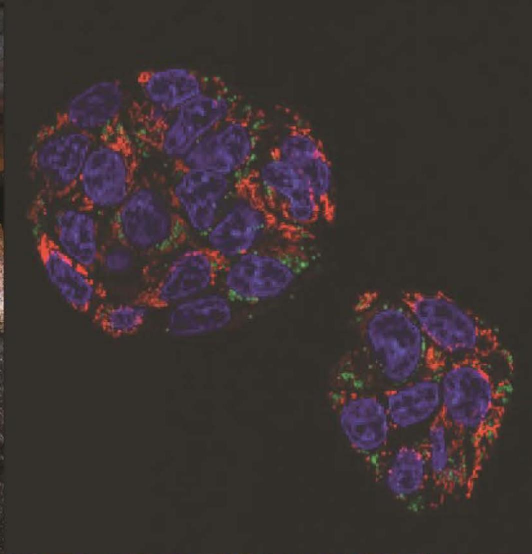

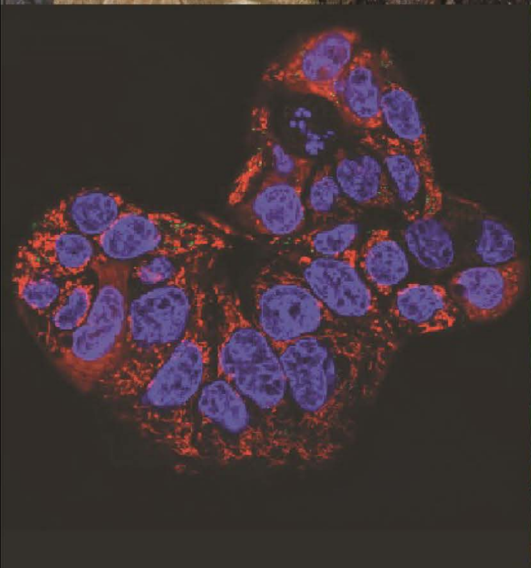

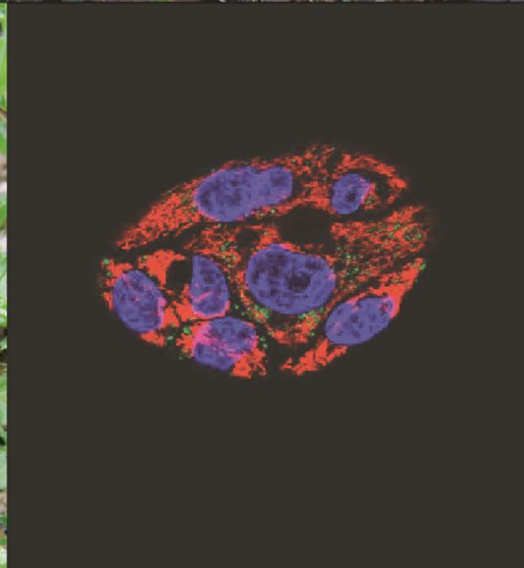

Cátia de Fátima Pires Gomes

Edible mushrooms as mediators of sustainability and human health

Master thesis in Biodiversity and Vegetal Biotechnology, supervised by Doctor Anabela Marisa Azul and Professor João Ramalho-Santos, presented at the Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra

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Edible mushrooms as mediators of sustainability and human health

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica da Doutora Anabela Marisa Azul (Centro de Neurociências e Biologia Celular), Doutor Paulo J. Oliveira (CNC), e com supervisão académica do Professor Doutor João Ramalho-Santos (Departamento de Ciências da Vida) da Universidade de Coimbra.

Cátia de Fátima Pires Gomes

UNIVERSIDADE DE COIMBRA

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The images presented in the cover of this work where used with the permission of the authors: *Ganoderma lucidum*, *Boletus edulis and Macrolepiota procera* by Anabela Marisa Azul, and visualization of mitochondrial network (in red) and nucleus (blue) in fluorescence microscopy, by using TMRE (Tetramethylrhodamine, ethyl ester) and Hoechst 33342 staining for nuclei, respectively, in human hepatocellular carcinoma cell line (HepG2).

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"*It doesn't matter how slowly you go as long as you do not stop*"

Confucius

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Abstract

Mushrooms are increasingly recognized as important foodstuffs for their nutritional benefits and bioactive compounds. Biological activities are present in both wild and cultivated mushrooms and include antioxidant, hypolipidemic, hypoglycemic, and hepatoprotective activities, among others. Non-alcoholic fatty liver disease (NAFLD) is characterized by hepatic lipid accumulation in the absence of excess alcohol intake and is a major public health issue, affecting 20–35% of the world population and accounting for about 2% of deaths annually. Despite the cellular mechanism not fully understood, it is well known that mitochondria are the sub-cellular mediators on liver disorder and disease progression, being involved on several metabolic pathways such as lipid homeostasis and increases in reactive oxygen species (ROS), which may, in turn, lead to a mitochondrial bioenergetics deficit and compromise hepatocyte recovery. Several mushroom species positively affect blood and liver lipid levels, and can thus be a promising strategy in the treatment of NAFLD and other metabolic disorders. However, cellular mechanisms underlying metabolic effects of mushrooms, and respective bioactive compounds, are not thoroughly understood.

The main goal of this study was to investigate the possible hepatoprotective effect of the medicinal species reishi (or Linghzi, *Ganoderma lucidum* (Curtis: Fr) P. Karst.) and the two edible mushrooms porcini (*Boletus edulis* Bull.), and "parasol mushroom" (*Macrolepiota procera* (Scop.) Singer), with a particular focus on mitochondrial function in a model of lipid-mediated injury.

First, the cytotoxic effect of the three mushroom ethanolic extracts was evaluated in human hepatocellular carcinoma cell line (HepG2) in order to estimate the safe concentration window, i.e. the mushroom concentrations that are not toxic to cells. Second, the cytotoxicity of palmitic acid (PA) in HepG2 cells was measured through the effect of this fatty acid on several cellular end points to optimize and validate a PA cytotoxicity-induced *in vitro* model. The cytotoxic effects of PA (without and with fructose), and mushroom extracts on cellular and mitochondrial bioenergetics function were measured using different end-points: metabolic activity (resazurin reduction assay), cell mass (sulforhodamine B assay) and ATP concentration (luminescent cell viability assay), ROS (mitochondrial superoxide anion detection using MitoSOX), and nuclear fluorescence staining (Hoechst). The selected concentrations of mushrooms were then used in order to

prevent PA toxicity on HepG2 cells, in order to obtain preliminary evidence of their potential protective effect on liver diseases.

The results demonstrated that all mushroom ethanolic extracts showed little toxicity; in fact, cellular/ mitochondrial toxicity was observed only at concentrations higher than 250 μg/mL for *G. lucidum*, 750 μg/mL for *M. procera* and 1000 μg/mL for *B. edulis*, supporting the safety associated to the consumption of the three species.

Regarding the PA cytotoxicity model, through the different end points, was defined as EC_{50} 500 μM. Then, the PA toxicity model was tested with the mushrooms, *Ganoderma lucidum* (62.5 μg/mL) contributed to a slight increase on metabolic activity and *B. edulis* and *M. procera* extracts presented similar profiles. The combination of *G. lucidum* with *M. procera* extracts (62.5 μg/mL + 125 μg/mL) appeared to prevent the PA cytotoxicity. However, there was no statistically significant protection of the mushroom extracts used on the end-points for cytotoxicity caused by PA. Our data *in vitro* does not support the use of *G. lucidum*, *B. edulis* and *M. procera* to prevent possible hepatotoxicity resulting from liver lipid overload. Similar negative results were obtained with resveratrol, a polyphenol recognized for its antioxidant activity. The results from the *in vitro* assays suggested that the cytotoxicity caused by PA might exceed a point of-no return, thus limiting the protection by different agents. Still, our data supports the future validation of dietary natural extracts and isolated phytochemicals from mushrooms in order to decrease the phenotype associated with fatty liver disease, as well the need to use proper *in vitro* models.

Keywords: mushrooms; mitochondria; antioxidants; cellular and mitochondrial function

Resumo

Os cogumelos são cada vez mais reconhecidos como alimentos relevantes, devido aos seus benefícios nutricionais e compostos bioativos. Tanto os cogumelos silvestres como os cogumelos cultivados apresentam atividades biológicas importantes, entre as quais antioxidante, hipolipídica, hipoglicémica e hepatoprotetora. A doença do fígado gordo não alcoólico (FGNA) é caracterizada pela acumulação de lípidos nos hepatócitos na ausência de consumo excessivo de álcool, sendo considerado um problema de saúde pública, que afeta 20 a 35% da população mundial e causa cerca de 2% das mortes por ano. Apesar de não serem conhecidos totalmente os mecanismos celulares envolvidos na doença, sabe-se que as mitocôndrias são importantes mediadores subcelulares nos distúrbios hepáticos e na progressão da doença, estando envolvidas em várias vias metabólicas, como na homeostasia lipídica e no aumento de espécies reativas de oxigénio (ROS), que pode conduzir a um défice na atividade bioenergética mitocondrial e a comprometer a recuperação dos hepatócitos. Várias espécies de cogumelos apresentam efeitos positivos nos níveis lipídicos do sangue e do fígado, apresentando-se assim como uma estratégia promissora no tratamento de FGNA e outros distúrbios metabólicos. No entanto, os efeitos metabólicos dos cogumelos e dos seus compostos bioativos nos mecanismos celulares ainda não são inteiramente compreendidos.

Este estudo teve como objetivo principal avaliar o possível efeito hepatoprotetor de três cogumelos, a espécie medicinal reishi (ou linghzi, *Ganoderma lucidum* (Curtis: Fr) P. Karst.), e duas excelentes comestíveis, o tortulho (*Boletus edulis* Bull.) e o frade (*Macrolepiota procera* (Scop.) Singer), com interesse na função e viabilidade mitocondrial num modelo de toxicidade mediado por lípidos.

Em primeiro, foi avaliado o efeito citotóxico dos três extratos etanólicos dos cogumelos na linha celular derivada de um hepatocarcinoma humano (HepG2), para estimar as concentrações seguras, ou seja, as concentrações não tóxicas para as células. Em segundo, pretendeu-se otimizar e validar um modelo *in vitro* de citotoxicidade de ácido palmítico (PA), avaliando-se a citotoxicidade do PA nas células HepG2, tendo em conta o efeito deste ácido gordo em vários ensaios celulares. Os efeitos na atividade bioenergética celular e mitocondrial na presença dos extratos de cogumelos e do PA (com e sem frutose) foram avaliados utilizando diferentes ensaios, avaliando a atividade metabólica (ensaio de redução da resazurina), a massa celular (ensaio de sulforodamina B), concentração de ATP (por luminescência), ROS (MitoSOX) e apoptose celular (coloração com Hoechst).

As concentrações selecionadas de cogumelos foram então usadas para prevenir a toxicidade do PA em células HepG2, de forma a obter evidências preliminares do seu potencial efeito protetor em doenças hepáticas.

Os resultados demonstraram que os extratos etanólicos dos cogumelos apresentam pouca toxicidade na alimentação, sendo que ao nível celular/mitocondrial, somente apresentaram toxicidade em concentrações superiores a 250 µg/mL para o *G. lucidum*, 750 µg/mL para o *M. procera* e 1000 µg/mL para o *B. edulis*, comprovando a segurança associada ao consumo das três espécies.

Em relação ao modelo de citotoxicidade com PA, através dos diferentes ensaios, foi definido como EC_{50}, 500 µM. Seguidamente, foi avaliado o efeito dos cogumelos no modelo de citotoxicidade com PA, *G. lucidum* (62,5 µg/mL) contribuiu para um ligeiro aumento na atividade metabólica e os extratos de *B. edulis* e *M. procera* apresentaram efeitos semelhantes. A combinação dos extratos de *G. lucidum* com *M. procera* (62,5 µg/mL + 125 µg/mL) parece prevenir a citotoxicidade PA. No entanto, os resultados revelam que os cogumelos não apresentaram um efeito protetor estatisticamente significativo na citotoxicidade causada pelo PA. Os resultados *in vitro* indicam que *G. lucidum*, *B. edulis* e *M. procera* não conseguiram prevenir a possível hepatotoxicidade resultante da acumulação de gordura no fígado. Similarmente, foram obtidos resultados negativos com o resveratrol, um reconhecido polifenol com atividade antioxidante. Os ensaios *in vitro* sugerem que a citotoxicidade causada pelo PA pode ter excedido o ponto de não retorno, limitando assim a proteção dos diferentes agentes. Ainda assim, os nossos resultados suportam uma futura validação de extratos naturais dietéticos e fitoquímicos isolados a partir de cogumelos, com o objetivo de diminuir o fenótipo associado à doença do fígado gordo, bem como a necessidade de adequar o modelo *in vitro*.

Palavras-chave: cogumelos; mitocôndria; antioxidantes; função celular e mitocondrial

List of abbreviations

AA	antimycin A
Acetyl-CoA	acetylcoenzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CAT	catalase
CTL	Control
Cyt c	Cytochrome c
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECACC	European collection of authenticated cell cultures
ETC	pH component of mitochondrial proton motive force
FBS	Fetal bovine serum
GPx	Glutathione peroxidase
GSH	Glutathione (reduced state)
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
IMM	Mitochondrial inner membrane
IMS	Intermembrane space
MCU	Mitochondrial calcium uniporter
MRC	Mitochondrial respiratory chain
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine nucleotide, reduced form
NADPH	Nicotinamide adenine nucleotide phosphate, reduced form
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
$-O_2\bullet$	Superoxide anion
OMM	Mitochondrial outer membrane

ONOO-	Peroxynitrite
OXPHOS	Oxidative phosphorylation
PA	Palmitic acid
PBS	Phosphate buffer saline
ROS	Reactive oxygen species
ROT	Rotenone
RS	Reactive species
SOD	Superoxide dismutase
SRB	sulforhodamine B
TCA	Tricarboxylic acid
VDAC	Voltage-dependent anion channel

1. Introduction

1.1. Fungi: Mushrooms

Fungi represent the second largest kingdom of eukaryotes on Earth. In 1991, a landmark paper from Hawksworth (1991) estimated that there are 1.5 million of fungal species. More recently, Blackwell (2011) estimated that there are 5.1 million of fungal species, while molecular tools beginning to reveal a hitherto unknown diversity (Peay *et al.*, 2016). Fungi range from yeast and slime molds to mushrooms (and truffles) being classified into major seven phyla, namely Ascomycota and Basidiomycota, ten subphyla, 35 classes, 12 subclasses, and 129 orders (Hibbet *et al.*, 2007).

Classically, Mycology has been considered a branch of Botany, however fungi are more closely related to animals than to plants (Baldauf and Jeffrey, 1993). Fungi and animal ancestors are believed to be simple aquatic forms that diverged about 1 billion years ago. The similarities between fungi and animals include the molecule of chitin, as the main structural component of the cell walls in fungi and as the exoskeleton in insects, for example. The large-scale DNA-sequencing datasets has been disclosing the magnitude of fungal diversity, which seems to be different to bacteria, animals and plants (Peay *et al.*, 2016). The structural unit – hyphae – represents a eukaryotic cell with a cell wall (as plants) unique to the fungi. The cell walls of hyphae are primarily composed of chitin, glucans and glycoproteins (Bowman and Free, 2006).

Fungi developed a multitude of unicellular and filamentous forms detailed for absorptive nutrition whereas animal branch evolved diverse organisms capable of feeding by ingestion (James *et al.*, 2006). Yet, the antiquity of fungi and the early history of eukaryotes may be about to be re-written. A 2.4-billion-year-old basalt containing fungus-like mycelium fossils has recently been discovered (Bengtson *et al.*, 2017), suggesting that fungal clade is considerably older than previously thought.

Mushrooms (and truffles) represent a large group of higher fungi, most belonging to the divisions Ascomycota and Basidiomycota, which fruiting bodies produce spores in a distinct structure called "ascoma" and "basidiome", respectively. Mushrooms was described by Chang and Miles (1992) as "a macrofungus with a distinctive fruiting body, which can be either epigeous (grow above the earth) or hypogeous (underground grow; i.e. truffles) and large enough to be seen with naked eye to be picked by hand". Mushrooms may include over 140 000 species (Lindequist *et al.*, 2005).

The morphological structure of mushrooms is commonly associated to umbrella-shaped fruiting bodies, with spores produced in gills (or lamella) located on the underside of the cap (or pileus) supported by a stipe (stem). However, fruiting bodies of higher fungi producing mushrooms can be very diversified, the umbrella-shaped fruiting bodies may have the hymenium with pores and spines, or false gills. There is also a high diversity of not gilled fruiting bodies, with hymenium incredibly varied in shape, from spherical (e.g. puffballs) to coral. The high diversity in morphology of mushrooms and truffles reflect the variability in chemical structure and physiology as well as their biology and ecology (Cheung, 2008).

1.1.1. Mushroom biology, ecology and sustainability

Mushrooms (and truffles) have two growth phases, the vegetative phase with mycelium growth and the sexual reproductive phase with the production of fruit bodies. The vegetative phase is accompanied by the hypha growth to form the mycelium, the fruit bodies promotes the production of spores (with similar functions as seeds in plants) (Cheung, 2008).

Fungi have diverse lifestyles, being major decomposers in certain ecosystems, essential symbiotic to many organisms, and parasites with ample dissemination (Azul *et al.*, 2014). Fungal habitats include soil, water, and organisms (Peay *et al.*, 2016). Some fungi establish symbiotic associations with roots of shrubs and trees, called mycorrhizas, the fungus helps the tree to obtain essential minerals from the soil and tree delivers energy-rich nutrients to fungal mycelium. Mycorrhizas are ancient fungal-plant symbiotic interactions with about 407 millions of years (Taylor *et al.*, 1992) and assumed of central importance to the early evolution of terrestrial ecosystems.

The symbiotic mushrooms (and truffles) establish mycorrhiza with trees, shrubs and occasionally herbs; the saprophytic mushrooms decompose dead organic material from plants, animals and litter. Most mushrooms are saprophytic and symbiotic, only very few are parasitic species (Peay *et al.*, 2016).

Mushrooms (and truffles) are traditionally considered non-wood forest products (NWFPs), with the cultivated (saprophytic) species rather than the edible wild fungi predominant in the market. The classification mushrooms and truffles separate the edible wild from cultivated products, in two main sections as a food product (prepared or preserved), or as

agriculture and forestry product (FAO 2017). Figures 1 and 2 illustrate the production (and yield) of mushrooms and truffles worldwide over the last two decades.

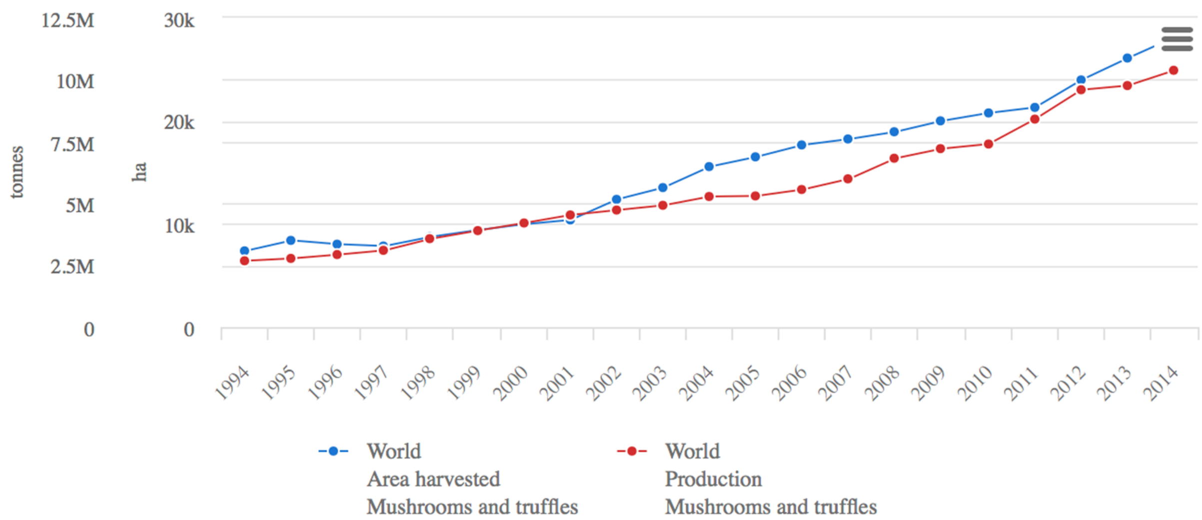


Figure 1 | World production and yield of mushrooms and truffles between 1994 and 2014. (source Food and Agriculture Organization of the United Nations, FAO).

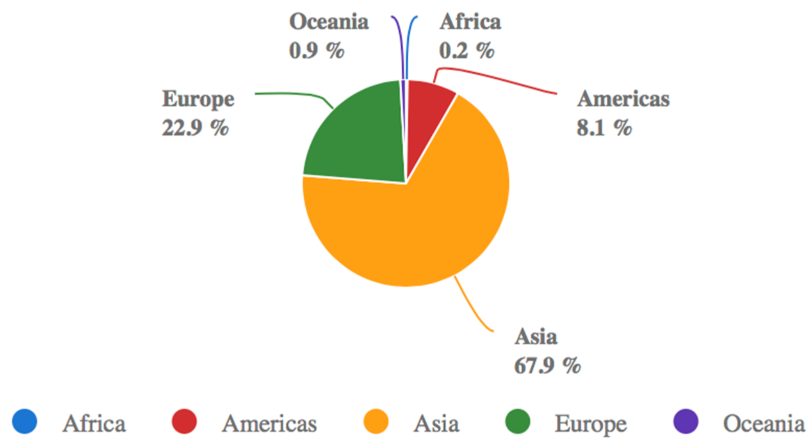


Figure 2 | Intercontinental mushrooms and truffles production between 1994 and 2014. (source Food and Agriculture Organization of the United Nations, FAO).

