

Regular Article

Anti-allergic Inflammatory Components from the Leaves of *Piper crocatum* Ruiz & Pav.

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Piper crocatum Ruiz & Pav. (*P. crocatum*), a traditional medicinal plant, has been shown to possess various pharmacological activities, including anticancer activity, antioxidant activity, antibacterial activity, anti-hyperglycemic activity, anti-allergic inflammatory activity and others. To identify the potential anti-allergic inflammatory effective constituents of *P. crocatum*, 13 single compounds were isolated from the methanol extract of *P. crocatum* leaves, and their structures were identified by contrasting their NMR spectroscopic data and previously published papers. First, the anti-allergic inflammatory activities of these single compounds were examined by accessing immune function related biomarkers such as nitric oxide (NO) and β -hexosaminidase. We found that the methanol extract and catechaldehyde (compound 1) potently suppressed NO production. Additionally, Western blot analysis showed that *P. crocatum* methanol extract and compound 1 suppressed the production of NO by reducing inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. Consistent with these observations, *P. crocatum* methanol extract and compound 1 remarkably decreased β -hexosaminidase release from RBL-2H3 cells stimulated with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA)-specific immunoglobulin E (IgE) antibodies. Furthermore, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay indicated that *P. crocatum* methanol extract and compound 1 exhibited no cytotoxicity to RAW264.7 and RBL-2H3 cells. Based on these findings, compound 1 is suggested as an active anti-allergic inflammatory component of *P. crocatum*.

Key words allergic inflammation; *Piper crocatum* Ruiz & Pav.; catechaldehyde; nitric oxide; inducible nitric oxide synthase; β -hexosaminidase

INTRODUCTION

Piper crocatum Ruiz & Pav. (*P. crocatum*) is one of medicinal plants commonly found in Indonesian rainforest. For a long time, *P. crocatum* has served as a folk medicine to treat various diseases based on experience. Some pharmacological activities of *P. crocatum* have been reported, such as cytotoxic effect and antimigration activity on metastatic breast cancer,¹⁾ antibacterial activity,^{2,3)} anti-hyperglycemic activity⁴⁾ and others.

Previous studies have demonstrated the inhibitory effect of *P. crocatum* ethanol and butanol extracts on proinflammatory cytokines production, such as tumour necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), nitric oxide (NO), and interleukin 6 (IL-6).^{5,6)} In addition, *P. crocatum* methanol extract was examined on the anti-allergic inflammatory effect by carrageenan-induced rat oedema test.⁷⁾ These studies make it pretty clear that *P. crocatum* has a potent anti-allergic inflammatory effect. However, the active anti-allergic inflammatory constituents of *P. crocatum* remain to be investigated.

In the present study, 13 single compounds were isolated from the methanol extract of *P. crocatum* leaves. To screen the active anti-allergic inflammatory constituents, we performed NO and β -hexosaminidase assays.

MATERIALS AND METHODS

General Experimental Procedures The NMR spectra were recorded using a BRUKER AVANCE III 600 (¹H, 600 MHz; ¹³C, 150 MHz) (Bruker Biospin GmbH, Karlsruhe, Germany), with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), rotating frame nuclear Overhauser effect spectroscopy (ROESY), and ¹H-¹H correlation spectroscopy (COSY) spectra were recorded using a pulsed field gradient. The high resolution-electrospray ionization (HR-ESI)-MS spectra were obtained using an Agilent 1200 LC-MSD Trap spectrometer (Agilent, Santa Clara, CA, U.S.A.). Preparative HPLC (prep-HPLC) was performed using a GILSON 321 pump, 151 UV/VIS detector (Gilson, VILLIERS-LE-BEL, France), and RStech HECTOR-M C₁₈ column (5-micron, 250 × 21.2 mm) (RS Tech Crop, Chungju, South Korea). Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), and C₁₈ resins. TLC was performed using a pre-coated silica gel 60F₂₅₄ and RP-18F_{254S} plates (both 0.25 mm, Merck), and the spots were detected with a solution of 10% H₂SO₄ under UV light.

Plant Material The plant samples were gathered from Cilendek Timur, Bogor, West Java, Indonesia, in August 2016 and taxonomically identified (Identification No. 1714/IPH.1.01/If.07/VIII/2016) as *Piper crocatum* Ruiz & Pav.

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(family Piperaceae) by Dr. Joeni Setijo Rahajoe at herbarium Laboratory, Research Center for Biology, Indoensian Institute of Sciences, Cibinong, West Java, Indonesia. A voucher specimen (BBRC-SMPLS-003) was deposited at the Herbarium of Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, West Java, Indonesia.

Extraction and Isolation The dried leaves of *P. crocatum* (2.6 kg) were refluxing extracted with MeOH (8 L) for 3 times. The total extraction (400.0 g) of MeOH was suspended in deionized water and partitioned with *n*-hexane, and water fraction. Then the water fraction was sequentially partitioned with EtOAc and *n*-BuOH, yielding EtOAc (1A, 16.1 g), *n*-BuOH (1B, 65.0 g) fractions. The EtOAc fraction was subjected to a silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (10:1:0, 9:1:0, 8:1:0, 6:1:0.1, 5:1:0.1, 4:1:0.1, 3:1:0.1, 2:1:0.1, MeOH 2.0 L for each step) to give 11 fractions (Frs. 1A-1-11). Fractions 5 and 6 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by medium-pressure liquid chromatography (MPLC) using a C₁₈ column to give 8 fractions (Frs. 2A-1-8). The fraction 2A-2 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **1** (20.0 mg). Fractions 7 and 8 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 8 fractions (Frs. 2B-1-8). Subfraction 2B-2 was separated using a silica gel column chromatography with a CHCl₃-MeOH (10:1) elution solvent to give compound **2** (23.0 mg). Subfraction 2B-8 was separated using a silica gel column chromatography with a CHCl₃-MeOH (20:1) elution solvent to give compound **8** (6.0 mg). Fraction 9 was isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give one fraction, and then it was isolated by prep-HPLC to give compound **3** (3.0 mg). The *n*-BuOH fraction was subjected to a silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (10:1:0, 9:1:0, 8:1:0, 6:1:0.1, 5:1:0.1, 4:1:0.1, 3:1:0.1, 2:1:0.1, MeOH 5.0 L for each step) to give 11 fractions (Fr. 2C-1-11). Fractions 2C-2 and -3 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 12 fractions (Frs. 1D-1-12). The fraction 1D-3 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **9** (3.0 mg). The fraction 1D-7 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **4** (4.9 mg). The fraction 1D-8 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **5** (3.6 mg). Fractions 2C-4 and -5 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 13 fractions (Frs. 1E-1-13). The fraction 1E-2 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **10** (15.0 mg). The fraction 1E-6 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **13** (30.4 mg). Fractions 2C-6 and -7 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 10 fractions (Frs. 1F-1-10). The fraction 1F-2 was separated by a Sephadex LH-20 column and eluted

by MeOH and its subfraction was isolated by prep-HPLC to give compounds **6** (3.0 mg), **11** (2.0 mg), and **7** (3.0 mg). The fraction 1F-5 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **12** (31.2 mg).

The TLC analysis of compounds **1-13** and extracts are shown in Supplementary Fig. 1.

