Stir-baked *Fructus gardeniae* (L.) extracts inhibit matrix metalloproteinases and alter cell morphology

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Abstract

Matrix metalloproteinases (MMPs) play vital roles in many pathological conditions, including cancer, cardiovascular disease, arthritis and inflammation. Modulating MMP activity may therefore be a useful therapeutic approach in treating these diseases. Qing-Kai-Ling is a popular Chinese anti-inflammatory formulation used to treat symptoms such as rheumatoid arthritis, acute hypertensive cerebral hemorrhage, hepatitis and upper respiratory tract infection. In this paper, we report that one of the components of Qing-Kai-Ling, *Fructus gardeniae*, strongly inhibits MMP activity. The IC\(_{50}\) values for the primary herbal extract and water extract against MMP-16 were 32 and 27 \(\mu\)g/ml, respectively. In addition, we show that the herbal extracts influence HT1080 human fibrosarcoma cell growth and morphology. These data may provide molecular mechanisms for the therapeutic effects of Qing-Kai-Ling and herbal medicinal *Fructus gardeniae*.

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**Keywords:** Matrix metalloproteinases (MMPs); Chinese herbal formulation; Stir-baked *Fructus gardeniae*; Three-dimensional (3-D) cell culture

1. Introduction

MMPs are a family of zinc dependent endopeptinases known to degrade ECM components (Johnson et al., 1998). According to their structural and functional properties, the MMP family can be subdivided into five groups: collagenases, gelatinases, stromelysins, membrane type (MT) and heterogeneous subgroup (Overall and López-Otín, 2002). Under normal physiological conditions, MMP activity is balanced by metalloproteinases (TIMPs). Under pathological conditions such as cancer, arthritis and autoimmune diseases, MMPs are abnormally activated. To effectively target MMPs in cancer and other acute or chronic diseases, many synthetic MMP inhibitors have been developed. These, however, sometimes carry undesirable side effects (Tonn et al., 1999). Because people are increasingly seeking better health through complementary and alternative medicine (Engel and Straus, 2002), there is increased interest in finding anti-tumor agents derived from natural sources, which may bear little or no toxicity compared with synthetic chemicals (Tang et al., 2003). Currently, researchers are focusing on developing nature-derived MMPIs which target specific MMPs.

Qing-Kai-Ling injection is a preparation of Chinese traditional medicine widely used in Chinese clinics for its anti-inflammatory and anti-fever properties. With medicinal herbs *Cholalic acid* (L.), *Concha pteriae* (L.), *Hyodeoxy cholic acid* (L.), *Cornu bubali* (L.), *Isatis root* (L.), *Baicalin* (L.), *Lonicera japonica* (L.) and *Fructus gardeniae* (L.) as its main components, Qing-Kai-Ling has been reported effective in treating diseases such as herpes simplex keratitis (HSK) infection, viral myocarditis, acute cerebral infarction, rheumatoid arthritis (RA) and edema (Ouyang and Liu, 2005; Pang and Wei, 2002). *Fructus gardeniae* is the ripe fruit of *Gardeniajasminoides Ellis* (family Rubiaceae), and before it is medicinally used, it is stir-baked to a brown or charcoal color. In Chinese clinics, this preparation is widely used to treat leukocytocemia, primary hepatic carcinoma, thyroid carcinoma, icterohepatitis, uterine cervix
cancer, coronary artery disease, etc. It has also been used as an anti-inflammatory medicine and to treat parenchyma injuries (Ni et al., 2006).

In an attempt to find MMPIs in traditional Chinese medicines used for their anti-inflammatory activity, we have screened a number of Chinese herbal formulations and herbal components for their effects on MMP. In this study, we propose that Qing-Kai-Ling has an inhibitory effect on MMPs. We demonstrate that the extract of one of Qing-Kai-Ling’s component, *Fructus gardeniae*, has the MMPs inhibitory activity. We also examine the influence of this herb’s extracts on HT1080 cell growth and morphology. Our results suggest that some components in *Fructus gardeniae* to regulate the activity of MMPs may be associated with the therapeutic effect of this herb and Qing-Kai-Ling injection.

2. Materials and methods

2.1. Materials

DQ-gelatin was purchased from Molecular Probes. The recombinant catalytic domains of human MMP-16 were expressed in *Escherichia coli*, purified and refolded in our lab (Shi et al., 2006). Gelatin was purchased from Sigma. Other reagents and solvents used in experiments are of analytical grade or reagent grade as appropriate. Qing-Kai-Ling injection was manufactured by Guanzhou Mingxing Pharmaceuticals and purchased from a local pharmacy.