The market associated to edible mushrooms cultivation is dominated by certain species, such as the "champignons" (*Agaricus* spp.), oyster mushroom (*Pleutorus* spp.), shitake (*Lentinus edodes*) or the medicinal reishi (or língzhi, *Ganoderma* spp.), and the wild species include namely *Boletus* spp., *Cantharellus cibarius*, *Tricholoma matsutake*, *Terfezia* spp. or *Tuber* spp. and other edible mushrooms and truffles collected in forests (FAO, 2017).

The mushrooms and truffles cultivation and exploration vary by region and countries all over the world. For example, the mushroom production in China in 2011 was estimated at about 26 million tons in fresh weight (Zhang *et al.*, 2014), which accounts for 80% of total production in the world. The production and cultivation of mushrooms and truffles represent an emergent critical support in forestry and bio-industry while providing an attractive economical return, ecosystems services (Azul *et al.*, 2014) and human health (Martel *et al.*, 2016).

Mushrooms (and truffles) are increasing recognized as a promising source for their nutritional benefits and bioactive compounds, namely polysaccharides, β-glucans and proteins, and secondary metabolites, such as phenolic compounds, terpenes, steroids offering potential solutions across human health and biotechnology (Erjavec *et al.*, 2012; Azul *et al.*, 2014). Besides, the fruiting bodies or mycelium can be manipulated to produce bioactive compounds in relatively shorter period of time than plants (Ferreira *et al.*, 2009). Ensuring long-term nutrition and ecological sustainability through mushrooms (and truffles) requires a better understanding of cellular mechanisms associated to bio-energetic functions, which are the basis of human health, but also the foundations to identify diets and conscious sustainable choices.

1.1.2. Nutrition and health benefits of mushrooms

Mushrooms have been valued as edible and medical provisions for a long time; in fact, ancient Greeks believed that mushrooms provided strength for warriors in battle (Valverde *et al.*, 2015), and the Romans considered them as the "Food of the Gods" (Mattila *et al.*, 2000).

The basic constitution of mushrooms is 90% water and 10% dry matter (Wani *et al.*, 2010). The dry matter includes proteins (27% to 48%), polyunsaturated fatty acids (72% to 85%), digestible carbohydrates (50 to 60%), high variety of minerals, such as potassium, chlorine, sulfur, magnesium, calcium and sodium, and vitamins D, B1, B2, B12, C, and E. The chemical constitution of mushrooms (and truffles) is very attractive from the nutritional point of view. The energetic value of fresh mushrooms is about 250 and 350 calories/kg, which is comparable to eggs, milk, and meat.

Mushrooms are also rich in bioactive compounds, such as ergosterols, lectins, phenolics and polyphenolics, polysaccharides, terpenoids, and volatile organic compounds, namely

carotenoids, flavonoids, and folates (Mattila *et al.*, 2000; Chang & Miles, 2004; Cheung, 2008; Kalač, 2009; Huang & Nie, 2015; Valverde *et al.*, 2015).

Mushrooms (and truffles) are increasingly recognized for their multiple biological activities, such antioxidant (Ferreira *et al.*, 2009; Yeh *et al.*, 2011; Yoon *et al.*, 2011), hepatoprotective (Soares *et al.*, 2013), antitumor and immunomodulator (Huang & Nie, 2015), anti-obesogenic (Martel *et al.*, 2016), antimicrobial (Ferreira *et al.*, 2015), ameliorated insulin resistance (Ma *et al.*, 2014), antiviral (Mattila *et al.*, 2000), antifungal and antiallergic (Lindequist *et al.*, 2005) effect, and as potential agents in the prevention of fatty liver diseases and other metabolic disorders (Lindequist *et al.*, 2005; Nagao *et al.*, 2010).

1.1.3. Antioxidant activity from mushrooms

Reduction of oxygen can result in the generation of reactive oxygen species (ROS), which, when in excess can damage cells (see section 1.2) and be a mediator in aging-associated cardiovascular and neurodegenerative diseases, as well as in many metabolic diseases (Valko *et al.*, 2007).

Similarly to several plants, several mushrooms have been reported to have antioxidant properties. In recent years, there is an increasing interest in natural antioxidants for the development of novel strategies to protect cellular functions (Grattagliano *et al.*, 2012; García-Ruiz *et al.*, 2015), acting to reduce excessive oxidative stress (Benfeito *et al.*, 2013). The synthetic antioxidants commonly used in bio-industry, namely BHT (butyl-hydroxytoluene), BHA (butyl-hydroxyanisol) and TBHQ (t-butyl-hydroxyquinone) have been associated to potential risks to health, namely by accelerating liver damage and carcinogenesis (Lobo *et al.*, 2010). The investigation on antioxidants from mushrooms will bring new insights about the potential benefits on an enriched-mushrooms diet. Besides, the antioxidant components may be produced in fruit bodies, mycelium and culture, extracted, and incorporated in food regime to prevent ROS agents in our body.

The main antioxidants found in mushrooms (Figure 3) include phenolic compounds (e.g. phenolic acids and flavonoids), carotenoids (e.g. β-carotene), polysaccharides, vitamins and derivatives (e.g. ascorbic acid, ergosterol and tocopherols), minerals (e.g. zinc and selenium), organosulfur, and nitrogen non-proteins compounds, among others (Ferreira *et al.*, 2009). The phenolic compounds in mushrooms are primarily phenolic acids, and they can be divided into hydroxybenzoic acids and hydroxycinnamic acids, which are derived from non-phenolic molecules benzoic and cinnamic acid, respectively (Ferreira *et al.*, 2009).

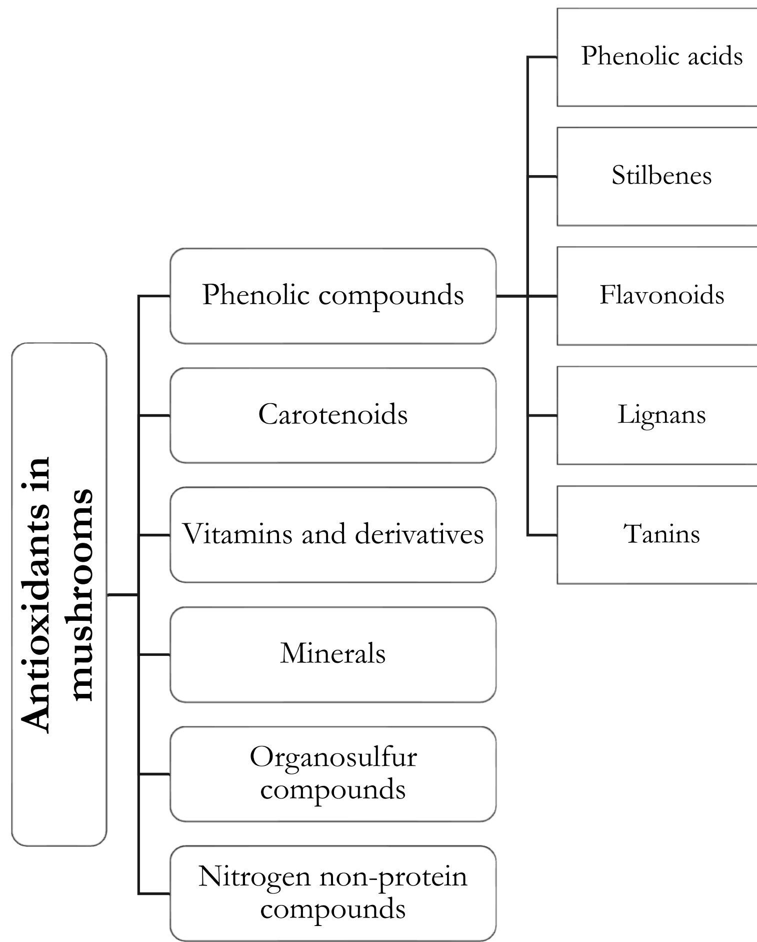


Figure 3 | Major classes of antioxidants identified in mushrooms. Adapted from Carocho & Ferreira (2013).

1.1.4. *Ganoderma lucidum* (Curtis: Fr.) P. Karst.

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Polyporales

Family: Ganodermataceae

Genus: *Ganoderma*

Species: *Ganoderma lucidum* (Curtis: Fr.) P. Karst

Description: *Cap* 6-12 cm diameter, initially irregularly elongated, afterwards with roughly a kidney-shaped form. *Surface* smooth with a shiny crust, yellowish red or brownish, sealing appearance. *Flesh* brownish, soft when young, sooner woody. *Pore surface*: 4-5 mm, circular to angular, white when young, turning brown with age. *Spore print*: ferruginous brown. *Stipe* 3-10x1-3 cm, vertical to eccentric, cylindrical, the same color as the hat; sometimes absent. *Odor:* not distinctive. *Taste:* rather bitter. **Ecological strategy:** Saprophytic of deadwood from hardwoods, or parasitic of living hardwoods.

Figure 4 | *Ganoderma lucidum* (Curtis: Fr.) P. Karst. Photo by Anabela Marisa Azul with permission.

Ganoderma lucidum (Curtis: Fr.) P. Karst, 1881, known as "Reishi" in China or "Linghzi" in India and Japan, has history of more than 2000 years of being used to promote health and longevity in many Asian countries (Sanodiya *et al.*, 2009). The species of *Ganoderma lucidum* have a worldwide distribution (Zhou *et al.*, 2015). However, the fruiting bodies are rather rare in nature. Typically, *G. lucidum* is saprophytic of deadwood, namely alder (*Alnus* sp.), ash (*Fraxinus* sp.), beech (*Fagus* sp.), birch (*Betula* sp.), chestnut (*Castanea* sp.), lime (*Tilia* sp.), maple (*Acer* sp.), *Magnolia* sp., oak (*Quercus* sp.), pear (*Pyrus* sp.) and poplar (*Populus* sp.) (Jong & Birmingham, 1992; Heleno *et al.*, 2012), or parasitic of living hardwoods.

Ganoderma lucidum products consumption is estimated at several thousand tons worldwide resulted from mushroom cultivation and have an estimated market value more than 2.13 billion Euros (Li *et al.*, 2013). The cultivation of *G. lucidum* fruiting bodies occurs mainly on wood logs and sawdust in plastic bags. However, there is also biotechnological production of *G. lucidum* mycelia in bioreactors, in both liquid media and solid substrates.

Nutritional value

Modern research has shown that *G. lucidum* has various medicinal properties, namely antioxidative, antiaging, anti-inflammatory, immunomodulatory, antitumor, anti-hypertensive and antiviral activities (Boh *et al.*, 2007; Sanodiya *et al.*, 2009). The therapeutic effects of *G. lucidum* are mainly due to their polysaccharides, triterpenoids and phenolic compounds (Mau *et al.*, 2002; Yang *et al.*, 2007), but also other several classes of bioactive substances, such as nucleosides, sterols, flavonoids and alkaloids, amino acids, steroids, oligosaccharides, proteins, mannitol, among others (Zjawiony, 2004).

Table 1 | Secondary metabolites identified in *Ganoderma lucidum* (Curtis: Fr.) P. Karst.

Metabolites	Quantity	Reference
p-Hydroxybenzoic acid (μg/g dw)	82 ± 4 (a)	Reis *et al.*, 2015
p-Coumaric acid (μg/g dw)	53 ± 3 (a)	
Cinnamic acid (μg/g dw)	40 ± 3 (a)	
Ganoderenic acid A Ganoderenic acid B Ganoderenic acid C Ganoderenic acid D Ganoderenic acid E	(c)	Komoda *et al*, 1985
Ganodermanondiol Ganodermanondiol	(a)	Fujita et al, 1986
Lucidumol B Lucidumol A	(a)	Min et al,1998
Ganoderiol A Ganoderiol C Ganoderiol D Ganoderiol G Ganoderiol H Ganoderiol E Ganoderiol I	(b)	Nishibota et al, 1988
20-Hydroxy lucidenic acid D2 20-Hydroxy lucidenic acid E2 20-Hydroxy lucidenic acid F 20-Hydroxy lucidenic acid N 20-Hydroxy lucidenic acid P	(b)	Akihisa et al, 2005

(a) methanol extraction; (b) ethanol extraction; (c) ethanol extraction divided in two fractions: chloroform and water

Polysaccharides, particularly beta-d-glucans, have been known to promote a protective effect against free radicals and reduce cell damage and inflammation (Wu *et al.*, 2016). In addition, polysaccharides have been recognized to exhibit antitumor effects through immunomodulation and anti-angiogenesis (Xu *et al.*, 2011; Suarez-Arroyo *et al.*, 2013). Triterpenoids have been reported to possess hepatoprotective (Wu *et al.*, 2015; 2016; Chiu *et al.*, 2017), anti-hypertensive, hypocholesterolemic effects, among others.

The table 1 illustrates the metabolites identified in *Ganoderma lucidum* through different types of extraction. Table 2 reflects the identification and quantification of triterpenic and phenolic acids in the ethanolic extracts of used in this work, by using high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC–DAD– ESI/MS) (see the supplementary information).

Table 2 | Identification and quantification of triterpenic and phenolic acids in *Ganoderma lucidum* ethanolic extracts.

Peak	Rt min)	UVλmax (nm)	$[M-H]^-$ and $[2M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)
Phenolic acids						
1	8.39	257	137	93	*p*-Hydroxybenzoic acid	tr
2	11.51	280	197	121	Syringic acid	0.026±0.001
Triterpenes						
3	29.06	259	533/1067	515,404,303	12-3,7,15-Trihydroxy-4-(hydroxymethyl)-11,23-dioxo-lanost-8-en-26-oic acid	0.18 ± 0.03
4	30.8	256	533/1067	515,497,453,423,319,303	12-Hydroxyganoderic acid C2	tr
5	31.27	252	529/1059	511,467	20-hydroxy-ganoderic acid AM1	0.015 ± 0.003
6	31.73	251	529/1059	511,499,481,467,438	2-deacetylganoderic acid H	tr
7	33.94	258	531/1063	513,451,401,385,304,301,286,249	Ganoderic acid η	0.99 ± 0.03
8	34.48	254	511/1023	493,449,413,405	Ganoderic acid F	0.63 ± 0.05
9	35.37	265	529/1059	511,467,449,437,317,301,300,263	12-hydroxyganoderic acid D	0.062 ± 0.001
10	35.95	251	515/1031	497,453,439,304,288,250	Ganoderic acid derivative	0.81 ± 0.02
11	36.84	256	517/1035	499 481, 456,438, 407, 304, 287	Ganoderic acid C2	0.428 ± 0.002
12	37.51	257	529/1059	511,481,467,451, 438, 424, 319, 303, 301	Ganoderic acid C6	1.82 ± 0.04
13	38.16	256	529/1059	511,481,467,451, 438, 424, 319, 303, 301	Ganoderic acid derivative	0.79 ± 0.03
14	38.46	256	531/1063	513,498,469,454,452,437,304,302,290,266	Ganoderic acid G	1.46 ± 0.04

15	38.75	248	513/1027	495,480,452,437,433,407,331,319,303,287,263,249,241	Ganoderenic acid B	2.24 ± 0.05
16	39.35	254	515/1031	497,453,439, 409, 304,288, 263, 250	Ganoderic acid B	2.75 ± 0.05
18	39.46	250	513/1027	495, 480, 451,433, 381,301,247	Ganoderic acid derivative	2.0 ± 0.1
19	39.66	261	513/1027	495, 480, 451,433, 381,301,247	Ganoderic acid AM1	0.67 ± 0.07
20	40.29	254	515/1031	497,480,454,436,302,301,285	Ganoderic acid A	1.42 ± 0.03
21	40.66	261	1143/571	553,511,510,468	Ganoderic acid H	6.71 ± 0.08
22	41.34	254	529/1059	511,493,467,449,434,419,421,317,301,299,285,263	Ganoderic acid derivative	1.30 ± 0.03
23	41.93	246	511/1023	493,478,449,435,431,405,335,329,301,286,283,261, 247,149	Ganoderenic acid D	1.37 ± 0.03
24	42.55	255	513/1027	495,451,437,433,407,301,286,284,261,247,149	Ganoderic acid D	1.05 ± 0.02
25	42.74	245	509/1019	491,476,461,447,429,417,300,299	Unknown	1.03 ± 0.04
26	43.15	256	511/1023	493,449,435,300,247	Ganoderic acid E	0.78 ± 0.01
27	43.95	255	569/1139	551,509,508,466	12-acetoxyganoderic acid F	2.421 ± 0.003
28	44.92	272	513/1027	451,437,433,422,301	Ganoderic acid J	0.104 ± 0.001
					Total triterpenoids	31.42 ± 0.08

Calibration curves used: 1-protocatechuic acid (y=214168x + 27102; R^2=0.999); 2- *p*-hydroxybenzoic acid (y = 208604x + 173056; R^2=0.999); 3-syringic acid (y=376056x + 141329; R^2=0.999); 4-ganoderic acid A (y=2539.7x + 16193; R^2=0.999). Characterization of ethanolic extract conducted with the collaboration of Isabel Ferreira at Laboratory of Applied Chemistry and Biochemistry (LQBA) in the School of Agriculture of Polytechnic Institute of Bragança (IPB).

1.1.5. ***Boletus edulis*** **Bull.**

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Boletales

Family: Boletaceae

Genus: *Boletus*

Species: *Boletus edulis* Bull.

Description: *Cap* up to 20cm diameter, first hemispherical, then convex to broadly convex. *Surface* smooth to finely tomentose, whitish brown when young, becoming brown with age. *Margin* usually lighter and with a distinctive whitish border. *Flesh* thick, firm and white when young, spongy with age, unchanging when bruised. *Tubes* 10-15 mm long, white to cream when young, then greenish yellow notched at the stipe. *Pores* 0.3-0.4 mm concolourous with tubes, polygonal or irregular. *Stipe* up to 15 cm long and 10 cm thick, firm ventricose or clavate, surface whitish to light brown, brown in middle and below, whitish in the base, covered with a whitish reticulum usually only from apex to the middle of the stipe. *Odor:* pleasant. *Taste:* mild nutty. **Ecological strategy:** ectomycorrhizal; fructification in late summer or autumn (Hall *et al.*, 1998) in northern hemisphere.

Figure 5 | *Boletus edulis* Bull, photo by Anabela Marisa Azul with permission.

Boletus edulis Bull., known as porcini and "the king bolete", is one of the most appreciated edible mushroom, and it is very popular in Europe, including Portugal, North America and Asia (Heleno *et al.*, 2011), in terms of occurrence and consumption. The crispy fruiting bodies are commonly eaten in pastas, soups or salads, in fresh or preserved (e.g. dried, frozen, soaked in vinegar).

Boletus edulis establishes symbiotic associations with roots of shrubs and trees. The plant hosts of *B. edulis* include deciduous trees, namely European chestnut (*Castanea sativa* Mill.), hazel

(*Corylus* sp.), beech (*Fagus* sp.), birch (*Betula* sp.), hornbeam (*Carpinus* sp.), lime (*Tilia* sp.), oak (*Quercus* sp.), poplar (*Populus* sp.) and sallow (*Salix* sp.); evergreen trees, such as cork oak (*Quercus suber* L.), holm oak (*Quercus rotundifolia* Lam.) and other oaks (*Quercus* sp.); conifers, such as spruce (*Picea* sp.), fir (*Abies* sp.), hemlock (*Tsuga* sp.), *Keteleeria* sp. and pines (*Pinus* sp.); or shrubs, such as *Cistus* spp. (Olivier *et al.*, 1997; Hall *et al.*, 1998; Águeda *et al.*, 2006; Mello *et al.*, 2006). *Boletus edulis* did not occur naturally in the southern hemisphere, although it has been accidentally introduced into South Africa and New Zealand (Wang *et al.*, 1995; Hall *et al.*, 1998)

The retail worldwide market associated to *B. edulis* is estimated at 20 000-100 000 tons/year and a value superior to 212.775 million Euros (Hall *et al.*, 1998). Controlled mycorrhization launches new perspectives on cultivation of this important commercial species (Hall *et al.*, 1998).

The nutritional and therapeutic effects of *B. edulis* are associated to the abundance of polysaccharides, amino acids, and vitamins (Heleno *et al.*, 2011) and reported to its antioxidant and antitumor activities (Luo *et al.*, 2012; Lemieszek *et al.*, 2013; Vamanu & Nita, 2013; Wang *et al.*, 2014).

The table 3 illustrates the metabolites identified in *Boletus edulis*, Heleno *et al.*, 2011, Ferreira *et al.* (2009) and Guo *et al.* (2012) reported other compounds with antioxidant activities.

Table 3 | Secondary metabolites identified in *Boletus edulis* Bull.

