Laccase purified from *Cerrena unicolor* exerts antitumor activity against leukemic cells

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Abstract. Chronic lymphocytic leukemia (CLL) is the most commonly observed adult hematological malignancy in Western countries. Despite the fact that recent improvements in CLL treatment have led to an increased percentage of complete remissions, CLL remains an incurable disease. *Cerrena unicolor* is a novel fungal source of highly active extracellular laccase (ex-LAC) that is currently used in industry. However, to the best of our knowledge, no reports regarding its anti-leukemic activity have been published thus far. In the present study, it was hypothesized that *C. unicolor* ex-LAC may possess cytotoxic activity against leukemic cell lines and CLL primary cells. *C. unicolor* ex-LAC was separated using anion exchange chromatography on diethylaminoethyl cellulose-Sepharose and Sephadex G-50 columns. The cytotoxic effects of ex-LAC upon 24- and 48-h treatment on HL-60, Jurkat, RPMI 8226 and K562 cell lines, as well as CLL primary cells of nine patients with CLL, were evaluated using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay. Annexin V/propidium iodide staining of Jurkat cells treated with ex-LAC was used to investigate apoptosis via flow cytometry. Ex-LAC induced changes in Jurkat and RPMI 8226 cells, as visualized by fluorescence and scanning electron microscopy (SEM). The XTT assay revealed high cytotoxic rates following treatment with various concentrations of ex-LAC on all the cell lines and CLL primary cells analyzed, with a half maximal inhibitory concentration ranging from 0.4 to 1.1 μg/ml. Fluorescence microscopy and SEM observations additionally revealed apoptotic changes in Jurkat and RPMI 8226 cells treated with ex-LAC, compared with control cells. These results were in agreement with the apoptosis analysis of Jurkat cells on flow cytometry. In conclusion, *C. unicolor* ex-LAC was able to significantly induce cell apoptosis, and may represent a novel therapeutic agent for the treatment of various hematological neoplasms.

Introduction

Chronic lymphocytic leukemia (CLL) is the most commonly observed hematological malignancy in Western countries (1-3). It represents 0.8% of all the neoplasms and almost 30% of all the leukemia cases documented worldwide (1-3). CLL affects particularly the elderly, since the median age at diagnosis is 72 years, although increased morbidity among younger patients has been recently reported (1-3). The incidence of CLL is 2-6 cases/100,000 patients/year, and it increases with age (1-3). The risk of malignancy development is double in men compared with women (1-3). CLL is a lymphoproliferative disorder that may be characterized by the accumulation of small, homogeneous, mature cluster of differentiation (CD)5^{+} CD19^{+} B-lymphocytes in the peripheral blood, bone marrow and secondary lymphoid organs (3,4). The clinical course of CLL is diverse. Certain patients display a favorable course of disease, with stable or slowly increasing lymphocytosis, long survival and no requirement for treatment, whereas other patients experience an aggressive, progressive course of CLL that requires immediate intensive therapy (3,4). The clinical heterogeneity observed in the course of CLL is due to genetic and epigenetic abnormalities (2-5). The microenvironment is additionally known to be involved in leukemogenesis (2-5). However, the pathological mechanism of CLL remains to be elucidated (2-5). Several prognostic factors have been described that are useful for risk stratification in CLL, including cytogenetic abnormalities (deletion of the chromosomes 11q and 17p), expression of CD38 and zeta-chain-associated protein kinase 70, and mutation status

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in immunoglobulin heavy chain variable region genes (2-5). Recent improvements in CLL treatment have led to an increased percentage of complete remissions. However, due to the advanced age of the patients and/or the presence of negative markers, relapse is expected in the majority of cases (2-5). Hence, the requirement for the development of novel therapies for the treatment of CLL remains.

Medicinal mushrooms have been extensively used in oriental medicine as a remedy against various disorders, including malignancies (6). To the best of our knowledge, of all the known species of mushrooms, 650 possess documented medicinal properties, and ~20 are currently in clinical use (6). Genera of mushrooms displaying antineoplastic activity include *Pleurotus, Phellinus, Ganoderma, Agaricus, Antrodia, Clitocybe, Cordyceps, Trametes, Calvatia, Xerocomus, Suillus, Schizophyllum, Flammulina, Funlia, Inonotus, Lactarius, Inocybe, Albatrellus, Fomes* and *Russula* (6-8). Extracts from these mushrooms contain bioactive compounds, including proteins, polysaccharides, glycosides, fats, volatile oils, alkaloids, phenols, tocopherols, folates, carotenoids, flavonoids, organic acids and ascorbic acid enzymes (6-8). These extracts are able to inhibit mitosis and angiogenesis, induce apoptosis and restrain proliferation of neoplastic cells (6-8).

Laccase [benzenediol:oxygen oxidoreductase, enzyme commission number, 1.10.3.2 (http://www.kegg.jp/dbget-bin/www_bget?ec:1.10.3.2); LAC] is part of the largest subgroup of blue multicopper oxidases, and exhibits the distinctive redox ability of copper ions, since it is capable of catalyzing the oxidation of an extensive range of aromatic substrates concomitantly with the reduction of molecular oxygen to water (9,10). The distribution of LAC is widespread among plants, fungi and bacteria (7). In particular, white-rot fungi have been identified to be the most efficient LAC producers (7,11-13). *Cerrena unicolor* has been established as the most effective fungal source of extracellular (ex)-LAC, with the highest activity reported to be 60,000 nkat/l (14). *C. unicolor* ex-LAC has been utilized in biodegradation, bioremediation, delignification and decolorization, although no data regarding its anticancer activity have been published to date (15).

The present study aimed to investigate the *in vitro* cytotoxicity of *C. unicolor* ex-LAC against leukemic cells. CLL cells were used as a model of disease in order to examine novel therapeutic agents, since they consist of two compartments: i) an accumulation compartment in the peripheral blood, followed by the spleen and liver; and ii) a proliferation compartment in the lymph nodes and bone marrow (4). No transgenic model or cell line of CLL currently exists (4). Therefore, several hematological cell lines were used in the present study, in addition to primary CLL cells, to evaluate the cytotoxic activity of *C. unicolor* ex-LAC against leukemic cells.

Materials and methods

Strain, medium, growth processing and preliminary separation of ex-LAC. *C. unicolor* (Bull.ex.Fr.) Murr, No. 139, was acquired from the Regensburg Culture Collection, Archaea Centre, University of Regensburg (Regensburg, Germany) and deposited in the fungal collection at the Department of Biochemistry of Maria Curie-Skłodowska University (Lublin, Poland) under the strain no. 139 (internal transcribed spacer

sequence deposited in the GenBank database under the accession no. DQ056858) (16). Fermenter scale cultivation was performed at 28˚C in a BioFlo® 310 fermenter (New Brunswick Scientific; Eppendorf, Hamburg, Germany) containing 2 l Lindenberg and Holm medium (Sigma-Aldrich, St. Louis, MO, USA) sterilized at 121˚C for 30 min (14). The fermenter was inoculated with crumbled fungal mats (10% of total volume), aerated at 1 l air/min and stirred at 100 rpm. Antifoam B emulsion (Sigma-Aldrich) was occasionally added to the fermenter cultures in order to disperse any foam formation. Cultures (10-day-old) were filtered through Miracloth (Calbiochem; EMD Millipore, Billerica, MA, USA) and utilized for subsequent assays. The beginning of the idiophase was determined according to the protocol recommended by Betina (17). The culture liquid obtained following mycelium separation was centrifuged (Sigma 4-16KS; SciQuip Ltd., Shropshire, UK) at 10,000 x g for 15 min. The supernatant was subdivided into two fractions via ultrafiltration, using Ultracel-10 membranes (EMD Millipore) incorporated into Pellicon 2 Mini cassettes holders (EMD Millipore). The fraction containing substances of molecular weight >10 kDa was used as the source of crude ex-LAC, and subsequently purified according to modified methods described by Rogalski and Janusz (16).

