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Large-scale and effective screening of Korean medicinal plants for inhibitory activity on matrix metalloproteinase-9

Un-Kyo Seo[a], Young-Ja Lee[a], June-Ki Kim[a], Byung-Yoon Cha[a], Dong-Wook Kim[a,1], Kyung-Soo Nam[b], Cheorl-Ho Kim[a,*]

[a] *Department of Internal Medicine, Biochemistry and Pathology, College of Oriental Medicine, Dongguk University, Kyungju City, Kyungbuk 780-714, Republic of Korea*
[b] *Department of Pharmacology, College of Medicine, Dongguk University, Kyungju, Kyungbuk 780-714, Republic of Korea*

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Abstract

Matrix metalloproteinase-9 (MMP-9) degrades type IV collagen constituting the major structural component of the basement membrane and extra cellular membrane. The enzymatic activity is found to be elevated in tumor tissues. With the aim of finding novel MMP-9 inhibitors from natural products, 87 extracts of oriental medicinal herbs, which are used as prescriptions for cancer treatment in traditional Korean medicine, were screened for their inhibitory activities towards MMP-9. It was found that most of the hexane and chloroform fractions as well as water extracts showed a weak inhibitory effect on MMP-9 activity at a concentration of 100 μg/ml. However, a strong inhibition was found in the butanol fractions of *Cinnamomum cassia* PRESL, *Magnolia obovata* THUEB., *Magnolia officinalis* REHD. et WILS., *Magnolia officinalis* REHD. et WILS. var. *biloba* REHD. et WILS., and *Euonymus alatus* (THUNB.) SIEB. with inhibitory activity (>90%) at a concentration of 100 μg/ml.

Keywords: Oriental medicinal herbs; Gelatine zymography; Matrix metalloproteinase-9 (MMP-9); Inhibitor; *Cinnamomum cassia*; *Magnolia officinalis*; *Euonymus alatus*

1. Introduction

Matrix metalloproteinases (MMPs) with a divalent Zn^{2+} at the active site constitute an important class of endoproteinases involved in extracellular matrix remodeling. Under normal physiological conditions, the proteolytic activities are controlled by maintaining a balance between synthesis of the active forms and inhibition of the same by tissue inhibitors of matrix metalloproteinases (TIMPs). In pathological conditions, this fine balance is tipped more towards degradation of proteins and proteoglycans leading to a loss of tissue integrity. Consequently, there has been significant interest in the development of MMP inhibitors with the understanding that such agents will be able to control the aberrant regulation of MMP production, thus leading to amelioration of various disease states, such as osteoarthritis and tumor metastasis (De et al., 1999). Several novel MMP inhibitors (i.e., Batimastat/BB-94 and Marimastat/BB-2516) have been developed and are currently being investigated in clinical trials (Sugita, 1999; Wojtowicz-Praga, 1999).

Abbreviations: MMP, matrix metalloproteinase; MMP-9, matrix metalloproteinase-9; TIMP, tissue inhibitors of matrix metalloproteinase; DMSO, dimethyl sulfoxide; BuOH, butanol; $CHCl_3$, chloroform; EtOAc, ethyl acetate; CC, (+)-catechin; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate (EGCG); DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline

* Corresponding author. Tel.: +82 54 770 2663; fax: +82 54 770 2281.
E-mail address: chkimbio@dongguk.ac.kr (C.-H. Kim).
[1] Present address: Department of Medicinal Plant Resources, Mokpo National University, Mokpo 534-729, Republic of Korea.

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Chemopreventive properties have been associated with the components of various natural products, including green tea polyphenols, resveratrol, limonene and organosulfur compounds from garlic (Kaegi, 1998). Especially, green tea polyphenols and one of its constituents (epigallocatechin gallate) caused a strong inhibition of the gelatinolytic activities of MMP-2 and MMP-9, and of the elastinolytic activity of MMP-12 (Demeule et al., 2000).