Metabolites	**Quantity**	**Reference**
Ash (g/100g dw)	5.53 ± 0.23	(Heleno *et al.*, 2011)
Proteins(g/100g dw)	21.07 ± 0.66	
Fat (g/100g dw)	2.45 ± 0.09	
Carbohydrates (g/100g dw)	70.96 ± 0.66	
Tocopherols (mg/g dry matter)	3.30×10^{-4}	(Ferreira *et al.*, 2009)
β-Carotene (mg/g extract)	2.73×10^{-3}	
Gallic acid (mg/100 g dw)	30.77 ± 1.283	(Guo *et al.*, 2012)
Protocatechuic acid (mg/100 g dw)	13.026 ± 0.508	
p-Hydroxybenzoic acid (mg/100 g dw)	4.15 ± 0.127	

The table 4 reflects the identification and quantification triterpenic and phenolic acids in the ethanolic extracts of used in this work, by using high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC–DAD– ESI/MS) (see the supplementary information).

Table 4 | Identification and quantification of phenolic acids in *Boletus edulis* ethanolic extracts.

Peak	Rt (min)	UV (nm)	λmax$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	Tentative identification	Quantification (mg/g)
1	30.82	276	147	103	Cinnamic acid	0.07 ± 0.01

Calibration curves used: cinnamic acid (y= 100000x + 222206; R^2=0.999). Characterization of ethanolic extract conducted with the collaboration of Isabel Ferreira at Laboratory of Applied Chemistry and Biochemistry (LQBA) in the School of Agriculture of Polytechnic Institute of Bragança (IPB).

1.1.6. *Macrolepiota procera* (Scop.) Singer

Kingdom: Fungi

Division: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Agaricaceae

Genus: *Macrolepiota*

Species: *Macrolepiota procera* (Scop.) Singer

Description: *Cap* 7–25 cm diameter, first spherical, becoming convex to plan-convex with age, and an obtuse umbo. *Surface* light brown to pale with brown scales regular patterned. *Flesh* whitish and soft. *Lamellae* free, densely crowded, thin, white when young, white to cream colored when mature. *Spore* print is white. *Stipe* 15-30 cm, enlarged to bulbous at the base with brown scales with herringbone pattern. *Ring* double and usually movable. *Odor:* nutty. *Taste:* sweet. **Ecological strategy:** saprophytic; fructification solitary or in small-scattered groups during late summer and beginning autumn in northern hemisphere, commonly in neglected pastureland, mainly in areas rich in nitrogen.

Figure 6 | *Macrolepiota procera* (Scop.) Singer. Photo by Anabela Marisa Azul with permission.

Macrolepiota procera, known by "parasol mushroom", or "frade", "marifusa" and "tortulho" in Portugal, is an edible species very appreciated in our country. *Macrolepiota procera* occurs solitary or in small-scattered groups during late summer and beginning autumn in meadows and forests. dominated by coniferous and *Fagaceous* trees in northern hemisphere. *M. procera* commonly appears in neglected pastureland, and has the ability to accumulate in its fruitbody toxic (Baptista *et al.*, 2009).

The nutritional and therapeutic effects of *M. procera* are associated to their abundance of proteins, carbohydrates, mineral contents (Baptista *et al.*, 2009), and phenolic compounds, namely flavonoids (Barros *et al.*, 2007, 2009) and cinnamic acid (Taofiq *et al.*, 2015). This compounds have been reported with anti-inflammatory (Taofiq *et al.*, 2015), antioxidant (Barros *et al.*, 2007, 2009) and anti-cancer (Arora & Simran, 2015) activities.

The table 5 indicates the metabolites identified in the parasol mushroom. Previous studies performed by Barros and collaborators (2009) focusing on phenolic components revealed the presence of 21.53 mg/kg of cinnamic acid in ethanolic extracts of *M. procera.*

Table 5 | Secondary metabolites identified in *Macrolepiota procera* (Scop.) Singer.

Metabolites	Quantity	Reference
Ash (g/100g dw)	0.65 ± 0.01	Palazzolo *et al.*, 2012
Proteins(g/100g dw)	3.01 ± 0.10	
Fat (g/100g dw)	0.20 ± 0.02	
Carbohydrates (g/100g dw)	2.26 ± 0.07	
Cinnamic acid (mg/kg dry matter)	21.53 ± 1.65	Barros *et al.*, 2009
Myristic acid		Muji *et al.*, 2011
Pentadecanoic acid		
Palmitoleic acid		
Palmitic acid		
Oleic acid + linoleic acid		
Stearic acid		
Ergosterol		
7,22-Ergostadienone		
Chalinasterol		
Macrocypin (cysteine protease inhibitor)		Saboti *et al.*, 2009
MpL (lectin)		Zurga *et al.*, 2014
Riboflavin* (Vit. B2)(mg/100g dw)	0,402 mg (27%)	Palazzolo *et al.*, 2012)
Niacin* (Vit. B3) (mg/100g dw)	3,607 mg (24%)	

* Metabolites detected on *Macrolepiota procera* (Scop.) Singer var. *procera*

The table 6 reflects the identification and quantification triterpenic and phenolic acids in the ethanolic extracts of used in this work, by using high-performance liquid chromatography coupled to photodiode array detector and mass spectrometer (HPLC–DAD– ESI/MS) (see the supplementary information).

Table 6 | Identification and quantification of phenolic acids in *Macrolepiota procera* ethanolic extracts.

Peak	**(min)**	**UV (nm)**	**λmax[M-H]$^-$ (*m/z*)**	**MS^2 (*m/z*)**	**Tentative identification**	**Quantification (mg/g)**
1	30.82	276	147	103	Cinnamic acid	0.386 ± 0.003

Calibration curves used: cinnamic acid (y= 100000x + 222206; R^2=0.999). Characterization of ethanolic extract conducted with the collaboration of Isabel Ferreira at Laboratory of Applied Chemistry and Biochemistry (LQBA) in the School of Agriculture of Polytechnic Institute of Bragança (IPB).

1.2. Mitochondria

1.2.1. Structure and function

In the late 19th century, mitochondria were described as microscopic bodies found in the cytoplasm of various cells in many different species (Lewis & Lewis, 1914). Nowadays, it is recognized that mitochondria are sub-cellular organelles present in every mammalian cell (with some exceptions), which may vary from a few organelles to a very large number per cell, especially in metabolically active organs such as liver, brain, cardiac and skeletal muscle (Manash *et al.*, 2006).

Mitochondria are the only organelles outside of the nucleus with their own genome, being capable of replicate itself independently from the nuclear genome (Moreira *et al.*, 2010). Moreover, they intervene in production of adenosine triphosphate (ATP), through oxidative phosphorylation (OXPHOS), but their function also include β-oxidation of fatty acids, metabolism of certain amino acids (Benard & Rossignol, 2008), calcium homeostasis, intermediate metabolism, and free radical production and regulation (Moreira *et al.*, 2010). Mitochondria are described as the powerhouses of the cells (Moreira *et al.*, 2010), as 95% of total energy requirements by cells are produced in mitochondria (Costa *et al.*, 2010). These organelles coordinate several metabolic pathways producing metabolites essential in cell life and death (Diogo *et al.*, 2011; Frezza, 2017). Structurally, mitochondria may differ across organs and tissues, ranging from "giant" spherical morphologies to "hyperfused" reticular networks. The alterations on mitochondrial volume and/or structure could affect the biochemical reactions in the mitochondrial matrix (Willems *et al.*, 2015).

Mitochondria consist in two-lipid bilayer membranes, the outer mitochondrial membrane (OMM) and the inner mitochondrial membrane (IMM), which has a large area and protrudes into the mitochondrial matrix compartment (Willems *et al.*, 2015). The OMM is constituted by cholesterol and is more permeable (Pereira *et al.*, 2009) due the presence of voltage-dependent anion channel (VDAC), non-selective channels responsible for the passage of low molecular-weight molecules between the cytoplasm and the intermembrane space (IMS) (Moreira *et al.*, 2010). Cristae are independent vesicular structures contained in the matrix which are very rich in proteins and which often bind to the mitochondrial inner membrane. The IMS separates the IMM and OMM, being partially connected via contact sites involved in cristae organization

(Willems *et al.*, 2015), and works as a reservoir of protons (H^+) establishing a electrochemical gradient, with an electrical component ($\Delta\psi$) coupled to a pH component (ΔpH), crucial for ATP production via ATP synthase (Pereira *et al.*, 2009).

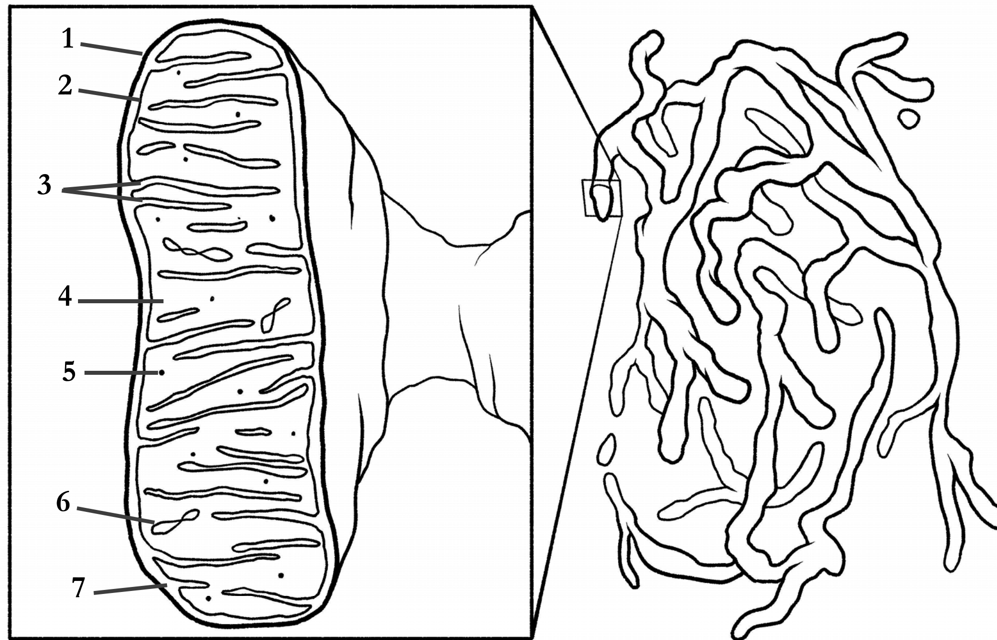


Figure 7 | Mitochondrial network. 1-Outer membrane; 2-Inner membrane; 3-Cristae; 4-Matrix; 5-Ribosome; 6-Mitochondrial DNA; 7-Intermembrane space. Image by Rui Tavares, with permission.

On the other hand, the IMM is rich in cardiolipin and an effective permeability barrier to cations and metabolites, which are transported through the membrane by specific proteins (Scatena *et al.*, 2007; Pereira *et al.*, 2009; Moreira *et al.*, 2010). The cristae, which as described above are independent structures (Mannella, 2006), are rich in enzymatic protein complexes, the electronic transport chain (ETC) and the oxidative phosphorylation (OXPHOS) (Pereira *et al.*, 2009). The ETC consists of four large and multi-subunit (I-IV) enzyme complexes and two mobile electron carriers (coenzyme Q and cytochrome c) (Benard & Rossignol, 2008), while OXPHOS involve the four enzymes complexes, which is coupled to complex V (ATP synthase), responsible for phosphorylation of ADP to ATP (Moreira *et al.*, 2010). Complex I, also named NADH-ubiquinone oxidoreductase, accepts electrons from the reduced form of NADH, which then are transported to oxidized ubiquinone. Buried in complex II (succinate-ubiquinone oxidoreductase), ubiquinone can also accept electrons from the reduced form of

$FADH_2$, originated from the conversion of succinate to fumarate. The electrons from reduced ubiquinone are transferred to cytochrome c reductase or complex III and cytochrome c mediates the electron transfer to cytochrome c oxidase (complex IV). Then, the electrons are transferred to the final electron acceptor, molecular oxygen (O_2), generating H_2O. The electron transport, which is linked to proton (H^+) efflux from mitochondrial matrix and resulting in a proton-motive force (PMF) is used to drive ATP generation by Complex V.

1.2.2. Mitochondrial dysfunction

The biosynthesis of amino acids, vitamins, lipids and many other intermediates, which are required for OXPHOS and cell well function, directly depends on mitochondria (Smith *et al.*, 2012). For this reason, mitochondrial dysfunction can cause augmented lipid peroxidation, nucleic acid oxidation and protein damage that may result in cell death (Pagano *et al.*, 2014).

Mitochondrial dysfunction can be caused by primary events, such as mutations in a gene encoded by mtDNA or a nuclear-encoded gene for a mitochondrial protein and/or secondary events resulted from outside stressors. Secondary mitochondrial dysfunctions are often related with external factors, such as increased oxidative stress, disruption of calcium (Ca^{2+}) homeostasis and defective mitochondrial ATP synthesis (Smith *et al.*, 2012). The dysregulation of Ca^{2+} homeostasis in cell, due to an increase and excessive mitochondrial Ca^{2+} accumulation lead to cell death, via dysregulation in ATP production and redox homeostasis (Brookes *et al.*, 2004).

Mitochondria are particularly vulnerable to oxidative damage, and the continuously metabolism of molecular oxygen may lead to oxidative stress and a mitochondrial dysfunction. Different studies identified some features commonly observed in *in vitro* and patients biopsies related with mitochondrial dysfunction and oxidative stress such as changes in ETC activities; decreased expression of enzyme associated with Krebs cycle and decreased levels of ATP (Pagano *et al.*, 2014).

1.2.3. Mitochondria: oxidative stress and antioxidant network

Increased body of evidences has indicated that mitochondrial dysfunction has a crucial role in early step of disease. Mitochondria consume nearly 85% to 90% of a cell´s oxygen to support oxidative phosphorylation by ATP synthesis (Szeto, 2006). For that reason, the oxidative

phosphorylation is the major source of energy supply and at the same time, a significant source of endogenous free radicals, which can be toxic especially during some pathologies (Willems *et al.*, 2015).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) can have deleterious effects in cells, some of those species being free radicals (Ferreira *et al.*, 2009). A free radical is an atom or molecule containing one or more impaired electrons in the outer orbital, generally unstable and very reactive (Carocho & Ferreira, 2013). ROS comprise free radicals derived from molecular oxygen such as singlet oxygen molecules ($^1O_2$), superoxide anions ($O_2^{\bullet-}$) and hydroxyl radicals ($^{\bullet}OH$) or non radical ROS such as hydrogen peroxide (H_2O_2) (Valko *et al.*, 2007; Singh *et al.*, 2008). In normal metabolism, it is estimated that aerobic cells may produce 10^{11} free radical species daily (Petersen *et al.*, 2007).

It is well established that ROS display a dual role depending on the concentration and intracellular site, as they can be the cellular worst enemy, but also an important flare gun to activate the cells own defenses. At low or moderate concentrations, ROS are required to maintain homeostatic signaling events as well as inducing cell proliferation and survival through the post-translational modifications of kinases and phosphatases (Martindale & Holbrook, 2002), being considered as true second messengers (Valko *et al.*, 2007). On the other hand, a diversity of endogenous factors such as metabolism and inflammation or exogenous environmental factors, such as chemical exposure, pollution or radiation can promote ROS overproduction, and a local imbalance between ROS production and antioxidant endogenous defenses can result in global oxidative stress state, which all may cause irreversible damage to DNA, proteins, and lipids (Martindale & Holbrook, 2002). Insufficient elimination by enzymatic and non-enzymatic endogenous antioxidants or both is often cause for increased local oxidative stress (Carocho & Ferreira, 2013; Sies, 2015).

The impact of oxidative stress damage on the organism depends on the type of oxidant, the intensity of its production and the ability of repair systems (Rahal *et al.*, 2014). For instance, evidence indicates that chronic and acute excess generation of ROS under pathophysiologic conditions represent a trigger to the development of several health problems (Pagano *et al.*, 2014), such as cancer, neurodegenerative, renal, cardiovascular, ageing-related processes and liver disorders (Valko *et al.*, 2007; Ferreira *et al.*, 2009; Sudheesh *et al.*, 2009; Carocho & Ferreira, 2013; Wu *et al.*, 2016).

Under normal conditions, cells have numerous strategies to alleviate the oxidative damage and maintain an equilibrium between ROS production and antioxidant defenses, such as physical protection and repair mechanisms, and mostly important an endogenous antioxidant defense systems. In this way, ROS are decomposed or neutralized by enzymatic or non-enzymatic antioxidants (Valko *et al.*, 2007). The enzymatic antioxidants comprise superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT), among others while non-enzymatic antioxidants include vitamin A and C, coenzyme Q10, uric acid and glutathione (GSH) (Valko *et al.*, 2007; Sudheesh *et al.*, 2009; Carocho & Ferreira, 2013).

1.3. "Love triangle": Mitochondria, Oxidative stress and NAFLD

Modern lifestyle, with sedentary behaviors and incorrect eating habits, contributes to several metabolic diseases. A clear example is non-alcoholic fatty liver disease (NAFLD) which is characterized by hepatic lipid accumulation in the absence of excess alcohol intake and include a wide spectrum of liver injury ranging from simple steatosis to steatohepatitis, fibrosis, and cirrhosis (Figure 8) (Ibrahim *et al.*, 2013; Lewis & Mohanty, 2010).

NAFLD was initially described to be almost exclusively a disease of adults, but nowadays, with an increasing prevalence of obesity in western countries, NAFLD is now also increasingly present in children (Browning & Horton, 2004). Currently, it is considered a major public health issue in Western countries, affecting 20–35% of people in the world and accounting for about 2% of deaths annually (Lewis & Mohanty, 2010; Rolo *et al.*, 2012; Ibrahim *et al.*, 2013).

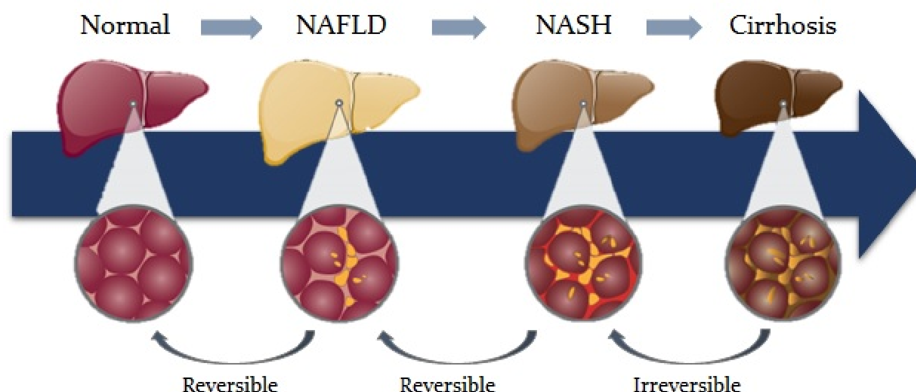


Figure 8 | Progression of Non-Alcoholic Fatty Liver Disease (NAFLD). Adapted from https://www.fxmedicine.com.au/content/non-alcoholic-fatty-liver-disease-nafld

The most common risk factors for NAFLD are obesity, type 2 diabetes mellitus, hyperlipidemia, and other common associations are hypertension and polycystic ovary syndrome. In the case of obese persons, the prevalence of NAFLD increases to 74% and individuals with NASH have a 20% prevalence of cirrhosis (Mehta *et al.*, 2002).

Most patients with NAFLD do not have any signs or symptoms of liver disease. The most common physical sign is hepatomegaly, which is present in up to 75% of the patients with NAFLD. Although, NAFLD is a multisystem disease, which affect several extra-hepatic organs and regulatory pathways (Byrne & Targher, 2015).

Since NAFLD is a multisystem disease (Byrne & Targher, 2015), currently there is no approved medical or surgical treatment (Ibrahim *et al.*, 2013) neither pharmacotherapy for NAFLD (Townsend *et al.*, 2017). Lifestyle modifications remain the mainstay of treatment, and one of the main changes is focused on dietary changes. The Mediterranean diet has been recommended as the diet of choice for the treatment of NAFLD, because besides promoting a long term weight reduction also improves metabolic status and steatosis (Zelber-Sagi *et al.*, 2017).

Steatosis is related with an accumulation of fat in the liver and oxidative stress leading to a mitochondrial dysfunction (Grattagliano *et al.*, 2011; García-Ruiz *et al.*, 2015; Yan *et al.*, 2016).

One the most important functions of mitochondria is lipid homeostasis. The catabolism of carbohydrates through glycolysis performed in the cytosol generates pyruvate, which can be converted to lactate under anaerobic conditions. However, in the presence of oxygen, pyruvate is converted into acetyl coenzyme A (acetyl-CoA) in the mitochondrial matrix (Pereira *et al.*, 2009). Acetyl-CoA enters the Krebs cycle, and generate several intermediates including NADH and succinate, which are then oxidized in the MRC, allowing for an ejection of protons across the inner mitochondrial membrane, and creating an electrochemical protonic gradient which can be used by Complex V to produce ATP (Pereira *et al.*, 2009).

In this process, mitochondria generate ROS, directly in the form of superoxide anion, and to prevent oxidative stress, there is a continuous balance between intrahepatic antioxidants and ROS production. However, when mitochondrial ROS production overcome the antioxidant defense network, ROS may cause damage to different structures, hindering the physiological oxidation of fatty acids (Mehta *et al.*, 2002).