Cell Culture and Reagents RBL-2H3 cells were obtained from Korea Cell Bank (Seoul, South Korean). RAW264.7 and IGEL b4 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). RAW264.7 and IGEL b4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. RBL-2H3 cells were maintained in minimum essential medium (MEM) containing 10% FBS and 1% penicillin-streptomycin. The cells were cultured in a humidified 37°C incubator with 5% CO₂. DMEM and FBS were obtained from Welgene (Gyeongsangbuk-do, South Korea) and MEM was purchased from Gibco (Scotland, U.K.). Quercetin and griess reagent were provided by Sigma-Aldrich (St. Louis, MO, U.S.A.). We purchased 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) from Invitrogen by Thermo Fisher Scientific (Eugene, OR, U.S.A.) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega (Madison, WI, U.S.A.), respectively.

Nitric Oxide Assay RAW264.7 cells were seeded (1×10^5 cells/well) in 48-well plates overnight. After treatment with drugs for 1 h, the cells were exposed to lipopolysaccharide (LPS) (500 ng/mL) for 24 h. The generation of NO in the culture supernatants was determined with griess reagent [1% sulfanilamide in 5% H₃PO₄, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride] for 30 min at room temperature. The absorbance was measured at 570 nm utilizing an enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, Winooski, VT, U.S.A.).

Western Blot Analysis To evaluate whether the decreased NO production was mediated by inducible nitric oxide synthase (iNOS) regulation, Western blotting was performed. RAW264.7 cells were seeded in 6-well plates (1×10^6 cells/well) in DMEM with 10% FBS at 37°C overnight. After treatment with *P. crocatum* methanol extract or compound **1** for 2 h, the cells were stimulated by LPS (200 ng/mL) for 18 h. Then the cells were lysed in an ice-cold cell culture lysis reagent (Promega) in the presence of the protease inhibitor cocktail (1:1000, Sigma-Aldrich). The protein concentration in the lysates was quantified with the Bradford reagent (Bio-Rad Laboratories, CA, U.S.A.). The denatured samples (20 µg) were size-separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS/T) at room temperature for 2 h, membranes were incubated with primary antibodies specific to iNOS (1:1000, Santa Cruz, CA, U.S.A.) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Santa Cruz, CA, U.S.A.) overnight at 4°C. Thereafter, membranes were washed three times with TBS/T and incubated with the secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by an ECL Western blot detection system (Advansta, Menlo Park,

CA, U.S.A.). The protein levels were quantitated with Image J software.

β -Hexosaminidase Assay RBL-2H3 cells were seeded in 48-well plates (3×10^5 cells/mL) in MEM with 10% FBS and immunoglobulin E (IgE) at 37°C overnight. After washing twice with siraganian buffer (pH 7.2, 119mM NaCl, 5mM KCl, 0.4mM MgCl₂·6H₂O, 25mM PIPES, 40mM NaOH), the cells were incubated in SBC buffer (siraganian buffer supplemented with 5.6mM glucose, 1mM CaCl₂, and 0.1% BSA). After treatment with methanol extract (30, 60 μ g/mL), compound **1** (10, 20 μ M) or the positive control quercetin (20 μ M) separately, the cells were stimulated with DNP-BSA (1 μ g/mL) (Thermo Fisher Scientific) for 30 min at 37°C. The cell supernatants (25 μ L) were collected and transferred to another 96-well plate. To determine the amount of released β -hexosaminidase, 25 μ L of the cell supernatants in the plate were incubated with 25 μ L of 1mM 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 0.1M sodium citrate (pH 4.5) at 37°C for 1h. The reactions were stopped with 200 μ L/well of carbonate buffer (0.1M Na₂CO₃ and 0.1M NaHCO₃, pH 10), and the absorbance was detected at 405 nm by using a microplate reader (BioTek).

MTS Assay RBL-2H3 and RAW264.7 cells were cultured in the 96-well plates (SPL life Sciences Co., Pocheon, Korea) (1×10^4 cells/well) overnight and then exposed to several concentrations of *P. crocatum* methanol extract or compound **1** for 24h. MTS solution was added to each well for another 1h following manufacturer's instructions. The absorbance was detected at 490nm with an ELISA microplate reader (BioTek).

Statistical Assay All data are expressed as mean \pm standard error of the mean (S.E.M.) of at least three independent experiments. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test or Student's *t*-test. $p < 0.05$ was considered statistically significant using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA,

U.S.A.).

RESULTS

Identification of the Single Compounds Isolated from Methanol Extract of *P. crocatum* Leaves Thirteen components were isolated from methanol extract of *P. crocatum* leaves, and their structures were identified as catechaldehyde (**1**),⁸⁾ gentisic acid (**2**),⁹⁾ benzyl β -D-glucoside (**3**),¹⁰⁾ methyl salicylate 2-*O*- β -D-glucopyranoside (**4**),¹¹⁾ β -phenylethyl β -D-glucoside (**5**),¹²⁾ cimidahurin (**6**),¹³⁾ hydroxytyrosol-1-glucopyranoside (**7**),¹⁴⁾ erigeside II (**8**),¹⁵⁾ syringin (**9**),¹⁶⁾ 3-[(1*E*)-3-hydroxy-1-propen-1-yl]-2,5-dimethoxyphenyl β -D-glucopyranoside (**10**),¹⁷⁾ 4-hydroxybenzoic acid β -D-glucosyl ester (**11**),¹⁸⁾ phenylmethyl 6-*O*- α -L-arabinofuranosyl- β -D-glucopyranoside (**12**),¹⁹⁾ and icariside D1 (**13**)²⁰⁾ by comparing their NMR spectroscopic data with previously published papers (Fig. 1).

Effect of Methanol Extract and Single Compounds of *P. crocatum* on NO Production in RAW264.7 Cells NO is generally regarded as one of the most important roles during the immune response.²¹⁾ Therefore, we examined NO production in LPS-induced RAW264.7 cells after the treatment of *P. crocatum* methanol extract to confirm its anti-allergic inflammatory activity. The results indicated that the methanol extract of *P. crocatum* potently suppressed NO production (Fig. 2A). Furthermore, to ensure the effective anti-allergic inflammatory constituents, 13 single compounds were isolated from methanol extract of *P. crocatum* leaves and their abilities to decay the NO production were detected. As appeared in Fig. 2B, only compound **1** significantly restrained NO production in LPS-induced RAW264.7 cells, and Bay11-7082 was used as a reference compound. Additionally, the inhibitory impact of compound **1** on NO production was in a dose-dependent manner (Fig. 2C). A large deal of NO is synthesized from

