

# Geraniin Suppresses the Expression of Cathepsin K in Osteoclasts

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## Research

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# Abstract

**Background:** Cathepsin K (CatK) plays a critical role in osteoclast-induced bone degradation, and it may be a potential therapeutic target for osteoporosis treatment. In our previous experiments, geraniin was reported to have anti-osteoporotic effects against OVX-induced rat osteoporosis and inhibitory effects on bone resorption in vitro. However, it is not known whether geraniin exhibits an inhibitory effect on CatK expression in vitro.

**Method:** In the present study, we investigated the effect of geraniin on the mRNA and protein expression of CatK in the primary osteoclasts isolated from rats. These cells were treated with different concentrations of geraniin, and the mRNA and proteins of CatK were quantified by in situ hybridization assay and immunocytochemical analysis.

**Results:** The results showed that different concentrations of geraniin ( $10^{-9}$  to  $10^{-7}$  mol/L) significantly decreased the values of integral optical density and mean optical density of CatK mRNA and protein expression in positive cells in a concentration-dependent manner, as determined by in situ hybridization and immunocytochemistry. Moreover, similar results were observed between  $10^{-7}$  mol/L geraniin and  $10^{-6}$  mol/L E-64.

**Conclusion:** These results demonstrated that geraniin can decrease the expression of CatK in osteoclasts. It may be a novel and potential CatK inhibitor came from natural plant. As a traditional Chinese herbal medicine, it may be used to treat osteoporosis in addition to its anti-inflammatory effect.

## Introduction

Osteoporosis is a systemic skeletal disease characterized by low bone mass and a deterioration of trabecular microarchitecture, resulting in increased bone fragility and their tendency to fracture. The mechanism of osteoporosis is the imbalance of bone resorption and bone formation, leading to physiological changes to the bone structure, which tend to induce bone fracture due to lower bone mineral density<sup>1</sup>. Osteoporosis is a major public health problem throughout the world, as the aging population continues to grow at an unprecedented rate. Currently affecting more than 10 million people in the United States, age-related osteoporosis is projected to impact approximately 27.6 million individuals in Europe<sup>2,3</sup>. Current drug treatments for osteoporosis treatment mainly contain one kind of drug that inhibits osteoclast activity and another that promotes osteoblast generation and promotes ossification. However, those medicines have different degrees of side effects. For example, bisphosphonates, a class of drugs that inhibit the activity of osteoclasts, are used to treat and prevent postmenopausal osteoporosis, but they can increase the risk of femoral fractures and atrial fibrillation<sup>4</sup>.

Recently, cathepsin K (CatK) inhibitors have been recognized for their potential to prevent and treat osteoporosis<sup>5</sup>. CatK degrades bone type I collagen, and it is a target for the pharmacological treatment of osteoporosis. CatK is a member of the papain family of cysteine proteases that comprises 11 human

family members (B, C, F, H, K, L, O, S, V, W, and Z). CatK is predominantly expressed in osteoclasts, a cell type in which the expression of CatB, L and S is relatively low. CatK plays a vital role in bone resorption due to its high expression in bone-degrading osteoclasts and its ability to cleave native type I collagen, a major component of bone<sup>6</sup>. *In vivo* and *in vitro* experiments have demonstrated that CatK is a key enzyme in the degradation of type I collagen, and inhibition of CatK activity is a potential approach for treating bone matrix metabolism disorders, especially bone over-resorption<sup>7,8</sup>.

## Methods

### 2.1 Preparation of geraniin

Geraniin (purity > 99%) was kindly provided by Professor Ji-kai Liu (Kunming Institute of Botany, Chinese Academy of Sciences, P.R China). In the *in vivo* experiments, geraniin was suspended in 0.5% sodium carboxymethyl cellulose before use. The compound was dissolved in DMSO and then diluted with phosphate-buffered saline (PBS) before the *in vitro* experiments. The final concentration of DMSO did not exceed 0.05%, a concentration that is not toxic to osteoclasts.

### 2.2 Isolation and culture of primary rat osteoclasts

Osteoclasts were isolated mechanically from the long bone of rats by a method described previously by Lakkakorpi *et al*<sup>14</sup>. Briefly, one-day-old rats were euthanized via cervical dislocation, and their femora were separated. The bones were then dissected away from adherent soft tissue under sterile conditions. The medullary contents were scraped from the bone cylinders in HEPES-buffered medium 199 supplemented with 15% foetal calf serum, 100 U/ml benzylpenicillin, and 100 U/ml streptomycin. The medullary bones were homogenized gently, and the cell suspension was collected in a centrifuge tube. The cell suspension was allowed to settle for 10 s before the supernatant was dropped onto the plastic substrate. This osteoclast-rich supernatant was collected again and filtered through a nylon mesh (70 µm). Following centrifugation, the cells were cultured under a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 30 min to allow the osteoclasts to subculture in 24-well plates directly. The cell culture plates were washed two times with a pre-warmed PBS solution to remove nonadherent cells, and the remaining adherent cells on the plates were continuously cultured. The cells were identified by their staining positive for TRAP and by their ability to form resorption pits on bovine bone slices. Osteoclasts were cultured and divided into six groups as follows (*n* = 4 per group): blank control group administered the complete culture media with no added substances (control group); geraniin groups treated with graded concentrations of geraniin (10<sup>-7</sup> mol/L, 10<sup>-8</sup> mol/L, 10<sup>-9</sup> mol/L, and 10<sup>-10</sup> mol/L) in osteoclasts (geraniin groups); positive control group treated with 10<sup>-6</sup> mol/L of E-64 (Sigma Chemical Co., St. Louis, MO, USA) in osteoclasts (E-64 group).

