



Clinacanthus nutans aqueous leaves extract exerts anti-allergic activity in preclinical anaphylactic models via alternative IgG pathway

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ABSTRACT

Ethnopharmacological relevance: Allergy is mediated by the crosslinking of immunoglobulins (Ig) -E or -G to their respective receptors, which degranulates mast cells, macrophages, basophils, or neutrophils, releasing allergy-causing mediators. The removal of these mediators such as histamine, platelet-activating factor (PAF) and interleukins (ILs) released by effector cells will alleviate allergy. *Clinacanthus nutans* (*C. nutans*), an herbal plant in Southeast Asia, is used traditionally to treat skin rash, an allergic symptom. Previously, we have reported that *C. nutans* aqueous leaves extract (CNAE) was able to suppress the release of β -hexosaminidase and histamine but not interleukin-4 (IL-4) and tumor necrosis factor- α (TNF- α) in the IgE-induced mast cell degranulation model at 5 mg/mL and above. We also found that CNAE could protect rats against ovalbumin-challenged active systemic anaphylaxis (OVA-ASA) through the downregulation and upregulation of certain metabolites using proton nuclear magnetic resonance ($^1\text{H-NMR}$) metabolomics approach.

Aim of the study: As allergy could be mediated by both IgE and IgG, we further evaluated the anti-allergy potential of CNAE in both *in vitro* model of IgG-induced macrophage activation and *in vivo* anaphylaxis models to further dissect the mechanism of action underlying the anti-allergic properties of CNAE.

Material & methods: The anti-allergy potential of CNAE was evaluated in *in vivo* anaphylaxis models of ovalbumin-challenged active systemic anaphylaxis (OVA-ASA) and IgE-challenged passive systemic anaphylaxis (PSA) using Sprague Dawley rats as well as IgG-challenged passive systemic anaphylaxis (IgG-PSA) using C57BL/6 mice. Meanwhile, *in vitro* model of IgG-induced macrophage activation model was performed using IC-21 macrophages. The release of soluble mediators from both IgE and IgG-mediated pathways were measured using enzyme-linked immunosorbent assay (ELISA). The signaling molecules targeted by CNAE were identified by performing Western blot.

Results: IgG, platelet-activating factor (PAF) and IL-6 was suppressed by CNAE in OVA-ASA, but not IgE. In addition, CNAE significantly suppressed PAF and IL-6 in IgG-PSA but did not suppress histamine, IL-4 and leukotrienes C_4 (LTC_4) in IgE-PSA. CNAE also inhibited IL-6 and TNF- α by inhibiting the phosphorylation of ERK1/2 in the IgG-induced macrophage activation model.

Conclusion: Overall, our findings supported that CNAE exerts its anti-allergic properties by suppressing the IgG pathway and its mediators by inhibiting ERK1/2 phosphorylation, thus providing scientific evidence supporting its traditional use in managing allergy.

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1. Introduction

Allergy is an immunological hypersensitivity response that includes anaphylaxis, urticaria, angioedema, asthma, and others (Ring, 2014). Globally, about 30–40% of the world population is affected by one form or another of allergy diseases, and due to rapid urbanization, the number

Abbreviations

¹ H-NMR	Proton nuclear magnetic resonance
Akt	Protein kinase B
AMPK	5' monophosphate-activated protein kinase
ERK	Extracellular signal-regulated kinase
FcγR	IgG receptor
FcεRI	High-affinity IgE receptor
Ig	Immunoglobulin
IgE-PSA	Immunoglobulin E-challenged passive systemic anaphylaxis
IgG-PSA	Immunoglobulin G-challenged passive systemic anaphylaxis
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
JNK	c-Jun N-terminal kinase
LTC ₄	Leukotriene C ₄
MAPK	Mitogen-activated protein kinase
MAPKAP K2	MAPK-activated protein kinase 2
OVA-ASA	Ovalbumin-challenged active systemic anaphylaxis
PAF	Platelet-activating factor
RBL-2H3	Rat basophilic leukemic cells
TNF-α	Tumor necrosis factor-alpha
UPLC-MS/MS	Ultra performance liquid chromatography-tandem mass spectrophotometry

of people affected is expected to increase (Pawankar et al., 2013; Pawankar et al., 2011). Allergies negatively affect one's quality of life – increased medical attention, disrupted sleep, or reduced sleep quality, increased susceptibility to sinus and skin infections, inability to focus on work or school etc. On the economic front, a country loses millions of dollars due to loss of work productivity of affected individuals (Grisanti and Grayson, 2018).

Anaphylaxis is a severe allergic reaction (Reber et al., 2017). It is an acute, life-threatening reaction that affects many systems caused by the sudden release of mediators from effector cells (Escribese et al., 2017). Anaphylaxis can be mediated through two pathways known as the classical immunoglobulin E (IgE) pathway and the alternative immunoglobulin G (IgG) pathway (Minai-Fleminger et al., 2009). In the IgE-dependent pathway, the crosslinking of antigen to antigen-specific IgE bound to high-affinity IgE receptor (FcεRI) found on mast cells and basophils leads to anaphylaxis. On the other hand, the crosslinking of antigen to antigen-specific IgG bound to IgG receptors (FcγRI, FcγRIIA, FcγRIIB, FcγRIIC, and FcγRIIIA) found on macrophages, neutrophils and basophils is responsible for the anaphylaxis reactions in the IgG-dependent pathway (Escribese et al., 2017). Upon crosslinking of IgE or IgG to their respective receptors and subsequent exposure to allergens, the effector cells will degranulate and release pro-inflammatory mediators that bring about allergy symptoms (Finkelman et al., 2016). These mediators are divided into early phase [histamine, platelet-activating factor (PAF)] and late phase mediators [interleukins (IL-), prostaglandins, and serotonin]. The early phase mediators are released because of the degranulation of effector cells. In contrast, the subsequent release of late-phase mediators is caused by the downstream activation of the respective signaling pathways (Reber et al., 2017). Falanga et al. (2012) postulated that the activation and degranulation of

both IgE and IgG-mediated pathways are similar as both Fcε and Fcγ receptors have identical γ subunits and they activate similar signaling molecules – Fyn, Ly, protein kinase B (Akt), p38, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) as they activate the same immunoreceptor tyrosine-based activation motif (ITAMs) found in the intracellular domains of both receptors (Crowley et al., 1996).

Thus far, the management of allergic diseases has relied on pharmacotherapy treatments that aim to alleviate the symptoms of allergy. The use of allergen immunotherapy has been promising - providing long term allergen desensitization and the possibility of curing allergic diseases. However, it still possesses IgE-mediated side effects and limited efficacy (Kun and Li, 2016). As a result, many people turn towards complementary treatments to manage their allergies. *Clinacanthus nutans* (*C. nutans*) is a perennial shrub found mainly in Southeast Asia and has been traditionally used to treat skin rashes, which is one of the symptoms of allergy. Previously, we reported that *C. nutans* aqueous leaves extract (CNAE) was able to reduce the release of β-hexosaminidase and histamine but not IL-4 and tumor necrosis factor-α (TNF-α) in IgE-mediated mast cell degranulation using RBL-2H3 cells (Kow et al., 2020). In another study using proton nuclear magnetic resonance (¹H-NMR) metabolomics approach, our group found that CNAE could protect rats against ovalbumin-challenged active systemic anaphylaxis (OVA-ASA) through the downregulation of lipid metabolism, carbohydrates, and signal transduction system as well as upregulation of citrate cycle intermediates, propanoate metabolism, amino acid metabolism and nucleotide metabolism (Khoo et al., 2018).

In this study, to better understand the anti-allergic effects of *C. nutans* aqueous leaves extract, we further evaluated the anti-allergy potential of CNAE in three models of anaphylaxis. The first model was OVA-ASA, where both IgE and IgG pathways were activated in this model upon the challenge of OVA. The other two models were the IgE and IgG-PSA models, in which only IgE or IgG pathway was activated respectively, upon the challenge of either IgE or IgG antibodies. In these models, ASA is induced with an allergen and an adjuvant injection, while PSA is induced by injecting antigen specific IgE or IgG and the antigen (Doyle et al., 2013). Lastly, the mechanism of action underlying the anti-allergic effects of CNAE was further confirmed by *in vitro* model of IgG-induced macrophage activation.

