

# Industrial and medicinal application of Reishi and Lion's Mane mushrooms

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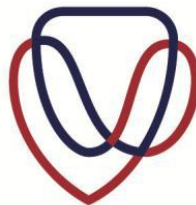
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# Chapter 1

## Literature review

### II. Abstract

Mushrooms are macro-fungi which can be identified according to their different shapes, sizes, colours, spore colour and chemical reactions. Medicinal mushrooms have been used for centuries for the treatment of various ailments while the high nutritional value add to their popularity. These fungi have been distinctively studied as a healthy source of food which are rich in protein. Recently there has specifically been an interest in bioactive compounds responsible for antimicrobial, anti-tumour, anti-diabetes and anti-hypercholesteraemic activities associated with mushrooms. Although *Ganoderma lucidum* is non-edible, it has been used for centuries due to its medicinal value and seems to possess the most medicinal properties. Another well-known edible mushroom, is *Hericium erinaceus* is currently scrutinized due to its ability to stimulate the growth of nerve growth factors. Therefore, it may be used as a natural alternative to treat the symptoms associated with Alzheimer's and cognitive decline. *Acacia mellifera* is considered to be an encroaching tree species in South Africa and may have economic benefits when used as substrate for the cultivation of *G. lucidum* to provide animal feed. In this review, the nutritional as well as medicinal values of *G. lucidum* and *H. erinaceus* will be addressed in order to highlight the advantages they hold for humankind and pave the way for alternative medicinal applications.

## 1.1 Introduction

Exotic mushrooms are macro-fungi identifiable according to the significant differences in their shapes, sizes, spore colour and chemical reactions (Chang 2009). Mushrooms are defined “as a macrofungus with a distinctive fruiting body, which can be epigeous or hypogeous and large enough to be seen with the naked eye and picked by hand” (Chang and Miles 1992). Exotic mushrooms are eukaryotic, heterotrophic fungi which include edible mushrooms as well as medicinal mushrooms. Fungi were first regarded as members of the Plant Kingdom, but are now recognized as a separate group, the Mycetea Kingdom (Smith et al. 2002). Over 12 000 mushroom species have been identified even though they are not a taxonomic group. Mushrooms are predominantly Basidiomycetes and are ecologically classified into three groups based on their lifestyle as saprophytes, parasites and mycorrhiza (Chang 2009).

Parasitic fungi grow on living organic material and thrive at their expense. Innumerable fungal species are both saprophytic and parasitic since they continue to feed off a dead host (Polese 2000; Smith et al. 2002). Mycorrhizal fungi live in true symbiosis with their host plant, generally a tree wherein essential micro-nutrients such as mineral salts are provided to their host in exchange for energy. Plants find these mineral salts, especially nitrates, the hardest to convert from the soil and can be obtained by making use of mushroom mycelium since it is in closer contact with the soil than the roots (Polese 2000). Saprophytes obtain nutrients from dead, organic material and most exotic mushrooms have been found to be saprophytic. These fungi are known to be primary decomposers due to the production of extracellular enzymes which results in the decomposition of woody structures (Polese 2000; Smith et al. 2002).

Exotic mushrooms can therefore be cultivated on substrates containing lignin due to their ability to break down complex lignocellulosic materials. As a result, mushrooms provide a way of returning carbon, nitrogen and hydrogen to the ecosystem (Chang and Miles 1992; Stamets 1993; Falandysz and Borovička 2013). Although mushrooms are predominantly saprophytes, there are exceptions such as *Pleurotus ostreatus* which seems to be a carnivorous mushroom (Barron and Thorn 1987; Chang 2009). In the case of *P. ostreatus*, nitrogen is believed to be obtained by means of digesting nematodes. This is achieved by the ability to secrete a nematoxin which results in immobilization of the nematode after which mycelium can penetrate and colonize within the nematode (Barron and Thorn 1987).

Throughout history, mushrooms have been used as a natural alternative for the treatment of various ailments (Smith et al. 2002). Nowadays macro fungi are known to be a source of



bioactive compounds of medicinal value (Chang 2009). They contain compounds with various properties including anticancer, antiviral, immunomodulating and hepatoprotective properties (Chang and Buswell 1996; Wasser 2011). All of these properties can be enjoyed by capsulation of liquid concentrates or dried, powdered mushrooms which are defined as mushroom nutraceuticals. It was suggested that these properties are due to the presence of immunomodulators which have the ability to modulate an immune response (Smith et al. 2002; El Enshasy and Hatti-Kaul 2013). This class of compounds belong mainly to polysaccharides ( $\beta$ -D-glucans), polysaccharide-protein complexes, proteoglycans, proteins and triterpenoids (Moradali et al. 2007). Among polysaccharides, especially  $\beta$  (1 $\rightarrow$ 3)-D-glucans and their protein derivatives polysaccharides play an important part in immunomodulating and antitumor activities. These important  $\beta$ -glucans are contained within the cell walls of edible mushrooms which can also act as antimicrobial agents, and reduce blood cholesterol and glucose levels (Manzi and Pizzoferrato 2000; Smith et al. 2002).

In addition to its medicinal value, mushrooms have been studied extensively as a healthy food source and are nutritionally well-balanced. They are rich in vitamins such as thiamine (B1), riboflavin (B2), ascorbic acid (vitamin C), ergosterol, biotin and niacin (Smith et al. 2002). A high protein (19-35%) and dietary fibre (3-35%) content on a dry weight basis are present, which contain all nine essential amino acids required by humans as well as improving digestive health, respectively. High proportions of iron, calcium, potassium, magnesium and phosphorus contribute to the valuable mineral content present. From a human health point of view, mushrooms are low in calories, fat and carbohydrates. Although mushrooms contain all the main classes of lipids, they are low in fat with 2-8% of dry weight. Fresh mushrooms contain 70-95% moisture, whereas it is about 10-13% on a dry weight basis (Chang 2009; Manzi and Pizzoferrato 2000; Smith et al. 2002).

In this review, exotic mushrooms consist of both edible and medicinal mushrooms. Edible mushrooms include *P. ostreatus* (Grey Oyster), *Lentinula edodes* (Shiitake), *Hericium erinaceus* (Lion's Mane) and *Grifola frondosa* (Maitake) which are all recognised for their medicinal properties. Two species, namely *G. lucidum* (Reishi) and *Trametes versicolor* (Turkey Tail) are purely medicinal mushrooms and regarded as non-edible mushrooms (Chang and Buswell 1996). However, this review will focus specifically on *G. lucidum* and *H. erinaceus* to explore their benefits and applications.

## 1.2 Exotic mushrooms in general

The medicinal value of higher Basidiomycetes mushrooms (both edible and medicinal) have been acknowledged and applied for centuries in China and Japan. Worldwide, the market value of specifically medicinal mushrooms and their derivatives was about U.S. \$1.2 billion in 1991. In 1994, it was about U.S. \$3.6 billion and estimated to be U.S. \$6.0 billion in 1999. In 1995, the market value of *G. lucidum*-based nutraceuticals alone was estimated at U.S. \$1 628.4 million (Chang and Buswell 1999).

As saprophytes, exotic mushrooms are commonly found on hardwood species, causing white rot (Goodell et al. 2009). These white-rot fungi possess both cellulolytic and lignin degrading enzymes, resulting in the utilization of lignin as a food source while leaving behind cellulose. Therefore they have the potential to degrade the entire wood structure when under the correct environmental conditions (Goodell et al. 2009). As a result, exotic mushroom cultivation can be used as an alternative for the breakdown of waste products as approximately 70% of agricultural and forest products are discarded as wastes (Chang 2009). A new discipline, namely applied mushroom biology, can convert these wastes into human food as well as produce mushroom nutraceuticals with many health benefits. Additionally, this discipline can be applied to the biota in order to create a pollution-free environment (Chang 1991).

An important group of these macro fungi are the polypore mushrooms which is defined as a group of fungi producing pores underneath the cap (Stamets 1993). This group includes *G. lucidum*, *G. frondosa* (Maitake) and *T. versicolor* (Turkey Tail) and stand in contrast to gill-producing mushrooms such as *L. edodes* (Shiitake), *H. erinaceus* (Lion's Mane) and *P. ostreatus* (Grey Oyster). Although both *G. lucidum* and *T. versicolor* are considered non-edible, *G. lucidum* has the most medicinal properties while *T. versicolor* is the most extensively studied medicinal mushroom (Hobbs 1995; Wasser 2005). In contrast to *G. lucidum* and *T. versicolor*, the Maitake mushroom (*G. frondosa*) is an edible polypore (Stamets 2005).

Commonly known as Maitake, *G. frondosa*, the most dominant property exhibited by this specific mushroom is the reduction of blood pressure as well as cholesterol (Stamets 2005). Other medicinal properties include anti-cancer, anti-diabetic and immunomodulating while it may also improve the health of HIV patients (Suzuki et al. 1984; Yamada et al. 1990; Kubo et al. 1994). In the late 1980's, the compound responsible for immune system enhancement has been identified as a water-soluble 1-6-monoglucosyl branched  $\beta$ -1,3 D-glucan, known as Grifolan (Adachi et al. 1988; Stamets 1993; Kurashige et al. 1997). This compound has been

found to be effective against various cancers such as breast, lung, liver, prostate and brain cancer (Stamets 1993; Kubo et al. 1994). Furthermore, *G. frondosa* also have the ability to reduce blood glucose, insulin, and chronic fatigue syndrome (CFS) as well prevent and treat diseases such as flu and diabetes (Kubo et al. 1994).

The Turkey Tail mushroom, scientifically known as *T. versicolor*, is known for its activity against various tumours and viruses as well as antioxidant properties (Stamets 2005). This species contains two important polysaccharopeptide compounds, both of which consist of  $\alpha$ -1,4 and  $\beta$ -1,3 glucoside linkages in the polysaccharide moieties and are found to be resistant to enzymatic proteolysis (Ng 1998). These two compounds are polysaccharopeptide krestin (PSK) and polysaccharopeptide (PSP) (Kobayashi et al. 1995; Collins and Ng 1997). This species seems to have an indirect effect on cervical and liver cancer which is caused by the human papillomavirus (HPV) and hepatitis C virus (HEP-C), respectively (Stamets 2012). Additionally, Turkey Tail mushrooms have antioxidant properties while they may also be useful against HIV-1 infection (Collins and Ng 1997). Finally, PSK and PSP present in Turkey Tail mushrooms also seem to have immunopotentiating activity (Ng and Chan 1997; Cui and Chisti 2003).

Currently, *L. edodes* (Shiitake) are the most popular mushroom regarding its medicinal properties, but the second most cultivated mushroom in the world (Chang and Buswell 1996). This specific species has been studied extensively to investigate its medicinal properties and various compounds have been identified (Çağlarirmak 2007). Lentinan, a water-soluble polysaccharide, is considered to be responsible for the majority of the medicinal properties such as anti-cancer, antimicrobial and anti-tumour properties. It is a protein-free polysaccharide present in both the fruiting bodies and mycelium and consists of  $\beta$ →(1-3)-D-glucopyranan with a branched chain of  $\beta$ →(1-6)-monoglycosyl (Çağlarirmak 2007). As an antimicrobial agent, lentinan inhibits the replication of Adenovirus type 12 and Abelson virus as well as inhibiting bacteria such as *Bacillus subtilis*, *Micrococcus luteus* and *Staphylococcus aureus* (Wasser and Weis 1999; Wasser 2005a; Rahman and Choudhury 2012). Even though lentinan does not have direct cytotoxic properties, it is deemed essential for the activation of a host-mediated response (Stamets 2005; Çağlarirmak 2007). Additionally, *L. edodes* have anti-oxidant properties and is capable of lowering blood serum cholesterol (BSC) (Wasser 2005b).

The Grey Oyster mushroom (*P. ostreatus*) contains various compounds such as lovastatin, pleuran, and an ubiquitin-like protein which is responsible for medicinal properties such as anti-cholesterol, anti-diabetic, antimicrobial, anti-oxidant, anti-tumour and immunomodulatory

properties (Bobek and Galbavý 2001). Lovastatin, a commercially available drug for the treatment of high cholesterol, has been approved in 1987 by the FDA (Bobek et al. 1998). However, isomers of this drug (3-hydroxy-3-methylglutaryl-coenzyme A reductase) are naturally produced by *P. ostreatus*. Pleuran, a novel  $\beta$ -glucan, is responsible for the antibacterial properties while a laccase isolated from *P. ostreatus* have antiviral activity due to inhibiting the entry and replication of hepatitis C virus (El-Fakharany et al. 2010). Additionally, *P. ostreatus* possibly have anti-HIV properties due to the presence of an ubiquitin-like protein which inhibits HIV-I reverse transcriptase activity by cleaving transfer RNA (Wang et al. 2000). This species are currently undergoing trials as a natural alternative for treatment of hyperlipidemia in HIV patients, but still needs to be confirmed (Abrams et al. 2011).

Recently there has been an increased interest in *H. erinaceus* (Lion's Mane) due to the presence of nerve growth factors (NGF) which may have application as a possible treatment of Alzheimer's disease since this compound seems to have the ability to regrow and rebuild myelin by stimulating neurons (Takei et al. 1989; Allen and Dawbarn 2006). Thus, NGFs may prevent neuronal death as well as maintain and organize neurons (Allen and Dawbarn 2006). Additionally, two low molecular weight compounds, namely erinacines and hericenones, are also present in Lion's Mane mushrooms and both of these compounds seem to induce NGF production (Kawagishi et al. 1994; Ma et al. 2010). As a result, we will focus on *H. erinaceus* in this review to further explore the benefits and possible applications of this species as a natural alternative for treating Alzheimer's disease and neuronal death.

As the mushroom with the most medicinal properties, *G. lucidum* has been used for centuries due to its health enhancing effects such as treatment of cancer as well as increasing longevity, resistance and recovery from diseases (Kino et al. 1989; El Enshasy and Hatti-Kaul 2013). Additionally, *G. lucidum* mushrooms have anti-oxidant properties, act as an antimicrobial agents and have strong immunomodulating properties while it may also have cholesterol, blood pressure and blood sugar lowering properties (Chang and But 1986; Lee et al. 2001; Smith et al. 2002; Wasser 2005a). Therefore, it is clear that *G. lucidum* has the most medicinal properties with the antimicrobial properties of microbiological interest and will be discussed in depth to further explore the benefits and possible applications.

### **1.3 Application of *G. lucidum* as medicinal fungus**

For over two millennia, this basidiomycete fungus has been recognised for its medicinal purposes by Chinese medicinal professionals (Stamets 1993; Wasser 2005a; Babu and

Subhasree 2008). In Latin, *lucidum*, refers to the shiny appearance of the fruiting body of this specific mushroom as it has a varnished appearance (Wasser 2005a). Naturally, *G. lucidum* grows in densely wooded mountains, but is rarely found since it mainly grows on decaying logs and tree stumps (Babu and Subhasree 2008).

Traditionally, *G. lucidum* has been widely used in Japan and China for the treatment of various ailments such as insomnia, cancer, hypercholesterolemia and hypertension resulting in *G. lucidum* to be considered as the mushroom with the most medicinal properties (Stamets 1993; Moncalvo 1996; Wasser 2005b). As treatment for cancer, *G. lucidum* seems to increase the production of cytokines and antibodies which results in the inhibition of various tumours (Battle et al. 1998; Mueller et al. 2000). Furthermore, *G. lucidum* has shown to act as antimicrobial agent since activity against *Helicobacter pylori* as well as HIV are exhibited (Moncalvo 1996; Wasser 2005a). Additionally, *G. lucidum* may also have anti-oxidant properties due to scavenging of free radicals (Chang and But 1986; Lee et al. 2001; Smith et al. 2002). Currently, *G. lucidum* is widely grown on a commercial scale and commonly applied as a dietary supplement (Stamets 1993; Wasser 2005b).

### 1.3.1 History of *G. lucidum* mushrooms

In China, *G. lucidum* is commonly known as Lingzhi (“spiritual potency”), which are regarded as the “Medicine of Kings” and is also called the “mushroom of immortality” (Babu and Subhasree 2008). The virtues of *G. lucidum* extracts have been handed down for generations and include a cancer cure, a symbol of happy augury, good fortune, good health, longevity, and even immortality. As a traditional Chinese medicine, *G. lucidum* has been recognized for treating bronchial asthma, coronary heart disease, dizziness, lengthy diseases, rhinitis and stomach ulcers (Jones 1998).

As one of the three well-known polypore mushrooms, *G. lucidum* was mentioned the first time during the era of China’s first emperor, Shih-huang of the Ch’in Dynasty (221-207 B.C.) (Wasser 2005a). This specific species has been endlessly represented in art since the Yuan Dynasty (1280-1368 A.D.) and subsequently representations of *G. lucidum* proliferated throughout Chinese literature and art (Wasser 2005a). Depictions of *G. lucidum* were even found on the facades of the Emperor’s palace in the Forbidden City in Beijing, China. In Japan, dried *G. lucidum* mushrooms were used as a talisman to ward off evil spirits in the home. In Northern America and Europe, this mushroom is known as one of the “artist’s conk” fungi (the true artist conk is *G. applanatum*) (Jones 1998; Wasser 2005a).

### 1.3.2 History of taxonomy within the *G. lucidum* genus

Although there are more than 2,000 known species, *G. lucidum* have been classified into six species which have all been studied extensively in order to investigate their potential health benefits (Hapuarachchi et al. 2015). This classification was based on the colour of the fruiting body: red (*Sekishi*), black (*Kokushi*), blue (*Seishi*), white (*Hakushi*), yellow (*Oushi*) and violet/purple-like (*Shishi*) and consequently assigned based on the triterpenoids patterns (Szedlay 2002) (**Table 1**).

The black (*G. sinensis*) and especially the red *G. lucidum* have demonstrated the most significant medicinal properties (Babu and Subhasree 2008). Both of these varieties are worldwide used as a health supplement with the red variety being the most commonly used and cultivated since black *G. lucidum* are deemed inferior to red *G. lucidum* (Babu and Subhasree 2008; Ulbricht et al. 2010). The reason being the lower polysaccharide content of black *G. lucidum* compared to the red variety (Babu and Subhasree 2008). It is the high polysaccharide content found in red *G. lucidum* that makes it particularly potent. Wild purple *G. lucidum* share similarities with red *G. lucidum* regarding appearance, but can be distinguished by the significant purple coloration in the heart of the cap. This specific type of *G. lucidum* is extremely rare which resulted in the limited research being done on the purple variety (Babu and Subhasree 2008).

In 1781, *G. lucidum* was first described and illustrated as *Boletus lucidus* by William Curtis (Curtis 1781). *Fungi Fenniae Exsiccati* (1865) by Karsten contained a specimen, *Polyporus lucidus* with rough basidiospores, but Curtis (1781) described *G. lucidum* based on material from Peckham, London, UK (Adaskaveg and Gilbertson 1986). The epithet was however sanctioned by Fries (1821).

**Table 1** Types of *G. lucidum*

Colour	TASTE	USE
BLACK	Salty	Improves lung function
BLUE	Sour	Improves eye sight and liver function
PURPLE	Sweet	Enhances function of eye joints, helps complexion
RED	Bitter	Aids internal organs and improves memory
WHITE	Hot	Protects kidneys
YELLOW	Sweet	Strengthen spleen function

(Szedlay 2002)

### 1.3.3 Taxonomy within the *G. lucidum* genus

Taxonomically, *G. lucidum* belongs to the Fungi Kingdom, Basidiomycota Division, Agaricomycetes Class, Polyporales Order, *G. lucidum*taceae Family and the *G. lucidum* Genus with a species name of *G. lucidum* (Adaskaveg and Gilbertson 1986). The *G. lucidum*taceae family consists of five genera, namely *G. lucidum* P. Karst 1881, *Amauroderma* Murril 1905, *Haddowia* Steyaert 1972, *Humphreya* Steyaert 1972 and *Polyporopsis* Audet 2010 (Richter et al. 2015). Since traditional taxonomy of *G. lucidum* is based on morphological traits, this genus was divided into two groups, namely the laccate (*G. lucidum* complex) and the non-laccate (*G. applanatum* complex) species (Zheng et al. 2009).

The *G. lucidum*taceae family with *P. lucidus* W. Curtis as its type species, was introduced by Donk (1948) and was known to be a laccate and stipitate white rot fungus (Moncalvo and Ryvarden 1997). The *G. lucidum*taceae family was predominantly classified by making use of morphological characteristics as well as a phonetic approach (Moncalvo 1996). As the grouping of organisms was based on a resemblance between morphological characteristics, a different classification system was proposed due to the assumption that morphologically similar taxa are also similar on genetic level (Moncalvo 1996). Based on that, this family was placed subsequently in the Polyporales order and Basidiomycotina division, resulting in the *G. lucidum* genus being a cosmopolitan genus (Schwarze and Ferner 2003; Cao and Yuan 2013). However, Karsten (1881) established the *G. lucidum* genus with *G. lucidum* (W. Curt, Fries) as the only species in this genus (Hapuarachchi et al. 2015).

There is clearly much taxonomic confusion in the *G. lucidum* species complex as the identification and restrictions of species are unclear due to the variety of morphological characteristics (Hapuarachchi et al. 2015). As a result, more than 290 taxonomic names have been published. Furthermore, *G. lucidum* have been described 13 times as a new species in Europe due to different authors as well as the variability in its morphological characteristics (Ryvarden 2000).

This resulted in the use of chemical and molecular methods to enable taxonomist to distinguish between different *G. lucidum* species. However, a model has been proposed by Moncalvo and co-workers to resolve systemic similarities in *G. lucidum*. This specific model uses both phylogenetic analysis (use of sequences of ITS and 26S rDNA) and morphological, ecological, cultural, and mating studies (Moncalvo et al. 1995a, 1995b; Hseu et al. 1996). Regardless of this model, the *G. lucidum* genus are still considered to be the largest of the polypore fungi due to the extensive variance in macro- and micro-morphological characteristics (Moncalvo and Ryvarden 1997).

#### **1.3.4 *G. lucidum* species complex**

In East Africa, due to a lack of a morphological solution to name different species in the *G. lucidum* species complex, all names of this complex was treated as the “*G. lucidum* group” by Ryvarden and Johansen (1980). This complex includes 12 taxa (**Table 2**) and the species are recognised as members of the *G. lucidum* species complex, known as the *G. lucidum sensu lato* complex. However, the taxonomy of the *G. lucidum sensu lato* complex have long been the subject of debate and the validity of its members are still being investigated as different opinions have been raised (Hapuarachchi et al. 2015).

The *G. lucidum sensu lato* species complex has been reported from East Asia (China, Japan and South Korea) as well as South and Southeast Asia (India, Indonesia, Philippines, Thailand and Vietnam) (Wang et al. 2012). Apart from Asia, other reported areas include East Africa (Ghana, Kenya and Tanzania), Europe (almost all the European countries), North America (Canada and U.S.A.), Oceania (Australia) and South America (Argentina, Brazil and Uruguay). However, due to phylogenetic analyses of the genus, *G. lucidum* collections of different areas are scattered in various separated lineages. Molecular phylogenetic analyses performed in the mid-nineties of the 20<sup>th</sup> century clearly indicated that *G. lucidum* collections in East Asia were in most cases not comparable to *G. lucidum* from Europe (Yang and Feng 2013). A study performed by Saltarelli and co-workers (2009) concluded that the European *G. lucidum*



species should be considered as *G. lucidum sensu stricto* as this species was firstly described in Europe (Moncalvo et al. 1995a; Buchanann 2001; Saltarelli et al. 2009).

**Table 2** Taxa belonging to *G. lucidum* complex.

Taxa	Reference
<i>G. lucidum tsugae</i> Murr.	Murril 1902
<i>G. lucidum valesiacum</i> Boud.	Murril 1908
<i>G. lucidum oregonense</i> Murr.	Murril 1908
<i>G. lucidum resinaceum</i> Boud.	Patouillard 1889
<i>G. lucidum pfeifferi</i> Bres.	Bazzalo and Wright 1982
<i>G. lucidum oerstedii</i> (Fr.) Torr.	Adaskaveg and Gilbertson 1986
<i>G. lucidum ahmadii</i> Stey.	Steyaert 1972
<i>G. lucidum multipileum</i> D. Hou.	Hou 1950
<i>G. lucidum sichuanense</i> J.D. Zhao & X.Q. Zhang.	Zhao et al. 1983
<i>G. lucidum lingzhi</i> Wu et al.	Cao et al. 2012
<i>G. lucidum sessile</i> Murrill.	Murril 1902
<i>G. lucidum zonatum</i> Murrill.	Murril 1902

(Ryvarden and Johansen 1980; Hapuarachchi et al. 2015)

### 1.3.5 *G. lucidum* species in China

The first report of *G. lucidum* in China was done by Patouillard (1907) after which more collections from different regions were reported by Teng in 1934 (Wang et al. 2012). Studies showed that *G. lucidum sensu stricto* was first distributed in both North and South Europe before it probably extended to China (Moncalvo et al. 1995a). Furthermore, analyses of ITS and 25S ribosomal DNA sequences indicated that *G. lucidum* species found in both Europe and China are not comparable. This has been confirmed by other authors (Moncalvo et al. 1995; Pegler and Yao 1996; Hong and Jung 2004), but misapplication of the name is yet to be corrected. For example, *G. lucidum* found in tropical Asia is actually *G. multipileum* and is not comparable with *G. lucidum sensu stricto* found in Europe, nor with “real” *G. lucidum* found in East Asia (Wang et al. 2009). However, the misapplication of *G. lucidum* to the Chinese species has a very short history, although it has become more dominant due to the successful cultivation of *G. lucidum* (Wang et al. 2012). Meanwhile, the distribution of true *G. lucidum* in China was confirmed after the use of *G. sichuanense* was proposed when referring to Chinese *G. lucidum* (Cao et al. 2012; Wang et al. 2012; Yang and Feng, 2013).

### 1.3.6 Northern American *G. lucidum* species

Four North American species were identified by Overholts (1953) and placed in the Friesian genus *Polyporus* instead of *G. lucidum*. The reason being that classification was based on geographical distribution, host-specificity, macroscopic morphology and spore characteristics (Adaskaveg and Gilbertson 1986). Various synonyms of *P. lucidus* such as *Ganoderma sessile*, *Ganoderma polychromum*, *Ganoderma zonatum* and *Ganoderma sulcatum* were considered by authors (Overholts 1953; Steyaert 1972; Moncalvo and Ryvarden 1997). However, *Ganoderma boninense* was suggested to be the correct name of the American *G. lucidum* species as Zhou et al. (2015) clearly distinguished *G. boninense* from *G. sessile* and *Ganoderma tsugae* (Moncalvo et al. 1995; Zhou et al. 2015). Therefore, originally described species from the USA need to be researched as most of the species are old and were never subjected to phylogenetic analyses (Zhou et al. 2015).

### 1.3.7 Confusion in the nomenclature of the *G. lucidum* genus

The identification of *G. lucidum* species have often been unclear, resulting in controversy regarding taxonomic classification (Moncalvo et al. 1995a). Due to the presence of heterogenic forms, taxonomic obstacles and inconsistencies, various *G. lucidum* species have been misnamed (Mueller et al. 2007). This species are genetically heterogeneous, caused by the crossing over of different generations and geographical areas (Miller et al. 1999; Pilotti et al. 2003). This resulted in a wide range of genetic variation which led to variation in listed morphological features, even within same species (Hong et al. 2001).

Identification of *G. lucidum* species is extremely difficult as a wide range of factors such as environmental factors, variability, inter hybridization and individual morphological preference have to be taken in account (Zheng et al. 2009). Since traditional taxonomic methods based on morphology are inconclusive, it can no longer be applied for establishing a stable classification system (Hseu et al. 1996; Hong et al. 2002). This resulted in an uncertain nomenclature due to different authors using different criteria regarding classification. Some authors are strictly focused on host-specificity, geographical distribution and macro morphology whereas other authors primarily focused on spore characteristics (Sun et al. 2006; Ekandjo and Chimwamurombe 2012).

Currently, the estimated amount of known *G. lucidum* species is about 80 (Kirk et al. 2008). As species and genus concepts are confused due to similar fungi found in *Fomes* (Fr.) Fr 1849, *Polyporus* P. Micheli 1729 and *Tomophagus* Murril 1905, the taxonomic situation within *G. lucidum* is still relatively unclear (Russell and Paterson 2006; Hapuarachchi et al., 2015). In order to develop a more stable taxonomy for the *G. lucidum* genus, it was suggested to make use of a combination of morphological, chemotaxonomic and molecular methods (Richter et al. 2015). Regardless of years of discussion and endless debates, the taxonomy of the *G. lucidum* complex still remain problematic (Hapuarachchi et al. 2015).

### 1.3.8 Diagnostic morphological characteristics of *G. lucidum*

A key diagnostic characteristic for the *G. lucidum* genus is the double walled basidiospores with interwall pillars (Smith and Sivasithamparam 2000). The reason being the uniqueness of morphology between polypores as they have two distinctive morphological properties, namely “*G. lucidumtoid*” and “amaurodermatoid” (Moncalvo 1996). Basidiospore morphology are therefore regarded to be an extremely important characteristic for distinguishing between taxonomic groups. Consequently, the division of *G. lucidumtaceae* in major groups was exclusively based on spore morphology. The “*G. lucidumtoid*” spore is defined by a thickened apex (*G. lucidum* Karst.) whereas “amaurodermatoid” spore walls are uniformly thickened (*Amauroderma* Murr.) (Moncalvo 1996).

Aside from basidiospores, the diverse pileus crust is another important characteristic for classification (Moncalvo 1996). It can be thick, strongly laccate and composed of pilocystidia as found in *G. lucidum* species group (*G. lucidum* subgen. *G. lucidum*); thin and shiny, but not strongly laccate with pilocystidia present (*Ganoderma colossum*); and dull with no pilocystidia present, as found in species such as *G. applanatum* - *G. australe* group (*G. lucidum* subgen. *Elfvingia*). Species with amaurodermatoid spores have been found to possess a dull pileus and lack a pilocystidia. Both Furtado (1965) and Steyaert (1972) attempted to distinguish between different types of dull pilei in both *G. lucidum* and *Amauroderma*, but both studies had inconclusive results (Moncalvo 1996).

Other important characteristics include an annual or perennial basidiocarp, stipitate to sessile, pileus (cap) surface with a thick, dull cuticle or shiny with a thin cuticle, cream coloured to dark red/brown, soft and spongy to firm-fibrous, cream coloured pore surface, 4-7 regular pores per mm, single or stratified tube layers, central or lateral stipe if present, pale to purplish brown stipe, dimitic hyphal system, generative hyphae with clamps, skeletal hyphae translucent to

brown coloured, non-septate hyphae, broadly to narrowly ellipsoid basidiospores with a truncate apex and apical germ pore, brown endospore and separated from translucent exospore by inter-wall pillars, negative reaction to Melzer's reagent, spores are 7-30 µm in length (Ryvarden 2004).

Furthermore, in a study conducted by Kapoor & Sharma (2004), *G. lucidum* were identified by following standard description of the species: fruiting bodies are usually large, stipitate, dimidiate, lateral and reddish brown in colour with the upper surfaces coated with a hard, shiny substance (Kapoor and Sharma 2014). Pileus is 2-5 cm broad with the surface often appearing varnished while the stipe is generally 0.5-2 cm thick. Produced basidiospores are brown, ovate, with a rounded base and truncate to narrowly rounded apex; 10-12 x 6.5-8 µm in size; slightly too strongly dimpled spore surface; wall composed of several layers. Outermost wall connected to inner wall by inter-wall pillars. The morphology of basidiocarps and basidiospores were also studied by Pegler and Young (1973) and Adaskaveg and Gilbertson (1986). In 1965, Furtado reported that an amorphous substance secreted by the hyphae responsible is for the varnished appearance of the basidiocarp of many polypores (Kapoor and Sharma 2014).

### **1.3.9 Distribution and natural habitat**

Considered to be the most medicinal mushroom, *G. lucidum* has been used for over 2000 years, regardless of being rarely found in nature (Stamets 2005; Hapuarachchi et al. 2015). As a wood-decaying fungus, *G. lucidum* causes white rot of a wide variety of trees with a worldwide distribution in green ecosystems (Stamets 2005; Kapoor and Sharma 2014; Hapuarachchi et al. 2015). As *G. lucidum* can survive under hot and humid conditions, it is usually found in both temperate and subtropical regions (Stamets 2005; Pilotti et al. 2004; Hapuarachchi et al. 2015). However, it is more common in tropical regions such as India and are therefore found more dominantly in Asia (Kapoor and Sharma 2014). As a result, *G. lucidum* is most commonly cultivated in China, Japan, Malaysia, Korea, Taiwan and North America (Kapoor and Sharma 2014).

As a saprophyte, *G. lucidum* is found on the widest range of hardwood species, including beeches, elms, oaks and even palms (Stamets 2005). However, some mycologists considered *G. lucidum* to behave parasitically toward palm trees, resulting in the depiction as a phytopathogenic fungus. Nevertheless, an emerging point of view is that *G. lucidum* is

facultative parasites, meaning they act as opportunistic parasite only when the tree is stressed or diseased (Stamets 2005).

### **1.3.10 Cultivation of *G. lucidum***

In ancient times, *G. lucidum* was found infrequently in nature which resulted in this fungus being highly cherished and expensive (Chang and Miles 1992). The sexual structures (basidiocarps) of *G. lucidum* found on living or dead trees, are known to form brackets. Commonly, two types of basidiocarps are produced, depending on the species: a laccate fruiting body with a shiny upper surface, or a non-laccate fruiting body with a dull upper surface (Smith and Sivasithamparam 2000; Pilotti et al. 2004). The preferred season for *G. lucidum* is during summer to early fall when temperatures are between 15-35°C (Stamets 2005).

Cultivation of *G. lucidum* has been acquired by using various different substrates as well as maintaining specific growth parameters such as temperature, light intensity, relative humidity, water content and air pH (Miles and Chang 2004). For mycelial growth, light and pH are of the most important parameters while depending on some factors such as temperature, culture media and nutrient elements (Kapoor and Sharma 2014). All of these factors were found to greatly influence the growth of *G. lucidum* in both field and laboratory conditions. Therefore, it is important to evaluate these factors in order to obtain optimum mycelial growth (Kapoor and Sharma 2014).

Artificial cultivation of *G. lucidum* was successfully achieved the first time in the early 1970's (Chang and Buswell 1999; Chang 2009). The basic substrate for artificial cultivation is hardwood sawdust supplemented with 20% wheat bran, 1% gypsum and 1% sucrose with a moisture content of 60-65% and pH 5.5-6.5 (Gurung et al. 2012). Coarse sawdust mixed with fine sawdust are preferred, resulting in increased aeration and water holding capacity in order to allow optimum colonization of mycelia. Alder wood supplemented with wheat bran was reported to be the best substrate for artificial cultivation of *G. lucidum* (Gurung et al. 2012). Regarding spawn production, cereal grains such as barley, maize, pearl millet, soybean and wheat can be used for spawn production with barley grains yielding the best results (Joshi and Sagar 2016).

Since the 1980's, the cultivation of *G. lucidum* has developed rapidly, especially in China (Chang and Buswell 1999; Chang 2009). Currently wood log, short wood segments, tree stumps, sawdust bags and bottle procedures are the most popular methods for commercial

cultivation of *G. lucidum*. In the case of wood logs or stumps, dowels (overgrown wood fragments) are used for inoculation after which logs or stumps are allowed to fruit under natural, uncontrolled conditions. However, sawdust bags and bottle procedures produce higher yields for a shorter time period (Chang and Buswell 1999; Chang 2009).

Optimum growth conditions for the cultivation of *G. lucidum* include temperature, humidity and oxygen (Stamets 1993). Spawn production require a temperature between 25-32°C. The optimum moisture content of sawdust substrate is 65-70%. For primordial induction, humidity should be maintained between 90-100%, 80-95% during cap formation and 30-40% during the final stages of fruit body development. For primordia formation, optimum temperature between 18-24°C is required whereas the optimum temperature for fruiting body development is between 21-27°C. High CO<sub>2</sub> levels are essential throughout cultivation as levels between 20,000-40,000 ppm are required for primordia formation. However, lower levels of < 2000 ppm are required for fruiting body formation. Light of 200-500 lux for 4-8 hours are required during primordial formation whereas light at 750-1500 lux at 12 hrs cycles are required for fruiting body development. By taking all of this in consideration, primordial formation take up to 14-28 days to be completed while fruiting body formation will take up to 60 days (Stamets 1993).

#### **1.3.11 Nutritional profile of *G. lucidum***

A *G. lucidum* extract (% of dry weight) is determined to consists of 7.3% protein, 11.1% glucose, 68.9% folin-positive material, and 10.2% minerals (K, Mg, and Ca are the major mineral components) (Wasser 2005a). A 100 g serving contains approximately 367 calories; 15.05 g protein; 3.48 g fat; 0.50 g polyunsaturated fat; 1.20 g total unsaturated fat; 0.27 g saturated fat, 71 g carbohydrates; 66.80 g dietary fibre; 0 mg cholesterol; 0 IU vitamin A; 0.06 mg vitamin B1; 2.70 mg pantothenic acid; 0 mg vitamin C; 66 IU vitamin D; 37 mg calcium; 1.30 mg copper; 13.0 mg iron, 760 mg potassium; 12.40 mg niacin; 1.59 mg riboflavin; 0.014 mg selenium; and 6 mg sodium. All of this contribute to the high nutritional value of *G. lucidum* (Stamets 2005).

#### **1.3.12 Medicinally important compounds and extractions**

Important compounds such as triterpenoids (triterpenes), β-D-glucans and ganomycin have been found in *G. lucidum* (Chang and But 1986). Triterpenoids present in *G. lucidum* are responsible for the bitter taste associated with *G. lucidum*. Reportedly, *G. lucidum* contains 40 ganoderic acids, 14 ganoderiols, 5 ganolucidic acids, and 15 lucidenic acids (Cole and

Schweikert 2003). Furthermore, two triterpenoids were described and five new 20-hydroxylucidenic acids were isolated from the basidiocarps (Akihisa et al. 2005).

Triterpenoids are comprised of four groups, namely the volatile mono- and sesquiterpenes (essential oils) (C10 and C15), less volatile diterpenes (C20), non-volatile triterpenoids and sterols (C30), and the carotenoid pigments (C40). Most investigations are focused on the less volatile triterpenoid (triterpene) and sterol forms (Paterson 2006). The chemical structure of triterpenes is based on the structure of lanosterol, an important intermediate. Stereochemical rearrangements of lanosterol are responsible for structural diversity and the physiochemical properties of over 130 lanostane-type triterpenoids which have been described since the first isolation of ganoderic acids A and B (Kim and Kim 1999).

Chemical components such as polysaccharides, proteins, amino acids, fatty acids, steroids, alkaloids, and phenolic compounds with potential nutritional and medicinal values have been reported (Mizuno 1995; Paterson 2006; Boh et al. 2007; Singh et al. 2013). These compounds are considered to be responsible for the antimicrobial, anti-tumour, anti-diabetic, anti-oxidant, anti-inflammatory, and immunomodulatory properties of *G. lucidum* (Paterson 2006; Cao et al. 2012; De Silva et al. 2012a, b; De Silva et al. 2013).

Isolated polysaccharides from *G. lucidum* are  $\beta$ -1,3- and  $\beta$ -1,6-D-glucans with a variety of physiochemical properties (Paterson 2006). Paterson (2006) stated that “the structure is  $\beta$ -1,3-D-glucopyranan with 1-15 units of  $\beta$ -1,6-monoglucosyl side chains”. Other important polysaccharides acting as alternative anti-tumour compounds include glycoproteins (polysaccharides and proteins), heteropolysaccharides and ganoderans A, B and C (Lindequist 1995). Increased effectiveness of anti-tumour activity are brought about by increased water solubility as a result of the high molecular weights of the compounds (Lindequist 1995). However, some water insoluble polysaccharides are also known to possess anti-tumour activity where branching have an effect on activity (Wang et al. 1993). Interestingly enough, a higher amount of bioactive water-insoluble polysaccharides compared to water-soluble polysaccharides were reported (Kim et al. 2003).

Although polysaccharides and triterpenoids are the most thoroughly investigated, other compounds such as sterols, lectins and proteins have also been described (Paterson 2006). Sterols found in *G. lucidum* are closely related to triterpenoids and were found to have potent cytotoxic activity as well as anti-bacterial effects (Paterson 2006). Ergosterol peroxide (5 $\alpha$ , 8 $\alpha$ -epidioxy-22E-ergosta-6, 22-dien-3 $\beta$ -ol) is a steroid derivative isolated from *G. lucidum* and

has been reported to enhance the inhibitory effect of linoleic acid on mammalian DNA polymerase (Mizushima et al. 1998b). Free sterols have been determined to contain mainly ergosterol and 24-methylcholesta-7, 22-trien-3-ol (Shim et al. 2004). In the case of proteins, Ling Zhi-8 (LZ-8) is a new polypeptide consisting of 110 amino acid residues with an acetylated amino terminus and has been isolated from the mycelium of *G. lucidum* (Paterson 2006). An antifungal protein, namely ganodermin, has been isolated by Wang and Ng (2006) and found to inhibit growth of *Botrytis cinerea*, *Fusarium oxysporum* and *Phylospora piricola*. Additionally, proteins in the form of enzymes have also been isolated and galactosidase has been purified from *G. lucidum* fruiting bodies (Paterson 2006). Therefore, it is clear that all the compounds present in *G. lucidum* are important and responsible for the exhibited medicinal properties.

### 1.3.13 Medicinal properties

Anti-cancer (including leukaemia), anti-oxidant and antimicrobial (including HIV) are of the most extensively studied medicinal properties *G. lucidum* claims to possess. Research concerning the anti-tumour and immunomodulating properties of *G. lucidum* have been reported as early as 1957 and recently, compounds responsible for anti-tumour properties have been studied more extensively (Paterson 2006). Several bioactive glucans have been isolated in the early 1980's and the mode of action of polysaccharides as an anti-tumour agent is believed to be an enhancement of host-mediated immunity instead of direct cytotoxicity (Wang et al. 1993; Kim et al. 2003). Remarkably, *G. lucidum* is known to be one of the most popular species due to the variety of medicinal properties regardless of being non-edible as a result of its bitter taste and indigestible structure (Chang and Buswell 1996). Recently *G. lucidum* has become recognized as an alternative adjuvant for treating leukaemia, carcinoma, diabetes, and hepatitis while it has traditional uses to combat migraines, hypertension, arthritis, asthma, gastritis, hypercholesterolemia and cardiovascular problems (Hobbs 1995; Wasser 2005b; Paterson 2006).

A study performed by Sliva (2006) investigated the effects of *G. lucidum* on cancer cells and observed the inhibition of proliferation as well as apoptosis in leukaemia, lymphoma and myeloma cells. The inhibition of acute myeloblastic leukaemia was associated with cell cycle arrest and apoptosis whereas lymphoma inhibition was mediated by the upregulation of expression. Therefore, *G. lucidum* appears to inhibit direct signalling pathways in different cancer cells (Sliva 2006). Triterpenoids have potential as anti-cancer agent due to their direct cytotoxicity activity against tumour cells as growth and invasive behaviour of cancer cells are



suppressed (Gonzalez et al. 2002; Li et al. 2005; Sliva 2006). Additionally, tumour growth have been found to be inhibited through the activation of host-mediated immune responses by stimulating the production of cytotoxic T lymphocytes from mononuclear leukocytes (Lieu et al. 2002). Furthermore, the production of interleukin 2 is promoted while anti-tumour effect is increased by the attachment of polyol groups to glucans (Sone et al. 1985; Lei and Lin, 1992; Ooi et al. 2002).

Recently, there has been an increased interest in polysaccharides associated with *G. lucidum* as they are responsible for stimulating the immune system resulting in cytokine production and activation of anti-cancer activities of immune cells (Sliva 2006). In a study performed on mice, a glycoprotein isolated from *G. lucidum* were found to stimulate the proliferation of mouse spleen lymphocytes which resulted in an increase in B cells, production of interleukin 2, secretion of immunoglobulin and expression of protein kinase. Additionally, bioactive polysaccharides may stimulate blood mononuclear cells which resulted in the production of cytokines, tumour necrosis factor, interferons and interleukins to be increased (Paterson 2006). Furthermore, active  $\beta$ -D-glucans also have anti-tumour activity as it acts by binding to serum-specific proteins (Hobbs 1995; Wang et al. 2002). This binding is known as polysaccharide-mediated potentiation of the immune system and results in the activation of macrophages, T-helper, natural killer (NK) as well as other effector cells. Subsequently, the production of cytokines and antibodies are increased (Battle et al. 1998; Mueller et al. 2000).

A *G. lucidum* polysaccharide peptide (GI-PP) identified by Cao and Lin (2006), demonstrated anti-tumour as well as potential anti-angiogenesis activity. The possible mechanisms of GI-PP action on anti-angiogenesis of tumours were elucidated while the induction of apoptosis seems to be the mechanism for inhibition of proliferation (Paterson 2006). The exposure of human lung carcinoma cells to high doses of GI-PP in hypoxia for 18 h resulted in a decrease in an important chemical indicator of cancer. Furthermore, the anti-angiogenesis of GI-PP may be due to the direct inhibition of the proliferation of vascular endothelial cells or the indirect decrease of growth factor expression (Cao and Lin 2006). In another study where the anti-proliferating activity of *G. lucidum* were investigated, profound activity against leukaemia, lymphoma and multiple myeloma cells were observed (Müller et al. 2006). Therefore, *G. lucidum* may have application as adjunctive therapy for the treatment of hematologic malignancies (Müller et al. 2006).

The immunomodulating effects of *G. lucidum* were observed in patients with advanced colorectal cancer, but further studies need to be performed in order to validate the results

(Chen et al. 2006). As immunomodulating agent, *G. lucidum* polysaccharide (GI-PS) have been associated with a wide range of immune modulating effects. Results obtained from a study performed by Zhu and Lin (2006) confirmed that GI-PS was a promising biological response modifier and immune potentiator (Zhu and Lin 2006). Isolated triterpenoids may also have strong immunomodulating properties due to the activation of immune effector cells such as natural killer cells, T cells and macrophages (Smith et al. 2002). This activation results in cytokine, interleukin, interferon, and tumour necrosis factor- $\alpha$  production (Smith et al. 2002).

Interestingly enough, recent research reported that anti-tumour and immunomodulating properties were closely related to its anti-oxidant properties as extracts were found to be effective in preventing DNA from strand breakage (Paterson 2006). Oxidation is essential for energy production to allow functioning of biological processes (Wang et al. 2013). However, oxidative stress is the result of over-production of reactive oxygen species (ROS) such as hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\bullet OH$ ) and singlet oxygen under pathological conditions (Guo et al. 2010; Hu et al. 2010; Luo et al. 2010; Sun et al. 2010; Wang et al. 2010; Xie et al. 2010). At physiological conditions, ROS may be required for normal cell function, but excessive amounts of ROS may damage important cellular components (DNA, lipids and proteins) as interactions with free radicals result in cellular deterioration and ageing (Wang et al. 2013). Furthermore, the over-production of ROS are believed to be a causative agent of diseases such as cancer, cardiovascular diseases, rheumatoid arthritis and atherosclerosis (Chen et al. 2009; Sun et al. 2009; Liu et al. 2010). Although most organisms possess anti-oxidant and repair systems, these systems are incapable of preventing the damage completely (Tommonaro et al. 2007; Tseng et al. 2008).

A study conducted by Sun et al. (2004) investigated the anti-oxidant activity shown by isolated peptides including polysaccharides, polysaccharide-peptide complex and phenolic components of *G. lucidum*. However, *G. lucidum* peptide (LZ-8) were reported to be the major anti-oxidant component due to a decrease in the oxidation of low density lipoproteins as well as the scavenging of reactive oxygen species (Sun et al. 2004; Paterson 2006). Additionally, triterpenoids also seem to play a role in anti-oxidant activity due to the scavenging of superoxide anions resulting in the interruption of the chain reaction of free radicals (Chang and But 1986; Lee et al. 2001). Furthermore, *G. lucidum* is capable of preventing oxidative damage caused by chemotherapy due to the ability of inhibiting hydroxyl radicals (Lee et al. 2001). Isolated polysaccharides may enhance free radical scavenging via macrophages as well as the activity of interleukins, tumour necrosis factors, and natural killer cells (Smith et al. 2002).

In a study performed by Wang et al. (2013), extracted polysaccharides from *G. lucidum* fruiting bodies were sulphated and carboxymethylated in order to investigate the free radical scavenging and immunomodulatory effects (Wang et al. 2013). The injection of the two derivatives in female mice resulted in an increase in the mouse thymus and spleen index which is an indicator of increased immunity. Additionally, the production of two of the most important enzymes of the anti-oxidant defence system, namely superoxide dismutase and glutathione peroxidase were effectively increased. Finally, it was reported that the anti-oxidant activity of sulphated polysaccharides are superior to that of carboxymethylated polysaccharides (Wang et al. 2013).

Since *G. lucidum* is directly active as an antimicrobial agent, antibacterial, antiviral and antifungal activity have been reported (Wasser 2005a). As an antibacterial agent, extracts of *G. lucidum* mushrooms have the ability to inhibit the growth of *Helicobacter pylori* which is mostly associated with gastro-intestinal diseases such as gastric carcinoma, peptic ulcers, and gastritis (Wasser 2005a). Moreover, activity against gram-positive bacteria were observed from fruiting body extracts (Kim et al. 1993). Methanol extracts of the mycelium as well as culture extracts inhibited *B. subtilis* while ethanol extracts from the mycelium were found to have anti-inflammatory activity (Kendrick 1985).

Activity against various viruses including HIV and herpes have been observed, resulting in an increased interest in the antiviral activity of *G. lucidum*. The activity of *G. lucidum* polysaccharides are reported to be linked to their anionic characteristics and can inhibit the very early stages of viral infection such as attachment and penetration (Shannon 1984). Additionally, antiviral activity increases with molecular weight or degree of sulfation which is defined as the enzyme-catalysed conjugation of a sulfo group to another molecule (Witvrouw et al. 1994). Isolated triterpenoids have been reported to be responsible for the antiviral activity against HIV due to the ability to inhibit the activity of DNA polymerase, HIV-1 reverse transcriptase, HIV-1 protease and HIV-2 protease (El-Mekkawy et al. 1998; Min et al. 1998; Wasser 2005a).

The antiviral activity against herpes simplex virus (HSV) have been associated with an acidic protein bound polysaccharide (APBP) (Paterson 2006). This activity appeared to be related to the binding of APBP with HSV-specific glycoprotein at the cell membrane (Huie and Di 2004). Herpes simplex virus type 1 (HSV-1) and type 2 (HSV-2) have been found to be responsible for a wide range of infectious diseases (Eo et al. 1999). Furthermore, HSV infections were

reported as a risk factor for human immunodeficiency virus (HIV) while HSV-2 are recognized as an oncogenic virus due to its ability to convert cells into tumour cells (Hook 3rd et al. 1992; Lapucci et al. 1993). In a previous study, HSV-1 and HSV-2 were reported to be sensitive to water soluble compounds such as protein-bound polysaccharides (Eo et al. 1999). Isolated polysaccharides responsible for antiviral or anti-tumour property were reported to be branched  $\beta$ -glucans with (1,3)- $\beta$ -, (1,4)- $\beta$ - and (1,6)- $\beta$ -linkages which may be responsible for the inhibitory effect on the proliferation of HSV *in vitro* (Mizuno et al. 1984). The protein and polysaccharide appeared to be bound as the protein moiety was not completely removed during purification. However, the entity of this binding is still uncertain and therefore the mechanism is not fully understood (Eo et al. 1999).

#### **1.3.14 Application as medicinal mushroom**

When all of the previously discussed properties are taken in consideration, it is clear that *G. lucidum* has tremendous value which resulted in an increased interest in the possible applications it may have. Commercially known as “Lingzhi”, *G. lucidum* has been widely used due to its health benefits and products are available as powders, coffee, tea, drinks, tablets, capsules, syrups and dietary supplements (Chang and Buswell 1999; Lai et al. 2004; Singh et al. 2013; Kapoor and Sharma 2014). These products have effectively been commercialized as a food and drug supplement for health benefits (Hapuarachchi et al. 2015). Hence the increased popularity of *G. lucidum* fruiting bodies as a dietary supplement in China, Japan and North America (Hapuarachchi et al. 2015).

Annually, the sale of *G. lucidum* derived products is estimated to be more than US\$ 2.5 billion in Asian countries, including China, Japan and Korea (Li et al. 2013; Kapoor and Sharma 2014). In 2002, about 4900-5000 tonnes was produced with approximately 3800 tonnes produced in China (Lai et al. 2004). Due to artificial cultivation, about 4300 tonnes of *G. lucidum* are annually being produced in over ten countries with USA being the biggest market for *G. lucidum* based nutraceuticals (Kapoor and Sharma 2014).

As a functional food, it is a popular remedy to prevent and treat immunological diseases such as cancer; blood pressure; hypercholesterolemia; hepatitis A, B and C; hypertension; diabetes; cardiovascular problems; rheumatism; ulcers; gastritis; nephritis; bronchitis; asthma and arthritis (Liu et al. 2002; Paterson 2006; Wang et al. 2012; Kapoor and Sharma 2014). Additionally, *G. lucidum* has the unique property of strengthening the immune system as well as promoting longevity (Kapoor and Sharma 2014).

The anti-tumour activity of *G. lucidum* has been investigated and may have application as adjunctive therapy for the treatment of hematologic malignancies as well as leukaemia (Müller et al. 2006). Additionally, isolated triterpenoids and polysaccharides may have application as an immunomodulating agent due to the activation of immune effector cells (Smith et al. 2002). Furthermore, *G. lucidum* may aid anti-oxidant and repair systems naturally found in most organisms due to their ability to scavenge reactive oxygen species (Sun et al. 2004; Paterson 2006; Tommonaro et al. 2007; Tseng et al. 2008). The capability of *G. lucidum* to inhibit hydroxyl radicals result in the possible application to prevent oxidative damage caused by chemotherapy (Lee et al. 2001). Finally, *G. lucidum* has medicinal application as an antimicrobial agent due to its ability to inhibit *H. pylori*, *B. subtilis*, gram-positive bacteria, HIV and HSV-1 and HSV-2 (Kendrick 1985; Kim et al. 1993; Eo et al. 1999).

An interesting application of *G. lucidum* as animal feed recently emerged due to its ability to degrade the lignocellulosic complex in wood. Lignin is a heterogeneous polymer occurring in woody structures and surrounds cellulose in woody cell walls by forming a matrix (ten Have and Teunissen 2001). As a result, hemicellulose and cellulose, known as holocellulose are protected against microbial depolymerisation. As white-rot fungi, *G. lucidum* is capable of completely breaking down lignin to CO<sub>2</sub> and H<sub>2</sub>O while also allowing access to holocellulose as a source of carbon and energy (Kirk and Farrell 1987; ten Have and Teunissen 2001). Enzymes capable of degrading the lignocellulosic complex such as  $\alpha$ -amylase,  $\beta$ -glucosidase, cellulase, laccase, lignin- and manganese peroxidase, and xylanase are produced by *G. lucidum*, contributing to its application. The cultivation of *G. lucidum* on encroaching wood such as *Acacia mellifera* may aid in relieving stress caused by the recent drought. In addition, land is cleared to allow grazing for animals. Composted spent mushroom substrate from *G. lucidum* have application as animal feed due to breaking down of lignin and cellulose, allowing animals to further digest the wood.

#### **1.4 *H. erinaceus***

*H. erinaceus* is an edible fungus with great significance in medicine and is commonly known as Lion's Mane due to the appearance of a mane of cascading white spines (Stamets 2005) (Sokół et al. 2016). Previously known as *Hydnum erinaceum*, this mushroom is one of only a few that produces a lobster or shrimp flavour when cooked. Although not common in Europe, this species is found throughout Asia and Northern America (Thongbai et al. 2015).

Both the fruiting bodies and mycelia of *H. erinaceus* have been recognized by traditional Chinese and Japanese healers for years for as both medicine and cuisine (Stamets 2005; Sokół et al. 2016). Traditional Chinese healers used it for medicinal purposes such as treating gastritis for centuries. Additionally, *H. erinaceus* were also prescribed by Chinese medicinal practitioners for the prevention of gastrointestinal cancer (Stamets 2005). Recently there has been a great interest in this species since *H. erinaceus* may be used as a natural alternative to treat symptoms of dementia and cognitive decline in the elderly (Mizuno 1999; Ueda et al. 2008).

Studies have been performed and *in vitro* studies of extracted compounds performed on mice exhibited promising results (Sokół et al. 2016). The extracts have been found to be of use in the treatment of cancer, hepatic disorders, and Alzheimer's and Parkinson's diseases. This is due to the production of low molecular compounds, known as nerve growth factors (NGF) (Takei et al. 1989; Allen and Dawbarn, 2006). Interestingly enough, this specific species have also been found to have antimicrobial activity against two fungal species, namely *Aspergillus* species and *Candida* species (Stamets 2005). The numerous bioactive compounds associated with *H. erinaceus* resulted in the development of food supplements and as alternative medicines. Regardless of various studies, the mode of action of these active compounds is however still unclear (Thongbai et al. 2015).

#### **1.4.1 History and taxonomy of *H. erinaceus***

Although *H. erinaceus* has a long history of traditional medicine in Asia, it was first described in Northern America (Thongbai et al. 2015). Detailed descriptions and illustrations are mostly from European countries and is also reported to be found in Southern America. According to the Global Biodiversity Information Facility (GBIF; <http://www.gbif.org/species/5248508>), *H. erinaceus* was also recorded from Australia, but interestingly enough there are no records from Asia, regardless of large-scale cultivation. No *Hericium* species have been recorded in Africa, where the genus *Dentipellis* Donk is the only related existing species (Hallenberg et al. 2012; Zhou and Dai 2013). A new taxon, namely *Hericium erinaceum* subsp. *erinaceo abietis*, was described in the late 1970's (Sokół et al. 2016). However, this taxon was different from the typical *H. erinaceus* by means of morphological traits of the fruiting bodies, spore size and mycelial growth rate since studies have proved the described subspecies to be a sterile hybrid between *H. erinaceus* and *H. abietis* (Burdshall Jr. et al. 1978).

The *H. erinaceus* species used to belong to the class Basidiomycetes, subclass Holobasidiomycetidae, order Hericiales and family Hericiaceae (Wojewoda 1998). However, Index Fungorum presents the currently adopted taxonomy described by Persoon (1797). Taxonomically, *H. erinaceus* (Bull.:Fr.) Pers. 1797 belongs to the Fungi Kingdom, Basidiomycota Division, Agaricomycetes Class, Russulales order, Hericiaceae family, and *Hericium* genus with a species name of *H. erinaceus* (Thongbai et al. 2015).

The epithet is often still being misspelt, even in the current literature, as “*erinaceum*” (Thongbai et al. 2015). The correct epithet “*erinaceus*” literally means “hedgehog” in Latin and was proposed by Buillard as it reminded him of this animal. Alternatively, *H. erinaceus* is also known as “Bearded Hedgehog” and “Hedgehog Mushroom”. In Japan, this fungus is known “Yamabushitake” which means “Mountain Priest”. In China, “Houtou” which means “monkey head”, is the common name for *H. erinaceus*. In other parts of the world, *H. erinaceus* is also known as “Monkey’s Mushroom”, “Bear’s Head”, “Hog’s Head Fungus”, “White Beard”, “Old Man’s Beard”, “Pom Pom” and “Bearded Tooth” (Thongbai et al. 2015).

