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ORIGINAL ARTICLE

Clinacanthus nutans Aqueous Extract Suppresses the Release of Histamine and β-Hexosaminidase in *in-vitro* Model of IgE-Mediated Mast Cell Degranulation

Audrey Siew Foong Kow[1], Lai Yen Fong[1], Leng Wei Khoo[2], Ji Wei Tan[1], Ming-Tatt Lee[3,4,5], Daud Ahmad Israf[1], Faridah Abas[2,6], Khozirah Shaari[6,7], Chau Ling Tham[1]

[1]Department of Biomedical Science, Faculty of Medicine and Health Sciences, [2]Department of Food Science, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 Serdang, [3]Faculty of Pharmaceutical Sciences, UCSI University, 56000 Kuala Lumpur, Malaysia, [4]Graduate Institute of Brain and Mind Sciences, College of Medicine, [5]Graduate Institute of Pharmacology, College of Medicine, National Taiwan University, Taipei, Taiwan, [6]Laboratory of Natural Products, Institute of Bioscience, [7]Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang, Malaysia

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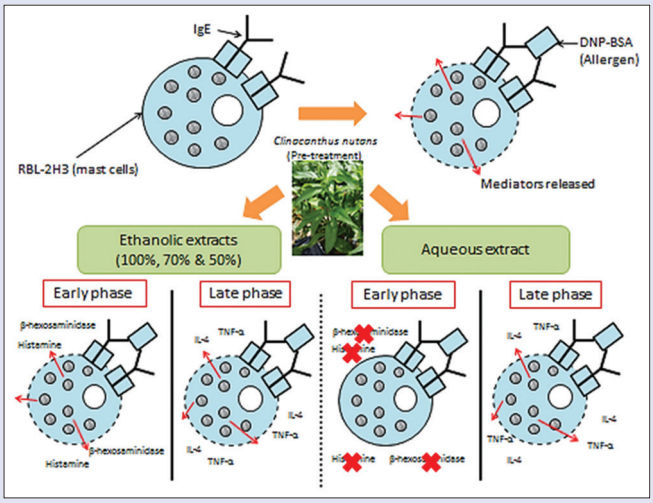
ABSTRACT

Introduction: *Clinacanthus nutans* (Burm. f.) Lindau, a shrub found in South East Asia, particularly in Malaysia, Thailand, and Indonesia has many potential medicinal uses. It is used as a traditional herbal medicine for treating many diseases including skin rashes. Skin rash often appear in an allergic reaction. Recently, we have shown that *C. nutans* aqueous extract has the ability to alleviate ovalbumin-induced active systemic anaphylaxis rats from anaphylaxis – the acute form of allergy. This present study is aimed at comparing the ability of ethanolic and aqueous extracts of *C. nutans* to suppress IgE-mast cell degranulation. **Materials and Methods:** IgE-presensitized rat basophilic leukemic (RBL-2H3) cells pretreated with *C. nutans* ethanolic (100% ethanolic, 70% and 50% aqueous ethanolic) or 100% aqueous extracts were challenged with dinitrophenyl-bovine serum albumin to analyze the release of early and late-phase pro-inflammatory mediators. **Results:** We found that at concentrations of 5 mg/ml and above, *C. nutans* aqueous extract was able to suppress the levels of β-hexosaminidase and histamine while suppression was not seen in ethanolic extracts pretreated RBL-2H3 cells. **Conclusion:** Hence, we proposed that *C. nutans* aqueous extract is more active compared to the ethanolic extracts in suppressing the mediators of IgE-mast cell degranulation.

Key words: *Clinacanthus nutans*, histamine, IgE-mediated mast cell degranulation, pro-inflammatory mediators, β-hexosaminidase

SUMMARY

- None of the ethanolic extracts of *Clinacanthus nutans* were able to suppress the release of early phase (β-hexosaminidase and histamine) nor late phase (interleukin-4 [IL-4] and tumor necrosis factor-alpha [TNF-α]) mediators
- *C. nutans* aqueous extract was able to suppress the release of β-hexosaminidase and histamine but not IL-4 and TNF-α
- *C. nutans* aqueous extract might be exerting its anti-allergy activity partly through IgE-mediated mast cell degranulation but the effect on IgG-mediated pathway remains unknown and should be further studied.



Abbreviations used: °C: Degree celsius; μm: Micrometer; μl: Microliter; μg/ml: Microgram/milliliter; mg/ml: Milligram/milliliter; nm: Nanometer; mM: Millimolar; g: Gram; min: Minute; h: Hour; CO_2: Carbon dioxide; mg/kg: Milligram/kilogram

Correspondence:

Dr. Chau Ling Tham,
Department of Biomedical Science, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia.
E-mail: chauling@upm.edu.my

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INTRODUCTION

Clinacanthus nutans (Burm. f.) Lindau is a perennial shrub that can be found in Malaysia, Indonesia, and Thailand and has been used traditionally by the locals to cure a variety of illnesses. Conventionally consumed as an herbal tea in Malaysia or as blended raw material in juice or used as an external treatment, this plant has gained much popularity as an anticancer treatment option among Malaysians.[1-3] In 2006, it was even regarded as a principal medicinal plant for primary health care by the Thai Ministry of Public Health to treat insect and snake bites, skin rashes, herpes simplex virus (HSV), and varicella zoster virus (VZV) lesions.[3-5] Besides being an effective treatment of HSV and VZV lesions and possesses anti-viral activity, *C. nutans* also potentially possesses

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anti-oxidant, anti-cancer, anti-inflammatory, and analgesic and anti-diabetic properties.[2,6]

Traditionally, the fresh leaves of *C. nutans* (Burm. f.) Lindau have been used to treat skin rashes, one of the symptoms of allergy.[6] Allergy is a hypersensitivity reaction caused by immunologic mechanisms that can be mediated by antibody or cell.[7] Typically, allergic reaction happens when there is a cross-linking of an allergen to the IgE antibodies bound to high affinity receptor FcεRI on mast cells. This in turn causes the mast cells to degranulate and release preformed mediators stored in cytoplasmic granules, cytokines, and chemokines. Some of these mediators include histamine, tumor necrosis factor-alpha (TNF-α), and interleukin-4 (IL-4) which are generally pro-inflammatory mediators that will enhance an allergic reaction.[8] Hence, in order to determine the anti-allergic property of *C. nutans*, the effect of different preparation of this plant – ethanolic and aqueous extracts were examined using an *in vitro* model of IgE-mast cell degranulation.

