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Original article

In vitro antileishmanial activity and apoptosis induction of *Pleurotus ostreatus* alcoholic extract on *Leishmania major*

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Abstract

Background and objectives: Leishmaniasis is caused by the genus *Leishmania*. Medications such as antimony compounds for the treatment of the disease are associated with limitations along with several side effects and disease recurrence; thus, evaluation of natural compounds with history of antimicrobial properties such as *Pleurotus ostreatus*, is of a great importance. The purpose of this study was to evaluate the apoptotic and leishmanicidal effects of *Pleurotus ostreatus* alcoholic extract on *Leishmania major* promastigote *in vitro*. **Methods:** Different concentrations of *Pleurotus ostreatus* extract (50, 100, 150, 200 and 250 µg/mL) were tested at 6, 24, 48 and 72 h on *Leishmania major* (MRHO/IR/75/ER) promastigotes. The leishmanicidal effects were determined using MTT [3-(4,5-dimethyl thiazolyl- 2)-2,5-diphenyle tetrazolium bromide] assay. Also, apoptosis induction was measured by flow cytometry and DNA fragmentation analysis. **Results:** The MTT results showed that leishmanicidal effect of *Pleurotus ostreatus* extract was dependent to extract concentration in a way that the lowest number of live promastigotes was obtained after treatment with 200 µg/mL of extract preparation at 72 h. The IC_{50} of *Pleurotus ostreatus* extract was 160±2 µg/mL. Flow cytometric analysis showed that the extract could induce apoptosis in promastigotes at its IC_{50}. Also, the result of gel electrophoresis showed that DNA fragmentation of treated promastigotes at the same concentration. **Conclusion:** The results indicated that *Pleurotus ostreatus* alcoholic extract have a strong toxic effect on cultivated *Leishmania* parasites. Based on these results *in vivo* studies using rodent models and human cutaneous leishmaniasis CL is recommended.

Keywords: apoptosis, flow cytometry, *Leishmania major*, MTT, *Pleurotus ostreatus*

Introduction

Leishmaniasis is a parasitic disease induced by an intracellular flagellate protozoan of the genus, *Leishmania*, and transmitted by the bite of sandflies [1]. Leishmaniasis is endemic in more than 98 countries worldwide, 350 million people are considered to be at risk; furthermore, 14 million people are infected and two million new cases are reported annually [2,3].

The different forms of the disease are cutaneous (CL), mucocutaneous (MCL) and visceral

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leishmaniasis (VL). In spite of being mostly self-limiting, CL leaves permanent scars on the skin, which may lead to long term social effects for the patient even after full healing. On the other hand, VL is the progressive form of the disease that can be lethal in human [1].

Glucantime is the first line drug for treatment of leishmaniasis; however, serious limitations such as toxicity and lack of efficacy in endemic areas, shows the need for new antileishmanial compounds; thus, the development of safe, potent and cost-effective antileishmanial agents are a critical public-health priority [4-6].

Numerous studies suggest that many plant extracts contain effective secondary metabolites, which have antimicrobial, antiparasitic and specifically antileishmanial properties [7-12] as sharif *et al.* have reported, leishmanicidal effect of *Artemisia aucheri* [13]. Also Valadares *et al.* proved that the *Agaricus blazei* Murill caused reduction of inflammation of *L. amazonensis* in infected mice foot pad [14], as well *Agaricus blazei murill* water extractwhich had led to the reduction the parasite burden in the mice infected with L. chagasi [15]. Mallick showed *Astraeus hygrometricus* extract induced apoptosis in *Leishmania* promastigotes [16].

One of the medicinal mushrooms is *Pleurotus. Ostreatus* a mushroom of Pleurotaceae family [17]. Researchers have shown that its ethanol extract has antioxidant activity in *in vitro* and *in vivo* tests [18]. Ethanol and methanol extracts of this fungus can elicit antibacterial activity against Gram-positive bacteria through cell membrane lysis, inhibition of protein synthesis, proteolithic enzymes and microbial adhesions and have strong inhibition effect of oxidative stress in liver and brain level [19-22].

