

Pleurotus ostreatus and *Ruscus aculeatus* Extracts Cause Non-Apoptotic Jurkat Cell Death

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Abstract

We tested the effect of ethanol extracts from *Ruscus aculeatus* (Asparagaceae) and *Pleurotus ostreatus* (Agaricales) on the survival and proliferation of the Jurkat cell line, a model of human acute T-cell lymphocytic leukemia. *R. aculeatus* extracts were toxic to both Jurkat cells and human primary lymphocytes. *P. ostreatus* extracts were significantly more toxic to Jurkat cells than primary lymphocytes, as seen by LDH release and MTT assays. Annexin-V detection as well as Bax, Bcl-2, Smac/Diablo, and Caspase-8 expression were largely unchanged by *P. ostreatus* extracts. Cell cycle analysis and CDK-4 expression revealed stop of Jurkat cell proliferation. Interestingly, both extracts cause a strong, dose-dependent decrease in p53 levels in Jurkat cells, consistent with toxicity and cell death occurring via an unknown, non-apoptotic mechanism.

Keywords: Acute lymphocytic leukemia, Jurkat, *Pleurotus ostreatus*, *Ruscus aculeatus*

1. Introduction

Acute lymphocytic leukemia (ALL), also known as acute lymphoid leukemia or acute lymphoblastic leukemia is a cancer of the lymphocytes that appears and advances very rapidly (Weber, 2007). ALL is the most common type of childhood cancer [American Cancer Society], hence it is an attractive therapeutic target.

There is a need for complementary and alternative approaches to standard cancer therapies (Weininger, Surana & Wang, 2010; Syljuasen & McBride, 1999). Natural products have promising results as potential treatment for cancer in general and ALL in particular (Jadhav *et al.*, 2007; Tai *et al.*, 2010; Abdel-Massih *et al.*, 2010; Davicino *et al.*, 2010).

Ruscus aculeatus, commonly known as butcher's broom, belongs to the Asparagaceae family and is native to the Mediterranean. Extracts from this plant have been used in traditional medicine (Guarrera, 2005). Studies have shown that *R. aculeatus* extracts are active against blood disorders such as venous insufficiencies, edema, orthostatic hypotension, and inflammation (Liu *et al.*, 2002; Huang *et al.*, 2008; Mimaki *et al.*, 1998). Notably, two saponin compounds found in the alcoholic extract of *R. aculeatus* inhibit the growth of the human promyelocytic leukemia cell line HL-60 (Lindequist, Niedermeyer & Julich, 2005).

The *Pleurotus* genus, also known as the oyster mushroom, which belongs to the family Agaricales, is a common edible mushroom widely used in folk medicine (Jayakumar, Ramesh, & Geraldine, 2006). Studies have shown that *P. ostreatus* extracts have antioxidant activity and can inhibit proliferation of human cancer cells (Jedinak & Sliva, 2008; Venkatakrishnan *et al.*, 2010; Wang, Gao & Ng, 2000). A lectin extract from *P. ostreatus* has activity against hepatoma and sarcoma cells (Lavi *et al.*, 2006). An aqueous polysaccharide extract induced a colon cancer cell line to undergo apoptosis (Sun & Liu, 2009), and water-soluble polysaccharides extracted from *P. ostreatus* have immunomodulating and antitumor effects (Schneider, Schwenk & Bornkamm, 1977).

The Jurkat cell line is a human acute T-cell lymphoblastic leukemia cell line used for interleukin-2 production and for testing the susceptibility of ALL to different drugs (Kendig & Tarloff, 2007).

In light of their previous tested effects on cancer cell lines, this study tested the ethanol extracts from *R. aculeatus* and *P. ostreatus* on the survival and proliferation of the Jurkat cell line by using biochemical and molecular approaches.

2. Material and Methods

2.1 Extract preparation

The plant *R. aculeatus* was collected from northern Lebanon, washed with water and dried. The shoots were ground and soaked in 80% ethanol with shaking for 1 week. The crude extract was filtered and evaporated in a rotoevaporator at room temperature, followed by concentration in a Centrivap Concentrator (Labconco, Missouri) until it was completely dry. The resulting powder was weighed, and dissolved in RPMI 1640 media to a density of 1000 mg/ml. The solution was then sterilized using a 0.45 µm pore size filter, and 1 ml aliquots were prepared and stored at -20°C for later use. On the day of the experiment, the aliquots were thawed and kept on ice until used.

