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Inhibitory Effects of (-)Epigallocatechin Gallate and Quercetin on Phorbol 12-Myristate 13-Acetate-Induced Secretion of Metalloproteinase-2 and Metalloproteinase-9*

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Matrix metalloproteinases (MMP) play an important role in the extracellular matrix (ECM) degradation underphysiological and pathological conditions. The present study examined the influence of (-)epigallocatechin gallate and quercetin on phorbol-12-myristate 13-acetate (PMA)-induced secretion of MMP-2 and MMP-9, when human umbilical vein endothelial cells (HUVEC) were treated with (-)epigallocatechin gallate and quercetin at supraphysiological concentrations of 25 μmol/L. No cytotoxicity was observed by MTT assay in response to a treatment with PMA in the presence of (-)epigallocatechin gallate and quercetin. Western blot analysis and gelatin zymography revealed that exposure of HUVEC to PMA enhanced the levels and gelatinolytic activities of pro and active forms of MMP-2 and active form of MMP-9. (-)Epigallocatechin gallate attenuated PMA-stimulated secretion of active forms of MMP-2 and MMP-9 concomitantly with a loss of activities of these enzymes, which was related to the decreased mRNA levels of MMP. Quercetin was more potent than (-)epigallocatechin gallate in alleviating MMP-9 protein secretion and activity with a decrease in MMP-9 mRNA accumulation. Taken together, the results indicated that (-)epigallocatechin gallte and quercetin exhibited inhibitory effects on MMP activity and may qualify as chemopreventive and cardiovascular protective agents.

Key words: Metalloproteinase-2, Matrix metalloproteinase-9, Phorbol-12-myristate 13-acetate, (-)Epigallocatechin gallate, Quercetin

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INTRODUCTION

The matrix metalloproteinases (MMP) play an important role in tumor invasion, angiogenesis and inflammatory tissue destruction.[1,2] Their proteolytic activity is regulated by activators or inhibitors such as membrane type MMP, urokinase-type plasminogen activator, specific tissue inhibitors of MMP (TIMP) and broad-spectrum inhibitors.[1,3,4] MMP-2, the most widely distributed MMP, plays an important role in the basement membrane turnover and is frequently overexpressed under pathological conditions.[5] Increased expression of MMP-2 and MMP-9 was observed in benign tissue hyperplasia and in atherosclerotic lesions.[2,6] These enzymes may contribute to a cell invasion-favoring matrix modification and thus to invasive aggressiveness of tumor cells.[7,8]

Polyphenols constitute one of the largest and most ubiquitous groups of phytochemicals. They are formed to protect plants from photosynthetic stress and reactive oxygen species.[9] Intensive interest has beenfocusing on the beneficial health effects of dietary polyphenols due to their anti-oxidative and anti-inflammatory activities.[10,11] Polyphenolic flavonoids differ in their anti-apoptotic and anti-atherogenic efficacy in hydrogen peroxide-treated human vascular endothelial cells.[12,13] We have shown that (-)epigallocatechin gallate and quercetin inhibited hydrogen peroxide- and oxidized LDL-induced apoptosis in human endothelial cells.[12,13] In addition, the flavones of luteolin and apigenin may hamper initial atherosclerotic events involving endothelial induction of cell adhesion

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molecules as anti-atherogenic agents.[14] In contrast, (-) epigallocatechin gallate and quercetin did not inhibit monocyte adhesion to cytokine-stimulated endothelium.[14] Consequently, these anti-apoptotic and anti-atherogenic features of polypenolic flavonoids appear to be related to their chemical structures.

(-)Epigallocatechin gallate, a major form of tea catechin, down-regulates vascular smooth muscle cell invasion through up-regulation of TIMP-2 expression to modulate MMP activity.[15] A green tea extract inhibits the activation of MMP-2 involved in vascular remodeling of atherosclerotic plaques in vascular smooth muscle cells.[16] (-)Epigallocatechin gallate and green tea hamper the MMP-mediated tumor angiogenesis and vascular tumor growth.[16,17] It has been also shown that the green tea extract reduces MMP-2 secretion and invasion in human ovarian cancer cell line SK-OV-3.[18] In addition, another bioflavonoid quercetin inhibits the invasion of murine melanoma B16-BL6 cells by decreasing pro-MMP-9 via the protein kinase C pathway.[19] Also quercetin inhibits MMP-1 expression in vascular endothelial cells, suggesting that itmight contribute to plaque stabilization.[20]

