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Aluminum ammonium sulfate dodecahydrate purified from traditional Chinese medicinal herb Korean monkshood root is a potent matrix metalloproteinase inhibitor

Yehua Shen · Sen Liu · Fenghai Jin · Tianyang Mu · Cong Li · Kun Jiang · Weihua Tian · Dahai Yu · Yingqi Zhang · Xuexun Fang

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Abstract Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases and key regulators for many physiological and pathological functions. The MMP inhibitors have been shown to modulate diseases such as cancer, inflammation, and cardiovascular diseases. In this paper we tracked the MMP inhibitory activities of the traditional Chinese medicinal herb Korean Monkshood Root. The purified active ingredient was identified by the elemental analysis, infrared spectrum (IR) and X-ray diffraction as aluminum ammonium sulfate dodecahydrate. This inorganic compound showed inhibitory activities toward a number of MMP family members. In particular, it has a strong inhibitory effect toward MMP-2 and MMP-9, with IC50 values of 0.54 and 0.50 μM, respectively. Further analysis suggested that the MMP inhibitory activity is mainly due to Al^{3+}. Cell viability assays using human fibrosarcoma HT1080 cells showed aluminum ammonium sulfate had minimal cyto-toxicity with a concentration up to 500 μM. However, within 50 μM, it exhibited significant inhibition of cell invasion. To our knowledge, there has been no previous report of inorganic form of the MMP inhibitor with strong inhibitory activity. Our results for the first time showed that aluminum ammonium sulfate is an inorganic form of MMP inhibitor with high potency, and can be used to interfere with MMP related cellular processes.

Keywords MMP inhibitors · Aluminum ammonium sulfate dodecahydrate · Inorganic MMP inhibitor

Y. Shen, S. Liu, F. Jin contribute equally to this paper.

Y. Shen · Y. Zhang (✉)
Biotechnology Center, School of Pharmacy, Fourth Military Medical University, Xi'an, Shaanxi 710069, People's Republic of China

Y. Shen · C. Li · W. Tian
Key Laboratory of Synthetic and Natural Functional Molecule Chemistry of Ministry of Education, College of Chemistry and Materials Science, Northwest University, Xi'an, Shaanxi 710069, People's Republic of China

S. Liu · F. Jin · T. Mu · K. Jiang · D. Yu · X. Fang (✉)
Key Laboratory of Molecular Enzymology and Enzyme Engineering of the Ministry of Education, Jilin University, Changchun, Jilin 130012, People's Republic of China
e-mail: fangxx@jlu.edu.cn

Introduction

MMPs are a family of zinc dependent endopeptinases responsible for the homeostasis of the extracellular matrix. Under normal physiological conditions, MMP activity is tightly regulated by tissue inhibitor of matrix metalloproteinases (TIMPs). Under many pathological conditions, such as cancer, cardiovascular diseases, arthritis, and neurodegenerative diseases, MMPs are disregulated, with their activities usually abnormally activated (Sternlicht and Werb 2001; Egeblad and Werb 2002; Sang et al. 2006). In cancer,

MMPs regulate molecular communication between tumor and stroma by affecting a variety of physiological processes and signaling events. The altered proteolysis leads to unregulated tumor growth, tissue remodeling, inflammation, tissue invasion, and metastasis (Sternlicht and Werb 2001; Egeblad and Werb 2002; Sang et al. 2006; Kessenbrock et al. 2010).

Many synthetic MMP inhibitors able to potently and selectively block the uncontrolled activity of MMPs have been developed in the aim of effectively control these enzymes in cancer and other diseases. However, these inhibitors carry undesirable side effects and failed the late stage clinical trials (Coussens et al. 2002; Fingleton 2008). Extremely potent MMP inhibitors, mostly broad-spectrum MMP inhibitors with strong zinc-binding groups such as the hydroxamate groups, is responsible for the poor MMP selectivity and inhibitory activity toward other zinc proteinases, and is likely the cause of poor clinical outcome of these compounds. Newer selective MMP inhibitors possess less avid zinc-binding group or no zinc-binding group and exploit the specificity of S1' cavity in MMPs. The current strategy of MMP inhibitor development emphasize more on the flexibility in the MMP active site and its impact in accommodating different inhibitor structures (Overall and Kleifeld 2006; Yiotakis and Dive 2008). The multiple complex roles these enzymes can play is now recognized. This knowledge has begun to impact new drugs in development. Two recently developed compounds bear specific inhibition to MMP12 and MMP13, The specific MMP13 inhibitor binds within the active site of MMP13 but without chelating the zinc. Another approach to designing inhibitors with better selectivity is to use a ''suicide inhibitor'' where the target is covalently modified by the inhibitor binding. An alternative strategy for obtaining specific inhibitors is to use an antibody to specific enzymes (Fingleton 2008; Overall and Kleifeld 2006; Yiotakis and Dive 2008).

As there is an increased interest in seeking better health through complementary and alternative medicine (Engel and Straus 2002), numerous new MMP inhibitors from natural sources have been reported (Sang et al. 2006; Mannello 2006; Dell'Aica et al. 2007). Thus developing nature-derived MMPIs is among the new strategies for seeking novel MMPIs.

Korean monkshood root (Guanbaifu in Chinese) is the stem tuber of *Aconitum coreanum (Levl.) Raipaics*. In traditional Chinese medicine, Korean monkshood root is commonly used for treating arthralgia, headache, convulsive epilepsy, coronary heart disease, ischemic arrhythmia, pyocutaneous disease, and anemogenous phlegm (http://www.wiki8.com/guanbaifu_74401/). It has also been reported to treat arthritis and physical injuries such as fractures with good results. A number of compounds have been isolated from this plant and studied for their biological activities and acehytisine, a diterpenoid alkaloid isolated from this herb, have been approved for the clinical use in China (http://www.wiki8.com/guanbaifu_74401/). However, little is known for the active ingredients in this herbal medicine or the molecular mechanisms that related to the effects in treating of the diseases mentioned above besides arrhythmia.