The mushroom *P. ostreatus* was cultivated adopting the "layer spawning" method. Freshly-harvested whole mushrooms were shade dried and finely powdered. A sample of 100 g of powder was extracted with 80% ethanol with shaking for 1 week. The same procedure used for *R. aculeatus* was adopted to obtain *P. ostreatus* aliquots.

2.2 Cell culture

Jurkat cells were suspended in RPMI 1640 medium containing 25 mM 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid (HEPES) and L-glutamine (Lonza, Belgium), supplemented with 10% Fetal Bovine Serum (FBS) (Sigma, USA) and 100 U/ml of penicillin-streptomycin (Lonza, Belgium). The cells were grown in a humidified incubator with 5% CO₂ at 37°C. Media was changed every second day, and the cells were passaged every four days at a ratio of 1:4.

2.3 Cytotoxicity assay

The cytotoxicity of each extract on the Jurkat cell line was assessed using the lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Takara Bio Inc., Japan). The assay was performed according to the manufacturer's protocol. The tested conditions were 1, 2, 4, 5, 8, 10, 16, 20, 32, 40, or 60 mg/ml of extract diluted in 1% FBS containing media, at 6 hrs, 24 hrs, or 48 hrs. For the 48 hrs period, the cells were fed at the 24 hrs time point. Each condition was done in triplicate, and the results shown represent the average of three independent experiments.

Normal lymphocytes from EDTA-anticoagulated peripheral blood of a normal human donor were isolated using the standard Ficoll-Paque Plus gradient method (GE Healthcare, USA) and subsequently washed twice in

complete RPMI 1640 medium. Cells were suspended in complete RPMI 1640 medium, following which the same procedure for the cytotoxicity test was used.

2.4 Metabolic activity test

Metabolically active cells were assessed using the Cell Proliferation Kit I (MTT) (Roche Applied Sciences, Germany). The assay was performed according to the manufacturer's protocol. The tested conditions were 1, 2, 4, 5, 8, 10, 16, 20 mg/ml of extracts diluted in 10% FBS-containing media, at either 24 or 48 hrs. Cells kept past 24 hrs were fed at the 24 hrs time point. Each condition was done in triplicate, and the results shown are the average of three independent experiments.

2.5 Annexin V-FITC

Cells were seeded at a density of 5×10^5 cells/ml in a 24-well cell culture plate and were incubated for 24 hrs. They were treated with 8, 10, or 16 mg/ml of *P. ostreatus* extract, or 8 or 10 mg/ml of *R. aculeatus* extract and incubated for 24 hrs. Cells were then harvested at a density of 5×10^5 cells/ml and washed twice with cold 1XPBS. The cells were gently suspended in a solution containing 100 μ l of Annexin V-Fluos labeling solution (Roche Applied Sciences, Germany), 5 μ l of fluorescein isothiocyanate labeled by Annexin V-FITC and 5 μ l of propidium iodide (PI). Cells were incubated in the dark at room temperature for 15 min before adding 500 μ l Annexin V-Fluos labeling solution to each well. Cells were then subjected to flow cytometry analysis using a BD FACSCalibur flowcytometer (Becton Dickinson, USA). CellQuest™ software (Becton Dickinson, USA) was used to analyze the data. Each condition was done in duplicate, and the represented results are the average of two independent experiments.

2.6 Cell cycle analysis

Cells were seeded at a density of 1×10^6 cells/ml in a 24-well cell culture plate and were incubated for 24 hrs. They were treated with 8, 10, or 16 mg/ml of *P. ostreatus* extract, or 8 or 10 mg/ml of *R. aculeatus* extract and incubated for 24 hrs. The cells were harvested at a density of 2×10^6 cells/ml and washed with cold 1XPBS. The cells were fixed in 70% ethanol and left at -20°C overnight. Cells were washed with cold 1XPBS, and then treated with RNaseA (Fermentas, Canada) for 1 hr 30 min at 37°C . The solution was centrifuged at 200 g and then washed twice with cold 1XPBS. The pellet was resuspended in 1XPBS and 67 μ g/ml PI (Invitrogen, USA) and incubated for 10 min in the dark and read on a FACScan flowcytometer (Becton Dickinson, USA). CellQuest™ software (Becton Dickinson, USA) was used to analyze the data. Each condition was done in duplicate, and the represented results are the average of three independent experiments.

