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RESEARCH ARTICLE

A comparison of *in vitro* anticancerous activity and mechanism of ethanolic extracts from different *Ganoderma* genus

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Abstract Five ethanolic extracts from the mycelia of *Ganoderma lucidum*, *G. tsugae*, *G. oerstedii*, *G. subamboinense,* and *G. resinaceum* were respectively studied on their anticancerous activities against leukemic HL-60 cell line in vitro. Results showed that all five extracts potently inhibited HL-60 proliferation. The extract from *G. lucidum* mycelia exerted the highest activity. Annexin V/PI bivariate flow cytometric analysis further revealed that the five extracts significantly induced early apoptosis in HL-60 cells. The results illustrate that not only *G. lucidum* but also other *Ganoderma* species can inhibit cancer cells, and their mechanisms are related to induction of apoptosis.

Keywords *Ganoderma*, anti-proliferation, apoptosis, HL-60

1 Introduction

Reishi, Lin-Zhi in Chinese, distributes a group of mushroom belonging to the genus of *Ganoderma* (Chen and Yu, 1990; Jong and Birmingham, 1992; Luo and Lin, 2002; Lin and Zhan, 2004). Recent study demonstrated that the triterpenes from the fruiting bodies of *G. lucidum* and *G. tsugae*, two species of *Ganoderma* genus, can effectively inhibit the proliferation of cancer cells *in vitro* and thus attracted intensive attention. However, *Ganoderma* has abundant species. According to Zhao Jiding's report in 1992, the number of species in Chinese sub genus only amounts to over 88. These rich resources of Ganoderma species are still not being well exploited (Lin, 2001).

On the other hand, the triterpenes from *Ganoderma* mycelia are also seldom studied. There are only few reports on several mycelial triterpenes that can inhibit the proliferation of hepatoma cells *in vitro* (Toth et al., 1983; Hu et al., 2002). Compared with the cultivation of fruiting bodies, mycelia can be produced in a large scale by using an industrial liquid fermentation technology. Therefore, study on mycelial triterpenes can provide useful information on the potential use of mycelia as an extraction material.

It is known that triterpene can be easily dissolved in organic solvents and therefore can be extracted by ethanol (Hu et al., 2002). Therefore, our study analyzed the ethanolic extracts from five different *Ganoderma* species mycelia that were cultivated using liquid fermentation. By comparing their triterpenes contents and *in vitro* anticancerous activities against leukemia HL-60 cells, the species that produced rich anticancerous triterpenes and was capable of inducing apoptosis was screened out. Our study provides preliminary data for further exploiting the *Ganoderma* resources.

2 Materials and methods

2.1 Materials and equipments

Five *Ganoderma* species: *Ganoderma lucidum*, *G. tsugae*, *G. oerstedii*, *G. resinaceum,* and *G. subamboinese* were collected in the Institute of Microbiology and Immunology, Shanghai Normal University, China and were authenticated by Dr. S.C. Jong. They were respectively abbreviated as "G", "T", "O", "R" and "S". Human promyelocytic leukemia HL-60 cell line was a generous gift from Dr. J. M. F. Wan's lab in the University of Hong Kong, China. Shimadzu UVPC2401 UV–visible spectrometer, Bio-Rad microplate reader, Olympus BX41-DP70 fluorescent microscope, BD FACS Calibur flow cytometry were used in this study. BD CELLQUEST and BD ModFIT softwares were used to process flow cytometric data.

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2.2 Methods

2.2.1 Liquid fermentation of Ganoderma

The mycelia of five different *Ganoderma* species was inoculated from slant cultures into a liquid medium that contained 5% carbon sources, 2% nitrogen sources, and minerals, respectively. The mycelia were cultivated at 25°C on a rotary shaker at 180 rpm for seven days. At the end, the culture was centrifuged and mycelia were collected and dried.

2.2.2 Extraction

The dried mycelia were extracted with 95% ethanol by sonication. After removal of mycelia by centrifugation, the triterpenes containing extracts were obtained under vacuum (Gu, 2002) and named as L (*G. lucidum*), T (*G. tsugae*), O (*G. oerstedii*), S (*G. subamboinense*), and R (*G. resinaceum*), respectively.