Preparation of fungal ex-LAC. Chromatography was performed using a chromatographic fast protein liquid chromatography system (BioLogic™ Low-Pressure Liquid Chromatography System; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The supernatant of culture fluid that was concentrated on the aforementioned Pellicon 2 Mini ultrafiltration system was next loaded on a diethylaminoethyl cellulose (DEAE)-Sepharose column of 2.5x15.0 cm (GE Healthcare Life Sciences, Chalfont, UK), which was pre-equilibrated with 20 mM Tris-HCl buffer (Sigma-Aldrich), pH 6.5. Proteins were eluted using a 0.1-0.5 M linear gradient of NaCl (Sigma-Aldrich) at a flow rate of 1 ml/min. The fraction containing LAC activity was collected and desalted on a Sephadex G-50 column of 5.0x20.0 cm (GE Healthcare Life Sciences). The purification processes were performed at 4˚C. The semi-pure ex-LAC solution was next lyophilized using FreeZone 12 Liter Console Freeze Dry System (Labconco Corporation, Kansas City, MO, USA).

Cell lines. Human chronic myeloid leukemia in blast crisis (K562) and human multiple myeloma (RPMI 8226) cell lines were acquired from the German Collection of Microorganisms and Cell Cultures, Leibniz-Institute DSMZ (Brunswick, Germany). Human acute promyelocytic leukemia (HL-60) and human T cell leukemia (Jurkat) cell lines were purchased from the American Type Culture Collection (Manassas, VA, USA). All the cell lines were cultured in RPMI 1640 medium (Biochrom; Merck Millipore, Darmstadt, Germany) supplemented with 10% fetal bovine serum (Biochrom; Merck Millipore) and 1% Penicillin-Streptomycin-Neomycin Solution (Sigma-Aldrich), and maintained in a humidified atmosphere with 5% CO_2 at 37˚C (New Brunswick™ Galaxy® 170R CO_2 Incubator; Eppendorf).

Peripheral blood samples. Upon obtaining written informed consent, peripheral blood was extracted from

Table I. Clinical characteristics of patients with chronic lymphocytic leukemia.

Clinical characteristic	Total patients, n (%)
Median age, years (range)	65 (47-82)
Gender	
Female	6 (66.67%)
Male	3 (33.33%)
Rai stage	
0	4 (44.44%)
I	4 (44.44%)
II	1 (11.11%)
ZAP-70, cut-off 20%	
Positive	2 (22.22%)
Negative	2 (22.22%)
Not available	5 (55.56%)
CD38, cut-off 30%	
Positive	4 (44.44%)
Negative	5 (55.56%)
IGHV status	
Mutated	3 (33.33%)
Not mutated	4 (44.44%)
Not available	2 (22.22%)
Cytogenetics	
Del(13q)	3 (33.33%)
Del(17p)	1 (11.11%)
No changes	6 (66.67%)

ZAP-70, zeta-chain-associated protein kinase 70; CD, cluster of differentiation; IGHV, immunoglobulin heavy chain variable region; Del, deletion.

nine patients with CLL, whose clinical characteristics are summarized in Table I. This study was approved by the Ethics Committee of the Medical University of Lublin (Lublin, Poland) (no. KE-0254/116/2012). Peripheral blood mononuclear cells (PBMCs) were isolated using Bicoll Density Centrifugation Media (Biochrom, Berlin, Germany). The viability of cells was >95%, as determined by trypan blue (Sigma-Aldrich) staining and quantification in a Neubauer chamber (Zeiss AG, Oberkochen, Germany). Immediately following isolation, cells were utilized in a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-te trazolium-5-carboxanilide (XTT; Sigma-Aldrich) assay.

Ex-LAC activity assay. Ex-LAC activity in the culture supernatant was measured spectrophotometrically at 525 nm with UV-160A spectrophotometer (Shimadzu Corporation, Tokyo, Japan), using syringaldazine (Sigma-Aldrich) as a substrate (18). Enzyme and substrate blanks were included in the assay. A unit (nkat) of ex-LAC activity was defined as the amount of enzyme catalyzing the production of 1 nmol/sec of colored product (quinone, ε^M = 65,000/M/cm) at 25°C and pH 7.4. The enzymatic activity of ex-LAC was expressed as nkat/mg of protein.

Protein concentration. Protein concentrations were determined using the Bradford reagent (Sigma-Aldrich), which is based on the color change of Coomassie brilliant blue G-250 dye in response to various concentrations of protein (the dye binds to primarily basic, especially arginine and aromatic amino acid residues), and bovine serum albumin (Sigma-Aldrich) as a standard (19). Protein concentration was determined following the addition of an acidic dye to the protein solution, and absorbance was measured at a wavelength of 595 nm using a UV-160A spectrophotometer (Shimadzu Corporation).

XTT assay. The cytotoxic effect of ex-LAC was measured using the In Vitro Toxicology Assay Kit, XTT based (Sigma-Aldrich). All cells were suspended in X-VIVO™ medium with or without phenol red (Lonza Group Ltd., Basel, Switzerland) and gentamicin (Lonza Group Ltd.). Cell lines were seeded onto 96-well plates (GenoPlast Biochemicals, Rokocin, Poland) at a concentration of $5x10^4$ cells/100 μl/well. PBMCs obtained from patients with CLL were dispensed at a concentration of $5x10^5$ cells/100 μl/well. Cells were exposed to different concentrations of ex-LAC ranging from 666.667 to 0.007 μg/ml. As a negative control, live cells were used, while as a positive control, cells treated with 0.1% Triton X-100 (Sigma-Aldrich) were used. XTT (25 μl) was added to all samples, prior to be incubated for 48 h in a humidified atmosphere with 5% CO_2 at 37°C. Optical densities (OD) were measured at 450 nm, using a background wavelength of 690 nm. OD measurements were performed every 24 h. Each sample was assayed in triplicate, and the entire experiment was performed three times. The results were expressed as half maximal inhibitory concentration (IC_{50}), and the percentage of cytotoxicity was calculated as follows:

Cytotoxicity = [1-(OD_s-OD_b)/(OD_c-OD_b)] x 100%,

where OD_s is the OD value of the assayed sample, OD_b is the OD value of the positive control and OD_c is the OD value of the live cells used as negative control.

