Trop J Nat Prod Res, November 2024; 8(11): 9027 - 9034
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

In vitro Anti-inflammatory and Anti-allergenic Effects of Thai Herbal Formula and its Isolates, Rhinacanthin-C and Rhinacanthin-N in RBL-2H3 Cells and RAW264.7 Macrophages

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ARTICLE INFO

Article history:
Received 07 October 2024
Revised 08 October 2024
Accepted 19 October 2024
Published online 01 November 2024

ABSTRACT

Herbal remedies are an alternative treatment for allergic skin inflammation, reducing reliance on conventional chemical orthodox drugs which may have potential risks and side effects. This study evaluated the effects of a Thai herbal formulation extract (HFE) on interleukin 4 (IL-4) production in RBL-2H3 cells, as well as its inhibitory effect on nitric oxide, tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), in RAW264.7 cells. Phytochemical analysis and high-performance liquid chromatography (HPLC) were also performed to identify the active compounds in HFE, which was prepared by maceration in 95% ethanol. IL-4 inhibition in RBL-2H3 cells was measured using an enzyme-linked immunoassay (ELISA). Nitric oxide inhibition was assessed with the Griess reagent, while TNF-α and IL-6 levels were also measured by ELISA. Cytotoxicity was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. The HFE inhibited IL-4 production (IC_{50}=48.73±0.45 µg/mL), and nitric oxide production (IC_{50}=67.95±9.80 µg/mL), with slight inhibition of TNF-α and IL-6. The main active compounds were rhinacanthin-C (1.65±0.05% w/w) and rhinacanthin-N (0.20±0.01% w/w), which showed the highest effects on IL-4 secretion, nitric oxide, and inflammatory cytokines. The HFE and its active compounds were nontoxic to human cell lines, with over 70% cell viability, except for rhinacanthin-C at 100 µg/mL. Overall, the HFE effectively inhibited IL-4 secretion in RBL-2H3 cells, reduced nitric oxide release, and slightly decreased TNF-α and IL-6 levels. Rhinacanthin-C and rhinacanthin-N significantly regulated IL-4, nitric oxide, TNF-α, and IL-6 production. These findings suggest that the HFE could help alleviate allergies and acute skin inflammation.

***Keywords*:** Allergic inflammation, Inflammatory cytokine, Allergy, Thai herbal formulation, Rhinacanthin

Introduction

Allergic skin inflammation is usually triggered by an allergen-induced immune response that exacerbates the inflammatory response. This process is primarily mediated through immunoglobulin E (IgE)-dependent pathways.[1] Clinically, it presents as urticaria and atopic dermatitis. Multiple variants of the disease are known to exist, each resulting from a different underlying cellular and molecular mechanism. The two main characteristics of allergic inflammation are increased numbers of activated CD4+ T helper type 2 (Th2) lymphocytes, which organize the infiltration of inflammatory cells to areas of allergen exposure, and IgE-dependent mast cell activation.[2]

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Citation: Choedchutirakul N, Panthong S, Sakpakdeejaroen I, Thongdeeying P, Taingthum A, Bunpean A. *In vitro* Anti-inflammatory and Anti-allergenic Effects of Thai Herbal Formula and its Isolates, Rhinacanthin-C and Rhinacanthin-N in RBL-2H3 Cells and RAW264.7 Macrophages. Trop J Nat Prod Res. 2024; 8(11):9027 – 9034
https://doi.org/10.26538/tjnpr/v8i11.10

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

Mast cells play a crucial role in allergic inflammation by binding specific IgE to the FcεRI receptor on their surface, contributing to increased levels of IgE in the serum and the activation of cytokine receptors. Upon activation, mast cells release various biochemical mediators, including histamine, serotonin, and proinflammatory cytokines such as IL-4, IL-5, IL-6, IL-13, TNF-alpha, and their granules into the extracellular space. These mediators are involved in numerous pathological processes, including allergic inflammatory skin conditions such as allergic contact dermatitis, hypersensitivity reactions, atopic dermatitis, and urticaria.[3] IL-4, which is produced by mast cells, enhances the ability of dendritic cells to promote TH2 polarization of naive T cells in response to antigens. This process contributes to the production of proinflammatory cytokines. Moreover, IL-4 binds to the -IL-4 receptor alpha on cutaneous cells, exacerbating allergic skin inflammation.[4] When activated, macrophages produce inflammatory mediators such as nitric oxide, TNF-α, and IL-6, which contribute to various inflammatory reactions. The symptoms include skin rash, itchiness, redness, swelling, and lesions.[5] Allergic skin inflammation develops when the skin encounters an allergen, such as chemicals, gluten, wheat protein, or alcohol. Allergens sensitize the skin and trigger a classic immune response characterized by immunotoxicity, irritation, and inflammation. This immune response involves the recognition of antigens by dendritic cells in the skin, which subsequently activate T cells and macrophages.[6]

Previous prevalence studies conducted in both China and Western countries revealed that 35.6% of respondents, mostly women, reported experiencing allergies. Among these individuals, 68.6% reported experiencing allergies and inflammation of the skin, as well as discomforts such as itching and burning.[7] In a primary care setting in

Thailand, skin diseases and subcutaneous tissue inflammatory symptoms included dermatitis (29.7%), urticaria, and erythema (13.9%).[8] Allergy symptoms such as urticaria and atopic dermatitis, are treated with antihistamines, while steroids are used for severe cases. However, some patients on these medications have reported side effects, including striae, rosacea, perioral dermatitis, atrophy, acne, and purpura.[9]