2.2.3 Determination of total triterpene contents

The determination method of total triterpene contents followed the literature (Wang and Gu, 2001).

2.2.4 Cancer cell culture

HL-60 cells were cultured in a RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 unit/mL penicillium, and 100 μg/mL streptomycin in an incubator supplied with 5% carbon dioxide and a humidified atmosphere at 37°C.

2.2.5 Comparison of anti-proliferation activity against HL-60 cells

HL-60 cells in logarithmic phase were seeded at a density of 1×10^5 cells/mL in 96-well plate. After 24 h, cells were treated with respective ethanolic extracts at a final concentration of 125 μg/mL for 72 h. The percentage of inhibition was determined by MTT assay and the results were read at 550 nm (Mosamann, 1983). The percentage of inhibition of control group was set as zero, and that of blank group which was free of cells was set as a hundred percent.

2.2.6 Detection of early apoptosis of HL-60 cells

HL-60 cells were stained with Annexin V-FITC conjugates and propidium iodide (PI), and subsequently analyzed by using flow cytometry according to the literature (Ranynal and Pollard, 1994). The combination of Annexin V-FITC and propidium iodide can discriminate early apoptotic cells (annexin V-FITC positive, PI negative), late apoptotic and/or necrotic cells (Annexin V-FITC and PI positive), and viable cells (unstained) (Ranynal and Pollard 1994).

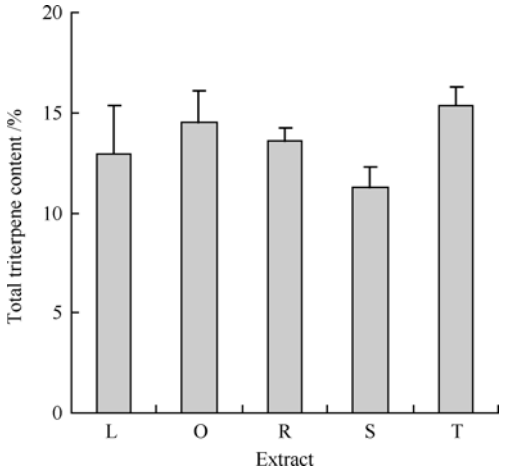
2.2.7 Statistics

SPSS 10.0 software was used for statistics and data was presented as mean ± SD.

3 Results

3.1 Comparison of total triterpene contents among five species

Colorimetric determination showed the average triterpene contents (13.5%) in ethanolic extracts from five *Ganoderma* species. Extract T from species *G. tsugae* had the highest total triterpene contents (15.4 ± 0.9%), while S from *G. subamboinese* the lowest (11.3±10.0%, n=3) (Fig. 1). The contents in a descent order were T, O, R, L, S, respectively.

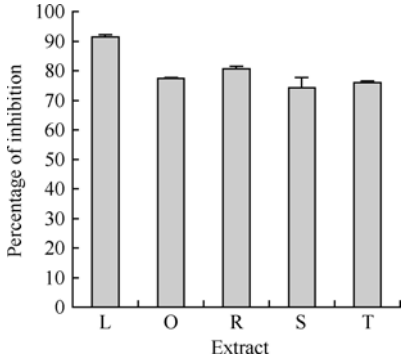


The total triterpene contents determined by colorimetric method; Data was showed as mean ±SD (n=3)

Fig. 1 Comparison of total tritepene contents of mycelial ethanolic extracts from five *Ganoderma* species

3.2 Comparison of anti-proliferation activity against HL-60 cells

MTT assay demonstrated that ethanolic extracts from all five species had significant inhibitory effect against HL-60 cells *in vitro*. Extract L from *G. lucidum* showed the strongest inhibition activity (91.4 ± 0.9%), while S the lowest (74.2±3.5%) (Fig. 2). The percentage of inhibition of five extracts in a descent order were L, O, T, S, and T, respectively, where the average value was 80.0%. Statistics showed the percentage of inhibition of L was significantly higher than those from the other four species (p<0.01).