Furthermore, the activity of MMP-9 has been implicated in a variety of tissue remodeling processes, including tumor invasion and metastasis both in experimental models and human malignancies (Partridge et al., 1997; Kim et al., 1998). Thus, it is known that MMP-9 is most closely linked with metastasis of cancer cell (Bernhard et al., 1994). The emergence of metastasis in organs distant from the primary tumor is the most devastating aspect of cancer. From this point of view, various inhibitors, such as inhibitors of angiogenesis and matrix metalloproteinase, are presently being developed as novel therapeutic drugs. Several traditional and herbal medicines, such as Keishi-ka-kei-to, Juzen-taiho-to, Shimotsu-to, Unsei-in, Hochu-ekki-to, Shosaiko-to, and Shichimotsu-koka-to have been so far reported to exhibit an anti-metastatic effect (Yano et al., 1994; Yoshikawa et al., 2000). However, there have been no systematic studies of herbal medicines in the metastatic setting with regard to MMP-9 activity using medicinal plant resources. Therefore, we have screened the Korean medicinal plants for their abilities to inhibit the MMP-9 activity in vitro.

2. Materials and methods

2.1. Materials

The plants were collected in Kyungju city, the Republic of Korea. The samples and voucher specimens are kept in the herbarium of the College of Oriental Medicine, Dongguk University. The plant samples (40 g) were extracted three times successively with 1000 ml methanol (MeOH) at 70 °C for 3 h. The methanol extracts were filtered, evaporated under vacuum and water was added. The w/w yield of extracts was about 7.4 ± 4.4%. The aqueous layer of the MeOH extract was partitioned with hexane (Hx) (3 ml × 100 ml) followed by chloroform ($CHCl_3$) (3 ml × 100 ml) and finally with butanol (BuOH) (3 ml × 100 ml). The respective fractions and water extracts were evaporated at 40 °C under reduced pressure. For the bioassay, the fractions and water extracts were dissolved in dimethyl sulfoxide (DMSO) and further diluted with incubation buffer.

For *Euonymus alatus*, the plant samples were extracted three times with methanol at 70 °C for 5 h. The extracts were filtered through a 0.45 μm filter and lyophilized. The w/w yield of extracts was about 2.25%. The methanol extract (45 g) was suspended in water (500 ml) and successively re-extracted by 500 ml each (three times) of Hx (yield: 10.1 g), $CHCl_3$ (yield: 11.6 g) and ethyl acetate (EtOAc) (yield: 13.4 g) and BuOH (yield: 3.9 g). All fractions, including the final remaining water fraction (yield: 9.0 g) were concentrated under reduced pressure using a rotary evaporator and then freeze dried.

The natural products (+)-catechin (C), (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC) and (−)-epigallocatechin gallate (EGCG) were obtained from Sigma Chemical Co. (St. Louis, Mo, USA).

2.2. Cell culture

Human hepatocellular carcinoma cell lines Hep3B, were obtained from the Korean cell line bank (Seoul, Korea). These cell lines were grown in Dulbecco's 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–air.

2.3. Gelatin zymography assay

Substrate gel zymography of the activity of MMP-9 was performed with a Mini-Protein II apparatus from Bio-Rad, according to a method described previously (Demeule et al., 2000; Choi et al., 2002). Cells were grown to sub-confluence and were rinsed with phosphate-buffered saline (PBS) and then incubated in serum-free medium for 24 h. The amount of gelatinase, such as MMP-9 in the serum-free medium was estimated and quantified by cell numbers. The serum-free medium was resuspended in a sample buffer, containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.00625% (w/v) bromophenol blue, then were agitated for 20 min at room temperature and loaded without boiling onto 7.5% acrylamide/bisacrylamide (29.2:0.8) separating gel, containing 0.1% (w/v) gelatin. Electrophoresis was carried out at a constant voltage of 100 V. After electrophoresis, the gels were soaked in 0.25% Triton X-100 (2 × 30 min) at room temperature and rinsed in NanoPure water. For gelatinase inhibition assays, the fractions, water extracts and EGCG were freshly solubilized in the Tris–HCl buffer used for developing the zymogram; the gel slab was cut into slices corresponding to the lanes which were put in different tanks and incubated at 37 °C for 20 h in the incubation buffer, containing 50 mM Tris–HCl (pH 7.6), 20 mM NaCl, 5 mM $CaCl_2$ and 0.02% Brij-58 with or without 100 μg/ml of the fraction, water extracts and 100 μM of EGCG. The slabs were stained for 15–30 min in 0.1% (w/v) Coomassie blue R-250 in 30% methanol and 10% acetic acid, and destained in the same solution without the Coomassie blue dye. After destaining, a light translucent band over a blue background was detected for gelatinase activity.

2.4. Densitometric and statistical analysis

The intensity of the bands obtained from zymogram studies was estimated with Gel-Print System (Core Bio Corp., Seoul, Korea). The values are expressed as mean ± S.E.