Natural products are being researched for various therapeutic purposes. Phytochemical compounds such as flavonoids, alkaloids, polyphenols, and naphthoquinones are reported to exhibit antiallergic and anti-inflammatory effects.[10] Thai herbal formulations, a natural product in Thailand, consists of *Allium ascalonicum* L., *Acanthus ilicifolius* L., *Bambusa blumeana* Schult. F., and *Rhinacanthus nasutus* (L.) Kurz are regarded as therapeutic options for allergic skin inflammatory conditions such as urticaria.[11]

These plant components have been shown to effectively inhibit inflammation and allergies. Chemical compounds found in *acanthus ilicifolius*, including alkaloids and phenylethanoids, have been shown to possess anti-inflammatory activity by regulating nuclear factor kappa B (NF-κB) and nitric oxide synthase.[12] *Allium ascalonicum* contains flavones and polyphenolic derivatives, such as quercetin, which inhibit *β*-hexosaminidase and IL-6.[13] *Bambusa blumeana* contains alkaloids, flavonoids, phenolics, and terpenoids, though no evidence currently supports its biological activities. These compounds also possess antioxidant properties which may help alleviate symptoms of immune-mediated inflammatory skin conditions.[14] *Rhinacanthus nasutus*, which consists of naphthoquinones such as rhinacanthin, has demonstrated anti-inflammatory effects in in vivo tests.[15] However, the use of Thai herbal formulations for urticaria or dermatitis has not been scientifically investigated. Therefore, proper preclinical research on this herbal medicine is necessary. This study evaluated the effect of a Thai herbal formulation extract (HFE) on anti-allergy activity, focusing on IL-4 production and inflammatory mediators such as nitric oxide, IL-6, and TNF-*α*.

Materials and Methods

Chemicals and reagents

Fetal bovine serum (FBS) Cat.no. 26140079, penicillin–streptomycin (10,000 units/mL) Cat.no. 15140122, minimum essential medium (MEM) Cat.no. 61100061, Dulbecco's Modified Eagle Medium (DMEM) Cat.no. 12800017, and 0.4% trypan blue solution Cat.no. 15250061 were purchased from Gibco (USA). Bovine serum albumin bovine Cat.no. A7906, anti-dinitrophenyl bovine serum albumin (DNP-BSA), DNP-specific IgE (monoclonal anti-DNP) Cat.no. D8406, 4-nitrophenyl N-acetyl-*β*-D-glucosaminide (PNAG) Cat.no. N9376, lipopolysaccharide from *Escherichia coli* O55:B5 (LPS) Cat.no. L4005, sulfanilamide Cat.no. S9251, and N-(1-naphthyl) ethylenediamine dihydrochloride Cat.no. N9125 were purchased from Sigma-Aldrich (USA). Diphenhydramine Cat.no. D4744 and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cat.no. M3297 were purchased from TCI (Japan). The ELISA kits for TNF-*α* Cat.no. DY410 and IL-6 Cat.no. DY406 were purchased from R&D Systems (USA), and the ELISA kit for IL-4 Cat.no. RAB0301 was purchased from Sigma-Aldrich (USA).

Plant materials, collection, and identification

The constituent plants of the Thai herbal formulation were collected in March 2022 from several parts of Thailand. *A. ascalonicum* L. was collected from Sisaket Province, *A. ilicifolius* L. and *R. nasutus* (L.) Kurz were collected from Lopburi Province, and *B. blumeana* Schult. F. was collected from Chachoengsao Province. The voucher specimens were deposited at the Princess Sirindhorn Plant Herbarium Building at Kasetsart University, Bangkok, Thailand, and the Thai Traditional Medicine Herbarium, Bangkok, Thailand. A description of the plant ingredients is provided in Table 1.

Preparation of crude extract [16]

Each fresh plant in the Thai herbal formulation was washed, air-dried until it became damp, and then cut into small pieces. From a total of 100 g of each plant, the specified proportions were combined. The mixture was macerated in 95% ethanol for three days at room temperature, with occasional stirring to enhance extraction. Afterward, the solution was filtered through Whatman No. 1 filter paper. The filtered extract was concentrated using an evaporator, then dried in a hot air oven at 45°C. Finally, the dried ethanol extracts were stored at -20°C until used.

Isolation and identification of biochemical markers [17]

The crude ethanol extract of *R. nasutus* was dissolved in dichloromethane and fractionated using vacuum chromatography on silica gel 60, with hexane as the initial eluent. The elution was then continued with increasing polarity by adding ethyl acetate, resulting in five separate fractions. Fraction 4 was separated using isocratic elution with a 1:1 mixture of chloroform and methanol on a Sephadex LH-20 column to obtain subfractions. These subfractions were then subjected to silica gel column chromatography, using a gradient of hexane, chloroform, ethyl acetate, and methanol, to isolate pure compounds.. The isolated compounds were identified based on their chemical structures using nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS).

Quantification of active compounds using high-performance liquid chromatography (HPLC)[18]

A total of 10 µL of the HFE was injected into a guard column composed of the same material as the analytical column, which was a C18 reverse-phase column (Phenomenex Luna, 5-micron C18(2), 100-angstrom pore size, 250 × 4.60 mm, 5 µm). The mobile phases consisted of water containing acetonitrile (A) and 0.1% phosphoric acid (B). The gradient elution profile was as follows: 75:25 (A/B) for 35 minutes, followed by a transition from 75:25 to 71.4:28.6 (A/B) over 3 minutes, then from 71.4:28.6 to 95:5 (A/B) over 2 minutes, and finally from 95:5 to 75:25 (A/B) over 3 minutes, with a 3-minute hold between each transition. The analysis was performed at room temperature with a wavelength of 254 nm and a flow rate of 1.0 mL/min. Rhinacanthins-C and N were used as reference markers, and quantitative HPLC analysis was conducted based on the peak area of each component.

