In Vitro Antitumor Activity of Endophytic Fungi Isolated From the Mexican Cactus *Pachycereus Marginatus* (DC.) Britton & Ros

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Abstract

Background: Arid zone plants such as cacti are known to harbor diverse groups of endophytic fungi, which represent potential sources of new compounds with anticancer properties. In the present study we isolated, identified, and characterized *Pachycereus marginatus* (DC.) Britton & Ros endophytic fungi with cytotoxic activity against murine and human tumor cell lines.

Methods: Endophytic fungi were isolated from *P. marginatus* stems. Methanol extracts were then obtained from fungi liquid cultures and their cytotoxic activity at concentrations ranging from 31 µg/ml to 250 µg/ml against murine L5178Y-R lymphoma, human colorectal adenocarcinoma HT-29, and human breast cancer MCF-7 was evaluated by the colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction assay, using the normal cells *Macacus rhesus* monkey epitelial kidney MA-104 and human peripheral blood mononuclear cells (PBMC) as controls. IC_{50} values were obtained and the selectivity index (SI) was calculated from the IC_{50} ratio of cancer cells and normal cells. Furthermore, molecular identification of fungi showing cytotoxic activity was determined by the internal transcribed spacer molecular marker.

Results: The *Cladosporium sp*. PME-H008 strain showed significant ($P < 0.01$) 94.3% and 36.8% cytotoxicity against L5178Y-R and HT-29 cells, respectively. The highest SI was observed by L5178Y-R cells with 2.4 and 2.9 for MA-104 and PBMC respectively. In addition, the *Metarhizium anisopliae* PME-H007 strain was more effective against MCF-7 with 55.8% cytotoxicity. The lowest IC_{50} was obtained with the *Aspergillus sp*. PME-H005 strain at 95.21 µg/ml against the MCF-7 cell line, followed by PME-H008 strain at 101 µg/ml against L5178Y-R cells.

Conclusion: *P. marginatus* endophytic fungi showed *in vitro* cytotoxic activity against murine and human tumor cell lines, without affecting normal cells.

Background

Cancer represents a serious public health issue and is currently considered the second cause of death worldwide, behind cardiovascular diseases. In 2020, around 9.9 million deaths by cancer were reported in the GLOBOCAN database [1]. Although radiation, surgery, immunological, hormone, and gene protocols are available, chemotherapy remains the most common method for cancer treatment [2]. However, the emergence of cancer cells resistant to antineoplastics, as well as the side effects of drugs, are major obstacles to chemotherapy success [3]. Therefore, it is essential to search for new drugs with marginal or absent side effects for the oncological patient [4].

Endophytic fungi have gained relevance in biotechnology as potential sources of new compounds with anticancer activity. Their rapid growth, culture conditions, high-cell density, easy genetic manipulation, and the possibility of scaling the production of compounds at an industrial level make them candidates for obtaining new antitumor drugs [5].

It has been proposed that the isolation of endophytic fungi, involving the selection of plants with ethnobotanical use, as well as those developing strategies for survival or growth under extreme environments [6], may lead to the discovery of endophytes that produce novel bioactive compounds. In this regard, arid zone plants such as cacti, establishes symbiotic relationships with different microorganisms, from which enzymes [7], antimicrobials [8], and anticancer compounds such as bikaverine [9] and triterpenes of the 24-homo-30-nor-cycloartane class [10] have been isolated. However, the biotechnological potential of fungi isolated from dessert plants is still limited [11].

Pachycereus marginatus, also called *Stenocereus marginatus* or *Cereus marginatus*, is a species of cactus endemic to Mexico belonging to the Cactaceae family, which is popularly known as chilayo, organ cactus or malinche [12]. In traditional medicine, it has been used for the treatment of gastrointestinal diseases [13] and diabetes [14]. Recent studies have demonstrated the antimicrobial [15] and anticancer activity of *P. marginatus* extracts in *in vitro* [16, 17] and *in vivo* models [18]. However, the antitumor potential of *P. marginatus* endophytic fungi has not yet been reported. Therefore, in the present

study, they were isolated and their cytotoxic activity against murine and human tumor cell lines and normal cells was evaluated.

Methods

Plant material

P. marginatus stems were collected in General Escobedo, Nuevo León, México (100°18'42.5"N 25°47 '52.5"W) in February 2020. It was identified by M.Sci. María del Consuelo González de la Rosa, Chief of the Herbarium of Facultad de Ciencias Biológicas at Universidad Autónoma de Nuevo León, México, with voucher specimen number 025588.