One way of survival of *Leishmania* parasite in the host body is the activity of the trypanotion reductase (TryR) enzyme. Therefore, this enzyme has been considered as a target for the design of many drugs [23]. It is expected that the discovery of some components with anti-trypanotion activity, would facilitate the development of the new anti-leishmanial drugs and it has been proven that the extracts drived from members of pleurotaceae family have had biological activities against TryR [24].

With regard to the importance of leishmaniasis and the mentioned various medical effects of *P. estreatus*, in this study the leishmanicidal activity of the methanol extract of *Pleurotus ostreatus* has been evaluated.

Experimental

Parasite culture

The cryopreserved form of *Leishmania major (MRHO/IR/75/ER)* was prepared from Department of Parasitology and Mycology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

Promastigotes were cultured in NNN (Novy-MacNeal-Nicollem) medium with 100 µg/mL streptomycin, 100 IU/mL penicillin and 100 µg/mL gentamycin and then for mass production were subcultured in RPMI-1640 (Gibco, UK) supplemented with 10% FBS (fetal bovine serum) [25].

Preparation of methanol extract of P. ostreatus

Alcoholic extract of *P. ostreatus* was prepared by maceration method. Briefly, 50 g of mushroom was smashed using a pounder. This grounded biomass was suspended in 400 mL of absolute methanol and incubated for 48 h at 200 rpm (using a shaker) and temperature of 30 °C. The suspension was filtered twice on Whatman No. 4 paper to remove the biomass. Concentrating the supernatant was done by a rotary evaporator at 50 °C in for 24 h and then it was stored at 4 °C [26].

Promastigote assay

The effect of *P. ostreatus* prepared extract on cultured promastigotes was evaluated by direct counting with Neubauer haemocytometer. For this purpose 2×10^5 parasite promastigotes/mL were seeded in a 24 well microtitre plate. These promastigotes were treated by different concentrations of *P. ostreatus* extract (50, 100, 150, 200, 250 µg/mL) and were incubated in 25±1 °C for 6, 24, 48 and 72 h. The live

promastigotes were counted and compared with control using linear regression.

MTT assay

The inhibitory effect of the *P. ostreatus* extract against *L.major* promastigotes was determined using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] test. The rate of living promastigotes had a direct relationship with the amount of purple formazan that was produced through the reduction of yellow MTT by the dehydrogenase enzyme in the inner mitochondrial membrane of the living cells. Briefly, the promastigotes (5×10^5 cells/mL; 300 µL) in stationary phase were seeded at 96-well micro titre plates and were treated with extract at different concentrations (50،100،150،200،250 µg/mL) and incubated at 24±2°C for 72 h . The controls were complete RPMI1640 medium with no parasites and complete RPMI 1640 medium with parasites without extract. The medium was discarded and the cells were incubated with MTT solution (5 mg/mL in PBS) for 4 h and the resulting formazan crystals were solubilized with 100 µL isopropanol. The absorption was measured using an ELISA reader [27].

Apoptosis assessment with flow cytometry

Apoptotic cells were detected by flow cytometry. Double staining was performed with Annexine-V FLUOS staining kit (Roche, Germany) according to the manufacturer protocol I. FITC-Annexin v and PI were used as cell markers. Briefly, the wells were treated with extract at IC_{50} equivalent to 160 µg/mL and were incubated at 24 °C. Promastigotes were washed by cold PBS and centrifuged at 1500 rpm for 10 min after 72 h, then the pellet were resuspended in binding buffer to a concentration of 1×10^5/mL of promastigotes. Thereafter, they were incubated at room temperature for 15 min in dark condition, with 10 µL of FITC-Annexin V in the presence of PI. At the end, the samples were analyzed with FACS Calibur flow cytometer (Becton Dickinson and Cell Quest software) and the percent of positive cells was determined for each sample [28].

