literature (Beltran-Garcia *et al.*, 1997; Fakeye *et al.*, 2000; Hirasawa *et al.*, 1999; Yoon *et al.*, 1994). The MIC values of some aqueous *G. lucidum* extracts (Yoon *et al.*, 1994) were comparable to the MIC values obtained in this investigation.

At extract concentrations greater than or the same as the MIC, the cell density (OD_{595}) decreased to below 'pre-extract addition' cell density (results not shown). As cell lysis is evident through a reduction in OD, this suggested that both the EtOAc and DCM extracts had bacteriolytic activity. To determine the exact mode of action in regards to disruption of the cell wall, further investigation would need to be performed, which is beyond the scope of this investigation. This investigation was limited to determining the bactericidal and/or bacteriostatic behaviour of the extracts, which is investigated and discussed further in the following Section (Section 6.3.2.7).

To this point, the activity exhibited by the DCM extract indicated that it had a bacteriostatic activity towards both *L. monocytogenes* and *S. epidermidis* and a bactericidal activity towards some of the other bacteria tested. The EtOAc extract also exhibited bactericidal activity towards a number of the test bacteria. In addition, the concentrations required by the EtOAc extract to impart bactericidal activity were observed to be far greater than the concentrations required by the DCM extract to have an equivalent effect on the same test bacteria.

From the MIC and MBC results, it can be concluded that the DCM extract appears to possess a greater biological activity towards the bacteria tested. Therefore, it is hypothesised that: a) the active compound/s is/are more concentrated in the DCM extract (assuming that the same compound is present in both the DCM and EtOAc extracts), or b) the compound causing the antibacterial activity in the DCM extract is more active than the compound that is causing the antibacterial activity in the EtOAc extract (assuming that there are different compounds which possess activity in both extracts). Further purification and preliminary identification of the active extracts is the topic of investigation Chapter 7.

6.3.2.7 Time Kill Assay

The bactericidal activities of the DCM and EtOAc extracts were examined against a selection of test organisms using time course growth assays as described in Section 3.13.4. Extract was added to the propagation broth, which contained the test organism, to achieve a final concentration that was equal to the MBC. If no MBC was determined for a particular bacterium, a final concentration that was equivalent to the MIC was used instead. The cultures were incubated at 37°C and the number of viable cells was determined after 0, 1, 2 or 3, and 4 hours of incubation. Control cultures without extract were also incubated under the same conditions.

Figure 6.9 displays the antibacterial effect of the DCM extracts on growing bacterial cells of *B. cereus*, *B. subtilis*, *L. monocytogenes*, *S. epidermidis*, and *S. pyogenes*. The DCM extract exhibited a bactericidal effect on both *B. cereus* and *B. subtilis*, reducing the viability by approximately 3-log and 2-log units, respectively, after 4 hours (Figures 6.9a,b). There was no reduction in viability of *L. monocytogenes* and *S. epidermidis* cells after 4 hours, as the log units were comparable to that at time zero (Figures 6.9c,d). This indicated that the DCM extract exhibited a bacteriostatic effect towards both *L. monocytogenes* and *S. epidermidis* and supported the conclusion of bacteriostatic activity of this extract toward these bacteria, since no MBC could be determined in the previous Section (Table 6.11).

Figure 6.9e shows the antibacterial effect of the EtOAc extract, as well as that of the DCM extract, on the growing cells of *S. pyogenes*. Both extracts appeared to have an

inhibitory effect against S. pyogenes, which maintained, but did not kill the bacterial cells over the 4 hour period examined. The time kill assay indicated that the DCM extract had a bacteriostatic effect on S. pyogenes, as the number of viable cells was comparable to that at time zero (the number of cells due to the inoculum). However, the EtOAc extract exhibited a slight bactericidal effect showing a reduction in cell viability of 0.5-log units after 4 hours. Previous results suggested that both extracts exhibited strong bactericidal qualities towards S. pyogenes, which was in contradiction to the time kill assay. The determination of an MBC (Table 6.11) and the observation of complete zones of inhibition in the disc diffusion assay (Table 6.8) indicated bactericidal activity. The viability of S. pyogenes after 24 hours of incubation was also determined in the time kill assay. This showed no viable cells in the cultures that had the DCM or EtOAc extract added (results not shown). It is hypothesised that the DCM and EtOAc extract exhibited a bactericidal effect on S. pyogenes some time after 4 hours of incubation. Prior to this, a bacteriostatic effect was exhibited by both extracts. To determine at what stage the activity changed from bacteriostatic to bactericidal, a time kill assay over a 24 hour period would need to be performed. This was not done in this investigation.

Figure 6.10 displays the antibacterial effect of the DCM and EtOAc extracts on growing bacterial cells of a number of *Clostridium* species. A time kill assay for the EtOAc extract against *C. butyricum* was not performed as this extract showed no inhibitory activity towards this organism (Table 6.10). In addition, results for the EtOAc extract against *C. perfringens* (cp9) are not shown. Problems were encountered in the propagation of this bacterium, so only results that showed good growth have been included. Such difficulty in growing *Clostridium difficile* dates back to the isolation and naming of this species, Latin name '*difficile*' meaning 'difficult', which referred to the unusual difficulty that was encountered in its isolation and study (Bergey, 1986).

Both the DCM and EtOAc extracts exhibited bactericidal activity towards the bacteria tested (Figure 6.10), although limited bactericidal activity towards *C. sporogenes* (Figure 6.10d) was observed, where after 4 hours, there was a decrease in viability by only 1-log unit for both extracts. After 24 hours, the viability had decreased to an undetectable level (results not shown), which indicated a slow bactericidal activity against *C. sporogenes*.



Figure 6.9. Time kill curves for the (\blacktriangle) DCM extract against (**a**) *Bacillus cereus*, (**b**) *Bacillus subtilis*, (**c**) *Listeria monocytogenes*, (**d**) *Staphylococcus epidermidis*, and both the (\blacktriangle) DCM extract and (\blacksquare) EtOAc extract against (**e**) *Streptococcus pyogenes*. Extracts from the mycelium of *Ganoderma* H1 that had been cultivated in Basal-G medium for 30 days. (\bigcirc) Growth of the organism in the absence of extract. The MBCs were used as the final concentration of extract in each assay and are listed in Table 6.11.



Figure 6.10. Time kill curves for the (\blacktriangle) DCM extract and (\blacksquare) EtOAc extract against: (a) *Clostridium* perfringens (cp8), (c) *Clostridium perfringens* (SUT), (d) *Clostridium sporogenes* (SUT) and (d) *Clostridium difficile* (cd1), and the (\checkmark) DCM extract against (b) *Clostridium perfringens* (cp9) and (e) *Clostridium butyricum*. Extracts from the mycelium of *Ganoderma* H1 that had been cultivated in Basal-G medium for 30 days. (\bigcirc) Growth of the organism in the absence of extract. The MBCs were used as the final concentration of extract in each assay and are listed in Table 6.11.

A strong bactericidal activity was exhibited by both extracts towards the *C. perfringens* isolates tested. The DCM extract reduced the viability of *C. butyricum* by 2.5-log units after 4 hours. A greater decrease in viability, by 3-log units and 4-log units, was exhibited by both extracts against *C. perfringens* (cp8) (Figure 6.10a) and *C. perfringens* (SUT) (Figure 6.10e), respectively. The DCM extract exhibited greater activity against the *C. perfringens* isolate (cp9) in that a rapid decrease in viability, to an undetectable level, was observed after 1 hour (Figure 6.10b). A similar, rapid bactericidal effect was also exhibited by both extracts against the clinical isolate *C. difficile* (cd1). Within 1 hour there was an undetectable level of viable cells (Figure 6.10f). Due to difficulties experienced in the propagation of the *C. difficile* isolates, as mentioned earlier, only one representative isolate was chosen for analysis.

The bactericidal or bacteriostatic activity exhibited by the DCM and EtOAc extracts were similar for the same bacterium. This suggested that the active compound was present in both extracts and was the same compound. The MBC determined for the extract against each test organism was used in the time course growth assays. Since the results of the MBCs for the DCM extract were less than that for the EtOAc extract, this led to the belief that the active compound was in a far greater concentration in the DCM extract.

Overall, the results of the time kill experiments correlated with those of the disc diffusion assays (Table 6.8 and Table 6.10) and the concentration response assays (Figure 6.8). The extracts that exhibited rapid bactericidal activity also showed large zones of inhibition (Table 6.10). The extracts that showed bacteriostatic behaviour were observed to show zones of incomplete inhibition (Table 6.8) and have dose inhibitory responses below the MIC₁₀₀ (Figure 6.8).

6.3.3 Antifungal Activity

The crude organic extracts, HEX, DCM, EtOAc and MeOH, from *Ganoderma* H1 were assessed for their antifungal activity using the disc diffusion test as described in Section 3.13.1. The organisms tested included; *Candida* sp., *Saccharomyces cerevisiae*, three yeast strains (2906, 6034 and 2119), *Mucor* sp. + and *Mucor* sp. -, *Aspergillus niger*, *Rhizopus* sp, *Penicillium* sp. and *Fusarium oxysporium* (Chapter 3, Table 3.4). An

extract volume of 6000 μ g was applied to each disc. No antifungal activity was observed for any of the extracts tested. To date there appears to be only one report of a *Ganoderma* species possessing antifungal activity. This is *G. annulare*, which was found to possess activity against two fungal species, *Microsporum canis* and *Trichophyton mentagrophytes*, at concentrations of 500 and 1000 μ g mL⁻¹, respectively (Smania *et al.*, 2003).

6.4 Summary

The liquid cultivated mycelial biomass of three Australian *Ganoderma* isolates, *Ganoderma* H1, *Ganoderma* H2 and *Ganoderma* H3, were investigated for their antibacterial activity. Initially, the three isolates were cultivated for 30 days in a standard fungal cultivation medium, MEB, to produce mycelial biomass. This biomass was then extracted using two methods. The first method employed a hot aqueous extraction technique (Section 3.11.1), while the second method employed extraction in a Soxhlet apparatus using organic solvents, in order of increasing polarity (Section 3.11.2). Preliminary antibacterial investigations were then performed on all extracts and an overall analysis of the active extracts performed.

The aqueous extracts from *Ganoderma* H1 and H2 were found to possess some antibacterial activity in preliminary investigations (Section 6.3.1.2). Incomplete inhibition was exhibited by *Ganoderma* H1 towards both *B. cereus* and *B. subtilis*, and by *Ganoderma* H2 towards *B. cereus*. The aqueous extracts from all three *Ganoderma* isolates exhibited strong antibacterial activity towards *S. pyogenes*. This is the first investigation reporting antibacterial activity of aqueous extracts from the liquid cultivated mycelium of Australian *Ganoderma* isolates.

Preliminary studies on the organic extracts from the three *Ganoderma* isolates found them to possess antibacterial activity towards a greater range of bacteria than the aqueous extracts (Section 6.3.1.3). Activity was observed for *Ganoderma* H1 and H2 against some Gram positive bacteria. However, there was no activity observed for any of the extracts from *Ganoderma* H3. At the concentrations examined, the organic extracts from *Ganoderma* H1 exhibited activity against a greater range of bacteria than those from *Ganoderma* H2. A more detailed investigation of the organic extracts from the mycelium of *Ganoderma* H1 was performed (Section 6.3.2). This focus was prompted by the observation that the extracts from *Ganoderma* H1 exhibited antibacterial activity towards the greatest number of bacteria. A review by Cowan (1999) reported that the most active components are generally water insoluble, hence, the expectation that low polarity organic solvents would yield more active extracts. This was the case in this investigation as the aqueous extracts exhibited less activity than the organic extracts. In addition, the extracts obtained using the less polar solvents, such as HEX, DCM and EtOAC, gave greater activity than those obtained using the highly polar solvent MEOH.

The mycelium of *Ganoderma* H1 was grown in three different cultivation media, PDB, MEB and Basal-G, to investigate the effect, if any, the media would have on the antibacterial activity. The HEX, DCM and EtOAc extracts exhibited activity against a number of Gram positive bacteria, whilst the MEOH extracts showed activity towards only two bacteria, namely *E. faecalis* and *S. pyogenes* (Table 6.8). There was no activity exhibited against any of the Gram negative bacteria tested. This was thought to be due to the difference in cell wall structure to Gram positive bacteria, which generally makes Gram negative bacteria resistant to greater number of compounds (Villee *et al.*, 1989).

All the DCM extracts exhibited activity towards *B. cereus*, *B. subtilis*, *E. faecalis*, *S. pyogenes* and *S. aureus*. In addition, the DCM(Basal-G) also inhibited *S. epidermidis* and *L. monocytogenes*. Overall, the DCM extract appeared to have promising inhibitory effects. Other reports within the literature have demonstrated that chlorinated extracts from different *Ganoderma* species possess cytotoxicity towards tumour cells (Gan *et al.*, 1998a), anticomplement activities towards the complement pathway (Min *et al.*, 2001), antihistamine release activities (Tasaka *et al.*, 1988a, 1988b), antinociceptive activities (Koyama *et al.*, 1997), as well as some antibacterial activity (Mothana *et al.*, 2003; Smania *et al.*, 1999), all *in vitro*. In this investigation, activity was also observed for the HEX and EtOAc extracts, but this activity was limited to one or two bacteria for extracts from mycelium cultivated in PDB.

Further investigation of the active extracts against some clinical MRSA and VRE isolates was investigated (Section 6.3.2.3). There was no inhibitory activity observed for any of the extracts against the VRE isolates (Table 6.8). For the MRSAs, the DCM(Basal-G), DCM(PDB), EtOAc(Basal-G) and EtOAc(PDB) extracts exhibited activity towards six isolates, while the DCM(MEB) and EtOAC(MEB) exhibited activity against one and five isolates, respectively (Table 6.8). This appears to be the first report on the inhibitory activity of a *Ganoderma* isolate against these types of clinical isolates.

Extracts from mycelium that had been grown in different cultivation medium exhibited different sized zones of inhibitions. This suggested that the cultivation media had an effect on the production of the active compound. Kim *et al.* (2002a) also found that the manipulation of the cultivation medium had an effect on the biological activity. In this study, the least amount of activity was exhibited from mycelium that had been grown in PDB and the greatest amount of activity was exhibited from mycelium grown in Basal-G medium (Table 6.8). This observation, combined with the outcome that Basal-G supported the greatest growth of the mycelium (Section 5.3.2.4) resulted in the choice of the Basal-G medium cultivation medium for further growth and examination of *Ganoderma* H1.

