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## Effects of Huaier aqueous extract on proliferation and apoptosis in the melanoma cell line A875

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### ABSTRACT

In recent years, an aqueous extract of the fungus *Trametes robiniophila* Murrill 1907 (Huaier) has been commonly used in China for complementary cancer therapy. However, the mechanisms of its anticancer effects are largely unknown. In the present study, we aimed to investigate the effects of Huaier extract on the inhibition of proliferation and promotion of apoptosis in a melanoma cell line, A875, and to explore the possible mechanisms of its anticancer effects. Cell proliferation was measured using a Cell Counting Kit-8 (CCK8) and PCNA-Western blot. The cell cycle distribution, and apoptosis levels were analyzed by flow cytometry, and Western blot was used to test the apoptotic pathways. We found that Huaier extract strongly inhibited cell proliferation of the A875 melanoma cells and induced G2/M arrest and apoptosis in a time- and dose-dependent manner. P53 expression was increased and cell apoptosis executed by caspase-3. Down-regulation of Bcl-2 and up-regulation of Bcl2-associated X protein (BAX) indicated that Huaier extract induced apoptosis through the mitochondrial pathway. As expected, the inhibitor Huaier decreased melanoma cell line A875 proliferation, and induced apoptosis in a time- and dose-dependent manner. Our findings indicate that Huaier extract is an effective complementary agent for cancer treatment of melanoma.

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### Introduction

Malignant melanoma is a high-grade malignant melanocytic tumor that occurs mostly in the skin. Malignant melanoma tumors occur throughout the world with an overall incidence per year of approximately 2 in 10,000. In China, incidence of malignant melanoma has gradually increased (Wang et al., 2005). There are multiple conventional strategies for melanoma treatment including: surgery, radiotherapy, chemotherapy, immunotherapy and other complementary therapies. However, these therapies cannot completely prevent recurrence and metastasis in cancer patients, and thus new drugs and new therapies are needed to treat these patients. Among the complementary therapies, traditional Chinese medicine (TCM) has a role in killing tumor cells in a more natural manner with fewer side effects (Pu et al., 2008). TCM has been used in China for more than three millennia. A retrospective analysis demonstrated that about 60% of anti-infective and anti-tumor

agents, either commercially available or in the late stages of development, are drugs of natural origin (Cragg and Newman, 1999).

*Trametes robiniophila* Murrill 1907 (Huaier) is a widely available fungus in China, which has been used in TCM for approximately 1600 years (Li et al., 2006), however, its anti-tumor properties were only recently discovered and it has been used in complementary therapy only in recent years. The fungus was extracted twice with hot water, and the free proteins and amino acids were eliminated using the Sevag method. After being dialyzed for 72 h, four times the volume of ethanol was added to the dialysis solution to precipitate the active ingredient. The HPLC and SDS-PAGE analysis confirmed that the active ingredient of Huaier extract (from Gaitianli Medicine Co. Ltd., Jiangsu, China) was a proteoglycan, which contained 41.53% polysaccharides, 12.93% amino acids and 8.72% water (Guo et al., 1992, 1993). Additionally, the inhibitory effect of the proteoglycan on murine sarcoma, liver cancer, Lewis lung cancer and breast cancer were investigated (Guo et al., 1992, 1993). The anti-tumor effects of Huaier extract displayed various biological activities such as promotion of apoptosis, anti-angiogenesis, drug resistance reversal, anti-metastasis and activation of the immune system. Although recent experimental data revealed the apoptotic effect of Huaier extract, the underlying mechanisms are not clear, and there may be other antitumor effects besides apoptosis. In the current study, we investigated the effects

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of Huaier extract on proliferation, apoptosis and anti-tumor effects in a melanoma cell line, A875.

