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Original article

The anti-allergic and anti-inflammatory effects of Benjakul extract (a Thai traditional medicine), its constituent plants and its some pure constituents using *in vitro* experiments



Sunita Makchuchit^a, Ruchilak Rattarom^b, Arunporn Itharat^{c,d,*}

^a Graduate School, Faculty of Medicine, Thammasat University, Klongluang, Pathumthani 12120, Thailand

^b Faculty of Pharmacy, Mahasarakham University, Kantarawichai, Maha Sarakham 44150, Thailand

^c Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Klongluang, Pathumthani 12120, Thailand

^d Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Klongluang, Pathumthani 12120, Thailand

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ABSTRACT

Benjakul (BJK), a Thai traditional medicine preparation, has long been used for balanced health, controlled abnormal of element in the body, carminative, and relief of flatulence. It is composed of five plants: *Piper interruptum* Opiz., *Piper longum* L., *Piper sarmentosum* Roxb., *Plumbago indica* L., and *Zingiber officinale* Roscoe. The ethanolic extracts of BJK, its five individual plants, and pure constituents of BJK were investigated for their anti-allergic activity using immunoglobulin E (IgE)-sensitized β-hexosaminidase in the rat basophilic leukemia-2H3 (RBL-2H3) cells and anti-inflammatory activity using lipopolysaccharide (LPS)-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) in the murine macrophage (RAW 264.7) cells. The ethanolic extracts of BJK showed anti-allergic activity ($IC_{50} = 12.69 \mu\text{g/ml}$) and exhibited potent NO inhibitory effect ($IC_{50} = 16.60 \mu\text{g/ml}$), but inactive on TNF-α release. Moreover, 6-shogaol and plumbagin, two pure compounds from BJK, showed higher anti-allergic activity than the ethanolic BJK extract with IC_{50} values of 0.28 and $4.03 \mu\text{g/ml}$, respectively. These compounds were significantly higher than chlorpheniramine (CPM), standard drug, with IC_{50} value of $17.98 \mu\text{g/ml}$. Determination of the anti-inflammatory activity by measuring the inhibition of NO production presented that plumbagin and 6-shogaol exhibited higher than crude BJK extract with IC_{50} values of 0.002 and $0.92 \mu\text{g/ml}$, respectively. In particular, plumbagin also showed higher anti-inflammatory than prednisolone, positive control, with IC_{50} value of $0.59 \mu\text{g/ml}$. 6-Shogaol also showed inhibitory effect on TNF-α release ($IC_{50} = 9.16 \mu\text{g/ml}$). These preliminary results may provide some scientific support for the use of BJK for the anti-allergic treatment and inflammatory disorders through the inhibition of NO production.

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1. Introduction

Allergy is a hypersensitivity reaction initiated by immunological mechanisms which result in an exaggerated response on exposure to normally harmless environmental substances, such as animal dander, house dust mites, foods, pollens, insects, cockroaches, mold spores, cosmetics, and chemical agents [1,2]. The prevalence and severity of allergic diseases, such as allergic

rhinitis, allergic conjunctivitis, asthma, atopic eczema, anaphylaxis, food, drug, insect allergy, and urticarial and angioedema have extended dramatically during the past few decades around the world [3].

Mast cells and basophils play an important role in the development of allergic and inflammatory disorders. Mast cells and basophils have the high-affinity for immunoglobulin E (IgE) because of specific IgE receptors (FcεRI) on their surface membranes [4]. When exposed to antigens, B cells and other plasma cells produce and release antigen-specific immunoglobulin E (IgE) antibodies that bind to high-affinity IgE receptors (FcεRI) on the surface membranes of mast cells or basophils. This reaction sensitizes mast cells and basophils to degranulate and release of inflammatory mediators and allergy related cytokines, including

* Corresponding author at: Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Klongluang, Pathumthani 12120, Thailand.

E-mail addresses: msunita_359@hotmail.com (S. Makchuchit), rutiluk_99@yahoo.com (R. Rattarom), iarunporn@yahoo.com (A. Itharat).

histamine, β -hexosaminidase, leukotrienes, proteases that trigger to allergy response [5–7]. The enzyme β -hexosaminidase has been extensively used to monitor mast cell degranulation since it is released in parallel with histamine [8]. The rat basophilic leukemia cell line as well as RBL-2H3 is sharing various similar characteristics with mast cells and is used as a model system for screening of sample for anti-allergic ability. Therefore, β -hexosaminidase is commonly used as a biomarker of mast cell or basophil activation for antigen-induced degranulation in RBL-2H3 cell line [9].

Inflammation is a complex pathophysiological process mediated by a variety of signaling molecules produced by macrophages, leukocytes, mast cells, etc. Macrophages play a central role in host defense against foreign agents by releasing various pro-inflammatory cytokines and inflammatory mediators such as nitric oxide (NO), tumor necrosis factor- α (TNF- α) and cyclooxygenase (COX-2) [10]. *NO, inorganic free radical, is synthesized from the amino acid L-arginine by a family of nitric oxide synthases (NOS). In the NOS family, inducible nitric oxide synthase (iNOS) is a main source of NO produced by macrophage during inflammation. Overproduction of NO has been involved in many diseases such as asthma, rheumatoid arthritis, and atherosclerosis [11,12]. Griess assay has been used to determine the inhibition of nitric oxide formation in RAW 264.7 cells induced by lipopolysaccharide (LPS) and interferon- γ (INF- γ) [13].

Benjakul (BJK) is one of Thai traditional medicine preparation in the National List of Essential Medicines [14], consisting of equal amounts of five plants. There are stems of *Piper interruptum* Opiz., fruits of *Piper longum* L., roots of *Piper sarmentosum* Roxb., roots of *Plumbago indica* L., and rhizomes of *Zingiber officinale* Roscoe. BJK is commonly used for balancing health, controlling abnormality of elements in the body, and relieving flatulence. Recently, there has been studied on phytochemical and biological activities on the ethanolic extract of BJK. The ethanolic extract from BJK preparation has resulted in the isolation of many pure compounds such as myristicin, plumbagin, methyl piperate, 6-shogaol, 6-gingerol, and piperine [15–17]. Furthermore, BJK has been demonstrated on biological activities such as cytotoxic activity against many types of cancer cells [18,19], antimicrobial activity [20], antimalarial activity [21], genotoxic activity [22], and anti-inflammatory activity [23]. However, the effects of BJK and some pure constituents on anti-allergic activity via IgE-stimulated β -hexosaminidase release and anti-inflammatory activity via LPS-stimulated NO production on cell based assay have not been reported. Thus, the aims of the present study were to investigate the inhibitory effect on β -hexosaminidase release by RBL-2H3 cells, and the inhibitory effects on NO production and TNF- α release by RAW 264.7 cells of the ethanolic crude extract of BJK, its constituent plants, and pure compounds of BJK using cell based assay.

