UNIVERSIDADE DE CAXIAS DO SUL

ÁREA DO CONHECIMENTO DE CIÊNCIAS DA VIDA

INSTITUTO DE BIOTECNOLOGIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA

NÍVEL DOUTORADO

Bioatividades de frações dos extratos de *Lentinula edodes* e *Pleurotus sajor-caju* em cultivo de células de mamíferos

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Caxias do Sul

2017

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Bioatividades de frações dos extratos de *Lentinula edodes e Pleurotus sajor-caju* em cultivo de células de mamíferos

Dissertação apresentada ao Programa de Pós Graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção de grau de Doutora em Biotecnologia.

Orientadores: Prof. Dr(a) Mariana Roesch Ely

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2017

F498b finimundy, Tiane cristine
Bioatividades de frações dos extratos de Lentinula edodes e Pleurotus sajor-caju em cultivo de células de mamíferos / Tiane cristine finimundy. – 2017.
124 f.: il.

Tese (Doutorado) - Universidade de Caxias do Sul, Programa de Pós-Graduação em Biotecnologia, 2017.
Orientação: Mariana Roesch-Ely, João Antonio Pêgas Henriques.
Coorientação: Aldo José Pinheiro Dillon.

1. Apoptose. 2. Cogumelos. 3. Pleurotus sajor-caju. 4. Lentinula edodes. 5. bioatividades. I. Roesch-Ely, Mariana, orient. II. Henriques, João Antonio Pêgas, orient. III. Dillon, Aldo José Pinheiro, coorient. IV. Título.

Elaborado pelo Sistema de Geração Automática da UCS com os dados fornecidos pelo(a) autor(a).

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TESE APROVADA EM 08 DE DEZEMBRO DE 2017

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Agradecimentos

Na realização da presente tese, contei com o apoio direto ou indireto de várias pessoas e instituições às quais estou profundamente grata. Quero deixar expresso os meus agradecimentos:

✓ Aos orientadores desta tese, Doutora Professora Mariana Roesch Ely, pela orientação prestada, pelo seu incentivo, disponibilidade e apoio que sempre demonstrou em todos esses anos e ao Doutor Professor João Antonio Pêgas Henriques pela sua colaboração e incentivo sempre que foi necessário, lhes agradeço.

✓ As orientadoras de Portugal Professora Doutora Isabel C.F.R Ferreira e a Professora Doutora Lillian Barros, quero manifestar o meu profundo reconhecimento, pelo nível de exigência pedagógica que sempre me colocaram e pelo estímulo que me motivaram para a realização do meu trabalho. Não posso esquecer os ensinamentos, e as valiosas contribuições que me deram para a concretização do meu objetivo. E por trabalharem em prol da ciência, do Centro e do Instituto. Aqui lhes exprimo a minha gratidão.

✓ Aos co-orientadores Doutor Professor Aldo José Pinheiro Dillon, pela sua disponibilidade ajuda e incentivo, e igualmente pelo seu apoio na elaboração deste trabalho. E ao Doutor Gustavo Scola, que mesmo estando longe sempre se disponibilizou para ajudar e contribuir para o desenvolvimento da tese bem como dos artigos, imensa gratidão.

✓ A todos os amigos e colegas que de uma forma direta ou indireta, contribuíram, ou auxiliaram na elaboração do presente estudo, pela paciência, atenção e força que prestaram em alguns momentos de turbulência, pelos "whatsapp" de apoio e de diversão. Para não correr o risco de esquecer algum não vou identificar ninguém, aqueles a quem este agradecimento se dirige saberão, desde já os meus agradecimentos.

✓ Não poderia deixar de agradecer à minha família por todo o apoio, segurança, pela força e pelo carinho que sempre me prestaram ao longo de toda a minha vida académica, principalmente na fase do intercâmbio, a qual sem o seu apoio teria sido impossível.

✓ Merece destaque, todos os pesquisadores do Instituto Politécnico de Bragança - Portugal, juntamente com o Centro de Pesquisa da Montanha (CIMO) ao Biochemcore e ao ao LQBA (Laboratório de Química e Bioquímica Aplicada, Escola Superior Agrária de Bragança), por toda a ajuda prestada em todos os experimentos. Meu muito obrigada, pela simpatia, paciência, bem como pela disponibilidade que sempre demonstraram em me ensinar tudo o que sabiam.

✓ Agradeço também a CAPES, por possibilitar a pesquisa e o intercâmbio realizado

Enfim, quero demonstrar o meu agradecimento, a todos aqueles que, de um modo ou de outro, tornaram possível a realização da presente tese, um sonho realizado. A todos, o meu sincero e profundo Muito Obrigado! Gratidão sempre!!!

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A375; Human malignant melanoma

aHSCs; activated Hepatic Stellate Cell

AO-EB; Acridine orange-Ethidium bromide

ATCC; American Type Culture Collection

Bak; protein pro-apoptotic

Bax; protein pro-apoptotic

Bcl-2; protein anti-apoptotic

Bcl-2-xL; protein anti-apoptotic

BID; protein pro-apoptotic

DAPI; 4′,6-Diamidine-2′-phenylindole dihydrochloride

DCFH-DA; 2′,7′-Dichlorofluorescin diacetate

DiOC6(3); 3,3′-dihexyloxacarbocyanine iodide

DMEM; Dulbecco's Modified Eagle Medium

DMSO; Dimethyl sulfoxide

DNA; Deoxyribonucleic acid

Fas; Cell surface death receptor

FBS; Fetal bovine sérum

FITC; fluorescein isothiocyanate

G0/G1; Gap 1 phase cell cycle or state of quiescence

G2/M; Gap 2 phase/Mitosis cell cycle

GA; Galic Acid

HCC; Hepatocellular carcinoma

HeLa; Human Epithelial cervix

HEp-2; Human epithelial larynx carcinoma

Hep3B; Human liver cells

HepG2; human liver hepatocellular cells

HMW; High Molecular Weight

HPLC; High performance liquid chromatography

HRP; Horseradish peroxidase

IAPs; Inhibitor apoptosis proteins

IC_{50}; The half maximal inhibitory concentration

Ig; Immunoglobulin

IGF; Insulin Growth Factor

LMW; Low Molecular Weight

MCL-1; protein anti-apoptotic

MCF-7; Human breast adenocarcinoma cell

MRC-5; Human normal lung fibroblasts cell

MTP; Mitochondrial permeability transition

MTT; (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NF-kappaB; Fator de necrose kappaB

NP40; Nonidet P-40

pAb; polyclonal Antibody

PBS; Phosphate-buffered saline

PDB; Protein Data Bank

PI; Propidium Iodide

PTP; Permeability transition pore

PVDF; Polyvinylidene difluoride

ROS; Reactive oxygen species

S; Synthesis phase cell cycle

SD; Standard Desviation

SDS; Sodium dodecyl sulfate

SMMC-7721; Human hepatocarcinoma cell

TBST; Tris-buffered saline and Tween

TNFR; Tumor Necrosis Factor Receptor

TNFα; Tumor Necrosis Factor alfa

TNF-β; Tumor Necrosis Factor beta

TRAIL; TNF-related apoptosis-inducing ligand

U937; Human lymphoma

UV; Ultraviolat

RESUMO

Os cogumelos têm vastas perspectivas como fontes de propriedades medicinais, pois são usados desde os tempos remotos para tratar doenças e promover a saúde em geral. Possuem uma quantidade de compostos bioativos interessantes o que despertou o interesse da área científica. O presente estudo tem como objetivo a avaliação das propriedades bioativas e da composição química e nutricional dos cogumelos *Pleurotus sajor-caju* e *Lentinula edodes*. A atividade antioxidante do extrato foi avaliada através de ensaios de DPPH, poder redutor, TBARS e betacaroteno. Foram realizadas atividade antitumoral nas células NCI-H460, HepG2, HeLa, MCF-7 PLP2 HCT-116 e MRC-5, e anti-inflamatória para o extrato etanólico de *P. sajor-caju* em Raw 264.7. A composição da amostra do *P. sajor caju* em tocoferóis foi determinada por Cromatografia Líquida de Alta Eficiência (CLAE) acoplada a um detector de arranjo de diodos (DAD). Os perfis em ácidos graxos e açúcares foram obtidos por cromatografia a gas acoplada a um detector de ionização de chama (CG-FID) e por CLAE acoplado a um detector de índice de refração (DIR). O *P. sajor-caju* mostrou ser fonte importante de antioxidantes principalmente fenóis (147,54 μg/g) e tocoferóis (615.9g/100g). β-tocoferol foi a forma encontrada em maiores quantidades. Este extrato apresentou atividade antioxidante, sendo esta mais significativa para os ensaios de inibição da peroxidação lipídica (valores de EC_{50} inferiores a 2,6 mg/mL). A determinação do perfil em macronutrientes demonstrou altos teores em proteínas (17,29 g/100 g) e carboidratos (76,18 g/100 g), apresentando baixos valores de lipídios (1,16 g/100 g). A análise da composição do ácidos graxos conduziu à quantificação de 22 moléculas, com predomínio dos ácidos graxos poli-insaturados, particularmente os ácidos oleico e linoleico, 17-61% e 20-54%, respectivamente. O ensaio de citometria de fluxo foi utilizado para a verificação do tipo de morte induzida, para ambos os cogumelos, que apresentaram resultados semelhantes, indicando morte por apoptose. Os estudos efetuados descrevem o potencial nutracêutico das espécies analisadas, bem como o *docking* molecular nos faz pensar em uma possível atividade pró-apoptótica e anti-inflamatória. O consumo de cogumelos *in natura* como em extratos podem exercer um efeito benéfico para a saúde e auxiliar no tratamento de determinadas doenças.

ABSTRACT

Mushrooms have vast prospects as sources of medicinal properties, since they have been used since ancient times to treat diseases and promote health in general. They have a number of interesting bioactive compounds which aroused the interest of the scientific area. The present study aims to evaluate the bioactive properties and the chemical and nutritional composition of the *Pleurotus sajor-caju* and *Lentinula edodes* mushrooms. The antioxidant activity of the extract was evaluated through DPPH, reducing power, TBARS and beta carotene assays. Antitumor activity was performed on NCI-H460, HepG2, HeLa, MCF-7, PLP2, HCT-116 and MRC-5 cells, and anti-inflammatory for the ethanolic extract of *P. sajor-caju* on Raw 264.7. The composition of the *P. sajor caju* sample in tocopherols was determined by High Performance Liquid Chromatography (HPLC) coupled to a fluorescence detector (DAD). The fatty acid and sugar profiles were obtained by gas chromatography coupled to a flame ionization detector (GC-FID) and by HPLC coupled to a refractive index detector (RID). *P. sajor-caju* showed to be an important source of antioxidants mainly phenols (147.54 μg / g) and tocopherols (615.9g / 100g). β-tocopherol was the form found in higher amounts. This extract showed antioxidant activity, which is more significant for lipid peroxidation inhibition assays (EC 50 values below 2.6 mg / mL). Macronutrient profile determination showed high levels of protein (17.29 g / 100 g) and carbohydrates (76.18 g / 100 g), presenting low lipid values (1.16 g / 100 g). Analysis of the composition of fatty acids led to the quantification of 22 molecules, with predominance of polyunsaturated fatty acids, particularly oleic and linoleic acids, 17-61% and 20-54%, respectively. The flow cytometry assay was used to verify the type of induced death for both mushrooms, which showed similar results, indicating death by apoptosis. The studies carried out describe the nutraceutical potential of the analyzed species, as well as the molecular *docking* makes us think of a possible pro-apoptotic and anti-inflammatory activity. The consumption of mushrooms *in nature* as in extracts can have a beneficial effect on health and help in the treatment of certain diseases.

1 INTRODUÇÃO

Há séculos que os cogumelos são consumidos por muitas culturas como fonte de alimentos devido à sua composição química e suas características organoléticas (Kalac 2013, Hoa *et al.* 2015, Sharif *et al.* 2017). Do ponto de vista nutricional, os cogumelos são alimentos saudáveis, uma vez que possuem uma quantidade significativa de fibras alimentares e são pobres em calorias e gorduras. Além disso, eles têm um bom teor de proteína (20-30% de matéria seca) que inclui a maioria dos aminoácidos essenciais e não-essenciais (Wasser 2002, Wasser 2011, Atri *et al.* 2013, sLi *et al.* 2015). Várias espécies são fontes de vitaminas lipossolúveis (vitaminas A, D, E e K) e ácidos graxos poliinsaturados (PUFA) (Barros *et al.* 2007). Os cogumelos mais consumidos em todo o mundo são *Agaricus bisporus* seguido de *Pleurotus spp* e *Lentinula edodes* (Cheung 2010).

O uso de cogumelos expandiu-se amplamente, e tem despertado o interesse da área científica, não só como alimento, mas também na área de produtos farmacêuticos, nutracêuticos e cosmecêuticos devido seus efeitos benéficos sobre a saúde humana (Ferreira *et al.* 2009, Wang *et al.* 2017). Eles contêm compostos bioativos de alto valor medicinal, como lectinas (Kumaran *et al.* 2017), polissacarídeos (Manna *et al.* 2017), fenólicos e polifenóis (Yahia *et al.* 2017), terpenos (Yaoita *et al.* 2014), ergosteróis (Barros *et al.* 2008) e compostos orgânicos voláteis (Barros et al., 2013), que são considerados como agentes responsáveis pelas suas atividades saudáveis (Venditti *et al.* 2017), incluindo antitumoral (Alonso *et al.* 2017), imunomodulador (Hsiao *et al.* 2016), antioxidante (Kimatu *et al.* 2017), anti-hipercolesterolemia (Anandhi *et al.* 2013), antivirais (Yan *et al.* 2015), antimicrobianos (Shameem *et al.* 2017), anti-inflamatória (Yuan *et al.* 2017) e antidiabéticos (Nyam *et al.* 2017). Neste contexto, a possibilidade de incluir cogumelos na nossa dieta, pode proporcionar benefícios desejáveis para a saúde,

indo além da nutrição básica (Ferreira *et al.* 2009), como por exemplo, diminuir o risco de câncer de mama (Zhang *et al.* 2009). Diversos produtos naturais derivados de plantas, fungos e animais, estão sendo utilizados como alternativa para reduzirem a resistência tumoral aos antineoplásicos e seus efeitos colaterais além de aumentar a seletividade ao tumor (Bordon 2014). Entretanto, pesquisas são necessárias no campo da genômica, proteômica, identificação e isolamento de metabolitos secundários e seu envolvimento na sinalização molecular que regem suas funções terapêuticas. Os cogumelos parecem ser uma possível fonte de interesse para colaborar para um maior entendimento relacionado aos efeitos desses compostos sobre células tumorais (Nobili *et al.* 2009, Sculier *et al.* 2015). Assim, no presente trabalho, os cogumelos *Pleurotus-sajur-caju* e *Lentinula edodes* foram avaliados quanto à sua composição química e bioatividade. A caracterização química foi alcançada por meio da avaliação de nutrientes (macronutrientes, açúcares, ácidos gordos e de tocoferóis) e não-nutrientes (ácidos orgânicos e ácidos fenólicos); a bioatividade dos extratos aquoso e hexânico foi avaliada em termos de potencial antioxidante, antimicrobiano, antitumoral e anti-inflamatório. Além disso, avaliou-se o mecanismo de morte celular através da citometria de fluxo, buscando evidências do mecanismo de ação destes compostos.

2 REVISÃO BIBLIOGRÁFICA GERAL

2.1 Cogumelos comestíveis

Os fungos constituem um grupo de organismos cosmopolita diverso, com uma ampla variedade de morfologias, metabolismos e habitats. Representam, assim, um dos maiores grupos taxonômicos com o maior número de espécies na natureza, aproximadamente 1,5 milhões de espécies (Todd *et al.* 2014). Dentre os fungos verdadeiros, o grupo Basidiomicota representa cerca de um terço das espécies de fungos. Eles são assim denominados por apresentarem uma estrutura característica denominada de basídio, estrutura protuberante onde são produzidos e armazenados os esporos **(Figura 1)** (Gehrig *et al.* 1998).

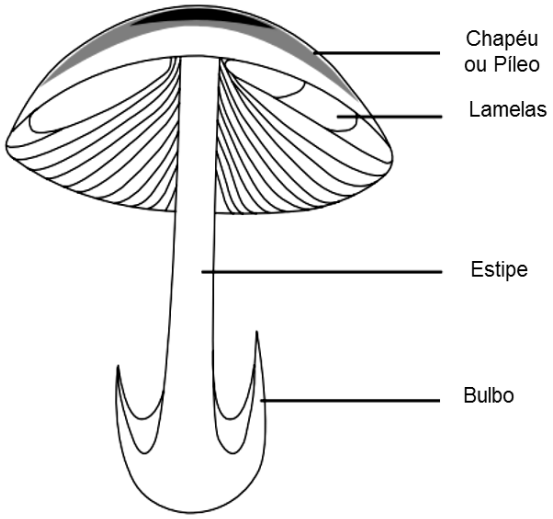

Figura 1 Estrutura de um corpo de frutificação, adaptado de Miles & Chang (2004)

.A estrutura chamada de cogumelo é na verdade apenas o corpo de frutificação do fungo, que após um período de crescimento é produzido pelo micélio estabelecido (Miles & Chang 2004, Moradali *et al.* 2007) (**Figura 2**).

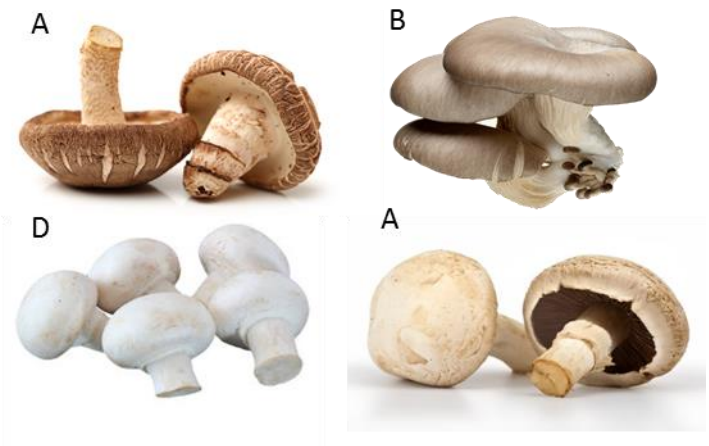

Figura 2 Alguns cogumelos comestíveis, observando-se o píleo e o estipe. A. *Lentinula edodes*, B. *Pleurotus* sp., C. *Champignon paris*, D. *Agaricus blaze*i. Fonte: https://thetruthaboutcancer.com/shiitake-mushroom/

De acordo com as estimativas atuais, os cogumelos constituem pelo menos 12 mil espécies em todo o mundo e dessas, 2000 espécies são relatadas como comestíveis. Cerca de 35 espécies de cogumelos comestíveis são comercialmente cultivadas, enquanto que existe cerca de 200 espécies selvagens utilizadas para fins medicinais (Sharma & Gautam 2015). Estes fungos de crescimento rápido têm tido um interesse nas últimas décadas, com a percepção de que são alimentos deliciosos, com elevado valor nutricional, e com interesse medicinal (Miles & Chang 2004). Os cogumelos não estão sendo consumidos só como alimentos, mas também utilizados como principais componentes bioativos na elaboração de suplementos dietéticos para melhorar a qualidade da vida humana (Jakopovich 2011) sendo considerados alimentos funcionais (Ribeiro *et al.* 2015). Isso reflete que os extratos de cogumelos podem ser alvo específico e estão sendo testados nos ensaios clínicos e também pelas empresas farmacêuticas para o desenvolvimento de alimentos funcionais já que, são fonte de vários compostos bioativos, como polissacarídeos (b-glucanos), fibras dietéticas, terpenos, peptidos, glicoproteínas, álcoois, elementos minerais, ácidos graxos insaturados, antioxidantes como compostos fenólicos, tocoferóis, ácido ascórbico etc. A presença de substâncias bioativas específicas torna os

cogumelos terapeuticamente valiosos desde o fortalecimento do sistema imunológico até a cura e prevenção de doenças, como doenças cardíacas, hipertensão, acidente vascular cerebral e câncer.

2.1.1 Pleurotus sajor-caju

Figura 3 *Pleurotus sajor-caju*. Fonte: https://www.revolvy.com

Os fungos do gênero *Pleurotus* são classificados como pertencentes ao filo Basidiomycota, classe Basidiomycetes, ordem Agaricales e família Pleurotaceae. Estudos realizados em diversas variedades de *Pleurotus* tem identificado proteoglicanas hidrossolúveis, uma glucana carboximetilada e uma α-glicana de baixo peso molecular (Sarangi *et al.* 2006). Trabalhos envolvendo a ação antitumoral desses polissacarídeos foram descritas por Liu *et al.* (2000) como encontradas no corpo de frutificação do cogumelo *P. sajor-caju* (Zhuang *et al.* 1993, Silveira *et al.* 2015). Além destes, alguns componentes flavorizantes que foram isolados, apresentaram atividade antioxidantes e antibacteriana (Gogavekar *et al.* 2014).

Estudos *in vivo* (Sun & Liu 2009) mostraram a relação entre parâmetros estruturais e atividade antitumoral. Chen *et al*. (2010) isolaram a proteína funcional PCP-3A do corpo de frutificação do cogumelo e mostraram que esta foi capaz de inibir o crescimento das células leucêmicas U937 por indução apoptótica. Lee *et al*. (2011) isolaram o pleurone, um polissacarídeo responsável pela atividade antitumoral e imunomoduladora. Silveira *et al.* (2014) isolaram um β-glucano com atividade antinflamatória.

2.1.2 Lentinula edodes

Figura 4 *Lentinula edodes*. Fonte: en.wikipedia.org/wiki/Shiitake

O cogumelo *Lentinula edodes* (Berk.) Pegler também é conhecido como shiitake, pertence a classe Basidiomicetos e a ordem Agaricales. A espécie é hoje em dia o segundo cogumelo comestível mais consumido no mundo, incorporado desde há muito nos hábitos alimentares dos povos asiáticos (Wasser & Weis 1999).

Este basidiomiceto tem sido pesquisado pelos benefícios medicinais que proporciona, mais notadamente a propriedade antitumoral, por produzir um polissacarídeo chamado lentinan, uma β-D-glucana (1→3) ramificada em C-6 por unidades de glucopiranose β(1→6) (**Figura 5**) (Kapoor 2014). Atua também como adjuvante nas terapias antineoplásicas (Sun *et al.* 2014).

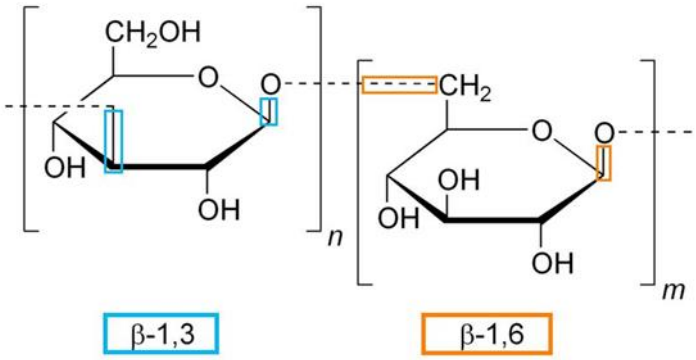

Figura 5 Polissacarídeos e suas possíveis ligações

Esses polissacarídeos têm recebido nos últimos anos uma crescente atenção devido à sua potente atividade antitumoral em vários tipos de cânceres humanos, com especificidade e seletividade sobre as células cancerígenas (Chang & Wasser 2012, Xu & Zhang 2015). Estudos também revelam atividade antitumoral direta em monoculturas *in vitro* (Kawagishi *et al.* 1989, Finimundy *et al.* 2013) ou através da ativação da resposta imune específica e não específica (Ooi & Liu 2000, Daba & Ezeronye 2003, Zhang *et al.* 2013). Uma nova proteína descoberta recentemente, C91-3, obtida pela extração do micélio do shiitake demonstrou ter ação contra as linhagens tumorais A549, H22 e contra o Sarcoma 180 não envolvendo o sistema imunológico, induzindo diretamente a célula a apoptose (Li *et al.* 2014).

2.1.3 Composição Química dos cogumelos

A composição química das espécies de cogumelos pode ser afetada por diversas variáveis tais como estrutura genética, cepas, estágio de maturação, condições ambientais, como a composição do solo, bem como a parte específica do cogumelo, método de preservação pós-colheita (procedimentos secos ou frescos) e processo de cozimento (Barros *et al.* 2007). Alguns compostos dão sabor aos cogumelos e podem ser classificados como derivados de octano e octensos, isoprenóides inferiores, aldeídos e cetonas, compostos sulfurosos (lencetionina) ou heterocíclicos e outros. A característica

aromática é atribuída a derivados de octano, 1-octeno e 2-octeno e álcoois e seus ésteres com ácidos graxos voláteis, ou cetonas (geralmente 3-octanona). Seu conteúdo aumenta durante sua secagem, já que são produzidos pela oxidação do ácido linoleico livre sob catálise com lipoxigenase e hidroperóxido liase (Kalac 2013).

As moléculas presentes nos cogumelos podem ser divididas em dois grandes grupos, que são os compostos de baixo peso molecular (quinonas, cerebrosides, isoflavonas, catecois, aminas, triacilglicerois, sesquiterpenos, esteróides, e selênio) e os compostos de alto peso molecular (homo e heteroglucanos, glicanos, glicoproteínas, glicopeptídeos, proteoglicanos, proteínas e complexos RNA-proteína) (**Tabela 1).**

Tabela 1. Compostos de baixo e alto peso molecular

Classe	**Baixo Peso Molecular**
Quinonas	Panepoxidone Cicloepoxidon Clavilactones (γ-L-glutaminil-4-hidroxi-2,5-benzoquinona) (E)-2-(4-hydroxi-3-metil-2-butenil)-hidroquinona
Cerebrosideos	(4E,8E)-N-D-2'-hidroipalmitoil-1-O-β-D-glucopiranosil-9-metil-4,8-sphingadienine (4E,8E)-N-D-2'-hidroxistearoil-1-O-β-D-glucopiranosil-9-metil-4,8-sphingadienin
Isoflavonas	Genistein
Catecois	6-(3,4-dihydroxystyryl)-4-hydroxy-2-pyrone
	Gerronemins
Aminas	2-aminophenoxazin-3-one Putrescine-1,4-dicinnamide
Trialcilglicerol	1-Oleoil-2-linoleoil-3-palmitoilglicerol
Sesquiterpenos	Iludin
Esterois	5,8-Epidioxi-24(R)-metilcolesta-6,22-dien-3-β ol Ergosterol Ergosta-4,6,8(14),22-tetraen-3-one

Classe	Alto peso molecular
Homoglucanas	Linear (1→3)-β-D-glucana
	(1→3)- β -D-glucana com (1→6)- β -D
	(1→3)- β - glucana
	(1 →6)- β -D-glucana com (1→4)- α
Heteroglucanas	Arabinoglucana
	Galactomannoglucana
	Manogalactoglucana
	Mannoxiloglucana
	Xiloglucana
Glucanas	Fucogalactana
	Glucogalactana
	Manogalactana
	Glucoxilana

Substâncias purificadas de diversos cogumelos têm apresentado quantidades variáveis de alcaloides, terpenos, flavonoides, ácidos graxos e proteínas (Dong *et al.* 2014, Konno *et al.* 2014, Konno *et al.* 2015). Estudos prévios demonstram que alcaloides e terpenos possuem atividade biológica antiproliferativa (Maistro *et al.* 2004) e a presença de proteínas demonstra atividade pró-apoptótica (Konno *et al.* 2014). Em contrapartida, a presença de flavonoides e ácidos graxos nesses extratos podem funcionar como imunoestimuladores (Nandi *et al.* 2014, sLi *et al.* 2015).

2.1.3.1 Valor Nutricional

A investigação da composição nutricional inclui a determinação de macronutrientes, como proteínas, aminoácidos, fibras dietéticas, lipídios, carboidratos, cinzas, bem como micronutrientes, nomeadamente vitaminas e minerais.

Segundo Mattila *et al.* (2001), os cogumelos possuem em torno de 90% de umidade e é um dos fatores mais importantes quando se trata de valor nutricional, pois este influencia diretamente na quantidade de material seco, e, desta forma, também na

quantidade dos nutrientes presentes. Em peso seco, os cogumelos contêm uma boa quantidades de carboidratos (~60%), fibras (~34%) e proteínas (~23%), incluindo todos os aminoácidos essenciais e, em menores quantidades, minerais e algumas vitaminas como riboflavina, niacina e folato, além de baixos teores de lipídios (~5%). Dentre esta pequena fração lipídica, a quantidade de ácidos graxos insaturados é maior que a de saturados, sendo que os ácidos palmítico, oléico e linolêico representaram quase a totalidade dos ácidos graxos estudados (Kalac 2013). Os ácidos graxos polinsaturados da família ω-6 e ω-3 possuem propriedades biológicas, mesmo em baixas concentrações (Ramprasath & Jones 2016) e são percursores biossintéticos dos eicosanóides (prostaglandinas). Estas moléculas de sinalização controlam vários sistemas no corpo humano, tendo efeito em doenças cardiovasculares, e possuindo um ótimo efeito anti-inflamatório (Flachs *et al.* 2011). Seu teor varia muito de acordo com o substrato usado, as condições de cultivo e frutificação e o estágio de desenvolvimento do cogumelo (Mattila *et al.* 2000).

A Vitamina E também conhecida como tocoferol, que compreende 8 isômeros que têm em comum na sua estrutura um núcleo fenólico bicíclico, o cromanol, além de uma cadeia lateral isoprénica (Jiang *et al.* 2001). Ela compreende quatro isoformas α, β, γ e δ-tocoferol, sendo a forma mais ativa o α-tocoferol (**Figura 6**) (Ferreira *et al.* 2009). Danos oxidativos podem ser inibidos pela ação antioxidante dessas vitaminas, juntamente com a glutationa, a vitamina C e os carotenóides, constituindo um dos principais mecanismos da defesa do organismo (Kamal-Eldin & Appelqvist 1996). Esta vitamina é um importante antioxidante lipofílico que mostrou efeitos positivos *in vivo* contra o envelhecimento (Fusco *et al.* 2007), fortalecendo o sistema imunológico (Rizvi *et al.* 2014).

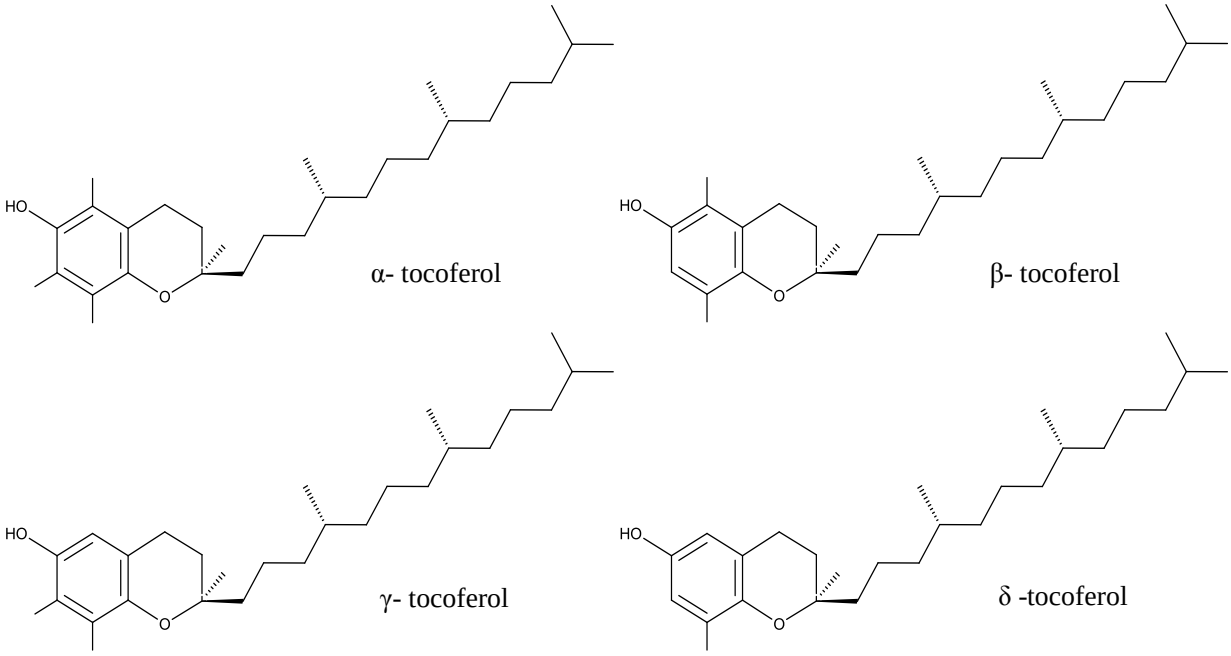

Figura 6. Tocoferóis e seus quatro isômeros

As fibras, por sua vez, também estão presentes em quantidades consideráveis, tanto na forma solúvel como insolúvel. As principais fibras insolúveis são a celulose e a lignina. Dentre as solúveis, a mais conhecida é o polissacarídeo β-glucana, constituído por ligações glicosídicas β(1→3) e β(1→6) (Manzi & Pizzoferrato 2000). De um modo geral, os cogumelos apresentam um alto teor proteico, podendo existir alguma variação entre as espécies ou dependendo do estado de maturação (Zhu *et al.* 2014). As proteínas constituem um importante componente funcional dos cogumelos tendo grande interesse no seu potencial farmacêutico (Xu *et al.* 2011). As lectinas são as proteínas mais investigadas estando comprovada a sua ação antiproliferativa, antitumoral e imunomoduladora (Singh *et al.* 2014). O conteúdo de aminoácidos essenciais dos cogumelos são de aproximadamente 34 a 47%, e os mais abundantes são a leucina, valina, glutamina, ácido glutâmico e ácido aspártico (Oyetayo *et al.* 2007).

2.1.4 Moléculas bioativas presentes nos cogumelos

Os cogumelos sintetizam uma grande variedade de compostos, sendo que os que possuem atividade são os metabólitos secundários (Lindequist 2013). Estes compostos, geralmente, apresentam baixo peso molecular e não são necessários para o crescimento e desenvolvimento do organismo, mas são importantes fatores de proteção no seu habitat natural frente a outros competidores (Shwab & Keller 2008). Consequentemente, muitos metabólitos secundários tendem a ser compostos que apresentam algum efeito tóxico ou inibitório sobre outros microrganismos (Rohlfs 2014) devido a essas propriedades biológicas. O interesse pelos compostos bioativos de origem natural tem crescido nas últimas duas décadas, de acordo com a eficácia de tais compostos contra várias doenças (Daletos *et al.* 2017), principalmente o câncer (Redondo-Blanco *et al.* 2017). As aplicações em ciência e tecnologia de alimentos também tiveram essa evolução, principalmente à evidência da correlação entre dieta e algumas doenças crônicas (Li *et al.* 2017).

Os primeiros medicamentos antitumorais desenvolvidos a partir de cogumelos tiveram como princípio ativo polissacarídeos do tipo β-glucanas, por isso muitos compostos de origem fúngica têm sido explorados para uso farmacêutico. Estes compostos apresentam diferentes estruturas moleculares e seus mecanismos bioquímicos responsáveis por suas propriedades bioativas ainda não são bem definidos (Liu *et al.* 2015). Eles possuem atividade antitumoral e imunoestimuladora, que são também eficazes na redução dos efeitos colaterais durante e nos tratamentos de quimioterapia e tratamentos por radiação, por interferirem nos efeitos regenerativos das células (Meng *et al.* 2016). Os três primeiros compostos biologicamente ativos desenvolvidos a partir de cogumelos medicinais foram os polissacarídeos, todos β-glucanos: Crestina (Krestin), a

partir de micélio da espécie *Coriolus versicolor* (Tsukagoshi *et al.* 1984), Lentinano (Lentinan) a partir de corpos de frutificação da espécie *Lentinula edodes* (Chihara *et al.* 1970) e esquizofilano (Schizophyllan) preparado a partir da espécie *Schizophyllum commune* (Komatsu *et al.* 1969). Nos últimos anos, as β-glucanas foram incluídas como estimuladores do sistema imunológico (Ann *et al.* 2014, Jeong *et al.* 2015), por serem reconhecidas pelo sistema imune inato através de alguns receptores de superfície celular como CR-3 (receptor 3 do complemento) (Vetvicka 2012), dectin-1 (Esteban *et al.* 2011, Bao *et al.* 2017) e lactosilceramida (Kimura 2013). Atualmente estudos de fase II estão sendo realizados na Alemanha, Canadá e Estados Unidos, utilizando os glucanos (β-glucanos) como adjuvante no tratamento de pacientes imunocomprometidos, e em tratamentos conjuntos com antineoplásicos (Thomas *et al.* 2017).

A **Tabela 2** mostra os tipos de glucanos isolados. O conhecimento sobre o potencial dos fungos na produção de metabólitos bioativos, e a possibilidade de melhoramento genético, análise química e farmacológica, leva-nos a considerar que os macrofungos (cogumelos) possuem um grande potencial bioativo (Lindequist *et al.* 2014).

Tabela 2. Cogumelos medicinais com β-glucanos como componentes ativos

Cogumelo	Nome Comum	Estrutura β-glucanos	Tipos de β-glucanos
Lentinus edodes	Shiitake	β-1,3;1,6-glucan	Lentinan
Schizophyllan commune	Schizophyllan	β-1,3;1,6-glucan	Schizophyllan (SPG)
Grifola frondosa	Maitake mushroom	β-1,3;1,6-glucan com xylose and manose	Maitake D-Fração
Coriolus versicolor	Yun Zhi	Ligação proteína β-1,3;1,6-glucan	PSP (peptideo polissacarideo), PSK (polissacarideo-K, krestin)
Ganoderma lucidum	Reishi	β-1,3;1,6-glucan	Ganoderma polissacarídeo

Agaricus blazei	Cogumelo do sol	Ligação proteina β-1,6-glucan	Agaricus polissacarideo
Pleurotus ostreatus	Cogumelo ostra	β-1,3-glucan com galactose e manose	Pleuran
Coprinus comatus	shaggy mane	β-1,3-glucan	Coprinus polissacarídeo

Os compostos fenólicos e ácidos orgânicos são conhecidos por influenciar as propriedades organoléticas dos alimentos (Valentao *et al.* 2005). Os fenólicos são um grupo de moléculas provenientes do metabolismo secundário das plantas que se caracterizam pela presença de um anel aromático com, pelo menos, um grupo hidroxila substituinte e uma cadeia lateral (Roche *et al.* 2017). Nos cogumelos, os ácidos fenólicos são os principais compostos encontrados e são responsáveis por um grande número de efeitos biológicos (Taofiq *et al.* 2015). Os ácidos fenólicos mais abundantes encontrados nos cogumelos são mostrados na **Figura 7**.