2. Materials and methods

2.1. Reagents and consumables

Fetal bovine serum (FBS) was purchased from Gibco by Life Technologies (Grand Island, NY, USA). RPMI-1640 was purchased from PAN-Biotech (Aidenbach, Bavaria, Germany). Monoclonal anti-dinitrophenyl antibody isotype IgE (DNP-IgE), aluminum hydroxide, albumin from chicken egg white (OVA), calcium chloride, magnesium chloride and Dulbecco's Phosphate Buffered Saline Modified without calcium chloride and magnesium chloride were purchased from Sigma Aldrich, Inc. (St. Louis, MO, USA). The 2,4-dinitrophenol bovine serum albumin (DNP-BSA) was purchased from Calbiochem (EMD Chemicals, Inc., San Diego, CA, USA). Purified anti-mouse CD16/32 antibody (Clone93) and purified goat anti-rat IgG (minimal x-reactivity) antibody (Poly4054) were purchased from BioLegend (San Diego, CA, USA). Bovine serum albumin (BSA), sodium chloride and 2-Mercaptoethanol were purchased from Amresco (Solon, OH, USA). CellTiter96® Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI, USA). Ethanol absolute was purchased from HmbG® Chemicals (Hamburg, Germany). Glycine, TRIS and sodium dodecyl sulphate were purchased from VWR Amresco Life Science (Solon, OH, USA). Potassium chloride (KCl) was purchased from HmbG® Chemicals, Hamburg, Germany. Methanol was purchased from Fisher Scientific (Loughborough, UK). Nylon syringe filter (0.22 μm) was purchased from Bioflow (Malaysia). Pierce™ BCA Protein Assay kit was purchased from EMD

Millipore Corporation (Burlington, MA, USA). Albumin fraction V (BSA) was purchased from Bio Basic Canada, Inc. (Markham, Ontario, Canada). Protease inhibitor cocktail and 40(w/v)% acrylamide/bis mixed solution (29:1) were purchased from Nacalai Tesque Inc. (Nakagyo-ku, Kyoto, Japan). Mini Trans-Blot filter paper and 4X Laemmli sample buffer were purchased from Bio-Rad (Hercules, CA, USA). TEMED was purchased from HiMedia Laboratories Private Limited (Mumbai, India). All primary antibodies (phospho and total) and secondary antibody anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). β -actin (C₄) HRP was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All inhibitors – PD98059 and SB203580 were purchased from Tocris Bioscience (Bristol, UK). Simply™ BLUEye Prestained protein ladder was purchased from Gene-DireX, Inc. (Cullman, AL, USA). WesternBright™ Sirius Advanta HRP substrate was purchased from Advanta (Menlo Park, CA, USA). PVDF membranes were purchased from Merck (Darmstadt, F. R., Germany). Anti-mouse CD16/32 (2.4G2) was purchased from BioXCell, West Lebanon, NH, USA. Ketamine and xylazine were purchased from Troy Laboratories PTY Limited, Glendenning, NSW, Australia. Food pellets were purchased from Gold Coin (Selangor, Malaysia). CV-6209 (chloride) was purchased from Cayman Chemical, Ann Arbor, MI, USA. BD Vacutainer® plasma tubes spray-coated with 150 USP units of sodium heparin were purchased from Becton, Dickinson and Company, Franklin Lakes, NJ, USA. ELISA kits for IgE (Cat. No. KA1951) and IgG (Cat. No. KA1952) were purchased from Abnova Corporation, Taiwan. EIA kit for histamine (Cat. No. A05890) was purchased from SPI-BIO, Montigny Le Bretonneux, France. ELISA kits for IL-4 (Cat. No. DY504) and IL-6 (Cat. No. DY506) and Magnetic Luminex assay kit – IL-6, -13 and TNF- α (Cat. No. LXSAMSM) were purchased from R&D Systems, Inc., Minneapolis, MN, USA. ELISA kit for PAF (Cat. No. 201552) was purchased from US Biologicals, Salem, MA, USA. EIA kit for LTC₄ (Cat. No. 501070) was purchased from Cayman Chemical, Ann Arbor, MI, USA. IL-6 (Cat. No. DY406-05) and TNF- α (Cat. No. DY410) ELISA kits were purchased from R&D Systems, Inc., Minneapolis, MN, USA.

2.2. *Clinacanthus nutans* (Burm. F.) lindau leaves aqueous extract preparation

Clinacanthus nutans (*C. nutans*) was collected from Sendayan Commodities Development Centre, Seremban, Negeri Sembilan, Malaysia. A voucher specimen of SK 2883/15 was assigned to the plant, and it was deposited at the herbarium of the Institute of Bioscience, Universiti Putra Malaysia. The plant was verified by Dr. Shamsul Khamis, an in-house botanist at the Institute of Bioscience, Universiti Putra Malaysia. Plant name was checked against <http://www.theplantlist.org>, ID kew-2727,901. *C. nutans* aqueous leaves extract (CNAE) was prepared at the Institute of Bioscience, Universiti Putra Malaysia by Khoo et al. (2015). Briefly, the whole plant of *C. nutans* was cleaned with water and wiped dry with tissues. The leaves were then separated from the stems and air-dried for 2 weeks under shade at 25 °C until a constant weight was met. Subsequently, the dried leaves were milled with a laboratory grinder (Waring, Conair Corporation, Stamford, CT, USA) and Retsch test sieve of 315 μ m (Retsch GmbH, Haan, Germany) to obtain uniform particle-sized plant powder. The ground samples were sonicated in the deionized water for 1 h without heating in the Nexul Ultrasonic Cleaner machine (NXP 1002, Shanghai Kudos, Shanghai, China) at a sample to solvent ratio of 1 g plant sample to 10 mL of deionized water. The extraction process was performed three times repeatedly. After that, the extract was filtered and vacuum evaporated to dryness, freeze-dried, and stored at 4 °C until further use. The final yield of CNAE was 3.980 g from 20.07 g of the ground dry sample. The CNAE extract was reconstituted with deionized water and diluted in deionized water to obtain the working *in vivo* doses and *in vitro* concentrations respectively. The metabolite profile of the plant had been established by Khoo et al. using ultra-performance liquid chromatography-tandem mass spectrophotometry (UPLC-MS/MS) and ¹H-NMR (Khoo et al., 2015; Khoo et al.,

2019). The following experiments were conducted using the same CNAE sample used in the metabolite profiling above. The chromatographic profiles of the active compounds are included as supplementary data.

2.3. Animals

Male Sprague Dawley rats (180–220 g) were used in the OVA-ASA and IgE-PSA models, while C57BL/6 mice (18–22 g) were used in the IgG-PSA model. All animals were used at a minimum age of 9 weeks, and all experiments received approval from the Universiti Putra Malaysia Institutional Animal Care and Use Committee (UPM/IACUC/AUP No. R080/2016). Animals were acclimatized for one week before all experiments. Animals were housed three animals per cage (rats) and six animals per cage (mice) in polycarbonate cages at 22–25 °C, 12 h light/dark cycle with food and water given *ad libitum*.

2.4. Ovalbumin-challenged active systemic anaphylaxis (OVA-ASA)

The effect of CNAE on OVA-ASA was evaluated based on previously described procedures by Guo et al. (2001), with slight modifications. Thirty-six (36) rats were randomly divided 6 per group into (i) Sham (S), (ii) Challenged (C), (iii) Ketotifen fumarate-treated (KF), (iv) CNAE-treated TG 1 (125 mg/kg), (v) CNAE-treated TG 2 (500 mg/kg) and (vi) CNAE-treated TG 3 (2000 mg/kg). Briefly, rats in the 'C', 'KF' and 'TG 1–3' groups were sensitized twice on days 0 and 6 with a 2 mL mixture of 1 mg OVA and 10 mg of aluminum hydroxide dissolved in PBS intraperitoneally. Rats in the 'S' group were administered with 2 mL of PBS during each pre-sensitization days intraperitoneally (Guo et al., 2001). On day 13, rats in 'TG 1–3' groups were pre-treated with different doses of CNAE (125, 500 and 2000 mg/kg) by oral administration at 10 mL/kg of body weight. As this was a preliminary study, the above doses were decided based on previous studies (Khoo et al., 2018; Pongphasuk et al., 2005; Thongharb and Tejasen, 1977) and chosen to represent a broad range of doses. In addition, Ketotifen fumarate (1 mg/kg), a mast cell stabilizer, was used as the positive control (Naik et al., 2013). It was administered intraperitoneally at 200 μ L/rat to rats in the 'KF' group. Rats in both 'S' and 'C' groups were administered with distilled water by oral administration at 10 mL/kg of body weight for 1 h (h) instead of CNAE or KF. After 1 h, rats in the 'C', 'KF' and 'TG 1–3' groups were challenged with 200 μ L of 1 mg OVA by intravenous tail vein injection while 200 μ L of PBS was administered to the 'S' group through the intravenous tail vein. After 1 h, all the rats were euthanized, and blood was collected by intracardiac puncture under anesthesia with ketamine/xylazine cocktail (91 mg/mL ketamine mixed with 9.1 mg/mL xylazine). The blood was centrifuged (High-Speed Brushless Centrifuge, MPW350-R, MPW Med. Instruments, Warsaw, Poland) at 3100 RPM for 15 min at 4 °C to obtain the plasma. All plasma samples were aliquoted and stored at –80 °C until further use.