The active role of mitochondria in oxidizing fatty acids creates an initially benign situation in which excess lipid leads to mitochondrial biogenesis and increased activity (Grattagliano *et al.*, 2012). Subsequently, the combination of lipid accumulation, oxidative stress, and other factors are the trigger for a lot of metabolic syndromes and/or diseases, such as obesity, type 2 diabetes, insulin resistance, cardiovascular and liver diseases (Park *et al.*, 2014; Wong *et al.*, 2014).

NAFLD can develop in a "multiple hit" event, in which the "first hit" involves the fat accumulation of fat in the liver and the "second hit" includes the toxicity of excessive oxidative stress resulting in inflammation and progression of NAFLD to NASH (Ibrahim *et al.*, 2013; García-Ruiz *et al.*, 2015). Mitochondrial dysfunction may induce both "hits", because mitochondria are involved in the β-oxidation of free fatty acids, and also being a major ROS (García-Ruiz *et al.*, 2015).

1.3.1. Biological model used – characterization of palmitic acid-induced toxicity

The HepG2 cell line, derived from liver biopsies of a 15-year-old Caucasian male with a differentiated hepatocellular carcinoma, and which retains biochemical and morphological properties characteristic of hepatocytes, is considered as a relevant *in vitro* hepatic cellular model for studying hepatocyte/liver injury through the role and biochemical effects of fat accumulation in the liver, dietary fatty acids or obesity. Moreover, is also considering a promising alternative to human hepatocytes to be used as cellular model for steatosis (Gómez-Lechón *et al.*, 2007).

Fatty acids can be divided into saturated and unsaturated fatty acids and their impact on cells is distinct. It has been reported that saturated fatty acids are more harmful to health than unsaturated fatty acids (Park *et al.*, 2014). The most common saturated fatty acid is palmitic acid (C16:0) (PA), which is found in animals, plants and microorganisms. The whole mechanism underlying PA toxicity remains understood but is known that the PA overloading induce apoptotic cell death, through activation of the mitochondrial apoptotic pathway (Dyntar *et al.*, 2001; Gómez-Lechón *et al.*, 2007), although other factors may jointly collaborate to increase PA toxicity, including excessive fructose intake (Zhao *et al.*, 2016).

1.4. Objectives

In recent years, evidences in experimental models of NAFLD raised the attention to two main classes of dietary antioxidants (polyphenols and carotenoids) contained in mushrooms, fruits and vegetables (Zelber-Sagi *et al.*, 2017). Due to the diverse characteristics of mushrooms, such as the production of a huge variety of compounds in short time with application in health and biotechnology.

This dissertation aimed to investigate the possible health beneficial effects of the medicinal species *Ganoderma lucidum* (Curtis) P. Karst. and the two edible mushrooms *Boletus edulis* Bull., and *Macrolepiota procera* (Scop.) Singer., against the toxicity caused by excessive PA treatment, mainly focusing in the cell viability.

This work had follow objectives:

i) Evaluation of the cellular/mitochondrial toxicity of mushrooms ethanolic extracts in human hepatocellular carcinoma cells (HepG2).

ii) Optimize and validate the PA-induced cytotoxicity model.

iii) Evaluate the hepatoprotective effects of mushrooms extracts against fatty acid-induced cytotoxicity in HepG2 cells.

The experimental design involved several functional end-points, aimed at testing the potential protector effect of mushrooms on cellular and mitochondrial function under PA overload conditions.

2. Material and Methods

2.1. Mushroom ethanolic extracts

Ethanolic extracts of *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera* where characterized by Professor Isabel Ferreira, Laboratory of Applied Chemistry and Biochemistry (LQBA) of Polytechnic Institute of Bragança (IPB). The mushroom samples and procedures to extract and analyse the individual triterpenes and phenolic compounds are supplied at section 7.

2.2. Common reagents

The common reagents include: Dulbecco's modified Eagle's medium (D5030), L-glutamine, glucose, sodium bicarbonate, sodium pyruvate, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), bovine serum albumin (BSA) (catalog #A1595), dimethyl sulfoxide (DMSO), sodium palmitate, BSA free fatty acid, sulforhodamine B sodium salt (SRB), trypan-blue solution, sodium chloride (NaCl) (Sigma-Aldrich Chemical Co., Saint Louis, MO, USA), fetal bovine serum (FBS), penicillin-streptomycin (10.000 U/mL), 0.05% Trypsin–EDTA (from Gibco-Invitrogen, Grand Island, New York, USA); acetic acid, methanol, magnesium chloride ($MgCl_2$), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium phosphate monobasic (NaH_2PO_4), sodium hydrogencarbonate ($NaHCO_3$), sodium hydroxide (NaOH) (from Merck, Whitehouse Station, New Jersey, USA), cellTiter-Glo® Luminescent Cell Viability Assay (from Promega Corporation), fluorescent probes Hoechst 33342 (from Molecular Probes, Life Technologies, New York, USA), and MitoSOX™ Red mitochondrial superoxide indicator (M36008) (from Molecular Probes, Life Technologies, New York, USA).

2.3. Solutions preparation

2.3.1. Mushroom ethanolic extracts solution

The ethanolic extracts of *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera* were solubilized in dimethyl sulfoxide cell culture grade (DMSO; > 99.5 %, EuroClone (COD. EMR385100)) and stored at -20 ºC until further use. The concentration of stock solutions was 27.1 mg/mL for *Ganoderma lucidum*, 100 mg/mL for *Boletus edulis*, and 50.2 mg/mL for

Macrolepiota procera. The extract solutions were then diluted in cell culture medium for assays with HepG2 cells.

2.3.2. Cell culture medium

Cell culture medium was composed of Dulbecco's Modified Eagle Medium (DMEM-D5030), supplemented with 5 mM glucose, sodium bicarbonate (3.7 g/L), HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (1.19 g/L), L-glutamine (0.876 g/L), sodium pyruvate (0.11 g/L), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin.

2.3.3. Bovine serum albumin (BSA) – conjugated palmitate

The BSA-conjugated palmitate (6:1) was prepared following two procedures, one based on the Agilent Technologies technical protocol, and a second with minor modifications to the standard preparation.

The BSA solution (0.83 mM) was prepared by dissolving 2.27 g of BSA in 100 mL of 150 mM NaCl. When completely dissolved, 50 mL of BSA solution was diluted with 50 mL of 150 mM NaCl. The final solution was filtered and the aliquots stored at –20ºC.

The palmitate sodium solution (5 mM) was prepared with 30.6 mg of sodium palmitate dissolved in 44 mL of 150 mM NaCl solution at 70 ºC. Then, 40 mL of 5 mM palmitate solution was transferred to 40 mL of 0.83 mM BSA solution and then incubated at 37 ºC during 1h. The pH was adjusted to 7.4 with 1 M sodium hydroxide (NaOH). The solution was filtered and aliquots were freezed at –20 ºC. This procedure was performed every 2 months.

2.3.4. Preparation of fructose solution

Fructose (2.97 g) was dissolved in 100 mL of MilliQ-purified water to obtained a stock solution with a concentration of 165 mM and stored at -20 ºC until further use.

2.3.5. Phosphate-buffered saline (PBS)

The PBS was prepared with 15.44 mM KH_2PO_4, 1.55 M NaCl, 27 mM KCl and 27.09 mM Na_2HPO_4, pH 7.2. PBS was prepared as a 10x solution.

2.3.6. Acetic acid solution in methanol, 1% (v/v)

The acetic acid solution 1% (v/v) was prepared with 5 mL of acetic acid added to 495 mL methanol. The homogenized solution was stored at −20°C.

2.3.7. Acetic acid solution in MilliQ-purified water, 1% (v/v)

For acetic acid solution 1% (v/v), 10 mL of acetic acid was added to 990 mL MilliQ-purified water. This solution was stored at room temperature.

2.3.8. Tris-NaOH

For Tris-NaOH, 10 mM, pH 10 0.64 g of Tris base was dissolved in 400 mL of MilliQ-purified water. pH was adjusted to 10.5 with 1 M NaOH and the solution was brought to a final volume of 500 mL with MilliQ-purified water.

2.3.9. Microscopy medium

The microscopy medium was composed by 12 mM NaCl, 3.5 mM KCl, 0.4 mM KH_2PO_4, 20 mM HEPES, 5mM $NaHCO_3$, 1.2 mM Na_2SO_4 and 15mM glucose microscopy media, prepared in MiliQ water and pH 7.4. This medium solution was filtered with a 0.2 porosity filter in sterile condition. The medium was used for fluorescent microscopy analyses.

2.3.10. Resazurin solution

The resazurin solution (1 mg/mL) was prepared in PBS (1×) solution. 10 mg of resazurin sodium salt was added to 10 mL of PBS and the solution was filtered with 0.2 μm pore filter and stored at -20 ºC.

2.3.11. Sulforhodamine B (SRB) solution

The SRB solution was prepared with 0.25 g of sulforhodamine B (SRB; Sigma, cat no. S9012) dissolved in 500 mL of 1% (v/v) acetic acid in MilliQ-purified water. The solution was maintained protected from light, at 4 °C.

2.3.12. MitoSOX solution

The MitoSOX 5 mM solution was prepared with 50 μg of MitoSOX for mitochondrial superoxide anion detection by dissolving in 13 μL of DMSO.

2.4. Cell Line

2.4.1. Initiating a new cell culture

To initiate a new cell culture, a frozen cell vial was thawed to a 100 mm plate with 10 mL of culture medium. Cells were incubated in a humid atmosphere with 5% of CO_2 at 37 °C, and after 24 hours, the medium was replaced to remove dead cells.

2.4.2. Assessment of cell proliferation

HepG2 cells were sub-cultured when approximately reached 80% confluence, the medium was removed and the plate was washed with PBS, to remove the remaining medium.

The cells were incubated with 0.05% trypsin/EDTA, in a humid atmosphere with 5% of CO_2 at 37 ºC, for at least 3 minutes until cells detached. Trypsin activity was inactivated by fresh medium, due the high content in proteins in medium.

Cells were centrifuged at 259×g for three minutes and resuspended in an adequate volume of media. After that, a 1:4 or 1:6 dilution was performed in a new 100 mm plate and cells were incubated in a humid atmosphere with 5% CO_2 at 37ºC, until used.

2.4.3. Cell counting

For the cell seeding in each experimental procedure, cells were counted previously. The cells are counted in a hemocytometer with trypan blue dye, using an optical microscope. Trypan blue is a dye that only penetrates non-viable cells, thus the dead cells stain blue and the viable cells do not take up dye. The number of viable cells per mL was calculated by using the following formula.

$$\textit{Cells}/\,\textit{m}\text{L} = \text{number of cells counted} \times 2 \times 10\,000$$

The number of cells counted corresponds to the average of the cells that are within the four quadrants in the hemocytometer. With the addition of Trypan blue, a dilution of cell volume occurs, so the number of cells is multiplied by the dilution factor, which was 2. Since the number of cells obtained in the hemocytometer was per 0.1 µL, we used a factor of 10,000 to obtain the number of cells per mL.

2.4.4. Assay planning

To analyze the toxicity of the *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera* extracts, HepG2 cells were plated at a density of 2.5×10^4 cells/well (75 343 cells/cm²). After 24h of seeding, cells were incubated with mushrooms extracts at concentrations ranging from 15.63 μg/mL to 500 μg/mL for *G. lucidum* extract and 31.25 μg/mL to 1000 μg/mL for *B. edulis* and *M. procera* extracts, and after incubating for 48h, viability assays were performed as above described. To Hoechst staining nuclei, HepG2 cells were plated at a density of 7.5×10^3 cells/well (22 603 cells/cm²), and then incubated with mushrooms extracts at concentrations of 15.63 μg/mL to 250 μg/mL for *G. lucidum* extract and 31.25 μg/mL to 500 μg/mL for *B. edulis* and *M. procera* extracts. After treatment time, HepG2 viability was evaluated through resazurin, SRB, CellTiter-Glo Luminescent assays, and Hoechst staining. The resazurin and SRB assays were performed in the same 96-well plate, while for CellTiter-Glo Luminescent assay, 96-opaque-walled multiwell and Hoechst staining on black 96-multiwell with glass bottom were used.

To evaluate the toxicity of PA, cells were plated at a density of a 2.5×10^4 cells/well (75 343 cells/cm²) in a 96 multi-well plate. After 48h of seeding, cells were incubated during 24 hours with PA at concentrations ranging from 1.95–750 μM. The effect of fructose on HepG2 cells was investigated through dose-response curves at concentrations ranging 3.13 – 50 mM and jointly with PA, being added simultaneously or 6h before or after PA addition. To investigate the effect of PA on mitochondrial superoxide anion content, MitoSOX assay was performed. For this experiment, after 48h of seeding, cells were incubated with PA or rotenone or antimycin for 24h.

To investigate the effect of mushrooms ethanolic extracts on PA-induced toxicity, after 24h of seeding, cells were pre-incubated with non-lethal concentration of each extract or the combination of them for 24 hours. Then, cell culture media was removed and adding fresh media with the desired PA concentration for 24 hours. After the treatment time, viability assays described above were performed. The effect of resveratrol on PA-induced toxicity was evaluated in same conditions. The Figure 9 illustrates the timeline and the assay planning design.

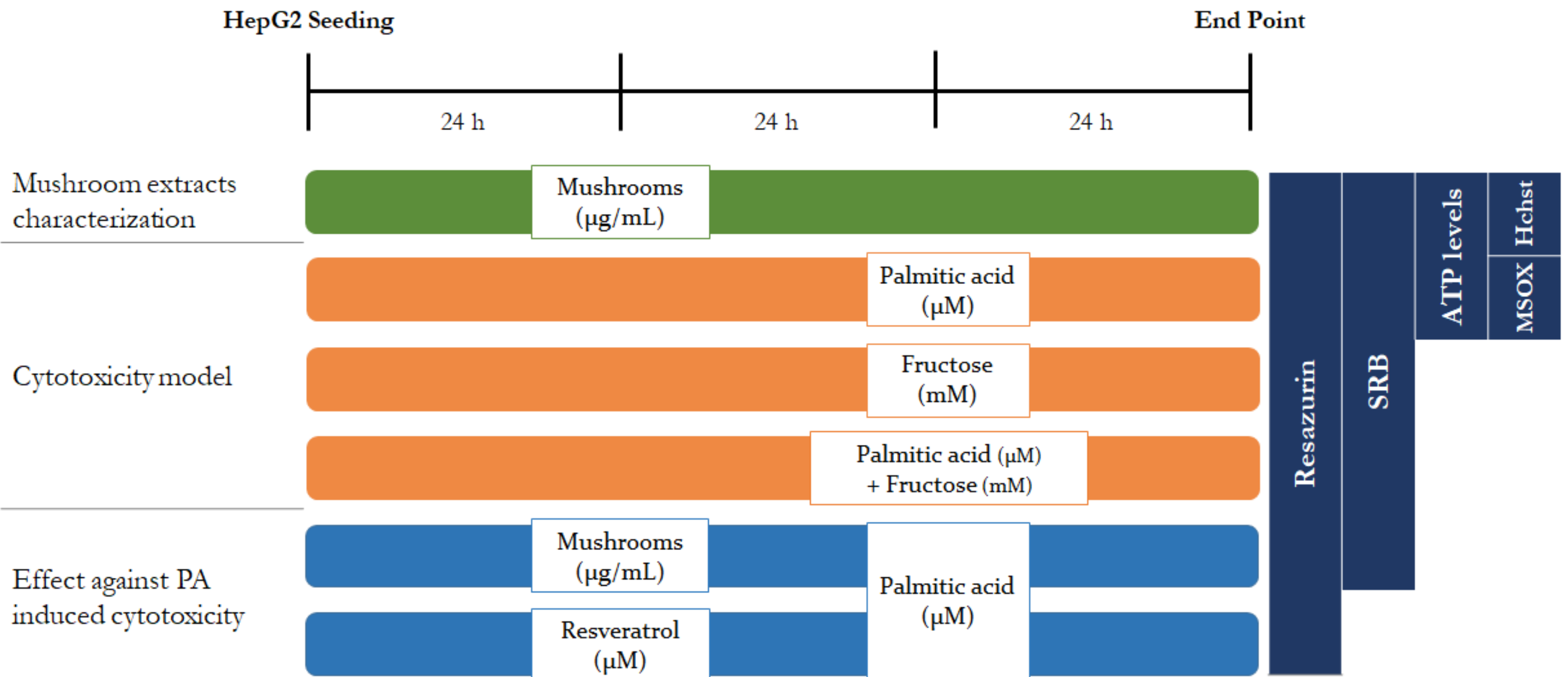


Figure 9 | Timeline and assay planning design. Assays were performed during 72h with different objectives, characterization of mushrooms extracts, palmitic acid-induced cytotoxicity (+ fructose) model and the effect of antioxidants of mushrooms and resveratrol on palmitic acid-induced cytotoxicity in HepG2. The assays were estimated using different end-points: metabolic activity (resazurin reduction assay), cell mass (sulforhodamine B assay), ATP levels (luminescent cell viability assay), ROS (MitoSOX-based detection of mitochondrial superoxide anion) and staining nuclei (Hoechst). Hchst: Hoechst staining for nuclei; MSOX: MitoSOX.

2.5. Cellular metabolic activity

2.5.1. Resazurin assay

Background: The resazurin assay allows the measurement of metabolic activity based on the reduction of resazurin to the fluorescent resorufin through the dehydrogenases in living cells. Resazurin (blue and nonfluorescent) acts as an intermediary acceptor of electrons, being reduced to resorufin (pink and highly fluorescent). The fluorescence is proportional to the number of metabolically active cells (O'Brien *et al.*, 2000; Silva *et al.*, 2016).

Procedure: 100 μL of 1 mg/mL of resazurin solution was added to 9.9 mL of culture medium, obtaining a final concentration of 10 μg/mL. Next, the culture medium was completely removed and cells were incubated at 37°C for 1 hour with 80 μL of resazurin (10 μg/mL) prepared in culture medium. The amount of resazurin reduced to resorufin was measured fluorometrically at an excitation wavelength of 540 nm and emission set at 590 nm in a Cytation 3 reader (BioTek Instruments, Inc.). The data are means ± SEM and are expressed as % of control (control = 100%). After that, the wells were washed with PBS (1×) and cells fixed with acetic acid in methanol to perform the SRB assay, allowing to have two distinct protocols in the same samples (Silva *et al.*, 2016).

2.5.2. Sulforhodamine B assay

Background: The sulforhodamine B assay measures the cell mass, based on the cellular protein content, after the different treatments (Vichai & Kirtikara, 2006; Silva *et al.*, 2016). Sulforhodamine is a pink dye that bind to basic amino acids of cellular proteins (Papazisis *et al.*, 1997; Vichai & Kirtikara, 2006) and the amount of dye extracted from stained cells is proportional to the cell mass. Despite given an indirect measure of the percentage of cell mass that survived to different treatments, SRB assay does not allow to distinguish between cell death and arrest of cell cycle (Vichai & Kirtikara, 2006).

Procedure: **Sulforhodamine B Solution** After treatment time, the medium was removed and wells were washed with PBS (1×). Then cells were fixed with 1% of acetic acid in ice-cold methanol during at least 2 hours at -20 ºC. Posteriorly, cells were incubated with 0.05% SRB in

1% acetic acid solution at 37 °C for 1 hour, and then washed to remove the excess of the dye with 1% acetic acid in H_2O. When wells were dried, 125 μL of Tris (pH 10) was added to resuspend the SRB dye. Finally, the absorbance was read at 510 nm and background measurement at 620 nm on multiwell plate reader (Cytation 3, BioTek Instruments, Inc.). The data are means ± SEM and are expressed as % of control (control = 100%).

2.5.3. CellTiter-Glo Luminescent

Background: The CellTiter-Glo Luminescent Cell Viability Assay indicates the ATP levels of metabolically active cells. The enzyme luciferase uses ATP as a co-factor, and acts on luciferin to produce oxyluciferin, in which energy is converted in luminescence. The luminescence produced is proportional to the amount of ATP present, and it relates directly to the number of living cells present in culture (Crouch *et al.*, 1993).

The luciferase reaction occurs only if cells are lysed. However, when cell membrane integrity is committed, the cells cannot synthesize ATP and endogenous ATPases rapidly degraded the existing nucleotides. To accurately measure ATP levels, the CellTiter-Glo Reagent contains a detergent to lyse the cells and ATPase inhibitors to stabilize the ATP. Thus, the stable form of luciferase catalysis the reaction and generates photons of light.

Procedure: The ATP production in living cells was measured using a commercial kit (Promega, Fitchburg, WI, USA) according to the manufacturer's protocols. The CellTiter-Glo Reagent is a mix of CellTiter-Glo buffer and CellTiter-Glo substrate. Firstly, CellTiter-Glo Buffer was transferred into the flask containing CellTiter-Glo substrate to reconstitute the lyophilized enzyme/substrate mixture. At the end of assay, an equal volume of CellTiter-Glo Reagent was added to cell culture medium present in each well (50 μL). Cells were incubated at 25 ºC for 2 min (constant shake) in a multi-well plate reader (Cytation 3, BioTek Instruments, Inc.) to induce cell lysis. Then, cells were incubated for more 10 min, at 25 ºC to stabilize luminescence signal, and luminescence was recorded. In parallel, an ATP standard curve was constructed with concentrations ranging 0.1 nM to 1×10^4 nM, to convert luminescence signal in nmol of ATP present in living cells. The data are means ± SEM and are expressed as % of control (control = 100%).

