

Anti-Cancer Effect of *Angelica Sinensis* on Women's Reproductive Cancer**Hong-Hong Zhu^{1,2,3,4}, Guo-Hui Huang^{1,*}, Patricia L. Tate^{1,*}, Lyndon L. Larcom¹**

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ABSTRACT:

Objective: Danggui, the root of *Angelica Sinensis*, has traditionally been used for the treatment of women's reproductive disorders in China for thousands of years. This study was to determine whether Danggui have potential anti-cancer effect on women's cancer and its potential mechanism.

Methods: Danggui was extracted by ethanol. The Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay was used to compare the effects of Danggui on human breast (MCF-7 and 7368) and cervical (CaSki and SiHa) cancer cells with its effects on normal fibroblasts (HTB-125). A revised Ames test was used to test for antimutagenicity. The standard strains of *Salmonella typhimurium* (TA) 100 and 102 were used in the test. Methyl methane sulfonate (MMS) and UV light were used as positive mutagen controls and ethanol and double distilled water (DDW) as controls. The SAS statistical software was used to analyze the data.

Results: Danggui was found to be much more toxic to all cancer cell lines tested than to normal fibroblasts. There was a significant negative dose-effect relationship between Danggui and cancer cell viability. Average viability of MCF-7 was 69.5%, 18.4%, 5.7%, 5.7%, and 5.0% of control for Danggui doses 0.07, 0.14, 0.21, 0.32, and 0.64 ug/ul, respectively, with a $P_{\text{trend}} < 0.0001$. Half maximal inhibitory dose (ID₅₀) of Danggui for cancer cell lines MCF-7, CaSki, SiHa and CRL-7368 was 0.10, 0.09, 0.10 and 0.07 ug/ul,

respectively. For the normal fibroblasts, ID_{50} was 0.58 ug/ul. At a dose of 0.32 ug/ul, Danggui killed over 90% of the cells in each cancer cell line, but at the same dose, only 12.3 % of the normal HTB-125 cells were killed. Revertants per plate of TA 100 decreased with the introduction of increasing doses of Danggui extracts with a $P_{trend} < 0.0001$ when UV light was used as a mutagen. There was no difference in revertants per plate between ethanol and DDW control groups.

Conclusions: Danggui could be used as a safe and effective adjuvant therapy to prevent and treat breast and cervical cancers. Anti-cancer effects may be due to its anti-mutagenicity. Danggui should be investigated as a potential adjuvant anti-cancer therapy for women's cancer treatment and prevention of recurrence.

Key words: *Angelica Sinensis*, Danggui, cancer, women's reproductive disorders

BACKGROUND:

Incidence of cancer is increasing with the aging of the population worldwide, as well as the increase of industrialization. The incidence rate of breast cancer continues to rise in all age groups and ranks first among all cancers occurring in women since the 1990s [1, 2]. Chemotherapy and radiotherapy are the two main therapies after surgery used for cancer treatment today, but have severe effects on quality of life and can even enhance the probability of developing new cancers. In traditional Chinese medicine, many plants, vegetables, and fruits are used to help prevent human cancers, and even treat some cancers, with relatively few to no side effects when compared to chemotherapy and radiotherapy. In the past years, herbs and herbal extracts have attracted increased attention as potential cancer preventive and therapeutic agents [3-10].

Danggui, the root of *Angelica sinensis*, has traditionally been used in the treatment of reproductive disorders including menstrual disorders such as amenorrhea, dysmenorrhea, anemia, premenstrual syndrome, and menopause for thousands of years in China and other Asian countries [11]. We hypothesized that Danggui may be effective in the prevention and treatment of reproductive cancers. Anti-mutagenicity is one of the important biological mechanisms for cancer prevention and control. Some natural herbs, like Danggui, may have anti-cancer effects because of an anti-mutagenic effect. In this study, the effects of Danggui on proliferation and survival of human breast and cervical cancer cells were compared with its effects on normal human fibroblasts, and the potential anti-mutagenicity of Danggui was explored using a revised Ames test.

