# STUDY OF ANTI-CANCER EFFECT OF WINTER WORM AND SUMMER GRASS ON MCF-7 HUMAN BREAST CANCER CELLS

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In Partial Fulfillment of the Requirements for the Degree

Master of Science

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by

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Dr. Qisheng Song, Thesis Supervisor

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The undersigned, appointed by the dean of the Graduate School,

have examined the thesis entitled

**STUDY OF ANTI-CANCER EFFECT OF WINTER WORM AND SUMMER GRASS ON MCF-7 HUMAN BREAST CANCER CELLS**

presented by Tongtong Xu,

A candidate for the degree of Master of Science,

And hereby certify that, in their opinion, it is worthy of acceptance.

Associate Professor Qisheng Song

Professor David Stanley

Professor Dennis Lubahn

Assistant Professor Michael Wang

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# STUDY OF ANTI-CANCER EFFECT OF WINTER WORM AND SUMMER GRASS ON MCF-7 HUMAN BREAST CANCER CELLS

Tongtong Xu

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## ABSTRACT

Winter worm and summer grass (WWSG) is one of the most valued traditional Chinese medicines for fighting cancer, increasing longevity, and improving immunity. It consists of the entomopathogenic fungus *Cordyceps sinensis* and its natural lepidopteran host *Hepialus armoricanus*. Using the water extract of *Cordyceps militaris*, a sibling species of *C. sinensis* cultivated on an artificial host the silkworm *Bombyx mori* pupae, I have found that the *C. militaris* extract inhibited growth of MCF-7 human breast cancer cells in a dose- and time-dependent manner, with $IC_{50}$ value at about 11 μg/ml when MCF-7 cells were incubated in the *C. militaris* extract-containing medium for 96 h. The inhibitory effect of the *C. militaris* extract on MCF-7 cells was irreversible. Real-time PCR analysis revealed that the *C. militaris* extract induced the transcriptional expression of early-response pro-apoptotic marker genes *Bax* and *Bim* in MCF-7 cells incubated with the *C. militaris* extract for 6 h or longer and induced the late-response apoptotic genes *Apaf-1* and *Caspase-7* in MCF-7 cells after 48 h incubation. The transcript of another gene *Cytc* which encodes cytochrome c, an essential element in mitochondria-mediated apoptosis, was also induced in MCF-7 cells after 48 h incubation with the *C. militaris* extract. The anti-apoptotic gene Bcl-2 transcript was suppressed in MCF-7 cells after 6 h

incubation with the *C. militaris* extract. All data indicate the *C. militaris* extract inhibited MCF-7 cells growth by sequentially inducing the expression of proapoptotic and apoptotic genes *Bim*, *Bax*, *Cytc*, *Apaf-1* and *Caspase-7*. It also inhibited the transcriptional expression of the anti-apoptotic gene *Bcl-2*, a gene encoding Bcl-2 which contributes to the initiation of this apoptosis pathway by activating Bax. The activation of caspases leads to the subsequent DNA fragmentation as detected in TUNEL system. Although the *C. militaris* extract also prevented MCF-10A normal breast cells from growth, MCF-10A cells quickly recovered to their normal growth rate when removed from the *C. militaris* extract-containing medium to fresh medium.

In addition to the apoptotic genes, the levels of the methyltransferase gene *DNMT1* and *DNMT3a* transcripts were also suppressed in MCF-7 cells incubated with the *C. militaris* extract for 6 h. This result indicates that less methylation in some tumor-suppressor genes may potentially lead to regained expression of these genes and subsequent inhibition of cancer cell growth.

In summary, these findings suggest that the *C. militaris* extract inhibits human breast cancer cell growth through an apoptosis cascade by inducing pro-apoptotic and suppressing anti-apoptotic marker gene expression. Moreover, the *C. militaris* extract reduced DNA methylation through the suppression of methyltransferase transcripts, leading to the recovery of tumor-suppressor genes and eventually inhibiting tumor cell growth.

# CHAPTER I: INHIBITORY EFFECT OF WINTER WORM AND SUMMER GRASS ON MCF-7 HUMAN BREAST CANCER CELL GROWTH

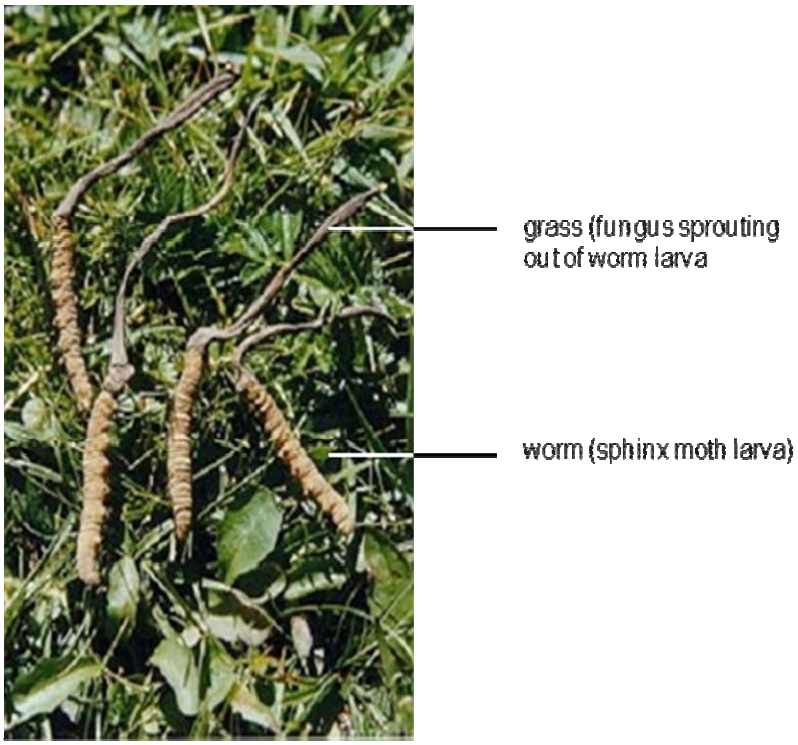
## INTRODUCTION

### *1. Winter worm and summer grass*

Winter worm and summer grass (WWSG) is a precious Chinese herbal medicine respected in Chinese medicine for thousands of years. The natural body of WWSG used in traditional Chinese medicine is a complex of Ascomycetes fungus *Cordyceps sinensis* and a larva of the sphinx moth *Hepialus armoricanus* (Holliday and Cleaver, 2005). In many publications and in this thesis, the fungus species name *C. sinensis* refers to the natural WWSG. WWSG is the English name translated from the Chinese "Dong Chong Xia Cao" word for word.

*C. sinensis* is a fungus with an annual appearance (Holliday and Cleaver, 2005). Native occurrence of this fungus is confined to the high Himalayan mountains across several Chinese provinces including Xi Zang, Sichuan and Qinghai, as well as neighboring regions in Nepal and India, the range of its natural host, the sphinx moth *H. armoricanus* thrives only above 3800 meters above sea level. The harvest season for this medicine is usually from April to August. The habitat of this herb is cold, grassy, alpine meadows on the mountains. It takes one year to form the natural body of WWSG. In natural environment, the sphinx moth *H. armoricanus* lays its eggs on the leaves of meadow bushes on high plateau during summer. When the larvae hatch from eggs, they

fall into earth. The fungus *C. sinensis* infects the larva underground and utilizes the nutrient in the larva from summer to winter. The larva becomes mummified during the winter and is called winter worm (Dong Chong in Chinese). In the next summer, the mycelium sprouts from the insect body and forms a mushroom-like structure. The mycelium grows like a grass above ground, so it is called summer grass (Xia Cao in Chinese). The mycelium of the fungus forms a fruit body. When the spores become mature, they spread out, fall into earth and infect the insect larva. The next cycle of WWSG production begins (Sharma, 2004) (Fig. 1).




(http://db.39kf.com/zhenjun_pic/PRB_1239.jpg)

**Fig. 1.** WWSG in natural conditions. This picture shows the WWSG used in traditional Chinese medicine. The dark brown herb-like part labeled "grass" is the fungus *C. sinensis* sprouting from the head of the sphinx moth larva. The yellowish part is the mummified larva of the sphinx moth *H. armoricanus* labeled as "worm".

*C. sinensis* was discovered by yak herders. They found their herd became more excited and ardent after they grazed the mushroom-like grass on high altitude in spring. This mushroom-like grass *C. sinesis* has been a traditional Chinese medicine ever since. Now it has come to be regarded as having many far-reaching medicinal effects. In traditional Chinese medicine, *C. sinensis* has been used to treat conditions including respiratory and pulmonary diseases, renal, liver, and cardiovascular diseases, hypo sexuality, and hyperlipidemia. It is also used in the treatment of immune disorders and as an adjunct to modern cancer therapies (chemotherapy, radiation treatment, etc.). The traditional consumption of this herb is to boil it with a variety of meats to make medicinal soup. Because of so many medicinal effects of this herbal medicine and the rarities and difficulties involved in harvesting it, *C. sinensis* has always been one of the most expensive "herbs" in traditional Chinese medicine. In ancient times, the high price of this herb excluded average people from getting access to it. In modern society, with the increased purchasing capability of Chinese people and high demand of this medicine in market, the price has recently increased to more than $2000 per pound, almost 50 times the price 10 years ago.

To make this herbal medicine more affordable to ordinary people, many modern cultivation techniques have been developed to cultivate this fungus and insect complex. By the mid 1980's, the majority of WWSG in market was artificially cultivated (Holliday and Cleaver, 2005).

### *2. Cultivated WWSG*

Because of the rarity and cost of this medicine, researchers have put great effort into studying the life cycle of the fungus *C. sinensis* in order to cultivate it under

laboratory conditions. In 1982, the strain Cs-4 was isolated successfully at the Institute of Materia Medica, Chinese Academy of Medical Sciences. This strain is the first commercial strain of *C. sinensis* isolated and has been widely used as a commercial product in China (Zhu, 1998). The fermentation product of Cs-4 has been shown to contain active components similar to those of natural Cordyceps but with relatively low concentration. Because of the traditional consumption of this herb, the whole WWSG with a "worm" of some type is more welcome by the consumers than just a fermented fungus. However, the study of this strain was confined only to the fermentation product without applying it to its natural host *H. armoricanus*.

There are two reasons for this limitation. First, the cultivation of the sphinx moth *H. armoricanus* under laboratory conditions takes more than 200 days to complete one generation. The long life cycle of the sphinx moth greatly increases the cost when applied to a big scale industrial production. Moreover, the fungus *C. sinensis* only infects the fourth instar larvae of the sphinx moth. Even if the larvae are available under laboratory conditions; it is very difficult to tell the fourth instar larvae from the third or fifth instar ones. Although many efforts have been made in the cultivation of natural WWSG, it is not feasible to culture the natural bodies under laboratory conditions.

Compared to the long life cycle of the sphinx moth, the mycelium of *C. sinensis* takes a much shorter time to be harvested. The fermentation of this fungus mycelium product has been applied to large scale industry production in many pharmacological plants for commercial production in China. Currently, there are three main types of WWSG in the market: the natural bodies, the fermented mycelia, and the cultivated

bodies of WWSG which use sibling fungus species as a substitute to infect insect larvae or pupae in the cultivation.

In addition to *C. sinensis*, there are many documented *Cordyceps* species being cultivated and used in medical treatment. These species include *C. militaris*, *C. sobolifera*, *C. subsessilus*, *C. ophioglossoides* and so on (Holliday and Cleaver, 2005). The cultivated bodies of WWSG are called "Yong Chong Cao" or "Bei Chong Cao" in Chinese, which use *C. militaris*, a sibling species of *C. sinensis*, as a substitute fungus and the silkworm *Bombyx mori* pupae as its host. After the silkworm pupae were sterilized and inoculated with *C. militaris*, the infected silkworm pupae were incubated under the defined laboratory conditions and harvested after about 60 days (Fig. 2).


**Fig. 2.** Cultivated winter worm and summer grass (*C. militaris* and *Bombyx mori*).

The cultivated bodies of WWSG have similar bioactive components and similar medicinal activities to the natural ones (Zhang and Liu, 1997). Because of the short

cultivation period and accessibility of silkworm pupae, the cultivated bodies of *C. militaris* and silkworm pupae are priced much lower and are more affordable to ordinary people when compared to the natural *C. sinensis* bodies.

### *3. Medical effect and usage of this medicine*

#### *3.1 Medical effect of C. sinensis*

*C. sinensis* has been used in China as a traditional medicine and tonic food to fight fatigue, night sweating, respiratory diseases, and many other diseases for almost 2000 years (Zhu, 1998). The first official record of this medicine was found in "Ben Cao Cong Xin" by Yi Lou Wu, a relatively complete and famous traditional Chinese medicine book, during the Qing Dynasty (1757 AD).

The traditional usage of this medicine is to boil this herb with meat to make soup. Taking this traditional usage as a reference, the water extract and organic solution extract of this medicine were investigated in many preclinical *in vivo* and *in vitro* experiments to study its medicinal effect. The water extract of *C. sinensis* has been examined to have anti-metastatic activity to accelerate the function of Kupffer cells (specialized macrophage in liver) in rats (Nakamura *et al.*, 1999). This anti-metastatic activity has been also examined in mice. When the mice were inoculated with Lewis lung carcinoma (LLC) and B16 melanoma (B16) cells, the orally administration of the *C. sinensis* water extract inhibits tumor metastasis in the injected mice (Zhang *et al.*, 2004; Nakamura *et al.*, 1999). Also the hot water extract of *C. sinensis* can modulate mouse immune system by activating macrophage and enhancing the secretion of IL-6 in mice (Koh *et al.*, 2002). Moreover, the water extract of *C. sinensis* has been demonstrated to have

hypocholesterolemic effects (lower the cholesterol level in mice) when the mice are fed with a cholesterol-enriched diet (Koh *et al.*, 2003).

In the *in vitro* studies, the hot water extract of *C. sinensis* inhibited the proliferation and differentiation of human leukemic U937 cells (Chen *et al.*, 1997). In addition to the water extract of *C. sinensis*, the ethyl acetate extract of *C. sinensis* showed the inhibitory effect on the proliferation of many cancer cell lines including human premyelocytic leukemia cell HL-60 (Zhang *et al.*, 2004), human breast cancer cell MCF-7, mouse melanoma cell B16 and human hepatocellular carcinoma cell HepG2 (Wu JY *et al.*, 2007). Other kinds of organic solution extracts of *C. sinensis* have also been investigated. The methanol extract of *C. sinensis* was found to have anti-tumor activities on K526, Jurkat (T lymphocyte), WM-1341 (human melanoma cell), HL-60, and RPMI-8226 (human myeloma cell) tumor cell lines (Bok *et al.*, 1999). The petroleum ether and ethanol extract of *C. sinensis* showed a significant inhibitory effect on the proliferation of MCF-7, B16, HepG2 and HL-60 cells (Wu JY *et al.*, 2007).

