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Xanthium strumarium suppresses degranulation and pro-inflammatory cytokines secretion on the mast cells

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비만세포에서의 창이자의 탈과립 및 pro-inflammatory cytokines 분비량에 미치는 영향

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Objective : Previously, the methanol extracts of the semen of *Xanthium strumarium* could involved anti-inflammatory effects in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells. We evaluated the anti-allergic effects of *X. strumarium* on rat basophilic leukemia (RBL-2H3) cells.

Methodes : To investigate the effect of *X. strumarium* on the phorbol 12-myristate 13-acetate (PMA) and calcium ionophore A23187-induced RBL-2H3 cells. The effects of *X. strumarium* on the degranulation and the pro-inflammatory cytokines secretion and expression from RBL-2H3 cells were evaluated with β-hexosaminidase assay, ELISA, and RT-PCR analysis. In addition, we examined the effects of *X. strumarium* on nuclear factor (NF)-κB activation and IκB-α degradation using Western blot analysis.

Results : *X. strumarium* inhibited degranulation and secretions and expressions of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-4 and cyclooxygenase (COX)-2, on stimulated RBL-2H3 cells, however, *X. strumarium* not affect cell viability. In stimulated RBL-2H3 cells, the protein expression level of nuclear factor-kappa B (NF-κB) was decreased in the nucleus by *X. strumarium*. In addition, *X. strumarium* suppressed the degradation of inhibitory protein IκB-α protein in RBL-2H3 cells.

Conclusion : These results suggest that *X. strumarium* inhibits the degranulation and secretion of pro-inflammatory cytokines through blockade of NF-κB activation and IκB-α degradation.

Key words : *Xanthium strumarium*, Cytokine, Cyclooxygenase (COX)-2, Nuclear factor-kappa B (NF-κB), IkappaB-alpha (IκB-α)

Introduction

The semen of *Xanthium strumarium* has been used to treat bacterial infections, diabetes, inflammatory diseases, such as rhinitis, empyema, and rheumatoid arthritis, in the Orient[1].

Tissue mast cells play a central role in inflammatory and particularly in immediate-type allergic reactions[2]. Cross linkage of IgE bound to high affinity receptors on mast cells not only results in the rapid release of autacoid mediators (histamine, serotonin, β-hexosaminidase), but also the sustained synthesis and release of cytokines, chemokines, and growth factors[3,4]. Tumor necrosis factor-alpha (TNF-α) is a pleiotropic pro-inflammatory cytokine and it has been suggested to induce tissue damage and it is also considered as major initiator of inflammation[5]. Interleukin (IL)-4, which is a pleiotropic cytokine and mainly produced by activated Th2 cells and inflammatory cells, plays a pivotal role in allergic inflammation[6-8].

Activated mast cells leads to phosphorylation of tyrosine kinase and mobilization of internal Ca^{2+}. This is followed by activation of protein kinase C, mitogen-activated protein kinases (MAPKs), nuclear factor-kappa B (NF-κB), and releasing of inflammatory cytokines[9,10]. NF-κB is a transcription factor controlling gene expression during inflammation, immunity, cell proliferation, stress response, and apoptosis[11]. NF-κB can function upstream of cyclooxygenase (COX)-2 to control the transcription of this gene[12]. COX-2 is induced in certain types of cells by variety of inflammatory stimulants[13,14].

In previous report, the methanol extracts of the semen of *X. strumarium* involved anti-inflammatory effects by nitric oxide (NO) production, prostaglandin E2 (PGE2) and TNF-α secretions and NF-κB activation in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells[15]. However, study on effect of *X. strumarium* in mast cells has not been identified. In this study, we investigated the anti-allergic effect of *X. strumarium* in rat basophilic leukemia (RBL-2H3) mast cells.

Materials and Methods

1. Preparation of water extract of *X. strumarium*

X. strumarium a one day dose for human adults were boiled with distilled water at 100 ℃, and the whole mixture is decocted until the volume is reduced by half. The extract water (500 ㎖) was filtered through 0.22 μm filter and the filtrate was freeze-dried (yield, 3.48 g) and kept at 4 ℃. The dried filtrate was dissolved in phosphate buffered saline (PBS) and filtered through 0.22 μm filter before use.

