Regular Article

Potential Therapeutic Agents, Polymethoxylated Flavones Isolated from *Kaempferia parviflora* for Cataract Prevention through Inhibition of Matrix Metalloproteinase-9 in Lens Epithelial Cells

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INTRODUCTION

Cataract is a common worldwide disorder leading to blindness, despite the option of surgery procedure in developed countries. Currently, there are no pharmacological therapy for inhibiting cataract formation. Thus, an understanding of the cellular and molecular mechanisms involved in cataract formation may lead to the development of therapeutic strategies for the prevention or treatment of cataracts.

Matrix metalloproteinases (MMPs) are a family of more than 25 genetically distinct but structurally related zinc-dependent endopeptidases that act as key regulators of tissue remodeling. MMPs are regulated by their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs), which control enzyme activity and proenzyme activation. MMPs and TIMPs in human eyes have been investigated by several experimental methods, and investigators reported the constitutive expression of multiple MMPs and TIMPs in various ocular tissues, including lenses. For example, the use of immunohistochemical and enzyme-linked immunosorbent assay (ELISA) analysis revealed that normal human lenses were found to express MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1, TIMP-2, and TIMP-3. In addition, Hodgkinson et al. found that membrane-type MMPs (MT-MMPs) genes were strongly expressed in human donor lenses. Of particular interest in the lens are the gelatinases A and B (MMP-2 and MMP-9), which are involved in corneal wound healing processes and cataract formation. In normal lenses, MMP-2 and MMP-9 are expressed at low levels; both are increased when the lens is stressed by radiation, oxidative stress, or transforming growth factor-β (TGF-β). In addition, evidence has accumulated to show that the expression of specific MMPs including MMP-2 and MMP-9 is induced in a variety of cataract phenotypes, such as anterior subcapsular cataract (ASC) and posterior capsular opacification (PCO).

Korol et al. demonstrated that MMP-9 knockout mice were resistant to TGF-β-induced ASC formation. These results suggested that the inhibition of MMP activity by MMP inhibitors may be useful for the prevention of specific forms of cataract, such as ASC and PCO.

Many naturally occurring compounds have become candidates for drug development and the subsequent clinical management procedures. Polymethoxylated flavones (PMFs) are found almost exclusively in plants of the *Citrus* genus. They are of particular interest owing to their broad spectrum of biological activities, which includes antitumor, anti-insulin resistance, and growth inhibitory activities. Recently, Korol et al. addressed the individual requirements of MMP-2 and MMP-9 in ASC formation through the use of MMP knockout mice and demonstrated that MMP-9 knockout mice were resistant to TGF-β-induced ASC formation. These results suggested that the consumption of PMFs isolated from *K. parviflora* may be an effective strategy to delay the development of cataracts, such as ASC and PCO.

Key words polymethoxylated flavone; *Kaempferia parviflora*; matrix metalloproteinase-9; lens epithelial cell; cataract; structure–activity relationship

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of *K. parviflora*, such as 3,5,7,3',4'-pentamethoxyflavone and 5,7,4'-trimethoxyflavone showed potent Sirtuin 1 (SIRT1) enzyme-stimulating and anti-glycation activities. As SIRT1 enzyme-stimulating and anti-glycation activities are reported to lead to the suppression of cataract formation,16) the intake of polymethoxylated flavones isolated from *K. parviflora* (KPMFs) may be able to delay cataract formation. However, the effect of KPMFs on MMPs activity in lens epithelial cells (LECs) has not been fully elucidated. In the present study, we investigated the effect of PMFs isolated from *K. parviflora* on MMP (gelatinase) activity in human lens epithelial cells.

