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Identification and evaluation of anti-inflammatory compounds from *Kaempferia parviflora*

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The rhizome of *Kaempferia parviflora* has been used in traditional Thai medicine. In this study, we identified and compared specific compounds from the hexane extract of *K. parviflora* with those from other Zingiberaceous plants by using gas chromatography–mass spectrometry. We identified 5,7-dimethoxyflavone (DMF), 5-hydroxy-3,7,3',4'-tetramethoxyflavone (TMF), estimated 3,5,7-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, and investigated their anti-inflammatory effects in rat basophilic leukemia (RBL-2H3) cells stimulated with an IgE antigen or a calcium ionophore. We found that DMF and TMF more potently inhibited antigen-induced degranulation than did nobiletin, a well-known anti-inflammatory agent. In addition, compared to RBL-2H3 cells stimulated with a calcium ionophore, those treated with DMF and TMF showed more marked inhibition of the degranulation and the production and mRNA expression of inflammatory mediators. These results suggest that DMF and TMF inhibit an early step in the high-affinity IgE receptor signaling cascade rather than intracellular calcium release and protein kinase C activation.

Key words: *Kaempferia parviflora*; inflammatory mediator; polymethoxyflavonoid; GC–MS analysis; anti-inflammation

The Zingiberaceae family includes *Curcuma aromatica*, *Curcuma zedoaria*, *Curcuma longa*, and *Kaempferia parviflora*. Curcumin from the rhizome of *C. longa*, also called autumn turmeric in Japan, has been widely reported to have anti-inflammatory,^{1,2)} anti-oxida-

tive,^{1,3,4)} anti-tumor,¹⁾ and liver-protective properties,⁵⁾ whereas *C. aromatica* and *C. zedoaria* have been well studied for their anti-tumor^{6,7)} and anti-inflammatory properties⁷⁾ as well as their anti-microbial activities.⁷⁾ *K. parviflora* has recently been used as a nutritional supplement in Japan because it is rich in anthocyanins, and the rhizome of *K. parviflora* has been used in traditional Thai medicine for the treatment of gout, aphthous ulcer, abscesses, allergy, and gastrointestinal disorders.⁸⁾ *K. parviflora* modulates the expression of multidrug resistance associated-proteins⁹⁾ and has an inhibitory effect on NO production and PGE₂ release by macrophage-like cells.⁸⁾ *K. parviflora* also contains 11 polymethoxyflavonoids, 7 of which have been identified as possessing inhibitory effects on degranulation in rat basophilic leukemia (RBL-2H3) cells.^{10–12)}

In this study, we systematically investigated the inhibitory mechanisms of extracts from *K. parviflora* on degranulation and inflammation and identified or estimated the *K. parviflora*-specific compounds in these extracts by comparing them with compounds in other Zingiberaceous plants by using gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS).

Materials and methods

Materials. *C. longa*, *C. aromatica*, and *C. zedoaria* were kindly provided by Okinawa Prefectural Agricultural Research Center (Okinawa, Japan). *K. parviflora* was obtained from Mr. Seirin Toyama in Tokashiki village (Okinawa, Japan). Ginger was purchased from a retail store (Osaka, Japan). The preparation of 5,7-dimethoxyflavone (DMF, Chromadex, CA, USA) was dissolved in 50% aqueous ethanol (EtOH) at 18 mmol/L, 5-hydroxy-3,7,3',4'-tetramethoxyflavone

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Abbreviations: DMF, 5,7-dimethoxyflavone; DNP, dinitrophenyl; IL, interleukin; MCP, monocyte chemotactic protein; TMF, 5-hydroxy-3,7,3',4'-tetramethoxyflavone; TNF, tumor necrosis factor.

(TMF, Extrasynthese, France) was dissolved in EtOH at 14 mmol/L, and 3',4',5,6,7,8-hexamethoxyflavone (nobiletin; Wako Pure Chemicals, Osaka, Japan) was dissolved in EtOH at 50 mmol/L. Wortmannin (Wako Pure Chemicals) and SKF96365 (Wako Pure Chemicals) were used as positive controls for the antigen-mediated¹³⁾ and calcium ionophore-stimulated¹⁴⁾ β -hexosaminidase assay, respectively. All other chemicals were of reagent grade.

Preparation of extracts from *C. longa*, *C. aromatica*, *C. zedoaria*, and *K. parviflora*. The fresh rhizomes were pulverized. Each sample (1 g) was extracted overnight with 50% aqueous EtOH (20 mL) at room temperature (RT) by using a shaker. After extraction, the solution was centrifuged at $10,000 \times g$ for 5 min, and the supernatant was collected and filtered through a 0.2- μ m-pore membrane. For *n*-hexane extracts, each sample (1 g) was extracted overnight with a 1:1 mixture (20 mL) by volume of *n*-hexane and phosphate-buffered saline (PBS) at RT by using a shaker. After extraction, the solution was centrifuged at $10,000 \times g$ for 5 min. An aliquot (1 mL) of each *n*-hexane layer was dried using nitrogen gas and dissolved in either dimethylsulfoxide (DMSO, 500 μ L) for the cell-based assay or methanol for the LC-MS analysis. Aliquots from each *n*-hexane layer were also used for GC-MS analysis.

Cell culture. RBL-2H3 cells (Health Science Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, CA, USA), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Sigma) as a growth medium at 37 °C under 5% CO₂.

β -Hexosaminidase assay. RBL-2H3 cells were inoculated into a 96-well plate (5×10^4 cells/well) and cultured overnight. The cells were incubated in a growth medium containing 50 ng/mL of mouse monoclonal anti-dinitrophenyl (DNP)-IgE (Sigma) for 2 h. After washing with modified Tyrode (MT) buffer containing 137 mmol/L NaCl, 2.7 mmol/L KCl, 1.8 mmol/L CaCl₂, 1 mmol/L MgCl₂, 5.6 mmol/L glucose, 20 mmol/L HEPES, and 0.1% bovine serum albumin at pH 7.3, each sample was diluted with MT buffer for a final EtOH concentration less than 0.5%, and a final DMSO concentration less than 0.25%. After incubation for 10 min, DNP-labeled human serum albumin (50 ng/mL final concentration, Sigma) was added and the cells were incubated for 3 h.^{15,16)} For experiments examining stimulation with a calcium ionophore, the cells were stimulated with the calcium ionophore A23187 (2.5 μ M final concentration, Wako Pure Chemicals) for 30 min without sensitizing with anti-DNP-IgE.¹⁷⁾ The supernatant was collected, and the cells were lysed with Passive Lysis Buffer (Promega, WI, USA). After incubating for 10 min, the supernatant or the cell lysate was mixed with 100 mmol/L citrate buffer (pH 4.5, 50 μ L) containing 3.3 mmol/L *p*-nitrophenyl-2-acetamide-2-deoxy- β -D-glucopyranoside, and this mixture was

incubated at 37 °C for 25 min. The reaction was terminated by adding 2 mol/L glycine buffer (pH 10.4, 50 μ L), and the absorbance at 405 and 650 nm, as reference wavelengths, was measured with a microplate reader (SpectraMax M2e, Molecular Devices Inc, Japan). The individual release of β -hexosaminidase (IH) was calculated as follows: IH = supernatant OD value of the cells/(the cell lysate OD value + supernatant OD value of the stimulated cells). The percentage of relative β -hexosaminidase release was calculated as follows: $100 \times \text{IHS} - \text{IHB} / \text{mean value of (IHC} - \text{IHB)}$, where IHS is the IH of the test samples, IHB is the IH of the blank, and IHC is the IH of the control.