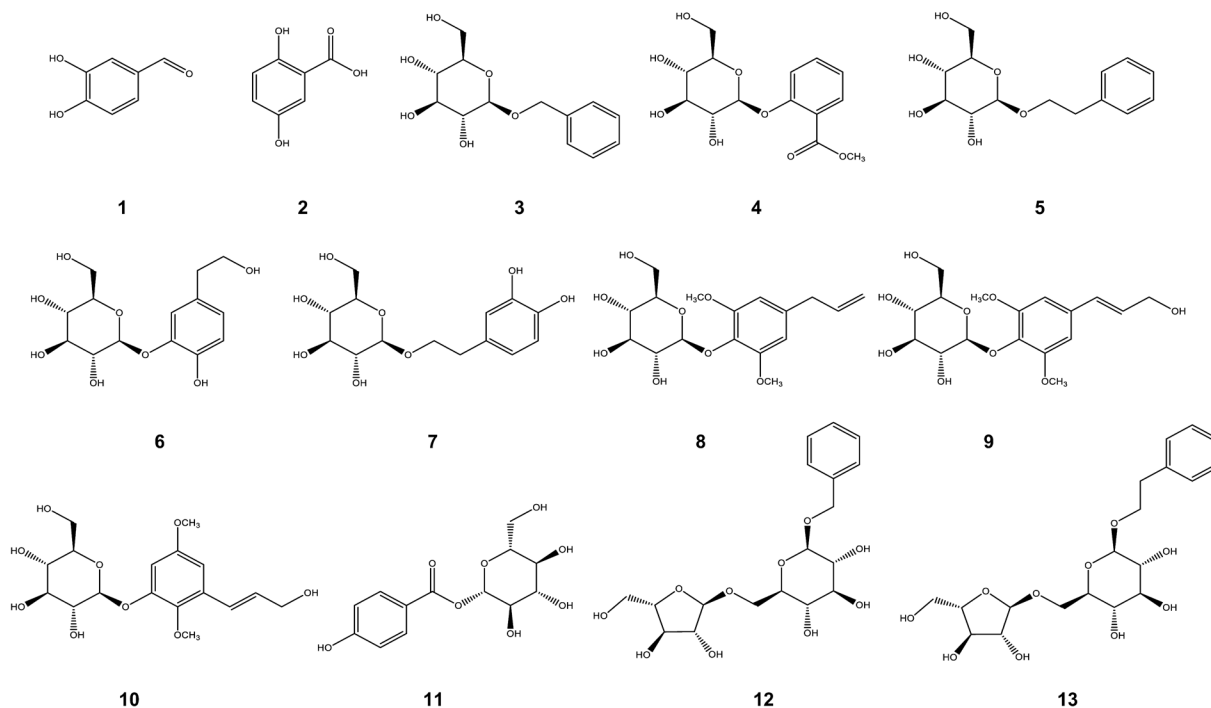


Fig. 1. Structures of Compounds **1**–**13** from the Leaves of *P. crocatum*

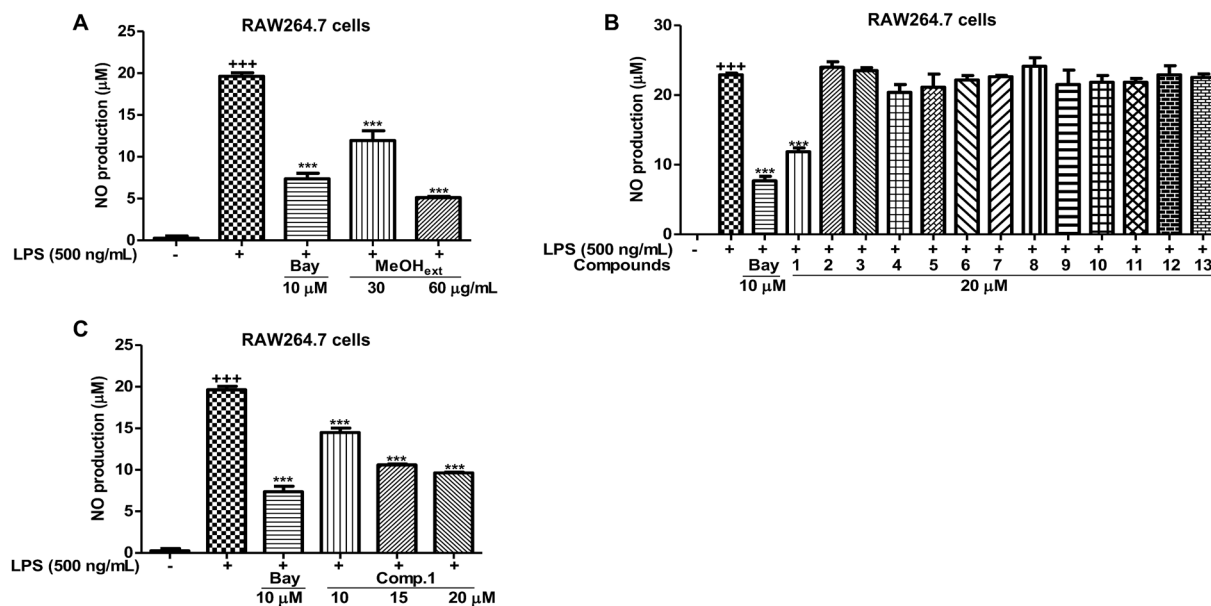


Fig. 2. Effect of Methanol Extract and Single Compounds of *P. crocatum* on NO Production in RAW264.7 Cells

The cells planted in 48-well plates overnight were exposed to LPS (500 ng/mL) for 24 h after treatment with (A) methanol extract (30, 60 µg/mL) of *P. crocatum*, (B) 13 single compounds (20 µM) isolated from *P. crocatum* methanol extract, (C) compound 1 (10, 15, 20 µM) or positive control drug Bay11-7082 (10 µM) for 1 h. The NO production in the culture supernatants was determined with griess reagent. Bay: Bay11-7082. Comp.1: compound 1. All experiments were performed at least three times. Results are expressed as mean ± S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test (***) $p < 0.001$ versus with LPS treated group; +++ means the significance compared with the mock cells).

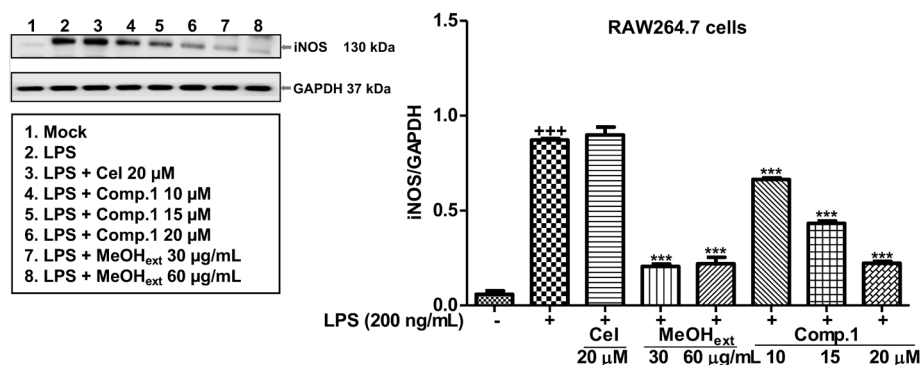


Fig. 3. Effect of Methanol Extract and Compound 1 of *P. crocatum* on iNOS Protein Expression in LPS-Induced RAW264.7 Cells

The cells were pretreated with *P. crocatum* methanol extract (30, 60 µg/mL), compound 1 (10, 15 and 20 µM) or celecoxib (20 µM) for 2 h, and then stimulated with LPS (200 ng/mL) for another 18 h. The iNOS protein expression was detected by Western blotting (left) and quantitated by Image J software (right). GAPDH was used as the protein loading control. Cel: celecoxib. Comp.1: compound 1. All experiments were performed at least three times. Results are expressed as mean ± S.E.M. The statistical differences were analyzed by Student's *t*-test (***) $p < 0.001$ versus with LPS treated group; +++ means the significance compared with the mock cells).

L-arginine by iNOS.²²⁾ Accordingly, we further investigated the expression level of iNOS by Western blot analysis. The results revealed that the methanol extract of *P. crocatum* and compound 1 significantly decreased iNOS expression (Fig. 3), which indicated that the downregulation of NO was caused by the decreased expression level of iNOS.