Isolation and morphological characterization of P. marginatus **endophytic fungi**

Stems were rinsed with tap water to eliminate dust and other contaminating material, and subjected to a disinfection protocol to remove epiphytes, which consisted of washing with 70% ethanol for 1 min, 2.5% sodium hypochlorite for 3 min, 70% ethanol for 30 s, and two rinses with sterile distilled water and one with PBS [19]. For the isolation of endophytic fungi, the previously disinfected plant tissue was cut into small pieces. One part was placed on the surface of Petri dishes with potato dextrose agar (PDA; Difco, Detroit, MI), Sabouraud dextrose agar (SA; Difco), and agar water added with penicillin-streptomycin (60 mg/L/100 mg/L) (Life Technologies, Grand Island, NY) to inhibit microbial growth and the other section was ground in PBS in a sterile mortar. Next, 100 µl of the sample were inoculated in the aforementioned culture medium by plate dispersion and the last wash with PBS was used as a negative growth control. Plates were then incubated at 20 ^{o}C for four weeks. Morphological characterization was determined from monosporic cultures of the isolates in PDA, recording radial growth, shape, size, color, edge, and type of mycelium.

Fermentation and production of methanolic extracts

For the extraction of secondary metabolites, 1 cm^2 fragments of isolate fresh cultures were individually inoculated in 250 ml flasks with 125 ml of potato and dextrose broth (PDB; Difco) and incubated for 30 d at 20 ^{o}C and 150 rpm (ET-4200, Tecnal Incubator, São Paulo, Brazil). After incubation, mycelium was separated by filtration and dried at 60°C, after which it was subjected to an extraction by maceration with methanol. Solvent was then removed with a rotary evaporator (Buchi R-3000; Brinkman Instruments, Inc., Westbury, NY). Extracts were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) at a final concentration of 25 mg/ml and kept at 4°C, until use.

Cell lines and culture conditions

The cell lines used in this study were L5178Y-R (ATCC CRL-1722) (murine lymphoma), HT-29 (ATCC HTB-38) (human colorectal adenocarcinoma), MCF-7 (ATCC HTB-2) (human breast cancer), and MA-104 (ATCC® CRL-2378.1™) (monkey kidney epithelial cells). Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood of healthy volunteer donors, using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA). Cells were maintained in RPMI-1640 medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies) and 1% antibiotic-antifungal solution (Life Technologies), except for MCF-7 cells that were grown in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies) supplemented with 10% FBS and 1% antibiotic-antifungal solution (Life Technologies). All cells were cultured at 37°C in an atmosphere of 5% CO_2.

Cytotoxic activity assay

L5178Y-R, HT-29, MCF-7, and MA-104 cell suspensions were cultured at a density of $1x10^4$ cells/well and PBMCs at $1x10^5$ cells/well for 24 h and treated with 31, 62.5, 125, and 250 µg/ml of methanol extracts for 48 h at 37°C in 5% CO_2. Cytotoxicity was evaluated by the colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT; Affymetrix, Cleveland, OH USA) reduction assay by adding 15 µL of MTT (0.5 mg/ml final concentration) and incubating at 37°C for additional 4 h. Formazan crystals were then dissolved with DMSO and optical densities (OD) were measured at 570 nm in a

MULTISKAN GO microplate reader (Thermo Fisher Scientific, Waltham, MA). The percentage of cytotoxicity was calculated as follows: % Cytotoxicity = 100-[(A570 in extract-treated cells/A570 in untreated cells) (100)], using 0.05 µg/ml vincristine sulphate (Hospira, Warwickshire, UK) as a positive control. Logarithmic scale concentrations were plotted against % cytotoxicity to determine IC_{50}. IC_{50} values were used to obtain the selectivity index (SI), for which the IC_{50} of normal cells was divided by the that of cancer cells [20].

