

Growth inhibition and apoptotic effects of total flavonoids from *Trollius chinensis* on human breast cancer MCF-7 cells

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Abstract. Dried flowers of *Trollius chinensis* have long been used as an important traditional Chinese medicine. Previous studies have demonstrated the ability of *T. chinensis* flavonoids to reduce the proliferation of human breast cancer MCF-7 cells. The present study further investigated the influence of *T. chinensis* flavonoids on the growth and proliferation of MCF-7 cells and observed clear inhibitory effects within the concentration range of 0.0991-1.5856 mg/ml. Apoptosis was triggered by *T. chinensis* flavonoids treatment that was evaluated by differential interference contrast software, the Hoechst 33258 method, scanning electron microscopy, hematoxylin/eosin staining and laser confocal light microscopy. Cells treated with *T. chinensis* flavonoids selectively reduced bcl-2 and NF- κ B expression and increased the expression of caspase-9 and caspase-3 indicating that the inhibition of cellular proliferation occurred through activation of a mitochondrial pathway. Taken together, the results confirmed the ability of *T. chinensis* flavonoids to inhibit cell proliferation.

Introduction

Breast cancer is the most common type of cancer in women. Over the past 30 years, the morbidity of breast cancer has increased at a rate of 3% each year in china (1). Surgery combined with chemotherapy and radiotherapy is the main therapeutic approach for this disease, but severe side effects occur in the course of these treatments. Therefore, effective antitumor therapeutic drugs with few side effects need to be developed.

The dried flowers of *Trollius chinensis* is used as a common traditional Chinese medicine. Modern pharmacological studies have shown that it possesses antimicrobial, antiviral (2),

anti-oxidative and anti-tumor activities and has been used widely to treat chronic tonsillitis and upper respiratory infections in clinical therapy (3).

Flavonoids as the major constituents of *T. chinensis* (4) possess antimicrobial, anti-inflammatory, hypotensive, antiviral, spasmolysis and antioxidant effects (*in vitro* and *in vivo*) (5-9). Currently, *T. chinensis* flavonoids are used for their antibacterial and antioxidant properties (10,11). A preliminary study demonstrated the strong inhibitory effects of *T. chinensis* flavonoids in K562, HeLa, EC-109 and NCI-H446 cells (12). *T. chinensis* flavonoids were also demonstrated to inhibit the proliferation of A549 cells in a dose-dependent manner, and the effect was associated with the expression of apoptosis-related genes (13). Another previous study showed the ability of *T. chinensis* flavonoids to dose-dependently inhibit the proliferation of MCF-7 cells, and telomerase activity decreased progressively with increasing drug concentrations (14); however, cellular apoptosis has not been verified from a morphological perspective, and the mechanism of action has not been clarified. Therefore, the current study was conducted to examine apoptosis induced by *T. chinensis* flavonoids in MCF-7 cells by different methods, including MTT assay, differential interference contrast (DIC), Hoechst staining, scanning electron microscopy, hematoxylin and eosin (HE) staining, Annexin-V/propidium iodide (PI) double-labeling and western blot analysis, in order to clarify the underlying biochemical mechanisms and facilitate clinical anti-tumor drug development.

Materials and methods

Drugs and reagents. 3-(4,5)-dimethylthiazolium-z-yl0-3,5-diphenyltetrazoliumromide (MTT) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-FITC/PI was manufactured by Beijing Zhaungmeng Science and Technology Limited Company (Beijing, China). Hoechst 33258 dye was obtained from Sigma-Aldrich. Total flavonoids were dissolved in DMSO and diluted with culture media immediately prior to use. Primary and secondary antibodies were manufactured by Beijing Boaosen Biotechnology Limited Company (Beijing, China). The *T. chinensis* flavonoids were prepared as previously described (15) with a purity of ~68%.

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Cell culture and experimental groups. MCF-7 breast cancer cells maintained at the Medical Genetics Department of

Beijing Cancer Institute were cultured in RPMI-1640 (Gibco, ThermoFisher Scientific, Inc., Waltham, MA, USA) medium supplemented with 10% fetal bovine serum (FBS), 100 μ M/ml gentamicin at 37°C in an atmosphere with 5% CO₂. Cells were subcultured and passaged at ~70-80% confluency. Cells in the logarithmic phase were used in all experiments. Total flavonoids were extracted from *T. chinensis*. Stock solutions, kept at -20°C, were diluted to the final concentrations with culture medium prior to use. The final DMSO concentration was below 0.2% in all experiments.