**MATERIALS AND METHODS**

**Materials** *K. parviflora* (rhizome) was collected in 2009 in Thailand. Bioactive fractions in *K. parviflora* (rhizome) were separated by partition using water and EtOAc and then repeated column chromatography15) and resulted in the isolation of polymethoxylated flavones as shown in Fig. 1. The human lens epithelial cell line, SRA01/04 was a kind gift from Dr. Nobuhiro Ibaraki (IBARAKI eye clinic, Tochigi, Japan). Antibodies against phospho-extracellular signal-regulated kinase (Erk)1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), and phospho-c-Jun N-terminal kinase (JNK) (Thr183/Tyr185) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Antibodies against TIMP-1, and TIMP-2, and β-actin and other chemicals were purchased from SigmaAldrich (St. Louis, MO, U.S.A.).

**Cell Culture and Treatment** SRA01/04 cells were incubated in low glucose (5 mM) Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen, Carlsbad, CA, U.S.A.) with 20% fetal bovine serum (Biowest, Nuaille, France) and penicillin/streptomycin/neomycin antibiotic mixture (Invitrogen) at 37°C with 5% CO₂. After reaching sub-confluence, they were treated up to 24 h with phorbol 12-myristate 13-acetate (PMA) in the presence or absence of polymethoxylated flavones isolated from *K. parviflora* in low glucose (5 mM) condition. The media were collected and centrifuged at 1200 rpm for 5 min. The supernatants were used as conditioned media, and the cells were used to prepare the cell fractions.

**Gelatin Zymography** For gelatin zymography, SRA01/04 were counted and seeded at 1 × 10⁵ cells per well with 6 well plate. After treatment, cell culture supernatants were analyzed for the presence of the secreted MMP-2 and MMP-9 gelatinases using zymography. Quantitative analyses were performed through the Image J software (NIH, Bethesda, MD, U.S.A.) and the IC₅₀ values (shown in Table 1) were estimated from the relative amounts of proMMP-9 at various concentrations (0.25, 1, 4, 16, or 64 µM) of test samples as previously reported.17)

**Real-Time RT-Quantitative (q)PCR** For RT-qPCR, SRA01/04 were counted and seeded at 2 × 10⁵ cells per dish with 60 mm dish. After treatment, Total RNA was extracted using ISOGEN reagent (Nippon Gene, Toyama, Japan), and first-strand cDNA was synthesized from 500 ng RNA using PrimeScript® RT reagent Kit (TaKaRa Bio Inc, Otsu, Japan) following manufacturer’s instruction. The concentrations of synthesized cDNA solution are finally 50 ng/µL. The cDNA solution was then used in qPCR reactions to analyze MMP-9 expression using SYBR green PCR master mix (TaKaRa Bio) as previously reported.14) The primers for qPCR were human MMP-9 (forward 5'-CCC TGG AGA CCT GAG AAC CAA-3', reverse 5'-CAT CTC TGCCAC CCG AGT GTA-3') and human β-actin (forward 5'-TGG CAC CCA GCA CA A TGA A-3', reverse 5'-CTA AGT CAT AGT CCG CCT AGA AGC A-3'). The standard PCR conditions included denaturation at 95°C for 30 s, followed by 40 cycles of extension at 95°C for 5 s and 64°C for 34 s. Threshold lines were automatically adjusted to intersect amplification lines in the linear portion of the amplification curves, and the cycles to threshold (Ct) were recorded automatically. Data were normalized to the expression of the endogenous control, β-actin. The reaction of qPCR was...
performed through Applied Biosystem 7500 Real time PCR System (Life Technologies, Carlsbad, CA, U.S.A.).

