Comparison of *in vitro* activities of selected *Ganoderma* species in relation to skin diseases

by

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DECLARATION

In accordance with rule G5.6.3, I hereby declare that the above-mentioned dissertation is my own work and that it has not previously been submitted for assessment to another University or for another qualification.

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Abstract

Ganoderma species of macrofungi have been reported to have a multitude of medicinal properties, however, there is limited information on this genus in South Africa. The goal of this study was to compare biological activities of selected *Ganoderma* spp. in relation to skin diseases with emphasis on the antibacterial, antioxidant, anti-inflammatory and cytotoxic *in vitro* activities. Ethanolic and aqueous extracts prepared from six samples of *G. lucidum* [two commercial products (MG-LZ8 and Medi Mushroom Reishi extract), polar, antler, fruiting body and mycelium] and ten cultivated *Ganoderma* spp. were screened for bioactivities. Antibacterial activity was assessed using the 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT) assay against skin pathogens (*Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae*). Cytotoxicity was determined using bisBenzamide H 33342 trihydrochloride/propidium iodide (Hoechst/ PI) staining on the B16F10 melanoma cell line. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing ability of plasma (FRAP) assays were used to investigate antioxidant activity. Immune modulatory effects included detection of phagocytic activity and phenotypic changes of RAW 264.7 murine macrophage cells. The phenotypic macrophage polarisation of the extracts on RAW 264.7 murine macrophage cells was tested by treating the cells with the extracts and measuring the fluorescence of cell surface markers. CD86 was used as indicator for the M1 phenotype and CD206 as a general marker for M2 phenotypes. The commercially available and cultivated *G. lucidum* extracts did not show antibacterial activity within the 0-2mg/mL concentration range tested. The commercially available MG-LZ8 *G. lucidum* extract expressed cytotoxic activity with an IC_{50} value of 21.26 µg/mL while the ethanolic conk fruiting body and mycelial *G. lucidum* extract showed a significant reduction in live cells, indicating anti-proliferative activity. *G. destructans* mycelial ethanol extract displayed anti-proliferative activity at a concentration of 200 µg/mL which shows great potential as a mycelial extract. All the *G. lucidum* extracts exhibited free radical scavenging abilities with the *Ganoderma* spp. showing little to no measurable activity. *G. lucidum* and *Ganoderma* spp extracts had similar responses without inflammatory activity for the concentration ranges tested. The extracts did not increase macrophage phagocytic activity using the pHrodo™ Green *E. coli*

BioParticles™ Conjugate. *G. lucidum* and *Ganoderma* spp. extracts induced macrophage polarisation toward the M2 phenotype, with the *G. lucidum* antler and conk fruiting body extracts displaying significant activity. This study illustrates one of the first investigations of the bioactivity of indigenous *Ganoderma* spp.; *G. destructans* type and *G. eickeri*, spp. nov. identified by Coeztee *et al* (2015).

Keywords: *G. lucidum*; *Ganoderma* species; antibacterial, anticancer; macrophage polarisation, M1; M2

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List of Abbreviations

µg	Microgram
µL	Microliter
µm	Micrometre
µM TE/g	µmol trolox equivalent/ g
5-FU	5-fluorouracil
AIDS	Acquired immunodeficiency syndrome
Aqueous extracts	Aq.
$BaCl_2$	Barium chloride
BBC	Basal cell carcinoma
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BW	Body weight
Ca^{2+}	Calcium ion
CO_2	Carbon dioxide
DAMPs	Damage-associated molecular patterns
DAPI	4′,6-diamidino-2-phenylindole
ddH_2O	double distilled water
Dimethyl sulfoxide	DMSO
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EGCG	Epigallocatechin gallate
Ethanolic	EtOH
FBS	Foetal bovine serum
$Fe2^+$	Ferrous ion
$Fe3^+$	Ferric ion
$FeSO_4$	Ferrous sulfate
FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing ability of plasma
g	grams
GLP	*Ganoderma lucidum* polysaccharides
Gl-PS	Glycopeptide derived from *G. lucidum*
GLSP	*G. lucidum* spore polysaccharide
H_2SO_4	Sulfuric acid
HIV	Human immunodeficiency virus
HPAE	High pressure assisted extraction

hrs	hours
IC50	Half maximal inhibitory concentrations
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IHME	Institute for Health Metrics and Evaluation
IL-10	PDGF
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
iNOS	Inducible nitric oxide synthase
INT	p-Iodonitrotetrazolium chloride
IRF-1	Interferon regulatory factor-1
IRF-8	Interferon regulatory factor-8
ITS	Internal transcribed spacer
KLF2	Krüppel-Like factor 2
KLF4	Krüppel-Like factor 4
LPS	lipopolysaccharides
LSU	Large subunit
LTR	Lysotracker Red
M1	Classically activated pathway
M2	Alternatively activated pathway
MAE	Microwave assisted extraction
MAMPs	microbial-associated molecular patterns
mg	Milligram
Mg^{2+}	Magnesium ions
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
min	Minutes
mL	Millilitre
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCF	Macrophage colony stimulating factor
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N/A	Not applicable
N2O3	dinitrogen trioxide
NADH	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
NHLS	National Health Laboratory Services

nm	Nanometres
NMU	Nelson Mandela University
NO	Nitric oxide
O_2	Oxygen
ºC	Degrees Celsius
PAMP	pathogen-associated molecular patterns
PBS	Phosphate buffer solution
PI	Propidium iodide
PRR	pattern recognition receptors
PS	phosphatidylserine
PSG-1	*Ganoderma atrum* polysaccharide
PUP	*Polyporus umbellatus* polysaccharides
RCF	Relative Centrifugal Force
RNA	Ribonucleic acid
ROS	reactive oxygen species
SD	Standard deviation
SOC 1	Suppressor of cytokine signalling proteins
spp.	Species
SSC	Squamous cell carcinoma
SSSS	*Staphylococcal* scalded-skin syndrome
TEAC	Trolox equivalent antioxidant capacity
TEF1-α	Translation elongation factor 1 alpha
TGF-β	Transforming growth factor beta
Th1	T Helper cells type 1
Th2	T Helper cells type 2
TLC	Thin layer chromatography plate
TLR	Toll like receptors
TNF-α	Tumor necrosis factor alpha
TPTZ	Tripyridyltriazine
TRITC	Tetramethylrhodamine
UAE	Ultrasound assisted extraction
University of Free State	UFS
UP	University of Pretoria
UT	Untreated
UV	Ultraviolet
VRE	Vancomycin resistant *Enterococci*
WHO	World Health Organisation
x g	Gravity

CHAPTER 1
LITERATURE REVIEW

1.1 INTRODUCTION

The skin is the largest, most complicated organ of the body and can repair itself and act as a sensory receptor to an array of sensations (Purohit and Solanki, 2013; Maranduca *et al,* 2020). The skin is susceptible to bacterial, viral and fungal infections, injury due to wounds and burns, and skin conditions which often arise from autoimmune diseases. Bacterial skin infections diverge clinically, varying from mild superficial folliculitis to necrotizing fasciitis (Sukumaran and Senanayake, 2016). The global emergence of resistant strains of bacteria has caused the treatment of these conditions to become increasingly difficult especially with the increase in nosocomial infections caused by methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant Enterococci (VRE) and multidrug resistant *Pseudomonas aeruginosa* (WHO, 2020, Sukumaran and Senanayake, 2016).

Immunity and wound healing are closely linked to the removal of pathogens and the prevention of infections. Wounds of all sizes present a route of entry for pathogens. Wounds may become infected at varying degrees with the worst case resulting in systemic infection and death. Thus, it is important that the body is capable of efficient and effective wound healing (Velnar *et al*., 2009; Coates *et al*., 2018).

With the increase in resistance to known treatments and last line defences, the search for new and better compounds that have the potential to treat skin infections has led to the exploration of the field of natural products research. Only a small percentage of South African macrofungi have been studied, especially in relation to *Ganoderma* spp. found in Southern Africa, leaving a vast knowledge base untouched (Oke *et al.*, 2022). Bioactivities of macrofungi found around the world have been reported to include cytotoxicity, apoptosis induction, hypoglycaemic, immune modulation, anti-HIV, hepatoprotective and anti-microbial (Lu, 2020, Niego *et al*., 2021).

The goal of this study was to compare biological activities of selected *Ganoderma* spp. in relation to skin diseases with emphasis on the antibacterial, antioxidant, anti-inflammatory and cytotoxic *in vitro* activities.

1.2. SKIN DISEASE BURDEN

There is a need for research to determine the burden of skin diseases in developing or resource poor areas and finding ways to reduce it. Many skin conditions are symptoms of more severe systemic illnesses, such as: HIV, diabetes and tropical diseases (Seth *et al.*, 2017; Flohr and Ray, 2021). A guideline for HIV associated skin conditions in both adults and children has been set out by the World Health Organisation (WHO) showing renewed interest in skin diseases as a priority (Seth *et al.*, 2017; Institute for Health Metrics and Evaluation (IHME), 2017).

The largest hindrance for resource poor countries is the cost of dermatologic treatment. As these diseases are often not seen as fatal or important many people suffer due to the lack of accessibility of medication and the inadequate treatment regimens being offered (Institute for Health Metrics and Evaluation (IHME), 2017; Flohr and Ray, 2021). Skin conditions are prevalent amongst individuals living in close quarters often with a lack of ventilation and adequate hygiene, which aids in the spread of skin infections (Hay *et al.*, 2006; Seth *et al.*, 2017). Individuals who suffer from skin conditions face harsh stigmas that may lead to a range of mental illnesses such as depression and anxiety; which hinders their social interactions. In many cultural settings people with skin conditions are often cast out of family dwellings and treated unfairly (Seth *et al.*, 2017).

1.3. BACTERIAL SKIN INFECTION AND COMMON PATHOGENS

The skin functions as the exterior interface of the body allowing it to act as a physical barrier against infection and it has several defence mechanisms, which reduce the probability of pathogens colonising the human skin (Boer *et al*., 2016). These mechanisms include; the nutrient poor and acidic environment as well as being mostly desiccated which does not support the growth of many microorganisms. Despite the harsh environment the skin is colonised by a multitude of microorganisms and has

been described as an ecosystem (Grice and Segre, 2012; Niego *et al*., 2021). This is known as the normal microflora; it is found on the skin surface and epidermal layer (Figure 1.1). However, certain types of microflora can penetrate deeper into the dermal layer without being pathogenic, for example within the sweat glands (Grice and Segre, 2012).

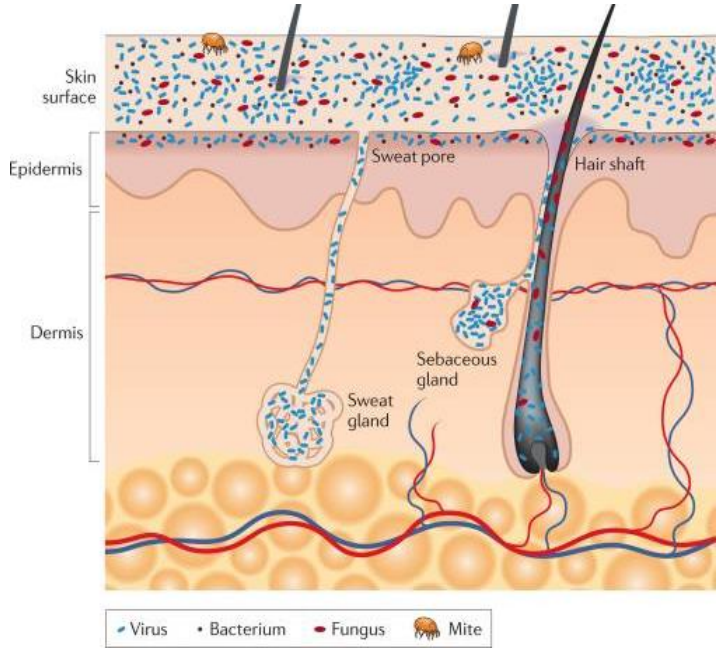

Figure 1.1: Microbial colonisation of the skin layers**.** Microbes may include; bacteria, fungi, viruses and mites. Most are restricted to the skin surface and epidermis while fungi and viruses may penetrate deeper into the dermis via sweat and sebaceous glands and hair follicles (Grice and Segre, 2012).

The area of the skin determines the type of microbial colonisation that takes place. There are several folds and invaginations that cause specialised niches. For example, the skin on the face is considered to have a thinner cornified layer, the keratinocytes contain less keratin and are not as densely packed (Boer *et al.*, 2016). The presence of several sweat and sebaceous glands and many blood vessels result in a more favourable environment for microorganisms which thrive in moist conditions and are capable of deriving nutrition from sebaceous secretions such as *Propionibacterium acnes* which causes acne (Fitz-Gibbon *et al., 2013*).

Propionibacterium spp. are also found in other highly sebaceous areas such as behind the ear, the back and forehead. These bacteria hydrolyse the triglyceride in the lipid rich sebum and release fatty acids (Grice and Segre, 2012*;* Fitz-Gibbon *et al., 2013*).

These acids decrease the pH and inhibit other skin microflora such as *Staphylococcus aureus* and *Streptococcus pyogenes*. However, coagulase negative Staphylococci and Corynebacteria can grow but are not prevalent. *Staphylococcus* spp. especially *S. aureus* and *Corynebacterium* spp. are most prevalent in moist areas such as the axillary vault (armpit), inside of the groin and the navel (Byrd *et al.*, 2018). *Staphylococcus* spp. can grow in humid areas with high salt content. The dry areas of the skin such as the appendages, with the forearms and hands particularly, have a larger diversity of microorganisms. These different organisms are often found to have lower numbers compared to the other niches of the skin as the skin conditions are harsher (Grice and Segre, 2012). The diversity can be attributed to the fact that the skin in these areas is exposed to a range of temperatures and in contact with many of the elements. Examples of bacteria found in these areas include those that belong to the Actinobacteria, Proteobacteria, Firmicutes and Bacteroidetes phyla (Chiller *et al*., 2001; Grice and Segre, 2012).

Factors which affect the diversity of the normal microflora depend on many other variables. These variables include age, gender, and ethnicity as well as several other factors shown in Figure 1.2. Age is one of the biggest contributing factors to the diversity of microorganisms (Shibagaki *et al.*, 2017). Babies are sterile while *in utero* and are colonised either during the birthing process or directly after with their microbial diversity which increases as they grow. The microbiome of the body then changes during puberty when the sebaceous glands become more active (Fitz-Gibbon *et al., 2013*). Furthermore, the microbiome develops as the individual becomes older with the elderly having a slightly different microbiome (Grice and Segre, 2012).

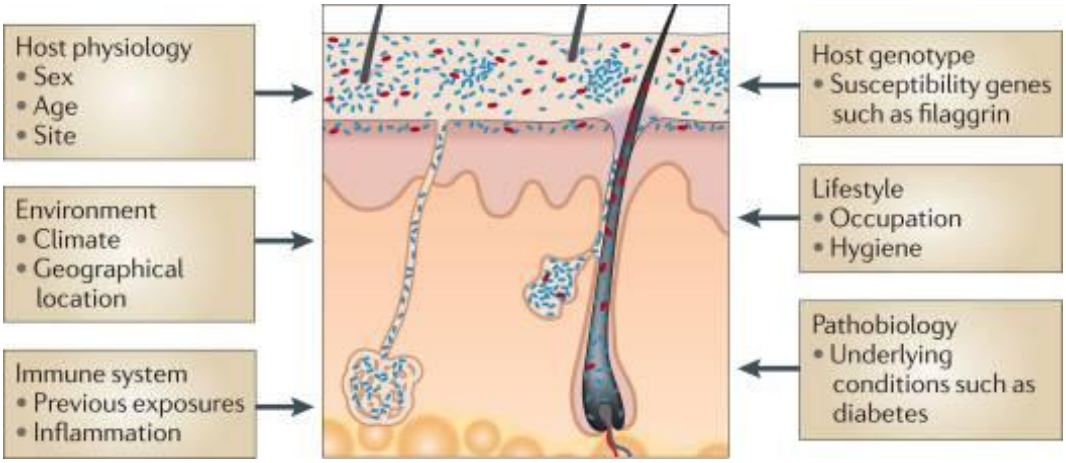

Figure 1.2: Factors that influence the colonisation and diversity of the microbiome. The factors determine the species type and amount of colonisation of certain bacteria as well as the prevention of invasion and infection by pathogenic microbes (Grice and Segre, 2012).

One of the easiest routes of infection occurs when there is a wound and the protective barrier of the skin is breached. There are five critical stages of wound infection, outlined in Figure 1.3 (Edwards and Harding, 2004). Normal microbiota as well as microbes introduced with the injury can result in contamination of the wound. Colonisation occurs when the wound is not properly sanitized and dressed allowing for microbial growth (Wysocki, 2002). Infection sets in when the microbes grow and expand to numbers greater than 10^5 colony forming units (Edwards and Harding, 2004). If not treated appropriately the infection becomes invasive; spreading and affecting surrounding areas until eventually reaching septicaemia. It is important to note that a wound is never just colonised by one bacterial species although one may be predominant (Edwards and Harding, 2004; Zabaglo and Sharman, 2022).

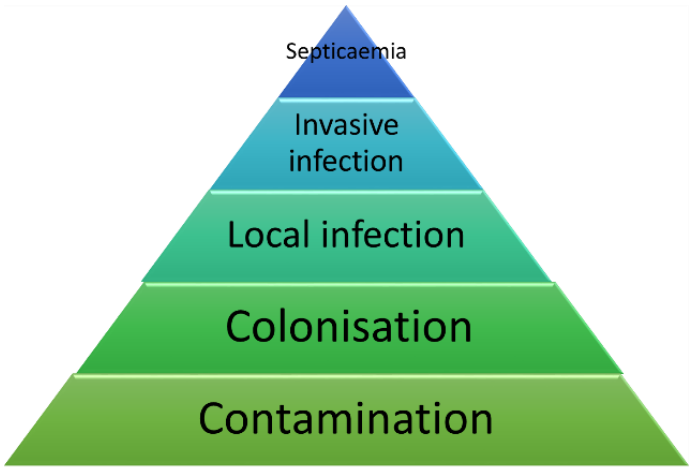

Figure 1.3: Stages of wound infection (Edwards and Harding, 2004)

1.3.1. *Staphylococcus aureus* and *Streptococcus pyogenes*

Staphylococcus aureus and more importantly MRSA are the leading causes of cutaneous and systemic infections (Turner *et al*.,2019). MRSA is a common nosocomial infection, it increases the mortality rate of hospital patients, especially those recovering from wounds and places a heavy financial burden on the hospital and patients (Turner *et al*.,2019). *S. aureus* generally colonises the skin of new-borns transiently and it may also be found in the anterior nares and skin of healthy individuals (Shibagaki *et al.*, 2017). It commonly causes superficial and deep dermal pathology in HIV-infected patients and may lead to life threatening complications (Hidron *et al*., 2010; Turner *et al*.,2019). *Streptococcus* spp. are commonly pathogenic on the skin as seen with *S. aureus*. *S. pyogenes* is the most frequent species to cause skin infections. *S. pyogenes* is not a resident skin bacterium but is reported to be asymptomatically carried in the throat of 15% of adolescents (Chiller *et al.,* 2001).

There are four main types of skin infections caused by these pathogens, namely ; epidermal, dermal, follicular and toxin mediated syndromes. Epidermal infections include infections such as impetigo and ecthyma. The first is most common in children and is highly communicable (Motswaledi, 2011). Bullous impetigo if left untreated, can cause staphylococcal scalded-skin syndrome (SSSS) which is mediated by an exfoliative toxin (Motswaledi, 2011). In ecthyma, ulcerations occur below the plaque formed in impetigo and results in scarring (Chiller *et al*., 2001).

Dermal infections such as erysipelas, cellulitis, necrotising fasciitis, are present when these pathogens infect the dermis and subcutaneous tissue. Erysipelas is caused by *S. pyogenes* which results in leucocytosis, (which is a major increase in white blood cells) and cavernous sinus thrombosis (Chiller *et al*., 2001). Cellulitis often includes complications such as lymphangitis, and if left unchecked necrotizing fasciitis and septicaemia occur. Perianal cellulitis found in young children is of special note because it results in painful defecation and bloody stool. Necrotizing fasciitis is a life threating infection of the subcutaneous tissue which can lead to necrosis of tissue after 48 hours (Gualtieri *et al*, 2021).

Follicular infections include folliculitis, furunculous and carbunculosis. *S. aureus* is the main causative agent for these types of infections. The first is the superficial infection of the hair follicle whereas the latter two are deeper set infections where carbuncles are multiple furuncles that form large abscesses (Ibler and Kromann, 2014; Winters and Mitchell, 2021). Toxin mediated syndromes of these pathogens are SSSS, toxic shock syndrome and scarlet fever. Systemic symptoms of these syndromes are mediated by super antigens (Chiller *et al*., 2001). They cause a large-scale T cell activation leading to the release of several cytokines that cause toxin induced symptoms such as strawberry tongue acral erythema and perineal erythema (Chiller *et al*., 2001; Winters and Mitchell).

1.3.2. *Pseudomonas aeruginosa*

P. aeruginosa is most commonly known for hospital acquired infection including pneumonia and ventilator acquired pneumonia (Pachori *et al*., 2019). This bacterium has shown multi-drug resistance through various mechanisms due to it having resistance genes to most of the mainstream antibiotics such as those encoding for β-lactamases (Mesaros *et al*., 2007). It is also well known to be the cause of severe wound and surgical infection and is considered an opportunistic pathogen often associated with immunocompromised or debilitated patients (Mesaros *et al*., 2007 Pang *et al*., 2019).

In terms of skin and wound infections the pathogens generally form three types of infections. The first is prosthetic material infections; this includes infections that are caused by the production of biofilms, which is a key virulence factor for the organism.

The pathogen colonises and forms a biofilm on the device and infects the patient at the point of entry of the device or where it is attached. The patients often suffer from erythema, pain and pustular discharge (Pachori *et al*., 2019). Secondly, there is the infection of burn wounds that can lead to serious complications. Burnt tissue has local immunosuppression making it more prone to infection. Biofilms are quick to form, and this raises problems with skin grafting as the grafting procedure can only begin once infection has been completely cleared (Purohit and Solanki, 2013). Infection of diabetes patients is the third most common *P. aeruginosa* infection, specifically causing severe foot ulcers (Sivanmaliappan and Sevanan, 2011). Diabetes is a key factor to delayed wound healing and *P. aeruginosa* prefers moist areas making it a large contributor to infections of wound areas and foot ulcers (Purohit and Solanki, 2013).

1.3.3. Enterococci

Enterococcus species, such as *E. faecium*, is an important nosocomial infection due to the increase in vancomycin resistance (O'Driscoll and Crank, 2015). After *S. aureus* and *P. aeruginosa* it is the leading cause of skin and soft tissue infections (Tong *et al*., 2015). *E. faecium* is a commensal organism of the gastrointestinal tract in humans and animals and may colonise asymptomatically before infection (Patel and Snydman, 2013).

It generally infects patients at the surgical site if wounds are not appropriately cleaned and dressed. It is also linked to catheter-associated bloodstream and urinary tract infections. However, the biggest concern of VRE is its role in the infection of transplant patients (Patel and Snydman, 2013; Nellore *et al*., 2019). These patients have an increased risk, compared to other infections, of mortality if they acquire nosocomial VRE bacteraemia. Nosocomial infections caused by VRE also have an increased treatment cost as it leads to prolonged hospital stays and the need for various antibiotic treatments (Patel and Snydman, 2013; Nellore *et al*., 2019).

1.4. WOUND HEALING

Wound healing takes place in four time-dependent phases (Velnar *et al*., 2009). Though the phases are time dependent the wound may be undergoing all four phases simultaneously in different areas. These phases include the coagulation and haemostasis phase followed by inflammation, proliferation and lastly wound remodelling phase presented in figure 1.4 (Velnar *et al*., 2009; de Oliveira Gonzalez *et al*., 2016).

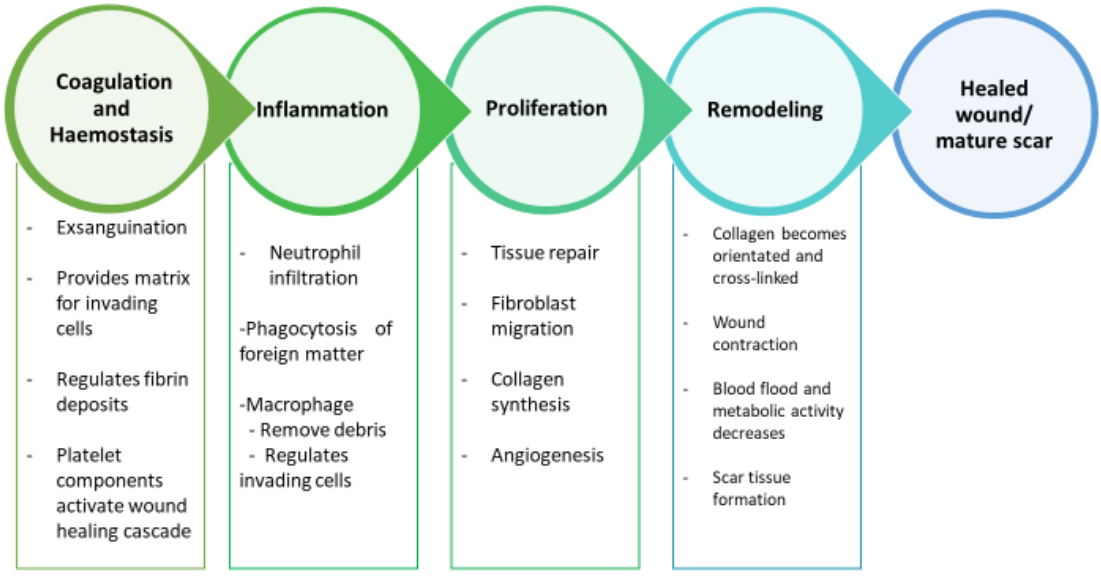

Figure 1.4: Stages of wound healing (Sinno and Prakash, 2013; de Oliveira Gonzalez, *et al*., 2016; Qing, 2017).

The first phase of wound healing is concerned with the stopping of blood flow and activating the wound healing cascade. The main function of the second phase (inflammation) is to remove foreign matter and regulate the invading cells. Proliferation phase involves tissue repair by new cells and the formation of new blood vessels. In the last phase the wound tissue becomes stronger and metabolic activity is reduced. These phases result in mature scar tissue (Velnar *et al*., 2009; Sinno and Prakash, 2013; de Oliveira Gonzalez *et al*., 2016; Qing, 2017). The process may differ in some tissues but generally follows the same course. Wound healing takes place within the wound as well as the surrounding tissue. The process is complex with interactions between varied immunological and biological systems (Velnar *et al*., 2009; de Oliveira Gonzalez *et al*., 2016).

This investigation focuses on the inflammatory phase of wound healing which has many functions, with the main function being prevention of infection. The inflammatory phase of wound healing has two phases; namely, the early and late phases (Velnar *et al*., 2009). The early inflammatory phase starts before the coagulation phase is fully complete by triggering the complement cascade and initiating molecular events which establishes the pathway for neutrophils to infiltrate the wound site. Neutrophils act as phagocytes by removing and destroying any invading bacteria as well as foreign particles and damaged tissue (Velnar *et al*., 2009; de Oliveira Gonzalez *et al*., 2016). They achieve these using proteases as well as free radical species (Yang *et al.*, 2020). An example of this is serine proteases which is a chymotrypsin-like protease that has specificity for a broad array of substrates (Stapels *et al*., 2015; Yang *et al.*, 2020). These proteases trigger reactions such as the generation of antimicrobial peptides, direct bactericide, the inactivation of bacterial virulence factors and helps form neutrophil extracellular traps (Stapels *et al*., 2015). After the duration of a few days the activity of the neutrophils gradually decreases. It is essential that the neutrophils are removed from the wound site as the failure to eliminate them may result in a perpetuated inflammatory state resulting in delayed tissue healing eventually leading to tissue damage (Velnar *et al*., 2009; de Oliveira Gonzalez *et al*., 2016). Removal is achieved by the neutrophils being extruded and being sloughed off as well as cell signalling causing apoptosis. The remnants are phagocytosed by macrophages signalling the late inflammatory phase (Velnar *et al*., 2009).

The late inflammatory phase of wound healing relies most heavily on macrophages. The macrophages last longer than neutrophils and are capable of working at a lower pH. They also play a key role in regulation of inflammation and provide tissue growth factors to activate fibroblast, keratinocyte and endothelial cell proliferation needed for the later phases (Velnar *et al*., 2009).

1.5. THE IMMUNE SYSTEM AND MACROPHAGE POLARISATION

When danger signals are triggered, either via external or internal sources it sparks the first line of defence, the innate immune system (Italiani and Boraschi, 2014). With the triggering of the innate immune system there is the recruitment of macrophages. These macrophages play a key role in the phagocytic clearing of pathogens and cell

debris. The macrophages are triggered to be activated along one of two pathways; known as the classical and alternative pathway (Roszer, 2015). Classical macrophage activation is termed the M1 pathway and the alternative pathway is known as M2 (Mosser and Edwards, 2008). The macrophage polarisation toward M1 or M2 activation is dependent on the cytokines released by Helper T cells, Th1 and Th2. (Martinez and Gordon, 2014). Table 1.1 describes activators that signal cytokines to bring about polarisation of a certain macrophage phenotype (Martinez and Gordon, 2014). If macrophages fail to polarise there are physiological effects that can lead to disease. One such example is chronic venous ulcers, where there is chronic inflammation due to the inability of macrophages to polarise from the M1 to M2 phenotype. In patients with severe burns, it is thought that a prolonged M2 macrophage activation by high levels of iron can lead to haemorrhaging and derailed tissue repair (Sica and Mantovani, 2012).

Table 1.1: Macrophage polarisation: Activators signal cytokines that bring about polarisation of a certain phenotype. Regulators ensure that neither phenotype has a prolonged reaction. Function of the phenotypes vary (Sica and Mantovani, 2012; Martinez and Gordon, 2014; Italiani and Boraschi, 2014; Roszer, 2015).

Macrophage phenotype	Activators	Regulators	Function
M1 Macrophages	Toll like receptors (TLR) ligands Interferon gamma (IFN-γ)	Interferon regulatory factors (IRF), for example IRF-1 and IRF-8 Suppressor of cytokine signalling proteins (SOC 1)	High expression levels of proinflammatory cytokines High levels of reactive nitrogen and oxygen intermediates Microbicidal (phagocytosis of microbes) Tumoricidal activity Initiation and sustaining of inflammation
M2 Macrophages	Fungal infection Immune complexes Apoptotic cells, Macrophage colony stimulating factor (MSCF) as Interleukin- IL-4, IL-13, IL-10 Transforming growth factor beta (TGF-β)	Krüppel-Like factor 4 and 2 (KLF4, KLF2) NF-κB	Removal of cellular debris via phagocytosis Producing extracellular matrix components as well as angiogenic and chemotactic factors and IL-10 Mitigate inflammatory response Promote wound healing

1.6. SKIN CANCER

Skin cancer is the leading form of cancer seen in South Africa and is second only to Australia with the highest incidence of malignant melanomas; especially within the white population. The high incidence of skin cancer is due to the exposure of year-round high solar ultra-violet light with HIV positive individuals at a higher risk (Wright *et al*., 2020). The incidence of skin cancer is estimated to increase the burden on an already strained health care system (Gordon *et al.*, 2016).

The most prevalent forms of skin cancer are represented in figure 1.5 (Gordon *et al.*, 2009). The prevalent types of cancer reported in South Africa, in white patients

followed by coloured and Asians/Indians; are squamous cell carcinoma (SCC), basal cell carcinoma (BCC) and cutaneous melanoma, with melanomas which have metastasized being the most prevalent in the black population (Gordon *et al.,* 2016; Tod *et al.*, 2019). BCC arises from basal keratinocyte cells and SCC from epidermal squamous keratinocyte cells (Cameron *et al.*, 2019). The former appears to stem more from intense short-term exposure whereas the latter is due to a more cumulative exposure. Both types tend to occur most on sun-exposed areas of the body; such as face, ears and extremities, however BCC has been reported to also occur on traumatised areas such as scars and burns (Gordon *et al.*, 2009). Melanoma is a form of skin cancer that begins in the melanocyte cells. It is less common than the other skin cancers however it is more dangerous as it has a higher risk of rapid spreading to other organs (Tod *et al.*, 2019).

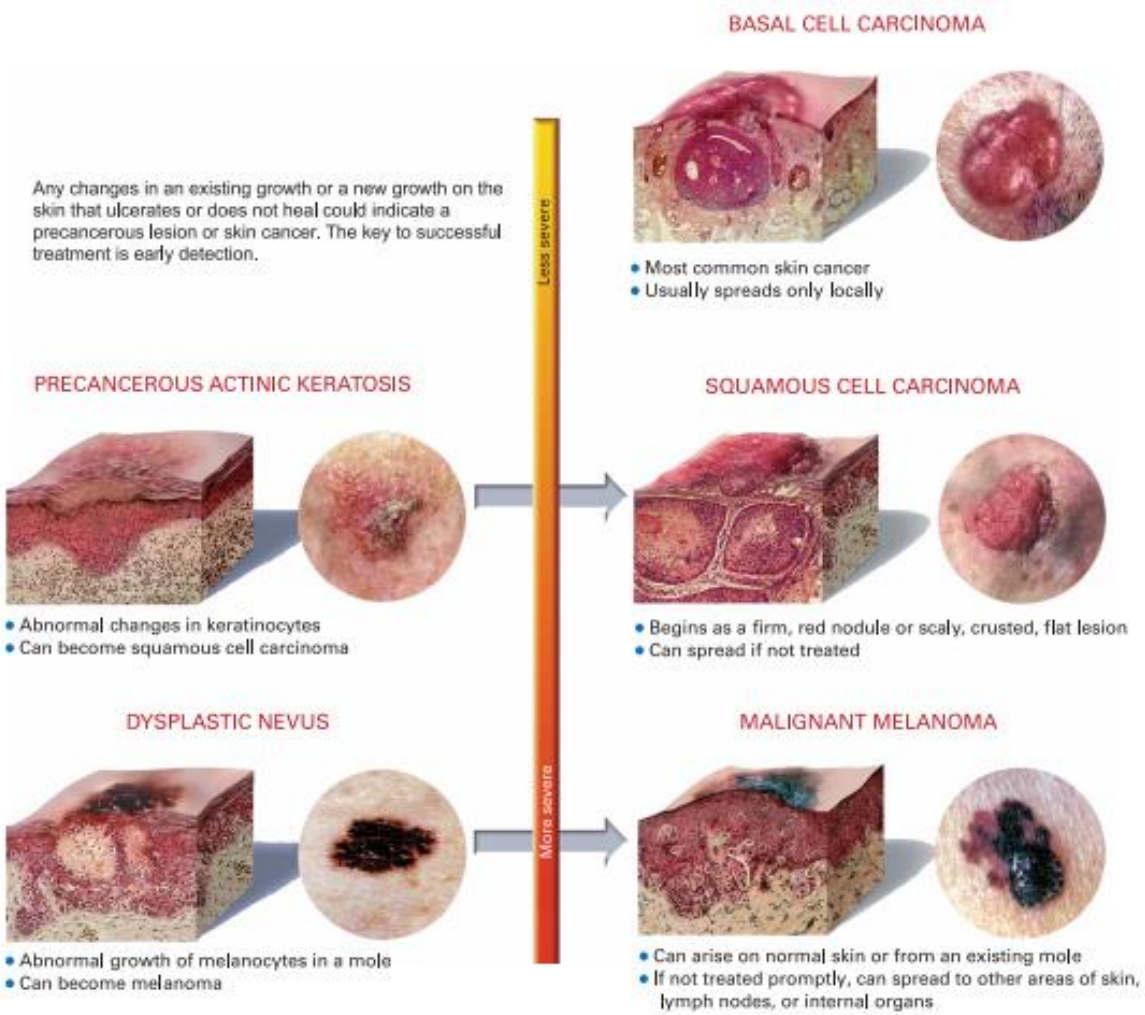

Figure 1.5: Most common forms of skin cancer; their appearance and where they occur within the dermal layers of the skin (Gordon *et al.*, 2009).

1.7. *GANODERMA* SPP.

Medicinal macrofungi have been used for a long time in traditional medicine in Asia and Africa. However, there has been a renewed interest in the use of natural products and mushrooms in the Western world (Lindequist *et al.*, 2005). Many species and their compounds remain undiscovered and need to be investigated. The mushrooms that have been studied have shown an array of biological activities (Lu, 2020).

Species of *Ganoderma* are well known for their medicinal properties and economic value. They are capable of decomposing lignin, cellulose and polysaccharides causing rot of hard wood; resulting in losses within the forest industry such as, oil palm and rubber (Hseu *et al*., 1996; Jargalmaa *et al*., 2017). Studies on *Ganoderma* spp. have revealed that they contain several bioactive components which impart anti-inflammatory, anti-tumour, antioxidant and anti-microbial activities (Hapuarachchi *et al*., 2017).

1.7.1. Taxonomy

Ganoderma spp. is a medicinal mushroom producing a group of frequently studied bioactive compounds. They belong to the kingdom of Fungi, division of Basidiomycota, class of Agaricomycetes, order Polyporales, family of Ganodermataceae and genus of *Ganoderma.* The taxonomy of *Ganoderma* spp. has been the subject of debate for decades and involved erroneous classification of different *Ganoderma* spp. as *Ganoderma lucidum*, the most prevalent *Ganoderma* spp. (Hseu *et al.*, 1996; Hapuarachchi *et al*., 2017; Hennicke *et al.*, 2016).

The genus of *Ganoderma* was originally established with *Ganoderma lucidum* as its only species. This genus grows as facultative parasites and can be found living as saprobes on the roots and lower regions of trees (Hseu *et al*., 1996; Hapuarachchi *et al*., 2017). Their sexual structures grow in the form of brackets of which there are two types depending on the species. Some species produce a laccate fruiting body with a shiny upper surface while the others have a non-laccate fruiting body forming a dull upper surface. This genus is widespread with species being found worldwide. They are capable of growth in both tropical and temperate regions (Hapuarachchi *et al*., 2017).