Molecular identification of P. marginatus endophytic fungi

Genomic DNA extraction was performed from monosporic cultures, using cetyltrimethylammonium bromide (CTAB; Sigma-Aldrich, St. Louis, MO) [21]. Purified DNA was then subjected to a PCR with the universal markers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') in a volume of 50 µl, using Ruby Taq Master mix 2X (Jena Bioscience, Jena, Germany), 100 ng of the DNA template, and 0.25 µM of each primer. The amplification program consisted of a denaturation cycle of 95°C for 5 min, 35 cycles of 94°C for 30 s, 60°C for 45 s, and 72°C for 90 s, followed by a final extension of 72°C for 8 min [22]. The PCR product was purified by the Agarose Gel Extraction kit (Jena Bioscience). Next, the product was sequenced with the ABI PRISM 310 TM Genetic Analyzer sequencer at the Synthesis and Sequencing Unit of the Institute of Biotechnology (IBT) of the UNAM, in Cuernavaca Morelos. Sequence analysis was performed using the NCBI BLAST nucleotide database.

Statistical analysis

Cytotoxicity results were expressed as mean ± SEM of three replicates per treatment from three independent experiments. Level of significance was evaluated by the Dunnett's *t* test. IC_{50} values were reported with ± 95% confidence intervals (95% CI). Statistical analyzes were performed using the Graph Pad Prism 7 program.

Results

Isolation of P. marginatus endophytic fungi

We isolated 10 filamentous fungi from *P. marginatus* stems. Isolates were morphologically characterized, mostly showing circular shape, filamentous edge, and flat mycelium. Radial growth at 3 d and 7 d was from 5.1 mm to 8.5 mm and 9.9 mm to 22.6 mm, respectively. Isolates first fermented in PDB, after which biomass methanol extraction was performed, obtaining yields ranging from 4.4–20.2%.

Cytotoxic activity of methanol extracts from P. marginatus endophytic fungi

Fungi methanol extracts were evaluated at concentrations ranging from 31 µg/ml to 250 µg/ml against tumor cell lines (L5178Y-R, HT-29, and MCF-7) and normal cells (MA-104 and PBMC). Five filamentous fungi showed cytotoxic effect against tumor cell lines (Fig. 1). Extracts were effective against L5178Y-R tumor cells, whereas the least susceptible cells were HT-29. The highest percentages of cytotoxicity were obtained at a concentration of 250 µg/ml. In this regard, PME-H008 extract caused the highest cytotoxicity against L5178Y-R and HT-29 tumor cell lines with 96.6% ($P < 0.01$) and 42.5% ($P < 0.05$) respectively. Furthermore, the PME-H007 extract caused the highest cytotoxic activity ($P < 0.01$) against MCF-7 cells with 55.8%. Regarding IC_{50}, the PME-H005 extract showed the lowest values with 95.21 µg/ml for the MCF7 cell line, followed by PME-H008 with 101 µg/ml for L5178Y-R cells, whereas for the HT-29 cell line, the extracts showed an IC_{50} higher than 291 µg/ml. Furthermore, MA-104 cell line was more susceptible compared with PBMC, being PME-H001 with IC_{50} of 437.7 µg/ml for the MA-104 cell line and PME-H002 with 409.8 µg/ml for PBMC the extracts that showed the lowest cytotoxicity. In contrast, the extract that showed the highest SI against L5178Y-R tumor cells was PME-H008 with values of 2.4 and 2.9, compared with MA-104 and PBMC respectively. For the MCF-7 cell line, we obtained an SI of 2.7 with the PME-H005 extract, compared with PBMC, and HT-29 showed values lower than 1.2 (Table 1).

Molecular identification of P. marginatus endophytic fungi with antitumor activity

We performed molecular identification of endophytic fungi that showed cytotoxic activity against tumor cell lines L5178Y-R, HT-29, and MCF-7 (Table 2). The amplified regions (ITS1-ITS4) were sequenced, and manually reviewed and analyzed using the Blast tool for fungi identification. The isolates PME-H001 and PME-H002 were identified as *Penicillium citricum* with 99.6% and 99.2% homology respectively. PME-H005 and PME-H008 were only identified up to gender with 99% and 97.4%, whereas PME-H007 was identified as *Metarhizium anisopliae* with 98.9% homology.