### 2.3 *In Situ* Hybridization Assay

The sequences of probes against CatK mRNA were 5'- TGGAA GAAGA CCCAC GGGAA GCAGT ACAAC AGCAA -3'; 5'- CATAc GTATG AGCTG GCCAT GAATC ACCTG GGAGA -3'; and 5'- TATAT GACCA CTGCC

TTCCA ATATG TGCAG CAGAA -3'. Hybridization was performed using a CatK mRNA kit (Boster Bioengineering Company, Wuhan, P.R. China) according to the instructions provided by the manufacturer. The endogenous peroxidase was inactivated by covering the slices with 0.6% hydrogen peroxide (v/v) in absolute methanol for 30 min at room temperature. mRNA fragments were exposed to pepsin diluted with 3% citric acid for digestion at 37°C for 2 min. After sufficient washing, 20 µL of prehybridization solution was added to the slices in a humidified chamber with 20% glycerol at 38-42 °C for 3 h. Then, 20 µL of the probe was added to the slices, and hybridization was performed at 38-42 °C for 18 h. The non-specifically bound probe was washed off with decreasing concentrations of standard saline citrate (SSC) starting at 2×SSC and ending with 0.2×SSC at 37 °C. Confining liquid was added to the slices that were then stored at 37 °C for 30 min. Anti-digoxin antibodies were pipetted onto slices, and the slices were incubated at 37 °C for 60 min followed by sufficient rinsing. Afterward, the slices were incubated with SABC peroxidase at 37 °C for 20 min. Then, the slices were treated with biotinylated-peroxidase and DAB in turn. After DAB staining for 20-30 min, the slices were dehydrated with alcohol, cleared with dimethylbenzene, sealed, and observed under a microscope (Olympus, Japan). The values of integral optical density (IOD) and mean optical density (MOD, equal to IOD divided by area of staining) of positive cells in the visual field were detected with a graphic analysis system (HPIAS-1000, Wuhan Champion Image Engineering Company of Tongji Medical University, Wuhan, P.R., China).

## 2.4 Immunocytochemical analysis

Immunocytochemistry analysis was conducted using a commercial kit (Zhongshan Golden Bridge Biological Company, Beijing, P.R., China) according to the manufacturer's directions. Briefly, the slices were coated with 0.1% poly-L-Lysine, and then one slice was put in each well of a 24-well plate. The cells were cultured in the 24-well plates with poly-L-Lysine-coated slices for 24 h. The slices were washed three times with PBS, fixed in 4% paraformaldehyde for 30 min, and treated with 3% H<sub>2</sub>O<sub>2</sub> for 10 min to inactivate endogenous peroxidase. The slices were incubated overnight using an anti-cathepsin K antibody at a 1:500 dilution and were finally stained with DAB. The slices were observed under a confocal microscope. The values of IOD and MOD for positive cells with brown nuclei were calculated as above.

## 2.5 Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.). Statistical comparisons were made by analysis of variance (ANOVA) and Student's *t*-test. Differences were considered statistically significant when *P* was less than 0.05.

# Results

## 3.1 Effect of geraniin on mRNA expression of CatK in osteoclasts

As shown in Fig. 2, geraniin (10<sup>-9</sup> to 10<sup>-7</sup> mol/L) significantly decreased the values of IOD and MOD of CatK mRNA levels in positive cells in a concentration-dependent manner compared to those of the control group (*P* < 0.01 for both). Additionally, 10<sup>-6</sup> mol/L E-64 showed a similar potency to 10<sup>-7</sup> mol/L geraniin.

### 3.2 Effect of geraniin on protein expression of CatK in osteoclasts

As shown in Fig. 3, geraniin ( $10^{-9}$  to  $10^{-7}$  mol/L) decreased the values of CatK protein IOD and MOD in positive cells in a concentration-dependent manner compared with those of the control group ( $P < 0.01$  for all). Additionally,  $10^{-6}$  mol/L E-64 had similar results to  $10^{-7}$  mol/L geraniin in reducing the protein expression of CatK compared with the control group.

## Discussion

Bone remodelling and bone metabolism are dynamic equilibrium processes between bone formation and bone resorption. The main characteristic of osteoporosis is that a relative or absolute increase of bone resorption mediated by osteoclasts exceeds a sustained decrease in bone formation induced by osteoblasts<sup>15</sup>. The main approach for treating osteoporosis is interference or inhibition of the bone resorption function of osteoclasts. In a previous study, we found that geraniin has an inhibitory effect on the bone-absorption ability of osteoclasts *in vitro*<sup>13</sup>. In this study, we confirmed that the inhibitory effect of geraniin in osteoclasts may also be closely related to its ability to down-regulate the mRNA and protein expression of CatK.

Osteoclasts attach to the surface bone and form a sealed bone absorption lacuna between the cell membrane and bone matrix in the process of bone resorption, and then an acidic environment is formed by H<sup>+</sup>-ATPase. CatK is the main cathepsin of osteoclasts in organic bone degradation. Bone resorption and collagen decomposition are inhibited by down-regulation of CatK expression via gene silencing<sup>16</sup>. The CatK precursor is secreted by osteoclasts and is activated in the bone resorption lacuna. During bone resorption, hydroxyapatite is dissolved by the acidic substance, while organic matrix components from the bone matrix are separated and degraded by CatK; early matrix degradation products together with CatK are taken up by osteoclasts. The main collagens of extracellular organic matter in the bone matrix are type I and type II, and type I accounts for approximately 90% of the collagen. CatK can degrade a variety of bone matrix proteins, including type I collagen, osteopontin, and osteonectin. CatK was found to bind native type I and II collagen at the N-terminus of its triple helix and then cleave bone collagen. Studies have found that the overexpression of CatK in transgenic mice causes increased bone turnover and reduced bone volume<sup>17, 18</sup>. Moreover, Pennypacker B reported that CatK-deficient mice have reduced osteoclastic bone resorption<sup>19</sup>. These results suggest that CatK may be a potential therapeutic target for bone resorption disorders such as osteoporosis.