2. Reagents

Phorbol 12-myristate 13-acetate (PMA), calcium

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ionophore A23187, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and ρ -nitro-phenyl-N-β-D-glucosaminide were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle's Medium (DMEM) containing L-glutamine (200 ㎎/L) and FBS were purchased from Hyclone (Logan, UT). TNF ELISA kit (BD OptEIA™ Rat TNF ELISA Set), IL-4 ELISA kit (BD OptEIA™ Rat IL-4 ELISA Set) and anti-COX-2 monoclonal antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). Anti-NF-κB, IκB-α, and β-actin polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Phosphatase labeled affinity purified antibody to rabbit IgG and BCIP/NBT phosphatase substrate were purchased from KPL (Gaithersburg, MD).

3. Cells culture

RBL-2H3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10 % (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/㎖ Penicillin and 100 ㎍/㎖ Streptomycin in a humidified incubator with 5 % CO_2. In all experiments, RBL-2H3 cells were treated for 1 h with the presence of the indicated concentrations of *X. strumarium* prior to stimulation with 50 nM PMA plus 1 μM A23187 in serum-free DMEM.

4. MTT assay

The cell viability of *X. strumarium* was assessed using the MTT assay[16] in the remaining cells after Griess reaction. The MTT solution (0.5 ㎎/㎖) was added to each well. After incubation for 3 h at 37 ℃ and 5 % CO_2, the supernatant were removed and formed formazan crystals in viable cells were measured at 540 nm with a microplate reader. The percentage of cell viability was calculated against untreated cells. All experiments were performed in triplicate well.

5. β -hexosaminidase assay

β-hexosaminidase was measured in both supernatant and pellet fractions using a previously reported method[17]. Briefly, RBL-2H3 cells (3×10^5 cells) were treated for 1 h with the presence of the indicated concentrations of *S. flavescens* prior to stimulation with 50 nM PMA plus 1 μM A23187 and incubated at 37 ℃ for 60 min. After stimulation, 50 ㎕ of each sample was incubated with 50 ㎕ of 1 mM ρ-nitro-phenyl-N-β-D-glucosaminide dissolved in 0.1 M citrate buffer, pH 5, in 96 well microtiter plate at 37 ℃ for 1 h. The reaction was terminated with 200 ㎕/well of 0.1 M carbonate buffer, pH 10.5. The plate was read at 405 nm in an ELISA reader. The inhibition percentage of β-hexosaminidase release was calculated using the following equation :

$$\beta\text{-hexosaminidase release (\%)} = \frac{A_{405}\text{ of sup.}}{A_{405}\text{ of sup.} + A_{405}\text{ of pellet}} \times 100$$

where is A_{405} is absorption of measured at 405 nm and sup. is supernatant.

6. Enzyme-linked immunosorbent assay for pro-inflammatory cytokines (TNF-α, IL-4)

Each cytokines concentration in RBL-2H3

cells were measured with commercially available Rat TNF, IL-4 ELISA kit (BD Biosciences), according to the manufacture's protocol. Color development was measured at 450 nm using an automated microplate ELISA reader.

7. Isolation of total RNA from cells and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated as per the manufacture's instructions. Briefly, cells were lysed additional Trizol reagent (Invitrogen, Carlsbad, CA) and the cell lysate was passed through the pipette several times. 0.2 ㎖ of chloroform was added per 1 ㎖ of Trizol reagent. The tubes were shaken vigorously and incubated at room temperature for 2-3 min. The samples were centrifuged at 14,000 g for 20 min. The aqueous phase was transferred to a fresh tube and RNA was precipitated by the addition of 0.5 ㎖ isopropanol. The RNA pellet was air-dried and resuspended in nuclease-free water. The concentration of RNA was estimated spectrophotometrically. Three microgram RNAs were reverse-transcribed using M-㎖V reverse transcriptase (Promega, Madison, WI). Single stranded cDNA was amplified by PCR with primers (Table 1).

