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A traditional Chinese medicine Huaier triggers G1 cell cycle arrest and apoptosis through cyclins-CDKs-CKIs machinery in MOLT4 cells

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Abstract *Objective:* The purpose of the study was to study the effect of Huaier, a traditional Chinese medicine, on the cell cycle adjustment in MOLT4 cells *in vitro*. *Methods:* We used MTT assay to test cell viability, flow cytometry to detect cell cycle and apoptosis and western blot to examine the expression of cell-cycle and apoptotic proteins in MOLT4 cells induced by Huaier. *Results:* Huaier could reduce the viability of MOLT4 cell by inducing G1 arrest and apoptosis. The induction of apoptosis after treatment with Huaier for 24 h was demonstrated in a dose- and time-dependent manner by flow cytometry analysis. G1 arrest induced by Huaier was modulated through the increased expression of Cdki proteins ($p21^{cip/waf1}$ and $p27^{kip1}$) with a simultaneous decrease in Cdk2, Cdk4, Cdk6, cyclin D1 and cyclin E expression. Huaier also induced Bax and Bcl-2 expression and activation of Caspase-3. *Conclusion:* It is firstly demonstrated that Huaier can inhibit proliferation of MOLT4 cells via G1 arrest and apoptosis. These results suggest that Huaier is a cell-cycle anti-cancer drug.

Key words Chinese medicine; apoptosis; cell cycle arrest; MOLT4

The normal cell cycle core machinery is a family complexes of catalytic components called cyclin-dependent protein kinases (Cdks) and regulatory subunits named cyclins [1]. Distinct cyclin–Cdk complexes sequentially phosphorylate their respective substrates, thereby driving the cell through different phases of the cell cycle. Three essential classes of cyclin–Cdk complexes include the D-type cyclins (cyclins D1, D2 and D3), which activate Cdk4 and Cdk6 to execute critical events in G1 phase, the E-type and A-type cyclins, which activate Cdk2 to drive the cell through S phase, and the A-type and B-type cyclins, which activate Cdk1 to direct structural and regulatory events in mitosis [2–4]. Two families of Cdk inhibitors (CKIs) are known as $p16^{INK4A}$ family (i.e. $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, $p19^{INK4D}$) and the p21CIP1 family (i.e. $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$) [4].

One hallmark of cancer is uncontrolled cell proliferation and/or insufficient apoptosis [5]. To inhibit cancer cell proliferation and induce apoptosis, most anti-tumor drugs were developed for targeting different cell cycle and apoptosis regulators [6–7].

Huaier, which come from an important traditional Chinese medicinal herb fungi growing on the Chinese scholar tree, has a long history as an effective anti-cancer drug in China. Being extracted from the trametes robiniophila murr, the major active ingredient of Huaier is a polysaccharide protein which consists of 6 kinds of monosaccharides and 18 kinds of amino acids [8].

In this study, we planned to investigate the biological function and molecular targets of Huaier in human cancer cells.

Materials and methods

Preparation of Huaier dissolution

Huaier was provided by Gaitianli Pharmaceutical Co.Ltd. (China). Five mg powder was dissolved in 1 mL water, and thus its initial concentration was 5 mg/mL (each culture well containing 1 mL RPMI 1640 medium).

Cell culture

The acute lymphocytic leukemia cell line MOLT4 (ATCC, USA) was cultured in a six-well plate with DMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. All media, supplements, and sera were purchased from GIBCO (USA). Cells were incubated in a 5% CO_2 humidified incubator at 37 ℃. The culture was diluted and re-plated every 3 days to keep them in an asynchro-