Growth inhibition assay. The effect of total flavonoids on cell growth was evaluated by using an MTT assay. Briefly, cells were seeded at 10⁵ cells/ml in 96-well plates for the assay. After culturing with flavonoids at different concentrations (0, 0.0991, 0.1982, 0.3964, 0.7928 or 1.5856 mg/ml) for various times (24, 48 or 72 h), 20 μ l of MTT was added to each well. Plates were incubated for another 4 h at 37°C, and then the medium was aspirated by pipetting and replaced with 100 μ l/well DMSO in order to dissolve the formazan produced by viable cells exposed to MTT. The absorbance reflecting the cell growth was measured at 490 nm with a microplate reader. All data were obtained from 4 independent experiments and expressed as the mean \pm standard deviation.

DIC microscopy and Hoechst33258 staining. Morphological changes were detected with Hoechst dye staining. Cells were seeded (10⁵ cells/ml) on coverslips overnight and then treated with flavonoids at different concentrations (0, 0.0991, 0.1982, 0.3964, 0.7928 or 1.5856 mg/ml) for 24 h. Thereafter, the coverslips were washed with PBS and fixed with 4% paraformaldehyde for 1 h. After washing the cells with PBS, images were captured with DIC software (Nikon, Tokyo, Japan). The cells on the coverslips were incubated with Hoechst 33258 staining solution for 30 min, washed 3 times for 2 min with PBS and mounted on microscope slides. A fluorescence microscope (Nikon) was used to capture images using an excitation wavelength of 340 nm. The entire experiment was performed at room temperature.

Scanning electron microscopy. Scanning electron microscopy was used for observation of morphological changes in apoptotic cells. MCF-7 cells were seeded in a 24-well plate at the density of 10⁵ cells/ml. Overnight, cells were treated with culture medium or total flavonoids at 0, 0.0991, 0.1982, 0.3964, 0.7928 and 1.5856 mg/ml. A total of 24 h later, the supernatant was discarded, and the cells were washed with PBS, fixed with glutaraldehyde for 24 h, washed 3 times with PBS for 1 min and then dehydrated in a gradient of alcohol solutions for 1 min at each concentration. Finally, the cells were soaked in tert-butyl alcohol twice for 10 min each time, dried with a vacuum pump for 24 h and metal spray-coated for 90 sec. The prepared specimen was observed under a scanning electron microscope.

HE staining. MCF-7 cells were seeded in a 6-well plate at 3x10⁵ cells/well in RPMI-1640 with 10% FBS. After 24 h, cells were treated with flavonoids at a concentration of 0, 0.0991, 0.1982, 0.3964, 0.7928 or 1.5856 mg/ml. Untreated cells served as the control group. After 24 h, supernatants were

discarded, and the cells were fixed with 1% paraformaldehyde for 24 h. After washing with PBS twice, cells were stained with hematoxylin for 15 min, differentiated in hydrochloric acid alcohol for 10 sec, treated with ammonium hydroxide for 20 sec and stained with eosin for 10 min, with rinses under running water after each step. Subsequently, the cells were subjected to gradient alcohol dehydration, and dimethylbenzene was used for rendering the slides transparent. Finally, the cells were mounted with resin and observed under a light microscope.

Laser scanning confocal microscopy. Laser scanning confocal microscopy was used for observation of cell morphological changes during apoptosis. MCF-7 cells were seeded in 6-well plates in RPMI-1640 media containing 10% FBS. After 24 h, the cells were exposed to flavonoids at concentrations of 0, 0.0991, 0.1982, 0.3964, 0.7928 and 1.5856 mg/ml for 24 h. The supernatant was discarded, and the cells were washed 3 times with PBS for 2 min each time. After incubation with 5 μ l Annexin V and 10 μ l PI for 10 min in the dark, cells were washed with PBS for 2 min and images were immediately captured under a laser scanning confocal microscope.