Regardless of the centuries of use, *Ganoderma* spp. are still being investigated. The Eurasia *Ganoderma* spp. have different phylogeny to those with the same name found in other places around the world. These differences were seen worldwide with *Ganoderma* spp. varying greatly between continents and geographic regions (Hapuarachchi *et al*., 2017). Hence, the *G. lucidum* complex was described consisting of a group of the genetically similar but not identical species, all bearing the *G. lucidum* taxonomic name with slight alterations (Hseu *et al.*, 1996; Hennicke *et al.*, 2016). Once the complex was established it became easier to identify different species of *Ganoderma*; with the knowledge of Southern Africa's species still being scarce. The taxonomy and current research will be further discussed in chapter 2.

1.7.2. *Ganoderma lucidum*

The most well-known and sought after *Ganoderma* spp. is *Ganoderma lucidum*. This species was first discovered in Asia and has been used in Asian traditional medicine for centuries. It is commonly known as Lingzi in China and Reishi in Japan. In traditional medicine, it was used to increase one's life span as well as boost youthful vigour and vitality (Mani *et al.*, 2016). Several products made from this popular mushroom are commercially available worldwide, including South Africa.

The taxonomy of the *Ganoderma* spp. is complex and many of the *Ganoderma* spp. have been misnamed due to heterogenic variation and conflicts on how the genus has been subdivided (Richter *et al*., 2014). Originally in China, *G. lucidum* was used to describe a collection of *Ganoderma* spp. from different regions (Hapuarachchi *et al*., 2017). Morphologically *G. lucidum* can be described as having a stipitate basidiocarp with the surface of the pileus being a brick coloured laccate, shiny, layer. The basidiospores tend to be ovate with a truncate apex. Spore sizes differ between regions where Europe and Africa are the most similar with the spores being smaller than those found in other regions (Hapuarachchi *et al*., 2017).

Being the most popular *Ganoderma* spp. several studies have been done to determine the pharmacological activities. Their polysaccharides, triterpenes, sterols, lectins and other proteins have been identified as bioactive compounds (Ferreira *et al*., 2015). It has shown the ability to activate immune effector cells and has shown an anticancer

effect to almost all the major cancers (Mani *et al*., 2016). Other pharmacological effects include, anti-microbial, hepatoprotective and neuroprotective activities (Ferreira *et al.*, 2015, Mani *et al*., 2016).

The recent rise in the awareness of the climate crisis and the drive to go natural has boosted the market for natural products (Mushtaq *et al.*, 2018). *G. lucidum* has infiltrated the cosmetic industry mainly as a skin-lightening agent. It is available across China, Korea and in smaller scale in the United States of America (USA), Asia and European countries (Hapuarachchi *et al.*, 2018). The lightening effect is achieved by the inhibition of tyrosinase, which plays a key role in the production of melanin. Melanin is the black pigment produced from tyrosine in epidermal melanocytes which determines the colour of skin and hair (Hsu *et al.*, 2016). Studies done by Kim *et al*, (2016) showed that ganodermanondiol extracted from *G. lucidum* significantly reduced the activity of tyrosinase and was most likely the agent responsible for the whitening effect offered by *G. lucidum*. There are several other cosmetic products containing extracts of this ancient healing mushroom; including products to increase hair growth in males, rejuvenating skin care products and those which claim to have wound healing and anti-inflammatory properties (Hapuarachchi *et al.*, 2018).

With exception of the centuries old tradition of using this mushroom as a form of health care, modern society has ensured that it will continue to boom (Mushtaq *et al.*, 2018). More than 7000 patents have been established amongst the *Ganoderma* spp. with *G. lucidum* being included in daily food such as soup, tea and wine as well as yoghurt. It is either consumed alone or in combination with herbs and berries to make soup and wine. Supplements containing the *Ganoderma* extract or powdered *Ganoderma* are sold worldwide. These supplements come in many forms, including capsules, teas and honey (Hapuarachchi *et al.*, 2018).

Despite the many advantages of *Ganoderma* spp. there are some draw backs. The largest one being economic loss of trees and perennial crops due to plant pathogenic *Ganoderma* spp., especially *Ganoderma boninense* which is the most aggressive plant pathogen of the genus (Wong *et al.,* 2012; Coetzee *et al.,* 2015). These pathogenic species are prevalent in tropical areas such as Africa, India, South Asia and tropical areas of North America. They contain lignocellulose decomposing

enzymes which weaken the structural integrity of the plants (Coetzee *et al.*, 2015). This causes the loss of forestry yields of plants and trees of economic importance such as; *Camillia sinensis* (tea), *Cocos nucifera* (coconut), *Elaeis guineensis* (oil palm) and *Hevea brasiliensis* (rubber) (Hapuarachichi *et al.*, 2018). However, their ability to decompose woody plants for nutrition could be used for bioremediation and bioenergy production (Coetzee *et al.,* 2015; Kües *et al.*, 2015).

1.7.3. *Ganoderma* spp. in Southern Africa

There are about 20 reported *Ganoderma* spp. based on morphological criteria in Southern Africa, however these species are not well documented, with only 11 being found in South Africa and only 6 of them being identified by DNA sequencing. These were *Ganoderma australe, G. enigmaticum, G. cf. cupreum, G. aff. austroafricanum, G. cf. resinaceum* and *G. destructans*. *G. austroafricanum* was once thought to be the predominant cause of root rot disease in *Jacaranda mimosifolia* in the city of Pretoria. However, Coetzee *et al*., (2015) showed that even though it was present it was not predominant in this area: they did, however, find a new species that they named *G. enigmaticum*. This species is similar in morphology to *G. lucidum* complex except the pileus is covered in a soft creamy non-poroid tissue and the basidiospores are ellipsoid. It has unique nucleotide polymorphisms at the Internal transcribed spacer (ITS) and Large subunit (LSU) gene regions.

The most prevalent *Ganoderma* spp. in the area was found to be *G. destructans*. It is similar in morphology to that of *G. enigmaticum*, however, the basidiospores are ovoid in shape and it has different culture conditions to that of *G. enigmaticum*. It can be separated based on its unique nucleotide polymorphisms in the same gene regions as *G. enigmaticum*. Figure 1.6 shows the phenotypic differences in the basidiocarp of these two species. Due to the recent discovery of these species not much research except phylogenetic studies have been done to date; to ensure that they are separate species (Coetzee *et al*., 2015). Studies by Tchotet Tchoumi *et al.,* (2019) recorded two new species found in South Africa, namely: *G. knysnamense* and *G. eickeri*. Both species were used in the present study and will therefore be one of the first investigations of their bioactivities.

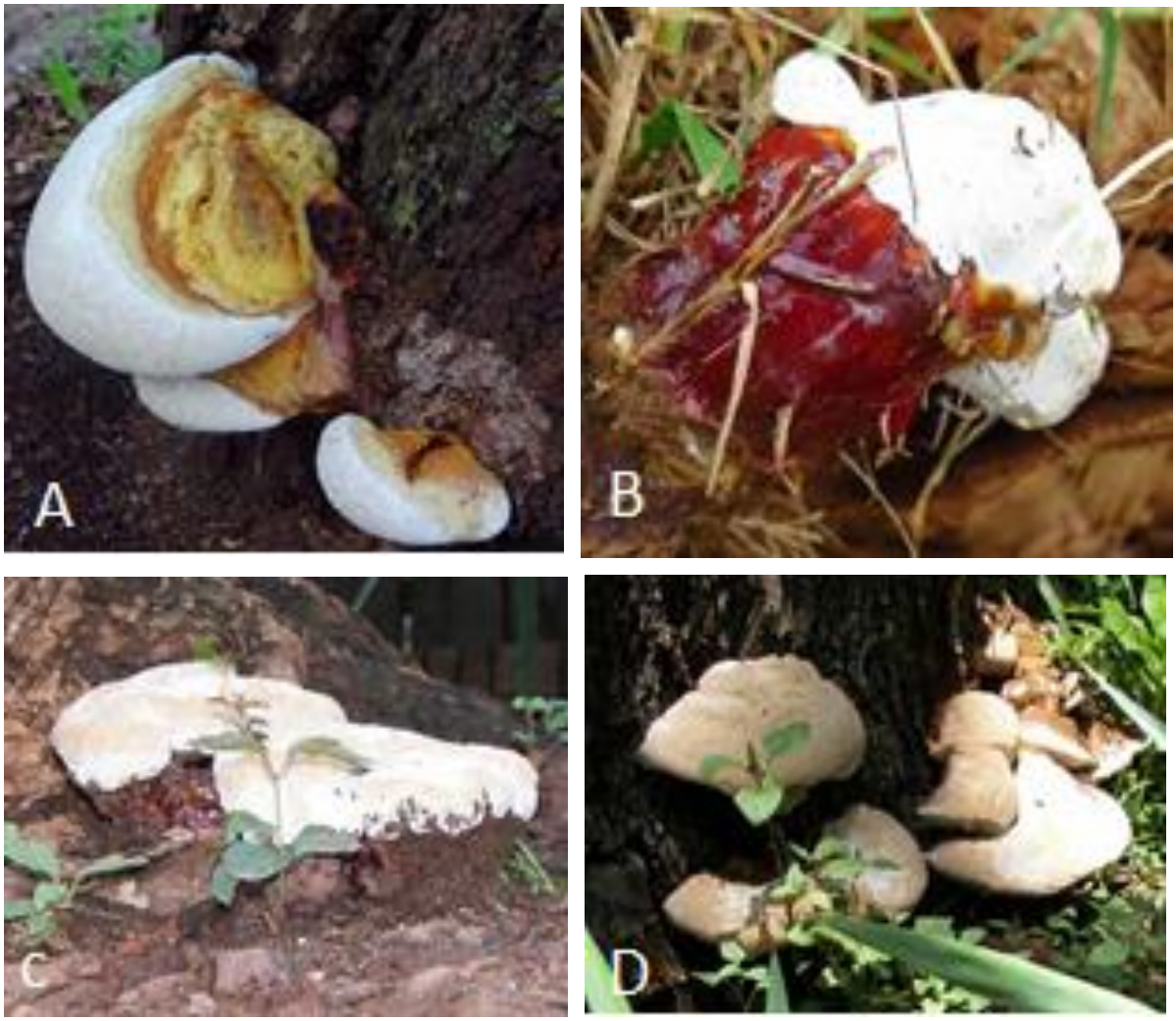

Figure 1.6: Basidiocarps of *G. enigmaticum* and *G. destructans*. [A] Young basidiocarp of *G. enigmaticum*. [B] Mature basidiome of *G. enigmaticum*. [C] Young basidiome of *G. destructans*. [D] Mature basidiocarp of *G. destructans* (Coetzee *et al*., 2015).

The bioactivities of *Ganoderma* spp. will be discussed in more detail in each chapter where relevant. This includes their cultivation, extraction, and phytochemical composition (Chapter two). The antibacterial, anti-cancer and antioxidant activities of *Ganoderma* spp. are discussed in chapters three and four respectively while chapter five investigates the immune-modulatory activity of the selected *Ganoderma* spp.

1.8. SIGNIFICANCE OF THE STUDY

Skin diseases are ranked fourth as the cause of non-fatal disease burden across the world according to the Global Burden of diseases report (Seth *et al*., 2017; Flohr and Hay, 2021). There is a need for new antimicrobials with the increase in prevalence of resistant bacteria associated with skin diseases such as MRSA, multidrug resistant *Pseudomonas* and vancomycin resistant *Enterococcus* spp. (Carpenter and Chambers, 2004). The growing population and increased resistance have made the exploration of natural sources such as *Ganoderma* spp. for bioactive agents to provide solutions pivotal.

Skin cancer research is lacking in South Africa although the risk of skin cancer to the South African population is increased due to the geographical position as well as the level of UV radiation (de Wet *et al*., 2020). Those with HIV have an increased risk of developing skin cancer and due to the prevalence of HIV-AIDS in the country the increasing demand for new chemotherapeutic agents needs to be addressed (Wright *et al*., 2020).

Although biological properties of *Ganoderma spp.* are documented throughout Europe and East Asia, there is limited information on this genus in South Africa (Tchotet Tchoumi *et al.*, 2019). The aim of this study was to evaluate and compare *in vitro* activities of South African *Ganoderma* spp. in relation to skin diseases.

1.8.1. Hypothesis

It was hypothesised that:

In vitro activities of selected *G. lucidum* commercial preparations and *Ganoderma* spp. have varying efficacy in the potential treatment of skin diseases and promotion of wound healing.

1.8.2. Objectives

Two commercial *Ganoderma lucidum* products and mycelia from ten *Ganoderma* spp. were used to:

I. Cultivate *Ganoderma* spp. and prepare extracts of cultivated species as well as selected commercial samples,

II. Detect activity against bacterial pathogens infecting skin and soft tissue (e.g., *Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa, Escherichia coli* and *Klebsiella pneumoniae*),

III. Investigate the potential cytotoxicity of the macrofungi extracts on the B16F10 melanoma cell line

IV. Ascertain the *in vitro* wound healing properties of the extracts by assessing their antioxidant activity, effects on inflammatory and phagocytotic responses and macrophage activation,

V. Compare the activities of commercially sold *Ganoderma* products with that of the cultivated *Ganoderma* spp. extracts.

CHAPTER TWO

Cultivation of *Ganoderma* spp. extracts

2.1 INTRODUCTION

The year 1881 saw the genus *Ganoderma* being established by Peter Adolf Karsten; with *Polyporus lucidus* (*Ganoderma lucidum* (Curtis) P. Karst), as the only species (Hapuarachchi *et al.*, 2018). *Ganoderma* spp. can grow in a wide range of climates worldwide, with their distribution being in both tropical and temperate regions (Tchotet Tchoumi *et al.*, 2019**).** They are basidiomycetes that are distinguished from other Polypores by having distinct double walled basidiospores. *Ganoderma* spp. live as facultative parasites and can be found on both living and dead tree trunks and branches (Henicke *et al.*, 2016; Hapuarachchi *et al.*, 2018). Most *Ganoderma* spp. are considered medicinal mushrooms and are therefore ingested. As a result of their hard fruiting bodies and bitter taste they are not considered edible mushrooms. However, they do act as an economic commodity due to their biological bioactivities and pathogenicity (Hapuarachchi *et al.*, 2018).

The taxonomy of the Ganodermataceae is often considered to be in a state of disarray with disagreement on the revision of the nomenclature, centuries after its discovery (Coetzee *et al.*, 2015; Jargalmaa *et al.*, 2017). The taxonomic studies of *Ganoderma* spp. originally relied on the analysis of the morphological characteristics for identification of the species. This process was prone to errors since the species proved to be highly variable and overlapping at the same time. The features were often attributed to the environmental conditions and the geographic origin of the species. This led to the same species found in different areas being given different nomenclature (Hapuarachchi *et al.*, 2015; Hennicke *et al.*, 2016). Following morphological identification, DNA sequencing and BLAST searches were included; however, the results were only reliable if the GenBank sequence submissions were accurately labelled (Jargalmaa *et al.*, 2017).

The *Ganoderma* spp. from the University of Pretoria (UP) that were used in this study were isolated using the method described in Tchotet Tchoumi *et al.,* (2019). Briefly,

they used the internal transcribed spacer (ITS) region to distinguish the general lineage and then species specific gene sequence regions such as the genes for ß-tubulin and Translation elongation factor 1 alpha (TEF1-α) were identified. Once all the data was analysed the differences between gene sequences were compared, using software such as MOTHUR1.38.0, BLAST and GenBank. This approach ensures that species which have similar characteristics are not correctly identified (Tchotet Tchoumi *et al.*, 2019).

Phytochemical analysis involves the identification of medicinally active substances found in natural products. These active substances include compounds such as: flavonoids, alkaloids, tannins, antioxidants, carotenoids, and phenolic compounds (Orole, 2016; Sande *et al.*, 2019). For *Ganoderma* spp. most studies report on the phytochemical analysis of *G. lucidum*. The presence and concentration of phytochemicals depend on the extraction method utilised. Shah *et al*., (2014) and Rakhee *et al.,* (2017) reported that the phytochemical composition of *G. lucidum* fruiting body and mycelium ethanol and aqueous extracts consisted of alkaloids, carbohydrates, saponins, proteins, phytosteroids, phenolic compounds and flavonoids (Table 2.1). In addition, Shah *et al.,* (2014) confirmed that extraction solvent used played a role in which compounds were present. Their study showed that methanol extraction contained the six phytochemical groups listed above while extraction by chloroform and ethyl acetate lacked certain compounds like tannins, saponins and polyphenols (Shah *et al.*, 2014).

Table 2.1: Phytochemical analysis of *G. lucidum* fruiting body (GLF) and mycelium (GLM) ethanol (Wb) and aqueous extracts (Aq) (Rakhee *et al.*, 2017).

Standard Phytochemical tests	***Observation***			
	GLF$_{Wb}$	***GLF$_{Aq}$***	***GLM$_{Wb}$***	***GLM$_{Aq}$***
1. Alkaloids				
a. Mayer's test	+ve	+ve	+ve	+ve
2. Carbohydrates				
a. Molish's test	+ve	+ve	+ve	+ve
b. Fehling's test	+ve	+ve	+ve	+ve
c. Benedict's test	+ve	+ve	+ve	+ve
3. Saponins				
a. Foam test	+ve	+ve	+ve	+ve
4. Protein				
a. Million's test	-ve	-ve	-ve	-ve
b. Biuret test	-ve	-ve	-ve	-ve
c. Ninhydrin test	+ve	+ve	+ve	+ve
5. Phytosteroids				
a. Liebermann-Burchard's test	+ve	-ve	+ve	-ve
6. Phenolic compounds and flavonoids				
a. Ferric chloride test	-ve	-ve	-ve	-ve
b. Lead acetate test	+ve	+ve	+ve	+ve
c. Magnesium and hydrochloric acid reduction test	-ve	-ve	-ve	-ve

The history of *Ganoderma* spp. stretches centuries with it playing an important role in Chinese and Japanese traditional medicine (Henicke *et al.*, 2016; Hapuarachchi *et al.*, 2018). The first use of Lingzi as an herb with medicinal value was recorded during the Han Dynasty between 206BCE- 220CE; it was described as the 'Mushroom of immortality''. It was later documented in the first Chinese Pharmacopeia as having several beneficial medicinal properties such as increase one's life span and energy levels, relieve the symptoms of asthma and used as liver tonic (Henicke *et al.*, 2016; Hapuarachchi *et al.*, 2018). Studies have demonstrated that *Ganoderma* spp. have many pharmacological effects (Jo *et al.*, 2009; Nahata, 2012; Henicke *et al.*, 2016) which will be discussed in greater detail in later chapters.

The cultivation of macrofungi requires several different growth conditions. Large scale production of *G. lucidum* fruiting body typically relies on cultivation in a mixture of sawdust and woodchips in bags or bottles (Jo *et al.*, 2013). The growth conditions often depend on the species or the place of origin of the sample (Jo *et al.*, 2009). For example, the same species from different geographical areas may require slightly different conditions for optimal growth. The optimal conditions for mycelial growth of most *Ganoderma* spp. include a temperature of between 25 - 30°C, a pH range of 5 - 9 and they can grow with a range of carbon and nitrogen sources (Jayasinghe *et al.*, 2008; Jo *et al.*, 2009). These conditions could be adjusted to produce a higher concentration of certain biological products. For example, Tang *et al.,* (2009) showed that adjusting the pH between 3 - 4.5 effectively increased the production of ganoderic acid. In the present study, the correct species of many of the isolates still had to be confirmed when they were received from the University of Pretoria (UP) and therefore it was increasingly important to grow them under constant growth conditions as the optimal conditions were unknown.

Extraction can be defined as the process of transferring a compound or compounds from a solid or liquid into a solvent or a different phase. There are several different forms of extraction; for example: solvent extraction, distillation, and sublimation (Azwanida, 2015; Truong *et al.*, 2018). Solvent extraction is by far the most common, especially for natural products and imitates extractions used in traditional medicine, which is why it was utilised in this study. Most solvent extractions follow the same process whereby the product is reduced to a crushed powder and exposed to a solvent. The solvent then ‘dissolves’ the solutes or crushed product and this results in the diffusing of the compounds found in the natural product into the solvent (Azwanida, 2015; Truong *et al.*, 2018). The objective of this chapter was to cultivate and prepare extracts from ten *Ganoderma* spp. and two commercial *Ganoderma lucidum* products.

2.2 MATERIALS AND METHODS

A variety of *Ganoderma* spp*.* samples were used in this study. Mycelial cultures were donated by Professor Coetzee from the University of Pretoria, *G. lucidum* conk fruiting body and mycelium samples were donated by Prof Viljoen from the University of the

Free State and *G. lucidum* antler samples were donated by Mushroom Guru. Commercial samples selected for this study are described in section 2.2.3.

2.2.1. Mycelial cultivation

The *Ganoderma* spp. mycelial cultures used in this study were donated by Professor Coetzee from the University of Pretoria. A PhD student from University of Pretoria used genetic techniques to identify the species, some of which were not previously described (Table 2.2) (Tchotet Tchoumi *et al.*, 2019). The cultures were grown as described by Leung *et al.,* (2006). The samples in Table 2.2 were first sub-cultured onto fresh malt extract agar plates. A small section of the original sample was placed on the agar plate aseptically and incubated at 28°C for 3-5 days in the dark, depending on the growth rate until it produced a mat of growth. These samples were then either used immediately for sub-culturing or stored at 4°C for later use. Due to the large mass needed for extraction, further sub-culturing took place in 2 L Erlenmeyer flasks. These flasks were prepared by adding 250 mL of malt extract broth (Sigma Aldrich). The flask containing the broth was sterilised by autoclaving. As before a section of the mycelial mat was transferred from the plate to the flask aseptically using tweezers. They were then incubated at room temperature in the dark for the duration of 14-21 days. The resulting mycelial mats were then placed on a clean dry surface and allowed to air dry before preparation of extracts. This process varied in duration depending on the density of the fungal mats.

Table 2.2: Isolates received from the University of Pretoria, identified by DNA sequencing and molecular analysis of the ITS gene sequences for *Ganoderma* spp. from South Africa (Tchotet Tchoumi *et al.*, 2019).

Isolate	Identity	Origin/ Province
CMW47760	*G. applanatum* [1]	Garden Route National Park
CMW47758	*G. applanatum*[2]	Garden Route National Park
CMW43670	*G. destructans* type	Gauteng
CMW25884	*G.* aff*. austroafricanum*	Limpopo
CMW49705	*G. eickeri,* spp. nov.	Limpopo
CMW49711	*G.* cf*. resinaceum* [1]	Gauteng
CMW50326	*G.* cf. *resinaceum*[2]	Kwa-Zulu Natal
CMW47755	*G. knysnamense,* spp. nov Type[1]	Garden Route National Park
CMW45100	*G. knysnamense,* spp. nov Type[2]	Garden Route National Park
CMW48134	*G.*cf*. cupreum*	Garden Route National Park

1, 2 – Denotes species which have different accession numbers on GenBank according to their ITS, ß- Tubulin and TEF1-α gene regions.

2.2.2 Extract preparation

Mycelia from *Ganoderma* spp. underwent a crude extraction using 80% ethanol. The commercial sample (2.2.3) underwent ethanol and aqueous (double distilled water; ddH_2O) extraction. The cultivated *Ganoderma* spp. resulted in a low yield of dried sample thus only ethanol extraction was used; the ethanol extraction was chosen as it was expected to yield extracts with higher bioactivity compared to the aqueous. In both the ethanol and aqueous extraction protocols the dry samples were submerged in liquid nitrogen and crushed to a fine powder using a mortar and pestle. The crushed samples were then weighed and placed in clean glass bottles. The aqueous extract ratio was 10 mL of ddH_2O to every 1g of crushed material. The ethanol extract was prepared at a ratio of 15 mL of 80% ethanol to 1g crushed sample. The extraction process (ethanol and aqueous) occurred over 24 hrs with constant stirring at room temperature (25±2°C) in the dark. The samples were decanted into 50 mL falcon tubes and centrifuged at 3000 rpm for 5 min. Supernatant was removed and filtered twice through Whatman no. 4 filter paper. The aqueous extracts were frozen at -80°C and lyophilised. The ethanol was evaporated at 60ºC using a BUCHI Rotavapor R-210 rotary evaporator (Switzerland). The ethanol extracts were freeze-dried using a Vertis SP scientific sentry 2.0 freeze dryer (Gardiner, NY, USA) and stored at 4°C in a desiccator in the dark until further use.

The yield of the extraction was calculated using the following formula:
(Uddin *et al.*, 2019)

Percentage yield = $\frac{Mass\ of\ extract\ (mg)}{Mass\ of\ dry\ matter\ (mg)} \times 100$

2.2.3 Commercial samples

At first the commercial samples were sourced from several different outlets including online stores. All these products claimed to contain *G. lucidum* crushed powder or extract, usually marketed as Reishi or Lingzi mushrooms. The products were investigated for their contents according to the packaging and ingredients listed. After consideration certain products were excluded due to factors such as: (i) the amount of extract or crushed powder could not be quantified accurately given the provided information and (ii) the presence of undefined fillers and preservatives.

One of the two commercial samples included in the study was the product Medi Mushroom Reishi Capsules shown in Figure 2.1(A). The product was purchased from the Faithful to Nature online store with each bottle containing 60 capsules (Available at https://www.faithful-to-nature.co.za/medi-mushrooms-Reishi-capsules). Each capsule contained 250 mg of *G. lucidum* crushed powder according to the packaging. The crushed powder was enclosed in a gelatine capsule which was removed, and the powder weighed. The crushed powder was then extracted according to the procedure described in 2.2.3. Aqueous and ethanolic extracts were produced.

The second commercial sample Ganoderma Gold was provided to us via Mushroom Guru™ (Pty) Ltd, Figure 2.1(B). The product contained a patented mixture of aqueous (polar) and ethanol (non-polar) extracts registered as MG-LZ8. The extracts were prepared from *G. lucidum* antlers grown by Mushroom Guru (Pty) Ltd. The antlers were grown under special conditions that differ from the general growth conditions used to produce conk fruiting bodies. The antlers are grown in conditions where the ventilation is controlled, and the carbon dioxide produced by the mushroom respiration can be controlled. The light conditions determine the shape and colour of the antler fruiting bodies; in darker conditions, they tend to grow towards the light, creating the elongated shape seen in Figure 2.1(C) (Jo *et al.*, 2013; Sudheer *et al.*, 2018). Mushroom Guru (Pty) Ltd provided us with a sample of their MG-LZ8 extract (polar

and ethanol combined), their Polar extract and a sample of their antlers Fig 2.1 (C-D). They did not have any of their ethanol extract available at the time. The *G. lucidum* antler fruiting bodies were extracted with water and ethanol using the protocol outlined in 2.2.2. It should be noted there was no monetary compensation for work done nor were the extracts and samples purchased from the company. The agreement was that results from the study concerning their product will be made available to the owners. All information about their product can be viewed on their website MushroomGuru; https://www.mushroomguru.co.za/.

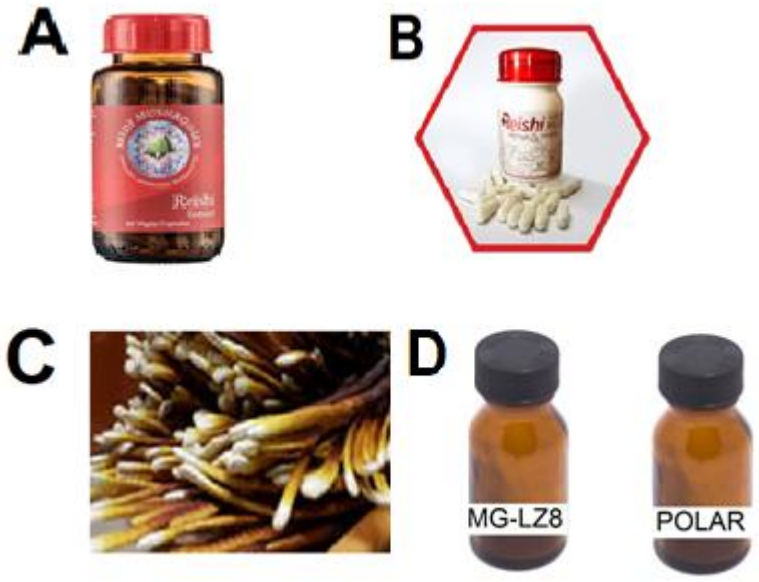

Figure 2.1: [A] Commercial sample Medi Mushroom™; [B] Commercial sample Ganoderma Gold™ Reishi capsules; [C] Antler fruiting bodies provided for the study; [D] Patented MG-LZ8 (combination of ethanol and aqueous extracts) and aqueous Polar extracts donated by the owners of Ganoderma Gold (Mushroom Guru (Pty) Ltd).

2.3. RESULTS

2.3.1 Cultivation of *Ganoderma* spp.

Figure 2.2 depicts selected examples of cultivated mycelia. Ten of the 16 samples were successfully cultivated while six of them were unsuccessful. This could possibly be attributed to suboptimal growth conditions for these species. The mycelial mats varied in colour and density with the duration of their growth varying between species. Most mycelial mats appeared white and fluffy as those seen in Fig. 2.2(A), while a few had a darker appearance such as *G*. cf*. resinaceum* in Fig. 2.2(B).

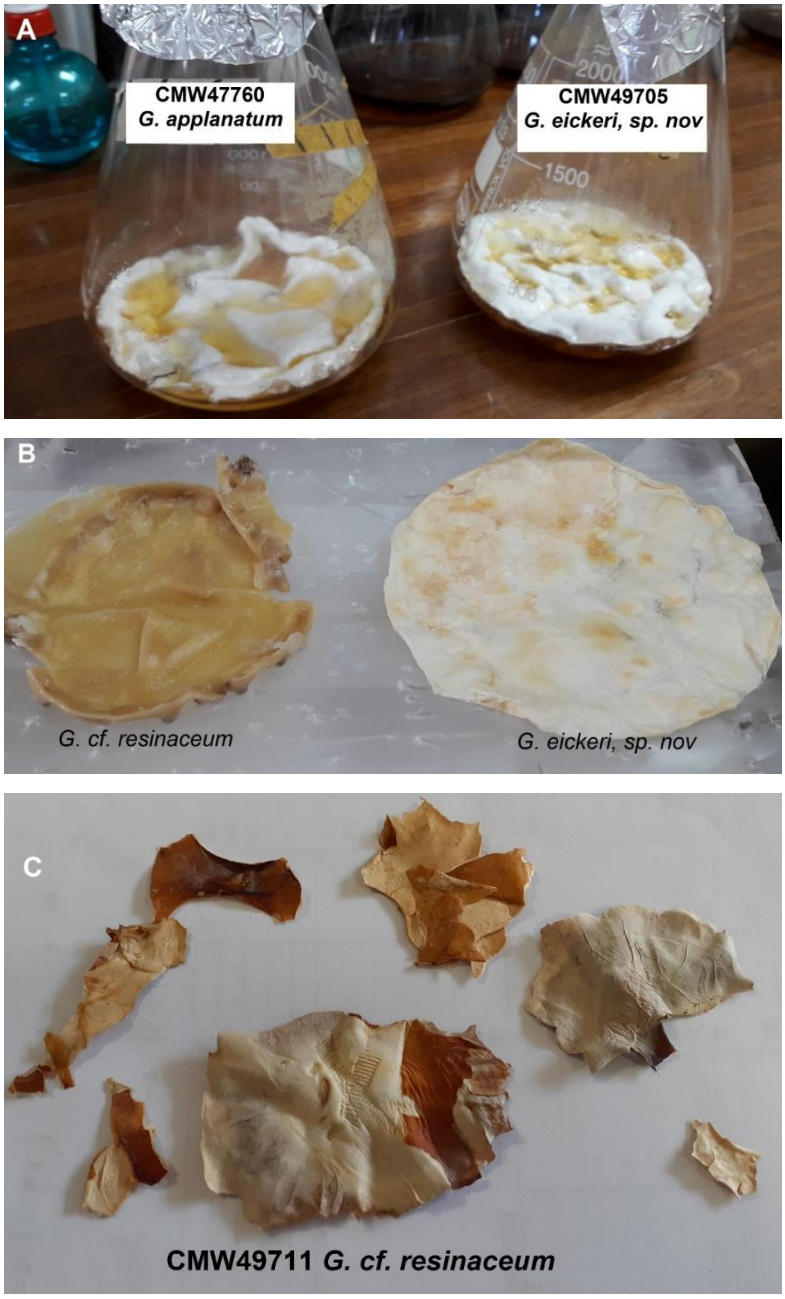

Figure 2.2: Cultivation of *Ganoderma* spp. mycelia. [A] Cultivation of CMW47760 *G. applanatum* and CMW49705 *G. eickeri,* spp. nov. in 2 L Erlenmeyer flask. [B] Mycelial mats removed after 21 days and placed on a parafilm layer with holes for moisture removal and the samples to air dry. [C] an example of CMW49711 *G.* cf. *resinaceum* air dried mycelium before extraction.

2.3.2 Extraction yield

When comparing the percentage yield for the different samples, the *G. lucidum* antlers [Mushroom Guru (Pty) Ltd] had the highest yields in both ethanol (11.6%) and water (8.34%), followed by Medi Mushroom capsules. According to the product label, these capsules contained pure, crushed *G. lucidum* without any additives and it is likely that

antlers were also used in their preparation, hence the high yield overall. Ethanolic extracts produced overall higher yields compared to the aqueous extracts. The yields from mycelia were lower than that of antlers and fruiting bodies (Table 2.3).

Table 2.3: *Ganoderma* extracts used in this study and percentage yield for the ethanolic (EtOH) and aqueous extracts (Aq.).

#Extract No./ Abbreviation	Extract	Sample Origins	Type of extract	% Yield (m/m)
1 (MG-LZ8)	*G. lucidum*	Prepared extract donated by MushroomGuru (Pty) Ltd	Mixture of ethanolic and hot aqueous	*N/A
2 (Polar)	*G. lucidum*	Prepared extract donated by MushroomGuru (Pty) Ltd	Aqueous	*N/A
3 (Ant EtOH)	*G. lucidum* – Antler fruiting body	Donated by MushroomGuru (Pty) Ltd	Ethanolic	11.6
4 (Ant Aq.)			Aqueous	8.34
5 (FB EtOH)	*G. lucidum* – Conk Fruiting body	Donated by UFS	Ethanolic	5.42
6 (FB Aq.)			Aqueous	3.42
7 (Myc EtOh)	*G. lucidum* – Mycelium	Donated by UFS	Ethanolic	4.37
8 (Myc Aq.)			Aqueous	2.98
9 (MM EtOh)	*G. lucidum* – Medi Mushroom Reishi capsules	Purchased from Faithful to Nature	Ethanolic	10.5
10 (MM Aq.)			Aqueous	5.11
11	*G. applanatum* [1]	Cultivated	Ethanolic	3.67
12	*G. applanatum*[2]	Cultivated	Ethanolic	3.58
13	*G. destructans* type	Cultivated	Ethanolic	2.32
14	*G.* aff*. austroafricanum*	Cultivated	Ethanolic	2.42
15	*G. eickeri,* spp. nov*.*	Cultivated	Ethanolic	1.36
16	*G.* cf*. resinaceum* [1]	Cultivated	Ethanolic	3.45
17	*G.* cf*. resinaceum*[2]	Cultivated	Ethanolic	3.24
18	*G. knysnamense,* spp. nov Type[1]	Cultivated	Ethanolic	2.35
19	*G. knysnamense,* spp. nov Type[2]	Cultivated	Ethanolic	2.43
20	*G.*cf*. cupreum*	Cultivated	Ethanolic	2.85

1, 2 – Denotes species which have different accession numbers on GenBank according to their ITS, ß- Tubulin and TEF1-α gene regions.

- Reference number of extract samples for use in upcoming chapters

*N/A - Not applicable as the prepared extracts were provided by Mushroom Guru (Pty) Ltd

Samples 11-20: mycelium cultures were donated by UP.

2.4 DISCUSSION

Natural products have been the backbone of medicine since their use in ancient traditional medicine; providing the active ingredients of thousands of pharmacological drugs in modern industry (Hennicke *et al.*, 2016, Zhang *et al.*, 2018). The first form of extraction in traditional medicines was often the production of tea/ infusions. The natural products would be steeped in warm or boiling water and left to diffuse into the water and strained afterwards. The product would either be drunk immediately or left to ferment with other products (Hapuarachchi *et al.,* 2018). Extraction evolved through the centuries and has now become a standardised process whereby targeted extraction can be used to extract specific compounds. There are several factors which affect the quality and outcome of the extract (Zhang *et al.*, 2018). These include the starting material of the sample, the solvent used and the extraction procedure (Pandey and Tripathi, 2014; Azwanida, 2015).

The antler fruiting body extracts (3 and 4) had the highest yield of all the extracts with 11.6 % and 8.34% for ethanol and water, respectively, while Medi mushroom Reishi capsules (9 and 10) had a yield of 10.5% and 5.11%. This was followed by the conk fruiting body (7 and 8), with 5.4% for the ethanolic extract and 3.42% for the aqueous. The lower yield of the conk compared to the antler fruiting body may be due to the hard outer covering along the surface of the conk. This hard covering was not only harder to macerate and crush than the antler but may contain insoluble material. A study by Hoq *et al.*, (2016) reported, in this case the conk fruiting body was older than that of the antler and may have negatively affected the extraction yield that the yield of *G. lucidum* was greatly affected by the maturity of the mushroom. The lower yield of the mycelium extracts was expected as the fruiting bodies formed a powder after maceration and crushing, similar to the Reishi capsule. In contrast the ground mycelium formed smaller coarse samples of varied sizes. The powdered samples resulted in smaller particles increasing the surface area in contact with the solvent. The increased surface contact increases efficiency of extraction, therefore the larger coarse particles of the mycelium would have had a less interactive surface area and decreased efficiency of extraction (Azwanida, 2015; Truong *et al.*, 2018).