MATERIALS AND METHODS:

Herb extract preparation: The studied Danggui root was purchased from a chain food market (Shanghai, P.R. China). One gram of Danggui was extracted by 2 ml ethanol alcohol for 24 hours which produced the first extract. The same amount of ethanol was added for another 24 hours to obtain the second extract. It was then concentrated at low temperature under vacuum. The extracts were stored at 4°C in a refrigerator. Thermal gravimetric analysis was used to obtain the dry weights of the extracts.

Cell lines: All the human cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). These included the MCF-7, a human epithelial breast

cancer cell line; CaSki, a human epidermoid cervical cancer cell line; SiHa, a human epithelial cervical cancer cell line; a CRL-7368, human fibroblast breast cancer cell line; and HTB-125, a line of the normal fibroblast cells from breast. The cells were cultured in either Eagle's essential medium, or RPMI-1640 medium as directed by ATCC. Media were supplemented with 10% fetal bovine serum (FBS). The medium for the HTB-125 cells was also supplemented with 30 ng/ml mouse epidermal growth factor. Media were purchased from Gibco Invitrogen (Carlsbad, CA) and BioWhittaker (Walkersville, MD).

Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay

Principle: MTS (Owen's reagent, 3-[4, 5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium, inner salt) is bio-reduced by dehydrogenase enzymes of living cells into a formazan product that is soluble in tissue culture medium. The absorbance of the formazan at 490nm can be measured directly from 96 well assay plates without additional processing. The absorbance is directly proportional to the number of viable cells. The advantages of this assay are that it is non-radioactive, fast, safe, and convenient.

Procedures: Cells were examined microscopically and washed with sterile phosphate buffered saline (PBS) A solution of 5% trypsin-EDTA in medium was added and the flask was incubated at 37 °C, 5% CO₂ for 5 minutes. This cell suspension was transferred to a centrifuge tube and 1.0 ml newborn calf serum was added to stop the action of trypsin. The mixture was centrifuged at low speed. The cell pellet was resuspended in 5-6 ml of the appropriate medium. The suspension was vortexed lightly and the cells were counted and divided into aliquots. Appropriate volumes of Danggui extract, ethanol, or medium were added to give the same dose of ethanol in the controls as in the Danggui-treated aliquots, and the same cell numbers in each aliquot. Fifty thousand cells were added to each well of a 96-well plate. Four wells were used for each treatment. The plates were incubated at 37 °C, 5% CO₂ for 2 days. The medium was removed and the cells were washed two times with PBS. The PBS was removed and 200 ul of fresh culture medium and 20 ul of the CellTiter 96 solution (Promega Corporation, Madison, Wisconsin) were added to each well. The plates containing cultures were incubated at 37 °C, 5% CO₂ for 4 hours. Absorbance at 490nm was measured using a Bio-RAD (Hercules, CA) Model 3550-UV Micro plate reader and the absorbance of the 4 wells containing the same samples were averaged. Cell Titer 96® Aqueous and MTS solutions were purchased from Promega Corporation (Madison, WI).

Salmonella Typhimarium (TA) culture: The standard strains of TA100 and TA102 were used in the Ames test. The two strains contain R plasmid, including pKM101, which increases chemical and spontaneous mutagenesis by enhancing an error-prone DNA repair system which is normally present in these organisms. TA100 can be used to detect mutagens that cause base-pair substitutions, primarily at the G-C pairs. TA102 contains multicopy plasmid, pAQ1, which carries the hisG42S mutation and a tetracycline resistance gene. TA100 was grown in the Master plates with the presence of ampicillin at a final concentration of 5 ug/ml, and TA102 was grown in 5 ug/ml ampicillin plus 2 ug/ml tetracycline. Culture medium was purchased from DIFCO (Franklin Lakes, NJ), and S9 fraction of Sprague-Dawley rat liver (5 ml per vial) was purchased from Molecular Toxicology Inc (Boone, NC).