In this paper, we report the inhibitory effects of MMPs by the water extract of this traditional Chinese herbal plant. We purified active ingredient and identified its structure as aluminum ammonium sulfate dodecahydrate. We further characterized the MMP inhibitory activity of this inorganic compound and analyzed the active component. Finally, we investigated its effect on cell invasion.

Materials and methods

Materials

Korean monkshood root was purchased from local Chinese medicine store. Macroporous resin D101 was obtained from Xi'an Lan-sen Resin Company. Recombinant human MMP-2, -9, -13, -14 and -16 were purchased from EMD Biosciences (CA, USA). DQ-gelatin was supplied by Molecular Probes, and Matrigel was obtained from Becton, Dickinson and Company, USA. Transwell was purchased from Corning Costar, USA. Other regents and solvents used in experiments are of analytical grade or reagent grade as appropriate.

Instruments

Elemental analysis was performed with vario EL III CHNS-O Element Analyzer (Elementar Analysensysteme GmbH, Germany), Equinox 55 FTIR/FTNIR Spectrometer (Bruker, Gernamy) was used for infrared spectrum (IR), X-ray diffraction was analyzed by Smart Apex IICCD (Bruker, Gernamy), and ICP-AES

was conducted using IRIS Advantage (Thermo Scientific, USA).

Methods

Separation of the active compound

Approximately 500 g of the Korean monkshood root were chopped into small pieces and boiled in 2,500 ml of water for 1 h, the procedure were repeated three times and the water extracts were combined. The extracts were concentrated to dryness under reduced pressure in a rotary evaporator (RE-52A, Shanghai Shen'an Instrument Co., China). Then the crude extracts were dissolved in ethanol–water (3:2, v/v), standing for 24 h, and the supernatant were collected, and this process was repeated twice. The supernatant was concentrated to dryness. Twenty grams of the dried sample was dissolved in 150 ml hot water and fractionated on a D101 resin, using methanol–water as eluent. The ratios of water–methanol were 1:0, 1:9, 2:3, 4:5 and 0:1 in turn. The fractions were named A1, A2, A3, A4 and A5. The inhibitory effects of these fractions to MMPs were detected. The inhibitory fraction (A1) was recrystallizated for several times and obtained the pure active compound.

Structural identification of the active compound

Intensity data were collected on a Bruker Smart APEX II CCD diffractometer with graphite-monochromated Mo Ka radiation ($k = 0.71073$ Å). Empirical absorption corrections were applied using the SADABS program. The intensities were corrected by Lorentz-polarization factors and empirical absorption. The structure was solved by direct methods using SHELXTL-97 program (Sheldrick 1997) and refined by the full-matrix least squares based on F2. All non-hydrogen atoms were refined anisotropically and the hydrogen atoms of organic ligands were generated geometrically. Crystal data and structural refinement parameters for this compound are summarized in Table 1. Selected bond distances and bond angles are listed in Table 2.

Determination of metal content in Korean monkshood root

The concentrations of metals in the Korean monkshood root were measured by inductively coupled

Table 1 Crystallographic Data for the active compound

Compound	$NH_4Al(SO_4)_2{\cdot}12H_2O$
Formula	$H_{28}AlNO_{20}S_2$
Formula weight	451.29
T (K)	296(2)
Crystal system	Cubic
Space group	*Pa-3*
a (Å)	12.2336(6)
b (Å)	12.2336(6)
c (Å)	12.2336(6)
α (°)	90
β (°)	90
γ (°)	90
V (Å^3)	1830.89(16)
Z	4
D_{calc} (g/cm^3)	1.637
Absorption coefficient (mm^{-1})	0.435
$F(000)$	948
Cystal size (mm)	0.31 × 0.21 × 0.15
Reflections collected	8,362
Unique reflections	554
Final R indices [$I > 2\sigma$ (I)]	R1 = 0.0527, wR2 = 0.1562
R indices (all data)	R1 = 0.0546, wR2 = 0.1583

Table 2 Selected bond lengths (Å) and bond angles (°)

Bond lengths (Å)	
Al(1)–O(1)	1.875(2)
Al(1)–O(1) #1	1.875(2)
Al(1)–O(1) #2	1.875(2)
Al(1)–O(1) #3	1.875(2)
Al(1)–O(1) #4	1.875(2)
Al(1)–O(1) #5	1.875(2)
Bond angles (°)	
O(1)–Al(1)–O(1)#1	89.51(9)
O(1)–Al(1)–O(1)#2	90.49(9)
O(1)#1–Al(1)–O(1)#2	180.00(14)
O(1)–Al(1)–O(1)#3	180.0
O(1)#1–Al(1)–O(1)#3	90.49(9)
O(1)#2–Al(1)–O(1)#3	89.51(9)
O(1)–Al(1)–O(1)#4	89.51(9)

Symmetry transformations used to generate equivalent atoms: #1 $x, -y + 1/2, z + 1/2$; #2 $-x + 1, y - 1/2, -z + 1/2$; #3 $-x + 1, -y + 1, -z$; #4 $x - 1/2, y, -z + 1/2$; #5 $-x + 1/2, -y + 1, z + 1/2$

plasma atomic emission spectrometry (ICP-AES). The sample was washed with distilled water and digested with HNO_3:$HClO_4$ (4:1, v/v). The optimized ICP experimental parameters are the following: incident power 1,150 W; Rf 27.12 MHz; atomization pressure 0.19 Mpa; integral time for low wave 20 s and high wave 10 s; sample uptake rate 1.85 ml min^{-1}. The analytical lines for the detected elements are: Al 237.312 nm, Cu 234.7 nm, Fe 238.2 nm, K 766.4 nm, Mg 285.2 nm, Mn 259.3 nm, Na 589.5 nm, Zn 213.8 nm.

