Research article

Water extract of *Rheum officinale* Baill. induces apoptosis in human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines.

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Abstract

Ethnopharmacological relevance: Rheum officinale Baill. (Da Huang) is one of the herbs commonly used in traditional Chinese medicine formulae against cancer. The traditional decotion is similar to the water extract used in the present study.

Aim of the study: The water extract of Da Huang was investigated to see if it possesses anticancer effects through apoptotic pathways.

Materials and methods: Human lung adenocarcinoma A549 and human breast cancer MCF-7 cell lines were treated with different concentrations of Da Huang water extract at different time intervals. Growth inhibition was detected by MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and colony formation assays; apoptosis was detected by cell morphologic analysis, DNA fragmentation analysis and COMET assay.

Results: Da Huang water extract was found to have significant growth inhibitory effects on both A549 and MCF-7 cell lines with IC_{50} values 620 ± 12.7 μg/ml and 515 ± 10.1 μg/ml, respectively. Growth inhibitory effects were dose- and time-dependent. A significant decrease in cell number, DNA fragmentation and single DNA strand breakages were observed in the Da Huang water-extract treated A549 and MCF-7 cells.

Conclusions: This suggests that the water extract of Da Huang exerts potential anticancer activity through growth inhibition and apoptosis on MCF-7 and A549 cells lines.

Keywords: *Rheum officinale* Baill.; Traditional Chinese medicine; Anticancer; Apoptosis; MCF-7; A549

1. Introduction

The use of herbal intervention is widespread in all regions of the developing world and is rapidly growing in developed countries (Cassileth, 1995; Yan et al., 2006). In spite of the extensive use of herbal therapies, there is insufficient scientific evidence validating their efficacy and safety. Thus, basic research aimed at elucidating the underlying mechanisms of any potential herbal effects are very important for the use of herbal medicine. Recently scientists have focused on the potential role of extracts of traditional Chinese medicinal herbs as alternative and complementary medications for cancer treatment. The extracts of Chinese medicinal herbs are often used together with traditional cancer therapy to improve the survival rate and quality of life, as the use of herbal extracts are much less expensive than the standard antineoplastic therapies currently available (Cha et al., 1997; Liu et al., 2001; Zou and Liu, 2003; Han et al., 2003; Hao et al., 2007).

Cancer is a significant worldwide health problem generally due to the lack of widespread and comprehensive early detection methods, the associated poor prognosis of patients diagnosed in later stages of the disease and its increasing incidence on a global scale. Indeed, the struggle to combat cancer is one of the greatest challenges of mankind. In industrialized countries, lung carcinoma is the leading cause of cancer death in men while breast carcinoma which is the most frequent cause of cancer death in women (Divisi et al., 2006). To date, the available treatment regimens are not able to achieve a cure, nor improve survival substantially, except in rare cases. Thus, new targets for prevention and new agents for therapy need to be identified.

Da Huang, *Rheum officinale* Baill., (in the family of *Polygonaceae*) is a Chinese herbal medicine that has been widely used as a laxative (Tsai et al., 2004), antiphlogistic (Moon et al., 2006; Wu, 1985) and haemostatic (Wang et al., 1985) agent in the treatment of obstipation, gastrointestinal indigestion, diarrhea and jaundice. In addition, it is one of the herbs commonly used in traditional Chinese medicine formulae prescribed to cancer patients (Li, 2001; Li et al., 2007). The major pharmacologic constituents of Da Huang (the rhizome and root portions of the plant) are anthraquinone and bianthrone derivatives. In addition, Da Huang has been reported to have anti-tumor activity with hepatocarcinoma (Cao et al., 2005). Notwithstanding these reports, its role as an anticancer agent has not been mechanistically established. To identify the possible biochemical pathways involved, and assess the therapeutic potential of Da Huang in cancer, we evaluated the effects of this herb on cell lines derived from the most common types of cancer found in males (lung cancer) and females (breast cancer). Therefore, we have selected the human lung adenocarcinoma A549 and the human breast carcinoma MCF-7 cell lines for use in this investigation. Additional studies using DNA fragmentation analysis and the COMET assay permitted determination of the role in which apoptosis may play in the anti-cancer activity of Da Haung.

2. Materials and methods

2.1. Herbs

The dried *Rheum officinale* Baill. (Da Huang) was purchased from Hip Shing Hong Ltd., Hong Kong SAR, China. The voucher sample (Lot. No. 07-823) of Da Huang herb was authenticated and stored in Dr. Peter H.F. Yu's laboratory, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR.