#### 1.4.2 Confusion in the nomenclature

Previously known as *Hydnum erinaceum* Fr., the taxonomy of *H. erinaceus* remained unchanged for the past decade (Thongbai et al. 2015). However, two species, namely *Hericium coralloides* and *Hericium abietis* are both similar to *H. erinaceus*, but are distinct in both their preferred habitat and form. Additionally, *H. coralloides* has been described under various synonyms, but didn’t have any effect on the taxonomy of the *Hericium* genus. Furthermore, the taxonomic situation of *Hericium cirrhatum* is still debated as this species is often being referred to as *Creolophus cirrhatum* by some mycologists and therefore considered to represent a separate genus (Thongbai et al. 2015). Recently, three new species were described with phylogenetic inference, namely *Hericium bharengense* and *Hericium yumthangense* from Himalaya, India (Das et al. 2011, 2013) as well as *Hericium rajchenbergii* from Argentina (Hallenberg et al. 2012).

It is easy to identify *H. erinaceus* in the mature state as the basidiomes consist of various single spines (Thongbai et al. 2015). Differentiation between species in the *Hericium* genus are done macroscopically by the presence of branched or unbranched hymenophore structures supporting various lengths of spines, occurrence of single spines or multiple clumps, and microscopically by amyloid basidiospores (Harrison 1973; Ginns 1985). However, basidiomes of *Hericium* often form a single clump from the primordia after which branches

only develop with age. This results in confusion as long-spine species may have short spines (1 cm in length or less) at their youngest age (Thongbai et al. 2015). In most cases, macromorphology of *H. erinaceus* is sufficient for identification. It is, however, difficult to differentiate certain growth forms of *H. erinaceus* from *H. coralloides* as both species have similar basidiospore sizes. Although *H. coralloides* basidiomes tend to be more branched, basidiomes with a massive body and long, graceful spines similar to *H. erinaceus* are known to exist, resulting in confusion. However, host substrates can be used as a way of identification as *H. coralloides* tends to occur on conifers while *H. erinaceus* are associated with deciduous trees. Furthermore, both *H. coralloides* and *H. erinaceus* can be cultivated on sawdust with the difference being that the spines of *H. coralloides* fork rather than emerge individually as in the case of *H. erinaceus* (Thongbai et al. 2015).

However, molecular methods can be applied to differentiate between species in the *Hericium* genus in order to clear up confusion in nomenclature (Thongbai et al. 2015). Identification of *H. erinaceus* can be done by making use of a set of polymerase chain reaction (PCR) primers specific to the internal transcribed spacer (ITS) nrDNA locus of *Hericium* species (Lu et al. 2002; Parfitt et al. 2005). Since studies have shown the close relation of the Hericiaceae family with different species of families Auriscalpiaecae, Echinadontaceae, Russulaceae, Schizophyllaceae and Stereaceae, the application of PCR made the verification of taxonomy possible. In a study conducted by Lu and colleagues, taxonomic identification and phylogenetic affiliation of representatives of the Hericiaceae family with other Holobasidiomycetidae were determined (Lu et al. 2002). As a result, Canadian strains previously known as *H. erinaceus* were isolated as a new taxon, namely *Hericium americanum*, as described in 1984 (Ginns 1985).

### **1.4.3 Diagnostic morphological characteristics of *H. erinaceus***

Consisting of downward, cascading, non-forking spines, *H. erinaceus* is typically white with the occurrence of discolouring to brown or yellowish brown when aged. Microscopic features include white spores; 4.5-5.5 by 4.0-4.5  $\mu\text{m}$  in diameter; smooth or minutely punctate-roughened; apiculate; short ellipsoid to sub globose; amyloid; smooth to slightly roughened and infrequent clamp connections. This species typically has a low spore load compared to other species (Hall and Stuntz 1971; Stamets 2005).

The basidiocarp of *H. erinaceus* is a solid knot of tissue; unbranched and long spines borne on the periphery of the carpophore ( $\pm 4$  cm) (Hall and Stuntz 1971). Furthermore, the basidia



are clavate, 40-50 x 5.3-6.0 µm and contains 4 spores. The cystidia is 43-52 x 5-7 µm; cylindrical; thick-walled, with denser contents than those of surrounding cells; and evenly distributed throughout the hymenial layer. The hyphae are 14µm; dimitic; moderately branched; thick-walled; curling and inter-woven; and clamp connections are present. *H. erinaceus* produces extensively branched fruiting bodies which are attached sideways to the substrate with a rounded or sub globose base (Gumińska and Wojewoda 1985; Stamets 1993). Since *H. erinaceus* mostly occurs on hardwoods, the long, peripheral spines and tuberculate sporocarp can be applied for identification (Hall and Stuntz 1971).

Mycelia of *H. erinaceus* are divided into two groups, namely monokaryotic and dikaryotic mycelia (Sokół et al. 2016). It has been found that only approximately 3% of monokaryotic mycelia are capable of yielding fruiting bodies while produced fruiting bodies were always smaller when compared to fruiting bodies produced by dikaryotic mycelia. A big difference between the two types are the production of hyphae with clamp connections in the case of dikaryotic mycelia while it is absent in the monokaryotic form. According to Sokół *et al* (2016), monokaryotic mycelia form 4 types of colonies, namely (*i*) thin, semi-airborne, showing a rapid growth comparable to that of a dikaryotic mycelium; (*ii*) compact, slower growing; (*iii*) thick and robust, characterized by very slow growth, and (*iv*) thin, with the slowest growth rate. Furthermore, monokaryotic mycelia is capable of producing chlamydospores (spindle-shaped, 6-8 x 8-10 µm in diameter) which may remain viable for approximately 7 years (Sokół et al. 2016).

#### **1.4.4 Distribution and Natural Habitat**

Although commonly found throughout the Northern Hemisphere, including Northern America, China and Japan, *H. erinaceus* is rarely found in tropical and polar regions (Doll 1979; Zhou and Liu 1988; Mao 1989; Stamets 1993; Grace and Mudge 2015). However, *H. erinaceus* is the most abundant species of the *Hericium* genus in the southern regions of the United States (Stamets 1993). In contrast, *H. erinaceus* is rarely found in Europe and was red-listed in 2003 in 13 of the 25 European countries as their natural habitats are deteriorating (Boddy et al. 2011; Govaerts et al. 2011). In Poland, all species from the Hericiaceae family are legally protected due to their rarity (Gumińska and Wojewoda 1985; Wojewoda 2003).

This specific species naturally occurs on dead or dying oak, walnut, beech, maple, sycamore, and other broadleaf trees. *H. erinaceus* is most commonly found on logs or stumps (Stamets 2005). Although *H. erinaceus* is predominantly a saprophytic mushroom, occasionally it may

also be considered as a weak parasite of trees as it is commonly found on dead or dying deciduous trees belonging to genera *Acer* sp., *Fagus* sp., *Juglans* sp., *Quercus* sp., and *Ulmus* sp. (Gumińska and Wojewoda 1985; Stamets 1993; Pegler 2003; Fora et al. 2009). However, the fruiting bodies of *H. erinaceus* may originate sometimes from knotholes or cracks of living hardwoods, indicating an endophytic lifestyle (Thongbai et al. 2015). In the UK, *H. erinaceus* commonly occurs on the central deadwood of trunks from September to December (Boddy and Wald 2003; Boddy et al. 2004, 2011).

#### **1.4.5 Cultivation of *H. erinaceus***

As *H. erinaceus* is rare to found in nature and habitats are threatened, artificial cultivation of this valued mushroom are increasing and was first documented in 1988 (Suzuki and Mizuno 1997). This species is known to be one of the few mushrooms that will produce well on walnut logs, regardless of decelerating cultivation due to the density of walnut wood (Stamets 2005). Preferred wood substrates include oak, elm, beech and other hardwood species while “paper” bark hardwoods such as alder and birch are not recommended. The preferred season of *H. erinaceus* is during the warm, wet months when temperatures are between 18-24°C (Stamets 2005).

Various substrates may be used for the cultivation of *H. erinaceus* such as sawdust supplemented with cereal bran and beech sawdust supplemented with wheat bran or corn meal (Kirchhoff 1996; Royse 1996; Oei 2003). High yields were obtained when wheat bran (20%), corn meal (7%) and glucose (3%) was added to sawdust as supplement (Siwulski and Sobieralski 2007; Siwulski et al. 2009). The process of primordia formation may be accelerated by adding an enriching substrate such as rice straw (20% of total substrate weight) of cultivars rich in nutrients (Zheng et al. 2002).

In China, large scale cultivation of *H. erinaceus* is achieved by making use of wood logs or stumps by inoculating these with dowels (wood fragments overgrown with *H. erinaceus* mycelium) after which they are incubated at high humidity (Sokół et al. 2016). Logs or stumps are allowed to fruit under natural, uncontrolled conditions which results in fruiting bodies to appear at various times ranging between several months to a year. However, the production of high yields with good quality fruiting bodies, require cultivation in bottles or bags containing sterilized substrates. Although cultivation in bags are cheaper and simpler, fruiting bodies are smaller compared to those growing from bottles, producing a single, large fruiting body (Stamets 1993; Sokół et al. 2016).

For mycelial growth of *Hericiium* species, various factors need to be taken in consideration (Sokół et al. 2016). Temperature and pH were found to be the most important parameters while depending on some factors such as light, moisture content and nutrient elements. The optimum temperature for mycelial growth is between 21-25°C while best growth was observed at pH 6 (Imtiaj et al. 2008). Optimum moisture content of substrate is 50-70%. Furthermore, mycelial growth is promoted by most carbon sources, except for lactose. Alanine proved to be the best nitrogen source while histidine was found to be the least advantageous source (Le Minh et al. 2009). According to Hu et al (2008) is the mycelial growth influenced by its enzymatic activity since *H. erinaceus* produces hydrolytic enzymes such as  $\alpha$ -amylase,  $\beta$ -amylase and protease to aid in the decomposition of lignin, cellulose, proteins and starch in the substrate (Kim et al. 2000b). Additionally, cellulose and laccase are also present with the latter having an effect on the growth period of *H. erinaceus* since high activity of laccase results in a shorter growth period (Hu et al. 2008; Sun et al. 2011; Fen et al. 2014).

Optimum growth parameters for the cultivation of *H. erinaceus* include temperature and humidity (Stamets 1993). Spawn production requires a temperature between 21-25°C with a relative humidity of 95-100%. The optimum moisture content of the substrate should be between 50-70% while CO<sub>2</sub> levels should be >5000-40 000 ppm with fresh air exchange every hour. For primordia formation, optimum temperature between 10-16°C is required whereas the optimum temperature for fruiting body development is between 18-24°C. It was suggested by Hu et al (2008) that a constant temperature of 23°C should be maintained for optimum yields of fruiting bodies. A relative humidity of 95-100% needs to be maintained for primordia formation while a lower humidity between 85-95% are required for fruiting body development. Relative low CO<sub>2</sub> levels between 500-700 ppm are required for primordia formation. However, higher levels of 500-1000 ppm are required for fruiting body formation. For both primordia and fruiting body formation, fresh air exchange every 5-8 hours are required. Light of 500-1000 lux are required during both primordial and fruiting body development. By taking all of this in consideration, primordial formation take up to 3-5 days to be completed while fruiting body formation will take up to 4-5 days (Stamets 1993).

#### **1.4.6 Nutritional profile**

A 100 g serving of *H. erinaceus* contains approximately 375 calories and 6.69 g moisture while providing 20.46 g protein; 5.06 g fat; 0.76 g saturated fat; 0.83 g polyunsaturated fat; 1.85 g total unsaturated fat; 61.80 g carbohydrates; 39.20 g dietary fibre; 0 mg cholesterol; 0 IU vitamin A; 0.16 mg vitamin B<sub>1</sub>; 2.26 mg B<sub>2</sub> (riboflavin); 7.40 mg B<sub>5</sub> (pantothenic acid); 0 mg

vitamin C; 57 IU vitamin D; 8 mg calcium; 1.66 mg copper; 6 mg iron; 11.80 mg niacin; 2.7 g potassium; 0.091 mg selenium; 4 mg sodium; and 5.99 g ash. Due to its lobster or shrimp flavour, it is often used as a meat alternative in soups (Stamets 2005).

#### **1.4.7 Medicinally important compounds and extractions**

Numerous studies conducted in recent years resulted in the isolation of various biologically active compounds from *H. erinaceus* (Qian et al. 1990; Kenmoku et al. 2001; Thongbai et al. 2015). Both the fruiting bodies and mycelia contain these compounds which have demonstrated therapeutic properties as well as pharmaceutical activity (Thongbai et al. 2015).

The most important bioactive compound include polysaccharides such as xylan, hericenones and erinacines which have been studied extensively for their potential and existing applications as functional foods and in pharmaceuticals (Mizuno and Nishitani 2013; Giavasis 2014; Sokół et al. 2016). Polysaccharides are mainly present in the cell walls of fungi and large quantities (approximately 20% of the biomass) are present in the fruiting bodies as well as mycelia (Lee et al. 2009). However, the total polysaccharide content in the fruiting bodies are 26.63% in contrast to 18.71% present in the mycelia (Mori et al. 1998). Four different polysaccharides, namely xylan, glucoxyllans, heteroxyloglucans and galactoxyloglucans were isolated from *H. erinaceus* (Mizuno et al. 1992). A new heteropolysaccharides (HEPF4) composed of D-fructose, D-galactose and D-glucose with 3-O-methyl rhamnose as minor component was isolated by Zhang et al. (2006, 2007). In a study conducted by Lee et al (2009), the crude water-soluble polysaccharides from the fruiting bodies were extracted and fractionated, yielding a  $\beta$ -1, 3-branched  $\beta$ -1, 6-glucan with a laminarin-like triple helix conformation (Lee et al. 2009). Alkaline extracts of the fruiting bodies resulted in a polysaccharide containing  $\beta$ -(1, 3)-linked D-glucopyranosyl residues with single galactose branches (Dong et al. 2006).

Aside from polysaccharides, various low-molecular weight secondary metabolites have been isolated from both the fruiting bodies and mycelia of *H. erinaceus* (Thongbai et al. 2015). These metabolites generally have poor water-solubility and organic solvents such as methanol or ethyl acetate are required in order to perform extractions. This also concerns erinacerins A and B, which are both fruiting body metabolites and seems to have no significant biological activities (Yaoita et al. 2005). Furthermore, various chlorinated aromatic compounds were isolated from submerged cultures and non-specific activities in biological systems are exhibited (Qian et al. 1990). Therefore, care should be taken that no significant amount is

present when dietary supplements are produced from *H. erinaceus* mycelia. Pyranones, such as erinapyrone A and B were also isolated from submerged cultures and found to have cytotoxic effects against HeLa S3 cells (Kawagishi et al. 1992; Ueda et al. 2009).

Two low-molecular weight compounds, known as hericenones and erinacines, isolated from the fruiting body and mycelium of *H. erinaceus*, respectively, may promote the biosynthesis of NGF (Takei et al. 1989; Ma et al. 2010). Recently, there has been an increased interest in possible alternatives for treating Alzheimer's disease which are caused by the functional deficiency of NGF and as such, plays a role in the advancement of the disease (Allen and Dawbarn 2006). NGF has potent biological activities, such as preventing neuronal death, promoting the outgrowth of neurite and is essential for maintaining and organizing neurons (Takei et al. 1989; Obara and Nakahata 2002). However, NGFs are proteins incapable of crossing the blood-brain barrier due to their size and being easily metabolized by peptidases (Ma et al. 2010). In contrast, hericenones and erinacines are capable of crossing the blood-brain barrier and may have application as treatment for Alzheimer's disease (Takei et al. 1989).

Aromatic compounds isolated from the fruiting body of *H. erinaceus* are known as hericenones. This compound is extracted with acetone, followed by "repeated chromatography of the chloroform-soluble fraction obtained by solvent partitions (chloroform and then ethyl acetate) of the extract with silica gel followed by HPLC with ODS column" (Ma et al. 2010). Benzyl alcohol derivatives, namely hericenones A, B, (Kawagishi et al. 1990), C, D, E, (Kawagishi et al. 1991), F, G, H, (Kawagishi et al. 1993), hericenones A-C (Alberto et al. 1995) and hericerin (Kimura et al. 1991) were isolated from *H. erinaceus* fruiting bodies. Hericenones A and B exhibited strong cytotoxic activity against HeLa cells while NGF biosynthesis were stimulated by hericenones C, D and E. According to Phan et al. (2014), the chain length and presence of double bonds in fatty acids have an effect on the activity of individual hericenones. In turn, hericenone E was found to have the greatest capacity for stimulating NGF biosynthesis due to the presence of two double bonds (Phan et al. 2014). Additionally, erinacerin A and B, 3-hydroxyhericenone F, hericenone I and hericenone J were also isolated from the fruiting bodies of *H. erinaceus* (Ma et al. 2010).

Erinacines A, B, C (Kawagishi et al. 1994), D (Kawagishi et al. 1996a), E, F, G (Kawagishi et al. 1996b), H, I (Lee et al. 2000), P (Kenmoku et al. 2000), Q (Kenmoku et al. 2002), J, K (Kawagishi et al. 2006), R (Ma et al. 2008) and erinacol (Kenmoku et al. 2004) were isolated from the mycelia of *H. erinaceus*. Consisting of angularly condensed five-, six-, and seven-

membered rings, erinacines are known to be cyathane-type diterpenoids (Ma et al. 2010). Erinacines A, B, C, E, F and H have exhibited potent inductive activity for the biosynthesis of NGF, both *in vivo* and *in vitro* (Kawagishi et al. 1994, 1996b; Kenmoku et al. 2000; Lee et al. 2000). Erinacine Q was isolated in 2002 by Kenmoku et al. (2002), who described its biosynthetic route to erinacine C (Kenmoku et al. 2002). Furthermore, Kenmoku et al. (2004) isolated both cyatha-3, 12-dien-14- $\beta$ -ol, known as erinacol, and 11-O-acetylcynthia A3, which were reported to be possibly related to biosynthesis of erinacine Q (Kenmoku et al. 2004). Erinacines are extracted with ethanol after which the extract is “fractionated by solvent partition between ethyl acetate and water followed by repeated silica gel chromatography and HPLC of the ethyl acetate extract” (Ma et al. 2010). Additionally, a biosynthetic intermediate of cyathane diterpenoids, namely cyatha-3, 12-diene and its isomer was also isolated from the mycelia of *H. erinaceus* (Ma et al. 2010).

However, a bioassay using mouse astroglial cells was performed by Mori et al. (2008), showed that the presence of erinacines resulted in greater amounts of NGF secreted than for hericenones. As a result, erinacines have been studied and found to be of value as potential alternatives for treating Alzheimer’s disease and peripheral nerve regeneration. Unfortunately, the detailed mechanism of NGF biosynthesis induction by erinacines remain unknown and further research are required (Shimbo et al. 2005).

Aside from the above-mentioned compounds, glycoside and six ergosterol derivatives were also isolated from dried fruiting bodies (Takaishi et al. 1991). Alternatively, ergosterol peroxide, a cytotoxic steroidal derivative, was isolated from the mycelia of *H. erinaceus* (Krzyczkowski et al. 2009). Furthermore, an alkaloid with anti-inflammatory activity, named hericirine was isolated as well as a new glycoprotein, namely HEG-5 with antineoplastic and hemagglutinating activities (Cui et al. 2014; Li et al. 2014d). Although *H. erinaceus* is one of the best medicinal mushrooms, new compounds are still being isolated and described, including ten new isoindolin-1 metabolites, namely erinacines C-L (Wang et al. 2015).

#### **1.4.8 Medicinal properties**

For centuries, *H. erinaceus* had application as anti-inflammatory medicine, immune booster, treatment of neurasthenia and general debility in Eastern Asia (Ying et al. 1987). Until recently the use of *H. erinaceus* as medication was only justified by the achievements and traditions of Chinese medicine (Sokół et al. 2016). However, the market in Europe and the USA are catching up since extracts (raw extracts or its fractions) were subjected to various tests to

exclude any harmful effects while confirming their health benefits, including treatment of Alzheimer's disease and leukaemia, enhancing mechanism of cancer cell apoptosis. Although various studies have been conducted, only preliminary clinical trials have been performed on humans. Regardless of this, the results of studies performed on animals are promising (Sokół et al. 2016).

Due to the production of erinacines and hericenones capable of inducing NGF production, *H. erinaceus* is commonly known for its nerve-regenerating abilities (Thongbai et al. 2015). As a highly-conserved protein, NGF is critical for survival as it is involved in promoting outgrowth of neurites, preventing neuronal death, maintaining and organizing neurons, supporting formation of synapses, and enhancing memory (Obara and Nakahata 2002). A deficiency in NGF plays a role in Alzheimer's disease which is "identified in patients by synaptic injury, deficiency of neurotransmitters, un-functioning and/or death of neuronal cells, and possibly by interference with the process of adult neurogenesis in the hippocampus", according to (Crews and Masliah 2010). However, NGF is unable of crossing the blood-brain barrier and are also easily metabolized by peptidases (Thongbai et al. 2015). In turn, hericenones and erinacines were investigated as they are low-molecular weight compounds capable of crossing the barrier, resulting in increasing the mRNA expression of NGF biosynthesis. Unfortunately the mechanism by which these compounds stimulate NGF production are still unknown and requires further investigation (Thongbai et al. 2015).

Hericenones A and B have shown significant cytotoxic effects against HeLa cells while hericenones C, D, E and H stimulated NGF synthesis *in vitro* (Thongbai et al. 2015). In contrast, other studies have reported the inability of hericenones C and D to increase synthesis of NGF in cell line 1321N1 (Mori et al. 2008). In the case of hericenone E, NGF synthesis was stimulated in rat pheochromocytoma (PC12) cells while also increasing the secretion of NGF. Additionally, phosphorylation on mitogen-regulated kinases or extracellular signal regulated kinases (MEK/ERKs) pathways was increased as well as protein kinase B (PKB) (Phan et al. 2014). However, hericenones F and G showed no stimulating activity on the synthesis of NGF under the same conditions (Kawagishi et al. 1991, 1993; Mizuno 1999). Furthermore, hericenones I, J and L were also identified with hericenone L showing cytotoxic effects against EC109 tumour cells (Ma et al. 2012). Regardless of all the evidence, the stimulating activity of hericenones on NGF biosynthesis is still heavily debated (Thongbai et al. 2015).

Erinacines are considered to have potential as alternative medicine for treating degenerative neuronal disorders as well as peripheral nerve regeneration (Thongbai et al. 2015). Erinacines

A, B, C, D, E, F, G, H and I have shown a strong stimulating activity on NGF biosynthesis in a study performed on murine astroglial cells with erinacine A exhibiting the strongest activity (Kawagishi et al. 1994, 1996a, b; Lee et al. 2000). As a result, NGF levels in the locus coeruleus and hippocampus of the rat were considerably increased, no increase in the cerebral cortex occurred (Shimbo et al. 2005).

Various studies have been performed in order to evaluate the mechanisms involved in the neuroprotective effects of erinacines and hericenones on the brain (Mori et al. 2008, 2009, 2011; Hazekawa et al. 2010; Phan et al. 2014). Erinacine A was found to have neuroprotection due to its ability of possibly acting as anti-inflammatory agent as well as preventing ischemic injury to neurons by making use of the global ischemic stroke model and the involved mechanisms (Lee et al. 2014). Additionally, potent nerve growth enhancing properties and the effective inhibition of neuronal cell death by means of phosphorylating p38 MAPK and CCAAT enhancer binding protein (C/EBP) and homologous protein (CHOP) as well as reducing levels of proteins containing nitrotyrosine was exhibited by erinacine A (Thongbai et al. 2015).

Abnormal accumulation of amyloid- $\beta$  peptide containing neurofibrillary tangles consisting of hyperphosphorylated Tau proteins are associated with patients with Alzheimer's disease (Murphy and Harry 2010). The endoplasmic reticulum (ER) is extremely important for inducing apoptosis, resulting in ER stress leading to the development of neurodegenerative diseases (Ueda et al. 2008). However, ER stress and amyloid- $\beta$  peptide toxicity seems to be reduced by isolated dilinoleoyl-phosphatidylethanolamine (DLPE) since neuronal death of neuro-2a cells are decreased by means of the protein kinase C pathway (Nagai et al. 2006). Furthermore, 3-hydroxyhericenone F seems to protect neuro-2a cells against neuronal death caused by ER stress (Ueda et al. 2008).

Myelin sheaths play an important role due to supporting and speeding up neural signals (Thongbai et al. 2015). Since these sheaths are wrapped around neuronal axons, damage to the structure leads to deterioration of the nerve system, resulting in neurodegenerative diseases. However, *H. erinaceus* has shown to effect the nerve tissue *in vitro*. Organic extracts from the fruiting bodies of *H. erinaceus* seems to have an enhancing effect on the myelination process (Moldavan et al. 2007). In a study conducted by Mori et al. (2008), it was reported that ethanol extracts have a stimulating effect on NGF biosynthesis by activating the c-Jun N-terminal kinases (JNKs) pathway (Mori et al. 2008).



In a separate study performed by Mori et al. (2009), the powder of air-dried *H. erinaceus* fruiting bodies, showed a significant prevention of cognitive impairment (Mori et al. 2009). In this preliminary clinical trial, *H. erinaceus* on 50-80 years old individuals with mild cognitive impairment was administered orally in a double-blind, parallel-group, placebo-controlled study. The cognitive ability of the *H. erinaceus* treated group have shown a significant increase on the cognitive function scale compared to those in the placebo group (Mori et al. 2009). Alternatively, an aqueous extract from *H. erinaceus* fruiting bodies, which were orally administrated, showed ability to promote the regeneration of a nerve-injury in an injured adult rat during the early stages of recovery (Wong et al. 2009, 2011). However, the mechanisms of hericenones and erinacines still needs to be determined and further studies are needed to confirm whether the compounds are capable of stimulating NGF synthesis in the brain *in vivo* (Thongbai et al. 2015).

Aside from neuroprotective activity, *H. erinaceus* also exhibited anti-tumour and immunomodulating activities (Thongbai et al. 2015). Various studies have been performed in order to evaluate the anti-tumour properties of *H. erinaceus* extracts on several cancers such as oesophageal-, intestinal-, pancreatic- and stomach cancers. Fewer side effects were reported for cancer patients treated with *H. erinaceus* compared to those being treated with chemotherapy and radiotherapy. Water-soluble polysaccharides have shown effectiveness against tumour cell lines *in vitro*, including oesophageal cancer (EC109), lymphoma (EL4), mammary carcinoma (MCF-7) and malignant hepatocytes (HepG2). Furthermore, various studies have reported the immunomodulatory effect of both aqueous and organic extracts of *H. erinaceus*, with the mechanisms of both activities seeming to rely on the same biochemical targets (Lee et al. 2010).

The anti-tumour activity of polysaccharides are characterised by activating different immune cells, such as expressing cytokines (IL-1 $\beta$  and TNF- $\beta$ ) and by activating nitric oxide (NO) production (Thongbai et al. 2015). In addition, the activation of c-Jun-N-terminal kinases (JNKs) revealed strong anti-tumour due to its involvement in apoptosis as well as suppressing the activity of nuclear factor kappa B (NF- $\kappa$ B) increasing in order to increase intracellular doxorubicin-mediated apoptotic signalling (Lee et al. 2010). Polysaccharides from aqueous extracts had shown anti-tumour activity against hepatocarcinoma while indirectly activating natural killer (NK) cells via the induction of IL-12 in splenocytes (Xu et al. 1994; Yim et al. 2007). HEP3  $\beta$ -D-glucans was characterized and anti-tumour activity on sarcoma 180 (S-180) in mice as well as artificial pulmonary metastatic tumour in was reported (Liu et al. 2000; Dong et al. 2006).

Moreover, these results have shown immunomodulating activities by increasing the amount cytotoxic cells, such as CD4+ cell, T lymphocytes (T cells) and macrophages (Thongbai et al. 2015). Thus, boosting the immune system by increasing the secretion of cytokines IL-10, IL-12 and IFN- $\gamma$  (Sheu et al. 2013). In a study conducted by Kim et al. (2011, 2013), CT-26 colon cancer was intra-cutaneous transplanted on the backs of Balb/c mice. By injecting the tumour daily for 2 weeks with a freeze-dried hot water extract and a microwave extract, there was a significant decrease of 38 and 41%, respectively, in the weight of the tumour (Kim et al. 2011, 2013). Therefore, the immune response is highlighted via increased phagocytosis of cytokines such as tumour-necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-1 $\beta$ , and interleukin-6 which enhance the activity of NK cells. Furthermore, a significant suppression of neo-angiogenesis inside the tumour was observed which mediated by decreasing pro-angiogenic factors, cyclooxygenase 2 (COX-2), vascular endothelial growth factor (VEGF), and 5-lipoxygenase (5-LOX). Additionally, NO production was restored up to 95-98% of normal levels and was observed in peritoneal macrophages (Kim et al. 2011, 2013).

Recently, erinacine D exhibited substantiate anti-tumour activity of on TNF- $\alpha$  and instigated inhibitory activity of NF- $\kappa\beta$ , which plays a vital role in the transcriptional regulation of adhesion molecules and various cytokines (Thongbai et al. 2015). Inhibitory activity of NF- $\kappa\beta$  was evaluated in human keratinocytes and found to have an IC<sub>50</sub> value of 9.7  $\mu\text{m}$  (Li et al. 2014a). However, results are yet to be reproduced *in vivo* and further evaluation of the results are needed as activity are low compared to commercially available drugs acting in the low nanomolar range in similar *in vitro* tests and (Thongbai et al. 2015).

Gastric cancer is the second most common cancer in the world and a 25% increase in all gastrointestinal (GI) cancers, including liver-, gastric- and colorectal cancers, has been documented by the National Cancer Institute in the USA (Dicken et al. 2005; Anand et al. 2008). Currently, the chemotherapeutic drug, fluorouracil (5-FU), is the main treatment for several types of cancer, but toxicity is severe (Thongbai et al. 2015). The potential of *H. erinaceus* extracts to treat gastrointestinal cancer in both *in vivo* and *in vitro* assays was investigated by Li et al. (2014a), who reported 5-FU to be less efficient and more toxic compared to *H. erinaceus* extracts (Li et al. 2014a). Furthermore, the effectiveness of extracts against colon cancer cells HT-29, and hepatic cancer cells HepG-2 and Huh-7 was confirmed in an *in vitro* study conducted by Li et al. (2014c). Additionally, water extracts as well as ethanol and ethyl acetate from *H. erinaceus* fruiting bodies have exhibited protective activity against gastric ulcers since isolated polysaccharides effectively inhibited *H. pylori*, the causative agent of many gastric disorders (Choi et al. 2012; Shang et al. 2013; Wong et al. 2013; Zhu et al.

2014) Interestingly enough, the immunomodulatory activity of *H. erinaceus* compounds may aid in overcoming bacterial infections by boosting the immune system of the human host (Thongbai et al. 2015).

Phenol-like and fatty acid-like compounds from *H. erinaceus* extracts act as antimicrobial agents against fungi and bacteria (Thongbai et al. 2015). Antimicrobial activity of water and alcohol extracts of *H. erinaceus* have been reported by Wang et al. (2001) after experiments were conducted on *Salmonella* Typhimurium TA98 in an *in vivo* assay by means of stimulating the immune system (Wang et al. 2001; Kim et al. 2012). A stronger anti-mutagenic activity was exhibited by the alcohol extract compared to the water extract while extracts produced from fruiting bodies also showed stronger activity than those produced from mycelia (Wang et al. 2001). However, Okamoto et al. (1993) reported antimicrobial activity of 4-chloro-3, 5-dimethoxybenzyl alcohol, 4-chloro-3, 5-dimethoxybenzaldehyde and chlorinated orcinol isolated from mycelial extracts (Okamoto et al. 1993). Hericene A, B, C and erinapyrone C exhibited moderate activity against gram-positive bacteria (Kawagishi et al. 1992; Alberto et al. 1995). In a study conducted by Eo et al. (2000) and Kim et al. (2000a), the gram-positive methicillin-resistant *S. aureus* (MRSA) was inhibited by mycelial extract of *H. erinaceus* with erinacine K showing direct inhibitory activity against MRSA (Kim et al. 2000a).

Anti-hyperglycemia effects of *H. erinaceus* extracts of mycelia and fruiting bodies were also reported in diabetic animals (Thongbai et al. 2015). In a study conducted by Vertesy et al. (1999), hericenol A, B and C were evaluated for therapeutic treatment of diabetes mellitus (Vertesy et al. 1999). The anti-hyperglycemia effects of fruiting body components, namely  $\alpha$ -D-glucan, D-arabinitol, D-threitol, and palmitic acid were also demonstrated (Wang et al. 2005; Hiwatashi et al. 2010). In addition, anti-hypercholesterolemic effects of these compounds were also exhibited due to a reduction in plasma total cholesterol, low-density lipoprotein cholesterol (LDL-C), triglycerides, phospholipid, atherogenic index and hepatic 3-hydroxy-3-methylglutaryl-coenzyme A (HMGCoA) reductase activity. The oral administration of *H. erinaceus* extracts demonstrated increased levels of plasma high-density lipoprotein cholesterol (HDL-C) in rats compared to the control group fed with saline (Yang et al. 2002, 2003; Wang et al. 2005).

The significant anti-hyperglycemia and anti-hypercholesterolemic effects of aqueous and methanol *H. erinaceus* extracts fed to streptozotocin-induced diabetic rats were demonstrated in various studies (Wang et al. 2005; Liang et al. 2013). Increased serum insulin levels and significantly lower elevation rates of blood glucose levels occurred in rats fed with a methanol

*H. erinaceus* extract compared to an untreated control group. Moreover, hypoglycemic effects were demonstrated by ethanol extracts of *H. erinaceus* due to the possible regulation of lipid metabolic gene expression of C57BL/6 in mice with diabetes mellitus (Hiwatashi et al. 2010). Although no clinical trials on humans have been conducted yet, various studies on *H. erinaceus* extracts have indicated the potential of treating metabolic disorders as well as preventing cardiovascular diseases (Thongbai et al. 2015).

Polysaccharides isolated from *H. erinaceus* have shown anti-ageing properties by enhancing the activity anti-oxidant enzymes (Xu et al. 2010). Lipopolysaccharide (LPSs) isolated from mycelia have shown significant anti-oxidant activities in mice by increasing hepatic glutathione levels (Jang et al. 2010). The pre-administration of  $\beta$ -glucans resulted in increased activities of anti-oxidant enzymes as well as decreasing levels of lipid peroxidation. Furthermore, anti-ageing activities were reported for  $\beta$ -glucans as they are capable of inhibiting matrix metalloproteinase (MMP)-1, and the tissue inhibitor of matrix metalloproteinase (TIMP)-1 activities in aged rat models (Ma et al. 2010; Xu et al. 2010). Additionally, methanol extracts from dried fruiting bodies are capable of anti-oxidant activity due to the presence of polyphenols (Mau et al. 2002; Huang et al. 2008; Mujic et al. 2010). Endo-polysaccharides isolated from ethanol extracts of mycelia, were reported to exhibit exceptionally high anti-oxidant activity *in vitro* (Zhang et al. 2012). Mycelial extracts were reported to exhibit the highest total phenol content as well as the highest ferric reducing antioxidant power (FRAP), resulting in hot water extracts of *H. erinaceus* mycelia being investigated in order to evaluate the anti-oxidant activity *in vitro* (Ferreira et al. 2009; Abdullah et al. 2012; Thongbai et al. 2015).

Methanol extracts from *H. erinaceus* proved to have hepatoprotective activity since three polysaccharide fractions, namely HEP40, HEP60 and HEP 80, showed protective action to liver cells (Choi et al. 2005; Zhang et al. 2012). The hepatoprotective activity observed *in vivo* are debated by many scientists to be related to the anti-oxidant activity observed *in vitro*. Furthermore, apoptosis of human hepatocellular carcinoma cells has been reported to be induced by *H. erinaceus* while supplementation with *H. erinaceus* could have a restraining effect on hepatic damage caused by severe alcohol exposure (Lee and Hong 2010; Liu et al. 2015). Methanol extracts of *H. erinaceus* mycelia also showed protective action on CCl<sub>4</sub>-induced hepatic damage (Choi et al. 2005). Therefore, *H. erinaceus* has application in preventing and treating various hepatic disorders (Zhang et al. 2012).

#### 1.4.9 Application as medicinal mushroom

All of the medicinal properties and compounds associated with *H. erinaceus*, contribute to its practical applications. High enzymatic activity for  $\alpha$ -amylase,  $\beta$ -glucosidase, cellulase, laccase, lignin- and manganese peroxidase, and xylanase are demonstrated by *H. erinaceus* (Ko et al. 2005). As a result, composted spent mushroom substrate from *H. erinaceus* has application for the commercial scale production of above-mentioned enzymes as it is a readily available and cheap source of enzymes to be used for bioremediation such as treating sewage in the paper and pulp industry (Wang et al. 1992; Phan and Sabaratnam 2012). Furthermore, *H. erinaceus* associated enzymes may have applications in the food industry since derived substrates from edible fungi are often regarded safe and more easily improved for industrial applications compared to enzymes from unidentified organisms (Thongbai et al. 2015).

Although research is still fairly new, *H. erinaceus* proved to be a good candidate for promoting health. Various commercial products and promising novel drugs, especially immunosuppressive agents are available in various forms (Thongbai et al. 2015). Due to the enhancement of the immune system, *H. erinaceus* is now being used as food supplement. Nevertheless, there are still some challenges regarding the development of pharmaceutical standards for extracting compounds from *H. erinaceus* fruiting bodies and mycelia. Sandwich biscuits containing *H. erinaceus* fruiting bodies have contributed to the therapeutic treatment and prevention of nutritional anaemia in pre-school children (Liu et al. 1992). Healthy beverages, including a sport drink and tea, was prepared from extracts of dried fruiting bodies and mycelia and demonstrated improved liver function as well as preventing of diabetes in China (Wang et al. 2005; Imtiaj et al. 2008; Qin et al. 2015). *H. erinaceus* tablets used in Chinese hospitals have proved to be effective anti-aging and anti-inflammatory agents as well as effectively treating oesophageal carcinoma and digestive tract ulcers while extending life expansion of cancer patients (Ying et al. 1987; Chen 1992; Liu et al. 1992; Poucheret and Rapior 2006; Li et al. 2014a).

In Malaysia, 100% pure *H. erinaceus* powder are capsulated and marketed as a supplement capable of promoting regeneration after peripheral nerve injury (Wong et al. 2013). In addition, the potential of *H. erinaceus* to stimulate synthesis of NGF resulted in *H. erinaceus* being placed on the list of "Nature's Nutrients for the Neurons" (Kawagishi et al. 2004). Various patents regarding inventions related to the application of hericenones and erinacines as nutraceutical or medicinal products for patients with neurological trauma have been submitted

in recent years. Recently, pharmaceutical products containing erinacerin A and B claimed to be protective agents for preventing dementia by stimulating NGF (Noh et al. 2004).

Since erinacines have good potential for treating neurological disorders, various studies have been conducted in order to investigate synthetic methods to provide erinacines for further exploitation (Ma et al. 2010). However, the biosynthesis of natural products is complex with many factors influencing the expression of many key genes responsible for synthesis. The construction of the 5-6-7 tricyclic core of erinacines is the key step for producing these natural products. Currently, a low yield, multi-step synthetic method is being used for producing large quantities of erinacines. However, this restricts their commercial application, resulting in fermentation being investigated for providing erinacines for industrial use (Ma et al. 2010).

Extensive research has been done on the anti-tumour activity of *H. erinaceus* extracts and may have applications in treating gastrointestinal-, colon- and hepatic cancer (Li et al. 2014a, b). Isolated polysaccharides and erinacine D are responsible for anti-tumour activity with the activation of different immune cells as the mode of action (Thongbai et al. 2015). Additionally, *H. erinaceus* extracts also have strong immunomodulating activity by boosting the immune system of the human host (Thongbai et al. 2015). Three polysaccharide fractions of *H. erinaceus* exhibited hepatoprotective activity while protective action on CCl<sub>4</sub>-induced hepatic damage were also reported (Choi et al. 2005). Additionally, hepatic damage caused by severe alcohol exposure may be restricted via supplementation with *H. erinaceus* extracts (Lee and Hong 2010; Liu et al. 2015). In turn, *H. erinaceus* has application in preventing and treating various hepatic disorders (Zhang et al. 2012).

Polysaccharides isolated from extracts of *H. erinaceus* exhibited both anti-hyperglycaemic and anti-hypercholesterolemic effects (Thongbai et al. 2015). Even though no human clinic trials have been performed, this species may have application for the treatment of metabolic disorders while also preventing cardiovascular diseases (Thongbai et al. 2015). In addition, anti-ageing activity was also exhibited by polysaccharides and polyphenols via enhancement of anti-oxidant enzymes (Mau et al. 2002; Huang et al. 2008; Mujic et al. 2010; Xu et al. 2010). Extracts from *H. erinaceus* have applications as antimicrobial agents due to the inhibitory activity of phenol-like and fatty acid-like compounds against fungi and bacteria (Thongbai et al. 2015). Furthermore, protective activity against gastric ulcers are due to the inhibition of the gram-negative bacteria, *H. pylori* (Choi et al. 2012; Shang et al. 2013; Wong et al. 2013; Zhu et al. 2014).

## 1.5 Conclusions

To conclude, both *G. lucidum* and *H. erinaceus* have an extensive range of medicinal properties, resulting in being used for centuries in Asia. Recently, the interest of these species increased in European as well as Western populations. In Northern America, mushroom extracts are sold without the need of being approved or registered.

For the cultivation of *G. lucidum* and *H. erinaceus*, various substrates can be used while different factors were found to greatly influence the growth of both species in both field and laboratory conditions. Therefore, it is important to evaluate these factors in order to obtain optimum mycelial and fruiting body growth (Kapoor and Sharma 2014).

A wide range of medicinal properties are exhibited by *G. lucidum* and *H. erinaceus*, adding to their potential as natural alternatives for the treatment of various ailments. Moreover, their nutritional value will further add to the overall benefits. Since they are low in fat with no cholesterol, they can be used as a natural alternative to lower cholesterol, blood pressure, and blood glucose levels. It can also be used as an alternative to treat diabetes since insulin levels are lowered. The high protein content (38-43%) of mushrooms compared to protein content of meats (17-22%) makes this food product a highly popular alternative to obtain protein under vegetarians. Their richness in vitamins and minerals can also be used as a natural alternative to prevent deficiencies and ailments linked to deficiencies. As a result, these species have a variety of applications, including providing nutraceutical and pharmaceutical products. Therefore, medicinal mushrooms and their derivatives as nutraceuticals/dietary supplements may help to alleviate suffering caused by various diseases (Chang and Buswell 1996).

Various clinical trials have been performed and *G. lucidum* seems to be effective against a wide range of viral infections such as HPV, HEC-V and HIV. Moreover, further research on antimicrobial agents of basidiomycetes are anticipated to lead to new antibiotics on the market (De Silva et al. 2013; Stadler and Hoffmeister 2015). Clinical trials performed in Asia (China, Korea, Japan and Malaysia), focusing on the effectiveness of bioactive compounds isolated from *H. erinaceus*, are now receiving much attention since promising potential of hericenones and erinacines capable of enhancing NGF synthesis were reported (Thongbai et al. 2015). These compounds are currently being scrutinized as they may be apply as natural alternative to treat the symptoms of Alzheimer's diseases and senility in the elderly. Moreover, clinical trials have also been done in order to determine whether certain medicinal mushroom species are effective against cancer. Clinical trials have proved *H. erinaceus* to be effective against

gastric and oesophageal cancer. The anti-tumour activity of *G. lucidum* are brought about by inhibiting DNA polymerase and post-translational modification of the Ras oncoprotein, or the stimulation of cytokine production (Mizushima et al. 1998a; Sliva 2006).

Furthermore, mushrooms can aid in recycling waste via bioremediation in order to reduce environmental pollution as well as producing animal feed (Stamets 2005). Both species produce various enzymes with various applications. For *G. lucidum*, enzymes can aid in the degradation of lignocellulosic substances in order to provide animal feed of high nutritional value. In the case of *H. erinaceus*, composted spent mushroom substrate can be applied to produce of high quantities of enzymes to be used in the food industry as well as for bioremediation.



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## Chapter 2

### Optimisation of growth conditions for the cultivation of medicinal *G. lucidum* and *H. erinaceus* mushrooms

#### III. Abstract

From literature, it is clear that both *Ganoderma lucidum* and *Hericium erinaceus* have various medicinal properties. In order to allow investigation of these medicinal properties, cultivation of *G. lucidum* and *H. erinaceus* had to be optimised. *Acacia mellifera* is considered to be an encroaching tree species in South Africa and may have economic benefits when used as substrate for the cultivation of *G. lucidum* to provide animal feed due to the ability of *G. lucidum* to break down complex lignocellulosic materials. As a result, the conversion of *A. mellifera* to animal feed may aid in relieving the stress of the drought. Modifications to various factors resulted in improve yields of *G. lucidum* when cultivated on *A. mellifera* wood and resulted in the application as animal feed which will be discussed and investigated further on in chapter 4. For *H. erinaceus*, cultivation on *A. mellifera* wood and pecan nut shells proved to be a sustainable method for removal of these waste products. Obtained yields for both *G. lucidum* and *H. erinaceus* were standardized and running costs were reduced. Cultivated *G. lucidum* and *H. erinaceus* can further be used to verify medicinal assumptions as well as aid in the sustainable removal of waste products. In this chapter, the optimum growth conditions for the cultivation of *G. lucidum* and *H. erinaceus* will be investigated as optimisation of cultivation for both species are important for further investigation of medicinal properties as well as possible applications.

## 2.1 Introduction

Mushrooms are macro-fungi which can be identified according to their different shapes, sizes, colours, spore colour and chemical reactions. For this chapter, the growth conditions for cultivation of medicinal *G. lucidum* and *H. erinaceus* were optimised. Although *G. lucidum* is non-edible and infrequently found in nature, it is considered to be the most superior medicinal mushroom and has been used for centuries due to its medicinal value (Stamets, 2005; Hapuarachchi et al. 2015). As a saprophyte and wood-decaying fungus, *G. lucidum* causes white rot of a wide variety of trees and are found on the widest range of hardwood species, including beeches, elms, oaks and even palms (Stamets 2005; Kapoor and Sharma, 2014; Hapuarachchi et al. 2015).

Various different substrates have been used for the cultivation of *G. lucidum* while also maintaining specific growth parameters such as temperature, light intensity, relative humidity, water content, air and pH (Miles and Chang 2004). For mycelial growth, light and pH are of the most important parameters while some factors such as temperature, culture media and nutrient elements are also essential (Kapoor and Sharma 2014). All of these factors were found to greatly influence the growth of *G. lucidum* in both field and laboratory conditions. Therefore, it is important to evaluate these factors in order to obtain optimum mycelial growth (Kapoor and Sharma 2014). Artificial cultivation of *G. lucidum* was successfully achieved the first time in the early 1970's (Chang and Buswell 1999; Chang 2009). Since the 1980's, the cultivation of *G. lucidum* has developed rapidly, especially in China (Chang and Buswell 1999; Chang 2009). Currently, wood logs, short wood segments, tree stumps, sawdust bags and bottle procedures are the most popular methods for commercial cultivation of *G. lucidum*. In the case of wood logs or stumps, dowels (overgrown wood fragments) are used for inoculation after which logs or stumps are allowed to fruit under natural, uncontrolled conditions. However, sawdust bags and bottle procedures produce higher yields within a shorter time period (Chang and Buswell 1999; Chang 2009).

Optimum growth conditions for the cultivation of *G. lucidum* include temperature, humidity and oxygen (Stamets 1993). Spawn production requires a temperature between 25-32°C with an optimum moisture content of 65-70%. For primordial induction, humidity should be maintained between 90-100%, 80-95% during cap formation and 30-40% during the final stages of fruiting body development. For primordia formation, optimum temperature between 18-24°C is required whereas the optimum temperature for fruiting body development is between 21-27°C. High CO<sub>2</sub> levels are essential throughout cultivation as levels between 20,000-40,000 ppm

are required for primordia formation. However, lower levels of < 2000 ppm are required for fruiting body formation. Light of 200-500 lux for 4-8 hours are required during primordial formation whereas light at 750-1500 lux at 12 hrs cycles are required for fruiting body development. By taking all of these in consideration, primordial formation takes up to 14-28 days to be completed while fruiting body formation will take up to 60 days (Stamets 1993).

As a saprophytic mushroom, *G. lucidum* is commonly found on hardwood species, causing white rot (Goodell et al. 2009). Since white-rot fungi possess both cellulolytic and lignin degrading enzymes, they have the potential to degrade an entire wood structure when under the correct environmental conditions, resulting in the utilization of lignin as a food source while leaving behind cellulose (Goodell et al. 2009). Therefore, *G. lucidum* can be cultivated on substrates containing lignin due to their ability to break down complex lignocellulosic materials. As a result, mushroom cultivation has application as an alternative method for the breakdown of agri-waste products as approximately 70% of agricultural and forest products are discarded as wastes (Chang 2009).

An encroaching tree species in South Africa, namely *A. mellifera*, is currently occupying large areas of farm land in the Northern Cape Province and has a detrimental impact on grazing land due to suppressing the growth of grass (Hagos 2001; Lukomska et al. 2010; Joubert et al. 2012; Lohmann et al. 2014; Fourie 2015). Furthermore, the loss of grazing and grassland have a detrimental effect on the availability of animal feed, especially during times of drought (Fourie 2015). Therefore, the conversion of the encroaching *A. mellifera* to animal feed by cultivation of *G. lucidum*, may aid in relieving the stress of the drought if the wood could be degraded sufficiently to expose the cellulose and hemicellulose within (Fourie 2015).

Another well-known edible mushroom, *H. erinaceus*, is currently being scrutinized due to its ability to stimulate the growth of nerve growth factors. Therefore, it may be used as a natural alternative to treat the symptoms associated with Alzheimer's and cognitive decline. This mushroom species naturally occurs on dead or dying oak, walnut, beech, maple, sycamore, and other broadleaf trees and are most commonly found on logs or stumps (Stamets 2005). Although *H. erinaceus* is predominantly a saprophytic mushroom, occasionally it may also be considered as a weak parasite of trees as it is commonly found on dead or dying deciduous trees belonging to genera *Acer* sp., *Fagus* sp., *Juglans* sp., *Quercus* sp., and *Ulmus* sp. (Gumińska and Wojewoda 1985; Stamets 1993; Pegler 2003; Fora et al. 2009). *H. erinaceus* is known to be one of the few mushrooms that will produce well on walnut logs regardless of decelerating cultivation due to the density of walnut wood (Stamets 2005). The preferred wood



substrates include oak, elm, beech and other hardwood species. However, “paper” bark hardwoods such as alder and birch are not recommended.

In China, large scale cultivation of *H. erinaceus* is achieved by making use of wood logs or stumps by inoculating these with dowels (wood fragments overgrown with *H. erinaceus* mycelium) after which they are incubated at high humidity (Sokół et al. 2016). Logs or stumps are allowed to fruit under natural, uncontrolled conditions which result in fruiting bodies to appear at various times ranging between several months to a year. However, the production of high yields with good quality fruiting bodies, require cultivation in bottles or bags containing sterilized substrates. Although cultivation in bags are cheaper and simpler, fruiting bodies are smaller compared to those growing from bottles, producing a single, large fruiting body (Stamets 1993; Sokół et al. 2016). Due to the rarity of *H. erinaceus* in nature and threatened habitats, artificial cultivation is increasing and was first documented in 1988 (Suzuki and Mizuno 1997). A wide variety of substrates such as sawdust supplemented with cereal bran and beech sawdust supplemented with wheat bran or corn meal may be used for the cultivation of *H. erinaceus* (Kirchhoff 1996; Royse 1996; Oei 2003). The preferred season of *H. erinaceus* is during the warm, wet months when temperatures are between 18-24°C (Stamets 2005).

Optimum growth parameters for the cultivation of *H. erinaceus* include temperature and humidity (Stamets 1993). For mycelial growth of *Hericium* species, various factors need to be taken in consideration (Sokół et al. 2016). Temperature and pH were found to be the most important parameters while depending on some factors such as light, moisture content and nutrient elements. The optimum temperature for mycelial growth is between 21-25°C while best growth was observed at pH 6 (Imtiaj et al. 2008). The optimum moisture content of the substrate is between 50 and 70%.

Spawn production requires a temperature between 21-25°C with a relative humidity of 95-100%. The optimum moisture content of the substrate should be between 50-70% while CO<sub>2</sub> levels should be >5000-40 000 ppm with fresh air exchange every hour. For primordia formation, optimum temperature between 10-16°C is required whereas the optimum temperature for fruiting body development is between 18-24°C. It was suggested by Hu et al. (2008) that a constant temperature of 23°C should be maintained for optimum yields of fruiting bodies. A relative humidity of 95-100% needs to be maintained for primordia formation while a lower humidity between 85-95% is required for fruiting body development. Relative low CO<sub>2</sub> levels between 500-700 ppm are required for primordia formation. However, higher levels of

500-1000 ppm are required for fruiting body formation. For both primordia and fruiting body formation, fresh air exchange every 5-8 hours is required. Light of 500-1000 lux is required during both primordial and fruiting body development. By taking all of these in consideration, primordial formation take up to 3-5 days to be completed while fruiting body formation will take up to 4-5 days (Stamets 1993).

## **2.2 Materials & Methods**

### **2.2.1 Medium development for pure cultures**

For the cultivation of *G. lucidum* and *H. erinaceus*, sorghum agar was prepared by milling sorghum to a powder. Three litres of distilled water (dH<sub>2</sub>O) and 15 g/l agar were added to 1kg of milled sorghum. Glucose (2%) was added to serve as carbon source. The mixture was microwaved until boiling occurred. The supernatant was used to fill Schott bottles up to 400 ml. The mixture was autoclaved for 15 minutes at 121°C and 100 kPa. After cooling down to 50°C, the medium was used to pour petri dishes.

### **2.2.2 Cultivation to obtain pure cultures**

Prepared sorghum agar was used to obtain pure cultures of *G. lucidum* (UFS-GL1) and *H. erinaceus* (UFS-L1). The isolates, cultivated on Potato Dextrose Agar (PDA), were received from the Mushroom Guru. Isolates were cut into squares with a sterile needle and placed on sorghum agar. The plates were incubated at 25°C for 14-21 days. Additionally, both strains were also transferred to PDA as back-up.

### **2.2.3 Preparation of first-generation spawn**

For the preparation of first-generation spawn, loose sorghum grains were soaked overnight in water with the addition of 2% gypsum. Drainage of excess water was achieved by spinning sorghum in a washing machine (SpeedyVac) at the lab. Coffee bottles were filled with 400 g sorghum and autoclaved as the preferred method for sterilization. Agar squares were cut with a sterile needle and added to the sorghum to produce spawn to be used as inoculum for second generation spawn bags. After inoculation, the bottles were incubated at 25°C for 1-2 weeks to allow full colonization to take place.

#### **2.2.4 Preparation of second-generation spawn bags**

For the preparation of second-generation spawn bags, the same method was used as with first-generation spawn. However, polypropylene bags instead of coffee bottles were used. Polypropylene bags were filled to a weight of 800 g and autoclaved as the preferred method for sterilization. First generation spawn (400 g) were divided (200 g) to inoculate two sorghum bags, respectively, to be used as inoculum for both *G. lucidum* and *H. erinaceus*. After inoculation, the bags were incubated at 25°C for 1-2 weeks to allow full colonization.

#### **2.2.5 Growth bag preparation**

Waste products such as pecan nutshells and encroaching *A. mellifera* (blackthorn wood) were desired substrates for cultivation. In the case of *G. lucidum*, *A. mellifera* was the desired substrate. Wood chips were soaked in water for 24 h. Drainage of excess water was achieved by spinning the substrates in a washing machine (SpeedyVac at the lab). Polypropylene bags were used as the whole growth block needs to be exposed to allow fruiting. The bags were filled to a weight of 800g (wet weight) and sterilized. However, for *H. erinaceus* cultivation, three different substrates, namely pecan nutshells, *A. mellifera* wood and a combination of pecan nutshells and *A. mellifera* wood were the desired substrates. Both *A. mellifera* and pecan nutshells are considered to be a waste product. In order to determine the effect of different substrates on yields, dry weight of substrates was measured and soaked separately for 4 hrs by making use of stockings. Dry weights for different substrates were as follow: ± 220g of pecan nutshells; ±110g of *A. mellifera* wood; and ±85g of *A. mellifera* wood and ± 75g of pecan nutshells for combined substrate. For drainage of excess water, spinning of substrates in a washing machine (SpeedyVac at the lab) was performed after which wet weights were determined. For *H. erinaceus*, coffee bottles were used in order to support fruiting bodies. The bottles were filled with the substrates and sterilized.

#### **2.2.6 Cultivation of medicinal mushrooms on different substrates**

Cultivation on different substrates was performed using a modified protocol from Stamets (1993). For artificial cultivation of *G. lucidum*, sterile substrate bags containing *A. mellifera* were inoculated with second generation spawn. The bags were incubated at 25°C for approximately 4-6 weeks to allow colonization until the appearance of mycelium growth onto bag. Bags were transferred to fruiting chamber to allow antler and cap formation. For fruiting of *G. lucidum*, black containers with aeration holes at the top and LED lights inside the box

(12 V and 6 500 K) were used to allow control over both the lighting and CO<sub>2</sub>/O<sub>2</sub> concentrations. For further control over CO<sub>2</sub>/O<sub>2</sub> concentrations and humidity, the aeration holes were covered with 3M Micropore Dressing Tape (24mm x 10m). This allowed control over high CO<sub>2</sub> concentrations needed during the initial stages for antler formation as the Micropore tape acted as selective membrane for oxygen transfer. As soon as antler formation was completed, higher O<sub>2</sub> concentrations were needed. The Micropore tape was removed as *G. lucidum* fruiting bodies grow in the direction of O<sub>2</sub> to escape the high CO<sub>2</sub> blanket at the bottom. During both antler and fruiting body formations, high humidity levels were achieved by wetting Perlite every 1-2 hrs while optimum light cycles of 4—8 hrs and 12 hrs were also incorporated for antler and fruiting body formation, respectively.

In the case of *H. erinaceus*, sterile coffee bottles were inoculated with second generation spawn. The bags were incubated at 25°C for approximately 4-6 weeks to allow formation of pins. As soon as pins appeared, the bags were transferred to a fruiting chamber for 7-15 days or until fruiting bodies appear.

### **2.2.7 Comparison of different substrates**

Selection of optimum substrates was based on calculations using wet and dry weights to compare yields.

