CYTOTOXICITY INDUCED BY EXTRACTS OF *Pisolithus tinctorius* SPORES ON HUMAN CANCER AND NORMAL CELL LINES—EVALUATION OF THE ANTICANCER POTENTIAL

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Fungi have been considered a potential source of natural anticancer drugs. However, studies on these organisms have mainly focused on compounds present in the sporocarp and mycelium. The aim of this study was to assess the anticancer potential of fungal spores using a bioassay-guided fractionation with cancer and normal cell lines. Crude extracts from spores of the basidiomycetous fungus *Pisolithus tinctorius* were prepared using five solvents/solvent mixtures in order to select the most effective crude extraction procedure. A dichloromethane/methanol (DCM/MeOH) mixture was found to produce the highest extraction yield, and this extract was fractionated into 11 fractions. Crude extracts and fractions were assayed for cytotoxicity in the human osteocarcinoma cell line MG63, the human breast carcinoma cell line T47D, the human colon adenocarcinoma cell line RKO, and the normal human brain capillary endothelial cell line hCMEC/D3. Cytotoxicity was assessed by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay. The results showed a reduction in cancer cell viability of approximately 95% with 4 of 11 fractions without a significant reduction in viability of hCMEC/D3 cells. Data demonstrated that spores of *P. tinctorius* might serve as an interesting source of compounds with potential anticancer properties.

Cancer is the foremost cause of mortality worldwide, and, despite progress in anticancer therapies, cancer treatment is still regarded as a major challenge in pharmacology. In the last decades, natural compounds have gained significant importance in treatment of cancer. Among organisms, fungi have been considered as an interesting source of natural drugs with potential anticancer properties (Borchers et al., 1999; Nishino et al., 2000; Moradali et al., 2007; Popovic et al., 2013; Liu et al., 2014). Used since antiquity in oriental civilizations, fungi were reported to limit or reverse tumor growth (Kidd, 2000), and some fungal compounds such as triterpenoids and triterpenes were shown to possess anticancer potential by inducing apoptosis in several tumor cells (Petronelli et al., 2009).

Pisolithus tinctorius (Basidiomycota) is a soil fungus commonly found in forest ecosystems and known to form ectomycorrhizal symbioses with plant roots (Sousa et al., 2011,

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2012; Oliveira et al., 2012). The pharmacological potential of this fungus was reported in several studies, in particular its antimicrobial potential (Tsantrizos et al., 1991; Ameri et al., 2011) and antiobesity effects (Ahn et al., 2007). Concerning the production of compounds with anticancer properties, the triterpene pisosterol isolated from the sporocarps (Montenegro et al., 2004) was found to exhibit antitumor activity against several human cancer cell lines (Montenegro et al., 2007, 2008; Burbano et al., 2009; Silva et al., 2010; Pereira et al., 2011), and thus this fungal species was considered as a potential source of anticancer compounds.

Studies on natural compounds from fungi and particularly from *P. tinctorius* were conducted using sporocarp and mycelium (Tsantrizos et al., 2001; Montenegro et al., 2004; Zamuner et al., 2005). However, no apparent studies focused on the anticancer potential of other structures such as spores. Considering that fungi are an interesting source of natural compounds and since *P. tinctorius* has a global distribution in forests, this study aimed to examine the anticancer potential of *P. tinctorius* spores by evaluating cytotoxicity in human cancer cell lines and normal cells.

MATERIALS AND METHODS

Pisolithus tinctorius Spores

Basidiocarps of *Pisolithus tinctorius* (Pers.) Coker & Couch were collected from a forest ecosystem in northern Portugal, cut into small pieces, and freeze dried for 48 h (FreeZone Freeze Dry System). The spores were then separated from the sporocarps by using a 1-mm sieve.

Pisolithus tinctorius Spores Crude Extracts

Five solvents/solvent mixtures were tested in order to identify the best extraction yield. Five hundred milligrams of spores was extracted separately with acetone, ethyl acetate (EtOAc), dichloromethane (DCM), and the mixtures DCM/MeOH (2:1) and EtOAc/MeOH (2:1) (Table 1). Spores suspensions were sonicated for 3 min and centrifuged at 6500 × g for 10 min. The pellets were reextracted three times and volumes were combined. Final extracts were filtered three times with Whatman filter paper number 1 and evaporated in a rotatory evaporator with reduced pressure (BUCCHI Rotavapor R-210, BUCCHI Vacuum Pump V-700). The crude extracts were chromatographed on a high-performance liquid chromatography (HPLC) system. This was performed with a Waters e2695 Separation Module and Waters 2998 Photodiode Array Detector, using a Lichrospher RP-C18 column (250 × 4 mm, 5 µm) kept at 30°C. According to the extraction yield, DCM/MeOH and EtOAc/MeOH extracts were resuspended in dimethyl sulfoxide (DMSO) at concentrations of 100, 10, and 1 mg/ml and assayed for anticancer potential as described below. Based on cytotoxicity results, DCM/MeOH extract was selected for fractionation.