## Materials and methods

The melanoma cell line A875 was purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Hyclone (Beijing, China). Fetal bovine serum (FBS) was supplied by Hyclone (Beijing, China). Cell Counting Kit-8 (CCK8) reagent was supplied by Dojindo (Kumamoto, Japan), Cycle Test Plus DNA Reagent Kit and FACS Calibur flow cytometry were supplied by Becton Dickinson (BD Biosciences, San Jose, CA, USA). Bcl-2 (1:1000), Bcl-2-associated X protein (BAX) (1:1000), Caspase-3 (1:1000) and p53 (1:1000) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-PCNA antibody (1:200) was purchased from Abcam (New territories, HK). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from KangChen (Shanghai, China). Anti-mouse IgG horseradish peroxidase (HRP) antibody (1:5000) was from ZhongShan Goldenbridge (Beijing, China). The pro-light-ning HRP agent for Western-blot analysis was from Tiangen Biotech Co. (Beijing, China). All other chemicals were from ZhongShan Goldenbridge (Beijing, China) and Hyclone (Beijing, China).

## Preparation of Huaier aqueous extract

Huaier electuary ointment was a gift from Gaitianli Medicine Co. Ltd. (Jiangsu, China). *Trametes* extract (1.76 g) was dissolved in 10 mL PBS buffer solution, and sterilized using a 0.22  $\mu\text{m}$  filter to obtain the 176 mg/mL stock solution suitable for long term storage at 4 °C (Campbell et al., 2002; Powell et al., 2003).

## Cell culture

Melanoma A875 cell lines were routinely cultured in DMEM medium supplemented with 10% FBS, under conditions of 5% CO<sub>2</sub> at 37 °C (Thermo Fisher Scientific, Marietta, OH, USA).

## CCK8 cell viability assay

The cells in suspension were digested using trypsin, the cell concentration adjusted to 4  $\times 10^5$ /mL, and 100  $\mu\text{L}$  of this cell suspension was placed in 96-well plates. The second day until after cell adherence, the Huaier extract stock solution was diluted to its final concentrations of 0, 2, 4, 8 and 16 mg/mL with complete DMEM medium. Each concentration was used in three parallel control wells, and CCK8 reagents were added after 24, 48, and 72 h. The cells were then incubated for 1 h at 37 °C. The plates were shaken for an additional 10 min and the absorbance values were read at 450 nm using a SpectraMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA).

## Effect of Huaier extract on cell morphology

Because the cells attached to the wall, cells in suspension were digested using trypsin and the final cell concentration was adjusted to 1  $\times 10^5$ /mL. The cell suspension (2 mL) was added to the 6-well plates. After incubation overnight, cells had attached to the side wall. The Huaier extract stock solution was diluted to final concentrations of 0, 2, 4, and 8 mg/mL using complete DMEM medium. Cells were incubated with the Huaier aqueous extract for 24, 48, or 72 h. The morphology of treated and negative-control cells was observed using an inverted microscope (Olympus, Tokyo, Japan).