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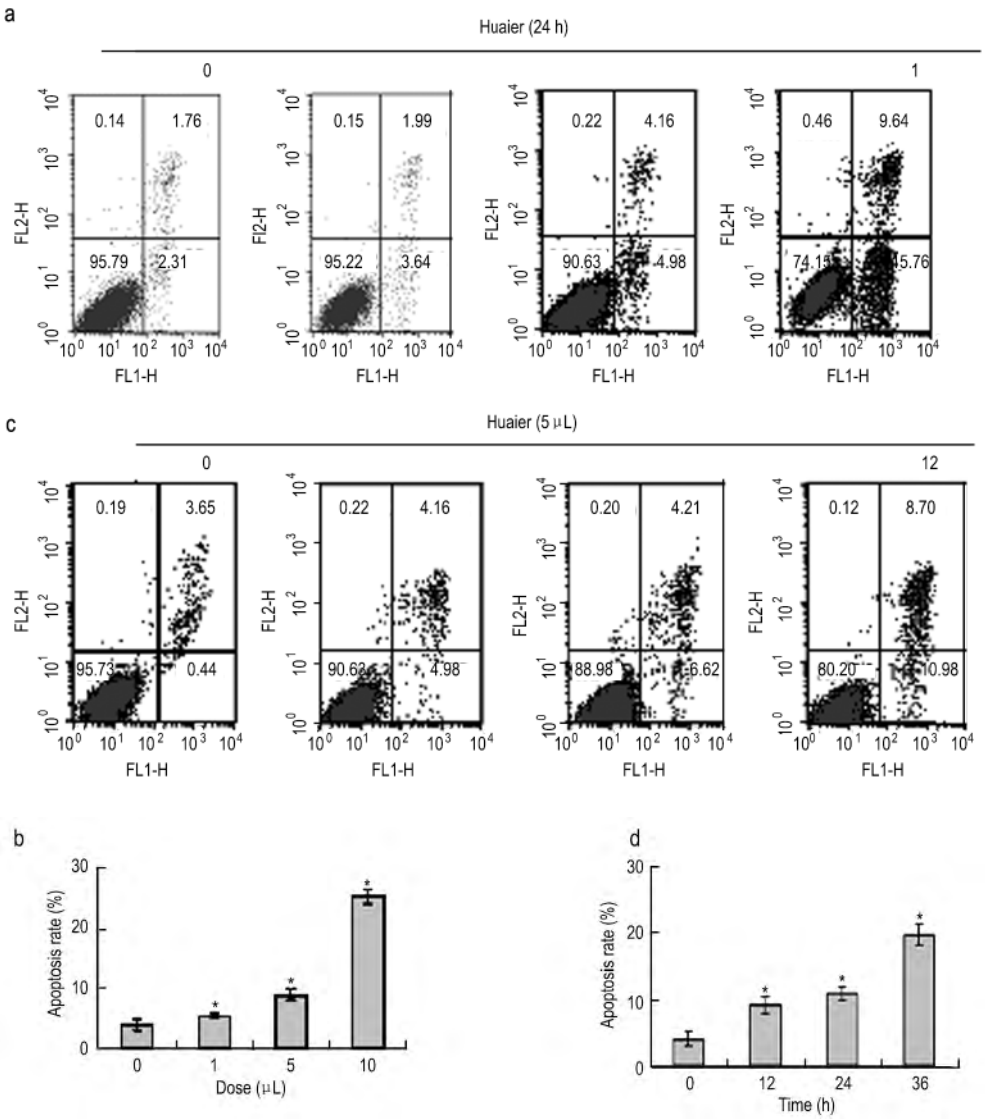

Fig. 1 Effect of Huaier on MOLT4 cells apoptosis with different doses or at different times. (a) and (c) showed Annexin V/PI assay was performed to detect apoptosis with different doses or at different times; (b) and (d) display histogram was used to show an increase of apoptosis rates as doses rised or time went by. * $P < 0.05$

nous and exponential phase of growth.

Huaier solution was added into 4 wells with 0 μL, 1 μL, 5 μL and 10 μL, respectively. After the cells grew for 24 h, they were then rinsed with phosphate buffered saline (PBS), centrifugated and harvested, while Huaier solution was also added to another 4 wells with 5 μL. After cells were incubated for 0 h, 12 h, 24 h and 36 h and then rinsed with PBS, centrifugated and harvested.

Flow cytometric analysis

Annexin V/PI assay for apoptosis

Five μL FITC-Annexin V and 10 μL PI were added to harvested fresh cells that had been resuspended in 100 μL cold binding buffer at a density of 10^6 cells/mL. The cells were then incubated at room temperature in the dark for 30 min, and finally detected by flow cytometry and analyzed with Cellquest software (FACSVantage, Becton Dickinson, USA).