2.7 Protein extraction and western blot

Cells were seeded at a density of 1×10^6 cells/ml in a 24-well cell culture plate and incubated for 24 hrs. Then they were treated with 1-20 mg/ml of *P. ostreatus* or *R. aculeatus* extract and incubated for 24 hrs. The cells were then harvested and washed with cold 1XPBS. Proteins were extracted from cells using a lysis buffer containing 25 mM Tris-HCl pH 7.6, 140 mM NaCl, 1% TritonX-100 (w/v), 0.5% SDS, and protease inhibitors. Lysates were left on ice for 40 min, then centrifuged at 14,000 g for 10 min at 4°C and the supernatant was collected and 15 μ l aliquots were stored at -20°C for later use. At the day of the experiment, 15 μ l of Laemmli Sample Buffer (Bio-Rad, USA) were added to each sample, and the tubes were put at 100°C for 5 min and were directly put on ice for 30 min. A volume of 30 μ l from each sample was loaded on a 12% polyacrylamide gel and run at 120 V for 1 hr 30 min. The proteins were electrically transferred to a methanol-activated PVDF membrane at 100 V for 1 hr 15 min on ice. The membrane was probed using antibodies against either Caspase-8 (1:500), β -actin (1:2000), p53 (1:2000), Smac/Diablo (1:4000), CDK-4 (1:500), Bax (1:1000) or Bcl-2 (1:500) (Abcam, USA). β -actin was used as a control for equal loading of protein samples. Represented results are the average of four different extractions from four independent experiments.

2.8 Statistical analysis

Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS) (Version 16, Chicago, IL) software. Outliers were identified by plotting a box and whisker plot, and one way ANOVA (Dunnet's test) was performed to compare means of data in different experiments.

3. Results

3.1 Effect on cell viability and cytotoxicity

The LDH Cytotoxicity Detection Kit detects the leakage of the cytoplasmic enzyme LDH into the extracellular medium upon damage to the cytoplasmic membrane so it can be used as an indicator of dead cells. After 6 hrs of *R. aculeatus* treatment, Jurkat cells did not show any significant release of LDH except at 40 mg/ml (Figure 1a). In

contrast, treatment with *P. ostreatus* extract showed a gradual increase in LDH release by Jurkat cells (Figure 1b). Moreover, after 24 and 48 hrs of treatment, both extracts showed a gradual, concentration- and time-dependent increase in LDH release from Jurkat cells (Figure 1). The inhibitory concentration (IC_{50} , concentration at which 50% of cells have released LDH) at 24 hrs was between 10 and 16 mg/ml for *R. aculeatus*, and between 16 and 20 mg/ml for *P. ostreatus*. The IC_{50} at 48 hrs was between 8 and 10 mg/ml for both *R. aculeatus* and *P. ostreatus*. Normal lymphocytes were used as control cells to assess the cytotoxicity of tested extracts at 24 and 48 hrs. *R. aculeatus* showed a similar cytotoxic effect to that of Jurkat cells whereas *P. ostreatus* showed much lower cytotoxicity on primary lymphocytes (data not shown).

In contrast to the LDH Cytotoxicity Detection Kit, the Cell Proliferation Kit I (MTT) detects living cells that are metabolically active. Extract activity at concentrations below IC_{50} in the cytotoxicity experiment were tested at 24 and 48 hrs. *R. aculeatus* extract causes a gradual decrease in the number of metabolically active cells. This decrease is both time and concentration dependant, reaching its lowest point for both time intervals at 16 mg/ml, while the IC_{50} is 5 mg/ml (Figure 2a). In contrast, *P. ostreatus* induced an increased metabolic activity of Jurkat cells, for both time-points, at low concentrations until 8 mg/ml. While the IC_{50} is between 10 and 16 mg/ml for the 48 hrs time-point, it reached a plateau between 10 and 20 mg/ml, without reaching the 50% mark after 24 hrs of treatment (Figure 2b).

3.2 Apoptosis and the cell cycle

In order to understand how these two extracts are affecting Jurkat cells, we used the Annexin V Flow Cytometry approach. We tested the concentrations that induced a change in cell behavior in the MTT assay, while still not inducing marked cell death neither in Jurkat cells, nor in normal lymphocytes in the LDH cytotoxicity assay. Our results show a significant decrease, with respect to the negative control, in the number of PI^{-}/A^{-} cells, no significant change in the number of PI^{-}/A^{+} cells, and a significant increase in the number of PI^{+}/A^{-} and PI^{+}/A^{+} cells when treating the cells with *R. aculeatus* (Table 1). In contrast, there is no significant change, with respect to the negative control, in the number of cells in any combination, except in the PI^{-}/A^{-} cells when treated with 16 mg/ml of *P. ostreatus* extract (Table 1).