The difference between the extraction yield of the cultivated samples could be attributed to the different textures of mycelial samples. Since the culture conditions of the mycelium were being kept constant between all samples, some isolates were not grown at their optimal conditions which may have affected the extraction yield (Jo *et al.*, 2009). As a result, some mycelia were more robust than others. The thicker mycelial mats were air dried to form harder sheets which were easier to crush and resulted in smaller particle sizes and increased yield. However, those which formed thinner, fragile mycelial mats with thin sheets broke apart easily but did not crush well, leaving larger particle sizes with reduced extraction efficiency yield (Azwanida, 2015; Truong *et al.*, 2018).

The solvent was chosen based on cost efficiency and ease of use. In addition, these solvents emulate the type of solvents that would be used in traditional medicine. Traditional medicine generally utilises water extraction or tinctures, which are a form of ethanol extraction (Hapuarachchi *et al.,* 2018). They are also less likely to complex or dissociate compared to other solvents and do not pose a health hazard such as chloroform or ether (Pandey and Tripathi, 2014). Both aqueous and ethanolic extraction allow for the release of most of the phytochemicals known to be present in *Ganoderma* spp. (Table 2.4) (Pandey and Tripathi, 2014). The higher yields for the ethanolic extracts can be expected as the 80% ethanol would extract a wider range of compounds, from polar to relatively non-polar, compared to water, which would only extract polar compounds (Truong *et al.*, 2018).

Table 2.4: Active components extracted by different solvents (Pandey and Tripathi, 2014).

Water	Ethanol	Methanol	Chloroform	Ether	Acetone
Anthocyanins	Tannins	Terpenoids	Terpenoids	Alkaloids	Phenol
Starches	Polyphenols	Saponins	Flavonoids	Terpenoids	Flavonols
Tannins	Polyacetylenes	Tannins		Coumarins	
Saponins	Flavanols	Xanthoxyllines		Fatty acids	
Terpenoids	Terpenoids	Quassinoids			
Polypeptides	Sterols	Lactones			
Lectins	Alkaloids	Flavones			
		Phenones			
		Polyphenols			

The results obtained from this study indicated that the extraction yield of samples is reliant on external factors such as the solvent used in the extraction process and the type of sample extracted. The most common form of extraction is solvent extraction. In this type of extraction the solvent used and the number of follow up extractions using the same or different solvents determine the compounds extracted (Ferreira *et al*., 2015). Practicality plays a large role in the choice of extraction; such as, the availability of equipment, solvents, the safety and the overall cost of the extraction process. Certain extractions such as microwave (MAE), ultrasound (UAE), and high pressure - assisted extraction (HPAE) used in the extraction of polyphenols are expensive and require specific equipment (Dobrincic *et al*., 2020).

There are many extraction methods which yield extracts with different compound profiles, for example table 2.4 portrays the various compounds extracted when using different solvents (Pandey and Tripathi, 2014). The samples growth condition needs to be taken into consideration as well, for example macrofungi which are grown in mycelial forms are harder to macerate than fruiting bodies due to the mycelia being soft and pliable. The mycelial maceration results in a larger particle size compared to what one would receive from fruiting body maceration therefore reducing the solvent to particle interaction which is needed to increase the yield of extraction (Azwanida, 2015). The effect of different starting material can be seen in this study, table 2.3, as there was a clear difference in the extraction yield of the mycelial samples (7,11-20) and the fruiting bodies (4-6), with the latter having a noticeably higher extraction yield.

The crude extraction performed in this study aimed to isolate non-specific compounds in the most natural form. For this investigation, the choice of aqueous and ethanolic

solvents were used as they mimic methods used in traditional medicine and can isolate the compounds reported to have bioactivity in *Ganoderma* spp., such as polyphenols and terpenoids (Pandey and Tripathi, 2014; Hapuarachchi *et al.*, 2018). Due to the time constraints of the project mycelial growth was the primary form of cultivation however for future studies a different cultivation approach may be more favourable. For example using the bed log or billet method for fruiting body, whereby the *Ganoderma* is cultured on a bed of sawdust or a wood log under controlled environmental conditions (Bijalwal *et al*., 2021). These culture methods lead to fruiting body formation which have a higher extract yield as seen in this study and other studies such as those of Azwanida, (2015) and Truong *et al.*, (2018). However, this is a longer process and time limitations need consideration (Hapuarachchi *et al.*, 2018). A different approach would be to optimise the growth conditions, such as creating a temperature and light stable environment with an optimal carbon to nitrogen ratio medium. However, since the species of the macrofungi samples were still being confirmed during the growth stages, the cultivation process had to be unified. Therefore, in future studies it would be advised that the species identity be determined first in order to ascertain and optimise a growth plan for each species. The solvent used in extraction is important and for future studies methanol would be advised as it is used to extract small molecules from mushrooms and it extracts most compounds known to be present in *Ganoderma* spp., table 2.4 (Carvajal *et al.*, 2012; Pandey and Tripathi, 2014).

In conclusion the extraction yield of the *Ganoderma* spp. extract is dependent on cultivation and the method of extraction used for each sample. Samples with different morphology and chemical composition should be cultivated and extracted using methods which best suit individual samples in order to achieve the best extraction yield for crude extraction. The chapters that follow will assess the antibacterial and wound healing properties of the crude extracts discussed above.

Chapter 3
Antibacterial activity of *Ganoderma* spp.

3.1 INTRODUCTION

The move to natural product research in terms of antimicrobial agents has heightened recently due to the global emergence of resistance. Methicillin resistant *Staphylococcus*, multi-drug resistant *P. aeruginosa* and vancomycin resistant Enterococci are of importance due to their transmission in the hospital setting. They are the frequent cause of nosocomial infections, causing harmful effects on immuno-comprised patients (Stulberg *et al.*, 2002, Sukumaran and Senanayake, 2016, Esposito *et al.*, 2017). The increase in drug resistance has led to a demand for new antibiotic treatments. Often treatment regimens rely on the combination of known antibiotics, which can be effective but also lead to many side effects (Stulberg *et al.*, 2002). Research on antimicrobial activities of natural products such as macrofungi would be a useful alternative to treatment of infections with drug resistant bacteria. There could be compounds from macrofungi which could act synergistically with known antibiotics (Stulberg *et al.*, 2002; Moussaoui and Alaoui, 2016).

Staphylococcus aureus, Streptococcus pyogenes, Pseudomonas aeruginosa and Enterobacter spp. represent the major skin and soft tissue pathogens (Sukumaran and Senanayake, 2016). Table 3.1 provides a summary of pathogenic bacteria and the major infections they cause. Antibiotic resistance has increased over recent years and caused increased mortality rates and thus increased the burden of cost on the health care systems (Stulberg *et al.*, 2002; Esposito *et al.*, 2017).

Table 3.1: Antibiotic resistance of common skin pathogens and their infections (Stulberg *et al.*, 2002; Sukumaran and Senanayake, 2016, Esposito *et al.*, 2017).

Bacteria	Prevalent skin infection/ disease	Source of infection	Resistance
Staphylococcus aureus	Nosocomial infection Impetigo Ecthyma Staphylococcal Scalded skin syndrome Cellulitis Boils and carbuncles Necrotising Fasciitis	Resident microbe on the skin Present in nasal nares Improper hygiene practices	Methicillin
Streptococcus pyogenes	Erysipelas Impetigo Ecthyma Cellulitis Boils and carbuncles Necrotising fasciitis	Direct contact with wounds, cuts and nasal discharge of infected individual	Erythromycin
Pseudomonas aeruginosa	Prosthetic material infection Burn wounds Mostly infects diabetic patients	Biofilms on surgical equipment Improper hygiene	All major β-lactam antibiotics
***Enterococcus* spp.**	Nosocomial infection, specifically surgical site infections Catheter associated infections	Improper hygiene contamination from the gastrointestinal tract	Vancomycin

Commercially sold Reishi products claim to have antimicrobial activity against a wide range of microorganisms; including viruses, fungi and bacteria (Quereshi *et al.*, 2014). Antimicrobial activity has been reported for a range of *Ganoderma* spp. with *G. lucidum* being the most widely studied (Kamble *et al.*, 2011). The compound inferring antimicrobial activity has yet to be elucidated. However, more than 150 triterpenes and 50 polysaccharides have been isolated and are therefore thought to be responsible for most bioactivity; either individually or in conjunction with each other (Quereshi *et al.*, 2014).

The efficacy of the antibacterial activity of tested *G. lucidum* extracts fluctuates in research. Differences are due to growth conditions, the extraction solvent and the assays used for antimicrobial activity determination (Shah *et al.*, 2014, Quereshi *et al.*, 2014). Eo *et al.*, (2000) documented antiviral activity against Herpes simplex virus while methanolic *G. lucidum* extracts had fungicidal effects against biofilm producing

species of *Candida* (Bhardwaj *et al.*, 2021). Ahmad *et al*., (2021) displayed that triterpenoids from *G.lucidum* produced antiviral effects through inhibiting various enzymes like neuraminidase and HIV-protease.

There are several methods to determine the antibacterial activity of natural products and compounds. A few of the most common assays include agar well diffusion, agar disc diffusion, the microbroth dilution method and the less common bioautographic method (Tenover, 2017). The agar well, disc diffusion, and bioautographic methods are qualitative as they are used to determine the presence or absence of antibacterial activity. The microbroth dilution method is quantitative as it can determine the minimal inhibitory concentration (MIC) (Valgus *et al.*, 2007).

The agar diffusion method relies on the principle of diffusion of the natural product or compound into the surrounding agar (Tenover, 2017). Firstly, a bacterial culture of a known concentration is spread on the surface of the agar. A well is punctured in the agar and the natural product or compound is placed in the well or impregnated on a disk which is placed on the surface of the agar containing the bacterial culture (Valgas *et al*., 2007; Hudzicki, 2009). If there is antibacterial activity there will be a clear zone around the edge of the well or disk where the bacteria were unable to grow, this is known as the zone of inhibition (Dahiya and Purkayastha, 2012). This assay is adequate for screening of antibacterial activity, however, it is not considered accurate for the determination of the MIC of natural products or compounds (Valgas *et al*., 2007).

In the direct bioautographic method, the natural product extract is applied to a thin layer chromatography plate (TLC) and developed to separate the extract into different compounds. The developed TLC plate is covered in a bacterial suspension, usually by spraying or dipping, and is loaded into a hermetically sealed polyethylene box and incubated (Choma and Grzelak, 2010; Dewanjee *et al*., 2015). After incubation, the TLC plate is sprayed with a growth detection dye such as p-Iodonitrotetrazolium chloride (INT) and incubated again. The incubation period for the bacterial suspension depends on the microorganism and the duration for the development of the dye depends on the temperature at which it is incubated (Dewanjee *et al*., 2015). Once the process is complete antibacterial activity is presented as a zone of inhibition. The

drawbacks of this assay include being a complicated and expensive procedure utilising specialised equipment needed for chromatography (Valgas *et al*., 2007; Choma and Grzelak, 2010).

The microbroth dilution method is considered as one of the most accurate forms of determining the MIC of natural products and compounds. The principle of this method relies on the addition of a specific concentration of the natural product or compound to the standardised culture in a 96 well microtiter plate (Valgas *et al*., 2007; Tenover, 2017). The product is diluted serially in the culture media before addition of the culture. It is then incubated for 24 hr and a growth detection dye is added, such as p-Iodonitrotetrazolium chloride (INT) (Valgas *et al*., 2007). INT is a tetrazolium dye used to measure the metabolic activity of cells (Berridge *et al.*, 2005) and was used to determine the antimicrobial activity of the extracts in the present study. This tetrazolium dye acts as an electron acceptor and when reduced it produces a formazan dye. The assay relies on the colour change from yellow (dye) to a pink/purple formazan compound. The colour change indicates the reduction of the dye. Tachon *et al*., (2009) suggest that NADH dehydrogenases found in active cells are most likely the reducing agent. The intensity of the formazan product is directly proportional to the number of active cells (Eloff *et al*., 1998, Lall *et al.*, 2013). The MIC is determined as the lowest concentration inhibiting growth, therefore where no colour change is detected. The advantage of this assay is that it is possible to test several samples with a wide concentration range using relatively inexpensive materials (Valgas *et al*., 2007).

This chapter focuses on the screening of the first ten extracts of *G. lucidum* from table 2.3 for antibacterial activity against common skin pathogens.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains

Clinical strains of common skin bacteria from the National Health Laboratory Service (NHLS) in Port Elizabeth were used for the antibacterial activity assays. These included Gram-positive (*S. aureus* and *S. pyogenes)* and Gram-negative (*P. aeruginosa, E. coli* and *K. pneumoniae*) bacteria. The bacterial cultures were maintained by growing them on selective media (MacConkey, blood agar and Brain

Heart infusion agar) at 37°C for 24 hrs while long term storage was on Microbank beads (Davies Diagnostics) at -80°C.

3.2.2 Control antibiotics

Gentamicin sulphate and vancomycin hydrochloride (Sigma, USA) were used as positive controls against Gram-negative and Gram-positive bacteria, respectively. The antibiotics were dissolved in double distilled water at stock concentrations of 2 mg/mL and filter sterilized (0.2 µm filter). Working concentrations of the antibiotics were prepared in Mueller-Hinton (MH) broth.

3.2.3 Macrofungal extracts

The following extracts, numbered from table 2.3, were tested; MG-LZ8 (1), polar (2), *G. lucidum* antler fruiting body (3 and 4), conk fruiting body (5 and 6) and mycelium (7 and 8) from the UFS as well as the Medi Mushroom Reishi extract (9 and 10). Cultivated mycelium extracts were not tested for antibacterial activity due to the low extraction yield. The ethanol extracts were dissolved in dimethyl sulfoxide (DMSO) and the aqueous extracts in double distilled water at a stock concentration of 100 mg/mL. Working concentrations were prepared at 4 mg/mL in MH broth.

3.2.4 Microplate p-Iodonitrotetrazolium chloride (INT) assay

This assay was carried out as described by Eloff (1998), using p-Iodonitrotetrazolium chloride (INT) dye. Bacterial growth/ viability was indicated by a colour change from yellow (dye) to a pink/purple formazan compound while no colour change implies inhibition of bacterial growth (non-viable bacteria) (Berridge *et al.*, 2005). The Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration (highest dilution) of the macrofungal extract that did not show visible growth when compared to the control (Shah *et al.*, 2014).

The bacterial inoculum was adjusted to a 0.5 McFarland standard [1.175% $BaCl_2$ (0.1175 g in 10 mL ddH_2O) and 1% H_2SO_4 (0.5 mL H_2SO_4 in 50 mL ddH_2O)] (absorbance at 600 nm = 0.08 - 0.1; equivalent to ~$1.5x10^8$ cells/mL) and 50 µL added to the wells of a sterile 96-well microtiter plate from columns 2 to 8. Macrofungal extracts (50 µL) were added to columns 1 and 2. This was followed by the transfer of 50 µL from column 2 to 3 and serial dilutions (two-fold dilutions) until column 8. The

concentration of extracts ranged from 15.6 – 2000 µg/mL. The following controls were prepared: (i) medium control (100 µL MH broth); (ii) macrofungal extract colour control (50 µL MH broth + 50 µL of highest macrofungal extract concentration); (iii) 4% DMSO control (50 µL MH broth + 50 µL 8% DMSO); and (iv) bacterial culture control (50 µL MH broth + 50 µL bacterial culture suspension). Plates were sealed with microplate sealing tape and incubated at 37°C for 24 h. INT (Sigma- Aldrich) was prepared at a working concentration of 0.2 mg/mL in dd$H_2$0 and filter sterilized (0.2 µm filter). INT (50 µL) was added to each well, and the plates were further incubated for 30-60 min at 37°C until there was a colour change in the bacterial culture control. The assays were performed three times in triplicate and interpreted visually.

3.3 RESULTS

3.3.1 Antibacterial activity

Visual interpretation of the MIC obtained using the INT assay revealed that the bacterial isolates were susceptible to the antibiotics used as positive drug controls. Vancomycin had a MIC of 2 µg/mL and 0.8 µg/mL against *S. aureus* and *S. pyogenes* respectively. Gentamycin was effective against *P. aeruginosa* with a MIC of 2 µg/mL, while the MIC for *E. coli* and *K. pneumoniae* was 0.8 µg/mL and 0.4 µg/mL, respectively. However, none of the *G. lucidum* extracts exhibited any antibacterial activity for the tested concentration range of up to 2000 µg/mL (Table 3.2). The DMSO control did not have any inhibitory effect on the growth of the respective bacteria.

Table 3.2: Antibacterial activity of *G. lucidum* extracts assessed by INT assay.

		MIC (µg/mL)				
		Bacterial isolates				
***Extract no.**	**Extract/ Antibiotic**	***S. aureus***	***S. pyogenes***	***P. aeruginosa***	***E. coli***	***K. pneumoniae***
1	MG-LZ8	>2000	>2000	>2000	>2000	>2000
2	Polar	>2000	>2000	>2000	>2000	>2000
3	Ant EtOH	>2000	>2000	>2000	>2000	>2000
4	Ant Aq.	>2000	>2000	>2000	>2000	>2000
5	FB EtOH	>2000	>2000	>2000	>2000	>2000
6	FB Aq.	>2000	>2000	>2000	>2000	>2000
7	Myc EtOH	>2000	>2000	>2000	>2000	>2000
8	Myc Aq.	>2000	>2000	>2000	>2000	>2000
9	MM EtOH	>2000	>2000	>2000	>2000	>2000
10	MM Aq.	>2000	>2000	>2000	>2000	>2000
Positive control	Vancomycin	2	0.8	N/A	N/A	N/A
Positive control	Gentamycin	N/A	N/A	2	0.8	0.4

***: Extracts are numbered according to reference numbers in table 2.3.**

3.4 DISCUSSION

Natural products are perceived to have fewer side effects, are relatively cost effective and are often more attractive to the general consumer than allopathic medicine. The increase in resistance to known antibiotics has spurred on the research of natural products extensively as an alternative form of therapy (Heinicke *et al.*, 2016, Moussaoui and Alaoui, 2016; Hapuarachchi *et al.*, 2018). The *G. lucidum* extracts tested did not exhibit activity against the selected bacteria, as can be seen in table 3.2. This was unexpected and contrary to findings of other researchers who demonstrated antibacterial activity of *G. lucidum* against *Bacillus cereus*, *B. subtilis*, *Enterobacter aerogenes, Staphylococcus aureus, E. coli*, *P. aeruginosa, S. pyogenes, Salmonella typhi* and *K. pneumoniae* (Quereshi *et al.*, 2010, Shah *et al.*, 2014; Ferreira *et al*., 2015; Sande *et al.*, 2019). Factors that may be contributing to the lack of activity observed include; the extraction method used to produce the extracts and the extract concentration ranges tested.

A study done by Shah *et al.*, (2014) used the INT microbroth dilution method with three of the same microorganisms. They found *G. lucidum* to have antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa*. However, for *S. aureus* the MIC was found to be above the limit of the concentration range tested (2.0 mg/mL) in this study with a MIC of 2.5 mg/mL for the ethyl acetate, chloroform and a methanol and ethyl acetate combination of *G. lucidum* extracts. *E. coli* had a MIC of 1.2 mg/mL. A lower MIC of 0.63 mg/mL was obtained for *P. aeruginosa* (Shah *et al*., 2014). The extracts with this activity against *E. coli* were both methanolic extracts. A comparison to other reports indicated that acetone extracts of *G. lucidum* had MIC values as high as 20.8 mg/mL for *S. aureus*, 8.17 mg/mL for *E. coli* and 21.3 mg/mL for *P. aeruginosa* (Quereshi *et al*., 2010). These MIC values are higher compared to those reported by Shah *et a*l, (2014), however there was a difference in the extraction solvent which could be the reason for the large discrepancies. Quereshi *et al.*, (2010) showed that an acetone extract of *G. lucidum* exhibited inhibitory activity against *K. pneumoniae* at 4.33 mg/mL which is well above the highest concentration tested in the present study. In more recent studies Celal (2019) found methanol and dichlormethane extracts of *G. lucidum* were microcidal at concentration between 50 – 200 µg/mL against MRSA, *E. faecalis, E. coli, P. aeruginosa*. These extracts were extracted in a Soxhelt extractor.

Many studies on the antimicrobial activity of *G. lucidum* did not include *S. pyogenes*, however a study of Kenyan *G. lucidum* showed the production of inhibitory zones when a hexane extract of 100 mg/mL was used in the agar diffusion method (Sande *et al.*, 2019).

The extraction procedure and solvents utilized in our study were chosen as they emulate the type of solvents that would be used in traditional medicine. Traditional medicines use water extraction or tinctures, which are a form of ethanol extraction (Hapuarachchi *et al.,* 2018). The traditional extracts produced are also less likely to complex or dissociate compared to other solvents and do not have a potential health hazard such as chloroform or ether (Pandey and Tripathi, 2014). Both aqueous and ethanolic extraction allow for the removal of most of the phytochemicals, (refer to table 2.4), known to be present in *Ganoderma* spp. (Pandey and Tripathi, 2014).

The discrepancies in the antibacterial activities reported by other researchers highlights the impact of the type of solvents employed in the extraction process. For example, the study by Shah *et al.*, (2014) compared extraction of *G. lucidum* using different solvents and methanol showed the highest level of antimicrobial activity. This implies that methanol resulted in the extraction of a compound which had antimicrobial activity (Shah *et al*., 2014) compared to that of the ethanol and aqueous extracts used in this investigation. Several studies on the phytochemical analysis of *G. lucidum* have shown that the macrofungi contain polysaccharides, glycosides, triterpenoids and phenolic compounds (Pandey and Tripathi, 2014, Sande *et al.*, 2019). Buddha *et al*., (2017) reviewed the antimicrobial and anti-parasitic activities of *Ganoderma* spp. and summarised that *Ganoderma* triterpenoids are the most likely active compounds that cause antimicrobial activity. Triterpenoids are usually extracted using heat and ultrasound assisted extraction techniques (Oludemi *et al*., 2017).

It is also important to take into consideration the effect of concentration of the extract being tested. Studies done by Kamble *et al.*, (2011), showed that mycelial methanol extracts had antibacterial activity using the agar diffusion method at a concentration of 100 mg/mL. They claim it to be a viable source of antimicrobial activity however at that concentration it would result in the patient ingesting or applying kilograms of *G. lucidum* to have the desired effect. Therefore, physiological outcomes/aspects need

to be considered. The other studies mentioned above (Shah *et al.*, 2014, Quereshi *et al.*, 2014, Sande *et al.*, 2019), found most of the MICs to be greater than the concentrations used in this study. Gertsch, (2009) described the importance of the concentration range tested as it determines the meaning of the results. The concentration of natural product extracts is of particular importance as most of the current reports of positive extract bioactivities are due to high concentrations of the extract. Natural products have evolved and are phylogenetically selected in a protein rich environment, creating secondary compounds which have an increased ability to facilitate protein interaction. This means that binding with natural products may be exponential instead of linear to concentration as it is currently treated in most research. Higher concentrations are more likely to generate a response as natural products tend to have more protein targets and the higher concentration may lead to non-specific binding. Therefore, the high concentrations used, and the antimicrobial results reported in studies such as; Sheena *et al*, (2008), Quereshi *et al*, (2010) and Sande *et al,* (2019) need to be reconsidered as to whether the activity at the particular concentration is physiologically viable.

The website of the commercial *Ganoderma* samples indicated that the products possessed antimicrobial activity, which was contradictory to the finding of this study. However, the information did not include the microbes tested nor the minimum inhibitory concentrations. Furthermore Loyd *et al.*, (2018) showed that several commercial Reishi products claiming to contain *G. lucidum* in fact contained a Reishi substitute or were supplemented with other *Ganoderma* spp. such as *G. applanatum sensu lato, G. australe sensu lato, G. curtisii, G. gibbosum, G. resinaceum sensu lato*, and *G. sessile*. *G. lucidum* from areas outside of Asia and China specifically are considered as Reishi substitutes (Loyd *et al*., 2018). As a consequence of the disarray in taxonomy and classification of the *Ganoderma* spp. many isolates of *G. lucidum* have been incorrectly identified in different regions (Jargalmaa *et al.*, 2017). Therefore, products that claim to contain *G. lucidum* known as Reishi often are not the Asian Reishi products known for their medicinal properties. These differences are likely to result in differences in the quality and quantity of the bioactive components of the products being sold as Reishi products (Loyd *et al.*, 2018). Newmaster *et al.*, (2013) reported 66% of the herbal samples they tested contained fillers and preservatives that were not listed on the packaging. These substances may affect the activity of the

products being sold and tested. Thus, the inactivity of the commercial products may also be attributed to the fact that they were not pure samples of *G. lucidum.* The samples would need to undergo genetic testing as well as chromatography to ensure they are in fact pure samples. Mushroom Guru ™ (Pty) Ltd guaranteed their prepared extracts contained pure *G. lucidum* meaning the lack of activity could be attributed to the limit of the concentration range or ineffective extraction of the active antimicrobial compounds.

In conclusion the type of extraction solvent used, and the concentration range tested are important factors in determining the antimicrobial activity of *Ganoderma* spp. Studies have shown that for *G. lucidum,* methanol extracts appear to have the most inhibitory action (Shah *et al*., 2014; Ferreira *et al.*, 2015). Future studies should include the analysis of methanol *G. lucidum* extracts to determine the antibacterial activity and active compound/s.

Chapter 4

Anti-cancer and antioxidant potential of *Ganoderma* spp. extracts

4.1 INTRODUCTION

Malignant melanoma is the most dangerous form of skin cancer resulting in the highest number of deaths and it is one of the fastest growing cancers worldwide (Lui and Sheikh, 2015). Melanoma is the uncontrolled growth of melanocytes which are found in the basal layer of the epidermis and are responsible for melanin production. Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are mostly treatable cancers with a wide range of treatment options such as superficial surgery, topical creams or gels containing 5-fluorouracil (5-FU) or imiquimod (Mantagna and Lopes, 2017, Orthaber *et al*., 2017). More invasive surgery followed by radiotherapy is needed for advanced BCC and SCC (Perez-Ordonez *et al.*, 2006). Metastatic or inoperable BCC is usually treated by oral administration of vismodegib or sonidegib (Koelblinger and Lang, 2018). Advanced SCC is treated with cemiplimab immunotherapy (Ahmend *et al.*, 2019).

Like BCC and SCC, the main causes of malignant melanomas are inheritance and UV exposure (Gordon *et al.*, 2016, Orthaber *et al*., 2017). The first line of treatment is surgical excision, which leads to a high survival rate. However, if metastasis occurs it changes the life expectancy to five years or less. Metastatic melanomas are treated with dacarbazine, however, there are newer therapeutic agents such as vemurafenib and dabrafenib which are not used as frequently (Lui and Sheikh, 2015).

Despite decades of advancements in skin cancer treatment, many challenges remain with current treatment regimens and there is still an increasing need for improvements. One of the challenges associated with many treatments is the lack of effectiveness over long periods of time due to cancer cell resistance (Lui and Sheikh, 2015). The chemotherapeutic agents also cause side effects, which include mild headaches to severely deteriorating the quality of life of patients (Herbst, 2019) the risk of birth defect especially in patients who use vismidegib and sonidegib for advanced BCC, and infertility caused by trametinib used to treat metastatic melanomas (Lui and Sheikh,

2015). Other general side effects include fatigue, photosensitivity, nausea and diarrhoea (Herbst, 2019). Resistance to dacarbazine increases to 75 – 80 % after 3 months of continued treatment.

Due to resistance and lack of treatment efficacy there is a need for research on novel chemotherapeutic agents. Natural products have long been known to exhibit antitumor or anticancer properties (Lui, 2007). Paclitaxel serves as well-established example of a plant derived chemotherapeutic agent. It currently serves in the treatment of breast, ovarian and lung cancer (Zhu and Chen, 2019). It was first isolated from the crude extract of *Taxus brevifolia* commonly known as the Pacific yew tree. It acts as a microtubule-targeting agent, restricting the movement of microtubes causing cell cycle arrest during mitosis (Zhu and Chen, 2019). Another example of plants utilised in the treatment of cancer includes the alkaloids vinblastine and vincristine from *Catharanthus roseus*, also known as the Madagascar periwinkle (Moudi *et al, 2013*). They have a similar mechanism of action to that of paclitaxel; whereby they disrupt cell cycle activity during the M phase by inhibiting tubulin polymerization causing cell cycle arrest leading to cell death (Thirumaran, *et al.*, 2007; Moudi *et al, 2013*). These compounds are structurally similar but differ in their spectrum of activity and toxicity (Moudi *et al, 2013*). Vincristine is used for the treatment of different cancers including patients with myeloma, rhabdomyosarcoma, neuroblastoma, Ewing's sarcoma, Wilm's tumor, chronic leukemia, thyroid cancer, brain tumours, and trophoblastic neoplasia (Thirumaran, *et al.*, 2007). In addition, vincristine is the main therapeutic agent in the treatment of non-Hodgkin’s lymphoma and Hodgkin’s disease with a higher effectiveness in paediatric tumours compared to adult tumours (Thirumaran, *et al.*, 2007; Moudi *et al, 2013*). Vinblastine is also used in the treatment of Hodgkin’s and non-Hodgkin’s lymphomas, however, is most frequently used for the treatment of testicular cancer and also used in conjunction with other chemotherapies in the treatment of breast cancer (Hait, *et al.,* 2015).

The phytochemical profile of *Ganoderma* spp. generally includes glycoproteins, polysaccharides, triterpenoids, meroterpenoids, sesquiterpenoids, steroids, alkaloids, benzopyran derivatives and benzoic acid derivatives. *G. lucidum* contains various biological active compounds such as triterpene, polysaccharides, proteins, amino acids, enzymes, vitamins, alkaloids, flavonoids, steroids and minerals and has been

extensively researched, with its anticancer properties being attributed to its polysaccharides and triterpenes (Ahmad, 2020). Their polysaccharides seem to play a larger role in their anticancer activity. The isolated polysaccharides include (1→3), (1→6)-α/ β-glucans, water-soluble heteropolysaccharides as well as glycoproteins (Sohretoglu and Huang, 2018). Studies have shown that their anticancer activity includes inhibition of tumour growth, metastasis and activation of T and B lymphocytes, macrophages and dendritic cells (Sohretoglu and Huang, 2018,). It has been confirmed that *G. lucidum* exhibits significant potency to prevent and treat different types of cancers such as breast, prostate, colon, lung and cervical (Ahmad, 2020), however, there is a lack of information on its activity against melanomas.

Oxygen plays a vital role in promoting wound healing and helps to prevent wound infection. However, reactive oxygen species (ROS) produced during oxidative phosphorylation has been seen to cause pathologic disease states if produced in excess (Shetty *et al*,, 2008). In response to the excess of free radicals, a recruitment of neutrophils, an activation of proinflammatory cytokine secretion that keeps the wound in a persistent inflammatory situation, and an alteration of the cellular response are triggered. Consequently, the healing process slows down, resulting in a hard-to-heal wound (Casado-Diaz *et al*., 2022). The main function of antioxidants is defending cells against ROS. Examples of ROS are superoxide anions, hydrogen peroxide and nitric oxide (Shetty *et al*, 2008; Rajasekaran and Kalaimagal, 2011). These products are formed when oxygen is partially reduced, becomes activated, and reacts with biomolecules, which have one, two or four electrons available. When these ROS products are overproduced, oxidative stress results. Oxidative stress is a dangerous condition for cells to be in, especially for extended periods of time as it leads to cell damage in many forms. The outcome of this damage can include cardiovascular dysfunction, neurodegenerative diseases, inflammatory diseases and delayed wound healing (Shetty *et al*, 2008; Rajasekaran and Kalaimagal, 2011).

Antioxidants inhibit ROS and prevent oxidative stress using several mechanisms (Lee and Lim, 2019). These include enzymes, which are capable of decomposing peroxides as well as proteins that take up transition metals. They are also capable of scavenging free radicals without it altering themselves into reactive oxygen species (Rajasekaran and Kalaimagal, 2011). Natural antioxidants are well known and are found in

abundance in vegetables and fruit. These natural products are known to have a high content of phenolic compounds and flavonoids (Rajasekaran and Kalaimagal, 2011; Celal, 2019). In *Ganoderma* spp. antioxidant activity has been reported to be due to the effect of triterpenoids which increase cellular antioxidant enzymes. They also produce a β-glucan-protein complex which acts to reduce ROS (Lee and Lim, 2019).

The aim of this chapter was to screen *G. lucidum* and other *Ganoderma* spp. extracts for cytotoxicity against B16F10 murine melanoma cells and investigate their antioxidant potential via the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and Ferric reducing ability of plasma (FRAP) assays.

4.2. MATERIALS AND METHODS

4.2.1 Cell lines/ culture maintenance

B16F10 murine melanoma cells were used to evaluate the toxicity of the *Ganoderma* spp. extracts as an indication of their anticancer potential. All the cell cultures were maintained in 10 cm cell culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and incubated at 37ºC in a humidified incubator with 5% CO_2.

4.2.2 Cytotoxicity screening

The extracts prepared as described in section 2.2.2. were screened for cytotoxicity against the B16F10 murine melanoma cell line by staining treated cells with Hoechst 33258 and propidium iodide. Refer to table 2.3 for the extract reference number and information. Analysis was performed using the Molecular Devices ImageXpress Micro XLS Widefield microscope for high content analysis as described by Pringle *et al.*, (2018). Hoechst 33258, [figure 4.1(A)] is a nuclear counterstain which fluoresces blue when it binds to double stranded DNA. Intact cell membranes are permeable to Hoechst 33258 and it therefore stains nuclei of live and dead cells. Propidium iodide (PI), [figure 4.1 (B)] is a nuclear counterstain but since it is excluded from living cells with intact membranes, it is used to detect dead cells which have lost their membrane integrity. It intercalates between the bases of DNA of dead cells and fluoresces red.

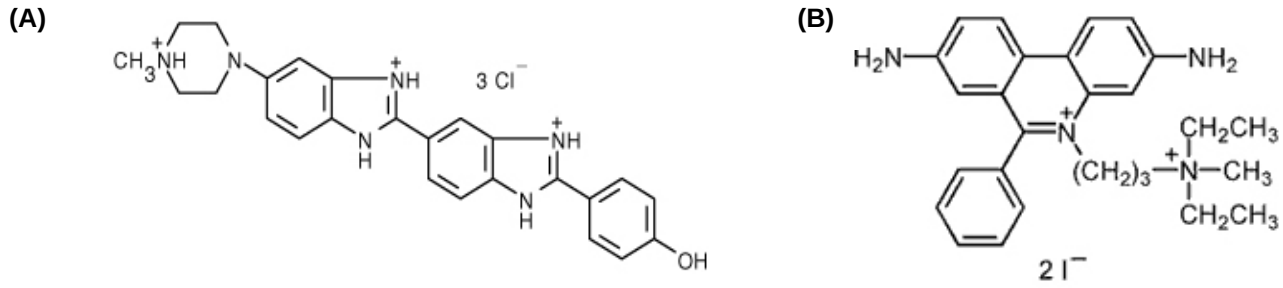

Figure 4.1: (A) Chemical structure of Hoechst 33258 and (B) Propidium iodide (ThermoFischer, 2019).

The B16F10 cells were seeded in a 96-well microtiter plate at a density of 4000 cells per well in 100 µL aliquots and incubated for 24 hrs to recover from trypsinisation and adhere to the plate. The *Ganoderma* spp. extract stocks (treatment) were prepared in pure dimethyl sulfoxide (DMSO) at 100 mg/mL and the working concentrations were diluted in culture medium directly before exposure to cells. The treatments were added to the appropriate wells in 100 µg/mL aliquots of the working concentrations of 50, 100 and 200 µg/mL of the *Ganoderma* spp. and *G. lucidum* extracts. Melphalan was used as positive control for the cytotoxicity assay while culture medium was used as a negative control. Melphalan is well known for its toxicity against cancer cells including melanomas. It enters the cell via a neutral amino acid transporter and is cytotoxic by inducing inter strand crosslinks in DNA and may also produce lesions in RNA, proteins and lipids. It does not have to be activated to be cytotoxic (Kuczma *et al.*, 2016). The cells were incubated with treatments for 48 hrs, after which the number of live and dead cells in each well were determined using the Hoechst 33342 and PI dual staining method. After treatment, the medium was removed from the well and 50 µL of Hoechst 33342 (5 µg/mL) was added to the well and incubated for 15 min at 37°C. Immediately before image acquisition, 50 µL of PI (100 µg/mL) was added to each well and imaged immediately to prevent overstaining of viable cells. Images were acquired using the Molecular Devices ImageXpress Micro XLS Widefield microscope for high content analysis equipped with a 10x plan fluor objective and DAPI and Texas Red filter sets for acquisition of Hoechst and PI, respectively. Nine image sites were acquired in each well, which covers roughly 70% of the total surface area of the well (Figure 4.2). Analysis was performed using the Multiwavelength Cell Scoring analysis module of MetaXpress® software to determine the total number of nuclei (stained with Hoechst) and number of dead cell nuclei (stained with Hoechst and PI) per well. For samples where the reduction in live cell numbers relative to controls exceeded 50%, IC_{50} values

were calculated using GraphPad Prism Version 5.0 (GraphPad Software, San Diego, USA).

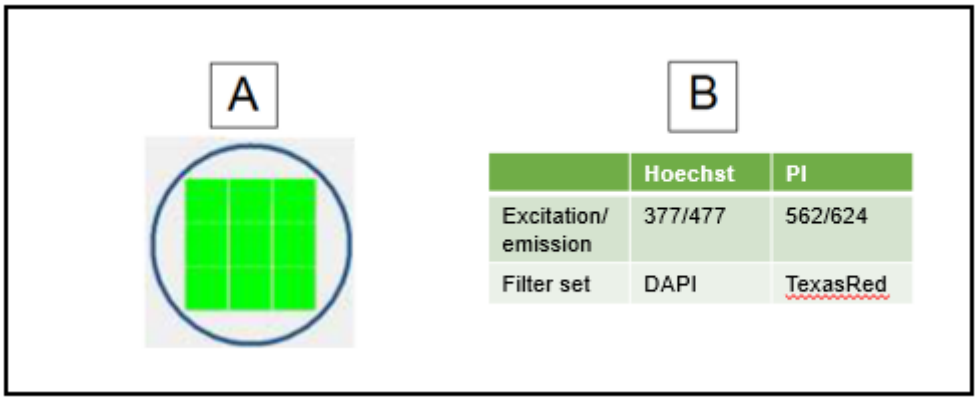

Figure 4.2. A - Spatial distribution of image sites acquired during cytotoxicity screening. The green represents the area that is covered by each image site of a single well, nine sites in total. B – Excitation/emission wavelengths and filters used to acquire data.