DCM/MeOH Crude Extract Fractionation

Spores (20.43 g) were extracted with DCM/MeOH (2:1) as already described. Fractionation was performed by vacuum liquid chromatography using a silica gel 60 (Merk) (0.015–0.04 mm mesh) as the stationary phase and using *n*-hexane as the initial eluent. Eleven fractions (A–K) with increasing polarity were prepared (Table 2). After evaporation in a rotatory evaporator with reduced pressure, dried fractions were resuspended in DMSO to a final concentration of 10 mg/ml.

Cancer Cell Lines

The anticancer potential of the crude extracts and fractions of *P. tinctorius* spores was tested against human osteosarcoma cell line MG63, human breast cancer cell line T47D, and human colon adenocarcinoma cell line RKO. The cytotoxicity was also evaluated using normal human brain capillary endothelial cells hCMEC/D3. RKO and MG63 cell lines were obtained from the American Type Culture

TABLE 1. Solvents and Solvent Mixtures Used to Prepare Crude Extracts, the Dried Amount Recovered, and the Respective Extraction Yield

Solvent/mixtures	Volume (ml)	Weight of spores (mg)	Extract dry weight (mg)	Extraction yield (%)
Acetone	75	500	26.5	5.3
DCM:MeOH (2:1)	75	500	83.8	16.8
EtOAc:MeOH (2:1)	75	500	58.0	11.6
EtOAc	75	500	21.8	4.4
DCM	75	500	35.2	7.0

Note. DCM, dichloromethane; MeOH, methanol; EtOAc, ethyl acetate.

TABLE 2. Fractionation of the Dichloromethane/Methanol Crude Extract in 11 Fractions, A–K

		Solvent volume (ml)			
Fraction	Solvent composition	Hexane (Hex)	Ethyl acetate (EtOAc)	Methanol (MeOH)	Total
A	100% Hex	250	0	0	250
B	20% EtOAc/80% Hex	120	30	0	150
C	40% EtOAc/80% Hex	90	60	0	150
D	50% EtOAc/50% Hex	75	75	0	150
E	60% EtOAc/40% Hex	60	90	0	150
F	80% EtOAc/20% Hex	30	120	0	150
G	100% EtOAc	0	250	0	250
H	75% EtOAc/25% MeOH	0	225	75	300
I	25% EtOAc/75% MeOH	0	137	413	550
J	100% MeOH	0	0	500	500
K	100% MeOH	0	0	500	500

Collection (ATCC), T47D cells were purchased from Sigma-Aldrich and hCMEC/D3 cells were kindly donated by Dr. P. O. Couraud (INSERM, France). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, GlutaMAX, Life Technologies), pH 7.4, supplemented with 10% fetal bovine serum, 2.5 μg/ml fungizone, and penicillin–streptomycin (100 IU/ml and 100 μg/ml, respectively). All culture reagents were purchased from Invitrogen. Cells were incubated at 37°C with a 5% CO_2 humidified atmosphere, and culture medium was renewed every 2 d. At 80–90% cell confluence, adherent cells were released with TrypLE Express Enzyme (Gibco).

Cytotoxic Assay

Cellular viability was assessed by reduction of 3-(4.5-dimethylthiazole-2-yl)-2.5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) (Correia et al., 2014). Cells were seeded in 96-well plates at a density of 3.3×10^4 cell/ml in 100 μl medium, and incubated for 24 h for cell adhesion. Cells were exposed to crude extracts and fractions in fresh medium for 24, 48, and 72 h. One microliter of each crude extract and fraction was diluted in 100 μl of medium, in order to test 1, 0.1, and 0.01 mg/ml for crude extracts and 0.1 mg/ml for fractions. After treatment, cells were incubated for 3 h at 37°C with 0.05 mg/ml MTT. The purple-colored formazan salts were dissolved in DMSO, and absorbance was read at 550 nm in a microplate reader (Bio-tek Synergy™ HT). The assays were run in triplicate and averaged. Cytotoxicity was expressed as percent cell viability, considering 100% viability in the control (cells treated with 1% DMSO).

Statistical Analysis

All statistical analyses were performed using the SPSS 20.0.0 software package (IBM SPSS Statistics, USA). Data were presented as mean ± standard deviation (SD) of three replicates. The criterion for significance was set at $p < .05$.