PCR amplifications were done in a 20 ㎕ PCR PreMix (Bioneer Co., Korea) containing 10 mM Tris-HCl, 40 mM KCl, 1.5 mM $MgCl_2$, 250 μM dNTP, 1 unit of Taq polymerase. Amplifications were carried out in a PCR machine (ASTEC PC802) using an initial denaturation at 95 ℃ for 5 min followed by 30 cycles (TNF-α, COX-1 : 35 cycles) of denaturation for 60 sec at 95 ℃, annealing for 60 sec at 52 ℃ (COX-1, COX-2 : 55 ℃) and extension for 60 sec at 72 ℃. This was concluded with a final extension for 7 min at 72 ℃. Amplicons were separated in 1 % agarose gels in 0.5× TBE buffer at 100 V for 30 min, stained with ethidium bromide and visualised under UV light. GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control to evaluate relative expressions of TNF-α, IL-4 and COX-2.

Table 2. Oligonucleotide primers used for PCR in this study.

Target gene	Oligonucleotide sequences (5' to 3' direction)	Expected size	Annealing temp.	Accession number
TNF-α	CGTCTACTCCTCAGAGCCCC TCCACTCAGGCATCGACATT	226 bp	52 ℃	NM012675
IL-4	AACACTTTGAACCAGGTCAC AGTGCAGGACTGCAAGTATT	330 bp	52 ℃	X16058
COX-1	ACTGGTCTGCCTCAACACCA CAAGGGTGAGACCCCAAGTT	223 bp	55 ℃	S67721
COX-2	TGACCAGAGCAGAGAGATGA CATAAGGCCTTTCAAGGAGA	250 bp	55 ℃	S67722
GAPDH	GGCCAAAAGGGTCATCATCT GTGATGGCATGGACTGTGGT	201 bp	52 ℃	NM017008

8. Western blot analysis

Cell extracts were prepared by detergent lysis procedure. Cells (5 × 10^6 cells) were scraped, washed once with PBS and resuspended in lysis buffer. Equal amounts of protein were separated electrophoretically using 10 % SDS-PAGE, and then the gel was transferred to nitrocellulose membranes. Blots were blocked for at least 2 h with 5 % non-fat dry milk. The blot was incubated with NF-κB, IκB-α, and β-actin polyclonal antibodies at 4 ℃ and secondary antibodies at room temperature were detected by the AP (BCIP/NBT phosphatase substrate) system according to the recommended procedure.

9. Preparation of nuclear extract

The treated cells were washed and centrifuged and then resuspended in hypotonic buffer and incubated on ice for 30 min. After centrifugation at 14,000 rpm, 4 ℃ for 20 min, the supernatant cytoplasmic protein was collected. The remnants were resuspended in extraction buffer, incubated on ice for 30 min, and then centrifuged (14,000 rpm, 4 ℃, 20 min), after which the supernatant nuclear extract was collected.

10. Statistical analysis

Data is presented as the Mean ± SE (standard error) of at least three separate experiments. Comparisons between two groups were analyzed using Student's t-test. *P* values less than 0.05 considered be statistically significant.

Results

1. *X. strumarium* inhibits degranulation on RBL-2H3 cells

We studied the effects of *X. strumarium* on degranulation in order to investigate anti-allergic effect. The measurement of degranulation, estimated by release of β-hexosaminidase assay from PMA plus A23187-induced RBL-2H3 cells. *X. strumarium* significantly inhibited β-hexosaminidase release (Fig. 1A). The inhibitory ratio of β-hexosaminidase release were 33.9 % with a dose of 1.0 ㎎/㎖ and 42.11 % with dose of 2.0 ㎎/㎖. However the cell viability was not affected by *X. strumarium* as measured by MTT assay (Fig. 1B).

2. *X. strumarium* inhibits production of TNF-α and IL-4 on RBL-2H3 cells

We investigated effect of *X. strumarium* on the production of pro-inflammatory cytokines. Cells were pre-treated with various concentration of *X. strumarium* and then PMA plus A23187 challenge for 8 h. *X. strumarium* significantly suppressed both cytokines into culture supernatant (Fig. 2A, 2B). The inhibitory ratio were 30.71 % with a dose of 2.0 ㎎/㎖ (TNF-α) and 82.97 % with dose of 2.0 ㎎/㎖ (IL-4).