The organic extracts from *Ganoderma* H1 mycelium, that had been cultivated in Basal-G growth medium, were investigated further against some *Clostridium* species, which included some clinical isolates (Section 6.3.2.5). The HEX, DCM and EtOAc extract exhibited strong antibacterial activity against all strains tested. The zones of inhibition against the anaerobic isolates were larger than the zones of inhibition against the anaerobic bacteria, including the spore forming aerobic bacteria. This indicated that the anaerobic bacteria were more susceptible to the extracts than the aerobic bacteria.

Small changes in disk content can produce rather minor changes in zone diameters i.e.: a two fold increase in disk potency generally increases zone sizes by 2 or 3 mm (Barry & Thornsberry, 1985). As the diameter of the zone increases radially, the areas of the zones increase by a much larger factor, which in turn, requires larger quantities of extract. Because of this, a more appropriate means of measurement than the disc diffusion assay would be to measure the area of the zone of inhibition, rather than the diameter. However, the diameter of the zone of inhibition is used most frequently when reporting inhibitory results using the disc diffusion assay (King & Brown, 2001). Since, the full effect of the extracts activity may not have been observed using the disc diffusion assays, other methods of analysis were used to determine the extract potency towards the test bacteria.

One method commonly used to describe the antimicrobial effectiveness of a compound is the MIC. A definition of the MIC is the lowest concentration of the compound capable of inhibiting the growth of the challenging organism (Mann & Markham, 1998). The MIC as well as the MBC was determined for the DCM and EtOAc extracts that showed strong antibacterial testing. These MIC and MBC values (Table 6.11) along with the concentration graphs (Figure 6.8) supported the idea that inhibitory action exhibited by the extract was concentration dependent and that the concentration would depend on the target bacteria. The MIC and MBC values determined were comparable to some MIC and MBC values for *Ganoderma* extracts reported in the literature (Yoon *et al.*, 1994).

Time kill assays confirmed the bacteriostatic or bactericidal activity of the extracts against the different test organisms. It was observed that the DCM and EtOAc extracts were predominantly bactericidal against the range of bacteria tested (Figure 6.9 and 6.10). However, as previously mentioned, this was highly dependent on the concentration of the extract and the target organism. The time kill assay also suggested that the active compound in the EtOAc and DCM was similar, as the rate of decrease in viability was the same for both extracts towards each bacterium.

The results reported in this Chapter indicate that the mycelium of the Australian *Ganoderma* isolate H1 contains a compound or some compounds that have antibacterial activity against some important Gram positive bacteria. The results also indicated that cultivation in the growth medium, Basal-G, supported the greatest production of mycelium and in turn, yielded the greatest quantity of active extract. It was observed that the less polar organic extracts appeared to have the greatest activity, suggesting that the active compound is organic and has a cyclic structure, as these type compounds are

nearly exclusively extracted by these solvents (Harbourne, 1973; Wagner *et al.*, 1984). To explore this further, additional testing and isolation of the organic compound was performed and is discussed in the following Chapter.

Chapter Seven

Preliminary Identification of Bioactive Components

7.1 Introduction

Thin layer chromatography (TLC) is one of the simplest and least expensive methods for detecting natural product constituents because the method is easy to perform, is reproducible and requires little equipment (Hostettmann, 1999). Advantages of TLC over other chromatographic techniques are that, parallel runs can be carried out for a series of spots on the origin line, and more complex mixtures with minimal purification can be analysed (thus, no blocking of sophisticated chromatography columns) because of the single use of a TLC plate (Sherma, 2002). Detection on TLC plates depends on the chemical structure of the compound and is based on the natural colour, fluorescence at UV-365 nm or UV absorption at UV-254 nm (fluorescence quenching on phosphor impregnated layers) of separated zones (Sherma, 2002). The use of selective or universal chemical spray reagents to determine classes of compounds is also widely reported (Krebs *et al.*, 1969; Wagner *et al.*, 1984).

Isolation of pure, pharmacologically active constituents from natural sources remains a long and tedious process (Hostettmann, 1999). A major focus for phytochemical analysis is the characterisation of an active agent responsible for some toxic or beneficial effect exhibited by a crude plant extract when tested against a living organism (Harbourne, 1973). Chemical screening can be performed to allow the localisation and targeted isolation of such constituents with potential activity. Bioassays are therefore essential for monitoring each stage of the extraction and separation procedures in order to follow the active material as it is purified (Harbourne, 1973). Bioautography is a technique that combines TLC with a bioassay *in situ*, and therefore, allows the localisation of active constituents in a complex matrix (Hamburger & Hostettmann, 1991). Such bioautographic methods include bioautography agar-overlay (Hostettmann, 1999) and direct bioautography (Horvath *et al.*, 2002).

A number of secondary metabolites have been purified and identified from *Ganoderma* species using column chromatography and HPLC/GC techniques (Gonzalez *et al.*, 1999, 2002; Kleinwachter *et al.*, 2001; Rosecke & Konig, 2000; Smania *et al.*, 1999). These secondary metabolites have generally been isolated, identified and then tested for bioactivity. In contrast, this chapter reports the bioassay-guided fractionation and

subsequent identification of the classes of compounds that exhibited antibacterial activity in the DCM extract from *Ganoderma* H1 (*G. cupreum*).

7.2 Overview

The components of the crude DCM extract of *Ganoderma* H1 were separated by TLC and observed by visualisation at UV-254 nm and UV-365 nm. The components were then assessed for antibacterial activity against *B. cereus*, using the bioautography agaroverlay method. Components that exhibited antibacterial activity were then separated by silica gel column chromatography following a bioassay-guided fractionation process. Fractions were subjected to TLC and the classes of compounds determined using universal and specific TLC detection reagents, UV-254 nm and UV-365 nm light. Finally, bioautographic analysis and comparison of R_f values with reference chromatograms provided information about the nature of the antibacterial constituents.

7.3 Results & Discussion

7.3.1 Crude Extract Composition and Active Components

Initial TLC analysis was performed to determine the number of different components within the crude DCM extract and to identify which component/s exhibited antibacterial activity. As the number and identity of extract components were not known, experimentation was required to determine: 1) a suitable solvent system, 2) time required for development of the plate, and 3) an optimal concentration of extract to be applied onto the TLC plate. Generally, these parameters were investigated simultaneously.

TLC was performed as described in Section 3.12.2. A number of TLC plates were spotted with increasing volumes (from 1 μ L to 15 μ L) of the DCM extract (2 μ g μ L⁻¹). Solvent systems investigated were HEX, CHCl₃, CHCl₃:EtOAc and CHCl₃:MeOH in varying ratios. The TLC plates were placed into solvent saturated TLC tanks, developed in the different solvent systems and then observed for the separation of components under UV-254 nm, UV-365 nm or visible light. CHCl₃:EtOAc (95:5), developed twice (TLC plate was developed once in the mobile phase, dried, and then developed a second time to give a better separation), was found to be the most suitable solvent system, yielding good separation of the different components in the DCM

extract. TLC plates were also subjected to a bioautography overlay assay as described in Section 3.13.5 and the specific components were observed for antibacterial activity. *B. cereus* was used in the bioautography assays, as this bacterium had previously displayed sensitivity to the crude DCM extract in the antibacterial assays (Chapter 6). TLC plates developed using CHCl₃:EtOAc (95:5 x 2) and observed at UV-254 and UV-365 nm are presented in Figure 7.1a,b,c. Bioautography results are shown in Figure 7.1d.

The term retention factor, R_{f} , is commonly used to describe the chromatographic behaviour of sample solutes. The R_{f} value for a given component is the distance it has travelled from the origin divided by the distance travelled by the solvent front (mobile phase) (Harbourne, 1973). The centre for each spot is the point taken for reference. $R_{f}x100$ values ($R_{f}x 100\%$) were calculated for comparison of spots and are reported in Table 7.1 in the following pages. Since silica gel retains the more polar compounds, the non-polar compounds were eluted first (and moved further up the TLC plate). Hence, the more polar the component the lower the R_{f} value, and the less polar the component the larger the R_{f} value. The R_{f} value of a component will not be the same when a different solvent system is used. For this reason, the R_{f} value can only be compared if the same mobile phase is used. Different mobile phases were employed throughout this investigation as the fractionation proceeded. Therefore, results in this Chapter only compare R_{f} values of components that have been separated in the same system at the same time.

The mobile phase, CHCl₃:EtOAc (95:5 x 2), resulted in the separation of eighteen components in the DCM extract (Table 7.1). Fourteen of these components fluoresced at UV-365 nm (Figure 7.1b) and an additional four components were observed to quench fluorescence at UV-254 nm (Figure 7.1c). Two of these eighteen components were seen in visible light (Table 7.1). The first, a yellow/orange spot ($R_f x 100 = 88$) also fluoresced green at UV-365 nm and the second, seen as a yellow arch (\cap) ($R_f x 100 = 59$) also quenched fluorescence at UV-254 nm.

The intensity and size of the spot on the TLC plate is a measure of the concentration of that component in a mixture (Harbourne, 1973). Therefore, relative concentrations of

the components can be estimated. It was clear that the DCM extract had components present in varying concentration, as different intensities of the components were observed under UV light (Figures 7.1b and 7.1c). The component that was eluted at an $R_{fx}100 = 69$, which fluoresced bright blue at UV-365 nm, was present in a much greater concentration than any other component in the DCM extract (Figure 7.1a,b). In addition, applying different volumes of the DCM extract onto TLC plates (1 μ L, 2 μ L, 5 μ L, 10 μ L, 15 μ L, 20 μ L and 30 μ L (not all results shown)) and observing the intensity and size of the components after development, allowed estimation of the relative concentrations of the different components. This process also identified additional spots that were present in the extract in small concentrations. When 1 μ L of the DCM extract was applied, ten components were observed (Figure 7.1a) and when a larger volume of extract was applied (15 µL), four extra components were observed at UV-365 nm (Figure 7.1b) and another four observed at UV-254 nm (Figure 7.1c). TLC plates spotted with 20 μ L or greater of the crude extract produced chromatograms with poorly resolved components (results not shown).

Components that had $R_f x 100$ values of 19, 8 and 6 were only just visible in UV-365 nm light when a 15 µL sample was applied to the TLC plate (Figure 7.1b). The resolution of these compounds was a little ambiguous and there may have been more compounds present in this part of the TLC plate. However, applying a larger volume of the extract (20 µL and 30 µL) decreased the resolution and smearing on the TLC plate was observed (results not shown). This smearing was due to the overloading of components that were present in high concentration (in this case the component that exhibited an $R_f x 100 = 69$), an effect commonly observed if some of the components in the mixture are present in too high a concentration (Harbourne, 1973). As a result, the less polar components eluted above this spot were affected by smearing and the separation of the components was not as clear.



Figure 7.1. TLC and bioautography of the DCM extract developed in a CHCl₃:EtOAc (95:5 x 2) solvent system. A 1 μ L sample of the DCM extract (2 μ g μ L⁻¹) was spotted onto the TLC plate, developed and the components visualised under (a) UV light at 365 nm. A 15 μ L sample of the DCM extract (2 μ g μ L⁻¹) was spotted onto the TLC plate, developed and the components visualised under (b) UV light at 365 nm, and (c) UV light at 254 nm. (d) Bioautography of the DCM extract using *Bacillus cereus* as the test bacterium. Clear zones of inhibition can be observed at an R_fx100 = -2 to 24, 29 and 37 to 58. All plates are aligned and the R_fx100 values of visible components listed.

Bioautography of the TLC plates showed the range of components that exhibited antibacterial activity (Figure 7.1d). The component present in the extract in the greatest concentration exhibited no activity ($R_f x 100 = 69$). In fact, there was no activity observed above this component, which eliminated the less polar components as the target compounds ($R_f x 100 = 90$, 88, 80, 75, 71 and 69) (Table 7.1). Activity was observed to be in components that had $R_f x 100$ values of no higher than 58 in this mobile phase, which corresponded with those beneath the component that quenched fluorescence at UV-254 nm (half moon, $R_f x 100 = 59$) (Figure 7.1c). It was difficult to determine exactly which compounds were active as the zones of inhibition were spread over a wide range ($R_f x 100$ range = 58 to 37, 29, 24 to -2) that included a number of components were in a too low a concentration to be visualised at UV-254 or UV 366 nm, hence, exact comparison with smaller volumes of extract was not performed. Slight

smearing caused by the high concentration of the component that exhibited an $R_f x 100 =$ 69 affected the clear separation of the components in this active region. As a result, zones of inhibition could not be matched to the corresponding components. In all, it appeared that the antibacterial activity was due to components that caused distinct fluorescence quenching at UV-254 nm and had little fluorescence at UV-365 nm (Table 7.1 and Figure 7.1b,c). The low $R_f x 100$ values of the active components in this mobile phase also suggested that they were slightly polar (Table 7.1).

Table 7.1

	Detection of Components in the DCM Extract ^a			
R _f x100 ^b	UV-365 nm	UV-254 nm	Visible	Bioactivity ^c
90	orange	black		
88	green	-	yellow/orange	
80	blue	black		
75	green	black		
71	-	black		
69	bright blue	light blue		
67	-	black		
65	green	-		
59	-	black (\cap)	orange (\cap)	
54	green	-		Activity
48	green	black		appeared in
43	blue	light blue		this region of
32	green	black		$K_f x 100$ values.
28	blue	light blue		30 tū 37, 20
24	-	black		-29,
19	blue	black		2710-2
8	blue	black		
6	blue	black		

TLC of the	crude	DCM	extract

^a Separation of the crude DCM extract was performed on silica gel TLC plates using the mobile phase CHCl3:EtOAc (95:5 x 2); ^b Rfx100, the distance the component traveled divided by the distance traveled by of the solvent front x 100; ^c The range of bioactivity was recorded over a number of R_f values.