Discussion

Endophytic fungi represent an important source of compounds with biological activity, such as phenolic acids, alkaloids, quinones, steroids, saponins, tannins, and terpenoids, which increases their potential in the identification of new compounds with antidiabetic, anti-inflammatory, antiviral, immunosuppressive, anti-arthritis, anti-oxidant, anti-microbial, and anti-cancer effects [23]. However, only less than 16% of the fungal species described have been cultured and studied and less than 5% of the total fungal species that have been characterized represent an important source of bioactive metabolites [24]. The distribution of certain populations of endophytic fungi is restricted to a species or family of plants, as well as to the genotype of the species, thus the presence of a specific population of fungi may determine the production of various secondary metabolites [25]. Therefore, due to the vast number of plant species in the world, different strategies have been devised to select the plants from which to isolate endophytes with biological activity, including the use of plants that have been exploited for human use in traditional medicine [26], as well as those with special strategies for their survival or that grow in extreme environments [6]. Plants that inhabit extreme environments, such as arid zones, are associated with endophytic fungi since they improve their performance and resistance against biotic and abiotic factors through the production of bioactive compounds [27]. However, the biotechnological potential of fungi isolated from this type of environment is still limited [11]. Therefore, this study reports for the first time the cytotoxic activity of endophytic fungi isolated from *P. marginatus*, a species of cactus endemic to Mexico, previously reported with anticancer activity [16–18]. In the present study, we isolated *Penicillium*, *Aspergillus*, and *Cladosporium* genera, which have been commonly isolated from plants that inhabit dry environments, such as cacti [19, 28, 29], whereas *Metarhizium* has been reported as a natural endophyte of legumes [30], conifers [31], herbs, and wildflowers [32]. We then report for the first time the isolation of *Metarhizium* as an endophyte of cacti.

Currently, anticancer resistance is a serious problem in oncology as in the case of breast cancer [33], colon cancer [34], and Non-Hodgkin lymphoma [35]. Therefore, it is essential to search and identify new compounds with antitumor activity, for which cancer cells are not resistant [36]. Various endophytic fungi have shown anti-cancer effect against hepatoma (HepG2), lung cancer (A-549), colorectal cancer (HCT-116, HT-29), breast cancer (MCF7), ovarian cancer (SKVO3), leukemia (HL-60), carcinoma (KB), cervical cancer (Hela), and lymphoma (L5178Y) [4].

The isolated strains of *P. citrinum* PME-H001 and PME-H002, showed differential cytotoxic activity, which is consistent with other studies evidencing that the medicinal properties of endophytic fungi vary, despite they belong to the same genus and are isolated from the same host [37]. On the other hand, the anticancer activity of this species has been reported against different tumor cell lines such as A549, Hela, HepG2, L5178Y, MOLT-4, MCF-7, BT-474, and MDA-MB-231, identifying different compounds responsible for the activity, such as penicillocitrin A, citriquinochroman, citrinin, scalusamide A, perinadine A, pencitrin, and pencitrinol [37–41].

Fungi of the genus *Aspergillus* are considered an important source of bioactive compounds with anticancer activity, among which are alkaloids, pyrones, polyketides, lactones, sterols, xanthones, anthraquinones, terpenes, peptides, depsipeptides, cyclic peptides, cytochalasins, enzymes, and proteins. They have been evaluated in different tumor cell lines such as MCF7, HL-60, K-562, A549, MOLT-4 and HEP-G2 [42]. Furthermore, the potential of *M. anisopliae* to produce anti-cancer compounds such as taxol with yields of 846.1 µg/L in liquid medium has been previously demonstrated by others [31] and destruxin B with IC_{50} values of 4.9 μM in A549 lung cancer cells [43].

Cladosporium sp. methanol extract caused the highest toxicity against MCF-7 cells, which agrees with a study reported by Raj *et al.* [44] showing the activity of taxol obtained from *C. oxysporum* extracts against the T47D breast cancer cell line, with an IC_{50} value of 2.5 µM, after 24 h of incubation.

Most anti-cancer drugs do not differentiate between tumor and normal cells, thus researchers investigate for new drugs that are selective for cancer cells, with minimal effects for other cells [5]. However, some endophytic fungal extracts such as *Acremonium* sp. and *Pestalotiopsis suffocata* are toxic against PBMCs with IC_{50} values of 13.4 and 12.2 µg/ml [45].

Endophytic fungi may play an important role in providing chemotherapeutic compounds with high specificity and minimal side effects. Therefore, the search for endophytic fungi from different habitats may provide an excellent avenue to discover new drugs and their application in the medical area, for the control of different human diseases.

Conclusions

Evaluation of *P. marginatus* endophytic fungi methanol extracts have revealed their potential as producers of bioactive compounds with antitumor activity, which may be used for the development of drugs for the treatment of cancer. The strain PME-H008 of *Cladosporium sp.* and PME-H007 from *Metarhizium anisopliae* have significant antitumor activity against lymphoma and breast cancer cells, which requires further investigation.