4.2.3 Antioxidant Activity

4.2.3.1 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

2-Diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical with a purple colour that acts as a radical scavenger. When DPPH encounters a compound, which can donate a hydrogen or transfer an electron the DPPH becomes discoloured, figure 4.3. The colour change is measured spectrophotometrically and the change in absorbance indicates the antioxidant capacity (Holtz, 2009). The composition of all reagents used in these assays can be found in the Appendix (section A).

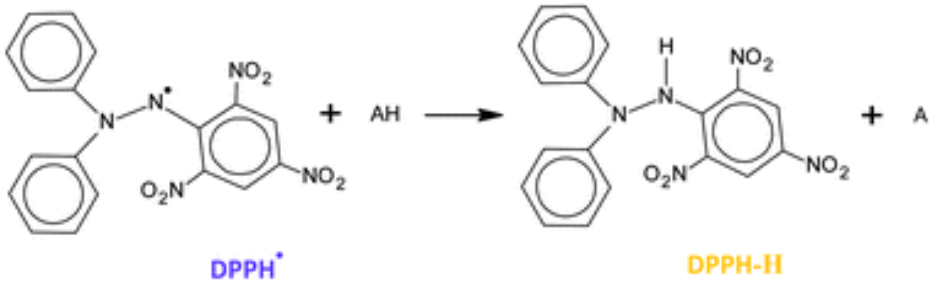

Figure 4.3: Chemical structure and principle of the DPPH assay (Pyrzynska and Pekal, 2013)

The extracts were prepared as described in section 2.2.2. Refer to table 2.3 for the extract reference number and information. Each extract was diluted to the appropriate concentrations (50, 100, 200 µg/mL) in TRIS-HCl buffer. Five microliters of the extract sample were transferred to each well in triplicate per concentration to a 96-well microtiter plate. Thereafter 120 µL of the TRIS-HCl buffer and 120 µL of the DPPH reagent were added. The plate was incubated for 20 min at room temperature and the absorbance read on a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) at 513 nm. The antioxidant potential was expressed as a percentage compared to the reference sample, i.e., DPPH in the absence of an antioxidant. Quercetin and epigallocatechin gallate (EGCG) were both used as positive controls for antioxidant activity. Both compounds are natural polyphenols that are well known as efficient antioxidants. Quercetin is a flavonoid extracted from plants and EGCG from green tea (=Ozgen *et al.*, 2016).

$$\%DPPH\ activity = \frac{Reference\ sample - Extract\ sample}{Reference\ sample} \times 100$$

4.2.3.2 Ferric reducing ability of plasma (FRAP) assay

The FRAP assay is a method to assess the antioxidant ability of compounds. The assay is based on the colour change, which occurs when Fe^{3+} -tripyridyltriazine (TPTZ) complex (colourless) is reduced to Fe^{2+}- TPTZ (blue) as illustrated in figure 4.4. This occurs at low pH and absorbance of the reduced complex can be measured at 593 nm.

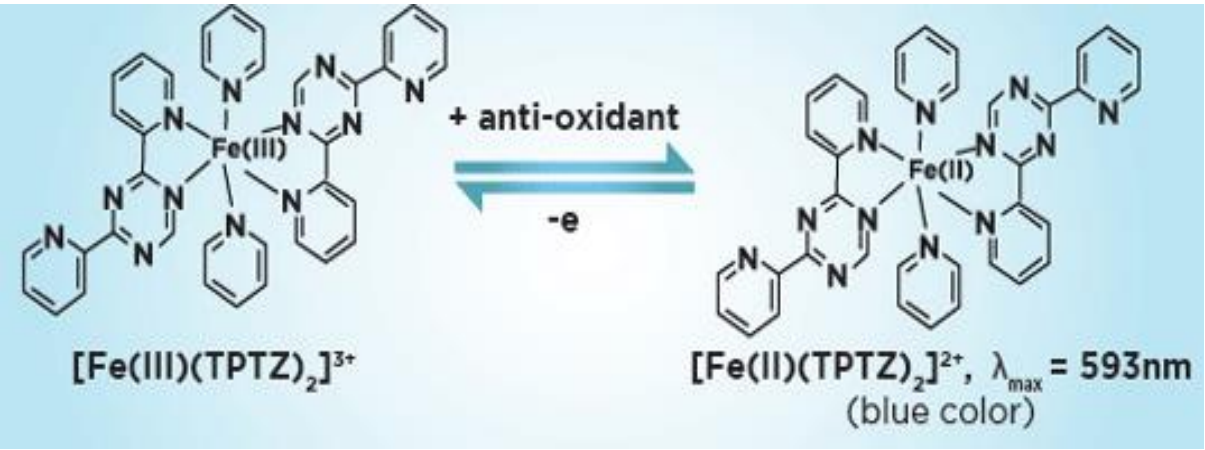

Figure 4.4: Principle of FRAP assay (Benzie and Strain, 1996).

Each extract was diluted in acetate buffer to 250, 500 and 1000 µg/mL. The extract sample (50 µL) was added to a single well in triplicate for each concentration in a 96-well microtiter plate followed by 200 µL of the FRAP reagent. This yielded final extract concentrations in the assay of 50, 100 and 200 µg/mL. The extract sample (50 µL) was added to a single well in triplicate for each concentration in a 96-well microtiter plate followed by 200 µL of the FRAP reagent. The plate was incubated for 10 min at room temperature, and the absorbance was read on BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA) at 593 nm. The activity was calculated by comparing it to a Trolox standard curve of known Fe^{2+} concentrations. The solutions used and the Trolox standard curve are indicated in the appendix section A and figure B1, respectively. EGCG was used as positive control for this assay.

4.2.4 Statistical analysis

All experiments were performed three times, each in triplicate for statistical analysis, except where otherwise stated. The two-tailed student t-tests assuming unequal variance was used to compare the means of the data sets using the data analysis package from Microsoft Excel. In cases where $p<0.05$ the data was considered statistically significant.

4.3 RESULTS

4.3.1. Cytotoxicity Screening

The cytotoxicity of the *G. lucidum* extracts (1 - 10) (Figure 4.5) and cultivated *Ganoderma* spp. (Figure 4.6) (refer to table 2.3 for extract number reference and information) were determined using the B16F10 melanoma cell line. The average number of live and dead cells were determined using the Hoechst/PI dual staining method. The dead cells were stained and counted but were minimal making the bars mostly invisible, with no significant difference when compared to the untreated cells. The general trend for most of the samples showed a small but statistically insignificant, dose-dependent decrease of live cells. One exception was Medi Mushroom Reishi extracts (9 and 10) which showed results consistent to those of the untreated control with the live cells staying relatively constant throughout the concentration range tested. The other exception was the significant dose-dependent decrease in live cells seen for the MG-LZ8 extract (1) as well as the highest concentration of the ethanolic extract of the fruiting body (5). The fruiting body extract (5) did not cause a 50% decrease in cells and therefore the IC_{50} value could not be calculated. MG-LZ8 (1) was tested at a concentration range of 5 – 500 µg/mL to enable the calculation of its IC50. The IC_{50} of the MG-LZ8 (1) extract was calculated to be 21.26 ± 2.15 µg/mL. The active MG-LZ8 (1) sample was able to decrease the number of viable cells without increasing the number of dead cells. This indicates potential cytostatic rather than cytotoxic activity, however, this would need to be further investigated. The two lower concentrations of the ethanolic and aqueous antler extracts (3, 4) caused a significant increase ($p<0.05$) in the live cell numbers. This indicates that at low concentration the extract may have proliferative activity and has the potential for further investigation.

The only extract from the cultivated *Ganoderma* spp. that showed any significant decrease in live cell numbers was *G. destructans* type (13) at 200 µg/mL ($p<0.05$). This was similar to the low numbers of dead cells resulting in barely visible bars observed for the *G. lucidum* extracts. This is the first description of cytotoxic activity of *G. destructans* type since its genetic identification by Coetzee *et al*., (2015).

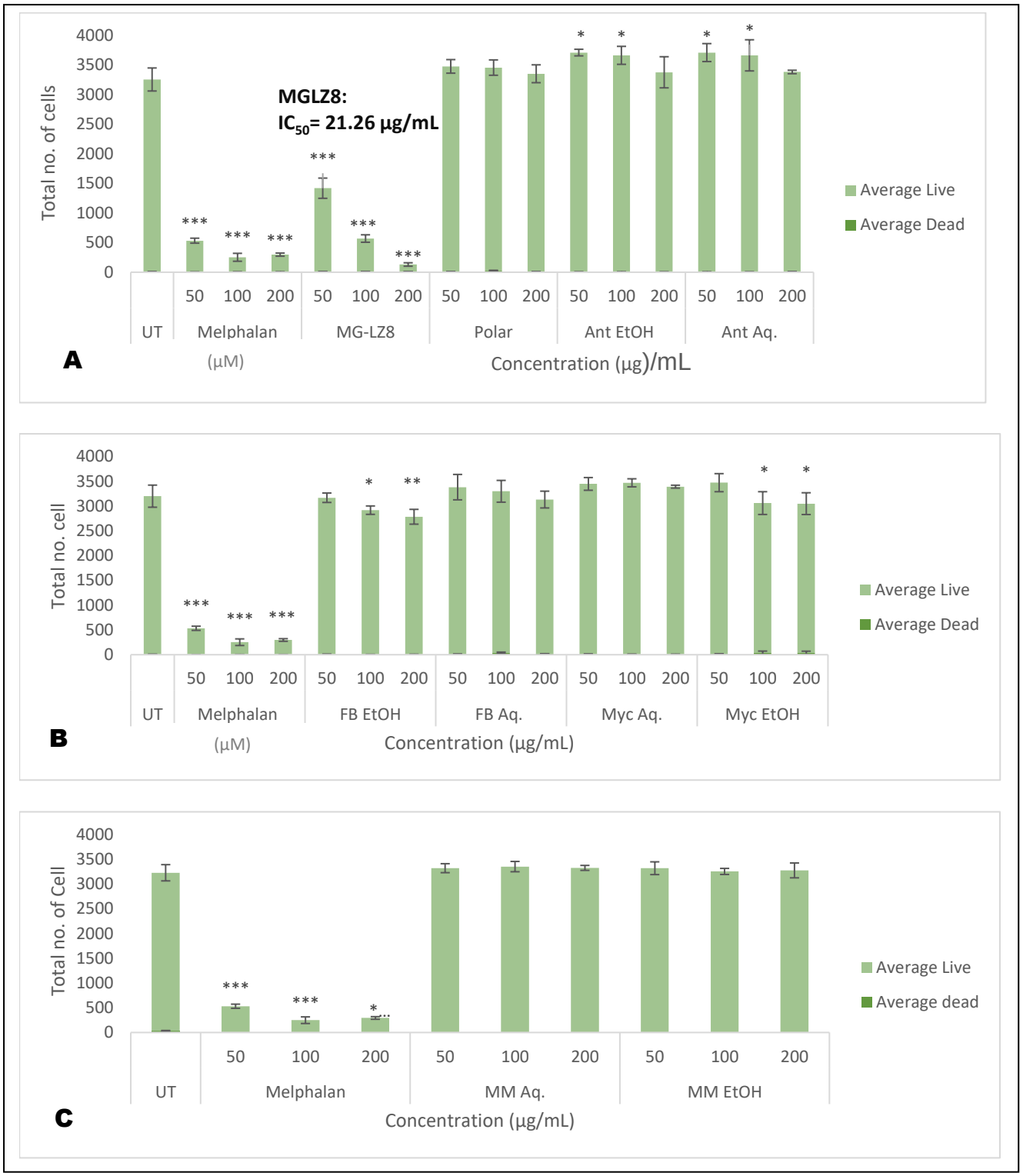

Figure 4.5: Cytotoxicity screening of *G. lucidum* extracts. (A) MG-LZ8, polar, antler (Ant) aqueous (Aq.) and ethanolic (EtOH) extracts. (B) Fruiting body (FB EtOH) and Mycelium (Myc) aqueous (Aq.) and ethanolic (EtOH) extracts. (C) Medi Mushroom Reishi (MM) capsule aqueous (Aq.) and ethanolic (EtOH) extract. The live and dead cell numbers were obtained from Hoechst 33342 and PI staining, respectively and presented as stacked bars with bars for live cells on top of dead cells, each with their own error bar. Dead cell numbers were very low and may be invisible on the graphs. Results reported as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to the untreated sample).

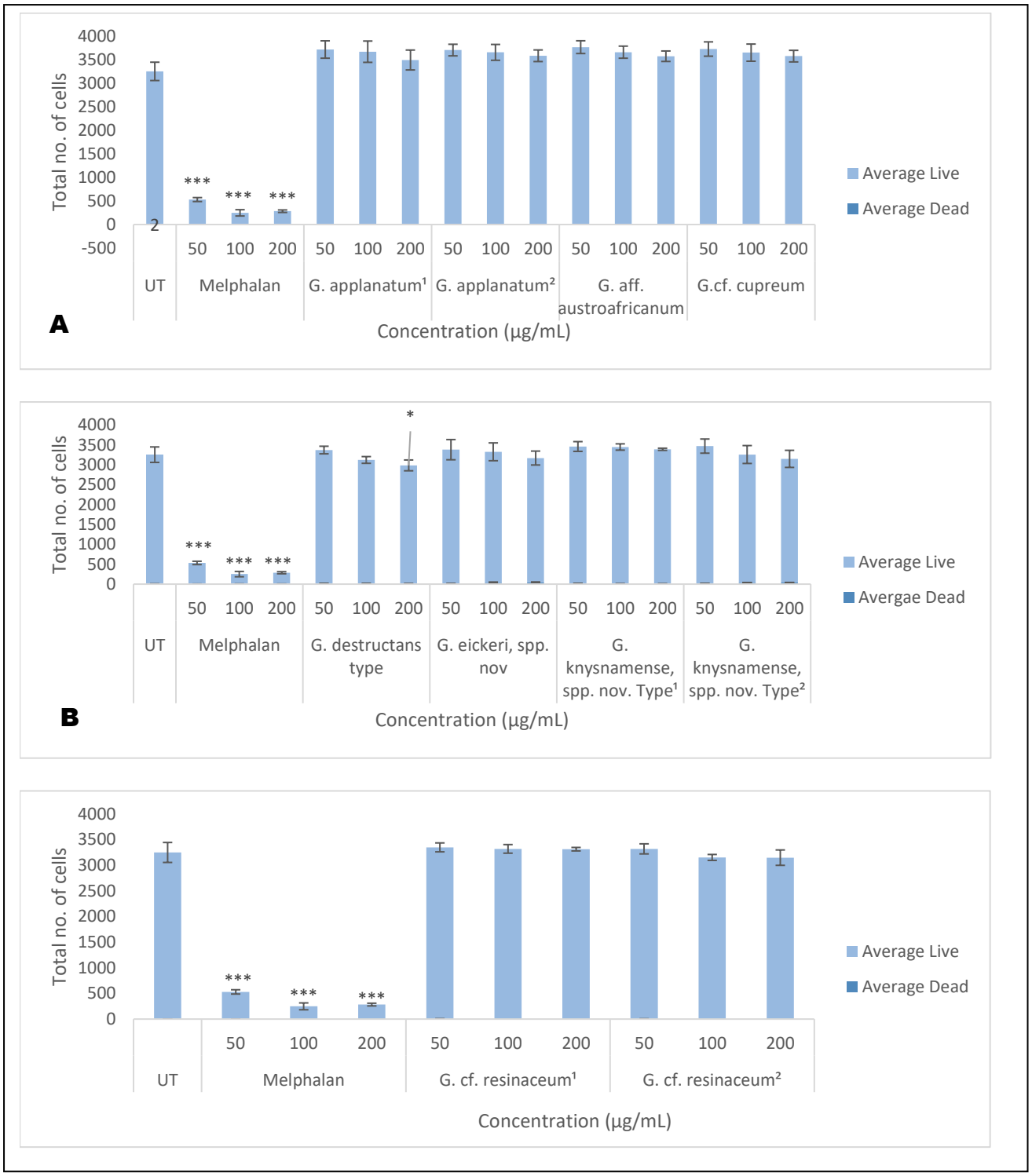

Figure 4.6: Cytotoxicity screening of cultivated extracts (A) *G. applanatum* [1], *G. applanatum*[2], *G.* aff. *austroafricanum, G.*cf. *cupreum,* (B) *G destructans* type, *G. eickeri,* spp. nov., *G. knysnamense,* spp. nov Type[1], *G. knysnamense,* spp. nov Type[2]. (C) *G.* cf.*resinaceum*[1], *G.* cf. *resinaceum*[2]. The live and dead cell numbers were obtained from Hoechst 33342 and PI staining respectively and presented as stacked bars with bars for live cells on top of dead cells, each with their own error bar. Dead cell numbers were very low and may be invisible on the graphs. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to the untreated (UT) sample).

4.3.2 Antioxidant activity

The DPPH assay was used to investigate the radical scavenging activity of *G. lucidum* and cultivated *Ganoderma* spp. extracts. All the *G. lucidum* extracts (1 - 10) showed a dose-dependent increase in radical scavenging activity. The ethanolic extracts generally showed higher radical scavenging activity than the corresponding aqueous extracts, except for the antler extracts (3 and 4) where the opposite trend was observed (Figure 4.7). MG-LZ8 (1) showed the highest activity of all the samples with an increase in activity from the 50 μg/mL (49 ± 8.5%) to the 100 μg/mL (69 ± 3.3%) and with the activity reaching a plateau at 200 μg/mL, the free radical scavenging activity was calculated to be the 69 ± 4.3%, the same as the previous concentration. This result was followed by the aqueous and ethanolic antler extracts (3 and 4) that originated from the same raw material used to produce MG-LZ8, the radical scavenging activity ranged from 43 – 69% and 36 – 64% respectively for the concentration range tested. The ethanolic fruiting body extract (5) at a concentration of 200 μg/mL had the radical scavenging activity of 63 ± 6.6% (Figure 4.7B) and was the only other sample with activity comparable to those in Figure 4.7A.

The *Ganoderma* spp. extracts (11 - 20) which were all prepared from cultured mycelia showed a similar trend of a dose-dependent increase in radical scavenging activity (Figure 4.8). However, the activities of all the *Ganoderma* spp. were lower compared to the mycelium extracts of *G. lucidum* (7 and 8) where the ethanolic extract 7 had the highest radical scavenging activity of 54 ± 4.0% at a concentration of 200 μg/mL, figure 4.5 B. The IC_{50} values were not calculated, however one can deduct from these results that the IC_{50} values of the *Ganoderma* spp. extracts would be above 200 μg/mL which would reduce their potential for significant physiological effects.

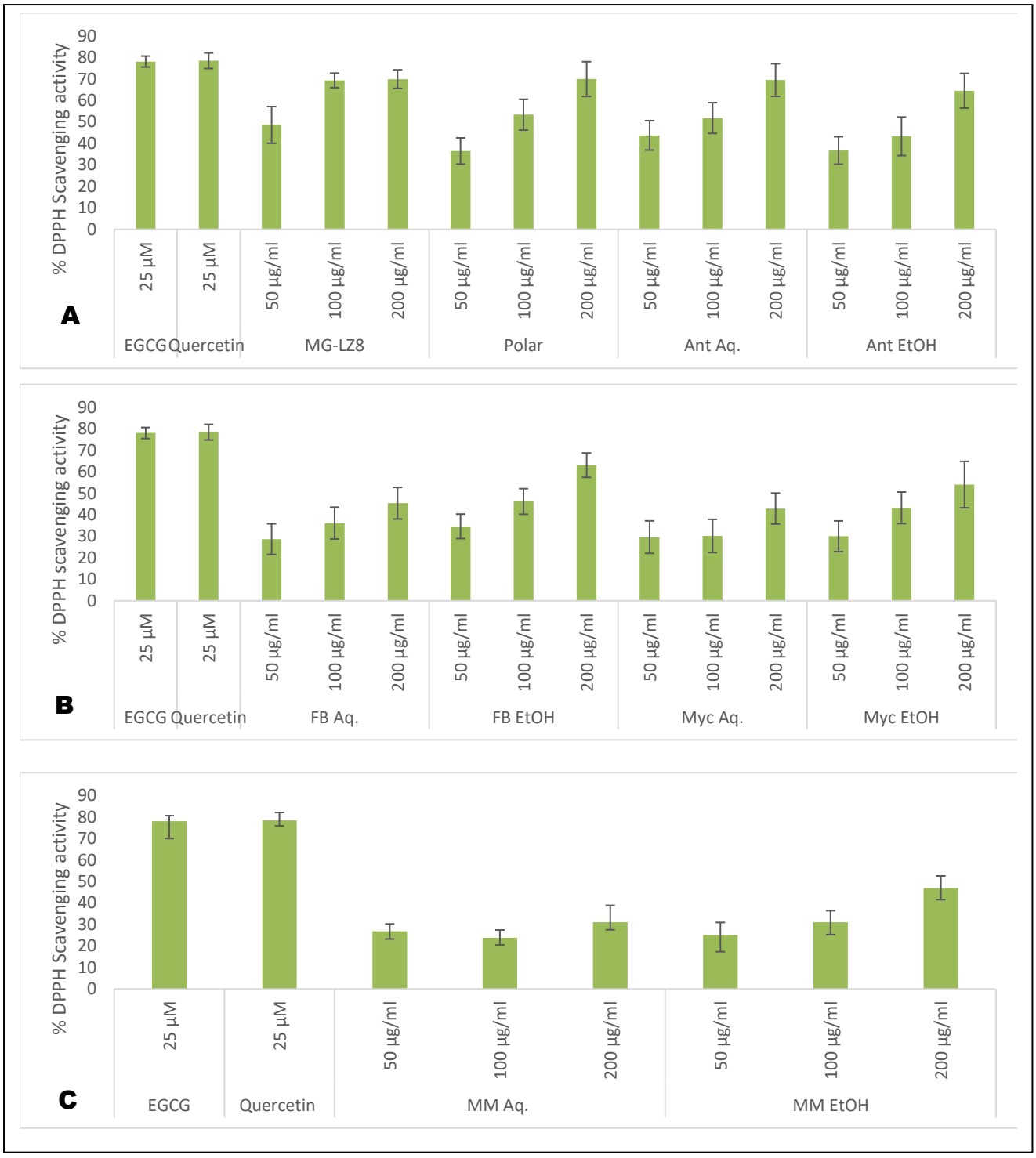

Figure 4.7: Antioxidant activity of *G. lucidum* extracts determined using the DPPH assay. (A) MG-LZ8, polar, antler (Ant) aqueous (Aq.) and ethanolic (EtOH) extracts. (B) Fruiting body (FB) and Mycelium (Myc) aqueous (Aq.) and ethanolic (EtOH) extracts. (C) Medi Mushroom (MM) Reishi capsule aqueous (Aq.) and ethanolic (EtOH) extract. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3)

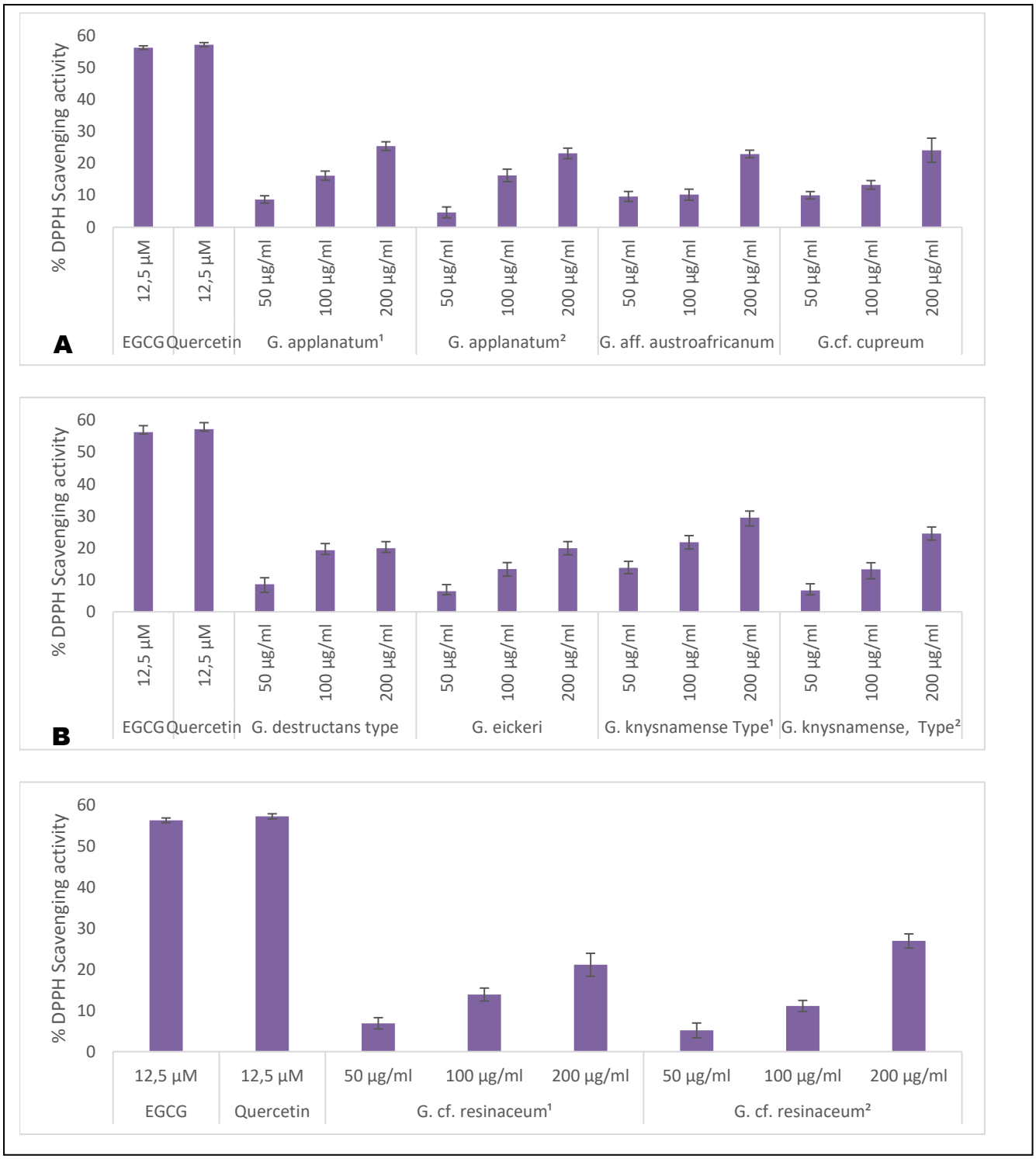

Figure 4.8: Antioxidant activity of cultivated extracts using the DPPH assay. (A) *G. applanatum* [1], *G. applanatum*[2],*G.* aff. *austroafricanum, G.*cf. *cupreum,* (B)- *G. destructans* type, *G. eickeri,* spp. nov., *G. knysnamense,* spp. nov Type[1], *G. knysnamense,* spp. nov Type[2]. (C)- *G.* cf. *resinaceum* [1], *G.* cf. *resinaceum*[2]. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3).

The ferric iron reducing ability of the *G. lucidum* (1-10) and *Ganoderma* spp. (11-20) extracts were low, as can be seen in Figures 4.9 and 4.10. A similar trend of a dose dependant increase can be seen in the FRAP assay compared to the DPPH assay; with the extracts showing similar results in terms of which extracts showed the highest activity. The *G. lucidum* extracts which displayed the highest activity were the MG-LZ8 (1) and polar extracts (2), at the highest concentration tested (200 µg/mL) the extracts had the ferric reducing ability of 26 ± 0.64 µmol trolox equivalent/ g and 24 ± 1.21 µmol trolox equivalent/ g respectively. The ferric reducing activity of the highest concentration (200 µg/mL) of ethanolic and aqueous antler extract (3, 4) as well as the polar extract (2) had a ferric reducing ability of 18 ± 0.64 µmol trolox equivalent/ g. The ethanolic extracts of the *G. lucidum* conk fruiting body (5) and mycelium (7) followed closely with a ferric reducing ability of 17 ± 0.64 µmol trolox equivalent/ g for both.

The mycelium extracts of the various *Ganoderma* spp. showed little to no ferric iron reducing ability. *G.* cf. *resinaceum* [1] (19) and *G.* cf. *resinaceum*[2] (20) showed the highest activity of the *Ganoderma* spp. in figure 4.10 C but the maximum was only 5 ± 0.84 µmol trolox equivalent/ g for both *G. cf. resinaceum* extracts compared to 17 ± 1.49 µmol trolox equivalent/g for the *G. lucidum* mycelium ethanolic extract (7) in figure 4.9 B. In general, all extracts displayed higher activity in the DPPH scavenging assay as compared to the FRAP assay. One can assume that at higher concentration more activity would be observed but the physiological relevance could be questioned.

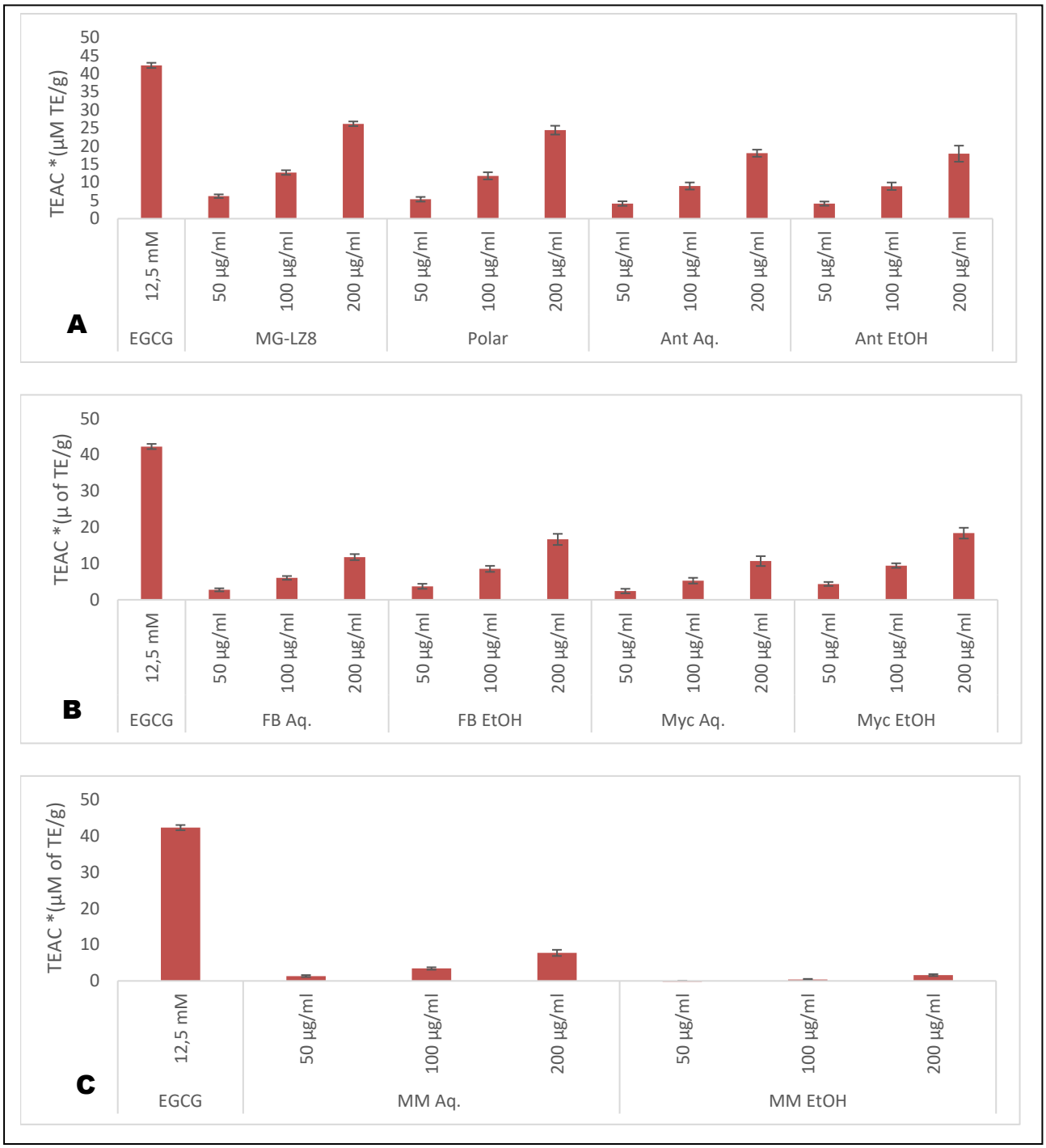

Figure 4.9: Trolox equivalent antioxidant capacity (TEAC) of *G. lucidum* extracts determined using the FRAP assay. (A) MG-LZ8, Polar, antler aqueous and ethanolic extracts. (B) Fruiting body and Mycelium aqueous and ethanolic extracts. (C) Medi Mushroom Reishi capsule aqueous and ethanolic extract. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3). *unit = µM of Trolox Equivalent per gram of extract

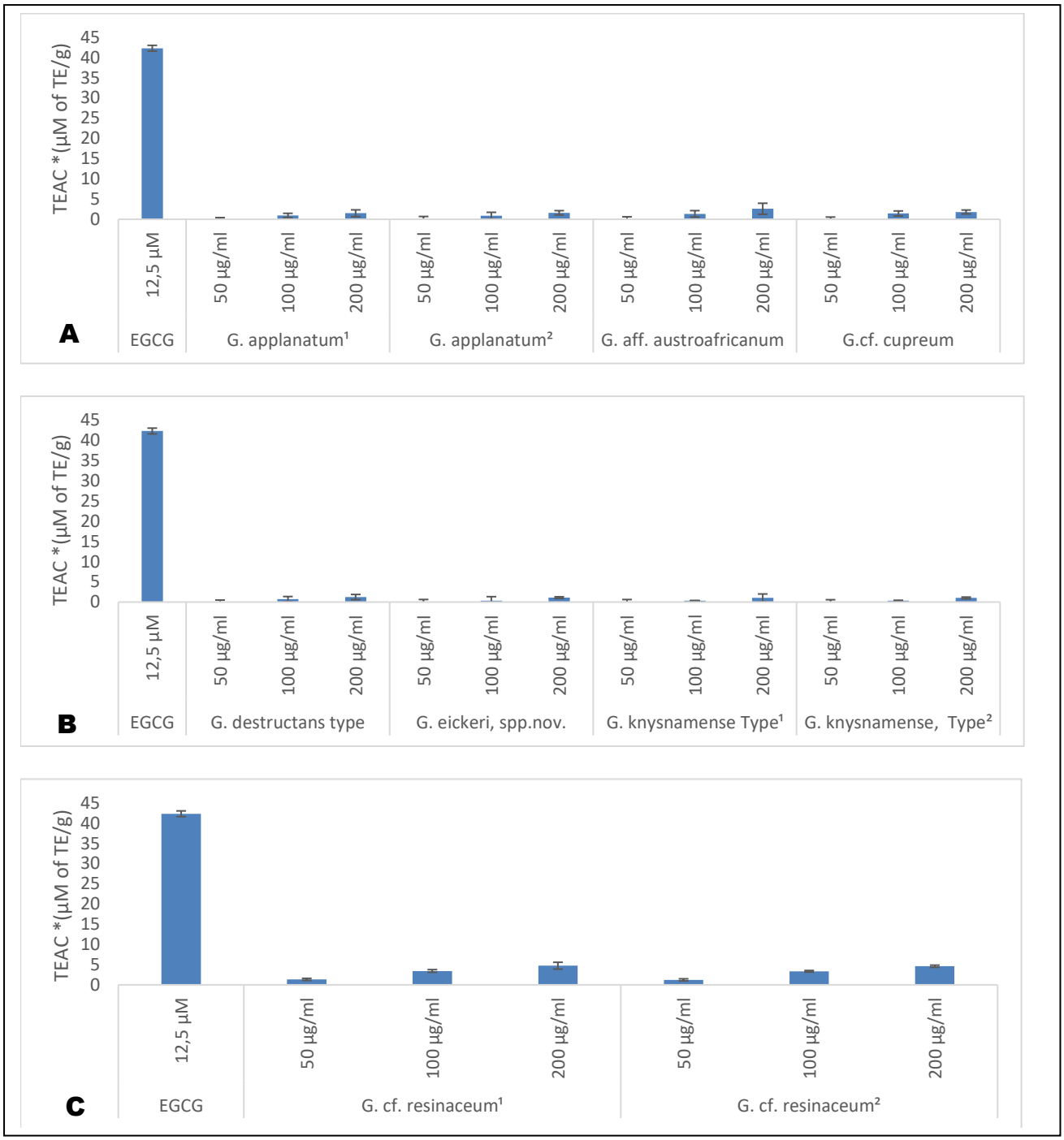

Figure 4. 10: Trolox equivalent antioxidant capacity of cultivated extracts using the FRAP assay. (A) *G. applanatum* [1], *G. applanatum*[2],*G.* aff. *austroafricanum, G.*cf. *cupreum,* (B) *G. destructans* type, *G. eickeri,* spp. nov., *G. knysnamense,* spp. nov Type[1], *G. knysnamense,* spp. nov Type[2]. (C) *G.* cf. *resinaceum* [1], *G.* cf. *resinaceum*[2]. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3). *unit = µM of Trolox Equivalent per gram of extract

4.4 DISCUSSION

The South African population has an increased risk of skin cancer and with the resistance to chemotherapeutic agents increasing, the need for new chemotherapeutic agents has become pivotal (de Wet *et al*., 2020). Most natural extracts when tested show some form of antioxidant activity, however this often does not correlate with the anticancer activity of the same extract (Yasueda *et al*., 2016). Antioxidant activity is important in the cell and body as it protects against oxidative stress. The cells have many of their own antioxidants, however, increased intake would help maintain cell integrity (Kattappagari *et al.*, 2015). For example, cancer and cancer treatments cause damage to normal cells which could be prevented or reduced if there is an increase in antioxidants (Lee and Lim, 2019, Kattappagari *et al.*, 2015). However, according to research on natural products and *Ganoderma* spp. indicate that there is no conclusive link between an extract having antioxidant activity and anticancer activity (Yasueda *et al*., 2016, Cör *et al.,* 2018).