Although *C. sinensis* has a long history of medicinal usage in China, the first introduction of this medicine to Western society was in 1726 in a scientific meeting held in Paris by a French Jesuit priest who recorded his experience with this herbal medicine during his staying in China. However, the first effective component in this fungus was not purified until over 200 years after the first introduction. In 1950, cordycepin, a derivative of the nucleoside adenosine believed to be the major active ingredient in Cordyceps, was first isolated from the culture of *C. militaris* by Cunningham (Cunningham *et al.*, 1950). Later, cordycepin was found in *C. sinensis* (Huang *et al.*, 2004). Cordycepin has anti-tumor activities on mouse melanoma B16-BL6 cells and

mouse Lewis lung carcinoma cells (Nakamura *et al.*, 2006). This purified compound also has apoptotic effect on OEC-M1, a human oral squamous cancer cell line (Wu WC *et al.*, 2007).

With more explorations in this medicine, many other components such as adenosine derivatives, ophicordin, polysaccharides, immunoporentiating galactomannan and anti-tumor sterols were identified and regarded as the effective components contributing to the medicinal effect of *C. sinensis* (Bok *et al.*, 1999). The purified polysaccharide has been shown to stimulate the function of phagocytes, inhibit tumor development, protect liver function, and protect pheochromocytoma PC12 cells against the free radical-induced neuronal cell toxicity (Li *et al.*, 2003).

### *3.2 Medicinal effect of C. militaris*

Because of the rarity and high price of natural WWSG, the cultivated *C. militaris* on the silkworm *B. mori* pupae was used widely to substitute the natural bodies (Zhang *et al.*, 1997). With the similar bioactive components, *C. militaris* has been found to have many similar medicinal functions as the natural *C. sinensis* does. The current market price for this cultivated *C. militaris* averages about 200 dollars per pound, one tenth the cost of *C. sinensis*,.

So far, *C. militaris* has been found to have anti-tumor activities in many cancer cell lines. *C. militaris* inhibits human glomerular mesangial cell proliferation induced by low dose native low-density-lipoproteins (LDL). This finding was explained by *C. militaris'* ability to improve renal function and reduce proteinuria (Wu *et al.*, 2000). The hot water extract of *C. militaris* also showed inhibitory effects on in vitro human premyelocytic leukemia HL-60 cell and sarcoma-180 cell proliferation, and in vivo

prolonged the life span of mice bearing sacoma-180 cell-induced ascites tumor (Lee *et al.*, 2003; Lee *et al.*, 2006). Moreover, the *C. militaris* extract has antiangiogenetic properties through inhibition in the proliferation of the human umbilical vein endothelial cell line HUVEC and the human fibrosarcoma cell line HT1080. This anti-angiogenic property could potentially apply to the treatment of some solid tumors (Yoo *et al.*, 2004). Another function of *C. militaris* is the regulation of glucose utilization. The water extract of *C. militaris* increases the disposal rate of glucose in skeleton muscles without altering insulin secretion (Choi *et al.*, 2004).

Although many investigations of the cultivated *C. militaris* have been performed, many medicinal effects and the mechanisms of the medicinal effects are still not well understood. In my project, I studied the anti-cancer effect of the cultivated *C. militaris* (the silkworm *B. mori* pupae as host) on MCF-7 human breast cancer cells and the mechanism of the inhibitory effect.

### *4. Project objectives*

One of the objectives of this project is to examine the anti-cancer effect of the cultivated *C. militaris* on the growth of MCF-7 human breast cancer cells.

In order to accomplish this, dose- and time-response assays were performed to test the anti-cancer effect of the *C. militaris* extract on MCF-7 cell growth. In this project, MCF-10A human breast cells were used as a control to evaluate the potential toxicity of the *C. militaris* extract on normal breast cell growth. The recovery assay was applied to demonstrate the different effects of the *C. militaris* extract on normal and cancer breast cells.

# MATERIALS AND METHODS

### *1. Cell culture*

MCF-7 human breast cancer cells, kindly provided by Dr. Dennis Lubahn at the University of Missouri-Columbia, were cultured in Minimum Essential Medium (MEM) (Sigma, St. Louis, MO, USA) supplemented with 5% Calf Serum and 100U/ml Penicillin/Streptomycin (Table 1). The final pH of the medium was adjusted to 7.4. MCF-7 cells were incubated at $37^0$C and 5% $CO_2$. The old medium was replaced with a fresh medium every two to three days to keep cells grow normally.

MCF-10A human breast cells, purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA), were cultured in Mammary Epithelial Growth Medium (MEGM) (Clonetics, San Diego, CA, USA) supplemented with Bovine Pituitary Extract (BPE) (Clonetics, San Diego, CA, USA) to a final concentration of 52 μg/ml and cholera toxin to 100 ng/ml (Sigma, St. Louis, MO, USA) (Table 2). MCF-10A cells were incubated at 37℃ and 5% $CO_2$. The old medium was replaced with a fresh medium every two to three days to keep cells grow normally.

### *2. C. militaris extract preparation*

The cultivated WWSG used was kindly provided by Dr. Chuanxi Zhang from Zhejiang University in China. To make this organism, the fungus *C. militaris* was inoculated and cultivated on the silkworm *B. mori* pupae (Holliday *et al.*, 2004). The dried bodies of mycelia and pupae were together chopped into small 1 mm pieces and then boiled in distilled water for 1 h. After centrifuge at 16,000 g for 20 min, the supernatant was collected into clean tubes and lyophilized. The lyophilized powder was

weighed and re-dissolved in distilled water to make a stock solution at 100 mg/ml. After being sterilized using a 0.22 μm syringe driven filter unit (Millipore, Bedford, MA, USA), the stock solution was aliquoted and stored at -80$^0$C until use.

***3. Dose and time response***

Both MCF-7 human breast cancer cells and MCF-10A normal breast cells were incubated for 48 h after the passage to ensure the cells were in the exponential growth phase when the assays were performed. Each passage was done when MCF-7 cells reached about 90% confluent. MCF-7 cells were collected with the treatment of 0.25% trypsin supplemented with EDTA for 5 min. When the cells were released from the flask, the reaction was stopped by adding the complete MEM medium and the cell suspension was collected into a 15 ml sterilized centrifuge tube. After centrifuge at 100 g for 5 min, MCF-7 cells were re-suspended in a fresh MEM medium and viable cells were counted using 0.4% Trypan blue. After making appropriate dilutions, about 5000 cells were added into each well of a 12-well cell culture plate. The cells were allowed to incubate for 6 h for synchronization before dose and temporal assays.

For the dose-response assay, the indicated amounts of the *C. militaris* extract were added to the culture medium of MCF-7 cells to make the final concentrations of the *C. militaris* extract in the culture medium at 0, 6.25, 12.5, 25, 50, and 100 μg/ml. The cells were incubated under the conditions described above for 96 h.

The cells were observed daily under a microscope for morphological change and collected at the end of 96 h incubation for viable cell count. Based on the result of the dose-response assay, a specific concentration of the *C. militaris* extract was selected for the time-response assay.

In the time-response assay, MCF-7 cells were incubated in the medium containing 50 μg/ml extract for the indicated time periods following the same protocol used in the dose response assay. After 0, 24, 48, 72 or 96 h incubation, the cells were observed for morphological change and collected for viable cell count as described above.

***4. Cell recovery assay of MCF-7 and MCF-10A***

MCF-10A human breast cells were treated with different concentrations of the *C. militaris* water extract to set a dose with an obvious inhibitory effect on MCF-7 human breast cancer cells, but no or little inhibitory effect on MCF-10A cell growth.

Both MCF-7 and MCF-10A cells were incubated in the presence or absence of 25 μg/ml *C. militaris* extract for 24, 48, 72, and 96 h. At the end of each incubation period, both MCF-7 and MCF-10A cells were examined for morphological change and for viable cell counts. In order to examine whether the inhibitory effect was reversible in both cell lines at the end of a 96 h incubation with the *C. militaris* extract, MCF-7 and MCF-10A cells were replaced with a fresh, extract-free medium and incubated for additional 96 h. At the end of the additional 96 h incubation, cell morphology was examined and viable cell number was recorded by using 0.4% Trypan blue.

**Table 1. A complete medium recipe for MCF-7 cells.**

| MEM complete medium | | |
|---|---|---|
| Distilled water | | 983 ml |
| Sigma M3024 (bottle) | 045K8305(Sigma, St. Louis, MO, USA) | 1 |
| $NaHCO_3$ | 7412KANP(Mallinckrodt, St. Louis, MO, USA) | 2.2 g |
| 2.5M HEPES | 051674(Fisher, Pittsburgh, PA , USA) | 4 ml |
| L-glutamine | GB126 (Sigma, St. Louis, MO, USA) | 0.292 g |
| Insulin (6 μg/ml) | 11070-73-8(Sigma, St. Louis, MO, USA) | 1 ml |
| Donor Bovine Serum | 16030074(Invitrogen, Carlsbad, CA, USA) | 53.2 ml |
| Penicillin (100U/ml) | 113K0521(Sigma, St. Louis, MO, USA) | 2.6 ml |
| Streptomycin (100 μg/ml) | 064K0546 (Sigma, St. Louis, MO, USA) | 2.6 ml |
| Phenol red | 04668(Fisher, Pittsburgh, PA , USA) | 10 mg |

**Table 2. A complete medium recipe for MCF-10A Cells.**

| MEGM (Mammary Epithelial Growth Medium) complete medium | | |
|---|---|---|
| MEGM | CC-3051(Fisher, Pittsburgh, PA, USA) | 500 ml |
| BPE (13mg/ml) | CC-4009(Fisher, Pittsburgh, PA, USA) | 2 ml |
| Insulin (5mg/ml) | 11070-73-8(Sigma, St. Louis, MO, USA) | 0.5 ml |
| Cholera toxin (100 μg/ml) | 131096-89-4 (Sigma, St. Louis, MO, USA) | 0.5 ml |

# RESULTS

### *1. Dose and time response*

After being incubated for 96 h in the MEM medium containing the indicated concentrations of the extract, or in the medium containing 50 μg/ml extract for the indicated time periods, MCF-7 cells were collected to evaluate the effect of *C. militaris* on cell growth. As shown in Fig. 3 and Fig. 4, the inhibitory effect of the *C. militaris* extract on MCF-7 cell growth was dose- and time-dependent. The inhibitory effect of the *C. militaris* extract could be detected at concentrations of as low as 6 μg/ml (Fig. 3b). However, the morphology of the cells treated with this dose of the *C. militaris* extract kept normal (Fig. 3a). $IC_{50}$ value is at around 11 μg/ml. At the concentration of 50 μg/ml, cell number in the treated group was only about 15% of that in the control. The maximum inhibitory effect occurred at the concentration of 100 μg/ml, where the cell number was approximate 5% of that in the control. The shape of the cells became rounded up, indicating the dying of the cells. Shrinkage and membrane blebbing could be observed in the remaining cells under microscope which implies apoptosis might occur in those cells.

Temporal response study revealed that when MCF-7 cells were incubated in the medium containing 50 μg/ml *C. militaris* extract for 24 h, the cell number in the treated group declined to about 82% of that in control (Fig. 4). The inhibitory effect of the extract became even greater with the increase in incubation time. At 48 h, the cell number in the treated sample was only about 28.2% of that in control. At the end of 96 h, the surviving cells in the treated group accounted only about 10% of those in control with abnormal morphology.

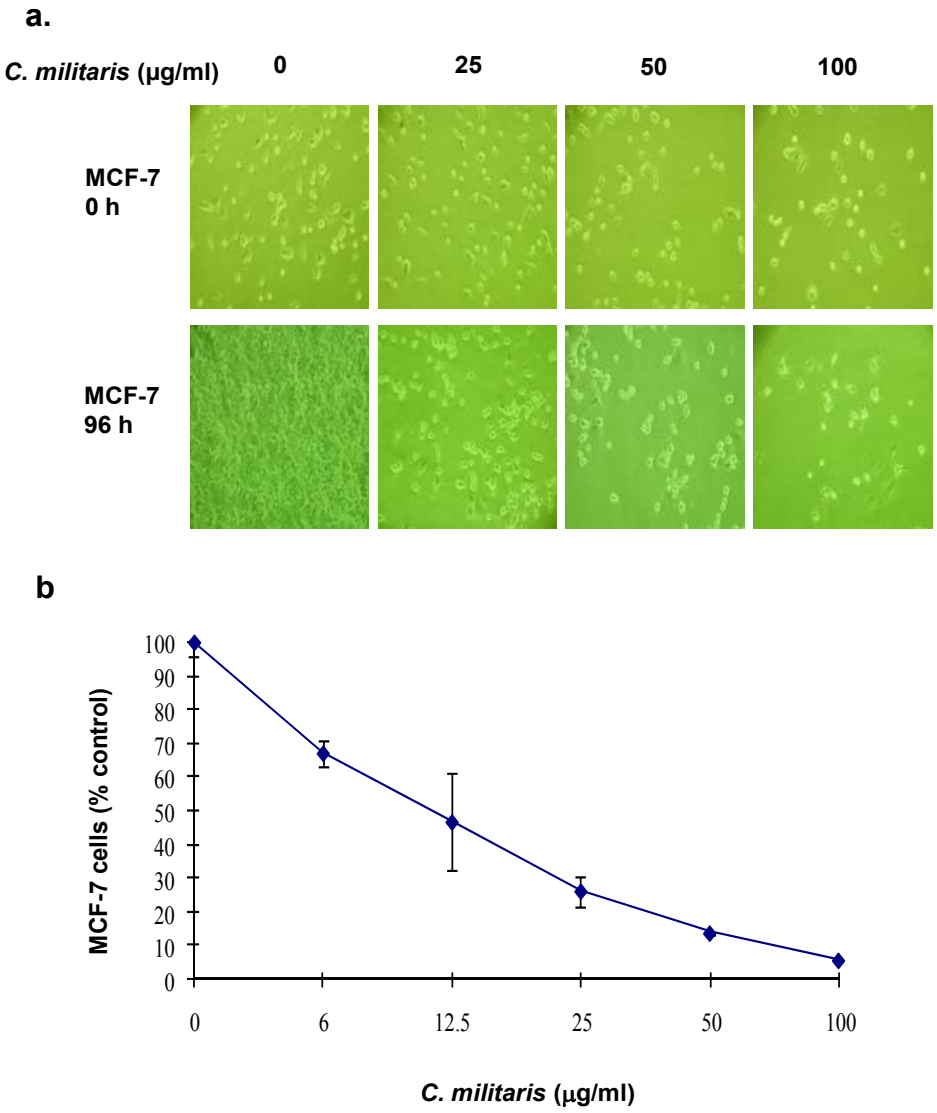



**Fig. 3.** Dose-response of MCF-7 cells to the aqueous extract of *C. militaris*. MCF-7 cells were incubated for 96 h in the presence or absence of the indicated concentrations of the extract. (a) Morphology of the MCF-7 cells incubated with the indicated concentrations (6, 12.5, 25, 50, and 100 µg/ml) of the *C. militaris* extract. (b) Dose response curve. The data represent the mean ± SD of three independent biological replicates.