Abbreviations

PDA: potato dextrose agar; SA: Sabouraud dextrose agar; PBS: phosphate buffered saline; PDB: potato and dextrose broth; DMSO: dimethyl sulfoxide; PBMC: peripheral blood mononuclear cells; FBS: fetal bovine serum; DMEM: Dulbecco's Modified Eagle Medium; MTT: 3- [4,5 - dimethylthiazol - 2 - yl] -2,5 - diphenyltetrazoliumbromide; OD: optical density; IC_{50}: half maximal inhibitory concentration; SI: selectivity index; DNA: Deoxyribonucleic acid; CTAB: cetyltrimethylammonium bromide; PCR: Polymerase chain reaction; ITS: Internal transcribed spacer region; NCBI: National Centre for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; SEM: Standard error of the mean.

Declarations

Experimental research and field studies on plants

The use of plant parts in present study compiles with international, national and/or institutional guidelines. Permissions or licenses to collect the plants used in the present study were not required, since they were of free access.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CR, KM, RG, PT contribute to study concept and design. Plant collection, identification and isolation of endophytic fungi from plant samples were carried out by JR and RR. JR extracted the fermented broth of endophytes in the supervision of QL. JR

carried out the anticancer experiments and results were analyzed by CR. Molecular analysis of isolated endophytes was carried out by JR and AO. RT and CRP contributed to the analysis/interpretation of data. JR wrote the manuscript. CR, KM, RG, edited the manuscript. All authors have read and approved the manuscript and agreed to be accountable for all aspects of the work.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

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Tables

Table 1. IC_{50} values (µg/ml) and SI of tumor cell lines treated with methanol extracts, compared with MA-104 and PBMC cells.

Isolate code	L5178Y-R		HT-29		MCF-7		MA-104	PBMC
	IC_{50}	SI*	IC_{50}	SI*	IC_{50}	SI*	IC_{50}	IC_{50}
PME-H001	269.4±1.4	1.6/1	348.1±1.1	1.2/0.8	1387±0.7	0.3/0.2	437.7±0.8	295.4±1.2
PME-H002	266.5±1.4	1.1/1.5	402.5±1	0.7/1	1244±0.6	0.2/0.3	295.4±1.4	409.8±1.2
PME-H005	166.2±1.8	0.7/1.5	291.6±1.2	0.4/0.9	95.21±1	1.2/2.77	123.5±1.3	264±1.5
PME-H007	132.9±1.5	1.8/0.7	291.7±1.3	0.8/0.7	114.7±1.3	2.1/1.8	245.9±1.9	215.8±1.6
PME-H008	101±1.5	2.4/2.9	301.1±1.2	0.8/0.9	337.5±1.3	0.7/0.8	250.2±1.2	298.8 ±1.4

*SI = MA-104/PBMC.

Table 2. Morphological characterization and molecular identification of *P. marginatus* endophytic fungi with cytotoxic activity.

Isolate code	Radial growth (mm)		Shape	Edge	Mycelium	Above color*	Reverse color*	Molecular identification	
	3 d	7 d						Identification based on the sequence ITS1-ITS4	Homology percentage
PME-H001	7.3	9.9	Circular	Filamentous	Flat	# 838B83	#F0E68C/ EEF3E2	*Penicillium citrinum*	99.6%
PME-H002	8.5	17.5	Circular	Filamentous	Flat	#838B83	#F0E68C/ EEF3E2	*Penicillium citrinum*	99.2%
PME-H005	8.1	17.9	Circular	Irregular	Flat	#838B83	#FFC125/ FEF0C9	*Aspergillus sp.*	99%
PME-H007	6.2	22.6	Circular	Filamentous	Flat	#006400/ FFFFFF	#CD9B10/ EEDC82	*Metarhizium anisopliae*	98.9%
PME-H008	5.1	15.2	Circular	Entire	Flate	#2F4F4F /EBECE4	#FEFEF2	*Cladosporium sp.*	97.4%

*The color of the colonies was defined with the color chart on the webpage http://www.webusable.com/coloursTable.htm

