Effects of Cinnamomi Cortex on the production of allergic and inflammatory mediators in RBL-2H3 and RAW264 cells.

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Abstract

Cinnamomi Cortex (cinnamon bark) is a major crude drug. In Japan, it is used in Kampo medicine to treat allergic and inflammatory diseases. In this study, we examined the anti-allergic and anti-inflammatory effects of Cinnamomi Cortex and attempted to identify its active components. Cinnamomi Cortex was extracted with 70% methanol (Cin70M) and separated into fractions via silica gel column chromatography (CC) or octadecylsilan (ODS) CC. In order to select fractions that have anti-allergic and anti-inflammatory activities, we evaluated the ability of the fractions to inhibit the expression of cytokine genes in vitro by using a rat basophilic leukemia (RBL-2H3) cell line and mouse macrophage (RAW264) cell line. Gene expression levels were assessed in both cell lines by using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), and the RBL-2H3 degranulation ratio was quantified via β-hexosaminidase assay. Cin70M and cinnamaldehyde (CNMA) inhibited degranulation and the mRNA expression of interleukins in RBL-2H3 cells. CNMA inhibited degranulation at 300 μM, and its inhibitory effects were slightly stronger than those of ketotifen which is a typical anti-allergic drug. In RAW264 cells, nitric oxide (NO) production and the mRNA expression of inflammatory cytokines were reduced by Cin70M and CNMA. In the NO production assay, 100 μM CNMA was found to significantly decrease NO production from activated RAW264 cells, and its activity was the same as that of 10 μM dexamethasone. The taste and scent of Cinnamomi Cortex were both attributed to CNMA. The results of the present study indicate that it is more beneficial for patients with allergic and/or inflammatory diseases to use Cinnamomi Cortex with a strong taste and scent.

Key words : Cinnamomi Cortex, cinnamon, cinnamaldehyde, anti-allergic effect, anti-inflammatory effect

Introduction

The prevalence of allergic diseases has increased worldwide in recent years. In Western countries, efficacious therapies and prophylactic interventions for allergic diseases are needed because of the high morbidity associated with these diseases and health care budgets[1]. Allergic diseases are also becoming increasingly prevalent in Japan[2-6]. Therefore, many researchers are extensively investigating the underlying mechanisms and therapeutics for these diseases[7].

Type I allergies, which are characterized by allergic reactions to dust, mites, and pollen, are initiated by the binding of antigens to the high affinity IgE receptor (Fc ε RI) on the surfaces of mast cells and basophils. The cross-linking of allergen-specific IgE leads to the release of histamine, leukotrienes, interleukins, and other mediators including chemokines, from mast cells (degranulation)[8]. Interleukin (IL)-4 and IL-13 are cytokines that induce the differentiation of naive helper T cells to type 2 helper T cells (Th2)[8]. Th2 cells, B cells, and mast cells are also activated by IL-4 and produce IgE antibodies and some

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cytokines. IL-13 has been shown to induce the secretion of the chemokines required for the recruitment of allergic effector cells in the lungs[9]. Allergies are essentially inflammatory diseases[10], and allergic inflammation involves many types of cells, particularly mast cells and macrophages. Activated macrophages produce NO, COX2, IL-1β, IL-6, and TNF-α. In the present study, we conducted experiments on a rat basophilic leukemia cell line (RBL-2H3) and mouse macrophage cell line (RAW264), which are frequently used in studies on allergies and inflammation.

Cinnamomi Cortex (cinnamon) is the dried bark of *Cinnamomum cassia* Blume (Lauraceae), which is widely used as a natural medicine in Asia and Europe[11]. In Japan, it used as a spice, but is mainly applied to Kampo medicines such as the kakkonto, shoseiryuto, and keishibukuryogan. The kakkonnto and shoseiryuto are administered to treat cold symptoms and rhinitis, while the keishibukuryogan is used for inflammation of the genitals.

The characteristics of Japanese Kampo medicines containing Cinnamomi Cortex prompted us to examine the anti-allergic and anti-inflammatory effects of cinnamon and identify the active components.