MATERIALS AND METHODS

Materials

Monoclonal anti-dinitrophenyl (DNP) antibody isotype IgE (IgE), 4-Nitrophenyl N-acetyl-β-D-glucosaminide (PNAG), calcium chloride ($CaCl_2$), magnesium chloride ($MgCl_2$), HEPES, and aluminum hydroxide were purchased from Sigma Aldrich, Inc., (St. Louis, MO, USA). DNP-Albumin Conjugate, Bovine (DNP-bovine serum albumin [BSA]) was purchased from Calbiochem (EMD Chemicals, Inc.,) (San Diego, CA, USA). Glucose and citric acid were purchased from Merck (Darmstadt, F. R., Germany). Sodium chloride (NaCl), BSA and 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) were purchased from Amresco (Solon, OH, USA). Potassium chloride (KCl) was purchased from HmbG® Chemicals (Hamburg, Germany). Glycine was from Bio Basic Canada, Inc., (Markham, Ontario, Canada). Eagle's Minimum Essential Medium (EMEM), sodium pyruvate, and fetal bovine serum (FBS) were purchased from Gibco by Life Technologies (Grand Island, NY, USA). Triton X-100 was purchased from Acros Organics (New Jersey, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Loughborough, UK). Nylon syringe filter (0.22 μm) was purchased from Bioflow (Malaysia). Enzyme-linked immunosorbent assay (ELISA) kits for TNF-α (Catalog number: DY510) and IL-4 (Catalog number: DY504) were purchased from R and D Systems, Inc., (Minneapolis, MN, USA). ELISA kit for histamine (Catalog number: A05890) was purchased from Bertin Pharma (formerly SPI-BIO) (Montigny Le Bretonneux, France).

Methods

Clinacanthus nutans extracts

All *C. nutans* – 100% ethanolic, 70% and 50% aqueous ethanolic extracts and 100% aqueous extract were prepared at the Institute of Bioscience, Universiti Putra Malaysia. The plant was collected from Sendayan Commodities Development Centre, Seremban, Negeri Sembilan, Malaysia, with voucher specimen SK 2883/15 deposited at the herbarium of the Institute of Bioscience, Universiti Putra Malaysia. The plant was verified by Dr. Shamsul Khamis, botanist at Institute of Bioscience, Universiti Putra Malaysia. Extracts preparation was done as described previously by Khoo *et al.*[9] Briefly, the whole plant was cleaned with water, wiped dry with tissue and the leaves were separated from the stems. The leaves were then air dried for 2 weeks under shade at 25°C until constant weight was met. A laboratory grinder (Waring, Conair Corporation, Stamford, CT, USA) was used to mill the dried leaves into fine powder and using Retsch (Retsch GmbH, Haan, Germany) test sieve of 315 μm, uniform particle-sized plant powder were obtained. The ground samples ranging from 20 to 25 g were then subjected to ultrasound-assisted extraction method whereby they were sonicated in the Nexul Ultrasonic Cleaner machine (NXP 1002) (Shanghai Kudos, Shanghai, China) for 1 h without heating. Sample to solvent ratio was kept constant at 1 g of plant sample to 10 ml of respective solvents. Extraction process was repeated thrice for each sample. Extracts were filtered and vacuum evaporated to dryness, freeze dried and stored at 4°C for further analysis. The final yield of each extract was recorded in Table 1. Ethanolic extracts of *C. nutans* were reconstituted in DMSO while aqueous extract was reconstituted in deionized water and filtered through 0.22 μm membrane before use in all assays.

Rat basophilic leukemic cells

Rat basophilic leukemic (RBL-2H3) cells ATCC® CRL-2256™ were purchased from the American Type Culture Collection (Manassas, VA, USA). RBL-2H3 cells were maintained in EMEM with 10% sodium pyruvate and 10% FBS at 37°C and 5% CO_2. Cells were subcultured every 2–3 days. The cells used for all assays were from passage 4 to 8.

Cytotoxicity assay

Cytotoxicity of *C. nutans* extracts was evaluated using the MTT assay according to procedures as described previously.[10] Briefly, RBL-2H3 cells (2×10^4 cells/well/100 μL) were seeded in 96-well plates overnight. Seeding concentration was determined by MTT optimization assay with optimal seeding range for RBL-2H3 cells for MTT between 0.5×10^4 and 2×10^4 cells/well/100 μL in 96-well plate (data not shown). Cells were treated with a range of concentrations (ethanolic extracts: 100–1000 μg/ml and aqueous extract: 0.1, 0.5, 1–8 mg/ml) for 24 h. After 24 h, the treatment solution was replaced with 100 μL of EMEM containing 10% FBS added with 20 μL of MTT solution (final concentration: 0.8 mg/ml) and incubated for 4 h. Subsequently, the solution was replaced with 100 μL of DMSO to dissolve the purple formazan formed and the plate was read at 570 nm using the automated microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA). Cell viability percentages were calculated based on Equation 1:

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

Measurement of β-hexosaminidase production

The measurement of β-hexosaminidase production was performed as previously described with slight modification.[11] Briefly, RBL-2H3 cells (4×10^4 cells/well/100 μL) were seeded into 96-wells tissue cultured plate overnight followed by presensitization with 0.1 μg/ml of IgE for 24 h. After that, cells were washed twice with Tyrode's buffer (NaCl, 130 mM; KCl, 5 mM; $CaCl_2$, 1.4 mM; $MgCl_2$, 1 mM; Glucose, 5.6 mM; HEPES, 10 mM; and BSA, 0.1%). Cells were then pretreated with *C. nutans* ethanolic or aqueous extracts at different concentrations for 4 h. Subsequently, cells were challenged with 1 μg/ml of DNP-BSA for 1 h at 37°C in a 5% CO_2 incubator. The reaction was stopped by incubating the plate at 4°C for 15 min. To quantitate the spontaneous release of β-hexosaminidase, 50 μL of supernatant was transferred into 96-well non-tissue culture-treated plate and incubated with 50 μL PNAG, 1 mM (dissolved in citric acid buffer, pH 4.5) for 1 h at 37°C followed by 50 μL of 400 mM glycine

Table 1: Yield of each *Clinacanthus nutans* extracts

Extract	Ground dry sample (g)	Yield (g)
50% aqueous ethanolic extract	20.11	4.80
70% aqueous ethanolic extract	20.09	3.25
100% ethanolic extract	25.03	1.31
Aqueous extract (100%)	20.07	3.98

buffer (pH 10.7) to stop the reaction. To quantitate the total release of β-hexosaminidase, cells were lysed with 100 µL of 0.1% Triton-X by re-suspending the cells several times before transferring 50 µL of the lysate and incubated as above. The plates were read at 405 nm using a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA) within 30 min. The percentage of β-hexosaminidase released was calculated based on Equation 2:

$$\text{Amount of } \beta-\text{hexosaminidase released } (\%) = \frac{(\text{Absorbance}_{\text{supernatant}})}{(\text{Absorbance}_{\text{supernatant + total release}})} \times 100 \quad (2)$$

Measurement of histamine production

To determine the effects of *C. nutans* ethanolic and aqueous extracts on the release of histamine in IgE-mediated mast cell degranulation, RBL-2H3 cells were presensitized and pretreated as above and challenged for 1 h.[11] Following that the supernatants were collected and analyzed by ELISA according to the manufacturer's protocol using a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA) at 405 nm.