Ames test: Salmonella stock culture was obtained from the Ames Laboratory ^[12]. A detailed description of how the test was performed was given by Maron and Ames in 1982 ^[12].

Methyl Methane Sulfonate (MMS) experiment-MMS as a positive mutagen control:

MMS was stored in a glove box under a hood at room temperature. One ml of overnight culture was diluted into 9 ml of Oxoid #2 medium and 1 ul MMS was added to 1 ml of diluted culture. One hundred ul was used for each sample. For each extract to be tested, the following tubes (in duplicate) are prepared: 100 ul cells + 100 ul H₂O, 100 ul cells + x ul extract (H₂O added to bring volume to 100 ul if necessary), 100 ul cells + 1 ul MMS +99 ul H₂O, 100 ul cells +1 ul MMS + x ul extract (H₂O added to bring volume to 100 ul if necessary). The tubes were gently vortexed to mix and placed at 37°C for 2 hours. Top agar was melted in a microwave and placed in 45°C water bath to cool. Five ml histidine/biotin (5 mM) mixture was added to top agar. Bottom agar plates were warmed to room temperature and then placed in 37°C incubator to dry for about 10 minutes. Top agar with cells was then poured onto bottom agar plates and spread by gentle shaking. Once the top agar had hardened, the plates were inverted and placed in 37°C incubator for 48 hours. Experiments had been done both with and without S9 fraction which was isolated from rat liver enzymes.

UV light experiment-UV light as a positive mutagen control: UV light with a sharp intensity maximum at 257 nm was used at a dose rate 60-70 W/m². A 1:10 dilution of cells was irradiated for 17 seconds (based on results from our lab). The irradiated cells were then separated in 100 ul aliquots, and assayed as in the MMS mutagenesis experiments.

Statistical Methods: The SAS statistical software programs were used to analyze the data. The number of revertants per plate of control was expressed as mean and standard deviation (SD). The viability in percentage of surviving cells of control was calculated as absorbance in the dose group and divided by the absorbance in the control group times 100. The percentage of revertants per plate of control was calculated by the average revertants per plate in Danggui group divided by the average revertants per plate in the normal control group times 100. General linear model (GLM) (F-test and Tukey method) was used to compare the absorbance or revertants per plate between the Danggui groups and the control groups with adjustment for multiple comparisons.

RESULTS:

Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay: The concentration of the Danggui extract was 32.0 ug/ul for the first extract, and 14.0 ug/ul for the second extract. Based on the actual concentrations, the dose of 0.07 ug/ul was obtained by adding 1.1 ul of a solution of 14.0 ug/ul Danggui extract to 220 ul of culture solution (200 ul of culture medium plus 20 ul of the CellTiter 96 solution). In a similar way, 0.14 and 0.21 ug/ul were prepared by adding 2.2 and 3.3 ul, respectively, to 220 ul of culture solution. For the doses of 0.32 ug/ul and 0.64 ug/ul, 2.2 ul or 4.4 ul of a solution of 32.0 ug/ul Danggui extract was added to 220ul of culture solution.

Figure 1 presents the viability in percentage of cells surviving of control at each dose for all tested cancer cell lines tested after treatment with different doses of ethanol extract from Danggui. The viability of all tested cancer cell lines decreased with increase in Danggui dose with a significant negative dose-effect relationship between the dose of Danggui extract and cancer cell viability. The average viability of MCF-7 was 69.5%, 18.4%, 5.7%, 5.7%, and 5.0% of control for Danggui doses 0.07, 0.14, 0.21, 0.32, and 0.64 ug/ul, respectively, with a $P_{\text{trend}} < 0.0001$. The average viability of SiHa was 83.2%, 33.2%, 11.5%, 3.5%, and

