South African Journal of Botany 113 (2017) 62–67

Contents lists available at ScienceDirect

South African Journal of Botany

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

Cytotoxicity of thirteen South African macrofungal species against five cancer cell lines

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ARTICLE INFO

Article history:
Received 18 November 2016
Received in revised form 4 June 2017
Accepted 28 July 2017
Available online xxxx

Edited by WA Stirk

Keywords:
Cytotoxicity
Macrofungi
Pycnoporus
Fomitopsis
Gymnopilus
HeLa
HT-29
MCF-7
MIA PaCa-2
PC-3
Apoptosis

ABSTRACT

Fungi are an important source of traditional medicine in Asian and Western countries. Globally, cancer is one of the leading causes of death with 22 million cases predicted by 2030. The cytotoxicity of 13 South African macrofungal species against HeLa, HT-29, MCF-7, MIA PaCa-2 and PC-3 cancer cell lines was measured using the 3-(4,5-dimethyl-2-thiazolul)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, and the IC_{50} values determined for the most cytotoxic macrofungal species. *Fomitopsis lilacinogilva* had IC_{50} values of 42.2, 55.1, 63, 48.8 and 79.8 μg/mL against the HeLa, HT-29, MCF-7, MIA PaCa-2 and PC-3 cancer cell lines, respectively. *Gymnopilus junonius* had IC_{50} values of 55.1 and 52.5 μg/mL against the HeLa and MIA PaCa-2 cancer cell lines, respectively. *Pycnoporus sanguineus* had IC_{50} values of 24.2, 48.1, 32.7, <10 and 28.6 μg/mL against the HeLa, HT-29, MCF-7, MIA PaCa-2 and PC-3 cancer cell lines, respectively. Apoptosis of HeLa cancer cells induced by *F. lilacinogilva*, *G. junonius* and *P. sanguineus* was confirmed using Hoechst and phalloidin staining. Morphological changes included condensed chromatin, membrane blebbing, loss of cytoskeletal arrangement and rounding up of cells, as well as multi- and micronuclei. Macrofungal research in South Africa remains a novel field, and this study provides interesting data on the cytotoxicity of South African macrofungal species against some of the most common cancers affecting humans worldwide.

1. Introduction

Fungi have been used for the past two thousand years in Asian countries (including China, Japan, Korea and Russia), and more recently in Western countries (including the USA and Canada) for their medicinal properties. It is estimated that 1.5 million species of fungi exist, of which approximately 140,000 species qualify as mushrooms. About 14,000 mushroom species have been described (Chatterjee et al., 2011). An estimated 2000 species are edible and 200 species have traditionally been gathered for food and medicinal usage (Erjavec et al., 2012).

Different mushroom species have been identified and shown to play an important role in the prevention and treatment of several human diseases. Mushroom polysaccharides, polysaccharide-peptides, polysaccharide-protein complexes, lectins, phenolics, fungal immunomodulatory proteins, ribosome inactivating proteins, antimicrobial proteins, ribonucleases and laccases are only a few of the bioactive agents isolated from mushrooms. Lenitinan, schizophyllan and krestin are mushroom derived polysaccharides used in Japan, China and Korea as immunoceuticals (Ng, 2004; Kozarski et al., 2011; Xu et al., 2011; Erjavec et al., 2012). Biological activities of mushrooms include antimicrobial, antioxidant, anti-inflammatory, antiallergic, antiatherogenic, hypocholesterolemic, hypolipidemic, antifibrotic, antidiabetic, antitumour, immunomodulatory and neuroprotection to name a few (Maiti et al., 2008; Das et al., 2010; Guillamon et al., 2010; Chatterjee et al., 2011, Sabaratnam et al., 2013).