## **2.3 Results & Discussions**

### **2.3.1 Mushroom isolation for pure cultures**

Pure mycelial cultures were obtained within 15 days after isolation of *G. lucidum* and *H. erinaceus* on sorghum-based medium (Stamets 1993). The cultivation of the mushrooms on this specific medium was successful with enhanced growth compared to standard cultivation media and both mushrooms were cultivated on the same medium instead of having to use different media. The sorghum based agar proved to be a better medium for the cultivation of these mushrooms than PDA and malt extract agar (MEA), as used by Stamets (1993).

## 2.3.2 Cultivation of medicinal mushrooms

### 2.3.2.1 G. lucidum

The cultivation of *G. lucidum* on *A. mellifera* wood was successful as antlers (**Fig. 1**) were obtained within 28 days and fruiting bodies (**Fig. 2**) were obtained after 65 days. Optimum growth conditions were achieved and contributed to the successful cultivation. These conditions include a spawn production temperature between 28-30°C and a moisture content of 68%; a humidity of 96%, 90% and 35% for primordial induction cap formation and final fruiting stages, respectively. Furthermore, optimum temperatures for primordia formation and fruiting body development were found to be 23°C and 27°C, respectively. The CO<sub>2</sub> levels were found to be 40,000 ppm and < 2000 ppm for primordia and fruiting body formation, respectively. Finally, 8hrs of light at 500 lux were used during primordial formation whereas light at 1500 lux at 12 hrs cycles was used for fruiting body development. Since *A. mellifera* wood is considered to be a waste product in South Africa, cultivation may have economic benefits. More importantly, *A. mellifera* wood has application as animal feed after cultivation since the complex and indigestible lignocellulosic complex are degraded. By determining the lignin and cellulose content before and after cultivation, it can be evaluated whether the complex was sufficiently degraded, resulting in the application as animal feed. Due to the use of waste products for cultivation, the costs have been reduced with more than 70% compared to cultivation on standard substrates.



**Fig. 1.** Antler formation of *G. lucidum*.



**Fig. 2.** Development of *G. lucidum* fruiting bodies on *A. mellifera* wood in laboratory conditions.

#### 2.3.2.2 *H. erinaceus*

The distinctive fruiting body of *H. erinaceus* resulted in the common name, the Lion's Mane mushroom. The cultivation of this mushroom on three different substrates was successful and optimum growth conditions contributed to the successful cultivation (**Fig. 3**). For evaluating the effect different substrates have on *H. erinaceus* cultivation, two waste products as well as a combination of these products were used for cultivation (**Fig. 3**). Both *A. mellifera* wood and pecan nut shells are currently considered to be waste products in South Africa and the use of these products as substrates for the cultivation of medicinal mushrooms, may have economic benefits and is considered to be a sustainable method of removal. Instead of using a liquid inoculation as described by Stamets (1993), spawn bags were prepared by using sorghum. Even though liquid inoculation of grain filled spawn jars is the preferred method, the use of spawn to inoculate substrate had no negative effect on the cultivation of *H. erinaceus* since the growth was successful and high yields were obtained.

Optimum conditions include weak, diffused light, a temperature of 25°C and a humidity of 95-100%, which were achieved by using Perlite. The use of transparent fruiting chambers did not have a negative effect on the growth of *H. erinaceus*.



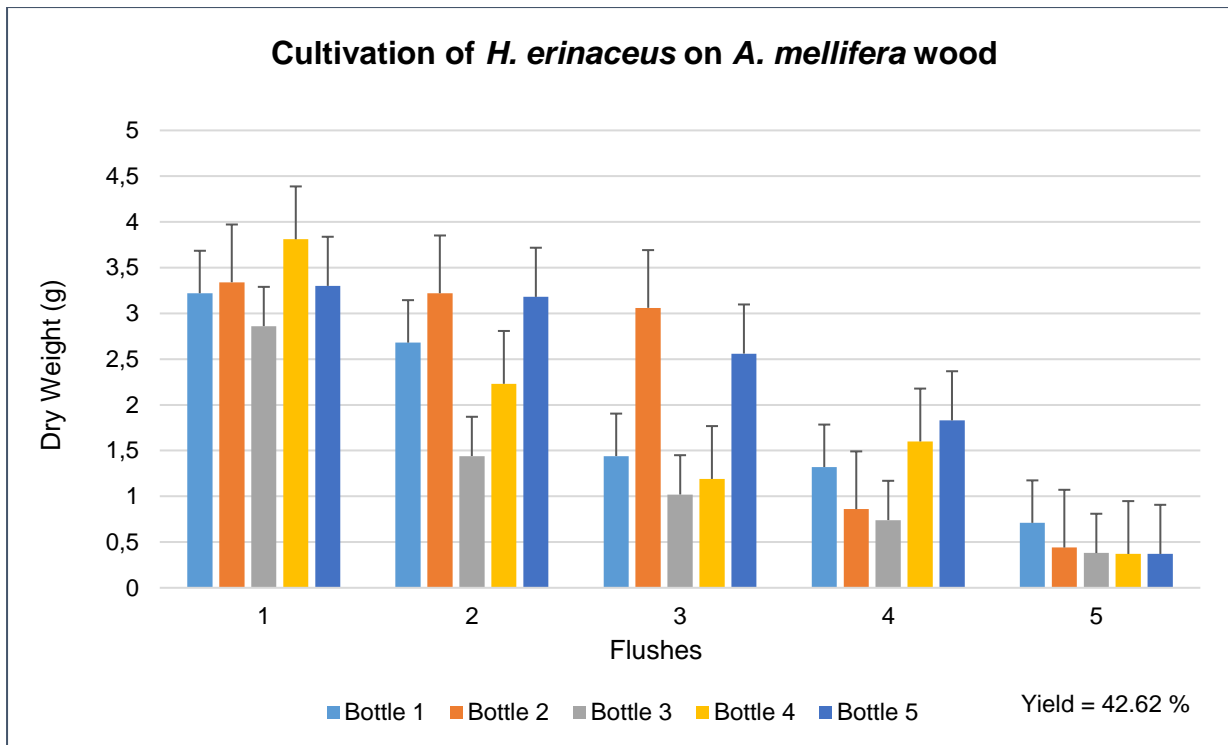


**Fig. 3.** Cultivation of *H. erinaceus* on different substrates with optimum growth conditions.

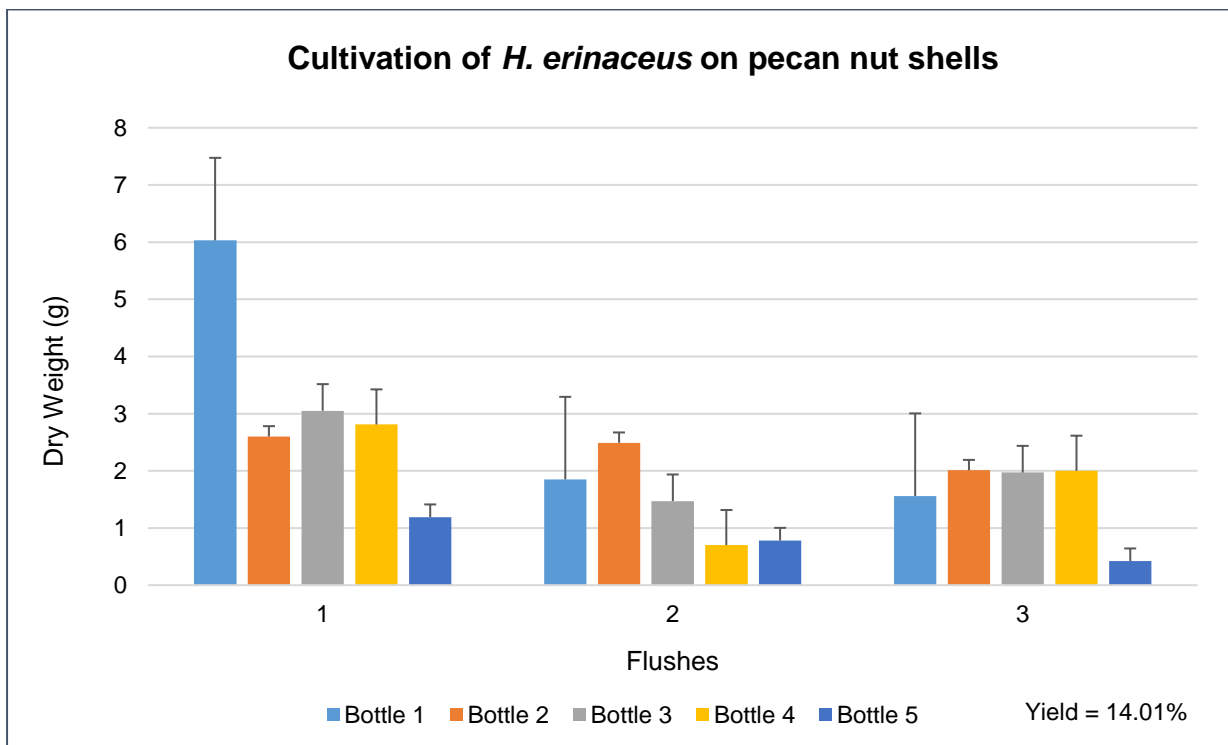
As depicted in **Fig. 4**, a dry yield of 42.62% was obtained for cultivation on *A. mellifera* wood whereas a dry yield of only 14.01% was obtained when cultivated on pecan nut shells (**Fig. 5**). When cultivated on a combination of *A. mellifera* wood and pecan nut shells, a dry yield of 42.01% was obtained (**Fig. 6**). By comparing **Fig. 4** and **Fig. 5**, it is clear that the different substrates had a significant effect on the yields obtained from *H. erinaceus* since the mean difference between the yields were 28.61%. Therefore, it is clear that *A. mellifera* wood is the preferred substrate as the highest yield were obtained.

In the case of cultivation on *A. mellifera* as well as on the combination, five flushes were obtained whereas only three flushes were obtained when cultivated on pecan nut shells only. When cultivated on *A. mellifera* only (**Fig. 4**), flushes 1-3 showed high yields of fruiting bodies as wet weights varied between 18.34 to 30.38 g whereas lower yields between 2.43 and 8.9 g were obtained during flushes 4 and 5. For cultivation of a combination of *A. mellifera* wood and pecan nut shells (**Fig. 6**), flushes 1-3 showed high yields of fruiting bodies as wet weights varied between 16.15 and 51.99 g while lower yields between 3.8 and 9.35 g were obtained for flushes 4-5. However, during the first flush for cultivation on pecan nut shells (**Fig. 5**), yields between 18.39 and 23.57g were obtained while significantly lower yields were obtained during the final flushes. The reason for pecan nut shells resulting in such low yields is unknown and further investigation is required. Therefore, it is clear that the first flushes resulted in higher yields than the last flushes, which is due to the depletion of nutrients.

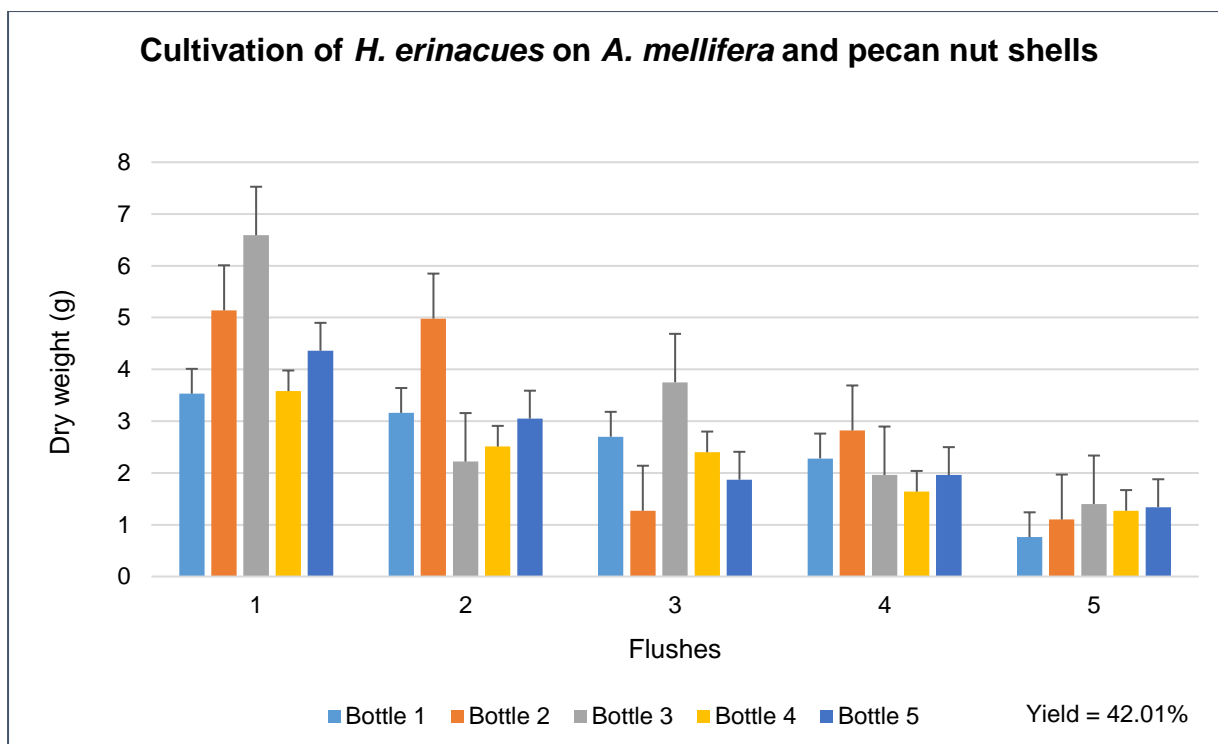




**Fig. 4.** Illustration of yields for *H. erinaceus* cultivation on *A. mellifera* wood.



**Fig. 5.** Yields obtained for *H. erinaceus* cultivation on pecan nut shells.



**Fig. 6.** Obtained yields for cultivation of *H. erinaceus* on a combination of *A. mellifera* wood and pecan nut shells.

## 2.4 Conclusions

Recently, there has been an increase in the popularity of medicinal mushrooms as a natural alternative to treat a wide range of ailments. Both *G. lucidum* and *H. erinaceus* have extensive applications and have been used for centuries in Japan and China. Throughout literature, the medicinal properties of these two species are mentioned with antimicrobial properties being specifically important. The reason being the possibility of serving as natural alternatives to treat bacterial infections in the case of antibiotic-resistant bacteria. In the case of *H. erinaceus*, various studies have been conducted to investigate the ability to treat symptoms of Alzheimer's disease naturally, but this is still ongoing.

Before medicinal properties can be investigated, cultivation of *G. lucidum* and *H. erinaceus* had to be optimised in order to perform various extractions. As cultivation requires pure cultures, the need for a single medium that allows cultivation of both *G. lucidum* and *H. erinaceus* was imperative. Therefore, sorghum agar was developed, resulting in better growth compared to YMA or MEA. Sorghum was used as it is considered to be an extremely good source of nitrogen and nutrients. Furthermore, it is considered to be a simpler medium as no additional supplements are required.

For *G. lucidum* cultivation various factors were taken in consideration and modified to improve yields on *A. mellifera* wood. These modifications were based on improved lighting and different lighting cycles, humidity control as well as CO<sub>2</sub> regulation in order to improve both antler and fruiting body formation. Due to sufficient breakdown of lignin, *A. mellifera* wood has application as animal feed which will be further investigated. In the case of *H. erinaceus*, humidity control resulted in high yields of fruiting bodies. Cultivation on *A. mellifera* wood and pecan nut shells proved to be a sustainable method for removal of these waste products.

For the cultivation of *G. lucidum* and *H. erinaceus*, the obtained yields were standardized and as a result, running costs were reduced. This reduction was more significant in the case of *A. mellifera* wood as it is currently a waste product in South Africa and there are no additional costs involved when used as a substrate. Due to sufficient yields and decreased costs, both *G. lucidum* and *H. erinaceus* fruiting bodies were pulverized and used for the development of capsules in collaboration with the pharmaceutical company, Sfera. Currently, AntiflamCare: Inflammation Support is commercially available and has been produced by using Reishi and Lion's Mane cultivated during this study. These capsules contain a combination of Reishi and Lion's Mane which are a formidable combination against inflammation. The addition of Athriblend® containing Glucosamine sulphate, Boswellin®, Curcumin C3 Complex® and Bioperine® support the medicinal mushrooms against inflammation.

From literature, it is clear that these species have various medicinal properties, rendering them great natural alternatives due to a rise in the need for natural alternatives to treat various ailments. Therefore, optimisation of *G. lucidum* and *H. erinaceus* cultivation are important for further investigation of medicinal properties as well as possible applications. Cultivated *G. lucidum* and *H. erinaceus* can be used to verify medicinal assumptions as well as aid in the sustainable removal of waste products.

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## Chapter 3

### Optimisation of extraction methods, evaluation of antimicrobial properties and hepatotoxicity assays

#### IV. Abstract

Fungi are considered to be an important source of traditional medicine in both Asian and Western countries. Different extraction methods were employed for extraction of valuable compounds of two South African macro-fungal species, namely *Ganoderma lucidum* and *Hericium erinaceus*. Preliminary separation of compounds was done with thin layer chromatography to proof the presence of multiple foreign compounds. The antimicrobial activity of the extracts was investigated against 12 different pathogens. Combined water and alcohol extracts of *G. lucidum* fruiting bodies and spent mushroom substrate (SMS) showed the most promising results. Hepatotoxicity assays of the extracts were performed on HepG2 cells using different parameters such as live/dead cells, lysosomes, mitochondrial dysfunction and steatosis to evaluate toxicity. A limited number of the extracts showed toxicity, with alcohol extracts proving to be more toxic compared to water extracts. Interesting data are presented in this study as the effect of extracts on antimicrobial activity were limited. Additionally, hepatotoxicity assays are considered to be novel and this study provides interesting data on the cytotoxicity of two macro-fungal species, *G. lucidum* and *H. erinaceus*.

### 3.1 Introduction

Various medicinal mushrooms have been screened for their medicinal properties, especially for anti-cancer application (Mizuno 1999). Additionally, some species have been found to be powerful immune system enhancers, leading to the potentiation of animal and human immunity against cancer (Borchers et al. 1999; Wasser and Weis 1999; Ikikawa 2000; Kidd 2000; Feng et al. 2001). Aside from China, the use of medicinal mushroom extracts is well documented in Japan, Korea, Russia and recently in the USA (Mizuno et al. 1995). However, the application of chemical technology for isolating relevant compounds to be used in controlled experiments only recently received attention. Although various species have yielded compounds with strong anti-cancer activity, only a small number of mushroom species have been used in clinical trials for assessing the anti-cancer potential in humans (Smith et al. 2002).

Various studies have been conducted in recent years to improve production and utilization of *G. lucidum* and *H. erinaceus* compounds. The major bioactive compounds include polysaccharides, sterols, lectins and proteins, with the first two being the most extensively studied. Although  $\beta$ -glucans are generally more abundant than other polysaccharides, they are still poorly characterised due to difficulty to isolate and identify (Askin et al. 2007). The major bioactive polysaccharides are  $\beta$ -1, 3- and  $\beta$ -1, 6 glucans, with a basic structure of  $\beta$ -1-3 D-glucopyranan with 1-15 units of  $\beta$ -1-6 monoglycosyl side chains (Mizuno et al. 1995). The isolation and identification of polysaccharides commonly involve various steps (Villares et al. 2012). Since it has been demonstrated that both the molecular structure and arrangement have a significant effect on the biological activity, various analytical methods are used for the interpretation of the chemical structures. It has been established that molecular weight, primary structure, solution conformation, and polymer charge may play a role in determining whether and with what affinity polysaccharides bind to receptors and exhibit biological activity (Villares et al. 2012). Therefore, the extraction of polysaccharides is thus extremely important for the application of both *G. lucidum* and *H. erinaceus* (Huang and Ning 2010).

Up until recently, numerous extraction methods have been developed with the main objective being to obtain higher yields of extracts at a lower cost. Extractions with organic solvents such as methanol (Escribano-Bailón et al. 1992; Fuleki and Da Silva 1997), ethanol (Kallithraka et al. 1997) and acetone (Vernhet et al. 1996) proved to be of value. However, these methods have the disadvantage of leaving residual solvent in the product, which is unacceptable for human application. As a result, researchers have been focusing on finding alternatives, such

as hot water extraction which is the most widely used traditional method (Huang and Ning 2010). Different classes of compounds are extracted when making use of hot water, but is however dependant on the temperature being used. Since the majority of compounds are effectively extracted while retaining their effectivity, the interest in hot water extractions are justified. Advantages of this extraction method include efficiency, low costs, clean, quick, and has the possibility of automation and selectivity due to the ability of modifying the polarity of the extraction medium (Askin et al. 2007).

For determination of compounds, UV spectrophotometry and high-performance liquid chromatography (HPLC) can be used (Askin et al. 2007). For determination of polysaccharides, glucose is often used as standard with the HPLC method for polysaccharide determination being gradually developed. For triterpenoid determination, oleanolic acid or ursolic acid is commonly used as the standard substance (Askin et al. 2007). Thin layer chromatography (TLC) can be applied for identifying compounds present in *G. lucidum* and *H. erinaceus*. As a solid-liquid technique, a solid (stationary phase) and a liquid phase are present as different compounds have different solubility and adsorption to the two phases between which they are to be partitioned.

Several triterpenoids and polysaccharides isolated from *G. lucidum* have been investigated regarding their physiological effects (Komoda et al. 1989; Shao 1992). Isolated triterpenoids from *G. lucidum* are responsible for important anti-cancer activity as they are capable of inhibiting the blood-supply to cancerous cells, resulting in the absence of oxygen and nutrients (Kimura et al. 2002). The main polysaccharides isolated from *G. lucidum* have been identified as ganoderan and ganopoly, both consisting of a  $\beta$ -linked glucose backbone with different patterns and degrees of branching (Villares et al. 2012). Isolated polysaccharides have been proven to have a wide range of medicinal properties, with immune system modulation being the most extensively studied. Specifically polysaccharides with  $\beta$ -linkages have been found to show significant anti-oxidant, antimicrobial and anti-tumour activity due to the activation of the immune system (Villares et al. 2012).

In the case of anti-tumour activity, various mechanisms such as anti-proliferation of cancer cells and induction of apoptosis, immune system regulation and anti-metastatic effects are involved in the mitogenic activity of fungal polysaccharides (Villares et al. 2012). Recently, there has been an increase in compounds isolated from *G. lucidum* as triterpenoids, polysaccharides and fungal immunomodulatory proteins (FIPs) exhibited anti-tumour activity by means of inhibiting DNA polymerase and post-translational modification of Ras oncoprotein



as well as stimulating cytokine production (Sliva 2006). For anti-oxidant activity, polysaccharides have shown activity for the scavenging of free radicals and superoxide radicals, inhibition of lipid peroxidation, and suppression of proliferation and oxidative stress (Villares et al. 2012). In addition, increased activity of enzymes such as superoxide dismutase and catalase, which are involved in removing ROS, are also exhibited by polysaccharides (Bishop et al. 2015).

The fruiting bodies of *H. erinaceus* contain bioactive substances such as aromatic compounds, sterols, diterpenoids, and polysaccharides (Huang and Ning 2010). Various studies have identified polysaccharides with immunomodulatory activity while aromatic compounds such as erinacines and hericenones have showed to promote the synthesis of nerve growth factors (NGF) (Ma et al. 2010). Additionally, hericenones also have cytotoxic effects against various cancer cell lines, but the mechanisms by which aromatic compounds exhibit anti-cancer activity are not fully understood (Li et al. 2015).

Cellomics, also referred to as high content analysis, are used to identify bioactive compounds (synthetic or from natural products) to aid in the development of new drugs as well as to investigate the molecular mode of action (van de Venter 2018). The ImageXpress® Micro XLS System is a wide-field automated microscope capable of fluorescence, transmitting light, and phase-contrast imaging and high content analysis of fixed- or live-cell assays, tissues and small organisms. It is equipped with a 4.66-megapixel scientific CMOS camera, objectives ranging from 10x-60x with optional phase contrast and a sample preparation station for the preparation of 384-well plates. The solid-state white-light engine is suitable for most fluorescence applications with excitation wavelengths ranging between 380-680 nm. The detection of chromogenic- and most fluorescent dyes are possible due to a colorimetric filter cube and eight fluorescence filter cubes. Environmental control allows regulation of temperature, humidity and CO<sub>2</sub> for high-content analysis and time lapse experiments over extended time periods. Finally, MetaXpress Imaging and Analysis software are being used by the instrument. Using automated imaging capabilities, morphological features are observed and recorded while changes in cell populations are quantified by the powerful software (van de Venter 2018).

Although there is a wide believe that herbal medicine is relatively safe as they are derived from nature, this belief is however untrue and misleading (van de Venter 2018). Several known toxic compounds have been isolated from nature and various reports highlighted the consequences from certain herbal remedies despite years of folk usage. In contrast to acute

toxicity being easily identified due to clear overt symptoms, idiosyncratic and chronic toxicity is unlikely to be identified unless fully investigated clinically. Moreover, herbal medicines have been shown to inhibit and/or induce drug-metabolizing enzymes. Herbal medicines are nowadays used together with conventional drugs, giving rise to the relatively modern phenomenon of kinetic and clinical drug interactions. Furthermore, due the continuous development of new drugs, potential drug interactions will continue to modify the safety landscape of herbal medicine (van de Venter 2018).

Similar to conventional drugs, the safety of herbal medicines also needs to be undergone both pre-clinical and clinical evaluation for toxicity (van de Venter 2018). Even though animal models (particularly rodent models) form an integral part of pre-clinical drug efficacy, it has been established that these models poorly portray toxicity in humans and therefore have little predictive value. Due to the availability of an enormous amount of herbal medicines, it is clear that clinical safety evaluation will be time-consuming and expensive. An achievable and more practical approach to explore potential adverse effects of herbal medicines, is the use of human derived cells for *in vitro* screening, provided there is confidence in the predictive capacity of such tests (van de Venter 2018).

A new *in vitro* approach for medicinal plant research, is hepatotoxicity assays (HCA) (van de Venter 2018). Since toxicity is regarded as the main reason for drug attrition, it presents a major challenge for the pharmaceutical industry. Therefore, the early identification of potential toxicity has become a key research objective for pharmaceutical companies. To this end, HCA has emerged as a suitable predictive tool for the identification of toxicity and currently represents the most promising approach to early drug development. The complex nature of macro-fungal extracts may be expected to interact simultaneously at various targets, resulting in the multi-parameter capacity of image cytometry to be well suited for early identification of toxicity. Moreover, HCS has developed extensively over the past few years, resulting in an increase in the most predictive endpoint parameters to accurately identify toxicity potential. For this chapter, hepatotoxicity assays for live/dead cells, lysosomes, mitochondrial dysfunction and steatosis will be investigated (van de Venter 2018).

### **3.1.1 Live/dead cells**

Live/dead cell assays are important for evaluating effects of compounds on cells (Immunochemistry Technologies). Hoechst 33342 is a popular cell-permeant, benzimidazole nuclear stain, emitting blue fluorescence when bound to dsDNA by binding to the minor groove

of adenine and thymine-rich sequences (Sabnis 2010). This dye is often used for visualization the nuclei of living cells. Additionally, Hoechst 33342 is used for cell cycle studies due to the ability to distinguish condensed pyknotic nuclei in apoptotic cells from living cells (Lalande et al. 1981; Burrell et al. 1992; Sabnis 2010).

In the case of dead cells, a popular red-fluorescent nuclear dye known as propidium iodide (PI) is used to evaluate cell viability (ThermoFisher). This dye is not permeant to live cells and can only enter the cell after cell death occurred, resulting in application for detecting dead cells as well as to differentiate necrotic, apoptotic and healthy cells (ThermoFisher; Suzuki et al. 1997). PI binds to DNA by intercalating between the bases with no or little preference to sequences (ThermoFisher).

### **3.1.2 Lysosomes**

Lysosomes are membrane-enclosed organelles containing a variety of enzymes capable of degrading proteins, nucleic acids, carbohydrates and lipids by means of engulfment (Kuehnel 2003). Since lysosomes function as the digestive system, it is responsible for degradation of material taken up from outside the cell as well as digestion of obsolete compounds of the cell itself. As enzymes responsible for hydrolysis require an acidic environment for optimal activity, the lysosome maintains a pH ranging from 4.5 to 5.0 by pumping in protons from the cytosol (pH 7.2) via proton pumps and chloride ion channels. In case of leakage, the rest of the cell is protected as the degradative enzymes of the lysosomes do not function well or at all in the alkaline environment of the cytosol (Kuehnel 2003).

In addition to degrading molecules, lysosomes are capable of conducting autophagy and phagocytosis (Cooper 2000; Kuehnel 2003). In the case of autophagy, damaged structures are removed through cooperation of phagosomes. According to Cooper (2000), “the first step of autophagy appears to be the enclosure of an organelle (e.g., a mitochondrion) in membrane derived from the endoplasmic reticulum”. As a result, the contents are digested due to the fusion of the autophagosome with the lysosome. For phagocytosis, large particles such as cell debris, aged cells, bacteria and virus particles are taken up and degraded by macrophages. Phagosomes (phagocytic vacuoles) are responsible for taking up these particles after which it fuses with lysosomes, resulting in digestion (Cooper 2000; Kuehnel 2003).

LysoTracker® Red is a highly selective dye for labelling and tracking acidic organelles in live cells. This dye consists of a fluorophore linked to a weak base that is only partially protonated

at neutral pH and can be detected using the Texas Red filter sets. As a deep red-fluorescent dye, live cells are effectively labelled at nanomolar concentrations as cell membranes are freely permeated (ThermoFisher).

### **3.1.3 Mitochondrial dysfunction – mitochondrial membrane potential**

Mitochondrial dysfunction comprises of two parts, namely mitochondrial membrane potential and mitochondrial mass (van de Venter 2018). The mitochondria are known to play a crucial role in various biochemical processes associated with life and death of eukaryotic cells. In the case mitochondrial membrane potential, oxidation of glucose and fatty acids by the mitochondrial respiratory chain results in a proton and pH gradient across the mitochondrial inner membrane. Subsequently, a transmembrane electrical potential gradient ( $\Delta\Psi_m$ ) is established. Under normal physiological conditions, a membrane-based proton pump is responsible for maintaining the electrochemical gradient, enabling ATP production for use in driving the energy requiring processes of cells. In the case of pancreatic  $\beta$ -cells, generated ATP helps to activate the secretion of insulin, thereby linking insulin secretion to blood glucose levels (van de Venter 2018).

Loss of  $\Delta\Psi_m$  results in an opening of the mitochondrial permeability transition pore, resulting in leakage of intermembrane proteins, including cytochrome c which facilitates apoptosis induction through the production of apoptosomes (van de Venter 2018). Cytochrome c is essential for production of the mitochondrial membrane potential as it promotes the pumping of protons into the inner-membrane space. This is achieved by shuttling electrons between complex II and IV in the electron transport chain. During apoptosis, cytochrome c is released into the cytosol thereby impairing the ability to shuttle electrons, resulting in a rapid dissipation of the mitochondrial membrane potential. Thus, loss of cytochrome c is associated with a drop in mitochondrial membrane potential and are therefore often used as a marker for apoptosis. For this reason, apoptotic drugs such as etoposide and melphalan can be used as positive controls when screening for changes in mitochondrial membrane potential. Furthermore, the activation of caspase has also been shown to accelerate the process of  $\Delta\Psi_m$  loss. Additionally, the production of reactive oxygen species (ROS) via a feedback mechanism also promote accelerates apoptosis and the rate of cell death (van de Venter 2018).

Detection of mitochondrial  $\Delta\Psi_m$  are achieved by making use of slow, lipophilic, cationic fluorescent dyes such as tetramethylrhodamine ethyl (TMRE) (van de Venter 2018). Although a delocalized positive charge is dispersed throughout their molecular structure, the lipophilic

solubility of TMRE allows them to be penetrate living cells. Due to its nature, TMRE redistributes across cell membranes according to the Nernst equation in a voltage dependant manner. The negatively charged mitochondria are entered where it accumulate according to the mitochondrial inner membrane potential. During apoptosis, the collapse of the mitochondrial  $\Delta\Psi_m$  results in the TMRE dye becoming evenly distributed throughout the cytosol as it is no longer accumulating inside the mitochondria. When dispersed in this manner, overall cellular fluorescence levels drop dramatically. This can easily be visualized/quantified by means of fluorescence microscopy/cytometry (van de Venter 2018).

### **3.1.4 Mitochondrial dysfunction – mitochondrial mass**

The number of mitochondria can change according to the energy demands of the cell (van de Venter 2018). This change is most notable during differentiation and proliferation. However, mitochondrial mass may also react to environmental factors such as toxins, which may either directly inhibit mitochondrial proliferation (such as drugs inhibiting mtDNA replication) or indirectly increase the energy demands of the cell, resulting in increased mitochondrial content in order to sustain survival of the cell (van de Venter 2018).

MitoTracker Green is a mitochondrial selective dye which stains mitochondria independent of membrane potential and are therefore used to assess the mitochondrial content of cells (van de Venter 2018). This fluorescent dye selectively accumulates in the mitochondrial matrix where it covalently binds to certain mitochondrial proteins by reacting with free thiol groups of cysteine residues. The concentration of the dye is approximately 300 times higher in the mitochondrial matrix than in the surrounding cytoplasm, thus selectively labelling proteins found in the mitochondrial inner membrane. Therefore, it is assumed that dye accumulation indicates increased mitochondrial proteins and thus increased mitochondrial mass (van de Venter 2018).

### **3.1.5 Steatosis**

The abnormal retention of lipids within a cell, is known as steatosis, also called lipid accumulation (Wilson et al. 2015). No single mechanism is responsible for steatosis as a variety of pathologies are responsible for disrupting normal lipid movement through the cell, causing accumulation. These mechanisms can be separated based on whether an oversupply of lipids, which cannot be removed quickly enough, are caused or whether a failure in lipid breakdown are caused. Failure of lipid metabolism can also cause impairment of mechanisms which usually would utilise and remove lipids, resulting in the accumulation of unused lipids.

Although mild cases of lipid accumulation may not have particular detrimental effect on cells, large accumulation can result in disruptions of the cell components, causing the cell to burst in severe cases (Wilson et al. 2015).

LipidTox Red was specifically developed to characterize the potentially toxic effects of compounds on lipid metabolism in mammalian cell lines. This dye has an extremely high affinity for neutral lipid droplets and can be detected via fluorescence microscopy (ThermoFisher).

## **3.2 Materials & Methods**

### **3.2.1 Medicinal mushroom, bacterial, and yeasts strains used**

South African isolates of two different medicinal mushrooms, namely *G. lucidum* (UFS-GL1) and *H. erinaceus* (UFS-L1) were obtained from the UFS fungal collection. Bacterial strains of *Bacillus cereus* (ATCC 10876), *Bacillus subtilis* (ATCC 6051), *Escherichia coli* (ATCC 25922), *E. coli* O157:H7 (SAIM M0770), *Klebsiella pneumonia* (ATCC 31488), *Listeria monocytogenes* (LMG 13305), *Salmonella* Diarizonae (ATCC 29934), *Salmonella* Typhimurium, *Staphylococcus aureus* (ATCC 6538) and *Streptococcus pyogenes* (ATCC 10389) were obtained from the UFS culture collection. Yeast strains of *Candida albicans* and *Cryptococcus neoformans* were obtained from the Unesco Mircen Yeast collection (UFS).

### **3.2.2 Hot water extraction of compounds in *G. lucidum* and *H. erinaceus***

Two different approaches to hot water extraction were performed to compare results and applicability for future reference. Macro-fungal material of *G. lucidum* and *H. erinaceus* was dried in a 25-30°C oven for 2-3 days and then briefly submerged in liquid nitrogen and crushed using a mortar and pestle. Water extracts were prepared by using a material: solvent ratio of 1: 15 (w/v) for 24 hrs at room temperature with frequent mixing. Extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant was filtered twice through Whatman filter under vacuum. Extracts were freeze-dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA) and stored in a desiccator in the dark at 4°C until further use.

The second water extraction method was performed in two phases to extract valuable compounds associated with *G. lucidum* and *H. erinaceus*. Water extracts were prepared by using a material: solvent ratio of 1: 15 (w/v). Macro-fungal material of *G. lucidum* and *H.*

*erinaceus* were again briefly submerged in liquid nitrogen and crushed using a mortar and pestle. Water was added and kept at a temperature of 90°C for 2 hrs after which the extracts were cooled down. For the second extraction step, the extracts were kept at a temperature between 65°C -90°C for 1h to allow further extraction of the compounds. Extracts were freeze-dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA) and stored in a desiccator in the dark at 4°C until further use.

### **3.2.3 Alcohol extraction on *G. lucidum* and *H. erinaceus***

Macro-fungal material derived from *G. lucidum* and *H. erinaceus* was dried at 25-30°C for 2-3 days and the dried cells briefly submerged in liquid nitrogen and crushed using a mortar and pestle. Ethanolic (80%) extracts were prepared by using a material: solvent ratio of 1: 15 (w/v) for 24 hrs at room temperature with frequent mixing. Extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant filtered twice through a Whatman filter under vacuum. Ethanol was evaporated using a BUCHI Rotavapor R-210 (Switzerland) at 50°C and extracts were freeze-dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA). Extracts were stored in a desiccator in the dark at 4°C until further use.

### **3.2.4 Two-step extraction of compounds in *G. lucidum***

A two-step extraction method involving both ethanol and water were performed in duplicate on the spent mushroom substrate (SMS) block, fruiting bodies and mycelia of *G. lucidum*.

SMS of *G. lucidum* was dried in a 25-30°C oven for 2-3 days after which it was milled. Ethanolic (80%) extracts were prepared by using a material: solvent ratio of 1: 5 (w/v) for 8 weeks at room temperature with frequent mixing (100, 07 g and 100, 15 g were weighed off and 500 ml ethanol was added, respectively). Extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant filtered twice through Whatman filter under vacuum. Ethanol extracts were stored in a desiccator in the dark at 4°C until further use. Water extracts were prepared by using a material: solvent ratio of 1: 5 (w/v) - 500 ml H<sub>2</sub>O was added to both Schott bottles containing SMS used for ethanol extracts. Water was evaporated to 400 ml by maintaining a temperature of 100°C. Water extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant filtered twice through Whatman filter under vacuum. Stored ethanol extracts were added to water extracts. Ethanol was evaporated using a BUCHI Rotavapor R-210 (Switzerland) at 50°C and extracts were freeze-dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA). Extracts were stored in a desiccator in the dark at 4°C until further use.

For the fruiting bodies and mycelia of *G. lucidum*, macro-fungal material was dried in a 25-30°C oven for 2-3 days. The material of *G. lucidum* was briefly submerged in liquid nitrogen and crushed using a mortar and pestle. Ethanolic (80%) extracts were prepared by using a material: solvent ratio of 1: 15 (w/v) for 2 weeks at room temperature with frequent mixing. Extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant was filtered twice through Whatman filter under vacuum. Ethanol extracts were stored in a desiccator in the dark at 4°C until further use. Water extracts were prepared by using a material: solvent ratio of 1: 15 (w/v) - 150 ml of H<sub>2</sub>O was added to Schott bottles containing *G. lucidum* fruiting bodies and mycelia used for ethanol extracts, respectively. Water was evaporated to 50 ml by maintaining a temperature of 100°C. Water extracts were centrifuged at 3, 000 rpm for 5 min, and the supernatant filtered twice through a Whatman filter under vacuum. Stored ethanol extracts were added to water extracts. Ethanol was evaporated using a BUCHI Rotavapor R-210 (Switzerland) at 50°C and extracts were freeze-dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA). Extracts were stored in a desiccator in the dark at 4°C until further use.

### **3.2.5 Thin layer chromatography on *G. lucidum* and *H. erinaceus***

One gram of crushed dried fruiting bodies of *G. lucidum* and *H. erinaceus* were placed in test tubes and covered with *n*-hexane (10 ml), respectively. The test tubes were placed in an ultrasonic bath for one hour. The *n*-hexane was decanted and evaporated under reduced pressure. TLC was used to separate the components of the *n*-hexane extract with an eluent of a Toluene: EtOAc ratio of 9:1. The above process was repeated another three times with the exception of using dichloromethane (10 ml), methanol (10 ml), and ethanol (10 ml) instead.

### **3.2.6 Extract preparation and identification of antimicrobial properties**

The freeze-dried extracts previously obtained were weighed and 1% DMSO was added. The pathogens were plated out in triplicate on nutrient agar (NA) to allow the growth of bacteria and yeasts. Extracts of *G. lucidum* and *H. erinaceus* were added by means of the spot-on-lawn method. Plates were incubated at 37°C for 24 h after which the zones of inhibition were determined.



### **3.2.7 Cultivation of HepG2 cells (hepatocytes)**

HepG2 cells were maintained in 10% fetal bovine serum (HighClone Laboratories, Utah, USA) in the presence of penicillin-streptomycin solution (100 µl/ml, Lonza, BioWhittaker, Verviers, Belgium). Cells were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 72 hrs.

### **3.2.8 Harvesting of HepG2 cells and preparation of extracts**

HepG2 cells were harvested by adding trypsin to tissue culture dish in order to break down proteins on the bottom of the dish. Serum was gently removed using a vacuum. Cells were washed with 5-10 ml DPBS/Modified to ensure all serum were removed. 1 ml trypsin was added to tissue culture dish and incubated at 37 °C for 15 min to allow breakdown of proteins to ensure all cells were released. Growth media to be used for wells were prepared by adding 500 µl Penicillin-Streptomycin solution, 500 µl non-essential amino acids and 5 ml Hyclone serum containing growth factors to a test tube after which MEM/EBSS was added to make media with a final volume of 50 ml. After incubation, HepG2 cells were harvested by adding 2 ml of growth media to cells in order to inactivate trypsin. 50 µl of cells were stained with Trypan Blue and counted using haemocytometer to determine cell viability. HepG2 cells were seeded in 96-well plates at cell densities of 5000 cells (100 µl/well). Plates were incubated in a humidified 5% CO<sub>2</sub> incubator at 37°C for 24 hrs in order to allow cells to attach to the bottom of the plate.

Stock concentrations of macro-fungal extracts were prepared in dimethyl sulfoxide (DMSO) at 100 mg/ml. Working concentrations (400 µg/ml) were prepared in complete media (MEM/EBSS, fetal bovine serum containing growth factors, penicillin-streptomycin solution, non-essential amino acids). A dilution series, ranging between 12.5 µg/ml and 200 µg/ml was prepared. Cells were treated with test samples (extracts) at the different concentrations. A negative control, with no addition of extracts and two positive controls namely Melphalan (chemotherapeutic drug) and Chloroquine (anti-malaria drug) were used at working concentrations ranging between 12.5 µg/ml and 200 µg/ml to allow comparison. The plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hrs.

### **3.2.8 Hepatotoxicity assay (live/dead cells)**

A hepatotoxicity assay of *G. lucidum* and *H. erinaceus* extracts for the evaluation of live/dead cells was performed. Harvested C3A cells were loaded in 96-well plate at a density of 5000

cells per well and allowed to attach overnight at 37°C. Cells were treated with 100 µl of indicated concentrations of test sample and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hrs. Spent culture medium was gently removed prior to staining and replaced with 100 µl PBS containing bisBenzamide H 33342 trihydrochloride (Hoechst 33342) at a concentration of 5 µg/ml. The cells were stained for 20 min at 37°C. Propidium Iodide was added to a final concentration of 10 µg/ml using 10 µl per well directly before imaging. Image acquisition was performed using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) at 20x magnification. Hoechst 33342 was acquired with the DAPI and Propidium Iodide with the PI filter sets. The images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software and Multi-wavelength Cell Scoring analysis module.

### **3.2.9 Hepatotoxicity assay (lysosomes)**

A hepatotoxicity assay of *G. lucidum* and *H. erinaceus* extracts for the evaluation of lysosome content was performed. Harvested C3A cells were loaded in 96-well plate at a density of 5000 cells per well and allowed to attach overnight at 37°C. Cells were treated with 100 µl of indicated concentrations of test sample and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hrs. A working concentration of 50 nM LysoTracker Red was prepared in PBS. Spent culture medium was aspirated from the wells and replaced with 100 µl of the LysoTracker solution after which the plates were incubated at 37°C for 30 min. After incubation, the dye solution was replaced with fresh PBS containing Hoechst 33342 (10 mg/ml in 1 ml DMSO) and incubated for a further 30 min before acquisition. Image acquisition was performed using an ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) at 20x magnification. Hoechst 33342 was acquired with the DAPI and LysoTracker Red with the Texas Red filter sets. The images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software and Multi-wavelength Cell Scoring analysis module.

### **3.2.10 Multi-parameter hepatotoxicity assay (mitochondrial dysfunction)**

A hepatotoxicity assay of *G. lucidum* and *H. erinaceus* extracts for the evaluation of mitochondrial dysfunction was performed. Harvested C3A cells were loaded in 96-well plate at a density of 5000 cells per well and allowed to attach overnight at 37°C. Cells were treated with 100 µl of indicated concentrations of test sample and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hrs. Spent culture medium were gently removed prior to staining and

wells were washed with 200 µl/well PBS. Tetramethylrhodamine ethyl (TMRE) stock solution (5 mg into 1 ml DMSO) were prepared and 1 µl TMRE were diluted into 200 µl RPMI (Roswell Park Memorial Institute) medium prior to staining. Mito-Tracker Green stock solution was prepared by adding 1 mM to 1ml DMSO. A staining cocktail was prepared by adding 5 ml PBS, 50 µl TMRE working stock, 1 µl Mito-Tracker Green and 2 µl Hoechst 33342 solution (10mg/ml in 1ml DMSO) to 5 ml of RPMI. Spent medium was aspirated from the wells and replaced with 50 µl staining solution after which the plates were incubated at 37°C for 30 min before acquisition before acquisition. Acquire images with ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) at 20x magnification. Hoechst 33342 Hoechst 33342 was acquired with the DAPI, TMRE with the FITC and Mito-Tracker Green with the TRITC filter sets. The images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software and Multi-wavelength Cell Scoring analysis module.

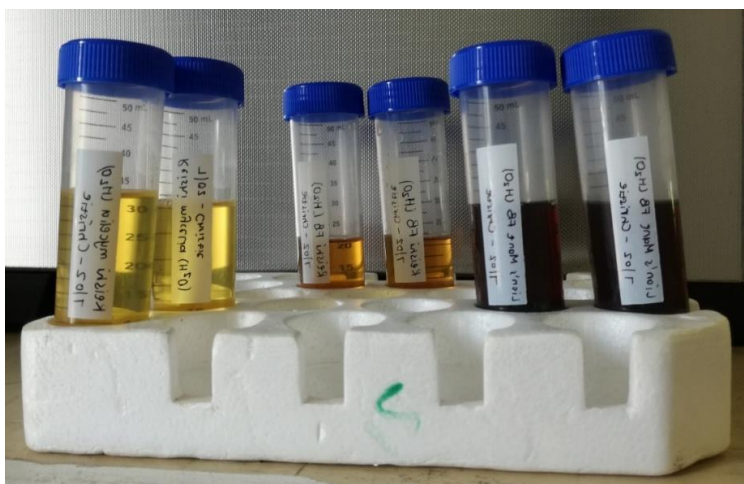
### **3.2.11 Hepatotoxicity assay (lipid accumulation)**

A hepatotoxicity assay of *G. lucidum* and *H. erinaceus* extracts for the evaluation of lipid accumulation in cells was performed. Harvested C3A cells were loaded in 96-well plate at a density of 5000 cells per well and allowed to attach overnight at 37°C. Cells were treated with treated with 100 µl of indicated concentrations of test sample and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 48 hrs. Spent culture medium were aspirated and wells were washed with 200 µl/well PBS. Cells were fixed for 15 min with 4% formaldehyde. A staining solution was prepared by adding 1 µl LipidTox Red and 2 µl Hoechst 33342 solution to 10 ml PBS. Fix solution was removed and replaced with 50 µl staining solution after which the plates were incubated at room temperature for 30 min before acquisition. Acquire images with ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) at 20x magnification. Hoechst 33342 was acquired with the DAPI and LipidTox Red with the Texas Red filter sets. The images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software and Multi-wavelength Cell Scoring analysis module.

## **3.3 Results & Discussions**

### **3.3.1 Water extracts**

Hot water extracts of *G. lucidum* mycelia and fruiting bodies as well as *H. erinaceus* fruiting bodies were obtained and used for evaluation of antimicrobial activity as well as to perform hepatotoxicity assays (**Fig. 1.**).



**Fig. 1.** Water extracts of *G. lucidum* mycelia and fruiting bodies and *H. erinaceus* fruiting bodies.

### 3.3.2 Alcohol extracts

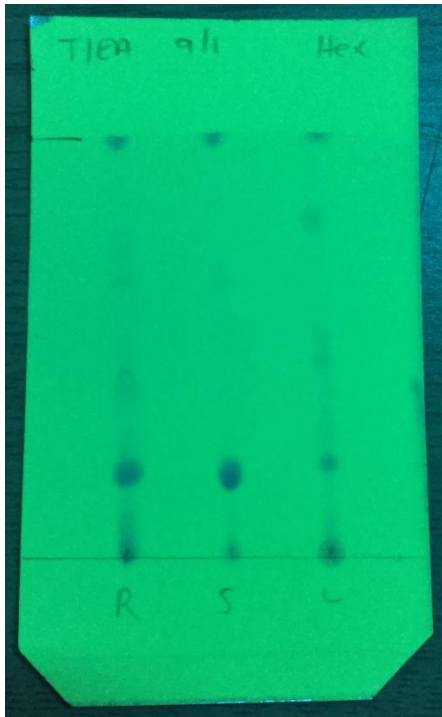
Alcohol extracts of *G. lucidum* mycelia and fruiting bodies and *H. erinaceus* fruiting bodies were obtained and used for evaluation of antimicrobial activity as well as to perform hepatotoxicity assays.

### 3.3.3 Two-step extraction of compounds in *G. lucidum* and *H. erinaceus*

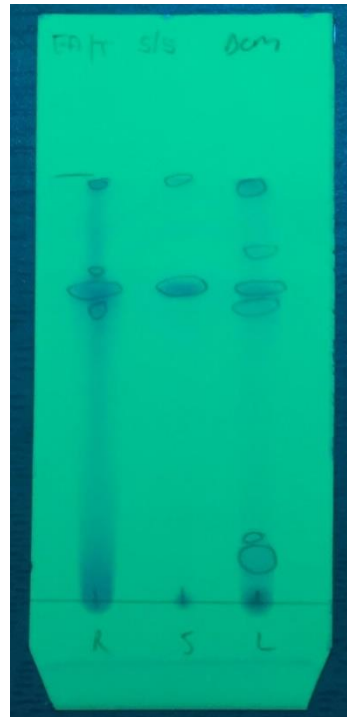
Combined alcohol and water extracts of *G. lucidum* mycelia, fruiting bodies and SMS were obtained and used for evaluation of antimicrobial activity as well as to perform hepatotoxicity assays.

### 3.3.4 Thin layer chromatography

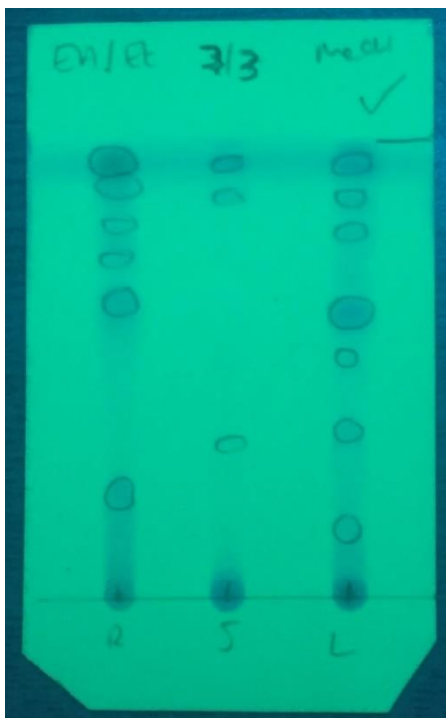
The first TLC (**Fig. 2**) showed the components of the *n*-hexane extract with an eluent of a Toluene: EtOAc ratio of 9:1. The components of the dichloromethane extract with an eluent of EtOAc: Toluene with a 5:5 ratio, can be seen in **Fig 3**. The methanol extract (**Fig. 4**) showed the most components in each of the samples with EtOAc: EtOH with a ratio of 7:3 as the eluent being used. For the ethanol extract (**Fig. 5**), an eluent of EtOAc: MeOH with a 7:3 ratio was used. Based on the variety of spots exhibited on each plate further separation and identification of compounds need exploration and will be performed in the future.



**Fig. 2.** n-hexane



**Fig. 3.** Dichloromethane



**Fig. 4.** Methanol



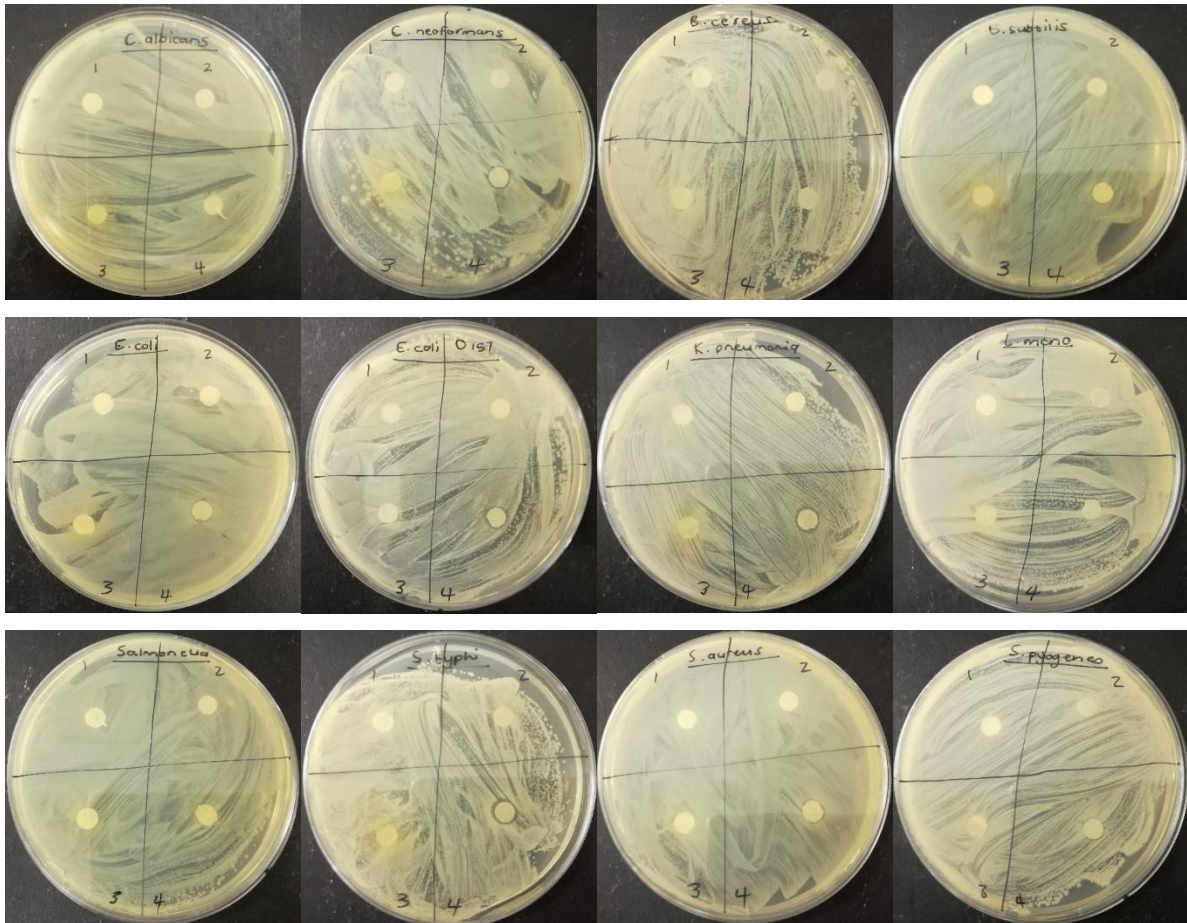
**Fig. 5.** Ethanol

### 3.3.5 Identification of antimicrobial activity

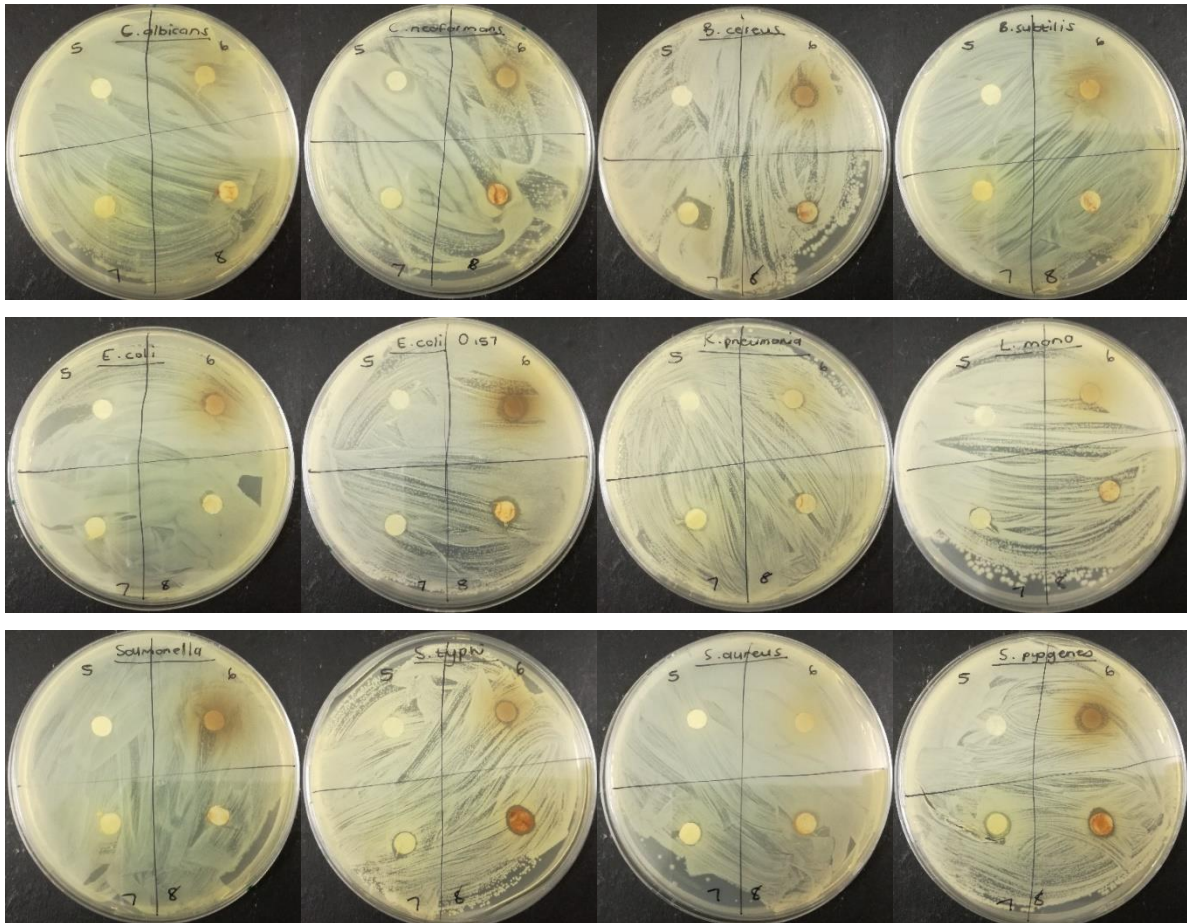
The different extracts of *G. lucidum* and *H. erinaceus* were evaluated to determine antagonistic effects on yeasts, gram-positive and gram-negative bacteria. **Fig. 6** depicts the inhibitory effect of the hot water and water extracts *G. lucidum* mycelia and fruiting bodies and *H. erinaceus* fruiting bodies. For this study, 1-3 on the agar plates represented the hot water extracts of *G. lucidum* mycelia, *G. lucidum* fruiting bodies and *H. erinaceus* fruiting bodies, respectively, whereas 4 represented the water extract of *G. lucidum* mycelia. No inhibitory effects were detected for the hot water extracts, which may be due to the shorter extraction time. For future work, the extraction time will be lengthened in order to allow extraction of all compounds. However, the water extract of *G. lucidum* mycelia (number 4 on plates) showed inhibitory effects against a variety of pathogens such as *C. neoformans*, *E. coli*, *E. coli* O157:H7, *K. pneumonia* and *S. Typhimirium*, *S. aureus* and *S. pyogenes*.