Western blot analysis. MCF-7 cells grown in RPMI medium with 10% FBS were treated with flavonoids at different concentrations. After 24 h, cells were harvested and disrupted in RIPA lysis buffer to extract total cellular proteins. The protein content was determined with a BAC protein determination kit (Wuhan Boshide Bio-engineering Limited Company, Wuhan, China). Total cellular proteins (50 μ g) were separated in 15% SDS-PAGE gels and transferred into PVDF membranes (Sigma-Aldrich) with a wet transfer system (Bio-Rad Laboratories). Membranes were blocked in 5% non-fat milk for 1 h at room temperature with 5% fat-free milk dissolved in PBST buffer. Thereafter, the membranes were probed with β -actin (cat. no. ZA109; Beijing Zoman Biotechnology Co., Ltd., Beijing, China), bcl-2 (cat. no. bs-0032R; BIOSS, Beijing, China), FasL (cat. no. bs-0216R; BIOSS), caspase-3 (cat. no. ZA135; Beijing Zoman Biotechnology Co., Ltd.), caspase-9 (cat. no. ZA137; Beijing Zoman Biotechnology Co., Ltd.), p53 (cat. no. ZA120; Beijing Zoman Biotechnology Co., Ltd.) and NF- κ B (cat. no. ZA131; Beijing Zoman Biotechnology Co., Ltd.) primary antibodies in TBST buffer containing 0.1% Tween-20 at 1:1,000 dilution for 24 h at 4°C, followed by exposure to horseradish peroxidase-conjugated secondary antibodies (Beijing Boasens Corporation, Beijing, China) for 90 min at temperature. The protein levels were visualized using a Western blot detection system, and immunoreactivities were detected with an enhanced colorimetric detection kit (Amplified Alkaline Phosphatase Goat Anti-Rabbit Immun-Blot Assay Kit, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Results

Effect of flavonoids of *T. chinensis* on MCF-7 cell viability. An MTT assay was performed to examine the effect of total flavonoids on cell growth. MCF-7 cells were treated with different concentrations (0, 0.0991, 0.1982, 0.3964, 0.7928 and 1.5856 mg/ml) of total flavonoids for 24, 48 and 72 h. As

Table I. Inhibition effect of *T. chinensis* flavonoids on MCF-7 cells detected by MTT method.

Concentration (mg/ml)	Cell growth inhibition rate by <i>T. chinensis</i> flavonoids (%)			
	0 h	24 h	48 h	72 h
0.0991	0	3.62±0.23 ^{a,d}	5.14±0.11 ^{a,e}	8.75±0.56 ^{a,f}
0.1982	0	24.8±0.55 ^{b,d}	27.61±0.54 ^{b,e}	31.76±0.69 ^{b,f}
0.3964	0	35.72±0.72 ^{c,d}	46.52±0.75 ^{c,e}	50.01±0.84 ^{c,f}
0.7928	0	48.03±1.34 ^{c,d}	73.49±1.51 ^{c,e}	77.92±1.61 ^{c,f}
1.5856	0	53.8±1.28 ^{c,d}	76.76±1.83 ^{c,e}	81.31±1.86 ^{c,f}

^aP<0.05, ^bP<0.01 and ^cP<0.001 for drug groups at different concentrations compared with control group, at the same time point. ^dP<0.05, ^eP<0.01 and ^fP<0.001 for different time points compared with control group, at the same drug concentration.

