

RESEARCH ARTICLE

Anti-allergic effect of a chloroform-soluble extract of *Cinnamomum cambodianum* in bone marrow-derived mast cells

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Abstract

Cinnamomum cambodianum has been used as a traditional medicine in Cambodia. Its effect on the bone marrow-derived mast cells (BMMCs) mediated allergic response remains unknown. In this study, a chloroform-soluble extract of *C. cambodianum* was evaluated for its effect on allergic mediators, including prostaglandin D₂ (PGD₂), leukotriene C₄ (LTC₄), β -hexosaminidase and cyclooxygenase-2 (COX-2) protein, in phorbol 12-myristate 13-acetate (PMA) plus calcimycin-stimulated BMMCs. The results revealed that the chloroform-soluble extract inhibited the production of interleukin-6, PGD₂ and LTC₄, and the expression of COX-2 in PMA plus calcimycin-stimulated BMMCs, implying a potential benefit of *C. cambodianum* in the treatment of allergy.

Keywords: *Cinnamomum cambodianum*, interleukin-6, prostaglandin D₂, leukotriene C₄, degranulation, bone marrow-derived mast cell

Introduction

Allergic pathogenesis involves an early phase reaction followed by a late phase reaction involving coordinated activities of mast cells, dendritic cells, macrophages and eosinophils.⁽¹⁾ Under allergic conditions, mast cells release a variety of substances such as histamine, cytokine and arachidonic acid metabolites (prostaglandins, leukotrienes) which are important factors in the regulation of allergic responses. Prostaglandin D₂ (PGD₂) and leukotriene C₄ (LTC₄) have emerged as key mediators of allergic inflammatory pathologies.⁽²⁾ Histamine, serotonin and serine proteases are released via exocytosis, while lipid mediators such as eicosanoids are released through activation of cyclooxygenase-2 (COX-2) pathways.⁽³⁾ As such, the pattern of cytokine expression largely determines the nature of the inflammatory response.⁽⁴⁾ Immediate-type allergic reactions are mediated by histamine released in response to the antigen cross-linking of immunoglobulin E (IgE) bound to Fc ϵ RI on mast cells. After activation via

the Fc ϵ RI, the mast cells start the process of degranulation, which results in the release of mediators such as arachidonic acid metabolites and an array of inflammatory cytokines.⁽⁵⁾

IL-6 is one of the most important mediators of inflammation and of the acute phase response.⁽⁶⁾ Further, in the nervous system, endocrine system and bone metabolism, interleukin-6 (IL-6) is associated with a variety of physiological events.^(7,8)

In PG biosynthesis, arachidonic acid is converted by constitutive COX-1 or by allergic cytokine inducible COX-2, which is the common substrate of terminal PG synthases, including PGD₂ synthase expressed in mast cells.^(9,10)

Cinnamomum cambodianum Lecomte (Lauraceae), a native plant of Cambodia, is a 10–15 m tall tree that grows in wet dense forests at medium elevations of southwestern Cambodia. The bark of this plant has been used traditionally as a warming stimulant, as

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carminative, anti-spasmodic and antiseptic medicines, and for the treatment of indigestion, menstrual pain, and tuberculosis.^(11,12) There has been no report of a phytochemical and pharmacological study for *C. cambodianum* so far. Due to its activity in an initial screening using bone marrow-derived mast cells, we evaluated the anti-allergic effects of chloroform-soluble extracts of *C. cambodianum* in a model of allergy.

In the present study, the chloroform-soluble extract of *C. cambodianum* was evaluated for its effect on degranulation, production of IL-6, PGD₂ and LTC₄, and COX-2 expression in BMMCs. In addition, the major secondary metabolites in this active extract were identified.

Materials and methods

Animals

Female BALB/c mice, 6–8 weeks of age (Daehan Biolink Co. LTD, Seoul, Korea), were used under protocols approved by the Institutional Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KRIBB).

Plant materials

Bark of *C. cambodianum* was purchased from a local store at Arousey market, Phnom Penh, Cambodia, in June 2008. Plant samples were identified by Prof. Yok Lin, Department of Biology, faculty of Science, Royal University of Phnom Penh, Cambodia. A voucher specimen (KRIBB OS01) was deposited at the herbarium of the KRIBB, Daejeon, Republic of Korea.