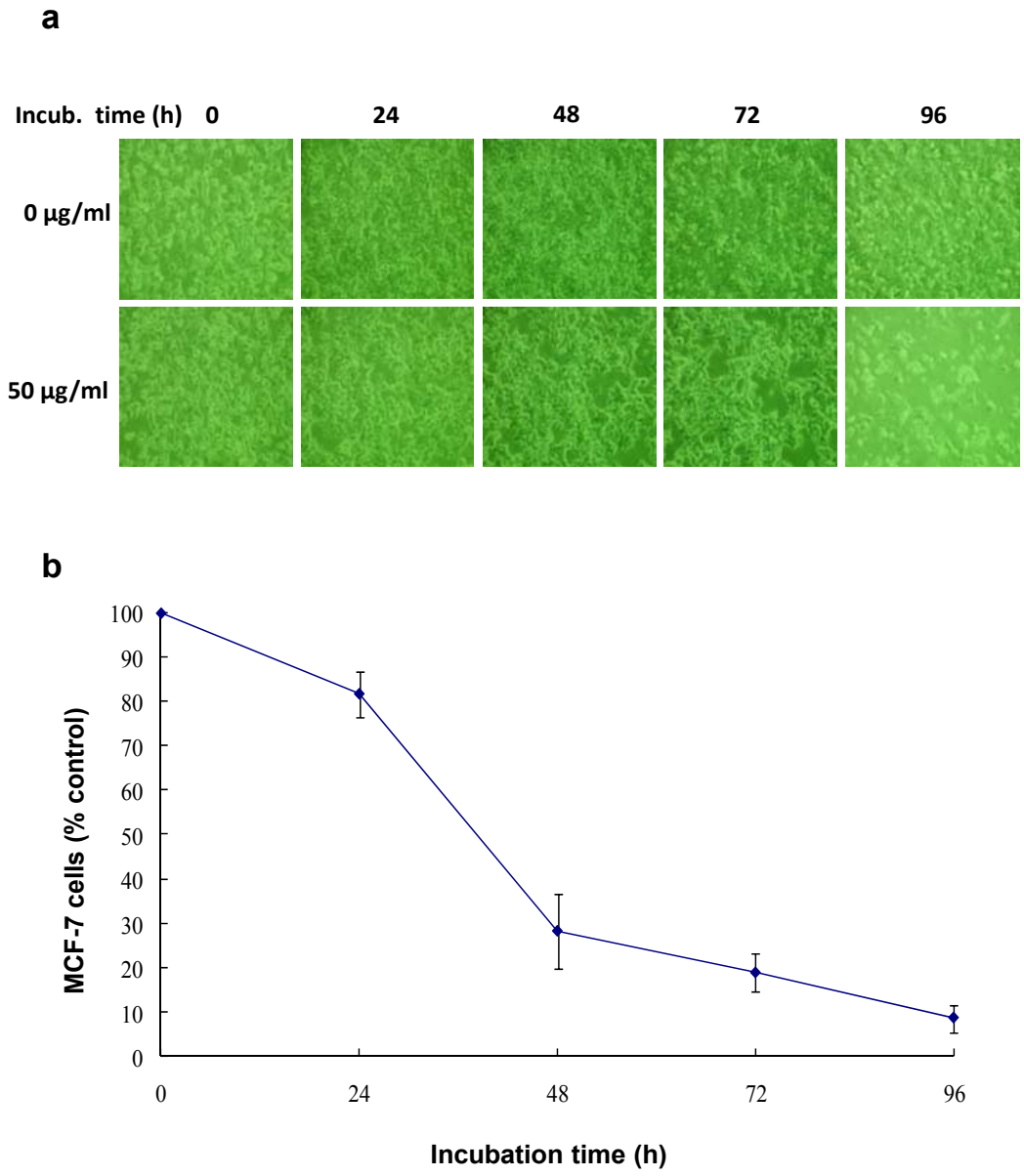
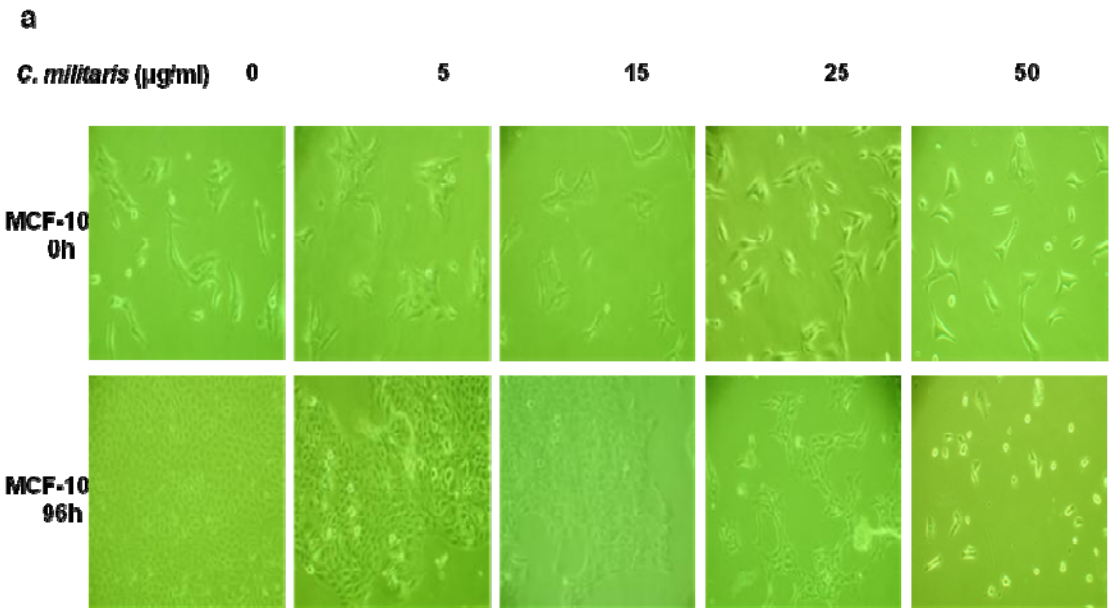



**Fig. 4.** Time-response of MCF-7 cells to the aqueous extract of *C. militaris*. MCF-7 cells were incubated for the indicated time periods in the presence or absence of 50 µg/ml extract. (a) Morphology of MCF-7 cells at different time periods. (b) Time response curve. The data represent the mean ± SD of three independent biological replicates.

### *2. Recovery assay of MCF-7 and MCF-10A*

To test whether the *C. militaris* extract has any effect on normal breast cells, MCF-10A human breast cells were incubated with different concentrations of the *C. militaris* extract to determine a dose effective on cancer cells and non-effective on normal cells (Fig. 5a). From the assay on MCF-10A cells, the concentration of 25 μg/ml *C. militaris* was chosen for the recovery assay.

MCF-10A normal breast cells were incubated side-by-side with MCF-7 breast cancer cells for the indicated time periods in the presence or absence of 25 μg/ml *C. militaris*. As shown in Fig. 5, the *C. militaris* extract had a similar, but less severe, inhibitory effect on the growth of MCF-10A normal breast cells when compared to MCF-7 breast cancer cells (Fig. 5a). At the end of initial 96 h, MCF-10A cell number still remained over two folds of MCF-7 (Fig. 5b) with normal morphology (Fig. 5a). When both MCF-10A and MCF-7 cells were removed from the *C. militaris* extract-containing medium and incubated for additional 96 h in the *C. militaris* extract-free fresh medium, MCF-10A quickly recovered to its normal growth rate while MCF-7 could not. At the end of additional 96 h incubation in the fresh medium, nearly all MCF-7 cells were dead, suggesting that the inhibitory effect of the *C. militaris* extract on MCF-7 was irreversible and lethal while the effect on MCF-10A was reversible.


a
C. militaris (µg/ml)
0
5
15
25
50
MCF-10
0h
MCF-10
96h

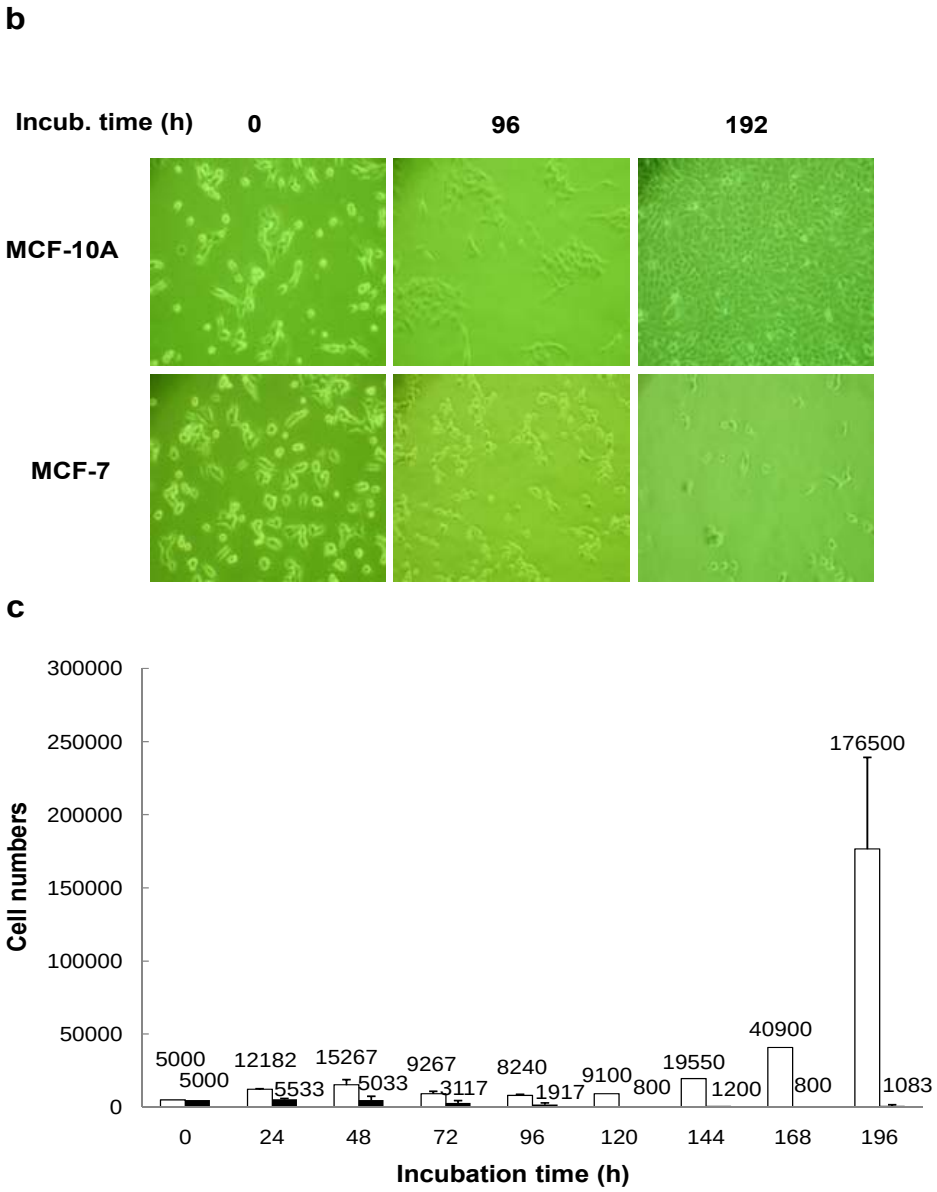



**Fig. 5.** (a) Morphology of MCF-10A incubated with or without the *C. militaris* extract to determine an appropriate dose for recovery assay. (b) Morphology of MCF-7 and MCF-10A incubated with 25µg/ml extract for 0 and 96 and for additional 96 h without the extract in a recovery assay. The pictures are the most representative ones of three independent biological replicates. (c) Time response in the recovery assay. The data represent the mean ± SD of two independent biological replicates except at 120, 144, and 168 h (one replicate). White bars represent the controls while the black bars indicate the treatments.

# DISCUSSION

We investigated the inhibitory effect of *C. militaris*, a cultivated substitute for traditional herbal medicine WWSG, on MCF-7 breast cancer cell growth. Our results show the *C. militaris* water extract inhibits MCF-7 cell growth in a dose- and time-dependent manner (Fig. 3 and 4). The results provide scientific evidence that *C. militaris* could be used as a supplement in cancer treatment. This is consistent with a recent report that the fermentation broth of *C. militaris* had an inhibitory effect on MCF-7 growth (Lin and Chiang, 2008).

The attractive character of an anti-cancer treatment does not lie in its capability to kill cancer cells but in its capability to target the cancer cells without harming or minimizing harm to healthy cells. In both traditional usage and clinical data, *C. militaris* has shown less side-effect when it is applied to carcinomatous subjects. In our investigation, MCF-10A normal breast cells were set up as a control in the recovery assay to evaluate the cyto-toxicity of the *C. militaris* extract on normal cells. Although the extract inhibited cancer cell growth, it had less adverse effects on MCF-10A normal breast cell growth than cancer cell growth (Fig. 5). This finding suggests that *C. militaris* is of a great potential to be used as a therapy of breast cancer in future.