2.2. Chemicals and Materials

Celltiter 96 aqueous MTS reagent was purchased from Promega Chemicals Co. (Madison, WI, USA). All other chemicals used were of analytical grade and were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Cell culture medium, Dulbecco's modified eagle medium (DMEM), and fetal bovine serum (FBS) were purchased from Gibco Laboratories (Grand Island, NY, USA). Human lung adenocarcinoma A549 cell line (CCL-185) and human breast carcinoma MCF-7 cell line (HTB-22) were purchased from The American Type Culture Collection (Manassas, VA, USA).

2.3. Extraction

Dried Da Huang herb was ground into fine powder. Da Huang powder (1 g) was weighed and mixed with 10 ml distilled water and shaken in a horizontal shaker at 37 ℃, 300 rpm for 2 hr. After 2 hr, the solution was centrifuged at 2735 ×g for 3 min. The supernatant was collected and the residue was re-extracted for two more times with same volume of distilled water. Finally, all of the supernatant was filtered using filter paper (Whatman no. 4). The filtered solution of Da Huang was lyophilized in

freeze dryer (Labconco, Freezone 6) for about 1 week and the Da Huang powder (water extract) was then stored at -20 ℃ until further use. The extraction yield was 12.36%.

2.4. Cell culture

The human breast carcinoma MCF-7 and human lung adenocarcinoma A549 cell lines were maintained in low glucose DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA) on culture plates at 37 ℃ in a humidified incubator with 5% carbon dioxide supplementation. The cells were fed every 2–3 days and subcultured once they reached 70–80% confluence.

2.5. MTS assay

The effects on inhibition of cell growth were measured by the MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] growth inhibition assay. The MTS growth inhibition assay was performed according to the instructions provided by the manufacturer (Promega). Briefly, the same number of cells was seeded into each well of a 96-well plate on day 1. On day 2, the cells were either treated with different concentrations of Da Huang water extract over different incubation periods (24, 48 and 72 hr), or remained as untreated controls. At the end of each time point, fresh complete medium containing 10 μl of MTS solution was added and further incubated for 2 hr. Optical density of each culture was then recorded at 490 nm using a microplate reader (Bio-Rad, model 550). Each experiment was performed in triplicate. Results are expressed as the percentage growth inhibition with respect to the untreated cells.

2.6. Morphological monitoring of Da Huang water extract treated cancer cells

The effects of 72-hour-treatment of Da Huang water extract (0 and 600 μg/ml) on the morphology of A549 and MCF-7 cells were recorded using a Nikon digital camera under an inverted microscope at ×200.

2.7. Soft agar colony formation assay

Soft agar colony formation assays were performed using a double-layer soft-agar method. In each well of a 6-well plate, 20,000 cells were mixed with Da Huang water extract (0, 100, 200, 300, 400, 600 and 800 μg/ml) and plated in top agar (0.5% agarose) over a base agar of 0.5% agarose. Cells were incubated for 7 to 14 days under standard conditions. Colonies with more than 50 cells were counted.

2.8. DNA fragmentation detection assay

Cell pellets (treated with 0 and 600 μg/ml of Da Huang water extract for 72 hr) were lysed with 600 μl nuclear lysis buffer (10 mM Tris-HCl pH 7.5, 400 mM NaCl, 100 mM EDTA, 0.6% SDS) and 10 μl RNase (4 mg/ml), and incubated in a 37 ℃ water bath for 5 min after gently mixing. 200 μl protein precipitation solution (6 M NaCl) was then added and chilled on ice for 5 min after the mixture was centrifuged at 17,779 ×g for 10 min (at room temperature) and the supernatant was collected. The supernatant was then mixed with 600 μl isopropanol and chilled on ice for 15 min. This mixture was then centrifuged at 17,779 ×g for 20 min, and the pellet was washed with 600 μl of 70% ethanol. The DNA pellet was air-dried, resuspended in 200 μl of Tris-EDTA buffer [10 mM Tris-HCl (pH 8.0) and 1 mM EDTA] and the concentration of DNA was determined spectroscopically. DNA was electrophoresed

on a 1.5% agarose gel at 100 V for 2 hr and analyzed. The fragmentized inter-nucleosomal DNA was visualized using a UV transilluminator.