## Cell-cycle detection

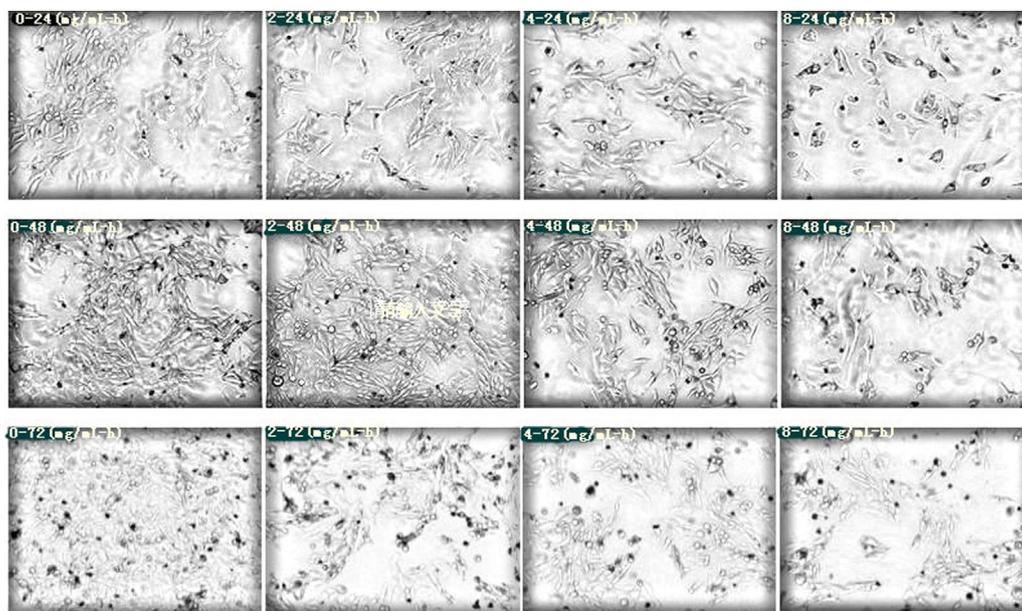
Cell-cycle analysis was performed using the standard method with some modifications (Cheng et al., 2004). The cells were digested using trypsin and then resuspended and washed twice with PBS buffer. The cell concentration was adjusted to 5  $\times 10^5$ /mL and 5 mL of the suspension was seeded in culture flasks. After incubation overnight, the cells had attached to the side wall, and the Huaier extract stock solution was diluted to final concentrations of 0, 4, and 8 mg/mL. Each concentration was used in three parallel control wells. Then, 1 mL was centrifuged and the supernatant was discarded. Solution A (containing trypsin in a spermine tetrahydrochloride detergent buffer for the enzymatic disaggregation of the solid tissue fragments and digestion of cell membranes and cytoskeletons) 250  $\mu\text{L}$  was added and incubated at room temperature for 10 min; solution B (containing trypsin inhibitor and ribonuclease A in citrate-stabilizing buffer with spermine tetrahydrochloride to inhibit the trypsin activity and to digest the RNA) 250  $\mu\text{L}$  was added and incubated at room temperature for 10 min, and then cold solution C (containing propidium iodide (PI) and spermine tetrahydrochloride in citrate stabilizing buffer). The PI stoichiometrically binds to the DNA at a final concentration of least 125  $\mu\text{g}/\text{mL}$  200  $\mu\text{L}$  was added. The resulting solution was filtered with a mesh filter, and analyzed after 48 and 72 h using flow cytometry. The cell cycle was analyzed using ModFit software obtained from CellQuest (BD Biosciences, San Jose, CA, USA).

## Detection of apoptosis in the cells by PI-Annexin-V staining

This assay was performed to detect cell apoptosis using an Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, harvested cells were resuspended in 100  $\mu\text{L}$  of the binding buffer to achieve a concentration of 1  $\times 10^6$ /mL. Then, 5  $\mu\text{L}$  Annexin V-FITC and 10  $\mu\text{L}$  propidium iodide (PI) (20  $\mu\text{g}/\text{mL}$ ) were added and the solution was incubated in the dark for 15 min at room temperature. Finally, 400  $\mu\text{L}$  of the binding buffer was added to each reaction tube before the cells were collected using FACS Calibur flow cytometry and analyzed using CellQuest software.

## Western blot analysis: determination of protein concentration using the BCA method

The protein concentration was adjusted to 2 mg/mL. SDS-PAGE gel (12%) and the electrophoresis solution were formulated and 10  $\mu\text{L}$  of the solution was placed into each well. The PVDF membrane was removed, washed with distilled water and placed in a blocking solution containing 5% non-fat dry milk and shaken for 1 h at room temperature. The first antibody was added, according to the dilution ratio recommended in the instructions and incubated overnight at 4 °C. The second antibody was then added at a ratio of 1:5000 and incubated for 1 h at room temperature on a shaker. The excess water in the PVDF membrane was absorbed with filter paper and ECL Plus A and B reagents were mixed in equal volumes on a fresh membrane. After 1 min, the surface membrane protein was turned down allowing full contact with this mixture. After 3 min, all the residue was removed, the membrane was covered with plastic wrap, and the protein expression was assessed using a Typhoon 9400 (GE Healthcare BioSciences AB, Uppsala, Sweden). GAPDH was used as the endogenous control. Cells in the control group were cultured in complete medium without Huaier extract. Electrophoresis and membrane transfer apparatus were Criterion Cell and PowerPac Universal Power Supply (Bio-Rad, Hercules, CA, USA), respectively.



**Fig. 1.** Huaier extract caused morphological changes in cells of the A875 melanoma cell line. Phase-contrast images of cells of the A875 melanoma cell line. A875 cells, before and after addition of Huaier (2, 4, 8 mg/mL) taken at 0 h, 24 h, 48 h and 72 h.