Measurement of cytokine production

Late-phase mediators – TNF-α and IL-4 were quantitated by ELISA. Cells were presensitized and pretreated as above but challenged for 6 h to induce the synthesis of TNF-α and IL-4.[11] The supernatants were collected and analyzed by ELISA according to the manufacturer's protocols using a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA) at 450 nm with subtraction at 570 nm.

Statistical analysis

All results were expressed as mean ± standard error of the mean of three independent experiments. Results were statistically analyzed by one-way ANOVA followed by Dunnett's *post hoc* analysis (GraphPad Prism, Version 5.02, La Jolla, CA, USA). Statistical differences were considered significant at $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$.

RESULTS

Clinacanthus nutans extract

Table 1 depicts the amount of ground sample (g) subjected to sonication and soaking in their respective solvents and their final yield (g).

Cytotoxicity assay

In order to determine the non-cytotoxic concentration range of *C. nutans* ethanolic and aqueous extracts, cytotoxicity profile for each extract was established as shown in Figure 1. RBL-2H3 cells were treated with a range of concentrations (a) ethanolic extracts – 100–1000 µg/ml and (b) aqueous extract – 0.1, 0.5, 1–8 mg/ml. All *C. nutans* ethanolic and aqueous extracts were not cytotoxic to RBL-2H3 after 24 h treatment even at the highest concentrations used. Although the percentage of viability of 8 mg/ml aqueous extract was not statistically different from the untreated cells, it was excluded from the subsequent experiments as the percentage of viability was lower than 80%.

Measurement of β-hexosaminidase production

Degranulation occurs within second, releasing a variety of preformed inflammatory mediators including β-hexosaminidase and histamine.[12] To quantify the amount of β-hexosaminidase that is being released on mast cell degranulation, RBL-2H3 cells were presensitized with IgE, pretreated with the ethanolic and aqueous extracts at different concentrations, and challenged by DNP-BSA. For all ethanolic extracts (100% ethanolic, 70% and 50% aqueous ethanolic), the concentration range used was between 100 and 1000 µg/ml and it was found that there was no inhibition in the release of β-hexosaminidase [Figure 2]. However, significant inhibition of β-hexosaminidase was recorded in the 100% aqueous extract at concentrations of 5 mg/ml (45.4%), 6 mg/ml (56.4%), and 7 mg/ml (66.1%). The inhibition was comparable to the positive control; ketotifen fumarate used which showed inhibition of 53.8%.

Measurement of histamine production

As one of the main preformed mediators released in allergic reactions, histamine is a short-lived vasoactive amine that causes an immediate increase in local blood flow and vessel permeability.[13] To quantify the amount of histamine that is being released on mast cell degranulation, RBL-2H3 cells were presensitized with IgE, pretreated with the ethanolic and aqueous extracts at different concentrations and challenged by DNP-BSA as shown in Figure 3. For all ethanolic extracts (100% ethanolic, 70% and 50% aqueous ethanolic), it was found that there was no inhibition in the release of histamine. Significant inhibition of histamine was recorded in the aqueous extract at concentrations of 5 mg/ml (11.2%), 6 mg/ml (34.6%), and 7 mg/ml (39.2%) which is comparable to ketotifen fumarate which showed significant inhibition of 32.9%.

Measurement of cytokines production

Late-phase mediators are produced as a result of mast cell activation after the aggregation of IgE to FcεRI receptors on mast cells.[14] To quantify the late-phase mediators of allergy such as IL-4 and TNF-α, RBL-2H3 cells were presensitized (overnight), pretreated (4 h), and induced (6 h) and the supernatant was collected for analysis by ELISA as shown in Figure 4 (IL-4) and Figure 5 (TNF-α). The amount of both IL-4 and TNF-α were not suppressed by all *C. nutans* ethanolic and aqueous extracts at all concentrations tested.

DISCUSSION

The use of natural products as medicine has a long history in Asia. The World Health Organization reported that more than 80% of the world's population look unto these products for their primary healthcare needs. Usually taken as raw material or boiled in water, natural products can be extracted using different solvents such as methanol, ethanol, or mixture of alcohol and water.[15] The choice of solvent to be used is decided based on the intended use of the extract.[16] In this study, *C. nutans* was extracted using ethanol and aqueous, respectively. Ethanolic extract was chosen as it had been used as a topical preparation in treating skin rashes by the Thais.[17] Apart from that, the ethanolic extract of *C. nutans* was being chosen too as it has shown potential anti-inflammatory property in a carrageenan-induced paw edema mice model and also in a bioactive screening; the 80% ethanol extract showed anti-inflammatory, anti-dengue virus, and immune-modulating properties.[18,19] On the other hand, aqueous solution was chosen as a solvent due to its traditional usage, whereby fresh *C. nutans* leaves are often blended into juice or taken as herbal tea. In addition, there was no current *in vitro* study on the aqueous extract of this plant apart from a toxicity study by Farsi *et al.*[20] As both ethanolic and aqueous extracts of *C. nutans* have been used traditionally to treat skin rash – a symptom of allergy and anaphylaxis, it brought us to investigate the possible anti-allergic property of this plant in an *in vitro* IgE-mediated mast cell degranulation model using RBL-2H3 cells. In addition, our research group has recently shown that in an ovalbumin-induced active systemic anaphylaxis (OVA-ASA) *in vivo* model, *C. nutans* aqueous extract was able to protect OVA-induced Sprague Dawley rats from anaphylaxis at 2000 mg/kg evaluated using the ^{1}H-NMR approach. The rats were protected from OVA-ASA through