Estimation of the components in *K. parviflora* by GC-MS and LC-MS. Samples were analyzed using a 6890N/5973N GC-MS system (Agilent Technologies, Japan), which included a DB-5 ms column (30 m $\times$ 0.25 mm i.d., 0.25- μ m film thickness, Agilent Technologies, Japan) and a helium carrier gas (flow rate, 1.0 mL/min). The GC-MS injection port was held at 250 °C and operated in splitless mode. The temperature program for the capillary column was started at 60 °C, and the temperature was increased after 1 min at 5 °C per min to 320 °C. The mass spectrometer was operated in electron-ionization mode with a source temperature of 230 °C and an ionization voltage of 70 eV. LC-MS was performed using the 1100SL system (Agilent Technologies, Japan), which included a CAPCELL PAK C₁₈ AQ column (150 mm $\times$ 3 mm i.d., 5 μ m, Shiseido, Japan), with a column temperature of 40 °C. The mobile phase consisted of 10 mmol/L ammonium acetate in water, and methanol (1:9 v/v) for isocratic elution at a flow rate of 0.3 mL/min at 40 °C. The retention times of DMF and TMF were 4.090 and 4.690 min, respectively. The mass spectrometer was operated in electrospray ionization positive mode with a nebulizer gas temperature of 350 °C, drying gas flow rate of 10 L/min, capillary voltage of 3000 V, measurement mode of SIM, and monitor ions of *m/z* 283.10 (DMF) and 359.10 (TMF).

RNA preparation and quantitative reverse transcriptase mediated polymerase chain reaction. RBL-2H3 cells were inoculated into a 24-well plate (2.5×10^5 cells/well) and treated by following the same procedure as that of the β -hexosaminidase assay. After the treatment, the total RNA was isolated from the cells by using an RNeasy kit (Qiagen NV, Netherland) and was then reverse-transcribed to prepare cDNA using a random primer (Takara Bio, Shiga, Japan) and a PrimeScript Reverse Transcriptase (Takara Bio) according to the manufacturer's instructions. An aliquot of cDNA was used as a template for quantitative polymerase chain reaction (PCR); the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) was used for amplification. The target cDNAs were amplified using Fast SYBR Green Master Mix (Applied Biosystems) and the PCR primer set for *Tnf- α* (RA008379, Takara Bio), *Il-4* (RA015145), *Mcp-1* (RA011410), and β -actin (RA015375). The relative expression level of the mRNA was normalized according to the amount of β -actin mRNA obtained.

Measurement of cytokines released from cells.

RBL-2H3 cells were inoculated into a 24-well plate (2.5×10^5 cells/well) and treated by following the same procedure as that of the β -hexosaminidase assay. After the treatment, the supernatant was collected. The amounts of tumor necrosis factor (TNF)- α , interleukin (IL)-4, and monocyte chemotactic protein (MCP)-1 in the supernatant were measured using the Procarta Cytokine Assay Kit (Affymetrix, Inc, CA, USA), and the multiplex immunoassay Luminex 200 system (Luminex, Co, TX, USA).

Statistical analysis. The results in Figs. 1, 7(D)–(F), and 8(D)–(F) were expressed as the mean $\pm$ SD and in Figs. 2 and 6 were expressed as mean $\pm$ SD. The data in Figs. 1 and 2 were analyzed using Dunnett's test. The data in Figs. 7(D)–(F), and 8(D)–(F) were analyzed using one-way ANOVA followed by Dunnett's test. Statistical analyses and the calculation of IC_{50} values were performed using GraphPad Prism 5.02 (GraphPad Software, San Diego, CA). Data were deemed statistically significant at values of $p < 0.05$, $p < 0.01$, or $p < 0.001$.

Results

Inhibitory effects of degranulation from *K. parviflora*

We first examined the effects of the extracts from *C. aromatica*, *C. longa*, *C. zedoaria*, *K. parviflora*, and *Zingiber officinale* (ginger) on degranulation in RBL-2H3 cells. Compared to the control, the *n*-hexane extract from *K. parviflora* significantly reduced the

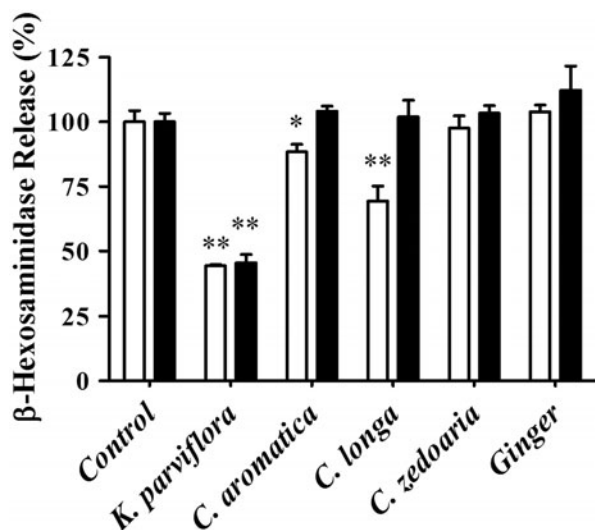


Fig. 1. Effects of the extracts from *Kaempferia parviflora*, *Curcuma aromatica*, *Curcuma longa*, *Curcuma zedoaria*, and *Zingiber officinale* (ginger) on the antigen-mediated degranulation in RBL-2H3 cells.

Notes: RBL-2H3 cells were sensitized with DNP-IgE for 2 h. Sensitized cells were incubated with the 50%EtOH extracts or *n*-hexane extracts from *C. aromatica*, *C. longa*, *C. zedoaria*, *K. parviflora*, and *Z. officinale* (each final concentration was 250 μ g/mL) and stimulated with antigen for 3 h. Released β -hexosaminidase was measured. Results are represented as mean $\pm$ SD, $n = 4$. Open bar, 50% EtOH extracts; closed bar, *n*-hexane extracts. Values are significantly different from the control at $*p < 0.01$ and $**p < 0.001$.