It has been recognized that CatK may play an important role in bone degradation induced by osteoclasts. Evidence suggests that CatK inhibitors may suppress bone resorption *in vivo* and *in vitro*<sup>20</sup>. Kumar et al<sup>21</sup> found that SB-462795 (relacatib) is a potent and oral small-molecule inhibitor of CatK that inhibits bone resorption both *in vitro* and *in vivo* in cynomolgus monkeys. SB-462795 inhibits endogenous CatK *in situ* in human osteoclasts and human osteoclast-mediated bone resorption with IC<sub>50</sub> values of ~ 45 nM and ~ 70 nM, respectively. Odanacatib is a prospective CatK inhibitor with a long pharmacokinetic half-life and strong selective inhibition in pre-clinical studies. Clinical trial results showed that odanacatib exerts

robust and sustained suppression of bone resorption biomarkers and increases lumbar spine bone mineral density (BMD) and total hip BMD in a dose-dependent manner in postmenopausal women<sup>22-24</sup>. Balicatib, another cathepsin K inhibitor,—which significantly prevents ovariectomy from bone mass changes in cynomolgus monkeys and reduces bone turnover—, has a therapeutic effect on osteoporosis in monkeys<sup>25</sup>. These studies showed that CatK is an important target for the treatment of hyperthyroidism in bone resorption, inhibitors of which represent a new mechanism for clinical treatment of osteoporosis in the future.

In this study, we used immunohistochemistry and in situ hybridization to explore the mRNA and protein expression of CatK in osteoclasts by integrating the luminosity, average grey level, and positive units. Our results showed that, compared with the normal group, geraniin significantly decreased the values of integral optical density and positive units and increased the mean optical density of the mRNA and protein expression of CatK in a concentration-dependent manner. These results demonstrated that geraniin reduces the mRNA and protein expressions of CatK. Moreover, in this study, we also found that the positive control drug E64 significantly inhibits the expression of CatK at the mRNA and protein level in osteoclasts, which is consistent with findings from previous studies<sup>26</sup>.

In conclusion, geraniin downregulates the expression of CatK in osteoclasts both at both the transcript and protein level. These results suggest that geraniin may be a novel and potent inhibitor of CatK. In future studies, we will investigate the effect of geraniin on CatK expression *in vivo* and its possible molecular mechanism.

## Conclusion

Geraniin, as a traditional Chinese herbal medicine, was mainly used to eliminate inflammatory. We found that the geraniin can decrease the expression of the key protein CatK in osteoclast-induced bone degradation in the primary osteoclasts isolated from rats significantly. This effect is similar to the effect of positive control E-64. It suggested that the geraniin may be a novel and potential CatK inhibitor came from natural plant and have potential medicinal development value for treating osteoporosis.

## Abbreviations

CatK: Cathepsin K

OVX: ovariectomized

PBS: Phosphate-buffered saline

DMSO: Dimethylsulfoxide

SSC: Standard saline citrate

SABC: Strept Avidin-Biotin Complex

DAB: 3,3'-Diaminobenzidine

IOD: Integral optical density

MOD: mean optical density

S.D.: Standard deviation

ANOVA: Analysis of variance

## **Declarations**

### **Availability of data and materials**

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

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### **Ethics approval and consent to participate**

This study was reviewed and approved by the ethics committee of Kunming Medical University.

### **Consent for publication**

Not applicable.

### **Competing interests**

The authors have no competing interests.