2. Materials and methods

2.1. Chemicals and reagents

Rat basophilic leukemia cell line (RBL-2H3) was purchased from American Type Culture Collection (ATCC® CRL-2256™, VA, USA). Murine macrophage cell line (RAW 264.7) was purchased from American Type Culture Collection (ATCC® TIB-71™, VA, USA). Anti-DNP IgE (Monoclonal Anti-DNP), anti-dinitrophenylated bovine serum albumin (DNP-BSA), *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (PNAG), albumin bovine fraction V power, D-(+)-glucose, chlorpheniramine, lipopolysaccharide (LPS), sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, phosphoric acid, thiazolyl blue tetrazolium bromide (MTT) and prednisolone were purchased from Sigma-Aldrich Inc. (MO,

USA). Calcium chloride dehydrate, citric acid monohydrate, magnesium chloride 6H₂O, potassium chloride, sodium carbonate and sodium bicarbonate were purchased from Merck (Darmstadt, Germany). Fetal bovine serum (FBS), minimum essential medium (MEM), penicillin-streptomycin (P/S), trypan blue, RPMI 1640 medium and trypsin-EDTA were purchased from Gibco BRL Life Technologies (NY, USA). Phosphate buffer saline (PBS) and piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES) were purchased from Amresco (OH, USA). Sodium chloride and sodium hydroxide were purchased from Univar (NSW, Australia). Commercial grade ethanol was purchased from Sasol Chemical Pacific LTD (Shenton, Singapore). Water was purified using a Milli-Q water purification system from Millipore (MA, USA). Analytical grade reagents (e.g. dimethyl sulfoxide, hydrochloric acid, isopropanol) were purchased from Labscan Limited (Bangkok, Thailand). Mouse TNF- α Quantikine ELISA Kit was purchased from R&D Systems Inc. (MN, USA). Piperine was purchased from Merck (Darmstadt, Germany). Plumbagin was purchased from Sigma-Aldrich (MO, USA). 6-Gingerol and 6-Shogaol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methyl piperate and Myristicin were isolated from BJK, as previously described [15].

2.2. Plant materials and preparation of the extracts

Piper interruptum Opiz. (stems), *Piper longum* L. (fruits), *Piper sarmentosum* Roxb. (roots), *Plumbago indica* L. (roots) and *Zingiber officinale* Roscoe. (rhizomes) were collected from Sakon Nakhon, Chanthaburi, Ratchaburi, Bangkok and Phetchabun provinces, Thailand, respectively. Authentication of plant materials were by comparison with specimens deposited at the herbarium of the Southern Center of Thai Medicinal Plants at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand.

Plant materials were washed with tap water to remove extraneous contaminants, sliced thinly, oven-dried at 50 °C (24 h), powdered with an electric-grinder and the powders stored in air-tight container at the room temperature until used. The powders in the same ratio of five plants were mixed to provide the BJK preparation. The ethanolic extracts of BJK preparation and each of five plants were obtained by macerating the powdered plant materials sequentially three times in 95% ethanol at room temperature for 3 days. Samples were filtered (Whatman no. 1), and the total solvent extract was then concentrated to dryness under reduced pressure (45 °C) using a rotary film evaporator. The ethanolic extracts were dried to constant weight and stored in air-tight glass container at –20 °C until used. The percentage yield (% w/w) for these are provided in Table 1 and chemical structures of pure compounds isolated from the ethanolic extract of Benjakul are shown in Fig. 1.

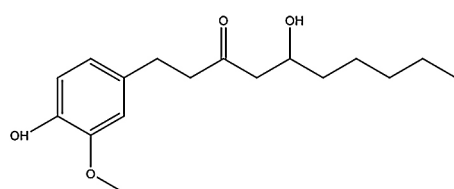
2.3. Determination of antigen-induced of β -hexosaminidase release from RBL-2H3 cells

The inhibition effect of the extract on the release of β -hexosaminidase from RBL-2H3 cells was evaluated by the following modified method [24]. The RBL-2H3 cells were cultured in MEM medium supplemented with 15% heat-inactivated FBS, 10,000 units/ml penicillin, and 10,000 μ g/ml streptomycin. The cells were maintained in an incubator at 37 °C in a humidified 5% CO₂/95% air atmosphere. The cells were seeded at a density of 2×10^5 cells/400 μ l/well in 24-well plates and allowed to adhere for 2 h at 37 °C in 5% CO₂. The cells were then sensitized with anti-dinitrophenyl-immunoglobulin E (anti-DNP IgE) (0.45 μ g/ml), and incubated at 37 °C in 5% CO₂ for 24 h. The cells were washed with 400 μ l of Siraganian buffer [buffer A; consisting of 119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM MgCl₂, 1 mM CaCl₂, 25 mM piperazine-*N,N*-bis(2-ethanesulfonic acid) (PIPES), 0.1% bovine

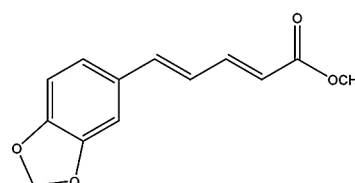
Table 1

Yields (%w/w) and voucher specimen number of Benjakul preparation, its constituents and pure compounds from the ethanolic Benjakul extract.