RESULTS

Assay to Select Extraction Solvents

Considering the spores extraction procedure, the mixtures DCM/MeOH and EtOAc/MeOH yielded the highest extraction (16.8% giving 83.8 mg of recovered dried weight extract, and 11.6% giving 58 mg of dry weight, respectively) (Table 1). These extracts were chromatographed using HPLC in order to record their chromatographic profile. The chromatographic profile of the DCM/MeOH extract is shown in Figure 1. These crude extracts were screened for cytotoxicity and results were similar, with a marked decrease in cell viability at 1 mg/ml (8% and 12% viable cells at 72 h, for DCM/MeOH and EtOAc/MeOH, respectively) (Figure 2). However, the extract obtained with the DCM/MeOH solution revealed a more pronounced cytotoxicity and was thus chosen for fractionation.

DCM/MeOH Extract Fractionation and Cytotoxicity

The fractionation of the DCM/MeOH crude extract rendered 11 fractions reflecting a segregation of compounds with increasing polarity (Table 2). Cytotoxic activity of the different fractions (except fraction B due to the small amount of material obtained) was tested against three human cancer cell lines and normal endothelial cell line for selectivity. The crude extract in all cancer cells decreased significantly cell viability, notably to 12% in RKO and MG63 cells and 6% in T47D cells after 48 h of exposure, as shown in Figure 3. However, the crude extract did not markedly affect to the same extent hCMEC/D3 cells, where a viability of approximately 100% was detected (Figure 3D). In the crude extract in all cell lines a reduction in cell viability was observed, depending on time of exposure. As shown in Figure 3A for MG63 cells, fraction J resulted in a decrease in cell viability depending upon time of exposure with cell viabilities of 83, 41, and 18% after 24, 42, and 72 h, respectively. A decrease in cell viability was noted in fractions D, F, and G, with only 10% of cells viable at the end of the experiment. A marked reduction in cell viability was observed in fractions F, G, and H, with 5% of viable cells after 72 h of incubation in T47D cells (Figure 3B). In RKO cells a decrease in cell viability was also more expressive in fractions F, G, and H (only 5% of the cells were viable), as illustrated in Figure 3C. In normal endothelial cells the results differed from those obtained with cancer cell lines (Figure 3D), and in all fractions the rate of viable cells was markedly higher than in cancer cell lines. The lowest rate of viable cells was found in fraction D with 61%. The rate of

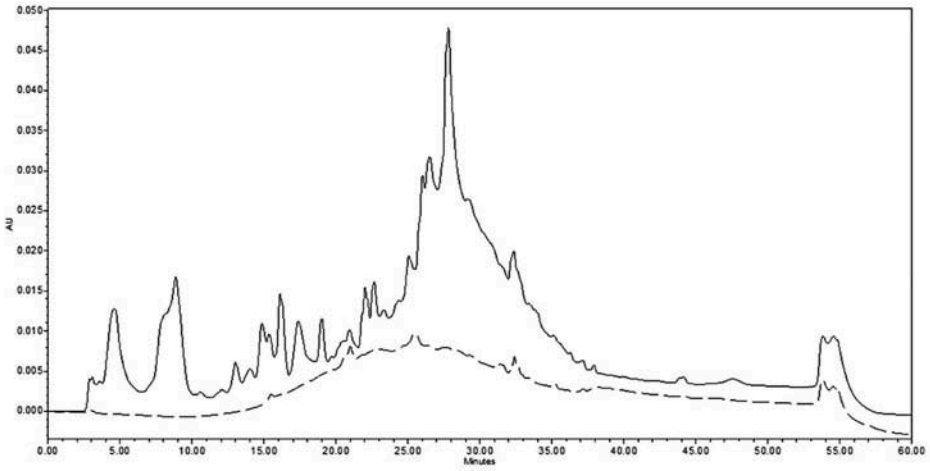

FIGURE 1. Full scan chromatogram of *Pisolithus tinctorius* spores dichloromethane/methanol crude extract. The dashed line corresponds to the blank injection and the continuous line to the crude extract.