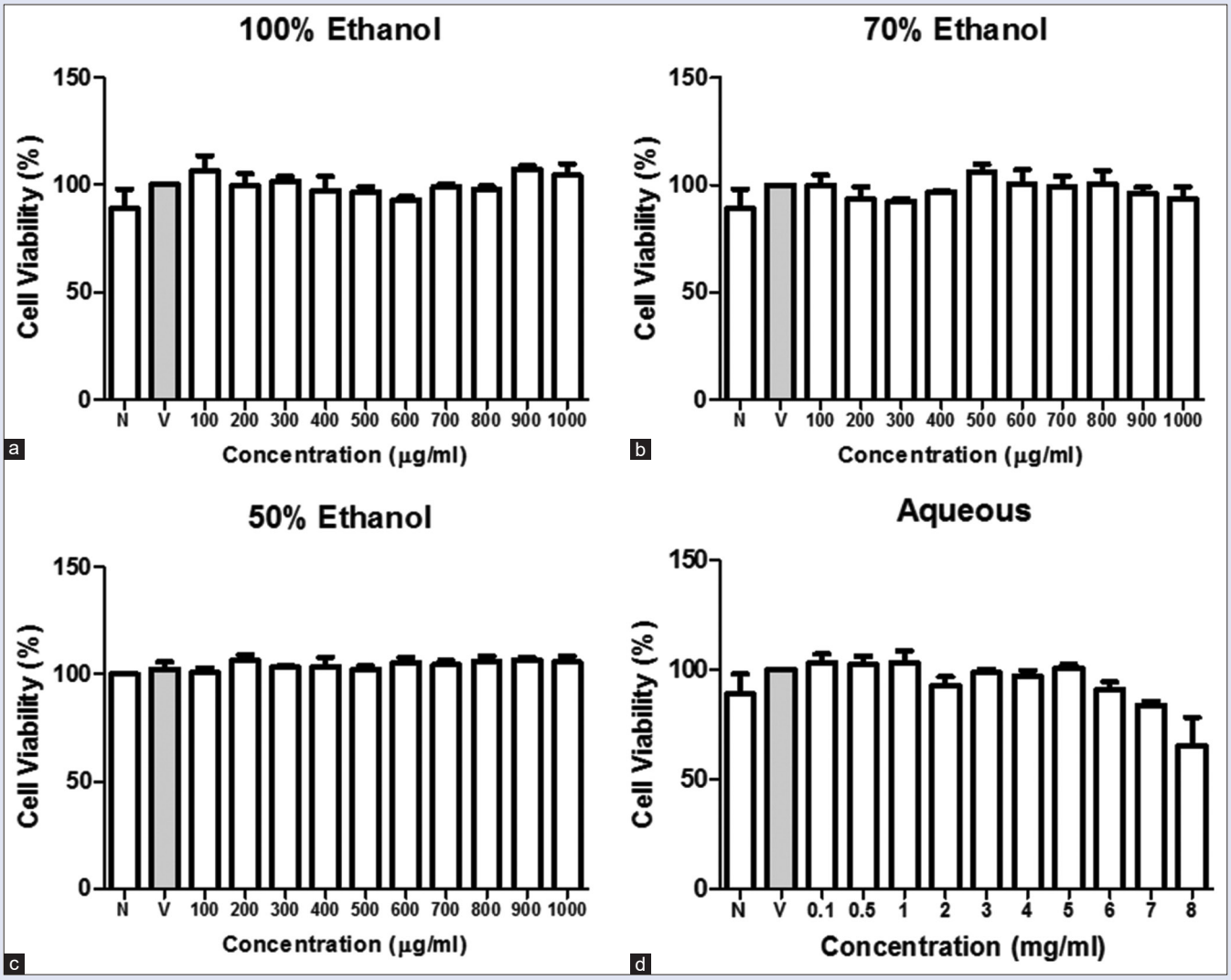

Figure 1: Cytotoxicity profiles of *Clinacanthus nutans* (a) 100% ethanolic, (b) 70% and (c) 50% aqueous ethanolic, and (d) 100% aqueous extracts. Rat basophilic leukemic cells were treated with different concentrations of *Clinacanthus nutans* ethanolic and aqueous extracts for 24 h and the cell viability was calculated on the untreated cells in percentage. Data were presented as mean ± standard error of the mean (n = 3). At all concentrations tested, rat basophilic leukemic cell viability was more than 80% except for 100% aqueous extract at 8mg/ml which showed 65% viability, but it was not significant when compared to the untreated cells, $P \geq 0.05$

the downregulation of lipid metabolism, carbohydrate, and signal transduction system and the upregulation of citrate cycle intermediates, propanoate, amino acid, and nucleotide metabolism.[21] However, in this OVA-ASA model, both the classical IgE and alternative IgG pathways of anaphylaxis could be activated. In this current study, our focus is on the classical IgE pathway whereby the aggregation of IgE to mast cell which leads to mast cell degranulation and activation play important roles in the pathogenesis of most allergy reactions. Mast cells degranulate as a result of the cross-linking of antigens to the IgE that were bounded to the FcεRI.[8,22] In our study, RBL-2H3 cells were presensitized with IgE, pretreated with the extracts and then challenged to degranulate with DNP-BSA. RBL-2H3 cells are widely used to study IgE-mediated mast cell degranulation as they have the characteristics of mucosal mast cells and basophils. They can be stimulated to degranulate just like primary mast cell and basophils either immunologically or nonimmunologically and functional FcεRI are found on their surface.[23]

Prior to determining the inhibitory effect of *C. nutans* extracts on IgE-mediated mast cell degranulation, cytotoxicity profiles of these extracts – 100% ethanolic, 70% and 50% aqueous ethanolic, and 100% aqueous were established to determine the safe concentration range that could be used for further assays. It is also to rule out that the inhibition was due to the effect of the extracts and not due to the toxic effect of the extracts on the cells. A broad-spectrum range of concentration was used and we found that at all concentrations tested, the ethanolic extracts were not cytotoxic to RBL-2H3 cells even at the highest concentration of 1000 µg/ml. Ethanolic extracts were dissolved in DMSO and the highest concentration of 1000 µg/ml was chosen based on its maximum solubility and taking into account the final concentration of DMSO that was safe for RBL-2H3 cells at 0.1%. As for the 100% aqueous extract, it was not cytotoxic to RBL-2H3 cells up to 7 mg/ml. However, it showed slight cytotoxicity at 8 mg/ml with cell viability of 65%. Even though there was no significant difference when compared to non-treated cells, 8 mg/ml was removed from subsequent assays as the cell viability was below 80%. Hence, for subsequent IgE-mediated mast cell degranulation assays, the concentration range chosen for all ethanolic extracts was from 100 to 1000 µg/ml while