Extraction, fractionation and isolation

Air-dried barks of *C. cambodianum* (5.0 kg) were extracted with MeOH at room temperature three times to obtain 450 g of solid extract. The MeOH extract was suspended in H₂O and then partitioned with CHCl₃, and EtOAc, successively, to give 70.0 g of CHCl₃-soluble extract (CC), and 56 g of EtOAc-soluble extract, respectively. The CHCl₃-soluble extract was subjected to silica gel column chromatography (10 × 80 cm, 230–400 mesh) with a stepwise gradient mixture of *n*-hexane-EtOAc (40:1 to 1:1, and CHCl₃-MeOH = 10:1 to 1:1, v/v) to yield 26 fractions (CC-1–CC-26). An active fraction, CC-13 (2.5 g), was crystallized to yield compound **2** (230 mg) and fraction (CC-13A). Fraction CC-13A (2.0 g) was chromatographed on a medium pressure liquid chromatography (MPLC) column (RP C-18; 3.5 × 45 cm, 40C₁₈-PREP), and eluted with MeOH-H₂O (3:1–100% MeOH, v/v) to yield 15 fractions (CC-13A-1–CC-13A-15). Fraction CC-13A-3 (100 mg) was further fractionated using silica gel MPLC (2.5 × 45 cm, 230–400 mesh) to obtain compound **1** (69 mg). The structures of compounds **1** and **2** were confirmed as methoxy eugenol (**1**) and marliolide (**2**) by the interpretation of MS and NMR spectroscopic data and by comparison with published values.

4-allyl-2,6-dimethoxyphenol (**1**)

Pale-yellow oil; ESI-MS *m/z* 195 [M+H]⁺; ¹H-NMR (400 MHz, CDCl₃): δ 3.31 (2H, d, *J* = 7.2 Hz, H-9), 3.86 (6H, s, ArOCH₃, H-7, H-8), 5.07 (2H, m, H-11), 5.41 (1H, s, H-1), 5.94 (1H, m, H-10), 6.40 (2H, s, H-2 & 6); ¹³C-NMR (100 MHz, CDCl₃): δ 40.3 (C-9), 56.2 (C-7, C-8), 105.1 (C-3, C-5), 115.6 (C-11), 131.0 (C-2, C-6), 132.9 (C-4), C-137.5 (C-10), 146.9 (C-1).⁽¹³⁾

Marliolide (**2**)

White amorphous powder; EI-MS *m/z* 310 [M]⁺; ¹H-NMR (400 MHz, CDCl₃) 4.80 (1H, d, *J* = 5.2 Hz, H-4), 4.52 (1H, dq, *J* = 5.2, 6.4 Hz, H-5), 1.44 (3H, d, *J* = 6.8 Hz, H-6), 6.91 (1H, t, *J* = 8.0 Hz, H-1'), 2.38 (2H, m, H-2'), 1.50 (2H, m, H-3'), 1.25 (brs, H-4'-H-13'), 0.87 (3H, t, *J* = 6.4 Hz, H-14'); ¹³C-NMR (100 MHz, CDCl₃) 170.2 (C-2), 130.4 (C-3), 67.7 (C-4), 79.1 (C-5), 14.1 (C-6), 147.8 (C-1'), 29.8 (C-2'), 28.4 (C-3'), 29.6 (C-4'), 29.3 (C-5'), 29.5 (C-6'), 29.6 (C-7'), 29.5 (C-8'), 29.4 (C-9'), 29.6 (C-10'), 29.4 (C-11'), 32.0 (C-12'), 22.8 (C-13'), 14.2 (C-14').⁽¹⁴⁾

Quantitative analysis of the major constituents in the chloroform-soluble extract using ultra-performance liquid chromatography (UPLC)

The chromatography was performed using an ACQUITY UPLCTM system (Waters Corporation, Milford, MA, USA). Chromatographic separations were performed on a ACQUITY BEH C18 chromatography column (2.1 × 100 mm, 1.7 μm) with a flow rate of 0.4 mL/min. The column temperature was maintained at 35°C, and the mobile phases A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The gradient duration program was as follows: 0 min, 40% B; 0–2 min, 40% B; 2–11 min, 40–100% B; wash to 13.5 min with 100% B; and a 1.5 min recycle time. The detection wavelength was set at 215 nm. It was found that the chloroform-soluble extract contained 138.2 mg/g of methoxyeugenol (*t*_R 2.19 min) and 83.9 mg/g of marliolide (*t*_R 9.69 min) by quantitative analysis (Figure 1).