Based on the literature evidence that (-) epigallocatechin gallate and quercetin may inhibit MMP-mediated tumor angiogenesis and vascular tumor growth,[16,17,19] the present study attempted to examine differential effects of (-)epigallocatechin gallate and quercetin on the activation of MMP-2 and MMP-9 in phorbol 12-myristate 13-acetate (PMA)-exposed human umbilical vein endothelial cells (HUVEC). PMA is the most commonly used phorbol ester, and causes an extremely wide range of effects in cells and tissues via binding to and activating protein kinase C. The relative levels and activities of MMP-2 and MMP-9 were determined by gelatin zymography and Western blot analysis.

MATERIALS AND METHODS

1. Materials

M199 medium chemicals, (-)epigallocatechin gallate, quercetin, human epidermal growth factor and hydrocortisone and PMA were obtained from Sigma-Aldrich Co. (St. Louis, MO), as were all other reagents, unless specifically statedelsewhere. Fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Cambrex Corporation (East Rutherford, NJ).

2. Preparation and Culture of Human Endothelial Cells

HUVEC were plated at 90~95% confluence in all experiments. In experiments for the PMA-induced secretion of MMP-2 and MMP-9, cells were incubated overnight with 25 μmol/L (-)epigallocatechin gallate and quercetin prior to an exposure to 50 ng/mL PMA in a serum-free HEPES-buffered M199.

HUVEC were isolated from human umbilical cords using collagenase as described elsewhere.[12,14] Endothelial cells were cultured at 37 ℃ humidified atmospheres of 5% CO_2 in air, and were incubated in 25 mmol/L HEPES-buffered M199 containing 10% FBS, 2 mmol/L glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin supplemented with 3 ng/mL human epidermal growth factor and 0.075 μmg/mL hydrocortisone. HUVEC were passaged at confluence and used within 10 passages and confirmed by their cobblestone morphology and uptake of fluorescent 1.1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated LDL.[21]

3. Gelatin Zymography

Gelatin zymography of MMP-2 and MMP-9 from culture supernatants was performed as previously described.[22,23] Briefly, culture supernatants were subjected to electrophoresis on 8% SDS-PAGE (0.3 mol/L Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.03% bromophenol blue) co-polymerized with 0.1% gelatin as the substrate. After electrophoresis was complete, the gel was incubated for 1 h at room temperature in a 2.5% Triton X-100 solution, washed in 50 mmol/L Tris-HCl buffer, pH 7.5 at 37 ℃ for 30 min, and incubated for 20 h in 50 mmol/L Tris HCl buffer, pH 7.5, containing 10 mmol/L $CaCl_2$ and 0.05% Brij-35. The gels were stained with 0.1% Coomassie Brilliant Blue G-250, 2% acetic acid and 45% methanol, and then destained with 30% methanol and 10% acetic acid. Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin. The pro and active forms of MMP-2, and pro form of MMP-9 were identified as bands at 72 kD, 68kD, and 92 kD, respectively, by the relation to the relative mobility of Sigma SDS-PAGE marker proteins.

4. Protein Isolation and Western Blot Analysis I

Whole cell extracts were prepared from HUVEC in 1 mol/L Tris-HCl, pH 6.8 lysis buffer containing 10% SDS, 1% -glycerophosphate, 0.1 mol/L Na_3VO4, 0.5 mol/L NaF and protease inhibitor cocktail.[24] Cell lysates containing equal amounts of total protein and equal volumes of culture supernatants were fractionated by electrophoresis on 8% SDS-PAGE gels and transferred onto a nitrocellulose membrane. Nonspecific binding was blocked by soaking themembrane in TBS-T buffer [0.5

mol/L Tris-HCl (pH 7.5), 1.5 mol/L NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 3 h. The membrane was incubated overnight at 4 ℃ with a primary antibody [monoclonal mouse anti-human MMP-2 antibody (1:1,000); monoclonal mouse anti-human MMP-9 antibody (1:1,000), R & D systems Inc., Minneapolis, MN]. After three washes with TBS-T buffer, the membrane was then incubated for 1 h with goat anti-mouse IgG horseradish peroxidase (1:5,000, Jackson ImmunoResearch Laboratories, West Grove, PA). The levels of pro/active MMP-2 and pro/active MMP-9 proteins wererdetermined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotech. Inc., Rockford, IL) and Konica X-ray film (Konica Co., Tokyo, Japan). Incubation with monoclonal mouse -actin antibody (1:5,000) was also performed for the comparative control.