**Statistical evaluation**

SPSS ver.17.0 software (IBM, Shanghai, China) was used for the statistical analysis, and the Student's *t*-test was used to analyze statistical differences. *P* < 0.05 was accepted as significant. The data were expressed as the mean ± standard deviation and the experiments were repeated three times.

**Results**

**Cell morphology change**

Morphological changes in the melanoma cell line A875 after treatment with Huaier extract at 0, 2, 4, and 8 mg/mL for 24, 48, and 72 h are shown in Fig. 1. The cell number decreased in a dose- and time-dependent manner compared with the untreated cells. The majority of the Huaier-treated melanoma A875 cells became enlarged, irregular-shaped and showed vacuolated changes in the cytoplasm. These morphological changes demonstrated cell damage after Huaier extract treatment.

**CCK8 assay to determine Huaier extract inhibition of cell proliferation**

To evaluate the effects of Huaier extract on the melanoma A875 cell line, we measured cell viability using the CCK8 assay after the cells were dose-dependently treated with Huaier extract for 24, 48, and 72 h. As shown in Fig. 2, Huaier extract significantly inhibited the viability of the melanoma A875 cell line after 48 and 72 h in a time- and dose-dependent manner at each concentration (2, 4, and 8 mg/mL) compared to that of the untreated cells (*P* < 0.05, respectively). Huaier extract (8 mg/mL) showed strong inhibition of cell viability independent of the treatment time.

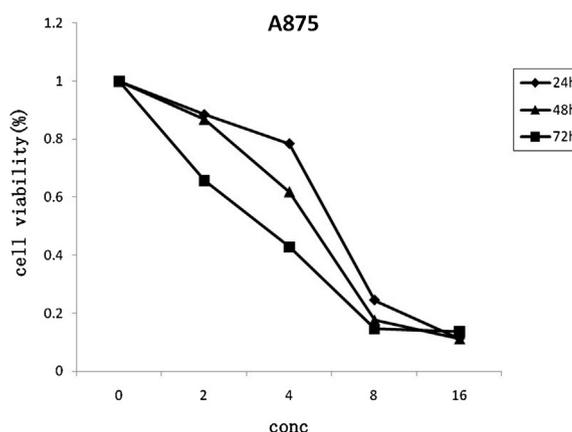
**Huaier extract treatment induces cell-cycle arrest**

The cell-cycle distribution of Huaier-treated melanoma A875 cells was analyzed using flow cytometry, in order to determine whether the inhibitory effect of Huaier extract was due to cell-cycle arrest. Before being processed and analyzed, melanoma A875 cells

were exposed to Huaier extract for a total of 48 or 72 h at drug concentrations of 4 or 8 mg/mL. Cell-cycle arrest was demonstrated in melanoma A875 cells exposed to Huaier extract through the increasing fraction of cells in the G2/M phase compared to that of the untreated cells (*P* < 0.05). The cells in the G2/M phase increased in a time- and dose-dependent manner compared with the control group. Huaier extract (8 mg/mL) showed more apparent inhibition (Table 1, Figs. 3 and 4). These results revealed that Huaier extract could inhibit melanoma A875 cell proliferation via cell-cycle arrest at the G2/M phase.

**Cell apoptosis analysis by flow cytometry**

Flow cytometry was used to detect intact cells, early apoptotic cells, late apoptotic cells or dead cells. After the Huaier extract treatment on melanoma A875 cells, late apoptosis or the cell death rate (UR, upper right quadrant – advanced stage apoptosis cell