Effect of Methanol Extract and Compound 1 of *P. crocatum* on β -Hexosaminidase Release in RBL-2H3 Cells β -Hexosaminidase, which is secreted from mast cells and basophils stimulated by antigens or degranulation inducers, is frequently considered as an indicator of anaphylactic reactions.²³⁾ To investigate whether methanol extract of *P. crocatum* or compound 1 affects mast cell degranulation, we measured the amount of released β -hexosaminidase in the supernatants of RBL-2H3 cells stimulated by IgE-antigen complex. With the treatment of methanol extract or compound

1, the released β -hexosaminidase from RBL-2H3 cells was obviously reduced, and quercetin was used as a reference compound (Fig. 4).

Effect of Methanol Extract and Compound 1 of *P. crocatum* on Cell Viability To ensure that the decreased levels of NO and β -hexosaminidase in RAW264.7 and RBL-2H3 cells were not caused by cell death in each condition, the cell viability was measured by MTS assay.²⁴⁾ The results showed that methanol extract and compound 1 did not have any cytotoxicity to RAW264.7 cells and RBL-2H3 cells (Figs. 5A, B). In addition, an increased cell growth was observed in 30 µg/mL of *P. crocatum* methanol extract-treated RAW264.7 cells (Fig. 5A).

DISCUSSION

Currently, more and more researchers focus on screening active constituents from medicinal plants. One of the potential medicinal plants is *P. crocatum*. Several studies have reported that *P. crocatum* exerts its anti-allergic inflammatory effect by the inhibition of several proinflammatory cytokines. However, the effective constituents of *P. crocatum* remain to be determined.

In order to ascertain the active anti-allergic inflammatory constituents of *P. crocatum*, we isolated 13 single compounds from its methanol extract and identified their structures (Fig. 1). First, we confirmed that methanol extract of *P. crocatum* was effective in suppressing LPS-induced NO production (Fig. 2A), which was consistent with previously reported paper.⁵ NO is known as an important biomarker during immune response.²⁵ Inhibition of NO production is considered to be one of the mechanisms of drugs for the treatment of

allergic inflammatory diseases. Therefore, we screened these single compounds by measuring the NO amount in the supernatants of LPS-induced RAW264.7 macrophages. Results implied that LPS-induced NO production was only apparently decreased with the treatment of compound **1** (Fig. 2B). The other compounds did not show inhibitory effect on the assay system. There is some structure difference between compound **1** and others. It may be a reason why only compound **1** showed significant inhibitory effect on both NO production and β -hexosaminidase release. More research is needed later to reveal structure–activity relationship. NO is synthesized from L-arginine, which was catalyzed by nitric oxide synthase (NOS). Three types of NOS have been identified in mammals and among them, iNOS is mainly responsible for the generation of NO during host defense response.²⁶ Consequently, we measured the effect of *P. crocatum* methanol extract and compound **1** on iNOS expression by Western blotting. The result suggested that methanol extract and compound **1** inhibited NO production through the inhibition of iNOS (Fig. 3).

β -Hexosaminidase is known as a component of mast cell granules.²⁷ To further confirm the anti-allergic inflammatory effect of *P. crocatum* methanol extract and compound **1**, we measured the amount of β -hexosaminidase released from IgE-antigen complex-stimulated RBL-2H3 cells. Similarly, result showed that methanol extract and compound **1** suppressed β -hexosaminidase release (Fig. 4), which was not caused by cell cytotoxicity (Figs. 5A, B).

Upon these findings, we consider that compound **1** (catechaldehyde) is an active constituent of *P. crocatum* to exert anti-allergic inflammatory effect. However, it should be noted that this study has isolated and examined only 13 single compounds. There may exist other effective compounds in *P. crocatum*. More research should be carried on to reveal the anti-allergic inflammatory mechanism of compound **1**. Catechaldehyde (also known as protocatechuic aldehyde), a natural compound in some medicinal plants, possesses multiple biological activities such as neuroprotective effect,²⁸ cardio-protective effect,²⁹ antioxidant stress effect. Previous researchers have determined the protective effect of catechaldehyde on renal inflammation.³⁰ However, the anti-allergic inflammatory mechanisms of catechaldehyde remain to be elucidated. Catechaldehyde was extracted from *P. crocatum* for the first time

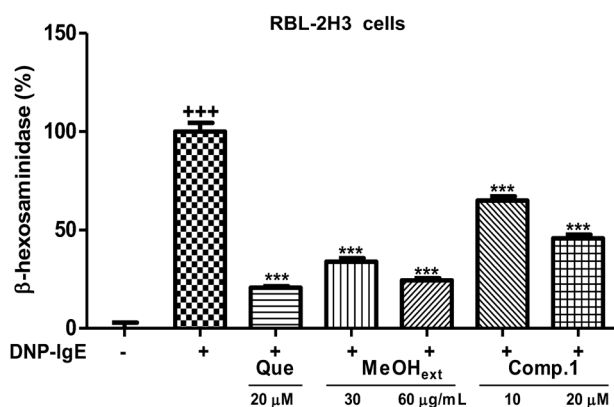


Fig. 4. Effect of Methanol Extract and Compound **1** of *P. crocatum* on β -Hexosaminidase Release in RBL-2H3 Cells Sensitized with DNP-IgE

RBL-2H3 cells were stimulated with DNP-IgE after treatment with methanol extract of *P. crocatum* (30, 60 μ g/mL), compound **1** (10, 20 μ M) or positive control quercetin (20 μ M) for 1 h. The amount of released β -hexosaminidase in cell supernatants was detected at 405 nm by using a microplate reader. Que: quercetin. Comp.1: compound **1**. All experiments were performed at least three times. Results are expressed as mean \pm S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test (***) $p < 0.001$ versus with DNP-IgE treated group; +++ means the significance compared with the mock cells.

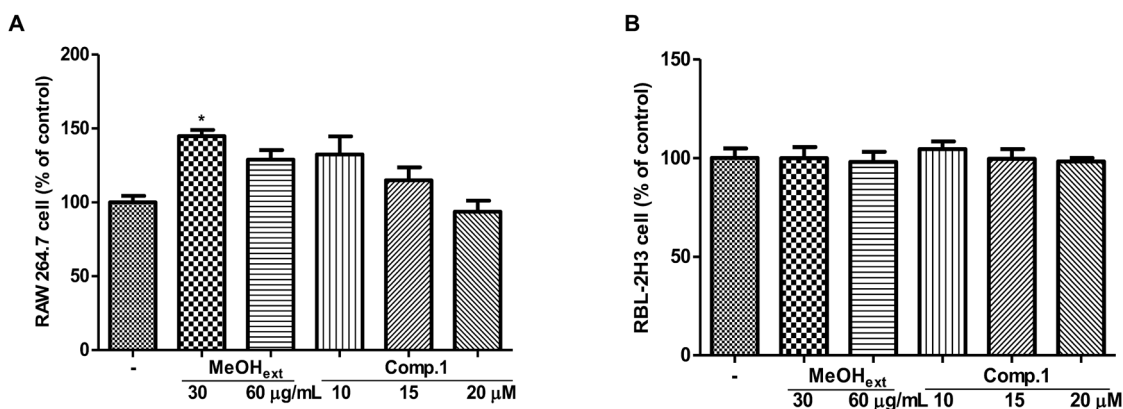


Fig. 5. Effect of Methanol Extract and Compound **1** of *P. crocatum* on Cell Viability