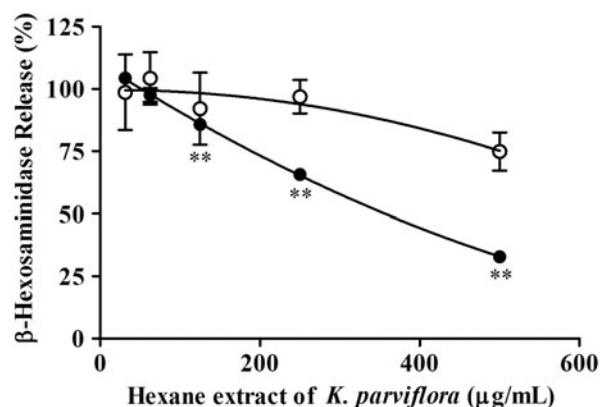


Fig. 2. Effects of the hexane extract from *Kaempferia parviflora* on the antigen-mediated or calcium ionophore-stimulated degranulation in RBL-2H3 cells as measured by β -Hexosaminidase release.

Notes: For antigen-mediated stimulation, RBL-2H3 cells were sensitized with DNP-IgE for 2 h, incubated with various concentrations of hexane extracts from *K. parviflora*, and stimulated with antigen (●) for 3 h. For calcium ionophore-stimulation, the cells were incubated with various concentrations of *n*-hexane extracts from *K. parviflora* without sensitizing with anti-DNP-IgE and stimulated with calcium ionophore A23187 (○) for 30 min. Results of β -hexosaminidase release are represented as mean $\pm$ SD, $n = 4$. Values are significantly different from the control at $*p < 0.01$ and $**p < 0.001$.

relative release rate of β -hexosaminidase to $45.5 \pm 3.2\%$ (mean $\pm$ SD; $p < 0.001$) (Fig. 1). In contrast, *n*-hexane extracts from *C. aromatica*, *C. longa*, *C. zedoaria*, and *Z. officinale* did not significantly alter the relative release rate of β -hexosaminidase as compared with the control. The relative released β -hexosaminidase activity compared with control was significantly reduced by the 50% EtOH extracts of *K. parviflora* ($44.3 \pm 0.5\%$, $p < 0.001$), *C. longa* ($69.4 \pm 5.8\%$, $p < 0.001$), and *C. aromatica* ($88.4 \pm 2.9\%$, $p = 0.004$).

Because these results suggested that the *n*-hexane extract from *K. parviflora* inhibited degranulation, we next examined the effects of this extract on antigen-mediated and calcium ionophore-stimulated degranulation in RBL-2H3 cells. We found that the relative released β -hexosaminidase activity with antigen compared with control was markedly reduced in a dose-dependent manner (maximally, $32.9 \pm 5.6\%$) while the calcium ionophore-stimulated release was moderately reduced ($74.8 \pm 10.2\%$), but not statistically different in RBL-2H3 cells by the *n*-hexane extract from *K. parviflora* (500 μ g/mL) (Fig. 2). The relative released β -hexosaminidase activity compared with control was reduced by Wortmannin (25 nM) which used as a positive control for the antigen-stimulated degranulation ($21 \pm 3.4\%$) and SKF96365 (25 μ M) which used as a positive control for the calcium ionophore-stimulated degranulation ($52 \pm 2.7\%$), respectively.

Identification of the possible anti-degranulation compounds in *n*-hexane extracts from *K. parviflora*

We analyzed the characteristics of the compounds in the *n*-hexane extract from *K. parviflora* by using GC-MS, and compared them to the characteristics of the compounds in the *n*-hexane extracts from *C. aromatica*, *C. longa*, *C. zedoaria*, *K. parviflora*, and *Z. officinale*. Only eight peaks were detected in the *n*-hexane extract

from *K. parviflora*, and five peaks were estimated by using a library search function to be 5,7-dimethoxy flavone (DMF), 3,5,7-trimethoxyflavone, 5-hydroxy-7,4'-dimethoxyflavone, 3,5,7,4'-tetramethoxyflavone, and 5-hydroxy-3,7,3',4'-tetramethoxyflavone (TMF) (Figs. 3–5). We quantified the amount of DMF and TMF in the *n*-hexane extract from *K. parviflora* as 556 and 193 $\mu\text{g/g}$, respectively, while the amount of DMF and TMF in the *n*-hexane extracts from *C. aromatica*, *C. longa*, *C. zedoaria*, and *Z. officinale* was less than 5 $\mu\text{g/g}$, each.

Inhibitory effects of polymethoxyflavonoids on degranulation

The inhibitory effects of DMF and TMF on degranulation were compared with those of nobiletin, a known anti-inflammatory compound in citrus species,¹⁸⁾ following antigen-stimulated or calcium ionophore A23187-induced release of β -hexosaminidase in RBL-2H3 cells. As shown in Fig. 6(A), for antigen-stimulated degranulation, TMF possessed the highest β -hexosaminidase inhibitory activity with an IC_{50} value of 1.8 μM , followed by DMF (IC_{50} = 19.2 μM) and nobiletin (IC_{50} = 195 μM). For calcium ionophore-induced degranulation, DMF reduced the relative release rate of β -hexosaminidase in a dose-dependent manner and possessed the

highest inhibitory activity with an IC_{50} value of 41.3 μM , while TMF and nobiletin reduced the relative release rate of β -hexosaminidase by less than 50% (Fig. 6(B)).

Inhibitory effect on production of inflammatory mediators by *K. parviflora* and polymethoxyflavonoids

We next investigated the effects of 50% EtOH or *n*-hexane extracts from *K. parviflora*, DMF, TMF, and nobiletin on production of cytokines by RBL-2H3 cells by measuring the release of the inflammatory cytokines TNF- α , IL-4, and MCP-1. We found that DMF, TMF, and nobiletin inhibited the production of TNF- α , IL-4, and MCP-1 in antigen-stimulated RBL-2H3 cells in a dose-dependent manner (Fig. 7(A)–(C)). The production of TNF- α was inhibited by approximately 100% with 50 μM of either DMF or TMF, and to 70% of the control with 50 μM of nobiletin. The production of IL-4 and MCP-1 was also inhibited by approximately 90% with 50 μM of DMF and TMF, while 50 μM of nobiletin inhibited approximately 83% of the IL-4 and 74% of the MCP-1 production. The 50% EtOH extract from *K. parviflora* inhibited the production of TNF- α by 95%, IL-4 by 84%, and MCP-1 by 67%, and the *n*-hexane extract from *K. parviflora* inhibited the production of TNF- α by 96%, IL-4 by 92%, and MCP-1 by 78%.