Western Blot Analysis For Western blot analysis, SRA01/04 were counted and seeded at $1 \times 10^5$ cells per well with 6 well plate. After treatment, the expression of TIMPs in the conditioned media was detected by Western blot analysis using specific rabbit anti-human TIMP-1 antibody (Sigma-Aldrich) and rabbit anti-human TIMP-2 antibody (Sigma-Aldrich) under reducing condition. The dilution rate of TIMP-1 and TIMP-2 antibody is 1000 times. Cell fractions had previously reported that treatment of PMA (10nM) augmented MMP-9 activity in several cells such as human fibrosarcoma HT1080 cells and human retinal Müller cells. We had previously reported that treatment of PMA (10nM) augmented MMP-9 activity in the human lens epithelial cell line, SRA01/04. Therefore, we examined the effect of KPMFs on the PMA-mediated activities of gelatinase through the analysis of conditioned media from KPMFs-treated cells by gelatin zymography. As shown in Fig. 2, although the strength of inhibitory activity was different, all KPMFs were found to reduce PMA-induced MMP-9 activity in SRA01/04 cells in a dose-dependent manner. Among the KPMFs, 5,7-dimethoxyflavone showed the most potent inhibition of PMA-stimulated MMP-9 activity (IC$_{50}$: 8.6 µM), followed by 3,5,7,3',4'-tetramethoxyflavone (IC$_{50}$: 25.5 µM) and then 3,5,7,4'-tetramethoxyflavone (IC$_{50}$: 35.2 µM). 3,5,7-Trime-thoxyflavone also inhibited MMP-9 activity, but to a lesser extent than other KPMFs (IC$_{50}$: >64 µM). However, KPMFs did not affect MMP-2 activity in PMA-stimulated SRA01/04 cells. As a result of statistical analysis, IC$_{50}$ values of 5,7-dimethoxyflavone was found to be significantly lower than 3,5,7,4'-tetramethoxyflavone ($p < 0.05$). On the other hand, IC$_{50}$ values between 5,7-dimethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone did not show no significant difference.

**Results**

**Effects of Polymethoxylated Flavones Isolated from K. parviflora on Gelatinase Activity** The activation of protein kinase C (PKC) is reported to be involved in cataract development through the inhibition of gap junction intercellular communication (GJIC), which enables the regulation of waste and nutrient transport to maintain lens transparency.

**Fig. 2. Effect of Polymethoxylated Flavones Isolated from Kaempferia parviflora on Phorbol Ester-Induced Gelatinase Activity in Human Lens Epithelial Cells**

SRA01/04 cell culture supernatants were analyzed for the gelatinase levels of MMP-2 and MMP-9 using gelatin zymography following treatment with 5,7-methoxylflavone (Compound 1) [A], 3,5,7-trimethoxyflavone (Compound 2) [B], 3,5,7,4'-tetramethoxyflavone (Compound 3) [C] or 3,5,7,3',4'-pentamethoxyflavone (Compound 4) [D] in the presence of PMA (10nM) for 24h.
flavone had comparable inhibitory action on the PMA-induced MMP-9 mRNA expression. On the other hand, 3,5,7-tetramethoxyflavone and 3,5,7,4’-tetramethoxyflavone were less active against PMA-induced MMP-9 mRNA expression compared to 5,7-dimethoxyflavone ($p < 0.05$). Among KPMFs, 5,7-dimethoxyflavone showed the most potent inhibition of PMA-stimulated MMP-9 activity and MMP-9 mRNA expression significantly different from 3,5,7,4’-tetramethoxyflavone ($p < 0.05$). The suppression strength on MMP-9 mRNA expression by 5,7-dimethoxyflavone was found to be significantly higher than 3,5,7,3’,4’-pentamethoxyflavone, and IC$_{50}$ values of 5,7-dimethoxyflavone tended to be lower than 3,5,7,3’,4’-pentamethoxyflavone although the difference was not significance. On the whole, the strength of inhibitory action on the MMP-9 mRNA expression by KPMFs tended to correlate with that of the inhibitory action on MMP-9 activity. These results suggested that the inhibitory effect of KPMFs on MMP-9 activity results in a reduction of MMP-9 mRNA expression.