2.5. IgE-challenged passive systemic anaphylaxis (IgE-PSA)

The effect of CNAE on IgE-PSA was evaluated based on previously described procedures by Tan et al. (2017). A total of 30 rats were used, whereby there were randomly divided 6 per group into (i) Sham (S), (ii) Challenged (C), (iii) Ketotifen fumarate-treated (KF), (iv) CNAE-treated TG 1 (500 mg/kg) and (v) CNAE-treated TG 2 (2000 mg/kg). Briefly, rats (C, KF, TG 1–2 groups) were pre-sensitized with 1 μ g/mL of IgE dissolved in PBS 24 h via the intraperitoneal route at 10 mL/kg while rats in the 'S' group were administered with PBS at 10 mL/kg body weight (Tan et al., 2017). On the following day, rats (TG 1–2 groups) were pre-treated with different doses of CNAE (500 and 2000 mg/kg) by oral administration at 10 mL/kg. Ketotifen fumarate (1 mg/kg), a mast cell stabilizer, was used as the positive control (Naik et al., 2013). It was administered intraperitoneally at 200 μ L/rat. Both 'S' and 'C' groups' rats were given distilled water orally at 10 mL/kg of body weight for 1 h instead of CNAE or KF. After 1 h, rats (C, KF, TG 1–2) were challenged

with 1 mg DNP-BSA, dissolved in 200 μ L of PBS via the intravenous injection of the tail vein. Rats in the 'S' group were administered with 200 μ L of PBS by intravenous injection of the tail vein. After 1 h, rats were euthanized, and blood was collected by intracardiac puncture under anesthesia with ketamine/xylazine cocktail (91 mg/mL ketamine mixed with 9.1 mg/mL xylazine). The blood was centrifuged (High-Speed Brushless Centrifuge, MPW350-R, MPW Med. Instruments, Warsaw, Poland) at 3100 RPM for 15 min at 4 °C to obtain the plasma. All plasma samples were aliquoted and stored at –80 °C until further use.

2.6. IgG-challenged passive systemic anaphylaxis (IgG-PSA)

The effect of CNAE on IgG-PSA was evaluated based on previously described procedures by [Falanga et al. \(2012\)](#) with slight modifications. Mice, totaling 36, were randomly divided 6 per group into (i) Sham (S), (ii) Challenged (C), (iii) Ketotifen fumarate-treated (KF), (iv) CNAE-treated TG 1 (125 mg/kg), (v) CNAE-treated TG 2 (500 mg/kg) and (vi) CNAE-treated TG 3 (2000 mg/kg). Before being challenged, mice (TG 1–3) were pre-treated with the different doses (125, 500 and 2000 mg/kg) of CNAE for 1 h. CV-6209 (PAF antagonist) was used as the positive control. CV-6209 (500 μ g/mL; 100 μ g dissolved in 200 μ L PBS) was administered intraperitoneally to the mice in the 'CV-6209' group 5 min before being challenged to anaphylaxis with 2.4G2 antibody ([Musio et al., 2009](#)). An hour prior to being challenged, 10 mL/kg of distilled water was orally administered to Group 'S' and 'C' mice. Mice in the 'C', 'CV-6209' and 'TG 1–3' groups were then challenged to anaphylaxis with 2.4G2 antibody intravenously through the tail vein (500 μ g in 200 μ L cold PBS (pH8); 2.5 mg/mL) and observed for 2 h. Mice in the 'S' group were administered with 200 μ L of PBS intravenously through the tail vein. After 2 h, blood was collected by intracardiac puncture under anesthesia with ketamine/xylazine cocktail (87.5 mg/kg ketamine mixed with 12.5 mg/kg xylazine, administered at 0.1 mL/20 g body weight). The blood was centrifuged (High-Speed Brushless Centrifuge, MPW350-R, MPW Med. Instruments, Warsaw, Poland) at 3100 RPM for 15 min at 4 °C to obtain the plasma. All plasma samples were aliquoted and stored at –80 °C until further use.

2.7. Quantification of pro-inflammatory mediators

The blood plasma collected was analyzed using enzyme-linked immunosorbent assay (ELISA) kits and Magnetic Illuminex assay according to the manufacturers' protocols. Absorbance was read on a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA). For OVA-ASA, the mediators quantified were IgE (representing the IgE pathway), IgG, PAF and IL-6 (representing the IgG pathway). On the other hand, histamine, LTC₄ and IL-4 were quantified for IgE-PSA, whereas PAF and IL-6 were quantified for IgG-PSA.

2.8. Cell culture

IC-21 cells ATCC®TIB-186™ were cultured in RPMI 1640 supplemented with 10% FBS at 37 °C and 5% CO₂. Cells were sub-cultured or assayed for experiment once they reached 90% confluency. Cells used for all assays were from passages 6 to 11.

2.9. Cytotoxicity assay

The cytotoxicity of CNAE was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay. IC-21 cells (5 $\times$ 10⁴ cells/well/100 μ L) were seeded in 96-well plates overnight. IC-21 cells were treated with different extract concentrations, from 1 to 10 mg/mL for 24 h. CNAE was reconstituted in deionized water and diluted in RPMI 1640 medium with 10% FBS to their final concentrations. Following the protocol from the manufacturer, 10 μ L of MTS solution was added to each well and incubated for 2 h at 37 °C in a 5% CO₂ incubator. After that, the

absorbance was read at 490 nm on the automated microplate reader (Molecular Devices, Versa Max, Sunnyvale, CA, USA). The percentage of cell viability was calculated using Equation (1):

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \right) \times 100 \quad (1)$$

2.9.1. Quantification of pro-inflammatory mediators

Pro-inflammatory mediators such as IL-6 and TNF- α were being analyzed by ELISA. Briefly, 5 $\times$ 10⁴ cells/well/100 μ L of IC-21 were seeded overnight in 96-well tissue cultured plate. Cells were then pre-treated with three non-cytotoxic CNAE concentrations or CV-6209 (1 μ M; positive control) for 4 h. Cells were then induced with antibodies that consisted of 25 μ g/mL Clone93 and 50 μ g/mL Poly4054 antibodies, which have been activated for 30 min at 37 °C beforehand. Cells were induced for 6 h in a 5% CO₂ incubator at 37 °C. After that, the supernatants were collected and analyzed by ELISA following the manufacturer's protocol. Absorbance was read at 450 nm with subtraction at 570 nm on an automated microplate reader (Molecular Devices, Versa Max, Sunnyvale, CA, USA).

2.10. Western blot

The mitogen-activated protein kinase (MAPK) signaling proteins of interest were being analyzed by Western blot. Briefly, 4 $\times$ 10⁵ cells/well/2.5 mL of IC-21 cells were seeded in a tissue-cultured 6-well plate overnight in a 37 °C incubator supplemented with 5% CO₂. Cells were then pre-treated with the highest concentration of CNAE (7 mg/mL) and the respective signaling proteins' inhibitors (p38 – SB203580 and ERK – PD98059) at 10 μ M for 4 h. After that, the cells were induced with the antibodies (concentration and preparation as earlier described) as follows: p38 – 30 min and ERK – 15 min. Cells were immediately dissociated using RIPA lysis buffer added with 1x protease inhibitor, and 15 μ g total cell lysate per sample was used to perform Western blotting. Proteins were loaded and separated over a 10% gradient SDS-polyacrylamide gel. Next, the proteins were transferred onto PVDF membranes and blocked for 1 h in 1x Tris-buffered saline, 0.1% Tween-20 (TBST) with 5% BSA. Subsequently, the membranes were incubated with the respective diluted primary antibodies overnight at 4 °C with gentle rocking. All antibodies were diluted as per the manufacturer's protocol in 1x TBST with 5% BSA. After the incubation, the membranes were washed thrice with 1x TBST with 10 min intervals per wash before incubating with the secondary antibody of HRP-linked anti-IgG anti-rabbit antibody in 1x TBST with 5% BSA solution for 1 h at room temperature with gentle rocking. The membranes were then re-washed three times before detection. Membranes were incubated with ECL chemiluminescence solution and then viewed on the Western blot documentation system (FusionFX, Vilber Lourmat, France). The protein ladder used was between 10 and 245 kDa for both ERK1/2 and p38 evaluations. The molecular weights for ERK1/2 are 42 kDa and 44 kDa respectively while for p38, it is 41 kDa.