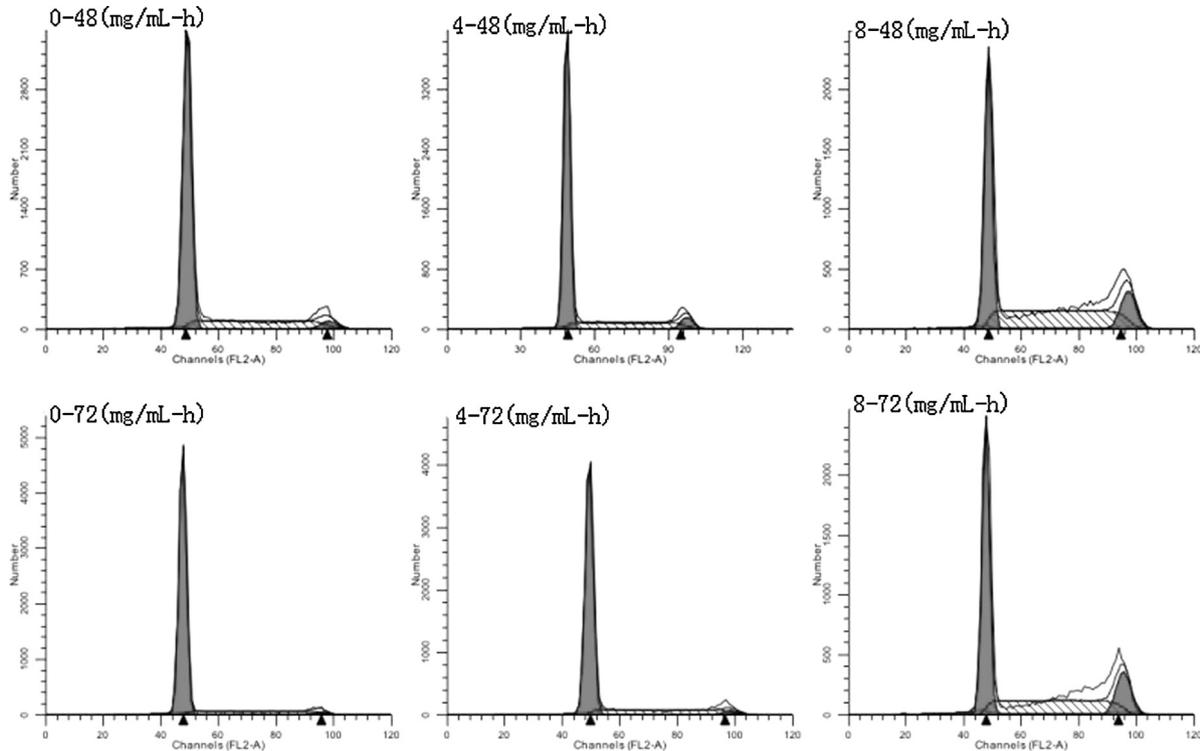


**Fig. 2.** Huaier extract suppresses cell proliferation viability of melanoma A875 cells. The effects of Huaier on cell proliferation viability was measured by CCK8 assay. Cells were treated with Huaier for 24, 48 and 72 h. Huaier extract significantly inhibited cell viability in a dose-dependent and time-dependent manner. The experiments were performed in triplicate and data presented as the mean ± SD of three separate experiments. Each concentration (2, 4, 8 mg/mL) treated with Huaier for 48 and 72 h was compared with that of the untreated cells, *P* < 0.05, respective.

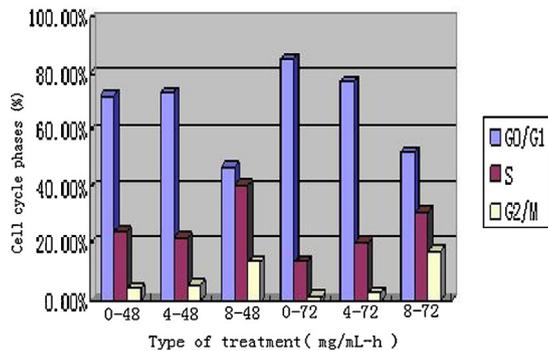
**Table 1**  
 Effects of Huaier extract on A875 cell-cycle.

	48-0(mg/mL-h)	48-4(mg/mL-h)	48-8(mg/mL-h)	72-0(mg/mL-h)	72-4(mg/mL-h)	72-8(mg/mL-h)
G0/G1 (%)	72.01	73.54	45.76	84.98	77.22	52.3
	72.18	72.92	46.44	84.89	77.91	52.35
	72.37	73.1	46.81	85.38	77.15	52.36
S (%)	24.14	21.28	41.78	13.59	20.15	32.48
	23.76	21.62	38.03	13.47	19.68	26.83
	23.84	21.7	41.42	13.72	20.16	32.97
G2/M (%)	3.85	5.17	12.46	1.43	2.62	15.22
	4.05	5.46	15.52	1.64	2.42	20.82
	3.79	5.20	11.77	0.91	2.69	14.67

In G2/M melanoma A875 cells were exposed to Huaier for a total of 48 and 72 h drug concentration of 4 and 8 mg/mL, as compared with that of the untreated cells,  $P < 0.05$ , respectively.