(A) RAW264.7 cells and (B) RBL-2H3 cells were seeded in 96-well plates overnight, and then were co-cultured with methanol extract of *P. crocatum* (30, 60 μ g/mL) or compound **1** (10, 15 and 20 μ M) for 24 h. The cell viability was performed by MTS assay. All experiments were performed at least three times. Results are expressed as mean \pm S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test (* $p < 0.05$ versus with mock cells).

and deemed to be an active anti-allergic inflammatory component of *P. crocatum*, which provided a valuable addition to the knowledge of the anti-allergic inflammatory mechanisms of *P. crocatum*.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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Anti-allergic Inflammatory Components from the Leaves of Piper crocatum Ruiz & Pav

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Regular Article

Anti-allergic Inflammatory Components from the Leaves of *Piper crocatum* Ruiz & Pav.Yue Gong,^{a,#} Hong Xu Li,^{b,#} Rui Hong Guo,^a Wahyu Widowati,^c Young Ho Kim,^b Seo Young Yang,^{*b} and Young Ran Kim^{*a}^a College of Pharmacy and Research Institute of Drug Development, Chonnam National University; Gwangju 61186, Republic of Korea; ^b College of Pharmacy, Chungnam National University; Daejeon 34134, Republic of Korea; and ^c Medical Research Centre, Faculty of Medicine, Maranatha Christian University; Bandung 40164, Indonesia.

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Piper crocatum Ruiz & Pav. (*P. crocatum*), a traditional medicinal plant, has been shown to possess various pharmacological activities, including anticancer activity, antioxidant activity, antibacterial activity, anti-hyperglycemic activity, anti-allergic inflammatory activity and others. To identify the potential anti-allergic inflammatory effective constituents of *P. crocatum*, 13 single compounds were isolated from the methanol extract of *P. crocatum* leaves, and their structures were identified by contrasting their NMR spectroscopic data and previously published papers. First, the anti-allergic inflammatory activities of these single compounds were examined by accessing immune function related biomarkers such as nitric oxide (NO) and β -hexosaminidase. We found that the methanol extract and catechaldehyde (compound 1) potently suppressed NO production. Additionally, Western blot analysis showed that *P. crocatum* methanol extract and compound 1 suppressed the production of NO by reducing inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-induced RAW264.7 macrophages. Consistent with these observations, *P. crocatum* methanol extract and compound 1 remarkably decreased β -hexosaminidase release from RBL-2H3 cells stimulated with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA)-specific immunoglobulin E (IgE) antibodies. Furthermore, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay indicated that *P. crocatum* methanol extract and compound 1 exhibited no cytotoxicity to RAW264.7 and RBL-2H3 cells. Based on these findings, compound 1 is suggested as an active anti-allergic inflammatory component of *P. crocatum*.

Key words allergic inflammation; *Piper crocatum* Ruiz & Pav.; catechaldehyde; nitric oxide; inducible nitric oxide synthase; β -hexosaminidase

INTRODUCTION

Piper crocatum Ruiz & Pav. (*P. crocatum*) is one of medicinal plants commonly found in Indonesian rainforest. For a long time, *P. crocatum* has served as a folk medicine to treat various diseases based on experience. Some pharmacological activities of *P. crocatum* have been reported, such as cytotoxic effect and antimigration activity on metastatic breast cancer,¹⁾ antibacterial activity,^{2,3)} anti-hyperglycemic activity⁴⁾ and others.

Previous studies have demonstrated the inhibitory effect of *P. crocatum* ethanol and butanol extracts on proinflammatory cytokines production, such as tumour necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β), nitric oxide (NO), and interleukin 6 (IL-6).^{5,6)} In addition, *P. crocatum* methanol extract was examined on the anti-allergic inflammatory effect by carrageenan-induced rat oedema test.⁷⁾ These studies make it pretty clear that *P. crocatum* has a potent anti-allergic inflammatory effect. However, the active anti-allergic inflammatory constituents of *P. crocatum* remain to be investigated.

In the present study, 13 single compounds were isolated from the methanol extract of *P. crocatum* leaves. To screen the active anti-allergic inflammatory constituents, we performed NO and β -hexosaminidase assays.

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MATERIALS AND METHODS

General Experimental Procedures The NMR spectra were recorded using a BRUKER AVANCE III 600 (¹H, 600MHz; ¹³C, 150MHz) (Bruker Biospin GmbH, Karlsruhe, Germany), with tetramethylsilane (TMS) as an internal standard. Heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond correlation (HMBC), rotating frame nuclear Overhauser effect spectroscopy (ROESY), and ¹H-¹H correlation spectroscopy (COSY) spectra were recorded using a pulsed field gradient. The high resolution-electrospray ionization (HR-ESI)-MS spectra were obtained using an Agilent 1200 LC-MSD Trap spectrometer (Agilent, Santa Clara, CA, U.S.A.). Preparative HPLC (prep-HPLC) was performed using a GILSON 321 pump, 151 UV/VIS detector (Gilson, VILLIERS-LE-BEL, France), and RStech HECTOR-M C₁₈ column (5-micron, 250 × 21.2 mm) (RS Tech Crop, Chungju, South Korea). Column chromatography was performed using a silica gel (Kieselgel 60, 70–230, and 230–400 mesh, Merck, Darmstadt, Germany), and C₁₈ resins. TLC was performed using a pre-coated silica gel 60F₂₅₄ and RP-18F_{254S} plates (both 0.25 mm, Merck), and the spots were detected with a solution of 10% H₂SO₄ under UV light.

Plant Material The plant samples were gathered from Cilendek Timur, Bogor, West Java, Indonesia, in August 2016 and taxonomically identified (Identification No. 1714/IPH.1.01/If.07/VIII/2016) as *Piper crocatum* Ruiz & Pav.

(family Piperaceae) by Dr. Joeni Setijo Rahajoe at herbarium Laboratory, Research Center for Biology, Indonesian Institute of Sciences, Cibinong, West Java, Indonesia. A voucher specimen (BBRC-SMPLS-003) was deposited at the Herbarium of Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, West Java, Indonesia.

Extraction and Isolation The dried leaves of *P. crocatum* (2.6 kg) were refluxed with MeOH (8 L) for 3 times. The total extraction (400.0 g) of MeOH was suspended in deionized water and partitioned with *n*-hexane, and water fraction. Then the water fraction was sequentially partitioned with EtOAc and *n*-BuOH, yielding EtOAc (1A, 16.1 g), *n*-BuOH (1B, 65.0 g) fractions. The EtOAc fraction was subjected to a silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (10:1:0, 9:1:0, 8:1:0, 6:1:0.1, 5:1:0.1, 4:1:0.1, 3:1:0.1, 2:1:0.1, MeOH 2.0 L for each step) to give 11 fractions (Frs. 1A-1-11). Fractions 5 and 6 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by medium-pressure liquid chromatography (MPLC) using a C₁₈ column to give 8 fractions (Frs. 2A-1-8). The fraction 2A-2 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **1** (20.0 mg). Fractions 7 and 8 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 8 fractions (Frs. 2B-1-8). Subfraction 2B-2 was separated using a silica gel column chromatography with a CHCl₃-MeOH (10:1) elution solvent to give compound **2** (23.0 mg). Subfraction 2B-8 was separated using a silica gel column chromatography with a CHCl₃-MeOH (20:1) elution solvent to give compound **8** (6.0 mg). Fraction 9 was isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give one fraction, and then it was isolated by prep-HPLC to give compound **3** (3.0 mg). The *n*-BuOH fraction was subjected to a silica gel column chromatography with a gradient of CHCl₃-MeOH-H₂O (10:1:0, 9:1:0, 8:1:0, 6:1:0.1, 5:1:0.1, 4:1:0.1, 3:1:0.1, 2:1:0.1, MeOH 5.0 L for each step) to give 11 fractions (Fr. 2C-1-11). Fractions 2C-2 and -3 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 12 fractions (Frs. 1D-1-12). The fraction 1D-3 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **9** (3.0 mg). The fraction 1D-7 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **4** (4.9 mg). The fraction 1D-8 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **5** (3.6 mg). Fractions 2C-4 and -5 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 13 fractions (Frs. 1E-1-13). The fraction 1E-2 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **10** (15.0 mg). The fraction 1E-6 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **13** (30.4 mg). Fractions 2C-6 and -7 were combined and isolated with a gradient of MeOH-H₂O (1:2, 1:1 and 100% MeOH) by MPLC using a C₁₈ column to give 10 fractions (Frs. 1F-1-10). The fraction 1F-2 was separated by a Sephadex LH-20 column and eluted