Table 1: Description of plant m**aterials**

Scientific name	Part used	Voucher specimen number
Allium ascalonicum	Rhizomes	BK No. 084791
Acanthus ilicifolius	Leaves	BK No. 084900
Bambusa blumeana	Leaves	TTM No. 0005495
Rhinacanthus nasutus	Leaves	BK No. 084901

Anti-allergy and anti-inflammatory activities in cell lines .
Inhibition of IL-4 release from RBL-2H3 cells [19]

RBL-2H3 ATCC CRL-2256, the basophilic leukemia cell line, was purchased from the American Type Culture Collection. The cells were cultured with complete MEM (MEM with 100 µg/mL streptomycin, 100 µg/mL penicillin, and 10% FBS), and they were subcultured every 5 days. Prior to testing, the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was used to assess the toxicity of the compound on RBL-2H3 cells to determine the cell viability rate. The cells were seeded into 24-well plates at a density of 2×10^5 cells/well and incubated for two hours at 37°C with 5% CO_2. Following a 24-hour incubation under the same conditions, the cells were sensitized with 40 µL/well of DNP-specific IgE (5 µg/mL). After incubation, the supernatant was removed, and the cells were washed twice with 400 µL/well of phosphate-buffered saline. Next, 160 µL of complete MEM was pipetted into each well, and the plate was incubated for 10 minutes. After that, different concentrations of extract (12.5, 25, 50, and 100µg/mL) were added to the cells, and the plate was incubated for 10 minutes. The antigen DNP-BSA (0.1 mg/mL) was added into the plate at a volume of 20 µL/well, and the mixture was incubated for 6 hours. IL-4 was detected by transferring the supernatant to an ELISA kit.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Diphenhydramine was used as a positive control at the final concentration of 12.5, 25, 50, and 100µg/ml.

Inhibition of inflammatory mediators in LPS-stimulated RAW264.7 cells [20, 21]

The RAW264.7 ATCC TIB-71 murine macrophage cell line was purchased from the American Type Culture Collection. It was cultured at 37°C in 5% CO_2 and kept in complete DMEM supplemented with 10% FBS, 100 µg/mL penicillin, and 100 µg/mL streptomycin. Every 4-5 days, the cells were subcultured.

Cytotoxicity measurement cells were seeded into 96-well plates and incubated for 24 hours at 37°C with 5% CO_2. After that, the supernatant was discarded, and different extract concentrations (12.5, 25, 50, and 100 µg/mL) were added with complete DMEM. The mixture was then incubated for 24 hours. Each well received 0.5 mg of MTT solution after incubation. After two hours, the supernatants were withdrawn and replaced with 100 µL/well of dimethyl sulfoxide to dissolve the formazan product. Finally, the optical density was measured at 570 nm. The results were expressed as viability rates. The extract concentration was not toxic to RAW264.7 cells; therefore, it was used for anti-inflammatory activity assays.

To determine nitric oxide production after cytotoxic testing, the cells were seeded into 96-well plates and incubated for 24 h. Then, the supernatant was replaced with 100 µL/well of LPS (10 ng/mL) and 100 µL/well of extracts (at concentrations of 12.5, 25, 50, and 100 µg/mL) in complete DMEM, and then the plates were incubated for 24 h. The supernatants (100 µL/well) were added to a new 96-well plate, and Griess reagent (100 µL/well) was added to each well to detect nitric oxide. The optical density was measured immediately at 570 nm.

The production of TNF-α and IL-6 was determined using RAW264.7 cells, which were cultured and activated with LPS to produce TNF-α and IL-6. The cells were then treated with the extracts following the same protocol described in the previous section. After treatment, the supernatants (100 µL/well) were collected to detect TNF-α and IL-6 production using an ELISA kit. Betamethasone was used as a positive control with final concentrations of 12.5, 25, 50, and 100µg/ml.

Statistical analysis

The mean ± standard deviation (SD) from a minimum of three experiments was used to express all data. GraphPad Prism 5 software was utilized to calculate the 50% inhibitory concentration (IC_{50}) values. Dunnett's multiple comparison test and one-way ANOVA were calculated using IBM SPSS statistics software to assess the statistical significance at p-value < 0.05.

Results and Discussion

HFE decreased IL-4 production

Exposure to the HFE at different concentrations from 12.5 to 100 µg/mL had no toxic effect on RBL-2H3 cells. The survival rate was more than 70%, indicating nontoxicity.[22] Treatment of IgE-stimulated RBL-2H3 cells for six hours with 12.5–100 µg/mL of the HFE resulted in concentration-dependent inhibition of IL-4 production (Figure 1).

The highest inhibitory effect, more than 50% inhibition of IL-4 release, was observed at a concentration of 100 µg/mL. HFE concentrations of 25 and 50 µg/mL HFE caused significant reductions in IL-4 production ($p < 0.05$) after six hours, while a 12.5 µg/mL concentration slightly decreased IL-4 production in RBL-2H3 cells. The HFE significantly suppressed IL-4 production with an IC_{50} value of 48.73 ± 0.45 µg/mL when compared to diphenhydramine. Diphenhydramine, an antihistamine, showed an inhibition effect on IL-4 production in a dose-dependent manner, as shown in Figure 2. The IC_{50} value of diphenhydramine was 33.06±0.50 µg/mL.