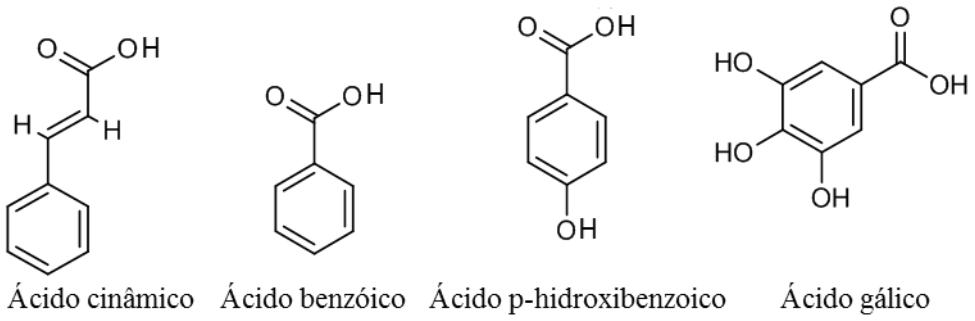

Figura 7. Alguns ácidos fenólicos mais abundantes nos cogumelos

Os ácidos orgânicos são compostos de baixa peso molecular que se caracterizam por conter um ou mais grupos carboxila na sua estrutura, o que lhes confere propriedades específicas na perceção do sabor dos alimentos e têm sido utilizados como controle da qualidade dos mesmos (Quitmann *et al.* 2014). Alguns destes ácidos possuem uma capacidade protetora contra várias doenças devido suas propriedade antioxidantes (Stajic

et al. 2013). Alguns estudos descrevem a presença dos ácidos orgânicos em cogumelos, nos corpos de frutificação e micélios de diferentes espécies (Valentao *et al.* 2005, Ribeiro *et al.* 2008). Os principais acidos orgânicos encontrados nos cogumelos são os ácidos oxálico, quinico, málico, cítrico e fumárico (Barros *et al.* 2013) como mostrado na **Figura 8**.

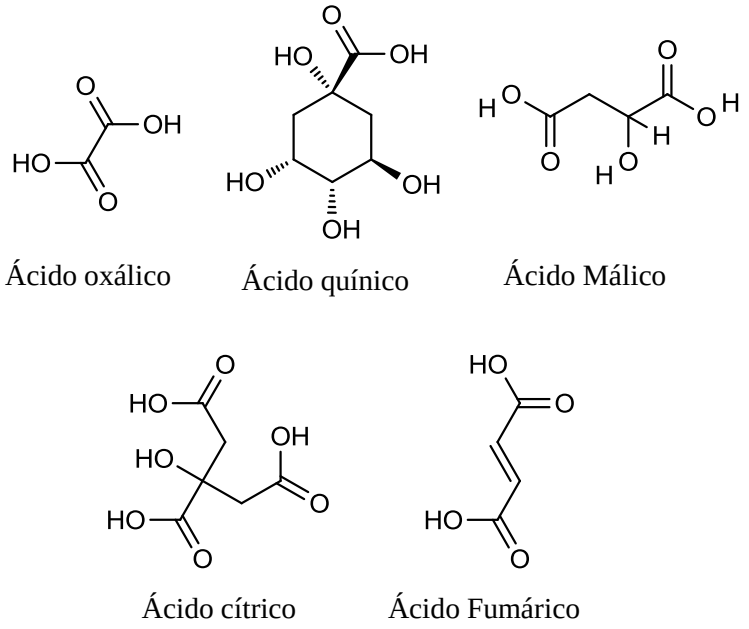

Figura 8. Principais ácidos orgâncios encontrados nos cogumelos.

2.1.5 Bioatividades dos cogumelos

Os cogumelos têm ajudado na melhora da saúde humana, pois além do seu reconhecimento como alimento nutritivo, eles são uma importante fonte de compostos biologicamente ativos com grande valor medicinal (Valverde *et al.* 2015). Várias espécies de cogumelos estão sendo estudadas com o intuito de desenvolver novas alternativas terapêuticas (Lindequist *et al.* 2014), pois são uma fonte rica em nutracêuticos (Kozarski *et al.* 2015, Venditti *et al.* 2016) responsáveis pela atividade antioxidante (Koutrotsios *et al.* 2017), antitumoral (Wasser 2017), antimicrobiana (Shameem *et al.* 2017) antiflamatória (Souilem *et al.* 2017). A **Tabela 2** mostra resumidamente algumas das atividades já estudadas em várias espécies de cogumelo.

Tabela 3. Atividades farmacológicas de substâncias extraídas de cogumelos

Efeito/doença	Substância	Espécie cogumelo	Mecanismo	Ano	Referência
Anticâncer (próstata)	Extrato não especificado	*Agaricus bisporus*	Indução da apoptose. Inibição da angiogênese, *in vivo*	2015	(Twardowski *et al.* 2015)
	Fração D	*Grifola frondosa*	Supressão da atividade de aromatase e da proliferação de células tumorais. Diminuição na produção de estrógeno.	2017	(Alonso *et al.* 2017)
Anticâncer (mama)	Triterpenos	*Pleurotus eryngii*	Supressão da sinalização Akt/NF-κB	2015	(Xue *et al.* 2015)
	Andosan	*Agaricus blazei*	Inibe a proliferação de células tumorais humanas *in vivo*	2016	(Hetland *et al.* 2016)
Anticâncer (coloretal)	Antroquinolol	*Antrodia camphorata*	Indução da expressão de proteínas pró--apoptóticas e inibição PI3K/AKT	2016	(Lin *et al.* 2017)
	Extrato aquoso	*Pleurotus sajor-caju*	Atividade antitumoral	2013	(Finimundy *et al.* 2013)
	Extrato micelial	*Lentinula edodes*	Efeitos antiproliferativos via indução da apoptose	2016	(Ishikawa *et al.* 2016)
Anticâncer (cervical)	Caldo de cultura	*Taiwanofungus camphoratus*	Atividade antitumoral e efeitos pré-clínicos positivos	2017	(Wang *et al.* 2017)
	Andosan™	*Agaricus blazei*	Aumento na produção de citocinas *in vitro* Redução na produção de IL-1-β (97%), TNF-α (84%), IL-17 (50%) e IL-2 (46%). Atividade antioxidante *in vivo*	2011	(Hetland *et al.* 2011)
Anticâncer (pulmonar)	Heteropolissacarideo	*Lentinula edodes*	Diminuição na produção de TNFα induzida por lipopolissacarídeos.	2016	(Xu *et al.* 2016)
Imunomodulação	Vários extratos	*Várias espécies*	Aumento de sobrevida e funções imunológicas melhoradas	2017	(Meng *et al.* 2016)

Imunomodulação (hipercolesterolemia)	Vários extratos	Várias espécies	Efeitos sobre células NK, macrófagos, células T e produção de citocinas. Ativação de vias mitogênicas por quinases (MAPK)	2016	(Lu *et al.* 2016)
Imunomodulação (câncer)		*Pleurotus sajor-caju*	Aumento da resistência à insulina, devido ao aumento na concentração de adiponectina	2015	(Ng *et al.* 2015)
Imunomodulação (doenças variadas)	Extrato não especificado	*Lentinula edodes*	Redução significativa na pressão sanguínea, glicose sanguínea, colesterol total e triglicérides (*in vivo*)	2016	(Spim *et al.* 2016)
Diabetes (tipo II)		*Pleurotus eryngii var. ferulae*	Efeito hipolipidêmico e de controle de peso via mecanismo envolvendo absorção de colesterol *in vivo*	2017	(Choi *et al.* 2017)
Doenças cardiovasculares	Polissacarideo	*Pleurotus ostreatus*	Protege contra a morte celular neuronal causada pela toxicidade do peptídeo beta-amiloide (A beta) e estresse oxidativo	2017	(Zhang *et al.* 2016)
	Polissacarideo	*Ganoderma lucidum*	Induz fator de crescimento (FGFR1) (in vitro e *in vivo*)	2017	(Huang *et al.* 2017)
Saúde mental e cognição	AbM extrato	*Agarius blazei Murill* (AbM)	Decresce concentrações de aspartato aminotransferase e alanina aminotransferase, normalizando as funções do fígado de pacientes com hepatite B	2008	(Hsu *et al.* 2008)
	Lectin	*Pleurotus ostreatus*	Antiviral e protetora do fígado em hepatite crônica	2017	(He *et al.* 2017)
	ganodermanol	*Ganoderma capense*	Inibição da protease HIV-1	2017	(Tan *et al.* 2017)
Hepatite B	Extrato aquoso enzimatico	*Agaricus bisporus*	Age no estágio inicial da replicação viral	2016	(Delgado-Povedano *et al.* 2016)
	Extrato não especificado	*Cordyceps* sp.	Inibe a proliferação e a diferenciação das células Th2 e reduz a expressão de	2016	(Wang *et al.* 2016)

			citocinas, em células mononucleares do sangue periférico. Alivia inflamação crônica por aumentar as concentrações de IL-10 (*in vivo*)		
Antiviral (HIV)	Extrato não especificado	*Lentinula edodes*	Inibe a proliferação e a diferenciação das células Th2 e reduz a expressão de citocinas. Alivia inflamação crônica por aumentar as concentrações de IL-10 (*in vivo*)	2016	(Kim *et al.* 2016)
Antiviral (HCV)	Fibra	*Pleurotus sajor-caju.*	Atenuam sintomas relacionados à constipação sem efeitos colaterais significativos (*in vivo*)	2012	(Parab *et al.* 2012)
Asma	Vários extratos	*Lignosus rhinocerusla*	Aumento da IgE	2014	(Johnathan *et al.* 2016)
Constipação	polissacarideo	*Pleurotus tuber-regium*	Aumento do bolo fecal	2014	(Chen & Cheung 2014)

2.1.5.1 Antioxidante

A formação de radicais livres está associada ao metabolismo normal do organismo, mas também com doenças degenerativas como o câncer. A ingestão de antioxidantes provindos de fontes naturais como frutas e vegetais podem minimizar a ação dos radicais livres e, consequentemente, reduzir os riscos de doenças **(Figura 9)** (Aziz & Karboune 2016). Existem diversos estudos recentes com cogumelos que comprovam sua atividade antioxidante, tanto pelo consumo *in natura* como através de extratos, como suplementos alimentares (Koutrotsios *et al.* 2017, Sanchez 2017, Sharif *et al.* 2017). Por isso, o aumento do consumo de fontes naturais de antioxidantes e os estudos desses compostos têm sido cada vez mais observados (Shashidhar *et al.* 2017).

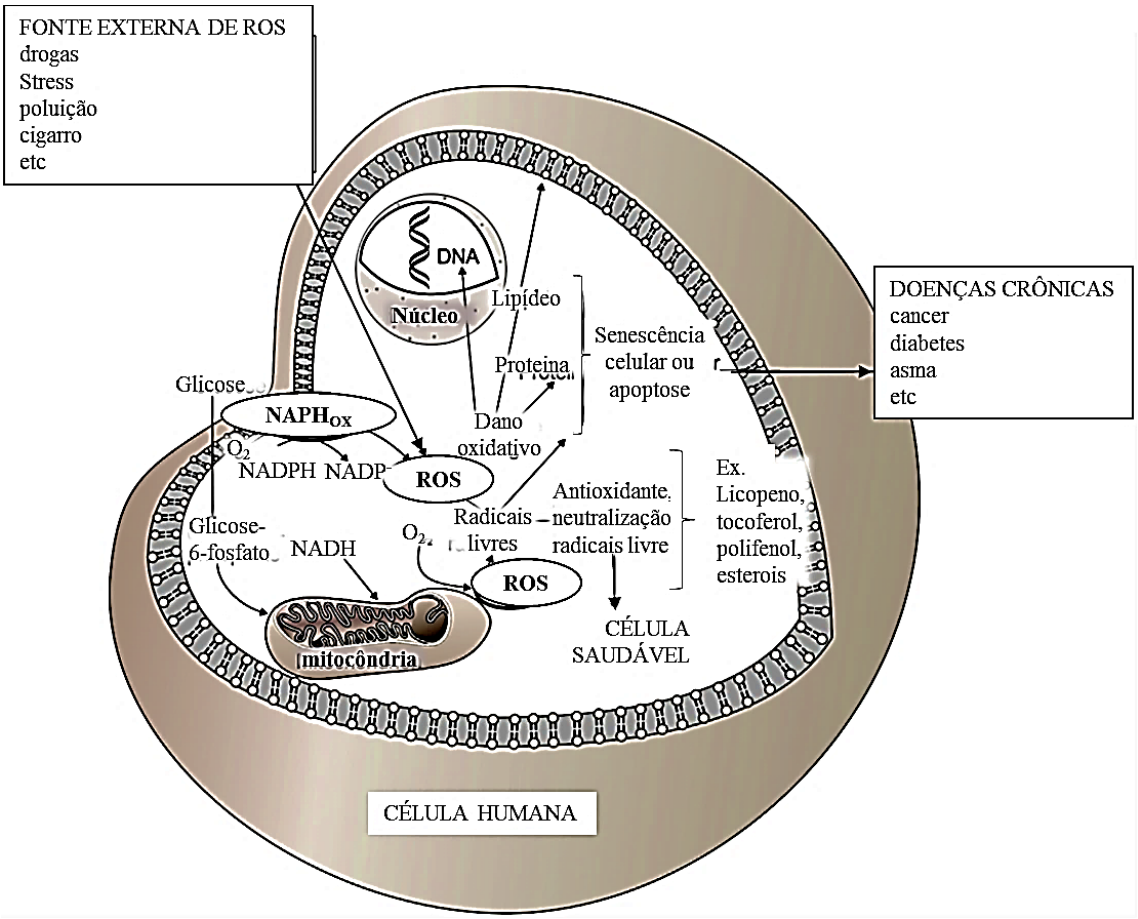

Figura 9. Representação esquemática de uma célula humana, que pode ser danificado por radicais livres gerados a partir de fontes internas e externas. Neutralizar os radicais livres com agentes antioxidantes é importante para manter uma célula saudável. Esquema baseado em Sánchez (2017).

2.1.5.2 Antimicrobiano

A descoberta dos antimicrobianos foi um dos avanços importantes na história médica moderna (Tomaras & Dunman 2015), mas o seu desenvolvimento não acompanhou o surgimento de novos agentes patogênicos (Nambiar *et al.* 2014). Apesar da enorme diversidade de compostos antibacterianos, a resistência bacteriana frente aos antibióticos de primeira escolha aumenta drasticamente (Alves *et al.* 2012) e apresenta um desafio para a ciência e a medicina (Lewis 2017). Estes compostos atuam de várias formas interferindo nos processos metabólicos ou nas estruturas do organismo **(Figura 10)** (Dehghan Esmatabadi *et al.* 2017). Entretanto os cogumelos demonstram um amplo espectro de atividades farmacológicas, incluindo atividade antimicrobiana contra bactérias e fungos (Heleno *et al.* 2013, Chandrasekaran *et al.* 2016), seus extratos podem modular a resposta imunitária do hospedeiro (Tanaka *et al.* 2016). Klancnik *et al.* (2017) demosntraram que extratos aquosos de cogumelos apresentaram atividade antimicrobiana contra bactérias gram positivas e gram negativas e antifúngica, juntamente com uma ação antiaderente.

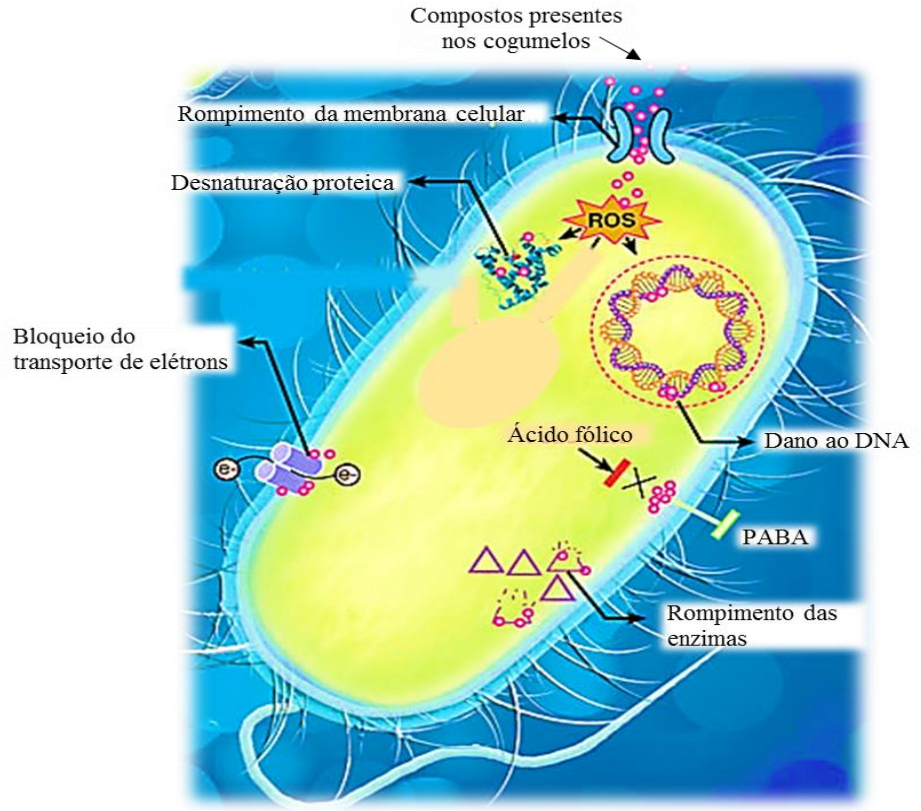

Figura 10. Provável mecanismo de ação antimicrobiana, adaptado de Blair *et al.* (2015).

2.1.5.3 Anti-inflamatório

Atualmente os fármacos anti-inflamatórios não esteróides (AINEs) são geralmente os medicamentos mais comumente administrados para reduzir a inflamação no organismo. Muitos estudos, no entanto, mostraram que a administração a longo prazo de AINEs tem potencial para efeitos colaterais significativos no trato gastrointestinal (Azab *et al.* 2016). Recentemente muitos esforços foram dedicados à descoberta de compostos anti-inflamatórios alternativos de origem vegetal como potenciais medicamentos naturais e seguros sem os efeitos colaterais prejudiciais dos AINE (Taofiq *et al.* 2016, Yuan *et al.* 2017). Os cogumelos podem ser uma alternativa já que apresentam propriedades antiinflamatórias devido seus compostos como polissacarideos, diterpenoides, triterpenoides, ergosterol e ácidos fenólicos (Elsayed *et al.* 2014).

2.1.5.4 Antitumoral

Atualmente as pessoas estão se direcionado para o consumo de substâncias naturais presentes em frutas, vegetais, óleos essenciais, fungos, entre outros, para trazer melhor benefício à saúde. (Elsayed *et al.* 2014, Turrini *et al.* 2014, Chen *et al.* 2015). Acredita-se que diversas dessas substâncias apresentem potencial valor na prevenção ao câncer ou atuem como agentes terapêuticos, induzindo a morte programada diferentemente do que ocorre com a maioria das terapias utilizadas atualmente (Simoben *et al.* 2015). Dessa forma, têm-se procurado identificar compostos isolados da flora e fauna brasileiras para alvos específicos contra o câncer, incluindo alvos moleculares e vias de sinalização que exercem seu feito anticancerígeno através de múltiplos mecanismos, incluindo inibição da proliferação, indução de apoptose, supressão de metástases, regulação da função imune e reversão de resistência a múltiplos fármacos.

O tratamento é um dos maiores desafios médicos da atualidade com avanços nas últimas décadas. As modalidades utilizadas clinicamente contra o câncer como remoção cirúrgica do tumor, seguido por radioterapia e principalmente quimioterapia e/ou hormônio terapia, são agentes anti-proliferativos que destroem preferencialmente células em divisão (Sculier *et al.* 2015), não sendo seletivos para células cancerosas. Outro importante efeito dessas terapias convencionais é a resistência tumoral em que o microambiente do tumor diminui a penetração de fármacos, conferindo vantagens proliferativas e antiapoptóticas às células sobreviventes, facilitando a resistência sem causar mutações genéticas e alterações epigenéticas (Sun 2016).

Os agentes tradicionais de quimioterapia bloqueiam a divisão celular e a replicação do DNA, modificando a dinâmica dos microtúbulos do fuso mitótico (Takei *et al.* 2011). Estes fármacos, tais como derivados de platina, análogos de nucleósidos, inibidores de topoisomerase, taxanos e alcalóides de vinca, são amplamente utilizados hoje em dia e

possuem bons efeitos curativos prolongando ligeiramente a sobrevida dos pacientes (Marsh & Liu 2009, Bordon 2014), no entanto, eles não são eficazes para todos os tipos de câncer (Bailon-Moscoso *et al.* 2014).

A investigação da atividade anticarcinogênica de isolados de cogumelos é relevante, considerando que esses compostos demonstram atividade antiproliferativa e citotóxica (Bukhari *et al.* 2014). Em um estudo realizado por Han *et al.* (2015), descreveram o provável mecanismo de ação dos extratos de cogumelos que levam a apoptose, indicando que esses extratos ativam a apoptose através da via mitocondrial **(Figura 11**). Uma vez que a via apoptótica mitocondrial é controlada pela família Bcl-2. Quando Bak e Bax são ativados, o citocromo c é liberado da mitocôndria e forma um apoptossomo com Apaf-1, o que resulta em ativação de caspase-7, caspase-9 e caspase-3, levando a célula a apoptose. Os extratos de cogumelos podem reduzir Bcl-2 e aumentar Bax diretamente para levar a célula a apoptose.

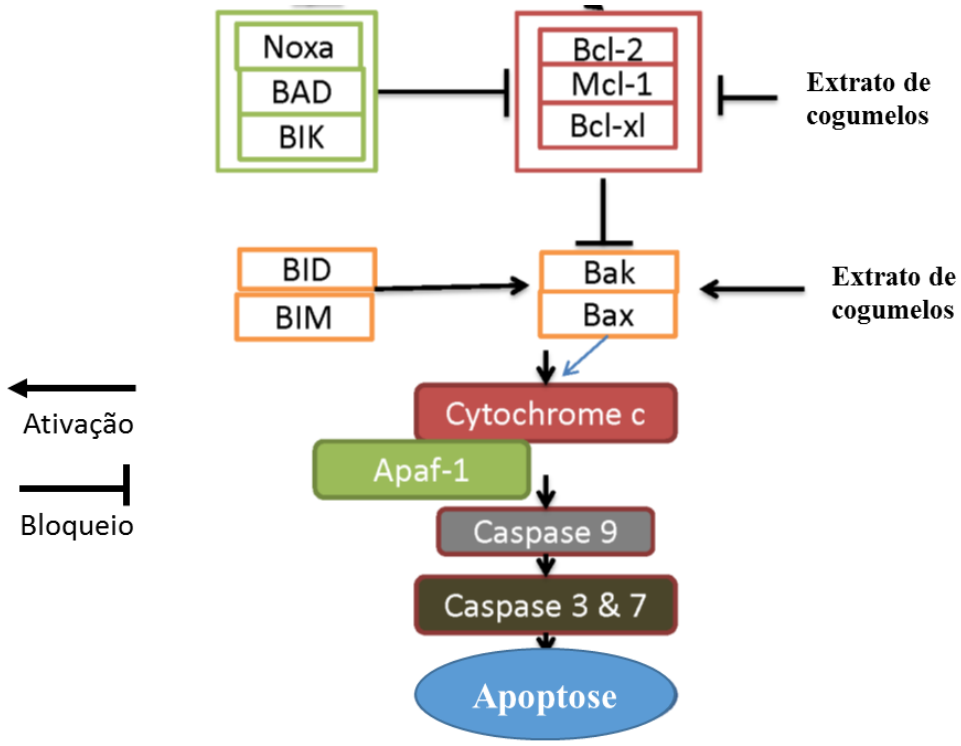

Figura 11. Provável sinalização molecular dos extratos de cogumelos que levam a apoptose, modificado de Han *et al.* (2015).

2.2 Terapia anticarcinogênica

Recentes pesquisas suportam a hipótese do papel central da apoptose na tumorigênese. Células malignas são caracterizadas pela redução da capacidade de sofrer apoptose na resposta a vários estímulos. A inativação do caminho pró-apoptótico ou ativação do caminho anti-apoptótico resulta na falha apoptótica, promovendo a sobrevivência das células transformadas (Zhang *et al.* 2015). Portanto, o desenvolvimento do câncer está associado ao rompimento do equilíbrio entre o crescimento e morte celular. O balanço entre a taxa de proliferação e de morte celular programada determina a taxa de crescimento do tumor. E alguma alteração entre esses dois fatores pode ser o elemento chave para a expansão descontrolada de tumores malignos e posteriormente, sua capacidade de invasão (Mester & Redeuilh 2008, Dalla Via *et al.* 2014).

A transformação maligna da célula normal é um processo multipassos que envolvem mudanças na atividade de diferentes genes e proteínas, resultando na perda do controle do ciclo celular e de um programa de diferenciação. Estes eventos celulares são geralmente mútuos e exclusivos, e são regulados por programas genéticos específicos, controlados pela ação sequencial e cooperativo de fatores de transcrição (Chokoeva *et al.* 2015). Oncogenes específicos tem sido identificados em tumores esporádicos, em outras palavras, numerosos genes que codificam proteínas envolvidas na regulação do ciclo celular são amplificados, superexpressos ou ativados, como c-Myc, ciclina D1, MDM2, HER-2 (Lacroix *et al.* 2006, Cerella *et al.* 2015). Dentre as proteínas que rigorosamente controlam o ciclo celular, a proteína p53 exerce importante função na atividade supressora de tumor por dois mecanismos: interrupção da proliferação e indução da morte celular programada (Vousden & Lu 2002). Assim, a p53 atua na proliferação celular, permitindo que sejam reparados danos espontâneos ou induzidos durante as fases G1-S do ciclo celular. Se houver falha dos mecanismos de reparo, os níveis de p53 aumentam e as

células são encaminhadas para apoptose. Nos tumores, mutações no gene P53 resultam na tradução de proteínas mutantes incapazes de corrigir danos genômicos e/ou levar a célula a apoptose (Labi & Erlacher 2015), fazendo com que as células tumorais não parem de se multiplicar.

As células tumorais adquiriram diversas maneiras de escaparem do processo de apoptose, quer por desregulação de mecanismos intracelulares ou por uma resposta imunológica ineficiente. O mais importante é a evidência de que mudando uma dessas condições, a morte celular por apoptose possa ser restabelecida (Yan *et al.* 2014).

2.3 Terapia Anti-inflamatória

Os anti-inflamatórios não-esteroides (AINEs) representam uma das classes de fármacos mais utilizadas no tratamento de inflamações agudas e crónicas (Bovill 2003). Entretanto eles podem causar efeitos colaterais significativos, tais como irritação da mucosa gástrica, toxicidade hepática e renal (Harirforoosh *et al.* 2013). Neste contexto, várias estratégias têm sido propostas de forma a evitar ou minimizar tais efeitos (Naito *et al.* 2011).

Os AINEs são classificados com base na sua estrutura química como salicilatos, propiônicos, enólicos, acéticos e derivados do ácido fenâmico; seletivos da cicloxigenase-2 (COX-2) inibidores da sulfonas e outros. Constituem um grupo heterogéneo de compostos, sendo constituídos a nível molecular por um ou mais anéis aromáticos ligados a um grupo funcional ácido (Bacchi *et al.* 2012). O principal mecanismo de ação dos AINEs consiste na inibição da COX (cicloxigenases) e consequente redução da conversão do AA (ácido araquidônico) em prostaglandinas (PGs) (Pereira-Leite *et al.* 2017).

As PGs são produtos originados a partir do AA o qual é obtido diretamente a partir da alimentação ou do ácido linoleico (**Figura 12**). O AA apresenta um papel regulador

chave na fisiologia celular. Consiste num ácido graxo composto por uma cadeia molecular de 20 carbonos sendo liberado a partir dos fosfolipídios das membranas celulares, através da ação da enzima fosfolipase A2 (FLA2) (Hwang *et al.* 2013). O AA uma vez liberado, ele pode seguir por duas vias de metabolização diferentes: a da lipoxigenase (LPO) e a da cicloxigenase (COX-1, COX-2 e COX-3). As lipoxigenases transformam o AA em leucotrienos (LT), principalmente em LTC4 e LTD4, sendo estes broncoconstritores. As cicloxigenases, por sua vez, formam tromboxanos (TX) e prostaglandinas: PGF2 (broncoconstritores), PGD2 (espasmógena) e PGE2 (broncodilatadores, vasodilatadores) (Patrignani & Patrono 2015).

A COX-1 é constitutivamente expressa e catalisa a produção de prostaglandinas que estão envolvidas em inúmeras funções fisiológicas, incluindo manutenção da função renal, proteção da mucosa no trato gastrointestinal e como pro-agregante tromboxano A2 nas plaquetas. Por outro lado, a expressão de COX-2 pode ser induzida por citocinas e outros mediadores inflamatórios em vários tecidos, incluindo células endoteliais, e acredita-se que tenha um papel na mediação de dor, inflamação e febre (Conaghan 2012). Entretanto, muitos dos efeitos tóxicos dos AINEs, tais como sangramentos e efeitos colaterais gastrointestinais, são atribuídos à inibição da COX-1 (Houston & Teach 2004) e maioria dos AINEs tradicionais são inibidores não seletivos de COX-1 e COX-2. Foi o reconhecimento e a compreensão das atividades da enzima COX-1 e COX-2 que se descobriu que uma droga que inibisse especificamente a COX-2 aliviaria a dor e a inflamação tão efetivamente quanto os AINE sem os efeitos colaterais tóxicos (Pereira-Leite *et al.* 2017).

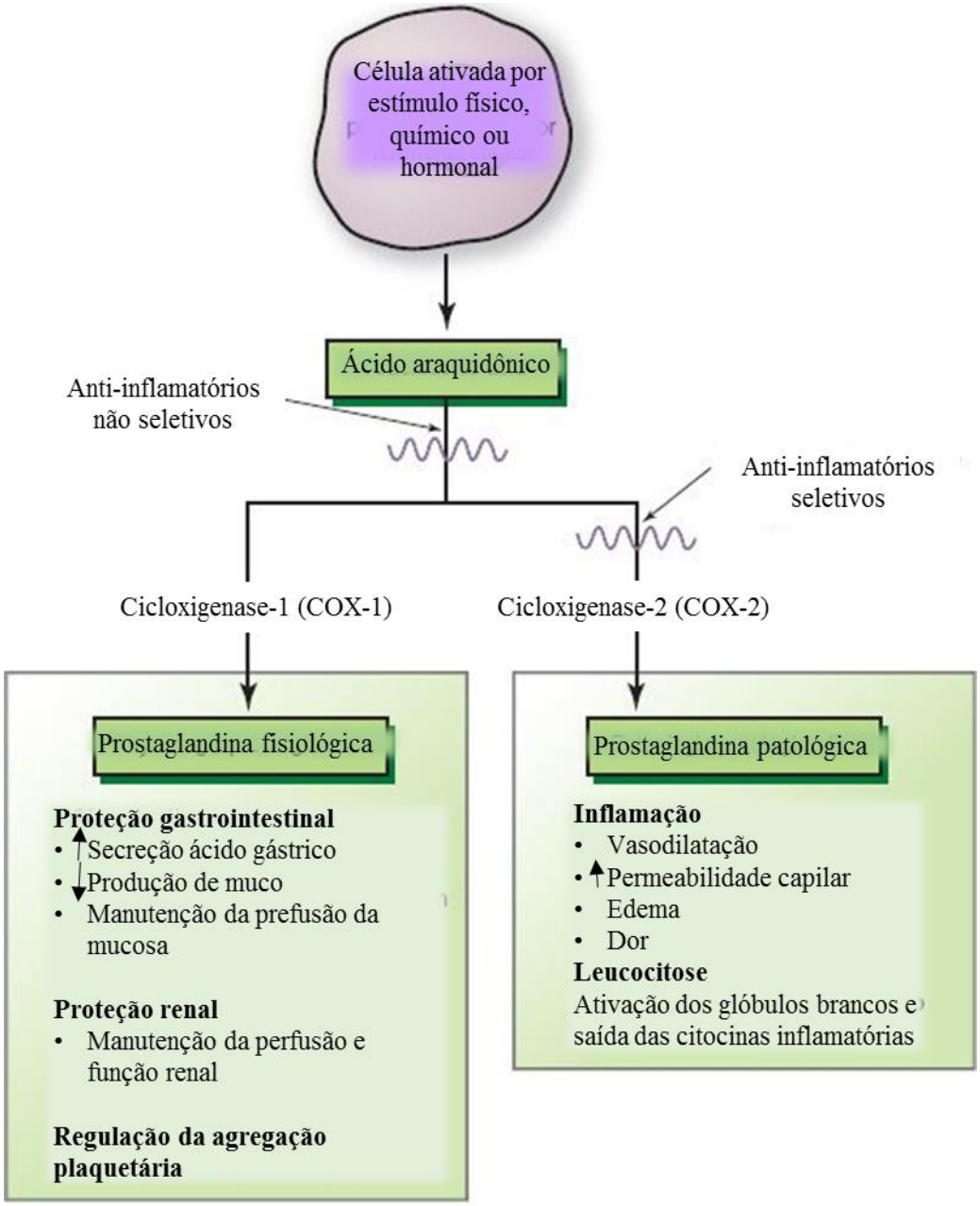

Figura 12. As vias metabólicas para o ácido araquidônico resultam na produção de prostaglandinas inflamatórias fisiológicas e patológicas. Os fármacos anti-inflamatórios podem inibir a COX-1 (não-seletivos) e a COX-2 (seletivos). Esquema modificado de Zarghi & Arfaei (2011).

2.4 Vias de sinalização de morte celular

Múltiplos sinais modulam a proliferação celular, sobrevida e morte celular e essa ação coordenada permite que a célula normal cresça e se divida até sua senescência (Kamal *et al.* 2014). Entretanto, as células tumorais perdem a capacidade de regular esses

sinais, resultando no descontrole de proliferação e ausência de morte celular, contribuindo para o desenvolvimento dos tumores. De modo geral, estímulos tóxicos ou deletérios a célula podem desencadear a morte celular por necrose ou apoptose, as quais são diferenciadas pela morfologia e vias bioquímicas celulares (Koff *et al.* 2015).

A necrose é definida como uma forma violenta de morte celular iniciada por estímulos ambientais que resultam em rápida desregulação da homeostasia. Durante a necrose, ocorre condensação da cromatina e a célula aumenta de volume, as mitocôndrias dilatam-se, juntamente com o retículo endoplasmático, e há desagregação dos ribossomos (Lebrec *et al.* 2015). Há alteração na permeabilidade da membrana, por diminuição nos níveis de ATP que tem como consequência comprometimento da bomba de Na^+/K^+ e de outros fenômenos que são ATP-dependentes. Isso resulta no rompimento de organelas e da membrana plasmática e liberação de componentes intracelulares, ocasionando uma reação inflamatória local (Huang & Freter 2015). A citólise é a estágio final da senescência celular evidente no processo de necrose (Saraste & Pulkki 2000).

Em contrapartida, a apoptose é um processo fisiológico altamente regulado de morte celular programada e desempenha um papel relevante na homeostase de diferentes tecidos em resposta a numerosos estímulos (Hail *et al.* 2006). É caracterizada por alterações no citoesqueleto que induzem contração celular, fragmentação do DNA, condensação da cromatina levando a aparência de núcleos picnóticos, formação de vesículas sem perda de integridade da membrana e sem resposta inflamatória (Koff *et al.* 2015). Ela é de crucial importância para o desenvolvimento embrionário, maturação do sistema imune, defesa contra infecções virais e eliminação de tumores.

Há duas vias principais de regulação da apoptose **(Figura 13**): 1) Apoptose mediada por receptores de morte (TNF, TNFR1, TRAMP, TRAIL e de Fas) presentes na membrana plasmática, denominada via extrínseca e 2) Apoptose mediada pela

mitocôndria denominada via intrínseca (Kim *et al.* 2015). Tanto a via extrínseca quanto a intrínseca possuem um grupo independente de caspases iniciadoras que convergem sinais para o mesmo grupo de caspases efetoras com finalidade de executar eventos intracelulares que resultarão na morte celular programada (Hajra & Liu 2004).