Materials and methods

Materials

The crude drug (Cinnamomi Cortex, Lot No. 252216) was obtained from Uchida-Wakanyaku Ltd. Monoclonal anti-dinitrophenyl mouse IgE isotype (anti-DNP IgE), albumin dinitrophenyl (DNP-HSA), dexamethasone, and lipopolysaccharide (LPS, *Escherichia c-oli* 0111:B4) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from GIBCO (Grand Island, NY, USA). The penicillin-streptomycin mixed solution and Eagle's minimal essential medium (MEM) were obtained from Nacalai Tesque. RBL-2H3 (as the mast cell line) and RAW264 (the murine macrophage-like cell line) were purchased from the Health Science Research Resources Bank and Cell Engineering Division of the RIKEN Bio Resource Center, respectively.

Cell culture

RBL-2H3 and RAW264 cells were grown in MEM containing 10% (v/v) heat-inactivated FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cultures were maintained at less than 5% CO_2 at 37°C in cell culture dishes. Cells were detached with trypsin-EDTA solution (Nacalai Tesque). After cells had been washed, they were resuspended in medium and used in subsequent experiments.

Sample preparation procedure

One hundred grams of cinnamon was powdered and extracted with 70% methanol by the percolation method. Some of the extract was evaporated to dryness (Cin70M). Methanol in the remaining portion was distilled to produce a water suspension, which was subjected to extraction with ether and ethyl acetate (Fig. 1). The ether fraction was repeatedly separated by silica gel column chromatography (CC) and ODS CC. A total of 0.39 g of cinnamaldehyde (CNMA) was successfully isolated from 0.50 g of the ether fraction.

Nitric oxide (NO) determination

Nitrite concentrations in the medium were measured as an indicator of NO production according to the Griess reaction method. A total of 1×10^5 RAW264 cells were plated on 24-well plates and pre-incubated overnight. Samples were added and incubated for 2hr. LPS was then added to the medium (at a final concentration of 10 ng/mL). Equal volumes of the culture medium and Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water) were mixed together. Absorbance at 540 nm was determined with a microtiter plate reader (infinite M200, TECAN, Switzerland), and the absorption coefficient was calibrated using a sodium nitrite solution standard.

β-Hexosaminidase secretion assay

The degranulation of RBL-2H3 cells was determined by measuring the release of the granule marker, β-hexosaminidase. RBL-2H3 cells were seeded on 24-well multiplates at a density of 2×10^5 cells per well and were sensitized overnight with 100 ng/mL DNP-specific IgE. The growth medium was replaced with 200 μL of HEPES buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 0.1% glucose, 0.1% BSA, and 10 mM HEPES, pH 7.4). IgE-sensitized RBL-2H3 cells were then exposed

to the anti-allergic drugs or crude drug extract for 15 min. After being incubated, the cells were stimulated with 100 ng/mL DNP-HSA for 2 hr. At the end of the experiment, 20 μL of the supernatant was incubated with an equal volume of the substrate solution (2 mM p-nitrophenyl-N-acetyl-β-D-glucosaminide resolved in 0.1 M citrate buffer (pH 4.5)) at 37°C for 1 hr. The reaction was stopped by adding 160 μL of stop solution (1:1 mixture 167 mM Na_2CO_3, $NaHCO_3$). Absorbance at 405 nm was measured with a microplate reader. In order to determine the total amount of β-hexosaminidase released, cells maintained as the control were lysed with 2 μL of Triton X-100 and the supernatant was incubated with the substrate using the same procedure.

Semi-quantification of cytokine mRNAs by the reverse transcription-polymerase chain reaction (RT-PCR)

RBL-2H3 cells were sensitized overnight with 100 ng/mL DNP-specific IgE on 24-well plates (5.0×10^5 cells/well). IgE-sensitized RBL-2H3 cells were treated with the extracts and anti-allergic drugs for 15 min and stimulated with 100 ng/mL DNP-HSA for 0 min to 8 hr. After being incubated, the cells were washed with ice-cold PBS.