Apoptosis analysis. For apoptosis analysis, Jurkat cells were treated with different concentrations of ex-LAC, and 0.1% Triton X-100 was added as a positive control. Following 48 h of incubation, all cells were washed using phosphate-buffered saline (PBS; Biochrom; Merck Millipore), resuspended in a binding buffer (Sigma-Aldrich) and stained with 5 μl Annexin V-fluorescein isothiocyanate (FITC) and 10 μl propidium iodide (PI), according to the manufacturer's protocol of the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich). Cells were incubated for 10 min in the dark, and immediately analyzed with FACSCalibur™ (BD Biosciences, San Jose, CA, USA). Live cells served as a negative control.

Visualization of Jurkat and RPMI 8226 cells using fluorescence and scanning electron microscopy (SEM). Jurkat and RPMI 8226 cells were incubated with ex-LAC for 48 h. Following incubation, all cells were washed with PBS, resuspended in a binding buffer and stained with 5 μl Annexin V-FITC and 10 μl PI, which were provided in the Annexin V-FITC Apoptosis Detection Kit. Cells subjected to SEM (VEGA3 LM; Tescan, Brno, Czech Republic) were pre-treated with 2.5% (v/v) glutaraldehyde (Sigma-Aldrich) for 2 h and 1% osmium tetroxide (Sigma-Aldrich) for 30 min.
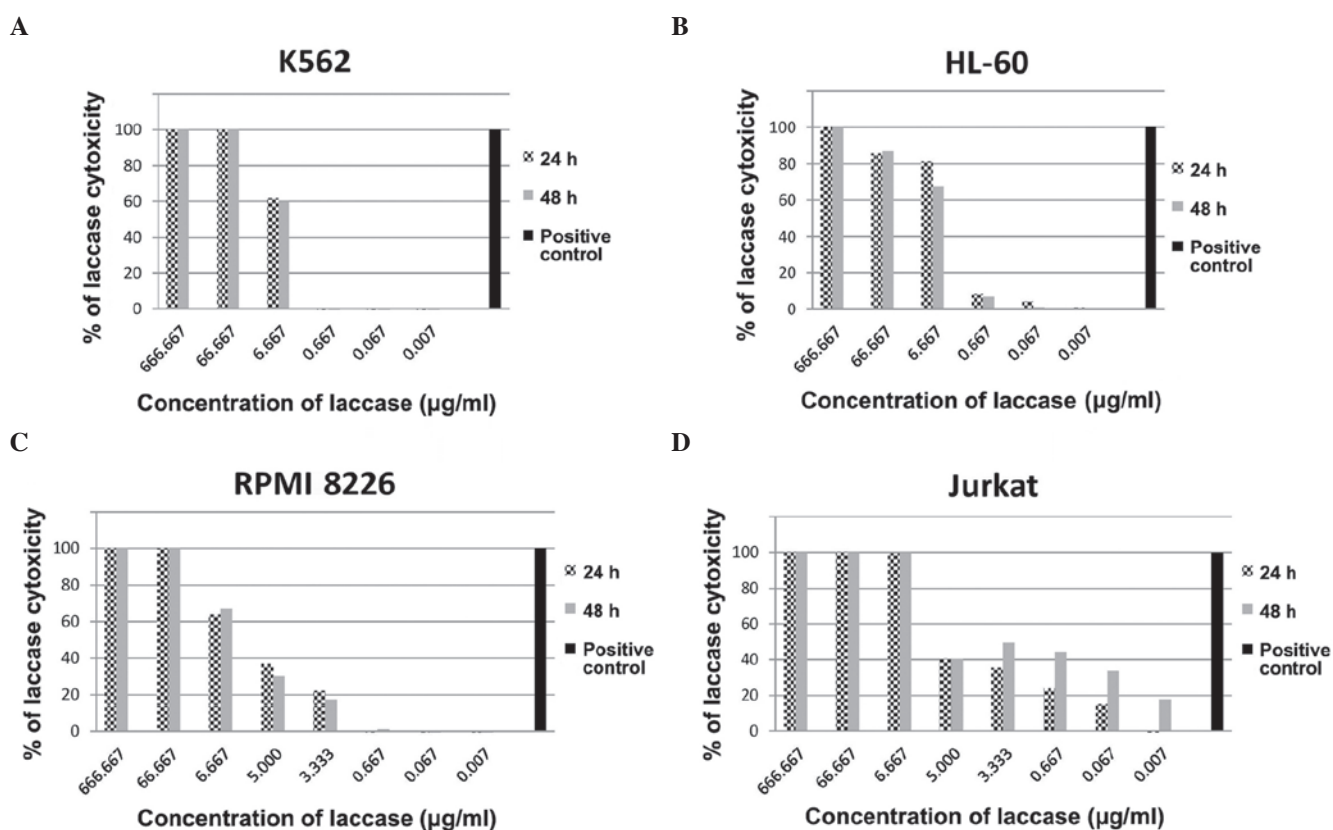
A

B

C

D

Figure 1. Cytotoxic effects of extracellular laccase on (A) K562, (B) HL-60, (C) RPMI 8226 and (D) Jurkat cell lines. The percentage of cytotoxicity with respect to the control was assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay.

The osmium-fixed cells were subsequently dehydrated using a series of graded ethanol solutions (Sigma-Aldrich) ranging from 10% to absolute ethanol, followed by air drying at room temperature and coating with gold (Sigma-Aldrich). Magnification x5,000 was used to observe the cells.

Results

C. unicolor ex-LAC preparation demonstrates enzymatic activity. Ex-LAC was isolated and partially purified from the idiophasic cultures of *C. unicolor* according to the method previously published by Rogalski and Janusz (16). Purification of *C. unicolor* ex-LAC was performed using ion exchange chromatography on DEAE-Sepharose and Sephadex G-50 columns. The enzyme isoforms Ia1, Ia2, Ib and IIa1 were recovered with a 65-92-fold increase in specific activity and a yield of 6.7, 27.5, 9.7 and 21.0%, respectively. The isoelectric points were in the range of 4.7-4.2 and the carbohydrate content in the purified enzymes was between 1.6 and 3.5% (16). A total of 1 mg lyophilized ex-LAC isoform mixture dissolved in 1 ml Milli-Q water (EMD Millipore) possessed an activity of 1,150,110 nkat and a protein concentration of 329 μg/ml.

Cytotoxicity of C. unicolor ex-LAC on the K562 cell line increases with concentration. To evaluate the cytotoxic effect of ex-LAC on cell lines, cells were treated with a range of concentrations of ex-LAC (666.667, 66.667, 6.667, 0.667, 0.067 and 0.007 μg/ml) for 24 and 48 h, and XTT assays were subsequently performed. The results indicated that

the cytotoxicity of ex-LAC on K562 cells increased with concentration (Fig. 1A). Cytotoxicity of 100.00% against K562 cells was observed following 24 and 48 h of incubation with 666.667 and 66.667 μg/ml ex-LAC. At a concentration of 6.667 μg/ml, the cytotoxic effect of ex-LAC on K562 cells following 24 h of incubation was 61.78%, and following 48 h of incubation the cytotoxic effect was 59.80%. No cytotoxic effect was detected on K562 cells following 24 and 48 h of incubation with 0.667, 0.067 and 0.007 μg/ml *C. unicolor* ex-LAC. The IC_{50} values of ex-LAC on K562 cells following 24 and 48 h of treatment were 0.8 and 1.0 μg/ml, respectively, as determined by XTT assay.