Flow cytometry analysis of the same concentrations and time intervals as tested in the Annexin V experiment reveal a significant increase in the number of Jurkat cells in the sub-G1 region and a significant reduction of cells in the G1 region when treated with *R. aculeatus* extract (Table 2). All *P. ostreatus* treatments did not show any significant change in any stage of the Jurkat cell cycle (Table 2).

3.3 Western blot

We next examined 2 proteins that are directly involved in apoptosis: caspase-8 for the extrinsic pathway and Smac/Diablo for the intrinsic pathway. Both proteins show little change over the range of tested treatments from both extracts (Figures 3 & 4). Jurkat cell CDK-4 protein levels also show little change over the range of tested treatments from *R. aculeatus* extract (Figure 3). With *P. ostreatus* treatment, this same protein shows a slight gradual increase between 1 and 4 mg/ml treatment, and almost disappears between 5 and 20 mg/ml, with the exception of the 8 mg/ml treatment (Figure 4). Finally we tested the anti-apoptotic protein Bcl-2, the pro-apoptotic protein Bax, and the tumor suppressor protein p53, all of which play a role in cell proliferation, apoptosis and cell cycle arrest. *R. aculeatus* extract causes the Bax protein to show a pattern of steady slight non-significant dose-dependent increase, while the Bcl-2 protein shows no change with increasing dose (Figure 3). With *P. ostreatus* treatment, both Bax and Bcl-2 show little variation over the range of tested concentrations (Figure 4). With both extracts the p53 protein shows a steady decrease with increasing dose, with significantly lower levels than the negative control at 10, 16 and 20 mg/ml (Figures 3 & 4).

4. Discussion

ALL is the most common type of childhood cancer [American Cancer Society]. In our study, we used the Jurkat cell line as a model of human T-cell ALL, to study the effect of ethanol extracts from *R. aculeatus* and *P. ostreatus* on the survival and proliferation of this type of cancer.

The results of the LDH experiment indicate first, that 6 hrs are not sufficient time for the extracts to exert their function, so we abandoned this time point in further experiments; second, they indicate that we should further investigate the 1-20 mg/ml concentrations in both extracts. They also indicate that *R. aculeatus* is not a good candidate for anticancer drug development since it showed a similar cytotoxic effect on normal human lymphocytes as that on Jurkat cells. However, because we are studying an extract that may contain many active compounds, and because it has been demonstrated that a number of molecules can interfere in the release of LDH into the cellular environment (Wang, Henning & Heber, 2010), further studies may refute this claim.

We tested the effect of these extracts on the metabolic activity of Jurkat cells, which is an indication of proliferating cells. The results of the MTT assay with *R. aculeatus* extract are consistent with previous results (Lindequist, Niedermeyer & Julich, 2005). In contrast, the increase in Jurkat cells' metabolic activity when treated with low concentrations of *P. ostreatus* might indicate that some compounds in the crude extract are working to induce either an increase in the mitochondrial activity, or an increase in the number of cells (proliferation), which is in turn increasing the total metabolic activity. Other groups reported similar results, while others did not see this increase at low concentrations of treatment (Venkatakrishnan *et al.*, 2010; Wang, Gao & Ng, 2000). These differences are most likely due to the difference in the cell lines and the concentrations of extracts used by these groups. Furthermore, these differences may also be due to the reduced sensitivity of the MTT assay as previously reported (Chen *et al.*, 1996).

Our flow cytometry experiments reveal that both extracts did not induce apoptosis however; at high concentrations they induce a non-apoptotic form of Jurkat cell death (Table 1), which is revealed in the significant increase in the number of cells in the sub-G1 and a decrease in the G1 regions when treated with *R. aculeatus* extract (Table 2).

