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Original article

Effects of *Clinacanthus nutans* Extracts on Cytokine Secretion in PMA-Induced U937 Macrophage Cells

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Abstract

Background and objectives: *Clinacanthus nutans* (Burm f.) Lindau (*C. nutans*) is a well-known traditional medicine in South East Asia and consists of abundant phytomedicinals properties. This study aimed to investigate the effects of *C. nutans* ethanol and aqueous extracts on interleukin-4 (IL-4) and interleukin-13 (IL-13) cytokines secretion in phorbol-12-myristate-13-acetate (PMA)--induced U937 macrophages. **Methods:** Sequential ultrasonic-assisted extraction was carried out using ethanol (ETOH) and water, by applying 1:10 ratio of leaves powder to the solvent volume. U937 cells were incubated with 25 nM PMA for 72 h to induce macrophage differentiation. The macrophage differentiation was assessed based on the cell morphological changes, cell viability and, CD14 and CD11b expression by using flow cytometry. The macrophages were incubated with both ETOH and aqueous extracts at 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL concentration for 48 h. The viability of the extract-treated cells was assessed using PrestoBlue cell viability assay and the IL-4 and IL-13 secretions were assessed by using Enzyme-Linked Immunosorbent Assay (ELISA). **Results:** PMA stimulation caused morphological changes of U937 cells from round-shaped, non-adherent to larger irregular-shaped, adherent cells, and a reduction of cells viability to 87%. CD14 expression was down-regulated from 7% to 4.5% upon PMA stimulation. CD11b expression was up-regulated from 16% in untreated cells to 38% in PMA-treated cells. ELISA results showed that 1 mg/mL of ETOH and AQ extracts stimulated 1200 and 1800 pg/mL IL-4 secretions, respectively. However, both extracts caused minimal IL-13 secretion. **Conclusion:** *Clinacanthus nutans* aqueous extracts stimulated IL-4 production higher than ETOH extract in PMA-induced U937 macrophages.

Keywords: *Clinacanthus nutans*; interleukin-4; macrophages; phorbol-12-myristate-13-acetate

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Introduction

C*linacanthus nutans* (Burm f.) Lindau (*C. nutans*) or commonly known in Malaysia as "Belalai Gajah" or "Sabah" Snake Grass is a medicinal plant that belongs to the Acanthaceae family. It has been popularly used as medicine in South East Asia, particularly Malaysia, Indonesia, China and Thailand. *Clinacanthus nutans* extracts were reported to have enormous phytomedicinal properties including anti-inflammatory for insect bites and allergic responses [1], antiviral activity against herpes simplex virus [2,3], anti-papillomavirus infection [4], anti-oxidant [5], and anti-proliferative selectively against cancer cell lines [6]. Furthermore, *C. nutans* extracts were reported to be safe when given continuously for 14 days with

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appropriate dosing in rats without adverse events or organ failure [7]. The *C. nutans* extracts have been tested for its immune modulating effects on cell proliferation and functions, cytokines expression, and apoptosis in experiments involving peripheral blood mononuclear cells, natural killer cells, neutrophils, and macrophages [1,8,9].

Macrophages are the tissue-resident professional phagocytes in innate immune system and are differentiated from the circulating peripheral blood monocytes [10]. Upon the action of cytokines and chemokines signalling, the monocytes leave the circulation system, differentiate into macrophages and migrate to the site of infection. Macrophage activation gives rise to two different types of macrophages, i.e. the classically activated M1 macrophages (CAM) or alternatively activated M2 macrophages (AAM) [11]. The CAM exhibits a Th1-like phenotype, which promotes inflammation and may potentially cause tissue destruction [12]. On the other hand, the AAM displays a Th2-like phenotype, which facilitates to resolve inflammation and promote wound healing [11]. The two prominent cytokines, IL-4 and IL-13, are produced during alternative activation. These cytokines trigger Th2-like responses, particularly in allergic, cellular and humoral responses to parasitic and extracellular pathogens [13].