### **Authors' Contributions**

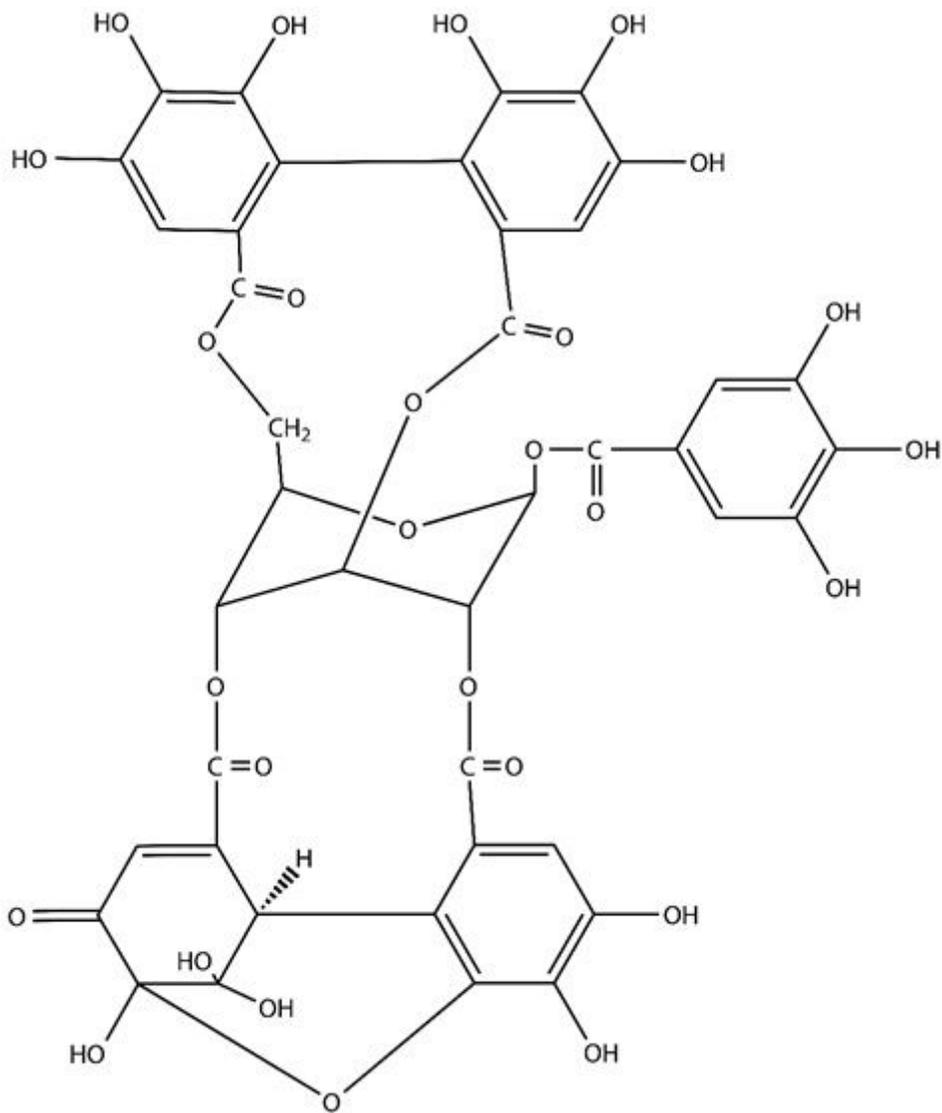
ZS and PC designed the analyses. XZ and GL performed the *in situ* hybridization assay and immunocytochemical analysis. XZ, BH, TL and YY isolated and cultured the rat primary osteoclasts. XZ, ZS and PC wrote the draft of the manuscript. All authors read and approved the final manuscript.

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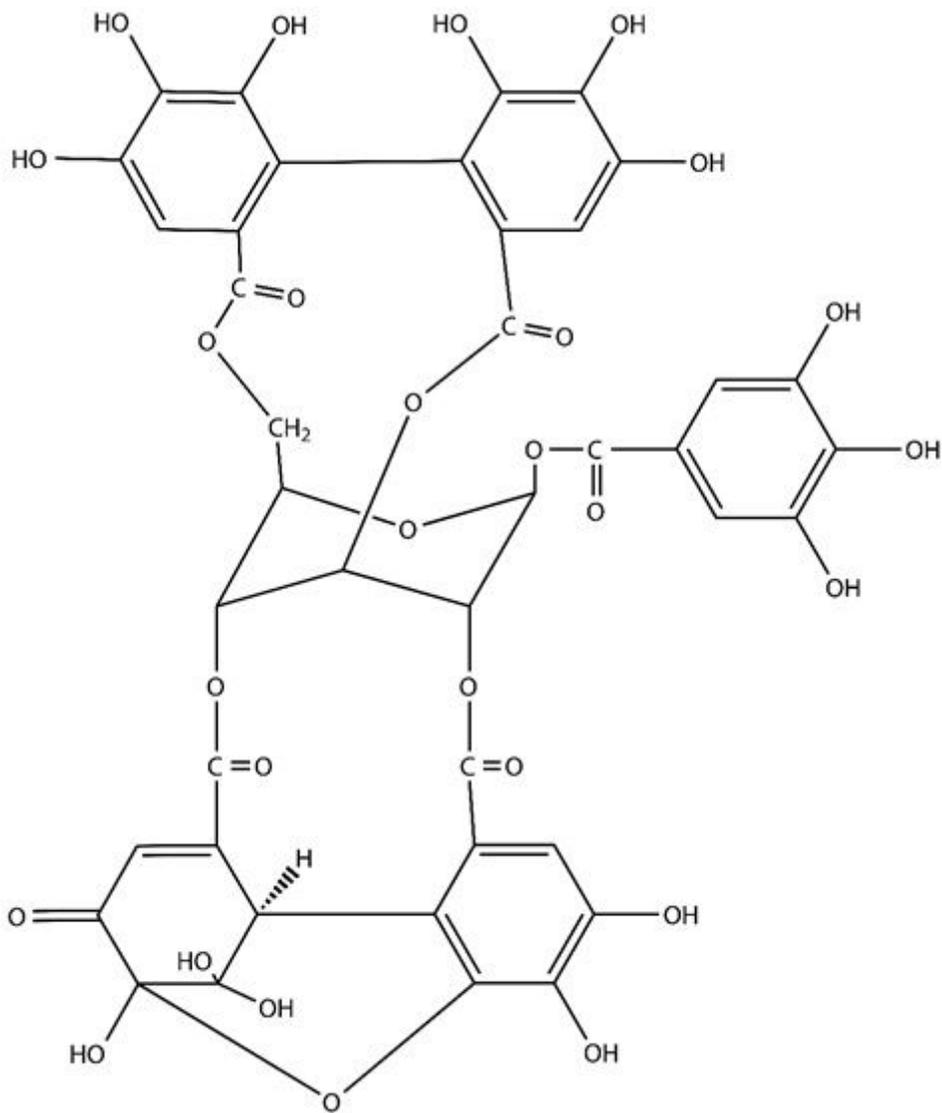
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## Figures