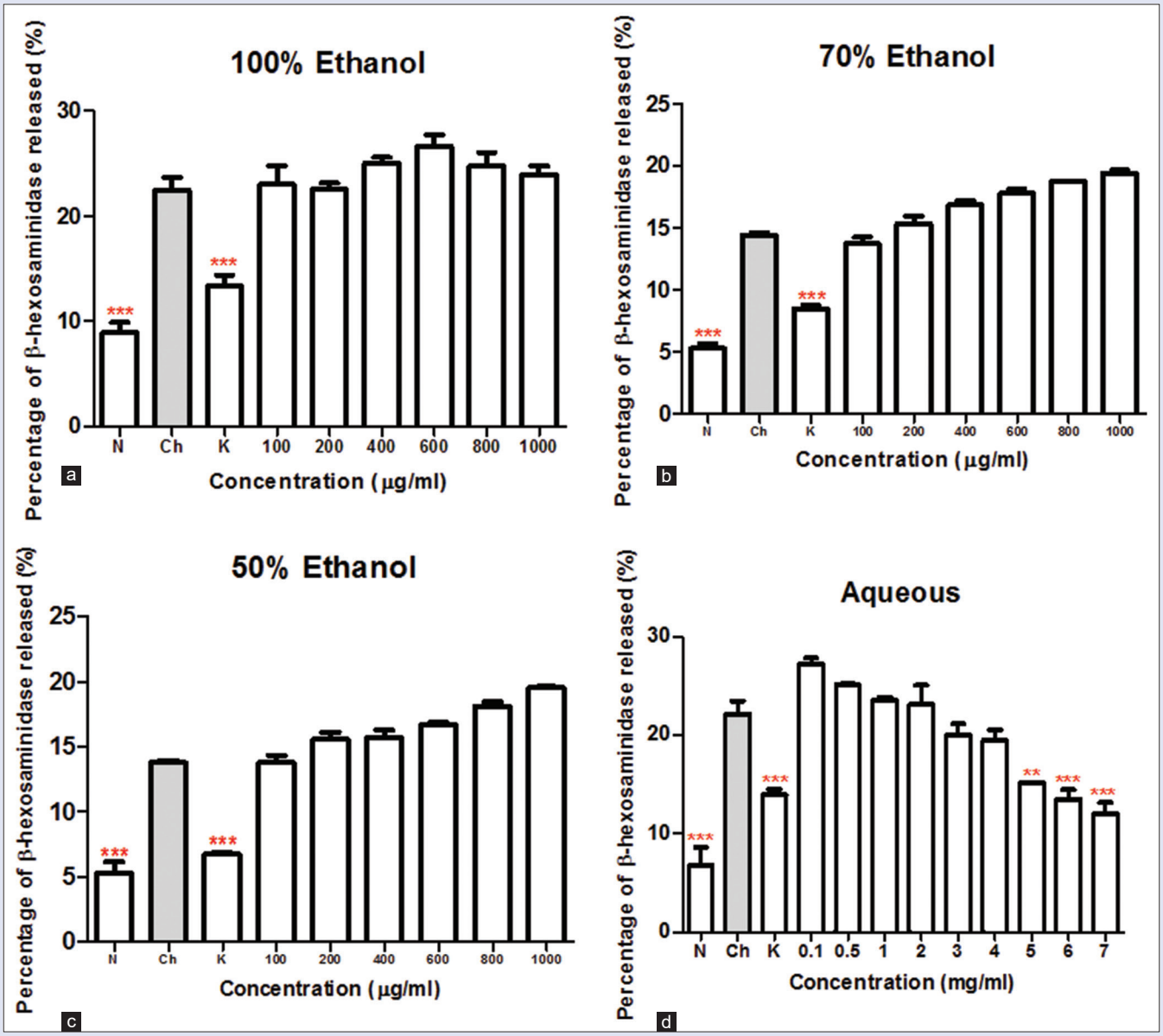

Figure 2: The effect of *Clinacanthus nutans* (a) 100% ethanolic, (b) 70% and (c) 50% aqueous ethanolic, and (d) 100% aqueous extracts on the release of β-hexosaminidase from IgE-mediated degranulated mast cells. Data were presented as mean ± standard error of the mean of three independent experiments in triplicate. Ethanolic extracts did not inhibit β-hexosaminidase even at 1000 µg/ml while significant inhibitions were recorded by aqueous extract at 5 mg/ml onward. Ketotifen fumarate (K) a clinically used mast cell stabilizer at 300 µM showed significant inhibition in all experiments. ***$P \leq 0.001$ significantly different from dinitrophenyl-bovine serum albumin-challenged control

for 100% aqueous extract, the concentrations chosen were 0.1, 0.5, and 1–7 mg/ml.

Based on the cytotoxicity profiles as assessed by MTT, we found that the extracts were non-toxic to the cells except a decreased viability was noted for cells treated with 8 mg/ml of *C. nutans* aqueous extract (excluded in subsequent assays). Following that we assessed the inhibitory effect of these extracts on the release of preformed mediators of IgE-mediated mast cell degranulation using the non-cytotoxic range of concentrations. Preformed mediators such as the lysosomal enzyme – β-hexosaminidase had been reported to be a suitable marker of inflammation as abnormal level of the enzyme has been found in a variety of inflammatory diseases.[24] In addition, it is also a widely accepted marker for mast cell degranulation reaction as it is stored alongside allergic mediators such as histamine and released when mast cells are immunologically stimulated.[22] Histamine has been regarded as the main mediator in most allergic diseases.[25] As shown in Figure 2, we found that there was no inhibition or reduction in the level of β-hexosaminidase in all ethanolic extract-treated cells. This indicated that the ethanolic extracts were not able to protect the mast cells from degranulating thus releasing β-hexosaminidase. On the other hand, it was found that there was significant inhibition of this enzyme by the aqueous extract at rather high concentrations of 5 mg/ml onward. Similar observations were noted in the quantification of histamine levels. We found that all the ethanolic extracts were not able to inhibit the release of histamine while significant inhibition was seen when the cells were pretreated with *C. nutans* aqueous extract at 5 mg/ml onward. Histamine, like β-hexosaminidase, is another preformed mediator that is stored in mast cell granules and they will be released on IgE-FcεRI aggregation acting

