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Huaier aqueous extract inhibits proliferation of breast cancer cells by inducing apoptosis

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Aqueous extract of Trametes robiniophila murr (Huaier) has been commonly used in China for cancer complementary therapy in recent years; however, the mechanisms of its anticancer effects are largely unknown. In the present study, we aim to investigate its inhibitory effect on both MCF-7 and MDA-MB-231 cells, and explore the possible mechanisms of its anticancer effect. Cell viability and motility were measured by MTT and invasive assays, migration and scratch assays *in vitro*, respectively. The distribution of cell cycle, PI-Annexin-V staining and Rhodamine 123 assay were analyzed by flow cytometry, and western blot were used to test the apoptotic pathways. We found that Huaier extract could strongly inhibit cell viability of MCF-7 and MDA-MB-231 cells in a time- and dose-dependent manner; however, MDA-MB-231 cells showed more susceptibility to the treatment. Furthermore, cell invasiveness and migration were also suppressed with exposure to Huaier extract. We also indicated that Huaier could induce G0/G1 cell-cycle arrest, p53 accumulation and activation selectively in MCF-7 cells. Inspiringly, the PI-Annexin-V staining assay and western blot analysis confirmed cell apoptosis executed by caspase-3. Decreased mitochondrial membrane potential by Rhodamine 123 assay and down-regulation of Bcl-2 and up-regulation of BCL2-associated X protein (BAX) indicated that Huaier induced apoptosis through the mitochondrial pathway. Caspase activation during Huaier-induced apoptosis was confirmed by pan-caspase inhibitor, Z-VAD-fmk. As expected, the inhibitor decreased Huaier-induced apoptosis in both cell lines. Based on our findings, Huaier can induce cell apoptosis in both ER-positive and ER-negative breast cancer cell lines and is an effective complementary agent for breast cancer treatment. (*Cancer Sci* 2010; 101: 2375–2383)

Worldwide, it is estimated that more than 1 million women are diagnosed with breast cancer every year, and it accounts for approximately 410 000 deaths per year.[1] Breast cancer is already the leading cause of cancer in southeast Asian women, and is second only to gastric cancer in east Asian women.[2] In some areas of China, the incidence of breast cancer is increasing by 5% per year, greater than that of the worldwide rate.[3] However, compared with other carcinomas, breast cancer has a better prognosis and over 5 million successful survivors comprise nearly 23% of the total cancer survivors in the USA. Although the 5-year survival is estimated at 98% and 94% for stage 1 and 2 localized disease,[4] respectively, the therapeutic options for advanced-stage breast cancers are still fairly limited.[5]

There are multiple conventional strategies for breast cancer treatment, including surgery, radiotherapy, chemotherapy, hormonotherapy and other complementary therapies.[6] However, these therapies can not totally prevent cancer patients from recurrence and metastasis, thus new drugs and new therapies are in great need for cancer patients. Among the complementary therapies, traditional Chinese medicine (TCM) has gained growing popularity for its novel role in killing tumor cells less intensively and more naturally.[7] Traditional Chinese medicine has a history of three millennia in Asia. Several years ago, a retrospective analysis demonstrated that 62% of anti-infective and antitumor agents either commercially available or in the late stages of development are drugs of natural origin.[8] Traditional Chinese medicine contains a variety of complex compounds such as alkaloids, steroids and proteoglycan[9,10] that can display special anticancer effects under a toxic level.

Trametes robiniophila murr (Huaier) is kind of officinal fungi in China and has been applied in TCM for approximately 1600 years[11]; however, its antitumor properties were found and used as a complementary therapy only in recent decades. The fungus was extracted twice with hot water, and the free proteins and amino acids were eliminated with the Sevag method. After being dialyzed for 72 h, four times the volume of ethanol was added to the dialysis solution to precipitate the effective ingredient. The HPLC and SDS-PAGE analysis certified the effective ingredient of Huaier extract was proteoglycan, which contains 41.53% polysaccharides, 12.93% amino acids and 8.72% water[12,13] (Table S1). Additionally, the inhibitory effect of the proteoglycan on sarcoma murine S_{180}, liver cancer H_{22}, lung cancer Lewis and breast cancer MCF-7 cells was investigated to confirm proteoglycan as the major active principal in the Huaier extract. However, the inhibitory effect of proteoglycan, although the most effective among all of the isolated ingredients, was less effective than the Huaier extract.[12,13] It is reasonable to speculate that the activities of the Huaier extract may be due to the synergistic or additive effect of these fractions. The antitumor effects of Huaier extract displayed various biological activities, for example, apoptosis, antiangiogenesis, drug resistance reversal, anti-metastasis and system immune activation. Although the recent experimental data displayed the apoptotic effect, the underlying mechanisms has not yet been studied in detail. Additionally, there are probably other antitumor effects besides apoptosis. In the current study, we investigated the antitumor mechanisms of Huaier in breast cancer cells.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco-BRL (Rockville, IN, USA). Fetal bovine serum (FBS) was supplied by Haoyang Biological Manufacturer Co., Ltd (Tianjin, China). Anti-Bcl-2 (1:250), BCL2-associated X protein (BAX) (1:500) and p53 (1:1000) antibodies were purchased from Dako Corp. (Carpinteria, CA, USA); phosphorylated p53 (1:1000) and Caspase-3 (1:1000) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-mouse IgG horseradish peroxidase (HRP) antibody (1:3000) was from ZhongShan Goldenbridge (Beijing, China). The pro-lighting HRP agent for western-blotting detection was from Tiangen Biotech CO., LTD (Beijing, China). All other chemicals were from Merck (Darmstadt, Germany) and Sigma-Aldrich (St Louis, MO, USA).

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Preparation of Huaier aqueous extract. Electuary ointment of Huaier was a gift from Gaitianli Medicine Co. Ltd (Jiangsu, China). Two grams of the electuary ointment was dissolved in 20 mL of complete medium and was sterilized with 0.22 μm filter to get the 100 mg/mL stock solution for long storage at −20°C.[14,15]

Cell culture. The two kinds of breast cancer cell lines (MCF-7 cells and MDA-MB-231 cells) and NIH3T3 fibroblast cells were obtained from American Type Culture Collection (ATCC), and routinely cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin under the conditions of 5% CO_2 at 37°C.