2.5.4. Hoechst staining for nuclei

Background: Morphological changes in nuclei chromatin condensation are a good indicator to complement the information obtained from all proliferation assay about cell well function. Hoechst 33342 is a cell-permeable DNA stain with a preference for sequences rich in adenine and thymine. This dye was used to marker nuclei in the different cells, when is excited by ultraviolet light, emits blue fluorescence.

Procedure: For microscopy measurements, 30 minutes before the end of treatment time, all culture medium was completely removed and replaced for microscopy medium supplemented with 1.2 mM $MgCl_2$ and 1.3 mM $CaCl_2$ at 37 ºC during 30 minutes containing Hoechst 33342 (1 μg/mL). After incubation time, the solution was removed and replaced for 100 μL of fresh microscopy media. All images were collected at 40× magnification using the InCell Analyser 2200 high-throughput imaging system.

2.5.5. MitoSOX-based detection of mitochondrial superoxide anion

Background: Mitochondrial superoxide anion is generated as a byproduct of oxidative phosphorylation. MitoSOX Red reagent is a fluorogenic dye specifically targeted to mitochondria of live-cell. In the mitochondria, this dye is oxidized by superoxide anion, but not by other reactive oxygen or nitrogen species, and produces red fluorescence. This reagent may enable to evaluate the effect of different agents on mitochondrial superoxide-induced production.

Procedure: After the treatment with palmitic acid, cells were washed with microscopy medium supplemented with 1.2 mM $MgCl_2$ and 1.3 mM $CaCl_2$. Then, cells were incubated with MitoSOX (5 μM). Changes in MitoSOX fluorescence was followed for 3 hours with readings each 100 seconds at 37 ºC (λex 510 nm; λem 580 nm) in a multi-plate reader (Cytation 3, BioTek Instruments, Inc.). Antimycin A (1 μM) and rotenone (1 μM) were added as a positive control to stimulate the maximal production of superoxide anion by the mitochondria. The changes in MitoSOX fluorescence were normalized in function of cell mass and the data are means ± SEM and are expressed as % of control (control = 100%).

2.6. Statistics analysis

Data analysis was performed with GraphPad Prism 6.0 Software (GraphPad Software, Inc., USA.) All results were expressed as mean ± SEM for the number of replicates in each experiment (n, indicated in the legend of respective figure). For statistical analysis of two means, the student's t test was performed. Multiple comparisons were performed using one-way and two-way ANOVA, with a Dunnet test for multiple comparisons. Comparisons with p value < 0.05 were considered as statistically significant.

3. Results and Discussion

3.1. Effect of ethanolic mushroom extracts in HepG2 cells

The effect of the ethanolic extracts of *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera* was initially investigated by performing several assays to evaluate relevant end points associated to the mitochondrial function in human hepatocytes-derived (HepG2) cell line: the resazurin to evaluate the metabolic activity, the sulforhodamine B (SRB) to estimate the cell mass, the CellTiter-Glo Luminescent to quantify the ATP levels, and the Hoechst staining for nuclei chromatin condensation. The main objective was to characterize the toxicity of the mushroom extracts in order to estimate the safe concentration window, and which concentration of the same extracts could be used to investigate a possible protective effect. The HepG2 cells were incubated with different mushroom extracts concentration, for *G. lucidum* (15.63 μg/mL; 31.25 μg/mL; 62.5 μg/mL; 125 μg/mL; 250 μg/mL and 500 μg/mL), and for *B. edulis* and *M. procera* (31.25 μg/mL; 62.5 μg/mL; 125 μg/mL; 250 μg/mL; 500 μg/mL and 1000 μg/mL) for 48h (Fig. 10).

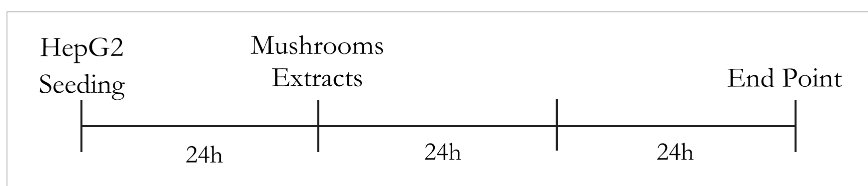


Figure 10 | Schematic representation of assay to evaluate the effect of mushrooms extracts in HepG2 cells.

3.1.1. Cellular metabolic activity

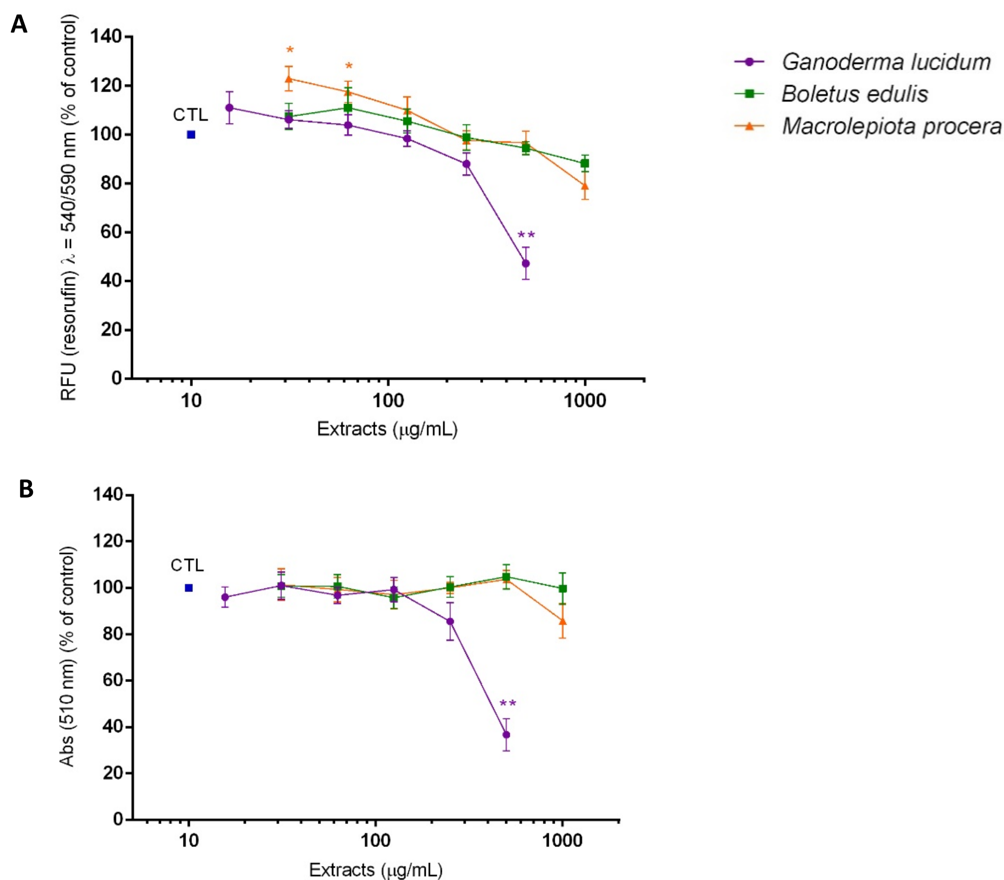
Regarding the effect on metabolic activity, the results show that *Ganoderma lucidum* at 500 μg/mL promoted a significant decrease of 50% in metabolic activity when compared with control (Fig. 11, A). On the other, the ethanolic extracts of *Boletus edulis* and *Macrolepiota procera* did not show any inhibitory effect on metabolic activity in comparison to control. Interestingly, *M. procera* at 31.25 μg/mL and 62.5 μg/mL contributed to a significant increase on metabolic activity.

3.1.2. Cell mass

Similarly, *G. lucidum* exhibited a toxic effect at the highest concentration 500 μg/mL, revealing a decrease of 60% in cell mass. The species *B. edulis* and *M. procera* also showed no effects on cell mass for all concentrations tested (Fig. 11, B), when compared to control. Contrary to the results in resazurin assay, *M. procera* at the lower concentrations, 31.25 μg/mL and 62.5 μg/mL, did not contribute to the increase of cell mass.

3.1.3. ATP levels

Ganoderma lucidum showed a significant effect at 62.5 μg/mL and 500 μg/mL with a decrease up to 50% in ATP levels in the highest concentration (Fig. 11, C). The species *B. edulis* showed a significant decrease on ATP levels at 125 μg/mL and 250 μg/mL, and *M. procera* promoted a significant decrease at 125 μg/mL. In case of the parasol mushroom, the ATP levels decreased 40 % when cells were incubated with the extracts at the highest concentration, 1000 μg/mL.



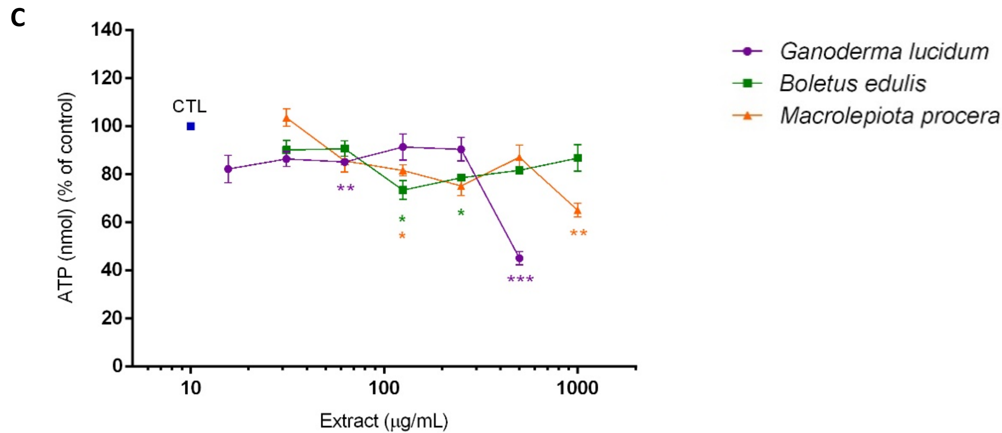


Figure 11 | Effect of mushrooms ethanolic extracts on metabolic activity (A), cell mass (B) and ATP levels (C) in HepG2 cells. The cells were incubated with different mushroom concentrations, ranging from 15.63 µg/mL to 500 µg/mL for *G. lucidum*, and from 31.25 µg/mL to 1000 µg/mL for *B. edulis* and *M. procera*. **A**) Metabolic activity was accessed by resazurin assay, n=6. **B**) Cell mass was determined using SRB assay, n=6. **C**) ATP levels were measured by CellTiter-Glo Luminescent assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, *(p<0.05); **(p<0.01); ***(p<0.001) represent statistical differences from CTL.

3.1.4. Hoechst staining for nuclei

Hoechst dye bind chromatin present in nucleus. In the cell death process, where chromatin condensation occurs, one can follow Hoechst fluorescence variation as consequence of chromatin condensation and nuclei fragmentation.

Ganoderma lucidum treatment promoted an evident decrease in the number of nuclei at concentration above 31.25 µg /mL, however the Hoechst 33342 fluorescence only increased 250 µg /mL, showing that the decrease in the number of nuclei was not directly related to cell death-associated chromatin condensation (Fig. 12, A). When cells were incubated with *Boletus edulis* there is inversely proportional with a decrease in the number of nuclei and an increase in nuclear chromatin condensation at the highest concentration, meanly that for 500 µg/mL of *B. edulis* probably cell death is occurring (Fig. 12, B). *Macrolepiota procera* had a higher number of nuclei at 125 µg/mL, after which there was a significant decrease of nuclei number at 500 µg/mL, most likely by increased by apoptosis (Fig. 12, C).

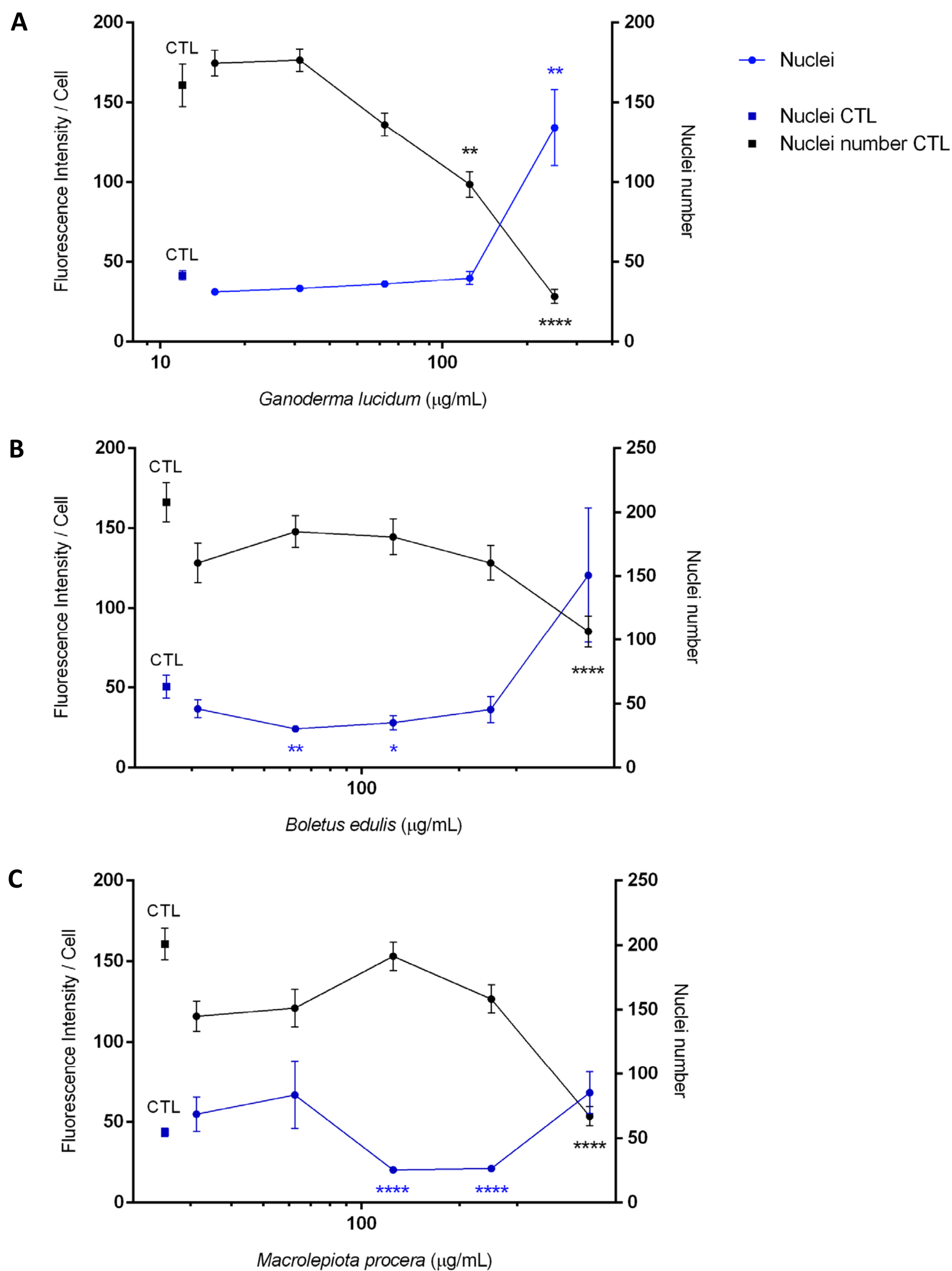


Figure 12 | Effect of *Ganoderma lucidum* (A), *Boletus edulis* (B) and *Macrolepiota procera* (C) ethanolic extracts on nuclei number and morphology of HepG2 cells. Cells were incubated with increasing concentrations of mushroom extracts, ranging from 15.63 µg/mL to 500 µg/mL for *G. lucidum* and from 31.25 µg/mL to 1000 µg/mL for *B. edulis* and *M. procera*. Nuclear morphology and nuclei number were determined by labeling cells with Hoechst 33342. Data are means ± SEM of four independent experiments and the results are expressed as fluorescence intensity of Hoechst 33342, for determination of nuclear morphology, and nuclei number for determination of cell number. Control (CTL) was assumed to be 100% and all results were expressed in function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, *(p<0.05); **(p<0.01); ***(p<0.001) and ****(p< 0,0001) represent statistical differences from CTL.

The table 7 summarizes the mushroom concentrations selected to the phase 2 of the work.

As mentioned before (Table 1), the *Ganoderma lucidum* extract used on this study predominantly contains syringic acid and triterpenes as major compounds. The main bioactive compounds of genus *Ganoderma* are triterpenoids assumed to have several pharmacological properties, such as hepatoprotective, cytotoxic, cholesterol-reducing, apoptosis and cell cycle regulation (Ferreira *et al.*, 2010; Bishop *et al.*, 2015; Wu *et al.*, 2016). Regarding the effect of syringic acid from *Ganoderma lucidum* on cellular models there is no data available. However, Heleno and collaborators (2015) refer several species of mushrooms with syringic acid, which exhibit antioxidant, antibacterial and hepatoprotective activities.

Table 7 | Mushroom concentrations selected.

Species	µg/mL
Ganoderma lucidum	15.63 – 250.00
Boletus edulis	62.50 – 1000.00
Macrolepiota procera	46.88 – 750.00

Previous studies conducted *in vitro* revealed that triterpenoids from *G. lucidum* were able to reduce cell growth in a dose-dependent in three cell lines: Caco-2, HeLa and HepG2 cells, assessed by MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay). Among the cell lines used, HepG2 cells were the most sensitive with less cell viability (Ruan *et al.*, 2015; Wu *et al.*, 2016) indicate that triterpenoids of *G. lucidum* at concentrations ≥400 µg/mL showed cytotoxicity effect by reducing cell viability of HepG2 cells. In this manner, *G. lucidum* extract present higher toxicity than other extracts at lower concentrations (500 µg/mL) and at concentrations >250 µg/mL cell viability decrease markedly (Fig. 11, A and B).

Boletus edulis presents the highest content of phenolic compounds among the wild mushrooms studied, namely *Cantharellus cibarius*, *Craterellus cornucopioides*, *Calocybe gambosa*, *Hygrosphorus marzuolus* and *Lactarius deliciosus*, and cultivated mushrooms like *Agaricus bisporus* and *Pleurotus ostreatus* (Palacios *et al.*, 2011).

Both *B. edulis* (Table 2) and *M. procera* (Table 3) revealed the presence of cinnamic acids, and there is no information about to effects of cinnamic acids from wild mushrooms *in vitro*. Liu *et al.* (2011) showed that cinnamic acid (with other phenolic compounds) in blueberries exhibited cytotoxicity on HepG2 cells at 120 µg/mL. Here, the assays with *B. edulis* and *M. procera* did not exhibit cytotoxicity until 1000 µg/mL (Fig. 11, A). Other studies with cinnamic derivatives extracted from plants also show very low toxicity profile (Sova, 2012).

However, at the highest concentration of *M. procera* extract there are significant differences in the quantification of ATP levels, for this reason was defined a new concentrations range, up to 750 µg / mL were used (Fig. 11, C). In addition, the commercial kit was used for the ATP quantification is more sensitive, so it will probably have a greater sensitivity in the detection of standard deviations, and probably for this reason there are some statistically significant values in intermediate concentrations of the three extracts.

3.2. Palmitic acid-induced toxicity model in HepG2 cells

3.2.1 Effect of fructose on palmitic acid-induced toxicity in HepG2 cells

Similarity to several studies, HepG2 cells were exposed to patho-physiologically relevant concentrations of palmitic acid to mimic the influx of excess free fatty acids into hepatocyte (Gómez-Lechón *et al.*, 2007; Joshi-Barve *et al.*, 2007; Izdebska *et al.*, 2017). The normal physiologic ratio of free fatty acids (FFA) to albumin is approximately 2:1, however, in disease states, serum FFA levels are elevated, yielding ratios as high as 7.5:1 (Joshi-Barve *et al.*, 2007). In our experiments were used palmitic acid conjugated with BSA free fatty acid (PA: BSA) ratio of 6:1. The BSA level used was close to physiological conditions.

The efforts to establish the palmitic acid (PA)-induced toxicity model in HepG2 cells involved the study of several factors. The first experiment also involved the evaluation of the interaction of PA with fructose (F), following the data on figure 13. Growing evidence suggests that an increasing intake of fructose to be a critical factor to hepatotoxic damage and metabolic adverse effects (Liu *et al.*, 2014). High-fat and high-sugar diets have been proposed to induce metabolic disorders, leading to hyperlipidemia and numerous liver lesions, including NAFLD. It has been reported that fat could induce a faster onset of metabolic symptoms with fructose than with sucrose or glucose (Zhao *et al.*, 2016).

The experimental design involved the assessment of a palmitic acid and fructose toxicity-induced model with palmitic acid (PA) + fructose (F) in HepG2 cells. To investigate if sugar (F) may predispose cells to PA toxicity or if this is aggravated by fructose, were used a combination of PA+F, varying concentrations for PA, from 1.95 μM to 750 μM, and a fixed concentration of 5.5 mM F, following figure 14.