2.9.COMET assay (Single cell gel electrophoresis, SCGE)

Drug-induced DNA damage was analyzed using the COMET assay as described in Klaude et al., 1996 and Singh et al., 1988, with modifications. Cell pellets (treated with 0, 400, 600 and 800 μg/ml of Da Huang water extract for 72 hr) were collected by centrifugation and re-suspended with 200 μl PBS and 800 μl of 1% low melting point (LMP) agarose. The mixture was then pipetted onto a frosted glass microscope slide pre-coated with a layer of 1.0% normal melting point agarose, prepared in PBS, covered with cover slips, and incubated at 4 °C for 10 min. After the LMP agarose solidified, the cover slips were gently removed, then 0.8% LMP agarose pre-coated cover slips were added and the slides were allowed to solidify at 4 °C for 10 min. After 10 min, the cover slips were removed and the cells were lysed in high salt solution (2.5 M NaCl, 10 mM Tris–HCl, 100 mM EDTA, pH 10, with 1 % Triton and 10% dimethyl sulfoxide added fresh) for one hour. The slides were then placed in a horizontal electrophoresis unit containing fresh buffer (1 mM EDTA, 300 mM NaOH pH 13) and incubated for 20 min to allow unwinding of DNA. Electrophoresis was then conducted in freshly prepared electrophoresis buffer (pH 13) for 20 min at 25 V and 300 mA (0.8 V/cm) at 4 ℃. Subsequently, the slides were gently washed with neutralization solution (0.4 M Tris–HCl, pH 7.5) for 20 min and stained with 20 μl ethidium bromide (15 μg/ml). Stained nucleoids were scored visually using a fluorescence microscope (Leica) equipped with a digital camera. 100 comets on 2 slides were acquired using the IM50 software image analysis system. Tail length was calculated and expressed in mean ± S.E.M.

2.10. Statistical analysis

All data are presented as means ± Standard Errors of Means (S.E.M). Statistical analysis was performed by Analysis of Variance (ANOVA) to detect significant differences in multiple comparisons with the Bonferroni post test: comparing all pairs of columns. A value of probability (p) < 0.05 was considered to be statistically significant. All statistical analyses were performed using Graph Pad Prism 4.02 for windows (GraphPad Software, San Diego California, USA).

3. Results

3.1. Inhibitory effect on cell growth

To test the grow inhibitory effects and estimate the IC_{50} values of Da Huang on A549 and MCF-7 cells, a wider concentration range (0 to 1000 μg/ml) of its water extract was used. The Da Huang water extract showed significant growth inhibitory effects on A549 and MCF-7 cells after 72-hour-treatment with IC_{50} values equal to 620 ± 12.7 μg/ml and 515 ± 10.1 μg/ml, respectively (Fig. 1). To investigate whether the anticancer effect of the Da Huang water extract is time- and concentration-dependent, A549 and MCF-7 cells were treated with different concentrations (0, 400, 600, 800 μg/ml) of Da Huang water extract for 24, 48 and 72 hr. The data indicate that the growth inhibitory effects of the Da Huang water extract on A549 and MCF-7 cells was both time- and concentration-dependent (Fig. 2A and 2B).

3.2. Morphological monitoring

The number of A549 and MCF-7 cells was decreased after 72-hour-treatment with the Da Huang water extract when compared with controls. The higher the concentration of the Da Huang water extract used to treat A549 and MCF-7 cells, the fewer cells were observed. Fig. 3 shows the representative photos of cells treated with either 0 or 600 μg/ml (a concentration close to the IC_{50} values) of Da Huang water extract. In the treatment groups, mild apoptotic behaviour scored as rounding and shrinkage of cells was detected (Fig. 3A and 3B).

3.3. Inhibition of colony formation

For the difference in the sensitivity of colony formation assay, a narrower concentration range with finer scale (0, 100, 200, 300, 400, 600 and 800 μg/ml) was

used. As shown in Fig. 4, Da Huang water extract treatment was found to exert a concentration-dependant inhibitory effect on the colony forming ability of both A549 and MCF-7 cells, with greater than 50% inhibition at 245 μg/ml and 250 μg/ml for A549 and MCF-7 cells, respectively.

3.4. Induction of chromosomal DNA fragmentation

To validate the Da Huang water extract-induced apoptotic responses in A549 and MCF-7 cells, chromosomal DNA from untreated and Da Huang water extract (600 μg/ml; a concentration close to the IC_{50} values) treated A549 and MCF-7 cells was isolated, extracted and resolved using agarose gel electrophoresis. The gel was analyzed under a UV transilluminator to detect the presence of any fragmented DNA. Da Huang water extract treatment resulted in inter-nucleosomal DNA cleavage in both A549 and MCF-7 as indicated by DNA laddering, while the inter-nucleosomal DNA from untreated cancer cells remained intact (Fig. 5).