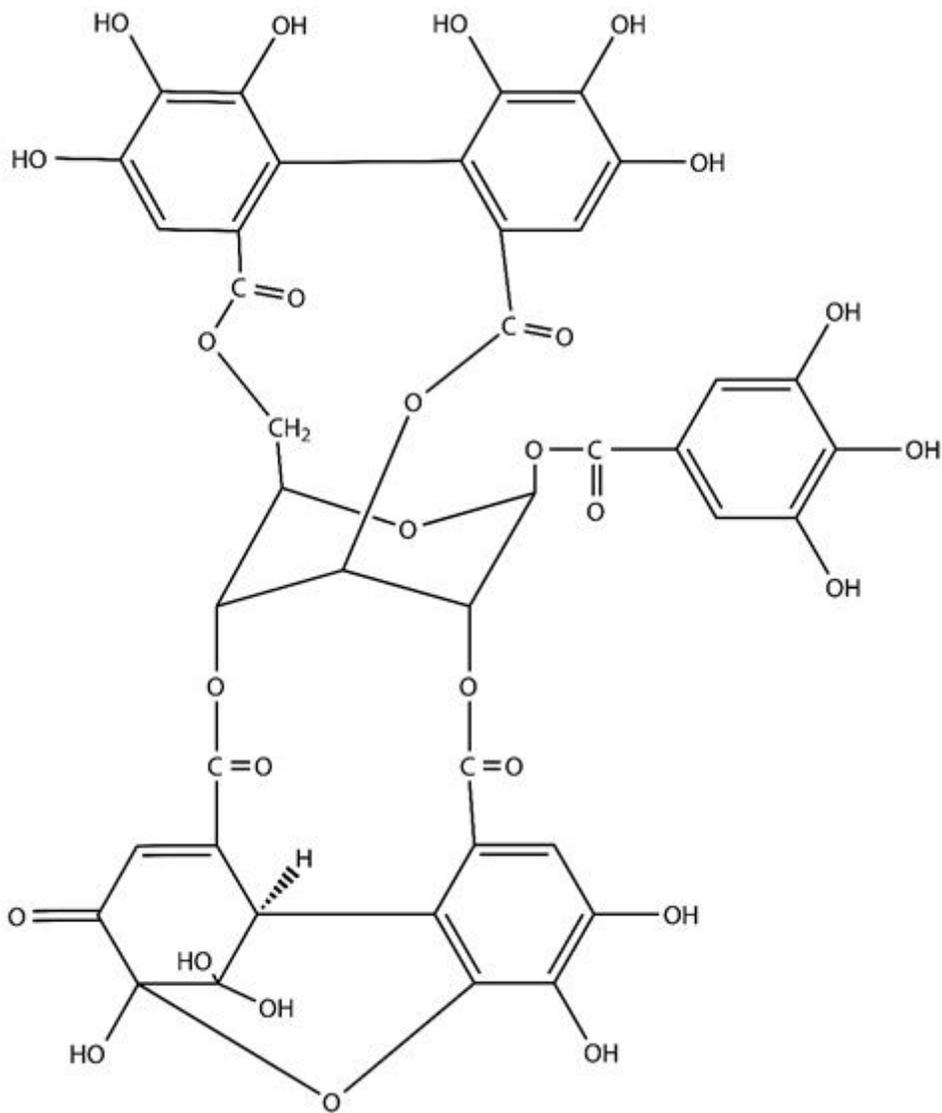
**Figure 1**

Chemical structure of geraniin.