RAW264 cells were treated with the extracts and anti-inflammatory drugs for 2 hr and stimulated with LPS for 8 hr. After being incubated, the cells were washed with ice-cold PBS. Total RNA was isolated from RBL-2H3 and RAW264 cells according to the manufacturer's instructions (Sepasol, Nacalai Tesque) . The synthesis of first-strand cDNA was performed using 1 μg of total RNA in 20 μL volumes with a random primer using the high capacity cDNA reverse transcription kit (Applied Biosystems). Primer pairs were selected based on previous studies and synthesized by Invitrogen (Table 1). The PCR conditions followed were those recommended by the manufacturer (TOYOBO). Cycle numbers and the annealing temperature were also shown in Table 1. PCR products were electrophoresed in 1.5% (w/v) agarose gels and stained with ethidium bromide. The detection and densitometric analysis of bands were performed using a Densitograph (ATTO). The sizes of the bands were confirmed by referring to molecular size markers (100 bp DNA Ladder Marker, Nacalai Tesque). The value of each

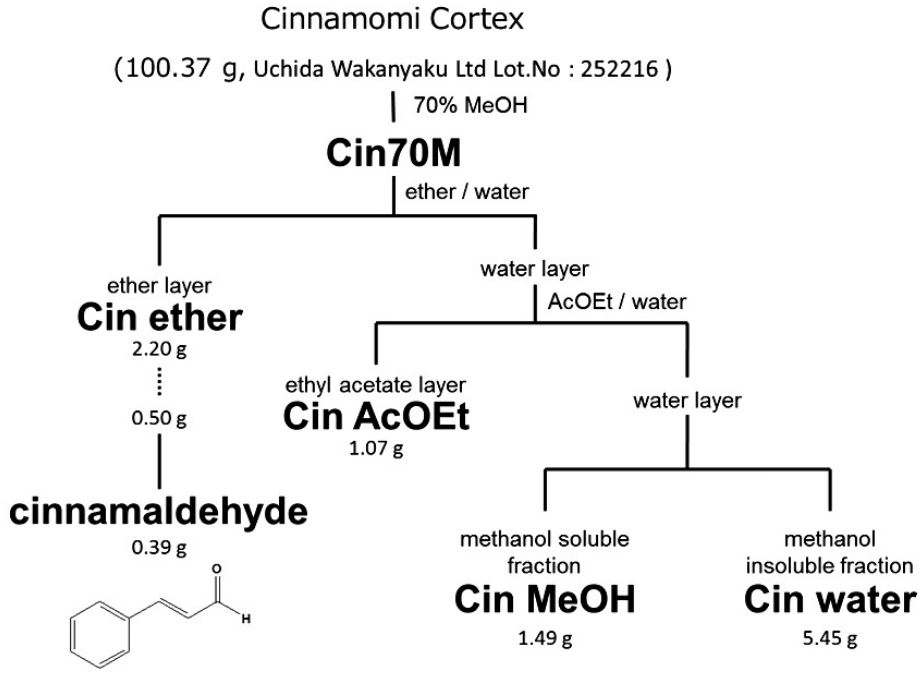

Fig. 1 Extraction and fractionation of Cinnamomi Cortex. The materials were extracted with 70% methanol to give 70% methanol extract (dryness : Cin70M) and others were distilled methanol away to make water fraction. The water fraction was divided with ether (Cin ether) and ethyl acetate. Cin ether is mainly composed of cinnamaldehyde and isolated it as active component.

cytokine mRNA was normalized to the amount of GAPDH mRNA, which was utilized as a housekeeping gene for each experimental condition.