2.11. Statistical analysis

All results are presented as mean $\pm$ Standard Error of the Mean (SEM) of three independent experiments (*in vitro*) and three to six animals per group (*in vivo*). Outlier values were excluded. ELISA results were generated using Four Parameter Logistic Curve (4-PL) (MyAssays, online data analysis tool). ImageJ (Rashband, 1997–2018) was used to analyze the densitometry of Western blot protein quantifications. Results were statistically analyzed using one-way ANOVA followed by Dunnett's *post-hoc* analysis. Statistical differences were considered significant at $p \leq 0.05$.

3. Results

3.1. Ovalbumin-induced active systemic anaphylaxis (OVA-ASA)

Both IgE and IgG pathways of anaphylaxis are activated in the OVA-ASA model. To discern the anti-allergy activity of CNAE, respective mediators of both pathways released upon being challenged were analyzed. It was found that at all CNAE doses, there was no significant inhibition of IgE level in the plasma (figure not shown) while the levels of IgG, PAF and IL-6 showed significant reductions (Fig. 1) compared to the 'C' group. The percentage of reductions for IgG were 78.4% (125 mg/kg), 90.1% (500 mg/kg) and 89.5% (2000 mg/kg); PAF, 167.4% (125 mg/kg), 167.2% (500 mg/kg) and 171.1% (2000 mg/kg); and for IL-6, 70.1% (125 mg/kg), 67.7% (500 mg/kg) and 92.6% (2000 mg/kg). In comparison, ketotifen fumarate at 1 mg/kg was able to reduce the amount of IgG by 88.1%, PAF by 147.2% and IL-6 by 66.6%. It is interesting to note that although induction of IgE was observed upon the OVA challenge, there was no significant difference compared to the 'Sham' group (figure not shown). There was also a slight reduction of IgE levels in the KF-treated but not CNAE-treated groups (figure not shown). KF is a mast cell stabilizer, and it was used as a positive control in this assay to ensure that the model is reliable and reproducible to analyze the anti-allergy effect of CNAE. Similarly, it was used for the same purpose

in the IgE-challenged passive systemic anaphylaxis model.

3.2. IgE-challenged passive systemic anaphylaxis (IgE-PSA)

The OVA-ASA analysis showed that CNAE does possess anti-allergy activity, but it did not indicate the targeted pathway because both the IgE and IgG-mediated pathways were triggered. Therefore, to determine the targeted pathway, IgE and IgG-PSA models were used to evaluate further the specific pathway targeted by CNAE. Also, as the IgE pathway was not being significantly induced in the OVA-ASA model, the IgE-PSA experiment was conducted to validate the effect of CNAE on the IgE-mediated pathway alone. In IgE-PSA, only the IgE pathway of anaphylaxis will be triggered. From the analysis, no significant inhibition of all three soluble mediators tested at all doses of CNAE was noted (figures not shown). Only ketotifen fumarate (the positive control) showed inhibition of histamine in the IgE-PSA experiment (figures not shown).

3.3. IgG-challenged passive systemic anaphylaxis (IgG-PSA)

Like the IgE-PSA model, only the IgG pathway of anaphylaxis will be triggered in the IgG-PSA model. Based on our earlier meta-analysis findings (Kow et al., 2019), PAF, IL-6 and TNF- α are a few of the most analyzed soluble mediators in IgG-challenged anaphylaxis. As shown in

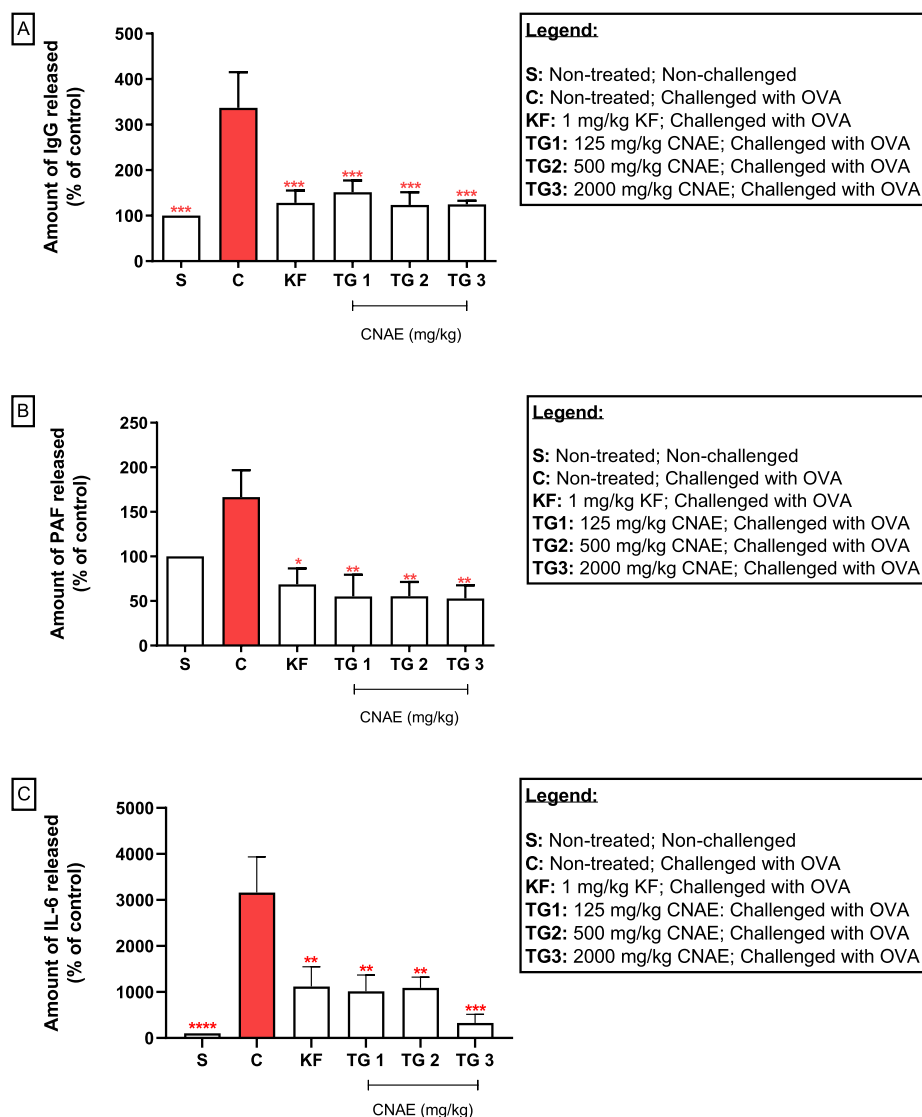


Fig. 1. Effect of CNAE on plasma A) IgG, B) PAF and C) IL-6 of OVA-ASA animals. Rats were pre-sensitized with OVA and AIOH₃ twice intraperitoneally on Days 0 and 6, pre-treated with CNAE for 1 h (oral) and challenged with OVA on Day 13 for 1 h intravenously. Plasma was collected and analyzed by ELISA. Data are presented as mean $\pm$ SEM where $n = 3-6$ and statistical analysis of one-way ANOVA and Dunnett's *post-hoc* was performed. Statistical differences were denoted as * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$.