Among the *G. lucidum* antler containing extracts (1 - 4), figures 4.5, only the MG-LZ8 (1) extract had a cytotoxic effect on the B16F10 cells. Mushroom Guru supplied an aqueous polar extract which had no cytotoxic activity which leads one to believe that the ethanolic extract found in the MG-LZ8 extract was responsible for the activity observed. However, the ethanolic antler extract (3) showed no cytotoxic activity. Since Mushroom Guru did not have any of their ethanolic extract available and they could not reveal their extraction method, they supplied antlers and an ethanolic extract was prepared as described in section 2.2.2. The differences in extraction procedure are likely the cause of the differences observed in the activity of the extract. Due to it being propriety information the exact extraction protocol and cultivation of the antler fruiting body used to produce MG-LZ8 extract is unknown, however, it stated on their website (https://www.mushroomguru.co.za/mg-lz8-reishi.htmL) to be a combination of an ethanol and hot water antler extraction. This combination makes it different from the polar extract (2) and both antler fruiting body extracts (3, 4) which had the same starting material, as well as the other *G. lucidum* and *Ganoderma* spp. tested. The hot water extraction is capable of extracting water soluble polysaccharides and triterpenes which do not solubilise at room temperature (Siani *et al*., 2014; Ferreira *et al*., 2015). The vast difference in the cytotoxic activities of the *G. lucidum* extract and the MG-

LZ8 is most likely associated with the starting material, antler fruiting body, and the combination of the ethanol and hot water extracts. The combination of these factors may work together in a complex manner to achieve the observed cytotoxic activity.

The ethanolic conk fruiting body extract (5) showed a significant (**p<0.01) decrease in the number of live cells for its highest concentration as well as the ethanolic mycelium extract with a significance of (**p<0.05) for the highest concentration tested (200 µg/mL), figure 4.5 B. This corresponds with other reports where *G. lucidum* exhibited anti-proliferative activity against B16F10 murine melanoma cancer cells (Barbieri *et al.*, 2016). Barbieri *et al.*, (2016) reported that their 70% ethanol extraction exhibited anti-proliferative activity against B16F10 murine melanoma cells. Their extract showed a 20% decrease in cells, compared to the control, for the concentrations ranging from 0.25 - 1.0 mg/mL. The fruiting body had a greater decrease in live cells compared to the mycelium which correlates with previous studies which suggest that extracts of fruiting bodies have a larger phytochemical profile compared to mycelial extracts (Azwanida, 2015). The MG-LZ8 extract (1) (figure 4.5 A) caused a decrease in the number of cells compared to that of the ethanolic conk fruiting body extract (5). The cultivation of the fruiting body plays an important role in its bioactivities, the antler fruiting body is produced through manipulating the light source and ventilation during the growth period. This type of fruiting body has been seen to have different biological activity, for example antioxidant activity, compared to the naturally occurring conk fruiting body (Sudheer *et al.*, 2018).

The commercial Medi Mushroom Reishi Capsule extracts (9, 10) figure 4.5 C showed no cytotoxic activity. The product description states that this product provides natural cancer and chemotherapy support (https://www.faithful-to-nature.co.za/medi-mushrooms-Reishi-capsules). This statement may be misleading, as many food supplements marketed do not describe how the desired effects are produced. This is possible as food supplements are not regulated and studies showed that the products often contain ingredients such as fillers and preservatives that are not listed or they contain *G. lucidum* Reishi substitutes (Newmaster *et al.*, 2013, Loyd *et al.*, 2018). These substitutes are *G. lucidum* from areas outside of Asia and China specifically (Loyd *et al*., 2018) with some products containing other *Ganoderma* spp., which are excluded on the product ingredient list (Loyd *et al*., 2018). Thus, without genetic testing

of the commercial sample to determine the *Ganoderma* spp. used and knowing the purity of the sample in terms of undefined ingredients of the product; one cannot confidently confirm that the Medi Mushroom Reishi Capsule extract contained *G. lucidum* or confer the activity one could expect from its extraction.

The extraction method is important as variations were seen between different samples of the same species and that fruiting bodies may show cytotoxicity. Due to time limitations fruiting bodies of the *Ganoderma* spp. could not be cultivated and only the mycelial samples from these different species were compared, figure 4.6. No cytotoxicity was seen for any of the mycelial samples except for *G. destructans* type (13) in figure 4.6 B. *G. destructans* type was first identified by Coetzee *et al.*, (2015) and there are no publications on its bioactivities. Hence, the anti-proliferative activity at a concentration of 200 μg/mL is the first report of bioactivity. However, the cytotoxic activity was at a lower concentration for a mycelial extract when compared to other reports; where concentrations were >1000 μg/mL (Kamble *et al*., 2011). This suggests the potential of *G. destructans* as a possible therapeutic agent and it should be investigated further. Future research should involve testing the fruiting bodies of *G. destructans* which may have higher activity. It would also be advised to use different solvents for extraction such as methanol or other more non-polar solvents for targeted extraction.

A study by Ruan *et al.*, (2015) tested six triterpenoids isolated from *G. lucidum* against three human carcinoma cell lines, Caco-2, HepG2, and HeLa cells. These triterpenoids; ganolucidic acid E, lucidumol A, ganodermanontriol, 7-oxo-ganoderic acid Z, 15-hydroxy-ganoderic acid S, and ganoderic acid all reduced cell growth. Ruan *et al.*, (2015) reported that the cell lines responded differently to the treatments indicating that the bioactive properties of the isolated compounds may differ between cells lines. Triterpenoids show cytotoxic effects directly on cancer cells whereas polysaccharides exert their effects by stimulating the immune system (Zhu *et al.,* 2007; Ruan *et al.*, 2015). Therefore, the cytostatic effects of the MG-LZ8 are likely due to triterpenoids found in the extract. Cytostatic agents are those which stop cancer cells from proliferating without killing the cells (Anttila *et al*., 2019). These types of agents are beneficial in many ways; in early stages of cancer, it prevents the metastases of

the cancer and allows for operable tumours to be removed. In late stages it can be used to prolong life expectancy and quality of life. However, it does not cause tumour shrinkage and needs to be administered over longer periods of time (Rixe and Fojo, 2007; Anttila *et al*., 2019). Rixe and Fojo, (2007) argued that all cytostatic agents are also cytotoxic as they cause cell cycle arrest which eventually leads to necrosis or apoptosis. The cytostatic effect of the MG-LZ8 extract would need to be further investigated by using a wider range of doses, times and exposure schedules to provide a clearer view on its anti-cancer potential.

The IC_{50} value calculated for MG-LZ8 (1) using a concentration range of 5 – 500 µg/mL was calculated to be 21.26 µg/mL. This value determines the half maximum inhibitory concentration needed to inhibit a biological function. The IC_{50} for MG-LZ8 is low indicating that it has potential as a future chemotherapeutic agent (Gertsch, 2009). The low IC_{50} indicates that at this concentration the therapeutic effects are less likely to be due to non-specific binding and more due to targeted interactions which directly affects cell proliferation thereby rarely affecting surrounding cells (Gertsch, 2009). With further investigation into the mechanism of action of MG-LZ8 cytostatic effects; it will be necessary to assess the effect of the extracts on normal cells. This is important as an effective chemotherapeutic agent has little to no effect on surrounding normal cells, as one of the aims of new chemotherapeutic agents is to reduce the side effects of current chemotherapeutic agents (Anttila *et al*., 2019).

G. lucidum is also used as an immune modulator. In-depth studies on specific compounds found within *G. lucidum* have shown that *Ganoderma* polysaccharides (*GL*P) and triterpeniods are likely responsible for anti-cancer activity (Zhu *et al,* 200; Ruan *et al.*, 2015). These *GL*P are thought to reduce tumours and cancer symptoms by stimulating the immune system. It does this by increasing the cytokine production and helping dendritic cells mature. It also improves the functioning of cytokine-induced killer cells and cytotoxic T lymphocytes (Sohretoglu and Huang, 2019).

The antioxidant activity of *G. lucidum* is well documented and ascribed to the phenolics it possesses (Mohsin *et al.*, 2011, Lin *et al*., 2015). All the *G. lucidum* (1 - 10) and *Ganoderma* spp. (11 - 20) extracts showed antioxidant activity using the DPPH assay (figures 4.7 and 4.8). The antler fruiting body extracts (3, 4) and the antler containing

extracts MGLZ-8 (1) and polar (2) extracts had slightly higher activity to those of conk fruiting body extracts (5, 6). Sudheer *et al.*, (2018) found similar results; where the antler extracts had higher antioxidant activity compared to the normal fruiting body and attributed it to the manipulation of light and ventilation used to grow the antler type fruiting body which resulted in different amounts and types of bioactive compounds. The difference in bioactive compounds were not elucidated in the Sudheer *et al.*, (2018) investigation however other studies showed that abiotic and biotic factors such as growing site, light, temperature and radiation can affect the bioactive compound profile (Cirak and Radusiene, 2019). There was a general dose-dependent increase in the activity with the ethanolic extracts showing a slightly higher activity for the *G. lucidum* conk fruiting body (5) and *G. lucidum* mycelium (7) activity compared to the aqueous extracts. The higher activity seen in the ethanol extracts compared to that of the aqueous extract is due to the ethanol being more likely to extract a higher concentration or wider range of phenols which are thought to be responsible for antioxidant activity of *G. lucidum* (Pandey and Tripathi, 2014).

The cultivated *Ganoderma* spp. extracts (11 - 20), figure 4.8, had lower antioxidant activity compared to the *G. lucidum* extracts. The *Ganoderma* spp. extracts were cultivated using a uniform procedure as the species used were still being genetically identified when they were received and confirmed later in the study. The general growth conditions used may have resulted in each species not growing in its optimal condition resulting in a low extraction yield (Jo *et al.*, 2009). This low extraction yield may have affected the quantity and quality of the bioactive components of the extracts (Hoq *et al.*, 2016). This means that the data in figure 4.8 may not be a true reflection of the antioxidant activity of each species. It would be recommended for future studies that growth conditions such as the carbon and light sources should be optimised for each species as well as allowing for the growth of fruiting bodies which could not be achieved in this investigation due to time constraints and the species identification being done concurrently to this study. This approach to future research would allow for a more holistic overview of the species bioactivities.

The mycelia extracts (7, 8) received from the University of Free state had a higher antioxidant activity for the highest concentration of 200 μg/mL, ethanolic (7) 54 ± 4.0%

and aqueous (8) 43 ± 8.0%, compared to that of the cultivated *Ganoderma* spp. where the highest antioxidant activity of 30 ± 2.60% was seen for *G. knysnamenses* type [1]. The species difference may have caused the difference in the antioxidant activity seen between the mycelial sample extracts however, Nagaraj *et al.*, (2014) showed that *G. applanatum* had similar antioxidant activity to *G. lucidum* which was not observed in this investigation. However, the growth conditions for the mycelium used in this study involved initial culture on agar followed by subculture in broth while the UFS mycelia samples (7, 8) where grown on wood chips. It has been well established that the growth condition; such as the substrate, the light and carbon source, influence the bioactivities of extracts (Cirak and Radusiene, 2019).'

Nagaraj *et al.*, (2014) used methanol, aqueous (hot water) and chloroform extracts; with the methanolic extracts showing the best result with an IC_{50} of 39.89 µg/mL. Mohsin *et al.*, (2011) also found that methanolic extracts of mycelia of wild *G. lucidum* portrayed the highest antioxidant activity compared to aqueous extracts. These studies (Mohsin *et al.,* 2011, Nagaraji *et al.*, 2014) show the importance of the solvent choice as it affects the bioactivities of the extracts produced. The solvent choice and extraction procedure used in current study imitated the home remedy method whereas the other extraction protocols focused on isolation of active compounds. There are few studies which focus on the bioactivity of other *Ganoderma* spp. apart from *G. lucidum* phylogenetic comparisons of *G. lucidum* and other species (Hapuarachchi *et al*., 2015; Hennicke *et al.*, 2016). This study is the first to report on bioactivities of *G. destructans* type*, G. eickeri,* spp. nov., *G. knysnamense,* spp. nov Type[1]*,* and *G. knysnamense,* spp. nov Type[2]. *G. destructans* type was first described by Coetzee *et al.*, (2015) and *G. eickeri,* spp. nov., *G. knysnamense,* spp. nov Type[1]*,* and *G. knysnamense,* spp. nov Type[2] was just recently isolated as species by Tchotet Tchoumi *et al.*, (2019).

The iron reducing ability of the extracts seen in figures 4.9 and 4.10 were low for the *G. lucidum* (1 - 10) extracts and barely present for the *Ganoderma* spp. (11 - 20) extracts. The results of the FRAP assay for the *G. lucidum* extracts (1 - 10) follow a similar trend to that of the DPPH assay whereby the antler fruiting body extracts (3, 4) and the antler containing MG-LZ8 (1) and polar extracts (2) had the highest activity of the G*. lucidum* extracts. This indicates that the antler fruiting body had increased activity compared to the conk fruiting body and this was most likely due to the

difference in growth conditions which leads to different bioactive profiles (Cirak and Radusiene, 2019). The antler containing extracts were followed by the ethanolic extracts of the conk fruiting body (5) and mycelium (7). This implies that the ethanolic extraction of these samples provided a wider range of phenols which are responsible for most antioxidant activity of natural products (Shahidi and Ambigaipalan, 2015). The results of the antioxidant activity in this study are lower than findings by Sa-ard *et al.*, (2015) for example who showed *G. lucidum* had ferric iron reducing activity. However, Sa-ard *et al.,* (2015) used protein extraction which could be the reason for observation of activity at an IC_{50} of 3.42 µg/mL Trolox equivalent (TE) of crude protein extract. Similarly, Sarnthima *et al.*, (2017) found FRAP activity of *G. lucidum* using protein extractions. Zengin *et al.*, (2015) found that *G. resinaceum* methanol extract had higher ferric reducing activity compared to *G. applanantum* at 36.40 mg TEs g^{-1} extract and 55.02 mg TEs g^{-1} respectively. The results in figure 4.10 showed similar results to that of Zengin *et al.*, (2015) as both *G.* cf. *resinaceum* extracts had higher ferric reducing activity to that of both *G. applanatum* extracts. The increased activity of the protein extracts seen in Sa-ard *et al.*, (2015) and Sarnthima *et al*., (2017) indicates that a targeted extraction of proteins results in a higher ferric reducing activity.

The experiments performed in this chapter reveal that not all commercial products possess the bioactivities they claim and that it is important to have stricter food supplement regulations in place. These regulations should ensure that all ingredients are listed and the evidence for the claims of bioactivities should be made readily available. The results of this chapter indicate that variability exists in the bioactivities among *Ganoderma* species and different structure of the same macrofungal species. Chapter 5 will delve into the immune modulating activity of *Ganoderma* species.

Chapter 5
Macrophage Function

5.1 INTRODUCTION

Macrophages were first identified by Elie Methchnikoff (1845-1916) as large phagocytic mononuclear cells that play a role in immunity (Merien, 2016., Atri *et al.*, 2018). The knowledge of the role and function of macrophages has increased significantly since their discovery however many aspects of their functions still remain a mystery. Macrophages are present in all tissues of the body and can change their phenotype according to the environment of the tissue or organ they are located in (Paul *et al.,* 2019). The different phenotypes result in many subsets of macrophages with multiple functions, with many of the exact functions remaining unknown (Murray *et al.,* 2014, Paul *et al.,* 2019).

Macrophages play a crucial role in both the innate and adaptative immunity. Innate immunity is a nonspecific defence mechanism that is activated immediately or soon after a pathogen is detected. Being nonspecific, it relies on a group of proteins and phagocytotic cells to recognise pathogen features in order to destroy them (Alberts *et al.,* 2002, Hirayama *et al*., 2018). The macrophages and dendritic cells in the innate immunity have pattern recognition receptors (PRR), which are surface receptors that recognise pathogen-associated molecular patterns (PAMPs). These PAMPs include; lipopolysaccharides (LPS) produced by gram negative bacteria, viral nucleic acid and or damage-associated molecular patterns (DAMPs) which are expressed by cells when they are exposed to harmful stimuli (Alberts *et al.,* 2002., Moroni *et al.,* 2019).

Adaptive immunity is specific and has features that allow the functional cells to ‘remember’ pathogens and enables them to launch a specific attack against pathogens they have encountered before. The adaptive immunity is slow to develop, with it taking approximately a week or more before the response is manifested and effective (Alberts *et al.,* 2002, Hirayama *et al*., 2018). In support of their defence against pathogens macrophages play an essential role in inflammation regulation (Paul *et al.,* 2019). They are responsible for resolving inflammation, play a role in wound healing and ensure that homeostasis is reached within the damaged tissue (Hirayama *et al*., 2018).

Macrophages are capable of changing their structure and function according to stimuli, this phenomenon is known as macrophage polarisation. (Atri *et al.,* 2018, Paul *et al.,* 2019). The polarisation of macrophages has been classified into two major clusters, these clusters include the classically activated M1 macrophages and the M2 alternatively activated macrophages (Murray *et al.,* 2014). The original classification of these two phenotypes was based on the different arginine metabolism observed in mice which had different T helper backgrounds, namely type 1 (Th1) and type 2 (Th2). Polarisation is mostly time dependant and is affected by the tissue type (Davis *et al.,* 2013, Atri *et al.,* 2018). The factors that affect polarisation include stimuli such as cytokines, growth factors and the pathogen derived molecules, (refer to table 1.1 for detailed stimuli examples). It is important to note that although the classification of the M1 and M2 phenotypes has been well established these phenotypes have high plasticity and are capable of phenotype loss, phenotypic switching and modulation (Paul *et al.,* 2019). In addition to M1- like and M2-like phenotypes, regulatory macrophages have recently been discovered to limit inflammation during immune responses (Murray *et al.,* 2014, Atri *et al.,* 2018).

Forming part of the innate immunity, macrophages are the first line of defence against pathogens; bacterial and viral pathogens in particular (Martinez and Gordon, 2014). They promote the Th1 polarisation of CD4 cells in humans; Th1 cells produce interferon gamma (IFN-γ) which is needed for the clearance of intercellular infection (Orecchioni *et al*., 2019). M1 macrophages respond to inflammatory environments and are signalled by interferons (IFN) and Toll-like Receptors (TLR). These macrophages have high phagocytotic activity and guide inflammatory responses by producing a wide array of cytokines and chemokines (Davis *et al.,* 2013, Orecchioni *et al*., 2019). These cytokines and chemokines result in the activation the Th 1 response, which in turn causes activation of the complement mediated phagocytosis and inflammation. These macrophages have high antigen presenting capacity and are characterised by their phagocytosis of microorganisms and debris in the early phases of healing (Martinez and Gordon, 2014, Atri *et al.,* 2018).

M2 macrophages primarily respond to fungal, helminthic, and parasitic infections and are induced via the Th2 response, Th2 cells produce interleukin 4 (IL-4), IL-5, and IL-13 as well as the macrophage colony-stimulating factor (M-CSF) (Atri *et al.*, 2018). They regulate proinflammatory cytokines and induce the production of anti-

inflammatory mediators; for example IL-4, IL-10 (Davis *et al.,* 2013, Orecchioni *et al*., 2019). They are mostly observed in repair mechanisms and are highly endocytic but are only partially phagocytic. In response to anti-inflammatory or regulatory responses they produce molecules which recruit neutrophils and T lymphocytes (Martinez and Gordon, 2014). There are three subsets of M2 macrophages that have been classified; these include; M2a, M2b and M2c. These subsets are stimulated by different molecules and express different surface markers (Atri *et al.,* 2018).

Nitric oxide (NO) is a molecule produced by macrophages and other immune cells such as dendritic cells. When macrophages are activated they produce nitric oxide (NO) from L-arginine, an amino acid, using the enzyme nitric oxide synthase as shown in Figure 5.1 (Schairer *et al.,* 2012, Vishwakarma *et al*., 2019).

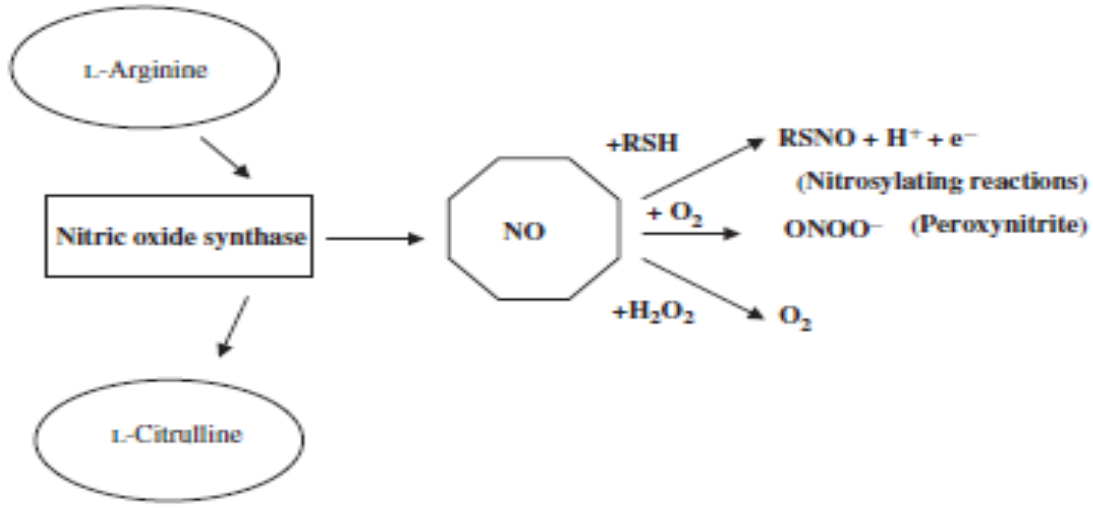

Figure 5.1: Synthesis of nitric oxide (Tripathi *et al.*, 2007)

NO has many functions some of which have nothing to do with the immune system. It is a vasodilator and acts as a vascular tone regulator as well as a neurotransmitter. However, its most well-known function is that of being a toxic agent against pathogens (Tripathi *et al*., 2007*,* Sivaloganathan and Brynildsen, 2020, Weigand *et al*., 2021). It does this in two ways; the first being at low concentration when it increases the activity and growth of immune cells such as; T-lymphocytes, antigen- presenting cells and neutrophils. Secondly, at high concentration it binds to DNA, proteins and lipids interrupting their function and disrupting cell function causing death of the pathogen

or cells (Schairer *et al.,* 2012, Sivaloganathan and Brynildsen, 2020). It also has a regulatory function by inducing or regulating the function and death of the host immune cells (Tripathi *et al.,* 2007). Like other reactive oxygen species (ROS), NO in high concentration can be toxic to the host cell. This prolonged inflammatory response can lead to many diseased states such as asthma, impaired wound healing and atherosclerosis (Tripathi *et al.,* 2007).

One of the primary functions of macrophages is phagocytosis and cells that are capable of phagocytosis are known as phagocytes. The process of phagocytosis involves several phases; the first involves the recognition of foreign entities and then the activation of the process for ingestion of particles (Fig 5.2). This is followed by the formation of the phagosome and lastly the phagosome maturation (Uribe-Querol and Rosales, 2020). A foreign entity or particles that are typically larger than 0.5 μm are targeted by phagocytes. Phagocytosis is necessary for the elimination of microorganism and apoptotic cells. Due to the wide range of target cells, phagocytes have a variety of cell receptors that allow them to recognise target cells and initialise phagocytosis. Examples of such cell receptors are IgG or IgA antibodies or complement components such as iC3b (Lindner *et al*., 2020, Uribe-Querol and Rosales, 2020). Once the target cells are recognised and the receptor binds to the ligand on the surface of the particle it signals the pathway that regulates the actin cytoskeleton. This leads to the remodelling of the actin cytoskeleton and lipids within the membrane of the cell and extend the membrane to cover the target particle. Once the particle is enclosed the membrane pinches off and the phagosome is formed (Rosales and Uribe-Querol, 2017). The phagosome matures to become a phagolysosome. It has an acidic and degradative environment, containing ROS and enzymes, which are capable of destroying the phagocytosed particle (Lindner *et al*., 2020, Uribe-Querol and Rosales, 2020).

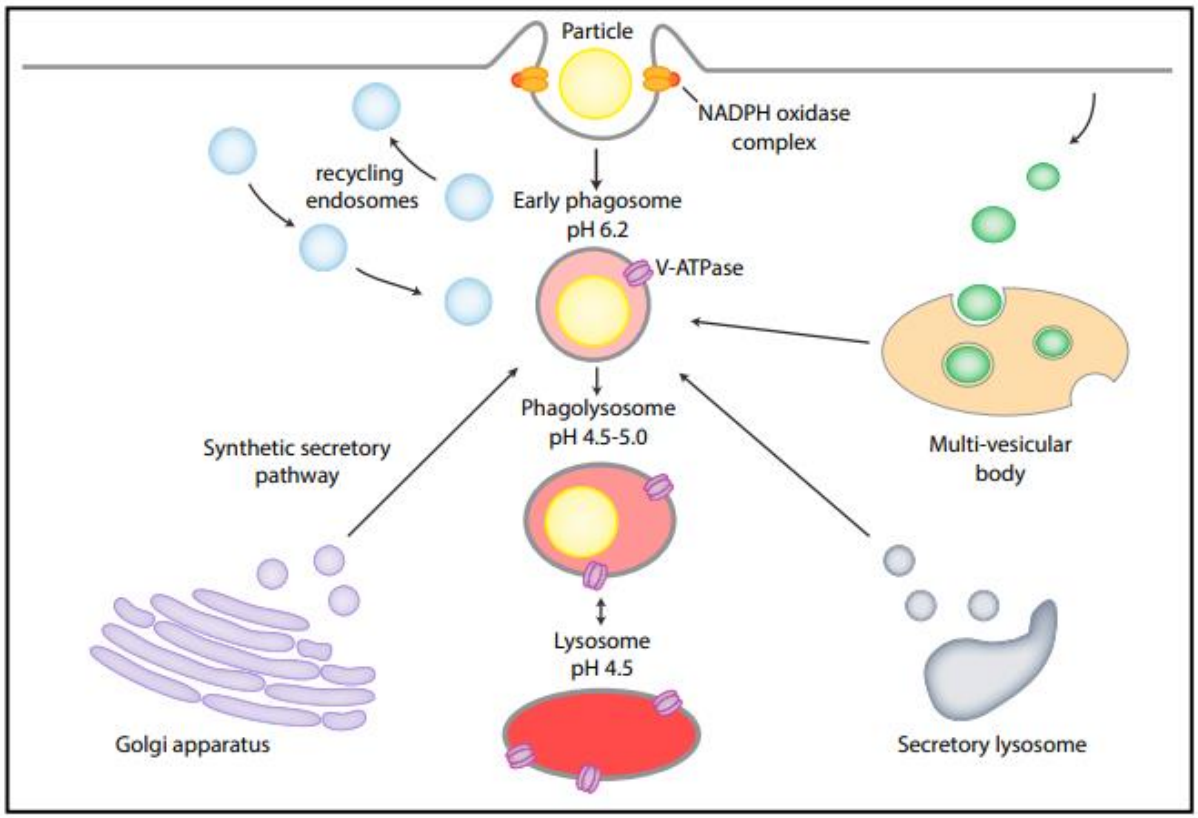

Figure 5.2: Summarised phagocytic pathway (Gordon[1], 2016)

The aim of this chapter was to determine the cytotoxicity of the *G. lucidum* and other *Ganoderma* spp. extracts on RAW 264.7 macrophage-like cells and determine their potential effect on macrophage function. This was assessed by analysing NO production in the presence and absence of LPS, determining the phagocytic activity in conjunction with acidic vacuole production and changes in the actin polymerisation. Lastly the effect of the macrofungal extracts on the macrophage phenotype changes was analysed.

5.2 MATERIALS AND METHODS

5.2.1 Cell Maintenance

The RAW 264.7 macrophage-like cells were maintained in 10 cm cell culture dishes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and incubated at 37ºC in a humidified incubator with 5% CO_2. The cells were sub-cultured when they reached 80% confluency.

5.2.2 Cytotoxicity

Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Holst-Hansen and Brünner (1998). MTT is a positively charged tetrazolium dye which is capable of penetrating eukaryotic cells. The principle of MTT assay is that viable cells with active metabolism are capable of reducing MTT to a purple coloured formazan product which has an absorbance of 540nm. Dead cells are unable to convert MTT and thus the colour change serves as a marker for viable cells (Riss *et al.,* 2016). The principle can be seen in figure 5.3.

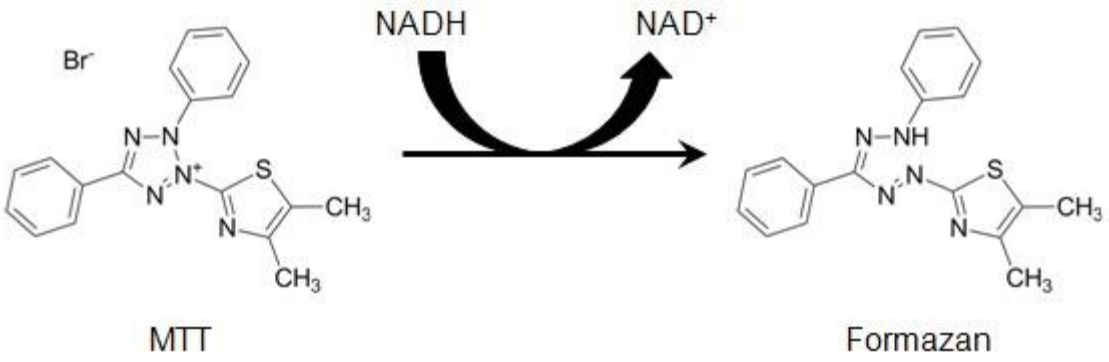

Figure 5.3: Chemical structure of MTT and the formazan product produced by viable cells (Riss *et al.,* 2016).

RAW 264.7 macrophages were seeded in 96-well plates at cell densities of 20 000 cells/ well. The cells were then incubated overnight at 37ºC in a humidified 5% CO_2 incubator to allow for attachment. Following the overnight incubation, the cells were treated with the *Ganoderma* spp. extracts 1 – 20 (table 2.3). Stock concentrations were prepared in DMSO at 100 mg/mL. The working concentrations were prepared in complete medium (DMEM and 10% FBS) and concentrations ranging between 0 - 200 µg/mL were tested. The cells were treated for 48 hours at 37ºC in a humidified 5% CO_2 incubator. The medium containing extract was gently aspirated prior to the addition of 100 µL MTT (0.5 mg/mL) and incubated for 3 hrs. Thereafter, the medium was aspirated, and the formazan product solubilized in 100 µL of DMSO. A DMSO vehicle control was included to ensure that it did not affect the viability of the cells. The absorbance was measured at 540 nm using a BioTek® PowerWave XS

Spectrophotometer (Winooski, VT, USA). The cell viability was calculated as a percentage of the untreated control.

5.2.3 Nitric oxide production

The NO production by treated RAW 264.7 macrophages was assessed using the Griess reaction. Pro-inflammatory activity was measured in naïve RAW 264.7 macrophages (not exposed to LPS) while anti-inflammatory activity was measured in cells activated with lipopolysaccharide (LPS). NO released into the medium can then be quantified by adding the Griess reagent which results in a colour change that can be measured spectrophotometrically (Vishwakarma *et al*., 2019). Figure 5.4 illustrates the Griess reaction, which is a two-step diazotization reaction in which dinitrogen trioxide (N_2O_3) generated from the formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to produce a diazonium ion which is then coupled to *N*-(1-napthyl) ethylenediamine to form an azo product that absorbs at 540 nm (Bryan and Grisham, 2007, Vishwakarma *et al*., 2019).

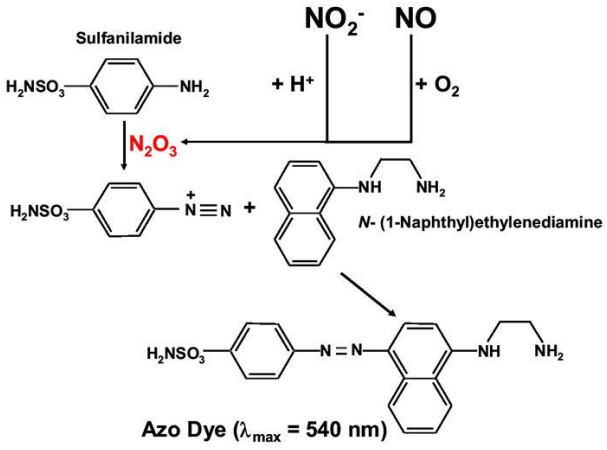

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Figure 5.4 Griess reaction mechanism used for the detection of nitric oxide production (Bryan and Grisham, 2007).

This assay was initiated by seeding a 100 µL of RAW 264.7 cells at a density of 40 000 cells per well into a 96-well microtitre plate and left to attach overnight at 37 °C. Thereafter the medium was gently aspirated, and cells were treated with 100 µL of the treatment (*Ganoderma* spp. extracts) at a concentration range of 0 – 200 µg/mL. Duplicate plates were produced where one was treated with a 100 µL of 200 ng/mL LPS (for activated macrophages) and the other 100 µL of complete medium was added (for naïve macrophages). These plates were incubated for a further 24 hours

at 37 °C. For the Griess reaction; 50 µL of the culture medium from each well was transferred into a new 96-well plate and 50 µL of Griess reagent (Sigma-Aldrich) was added to each well. The plates were incubated for 10 minutes in the dark at room temperature after which the absorbance was measured at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). Amino-guanidine (Sigma-Aldrich) at a concentration of 100 µg/mL was utilised as a positive control for NO inhibition.

5.2.4. Image Cytometry techniques

Fluorescence micrographs were captured using Molecular Devices ImageXpress Micro XLS Widefield microscope for high content analysis. Image analysis was performed using the Multiwavelength Cell Scoring analysis module of the MetaXpress® software (Molecular Devices).

5.2.4.1 Phagocytic activity and acidic vesicle formation

The phagocytic capability of RAW 264.7 macrophage-like cells was assessed using the pHrodo™ Green BioParticles® Conjugate (Thermo Fisher Scientific). The pHodo™ particle contains unopsonised *Escherichia coli* particles that are conjugated to pHrodo™ Green. pHrodo™ Green is a dye which is fluorogenic and only fluoresces when in an acidic environment and the intensity increases with the decrease in the pH. At a neutral pH, like outside of the cell, the conjugate does not fluoresce (Kapellos *et al*., 2016). In addition to the phagocytic activity the acidic vacuole formation was assessed using LysoTracker® Red (Thermo Fisher Scientific). LysoTracker® Red dye is used to track and selectively label acidic organelles in live cells. The probes consist of a fluorophore that is linked to a weak base that is only partially protonated at a neutral pH and this allows it to freely permeate the cell membranes to label live cells (Thermo Fisher Scientific).

To assess the phagocytic and acidic vacuole formation 100 µL of RAW 264.7 macrophage-like cells were seeded into a 96-well plate at 20 000 cells per well in DMEM without phenol red supplemented with 10 % FBS and left to attach overnight at 37°C. The cells were treated with 50 µL of the extracts at a concentration range of

0 – 200 µg/mL for 24 hrs at 37°C. After the 23rd hour of the incubation LPS at 200 ng/mL was added to the appropriate wells for an hour to serve as a positive control for the stimulation of phagocytosis. Thereafter 20 µL of pHrodo™ Green BioParticles (20 µg/mL) was added and incubated at 37°C for 2 hrs followed by the addition of LysoTracker® Red at 50nM. Image acquisition as described in section 5.2.4.4 and analysis was performed using the MetaXpress® software (Molecular Devices).

5.2.4.2 Phalloidin Staining

The RAW 264.7 macrophage cells were seeded and treated as explained in section 5.2.4.1. After the phagocytic and lysotracker red staining was imaged the culture medium was aspirated, cells fixed by adding 100 µL of 4% formaldehyde, incubated for 15 min at room temperature in the dark, and washed with DPBS. The cells were stained with phalloidin-TRITC (1 µM; 100 µL per well) for 15 min at 37 ºC in the dark. Subsequently, the nuclei of cells were stained with Hoechst 33342 (5 µg/mL, 100 µL per well). Image acquisition as described in section 5.2.4.4 and analysis was performed using the MetaXpress® software (Molecular Devices).

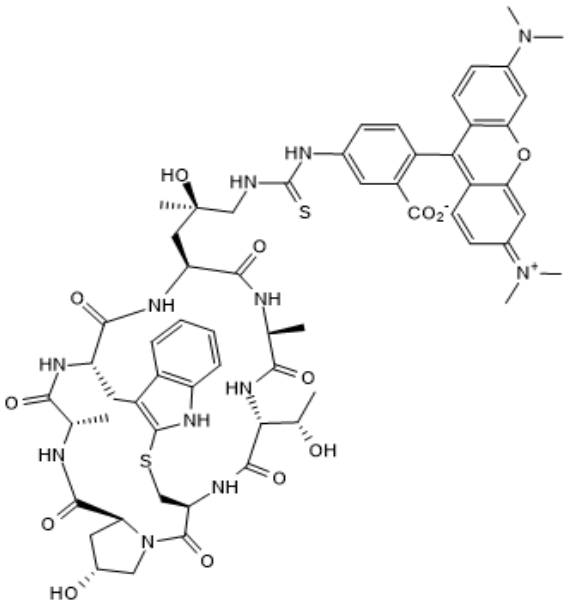

Figure 5.5. Chemical structure of Phalloidin-TRITC (RNDSystems, 2022)

5.2.4.3. Analysis of M1/M2 phenotypic changes in macrophages

Macrophages are known to have high plasticity and are capable of changing their phenotypes according to the signals they receive from the immune system. These different macrophage phenotypes result in macrophages which have different cell surface receptors which can be analysed using antibodies that are specific for a particular macrophage type. M1 and M2 macrophage activation was assessed using the Alexa-Fluor® anti-mouse CD86 and the APC anti-mouse CD206 antibodies from BioLegend®, respectively.