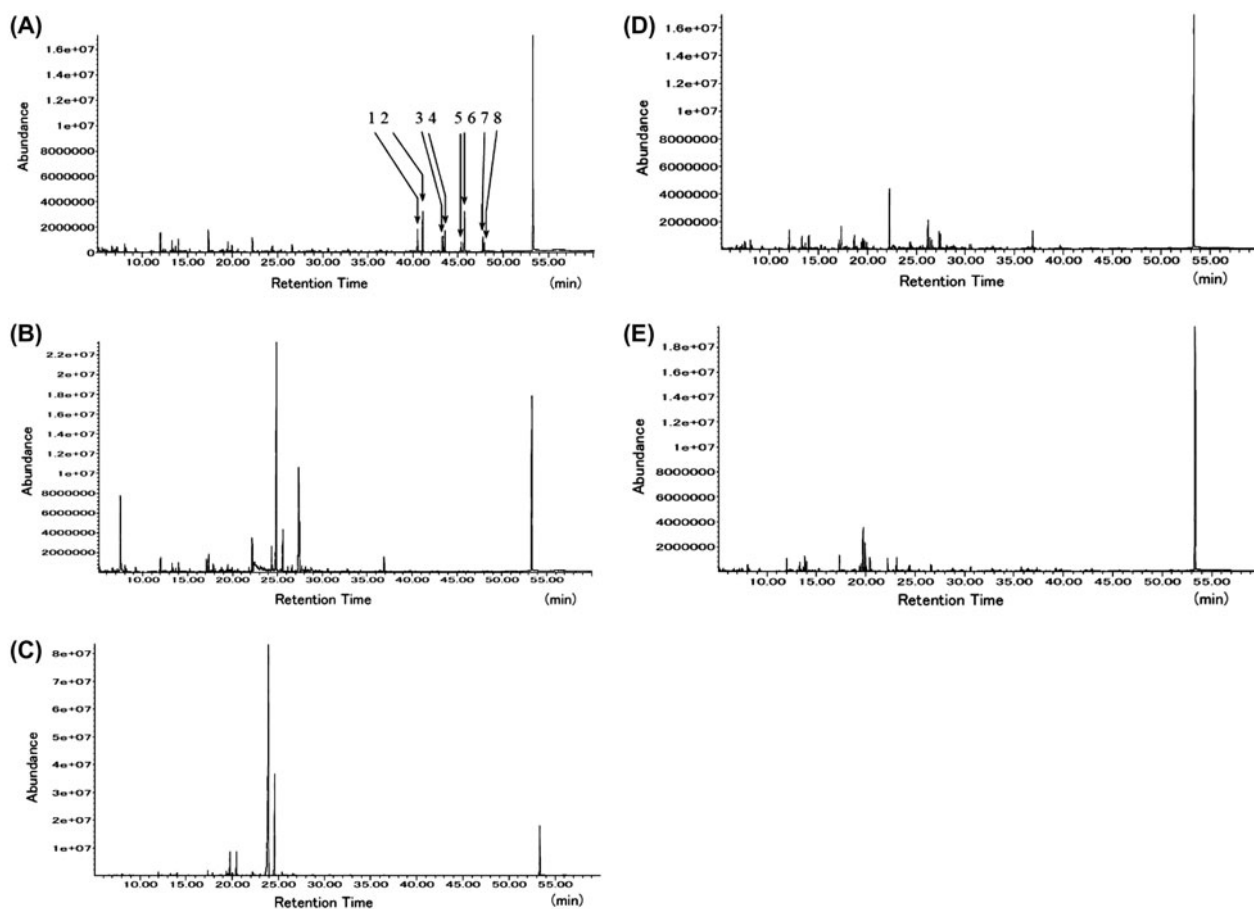


Fig. 3. GC-MS total ion chromatogram of hexane extracts from *Kaempferia parviflora*, *Curcuma aromatica*, *Curcuma longa*, *Curcuma zedoaria*, and *Zingiber officinale* (Ginger).

Notes: Results represent *K. parviflora* (A), *C. aromatica* (B), *C. longa* (C), *C. zedoaria* (D), and *Zingiber officinale* (E). Compound numbers and retention time (r.t.) represent the following: 1 = unknown (r.t. 40.479 min); 2 = unknown (r.t. 41.106 min); 3 = 5,7-dimethoxyflavone (DMF) (r.t. 43.283 min); 4 = 3,5,7-trimethoxyflavone (r.t. 43.555 min); 5 = 5-hydroxy-7,4'-dimethoxyflavone (r.t. 45.345 min); 6 = unknown (r.t. 45.713 min); 7 = 3,5,7,4'-tetramethoxyflavone (r.t. 47.775 min); 8 = 5-hydroxy-3,7,3',4'-tetramethoxyflavone (TMF) (r.t. 47.936 min).