Cell culture

HT1080 human fibrosarcoma carcinoma cell line was maintained at our lab. The cells were grown in Dulbecco modified Eagle medium (DMEM) containing 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 10% fetal bovine serum at 37°C in 5% CO_2.

Metalloproteinase assays

MMP enzymatic activities were measured using the quenched fluorescein conjugate-DQ gelatin. The assays were conducted in 50 mM HEPES buffer (pH 6.8), containing 200 mM NaCl, 10 mM $CaCl_2$, 20 μM $ZnCl_2$, 10 mM $MgCl_2$ and 0.01% Brij-35. Increasing concentrations of each extract were added to a reaction mixture with constant concentration of each MMP, and incubated at 37°C for 30 min. The reactions were started by addition of 1 μl of 200 μg/ml DQ-gelatin substrate. The inhibitory efficiency was determined by comparing the relative activity of enzyme in the presence and absence of the different extract samples. The fluorescence was measured using FLX800 fluorescence microplate reader (Bio-Tek) with excitation at 495 nm and emission at 515 nm. All assays were performed in 96-well flat-bottom microtiter plates. Background fluorescence was subtracted from each measurement.

MTT assay

The extracts of Korean monkshood root were examined for their anti-proliferation activities on HT1080 cells using the MTT assay. Briefly, the cells were plated in 96-well culture plates at a density of 5×10^4 cells/well in DMEM culture medium. The extracts were added to various final concentrations (for control, 0 μg/ml). After 24 h of culture, 50 μl MTT (0.5 mg/ml) reaction solution (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide) was added to each well and the plates were placed in an incubator for 4 h (37°C and 5% CO_2). When the purple precipitate is clearly visible under the microscope, add 5 μl of acidic isopropanol (0.04 M HCl in isopropanol) to the wells. The optical density was read at a wavelength of 570 nm in a microplate reader. All determinations confirmed by replication in at least three identical experiments.

Invasion assays

The ability of cells to invade through *Matrigel*-coated filters was measured in Transwell (BD Biosciences). Eight-micrometer Transwell filters were coated with 300 ng (30 μl *Matrigel* added into 70 μl DMEM) *Matrigel* (BD Biosciences) per filter (24-well plate). DMEM supplemented with 20% fetal bovine serum was placed in the lower chamber. HT1080 cells (10^5 cells per well) were placed in the upper chamber of the serum-free medium. After 24 h of incubation in the presence of various concentrations of aluminum ammonium sulfate, cells that had migrated to the lower surface of the filter were fixed, stained with Giemsa, and counted. Values for invasion were determined by calculating the average number of migrated cells per mm^2 over five fields per assay and expressed as an average of duplicate determinations.

Results

Korean monkshood root extract inhibit MMP activities

The water extract of the Korean monkshood root were measured for the inhibitory effects on the activities of MMP-2, MMP-9, MMP-14 and MMP-16 toward DQ-gelatin, a commonly used fluorescent substrate for MMPs. The extract strongly inhibited MMP-2, MMP-9, MMP-14 and MMP-16, in a dose dependent manner (Fig. 1).

Purification of the active MMP inhibitory compound in Korean monkshood root

The water extract of Korean monkshood root had shown strong inhibitory effect to MMPs. The water