Cell viability assay. Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The MCF-7 (3×10^3 cells/well) and MDA-MB-231 (1.5×10^3 cells/well) cells were cultured in 5% CO_2 at 37°C in DMEM medium (containing 10% FBS, 100 μg/mL penicillin and 100 U/mL streptomycin) in 96-well plates. After incubation overnight, the DMEM medium in each well was replaced with different concentrated solutions and incubated for 24, 48 or 72 h individually. Afterwards, 20 μL of MTT (5 mg/mL in PBS) was added to each well and the cells were incubated for another 4 h at 37°C. The supernatants were then aspirated carefully and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The plates were shaken for an additional 10 min and the absorbance values were read by the Microplate Reader (Bio-Rad, Hercules, CA, USA) at 570 nm.[16] The stock solution of Huaier extract was diluted at final concentrations of 0, 2, 4, 8 and 16 mg/mL with complete DMEM medium.

Effect of Huaier on cell morphology. The MCF-7 and MDA-MB-231 cells were incubated with the Huaier aqueous extract at a concentration of 4 mg/mL for 24 and 48 h. The morphology of treated and negative-control cells was observed at 24 and 48 h under an Olympus light microscope and photomicrographs were taken with an Olympus digital camera (Tokyo, Japan).

Invasion assays. The *in vitro* invasive assay was performed using the Transwell system (24 wells, 8 μm pore size with polycarbonate membrane; Corning Costar, Lowell, MA, USA) to determine the change to the invasiveness of MDA-MB-231 cells by Huaier. Cells were starved in serum-free medium for 12 h at 37°C. The polycarbonate membranes were coated with 40 μL matrigel (1.5 mg/mL; BD Biosciences, San Jose, CA, USA) at 37°C for 2 h to form a reconstituted basement membrane. Five hundred microlitres of balanced mixture of the conditional medium from NIH3T3 fibroblasts and the complete medium was added to the lower well of each chamber and 1×10^5 of MDA-MB-231 cells were added to the upper wells. The control group cells were suspended in 100 uL of serum-free medium containing 0.1% BSA, while in the test group the medium also contained 4 mg/mL Huaier. After incubation for 18 h, the upper surface of the membrane was swiped with cotton swabs to remove the attached cells. The upper wells were then placed in a

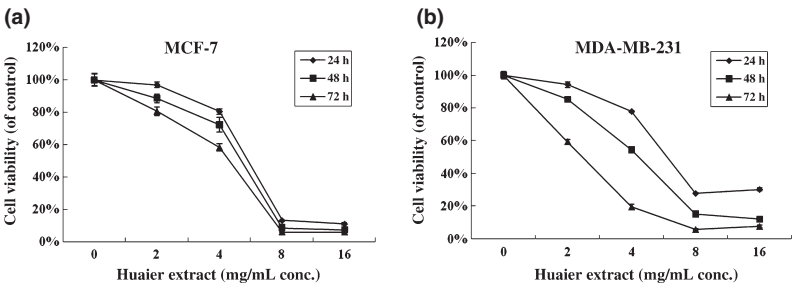

Fig. 1. Huaier extract suppresses cell viability of MCF-7 and MDA-MB-231 cells. The effect of Huaier on cell viability was measured by MTT assay. (a) MCF-7 and (b) MDA-MB-231 cells were treated with Huaier for 24, 48 and 72 h. Huaier extract significantly inhibited cell viability of both cell lines in a dose- and time-dependent manner. The experiments were performed in triplicate and data presented as the mean ± SD of three separate experiments.

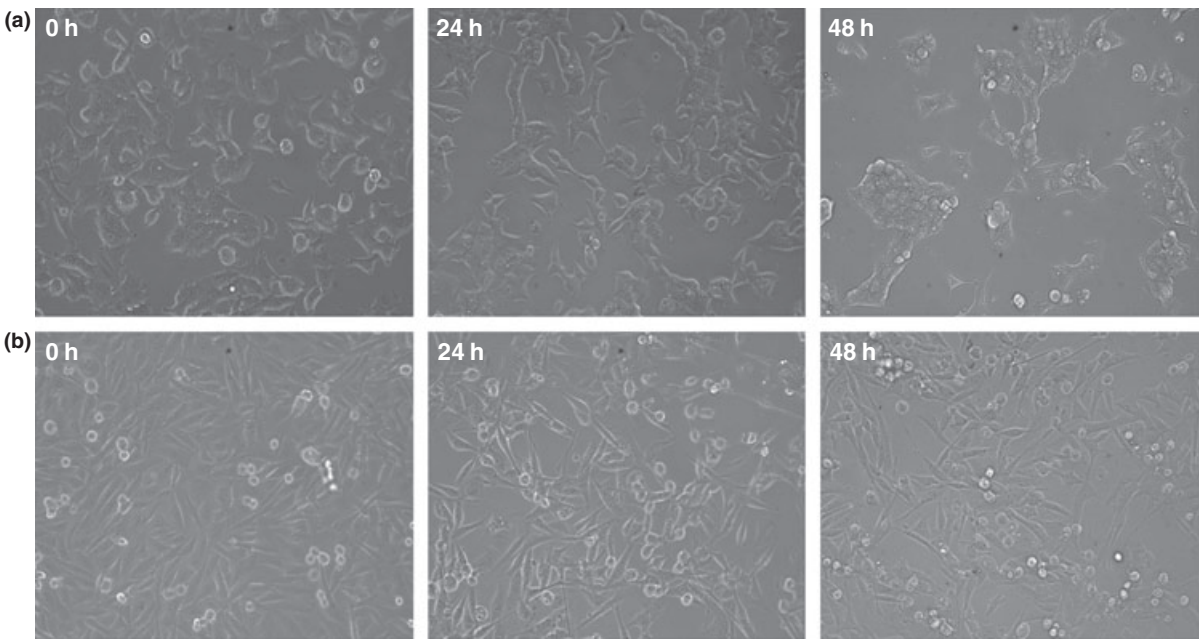

Fig. 2. Huaier extract caused a morphology change of MCF-7 and MDA-MB-231 cells. Phase-contrast images of (a) MCF-7 and (b) MDA-MB-231 cells before and after addition of Huaier (4 mg/mL) taken at 0, 24 and 48 h.