Preparation and activation of bone marrow-derived mast cells (BMMCs)

Bone marrow cells from male BALB/c mice were cultured for up to 10 weeks in 50% enriched medium (RPMI 1640 containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal calf serum) and 50% WEHI-3 cell conditioned medium as a source of IL-3. After 3 weeks, >98% of the cells were BMMCs when checked by a previously described procedure.⁽⁸⁾

Drugs and chemicals

RPMI 1640, penicillin and streptomycin were obtained from Hyclone (Hyclone Labs Logan, UT). Bovine serum albumin, phorbol 12-myristate 13-acetate (PMA), calcium ionophore (A23187) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-thiazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). COX-2,

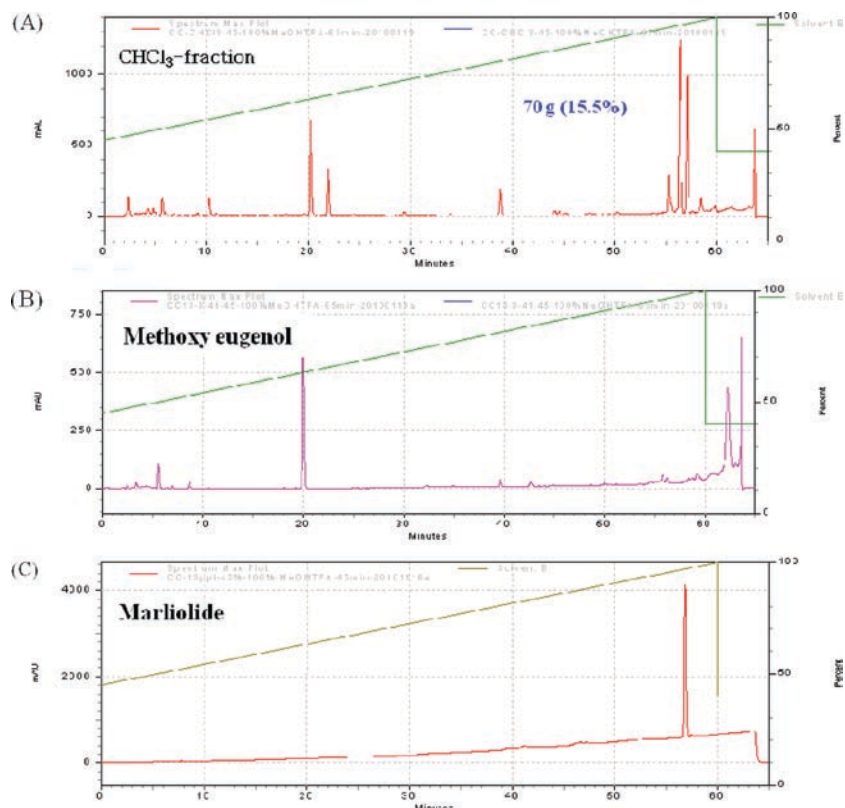


Figure 1. UPLC Chromatograms of CC (A), methoxy eugenol standard (B) and marliolide standard UPLC indicated that methoxy eugenol and marliolide of CC are detected at *t*_R 2.19 min and 9.69 min.

AKT, p-AKT monoclonal antibodies and peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). An RNA extraction kit was purchased from iNtRON (iNtRON Biotech, Korea). COX-2, IL-6 and β -actin oligonucleotide primers were purchased from Bioneer (Bioneer Corporation, Korea).