2.2. Extracts of *Fructus gardeniae*

Stir-baked *Fructus gardeniae* (1 kg) was boiled in water three times, and the resulting decoctions pooled. This solution was then clarified by centrifugation and filtration, with the resulting filtrate being the primary extract. A part of this extract was further extracted in turn with petroleum ether, chloroform, ethyl acetate and n-butyl alcohol. The water phase after the last extraction constituted the final water fraction. All fractions were then concentrated under vacuum using a rotary evaporator, and the solvents were vaporized. We obtained five extracted fractions: petroleum ether extract (25 mg), chloroform extract (1.8 g), ethyl acetate extract (2.3 g), n-butyl alcohol extract (3.7 g) and final water extract (7.9 g).

2.3. Cell culture

Human fibrosarcoma carcinoma cell line HT1080 was cultured in Dulbecco’s modified eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) supplemented with penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37 °C with 5% CO₂.

2.4. Matrix metalloproteinase activity assay

An FLx800 fluorescence microplate reader (Bio-Tek) was used to measure the MMP activity and DQ-gelatin was used as the substrate. Kinetics assays were performed in 50 mM HEPES buffer (pH 7.5), with 0.2 M NaCl, 1 mM CaCl₂, 20 μM ZnSO₄ and 0.05% Brij-35 at 37 °C (Hurst et al., 2004). For the inhibition assays, either diluted Qing-Kai-Ling injection solution or the *Fructus gardeniae* extracts were incubated with an appropriate quantity of MMPs for 45 min before adding the fluorescence substrate, to ensure equilibrium. The extent of inhibition was determined using the initial rates with and without the inhibitor, respectively.

2.5. Cell viability (MTT) assay

HT1080 cells were seeded in 96-well plates at a density of 1 × 10⁴ cells/well in 200 μl DMEM containing 2% fetal bovine serum. Twenty-four hours after seeding, the medium was removed and the cells were incubated for 2 days with DMEM containing 2% FBS in the absence or presence of various concentrations of *Fructus gardeniae* extracts. Two days later, 20 μl (5 ng/ml) MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was added to each well and the plates were placed in an incubator at 37 °C in 5% CO₂ for 4 h. The medium was then replaced by 200 μl DMEM, and after 20 min, the absorbance was recorded at 490 nm.

2.6. Three-dimensional (3-D) culture of HT1080 cells

The 3-D culture model was established as described previously (Frisk et al., 2005). Briefly, HT1080 cells (1.5 × 10³) were suspended with a neutralized solution of type I collagen (200 μg/ml) and 1/5 volume of a 5 × DMEM. The cell suspension containing various final concentrations of *Fructus gardeniae* extracts was added to 24-well plates and kept at 37 °C until gelled. The plates were then incubated at 37 °C for 48 h. The results were observed on an Olympus IX-51 microscope.

3. Results

The inhibitory effects of Qing-Kai-Ling injection on MMP-2, MMP-9 and MMP-16 were measured. As shown in Fig. 1, 1 μl of...
Fig. 2. Dose-dependent inhibition effect of the Qing-Kai-Ling on MMP-16. The Qing-Kai-Ling injection was diluted 30-fold, and various quantities of this solution were added to a 100 μl reaction mix.

Fig. 3. Dose-dependent inhibition effect of the primary extract of *Fructus gardeniae* on MMP-16. 10-fold diluted Qing-Kai-Ling injection solution strongly suppressed the activity of MMP-2, MMP-9, and especially MMP-16 in a 100 μl reaction system. The dose-dependent inhibitory effect of Qing-Kai-Ling injection on MMP-16 is shown in Fig. 2.

From testing the herbal mix, we went on to test one of its components, *Fructus gardeniae*, which is clinically used to treat inflammation. The primary water extract strongly suppressed MMP-16 activity in a dose-dependent manner, with an IC\textsubscript{50} value of 32 μg/ml (Fig. 3). This extract was further extracted by organic reagents as described in materials and methods, and the polarity of these organic extracts was found to increase gradually from petroleum ether to water. The organic extracts’ MMP-16 inhibitory effects were evaluated and the results are shown in Table 1. No inhibitory activity was found in these extracts. The final water extract showed inhibitory activity for MMP-16, as shown in Fig. 4.

MTT assays were performed using HT 1080 cells to determine whether these extracts can have a toxic effect on cells. Fig. 5 shows a comparison of cell growth over a period of 2 days.