Concentration-dependent cytotoxic activity of C. unicolor ex-LAC is observed in the HL-60 cell line. The cytotoxicity of various concentrations of ex-LAC on HL-60 cells was established using an XTT assay. Following 24 h of incubation of HL-60 cells with 666.667, 66.667, 6.667, 0.667, 0.067 and 0.007 μg/ml ex-LAC, the percentage of cytotoxicity observed was 100.00, 85.63, 81.55, 8.53, 4.39 and 1.11%, respectively (Fig. 1B). The IC_{50} value corresponding to 24-h treatment was 0.5 μg/ml. Similar results were obtained following 48 h of HL-60 incubation with ex-LAC. A total of 100.00% cytotoxicity was observed following incubation with 666.667 μg/ml ex-LAC for 48 h, 87.00% following incubation with 66.667 μg/ml and 67.51% following incubation with 6.667 μg/ml ex-LAC. Cytotoxicity of 6.94, 1.34 and 0.32% was observed following 48 h of incubation with 0.667, 0.067 and 0.007 μg/ml ex-LAC, respectively (Fig. 1B). Following 48 h

Table II. Percentage of live, apoptotic and necrotic Jurkat cells following 48-h treatment with *Cerrena unicolor* ex-LAC, as determined by flow cytometry using Annexin V and propidium iodide staining.

Concentration of ex-LAC, μg/ml	Live cells, %	Apoptotic cells, %	Necrotic cells, %
666.667	2.20	95.67	2.13
66.667	3.25	95.87	0.88
6.667	0.82	98.00	1.18
5.000	1.24	97.05	1.71
3.333	1.65	96.76	1.59
0.667	0.68	98.90	0.42
0.067	7.95	90.24	1.81
0.007	8.86	88.98	2.16
Control	99.93	0.05	0.02

ex-LAC, extracellular laccase.

of treatment, the IC$_{50}$ value of ex-LAC on HL-60 cells was 0.9 μg/ml.

C. unicolor ex-LAC decreases viability of the RPMI 8226 cell line. The RPMI 8226 cell line was incubated with a series of dilutions of ex-LAC, and the cytotoxic effect caused by ex-LAC in these cells was also observed to be dose-dependent (Fig. 1C). Following 24- and 48-h treatment of cells with 666.667 and 66.667 μg/ml ex-LAC, 100.00% cytotoxicity was observed, while 6.667 μg/ml ex-LAC achieved 63.84 and 66.96% cytotoxicity following 24 and 48 h of incubation, respectively. Cytotoxicity of 36.77 and 30.20% was observed following treatment with 5.000 μg/ml ex-LAC for 24 and 48 h, respectively. Following 24 and 48 h of incubation with 3.333 μg/ml ex-LAC, the observed cytotoxicity on RPMI 8226 cells was 21.97 and 17.59%, respectively. Cytotoxicity of 1.03% was noted following 48 h of treatment with 0.667 μg/ml ex-LAC. By contrast, no effect was observed following 24 h of incubation with 0.667 μg/ml ex-LAC. Similarly, no decrease in RPMI 8226 cell viability was observed following 24 and 48 h of incubation with 0.067 and 0.007 μg/ml ex-LAC. The IC$_{50}$ values obtained following 24- and 48-h treatment with ex-LAC were 0.9 and 1.1 μg/ml, respectively. The IC$_{50}$ values following 24- and 48-h treatment with ex-LAC were similar for all cell lines used (K562, 0.8 and 1.0 μg/ml; HL-60, 0.5 and 0.9 μg/ml; RPMI8226, 0.9 and 1.1 μg/ml; and Jurkat, 0.8 and 0.4 μg/ml). The differences in IC$_{50}$ values observed result from the fact that each cell line originates from a different neoplasm and different cell lineages. Jurkat cells are derived from a human T cell leukemia line and T cells are considered the most treatment resistant cell type (20).

Cytotoxic activity of C. unicolor ex-LAC is observed in the Jurkat cell line. The cytotoxic activity of ex-LAC was additionally investigated in the Jurkat cell line via XTT assay (Fig. 1D). Following 24 h of incubation with 666.667 and 66.667 μg/ml ex-LAC, 100.00% cytotoxicity was observed in Jurkat cells. Treatment with 6.667 μg/ml ex-LAC resulted in 99.28% cytotoxicity, while 24-h treatment with 5.000 and 3.333 μg/ml ex-LAC resulted in 40.39 and 35.22% cytotoxicity, respectively. Cytotoxicity of 24.00 and 15.14% was observed

following 24-h incubation with 0.667 and 0.067 μg/ml ex-LAC, respectively. No effect on Jurkat cell viability was observed with a concentration of ex-LAC of 0.007 μg/ml. By contrast, incubation with 666.667 μg/ml ex-LAC for 48 h resulted in 100.00% cytotoxicity. Similarly, concentrations of 66.667 and 6.667 μg/ml had a cytotoxic effect on Jurkat cells of 99.85 and 100.00%, respectively. Similar results were obtained following 48 h of incubation with 5.000, 3.333 and 0.667 μg/ml ex-LAC, which achieved cytotoxicities of 40.35, 49.46 and 44.36%, respectively. Jurkat cells treated with 0.067 and 0.007 μg/ml ex-LAC for 48 h exhibited a percentage of cell death of 33.80 and 17.91%, respectively. The IC$_{50}$ values for 24- and 48-h incubation with ex-LAC were 0.8 and 0.4 μg/ml, respectively.

C. unicolor ex-LAC induces morphological changes in the RPMI 8226 cell line. Morphological changes in RPMI 8226 cells following 48 h of treatment with various concentrations of ex-LAC were observed under fluorescence microscopy and SEM (Fig. 2). Contrarily to untreated RPMI 8226 cells, which possessed a regular, oval shape (Fig. 2A), SEM revealed cell volume shrinkage, membrane blebbing and apoptotic body formation in RPMI 8226 cells treated with ex-LAC (Fig. 2B). For apoptosis detection, ex-LAC-treated and untreated cells were stained with Annexin V and PI, and visualized under a fluorescence microscope (E-800; Nikon Corporation, Tokyo, Japan). In comparison with untreated cells (Fig. 2C), cells treated with ex-LAC displayed apoptotic-like changes, including condensation and fragmentation of nuclei, in addition to cytoplasm condensation (Fig. 2D).