3.5. Induction of single-strand DNA damage

To study the DNA damage in the Da Huang water extract treated A549 and MCF-7 cells after 72-hour-treatments, the COMET assay was performed. The COMET assay is a sensitive method used to monitor single strand (ss) DNA breaks at the single-cell level. Any DNA damage is represented as a tail length (tail migration) of the DNA strand. When A549 and MCF-7 cells were treated with 0, 400, 600 and 800 μg/ml of the Da Huang water extract for 72 hr, ssDNA damage was significant as indicated by the increased tail length observed in the Da Huang water extract treated cells compared with the controls (Fig. 6A and 6B). Moreover, the DNA damage induced by the Da Huang water extract was dose-dependent.

4. Discussion

Chinese medicinal herbs have been widely used and historically well documented over hundreds of years in Asia. However, there is insufficient scientific evidence concerning the mechanisms of action of extracts from these Chinese medicinal herbs. We therefore initiated this pilot study as a platform on which to launch investigations into the cellular mechanisms which mediate the anticancer effects of Da Huang, one of the best known and widely prescribed Chinese medicinal herbs, on A549 and MCF-7 cells.

The present study provided the first examination on Da Huang's antiproliferation effects. We demonstrate for the first time that the Da Huang water extract has strong dose- and time- dependent antiproliferative activity on A549 and MCF-7 cells in culture, with an IC_{50}, 620 ± 12.7 μg/ml and 515 ± 10.1 μg/ml, respectively (Fig. 1 & Fig. 2). This dose-dependent growth inhibitory effect was also confirmed in the colony formation assay (Fig. 4). These observations strongly suggest that at least some of the active components of this plant can be found in the aqueous fraction (which is the common formulation for human consumption in China). Since a crude water extract was used in this study, it is not possible to attribute the reported effects to specific compounds. In addition, it cannot be ruled out that the overall reported effects were contributed by the interactions between the various compounds in the extract. Thus, future work involving separation, purification and bioassays on purified compounds may unveil the true nature and extent of their potency for anti-cancer effects observed *in vitro*.

Two cancer cell lines derived from different tissues were used in the current study. Although the extent of the inhibition of colony formation exerted by Da Huang water extract was almost identical in both cell lines (Fig. 4), a wider variation among the cell lines (~20%) was observed in the MTS assay (Fig. 1). In the cell viability assay, MCF-7 cells showed more sensitivity to Da Huang water extract-induced growth inhibition than A549 cells, as indicated by the dose response curve for the A549 cells shifted to the right of that for the MCF-7 cell line. The variable sensitivity of these cell lines to Da Huang extract may suggest that Da Huang has a general function in suppressing cancerous cell growth but may act through multiple pathways. In fact, MCF-7 cells differ from A549 cells in the unique characteristic of being deficient in caspase-3 (Jänicke et al., 1998). It is certainly plausible then, that Da Huang acts through different pathways in these two cell lines.

The colony formation assay was developed to detect the growth inhibitory effects of compounds on the anchorage-independent growth of colonies (Price, 1986). It is a more sensitive parameter of toxicity because the number of colonies formed is assessed when the cells are in a state of proliferation. In this study, A549 and MCF-7 cells were able to grow in soft agarose, and which a number of colonies were observed in the controls. The Da Huang water extract inhibited colony formation in both the A549 and MCF-7 cell lines in a dose-dependent manner (Fig. 4). This suggests that Da Huang water extract inhibits the anchorage-independent growth of A549 and MCF-7 cells.

Apoptosis, or programmed cell-death, is a normal physiologic process that occurs during embryonic development and in the ongoing process of tissue homeostasis in

the adult animals. Any dysregulation of apoptosis can result in abnormality, disease and death (Wilson et al., 1998). The hallmarks of apoptosis include chromatin condensation, DNA fragmentation to nucleosome-sized pieces, membrane blebbing, cell shrinkage and compartmentalization of dead cells into membrane-enclosed vesicles or apoptotic bodies (Darzynkiewicz et al., 1997). Cancer is a result of uncontrolled cell proliferation as well as the dysregulation of apoptosis (Han et al., 2007). It has been suggested that cancer chemotherapeutic as well as chemopreventive agents exert part of their pharmacological effects by triggering apoptotic cell death. The induction of apoptosis or arrest of cell cycle progression in tumor cells has become an indicator of tumor treatment response (Parker et al., 1998). We show in this study that Da Huang water extract induces several features of apoptosis, such as DNA fragmentation (Fig. 5) and ssDNA breakage (Fig. 6A and 6B) in both cell lines. These data strongly suggest that Da Huang's pharmacological effects may be brought about through the induction of apoptosis in the cell lines examined.