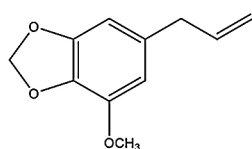
Plant species and pure compounds	Family name	Voucher specimen number	%Yield
<i>Piper interruptum</i> Opiz.	Piperaceae	SKP 146 16 09 01	2.47
<i>Piper longum</i> Linn.	Piperaceae	SKP 146 16 03 01	10.89
<i>Piper sarmentosum</i> Roxb.	Piperaceae	SKP 146 16 19 01	6.21
<i>Plumbago indica</i> Linn.	Plumbaginaceae	SKP 148 16 09 01	10.61
<i>Zingiber officinale</i> Roscoe.	Zingiberaceae	SKP 206 26 15 01	4.30
Benjakul preparation	–	–	11.10
6-Gingerol	–	–	0.04 ^a
Methyl piperate	–	–	0.10 ^a
Myristicin	–	–	0.01 ^a
Plumbagin	–	–	0.13 ^a
Piperine	–	–	1.12 ^a
6-Shogaol	–	–	0.04 ^a

^a Percent of yield was isolated from the ethanolic Benjakul extract [15].

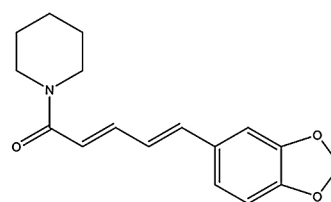
6-Gingerol



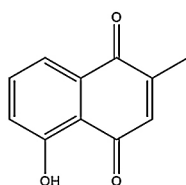
Methyl piperate



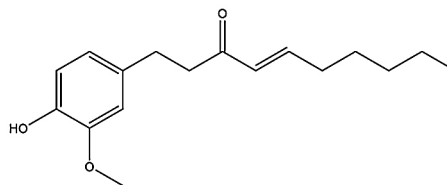
Myristicin



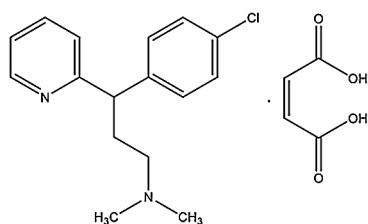
Piperine



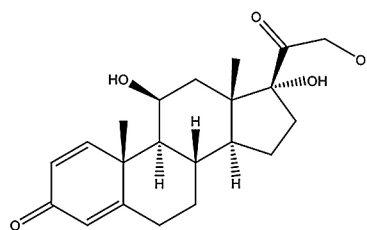
Plumbagin



6-Shogaol



Chlorpheniramine maleate



Prednisolone

Fig. 1. Chemical structures of pure compounds isolated from the ethanolic extract of Benjakul preparation and chemical structures of the standard drugs.

serum albumin (BSA) and 40 mM NaOH, pH 7.2], and then incubated in 160 μ l of Siraganian buffer (buffer A) for an additional 10 min at 37 °C in 5% CO₂. Various concentrations of test samples (20 μ l) were then added to each well and incubated for 10 min, followed by addition of 20 μ l of antigen (DNP-BAS, final concentration is 10 μ g/ml) for 20 min at 37 °C in 5% CO₂ to stimulate the cells into producing allergic reactions. Aliquots (50 μ l) of supernatant were transferred into 96-well plates and incubated with 50 μ l of substrate (PNAG) (1 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37 °C in 5% CO₂ for 2 h. The reaction was stopped by adding 200 μ l of a stop solution (0.1 M Na₂CO₃/NaHCO₃, pH 10.0). The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in dimethyl sulfoxide (DMSO), and the solution was added to Siraganian buffer (final DMSO concentration was 0.1%) (Table 2).

The inhibition (%) of release of β -hexosaminidase by the test samples was calculated by the following equation, and IC₅₀ values were calculated from the Prism program.

$$\text{Inhibition (\%)} = \left[1 - \frac{T - B - N}{C - B - N} \right] \times 100$$

where Control (C): DNP-BSA (+), test sample (–); Test (T): DNP-BSA (+), test sample (+); Blank (B): DNP-BSA (–), test sample (+); Normal (N): DNP-BSA (–), test sample (–).

2.4. Determination of LPS-induced NO production from RAW 264.7 cells

The inhibition effect of the extract on NO production from RAW 264.7 cells was evaluated by the following modified method [25]. The RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10,000 units/ml penicillin, and 10,000 μ g/ml streptomycin. The cells were maintained in an incubator at 37 °C in a humidified 5% CO₂/95% air atmosphere. The cells were seeded at a density of 1×10^5 cells/100 μ l/well in 96-well plates and allowed to adhere for 24 h at 37 °C in 5% CO₂. After that, the medium was replaced with fresh medium (100 μ l/well) containing 10 ng/ml of LPS and treated with various concentrations of test samples (100 μ l/well) for 24 h at 37 °C in 5% CO₂. After LPS stimulation for 24 h, NO production was determined by the Griess reagent. Aliquots (100 μ l) of supernatant were transferred into 96-well plates and mixed with the same volume of Griess reagent (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric

acid). The absorbance was measured with a microplate reader at 570 nm. After aliquots supernatant, cell viability was determined by the MTT assay. Briefly, 10 μ l of MTT solution (5 mg/ml) was added to each well, and 96-well plates were incubated for 2 h at 37 °C in 5% CO₂. Then, the medium was removed, and 100 μ l of isopropanol containing 0.04 M HCl was added to each well to dissolve the formazan dye in the cells. The absorbance was measured with a microplate reader at 570 nm. The test samples were considered to be cytotoxic when the optical density of the sample group was less than 70% of that in the control group. The test sample was dissolved in DMSO, and the solution was added to RPMI 1640 medium (final DMSO concentration was 0.2%) (Table 3).

The inhibition (%) of production of NO by the test samples was calculated by the following equation, and IC₅₀ values were calculated from the Prism program.

$$\text{Inhibition (\%)} = \left[\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right] \times 100$$

2.5. Determination of LPS-induced TNF-alpha release from RAW 264.7 cells

The inhibition effect of the extract on the release of TNF- α from RAW 264.7 cells was evaluated using mouse TNF-alpha quantikine ELISA kit according to the manufacturer's instructions. In brief, RAW 264.7 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10,000 units/ml penicillin, and 10,000 μ g/ml streptomycin. The cells were maintained in an incubator at 37 °C in a humidified 5% CO₂/95% air atmosphere. The cells were seeded at a density of 1×10^5 cells/100 μ l/well in 96-well plates and allowed to adhere for 24 h at 37 °C in 5% CO₂. After that, the medium was replaced with RPMI 1640 medium containing 10 ng/ml of LPS (100 μ l/well) and treated with various concentrations of test samples (100 μ l/well) for 24 h at 37 °C in 5% CO₂. After 24 h, the culture supernatant was transferred into 96-well ELISA plate and then TNF-alpha concentration was determined. The absorbance was measured with a microplate reader at 405 nm. The test sample was dissolved in DMSO, and the solution was added to RPMI 1640 medium (final DMSO concentration was 0.2%) (Table 4).