DNA fragmentation

The hallmark of apoptotic cell death is DNA fragmentation into nucleosomal units. An apoptotic DNA ladder kit (Genetbio) was used to extract DNA from apoptosis-induced *L. major* promastigotes by mentioned different concentrations of extract for 72 h. Quantitative analysis of DNA fragmentation was carried out by agarose gel electrophoresis using Tris–acetate–EDTA (pH 8.0) running buffer at 80 V and was visualized under UV light [29].

Statistical analysis

To compare the means of different treatment groups, statistical analysis was performed by one way ANOVA and multiple comparison Tukey-Kramer test. All statistical analysis were done using SPSS software, version 16 was considered as significant (p values <0.05).

Results and Discussion

The cytotoxic potential of *P. ostreatus* extract on *L. major* promastigotes was measured by direct counting of live promastigtes after parasite exposure by various mentioned concentrations of extract at different times. The number of live *L. major* promastigotes in the presence of different concentrations of *P. ostreatus* extract in comparison with control group have been showed in table 1. *P. ostreatus* extract showed activity against promastigotes in a time and concentration-dependent manner.

Antileishmanial effects of different concentrations of *P. ostreatus* (0, 50, 100, 150, 200, 250 µg/mL) were assessed after 72 h of exposure (figure 1). The effect of extract at 200 µg/mL was significantly higher than other concentrations. IC_{50} was calculated to be 160±2 µg/mL. Flow cytometric analysis was performed after processed samples were labeled with FITC-AnnexinV FLUOS and propidium iodide. The percentages of apoptotic and necrotic cells were determined 39.6% and 0.21% at IC_{50} of *P. ostreatus* extract after 72 h treatment, respectively (figure 2).

Table 1. The number of live promastigotes (1×10^6) at different concentrations of extract

Concentration (µg/mL)	The number of live promastigotes (1×10^6)			
	6h	24h	48h	72h
50	0.61±0.11	0.61±0.11	0.48±0.02	0.48±0.02
100	0.55±0.04	0.55±0.04	0.44±0.04	0.44±0.04
150	0.54±0.03	0.54±0.03	0.49±0.07	0.43±0.07
200	0.53±0.02	0.34±0.00	0.31±0.06	0.27±0.05
250	0.56±0.03	0.45±0.00	0.38±0.04	0.26±0.01
Control	0.62±0.06	0.66±0.01	0.7±0.11	0.72±0.11

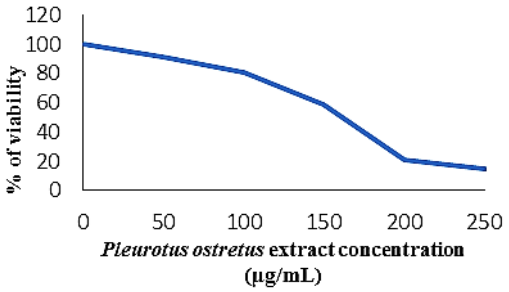

Figure1. Cytotoxic activity of *Pleurotus ostreatus* extract on *Leishmania major* promastigotes