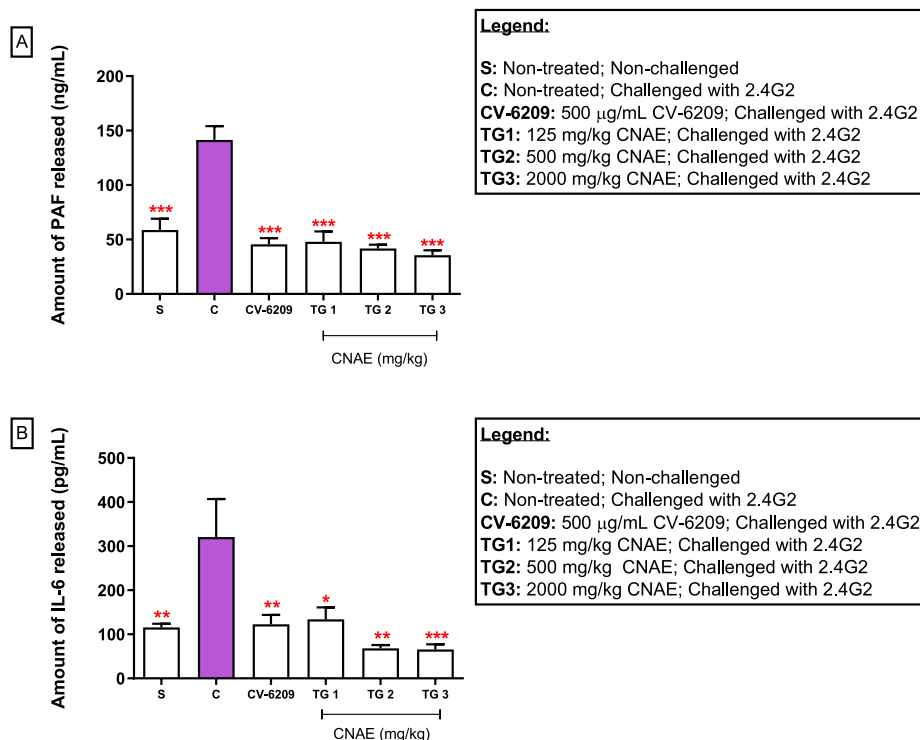


Fig. 2. Effect of CNAE on plasma A) PAF and B) IL-6 of IgG-PSA animals. Mice were pre-treated with CNAE for 1 h (oral) and challenged with 2.4G2 antibodies for 2 h (intravenously). Plasma was collected and analyzed with a magnetic Luminex assay. Data are presented as mean $\pm$ SEM where $n = 6$. One-way ANOVA and Dunnett's *post-hoc* analyses were performed. Statistical differences were denoted as $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

Fig. 2, there were significant reductions of both PAF and IL-6 levels in all CNAE pre-treated mice. The percentage of reductions for PAF were 113.2% (125 mg/kg), 120.7% (500 mg/kg) and 128.2% (2000 mg/kg) while for IL-6 were 91% (125 mg/kg), 123.2% (500 mg/kg) and 124.4% (2000 mg/kg). On the other hand, CV-6209 reduced the amount of PAF by 116% and IL-6 by 96.4%, respectively. In this analysis, CV-6209 which is a PAF antagonist was used as a positive control for the assay to ensure that the chosen model is reliable and reproducible to evaluate the anti-allergy activity of CNAE.

3.4. Cytotoxicity

Cytotoxicity analysis was performed to ensure that the anti-allergy effect of CNAE on IgG-induced macrophage was attributed to the extract alone and not due to its cytotoxic effect on the cells. As shown in Fig. 3, the extract was non-cytotoxic to IC-21 cells from 1 to 7 mg/mL, with more than 90% cell viability recorded. At concentrations of 8–10 mg/mL, the extract was significantly cytotoxic to the cells as the cell viabilities at these higher concentrations dropped to between 65% and 72%. From this analysis, three non-cytotoxic concentrations were

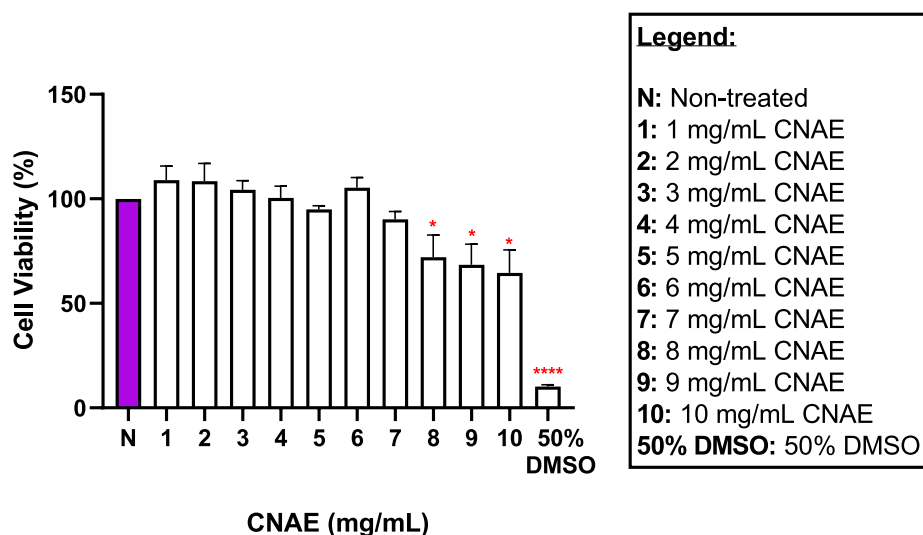


Fig. 3. Cytotoxicity profile of IC-21 cells after 24 h treatment with CNAE analyzed by MTS assay. Cells were treated with CNAE at a concentration range of 1–10 mg/mL for 24 h. Data are presented as mean $\pm$ SEM where $n = 3$. Data were analyzed with one-way ANOVA and Dunnett's *post-hoc*. Statistical differences were denoted as $*p \leq 0.05$ and $****p \leq 0.0001$.

chosen to evaluate their effect on the soluble mediators released upon IgG induction.

3.5. IgG-induced macrophage activation

Our previous experiments showed that CNAE exerted its anti-allergic effects via IgG-mediated pathway, evidenced by the suppression of soluble mediators involved in the IgG-mediated pathway such as PAF and IL-6. In this IgG-dependent pathway, macrophages are the major effector cells, and IgGs are the immunoglobulins involved (Escribese et al., 2017). When IgGs crosslink with their receptors on macrophages, a wide variety of soluble mediators that cause allergy symptoms will be released into the system. Hence, the effects of CNAE on these soluble mediators released by IC-21 macrophages were analyzed. All three concentrations of CNAE – 1.75, 3.5 and 7 mg/mL recorded significant IL-6 and TNF- α inhibitions, as shown in Fig. 4. The percentage of suppression of IL-6 were 88.4% (1.75 mg/mL), 96.9% (3.5 mg/mL) and 99.8% (7 mg/mL); whilst for CV-6209 (positive control) at 1 μ M, the suppression was 88.8%. CNAE was also able to inhibit the release of TNF- α – 91.4% (1.75 mg/mL), 103.7% (3.5 mg/mL) and 87.7% (7 mg/mL); comparatively the percentage of inhibition by CV-6209 was 57.4%.

3.6. The anti-allergy mode of action of CNAE

Several pathways and kinases govern the activation and degranulation of macrophages after IgG aggregation to Fc γ receptors. Fig. 5A showed the inhibition of ERK phosphorylation by 88.7%, while the specific inhibitor PD98059 recorded an inhibition of 95.4% compared to the induced group. Unexpectedly, an increase in the phosphorylation of p38 kinase was seen in CNAE pre-treated IgG-induced cells, as depicted in Fig. 5B. Whereas SB203580, the specific inhibitor of p38 kinase, was able to inhibit the kinase's phosphorylation by 59.6% compared to the induced group, as shown in Fig. 5B.