![Table 1](image-url)

Table 1 Inhibitory activities of different solvent extraction components of *Fructus gardeniae* on MMP-16

<table>
<thead>
<tr>
<th>Samples</th>
<th>Primary extract</th>
<th>Petroleum ether extract</th>
<th>Chloroform extract</th>
<th>Ethyl acetate extract</th>
<th>N-butyl alcohol extract</th>
<th>Water extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
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Each experiment was performed three times, “+” means strongly inhibitory to MMP-16 and “−” means none or slight inhibition to MMP-16.
of culture with various concentrations of primary and final water extracts. The values for all concentrations of the extracts were similar to that of the control, indicating that cell viability was not affected.

The behavior and morphology of cells cultured in a 3-D system is quite different from that observed in the 2-D system (Hotary et al., 2003). To further investigate the effects of these extracts on HT1080 cells, we seeded the same density of cells suspended in various final concentrations of the extracts into a 3-D collagen gel. After 48 h, the control cells (without extracts) stretched out in the gel and formed many branched and elongated structures in the type I collagen matrix (Fig. 6A). In contrast, the branched structure of the cells with the primary water extract (25 μg/ml) was reduced (Fig. 6B), and when the extract concentration increased to 100 μg/ml, the branched structure disappeared (Fig. 6C), and the cells change to a spherical shape. Similar changes occurred in the final water extract group (Fig. 6D–F). These results suggest that the primary and final water extracts of *Fructus gardeniae* influenced cell shape and morphology.

![Fig. 6. Three-dimensional (3-D) cultures of HT1080 cells: (A) control group without extract; (B) 25 μg/ml of the primary extract; (C) 100 μg/ml the primary extract; (D) 50 μg/ml of the final water extract; (E) 100 μg/ml of the final water extract; (F) 150 μg/ml of the final water extract.](image-url)
4. Discussion

Traditional Chinese medicine with anti-inflammation and anti-tumor properties is used extensively in Chinese clinical practice (Mathieu, 2005). Understanding the molecular mechanisms of these herbal medicines in pathological conditions is, therefore, of great significance. Qing-Kai-Ling injection is composed of eight traditional Chinese herbs and has been used in treating a number of diseases. In this paper, we report that this treatment suppresses the activity of MMPs. We also studied the inhibitory effect of one of its components, stir-baked *Fructus gardeniae*, on MMP-16. We found that the MMP inhibitory activity was confined to the primary and final water extract fractions of this herb.

To evaluate the effect of these extracts on cell viability and growth, we used the MTT cell viability assay and the 3-D type I collagen gel cell culture system. The 3-D system provides an environment closer to the in vivo situation for cancer cells (Baker et al., 2002). We found that cell viability was not affected by the primary and final water extracts. However, the branching structure of HT1080 cells in the 3-D matrix was inhibited. Since these extracts also strongly inhibit MMP-16 activity, we surmise that MMP inhibition may be involved in the change in cell morphology, which may in turn lead to an alteration of cell behavior and function.

MMPs, a family of zinc-dependent endopeptinas, participate in many physiological and pathological processes. An imbalance between the inhibition and activation of MMPs relates to the pathology of such diseases as osteoarthritis, rheumatoid arthritis, tumor metastasis, cardiovascular disease and congestive heart failure (Yoshihara et al., 2000; Spinale et al., 1999; Stricklin and Welgus, 1983). Finding MMP inhibitors to treat these prevalent diseases has been an active area of research. Until recently, MMPIs entering clinical trial were synthetic in origin, and their undesirable side-effects led to failure of the trials (Overall and López-Otín, 2002). Attention turned to natural sources for MMPIs. Ha and Lee reported that *Uncariae Ramulus Cum Uncis* extract inhibit MMP-2 and MMP-9 activities (Ha et al., 2004). Cha and Park showed that the methanol extract of *Euonymus alatus* inhibits MMP-9 (Cha et al., 2003), and later Park and Kim reported that caffeic acid from *Euonymus alatus* inhibits MMP-9 (Park et al., 2005). EGCG, green tea polyphenols (Demeule et al., 2000) and caffeic acid (Park et al., 2005) are among the compounds successfully extracted from herbs and identified to as MMP inhibitors. Our research showing the effect of *Fructus gardeniae* on MMP activity and cell morphology indicates that MMP inhibition may contribute to the therapeutic mechanism of this herbal medicine, and that its extracts may serve as a basis for further isolation and identification of effective MMP-inhibitory compounds.

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References