5. Conclusions

The present results provided evidence for the potential use of Da Huang water extract in the prevention, or even treatment of human lung and breast cancers. The findings indicated that Da Huang significantly inhibited the proliferation of A549 and MCF-7 cells *in vitro*, confirmed by the cell viability and colony formation assays. Da Huang's cytotoxic activity is likely to be due to the induction of apoptosis, as demonstrated by the stereotypical morphologic changes, DNA fragmentation, and COMET assay results. These combined results suggest that the water extract of Da Huang may be an attractive alterative to manage human lung and breast cancer. *In vivo* studies in animal

models and humans would provide more evidence of Da Huang's anticancer effects and further therapeutic value. Additional analytical and molecular level (i.e., pathway) investigations are needed to identify the active components in Da Huang which induce inhibition of growth and to further dissect the molecular mechanisms involved in mediating this activity in A549 and MCF-7 cells.

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Figure legends

Fig. 1. Growth inhibitory effect of Da Huang water extract on A549 and MCF-7 cells after 72hr-treatment. Data are expressed as mean ± S.E.M., n=3. $^{**}p < 0.01$ and $^{***}p < 0.001$ represent significant differences when compared with its corresponding concentration in A549.

Fig. 2. Growth inhibitory effect of Da Huang water extract (0, 400, 600 and 800 μg/ml) on (A) A549 and (B) MCF-7 cells after 24hr-, 48hr- and 72hr- treatments. Data are expressed as mean ± S.E.M., n=3. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ represent significant differences when compared with the control group.

Fig. 3. Representative photos to show the morphology of Da Huang water extract (0 and 600 μg/ml) treated (A) A549, and (B) MCF-7 cells after 72hr-treatment using bright field microscopy (×200). Both cells treated with Da Huang water extract demonstrated rounding and shrinkage of cells (indicated by arrows).

Fig. 4. Inhibitory effect of Da Huang water extract (0, 100, 200, 300, 400, 600 and 800 μg/ml) on formation of colonies for A549 and MCF-7 cells after 10 day-treatment. Data are expressed as mean ± S.E.M., n=3.

Fig. 5. Chromosomal DNA of Da Huang water extract treated- or untreated- A549 and MCF-7 cells following electrophoresis through a 1.5% agarose gel. Lane 1: 100bp DNA Marker; lane 2: DNA from untreated A549 cells; lane 3: DNA from 600 μg/ml Da Huang water extract treated A549 cells; lane 4: DNA from

untreated MCF-7 cells; lane 5: DNA from 600 μg/ml Da Huang water extract treated MCF-7 cells. All A549 and MCF-7 cells were treated with Da Huang water extract for 72hr.

Fig. 6. Effect of Da Huang water extract (0, 400, 600 and 800 μg/ml) on DNA damage in (A) A549, and (B) MCF-7 cells after 72hr-treatment represented as comet tail length (μm). Data are expressed as mean ± S.E.M., n=100. ***$p < 0.001$ represent significant differences when compared with the control group.