5. RNA Isolation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from HUVEC using a commercially available Trizol reagent kit (Invitrogen Co., Carlsbad, CA) after culture protocols. The RNA (5 μg) was reversibly transcribed in a Tris buffer [50 mmol/L Tris-HCl, pH8.3, 75 mmol/L KCl, 3 mmol/L $MgCl_2$, 110 mmol/L dithiothreitol, 10 mmol/L dNTP] with 200 units of reverse transcriptase and 10 μmol/L oligo-(dT)15 primer (Bioneer Co., Korea). The expressions of the mRNA transcripts of MMP-2 (forward primer: 5'-CTTCCCCCGCCAGCCCAAGTGGG-3', reverse primer: 5'-GGTGAACAGGGCTTCATGGGGGC-3'), MMP-9 (forward primer: 5'-ACGTGGACATCTTCGACGC-3', reverse primer: 5'-CGAACCTCCAGAAGCTCTGC-3'), and β-actin (forward primer: 5'-GACTACCTCATGAAGATC-3', reverse primer: 5'-GATCCACATCTGCTGGAA-3') were evaluated by RT-PCR as previously described with slight modification.[24] The PCR was performed in 50 μL of 10 μmol/L Tris-HCl (pH 9.0), 50 μmol/L KCl, 0.1% Triton X-100, 25 mmol/L $MgCl_2$, 10 mmol/L dNTP, 2.5 units of *Taq* DNA polymerase (Promega Co., Madison, WI), 10 μmol/L of each primer and was terminated by heating at 95 ℃ for 5 min. After 30 thermocycles of 30 s at 94 ℃, 30 s at 55 ℃ and 1 min at 72 ℃, electrophoresis of the PCR products (25 μL) on 1.5% agarose gel, the bands were visualized using an UV transilluminator (Amersham Pharmacia Biotech., Piscataway, NJ) and gel photographs were obtained. The absence of contaminants was routinely checked by the RT-PCR assay of negative control samples without a primer addition.

6. Data Analysis

Values wererepresented as means ± S.E.M. of separate experiments. Statistical analyses were conducted using Statistical Analysis Systems statistical software package version 6.12 (SAS Institute Inc., Cary, NC). Significance was determined by one-way ANOVA followed by Duncan multiple range test for multiple comparisons and *P*-values less than 0.05 were considered as statistically significant.

RESULTS

1. Expression of MMP-2 and MMP-9

Western blot analysis was used to address that 25 μ mol/L (-)epigallocatechin gallate and quercetin block the PMA-stimulated expression of MMP-2 and MMP-9. It should be noted that (-)epigallocatechin gallate and quercetin at 25 μmol/L in the presence of PMA showed no endothelial cytotoxicity, evidenced by MTT assay (data not shown). Expression of these proteins was

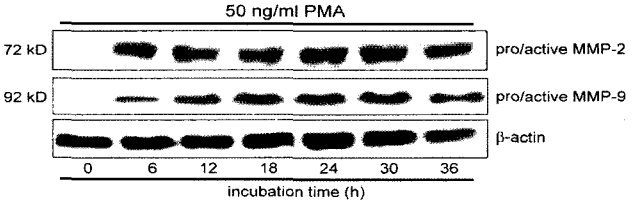

Fig. 1. Western blot data showing time course responses of synthesis of pro/active MMP-2 and pro/active MMP-9 to PMA in HUVEC.
After HUVEC culture protocols with 50 ng/mL PMA for 24 h, cell extracts were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against pro/active MMP-2 or pro/active MMP-9 (3 separate experiments). β-Actin protein was used as an internal control.