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new plate and the cells adhering to the lower surface were fixed in 95% ethanol for 10 min. Finally, the cells were stained with Giemsa. The total number of cells invading and adhering to the lower surface was acquired in six representative fields using an Olympus light microscope.[17–19]

Migration assays. The migration assay was performed in the same way as the invasion assay above except that the membrane was not coated with Matrigel. MDA-MB-231 (1×10^5 cells/well) and MCF-7 (1×10^5 cells/well) cells were added to the upper wells, and the culture medium in the upper wells of the control and test groups was the same as the invasive assay. After incubation for 5 h at 37°C, the cells were stained and counted in the same way as in the invasion studies.[17]

***In vitro* scratch assay.** To evaluate cell mortality affected by the Huaier aqueous extract, a scratching assay of the MCF-7 and MDA-MB-231 cells was performed. After harvesting by trypsinization with 0.1% trypsin and 0.1% EDTA, the MCF-7 and MDA-MB-231 cells were seeded in the 24-well plates at a density of 4×10^5 and 2×10^5, respectively. Reference points near the ''scratch'' were marked to guarantee the same area of image acquisition and placed in plates at 37°C. Cells were grown in complete medium to a confluent monolayer for 2 days, and were scraped by p20 pipette tips to create a straight-line cell-free ''scratch''. Each well was washed twice with PBS to remove debris and to smooth the edge of the scratch and then replaced with the serum-free Huaier aqueous extract (4 mg/mL). After that, the migration of cells in the ''scratch'' was photographed at the matching reference points with phase-contrast microscope for the first image, and the following images were taken at intervals of 12 h.[20,21] The images were analyzed quantitatively by ImageJ; the distances between the two edges of the scratch were measured at the reference points and analyzed statistically.

Cell-cycle analysis. Cell-cycle analysis was performed using the standard method[22] with some modifications. Briefly, 5×10^5 cells/well were seeded in 6-well plates and starved in serum-free medium at 37°C. After 12 h starvation, the cells were treated with Huaier solution and complete medium for 24 or 48 h. The cells were then trypsinized, washed with cold PBS and fixed overnight with 70% cold ethanol containing 3% FBS at −20°C. The next day, the fixed cells were centrifuged at 1200*g*. for 1 min, and washed with PBS twice. After that, the cell plates were resuspended with 200 μL RNase A (1 mg/mL) at 37°C for 10 min, followed by the addition of 300 μL propidium iodine (PI, 100 μL/mL) to stain the DNA of cells in the dark. After 20 min incubation at room temperature, the DNA contents of the cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data was analyzed by ModFitLT V2.0 software (Becton Dickinson).