Figures

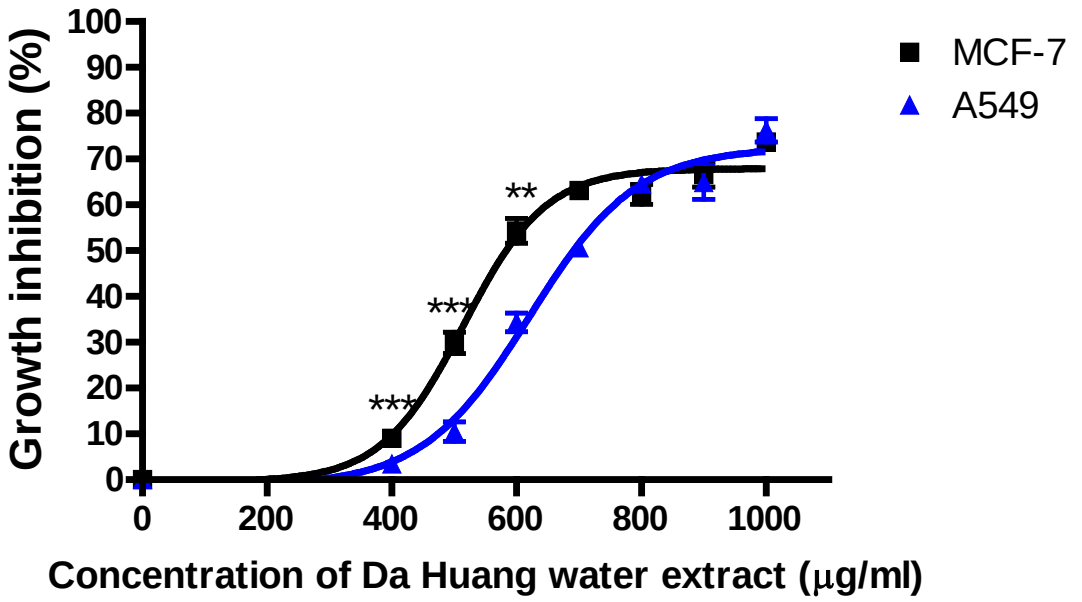
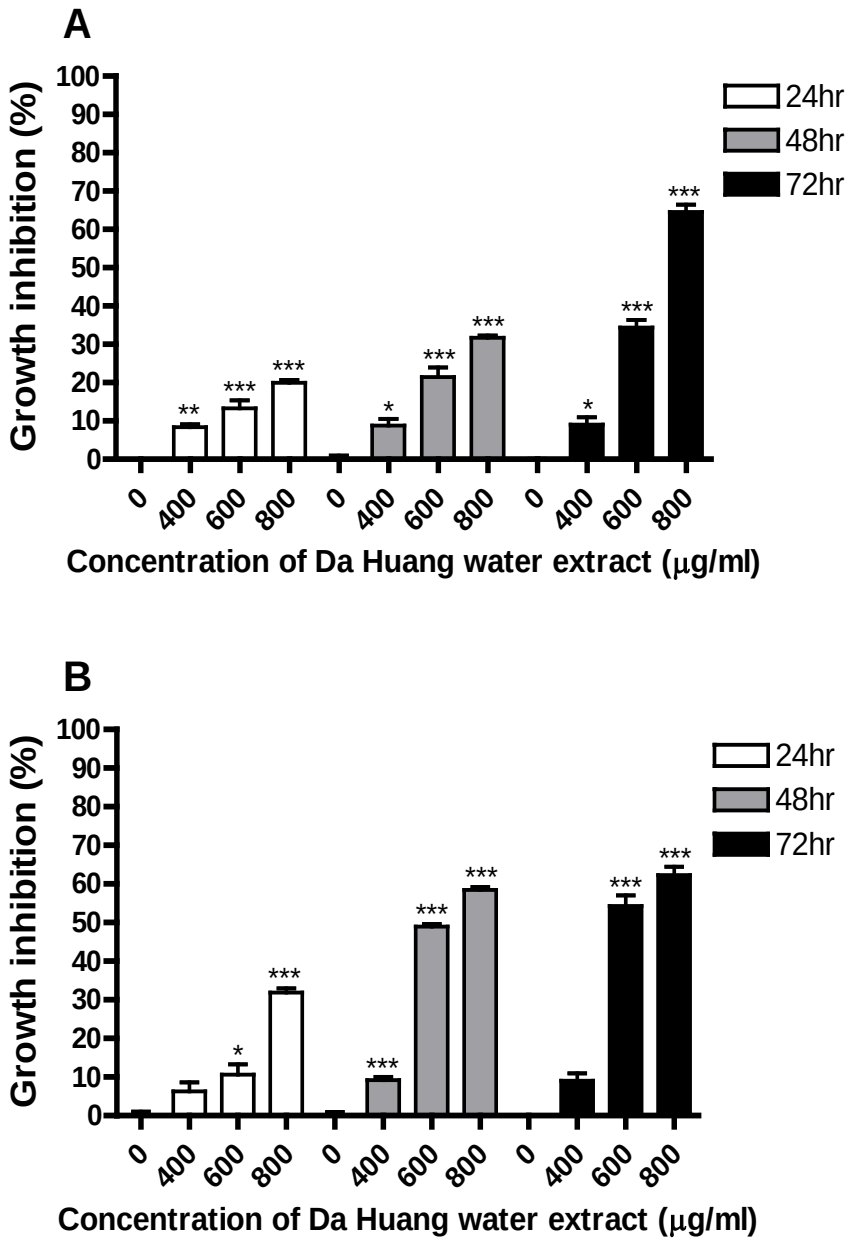


Fig. 1.