**Fig. 3.** Arrest of cell-cycle progression at G2/M in response to Huaier treatment.



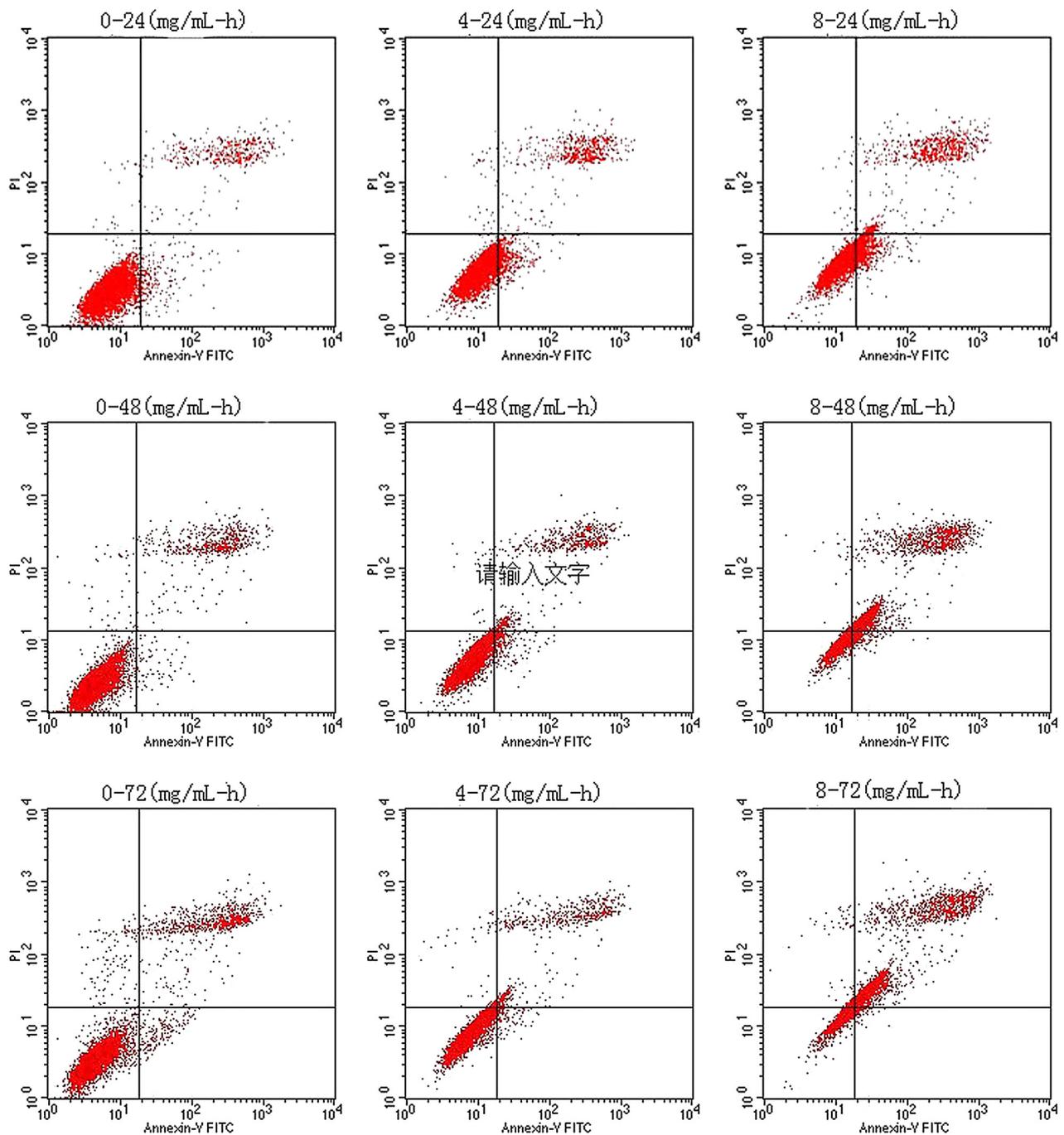
**Fig. 4.** Arrest of cell-cycle progression at G2/M in response to Huaier treatment. Huaier-induced G2/M arrest in A875 cells. The distribution of the cell cycle of A875 cells was assessed by flow cytometry. The data presented are the mean  $\pm$  SD of three independent experiments.