PI assay for cell cycle analysis based on DNA content and distribution

Harvested cells were re-suspended in 1 mL of 0.1% sodium citrate containing 0.3% NP-40, 0.0002 mg/mL RNase and 50 μg/mL propidium iodide, and then were incubated on ice in the dark for 30 min. Cell cycle were analyzed on a FACSort with Cellquest (Becton Dickinson, USA) and ModFit software (Verity Software House, USA).

γH2AX for DNA damage

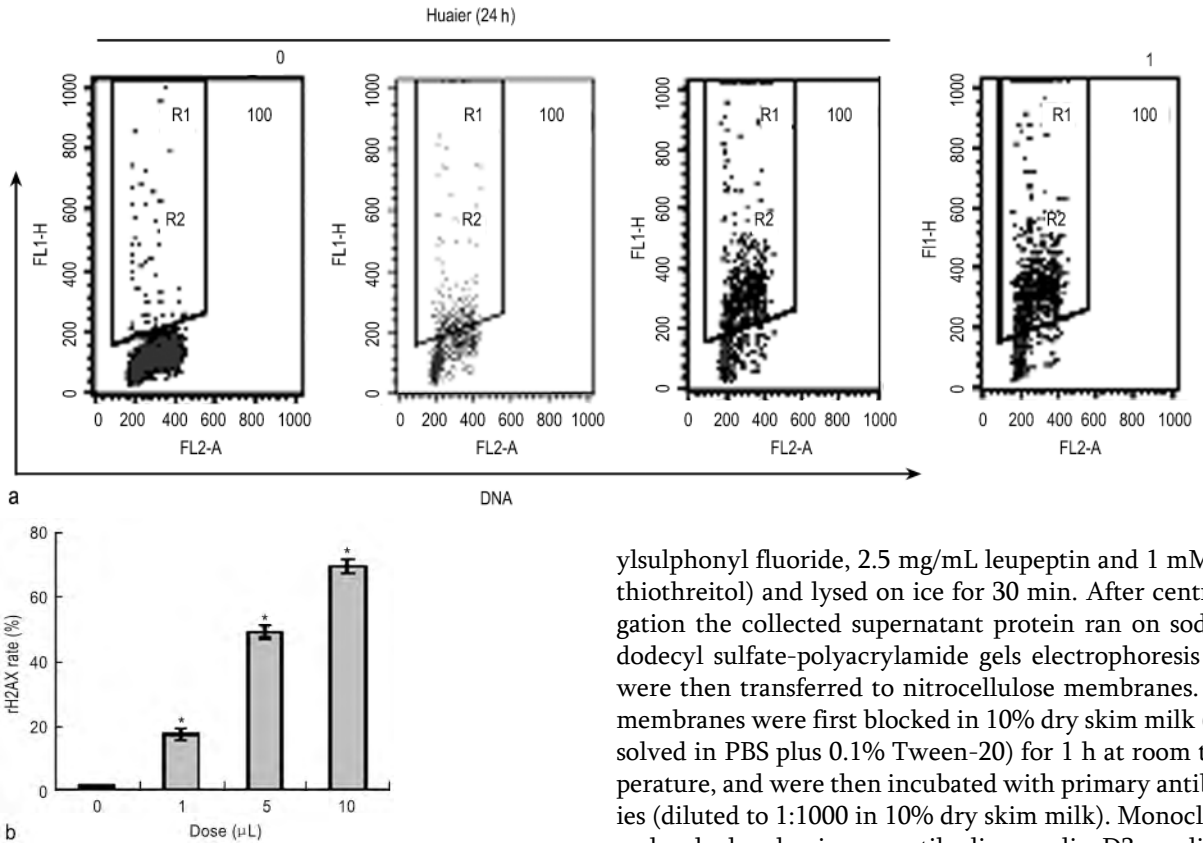

Fig. 2 Effect of different doses Huaier on MOLT4 cell cycle distribution after 24 h. (a) PI assay was performed to detect every percentage of G1, S and G2/M; (b) histogram was used to show an increase of G1 percentage and a decrease of G2/M percentage. * $P < 0.05$