The fact that neither the proteins that are directly involved in apoptosis (Caspase-8 and Smac/Diablo), nor the ones that are indirectly involved in apoptosis (Bcl-2 and Bax), showed any significant change with increasing concentration of either extract, confirms the flow cytometry results and the claim that these extracts are not inducing apoptotic cell death. The observed result that *P. ostreatus* induces Jurkat cells to increase the expression of the CDK-4 protein, at low concentrations, may be the reason behind the increase in the MTT assay. At high concentrations this protein is inhibited which indicates, along with the insignificant change in cell cycle, a stop of proliferation and later death by a non-apoptotic process or senescence and thereby reduced metabolic activity. Finally the observed decrease in p53 level upon treatment with both extracts may be a result of cell cycle arrest, as suggested previously (Cheng & Hass, 1990). It was also shown that the p53 protein is highly mutated in the Jurkat cell line with missense or nonsense mutations. These mutations render the protein inert for both inducing apoptosis and inducing cell cycle arrest (Cheng & Hass, 1990), indicating that this down regulation effect may be a result of increased sensitivity of the cells to undergo death, instead of being a cause of cell death on its own. Taken together, these results indicate that both extracts cause non-apoptotic Jurkat cell death at high concentrations. The main difference is that *R. aculeatus* extract causes death of normal lymphocytes as well. In contrast, *P. ostreatus* extract causes stop of proliferation of Jurkat cells, and has no effect on normal lymphocytes.

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Table 1. Flow cytometry annexin V analysis

	PI ⁻ /A ⁻ (%)	PI ⁻ /A ⁺ (%)	PI ⁺ /A ⁻ (%)	PI ⁺ /A ⁺ (%)
Untreated	85±6.6	7±5	0.2±0.2	1±1
P8	81±8.4	10±6	0.7±0.6	2±1
P10	83±7	9±5	0.6±0.6	2±1.3
P16	79±2.5	9±4	6±0.8*	2±1
R8	73±3.8*	4±1.1	10±1.3*	10±4*
R10	72±2.7*	4±1	13±1.3*	9±2*

P8: *P. ostreatus* 8 mg/ml, P10: *P. ostreatus* 10 mg/ml, P16: *P. ostreatus* 16 mg/ml, R8: *R. aculeatus* 8 mg/ml, R10: *R. aculeatus* 10 mg/ml.

Each value is the mean±SEM of two separate experiments done in duplicate.

* Statistically significant values with a p value of 0.05 with respect to the untreated samples.

Table 2. Flow cytometry cell cycle analysis

	Sub-G1	G1 (%)	S (%)	G2/M
Untreated	11±1.9	44±2	22±4	11±3.5
P8	13±1.3	44±3.5	20±3.5	7±1.5
P10	14±1.4	42±3.3	19±3.3	9±2.8
P16	17±0.9	40±3	20±3.6	10±3
R8	24±3.6*	31±2*	26±4.5	11±5
R10	27±2.7*	30±2*	25±4	10±4

P8: *P. ostreatus* 8 mg/ml, P10: *P. ostreatus* 10 mg/ml, P16: *P. ostreatus* 16 mg/ml, R8: *R. aculeatus* 8 mg/ml, R10: *R. aculeatus* 10 mg/ml.

Each value is the mean±SEM of three separate experiments done in duplicate.

* Statistically significant values with a p value of 0.05 with respect to the untreated samples.