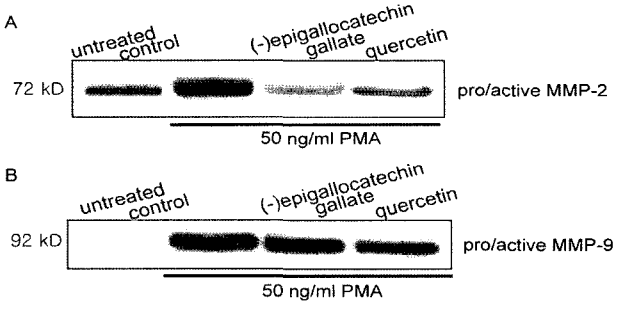

Fig. 2. Inhibition of PMA-activated endothelial secretion of pro/active MMP-2 (A) and pro/active MMP-9 (B) by (-)epigallocatechin gallate and quercetin.
After HUVEC culture protocols with 50 ng/mL PMA for 24 h in the presence of 25 μmol/L (-)epigallocatechin gallate and quercetin, equal volumes of culture supernatants were subjected to 8% SDS-PAGE and Western blot analysis with a primary antibody against pro/active MMP-2 or pro/active MMP-9 (3 separate experiments). β-Actin protein was used as an internal control.

markedly induced 6 h after activation by PMA in stimulated cells over the quiescent cells, and remained elevated up to 36 h (Fig. 1).

As expected, PMA strikingly elevated MMP-2 secretion within 24 h (Fig. 2A). PMA-exposed cells treated with 25 μmol/L (-)epigallocatechin gallate proved a marked inhibition of MMP-2 secretion. Quercetin- and PMA-exposed cells exhibited a substantial inhibition of MMP-2 secretion (Fig. 2A). In contrast, the MMP-9 secretion enhanced by PMA was feebly down-regulated in (-)epigallocatechin gallate-treated HUVEC (Fig 2B). Quercetin was more potent in attenuating the PMA-induced MMP-9 secretion.

2. Gelatinolytic Activity of MMP-2 and MMP-9

When HUVEC were treated with PMA up to 36 h under serum-free conditions, gelatin zymography revealed that the level and gelatinolytic activity of the active form of MMP-2 secreted in culture supernatants were increased within 24 h (Fig. 3A). It was shown that MMP-2 appeared to be substantially released as a pro form without an addition of PMA. In addition, the MMP-9 activationwas steadily increased 12 h after treatment with PMA (Fig. 3B).

Fig. 4 shows a representative zymogram of pro-MMP-2 and active forms of MMP-2 and MMP-9 in culture supernatants secreted from cells treated with (-)epigallocatechin gallate and quercetin. (-)Epigallocatechin gallate attenuated the gelatinolytic activity of pro-MMP-2 increased by 24 h treatment with PMA and abolished activation of MMP-2 (Fig. 4A). Also quercetin substantially mitigated the elevated activity of pro-MMP-2 and MMP-2. In contrast, quercetin proved full inhibition of activation of MMP-9, while (-)epigallocatechin gallate moderately inhibited the MMP-9 activity (Fig. 4B)

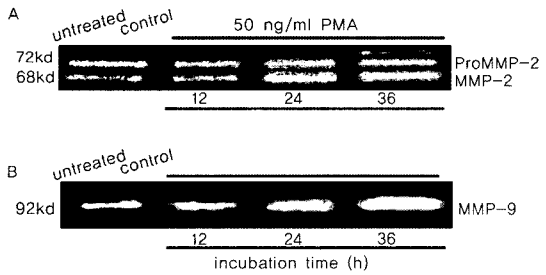

Fig. 3. Gelatin zymography showing time course responses of enzyme activities of pro-MMP-2/active MMP-2 (A) and active MMP-9 (B) in culture supernatants secreted from HUVEC after treatment with 50 ng/mL PMA.

Collected culture supernatants were subjected to electrophoresis on 8% SDS-PAGE co-polymerized with 0.1% gelatin as the substrate (3 separate experiments). Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin.