3. *X. strumarium* inhibits expression of TNF-α and IL-4 mRNA

We tried to determine whether it also inhibits expression of pro-inflammatory cytokines (TNF-

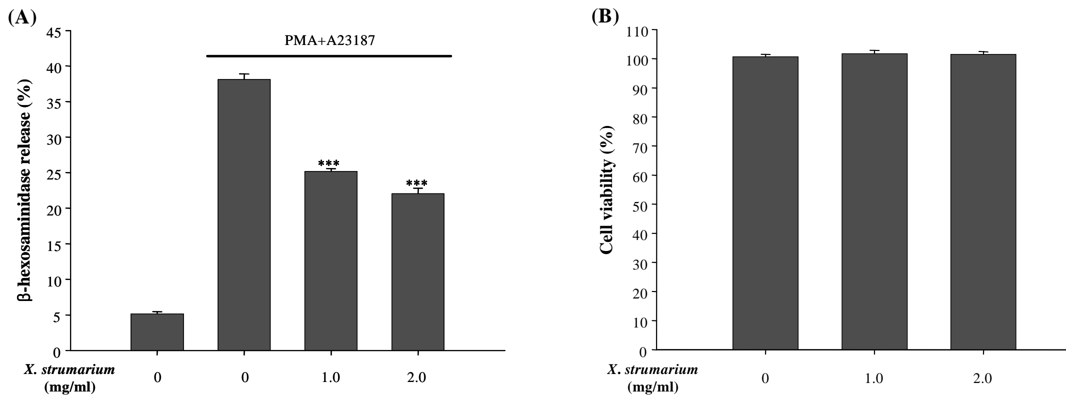

Fig. 1. Effects of *X. strumarium* on degranulation (A) and cell viability (B) on RBL-2H3 cells. (A) Cells were treated with the indicated concentration of *X. strumarium*. Degranulation was assessed by β-hexosaminidase release into the supernatant. β-hexosaminidase released into the medium is presented as mean ± SE (n=4). *** P < 0.005; significantly different from the stimulated group. (B) Cell viability was evaluated by MTT assay. Data represent the mean ± SE of four independent experiments.

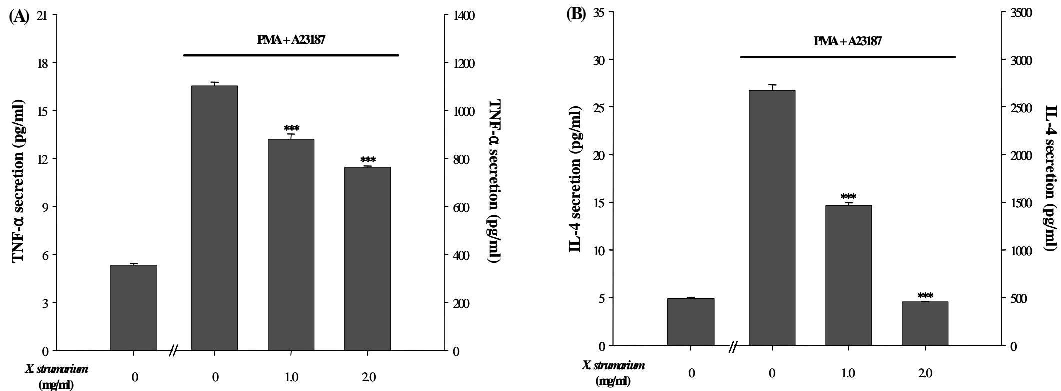

Fig. 2. Effects of *X. strumarium* on production of TNF-α and IL-4 on RBL-2H3 cells. TNF-α (A) and IL-4 (B) concentration was measured from cell supernatants using ELISA method. Vertical bars represent as the mean ± SE from 4 wells. *** P < 0.005; significantly different from the stimulated group.

α and IL-4) mRNA. RT-PCR analysis indicated that the expression of the both mRNA were decreased significantly by *X. strumarium* (Fig. 3).

4. *X. strumarium* inhibits expression of COX-2 mRNA

We also determined that whether *X. strumarium* can modulate COX-2 mRNA expression. Cells were pre-treated with various concentration of