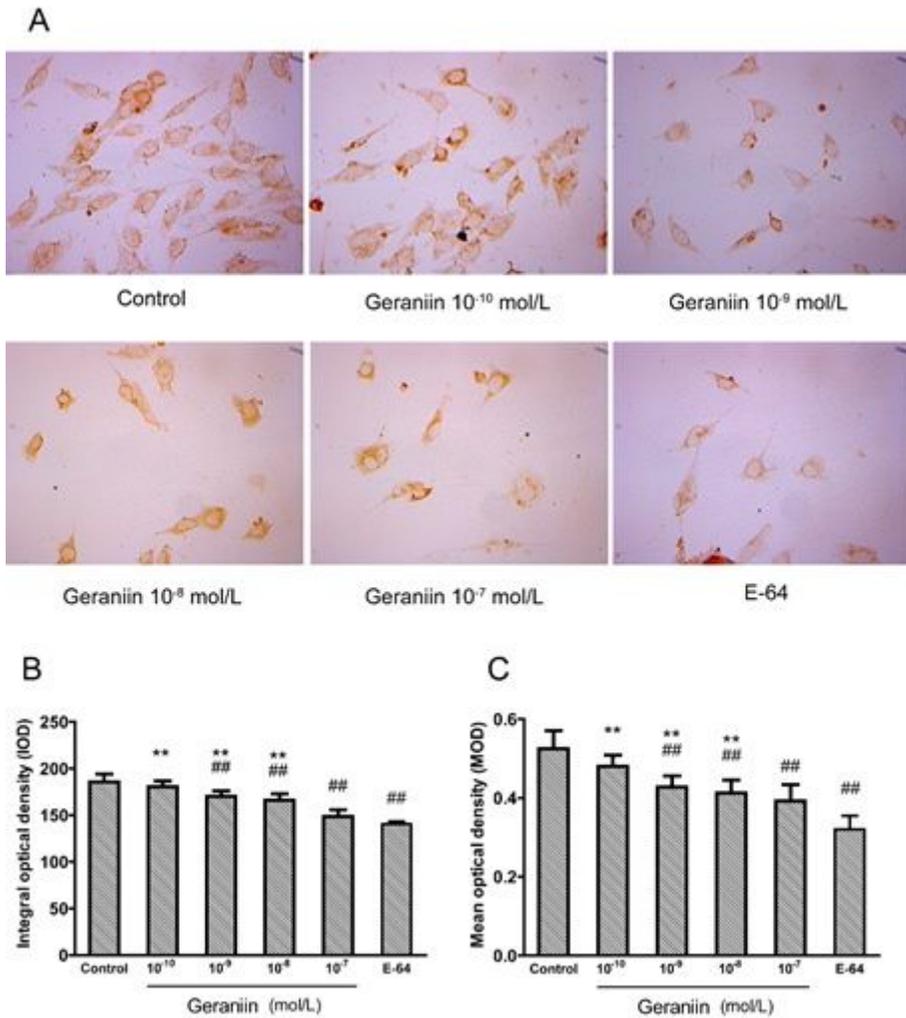
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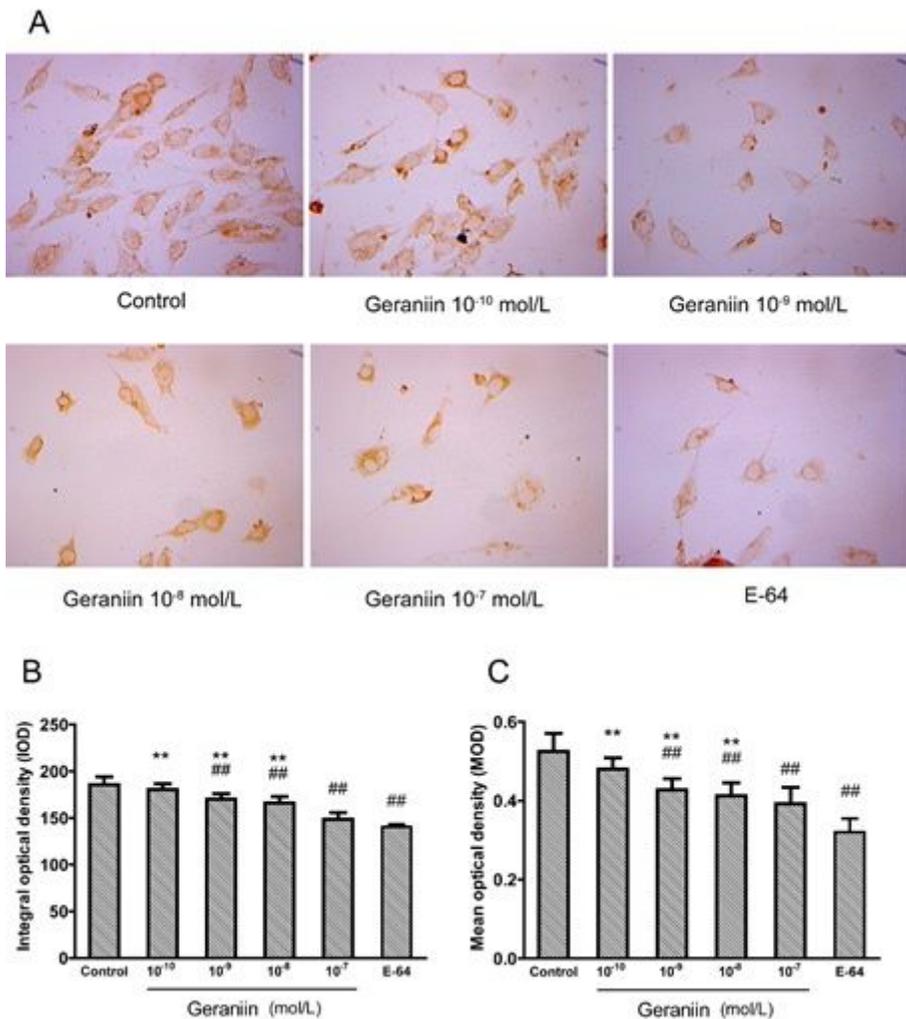
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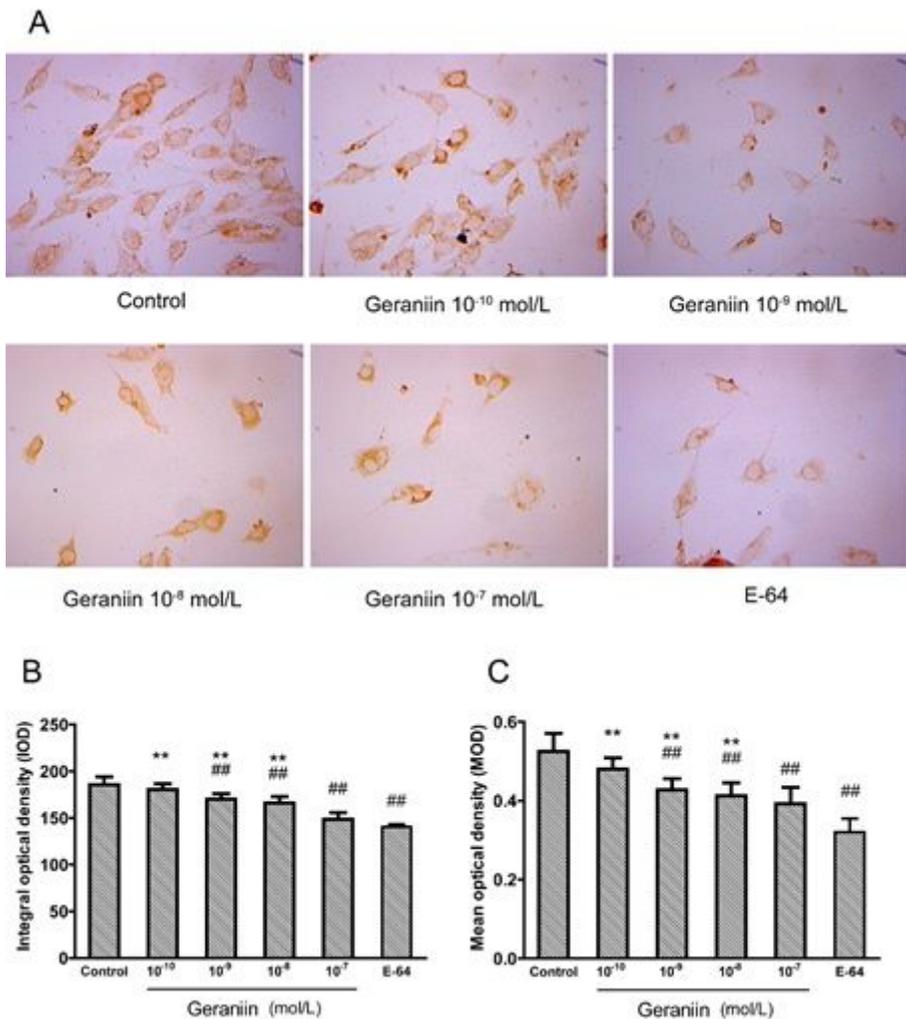
**Figure 2**