Figures

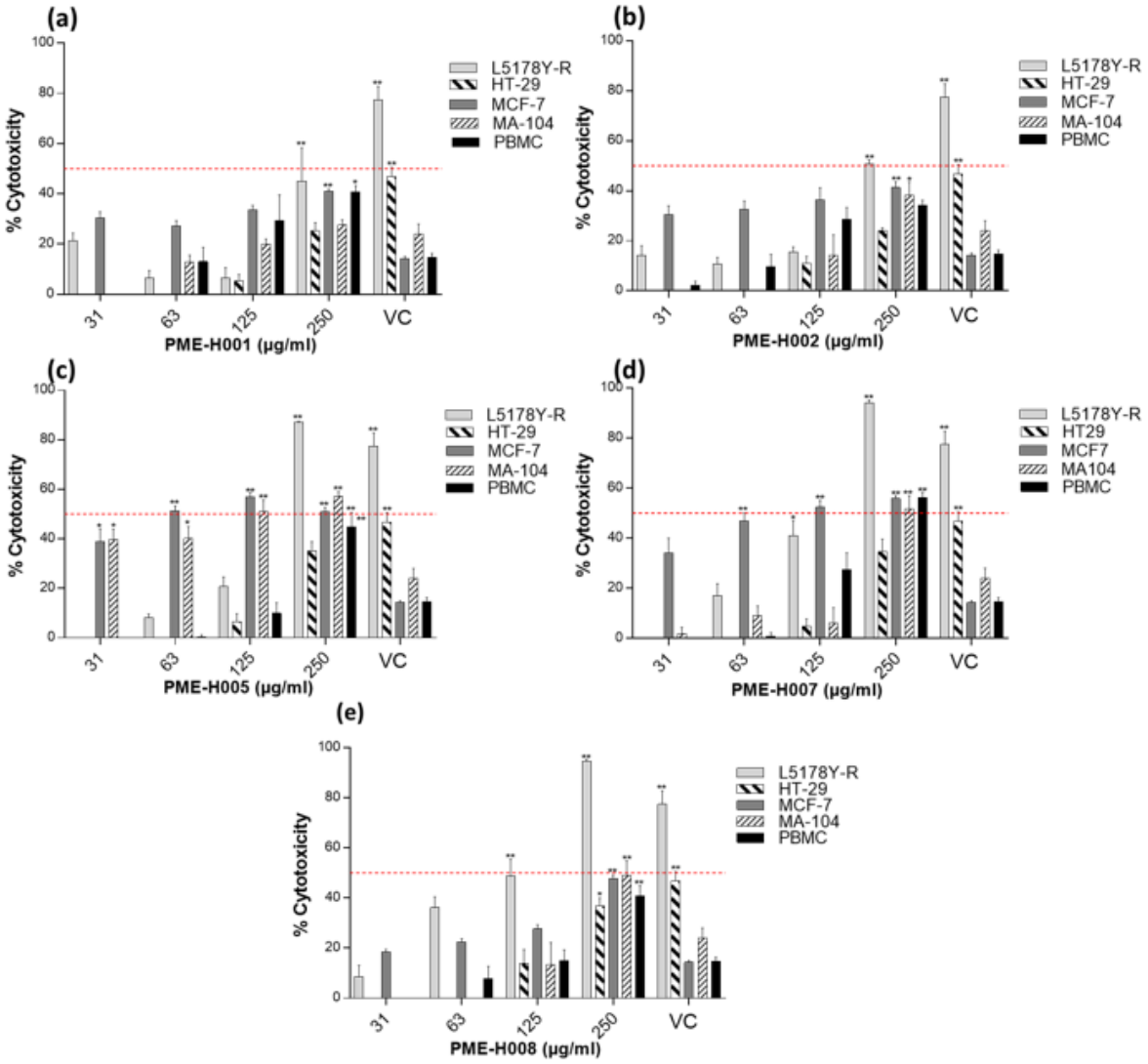

Figure 1

Cytotoxic activity of methanol extracts from P. marginatus endophytic fungi against tumor and non-tumor cells. L5178Y-R, HT-29, MCF-7, and MA-104 cells were cultured at 1x104 cells/well and PBMC at 1x105 cells/well for 24 h and treated with 31 mg/ml to 250 µg/ml of methanol extracts for 48 h, as detailed in the text. Cytotoxicity was evaluated by the MTT reduction assay and ODs measured at 570 nm, using 0.05 µg/ml vincristine sulphate as a positive control. Dotted line represents the IC50. Data represent mean ± SEM of triplicates from three independent experiments. *, P < 0.05; ** P < 0.01, as compared with untreated control. OD for untreated control was 1.39 ± 0.12.