A via extrínseca é iniciada por associação de monômeros de diferentes receptores de morte na membrana plasmática e, ao se agruparem, promovem o recrutamento de proteínas adaptadoras. Dentre os receptores na membrana plasmática, o receptor fas/CD95 recruta procaspase 8 e/ou procaspase 10, e a consequente elevação nos níveis de procaspases próxima a membrana garantem interação dessas caspases inativas com proteínas adaptadoras associadas a fas/CD95. A interação desse complexo proteico promove a autocatálise das procaspases que se tornam nesse momento em caspases iniciadoras ativadas. A ativação proteolítica sequencial de outras caspases culmina na ativação de caspases efetoras 3, 6 e 7 que estão no citoplasma (Fulda 2014). Ainda, a ativação de outros receptores de morte na membrana plasmática, especialmente do receptor de estresse celular, bem como a ação das caspases iniciadoras 8 e 10, integram sinais de apoptose de via extrínseca aqueles de via intrínseca. Assim, é preciso avaliar sinais na membrana plasmática capazes de agir sinergicamente com sinais mitocondriais, na indução de apoptose (Hail *et al.* 2006). Na via intrínseca, diversos sinais atuam modulando a permeabilização da membrana mitocondrial externa. O caminho mitocondrial pode ser ativado por vários estímulos incluindo hipóxia, espécies reativas de oxigênio, irradiação ultravioleta ou gama, deprivação de fatores de crescimento e vários compostos citotóxicos, resultando na ativação de proteínas pró-apoptóticas (Roy *et al.* 2014). Estas proteínas oligomerizam e induzem a permeabilização da membrana externa mitocondrial através da formação de canais que causam a liberação e redistribuição de pequenos íons, solutos, metabólitos, citocromo c e da proteína 14-kDa

carreadora de elétrons da cadeia respiratória para o citosol. O citocromo c liberado no citosol é necessário como co-fator e rapidamente se associa com a região C-terminal a uma proteína adaptadora (Apaf-1), esta interação facilita a ligação ao dATP e pro-caspase 9 formando um complexo chamado de apoptossomo, e através da clivagem proteolítica a caspase 9 se torna ativa e subsequentemente ativando outras caspases (Waterhouse *et al.* 2002, Fulda 2014).

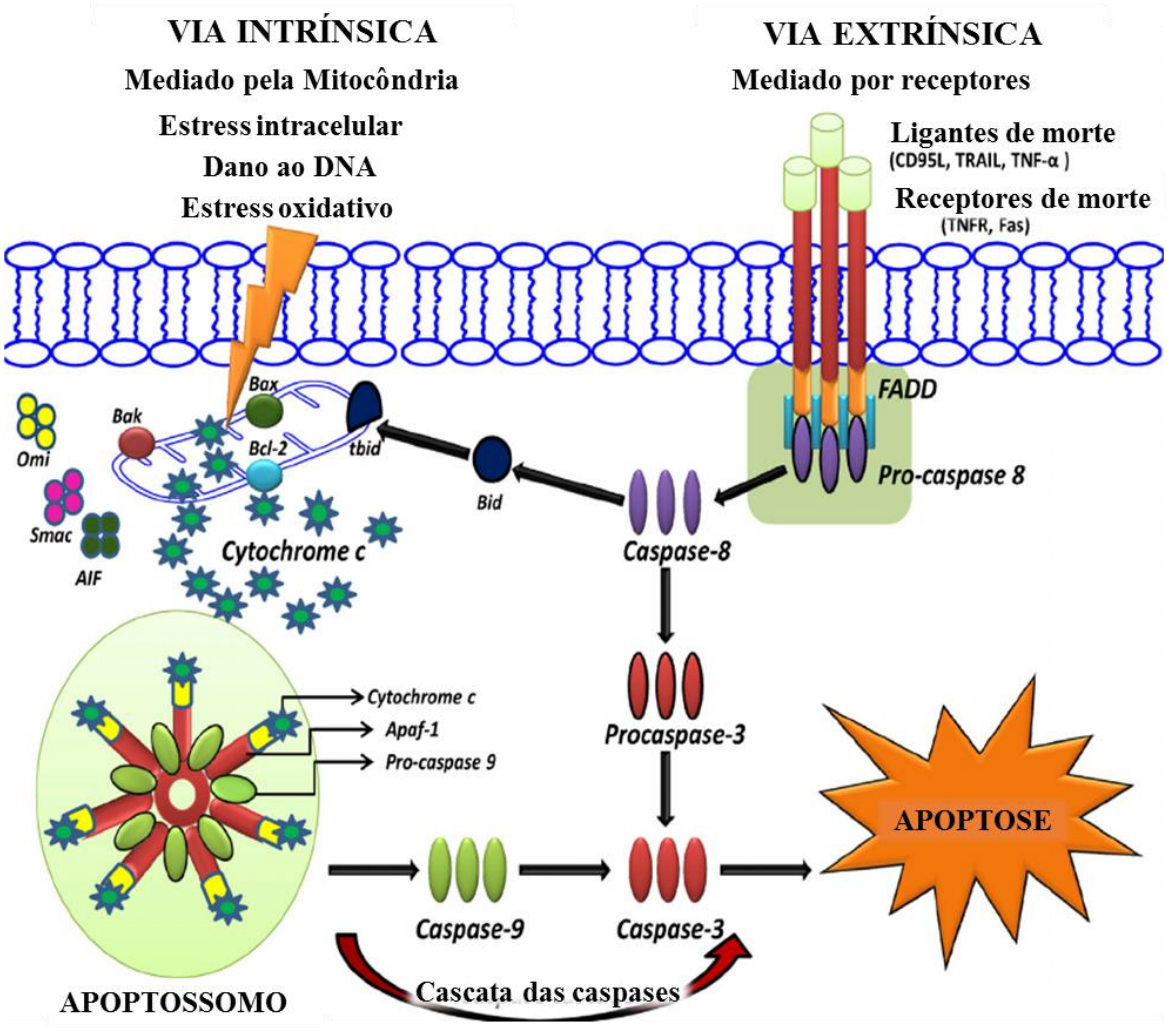

Figura 13. Principais vias de regulação da apoptose (Beesoo *et al.* 2014).

As caspases clivam proteínas envolvidas na regulação do citoesqueleto, incluindo gelsolina, fodrina, Gas-2, proteína quinase de adesão focal (FAK), proteína quinase p21,

isoformas da proteína quinase C e Mekk-1. A destruição da rede de microtúbulos provoca o arredondamento e deslocamento da célula do tecido. Várias alterações do núcleo apoptótico são dependentes da ação da caspase-6, caspases que degradam as laminas A, e C do invólucro nuclear. O rompimento dessas estruturas parece facilitar o acesso e degradação das fitas de DNA na região internucleossomal pela nuclease CAD (caspase activated deoxyribonuclease). Em células não apoptóticas, CAD está presente como um complexo inativo com ICAD (inhibitor of caspase activated deoxyribonuclease). Após a indução da apoptose, ICAD é inativada pelas caspases 3 e 7, deixando CAD livre para funcionar como uma nuclease (Enari *et al.* 1998).

Outro fator mitocondrial pró-apoptótico é o Smac/DIABLO que atua inibindo as IAPs (inhibitor apoptosis protein) de bloquear a atividade das caspases. As IAPs pertencem uma família de proteínas com atividade anti-apoptótica que atuam inibindo as caspases. Após dano mitocondrial, a Smac/DIABLO é liberada do espaço intermembrana para o citoplasma, juntamente com o citocromo c. Enquanto o citocromo c liga-se à APAF-1 e ativa diretamente a caspase-9, Smac/DIABLO remove as IAP de sua ligação inibitória com as caspases (GRIVICICH, 2007). A permeabilização de membrana mitocondrial e liberação de citocromo c são fundamentais para os sinais de via intrínseca, as quais dependem da modulação de proteínas anti e pró-apoptóticas da família Bcl-2 (Czabotar *et al.* 2014).

A família Bcl-2 possui cerca de vinte proteínas já identificadas, cada uma delas com duas ou mais isoformas. Dentre essas proteínas, Bax, Bak, Bcl-Xs, Bok, Bad, Bid exercem função pro-apoptóticas enquanto as proteínas Bcl-2, Bcl-XL, Bcl-w, Mcl-1 têm função anti-apoptótica. Na mitocôndria, durante a apoptose, algumas modificações dessas proteínas podem ocorrer. É conhecido que, durante a apoptose, membros da família Bcl-2 podem ser fosforilados (Bcl-2 e Bad), clivados (Bid) e podem ter mudanças de

conformação e oligomerização (Bax e Bak). Os sinais mitocondriais anti ou pró-apoptóticos estão em equilíbrio e a predominância de um desses sinais, conduz, respectivamente, a sobrevida ou morte celular programada (Moldoveanu *et al.* 2014). A interação de proteínas anti e pró-apoptóticas, ocorre por domínio hidrofóbicos gerados pelas α hélices de BH1, BH2 e BH3. Proteínas BH3 (Bid e Bim), interagem com proteínas pró-apoptóticas da família Bcl-2, como Bak e Bax e essa interação induz a ativação/oligomerização (Hail *et al.* 2006). Assim, no equilíbrio anti e pró-apoptótico são modulado por vias que se complementam, nas quais ocorre a associação das proteínas Bcl-2/Bax e Bcl-xL/Bak. Se o estímulo for anti-apoptótico, a ligação de Bcl-2/Bax e Bcl-xL/Bak é mantida, impedindo a formação do poro na membrana mitocondrial e liberação de citocromo-c e das IAPs. Entretanto, por estímulo pró-apoptótico, a ativação de Bax e Bak ocorre pela forma clivada de Bid (tBid), seguida da oligomerização Bax e Bak na membrana mitocondrial, com subsequente liberação das proteínas apoptogênicas (citocromo c) e consequente indução de morte celular (Roy *et al.* 2014). Este processo parece envolver modificações na atividade de duas enzimas: (1) a ativação da enzima fosfolipídio scramblase, que aumenta o movimento bidirecional de lipídeos através da dupla camada da membrana, um movimento conhecido como "flip-flop" e (2) inativação da enzima aminofosfolipideo translocase, que retornaria a PS para a camada interna da membrana citoplasmática. Estas alterações enzimáticas irreversíveis parecem estar relacionadas com a atividade da enzima caspase-3. Além disso, a expressão de fosfatidilserina (PS) parece ser um evento crítico para o reconhecimento e remoção destas células por macrófagos (**Figura 14).** Apesar de muitos outros receptores participarem deste processo, na ausência de PS a fagocitose de células apoptóticas é mínima (Dan *et al.* 2015). A perda de assimetria do fosfolipídio de membrana contribui para facilitar o

reconhecimento de células apoptóticas por fagócitos pela exposição de outros ligantes que estão obscurecidos nas células viáveis (Arthur *et al.* 2015).

No entanto, a morte celular apoptótica não é a única modalidade de morte celular. As pesquisas recentes sugerem uma relação entre a apoptose, necrose e a autofagia. De fato, dependendo do contexto celular e do estímulo de morte, a apoptose e a necrose podem cooperar de uma forma equilibrada envolvendo também a autofagia. O estado metabólico celular é um determinante na escolha de qual tipo de morte pode ser desencadeada em resposta a tensões farmacológicas. Por isso, é necessário compreender todas as vias de morte celular existentes: seu papel no metabolismo celular, bem como os mediadores reguladores fundamentais envolvidos, para desenhar novas estratégias para combater o câncer (Radogna *et al.* 2015).

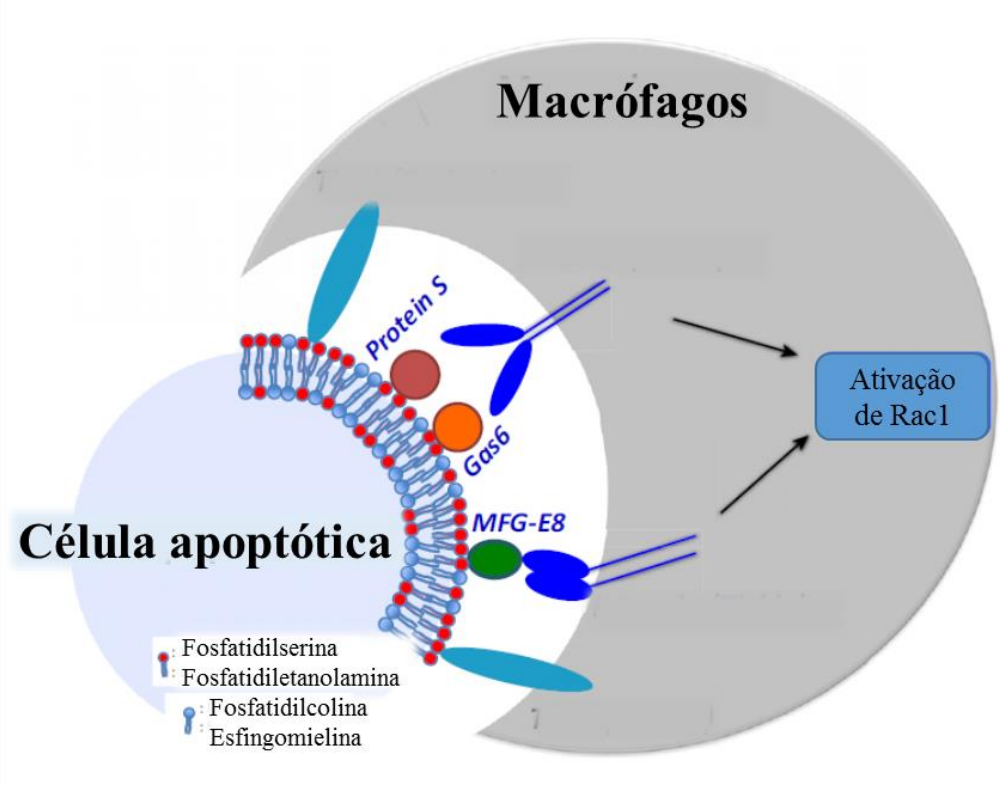

Figura 14. Macrófagos se ligando a fosfatidilserina externalizada da célula apoptótica (Segawa & Nagata).

2.5 Screening molecular – *Docking*

Docking molecular, ou simplesmente *docking*, é o processo de se encontrar o melhor ajuste para o encaixe entre duas moléculas tridimensionais como por exemplo interações proteína-proteína, proteína-ligante ou proteína-DNA. O sistema de "*docking*" macromolecular proteína-ligante consiste de duas etapas: um algoritmo de busca conformacional que envolva todos os graus de liberdade do ligante (translacional, rotacional e conformacional) e uma função de pontuação que ranqueie as prováveis posições espaciais para um composto ou vários provenientes de uma grande biblioteca Há vários programas que permitem realizar essa busca de forma automatizada (Pagadala *et al.* 2017).

Os primeiros procedimentos de *docking* começaram a acontecer logo depois do desenvolvimento dos programas gráficos moleculares, dado que eles permitiram aos pesquisadores manipular duas moléculas e encontrar diferentes conformações que pareciam complementares geométrica e quimicamente. Os programas de *docking* molecular permitiram a automação da busca e um modo mais objetivo de avaliar o ajuste entre duas moléculas.

O processo de *docking* pode ser dividido em três partes:

- planejamento do experimento *in silico* (realizado utilizando programas e simulações computacionais);
- realização do "experimento" com objetivo de formar complexos;
- avaliação dos resultados obtidos no experimento.

Para a parte de planejamento, necessita-se primeiro buscar quais as moléculas serão estudadas e as informações disponíveis sobre as mesmas. A escolha também depende da disponibilidade de dados cristalográficos das moléculas envolvidas. No caso dos receptores biológicos, a consulta será realizada no PDB (Protein Data Bank). Depois da

escolha das moléculas, procura-se o sítio de ligação a ser estudado, que será o centro de uma esfera de raio R, em torno do qual os programas computacionais realizam sua busca. E o programa computacional escolhido, fará todos os cálculos necessários relacionados a energia de ligação, ligações de hidrogênio, entre outros (Abdolmaleki *et al.* 2017).

Há basicamente três possibilidades metodológicas utilizadas pelos programas computacionais: o *docking* rígido, o *docking* semiflexível e o *docking* flexível. No primeiro caso, as duas moléculas, o farmacóforo e a molécula de interesse, são consideradas como corpos rígidos e o programa busca a melhor orientação de um corpo em relação a outro. No semiflexível, permite-se que o ligante possa variar seus ângulos torsionais. No último caso, permite-se que o receptor também sofra flexibilização parcial ou total. Os programas computacionais variam também no que diz respeito à sua essência metodológica de tal forma que a busca de conformações pode ser feita utilizando métodos de mecânica molecular, métodos estocásticos e algoritmos genéticos ou outros. Outro fator que também varia de programa para programa é a chamada "função escore" que determina a melhor orientação e/ou conformação das moléculas. A função escore pode estar baseada em métodos empíricos, campos de força ou potenciais variados (de Ruyck *et al.* 2016). No *docking* rígido, o algoritmo de busca explora diferentes posições para o ligante no sítio ativo do receptor (**Figura 15**). O *docking* de ligantes flexíveis adicionalmente, explora os graus de liberdade torcionais do ligante nesse processo (Brooijmans & Kuntz 2003). Os resultados obtidos da energia em função da conformação mostram que a mínima energia sempre resulta no melhor resultado receptor-ligante.

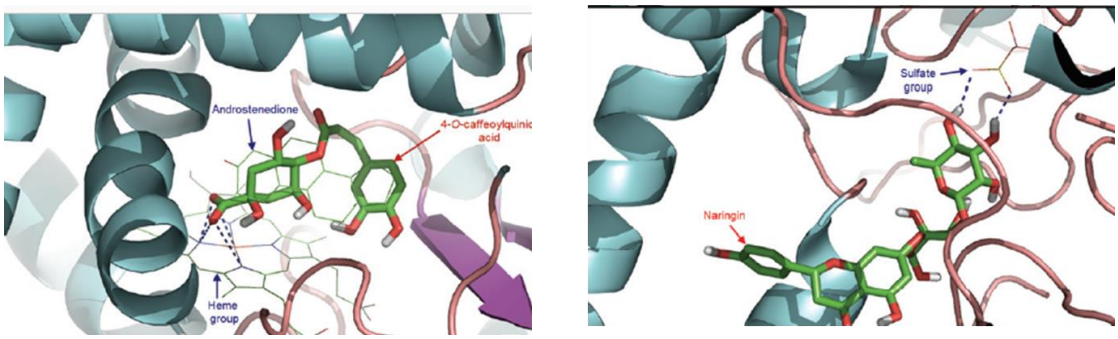

Figura 15. Exemplos de *docking* moleculares rígidos (Froufe *et al.* 2011)

Froufe *et al.* (2011) avaliaram *in silico* a atividade antitumoral dos compostos de baixo peso molecular presente nos cogumelos, utilizando como alvo as proteínas específicas do câncer de mama, aromatase, estrona sulfatase e 17β-Hidroxiesteroide Desidrogenase-1, sugerindo que os compostos de baixo peso molecular apresentam atividade antitumoral contra o câncer de mama. Os derivados do ácido cinâmico foram o que melhor inibiram essas proteínas. Estudos recentes estão investigando o efeito inibidor da tirosinase, com substâncias presentes nos cogumelos, e estão demonstrando que algumas dessas substâncias são promissoras para o desenvolvimento de novos fármacos (Ashraf *et al.* 2017, Larik *et al.* 2017). Xiong *et al.* (2016) descreveu que a apigenina, um polifenol encontrados no cogumelo *Agaricus bisporus,* apresentou uma forte inibição contra a proteína polifenoloxidase pela simulação computacional de *docking*. Já Alves *et al.* (2014), avaliaram a atividade antimicrobiana dos cogumelos com 34 compostos presentes que possuem afinidade com as proteínas bacterianas e são alvos conhecidos para alguns antibióticos. Os resultados demonstraram que os extratos de cogumelos podem ser uma alternativa contra microrganismos patogênicos resistentes aos tratamentos convencionais.

3 OBJETIVOS

Objetivo geral

Caracterização de extratos dos cogumelos *Pleurotus sajor-caju* e *Lentinula edodes* quanto a sua composição química, capacidade antioxidante, e bioatividades *in vitro* e *in silico.*

Objetivos específicos

Para o *Lentinula edodes* (LE-01),

- Avaliar a presença de ácidos fenólicos no extrato aquoso;
- Verificar a citotoxicidade nas células de câncer de laringe (Hep-2) e na célula normal de pulmão (MRC-5);
- Verificar a morfologia celular;
- Avaliar a expressão de marcadores biológicos envolvidos na regulação do ciclo celular, apoptose e proliferação celular nas células MRC-5 e Hep-2;

Para o *Pleurotus sajor-caju* (PS-2001)

- Obter diferentes frações de extratos com diversos solventes;
- Otimizar os métodos de extração para compostos fenólicos;
- Avaliar a presença de ácidos fenólicos no extrato etanólico;
- Caracterizar quimicamente a fração hexânica;
- Verificar a citotoxicidade da fração hexânica nas células de câncer coloretal (HCT-116^{wt}) e nas células HCT-116 deletadas para a expressão de -p21, -p53 e -Bax e na célula normal de pulmão (MRC-5);
- Verificar a morfologia celular;

- Avaliar a expressão de marcadores biológicos envolvidos na regulação do ciclo celular, apoptose e proliferação celular nas células MRC-5 e HCT-116 ^{wt};
- Verificar a citotoxicidade do extrato etanólico nas células tumarais HepG-2, MCF-7, NC-H460, HeLa e na célula normal de fígado de porco (PLP2);
- Avaliar o potencial antioxidante do extrato etanólico;
- Verificar a atividade anti-inflamatória e antimicrobiana do extrato etanólico;
- Realizar modelos *docking*; para a atividade antitumoral do extrato etanólico e para a atividade anti-inflamatória do extrato hexânico;

4 RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de capítulos, os quais correspondem a três artigos científicos. O primeiro artigo, intitulado ***Extrinsic and Intrinsic Apoptotic Response Induced by Shiitake Medicinal Mushroom Lentinus edodes (Agaricomycetes) Aqueous Extract Against Larynx Carcinoma Cell Line*** está publicado na revista "*International Journal of Medicinal Mushrooms",* DOI: 10.1615/IntJMedMushrooms.2018025400. O segundo, ***Multifunction of Pleurotus sajor-caju a high nutrition food and a source of bioactive supplements*** está publicado na revista "*Food Chemistry"* DOI: 10.1016/j.foodchem.2017.10.088. E o terceiro, ***Apoptosis induction by Pleurotus sajor-caju (Fr.) Singer extracts on colorectal cancer cell lines***, está publicado na revista "*Food Chemical and toxicology*", DOI: 10.1016/j.fct.2018.01.015.

CAPÍTULO I Extrinsic and Intrinsic Apoptotic Response Induced by Shiitake Medicinal Mushroom *Lentinus edodes* (Agaricomycetes) Aqueous Extract Against Larynx Carcinoma Cell Line

CAPÍTULO II Multifunction of *Pleurotus sajor-caju* a high nutrition food and a source of bioactive supplements

CAPÍTULO III Apoptosis induction by *Pleurotus sajor-caju* (Fr.) Singer extracts on colorectal cancer cell lines

4.1 CAPÍTULO I

Extrinsic and Intrinsic Apoptotic Responses Induced by Shiitake Culinary-Medicinal Mushroom *Lentinus edodes* (Agaricomycetes) Aqueous Extract against a Larynx Carcinoma Cell Line

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ABSTRACT: Cumulative evidence from research studies has shown that the shiitake culinary-medicinal mushroom, *Lentinus edodes*, is an excellent source of natural antitumor agents and is capable of inhibiting cancer cell growth. However, the cell signaling pathway that leads tumor cells to apoptosis is not well understood because many chemical compounds may be acting. This study investigated the chemopreventive effects of an *L. edodes* aqueous extract on human HEp-2 epithelial larynx carcinoma cells and normal human MRC-5 lung fibroblasts by identifying proliferative and apoptotic pathways. The chemical characterization of the dry powder was assessed by high-performance liquid chromatography. Antiproliferative and proapoptotic effects induced by the extract were evaluated by assessing proliferative markers, cell sorting through flow cytometry, and expression levels of apoptotic proteins with Western blotting. The results suggest that inhibition of cell proliferation was more prominent in HEp-2 than in MRC-5 cells. Cell death analysis showed the appearance of cell populations in the sub-G_1 phase, with late apoptotic signaling increased in a dose-dependent manner. In addition, the aqueous extract induced depolarization of mitochondria, activating the generation of intracellular reactive oxygen species in HEp-2 cells. These observations suggest that *L. edodes* extract may exert a chemopreventive effect, regulating mitotic induction of apoptogenic signals. These findings highlight the mushroom's pharmacological potential in cancer treatment.

KEY WORDS: cytotoxicity, flow cytometry, HEp-2, *Lentinus edodes*, medicinal mushrooms, multitarget

ABBREVIATIONS: AO/EB, acridine orange/ethidium bromide; **DCFH-DA**, 2′,7′-dichlorofluorescin diacetate; **DMEM**, Dulbecco's modified Eagle's medium; **FITC**, fluorescein isothiocyanate; **GA**, galic acid; **HPLC**, high-performance liquid chromatography; **HRP**, horseradish peroxidase; **IC_{50}**, half-maximal inhibitory concentration; **PBS**, phosphate-buffered saline; **PI**, propidium iodide; **ROS**, reactive oxygen species; **TNF**, tumor necrosis factor

I. INTRODUCTION

The shiitake culinary-medicinal mushroom, *Lentinus edodes* (Berk.) Singer (Marasmiaceae, Agaricomycetes), is a species with a long history of use in folk medicine to treat disease, especially in countries such as Japan, China, India, and Korea. The chemical characterization of this mushroom is dependent on environmental variation, the substrate, growing conditions, and the fruiting and fungal development stages.[1] Various preparations of this mushroom (especially polysaccharide lentinan) have been shown to exert anticancer activity and seem to be potent alternative agents for chemoprevention and immune system regulation.[2,3]

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L. edodes is also known to have a multifaceted spectrum of beneficial effects, such as antioxidant[4] and anti-inflammatory[5] properties against several diseases and cancers.[6] Antioxidant activity is correlated with different components such as tocopherols, carotenoids, ascorbic acid, and total phenolics.[7,8] Phenolic compounds, particularly flavonoids, have been suggested to protect human cells from the development of oxidative stress and the progression of cancer, aging, and cardiovascular disease.[9] Different studies demonstrated the important effect of phenolic acids in inducing death of tumor cells.[10,11]

So far, some studies have indicated that molecules present in *L. edodes* extracts can alter the expression of signals within tumor cells by delaying the cell cycle and inducing apoptosis.[12] These studies indicate that substances contained in mushrooms can interfere with tumor progression through a variety of mechanisms, for example, by enhancing the host's antioxidant capacity.[13] According to Patel and Goyal,[14] polysaccharide components isolated from mushrooms exhibited potent antineoplastic activity. These polysaccharides have received growing attention in recent years because of their antitumor activity against a wide variety of human cancers, with high specificity and selectivity for HepG2, hepatocellular carcinoma, Hep3B, activated hepatic stellate, U937, MCF-7, A375, and HeLa cell lines, among others.[15–17] Although activation of specific and nonspecific immune response has been linked to tumor development,[18,19] studies point out direct activity of extracts on tumors through mechanisms of apoptosis that depend on 2 fundamental pathways: the mitochondrial pathway and the extrinsic pathway, which involves cell death receptors.[20,21] Studies have suggested involvement of autophagy in cell death,[22,23] and it may also induce necroptosis through reactive oxygen species (ROS) signaling in cancer cells.[24] So, it is crucial to explore how *L. edodes* constituents may regulate the cell dynamics and reduce cancer cell proliferation. A better understanding of how the compounds present in *L. edodes* can regulate the molecular mechanisms that directly or indirectly control apoptosis and the development of more specific and less harmful therapies is recommended. Identification of a specific drug or compound that targets the apoptotic machinery to reduce the growth and proliferation rates of cancer cells would be an effective strategy to slow cancer progression.[6,25,26]

While prior research suggests that mushrooms have an effect on the apoptotic pathway, this study was designed to investigate the biological effect on the regulation and inhibition of cancer cell proliferation and to understand the death signaling involved in this regulation. Therefore, cytotoxic effects and apoptosis induction were assessed against the well-known classic HEp-2cancer cell line, as well as the selectivity of the extract against this tumor.

II. MATERIALS AND METHODS

A. Mushroom Sample Preparation

L. edodes fruiting bodies were obtained from a commercial producer in Caxias do Sul, Rio Grande do Sul, Brazil, and inoculated with spawn from Brasmicel, Suzano, São Paulo, Brazil. These were dried in an oven at 50°C and ground to powder with a knife mill. The powder obtained was used for extraction, which was carried out using 10% (w/v) distilled water at 22°C in a rotational shaker for 1 hour. The extract was then filtered using Whatman no. 1 filter paper, ultrafiltrated using 0.22-μm units in a laminar flow chamber, and stored at −20°C until use, according to the process described by Zhuang et al.,[27] with slight modifications. The amount of moisture was determined based on loss upon drying, in accordance with the method described by Ileleji et al.,[28] and showed 96.53% humidity.

B. Chemicals

All solvents used for the analysis were analytical grade. Methanol and formic acid [85% (v/v)] for high-performance liquid chromatography (HPLC) were purchased from Merck. The ultrapure water was Milli-Q

(Eppendorf). All standards, gallic acid (GA), catechin, epicatechin, rutin, ferulic acid, naringin, hesperidin, myricetin, resveratrol, quercetin, vitexin, apigenin, and kaempferol were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were acquired from Sigma-Aldrich. We also used a Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich); the antibodies anti-Bax (1:500), anti-Bcl-2 (1:500), anti-caspase-3 (1:500), and anti-β-actin (1:1000) (all from Abcam); and a Human Apoptosis Antibody Array Kit (Abcam).

C. HPLC Determination of Total Phenolics

Total phenolic content was determined using HPLC with an HP 1100 reciprocating quaternary system of pumps connected to a column. The ultraviolet absorption spectra of standards and samples were recorded at 210 nm. The mobile phase consisted of water containing 2% acetic acid (solvent A) and methanol (solvent B). The mobile phase was generated through a gradient pump system, with 90% of solvent A and 10% of solvent B for 0 to 5 minutes, and 60% of solvent A and 40% of solvent B for 45 to 50 minutes. Samples, standards solutions, and the mobile phase were degassed and filtered through a 0.45-μm membrane filter (Millipore). Chromatographic analysis was carried out at room temperature in triplicate. The compounds were identified by chromatographic comparison with the standards. Quantification was based on calibration curves obtained from commercial standards of each compound.[29]

D. Cell Culture and Treatment

The human HEp-2 larynx carcinoma tumor cell line and the normal human MRC-5 lung cell line, obtained from the American Type Culture Collection, were cultured in DMEM (Sigma-Aldrich). The media were supplemented with 10% fetal bovine serum (Gibco-BRL, Grand Island, NY), 100 mg/L streptomycin, and 100 IU/mL penicillin at 37°C in a humidified atmosphere with 5% CO_2. Both cell lines were plated in 96-well plates at a density of 7×10^4 cells/well and cultured for 24 hours. After 24 hours, cells were treated with *L. edodes* aqueous extract using serial dilution (0.2, 0.5, 1, 2, 5 mg/mL) to give a final volume of 200 μL/well. Medium without the extract served as the control. Plates were also treated for 24 hours with serum-free medium in the same conditions, and cell viability was assayed. The half-maximal inhibitory concentration (IC_{50}) of the extracts was defined as the concentration producing a 50% decrease in cell growth.[30]

E. MTT Assay

After treatment with the extract, 100 μL MTT (1 mg/mL) in DMEM solution was added to each well and the plate was incubated for another 2 hours. The media containing MTT were removed, and 100 μL dimethyl sulfoxide solution was added to each well and mixed thoroughly to dissolve the formed formazan crystals. After 30 minutes of incubation, light absorption was measured at 570 nm using a SpectraMax M2/M2e microplate reader (Molecular Devices). Viability was expressed as the percentage of absorbance of treated cells compared with that of control cells.[31]

F. DAPI and Acridine Orange/Ethidium Bromide Staining

Changes in chromatin organization upon treatment with *L. edodes* aqueous extract were determined microscopically by staining with DAPI and acridine orange/ethidium bromide (AO/EB) dual stain. HEp-2 cells (7×10^4 cells/well) were grown on coverslips placed in 24-well plates. After 24 hours of treatment with the extract, cells were washed with phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde

for 15 minutes at room temperature, then washed again with PBS. The fixed cells were incubated with 1 mg/mL DAPI solution for 5 minutes at room temperature in the dark. After washing twice with PBS, cells were examined under fluorescence microscopy. Processed cells were observed using an Olympus BX43 microscope to determine nuclear morphological changes, according to the method described by He et al.[32] The apoptotic cells were identified based on the presence of highly condensed chromatin or fragmented nuclei. For AO/EB staining, after treatment with indicated concentrations of *L. edodes* for 24 hours, the cell suspension from each well was separated into a vial. The vials were centrifuged at 1200 rpm for 5 minutes. The pellets obtained were washed once with PBS, stained with AO/EB solution (25 μL PBS and 2 μL AO/EB dye [100 μg/mL]), incubated for 5 minutes, and observed under an Olympus BX43 microscope. Morphological changes were determined according to the method described by Kasibhatla et al.[33]

G. ROS Generation Measurement and Changes in Mitochondrial Membrane Potential

ROS generation was analyzed by flow cytometry using 2′,7′-dichlorofluorescin diacetate (DCFH-DA). Cells were treated with *L. edodes* extract at different concentrations (1 and 2 mg/mL) for 24 hours, suspended in PBS, and incubated with 10 μmol/L DCFH-DA at 37°C for 30 minutes. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein by nonspecific cellular sterases and the subsequent oxidation of dichlorodihydrofluorescein by peroxides was measured by flow cytometry (BD FACSCalibur; BD Biosciences, San Jose, CA).[34]

The uptake of the cationic fluorescent dye probe DiOC6(3) (2 μL of 2 μmol/L stock solution in dimethyl sulfoxide) was used to evaluate mitochondrial membrane potential. HEp-2 cells were seeded at 1×10^6 cells/well into 6-well plates. After 24 hours of incubation, cells were treated with *L. edodes* extract at different concentrations (1 and 2 mg/mL) for 24 hours. Untreated controls and treated cells were harvested and washed twice with PBS. The cell pellets were then resuspended in 2 mL fresh incubation medium containing DiOC6 and incubated at 37°C in a thermostatic bath for 30 minutes. HEp-2 cells were separated by centrifugation, washed twice with PBS, and analyzed by flow cytometry (BD FACSCalibur).[34]

H. Flow Cytometric Analysis of Cell Cycle and Apoptosis

Briefly, 1×10^6 HEp-2 cells/well were seeded in a 6-well plate and left in an incubator for 24 hours. Cells were then treated with *L. edodes* (1 and 2 mg/mL) for 24 hours. After suspension in PBS (800 μL) and propidium iodide (PI; 200 μL), cell cycle distribution of 10,000 cells was recorded by flow cytometry (BD FACSCalibur), and the percentages of cells at the G_0/G_1, S, and G_2/M phases were analyzed using FlowJo 10.0 software, according to the method described by Wlodkowic et al.[34]The extent of apoptosis was measured through the use of an Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich), as described in the manufacturer's instructions. After treatment, cells were collected, washed twice with PBS, gently resuspended in Annexin V binding buffer, incubated with Annexin V–fluorescein isothiocyanate (FITC)/PI in the dark for 15 minutes, and analyzed by flow cytometry (BD FACSCalibur). The fractions of the cell population in different quadrants were measured using quadrant statistics with FlowJo 10.0 software. The lower left quadrant contained intact cells, the lower right quadrant apoptotic cells, and the upper right quadrant necrotic or postapoptotic cells.

I. Apoptosis Antibody Array Membrane Analysis

Relative levels of 43 human apoptosis-related proteins were detected and analyzed using a human array kit (no. ab134001; Abcam), according to the manufacturer's instructions. Briefly, the membrane containing

immobilized apoptosis-related antibodies was blocked with bovine serum albumin on a rocking platform at room temperature for 2 hours. The membrane was then incubated with lysates of untreated or treated HEp-2 cells (with *L. edodes* [1 mg/mL] for 24 hours) along with a detection antibody cocktail overnight at 2–8°C. The membrane was incubated with streptavidin horseradish peroxidase (HRP) conjugate, followed by chemiluminescent detection reagent. The membrane was scanned using an ImageQuant LAS 500 imager (GE Healthcare Life Sciences). Pixel density was determined in each spot volume, corrected for background, and expressed as fold change (treated vs. untreated cells). Was used ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij) with the Protein Array Analyser plugin.[35]

J. Western Blotting Analysis

To evaluate the expression of intracellular proteins related to apoptosis, HEp-2 cells were treated with the *L. edodes* extract at different concentrations (1 and 2 mg/mL) for 24 hours. To isolate total protein fractions, the cells were collected, washed twice with ice-cold PBS, and lysed using cell lysis buffer (nonidet P-40, 20 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% Triton X-100, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L EDTA, and protease inhibitor). The lysates were collected by scraping them from the plates and then centrifuging at 10,000 rpm at 4°C for 5 minutes. Total protein samples were loaded on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA) for 1 hour. Membranes were blocked with blocking solution (5% powdered milk in Tris-buffered saline and Tween) at room temperature for 1 hour. Next, the membranes were incubated for 1 hour at room temperature with anti-human Bcl-2 rabbit (1:1000 dilution; Abcam), anti-human Bax rabbit (1:1000 dilution; Abcam), and anti-human caspase-3 rabbit (1:500 dilution; Abcam) polyclonal antibodies, or with anti-human β-actin mouse antibody (1:1500). After washing, the membranes were incubated for 1 hour at room temperature with HRP-linked anti-mouse immunoglobulin (1:1000 dilution; Amersham) for β-actin, or HRP-linked anti-rabbit immunoglobulin (1:50,000 dilution; Amersham) for Bax, Bcl-2, and Caspase-3. Immunoblotting was performed using ECL Prime Western Blotting Detection Kit (Amersham). Chemiluminescence was visualized and detected using an ImageQuant LAS 500 imager (GE Healthcare Life Sciences).

K. Statistical Analyses

The results were expressed as the mean ± standard deviation of 3 independent experiments performed in triplicate and were used to assess the normal distribution of data. Between-group differences in mean values were assessed by 1-way analysis of variance, followed by the Tukey post hoc test. Kruskal-Wallis 1-way analysis of variance on ranks was used for data that failed the normality test. Statistical significance of mean differences was accepted at $P < 0.05$. All statistical analyses were performed using SPSS statistical software (version 23.0 for Windows; SPSS Inc., Chicago, IL).

III. RESULTS

A. Phenolic Acids in *L. edodes* Aqueous Extract

The phenolic compounds were characterized according to their retention time and comparison with authentic standards, when available. For quantitative analysis, a calibration curve was obtained through injection

of known concentrations (5–100 µg/mL) of different standard compounds. The correlation coefficients (R^2) of the calibration curves were higher than 0.99.