Effect of geraniin on mRNA expression of CatK in osteoclasts. Representative images of in situ hybridization for MMP-9 mRNA (A). Geraniin (10<sup>-9</sup>-10<sup>-7</sup> mol/L) reduced the CatK mRNA IOD (B) and MOD (C) values in positive cells in a concentration-dependent manner. The results represent the mean ± SEM of three experiments. ## P < 0.01 versus control group, \*\* P < 0.01 versus E-64 group. One-way ANOVA followed by Student's t-test.



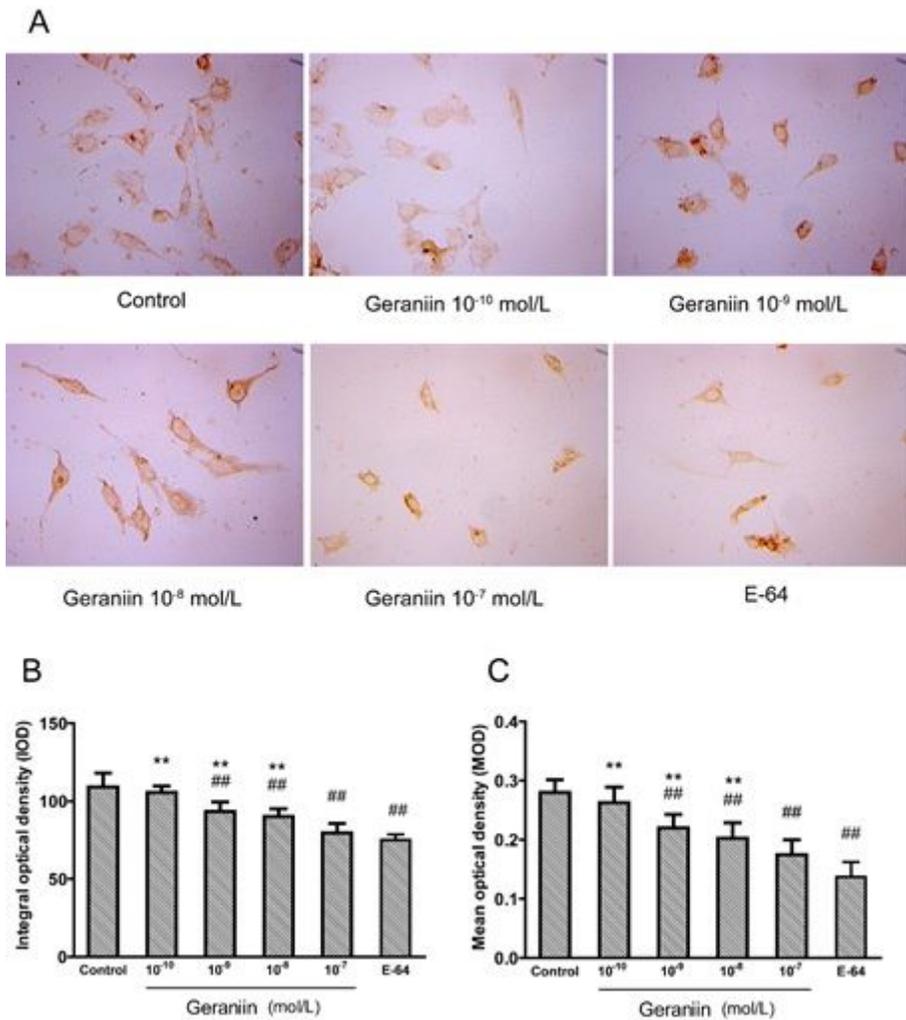
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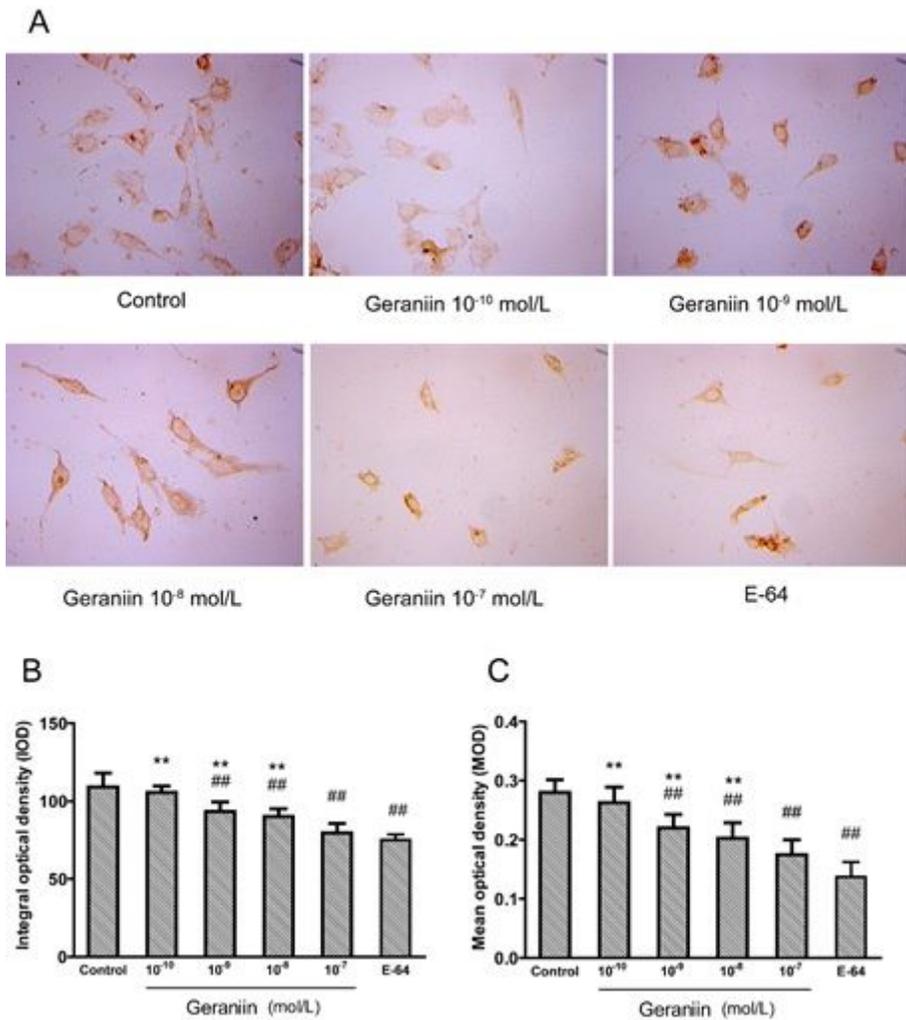