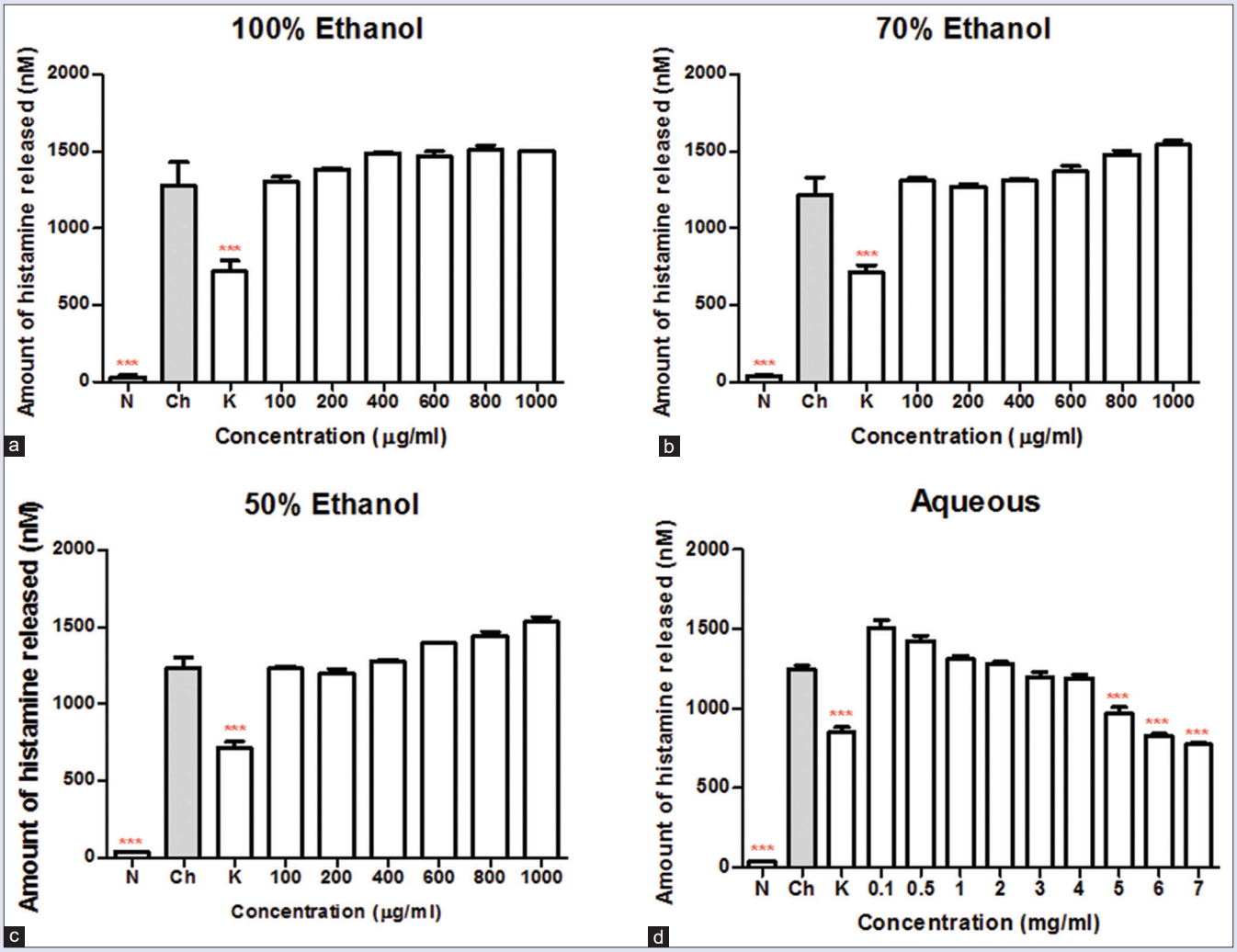

Figure 3: The effect of *Clinacanthus nutans* (a) 100% ethanolic, (b) 70% and (c) 50% aqueous ethanolic, and (d) 100% aqueous extracts on the release of histamine from IgE-mediated degranulated mast cells. Data were presented as mean ± standard error of the mean of three independent experiments in triplicate. Ethanolic extracts did not inhibit histamine even at 1000 µg/ml while significant inhibitions were recorded by aqueous extract at 5mg/ml onward. Ketotifen fumarate (K), a clinically used mast cell stabilizer at 300 µM showed significant inhibition in all experiments. ***$P \leq 0.001$ significantly different from dinitrophenyl-bovine serum albumin -challenged control

on histamine H1 receptors causing allergic responses.[25] The results on histamine further confirmed the ability of *C. nutans* aqueous extract in inhibiting the release of preformed mediators in IgE-mediated mast cell degranulation. A plausible explanation for the differences in the inhibitory effects between ethanolic and 100% aqueous extracts could be due to the different metabolites that are present in the extracts. From the findings by Khoo *et al.,* all these primary and intermediate metabolites – valine, alanine, proline, glutamate, glutamine, tryptophan, betaine, hypoxanthine, formic acid, acetic acid, fumaric acid, malonic acid, succinic acid, phenylacetic acid, and choline were found in all the ethanolic and 100% aqueous *C. nutans* extracts.[21] Common secondary metabolites that were found in both ethanolic and 100% aqueous extracts were trigonelline, clinamide A, luteolin, orientin, isoorientin, vitexin, schaftoside, isoschaftoside gendarucin A (flavones); 3-*O*-methylgallic acid; and syringic acid (phenolic acids). Interestingly, it was found that only 100% aqueous *C. nutans* extract possessed propionic acid (primary metabolite); lupeol which is a triterpenoid; β-sitosterol, stigmasterol and stigmasterol-β-D-glucoside of the phytosterol family; clinacoside A (sulfur-containing glucoside compound) and clinamide C, a sulfur-containing compound.[21] It is important to note that these metabolites were identified from ethanolic and 100% aqueous *C. nutans* extracts that were extracted through air-dried and sonication method which was employed in this study. Alkaloid, flavonoids, triterpenoids, diterpenes, and phytosterol have also been found in the leaf water extract of *C. nutans* from Indonesia, which corresponds to the above findings.[26] It is interesting to note also that some of these metabolites were found in the hexane fraction of methanol extract such as lupeol, β-sitosterol and stigmasterol; stigmasterol-β-D-glucoside in methanol fraction of 96% ethanol extract of sun-dried macerated aerial section of the plant; clinacoside A in butanol soluble and aqueous soluble portion of methanol extract aerial section and clinamide C in 80% ethanol extract of aerial, air-dried plant sample but not in 100% ethanol, 70% and 50% aqueous ethanol extracts.[26] These differences thus highlight the different ability of the ethanolic and aqueous extracts in suppressing the mediators of allergy tested in this study. However, these unique metabolites found in the 100% aqueous extract have not been reported to have anti-allergy activity; hence, it is worthy to isolate them and study them for their possible anti-allergy activity in the future.