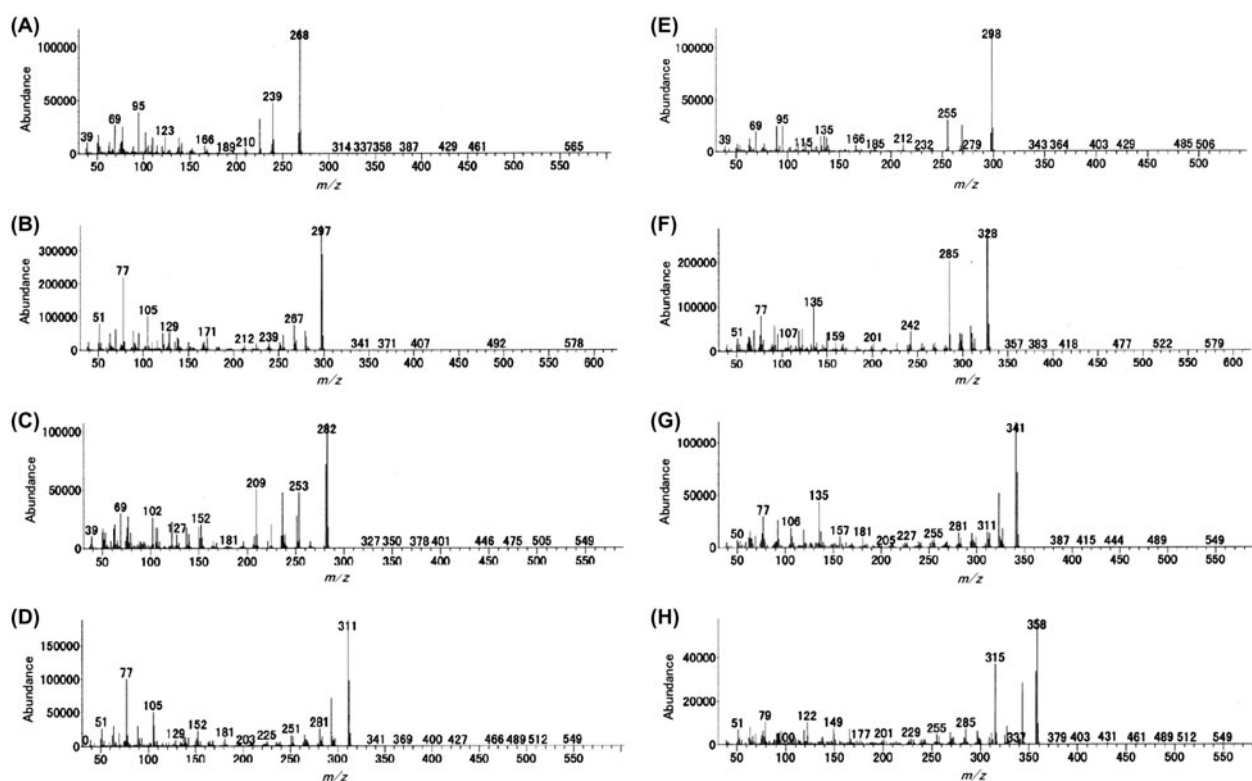
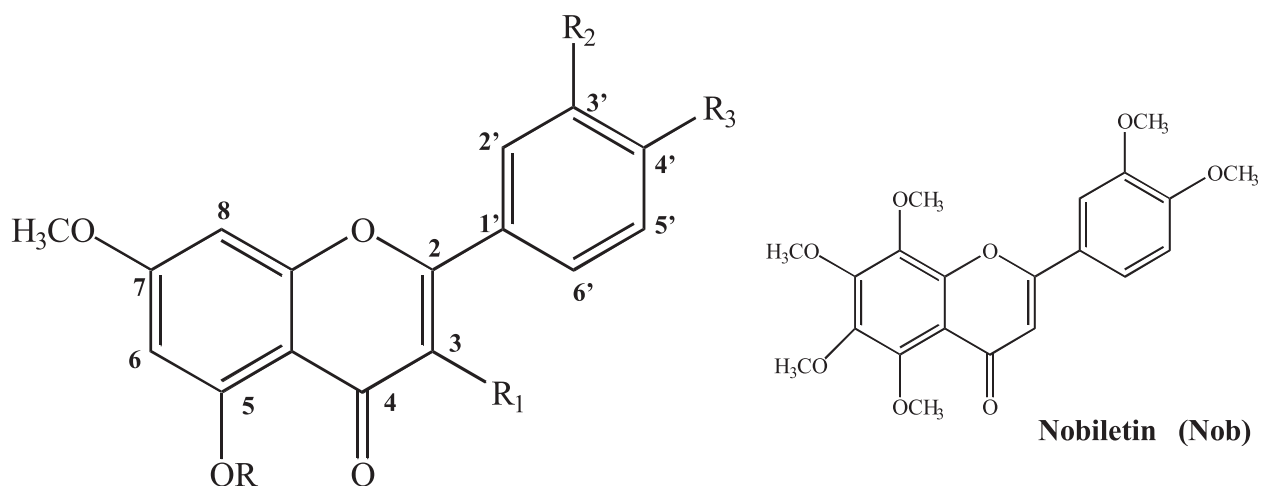


Fig. 4. The MS spectra of characteristic peaks in the *n*-hexane extract from *K. parviflora*.

Notes: Peak numbers are represented in Fig. 3(A). (A) peak number 1; (B) peak number 2; (C) peak number 3; (D) peak number 4; (E) peak number 5; (F) peak number 6; (G) peak number 7; (H) peak number 8.



Compound	R	R ₁	R ₂	R ₃
1 unknown	-	-	-	-
2 unknown	-	-	-	-
3 5,7-dimethoxyflavone (DMF)	CH ₃	H	H	H
4 3,5,7-trimethoxyflavone	CH ₃	OCH ₃	H	H
5 5-hydroxy-7,4'-dimethoxyflavone	H	H	H	OCH ₃
6 unknown	-	-	-	-
7 3,5,7,4'-tetramethoxyflavone	CH ₃	OCH ₃	H	OCH ₃
8 5-hydroxy-3,7,3',4'-tetramethoxyflavone (TMF)	H	OCH ₃	OCH ₃	OCH ₃

Fig. 5. Structures of compounds identified from *Kaempferia parviflora*, and nobiletin.

Note: Peak numbers are represented in Fig. 3.

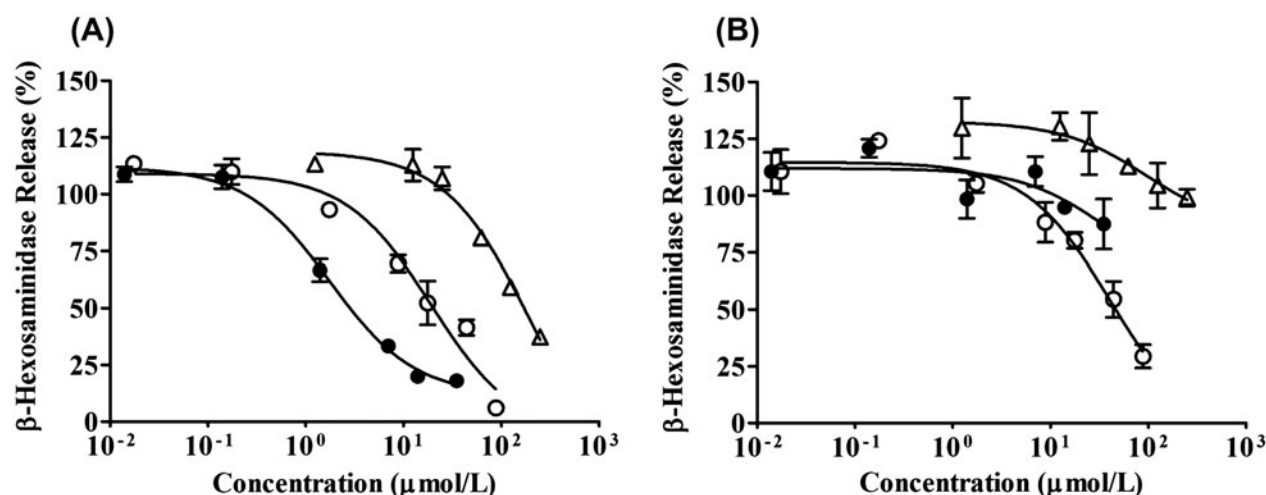


Fig. 6. Effects of DMF, TMF, and nobiletin on the antigen-mediated or calcium ionophore-stimulated degranulation in RBL-2H3 cells as measured by β -Hexosaminidase release.