Although the inhibitory effect of the *C. militaris* extract on MCF-7 breast cancer cell growth was reported recently (Lin and Chiang, 2008), there is no published data on the mechanism of this inhibitory effect. Because of this, the second objective of our project is to investigate the mechanism of the inhibitory effect of the *C. militaris* extract on breast cancer cell growth. The abnormal morphology of shrinkage in MCF-7 cells treated with the extract suggests that MCF-7 cells might experience an apoptotic process.

To test this hypothesis, we selected the representative pro-apoptotic marker genes and anti-apoptotic marker genes to study the mechanism of this inhibitory effect, which will be discussed in next chapter.

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# CHAPTER II: THE EFFECT OF THE C. MILITARIS EXTRACT ON APOPTOTIC PATHWAY AND DNA METHYLATION IN MCF-7 CELLS

## INTRODUCTION

### *1. Winter worm and summer grass vs. cancer*

As described in the previous chapter, WWSG has been widely used as a supplement in cancer treatment in China. The anti-cancer effects of this medicine are attracting more and more attentions. Compared to the natural *C. sinensis*, the cultivated *C. militaris* has been studied in only a few cancer cell lines. So far, the anti-cancer effect of *C. militaris* has been investigated in leukemia HL-60 cell line, human leukemia U937 cell line, human umbilical vein endothelial cell line HUVEC, and human fibrosarcoma cell line HT1080 (Lee *et al.*, 2006; Yoo *et al.*, 2004; Park *et al.*, 2005) and in MCF-7 human breast cancer cells (Lin and Chiang, 2008).

Although Lin and Chiang (Lin and Chiang, 2008) have shown the inhibitory effect of the fermentation broth of *C. militaris* mycelia on MCF-7 breast cancer cell growth, the mechanisms of the inhibitory effects have not been investigated in their researches. The purpose of this project was not only to investigate the inhibitory effect of the cultivated *C. militaris* (*C. militaris* infects the silkworm pupae) water extract on MCF-7 cell growth but also to explore the mechanism of this inhibitory effect. In Lin and Chiang's investigation, the $IC_{50}$ value of the *C. militaris* mycelia fermentation broth in the medium of *Radix astragali* was 36.9 μg/ml, which is as three times as the $IC_{50}$ value of the *C. militaris* complex water extract used in this project (11 μg/ml). Since the fungus

used in this project was cultivated on insect pupae rather than liquid medium, in the process of parasitism, the defense of insect pupae might produce some bio-active components that contribute to the anti-cancer effect of this complex. For the fungus cultivated in the liquid medium with *Radix astragali*, the components from the insect-fungus interaction could not be produced.

In addition to *C. militaris*, the mycelia of *C. sinensis* has also been investigated in the cyto-toxicity test of several cancer cell lines including MCF-7 cell line (Wu JY *et al.*, 2007). In Wu's investigation, the $IC_{50}$ value of the hot water extract of *C. sinensis* mycelia was higher than 200 μg/ml, which is much higher than the $IC_{50}$ value of the cultivated *C. militaris* water extract. Based on the previous anti-cancer experiment performed in MCF-7 cells, the *C. militaris* extract used in this project has the highest quality in terms of $IC_{50}$ value.

The regular treatments of cancer include surgery, chemotherapy, radiation therapy, and complementary medicine. All therapies are aimed at removing cancer from tissues or killing the cancer cells. But most therapies, such as chemotherapy and radiation, kill cancer cells at the cost of destroying the normal cells at the same time. In order to reduce side-effects, therapies targeted specifically on intra-cellular targets of cancer cells have attracted more attention and have been studied intensively. The realization of apoptosis and exploitation of apoptotic genes have become potential strategies for cancer therapies (Lowe and Lin, 2000).

Many alternative medicines used for cancer treatment have been shown to inhibit cancer cell proliferation by inducing apoptosis. For example, ginseng was found to have anti-proliferation effect on human prostate cancer LNCaP by inducing apoptosis (Liu *et*

*al.*, 2000). The traditional Indian medicine *Rasagenthi lehyam* inhibits lung cancer cell proliferation (A-549 and H-460 cell lines) through regulating the apoptotic genes, which eventually initiate apoptosis (Rama *et al.*, 2005). Even *C. militaris* has been found to have inhibitory effect on leukemia cell proliferation by inducing apoptosis (Park *et al.*, 2005).

In the United States, breast cancer is listed as the No. 2 cause for female mortality after lung cancer. The National Cancer Institute estimates the numbers of new cases from breast cancer in the United States in 2008 are 182,460 for females and 1990 for males (http://www.cancer.gov/cancertopics/types/breast). The death estimations are 40,480 for female and 450 for male. Investigating the anti-cancer effects of WWSG on breast cancer cells is a promising research area that will potentially lead to successful breast cancer treatment in future.

### *1.1 Apoptosis*

Apoptosis, also called a programmed cell death, is a biological process in cells to destruct cells by endogenous mechanism. Kerr *et al.* (1972) found the cells underwent a characteristic morphology when dying and gave this type of cell death a term–apoptosis. Apoptosis, a Greek word meaning "falling away", was used in ancient Greek poems to describe the dropping of petals from flowers or leaves from trees. Some modern bilingual dictionaries, however, translate apoptosis as "wandering" or "aberrant."

The early postulate of apoptosis existed in embryology. It was assumed that controlled cell death occurred during developmental stages to balance cell division (Kerr *et al.*, 1987). Some examples were investigated to show that apoptosis played a role in developing central nervous system, deleting unnecessary tissue in the process of organ

development, and deleting auto-immune cells in immature thymus so the immune system could achieve self-tolerance. Apoptosis was also seen in growing and regressing tumors (Kerr *et al.*, 1987).

With the concept that cells follow a certain pathway to suicide in the whole process of life, the idea of regulated cell destruction in cancer cells suggested a possible therapeutic intervention in clinical treatment (Fadeel and Orrenius, 2005). Many anti-cancer therapies, including the usage of alternative medicines, are aimed at promoting apoptosis in cancer cells (Debatin, 2004).

### *2. Apoptosis in cancer*

The study of apoptosis starts from the morphological observation such as cell shrinkage, membrane blebbing, chromatin condensation, and nuclear fragmentation ( Wyllie *et al.*, 1980; Kerr *et al.*, 1994). With more understanding of apoptosis and apoptotic genes, investigators became aware of the importance of apoptosis in carcinogenesis. It has been found that the mutation of apoptosis-related genes can disrupt apoptosis and even facilitate carcinogenesis. For example, lymphoproliferative disorders or cancers could be induced by mutating the Fas/CD95 receptor which controls the elimination of cells by apoptosis in immune system (Beltinger *et al.*, 1998). With the transgenic and knockout techniques provided, the direct evidence that the disruption of apoptosis can promote cancer development was demonstrated. In transgenic mice that over-express the anti-apoptotic protein Bcl-2, the over-expressed Bcl-2 protein could accelerate the development of mammary tumors (Jäger *et al.*, 1997).

Apoptosis is a process of biochemical action consisting of many key components. At the molecular level, many pro-apoptotic genes involve in apoptotic cascade and many anti-apoptotic genes manipulate the cascade. The pro-apoptotic genes are those whose over-expressed products facilitate apoptotic pathway, while the anti-apoptotic genes have over-expressed products inhibiting apoptotic pathway. Based on their sequential roles in the apoptotic pathway, they could be divided into three types: 1) early-response apoptotic genes including *Bax*, *Bim*, *Bid*, *Bak*, etc; 2) late-response apoptotic genes including *Cytc*, *Apaf-1*, etc; and 3) the genes in the terminal phase of apoptotic pathway including various caspases like *caspase 9*, *caspase 3*, *caspase 7*, and so on. In this introduction, five apoptotic genes, including early- and late-response genes, will be discussed.

***Bcl-2***

The *Bcl-2* (B-cell lymphoma 2) gene was first identified in a leukemia cell line and was later found widely existed in follicular lymphoma (Tsujimoto *et al.*, 1985). In follicular lymphoma, the *Bcl-2* gene was found to inhibit cell death rather than promote proliferation. This discovery implied the impaired apoptosis is a crucial step in tumorigenesis (Cory and Adams, 2002). The defect in apoptosis pathways contributes to many diseases including cancer, autoimmunity and degenerative disorders (Thompson, 1995). The *Bcl-2* gene behaves as an oncogene to promote cell survival (Vaux *et al.*, 1988).

In higher eukaryotes, Bcl-2 has at least 20 homologues (Festjens *et al.*, 2004). Bcl-2 and its relatives, Bcl-xL and Bcl-w, potently inhibit apoptosis in response to many cytotoxic insults. Bcl-2 has a hydrophobic carboxy-terminal domain which helps it target the intracellular membranes: the outer mitochondrial membrane, the endoplasmic

reticulum, and the nuclear envelope. Bcl-2 is an integral membrane protein even in healthy cells, and is required in kidney and melanocytes stem cells and mature lymphocytes (Janiak *et al.*, 1994; Veis *et al.*, 1993).

The structure of Bcl-2 determines the mechanism of its action. Bcl-2 heterodimerizes with Bax, a member of Bcl-2 family, which shares homology with Bcl-2 protein in conserved regions including BH1 (Bcl-2 homology 1) and BH2 (Bcl-2 homology 2) domains. *Bax* is a pro-apoptotic gene whose over-expression promotes cell death. When Bcl-2 is over-expressed, it heterodimerizes with Bax and disables Bax function, repressesing cell death (Oltvai *et al.*, 1993). In mammalian cells the biochemical study demonstrated that Bcl-2 promotes cell survival by blocking the activation of caspases (Knudson and Korsmeyer, 1997).

The anti-apoptosis capability of Bcl-2 was demonstrated in both *in vivo* and *in vitro* experiments. The over-expressed Bcl-2 prolonged the cytokine-dependent cell survival upon cytokine withdrawal and kept the cells at $G_0$ stage (Vaux *et al.*, 1988; Hockenbery *et al.*, 1990; Korsmeyer, 1992). Bcl-2 protected T-cells against many apoptotic signals (Sentman *et al.*, 1991; Strasser *et al.*, 1991). In transgenic mice that over-express Bcl-2, follicular hyperplasia or high-grade monoclonal lymphomas was induced when the transgene Bcl-2 was expressed in B lymphocytes. When the transgene targeted to T cells, one-third of the mice developed peripheral T cell lymphomas.

***Bax***

*Bax*, a pro-apoptotic gene belonging to Bcl-2 family, produces a 21-kDa protein. The over-expression of *Bax* gene could accelerate apoptotic death in response to a death signal (Chao and Korsmeyer, 1998). Bax is widely expressed in tissues and exists in cells

as a stable soluble protein in cytosol. Just like Bcl-2, Bax is an integral membrane protein located in intracellular membranes including mitochondria. When stimulated with apoptotic signals, Bax protein moved to mitochondrial membrane within 30 min, which could be detected under confocal microscope with labeled GFP fluorescent tag (Wolter *et al.*, 1997).

When the cells are stimulated by apoptotic signals, Bid (another member of Bcl-2 family) binds with Bax on the outer membrane of mitochondria and facilitates the conformational change of Bax, changing the permeability of the mitochondria outer membrane. As a result, cytochrome c is released from mitochondria into the cytosol (Crompton, 2000). The released cytochrome c participates in the next step in the apoptotic cascade.

Prolonged cell survival by eliminating *Bax* was demonstrated in embryonic fibroblast treated with chemotherapeutic agents. The elimination of *Bax* blocked about half the cell death (McCurrach *et al.*, 1997). The mutation of *Bax* gene was noted in about 20% of human hematopoietic malignancy lines (Meijerink *et al.*, 1998) and was found to exist in about 50% human colon adenocarcinomas. Mutations of pro-apoptotic gene *Bax* in human malignancies support the conclusion of Bax protein as a cell death suppressor (Rampino, 1997; Chao and Korsmeyer, 1998).

***Bim***

*Bim* is another pro-apoptotic marker gene. Bim protein, a member of Bcl-2 family who shares only the Bcl-2 homology-3 (BH-3) domain with Bcl-2 protein known as BH3-only protein, is not only a critical regulator in immune cell homeostasis, but also an apoptotic inducer in cell development. Bim has three isoforms: BimEL (196 a.a), BimL

(140 a.a) and BimS (110 a.a) (Abrams *et al.*, 2004; O'Connor *et al.*, 1998). All of the three isoforms are potent to induce apoptosis in cancer cells. Bim-null lymphocytes were refractory to some apoptotic stimuli, such as cytokine deprivation, calcium ion flux, and microtubule perturbation (Wang, 2001). Mice lacking Bim showed defects in apoptotic response in the immune system to the apoptotic signals (Bouillet *et al.*, 1999). BimS is the most potent inducer of apoptosis (Abrams *et al.*, 2004; Li *et al.*, 2005) and it recruits Bax upon stimulation. (Weber *et al.*, 2007).

The BH-3 only protein, like Bim, breaks up the interaction of Bax and Bcl-2 to remove the block of apoptosis (Willis *et al.*, 2007). Although there is no direct evidence for this prediction, no Bax-dependent apoptosis occurred when BH-3 only proteins were taken away (Kim *et al.*, 2006; Willis *et al.*, 2007). In Jurkat T cells, caspase has a feedback amplification capability to cleave the phosphorylated BimEL into a truncated fragment which has higher affinity to Bcl-2 and activates apoptotic activity. BimEL has the phosphorylated and nonphosphorylated forms in healthy cells (Chen and Zhou, 2003).

***Cytc***

Cytochrome c is a very important component in oxidative phosphorylation in mitochondria facilitating production of ATP in electron transport chains. The bad side of cytochrome c for cells was first indicated by Liu *et al.* (1996). When they tried to purify the factors from cytosol that induced proteolytic processing and caspase activation, cytochrome c was found to be the required factor.

Cytochrome c is encoded as an apocytochrome c and becomes a mature protein when it crosses the outer membrane of mitochondria and combines with heme. Cytochrome c resides between the outer and inner membrane of mitochondria (Reed,

1997). When there is a Bax-dependent apoptotic pathway being actived, cytochrome c will be released from the intermembrane of mitochondria into cytosol to relay the cell death process. The released cytochrome c initiates apoptotic pathway by combining with and activating the next important factor, Apaf-1, in the mitochondria-mediated apoptotic pathway (Heimilch *et al.*, 2004; Jiang and Wang, 2004).

***Apaf-1***

Apaf-1 refers to apoptotic protease activating factor-1. Cytochrome c behaves as a dock protein to bind two purified proteins, Apaf-1 and Apaf-3, as soon as it is released from mitochondria into cytosol (Liu *et al.*, 1996). Apaf-3 is identified as caspase-9 in human cells (Li, 1997).