MTT assay for cell viability

Cell viability was examined by MTT assay. BMMCs were seeded at a density of 1×10^5 /mL in 96-well plates (Nunc, Denmark). Each group had a non-treated group as aa control. CC (20 μ g/mL) was added to each well and incubated for 24 h at 37°C, 5% CO₂. MTT solutions (5 mg/mL) were added to each well and then cells were cultured for another 4 h. The supernatant was discarded and 100 μ L of dimethyl sulfoxide was added to each well. The optical density was read at 590 nm. Cytotoxicity was calculated by subtracting from one the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells.

Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded at a density of 1×10^6 cells/mL in 24 well tissue culture plates and then pretreated with various concentrations of CC (5, 10 and 20 μ g/mL) for 30 min prior to PMA (50 nM) plus A23187 (1 μ M) stimulation. ELISA plates (BD labware, MD, USA) were then coated overnight at 4°C with anti-mouse IL-6 antibody diluted in coating

buffer (0.1 M carbonate, pH 9.5) and then washed three times with PBS containing 0.05% tween 20. Non-specific protein binding sites were then blocked by incubating the plates with assay diluent (PBS containing 10% fetal bovine serum, pH 7.0) for at least 1 h. Immediately following the incubation, each sample and IL-6 standard was added to the wells, and the plates were then incubated for 6 h. Next, working detector (biotinylated anti-mouse IL-6 monoclonal antibody and streptavidin-horseradish peroxidase reagent) was added to each well and the plates were incubated for an additional 1 h. Substrate solution (tetramethylbenzidine) was then added to the wells, after which the plates were incubated in the dark for 30 min. Next, the reaction was stopped using stop solution (2 N H₃PO₄) and the absorbance at 450 nm was measured. All standards and samples were assayed in duplicate.

Determination of PGD₂ and LTC₄ levels

BMMCs were activated with PMA (50 nM) plus A23187 (1 μ M) at 37°C for 1 h in the presence or absence of CC dissolved in PBS. The supernatants were isolated for further analysis by enzyme immunoassay (EIA). PGD₂ and LTC₄ were determined using an EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Data are reported as the arithmetic mean of triplicate determinations.

Assay of β -hexosaminidase release

β -hexosaminidase (β -HEX), a marker of mast cell degranulation, was quantitated by spectrophotometric analysis

of the hydrolysis of p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside hydrolysis (Sigma Chemical Co.). Briefly, after harvesting supernatant, cells were lysed in the same volume of medium by freeze and thaw three times. Ten milliliter of the BMMCs lysate or supernatant samples were mixed with 50 mL of β -HEX substrate solution (1.3 mg/mL p-nitrophenyl-2-acetamido-2-deoxy- β -D-glucopyranoside in 100 mM sodium citrate, pH 4.5) in each well of 96-well plates and then incubated at 37°C for 60 min. The reaction was stopped by adding 150 μ L of 0.2 M Glycine-NaOH (pH 10.7). The absorbance at 410 nm was measured in a micro plate reader. The percentage of β -HEX released into the supernatant was calculated by the following formula: $[S/(S-P)] \times 100$, where *S* and *P* are the β -HEX contents of supernatant and cell pellet.

Western blot analysis

Protein expression was assessed by western blot analysis according to standard procedure. BMMCs were cultured in 60 mm culture dishes (3×10^6 /mL), and pretreated with various concentrations of CC (10 and 20 μ g/mL). After 30 min, PMA (50 nM) plus A23187 (1 μ M) were added to the culture medium and the cells were incubated at 37°C. Immunoreactivity was detected using enhanced chemiluminescence (Amersham, Milan).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated using an easy-BLUE™ RNA extraction kit according to the manufacturer's instructions. Briefly, total RNA (2 μ g) was converted to cDNA by treatment with 200 units of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 h. The reaction was then stopped by incubating the solution at 70°C for 15 min, after which 3 μ L of the cDNA mixture was used for enzymatic amplification. PCR was then performed using a reaction mixture comprised of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, 0.2 mM dNTPs, 2.5 units of Taq DNA polymerase, and 0.1 μ M each of primers specific for COX-2, and β -actin. The amplification conditions were as follows: denaturation at 94°C for 3 min for the first cycle and then 35 cycles of 94°C for 45 s, annealing of COX-2 at 53°C for 45 s annealing of IL-6 at 57°C for 45 s with a final extension at 72°C for 7 min. The PCR products were then electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The primers used were 5'-GCCCAACAATACAAATGACCCTA-3' (sense) and 5'-TCCTGTTGTTTCTATTTCCTTTGT-3' (antisense) for IL-6, 5'-ACTCAGTTTGTTGAGTCATTC-3' (sense) and 5'-ATTAGTACTGTAGGGTTAATG-3' (antisense) for COX-2, 5'-TGAAGATCCTGACCGAGCGT-3' (sense) and 5'-ACGCAGCTCAGTAACAGTCCG-3' (antisense) for β -actin.