HFE significantly inhibited nitric oxide release, IL-6, and TNF-α production in LPS-stimulated RAW264.7 cells

At all concentrations, the HFE showed no toxicity on RAW264.7 cells. The effect of HFE on nitric oxide and TNF-α levels in RAW264.7 cells was concentration-dependent, while the effect on the IL-6 level was not (Figure 3). Moreover, 50 and 100 µg/mL concentrations of the HFE caused a significant decrease in nitric oxide and TNF-α levels ($p < 0.05$) after 24 h of treatment. Although 25, 50, and 100 µg/mL concentrations significantly inhibited IL-6 production, their inhibitory effect appeared to be similar. Even at the maximum dose, the HFE's ability to inhibit the production of TNF-α and IL-6 was less than 50%. In the case of nitric oxide production, the IC_{50} was 67.95 ± 9.80 µg/mL. Betamethasone was used as a positive control and effectively inhibited the production of nitric oxide, IL-6, and TNF-α in a concentration-dependent manner, with IC_{50} values of 27.76±4.55, 28.57±0.85, and 98.05±0.73 µg/mL, respectively (Figure 4). Comparisons were made to cell lines that were stimulated without treatment to assess the efficacy of the HFE and betamethasone treatment.

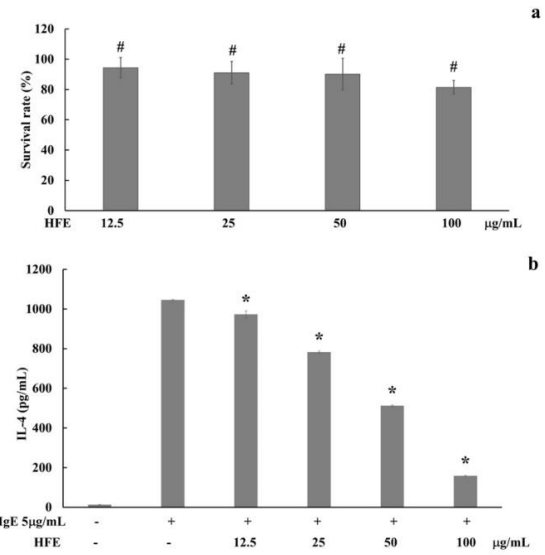

Figure 1: Effect of HFE on (a) mean of cytotoxicity ($p > 0.05$) and (b) IL-4 production of RBL-2H3 cells, *$p < 0.05$ compared with IgE-stimulated condition without HFE treatment.

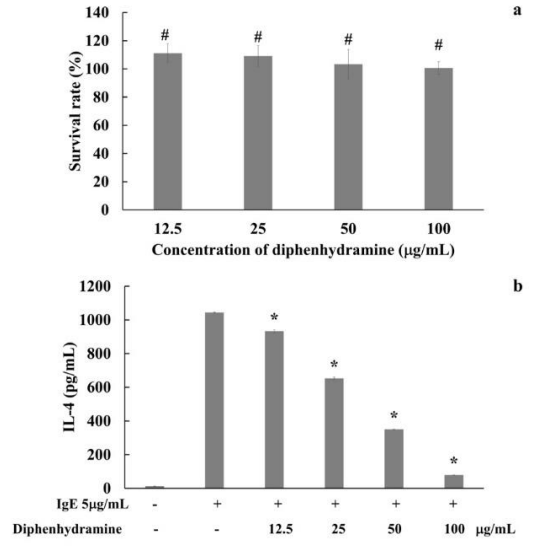

Figure 2: Effect of diphenhydramine (12.5-100 µg/mL) on (a) mean of cytotoxicity ($p > 0.05$) and (b) IL-4 production of RBL-2H3 cells, *$p < 0.05$ compared with IgE-stimulated condition without diphenhydramine treatment.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

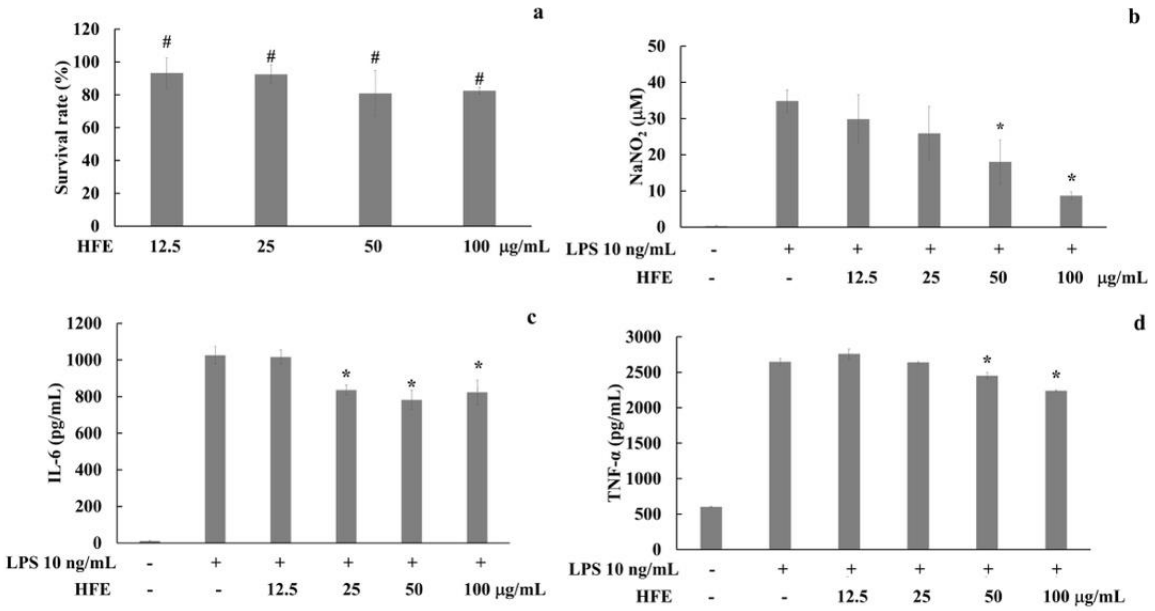

Figure 3: Effect of HFE on (a) mean of cytotoxicity ($p > 0.05$), (b) nitric oxide, (c) IL-6, and (d) TNF-α production in LPS-stimulated RAW264.7 cells. *$p < 0.05$ in comparison to LPS-stimulated condition without HFE treatment.