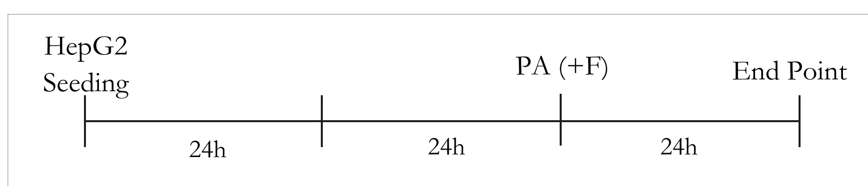
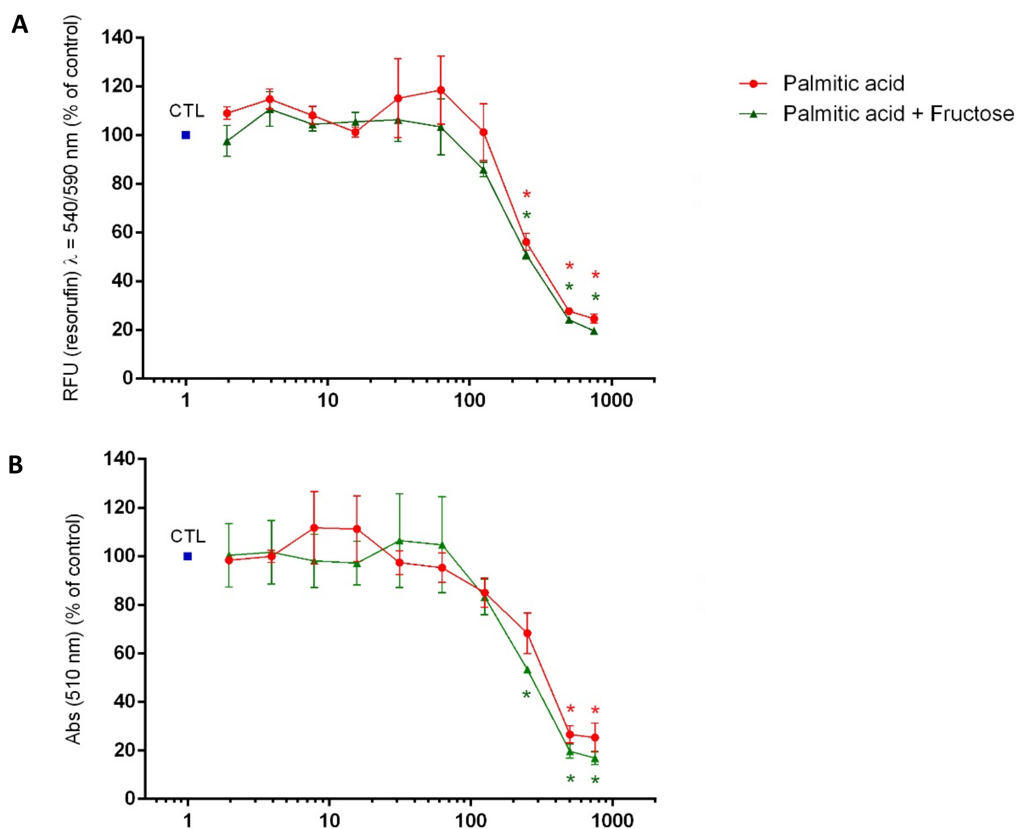


Figure 13 | Schematic representation of the assay to evaluate the effect of palmitic acid (PA) in the presence or absence of fructose (F) in HepG2 cells.

The effects of PA (+F) were evaluated by measuring changes in metabolic activity (Fig. 14, A), cell mass (Fig. 14, B), and the ATP levels (Fig. 14, C). The results showed a clear pattern among the three different assays, with PA-inducing a dose dependent decrease in metabolic activity, cell mass and ATP levels. PA-induced cytotoxicity was observed at concentration above 250 µM. Surprisingly, the presence of fructose (5.5 mM) did not alter (positively or negatively) the toxic effects of PA.

The metabolic activity and the cell mass decreased about 80% at the higher concentrations of PA and the combination of PA + F (500 µM and 750 µM). The ATP levels revealed a decrease up to 90% at the higher concentrations of PA and PA + F, 500 µM and 750 µM.

Gómez-Lechón and collaborators (2007) demonstrated that the increasing of PA concentration induces the cytotoxicity in HepG2 cells and human hepatocytes, associated with increased liver injury. Despite different time points and concentrations concerning PA treatment can be found in literature, our data is consistent with majority of literature that describes cytotoxic effects after PA treatment for concentrations above 250 µM.



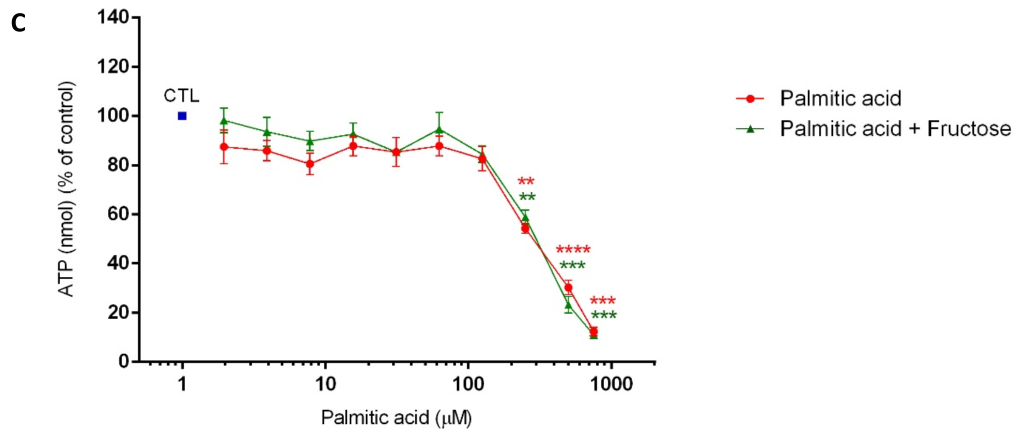


Figure 14 | Effect of palmitic acid (PA) in the presence or absence of fructose (5.5 mM) on metabolic activity (A), cell mass (B) and ATP levels (C) in HepG2 cells. The cells were treated with increasing PA concentrations, ranging from 1.95 µM to 750 µM for 24h. The proportion between PA and BSA was 6:1. **A)** Metabolic activity was determined using resazurin assay, n=4. **B)** Cell mass was determined using SRB assay, n=4. **C)** ATP levels were measured using CellTiter-Glo Luminescent assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, *(p<0.05), **(p<0.01), ***(p<0.001) and ****(p< 0,0001) represent statistical differences with the CTL.

3.2.2 Effect of palmitic acid and fructose in HepG2 cells

Next, the effect of fructose alone at different concentrations (3.13-50 mM) with 24 h incubation and adding fructose at different times with a fixed concentration of PA (250 µM) was evaluated (Fig. 15).

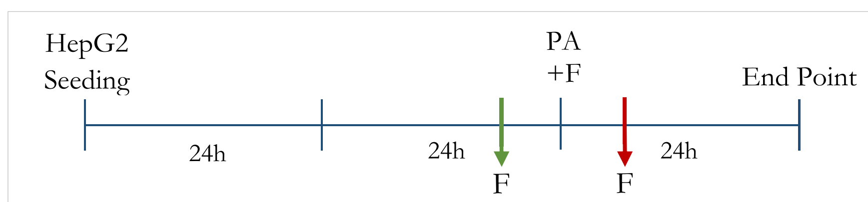


Figure 15 | Schematic representation of the assay to evaluate the potential cumulative effect of fructose (F) on palmitic acid toxicity in HepG2 cells.

When cells were incubated with fructose alone, there was a slight decrease on metabolic activity (Fig. 16, A) in concentrations of 3.13 and 6.25 mM and an increase over the control at the highest 12.5 to 50 mM concentrations. The mixture of PA (250 µM) with different

concentrations of fructose had a similar effect on metabolic activity, both when fructose was added 6h before or after PA.

The cell mass in the presence of fructose increased to 50 mM and at the remaining concentrations resembled of the control (Fig. 16, B). Addition of fructose 6h before PA promoted an increase in cell mass at all concentrations used, while addition after PA decreased the number of cells to 25 mM fructose and at 50 mM an increase was seen.

Fructose did not show a significant effect on the worsening of the PA effect in HepG2, as the results obtained, Zhao *et al.*, 2016 did not have a significant cytotoxicity (MTT assay) when cells were treated with different combinations of fructose in the presence or absence of FFAs.

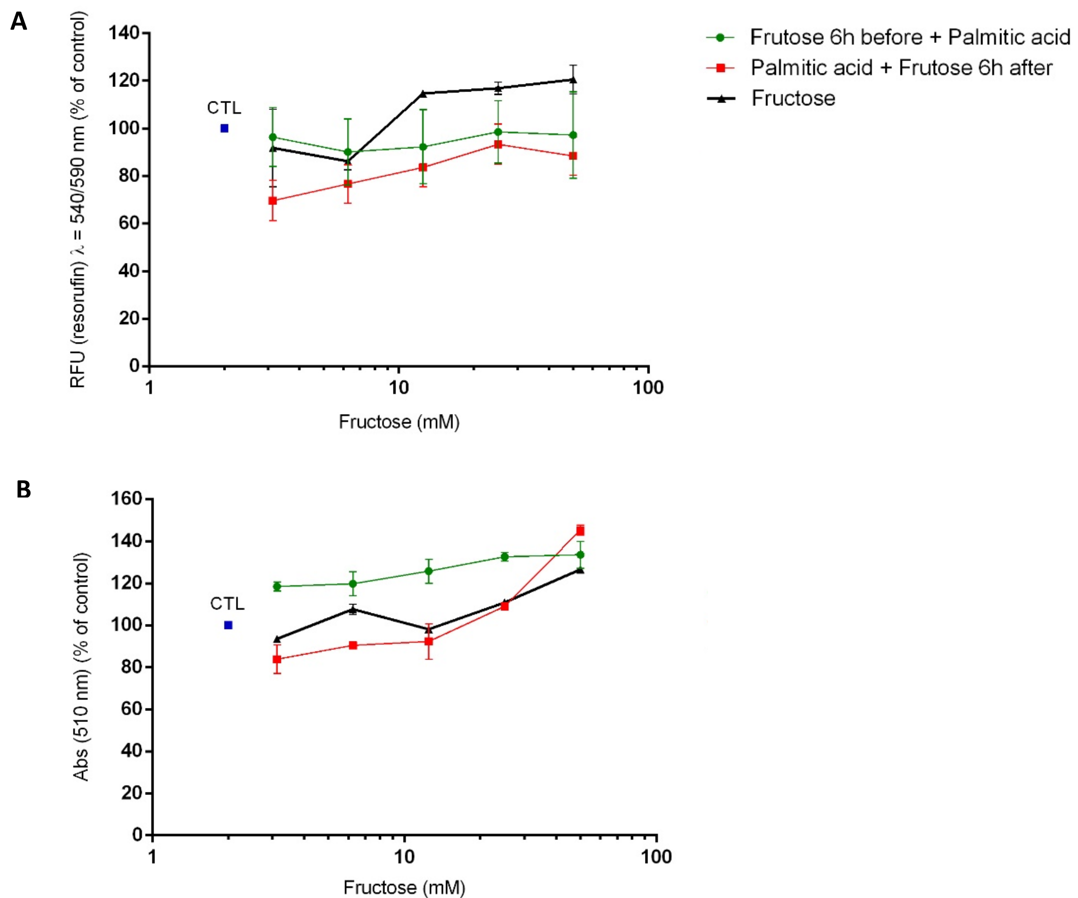


Figure 16 | Effect of PA (+ F) on metabolic activity (A) and cell mass (B) in HepG2 cells. The cells were treated with different fructose concentrations, ranging from 3.13 mM to 50 mM and PA (250 μM). The proportion between PA and BSA was 6:1. Control (CTL) was assumed the 100% and all the other experiments were expressed in function of this control ± SEM, n=2.

3.2.3 MitoSOX-based detection of mitochondrial superoxide anion

Overload of free fatty acids, namely PA, is described to induce mitochondrial dysfunction and ROS production. Thus, the effect of PA on mitochondrial ROS was evaluated through the measurement of alterations in MitoSOX oxidation rate by hepatic cells. The assay conditions to estimate the effect of PA on mitochondrial superoxide anion content in HepG2 cells are represented in figure 17. Rotenone (ROT) and Antimycin A (AA) were used as control, in order to induce maximal superoxide anion production by mitochondria.

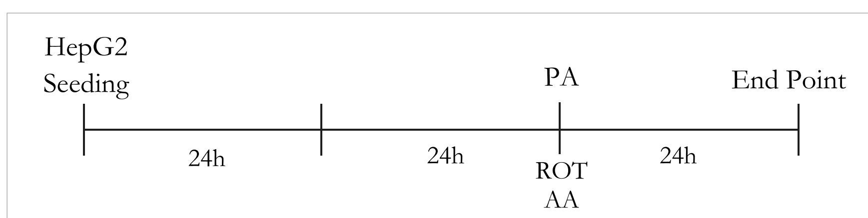


Figure 17 | Schematic representation of the assay to evaluate the effect of PA on ROS production through MitoSOX-based detection of mitochondrial superoxide anion.

The oxidation of MitoSOX was followed during 3h and the results revealed that only cells incubated with 250 μM and 500 μM of PA increased the MitoSOX oxidation signal (Fig. 18). As expected, when superoxide anion production was stimulated by inhibiting ETC with ROT (1 μM) and AA (1 μM), both mitochondrial complex I and complex III inhibitors, the oxidation rate of MitoSOX increased. However, is interesting to notice that cells treated with PA (500 μM) presented a MitoSOX oxidation rate higher than ROT or AA themselves, meaning that in this particular case, MitoSOX oxidation may occur at non-mitochondrial sites (for example, when mitochondria are depolarized).

Next, the rate of MitoSOX oxidation in cells was determined after PA treatment. To do so, we analysed the linear phase of plots show in figure 19. This figure represents the PA-dependent alterations in MitoSOX oxidation rate in hepatic cells and allows determine the EC_{50} value of 268±36 μM. Moreover, the data shows that treating cells with 250 μM and 500 μM of PA dramatically increased MitoSOX oxidation rate approximately 700 and 2200x, when compared to control.

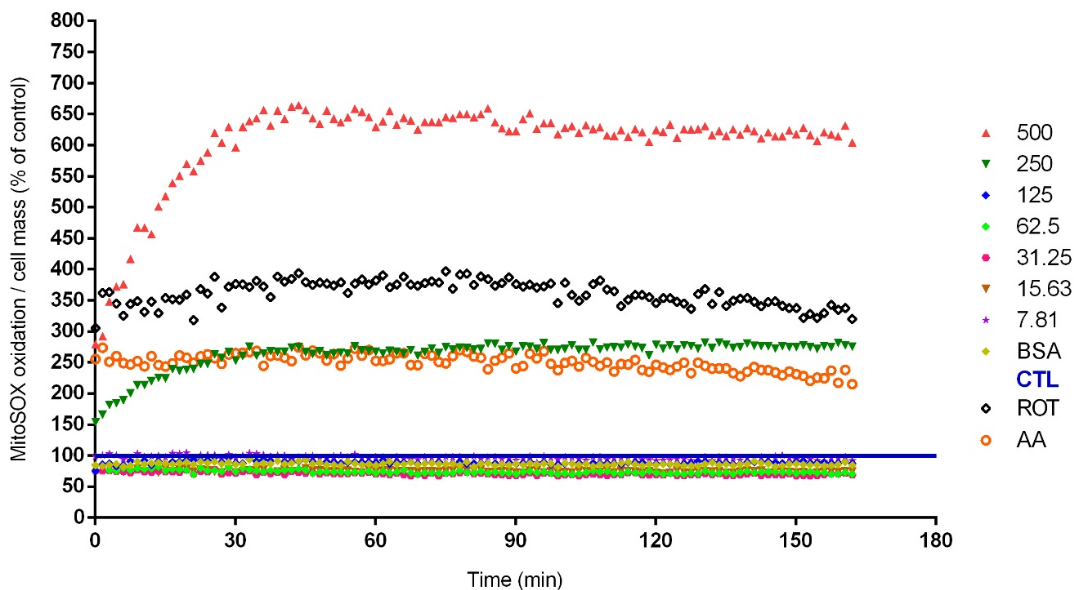


Figure 18 | Effect of Palmitic acid (PA) on mitochondrial superoxide anion in HepG2 cells. Cells were treated with PA, concentrations ranging from 7.81 μM to 500 μM and, the time-dependent variations of MitoSOX oxidation were determined fluorometrically. The proportion between PA and BSA was 6:1. ROT and AA (1 μM) were used as controls for maximal mitochondrial superoxide production. Control (CTL) was assumed the 100% for variations in MitoSOX oxidation per cell mass and all the other experiments were expressed in function of this control ± SEM, n=4, except ROT and AA, n=2.

Higher concentrations of PA promoted subsequent release of toxic factors such as reactive oxygen species (Joshi-Barve *et al.*, 2007; Klupp *et al.*, 2016). Despite our results demonstrated that higher concentrations of PA promote the increase of superoxide production, when cells were treated with PA (500 μM), the rate of MitoSOX oxidation was even higher than when cells were treated with ROT and AA. Higher concentrations of PA, i.e. 500 μM, may induce a loss of mitochondrial membrane potential and MitoSOX can be exposed to cytoplasmatic ROS. Although a clear and dramatic increase in MitoSOX oxidation rate, this increment cannot ensure that is exclusively due to an increase in mitochondrial superoxide anion production.

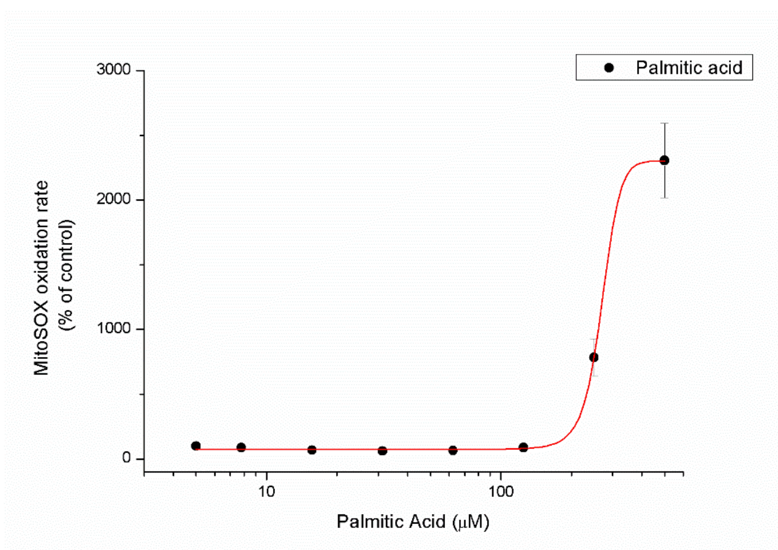


Figure 19 | MitoSOX oxidation rate of cells treat with increasing concentrations of palmitic acid (PA). The slope of the linear phase for mitochondrial superoxide anion per cell mass was attained. Control (CTL) was assumed the 100% for time-dependent variation on MitoSOX oxidation signal and all the other experiments were expressed in function of this control ± SEM, n=4.

By using four different end-points for toxicity, we characterized and determined the respective EC_{50} (Table 8) to evaluate the toxicity of PA in our cell model. These concentrations can be used in the future to evaluate the effects of mushrooms extracts or other isolated phytochemicals on PA-induced toxicity.

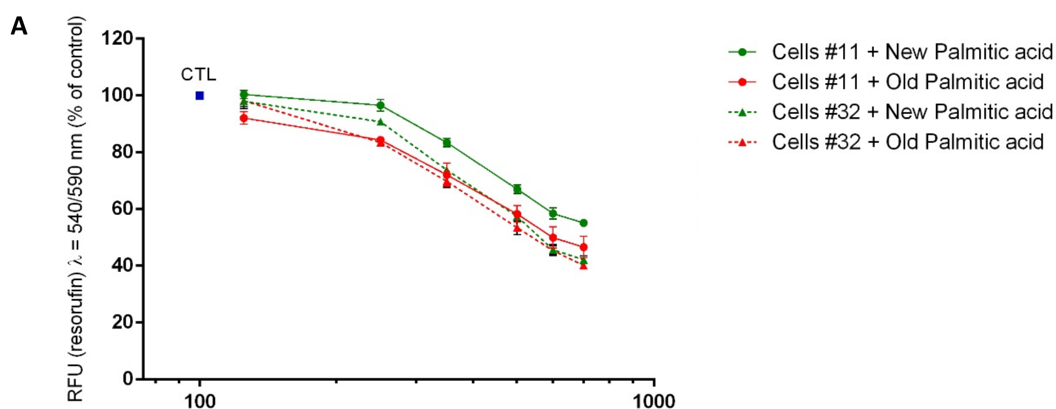
Table 8 | Palmitic acid concentrations presenting 50% of the effects (EC_{50}) on different end-points evaluated.