Cancer is characterized by the rapid proliferation of abnormal cells containing mutations, which can affect any part of the body and spread to other parts of the body/organs. The spread of cancer from a primary to a secondary site is known as metastases. Cancer accounted for about 8.2 million deaths in 2012; making it one of the leading causes of death worldwide. Cancers claiming the most lives include lung, liver, stomach, colorectal and breast cancer. More than 60% of the world's new annual cancer cases occur in Africa, Asia and Central and South America, which accounts for 70% of the world's cancer deaths. It is forecasted that annual cancer cases will rise from 14 million in 2012 to 22 million in 2030 (WHO). The top five cancers affecting South

Abbreviations: DMSO, dimethyl sulfoxide; DPBS, Dulbecco's phosphate buffered saline; HeLa, human cervical adenocarcinoma; HT-29, human colorectal adenocarcinoma; MCF-7, human breast adenocarcinoma; MIA PaCa-2, human pancreatic ductal adenocarcinoma; MTT, 3-(4,5-dimethyl-2-thiazolul)-2,5-diphenyl-2H-tetrazolium bromide; PC-3, human prostate adenocarcinoma; Vero, normal African green monkey kidney cells.

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http://dx.doi.org/10.1016/j.sajb.2017.07.010
0254-6299/

African men are prostate, unknown origin, lung, Kaposi sarcoma and colorectal cancer. The top five cancers affecting South African women are breast, cervical, unknown origin, Kaposi sarcoma and colorectal cancer. Unknown origin means that it was not possible to determine where the cancer originated in the body (CANSA).

Macrofungal species with known cytotoxic and/or anticancer properties include *Ganoderma lucidum* (reishi), *Pleurotus ostreatus* (oyster), *Cordyceps militaris*, *Hericium erinaceus* (lion's mane), *Inocybe umbrinella*, *Lentinula edodes* (shiitake), *Grifola frondosa* (maitake), *Inonotus obliquus*, *Schizophyllum commune*, and others (Patel and Goyal, 2012).

Compared to animals and plants, fungi have been poorly documented and studied due to collection and identification difficulties. The pharmacological potential of macrofungi remains largely unexplored. The main aim of this study was to identify South African macrofungal species with cytotoxic properties against five cancer cell lines, namely cervical-, colorectal-, breast-, pancreatic- and prostate cancer.

2. Materials and methods

2.1. Materials/reagents/chemicals

HeLa (human cervical adenocarcinoma) cancer cell line was purchased from Cellonex (South Africa). HT-29 (human colorectal adenocarcinoma) and MCF-7 (human breast adenocarcinoma) cancer cell lines were purchased from Highveld Biological (South Africa). PC-3 (human prostate adenocarcinoma) and MIA PaCa-2 (human pancreatic ductal adenocarcinoma) cancer cell lines were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Vero (African green monkey kidney) normal cell line was purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA). 3-(4,5-dimethyl-2-thiazolul)-2,5-diphenyl-2H-tetrazolium bromide (MTT), melphalan, cisplatin, bisBenzimide H33342 trihydrochloride and phalloidin tetramethylrhodamine B isothiocyanate were purchased from Sigma (St. Louis, MO, USA).

2.2. Macrofungal material and preparation of extracts

Only fruiting bodies of the various species were collected. *Amanita foetidissima* D.A. Reid & Eicker. was collected in Despatch, Eastern Cape, South Africa. *Fomitopsis lilacinogilva* (Berk.) J.E. Wright & J.R. Deschamps, *Ganoderma lucidum* (Curtis) P. Karst, *Gymnopilus junonius* (Fr.) P.D. Orton, *Hypholoma fasciculare* (Huds.:Fr.) P. Kumm., *Lenzites elegans* (Spreng.) Pat., *Pisolithus tinctorius* (Pers.) Coker & Couch, *Pycnoporus sanguineus* (L.) Murrill, *Russula capensis* A. Pearson, *Schizophyllum commune* Fries, *Stereum hirsutum* (Wild.) Pers. and *Imleria badia* (Fr.) Vizzini were collected in Plettenberg Bay/Knysna, Western Cape, South Africa. *Pleurotus ostreatus* (Jacq.Ex Fr.) P. Kumm. was bought at a local supermarket. The identity of these mushrooms was confirmed morphologically using South African mushroom guides (Gryzenhout, 2010; Branch, 2001; van der Westhuizen and Elcker, 1994; Levin et al., 1987) and confirmed by the Mushroom Guru (Pty) Ltd (Somerset West, South Africa, Fig. 1). Voucher specimens were deposited in the Nelson Mandela Metropolitan University (Port Elizabeth, South Africa) herbarium (Table 1). Macrofungal material was dried in a 25–30 °C oven for 2–3 days. Macrofungal material was briefly submerged in liquid nitrogen and crushed using a mortar and pestle. Ethanolic (80%) extracts were prepared by using a material: solvent ratio of 1:15 (w/v) for 24 h at room temperature with frequent mixing. Extracts were centrifuged at 1800 ×*g* for 5 min, and the supernatant filtered twice through Whatman No. 1 filter paper under vacuum. Ethanol was evaporated using a BUCHI Rotavapor R-210 (Switzerland) rotary evaporator at 50 °C and the extract freeze dried using a VirTis SP Scientific sentry 2.0 freeze dryer (Gardiner, NY, USA). Macrofungal extracts were stored in a desiccator in the dark at 4 °C until further use. Table 1 summarizes the common names, voucher specimen numbers, status, usage and percentage yield of ethanol extracts of the 13 South African macrofungal species used in this study.