From **Fig. 7** the inhibitory effects of the water and alcohol extracts can be seen. For this study, 5-6 on the agar plates represented the water extracts of *G. lucidum* fruiting bodies and *H. erinaceus* fruiting bodies, respectively, whereas 7-8 represented the alcohol extracts of *G. lucidum* mycelia and *G. lucidum* fruiting bodies. Inhibitory effects for the water extracts of *G. lucidum* fruiting bodies (number 5 on plates) were limited as inhibition was only detected against *C. neoformans* and *S. aureus* whereas water extracts of *H. erinaceus* fruiting bodies (number 6 on plates) showed inhibition against *C. neoformans*, *B. cereus*, *E. coli*, *E. coli* O157:H7, *K. pneumonia*, *L. monocytogenes*, *S. Typhimirium* and *S. pyogenes*. Good inhibitory effects were detected for the alcohol extracts of both *G. lucidum* mycelia (number 7 on plates) and *G. lucidum* fruiting bodies (number 8 on plates) against most of the pathogens evaluated such as *C. neoformans*, *B. cereus*, *E. coli*, *E. coli* O157:H7, *K. pneumonia*, *L. monocytogenes*, *S. Typhimirium*, *S. aureus* and *S. pyogenes*.

In the case of **Fig. 8**, the combined extracts of *G. lucidum* mycelia, fruiting bodies and SMS were represented by 9-11, respectively. The combined extract of *G. lucidum* fruiting bodies (number 10 on plates) showed the most promising results with inhibition against all pathogens tested except for *C. albicans* and *B. subtilis* followed by the combined extracts of *G. lucidum* SMS (number 11 on plates) with inhibition against all pathogens tested except for *C. albicans*, *C. neoformans*, *B. subtilis* and *Salmonella*. In the case of the combined extract of *G. lucidum* mycelia (number 9 on plates), inhibition was detected against all pathogens tested except for *C. albicans*, *B. subtilis*, *Salmonella* and *S. aureus*.

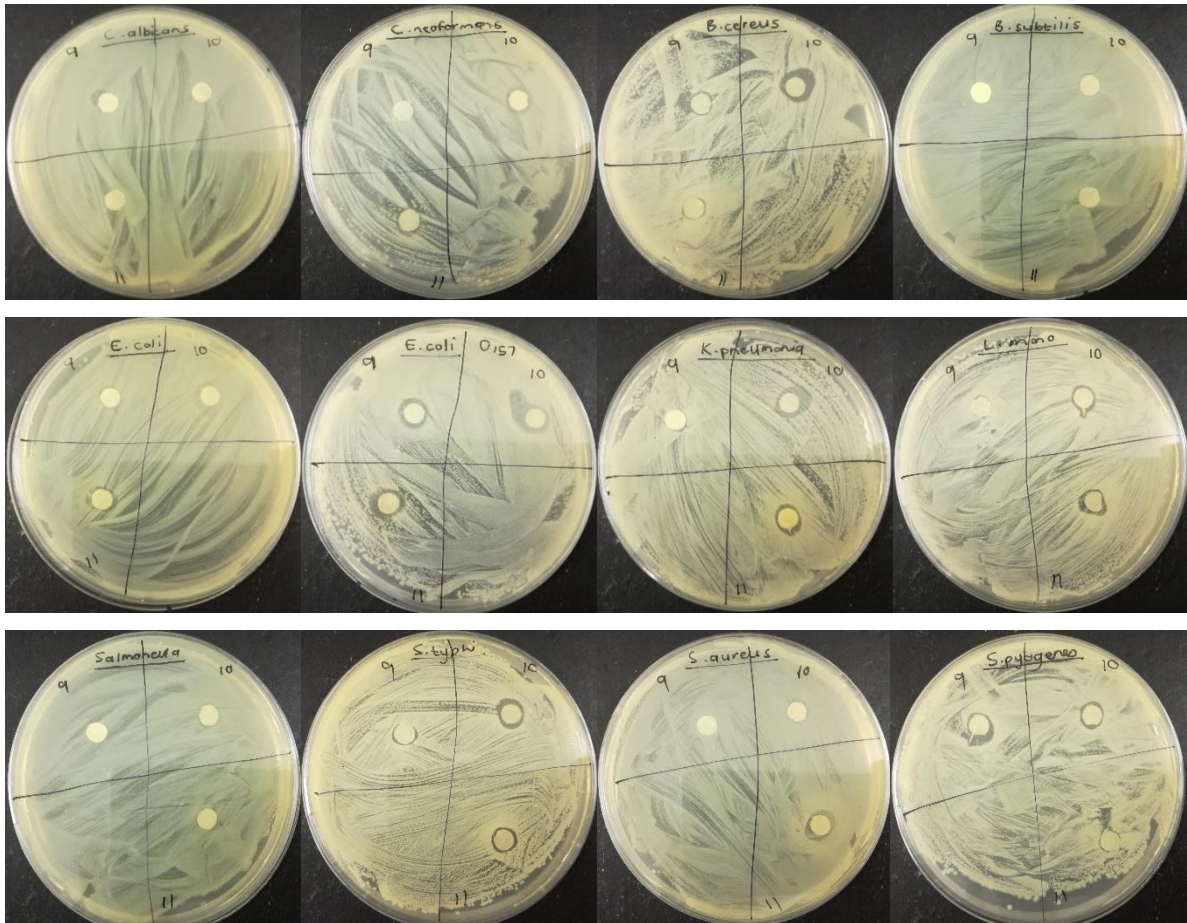


**Fig. 6.** 1-3: Hot water extracts of *G. lucidum* mycelia, *G. lucidum* fruiting bodies and *H. erinaceus* fruiting bodies. 4: Water extract of *G. lucidum* mycelia.



**Fig. 7. 5-6:** Water extracts of *G. lucidum* fruiting bodies and *H. erinaceus* fruiting bodies. 7-8: Alcohol extracts of *G. lucidum* mycelia and *G. lucidum* fruiting bodies.





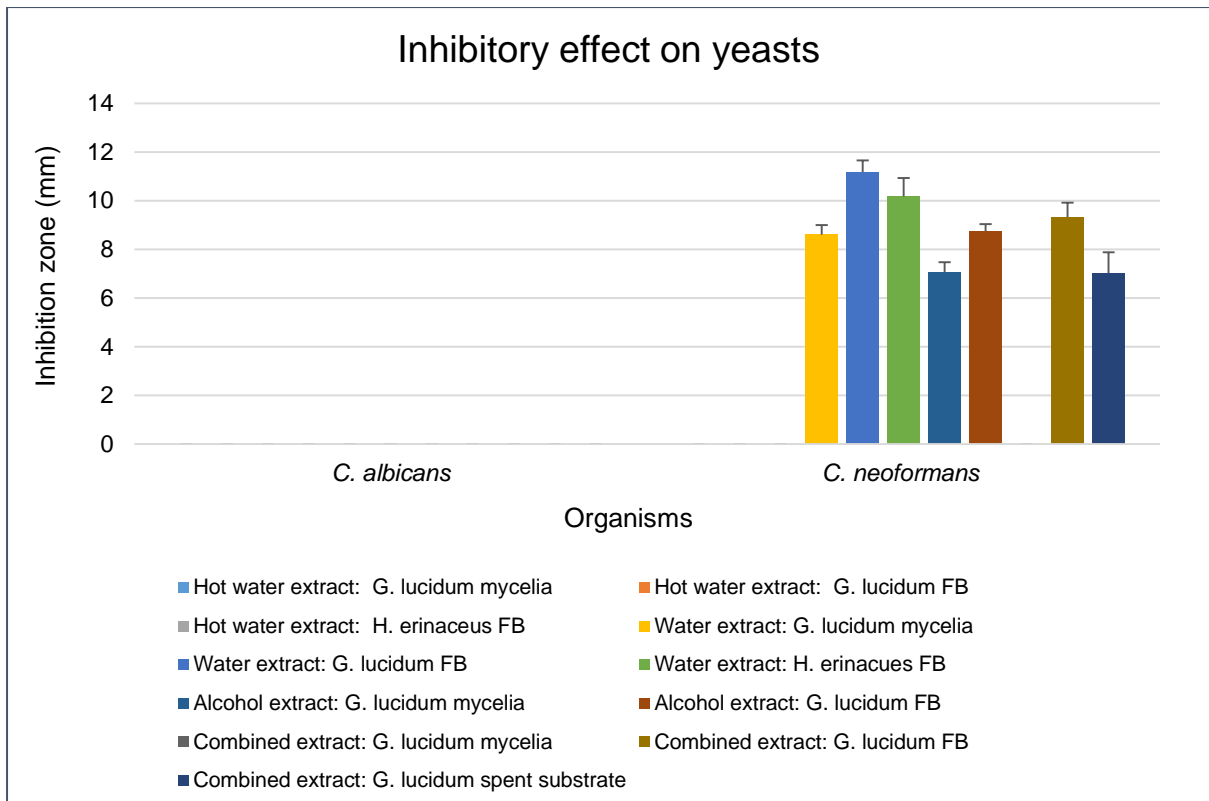
**Fig. 8.** 9-11: Combined extracts of *G. lucidum* mycelia, fruiting bodies and SMS.

In the case of yeasts, no inhibitory effect on *C. albicans* was detected by any of the extracts whereas good results were detected for activity against *C. neoformans*. From **Fig. 9**, none of the hot water extracts showed inhibitory effects. However, the water extract of *G. lucidum* fruiting bodies showed the best inhibitory effect followed by the water extract of *H. erinaceus* fruiting bodies. Both the water and alcohol extract of *G. lucidum* mycelia showed inhibitory effects although the combined extract of *G. lucidum* mycelia showed no effect. Furthermore, the combined extracts of *G. lucidum* fruiting bodies and spent substrate showed good inhibitory effects.

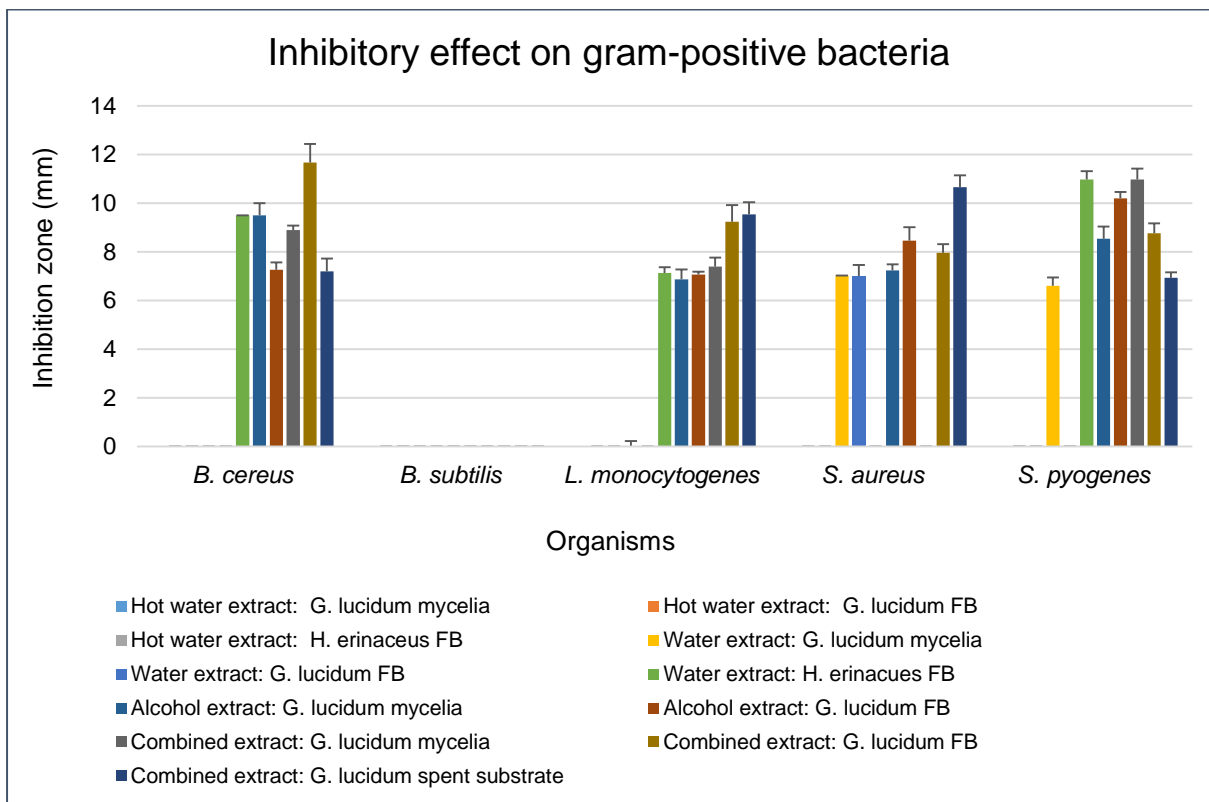
Similar inhibitory effects were detected against gram-positive bacteria. From **Fig. 10**, none of the extracts showed inhibitory effects against *B. subtilis*. Again, none of the hot water extracts resulted in positive results. It is interesting to note that the combined extract of *G. lucidum* fruiting bodies and spent substrate showed similar results as inhibitory effects against *B. cereus*, *L. monocytogenes*, *S. aureus* and *S. pyogenes* were detected while the combined extract of *G. lucidum* mycelia only showed inhibitory effect against *B. cereus* and *L. monocytogenes*. This is important as spent substrate which is considered to be agricultural

waste, have similar positive effects and can therefore be applied as animal feed, thus utilising waste. Inhibitory effects against *L. monocytogenes* were detected for various extracts, including all three combined extracts of the different growth phases for *G. lucidum*. Water extract of *G. lucidum* mycelia showed positive results against *S. aureus* as well as *S. pyogenes* whereas the water extract of *G. lucidum* fruiting bodies only showed inhibitory effect against *S. aureus*. Furthermore, positive effects against *B. cereus*, *L. monocytogenes* and *S. pyogenes* were detected for the water extract of *H. erinaceus* fruiting bodies. Alcohol extracts of *G. lucidum* mycelia and fruiting bodies showed inhibitory effect against all gram-positive bacteria except for *B. subtilis*.

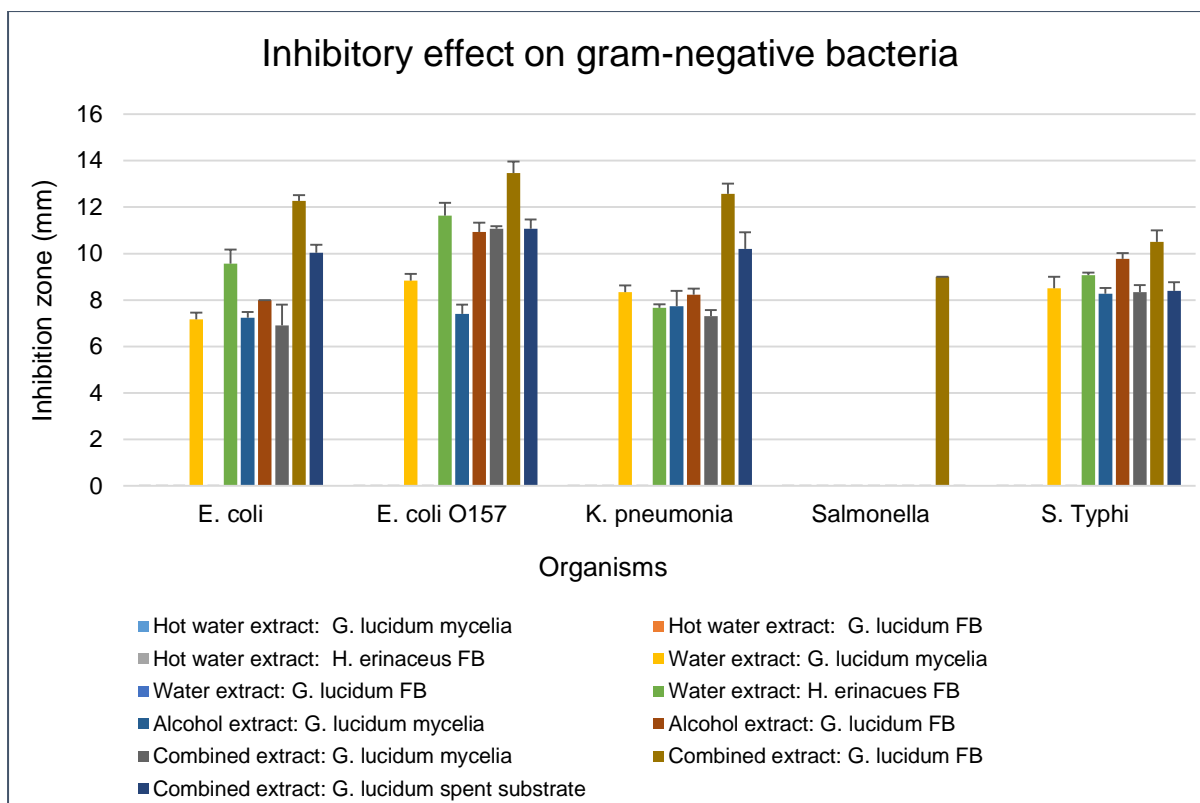
Good inhibitory effects were also detected against gram-negative bacteria. From **Fig. 11**, none of the hot water extracts resulted in positive results. Also, none of the extracts, except for the combined extract of *G. lucidum* fruiting bodies showed inhibitory effects against *Salmonella*. It is again interesting to note that the combined extracts of the different growth phases for *G. lucidum* showed positive results against all five gram-negative bacteria (*E. coli*, *E. coli* O157:H7, *K. pneumonia*, *Salmonella* and *S. Typhi*) used for evaluation of antibacterial activity. Water extracts of *G. lucidum* mycelia and *H. erinaceus* fruiting bodies showed positive results against all gram-negative bacteria, except for *Salmonella* while no inhibitory effects were detected for the water extract of *G. lucidum* fruiting bodies. Alcohol extracts of both *G. lucidum* mycelia and fruiting bodies showed inhibitory effect against all gram-negative bacteria except for *Salmonella*.



**Fig. 9.** Inhibitory effect of different extracts on yeasts.



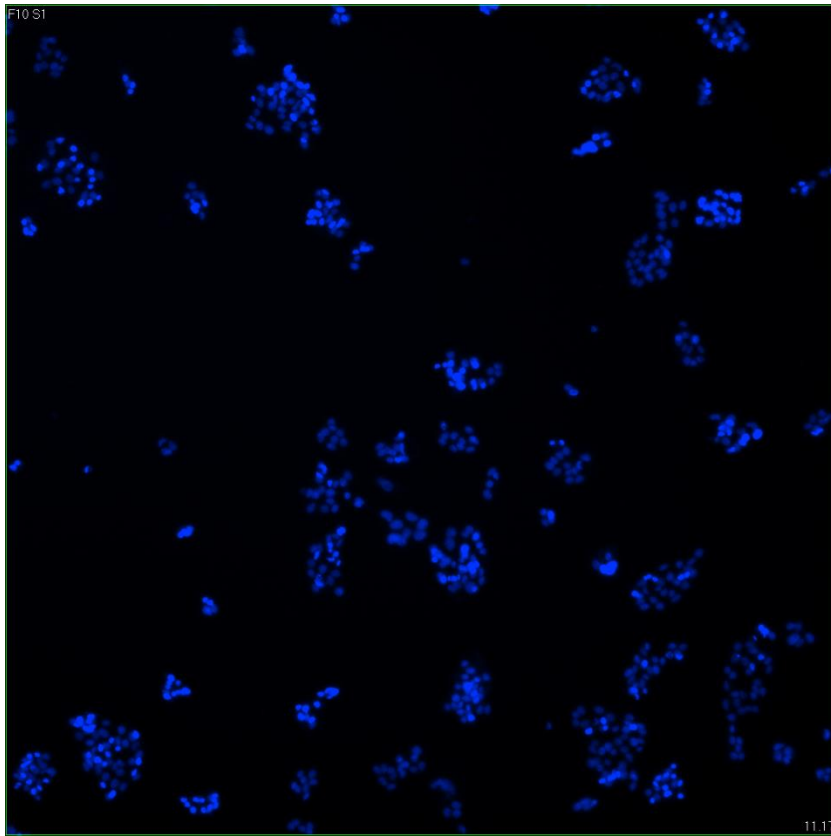
**Fig. 10.** Inhibitory effect of different extracts on gram-positive bacteria.



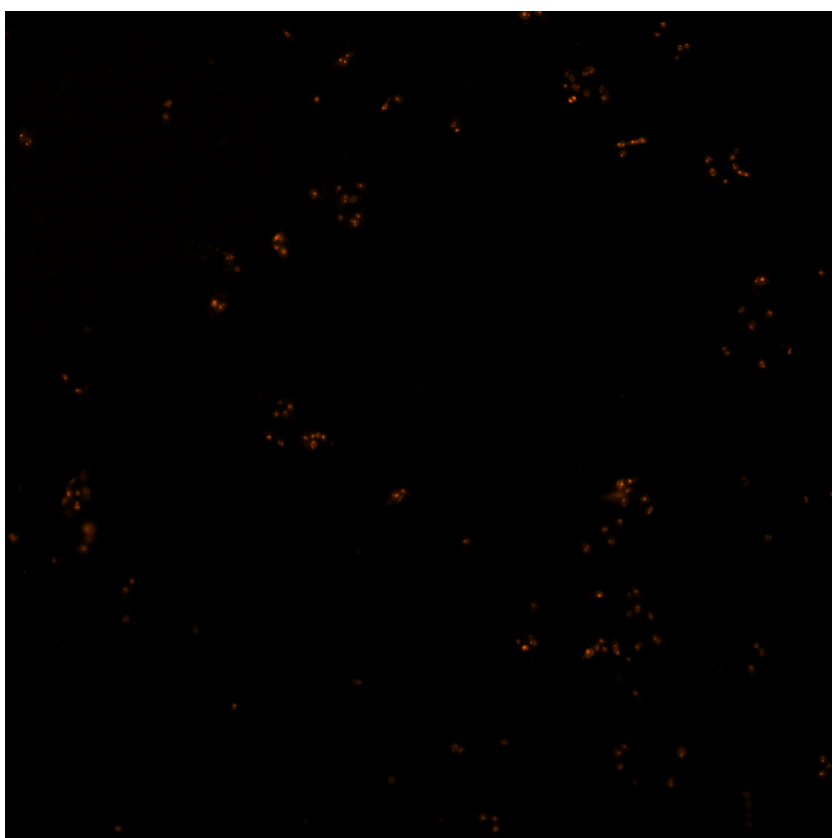
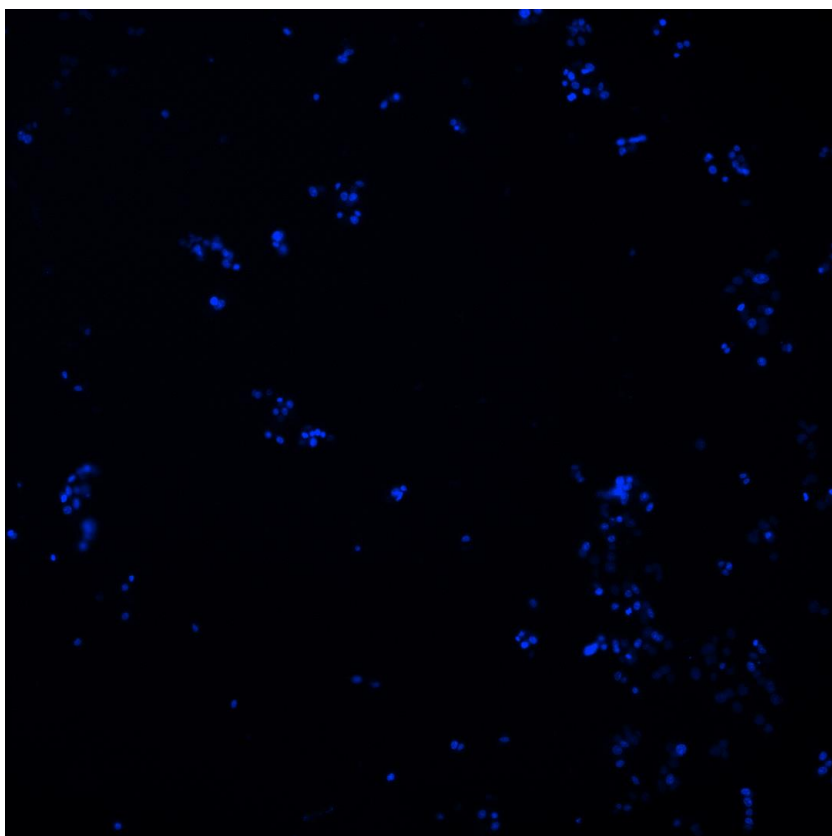
**Fig. 11.** Inhibitory effect of different extracts on gram-negative bacteria.

### 3.3.6 Hepatotoxicity assay (live/dead cells)

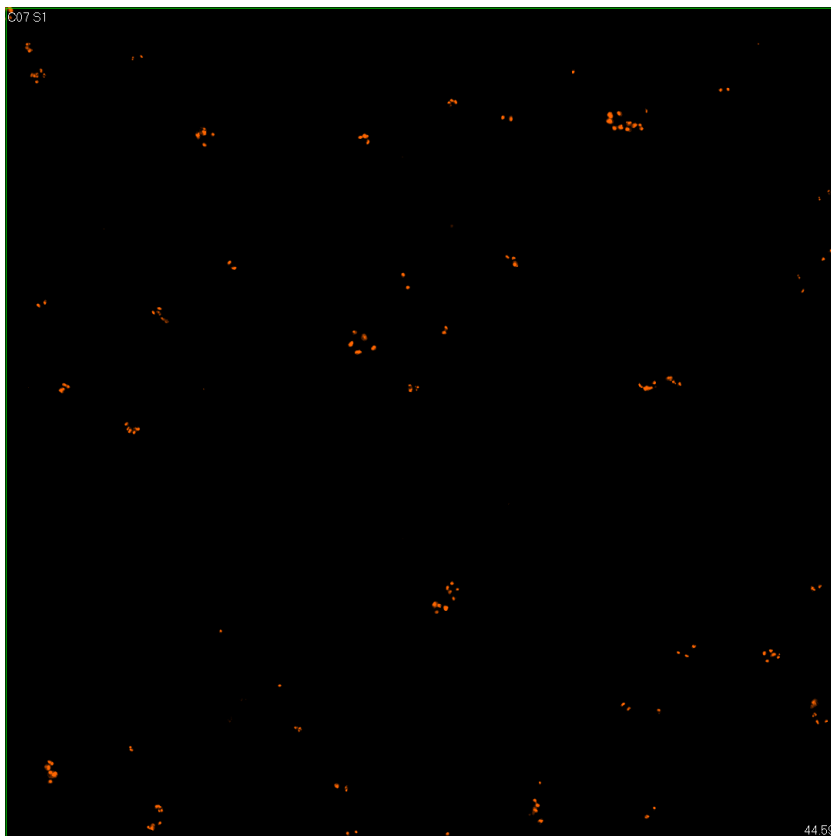
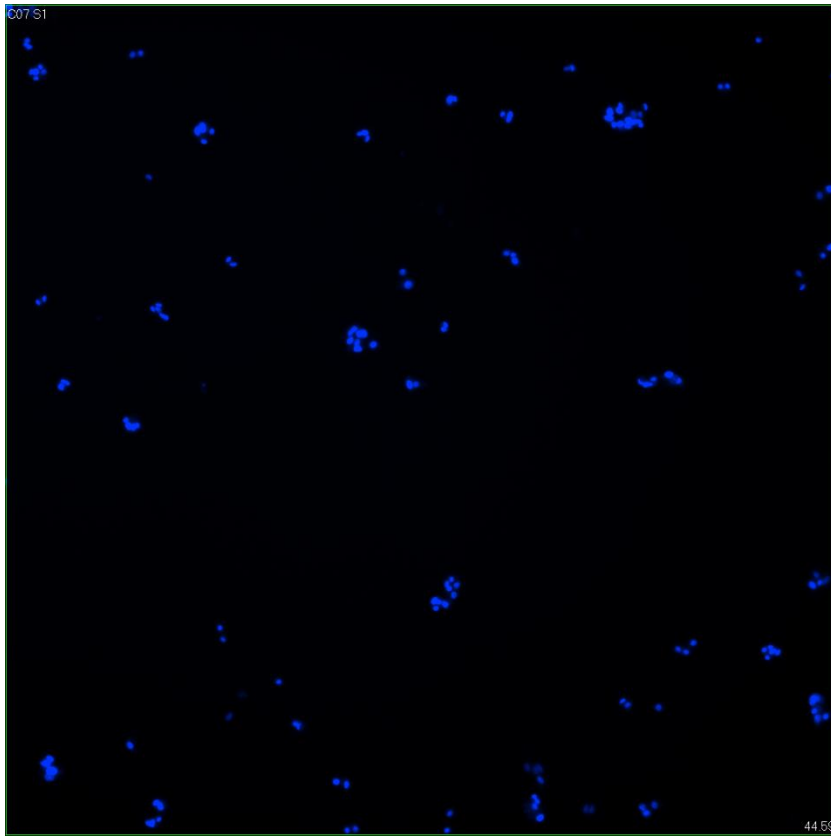
When compared to the negative control (**Fig. 12**), the positive control, Melphalan (**Fig. 13**), did show a significant decreased number of nuclei (live cells) and increased number of dead cells. In the case of dead cells, PI is only taken up by the cell after the cells had died, resulting in an increased number of dead cells for the positive control, Melphalan (**Fig. 13**). For the live/dead cells assay, only the images of wells C7, D8 and E8 are shown (letters indicate different concentrations while numbers indicate extracts). The alcohol extracts of *G. lucidum* fruiting bodies (**Figs. 14-16**) at different concentrations (50, 25, and 12.5 µg/ml) showed a slight decrease in the nuclei (live cells) while only a few numbers of dead cells were detected as PI was only taken up by a few cells when compared to the negative control (**Fig. 12**). From **Fig. 14**, the effect of the alcohol extract *G. lucidum* fruiting bodies at a concentration of 50 µg/ml can be seen and more toxicity was detected compared to the lower concentrations (**Figs. 15-16**).



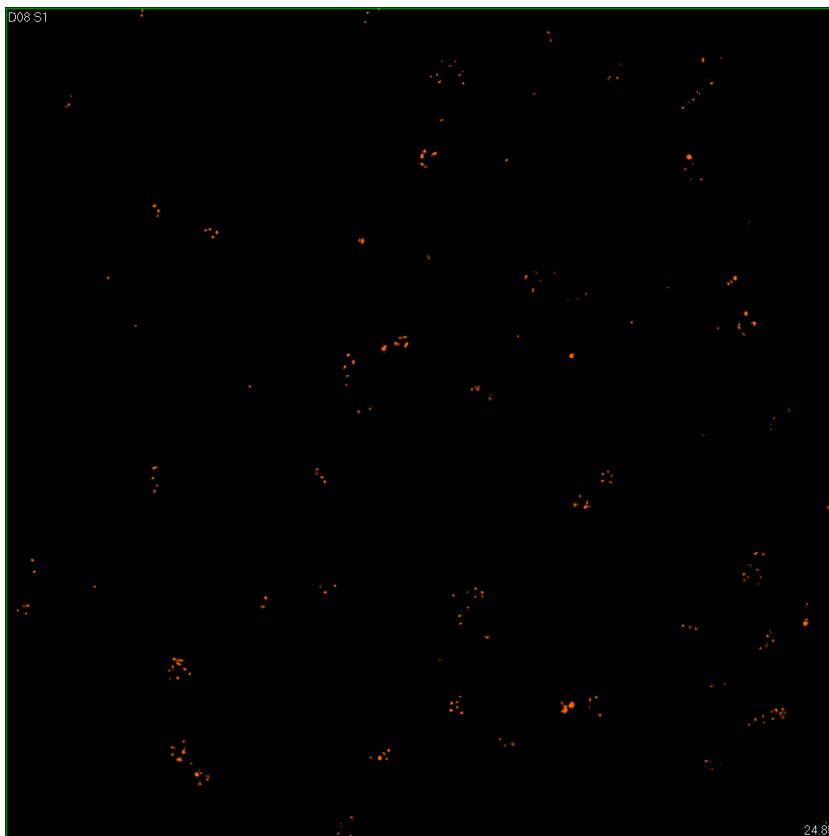
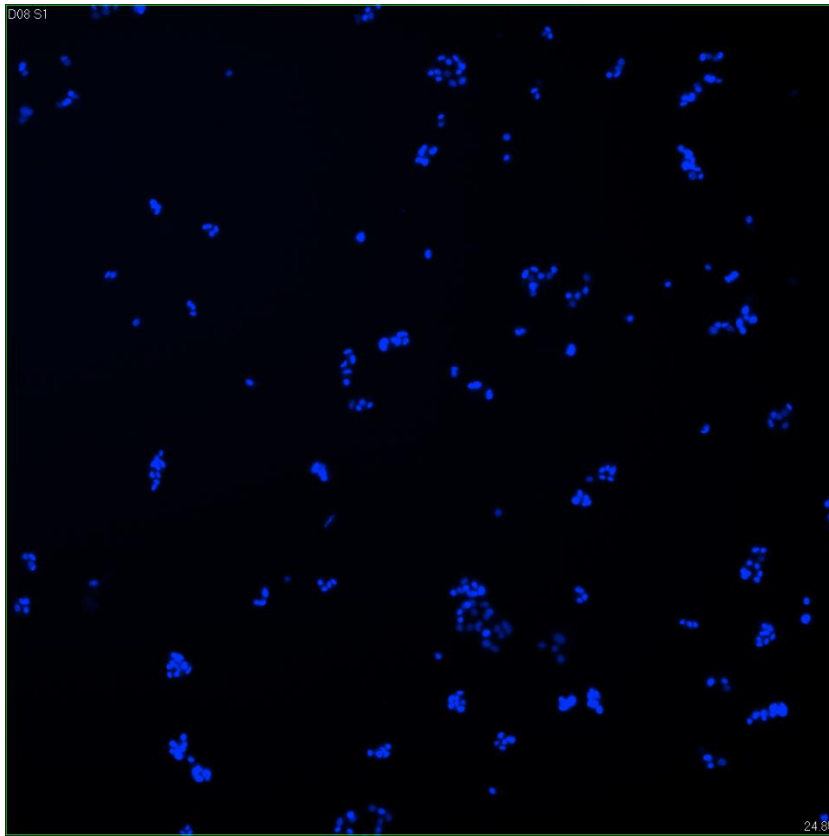
**Fig. 12.** Nuclei (live cells) and dead cells for the negative control (F10).



**Fig. 13.** Nuclei (live cells) and dead cells for the positive control (Melphalan).

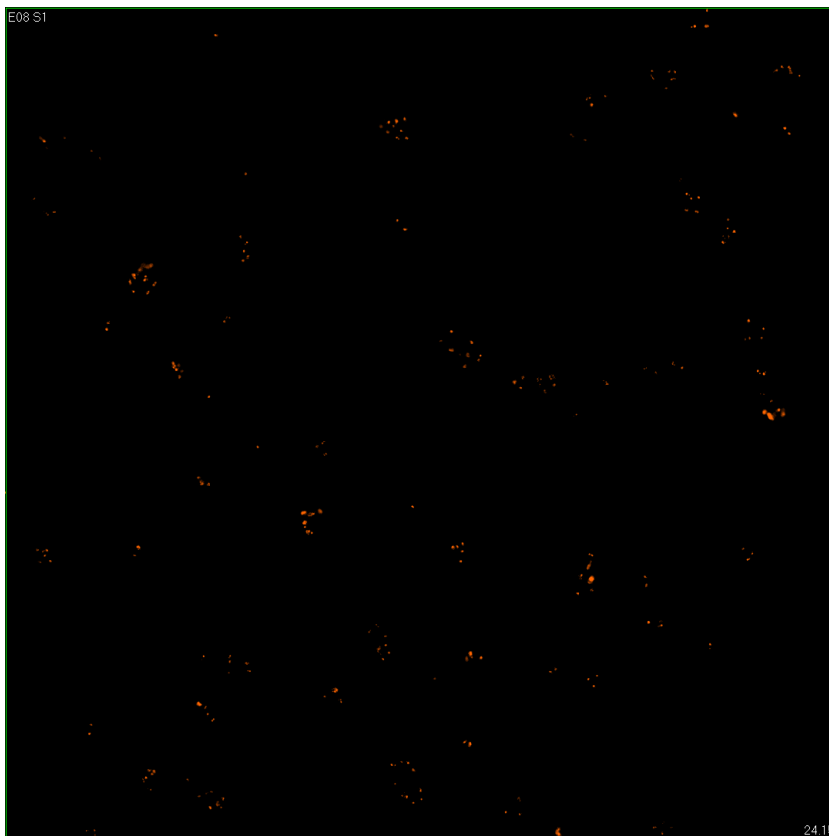
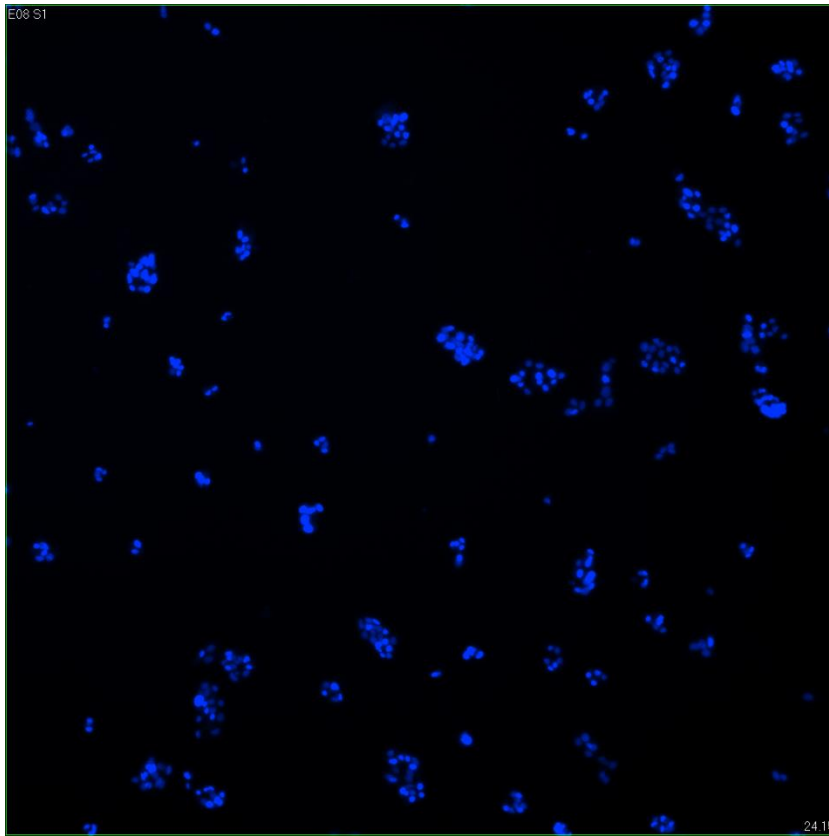


**Fig. 14.** Nuclei (live cells) and dead cells for C7, alcohol extract of *G. lucidum* fruiting bodies (50 µg/ml).



**Fig. 15.** Nuclei (live cells) and dead cells for D8, alcohol extract of *G. lucidum* fruiting bodies (25 µg/ml).

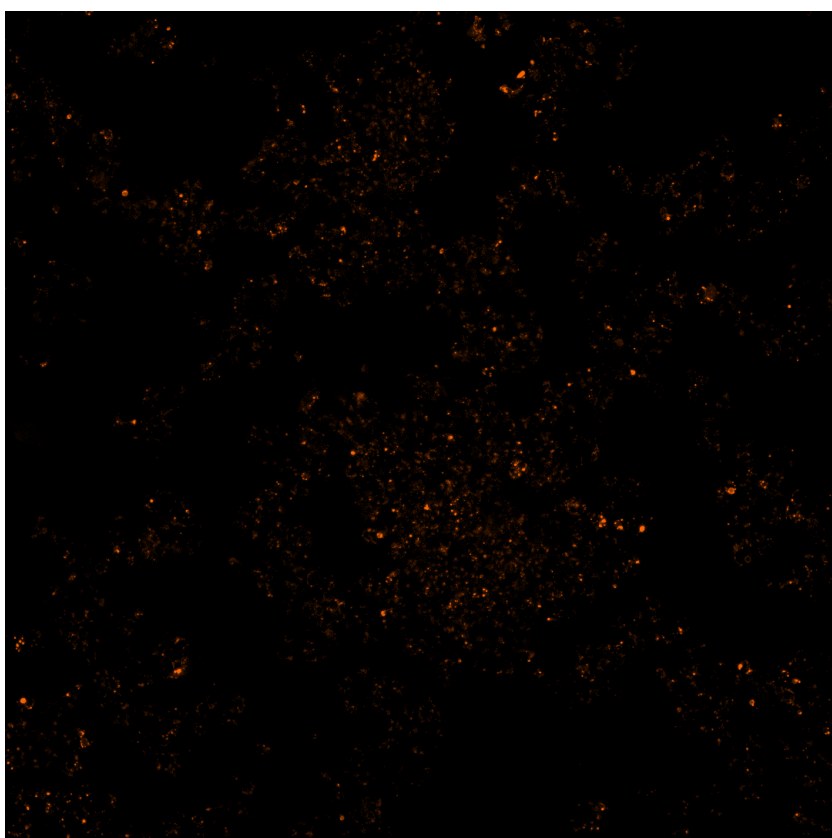
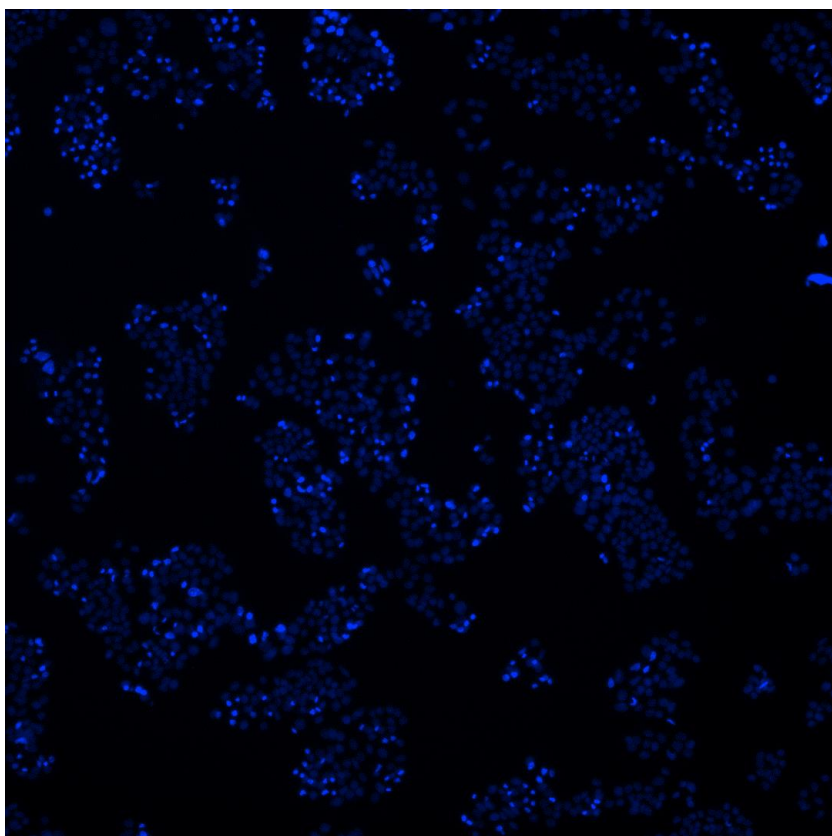




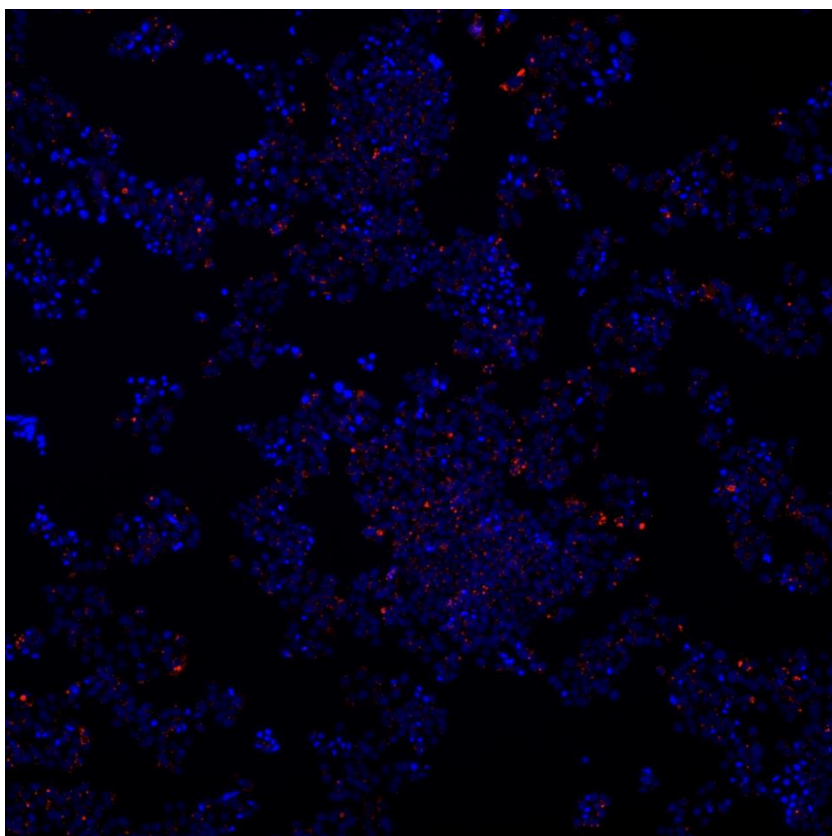
**Fig. 16.** Nuclei (live cells) and dead cells for E8, alcohol extract of *G. lucidum* fruiting bodies (12.5 µg/ml).

### 3.3.7 Hepatotoxicity assay (Lysosomes)

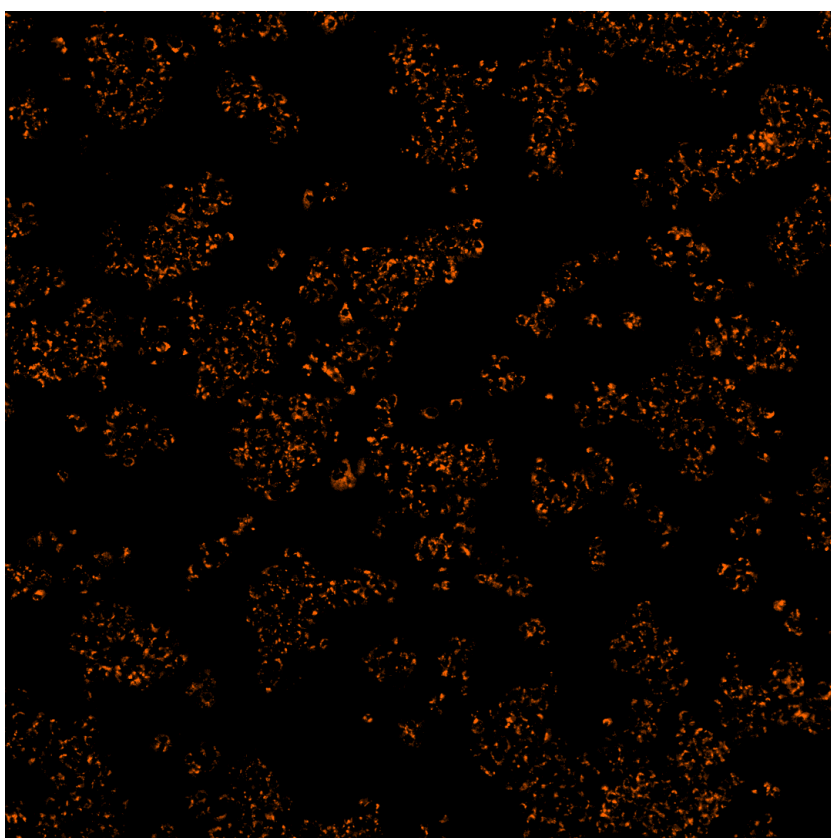
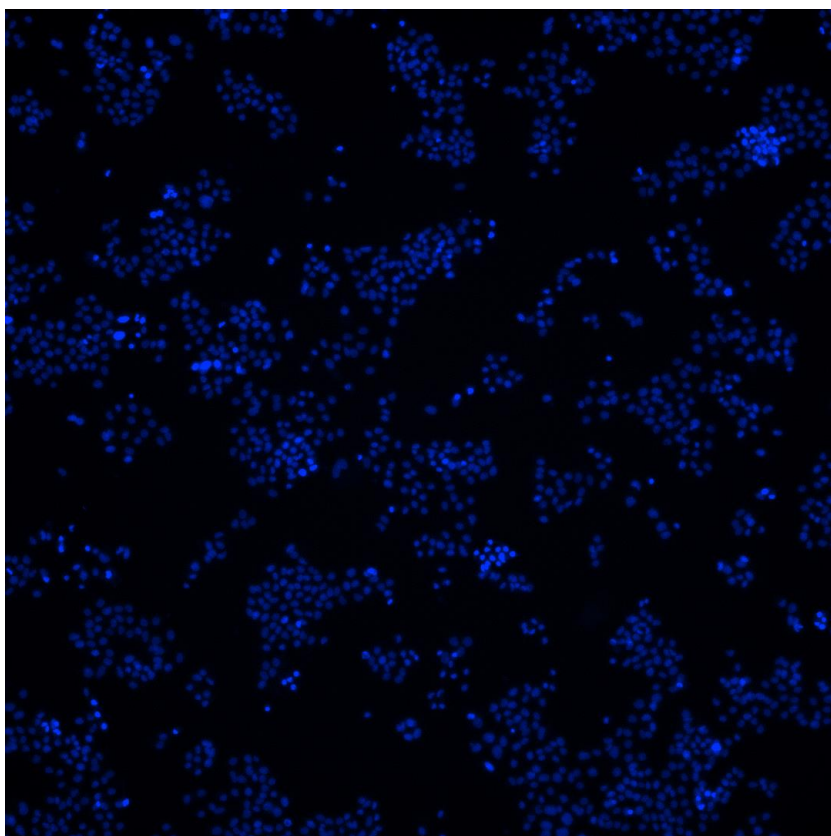
In the case of lysosomes, LysoTracker Red was used to stain lysosomes which can be seen as brightly red fluorescent cells. By comparing the negative (**Fig. 17**) and the positive control (**Fig. 18**), an increase in lysosomes was detected for the positive control, Melphalan. From the combined images of the negative (**Fig. 19**) and positive controls (**Fig. 20**), the increased number of lysosomes compared to the number of nuclei (live cells) can clearly be seen for the positive control, melphalan. For the lysosome assay, only the images of wells B5 and D5 are shown (letters indicate different concentrations while numbers indicate extracts). For the combined extract of *G. lucidum* fruiting bodies (**Figs. 21-24**) at different concentrations (100 and 25 µg/ml), the combined extract of *G. lucidum* fruiting bodies (**Figs. 21-24**) showed no increase in the lysosome content at either of the concentrations. No increase in dead cells was detected for the combined extract of *G. lucidum* fruiting bodies (**Figs. 21-24**) compared to the positive control, Melphalan (**Fig. 18**). Therefore, the extract resulted in no toxicity.



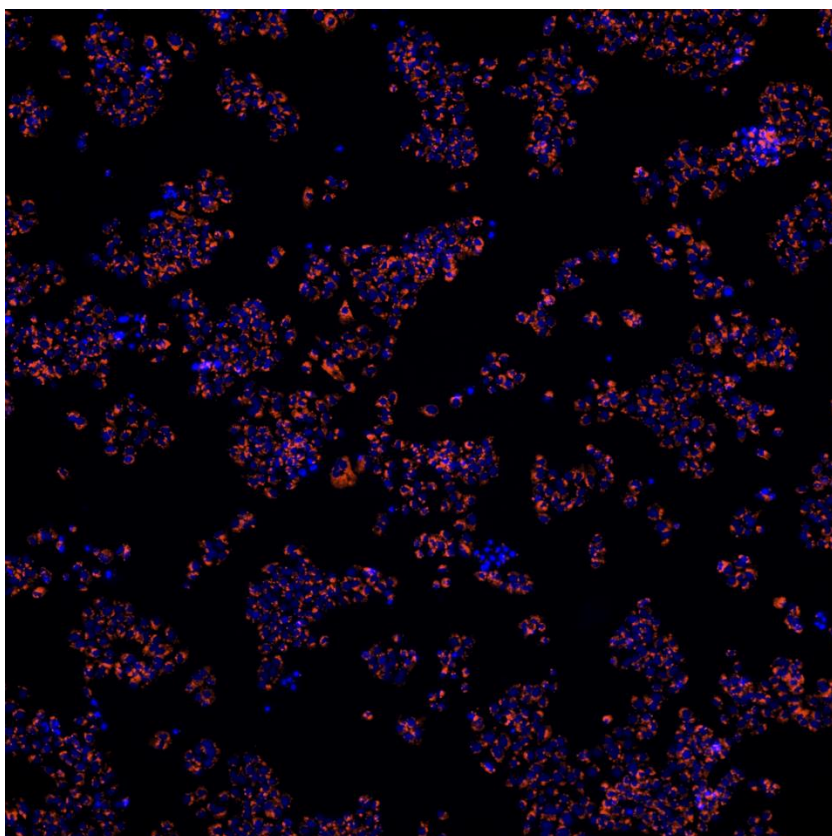
**Fig. 17.** Nuclei (live cells) and lysosomal content for the negative control.



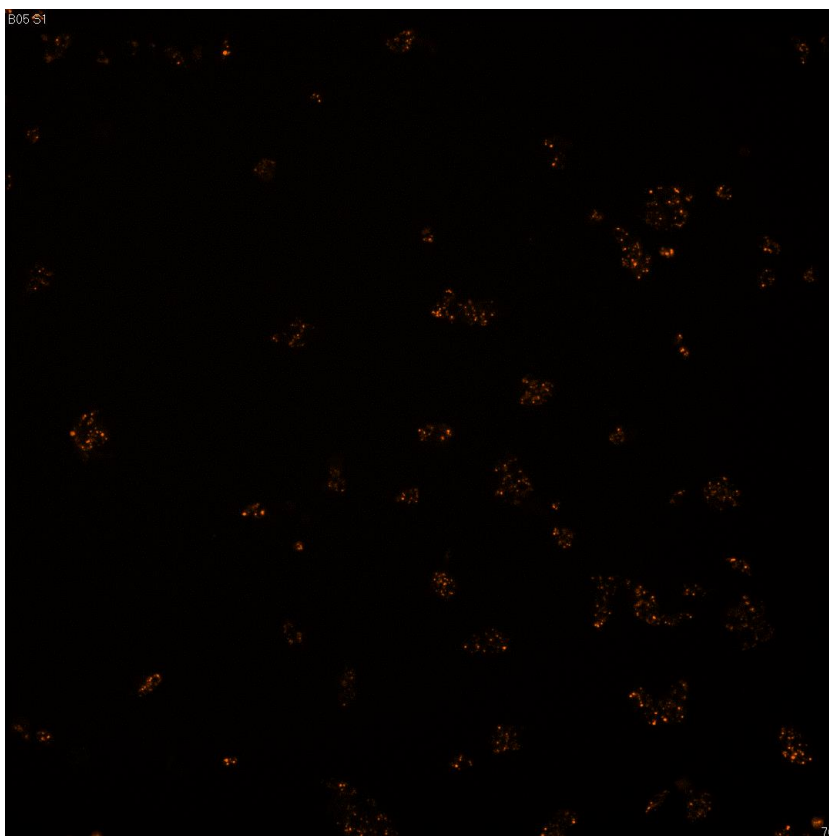
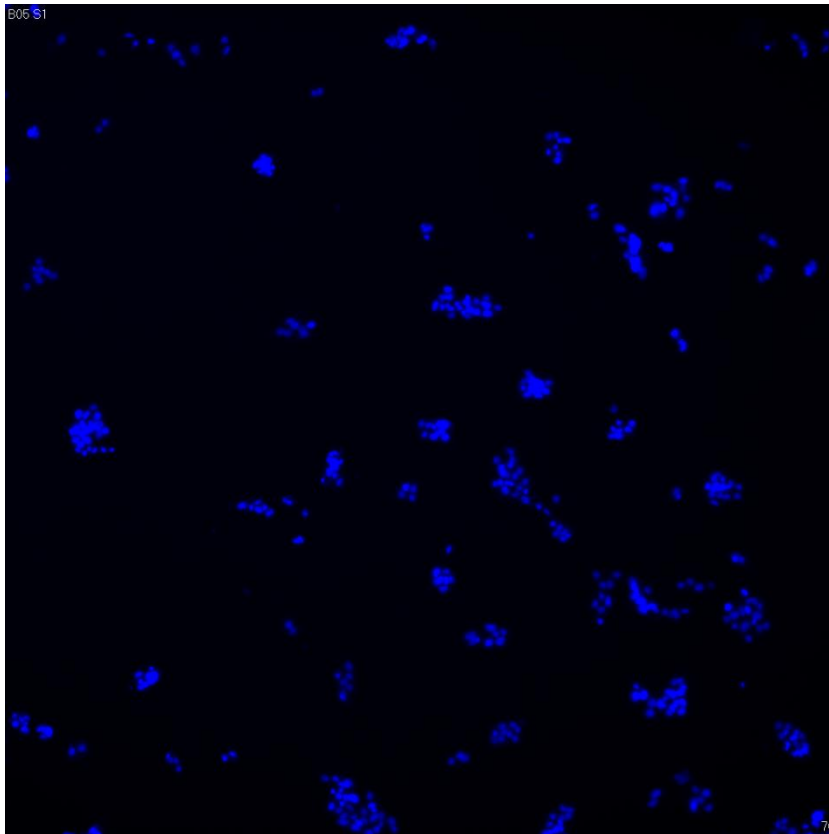
**Fig. 18.** Combined image of nuclei (live cells) and lysosomal content for the negative control.