percentage) and the early apoptosis rate (LR, lower right quadrant – prophase apoptosis cell percentage) were increased in a time- and dose-dependent manner (Fig. 5 and Table 2). Apoptotic cells (UR+LR – all apoptosis cell percentage) were exposed to Huaier extract for a total of 24, 48, and 72 h at a drug concentration of 8 mg/mL compared to that of the untreated cells ( $P < 0.05$ ).

**Proliferation-related PCNA changes after Huaier extract treatment and Western blot analysis**

PCNA protein expression was assessed using Western blot. PCNA protein expression in the cells treated with Huaier extract decreased in a time- and dose-dependent manner compared with the control group (Fig. 6).

Because the activation of p53 can lead to either cell-cycle arrest and DNA repair or apoptosis, we tested the expression of p53 using Western blot to detect the apoptotic pathway-associated proteins. The expression of p53 was up-regulated in a time- and dose-dependent manner. To investigate further the Huaier apoptosis-causing mechanisms, the expression of Bcl-2,

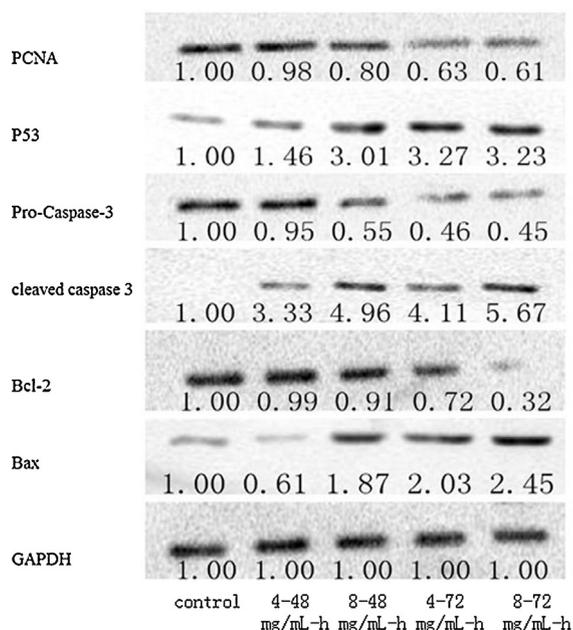


**Fig. 5.** Flow cytometric analysis of PI-Annexin-V to quantify Huaier-induced apoptosis in A875 cells. Dot plots of A875 cells with Huaier treatment at 0, 4 and 8 mg/mL for 24, 48, and 72 h. The results shown are representative of three independent experiments.

**Table 2**  
 Percentage of quadrant distribution (QD) in flow cytometry assay.

	Control	24 h-4	24 h-8	Control	48 h-4	48 h-8	Control	72 h-4	72 h-8
UL	0.29 ± 0.05	0.51 ± 0.09	0.94 ± 0.59	0.98 ± 0.40	1.01 ± 0.44	3.42 ± 0.37	1.79 ± 0.45	2.09 ± 0.37	8.49 ± 0.09
UR	5.04 ± 0.20	6.41 ± 0.14	11.43 ± 1.16	6.54 ± 0.29	8.01 ± 0.28	48.84 ± 2.43	9.09 ± 0.46	7.86 ± 0.28	66.34 ± 0.95
LL	92.92 ± 0.63	89.46 ± 2.01	77.28 ± 5.92	91.66 ± 0.13	87.60 ± 0.48	40.24 ± 2.75	87.40 ± 0.35	88.95 ± 0.45	23.86 ± 0.87
LR	1.75 ± 0.49	3.62 ± 1.98	10.36 ± 5.18	0.81 ± 0.02	3.38 ± 0.21	7.50 ± 0.81	1.72 ± 0.22	1.09 ± 0.12	1.30 ± 0.10

The data presented are the mean ± SD of three independent experiments. LL, lower left; LR, lower right; UL, upper left; and UR, upper right. Apoptotic cells were in UR + LR, melanoma A875 cells were exposed to Huaier for a total of 24, 48 and 72 h. drug concentration of 8 mg/mL, as compared with that of the untreated cells,  $P < 0.05$ , respectively.