Harvested cells were fixed by 1% formaldehyde in PBS without methanol on ice for 15 min, and rinsed, then centrifugated. The collected cells were fixed by ice-cold 80% ethanol at –20 ℃ for at least 24 h. These cells were washed with PBS twice and permeated with 0.5% Triton X-100 in PBS on ice for 5 min. After centrifugation, they were incubated overnight at 4 ℃ in the presence of primary antibody γH2AX [BD PharMingen; diluted in PBS containing 1% bovine serum albumin (BSA)]. The next day cells were rinsed and incubated with the secondary FITC-conjugated antibody (DAKO, Denmark; diluted in PBS containing 1% BSA) for 30 min. Finally, cells were rinsed and resuspended in propidium iodide solution (50 μg/mL PI) and incubated at room temperature for 30 min. Cell fluorescence was measured by a FACSVantage flow cytometry (Becton Dickinson).

Western blotting detection

Harvested cells were re-suspended in lysis buffer (50 mM HEPES, pH 7.4, 250 mM NaCl, 2 mM ethyleneglycoltetraacetic acid, 2 mM ethylenediamine-tetraacedtic acid, 1 mM NaF, 0.1 mM vanadate, 0.1 mM phenylmethylsulphonyl fluoride, 2.5 mg/mL leupeptin and 1 mM dithiothreitol) and lysed on ice for 30 min. After centrifugation the collected supernatant protein ran on sodium dodecyl sulfate-polyacrylamide gels electrophoresis and were then transferred to nitrocellulose membranes. The membranes were first blocked in 10% dry skim milk (dissolved in PBS plus 0.1% Tween-20) for 1 h at room temperature, and were then incubated with primary antibodies (diluted to 1:1000 in 10% dry skim milk). Monoclonal and polyclonal primary antibodies: cyclin D3, cyclin E, CDK2, CDK4, CDK6, p16, p21, p27, Bcl-2, Bax and Beta-actin antibodies (BD PharMingen) overnight at 4 ℃. Next day the membranes were rinsed twice and incubated with the horseradish peroxidase-coupled secondary antibody (diluted to 1:5000 in 10% dry skim milk. Wuhan Boster Biological Technology Ltd., China) at room temperature for 2 h. Detection was performed by ECL system (Amersham Pharmacia, Tokyo).

Statistical analysis

The study was repeated thrice. The statistical significance of all results was evaluated by paired Student's *t*-test. Data were presented with $P < 0.05$ accepted as significance.

Results

Huaier treatment induces a dose- and time-dependent apoptosis in MOLT4

To examine its apoptotic effect on MOLT4 cells, different dosages of Huaier (0 μL, 1 μL, 5 μL, 10 μL) were treated for 24 h, and cell apoptosis was determined by Annexin V/PI assay. With the increased dosage of Huaier, the percentage of cell apoptosis also increased from 4.07% to 25.40% (Fig. 1a and 1b). In addition, apoptosis at different time courses (0 h, 12 h, 24 h, 36 h) was detected. Similarly, as the time went by, the apoptosis rate increased