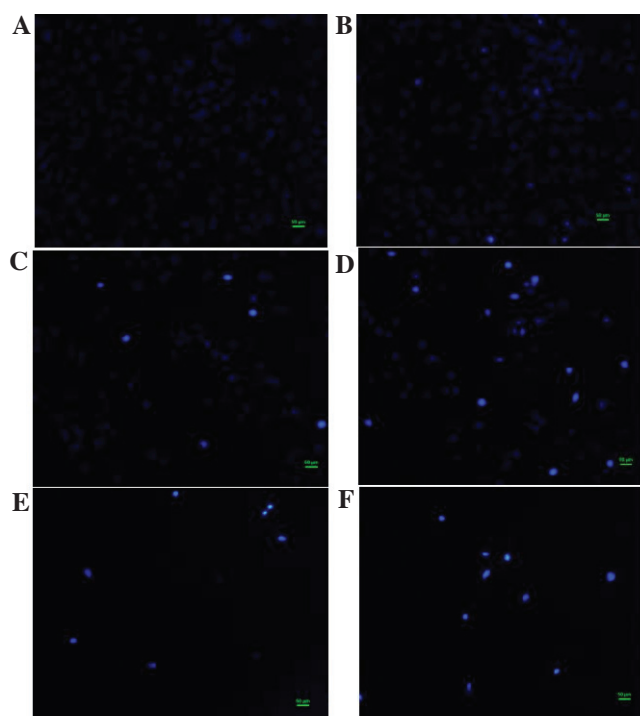


Figure 1. The apoptosis and junction changes between MCF-7 cells detected using differential interference contrast software (x400 magnification). (A) Control group, (B) 0.0991, (C) 0.1982, (D) 0.3964, (E) 0.7928 and (F) 1.5856 mg/ml *T. chinensis* flavonoids.

presented in Fig. 1, the survival of MCF-7 cells reduced in a time-dependent and dose-dependent manner. At 72 h, the maximum inhibition ratio reached up to 81.31% at the drug concentration of 1.5856 mg/ml (Table I).

DIC microscopy analysis. In the control group, normal fusiform-shaped cells were connected and radiated outward, adhering tightly to the cover glass. With a low concentration of flavonoids (0.0991 mg/ml), the shape of cells started to change. Upon exposure to increased flavonoid concentrations (0.1982 and 0.3964 mg/ml), the cells rounded up with loosened cell junctions and appeared to be vacuolated. At high concentrations of flavonoids (0.7928 and 1.5856 mg/ml), fractured junctions, swelled nuclei, plasmolysis and a surge in the number of desquamated cells was observed (Fig. 1).

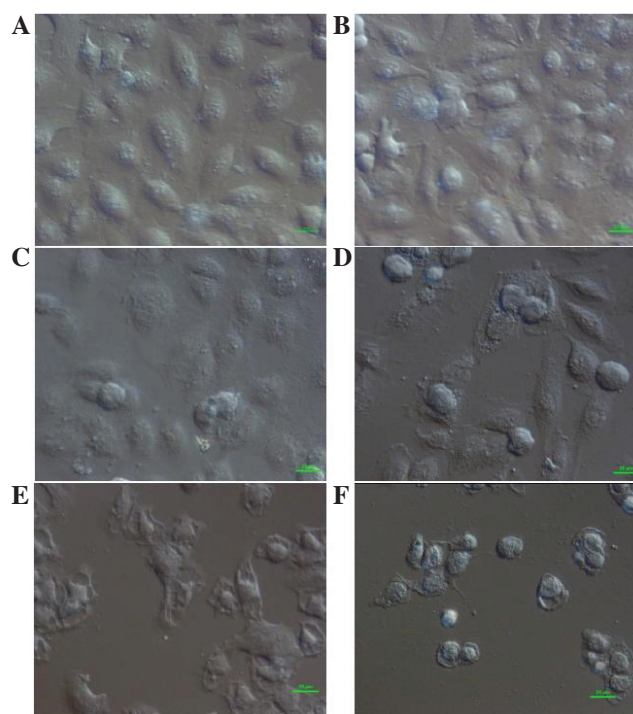


Figure 2. The effect of *T. chinensis* flavonoids on MCF-7 cell morphology detected by Hoechst 33258 method. (A) Control group, (B) 0.0991, (C) 0.1982, (D) 0.3964, (E) 0.7928 and (F) 1.5856 mg/ml *T. chinensis* flavonoids.

Effect of flavonoids of *T. chinensis* on nuclear morphology of MCF-7 cells. To examine the cell death induced by total flavonoids, the nuclear morphology of dying MCF-7 cells stained with Hoechst 33258 dye was observed. In the control group, the nuclei of cells had a regular shape and showed a uniform distribution of low-density fluorescence. In the group exposed to a low concentration of flavonoids (0.0991 mg/ml), the nuclei of a proportion cells appeared darker than those of normal cells, indicating apoptotic cell death. In groups treated with flavonoids in the middle of the concentration range (0.1982 and 0.3964 mg/ml), cell numbers gradually decreased, while the proportion of cells with hyperchromatic nuclei increased. With high concentrations of flavonoids (0.7928 and 1.5856 mg/ml), MCF-7 cell numbers fell sharply, fragmented nuclei appeared and typical apoptotic characteristics became more apparent (Fig. 2).