	PA (EC_{50})
Metabolic activity	194.75 (+/- 15)
Cell mass	210.31 (+/- 21)
ATP	320.79 (+/- 89)
ROS production	268.90 (+/- 36)

Free Fatty Acids Toxicity: Facing a problem…

Cellular mechanisms by which free fatty acids exert toxicity are not fully understood. During the following experiments, we faced a problem concerning the reproducibility of PA-induced toxicity (data not shown). In fact, a new batch of HepG2 cells (EACC) was acquired, which showed different susceptibility to PA-induced toxicity. Although the data was not conclusive, the cells showed different cellular ATP levels and susceptibility to oligomycin (complex V – ATP synthase - inhibitor) and FCCP (uncoupler) (data not shown). Facing this problem, and because new HepG2 acquired from EACC were used, it was necessary to test whether the PA-induced toxicity was affected by other parameters such as cell passage (#11 and #32). Moreover, we also tested if and whether slight different PA protocol preparation (1 mM and 5 mM) and if used fresh (new) vs. frozen (old) preparations may affect its cellular toxicity. Finally, also were tested if and whether the presence or absence of foetal bovine serum (FBS) on cell culture medium may affect PA-induced toxicity.

In figure 20, the effect of cell passages and palmitic acid (new vs. old) on PA-induced toxicity is depicted, assessed through variation in metabolic activity (A) and cell mass (B). In both experiments, the data showed that neither cell passages nor PA significantly affected PA-induced toxicity in HepG2 cells. It has been reported that cells can alter their metabolism with the increase of the number of passages suggesting that the number of passages should not go beyond 15–18 continuous subcultures (Walker, 2015), which did not happen in our study (Fig. 20, A and B). However, we observed that higher concentrations (600 and 700 μM) were necessary to promote a decrease of ±50% in metabolic activity and cell mass, in comparison with the control, which also reinforce the idea that these cells may have different energetic efficiency.



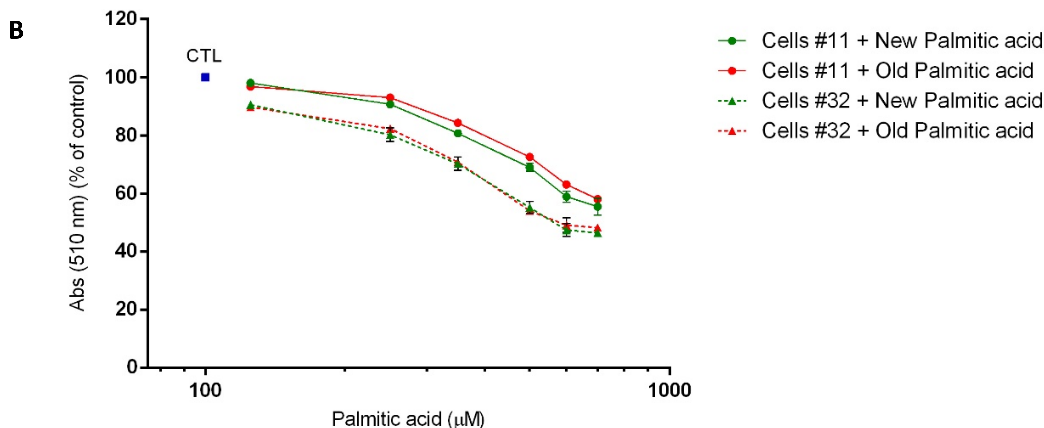


Figure 20 | Effect of palmitic acid on cellular metabolic activity (A) and cell mass (B), considering cells on different passages (#11 and #32) and different PA preparations. Cells were treated with different concentrations of PA, ranging 125 µM to 700 µM, for 24h. New PA refers to fresh PA and Old PA was prepared and stored at – 20 °C. Both with 6:1 PA: BSA. **A)** Metabolic activity was determined using resazurin assay, n=4. **B)** Cell mass was determined using SRB assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by two-way ANOVA followed by a multiple Tuckey test; no statistical differences were obtained.

Next, the effect of the presence or absence of (FBS) in cell culture medium and PA prepared from different protocol preparations (1mM and 5mM) were tested. Figure 21 shows the effect of FBS on PA-induced toxicity, through variation in metabolic activity (A) and cell mass (B). Results revealed a difference in metabolic activity (A) and cell mass (B) to the PA-induced toxicity conditions: 1 mM and 5 mM PA, but not influenced by the presence/absence of FBS in the medium. FBS is a medium supplement, which provide cells nutrients, growth factors and hormones necessary for survival and proliferation. However, it was reported that FBS could influenciate cell viability per se in other cell lines, such as SH and N27 cells, this was not observed in HepG2 cells. (Thomas *et al.*, 2015). Again, higher concentrations of PA were necessary to promote a decrease of ±50% in metabolic activity and and cell mass, in comparison with the control. For that reason, a new EC_{50} for the PA was established, being 750 µM the concentration used for the next experiments. In fact, these amounts of FFA (700 µM) are commonly observed in patients suffering from metabolic disorders, such as obesity and type 2 diabetes (Leekumjorn *et al.*, 2008).

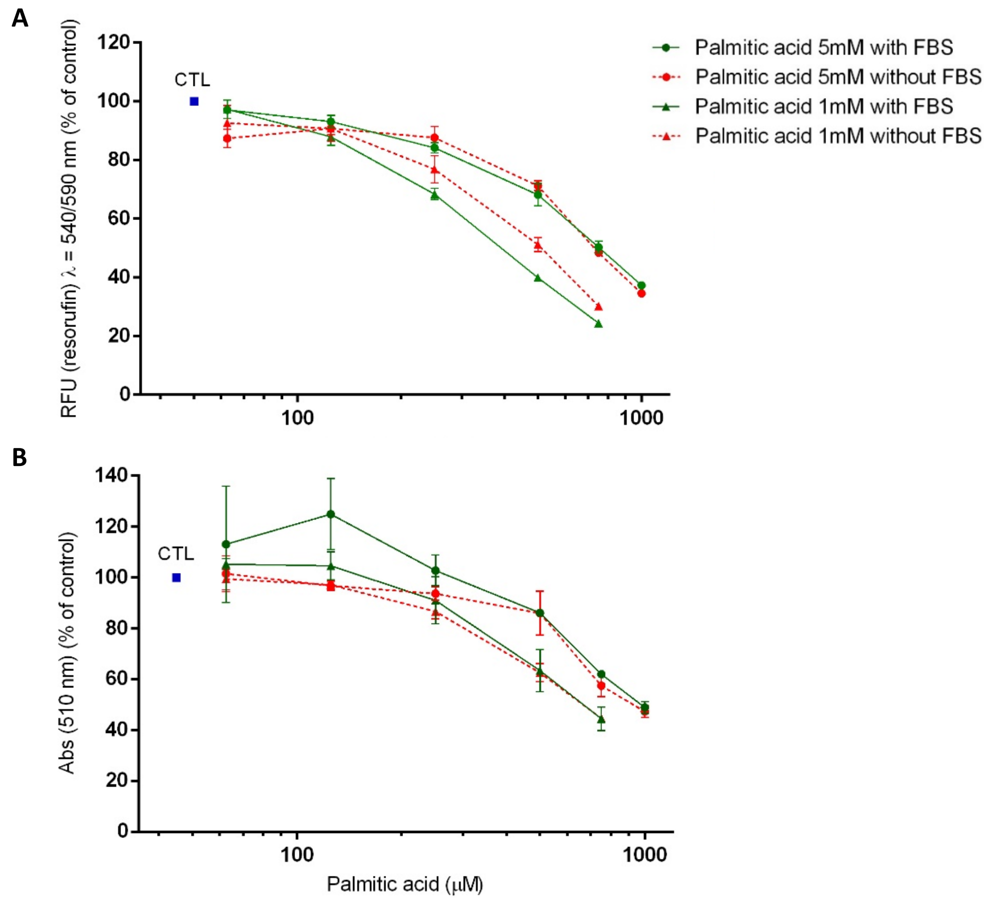


Figure 21 | Effect of palmitic acid on cellular metabolic activity (A) and cell mass (B), considering the presence or absence of FBS and different PA preparation protocols. Different PA (1 mM or 5 mM) stock solutions were prepared and then cells were treated with PA, with concentrations ranging 62.5 μM to 1000 μM, for 24h. For both 1mM and 5mM, the ratio PA:BSA remains 6:1. **A)** Metabolic activity was determined using resazurin assay, n=4. **B)** Cell mass was determined using SRB assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by two-way ANOVA followed by a multiple Tuckey test; no statistical differences were obtained.

3.3. Effect of mushroom extracts on PA-induced toxicity in HepG2 cells

Mushrooms have been used for centuries in traditional medicine due to beneficial effects on human health, such as hepatoprotective, anti-obesity proprieties and antidiabetic. These beneficial proprieties were associated with the presence of antioxidants.

Given the potential beneficial effects of mushrooms extracts on human health, we tested if and whether *G. lucidum*, *B. edulis*, *M. procera* extracts may be beneficial to prevent the progression of NAFLD, *in vitro* simulated by overloading HepG2 cells with PA. The effects of mushroom extracts on the toxicity of PA in HepG2 cells was evaluated following the assay conditions indicated in figure 22. Cells were first incubated 24 h with the selected concentrations of mushroom extracts and then with PA added at 750 μM and 500 μM, for 24h more. We also tested here the effects of the presence or absence of FBS in PA-induced toxicity (Fig. 23). After treatment time, the effects of mushroom extracts on PA-induced toxicity were assessed through resazurin assays, measured as changes in cellular metabolic activity.

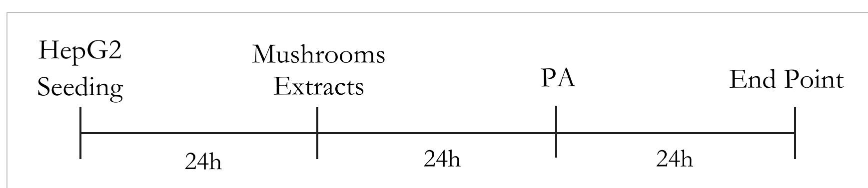


Figure 22 | Schematic representation of the assay to evaluate the effect mushroom extracts on PA-induced toxicity in HepG2 cells.

HepG2 cells were treated with 750 μM PA, which promoted a significant decrease on metabolic activity of about 60%, when compared to control. Although pre-treating cells with lower concentrations of *Ganoderma lucidum* (62.5 μg/mL) contributed to a slight increase on metabolic activity, at higher concentrations (250 μg/mL), *G. lucidum* increased the PA-induced toxicity effects.

Regarding, *B. edulis* and *M. procera*, both extracts presented similar profiles. By pre-treating cells with lower concentrations (15.31 μg/mL), mushrooms extracts appeared to slightly increase PA-induced toxicity; however as mushrooms concentrations increased, these effects tended to disappear and the effect of mushrooms extracts on PA-induced toxicity was similar to that of PA alone.

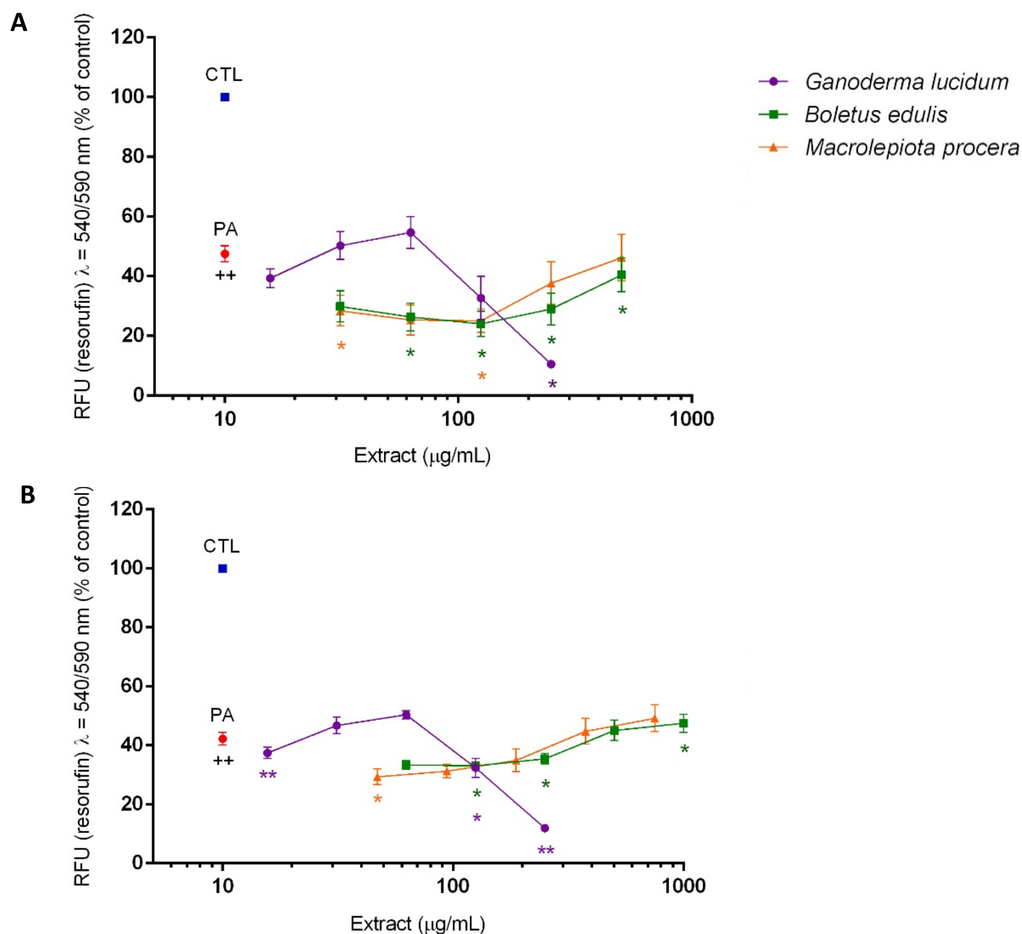


Figure 23 | Effect of mushrooms ethanolic extracts on PA-induced toxicity (750 µM) in HepG2, in the absence and presence of FBS in the cell culture medium, (A) and (B) respectively. The cells were incubated with different concentrations of mushroom extracts, ranging 15.63 µg/mL to 250.00 µg/mL for *G. lucidum* extract; 62.50 µg/mL to 1000.00 µg/mL for *B. edulis* and 46.88 µg/mL to 750.00 µg/mL for *M. procera*, during 24h. After the incubation, PA was diluted in medium in absence of FBS **(A)** and a medium with FBS **(B)**. Metabolic activity was determined using resazurin assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, ++($p<0.01$) represent statistical differences from control (CTL) and *($p<0.05$); **($p<0.01$) represent statistical differences from palmitic acid (PA).

As expected, both PA and mushroom extracts exhibited similar toxicity profiles in the presence or absence of FBS in the cell culture medium (Fig. 23, A and B).

The results did not support a protective effect of mushrooms extracts on PA-induced toxicity. These results may be due to several factors: 1) despite mushrooms have been described to

present beneficial effects on some metabolic disorders (Chang *et al.*, 2015; Wu *et al.*, 2016; Xiao *et al.*, 2017), NAFLD (*in vitro* simulated by overloading cells with PA) is a multisystemic disease with different cellular hallmarks that might not be counteracted by mushrooms extracts; 2) despite in this study PA concentrations (750 μM) was used, similar to those commonly found in some metabolic syndromes, such as obesity and diabetes type-2 patients, there is few *in vitro* studies using such amount of free fatty acids (Leekumjorn *et al.*, 2008). In fact, when cells were incubated with 750 μM PA, with or without FBS, a decrease up to 60% in metabolic activity was observed (Fig. 23, A and B).

For this reason, it was hypothesized that concentrations of PA used could be too high and possibly representing a point of no-return for cells to revert the cell death signalling pathways involved in PA-induced toxicity; and 3) accounting that this disease develops in a "multiple hit" event, the "first hit" involves the fat accumulation in the liver, while the "second hit" includes oxidative stress resulting in inflammation and progression of NAFLD to NASH (Ibrahim *et al.*, 2013; García-Ruiz *et al.*, 2015), may not were excluded that by using antioxidants-enrich mushrooms extracts, probably their action is only on the "second hit" (oxidative stress). Consequently, the PA-induced cellular effects may not be inhibited by the mushroom extract.

Next, several experiments were performed in order to test if and whether some of the reasons mentioned above might be valid. First, the effect of mushrooms extracts on PA-induced toxicity at 500 μM were tested. HepG2 cells were treated with 500 μM PA, which promoted a significant decrease on metabolic activity of about 40%, when compared to control. Although pre-treating cells with lower concentrations of *Ganoderma lucidum* (62.5 μg/mL) contributed to a slight increase on metabolic activity, at higher concentrations (250 μg/mL), *G. lucidum* increased the PA-induced toxicity effects.

Regarding, both *B. edulis* and *M. procera* extracts presented similar profiles. By pre-treating cells with lower concentrations (15.31 μg/mL), mushrooms extracts seem to slightly increase the PA-induced toxicity; however with mushrooms concentrations increase, these effects tends to disappear and the effect of mushrooms extracts on PA-induced toxicity is similar to that of PA alone.

As were expected, both PA and mushroom extracts exhibited similar toxicity profiles in the presence or absence of FBS in the cell culture medium (Fig. 24, A and B).

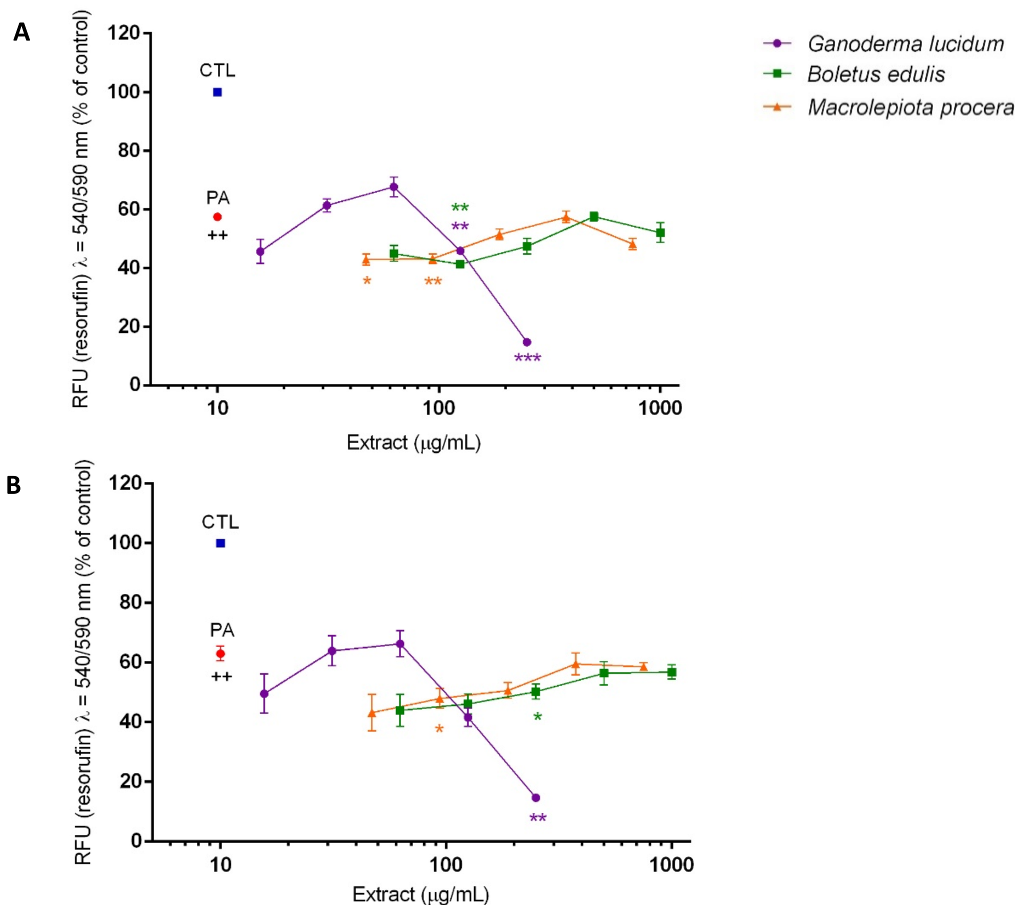


Figure 24 | Effect of mushroom extracts on PA-induced toxicity 500 µM in HepG2 cells, in the presence and absence of FBS in the cell culture medium, (A) and (B) respectively. The cells were incubated with different concentrations of mushroom extracts, ranging 15.63 µg/mL to 250.00 µg/mL for *G. lucidum* extract; 62.50 µg/mL to 1000.00 µg/mL for *B. edulis* and 46.88 µg/mL to 750.00 µg/mL for *M. procera*, during 24h. After the incubation, PA was diluted in medium without (**A**) or with (**B**) FBS for 24 h. Metabolic activity was determined using resazurin assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, $^{++}$(p<0.01) represent statistical differences from control (CTL) and *(p<0.05); **(p<0.01); ***(p<0.001) represent statistical differences from PA.