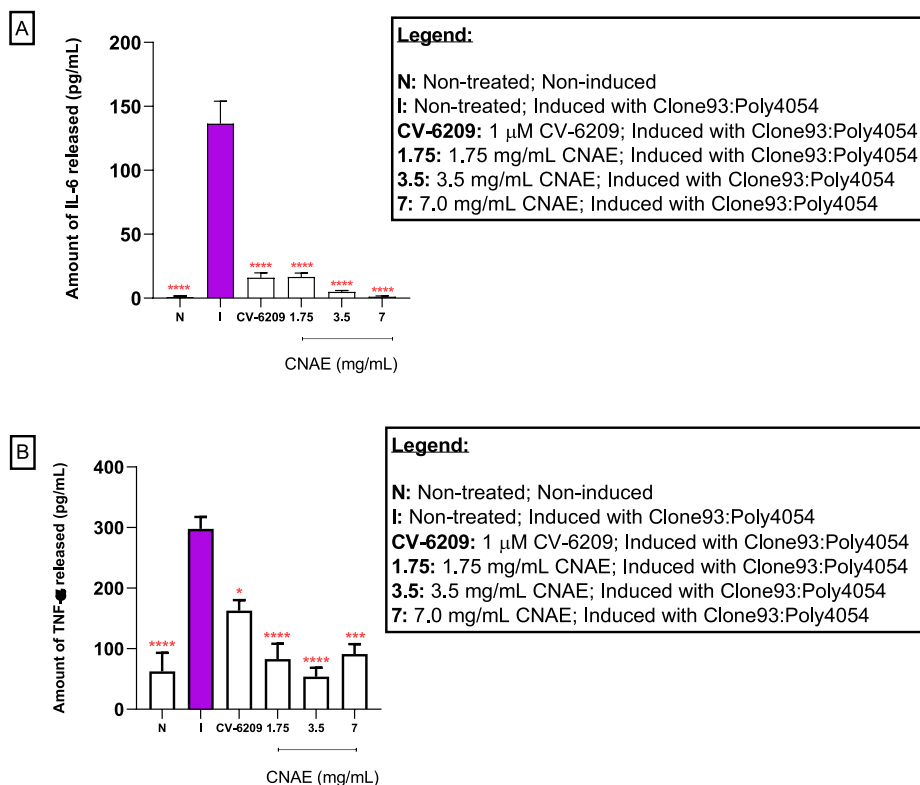


Fig. 4. Effect of CNAE on the release of A) IL-6 and B) TNF- α upon IgG-induced macrophage activation. IC-21 macrophages were pre-treated with different concentrations of CNAE extract for 4 h and subsequently challenged with Clone93:Poly4054 antibodies for 6 h. Supernatants were then collected and analyzed by ELISA. CV-6209 (1 μ M) used as the positive control is a PAF inhibitor. Data are presented as mean $\pm$ SEM where $n = 3$ and analyzed with one-way ANOVA and Dunnett's *post-hoc*. Statistical differences were denoted as * $p \leq 0.05$ *** $p \leq 0.001$ and **** $p \leq 0.0001$.

4. Discussion

In this study, the anti-allergic activity of *C. nutans*, an herbal plant traditionally used to treat skin rash – a symptom of allergy was investigated using *in vivo* model of anaphylaxis. Anaphylaxis is an acute, potentially fatal systemic allergic reaction with varied clinical presentations and mechanisms. In terms of mechanisms, anaphylaxis can be mediated via two pathways known as the IgE and IgG pathways. IgE-mediated anaphylaxis is well established and is the main anaphylactic pathway. However, increasing evidence obtained from animal models supports the existence of a second pathway. Incidences of IgG-mediated anaphylaxis in humans are not conclusive, but PAF has shown to be released from activated macrophages that have IgG receptors and subsequently activate mast cells *in vitro* and there were cases of non-IgE dependent anaphylaxis involving omalizumab, an IgG anti-IgE antibody and infliximab (Kajiwara et al., 2010; Khan and Kemp, 2011). As IgG-mediated anaphylaxis has been proven to occur in murine and, Fc γ IIB and Fc γ IIIC receptors are found on mast cells and macrophages respectively of both mice and humans, it was postulated that IgG-mediated anaphylaxis could happen in humans too (Finkelman, 2007).

Based on our previous findings on the anti-allergic activity of CNAE in an *in vitro* model of IgE-mediated mast cell degranulation and a rodent model of OVA-ASA using ^{1} H-NMR metabolomics approach, we found that CNAE significantly reduced both β -hexosaminidase and histamine at 5 mg/mL and above in the *in vitro* model (Kow et al., 2020) and, OVA-ASA rats were alleviated from anaphylaxis due to the down-regulation of carbohydrates and lipid metabolism as reported by Khoo et al. (2018). We subsequently assessed the anti-allergic effect of CNAE in *in vivo* models mimicking anaphylaxis to understand better the anti-allergic effects of CNAE and its underlying mechanism of action by focusing on the IgE and IgG-mediated pathways. A broad range of CNAE doses (125, 500 and 2000 mg/kg) was evaluated according to previous studies by Khoo et al. (2018). A 14-day acute toxicity profile performed by Khoo et al. (2018) revealed that a single dose of 5000 mg/kg CNAE

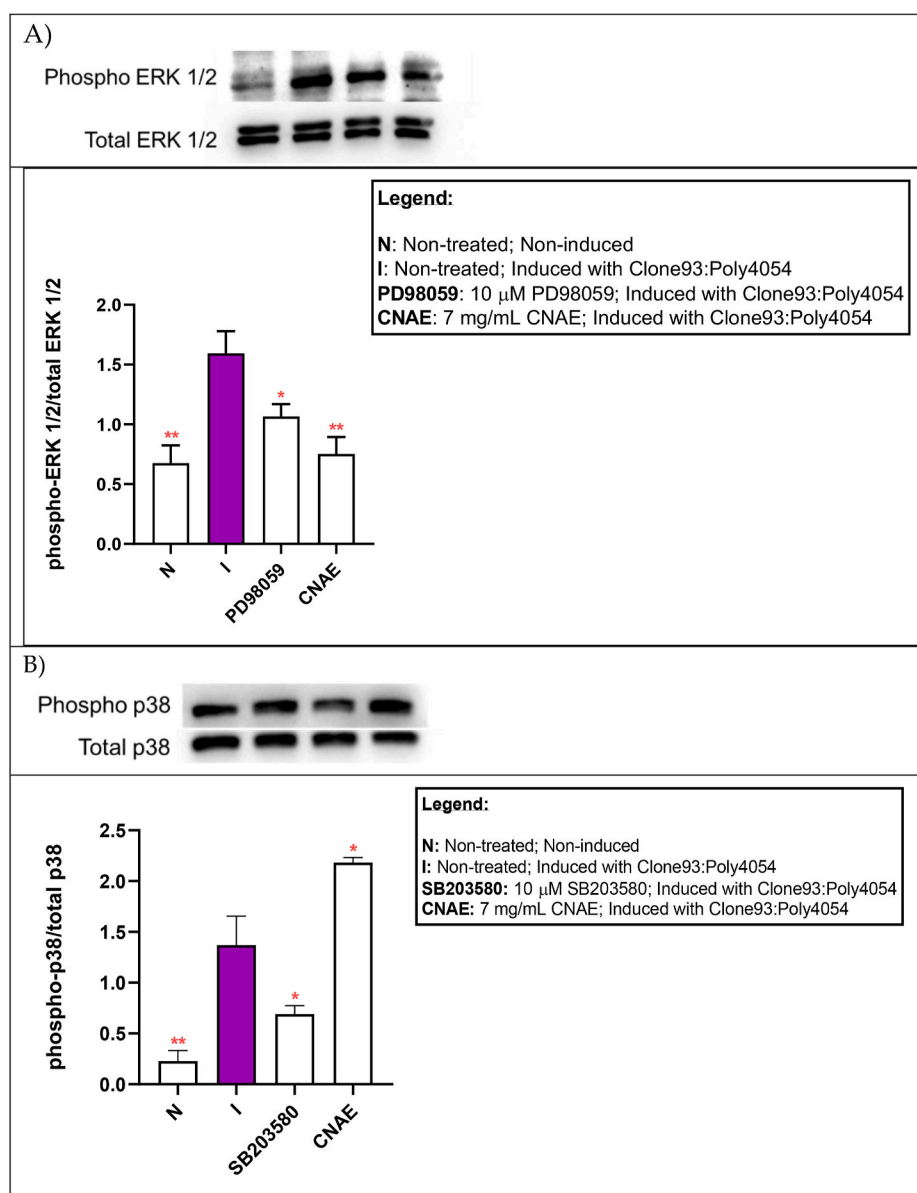


Fig. 5. Effect of CNAE on A) ERK 1/2 kinase and B) p38 kinase by Clone93:Poly4054 (IgG antibodies) induced IC-21 cells after 4 h pre-treatment analyzed by Western blotting. IC-21 cells were induced for 15 min and 30 min respectively. Data are presented as mean $\pm$ SEM where $n = 3$ and analyzed with one-way ANOVA and Dunnett's *post-hoc*. Statistical differences were denoted as $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

was not toxic to the experimental rats. From the $^1\text{H-NMR}$ analysis of the OVA-ASA model, the anti-allergy effects were better in the 500 and 2000 mg/kg CNAE treated group of animals, suggesting better protective effects at higher doses (Khoo et al., 2018). Similar findings were also reported by Thongharb and Tejasen (1977), where 2000 mg/kg of 95% ethanol extract of *C. nutans* showed only a moderate anti-venom effect. Pongphasuk et al. (2005) also reported that 95% ethanol extract of *C. nutans* at 5000 mg/kg showed moderate anti-inflammatory effect compared to the other herbal extracts.