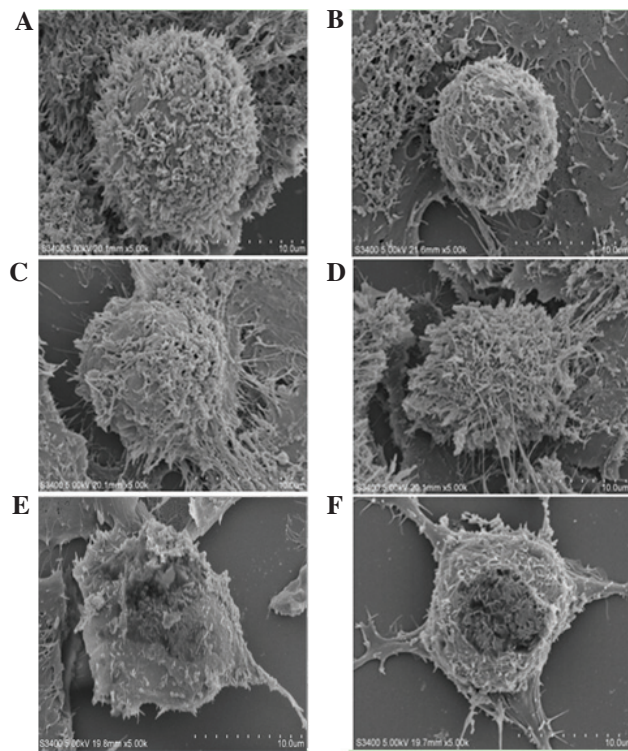


Figure 3. The apoptotic morphological changes of MCF-7 cells detected by scanning electron microscope. (A) Control group, (B) 0.0991, (C) 0.1982, (D) 0.3964, (E) 0.7928 and (F) 1.5856 mg/ml *T. chinensis* flavanoids.

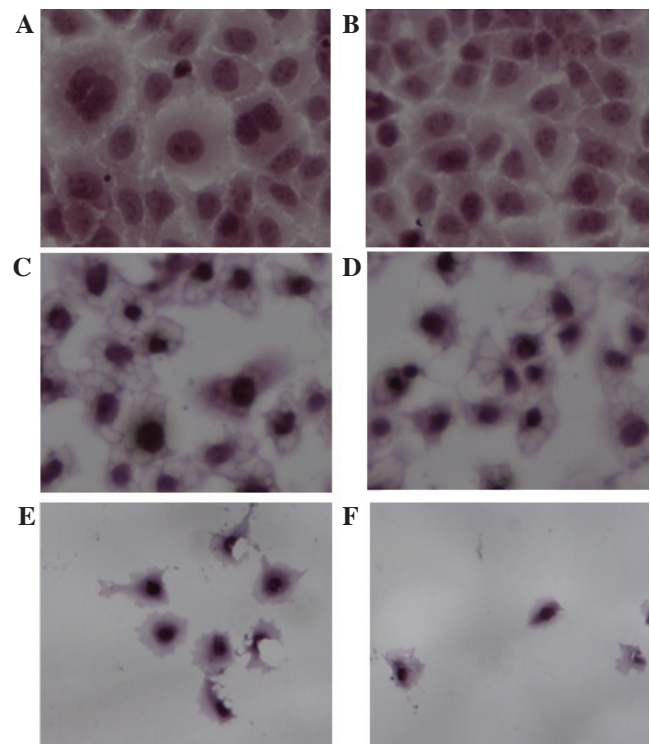


Figure 4. The nuclear and cytoplasmic changes of MCF-7 cells detected by hematoxylin and eosin method (x400, magnification). (A) Control group, (B) 0.0991, (C) 0.1982, (D) 0.3964, (E) 0.7928 and (F) 1.5856 mg/ml *T. chinensis* flavanoids.