4.0% of control for Danggui doses 0.07, 0.14, 0.21, 0.32, and 0.64 ug/ul, respectively, with a $P_{\text{trend}} < 0.0001$. There was no significant difference between absorbance value of the double distilled water control in medium and the ethanol control for any of the cell lines ($P_{\text{Tukey}} = 0.14$ for MCF-7, 0.95 for CaSki, 1.00 for SiHa, and 0.86 for 7368). There were not enough 7368 cells available to test the higher doses of Danggui. This cell line is particularly difficult to culture. We also examined the cell toxicity of Danggui to the normal cells - HTB-125 normal fibroblast cells. The average viability of HTB-125 was 90.2%, 85.3%, 87.7%, and 47.9% of control for Danggui doses 0.14, 0.21, 0.32, and 0.64 ug/ul, respectively, with a $P_{\text{trend}} < 0.0001$.

The half maximal inhibitory doses (ID_{50}) in ug/ul of Danggui on the different cell lines were 0.10 for MCF-7, 0.09 for CaSki, 0.10 for SiHa, 0.07 for CRL-7368 and 0.58 for HTB-125. From the ID_{50} values, the toxicity of Danggui for the cancer cells was almost 6 times greater than for the normal fibroblasts. At a dose of 0.32 ug/ul, Danggui killed more than 90% cancer cells: 93.2% MCF-7, 94.2% CaSki, 96.4% SiHa, but only killed 12.3% of the normal fibroblasts.

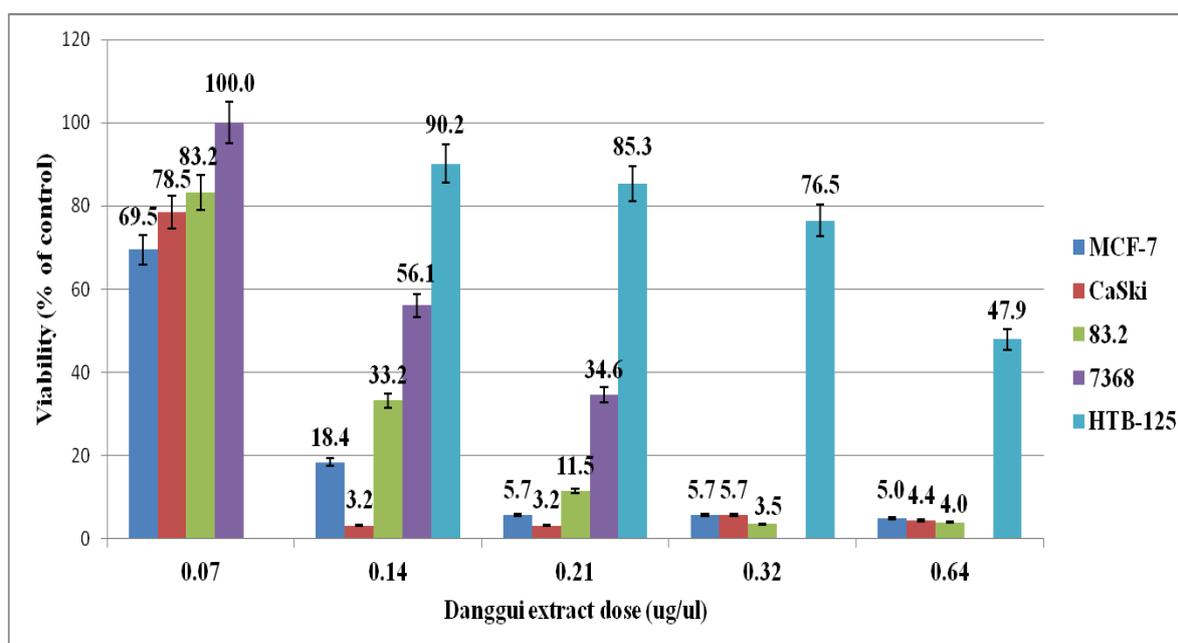


Figure 1. Average viability (% of control) of human cancer (MCF-7, CaSki, SiHa, and 7368) and normal (HTB-125) cells after treatment with different doses (ug/ul) of ethanol extract from Danggui in Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay.