Apaf-1 has a caspase-recruitment domain (CARD) (Zou, 1999). When cytochrome c is released into the cytosol, it binds with Apaf-1. This combination increases the affinity of Apaf-1 for dATP/ATP by almost ten-fold at the nucleotide-binding domain of Apaf-1. The binding of dATP/ATP to the Apaf-1/cytochrome c complex facilitates the oligomerization to form an apoptosome of Apaf-1 and cytochrome c multimers (Zou, 1999; Jiang and Wang, 2000). In the apoptosome, the exposed CARD domain recruits caspase-9 and triggers the autoactivation of caspase-9 (Wang, 2001).

The importance of *Apaf-1* was implied in gene knockout experiments. The gene knockout mice failed to respond to the apoptotic signals induced by UV, γ-irradiation, and treatment with chemotherapeutic drugs (Li *et al.*, 2000). In the *Apaf-1* knockout cells, no apoptosis could be induced even though cytochrome c was released from mitochondria into cytosol (Yoshida *et al.*, 1998).

***Caspase-7***

Caspase-7 is structurally similar to caspase-3 and has the same catalytic function in the apoptosis cascade. Both of them are essential proteases in the terminal phase of apoptosis and act as enzymes disrupting the integrity of mitochondria membranes increasing the permeability of mitochondria membranes. Consequently, the intermembrane proteins such as cytochrome c will be released to the cytosol to participate in the activation of caspase. Thus the caspases in the apoptosis cascade accelerate the apoptotic process by a self-amplifying system. (Marzo *et al.*, 1998; Chandler *et al.*, 1998; Liang *et al.*, 2001). In the mitochondria-mediated apoptotic pathway, the activated caspase-9 activates procaspase-3 into caspase-3. In the case of null-caspase-3 system, caspase-7 takes part in the activation as well (Cohen, 1997; Liang *et al.*, 2001).

Human breast cancer cells, MCF-7, does not express caspase-3 because of the 47-base pair deletion in the exon of caspase-3 gene (Janicke *et al.*, 1998). Upon apoptotic stimulation, MCF-7 cells also experience apoptotic morphological and physiological change. Caspase-7 is activated by capase-9 and then induces caspase-6 activation which eventually cleavages nuclear lamina and triggers cell death (Orth *et al.*, 1996; Liang *et al.*, 2001).

In summary, one mitochondria-mediated apoptotic pathway involving all the apoptotic marker genes we described above could be summarized in Fig. 6. The over-expressed Bax induced by certain apoptotic stimuli facilitates cytochrome c release from mitochondria into cytosol. The released cytochrome c combines with Apaf-1 to form an apoptosome to further combine with and activate caspase-9. The activated caspase-9

activates caspase-7 sequentially in MCF-7 cells. Then the activated caspase-7 in turn activates caspase-6 eventually leading to apoptosis in MCF-7. Bcl-2 acts as an anti-apoptotic protein to block Bax and caspases activities. Meanwhile, Bim activates Bax by recruiting and activating Bax upon the stimulation. Although there is no direct evidence, Bim might also promote the apoptotic pathway by intervening in the interaction of Bcl-2 and Bax.

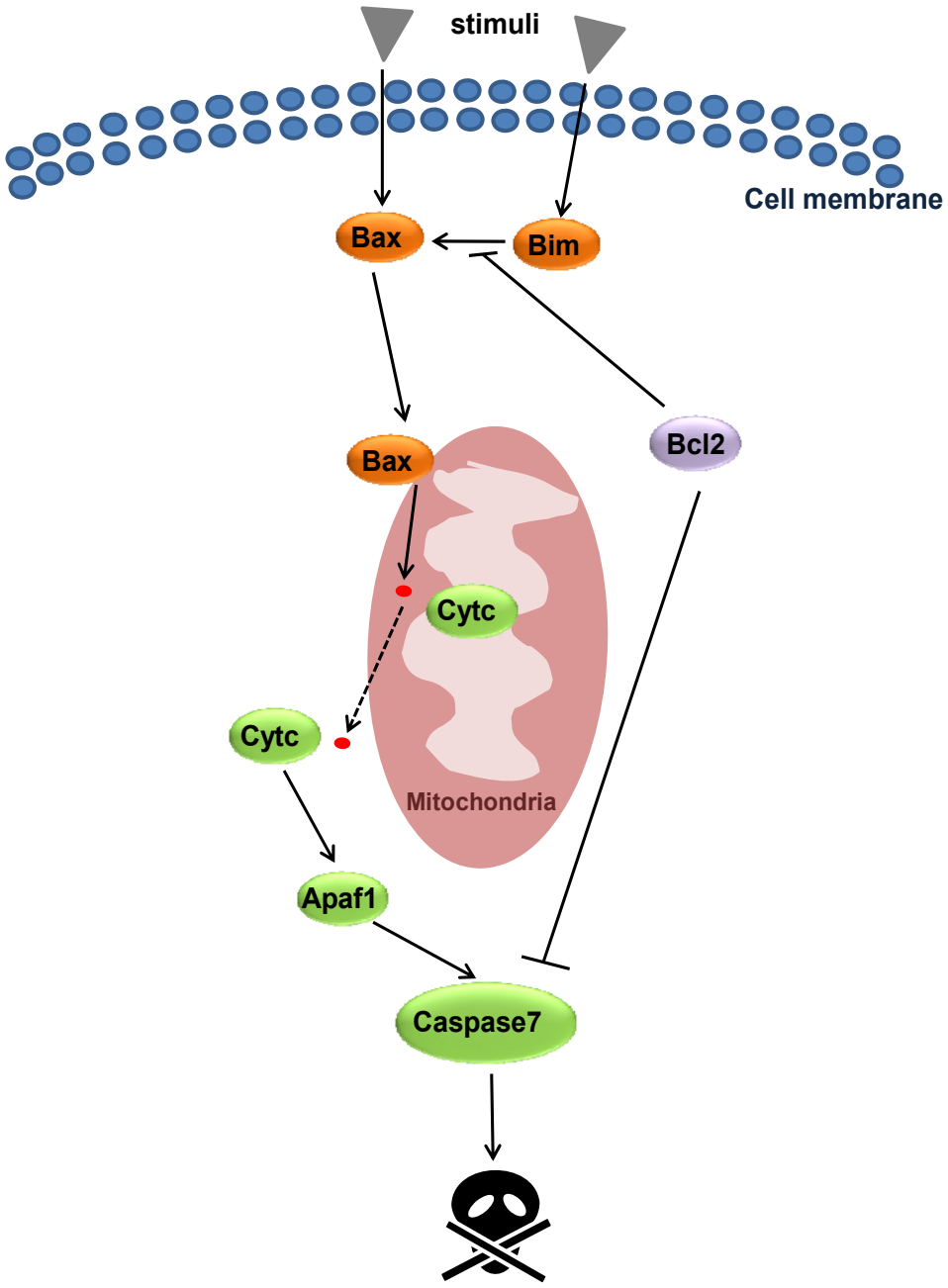



**Fig. 6.** Schematic apoptotic cascade in MCF-7 cells.

### *3. DNA methylation in cancer*

DNA methylation is an epigenetic modification by the addition of methyl group from the methyl donor S-adenosyl-methionine (SAM) to the 5-position of cytosine of the CpG-rich regions called CpG island which is a region containing high frequency of cytosine-guanine dinucleotide (The "p" in CpG notation refers to the phosphodiester bond between the cytidine and the guanosine) in mammalian DNA. DNA methylation usually associates with transcriptional silencing of gene expression in mammalian cells (Baylin, 2000). When the methylation happens on CpG island located in gene promoter, the gene transcription will be silenced as a result of the methylation. Approximately about half of all the genes in human contain CpG islands and these islands are often found at the promoter area of these genes (Cross and Bird, 1995).

In cancer cells, there is a co-existence of hypermethylation and global hypomethylation. There are two mechanisms accounting for the transcription inhibition via DNA methylation. In the first mechanism, the CpG dinucleotides are methylated by methyltransferase in the gene promoter area. Transcription factors which are sensitive to the methylated CpG dinucleotides are not able to bind to the promoter region to initiate transcription. The other mechanism is through the actions of methyl-CpG binding protein (MeCP) and methyl-CpG bingding domain (MBD) proteins. With the recruitment of methylated DNA, MeCP and MBD bind specifically to the methylated DNA and cumber the binding of transcription factors to the methylated DNA, hence inhibit the transcription process (Hendrich and Bird, 1998; Luczak and Jagodzinski, 2006). These two mechanisms are demonstrated in Fig. 7.

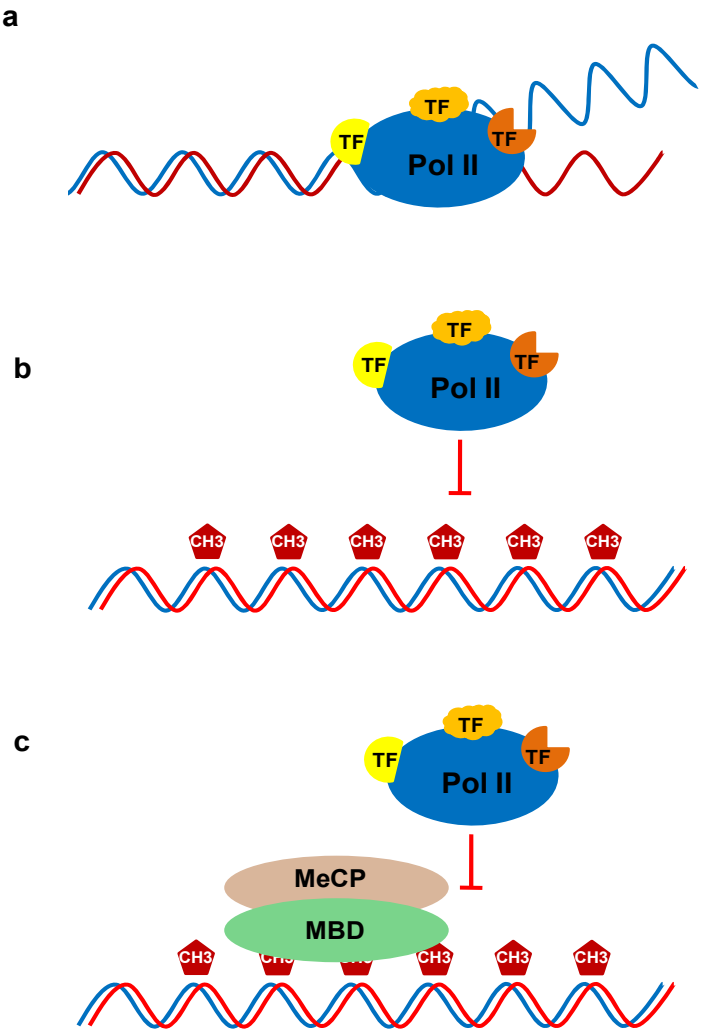



**Fig. 7.** The mechanisms of transcription inhibition by DNA methylation. (a) Transcription under the action of transcription factors (TF) and RNA polymerase II (Pol II). (b) The methylated DNA prevents the binding of TFs (c) Methylated DNA binds m5CpG binding (MeCPs) and m5CpG-binding domain (MBDs) proteins to make a spatial obstacle preventing TFs binding.

Many genes were methylated in breast cancer cells and the methylated genes were shown to contribute to tumorigenesis. There are four groups of genes found to be methylated in cancer cells. The first group is the tumor-suppressor genes such as *P16* which is found to be methylated and silenced in human breast cancer cells leading to the lost control of cell growth (Herman *et al.*, 1995; Szyfa *et al.*, 2004). Another group is the damage response genes, for example, the *BRCA-1* gene involved in repairing the damaged DNA in normal cells (Niwa *et al.*, 2000). The third group is the steroid receptor gene family members such as *ER* (estrogen receptor). In some breast cancer patients, ER expression is lost due to the methylation at CpG island in *ER* promoter (Yan *et al.*, 2001). The last group includes the genes encoding cell adhesion and cell surface molecules. In breast cancer cells, the gene encoding E-cadherin, which governs the cell-cell adhesion in tissue, was detected to be methylated before the invasion of cancer cells into the normal tissues during the malignant progression (Graff *et al.*, 2000).

DNA methylation in cytosine is catalyzed by DNA-cytosine methyltransferase (DNMT). In this chapter, there are three members of *DNMT* gene family being discussed: *DNMT1*, *DNMT3a* and *DNMT3b* (Bestor, 2000).

***DNMT1***

In mammals, about 3-6% of cytosines are methylted. In human genome, about 70-80% of CpG sites are methylated under the catalysis of DNA-cytosine methyltransferase-1 (*DNMT1*). The human *DNMT1* gene encodes a protein DNMT1 of 200 kDa. The methyltransferase catalytic domain sites in the C-terminal region of this protein which catalyzes methylation from the hemimethylated DNA during cell division (Bestor, 1988). In addition to the C-terminal domain, the N-terminal domain alone could suppress

transcription in vitro. The deacetylation capability of the N-terminal region of this protein was found to remove the acetyl tail from histones to form a transcriptional inactivate chromatin (Fuks *et al.*, 2000). Also part of the N-terminal domain binds to a transcriptional co-repressor that represses transcription (Rountree *et al.*, 2000). Although the increased expression of DNMT1 was found in many cancer cells, the methylation pattern of CpG island in certain genes keeps unchanged with the knockout of *DNMT1* gene, suggesting that other possible DNMTs exist functionally (Rhee, 2000; Li *et al.*, 1992).

***DNMT3***

Two isoforms DNMT3a and DNMT3b are essential for the *de novo* methylation and are highly expressed in mammalian cells. The *de novo* isoforms of DNMT3a and DNMT3b were isolated from mice in 1999 (Okano *et al.*, 1999). The expression of DNMT3a is ubiquitous and could be detected in most adult tissues but DNMT3b is expressed highly only in testis, thyroid and bone marrow (Xie *et al.*, 1999). The over-expression of both DNMT3a and DNMT3b were found in many types of tumor cells but the inactivation of either DNMT3a or DNMT3b does not change the methylation profile in the cells, suggesting the redundant effects of these two isoforms (Xie *et al.*, 1999).