Scanning electron microscopy analysis. MCF-7 cells in the control group were firmly adherent and covered with abundant microvilli. Those cells were connected tightly with neighboring cells and extended in all directions. In the low flavonoid concentration group (0.0991 mg/ml), cells with gap junctions shrank and showed decreased surface microvilli. In the middle concentration group (0.1982 and 0.3964 mg/ml), characteristics of apoptosis became marked. With high concentrations of flavonoids (0.7928 and 1.5856 mg/ml), the microvilli nearly completely disappeared, and cell membranes collapsed (Fig. 3).

HE staining analysis. In the control group, cells were in good condition and able to grow adhering to the cover glass. The nucleoplasm was uniformly stained, and mitoses could be observed. The nucleolus and nuclear membrane profiles also could be seen. At a low concentration (0.0991 mg/ml), treatment with total flavonoids halted cell division. With flavonoids in the middle range of concentrations (0.1982 and 0.3964 mg/ml), intercellular spaces dilated. However, cells with condensed cytoplasm maintained their plasma membrane integrity. The cells shrank and appeared increasingly deformed or dehydrated. At high concentrations of flavonoids (0.7928 and 1.5856 mg/ml), adherent cells were drastically reduced in number and cells were no longer intact. Cells treated with high flavonoid concentrations (0.7928 and 1.5856 mg/ml) were more hyperchromatic than the cells of the middle concentration group (0.1982 and 0.3964 mg/ml). The HE result revealed shows swollen nucleoli and fractured junctions, which was in accordance with what DIC and scanning electron microscopy results demonstrated (Fig. 4).

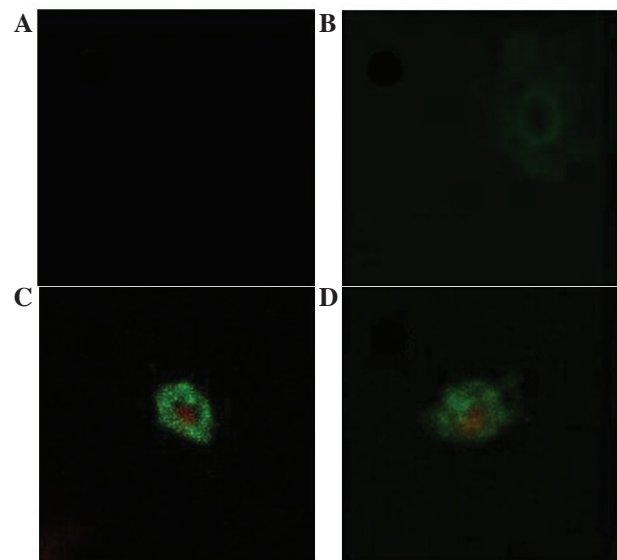


Figure 5. The apoptotic morphological change of MCF-7 cells detected by laser confocal microscopy. (A) Control group, (B) 0.0991, (C) 0.1982, (D) 0.3964 mg/ml *T. chinensis* flavanoids.

Detection of apoptosis by Annexin V-FITC/PI staining. In the control group, MCF-7 cells were not readily stained by Annexin V or PI. In the low flavonoid concentration group (0.0991 mg/ml), cells in the early stage of apoptosis were seen. Plasma cell membranes labeled green by Annexin V appeared gradually. In the middle concentration range of flavonoids (0.1982 and 0.3964 mg/ml), the integrity of the cytomembrane was no longer intact and stained green, while the nucleus was dyed red. In the high flavonoid concentration