It was apparent that the non-active compounds were present in a much higher concentration than the active (target) compounds. To perform a preliminary identification of the classes of compounds, a high enough concentration of the active components was required to enable detection by spray reagents. In addition, clear separation of the active components by TLC was also required, so that other

components would not interfere in the identification process. Therefore, to obtain sufficient quantities of active components and to remove the non-active less polar compounds, bioassay-guided fractionation using silica gel column chromatography was performed and is reported in the following sections. It is important to note that the aim of this chapter was to report only the class of compounds that were exhibiting the antibacterial activity and thus, full purification and identification of the active components was not performed.

7.3.2 Bioassay-Guided Fractionation of the DCM Extract

Bioassay-guided fractionation of the DCM extract was performed to remove components in the mixture that exhibited no antibacterial activity. The fractionation procedure was performed as described in Section 3.12.1. Crude DCM extract was applied to the top of a silica gel column and the components eluted using a stepwise gradient starting with CHCl₃:MeOH (99:1) and ending with CHCl₃:MeOH (2:8) (A series, Figure 7.2). An insoluble layer on the extract was removed prior to fractionation. This layer was also tested for bioactivity in the previous chapter (Section 6.3.2.1), however, no activity was observed and it was the soluble layer that exhibited activity.

During the first step of the fractionation process, thirty fractions, each consisting of 5 mL, were collected, evaporated to dryness and then resuspended in an appropriate solvent, depending on where the fraction was eluted from the column. Each fraction was subjected to a preliminary bioautographic evaluation (Section 3.13.5) to observe which fractions exhibited antibacterial activity. The fractions that exhibited antibacterial activity were spotted onto a TLC plate, developed in an appropriate solvent system (determined by trial and error) and then visualised at UV-365 nm and UV-254 nm. Fractions that contained the same components and exhibited antibacterial activity were pooled. Active fractions that were of interest were subjected to the fractionation process for a second time. This further purified the fractions so that a greater separation could be achieved and, in turn, detection of the class of compound could be carried out without the interference of some non-target compounds. A flow diagram representing the fractionation process is presented in Figure 7.2.



Figure 7.2. Diagram of the fractionation process of the DCM extract; Fr, fraction; * Fraction exhibited antibacterial activity.

7.3.2.1 Fractionation No. 1

The first fractionation of the crude DCM extract by column chromatography yielded thirty fractions (Figure 7.2). A preliminary bioautographic evaluation was performed on each fraction by spotting them onto a silica gel plate and evaluating their bioactivity without development of the plate (Figure 7.3). This indicated which fractions contained active components. As a result, the non-active fractions could be eliminated from further TLC and bioautography analysis.

Figure 7.3 displays the results of the preliminary bioautography assay. Zones of inhibition were observed for thirteen fractions: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 22, 23 and 24. Fractions 1 to 8, 25 to 27 and 28 to 30 exhibited no activity. Fraction 9 exhibited only slight activity. The largest zones were exhibited by fractions 10, 11 and 12, which suggested that there was either a concentrated amount of the active compound or that there were a number of active compounds present. Although fractions 22 to 24 exhibited antibacterial activity, the zones of inhibition appeared be less intense when compared to the other active fractions.

.1	2	3.	4	5	6
.7	8	9	10	11	12
13	14 •	15	16 •	17 •	18
19	20	21	22	23	24
25	26	27	C O	D1	D2
.28	.29	.30		.D3	.D4

Figure 7.3. Bioautography of the DCM extract fractionation; Thirty fractions in total; 10 μ L of each 5 mL fraction was spotted onto the TLC plate. The plate was overlayed with *Bacillus cereus*, incubated overnight and then sprayed with MTT; Fraction 1 to Fraction 30 are numbered on the plate; C, positive control consisting of the crude DCM extract; **D1 - D4**, DCM solvent blank.

In the previous chapter (Chapter 6), it was suggested that there might be more than one active compound present in the crude DCM extract. The preliminary bioautography assay supported this suggestion, as antibacterial activity was seen in a number of fractions eluted at the start and end of the fractionation process, whereas there was no activity in the fractions (compounds) that were eluted in the middle (Figure 7.3).

Separation of the active (9 to 18, 22 to 24) and non active fractions (1 to 8, 19 to 21) on TLC using different solvent systems were performed (results not shown). Fractions that contained similar components were pooled into seven fractions (designated the 'A series') (Figure 7.2). Of the A series, only four fractions exhibited antibacterial activity (A2, A3, A4 and A6) (Figure 7.2). Fraction A6 exhibited weak antibacterial activity compared to the other active fractions, thus, was not assessed further for the identity of its components. Components within fractions A1, A5 and A7 exhibited no antibacterial activity when examined via bioautography (results not shown). Fractions A2, A3 and A4 displayed a number of components that showed zones of inhibition, which indicated that there was more than one active component present in the crude DCM extract. However, it was not possible to align the zones with the appropriate component, as there appeared to be several new components (results not shown). These new active components appeared to be present in a low concentration and either fluoresced at UV-365 nm and/or quenched fluorescence at UV-254 nm.

It is likely that there are many components present in crude extracts that are present in very low concentration (Harbourne, 1973). Therefore, the process of fractionation will separate and concentrate these components. As the fractionation proceeded in this investigation, a greater number of components appeared in the crude extract than the eighteen observed in the initial screening (Section 7.3.1). It also became apparent that some of these new components exhibited antibacterial activity, although, some exhibited only slight activity, which indicated either low antibacterial activity or simply the presence of the component in a low concentration. Identification of the classes of all the compounds that exhibited activity would be a time consuming task, since solvent systems and spotting concentrations would need to be optimised, and fractionation processes repeated a number of times. Therefore, a decision was made to focus on the components that exhibited the strongest antibacterial activity, or appeared to be present in greater concentration. Only classes of compounds from the components that exhibited strong antibacterial activity were investigated further.

7.3.2.2 Fractionation No. 2

Three fractions from the A series (A2, A3 and A4) that exhibited the strongest activity were subjected to silica gel column chromatography a second time (Section 3.12.1), to

purify the components further so a better separation could be achieved. Fraction A2 was fractionated into five fractions (B1 to B5) (Figure 7.2) and have been designated the 'B series' to aid in discussion. The fractionation of A3 and A4 yielded some similar fractions with similar components (results not shown). These were pooled. In all, six further fractions were produced from A3 and A4 (C1 to C6) and have been designated the 'C series' (Figure 7.2) and are discussed in the latter half of this section. Components in the new fractions were separated by TLC and visualised at UV-254 nm and UV-365 nm (Figures 7.4a,b and 7.5a,b (discussed later)). Bioautography was also performed on TLC plates alongside the reference TLC plates to determine which components exhibited activity (Figure 7.4c and 7.5c (discussed later)). The same amount of each fraction was spotted onto the TLC plates (100 μ g) and relative concentrations/activities of the active components were compared.

Suitable mobile phase conditions and appropriate fraction concentrations to be applied to the TLC system were again determined by experimentation. Two different solvent systems were employed for the different series. The B series used a mobile phase of CHCl₃:MeOH (97:3) and the C series used a mobile phase of CHCl₃:MeOH (97:3) and the C series used a mobile phase of CHCl₃:MeOH (90:10). Although separation of most of the components in the B series was achieved, the target active components exhibited low R_f values and separated poorly from each other (Figure 7.4). However, the primary purpose was to identify the number of components within the fractions, and therefore, this mobile phase was determined to be the most suitable. Since it is ideal that the components of interest should elute with an R_f value equal to or greater than 0.25 to 0.40 (Harbourne, 1973; Wagner *et al.*, 1984), a slightly more polar mobile phase (CHCl₃:MeOH (95:5)) was used for the identification of the classes of compounds in the next section (Section 7.3.3). $R_f x100$ values for components in the B series and the C series are presented in Table 7.2 and Table 7.3 (presented in the latter part of this section), respectively.

The separation of fractions B1 to B5 using the mobile phase CHCl₃:MeOH (97:3) yielded a number of components that fluoresced at UV-365 nm (Figure 7.4a). However, none of these fluorescent compounds exhibited antibacterial activity (Figure 7.4c). In fact, it appeared that antibacterial activity was again limited to the components that

quenched fluorescence at UV-254 nm or components that did not exhibit fluorescence of any kind (Figure 7.4).



Figure 7.4. TLC separation of fractions B1 to B5. Components on the TLC plates were visualised at (a) 365 nm and (b) 254 nm. Corresponding active compounds were observed by (c) bioautography of the TLC plate. TLC plates were developed in CHCl₃:MeOH (97:3); $R_f x100$ values of relevant spots are shown on the plates.

Fraction B1 showed no antibacterial activity. Components in fractions B2 to B4 showed three zones of inhibition that had the same $R_f x 100$ values at 20, 9 and 0 (Figure 7.4c and Table 7.2). When bioautography assays was compared to reference plates, there appeared to be no compounds that had similar R_f values at either UV-365 nm or UV-254 nm (Figure 7.4). In contrast, fraction B5 displayed a component that strongly quenched fluorescence ($R_f x 100 = 17$). This component also appeared to exhibit antibacterial activity (Figure 7.4c), although the activity was not defined to a particular spot as there were no distinct zones of inhibition and instead, a zone of inhibition that ranged from an $R_f x 100 = 20$ to the origin was observed. At the same $R_f x 100$ in fractions B2 to B4 ($R_f x 100 = 17$) no activity was observed and instead, activity was observed just above ($R_f x 100 = 20$) and below ($R_f x 100 = 12$) this spot (Figure 7.4). Overall, the entire inhibition region exhibited by fraction B5 displayed slight fluorescence quenching at UV-254 nm (Figure 7.4b).

	Detection of Components in the DCM Fractions ^a			
R _f x100 ^b	UV-365 nm	UV-254 nm	Fraction ^c	Bioactivity^d
77	blue	-	B1	-
68	bright blue	bright blue	B1, B2, B3, B4, B5	-
59	blue	-	B1, B2	-
52	green/ light blue	-	B1, B2, B3, B4	-
43	corn flour blue	-	B1, B2, B3, B4	-
38	purple	-	B1, B2, B4	-
32	bright green	-	B1	-
20	-	-	B2, B3, B4, B5	yes
17	-	black	B5	yes
12	-	-	B2, B3, B4, B5	yes
9	-	black	B5	yes
0	-	black	B2, B3, B4, B5	yes

 Table 7.2

 TLC of the DCM fractions B1 to B5

^a Separation of fractions B1 to B5 was performed on silica gel TLC plates using the mobile phase CHCl₃:MeOH (97:3); ^b $R_{fx}100$, the distance the component moved divided by the distance of the solvent front x 100; ^c Fractions that exhibited the component; ^d Distinct zones of inhibition were exhibited, except for fraction B5, which exhibited a wide range of inhibition; -, indicates no components or bioactivity was observed/detected.

Comparing fractions B2 to B5, they all appeared to exhibit activity within the same regions. Whilst fractions B2 to B4 showed distinctive zones, fraction B5 showed a larger zone of inhibition, which suggested either a larger amount of the active component or a greater number of active compounds. However, the shape of the zone of inhibition for fraction B5 suggested that it was more likely to be three separate compounds at the same R_f values as those exhibited by B2, B3 and B4 ($R_f x 100 = 20, 12$ and 0), suggesting that the same active components were present in these fractions.

Fraction B5 quenched fluorescence at UV-254 nm. This fluorescence quenching was also observed in fractions B2 to B4 when a larger volume of sample was applied to the TLC plate (results not shown). However, the larger sample size resulted in overloading and in turn smearing of the sample on the TLC plate, and one large zone of inhibition rather than three separate zones was observed (results not shown). In order to obtain sharply resolved zones, the quantity of material applied to the chromatogram should be as small as possible. However, rather large sample volumes are necessary for the

detection of substances that are present in low concentrations, which inevitably results in broadening and overlapping of zones (Wagner *et al.*, 1984).

The mobile phase CHCl₃:MeOH (97:3) effectively separated the less polar components that fluoresced at UV-365 nm. The more polar active components, which did not fluoresce and had lower R_f values, were not separated well from each other. It is known that if components are similar in structure they will migrate close to each other on silica gel and be difficult to separate (Harbourne, 1973). This appeared to be the case in this investigation, as distances between active components did not change when a number of different solvent systems were investigated (results not shown). In addition, when a more polar solvent was used to separate fractions B1 to B5, they were eluted at R_f values similar to that of the components that gave colour fluorescence at UV-365 nm, which led to difficulty in assigning antibacterial activity to particular compounds.

Overall, it was observed that the active components in fractions B2 to B4 did not fluoresce at UV-365 nm and exhibited little fluorescence quenching at UV-254 nm. It is quite common for some natural plant and fungal compounds to neither fluoresce at UV-365 nm nor quench fluorescence at UV- 254 nm (Harbourne, 1973; Wagner *et al.*, 1984). This makes detection and identification of such compounds difficult.

The C series fractions (C1 to C3) were separated using the solvent system CHCl₃:MeOH (90:10) (Figure 7.5). Bioautography of the TLC plate showed that the concentration of the active components in fraction C1 was small, whilst fractions C2 to C4 contained higher concentrations. This was indicated by a slight zone of inhibition and strong zones of inhibition, respectively (Figure 7.5c). The components in fraction C7 exhibited no antibacterial activity. The slight figure eight shape exhibited by the zone of inhibition of fraction C2 suggested that there were two active components present (Figure 7.5c). The first zone of inhibition ($R_f x 100 = 42$) in fraction C2 also quenched UV light at 254 nm (Figure 7.5b) and appeared as a blue crescent spot when viewed under UV light at 365 nm (Figure 7.5a). There was also activity seen beneath this component ($R_f x 100 = 32$), which appeared to be the same as that observed for one of the active components in fraction C4 (Figure 7.5c). However, at this $R_f x 100$ (32), fraction C2 did not fluoresce and quench fluorescence at UV-365 and UV-254 nm,

respectively, as was exhibited by the same component in fraction C4 (Table 7.3). This suggested that the active component was in a much greater concentration in fraction C4 than C2, or indeed a different component with identical R_f value. However, bioautography zones suggested the active component was in a similar concentration (Figure 7.5).



Figure 7.5. TLC of DCM fractions C1 to C7. Compounds on the TLC plates were visualised at (a) 365 nm and (b) 254 nm. Corresponding active compounds were observed by (c) bioautography of the TLC plate. TLC plates were developed in CHCl₃:MeOH (90:10); R_fx100 values have been labelled to the left of spots of interest.