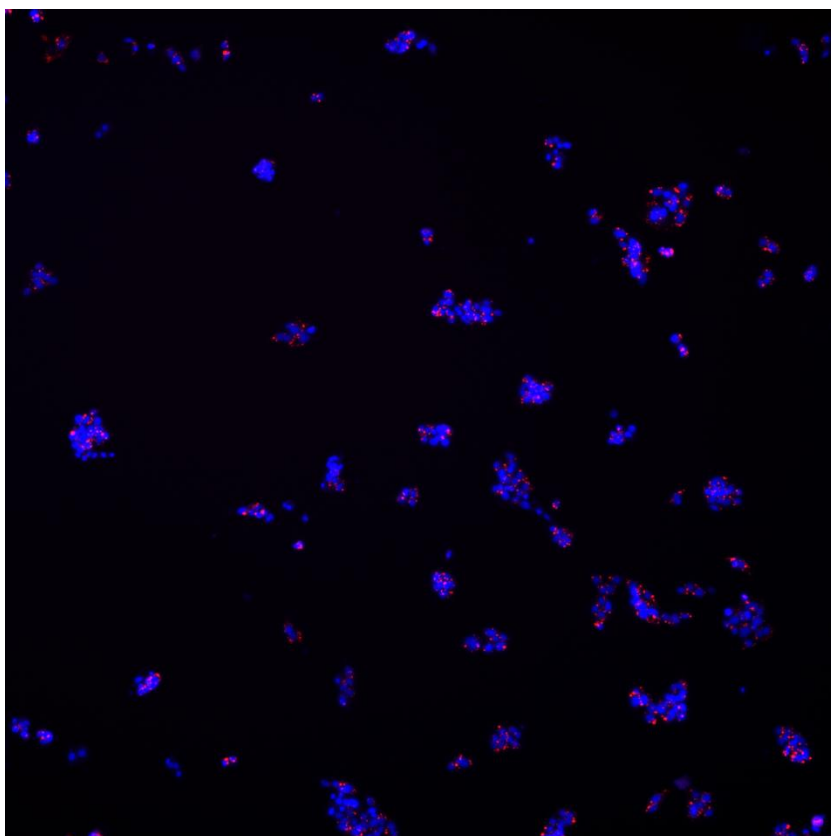
**Fig. 19.** Nuclei (live cells) and lysosomal content for the positive control, Melphalan.



**Fig. 20.** Combined image of nuclei (live cells) and lysosomal content for the positive control, Melphalan.

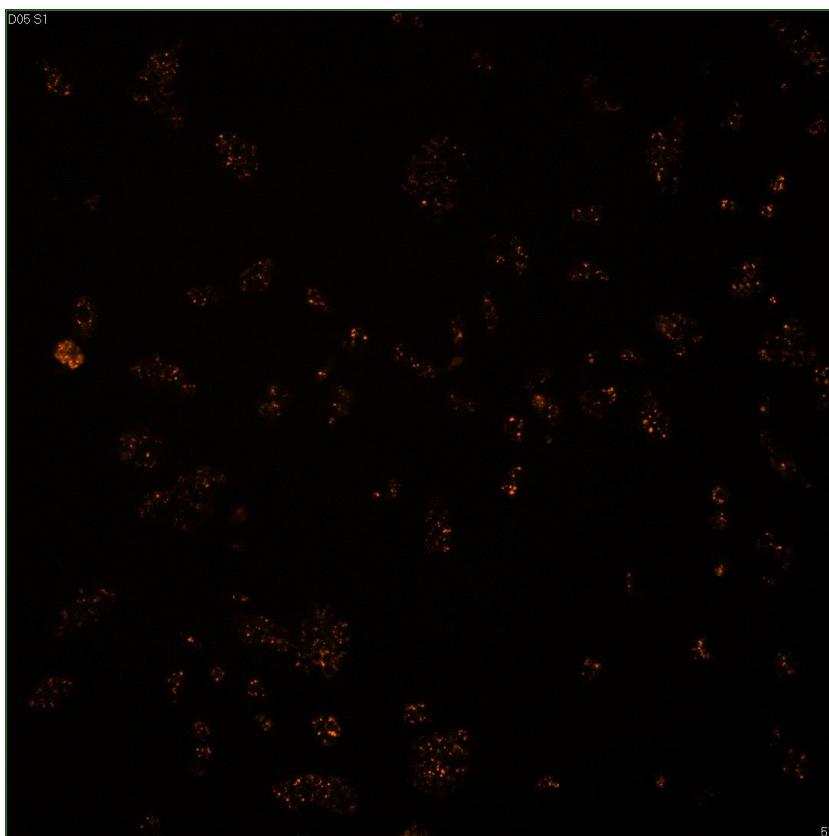
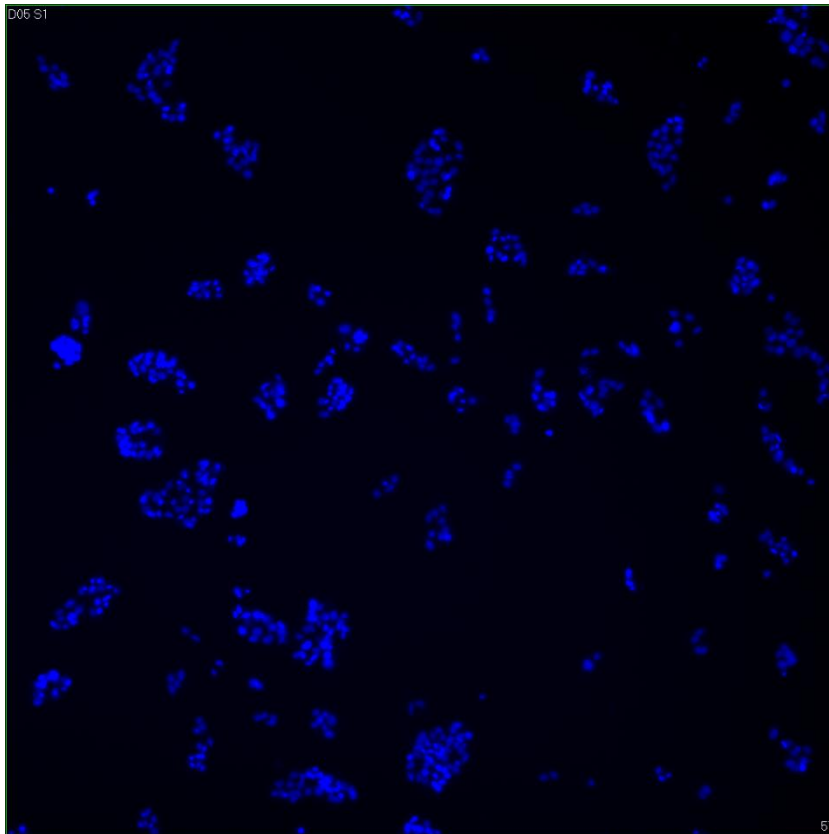


**Fig. 21.** B5 - Nuclei (live cells) and lysosomal content for the combined extract of *G. lucidum* fruiting bodies (100 µg/ml).

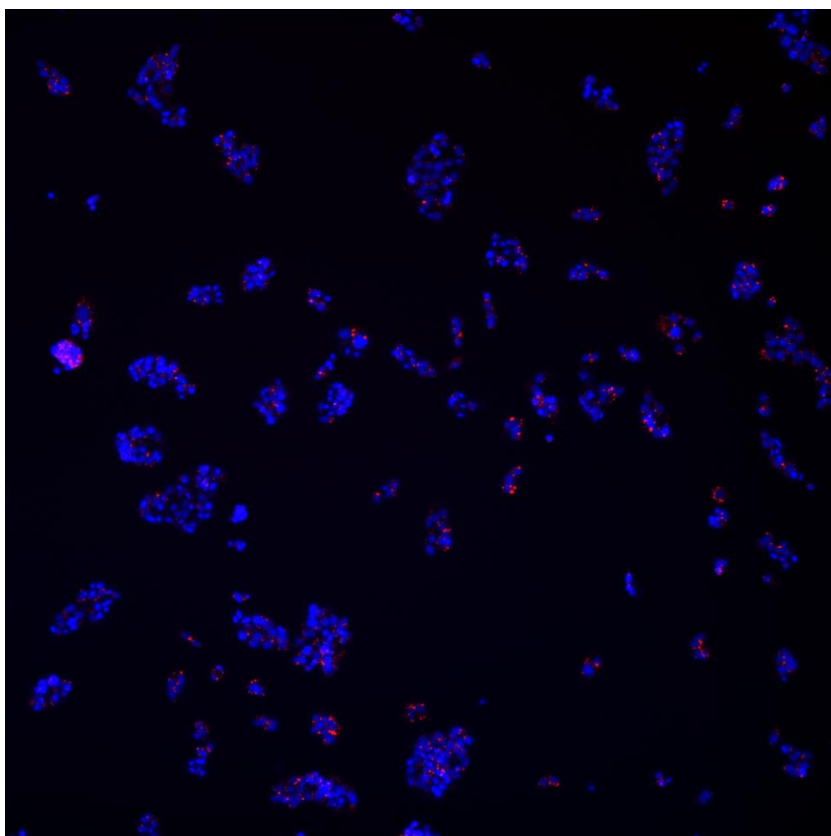


**Fig. 22.** Combined image of nuclei (live cells) and lysosomal content for *G. lucidum* fruiting bodies (100 µg/ml).





**Fig. 23.** D5 – Nuclei (live cells) and lysosomal content for the combined extract of *G. lucidum* fruiting bodies (25 µg/ml).



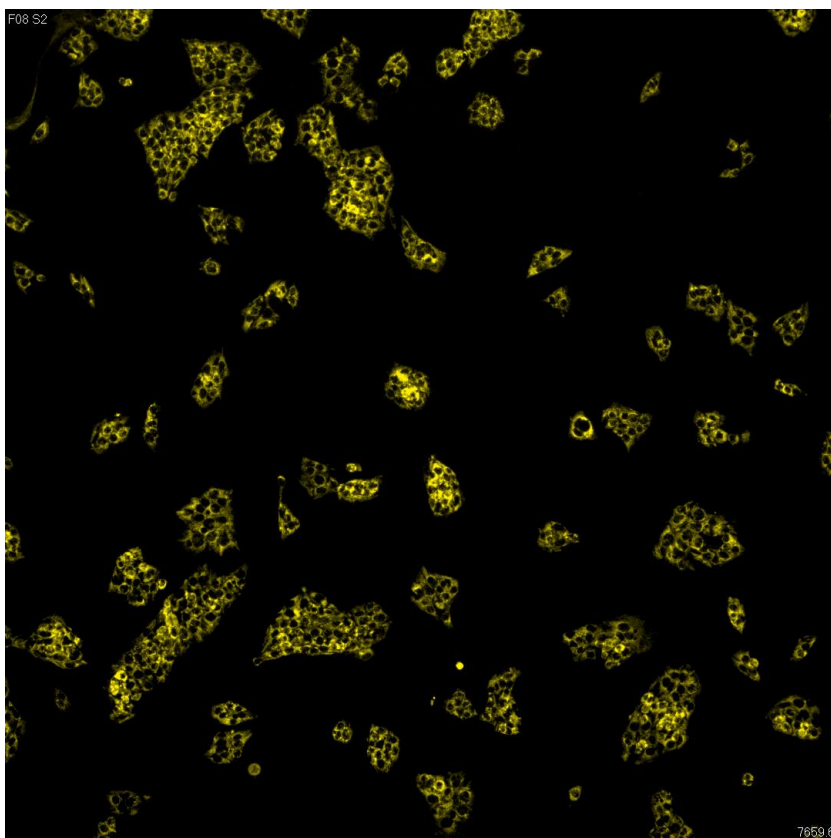
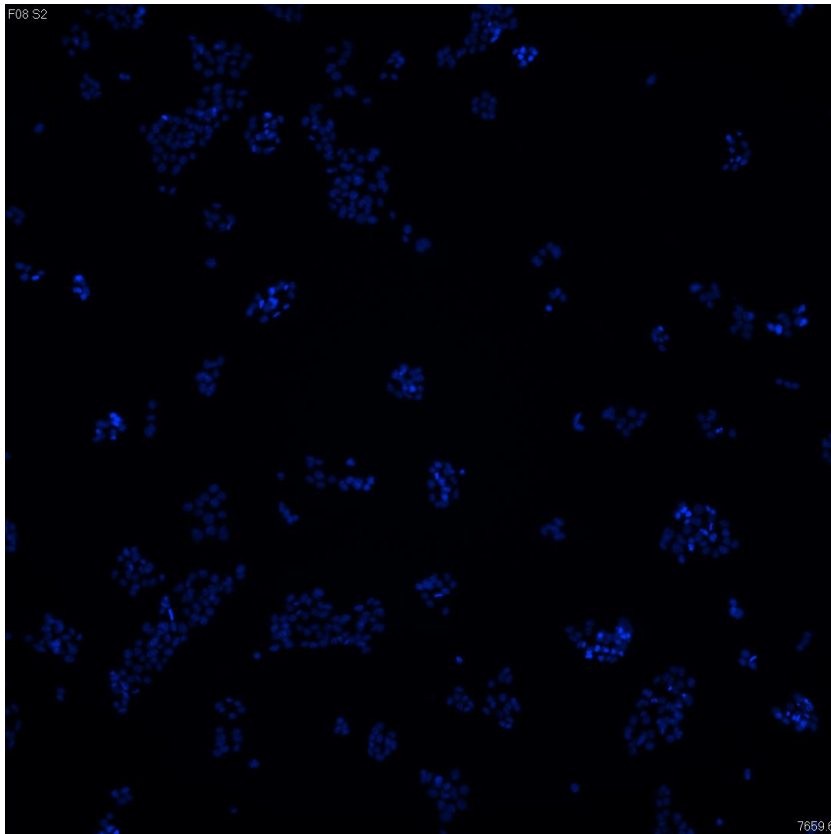
**Fig. 24.** Combined image of nuclei (live cells) and lysosomal content for the combined extract of *G. lucidum* fruiting bodies (25 µg/ml).

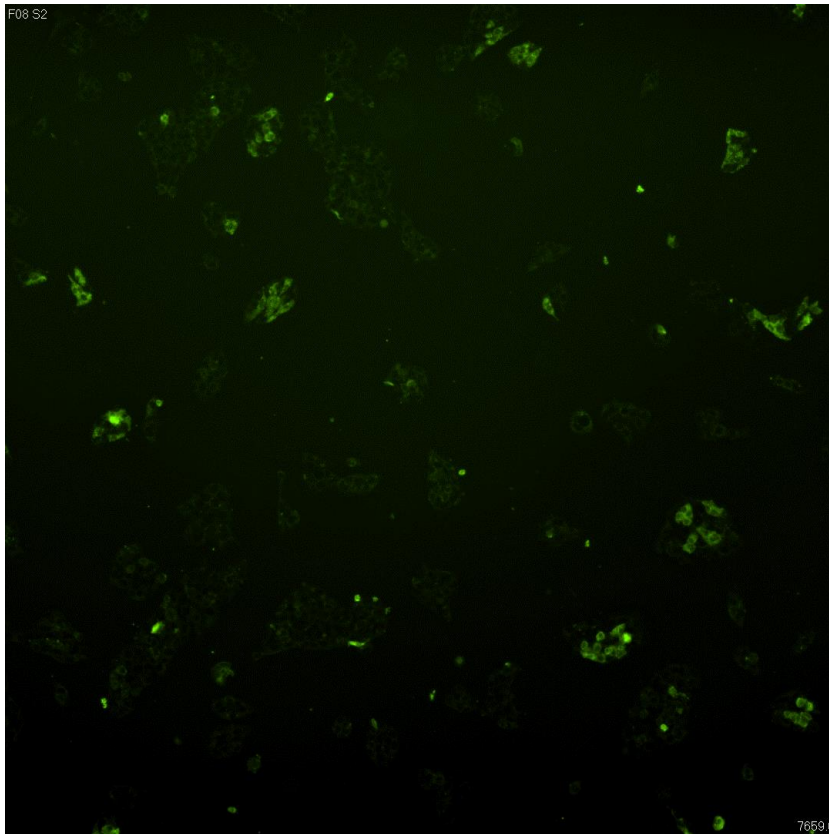
### 3.3.8 Multi-parameter hepatotoxicity assay (Mitochondrial dysfunction)

For the mitochondrial dysfunction assay, only the images of wells C12 and D9 are shown (letters indicate different concentrations while numbers indicate extracts). Hoechst 33342 was used to stain the nuclei blue. When comparing the negative control (**Fig. 25**) to the positive control, Melphalan (**Fig. 26**). Both the combined extract of *G. lucidum* SMS (**Fig. 27**) and the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 28**) at a concentration of 50 µg/ml and 25 µg/ml, respectively, had no effect on nuclei, resulting in no hepatotoxicity.

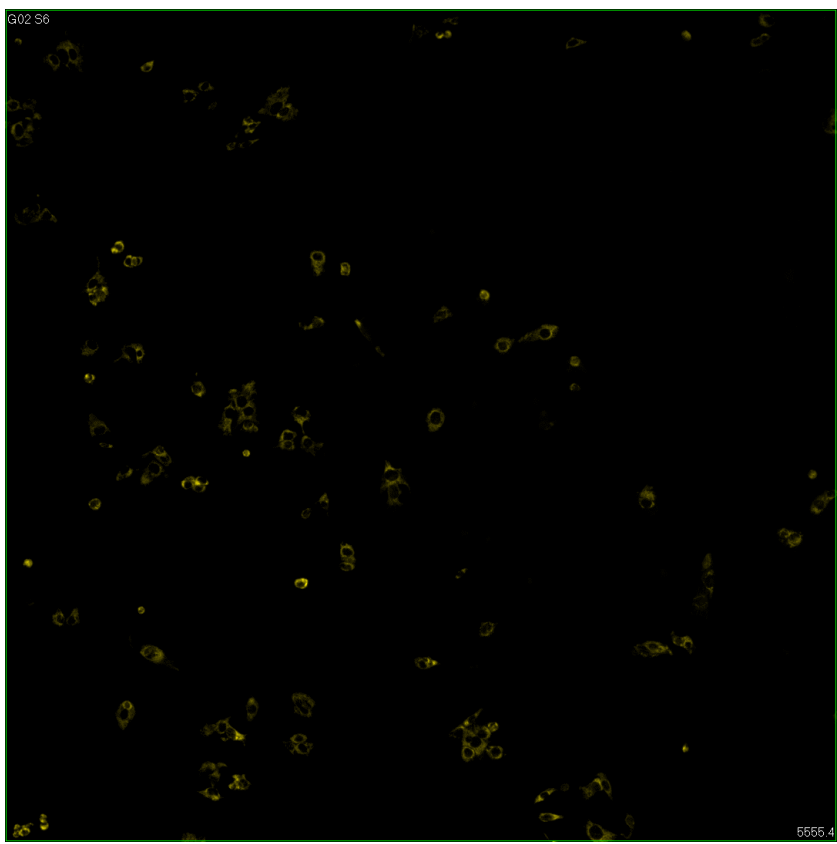
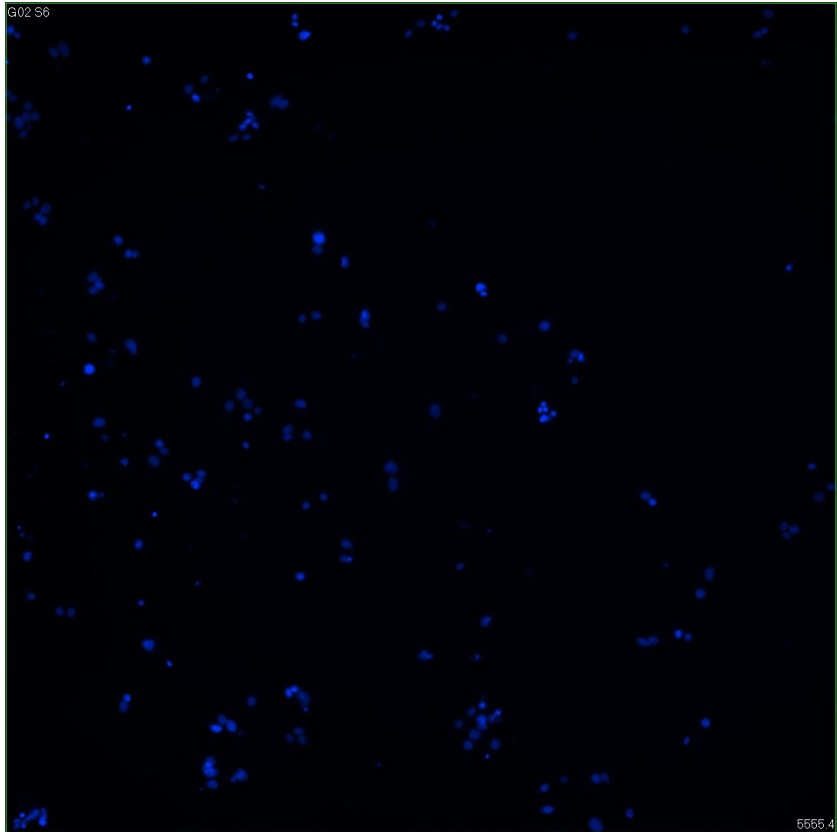
In the case of mitochondrial membrane potential, TMRE was used for staining the cells yellow. When compared to the negative control (**Fig. 25**), a decrease in intensity can be seen for the positive control, melphalan (**Fig. 26**) whereas a slight decrease in the mitochondrial membrane potential can be detected for both the combined extract of *G. lucidum* SMS (**Fig. 27**) and the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 28**) at a concentration of 50 µg/ml and 25 µg/ml, respectively. Thus, no hepatotoxicity was detected for both extracts.

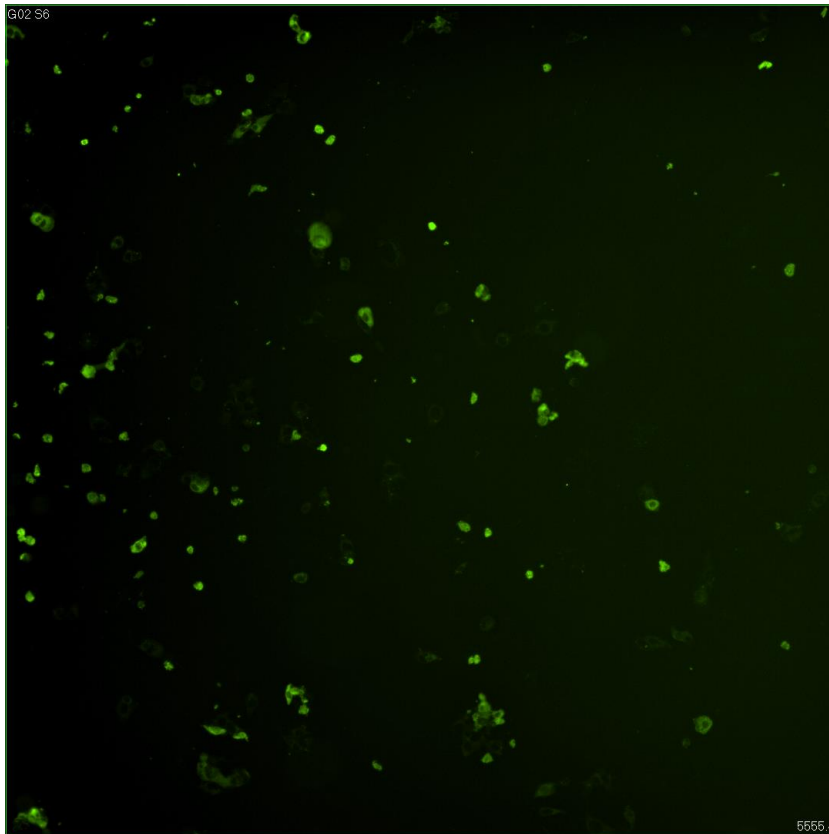
The mitochondrial membrane was stained green using Mito-Tracker Green, which stains mitochondria independent of the membrane potential. When compared to the negative control (**Fig. 25**), a significant increase in the mitochondrial membrane was detected for the positive control, Melphalan (**Fig. 26**). This increase is due to cells trying to compensate for the loss of membrane potential. When compared to the negative control (**Fig. 25**), the combined extract of *G. lucidum* SMS (**Fig. 27**) and the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 28**) at concentrations of 50 µg/ml and 25 µg/ml, respectively, seemed to only result in a slight increase in mitochondrial membrane.



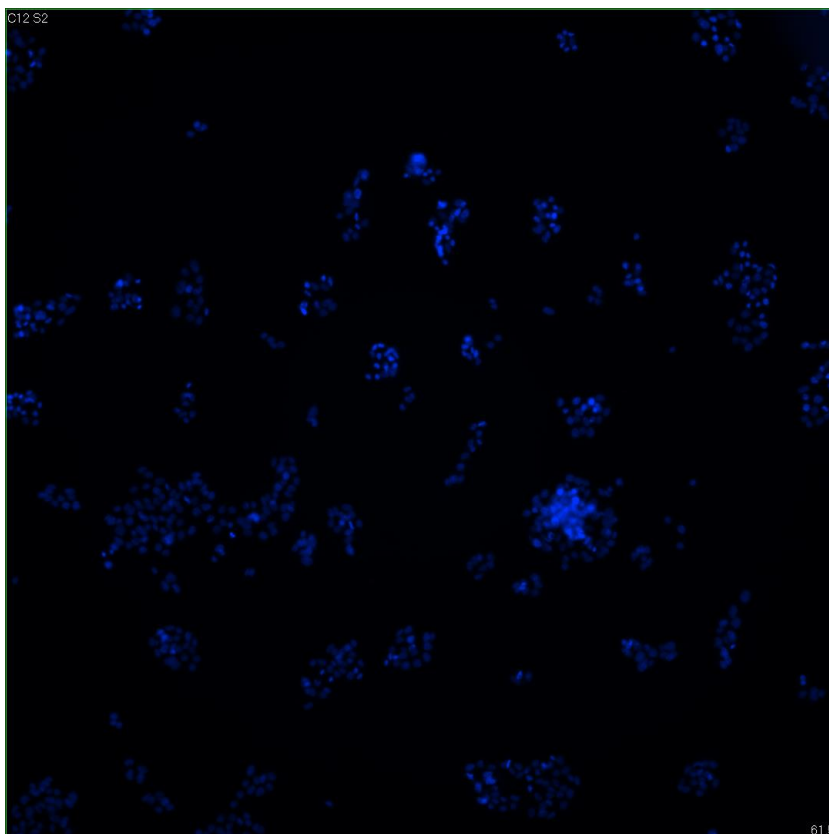


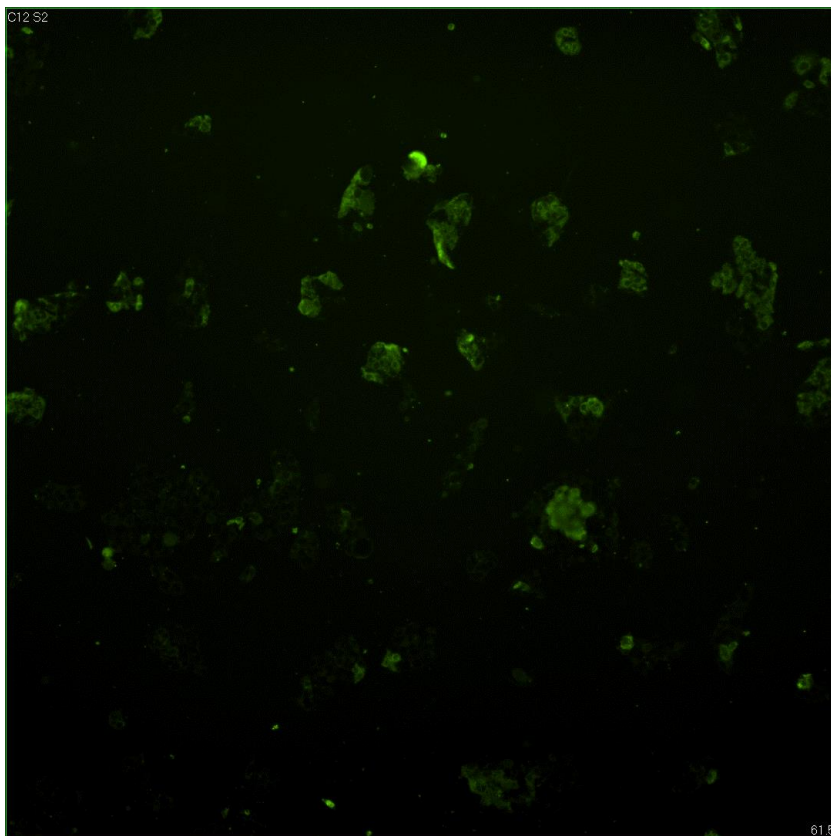
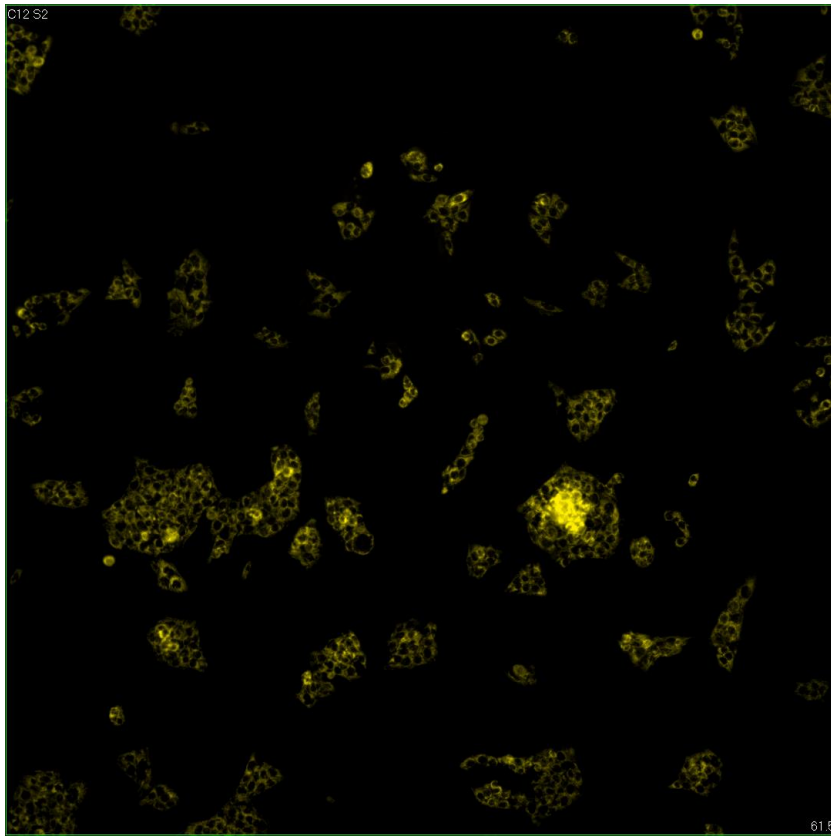
**Fig. 25.** Images of the nuclei (blue), mitochondrial potential (yellow) and mitochondrial membrane (green) for the negative control (F8).





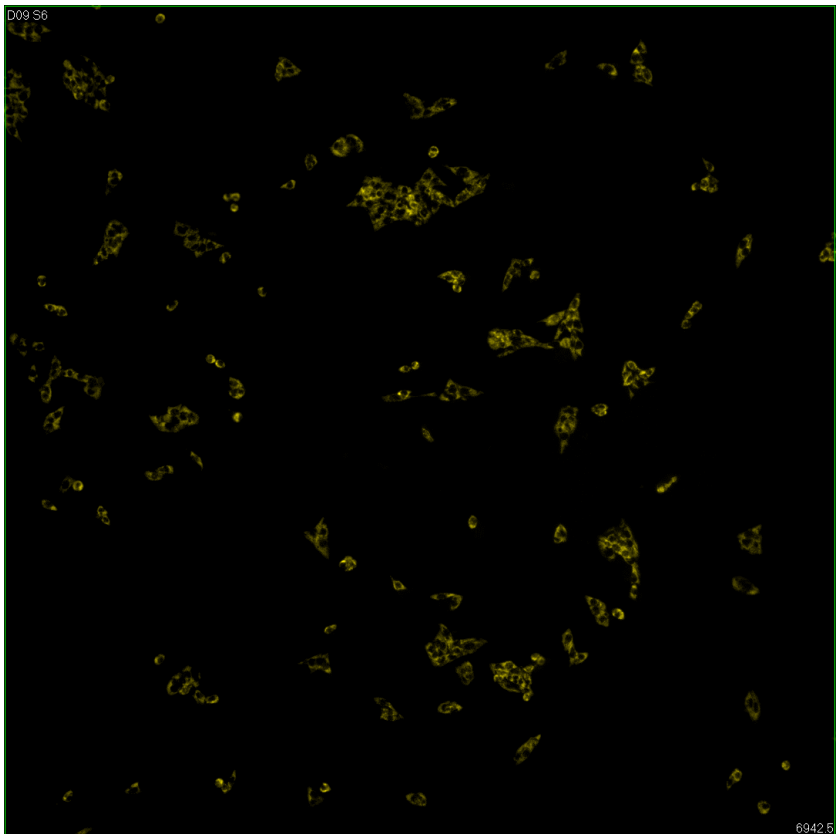
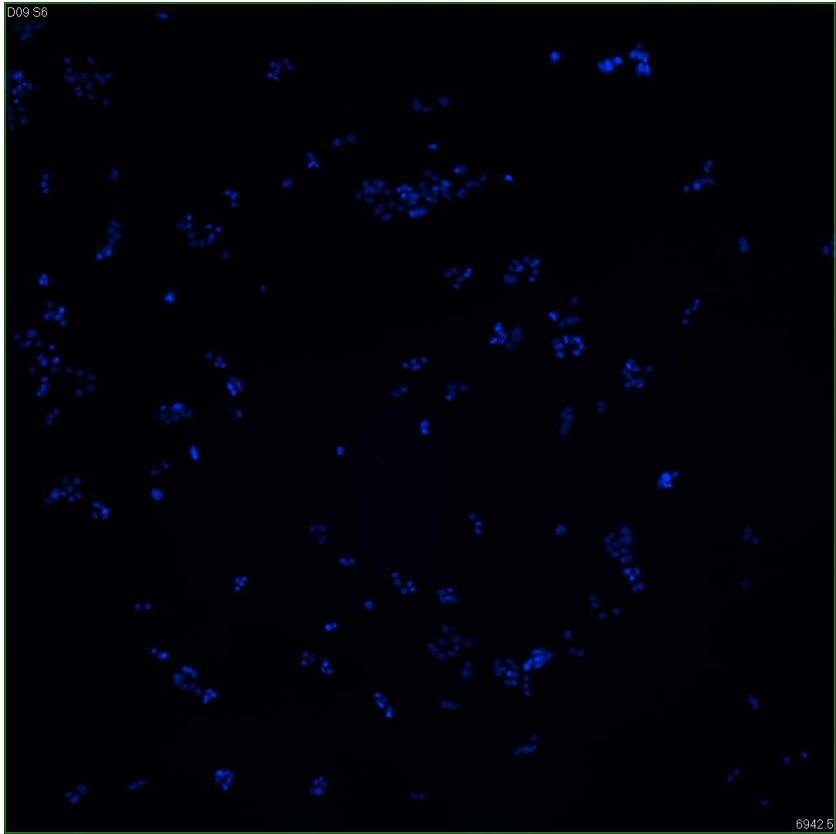
**Fig. 26.** Images of the nuclei (blue), mitochondrial potential (yellow) and mitochondrial membrane (green) for the positive control, Melphalan (G2).

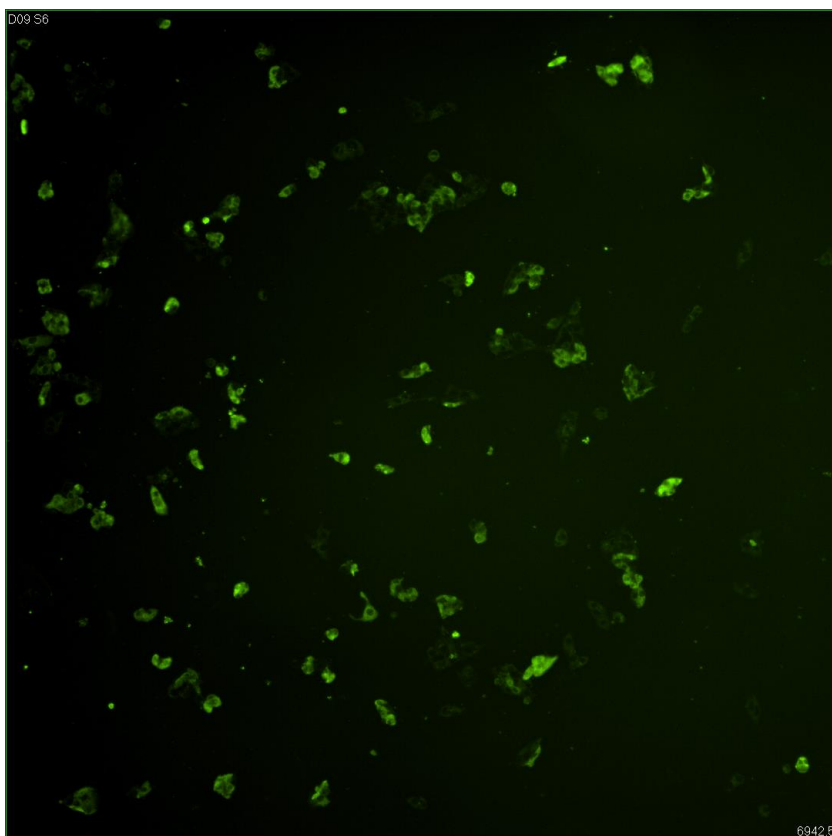




**Fig. 27.** Images of the nuclei (blue), mitochondrial potential (yellow) and mitochondrial membrane (green) for C12, combined extract of *G. lucidum* SMS (50 µg/ml).



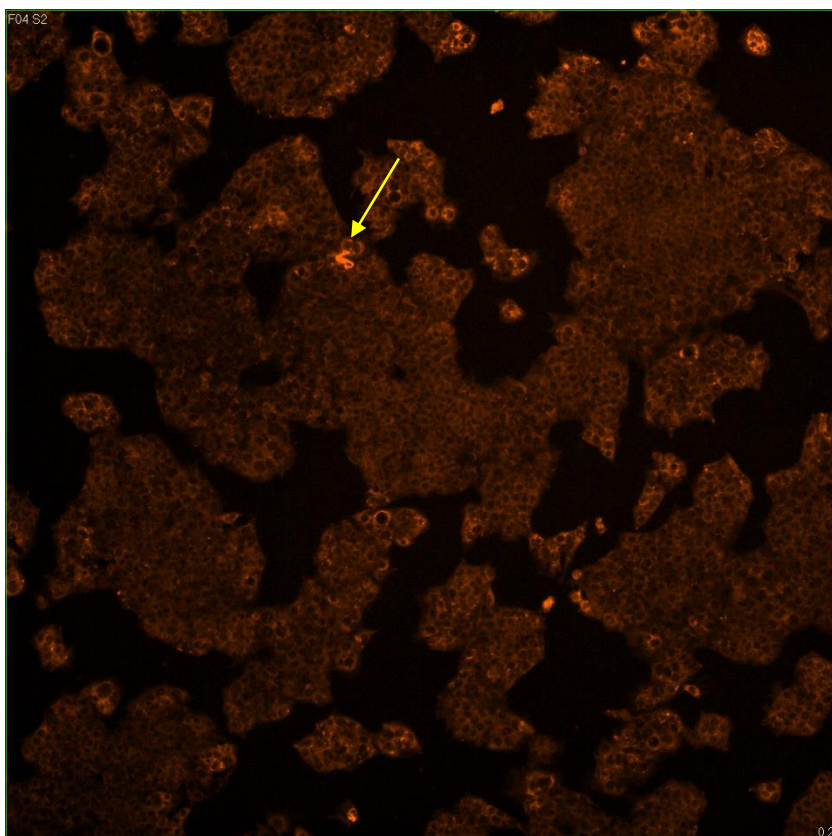
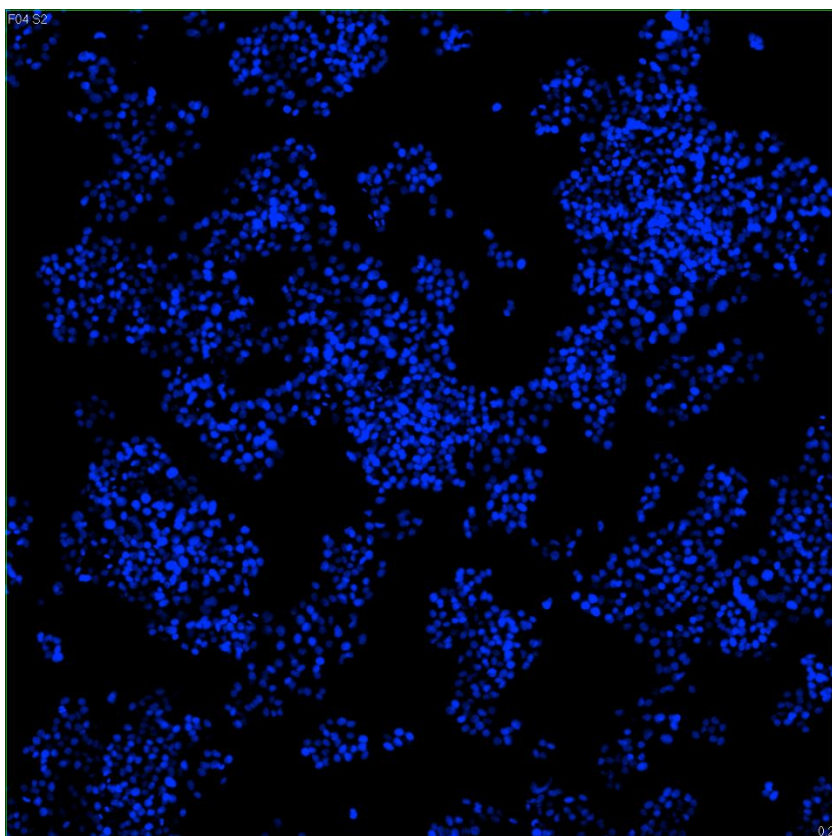




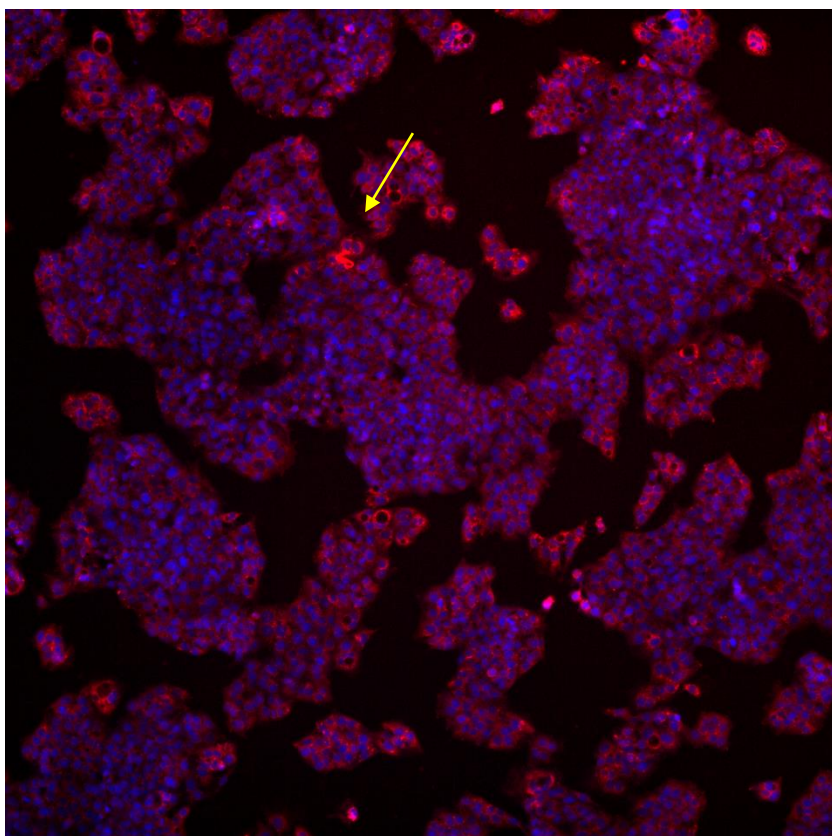
**Fig. 28.** Images of the nuclei (blue), mitochondrial potential (yellow) and mitochondrial membrane (green) for D9, alcohol extract of *G. lucidum* fruiting bodies (25 µg/ml).

### 3.3.9 Hepatotoxicity assay (lipid accumulation/steatosis)

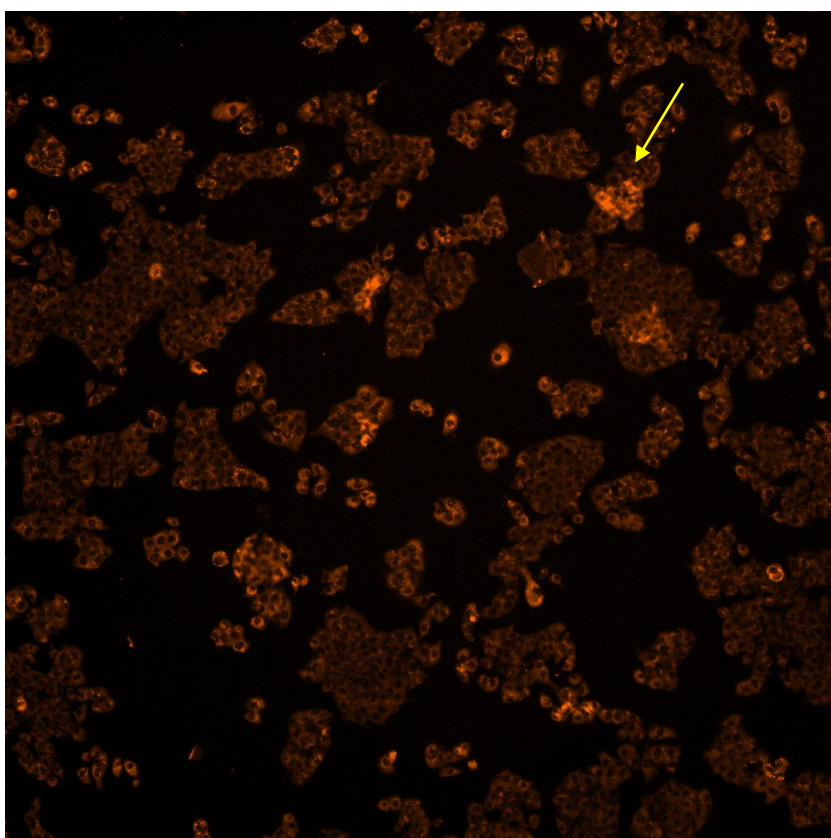
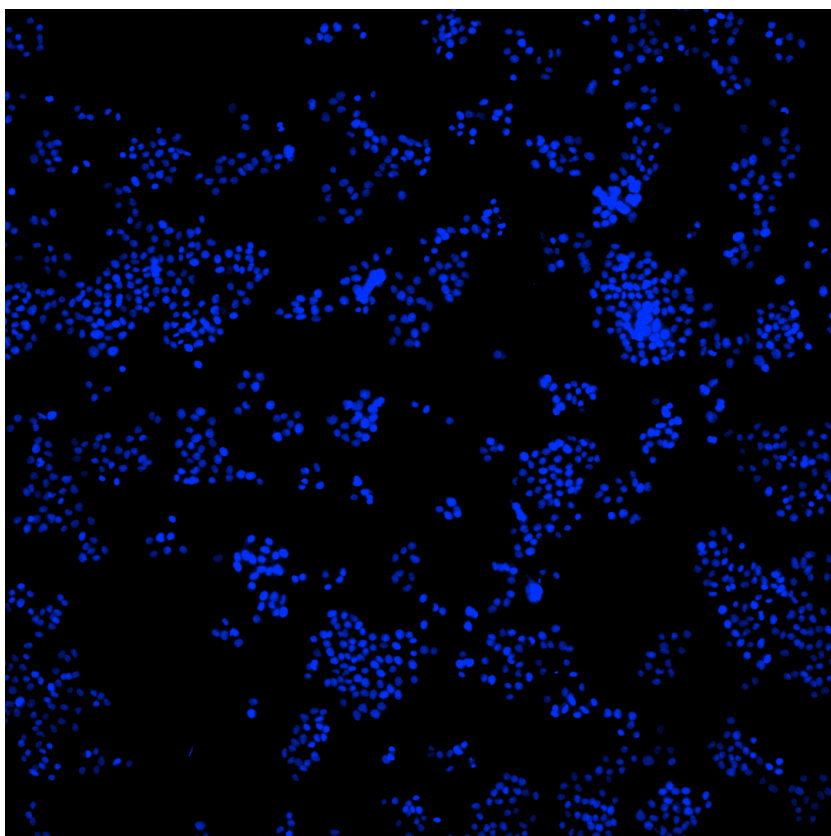
For lipid accumulation (steatosis), only the images of wells D7 and E7 are shown (letters indicate different concentrations while numbers indicate extracts). LipidTox Red was used to stain lipids accumulating in the cells red while Hoechst 33342 was used for staining nuclei blue. When compared to the negative control (**Fig. 29**), the positive control, Chloroquine (**Fig. 31**), showed an increase in accumulated lipids. From the combined images of the negative (**Fig. 30**) and positive controls (**Fig. 32**), the increased amount of accumulated lipids compared to the number of nuclei (live cells) can clearly be seen for the positive control, Chloroquine (**Fig. 32**). The alcohol extract of *G. lucidum* fruiting bodies (**Figs. 33 and 35**) at different concentrations (25 and 12.5 µg/ml) showed no increase in the accumulation of lipids resulting in no toxicity of the extracts. From the combined images (**Figs. 34 and 36**), no increase in lipid accumulation or decrease in nuclei content were detected for the alcohol extract of *G. lucidum* fruiting bodies at different concentrations (25 and 12.5 µg/ml).



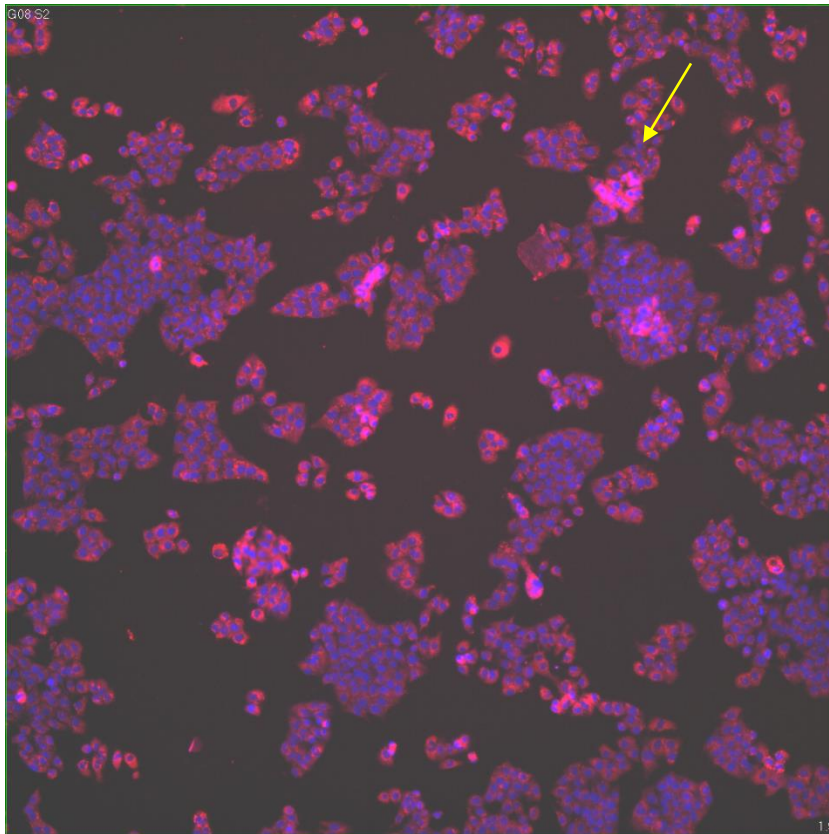
**Fig. 29.** Images of nuclei (blue) and lipid accumulation (red) for the negative control (F4).



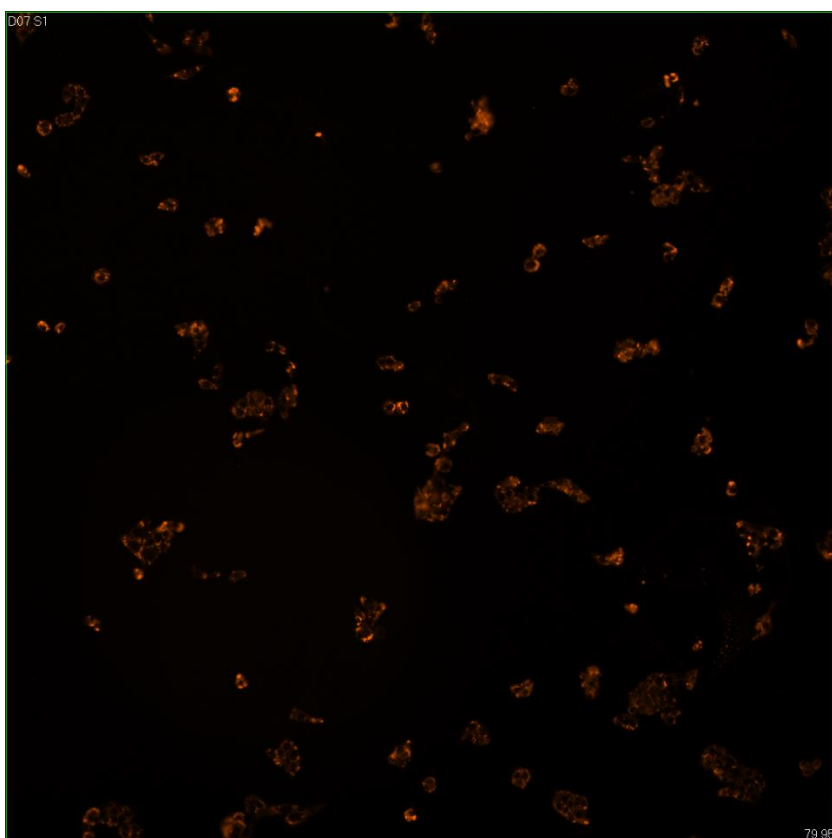
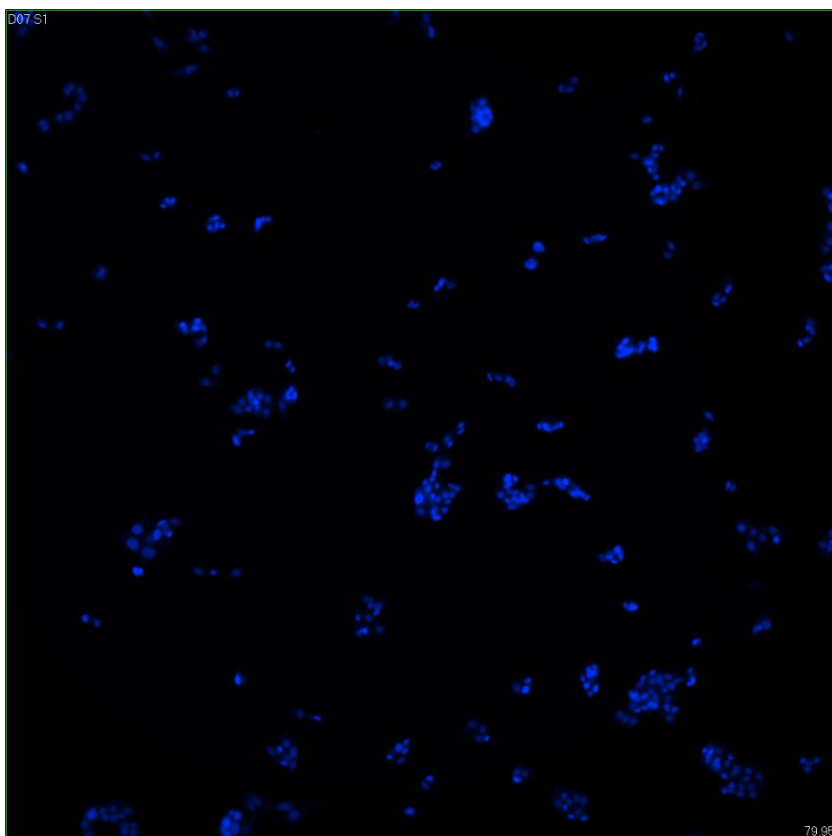
**Fig. 30.** Combined image of nuclei (blue) and lipid accumulation (red) for the negative control (F4).



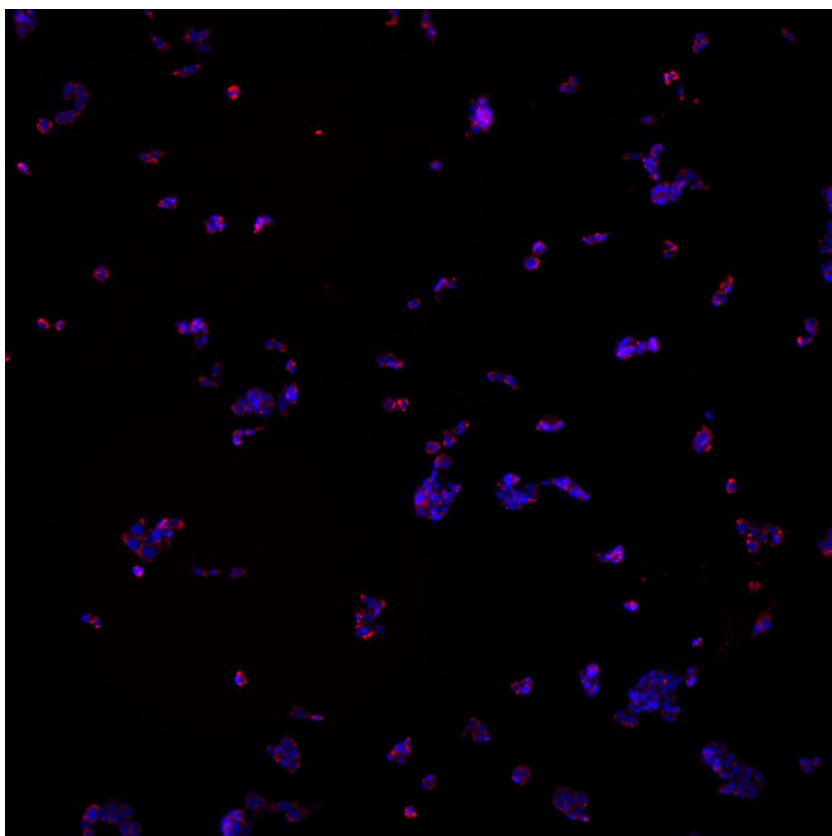
**Fig. 31.** Images of nuclei (blue) and lipid accumulation (red) for the positive control, Chloroquine (G8).