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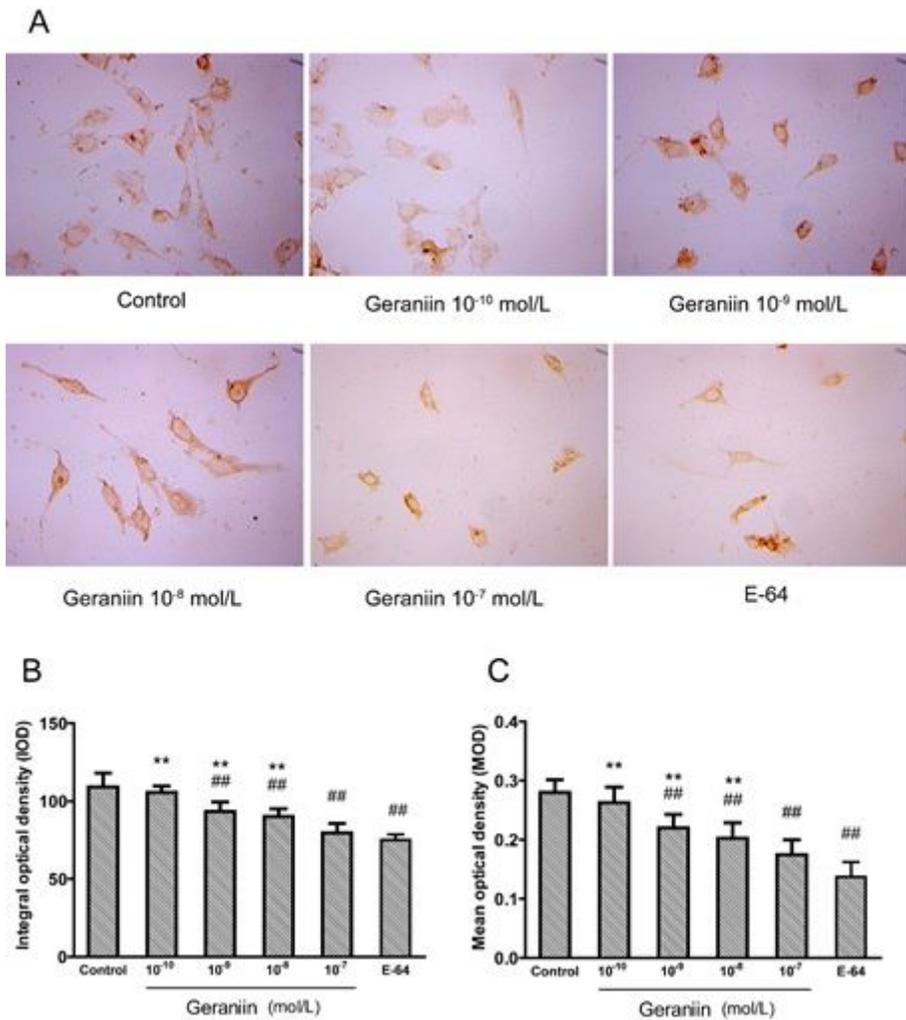
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Effect of geraniin on protein expression of CatK in osteoclasts. Representative images of immunocytochemistry staining for MMP-9 protein expression (A). Geraniin ( $10^{-9}$ - $10^{-7}$  mol/L) reduced the CatK protein IOD (B) and MOD (C) values in positive cells in a concentration-dependent manner. The results represent mean  $\pm$  SEM of three experiments. ##  $P < 0.01$  versus control group, \*\*  $P < 0.01$  versus E-64 group. One-way ANOVA followed by Student's t-test.



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