by MeOH and its subfraction was isolated by prep-HPLC to give compounds **6** (3.0 mg), **11** (2.0 mg), and **7** (3.0 mg). The fraction 1F-5 was separated by a Sephadex LH-20 column and eluted by MeOH and its subfraction was isolated by prep-HPLC to give compound **12** (31.2 mg).

The TLC analysis of compounds **1-13** and extracts are shown in Supplementary Fig. 1.

Cell Culture and Reagents RBL-2H3 cells were obtained from Korea Cell Bank (Seoul, South Korean). RAW264.7 and IGEL b4 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). RAW264.7 and IGEL b4 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. RBL-2H3 cells were maintained in minimum essential medium (MEM) containing 10% FBS and 1% penicillin-streptomycin. The cells were cultured in a humidified 37 °C incubator with 5% CO₂. DMEM and FBS were obtained from Welgene (Gyeongsang-si, Gyeongsangbuk-do, South Korea) and MEM was purchased from Gibco (Scotland, U.K.). Quercetin and griess reagent were provided by Sigma-Aldrich (St. Louis, MO, U.S.A.). We purchased 2,4-dinitrophenylated bovine serum albumin (DNP-BSA) from Invitrogen by Thermo Fisher Scientific (Eugene, OR, U.S.A.) and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) from Promega (Madison, WI, U.S.A.), respectively.

Nitric Oxide Assay RAW264.7 cells were seeded (1×10^5 cells/well) in 48-well plates overnight. After treatment with drugs for 1 h, the cells were exposed to lipopolysaccharide (LPS) (500 ng/mL) for 24 h. The generation of NO in the culture supernatants was determined with griess reagent [1% sulfanilamide in 5% H₃PO₄, 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride] for 30 min at room temperature. The absorbance was measured at 570 nm utilizing an enzyme-linked immunosorbent assay (ELISA) microplate reader (BioTek, Winooski, VT, U.S.A.).

Western Blot Analysis To evaluate whether the decreased NO production was mediated by inducible nitric oxide synthase (iNOS) regulation, Western blotting was performed. RAW264.7 cells were seeded in 6-well plates (1×10^6 cells/well) in DMEM with 10% FBS at 37 °C overnight. After treatment with *P. crocatum* methanol extract or compound **1** for 2 h, the cells were stimulated by LPS (200 ng/mL) for 18 h. Then the cells were lysed in an ice-cold cell culture lysis reagent (Promega) in the presence of the protease inhibitor cocktail (1:1000, Sigma-Aldrich). The protein concentration in the lysates was quantified with the Bradford reagent (Bio-Rad Laboratories, CA, U.S.A.). The denatured samples (20 µg) were size-separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, U.S.A.). After blocking with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS/T) at room temperature for 2 h, membranes were incubated with primary antibodies specific to iNOS (1:1000, Santa Cruz, CA, U.S.A.) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Santa Cruz, CA, U.S.A.) overnight at 4 °C. Thereafter, membranes were washed three times with TBS/T and incubated with the secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized by an ECL Western blot detection system (Advansta, Menlo Park,

CA, U.S.A.). The protein levels were quantitated with Image J software.

β -Hexosaminidase Assay RBL-2H3 cells were seeded in 48-well plates (3×10^5 cells/mL) in MEM with 10% FBS and immunoglobulin E (IgE) at 37 °C overnight. After washing twice with siragian buffer (pH 7.2, 119mM NaCl, 5mM KCl, 0.4mM MgCl₂·6H₂O, 25mM PIPES, 40mM NaOH), the cells were incubated in SBC buffer (siragian buffer supplemented with 5.6mM glucose, 1mM CaCl₂, and 0.1% BSA). After treatment with methanol extract (30, 60 μ g/mL), compound **1** (10, 20 μ M) or the positive control quercetin (20 μ M) separately, the cells were stimulated with DNP-BSA (1 μ g/mL) (Thermo Fisher Scientific) for 30 min at 37 °C. The cell supernatants (25 μ L) were collected and transferred to another 96-well plate. To determine the amount of released β -hexosaminidase, 25 μ L of the cell supernatants in the plate were incubated with 25 μ L of 1mM 4-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich) in 0.1M sodium citrate (pH 4.5) at 37 °C for 1h. The reactions were stopped with 200 μ L/well of carbonate buffer (0.1M Na₂CO₃ and 0.1M NaHCO₃, pH 10), and the absorbance was detected at 405 nm by using a microplate reader (BioTek).

MTS Assay RBL-2H3 and RAW264.7 cells were cultured in the 96-well plates (SPL life Sciences Co., Pocheon, Korea) (1×10^4 cells/well) overnight and then exposed to several concentrations of *P. crocatum* methanol extract or compound **1** for 24h. MTS solution was added to each well for another 1h following manufacturer's instructions. The absorbance was detected at 490 nm with an ELISA microplate reader (BioTek).

Statistical Assay All data are expressed as mean \pm standard error of the mean (S.E.M.) of at least three independent experiments. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test or Student's *t*-test. $p < 0.05$ was considered statistically significant using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA,

U.S.A.).

RESULTS

Identification of the Single Compounds Isolated from Methanol Extract of *P. crocatum* Leaves Thirteen components were isolated from methanol extract of *P. crocatum* leaves, and their structures were identified as catechaldehyde (**1**),⁸⁾ gentisic acid (**2**),⁹⁾ benzyl β -D-glucoside (**3**),¹⁰⁾ methyl salicylate 2-*O*- β -D-glucopyranoside (**4**),¹¹⁾ β -phenylethyl β -D-glucoside (**5**),¹²⁾ cimidahurin (**6**),¹³⁾ hydroxytyrosol-1-glucopyranoside (**7**),¹⁴⁾ erigeside II (**8**),¹⁵⁾ syringin (**9**),¹⁶⁾ 3-[(1*E*)-3-hydroxy-1-propen-1-yl]-2,5-dimethoxyphenyl β -D-glucopyranoside (**10**),¹⁷⁾ 4-hydroxybenzoic acid β -D-glucosyl ester (**11**),¹⁸⁾ phenylmethyl 6-*O*- α -L-arabinofuranosyl- β -D-glucopyranoside (**12**),¹⁹⁾ and icariside DI (**13**)²⁰⁾ by comparing their NMR spectroscopic data with previously published papers (Fig. 1).