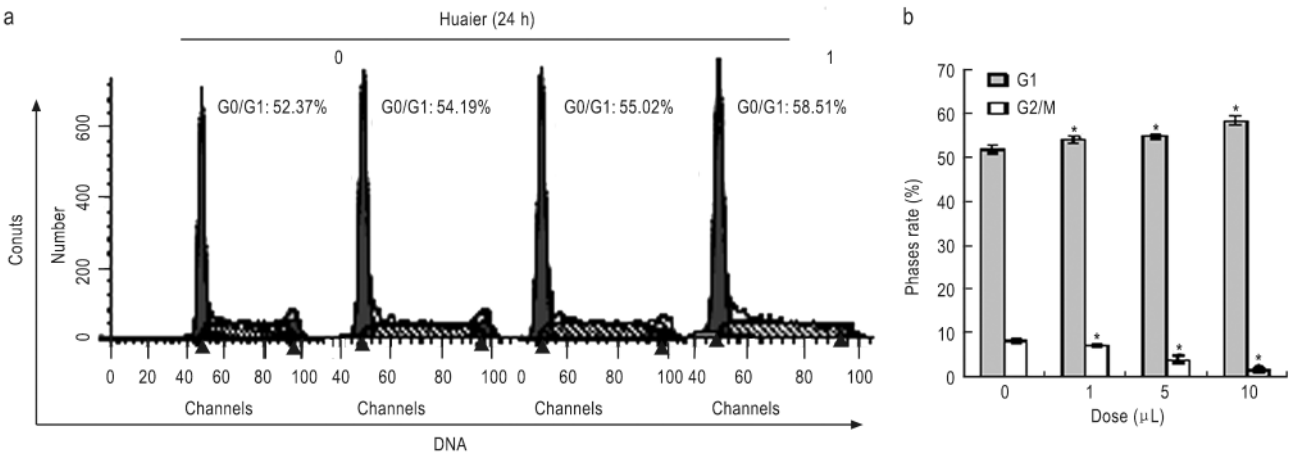

Fig. 3 Effect of different doses Huaier on MOLT4 cells DNA damage after 24 h. (a) γH2AX/DNA multiparameter assay was performed to detect the expression of γH2AX foci; (b) histogram was used to show an aggravation of DNA damage degree as doses increased. * $P < 0.05$

from 4.09% to 19.68% (Fig. 1c and 1d).

To examine the effect of Huaier on cell proliferation, we analyzed cell cycle distribution of MOLT4 cells treated at different dosages (0 μL,1 μL, 5 μL ,10 μL) for 24 h. Flow cytometry data showed that cell cycle was arrested in G1 phase, which was indicated by increasing in percentage G1 phase from 52.37% to 58.51% with a dose-dependent manner and concomitant decreasing in the percentage of G2/M cells from 8.22% to 1.50%, whereas the S phase did not change significantly (Fig. 2).

To examine the DNA damage induced by Huaier, we measured the expression of γH2AX protein by multi-parameter flow cytometric analysis. After MOLT4 cells were treated with Huaier (0 μL, 1 μL, 5 μL, 10 μL) for 24 h, the expression of γH2AX protein increased significantly compared to control treatment (from 0.96% to 69.71%, Fig. 3).

We further determined the molecular mechanism of Huaier in inducing G1 arrest and apoptosis. Expression of G1- and apoptosis-associated proteins in MOLT4 cells treated with Huaier was detected by Western blotting. Our data showed that Huaier induced a dramatic upregulation of p16, p21 and p27 and downregulation of cyclin D3, cyclin E, CDK2, CDK4 and CDK 6 in a dose-dependent manner (Fig. 4). The apoptosis-associated Bcl-2 family includes anti-apoptotic (such as Bcl-2) and pro-apoptotic proteins (such as Bax) [9]. Western blotting analysis showed that the expression of Bax protein was significantly increased, whereas the expression of Bcl-2 protein was markedly decreased in the cells treated with Huaier (Fig. 4). The ratio of Bax/Bcl-2 showed a significant increase, which is believed to induce apoptosis in MOLT4 cells [10].

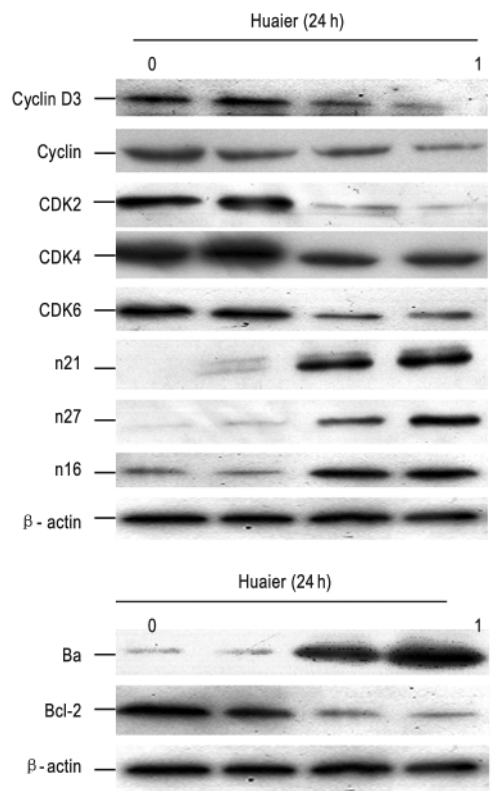

Fig. 4 Effect of different doses Huaier on the expression of G1- and apoptosis-associated proteins after 24 h by Western blot analysis