Next, the possible additive or synergistic effects of mushroom extracts on PA-induced toxicity in HepG2 cells were tested, which were assessed through resazurin, measured as changes in cellular metabolic activity (Fig. 25, A) and SRB assays measured as alterations in cell mass (Fig. 25, B). Three combinations of mushrooms extracts were tested: *G. lucidum* (0.49 µg/mL - 250 µg/mL) with *B. edulis* (0.98 µg/mL – 500 µg/mL); *G. lucidum* (0.49 µg/mL - 250 µg/mL) with

M. procera (0.98 μg/mL – 500 μg/mL) and *B. edulis* (0.98 μg/mL – 500 μg/mL) with *M. procera* (0.98 μg/mL – 500 μg/mL).

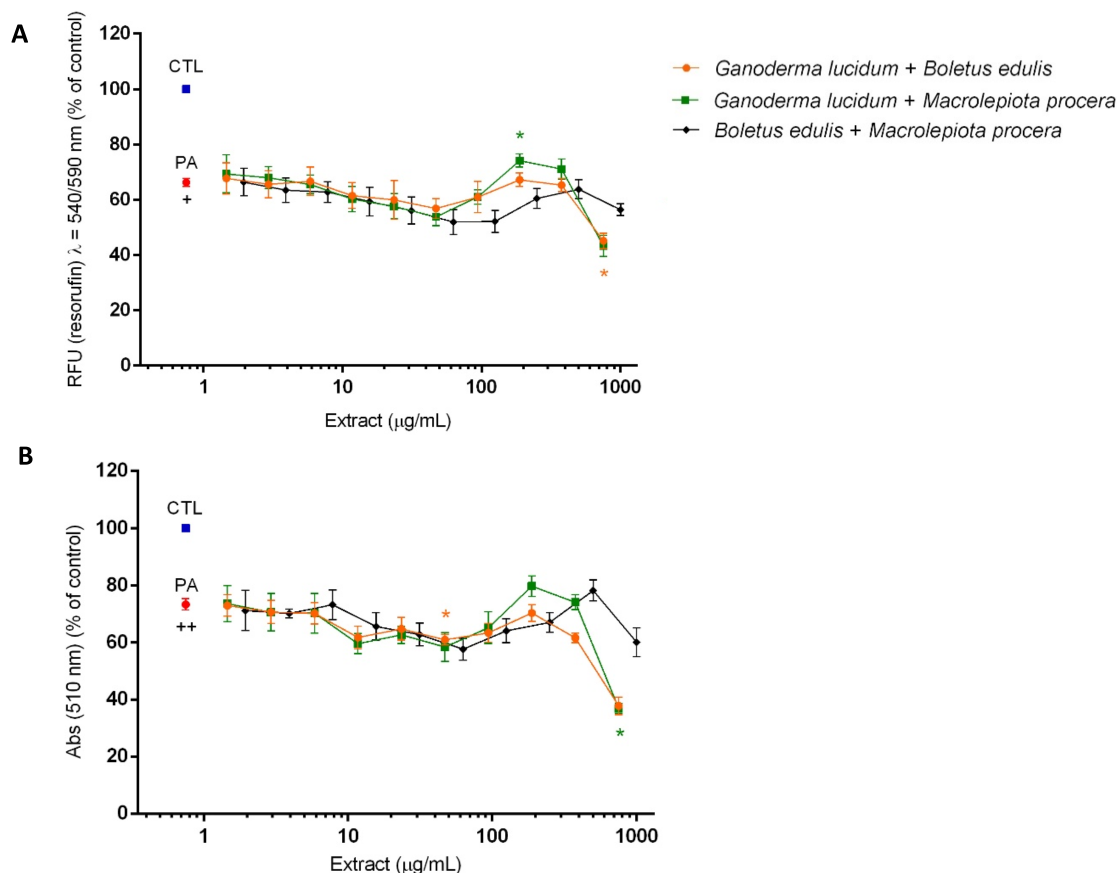


Figure 25 | Effect of combination of two mushrooms ethanolic extracts on PA-induced toxicity (500 μM), was accessed cellular metabolic activity (A) and cell mass (B). HepG2 cells were incubated with increasing concentrations of combination extracts, *G. lucidum* (0.49 μg/mL – 250.00 μg/mL) with *B. edulis* (0.98 μg/mL – 500.00 μg/mL); *G. lucidum* (0.49 μg/mL – 250.00 μg/mL) with *M. procera* (0.98 μg/mL – 500.00 μg/mL); *B. edulis* (0.98 μg/mL – 500.00 μg/mL) with *M. procera* (0.98 μg/mL – 500.00 μg/mL) during 24h. **A)** Metabolic activity was determined using resazurin assay, n=4. **B)** Cell mass was determined using SRB assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, $^{+}$(p<0.05); $^{++}$(p<0.01) represent statistical differences from control (CTL) and *(p<0.05) represent statistical differences from palmitic acid (PA).

HepG2 cells were treated with 500 μM PA, which promoted a significant decrease on metabolic activity and on cell mass of about 35% and 30%, when compared to control.

Regarding *G. lucidum* + *B. edulis* combination extract, both assays followed the same trend.

By pre-treating cells with increasing concentrations, the extracts did not show any preventive effect on PA-induced toxicity, as measured by resazurin and SRB assays. Moreover, for the highest concentration used (250 μg/mL + 500 μg/mL), the combination of both extracts significantly increased the PA-induced toxicity.

Regarding *B. edulis* + *M. procera* combination extracts, also both assays followed the same trend, meaning that by pre-treating cells with increasing concentrations, there was no preventive effects on PA-induced toxicity, as measured in resazurin and SRB assays.

Interestingly in the *G. lucidum* + *M. procera* combination, these extracts (62.5 μg/mL + 125 μg/mL) significantly prevented the PA-induced toxicity, as measured by the resazurin assay; however this was not confirmed by the SRB assay. Moreover, with increased mushrooms concentrations, these effects disappeared and for the highest concentration used (250 μg/mL + 500 μg/mL), the combination of both extracts significantly increased the PA-induced toxicity.

Resazurin and SRB assays are both used as an indirect measure of cell viability. Further, these assays may complement each other as they measure different end-points, metabolic activity and cell mass, respectively. The difference in *G. lucidum* + *M. procera* combination extract may be explained by the fact that, despite the cell mass was not altered, the cell treated with both extracts presented improved metabolic activity.

Finally, the possible additive or synergistic effects of three mushroom extracts under study on PA-induced toxicity on HepG2 cells were tested, which were assessed through resazurin, measured as changes in cellular metabolic activity, and SRB assays measured as alterations in cell mass. HepG2 cells were treated with 500 μM PA, which promoted a significant decrease on metabolic activity and on cell mass of about 35% and 30%, when compared to control.

Regarding the combination of three extracts (*G. lucidum* + *B. edulis* + *M. procera*), both assays followed the same trend, meaning that by pre-treating cells with increasing concentrations, the extracts did not show any preventive effect on PA-induced toxicity, as measured in resazurin (Fig. 26, A) and SRB (Fig. 26, B) assays. Moreover, for the highest concentration used (250 μg/mL + 500 μg/mL + 500 μg/mL), the combination of three extracts significantly increased the PA-induced toxicity. From the data, we can conclude that mushrooms extracts did not

present a protective effect on PA-induced toxicity, even when lower PA concentration were used.

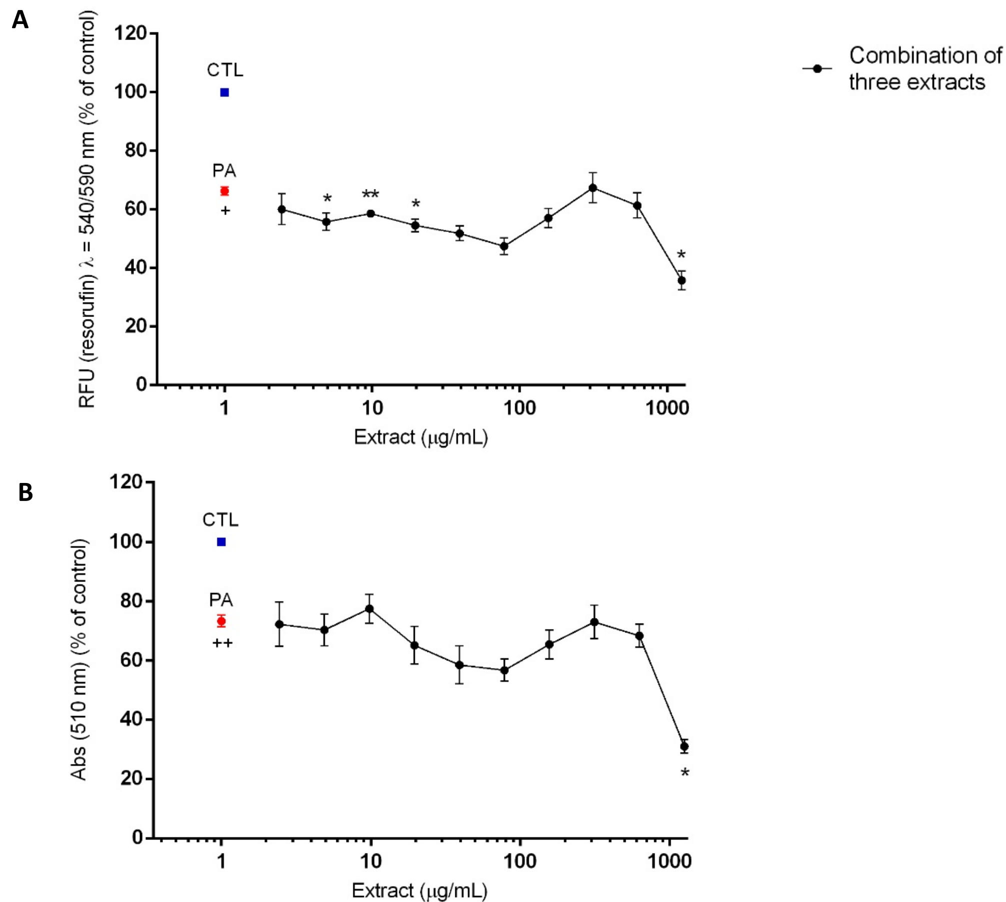


Figure 26 | Effect of combination of three mushrooms ethanolic extracts on PA-induced toxicity (500 µM), was accessed cellular metabolic activity (A) and cell mass (B). HepG2 cells were incubated with increasing concentrations of combination extracts, *G. lucidum* (0.49 µg/mL – 250.00 µg/mL) with *B. edulis* (0.98 µg/mL – 500.00 µg/mL) and *M. procera* (0.98 µg/mL – 500.00 µg/mL) during 24h. **A)** Metabolic activity was determined using resazurin assay, n=4. **B)** Cell mass was determined using SRB assay, n=4. Control (CTL) was assumed to be 100% and all results were expressed as function of CTL ± SEM. Data were analyzed by one way ANOVA followed by a multiple Dunnet test, $^{+}$(p<0.05); $^{++}$(p<0.01) represent statistical differences from control (CTL) and *(p<0.05); **(p<0.01) represent statistical differences from PA.

NASH is mainly characterized by fat accumulation and chronic inflammation and oxidative stress in hepatic cells (Hetherington *et al.*, 2016). In our study, there is an evident decrease in cell viability, measured as metabolic activity and/ cell mass of hepatic cells, which may not truly correlate to what happens in hepatic steatosis. Regarding this topic, we hypothesize that a

possible protective activity may be observed by lowering further the amount of free fatty acids added to hepatic cells, i.e., 250 μM or 150 μM, which is more in agreement with literature describing *in vitro* studies using free fatty acids, such as palmitic acid or oleic acid (Joshi-Barve *et al.*, 2007; García-Ruiz *et al.*, 2015; Izdebska *et al.*, 2017).

Effect of resveratrol on PA-induced toxicity model

In order to test the hypothesis that antioxidants-enrich mushrooms extracts probably only act on the "second hit" (oxidative stress) explaining why PA-induced cellular effects may not be inhibited by the potential antioxidant activity of mushrooms, we next used resveratrol. Resveratrol is well known for its health-beneficial properties, such as the improvement of glucose tolerance and insulin sensitivity, the reduction of the concentration of serum lipids, and for being a potent antioxidant (Izdebska *et al.*, 2017). Despite most cellular effects of resveratrol have been described to be due to its antioxidant activity, resveratrol has other cellular targets such as SIRT1, AMPK, SREBP-1c and FAS (Aguirre *et al.*, 2014). Resveratrol has also the ability to change metabolic needs and redox state of the cells, by regulating several antioxidant signaling pathways. Moreover, the beneficial effects of resveratrol in the prevention of liver steatosis have also been reported (Aguirre *et al.*, 2014).

In our model, beneficial effects of increasing concentrations of resveratrol (0.195 μM to 100 μM) were tested against PA-induced cytotoxicity on HepG2 cells (Fig. 27).

HepG2 cells were treated with 500 μM PA during 24 hours, which promoted a decrease on metabolic activity of about 30% and 25%, respectively, when compared to control. By pre-treating cells with increasing concentrations of resveratrol not only did not show any preventive effect on PA-induced toxicity, as for the highest concentration used (100 μM), resveratrol treatment significantly increased the PA-induced toxicity, as measured in resazurin assay.

Very recently, Izdebska and collaborators (2017) demonstrated that resveratrol (20 μM) increased the viability of HepG2 cells exposed to 100 μM PA. However, when cells were incubated with 200 μM PA in combination with 20 μM resveratrol, a decrease up to 47.4% in cell viability occurred, contrarily to the results obtained with the PA or with resveratrol alone.

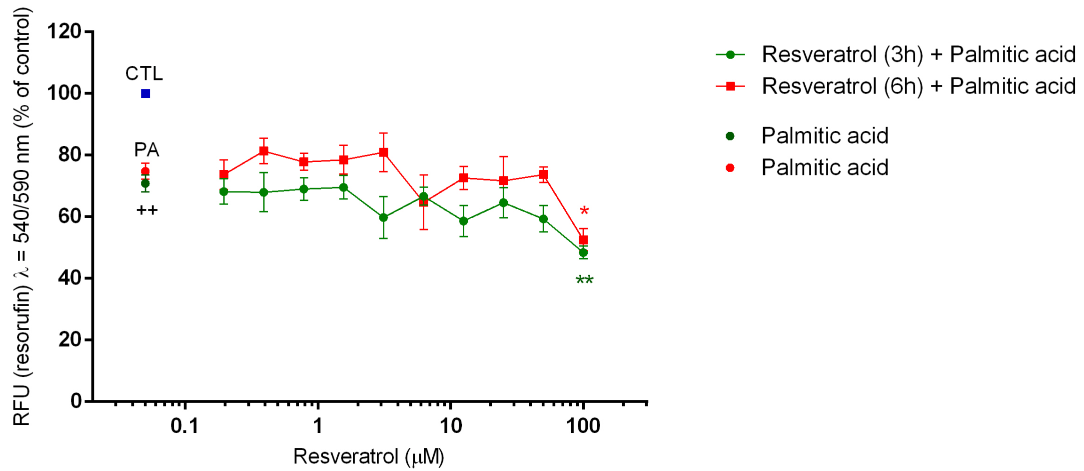


Figure 27 | Effect of resveratrol on PA-induced toxicity (500 μM), evaluated through variations on cellular metabolic activity of HepG2 cells. The cells were incubated with concentrations of resveratrol ranging from 0.195 μM to 100 μM, during 3h and 6h, and PA (500 μM) diluted in medium without FBS over an incubation period of 24h. Control (CTL) was assumed the 100% and all the other experiments were expressed in function of this control ± SEM, n=4. Data were analyzed by one-way ANOVA followed by a multiple Dunnet test, ++(p<0.01) represent statistical differences from control (CTL) and *(p<0.05); **(p<0.01) represent statistical differences from PA.

4. Final conclusions

The main goal of this study was to study the possible hepatoprotective effect of *Ganoderma lucidum* (Curtis: Fr) P. Karst., *Boletus edulis* Bull. and *Macrolepiota procera* (Scop.) Singer, with particular focus on mitochondrial function in a lipid-mediated injury model using the mushroom ethanolic extracts and human hepatocellular carcinoma cell line (HepG2).

The data contributed to confirm that the three mushrooms species are relatively safe as they show cellular/mitochondrial toxicity at concentrations higher than 250 µg/mL for *Ganoderma lucidum*, 750 µg/mL for *Macrolepiota procera* and 1000 µg/mL for *Boletus edulis*. The cytotoxic profile of *G. lucidum* showed higher toxicity to hepatic cells probably due to the presence of triterpenoids. Still, mushrooms extracts have the potential to be used in human health as they presented a good safe concentration window, i.e., range of nontoxic concentrations in which no cytotoxic effects were observed.

The *in vitro* model of lipid-mediated injury was measured through the effect of palmitic acid (PA) (without and with fructose) on cellular and mitochondrial bioenergetics function by using different end-points: metabolic activity (resazurin reduction assay), cell mass (sulforhodamine B assay) and ATP concentration (luminescent cell viability assay), ROS (mitochondrial superoxide anion detection using MitoSOX), and nuclear fluorescence staining (Hoechst). The selected concentrations of palmitic acid were then used in order to evaluate the protective effect of the mushrooms.

The three mushrooms *G, lucidum*, *B. edulis* and *M. procera*, at 62.5 µg/mL, contributed to a slight increase on metabolic activity in response to PA-mediated injury. Also the combination of *G. lucidum* at 62.5 µg/mL with *M. procera* at 125 µg/mL appeared to promote a positive response to lipid-mediated injury, but no statistically significant. Similar disappointing results were obtained when the PA-induced toxicity on HepG2 cells model was tested with the antioxidant resveratrol. The results *in vitro* assays suggested that the cytotoxicity caused by PA may exceed a point of-no return, thus limiting the protection by different agents.

Still, our data supports the future validation of dietary natural extracts and isolated phytochemicals from mushrooms in order to decrease the phenotype associated with fatty liver disease, as well the need to use proper *in vitro* models.

5. Future Perspectives

Facing the inconclusive results obtained for *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera* in preventing PA-induced toxicity, the future work will attempt to test new several hypotheses:

1) Lower concentration of free fatty acids, in which PA overload can induce minimal loss of cell viability but capable to induce hepatic steatosis; or combination of PA with tumor necrosis-factor-alpha, to add the inflammation component to our model.

2) Different end-points. Loss of cell viability may not reproduce *in vitro* what really happens in *in vivo* systems. So, different end-points should be considered, i.e. lipid accumulation and/or quantification of triglycerides (TG).

3) Different extract fraction from *Ganoderma lucidum*, *Boletus edulis* and *Macrolepiota procera*, namely a comparison of methanolic or ethanolic extracts and the influence of respective polyphenols or polysaccharides fractions.

4) Use alternative cell models, since HepG2 is a hepatoma cell line, with the caveat that some mushrooms extracts presenting antitumor effect.

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7. Supplementary information

7.1. Mushroom samples

Boletus edulis Bull. and *Macrolepiota procera* (Scop.) Singer were collected in Bragança (Northeast Portugal), in autumn 2009. Taxonomic identification of sporocarps was made according to several authors (Moser, 1983; Alessio, 1985; Bon, 1988; Courtecuisse and Duhem, 2005), and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança. The samples were lyophilised, (LabConco, Frezone -105 °C, 4.5 L Cascade Benchtop Freeze Dry System, Kansas, MO, USA). The fruiting bodies of *Ganoderma lucidum* were provided by Bioreishi - Agricultura Biológica, Lda, Portugal (Reishi producers; Batalha, Portugal) as dry material.

All the specimens were reduced to powder (20 mesh) and mixed to obtain homogeneous samples. For all the experiments three independent samples were analysed and all the assays were carried out in triplicate. The results are expressed as mean values ± standard deviation (SD).

7.2. Mushroom extract preparation

Each mushroom sample (~3 g) was extracted using an ethanol:water (80:20; 30 ml) mixture at -20°C for 2h. After 15 min in an ultrasonic bath, the extract was centrifuged at 4000*g* for 10 min and filtered through Whatman nº 4 paper. The residue was then extracted with two additional 30 ml portions of the ethanol:water mixture. The combined extracts were evaporated at 40 ºC under reduced pressure to remove ethanol (rotary evaporator Büchi R-210).

7.3. Individual triterpenes and phenolic compounds composition

For the individual phenolic and triterpenoid compounds characterization, the obtained extracts were dissolved in ethanol:water (80:20; 10 mg/mL), filtered through a 0.22 μm nylon syringe filter and analyzed by HPLC-DAD-ESI/MSn in a Dionex Ultimate 3000 UPLC system (ThermoScientific, San Jose, CA, USA). The equipment consisted of a diode array detector coupled to an electrospray ionization mass detector, a quaternary pump, an auto-sampler (kept at 5 ºC), a degasser and an automated thermostated column section (kept at 35ºC). Waters Spherisorb S3 ODS-2 C_{18} (3 μm, 4.6 × 150 mm, Waters, Milford, MA, USA) column was used.

The solvents used were (A) 0.1% formic acid in water and (B) acetonitrile. The gradient elution applied was: 15% B (0-5 min), 15% B to 20% B (5-10 min), 20-25% B (10-20 min), 25-35% B (20-30 min), 35-50% B (30-40 min), the column was then re-equilibrated, using a flow rate of 0.5 mL/min. Data was collected simultaneously with DAD (280 nm) and in negative mode detection on a Linear Ion Trap LTQ XL mass spectrometer (ThermoScientific, San Jose, CA, USA), following a procedure previously described by Bessada, Barreira, Barros, Ferreira, & Oliveira, (2016). Xcalibur® data system (ThermoScientific, San Jose, CA, USA) was used in data acquisition. For identification, retention times, UV-VIS and mass spectra were compared with available standards; when not available data from literature was used to tentatively identify the remaining compounds. Quantification was based on calibration curves of available phenolic and triterpenoid standards constructed based on the UV signal. Compounds with unavailable commercial standards were quantified using the calibration curve of the most similar available standard. The results were expressed as mg/g of extract.