Briefly, RAW 264.7 macrophages were seeded into 12-well plates at a seeding density of 200 000 cells per well and left to attach overnight at 37°C in a humidified 5% CO_2 incubator. The cells were then treated with the *Ganoderma* spp. extracts at concentrations ranging from 0 – 200 µg/mL and incubated for 24 hours. LPS at a concentration of 200 ng/mL and curcumin at a concentration of 20 µg/mL were included as positive controls for CD86 (M1 phenotype) and CD206 (M2 phenotype), respectively. The cells were scraped from the wells with sterile plastic scrapers and transferred into Eppendorf tubes. Thereafter they were washed twice with 500 µL PBS (+ Ca^{2+}/Mg^{2+}) by centrifugation at 500 rcf for 5 minutes. The cells were blocked with 500 µL of 1% BSA in PBS for 15 minutes at room temperature and washed again. The antibodies were prepared using 1% BSA in PBS using a 1:300 dilution for CD86 and a 1:400 dilution for CD206. Samples were stained with 100 µL of diluted antibody for 30 minutes at 37°C in the dark. An isotype control for each fluorophore was included using a 1:400 dilution for the APC-conjugate and a 1:300 dilution for the Alexa-Fluor® conjugate. The cells were washed twice as before and resuspended in 350 µL of PBS (+ Ca2+/Mg2+). A 175 µL aliquot of each sample was transferred into a 96-well microtitre plate and nuclei counterstained with 5 µg/mL Hoechst 33342. The cells were allowed to settle to the bottom of the plate for an hour before image acquisition as described in section 5.2.4.4 and analysis was performed using the MetaXpress® software (Molecular Devices).

5.2.4.4. Image acquisition and analysis

All fluorescence images were acquired using the ImageXpress Micro XLS (Molecular Devices). Image analysis was performed using the Multiwavelenth Cell Scoring module of MetaXpress software. LysoTracker® Red and the pHrodo™ Green

BioParticles® use a 20X magnification of nine sites (figure 3.4) to record the mean cell integrated intensity. Fluorescent micrographs for the phalloidin staining used the same nine image sites per well (Figure 3.4) configuration conjunction with a 40X magnification. Fluorescence micrographs used to image the macrophage phenotypic changes used a 10X magnification of nine image sites per well (Figure 3.4) to determine the percentage of cells stained positive for CD86 and CD206. The ImageXpress Micro XLS instrument configuration for each assay is summarised in Table 5.1.

Table 5.1 Instrument configurations for ImageXpress Micro XLS (Molecular Devices)

Assay	Magnification	Dye utilised	Filter Cube	Excitation/ Emission
Phagocytic activity	20x	pHrodo™ Green	FITC	532
Acidic Vesicle formation	20x	LysoTracker® Red	Texas Red	562/624
Phalloidin Staining	40x	phalloidin-TRITC	TRITC	543/593
Analysis of M1 phenotypic changes	10x	Alexa-Fluor® 448	FITC	482/536
Analysis of M2 phenotypic cha	10x	APC	Cy5	628/692

5.3 RESULTS

5.3.1 Effect of *Ganoderma* spp. extracts on macrophage function of Raw 264.7 cells

The macrophage function of the RAW 264.7 cells were determined by their nitric oxide (NO) and phagocytotic activity as well as their ability to undergo phenotypic polarisation. The results for each extract were grouped together into a composite figure and is represented in figures 5.6 – 5.25 to give a clear view of all its effects. Figures 5.6 – 5.15 were all grouped together to represent the results obtained with the *G. lucidum* commercial samples, antler-, conk fruiting body and mycelium extracts (1 – 10). Results for the cultivated *Ganoderma* spp. mycelium extracts (11 – 20) were grouped together as Figures 5.16 – 5.25. The positive control results are identical in all these figures.

The cell viability after treatment with the extracts was determined by the MTT assay. None of the extracts except MG-LZ8 (1) had a negative effect on cell viability in the range of 50 – 200 μg/mL (figures 5.7 – 5.25 A). The concentration range for the MG-LZ8 (1) extract had to be adjusted to 12.5, 25 and 50 μg/mL, (figure 5.6 A), as higher concentrations were cytotoxic (not shown). The highest concentration used for the macrophage experiments was therefore 50 μg/mL for MG-LZ8 and 200 μg/mL for all the other extracts.

The pro- and anti-inflammatory activity of the different extracts were tested by treating cells with each extract at the selected non-toxic concentrations, in the absence of LPS (figure 5.6 - 5.15 B) or presence of LPS (figure 5.6 – 5.15 C) for 24h before quantifying the amount of NO released by the cells. As expected for the pro-inflammatory effects, (figure 5.6 B), LPS caused a significant increase in NO production compared to control cells, while all extracts had no effect on activation of the macrophages. In the anti-inflammatory assay (figure 5.6 – 5.15 C), the positive control aminoguanidine (AG) significantly reduced the LPS-induced NO production. None of the extracts showed a reduction in LPS-induced NO production.

The phagocytotic activity was measured by means of Phrodo Green *E. coli* bioparticles and Lysotracker Red (LTR). M1 macrophages are highly phagocytic because one of their functions is to remove invading pathogens. Once endocytosed, the phagosomes will fuse with lysosomes to become phagolysosomes with a low intra-vesicular pH (Lee

et al., 2020). Lysotracker Red is a fluorescent dye that specifically stains acidic organelles in live cells and can be used to track the development of phagolysosomes (Chen *et al.,* 2015). LTR staining was therefore used as confirmation of the Phrodo Green results. There was an increase in LTR and Phrodo Green *E. coli* bioparticle staining respectively, with LPS (figure 5.6 -5.15 D and E). In addition to LTR and Phrodo Green particle staining, polymerised actin was also stained using phalloidin conjugated to a fluorophore. Actin polymerisation is an essential process in endocytosis (and therefore also phagocytosis) because it facilitates the formation of membrane protrusions and invagination in order to engulf the particle (Akamatsu *et al.,* 2020). It was expected that there would be an increase in phalloidin staining when phagocytosis is enhanced, but this was not observed with LPS (figure 5.6 - 5.15 F).

The results in figure 5.6 D reflect that the MG-LZ8 extract reduced the number of acidic vesicles found in the cells compared to the untreated controls. The phagocytic activity of the cells was, however not affected by treatment with the extract as they appeared to have the same level of activity as the untreated cells, figure 5.6 E. The phalloidin staining in figure 5.6 F showed that there was no change in the filamentous actin (F-actin) content of the treated cells compared to the untreated cells.

Phagocytosis results for the antler (figure 5.8 and 5.9 D) and conk fruiting body (figures 5.10 and 5.11 D) ethanolic and aqueous extracts were very similar to that of MG-LZ8 where a significant decrease was observed in LTR staining compared to control cells, without any changes in Phrodo Green or phalloidin staining. The remaining extracts of the *G. lucidum*, such as the polar extract (figure 5.7 D), the mycelia extracts (figures 5.11 and 5.12 D) and the Medi Mushroom extracts (figures 5.13 and 5.14 D) induced little to no significant changes in any of these parameters associated with phagocytosis.

The M1 and M2 polarisation was determined by exposing the RAW 264.7 macrophages to the extracts and then determining their CD86 and CD206 expression by immunofluorescent staining. The integrated intensity of each was compared to the untreated sample, expressed as a percentage with the untreated sample set as 100%. LPS and curcumin were used as positive controls for M1 (CD86) and M2 (CD206) polarisation, respectively. One can see that the CD206 marker was detected at significantly higher levels in curcumin treated cells (220%) compared to controls

(100%), while CD86 was upregulated to 130% in LPS treated cells (figure 5.6 G). MG-LZ8 gave a dose dependant increase in CD206 with the highest concentration having an intensity of 106 % higher than the control (i.e., 206% vs 100%). This is similar to that of the positive control curcumin with an intensity of 120% above that of the control cells (i.e., 220% vs 100%).

Similar results of M2 polarisation were observed for the polar (figure 5.7 G) and antler extracts (figures 5.8 and 5.9 G). The conk fruiting body extracts (figures 5.10 and 5.11 G) showed the highest levels of CD206 upregulation of all the extracts at all 3 concentrations tested. The mycelia (figures 5.11 and 5.12 G) and Medi Mushroom extracts (figures 5.13 and 5.14 G) did not induce any significant changes in the CD206 or CD86 marker detection compared to the untreated control.

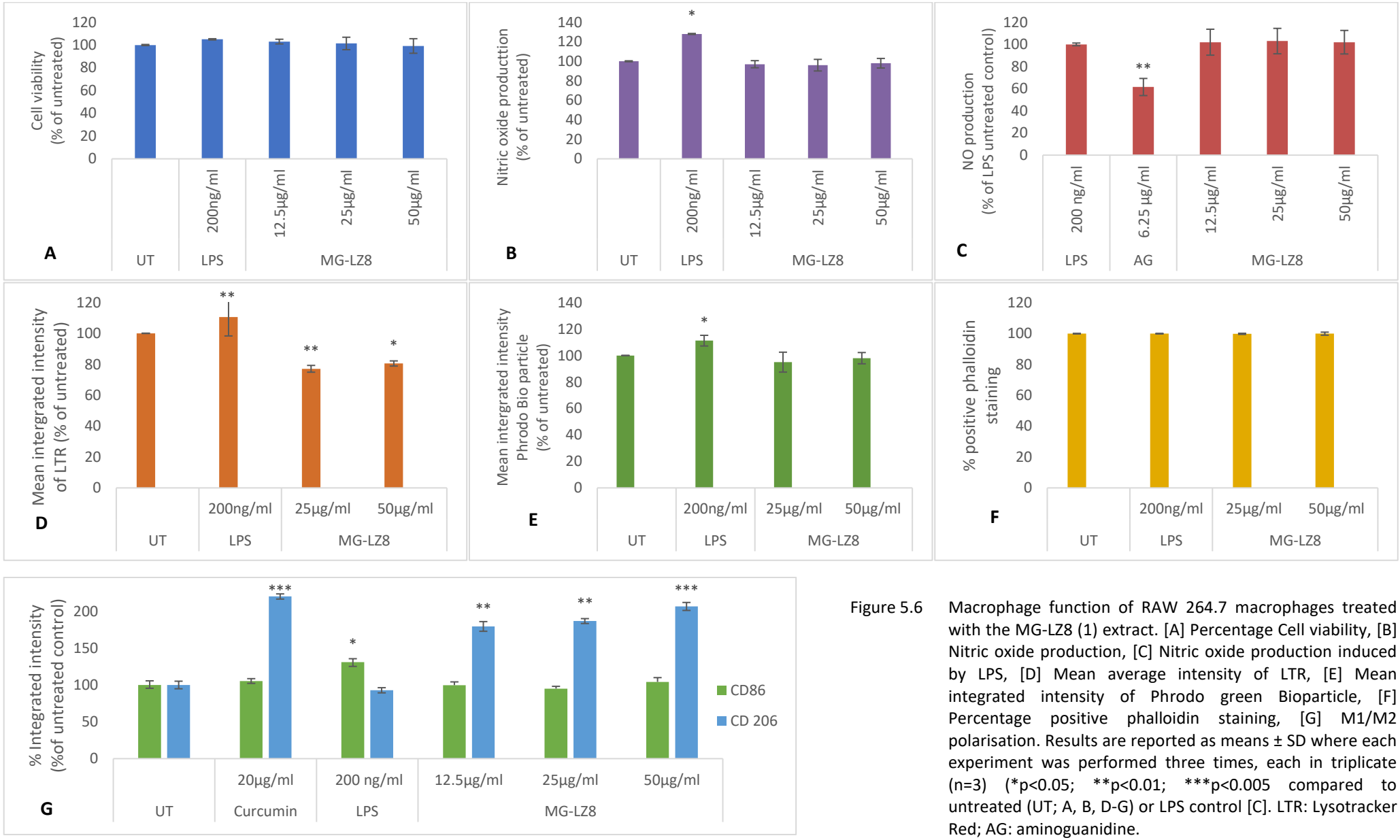

Figure 5.6 Macrophage function of RAW 264.7 macrophages treated with the MG-LZ8 (1) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. Results are reported as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated (UT; A, B, D-G) or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

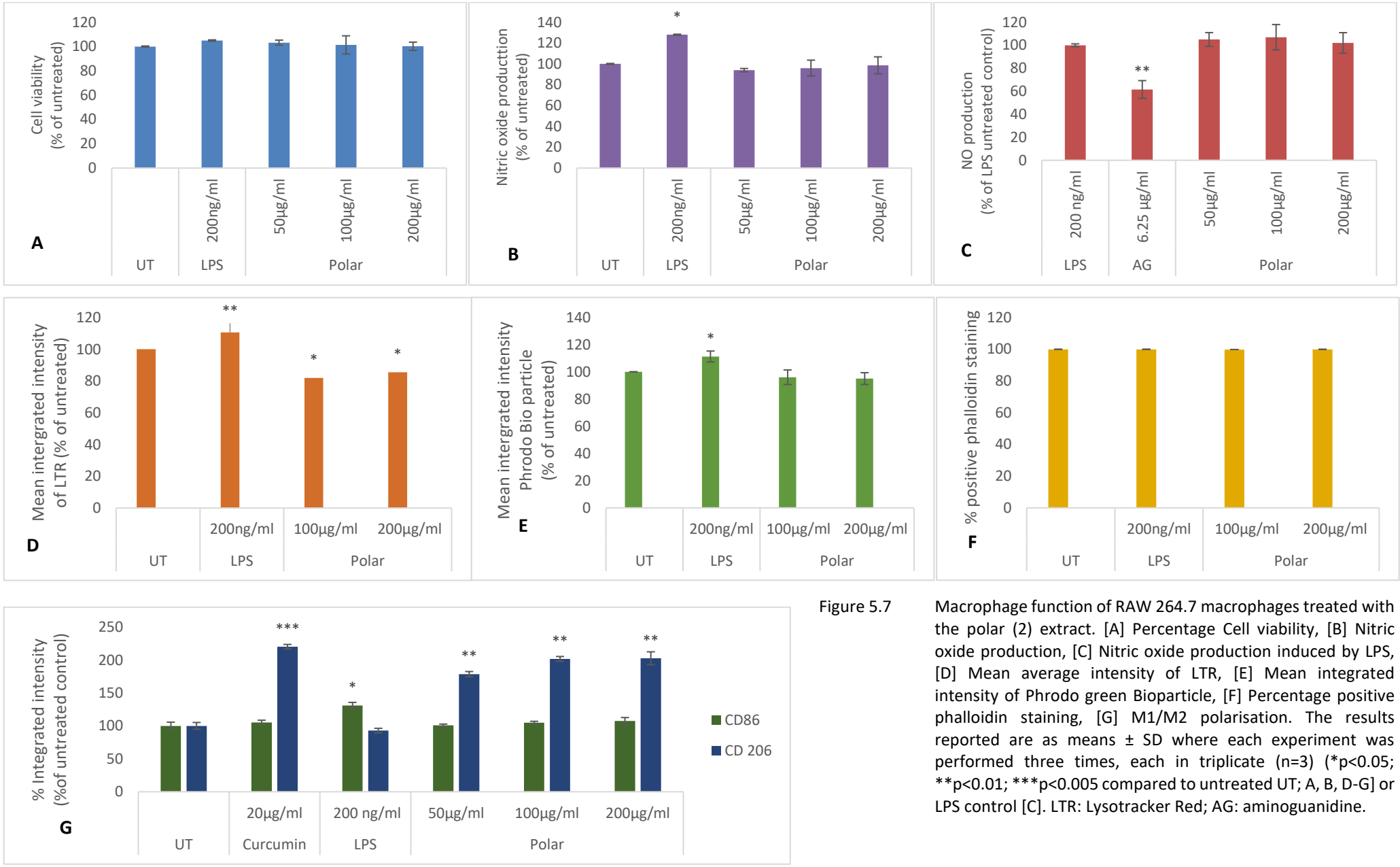

Figure 5.7 Macrophage function of RAW 264.7 macrophages treated with the polar (2) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

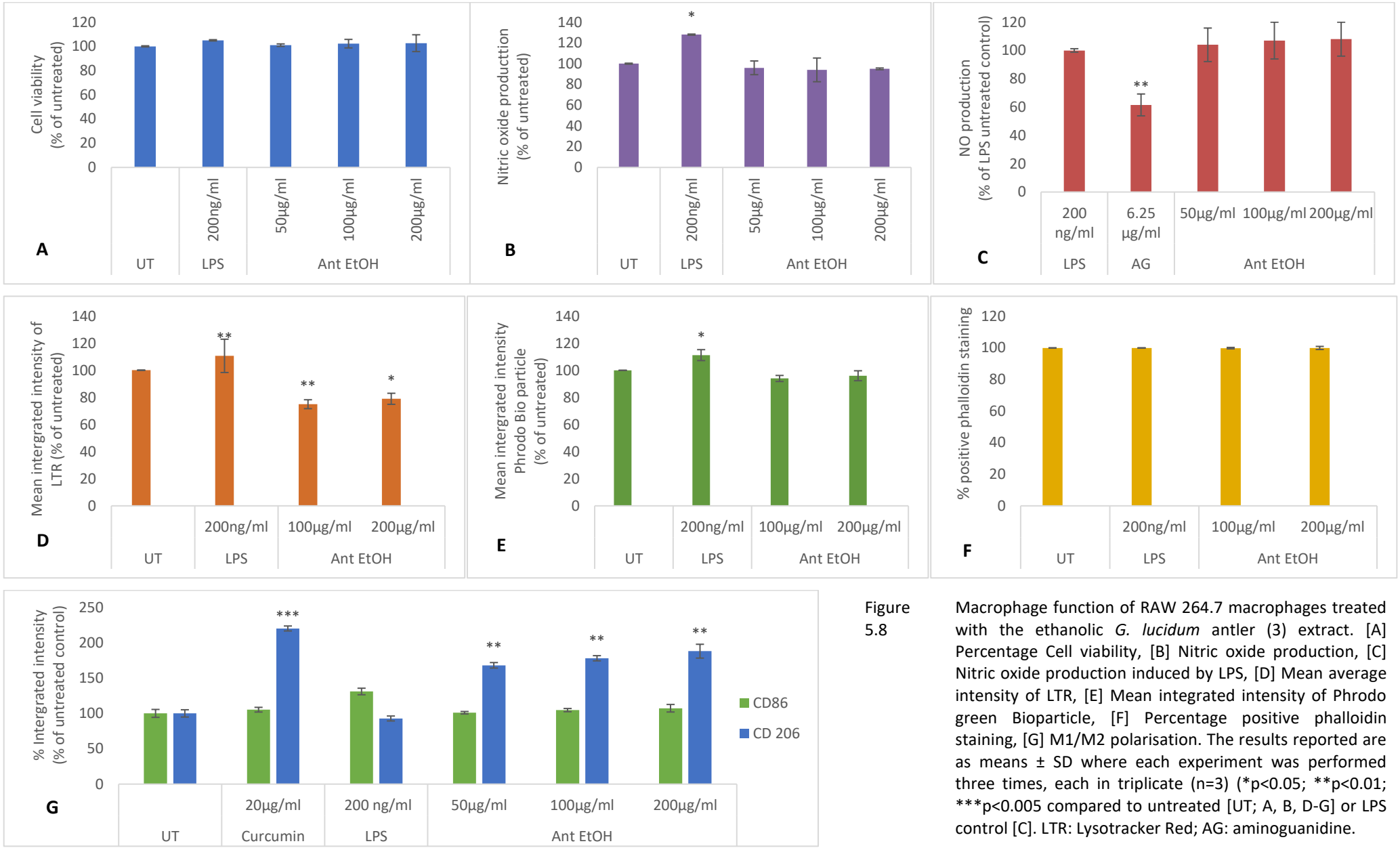

Figure 5.8

Macrophage function of RAW 264.7 macrophages treated with the ethanolic *G. lucidum* antler (3) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

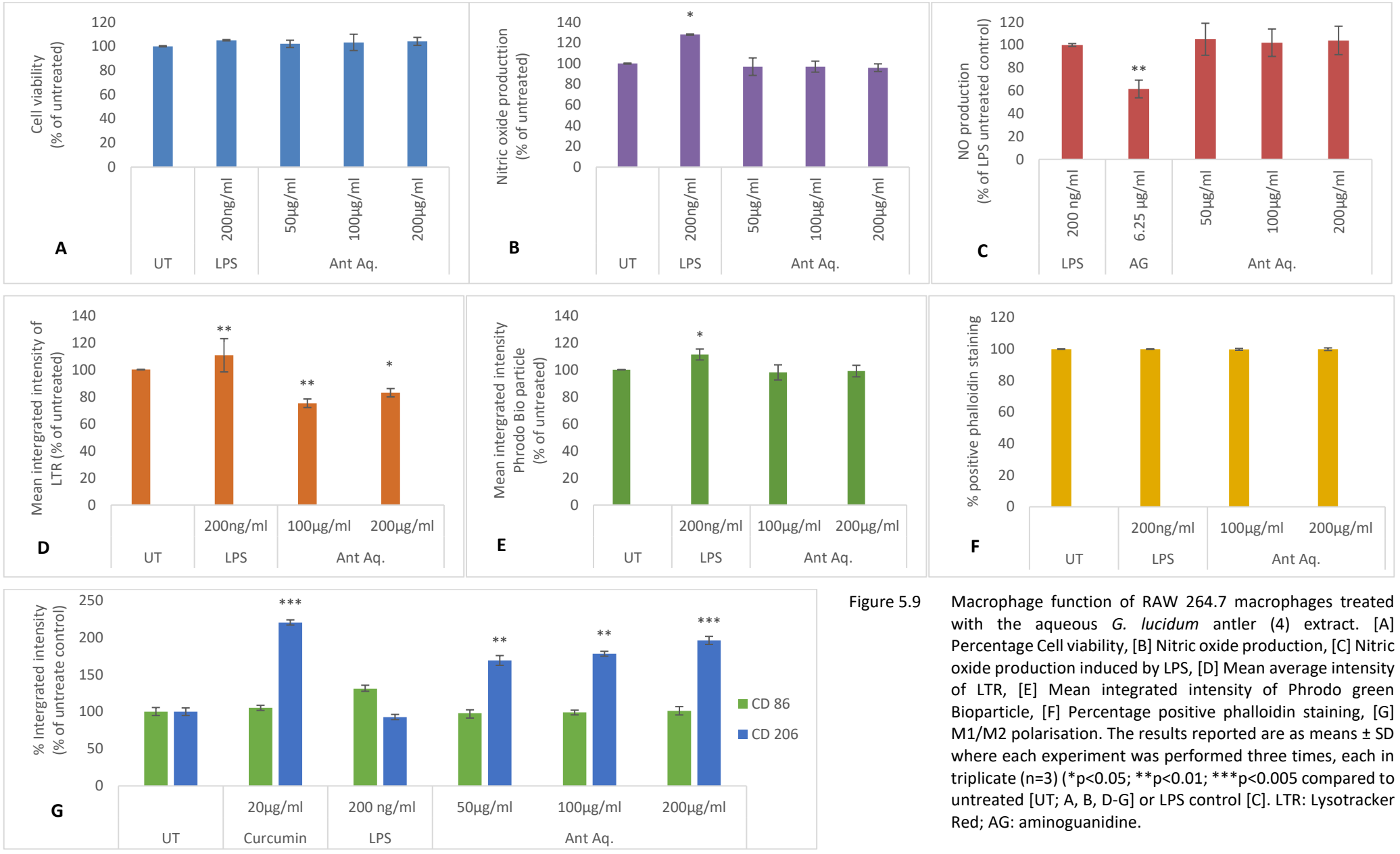

Figure 5.9 Macrophage function of RAW 264.7 macrophages treated with the aqueous *G. lucidum* antler (4) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

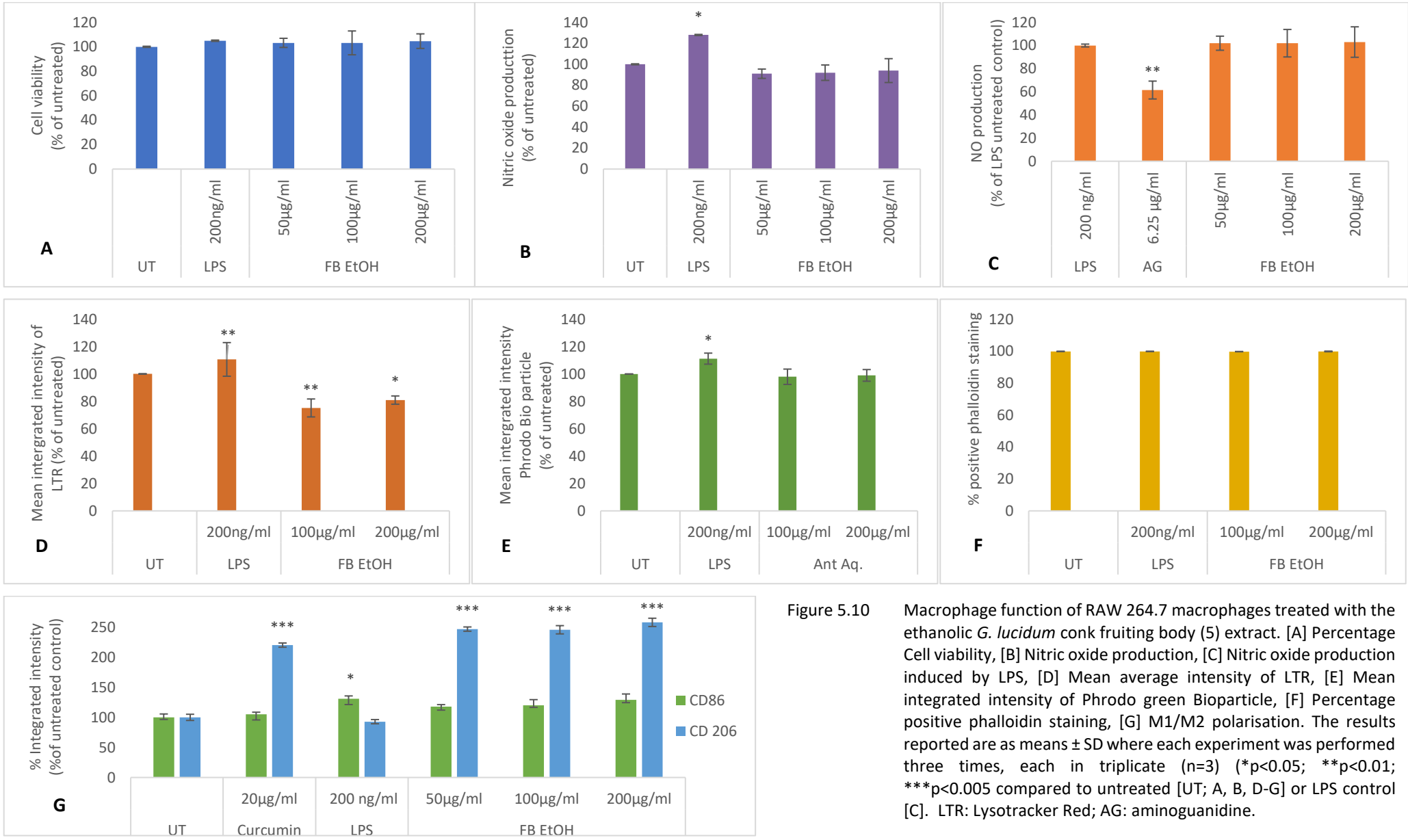

Figure 5.10 Macrophage function of RAW 264.7 macrophages treated with the ethanolic *G. lucidum* conk fruiting body (5) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

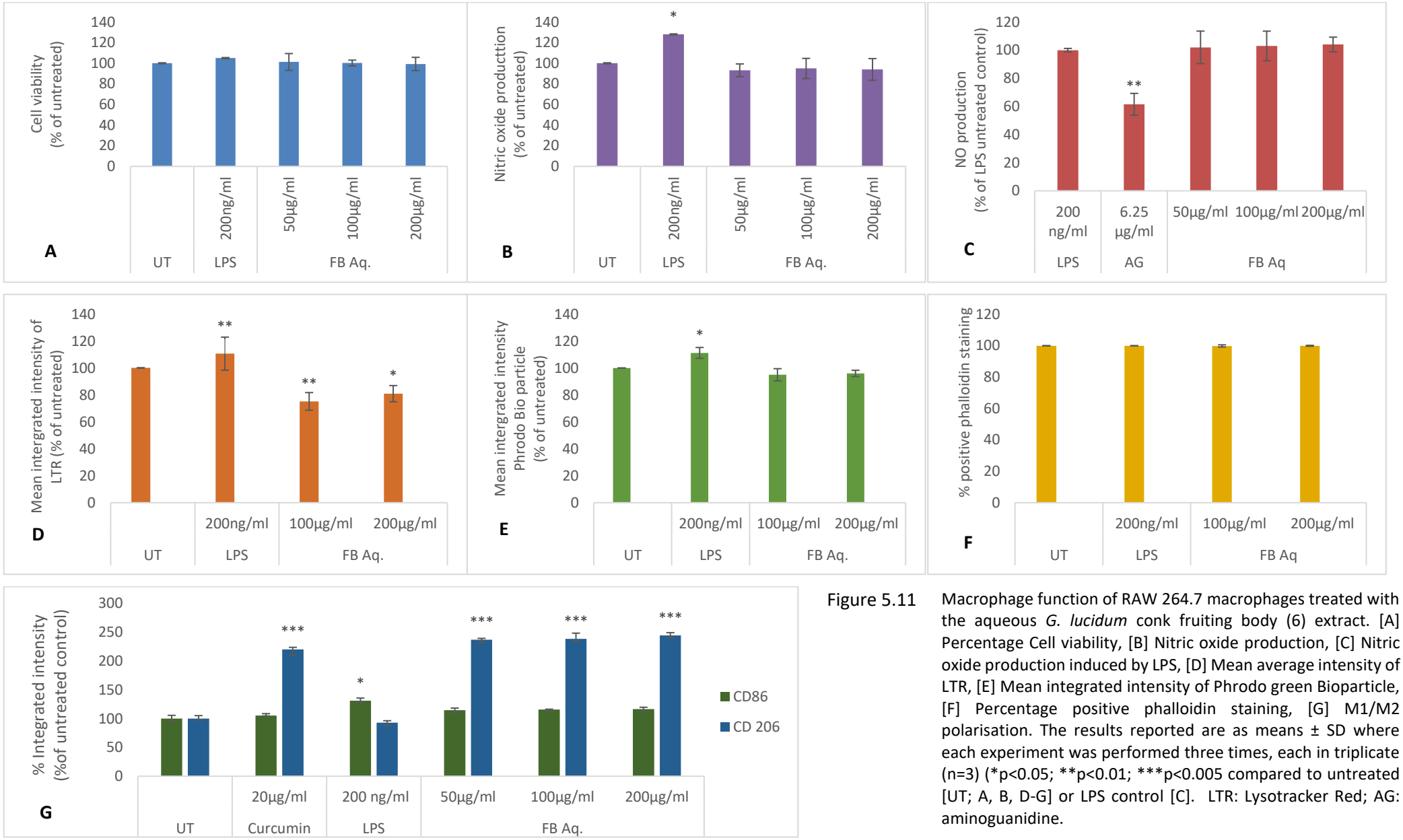

Figure 5.11 Macrophage function of RAW 264.7 macrophages treated with the aqueous *G. lucidum* conk fruiting body (6) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

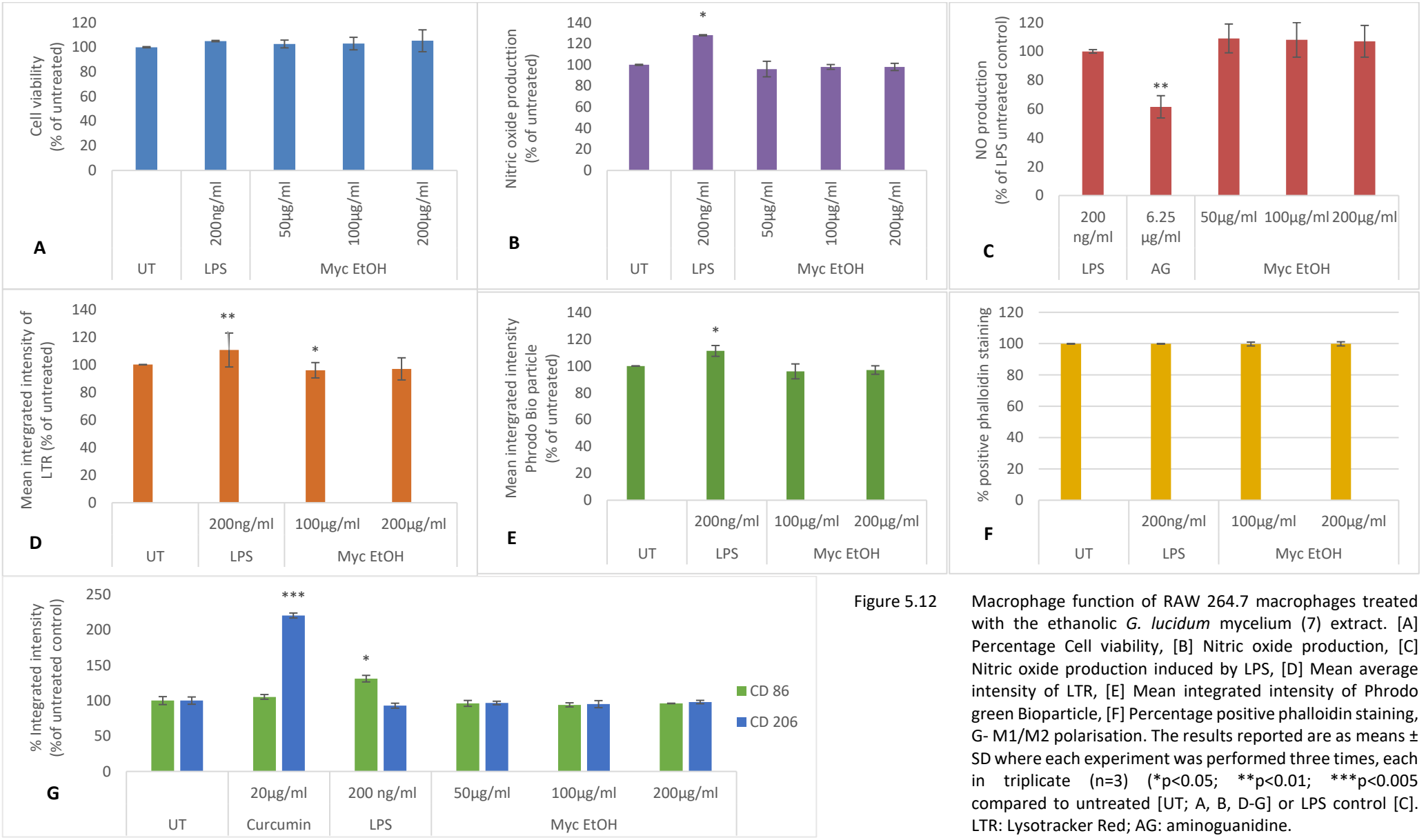

Figure 5.12 Macrophage function of RAW 264.7 macrophages treated with the ethanolic *G. lucidum* mycelium (7) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, G- M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

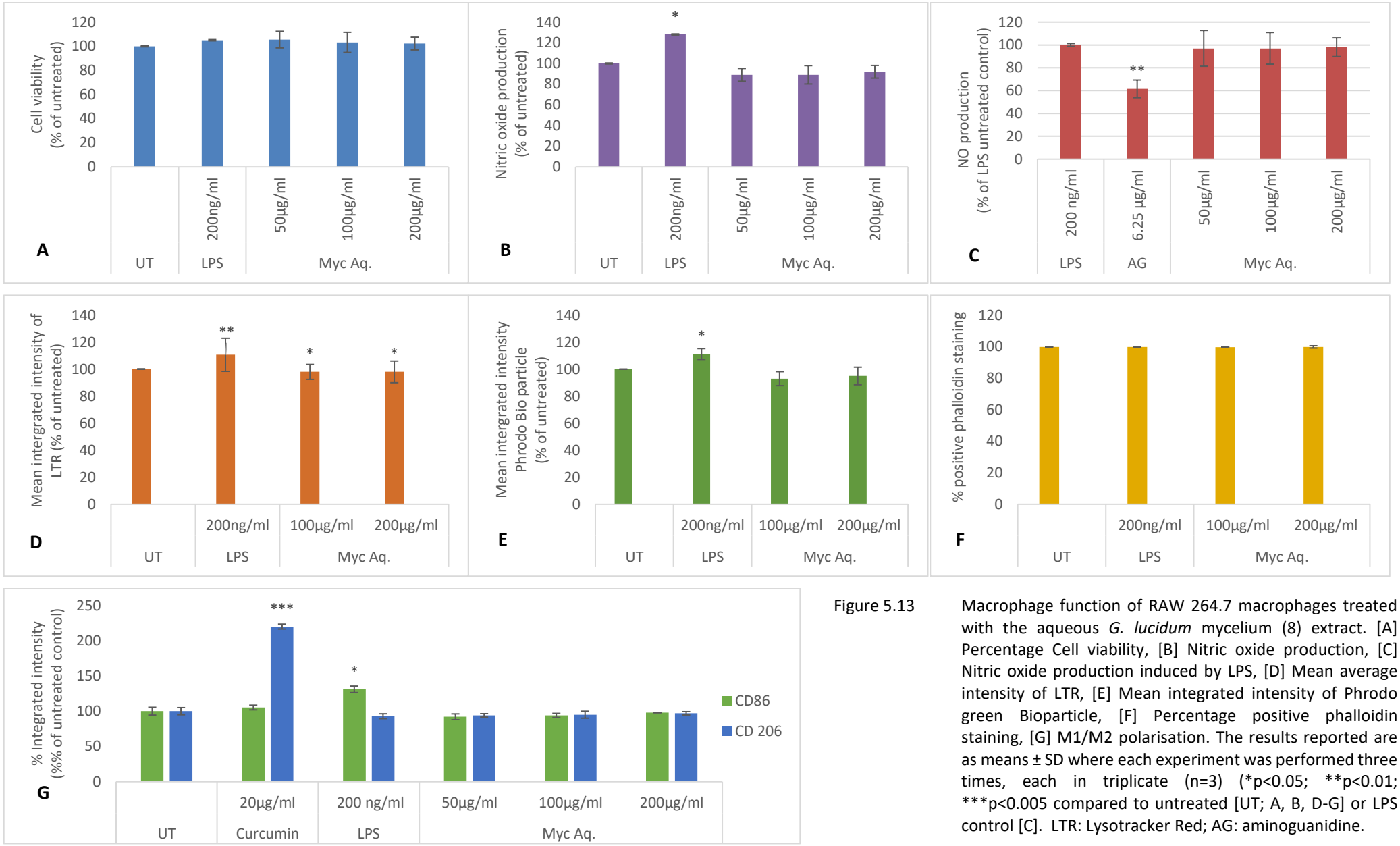

Figure 5.13

Macrophage function of RAW 264.7 macrophages treated with the aqueous *G. lucidum* mycelium (8) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