***4. Project objectives***

The objective of this chapter is to investigate the change of the apoptotic gene transcripts and DNA-cytosine methyltransferase (DNMT) gene transcripts in MCF-7 incubated with the *C. militaris* extract to study the possible apoptotic cascade and DNA methylation induced by the extract treatment. In order to accomplish this objective, the

transcriptional expression profiles of three early-response apoptotic genes-*Bcl-2*, *Bax* and *Bim* and three late-response genes-*Cytc*, *Apaf-1* and *caspase-7* were investigated using real-time PCR. And a TUNEL system was used to detect DNA fragmentation, a final stage in the apoptotic process induced by the activated caspase, in both MCF-7 cancer cells and MCF-10A normal cells to confirm the hypothesis that different inhibitory mechanisms were induced in cancer and normal cells. The decreased levels of *DNMT* transcripts- *DNMT1*, *DNMT3a* and *DNMT3b* in MCF-7 human breast cancer cells induced by the *C. militaris* extract was also investigated using real-time PCR.

# MATERIAL AND METHODS

## *1. Real-time PCR analysis of apoptotic marker genes*

MCF-7 cells were collected after 6, 12, 24 and 48 h incubation in medium containing 50 µg/ml *C. militaris* extract. Total RNA was isolated using TRIZOL® (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol and RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. The extracted total RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) to eliminate any potential contamination of genomic DNA. About 2 µg total RNA from each sample was used for the first-strand cDNA synthesis. The first-strand cDNA synthesis was primed using oligo (dT) based on the SupperScript™ First-Strand synthesis kit (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was used as template for estimation of gene transcription in MCF-7 cells by real-time PCR.

cDNAs of apoptotic marker genes (*Baxα* , *Baxβ*, *BimEL*, *BimL*, *BimS*, *Apaf-1* and *Caspase-7*), *Cytc* and anti-apoptotic marker gene *Bcl-2* were amplified using gene specific primers (Table. 3). *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to normalize marker gene PCR products. Real-time PCR amplification and analysis were carried out on Applied Biosystems 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA). The final volume of reaction was 25 µl using ABI SYBR Green Supermix (ABI, Foster City, CA, USA). PCR program was: hold at $95^0$C for 10 minutes; 2-temperature cycle repeats for a total of 40 times, $95^0$C for 15 seconds, $60^0$C for 1 minute. The specificity of SYBR green PCR signal was confirmed by

melting curve analysis and agarose gel electrophoresis. The mRNA expression was quantified using the comparative CT (Cross Threshold, the PCR cycle number that crosses the signal threshold) method (Liverk and Schmittgen, 2001). The CT of the housekeeping gene GAPDH was subtracted from CT of the target gene to obtain ΔCT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT is equal to $\Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}}$. Three biological replicates were performed in real-time PCR.

**Table 3. Primers for real-time PCR amplification of apoptotic genes.**

| Gene name | Forward | Reverse |
|---|---|---|
| *Bcl-2* | 5'-acatttcggtgacttccgcatca-3' | 5'-gcttccagacattcggagac-3' |
| *Bax α* | 5'-ttctgacggcaacttcaact-3' | 5'-tcttccagatggtgagtgag-3' |
| *Bax β* | 5'-cgaactggacagtaacatg-3' | 5'-ccaatgtccagcccatgat-3' |
| *BimEL* | 5'-aattgcagcctgcggagag-3' | 5'-tgctgggctcctgtctgtg-3' |
| *BimL* | 5'-gatgtaagttctgagtgtg-3' | 5'-atagtaagcgttaaactcgt-3' |
| *BimS* | 5'-tgatgtaagttctgagtgt-3' | 5'-tcgtaagataaccattcgt-3' |
| *Cytc* | 5'-tatgaagcgttcccagtgc-3' | 5'-agtgctcggattacaggcgtg-3' |
| *Apaf-1* | 5'-gtgcctcagggtagcagtg-3' | 5'-gcctgctaaatccaagact-3' |
| *Caspase-7* | 5'-tcagtggatgctaagccagac-3' | 5'-cgaacgcccatacctgtcact-3' |
| *GAPDH* (control) | 5'-taaagggcatcctgggctacact-3' | 5'-ttactccttggaggccatgtagg-3' |

### *2. Real-time PCR analysis of methyltransferase genes*

MCF-7 cells were collected after 6, 12, 24 and 48 h incubation in medium containing 50 μg/ml *C. militaris* extract. Total RNA was isolated using TRIZOL® (Invitrogen, Carlsbad, CA, USA) according to the manufacture's protocol and RNA concentration was determined by measuring the absorbance at 260 nm in a spectrophotometer. The extracted total RNA was treated with RNase-free DNase I (Promega, Madison, WI, USA) to eliminate any potential contamination of genomic DNA. About 2 μg total RNA from each sample was used for the first-strand cDNA synthesis. The first-strand cDNA synthesis was primed using oligo (dT) based on the SupperScript$^{TM}$ First-Strand synthesis kit (Invitrogen, Carlsbad, CA, USA). The synthesized cDNA was used as template for estimation of gene transcription in MCF-7 cells by real-time PCR.

cDNAs of *DNMT1, DNMT3a* and *DNMT3b* in MCF-7 breast cancer cells were amplified using gene specific primers (Table. 4). *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to normalize marker gene PCR products. Real-time PCR amplification and analysis were carried out on Applied Biosystems 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA). The final volume of reaction was 10 μl using ABI SYBR Green Supermix (ABI, Foster City, CA, USA). PCR program was: hold at $95^0$C for 10 minutes; 2-temperature cycle repeats for a total of 40 times, $95^0$C for 15 seconds, $60^0$C for 1 minute. The specificity of SYBR green PCR signal was confirmed by melting curve analysis and agarose gel electrophoresis. The mRNA expression was quantified using the comparative CT (Cross Threshold, the PCR cycle number that crosses the signal threshold) method (Liverk and Schmittgen, 2001). The CT

of the housekeeping gene GAPDH was subtracted from CT of the target gene to obtain ΔCT. The normalized fold changes of the target gene mRNA expression were expressed as $2^{-\Delta\Delta CT}$, where ΔΔCT is equal to $\Delta CT_{\text{treated sample}} - \Delta CT_{\text{control}}$.

**Table 4. Primers for real-time PCR amplification of methyltransferase genes.**

| Gene name | Forward | Reverse |
|---|---|---|
| *DNMT1* | 5'-ccctttccaaacctcgcacg-3' | 5'-ggactcatccgatttggctct-3' |
| *DNMT3a* | 5'-gggcattcaggtggaccgctaca-3' | 5'-cttgcgagcagggttgacgat-3' |
| *DNMT3b* | 5'-cccaatcctggaggctatc-3' | 5'-gctgggctttctgaacgagtc-3' |
| *GAPDH* (control) | 5'-taaagggcatcctgggctacact-3' | 5'-ttactccttggaggccatgtagg-3' |

***3. TUNEL detection of apoptotic signal in MCF-7 and MCF-10A***

Based on the result in real-time PCR, apoptosis might be induced in MCF-7 cells after *C. militaris* extract treatment. In order to confirm real-time PCR result, the DeadEnd™ Fluorometric TUNEL System (Promega, Madison, WI, USA) was employed to detect the apoptotic signal in MCF-7 and MCF-10A cells incubated for the indicated time periods in medium containing 50 μg/ml extract. The DeadEnd™ Fluorometric TUNEL System is designed for specific detection and quantification of apoptosis cells within a cell population. In many cell types, apoptosis is characterized by the generation of DNA fragments through the action of endogenous endonuclease (Schwartzman and Cidlowski, 1993; Walker *et al.*, 1991; Oberhammer *et al.*, 1993; Roy *et al.*, 1992). The

DeadEnd™ Fluorometric TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP[(a)] at 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). rTdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (Gavrieli *et al.*, 1992). The fluorescein-12-dUTP-labeled DNA can then be visualized directly by fluorescence microscopy.

The cells were cultured on slides for different time periods and stained with the TUNEL kit according to the manufacture's protocol. PI (propidium iodide) was used for nuclear staining according to the manufacture's protocol. The apoptotic signal was observed under the confocal microscope (BioRad Radiance 2000 Confocal System) at the MU Cytology Core.

# RESULTS

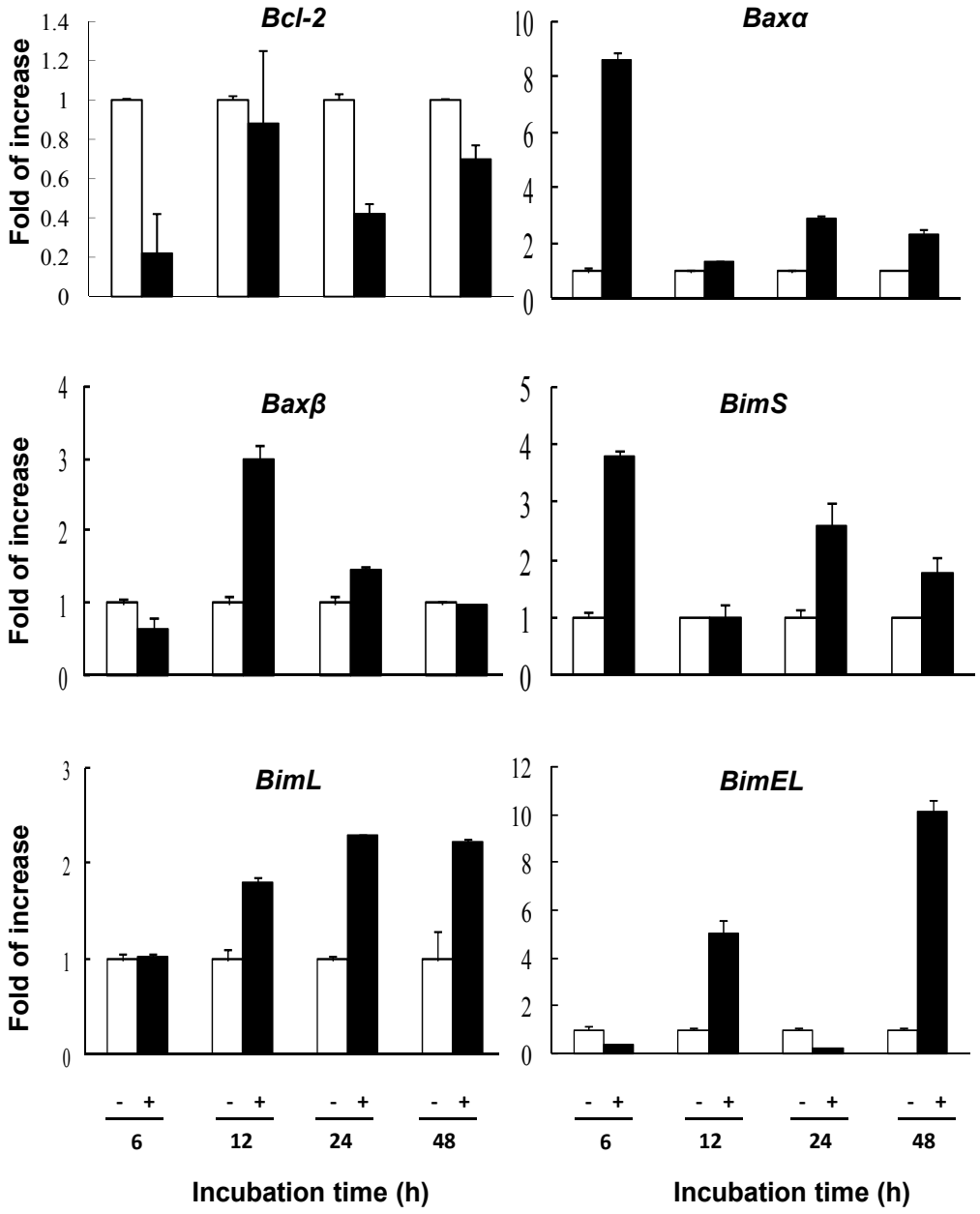
### *1. Real-time PCR analysis of apoptotic marker genes*

To investigate the mechanism of the inhibitory effect, Cytc and five breast cell apoptotic marker genes *Bcl-2*, *Bax*, *Bim*, *Apaf-1* and *Caspase-7* were amplified using real-time PCR. As shown in Fig. 8a, two isoforms of *Bax* and three isoforms of *Bim* transcripts were induced in MCF-7 cells incubated with the *C. militaris* extract. *Baxα* and *BimS*, two pro-apoptotic genes, were increased by approximate 8 and 4 fold respectively at 6 h. The transcripts of *Baxβ*, *BimL* and *BimEL* were increased by approximate 3, 2 and 5 fold respectively when MCF-7 cells were incubated with the *C. militaris* extract for 12 h. *Baxβ* transcript began to decrease after 12 h and declined to the basal level after 48 h treatment. The transcript of *BimEL* had a second peak of 10 fold when MCF-7 cells were incubated with the *C. militaris* extract for 48 h. The transcript of *BimL* kept on increasing and remained at about 2 fold after 12 h.

As shown in Fig. 8b, *Cytc*, whose product involves in later phase of apoptosis, was increased by 8 fold when MCF-7 was incubated with the *C. militaris* extract for 48 h, suggesting that the increased expression of *Bax* gene product led to the increased expression of cytochorme c in MCF-7. Cytochrome c is released from mitochondria into the cytosol to induce the apoptotic cascade. The later-response apoptotic gene *Apaf-1* was also detected at a 2-fold increase in transcript level in MCF-7 cells after 12 h incubation with the *C. militaris* extract. After 48 h incubation, the transcript of *Apaf*-1 increased by about 7-fold, indicating the activation of *Apaf-1* in apoptosis pathway after cytochrome c release. *Caspase-7* transcript in the terminal phase of apoptosis increased almost 2 fold in

MCF-7 after 48 h incubation with the extract. This further confirms the apoptosis pathway in MCF-7 induced by the *C. militaris* extract via activated caspases. Moreover, the level of the anti-apoptosis marker gene *Bcl-2* transcript was suppressed with the *C. militaris* extract treatment (Fig. 8a). After 6 h incubation, the transcript of *Bcl-2* in MCF-7 cells incubated with the extract was only 20% of that in control MCF-7 cells. The decreased expression of anti-apoptotic gene and increased expression of pro-apoptotic and apoptotic genes suggest that apoptosis is induced in MCF-7 cells incubated with the *C. militaris* extract.