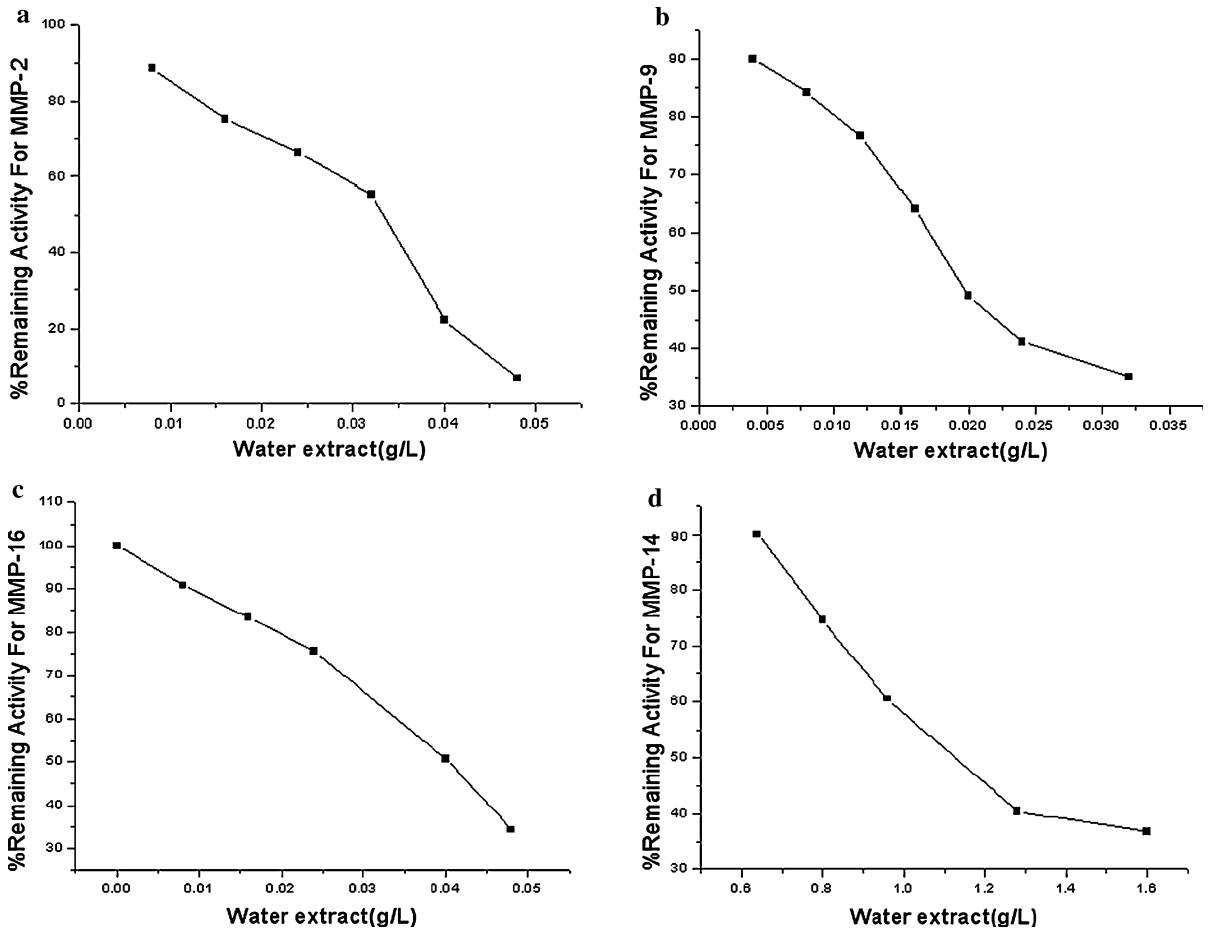

Fig. 1 Dose-dependent inhibition of final water extract on enzymatic activities of MMPs. **a** MMP-2, **b** MMP-9, **c** MMP-16, **d** MMP-14. Increasing volumes of water extract were used in the reactions to measure MMP activities as described in "Materials and methods" section. The concentration of the water extract and the remaining activities for each MMP was indicated in the graph

extract was precipitated using ethanol and filtered. The filtrate showed a good inhibitory activity to MMPs, while the precipitation mainly composed of polysaccharide and protein was no inhibitory effect. Then the filtrate was concentrated and separated by using the D101 macroporous adsorption resin. The sample in the resin was eluted with methanol–water and the ratios of water–methanol were 1:0, 1:9, 2:3, 4:5 and 0:1 in turn. The fractions were named A1, A2, A3, A4 and A5, and the inhibitory effects of these fractions to MMPs were detected. Only A1 fraction showed strong inhibitory activity of MMPs. In contrast, there were no inhibitory effect of the fraction A2, A3, A4 and A5 on the MMP activity. These results indicated that the active MMP inhibitory compound of Korean monkshood root was in the fraction of water solution, and not in the fractions with weak polarity. The fraction A1 was recrystallizated for several times and a white pure compound was obtained and further characterized.

Structure determination of the active MMP inhibitory compound

The active compound from *Korean monkshood root* has strong inhibitory effects on many MMPs, of which structure was identified by the elemental analysis, infrared spectrum (IR) and X-ray diffraction. The elemental analysis showed there was almost no carbon in the sample with excluding organic compounds. Figure 2 presented the characteristic infrared absorption of this compound. There was no peak in 3,000 cm^{-1}, the characteristic absorption peak of

methyl, which further support the speculation that it might not be organic matter. In the 3,500–3,000 cm^{-1} region, 3402.71 cm^{-1} exhibited a strong stretching vibration, which is the characteristic absorption of water hydroxyl and amino groups. The compound contained coordinated water in the crystallization which confirmed by the characteristic absorption of coordinated water at 1635.25 cm^{-1}.

To confirm the cation and anion in this active inorganic substance, it was detected with ICP-AES. The results showed that this compound only has Aluminum ion without other metal ions. The reaction of this compound with NaOH displayed blue with wet pH test paper, which proved the existence of NH_4^+. The existence of SO_4^{2-} was proved by that the solution reacted with $BaCl_2$ resulted white insoluble precipitate in nitric acid.

The X-ray analysis revealed that this compound was aluminum ammonium sulfate dodecahydrate ($NH_4Al(SO_4)_2 \cdot 12H_2O$), belonging to space group *Pa-3* and indicating a high symmetry. The asymmetric unit consists of one-third of the sulfate ion, one-sixth of aluminum hexahydrate ion, one-sixth of the ammonium cation and one lattice water (as the ammonium cation located at a special symmetric position, the hydrogen atoms on it cannot be determined accurately). Table 1 summarized the crystal data and structural refinement parameters for this compound and Table 2 listed selected bond distances and bond angles. In Fig. 3, the aluminum ion is six coordinated to four water molecules in the equatorial plane, and another two water molecules in the axial position to give an octahedral geometry. In addition, there are cationic ammonium ion and anti-water