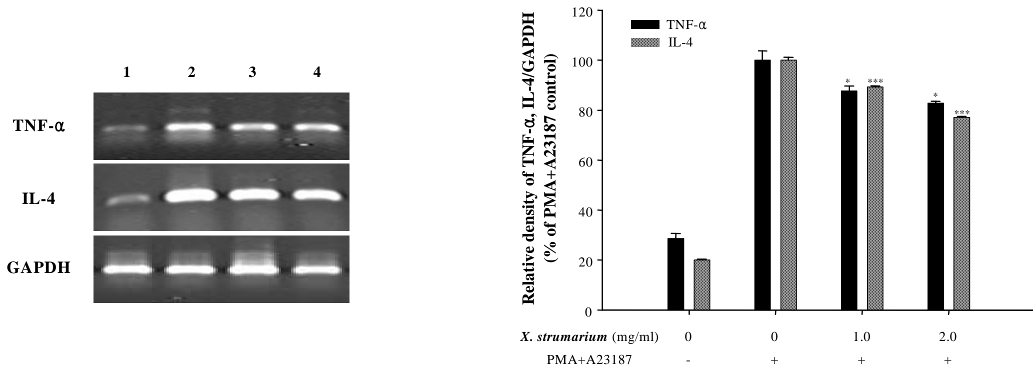

Fig. 3. Effects of *X. strumarium* on TNF-α and IL-4 mRNA expression on RBL-2H3 cells. Total RNA was isolated, TNF-α and IL-4 mRNA expression was detected by RT-PCR analysis. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. *X. strumarium* 1.0 ㎎/㎖ + stimulus; lane 4. *X. strumarium* 2.0 ㎎/㎖ + stimulus. * $P < 0.05$, *** $P < 0.005$; significantly different from the stimulated group.

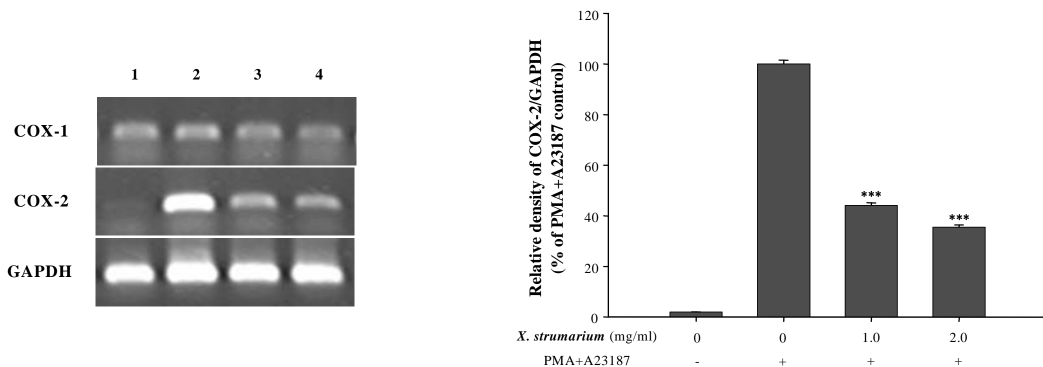

Fig. 4. Effects of *X. strumarium* on COX-2 mRNA expression on RBL-2H3 cells. Total RNA was isolated, COX-2 mRNA expression was detected by RT-PCR analysis. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. *X. strumarium* 1.0 ㎎/㎖ + stimulus; lane 4. *X. strumarium* 2.0 ㎎/㎖ + stimulus. *** $P < 0.005$; significantly different from the stimulated group.

X. strumarium and then PMA plus A23187 challenge for 4 h. RT-PCR analysis indicated that the expression of the COX-2 mRNA were decreased significantly by *X. strumarium* but, COX-1 mRNA expression showed no change after such treatment (Fig. 4).

5. *X. strumarium* reduce the activation of NF-κB and the degradation of IκB

We examined the effects of *X. strumarium* on NF-κB activation in nuclear protein and IκB degradation in cytoplasmic protein. RBL-2H3

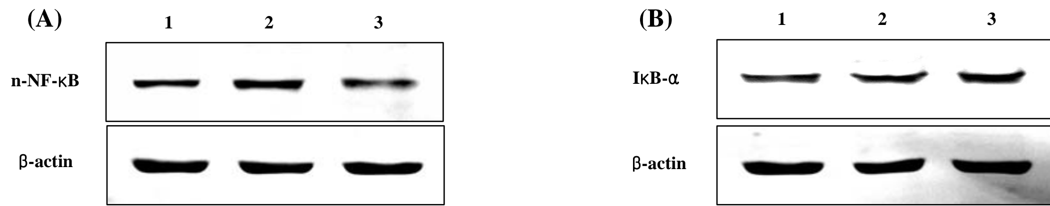

Fig. 5. Effects of *X. strumarium* on the NF-κ B activation (A) and the Iκ B degradation (B) on RBL-2H3 cells. (A) The cell extract were assayed Western blot analysis for NF-κ B in nuclear extracts (n-NF-κ B). (B) The cell extract were assayed Western blot analysis for Iκ B-α in cytoplamic extract. Lane 1. negative control group; lane 2. positive control group (only treated stimulus); lane 3. X. strumarium 2.0 ㎎/㎖ + stimulus.