Apoptotic changes in the Jurkat cell line are induced by ex-LAC. In order to confirm the results obtained by XTT assay, Jurkat cells were analyzed using Annexin V/PI staining and flow cytometry following 48 h of incubation with ex-LAC (Table II and Fig. 3). Compared with the control, the percentage of live cells (R4) was decreased, and the percentage of apoptotic cells (R3+R5) was increased for all the concentrations of enzyme tested (Table II). The frequency of live and apoptotic cells in the untreated control was 99.93 and 0.05%, respectively (Fig. 3A). At a concentration of 666.667 μg/ml, the percentage of live
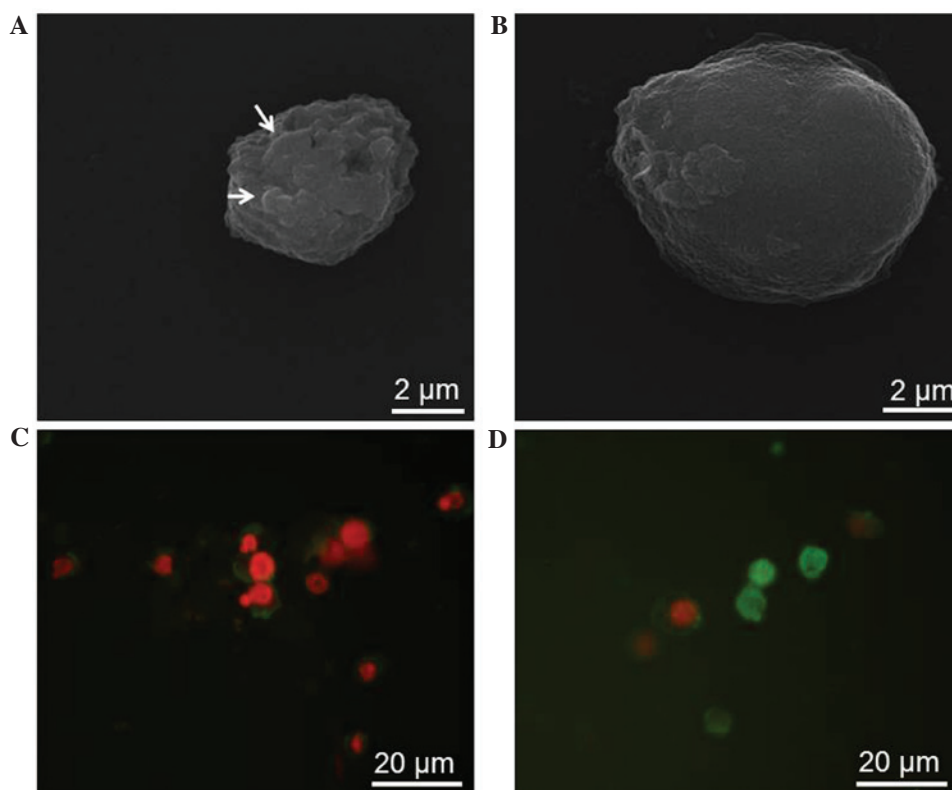
Figure 2. Analysis of the effects of ex-LAC on RPMI 8226 cells following 48 h of treatment. (A) Changes in cells incubated with 666.667 µg/ml ex-LAC and (B) untreated control cells were visualized by SEM. (C) The effects of treatment with 666.667 µg/ml ex-LAC on RPMI 8226 cells were analyzed by fluorescence microscopy, and compared with (D) untreated cells. Propidium iodide staining (red) indicates apoptotic/necrotic cells. Annexin V-fluorescein isothiocyanate staining (green) indicates viable cells. White arrows indicate apoptotic changes in cells. ex-LAC, extracellular laccase; SEM, scanning electron microscopy.
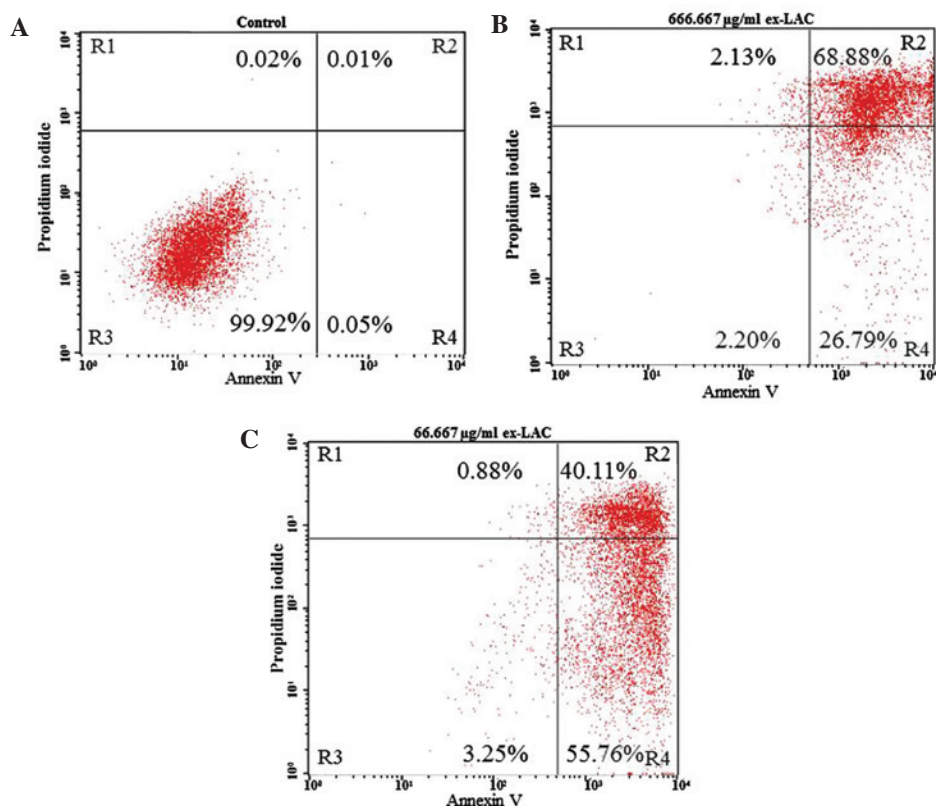