Viability in percentage of control is calculated as 100 (the average absorbance of cancer cells treated by the Danggui extract dose divided by average absorbance for control). The number above the color bar is average viability with a black error bar for 95% confidence interval (CI). The 95% CIs of viability for 0.07, 0.14, 0.21, 0.32, and 0.64 ug/ul were, respectively, (59.3, 75.6), (4.2, 27.0), (0.0, 9.0), 4.5, 7.6), and 4.8, 5.1) for MCF-7 with a P for trend < 0.0001 , (73.4, 83.0), 2.0, 4.0), 3.0, 3.3), 2.1, 9.0), and (4.2, 4.7) for CaSki with a P for trend < 0.0001 , (57.2, 99.0), 0.0, 58.1), 10.0, 14.0), (2.4, 4.2), and (3.0, 4.6) for SiHa with a P for trend < 0.0001 , (100.0, 100.0), (42.7, 65.0), and (29.5, 37.9) for 7368 with a P for trend < 0.0001 (There were not enough 7368 cells available for treatment with the 0.32 and 0.64 ug/ul doses of Danggui extract), and (67.8, 100.0), (49.8, 100.0), (62.6, 100), and (34.3, 59.9) for HTB-125 with a P for trend < 0.0001 (HTB-125 was not used to test the 0.07 ug/ul dose of Danggui extract since Danggui has no direct effect on HTB-125 at this dose).

Ames Tests: The number of Salmonella TA100 revertants per plate significantly decreased with increasing dose of Danggui extracts in UV light experiment (Figure 2). There was no difference in revertants per plate between the ethanol and double distilled water control groups ($P = 0.95$). Average revertants per plate of TA100 were 417.5, 363.0, 317.0, 297.0, 196.0, 146.0, and 99.5 for Danggui 0 (control), 1, 2, 5, 10, 15, and 22 ug/ul, respectively, with a $P_{\text{trend}} < 0.0001$.

The number of TA100 revertants per plate after treatment with and without MMS and doses of the different Danggui extract are shown in Figure 3. There was no difference in revertants per plate between the ethanol and double distilled water control groups.