The inhibition (%) of release of TNF-alpha by the test samples was calculated by the following equation, and IC₅₀ values were calculated from the Prism program.

$$\text{Inhibition (\%)} = \left[\frac{\text{OD}_{\text{control}} - \text{OD}_{\text{sample}}}{\text{OD}_{\text{control}}} \right] \times 100$$

Table 2

Inhibition on β -hexosaminidase release of ethanolic extracts and pure compounds from Benjakul preparation from RBL-2H3 cells.

Crude extracts and pure compounds	Inhibition of β -hexosaminidase release (%) from RBL-2H3 cells at various concentrations (n = 3) (μ g/ml)						IC ₅₀ $\pm$ SEM, μ g/ml (μ M data for pure compound)
	0.01	0.1	1	10	50	100	
<i>P. interruptum</i>	–	–	–52.02 $\pm$ 26.66	25.99 $\pm$ 5.60	63.56 $\pm$ 4.76	93.85 $\pm$ 2.64	14.13 $\pm$ 0.92
<i>P. longum</i>	–	–	–40.76 $\pm$ 6.34	24.50 $\pm$ 5.78	65.11 $\pm$ 9.14	100.54 $\pm$ 3.69	15.82 $\pm$ 2.00
<i>P. sarmentosum</i>	–	–	–29.53 $\pm$ 6.61	18.78 $\pm$ 1.36	46.49 $\pm$ 1.77	81.05 $\pm$ 4.63	21.73 $\pm$ 2.14
<i>P. indica</i>	–	–	–55.37 $\pm$ 16.44	31.42 $\pm$ 5.21	85.25 $\pm$ 11.52	102.83 $\pm$ 1.91	13.31 $\pm$ 1.78
<i>Z. officinale</i>	–	–	–29.86 $\pm$ 18.01	35.19 $\pm$ 5.15	70.12 $\pm$ 3.23	84.14 $\pm$ 3.21	12.93 $\pm$ 1.28
Benjakul preparation	–	–	–23.42 $\pm$ 5.12	37.37 $\pm$ 4.92	68.16 $\pm$ 9.61	89.65 $\pm$ 4.40	12.69 $\pm$ 1.25
6-Gingerol	–	–	–27.87 $\pm$ 19.81	27.15 $\pm$ 3.42	71.89 $\pm$ 4.12	93.57 $\pm$ 5.58	18.30 $\pm$ 3.38 (62.16 μ M)
Methyl piperate	–	–	–4.85 $\pm$ 10.75	40.01 $\pm$ 4.75	77.87 $\pm$ 3.29	90.59 $\pm$ 0.81	12.44 $\pm$ 1.09 (53.57 μ M)
Myristicin	–	–	–45.83 $\pm$ 7.43	0.34 $\pm$ 0.91	31.82 $\pm$ 4.90	75.29 $\pm$ 2.38	80.80 $\pm$ 2.80 (420.37 μ M)
Plumbagin	–	–	15.65 $\pm$ 14.79	101.81 $\pm$ 0.97	102.58 $\pm$ 1.51	102.31 $\pm$ 0.79	4.03 $\pm$ 0.78 (21.42 μ M)
Piperine	–	–	–48.92 $\pm$ 23.94	30.52 $\pm$ 2.83	65.05 $\pm$ 9.22	80.52 $\pm$ 2.09	14.14 $\pm$ 1.46 (49.55 μ M)
6-Shogaol	–44.82 $\pm$ 16.45	9.92 $\pm$ 4.42	30.94 $\pm$ 5.99	68.23 $\pm$ 4.69	–	–	0.28 $\pm$ 0.11 (1.01 μ M)
Chlorpheniramine	–	–	–38.89 $\pm$ 8.01	17.82 $\pm$ 1.01	68.35 $\pm$ 1.50	91.33 $\pm$ 1.22	17.98 $\pm$ 0.78 (46.00 μ M)

NT (–) means not tested.

Table 3
Inhibition on LPS-induced NO production of ethanolic extracts and pure compounds from Benjakul preparation from RAW 264.7 cells.

Crude extracts and pure compounds	Inhibition of NO production (%) from RAW 264.7 cells at various concentrations (n=3) (μg/ml)												IC ₅₀ ± SEM (μg/ml)
	0.00001	0.0001	0.001	0.01	0.1	1	10	15	20	30	50	100	
<i>P. interruptum</i>	-	-	-	-	-6.00 ± 6.07	4.37 ± 3.04	27.86 ± 1.80	-	48.40 ± 3.46	62.65 ± 5.25	-	-	21.59 ± 1.69
<i>P. longum</i>	-	-	-	-	-4.30 ± 1.65	3.77 ± 1.33	27.81 ± 1.69	-	48.79 ± 5.94	63.67 ± 8.93	-	-	22.28 ± 2.79
<i>P. sarmentosum</i>	-	-	-	-	-	-8.99 ± 0.82	20.94 ± 3.72	-	-	-	84.19 ± 6.21	94.93 ± 1.18	22.42 ± 3.61
<i>P. indica</i>	-	-	-	-	-3.49 ± 4.33	0.82 ± 3.50	30.85 ± 15.47	-	78.87 ± 7.32	92.09 ± 1.31 ^a	-	-	14.06 ± 2.91
<i>Z. officinale</i>	-	-	-	-	-7.14 ± 1.18	-1.86 ± 1.30	37.58 ± 3.43	54.16 ± 1.61	-	-	-	-	13.44 ± 0.78
Benjakul	-	-	-	-	-4.20 ± 5.14	4.97 ± 3.45	28.83 ± 8.10	-	59.86 ± 4.74	76.93 ± 3.42	-	-	16.60 ± 1.85
6-Gingerol	-	-	-	-	-	-0.45 ± 1.27	9.45 ± 2.01	-	-	20.75 ± 4.32	34.56 ± 5.34	70.74 ± 3.94	72.25 ± 7.75 (245.42 μM)
Methyl piperate	-	-	-	-	-	-	0.88 ± 2.64	-	-	-	-	-	>10
Myristicin	-	-	-	-	-	-	-	-	-	-	-	35.89 ± 10.83	>100
Plumbagin	8.45 ± 2.94	25.65 ± 18.94	51.10 ± 12.69	77.08 ± 7.66	88.57 ± 3.20 ^a	-	-	-	-	-	-	-	0.002 ± 0.002 (0.01 μM)
Piperine	-	-	-	-	-	0.61 ± 0.98	18.48 ± 0.26	-	-	40.08 ± 0.71	64.66 ± 1.38	89.94 ± 4.38 ^a	38.89 ± 0.79 (136.29 μM)
6-Shogaol	-	-	-12.98 ± 3.79	-8.01 ± 2.25	-3.97 ± 1.44	5.09 ± 0.43	81.95 ± 9.02	-	-	-	-	-	0.92 ± 0.31 (3.33 μM)
Prednisolone	-	-	-	-	39.71 ± 5.79	62.78 ± 8.89	70.42 ± 9.10	-	-	75.95 ± 8.32	81.47 ± 6.19	-	0.59 ± 0.32 (1.64 μM)