Table 7.3

TLC of the DCM fractions C1 to C7

	Detection of Components in the DCM Fractions ^a			
R _f x100 ^b	UV-365 nm	UV-254 nm	Fraction ^c	Bioactivity ^d
0.70	bright blue	Black	C1	-
0.52	blue	Black	C3	yes
0.42	blue moon (\cap)	Black	C2, C3	yes
0.32	blue	Black	C2, C4, C5	yes
0.25	blue	Black	C5, C6	yes
0.22	purple	-	C5, C6	-
0.18	green	Black	C5, C6	-
0.14	dark purple	-	C5, C6	-

^a Separation of fractions C1 to C7 was performed on silica gel TLC plates using the mobile phase CHCl₃:MeOH (90:10); ^b R_fx100 , the distance the component moved divided by the distance of the solvent front x 100; ^c Fractions that contained the component; ^d Zones of inhibition exhibited. Note shaded components exhibited a zones of inhibition over a range; -, indicates no components or bioactivity was observed/detected.

Fraction C3 exhibited strong activity at an $R_f x 100 = 52$ (Figure 7.5c), which correlated with a blue spot seen at UV-365 nm (Figure 7.5a). This active spot also quenched fluorescence at UV-254 nm (Figure 7.5b). In addition, the shape of the zone of inhibition exhibited by fraction C3 combined with the observation that the zone overlapped that exhibited by fraction C2, suggested that there were two active components present in this fraction. The second active component appeared to have an $R_f x 100 = 42$, the same as that seen in fraction C2. On repeat bioautographic analyses using smaller concentrations of fraction C3, it was observed that the activity was actually due to three components (results not shown). However, separation of these three components was difficult and therefore, three distinct zones of inhibition were not easily observed. Bioautography of fraction C3, which is discussed in the following section with regards to compound identification, showed zones of inhibition that suggested there were three active components present (Figure 7.6, in Section 7.3.3).

Fractions C5 and C6 were combined as they were observed to contain the same four components seen at UV-365 nm, a blue spot ($R_f x 100 = 25$), purple spot ($R_f x 100 = 22$), green spot ($R_f x 100 = 18$) and a dark purple spot ($R_f x 100 = 14$). The purple spots are difficult to see in the figure presented, as the colour is similar to the background colour of the TLC plate (Figure 7.5a). The component that fluoresceed blue at UV-365 nm ($R_f x 100 = 25$) was the only spot to quench fluorescence at UV-254 nm (Figure 7.5b) and exhibit a zone of inhibition (Figure 7.5c). A slight zone of inhibition, exhibited at an $R_f x 100 = 25$, was also observed in fraction C4, which suggested that some of this component was also contained in this fraction.

Fractions that appeared to contain more than one active component were subjected to different TLC solvent systems in an attempt to separate the active components (results not shown). This resulted in the active components moving further up the plate yielding larger R_f values, but the relative distance between the active spots did not change. This strongly suggested that some of the active components were very similar in structure. When components are similar in structure they can be very difficult to identify as the separation systems required become complex (Harbourne, 1973). However, as this investigation was only interested in the class of compounds present that exhibited activity rather than absolute identification of the compounds, complete separation of the

active components was not necessary. If complete identification of the active compounds were to be performed, further separation and purification using HPLC and other chromatographic techniques would be required. Preliminary identification using specific and universal detection reagents on TLC plates is discussed in the following section.

7.3.3 Detection of Classes of Compounds

Fractions B2, B3, B4, B5, C2, C3, C4 and C5/6 were selected for the determination of the classes of compounds of active components. These fractions were chosen as they covered all components that exhibited antibacterial activity in the final stages of the fractionation process. Fractions within the B series and C series were subjected to silica gel TLC using the mobile phase CHCl₃:MeOH (95:5) and CHCl₃:MeOH (90:10), respectively, as described in Section 3.12.2. R_fx100 values of the active components and in turn the corresponding colour reactions were determined and compared. A number of TLC plates were spotted with the fractions, developed, dried and then sprayed with different spray reagents to determine the presence of classes of compounds (Section 3.14). Bioautography was also performed on separate TLC plates as described in Section 3.13.5 and photos of plates are shown in Figure 7.6. Components that exhibited bioactivity and their corresponding colour reactions determined using each spray reagent are presented in Table 7.4.

Since there appear to be few reports on using spray reagents for the preliminary chemical identification of constituents in basidiomycetous fungi, the spray reagents chosen were those that have been used in the phytochemical identification of plant constituents (Ahmad & Beg, 2001; Ebi & Kamalu, 2001; van der Watt & Pretorius, 2001). Preliminary identification of active constituents in plants using TLC spray reagents has been performed for many years and only recently have the same techniques been used in the identification of constituents in *Ganoderma* species (Aryantha *et al.*, 2002). Spray reagents used have been listed in the Materials and Methods chapter (Section 3.14.1). In the case where a first spray reagent detected a certain class of compound, a second spray reagent was then used for confirmation.
Figure 7.6 displays the bioautography results of the B and C series fractions separated using the mobile phase CHCl₃:MeOH (95:5) and CHCl₃:MeOH (90:10), respectively. Three active components were observed in the B series at R_fx100 values of 55, 49 and 32. Another five components that exhibited activity were observed in the C series fractions at R_fx100 values of 60, 52, 42, 32 and 25. These eight active components were the focus of class identification using detection reagents. Separation of these active components was attempted using different solvent systems but did not prove successful, which suggested that some of the components were similar in structure. Nevertheless, for the purpose of class identification, this was not necessary as the active components had different R_fx100 values.



Figure 7.6. Bioautography agar overlay of the B series and C series fractions from the DCM extract. R_fx100 values are shown to the left of active components. (a) B series fractions (B2 to B5) separated by TLC using the mobile phase CHCl₃:MeOH (95:5) and (b) C series fractions (C2 to C5) separated using the mobile phase CHCl3:MeOH (90:10); Note that R_fx100 values cannot be compared between plates since they were developed in different solvent systems.

Table 7.4 shows R_fx100 values and the corresponding colour reaction for the eight components that exhibited antibacterial activity. For ease of discussion, the active components in the B series that exhibited R_fx100 values of 55, 49 and 32 have been numbered 1, 2 and 3, respectively, and active components in the C series exhibiting R_fx100 values of 60, 52, 42, 32 and 25 have been numbered 4, 5, 6, 7 and 8, respectively. In addition, rather than separately discussing the results of each spray reagent reaction for the different active components, the different classes of compounds

have been grouped and discussed. This enabled the identification of the classes of compounds to be performed by taking into account all the results obtained from the detection reagents, as well as from observations at UV-365 or UV-254 nm.

Identification of Active Components in DCM Fractions											
	B series ^a			C series ^b							
Component	1	2	3	4	5	6	7	8			
R _f x100	55	49	32	60	52	42	32	25			
UV-254 nm	-	Q	Q	Q	Q	Q	Q	Q			
UV-365 nm	-	-	-	-	blue	blue arch	blue	green			
Iodine ^c	√	√	~	\checkmark	✓	\checkmark	✓	\checkmark			
AS	green red*	purple	purple	blue	purple	purple	purple	purple			
VS	violet brown*	pink orange*	violet brown*	purple _*	purple	violet- pink	purple	blue			
LBr	blue orange*	✓ white*	blue orange*	✓ white*	green white*	blue orange*	green white*	✓ white*			
BB	\checkmark	-	\checkmark	-	✓	\checkmark	\checkmark	-			
Folins	blue	-	-	-	blue	blue	blue	-			
FBS	brown	-	brown	-	brown	brown	brown	-			
NP-PEG	-	-	-	-	-	-	-	-			
КОН	-	-	-	-	-	-	-	-			
AlCl ₃	-	-	-	-	-	-	-	-			
Dragendorff	-	-	-	-	-	-	-	-			
Kedde	-	-	-	-	-	-	-	-			
Fluorescein	-	-	-	-	-	-	-	-			

Table 7.4 Spray reagent detection of active components in the DCM extract

^a Components in the B series fractions were separated using the mobile phase CHCl₃:MeOH (95:5); ^b Components in the C series fractions were separated using the mobile phase CHCl₃:MeOH (90:10); Q, quenching of fluorescence; Reaction colours noted were observed in visible light; * Colour observed at UV-365 nm; ^c When exposed to iodine vapour, yellow zones were observed if the component contained conjugated double bonds; -, no change or colour reaction; Detection reagents (Section 3.14.1): AS, Anisaldehyde-sulphuric acid; VS, vanillin sulphuric acid; LBr, Leibermann-Burchard; BB, Berlin blue; FBS, Fast blue salt reagent; NP-PEG, natural products - polyethylene glycol.

7.3.3.1 Alkaloids and Lipids

The appearance of yellow/brown spots in visible light immediately after spraying with Dragendorff's reagent indicates the presence of alkaloids (Wagner *et al.*, 1984). The appearance of yellow zones after spraying with 0.01% (v/v) fluorescein indicates the presence of lipids (Harbourne, 1973; Krebs *et al.*, 1969). Neither of these colours was observed after spraying with the two reagents (Table 7.4), thus eliminating alkaloid and lipid type compounds as the active constituents. The largest, most intense spot referred

to earlier on in the fractionation process (Section 7.3.1), however, did not exhibit antibactierial activity, was determined to be an alkaloid type compound. This spot appeared yellow/orange in visible light after spraying with Dragendorff's reagent, as well as fluoresced bright blue at UV-365 nm (results not shown), which is indicative of alkaloids (Harbourne, 1973; Wagner *et al.*, 1984).

7.3.3.2 Phenols

A number of detection reagents have been developed that can detect phenolic compounds. Such detection includes the presence of blue spots in visible light after spraying with Folins reagent and the appearance of red-brown zones in visible light after spraying with Fast Blue Salt reagent (FBS) (Wagner *et al.*, 1984), or the appearance of intense green, purple, blue or black colour in visible light after spraying with Berlin Blue (BB) reagent (Harbourne, 1973). The majority of the active constituents in the fractions gave positive colour reactions with these spray reagents, indicating the presence of phenolics (Table 7.4). However, components no. 2, 4 and 8 (R_fx100 = 49, 60 and 25, respectively) did not react with any of these reagents (Table 7.4), which suggested that these compounds were not phenolic structures or structures that contained phenolic units.

Phenolic compounds absorb in short UV wavelength, which is observed as dark spots on the plates (Harbourne, 1973). Although components 2 and 3 ($R_fx100 = 49$ and 32, respectively) quenched fluorescence at UV-254 nm, other structures are also known to cause this effect. Components 4 and 8 also quenched fluorescence at UV-254 nm (results not shown) and, as previously observed, did not have any positive phenolic reactions. Components 5, 6 and 7 ($R_fx100 = 52$, 42, 32, respectively) were observed to quench fluorescence at UV-254 nm (Table 7.4). They also exhibited positive reactions with all the phenolic detection reagents, suggesting that they were phenolic structures or contained phenolic units.

7.3.3.3 Flavonoids and Coumarins

Coumarins and flavonoids are phenolic structures (Cowan, 1999) and have been shown to exhibit antibacterial activity from plants (Ahmad & Beg, 2001; Nostro *et al.*, 2000). However, there appear to be no reports of fungal flavonoids or coumarins exhibiting

antibacterial activity. This was also the case in this investigation. The active components did not react with either KOH or AlCl₃, indicating that they were neither coumarin nor flavonoid type compounds (Table 7.4). This was confirmed using the spray reagents NP-PEG, which intensifies the fluorescence of native coumarins at UV-365 nm, and FBS, which reacts with flavonoids to produce blue to blue violet zones in visible light (Table 7.4) (Krebs *et al.*, 1969; Wagner *et al.*, 1984).

A number of coumarins and flavonoids were observed in earlier fractions of the crude DCM extract, as indicated by their fluorescence of bright blue, blue, purple and green at UV-365 nm (Harbourne, 1973). However, none of these compounds exhibited antibacterial activity in earlier assays and thus were not included at this stage of the investigation.

7.3.3.4 Anthracene Derivatives

KOH and NP-PEG are also used for the detection of anthracene derivatives. When the TLC plate is sprayed with KOH, anthraquinones appear red in visible light and exhibit red fluorescence in UV-365 nm, whilst anthrones and anthronols appear yellow in visible light and exhibit yellow fluorescence in UV-365 nm (Wagner *et al.*, 1984). When NP-PEG is used as the detection reagent, anthracene derivatives give intense yellow fluorescence at UV-365 nm (Wagner *et al.*, 1984). No active components yielded these colours when these spray reagents were applied, indicating that they were not derivatives of anthracene (Table7.4). In addition, there was no yellow or red to brown fluorescence exhibited by the active components at UV-365 nm, which also indicated they were not anthracene derivatives (Wagner *et al.*, 1984).

7.3.3.5 Terpenoids

Non-specific detection of terpenoids can be performed by spraying the plate with vanillin-sulphuric acid reagent (VS), anisaldehyde-sulphuric acid reagent (AS) or Liebermann-Burchard reagent (LB), heating the plate at 100°C for 5 to 10 minutes and then observing for colours in visible light (Wagner *et al.*, 1984). All active components exhibited distinct colours, ranging from violet/purple to blue to green, with these detection reagents, indicating the presence of terpenoids (Table 7.4). The colour

reactions with these reagents were strong, suggesting that the active components were terpenoid type.

Natural terpenoids have cyclic structures with one or more functional groups (e.g. hydroxyl, carbonyl) (Harbourne, 1973). They have conjugated double bond systems that will appear as yellow zones in visible light when exposed to iodine vapour (Wagner *et al.*, 1984). All active components displayed this reaction when exposed to the vapour, confirming that they were compounds that contained conjugated double bonds (Table 7.4). This confirmed the positive reactions obtained with the terpenoid universal detection reagents, including VS, As and LBr reagents (Table 7.4). Phenolic units can also be encountered in terpenoids (as a functional group) (Harbourne, 1973) and may explain why there were positive reactions with some of the detection reagents that detect phenolic compounds. In addition, the colour reactions observed for phenolics were less intense than those for terpenoids, which suggested that they were a substituent attached to the main terpenoid compound.