GA was the major component found in the extract (289.15 mg/g), followed by epicatechin (2.89 mg/g) and apigenin (0.09 mg/g), through HPLC analysis. High-performance liquid chromatograms of the *L. edodes* extract and molecular spatial representation of the major compounds (GA, epicatechin, and apigenin) are presented in Fig. 1.

B. *L. edodes* Decreased Cell Viability and Induced Apoptosis in HEp-2 Cells

First, the antiproliferative properties of the *L. edodes* extract were predetermined using the MTT assay. The IC_{50} was used as a parameter for cytotoxicity. The *L. edodes* extract presented a higher antiproliferative effect on HEp-2 tumor cells (IC_{50} = ~1 mg/mL) when compared with that of nonmalignant (MRC-5) cells (Fig. 2A). The difference in stage at cell death and the percentage of apoptotic cells among HEp-2 cells after treatment with 2 mg/mL showed a higher amount of cells undergoing late apoptosis compared with untreated cells (Fig. 2B). The morphological changes observed upon DAPI staining were cell

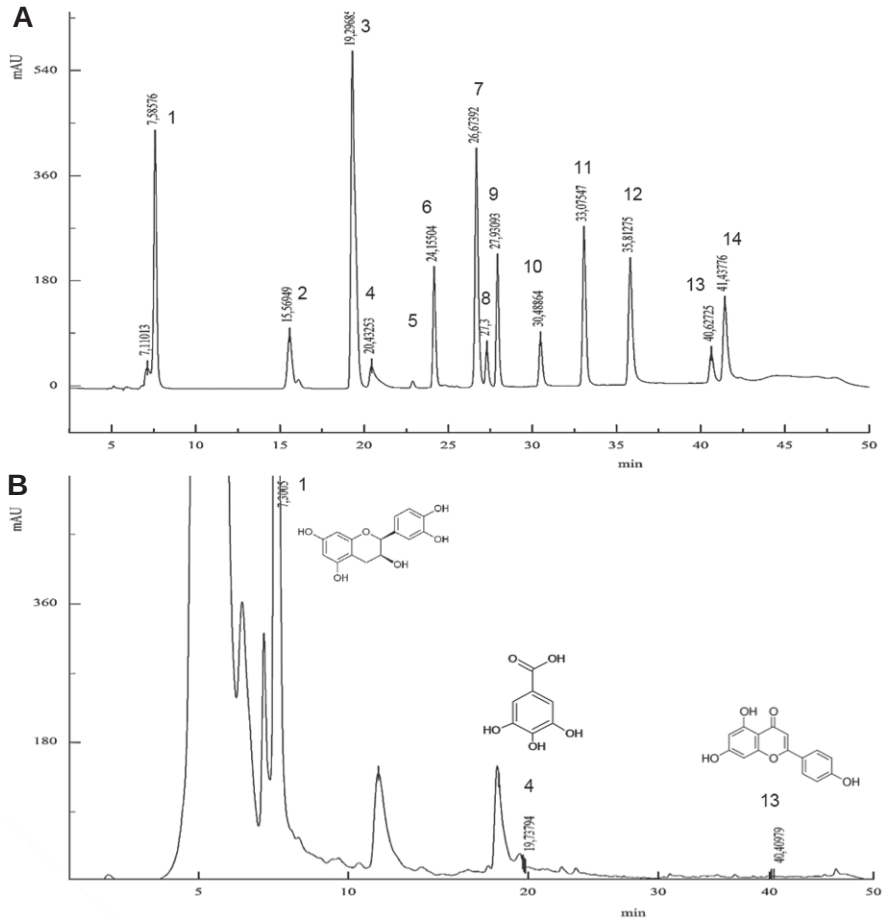

FIG. 1: High-performance liquid chromatograms of *Lentinus edodes* (A) and separation of a standard mixture of phenolic compounds (B): 1, gallic acid; 2, catechin; 3, chlorogenic acid; 4, epicatechin; 5, vitexin; 6, rutin; 7, ferulic acid; 8, naringin; 9, hesperidin; 10, myricetin; 11, resveratrol; 12, quercetin; 13, apigenin; and 14, kaempferol.

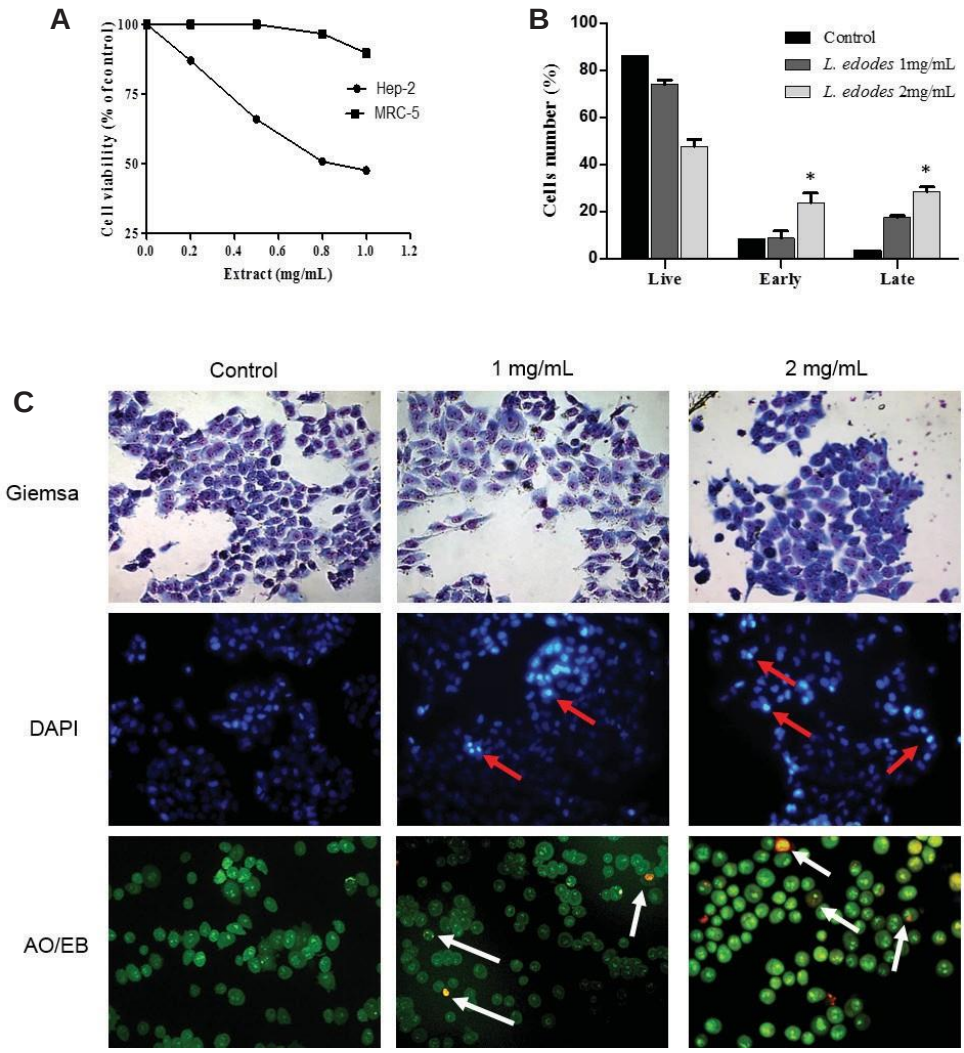

FIG. 2: (A) Cytotoxic effects of *Lentinus edodes* extract (1–2 mg/mL) on HEp-2 and MRC-5 cells after 24 hours of treatment based on the MTT assay. (B) Percentage of apoptosis determined by ethidium bromide/acridine orange (EB/AO) staining. All experiments were repeated at least 3 times. Error bars represent the standard deviations of 3 independent experiments (*$P < 0.05$). (C) HEp-2 cells were treated with 1 and 2 mg/mL of *L. edodes* aqueous extract for 24 hours, and the percentage of apoptotic cells was determined. Red arrows indicate cells with chromatin condensation, and white arrows indicate cells undergoing apoptosis. Cells were stained with Giemsa to analyze morphology. Chromatin condensation was evaluated through DAPI staining and AO/EB staining was processed to evaluate apoptotic induction. A total of 250 cells were scored and counted for each treatment.

shrinkage, chromatin condensation, cell volume reduction, and cytoplasmic bleb formation. In the assay, AO/EB stain can be observed early apoptotic HEp-2 treated in the 1 mg/mL concentration and late-stage apoptotic cells were asymmetrically localized with orange nuclear staining at 2 mg/mL (Fig. 2C, arrows). The percentages of apoptotic and necrotic cells using Annexin V-FITC/PI, as determined with cytometric analysis, are shown in Fig. 3. Late apoptosis and/or necrosis events ranged from 20.3% to 33.1%, and early apoptosis occurred in a concentration-dependent manner (1–2 mg/mL, respectively). Data points were scattered and moved to quadrant 2 in a dose-dependent manner when HEp-2 cells were treated with the extract, indicating that the cells were transferred to the final stage of apoptosis.

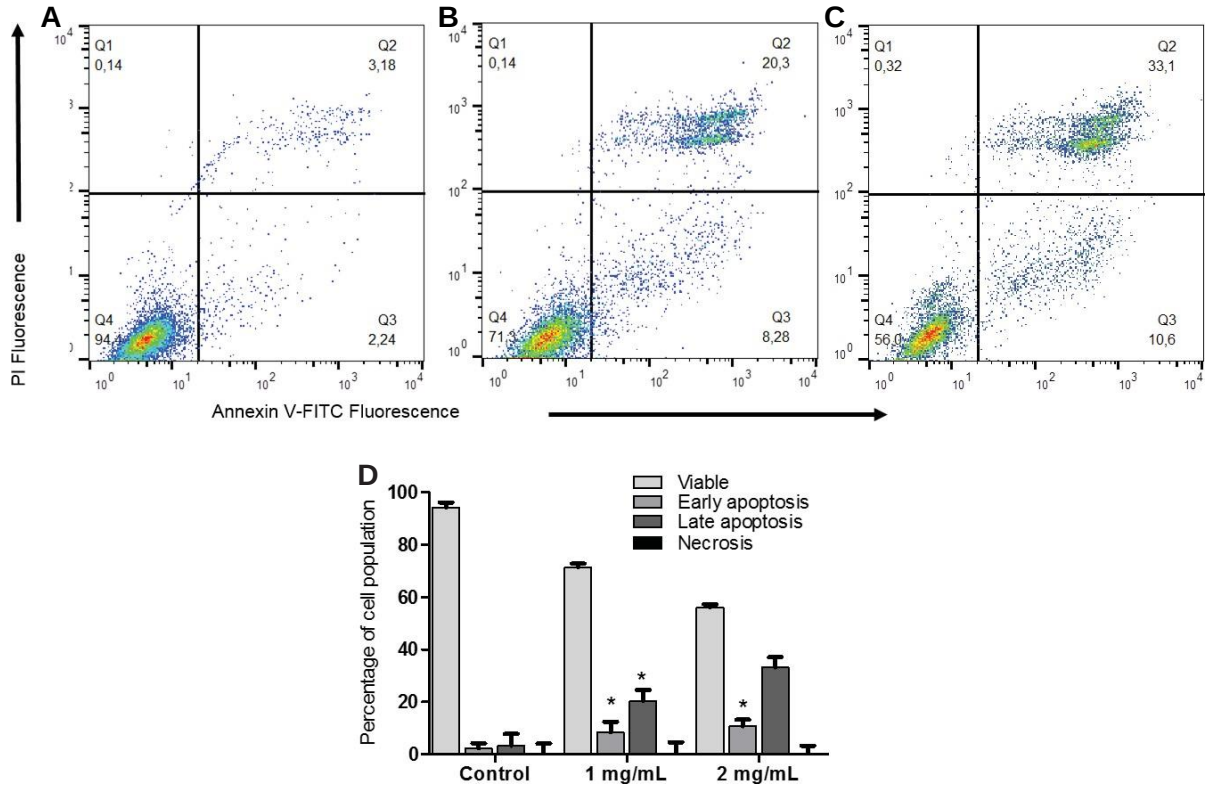

FIG. 3: Apoptosis analysis in HEp-2 cells using Annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) though cytometry analysis for control and treated samples. (A–C) The early apoptotic events (Annexin+/PI−) are shown in the lower right quadrant (Q4) of each panel. Quadrant 2 (Q2) represents Annexin+/PI+ late stage of apoptosis/necrosis. Graphs show results from the control (A) and with treatment with 1 mg/mL (B) and 2 mg/mL (C) of *Lentinus edodes*. (D) Quantitative results from flow cytometry. Data represent the mean ± standard error of the mean from at least 3 independent experiments. **P* < 0.01 vs. control.

C. *L. edodes* Induced ROS Production and Increased Mitochondrial Depolarization and the Sub-G_1 Population in HEp-2 Cells

Flow cytometry analysis demonstrated the occurrence of cellular alterations, possibly due to peroxide and superoxide accumulation in HEp-2 cells treated with the *L. edodes* extract (Fig. 4). HEp-2 cells treated with the *L. edodes* extract (1–2 mg/mL) for 24 hours demonstrated a concentration-dependent increase in ROS production (Fig. 4A–C). The production of ROS by untreated cells was 36.8%, and after treatment with the extract, production of ROS increased to 58% (1 mg/mL). With 2 mg/mL, ROS were reduced, probably due to a loss of mitochondrial function. To evaluate the effects of the *L. edodes* extract on the mitochondrial apoptotic pathway, the mitochondrial membrane potential ($\Delta\psi m$) in HEp-2 cells treated with 1 and 2 mg/mL extract was measured using DiOC6 as a fluorescent dye. The loss of mitochondrial permeability transition (transmembrane potential) was found to be associated with mitochondrial dysfunction leading to apoptosis. The results showed that the amount of depolarized mitochondria increased significantly after *L. edodes* treatment (1–2 mg/mL)—by 59.4% and 60.6%, respectively—when compared with control cells (46.3%) (Fig. 5A–C).

The induction of apoptosis and cell cycle arrest were evaluated further. Cells treated with *L. edodes* at 1 and 2 mg/mL for 24 hours resulted in a minor population of cells in the G_2/M phase and a significant

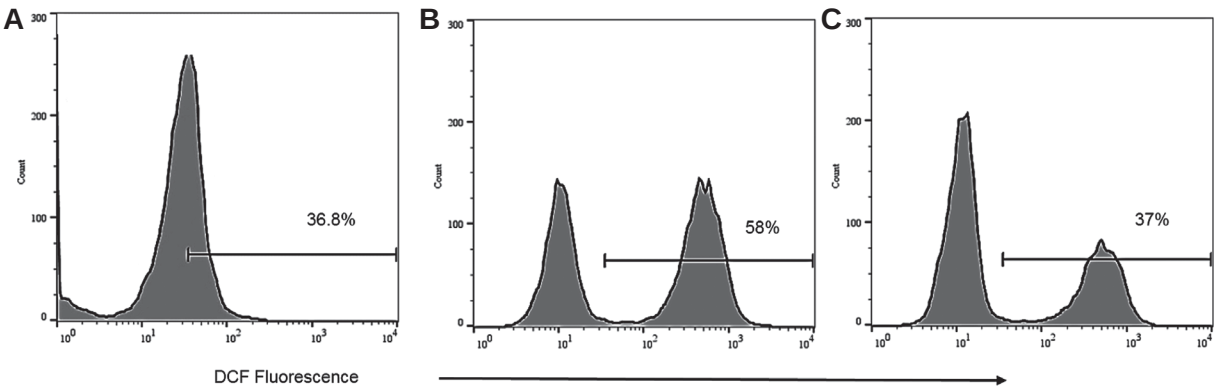

FIG. 4: Cytometry evaluation indicating reactive oxygen species formation in HEp-2 cells before and after extract exposition: control (A), treatment with 1 mg/mL *Lentinus edodes* (B), and treatment with 2 mg/mL of *L. edodes* (C). DCF, dichlorofluorescin.

concentration-dependent accumulation of cells in the sub-G_1 fraction, from 1.5% in the untreated control cells to 16.8% (1 mg/mL) and 36.5% (2 mg/mL) in cells treated with the extract (Fig. 6).

D. Cell Death Induced by *L. edodes* Treatment Through the Activation of Intrinsic and Extrinsic Pathways

Changes in major markers responsible for the apoptotic signaling pathway, including Bax, Bcl-2, Bim, cytochrome c, caspase-3, and caspase-8, are shown in Fig. 7A. Treatment with the *L. edodes* extract resulted in the modulation of protein levels in HEp-2 cells treated both in proapoptotic proteins such as the antiapoptotic proteins, compared with untreated HEp-2 cells (Fig. 7B). To confirm and validate the protein array results, the expression of Bax, Bcl-2, and caspase-3 was further determined using Western blotting. Treatment with the *L. edodes* extract resulted in upregulation of Bax and caspase-3 in a concentration-dependent manner (Fig. 8A). As shown in Fig. 8B, analysis revealed increased relative expression Bcl-2, caspase-3, and Bax in *L. edodes*–treated HEp-2 cells.

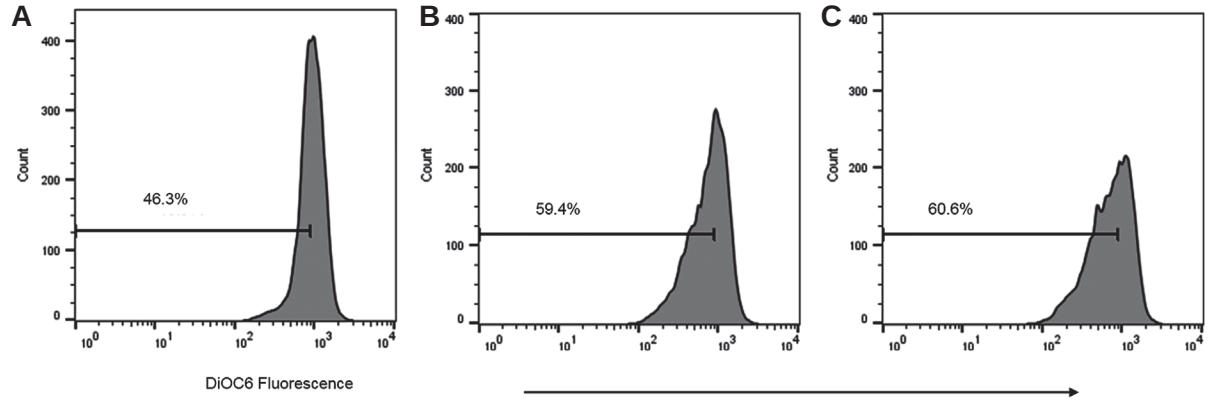

FIG. 5: Mitochondrial membrane potential of HEp-2 cells after treatment with mushroom extracts using $DiOC_6(3)$ staining through cytometry: control (A), treatment with 1 mg/mL *Lentinus edodes* (B), and treatment with 2 mg/mL *L. edodes* (C).

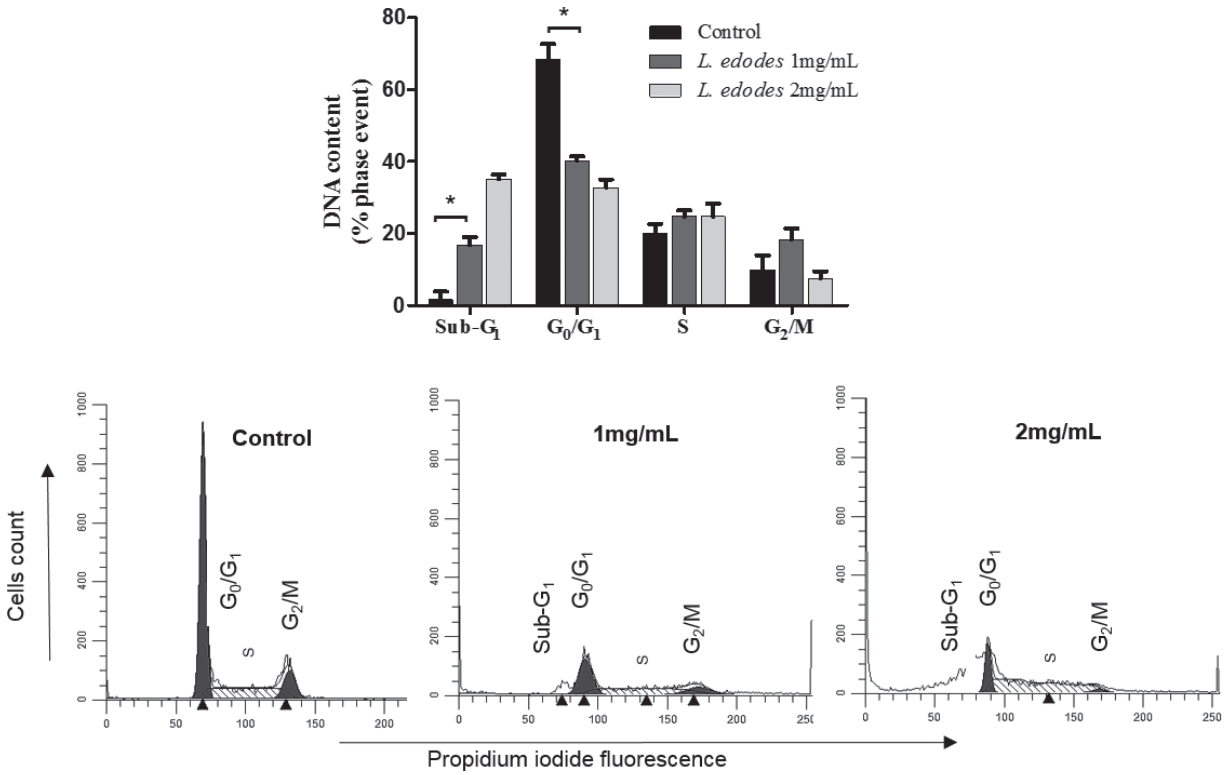

FIG. 6: Cell cycle distribution percentage of HEp-2 cells after treatment with different concentrations of *Lentinus edodes* compared with the control. The results of the cell cycle distribution analysis by flow cytometry were analyzed using ModFit software. Treatment with *L. edodes* induced the appearance of cells in the sub-G_1 phase in a concentration-dependent manner. The percentages of cell populations of different cell cycle phases are shown in the top graph (*$P < 0.01$ vs. the control). Data are presented as the mean ± standard deviation of 3 independent experiments with similar results.

IV. DISCUSSION

Many studies have shown antiproliferative effects of *L. edodes* extracts on several tumor cell lines.[21,36,37] However, the exact molecular mechanisms by which the extracts induce apoptosis have not been fully understood until now, probably because of the synergistic effects of the compounds present in this complex mixture. In this study, we assessed the proapoptotic and antiproliferative effects of the *L. edodes* extract on human HEp-2 larynx carcinoma cells. In addition, we evaluated ROS-dependent extrinsic and intrinsic death pathways.

It is well known that *L. edodes* has a very complex chemical composition, as it comprises a large variety of molecules and their derivatives, including low molecular weight compounds (e.g., quinones, cerebrosides, isoflavones, catechols, amines, triacylglycerols, sesquiterpenes, steroids) and high molecular weight compounds (e.g., homopolysaccharides and heteropolysaccharides, glycoproteins, glycopeptides, and proteins).[38] The *L. edodes* aqueous extract presents many polar substances, among which many are of low molecular weight.[39] The extract contains a variety of compounds derived from secondary metabolism, such as phenolic compounds, triterpenoids,[40] proteins,[41] and steroids, which are unique to each mushroom and have specific effects in humans. These compounds are involved in the regulation and restoration of antioxidant levels in biological systems, regulating redox alterations induced by oxidative processes in

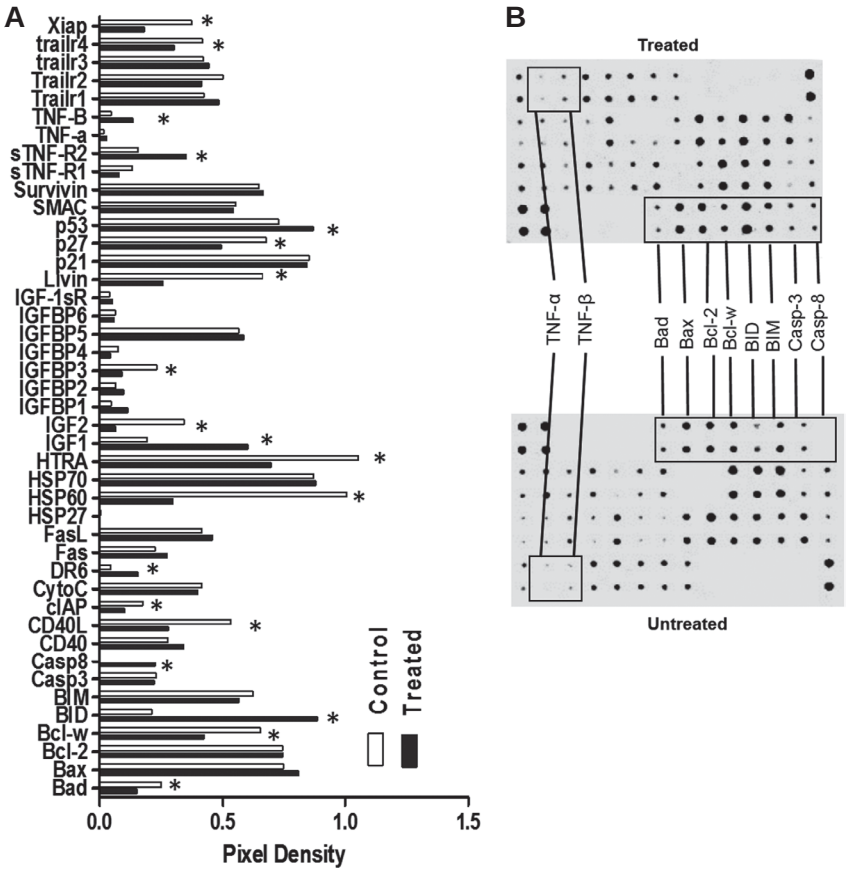

FIG. 7: Modulation of apoptosis-related proteins before and after *Lentinus edodes* extract exposition in HEp-2 cells. (A) Pixel densities of apoptosis-related proteins identified from array analysis of HEp-2 cell in response to treatment with a concentration of 1 mg/mL for 24 hours, showing changes in levels of apoptosis protein compared with the untreated group (*$P < 0.05$). (B) Template demonstrating the location of spots of treated (top) and untreated (bottom) cell sample groups representing the 43 apoptosis-related proteins and locations of the spots of expression apoptosis-related proteins.

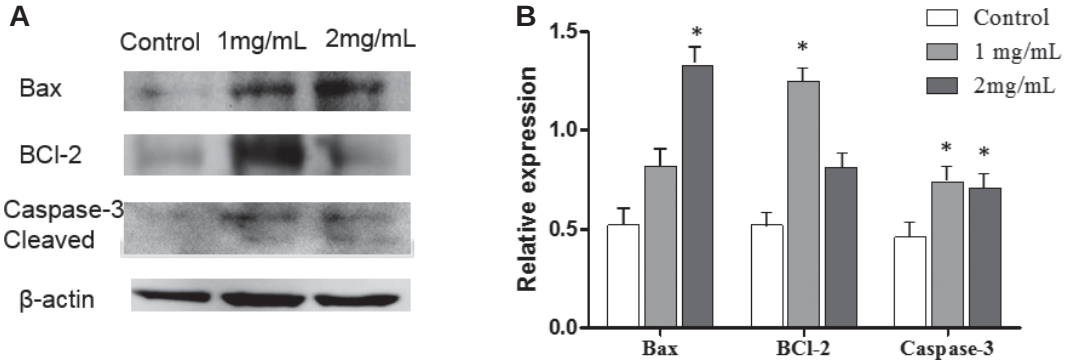

FIG. 8: Effect of *Lentinus edodes* on cell cycle regulators in human HEp-2 larynx cell carcinoma and expression of Bax, Bcl-2, caspase-3, and β-actin treatment with 1 and 2 mg/mL doses of *L. edodes* aqueous extract for 24 hours. (A) Western blotting showing the expression of the proteins. (B) Quantitative analysis of Bax, Bcl-2, and caspase-3 protein levels (*$P < 0.05$). For normalization of protein levels, β-actin was used as the loading control.

the body.[42] These properties may be related to phenolic compounds presented in the aqueous extract.[43] GA was one of the most prevalent phenolic acids found in the *L. edodes* extract. Several studies have indicated that GA is capable of modifying the molecular metabolism of cells.[44–46] GA has also been found to induce morphological alterations, as well as early and late apoptotic events in SMMC-7721 cells with increasing GA concentration.[47] Apigenin, another component of the *L. edodes* extract, has been reported to inhibit the growth of pancreatic cancer cells in the G_2/M phase[48] and to promote the induction of apoptosis in breast cancer cells.[49] Apigenin has also been shown to inhibit the proliferation and invasion of osteosarcoma cells[50] and activate apoptosis in hepatocellular carcinoma[51] and prostate cancer.[52] According to Chang et al.,[53] this molecule promotes cell death by necroptosis and induces oxidative stress through the accumulation of ROS. In this study, we demonstrated that treatment with *L. edodes* decreased viability of and induced significant morphological alterations to HEp-2 cells in a concentration- and time-dependent manner. An increased number of Annexin V–PI-positive cells and an augmented number of cells in the sub-G_1 phase were also observed, suggesting that the *L. edodes* extract was able to induce cell death through either apoptosis, necrosis, or a combination of both processes.

According to You et al.,[54] an alkali-soluble polysaccharide isolated from *L. edodes* induces apoptosis through microtubule depolymerization, with relative percentages of cells in the sub-G_1 phase. Thus, these effects could be due to a wide variety of low and high molecular weight compounds present in the crude *L. edodes* extract that produce a synergistic biological effect.[55]

Mitochondrial damage is an important early event in apoptosis.[56,57] It has been suggested that mitochondrial ROS is a significant mediator of apoptosis. When ROS generation is not controlled and balanced with antioxidant proteins, such as the endogenous and exogenous antioxidant system, ROS accumulation in the mitochondria can lead to depolarization of the mitochondrial membrane. As a result, the permeability transition pore may form, releasing mitochondrial cytochrome c into the cytosol, which activates a series of downstream target proteins and apoptotic events.[58,59] In this study, *L. edodes* treatment stimulated a significant increase in ROS production in HEp-2 cells, in a concentration-dependent manner, when compared with the control cells. Large amounts of intracellular ROS are sufficient to trigger different cellular responses such as senescence, apoptosis, cell cycle arrest, or necrosis, depending on the intensity of oxidative damage.[60] ROS initiate the cell death processes affecting signaling cascade,[61] and tumor necrosis factor (TNF)-α–induced nuclear factor-κB activation can reduce ROS levels[62]; this explains the reduction in ROS at the higher treatment concentration (2 mg/mL) (Fig. 4). To evaluate whether *L. edodes*–induced ROS production in HEp-2 cells could be due to a loss of mitochondrial membrane potential, variations of this potential were explored. The results demonstrated that the number of depolarized mitochondria significantly increased after *L. edodes* treatment compared with the control. Mitochondrial dysfunction and decreased mitochondrial membrane potential are early events preceding phosphatidylserine externalization and caspase activation associated with apoptosis.[56,63] Our results suggest that increased levels of ROS and compromised mitochondrial membrane potential in HEp-2 cells treated with *L. edodes* are crucial events responsible for inducing cell death by apoptosis.

A defective apoptotic pathway, particularly too little apoptosis, is a hallmark of cancer, making it an area of much interest in cancer research. Two major pathways that lead to apoptosis are the extrinsic and intrinsic pathways. The extrinsic pathway occurs via transmembrane receptors that belong to the TNF receptor superfamily, including the Fas cell surface death receptor and the TNF-related apoptosis-inducing ligand receptor. The activation of these transmembrane receptors by their corresponding ligands induces the initiation and activation of procaspase 8. The activated form of the enzyme, caspase 8, initiates apoptosis by activating downstream targets.[64] In contrast, the intrinsic pathway of apoptosis is initiated within a cell. Intense cellular stresses such as DNA damage, growth factor alterations, and oxidative stress (ROS production) are some of the internal stimuli that trigger the intrinsic pathway. This pathway is tightly

regulated by the Bcl-2 superfamily, which includes both proapoptotic and antiapoptotic proteins. Any shift in the balance between these proteins changes mitochondrial permeability and eventually leads to the execution of apoptosis through the release of proapoptotic proteins such as cytochrome c into the cytoplasm, caspase-9 activation, and subsequently the activation of caspase-3 and caspase-7.[65] In this study, the *L. edodes* extract increased the expression of several proteins involved in both the intrinsic and the extrinsic pathways of apoptosis.

The mitochondrial pathway is one of the major mechanisms involved in apoptosis. The Bcl-2 family of proteins is divided into proapoptotic proteins (e.g., Bax and Bid) and antiapoptotic proteins (e.g., Bcl-2). However, our Western blotting results for the lowest treatment concentration (1 mg/mL) indicated an increase in Bcl-2 expression, which was downregulated at the highest treatment concentration (Fig. 8A). In this study, the relative expression of Bax and Bcl-2 increased in HEp-2 cells with *L. edodes* treatment, suggesting increased apoptotic events. However, an increase in cytochrome c was not observed, which may be attributable to the complex mixture of several chemical compounds present in the total extract. That may be regulating cytochrome c level, it suggests that the *L. edodes* extract induces apoptosis through the extrinsic pathway to a greater extent than the intrinsic pathway, because the proteins involved in extrinsic apoptotic pathway, such as Fas, caspase-8, family TNF, TNF-related apoptosis-inducing ligand, and insulin growth factor, were also found to be significantly increased in treated HEp-2 cells.

Increased caspase-8 levels suggest the involvement of an extrinsic pathway of apoptosis. In this study, the levels of TNF-α and TNF-β were significantly increased in HEp-2 cells after treatment with the *L. edodes* extract. TNF-α and TNF-β have been shown to induce apoptosis through activation of caspase 8.[66] However, in almost all cell types, activation of caspase-8 is not sufficient to induce apoptosis. In this study, the caspase pathway was found to be activated by increases in both caspase-8 and caspase-3 protein levels, suggesting that both the intrinsic and the extrinsic pathways were activated by the *L. edodes* extract. According to Han et al.[20] and Rouhana-Toubi et al.,[67] compounds isolated from mushrooms have been shown to alter the mitochondrial pathway machinery of apoptosis by increasing caspase-3 and caspase-9 expression, increasing cytochrome c release, and decreasing Bcl-2.

The findings of this study should be interpreted in light of its limitations. This study examined only a limited number of factors that are affected by or involved in the modulation of the apoptotic pathway in HEp-2 cells by *L. edodes*. Examining a broader range of factors may help further elucidate the role of mitochondrial dysfunction and apoptotic activation induced by the extract. Moreover, we used HEp-2 cells, which are an established model for human laryngeal carcinoma. Examining the effect of the *L. edodes* extract in different human laryngeal carcinoma cells will expand our knowledge regarding the potential effects of these molecules in targeting this particular type of cancer.

V. CONCLUSIONS

Chemical characterization of the *L. edodes* extract showed that the major constituent was GA. We believe that these molecules and all other compounds present in the extract presented, because of their synergism, higher selectivity for HEp-2 tumor cells when compared with the effects on MRC-5 noncancer cells. In addition, the *L. edodes* extract stimulated inhibition of cell cycle progression through molecular events associated with activation of apoptotic machinery in HEp-2 cells. Treatment with the *L. edodes* extract showed an increase in the expression levels of TNF-α and TNF-β, promoting apoptosis by activating caspase-8. It is interesting to note that our results suggest that both the extrinsic and the intrinsic pathways were activated by the extract, inducing cell death along with regulating Bax and caspase-8. These findings are still preliminary, and more experimental support needs to be provided. So far, our results suggest that the polysaccharides/phenolic compounds in *L. edodes* induce direct selective cytotoxicity and

antiproliferative effects in HEp-2 cells in a dose-dependent manner. Furthermore, these results highlight the importance of the molecular changes induced by the *L. edodes* extract, suggesting that its effect might be considered as an anticancer agent for potential development of chemopreventive drugs.

ACKNOWLEDGMENTS

This work was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

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4.2 CAPÍTULO II

Food Chemistry 245 (2018) 150–158

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Multifunctions of *Pleurotus sajor-caju* (Fr.) Singer: A highly nutritious food and a source for bioactive compounds

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ARTICLE INFO

Keywords:
Pleurotus sajor-caju
Nutrients
Extraction optimization
Phenolic acids
Bioactive properties
COX-2

ABSTRACT

A study with *Pleurotus sajor-caju* was conducted to: evaluate the nutritional and chemical composition of the fruiting bodies; optimize the preparation of bioactive phenolic extracts; and characterize the optimized extract in terms of bioactive compounds and properties. *P. sajor-caju* revealed an equilibrated nutritional composition with the presence of hydrophilic (sugars and organic acids) and lipophilic (tocopherols and PUFA) compounds. *p*-Hydroxybenzoic, *p*-coumaric and cinnamic acids were identified in the extract obtained with ethanol (30 g/l ratio) at 55 °C for 85 min. This extract showed antioxidant properties (mainly reducing power and lipid peroxidation inhibition), antibacterial activity against MRSA and MSSA and cytotoxicity against NCI-H460, MCF-7 and HeLa. Furthermore, as the extract showed capacity to inhibit NO production in Raw 264.7 macrophages, molecular docking studies were performed to provide insights into the anti-inflammatory mechanism of action, through COX-2 inhibition by the phenolic acids identified.