Effect of Methanol Extract and Single Compounds of *P. crocatum* on NO Production in RAW264.7 Cells NO is generally regarded as one of the most important roles during the immune response.²¹⁾ Therefore, we examined NO production in LPS-induced RAW264.7 cells after the treatment of *P. crocatum* methanol extract to confirm its anti-allergic inflammatory activity. The results indicated that the methanol extract of *P. crocatum* potently suppressed NO production (Fig. 2A). Furthermore, to ensure the effective anti-allergic inflammatory constituents, 13 single compounds were isolated from methanol extract of *P. crocatum* leaves and their abilities to decay the NO production were detected. As appeared in Fig. 2B, only compound **1** significantly restrained NO production in LPS-induced RAW264.7 cells, and Bay11-7082 was used as a reference compound. Additionally, the inhibitory impact of compound **1** on NO production was in a dose-dependent manner (Fig. 2C). A large deal of NO is synthesized from

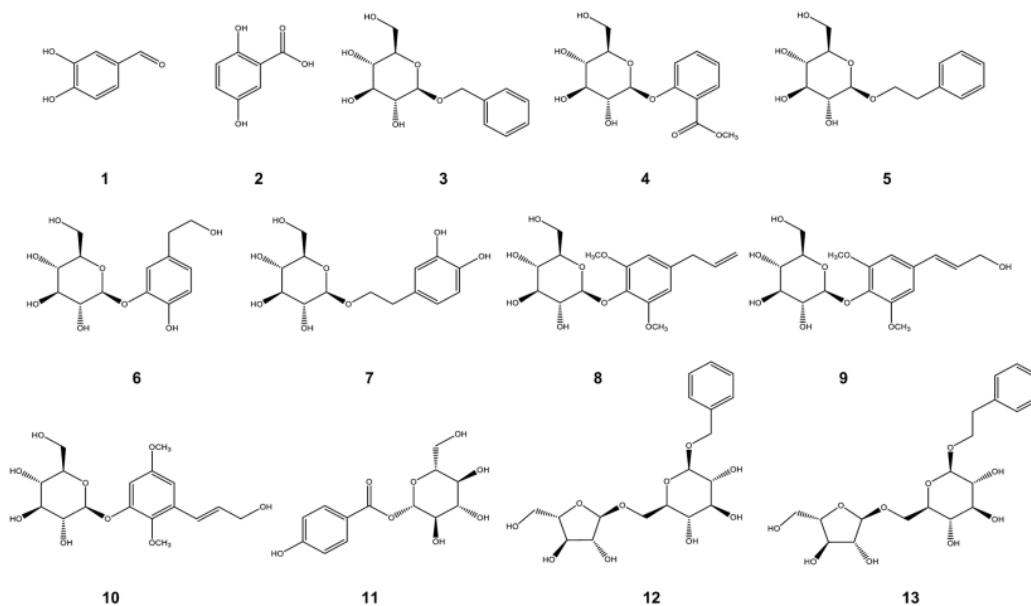


Fig. 1. Structures of Compounds 1–13 from the Leaves of *P. crocatum*

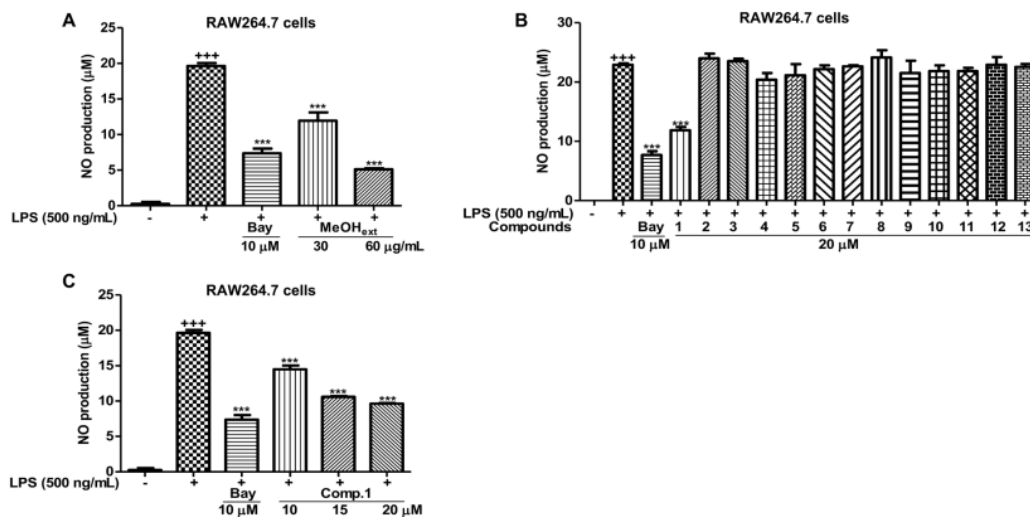


Fig. 2. Effect of Methanol Extract and Single Compounds of *P. crocatum* on NO Production in RAW264.7 Cells

The cells planted in 48-well plates overnight were exposed to LPS (500 ng/mL) for 24 h after treatment with (A) methanol extract (30, 60 µg/mL) of *P. crocatum*, (B) 13 single compounds (20 µM) isolated from *P. crocatum* methanol extract, (C) compound 1 (10, 15, 20 µM) or positive control drug Bay11-7082 (10 µM) for 1 h. The NO production in the culture supernatants was determined with griess reagent. Bay: Bay 11-7082. Comp.1: compound 1. All experiments were performed at least three times. Results are expressed as mean ± S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test (***) $p < 0.001$ versus with LPS treated group; +++ means the significance compared with the mock cells).

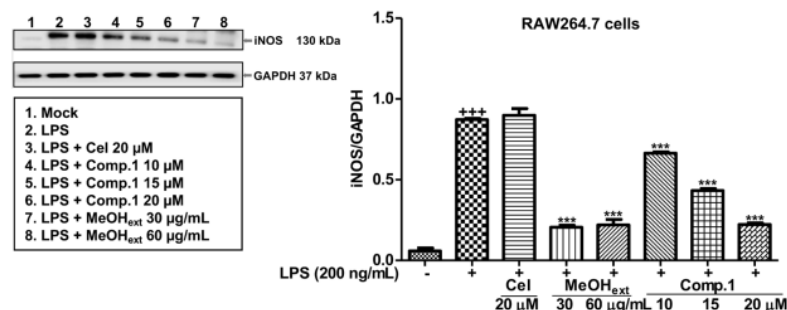


Fig. 3. Effect of Methanol Extract and Compound 1 of *P. crocatum* on iNOS Protein Expression in LPS-Induced RAW264.7 Cells

The cells were pretreated with *P. crocatum* methanol extract (30, 60 µg/mL), compound 1 (10, 15 and 20 µM) or celecoxib (20 µM) for 2 h, and then stimulated with LPS (200 ng/mL) for another 18 h. The iNOS protein expression was detected by Western blotting (left) and quantitated by Image J software (right). GAPDH was used as the protein loading control. Cel: celecoxib. Comp.1: compound 1. All experiments were performed at least three times. Results are expressed as mean ± S.E.M. The statistical differences were analyzed by Student's *t*-test (***) $p < 0.001$ versus with LPS treated group; +++ means the significance compared with the mock cells).

L-arginine by iNOS.²²⁾ Accordingly, we further investigated the expression level of iNOS by Western blot analysis. The results revealed that the methanol extract of *P. crocatum* and compound 1 significantly decreased iNOS expression (Fig. 3), which indicated that the downregulation of NO was caused by the decreased expression level of iNOS.