Statistical analysis

Data from the experiments are presented as the mean $\pm$ S.E.M. The level of statistical significance was determined

by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. *P* values less than 0.05 were considered to be significant.

Results

Effects of CC on PMA and A23187-induced IL-6 expression

To evaluate the effects of CC on the production of IL-6, we pretreated cells with CC before stimulation with PMA (50 nM) plus A23187 (1 μ M) for 2 h, after which the samples were analysed by ELISA. As shown in Figure 2, the level of IL-6 was considerably higher after stimulation with PMA plus A23187 in BMMCs. Pretreatment of cells with CC (5, 10 and 20 μ g/mL) inhibited these increases in a concentration-dependent and statistically significant manner. The cytotoxic effects of CC were evaluated in BMMCs by MTT assay. Treatment with CC at concentrations of up to 20 μ g/mL did not affect the viability of BMMCs (data not shown).

Effects of CC on PMA and A23187-Induced PGD₂ and LTC₄ production

After stimulation with PMA plus A23187, cell-culture media were harvested to measure PGD₂ production. CC

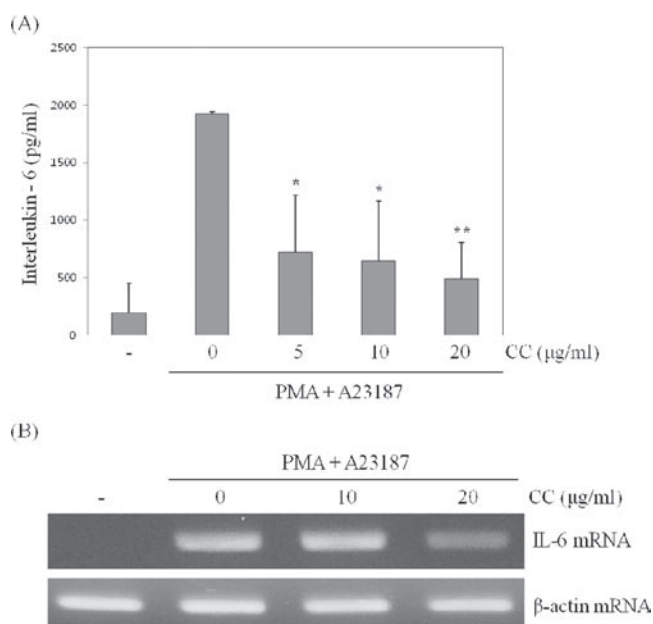


Figure 2. Effect of CC on the expression of IL-6 in PMA + A23187-stimulated BMMCs. (A) BMMCs were pretreated with the indicated concentrations of CC for 30 min prior to being incubated with PMA + A23187 for 2 h. The culture supernatant was then isolated and analysed for IL-6 production by ELISA. (B) IL-6 mRNA expression in BMMCs was assessed by RT-PCR. Cells were pretreated with the indicated concentrations of CC for 30 min before being incubated with PMA + A23187 for 2 h. β -actin mRNA was analysed in parallel to confirm equal cDNA preparation. Statistical significance: **P* < 0.05, as compared to the stimulated group. Significant differences between treated groups were determined using the Dunnett's *t*-test. Values shown are the mean $\pm$ S.E. of duplicate determinations from three separate experiments.