MTT assay was applied to determine the antiproliferation activity of ethanol extracts from five *Ganoderma* species;
Cells were treated with respective extracts at a concentration of 125 μg/ml for 72 h;
Data showed as mean±SD (n = 6)

Fig. 2 Comparison of antiproliferation activity against HL-60 among mycelial ethanol extracts from different *Ganoderma* species

3.3 Results of apoptosis detection

The exposure of phosphatidylserine (PS) on the outer leaflet of the cytoplasmic membrane was a hallmark which occurs early in the apoptotic process (Martin et al., 1995). The exposed PS can be detected by binding Annexin V. At the early apoptotic stage, the cell membrane is intact, while at the late apoptotic stage the cell loses its membrane integrity and the PI can permeate into the cell and stain the nucleus. The combination of Annexin V-FITC and PI allows for the discrimination of early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic and/or necrotic cells (Annexin V-FITC and PI positive) and viable cells (unstained). In our test, HL-60 cells were treated with *Ganoderma* ethanolic extracts at a concentration of 125 μg/mL for 48 h. Cells were subsequently stained with Annexin V-FITC and PI, and examined by flow cytometry. It was found that all five ethanolic extracts induced apoptosis in HL-60 cells (Fig. 3). The percentages of early apoptotic cell subsets among total population were 12.7% (L), 15.8% (O), 12.3% (S), 10.5% (R), and 8.7% (T), respectively, which were all significantly higher than that of the control (3.5%). While the late apoptotic or necrotic percentages were not significantly different (Table 1). The results were further confirmed by using acridine orange/ethidium bromide bi-staining for morphological observation (data not shown).

HL-60 cells were treated with *Ganoderma* ethanolic extracts at a concentration of 125 μg/mL for 48 h, and subsequently stained with Annexin V-FITC and PI, and assayed by flow cytometry. Cell Quest software was applied to analyze the results. Early apoptotic cells (Annexin V positive, PI negative) appeared in low-left quadrant, late apoptotic and/or necrotic cells (Annexin V and PI positive) in the upper-left quadrant, and viable cells (both Annexin V and PI negative) in the lower-right quadrant.

Table 1 The apoptosis and necrosis of HL-60 induced by ethanol extracts from *Ganoderma* mycelia

Group	Viable /%	Early apoptosis /%	Late apoptosis and/or necrosis /%
Control	90.2	3.5	2.3
L	80.3	12.7	1.4
O	78.2	15.8	1.8
S	83.1	12.3	0.9
R	81.2	10.5	0.9
T	82.4	8.7	1.1

4 Discussion

Ganoderma triterpene is the main component responsible for the anticancerous activity of *Ganoderma* (Su et al., 2000; Gonzalez et al., 2002). Except for a few from *G. tsugae* and *G. concinna*, most reported anticancerous triterpenes are from *G. lucidum* (Wu et al., 2001; Gao et al., 2002; Luo and Lin, 2002). In this study, we cultivated the mycelia of five different *Ganoderma* species including *G. lucidum, G. tsugae, G. oerstedii, G. resinaceum,* and *G. subamboinense* using liquid fermentation method, and extracted the triterpenes using ethanol. The triterpenes contents of these extracts were analyzed and their anticancerous activity *in vitro* was also studied. Except for the common triterpenes from *G. lucidum* and *G. tsugae*, the triterpene-enriched ethanolic extracts from *G. oerstedii, G. resinaceum, G. subamoinense* mycelia also exhibit anticancerous activities. It is known that the induction of apoptosis is an effective means in cancer treatment. Our Annexin V assay results indicate that the anticancerous activities of five ethanolic extracts from L, O, R, S, and T species are related to induction of apoptosis in HL-60 cells but not necrosis.