^a Cytotoxic effect was observed; NT (-) means not tested.

2.6. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) of three independent experiments; for each experiment samples at each concentration were tested in triplicate. The IC₅₀ values were calculated using the Prism program. Data were analyzed by using one-way ANOVA and Dunnett's multiple comparison tests. The level of statistical significance was taken at $p < 0.05$.

3. Results

3.1. Determination of antigen-induced of β-hexosaminidase release from RBL-2H3 cells

The inhibitory effects of the ethanolic extracts of BJK, five of its individual plant constituents, and pure compounds from BJK on antigen-induced degranulation in IgE-sensitized RBL-2H3 cells were measured with β-hexosaminidase as degranulation bio-marker and compared to the standard anti-allergy drug as chlorpheniramine. As the results shown in Fig. 2, the ethanolic crude extract from BJK preparation possessed the most potent anti-allergic activity, followed by *Z. officinale*, *P. indica*, *P. interruptum*, *P. longum*, and *P. sarmentosum* (IC₅₀ values of 12.69 ± 1.25, 12.93 ± 1.28, 13.31 ± 1.78, 14.13 ± 0.92, 15.82 ± 2.00, and 21.73 ± 2.14 μg/ml, respectively). Additionally, 6-shogaol, one of pure compounds isolated from BJK preparation, exhibited the highest anti-allergic activity, with IC₅₀ value of 0.28 ± 0.11 μg/ml (1.01 μM). The other compounds were plumbagin, methyl piperate, piperine, and 6-gingerol also showed strong anti-allergic activity, with IC₅₀ values of 4.03 ± 0.78, 12.44 ± 1.09, 14.14 ± 1.46, and 18.30 ± 3.38 μg/ml (or 21.42, 53.57, 49.55, and 62.16 μM), respectively. Whereas, myristicin possessed mild inhibitory activity (IC₅₀ = 80.80 ± 2.80 μg/ml, 420.37 μM). Compared to chlorpheniramine (positive control drug with IC₅₀ value of 17.98 ± 0.78 μg/ml, 46.00 μM), 6-shogaol and plumbagin had significantly decreased releasing of β-hexosaminidase from RBL-2H3 cells compared with positive control (CPM) ($p < 0.05$, Fig. 2). By contrast, treatment with myristicin significantly increased the β-hexosaminidase release from RBL-2H3 cells ($p < 0.05$, Fig. 2).

3.2. Determination of LPS-induced NO production from RAW 264.7 cells

The ethanolic extracts of BJK, five of its individual plant constituents, and pure compounds from BJK were tested for anti-inflammatory activity against LPS-induced NO production and compared to the standard anti-inflammatory drug as prednisolone. As the results shown in Fig. 3, plumbagin was isolated from BJK preparation exhibited the most potent anti-inflammatory activity with IC₅₀ value of 0.002 ± 0.002 μg/ml (0.01 μM) and higher than prednisolone (positive control) (IC₅₀ = 0.59 ± 0.32 μg/ml, 1.64 μM). In addition, 6-shogaol also exhibited the potent anti-inflammatory activity (IC₅₀ = 0.92 ± 0.31 μg/ml, 3.33 μM). It was found that piperine was shown moderate activity with IC₅₀ value of 38.89 ± 0.79 μg/ml (136.29 μM), whereas the activity of 6-gingerol was shown mild activity on NO production (IC₅₀ = 72.25 ± 7.75 μg/ml, 245.42 μM). Moreover, the ethanolic crude extracts of *Z. officinale*, *P. indica*, BJK preparation, *P. interruptum*, *P. longum*, and *P. sarmentosum* possessed the high potent anti-inflammatory activity, with IC₅₀ values of 13.44 ± 0.78, 14.06 ± 2.91, 16.60 ± 1.85, 21.59 ± 1.69, 22.28 ± 2.79, and 22.42 ± 3.61 μg/ml, respectively. Cytotoxicity also tested by MTT assay to ensure that inhibitory effect of NO production was not due to cell death (data not shown). For NO assay, ZIO, PLI, BJK, PII, PIL, PIS, piperine, and 6-gingerol had

Table 4Inhibition on LPS-induced TNF- α release of ethanolic extracts and pure compounds from Benjakul preparation from RAW 264.7 cells.