1. Introduction

Pleurotus sajor-caju (Fr.) Singer (Pleurotaceae, Basidiomycetes higher) is a tasty mushroom with high nutritional value, therapeutic properties and diverse environmental and biotechnological applications (Han, Ahmad, & Ishak, 2016). It is nutritionally rich in protein, fibre, minerals and vitamins, and is low in calories with very low lipid or starch contents. This mushroom has also been widely used in traditional medicine because of its various bioactive properties, including antiviral (Teplyakova & Kosogova, 2016), antibacterial (Heleno et al., 2015), antifungal (Alves et al., 2013), antiparasitic (Ademola & Odeniran, 2017), antitumor (Ferreira et al., 2015), antioxidant (Ferreira, Barros, & Abreu, 2009), antihypertensive (through active ingredients that affect the renin-angiotensin system) (Chang & Wasser, 2012), antidiabetic, preventing hyperglycemia and insulin resistance (Kanagasabapathy et al., 2012) and anti-inflammatory (Taofiq et al., 2016).

P. sajor-caju has been receiving great interest in research because of its biologically active compounds, such as polysaccharides (e.g. pleurane, a β-glucan), proteoglycans, phenolic acids, terpenes, proteins, and sterols, becoming a source of nutraceuticals (Duru & Cayan, 2015). Phenolic compounds constitute one of the several categories with proven antioxidant effects, scavenging free radicals present in the body. The most abundant phenolic compounds reported in edible mushrooms belongs to the phenolic acids family, such as *p*-coumaric, *p*-hydroxybenzoic and protocatechuic acids (Barros, Duenas, Ferreira, Baptista, & Santos-Buelga, 2009).

The extraction of bioactive compounds depend on the type of solvent, time and temperature used, as well as on the complexity of molecules, and should be optimized to obtain a higher overall efficiency of the target components (Heleno et al., 2016). According to Vongsak et al. (2013), heat assisted extraction (HAE) is the most used method for the extraction of phenolic compounds, which is simpler, more convenient and less costly in terms of instrumentation. However and as previously mentioned, it should be optimized to maximize the extraction of the bioactive compounds, monitoring the extraction yield by applying the Response Surface Methodology (RSM), where the effects of extremely important variables, such as time, temperature and solvent, are considered. The optimized bioactive extracts can be incorporated into supplements, nutraceuticals or functional foods (Milić, Rajković,

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http://dx.doi.org/10.1016/j.foodchem.2017.10.088
Received 10 July 2017; Received in revised form 30 September 2017; Accepted 16 October 2017
Available online 17 October 2017

Stamenković, & Veljković, 2013). Therefore, the aim of the present work was to highlight the diverse functional properties of fresh *P. sajor-caju*, taking advantage of its high nutritional value, as well as used to extract high valuable bioactive compounds for supplements' formulations.

2. Material and methods

2.1. Mushroom sample

Fresh samples of *Pleurotus sajor-caju* (Fr.) Singer (OS-2001) were purchased from a local producer in the South of Brazil. The botanical identification was confirmed by Ronaldo Adolfo Wasum, Professor of the University of Caxias do Sul. The fruiting bodies were dried at 30 °C in an oven, reduced to a fine dried powder (~20 mesh), mixed to obtain homogenous samples and stored in a desiccator, protected from light, until further analysis.

2.2. Standards and reagents

HPLC grade acetonitrile (99.9%) was purchased from Fisher Scientific (Lisbon, Portugal). HPLC grade methanol was obtained from Lab-Scan (Lisbon, Portugal). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), phenolic acid standards (gallic, *p*-hydroxybenzoic, *p*-coumaric, protocatechuic and cinnamic acids), fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U), sugars (D(−)-fructose, D(−)-mannitol, D(+)-raffinose pentahydrate, and D(+)-trehalose), sulforhodamine B, trypan blue, trichloroacetic acid (TCA), tris lipopolysaccharide (LPS) and dexamethasone were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Dulbecco's modified Eagle's medium (HyClone), Hank's balanced salt solution (HBSS) and all the additional culture media components were purchased from Gibco Invitrogen Life Technologies (Paisley, UK). RAW264.7 cells were acquired from ECACC ("European Collection of Animal Cell Culture") (Salisburg, UK), Griess reagent system kit was purchased from Promega (Madison, WI, USA).

The culture media Muller Hinton broth (MHB) and Tryptic Soy Broth (TSB) were obtained from Biomerieux (Marcy l'Etoile, France). Blood agar with 7% sheep blood and Mac Conkey agar plates were purchased from bioMérieux (Marcy l'Etoile, France). The dye *p*-iodonitrotetrazolium chloride (INT) was also purchased from Sigma-Aldrich (St. Louis, MO, USA) and was used as microbial growth indicator. All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI PureWater Systems, Greenville, SC, USA).

2.3. Analysis of the nutritional composition

2.3.1. Nutritional value

Samples were analyzed for macronutrient composition (protein, carbohydrates, fat, moisture and ash) using AOAC procedures (AOAC, 2016). Crude protein content (N × 4.38) was estimated by the macro-Kjeldahl method. Crude fat was determined using a Soxhlet apparatus by extracting a known weight of sample with petroleum ether. The ash content was determined by incineration at 600 ± 15 °C. For moisture the sample was held in the oven to constant weight. Total carbohydrates were calculated by difference and total energy was calculated according to the following equation: Energy (kcal) = 4 × (g protein + g carbohydrates) + 9 × (g fat).

2.3.2. Free sugars

Sugars were analyzed by high performance liquid chromatography coupled to a refractive index detector (HPLC-RI, Knauer, Smartline system 1000; Berlin, Germany), using a previously described procedure (Heleno et al., 2015). The chromatographic separation was achieved with a Eurospher 100-5 NH_2 column (4.6 × 250 mm, 5 µm, Knauer, Berlin, Germany) operating at 30 °C (7971 R Grace oven). The mobile phase was acetonitrile/deionized water, 70:30 (*v/v*) at a flow rate of 1 ml/min. Quantification and identification was based on the RI signal response of each standard, using raffinose as the internal standard (IS), and the results were expressed in g per 100 g of dry weight (dw).

2.3.3. Organic acids

Organic acids were determined by ultra-fast liquid chromatography coupled to a photo diode array detector (UFLC-DAD), following a previously described methodology (Heleno et al., 2015). Separation was achieved on a SphereClone reverse phase C_{18} column (4.6 × 250 mm, 5 µm, Phenomenex, Torrance, CA, USA) thermostatted at 35 °C. The elution was performed with sulphuric acid (3.6 mM) using a flow rate of 0.8 ml/min. The identification was performed by comparing relative retention times and UV spectra with commercial standards. Quantification was accessed by area comparison of the peaks recorded at 215 nm with calibration curves obtained from commercial standards and results were expressed in g per 100 g dw.

2.3.4. Fatty acids

Fatty acids (obtained after Soxhlet extraction) were determined by gas chromatography with flame ionization detection (GC-FID)/capillary column, following a methodology previously described by Heleno et al. (2015). Separation was achieved in a Macherey–Nagel column (50% cyanopropyl-methyl-50% phenylmethylpolysiloxane, 30 m × 0.32 mm i.d. × 0.25 µm d_f, Düren, Germany). The oven temperature program was as follows: the initial temperature of the column was 50 °C, held for 2 min, then a 30 °C/min ramp to 125 °C, 5 °C/min ramp to 160 °C, 20 °C/min ramp to 180 °C, 3 °C/min ramp to 200 °C, 20 °C/min ramp to 220 °C and held for 15 min. The carrier gas (hydrogen) flow-rate was 4.0 ml/min (0.61 bar), measured at 50 °C. Split injection (1:40) was carried out at 250 °C. Fatty acid identification was made by comparing the relative retention times of FAME peaks from samples with standards. Fatty acids were processed using Clarity 4.0.1.7 Software (DataApex, Podohradska, Czech Republic), identification was performed by comparing the relative retention times from samples with standards and results were expressed in relative percentage (%) of each fatty acid.

2.3.5. Tocopherols

Tocopherols were determined using a HPLC system (Smartline, Knauer, Germany), coupled to a fluorescence detector (FP-2020; Jasco, Japan) programmed for excitation at 290 nm and emission at 330 nm, following a procedure previously described (Heleno et al., 2015). The chromatographic separation was achieved with a Polyamide II (4.6 × 250 mm, 5 µm, YMC Waters, Lisbon, Portugal) normal-phase column, operating at 30 °C. The mobile phase used was a mixture of *n*-hexane and ethyl acetate (70:30, *v/v*) at a flow rate of 1 ml/min. Data was processed using Clarity 2.4 Software (DataApex). Compounds were identified by chromatographic comparisons with commercial standards and quantification was based on the fluorescence signal response, using an internal standard method. Tocopherols content in mushrooms were expressed in µg per 100 g dw.

2.4. Solid-liquid extraction of the P. sajor-caju samples. Heat assisted extraction (HAE)

HAE is the conventional solid-liquid extraction method for researcher and industries to obtain compounds from plant matrices by stirring the sample in a solvent for a certain time and at a specific temperature (Heleno et al., 2016). The solid-liquid heat assisted extractions were performed by placing the dry powdered samples (600 mg) in an extraction vial with 20 ml of solvent at a fixed solid/liquid ratio (30 g/l) and placed in a thermostatic water bath under continuous electro-magnetic stirring (500 rpm, CIMAREC Magnetic Stirrer) at each of the given *t*, *T* and *S* conditions defined by the RSM design (Table A1).

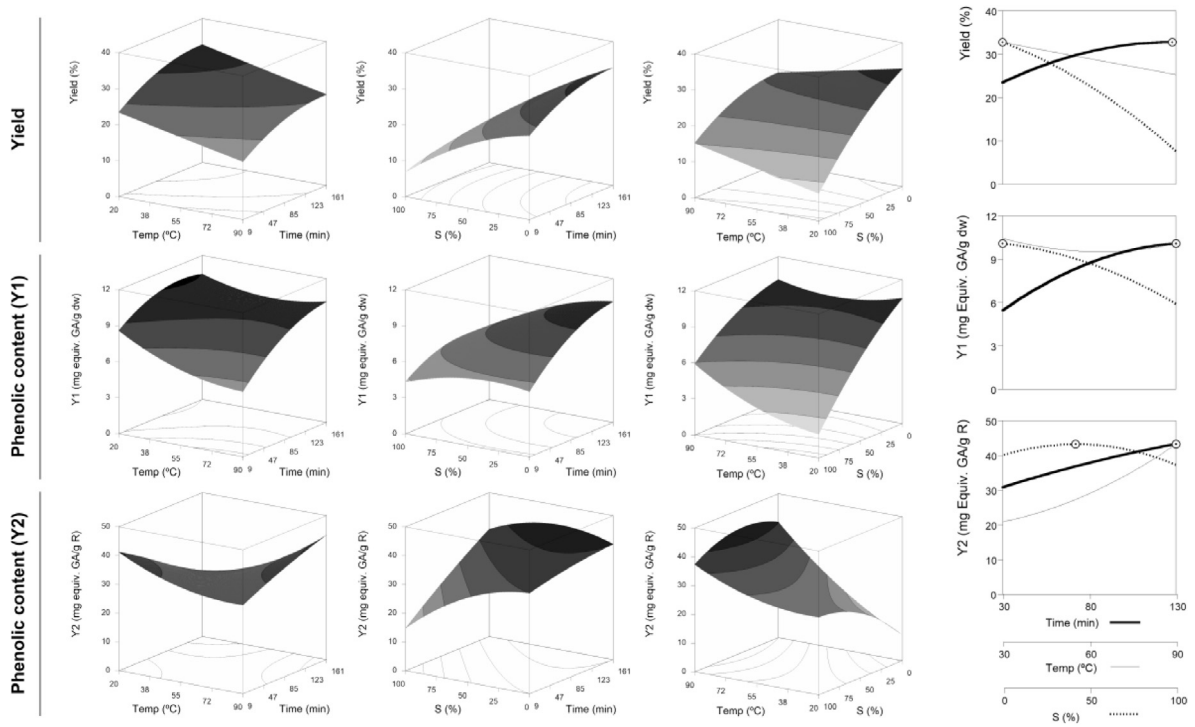

Fig. 1. The left figure shows an illustration of the joint graphical 3D analysis in terms of the extraction behaviour for each of the response criteria assessed. The right figure shows the final summary of the effects of all variables assessed in a 2D format, in which the dots (⊙) presented alongside each line highlight the location of the optimum value. Lines, dots and each one of the net surfaces are generated by the theoretical second order polynomial models of Eqs. (2), (3) and (4).

2.5. Optimization of the conditions to extract bioactive phenolics

2.5.1. Experimental design

Variables and ranges were selected by preliminary analysis. Through the analysis of these experimental results (data not shown), time (t, from 10 to 160 min), temperature (*T*, from 20.0 to 90.0 °C) and water-ethanol solvent proportions (*S*, from 0 to 100%) were chosen as the relevant variables and ranges for the RSM design. The combined variables effect on the HAE were studied using a *circumscribed central composite design* (*CCCD*) with five levels for each one (Table A1).

2.5.2. Responses criteria used to evaluate the extraction process

After the extraction at each given condition, the mixture was filtered (Whatman n° 4 paper) and centrifuged (4800*g* for 10 min). The pellet was discarded and the supernatant was collected and divided in two parts to analyze separately the main responses for the optimization procedure:

- One part was used to quantify the yield of residual material extracted (R) expressed as percentage (%) of the *P. sajor-caju* dry material used;
- The second part of the supernatant was evaporated, lyophilized, and then employed to quantify spectrophotometrically (UV-160A; Shimadzu Corporation, Kyoto, Japan) the phenolics. The phenolic content (Ph) was estimated by a colourimetric procedure. Briefly, a *P. sajor-caju* extract solution (1 ml) in the concentration of 30 g/l was mixed with Folin-Ciocalteu reagent (5 ml, previously diluted with water 1:10, v/v) and sodium carbonate Na_2CO_3 (75 g/l, 4 ml) in triplicate. The tubes were vortex for 15 s and allowed to stand for 30 min at 40 °C for colour development. Absorbance was then measured at 765 nm. Gallic acid (9.4–150 μg/ml) was used to obtain the standard curve. Results were expressed as mg of gallic acid equivalents per g of *P. sajor-caju* dw (mg GAE/g dw).

Therefore, three responses are used as criterion for optimization purposes: 1) the R yield extracted (in% and described as *yield*); 2) the phenolic content in the *P. sajor-caju* dry material (in mg GAE/g dw and described as *Ph-Y_1*); and 3) an additional response that depends on the other two responses can be computed for the phenolic content in the R (in mg GAE/g R and described as *Ph-Y_2*).

2.5.3. Mathematical model development and statistical assessment

All analysis were performed in *Microsoft Excel*. Models were fitted by the '*Solver*' macro by minimization of the sum of quadratic differences between observed and model-predicted values using the following second-order polynomial equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (1)$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables. The parametric confidence intervals were determined via '*SolverAid*' macro (Prieto, Curran, Gowen, & Vázquez, 2015) and final model reliability was confirmed by applying the following criteria: a) the Fisher *F*-test ($\alpha = 0.05$) to test model consistency; b) assessment of the parameter and model prediction uncertainties by the '*SolverStat*' macro (Prieto, Murado, & Vázquez, 2014); and c) R2 coefficient to explain the proportion variability of the dependent variable obtained by the model.

2.5.4. Optimization procedure of the variables to maximize the responses

The second-order polynomial model produced for each of the responses was used for variables optimization and response maximization by a simplex method using the '*Solver*' macro *in Microsoft Excel* (Albuquerque et al., 2017). Limitations were made to the variable coded values to avoid unnatural conditions (*i.e.*, times lower than 0).

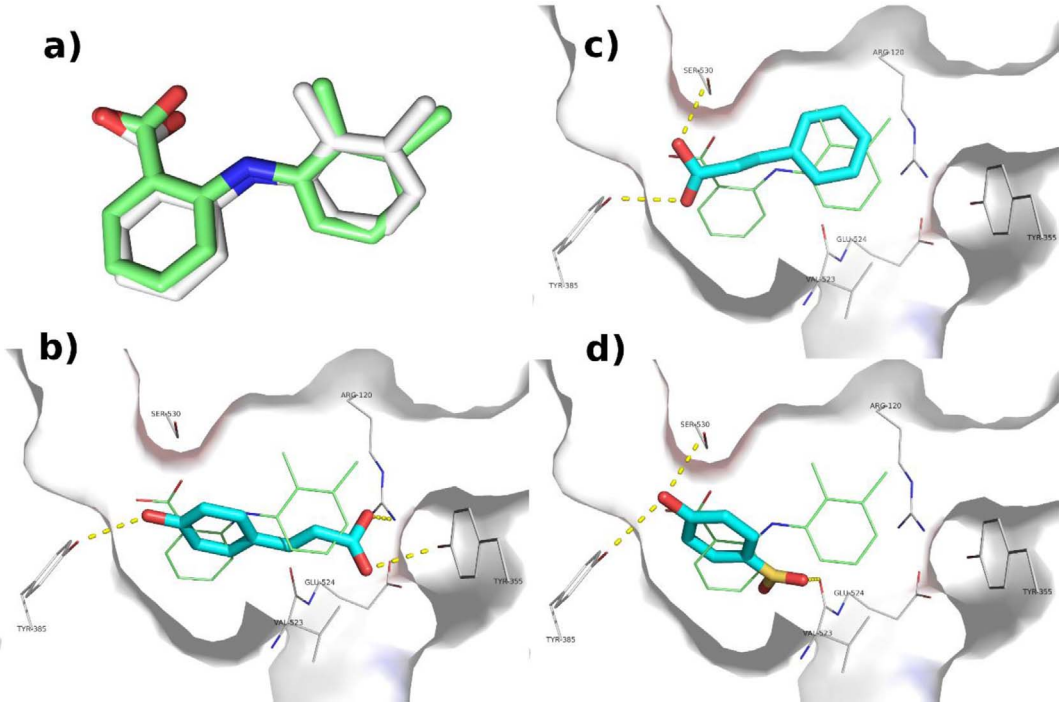

Fig. 2. Docking conformation of the phenolic acids against COX-2 structure: a) superimposition between mefenamic acid experimental representation (green colour) and docked conformation (white colour); docked conformation of (b) cinnamic acid, (c) *p*-coumaric acid and (d) *p*-hydroxybenzoic acid, (sticks and balls; cyan colour). For comparison, the experimental representation of mefenamic acid is also included (wire representation; green colour). Discovered H-bonds (trace lines, yellow colour) and key COX-2 residues are also presented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Chemical characterization of the optimized extract

The optimized conditions of the extraction procedure that maximize the response criteria used were dissolved in 100% ethanol (20 mg/ml), filtered through a 0.22 μm nylon syringe filter and further analyzed by HPLC-DAD-ESI/MSn on a Dionex Ultimate 3000 UPLC (ThermoScientific, San Jose, CA, USA) system following a procedure previously reported by Svobodova et al. (2017). The equipment consisted of a diode array detector coupled to an electrospray ionization mass detector, a quaternary pump, an auto-sampler (kept at 5 °C), a degasser and an automated thermostated column section (kept at 35 °C). Waters Spherisorb S3 ODS-2 C_{18} (3 μm, 4.6 × 150 mm, Waters, Milford, MA, USA) column provided chromatographic separations. 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 were collected simultaneously with DAD (280 and 370 nm) and in negative mode detection on a Linear Ion Trap LTQ XL mass spectrometer (ThermoScientific, San Jose, CA, USA). Sheath gas (nitrogen) was kept on 50 psi. Other parameters settings: source temperature 325 °C, spray voltage 5 kV, capillary voltage −20 V, tube lens offset −66 V, collision energy 35 arbitrary units. The full scan captured the mass between *m/z* 100 and 1500. Xcalibur® data system (ThermoScientific, San Jose, CA, USA) was operating the data acquisition. For identification, retention times, UV–VIS and mass spectra were compared with available standards and data from literature were used to tentatively identify the remaining compounds. Calibration curves of available phenolic standards were constructed based on the UV signal to perform quantitative analysis. The results were expressed as μg/g of extract.

2.7. Bioactive properties of the optimized extract

The extract obtained in the optimized conditions of the extraction procedure that maximize the response criteria used were used to evaluate the bioactive properties

2.7.1. General preparation of stock solutions for bioactivity analysis

The optimized extract was re-dissolved in ethanol/water (80:20, v/v) and water at 20 and 100 mg/ml for antioxidant and antimicrobial assays, respectively. For cytotoxicity and anti-inflammatory activity evaluation, the extract was re-dissolved in water, with a final solution of 8 mg/ml. The stock solutions were diluted to different concentrations to be submitted to the following assays.

2.7.2. Antioxidant activity

The antioxidant activity was evaluated by DPPH radical-scavenging activity, reducing power, inhibition of β-carotene bleaching in the presence of linoleic acid radicals and inhibition of lipid peroxidation using TBARS in brain homogenates. The extract concentrations providing 50% of antioxidant activity or 0.5 of absorbance (EC_{50}) were calculated from the graphs of antioxidant activity percentages (DPPH, β-carotene bleaching and TBARS assays) or absorbance at 690 nm (reducing power assay), against extract concentration (Svobodova et al., 2017). Trolox was used as positive control.

2.7.3. Cytotoxic activity in tumour and non-tumour cells

The cytotoxicity was determined using four human tumour cell lines, HeLa (cervical carcinoma), HepG2 (hepatocellular carcinoma), MCF-7 (breast adenocarcinoma) and NCI-H460 (non-small cell lung cancer). The cell growth inhibition was measured using sulforhodamine B (SRB) assay, where the amount of pigmented cells is directly proportional to the total protein content and, therefore, to the number of bound cells (Svobodova et al., 2017). For hepatotoxicity evaluation, a freshly harvested porcine liver, obtained from a local slaughter house, was used in order to obtain the cell culture, designated as PLP2. The results were expressed in GI_{50} values; sample concentration that inhibited 50% of the net cell growth and ellipticine was used as positive control.

2.7.4. Antibacterial activity

Bacteria strains were clinical isolates obtained from patients hospitalized

Table 1
Nutritional composition of *P. sajor caju* fruiting bodies expressed in dry weight basis (mean ± SD).

Nutritional value	
Moisture (g/100 g)	91.06 ± 0.09
Ash (g/100 g)	5.4 ± 0.5
Proteins (g/100 g)	17.29 ± 0.06
Fat (g/100 g)	1.16 ± 0.03
Carbohydrates (g/100 g)	76.2 ± 0.4
Energy (kcal/100 g)	384 ± 2
Sugars Content (g/100 g)	
Mannitol	3.2 ± 0.2
Trehalose	10.1 ± 0.5
Total sugars	13.3 ± 0.7
Organic acids Content (g/100)	
Oxalic acid	1.18 ± 0.05
Malic acid	1.80 ± 0.08
Fumaric acid	0.37 ± 0.03
Total organic acids	3.4 ± 0.2
Tocopherols (μg/100 g)	
α-Tocopherol	3.4 ± 0.8
β-Tocopherol	682 ± 6
δ-Tocopherol	19 ± 3
Total tocopherols	704 ± 3
Fatty acids (%)	
C6:0	0.24 ± 0.04
C8:0	0.080 ± 0.001
C10:0	0.042 ± 0.004
C12:0	0.115 ± 0.002
C14:0	0.45 ± 0.04
C14:1	0.241 ± 0.007
C15:0	2.48 ± 0.03
C16:0	17.17 ± 0.04
C17:0	0.477 ± 0.007
C18:0	3.79 ± 0.07
C18:1n9	16.11 ± 0.03
C18:2n6	53.7 ± 0.2
C18:3n6	0.101 ± 0.007
C18:3n3	0.084 ± 0.002
C20:1	0.49 ± 0.02
C20:2	0.42 ± 0.04
C20:3n3 + C21:0	0.276 ± 0.004
C20:5n3	0.10 ± 0.01
C22:0	1.39 ± 0.09
C22:1n9	0.163 ± 0.006
C23:0	0.32 ± 0.01
C24:0	0.73 ± 0.06
C24:1	1.05 ± 0.01
SFA	27.3 ± 0.2
MUFA	18.05 ± 0.01
PUFA	54.7 ± 0.2

Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Lauric acid (C12:0); Myristic acid (C14:0); myristoleic acid (C14:1); Pentadecanoic acid (C15:0); Palmitic acid (C16:0); Heptadecanoic acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); γ-Linolenic acid (C18:3n6); α-Linolenic acid (C18:3n3); Eicosenoic acid (C20:1); Eicosadienoic acid (C20:2); Eicosatrienoic acid (C20:3n3); Eicosapentaenoic acid (C20:5n3); Behenic acid (C22:0); Docosenoic acid (C22:1n9); Tricosanoic acid (C23:0); Lignoceric acid (C24:0); Tetracosenoic acid (C24:1). SFA-Saturated fatty acids; MUFA-Monounsaturated fatty acids; PUFA-Polyunsaturated fatty acids.

in various departments at the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Four Gram-positive bacteria (*Enterococcus faecalis* isolated from urine, *Listeria monocytogenes* isolated from cerebrospinal fluid, MSSA: methicillin-sensitive *Staphylococcus aureus* isolated from wound exudate and MRSA: methicillin-resistant *Staphylococcus aureus*, isolated from expectoration), and six Gram-negative bacteria (*Pseudomonas aeruginosa* isolated from expectoration, *Escherichia coli, Escherichia coli* spectrum extended producer of β-lactamases (ESBL), *Klebsiella pneumoniae, Klebsiella pneumoniae* ESBL and *Morganella morganii*, all isolated from urine). All the tested bacteria were placed to grow in the appropriate fresh medium, 24 h, and kept in the oven at 37 °C before analysis in order to maintain the exponential growth phase (Svobodova et al., 2017).

The determination of the minimal inhibitory concentration (MIC) was performed on a 96-well microplate by the method *p*-iodonitrotetrazolium chloride (INT) colourimetric assay (Svobodova et al., 2017). Initially, 40 μl of the extract solution was added to 150 μl of medium TSB or MHB, according to the bacteria requirements. Afterwards, 10 μl of inoculum (1.5×10^8 CFU/ml) was added to all 96-wells thus achieving a concentration range of 20–0.156 mg/ml. The plates were then incubated at 37 °C, for 24 h, in an oven (Jouan, Berlin, Germany). The MIC of the samples was determined after adding INT (0.2 mg/ml) and after incubation at 37 °C for 30 min. Viable microorganisms reduced the yellow dye to a pink colour. MIC was defined as the lowest extract concentration that prevented this change, thus showing complete inhibition of bacterial growth.

Microorganism's identification and susceptibility tests were performed using the MicroScan panels (MicroScan®; Siemens Medical Solutions Diagnostics, West Sacramento, CA, USA) by the microdilution method (Svobodova et al., 2017). The interpretation criteria were based on Interpretive Breakpoints as indicated in Clinical and Laboratory Standards Institute (CLSI, 2008) and in the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2013).

2.7.5. Anti-inflammatory activity

2.7.5.1. NO production inhibition. The LPS-induced NO production by Murine macrophage (RAW 264.7) cell lines was determined as nitrite concentration in the culture medium (Svobodova et al., 2017). Dexamethasone (50 μM) was used as a positive control. The mouse macrophage-like cell line RAW 264.7 stimulated with LPS was used in the assay. Nitric oxide (NO) production was studied with Griess Reagent System kit. Results were expressed as IC_{50} values (μg/ml) equal to the sample concentration providing a 50% inhibition of NO production. Dexamethasone was used as positive control.

2.7.5.2. Molecular docking. The selected COX-2 crystal structure was obtained from the Protein Data Bank (PDB entry: 5KIR). The co-crystallized ligand, in this case mefenamic acid, was separated from the corresponding protein structure and used to perform the docking validation. All crystallized water molecules were removed from the structure. AutoDockTools1.5.2 (ADT) (Morris, Huey, & Olson, 2008) was then used to assign polar hydrogens, add Gasteiger charges and save the protein structure in PDBQT file format. The docking grid was defined using ADT. The X,Y,Z center coordinates and X,Y,Z grid dimensions were selected and used for all docking simulations. The 2D structure of the three studied compounds (*p*-hydroxybenzoic, *p*-coumaric and cinnamic acids), were drawn using ACD/ChemSketch Freeware 12.0 software. VegaZZ software was then used to perform 2D to 3D conversion and to save the structures in PDB file format. Finally, ADT was used to convert PDB to PDBQT file format. All docking simulations were performed using AutoDock Vina software (Trott & Olson, 2010), using an exhaustiveness parameter of 16. Docking conformation analysis and image preparation was performed using PyMOL software (Delano, 2002). Autodock Vina was subsequently used to perform all docking simulations for each structure in the co-crystallized ligands, and with the necessary dimensions to encompass the ligand binding site. All figures with structure representations were produced using PyMOL.

3. Results and discussion

3.1. Nutritional value of the fruiting bodies

Nutritional composition and energetic contribution of *Pleurotus sajor-caju* (PSC) are shown in Table 1. The overall macronutrients balance is in accordance with literature (Obodai et al., 2014) reported a moisture value of 90 g/100 g, while described 89.58 g/100 g. The fat content found was also according to Goyal, Grewal, and Goyal (2006) (1.96 g/100 g). According to Gogavekar et al. (2014), the carbohydrates content mainly includes glucose,

Table 2
Experimental RSM results of the *CCCD* for the optimization of the three main variables involved *t* (X_1), *T* or *P* (X_2) and *S* (X_3) for the three response value formats assessed (*yield*, *Ph-Y*$_1$ and *Ph-Y*$_2$). Parametric results of the second-order polynomial equation of Eq. (1) in terms of the extraction behaviour of the three response value formats (*yield*, *Ph-Y*$_1$ and *Ph-Y*$_2$). The parametric subscript 1, 2 and 3 stands for the variables involved *t* (X_1), *T* or *P* (X_2) and *S* (X_3), respectively. Analysis of significance of the parameters ($\alpha = 0.05$) are presented in coded values.

Variable coded values			Experimental responses		
X_1	X_2	X_3	*Yield*	*Ph-Y*$_1$	*Ph-Y*$_2$
−1	−1	−1	21.67	7.24	33.40
1	−1	−1	53.83	8.56	15.90
−1	1	−1	18.50	5.02	27.13
1	1	−1	22.83	8.03	35.16
−1	−1	1	18.33	5.99	32.66
1	−1	1	19.67	6.94	35.28
−1	1	1	18.17	5.99	32.97
1	1	1	21.73	7.21	33.20
−1.68	0	0	18.50	6.03	32.60
1.68	0	0	20.83	6.33	30.40
0	−1.68	0	20.00	6.68	33.39
0	1.68	0	24.33	9.08	37.30
0	0	−1.68	31.83	11.40	35.81
0	0	1.68	7.33	2.56	34.87
0	0	0	21.83	6.82	31.24
0	0	0	22.83	6.83	29.89
0	0	0	22.67	7.18	31.69
0	0	0	24.17	7.46	30.85
0	0	0	22.00	7.28	33.08
0	0	0	22.00	7.49	34.05

Parametric responses

Fitting coefficients		Yield	Ph-Y_1	Ph-Y_2
Intercept	b_0	22.58 ± 1.10	7.17 ± 0.64	31.60 ± 1.87
Linear effect	b_1	1.45 ± 0.26	0.51 ± 0.03	−0.76 ± 0.16
	b_2	*ns*	*ns*	1.30 ± 0.26
	b_3	−4.00 ± 0.86	−1.42 ± 0.03	*ns*
Quadratic effect	b_{11}	−0.86 ± 0.13	−0.34 ± 0.02	*ns*
	b_{22}	*ns*	0.26 ± 0.02	1.11 ± 0.11
	b_{33}	−0.89 ± 0.23	−0.25 ± 0.02	−1.56 ± 0.11
Interactive effect	b_{12}	*ns*	*ns*	2.89 ± 0.13
	b_{13}	−0.77 ± 0.13	*ns*	1.54 ± 0.17
	b_{23}	1.33 ± 0.13	0.38 ± 0.06	−1.85 ± 0.19

trehalose, mannitol, glycogen and some other polysaccharides with recognized therapeutic effects. Gupta, Sharma, Saha, and Walia (2013), also described mannitol and trehalose as the major sugars in this mushroom species. Malic, oxalic and fumaric acids were the organic acids found in PSC, with the prevalence of the malic acid (Table 1). However, the profile of organic acids described by Obodai et al., 2014 was slightly different since the authors did not detect malic acid. Twenty-three fatty acids were quantified in PSC and the most abundant was linoleic acid (C18:2n6). A prevalence of PUFA over SFA was observed and the global percentages obtained are very similar to those described in other studies (Kayode et al., 2015; Obodai et al., 2014). Saturated fatty acids (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) are shown in Table 1. According to Khan and Tania (2012), many environmental factors affect the lipid content, that is naturally low in mushrooms. Nevertheless, they are sources of some essential fatty acids for the human body. PSC also contains tocopherols, mainly β-tocopherol; γ-tocopherol was not found, which is in agreement with the results described for other Pleurotus species (Yang, Lin, & Mau, 2002). Tocopherols are natural antioxidants because they act as free peroxyl radical scavengers.

3.2. Optimized conditions to extract bioactive phenolics

Heat assisted extraction (HAE) is a conventional solid-liquid extraction method frequently used to obtain compounds from plant matrices. It is a simple technique with lower requirements in terms of equipment, but very often involves long time periods and high temperatures. The extraction of target compounds from matrices requires a specific consideration due to the intrinsic nature and stability of these target compounds (Heleno et al., 2016). Therefore, to maximize the response extraction of these compounds, it is essential to identify the response effects caused by the main variables involved using the minimum time, energy and solvent consumption and designing the most cost-effective and profitable extraction system. The RSM design allows optimizing all the variables simultaneously considering interactive reactions and to predict the most efficient conditions (Albuquerque et al., 2017).

3.2.1. Mathematical models derived from the RSM for a CCCD with three variables and statistical assessment

The results obtained by the statistical *CCCD* are shown in the first part of Table 2 for each one of the response criteria used. By fitting the second-order polynomial model of Eq. (1) to the obtained responses using nonlinear least-squares estimations, the parametric values are obtained and presented in Table 2. Therefore, the resulting models for each response format are as follows:

$$Y_{Yield} = 22.58 + 1.45t - 4.0S - 0.86t^2 - 0.89S^2 - 0.77tS + 1.33TS \quad (2)$$

Table 3
Operating optimal conditions of the variables involved that maximize the response values of the RSM in individual, relative and global terms. Response criteria comprise the following: 1) % yield of extraction of the residue (R); and 2) spectrophotometric quantification of total phenolic (Ph) in both response format values (*Ph-Y_1* and *Ph-Y_2*).

Criteria	Optimal variable conditions			Optimum response	
	X_1: t (min)	X_2: T (°C)	X_3: S (%)		
A) Individual optimal variable conditions					
Yield	157.4	20.0	0.0	32.75	%
Ph-Y_1	160.7	90.0	0.0	10.08	mg GAE/g dw
Ph-Y_2	160.7	90.0	42.1	43.30	mg GAE/g R
B) Relative optimal variable conditions using response analysis of pH					
Yield	157.4	20.0	0.0	32.75	%
Ph-Y_1	160.7	90.0	15.6	9.8	mg GAE/g dw
Ph-Y_2				42.0	mg GAE/g R
C) Global optimal variable conditions					
Yield	140.6	90.0	72.4	20.61	%
Ph-Y_1				7.7	mg GAE/g dw
Ph-Y_2				39.7	mg GAE/g R

$$Y_{Ph-Y_1} = 7.17 + 0.51t - 1.42S - 0.34t^2 + 0.26T^2 - 0.25S^2 - 0.38TS \quad (3)$$

$$Y_{Ph-Y_2} = 31.6 - 0.76t + 1.3T + 1.11T^2 - 1.56S^2 + 2.89tT + 1.54tS - 1.85TS \quad (4)$$

Those coefficients, which showed confidence interval values ($\alpha = 0.05$) higher than the parameter value, were consider as non-significant (ns) and were not used for the model development.

Overall, the response patterns for the three response criteria formats (*yield*, *Ph-Y_1* and *Ph-Y_2*) show a relatively high complexity of the possible sceneries. For each response criteria, the linear and quadratic effects are found to play an important and significant role, meanwhile regarding the interactive effects, only the *Ph-Y_2* response showed significant effects for all the possible combinations.

Fig. 1, part A, shows the extraction results for the three response criteria formats used. The 3D figures are organized in three rows, each row contains each one of the responses obtained. Each row shows the 3D surface plots for the three possible variable combinations produced by the described Eqs. (2), (3) and (4). The binary action between variables is presented when the excluded variable is positioned at the center of the experimental domain (see Table A1, Supplementary material). In almost all combinatory 3D responses of Fig. 1, the responses increases to an optimum value and then decreases as a function of each of the assessed independent variables. Therefore, in almost all combinations, an absolute optimum can be found at one single point along with the response, allowing computing the conditions that lead to the absolute maximum.

In statistical terms, the tests used to assess the competence of the models showed that the non-significant parameters of both RSM approaches (Table 2) did not improve the reached solution and, in contrast, all significant parameters were highly consistent ($p < .01$). This was also verified by the achieved high R^2 and R^2_{adj} values, indicating the percentage of variability explained by the model. The distribution of the residuals presented in was arbitrarily around zero and no group of values or autocorrelations were observed. Additionally, the agreement between the experimental and predicted values implies an acceptable explanation of the results obtained by the independent variables used. Therefore, the models developed in Eqs. (2), (3) and (4) are completely functional and adequate to be used for prediction and process optimization.

3.2.2. Numerical optimal conditions that maximize the extraction

Once the models are validated, the numerical optimal conditions that maximize the extraction can be assessed. By applying a simple procedure inserting restrictions to the experimental ranges, the optimal conditions that maximize the responses criteria individually, relative or globally are achieved and presented in Table 3. The values found are discussed next:

- The optimal individual variable conditions that maximize each of the response criteria used were: for the yield 157.4 min, 20.0 °C and 0.0% of S (or 100% of water) producting 32.75% of R; for the *Ph-Y_1* 160.7 min, 90.0 °C and 0.0% of S producting 10.08 mg GAE/g dw; and for the *Ph-Y_2* 160.7 min 90.0°C and 42.1% of S producting 43.30 mg GAE/g R.
- The relative optimal variable conditions for the response analysis of the Ph content were: for the yield 160.7 min, 90.0 °C and 15.6% of S producting 9.8 mg GAE/g dw (*Ph-Y_1*) and 42.0 mg GAE/g R (*Ph-Y_2*).
- The global optimal variable conditions for all the response criteria were 140.6 min, 90.0 °C and 72.4% of S producing 20.61% (yield), 7.7 mg GAE/g dw (*Ph-Y_1*) and 39.7 mg GAE/g R (*Ph-Y_2*).