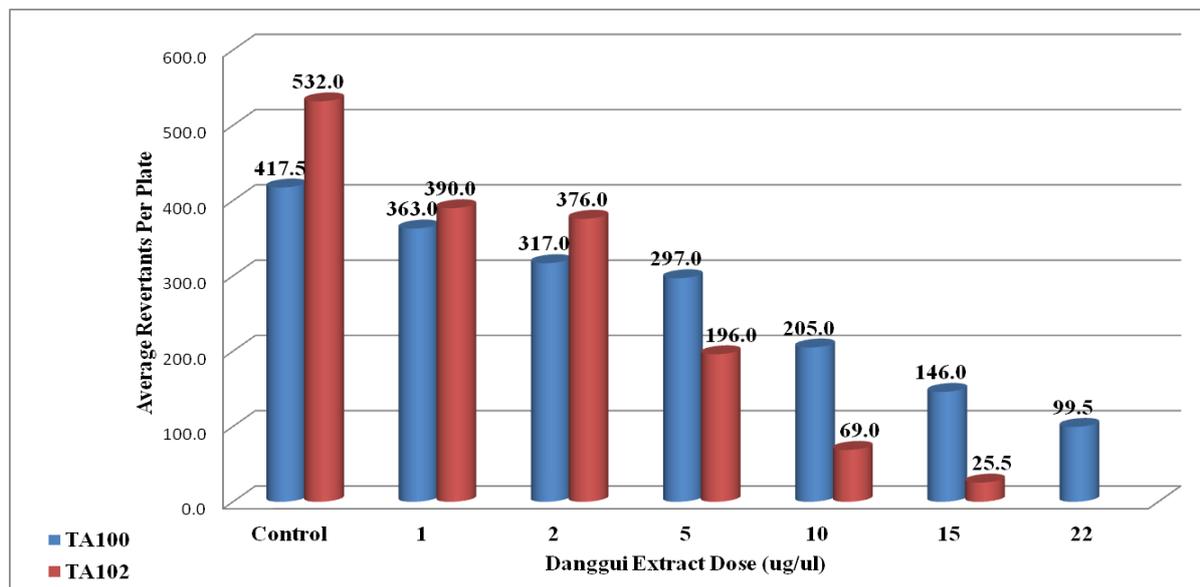


Figure 2 Average revertants per plate of the standard strains of Salmonella Typhimarium (TA) 100 and TA 102 treated with Danggui extracts in UV light experiment by revised Ames test.

Standard Deviation for Danggui extract dose in 0.0 (control), 1, 2, 5, 10, 15, and 22 ug/ul was, respectively, 195.9, 14.1, 8.5, 26.9, 15.6, 1.4, and 40.3 revertants per plate of TA 100 with a $P_{\text{trend}} < 0.0001$ and 79.2, 36.8, 22.6, 11.3, 12.7, and 5.0 revertants per plate of TA 102 with a $P_{\text{trend}} < 0.0001$ (TA 102 was not used to test Danggui extract dose in 22 ug/ul because the 15 ug/ul dose showed a big enough effect).

The Number of TA100 revertants per plate in Danggui groups was not different when compared to control groups. The percent of TA100 revertants per plate of control in 10.00 ug/ul Danggui group was still 92.90%. But after treatment with MMS, the number of TA100 revertants per plate was reduced to about 76% ($=404/531.5$) of control at a Danggui concentration 10 ug/ul ($P = 0.02$ compared to control group). Due to limited amount of Danggui extract, this study was not able to test the effect of higher dose on TA100. The results were the same between with S9 and without S9 fraction.

For TA102, the number of revertants per plate was about 53% of control for a Danggui concentration 8 ul and 56% of control for MMS plus 8 ul Danggui compared to the control group (Figure 4). There was no difference in revertants per plate between the ethanol and double distilled water control groups. The results are similar with S9 to without S9 fraction (Data are not shown in Figure 4).