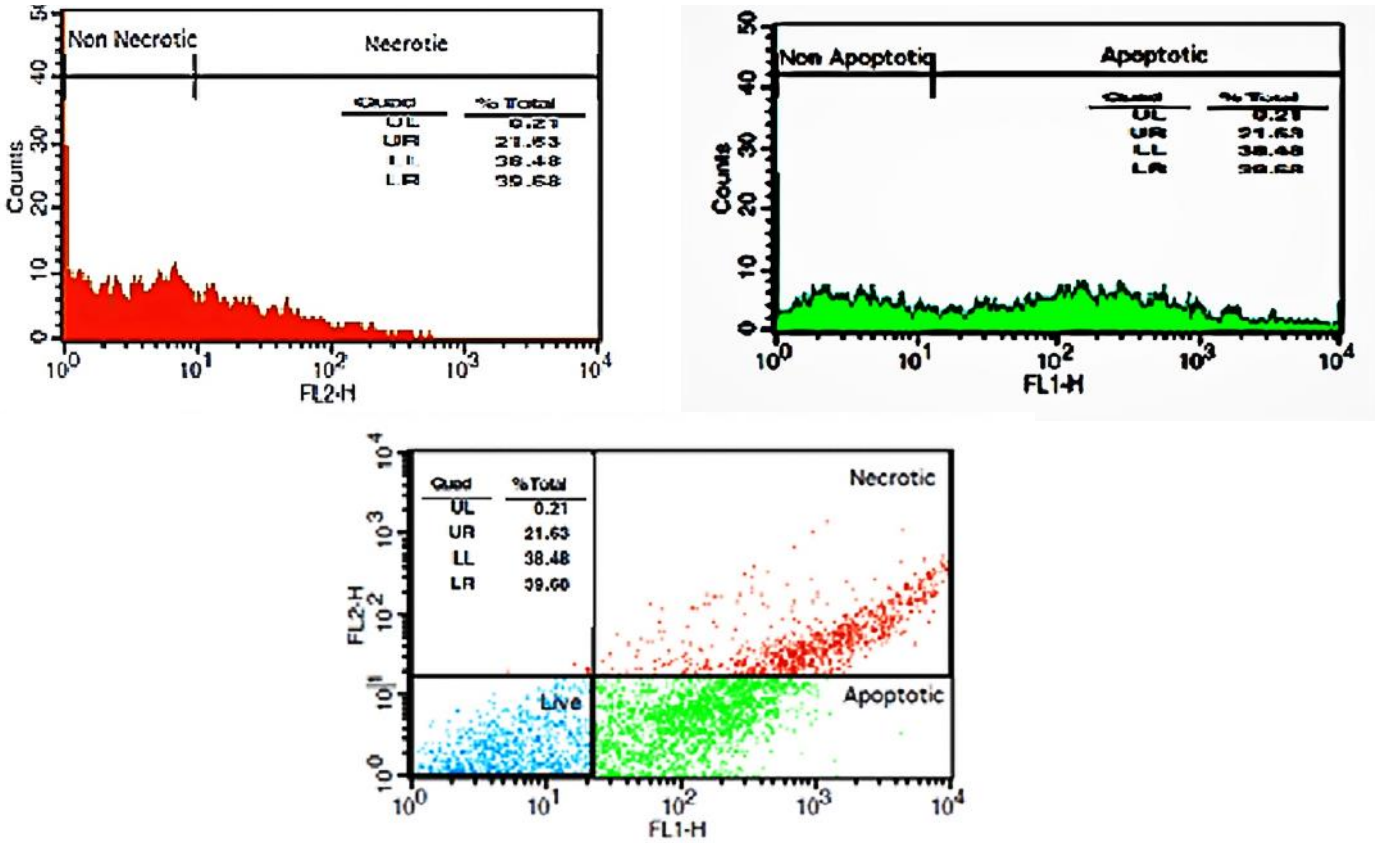

Figure 2. Flow cytometric analysis of promastigotes following treatment with 200 µg/mL *Pleurotus ostreatus* extract and after labeling with annexin-V and PI. Lower right region (LR) belongs to apoptotic cells (annexin-positive) and upper right region (UR) belongs to necrotic cells (PI-positive). Upper left region (UL) belongs to banded cell region with annexin and PI, and lower left region (LL) belongs to survived cells. FL2 is propidium iodide and FL1 is Annexin V.

Necrotic process of promastigotes of *L. major* was confirmed by presence of fragmented DNA in agarose gel electrophoresis. After 72 h treatment with 200 µg/mL of *P. ostreatus* extract, the degree of fragmented DNA was comparable to the control and untreated cells which did not show DNA fragmentation (figure 3).