Notes: For antigen-mediated degranulation, RBL-2H3 cells were sensitized with DNP-IgE for 2 h, incubated with various concentrations of DMF (○), TMF (●), and nobiletin (Δ) and stimulated with antigen for 3 h (A). For calcium ionophore-mediated degranulation, the cells were incubated with various concentrations of DMF (○), TMF (●), and nobiletin (Δ) and stimulated with calcium ionophore A23187 for 30 min without sensitizing with anti-DNP-IgE (B). Results of β -hexosaminidase release are represented as the mean $\pm$ SD, $n = 4$.

DMF, TMF, and nobiletin also inhibited the production of the inflammatory mediators in RBL-2H3 cells stimulated with a calcium ionophore (Fig. 8(A)–(C)). The production of TNF- α was inhibited with 50 μ M of DMF (54%), TMF (77%), or nobiletin (49%). The production of IL-4 and MCP-1 was also inhibited with 50 μ M of DMF by approximately 96% for IL-4 and by 89% for MCP-1, with 50 μ M of TMF by 75% for IL-4 and MCP-1, and with 50 μ M of nobiletin by 80% for IL-4 and 84% for MCP-1. The 50% EtOH extract from *K. parviflora* inhibited the production of TNF- α (49%), IL-4 (34%), and MCP-1 (42%). Although the *n*-hexane extract from *K. parviflora* inhibited the production of TNF- α by only 22%, it had no inhibitory effects on production of IL-4 and MCP-1.

The inhibitory effects of the 50% EtOH extract and the *n*-hexane extract from *K. parviflora*, DMF, TMF, and nobiletin on the expression of *Tnf- α* , *Il-4*, and *Mcp-1* mRNA levels were similar to the inhibition in production of the respective proteins (Fig. 7(D)–(F)). These inhibitory effects on *Tnf- α* , *Il-4*, and *Mcp-1* mRNA in RBL-2H3 cells stimulated with a calcium ionophore were weak compared with those following stimulation with an antigen (Fig. 8(D)–(F)). Wortmannin, which was used as a positive control for inhibition of the antigen-mediated degranulation in RBL-2H3 cells, had no effect on the production of TNF- α but inhibited *Tnf- α* , *Il-4*, and *Mcp-1* mRNA expression. SKF96365, which was used as a positive control for inhibition of the calcium ionophore-stimulated degranulation, also had no effect on *Tnf- α* , *Il-4*, and *Mcp-1* mRNA expression but inhibited the production of these inflammatory mediators.

Discussion

We obtained *K. parviflora* specific compounds from the 50% EtOH or the *n*-hexane extracts from *K. parviflora* and found that *K. parviflora* more potently inhibited

degranulation than did compounds from the other tested members of the Zingiberaceae family (Fig. 1). It has been reported that the polymethoxyflavonoids in the ethanol extract from *K. parviflora* inhibit antigen-stimulated degranulation in RBL-2H3 cells.^{10,11} These results suggest that *K. parviflora* contains polymethoxyflavonoids as well as other compounds that suppress the degranulation, and that these compounds are differentially contained within *K. parviflora* rather than within other Zingiberaceae plants.

It is well known that the cross-linking of IgE-bound high-affinity IgE receptors (Fc ϵ RI) by an antigen initiates an intracellular biochemical cascade followed by degranulation in mast cells. This intracellular signal transduction cascade involves the hydrolysis of phosphatidylinositol-4,5-bisphosphate to inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate mediates the initial release of intracellular calcium, and diacylglycerol activates protein kinase C (PKC). The release of calcium ions (Ca²⁺) from intracellular Ca²⁺ stores causes a Ca²⁺ influx via Ca²⁺ release-activated Ca²⁺ (CRAC) channels.^{19,20} Therefore, to investigate the effects of the *n*-hexane extract from *K. parviflora*, we examined the release of β -hexosaminidase in cells sensitized with DNP-IgE and stimulated with an antigen, which mimicked the intravital degranulation of mast cells caused by the antigen-antibody reaction. On the other hand, to investigate the inhibitory effects of the *n*-hexane extract from *K. parviflora* after activation of PKC in the intracellular signal transduction cascade, we examined the β -hexosaminidase assay stimulated with the calcium ionophore A23187, which directly transports extracellular Ca²⁺ into mast cells to induce degranulation (Fig. 2). We found that the *n*-hexane extract from *K. parviflora* inhibited the antigen-stimulated degranulation more strongly than the calcium ionophore-stimulated degranulation. These results indicate that this extract inhibits a step in the intracellular signal transduction cascade prior to the extracellular calcium release and PKC activation.