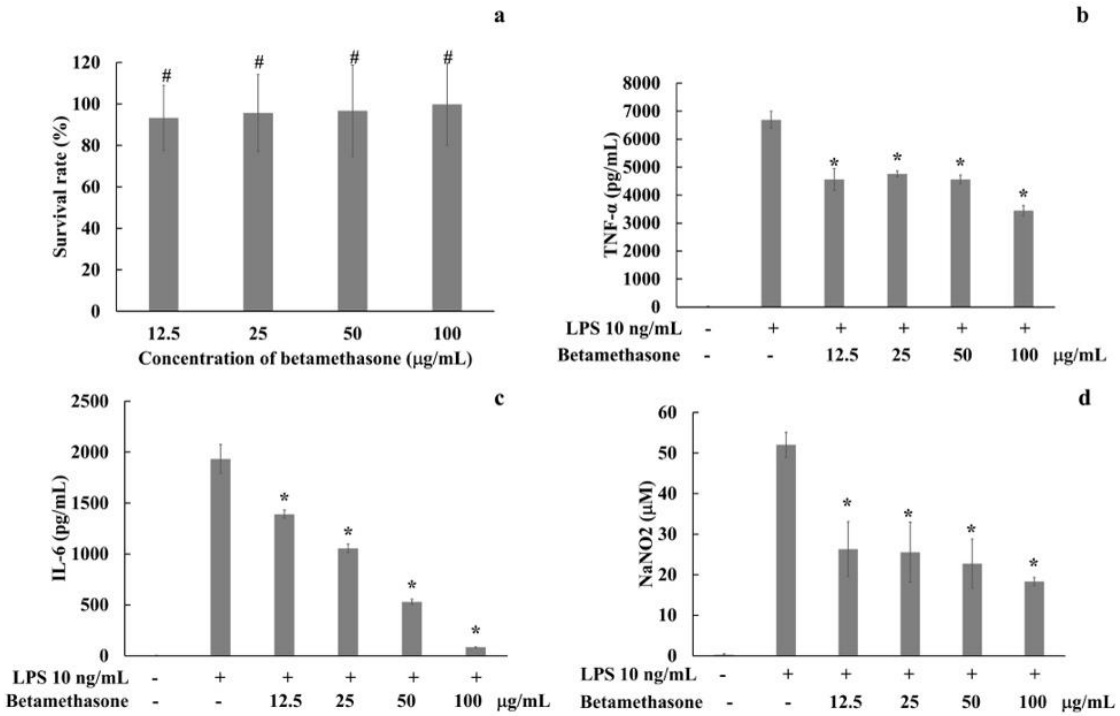

Figure 4: Effect of betamethasone (12.5-100 µg/mL) on **(a)** mean of cytotoxicity ($p > 0.05$), **(b)** nitric oxide, **(c)** IL-6, and **(d)** TNF-α production in LPS-stimulated RAW264.7 cells. *$p < 0.05$ in comparison to LPS-stimulated condition without betamethasone treatment.

Isolation and quantification of chemical compounds

The HPLC method was used to quantify active compounds in the HFE. Quantitative estimation revealed peaks in the HPLC chromatogram, as shown in Figure 5. However, when comparing the HPLC chromatogram of HFE with plant constituents, the HPLC chromatogram of *R. nasutus* was similar to that of HFE. Consequently, we isolated two chemical compounds from *R. nasutus* extract as marker compounds. A confirmation was analyzed using nuclear magnetic resonance (NMR) and mass spectrometry. The NMR results of the isolated compounds were compared to the previously

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

reported data. Compound 1 was isolated as a red-brown crystal that was described as rhinacanthin-N.[23] The molecular formula of compound 1 was determined as $C_{27}H_{24}NaO_7$ from ESI mass-spectrometry, with a molecular ion peak at 3000 m/z. The exact mol
ecular weight of compound 1 was 483.1414. Examination of the ^1H and ^{13}C-NMR spectra showed 25 signals representing 27 carbons and four aromatic rings at δ 6.99 (CH-3′) together with nine methines (CH), two methylenes (CH_2) at δ 2.80 (^2H, s) C-11′, 4.17 (2H, s) C-13, seven methyls (CH_3) at δ 1.15 (3H, s) C-12b, and a methoxy with a signal at δ 3.86 (3H, s) C-4′a. The 1-hydroxynaphthoyl structure was constructed with carbonyl (C-1, C4) and hydroxy (C-2, C3) groups. An ester connector was observed between 1,4-naphthoquinone and the *β*-naphthoic, coupled to a signal from a methoxyl structure at C-4′. Compound 2 was a yellow oily liquid. The molecular formula of compound 2 was determined as $C_{25}H_{30}NaO_5$, and its molecular weight was 433.1985. Compound 2 was identified as rhinacanthin-C, and the ^1H and ^{13}C-NMR spectra of rhinacanthin-C were confirmed according to the previous report.[24] The results showed that 29 signals were detected, indicating the presence of two aromatic rings at C-5, C-6 δ 8.08 (1H, dd 7.6, 1.0), CH-7 δ 7.75 (1H, dt 7.6, 1.3), CH-8 7.67 (1H, dt 7.6, 1.2), CH-9 8.11 (1H, dd 7.6, 1.0), CH-9a, CH-10, CH-10a, which accounted for six methines (CH), four ethyls (CH_2) at δ 2.70 (2H, s), 2.17 (2H, q 7.62), 2.01 (1H, q 7.35), nine methyls (CH_3) at δ 1.02 (6H, s), 1.56 (3H, brs 3.75), 1.79 (3H, brs 0.87), 1.58 (3H, s), and 10 carbons. The two aromatic proton correlations between the signal at δ 2.70 (H-4) and three signals [δ 121.8 (C- 4a), 154.2 (C-10a), 184.8 (C-5)] in the HMBC spectrum of compound 1 established the connection of the 2,2-dimethylpropyl group to the naphthoquinone at C-4a.