Terpenoids encompass a number of compounds including: volatile mono- and sesquiterpenes (C15) (essential oils) through to the less volatile diterpenes (C20) to the involatile triterpenoids and sterols (C30) and carotenoid pigments (C40) (Harbourne, 1973). Unfortunately there is no sensitive universal reagent for the detecting classes of terpenoids (Harbourne, 1973). Thus, differentiation of the different types of terpenoids is generally impossible by using TLC spray reagents.

Triterpenes have been detected in *Ganoderma* by spraying with LBr reagent and observing for fluorescence at UV-365 nm (Aryantha *et al.*, 2002). In this investigation, all the active components fluoresced white or orange at UV-365 nm after spraying with this detection reagent, which suggested the active components were triterpenes. In addition, when sprayed with the same reagent and viewed in visible light, triterpenoids and sterols appear a blue to green colour (Harbourne, 1973), which was also observed for components 1, 3, 5, 6 and 7 (Table 7.4). However, further testing would need to be performed to confirm this, which was beyond the scope of this investigation. An interesting observation, however, is that most of the pharmacologically active

compounds isolated from *Ganoderma* species have been triterpenoids (Chairul *et al.*, 1991; Gao *et al.*, 2002a; Kimura *et al.*, 2002; Wu *et al.*, 2001).

Two types of triterpenes that can be specifically detected are cardiac glycosides and saponins (Harbourne, 1973; Wagner *et al.*, 1984). Cardiac glycosides are detected by the appearance of pink zones in visible light after spraying with Kedde reagent (Harbourne, 1973). When TLC plates containing active fractions were sprayed with this reagent some of the compounds turned pink (results not shown). However, none of these pink spots correlated with those of the active components, indicating that the active components were not cardiac glycosides. There is no specific detection of saponins using spray reagents, but they have soap like properties and are generally observed if the solution that contains the extract foams (Harbourne, 1973). Foaming of the extract did not occur, which eliminated the presence of saponins. However, component 1 did not fluoresce at UV-365 nm or quench fluorescence at UV-254 nm (Table 7.4), which are properties associated with saponins (Harbourne, 1973).

7.3.3.6 Preliminary Identification of the Active Components

Overall, the results indicated that the active components were terpenoid type compounds, some with phenolic groups attached. A summary of the results are displayed in Table 7.5, which shows that all components were terpenoids type structures containing conjugated bond systems. Components 1, 3, 5, 6 and 7 also contained phenolic groups attached to the main terpenoid structure. Compounds that have similar structures tend to migrate close to each other using TLC, making them difficult to separate (Harbourne, 1973). Thus, the preliminary identification of all compounds into the same class of compounds may explain why there was difficulty in separating the active components in the fractionation processes (Section 7.3.2).

There were other characteristics of the extract and resulting fractions that supported the classification of the active components into the class of terpenoids. Such factors include: 1) Terpenoids are lipid soluble and often difficult to characterise on a microscale level since all (except carotenoids) are colourless (Harbourne, 1973). This was the case in this investigation, as the fractionation process proceeded the active fractions became clear and there was no detection in visible or UV light; and, 2)

Terpenoids are generally extracted from plants by light petroleum, ether or chloroform and can be separated on silica gel chromatography using the same solvents (Cowan, 1999; Harbourne, 1973). The DCM extract in this investigation exhibited the strongest antibacterial activity and partial separation of components in the extract was performed on silica gel using similar solvents.

Identification of Active Components in DCM Fractions											
	B series ^a			C series ^b							
Component	1	2	3	4	5	6	7	8			
R _f x100	55	49	32	60	52	42	32	25			
UV-254 nm	-	+	+	+	+	+	+	+			
UV-365 nm	-	-	-	-	+	+	+	+			
CONJUGATED DOUBLE	+	+	+	+	+	+	+	+			
BONDS											
ALKALOIDS	-	-	-	-	-	-	-	-			
LIPIDS	-	-	-	-	-	-	-	-			
TERPENOIDS	+	+	+	+	+	+	+	+			
Saponins	-	-	-	-	-	-	-	-			
Cardiac glycosides	-	-	-	-	-	-	-	-			
PHENOLICS	+	-	+	-	+	+	+	-			
Coumarins	-	-	-	-	-	-	-	-			
Flavonoids	-	-	-	-	-	-	-	-			

 Table 7.5

 Summary of the class of compounds for the active components present in the DCM extract

^a Components in the B series fractions were separated using the mobile phase CHCl₃:MeOH (95:5);

^b Components in the C series fractions were separated using the mobile phase CHCl₃:MeOH (90:10); +, positive detection; -, not detected;

There have been reports of volatile oils and sesquiterpenes from plants that have been shown to exhibit antibacterial activity (Anke & Sterner, 1991; Dorman & Deans, 2000). Sesquiterpenes from the mycelial culture of two basidiomycetous fungi, *Flammulina velutipes* and *Resupinatus leightonii*, have also been shown to exhibit antibacterial activity (Eilbert *et al.*, 2000; Ishikawa *et al.*, 2001). However, the likelihood that the active components were mono-terpenoids or sesquiterpenoids (essential oils) was very low, as preliminary analysis of the active fractions by GC-MS showed no volatile compounds (results not shown). Since, essential oils are highly volatile (Dorman & Deans, 2000; Harbourne, 1973) they should be relatively easy to detect by GC-MS. In addition, there have been no reports on the isolation of sesquiterpenes from *Ganoderma* species (Mothana *et al.*, 2000).

Diterpenes and triterpenes are found to exist in the resins and barks of trees or the latex of herbaceous plants (Harbourne, 1973). *Ganoderma cupreum* is known to be a woody fungus with a latex context (Smith & Sivasithamparam, 2003) and therefore might exude these types of terpenoids. They generally have a protective function in nature (Harbourne, 1973), and studies have shown that various diterpenes and triterpenes extracted from other sources have exhibited antibacterial activities (Pinducciu *et al.*, 1995; Savluchinske Feio *et al.*, 1999; Wilkens *et al.*, 2002). In addition, it has been reported that there is no single TLC system available for their resolution, and they are not volatile (Harbourne, 1973).

The main constituents isolated from *Ganoderma* species have been triterpenoids, which include steroidal compounds (Chen & Chen, 2003; Kleinwachter *et al.*, 2001; Rosecke & Konig, 2000; Su *et al.*, 2000, 2001). Many of these compounds have been shown to possess antitumour activity (Gan *et al.*, 1998a; Lin *et al.*, 1991; Wu *et al.*, 2001), as well as antioxidant properties (Zhu *et al.*, 1999). However, there appear to be only a few investigations on the antibacterial properties of such compounds (Li *et al.*, 2000; Mothana *et al.*, 2000; Smania *et al.*, 1999).

Overall, the active constituents were determined to be in the class of terpenoids, and most likely to be triterpenes, given the number of triterpenes from *Ganoderma* species with reported biological activity (Chairul *et al.*, 1991; El-Mekkawy *et al.*, 1998; Giner-Larza *et al.*, 2000; Kimura *et al.*, 2002). However, to confirm this, further studies would need to be performed. To determine the structures, the fractions would need to be further purified by HPLC and identified using other spectroscopic techniques such as NMR, GC-MS, UV and IR. This was beyond the scope of this investigation as the primary purpose of this investigation was only to identify the class of compound. Therefore, this chapter reported the preliminary identification of some of the major active components found in the DCM extract from *Ganoderma cupreum*, and is one of the first accounts of *Ganoderma* terpenoid compounds exhibiting antibacterial activity.

7.4 Summary

Putative chemical identification (class of compounds) of antibacterial components in the DCM extract from the mycelium of an Australian *Ganoderma* isolate was performed.

This was achieved using a bioassay-guided fractionation process, which utilised TLC and an agar-overlay bioautographic technique. Only the components that exhibited antibacterial activity were of interest. Detection of the components was based on fluorescence at UV-365 nm or UV absorption at UV-254 nm, as well as the use of selective or universal chemical spray reagents.

TLC was chosen as the means for analysis as it is a simple, fast and reproducible means of detecting constituents in a crude extract (Hostettmann, 1999). Initial analysis by TLC determined there to be eighteen components present in the crude DCM extract (Section 7.3.1). Bioautographic analysis of these components indicated that the antibacterial activity was restricted to the less polar components in the extract (Figure 7.1). The number of biologically active components could not be determined in initial stages due to the low concentration of the components exhibiting activity. After further purification, it was observed that there were more than eighteen components present in low concentrations, they were only detected in the later stages of the fractionation and purification process. However, since only the biologically active components were of interest, the absolute number of components in the extract was not considered to be of high importance.

Partial purification of the extract was performed by silica gel column chromatography and a stepwise gradient, beginning with CHCl3:MeOH (99:1) and ending with CHCl₃:MeOH (2:8). The first purification yielded thirty fractions and the fractions that contained like compounds were combined to make seven final separate fractions from the first purification (A series) (Figure 7.2). Analysis of the fractions for antibacterial activity demonstrated that four of these, A2, A3, A4 and A6, exhibited activity (results not shown). Fractions A2, A3 and A4, showed strong activity and were subjected to a second purification step.

A second purification using the same system was performed on fractions A2, A3 and A4 (Section 7.3.2.2). This led to eleven further fractions, of which eight fractions, B2 to B5 and C2 to C5, had components that displayed antibacterial activity (Figure 7.4 and Figure 7.5). The active components within these fractions were then separated and

analysed by TLC using two solvent systems. Fractions B2 to B5 were separated using $CHCl_3$:MeOH (95:5) and fractions C2 to C4 were separated using $CHCl_3$:MeOH (90:10). Bioautographic analysis of the eight fractions yielded eight active components (Figure 7.6).

Finally, a number of TLC spray reagents were used to identify the chemical class of active compounds (Section 7.3.3). These reagents, combined with the localisation of the active components in the bioautography assay, determined that the eight active components were terpenoids. In addition, five of the active components were determined to be terpenoid type compounds with phenolic groups (Table 7.4 and Table 7.5). This is one of the first accounts of the isolation of antibacterial terpenoid type compounds from the mycelium of *Ganoderma cupreum*.

Chapter Eight



8.1 Identification of Three Australian *Ganoderma* Isolates

Three Australian *Ganoderma* species, which had been isolated from the Cairns State Forest by a local mushroom cultivator, were confirmed to be from the Family Ganodermataceae. Using molecular tools and evolutionary studies, the three species were identified to be *Ganoderma cupreum* (H1), *Ganoderma weberianum* (H2) and *Ganoderma* species (H3) (Section 4.3.3.4). The third isolate, *Ganoderma* H3, could not be classified to a particular species using molecular methods, as the available sequence data in the *Ganoderma* database did not contain sufficiently closely related sequences to allow identification. However, traditional methods have identified this isolate as an Australian *G. boninense* (personal communication, Dr. Brendan J. Smith, CSIRO, Canberra, ACT, Australia). The speciation of this *Ganoderma* will require additional sequence data of Australian *G. boninense* isolates.

With reports suggesting that many *Ganoderma* species have been misnamed (Hseu & Wang, 1991; Ryvarden, 1991), the correct identification of the three Australian *Ganoderma* isolates was an important first step in this investigation. The nucleotide sequence data (entire ITS I to ITS II region) for the three species were given accession numbers and submitted for future reference into GenBank, a universal sequence data bank that is accessible worldwide. Accession numbers are: *Ganoderma cupreum* (H1), AY569450; *Ganoderma weberianum* (H2) AY569451, and; *Ganoderma species* (H3), AY569452. This is a major contribution to the database on *Ganoderma* species, particularly from the Asia-Pacific region, and will ultimately aid in establishing a universal classification system for *Ganoderma*. In addition, this will aid in the clarification of misidentified species and enable correct identification of new species in the future.

8.2 Storage and Growth of Three Australian *Ganoderma* Isolates

Long term and short term storage conditions of the three *Ganoderma* isolates were identified. The importance of suitable long term storage conditions was so the fungi would remain viable throughout this investigation, as well as for future reference and use. The most suitable method determined for the long term storage of the isolates was on balsa wood chips, stored at -80° C for periods of up to 2 years without subculture (Section 5.3.1.1). However, long term storage resulted in decreased growth after

subculture of both *Ganoderma* H1 and H3. Short-term storage, which was investigated to allow for rapid subculture throughout this investigation, was determined to be best on agar slants up to a maximum of eight weeks for the three isolates (Section 5.3.1.2). In addition, it was important to store *Ganoderma* species H1 and H3 away from direct light, as light was observed to have a detrimental effect on the viability of the mycelium. The viability studies highlighted that closely related species can require different storage conditions and the importance of determining appropriate conditions for the survival of each fungus.

The optimal temperature and pH for the mycelial growth of the three isolates were determined to be 30°C and an initial medium pH of 6 (Section 5.3.2.1). This is similar to what has been reported for other *Ganoderma* species (Yang & Liau, 1998) and agrees with the observation that *Ganoderma* species are generally isolated from tropical regions (Moncalvo & Ryvarden, 1998). However, the growth behaviour generally varied between the three isolates. The temperature range over which the fungi grew (Section 5.3.2.1) as well as their growth rates (Section 5.3.2.2) were different, again highlighting that there can be differences between closely related species.

A liquid growth medium, which contained phosphates, organic nitrates and minerals, was found to support the best mycelial growth of the three Australian isolates (Section 5.3.2.3) and the carbohydrate source within this medium affected mycelial biomass production (Section 5.3.2.4). It was observed that glucose supported the greatest growth of both *G. cupreum* (H1) and *G. weberianum* (H2), and galactose and fructose supported the greatest growth of *Ganoderma* species (H3) (Section 5.3.2.4). These observations were generally consistent with other reports that have noted that different species can utilise different nutrient sources (Griffin, 1994; Sone *et al.*, 1985; Tang & Zhong, 2002).

Some important growth factors, including pH, temperature and carbohydrate source, for the three isolates were identified. This enabled the rapid production of the mycelial biomass to produce sufficient quantities for further investigation of the antibacterial activities of the mycelium. These growth conditions provided evidence that fungi from different regions around the world may require different growth conditions as well as the notion that different species from within the same genus may also require different growth factors. In addition, these growth conditions have added to the wealth of information currently available on the growth and cultivation of *Ganoderma*, but more importantly contributed to information on the specific species, *G. cupreum* (H1), *G. weberianum* (H2), and possibly *G. boninense* (H3).