**a**


Bcl-2
Baxα
Baxβ
BimS
BimL
BimEL
Fold of increase
Fold of increase
Fold of increase
- + - + - + - +
6 12 24 48
Incubation time (h)
- + - + - + - +
6 12 24 48
Incubation time (h)

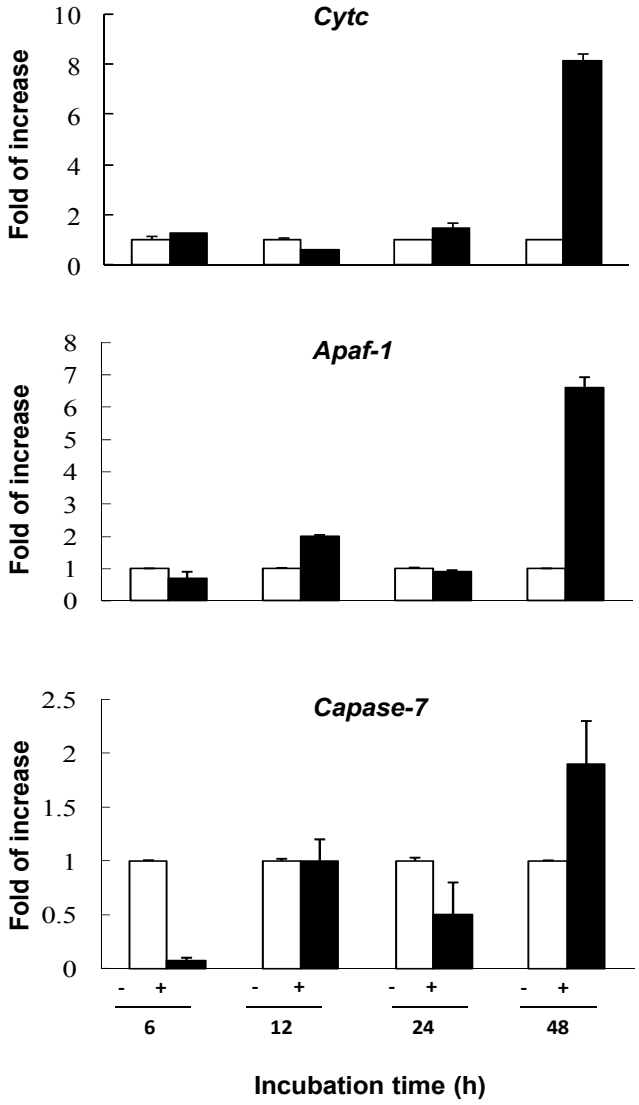



**Fig. 8.** Real-time PCR analysis of apoptotic marker gene transcripts in MCF-7 cells. (a) The transcriptional expression of early-response apoptotic marker genes *Bcl-2*, *Bax* and *Bim* in MCF-7 incubated with the extract for the indicated time periods. (b) The transcriptional expression of late-response apoptotic genes *Cytc*, *Apaf-1* and *Caspase-7* in MCF-7 incubated with the extract for the indicated time periods. Each histogram bar represents the mean ± SD of three independent biological replicates. White bars represent the controls while the black bars indicate the treatments.

### *2. Real-time PCR analysis of methyltransferase genes*

Three *DNMT* (DNA-cytosine methyltransferase) genes –*DNMT1, DNMT3a* and *DNMT3b* were amplified using real-time PCR to investigate the possible mechanism of the inhibitory effect inducing by the *C. militaris* extract in MCF-7 cells. As shown in Fig. 9, *DNMT1* and two isoforms *DNMT3* (*DNMT3a* and *DNMT3b*) transcripts were suppressed in MCF-7 cells incubated with the *C. militaris* extract for 6 h (Fig. 9). *DNMT1* transcript was suppressed by 58% when compared with that in control at 6 h. After 6 h, *DNMT1* transcript began to increase and had a peak of 8 fold when MCF-7 cells incubated with the *C. militaris* extract for 48 h. *DNMT3a* transcript was only about 30% of control when MCF-7 cells were incubated with the extract for 6 h. This suppression continued until 24 h, when the level of *DNMT3a* transcript increased to 2 fold of control and kept at that level at 48 h. The *C. militaris* extract had little suppression effect on the level of *DNMT3b* transcription. The transcript of *DNMT3b* was not suppressed by the *C. militaris* extract. In MCF-7 cells incubated with the *C. militaris* extract, *DNMT3b* transcript kept at around the basal level during first 24 h treatment and began to increase to almost 2 fold after 48 h incubation.

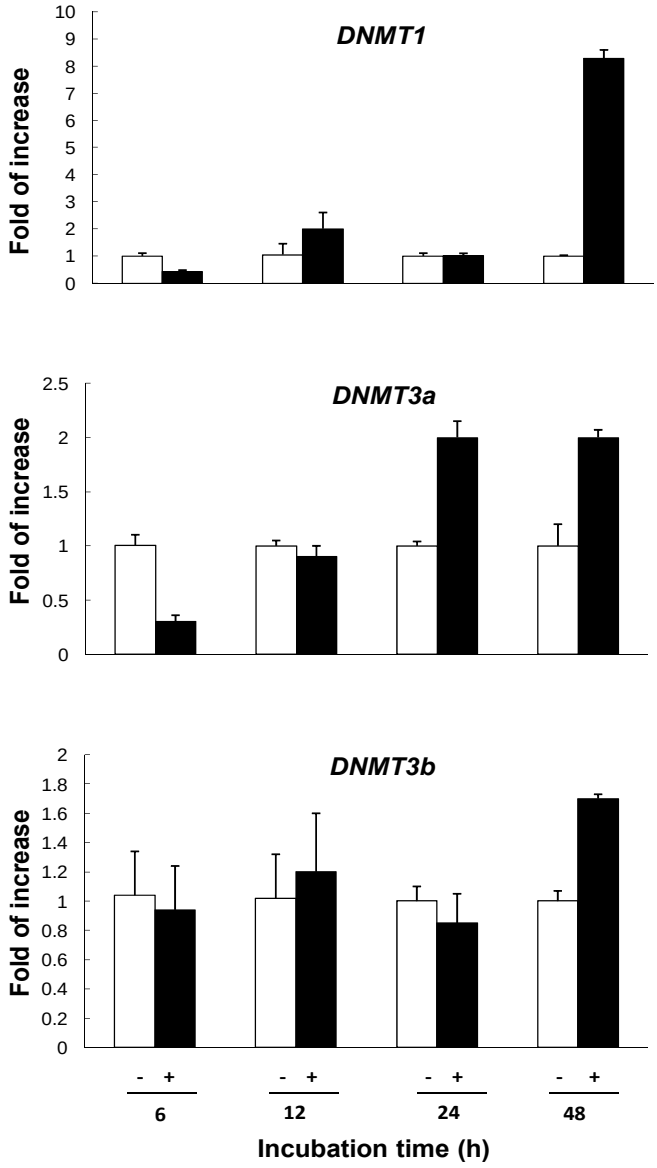



**Fig. 9.** Real-time PCR analysis of DNA-cytosine methyltransferase (DNMT) gene transcripts in MCF-7 cells. MCF-7 cells were incubated in the presence or absence of 50 µg/ml *C. militaris* extract for the indicated time periods. Total RNA was extracted for real time PCR amplification of *DNMT1, DNMT3a* and *DNMT3b* transcripts in the treated and control cells. *GADPH* transcript was used as an internal control for normalization. Each histogram bar represents the mean ± SD of triple replicates in one independent biological replicate. The blank bars represent *DNMT* transcript expression in control cells and the black bars represent *DNMT* transcript expression in treated cells.

### *3. TUNEL detection of apoptotic signal in MCF-7 and MCF-10A*

As shown in Fig. 10, apoptotic signal was detected using TUNEL system in MCF-7 cells incubated in medium containing the extract at 72 h after the treatment (Fig. 10a-f). The apoptotic signals were detected as green fluorescent signal under the confocal microscope and the PI nuclear staining was detected as red fluorescent signal. Multiple nucleoli were observed in control MCF-7 cells (Fig. 10a-c). After incubation with the *C. militaris* extract for 72 h, the nucleoli disappeared and apoptotic signal was detected in MCF-7 cells (Fig. 10d-f). It could be inferred that the *C. militaris* extract inhibits MCF-7 cell growth by inducing apoptotic gene expression (Fig. 8), which leads to the activation of caspases and DNA fragmentation in MCF-7 after 72 h treatment. Apoptotic signal was not detected in the normal MCF-10A breast cell incubated with the extract for 72 h or longer (Fig. 10g-l).

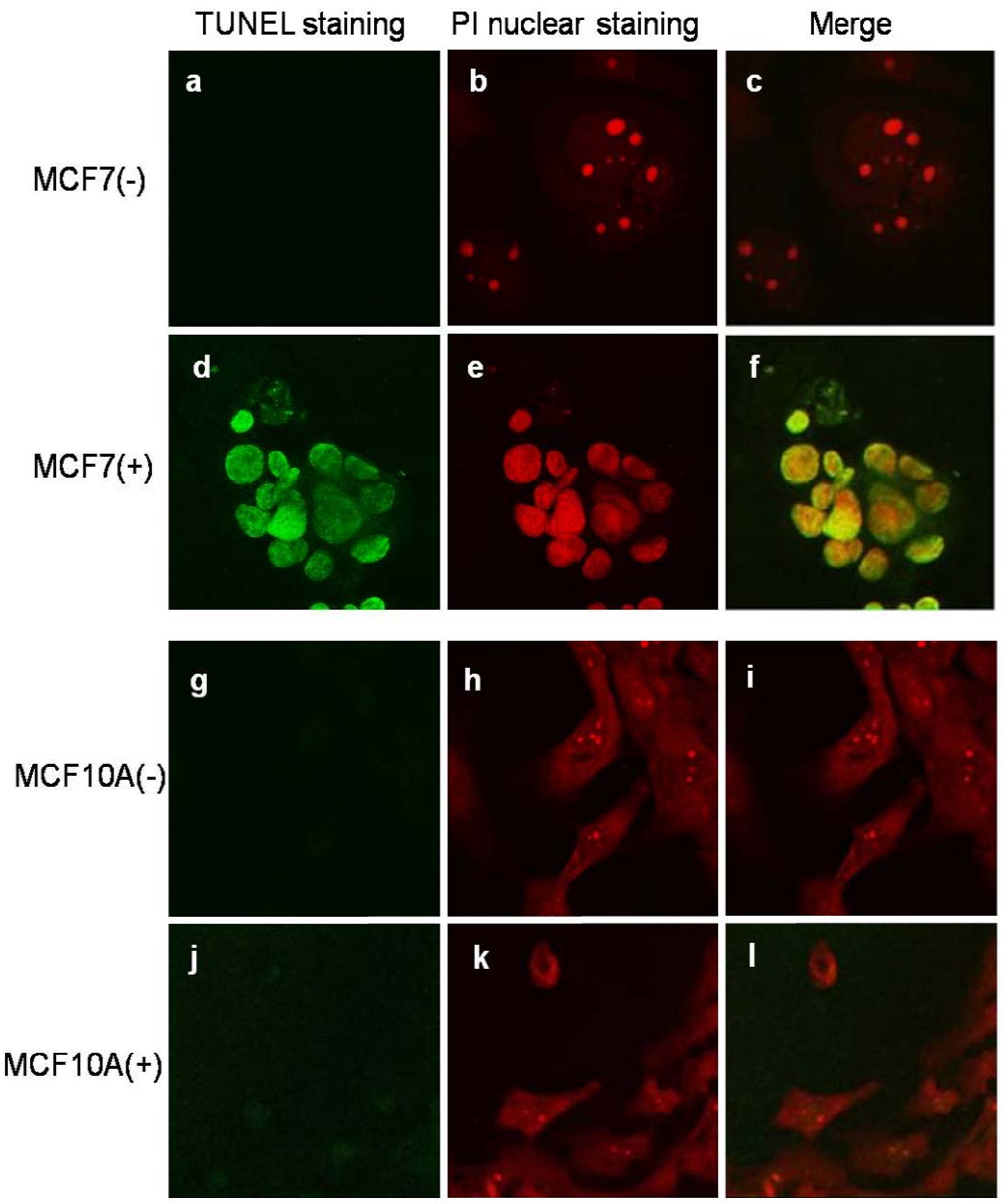



**Fig. 10.** TUNEL detection of apoptotic signal in the control and *C. militaris* extract-treated MCF-7 breast cancer cells (a-f) and MCF-10A breast cells (g-l). MCF-7 and MCF-10A cells were incubated in the presence or absence of 50 μg/ml extract for 72 h and subjected to TUNEL staining. The green signal indicates the apoptotic signal (a and d for MCF-7; g and j for MCF-10A), the red signal indicates the PI staining in nucleus (b and e for MCF-7; h and k for MCF-10A). TUNEL and PI staining were merged for the purpose of comparison (c, f, i and l). The pictures are the representative ones from three independent biological replicates.

# DISCUSSION

### *1. Effect of the C. militaris extract on apoptotic pathway in MCF-7*

We investigated the mechanism of the anti-cancer effect of the *C. miliatris* extract on MCF-7 breast cancer cells by examining transcriptional changes of three early-response apoptotic genes (two early-response pro-apoptotic marker genes *Bax* and *Bim* and one anti-apoptotic marker gene *Bcl-2*) and three late/terminal phase response genes (two later-response genes, *Cytc* and *Apaf*-1, and one involving in the terminal phase of apoptosis, *caspase*-7). Real-time PCR analysis showed clearly that the *C. militaris* extract indeed increased the levels of *Bax*, *Bim*, *Cytc*, *Apaf*-1 and *Caspase*-7 transcripts while inhibiting the level of *Bcl*-2 transcript in MCF-7 cells (Fig. 8).

Over-expression of *Bax* and *Bim* implied the increased translation of Bax and Bim protein, which induces apoptosis in MCF-7. Upon the apoptotic stimulation, the pro-apoptotic marker gene *Bax* undergoes over-expression and the over-expressed Bax protein undergoes conformational change in the putative pore-forming domain composing α-helices-5 and -6 and moves to the mitochondria outer membrane (Heimlich *et al.*, 2004; Wolter *et al.*, 1997). The α-helices insert into mitochondrial membranes and form a pore to facilitate intermembrane cytochrome c releasing into the cytosol. The induced expression of *Cytc* transcript indicates the compensation of cytochrome c in mitochondria after the release, confirming this apoptosis cascade at transcriptional level.