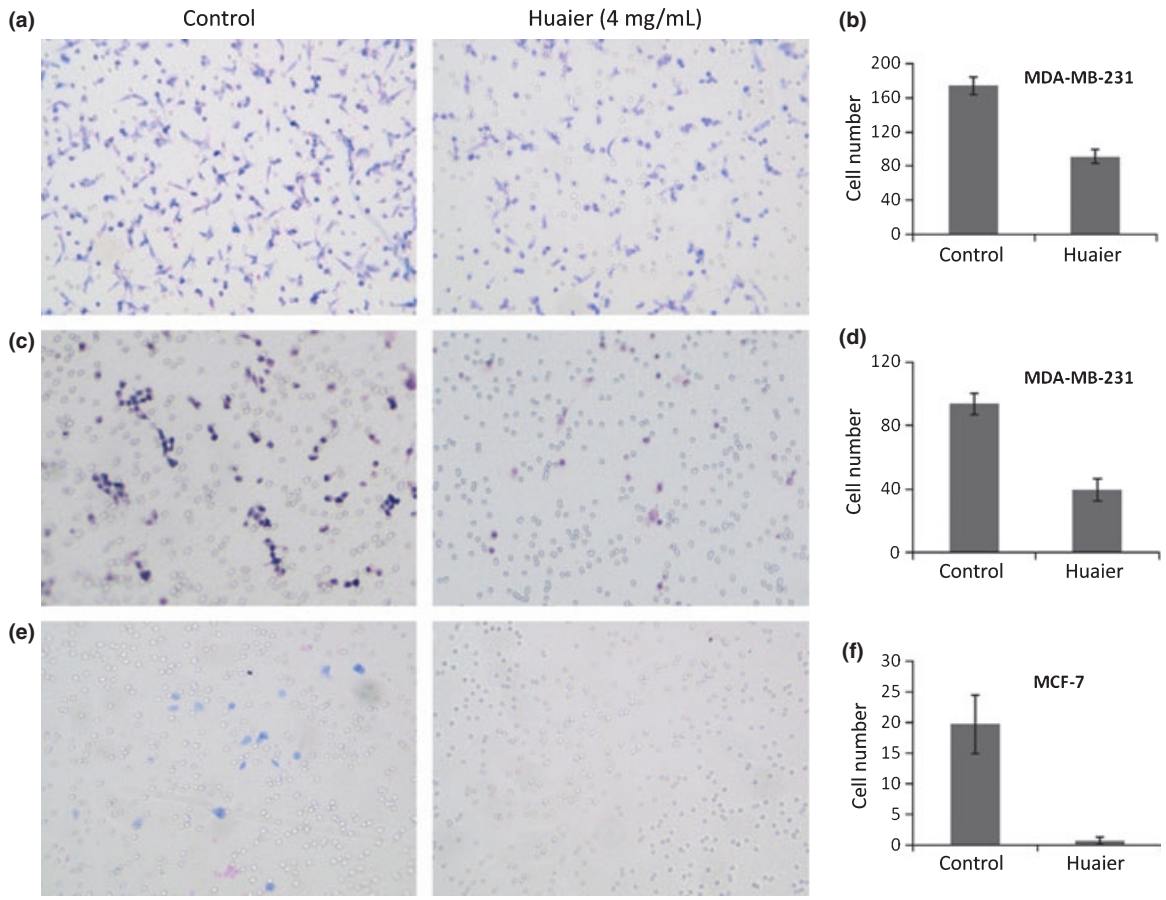

Fig. 3. Cell mobility was strongly inhibited by Huaier extract (4 mg/mL) in invasive and migration assays. (a) Invasive assay of MDA-MB-231 cells using the Transwell system, which shows the inhibitory effect of 18 h of Huaier treatment on invasiveness. (b) The number of successfully invading MDA-MB-231 cells with or without Huaier treatment. (c) Migration assay of MDA-MB-231 cells after 5 h of treatment with Huaier. (d) The number of MDA-MB-231 cells, which successfully matrigel through membrane, was compared between control and Huaier-treated group. (e) Migration assay of MCF-7 cells after 5 h of treatment with Huaier. (f) The cell number decrease of the MCF-7 cells indicates the great inhibitory effect of Huaier. Giemsa was used as a staining agent and cell numbers were counted at six representative fields. Data is presented as mean ± SD.

Identification of apoptosis by PI-Annexin-V staining. This assay was performed to detect cell apoptosis with an Annexin V-FITC Apoptosis Detection Kit (JingMei Biotech, Beijing, China), following the instructions from the manufacturer. In brief, harvested cells were resuspended in 100 μL of the binding buffer to achieve a concentration of 1×10^6/mL. Then, 5 μL Annexin V-FITC and 10 μL propidium iodide (PI) (20 μg/mL) were added and incubated in the dark for 15 min at room temperature. Finally, 400 μL of the binding buffer was added to each reaction tube before the cells were analyzed by FACScan flow cytometry. The data was analyzed by WinMDI V2.9 software (The Scripps Research Institute, San Diego, CA, USA).[22]

Mitochondrial membrane potential (MMP) assay. After treatment with series concentrations of Huaier, the MDA-MB-231 and MCF-7 cells were harvested and suspended in PBS. One microlitre rhodamine 123 (5 μg/mL) was added and the cells were incubated for 1 h in the dark at 37°C. The cells were then washed twice with PBS and resuspended. The mitochondrial membrane potential was measured by FACScan flow cytometry of 10 000 cells and shown as mean fluorescence intensity (MFI).

Western blot analysis. The MDA-MB-231 and MCF-7 cells were treated with Huaier extract in gradient time (24 and 48 h) and concentrations (4 and 8 mg/mL). The proteins of distinctively treated cells were collected and lysed in lysis buffer in the presence of protease inhibitors.[23] Eighty micrograms of protein were separated by 12% SDS-PAGE and electroblotted to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat milk, the membranes were incubated overnight at 4°C with the primary antibodies, followed by labeling with the secondary antibody. Protein bands were visualized using the Pro-lighting HRP agent. β-actin was used as the endogenous control and the control cells was cultured in the complete medium without Huaier.

Statistical evaluation. The software SAS V9.1 (SAS Institute Inc., Cary, NC, USA) was used for statistical analysis, and Student's *t*-test was used to analyze the statistical difference. $P < 0.05$ was accepted as significant. The data were expressed as mean ± standard deviation and the experiments were repeated three times.

Results

Huaier inhibits cell viability and causes cell morphology change in both MCF-7 and MDA-MB-231 cells. To evaluate the effect of Huaier extract on MCF-7 and MDA-MB-231 breast cancer cells, we measured cell viability using the MTT assay after the cells were dose-dependently treated with Huaier for 24, 48 and 72 h. As shown in Figure 1, Huaier significantly inhibited viability of both MCF-7 (Fig. 1a) and MDA-MB-231 (Fig. 1b) in a time- and dose-dependent manner. Both in the MCF-7 and MDA-MB-231 cells, a sharp decrease in cell viability was present at 8 mg/mL independent of the treatment time. However, MDA-MB-231 cells indicated the cytotoxic effect more evidently at 48 and 72 h. The viability of MDA-MB-231 cells after 4 mg/mL exposure for 72 h decreased by almost 80%, much higher than 42% in the MCF-7 cells.

Morphology change of MCF-7 and MDA-MB-231 cells after treatment with Huaier at 4 mg/mL for 24 and 48 h is shown in Figure 2. Compared with the untreated cells, the majority of the Huaier-treated MCF-7 cells became enlarged, irregular-shaped, spinous and had a vacuolized change of cytoplasm (Fig. 2a), while numerous MDA-MBM-231 cells became extremely elongated and showed the special ''wiredrawing'' morphology (Fig. 2b). These morphology changes demonstrated cell damage after Huaier treatment.

Cell motility decreases due to exposure to Huaier extract. To explore whether or not Huaier extract affected cell motility, invasive, migration and scratch assays were carried out