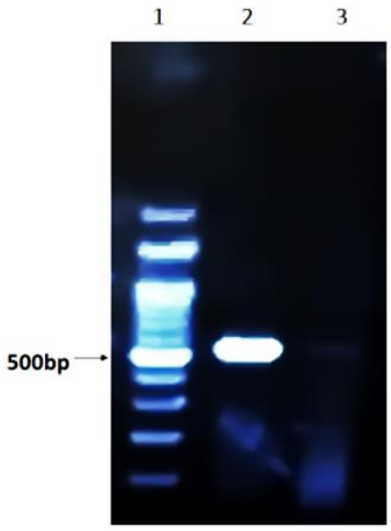

Figure 3. . Demonstration of DNA fragmentation of promastigotes treated with *Pleurotus ostreatus* extract by agarose gel electrophoresis. Line 1: DNA molecular size marker (1 kb DNA ladder), line 2: control untreated DNA and line 3: promastigotes DNA fragmentation after 72 h treatment at IC_{50} of *Pleurotus ostreatus* extract

Cutaneous leishmaniasis (CL) is caused by *Leishmania major* and endemic in east, central and southern parts of Iran [4]. Since antileishmanial chemotherapy being used for leishmaniasis has limitations including high cost, difficulty to manage, high toxicity and resistance, there is an urgent need for new, safe, more effective and economically feasible drugs for the treatment of leishmaniasis [6].

Pleurotus ostereatus belonging to Pleurotaceae family, is commonly refer to an oyster-shared cap spanning 5 to 25 cm from white to dark brown. The Pleurotus genus has several species such as *P. sajorcaju, P. florida, P. flabellatus, P. highbing, P. cystidiosus, P. sapidus, P. eryngii, P. tuberegium, P. ulmarium, P. pulmonarius, P. citrinopileatus, P. geesteranus* and others [30].

Kazunori Ike *et al.* suggested that the hot-water extract of *P. eryngii* induced Th1 immunity by acting as an immune stimulator [31].

Angel Ramos *et al*. have shown that *P. ostreatus* has a trypanocidial activity and although the ergostrol peroxide of this mushroom showed an inhibitory act on *Trypanosoma cruzi*, it has no lytic action on erythrocytes and no cytotoxic effect on mammalian cells at concentrations higher than 1600 µg/mL [32].

According to the mentioned studies, in present study leishmanicidal effects of *Pleurotus ostreatus* alcoholic extract on promastigotes of *L.major* was examined for the first time.

In this research we found that *P. ostreatus* extract was able to induce a lethal effect on *L. major* promastigotes with numerous nuclear and membrane feature of apoptosis, including DNA fragmentation and phosphatidylserine exposure.

The biomedical pathways that mediated apoptosis in kinetoplastids are still unknown [33]. Caspases are the main protolithic enzymes that interfere in apoptosis in mammalian cells [34]. The interference of other cysteine proteases like calpains, cathepsins, and serine proteases or the proteasome, have also been suggested [35].

In the current study, parasite treatment was directed toward reduction of the parasite number *in vitro* and regarding to other features such as exposure of phosphatidylserine and DNA fragmentation, probably the extract of *P. ostreatus* did not kill promastigotes *of L. major* by its toxicity mechanism, but through apoptosis.

We used direct counting live promastigotes and for the best results, we used MTT assay. Comparing the results of the two methods, MTT assay was more sensitive and thus there were some little differences between the obtained results *i.e.* in concentrations of 100 and 150 µg/mL; however, reducing the live promastigotes by increasing the concentration, shows that the final conclusion is correct.

The results revealed that *P. ostreatus* extract induced apoptosis in promastigotes of *L. major* in a concentration and time dependent manner. By comparing the results of different concentrations it seemed that perhaps the inhibitory effect of the extract at low concentrations e.g. 50 and 100 µg/mL was greater than its lethal effect.

Also, apoptosis induction was 39.6% by flow cytometry in IC_{50} at 72 h treatment and the gel

electrophoresis tests showed DNA fragmentation of promastigotes at the same concentration.
The leishmanicidal effect of *Pleurotus ostreatus* alcoholic extract was confirmed by MTT, flow cytometry and gel electrophoresis methods. In order to achieve a good therapeutic option for treatment of leishmaniasis it is suggested to design trials on murine and then human CL with optimized doses of *Pleurotus ostreatus* alcoholic extract.

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Declaration of interest
The authors declare that there is no conflict of interest. The authors alone are responsible for the content of the paper.

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