A
Growth inhibition (%)
100
90
80
70
60
50
40
30
20
10
0
24hr
48hr
72hr
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***
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0
400
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Concentration of Da Huang water extract (μg/ml)
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Growth inhibition (%)
24hr
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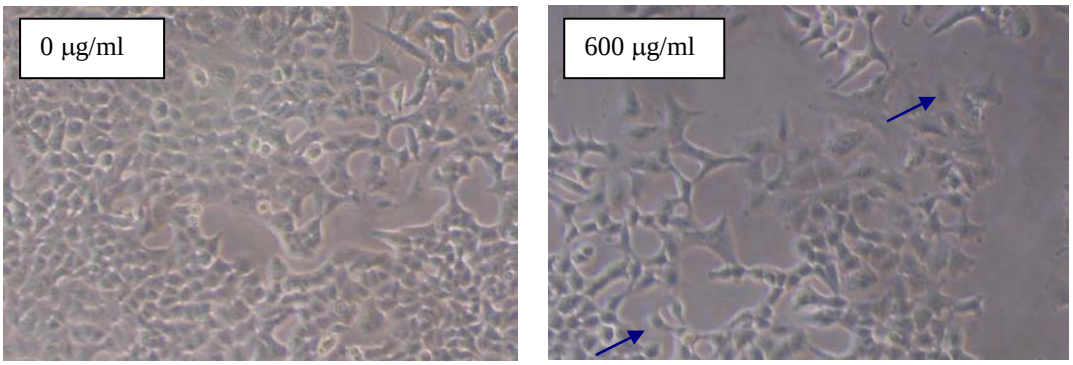
Concentration of Da Huang water extract (μg/ml)

Fig. 2.

A



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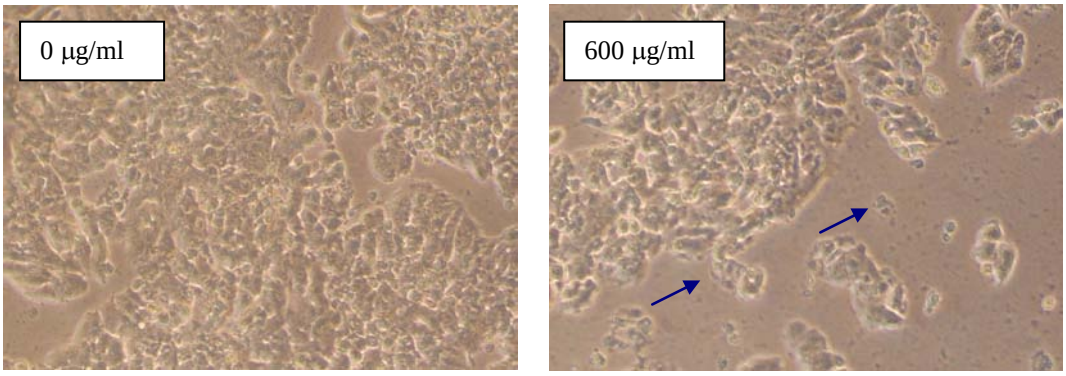


Fig. 3.

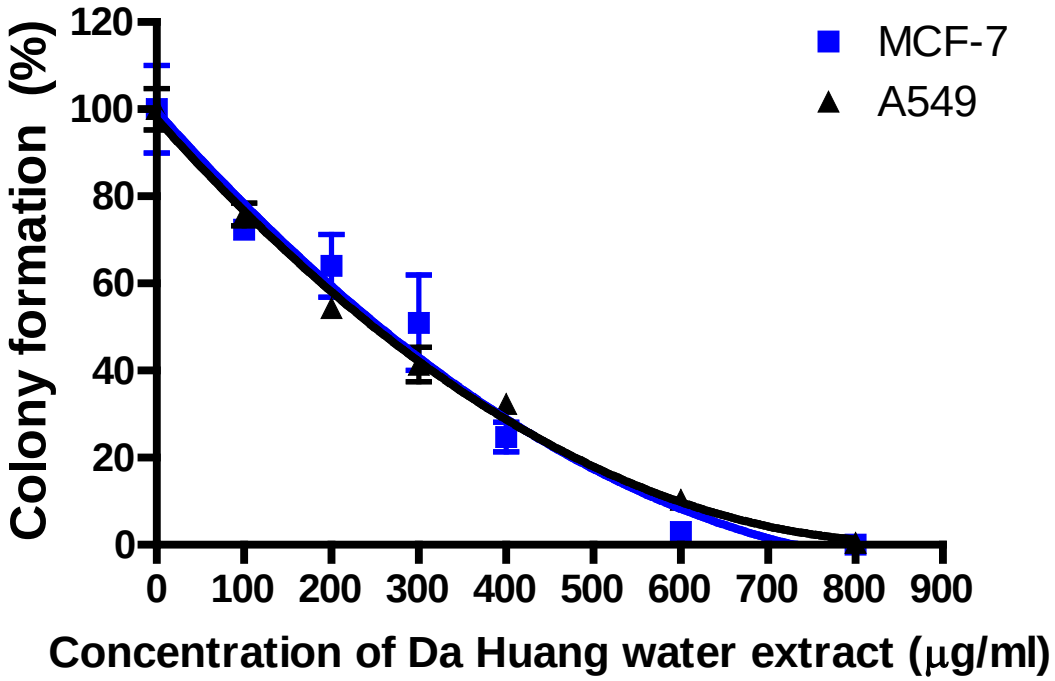

Colony formation (%)
120
100
80
60
40
20
0
0
100
200
300
400
500
600
700
800
900
Concentration of Da Huang water extract (μg/ml)
MCF-7
A549

Fig. 4.