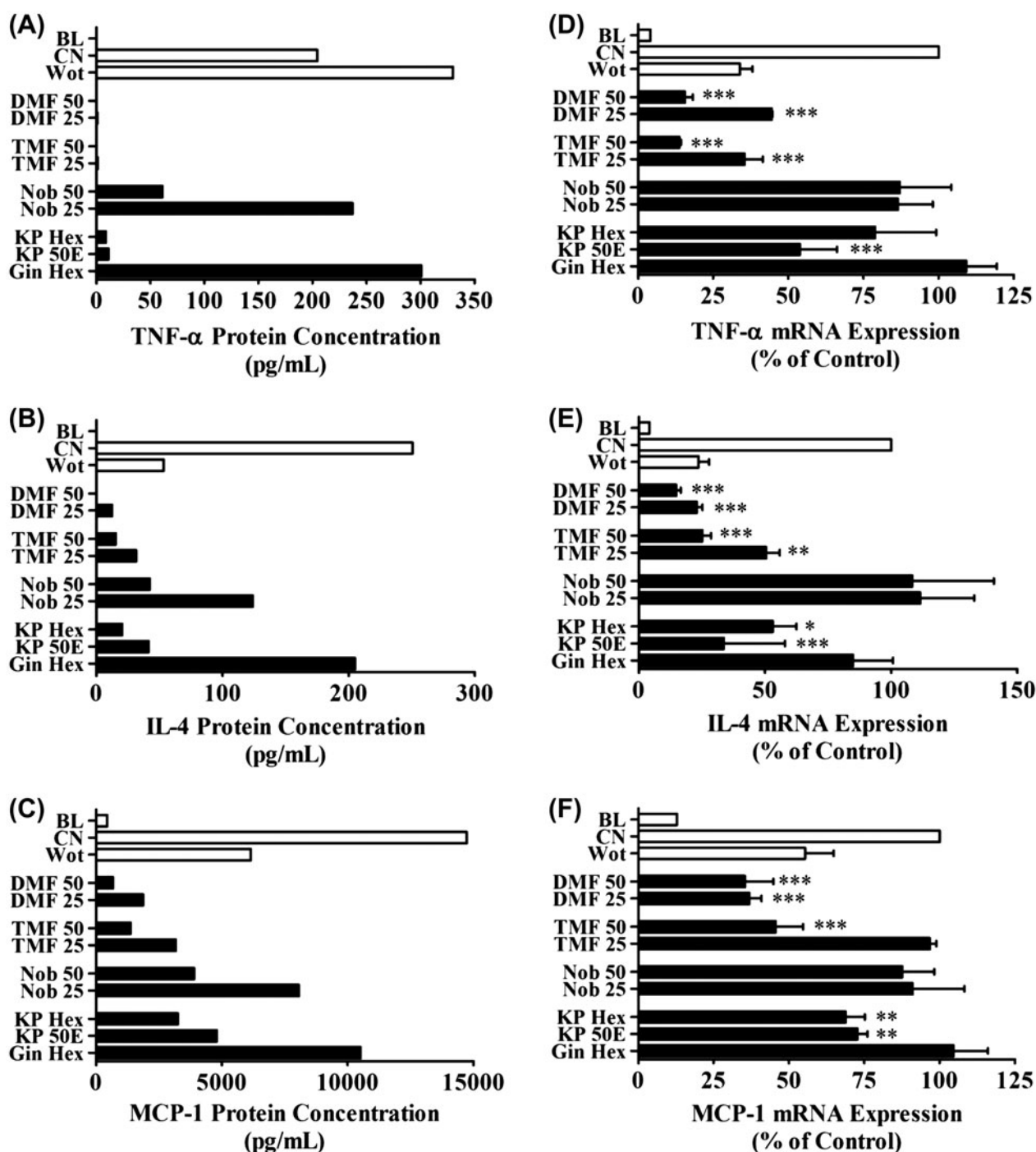


Fig. 7. Effects of extracts from *Kaempferia parviflora*, *Zingiber officinale*, and polymethoxyflavonoids on production of the inflammatory mediators and on mRNA expression in antigen-stimulated RBL-2H3 cells.

Notes: RBL-2H3 cells were sensitized with DNP-IgE for 2 h and then incubated with various concentrations of 50% EtOH or *n*-hexane extracts from *K. parviflora*, *Z. officinale*, or DMF, TMF, or nobilentin and stimulated with antigen for 1 h. The amounts of TNF- α (A), IL-4 (B), and MCP-1 (C) were measured by the multiplex immunoassay Luminex 200 system. For mRNA expressions, sensitized cells were similarly incubated but stimulated with antigen for 30 min. The mRNA levels of *Tnf- α* (D), *Il-4* (E), and *Mcp-1* (F) were measured by quantitative reverse transcriptase-PCR, normalized to the levels of β -actin (the internal standard), and expressed as a percentage of the control values. Numbers on the Y-axis legend indicate compound concentrations in μ M; BL, blank; CN, control; Wot, wortmannin; Nob, nobilentin; KP Hex, the hexane extract from *K. parviflora* (250 μ g/mL); KP 50E, the 50% EtOH extract from *K. parviflora* (250 μ g/mL); Gin Hex, the hexane extract from *Z. officinale* (250 μ g/mL). Results are represented as mean; $n = 2$ (A)–(C), and mean $\pm$ SD; $n = 3$ (D)–(F). Values in D–F are significantly different from the CN at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

We next estimated the identity of the specific compounds from the *n*-hexane extracts of *K. parviflora* and of other Zingiberaceae plants by using GC–MS and found 5 polymethoxyflavonoids (Figs. 3–5). It was reported that these *K. parviflora* specific polymethoxyflavonoids inhibit degranulation.¹¹⁾ In addition,

quantitative determinations clarified that DMF and TMF specifically and more abundantly exist in *K. parviflora* as compared with other Zingiberaceae plants. It has not been reported previously that DMF inhibits antigen-mediated and calcium ionophore-stimulated degranulation in RBL-2H3 cells. Therefore, our