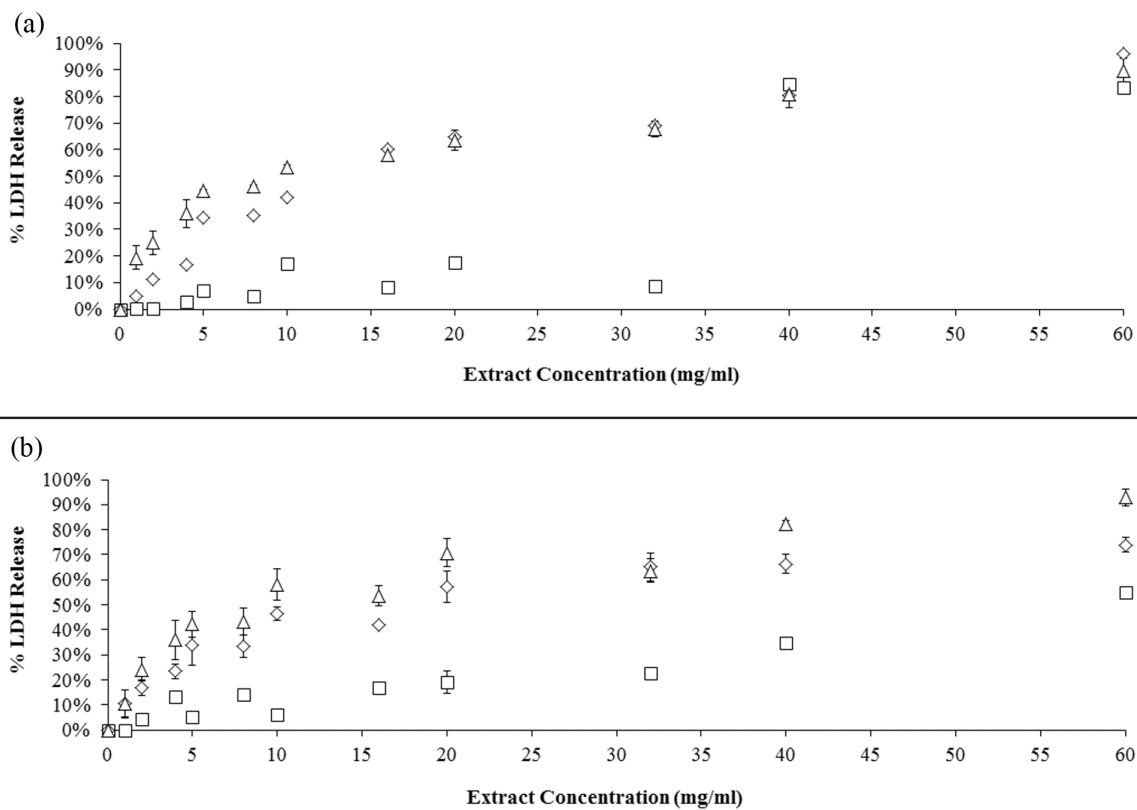


Figure 1. LDH release from Jurkat cells

LDH release from Jurkat cells after treatment with different concentrations of *R. aculeatus* (a), and *P. ostreatus* (b) at different time points. Error bars represent standard error of the mean of three independent experiments. ■ 6 hrs, ◆ 24 hrs, ▲ 48hrs.

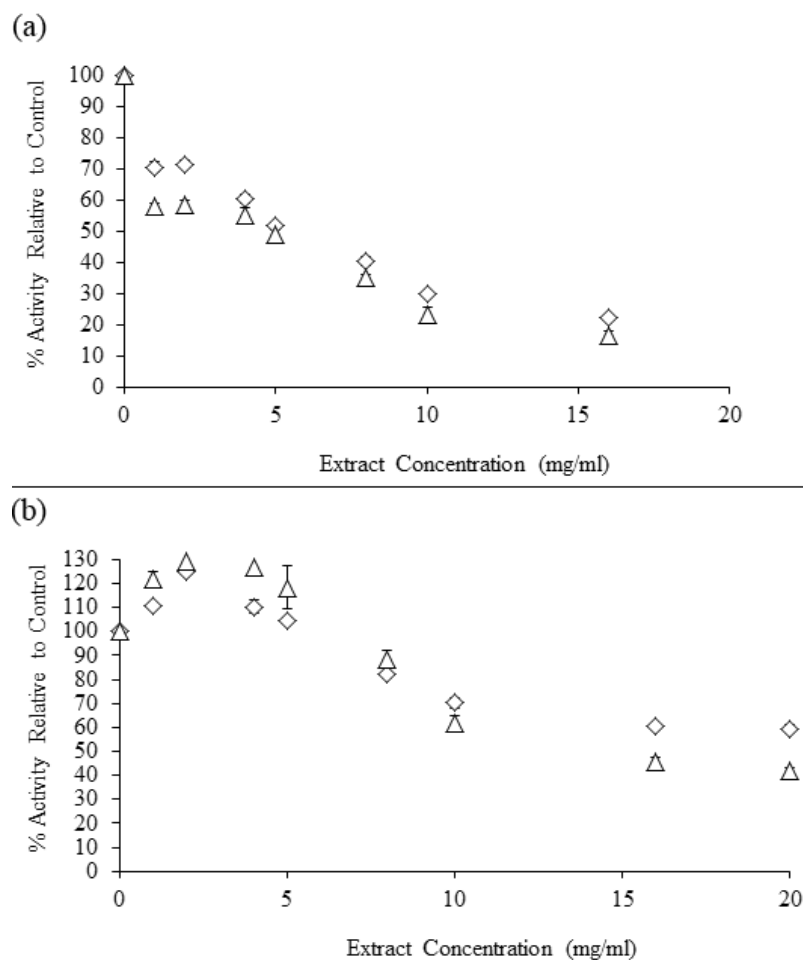


Figure 2. MTT assay for the detection of metabolically active Jurkat cells

MTT assay for the detection of metabolically active Jurkat cells after treatment with different concentrations of *R. aculeatus* (a), and *P. ostreatus* (b) at different time points. Error bars represent standard error of the mean of three independent experiments. ♦ 24 hrs, ▲ 48hrs. The results of these experiments are represented as percent of cell metabolic activity relative to the untreated control.