8.3 Antibacterial Properties

The antibacterial activity of the mycelium from the three Australian *Ganoderma* isolates was investigated. Two extraction processes were employed, a hot water and organic extraction (Chapter 6). Preliminary results indicated that both the aqueous and organic extracts from *G. cupreum* (H1) possessed activity against a greater range of bacteria than the other *Ganoderma* isolates, and thus, *Ganoderma* H1 was used for a more indepth investigation. Hot water extracts exhibited activity against some Gram positive bacteria (Section 6.3.1.2). However, organic solvent extracts exhibited antibacterial activity against a greater range of Gram positive bacteria (Section 6.3.1.4) of which the DCM extract appeared to have the strongest antibacterial effect. Other reports have also shown chlorinated solvent extracts from different *Ganoderma* species to possess biological activity towards Gram positive bacteria (Mothana *et al.*, 2003; Smania *et al.*, 1999). Activity was also observed for the HEX and EtOAc extracts, but this activity was limited to one or two bacteria for extracts from mycelium cultivated in PDB. No activity was exhibited against the Gram negative bacteria for any of the extracts.

The medium that supported the greatest mycelial growth of *G. cupreum* (H1) (Basal-G) also yielded extracts with the greatest antibacterial activity (Section 6.3.2.2), thus, the selection of this growth medium for further investigations of *G. cupreum* (H1). The HEX, DCM and EtOAc extracts from this fungus exhibited antibacterial activity towards some Gram positive aerobic/aerotolerant bacteria, *B. cereus, B. subtilis, E. faecalis, S. pyogenes, S. aureus S. epidermidis* and *L. monocytogenes*, as well as some *Clostridium* species, *C. perfringens, C. sporogenes*. In particular, the antibacterial activity exhibited against a number of clinically important pathogens, *Clostridium difficile*, and MRSAs, was of great significance, as these bacteria are among those that are reported to have resistance to a number of currently available antibiotics (Lipsitch & Samore, 2002; Swartz, 2000).

The effective concentrations of the most active organic extracts were investigated by means of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays (Section 6.3.2.6). HEX extracts were not investigated further due to their insolubility in the broth medium cultures. It was observed that a lower concentration was required to kill the *Clostridium* isolates than the other bacteria, indicating that the anaerobic bacteria were more susceptible to the extracts (Section 6.3.2.6). The MIC and MBC values (Table 6.11) along with the concentration dose graphs (Figure 6.8a-w) indicated that the inhibitory action exhibited by the extract was concentration dependent and that the concentration would depend on the target bacteria. Time course growth assays confirmed the bacteriostatic or bactericidal activity of the extracts against the different test organisms. The DCM and EtOAc extracts were predominantly bactericidal against the range of bacteria tested (Figure 6.9 and 6.10). However, this was highly dependent on the concentration of the extract and the target organism.

This is the first report of antibacterial activity of extracts from the liquid cultivated mycelium of *Ganoderma*.

8.4 Identification of Active Components

Preliminary identification of the components that exhibited antibacterial activity from the DCM extract was carried out (Chapter 7). Bioassay guided fractionation partially purified more than one active compound that was present in the DCM crude extract (Section 7.3.2). These active compounds were determined to be terpenoid in structure, some with phenolic groups attached. This preliminary identification is in general consensus with reports in the literature that triterpenes (a subclass of terpenoids) are a major group of constituents isolated from *Ganoderma* that have been shown to possesses biological activity (Kim & Kim, 1999a; Kimura *et al.*, 2002; Mizuno *et al.*, 1995c; Wu *et al.*, 2001).

It is most likely that the active compounds isolated from this Australian *Ganoderma* species are triterpenoid type compounds. However, until further structure elucidation is performed, one can only speculate as to the type of terpenoid present. To identify the exact type of compound, further work is required which would involve complete

purification of the active compounds and further analysis using chromatographic techniques. However, this is beyond the scope of this investigation.

This is the first account of the isolation of antibacterial terpenoid type compounds from the mycelium of *Ganoderma cupreum*.

8.5 Close

This investigation provided an insight to the growth and activity of three Australian *Ganoderma* species. It demonstrated the importance of establishing viability and culture conditions for new isolates. In addition, it demonstrated that liquid cultivated mycelium is a valuable source of bioactive compounds, and with this comes the possibilities of culture medium manipulation to increase bioactivity or bioactive substance production.

Finally, the findings from this investigation are particularly interesting from a commercial aspect. Potential possibilities exist to establish research and development of bioactive metabolites from 'indigenous' *Ganoderma* species. Consequently, the need to comply with Australian Quarantine laws is not warranted, as the *Ganoderma* species that could be used in the production of such metabolites could be taken from the those already found naturally within the Australia environment.

Glossary

All glossary definitions are taken from the Oxford Dictionary of Biochemical and Molecular Biology (Anonymous, 1997), unless otherwise stated.

Agglutinate to cause the clumping of cells, particles, etc, or to undergo such clumping.

- Angiogenesis the formation of blood vessels, whether during embryogenesis, tissue repair, or invasive growth of tumours.
- Antibacterial any of numerous substances produced by living microorganisms (and some plants) that are able to selectively and at low concentrations to destroy or inhibit the growth of bacterial organisms.
- **Antibiotic** any of numerous substances produced by living microorganisms (and some plants) that are able to selectively and at low concentrations to destroy or inhibit the growth of other organisms.
- **Antibody** any **glycoprotein** that is capable of combining noncovalentely, reversibly and in a specific manner with a corresponding **antigen**.
- Anticomplement see *adj* complement
- **Antifungal** any of numerous substances produced by living microorganisms (and some plants) that are able to selectively and at low concentrations to destroy or inhibit the growth of fungal organisms.
- Antiherpetic any agent having the ability to destroy or inhibit the herpes virus.
- Antigen any agent that stimulates the production of a specific **antibody** or antibodies that can combine with the antigen.
- Antihistamine any drug or other agent that antagonises an action of histamine on the body. Antihistamines are used in the treatment of immediate type hypersensitivity.
- **Antiinflammatory** any drug or other agent that is able to inhibit the natural defence reaction (inflammation) of vertebrate tissue to infection or to injury.
- Antimetatstatic see *adj* metastasis.
- Antimicrobial describing a drug, antibiotic agent, physical process, radiation, etc. that is inimical to microbes. Any substance having antimicrobial properties.
- Antinociceptive see *adj* nociceptive.
- Antioxidant any substance (often an organic compound) that opposes oxidation or inhibits reactions brought about by dioxygen or peroxides
- Antithrombotic any substance that has the ability to inhibit the process of thrombus

Antiviral activity see *adj* virus.

- **Apoptosis** cell death that may either occur by accident, cell necrosis, or by an intracellular controlled process characterised by a condensation, and subsequent fragmentation of the nucleus during which the plasma membrane remains intact.
- Atheroma a condition or process affecting blood vessels in which plaques form beneath the inner lining
- Atherosclerosis a degenerative condition affecting arteries in which there is **hyperplasia** of the outer coat and fatty degeneration of the middle coat of the arteries due to **atheroma**.
- **ATPase** *abbr. for* adenosine triphosphate. A universally important coenzyme an enzyme regulator. Reactions in which is participates are often driven in the direction to hydrolysis of ATP.
- Basidiocarp the fruiting body (mushroom cap) of a fungus
- **Bioinformatics** the science concerned with the structure and properties of biological scientific information
- **Biological activity** any activity of a substance that is demonstratable in living organisms.
- **Biological response modifiers** *abbr*. **BRM** any substance that has the ability to modulate the host's biological response by a stimulation of the immune system that may result in various therapeutic effects (Zhou & Gao, 2002).
- Cancer any malignant neoplasm.
- **Carcinogenesis** the process(es) involved in the production of **cancers**, including the action of **carcinogens** on living cells.
- **Carcinogen** any agent that directly or indirectly induces the transformation of a normal cell into a **neoplastic** cell.
- **Cell mediated immunity** specific immunity that depends on the presence of **T lymphocytes**. It is important in the organism's defence against viral and some bacterial infections.
- **Clade** a monophyletic taxon. A group of organisms or genes that contains that include the most recent common ancestor of all its members (Brinkman & Leipe, 2001).
- **Cladistics** a method of classification that attempts to reconstruct a **phylogeny** using characters that are unique to each taxonomic group. The term **cladistic** is currently used to describe an evolutionary or phylogenetic tree based on gene sequence similarities and gene association.
- **Cloning vector** the DNA of any transmissible agent into which a segment of foreign DNA can be spliced in order to introduce the foreign DNA into cells of the agent's normal host and promote its replication and transcription therein.
- **Complement** a system of plasma proteins, found in the blood of vertebrates that acts as an effector mechanism in immune defence against infection by microorganisms. The activation products of complement components cause lysis of antigenic cells, attract phagocytic cells to the site of activation, and assist the uptake and destruction of antigenic cells by phagocytes.

- **Concanavalin** A *abbr. Con* A an agglutinating and mitogenic protein that constitutes the major component of the subsidiary globulin fraction obtained from the ripe seeds of the jack bean.
- **Cutex** the outer, shiniest layer of the fruiting body. Containing an outer layer. Generally referred to as *laccate*.
- **Cytokine** any of a varied group of proteins that are released by mammalian cells and act on other cells through specific receptors. They elicit from the target cell a variety of responses depending on the cytokine and target cell.
- Cytotoxic causing cell death.
- Cytotoxicity the attribute of being cytotoxic
- Deoxyribonuclease I an enzyme that catalyses the endonucleolytic cleavage of DNA.
- **DNA polymerase (\beta and \alpha)** common name for either two of categories of enzymes that catalyse the synthesis of DNA from deoxyribonuclease triphosphates in the presence of nucleic acid primer.
- **Endonuclease** any enzyme from a large group of phosphoric diester hydrolases, forming sub-classes, that catalyse the hydrolysis of nonterminal diester linkages in polynucleotides to yield **oligonucleotides**.
- Extracellular present outside a cell, expelled from a cell or happening outside a cell.
- **Fruiting body** the fruit of the mushroom, visible to the naked eye. Other names include: conk, mushroom, basidiocarp.
- **Glycoprotein** any protein that contains covalently bound glucose residues other than as a moiety of nucleic acid.
- Haemagglutination the agglutination of red blood cells.
- Haemophiliac having any of various hereditary disorders in which there is a deficiency or defect in certain of the blood coagulation factors resulting in prolonged bleeding following injury
- Haemostasis the stopping of bleeding or the arrest of the circulation to an organ or part
- Hepato+ denoting or pertaining to the liver.
- **Hepatoprotective activity** the ability of a substance to have protective abilities toward the liver.
- **Histamine** a compound present in many mammalian tissues. It is a potent vasodilator, increases capillary permeability, causes contraction of smooth muscle, plays a role in the regulation of gastric secretion and acts as a mediator in allergic and anaphylactic conditions.
- **Hypercholesterolaemia** the presence of higher than normal concentrations of cholesterol in the blood.
- **Hyperplasia** the increase in size of a tissue or organ resulting from an increase in the total number of cells present. The part thus affected retains its normal form.
- **Hypersensitive** having an abnormally great sensitivity, especially to an allergen, drug or other antagonist.

Hypersensitivity the state or condition of being hypersensitive.

- **Hypertension** the state or condition of having a higher than normal arterial blood pressure.
- Hypoglycaemea the presence of an abnormally low blood glucose concentration.
- Hypoglycaemic having abnormally low blood glucose concentration.
- **Hypolipidemic activity** substances that have the ability to inhibit cholesterol biosynthesis and cholesterol.
- **Hypotension** the state or condition of having a lower than normal arterial blood pressure. *Hypotensive* adj.
- **IFN-***γ abbr. for* **interferon**. Suffixed Greek letters are added to differentiate the main classes.
- Immuno+ indicating immune or immunity.
- Immunoinitiators see *biological response modifier* (Zhou & Gao, 2002)
- **Immunomodulator** any agent that alters the extent of the immune response to an antigen, by altering the antigenicity of the **antigen** or by altering in a nonspecific manner the specific reactivity of the non-specific effector mechanism of the host. *Immunomodulatory* adj.
- **Immunostimulant** any agent that non-specifically enhances the immunologically specific reactivity of an animal to an antigen and also the animal's non-specific effector mechanisms. *Immunostimulatory adj.*
- Immunosuppression the suppression of immune responses to antigens.
- Immunosuppressive able to cause immunosuppression.
- **Interferon** any member of a group of proteins that form a closely related group to nonviral proteins that are produced and liberated by animal cells following exposure to a variety of inducing agents. They are not normally present in noninduced cells. They exert non-specific antiviral activity through cellular metabolic processes involving the synthesis of both RNA and protein.
- **Interleukin** any member of a heterogeneous group of **cytokines** that have the ability to act as signalling molecules between different populations of leukocytes. Several classes have been defined, each is designated by a suffixed Arabic numeral, e.g. *Interleukin 2*
- Interleukin 2 produced by T cells in response to antigenic or mitogenic stimulation.

Intracellular within a cell or cells.

Laccate see cutex

Leukocytes any white blood cell.

Ling Zhi Chinese name for Ganoderma lucidum.

- Lyophilic solvent preferring
- **Lypholisation** the process of rendering lyphile or lyophilic. The term is now specifically applied to freeze drying in which, by use of vacuum, water is removed from ice by sublimation.

- **Macrophage** any cell of the mononuclear phagocyte system that is characterised by its ability to phagocytose foreign particulate and colloidal material.
- **Metastasis** the transfer of disease, especially tumour cells, from one part of the body or another by way of the natural passages (i.e. blood vessels, lymphatics) or by direct continuity.

Mitogen any substance or agent that induces or stimulates mitosis.

Mitogenic activity having the ability to induce or stimulate mitosis.

Monophyletic derived from the same origin

Necrosis the death of a portion of tissue as a result of disease or injury.

Neutriceutical any substance that may be considered a food or part of a food and provides medical or health benefits including the prevention and treatment of disease.

Neoplasm any new and morbid formation of tissue; a tumour.

Nociceptor any receptor that reacts to painful stimulus.

Oligonucleotides any molecule that contains a small number of nucleotide units connected by phosphodiester linkages.

Oncogenic (of an agent) causing the formation of a neoplastic tumour or tumours

Phylogeny the evolutionary history of an organism or group of related organisms

Phylogram (phylogenetic tree) a graphical representation of the putative evolutionary relationships of a group of organisms

Platelets the smallest of the blood cells. The main function are in haemostasis.