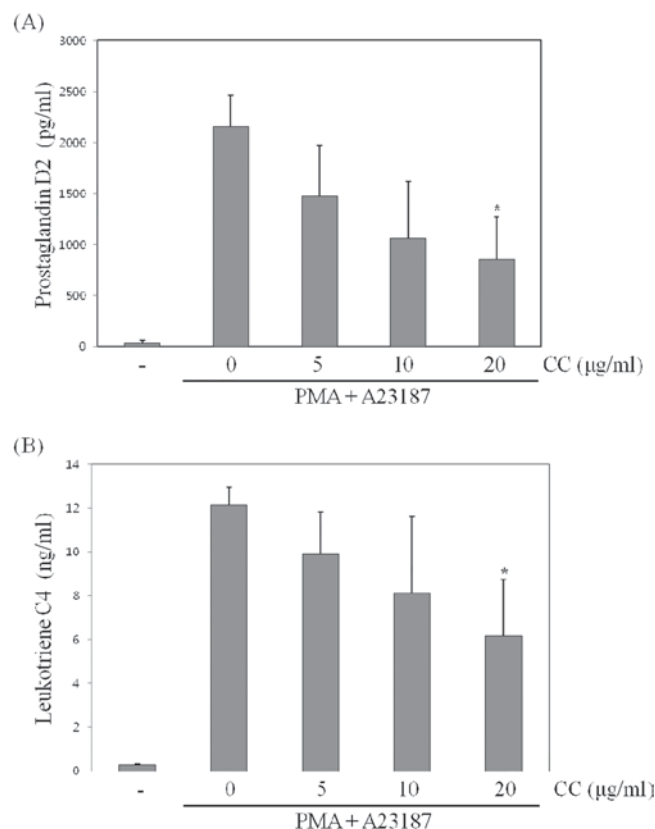


Figure 3. Effects of CC on degranulation and the production of PGD₂ and LTC₄ in PMA plus A23187-stimulated BMMCs. (A) Effect of CC on the production of PGD₂ in PMA and A23187-stimulated BMMCs. (B) LTC₄ production. BMMCs were pretreated with the indicated concentrations of CC for 30 min before being incubated with PMA and A23187 for 0.5 h.

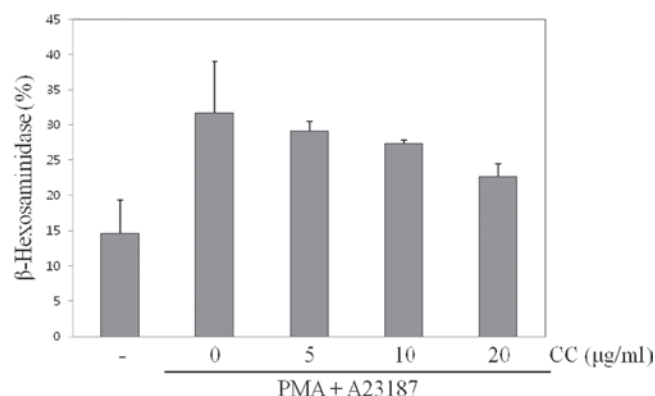


Figure 4. Effects of CC on PMA plus A23187-induced COX-2 protein and mRNA expression in BMMCs. (A) BMMCs were pretreated with the indicated concentrations of CC for 30 min prior to being incubated with PMA (50 nM) plus A23187 (1 µM) for 2 h. Equal amounts of protein (20 µg) were then separated by SDS-polyacrylamide gel electrophoresis and immunoblotted with COX-2 antibodies. Equal loading of protein was verified by β-actin. (B) COX-2 mRNA expression in BMMCs was assessed by RT-PCR. Cells were pretreated with the indicated concentrations of CC for 30 min before being incubated with PMA (50 nM) plus A23187 (1 µM) for 2 h. β-actin mRNA was analysed in parallel to confirm equal cDNA preparation.

significantly inhibited PGD₂ production in PMA plus A23187-induced BMMCs, in a dose-dependent manner (Figure 3A). Also, an inhibitory effect of CC on LTC₄