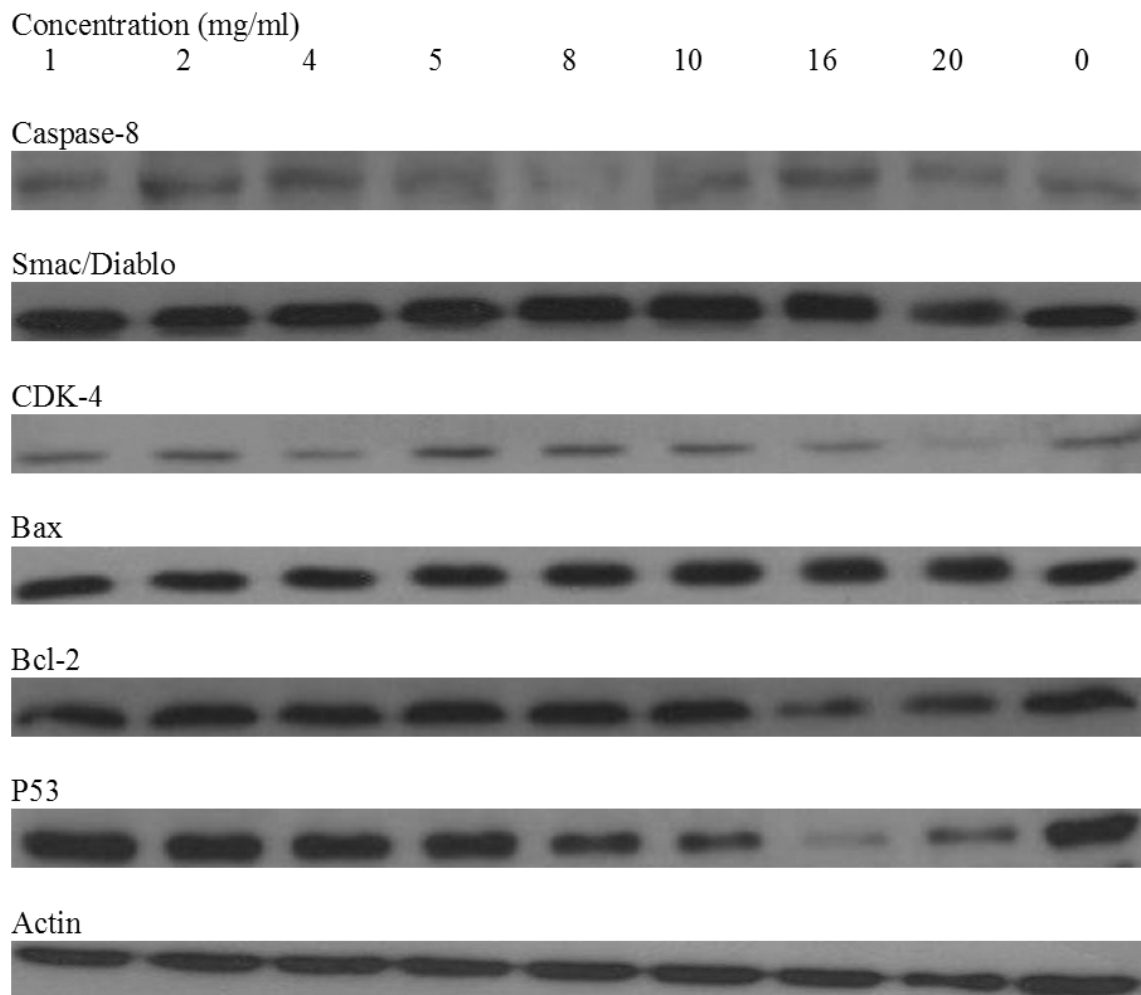


Figure 3. Western blot results for Jurkat cells treated with different concentrations of *R. aculeatus* extract. Lane 1: 1 mg/ml, Lane 2: 2 mg/ml, Lane 3: 4 mg/ml, Lane 4: 5 mg/ml, Lane 5: 8 mg/ml, Lane 6: 10 mg/ml, Lane 7: 16 mg/ml, Lane 8: 20 mg/ml, and Lane 9: the negative control which contains extracted proteins from untreated cells.