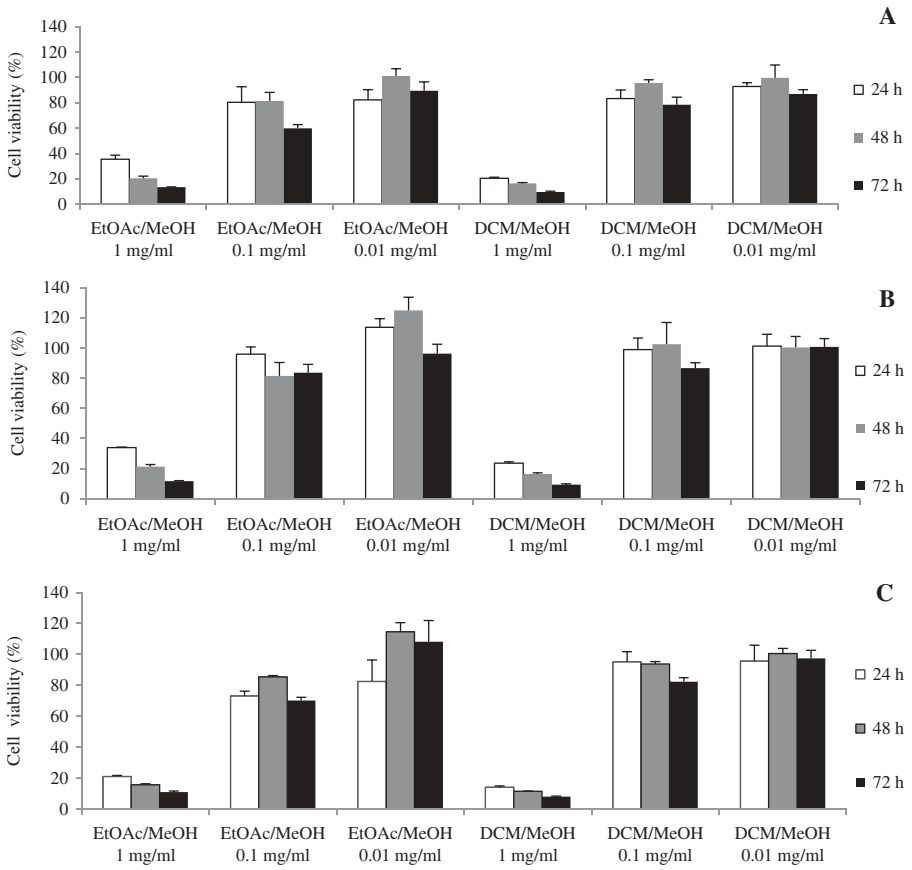

FIGURE 2. Cytotoxicity of ethyl acetate (EtOAc)/methanol (MeOH) (2:1) and dichloromethane (DCM)/methanol (MeOH) (2:1) crude extractions at different concentrations and exposure times to MG63 (A), T47D (B), and RKO (C) cell lines. Values are present as means ± SD of triplicate analyses and expressed as a percentage of cell viability compared with the control (cells treated with 1% dimethyl sulfoxide).

viable cells at 48 h was numerically lower when compared to 24 h.

DISCUSSION

A large number of compounds that may be useful for human cancer treatment have been identified from fungal species (Nishino et al., 2000). The triterpene pisosterol isolated from sporocarps of *P. tinctorius* was identified by Montenegro et al. (2004) and in further studies conducted in 2007 and 2008 by the same authors, demonstrating cytotoxicity to several cancer cell lines. However, there are no apparent data available regarding bioactivity potential of spores of this fungus. To our knowledge this is the first study to demonstrate cytotoxic potential of *P. tinctorius* spores extracts and fractions on cancer cell lines.

Of the five types of solvents/solvent mixtures used to perform the crude extraction, the DCM/MeOH mixture yielded highest extraction (Table 1). The results concerning cytotoxicity of crude extract showed that this extract was able to inhibit cell viability in all studied cancer cell lines. This is supported by data obtained with the fractions, since the crude extract represents a mixture of the compounds that were then separated in the fractions. Fractions D, F, G, and H were the ones where the lowest rate of viable cells was noted, suggesting the presence of compounds with potent cytotoxic activity. However, data demonstrated that different cancer cell lines were not markedly affected to the same extent