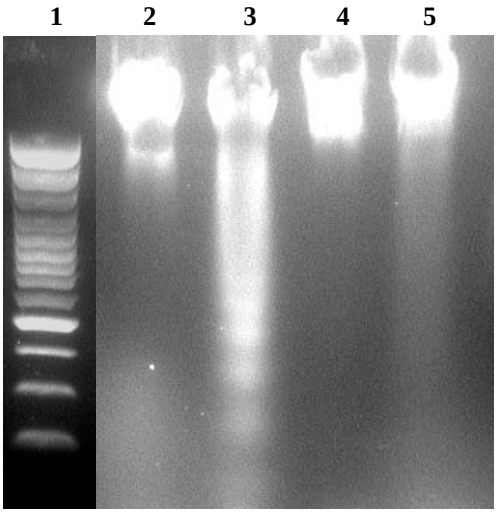
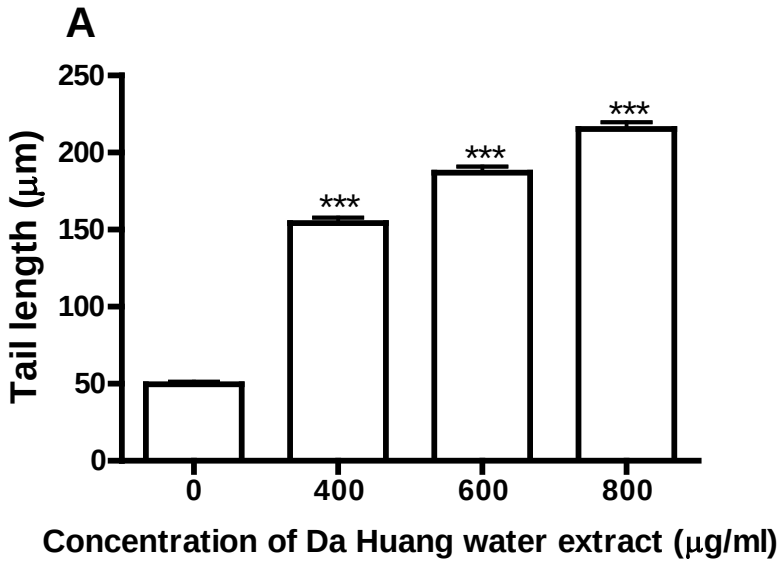


Fig. 5.


A
250
200
150
100
50
0
Tail length (μm)
***
***
***
0
400
600
800
Concentration of Da Huang water extract (μg/ml)

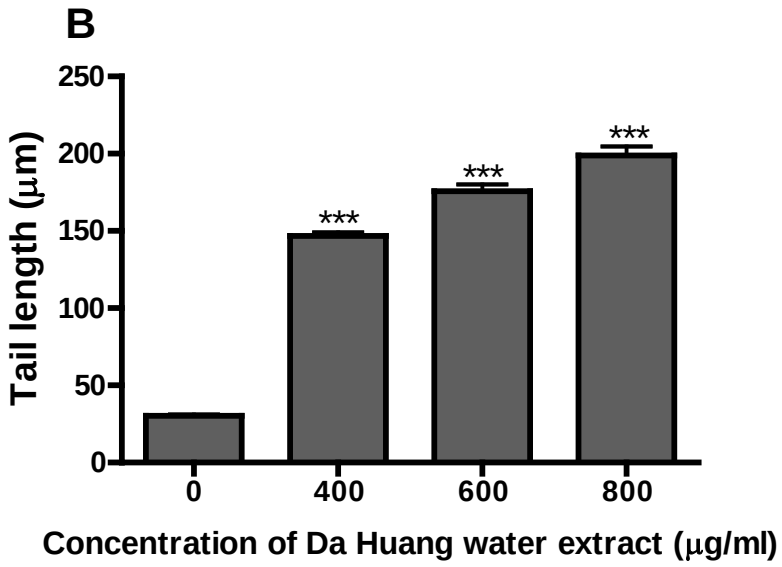

B
250
200
150
100
50
0
Tail length (μm)
***
***
***
0
400
600
800
Concentration of Da Huang water extract (μg/ml)

Fig. 6.