Table 1 Primer sets used in this experiment

Cell type	primer name	primer sequence	annealing temperature	PCR cycles
RBL-2H3 RAW264	GAPDH F	TTCACCACCATGGAGAAGGC	65	21
	GAPDH R	GGCATGGACTGTGGTCATGA		
RBL-2H3	IL-4 F	ACCTTGCTGTCACCCTGTTC	65	30
	IL-4 R	TTGTGAGCGTGGACTCATTC		
RBL-2H3	IL-13 F	TCTCGCTTGCCTTGGTGGTC	65	28
	IL-13 R	CATCCGAGGCCTTTTGGTTACAG		
RAW264	iNOS F	GTTCCAGCATATCTGCAGAC	55	28
	iNOS R	CACAACTGGGTGAACTCCAA		
RAW264	COX2 F	AACCGTGGGGAATGTATGAGCA	65	26
	COX2 R	AACTCTCTCCGTAGAAGAACCTTTTCCA		
RAW264	IL-1β F	ATGGCAACTGTTCCTGAACTCAACT	65	26
	IL-1β R	ATATTCTGTCCATTGAGGTGGAGAGCT		
RAW264	IL-6 F	TGGAGTCACAGAAGGAGTGGCTAAG	55	28
	IL-6 R	CATCTGGCTAGGTAACAGAATATTTATATC		
RAW264	TNF-α F	TACTGAACTTCGGGGTGATCGGTCC	65	26
	TNF-α R	CAGCCTTGTCCCTTGAAGAGAACC		

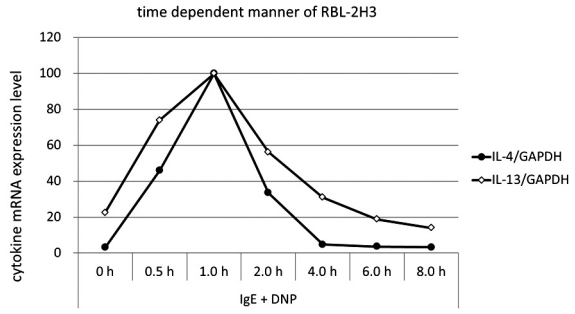

Fig. 2a

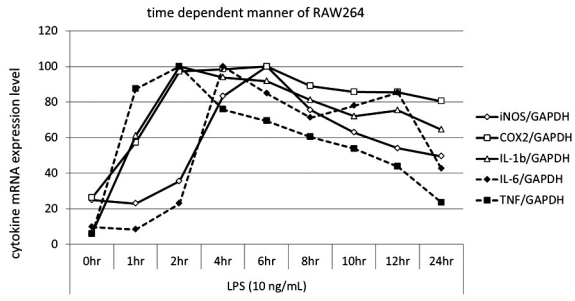

Fig. 2b

Fig. 2 Time dependent manner of stimulated RBL-2H3 and RAW264.

Fig. 2a RBL2H3 stimulated by antigen (sensitized anti-DNP IgE antibody for overnight and antigen is used DNP-HSA). IL-4 and IL-13 gene expression reached to maximum level after 1 hr of stimulation and quickly tapered. Fig. 2b RAW264 stimulated by LPS (10 ng/mL). COX-2, IL-1*β* and TNF-*α* gene expression reached to maximum level after 2 hr of stimulation and slowly tapered. IL-6 and iNOS gene reached to muximum level after stimulated 4 hr and 6 hr respectively.

Results

Time-dependent effects of the stimulant on inflamma tory cytokine mRNA expression in RBL-2H3 and RAW264 cells

RBL-2H3 and RAW264 cells were stimulated with DNP-HSA or LPS, respectively, in order to determine the time-dependent effects of the stimulant. RT-PCR reactions were performed to amplify GAPDH, IL-4, and IL-13 gene mRNAs. RAW264 cells were incubated with