cells were treated with *X. strumarium* and PMA plus A23187 for 2 h. *X. strumarium* inhibited the PMA plus A23187-induced the nuclear translocation of NF-κB (Fig. 5A). RBL-2H3 cells were treated with *X. strumarium* and PMA plus A23187 for 30 min. As shown in Fig. 5B, PMA plus A23187 treatment caused degradation of IκB-α. However, *X. strumarium* suppressed degradation of IκB-α.

Discussion and Conclusion

Mast cells and basophils play important roles in the pathogenesis of allergic diseases through the release of inflammatory mediators such as histamine and several cytokines[18]. The RBL-2H3 mast cell line has been widely used as a convenient model system to study regulated secretion in mast cells. The high affinity receptor for IgE (FcεRⅠ) is abundant on the surface of these cells, and aggregation of IgE-FcεRⅠ complexes by multi-valent antigen generates a complex cascade of intracellular events leading to degranulation and consequent release of chemical mediators of allergic response including histamine, serotonin, and β-hexosaminidase[19,20,17].

The β-hexosaminidase assay has been widely used to monitor RBL-2H3 cell degranulation[21-23], and this assay is convenient method for the study of signal transduction mechanisms leading to exocytosis[24], as well as for monitoring the capacity of potential new drugs to block mast cell activation and degranulation[25,26]. TNF-α promotes inflammation, leukocyte infiltration, granuloma formation, and tissue fibrosis. And this cytokine is through to be an initiator of cytokine-related in inflammatory states by stimulation cytokine production in other types of cells[27,28]. IL-4 has been proposed as a molecular target for the control of allergic diseases. Indeed, interference with IL-4 expression has provided great possibilities in the development of therapeutics for allergic diseases[29,30]. The present study showed that *X. strumarium* pre-treatment profoundly affected PMA plus A23187-induced degranulation of

RBL-2H3 cells. Also we found that the *X. strumarium* significantly suppressed TNF-α and IL-4 secretions and both cytokines mRNA expression in activated RBL-2H3 cells. These results may suggest *X. strumarium* have anti-allergic action.

COX is involved in the inflammatory process and catalyzes the rate-limiting step in synthesis of prostaglandins from arachidonic acid. COX exists in two isoforms; COX-1 and COX-2[31]. COX-1 is expressed constitutive in most tissues and appears to be responsible for maintaining normal physiological functions. Whereas, COX-2 is detected in only certain types of tissues and is induced transiently by growth factors, pro-inflammatory cytokines, tumor promoters, and bacterial toxins[32,33,15]. In this study, the expression of COX-2 mRNA and protein was decreased by treatment of *X. strumarium*.

The activation of the NF-κB transcription family plays an important role in inflammation through its ability to induce the tracscription of pro-inflammatory genes[34]. In unstimulated cells, NF-κB is sequestered in the cytoplasm by tightly bound inhibitors (IκB-α, IκB-β, IκB-ε). The inhibitors are phosphorylated and rapidly degraded, allowing NF-κB to translocate into the nucleus and activate target genes. IκB-α is phosphorylated on serine residues by the multicomponent IκB kinase (IKK) containing 2 catalytic subunits (IKKα and IKKβ) and one regulatory subunit (IKKγ)[35-39]. *X. strumarium.* suppressed translocation into the nucleus of NF-κB and inhibited degradation of IκB-α.

In summary, the results of the present study evaluate that *X. strumarium* significantly reduces β-hexosaminidase release, pro-inflammatory cytokines production and mRNA expression by blocking NF-κB activation via suppressing the IκB degradation in PMA plus A23187-stimulated RBL-2H3 cells. Overall our results suggest that water extract of *X. strumarium* is a specific inbibitor of degranulation and NF-κB activation in mast cells, which is a potential drug for the treatment of allergic diseases.

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