- **Polypore** a fungus that is a member of the Polyporaceae Family. Produces spores in pores found on the underside of the mushroom. A typically tough, woody mushroom that is generally found on logs, stumps, or other dead wood (Zjawiony, 2004).
- **Promyelocytic leukaemia** the promotion of neoplastic diseases of the leukocytes within the bone marrow.
- **Recombinant DNA** a fragment of DNA that has been inserted into a cloning vector, thereby leading to its use in the isolation of a clone of cells characterised by the presence of the fragment.
- **Resistance** the ability of a living organism, particularly a bacterium, to resist the effect of a disadvantageous environment or substance, especially an antibiotic.
- **Restriction enzyme** any of a group of enzymes that cleaves molecules of DNA internally at specific base sequences. Alternative name *restriction endonuclease*.
- **Reverse transcriptase** a DNA polymerase enzyme, found particularly in retroviruses and possibly in normal animal cells that uses either DNA or RNA as a template.
- **RNA polymerase** an enzyme that utilises ATP, GTP, CTP and UTP to synthesise RNA from a DNA or RNA template
- Sarcoma a malignant tumour of connective tissue or of its derivatives. Sarcoma 180 is a commonly used tumour for *in vitro* experimentation.

Stipe the stem or stalk of the mushroom.

- **Terminal deoxynucleotidyl transferase** an enzyme commonly isolated from calf thymus that catalyses template-independent addition of deoxynucleoside phosphates to the 3'-OH group on a DNA fragment.
- Therapeutic effect an agent that has a beneficial effect on a substance.
- Thrombus a blood clot forming within a blood vessel.
- **T lymphocyte** any of a calls of lymphocytes that undergo maturation and differentiation in thymus, they are responsible for immune reactions involving cell-cell interaction.
- **TNF-\alpha abbr. (Tumour necrosis factor)** either of two structures and functionally related proteins. TNF- α is produced mainly by monocytes and macrophages, and is involved in the activation of a large array of cellular genes and also multiple signal transduction pathways, kinases and transcription factors.
- **Tumour** a mass of proliferating cells lacking to varying degrees, normal growth control.
- Virus a noncellular infective agent that is capable of reproducing only in an appropriate host cell.

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Appendix 1

~	1		azamaaamm						~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
G.	weberianum CBS 219.3	TCGAGTTTT-	-GACTGGGTT	GTAGC-TGGC	CITICCGAGGC	AT-GIGCACG	CCCTGCTCA-	TCCACTC-TA	CACCIGIGCA
G.	weberianum RSH 0821				• • • • • • • • • • •				
G.	weberianum CCRC 37081								
G .	weberianum DFP 8401								
G.	weberianum DFP 8405								
G.	weberianum DFP 4483								
G	SD SUT H2	-	-	-		-	-	_	
G	ap OFRT 8147 1		-	-		-		G -	
c.	CD OEDT 9647 1		-			-			
с. а	SP QFRI 8047.1	•••••					•••••		
G.	sp DAR /3//9							G	
G.	sp UWA 8						· · · · · · · · · · -	G	
G.	tsugae RSH J2		C						
G.	tsugae RSH 1109		C						
G.	tsugae RSH BLC		C					–	
G	teugae HMAS 60537		- C	-		-		-	
с.	cinongo ZUANC 1734								
G.	SINENSE ZHANG 1734								
G.	sinense RSH 0109					.CT			
G.	cupreum DFP 4336				A	T			
G.	cupreum QFRI 8678.1				A	T			
G.	cupreum DFP 3896				A	T			
G.	sp SUT H1				A	Т		–	
G	incregatum SE 3	с -	-	-		-		-	
с.	ingragatum DAD 72792	c							
G.	Inclassaculi DAR 73765								
G.	adspersum CBS 351.74				A			GC	
G.	adspersum IMI 157816				A			GC	
G .	<i>autrale</i> DAR 73781					.C			
G.	australe UWA 92	.G				.C			
G	australe IIWA 108	-	-	-		C-	-	-	
с.	australe DED 3384		-	_		C-			
с.	and CE 1 Domith								
ч. С	P OT I DOMILLI				• • • • • • • • • • •				
G.	sp SUT H3				· · · · · · · · · · · · · · · · · · ·	c			
F.	rosea JMM T92-10		-A.T		ATA	A	TG.T.T.A		
G.	weberianum CBS 219.3	CTTACTGTG-	GGTT-TCAAA	CGTCGTA-AA	GCGAGTCTCT	TTACCG	AGCT-TGTAG	AGCGGCGT	CTGT-GCCTG
G	weberianum RSH 0821	-	_	_			_		_
c.	Weberianum CCPC 27091		_				_		
с. а	weberranum CERC 57081	•••••							
G.	weberianum DFP 8401				· · · · G · · · · · ·				····
G.	weberianum DFP 8405				G	C			
G.	weberianum DFP 4483				G	CT.			
G.	sp SUT H2				G	C			
G.	sp QFRI 8147.1		CG.	TCG	A		GC.CG.	T	
G	SD OFRT 8647 1	_	C = G	TCG	A G C		G =C CG	-T	_
с.	ap DAR 73770			TCG	A		c c.cc.		
G.	SP DAR 73779			TCG	AGC		G=CG.		
G.	SP UWA 8	G	CG.	T.C	AGC		GC.CG.	T	
G.	tsugae RSH J2		CG.	TTGCGG	AC		GCG.	A-TA	
G.	<i>tsugae</i> RSH 1109	C	CG.	TTGCGG	AC		GCG.	A-TA	
G .	<i>tsugae</i> RSH BLC		CG.	TTGCGG	AC		GCG.	A-TA	
G.	tsugae HMAS 60537		CG.	TTGCGG	AC		GCG.	A-TA	
G	sinense ZHANG 1734	_	- A GGG	-C - G-G	G CTC	G G	-C GA		_
c.	cipence PSU 0109		-7 000	-C - G-G	G CTC	G	-C CA		
с. а	SINCIPE RSN 0105	•••••	A.000	-cg-g.	G.CIC		GA		
G.	cupreum DFP 4336		A.GG.	TCGG-G.	G.CTC	T.CG.G.	GA	T	
G.	cupreum QFRI 8678.1		A.GG.	TCGG-G.	G.CTC	T.CG.G.	GA	T	
G.	cupreum DFP 3896		A.GG.	TCGG-G.	G.CTC	T.CG.G.	GA	T	
G.	sp SUT H1		A.GG.	TCGG-G.	G.CTC	T.CG.G.	GA	T	
G.	incrassatum SE 3		TA.GGG	TCGG	AG.CTCG.	T.ATT	GC.	AC	TC
G.	incrassatum DAR 73783		TA.GGG	TCGG	A.G.CTCG.	T.ATT	G	AC	тт
<i>c</i>	adaparaum CBC 3E1 74		TA C C	TCC CC	7 7 C C CC	T (1) TT	c c	70	m m
с. а	adspersum CDS 551.74	•••••	IA.G.G	TCGCG	A.AG.C.CG.	ICAII	g	AC	
G.	adspersum IMI 157816		IA.G.G	ICGCG	A.AG.CICG.	ICAII	GG.	AC	11
G.	<i>autrale</i> DAR 73781		TA.GGG	TCGCG	AG.CTCG.	T.ATT	TG.	AC	ТТ
G.	<i>australe</i> UWA 92		TA.GGG	TCGCG	AG.CTCG.	T.ATT	TG.	AC	ΤΤ
G.	<i>australe</i> UWA 108		TA.GGG	TCGCG	AG.CTCG.	T.ATT	GG.	AC	ΤΤ
G.	australe DFP 3386		TA.GGG	TCGCG	AG.CTCG.	T.ATT	GG.	AC	ΤΤ
G.	sp SE 1 BSmith		AT.G.	TCGTG-G.	.TCTCG.	TCGTGA	TCGA		
G.	SD SUT H3		ATGG.	TTGG-T.	A		A		.C
5	rogen JMM T92-10	Ψ Ψ λ_	С – тт	TCA _ CA	7 7 TOT	T'A			77 T
± .	10500 0111 152 10	1		10A0A			0.1.101404		
a	unhamineum (DC 210 2								
G.	Weberranum CB5 219.5	CGIII-AICA	CAAACICIAI	AAA-GIAIIA	GAAIGIGIAI	IGCGAIGIAA	CGCAICIAIA	IA	
ч.	weberianum GCDG 20001		• • • • • • • • • • •		• • • • • • • • • • •	• • • • • • • • • • •	• • • • • • • • • • •	••	
ч. а	weberianulli CCRC 3/081		••••••			• • • • • • • • • • •	• • • • • • • • • • •	••	
G.	Weberianum DFP 8401		· · · · · · · · · · · · · · · · · · ·					••	
G.	weberianum DFP 8405			C.					
G.	weberianum DFP 4483		C					••	
G.	sp SUT H2			C.					
G.	sp QFRI 8147.1			C.		C			
G.	SD OFRI 8647.1			- C .		С			
G	gn DAR 73779	-		- C		с			
с.	ap IIIIA 9			· · · · · · · · · · · · · · · · · · ·		c		••	
G.	SP UWA 8					C		••	
G.	tsugae RSH J2			AC.	• • • • • • • • • • •	• • • • • • • • • • •	.A	••	
G.	<i>tsugae</i> RSH 1109			AC.			.A	••	
G.	tsugae RSH BLC			AC.			.A		
G.	tsugae HMAS 60537			AC			.A		
G	sinense ZHANG 1734	т т	A	C					
с. С	cinence RSH 0100		π						
д. С	CURROUM DED 4226		A					••	
G.	Cupreum DFP 4336		AT.	A				••	
G.	<i>cupreum</i> QFRI 8678.1	TA.	AT.	A				••	
G.	cupreum DFP 3896	TA.	AT.	A					
G.	sp SUT H1	T.CA.	AT.	A					
G	incrassatum SE 3			C					
 G	incrassatum DAP 73783	-		- C					
с.	adaparaum CDC 251 74	 T						••	
ч. С	adaparaum TMT 155016			· · · · ² · · · · · · ·				••	
G.	auspersum IMI 157816					• • • • • • • • • • •		••	
G.	autrale DAR 73781		C					• •	
G.	australe UWA 92		C						
G.	australe UWA 108		C						
G.									
	australe DFP 3386		C						
<i>G</i> .	australe DFP 3386 sp SE 1 BSmith	 т	C		т				
G. G	australe DFP 3386 sp SE 1 BSmith sp SUT H3	 Т	C A	····-	T			••	
G. G. F	australe DFP 3386 sp SE 1 BSmith sp SUT H3 rosea_IMM_T92-10		C A A	 AC.	Т т		·····································	· · · · · · · · · · · · · · · · · · ·	

Appendix 1A. ITS 1 region alignment of 33 taxa, dots are conserved bases, dashes are gaps.