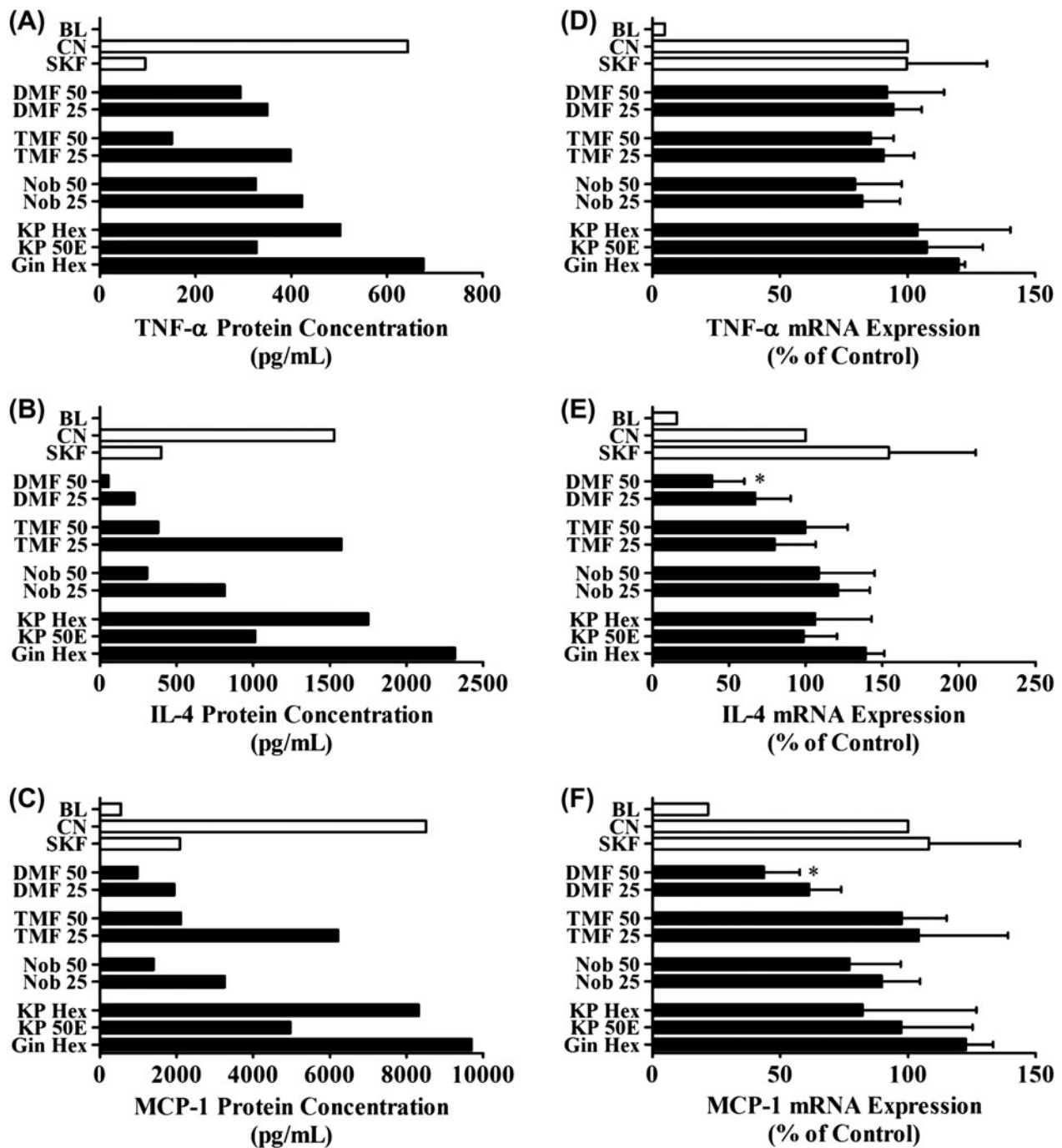


Fig. 8. Effects of extracts from *Kaempferia parviflora*, *Zingiber officinale*, and polymethoxyflavonoids on production of the inflammatory mediators and on mRNA expression in calcium ionophore-stimulated RBL-2H3 cells.

Notes: RBL-2H3 cells were incubated with various concentrations of 50% EtOH or hexane extracts from *K. parviflora*, *Z. officinale*, or DMF, TMF, or nobiletin and stimulated with A23187 for 3 h. The amounts of TNF- α (A), IL-4 (B), and MCP-1 (C) were measured by the multiplex immunoassay Luminex 200 system. For mRNA expressions, RBL-2H3 cells were similarly incubated but stimulated with A23187 for 15 min. The mRNA levels of *Tnf- α* (D), *Il-4* (E), and *Mcp-1* (F) were measured by quantitative reverse transcriptase-PCR, normalized to the levels of β -actin (the internal standard), and expressed as a percentage of the control values. Numbers on the Y-axis legend indicate compound concentrations in μ M; BL, blank; CN, control; SKF, SKF96365; Nob, nobiletin; KP Hex, the hexane extract from *K. parviflora* (250 μ g/mL); KP 50E, the 50% EtOH extract from *K. parviflora* (250 μ g/mL); Gin Hex, the hexane extract from *Z. officinale* (250 μ g/mL). Results are represented as mean; $n = 2$ (A)–(C), and mean $\pm$ SD; $n = 3$ (D)–(F). Values in D–F are significantly different from the CN at $*p < 0.05$.

results first suggest that polymethoxyflavonoids including DMF and TMF mediate the anti-degranulation effect of *K. parviflora*.

Many studies have demonstrated that flavonoids have anti-inflammatory properties.^{19,21,22} Thus, we examined the inhibitory effects of DMF, TMF, and nobiletin on antigen-stimulated or calcium ionophore A23187-induced release of β -hexosaminidase in RBL-2H3 cells.

As shown in Fig. 5, DMF, TMF, and nobiletin inhibited the degranulation associated with antigen stimulation more potently than that associated with calcium ionophore stimulation, and DMF and TMF were more potent than nobiletin.

Mast cells are associated with the immediate hypersensitive response to antigens resulting from the release of chemical mediators such as histamine from secretory

granules. Thus, we examined the effects of polymethoxyflavonoid and extracts from *K. parviflora* on the secretion of TNF- α , which is secreted in the early stages of inflammation and induces the production of cytokines such as IFN- γ and IL-6, also activates cell signals involved in inflammation.^{23,24} IL-4 plays an important role with the T helper cells (T_H2) and cytokines by promoting the IgE synthesis,^{24,25} and MCP-1, which works as a chemo-attractant for monocytes and basophils.²⁶ As shown in Figs. 7 and 8, DMF and TMF more potently inhibited the TNF- α , IL-4, and MCP-1 secretion by antigen-stimulated RBL-2H3 cells than did nobiletin. However, DMF and TMF showed weaker inhibition against TNF- α , IL-4, and MCP-1 secretion induced by the calcium ionophore than by that induced by the antigen. The inhibitory effect of the 50% EtOH extract and that of the *n*-hexane extract from *K. parviflora* was similar against the antigen-stimulated secretion of TNF- α , IL-4, and MCP-1 in RBL-2H3 cells. On the other hand, only the 50% EtOH extract inhibited the TNF- α , IL-4, and MCP-1 secretion in RBL-2H3 cells stimulated with the calcium ionophore. These results suggest that unknown components within only the 50% EtOH extract from *K. parviflora* may possess the anti-inflammatory effects.

We also showed that suppression of *Tnf- α* , *Il-4*, and *Mcp-1* mRNA expression by polymethoxyflavonoids and extracts from *K. parviflora* was weak in comparison with the suppression of their respective proteins following stimulation with a calcium ionophore in RBL-2H3 cells. For example, DMF and TMF did not inhibit the inflammatory mediators mRNA expression but did inhibit their protein secretion. Therefore, DMF and TMF inhibit the secretion mechanisms of inflammatory mediators rather than the pathway from Ca²⁺ influx to transcriptional activation of inflammatory mediators.

In contrast with the calcium ionophore stimulation of RBL-2H3 cells, we showed that for antigen stimulation, the suppression of *Tnf- α* , *Il-4*, and *Mcp-1* mRNA expression was almost the same as the suppression of their protein levels by polymethoxyflavonoids and extracts from *K. parviflora*. DMF and TMF inhibited the secretion of TNF- α , IL-4, and MCP-1, indicating that DMF and TMF reduce the secretion of inflammatory mediators as well as inhibit calcium ionophore stimulation in RBL-2H3 cells. In addition, DMF and TMF reduced the mRNA expression of *Tnf- α* , *Il-4*, and *Mcp-1*, suggesting that DMF and TMF inhibit the signal transduction from the cross-linking of the bound IgE-Fc ϵ RI complex to the Ca²⁺ influx.

In conclusion, extracts from *K. parviflora* exerted anti-degranulation and anti-inflammatory effects in RBL-2H3 cells. We first reported to approach the characteristic components included in only *K. parviflora* from comparison of five different Zingiberaceous plants using the extraction method with different polarities. We identified or estimated *K. parviflora*-specific polymethoxyflavonoids such as DMF and TMF from the *n*-hexane extract by comparing them with other extracts from Zingiberaceous plants using GC-MS and LC-MS analysis. And we found that DMF inhibits degranulation in RBL-2H3 cells, and its activity is almost same as that of TMF. Furthermore, DMF and TMF inhibited

a step early in signal transduction rather than inhibiting intracellular calcium release.

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