Figure 3. Cytotoxic effect of ex-LAC on Jurkat cells. (A) Control cells. (B) Cells treated with 666.667 µg/ml ex-LAC. (C) Cells treated with 66.667 µg/ml ex-LAC. Cytotoxicity was assessed by staining the cells with Annexin V and PI prior to being subjected to flow cytometry analysis. In each graph, the lower left quadrant (R4) indicates viable cells (Annexin V⁻PI⁻); the upper left quadrant (R2) represents necrotic cells (Annexin V⁻PI⁺); the lower right quadrant (R5) indicates early apoptotic cells (Annexin V⁺PI⁻); and the upper right quadrant (R3) represents late apoptotic cells (Annexin V⁺PI⁺). ex-Lac, extracellular laccase; PI, propidium iodide.
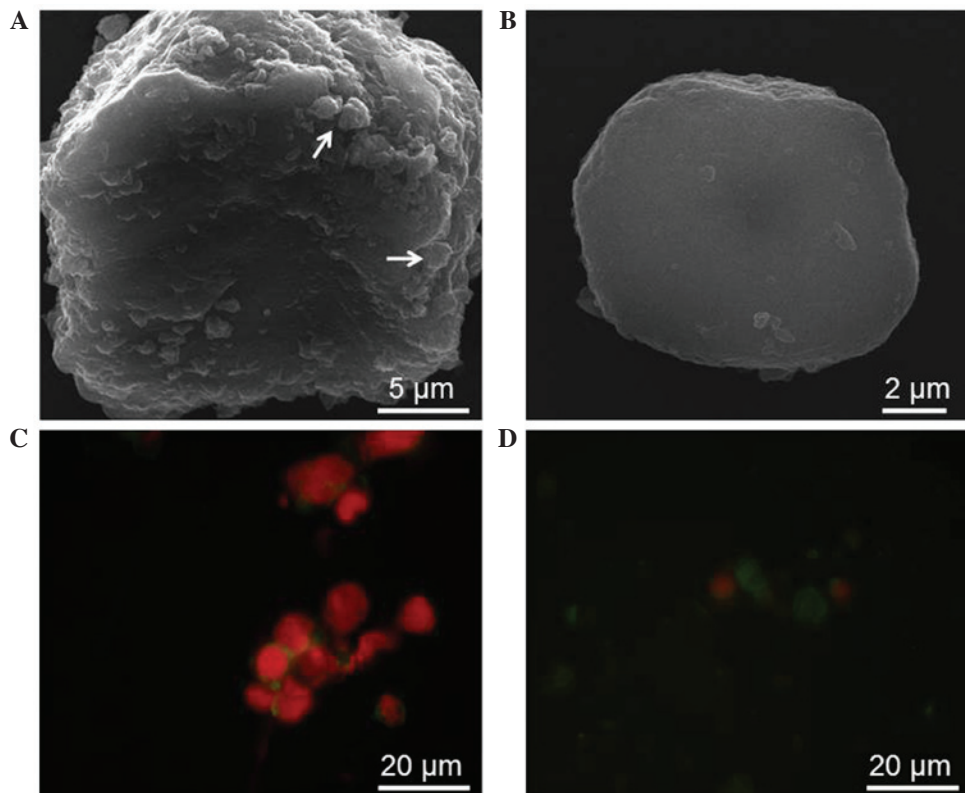
Figure 4. Analysis of the effects of ex-LAC on Jurkat cells following 48 h of treatment. (A) Changes in cells incubated with 666.667 µg/ml ex-LAC were visualized by SEM.(B) Untreated control cells were visualized by SEM. (C) Fluorescence microscopy analysis of the effects of treatment with 666.667 µg/ml ex-LAC on Jurkat cells, compared with (D) untreated cells. Propidium iodide staining (red) indicates apoptotic/necrotic cells. Annexin V-fluorescein isothiocyanate staining (green) indicates viable cells. White arrows indicate apoptotic changes in cells. ex-LAC, extracellular laccase; SEM, scanning electron microscopy.

vs. apoptotic cells was 2.20 vs. 95.67% (Fig. 3B), while for a concentration of 66.667 µg/ml these percentages were 3.25 and 95.87%, respectively (Fig. 3C). At a concentration of ex-LAC of 6.667 µg/ml the percentage of live vs. apoptotic cells was 0.82 vs. 98.00%, while it was 1.24 vs. 97.05% at 5.000 µg/ml ex-LAC. At a concentration of 3.333 µg/ml, the percentage of live vs. apoptotic cells were 1.65 vs. 96.76%, while these percentages were 0.68 vs. 98.90%, 7.95 vs. 90.24% and 8.86 vs. 88.98% for concentrations of ex-LAC of 0.667, 0.067 and 0.007 µg/ml, respectively (Table II).

SEM and fluorescence microscopy were used to observe the morphology of control cells and Jurkat cells undergoing treatment with various concentrations of ex-LAC (Fig. 4). Whereas control cells possessed an oval, regular shape (Fig. 4A), SEM observation of Jurkat cells following 48 h of incubation with ex-LAC revealed several characteristics of apoptosis, including volume shrinkage and apoptotic body formation (Fig. 4B). Ex-LAC-treated and untreated Jurkat cells were stained with Annexin V and PI prior to be observed under fluorescence microscope (E-800; Nikon Corporation). Contrarily to Jurkat control cells (Fig. 4C), cells incubated with ex-LAC for 48 h displayed nucleus shrinkage and fragmentation, as well as significant cytoplasm condensation (Fig. 4D).

Cytotoxicity of ex-LAC is additionally exerted against primary CLL cells. The cytotoxic activity of *C. unicolor* ex-LAC was also assessed in PBMCs derived from nine patients with CLL via XTT assay (Fig. 5). Based on the results obtained in the established cell lines, three concentrations of enzyme were

selected for 24- and 48-h incubation with primary CLL cells. Following 24 h of incubation with 66.667, 6.667 and 3.333 µg/ml ex-LAC, the median cytotoxic effect observed was 100.00, 69.06 and 42.85%, respectively. Following 48 h of incubation with 66.667, 6.667 and 3.333 µg/ml ex-LAC, a median cytotoxicity of 100.00, 75.99 and 70.26% was observed, respectively. The IC_{50} values of ex-LAC on CLL cells were 0.7 and 0.9 µg/ml following 24 and 48 h of incubation, respectively.

Discussion

It has been reported that *C. unicolor* may be a source of active ex-LAC (21). Thus, we hypothesize that *C. unicolor* may be exploited to produce high amounts of this biologically active substance with pharmacological potential. The characterization of *C. unicolor* ex-LAC isoforms has been previously described (22). In the present study, the enzyme isoforms Ia1, Ia2, Ib and IIa1 were recovered with a 65-92-fold increase in specific activity and a yield of 6.7, 27.5, 9.7 and 21.0%, respectively. The isoelectric points were in the range of 4.7-4.2, and the carbohydrate content in the purified enzymes was 1.6-3.5%. The specific activity of this isoform mixture was 3,495.4 nkat/mg of protein. In comparison with a number of alternative sources of ex-LAC, *C. unicolor* produces the enzyme with high activity without requiring additional supplementation such as aromatic compounds (21,23).

C. unicolor ex-LAC is currently utilized in biodegradation, bioremediation, delignification and decolorization
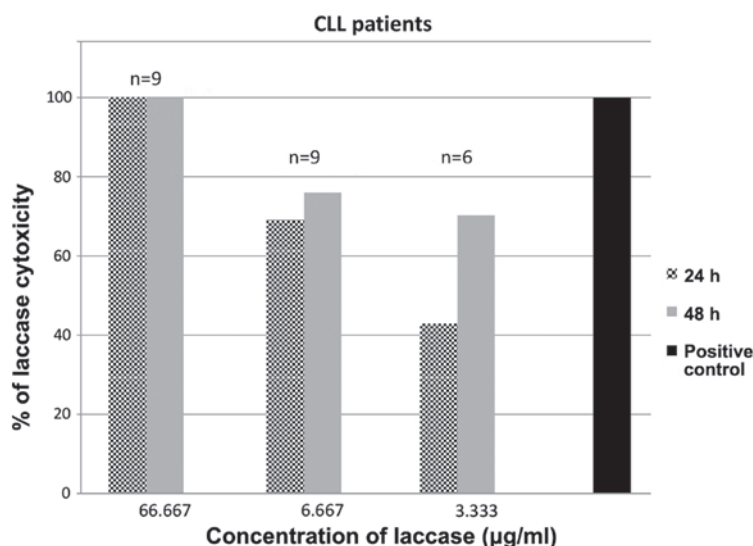
Figure 5. Cytotoxic activity of extracellular laccase on peripheral blood mononuclear cells of patients with chronic lymphocytic leukemia. The percentage of cytotoxicity with respect to the control was assessed by 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide assay.

processes (15). However, to the best of our knowledge, there are no reports to date regarding its anti-leukemic activity. In the present study, it was identified that ex-LAC purified from *C. unicolor* possessed cytotoxic activity against several hematological malignancies and primary CLL cells using an XTT assay, and these findings were confirmed by apoptosis analysis using flow cytometry, and additionally visualized under SEM and fluorescence microscopy.