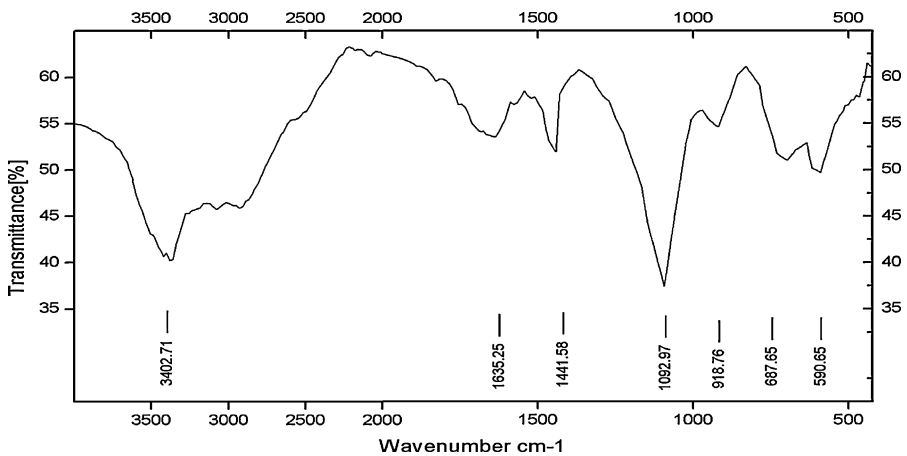

Fig. 2 The IR absorption spectrum of the monomer

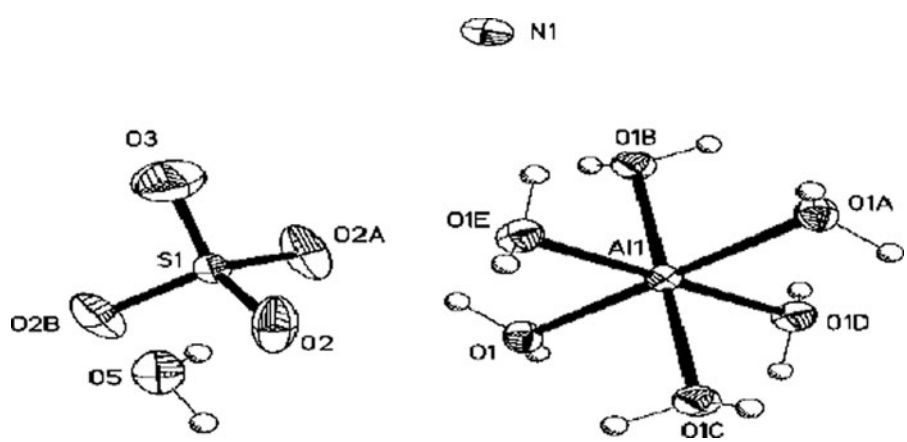

Fig. 3 The molecular structure of the monomer

sulfate anion. The crystal cell contains six crystal water. Thus it was confirmed that the main MMPIs isolated from Korean monkshood root was aluminum ammonium sulfate dodecahydrate.

The content of metallic elements in *Korean monkshood root*

To explain the reason that $NH_4Al(SO_4)_2{\cdot}12H_2O$ was purified from Korean monkshood root, the ICP-AES was used to determine the content of major metallic elements in this herbal medicine. The results were presented in Table 3.

There were a number of metallic elements in Korean monkshood root, the contents of which in order are Al > Ca > Fe > K > Mg > Na > Zn > Cu > Mn. The content of Al reached 2560.00 mg/kg, which is much higher than the other metallic elements. This result indicated that Korean monkshood root is enriched in Al element.

The inhibitory effect of $NH_4Al(SO_4)_2{\cdot}12H_2O$ on MMPs

In order to investigate the inhibitory effect of this compound on MMPs, MMP-2, -3, -9, -13, and -16 were tested. The results are shown in Table 4. This active compound has strong inhibitory effect on MMPs, especially on the gelatinases MMP-2 and MMP-9, of which the IC_{50} was 0.54 and 0.50 μM, respectively.

Determine the active MMP inhibitory component of $NH_4Al(SO_4)_2{\cdot}12H_2O$

To find the active MMP inhibitory component of $NH_4Al(SO_4)_2{\cdot}12H_2O$, a series of salts, containing the NH_4^+, Al^{3+} or SO_4^{2-}, were selected to test the inhibitory effect on MMP-2, -9 and -16. Table 5 shows the IC_{50} values of these salts. The salts containing Al^{3+} ions, such as $NH_4Al(SO_4)_2$, $KAl(SO_4)_2$, $Al_2(SO_4)_3$, $AlCl_3$ and $Al(NO_3)_3$ showed strong inhibitory action. However, the salts without Al^{3+} ions, regardless of SO_4^{2-} salts or NH_4^+ salts, showed no inhibitory effect on these MMPs. These data suggested that the active group of $NH_4Al(SO_4)_2{\cdot}12H_2O$ for MMP inhibition was Al^{3+}.

Effect of $NH_4Al(SO_4)_2$ on cell viability and *Matrigel* invasion

To evaluate the viability of HT1080 cells after exposure to $NH_4Al(SO_4)_2$, cells were treated with up to 500 μM of $NH_4Al(SO_4)_2$ and cell viability was measured using an MTT assay. As shown in Fig. 4, $NH_4Al(SO_4)_2$ had a dose-dependent effect on the viability of the cells. However, 50 μM of $NH_4Al(SO_4)_2$ had minimal effect on the viability of HT1080 cells.

As MMPs participates the invasion process of many tumor cells, we further investigated if $NH_4Al(SO_4)_2$ impairs invasion of HT1080 cells. An in vitro invasion

Table 4 The IC_{50} of $NH_4Al(SO_4)_2{\cdot}12H_2O$ on MMPs

MMPs	IC_{50}(μM)
MMP-2	0.54
MMP-3	11.88
MMP-9	0.50
MMP-13	4.80
MMP-16	3.40

Table 5 Analysis of the active component of $NH_4Al(SO_4)_2$ on MMP-2, -9, and -16

	IC_{50} (μM)		
	MMP-2	MMP-9	MMP-16
Compound			
$NH_4Al(SO_4)_2$	0.54	0.50	3.40
$KAl(SO_4)_2$	0.47	0.43	2.99
$Al_2(SO_4)_3$	0.22	0.27	1.56
$AlCl_3$	1.27	0.86	10.00
$Al(NO_3)_3$	0.75	0.83	4.00
$(NH_4)_2SO_4$	No inhibition	No inhibition	No inhibition
K_2SO_4	No inhibition	No inhibition	No inhibition