0.	weberianum CCPC 37081	AATCTTCAAC	TT-ACCCACC	TTTCCC	CCTTTT-CTA	-GGCTTGGAC	TT-CCACCCT	TGTCGGTC	GTGTTTC
G.	weberianum CBS 219.3						N	N.	
G.	weberianum RSH 0821							N.	
G.	weberianum DFP 8401		A		C			.TC.	
G.	weberianum DFP 4483		A		C			.TTC.	
G.	weberianum DFP 8405		A		C			C.	
G.	sp SUT H2		A		NT			C.	
G.	sp UWA 8		CAAG.T	GT	CC			.TC.	.CTCG
G.	sp DAR 73779		CAAG.T	GT	C			.TC.	.CTCG
G.	sp QFRI 8147.1		CAAG.T	GT	C			.TC.	.CTCG
G.	sp QFRI 8647.1		CAAG.T	GT	C			C.	TCG
G.	tsugae RSH J2	N	CAAG.T	· · · · ·	TNGT			C.	T-A
G.	tsugae RSH BLC	N	CAAG.T	· · · · ·	T.GT			C.	T-A
G.	tsugae HMAS 60537	N.NN	CAAG.T	· · · · ·	T.GT			C.	T-A
G.	tsugae RSH 1109		CAAG.T		T.GT			C.	T-A
G.	sinense RSH 0109		CAAGGT	C. TGTAA	A.GCTTN.				-CTATA
G.	sinense ZHANG 1734		CAAGGT	C. TGTAA	A.GCTT				-CTATA
G.	cupreum QFRI 8678.1	• • • • • • • • • • •	CAAG.T	TAA	T.CGT				-CT.GATT
G.	Cupreum DFP 3896		CAAG.T	TAA	T.CGT				-CT.GATT
G.	cupreum DFP 4336		CAAG.T	TAA	T.CGT				-CT. ATT
G.	sp SUT HI		CAAG.N	TTAA	T.GGT				-CT.GATT
G.	incrassatum DNR 72782		CAAT	.C.IAIG.	CC.I				CCT.A.A-A
G.	Incrassacum DAR 73783		CAAI	.C.IAIG.	CC.I				CCI.A.A-A
G.	adspersum CBS 551.74		AG.I	.C.IGCGA	···-·	G			-CIAAA
с. С	auspersum IMI 157616		AG.I	C TC CC	ст	G			CT AAA
G.	autrale DAR /3/81			CIGCG.	C T			C-	-CIAAC
с. С	australe DED 3396		AAG.I	C.IGCG.	C				-CIAAC
с. С	australe UFF 3300		AAG.T	CGCG.	C T	-			-CT 777 -C
с. С	an SE 1 RSmith		мас.т С = ллт т	CUGUG.	 m	-			
G.	an SIIT H3		C = AAG T	222722	т сс ст		_	C	- T - ATC
э. F	rosea JMM T92-10	ТА	TAAG		TTAG. TA	A			-CC.AAT
÷ •	10000 0111 192-10		inng		. ING IA		GA		
G	weberianum CCPC 27001	GGT COOO	ርርሞርሞሞአ አ አ ሞ	ദ്വമ - നനമരന്ന	ጥር ልጥጥ - ሮሮጥጥ	GCGCATCCC	പ്പപപ്പവുന്നവും	GATA AT- CTC	TACCACCCCA
G.	weberianum CBS 219 3		CCICIIAAAI	-		- 500 AT COG	2101000101	-	ADJEJACGA
G.	weberianum PSH 0821								N
G .	weberianum DFP 8401			= .				=	C
G.	weberianum DFP 4483			_					C N
G.	weberianum DFP 8405			-	-			-	C
G.	sp SUT H2								C
G.	sp UWA 8	C		T	G			A	T.C
G.	SD DAR 73779	C			G			A	
G.	sp OFRI 8147.1	C			G	.T		A	T.C
G.	sp OFRI 8647.1	C			G			A	T.C
G.	tsugae RSH J2				G				CGCT
G.	tsugae RSH BLC				G				CT
G.	tsugae HMAS 60537				G				C
G.	tsugae RSH 1109				G	.N			N
G.	sinense RSH 0109		C			T	T.G	C	
G.	sinense ZHANG 1734		C			T	T.G		
G.	cupreum QFRI 8678.1		C		G	C	T.G		C
G.	cupreum DFP 3896		C	A	G	C	T.G		C
G.	cupreum DFP 4336		C	T	G	C	T.G		C
G	sp SUT H1		C		G	C	T.G		C
۰.					G	C	${\tt T.G.\ldots.}$		C
G.	incrassatum SE 3				G -	C	TG		C
G. G.	incrassatum SE 3 incrassatum DAR 73783						1.0		
G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74				G	G	T.G		C
G. G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816	· · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·	 	G	G	T.G T.G	· · · · · · · - · · · ·	C
G. G. G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·		G G T	G G	T.G T.G G	· · · · · · · · · · · · · · · · · · ·	C C TC
G. G. G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92	· · · · · · · · · · · · · · · · · · ·	· · · · · · · · · · · · · · · · · · ·		G G T T	G G 	T.G T.G G	·····	C TC TC
G. G. G. G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe DFP 3386		· · · · · · · · · · · · · · · · · · ·		G G T T		T.G T.G G G	· · · · · · · · · · · · · · · · · · ·	C TC TC TC
G. G. G. G. G. G. G. G. G. G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe DFP 3386 australe UWA 108						T.G T.G .G .G .G	· · · · · · · · · · · · · · · · · · ·	C TC TC TC TC
G. G. G. G. G. G. G. G. G. G. G. G. G. G	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe DFP 3386 australe UWA 108 sp SE 1 BSmith op SUT W2	· · · · · · · · · · · · · · · · · · ·	c.	· · · · · · · · · · · · · · · · · · ·			T.G T.G G G T.G T.C.	· · · · · · · · · · · · · · · · · · ·	C TC TC TC TC C
G. G. G. G. G. G. G. G. G. G. G. G. G. G	incrassatum DE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe UWA 92 australe UWA 108 sp SE 1 BSmith sp SUT H3						T.G T.G .G .G .G T-G T.G		C- C. TC. TC. TC. TC. C. C.
G. G. G. G. G. G. G. G. G. G. G. G. G. G	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe DFP 3386 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10			A			T.G T.G .G .G T-G T.G		C- C TC TC TC
G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe DFP 3386 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10			· · · · · · · · · · · · · · · · · · ·			T.G T.G .G .G .G T-G T.G		C- C TC TC TC
G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.	incrassatum DE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe UWA 92 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCRC 37081						T.G T.G .G .G T.G T.G GACAAAT		
G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe UWA 108 sy SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCRC 37081 weberianum RSH 0821						T.G T.G .G .G T.G T.G GACAAAT		
G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.	incrassatum DE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 australe DAR 73781 australe DVA 92 australe DVP 3386 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCRC 37081 weberianum CBS 219.3 weberianum DPP 8401			GCGAGCT	. G		T.G T.G .G .G T-G T-G GACAAAT 		
G G G G G G G G G G G G G G G G G G G	incrassatum DER 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe UWA 92 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCR 37081 weberianum CSS 219.3 weberianum DFP 8401 weberianum DFP 8403		GTTTTG	GCGAGCT	G G T T T GC.T GC.T ACT. TCTAACC-GT		T.G T.G .G .G T-G T.G GACAAAT -GC.		C TC TC TC CC C CC.AT.G
G G G G G G G G G G G G G G G G G G G	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 autrale DAR 73781 australe UWA 92 australe UWA 108 sy SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCRC 37081 weberianum RSH 0821 weberianum RSH 0821 weberianum DFP 4483 weberianum DFP 4483		GTTTTG	GCGAGCT	G 		T.G T.G .G .G T-G T.G T.G GACAAAT 		C TC TC TC TC C C
G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.G.	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum IMI 157816 australe DAR 73781 australe UWA 92 australe UWA 92 australe UWA 108 sp SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CCRC 37081 weberianum CSS 219.3 weberianum RSH 0821 weberianum DFP 4403 weberianum DFP 4403 weberianum DFP 4405 sp SUT H2		GTTTTG CC GC	GCGAGCT			T.G T.G .G T.G T.G T.G GACAAAT -G CG CG C.		C TC TC TC TC C C CG
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	incrassatum SE 3 incrassatum DAR 73783 adspersum CBS 351.74 adspersum CBS 351.74 australe UWA 92 australe UWA 92 australe UWA 108 sy SE 1 BSmith sp SUT H3 rosea JMM T92-10 weberianum CBS 219.3 weberianum CBS 219.3 weberianum RSH 0821 weberianum DFP 8401 weberianum DFP 8401 weberianum DFP 4483 weberianum DFP 8405 sp SUT H2 sp UWA 8 sp DAR 73779 sp QFRI 8147.1 sp QF		GTTTTG 	G CGAGCT 	G	G	T.G T.G G G T.G T.G T.G T.G T.G T.G T.G T.G C -G -C. -G. -G -C. -G -C. -G. -C. -G -C. -G. -C. -C. -G -C. -C. -G -C. -C. -G -C. -C. -G -C. -C. -C. -C. -C. -C. -C. -C. -C.		C TC TC TC TC C C C C.AT.G

Appendix 1B. ITS 2 alignment of 33 taxa, 224 taxa, dots are conserved bases, dashes are gaps.

Appendix 2

		Average Mycelial Growth at Different pH and Temperatures ^a													
		10°C		15°C		20°	С	25°	C	30°C		37°C		45°	С
	pН	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD
Ganoderma H1	3	0.00	0.00	1.75	0.00	3.50	0.00	11.33	2.08	20.67	2.08	6.67	0.58	0.00	0.00
	4	0.00	0.00	1.92	0.14	3.67	0.29	19.67	2.08	29.67	1.53	10.50	0.50	0.00	0.00
	5	0.00	0.00	2.50	0.00	3.58	0.52	20.67	1.15	29.33	3.06	11.33	0.58	0.00	0.00
	6	0.00	0.00	1.83	0.14	4.08	0.52	18.33	1.53	33.33	2.08	11.67	0.58	0.00	0.00
	7	0.00	0.00	2.08	0.14	4.00	0.00	19.33	1.53	31.33	2.31	12.08	0.14	0.00	0.00
	8	0.00	0.00	2.42	0.38	3.92	0.14	22.00	3.46	33.17	0.76	12.67	0.58	0.00	0.00
	9	0.00	0.00	1.67	0.58	4.17	0.29	25.67	1.53	25.17	1.89	9.83	0.76	0.00	0.00
Ganoderma H2	3	0.00	0.00	2.67	0.58	7.50	0.00	26.83	0.76	32.17	0.76	8.83	0.76	0.00	0.00
	4	0.00	0.00	3.50	0.50	10.00	0.00	31.00	0.87	39.17	0.76	14.17	1.44	0.00	0.00
	5	0.00	0.00	4.33	0.58	13.67	0.58	38.00	1.00	40.00	0.00	14.33	0.58	0.00	0.00
	6	0.00	0.00	4.67	0.58	13.33	0.58	37.67	1.53	43.33	2.89	12.00	1.00	0.00	0.00
	7	0.00	0.00	4.00	0.00	12.00	0.00	36.33	2.08	40.00	0.00	13.17	0.29	0.00	0.00
	8	0.00	0.00	3.50	0.00	11.67	0.58	36.83	1.26	40.00	0.00	11.67	1.53	0.00	0.00
	9	0.00	0.00	2.50	0.50	7.50	0.50	25.17	2.75	34.67	0.58	11.00	1.00	0.00	0.00
Ganoderma H3	3	2.67	0.58	7.33	0.58	25.17	0.76	28.33	2.89	1.00	0.00	0.00	0.00	0.00	0.00
	4	3.00	0.00	9.00	0.00	28.33	0.58	34.67	0.58	0.67	0.29	0.00	0.00	0.00	0.00
	5	4.67	0.58	12.33	0.58	29.00	3.61	35.33	1.44	1.00	0.00	0.00	0.00	0.00	0.00
	6	5.33	0.58	14.00	0.00	32.00	1.00	36.00	1.00	0.75	0.25	0.00	0.00	0.00	0.00
	7	4.67	0.58	12.50	0.50	30.50	0.87	35.67	0.58	1.17	0.29	0.00	0.00	0.00	0.00
	8	2.67	0.58	8.67	0.58	37.67	1.15	29.00	2.65	0.92	0.14	0.00	0.00	0.00	0.00
	9	2.50	0.50	8.00	0.00	19.00	3.61	22.50	2.18	0.17	0.29	0.00	0.00	0.00	0.00

Appendix 2 Temperature and pH growth profiles of Basidiomycetous fungi.

^a Mycelial growth was performed on PDA plates.; Mycelial measurements were recorded on the fifth day of growth.; Average, mycelial radial growth (mm) was an average of triplicate growth plates, and were measured on the fifth day of growth; STD, standard deviation of mycelial growth.

		Average Mycelial Growth at Different pH and Temperatures ^a													
		10°C		15°C		20°	С	25°C		30°C		37°C		45°C	
	pН	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD	Average	STD
Ganoderma	3	0.50	0.50	3.17	0.58	7.33	1.44	12.17	1.44	4.50	0.50	0.00	0.00	0.00	0.00
lucidum	4	0.33	0.58	3.00	1.73	10.83	1.76	15.67	1.53	5.17	0.29	0.00	0.00	0.00	0.00
	5	0.33	0.29	6.00	0.00	9.67	2.89	12.67	1.53	3.67	0.58	0.00	0.00	0.00	0.00
	6	0.17	0.29	4.50	0.71	9.67	0.29	12.67	2.31	4.00	0.87	0.00	0.00	0.00	0.00
	7	0.17	0.29	3.75	0.25	6.67	2.08	13.00	0.00	3.25	0.35	0.00	0.00	0.00	0.00
	8	0.00	0.00	1.50	0.00	7.67	1.15	11.00	2.65	3.50	0.50	0.00	0.00	0.00	0.00
	9	0.00	0.00	1.00	0.00	2.67	2.08	3.83	2.47	2.00	0.00	0.00	0.00	0.00	0.00
Ganoderma	3	0.00	0.00	3.67	0.58	10.00	0.00	14.67	0.58	23.00	0.00	27.67	0.58	18.33	2.08
applanatum	4	0.00	0.00	6.67	0.58	15.00	0.00	29.67	1.53	35.67	6.66	40.00	0.00	32.67	0.58
	5	0.50	0.00	6.33	0.58	15.50	0.50	29.67	0.58	39.67	0.58	40.00	0.00	31.33	3.06
	6	0.33	0.29	6.33	0.29	13.67	1.15	29.00	1.00	40.00	0.00	40.00	0.00	31.00	2.18
	7	0.17	0.29	5.67	0.29	14.33	1.15	27.33	0.58	39.33	0.58	40.00	0.00	33.33	0.58
	8	0.00	0.00	5.67	1.15	12.33	0.58	27.00	0.00	36.33	0.58	40.00	0.00	25.67	2.08
	9	0.00	0.00	1.17	0.29	7.67	0.58	15.00	0.50	20.00	0.00	24.33	1.15	12.50	3.54
Pycnoporus	3	0.50	0.71	2.67	0.58	8.33	0.58	15.33	0.58	20.33	1.53	22.33	2.08	20.33	0.58
cinnabarinus	4	0.17	0.29	4.00	0.00	13.00	2.00	25.67	1.53	34.00	2.00	39.67	0.58	34.67	0.58
	5	0.67	0.29	3.33	0.58	12.33	0.29	25.00	1.73	36.00	1.00	39.33	0.58	33.00	1.00
	6	1.00	0.00	4.00	1.00	12.00	0.00	25.67	1.15	34.33	1.15	37.67	0.58	31.33	3.21
	7	0.50	0.00	6.00	0.00	13.67	0.58	23.00	1.00	22.67	16.20	34.67	0.58	29.00	1.73
	8	0.00	0.00	4.67	0.58	11.00	1.73	21.67	1.53	30.00	1.00	33.33	0.58	29.00	1.32
	9	0.00	0.00	0.50	0.00	4.33	0.58	10.67	0.58	15.00	0.00	14.33	1.53	15.00	1.00

Appendix 2... continued Temperature and pH growth profiles of Basidiomycetous fungi.

^a Mycelial growth was performed on PDA plates.; Mycelial measurements were recorded on the fifth day of growth.; Average, mycelial radial growth (mm) was an average of triplicate growth plates, and were measured on the fifth day of growth; STD, standard deviation of mycelial growth.

Appendix 3

Percentage Inhibition of the Clostridium difficile isolates by EtOAc C. difficile (cd8) C. difficile (cd1) C. difficile (cd14) Concentration $(\mu g m L^{-1})$ Mean SD Mean SD Ν Mean SD Ν Ν 12000 100.00 1.12 3 100.00 1.20 3 99.97 1.29 3 95.69 1.07 3 93.05 1.17 3 90.93 0.76 3 6000 3000 96.19 1.4 3 92.48 0.86 3 93.90 2.14 3 91.39 2.59 3 1500 93.05 Median 96.19 92.65 Maximum 100.00 100.00 99.97 94.05 Mean 97.29 95.18 Lower 95% CI 91.44 84.78 87.43 Upper 95% CI 103.10 105.6 100.7 T test 71.49 39.38 45.23 2 2 df 3 .0002 .0006 <.0001 P value Significant YES YES YES (alpha = 0.05)

Appendix 3

Student T test of MIC of the EtOAc extract

N, number of data measured; SD, standard deveiation; CI confidence interval; T-test, student T-test; df, distribution frequency; P value, two tailed probability value