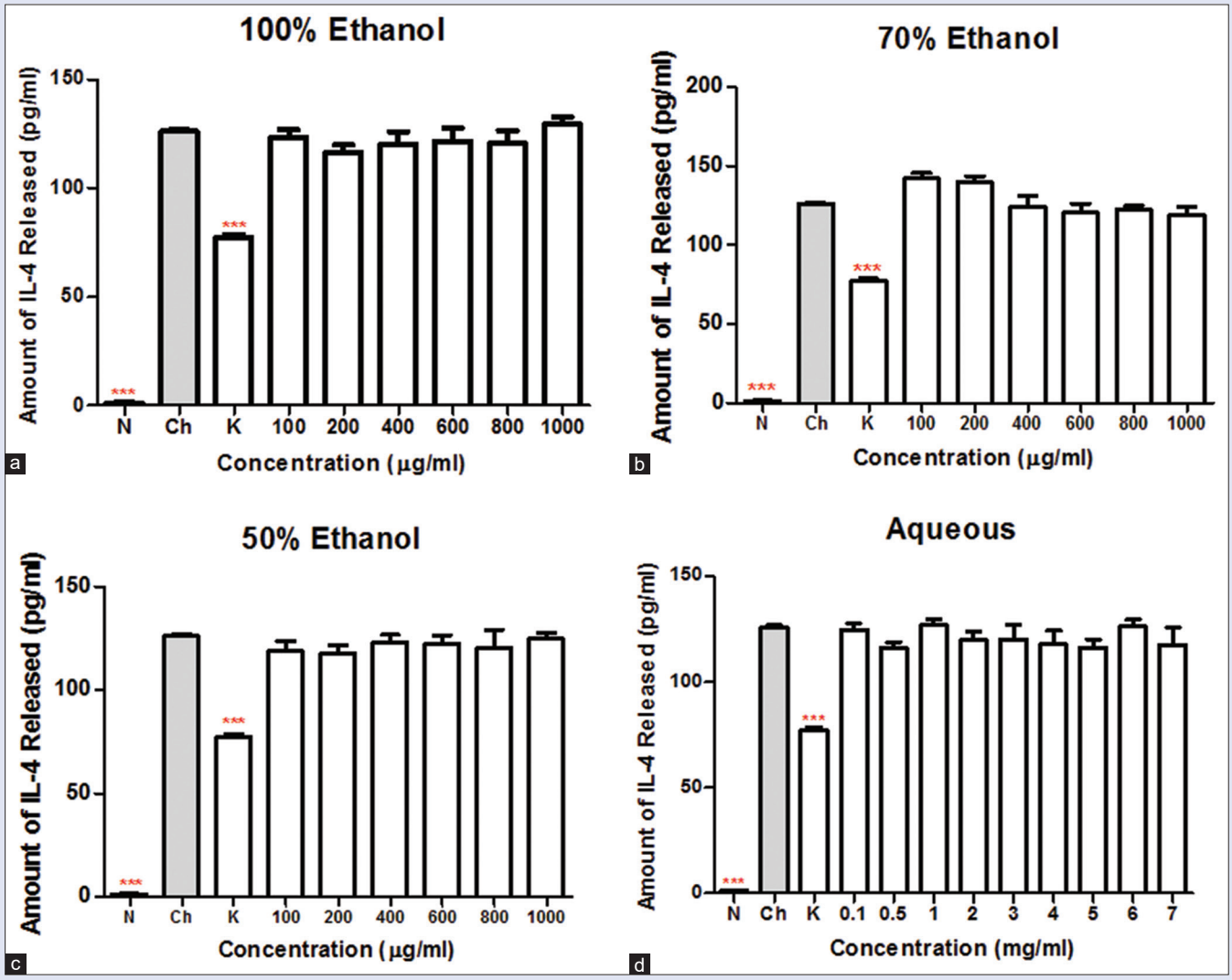

Figure 4: The effect of *Clinacanthus nutans* (a) 100% ethanolic, (b) 70% and (c) 50% aqueous ethanolic, and (d) 100% aqueous extracts on the release of interleukin-4 from IgE-mediated degranulated mast cells. Data were presented as mean ± standard error of the mean of three independent experiments in triplicate. All ethanolic and aqueous extracts did not inhibit the release of interleukin-4 even at 1000 µg/ml (ethanolic) and 7 mg/ml (aqueous). Ketotifen fumarate (K), a clinically used mast cell stabilizer at 300 µM showed significant inhibition in all experiments. ***$P \leq 0.001$ significantly different from dinitrophenyl-bovine serum albumin -challenged control

Apart from preformed mediators, the pathogenesis of allergy can be enhanced by late-phase mediators such as cytokines and chemokines. The inhibition of late-phase mediators such as IL-4 and TNF-α was hence analyzed in *C. nutans* ethanolic and aqueous extract-treated RBL-2H3 cells. It was found that none of these extracts were able to inhibit the amount of IL-4 and TNF-α that were being released on IgE-mast cell degranulation. No inhibition was recorded at all concentrations. This indicated that the *C. nutans* ethanolic and aqueous extracts were not able to arrest the late-phase allergy reactions that were mediated by IgE as other signaling pathways are still being activated to produce IL-4 and TNF-α. Mast cell activation that causes the degranulation and cytokine production is governed by a complex series of intracellular signaling. Apart from the activation that results from the initial aggregation of IgE-FcεRI, downstream signaling pathways of LYN/SYK-LAT-PLCγ and FYN-GAB2-PI3K also play important role in the degranulation process and production of cytokines and chemokines.[14] Although both of these pathways are also involved in the degranulation process, different proteins are being activated in the production of cytokines and chemokines. Hence, it could be possible that these downstream pathways that activate the proteins responsible for the production of cytokines and chemokines were not inhibited by *C. nutans* extracts leading to the release of IL-4 and TNF-α by the *C. nutans*-treated IgE-DNP-BSA – challenged-RBL-2H3 cells.