These data suggest that besides *G. lucidum* and *G. tsugae*, other *Ganoderma* species can also be the candidate for screening anticancerous agents, and the triterpenes from liquid fermentation cultivated mycelia can significantly inhibit cancer cells proliferation *in vitro*, just like those from fruiting bodies. Therefore, the mycelia cultivated in liquid fermentation in a factory may be the replacement of agricultural-growth fruiting bodies and can be used as a potential material for developing anticancer drugs.

Our data also show that the cancer inhibition activities of ethanol extracts are not closely correlated with their total triterpene contents. For instance, extract T has a high total triterpene contents (15.4±0.9%), but its inhibition rate is only 76.0±0.4%, less than the mean of five extracts (80.0%). Extract L has a high inhibition rate (91.4±0.9%), but its total triterpene content is only 12.9±0.4%, less than the mean (13.5%). The possible reason is that the quantification method for triterpene content cannot reflect the amount of specific anticancerous triterpenes in a complex triterpenes mixture. Therefore, we suggest it is

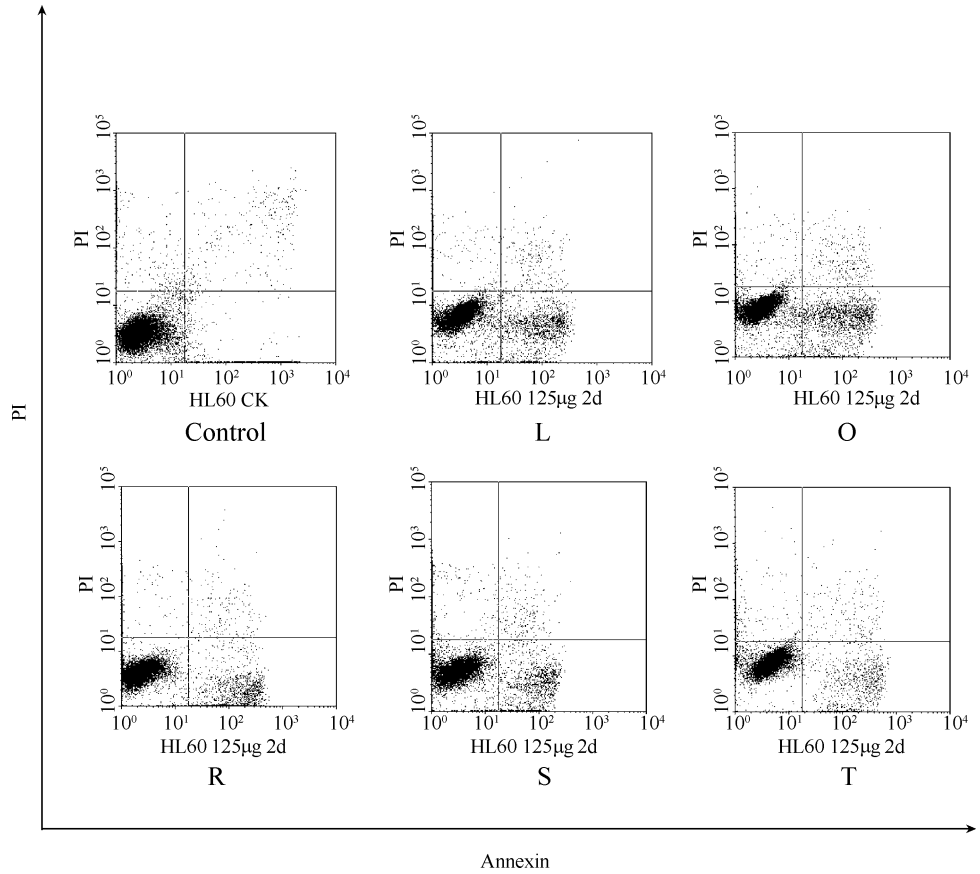


Fig. 3 The Annexin V/PI analysis on the HL-60 death induced by mycelial ethanolic extracts from five *Ganoderma* species

necessary to screen the bioactivity, in addition to determination of total triterpene contents, to study the complex triterpenes that the components have not been clearly identified and quantified. The detailed anticancerous components in these triterpenes enrich extracts need to be further identified.

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