Effect of Methanol Extract and Compound 1 of *P. crocatum* on β -Hexosaminidase Release in RBL-2H3 Cells β -Hexosaminidase, which is secreted from mast cells and basophils stimulated by antigens or degranulation inducers, is frequently considered as an indicator of anaphylactic reactions.²³⁾ To investigate whether methanol extract of *P. crocatum* or compound 1 affects mast cell degranulation, we measured the amount of released β -hexosaminidase in the supernatants of RBL-2H3 cells stimulated by IgE-antigen complex. With the treatment of methanol extract or compound

1, the released β -hexosaminidase from RBL-2H3 cells was obviously reduced, and quercetin was used as a reference compound (Fig. 4).

Effect of Methanol Extract and Compound 1 of *P. crocatum* on Cell Viability To ensure that the decreased levels of NO and β -hexosaminidase in RAW264.7 and RBL-2H3 cells were not caused by cell death in each condition, the cell viability was measured by MTS assay.²⁴⁾ The results showed that methanol extract and compound 1 did not have any cytotoxicity to RAW264.7 cells and RBL-2H3 cells (Figs. 5A, B). In addition, an increased cell growth was observed in 30 µg/mL of *P. crocatum* methanol extract-treated RAW264.7 cells (Fig. 5A).

DISCUSSION

Currently, more and more researchers focus on screening active constituents from medicinal plants. One of the potential medicinal plants is *P. crocatum*. Several studies have reported that *P. crocatum* exerts its anti-allergic inflammatory effect by the inhibition of several proinflammatory cytokines. However, the effective constituents of *P. crocatum* remain to be determined.

In order to ascertain the active anti-allergic inflammatory constituents of *P. crocatum*, we isolated 13 single compounds from its methanol extract and identified their structures (Fig. 1). First, we confirmed that methanol extract of *P. crocatum* was effective in suppressing LPS-induced NO production (Fig. 2A), which was consistent with previously reported paper.⁵⁾ NO is known as an important biomarker during immune response.²⁵⁾ Inhibition of NO production is considered to be one of the mechanisms of drugs for the treatment of

allergic inflammatory diseases. Therefore, we screened these single compounds by measuring the NO amount in the supernatants of LPS-induced RAW264.7 macrophages. Results implied that LPS-induced NO production was only apparently decreased with the treatment of compound **1** (Fig. 2B). The other compounds did not show inhibitory effect on the assay system. There is some structure difference between compound **1** and others. It may be a reason why only compound **1** showed significant inhibitory effect on both NO production and β -hexosaminidase release. More research is needed later to reveal structure–activity relationship. NO is synthesized from L-arginine, which was catalyzed by nitric oxide synthase (NOS). Three types of NOS have been identified in mammals and among them, iNOS is mainly responsible for the generation of NO during host defense response.²⁶⁾ Consequently, we measured the effect of *P. crocatum* methanol extract and compound **1** on iNOS expression by Western blotting. The result suggested that methanol extract and compound **1** inhibited NO production through the inhibition of iNOS (Fig. 3).

β -Hexosaminidase is known as a component of mast cell granules.²⁷⁾ To further confirm the anti-allergic inflammatory effect of *P. crocatum* methanol extract and compound **1**, we measured the amount of β -hexosaminidase released from IgE-antigen complex-stimulated RBL-2H3 cells. Similarly, result showed that methanol extract and compound **1** suppressed β -hexosaminidase release (Fig. 4), which was not caused by cell cytotoxicity (Figs. 5A, B).

Upon these findings, we consider that compound **1** (catechaldehyde) is an active constituent of *P. crocatum* to exert anti-allergic inflammatory effect. However, it should be noted that this study has isolated and examined only 13 single compounds. There may exist other effective compounds in *P. crocatum*. More research should be carried on to reveal the anti-allergic inflammatory mechanism of compound **1**. Catechaldehyde (also known as protocatechuic aldehyde), a natural compound in some medicinal plants, possesses multiple biological activities such as neuroprotective effect,²⁸⁾ cardioprotective effect,²⁹⁾ antioxidant stress effect. Previous researchers have determined the protective effect of catechaldehyde on renal inflammation.³⁰⁾ However, the anti-allergic inflammatory mechanisms of catechaldehyde remain to be elucidated. Catechaldehyde was extracted from *P. crocatum* for the first time

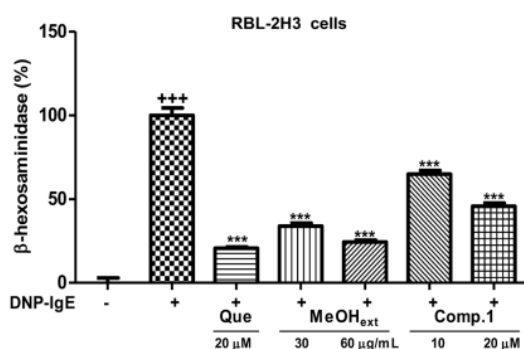


Fig. 4. Effect of Methanol Extract and Compound **1** of *P. crocatum* on β -Hexosaminidase Release in RBL-2H3 Cells Sensitized with DNP-IgE

RBL-2H3 cells were stimulated with DNP-IgE after treatment with methanol extract of *P. crocatum* (30, 60 μ g/mL), compound **1** (10, 20 μ M) or positive control quercetin (20 μ M) for 1 h. The amount of released β -hexosaminidase in cell supernatants was detected at 405 nm by using a microplate reader. Que: quercetin; Comp.1: compound **1**. All experiments were performed at least three times. Results are expressed as mean \pm S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test ($***p < 0.001$ versus with DNP-IgE treated group; +++ means the significance compared with the mock cells).

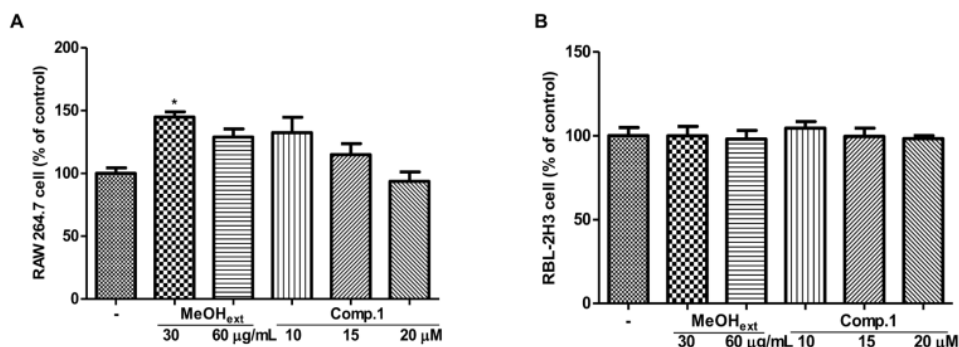


Fig. 5. Effect of Methanol Extract and Compound **1** of *P. crocatum* on Cell Viability

(A) RAW264.7 cells and (B) RBL-2H3 cells were seeded in 96-well plates overnight, and then were co-cultured with methanol extract of *P. crocatum* (30, 60 μ g/mL) or compound **1** (10, 15 and 20 μ M) for 24 h. The cell viability was performed by MTS assay. All experiments were performed at least three times. Results are expressed as mean \pm S.E.M. The statistical differences were analyzed by one-way ANOVA followed by the Tukey test ($*p < 0.05$ versus with mock cells).

and deemed to be an active anti-allergic inflammatory component of *P. crocatum*, which provided a valuable addition to the knowledge of the anti-allergic inflammatory mechanisms of *P. crocatum*.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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