From our findings, *C. nutans* aqueous extract was able to suppress the release of β-hexosaminidase and histamine. This finding was parallel to the finding by our research group which identified that the aqueous extract recorded the highest nitric oxide (NO) inhibition activity in lipopolysaccharide-induced RAW 264.7 macrophages.[21] Similarly, NO, a pro-inflammatory mediator like β-hexosaminidase and histamine, is also one of the markers of interest in human inflammatory and allergic conditions.[27] As mentioned above, our research group has found that in an OVA-ASA *in vivo* model which is mediated by both IgE and IgG pathways, *C. nutans* aqueous extract was able to protect OVA-challenged rats from anaphylaxis at 2000 mg/kg. Paralleled to our *in vitro* findings, *C. nutans* aqueous extract seemed to be exerting its anti-allergic protective effect at rather high concentrations or dosage. However, in the OVA-ASA

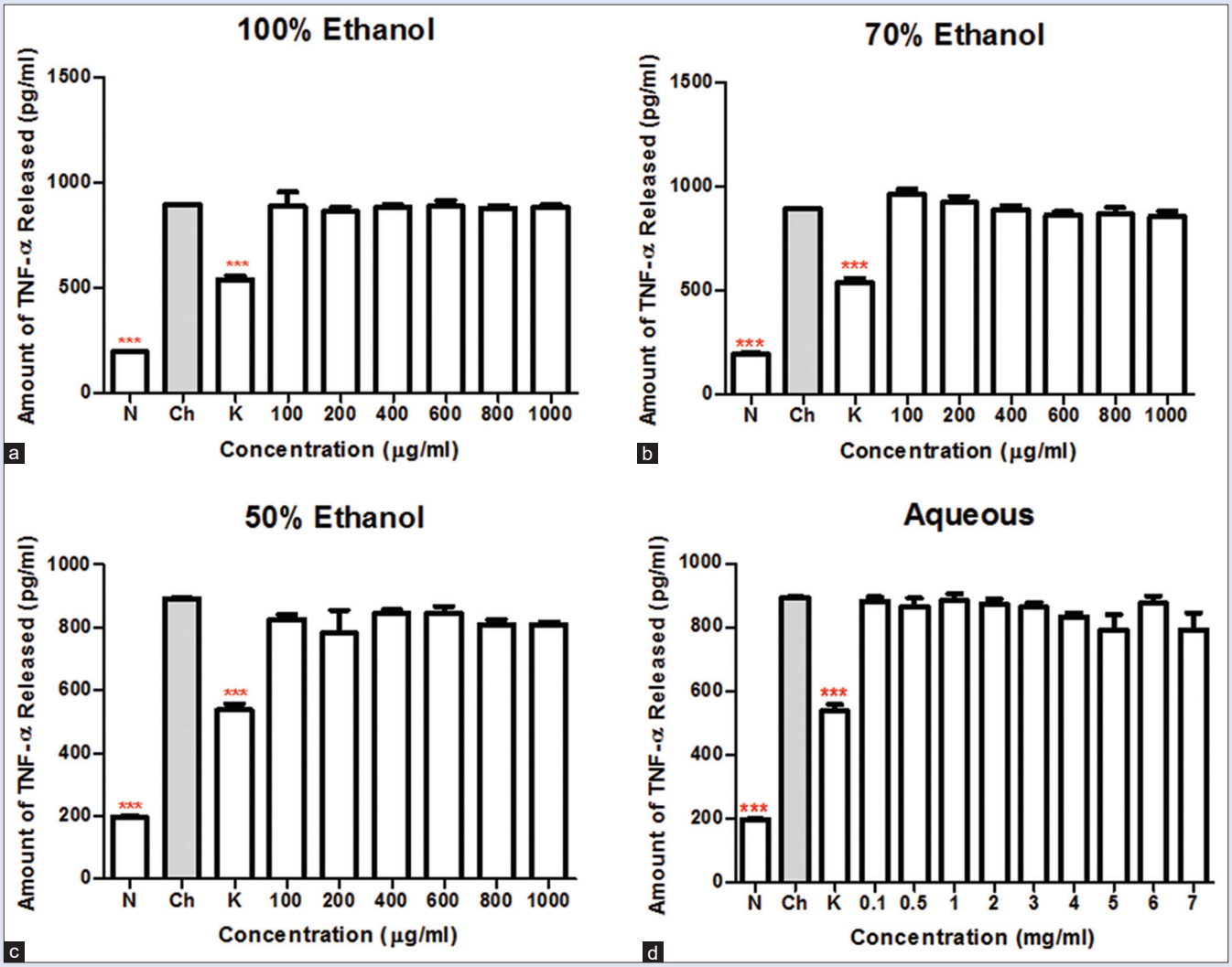

Figure 5: The effect of *Clinacanthus nutans* (a) 100% ethanolic, (b) 70% and (c) 50% aqueous ethanolic, and (d) 100% aqueous extracts on the release of TNF-α from IgE-mediated degranulated mast cells. Data were presented as mean ± standard error of the mean of three independent experiments in triplicate. All ethanolic and aqueous extracts did not inhibit the release of tumour necrosis factor-alpha even at 1000 µg/ml (ethanolic) and 7 mg/ml (aqueous). Ketotifen fumarate (K), a clinically used mast cell stabilizer at 300 µM showed significant inhibition in all experiments. ***$P \leq 0.001$ significantly different from dinitrophenyl-bovine serum albumin -challenged control.

study, the protective effect of this extract could not be determined specifically due to the activation of both IgE and IgG pathways. Even though IgE, mast cells and histamine have been regarded as the main players of most allergy cases,[28] allergy is not solely a histamine-related condition as it is dependent on other factors such as the production of cytokines and the involvement of other immune-competent cells which explained the continuous released of IL-4 and TNF-α.[25] Besides that, allergy could also be mediated by the cross-linking of IgG and its receptor Fc gamma and platelet activating factor.[28] This in turn led us to further study the anti-allergic property of *C. nutans* aqueous extract in the IgG-mediated pathway.

CONCLUSION

From the screening test, we identified that *C. nutans* aqueous extract as the most active extract compared to the ethanolic extracts in suppressing the release of preformed mediators of IgE-mediated mast cell degranulation – β-hexosaminidase and histamine, but it did not suppress the release of late-phase mediators – IL-4 and TNF-α. Based on our findings, *C. nutans* aqueous extract may possess anti-allergic property by suppressing the early phase of IgE-mediated mast cell degranulation which could be attributed to the different metabolites that are present in the 100% aqueous extract such as lupeol, β-sitosterol, stigmasterol, stigmasterol-β-D-glucoside, propionic acid, clinacoside A, and clinamide C.

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Conflicts of interest

There are no conflicts of interest.

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