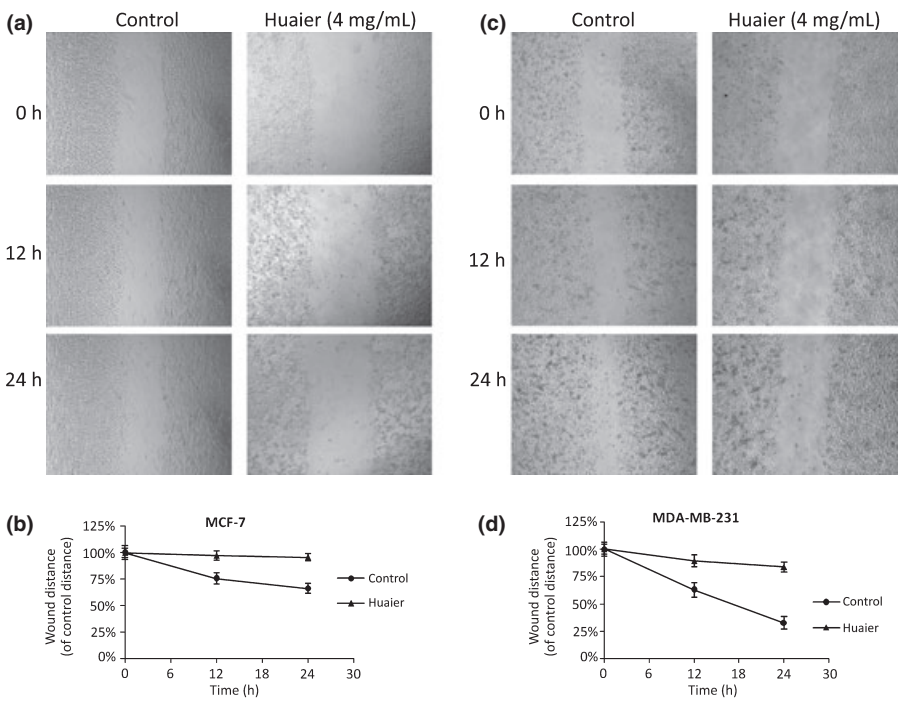

Fig. 4. A scratch assay showed a delayed process of wound healing. (a) Images of untreated and 4 mg/mL Huaier-treated MCF-7 cells were taken at 0, 12 and 24 h. (b) Wound healing was defined as the percentage of the starting distance between the two edges of each wound. The Huaier-treated MCF-7 cells showed a slower rate of wound closure than the control cells. (c) Images of the MDA-MB-231 cells with and without administration of Huaier (4 mg/mL) taken at 0, 12 and 24 h. (d) The distance between the two edges of the scratch in the Huaier-treated well was greater than that of control.

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in vitro. MCF-7 cells were reported as a noninvasive cell line compared with MDA-MB-231,[24] therefore, MCF-7 cells were not applied in the invasive assay mentioned above. *In vitro* invasion assays were conducted to compare the invasive potential of Huaier-treated and -untreated MDA-MB-231 cells. Figure 3a,b revealed that the number of invading cells through the matrigel-coated membrane was significantly greater than that of the cell's counterparts.

Migration and scratch assays *in vitro* were performed to determine the cell migration ability from different aspects, and both of the assays showed consistent results. As indicated in Figure 3c,d, 4 mg/mL Huaier-treated MDA-MB-231 cells showed significantly inhibited migrational movement (39.67 ± 7.02) in comparison with the untreated cells (93.67 ± 6.66; $P = 0.0006$). Similarly, migration of MCF-7 cells was greatly suppressed with the same concentrated Huaier extract (0.67 ± 0.58) compared with the control cells (19.67 ± 4.73; $P = 0.0188$) (Fig. 3e,f).

The scratch assay is displayed in Figure 4; a straight wound area was generated in each culture well at the beginning and administration of Huaier (4 mg/mL) showing a delayed process of wound closure compared with the untreated cells.

Huaier treatment induces cell-cycle arrest in MCF-7 cells but not in MDA-MB-231 cells. Cell-cycle distribution of Huaier-treated MCF-7 and MDA-MB-231 cells was analyzed by flow cytometry, aiming to determine whether the inhibitory effect was due to cell-cycle arrest. Before being processed and analyzed, both kinds of cells were exposed to Huaier for a total of 24 or 48 h. As shown in Figure 5, MCF-7 cells exposed to Huaier extract showed G0/G1 arrest by increasing the fraction of the G0/G1 phase, as compared with that of the untreated cells. The exposure of Huaier also indicated a concomitant decrease in the fraction of the S phase. These results revealed that Huaier could hold up MCF-7 cell proliferation via cell-cycle arrest at the G0/G1 phase. Nevertheless, the distribution of Huaier-treated MDA-MB-231 cells, as assessed by flow cytometry, was similar to the untreated control cells (data not shown).

Cell apoptosis analyzed by PI-Annexin-V staining and MMP assay. Detection between the intact cells, early apoptotic cells and late apoptotic cells or dead cells could be carried out with PI-annexin-V double staining[25]; thus, we performed this assay to further explore cell apoptosis. Figure 6 and Table 1 show that after the Huaier treatment, late apoptosis or cell death rate and the early apoptosis rate were increased in a time- and dose-dependent manner in both MCF-7 and MDA-MB-231 cells. To confirm caspase activation during Huaier-induced apoptosis, we also investigated the effect of pan-caspase inhibitor, Z-VAD-fmk, on the induction of apoptosis. As expected, the inhibitor decreased Huaier-induced apoptosis in both MCF-7 and MDA-MB-231 cells.

Apoptosis is associated with a loss of MMP[26]; therefore, we used rhodamine 123 to examine the loss of MMP. Figure 7 showed that Huaier treatment resulted in a greater decrease in MFI in a time- and dose-dependent manner compared with the control group, indicating that Huaier has a considerable effect on mitochondria and causes apoptosis in both cell lines.

Mechanisms of cell cycle arrest and apoptosis after Huaier treatment. Since activation of p53 can lead to either cell cycle arrest and DNA repair or apoptosis, we tested the expression of p53 and phosphorylated-p53 (p-p53) with western blot. As shown in Figure 8a, the expression of p53 and p-p53 were up-regulated in a time- and dose-dependent manner, indicating p53 accumulation and activation in response to Huaier treat-