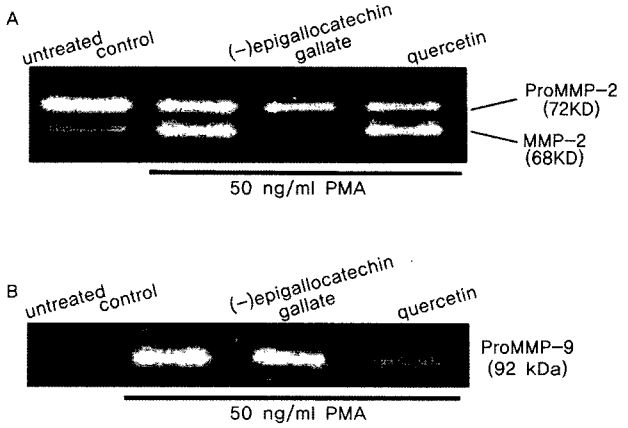

Fig. 4. Inhibitory effects of (-)epigallocatechin gallate and quercetin on gelatinolytic activities of pro-MMP-2/active MMP-2 (A) and active MMP-9 (B).

After HUVEC were incubated with 50 ng/mL PMA for 24 h in the presence of 25 μmol/L (-)epigallocatechin gallate and quercetin, culture supernatants were collected and subjected to gelatin zymography using 8% SDS-PAGE co-polymerized with 0.1% gelatin (3 separate experiments). Gelatinolytic activities were detected as unstained bands against the background of Coomassie blue-stained gelatin.

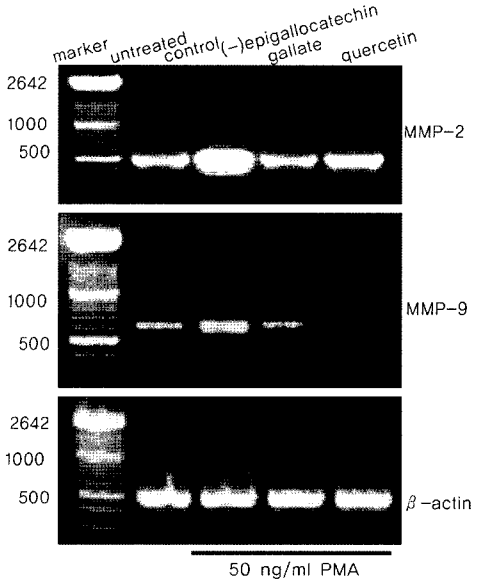

Fig. 5. RT-PCR data showing the steady-state mRNA transcriptional levels of MMP-2 and MMP-9 in (-)epigallocatechin gallate- or quercetin-treated and PMA-stimulated HUVEC.

Confluent HUVEC were incubated with 50 ng/mL PMA for 24 h in the presence of 25 μmol/L (-)epigallocatechin gallate and quercetin. β-Actin gene was used as an internal control for the co-amplification with MMP-2 and MMP-9 (3 separate experiments).

3. PMA-Stimulated Transcription of MMP

The RT-PCR data showed that PMA enhanced mRNA contents of MMP-2 and MMP-9 at 24 h (Fig. 5). The mRNA accumulation of MMP-2 in PMA-exposed HUVEC treated with (-)epigallocatechin gallate or quercetin was reduced. (-)Epigallocatechin gallate was more potent than quercetin in mitigating mRNA expression of MMP-2 and

still effective in inhibiting the mRNA expression of MMP-9 (Fig. 5). The MMP-9 mRNA content was completely dropped by a treatment with quercetin. Taken together, the data suggest that the inhibition of PMA-induced MMP activity in response to (-)epigallocatechin gallate and quercetin occurred concomitantly with loss of MMP protein synthesis (Fig. 2), which was related to down-regulation of MMP gene transcription.

DISCUSSION

Four major observations were extracted from this study. 1) (-)Epigallocatechin gallate at the non-toxic dose of 25 μmol/L, substantially attenuated endothelial secretion and gelatinolytic activity of MMP-2 and MMP-9 proteins elevated by PMA. 2) Supraphysiological quercetin also reduced the levels and activities of these activated proteins of HUVEC. 3) (-)Epigallocatechin gallate and quercetin appeared to inhibit the synthesis of MMP proteins via a direct modulation at their gene transcriptional levels. 4) (-)Epigallocatechin gallate was more potent in inhibiting PMA-induced MMP-2 secretion, while quercetin was more effective in blocking the elevated MMP-9 level. These overall observations demonstrate that (-)epigallocatechin gallate and quercetin have the potential capability to prevent angiogenesis, inflammation and atherosclerosis involving ECM degradation. The ability to block endothelial MMP secretion argues for a therapeutic target of actions of (-)epigallocatechin and quercetin under these pathological conditions.