Effects of Polymethoxylated Flavones Isolated from K. parviflora on MAPKs Phosphorylation

To examine the molecular mechanisms underlying the MMP regulation by KPMFs, we examined the effects of KPMFs on the phospho-MAPKs expression in PMA-stimulated SRA01/04 cells by using Western blotting. As shown in Fig. 4, PMA increased the phospho-MAPKs (Erk1/2, p38, and JNK) in SRA01/04 cells. 5,7-Dimethoxyflavone significantly reduced the phosphorylation of Erk1/2 ($p < 0.01$), which was different to the effects of the other three KPMFs. 3,5,7,4’-tetramethoxyflavone and 3,5,7,3’,4’-pentamethoxyflavone were also found to significantly inhibit the PMA-induced phosphorylation of p38 ($p < 0.001$) and JNK ($p < 0.05$). Both 3,5,7,4’-tetramethoxyflavone and 3,5,7,3’,4’-pentamethoxyflavone were also found to significantly inhibit the PMA-induced phosphorylation of p38 and JNK, comparable with 5,7-dime-

Fig. 3. Effect of Polymethoxylated Flavones Isolated from Kaempferia parviflora on Phorbol Ester-Induced MMP-9 mRNA Expression in Human Lens Epithelial Cells

Total RNA isolated from SRA01/04 cells were analyzed for the mRNA expression of MMP-9 and β-actin using RT-qPCR following treatment with polymethoxylated flavones (64 µM) isolated from Kaempferia parviflora in the presence of PMA (10 nM) for 24 h. Data are shown as means ± S.D. of four independent experiments. * Significantly different from PMA-treated cells ($p < 0.05$). # Significantly different from 5,7-dimethoxyflavone-treated cells ($p < 0.05$).

Fig. 4. Effect of Polymethoxylated Flavones Isolated from Kaempferia parviflora on Phosphorylated MAPKs in Phorbol Ester-Treated Human Lens Epithelial Cells

Western blot analysis revealed phospho-Erk1/2 [A], phospho-p38 [B], phospho-JNK1/2 [C] or β-actin. SRA01/04 cells were pretreated with polymethoxylated flavones isolated (64 µM) from Kaempferia parviflora for 2 h, and then treated with PMA (10 nM) for another 20 min. Data are shown as means ± S.D. of four independent experiments. *, ** and *** significantly different from PMA-treated cells ($p < 0.05$, 0.01 and 0.001, respectively).
thoxyflavone. However, the inhibitory effect of 3,5,7-trime-thoxyflavone on the PMA-induced phosphorylation of p38 and JNK by tended to be weaker than that of the other three KPMFs (Fig. 5).

Effects of Polymethoxylated Flavones Isolated from Kaempferia parviflora on TIMPs Production