Anticancer properties have been reported for several fungal extracts, including *Funalia trogii* (7,8). Unyayar *et al* (8) measured the inhibition of growth of the HeLa human cervical cancer cell line and normal fibroblasts following 96 h of treatment with *F. trogii*. The observed growth inhibition on HeLa cells was 27.2% for 1 μl *F. trogii* extract, 39.7% for 3 μl, 56.1% following treatment with 5 μl, 59.5% in the presence of 7 μl extract and 71.5% following incubation with 10 μl extract, as evaluated by methyl thiazolyl tetrazolium assay (8). *F. trogii* extract contains LAC, glutathione reductase and peroxidase, which have been documented to be actively involved in cytotoxicity (8). Rashid *et al* (7) additionally confirmed the antitumor activity of *F. trogii* extract against the HMEC-1 human microvascular endothelial cell line using a trypan blue exclusion assay (7). The highest toxicity observed in the assay corresponded to 96-h treatment with 2.5 mg/ml extract, while no toxicity was observed towards fibroblasts and non-proliferating cells under those conditions. The results of the XTT assay performed in the present study indicated that *C. unicolor* ex-LAC induced cytotoxicity on HL-60, K562, RPMI 8226, Jurkat and primary CLL cells in a dose-dependent manner. The antitumor properties of LAC and peroxidase identified in *F. trogii* are due to the presence of natural quinone substances produced by the action of these enzymes on a lignin substrate (8). Since all tumor cells are rich in quinones and quinone-like molecules, extracts containing LACs and/or peroxidases are able to selectively convert these molecules into toxic substances that cause apoptosis of cells (8). The pro-oxidative and antibacterial properties of ex-LAC from *C. unicolor* have been previously described (24). In that study, the potential

of ex-LAC for production of reactive oxygen species was investigated chemiluminometrically, and a marked pro-oxidative action of the enzyme was identified (24). Estimation of ex-LAC toxicity using a Microtox® detection system demonstrated that the exposure of the marine bacterium *Vibrio fischeri* to ex-LAC caused 38 and 51% cell damage following 5 and 15 min of incubation, respectively (24). Ex-LAC has also been reported to be effective against *Escherichia coli* (24). The apoptosis analysis of ex-LAC extracted from *C. unicolor* conducted in the present study additionally demonstrated apoptosis of Jurkat cells in the presence of various concentrations of ex-LAC, compared with untreated control cells. Apoptotic changes in Jurkat and RMPI 8226 cells caused by *C. unicolor* ex-LAC were also observed under SEM and fluorescence microscopy, thus confirming the above findings.

Lau *et al* (25) and Unyayar *et al* (8) have independently documented the cytotoxic activity of *Coriolus versicolor* extracts. Lau *et al* (25) observed significant dose-dependent inhibitory effects on the proliferation of the Raji human Burkitt's lymphoma B-cell line and the NB-4 and HL-60 human acute promyelocytic leukemia cell lines treated with *C. versicolor* extract. In that study, >90% inhibition was detected following 72 h of treatment. For the Raji lymphoma cell line, the IC$_{50}$ value of *C. versicolor* extract was 253.8 μg/ml, while for the NB-4 and HL-60 cell lines, the IC$_{50}$ values were 269.3 and 147.3 μg/ml, respectively. The results of the present study revealed that *C. unicolor* ex-LAC was able to inhibit proliferation of human leukemic cell lines in a dose-dependent manner at lower dosages than those documented by Lau *et al* (25), since the IC$_{50}$ values following 48 h of treatment were 0.9, 0.4, 1.1 and 1.0 μg/ml for HL-60, Jurkat, RPMI 8226 and K562 cells, respectively. Furthermore, the inhibitory effect of *C. versicolor* extracts on the HeLa cell line demonstrated by Unyayar *et al* (8) was lower than the activity of *C. unicolor* ex-LAC identified in the present study, since 1 μl *C. versicolor* extract caused 27.5% growth inhibition in HeLa cells, and the maximum inhibition observed was 45.5% following treatment with 10 μl extract (8). The results of the present study indicated

an inhibitory effect of *C. unicolor* ex-LAC of ~100.00% at a concentration of 666.667 μg/ml on all the cell lines tested.

Anti-leukemic activity of agaritine, a β-glucan isolated from *Agaricus blazei*, was reported by Endo *et al* (26). In that study, the 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2 ,4-disulfophenyl)-2H-tetrazolium assay was used to evaluate the inhibitory effect of agaritine from *A. blazei* on the U937 human leukemic monocyte lymphoma, MOLT-4 human acute lymphoblastic leukemia, HL-60 and K652 cell lines following 48 h of treatment. The authors observed that the viability of all the cell lines tested decreased with increasing concentrations of agaritine. Agaritine suppressed cell growth in U937, MOLT-4, HL-60 and K562 cells with an IC_{50} value of 2.7, 9.4, 13.0 and 16.0 μg/ml, respectively (26). By contrast, in the present study, following 48 h of incubation of HL-60 and K562 cells with *C. unicolor* ex-LAC, the IC_{50} values measured (0.9 and 1.0 μg/ml, respectively) were lower than those reported by Endo *et al* (26), indicating increased activity of *C. unicolor* ex-LAC compared with *A. blazei* agaritine.

Chen *et al* (27) investigated the antitumor and immuno-modulatory effects of PCP-3A, a non-lectin glycoprotein extracted from the mushroom *Pleurotus citrinopileatus* (27). Trypan blue exclusion assay revealed 37-64% growth inhibition of U937 cells following 72 h of incubation with 25 μg/ml PCP-3A. In addition, *P. citrinopileatus* PCP-3A was able to stimulate the secretion of tumor necrosis factor α, interleukin-2 and interferon-γ by CD4$^+$ T cells, which indirectly suppressed the growth of U937 cells, indicating that PCP-3A possessed antitumor and immunomodulatory activities (27). By contrast, *C. unicolor* ex-LAC inhibited the growth of K562, Jurkat and RPMI 8226 cells by 100.00% at a concentration of 66.6667 μg/ml, while 87.00% cytotoxicity was observed in the HL-60 cell line at a concentration of 66.667 μg/ml, as assessed by XTT assay in the present study. Furthermore, 6.667 μg/ml *C. unicolor* ex-LAC exhibited a higher cytotoxic rate against the tested cell lines than 25 μg/ml *P. citrinopileatus* PCP-3A, with cytotoxic rates of 67.51, 59.80, 100.00 and 66.96% for HL-60, K562, Jurkat and RPMI 8226 cells, respectively. Additionally, *C. unicolor* ex-LAC was demonstrated to induce apoptosis, which was further confirmed in Jurkat and RPMI 8226 cell lines using flow cytometry and microscopic techniques in the present study.