All of the three isoforms of Bim: BimEL (196 a. a), BimL (140 a. a) and BimS (110 a. a) induce apoptosis in cancer cells (Abrams *et al.*, 2004). The isoform BimS is expressed only in the cells undergoing apoptosis and is regarded as the most potent

isoform of Bim to induce apoptosis (Abrams *et al.*, 2004; Li *et al.*, 2005; Weber *et al.*, 2007). In BimS-induced apoptosis in epithelial cells, BimS recruited and activated Bax and facilitated the release of cytochrome c from mitochondria to cytosol (Weber *et al.*, 2007). The induction of *BimS* transcript by 6 h suggests the over-expressed Bim contributes to apoptosis induction by activating Bax in MCF-7 cells incubated with the *C. militaris* extract. This result is similar to the induced expression of *Bim* at the mRNA level in breast cancer cells treated with Smad4 (Li *et al.*, 2005). In addition to *BimS*, the other two homologous *BimEL* and *BimL* transcripts were also detected to be induced in MCF-7 cells after 12 h incubation. Although the capabilities of apoptotic induction of BimEL and BimL are weaker than BimS, the cleaved phosphorylated BimEL fragment acts as an apoptotic inducer by combining with Bcl-2. BimL also contributes to apoptosis induction although it has the least apoptosis induction capability among these three homologous. Hence, in MCF-7 cells incubated with the *C. militaris* extract, *Bim* is induced and initiates apoptosis through activating *Bax* and suppressing *Bcl*-2.

Bcl-2 acts as a suppressor of apoptosis through the combination with Bax to block this apoptosis inducer (Oltvai *et al.*, 1993) and inhibits caspase activation (Pratt and Niu, 2003). The decrease expression of *Bcl-2* detected in MCF-7 as early as 6 h incubation with the *C. militaris* extract suggests decreased expression of Bcl-2 in MCF-7 cells, indicating the inducing of apoptosis from another angle of lacking suppressor.

The released cytochrome c associates with Apaf-1 and then initiates apoptosis through the activation of caspase 9 (Li *et al.*, 2005). The sharp increase of Apaf-1 transcript in the later stage (48 h) from real-time PCR implied this combination and activation of Apaf-1 in the apoptosis pathway. Upon all the elements (cytochrome c,

Apaf-1 and dATP/ATP) assembled to form apoptosome, caspase-9 is recruited and auto-activated on this apoptosome. Because of the lacking caspase-3 expressed in MCF-7 cells, the mitochondria-mediated apoptosis pathway in MCF-7 bypasses caspase-3 and goes through caspase-7. The activated caspase-7 then activates caspase-6, which eventually induces apoptosis in these cells (Liang *et al.*, 2001). The increased level of *Cytc*, *Apaf*-1 and *caspase*-7 transcripts as shown in Fig. 8 suggests that the complete apoptotic cascade starting from the induction of early-apoptotic element Bax to the terminal-apoptotic component caspase-7 does occur in MCF-7 incubated with the *C. militaris* extract for 6 h or longer. The activation of caspase-7 leads to DNA degradation as detected by TUNEL system in MCF-7 breast cancer cells incubated with the extract (Fig. 10). From the result of real-time PCR, transcripts of the apoptotic genes were induced as early as 6 h after the treatment, but the DNA fragmentation was detected 72 h after the treatment. The reason for the DNA fragmentation as detected by TUNEL lagging behind apoptotic gene transcription as seen in real-time PCR could be due to the time needed to respond to the signal in the apoptosis cascade.

In the investigation of the cyto-toxicity of this herb on MCF-10A normal breast cells, although the growth of MCF-10A was slow down or partially arrested, the morphology of MCF-10A cells kept normal even after 96 h incubation in the extract-containing medium. MCF-10A cells were able to resume to its normal growth rate under the current experimental condition after being removed from the extract-containing medium (Fig. 5b). However, the effect of the *C. militaris* extract on MCF-7 was irreversible and lethal. MCF-7 cells were not able to recover at all after 96 h incubation in the extract-free medium in the recovery assay. This prompted us to speculate that the

inhibitory effect of the *C. militaris* extract on MCF-10A normal breast cells is presumably due to cell growth arrest rather than cell death resulting from apoptosis as observed in MCF-7 breast cancer cells.

This speculation is also supported by the results from TUNEL staining of MCF-10A cells. There is no apoptotic signal observed in MCF-10A normal breast cells incubated with the extract that displayed cell arrestment. This result could in some way explain the results in the recovery assay: there is no typical cellular feature of apoptosis but slow growth of MCF-10A cells. These data suggest that the *C. militaris* extract interferes or inhibits certain pathways related to cell growth in MCF-10A rather than induces cell death. While in the breast cancer cells, the *C. militaris* extract induces apoptotic genes, which eventually lead to cancer cell death. It could be inferred that the retarded growth of MCF-10A by the *C. militaris* extract is due to the reversible inhibition of certain pathway related to cell cycling. At this point, we do not have direct evidences to support which pathway is actually arrested by the *C. militaris* extract in MCF-10A cells.

As reported in literatures, apoptosis is one of the major types of cell death (Nelson and White, 2004). The induction of apoptosis has been demonstrated as an inhibitory mechanism in cancer cells, including human breast cancer cells (Zhu *et al.*, 1998; Liu *et al.*, 2000; Fesik, 2005; Rama *et al.*, 2005; Yu *et al.*, 2006). But so far there is no report to identify the induction of apoptosis by the *C. militaris* extract in MCF-7 breast cancer cell although a recent report indicated the inhibitory effect of the *C. militaris* extract on MCF-7 cell growth (Wu *et al.*, 2007). The data from the present study demonstrated clearly that inhibition of MCF-7 growth by the *C. militaris* extract was via induction of apoptosis, as

evidenced by the over-expressed early-response pro-apoptotic marker genes and late-response genes in real-time PCR analysis (Fig. 8) and the detection of DNA fragmentation using TUNEL system (Fig. 10).

We could not exclude the possibility that other mechanisms might also contribute to the inhibition of cancer cell growth by the *C. militaris* extract since the extract contains multiple components. Although cordycepin (Seiv *et al.*, 1969; Wu *et al.*, 1972) and several recently-isolated compounds from Cordyceps have been showed to have anti-cancer and anti-virus activities (Bunyapaiboonsri *et al.*, 2007), the effective anti-cancer compounds still remain unknown. Isolation and identification of the active component(s) from the *C. militaris* extract will be a very promising investigation.

In summary, in my experiments, the increase or decrease of apoptotic gene transcripts in real-time PCR analysis suggests the importance of the apoptotic pathway in MCF-7 cells incubated with the *C. militaris* extract. When MCF-7 cells receive the apoptotic stimuli from the *C. militaris* extract, over-expressed Bax initiates the apoptosis cascade resulting in the release of cytochrome c from mitochondria into cytosol. The released cytochrome c activates caspase-9 subsequently. Then the activated caspase-9 activates caspase-7 and caspase 6, eventually leading to apoptosis. At the same time, the over-expressed Bim recruits and activates Bax. The decreased expressed Bcl-2 also contributes to Bax activation by minifying the effective Bcl-2. Both the increase in Bim and decrease in Bcl-2 contribute a positive feedback of Bax activation and facilitate the apoptosis cascade. Our finding may help to better understand the mechanism of the *C. militaris* extract effect on breast cancer cell growth and thus provide a potential therapy to breast cancer in future.

### *2. Effect of C. militaris extract on DNA methylation in MCF-7*

The general level of 5-methylcytosine in breast cancer cells is lower than that in normal cells. This situation is named as global hypomethylation in tumor cells. The global hypomethylation always happened in the genes encoding proteins that involve in cancer cell proliferation, invasion and metastasis (Luczak and Jagodzinski, 2006). However, there is also a hypermethylation at CpG islands of some tumor-suppressor genes in cancer cells. Because of the hypomethylation in tumor-inducer genes and hypermethylation in tumor-suppressor genes in cancer cells, less methylation mainly has effect on the hypermethylated tumor-supressor genes, assisting re-expression of these genes. The more tumor-suppressor gene expressed, the more tumor inhibition occurred.

In MCF-7 breast cancer cell, there are two tumor-suppressor genes *RAR-β2* and *GSTP1* being methylated and not expressed during the tumorigenesis (Yang *et al*., 2001; Widschwendter *et al.*, 2000). RAR-β2 encodes retinoic acid receptor beta, one of thyroid-steroid hormone receptor superfamily members which are transcription regulators activated by ligand binding (Chambon, 1996; Minucci and Pelicci, 1999). RAR-β limits the growth of tumor in breast. The other gene *GSTP1* encodes corresponding glutathione (GST) in cytosol. GST is a family of enzymes who have play important roles in detoxification of a wide range of xenobiotics and chemotherapeutic agents (Esteller *et al.*, 1998). GSTs conjugate and inactivate the electrophilic carcinogens.

DNMTs were found to be highly expressed in cancer cells compared to those in normal cells (Robertson *et al.*, 2000; Girault *et al.*, 2003).

From the real-time PCR, *DNMT1* transcript in MCF-7 cells was greatly suppressed by the *C. militaris* extract after 6 h incubation. The decreased transcript

suggests the decreased expression of DNMT1 in breast cancer cells. In the progression of tumorigenesis, DNMT1 acts as a methyltransferase to methylate the tumor-suppressor genes. When the amount of DNMT1 decreased as a result of the *C. militaris* extract treatment in MCF-7, the methylated tumor-suppressor genes will be re-expressed by less methylation, thus inhibiting cancer cell growth.

However, DNMT1 is not the sole effective component to methylate tumor-related genes. DNMT3a and DNMT3b do have *de novo* methyltransferase activity. The suppression of DNMT3a transcript indicates the downregulation of DNMT3a in MCF-7 cells by the *C. militaris* extract (Fig. 9). There is no suppression of DNMT3b detected in MCF-7 cells incubated with the *C. militaris* extract. Although DNMT3a and DNMT3b are responsible to methylate genomic DNA (Das and Singal, 2004), they contribute less to keep the methylation during the cell division when compared to DNMT1. DNMT1 was found to be a component of DNA replication complex (Szyf *et al.*, 2004). In human genome, DNMT1 performs as an enzyme to methylate cytosine residues in the newly synthesized DNA (Bestor, 1988; Bestor and Verdine, 1994; Momparler, 2003). Since the DNMTs were found to be expressed highly in breast cancer cells, the methylation of specific cancer-suppressor genes must occur to maintain the tumor growth. In order to keep the methylation in cancer cells, methyltransferase (DNMT1) play an essential role to add methyl group to the newly synthesized DNA strains. The greatly decreased expression of DNMT1 transcript in the real-time PCR suggests the disruption of methylation in the breast cancer cells by the *C. militaris* extract. Consequently, with less methylation, the tumor-suppressor gene will be re-expressed and the growth of MCF-7 cell will be suppressed. This result could also explain the inhibitory effect of the *C.*

*militaris* extract on MCF-7 cell growth in the previous experiments as reported in Chapter 1. However, there is an inconsistence in DNMT3a and DNMT3b suppression studies in literatures. This inconsistence might be due to the specific distribution of DNMT3b in certain types of cells such as leukemia, melanoma, and colorectal cells. From investigation by other groups, DNMT3a and DNMT3b could play a redundant role that either of them could enact the methyltransferase activity (Xie et al, 1999).

Based on the literature reports and our current real-time PCR result, it could speculate that the downregulation of DNMT1 and DNMT3a in MCF-7 by the *C. militaris* extract assists the tumor-suppressor genes to regain the function and contributes to the anti-cancer effect in MCF-7 cells.

To summarize all the data we currently have, the water extract of *C. militaris* inhibits human breast cancer cell growth by activating the early-response pro-apoptotic gene *Bax* and *Bim* and subsequently activating the late-response apoptotic genes in mitochondria-mediated apoptotic cascade eventually leading DNA fragmentation and cell death. In this pathway, the anti-apoptotic gene is suppressed to facilitate the accomplishment of the cascade. The anti-cancer effect of the *C. militaris* extract could also be the result of DNA-cytosine methyltransferase gene suppression, resulting in the expression of tumor suppressor and inhibition of tumor growth.

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# CHAPTER III: SUMMARY AND PERSPECTIVES ON FUTURE RESEARCH

Although anti-cancer effect of *C. militaris* has been studied in several cancer cell lines including MCF-7 human breast cancer cell line (Lee et al., 2006; Yoo et al., 2004; Park et al., 2005; Lin and Chiang, 2008), the anti-cancer effect of *C. militaris* (growing on the silkworm pupae) on MCF-7 cell growth and the mechanism of the anti-cancer effect of *C. militaris* extract have not been investigated. From the investigations of this project, the dose- and time-dependent inhibitory effects of the *C. militaris* extract on MCF-7 cell growth is through the induction of apoptosis by activating pro-apoptotic genes (*Bax, Bim, Apaf-1,* and *Caspase-7*) and anti-apoptotic gene (*Bcl-2*). The down-regulation of DNA-cytosine methyltransferase gene (*DNMT1, DNMT3a* and *DNMT3b*) also potentially contributes to the cancer cell death through the less methylation on cancer-suppressor genes. But this hypothesis need to be further confirmed.

The *C. militaris* extract has also been shown to arrest MCF-10A normal breast cell growth, but MCF-10A cell was able to recover to its normal growth rate after being incubated with the extrct-free medium for 96 h. Despite the DNA fragmentation was not detected in normal breast cells after 72 h of incubation in extract-containing medium, the apoptotic gene transcripts in the treated normal breast cells need to be investigated in order to confirm that the effect is not through the induction of apoptosis.

The extract used in this project contains multiple components. In order to make a further investigation of this herbal medicine, the extract needs to be fractioned using HPLC. The effective fraction(s) need to be identified using the dose- and time-response

assays on breast cancer and normal cells. It will contribute a lot to the development of new medicine if the purified component(s) is identified using GS/MS. Also, the mechanism of the inhibitory effect is worth being investigated. In the study of anti-cancer mechanism, more techniques could be involved in addition to the real-time PCR, such as proteomic approach including 2D-gel electrophoresis and MALDI-TOF analysis that could identify the differentially proteins between the treated and control cells, with a potential leading to discovery of new mechanisms involved in cancer inhibition. Moreover, more cancer cell lines could be tested to explore the anti-cancer effect of this herbal medicine, using other types of extracts (ethyl acetate, ethanol extract and petroleum ether, etc.) in addition to water extract. In order to show the clinical value of this herbal medicine, the *in vivo* studies in mice or rats could also be performed.

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***Cordyceps militaris* inhibits MCF-7 breast cancer cell proliferation by inducing apoptotic gene expression** Tongtong Xu, Shiheng An, Yaning Sun, Songjie Wang, Chuanxi Zhanga and Qisheng Song (submitted)