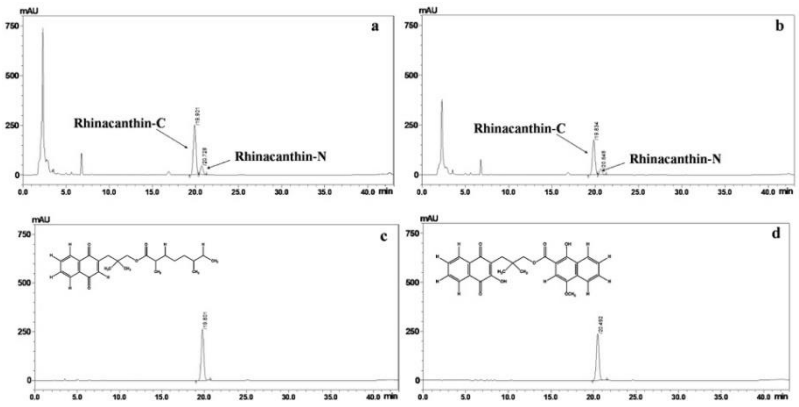

Figure 5: HPLC chromatograms of (a) 10 mg/mL of HFE, (b) 3 mg/mL of *R. nasutus* extract, (c) 200 µg/mL of standard rhinacanthin-C, and (d) standard rhinacanthin-N.

Rhinacanthin-C and rhinacanthin-N inhibited IL-4 and inflammatory cytokines

The effect of isolated rhinacanthin-C and rhinacanthin-N from *R. nasutus*, on IL-4 inhibition in RBL-2H3 cells was assessed at a concentration range of 1.56–50 µg/mL, which was not cytotoxic. These compounds inhibited IL-4 secretion in a concentration-dependent manner. They exhibited significant effects at all concentrations ($p < 0.05$). Rhinacanthin-N had a lower IC_{50} value than rhinacanthin-C in inhibiting IL-4 production in RBL-2H3 cells (Figure 6). The IC_{50} values of rhinacanthin-C and rhinacanthin-N for inhibiting IL-4 production were 15.35 ± 0.55 and 4.65 ± 0.02 µg/mL, respectively.

Rhinacanthin-C was toxic to RAW264.7 cells at 100 µg/mL but exhibited no toxic effect at lower concentrations. Moreover, rhinacanthin-N exhibited no cytotoxic effect at all tested concentrations (Figure 7). All tested isolates that didn't cause any harm to the cells showed a significant ($p < 0.05$) inhibitory effect, particularly against nitric oxide production (Figure 8). The IC_{50} values of rhinacanthin-C and rhinacanthin-N were 34.88 ± 4.87 and 41.41 ± 1.77 µg/mL, respectively. Moreover, rhinacanthin-C and rhinacanthin-N could inhibit TNF-*α* production at a concentration range of 50–100 µg/mL. Rhinacanthin-N inhibited TNF-*α* production in a concentration-dependent manner. Treatment with 12.5–50 µg/mL of rhinacanthin-C and 50–100 µg/mL of rhinacanthin-N inhibited IL-6 production. However, the IC_{50} values of compounds that could not be calculated were more than the maximal tested concentration.

Allergy represents an exaggerated immune response triggered upon re-exposure to allergens. During cascade induction, white blood cells, including mast cells and basophils, initiate the degranulation process and the subsequent release of histamine, IL-4, TNF-*α*, IL-6, IL-1, and IFN-γ. After exposure to allergens IgE binding FcεRI receptors induce mast cell degranulation and produce IL-4 responses that act on B-cells to promote IgE antibody production as well.[25] Notably, IL-4 is pivotal in modulating the activation and functionality of T-helper cells, monocytes, and macrophages, thereby orchestrating the release of various inflammatory mediators.[26] Consequently, inflammatory processes lead to the release of nitric oxide, TNF-*α*, IL-1, IL-6, and other cytokines by immune cells, contributing to the manifestation of allergic and inflammatory symptoms. Therefore, extensive research is needed to assess the efficacy of herbal interventions in alleviating such symptoms.[5]

We explored the ability of a Thai HFE to ameliorate inflammation and allergy. Assessment of the biological activity of the HFE revealed its capacity to inhibit the release of IL-4 and nitric oxide. Moreover, it slightly inhibited IL-6 and TNF-*α* production. The isolates from HFE included *A. ascalonicum*, *R. nasutus*, *A. ilicifolius*, and *B. blumeana*, which have been shown to potently inhibit proallergic and proinflammatory cytokines.[27-30] Plant phytochemicals exhibit pharmacological effects. Compounds such as anthraquinones, flavonoids, and volatile oils, have been detected in HFEs. These chemical compounds have been found to reduce IL-4 levels and nitric oxide production.[31, 32]

Analysis of the phytochemical compositions revealed that rhinacanthin-C and rhinacanthin-N were the predominant bioactive compounds in the HFE. Rhinacanthin is an anthraquinone compound present in *R. nasutus*.[33] Our study showed that rhinacanthin-C and rhinacanthin-N inhibited the production of IL-4 , IL-6, TNF-*α*, and nitric oxide, consistent with previous findings.[34] Interestingly, rhinacanthin, particularly at low doses, can potentially inhibit IL-4 production and may reduce allergic inflammatory response.