In all cases, the conditions that lead to the optimal values were experimentally tested in order to ensure the accuracy of the presented results. Fig. 1, part B, shows the summarized individual 2D responses as a function of the defined variables to illustrate the most favourable conditions. The line represents the variable response pattern when the others are located at the optimal global conditions (presented in the third part of Table 3). The dots (⊙) presented alongside the line highlight the location of the optimal value. When combining, the information produced by the three responses criteria (*yield*, *Ph-Y_1* and *Ph-Y_2*), the complete behaviour of each relevant variable influencing the responses is defined in precise form regarding the Ph extraction from *P. sajor-caju*.

3.3. Chemical characterization of the optimized extract

PSC extract obtained in the optimized conditions was analyzed using HPLC-DAD-ESI/MS. *p*-Hydroxybenzoic, *p*-coumaric and cinnamic acids were identified in the extract at the following concentrations, respectively: 66 ± 4, 43.7 ± 0.9 and 37.5 ± 0.3 μg/g extract (Table A2). The presence of these compounds was reported in many other mushrooms species (Nattoh, Musieba, Gatebe, & Mathara, 2016). However, according to Carrasco-González, Serna-Saldívar, and Gutiérrez-Uribe (2017), mushrooms' chemical composition varies drastically with the substrate where they grow.

3.4. Bioactive properties of the optimized extract

3.4.1. Antioxidant activity

Four *in vitro* assays were used to evaluate the antioxidant properties of the PSC extract: DPPH scavenging activity, reducing power, β-

Table 4
Antioxidant, cytotoxic, antibacterial and anti-inflammatory activities of *P. sajor-caju* (mean ± SD).

Antioxidant EC_{50} values (mg/ml)[A]		**Antimicrobial** MIC values (mg/ml)	
DPPH scavenging activity	13.3 ± 0.3	**Gram negative bacteria**	
Reducing power	2.06 ± 0.08	*Escherichia coli ESBL*	> 20
β-carotene bleaching inhibition	8.3 ± 0.4	*Escherichia coli*	> 20
TBARS	2.61 ± 0.08	*Klebsiella pneumonia*	> 20
Cytotoxic GI_{50} values (μg/ml)[B]		*Klebsiella pneumoniae ESBL*	> 20
MCF-7 (breast carcinoma)	250 ± 22	**Gram positive bacteria**	
NCI-H460 (non-small cell lung carcinoma)	246 ± 18	*Enterococcus faecalis*	> 20
HeLa (cervical carcinoma)	308 ± 22	*Listeria monocytogenes*	> 20
HepG2 (hepatocellular carcinoma)	> 400	*Pseudomonas aeruginosa*	> 20
Hepatotoxic GI_{50} values (μg/ml)[B]		*Morganela Morganii*	> 20
PLP2	> 400	MRSA	10
Anti-inflammatory IC_{50} value (μg/ml)[C]		MSSA	10
Raw 264.7 macrophages (NO inhibition)	339 ± 15		

EC_{50} values correspond to the extract concentration achieving 50% of antioxidant activity or 0.5 of absorbance in reducing power assay. GI_{50} values correspond to the extract concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2. IC_{50} value corresponds to the extract concentration responsible for 50% inhibition of NO production. A – Trolox EC_{50} values: 62.98 μg/ml (DDPH); 45.71 μg/ml (reducing power); 10.25 μg/ml (β-carotene bleaching inhibition). B – Ellipticine GI_{50} values: 1.21 mg/ml (MCF-7); 1.03 mg/ml (NCI-H460); 0.91 mg/ml (HeLa); 1.10 mg/ml (HepG2) and 2.29 mg/ml (PLP2). C – Dexamethasone IC_{50} value = 16 ± 2 μg/ml (Raw 264.7). MIC values correspond to the minimal sample concentration that inhibited the bacterial growth.

carotene bleaching and lipid peroxidation (TBARS) inhibition; the results are shown in Table 4. The lowest EC_{50} values (highest antioxidant activity) were obtained in the reducing power and TBARS assays. According to Ferreira et al. (2009), the secondary metabolites produced by mushrooms, including phenolic acids, have a strong ability as free radical scavengers. The bioactivity of these compounds is correlated to their chemical structure consisting of an aromatic ring with hydroxyl substituents (Reis, Martins, Barros, & Ferreira, 2012). Therefore, PSC extract can help in the control of oxidative damage caused by free radicals.

3.4.2. Cytotoxicity in tumour and non-tumour cells

The results of the cytotoxicity of the PSC extract on human tumour cell lines and on non-tumour cells are shown in Table 4. The extract was able to inhibit the cell growth of NCI-H460 (non-small cell lung carcinoma), MCF-7 (breast carcinoma) and HeLa (cervical carcinoma), but did not have effects on HepG2 cell line (hepatocellular carcinoma). Furthermore, the extract did not reveal toxicity for non-tumour cells (primary PLP2), up to the maximal concentration tested (400 μg/ml). Some studies have also demonstrated that PSC methanolic extract had effects on U937 (Panthong, Boonsathorn, & Chuchawankul, 2016) and sarcoma 180 (Assis et al., 2013).

3.4.3. Antibacterial activity

Mushrooms have been reported to have higher activity against Gram-positive bacteria (Reis, Martins, Vasconcelos, Morales, & Ferreira, 2017). Our study confirmed this information, as the extract was only active against MRSA and MSSA with MIC values of 10 mg/ml (Table 4). Nonetheless, Ahmad et al. (2012) reported antimicrobial activity of PSC ethanolic extracts against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *B. atrophaeus*, *Staphylococcus aureus* and *Klebsiella pneumoniae*, while Gogavekar et al. (2014) described the activity of methanolic extracts against *E. coli*, *Salmonella typhimurium*, *B. cereus*, *S. aureus* and *Micrococcus luteus*. However, it should be highlighted that the tested bacteria are collection microorganisms and not clinical isolates, as the ones used in the present study.

3.4.4. Anti-inflammatory activity

PSC extract was able to reduce NO production in murine RAW 264.7 macrophage cells (Table 4). Similar results were observed for other *Pleurotus* species (Carrasco-González et al., 2017).

Recently, the interaction mechanism of several phenolic acid derivatives (anthranilic acid derivatives) with COX-2, a known anti-inflammatory target, was elucidated (Orlando & Malkowski, 2016). Hypothesizing that the anti-inflammatory activity observed could be related to COX-2 inhibition, we perform *in silico* docking studies of the three major phenolic acids, found in *P. sajor-caju*, against COX-2. An initial docking study was performed, using the co-crystallized mefenamic acid against the selected PDB structure (PDB entry: 5KI5). A near perfect superimposition between the predicted and the experimental mefenamic acid structure was observed (Fig. 2a), demonstrating that the docking methodology used was adequate for this study. We subsequently performed docking simulations using the three major phenolic acids and a possible COX-2 mechanism of inhibition is presented (Fig. 2b-c). The predicted interaction between cinnamic acid and COX-2 is mainly stabilized by two hydrogen bonds (H-bonds) between the carboxyl group of cinnamic acid and two COX-2 residues (Tyr385 and Ser530), while the phenyl ring occupies the same interaction space as mefenamic acid (Fig. 2b). *p*-Coumaric acid is also predicted to form an H-bond between its hydroxyl group and Tyr385 while also forming a second H-bond with Tyr355. Finally, *p*-hydroxybenzoic acid is predicted to form H-bonds with Tyr385 and Ser530, while also forming a third H-bond with the peptide bond between Val523 and Glu524. Although the predicted interaction conformations need to be experimentally validated, this docking analysis demonstrates that COX-2 is a plausible target for the phenolic acids discovered in *P. sajor-caju,* and that the anti-inflammatory activity observed can possibly be a result of a synergistic effect between different phenolic acids or phenolic acid derivatives.

4. Conclusions

The effects of three independent variables (*t*, *T* and *S*) was optimized for maximizing yield and Ph compounds extraction from *P. sajor-caju* using HAE. RSM methodology was applied using a *CCCD* of three factors with five-levels and second-order polynomial models, which were successfully designed and experimentally verified, showing that the studied variables have significant effects on the responses. In all cases, the used RSM models were statistically significant and their application allowed the optimization, resulting in enriched extracts in Ph compounds. Afterwards, the extracts were analyzed in terms of its chemical composition an in terms of its bioactive properties. The results produced of the enriched extracts in phenolic compounds shows a promising and broad range of bioactivities, with potential application in different industrial fields (food, pharmaceutical and cosmetic sectors).

Acknowledgements

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Program PT2020 for financial support to CIMO (UID/AGR/00690/2013) and for L. Barros and R. Calhelha contracts. T.C. Finimundy thanks CAPES Foundation, Ministry of Education of Brazil (CAPES fellow, process number 88881.134581/2016-01). To Xunta de Galicia for financial support for the post-doctoral researcher of M.A. Prieto.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2017.10.088.

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4.3 CAPÍTULO III

Food and Chemical Toxicology 112 (2018) 383–392

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Apoptosis induction by *Pleurotus sajor-caju* (Fr.) Singer extracts on colorectal cancer cell lines

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ARTICLE INFO

Keywords:
Pleurotus sajor-caju
Sterols
Cytotoxicity
Apoptosis

ABSTRACT

Pleurotus sajor-caju (PSC) is an edible mushroom used in food supplements, presenting antitumor properties through induction of cell death pathways. The PSC potential against colorectal cancer was analyzed by exposing HCT116^{wt} cells to different PSC extracts. The PSC n-hexane extract (PSC-hex) showed the highest cytotoxicity effect (IC_{50} value 0.05 mg/mL). The observed cytotoxicity was then associated to apoptosis-promoting and cell cycle-arrest pathways. PSC-hex was able to induce apoptosis related to breakdown of mitochondrial membrane potential and ROS generation. The absence of cytotoxicity in HTC116^{-p53} and HTC116^{-Bax} cells, alongside with an increase in p53, Bax and Caspase-3 expression, and decrease in Bcl-2 expression, supports that the pro-apoptotic effect is probably induced through a p53 associated pathway. PSC-hex induced cell cycle arrest at G2/M in HCT116^{wt} without cytotoxicity in HTC116^{-p21} cells. These findings suggest that a p21/p53 cell cycle regulation pathway is probably disrupted by compounds present on PSC-hex. Identification of the major components was then performed with ergosta-5,7,22-trien-3β-ol representing 30.6% of total weight. In silico docking studies of ergosta-5,7,22-trien-3β against Bcl-2 were performed and results show a credible interaction with the Bcl-2 hydrophobic cleft. The results show that PSC-hex can be used as supplementary food for adjuvant therapy in colorectal carcinoma.

1. Introduction

Colorectal cancer (CRC) is the third most common type of cancer and fourth leading cause of cancer death in the world as about 690,000 people annually die for this disease (Arnold et al., 2017). Clinically, chemotherapy and radiation are commonly used before or after surgery, even so 40% of all patients develop metastasis and presented recidives (Bahrami et al., 2018). CRC is classified by its clinicopathological characteristics, but clinical outcomes and drugs response, molecular characteristics and prognosis are uncertain and heterogeneous (Rodriguez-Salas et al., 2017). The molecular changes associated to tumor progression are attributable to genomic instability that shows common alteration of critical pathways. These include TP53, PI3CA, TGF-β, and Epithelial-to-mesenchymal transition (EMT) genes. These molecular events allows modifications such as loss of control of cell growth, increases cell-survival and cell-proliferation, promotes invasion, inhibits apoptosis, promotes epithelial to mesenquimal-transition and angiogenesis (Rodriguez-Salas et al., 2017).

The identification of consensus molecular subtypes (CMS) have clinical relevance independent of cancer stage and is heavily influenced by the tumor microenvironment, are divided into CMS1 (MSI immune), CMS2 (canonical), CMS3 (metabolic) and CMS4 (mesenchymal). These CMS subtypes are represented *in vitro* model systems, providing resource for preclinical studies in CRC (Berg et al., 2017). CMS4 tumors tended to be diagnosed at more advanced stages (III and IV), and one of the cell lines representing *in vitro* this subtype is HCT116.

Therefore there is a constant demand to investigate new and effective anti-CRC drugs to improve survival while maintaining health-related quality of life (Quidde et al., 2016; Rejhová et al., 2017). Diet appears to be one of the ways by which carcinogenic agent instigate the

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https://doi.org/10.1016/j.fct.2018.01.015
Received 4 December 2017; Received in revised form 9 January 2018; Accepted 10 January 2018
Available online 11 January 2018
0278-6915/

DNA repair modulating the intensity of early carcinogenic events (Rejhová et al., 2017). Several natural chemopreventive food compounds are being evaluated as edible and medicinal supplements, including the ones found in mushrooms, with antitumor substances already been identified in many species (Alonso et al., 2017; Gogavekar et al., 2014; Jin et al., 2016; Santesso and Wieland, 2016). Antiproliferative activity of *Pleurotus sajor-caju* (Fr) Singer extracts against human tumor cell lines (Hep-2, HeLa, Sarcoma 180, among others) has been reported (Assis et al., 2013). There is a major interest in the use of mushrooms and/or extracts as dietary supplements, given the beneficial properties as enhancers of immune function and promoters of health (Lo et al., 2012; Roncero-Ramos and Delgado-Andrade, 2017; Tanaka et al., 2016).

Different molecules found in fruiting bodies have been reported to present antitumor potential (Greeshma et al., 2016; Ren et al., 2012). Some examples are polysaccharides, glycoproteins, proteoglycans, proteins, quinones, cerebrosides, isoflavones, catechols, triacylglycerols, phenolic acids, terpenes, and steroids (Ferreira et al., 2010; Villares et al., 2012). Evidences support the theory of antitumoral properties of sterols, including inhibition of tumor growth and stimulation of apoptosis (Kikuchi et al., 2017). According to Barreira and Ferreira (2015), the mechanism underlying the inhibition of cell growth could be similar to the mechanism associated with protein kinase C activity. Understanding the mechanism of apoptosis has important implications in the prevention and treatment of many diseases, in particular cancer. The Bcl-2 family of proteins are apoptotic regulators that control cell survival (Opferman and Kothari, 2018).

So, it is important to explore how *Pleurotus sajor caju* (PSC) constituents may regulate the cell dynamics and reduce cell proliferation. Identification of isolated compounds or extracts that induce the apoptotic cascade to reduce proliferation rates of cancer cells would be an effective strategy to control cancer progression (Venkatesh Gobi et al., 2018). The aim of this study was to investigate the effect of PSC extracts, specifically the PSC n-hexane extract (PSC-hex) in the proliferation of colorectal cancer cells (HCT116^{wt}, $^{-Bax}$, $^{-p21}$ and $^{-p53}$), and to correlate the observed anti-proliferation activity to activation of pro-apoptotic and/or cell arrest regulation pathways. To verify cell selectivity, MRC-5 cell line was used, a lung fibroblast cell line origin that is associated with the initiation of epithelial-mesenchymal transition and metastasis. The identification of the main PSC -hex components was also obtained, and *in silico* docking studies were performed to provide clues on the potential mechanism of action of the PSC n-hexane extract by the inhibition of the Bcl-2, an anti-apoptotic protein.

2. Material and methods

2.1. Experimental materials

Trypsin and RNase A were from Gibco Invitrogen Co. (Paisley, UK). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were acquired from Hyclone Lab Inc. (USA). Acetic acid, dimethylsulfoxide (DMSO), propidium iodide (PI) and phenolic standards were from Sigma Chemical Co. (St. Louis, USA). DiOC6(3) (3,3′-Dihexyloxacarbocyanine Iodide (TermoFisher, Eugene,OR). Dichlorodihydrofluorescein (DCFH) and DAPI 4′,6-diamidino-2-phenylindole were purchased from Sigma-Aldrich (St. Louis, USA). Tricloroacetic acid (TCA) and Tris-Base were from Merck (Darmstadt, Germany). Folin-Ciocalteu's phenol reagent (Merck) was used for the determination of total phenols. All standards were purchased from Sigma-Aldrich, FITC Annexin V Apoptosis Detection Kit (Sigma-Aldrich, MO, USA), anti-Bax (Abcam, 1:500), anti-BCl-2 (Abcam, 1:500), anti-caspase-3 (Abcam, 1:500), anti-β-actin (Abcam, 1:1000), Human Apoptosis Antibody Array Kit (Abcam).

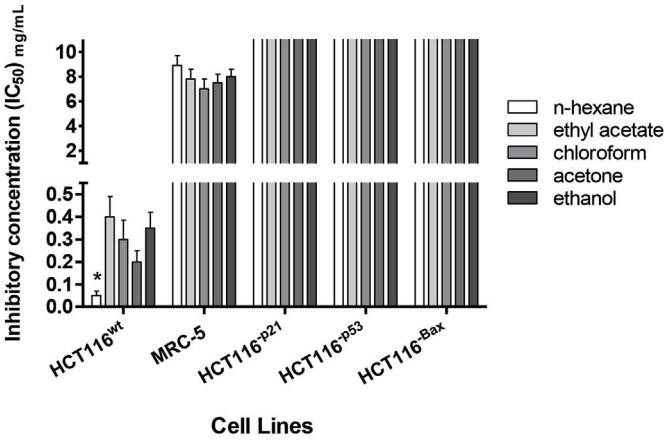

Fig. 1. Results the cytotoxic effect from MTT assay after 24 h incubation for the different extracts in HCT116^{wt}, HCT116^{-Bax}, HCT116^{-p53} and HCT116^{-p21} cell lines and lung human cell line (MRC-5). Data are the mean ± S.D. of three independent experiments. * $p < .05$ versus control.

2.2. Sample preparation

Samples of *Pleurotus sajor-caju* fruiting bodies (edible mushrooms) were collected in farm located 29°09′48.9″S 51°36′56.5″W, at Rio Grande do Sul state, in the southern region of Brazil, in autumn 2015. The extracts were obtained using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA), comprising an ultrasound power of 500 W, at a frequency of 20 kHz, equipped with a digital timer, following a protocol previously described by Heleno et al. (2016). The lyophilized powdered samples (10 g) were extracted with 100 mL of each selected solvent (n-hexane, chloroform, ethyl acetate, ethanol and ethanol/water (1:1, v/v)) into the ultrasonic device at the temperature of 20 °C, for 30 min. After extraction, the mixture was filtered and the solvent lyophilized. For the cytotoxicity assay, five dried extracts obtained were dissolved in ethanol/water (25:75, v/v).

2.3. Cell culture

HCT116^{wt} and MRC-5 cells were acquired from ATCC (American Type Culture Collection, United States), and HCT116^{-Bax}, HCT116^{-p21}, HCT116^{-p53} were supplied by Dr. Annette K. Larsen (Laboratory of Cancer Biology and Therapeutics, Paris, France). All the reagents were of ultrapure grade. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA). HCT116^{wt} (human colorectal carcinoma), HCT116^{-Bax}, HCT116^{-p21}, HCT116^{-p53} and MRC-5 (Fibroblast lung) were routinely maintained as adherent cell cultures in DMEM medium with 10% heat-inactivated FBS, in a humidified incubator at 37 °C with 5% CO_2. Cell cultures were periodically tested for Mycoplasma contamination. HCT116 cell lines were authenticated at DNA Diagnostics Center (DDC) from Public Health England (PHE) using short tandem repeat (STR) methodology and reference sample comparison to ATCC STR profile database. All assays were performed with cells in exponential growth, with viabilities over 90% and repeated at least in three independent experiments.

2.4. Cell viability (MTT assay)

All cells were evaluated according to the procedure adopted in the NCI's (National Cancer Institute) *in vitro* anticancer drug screening, which uses MTT assay to assess cell survival (Denizot and Lang, 1986). Briefly, the cell line was plated at an appropriate density (5×10^4 cells/well) in 96-well plates and allowed to attach for 24 h. Cells were then treated for 24 h with various concentrations of the extracts. Following this period, the adherent cells were incubated with MTT for 2 h. The crystal formazan was solubilized with DMSO and the absorbance was

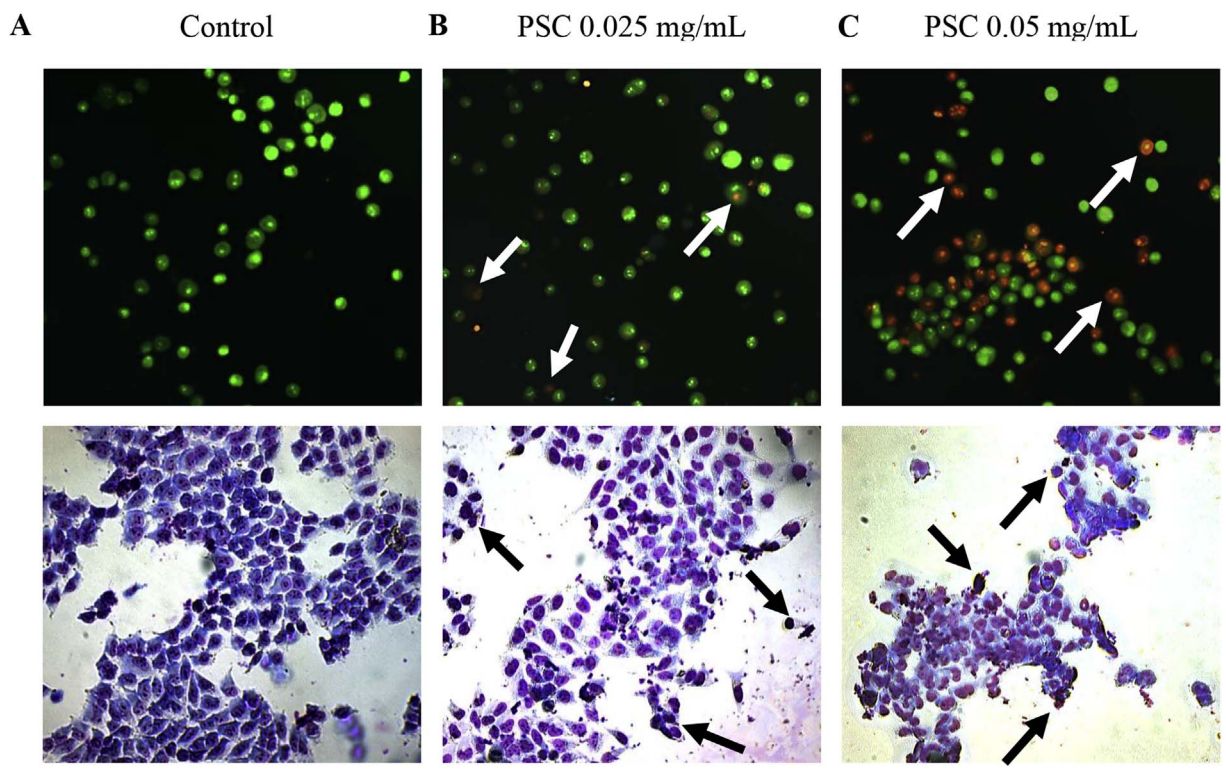

Fig. 2. Results dual stain OA/EB and Giemsa in HCT116^{wt} cell line. (A). Negative control (ethanol) group: circular green nucleus uniformly distributed in the center of the cell. (B) PSC 0.025 mg/mL (early apoptotic cells): nucleus showed yellow-green fluorescence staining and concentrated into a crescent or granular shape. (C) PSC 0.05 mg/mL (late apoptotic cells): the nucleus of cell showed orange or red fluorescence staining and gathered in concentration and located in bias and necrotic cells volume was increased, showing uneven red fluorescence and an unapparent outline. and the color observed after fluorescence staining in red indicate late apoptosis and yellow early events of apoptosis. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

measured at 540 nm in a microplate reader SpectraMAX M2/M2e (Molecular Devices, USA). The anti-proliferative activity of the compounds was inferred from the MTT assay by comparing the absorbance of the wells containing extract-treated cells with the absorbance of the wells containing untreated cells (Tolosa et al., 2015). Three to six independent experiments were performed in duplicate, the results were expressed as mean values ± standard deviation (SD) and IC_{50} (dose causing 50% cell death).

2.5. Acridine orange/ethidium bromide staining

The changes in chromatin organization, apoptotic cells or fragmented nuclei upon treatment with PSC-hex was determined microscopically by acridine/orange-ethidium bromide (AO-EB) dual staining. HCT116 cells (7×10^4 cell/well) were grown in 24-well plates. After 24 h of extract treatment, the cells were detached and the suspension from each well was separated in vials. The vials were centrifuged at 1200 rpm for 5 min. The pellet obtained was washed once with PBS, stained with AO/EB solution with 25 µL PBS and 2 µL AO/EB dye, incubated for 5 min, and observed under fluorescence microscope (BX43 - Olympus). Morphological changes were determined according to Pajaniradje et al. (2014).

2.6. ROS accumulation and mitochondrial membrane potential ($\Delta\psi m$) measurement

Reactive Oxygen Species (ROS) generation was analyzed by flow cytometry using DCFH-DA. Cells were treated with PSC-hex for 24 h, suspended in PBS and incubated with 10 µM DCFH-DA at 37 °C for 30 min. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by non-specific cellular sterases, and the subsequent oxidation of DCFH by peroxides was measured by means of flow cytometry (BD FACScalibur, San Jose, California). The uptake of the cationic fluorescent dye 3,3′-dihexyloxacarbocyanine iodide (DiOC6(3)) (2 µL of 2 µmol/L stock solution in dimethyl sulfoxide [DMSO]) was used for the evaluation of mitochondrial membrane potential (Aranda et al., 2013). Cell treatment was performed as in ROS experiments. Untreated controls and treated cells were harvested and washed twice with PBS. The cell pellets were then re-suspended in 2 mL of fresh incubation medium containing DiOC6 and incubated at 37 °C in a thermostatic bath for 30 min. HTC116 cells were separated by centrifugation, washed twice with PBS, and analyzed by flow cytometry using FL1 channel (488/533 nm) (BD FACScalibur, San Jose, Califórnia) (Wlodkowic et al., 2009).

2.7. Apoptosis and cell cycle analysis by flow cytometry

Induced apoptosis was assayed by the Human Annexin V-FITC/PI apoptosis Kit (Sigma-Aldrich, MO, USA), according to the manufacturer's instructions. The fraction of the cell population in different quadrants was measured using quadrant statistics with the FlowJo 10.0 software (LLC, Ashland, Ore). For the analysis of cell cycle phase distribution, HCT116^{wt} cells were plated at 1.5×10^5 cells/mL in 6-well plates and left incubating for 24 h. Cells were then incubated with complete medium only, medium with the control solvent ethanol/water (25:75 v/v) or with PSC-hex at IC_{50} (0.05 mg/mL) and half IC_{50} (0.025 mg/mL), previously determined by the MTT assay (Monks et al., 1991). Cells were harvested following 24 h incubation with the extract and further processed for either cell cycle analysis or apoptosis detection. For cell cycle analysis, cells were fixed in 70% ethanol for 10 min at room temperature. After centrifugation cells were incubated with PI (5 µg/mL) and RNase A in PBS (100 µg/mL) for 30 min on ice (Pozarowski and Darzynkiewicz, 2004). Cellular DNA content (for cell cycle distribution analysis and presence of sub-G1 peak, suggestive of

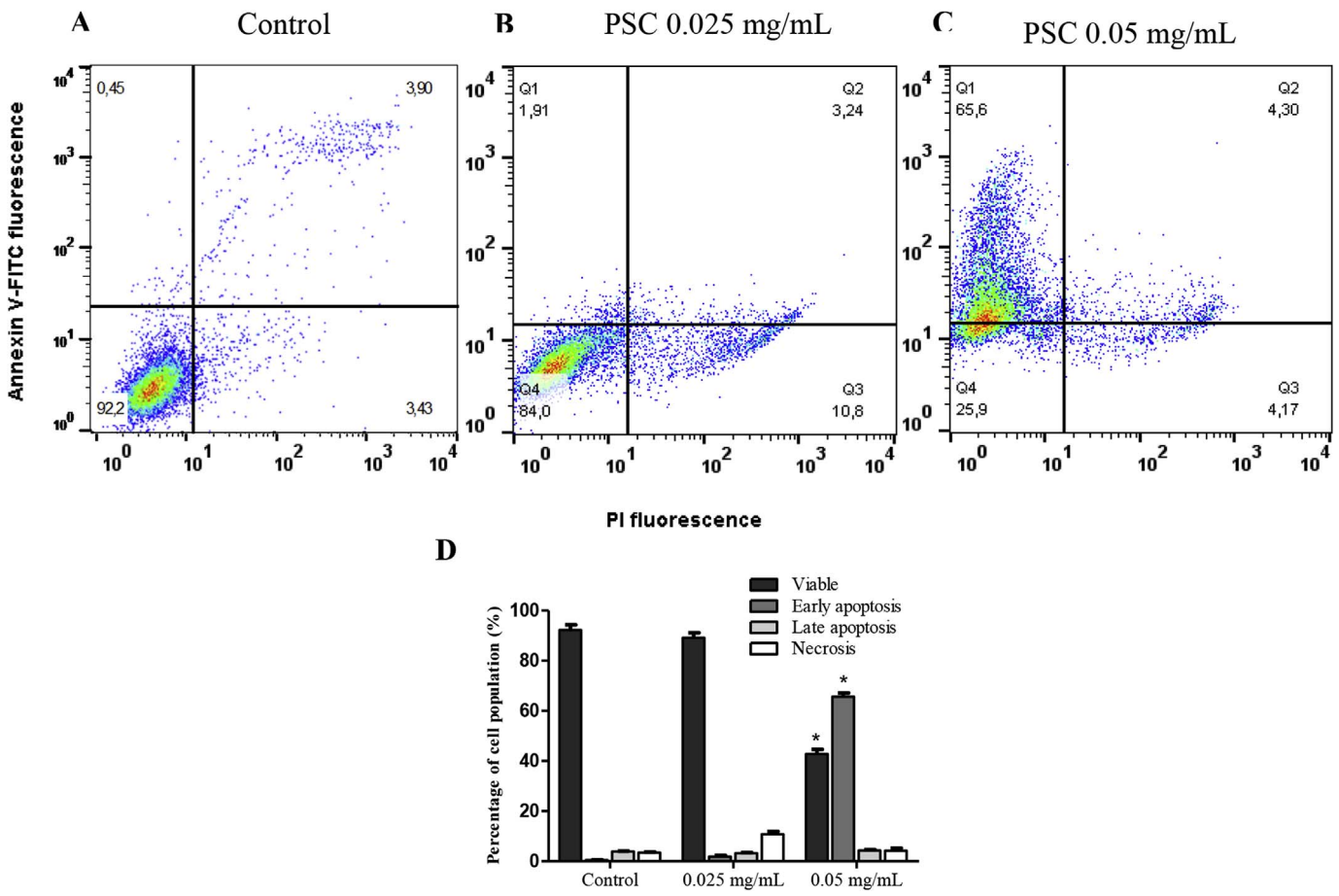

Fig. 3. Apoptosis analysis in HCT116^{wt} cells using annexin V-FITC/PI though cytometry analysis for control and treated samples. The early apoptotic events (Annexin + /PI-) are shown in lower right quadrant (Q1) of each panel. Quadrant (Q3) represents Annexin + /PI + late stage of apoptosis/necrosis. A) Control; (B, C) treatment with 0.025 mg/mL and 0.05 mg/mL of PSC respectively. D) Quantitative results from flow cytometry. Results are the mean ± SD of three to six independent experiments performed in duplicate. *Values statistically significantly ($P < .05$) different when compared to blank.

apoptosis induction) and measurement of phosphatidylserine externalization were analyzed using FL1 channel (488/533 nm) and FL3 channel (488/670 nm) an BD FACScalibur, San Jose, Califórnia, plotting at least 10,000 events per sample (Pietkiewicz et al., 2015). Three to six independent experiments were performed in duplicate and the results were expressed as mean values ± standard deviation (SD). ρ values < 0.05 were considered as statistically significant.

2.8. Apoptosis antibody array membranes analysis

Relative levels of 43 human apoptosis-related proteins were detected and analyzed using a human array kit, according to the manufacturer's instructions (Abcam, #ab134001). Briefly, the membrane containing immobilized apoptosis-related antibodies was blocked with bovine serum albumin for 2 h on a rocking platform at room temperature. The membrane was then incubated with lysates of untreated or treated HCT116^{wt} cells (IC_{50}), along with Detection Antibody Cocktail overnight at 2 °C to 8 °C. The membrane was incubated with streptavidin horseradish peroxidase conjugate followed by chemiluminescent detection reagent. The membrane was scanned using ImageQuant LAS 500 (GE Healthcare life sciences). According Schneider et al. (2012) the pixel density in each spot volume was determined, corrected for background and expressed as fold change (treated vs. untreated cells) using ImageJ version 1.46 software (NIH, Bethesda, MD; http://imagej.nih.gov/ij). Protein Array Analyzer plugin (available at image.bio.methods.free.fr/ImageJ/?Protein-Array-Analyzer-for-ImageJ.html, last accessed Oct 8, 2015) (Carpentier, 2014).

2.9. Western blotting analysis

To evaluate the expression levels of intracellular proteins related to apoptosis, HCT116^{wt} cells were treated with PSC-hex (IC_{50}) for 24 h. For the isolation of total protein fractions, the cells were collected, washed twice with ice-cold PBS, and lysed using cell lysis buffer (NP40, 20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, protease inhibitor). The lysates were collected by scraping from the plates and then centrifuged at 10,000 rpm at 4 °C for 5 min. Total protein samples were loaded on a 12% of SDS polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) for 1 h. Membranes were blocked at room temperature for 1 h with blocking solution (5% power milk in TBST). Next, the membranes were incubated for 1 h at room temperature with antihuman Bcl-2 rabbit pAb (1:1000 dilution; Abcam), antihuman Bax rabbit pAb (1:1000 dilution; Abcam), antihuman Caspase-3 rabbit pAb (1:500 dilution; Abcam) or antihuman β-actin mouse (1:1500). After washing, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-linked antimouse Ig (1:1000 dilution; Amersham) for β-actin or HRP-linked antirabbit Ig (1:50,000 dilution; Amersham) for Bax, Bcl-2 and Caspase-3. Immunoblots were performed using ECL prime Western blotting detection kit (Amersham). Chemiluminescence visualization and detection was performed using ImageQuant LAS 500 (GE Healthcare life sciences).

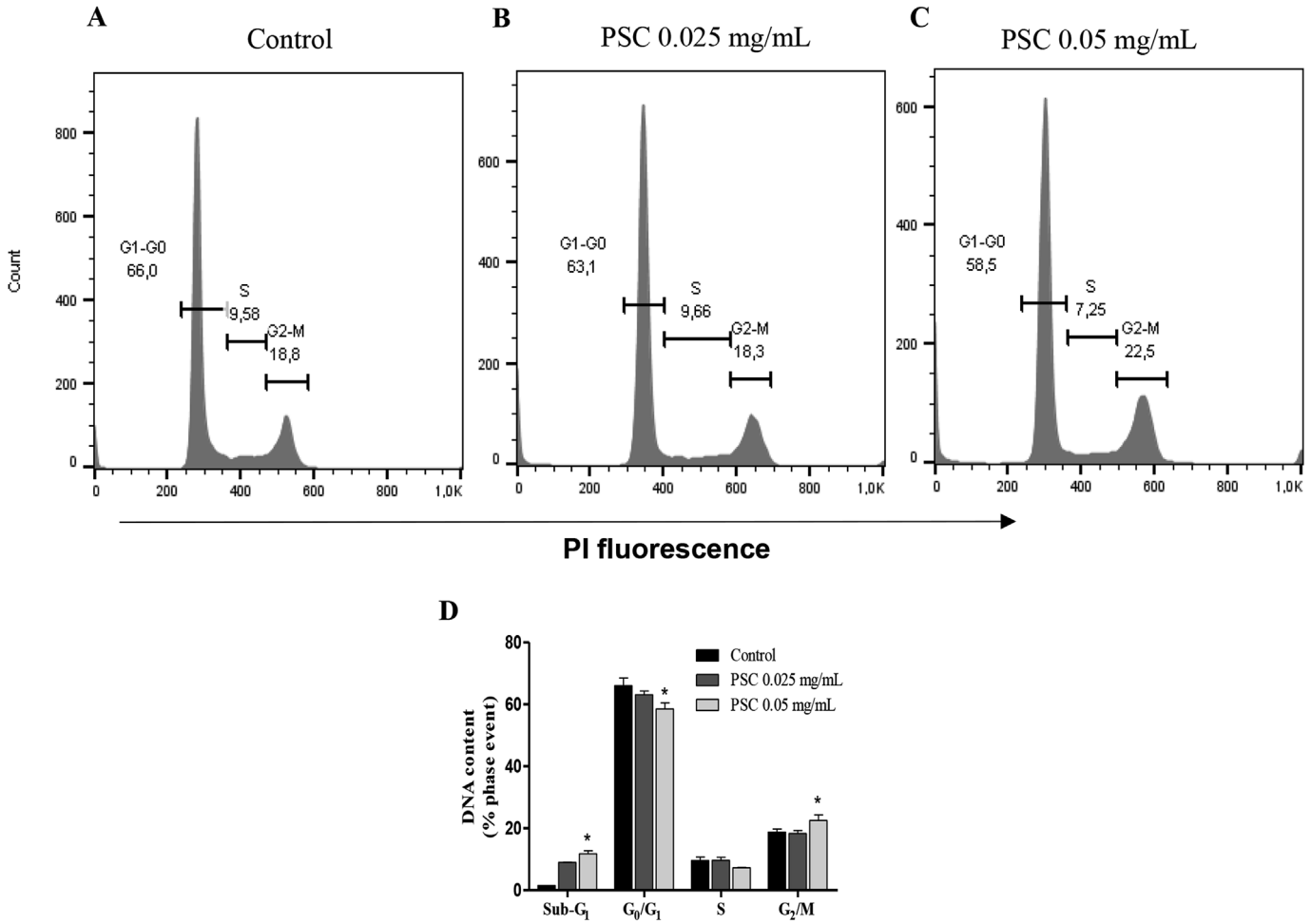

Fig. 4. Cell cycle distribution percentage of HCT116^{wt} cells after treatment with different concentrations of PSC (B,C) compared to control (A). The results of the cell cycle distribution analysis by flow cytometry were analyzed by ModFit. D) The percentages of cell populations of different cell cycle phases are shown. Results are the mean ± SD of three to six independent experiments performed in duplicate. *Values statistically significantly ($P < .05$) different when compared to blank.