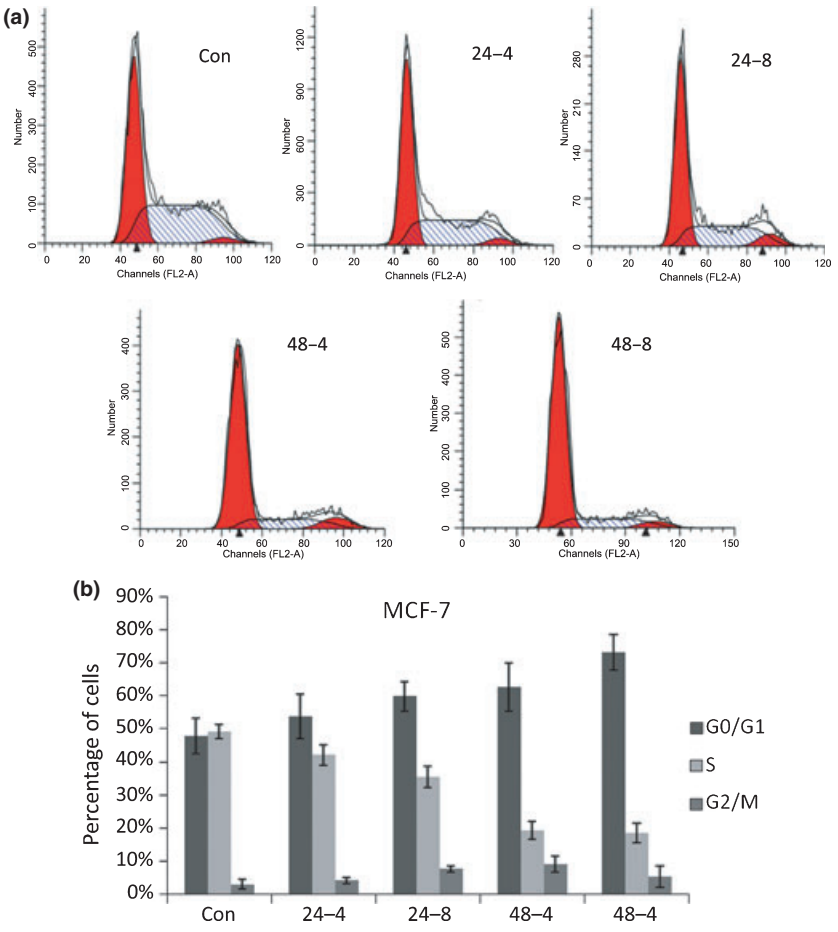

Fig. 5. Arrest of cell-cycle progression at G0/G1 in response to Huaier treatment. (a) Huaier-induced G0/G1 arrest in MCF-7 cells. (b) The distribution of the cell cycle of MCF-7 cells was assessed by flow cytometry after staining with propidium iodide (PI). The data presented are the mean ± SD of three independent experiments. con, control.

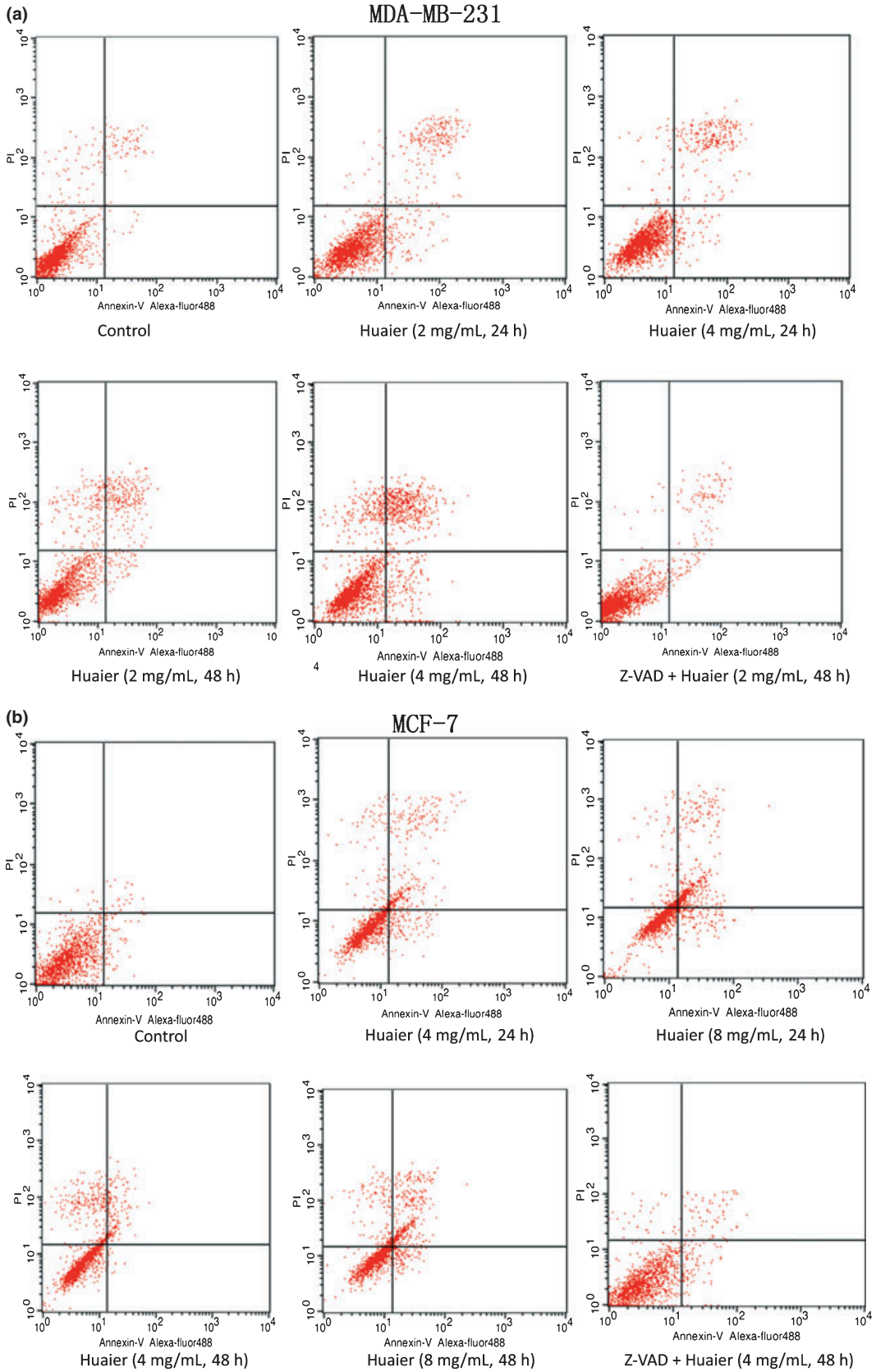

Fig. 6. Flow cytometric analysis of PI-Annexin-V to quantify Huaier-induced apoptosis in MCF-7 and MDA-MB-231 cells. (a) Dot plots of MDA-MB-231 cells with Huaier treatment at 0, 2 and 4 mg/mL for 24 or 48 h. Z-VAD-fmk was added to the culture medium 1 h before the treatment with 2 mg/mL 48 h Huaier extract. (b) Dot plots of MDA-MB-231 cells with Huaier treatment at 0, 4 and 8 mg/mL for 24 or 48 h. Z-VAD-fmk was added to the culture medium 1 h before the treatment with 4 mg/mL 48 h Huaier extract. The results shown are representative of three independent experiments.