Although the anti-allergy effect of CNAE on IgE pathway was not significant even at 2000 mg/kg, interestingly, the anti-allergy effect of CNAE on IgG pathway was prominent as low as 125 mg/kg. In the OVA-ASA model, both IgE and IgG pathways of anaphylaxis are activated. CNAE did not suppress the release of IgE in Sprague Dawley rats, while there was suppression in the levels of IgG, PAF and IL-6. These results showed that CNAE does potentially possess anti-allergic properties. However, further confirmation is required to determine CNAE's anti-allergy properties as the other soluble mediators involved in the IgE-

mediated pathway, such as histamine and IL-4, could not be detected in the plasma of the test animals. Despite the inability to detect histamine and IL-4 in the plasma of the test animals anaphylaxis still took place as Guo et al. (2001) previously reported that active systemic anaphylaxis could still be triggered in mast cell-deficient rats. Oettgen et al. (1994) have also reported that despite being lack of IgE producing *Cε* gene, their homozygous null mutated mice were still able to display anaphylactic reactions upon antigen challenge.

The plasma and urine samples collected from these test animals were subjected to a metabolomics study performed by our research group (Khoo et al., 2018). The metabolomic profile revealed upregulation of carbohydrates (glucose, myo-inositol, galactarate) and lipid metabolisms (glycerol, choline, sn-glycero-3-phosphocholine) in anaphylactic rats. Upon pre-treatment with 2000 mg/kg of CNAE, choline and sn-glycero-3-phosphocholine levels were downregulated. Choline is an essential component of phosphatidylcholine lipids, and PAF (1-O-Hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) is one of them (Chalcraft et al., 2014). The low level of choline corresponded with our current

finding where there was a decrease in PAF level in the plasma of CNAE-treated rats (Fig. 1B). Even though the potential protective effect of CNAE on OVA-ASA was supported by the metabolomics findings of Khoo et al. (2018), it did not suggest the specific targeted pathway of protection and the immunological modulation underlying the anti-anaphylactic effects of CNAE. Hence, both IgE-PSA and IgG-PSA models were used to evaluate the anti-anaphylactic effects of CNAE further.

Two different animal species from the rodent family – Sprague Dawley rats and C57BL/6 mice were used in the IgE- and IgG-PSA models respectively. The use of rats in both OVA-ASA and IgE-PSA models are well supported by previous studies (Yamamoto et al., 1984; Carvalho et al., 2003; Kuda et al., 2019; Ufkes et al., 1983; Pellón et al., 1994; Oka et al., 2012). Unfortunately, there was no established rat model for IgG-PSA to our best knowledge. Hence, mice were used for IgG-PSA in reference to the study by Falanga et al. (2012) which used the C57BL/6 mice. Although animals of different species were used for the IgE-PSA and IgG-PSA models, both species are from the rodent family and have been proven to be genetically similar (Rat Genome Sequencing Project Consortium, 2004). In the IgE-PSA model, male Sprague Dawley rats were sensitized with IgE and challenged to anaphylaxis with the allergen DNP-BSA to specifically trigger the IgE pathway. Histamine is an early phase mediator released by mast cells upon aggregation. In contrast, the release of late-phase mediators such as IL-4 and LTC₄ results from downstream activation of phospholipase A₂, cyclooxygenases and lipoxygenases (Peavy and Metcalfe, 2008). Although CNAE had been previously reported to inhibit *in vitro* model of IgE-mast cell degranulation, however, the effect of CNAE was seen only on early phase mediators such as histamine and β -hexosaminidase at concentrations of CNAE of 5 mg/mL and above without affecting the release of late-phase mediators including IL-4 and LTC₄. In this model, the levels of histamine, IL-4 and LTC₄ in the CNAE-treated groups were like the challenged group, even at 2000 mg/kg. Our finding in IgE-PSA has once again confirmed the observations in the OVA-ASA model that CNAE could not inhibit the release of IgE-mediated pathway mediators. These findings have collectively led us to postulate that the anti-anaphylactic activity of CNAE was not exerted via the IgE pathway.

In the IgG-PSA model, C57BL/6 mice were challenged to anaphylaxis with a 2.4G2 antibody. We found significant reductions in PAF and IL-6 in the plasma of the CNAE-treated animals. The reduction of these soluble mediators has further supported our previous findings from the OVA-ASA model. PAF is regarded as the key mediator in the IgG-mediated anaphylaxis (Finkelman, 2007), and it also indicates the severity of an anaphylactic reaction (Vadas et al., 2008). Hence, reducing the amount of PAF could halt the severity and progression of anaphylaxis (Vadas et al., 2008). Ishii et al. (1998) also found that PAF-receptor deficient mice did not develop anaphylaxis when challenged with OVA. A few organic and synthetic PAF inhibitors were reported to have the ability to inhibit the biological activities of PAF. Ginkgolides from *Ginkgo biloba* extracts, alpha-bulnane from *Pogostemon cablin*, yangambin from *Ocotea duckei* Vattimo and lipid extracts from *Urtica dioica* have been shown to act as PAF inhibitors, and these plants have been used traditionally as an allergy relief (Papakonstantinou et al., 2017). Rupatadine, a second-generation H₁-antihistamine that can inhibit both histamine H₁- and PAF receptors, is a synthetic PAF indicator that has shown positive results in alleviating symptoms of allergic rhinitis and chronic urticaria (Mullol et al., 2015). Besides reducing the level of PAF, CNAE was also able to reduce the levels of IL-6. Stone et al. (2009) reported that elevated serum IL-6 was found in anaphylactic patients, which correlated to the severity of the reaction.

Ketotifen fumarate, a mast cell stabilizer was used in the OVA-ASA and IgE-PSA models because mast cells are the main effector cells that are activated to release histamine and other pro-inflammatory mediators that bring about the symptoms of allergy and anaphylaxis in these models. Ketotifen fumarate can prevent mast cells from degranulating and thus the release of the pro-inflammatory mediators including

histamine and IL-4 (Mita and Shida, 1995). CV-6209, a PAF antagonist was used as the positive drug control in the IgG-PSA model because PAF is the key proinflammatory mediator in the IgG anaphylaxis pathway (Musio et al., 2009). Based on the study of Musio et al. (2009), CV-6209 was reported to reduce the incidences of anaphylaxis in their mice that are mast cells or histamine deficient. Ketotifen fumarate and CV-6209 showed better effects compared to CNAE based on their pharmacological titers and it is highly attributed to the fact that both drugs are pure compounds whilst CNAE is an extract. It is important to note that the aim of this study was to evaluate the potential anti-allergy activity of CNAE in the hope that it could be developed as a complementary herbal medicine instead of an alternative to the current and proven anti-allergy medications that have been used for many years.

As of now, our findings show that CNAE exerts anti-anaphylactic effects via IgG-mediated pathway. However, the underlying mechanism of action remains unknown. Macrophages are the primary effector cells involved in the IgG-mediated pathway, and the immunoglobulins involved are IgGs (Escribese et al., 2017). The crosslinking of IgG to Fc γ found on macrophages causes the activation of the cells. The activated cells release pro-inflammatory mediators that trigger allergy symptoms when triggered by an allergen. In our model, IgG antibodies, Clone93 and Poly4054, were used to activate the IC-21 macrophages to release these pro-inflammatory mediators directly. Before we evaluate the anti-allergic properties of CNAE, a cytotoxicity assessment was carried out, and from there, we found that CNAE is non-cytotoxic to IC-21 macrophages from 1 to 7 mg/mL. With this analysis, three non-cytotoxic concentrations (1.75, 3.5 and 7 mg/mL) were chosen to evaluate their anti-allergic effect on IgG-induced macrophage activation assays. It was observed that there were significant reductions in the amount of IL-6 and TNF- α released by IC-21 macrophages even at 1.75 mg/mL after the cells were being pre-treated with CNAE. The significant reductions were comparable to the CV-6209, a potent PAF receptor antagonist. Both IL-6 and TNF- α have been identified as the few common soluble mediators studied in the alternative IgG pathway. The parallel decrease of both soluble mediators is possible because TNF- α could reduce the signal transduction of IL-6 through the internalization of gp130. gp130 is the signal transducer of IL-6, and the dimerization of gp130 will bring about the initiation of several signaling pathways to produce IL-6 (Schaper and Rose-John, 2015).