Numerous studies have previously shown that polyphenolic flavonoids present in fruits and vegetables, and beverages derived from plants such as cocoa, red wine and tea, have substantial antioxidant abilities under various oxidative circumstances.[25~27] There is compelling evidence that the distinct structures of flavonoids appear to be partially responsible for their antioxidant activities.[28,29] Among the numerous plausible mechanisms by which polyphenols may confer cardiovascular protection, improvement of the endothelial function and inhibition of angiogenesis and cell migration and proliferation in blood vessels have been the focus of recent studies. Themechanisms triggered by polyphenols with specific structures contribute to the vasoprotective, anti-angiogenic, anti-atherogenic, vasorelaxant and anti-hypertensive effects found in animals and in patients.[30] In addition, thecurrent evidence suggests that there are multiple targets for cancer chemoprevention by green tea and/or its polyphenolic constituents by modulating novel pathways involved in angiogenesis and metastasis.[31]

The role and involvement of MMP in the angiogenic and metastatic cascades have been shown by molecular genetic studies employing knock-out or transgenic animals and tumor cell lines over-expressing or down-'regulating a specific MMP.[32] In particular, the gelatinolytic MMP-2 and MMP-9 are abundantly expressed in various malignant tumors, play an active role in angiogenesis, and may also influence the process of atherosclerotic lesion formation. One of the earliest events in the metastasis of cancer is the invasion through the basement membrane and proteolytic degradation of the ECM proteins. Accordingly, given the clear implications of MMP in many human cancers, MMP remain as important targets of cancer therapy. Considerable interest is increasing with regard to the use of dietary botanical supplementsfor chemopreventive and neuro- and cardiovascular-protective effects.[33] Indeed, dietarybotanicals such as green tea polyphenols and grape seed proanthocyanidins have been suggested as MMP inhibitors and as cancer chemo-therapeutic agents.[31,34]

Polyphenolic flavonoids have been shown in vitro to profoundly affect ECM turnover by regulating expression and activity of gelatinases at the pre- and post-transcriptional levels, suggesting that they could have a beneficial effect in connective tissue destruction and remodeling.[35] The present studydemonstrated that (-)epigallocatechin gallate and quercetin inhibited endothelial secretion and gelatinolytic activity of MMP-2 and MMP-9 activated by a phorbol ester by modulating the transcriptional expression of these proteins. The respective magnitudes of (-)epigallocatechin gallate and quercetin in inhibiting MMP-2 and MMP-9 were not the same, which denotes that there are different structural requirements for the inhibitory effects on MMP-2 and MMP-9 proteins. It has been reported that catechins are able to modulate the gelatinolytic activity of MMP-9 by reducing its release from macrophages and that gallocatechins decrease MMP-9 secretion by lowering MMP-9 promoter activity and mRNA levels.[36] The structural requirements for the MMP-9 inhibition are different from those necessary for targeting gene expression.[36] In addition, both red wine polyphenols and green tea polyphenols prevent effectively the thrombin-induced activation of MMP-2 in vascular smooth muscle cellsby both redox-sensitive and redox-insensitive mechanisms.[37]

In summary, the present study has shown that (-)epigallocatechin gallate and quercetin inhibited expression and activity of gelatinases, MMP-2 and MMP-9 at the transcriptional levels, although the magnitudes of

(-)epigallocatechin gallate and quercetin in inhibiting these gelatinases were not the same. In recent years, much consideration has been given to the role of polyphenols in preventing degenerative diseases such as cancer and cardiovascular diseases. This study provides the evidence that (-)epigallocatechin gallate and quercetin havethe potential capability to prevent angiogenesis, inflammation and atherosclerosis involving ECM degradation. It is deemed that there are multiple targets for chemoprevention and treatment of cardiovascular diseases by (-)epigallocatechin gallate and quercetin. Further studies are necessary to identify novel pathways that could be exploited for prevention and/or treatment of cancer and cardiovascular diseases by (-)epigallocatechin gallate and quercetin

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