Crude extracts and pure compounds	Inhibition of TNF- α release (%) from RAW 264.7 cells at various concentrations ($n=3$) ($\mu\text{g/ml}$)									IC ₅₀ $\pm$ SEM ($\mu\text{g/ml}$)
	0.01	0.1	1	10	15	20	30	50	100	
<i>P. interruptum</i>	–	–	15.94 $\pm$ 1.39	34.68 $\pm$ 3.29	–	46.98 $\pm$ 3.86	57.84 $\pm$ 1.55	–	–	21.84 $\pm$ 3.26
<i>P. longum</i>	–	–	–	–	–	–	47.01 $\pm$ 0.36	–	–	NA
<i>P. sarmentosum</i>	–	–	25.65 $\pm$ 1.99	37.22 $\pm$ 2.51	–	–	–	55.02 $\pm$ 2.04	81.90 $\pm$ 0.89	30.31 $\pm$ 8.07
<i>P. indica</i>	–	–	–	–	–	–0.31 $\pm$ 1.35	–	–	–	NA
<i>Z. officinale</i>	–	–	–	–	29.77 $\pm$ 1.04	–	–	–	–	NA
Benjakul	–	–	–	–	–	–	44.26 $\pm$ 2.12	–	–	NA
6-Gingerol	–	–	–30.95 $\pm$ 15.70	–9.35 $\pm$ 15.55	–	–	–	38.04 $\pm$ 2.14	72.98 $\pm$ 1.54	67.83 $\pm$ 2.45 (230.41 μM)
Methyl piperate	–	–	–	–	–	–	–	–	33.11 $\pm$ 1.29	>100
Myristicin	–	–	–22.08 $\pm$ 13.10	–6.20 $\pm$ 9.09	–	–	–	52.31 $\pm$ 1.30	75.54 $\pm$ 0.88	47.81 $\pm$ 1.11 (248.74 μM)
Plumbagin	35.31 $\pm$ 1.67	70.91 $\pm$ 0.98 ^a	–	–	–	–	–	–	–	NA
Piperine	–	–	–	–	–	–	–	21.77 $\pm$ 4.68	18.11 $\pm$ 0.30 ^a	NA
6-Shogaol	–39.40 $\pm$ 13.65	–29.67 $\pm$ 8.79	–14.43 $\pm$ 5.98	68.26 $\pm$ 1.09	–	–	–	–	–	9.01 $\pm$ 0.31 (32.60 μM)
Prednisolone	–	22.06 $\pm$ 5.86	28.05 $\pm$ 3.27	56.16 $\pm$ 3.78	–	–	–	80.86 $\pm$ 1.76	–	6.72 $\pm$ 2.51 (18.64 μM)

^a Cytotoxic effect was observed; (–) means not tested; NA means not active.

significantly increased levels of NO production compared with prednisolone, as positive control ($p < 0.05$, Fig. 3).

3.3. Determination of LPS-induced TNF- α release from RAW 264.7 cells

The ethanolic extracts of BJK, five of its individual plant constituents, and pure compounds from BJK were tested for anti-inflammatory activity against LPS-induced TNF- α release and compared to the standard anti-inflammatory drug as prednisolone. As the results shown in Fig. 4, It was found that 6-shogaol presented the most potent TNF- α release with IC₅₀ value of 9.16 $\pm$ 0.48 $\mu\text{g/ml}$ (33.14 μM) but lower than prednisolone (positive control) (IC₅₀ = 5.77 $\pm$ 4.03 $\mu\text{g/ml}$, 16.01 μM), followed by *P. interruptum*, *P. sarmentosum*, myristicin, and 6-gingerol, with IC₅₀ values of 19.81 $\pm$ 4.41, 34.33 $\pm$ 12.12, 47.52 $\pm$ 1.86 (247.23 μM), and

65.47 $\pm$ 1.13 (222.39 μM) $\mu\text{g/ml}$, respectively. The results demonstrated that PIS, myristicin, and 6-gingerol had significantly increased the level of TNF- α by the LPS-stimulated RAW 264.7 cells compared with prednisolone ($p < 0.05$, Fig. 4).

4. Discussion

Hypersensitivity allergic reaction type I is an IgE-mediated immune response. This class of antigens induces the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophils [1,2,4,26], and involve two phases, a biphasic early-phase, and a late-phase. The early phase of allergy occurs within minutes after allergen exposure and the mediators such as histamine and serotonin are released from the cell. The late phase of allergy occurs within 4–6 h, and involves secretion of cytokines,

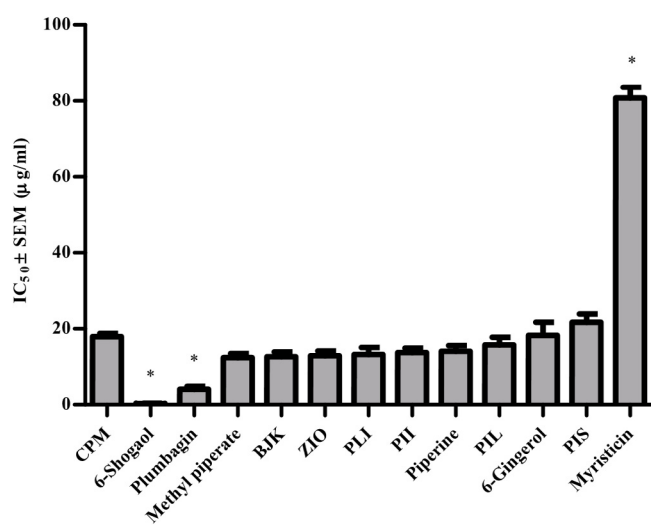


Fig. 2. Effect of ethanolic extracts of BJK, its plant constituents, and its pure compounds on β -hexosaminidase release in RBL-2H3 cells. Data were analyzed by using one-way ANOVA and Dunnett's multiple comparison tests. Results are presented as the IC₅₀ $\pm$ SEM ($\mu\text{g/ml}$) values ($n=3$). * Significant differences ($p < 0.05$) compared with CPM (positive control). BJK: Benjakul; CPM: chlorpheniramine; PII: *P. interruptum*; PIL: *P. longum*; PIS: *P. sarmentosum*; PLI: *P. indica*; ZIO: *Z. officinale*.