2.3. Cell culture conditions

HeLa, HT-29 and MCF-7 cells were routinely maintained in RPMI1640 medium (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum (HighClone Laboratories, Inc., South Logan, Utah, USA) in the presence of penicillin-streptomycin (100 U/mL, Lonza, BioWhittaker, Verviers, Belgium). MIA PaCa-2 cells were routinely maintained in Eagle's minimum essential medium (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum and 1% non-essential amino acids (Sigma, St. Louis, MO, USA) in the presence of penicillin-streptomycin. PC-3 cells were routinely maintained in F12 Kaighn's modification medium (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum in the presence of penicillin-streptomycin. Vero cells were routinely maintained in low glucose DMEM (HighClone Laboratories, Inc., South Logan, Utah, USA) supplemented with 10% fetal bovine serum in the presence of penicillin-streptomycin. Cells were incubated in a humidified 5% CO_2 incubator at 37 °C.

2.4. Cytotoxicity and IC_{50} determination

HeLa, HT-29, MCF-7, MIA PaCa-2, PC-3 and Vero cells were seeded in 96-well plates at cell densities of 6000 cells/100 μL/well. Cells were allowed to attach overnight at 37 °C and 5% CO_2 in a humidified incubator. Stock concentrations of macrofungal extracts were prepared in dimethyl sulfoxide (DMSO) at 100 mg/mL. Working concentrations (400 μg/mL) were prepared in complete medium (i.e. medium, fetal bovine serum and penicillin-streptomycin in presence/absence of non-essential amino acids) and dilutions, ranging between 10 and 200 μg/mL, tested. Melphalan and cisplatin were used as positive controls at working concentrations ranging between 1.56 and 200 μM. DMSO (0.25%, v/v) was used as a negative/vehicle/solvent control. Cells were treated for 48 h at 37 °C and 5% CO_2 in a humidified incubator.

Cell viability was determined using the MTT assay as described by Holst-Hansen and Brünner (1998), and the absorbance read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA). IC_{50} values were determined using GraphPad Prism Version 5.01 (GraphPad Prism Software, San Diego, USA).

2.5. Hoechst and phalloidin staining for apoptosis

HeLa cells were seeded in 96-well plates at cell densities of 10,000 cells/100 μL/well. Cells were allowed to attach overnight at 37 °C and 5% CO_2 in a humidified incubator. Stock concentrations of macrofungal material were prepared in DMSO at 100 mg/mL. Ethanol extracts of *F. lilacinogilva*, *G. junonius* and *P. sanguineus* were tested at IC_{50} concentrations (see Table 2). Melphalan (65.9 μM) was used as positive control. DMSO (0.25%, v/v) was used as a negative/vehicle/solvent control. Cells were treated for 48 h at 37 °C and 5% CO_2 in a humidified incubator. For phalloidin staining, cells were fixed in formaldehyde (37%, 20 μL/well) for 15 min at room temperature and washed three times with Dulbecco's phosphate buffered saline (DPBS; with Ca^{2+}/Mg^{2+}, 200 μL). Cells' filamentous actin cytoskeletons were stained with phalloidin-TRITC (1 μM; 100 μL) in the dark for 15 min at 37 °C, followed by washing (two times) with DPBS. Nuclei were stained with Hoechst 33342 (5 μg/mL, 100 μL) and images taken with a Molecular Devices ImageXpress® Micro XLS widefield microscope (CA, USA) using the TRITC and DAPI filters, and 40x objective.