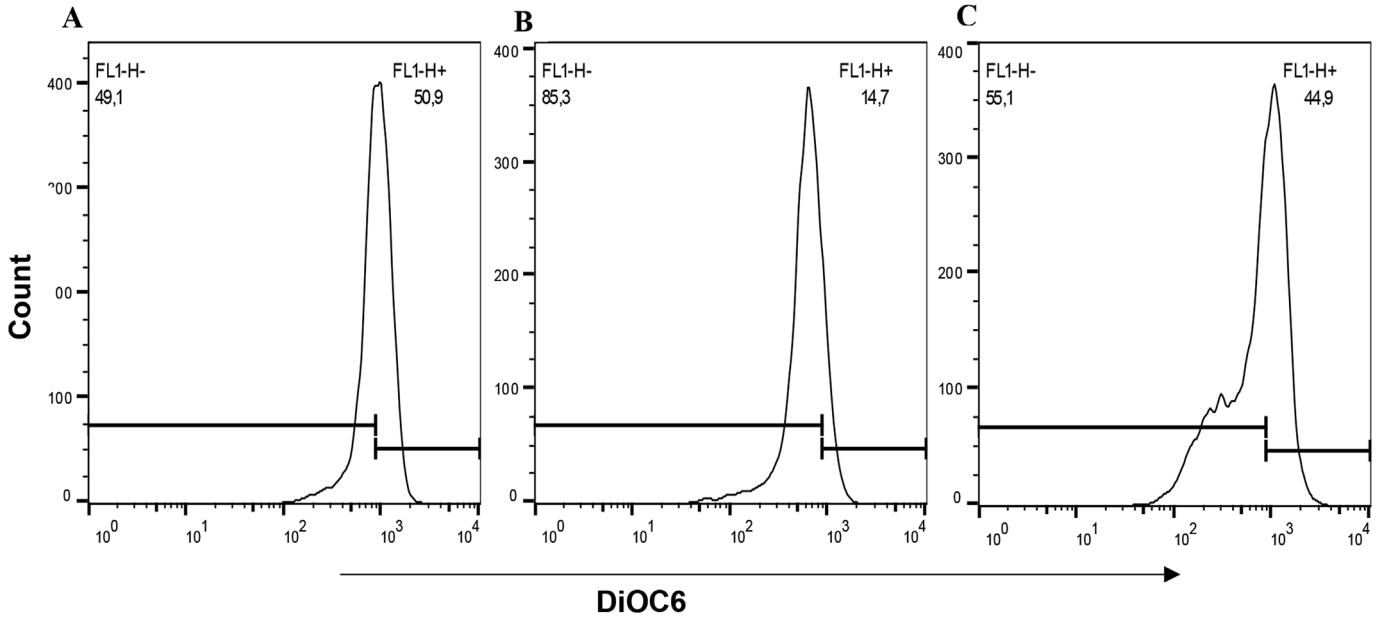

Fig. 5. Mitochondrial membrane potential of HCT116^{wt} cells after treatment with extracts of mushrooms using DiOC6(3) staining through cytometry. Cells with decreased fluorescence (increase to the left of the graph), present mitochondria depolarization A) Control; (B, C) treatment with 0.025 mg/mL and 0.05 mg/mL of PSC, respectively. Results are the mean ± SD of three to six independent experiments performed in duplicate.

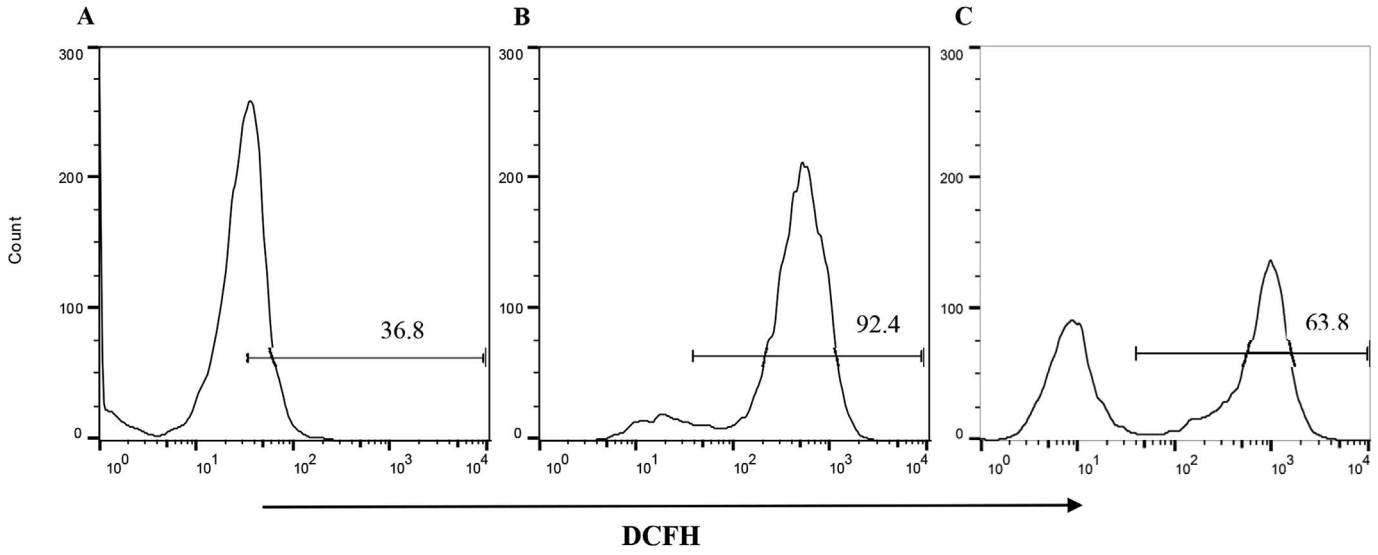

Fig. 6. Cytometry evaluation indicating ROS formation in HCT116^{wt} cells before and after extract exposition. Cells with increased fluorescence (increase to the right of the graph), present accumulation of ROS A) Control; (B, C) treatment with 0.025 mg/mL and 0.05 mg/mL of PSC, respectively. Results are the mean ± SD of three to six independent experiments performed in duplicate.

2.10. Gas chromatography-mass spectrometry (GC-MS)

PSC-hex was chemically characterized by GC-MS after sample derivatization. This process consisted on the derivatization of hydroxyl and carboxyl groups of the extracted compounds with 300 µL of *N,O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA; PanReac AppliChem, Barcelona, Spain) by heating the resulting mixture at 70 °C for 1 h prior to analysis. The derivatized sample was analyzed by GC-MS following a protocol previously described by Falcão et al. (2016), consisting on a Perkin Elmer system with a Clarus® 580 GC module and a Clarus® SQ 8 S MS module, equipped with DB-5MS fused-silica column (30 m × 0.25 mm i.d., film thickness 0.25 µm; J & W Scientific, Inc.). Oven temperature was programmed, 45–175 °C, at 3 °C/min, subsequently at 15 °C/min up to 300 °C, and then held isothermal for 10 min; injector and detector temperatures were 280 °C. The transfer line temperature was 280 °C; ion source temperature, 220 °C; carrier gas, helium, adjusted to a linear velocity of 30 cm/s; split ratio, 1:40; ionization energy, 70 eV; scan range, 40–300 u; scan time, 1 s. The software Turbomass (software version 6.1.0, Perkin Elmer, Shelton, CT, USA) for Windows was used for data acquisition. The identity of the components was assigned by comparison of their retention indices, relative to C7-C40 n-alkane indices and GC-MS spectra from a commercial MS database (NIST).

2.11. Molecular docking

A Bcl-2 crystal structure was selected and obtained from the Protein Data Bank (PDB entry: 4LXD). The Bcl-2 protein structure was prepared for docking by removing all crystallized water molecules and the Venetoclax inhibitor. AutoDockTools1.5.2 (ADT) (Morris et al., 2008) was then used to assign polar hydrogens, add Gasteiger charges and save the protein structure in PDBQT file format. A docking grid was selected using ADT in order to encompass completely the ligand binding site. The X,Y,Z grid center coordinates selected were 23.3, 32.7 and 13.1 respectively, and the X,Y,Z grid dimensions used were of 20 by 20 by 20 Å. The 2D structure of the studied sterol (Ergosta-5,7,22-trien-3β-ol), was drawn using ACD/ChemSketch Freeware 12.0 software. VegaZZ software was then used to perform 2D to 3D conversion and to save the structures in PDB file format (Pedretti et al., 2004). Finally, ADT was used to convert PDB to PDBQT file format. All docking simulations were performed using AutoDock Vina software (Trott and Olson, 2010), using an exhaustiveness parameter of 32. Docking conformation analysis and image preparation was performed using PyMOL software (Delano, 2002).

2.12. Statistical analysis

All of the data were expressed as mean ± SD. Differences between groups were determined by using the Student's *t*-test, and different groups were compared using one-way ANOVA followed by Tukey multiple comparison to evaluate the differences between two groups under multiple conditions. Statistical analysis was performed using SPSS21.0 software. $P < .05$ was considered statistically significant.

3. Results and discussion

3.1. PSC extract exhibited anti-proliferative activity against the HCT116^{wt} cells

In order to evaluate the anti-proliferative activity of PSC, five cell lines (HCT116^{wt}, HCT116^{-Bax}, HCT116^{-p21}, HCT116^{-p53} and MRC-5) were exposed to different concentration of PSC extracts for 24 h using a variation of solvent polarity (hexane, chloroform, ethyl acetate, ethanol and ethanol/water (1:1)). According Joana Gil-Chávez et al. (2013), these organic solvents can be used for the extraction of both polar and nonpolar compounds such as fatty acids, alkaloids, organochlorine, phenols, aromatic hydrocarbons and oils, among others. As shown in Fig. 1 a batch of different conditions crude extract were investigated. The results revealed PSC n-hexane extraction as the most promising condition, with an IC_{50} value of 0.05 mg/mL in HCT116^{wt} followed by PSC acetone extract with an IC_{50} value of 0.2 mg/mL. This result is probably due to the amount of lipophilic compounds extracted in this extract, corroborating with Sang et al. (2006) that they also presented better activity against human cancer cells including colon carcinoma (Caco-2), breast carcinoma (MCF-7) and acute myeloid leukemia (HL-60) cells. The other extracts might require very high concentrations to obtain the same effect as the n-hexane extract, which would make it unusable.

Interestingly, while the PSC-hex was able to induce cytotoxic effects on human colorectal carcinoma cells (HCT116^{wt}), it showed practically no anti-proliferative activity in non-tumor MRC-5 cells (Fig. 1). The anticancer activity of the n-hexane extract was attributed to high sterol content. Their selectivity may be related to inhibition of the mitochondrial complex I, which according to Kalyanaraman et al. (2018),

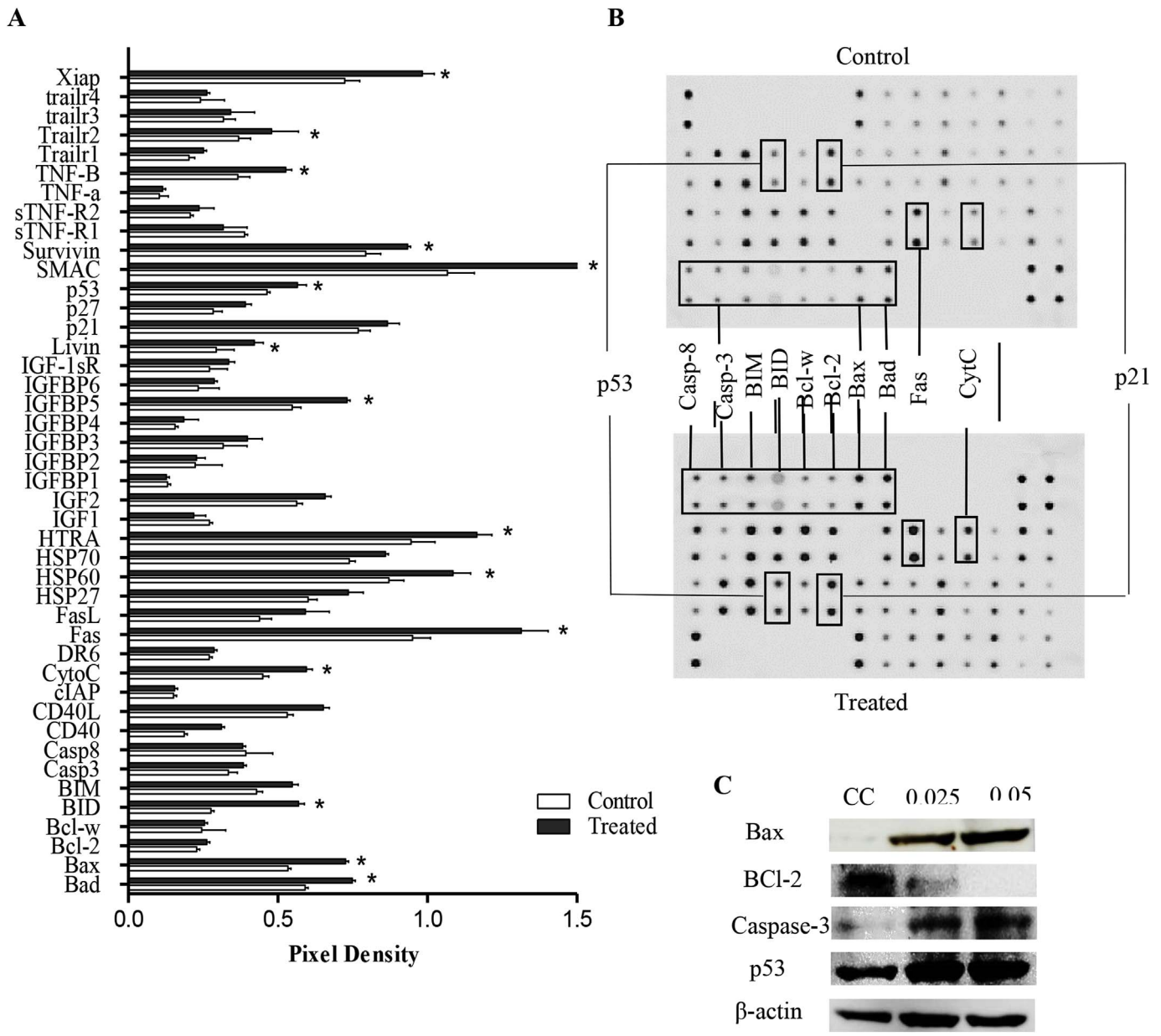

Fig. 7. Modulation of apoptosis-related proteins before and after PSC extract exposition in HCT116^{wt} cells treatment with 0.025 and 0.05 mg/mL A) Pixel densities of apoptosis-related proteins identified from array analysis of HCT116^{wt} cell in response to treatment of 24 h at a concentration of 0.05 mg/mL, showing changes in levels of apoptosis protein compared to untreated group *ρ < 0.05. B) Template demonstrating the location of spots of treated (below) and untreated (above) cell sample group representing the 43 apoptosis-related proteins and location of the spots of the expression apoptosis-related proteins, C) Western blotting showing the expression levels of the proteins. For normalization of protein levels was used β-actin loading control. Results are the mean ± SD of three to six independent experiments performed in duplicate. *Values statistically significantly (P < .05) different when compared to blank.

lipophilic compounds may be a class of therapeutic drugs that inhibit mitochondrial bioenergetics, mitochondrial respiration in tumor cells at relatively non-toxic concentrations. These experiments suggest that the PSC-hex promotes its cytotoxicity by inhibiting tumor-associated signaling pathways. This assumption was tested by performing the same experiments using HCT116 cell lines with deleted tumoral-associated proteins: Bax (HCT116^{-Bax}), p21 (HCT116^{-p21}) and p53 (HCT116^{-p53}). The results were drastic, with no observed anti-proliferation activity on any HCT-116 protein deficient cell lines, after exposure to PSC extracts (Fig. 1). These results suggest that the PSC-hex components disrupt multiple tumoral signaling pathways, including growth arrest (associated to proteins p21 and p53) and/or apoptosis pathways (associated to proteins Bax, Bcl-2 and p53). These results are in line with recent findings, showed that many natural compounds found in mushrooms have pro-apoptotic activities (Yang et al., 2016). Moreover, literature supports that mushroom constituents such as sterols may contribute to the antitumoral effects observed in this study (Heleno et al., 2015). Considering these results, the follow up studies were all performed using 0.05 mg/mL (IC_{50} value) and 0.025 mg/mL (half the IC_{50} value) concentrations of the PSC-hex applied to HCT116^{wt} adenocarcinoma model cell line.

3.2. PSC-hex effect on apoptosis and cell cycle in HCT116^{wt}

Apoptotic programmed cell death is characterized by various morphological and biochemistry changes (Hird et al., 2015). To evaluate the effect of PSC-hex in inducing apoptosis and changes in cell morphology, the Giemsa and OA/EB staining protocol was used, after exposition of HCT116^{wt} cells to 0.025 and 0.05 mg/mL of extract concentration (Liu et al., 2015). Few early-stage apoptotic events were

Table 1
Identification of the compounds present in *P. sajor-caju* non-polar extract (relative abundance %, mean ± standard deviation).

Peak	Identification	RT (min)	LRI	Peak Area (%)
1	Tetradecanoic acid (myristic acid; C14:0)	43.56	1839.14	0.19 ± 0.01
2	D-Mannitol	45.25	1630.50	2.9 ± 0.1
3	Butane	45.38	1738.83	2.67 ± 0.04
4	N-Pentadecanoic acid (C15:0)	45.64	1865.77	3.48 ± 0.09
5	Hexadecanoic acid (palmitic acid, C16:0)	46.98	2021.41	23.2 ± 0.1
6	(z)-9-octadecenoic acid (oleic acid, C18:0)	47.34	1980.35	0.32 ± 0.01
7	Octanoic acid (caprylic acid, C8:0)	47.90	2122.40	0.246 ± 0.003
8	9,12-Octadecadienoic acid (linoleic acid)	48.48	2189.84	24.81 ± 0.03
9	(z)-9,17-Octadecadienal	50.16	2085.69	1.95 ± 0.05
10	Cyclopenta[a.d]cyclo-octen-5-one	50.90	2554.49	0.21 ± 0.01
11	D-(+)-Trehalose	51.52	1940.12	1.34 ± 0.05
12	3,7,11,15-tetramethylhexadeca-2,6,10,14-tetraen-1-ol	51.98	2506.66	0.391 ± 0.005
13	2-α-Mannobiose	52.20	1955.18	1.25 ± 0.03
14	α-D-Glucopyranosiduronic acid	53.05	1974.00	0.96 ± 0.02
15	9(11)-Dehydroergosteryl benzoate	53.19	3275.92	0.44 ± 0.01
16	3α,5α-Cyclo-ergosta-7,9(11),22-triene-6β-ol isomer 1	54.57	3194.11	0.220 ± 0.008
17	Ergosta-5,7,9(11),22-tetraen-3-yl 4-methylbenzenesulfonate	54.68	3316.01	0.78 ± 0.03
18	Ergosta-5,7,22-trien-3β-ol	55.43	3338.31	30.6 ± 0.4
19	3β,22E-ergosta-7,22-dien-3	55.51	3225.26	0.99 ± 0.01
20	Cholesta-5,7-dien-3-ol acetate (3β)	55.90	3340.14	1.28 ± 0.07
21	Cholestan-26-oic acid	56.09	3513.09	1.18 ± 0.03
22	3α,5α-Cyclo-ergosta-7,9(11),22-triene-6β-ol isomer 2	56.27	3479.49	0.60 ± 0.02

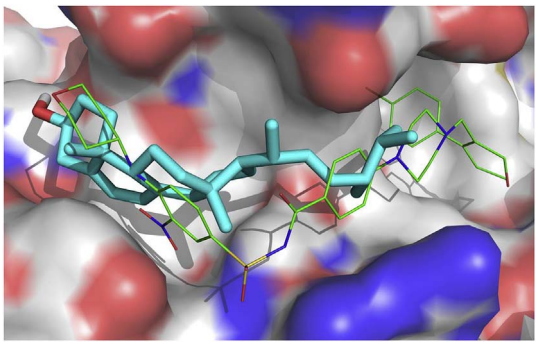

Fig. 8. Representation of Bcl-2 structure with docking conformation of Ergosta-5,7,22-trien-3β (cyan color, sticks and balls representation) and the co-crystallized inhibitor Venetoclax (green color, wire representation). Representation prepared using Pymol. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detected in the negative control (Fig. 2), while early-stages of apoptosis, marked by crescent-shaped or granular yellow green AO nuclear staining, were detected after cell exposition to 0.025 mg/mL of the extract (Fig. 2B). Late-stage of apoptosis, with condensation of chromatin, was observed after 0.05 mg/mL exposition, as indicated by localized orange and red nuclear EB staining (Fig. 2 C). All necrotic cells increased in volume and showed red fluorescence at their periphery.

To further analyze the effect of the PSC-hex in promoting apoptosis, the Annexin V and PI staining assay was performed (Fig. 3). After exposition to PSC-hex, the number of HCT116^{wt} cells in early apoptosis increased from 0,45% (Fig. 3A, **control**), to 65,6% (Fig. 3C, **0.05 mg/mL);** while the number of viable cells decreased from 92,2% (Fig. 3A, **control**), to 25,9% (Fig. 3C, **0.05 mg/mL)**. Both staining protocols confirm that PSC-hex exerts the observed cytotoxicity, at least partially, through a pro-apoptotic pathway.

Analysis of the effect of PSC-hex on HCT116^{wt} cell cycle was also performed by flow cytometry and results show an increase of cells in the G2/M transition phase, with a concomitant decrease of cells in the G1 and S-phases (Fig. 4). PSC-hex therefore seems to be an inducer of G2/M cell cycle arrest. A significant accumulation of cells in the sub-G1 fraction was also observed, indicating that the extract induces apoptosis (Fig. 4D).

3.3. PSC-hex activated depolarization of the mitochondrial membrane potential and ROS accumulation in HCT116^{wt} cells

The loss of Mitochondrial Membrane Potential (ΔΨm) is usually an indicator of changes in the permeability of the mitochondrial membrane, a process that is regulated by the Bcl-2 protein family (Ashkenazi et al., 2017). In addition to the loss of ΔΨm, changes in permeability can lead to the release of apoptosis factors such as cytochrome c, which triggers the activation of caspase-9 followed by activation of effector caspase-3, finally leading to apoptosis (Birkinshaw and Czabotar, 2017). The study of possible fluctuations in △Ψm in the presence of PSC-hex was thus performed by flow cytometry, using the DiOC6(3) stain protocol (Fig. 5). Compared with the untreated control (Fig. 5A), both 0.025 and 0.05 mg/mL PSC-hex concentrations (Fig. 5B–C) promoted a decrease in fluorescence, indicating an induced ΔΨm depolarization in HCT116^{wt} cells. These results suggest that PSC-hex might be promoting apoptosis through pathways associated with increase in mitochondrial permeability.

Cellular ROS production has been suggested as a possible cause for ΔΨm depolarization and subsequent induction of apoptosis and cell death (Chang et al., 2017). Many chemotherapeutic agents may be selectively toxic to tumor cells, because they increase oxidant stress beyond tumor cell support (Lee et al., 2016). Previous studies indicate that production of ROS is a relevant factor for regulating apoptosis (Al-Khayal et al., 2017; Hu et al., 2016). To investigate if the mitochondrial dysfunction observed in HCT116^{wt} cells is promoted by ROS production, a flow cytometry assay using DCF-DA stain was used to measure ROS levels. As shown in Fig. 6, the levels of H_2O_2 and $O_2^{\bullet -}$ in cells treated with 0.025 mg/mL and 0.05 mg/mL of PSC-hex, were elevated by 3-fold and 2-fold respectively, compared to the untreated control cells. These results indicate that apoptosis induced by PSC-hex may be strongly associated with ROS accumulation.

3.4. Intrinsic signaling pathway modulated apoptosis in HCT116^{wt} cells

To investigate the pathways by which PSC-hex may inducing apoptosis in HCT116^{wt}, we performed determination of apoptosis-related proteins using the Proteome Profiler Array (Human Apoptosis Antibody Array Kit, Abcam, #ab13400). HCT116^{wt} cells exposed to 0.05 mg/mL of PSC-hex, showed a significantly altered expression profile of apoptosis-related proteins. Increased expression was observed for several apoptotic related proteins including Fas, HSP 60, HSP 70, Xiap, HTRA, Survivin, Smac, caspase-3, Cytochrome-c, p53, Bax, Bad, Bid and Bim (Fig. 7A and B).

To confirm and validate the protein array results, the expression levels of Bax, Bcl-2, caspase-3 and p53 was further determined using western blot analysis. The results indicate up-regulation of Caspase-3, Bax and p53, while Bcl-2 showed down-regulation in HCT116^{wt} (Fig. 7C). p53 has been shown to play a critical role in intrinsic tumor suppression pathways, via apoptosis induction and cell cycle arrest pathways (Napoli and Flores, 2017). One of the multiple effects of p53 is to promote apoptosis by disrupting the Bax/Bcl-2 complex and consequent activation of caspase 3. The observed increase in Bax and p53 expression and decrease Bcl-2 expression, provides evidence that the PSC-hex probably promotes its pro-apoptotic activity by activating the p53 mediated pro-apoptotic pathway. This assumption is corroborated by the observed decrease in ΔΨm, a wallmark in p53 mediated induction of apoptosis. For cell cycle arrest, p53 exerts its effects through

cyclin-dependent kinase (CDK) inhibitor p21, leading to cell cycle arrest (Kim et al., 2017). Cell cycle analysis showed that PSC-hex induced cell cycle arrest at the G2/M phase, suggesting a blockage of cell proliferation of the HCT116^{wt}, which might be regulated by p53 by activating p21. This assumption is corroborated by the absence of cytotoxicity when HTC116^{-p21}, HTC116^{-p53} and HTC116^{-Bax} cells were used.

3.5. Chemical composition of PSC-hex

Table 1 shows the results regarding the identification and relative percentage of the twenty-two volatile compounds present in the PSC-hex. All compounds were identified with tetradecanoic acid (myristic acid, C14:0), pentadecanoic acid (C15:0), hexadecanoic acid (palmitic acid, C16:0), 9,12-octadecadienoic acid (linoleic acid, C18:2) and ergosta-5,7,22-trien-3β-ol being the main compounds identified in the extract, with the latter one as most abundant (30.6% of total compounds). These results are in accordance with those previously reported by others authors studying a similar extract from the *Pleurotus ostreatus* (Priya et al., 2012; Mohamed and Farghaly, 2014). Kayode et al. (2015) identified fatty acids and Usami et al. (2014) other types of compounds, mainly volatile, in *Pleurotus sajor-caju*.

3.6. Docking simulation of ergosta-5,7,22-trien-3β against Bcl-2

Bcl-2 is a well-known therapeutic target for anti-tumoral compounds. Known inhibitors promote apoptosis by occupying a hydrophobic cleft in the Bcl-2 structural thus preventing binding of Bcl-2 to pro-apoptotic protein partners including Bax (Radha and Raghavan, 2017). PSC-hex has shown to induce apoptosis by regulating expression of several apoptotic-related proteins, including down-regulating on of Bcl-2 expression. However an alternative pathway for inducing apoptosis might be accomplished by direct interaction of the compounds observed in PSC-hex with Bcl-2 (Table 1). Because ergosta-5,7,22-trien-3β comprises 30% of the extract content, we performed *in silico* docking simulations of this compound against Bcl-2, targeting the hydrophobic interaction cleft. The ergosta-5,7,22-trien-3β docked conformation fitted nicely in the Bcl-2 hydrophobic cleft, in a similar fashion to Venetoclax, a known Bcl-2 inhibitor (Fig. 8) (Souers et al., 2013), with an experimental IC_{50} value of 58 nM and currently in clinical trials against different types of tumors (Cang et al., 2015). Ergosta-5,7,22-trien-3β only interacts with a partial section of the hydrophobic cleft, so we do not expect its inhibition ability to be as high as Venetoclax, still this docking analysis demonstrate that activation of apoptosis by PSC-hex may be, at least partially, through direct inhibition of Bcl-2 by ergosta-5,7,22-trien-3β.

4. Conclusion

PSC-hex was able to generate several molecular responses on HCT116^{wt} colorectal cancer cell line, such as inducing initial apoptosis through the intrinsic pathway with upregulation of caspase-3 and Bax, with cell cycle arrest in G2/M, ROS accumulation and mitochondrial membrane depolarization. *P. sajor-caju* can emerge as an important nutraceutical and pharmacological natural source, in which steroid compounds appear to play a role on the observed cytotoxic and apoptotic effects here reported.

Due to the clinical and molecular heterogeneity of CRC, therapeutic modalities with new approaches to cancer therapy are essential. Many tumors have defects in activation of apoptosis because of overexpression of Bcl-2 pro-survival proteins or by inactivation of the p53 pathway (Adams and Cory, 2018). There is evidence that mushroom extracts have direct cytotoxic effects on cancer cells, which partially explains the *in vivo* effect on reducing tumor growth and anti-metastasis effect (Arata et al., 2016; Tangen et al., 2015). One of the mechanisms is induction of apoptosis through the mitochondrial pathway, increasing Bax and decreasing Bcl-2 expression (Liang et al., 2014). Because extracts act on this signaling pathway, nanotechnology can be used to pack mushroom extracts for more efficient delivery. Another alternative is dietary supplements in combination with conventional therapy. The use of combined therapy works on multiple metabolic pathways: it reduces the development of resistance to anticancer drugs, increases the sensitivity to the effect of chemotherapeutics, reinforcing the effectiveness of concentrations and minimizes adverse effects.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

The authors thank Dr. Larsen (Institut National de la Santé et de la Recherche Médicale) for the gift of HCT116^{-Bax}, HCT116^{-p21} and HCT116^{-p53} cells. The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) and FEDER under Programme PT2020 for financial support to CIMO (UID/AGR/00690/2013) and L. Barros contract. T.C. Finimundy thanks CAPES Foundation, Ministry of Education of Brazil (CAPES fellow, process number 88881.134581/2016–01). This work is supported by a grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.fct.2018.01.015.

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5 DISCUSSÃO GERAL

Os cogumelos têm sido utilizados tradicionalmente para diferentes aplicações, especialmente nos países orientais e direcionado a terapias tradicionais. Já nos países ocidentais, esse consumo aumentou somente nas últimas décadas (Lindequist *et al.* 2014). Eles têm sido usados por muitos anos como chás, alimentos nutricionais, como fonte de proteínas (incluindo todos os aminoácidos essenciais), possuindo baixo teor de lipídeos e também, devido a sua fragrância e textura especiais. Os cogumelos contêm quantidades relativamente elevadas de glicídios e fibras e quantidades significativas de vitaminas, nomeadamente tiamina, riboflavina, ácido ascórbico, niacina e sais minerais (Kalac 2013).

Os compostos naturais com atividade biológica estão normalmente presentes em plantas, cogumelos e outras fontes naturais como por exemplo, algas, crustáceos, etc. Em estudos com cogumelos, vários pesquisadores atribuem sua atividade antitumoral à capacidade imunoestimuladora dos extratos de micélios e corpos de frutificação (Meng *et al.* 2016, Chatterjee *et al.* 2017). Substâncias obtidas a partir de muitas espécies de cogumelos vêm demonstrando atividades medicinais importantes em estudos científicos (Afrin *et al.* 2016, Kalaras *et al.* 2017). Dependendo do tipo de solvente utilizado, se obtêm compostos diferentes, com propriedades distintas. Pode-se verificar neste trabalho, que com o extrato hexânico do *Pleurotus sajor-caju*, obtivemos uma melhor atividade antitumoral devido a presença de esteróis, enquanto para o extrato etanólico, obtivemos uma atividade anti-inflamatória devido a presença de ácidos fenólicos. Enquanto que para o extrato aquoso do *Lentinula edodes*, se verificou uma atividade antitumoral devido a alguns ácidos fenólicos identificados. Pode-se iniciar a extração através da partição por solventes orgânicos de polaridade crescente. Esse método de extração líquido/líquido,

resultará na separação dos principais metabólitos secundários (Tomsik *et al.* 2016). Solventes mais apolares são passíveis de extrair substâncias como lipídios, ceras, furanocumarinas, entre outras. Já solventes mais polares apresentam uma composição final de substâncias como taninos, alcaloides, proteínas, fenólicos (Goldsmith *et al.* 2014).

Os fenólicos são compostos gerados como metabólitos secundário nas plantas e nos fungos, sendo considerado um dos grupos mais importantes associados ao poder antioxidante, já tendo sido descrita sua capacidade de quelar metais e inibir a lipoxigenase e radicais livres (Heleno *et al.* 2015). A procura de fontes naturais e um melhor conhecimento das espécies de cogumelos presentes na biodiversidade brasileira e suas propriedades medicinais, permitem o desenvolvimento de novos medicamentos ou nutracêuticos para a prevenção e tratamento de enfermidades com propriedades antitumorais, antioxidantes, anti-inflamatórias e antimicrobianas (Money 2016).

Um dos objetivos propostos neste estudo foi avaliar extratos de cogumelos do filo Basidiomicetos quanto as suas propriedades químicas e antioxidantes e relacionar com a atividade biológica. No, **Capítulo I**, utilizou-se extrato aquoso do *L. edodes*, dando seguimento aos trabalhos anteriores do grupo (ANEXO I). Neste estudo avaliou-se a citotoxicidade celular, sem a geração de resíduos tóxicos, com extrações que se aproximassem a infusões de chás utilizados na medicina popular. O *L. edodes* possue vários compostos que apresentam propriedades imunoestimuladoras e antioxidantes. Os resultados obtidos do extrato aquoso deste cogumelo em concentrações de 2mg/mL apresentou duas vias de morte, tanto apoptose como necrose nas células de carcinoma de laringe e na sua caracterização química, foi identificado somente 3 ácidos fenólicos. Já se sabe que sua atividade antitumoral já é reconhecida em diversos países (Borchers *et al.* 2008), no entanto, a literatura mostra um número limitado de artigos que trabalham com

extratos aquoso. Devido a isso, posteriormente se fez um screening de solventes para a realização dos extratos e seguiu-se com o estudo do *P. sajor-caju,* por ser o tipo de cogumelo menos estudado na literatura.

Na segunda parte do trabalho, **capítulo II**, verificou-se que o extrato orgânico do cogumelo *P. sajor-caju*, não aquoso, de composição etanólica, teve um melhor resultado em termos de extração de compostos. A literatura mostra que aproximadamente 70% dos estudos com cogumelos utilizam extrato etanólico, inferindo em uma melhor extração dos compostos ativos, como por exemplo os ácidos fenólicos. Segundo Gogavekar *et al.* (2014), o *P. sajor-caju* auxilia na manutenção da saúde, pois é fonte importante de antioxidantes tais como fenóis e tocoferóis, que podem ser utilizados no combate a doenças, em aplicações dermatológicas, cosméticas, assim como em suplementos na indústria alimentar devido à sua atividade bloqueadora de radicais livres e capacidade de inibição da peroxidação lipídica. Suas frações parecem manifestar ação anticâncer de maneira indireta, pela ativação da resposta imunológica específica e não específica do hospedeiro, ou promovendo a morte das células tumorais por impedir a angiogênese (Assis *et al.* 2013). Silveira *et al.* (2015), demonstraram que um exopolissacarideo isolado do *P. sajor-caju* teve uma atividade antinociceptiva e anti-inflamatória. O trabalho apresentado neste capítulo busca relacionar mecanismo de ação de morte celular na célula de carcinoma de intestino (HCT-116) utilizando um extrato etanólico de *P. sajor caju*. A apoptose como sido reportada na literatura como via principal de morte celular em outras espécies de Pleurotus (Cui *et al.* 2014, Cui *et al.* 2016). Neste capítulo verificou-se também diversas vias de metabolismo celular, proliferação e morte celular. O extrato apresentou atividade citotóxica, anti-inflamatória e antimicrobiana para bactérias gram-positivas, fato que reforça a ideia dos compostos fenólicos contribuírem significativamente para essas bioatividades. Em particular, o ácido cinâmico (composto

encontrado em abundância) já mostrou inibir o crescimento de uma linhagem de células tumorais humanas (NCI-H460) e, quando combinado com os ácidos catequínico e hidroxibenzóico, conduz a um forte decréscimo do número de células viáveis, sugerindo um possível efeito concomitante destes compostos (Vaz *et al.* 2012). De acordo com os fenólicos encontrados, realizou-se um estudo mais profundo em relação a atividade anti-inflamatória, com um ensaio *in silico* por *docking* molecular. Aproximadamente 20% dos artigos apresentados na literatura estudaram a atividade anti-inflamatória relacionada aos extratos etanólicos e metanólicos destes cogumelos. Conforme apresentado neste capítulo, confirmou-se a provável atividade anti-inflamatória seletiva (inibição de COX-2).

A terceira parte deste trabalho apresentada no **capítulo III** evidenciou que os extratos de *n*-hexano de *P. sajor-caju* revelaram melhores atividades citotóxicas na linhagem HCT-116^{wt} de carcinoma coloretal, apresentando menores valores de $IC_{50,}$ e para a célula MRC-5, apresentou os maiores IC_{50}, com valores acima de 10mg/mL. Enquanto para as células HCT-116^{-p21}, HCT-116^{-p53} e HCT-116^{-Bax}, não apresentou citotoxicidade para os diversos extratos testados. A via de morte celular prevalente, foi a apoptose, com uma possível parada de ciclo celular em G2-M, despolarização da membrana mitocondrial e acúmulo das espécies reativas de oxigênio.