Discussion

Huaier used to be extracted for the treatment of many kinds of carcinomas in the practice of Chinese traditional medicine, such as liver cancer [11], breast cancer [12], lung cancer [13]. Besides, we found it have also the same effect on colorectal cell line SW480 and human leukemic cell line HL-60 (data not shown), but better effect on MOLT4 cells. It is reported that Huaier can induce cells to secrete

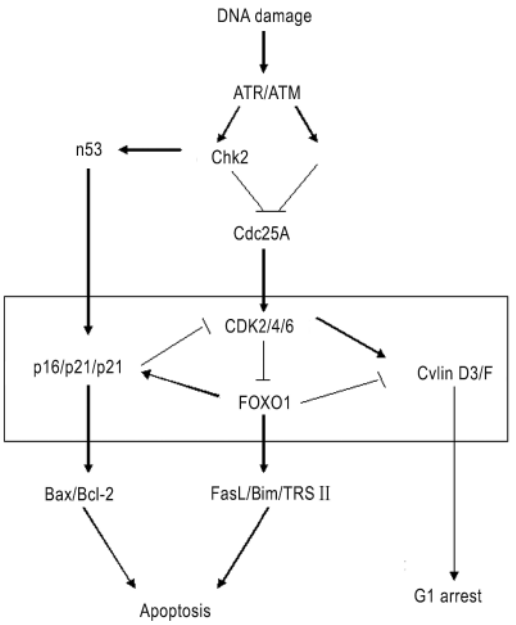

Fig. 5 Huaier triggers G1 cell cycle arrest and apoptosis probably through cyclins-CDKs-CKIs machinery in MOLT4 cells

IFN-α and IFN-γ, then activate NK cells, and finally in turn kill cancer cells [14].

In our study, we showed that Huaier could inhibit cancer cell proliferation by triggering G1 cell cycle arrest and induce cell apoptosis, which provided the *in vitro* evidence for its anti-tumor effect. Huaier-induced DNA Stranded Break indicated by γH2AX upregulation was measured by flow cytometry (Fig. 3). It can activate DNA checkpoints including Ataxia telangiectasia mutated (ATM) and/or ATM and Rad 3-related (ATR) [15–17], which inhibits CDKs to arrest cell cycle progression, allowing cells to repair the damaged DNA or removing those cells by inducing apoptosis whose DNA damage can not be repaired [18–20]. ATM and ATR were shown to activate Chk1 and Chk2 respectively [21–23], and the latter can cause ubiquitin-dependent degradation of protein phosphatase Cdc25A, which inhibits CDK2 [24–25], CDK4 and CDK6 (Fig. 5). It was reported that CDK2 phosphorylates FOXO1 at ser249 in vivo and inhibit FOXO1 function [26–27]. FOXO1, as a transcription factor, on the one hand up-regulates p21 [28], p27 [29–30] and p16; on the other hand, it represses cyclin D3 expression [31–32]. Our data showed that in Huaier-treated cells, CDK2 was down-regulated; p21, p27, and p16 were up-regulated, as well as cyclin D3 was down-regulated, which is consistent with previous studies.Activated FOXO1 can also trigger a complicated network of mitochondria apoptotic signaling, such as Bax/Bcl-2 pathway [11] activated indirectly by p21, p27, and p16 [28–30, 33–34] (Fig. 4), and/or death receptor apoptotic signaling, such as FasL/Bim/TRAIL pathway activated directly by FOXO1 [26–27]. Both apoptotic signaling can activate common downstream caspase-3 to trigger apoptosis (Fig. 5).

In summary, we have shown that Huaier can effectively inhibit cell proliferation by inducing G1 arrest through the down-regulation of CDK2/CDK4/CDK6, and cyclin D3/cyclin E, together with the up-regulation of CKIs including p16/p21/p27. Apoptosis was accompanied by the G1 cell cycle arrest. To our knowledge, this is the first study that shows the involvement of the cyclins–CDKs–CKIs machinery during the G1 arrest and apoptosis of MOLT4 cells treated with Huaier. These findings indicate that Huaier could be a potential therapeutic anti-cancer agent. In our future study, we will further purify the Huaier's monosomy effective ingredient and elucidate the mechanism of Huaier-triggered both mitochondria- and death receptor-mediated apoptotic pathways.

Conflicts of interest

The authors indicated no potential conflicts of interest.

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