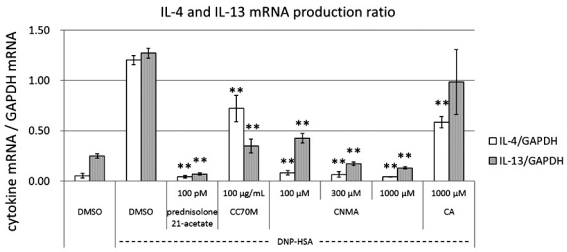

Fig. 3a

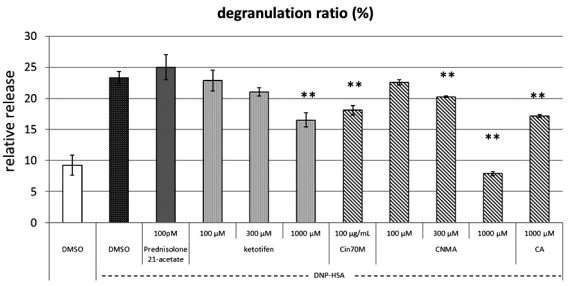

Fig. 3b

Fig. 3 Effects of Cin70M, CNMA and CA on the increases in IL-4, IL-13 mRNA and degranulation induced by Fcreceptor cross-linking. RBL-2H3 cells were sensitized with anti-DNP IgE anti-body and treated with Cin70M and cinnamaldehyde (CNMA) and cinnamic acid (CA). And then, it was stimulated with 100 ng/mL DNP-HSA.

Fig. 3a The IgE-sensitized RBL-2H3 cells were treated with indicated concentrations of extract or constituents which derived from Cinnamomi cortex. Results of densitometric analysis are shown as cytokine mRNA/GAPD-H mRNA ratio. Fig. 3b The enzymatic activities of released *β*-hexosaminidase were measured. Values are plotted as percentages of total *β*-hexosaminidase content determined by dissolving cells with 0.1% Triton-X100. Values represent means ±SD of three determinations.

** $P < 0.01$ compared with the DMSO + DNP-HSA group (student's *t*-test).

LPS (10 ng/mL) for different times, and RT-PCR reactions were performed to amplify GAPDH, iNOS, COX-2, IL-1β , IL-6, and TNF- α gene mRNAs, the most extensively studied inflammatory cytokines enhanced by LPS with RAW264 macrophages. The expression of various cytokine mRNAs in RBL-2H3 cells was detectable 30 min after the addition of DNP-HSA and peaked 1 hr after the incubation (Fig. 2a). COX2, IL-1β, and TNF-α gene expression levels rapidly increased in RAW264 cells 1 hr after the LPS stimulation, whereas those of iNOS and IL-6 slowly increased and reached a maximum after 4 hr (Fig. 2b).

Effects of Cin70M and CNMA on antigen-induced IL-4 and IL-13 mRNA expression and degranulation in RBL-2H3 cells

When RBL-2H3 cells were sensitized and stimulated, the production of IL-4 and IL-13 mRNAs markedly increased. The pretreatment with prednisolone 21-acetate markedly suppressed the expression of both mRNAs. The pretreatment with Cin70M and CNMA suppressed increases in in the expression of IL-4 and IL-13 mRNAs in a concentration-dependent manner (Fig. 3a).

Figure 3b shows the results of the degranulation test. Unlike the expression of mRNAs, prednisolone 21-acetate did not inhibit the release of β-hexosaminidase. Ketotifen is a well-known second-generation antihistamine that inhibits the degranulation of mast cells. In our system, ketotifen decreased the release of β-hexosaminidase to 28% at 1000 μM, whereas CNMA suppressed it to 67% at 1000 μM.

Effects of Cin70M and CNMA on NO production and inflammatory mRNA expression in RAW264 cells stimulated with LPS

In order to evaluate the inhibitory effects of the cinnamon extract on LPS-mediated pro-inflammatory mediators, Cin70M and CNMA were investigated in cells stimulated with LPS. Cin70M and CNMA both markedly reduced the production of NO at concentrations of 100 mg/mL and 300 μM, respectively (Fig. 4a). We also investigated the effects of Cin70M and CNMA on the expression of iNOS mRNA, which induces inflammatory reactions, and found that both suppressed its expression (Fig. 4b). Figure 5 shows that CNMA markedly suppressed the expression of inflammatory cytokine mRNAs. Similar to its effects on the expression of iNOS mRNA, CNMA reduced the expression of inflammatory cytokine mRNAs (COX2, IL-1β, IL-6, and TNF-α) at 300 μM.