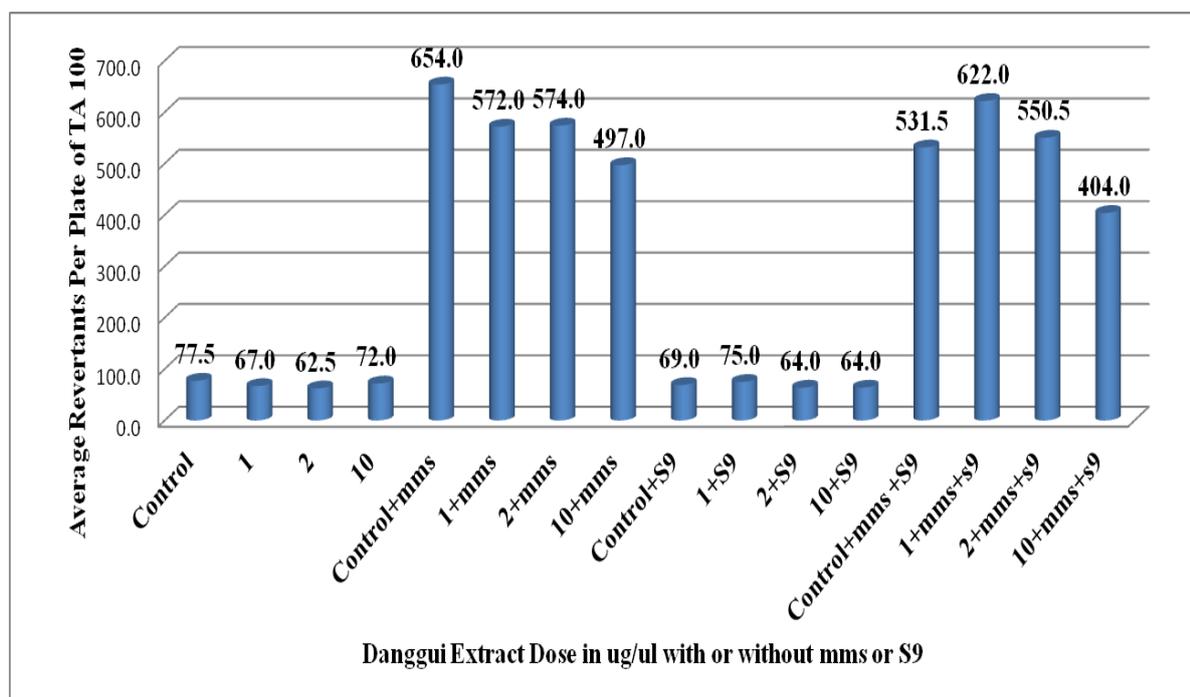


Figure 3. Average revertants per plate of the standard strains of Salmonella Typhimarium (TA) 100 treated with Danggui extracts in methyl methane sulfonate (mms) experiment with or without S9 by revised Ames test.

Standard Deviation for Danggui extract dose in 0 (control), 1, 2, 10 ug/ul, 0+mms, 1+mms, 2+mms, 10+mms, 0+S9, 1+S9, 2+S9, 10+S9, 0+mms+S9, 1+mms+S9, 2+mms+S9, and 10+mms+S9 was, respectively, 10.6, 5.7, 14.9, 2.8, 14.1, 62.2, 65.1, 7.1, 11.3, 12.7, 1.4, 2.8, 0.7, 25.5, 41.7, and 7.1 revertants per plate of TA 100.