Tsai *et al* (28) reported a novel non-lectin glycoprotein (HM-3A) purified from *Hypsizygus marmoreus* with anti-leukemic activity against the U937 cell line (28). The antiproliferative effect of HM-3A increased with concentration. As demonstrated by trypan blue exclusion assay, HM-3A induced cytotoxicity against U937 cells at concentrations ranged between 12.5 and 100.0 μg/ml, following treatment for 24-72 h. At 100.0 μg/ml, HM-3A led to 96.2% growth inhibition of U937 cells within 72 h (28). Similar cytotoxic rates were observed in the current study, as 100.00% cytotoxicity was achieved at concentrations of 666.667 and 66.667 μg/ml *C. unicolor* ex-LAC in K562, Jurkat and RPMI 8226 cell lines. In HL-60 cells, growth inhibition at 666.667 and 66.667 μg/ml *C. unicolor* ex-LAC was 100.00 and 87.00%, respectively. Therefore, the results of the present study indicated that *C. unicolor* ex-LAC possessed similar anti-leukemic activity to *H. marmoreus* HM-3A.

PNAP, a novel protein with antitumor activity towards HeLa cells and the MCF7 breast cancer cell line, was isolated from *Pholiota nameko* by Zhang *et al* (29). The results of a trypan blue exclusion assay conducted by these authors revealed dose-dependent antiproliferative effects of PNAP following 24 h of treatment, with an IC_{50} value of 9.97 μM for MCF7 cells and 12.11 μM for HeLa cells. The authors observed that the cytotoxic effect of PNAP on cancer cell lines was significantly increased, compared with the effect on normal cells (29). In the present study, the IC_{50} values observed following 24 h of incubation with *C. unicolor* ex-LAC were 0.5, 0.8, 0.9, 0.8 and 0.7 μg/ml for HL-60, Jurkat, RPMI 8226, K562 and CLL primary cells, respectively. Flow cytometric analysis of apoptosis performed by Zhang *et al* (29) following 48 h of incubation of MCF7 cells with 5, 10 and 15 μM PNAP revealed the presence of 5.29, 10.05 and 22.88% apoptotic cells, respectively, whilst the apoptotic rate in non-treated cells was 3.23%. By contrast, in the present study, apoptotic rates ranging from 88.98 to 98.90% were observed in Jurkat cells following 48 h of ex-LAC treatment, depending on the concentration of ex-LAC used. Furthermore, Zhang *et al* (29) measured the accumulation of mitochondrial cytochrome *c* release into the cytosol, and observed that the cytosol from untreated cells contained low amounts of cytochrome *c*, in contrast to MCF7 cells incubated with PNAP, where cyto-chrome *c* was significantly accumulated. Similarly, reduced levels of cytochrome *c* in the mitochondrial fraction were also detected. Release of cytochrome *c* resulted in the activation of caspase-mediated apoptosis (29). In the present study, SEM and fluorescence microscopy images of ex-LAC-treated Jurkat and RPMI 8226 cells also indicated apoptosis involving cell volume and nucleus shrinkage, apoptotic body formation and fragmentation, as well as significant cytoplasm condensation.

To the best of our knowledge, a limited number of LACs of fungal origin with anti-malignant properties have been described thus far. Zhang *et al* (29) reported antiprolif-erative activity of *Clitocybe maxima* LAC against HepG2 and MCF7 hepatocellular carcinoma cells, and inhibitory activity towards human immunodeficiency virus (HIV)-1 reverse tran-scriptase (30). At concentrations of 2.5, 5.0, 10.0 and 20.0 μM, purified LAC from *C. maxima* inhibited the proliferation of HepG2 cells by 9.1, 20.4, 43.0 and 80.5%, respectively, and inhibited cell growth in MCF7 cells by 40.2, 75.3, 90.2 and 95.4%, respectively. The IC_{50} values against HepG2 and MCF7 cells were 12.3 and 3.0 μM, respectively. The authors observed that *C. maxima* LAC reduced the activity of HIV-1 reverse transcriptase with an IC_{50} value of 14.4 μM, and the percentage of inhibition of HIV-1 reverse transcriptase activity at 5, 10 and 20 μM *C. maxima* LAC was 13.7, 35.1 and 70.4%, respectively. Zhang *et al* (31) purified LAC from the white-rot fungus *Abortiporus biennis* and proved its antitumor activity against HepG2 and MCF7 cells, as well as observing its inhibi-tory activity against HIV-1 reverse transcriptase, achieving IC_{50} values of 12.5, 6.7 and 9.2 μM, respectively (31). Hu *et al* (32) demonstrated that LAC extracted from the fruiting bodies of *Agrocybe cylindracea* species possessed antiproliferative activity against MCF7 and HepG2 cell lines (32). The authors noted that *A. cylindracea* LAC additionally possessed HIV-1 reverse transcriptase inhibitory activity, with percentages of inhibition at 3.2, 8.0 and 20.0 μM of 15.7, 40.2 and 62.5%, respectively, and a calculated IC_{50} value of 12.7 μM. The cytotoxicity of *A. cylindracea* LAC towards HepG2 cells was

7.8, 30.2, 46.4 and 78.5% at concentrations of 1.2, 2.5, 5.0 and 10.0 μM, respectively, with an IC_{50} value of 5.6 μM. In the case of MCF7 cells, the IC_{50} value was 6.5 μM, and the percentage of inhibition was 7.2, 22.7, 41.3 and 70.6% at concentrations of 1.2, 2.5, 5.0 and 10.0 μM, respectively (32). In the present study, ex-LAC isolated from *C. unicolor* induced a higher cytotoxic effect towards HL-60, Jurkat, RPMI 8226 and K562 cells than *A. cylindracea* LAC did in the above previous study, since *C. unicolor* LAC demonstrated IC_{50} values of 0.5 (0.01 μM), 0.8 (0.014 μM) and 0.9 (0.016 μM) μg/ml for HL-60, Jurkat, RPMI 8226 and K562 cells, respectively.

To the best of our knowledge, the present study represents the first report to investigate the anti-leukemic activity of ex-LAC isolated and partially purified from idiophasic cultures of *C. unicolor*. The present study provided novel data concerning the isolation and chemical characterization of bioactive compounds of the white-rot fungus *C. unicolor*. The cytotoxic effect of ex-LAC extracted from this fungus was demonstrated on HL-60, Jurkat, RPMI 8226 and K562 cell lines, as well as CLL primary cells, using XTT assay. Additional analysis of Jurkat and RPMI 8226 cells revealed that *C. unicolor* ex-LAC was able to induce apoptosis of leukemic cells, even at low concentrations. Compared with other compounds of fungal origin, the IC_{50} values of *C. unicolor* ex-LAC were reduced, indicating high anti-malignant activity of this enzyme. In conclusion, the results of the present study suggest that *C. unicolor* ex-LAC should be considered as a novel therapeutic agent for the treatment of hematological malignancies.

Acknowledgements

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