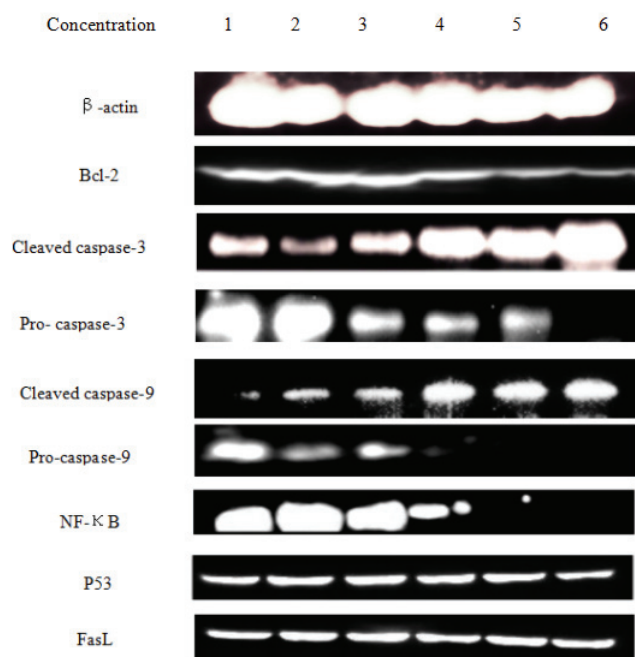


Figure 6. The expression of protein in MCF-7 cells treated with *T. chinensis* flavonoids detected using western blot analysis. 1, control group; 2, 0.0991 mg/ml; 3, 0.1982 mg/ml; 4, 0.3964 mg/ml; 5, 0.7928 mg/ml; 6, 1.5856 mg/ml.

group (0.7928 and 1.5856 mg/ml), both the cytomembrane and nucleus were fragmented (Fig. 5).

Effect of *T. chinensis* flavonoids on levels of apoptosis-associated proteins. In order to study the mechanism of flavonoid-induced apoptosis in MCF-7 cells, the expression of caspases, fasL, p53 and bcl-2 were determined. After 24 h of exposure to flavonoids, the expression of cleaved caspase-3 and caspase-9 increased in a dose-dependent manner. Bcl-2 is a crucial determinant of cell apoptosis, and a decrease in its expression was observed in the flavonoid-treated cells. This observation suggests that an intrinsic apoptotic signaling contributed to the damaging effects of flavonoids on MCF-7 cells. Total flavonoids also down-regulated the expression of NF- κ B, which in turn could down-regulate the expression of bcl-2. By contrast, the expression levels of p53 and fasL were not markedly changed. These findings suggest that induction of the mitochondrial pathway is crucial for flavonoid-induced apoptosis of MCF-7 cells (Fig. 6).

Discussion

In the present study, *T. chinensis* flavonoids were shown to clearly inhibit the growth and proliferation of MCF-7 cells within a certain range of concentrations (0.0991-1.5856 mg/ml) by the MTT assay. The inhibitory effect increased as the concentration or treatment time of flavonoids increased. Although the inhibition of MCF-7 cells increased with time (24 h \leq 48 h \leq 72 h) when treated with flavonoids at the same concentration, a direct proportional relationship between the inhibition ratio and time was not observed. According to previous evaluation criterion (16), the effect is weak when inhibition rate is $<$ 30%; The effect is strong when inhibition rate is $>$ 50%. And the effect is modest when inhibition rate is between 30 and 50%. In the present study, the inhibition rate was 58.3%

when the concentration was 1.5856 mg/ml at 24 h. The effect may therefore be described as strong and the typical apoptosis characteristics were apparent at 24 h, therefore 24 h was selected as an appropriate time-point for further analyses.

The findings of the present study are in line with those of Sun *et al* (14) reporting that total flavonoids in *T. chinensis* could inhibit the proliferation of MCF-7 cells. This finding was confirmed in the present study with additional morphological observations and the corresponding mechanism was investigated.

The DIC results demonstrated that particles appeared on the surface of cells treated with drugs. 'Bubble up' demonstrated that cytoskeletal structure was damaged. Ji *et al* (17) observed the morphological changes in MCF-7 and MDA-MB-231 cells treated with Huaier Granule (a traditional Chinese medicine) by DIC software, and observed morphological changes induced by flavonoids that were indicative of apoptosis in MCF-7 cells.

The Hoechst results demonstrated that the nuclei of cells treated with drugs appeared darker than that of normal cells and fragmented nuclei appeared. Wang *et al* (18) studied the apoptotic effect of genistein on breast cancer cells by the Hoechst 33258 method and similarly to the present study, the authors observed that MCF-7 cells treated with drugs shows apoptotic morphological characteristics.

The scanning electron microscope results indicated that the microvilli on the surface of cells treated with drug of different concentrations were reduced. In the high dose group (0.7982, 1.5856 mg/ml), cell membrane collapse was observed, appearing as a hole-like structure. The disruption of the cell membrane indicates cells necrosis. The typical apoptosis characteristics observed in the present study are in line with what Li *et al* (19) observed, in a previous study on the apoptosis of breast cancer cells induced by Herceptin. Therefore, flavonoids could induce the apoptosis of MCF-7 cells.