**Fig. 6.** Effects of Huaier extract on expression of PCNA, p53, caspase-3, Bcl-2, Bcl2-associated X protein (BAX) proteins. Expression of GAPDH was used as an internal control. A875 cells were treated with 4 mg/mL for 24 or 48 h and 8 mg/mL for 24 or 48 h. The expression of each protein was quantified as the densitometry value analyzed by Image. The figure shown is representative of three independent experiments.

BAX, pro-caspase-3 and cleavage caspase-3 was measured using Western blot. Treatment with Huaier extract suppressed Bcl-2 expression and up-regulated BAX expression in a time- and dose-dependent manner (Fig. 6). Thus, the ratio of Bcl-2 to BAX was decreased, suggesting a mitochondrial mediated apoptosis. For evaluation of the Huaier-induced apoptosis executor pathway, pro-caspase-3 and cleaved caspase-3 were also measured using Western blot. Caspase-3 activation was significantly increased after Huaier extract treatment, resulting in increased cleavage caspase-3 expression and decreased pro-caspase-3 expression.

## Discussion

Recently, TCM products have been increasingly recognized as new drugs, and more TCM products have been found to induce apoptosis (Thatte et al., 2000; Yufei et al., 2008). In this study, we used CCK8 and Western blot detection of PCNA, and confirmed that Huaier extract inhibits melanoma A875 cell proliferation. Huaier extract changed the number and morphology of melanoma A875 cells and it inhibited melanoma A875 cell proliferation via cell-cycle arrest at the G2/M phase. Huaier extract also promoted apoptosis in a time- and dose-dependent manner compared with the control group. Using Western blot, we explored the apoptosis pathway promoted by Huaier extract. Two main pathways involved in the process have been investigated. One pathway is the death-receptor pathway and the other is the mitochondrial pathway. The latter has been considered an important mediator of cell apoptosis in mammals. Mitochondria are the target of several molecular pro-apoptotic signal transduction pathways and are also the integrating components of the cell death pathway. Apoptosis induced by some anticancer agents constitutes one aspect of the treatment effect. P53 tumor suppressor genes can influence the cell cycle progression, DNA damage repair, genomic stability and influence the apoptotic pathway by promoting apoptosis (Yonish-Rouach et al., 1991; Lowe et al., 1993a, 1993b). P53 gene mutations exist in many human tumors. The complete caspase-3 protein does not have

proteolytic enzyme activity; however, hydrolyzed caspase-3 cleavage fragments have proteolytic activity, and can induce apoptosis through the cascade reaction (Hengartner, 2000). Caspase-3 is one of the key apoptosis executors because most of the factors that trigger apoptosis ultimately lead to apoptosis through the caspase-3-mediated signaling pathway (Jin et al., 2010; Rao et al., 2001). Bcl-2 is mainly distributed in the mitochondrial membrane and cytoplasm. Bcl-2 is an intracellular anti-apoptotic factor that can stabilize the mitochondrial membrane, prevent mitochondrial caspase release and deter the oxygen free radical-induced apoptosis signaling pathway (Bagci et al., 2006). Bax is distributed in the cytoplasm and can also promote apoptosis (Adams and Cory, 1998). Our study demonstrated that in melanoma A875 cells exposed to Huaier aqueous extract, cell proliferation was inhibited; the enhanced P53 expression induced cell cycle arrest at the G2/M phase, decreased the anti-apoptotic factor Bcl-2 expression, increased the pro-apoptotic factor Bax expression, decreased caspase-3 protein expression, increased cleavage-caspase-3 expression, promoted melanoma A875 cell apoptosis through the mitochondrial pathway, and thus inhibited cell proliferation and viability.

Given that Huaier aqueous extract may play a novel role as a complementary medicine in melanoma treatment, future research on its anticancer mechanisms is required. Additionally, the interaction between Huaier extract and routine conventional therapies, such as chemotherapy, surgical therapy, radiation and immunotherapy should be considered.

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