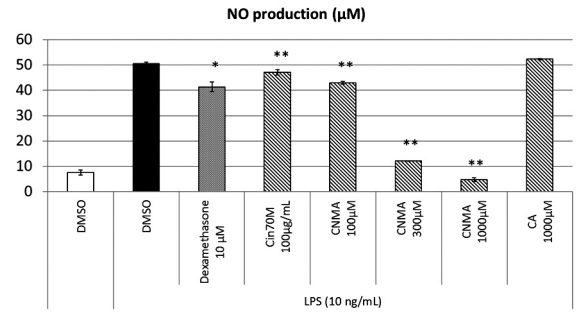

Fig. 4a

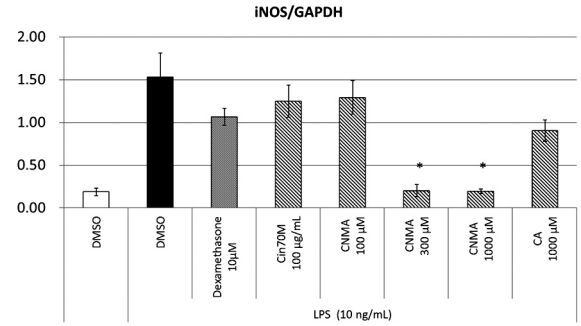

Fig. 4b

Fig. 4 Effects of Cin70M, CNMA and CA on the RAW264 NO production and iNOS mRNA ratio induced by LPS (Lipopolysaccharide). RAW264 cells were stimulated with 10 ng/mL LPS.

Fig. 4a Time course of change in NO production level was analyzed with Griess method. RAW264 cells were treated with indicated concentrations of extract or constituents which derived from Cinnamomi cortex. Fig. 4b Time course of change in these mRNA levels were analyzed with the semi-quantitative RT-PCR method. RAW264 cells were treated with indicated concentrations of extract or constituents which derived from Cinnamomi cortex. Results of densitometric analysis are shown as iNOS mRNA/GAPDH mRNA ratio. Values represent means ±SD of three determinations.

Significantly different from LPS+DMSO group values: * $p < 0.05$, ** $p < 0.01$, as analyzed by student's t-test method.

Discussion

CNMA, a typical phenylpropanoid, is known to exhibit many pharmaceutical activities including sedative, anti-inflammatory, and antibacterial. We herein demonstrated that CNMA had anti-allergic and anti-inflammatory effects. Cinnamic acid (CA), which is the oxidative product of CNMA, is a starting material for the biosynthesis of many natural compounds, but does not have any anti-allergic or anti-inflammatory effects, which indicates that the aldehyde group is an important factor in the medicinal effects described above. Yuan et al. examined the effects of the i.v. and oral administration of CNMA in Fischer 344 rats, and found that the biological half-life of CNMA following its i.v. administration was 1.7 hr, while its oral bioavailability was less than 20%[12]. The effective blood level of CNMA cannot be maintained by oral administration because of its biological behaviors, such as low bioavailability and rapid metabolism to its inactive form, CA. Therefore, a route of administration other than orally needs to be selected for Cinnamomi Cortex in order to maintain the efficacy of CNMA.

Chlorogenic acid, one of the major phenylpropanoids, is contained in coffee and many other plants. Chlorogenic acid has been shown to inhibit the production of NO and expression of COX-2 mRNA, thereby suppressing NF-κB and JNK/AP-1[13]. CNMA appears to exhibit anti-inflammatory effects using a similar mechanism. Chao et al. previously reported that CNMA decreased the LPS-mediated activation of JNK1/2 and ERK1/2 in J774A.1 macrophages[14]. Matsuda et al. also demonstrated its anti-allergic effects through the suppression of JNK in IgE-mediated allergic reactions in RBL-2H3 cells[15]. Although anti-allergic phenylpropanoids have not yet been examined in RBL-2H3 cells, chlorogenic acid inhibited JNK/AP-1 and CNMA also exhibited anti-allergic effects through the same mechanisms.