Previous studies have suggested that anthraquinones exert anti-inflammatory effects and can modulate immune responses.[35] Rhinacanthin-C and rhinacanthin-N have been reported to suppress nitric oxide release with IC_{50} values of 1.8 and 3.0 µM, respectively. However, they did not inhibit TNF-*α* production in RAW264.7 cells.[36] Notably, rhinacanthin-C reduced inflammation in rats using carrageenan-induced paw edema assay.[15] It attenuated LPS-activated

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

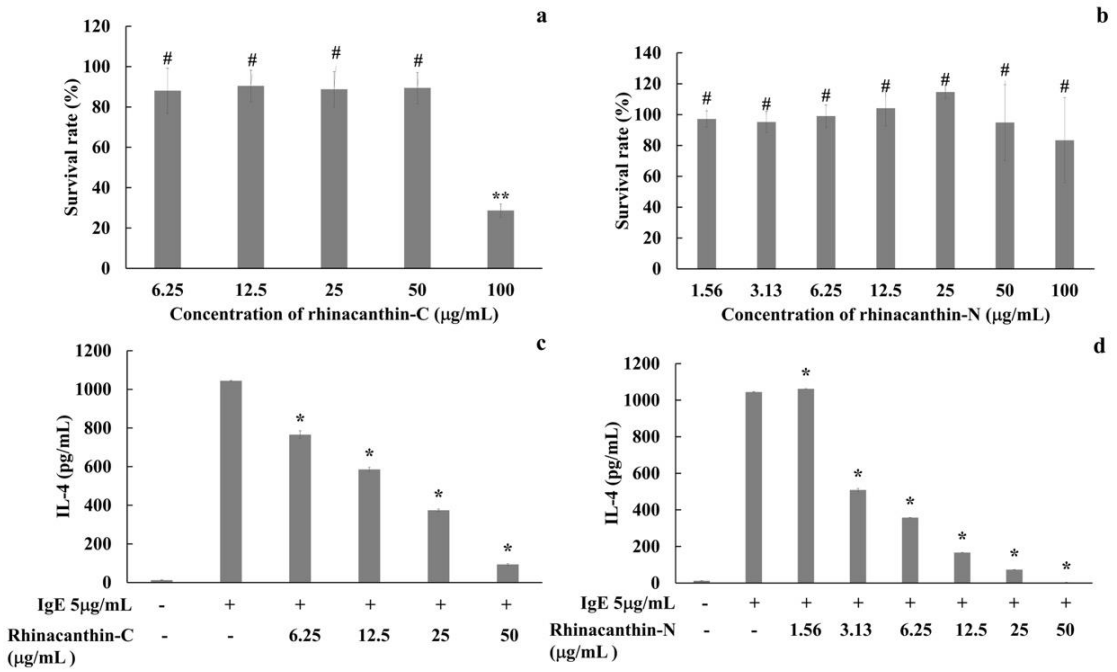

Figure 6: Effect of rhinacanthin-C and rhinacanthin-N on (a,b) cytotoxicity ($p > 0.05$) while **$p<0.05$ compared with other concentrations and (c,d) IL-4 production in RBL-2H3 cells. *$p < 0.05$ compared with IgE-stimulated condition without compound treatment.

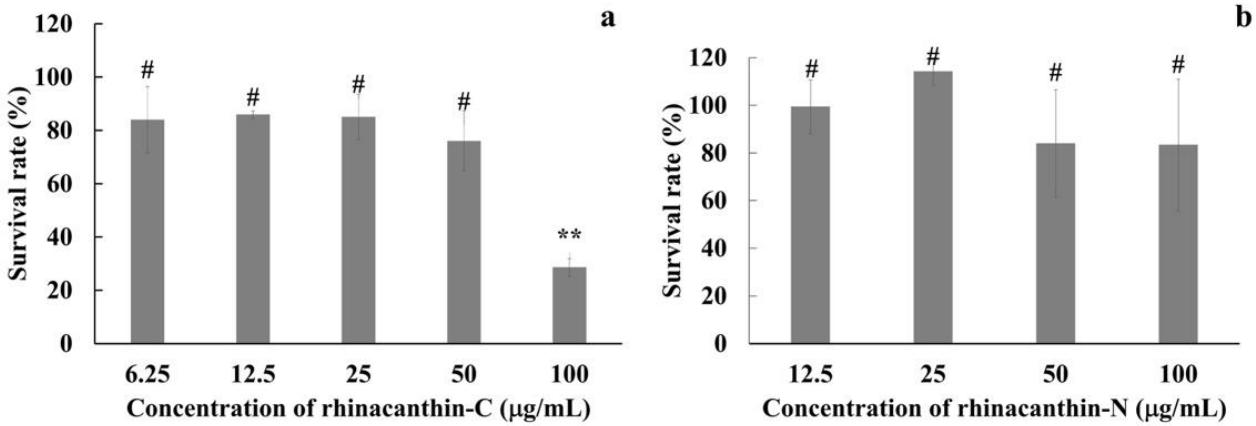

Figure 7: Cytotoxicity effect of (a) rhinacanthin-C and (b) rhinacanthin-N on RAW264.7 cells, the same symbol is not significantly different from each other ($p > 0.05$) while **$p<0.05$ compared with other concentrations.