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Table 1. Percentage of quadrant distribution (QD) in PI-Annexin-V staining apoptosis assay

QD	Control	24–2	24–4	48–2	48–4	Z–vad+48–2
MDA-MB-231 cell line						
UL	2.62 ± 0.10	0.91 ± 0.05	4.08 ± 3.40	6.78 ± 0.21	8.45 ± 1.87	0.43 ± 1.01
UR	2.41 ± 0.38	8.18 ± 0.52	8.84 ± 0.44	10.64 ± 0.31	14.22 ± 3.50	3.21 ± 0.29
LL	94.71 ± 0.57	87.23 ± 0.75	84.41 ± 0.61	80.84 ± 0.77	72.02 ± 6.25	94.47 ± 0.18
LR	0.26 ± 0.11	3.67 ± 0.45	3.51 ± 0.22	1.74 ± 0.25	5.30 ± 1.32	1.89 ± 0.13
QD	Control	24–4	24–8	48–4	48–8	Z–vad+48–4
MCF-7 cell line						
UL	0.91 ± 0.20	8.06 ± 0.73	9.77 ± 0.60	14.34 ± 0.31	9.94 ± 0.19	2.16 ± 0.17
UR	1.49 ± 0.19	11.89 ± 2.63	21.47 ± 0.25	6.47 ± 0.17	19.28 ± 0.25	2.99 ± 0.18
LL	93.11 ± 0.07	75.83 ± 3.34	63.54 ± 0.47	78.45 ± 0.13	65.05 ± 0.83	90.48 ± 0.11
LR	4.49 ± 0.34	4.23 ± 1.36	5.22 ± 0.18	0.75 ± 0.05	5.73 ± 0.42	4.36 ± 0.33

The data presented are the mean ± SD of three independent experiments. LL, Lower Left; LR, Lower Right; UL, Upper Left; UR, Upper Right.

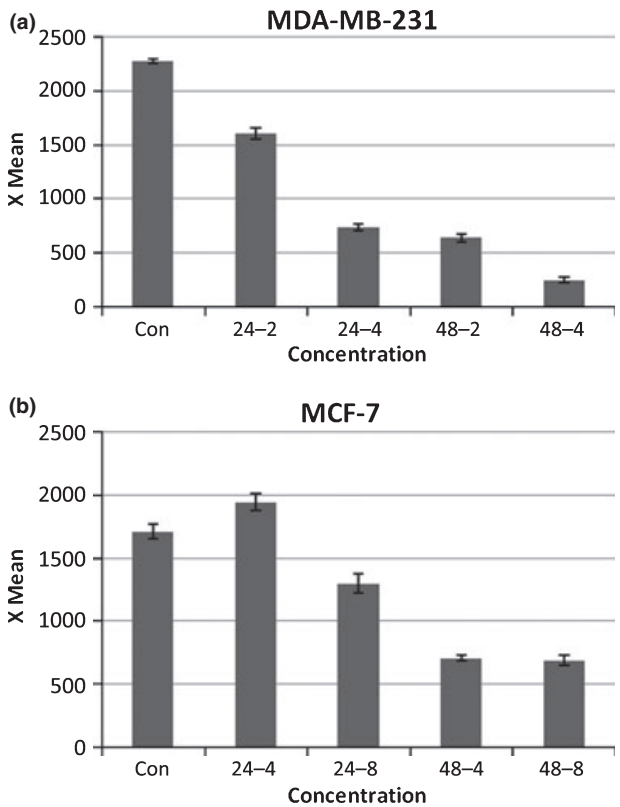

Fig. 7. Mitochondrial membrane potential (MMP) analysis by rhodamine 123 assays. After treatment of the series with Huaier extract, the MMP of both (a) MDA-MB-231 and (b) MCF-7 cells decreased in a time- and dose-dependent manner. The mean fluorescence intensity is shown on each graph and presented as mean ± SD of three independent experiments. con, control.

ment in MCF-7 cells. However, no obvious accumulation and activation of p53 were observed in MDA-MB-231 cells (Fig. 8b).

To further investigate the mechanisms of Huaier causing apoptosis, the expression of Bcl-2, BAX, pro-caspase-3 and cleavage caspase-3 was measured by western blot. As indicated in Figure 8, the MCF-7 cell line was Bcl-2 high-expressed but the MDA-MB-231 cell line was low. Treatment with Huaier could suppress the Bcl-2 expression and up-regulate BAX expression in a time- and dose-dependent manner. Thus, the ratio of Bcl-2 to BAX was decreased, leading to mitochondrial-mediated apoptosis.

For evaluation of the executor pathway of Huaier-induced apoptosis, pro-caspase-3 and cleavaged caspase-3 were also measured by western blot. As shown in Figure 8c,d, activation of caspase-3 was significantly increased in both MCF-7 and MDA-MB-231 cells after Huaier treatment, resulting in increased expression of cleavage caspase-3 and decreased expression of pro-caspase-3.

Discussion

Recently, TCM has become a rich source for finding new drugs, and more and more TCM are found to be able to induce apoptosis.[27] In our study, we found that the Huaier aqueous extract could inhibit cell viability and mobility, and cause cell morphology changes as well. The MTT assays showed that the viability inhibitory effect was represented in a time- and dose-dependent manner; Huaier showed a more evident cytotoxic effect on MDA-MB-231 than MCF-7 cells. In addition, some typical morphology changes were observed after treatment with Huaier, indicating cell damage. Our results also demonstrated that Huaier acted as an inducer to arrest the MCF-7 cell cycle at the G0/G1 phase via p53 activation, but not in MDA-MB-231 cells. The p53 tumor suppressor protein plays a major role in the cellular response to DNA damage and other genomic aberrations. Activation of p53 can lead to cell-cycle arrest or apoptosis. Phosphorylated-p53 is one of the p53 activation forms.[28] Thus, the up-regulation of p53 and p-p53 can explain cell-cycle arrest in the G0/G1 phase in MCF-7 cells, in which p53 is a wild type. However, in MDA-MB-231 cells, p53 is in mutant form and was dysfunctional.[29]

Generally, breast cancer cells can be divided into two categories according to their estrogen receptor (ER) status; the MCF-7 cell line is ER-positive and the MDA-MB-231 cell line is ER-negative.[30] The former breast cancer cell line usually has a better prognosis,[31] whereas approximately one-third of breast cancers are ER-negative with a worse prognosis.[32] Moreover, MCF-7 cells were considered as a noninvasive cell line compared with the moderately invasive MDA-MB-231 cells.[24] Therefore, finding new agents that are effective in both ER-positive and ER-negative breast cancers is important. Inspiringly, the PI-Annexin-V staining assay and western blot analysis confirmed cell apoptosis executed by caspase-3 in both cell lines.