MMPs are proteolytic enzymes controlled by a family of natural antagonists, TIMPs. TIMP-1 exhibits a greater inhibition for MMP-9 compared to other MMPs, and TIMP-2 strongly binds to proMMP-2 to form a complex that is significant in the cell surface activation of proMMP-2. Finally, we examined the effects of KPMFs on the TIMP-1 and TIMP-2 production in PMA-stimulated SRA01/04 cells. As shown in Fig. 6, we found that SRA01/04 cells constitutively produce both TIMP-1 and TIMP-2. Treatment with PMA remarkably augmented TIMP-1 production. Among the KPMFs, 5,7-dimethoxyflavone, 3,5,7-trimethoxy-flavone, and 3,5,7,3’,4’-pentamethoxyflavone were found to inhibit PMA-induced TIMP-1 production, although the activity of 3,5,7,4’-tetramethoxyflavone was lower. These results suggested that KPMFs may not exert inhibitory effect against MMP-9 activity through the augmentation of TIMP-1 production in PMA-stimulated SRA01/04 cells. TIMP-2 production was slightly enhanced by PMA, and 3,5,7,4’-tetramethoxyflavone and 3,5,7,3’,4’-pentamethoxyflavone remarkably augmented the production of TIMP-2 in PMA-stimulated SRA01/04 cells. However, 5,7-dimethoxyflavone and 3,5,7-trimethoxyflavone did not affect the PMA-stimulated TIMP-2 production. To ensure the equal loading between each sample, we examined the total protein concentration in culture media. We demonstrated that there is no significant different between protein concentrations in culture media collected from PMA and/or KPMFs-treated cells (Table 2). In addition, we performed Ponceau S staining using the membranes prepared from conditioned media after treatment of KPMFs in PMA-stimulated SRA01/04 cells. As a result, the expressions of Ponceau S-binding protein on membrane were found to be approximately same between each sample (see Supplementary Materials). We concluded that these results could guarantee the equal loading and transfer efficiency between each sample for Western blotting of TIMPs.
The number and position of the methoxy groups of polymethoxylated flavones are reported to be closely associated with their biological activity. In the present study, we found that 5,7-dimethoxyflavone showed the most potent inhibitory effects on MMP-9 activity and mRNA expression compared with other KPMFs. On the other hand, our results also demonstrated that the addition of the methoxy group at the 3-position of the C-ring tended to lead to a significant reduction in the inhibitory effects on MMP-9 activity and mRNA expression by comparing the strength of inhibitory action between 5,7-dimethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone in PMA-stimulated SRA01/04 cells. These results suggested that another signal transduction pathway may be associated with the additional inhibitory effects of 3,5,7,4'-tetramethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone on MMP-9 mRNA expression. However, the molecular mechanism of this reduction in the inhibitory effects on MMP-9 mRNA expression by KPMFs with a methoxy group at the 3-position of the C-ring remains to be fully elucidated. In this study, 3,5,7-trimethoxyflavone showed the weakest inhibitory action against MMP-9 mRNA expression but suppressed p38 and JNK phosphorylation to a similar extent as 3,5,7,4'-tetramethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone in PMA-stimulated SRA01/04 cells. These results suggested that another signal transduction pathway may be associated with the additional inhibitory effects of 3,5,7,4'-tetramethoxyflavone and 3,5,7,3',4'-pentamethoxyflavone on MMP-9 mRNA expression. Taiyab et al.29 recently demonstrated that β-catenin/CBP-dependent signaling was involved in the regulation of MMP-9 expression in rat lens epithelial explants. The investigation to clarify the effect of KPMFs with a methoxy group at the 3-position of the C-ring on β-catenin/CBP-dependent signaling is a challenge for the future. Furthermore, Nakata et al.30 demonstrated that a 4'-methoxy moiety on the B-ring in KPMFs was essential for SIRT1 enzyme-stimulating and anti-glycation activities. In the present study, the KPMFs with 4'-methoxy moiety on B-ring in KPMFs showed significant inhibition on PMA-induced MMP-9 mRNA expression. Therefore, the presence of a 4'-methoxy group may be necessarily required for not only SIRT1 enzyme-stimulating and anti-glycation activities but also the inhibition of MMP-9 activity. Yenjai et al.30 structurally modified 5,7-dimethoxyflavone and synthesized more polar derivatives, such as phenolic or oxime compounds, which were shown to have greater antifungal activity against Candida albicans than their parent compound, 5,7-dimethoxyflavone. In future, it should be investigated if these structural modifications of 5,7-dimethoxyflavone affect the inhibitory effect against MMP-9 activity.

In conclusion, MMPs may be a promising target that exerts multiple actions on specific cataract phenotypes, such as ASC and PCO. In this study, KPMFs inhibited MMP-9 activity through the suppression of MAPKs-mediated MMP-9 mRNA expression. Therefore, the dietary intake of KPMFs may have therapeutic value for the prevention or treatment of fibrotic cataracts such as ASC and PCO. This study has provided novel findings related to the specific chemical structures of KPMFs that enhance the inhibitory effects on MMP-9 activity; 5,7-dimethoxyflavone was found to be the most effective for the inhibition of MMP-9 activity. The delivery of KPMFs to lens presents challenges in the prevention or treatment of cataracts, and the delivery of KPMFs directly to the LECs from the intraocular lens may offer a very promising solution to reduce the incidence of secondary cataract formation. Further insights into the bioavailability of KPMFs are necessary to evaluate their pharmacological action within target tissues. The dietary intake of KPMFs may delay the cataract formation.

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Supplementary Materials The online version of this article contains supplementary materials.

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