Table 3 The contents of metallic elements in *Korean monkshood root*

Element	Al	Ca	Fe	K	Mg	Na	Zn	Cu	Mn
Content (mg kg^{-1})	2560.00	959.94	141.30	108.32	75.40	43.16	8.57	2.23	0.78

assay using a Transwell chamber coated with a reconstituted basement membrane (EHS *Matrigel*) was performed to examine the anti-invasive effects of $NH_4Al(SO_4)_2$. The Transwell invasion assay showed that $NH_4Al(SO_4)_2$ in the concentration ranges of 0–50 μM exerted a dose-dependent inhibition of human HT-1080 cells invasion through *Matrigel* (Fig. 5). The cells that penetrated through *Matrigel* was 85.47, 72.58, and 62.58% compared to the no $NH_4Al(SO_4)_2$ control for 10, 20, and 50 μM $NH_4Al(SO_4)_2$, respectively, while no effect was observed to the viability of the cells at these concentrations (Fig. 4).

Discussion

In the present study, we showed the water extract of Korean monkshood root significantly inhibit the activity of several members of the MMP family (Fig. 1). The inhibition activities towards these MMPs remained in the water fraction of the extract after the fractionation processes. Surprisingly, the active compound is an inorganic salt, aluminum ammonium sulfate dodecahydrate identified by the elemental analysis, infrared spectrum (IR) and X-ray diffraction (Figs. 2, 3). The MMP inhibitory activities by this inorganic salt was strong as the IC50 values are in micro-molar to sub micro-molar range (Table 4). The compound exhibited some selectivity towards MMPs, it showed stronger inhibition to MMP-2 and MMP-9 than other MMPs examined (Table 4). Further analysis revealed that the Al^{3+} was mostly responsible for the MMP inhibition, as a number of the salts bearing Al^{3+} showed strong MMP inhibitory activities. Finally, aluminum ammonium sulfate showed low cytotoxicity to HT1080 human fibrosarcoma cells, and it inhibited cell invasion similar to other unknown MMP inhibitors.

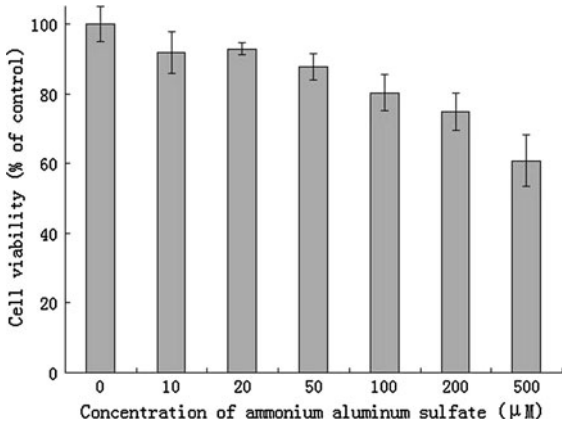

Fig. 4 MTT assay. 1×10^4 cells containing 2% fetal bovine serum with various final concentrations of $NH_4Al(SO_4)_2$ were incubated in 96-plates well for 2 days

Natural compounds are becoming a popular source for the discovery of MMP inhibitors in recent years. Anti-MMPs properties have been reported from the components of various natural products (Sang et al. 2006; Mannello 2006; Dell'Aica et al. 2007). Epigallocatechin gallate (EGCG) from green tea can act as a potent inhibitor of matrix metalloproteinases (Demeule et al. 1478). Caffeic acid (CA), which found in fruits, vegetables, wine, olive oil, and coffee (Shahidi and Naczk 1995), has inhibitory activities towards a number of enzymes including MMPs (Park et al. 2005). It has also been reported with anti-tumor activity (Tanaka et al. 1993) and anti-inflammatory properties (Michaluart et al. 1999). A CA derivative, 5-caffeoylquinic acid (chlorogenic acid; CHA), has been isolated from the stem barks of Euonymus alatus (Jin et al. 2005), which have been utilized as a traditional medicine for cancer treatment (Lee et al. 1993). CHA also exerts a strong inhibitory effect against MMP-9 activity in a concentration-dependent manner (Jin et al. 2005). Flavonoids have also been found to have inhibitory activities toward MMPs. Some flavonoids including 5-hydroxyflavone, luteolin 7-*O*-glucoside were shown to inhibit MMP-2 and MMP-9 in the micromolar range (Ende and Gebhardt 2004).

Despite these progresses in the nature compound as MMP inhibitors, to our knowledge, no inorganic compound has been isolated from herbal medicines as MMP inhibitors. Here, we isolated and identified the active compound in the water extracts of Korean monkshood root as $NH_4Al(SO_4)_2{\cdot}12H_2O$, and demonstrated it as an inorganic non-chelator compound with high MMP inhibitory activity. Although $NH_4Al(SO_4)_2{\cdot}12H_2O$ was isolated from Korean monkshood root after a series of purification steps, whether this inorganic compounds exists in the plant is still not verified. As it was demonstrated by ICP-AES that the Korean monkshood root is enriched in Al element, it will be interesting to know the real form of existence of aluminium in the plant from a biological point of view.