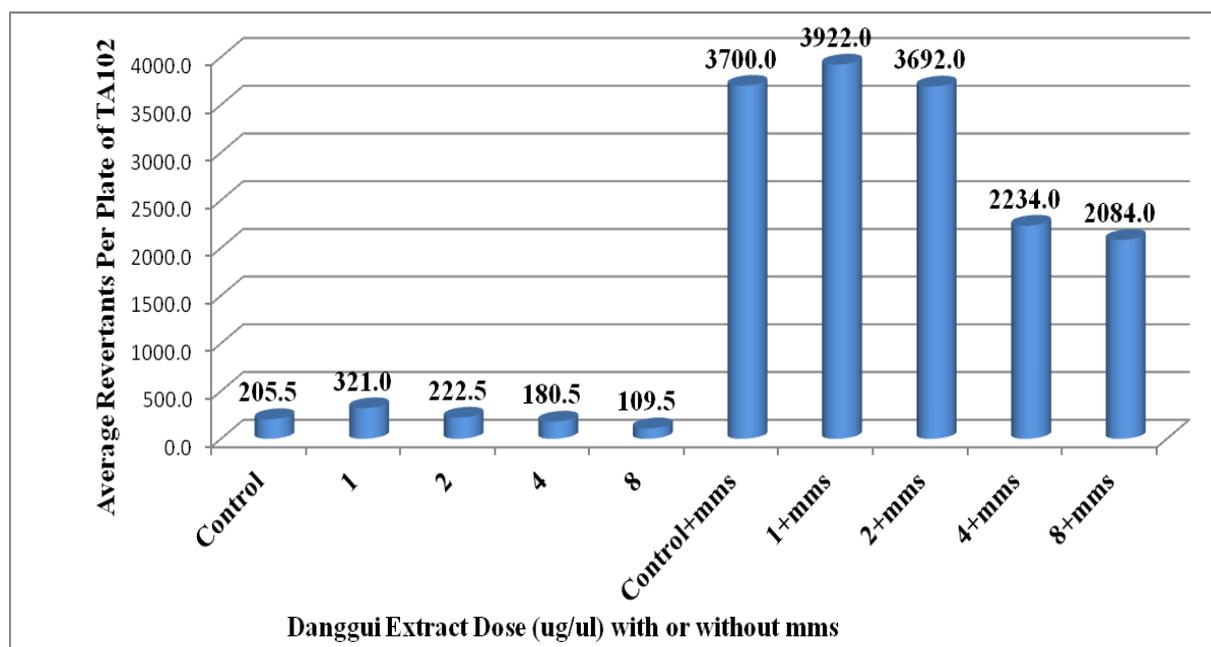


Figure 4. Average revertants per plate of the standard strains of Salmonella Typhimarium (TA) 102 treated with Danggui extracts in methyl methane sulfonate (mms) experiment by revised Ames test.

Standard Deviation for Danggui extract dose in 0 (control), 1, 2, 4, 8 ug/ul, 0+mms, 1+mms, 2+mms, 4+mms, and 8+mms was, respectively, 23.3, 11.3, 2.1, 23.3, 24.8, 311.1, 39.6, 127.3, and 458.2 revertants per plate of TA 102.

DISCUSSION:

The results indicate that Danggui is significantly more inhibitive toward the proliferation of human breast and cervical cancer cells than toward the normal fibroblasts. Thus, Danggui could prove to be a valuable agent for chemoprevention and/or adjuvant cancer treatment. Chemical analysis of Danggui might yield even more potent compounds for therapy. Also, animal studies and other anti-cancer abilities such as apoptosis and anti-migration can be done to further verify the anti-cancer effects of Danggui on women's reproductive cancer in future studies.

The data indicated an excellent inverse dose-response relationship between Danggui dose and cancer cell viability. Metabolism of cancer cells was effectively shut down at a concentration of 0.21ug/ul for MCF-7, 0.14 ug/ul for CaSki, and 0.32 for SiHa, but at the highest of these concentrations more than 85% of normal fibroblasts still survive. The ID₅₀ of the Danggui extract was almost six times greater for the normal HTB-125 cell line than for the human cancer cells.

This study indicated that Danggui has anti-mutagenic activity. Our findings found an excellent dose-response relationship between Danggui extract and anti-mutagenicity on Salmonella TA100 in UV light experiment. The percent of TA100 revertants per plate of control in 10.00 ug/ul Danggui only group was still 92.9% without S9 fraction and 92.8% with S9 fraction (Figure 3). This indicates that Danggui *per se*, even in a high dose, does not directly kill TA100, but can kill some TA102 (Percent of control of TA102 in a dose of 8.00 ug/ul Danggui only group was only 53.28%) Danggui may protect Salmonella from the mutagenic damage of UV light. In MMS experiment, anti-mutagenicity of Danggui was shown on the TA100 strain of Salmonella, but not for TA102 strain. S9 did not alter this effect since results of addition of S9 fraction did not affect the magnitude of this anti-mutagenic effect.

CONCLUSION:

Danggui inhibits the proliferation of human breast and cervical cancer cells while sparing most of the normal cells. This suggests that Danggui may be an effective and safe alternative medicine to prevent human breast and cervical cancers, or to be used as an adjuvant for their treatment. Danggui deserves to be further investigated with regard to anti-cancer effect and can be developed as an adjuvant anti-cancer therapy for women's reproductive cancer treatment.

Competing interests: Authors have no competing interests with anyone or any organizations.

Authors' contributions:

Dr. Hong-Hong Zhu designed and conducted the study, performed data analysis and interpretation, and drafted, revised and edited the entire manuscript; Dr. Guo-Hui Huang supervised and co-conducted the MTS experiments, and performed quality assurance and control on the MTS portion; Dr. Patricia L. Tate supervised and co-conducted the Ames tests and performed quality assurance and control on the Ames test portion; Dr. Lyndon L. Larcom supervised and co-designed the entire study, and in addition, supervised data interpretation and performed quality assurance and control on the study as a whole, and also co-revised and edited the manuscript in its entirety.

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