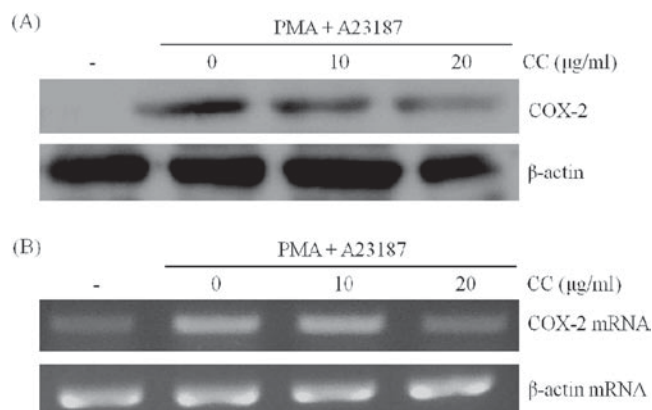


Figure 5. Effect of CC on the release of β-hexosaminidase in PMA plus A23187-stimulated BMMCs. BMMCs (1 × 10⁶ cells/mL) were pretreated with CC (5, 10 and 20 µg/mL) 30 min prior to stimulation with PMA (50 nM) plus A23187 (1 µM). At 90 min after PMA plus A23187 stimulation, the β-HEX released into the supernatant cell lysate was measured by ELISA. The procedure is described in detail in the Materials and methods section. All data are the arithmetic mean of triplicate determinations.

production in the PMA plus A23187-induced BMMCs was observed (Figure 3B). These results show that CC can inhibit PGD₂ and LTC₄ production in BMMCs stimulated by PMA plus A23187.

Inhibitory effects of CC on PMA plus A23187-induced degranulation

When mast cells are activated by various stimuli, the release of histamine occurs in a fashion similar to that of β-HEX, which is a degranulation marker. We investigated the effects of CC on PMA plus A23187-induced β-HEX release using an ELISA. Pretreatment of BMMCs with CC at concentrations of 5, 10 and 20 µg/mL did not give significant reduction in β-HEX release when compared to the stimulated group (Figure 4).

Effects of CC on PMA plus A23187-induced COX-2 expression

RT-PCR analysis was performed to determine if the inhibitory effects of CC on PGs were related to modulation of COX-2 expression. COX-2 mRNA was strongly expressed in response to PMA plus A23187 in BMMCs, while the treatment of CC at a dose of 20 µg/mL to BMMCs significantly inhibited COX-2 expression (Figure 5).

Discussion

Mast cell activation is initiated through cross-linking of IgE and the surface receptors for IgE (FcεRI) as well as by anaphylatoxins, cytokines, bacterial and viral antigens, and leads to allergic reactions, degranulation and release of allergic mediators including histamine PGD₂, and tryptase.^(15,16) It has been reported that mast cells can be a good source of ILs such as IL-6 and IL-8.⁽¹⁷⁾ PMA and A23187 (a calcium ionophore) have been used to trigger allergic reactions, stimulating the

production of histamine, IL-6, LTC₄, and PGD₂ in mast cells.^(18,19) In PMA plus A23187-stimulated BMMCs, CC decreased IL-6, LTC₄ and PGD₂ production (Figures 2 and 3). In addition, based on measurement of the amount of β -hexosaminidase, parallel to measurement of histamine in PMA plus A23187-stimulated BMMCs, the release of histamine, a biological marker of the degranulation reactions,^(20,21) was lessened by CC treatment (Figure 4). Further, the effect of CC on COX-2 protein expression was investigated in PMA plus A23187-stimulated BMMCs and the results are presented in Figure 5. RT-PCR and western analyses demonstrated that COX-2 mRNA and protein expression is blocked by CC treatment, implying a correlation with the reduction of PGD₂.^(22,23)

Furthermore, methoxy eugenol and marliolide were identified as main components of CC (see supporting material). The two isolated compounds were evaluated for their inhibitory activity against IL-6 expression in BMMCs but were inactive (IC₅₀ > 10 μ M). Thus, the effects of CC may be derived from other principles in the extract.

In conclusion, the chloroform-soluble extract of *C. cambodianum* inhibited IL-6 production in PMA plus A23187-stimulated BMMCs; IL-6, LTC₄ and PGD₂ production was decreased. Thus, it seems that CC possesses anti-allergic potential. Further research is required to purify and identify the structure of the active principles included in this fraction.

Declaration of interest

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