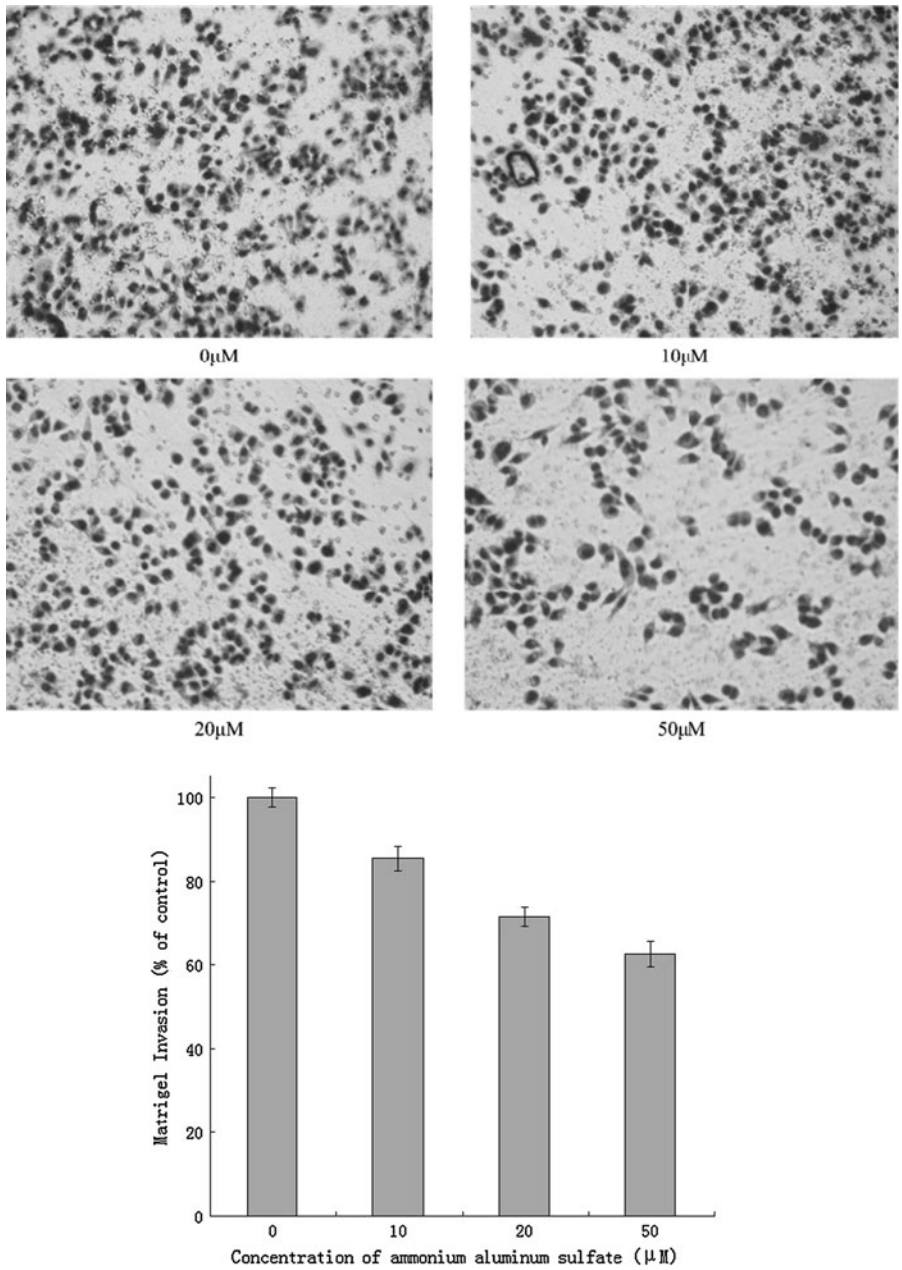

Fig. 5 Matrigel invasion assay was carried out with $NH_4Al(SO_4)_2$ (0, 10, 20, and 50 μM). After 24 h incubation, the cells that invaded to the underside of the filters were visualized under the microscope (*upper panel*) and the total number of cells were counted (*lower panel*). Values obtained were calculated by averaging the total number of cells from three filters. Results are the means ± SD of three independent experiments

Inorganic salt MMP inhibitors had rarely been reported: de Souza et al. analyzed MMP-2 and MMP-9 in the conditioned media of gingival explants by gelatin zymography and showed inhibition of MMP-2 and MMP-9 by $ZnSO_4$ with half inhibition concentration of 15 and 40 μM, respectively whereas $CuSO_4$, $HgSO_4$ and $SnCl_2$ showed less efficient inhibition potential (de Souza et al. 2000). In another case, Ziouti et al. (2006) showed 5 mM barium chloride selectively inhibited MMP-2 but not MMP-9 by zymography, while other bivalent metal salts such as Fe, Ni, Co, Mn, Cd, Hg, and Zr, showed not inhibitory effect on the activity of MMP-2, and $SrCl_2$ slightly suppressed MMP-2 activity.

We have also analyzed the active component in $NH_4Al(SO_4)_2{\cdot}12H_2O$ and demonstrated the Al^{3+} is mainly responsible for the MMP inhibition. The method we used for the MMP activity assay is fluorescent substrate method. However, when zymography was performed to verify the MMP inhibition of

$NH_4Al(SO_4)_2$ with active form of the MMP-2 and MMP-9 or the MMPs in the HT1080 cell culture media, to our surprise, $NH_4Al(SO_4)_2$ only showed apparent inhibition to MMP-2 and MMP-9 at very high concentrations ($\geq$500 μM, data not shown). Therefore, a huge discrepancy exists of the extent of MMP inhibition by $NH_4Al(SO_4)_2$ between using zymography method and using fluorescent substrate method. Although it is not clear for the cause of this discrepancy, we suspect that it may be caused by the differences in the steps of these two methods. In zymography method, the re-natured MMPs were pre-exposed readily able to digest gelatin after the removal of SDS. The Al^{3+} could only get access to the MMPs after the electrophoresis. While when fluorescent substrate method was used, the Al^{3+} salt is pre-incubated with MMPs before the fluorescent substrate is added. Therefore it is likely that the fluorescent substrate method achieve better inhibition of the MMPs by the Al^{3+} salt.