nitric oxide production and amyloid-β peptide in rat hippocampal neurons, and decreased proinflammatory cytokine production, including IL-6 and TNF-α in BV-2 cells.[37]

The mechanism of action of HFE most likely involves preventing mast cells or RBL-2H3 from degranulating upon allergen exposure by preventing IgE from cross-linking to FcεRI receptors, which in turn prevents the production of the cytokine IL-4.[38] Analysis of the mechanisms underlying allergic reactions revealed distinct cytokine involvement in acute and chronic inflammation. Specifically, IL-4 and nitric oxide are implicated in acute inflammation, whereas TNF-α and IL-6 are associated with chronic inflammation.[39, 40] The mechanism involved a reduction in signaling molecules during T cell-mediated immunity in macrophages, including IL-6 and nitric oxide, which inhibits inflammation.[41] Additionally, HFE may immunomodulate RAW264.7 cells by inhibiting the toll-like receptor 4 (TLR4) signaling that activates the NF-κB pathway. The inactivation of NF-κB leads to the inhibition of nitric oxide, IL-6, and TNF- α and indicates its immunomodulatory potential.[42]

This study supports the ethnomedicinal use of Thai herbal formulas. HFE demonstrated anti-allergic and anti-inflammatory activities by inhibiting IL-4 production and other inflammatory mediators. Rhinacanthin-C and rhinacanthin-N, which block inflammatory and allergy-related cytokines, are potent modulators of inflammation. These findings indicate that HFE is a promising antiallergic agent. However, our results were obtained from *in vitro* studies, and the safety profile of HFE was not assessed. Therefore, further research is needed to investigate the safety and efficacy of HFE. Additionally, the molecular mechanisms underlying its anti-allergic and anti-inflammatory activities should be explored in *in vivo* models.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

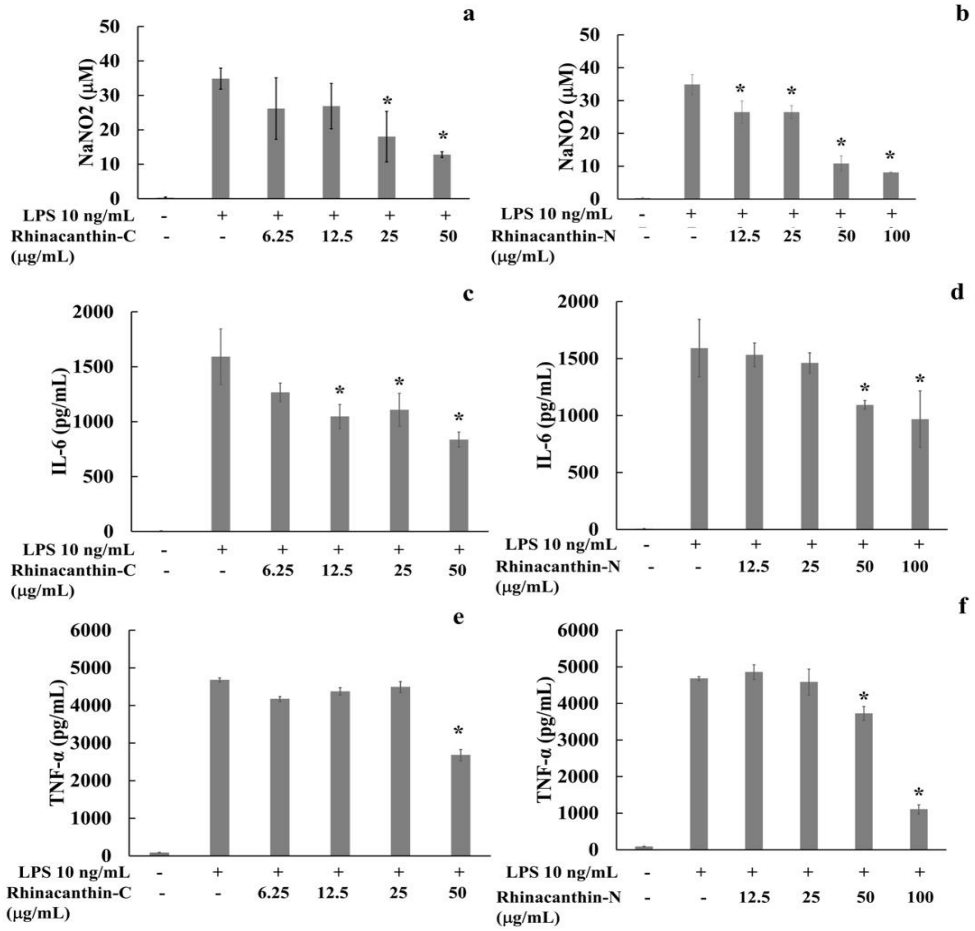

Figure 8: Inhibition effect of rhinacanthin-C and rhinacanthin-N on **(a-b)** nitric oxide, **(c-d)** IL-6, and **(e-f)** TNF-*α* production in LPS-stimulated RAW264.7 cells. *p < 0.05 when compared with LPS stimulated condition without treated compounds.

Conclusion

The Thai herbal formula (HFE) inhibited IL-4 production in RBL-2H3 cells. It also reduced nitric oxide production and slightly decreased the production of inflammatory cytokines such as TNF-*α* and IL-6 in RAW264.7 cells. The active chemical constituents of HFE primarily consisted of higher levels of rhinacanthin-C than rhinacanthin-N. Nonetheless, both compounds effectively regulated IL-4 secretion, nitric oxide levels, and the production of TNF-*α* and IL-6.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article are original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This study was supported by the Faculty of Medicine, Thammasat University (grant no. TP. 2-35/2565).

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