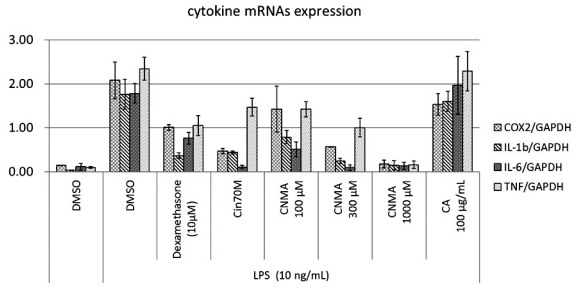

Fig. 5 Effects of Cin70M, cinnamaldehyde on the increases in COX-2, IL-1beta, IL-6 and TNF-alfa mRNA induced by LPS stimulation. RAW264 cells were stimulated with 10 ng/mL of LPS. Time course of change in these mRNA levels were analyzed with the semi-quantitative RT-PCR method. RAW264 cells were treated with indicated concentrations of extract or constituents which derived from Cinnamomi cortex. Results of densitometric analysis are shown as cytokine mRNA/GAPD-H mRNA ratio. Values represent means ±SD of three determinations.

In the present study, Cinnamomi Cortex not only reduced the expression of IL-4 and IL-13 mRNAs, but also suppressed degranulation. In allergic rhinitis patients, histamine and IL-4 have been implicated in sneezing and the production of nerve growth factor (NGF), which causes supersensitization to the allergen in the nasal mucosa, respectively[16, 17]. In the future, the anti-allergic and -inflammatory effects of Cinnamomi Cortex should be further investigated *in vivo* to clarify its effectiveness in the treatment of allergic rhinitis, such as reducing the frequency of sneezing and suppressing NGF production in the nasal mucosa.

CNMA was previously identified as the characteristic flavoring compound of cinnamon that gives it its sweet and pungent taste[11], and we herein demonstrated that CNMA exhibited anti-allergic and anti-inflammatory effects. Some Materia Medicas indicated that high quality cinnamon has a strong scent, sweetness, and pungency[18-20]. Hamano et al. compared cinnamon produced in Vietnam and China, and found that Vietnamese cinnamon contained higher levels of CNMA than Chinese cinnamon[21]. Therefore, when Cinnamomi Cortex is administered for its anti-allergic effects, such as in the kakkonto and shoseiryuto, or its anti-inflammatory effects, such as in the keishibukuryogan, the results of the present study suggest that strong smelling Cinnamomi Cortex produces superior effects.

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RBL－2H3およびRAW264細胞株を用いたケイヒの抗アレルギー作用および抗炎症作用の検討

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和文要旨

桂皮（ケイヒ）は日本薬局方に収載される生薬で，抗アレルギーや解熱鎮痛炎症薬とみなされる漢方処方に配剤される．我々はケイヒの抗アレルギー作用および抗炎症作用について調査し，活性成分の同定を行った．ケイヒを70％メタノールで抽出し（Cin70M），シリカゲルカラムクロマトグラフィー（CC）およびオクタデシルシリルCCを用いて成分を分離した．分離した画分はin vitroでの実験としてラット好塩基球性白血病細胞株（RBL－2H3）およびマウスマクロファージ細胞株（RAW264）を用い，それらが産生するサイトカインmRNAの発現率によって抗アレルギーおよび抗炎症活性を示す画分を明らかにした．また，抗アレルギー作用においては，RBL－2H3の脱顆粒率を測定するために細胞培養液中のβ－ヘキソサミニダーゼも測定した．Cin70Mとシンナムアルデヒド（CNMA）はRBL－2H3細胞における脱顆粒およびインターロイキンのmRNA発現を阻害した．CNMAは300μMで脱顆粒を抑制し，その抑制作用はポジティブコントロールとして用いたケトチフェンより僅かに強かった．同様にCin70MとCNMAはRAW264が産生した一酸化窒素（NO）および炎症性サイトカインmRNA発現を抑制した．特にNO産生についてはCNMA 100μMで10μMのデキサメタゾンと同等の抑制作用を示した．ケイヒの特徴である甘い味と芳香は明らかにした活性成分の一つであるCNMAに由来する．今回の結果から，アレルギー疾患や炎症性疾患の患者に対し，ケイヒを含む漢方薬が処方された際には甘い味と芳香が強いケイヒを選んだ方が良いことが示された．

キーワード：ケイヒ，シナモン，シンナムアルデヒド，抗アレルギー作用，抗炎症作用