As both IgG-Fc γ and IgE-Fc ϵ RI aggregation and cell activation shared similar pathways, it is not surprising that the suppression of both IL-6 and TNF- α was attributed to the inhibition of the phosphorylation of ERK1/2 kinase of the MAPK pathway (Falanga et al., 2012). ERK1/2 kinase was found to play a role in regulating IgE-mediated mast cell activation and degranulation, causing the release of eicosanoids and soluble mediators. Hence the activation of the ERK pathway in IgG-induced IC-21 cells will result in similar responses, whereby there will be a production of pro-inflammatory mediators. As inhibition of ERK1/2 kinase phosphorylation that leads to the suppression of IL-6 and TNF- α in IgG-induced macrophages was observed, it was hypothesized that this contributed to the anti-allergy activity of CNAE. The suppression of ERK1/2 kinase phosphorylation could be due to the activation of 5' monophosphate-activated protein kinase (AMPK), which has anti-inflammatory action. Hwang et al. (2014) reported that AMPK could block the production of pro-inflammatory mediators in parallel to ERK1/2 specific inhibitors – U0126 or PD98059. These inhibitors could inhibit the phosphorylation of ERK1/2, JNK and I κ B kinases (IKK) and soluble mediators such as β -hexosaminidase, LTC₄, PGD₂, TNF- α and IL-6, which were also seen in activated AMPK (Hwang et al., 2014). Surprisingly, an up-regulation of p38 kinase was also observed. p38 pathway is the main contributor that causes the release of pro-inflammatory soluble mediators when activated. However, due to the reduction of IL-6 and TNF- α , it was postulated that there could be an inhibition in the downstream kinase known as the MAPK-activated protein kinase 2 (MAPKAP K2), a substrate of p38 α MAPK, but this requires further analysis in the future (Kumar et al., 2003). MAPKAP K2 is

directly regulated by the phosphorylation of p38 MAP kinase and the p38 α /MAPKAP K2 pathway plays an important role in the production and signaling of inflammatory cytokines (Kotlyarov et al., 1999; Kumar et al., 2003). MAPKAP K2-deficient mice were found to have lower IL-6 and TNF levels when induced by lipopolysaccharides as mediated by a mechanism that involves messenger RNA turnover and protein translation (Kumar et al., 2003). Deficiency of MAPKAP K2 was also reported to block the production of short-lived mRNAs of inducible cytokines IL-1, IL-8 and TNF stated Kumar et al. (2003). Therefore, the possibility of the reduced IL-6 and TNF- α in this IgG-induced macrophage activation model was due to down regulation of MAPKAP K2, downstream substrate of p38.

Based on the analyses performed, we propose that CNAE does possess anti-allergy activity. Based on the chromatographic profile of CNAE, several metabolites were identified (Khoo et al. 2019). Among all, flavones are secondary metabolites that are under the flavonoids group. Apart from possessing antioxidant and anti-inflammatory activities, flavonoids were also reported to exert anti-allergy activities (Kawai et al., 2007). In CNAE, flavones such as orientin, isoorientin, vitexin, isovitexin and schaftoside were present. With reference to Teshima et al. (1997) analysis, Khoo et al. (2019) further quantified these five major flavones that are present in *C. nutans*. In general, numerous studies showed that these flavones have anti-inflammatory activity. There were able to suppress the production of TNF- α , IL-6, IL-8 and IL-1 β among other proinflammatory mediators (Khoo et al., 2019). Hence, we postulate that the anti-allergic activity of CNAE could be attributed to these flavones. Although anti-allergy studies involving these flavones are still in its infancy, there are emerging studies showing that these flavones could have anti-allergic activities too. In a study by Dhakal et al. in 2020, orientin was shown to significantly inhibit IgE-mediated mast cell degranulation by reduction of intracellular calcium levels and the production of proinflammatory cytokines in mast cells (Dhakal et al., 2020). On the other hand, isoorientin from *Phyllostachys edulis* and baicalin from *Scutellaria baicalensis* combined showed favorable anti-allergic effects supported by the significant reduction of histamine and leukotriene levels from mast cells activated by anti-OVA/OVA binding (Kim et al., 2010). Isoorientin also was able to alleviate OVA-induced allergic asthma by reducing IL-4, IL-5, IL-13, malondialdehyde, nitric oxide and reactive oxygen species as reported by Liang et al. (2022). Vitexin from *Spirodela polyrhiza* (L.) Schleid was able to inhibit the degranulation of RBL-2H3 cells (Kim et al., 2018). Zhang et al. (2021) discovered that the isovitexin from *Celtis sinensis* Pers. was able to suppress ginkgolic acids-induced contact dermatitis in their mice model. To our understanding schaftoside has not been tested for its anti-allergy activity yet. However, due to it being a substituted apigenin (substituted by beta-D-glucopyranosyl and alpha-L-arabinopyranosyl), it is postulated to have anti-allergy activity too. A recent study by Park et al. (2020) revealed the anti-allergy activity of apigenin by suppressing the phosphorylation of Lyn, Syk, phospholipase C γ 1, ERK and JNK in addition to the expression of high-affinity IgE receptor (Fc ϵ 1 α) and cytokines such as TNF- α , IL-4, IL-5, IL-6, IL-13, and COX-2 in IgE-DNP-BSA-induced RBL-2H3 cells.

Lastly, it is important to highlight that this present study does possess several limitations that could be further improved in future studies. OVA was used as the anaphylactic inducer in our active systemic anaphylaxis experiment as it is a common allergen used in many studies. According to Guo et al. (2001), anaphylaxis reaction was apparent when the animals were challenged with OVA, which recorded a drop in mean arterial pressure. In the other studies utilizing OVA, the release of mediators involved in IgE-mediated pathway such as histamine, IL-4 or mouse mast cell proteinases was detected in the serum of the test animals (Xu et al., 2008; Liu et al., 2018). However, histamine and IL-4 could not be detected in our OVA-ASA model. As a future recommendation, other inducers such as compound 48/80 or *Bordetella pertussis* toxin could be used to induce active systemic anaphylaxis. Also, as this was a preliminary study, only one time point - 1 h was chosen for the

pre-treatment in *in vivo* analysis. Further evaluations with different time points could be assessed as the increase duration will allow for better metabolization and distribution of CNAE in the body. Additionally, to demonstrate the concentration- or dose-dependent effects of CNAE, future studies may consider evaluating its anti-allergic properties by using lower concentrations or doses of CNAE in both *in vitro* and *in vivo* experiments. Furthermore, as the inhibitory effects exerted by 2000 mg/kg CNAE, and the lower doses of 125 mg/kg and 500 mg/kg were comparable, further analysis could analyze the anti-allergy activity of CNAE at lower than 125 mg/kg so that it could be translated for human usage in the future. Doing this will help determine the most effective concentration and dose of CNAE in the management of allergic diseases. In addition, further evaluation of CNAE at 2000 mg/kg as a topical cream to treat skin rash that is induced by IgG or IgG-induced contact dermatitis could be carried out as this high dose was used in a study by Gupta et al. (2013). In this study 2000 mg/kg of methanolic extract of *Cassia tora* L. leaves was formulated into a topical oil in water cream and it prevented ultraviolet-B light-induced oxidative stress in rats (Gupta et al., 2013). A couple of clinical studies of incorporating 5% of *C. nutans* as a cream formulation had also been carried out by Charuwichitratana et al. (1996) and Sangkitporn et al. (1995) indicating its possibility to be formulated into a cream.

5. Conclusions

In the OVA-ASA model, CNAE was found to reduce the levels of IgG, PAF and IL-6 but not IgE. Apart from that, CNAE was also able to reduce the release of PAF and IL-6 in the IgG-PSA model. However, CNAE could not reduce the levels of histamine, IL-4 and LTC $_4$ in the IgE-PSA model. The *in vitro* IgG-induced macrophage activation analysis found reductions of IL-6 and TNF- α in CNAE pre-treated IC-21 macrophages by inhibiting the phosphorylation of ERK1/2 kinase of the MAPK pathway. All in all, we postulated that CNAE could potentially be a traditional herbal candidate in targeting the IgG pathway of allergy and possibly a PAF inhibitor.

Author contributions

Conceptualization and Methodology, all authors; Experimental Performance, ASFK, LWK and JWT; Data Analysis and Interpretation, ASFK, Writing-Original Draft Preparation, ASFK and CLT, Writing-Review and Editing, all authors; Supervision, CLT, FA, MTL, DAI and KS. All authors have read and agreed to the published version of the manuscript.

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CRediT authorship contribution statement

Audrey Siew Foong Kow: Conceptualization, Methodology, Writing – review & editing, Formal analysis, Writing – original draft. **Leng Wei Khoo:** Conceptualization, Methodology, Writing – review & editing. **Ji Wei Tan:** Conceptualization, Methodology, Writing – review & editing. **Faridah Abas:** Conceptualization, Methodology, Supervision, Funding acquisition. **Ming-Tatt Lee:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Daud Ahmad Israif:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Khodzirah Shaari:** Conceptualization, Methodology, Writing – review & editing. **Chau Ling Tham:** Supervision, Conceptualization, Funding acquisition, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jep.2022.116003>.

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