Apoptosis induced by some anticancer agents constitutes one aspect of treatment effect. Two major pathways involved in the process have been investigated in great depth. 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. Our results in the MMP assay also

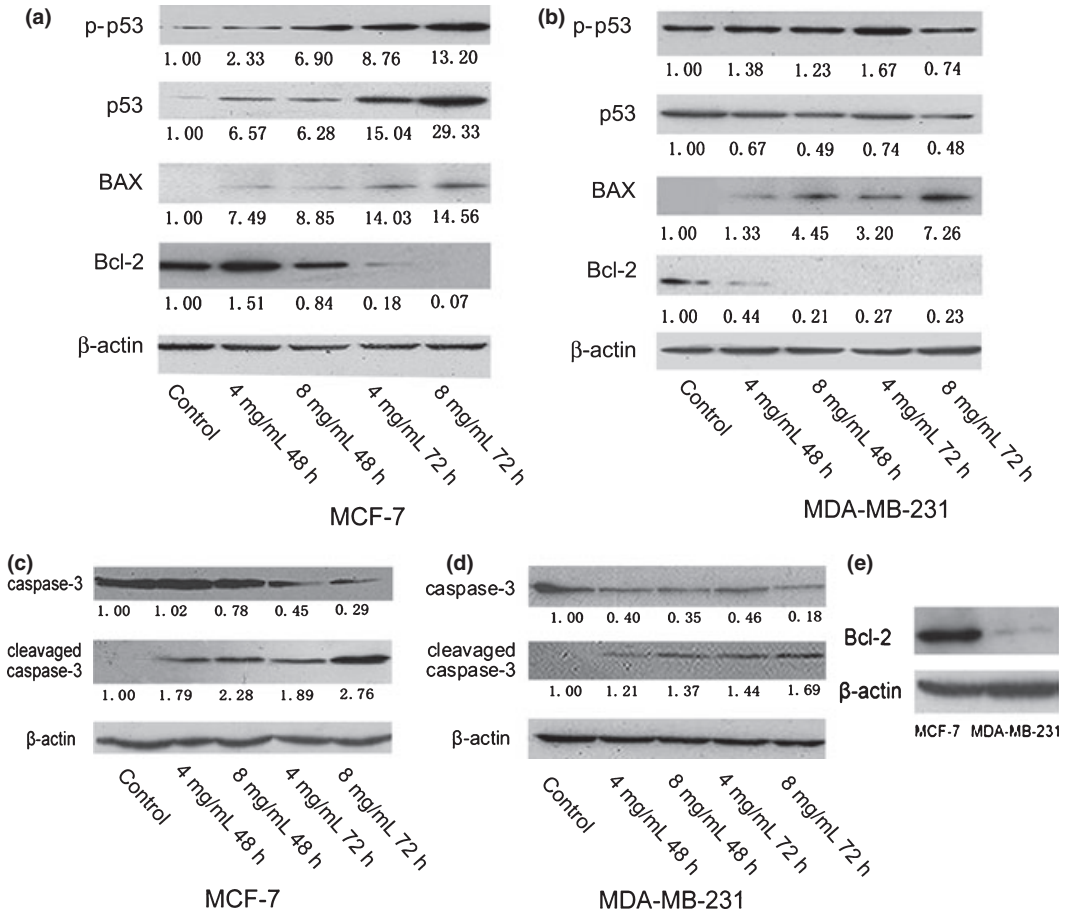

Fig. 8. Effects of Huaier extract on expression of p53, phosphorylated p53 (p-p53), Bcl-2, BCL2-associated X protein (BAX) and caspase-3 proteins. Expression of β-actin was used as an internal control. (a,c) MCF-7 cells and (b,d) MDA-MB-231 cells were treated with 4 mg/mL for 24 or 48 h and 8 mg/mL for 24 or 48 h. The expression of each protein was quantified as the densitometry value analyzed by ImageJ. (e) Different expression of Bcl-2 in MCF-7 and MDA-MB-231 cells. The figure shown is representative of three independent experiments.

confirmed decreased MMP on Huaier-induced apoptosis. In the mitochondrial pathway, Bcl-2 family members shoulder the responsibility of regulation of apoptosis in different situations, including the anti-apoptotic Bcl-2 protein and pro-apoptotic BAX protein.[33] As for the two distinctive cell lines applied in this study, ER-positive MCF-7 cells showed Bcl-2 high-expressed, but in ER-negative MDA-MB-231 cells the Bcl-2 expression level is very low. This result is in agreement with a previous investigation[34] and verified that the Bcl-2 expression displayed a positive correlation with ER status.[35] Furthermore, it is not hard to infer the reason for the viability difference between the two cell lines after the same treatment, because the ER-positive cells have an excess of anti-apoptotic Bcl-2 protein, which assists in its resistance to anticancer drugs.[36–38]

Given that Huaier aqueous extract may play a novel role as a complementary medicine in breast cancer treatment, especially in triple-negative and advanced breast cancer, future research on its anticancer mechanisms needs to be done. Additionally, we should also consider the interaction between Huaier extract and routine conventional therapies, such as chemotherapy, endocrine therapy and radiation.

In summary, our results showed that extract of Huaier could inhibit cell proliferation by inducing apoptosis and cell-cycle arrest in breast cancer cells. These results contribute to the understanding of the anticancer activity of Huaier. Recently, combination treatment with Huaier extract for breast cancer has already been under investigation and we look forward to the results from the clinical trial with great interest.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. The chemical composition of proteoglycan extracted from Huaier.

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