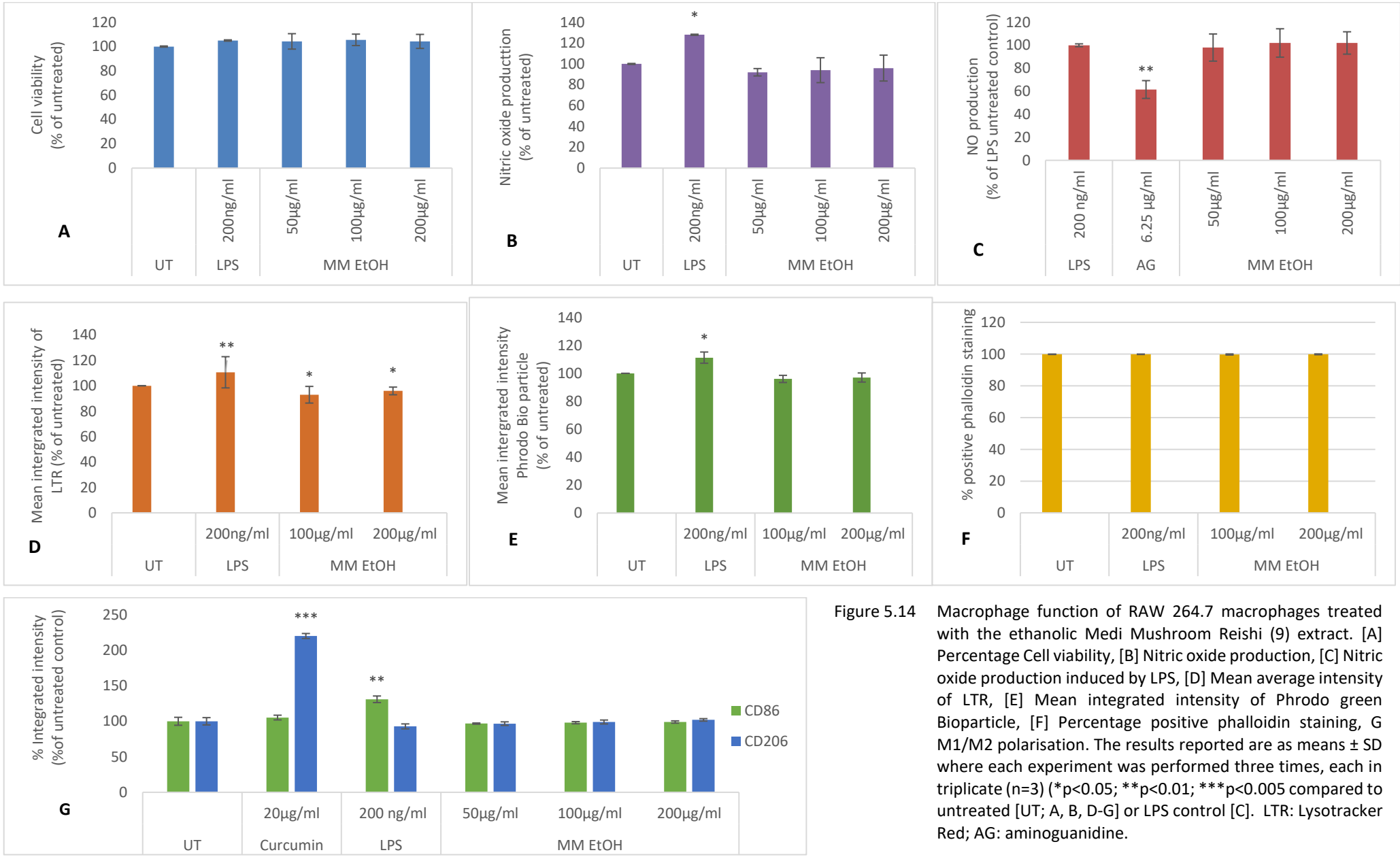

Figure 5.14 Macrophage function of RAW 264.7 macrophages treated with the ethanolic Medi Mushroom Reishi (9) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, G M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

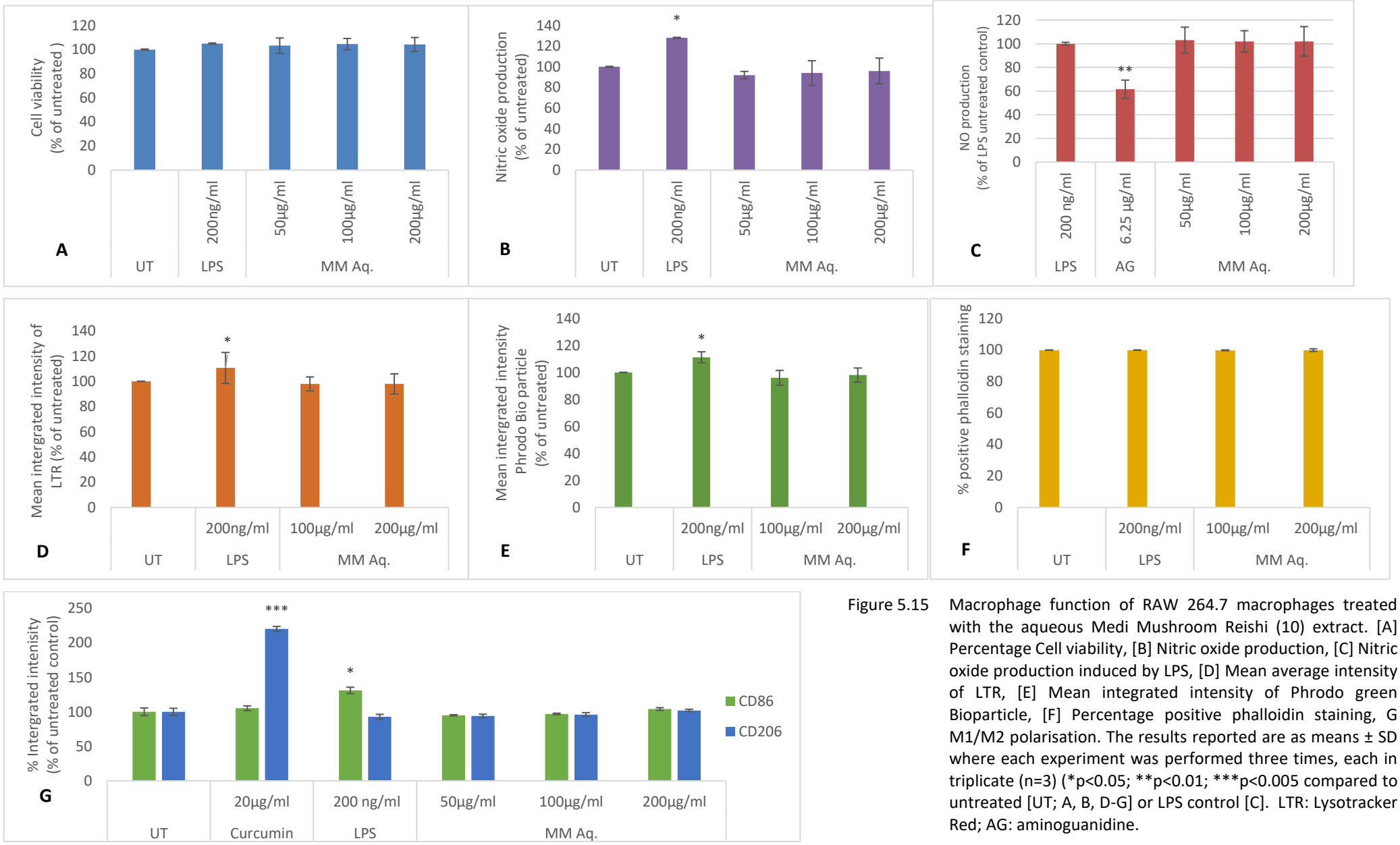

Figure 5.15 Macrophage function of RAW 264.7 macrophages treated with the aqueous Medi Mushroom Reishi (10) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, G M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

Figures 5.16 to 5.25 depict the cultivated *Ganoderma* spp. extracts (11-20). All these species were in mycelia form and underwent the same assays as the *G. lucidum* extracts (1 – 10) stated above. These cultivated extracts showed similar results to that of the commercially available *G. lucidum* extracts. *Ganoderma* spp. extracts were non-cytotoxic with no pro- or anti-inflammatory effects and did not affect the phagocytotic or actin activity of the RAW 264.7 macrophage cells.

The acidic vesicle formation followed a similar trend to that of the *G. lucidum* extracts seen above with a decrease in acidic vesicle formation compared to the untreated control for some of the species. A significant decrease was seen for *G. applanatum*[1] (figure 5.16 D), *G. applanatum* [2] (figure 5.17 D), *G. destructans* type (figure 5.18 D), *G. eickeri,* spp. nov (figure 5.20 D) and *G.* cf*. cupreum* (figure 5.25 D). *G*. aff. *austroafricanum* (figure 5.19 D)*, G.* cf. *resinaceum* [1] (figure 5.21 D) , *G.* cf. *resinaceum* [2] (figure 5.22 D)*, G. knysnamense,* spp. nov. type [1](figure 5.23 D) and *G. knysnamense,* spp. nov. type [2] (figure 5.24 D) showed no significant changes in the acidic vesicle formation compared to the untreated control.

As previously described, the M1 and M2 polarisation was determined by exposing the RAW 264.7 macrophages to the extracts and then determining their CD86 and CD206 expression by immunofluorescent staining; with CD86 being indicative of a M1 phenotype and CD206 a M2 phenotype. LPS and curcumin were used as positive controls for M1 (CD86) and M2 (CD206) polarisation, respectively. The *Ganoderma* spp. extracts that showed a significant change in the expression of the respective markers had a bias towards the M2 marker CD206. These extracts included the *G. applanatum* [1] (figure 5.16 G)*. G. applanatum* [2] (figure 5.17 G)*, G. destructans* type [1] (figure 5.18 G)*, G. eickeri,* spp. nov. (figure 5.20 G)*, G.* cf. *resinaceum* [1] (figure 5.21 G)*, G*. cf. *resinaceum* [2] (figure 5.22 G) and *G. cf. cupreum* (figure 5.25 G)*. G. aff. austroafricanum* (figure 2.19 G) was the only extract that showed no significant change in either the M1 or M2 marker. Interestingly *G. knysnamense*, spp. nov. type[1] (figure 5.23 G) and *G. knysnamense*, spp. nov. type [2] (figure 5.24 G) showed a significant increase in both the M1 (CD86) and M2 (CD206) markers, however the M2 marker still showed higher intensities compared to that of the M1 marker indicating that the *Ganoderma* spp. like the *G. lucidum* extracts are biased towards the M2 phenotype.

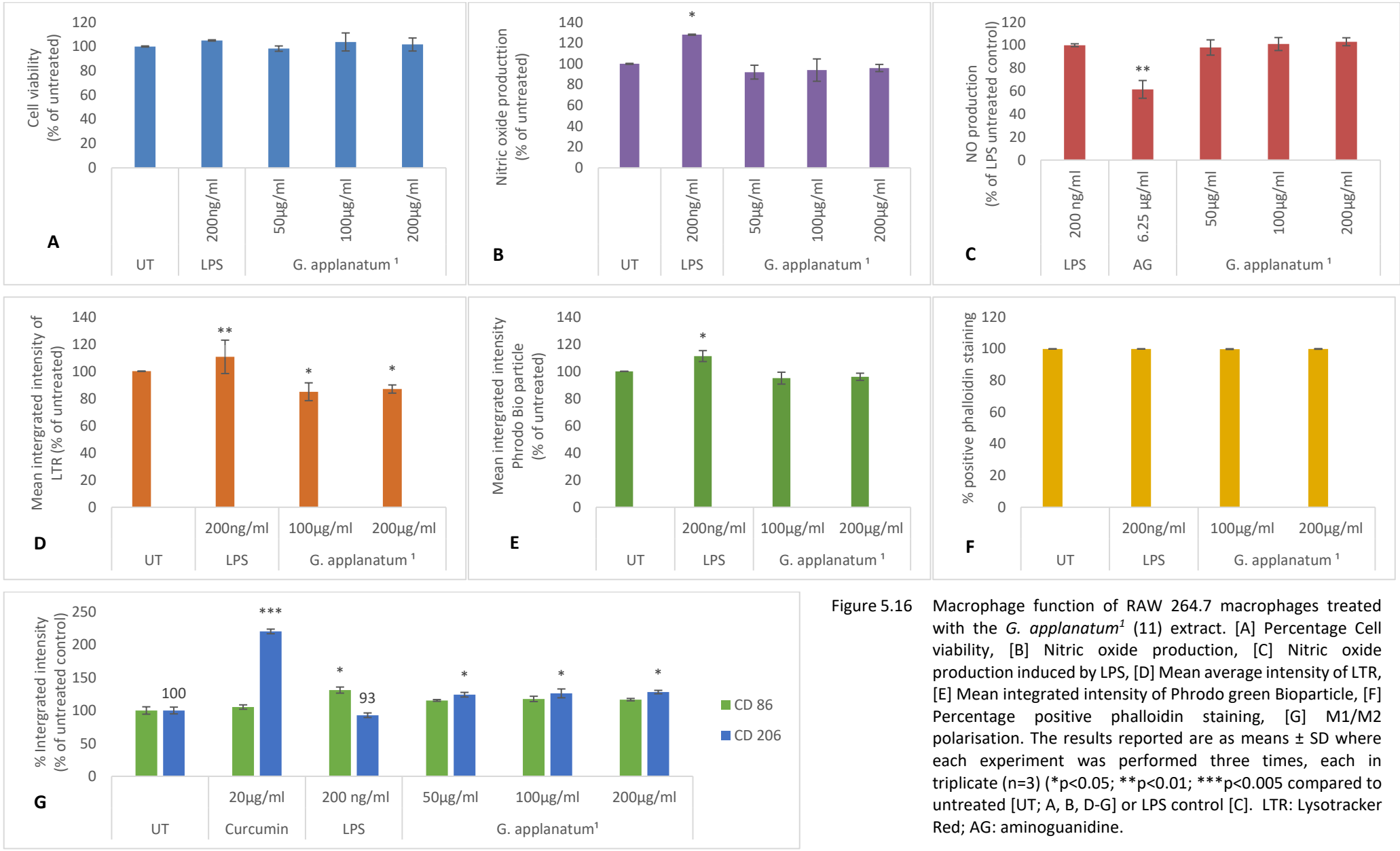

Figure 5.16 Macrophage function of RAW 264.7 macrophages treated with the *G. applanatum*[1] (11) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

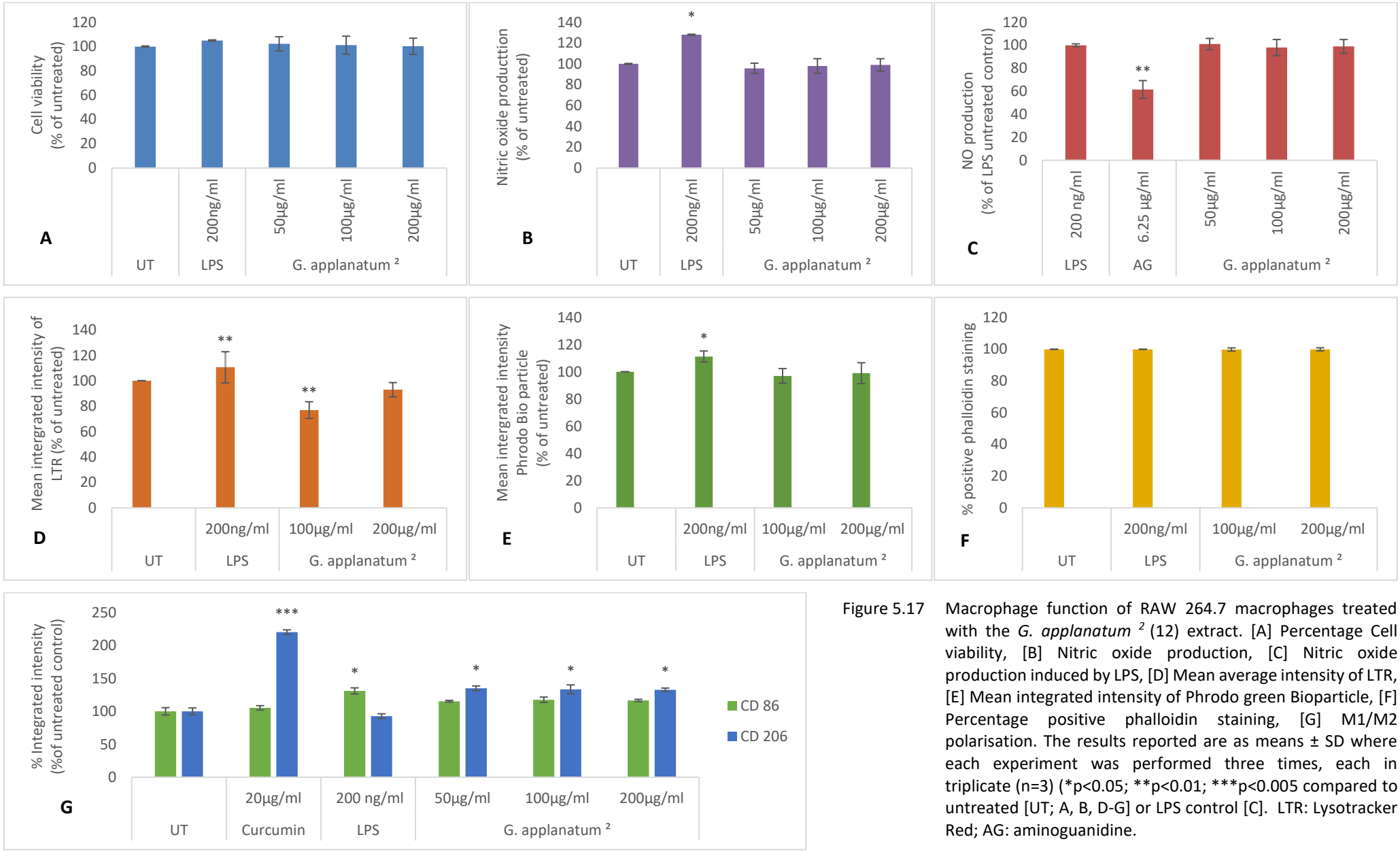

Figure 5.17 Macrophage function of RAW 264.7 macrophages treated with the *G. applanatum* ² (12) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

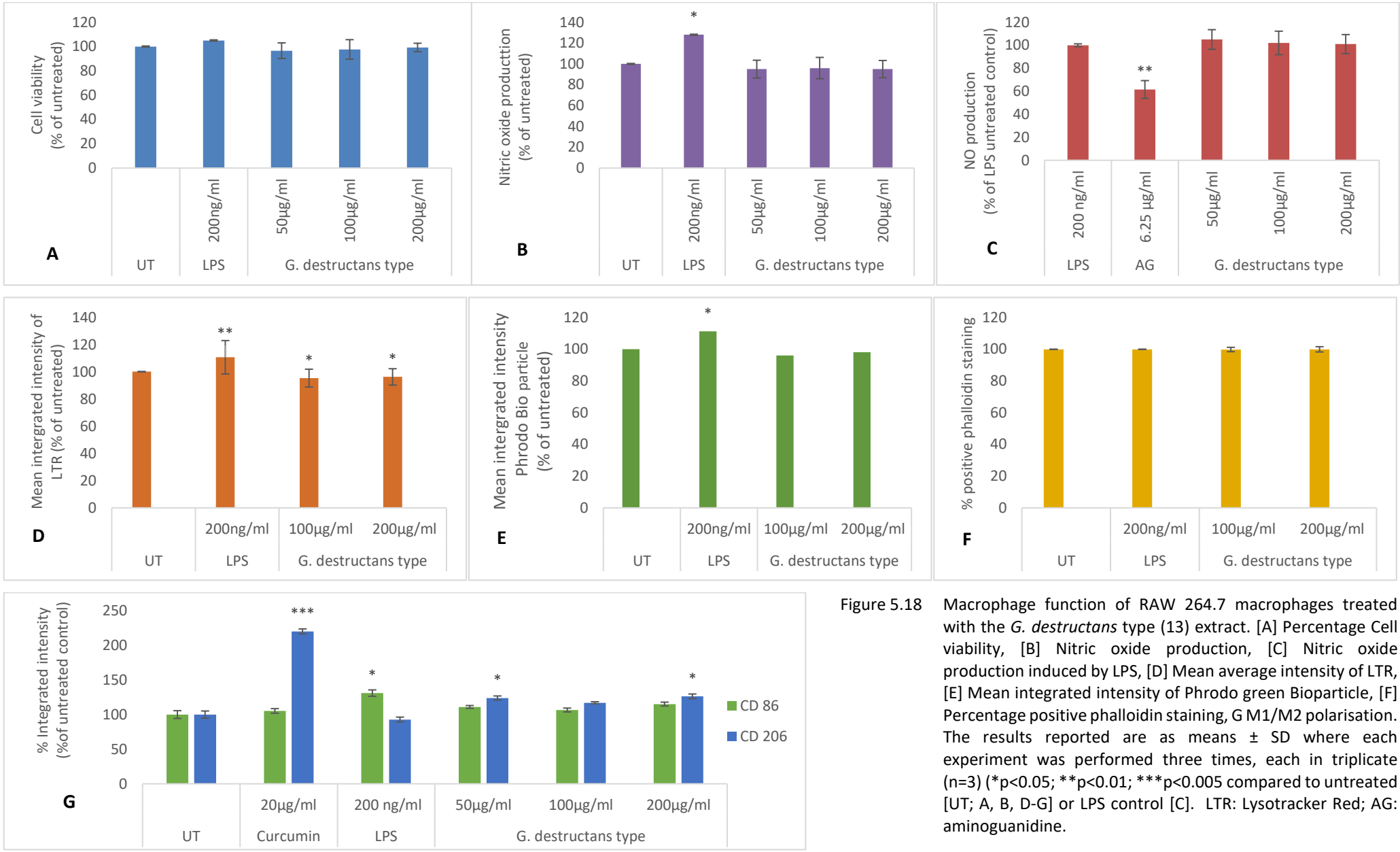

Figure 5.18 Macrophage function of RAW 264.7 macrophages treated with the *G. destructans* type (13) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, G M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

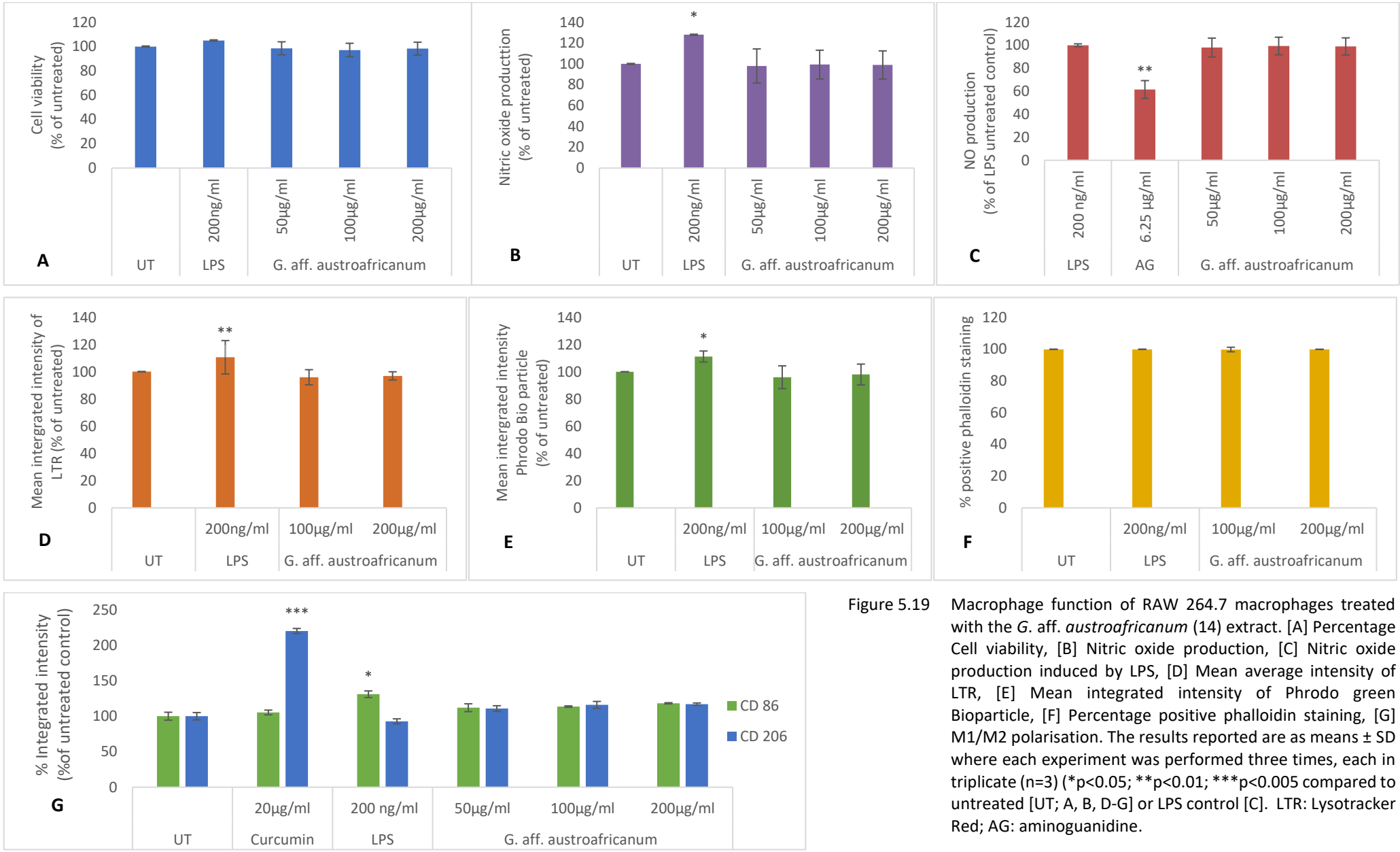

Figure 5.19 Macrophage function of RAW 264.7 macrophages treated with the *G.* aff. *austroafricanum* (14) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

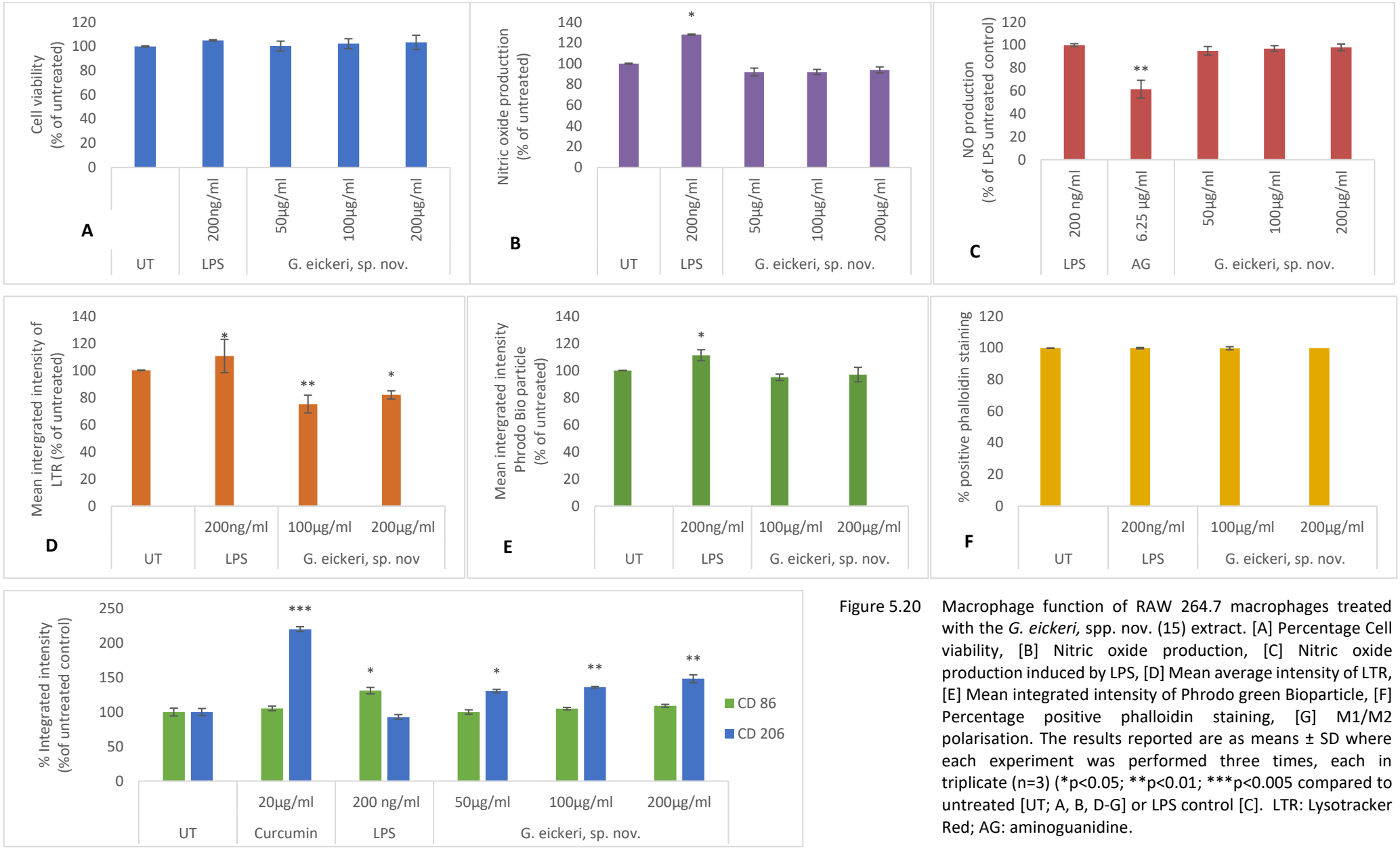

Figure 5.20 Macrophage function of RAW 264.7 macrophages treated with the *G. eickeri,* spp. nov. (15) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

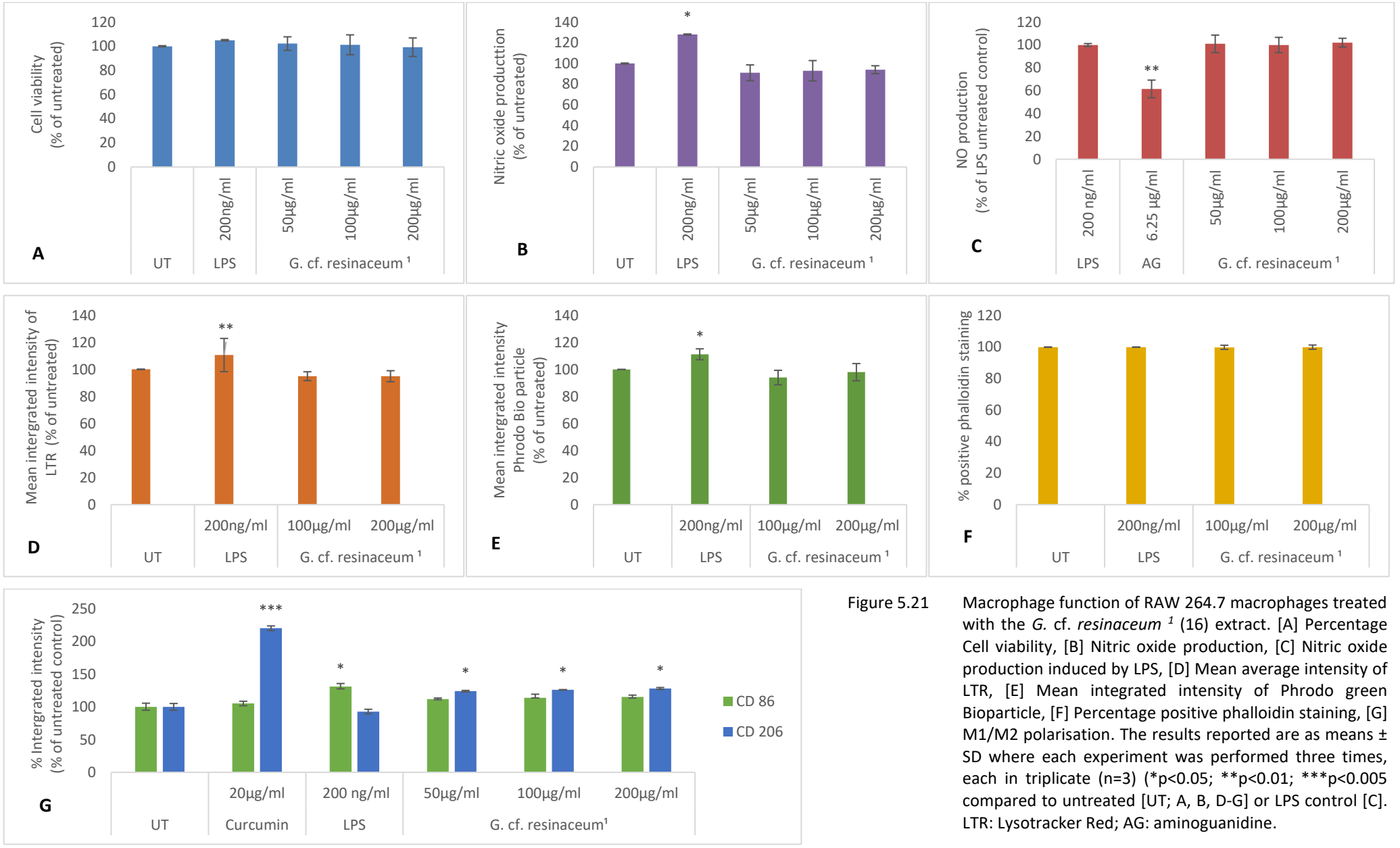

Figure 5.21 Macrophage function of RAW 264.7 macrophages treated with the *G.* cf. *resinaceum* [1] (16) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

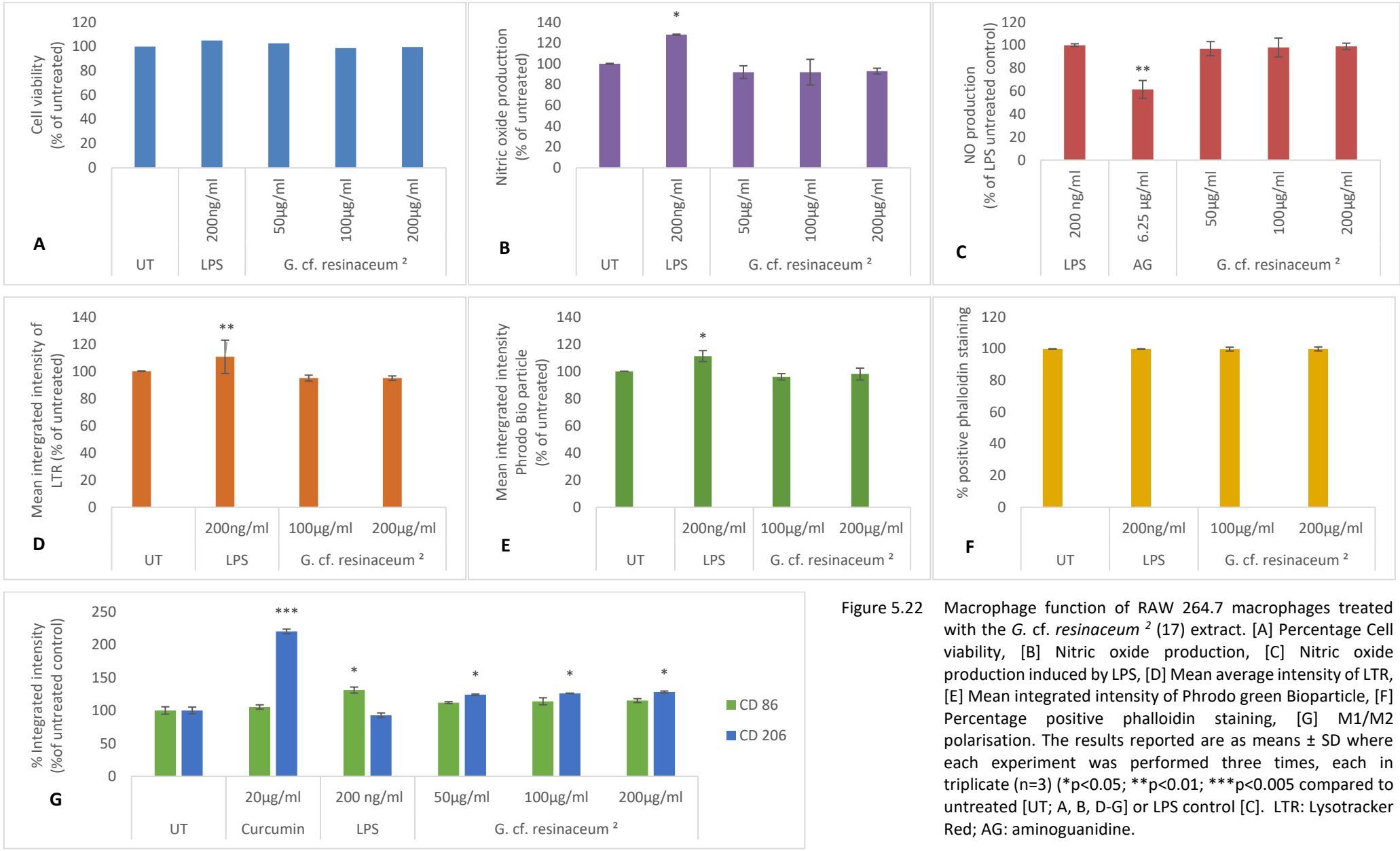

Figure 5.22 Macrophage function of RAW 264.7 macrophages treated with the *G.* cf. *resinaceum* [2] (17) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