**Fig. 32.** Combined image of nuclei (blue) and lipid accumulation (red) for the positive control, Chloroquine (G8).

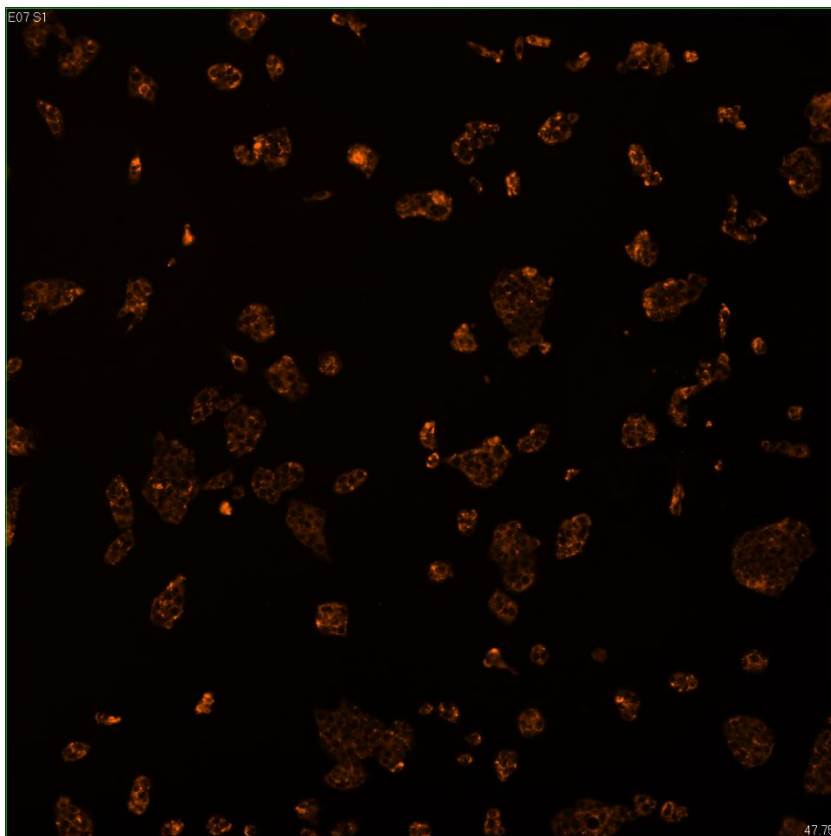
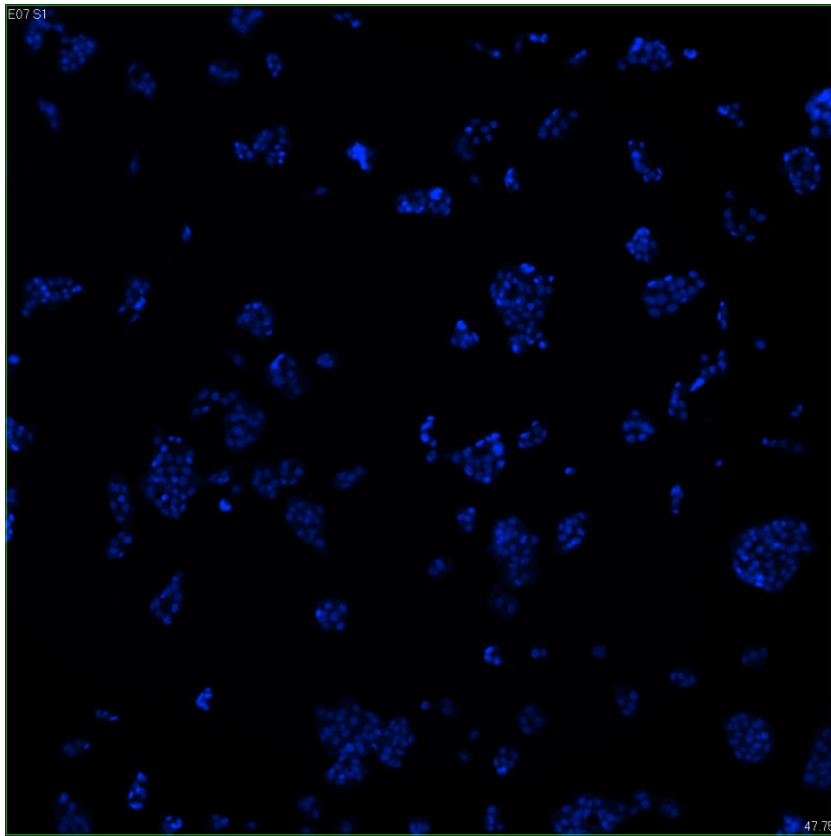


**Fig. 33.** Images of nuclei (blue) and lipid accumulation (red) for D7, alcohol extract of *G. lucidum* fruiting bodies (25 µg/ml).

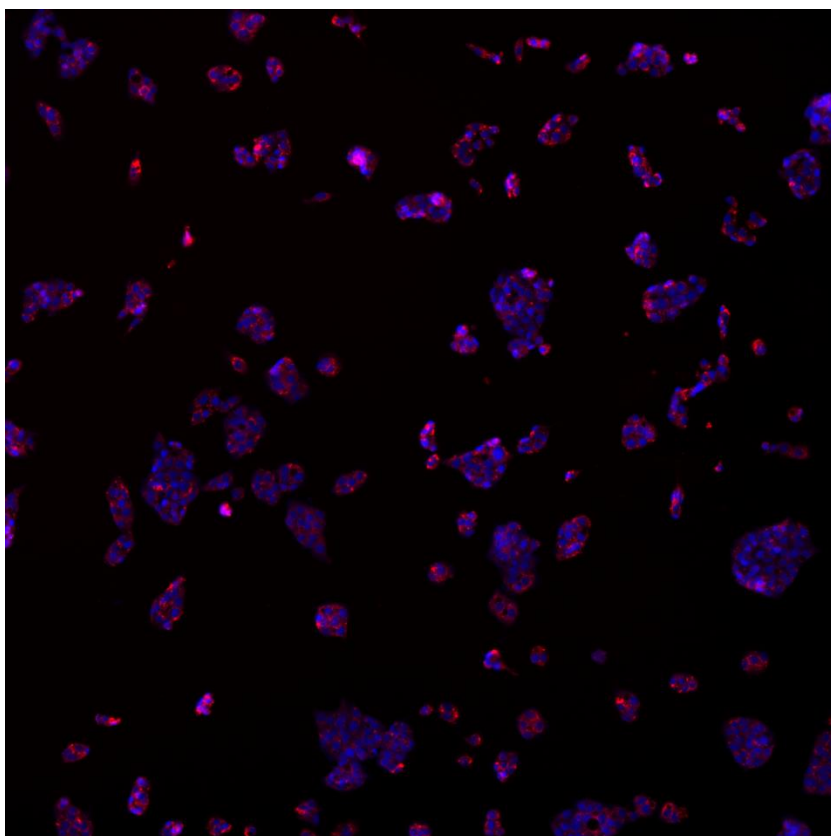


**Fig. 34.** Combined image of nuclei (blue) and lipid accumulation (red) for D7, alcohol extract of *G. lucidum* fruiting bodies (25 µg/ml).





**Fig. 35.** Images of nuclei (blue) and lipid accumulation (red) for E7, alcohol extract of *G. lucidum* fruiting bodies (12.5 µg/ml)



**Fig. 36.** Combined image of nuclei (blue) and lipid accumulation (red) for E7, *G. lucidum* fruiting bodies (12.5 µg/ml).

### 3.3.10 Analysis of plates

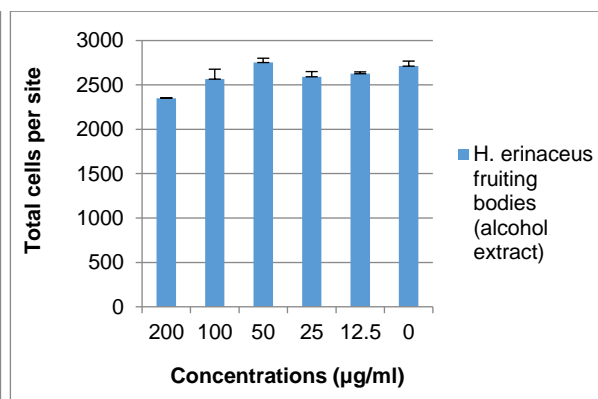
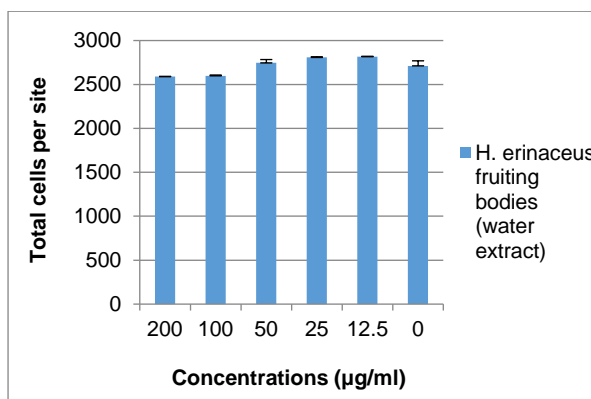
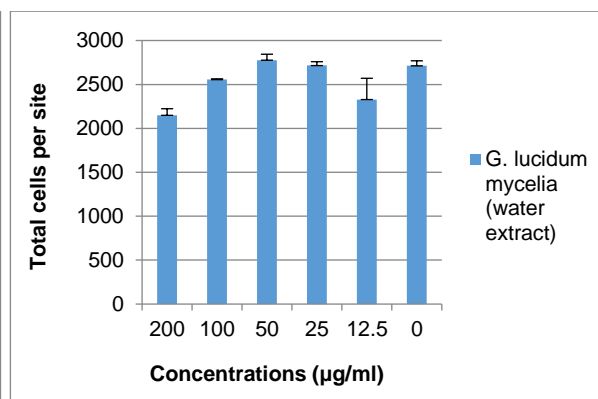
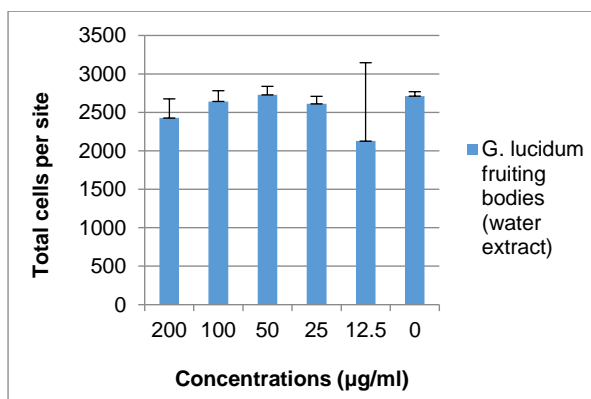
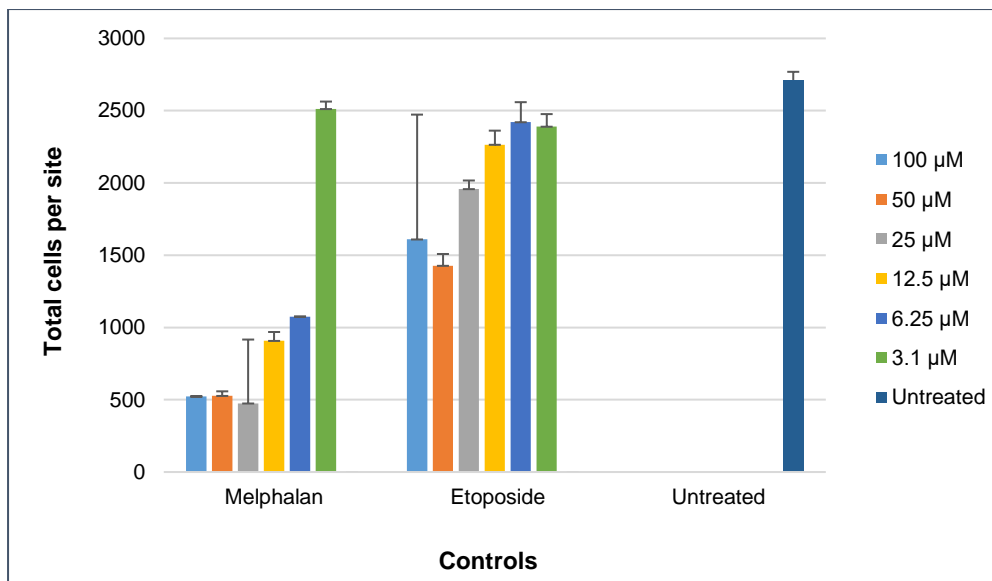
#### 3.3.10.1 Live/dead cells

After image acquisition using ImageXpress Micro XLS Widefield High-Content Analysis System (Molecular Devices®) at 20x magnification, the images were analysed using the MetaXpress® High-Content Image Acquisition & Analysis Software and Multi-wavelength Cell Scoring analysis module.

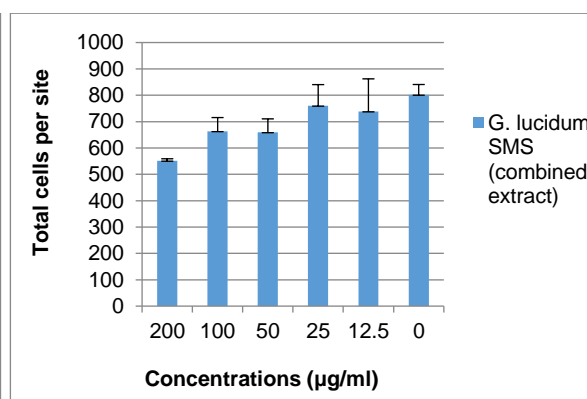
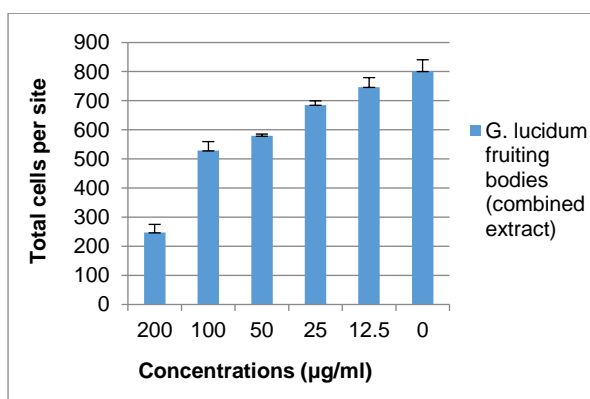
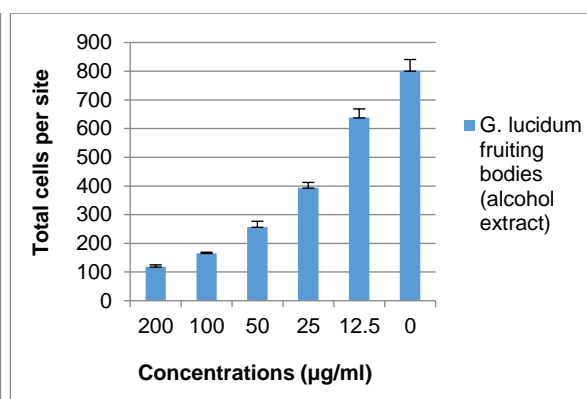
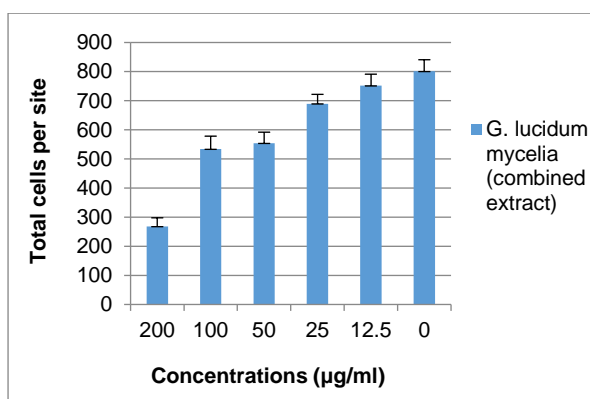
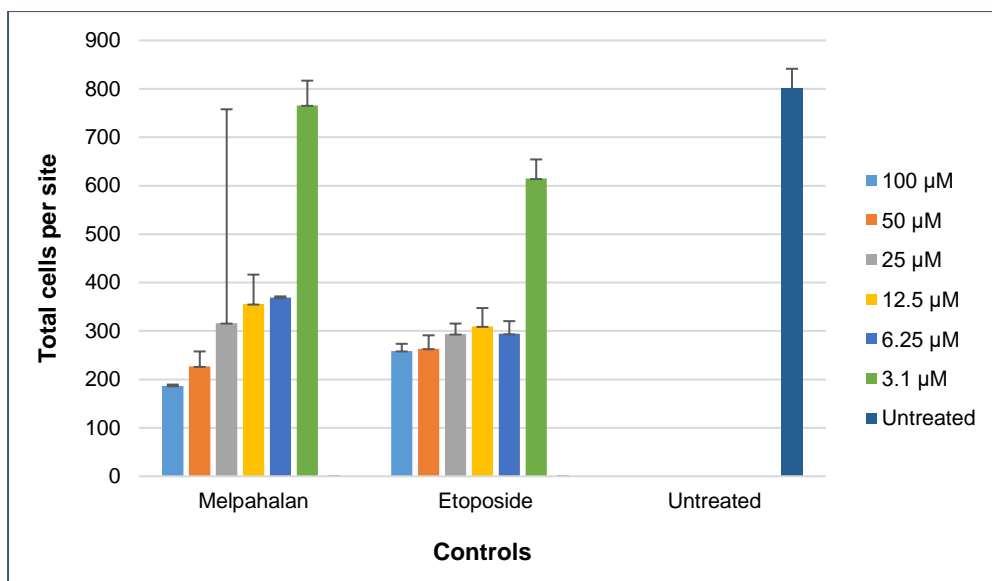
Different plates were used, thus resulting in different effects of controls. From **Fig. 37**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For this part, 0 µg/ml refers to the negative control for comparison to show any effect that may have occurred. From **Fig. 37**, no significant effect of the water extract of *G. lucidum* fruiting bodies can be detected for the total cells per site at high concentrations, compared to the negative control (0 µg/ml). However, a decrease was detected for the lowest concentration (12.5 µg/ml), but this might be due to contaminated wells and further

investigation is required. Although no significant effect was detected for the water extract of *G. lucidum* mycelia (**Fig. 37**) at concentrations between 100-25 µg/ml, a decrease in total cells per site for concentrations of 200 and 12.5 µg/ml were detected. This was unexpected and might be due to contaminated wells and further investigation is required. For the water extract of *H. erinaceus* fruiting bodies (**Fig. 37**), no decrease in total cells per site were detected for any of the concentrations compared to the negative control (0 µg/ml). Moreover, concentrations of 50, 25 and 12.5 µg/ml showed an increase in total cells per site and further investigation is required. For the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 37**), no significant effect was detected apart from the highest concentration of 200 µg/ml resulting in a slight decrease of total cells per site.

From **Fig. 38**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to show any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 38**), a significant decrease of total cells per site were detected for the highest concentration (200 µg/ml) was detected while only a slight decrease in total cells per site were detected for concentrations between 100 and 12.5 µg/ml when compared to the negative control (0 µg/ml). In contrast to the water extract, the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 38**) proved to be more toxic than the water extract as a significant decrease in total cells were detected at concentrations between 200 and 25 µg/ml. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 38**), only a slight decrease in total cells per site were detected while a significant decrease was detected for the 200 µg/ml concentration when compared to the negative control (0 µg/ml). The combined extract of *G. lucidum* SMS (**Fig.38**) showed no significant effect apart from the highest concentration of 200 µg/ml resulting in a slight decrease of total cells per site.



**Fig. 37.** Effect of controls and different extracts at different concentrations on total cells per site (plate1).

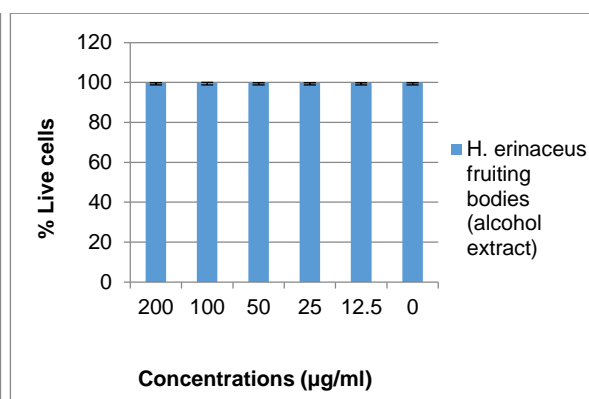
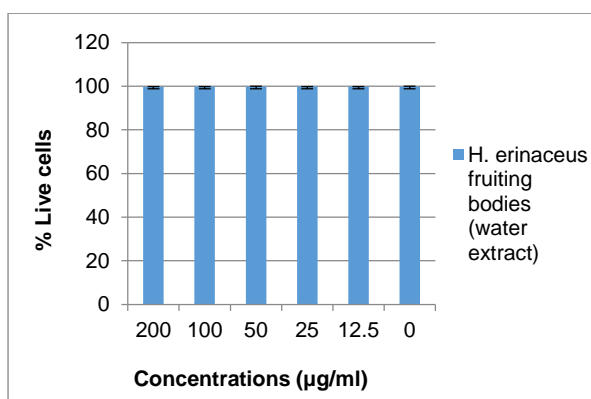
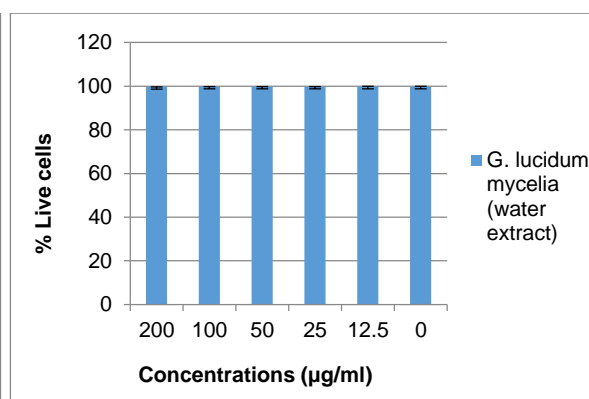
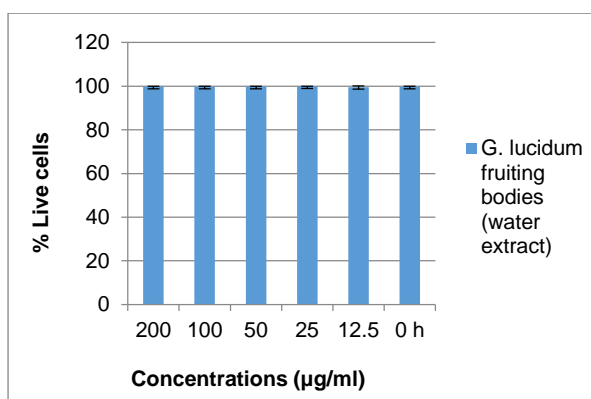
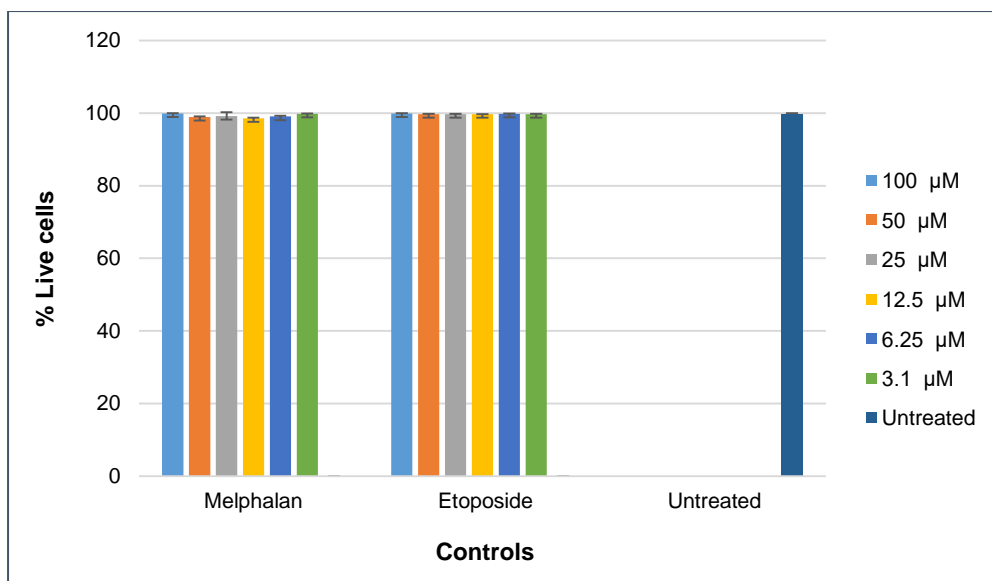


**Fig. 38.** Effect of controls and different extracts at different concentrations on total cells per site (plate 2).

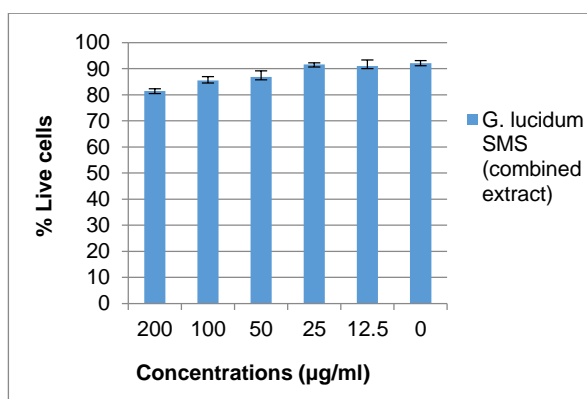
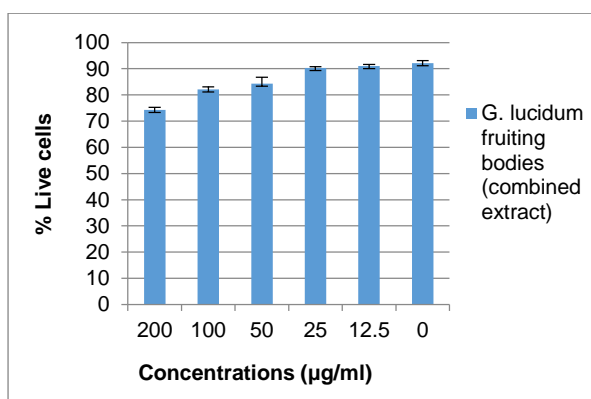
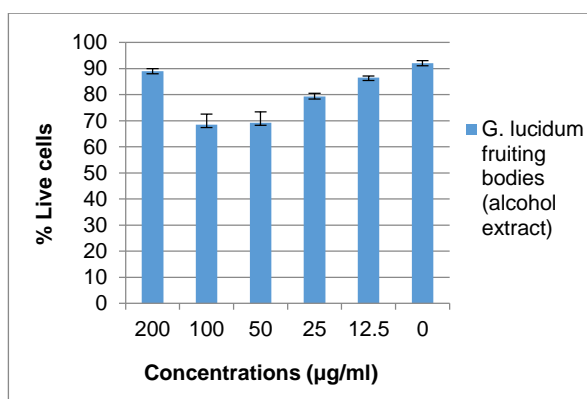
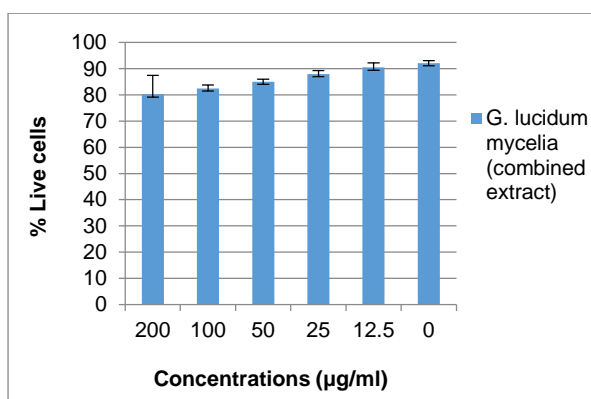
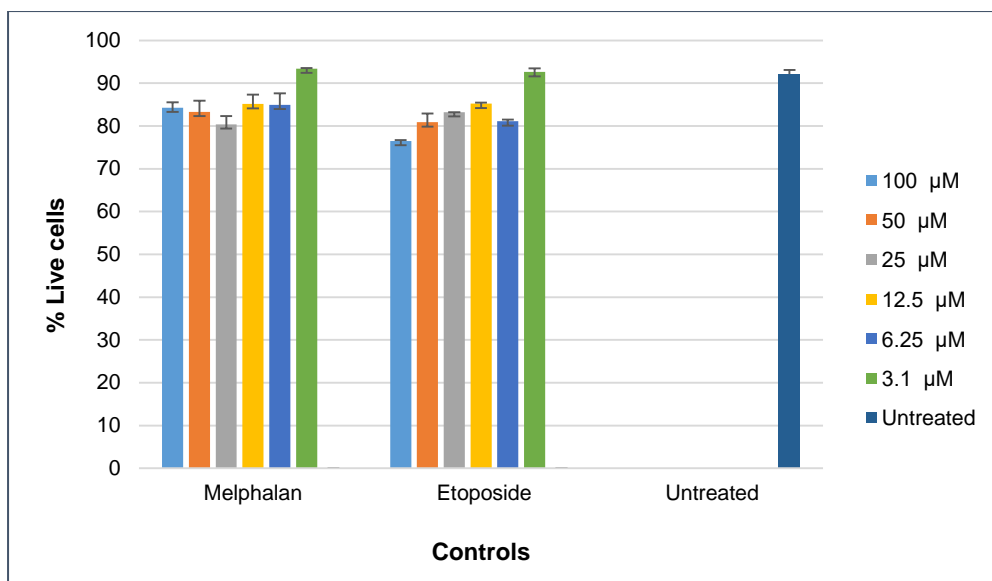
Different plates were used, thus resulting in different effects of controls. From **Fig. 39**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For this part, 0 μg/ml refers to the negative control for comparison to show any effect that may have occurred. From **Fig. 39**, no significant effect of the water extract of

*G. lucidum* fruiting bodies can be detected for the % live cells for any of the concentrations compared to the negative control (0 µg/ml). No decrease in % live cells were detected for the water extract of *G. lucidum* mycelia (**Fig. 39**) at any of the concentrations. For both the water and alcohol extracts of *H. erinaceus* fruiting bodies (**Fig. 39**), no decrease in % live cells were detected for any of the concentrations compared to the negative control (0 µg/ml).

From **Fig. 40**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to show any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 40**), only a slight decrease in % live cells were detected for all the concentrations with the highest concentration of 200 µg/ml causing the most significant decrease in % live cells. For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 40**), a significant decrease in % live cells were detected for concentrations of 100 and 50 µg/ml while concentrations at 25 and 12.5 µg/ml showed a slight decrease in % live cells, which might be due to contaminated wells. However, for the 200 µg/ml concentration, an increase in % live cells were detected and further investigation is required. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 40**), no decrease in % live cells were detected for concentrations of 25 and 12.5 µg/ml while only a slight decrease in % live cells for concentrations of 100 and 50 µg/ml with the most significant decrease in % live cells detected for 200 µg/ml concentration. The combined extract of *G. lucidum* SMS (**Fig. 40**) showed no significant effect apart from the highest concentration of 200 µg/ml resulting in a slight decrease of % live cell.



**Fig. 39.** Effect of controls and different extracts at different concentrations on % live cells (plate1).



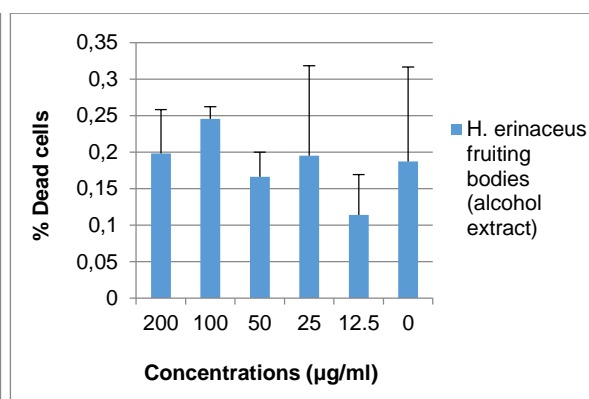
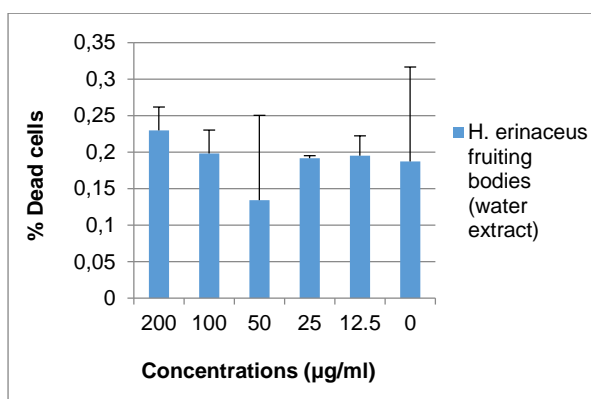
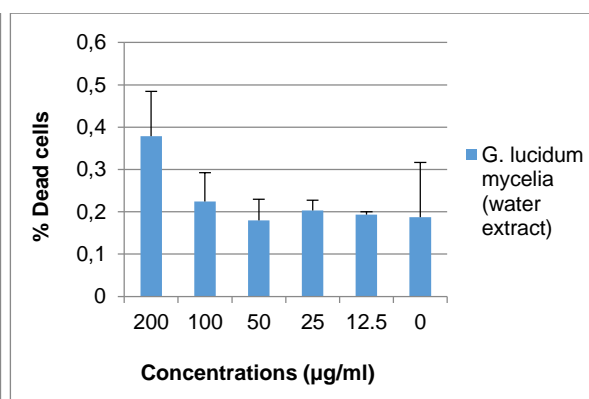
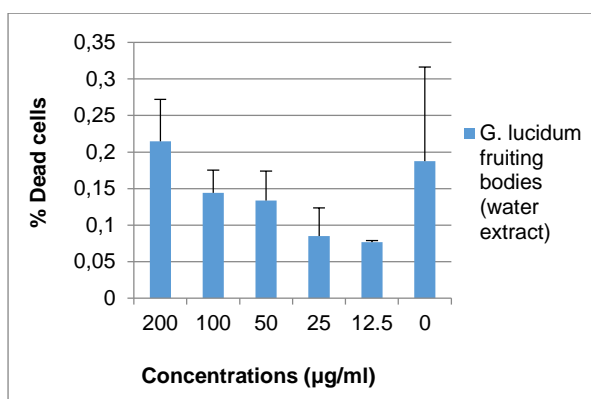
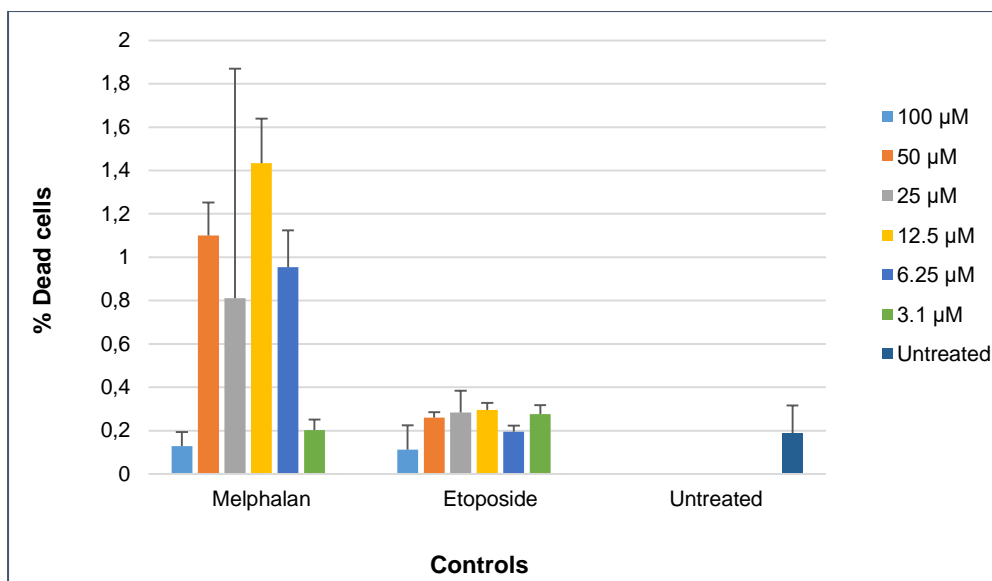
**Fig. 40.** Effect of controls and different extracts at different concentrations on % live cells (plate 2).

Different plates were used, thus resulting in different effects of controls. From **Fig. 41**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 μg/ml refers to the negative control to indicate any effect that may have occurred. From **Fig. 41**, a decrease in % dead cells were

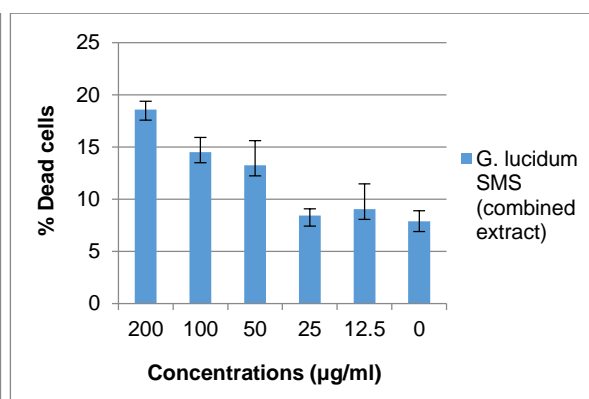
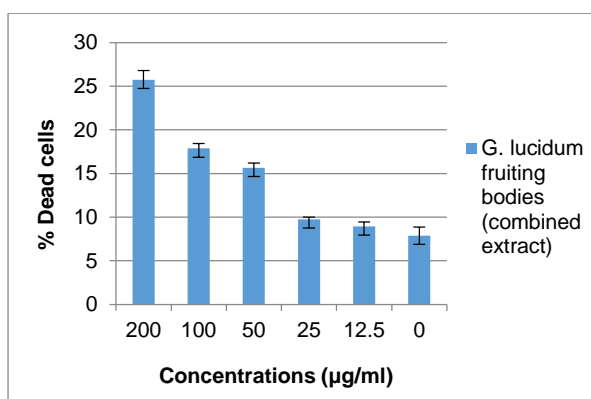
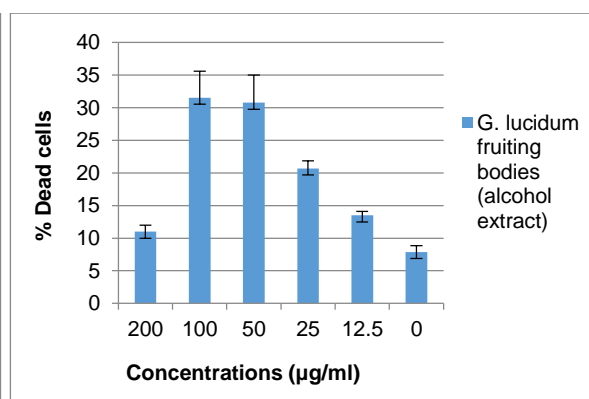
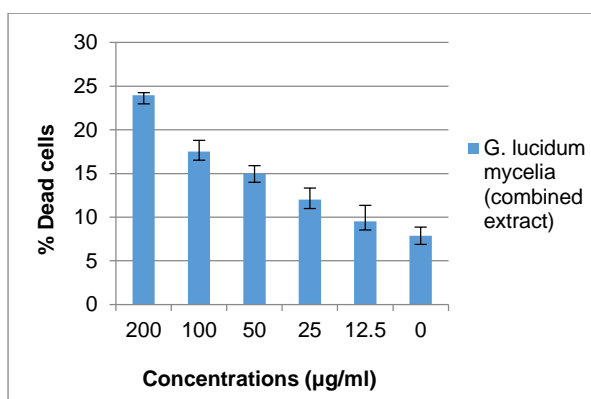
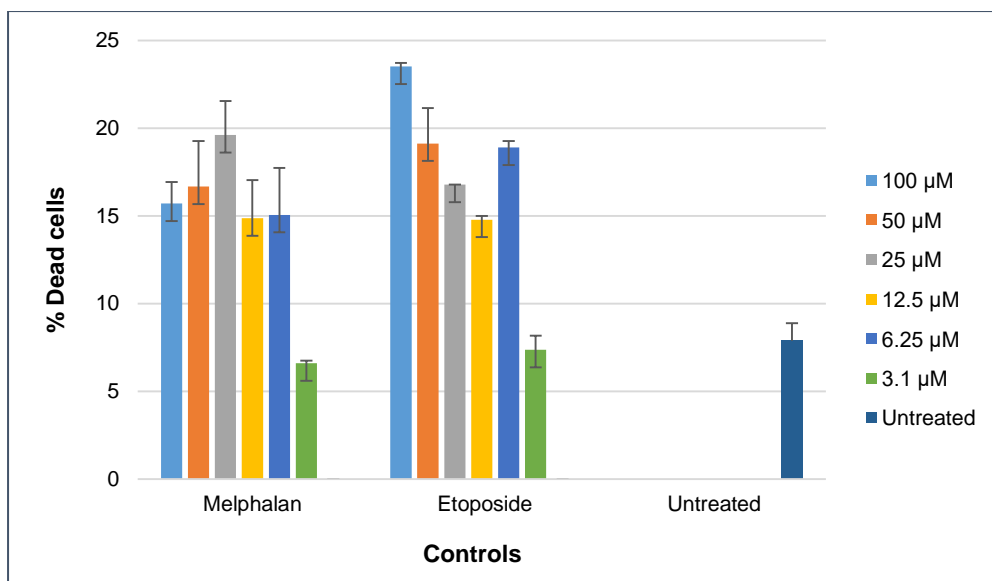


detected for concentrations of 100, 50 and 25 µg/ml for the water extract of *G. lucidum* fruiting bodies with the most significant decrease in % dead cells at 12.5 µg/ml concentration. However, the 200 µg/ml concentration showed a slight increase in the % dead cells when compared to the negative control (0 µg/ml). Although no significant effect on % dead cells were detected for the water extract of *G. lucidum* mycelia (**Fig. 41**) at concentrations between 100 and 12.5 µg/ml concentrations, the 200 µg/ml concentration showed an increased amount of % dead cell. For the water extracts of *H. erinaceus* fruiting bodies (**Fig. 41**), the 50 µg/ml concentration showed the smallest percentage of dead cells compared to the negative control (0 µg/ml) whereas the 200 µg/ml concentration showed the highest percentage of dead cells. In the case of the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 41**), the smallest percentage of dead cells were detected for the 50 and 12.5 µg/ml concentrations with the 100 µg/ml concentration showing the highest percentage of dead cells.

For **Fig. 42** the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 42**), the highest concentration of 200 µg/ml resulted in the most significant increase in % dead cells while the lower concentrations resulted in lower percentage of dead cells. Only a slight decrease in % live cells were detected for all of the concentrations with of the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 42**), the highest concentration of 200 µg/ml together with the lowest concentration of 12.5 µg/ml resulted in the lowest percentage of dead cells while a significant increase in % dead cells were detected for concentrations of 100, 50 and 25 µg/ml. This might be due to contaminated wells. However, for the 200 µg/ml concentration, an increase in % live cells were detected and further investigation is required. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 42**), concentrations of 25 and 12.5 µg/ml showed no significant increase in % dead cells while the most significant increase in % dead cells detected for the 200 µg/ml. The combined extract of *G. lucidum* SMS (**Fig. 42**) showed no significant effect on % dead cells for concentrations of 25 and 12.5 µg/ml while the highest concentration of 200 µg/ml resulting in the highest percentage of dead cells when compared to the negative control (0 µg/ml).



**Fig. 41.** Effect of controls and different extracts at different concentrations on % dead cells (plate 1).



**Fig. 42.** Effect of controls and different extracts at different concentrations on % dead cells (plate 2).

### 3.3.10.2 Lysosomal content

For evaluation of the lysosomal content, the integrated intensity was used to determine the number of lysosomes as well as their brightness (the lower the pH, the brighter LysoTracker Red will fluoresce). Furthermore, integrated intensity takes cell sizes in account and will

increase with size or when high amounts of lysosomes are present as it results in a bigger area being stained.

Different plates were used, thus resulting in different effects of controls. From **Fig. 43**, the effect of the negative control and two positive controls (Melphalan and Chloroquine) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. From **Fig. 43**, no increase in the mean integrated intensity in the cytoplasm (relative fluorescent units) was detected for the water extract of *G. lucidum* fruiting bodies for any of the concentrations when compared to the negative control (0 µg/ml). For the water extract of *G. lucidum* mycelia (**Fig. 43**), a significant increase was detected in the mean integrated intensity in the cytoplasm for the highest concentration of 200 µg/ml while concentrations of 25 and 12.5 µg/ml showed no significant increase. For the water extracts of *H. erinaceus* fruiting bodies (**Fig. 43**), no increase in the mean integrated intensity in the cytoplasm was detected for any of the concentrations when compared to the negative control (0 µg/ml). In the case of the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 43**), an increase in the mean integrated intensity in the cytoplasm was detected for all of the concentrations with the 200 µg/ml concentration showing the highest increase, resulting in an increase in lysosomal content when compared to the negative control (0 µg/ml).

For **Fig. 44** the effect of the negative control and two positive controls (Melphalan and Chloroquine) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 44**), the highest concentration of 200 µg/ml resulted in the most significant increase in mean integrated intensity in the cytoplasm followed by the 100 µg/ml concentration. The lower concentrations of 50, 25 and 12.5 µg/ml resulted in a slightly increased mean integrated intensity in the cytoplasm, but no toxicity as the increase is not significant enough to cause toxicity. For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 44**), the highest concentrations of 200 and 100 µg/ml showed no increase in the mean integrated intensity in the cytoplasm while the lowest concentration of 12.5 µg/ml resulted in a slight increase. However, the concentrations of 50 and 25 µg/ml showed a significant increase, which might be due to contaminated wells and further investigation is required. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 44**), concentrations of 200 and 12.5 µg/ml showed no significant increase in mean integrated intensity in the cytoplasm while the most significant increase was detected for the 100, 50 and 25 µg/ml concentrations. The

combined extract of *G. lucidum* SMS (Fig. 44) showed a significant effect on the mean integrated intensity in the cytoplasm for all of the concentrations and further investigation is required to evaluate lysosomal content.

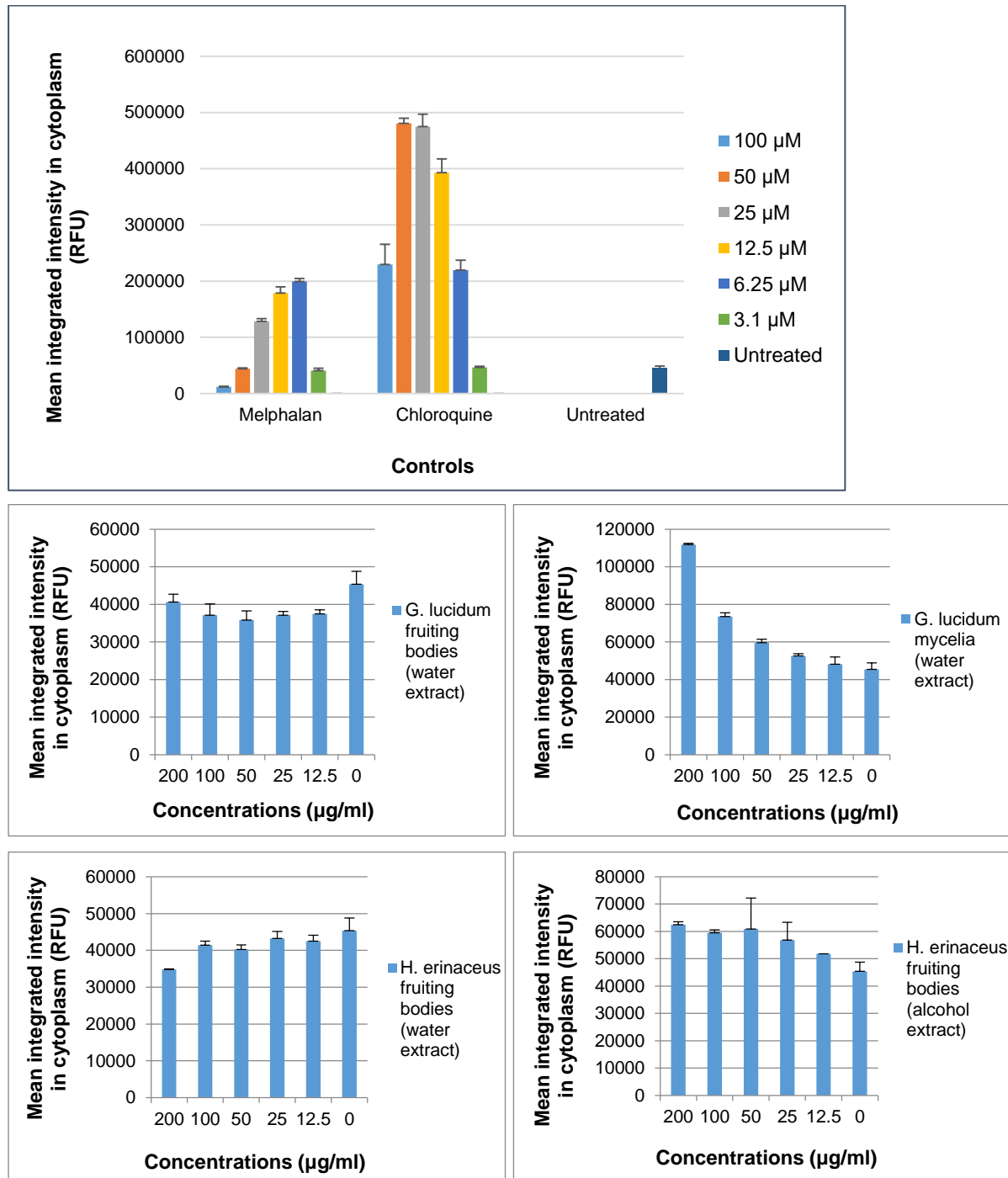
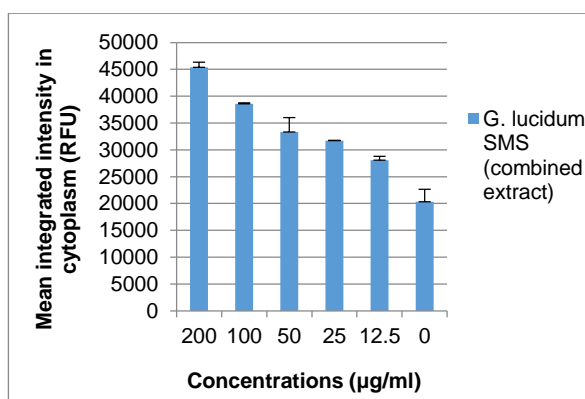
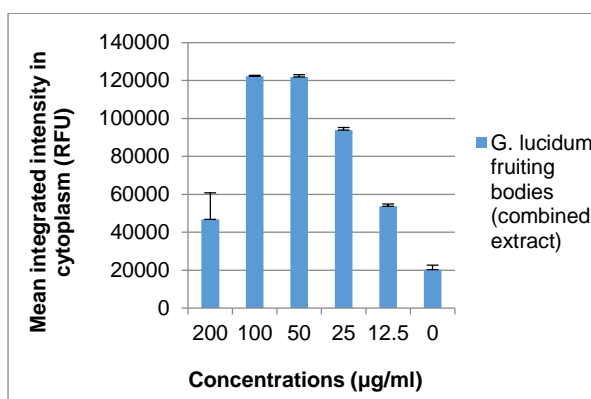
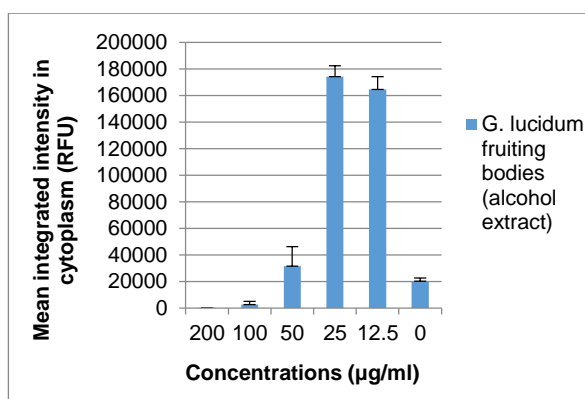
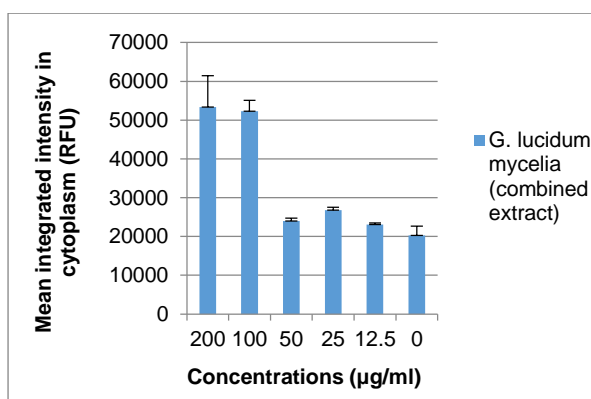
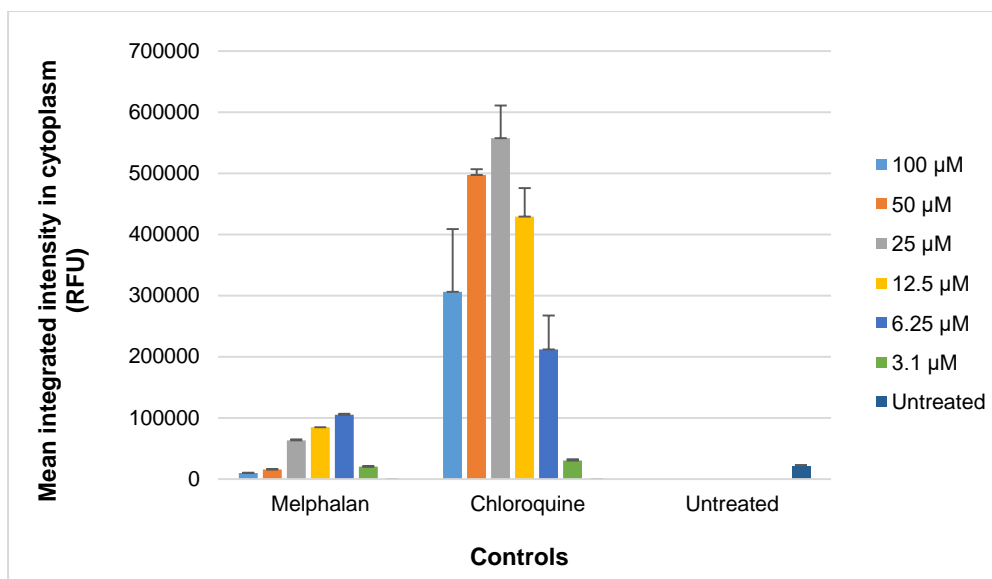


Fig. 43. Effect of controls and different extracts at different concentrations on lysosomal content (plate 1).



**Fig. 44.** Effect of controls and different extracts at different concentrations on lysosomal content (plate 2).

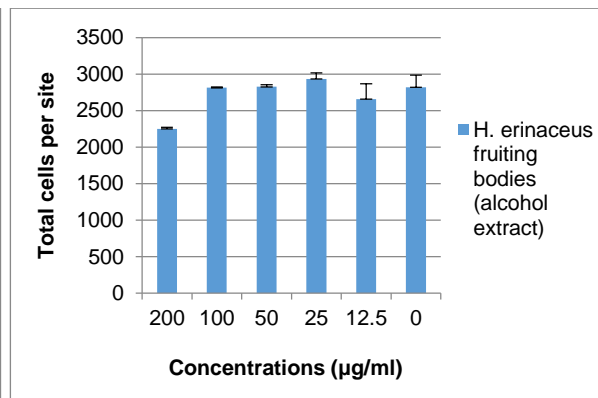
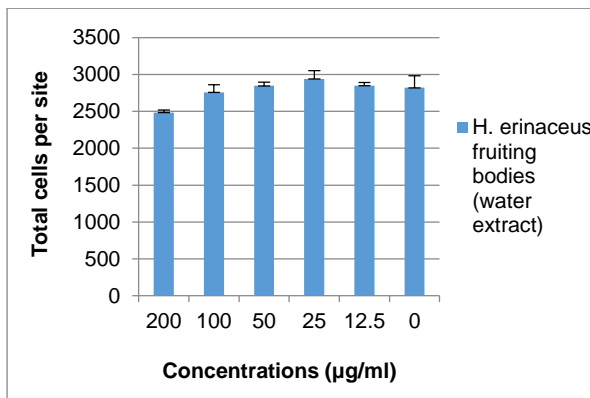
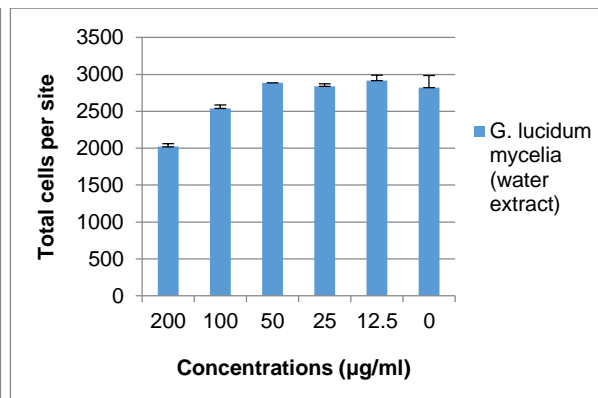
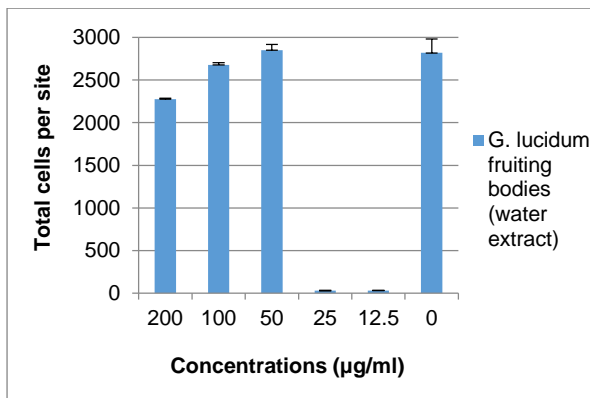
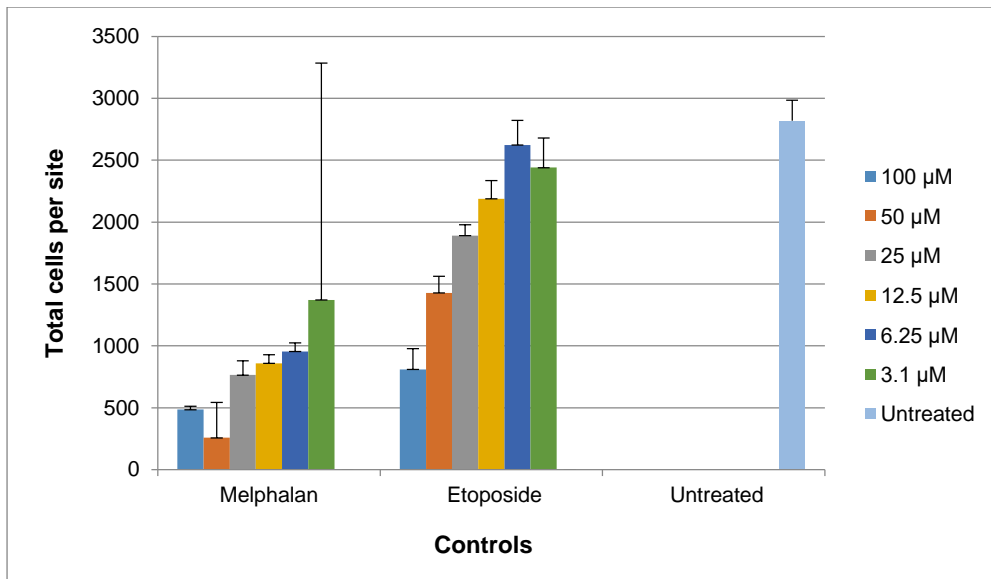
### 3.3.10.3 Mitochondrial dysfunction

For evaluation of the mitochondrial potential, TMRE was used and the average intensity was used as the average of all pixels in that object is taken in account. Average intensity was therefore used as the brightness of the mitochondria is measured. For the mitochondrial membrane (mass), mean stain area was used as the number of mitochondria are determined.