The HE results indicated that in the low and middle flavonoid dose group (0.0991, 0.1982, 0.3964 mg/ml), there were shrunken nuclei of cells dyed black-blue and intercellular gap junctions were fractured, indicating the apoptosis of cells. In the high dose group (0.7928, 1.5856 mg/ml), the cells were no longer intact and the cell membrane was disrupted indicating cell necrosis. The typical apoptosis characteristics that were observed using HE methods were in accordance with what Li observed (20), in a previous study investigating the apoptosis of lung adenocarcinoma A549 cells induced by soy isoflavone and vinorelbine. These results provide further evidence that flavonoids may induce apoptosis in MCF-7 cells.

The results of laser confocal microscopy indicated that *T. chinensis* flavonoids at the concentration of 0.0991, 0.1982 and 0.3964 mg/ml inhibit MCF-7 cell proliferation by inducing the apoptosis of cells. However, *T. chinensis* flavonoids at the concentration of 0.7982 and 1.5856 mg/ml inhibit the proliferation of MCF-7 cells by inducing the necrosis of cells.

The abovementioned results reveal that morphological changes in MCF-7 cells occur via multiple pathways and from multiple perspectives, leading to the apoptosis of cells. Cell apoptosis is affected by precise control and interaction of regulatory factors, and may be activated by extracellular and intracellular stimulation. Although the apoptotic signals of cells were different, the apoptotic morphology of the cells were the same (21). A large proportion of antineoplastic drugs kill cancer cells via starting the cell apoptosis mechanism (22).

Mitochondria are the central control sites for one mechanism of apoptosis (23). DNA injury activates the mitochondrial pathway of apoptosis, and NF- κ B inhibits cellular apoptosis via down-regulation of bcl-2 (24). Furthermore, bcl-2 expression is able to influence mitochondrial membrane depolarization (25). Caspases form a proteolytic network within the cell whereby upstream initiator caspases are activated early in the apoptotic process (e.g., caspase-8 and caspase-9) and then activate other downstream caspases (e.g., caspase-3) (26). The activation of caspase-9 is an indicator of the activation of the mitochondrial pathway of apoptosis.

FasL, ligand of FasL, combined with FasL on the surface of cells activating caspase-8 and -10, when exogenous signals stimulate. Then downstream caspase-6 and -7 are activated, caspase-3 activation is also induced triggering the apoptosis of cells (27). p53 serves a prominent role in cell apoptosis and is an important cancer suppressor gene. The main biological activity of p53 is to identify DNA injury and to repair DNA damage. If the repair fails, p53 induces cell apoptosis to prevent the development of tumor (28,29). p53 down-regulates the expression of bcl-2 to induce the apoptosis of MCF-7 cells (30).

The results of the present study demonstrate that FasL and p53 protein expression in MCF-7 cells was not changed significantly as drug concentration increased. These results indicate that flavonoids do not influence the expression of FasL and p53 protein.

In the present study, flavonoids may have down-regulated the expression of NF- κ B and bcl-2 and no expression of NF- κ B protein was observed in flavanoid-treated breast cancer cells. These results indicate that the decrease in NF- κ B and bcl-2 protein levels promotes the expression of caspase-9 and caspase-3. In addition, the expression levels of caspase-9 and -3 were up-regulated, which suggests that upstream caspase-9 protein increased to increase the expression of downstream caspase-3, the activation of caspase-3 resulting in the degradation of cytoskeleton and DNA strand breaks inducing MCF-7 apoptosis.

In conclusion, *T. chinensis* flavonoids were shown to suppress the growth and induce apoptosis of MCF-7 cells. Their effects in down-regulating the expression of NF- κ B may serve to down-regulate expression of Bcl-2, which participates in the mitochondrial pathway involving caspase-3 and caspase-9 to induce cell apoptosis. There is no change in FasL and p53 protein expression, so the FasL and p53 protein could not lead to the apoptosis of EC-109 cells. Therefore, *T. chinensis* flavonoids may have the potential to be developed as a novel anti-breast cancer drug.

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