An important question need to be answered is the mechanism of the inhibition by Al^{3+}. How does it interact with MMPs? Does it bind to a specific site in the enzyme? Dose the binding cause the conformation change of the enzyme? Moreover, besides 3 valent aluminum cations, do other metal ions have the inhibitory activity toward MMPs? The answers to these questions may provide valuable information to the research on the MMP inhibitors. Currently, we are investigating the mechanism of this inhibition in a structural basis as well as studying the effect of other metal ions on the MMP activities.

Finally, the therapeutic benefits of Korean monkshood root as well the applicability of aluminum in medical use must be carefully examined and not be overly interpreted. Aluminum is known for its neurotoxicity and is associated with altered membrane function of the blood–brain barrier (Banks and Kastin 1989). Studies have also raised possible connection of aluminum salts to diseases such as breast and lung cancers, suggesting the adverse effects of accumulation of aluminum in human tissues (Exley et al. 2007; Rønneberg and Andersen 1995). Therefore, the toxicity of aluminum containing Korean monkshood root must be thoroughly evaluated for its clinical use.

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References

Banks WA, Kastin AJ (1989) Aluminum-induced neurotoxicity: alterations in membrane function at the bloodbrain barrier. Neurosci Biobehav Rev 13:47–53

Coussens LM, Fingleton B, Matrisian LM (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulation. Science 295:2387–2392

de Souza AP, Gerlach RF, Line SR (2000) Inhibition of human gingival gelatinases (MMP-2 and MMP-9) by metal salts. Dent Mater 16:103–108

Dell'Aica I, Caniato R, Biggin S, Garbisa S (2007) Matrix proteases, green tea, and St. John's wort: biomedical research catches up with folk medicine. Clin Chim Acta 381:69–77

Demeule M, Brossard M, Page M, Gingras D, Beliveau R (2000) Matrix metalloproteinase inhibition by green tea catechins. Biochim Biophys Acta 1478:51–60

Egeblad M, Werb Z (2002) New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2:161–174

Ende C, Gebhardt R (2004) Inhibition of matrix metalloproteinase-2 and -9 activities by selected flavonoids. Planta Med 70:1006–1008

Engel LW, Straus SE (2002) Development of therapeutics: opportunities within complementary and alternative medicine. Nat Rev Drug Discov 1:229–237

Exley C, Charles LM, Barr L, Martin C, Polwart A, Darbre PD (2007) Aluminium in human breast tissue. J Inorg Biochem 101:1344–1346

Fingleton B (2008) MMP inhibitor clinical trials—the past, present, and future. The cancer degradome—proteases and cancer biology. Springer, New York, pp 759–785

Jin UH, Lee JY, Kang SK, Kim JK, Park WH, Kim JG, Moon SK, Kim CH (2005) A phenolic compound, 5-caffeoylquinic acid (chlorogenic acid), is a new type and strong matrix metalloproteinase-9 inhibitor: isolation and identification from methanol extract of *Euonymus alatus*. Life Sci 77:2760–2769

Kessenbrock K, Plaks V, Werb Z (2010) Matrix metalloproteinases: regulators of the tumor microenvironment. Cell 141:52–67

Lee H, Kim HK, Ha TY (1993) Anti-tumor effect of winged Euonymus against chemically induced and malignant cell implanted-tumors in mice. Korean J Immunol 15:243–253

Mannello F (2006) Natural bio-drugs as matrix metalloproteinase inhibitors: new perspectives on the horizon? Recent Pat Anticancer Drug Discov 1:91–103

Michaluart P, Masferrer JL, Carothers AM, Subbaramaiah K, Zweifel BS, Koboldt C, Mestre JR, Grunberger D, Sacks PG, Tanabe T, Dannenberg AJ (1999) Inhibitory effects of caffeic acid phenethyl ester on the activity and expression of cyclooxygenase-2 in human oral epithelial cells and in a rat model of inflammation. Cancer Res 59:2347–2352

Overall CM, Kleifeld O (2006) Towards third generation matrix metalloproteinase inhibitors for cancer therapy. Br J Cancer 94:941–946

Park WH, Kim SH, Kim CH (2005) A new matrix metalloproteinase-9 inhibitor 3,4-dihydroxycinnamic acid (caffeic acid) from methanol extract of *Euonymus alatus*: isolation and structure determination. Toxicology 207:383–390

Rønneberg A, Andersen A (1995) Mortality and cancer morbidity in workers from an aluminium smelter with prebaked carbon anodes—part II: cancer morbidity. Occup Environ Med 52:250–254

Sang QX, Jin Y, Newcomer RG, Monroe SC, Fang X, Hurst DR, Lee S, Cao Q, Schwartz MA (2006) Matrix metalloproteinase inhibitors as prospective agents for the prevention and treatment of cardiovascular and neoplastic diseases. Curr Top Med Chem 6:289–316

Shahidi F, Naczk M (1995) Food phenolics, sources, chemistry, effects, applications. Technomic Publishing Company, Lancaster

Sheldrick GM (1997) SHELXL-97, Program for X-ray Crystal Structure refinement. University of Gottingen, Germany

Sternlicht MD, Werb Z (2001) How matrix metalloproteinases regulate cell behavior. Annu Rev Cell Dev Biol 17:463–516

Tanaka T, Kojima T, Kawamori T, Wang A, Suzui M, Okamoto K, Mori H (1993) Inhibition of 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis by the naturally occurring plant phenolics caffeic, ellagic, chlorogenic and ferulic acids. Carcinogenesis 14:1321–1325

Yiotakis A, Dive V (2008) Third-generation MMP inhibitors: recent advances in the development of highly selective inhibitors. The cancer degradome—proteases and cancer biology. Springer, New York, pp 787–810

Ziouti N, Mastronikolis NS, Andonopoulos AP, Georgakopoulos CD, Vynios DH (2006) Selective inhibition of MMP-2 (Gelatinase A) by Barium Chloride. Anal Biochem 350:159–161