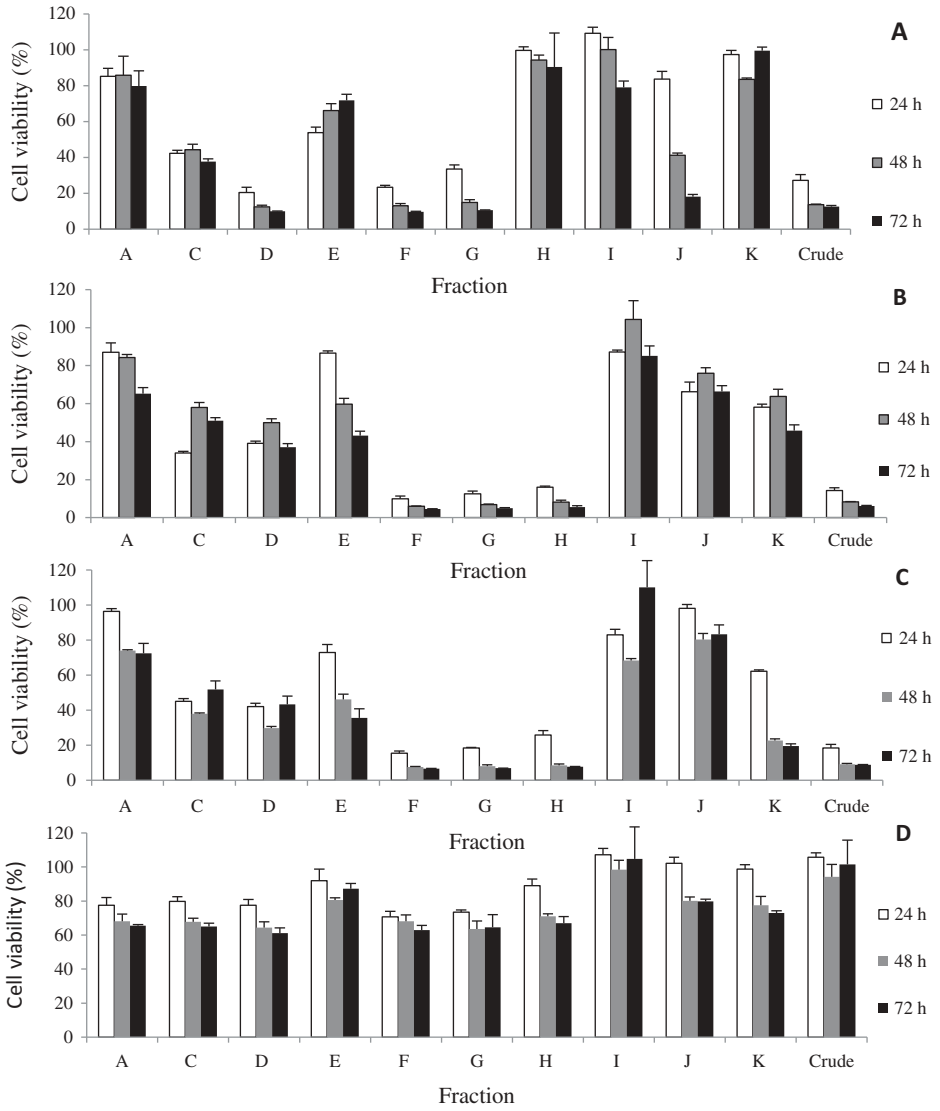

FIGURE 3. Effects of *Pisolithus tinctorius* spores crude and fraction extracts on the viability of MG63 (A), T47D (B), RKO (C), and hCMEC/D3 (D) cells. The cells were exposed for 24, 48, and 72 h at a concentration of 0.1 mg/ml for fractions A–K and 1 mg/ml for the crude extract. Values are presented as means ± SD of triplicate analyses and expressed as a percentage of cell viability compared with the control (cells treated with 1% dimethyl sulfoxide). Fraction B was not tested due to the small amount of material obtained in the fractionation procedure.

when exposed to the same fraction. As an example, in fraction H 5% of viable cells in RKO and T74D after 48 h of incubation was obtained, while in MG63 the rate of viable cells was 90%. This indicates that the cytotoxic compounds that are present in fraction H are selective toward RKO and T74D. This is an interesting finding, since selective toxicity to different cell lines suggests a specific mode of action, which in the case of cancer treatment is preferable. In hCMEC/D3 cell lines exposed to D, F, G, and H fractions, the number of viable cells was significantly higher than that found in cancer cell lines, indicating that the fractions are more cytotoxic to cancer cells than to normal cells, reinforcing the notion of the presence of compounds with anticancer potential in these fractions.

Considering compounds already isolated from *P. tinctorius*, Montenegro et al. (2008)

evaluated the antitumor activity of pisosterol in vivo using sarcoma 180 in mice. The tumor growth inhibition ratio was 43% and some damage to liver and kidney epithelium tissue was observed; however, Montenegro et al. (2008) considered that these damages might be reversible. This compound may also be present in fractions D, F, G, and H and consequently in *P. tinctorius* spores, which might explain the cell viability ratio of 61% in hCMEC/D3 cells. Data demonstrated that the spores of *P. tinctorius* are an interesting source of compounds with anticancer potential and pave the way for discovery of new fungal sources of anticancer compounds. Further studies are already in progress to identify compounds present in the fractions F, G, and H and that may be responsible for selective toxicity against the tumor cells.

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