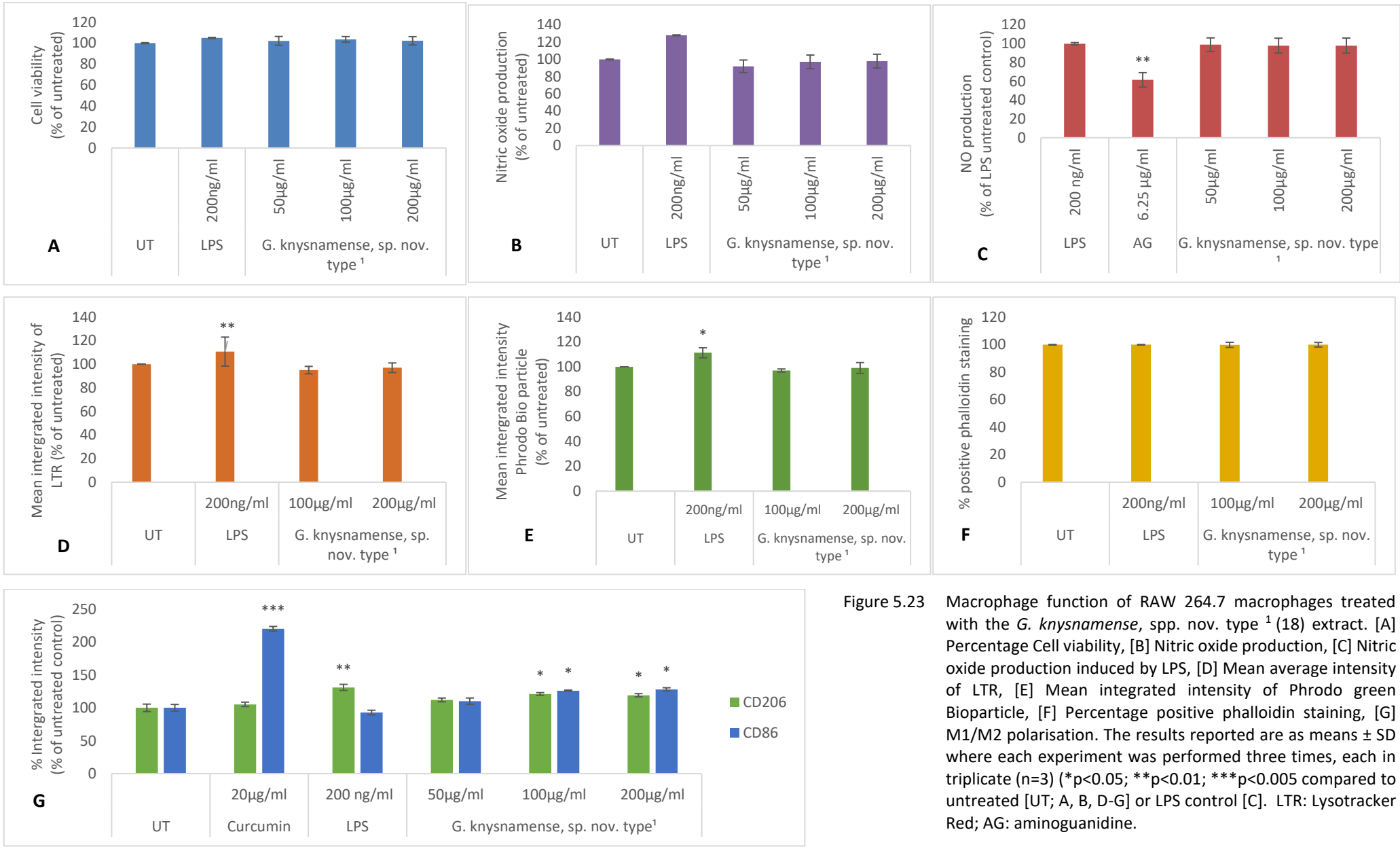

Figure 5.23 Macrophage function of RAW 264.7 macrophages treated with the *G. knysnamense*, spp. nov. type [1] (18) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

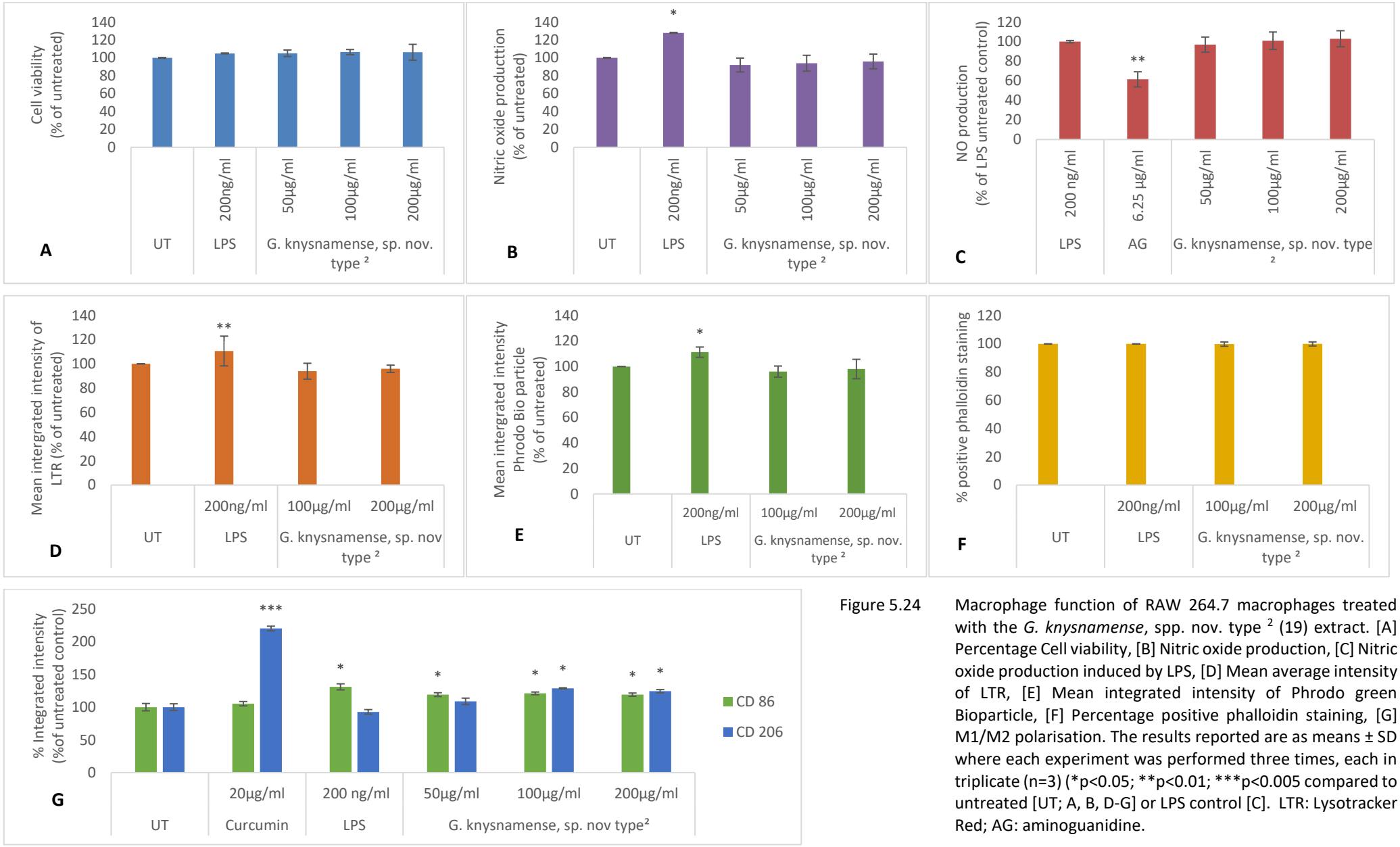

Figure 5.24 Macrophage function of RAW 264.7 macrophages treated with the *G. knysnamense*, spp. nov. type ² (19) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, [G] M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

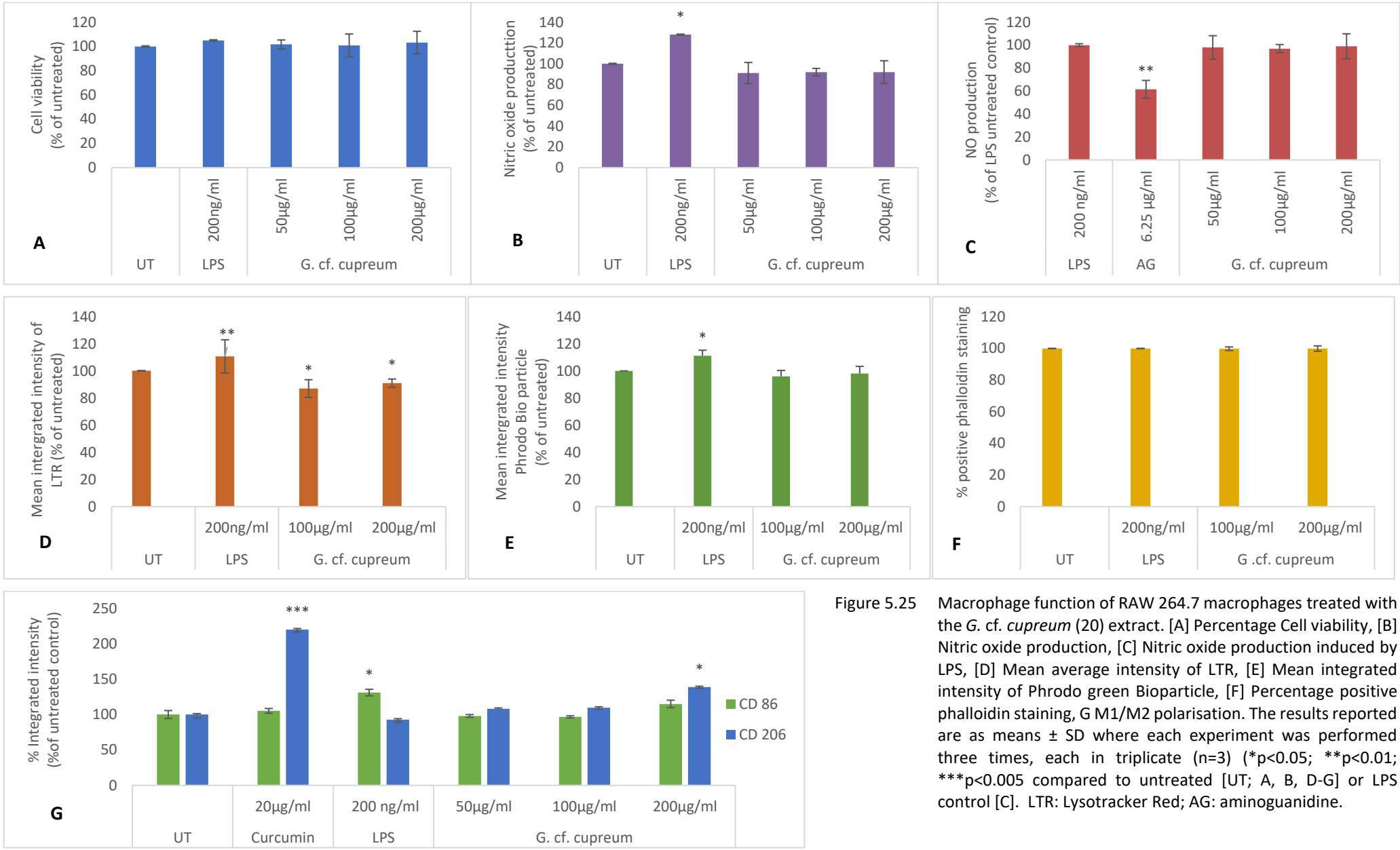

Figure 5.25 Macrophage function of RAW 264.7 macrophages treated with the *G.* cf. *cupreum* (20) extract. [A] Percentage Cell viability, [B] Nitric oxide production, [C] Nitric oxide production induced by LPS, [D] Mean average intensity of LTR, [E] Mean integrated intensity of Phrodo green Bioparticle, [F] Percentage positive phalloidin staining, G M1/M2 polarisation. The results reported are as means ± SD where each experiment was performed three times, each in triplicate (n=3) (*p<0.05; **p<0.01; ***p<0.005 compared to untreated [UT; A, B, D-G] or LPS control [C]. LTR: Lysotracker Red; AG: aminoguanidine.

5.4 DISCUSSION

This chapter describes the effect on macrophage function after treatment of RAW 264.7 cells with commercially available or cultivated *Ganoderma* spp. with the focus being on macrophage function in the wound healing process. Macrophages play key roles in the inflammation, proliferation and remodelling phase of wound healing with the macrophages transitioning between phenotypes.

When studying macrophages, it is important to note that there is evidence showing that macrophages evolve throughout the macrophage spectrum with cells presenting a variety of M1 and M2 characteristics and that heterogenous populations can exist at any given time depending on the cellular environment (Krzyszczyk *et al.,* 2018, Song *et al.,* 2021). In order to determine the macrophage function of treated RAW 264.7 macrophages the pro-inflammatory and anti-inflammatory activities were determined, along with the phagocytic ability and determining the macrophage phenotype present when treated with the extracts.

Overall, there is a general trend among the *Ganoderma* spp. commercial products and cultivated samples with most extracts showing similar results where some species showed higher activity than others. Hence, the results will be discussed collectively with reference to extracts which showed activity compared to those that did not and evidence from literature.

G. lucidum extracts MG-LZ8 (figure 5.6), polar extract (figure 5.7), antler ethanolic and aqueous (figure 5.8 and 5.9) and the conk fruiting body extracts (figure 5.10 and 5.11) all showed similar results. The extracts showed no increase in nitric oxide production in figure B of each respective figure indicating that the extracts did not exhibit a pro-inflammatory response whereby nitric oxide is elevated as seen in the cells treated with the positive control LPS. Next, the response of the LPS-activated cells was observed. LPS binds to toll-like receptors (TLR) on macrophages and activates the inflammatory response; it does this by increasing iNOS levels and thereby increasing NO production which was used to determine

if the extracts exhibited anti-inflammatory activity (Wang *et al*., 2021). In figures 5.6 and 5.7C there were no significant changes indicating that the extracts did not exhibit an anti-inflammatory response. Most literature claims *G. lucidum* extracts to be anti-inflammatory for example Barbieri *et al.,* (2017) showed that a DMSO *G. lucidum* extract significantly reduced the amount of pro-inflammatory cytokines such as Interleukin-8 (IL-8) and IL-6, by 22% at a concentration of 2 mg/mL and up to 52% at 5 mg/mL. Hu *et al., (*2020) showed similar results where *G. lucidum* triterpene extracts showed anti-inflammatory results by reducing the cytokines TNF-α and IL-6 released in response to LPS induced inflammation. Barbieri *et al.,* (2017) utilised a much higher concentration than those investigated in this study while Hu *et al*. (2020) used triterpenes extracted from ethanolic *G. lucidum* extracts. The use of higher concentration (2 and 5 mg/mL) brings into question the validity of these findings when considering the physiological relevance (Gertsch, 2009). Effects of high extract concentrations may include non-specific interactions with proteins or changes in osmolarity of the culture medium to name just a few. Furthermore, the use of targeted extractions has yielded significant and positive results compared to those of crude extracts as they may be enriched in active compounds (Gertsch, 2009). Therefore, although anti-inflammatory activity was expected for the *G. lucidum* extracts it is not surprising that crude extracts used at low concentrations did not exhibit any activity.

The phagocytic activity was determined using the pHrodo™Green *E. coli* BioParticles™ Conjugate which utilises microbial-associated molecular patterns (MAMPs) which are general structural patterns associated with pathogens, as a trigger to stimulate phagocytosis (Thermo Fisher Scientific). None of the *G. lucidum* extracts (figure 5.6 – 5.15 E) showed any increase in phagocytic activity. The research on phagocytic activity of *G. lucidum* varies in literature. For example, a study by Cia *et al.,* (2017) showed that a *G. lucidum* polysaccharides (GLP) extract at a concentration of as low as 1 μg/mL reduced phagocytosis. On the other hand; Huang *et al.,* 2019 found that GLP in conjunction with *Polyporus umbellatus* polysaccharides (PUP) hot water extract

of water soluble polysaccharides increased phagocytosis *in vivo* at concentrations of 360 mg/kg body weight (mg/kg BW) orally for 30 days. This result is similar to previous studies for example Wu *et al.,* (2009), and Sugiyama *et al.*(2018) showed enhanced phagocytosis in the presence of LPS. The inactivity seen in this investigation may be due to the cell environment as well as using crude extracts which have different interactions compared to extracts that have been optimised for a particular compound or compounds. For example, hot water extracts are typically used to extract water soluble polysaccharides, however this is a lengthy procedure at high temperatures with low yields. To increase yield, techniques such as Ultrasound Microwave Assisted Extraction (UMAE) and Ultrasonic Assisted Extraction (UAE) can be utilised, however these techniques require specialised equipment and are not cost effective. Ethanol extraction of water soluble polysaccharides as seen in Parepalli *et al*., (2020) requires a step by step extraction with the addition of phosphate buffer solution and polysaccharide purification. This study showed that the ethanol extraction had higher yields compared to the traditional hot water extraction (Parepalli *et al*., 2020). Thus, for future studies to determine the effect of the *G. lucidum* and *Ganoderma* spp. extracts of phagocytic activity it would be advisable to perform an ethanol extraction for polysaccharides.

The effects of the extracts on macrophage polarisation were tested using cell surface markers. CD86 was used as indicator for the M1 phenotype and CD206 as a general marker for M2 phenotypes. The MG-LZ8, polar, antler and conk fruiting body ethanolic and aqueous extracts (figure 5.6 – 5.11 G) all showed a predominant bias toward the M2 phenotype with levels of CD206 marker detection being similar to the curcumin positive control for the higher concentrations (100 μg/mL and 200 μg/mL) of the extracts tested. MG-LZ8 at a concentration of as little as 12.5 μg/mL showed similar results to that of the positive control with the conk fruiting body ethanolic and aqueous extracts showing a higher level of the CD206 marker than any of the other extracts and positive control. The lowest concentration of 50 μg/mL for ethanolic extract had a relative intensity of 247% and the aqueous extract having an intensity of 237%

compared to 100% for the untreated control. Literature on the effects of *G. lucidum* on macrophage polarisation are contradictory to those observed in this study. For example, Song *et al.,* (2021) showed that *G. lucidum* spore polysaccharide (GLSP) promoted a phenotypic change towards the M1 phenotype with an increase in inflammatory factors and cytokines. Another example is that of Sun *et al.,* (2017) where glycopeptide derived from *G. lucidum* (*Gl*-PS) promoted M1 polarisation and Lui *et al.,* (2022) demonstrated that *Ganoderma atrum* polysaccharide (PSG-1) modulated the M1/M2 polarisation with a tendency to promote M1 polarisation.

The common denominator amongst these extracts which showed contradictory results to those observed in this study was that the extracts were prepared to extract specific compounds, in this case polysaccharides. This investigation utilised a crude extract which would have completely different reactions within the cellular environment compared to that of specific compound-enriched extracts (Ferreira *et al.,* 2015, Wang *et al.,* 2019, Samba *et al.,* 2022).

The lack of phagocytic activity seen can be attributed to the fact that the extracts showed a bias toward M2 polarisation. As M2 macrophages do phagocytose, however, they are generally not triggered by MAMPs as M1 macrophages are. The *E. coli* in pHrodo™ Green *E. coli* BioParticles™ Conjugate used in this investigation contain distinct molecular patterns (MAMPs) that are recognised by pattern recognition receptors (PRRs) on the surface of macrophages. These trigger phagocytosis in M1 macrophages. M2 macrophages recognise so-called “eat-me” signals on apoptotic and dead cells, the most well-known is phosphatidylserine (PS), a membrane phospholipid that is only exposed on the surface of the cell in response to apoptotic signals (Hochreiter-Hufford and Ravichandran, 2013). If the macrophages were polarised towards the M2 phenotype they would not have been stimulated to phagocytose the conjugated *E. coli* particles used to detect phagocytosis. M2 macrophages are involved in the later stages of wound healing with their main function being the clearing of debris of apoptotic cells and acting as anchors at the start of the proliferation and

vascularisation stage (Ogle *et al.,* 2016). The lack of anti-inflammatory activity by *G. lucidum* extracts (figure 5.6 – 5.11 C) in combination with M2 polarisation seen in figures 5.6 – 5.11 G suggest that the macrophage phenotype is most likely the M2a phenotype. The M2a macrophage phenotype is what was traditionally considered the wound healing macrophage as it secretes collagen precursors and factors which stimulate fibroblasts for the proliferation phase. They are also involved in angiogenesis as they secrete high levels of Platelet-derived growth factor (PDGF) (Krzyszczyk *et al.,* 2018).

As seen in the previous section, *G. lucidum* ethanolic mycelia (figure 5.12) and aqueous extract (figure 5.13) as well as the ethanolic Medi mushroom (figure 5.14) and aqueous Medi mushroom (figure 5.15) extracts showed little to no activity for any of the tested parameters. This lack of activity could be attributed to the starting material as mycelium extracts have been shown to exhibit little to no activity when compared to their fruiting body counter parts (Azwanida, 2015; Truong *et al.,* 2018). The lack of activity seen from the Medi mushroom extracts is most likely due to the fact that the amount of actual *G. lucidum* in the powder formulation of the original sample is not expressly stated as it is not a regulated pharmaceutical product and therefore may not contain high concentrations of *G. lucidum* or may not contain *G. lucidum* but instead a substitute such as those studied in Loyd *et al.,* (2018).

The *Ganoderma* spp. mycelium extracts seen in figures 5.16 – 5.25 displayed similar results to those of the *G. lucidum* extracts (figures 5.6 – 5.15). None of the extracts in figures 5.16 – 5.25A were cytotoxic against RAW 264.7 macrophages at the concentrations tested (50 – 200 μg/mL) nor did they exhibit significant pro-inflammatory or anti-inflammatory activities in figures 5.16 – 5.25 B and C. There is little to no research done on *Ganoderma* spp. apart from *G. lucidum* and this is mostly attributed to the incorrect and misleading nomenclature found within the species. However, in the limited research Sipping *et al.,* (2022) found that polysaccharide-rich fractions from *G. resinaceum* increased pro-inflammatory cytokines and decreased anti-inflammatory cytokines. Raseta *et al.,* (2020)

reported that chloroform extracts of *G. resinaceum* mycelium had strong anti-inflammatory activity at concentrations of 41 μg/mL which is contradictory to the results seen in this study in figure 5.21 and 5.22. Both studies on *G. resinaceum* use different extraction to that utilised in this study. Sipping *et al.,* (2022) used polysaccharide-enriched fractions and Raseta *et al.,* (2020) used a chloroform extract which are both more likely to have specific interactions compared to the crude extracts which may explain the lack of activity seen in this investigation for *G. resinaceum*. Similarly, OsiNska-Jaroszuk *et al.,* (2014) found that ethanolic *G. applanatum* exopolysaccharides extract at concentration of 328.5 μg/mL increased the secretion of pro-inflammatory Interleukin 6 (IL-6). This is unlike the results seen for *G. applanatum*[1] (figure 5.16) and *G. applanatum*[2] (Figure 5.17) which displayed no inflammatory activity. When comparing these two studies, OsiNska-Jaroszuk *et al.,* (2014) used the exopolysaccharide extracted from the supernatant of *G. applanatum* cultures to produce ethanolic extracts and used a concentration range above those tested in this study which provides key evidence for the disparities observed.

Similar to the inflammatory activity, the *Ganoderma* spp. mycelium extracts (figures 5.16 – 5.25 E) showed no change in the phagocytic activity compared to the untreated control. All the *Ganoderma* spp. (figure 5.16 – 5.25 G) except *G.* aff. *austroafricanum* (figure 5.19 G) showed significant increases in the intensity of the CD206 marker. One can deduce from this that the extracts caused the macrophages to polarise toward the M2 phenotype, as was seen with the *G. lucidum* extracts (figure 5.6 – 5.11 G). Most reports on *Ganoderma* spp. besides *G. lucidum* are focused on phylogenetic studies of this vast genus, focusing on the molecular detection and identification of species found across the globe, with sparse literature on macrophage function for comparison. Specifically, the macrophage polarisation has only focused on *G. lucidum* extracts with various extraction methods (Sun *et al*., 2017; Song *et al.,* 2021). Thus, the activity seen in this study will be some of the first noted activity for extracts like *G. eickeri,* spp. nov (figure 5.20) which showed the highest CD206 of all the *Ganoderma* spp. extracts tested. *G. eickeri,* spp. nov (figure 5.20) is indigenous to South Africa

and was first described by Coetzee *et al.,* (2015). The fact that the mycelium extracts showed significant activity for the M2 phenotype presents a unique opportunity for further study whereby the sample can be grown to the fruiting body stage as well as for testing of the culture media to ascertain any other biological activity these species may have.

All the *G. lucidum* extracts (figures 5.6 – 5.14 D) except the aqueous Medi Mushroom extract showed a decrease in acidic vesicle production. This decrease in acidic vesicle production is also observed for *G. applanatum*[1] (figure 4.16 D), *G. applanatum*[2] (figure 5.17 D), *G. destructans* type (figure 5.18 D) *G. eickeri,* spp. nov (figure 5.20 D) and *G.* cf*. cupreum* (figure 15.25 D). One possible explanation of this could be the method used for the detection of these acidic vesicles. LysoTracker probes tend to accumulate intracellularly if used for prolonged periods which causes the intracellular pH to increase which in turn may result in enhanced quenching of the fluorescent dye (Chen *et al.,* 2015). Therefore, it is advised that the acidic vesicle formation assay be repeated in a more time efficient manner or with a more sensitive probe.

Overall, the RAW 264.7 macrophages treated with *G. lucidum* extracts and the *Ganoderma* spp. extracts showed little to no significant change in the macrophage function except for the predominant M2 phenotype polarisation by most of the extracts tested. Further study and confirmation of the M2 polarisation could lead to the potential of these extracts becoming key players in the treatment of chronic wound healing where patients suffer from a prolonged M1 inflammatory response. The extracts of mycelia *G. eickeri,* spp. nov (figure 5.20) MG-LZ8 (figure 5.6) and *G. lucidum* conk fruiting body (Figure 5.8 and 5.9) provided by the UFS research group show great promise as potential treatment regimens and warrant further investigation.

CHAPTER SIX
CONCLUSION

6.1. THE RESEARCH IN PERSPECTIVE

G. lucidum is well known for its medicinal properties and has been extensively studied. It has been shown that *G. lucidum* has wound healing activity and toxicity to a wide variety of cancer cells but due to misclassification/ misidentification, other Ganoderma spp. have not been studied. This study assessed the wound healing potential and cytotoxic activity against melanoma cells of ten *G. lucidum* samples and *Ganoderma* spp. and screened their antibacterial activity against selected pathogens.

The yield of the prepared extracts varied greatly amongst the samples extracted with the *G. lucidum* ethanolic antler sample having the highest extraction yield. The crude extraction method with ethanol or water as solvents utilized in this investigation was chosen as it emulates the extraction method used in natural/ traditional medicine. Although this may not be the best approach from a phytochemical perspective, the aim of the study was to compare samples of different origin and species and not to isolate active compounds.

G. lucidum extracts were screened for their antibacterial activity against Gram-positive (*S. aureus* and *S. pyogenes)* and Gram-negative (*P. aeruginosa, E. coli* and *K. pneumoniae*) bacteria. None of the ethanolic or aqueous extracts exhibited anti-bacterial activity at the concentration range tested in this investigation. According to literature, *G. lucidum* macrofungi showed high levels of antibacterial activity. , however, the concentration ranges used in those studies were higher than those tested in this study (0 – 2 mg/mL) for crude extracts. The use of high concentrations at initial screening level is accepted as it acts as a simple check for bioactive compounds. However, the physiological relevance of the concentration required to observe significant bioactivity needs to be considered, which is why only physiologically relevant lower concentrations were screened. The extraction method plays an important role in the antibacterial activity of

extracts. For example, different solvents create extracts with unique chemical profiles and thus exhibit different biochemical effects. The difference in bioactivity profile compared to findings in most literature, coupled with the concentration tested is the most plausible reason for the lack of antibacterial activity.

The MG-LZ8 (1) extract provided by Mushroom Guru (https://www.mushroomguru.co.za/) displayed a reduction of live B16F10 murine melanoma cells with an IC_{50} of 21.26 µg/mL. The low numbers of dead cells detected by the Image Xpress indicated that the MG-LZ8 extract most likely had a cytostatic rather than cytotoxic effect. The other antler containing extracts (2 – 4) did not exhibit any cytotoxic or cytostatic activity suggesting that the double extract (ethanol and hot water extraction) combination worked together as a complex to achieve the cytostatic activity. The *G. lucidum* ethanolic conk fruiting body extract and the ethanolic mycelium showed significant reduction in cells at the highest concentrations tested (200 mg/mL). Commercial natural medicinal products often claim a wide variety of medicinal benefits but as they are not legally regulated they tend to fall short when tested for the bioactivities. In this case the Medi Mushroom Reishi Capsule (9, 10) showed no potential anti-cancer activity. This often happens as many commercial products contain Reishi substitutes. *G. destructans* (13) was the only *Ganoderma* spp. mycelium where a significant reduction in live cells was observed. This is the first reported cytotoxic activity for this species since its discovery by Coetzee *et al.,* (2015). It is a well-known fact and confirmed throughout this investigation that mycelium extracts tend to have lower activities than fruiting bodies. The positive result obtained with *G. descructans* (13) mycelium extract is therefore promising and represents a potential for future study.

Antioxidant activities were measured via the DPPH and FRAP assays. While the results varied between the two assays, the same general trend was observed for the *G. lucidum* extracts and *Ganoderma* spp. extracts. For both assays the antler containing extracts (1 – 4) had the highest observed antioxidant activity followed closely by the conk fruiting body extracts (5, 6). The ethanolic extracts exhibited

higher antioxidant activity than their aqueous counterparts. This can be attributed to the fact that ethanol is more likely to extract a higher concentration or wider range of phenols which are thought to be responsible for antioxidant activity of *G. lucidum*. The cultivated *Ganoderma* spp. mycelium extracts (11 - 20) had lower antioxidant activity compared to the *G. lucidum* extracts. The uniform cultivation of the mycelium extracts leads to low extraction yield which may have affected the quantity and quality of the bioactive components of the extracts, thereby affecting the activity of the *Ganoderma* spp. mycelia.

In terms of macrophage function there was a general trend throughout the *G. lucidum* extracts as well as the *Ganoderma* spp. extracts. Overall little to no inflammatory or phagocytic activity was seen in the extract treated cells when compared to the appropriate controls. The extract treated cells showed no changes in nitric oxide levels in naïve (absence of LPS) or LPS-activated macrophages. The extract treated cells (1 – 20) therefore had no pro- or anti-inflammatory activity. The extract treated cells (1 – 20) furthermore showed little to no change in the phagocytic activity; this was accompanied with no change in actin polymerisation and acidic vesicle formation.

Interesting results were obtained when macrophage phenotype polarisation in response to the extracts showed a predominant bias toward the M2 phenotype. The most significant activity observed is that of the antler containing *G. lucidum* extracts (1 – 4) and the conk fruiting body extracts (5, 6). All the *Ganoderma* spp. (11 – 20) except *G*. aff. *austroafricanum* (14) showed a significant increase in the intensity of the CD206 marker. One can deduce that the extracts caused the macrophages to polarise toward the M2 phenotype, as observed for the *G. lucidum* extracts. The lack of phagocytic and inflammatory activities with the M2 cell marker being predominant, suggest that the extracts caused the polarisation of the M2a phenotype, which is considered the wound healing macrophage as it secretes collagen precursors and factors which stimulate fibroblasts for the proliferation phase.

6.2. POTENTIAL FOR FUTURE DEVELOPMENT OF THE WORK

The potential for physiological and pharmacologic relevance of natural products like the *Ganoderma* spp. can be seen in this study, however for future studies species confirmation should also considered prior to cultivation so that the growth conditions such as the carbon and light sources can be optimised for each species as well as allowing for the growth of fruiting bodies which could not be achieved in this investigation due to time constraints. This would allow for a more holistic overview of the species bioactivities as well as the fruiting bodies producing better extract yields. In addition, the solvent and extraction method should be specific to a targeted compound or a particular range of compounds. For example, non-polar solvents such as methanol are best for the extraction of phenols which act as antioxidants (Alara *et al.,* 2021).

The low IC_{50} for MG-LZ8 indicates that it has potential as a chemotherapeutic agent. The mechanism of action of MG-LZ8 cytostatic effects should be elucidated in future studies. It would be useful to assess the effect of the MG-LZ8 extract on normal cells and test other parameters such as the mechanism of stasis and the active compound(s) (Anttila *et al.,* 2019). *G. destructans* mycelia extract showed potential as a future chemotherapeutic agent as it demonstrated cytotoxic activity at a concentration of 200 µg/mL. The mycelium extracts had lower activities and it may be useful to grow the *Ganoderma* spp to fruiting body stage and ascertain whether there is an increase in activity from a fruiting body extract.

Although *in vitro* studies are an important part of the screening and testing for bioactivities of natural compounds such as *Ganoderma* spp, the human body is complex and positive *in vitro* results are not always applicable *in vivo*. The positive results for *G*. aff. *austroafricanum* seen in this study and future research should be confirmed *in vivo* before the true potential of these active extracts can be cemented.

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Appendix

Section A: Reagent Preparation

The reagents required for the FRAP assay were prepared as follows:

- Acetate buffer (300 mM)
  Dissolve 3.1 g sodium acetate in 800 mL distilled water; add 16 mL glacial acetic acid and fill to 1 L with distilled water. Store at 4 °C.
- Dilute HCl (40 mM)
  Add 1.5 mL HCl to 500 mL distilled water.
- TPTZ (10 mM)
  Dissolve 0.031 g TPTZ in 10 mL dilute HCl in a water bath at 50 °C; freshly prepared.
- Ferric chloride (20 mM)
  Dissolve 0.108 g $FeCl_3 \cdot 6H_2O$ to 20 mL distilled water; freshly prepared.
- FRAP reagent
  The FRAP reagent should be prepared fresh on the day. Add 20 mL sodium acetate buffer (300 mM), 2 mL TPTZ (10 mM), 2 mL $FeCl_3$ (20 mM in distilled water; freshly prepared) and 2.4 mL distilled water.

The reagents required for the DPPH assay were prepared as follows:

- Tris-HCl buffer (50 mM)
  Dissolve 0.606 g Tris in 80 mL distilled water; adjust the pH to 7.4 using HCl then fill to 100 mL with distilled water.
- DPPH (0.1 mM)
  Dissolve 0.002 g DPPH in 50 mL ethanol; prepare freshly and protect from light.

Section B: Trolox standard curve

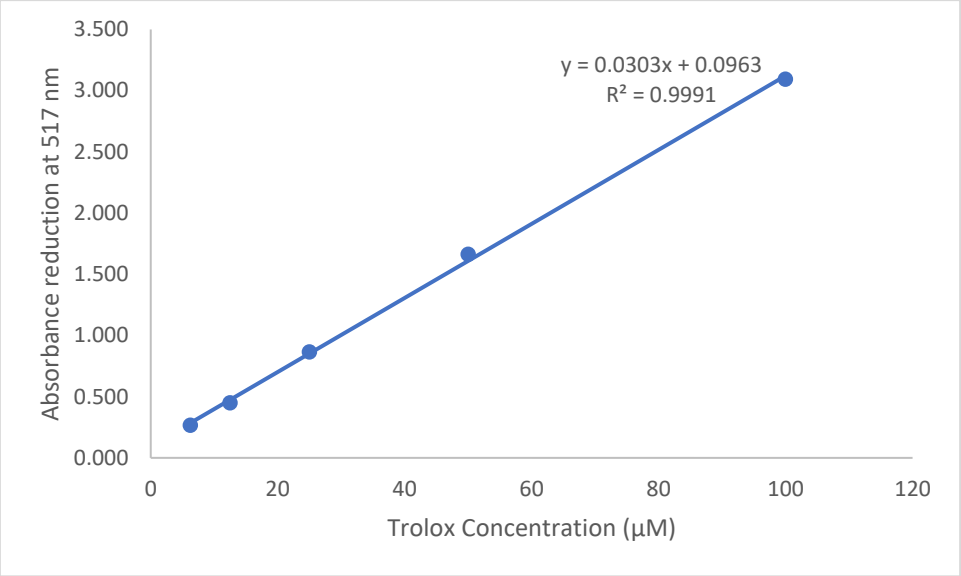

Figure B1: Trolox standard curve

Section C: Turnitin Report

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