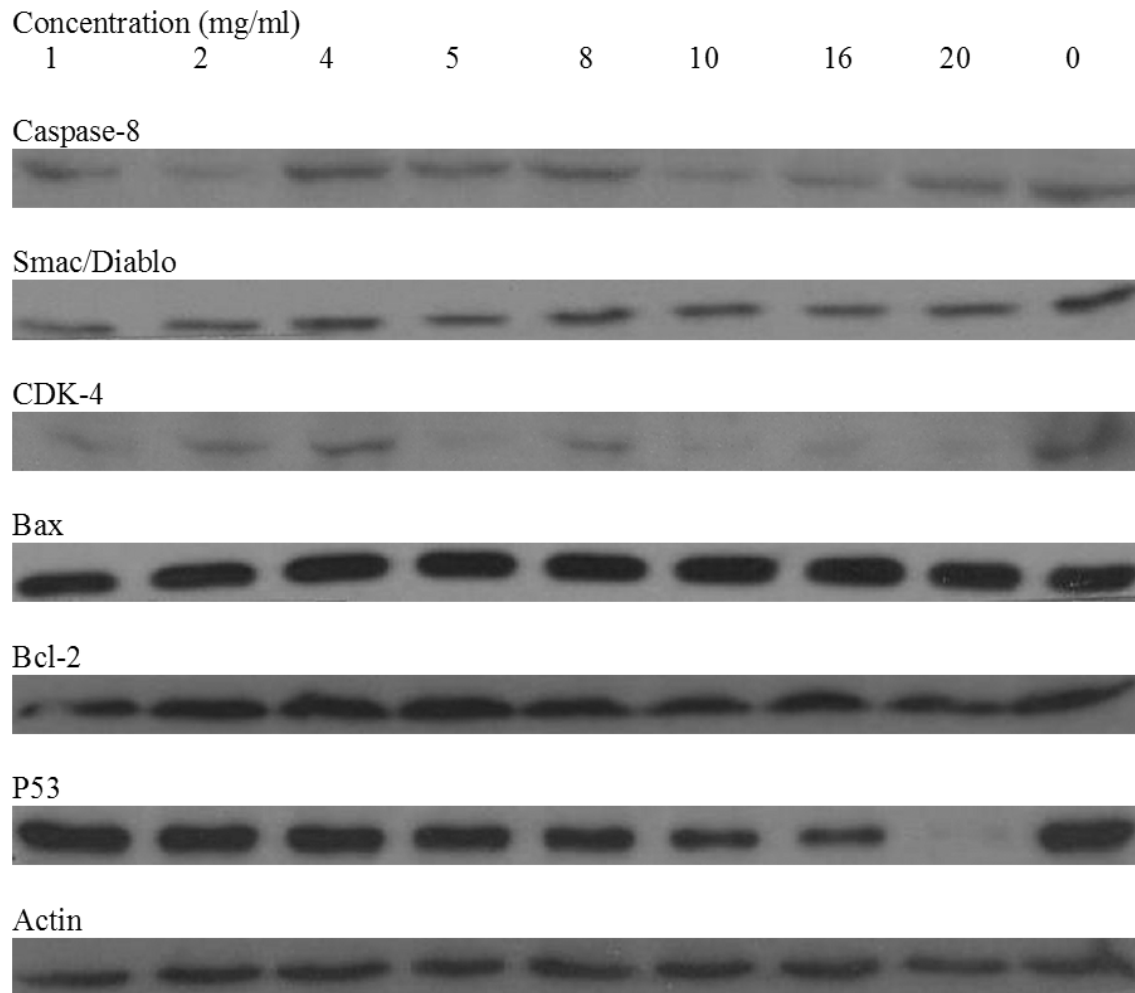


Figure 4. Western blot results for Jurkat cells treated with different concentrations of *P. ostreatus* extract
Lane 1: 1 mg/ml, Lane 2: 2 mg/ml, Lane 3: 4 mg/ml, Lane 4: 5 mg/ml, Lane 5: 8 mg/ml, Lane 6: 10 mg/ml, Lane 7:
16 mg/ml, Lane 8: 20 mg/ml, and Lane 9: the negative control which contains extracted proteins from untreated
cells.