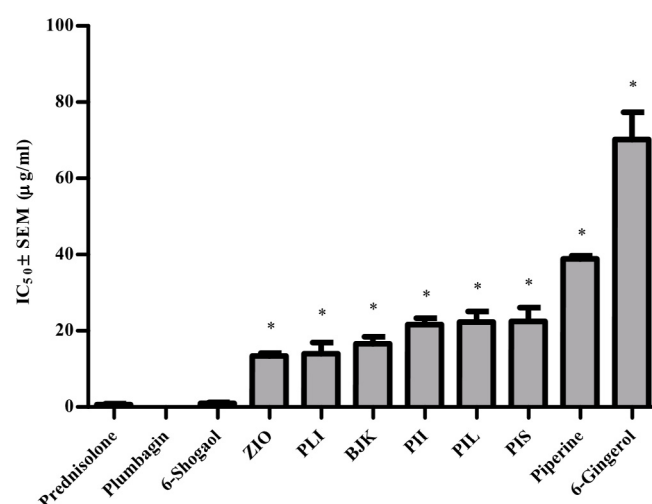


Fig. 3. Effect of ethanolic extracts of BJK, its plant constituents, and its pure compounds on NO production in LPS-stimulated RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's multiple comparison tests. Results are presented as the IC₅₀ $\pm$ SEM ($\mu\text{g/ml}$) values ($n=3$). * Significant differences ($p < 0.05$) compared with prednisolone (positive control). BJK: Benjakul; PII: *P. interruptum*; PIL: *P. longum*; PIS: *P. sarmentosum*; PLI: *P. indica*; ZIO: *Z. officinale*.

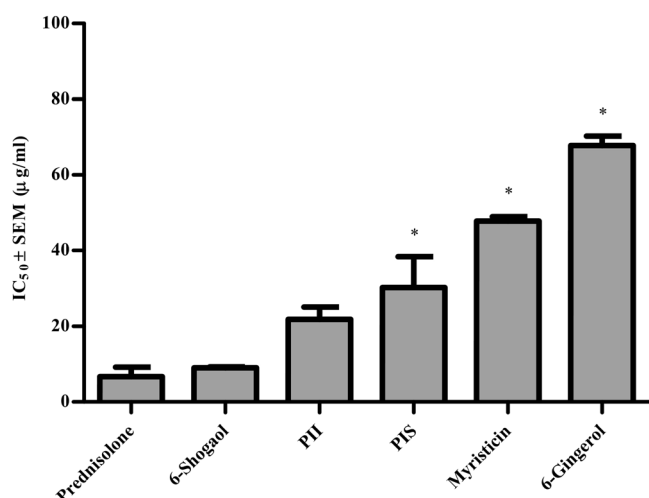


Fig. 4. Effect of ethanolic extracts of plant constituents from BJK, and its pure compounds on TNF- α activity in LPS-stimulated RAW 264.7 cells. Data were analyzed by using one-way ANOVA and Dunnett's multiple comparison tests. Results are presented as the $IC_{50} \pm SEM$ ($\mu g/ml$) values ($n=3$). * Significant differences ($p < 0.05$) compared with prednisolone (positive control). PII; *P. interruptum*; PIS; *P. sarmentosum*.

such as tumor necrosis factor- α (TNF- α) and interleukin-4 (IL-4) [4,26]. β -Hexosaminidase is stored in secretory granules of mast cells and basophils and is released in parallel with histamine and serotonin upon antigen crosslinking with specific high-affinity receptors, Fc ϵ RI. Therefore, the release of β -hexosaminidase is commonly used as an indicator to evaluate the degranulation in RBL-2H3 cells. This assay is a convenient tool for monitoring the capacity of potential drugs to block mast cell activation and degranulation [8,9]. BJK is a Thai Traditional preparation which used for adaptogen in Thailand and it is used before changing weather such as change from summer to be rainy season or rainy reason to be winter or winter to be summer. The weather changing will make human be sick especially allergy. Thus, BJK was proven about allergic assay and it exhibited the highest anti-allergic activity and it showed higher anti-allergic activity than each plant and chlorpheniramine as positive control. This result showed that it is effect form synergistic effect of five plants which are components in this preparation. In addition, five plant extracts as component of BJK also showed higher anti-allergic effect than chlorpheniramine except *Piper sarmentosum*. This discovery is the first report on anti-allergic effect of BJK extract. Some individual plant components of BJK preparation such as *P. interruptum*, *P. longum*, *P. sarmentosum*, and *P. indica* are also first report.

Z. officinale, only one plant in BJK preparation was reported on anti-allergic effect. Its ethanolic extract inhibited β -hexosaminidase release from RBL-2H3 cells ($IC_{50} = 40.3 \mu g/ml$) [27]. However, the ethanolic extract of *Z. officinale* showed stronger anti-allergic activity ($IC_{50} = 12.93 \mu g/ml$) than the previous study [27]. It depends on different seasons of plant collection, geographic collection of place and the method of preparation of the extracts. *In vivo* study, 2% ginger diet was also reported that it suppressed allergic effect on ovalbumin (OVA)-induced allergic rhinitis [28]. Moreover, the ethanolic extract (500 mg/kg) and aqueous extract (700 mg/kg) of ginger rhizomes inhibited Th2-mediated immune responses (IL-4 and IL-5 cytokines) by using OVA-induced allergic asthma in mouse model [29]. The aqueous extract of ginger (360 mg/kg) suppressed allergen-induced airway inflammation by inhibits Th2-mediated immune responses [30]. These all results can support that BJK can used to be anti-allergic drug instead of modern drug such as chlorpheniramine because it is possible that

BJK can be used as alternative drug for anti-allergy. However, some factors can affect the amount of active compound. So, the adequate of quality of herbal should be concerned. 6-Gingerol, 6-shogaol, and piperine, pure compounds isolated from BJK preparation, had ever been reported on anti-allergic ability but plumbagin, methyl piperate and myristicin were reported as the first time on anti-allergic activity. Both 6-gingerol and 6-shogaol, major constituents of ginger, showed the potential anti-allergic activity using the degranulation model of RBL-2H3 cells [31]. In another study, 6-gingerol has been inhibited both OVA-induced airway inflammation [30] and OVA-induced allergic rhinitis [28]. In addition, in previous study, piperine that isolated from *Fructus piperis* inhibited the expression of cytokines such as IL-4, IL-13, and TNF- α , and suppressed β -hexosaminidase and histamine release from RBL-2H3 cells [32]. However, these six compounds which were isolated from the ethanolic extract of BJK showed higher anti-allergic activity than chlorpheniramine except 6-gingerol and myristicin. Thus, these compounds have synergistic effect on BJK extract especially, piperine as a main compound and methyl piperate as derivative of piperine had high yield in the ethanolic BJK extract also showed higher anti-allergic activity than chlorpheniramine. Methyl piperate and plumbagin are the first report on high anti-allergic activity. These four compounds (6-shogaol, piperine, plumbagin, and methyl piperate) should be used to marker for analysis and quality control on the ethanolic extract.