Mitochondrial toxicity is characterised by a decrease in mitochondrial potential (decrease in TMRE fluorescence) and a corresponding increase in mitochondrial membranes (increase in MTG fluorescence) as the cell try to compensate for the loss of membrane potential by increasing the amount of membranes.

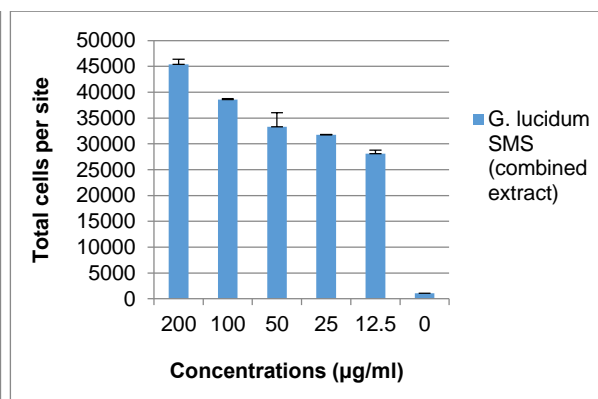
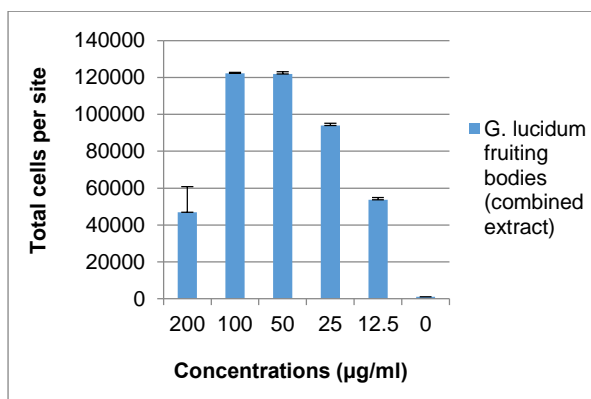
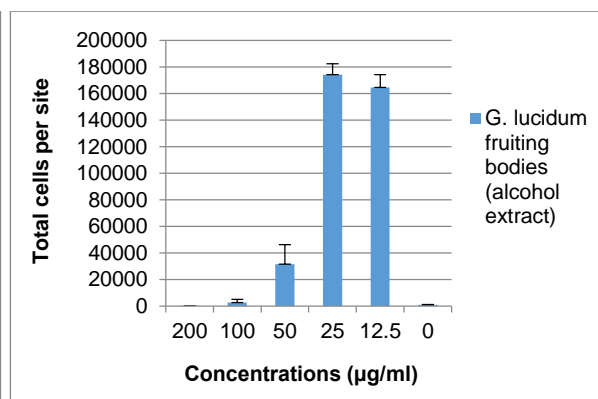
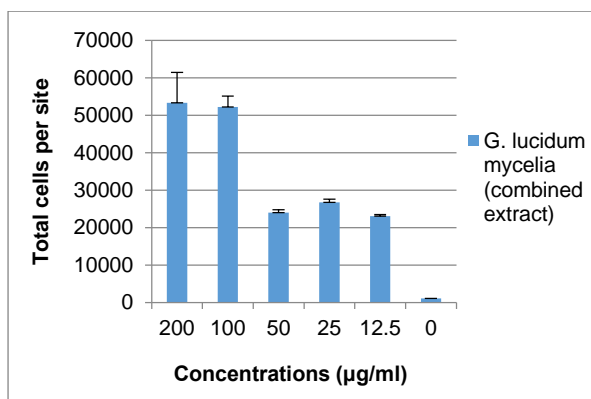
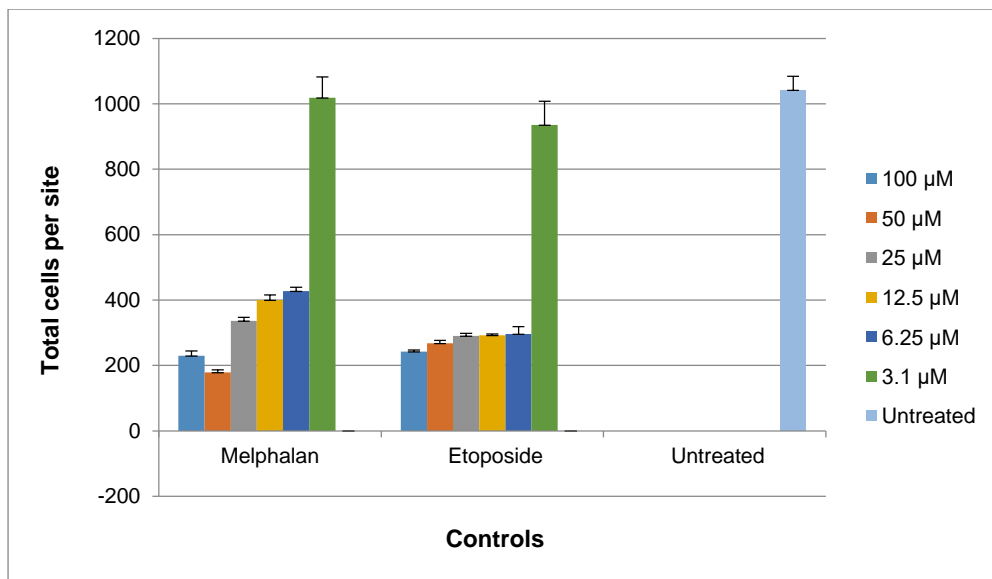
Different plates were used, thus resulting in different effects of controls. From **Fig. 45**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. From **Fig. 45**, no significant decrease in the total cells per site were detected for the 100 and 50 µg/ml concentrations followed by the 200 µg/ml concentration. However, no total cells per site were detected for the water extract of *G. lucidum* fruiting bodies at concentrations of 25 and 12.5 µg/ml. This was unexpected and might be due to contaminated wells. For the water extract of *G. lucidum* mycelia (**Fig. 45**), concentrations of 50, 25 and 12.5 µg/ml showed no significant effect on the total cells per site while a significant decrease was detected in the total cells per site for the highest concentration of 200 µg/ml followed by the 100 µg/ml concentration. For the both the water and alcohol extracts of *H. erinaceus* fruiting bodies (**Fig. 45**), no significant effect on the total cells per site were detected for concentrations of 100, 50, 25 and 12.5 µg/ml when compared to the negative control (0 µg/ml) while the 200 µg/ml concentration only showed a slight decrease.

For **Fig. 46** the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 46**), a significant increase in total cells were detected for all of the concentrations when compared to the negative control (0 µg/ml). For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 46**), the highest concentrations of 200 and 100 µg/ml showed no significant effect on the total cells per site while the lower concentrations of 25 and 12.5 µg/ml resulted in a significant increase. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 46**), total cells per site increased for all of the concentrations with the 100 and 50 µg/ml concentrations having the most significant effect. The combined extract of *G. lucidum* SMS (**Fig. 46**) also showed a significant effect on the total cells per site for all of the concentrations, with the highest concentration of 200 µg/ml resulting in the highest number of total cells per site.



**Fig. 45.** Effect of controls and different extracts at different concentrations on total cells per site (plate 1).



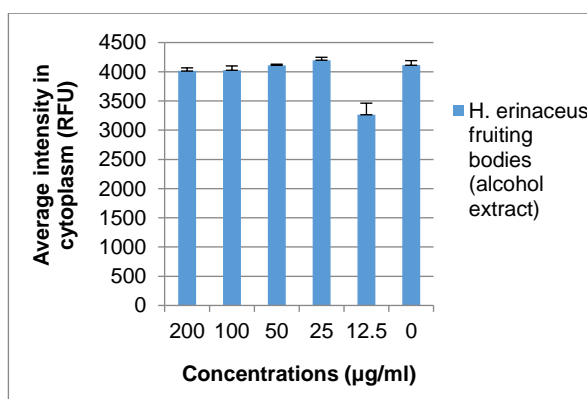
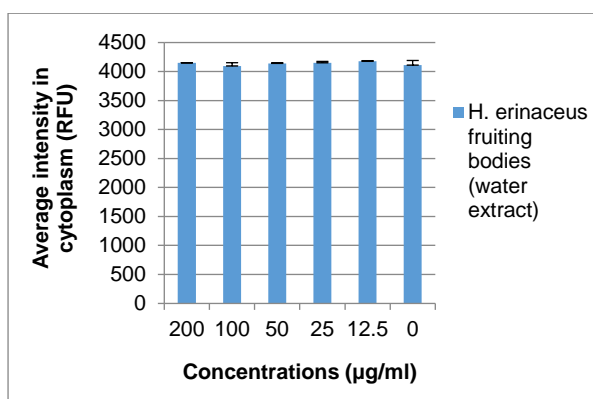
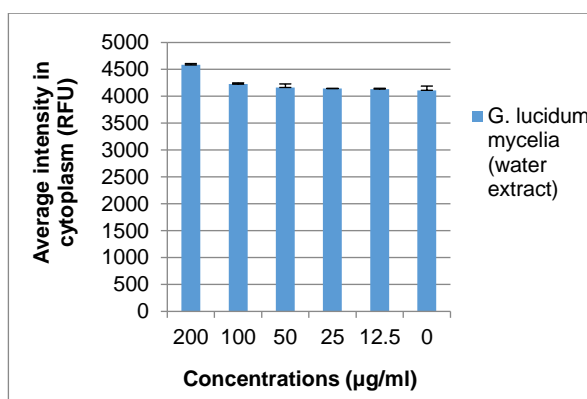
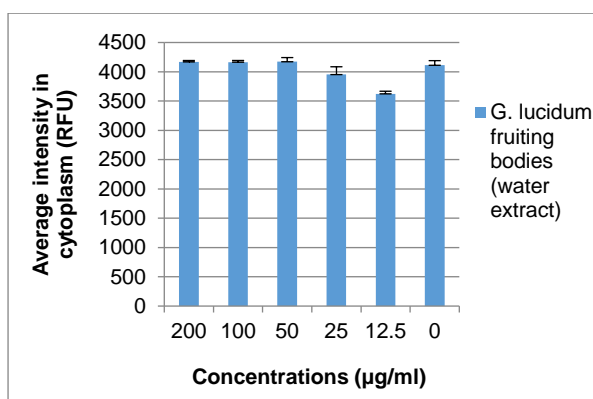
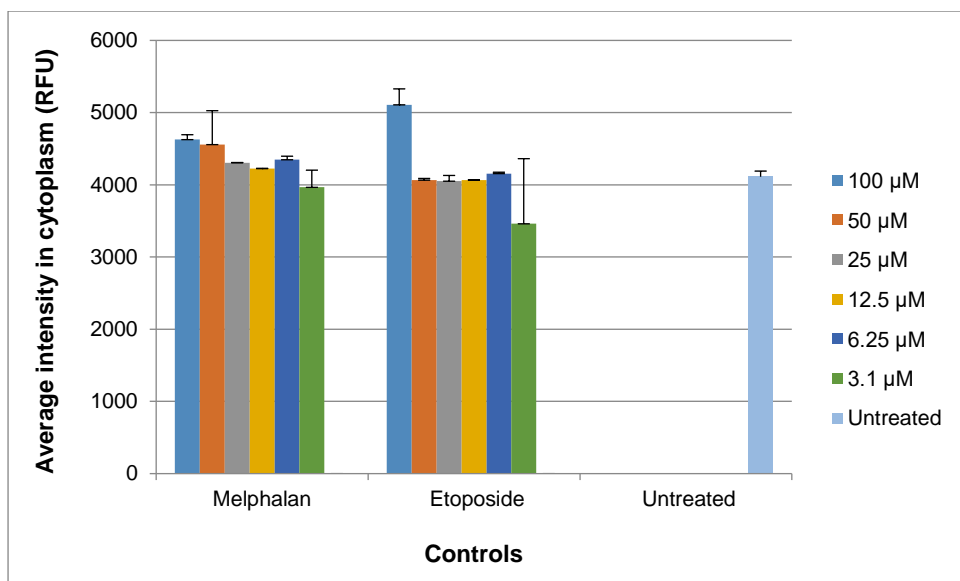


**Fig. 46.** Effect of controls and different extracts at different concentrations on total cells per site (plate 2).

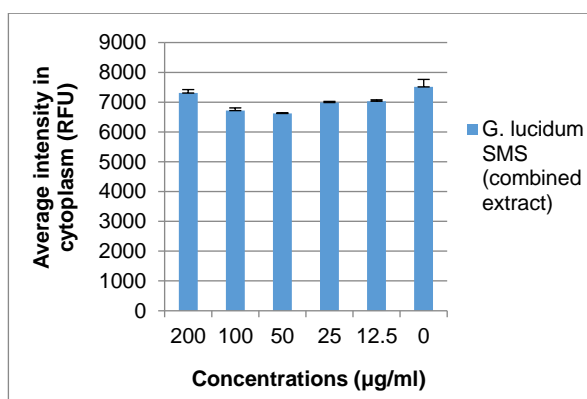
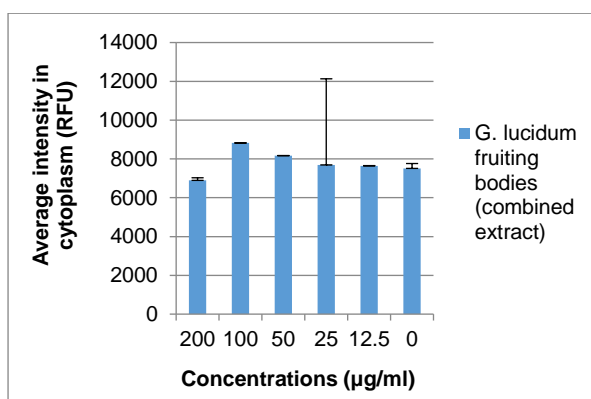
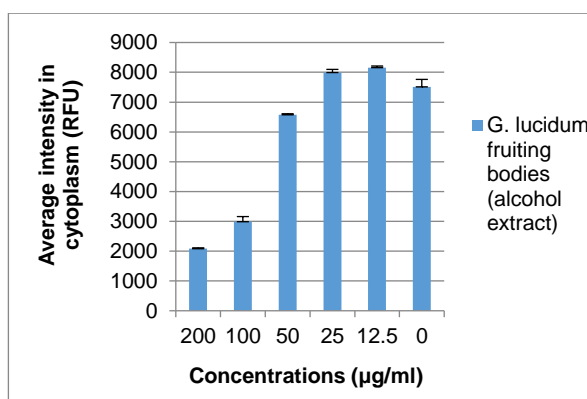
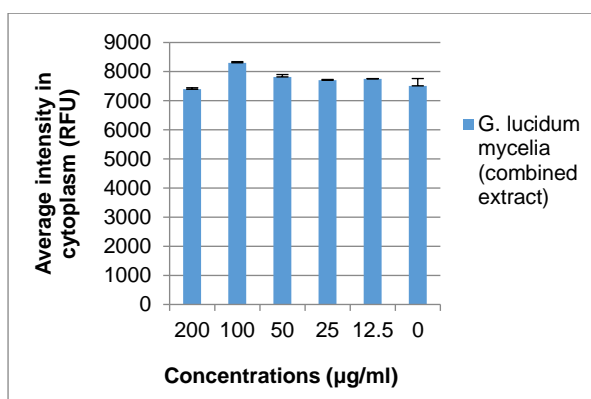
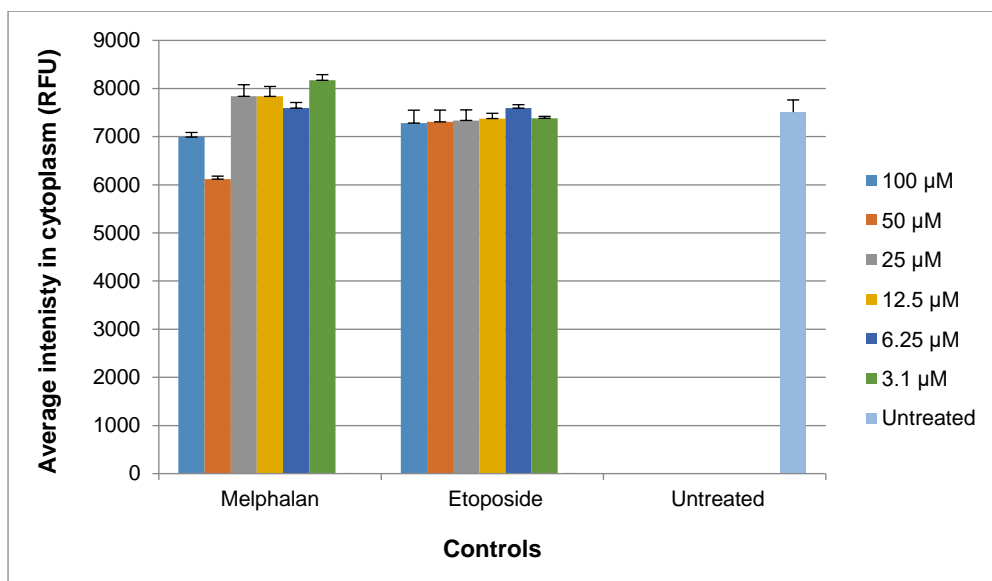
Different plates were used, thus resulting in different effects of controls. From **Fig. 47**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0  $\mu$ g/ml refers to the negative control to indicate any effect that may have occurred. From **Fig. 47**, no significant effect on the average

intensity in the cytoplasm were detected for the water extract of *G. lucidum* fruiting bodies at concentrations of 200, 100, 50 and 25 µg/ml. However, a decrease in the average intensity in the cytoplasm were detected were for the 12.5 µg/ml concentration, resulting in a decrease in mitochondrial potential. For the water extract of *G. lucidum* mycelia (**Fig. 47**), none of the concentrations showed significant effect on the average intensity in the cytoplasm with the 200 µg/ml concentration resulting in a small increase. Thus, increased mitochondrial potential. For the water extract of *H. erinaceus* fruiting bodies (**Fig. 47**), no significant effect on the average intensity in the cytoplasm were detected for any of the concentrations. For the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 47**), only the 12.5 µg/ml concentration resulted in a decrease in the average intensity, and a decrease in mitochondrial potential, when compared to the negative control (0 µg/ml) while the other concentration showed no significant effect.

For **Fig. 48** the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 48**), no significant effect on the average intensity in the cytoplasm were detected for any of the concentrations, except for the 100 µg/ml concentration resulting in an increase when compared to the negative control (0 µg/ml). For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 48**), the lower concentrations of 25 and 12.5 µg/ml showed no significant effect on the average intensity in the cytoplasm while the highest concentrations of 200 and 100 µg/ml resulted in a significant decrease in mitochondrial potential due to the decreased intensity. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 48**), no significant effect on the average intensity in the cytoplasm were detected for any of the concentrations, except for the 100 µg/ml concentration resulting in an increase when compared to the negative control (0 µg/ml). The combined extract of *G. lucidum* SMS (**Fig. 48**) also showed a significant effect on the average intensity in the cytoplasm as all of the concentrations resulted in a decrease in the intensity, except for the 200 µg/ml concentration having no effect.



**Fig. 47.** Effect of controls and different extracts at different concentrations on mitochondrial potential by measuring the average intensity in cytoplasm (plate 1).

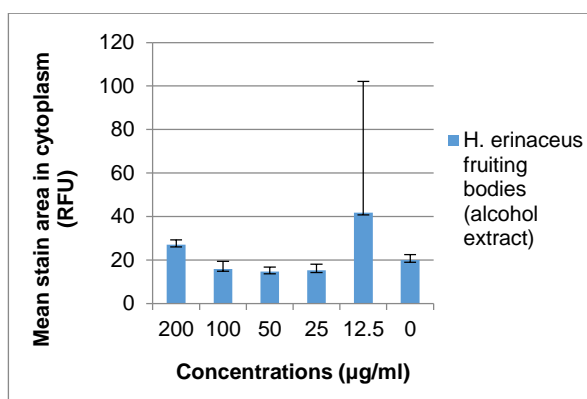
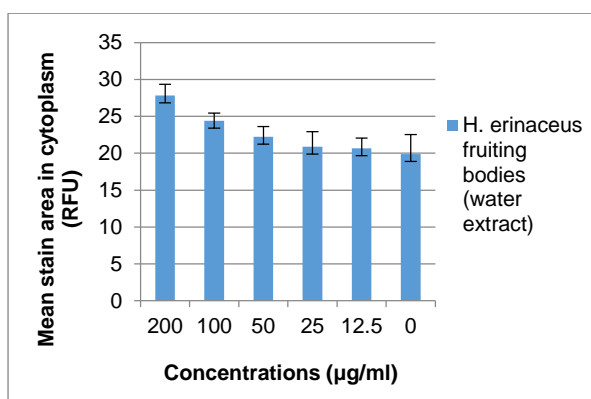
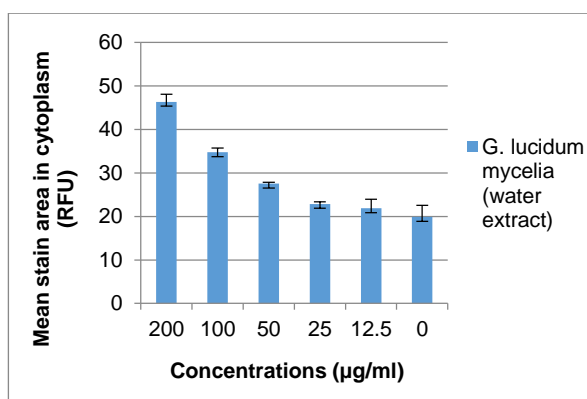
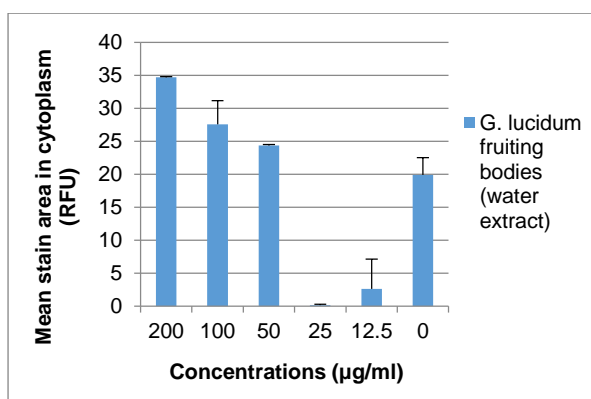
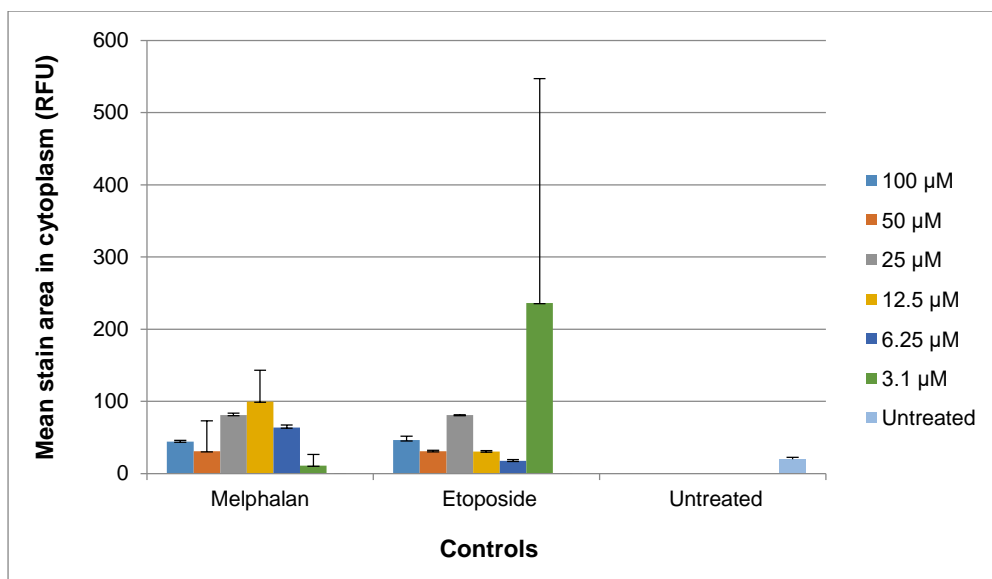


**Fig. 48.** Effect of controls and different extracts at different concentrations on mitochondrial potential by measuring the average intensity in cytoplasm (plate 2).

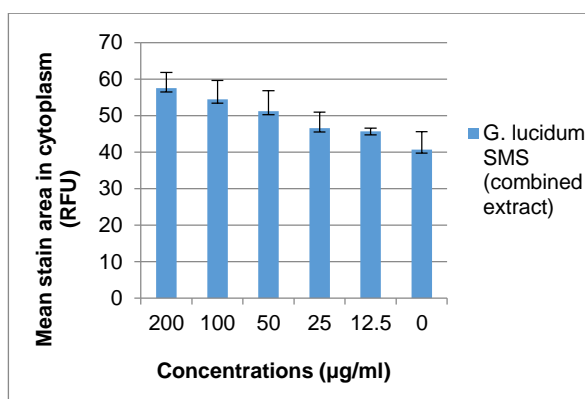
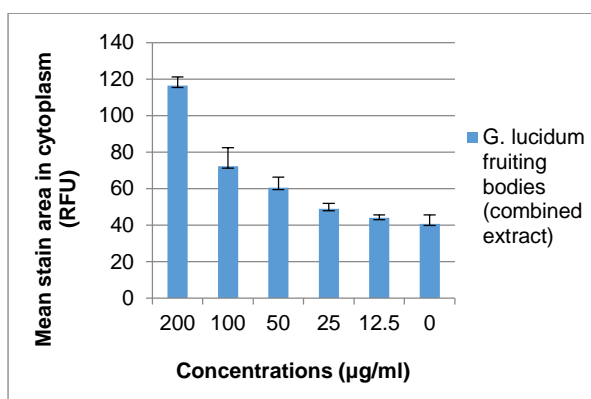
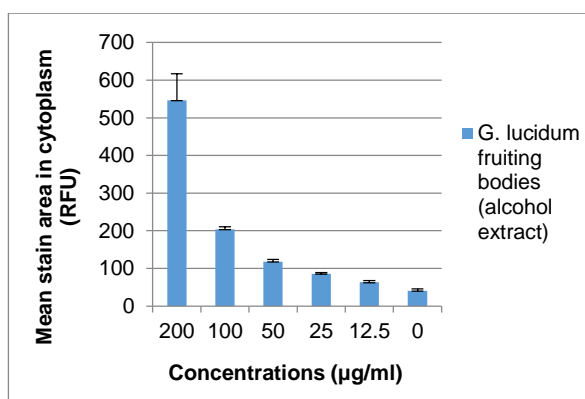
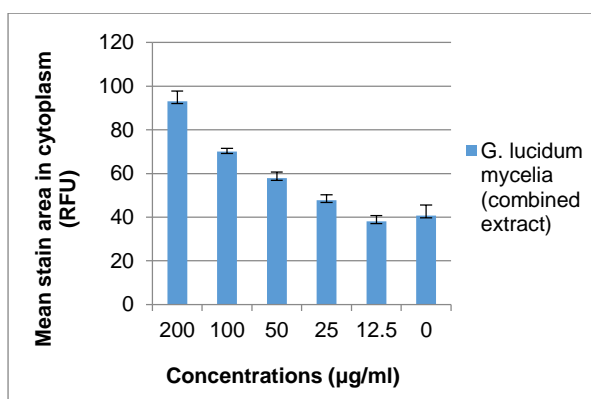
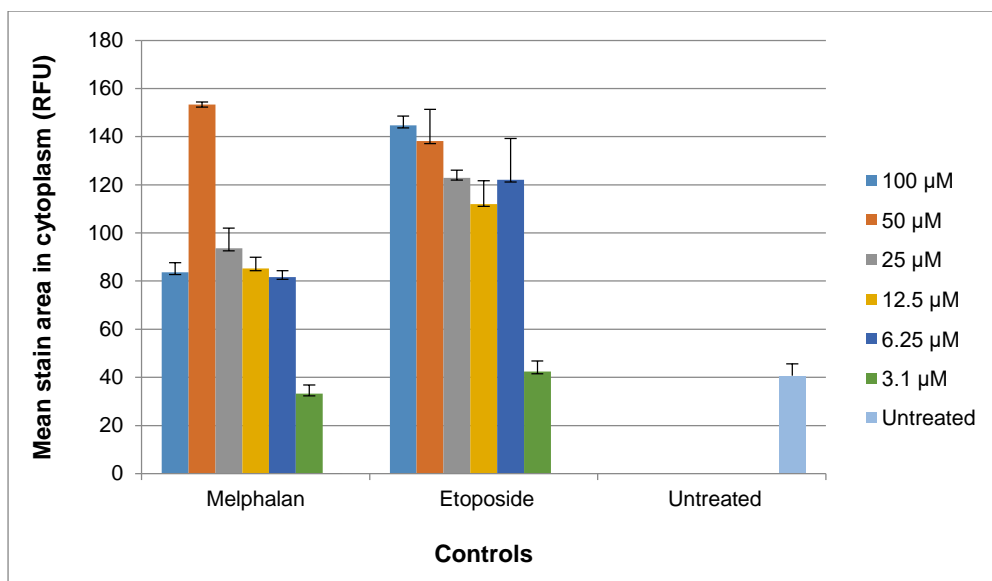
Different plates were used, thus resulting in different effects of controls. From **Fig. 49**, the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0  $\mu$ g/ml refers to the negative control to indicate any effect that may have occurred. From **Fig. 49**, an increase in the mean stain area

in the cytoplasm was detected for the 200, 100 and 50 µg/ml concentrations for the water extract of *G. lucidum* fruiting bodies with concentrations of 25 and 12.5 µg/ml resulting in a decrease. However, no toxicity as an increase in mitochondrial membranes may be due to cell division as there is no corresponding decrease of mitochondrial potential and increase in mitochondrial membrane. For the water extract of *G. lucidum* mycelia (**Fig. 49**), concentrations of 200, 100 and 50 µg/ml showed a significant increase in the mean stain area in cytoplasm while the lower concentrations (25 and 12.5 µg/ml) had no significant effect when compared to negative control (0 µg/ml). For the water extract of *H. erinaceus* fruiting bodies (**Fig. 49**), no significant effect on the mean stain area in cytoplasm were detected for the lower concentrations (25 and 12.5 µg/ml) while the 200 µg/ml concentration showed an increase in the mean stain area in cytoplasm followed by the 100 and 50 µg/ml concentrations, respectively. For the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 49**), the 12.5 µg/ml concentration resulted in a significant increase in the mean stain area in the cytoplasm while the 100, 50 and 25 µg/ml concentrations rather showed a decrease than an increase when compared to the negative control (0 µg/ml).

For **Fig. 50** the effect of the negative control and two positive controls (Melphalan and Etoposide) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 50**), all of the concentrations resulted in an increase in the mean stain area in the cytoplasm, except for the 12.5 µg/ml concentration, which resulted in a decrease. For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 50**), the 200 µg/ml concentration showed an increase in the mitochondrial membranes (mean stain area in cytoplasm), resulting in mitochondrial toxicity as a corresponding decrease in mitochondrial potential (average intensity in cytoplasm) was detected in **Fig. 48**. However, no toxicity was detected for any of the other concentrations. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 50**), no significant effect on the mean stain area in the cytoplasm were detected for any of the lower concentrations, except for the 200 and 100 µg/ml concentrations resulting in an increase when compared to the negative control (0 µg/ml), but no toxicity. The combined extract of *G. lucidum* SMS (**Fig. 50**) also showed an increase in the mean stain area for all of the concentrations, but no toxicity was detected.



**Fig. 49.** Effect of controls and different extracts at different concentrations on mitochondrial membrane by measuring the mean stain area in the cytoplasm (plate 1).



**Fig. 50.** Effect of controls and different extracts at different concentrations on mitochondrial membrane by measuring the mean stain area in the cytoplasm (plate 2).

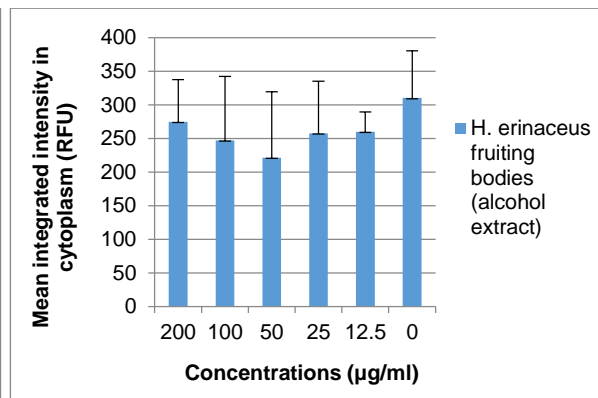
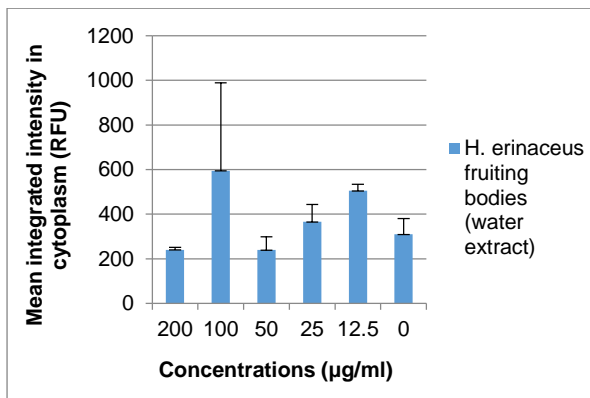
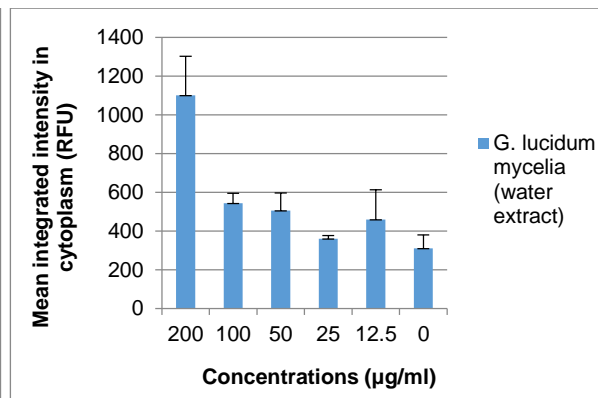
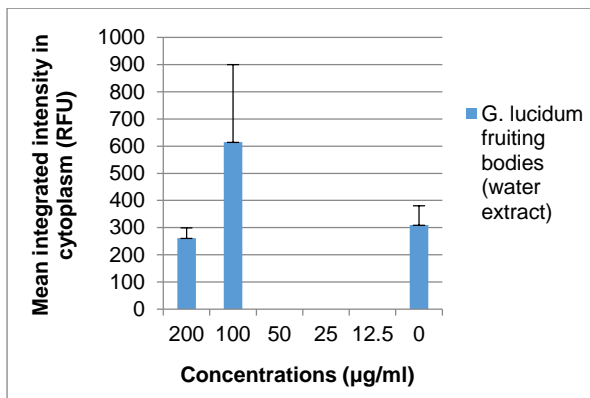
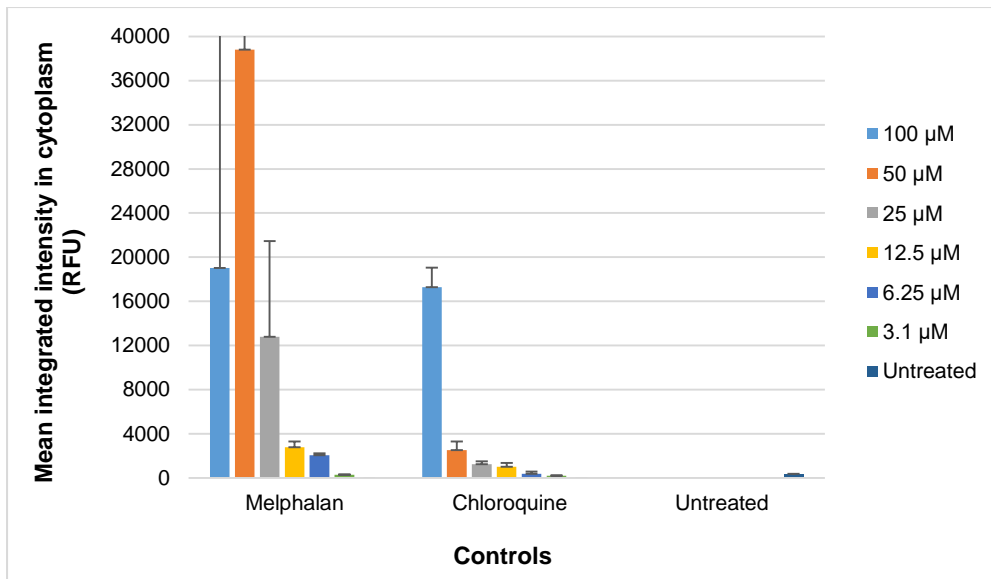
#### 3.3.10.4 Steatosis

Different plates were used, thus resulting in different effects of controls. From **Fig. 51**, the effect of the negative control and two positive controls (Melphalan and Chloroquine) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 μg/ml refers to the negative control to

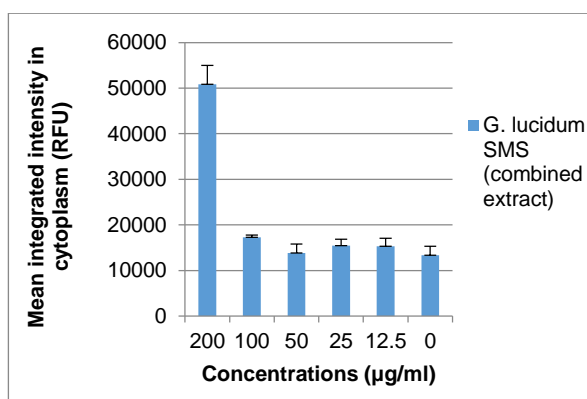
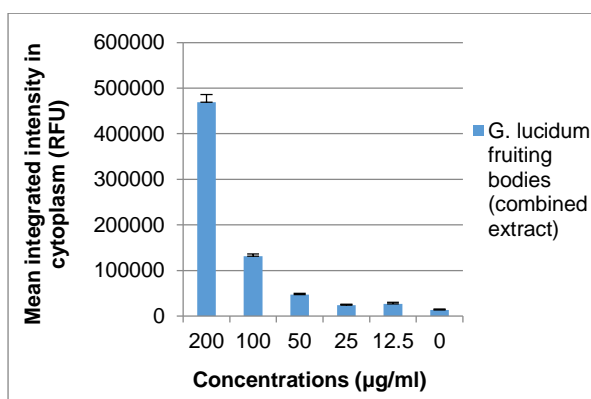
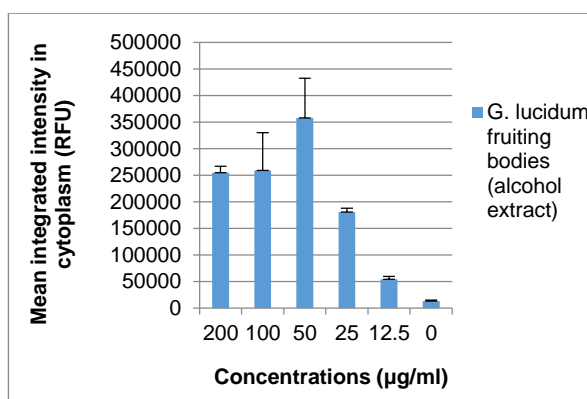
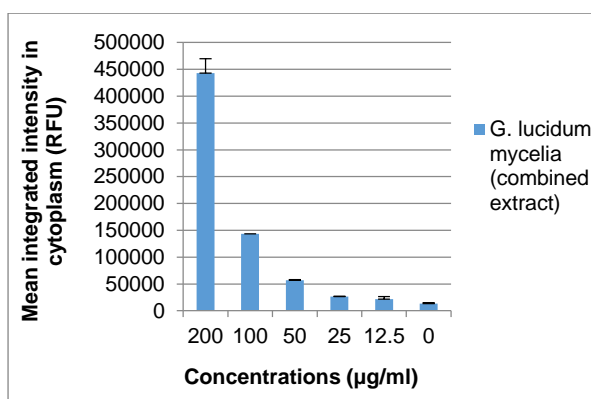
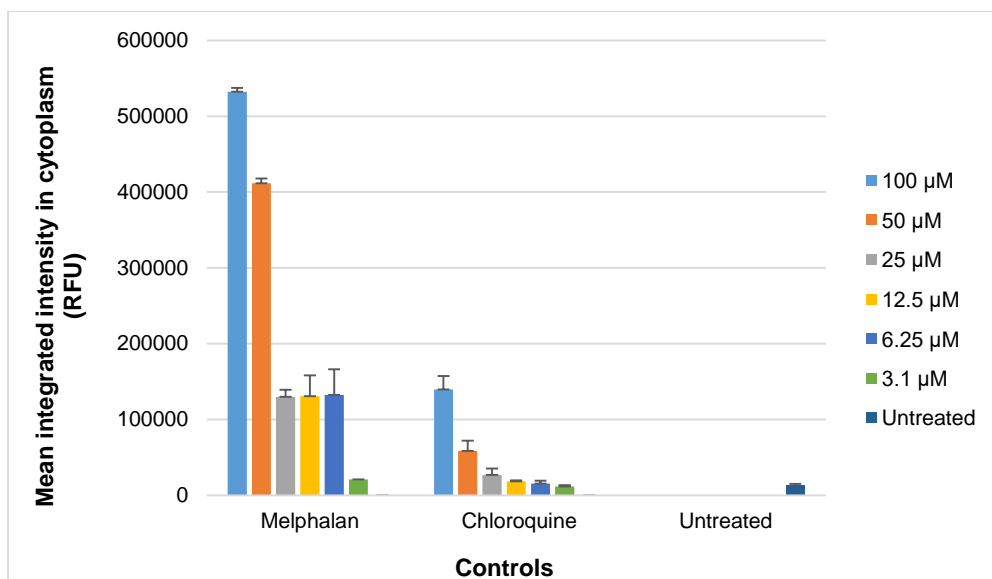
indicate any effect that may have occurred. From **Fig. 51**, only the 200 and 100 µg/ml concentrations can be seen for the water extract of *G. lucidum* fruiting bodies as the data of the other wells had to be disregarded due to contamination. For the 200 µg/ml concentration, no increase in the mean integrated intensity was detected whereas an increase was detected for the 100 µg/ml concentration, which may be accumulated lipids. For the water extract of *G. lucidum* mycelia (**Fig. 51**), the 200 µg/ml concentration showed the most significant increase in the mean integrated intensity compared to the increase caused by 100, 50 and 12.5 µg/ml concentrations. In contrast, no increase in the mean integrated intensity was detected for the 25 µg/ml concentration. For the water extract of *H. erinaceus* fruiting bodies (**Fig. 51**), no significant effect on the mean integrated intensity in the cytoplasm were detected for the 200 and 50 µg/ml concentrations while the 100 µg/ml concentration showed a significant increase in lipid accumulation followed by the 12.5 and 25 µg/ml concentrations, respectively. For the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 51**), none of the concentrations resulted in an increase in the mean integrated intensity in the cytoplasm when compared to the negative control (0 µg/ml). Thus, no hepatotoxicity for the alcohol extract of *H. erinaceus* fruiting bodies (**Fig. 51**).

For **Fig. 52** the effect of the negative control and two positive controls (Melphalan and Chloroquine) at different concentrations can be seen for comparison with the different extracts at different concentrations. For comparison of the extracts, 0 µg/ml refers to the negative control to indicate any effect that may have occurred. For the combined extract of *G. lucidum* mycelia (**Fig. 52**), all of the concentrations showed an increase in the mean integrated intensity with the 200 µg/ml concentration showing the most significant increase, resulting in possible toxicity due to the increase, but further investigation is required and the experiment will be repeated. For the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 52**), all of the concentrations showed an increase in the mean integrated intensity with the 50 µg/ml concentration showing the most significant increase, resulting in possible toxicity due to the increase, but further investigation is required and the experiment will be repeated. For the combined extract of *G. lucidum* fruiting bodies (**Fig. 52**), no toxicity was detected for the lower concentrations (50, 25, 12.5 µg/ml), but toxicity was detected for the 200 and 100 µg/ml concentrations due to the increase in the mean integrated intensity. However, further investigation is required. The combined extract of *G. lucidum* SMS (**Fig. 52**) showed no increase in the mean integrated intensity for all of the concentrations, except for the 200 µg/ml concentration when compared to the negative control (0 µg/ml).





**Fig. 51.** Effect of controls and different extracts at different concentrations on mitochondrial membrane by measuring the mean integrated intensity in the cytoplasm (plate 1).



**Fig. 52.** Effect of controls and different extracts at different concentrations on mitochondrial membrane by measuring the mean integrated intensity in the cytoplasm (plate 2).

### 3.4 Conclusions

Medicinal mushrooms are considered to be an important source of bioactive polysaccharides which are regarded as biological response modifiers due to their ability to enhance the immune system (Villares et al. 2012). As a result, medicinal mushrooms have application to prevent

and treat various diseases. Cancer, cardiovascular, and bacterial infections are among the most studied diseases treated with polysaccharides extracted from medicinal mushrooms. Results obtained from conducted studies resulted in the possible application of bioactive polysaccharides for treatment of these diseases (Villares et al. 2012). For the identification of compounds, separation and isolation of each extract using TLC should be undertaken in future but it will be a tedious task. Additionally, fingerprinting will be performed to identify and isolate compounds for further investigation.

The antimicrobial properties of *G. lucidum* and *H. erinaceus* are of microbiological importance, resulting in the application of various extraction methods in order to evaluate antimicrobial activity of the various growth phases. Although hot water extracts showed no inhibitory effect against the various pathogens used to evaluate antimicrobial activity, various factors have to be considered. The extraction times might have been too short while some of the compounds such as triterpenoids are water-insoluble and can only be extracted with alcohol. Both alcohol and water extracts showed good antimicrobial activity, with *G. lucidum* fruiting bodies showing the best inhibitory effect. Combined extracts were performed on the three growth phases of *G. lucidum*, namely mycelia, fruiting bodies and SMS. Consequently, the combined extracts of *G. lucidum* fruiting bodies and SMS showed similar positive results against a variety of pathogens. This is due to extraction of both water-soluble and water-insoluble compounds, such as polysaccharides and triterpenoids. As a result, SMS have application as animal feed due to antimicrobial activity, thus utilizing waste.

Herbal medicines also require pre-clinical and clinical trials for safe use. Although animal models are important for pre-clinical testing, it poorly portrays human toxicity. As a result, *in vitro* screening using human derived cells are considered to be a more practical approach to identify possible toxicity as it is the main reason for drug attrition and poses major challenges for the pharmaceutical industry. As a result, early identification is a key research objective. HCA emerged as a predictive tool for identifying toxicity and represents the most promising approach to early drug development. Various parameters can be investigated by staining cells followed by analysis with the ImageXpress Micro XLS Widefield High-Content Analysis System. This microscope is specifically used for high content analysis as bioactive compounds are identified and mode of actions can be investigated. The morphological features are observed and recorded using the automated imaging capabilities and software quantifies changes in cell populations.

Initial hepatotoxicity assays of extracts of the different growth phases for *G. lucidum* and *H. erinaceus* showed some toxicity. In general, alcohol extracts were also found to exhibit more toxicity compared to water extracts and further investigations will be done to determine the reason for toxicity. An increase in the lysosomal content was detected for the water extract of *G. lucidum* mycelia, the alcohol extract of *H. erinaceus* fruiting bodies and the combined extract of *G. lucidum* mycelia at a concentration of 200 µg/ml. In addition, all of the concentrations for the combined extract of *G. lucidum* SMS resulted in a significant increase in the lysosomal content and further investigation is required.

Mitochondrial toxicity was detected for the alcohol extract of *G. lucidum* fruiting bodies (**Fig. 52**) at a 200 µg/ml concentration as an increase in the mitochondrial membranes (mean stain area in cytoplasm) and a corresponding decrease in mitochondrial potential (average intensity in cytoplasm) was detected. Further investigation will be done by repeating the procedure to evaluate results. No mitochondrial toxicity was detected for any of the other extracts as no corresponding decrease in mitochondrial potential and increase in mitochondrial membrane were detected for any of the extracts. In the case of steatosis (lipid accumulation), toxicity was detected at high concentrations (100 and 200, respectively) for the water extracts of *G. lucidum* fruiting bodies and mycelia. Additionally, the water extract of *H. erinaceus* fruiting bodies showed a significant increase in lipid accumulation at 100 µg/ml concentration followed by the 12.5 and 25 µg/ml concentrations, respectively. Furthermore, the 50 µg/ml concentration of the alcohol extract of *G. lucidum* fruiting bodies showed an increase while the 200 µg/ml concentration of both the combined extract of *G. lucidum* mycelia and fruiting bodies showed the most significant increase, resulting in possible toxicity due to the increase.

Further investigation is required as this was just a preliminary study in order to evaluate the antimicrobial effects and whether different extracts at different concentrations are highly toxic or not to human derived cells by making use of *in vitro* screening. By taking all of the results in account, none of the extracts proved to be highly toxic, paving the way for further investigations and improving health in a natural manner.

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## Chapter 4

### Production and evaluation of animal feed using spent *G. lucidum* growth blocks

#### V. Abstract

In recent years, mushrooms have demonstrated a great impact on agriculture and the environment, resulting in their potential for generating a great socio-economic impact in human welfare on local, national, and global levels. Aside from the advantages, the unutilised product of mushroom cultivation, namely spent mushroom substrate (SMS), is increasing annually. Therefore, new methods of disposal need to be explored and investigated to evaluate feasibility and sustainability. One such solution is the application of SMS as animal feed after delignification of encroaching wood species such as *Acacia mellifera* following mushroom cultivation due their ability to degrade the lignocellulosic complex of wood. As a result, the objective of this chapter was to evaluate the effect of animal feed supplemented with SMS on ruminants by considering growth, % fat, total fatty acids, fatty acid ratios and colour of meat. This study found that ruminants from the experimental groups showed improved growth compared to the control groups. Furthermore, total fatty acids and fatty acid ratios of the experimental groups were considered to be more acceptable than the control groups. Additionally, meat from the experimental groups were more favourable to consumers when various parameters ( $L^*$ ,  $a^*$ ,  $b^*$ , hue and saturation index) were taken into consideration. Consequently, the application of SMS as animal feed were considered to be successful and should be further investigated.



## 4.1 Introduction

Parasitic fungi grow on living organic material and thrive at their expense as they deprive their host from nutrients (Chang 2009). Mycorrhizal fungi live in true symbiosis with their host plant, generally a tree wherein essential micro-nutrients such as mineral salts are provided to their host in exchange for energy. Plants find these mineral salts, especially nitrates, the hardest to convert from the soil and can be obtained by making use of mushroom mycelium since it is in closer contact with the soil than the roots (Polese 2000). Saprophytes obtain nutrients from dead, organic material and most exotic mushrooms have been found to be saprophytic. These fungi are known to be primary decomposers due to the production of extracellular enzymes which results in the decomposition of woody structures. Innumerable fungal species are both saprophytic and parasitic since they continue to feed off a dead host (Polese, 2000; Smith et al. 2002). For example, *Ganoderma lucidum* is commonly known to act as both saprophyte and parasite (Chang 2009). Exotic mushrooms can therefore be cultivated on substrates containing lignin due to their ability to break down complex lignocellulosic materials. As a result, mushrooms provide a way of returning carbon, nitrogen and hydrogen to the ecosystem (Falandysz and Borovička 2013; Chang and Miles 1992; Stamets 1993).

Saprophytic mushrooms are currently the most cultivated mushrooms as they are heterotrophic for carbon compounds and are devoid of vascular phloem and xylem (Chang and Wasser 2016). In addition, mushrooms contain chitin and no chlorophyll in their cells walls, resulting in the absence of photosynthesis. Instead, mushrooms rely on organic matter produced by green plants in their immediate environment to obtain nutrients. Mushrooms synthesize and secrete relevant hydrolytic and oxidative enzymes in order to degrade complex organic matter to generate simpler compounds, which can be absorbed for their nutrients (Chang and Wasser 2016; Miles and Chang 2004). In addition, mushrooms can degrade lignocellulosic matter due to the production of enzymes in order to obtain nutrients (Buswell and Chang 1994; Buswell et al. 1996).

Approximately 70% of agricultural and forest materials are non-productive and are regarded as lignocellulosic wastes, which mainly consist of three organic compounds, namely cellulose, hemicellulose and lignin (Chang 1987, 1989; Chang and Wasser 2016). Brewery spent grain, cereal straw, coffee pulp, spent ground coffee, cotton seed hull, sawdust and wood chips are commonly discarded as wastes. Since lignocellulosic wastes have insignificant commercial value and no food value, careless disposal of these wastes by dumping or burning in the environment are bound to cause pollution and, consequently, lead to health hazards (Stamets

2005). Up to date, complex and insoluble lignocellulosic wastes have been chemically treated in order to increase the digestibility and potential nutritional value (Chang and Wasser 2016). However, chemical treatment, for example, with diluted hydrochloric acid and calcium chloride, are expensive and time consuming. Consequently, there has been an increased interest in the potential of mushroom cultivation to improve the nutritional value of these waste products as well as promote equitable economic growth as it is abundantly available (Chang and Wasser 2016).

Belonging to the Fungi Kingdom, both edible and medicinal mushrooms can be cultivated on these waste materials (Chang and Wasser 2016). As a result, mushrooms can greatly benefit environmental conditions as spent mushroom substrate has application in environmental bioremediation, as a soil conditioner, and as animal feed or fodder for livestock. Furthermore, mushroom cultivation could aid in reducing environmental pollutants as mycelia remove and break down contaminants, resulting in the absorption of the pollutants (Stamets 2005; Dai 2010; Miller 2013). After harvesting the mushrooms, lignocellulosic substrates have application as compost with high quantities of nitrogen-rich materials as well as partly decomposed lignocellulosic components. Therefore, sustainable research has been performed on mushroom biotechnology, known as the “white-agricultural evolution” or “non-green revolution” (Chang and Wasser 2016).

Recently, there has been increased interest in the addition of beneficial products to animal feed due to increased feed costs as well as environmental issues such as droughts (Park et al. 2012). Although the cultivation of mushrooms on lignocellulosic wastes have its advantages, spent mushroom substrate (SMS) are also considered to be a waste product. In Korea, the production of approximately of 300 000 metric tons of SMS presents an economic problem due to costs associated with disposal (Park et al. 2012). As the mushroom industry is gradually increasing, the volume of SMS generated annually is also increasing (Phan and Sabaratnam 2012).

New solutions for the disposal of SMS need to be explored and the potential of using SMS for the production of value-added products have been discussed extensively (Phan and Sabaratnam 2012). One of them is the production of lignocellulosic enzymes such as cellulase, hemicellulose, laccase, lignin peroxidase and xylanase. After harvesting of mushrooms, SMS could be more easily digested by ruminants due to the enzymatic degradation during mushroom cultivation (Streeter et al. 1982; Adamović et al. 1998). Thus, being considered to be a product of interest as it may have application as animal feed for

ruminants (Park et al. 2012). However, the substrate used for the cultivation of mushrooms contains cellulose, lignin and low quantities of protein (Phan and Sabaratnam 2012). As a result, it was declared to be unsuitable for application as animal feed. Therefore, various studies have been performed on the viability of SMS as animal feed and the effects of fibre on ruminants were demonstrated, thereby proving the use of SMS in ruminant diets should be reconsidered (Kim et al. 2010; Lee et al. 2008; Oh et al. 2010; Xu et al. 2010). Nevertheless, limited data on SMS as dietary supplement are available, particularly regarding the biological effect of SMS on ruminants and research are ongoing (Park et al. 2012).

Species of *Ganoderma*, especially *G. lucidum*, are capable of degrading lignin as well as improve the *in vivo* digestibility of dry matter. Over time, the ash content, crude protein and fat content increase, but not the cell wall components (Streeter et al. 1981, 1982; Zadrazil and Puniya 1995). According to Adamović *et al.* (1998), the reduction in hemicellulose is the most notable change in the composition of SMS followed by cellulose and lignin, proving the enzymatic degradation of the cell wall components by enzymes secreted during mushroom cultivation. As a result, *in vivo* dry matter digestibility (IVDMD) of SMS are directly increased as ruminant feed. Additionally, high quantities of polysaccharides, vitamins and valuable trace minerals such as Ca, Fe, Mg and Zn are produced by SMS (Medina et al. 2009; Paredes et al. 2009; Zhu et al. 2012). However, studies have indicated that a high ash content could deplete minerals available in the feed (Kakkar et al. 1990; Kakkar and Dhanda 1998). Furthermore, lignin degradation by mushrooms results in the presence of phenolic compounds, which could have a negative effect on digestibility. In order to formulate a diet which includes SMS, factors such as animal species, mushroom strains, cell wall components, digestibility, voluntary intake and nutrition level of the SMS must be taken into consideration before SMS could be utilised as supplement to animal feed (Langar et al. 1982; Phan and Sabaratnam 2012).

Lately there has been a great interest in the application of spent *G. lucidum* substrate as animal feed due to the ability to degrade the lignocellulosic complex in wood. Lignin is a heterogeneous polymer occurring in woody structures and surrounds cellulose in woody cell walls by forming a matrix (ten Have and Teunissen 2001). As a result, hemicellulose and cellulose, known as holocellulose are protected against microbial depolymerisation. As a white-rot fungus, *G. lucidum* is capable of completely breaking down lignin to CO<sub>2</sub> and H<sub>2</sub>O while also allowing access to holocellulose as a source of carbon and energy (Kirk and Farrell 1987; ten Have and Teunissen 2001). Important enzymes responsible for degradation of the lignocellulosic complex such as  $\alpha$ -amylase,  $\beta$ -glucosidase, cellulase, laccase, lignin- and

manganese peroxidase, and xylanase are produced by *G. lucidum*, contributing to its application (ten Have and Teunissen 2001).