Poucos artigos na literatura avaliaram o potencial de extratos hexânicos de cogumelos. A partir deste extrato, por ter baixa polaridade, foram identificadas substâncias como os esteróides, ácidos graxos e terpenos. Esses compostos possuem bioatividades importantes, como potencial atividade antitumoral. Assim, estudou-se *in silico,* por ferramentas de *docking*, a atividade bloqueadora do esteroide mais abundante no extrato frente a proteína anti-apoptótica Bcl-2.

Perante o grande espectro de bioatividades relatadas na literatura, os cogumelos fazem parte de um grupo de importantes fontes de substâncias nutracêuticas, definidas como "alimento ou parte de um alimento que proporciona benefícios a saúde, incluindo a prevenção e/ou tratamento da doença". Tais produtos podem abranger desde os nutrientes isolados, suplementos dietéticos na forma de cápsulas e até alimentos processados como cereais, sopas e bebidas (Gul *et al.* 2016). Além disso, também são importantes como grupos de alimentos funcionais, que são definidos como "alimento que melhora a saúde e o bem-estar, e/ou que reduz o risco de doenças através da sua intervenção benéfica ao nível das funções orgânicas". (Salami *et al.* 2013, Aronson 2017). Estas áreas são relativamente novas no Brasil, contudo, o aumento do aparecimento dos alimentos funcionais e suplementos alimentares no mercado torna mais difícil a distinção entre farmacologia e nutrição (Lajolo 2007, Neves *et al.* 2015). Tradicionalmente, os produtos farmacêuticos têm sido usados no tratamento de doenças crônicas ou para aliviar os seus sintomas. A nutrição, por outro lado, têm como objetivo fundamental prevenir doenças, fornecendo ao corpo um equilíbrio ideal de macro e micronutrientes. Os medicamentos são cada vez mais usados para diminuir os fatores de risco e, assim, prevenir doenças crônicas. Justamente por isso, os cogumelos podem estar na interface farmacologia-nutrição pois tem sido consumidos não apenas como parte de uma dieta normal mas, também, pelas suas propriedades nutricionais e medicinais (Kozarski *et al.* 2015).

Em suma, o consumo de cogumelos e a exposição a diferentes formulações a base de cogumelos podem trazer benefícios a saúde, como reduzir os fatores de risco para o desenvolvimento de doenças, tanto em monoterapia como em combinação com outras drogas de prescrição. A tendência é que cada vez mais estudos sejam conduzidos para avaliar potenciais atividades medicinais na inclusão de cogumelos na alimentação,

estimulando seu consumo e sua produção já que eles podem ser consumidos como parte de uma dieta normal beneficiando funções nutricionais básicas e trazendo efeitos benéficos na redução do risco de doenças crônicas. Várias drogas antineoplásicas ainda possuem limitação quanto ao uso por fatores como baixo potencial, baixa solubilidade em água, toxicidade e resistência tumoral (Mouhid *et al.* 2017). Sendo assim, faz-se necessários o desenvolvimento de agentes antitumorais alternativos, mais efetivos e seguros (Ali *et al.* 2012). Como consequência, novos produtos biotecnológicos serão desenvolvidos em decorrência dos avanços recentes nas áreas de produção e purificação de substâncias medicinais a partir de cogumelos ou cultivos miceliais, refletindo em benefícios econômicos e ecológicos e contribuindo para uma vida mais saudável dos indivíduos.

6 CONCLUSÃO

Para o cogumelo *Lentinula edodes*, o extrato aquoso não se mostrou muito promissor já que apresentou duas vias de morte celular, apoptose e necrose, e a caracterização química ficou limitada na identificação de 3 ácidos fenólicos.

Para o cogumelo *Pleurotus sajor-caju*, diferentes extratos foram obtidos, mostrando que pela avaliação *in vitro*, o extrato n-hexanico apresentou uma melhor atividade anti-proliferativa que foi comprovada com a avaliação *in silico* como inibidor da Bcl-2 pelo seu esterol majoritário. Através de uma otimização nos métodos de extração para compostos fenólicos, o extrato etanólico apresentou atividade citotóxica e uma boa atividade antioxidante podendo auxiliar na proteção da peroxidação lipídica. A otimização da extração resultou na identificação de 3 compostos majoritários de ácidos fenólicos. *In vitro* mostrou ter uma boa atividade anti-inflamatória que foi comprovada *in silico* através do bloqueio da proteína específica da cascata inflamatória COX-2 pelos ácidos fenólicos identificados.

Em suma, podemos concluir que os extratos de cogumelos são promissores para o desenvolvimento de suplementos alimentares, bem como seu consumo *in natura*, auxiliando no tratamento e prevenção de doenças.

7 PERSPECTIVAS

Futuros estudos podem conduzir pesquisas com foco em:

- obter diferentes condições de extração de cogumelos e possíveis fracionamentos de extratos, a fim de obter frações mais purificadas;
- isolar e quantificar compostos responsáveis pelas propriedades bioativas, em particular antioxidantes, tais como compostos fenólicos, tocoferóis, ácido ascórbico, carotenóides, ácidos graxos e açúcares;
- investigar diferentes vias de sinalização celular e ampliar o número de ensaios com linhagens celulares, a fim de se elucidar melhor os mecanismos de ação dos extratos ou frações investigadas;
- avaliar potenciais atividades medicinais da inclusão de cogumelos na alimentação, estimulando o consumo e a sua produção;
- desenvolvimento de novos produtos biotecnológicos nas áreas de produção e purificação de substâncias medicinais a partir de cogumelos ou cultivos miceliais.

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ANEXOS

ANEXO I

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Aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* exhibit high antioxidant capability and promising in vitro antitumor activity

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ARTICLE INFO

Article history:
Received 1 May 2012
Revised 1 November 2012
Accepted 5 November 2012

Keywords:
Lentinula edodes
Pleurotus sajor-caju
Hep-2
HeLa
Tumor cell lines

ABSTRACT

Mushroom extracts are increasingly sold as dietary supplements because of several of their properties, including the enhancement of immune function and antitumor activity. We hypothesized that soluble polar substances present in mushroom extracts may show antioxidant and anticancer properties. This report shows that Brazilian aqueous extracts of *Lentinula edodes* and *Pleurotus sajor-caju* exert inhibitory activity against the proliferation of the human tumor cell lines laryngeal carcinoma (Hep-2) and cervical adenocarcinoma (HeLa). Cell viability was determined after using 3 different temperatures (4°C, 22°C, and 50°C) for mushroom extraction. Biochemical assays carried out in parallel indicated higher amounts of polyphenols in the *L edodes* extracts at all extraction temperatures investigated. The scavenging ability of the 2,2-diphenyl-1-picrylhydrazyl radical showed higher activity for *L edodes* extracts. Superoxide dismutase–like activity showed no statistically significant difference among the groups for the 2 tested extracts, and catalase-like activity was increased with the *L edodes* extracts at 4°C. The results for the cytotoxic activity from *P sajor-caju* extracts at 22°C revealed the half maximal inhibitory concentration values of 0.64% ± 0.02% for Hep-2 and 0.25% ± 0.02% for HeLa. A higher cytotoxic activity was found for the *L edodes* extract at 22°C, with half maximal inhibitory concentration values of 0.78% ± 0.02% for Hep-2 and 0.57% ± 0.01% for HeLa. Substantial morphological modifications in cells were confirmed by Giemsa staining after treatment with either extract, suggesting inhibition of proliferation and induction of apoptosis with increasing extract concentrations. These results indicate that the aqueous extracts of Brazilian *L edodes* and *P sajor-caju* mushrooms are potential sources of antioxidant and

Abbreviations: CAT, catalase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; Hep-2, cell line human laryngeal carcinoma; HeLa, cell line human cervical adenocarcinoma; $IC_{50(\%)}$, half maximal inhibitory concentration; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); SOD, superoxide dismutase.

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http://dx.doi.org/10.1016/j.nutres.2012.11.005

anticancer compounds. However, further investigations are needed to exploit their valuable therapeutic uses and to elucidate their modes of action.

1. Introduction

Mushrooms have long attracted great interest for use in foods and biopharmaceuticals [1]. Hot water-soluble fractions known as decoctions and essences from medicinal mushrooms, such as *Ganoderma lucidum* (Reishi), *Inonotus obliquus* (Chaga), and *Lentinula edodes* (Shiitake), have been collected and used as alternative medicines for hundreds of years in Korea, China, Japan, and eastern Russia [1]. Numerous molecules synthesized by macrofungi are known to be bioactive, including polysaccharides, glycoproteins, terpenoids, and lectins [2]. A wide variety of naturally occurring substances have been shown to protect against tumor development [3] and inflammatory processes [4]. Recent scientific evaluations of macrofungi, such as mushrooms and entomopathogenic fungi, have confirmed the efficacy of extracts from either the fruiting bodies or mycelia of these species in inhibition of the proliferation of various cancer cells lines [1,5].

Edible mushrooms have been reported to generate beneficial effects for health and in the treatment of disease through their immunomodulatory and antineoplastic properties [6,7]. The Shiitake mushroom has served as a model for investigating functional fungi properties and isolating pure compounds for pharmaceutical use [8]. Water extracts of the Shiitake fruiting body have been shown to prevent tumor growth in mice [9,10]; however, most studies have focused on the antioxidant capacity of polyphenolic compounds in *L edodes* [11,12]. A great number of polysaccharides have been isolated from basidiomycetes [13], representing homopolymers and heteropolymers primarily from β-configuration glucans. Glucans containing both α-configuration and β-configuration are less represented in basidiomycetes [2,14]. *Pleurotus* species are promising as medicinal mushrooms and exhibit hematological, antiviral, antitumor, antibacterial, hypocholesterolic, and immunomodulatory activities [15] as well as antioxidant properties [16-18]. Approximately 40 species of the genus *Pleurotus*, also known as oyster mushrooms, have been reported, including *Pleurotus florida* and *P sajor-caju*; all are commonly available, edible mushrooms, and detailed structural characterizations of their isolated polysaccharides have been reported [19]. These 2 mushrooms were selected for our study to compare their antitumor properties and to investigate the differences between their mechanisms of action. In addition to the well-known *Agaricus blazei*, *L edodes* has been extensively studied and has shown excellent biological properties. However, few studies have been performed with *P sajor-caju* to evaluate its in vitro biological activity, and all have involved nonaqueous (low temperature)–based extracts. Until now, most experimental work with mushroom extracts has been performed using hot water–based extracts or ethanol/methanol extracts [20-22].

Our work is a preliminary and pioneering study assessing the antioxidant and antitumor activity of Brazilian *L edodes* and *P sajor-caju* mushrooms. To address our hypothesis that soluble polar substances found in mushrooms may have antioxidant and anticancer properties, this article investigated the biological potential of low temperature (<50°C) aqueous extracts. The samples were tested for total polyphenol content, 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability, and superoxide dismutase (SOD)– and catalase (CAT)–like activities as well as the ability to inhibit the proliferation of the human tumor cell lines laryngeal carcinoma (Hep-2) and cervical adenocarcinoma (HeLa) using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and observing in situ morphological alterations.

2. Methods and materials

2.1. Mushroom grown and extract preparation

The Brazilian *L edodes* and *P sajor-caju* mushrooms were grown in South Brazil in the region known as Serra Gaúcha. Fruiting bodies of *L edodes* and *P sajor-caju* were initially chopped and oven dried at 50°C until a dry weight was recorded, and then they were ground into powder with a knife mill. The humidity values obtained were 10.41% in *P sajor-caju* and 14.37% in *L edodes*, and the method used was that described by Burrows [23] with modifications. Extraction was carried out using distilled water at 4°C, 22°C, and 50°C in a rotational shaker for 1 hour. The extracts were than separated and filtered using filter paper, sterilized using 0.22-μm filter units in a laminar flow chamber and stored at –20°C until used, using the procedure of Zhuang et al [24] with modifications.

2.2. Phenolic content and antioxidant activity of extracts

The total phenol content of the extracts was determined using the modified Folin-Ciocalteu colorimetric method as described in Singleton and Rossi [25]. Total phenol content was determined by comparison with a catechin standard curve (0.3-10 mg% catechin; Sigma Chemical Co, São Paulo, Brazil). The total phenolic contents are expressed as %mg of the catechin equivalent.

The antioxidant activity of 6 different extracts was determined by in vitro assays. The radical scavenging activity of DPPH was measured using a method modified by Yamaguchi et al [26] in which 200 μL of the extracted solutions (1%-10% wt/vol) were added to 800 μL of Tris-HCl buffer (100 mM, pH 7.0) containing 1 mL of DPPH solution (500 μM dissolved in ethanol). Tubes were stored in the dark at room temperature for 20 minutes, and the absorbance was measured at 517 nm (model UV-1700 spectrophotometer; Shimadzu, Kyoto, Japan). The result was expressed in half maximal inhibitory concentration ($IC_{50(\%)}$) (amount of extract necessary to scavenge 50% of DPPH radical). Superoxide dismutase–like activity was determined spectrophotometrically by measuring the inhibition of the autocatalytic adrenochrome formation rate at 480 nm in a reaction medium containing 1 mmol/L adrenaline (pH

2.0) and 50 mmol/L glycine (pH 10. 2) as well as different volumes of 10% (wt/vol) solutions of the extracts. This reaction was performed at 30°C for 3 minutes [27]. The results were expressed as the $IC_{50(\%)}$ in microliters of the sample required to inhibit 50% the formation of adrenochrome [28]. Catalase-like activity was assessed using 10% (wt/vol) solutions of the extracts to determine the rate of decomposition of hydrogen peroxide at 240 nm. The results are expressed as nanomole H_2O_2 decomposed/minute [29].

2.3. *Cytotoxic assay*

The Hep-2 and HeLa cell lines were cultured in Dulbecco's Modified Eagle Medium supplemented with antibiotics and 10% fetal bovine serum [Gibco BRL; Life Technologies (Van Allen Way, Carlsbad, CA, USA)] at 5% CO_2 and 37°C. For the assessment of the cytotoxic activities of *L edodes* and *P sajor-caju* extracts, using the procedure of Alley et al [30], the cells were seeded in 96-well flat-bottom microplates at a density of approximately 7×10^4 cells/well in 10% fetal bovine serum Dulbecco's Modified Eagle Medium. After cell attachment, serial dilutions of the extracts in the culture medium were prepared, added to the cells for 1 hour, and removed, followed by incubation for 24 hours in extract-free medium.

Cell proliferation was determined by the tetrazolium salt method using MTT [31]. Briefly, 7×10^4 cells/well were cultured in 96-well plates and treated for 1 hour with increasing extract concentrations (0.05%-1.5%) at 37°C. Doxorubicin was used as a positive control. After incubation with MTT solution at room temperature for 2 hours, dimethyl sulfoxide was added, the cells were harvested, and absorption was determined at 540 nm. At least 3 independent experiments were performed for each experimental cell line, and $IC_{50(\%)}$ values (dose causing 50% cell survival) were determined as the means and SD [32].

2.4. *Morphological examination of cancer cells*

The morphology of the Hep-2 and HeLa cell lines was monitored using an inverted microscope under a conventional Giemsa staining protocol [33,34]. Changes in the cellular morphology were observed and documented after being treated for 1 hour with the aqueous extracts of *L edodes* and *P sajor-caju*, followed by cultivation for 24 hours in extract-free medium. The negative control group was treated with distilled water instead of extract for the same period.

2.5. *Statistical analyses*

The results are expressed as the means ± SD of each group. Analysis of variance followed by the Tukey post hoc test was used to test for differences among the treatment groups in triplicate. Statistical analyses were performed using SPSS 19.0 (Armonk, NY, USA). The level of significance was uniformly set at $P < .05$.

3. Results

The data for the polyphenol contents, antioxidant activities using DPPH, and the SOD-like and CAT-like activities of *L edodes* and *P sajor-caju* extracts are presented in Table 1 and indicate that the extracts of these mushrooms prepared at temperatures lower than 50°C contain polyphenol substances that are the potential sources of antioxidant activity.

The cytotoxic activity assays for the cell line Hep-2 after treatment with *P sajor-caju* extracts prepared at 4°C revealed an $IC_{50(\%)}$ of 0.23% ± 0.08%, and the mushroom extracts prepared at 22°C and 50°C showed proportionately increased values of 0.64% ± 0.02% and 1.17% ± 0.03%, respectively. When HeLa cells were treated with 4°C extracts of *P sajor-caju*, an $IC_{50(\%)}$ of 0.31% ± 0.01% was observed, which is higher than half of the maximal inhibitory concentration observed with the 22°C extracts, $IC_{50(\%)}$ of 0.25% ± 0.02%, and lower than the 50°C tested extract, $IC_{50(\%)}$ of 1.21% ± 0.01% (Table 2). The concentrations used for the HeLa and Hep-2 cells ranged from 0.05% to 1.5% (wt/vol) for the *P sajor-caju* extracts, and the data are given in Fig. 1.

Using the cell line Hep-2 after treatment with mushroom extracts derived at 4°C from *L edodes*, an $IC_{50(\%)}$ of 0.46% ± 0.08% was obtained. The extracts obtained at 22°C showed an $IC_{50(\%)}$ of 0.78% ± 0.02%, and those derived 50°C showed $IC_{50(\%)}$ of 1.03% ± 0.04%. The HeLa cell treatment using *L edodes* extracts at 4°C showed an $IC_{50(\%)}$ of 0.74% ± 0.02%, a higher value than that from the extract obtained at 22°C, which showed an $IC_{50(\%)}$ of 0.57% ± 0.01%. However, the $IC_{50(\%)}$ for *L edodes* extracts at 4°C was lower than half the maximal inhibitory concentration rate observed for the extracts derived at 50°C, with an $IC_{50(\%)}$ of 0.91% ± 0.07%. The concentrations used for the HeLa and Hep-2 cells ranged from 0.05% to 1% (wt/vol) for the *L edodes* extracts, and variances are depicted in Fig. 2.

The effect on tumor cell survival after mushroom extract treatment varied not only according to the temperature used for extraction but also according to the extract concentration, as shown in Fig. 1 (*P sajor-caju*) and Fig. 2 (*L edodes*). The changes in cell morphology following treatment with both mushroom extracts included a series of cellular modifications, including cell shrinkage, which suggests the induction of apoptosis as a consequence of exposure to the extract (Fig. 3). We monitored changes in the cell morphology under the microscope and observed that cell modifications were observed initially in the group treated with *P sajor-caju* extracts, 30 minutes after extract incubation. In contrast, cellular modifications in samples treated with *L edodes* extract were only detectable after 1 hour of incubation, after the cells had been cultured with free-extract medium for 24 hours. Furthermore, treatment with *L edodes* extracts caused less extreme changes (Fig. 3B and F) compared with incubation with *P sajor-caju* extracts (Fig. 3D and H). Samples treated exclusively with water are shown as the negative controls in Fig. 3A, C, E, and G and show uniform cell morphology.

4. Discussion

Most studies using mushroom extracts to test in vitro biological responses have focused on nonaqueous extraction protocols. Here, we provide experimental evidence that a water-based extraction protocol is sufficient to assess the inhibitory effects on tumor cells; this suggests the existence of active, polar constituents in mushroom extracts, which supports the hypothesis of our study. Previously, we have

Table 1 – Total polyphenol content and antioxidant activity of *P sajor-caju* and *L edodes* using different extraction temperatures

Samples		Total polyphenol content (mg% catechin)	DPPH scavenging ability ($IC_{50(\%)}$) *	SOD-like activity ($IC_{50(\%)}$) †	CAT-like activity (nmol H_2O_2/min)
P sajor-caju	4°C	35.22 ± 0.28[a]	10.38 ± 0.19[d]	4.30 ± 0.33[a]	3.19 ± 0.80[ab]
	22°C	34.85 ± 0.40[a]	9.68 ± 0.15[c]	4.38 ± 0.66[a]	3.75 ± 0.01[bc]
	50°C	35.98 ± 0.14[a]	9.01 ± 0.14[b]	6.76 ± 0.39[a]	3.56 ± 0.27[ab]
L edodes	4°C	55.26 ± 0.51[b]	3.31 ± 0.05[a]	5.54 ± 0.86[a]	6.28 ± 0.13[d]
	22°C	55.29 ± 0.47[b]	3.28 ± 0.05[a]	4.34 ± 1.44[a]	5.06 ± 0.27[cd]
	50°C	55.63 ± 0.08[b]	3.45 ± 0.06[a]	4.72 ± 0.20[a]	2.25 ± 0.01[a]

The results are presented as the means ± SD. The results represent the averages of 3 independent experiments performed in triplicate. Different letters represent different values for each assay, according to analysis of variance and post hoc Tukey tests ($P < .05$).

* Concentration (percentage) of the samples needed to scavenge 50% of the DPPH radicals.

† Microliter of the extracts needed to inhibit 50% of the formation of adrenochrome.

tested a 100°C extraction protocol using decoction and observed no significant difference in the viability of tumor cell lines after treatment for 1 hour. The absence of extract activity at high temperatures may be related to a decrease in the concentration of polysaccharides caused by internal β-glucanase activity [35].

Different extraction temperatures showed no influence on the content of total polyphenols. *L edodes* extracts, however, presented a higher concentration of these compounds than those of *P sajor-caju*. Phenolic compounds are important to provide protection against several degenerative diseases in humans, including brain dysfunction, cancer, and cardiovascular diseases [36,37]. The best described property of almost every group of polyphenols is their capacity to act as antioxidants, which can scavenge free radicals and reactive oxygen species [38,39]. In fact, higher scavenging activity for the DPPH radical was observed for the extracts of *L edodes*, which presented a higher CAT-like activity (using the 4°C and 22°C extraction methods) than those of *P sajor-caju*. With the 50°C extraction protocol, a significant decrease in CAT-like activity was observed for the extract of *L edodes*. This effect is most likely due to a chemical alteration in the active compounds present in this mushroom caused by the use of a higher temperature during extraction. The high antiproliferative activity exhibited by the *P sajor-caju* extract may be a result of its specific proteins, terpenoids, steroids, fatty acids, and phenolic compounds. This extract was also shown to possess bioactive effects that may be relevant for health homeostasis, such as immunomodulation, antihypertension, cytotoxic, antibacterial, and pro-oxidative effects [40,41]. No major difference in SOD-like activity was found between the 2 mushrooms. Superoxide dismutase and CAT enzymes have an important role in maintaining the physiologic redox equilibrium. Superoxide dismutase catalyzes the dismutation of the superoxide anion ($O_2^{\bullet-}$) in H_2O_2, and CAT catalyzes the direct decomposition of H_2O_2 to ground-state O_2 [42].

A positive correlation between substances with antioxidant activity and the inhibition of tumor cell proliferation is well established, suggesting that the antioxidant properties of extracts influence anticancer activity. Yet, the underlying mode of action still remains to be elucidated. Antioxidant activity is related directly to the reactive species involved in many aspects of carcinogenesis as well as the mechanisms of carcinogenesis, including proliferation, induction of senescence, apoptosis facilitation triggered by other agents, the suppression of apoptosis, and the initiation of DNA damage [43].

According to the cytotoxic activities, the *P sajor-caju* extracts were more effective against tumor cells compared with the extracts of *L edodes*, with the exception of the extracts prepared at 50°C. We observed a great variation in cell viability after incubation with the extracts of *P sajor-caju* and *L edodes* within the concentration range from 0.05% to 1.5%. Inhibitory effects were greater in tumor cells treated with *P sajor-caju* extracts obtained at 4°C and 22°C in both cell lines tested. In contrast, a higher concentration of *P sajor-caju* extracts prepared at 50°C was needed to achieve similar inhibition of tumor cell viability compared with the extracts prepared at lower temperatures. *P sajor-caju* aqueous extracts obtained at 50°C showed a lower cytotoxic activity than those from treatment with *L edodes* aqueous extracts. Consequently, lower concentrations of *L edodes* were needed to achieve the same 50% inhibitory concentration rate.

To further investigate the kinetics and mechanisms of action for the extracts, we monitored the changes in cell morphology by microscopic inspection during and after the treatment with the extracts obtained at 22°C. A comparison of the inhibition rate revealed that the extracts derived from *P*

Table 2 – Cytotoxic activity $IC_{50(\%)}$ of *P sajor-caju* and *L edodes* extracts using different extraction temperatures

		Cytotoxic activity $IC_{50(\%)}$ [a]		
		4°C	22°C	50°C
P sajor-caju	Hep-2	0.23 ± 0.08[a]	0.64 ± 0.02[b]	1.17 ± 0.03[c]
	HeLa	0.31 ± 0.01[a]	0.25 ± 0.02[a]	1.21 ± 0.01[c]
L edodes	Hep-2	0.46 ± 0.08[a]	0.78 ± 0.02[c]	1.03 ± 0.04[d]
	HeLa	0.74 ± 0.02[c]	0.57 ± 0.01[b]	0.91 ± 0.07[c]

The results represent the averages of 3 independent experiments performed in triplicate. The relative expression levels are presented as the *n*-fold increases compared with the control group across the same lines, and different superscript lowercase letters indicate the results that were significantly different with $P < .05$ by analysis of variance and post hoc Tukey tests. The results are presented as the means ± SD.

[a] Cytotoxic activity was assessed by MTT assay. The $IC_{50(\%)}$ values (dose causing 50% cell death) were calculated using dose–response curves for each condition.

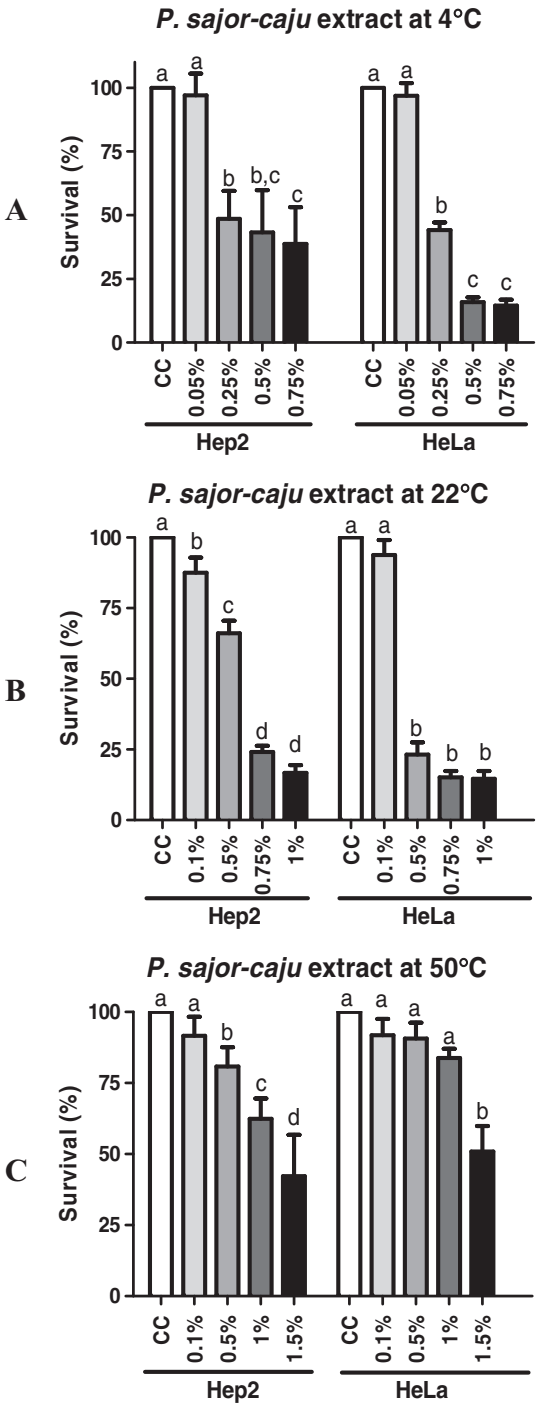

Fig. 1 – Effect of water extract from *P sajor-caju* mushroom grown in different concentrations on the of Hep-2 and HeLa cells. Values are expressed in means ± SD, and statistical analyses used Tukey test. A, Extract at 4°C; B, Extract at 22°C; C, Extract at 50°C. Different letters represent statistical significance among groups.

sajor-caju were more effective in inducing cytotoxicity in cells, thereby causing changes after 1 hour of treatment, whereas morphological modifications caused by *L edodes* were only observed 24 hours after treatment during incubation with the extract-free medium.

According to Ooi and Liu [44], morphologic analysis of cells indicates that the mushroom extract may initiate apoptotic mechanisms to trigger cell death. Tumor growth is known to be regulated by the balance between cell proliferation and apoptosis. Deregulated cell proliferation and suppressed cell death together provide the underlying basis for neoplastic

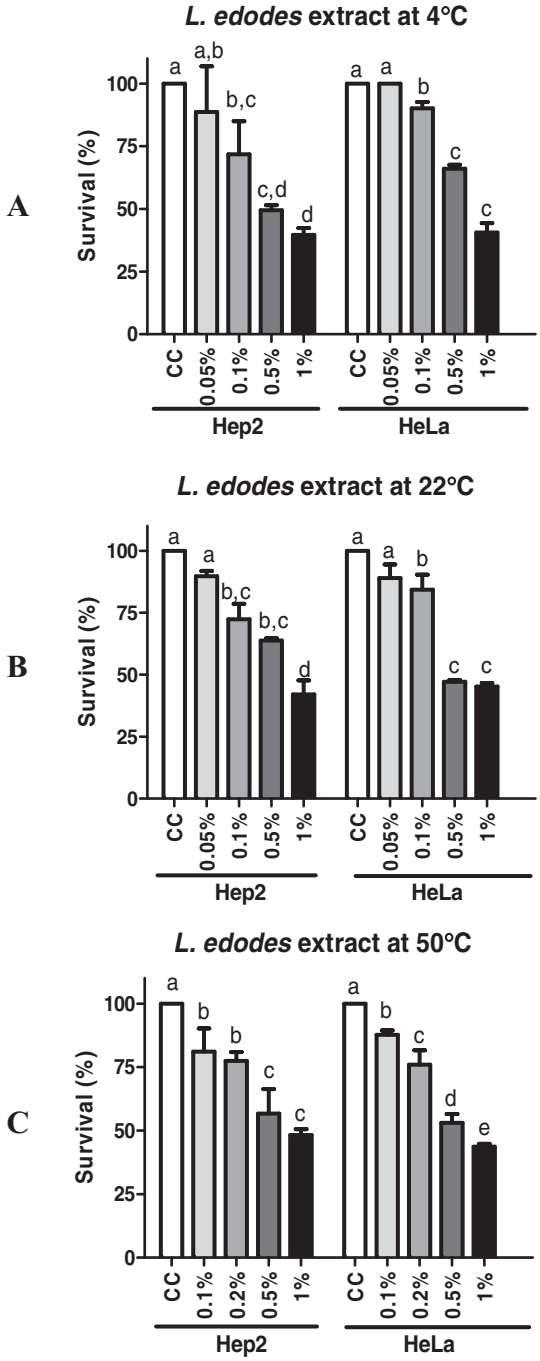

Fig. 2 – Effect of water extract from *L edodes* mushroom grown in different concentrations on the of Hep2 and HeLa cells. Values are expressed in means ± SD, and statistical analyses used Tukey test. A, Extract at 4°C. B, Extract at 22°C. C, Extract at 50°C. Different letters represent statistical significance among groups.

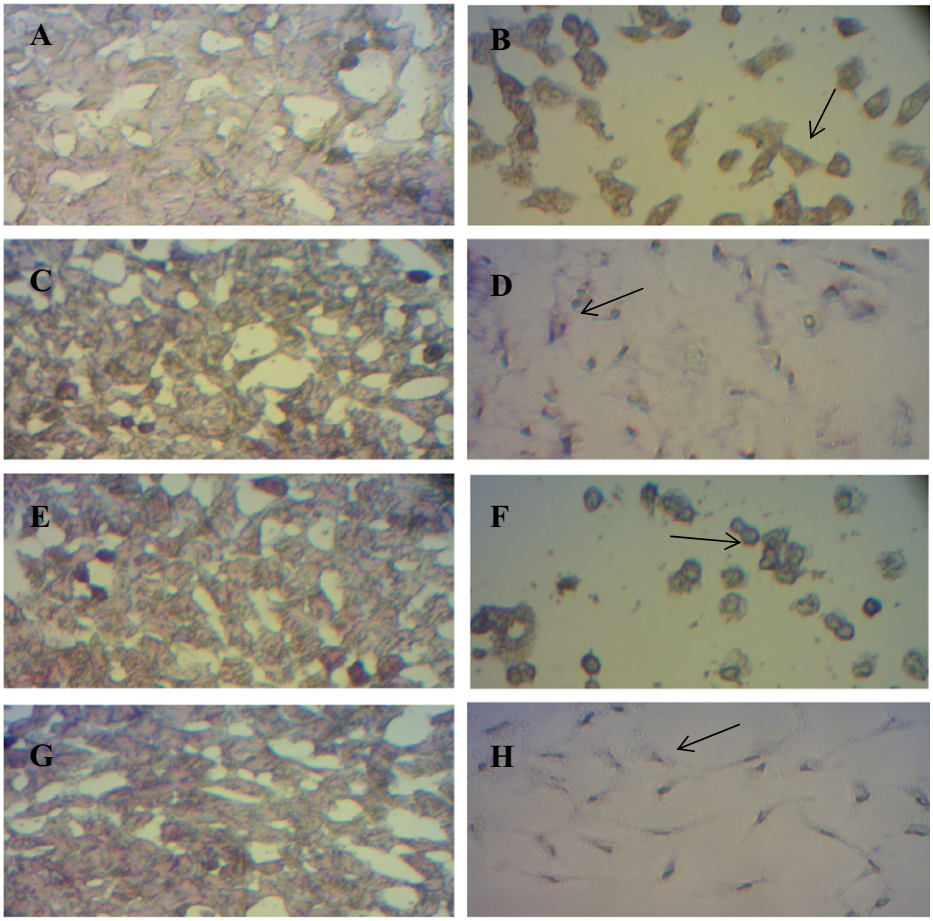

Fig. 3 – Morphological analysis of cell lines after mushroom extract treatment using the 22°C extraction protocol: Hep-2—control (A); Hep-2—$IC_{50(\%)}$ *L edodes* extract (B); Hep-2—control (C); Hep-2—$IC_{50(\%)}$ *P sajor-caju* extract (D); HeLa—control (E); HeLa—$IC_{50(\%)}$ *L edodes* extract (F); HeLa—control (G); and HeLa—$IC_{50(\%)}$ *P sajor-caju* extract (H). The arrows in "B," "D," "F," and "H" show representative cells after treatment with the extract from *L edodes* and *P sajor-caju*.

transformation and malignant progression. Consequently, 1 essential strategy for cancer therapy is to target lesions that suppress apoptosis in tumor cells [45]. It has been found that many cancer chemotherapeutic drugs exert anticancer effects on malignant cells by inducing apoptosis [5,46].

Although the underlying mode of action of the mushroom extracts on tumor cell physiology is still unclear, water-soluble heteroglycans and insoluble β-glucans have already been isolated from this species and may be involved in their antitumor activity [47,48]. So far, β-glucans are well known for their biological activity specifically related to the immune system, which differs from the mechanisms observed for conventional chemotherapeutic agents [20,49]. However, activating and reinforcing the host immune system seem to be the best strategy for inhibiting the growth of cancer cells [50-52].

According to Fang et al [21] and Wu et al [53], effective adjuvant substances from edible or medical mushrooms are capable of activating the cellular apoptotic response in cancer. One example is lectin [54], which is isolated from *Lentinula* species and potently inhibits the growth of sarcoma and hepatoma cells and prolongs the life spans of tumor-bearing mice [55,56]. Lentinan, from *L edodes*, is currently used in the clinic (ie, 0.5-1.0 mg lentinan per day, intravenous), especially in Japan and China, as an adjuvant tumor therapy for other cancer therapies such as surgical resection, radiotherapy, and chemotherapy [8,57-59]. Many interesting biological activities of lentinan have been investigated, including the activation of nonspecific inflammatory responses, such as acute phase protein production [60], vascular dilation, and hemorrhage-inducing factors in vivo [61,62] as well as the activation and generation of helper and cytotoxic T cells [63]. There are many other examples of isolated mushroom compounds with beneficial properties against cancer [8]. Maity et al [47] observed the in vitro activation of peritoneal macrophages stimulated with different concentrations of the heteroglycan isolated from *Pleurotus ostreatus*. Zhuang et al [24] discovered a protein present in *P sajor-caju* that showed antitumor effects [15,64] and antioxidant properties [22,65]. A ribonuclease isolated from *P sajor-caju* presented antimicrobial, antimitogenic, and antiproliferative activities [66]. Polysaccharides of other mushrooms that have been investigated include schizophyllan [67], active hexose-correlated compounds, maitake D-fraction [68], polysaccharide-K and polysaccharide-P [50],

scleroglucan [69] and grifolan [70], among others, and glucans always present strong antitumor effects [71].

Our results provide experimental evidence that aqueous extracts of the mushrooms *L edodes* and *P sajor-caju* grown in South Brazil are potential sources of antioxidant and anticancer compounds. We used low temperature (<50°C) water extracts and achieved striking results, in contrast to previous experiments using hot water–based extracts. Therefore, it is necessary to prepare extracts immediately before their use to guarantee the stability of the components. Further investigations, however, are needed to explore the biological properties of *L edodes* and *P sajor-caju* and to elucidate the molecular mode of action against tumor cell proliferation and the induction of apoptosis. It is known that most drugs isolated for cancer therapy are not cancer specific and, therefore, may be highly toxic to normal tissues, leading to serious adverse effects. Mushroom extracts might be considered alternative sources for adjuvant cancer therapy, as they have no adverse effects, activate the cells of the immune system, and reduce free radicals. Further studies, however, including the isolation and chemical characterization of the major compounds that contribute to the promotion of the immune system and to the inhibition of carcinogenesis, are needed and may generate new targets for therapy.

Acknowledgment

This work is supported by a grant from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.nutres.2012.11.005.

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