Inflammation is a complex process in which immune cells and their soluble factors work together to generate protective responses against infections and cell damage. Further, the inflammatory process involves pro-inflammatory cytokines, chemokines, and inflammatory immune cells [33]. Activation of macrophage is related to release pro-inflammatory mediators and cytokines, such as nitric oxide (NO), prostaglandin E_2 (PGE $_2$), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), interleukine-1 beta (IL-1 β), and interleukin-6 (IL-6). Therefore, overexpression of these mediators and cytokines that can leads to tissue injury, cell death, and multiple organ failure [34] and relate to many diseases including asthma, cancer, osteoarthritis, rheumatoid arthritis, and diabetes [35].

In the present study, we have investigated the anti-inflammatory effects of BJK preparation, individual plant components of BJK preparation, and pure compounds of BJK preparation in LPS-induced NO and TNF- α . We firstly found that myristicin demonstrated the moderate potent anti-inflammatory activity on LPS-induced TNF- α . Moreover, 6-shogaol, plumbagin, piperine, and 6-gingerol which were isolated from the ethanolic BJK extract that compounds had ever been reported for in the anti-inflammatory activity [36–38]. In present study, we also found that 6-shogaol displayed strongly potent inhibitory activities of NO production and TNF- α release in LPS-activated macrophages. Interestingly, our finding showed the results as well as previous report that 6-shogaol exhibited the inhibition of NO production in LPS-stimulated RAW 264.7 cells [36,39]. In another report, 6-shogaol also showed inhibition of LPS-induced inflammation in cell based assay, such as inhibition of iNOS and COX-2 genes [40,41]. Therefore, 6-shogaol decreased the level of inflammatory cytokines and both COX-2 and iNOS expression in brain of MCAO-induced mice [42]. As another pure compound, plumbagin, a major pure compound of *Plumbago zeylanica*, was found to possess the most anti-inflammatory activity by inhibition of NO in RAW 264.7 cells with potency comparable to prednisolone. Recently, *in vitro* study, plumbagin showed the effect on TNF- α production [37,43], IL-1 β , IL-6 and iNOS [37] in RAW 264.7 cells. A recent study reported that piperine, a pure constituent of *P. longum*, possessed inhibition of TNF- α induced activation of NF- κ B by blocking I κ B α kinase activation in endothelial cells [38], inhibition of PMA-induced COX-2 expression by blocking the Akt and ERK MAP kinase

pathways in RAW 264.7 cells [44] and inhibition of LPS-induced endotoxin shock through inhibition of type 1 IFN production in C57BL/6 mice [45]. In addition, the inhibitory effect of 6-gingerol on anti-inflammatory activity has been reported both *in vitro* [36,39] and *in vivo* [46] studies. There were already intensively studies in many published paper about the suppressive effects of 6-shogaol and plumbagin on allergy and inflammation. In this study, we confirm the results of 6-shogaol and plumbagin showed strong inhibitory effects of anti-allergy and anti-inflammation. Moreover, 6-shogaol is presented in *Z. officinale* and plumbagin is presented in *P. indica*. Both compounds are presented in the BJK extract. It also explained that compounds made the BJK extract showed anti-allergic and anti-inflammatory effects. In addition, our results also showed that the concentration of BJK extract between 10 and 20 µg/ml displayed both anti-allergic effect and anti-inflammatory effect, which suggested that BJK extract has an inhibitory effect on IgE-stimulated β-hexosaminidase release associated with inhibiting inflammation via LPS-stimulated NO production because when allergen come into the body and then the body responds to the allergen, releasing antibodies and triggering inflammation.

Meanwhile, we found that the ethanolic BJK extract presented the inhibitory effect of NO production but not TNF-α activity. Likewise results on inhibitory effect of BJK have been reported *in vitro* study [23]. *In vivo* study, the ethanolic extract of BJK exhibited inhibitory effect on the acute phase of inflammation in ethyl phenylpropionate (EPP)-induced ear edema and carrageenan injection induced paw edema in rats. In the chronic inflammatory model, it was found that the ethanolic extract of BJK possessed a steroidal-like effect. Moreover, the ethanolic extract of BJK exhibited an analgesic effect on the formalin test in mice [47]. On the other hand, several studies have been reported that some plant composing BJK not only exhibited anti-inflammatory activity *in vitro* study, but also have been exhibited *in vivo* study. For instance, *in vitro* studies, the methanolic extract of *Z. officinale* showed strongly inhibitory effect of the release of IL-1β in human peripheral blood mononuclear cells (PBMCs) (IC₅₀ = 3.17 µg/ml) [48], COX-1 (IC₅₀ = 20.0 µM) and COX-2 (IC₅₀ = 7.5 µM) [49]. The ethanolic extract of both *P. longum* fruits and *P. indica* roots showed potent inhibitory effect on NO production with IC₅₀ values of 9.98 and 9.74 µg/ml, respectively [50].

In vivo studies, the water extract of *Z. officinale* also reduce the production of IFN-γ, IL-6, iNOS, COX-2 [51], and prostaglandin E₂ (PGE₂) [52]. Recent study has reported that the ethanolic extract of *P. interruptum* stems, *P. chaba* fruits [53], and *P. sarmentosum* roots [54] have exhibited the inhibitory effect on ethyl-phenylpropionate-induced ear edema in rat, reduced the paw edema after carrageenan injection, and decreased transudative weights using cotton pellet-induced granuloma formation in rat. Although the BJK extract and some pure compounds showed the anti-allergic and anti-inflammatory activities but the molecular mechanism did not evaluated. So, it might be considered as a limitation of our study.

5. Conclusion

The results of present study suggest that BJK contains anti-allergic and anti-inflammatory activities *in vitro* studies. Furthermore, these results provide some scientific support for the use of this preparation in Thai traditional medicine as an anti-allergic and anti-inflammatory drug preparation. Particularly, 6-shogaol and plumbagin are pure compounds with potent anti-allergy and anti-inflammatory properties. We recognize that there are need to further explore its mechanisms of action and pharmacological properties. In addition, these results could give the knowledge for further use of Thai traditional medicine in pharmaceutical area and

developed into commercial drug for treatment of allergic and inflammatory disorders.

Conflict of interests

The authors declare that they have no conflict of interests.

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