Lignin forms the structural matrix of wood and is therefore responsible for providing rigidity. Additionally, lignin protects cellulose and hemicellulose. According to Hammel (1997), the water insolubility of lignin is responsible for limiting its availability to lignolytic systems, resulting in degradation being a slow process (Hammel 1997). Two types of extracellular enzymatic systems, namely the hydrolytic and the ligninolytic system are presented by saprophytic mushrooms (Sánchez 2009). The hydrolytic system produces hydrolases responsible for degradation of polysaccharides whereas the ligninolytic system degrades lignin (Sánchez 2009). Since wood is a relatively poor source of nitrogen and minerals, white-rot fungi are able to transport both nitrogen and minerals through their mycelia. Since mushroom mycelia are capable of producing and secreting enzymes, lignin are degraded into smaller compounds (Fourie 2015). When lignin is degraded, important polysaccharides such as cellulose and hemicellulose are exposed, which form a significant part of animal feed and are mostly responsible for energy intake. Thus, delignification of wood is the key to converting woody biomass to animal feed which can be metabolized by ruminants. Cultivation of *G. lucidum* generates a large amount of SMS, which have application as animal feed when lignocellulosic complex are degraded (Zhou et al. 2012). As a result, agricultural and forest wastes can be converted into useful matter (Zhou et al. 2012).

Currently, *A. mellifera* is considered to be an encroaching tree species in South Africa as large areas of farm land in the Northern Cape Province are suffering due to the detrimental impact it has on grazing land by suppressing growth of grass (Hagos 2001; Joubert et al. 2012; Lohmann et al. 2014; Lukomska et al. 2014; Fourie 2015). As a result, grazing and grassland are lost due to the encroaching *A. mellifera* in arid area, leading to decreased availability of animal feed, especially during times of drought (Fourie 2015). The encroaching *A. mellifera* occurs in bushveld or semi-desert areas and are described as either a large shrub or small tree (Fourie 2015). As a drought tolerant tree, this bush is extremely hardy when matured. The trees mainly reach a height of 3 m and multiple stems are formed, with stems smaller in diameter. As two thorns are produced at each node of the low spreading branches, *A. mellifera* are considered to be extremely thorny. Moreover, impenetrable thickets are formed where grazing exits. Hence, making the land useless as animals cannot reach the land covered with *A. mellifera*, nothing else grows underneath and grazing animals cannot utilize the *A. mellifera* itself (Fourie 2015).

Up to date, chemical, biological and mechanical control are the three methods employed by farmers as a way of eradicating *A. mellifera* (Fourie 2015). In the case of chemical control, arboricide and manual removal are currently used. However, arboricides have an extremely negative effect on other tree species such as camelthorn trees. For biological control, Boer goats are used to maintain encroaching *A. mellifera* at a minimum. Unfortunately, this is not a viable option as Boer goats are incapable of completely eradicating *A. mellifera*, deeming the use of unwanted de-bushing necessary. The mechanical control of *A. mellifera* requires the use of large bush harvesters which cut the tree at ground level, resulting in a mass of freshly cut wood chips, sawdust, twigs and leaves. However, this method is not feasible due to the cost associated with the harvesters and transportation of the materials (Fourie 2015). Due to the costs associated with chemical, manual and mechanical de-bushing, a system of planned burning was recommended by Lohmann et al. (2014). However, farmers were found to be hesitant due to various risks accompanying this method. Consequently, the idea of converting harvested encroaching *A. mellifera* to animal feed was suggested, if the wood could be degraded sufficiently to expose the cellulose and hemicellulose within (Fourie 2015). As a result, mushroom cultivation took centre stage due to these methods being unsustainable (Fourie 2015).

For *A. mellifera* wood to be utilized, the lignocellulosic structure needs be degraded aerobically at such a level to allow nutrients to be available as well as improve digestibility (Fourie 2015). Currently, chemical, physical and physiochemical methods are employed for delignification of wood. A summary of the advantages and disadvantages of these methods are highlighted in the table (**Table 1**) below.

**Table 1.** Comparative information on different delignification treatments (Fourie 2015).

Treatment		Advantages	Disadvantages	Reference
Physical	Grinding	Lower cellulose crystallinity, resulting in cellulose being better degradable by enzymes.	Limited feasibility to be applied on farm – requires machines or industrial processes.	Sarnklong et al. 2010.
	Soaking			
	Pelletising			
Chemical	Sulphuric acids	Lower cellulose crystallinity and high sugar yields.	Expensive and environmental pollution.	Hendriks and Zeeman 2009; Agbor et al. 2011; Sarkar et al. 2012.
	Nitric acids			
	Sodium hydroxide	Improved digestibility.	Expensive and environmental pollution.	Sarnklong et al. 2010; Chaturvedi and Verma 2013.
	Potassium hydroxide			
	Urea	Cheap, safe, adds nitrogen.	Very small improvement on dry matter degradability.	Sarnklong et al. 2010.
	Hydrogen peroxide	Simple process.	Non-selective, causing loss of cellulose and hemicellulose.	Hendriks and Zeeman 2009.
	Peracetic acid			
Physio-chemical	Combination of heat, moisture, pressure and chemicals	Time-saving.	High energy demands, requires special equipment, production of toxic wastes.	Hendriks and Zeeman 2009; Agbor et al. 2011; Sarkar et al. 2012.
Biological	Fibrolitic enzymes	Improved degradability of 13%.	Extraction of enzymes expensive.	Rodrigues et al. 2008.
	Fungi	Cheap, safe, simple, adds fungal mass and organic matter, energy saving, reduce chemicals, environmentally friendly.	Time consuming, requires controlled environment for fermentation.	Akhtar et al. 1992; Howell et al. 2009; Rasmussen et al. 2010; Giles et al. 2011; Tian et al. 2012.

Both brown- and white-rot fungi are capable of degrading lignin (Fourie 2015). However, white-rot fungi such as saprophytic mushrooms only degrade lignin during colonisation whereas polysaccharides are only degraded when fruiting occurs (Jalč 2002). *A. mellifera* may have economic benefits when used as substrate for the cultivation of *G. lucidum*. As mentioned earlier, composted SMS from *G. lucidum* have application as animal feed due to the degradation of lignin and cellulose, allowing animals to further digest the wood. Thus aiding in relieving stress caused by the recent drought as well as clearing land to allow grazing for animals (Jalč 2002; Fourie 2015).

In addition, application of feed may be beneficial to ruminants due to improved nutritional values as well as medicinal properties brought about by the mushroom species. In order to evaluate the effect of above-mentioned feed on sheep, various parameters such as % fat, % fat free dry matter and % moisture, % total fatty acids and three fundamental colour coordinates ( $L^*$ ,  $a^*$  and  $b^*$ ) have to be considered when meat are evaluated. In the case of % total fatty acids, oleic acid is considered to be the most abundant fatty acid, followed by palmitic acid (Costa et al. 2015). Therefore, the aim of this chapter is to evaluate the practical application of SMS as animal feed and energy feedstock as well as the effect of the feed on meat.

## **4.2 Materials & Methods**

### **4.2.1 *A. mellifera* used for *G. lucidum* cultivation**

*A. mellifera* was obtained from the Northern Cape farm, Geelkoppies. The material for this study was harvested in the winter when trees were dormant and no fresh growth occurred. Only the upper parts of the trees were used for the study, resulting in the absence of any leaves.

### **4.2.2 Preparation of spawn**

First generation and second-generation spawn were produced. For the preparation of first-generation spawn, sorghum was soaked overnight in water with the addition of 2% gypsum. Drainage of excess water was achieved by spinning sorghum in a washing machine (SpeedyVac at the lab). Coffee bottles were filled with 400 g sorghum and autoclaved as the preferred method for sterilization. Agar squares of *G. lucidum* (UFS-GL1) were cut with a sterile needle and added to the sorghum to produce spawn to be used as inoculum for second generation spawn bags. After inoculation, the bottles were incubated at 25°C for 1-2 weeks to allow full colonization to take place. In the case of second-generation spawn bags, sorghum was soaked overnight in water with the addition of 2% gypsum after which drainage of excess water was achieved by spinning the sorghum in washing machine (SpeedyVac at the lab). Polypropylene bags were filled to a weight of 800 g and autoclaved as the preferred method for sterilization. First generation spawn (400 g) were divided (200 g) to inoculate two sorghum bags, respectively, to produce second generation spawn to be used as inoculum for *G. lucidum* cultivation. After inoculation, the bags were incubated at 25°C for 1-2 weeks to allow full colonization.

### 4.2.3 Growth bag preparation

For cultivation of *G. lucidum*, encroaching *A. mellifera* was the desired substrate. Branches were chipped with a hammer mill into smaller pieces. The milled wood was soaked in water for 24 h. Drainage of excess water was achieved by spinning the substrates in a washing machine (SpeedyVac at the lab). Polypropylene bags were filled to a weight of 800 g and sterilized.

### 4.2.4 Cultivation of *G. lucidum* on *A. mellifera*

For artificial cultivation of *G. lucidum* (UFS-GL1), sterile substrate bags containing *A. mellifera* were inoculated with second generation spawn. The bags were incubated at 25°C for approximately 4-6 weeks to allow colonization until the appearance of mycelial growth onto bag. Bags were transferred to fruiting chamber to allow antler and cap formation. For the artificial cultivation of *G. lucidum*, black containers with aeration holes at the top and LED lights inside the box (12 V and 6 500 K). For antler formation, 4-8 h light cycles were incorporated whereas 12 hrs light cycles were required for fruiting body formation. Aeration holes covered with 3M Micropore Dressing Tape (24mm x 10m) contributed to control over CO<sub>2</sub>/O<sub>2</sub> concentrations due to acting as selective membrane for oxygen transfer. Furthermore, antler production of *G. lucidum* was further improved by using an optimum temperature of 18-24°C and a humidity of 95-100%. For fruiting body formation, a change in temperature was required as optimum temperatures were 21-27°C with a humidity of 90-95%. During both antler and fruiting body formations, maintenance of high humidity levels was achieved by wetting Perlite every 1-2 hrs.

### 4.2.5 Preparation of animal feed

For preparation of animal feed, *G. lucidum* mushrooms were harvested and spent mushroom substrate were dried and milled. Feed consisting of lusern: corn with a ratio of 70:30 was prepared and SMS was added as a supplement (30%), using a ratio of 15kg supplement: 50 kg feed (**Fig. 1**).





**Fig. 1.** Preparation of animal feed.

#### **4.2.6 Selection of ruminants**

For this experiment, 25 sheep were weighed using a Libra Measuring Instrument scale after which a group of sheep of the same weight (ranging between 29 and 30 kg) were selected and divided into control and experimental groups. This was done to aid in maintaining a uniform amount of feed (**Fig. 2**). The sheep were tagged (UFS Christie) accordingly: white tags for control and orange for experimental (**Fig. 3**). To prevent any interference that may be beneficial or detrimental, pens in the same environment were prepared after which lambs were randomly distributed into two pens in accordance with two treatments. Camps in the same environment were prepared (**Fig. 4, 5, 6**) to prevent any interference that may be beneficial or detrimental.



**Fig. 2.** Weighing and Selection.



**Fig. 3.** Tagging.



**Fig. 4.** Pens in same environment.



**Fig. 5.** Sheep in pens.



**Fig. 6.** Separate pens for separation of control and experimental groups.

#### **4.2.7 Prepping of ruminants**

The whole group of sheep were vaccinated with Multivax P Plus and Embavit as per usual procedure. The sheep were vaccinated with Multivax P Plus for protection against *Clostridium* spp. and *Pasteurella* spp. (**Fig. 7**) while Embavit (**Fig. 8**) was orally administered as a liquid

vitamin with a dosage rate of 5 ml diluted solution per 50 kg live mass. Additionally, Swavet Oradose A (Fig. 9) was administered as vitamin A supplement with a dosage rate of 2 ml per 10 kg live mass for prevention and treatment of vitamin A shortage. 100 ml was added to 100 ml of water and orally administered. For protection against parasites, the sheep were also injected with Ivermax at 1% concentration as anti-parasitic remedy (Fig. 10).

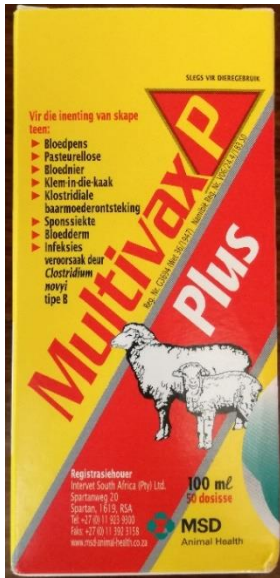


Fig. 7. Multivax P Plus



Fig. 8. Embavit



Fig. 9. Swavet Oradose A



Fig. 10. Ivermax

#### 4.2.8 Feeding of ruminants

Sheep were fed in the morning for 41 days. The treatments were: sheep fed with feed consisting of 30:70 ratio, corn: lusern (control group) and the same feed supplemented with 30% SMS (experimental group). For research purposes, the sheep were weighed individually at daily intervals in the morning before feeding, using a Libra Measuring Instrument scale.

#### 4.2.9 Analysis of meat

For analysis, sheep of each treatment were slaughtered on the same day and meat samples were sent for various analysis. Microbial analysis was also performed on the stomach content of both the control and experimental groups. Three different dilutions ( $10^{-6}$ ,  $10^{-9}$  and  $10^{-12}$ ) were plated out to determine total counts (Plate Count Agar), coliforms (VRB-Mug), *S. aureus* (Baird Parker Agar), Lactobacilli (MRS) as well as *L. monocytogenes* in Listeria Broth.

For the determination of the fatty acid profile, the following procedure was followed:

A 5 g meat sample was removed from the middle from loin chops per treatment. Total lipid from muscle was quantitatively extracted, according to the method of Folch et al. (1957), using chloroform and methanol in a ratio of 2:1. An antioxidant, butylated hydroxytoluene was added at a concentration of 0.001 % to the chloroform: methanol mixture. A rotary evaporator was used to dry the fat extracts under vacuum and the extracts were dried overnight in a vacuum oven at 50°C, using phosphorus pentoxide as a moisture adsorbent. Total extractable intramuscular fat was determined gravimetrically from the extracted fat and expressed as percent fat (w/w) per 100 g tissue. The extracted fat from feed and muscle was stored in a polytop (glass vial, with push-in top) under a blanket of nitrogen and frozen at -20°C pending fatty acid analyses.

A lipid aliquot (20 mg) of feed and muscle lipid were converted to methyl esters by base-catalysed transesterification, in order to avoid conjugated linoleic acid (CLA) isomerisation, with sodium methoxide (0.5 M solution in anhydrous methanol) during 2 h at 30 °C, as proposed by Alfaia et al. (2007), Kramer et al. (2002) and Park et al. (2001). FAMES (% total fatty acids) from feed and muscle were quantified using a Varian GX 3400 flame ionization GC, with a fused silica capillary column, Chrompack CPSIL 88 (100 m length, 0.25 mm ID, 0.2 µm film thicknesses). Analysis was performed using an initial isothermal period (40°C for 2 minutes). Thereafter, temperature was increased at a rate of 4°C/minute to 230°C. Finally,

an isothermal period of 230°C for 10 minutes followed. FAMES n-hexane (1µl) were injected into the column using a Varian 8200 CX Autosampler. The injection port and detector were both maintained at 250°C. Hydrogen, at 45 psi, functioned as the carrier gas, while nitrogen was employed as the make-up gas. Varian Star Chromatography Software recorded the chromatograms.

Fatty acid methyl ester samples were identified by comparing the retention times of FAME peaks from samples with those of standards obtained from Supelco (Supelco 37 Component Fame Mix 47885-U, Sigma-Aldrich Aston Manor, Pretoria, South Africa). Conjugated linoleic acid (CLA) standards were obtained from Matreya Inc. (Pleasant Gap, Unites States). These standards included: cis-9, trans-11; cis-9, cis-11, trans-9, trans-11 and trans-10, cis-12-18:2 isomers. All other reagents and solvents were of analytical grade and obtained from Merck Chemicals (Pty Ltd, Halfway House, Johannesburg, South Africa). Fatty acids were expressed as the proportion of each individual fatty acid to the total of all fatty acids present in the sample. The following fatty acid combinations were calculated: omega-3 (n-3) fatty acids, omega-6 (n-6) fatty acids, total saturated fatty acids (SFA), total monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), PUFA/SFA ratio (P/S) and n-6/n-3 ratio.

For the analysis of the colour of loin chops, the following method was followed:

Two loin chops from each treatment were used for colour measurements. Colour ( $L^*$ ,  $a^*$  and  $b^*$  values) of muscle was assessed in 9-fold using a Minolta chromometer. Chroma or saturation index (SI) which is related to colour intensity of meat, was calculated according to the formula:  $SI = (a^{*2} + b^{*2})^{0.5}$  for muscle (Ripoll et al. 2011). Hue angle was calculated according to the formula  $\tan^{-1}(b^*/a^*)$  (Ripoll et al. 2011).

## 4.3 Results & Discussions

### 4.3.1 Cultivation of *G. lucidum*

The cultivation of *G. lucidum* on *A. mellifera* wood was successful as antlers (**Fig. 11**) were obtained within 28 days and fruiting bodies (**Fig. 12**) were obtained after 65 days.



**Fig. 11.** Antler formation of *G. lucidum*.



**Fig. 12.** Fruiting body development of *G. lucidum*.

#### **4.3.2 Preparation of animal feed**

Production of animal feed consisting of corn and lusern (30:70 ratio) supplemented with 30% milled mushrooms was successful.

### **4.3.3 Selection of ruminants**

Successfully selected a group of sheep of the same weight (ranging between 29 and 30 kg) to aid in maintaining a uniform amount of feed. Preparation of camps in the same environment prevented any interference that may be beneficial or detrimental.

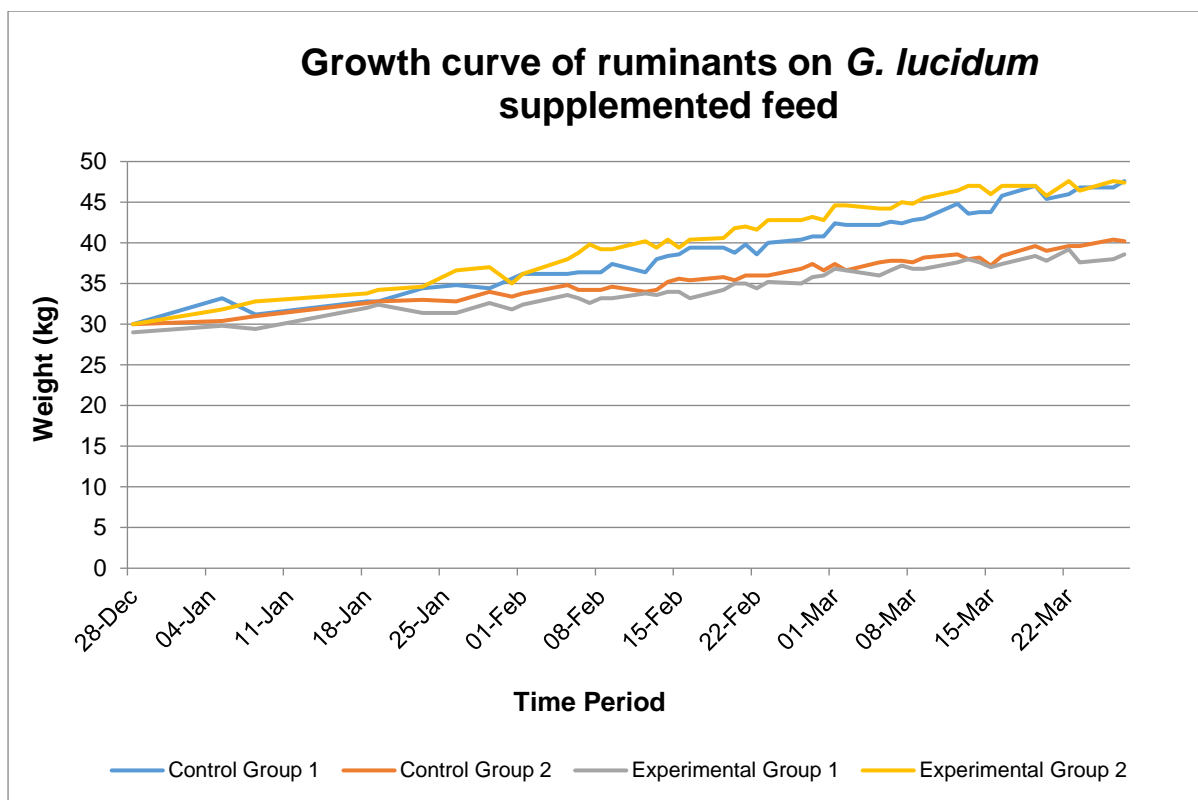
### **4.3.4 Prepping of ruminants**

Vaccination of sheep with Multivax P Plus was successful as no infections caused by *Clostridium* spp. and *Pasteurella* spp were detected. In addition, no vitamin A shortage was detected nor any parasitic infection.

### **4.3.5 Feeding of ruminants**

From **Fig. 13** it is clear that experimental group 4 showed improved growth compared to control group 1. Both sheep 2 and 3 were ewes while sheep 1 and 4 were rams. Furthermore, rams were considered to grow faster compared to ewes. As a result, increased growth was sooner observed in the rams compared to the ewes.





**Fig. 3.** Growth of both experimental and control animals on feed supplemented with *G. lucidum*.

#### 4.3.6 Analysis of meat

For **Fig. 14**, teeth of ruminants indicate that they were still lambs as deciduous teeth were still present. Lambs fed with feed supplemented with SMS, showed more fat surrounding organs compared to the control group (**Fig. 15**). Although this study was focused on the application of SMS as a supplement to animal feed, carcass weights were also considered. Results obtained showed no significant differences for the control and experimental groups (**Fig.16**). Ruminants 1 (control) and 4 (experimental) were rams while ruminants 2 (control) and 3 (experimental) were ewes. Meat from experimental groups were graded as A2 meat whereas meat from control groups were graded as A3. Microbial analysis was also performed on the stomach content of both the control and experimental groups (**Table 2**). Significant total counts were observed at a dilution of  $10^9$  whereas no coliforms, *S. aureus* and *L. monocytogenes* were present at any of the dilutions. In the case of *Lactobacilli*, a significant increase was observed at the  $10^6$  dilution for the experimental group (>300) compared to the control group (**Table 2**). This may be an indicator of probiotics and natural flora (**Table 2**). As a result, digestion of food may be improved. However, further investigation is required.

For the proximate analysis of the meat samples, % fat, % fat free dry matter and % moisture was determined. From **Fig. 17**, meat from the experimental group (sheep 3) had the highest fat %, compared to the control group (sheep 1 and 2), which is desired. Approximately the same results were obtained for % fat free dry matter as well as % moisture (**Fig. 17**).

For determination of the % total fatty acids, oleic acid was found to be the most abundant fatty acid for both the experimental and control group (**Fig. 18**). The experimental group (sheep 3) had the highest amount of oleic acid with a value of 41.25 followed by the control group (sheep 1 and 2) with values of 39.06 and 40.49, respectively. Palmitic acid with values of 27.95, 26.96 and 25.02 were observed for the control group (sheep 1 and 2), respectively, followed by the experimental group (sheep 3). In comparison,  $\gamma$ -linolenic, capric, lauric, arachidic acids with values varying between 0.0 and 0.06 were observed. These results indicated a positive effect of feed supplemented with SMS on the meat as higher amounts of the unsaturated fatty acid oleic acid was observed for the experimental group compared to the control groups. In addition, the lowest amount of the saturated fatty acid, palmitic acid was observed for the experimental group compared to the control groups (**Fig. 18**).

From **Fig. 19** a positive effect of feed supplemented with SMS on the meat were observed as meat sample 2 (control group) were found to contain the most total saturated fatty acids (SFA) with a value of 51.25 followed by the experimental group (sample 3) with a value of 49.39 and sample 1 with a value of 48.89 (**Fig. 19**). The lowest amount of the saturated fatty acid, palmitic acid was observed for the experimental group compared to the control group. High amounts of SFA are undesired as it increases the risk of cardiovascular disease. Therefore, meat with lower amounts of short-chain SFAs are desired (Lôbo et al. 2014).

For the total mono unsaturated fatty acids (MUFA), the control group (sample 1 and sample 2) had the lowest content with values of 42.21 and 42.93, respectively, whereas the experimental group (sample 3) had the highest value at 43.59 MUFA (**Fig. 19**). Higher amounts of the unsaturated fatty acid oleic acid were observed for the experimental group compared to the control groups, which is desired as MUFAs provide protection against cardiovascular disease due to increased membrane fluidity when compared to saturated fats (Lôbo et al. 2014).

In the case of total poly unsaturated fatty acids (PUFA), control groups (sample 1 and 2) contained 8.90 and 6.33 PUFA, respectively, compared to the experimental group (sample 3) with a value of 7.02 (**Fig. 19**). Although the experimental group showed lower PUFA contents

compared to the control group, the value is still within the desired range and may aid in decreasing the possibility of coronary heart disease and type 2 diabetes (Wood et al. 2005).

Experimental and control groups exhibited similar results for both omega-6 and omega-3 fatty acids as higher amounts of both omega-6 and omega-6 fatty acids were observed for the control group compared to the experimental group (**Fig. 19**). For total omega-6 fatty acids (*n*-6), a significantly higher value of 7.87 were observed for the control group (sample 1) whereas a value of 6.14 were obtained for the experimental group (sample 3). Additionally, a value of 5.48 were observed for the control group (sample 2) (**Fig. 19**). In the case of total omega-3 fatty acids (*n*-3), a lower value at 0.88 were observed for the experimental group (sample 3) compared to the control group (sample 1) with a value of 1.03 while a value of 0.84 were observed for the control group (sample 2) (**Fig. 19**). Both omega-6 fatty acids (*n*-6) and omega-3 fatty acids (*n*-3) are polyunsaturated fatty acids which are considered to be essential fatty acids as humans are incapable of synthesizing it. Linoleic acid is the shortest-chained omega-6 fatty acids and is believed to contain anti-inflammatory activity. Shorter-chain omega-3 fatty acids  $\alpha$ -linoleic acid (ALA) can be obtained through diet and used to produce more important long-chain omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These omega-3 fatty acids are believed to improve blood lipid levels and as result, the risk of coronary disease is decreased.

For PUFA: SFA ratio, control group (sample 1) had a higher value of 0.18 than the experimental group (sample 3) with a value of 0.14 followed by control group (sample 2) with a value of 0.12 (**Fig. 19**). In the case of PUFA: SFA, it has been recommended to consume food with the highest ratio of PUFA: SFA. Even though the experimental group had a slightly lower PUFA: SFA ratio than the control groups, it is not a significant difference. For PUFA/ MUFA, control groups (sample 1 and 2) had values of 0.21 and 0.15, respectively whereas the experimental group (sample 3) had a value of 0.16 (**Fig. 19**). For the *n*6/*n*3 ratio, control groups (sample 1 and 2) had values of 7.63 and 6.51, respectively whereas the experimental group (sample 3) had a value of 6.98 (**Fig. 19**). The desired *n*6/*n*3 ratio is considered to be 4:1 with 4 omega-6 fatty acids for each omega-3 fatty acid consumed.

The lowest value for the atherogenic index of plasma (AIP) was observed for the experimental group (sample 3) with a value of 0.55, whereas control groups (sample 1 and 2) had values of 0.60 and 0.61, respectively (**Fig. 19**). The AIP reflect the true relationship between protective and atherogenic lipoprotein and has been shown to be a strong marker to predict the risk of atherosclerosis and coronary heart disease (Nwagha et al. 2010; Dobiášová et al. 2011). An

AIP value of under 0.11 is associated with low risk of cardiovascular disease while values between 0.11 to 0.21 and higher than 0.21 are associated with intermediate and increased risks, respectively (Dobiášová 2006; Dobiášová et al. 2011). However, results obtained showed significantly higher AIP values and further investigation is required.

The desaturase index has been established to be based on the relationship between the substrate and product for  $\Delta^9$  desaturase (Lôbo et al. 2014). It was suggested that an increase in MUFA concentration, especially regarding increased oleic acid concentration, might be an indicator of desaturase activity (Bauman et al. 1999; Dinh et al. 2010). Desaturase index values of 2.43 and 2.14 were observed for the control groups (sample 1 and 2) while a value of 2.16 were obtained for the experimental group (sample 3) (**Fig. 19**). Although higher MUFA values were obtained for the experimental group, a higher desaturase index value was obtained for the control group when compared to the experimental group. As a result, further investigation is required.

Three fundamental colour coordinates, namely  $L^*$ ,  $a^*$  and  $b^*$  are used to evaluate colour of meat samples (Priolo et al. 2001).  $L^*$  is the lightness and is a measure of the light reflected (100 = all light reflected; 0 = all the light absorbed) while  $a^*$  (positive red, negative green) and  $b^*$  (positive yellow, negative blue) are the other coordinates (Priolo et al. 2001). In a study conducted by Khlijji et al. (2010), it was suggested that when  $a^*$  (redness) and  $L^*$  (lightness) values are equal to or exceed 9.5 and 34, respectively, on average consumers will consider the meat colour acceptable (Khlijji et al. 2010). However, to have 95% confidence that a randomly selected consumer will consider a sample acceptable,  $a^*$  and  $L^*$  values must be much higher (14.5 and 44, respectively). For aged meat, when  $a^*$  value is equal to or greater than 14.8, on average consumers will consider the meat acceptable. However, the  $a^*$  value has to be increased to 21.7 to be 95% confident that a randomly selected consumer will consider a sample acceptable (Khlijji et al. 2010).

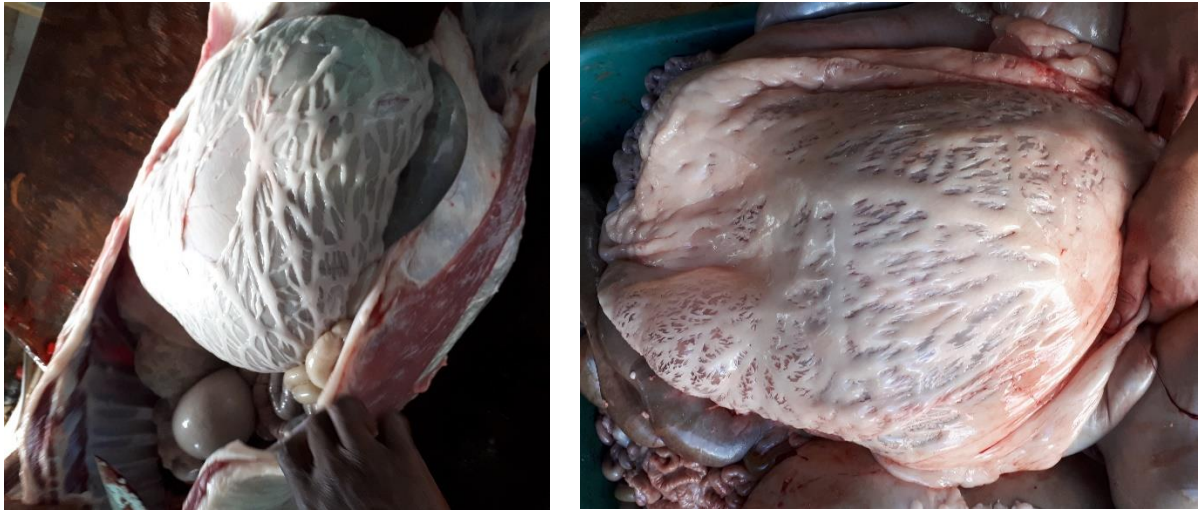
It has been advised by Wyszecki and Styles (1982) to define colour in terms of lightness ( $L^*$ ), chroma and hue (Wyszecki and Styles 1982). Lightness is described as the brightness or darkness of a colour and is described by Acton and Dawson (2004) as “a scaled proportion of the light energy reflected by or transmitted from the sample relating to achromatic white-to-grey-to-black” (Acton and Dawson 2004). Chroma (also saturation) refers to the strength of the colour, for example how dull or vivid a colour is (Hunt et al. 1991, 2012) and is described by Acton and Dawson (2004) as “the intensity or the amount of hue departure from grey of the same lightness.” As a result, coordinates  $a^*$  and  $b^*$  alone cannot describe chroma since hue

intensity is a function of the line connecting coordinates  $a^*$  and  $b^*$  with the core  $L^*$  coordinate (Acton and Dawson 2004). Hue refers to the name of the colour (red, yellow, blue, green, etc.) and is developed by the reflecting specific wavelengths from a meat surface back to the detector (Hunt et al. 1991, 2012). Furthermore, hue is described by Acton and Dawson (2004) as “the angular specification for the colour perceived as red, yellow, blue, and green” and is the angle formed from the centre core ( $L^*$ ) with coordinates  $a^*$  and  $b^*$ . In 2008, Ripoll and co-workers indicated hue to be a good indicator of discolouration (Ripoll et al. 2008).

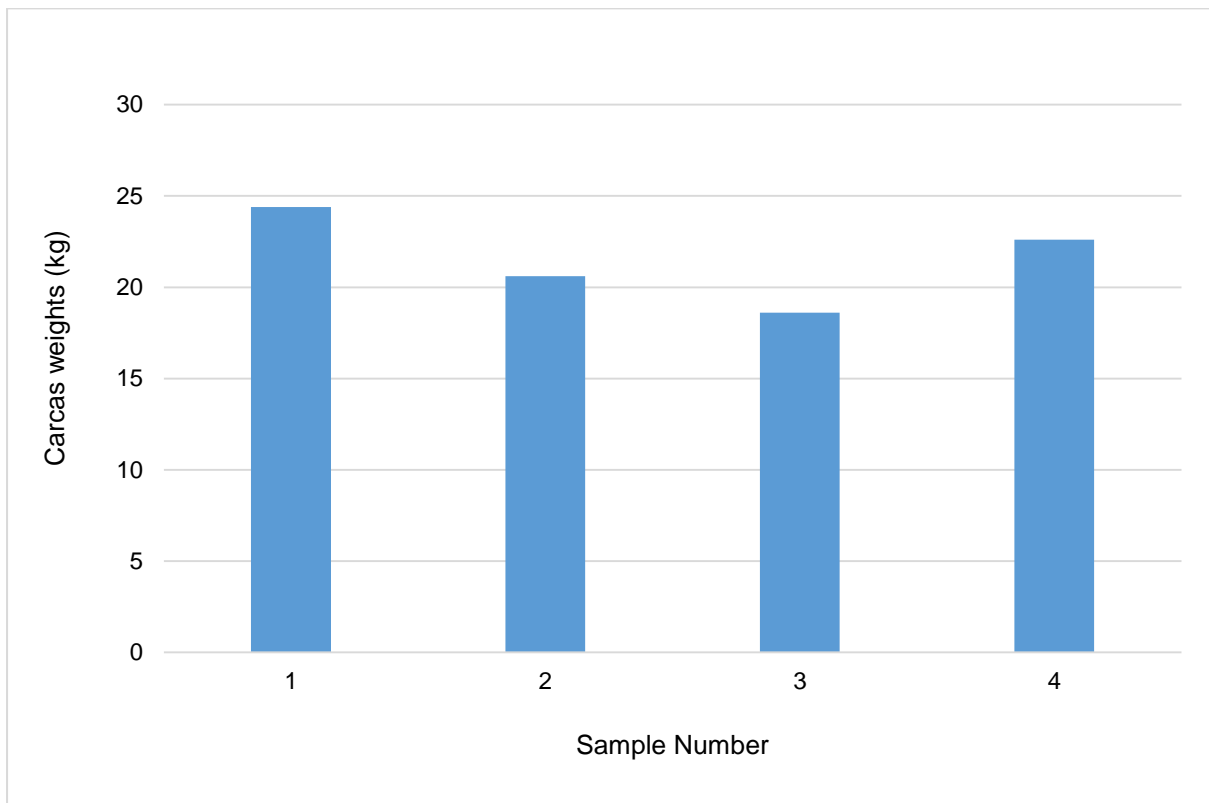
From **Fig. 20**, the following results were obtained regarding the colour of the loin chops: For  $L^*$ , sample 4 showed to best results with 47.63 compared to sample 3 (47.37) and 2 (44.26) (**Fig. 20**). For  $a^*$ , sample 4 showed to best results with 20.52 compared to sample 3 (18.08) and 2 (17.60) (**Fig. 20**). When these two parameters are taken in account, meat from the experimental group is considered to be most in accordance with guidelines regarding acceptability for consumers. For  $b^*$ , sample 3 showed to best results with 10.11 compared to sample 4 (11.02) and 2 (9.30) (**Fig. 20**). In the case of chroma (saturation index), the best result was obtained for sample 4 with a value of 22.86 compared to sample 3 (20.75) and sample 2 (19.95) (**Fig. 20**). For the hue angle, sample 3 showed the most promising result with a value of 29.25 followed by sample 2 (28.31) and sample 4 (25.91) (**Fig. 20**).



**Fig. 4.** Teeth of ruminant indicating deciduous teeth, still lambs.



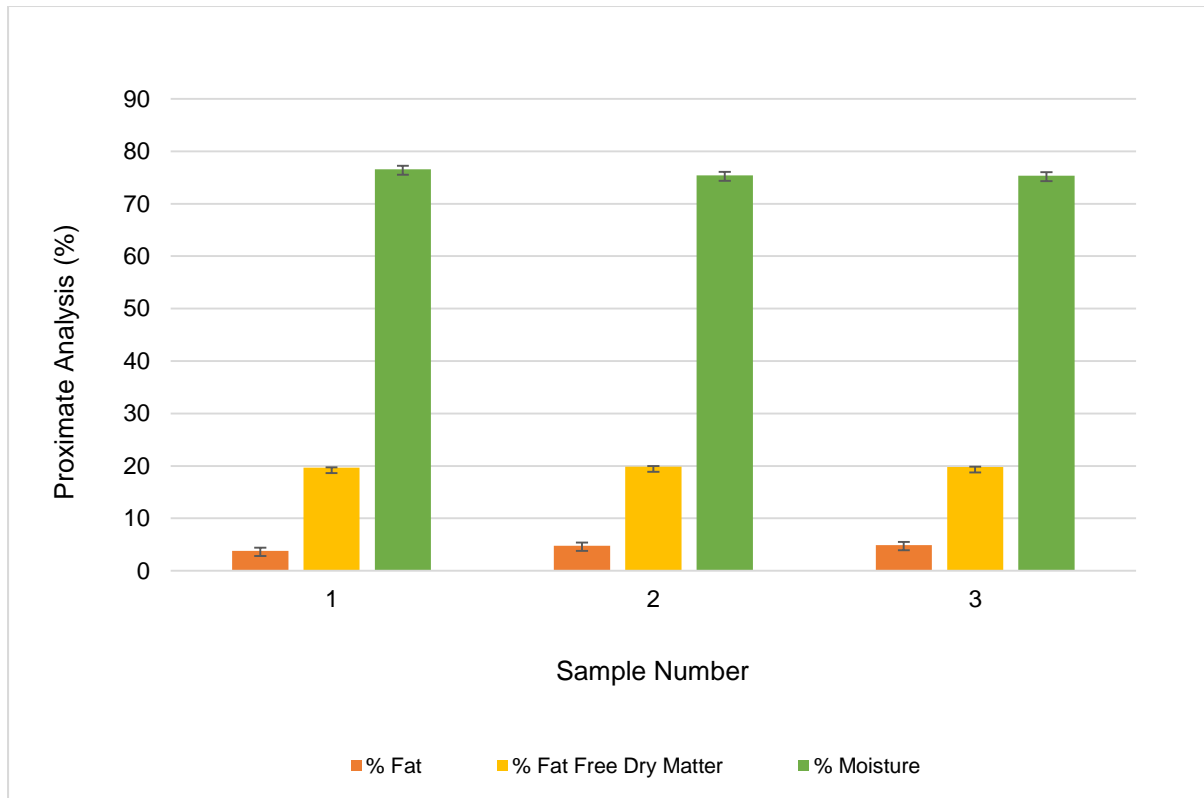
**Fig. 5.** Fat surrounding organs of control and experimental groups, respectively.



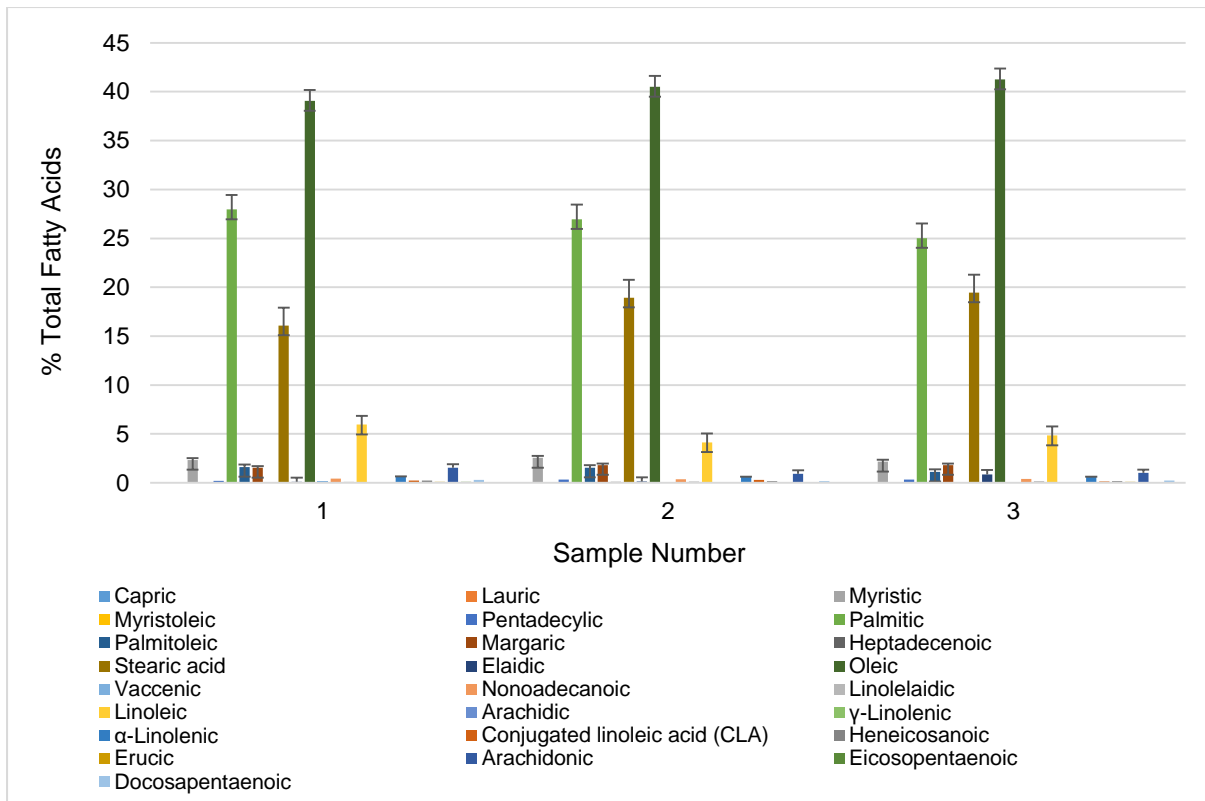
**Fig. 6.** Indication of carcass weights for control (numbers 1 and 2) and experimental (3 and 4) groups.

**Table 2.** Microbial analysis of stomach of control and experimental groups.

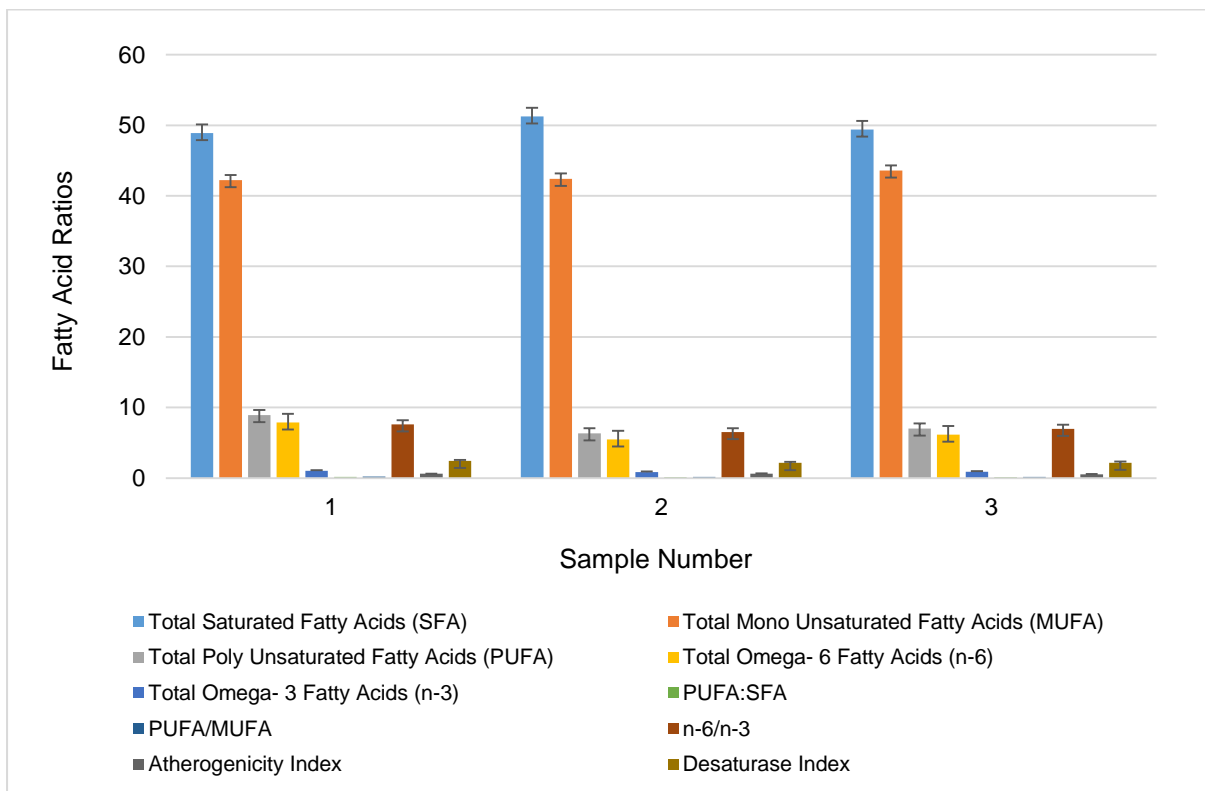
	<b>Sample Number</b>	Total Counts	Coliforms	<i>S. aureus</i>	<i>Lactobacilli</i>	<i>L. monocytogenes</i>
<b>Control</b>	1	194 x 10 <sup>9</sup>	<10 <sup>6</sup>	<10 <sup>6</sup>	180 x 10 <sup>6</sup>	0
	2	22 x 10 <sup>9</sup>	<10 <sup>6</sup>	<10 <sup>6</sup>	84 x 10 <sup>6</sup>	0
<b>Experimental</b>	3	88 x 10 <sup>9</sup>	<10 <sup>6</sup>	<10 <sup>6</sup>	340 x 10 <sup>6</sup>	0
	4	100 x 10 <sup>9</sup>	<10 <sup>6</sup>	<10 <sup>6</sup>	346 x 10 <sup>6</sup>	0



**Fig. 7.** Proximate analysis in percentage indicating % fat, % dry fat free matter and % moisture of meat samples.

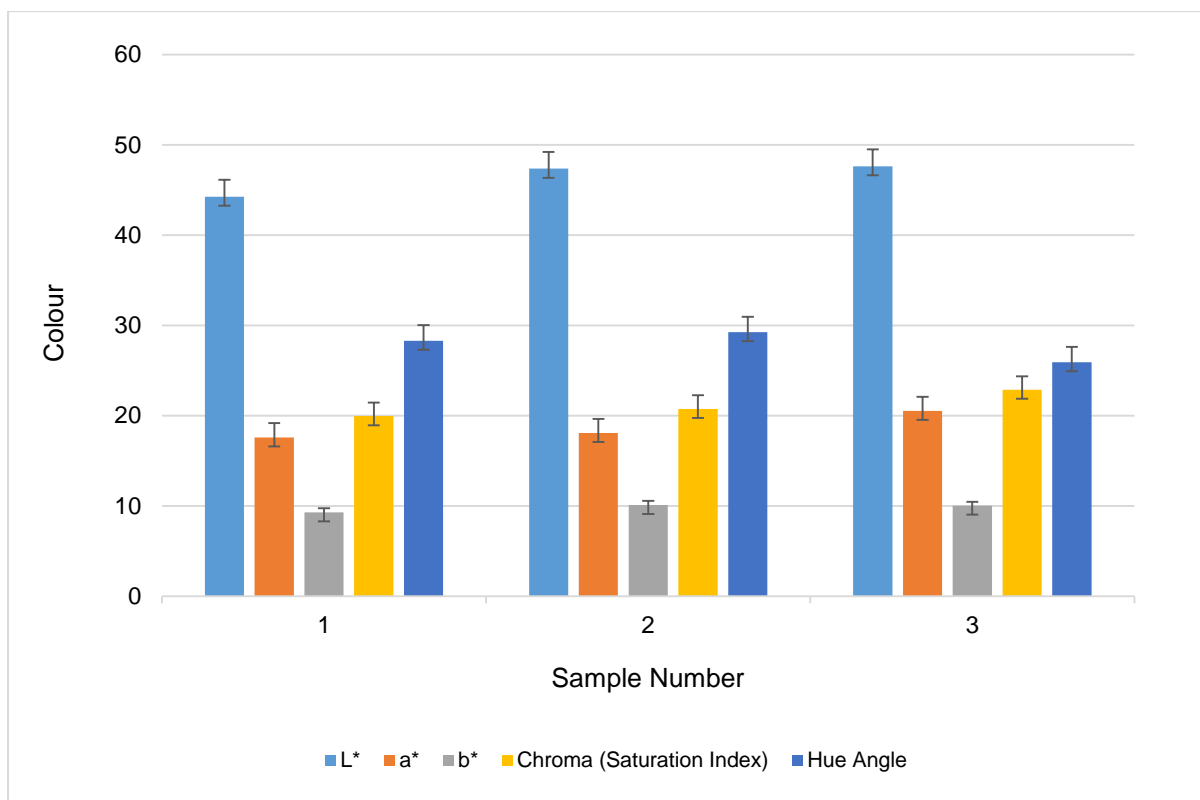


**Fig. 8.** Indication of % total fatty acids in the meat samples.



**Fig. 9.** Indication of the fatty acid ratios in the meat samples.





**Fig. 10.** Results of the colour of the meat samples using five different parameters.

#### 4.4 Conclusions

The world population is continuing to increase, resulting in an increase in lignocellulosic biomass, which is to a large extent considered to be insignificant or of no commercial value in its original form (Chang 2009). Although research is ongoing for utilization of various core products, very few research are available for utilization of resulting waste products. One such example is the utilization of *A. mellifera* wood, which is considered to be an encroaching wood species in South Africa. Clearing of lands results in large amounts, which need to be disposed. Careless disposal to the surrounding environment by burning are bound to contribute to environmental pollution and consequently cause health hazards (Chang 2009).

In the event of proper utilization of *A. mellifera* wood, by application for cultivation of mushrooms, economic growth could be promoted (Chang 2009). After harvesting of mushrooms, spent mushroom substrate (SMS) have further application as animal feed, resulting in full utilization of waste products. Enzymes produced by mushrooms aid in the delignification process, resulting in degradation of lignocellulosic materials for growth and fruiting. For this study, *G. lucidum* was cultivated on *A. mellifera* wood in order to evaluate applications and various factors were taken in consideration and modified to improve yields

on *A. mellifera* wood. These modifications were based on improved lighting and different lighting cycles, humidity control as well as CO<sub>2</sub> regulation in order to improve both antler and fruiting body formation. Due to sufficient breakdown of lignin, the further application of *A. mellifera* wood as animal feed were investigated. As animal feed, SMS was added as supplement and preliminary experiments were conducted to evaluate effect of SMS as supplement on growth of ruminants. Sheep fed with supplemented feed showed more consistent growth compared to the control groups fed on feed consisting only of lucern and corn. In addition, microbial analysis of stomach contents of both control and experimental groups showed a significant increase in *Lactobacilli* counts for the experimental group. This is beneficial as it aids in digestion of food minimizing pathogen multiplication.

For the proximate analysis of the meat samples, meat from the experimental group (sheep 4) had the highest fat %, compared to the control group (sheep 2 and 3), which is desired as it aids in protecting organs. Approximately the same results were obtained for % fat free dry matter as well as % moisture. In the case of the % total fatty acids, the feed supplemented with SMS seemed to lower the SFAs as the lowest amount of the saturated fatty acid, palmitic acid was observed for the experimental group, which is desired. Furthermore, higher amounts of MUFAs were observed for the experimental groups. The unsaturated fatty acid, oleic acid was found to be the most abundant fatty acid for both the control and experimental groups, with a slightly higher value for the experimental group. Low amounts of PUFAs were found for both the experimental groups and control groups, with slightly higher values for the experimental groups. When colour of the meat was evaluated, L\*, a\* and b\* as well as hue and saturation index were the parameters to be considered. Meat from the experimental group were found to be the most in accordance with guidelines regarding acceptability for consumers. As a result, meat from the experimental group were more favourable than meat from the control group. It can therefore be concluded that feed supplemented with SMS have a positive effect when compared to traditional feed.

Aside from mushroom cultivation and animal feed, the enzymes recovered from SMS have potential uses for bioremediation of pollutants as well as other biotechnological purposes. However, the potential of SMS needs to be further investigated to evaluate the applications of SMS and SMS-related products (Phan and Sabaratnam 2012).

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## Chapter 5

### Summary

Medicinal mushrooms have had applications for centuries as an alternative treatment of various ailments. Their nutritional value adds to their popularity and has been extensively studied as a healthy source of food which are rich in protein. Two medicinal mushrooms, *Ganoderma lucidum* and *Hericium erinaceus* have been scrutinized in recent years due to the presence of bioactive compounds. Although a non-edible mushroom, *G. lucidum* is considered to possess the most medicinal properties and cultivation may have the potential of providing animal feed. The edible *H. erinaceus* is currently scrutinized as it may have application as a natural alternative to treat the symptoms associated with Alzheimer's and cognitive decline. This study focused primarily on highlighting the advantages both mushrooms hold for humankind and pave the way for alternative medicinal applications.

In chapter 2, the optimum growth conditions for the cultivation of *G. lucidum* and *H. erinaceus* have been investigated in order to allow exploitation of their medicinal and economic benefits. In this chapter, pure cultures of *G. lucidum* and *H. erinaceus* were required with the need for a single medium allowing cultivation of both species being imperative. Sorghum agar was developed and found to be a superior growth medium compared to MEA or YMA. For cultivation of *G. lucidum*, the encroaching tree species, *Acacia mellifera*, were used as substrate for cultivation in order to provide animal feed. Various factors, such as improved lighting and different lighting cycles, humidity control and CO<sub>2</sub> regulation were taken in consideration and modified to improve yields. Cultivation of *H. erinaceus* was conducted on both *A. mellifera* wood and pecan nut shells, which proved to be a sustainable method for removal of these waste products. Factors such as humidity control resulted in high yields of *H. erinaceus* fruiting bodies. As running costs were reduced, both *G. lucidum* and *H. erinaceus* were used for the development of medicinal applied capsules in collaboration with the pharmaceutical company, Sfera Nutraceuticals.

After optimization of growth conditions, extraction methods, antimicrobial activity and toxicity assays were investigated in chapter 3. Water, alcohol and a combined extract of water and alcohol were performed on the different growth stages of *G. lucidum* and *H. erinaceus*, namely mycelia, fruiting bodies and spent mushroom substrate (SMS). Various studies have been conducted on the applications of bioactive polysaccharides. Thin layer chromatography (TLC) was applied for preliminary separation of compounds. For the identification of compounds, separation and isolation of each extract using TLC should be undertaken in future while



fingerprinting can be performed to identify and isolate compounds for further investigation. The antimicrobial activity of the extracts was investigated against 12 different pathogens. The hot water extracts showed no inhibitory effect against the various pathogens, but various factors have to be considered. The extraction times might have been too short while some of the compounds such as triterpenoids are water-insoluble and can only be extracted with alcohol. Alcohol extracts of *G. lucidum* fruiting bodies showed the best inhibitory effect. In addition, combined water and alcohol extracts were performed on the three growth phases of *G. lucidum*, with the combined extracts of *G. lucidum* fruiting bodies and spent mushroom substrate (SMS) showing the most promising results. It can be explained due to the extraction of both water-soluble and water-insoluble compounds, such as polysaccharides and triterpenoids. As a result, SMS have application as animal feed due to antimicrobial activity, thus utilizing waste. Pre-clinical and clinical trials are required to ensure safe use of herbal medicines. Human toxicity is poorly portrayed by animal models, resulting in the use of *in vitro* screening using human derived cells. Various parameters such as live/dead cells, lysosomes, mitochondrial dysfunction and steatosis can be investigated by staining cells followed by analysis with the ImageXpress Micro XLS Widefield High-Content Analysis System. Hepatotoxicity assays of the different extracts at different concentrations were performed on HepG2 cells using above-mentioned parameters. Some of the extracts showed toxicity, with alcohol extracts proving to be more toxic when compared to water extracts. This study provided interesting data on the cytotoxicity of two South African macro-fungal species, *G. lucidum* and *H. erinaceus*. As hepatotoxicity assays are considered to be novel, further investigation is required as this was just a preliminary study in order to evaluate the antimicrobial effects and whether different extracts at different concentrations are highly toxic or not to human derived cells by making use of *in vitro* screening. By taking all of the results in account, none of the extracts proved to be highly toxic, paving the way for further investigations and improving health in a natural manner.

New methods of waste disposal are required with the application of *G. lucidum* SMS as animal feed after delignification taking centre stage. The use of encroaching wood species such as *A. mellifera* following mushroom cultivation was investigated in chapter 4. Enzymes produced by mushrooms aid in the delignification process, resulting in degradation of lignocellulosic materials for growth and fruiting. Due to sufficient breakdown of lignin, *A. mellifera* wood had application as animal feed and SMS were added as supplement. Ruminants from experimental group showed improved and more consistent growth compared to the control groups fed on feed consisting only of lucern and corn. In addition, microbial analysis of stomach contents showed a significant increase in *Lactobacilli* counts for the experimental group compared to

the control groups. Additionally, the effect of animal feed supplemented with SMS on ruminants was also investigated by weight gaining, % fat, total fatty acids, fatty acid ratios and colour of meat. In general, total fatty acids and fatty acid ratios of the experimental groups were considered to be more acceptable compared to the control groups. Furthermore, consumers considered meat from the experimental groups to be more favourable when various parameters ( $L^*$ ,  $a^*$ ,  $b^*$ , hue and saturation index) were taken into consideration. As a result, meat from the experimental group were found to be the most in accordance with guidelines regarding acceptability for consumers. Consequently, the application of SMS as animal feed were considered to be successful and should be further investigated. It can therefore be concluded that feed supplemented with SMS have a positive effect when compared to traditional feed.