It has been demonstrated that extracts prepared from *C. nutans* polar solvents exhibited promising anti-inflammatory properties in vitro and in vivo. However, most of these studies used methanol, ethanol, *n*-butanol extracts, and only few used water or aqueous extract [14,15]. In addition, most of the studies were carried out on neutrophils [1,15,16] whereas studies on macrophages were limited [9,17]. Therefore, this study was carried out to elucidate and compare the effects of ethanol and aqueous extracts of *C. nutans* on PMA-induced U937 macrophage cells. In specific, we investigated the effects of these extracts on cells viability and, IL-4 and IL-13 cytokines secretion. Since IL-4 and IL-13 are pro-M2-Th2 cytokines that generate alternative macrophages, this study shall provide an insight on the extracts' ability in modulating M2 macrophage generation, and prospectively, the extracts potential in anti-inflammatory activity.

Material and Methods

Ethical considerations

All experimental procedures, which involved in vitro cell line work, were conducted following ethics and good practices in research. No human participants nor identifiable human tissue, biological samples, nor animals were used in this study. The human monocytic cell line U937 (ATCC® CRL-1593.2™) was purchased from American Type Culture Collection (ATCC, Virginia, USA).

Chemicals

Cell culture reagents including Roswell Park Memorial Institute (RPMI) 1640 medium were purchased from Nacalai Tesque (Japan), fetal bovine serum (FBS) was purchased from JR Scientific Inc. (USA), L-glutamine and penicillin-streptomycin solutions were purchased from Hyclone (USA). Phorbol-12-myristate-13-acetate (PMA) and Trypan Blue Solution 0.4% were purchased from Sigma-Aldrich (USA) and Gibco (USA), respectively. All antibodies, which are mouse anti-human CD14, mouse anti-human CD11b, mouse IgG2a, κ and mouse IgG1, κ isotype controls, were from BD Biosciences (USA). PrestoBlue™ Cell Viability Reagent was purchased from Invitrogen™ (USA) while IL-4 and IL-13 pre-coated ELISA plates were from R&D Systems (USA).

Collection and extraction of *C. nutans*

C*linacanthus nutans* was collected from Manjung, Perak, Malaysia in September 2018. The botanical identification of the plants was verified by the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia, Penang (Voucher no.: USM Herbarium 11465). The leaves were parted from the stem, washed and cleaned and dried in an oven at 40 - 45 °C for 5–7 days. The dried leaves were pulverised into fine powder and weighed at 100 g for ultrasonic-assisted maceration. Sequential ultrasonic-assisted extraction was carried out using ethanol (ETOH) and water at room temperature for 30 min. The ratio of powder weight to solvent volume was 1:10. The macerated leaves were centrifuged at 1250 g and the supernatant was evaporated off using rotary evaporator. The final aqueous extract was obtained using freeze-drying method. Both extracts were tightly sealed with parafilm and kept at 4 °C. The extract yield percentage was calculated using the following equation:

$$\text{Percentage yield} = [\text{Weight of extract (g)} \div \text{Weight of leaves (g)}] \times 100\ \%$$

Differentiation of human U937 monocytic cells to macrophages

The human monocytic cell line U937 (ATCC® CRL-1593.2™) were maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin-streptomycin solutions, in a 5% (v/v) CO_2-supplied incubator at 37 °C. The medium was changed every 3–4 days. For macrophage differentiation, approximately 1×10^6 cells were incubated with 25 nM PMA for 72 h. Cells cultured without the addition of PMA were used as control. After three days of PMA induction, the cells morphological changes were observed under an inverted phase-contrast microscope and the cells viability assay was carried out using Trypan Blue Solution, 0.4%.

Assessment of cell viability

On the third day of incubation with PMA the cells viability was assessed using Invitrogen™ Countess™ Automated Cell Counter (Thermo Fisher Scientific, USA).

Flow cytometry

Flow cytometric analysis was carried out to assess macrophage differentiation. The U937 monocyte-derived macrophages were stained with mouse anti-human CD14 and anti-human CD11b antibodies, including their corresponding IgG2a, κ and IgG1, κ isotype control antibodies. All antibodies were from BD Biosciences, USA. About 1×10^6 cells were resuspended with 200 µL PBS in FACS tubes. The cells were stained with 5 µL of each antibody, incubated for 45 min on ice, shielded from light. Next, the cells were washed twice with PBS and centrifuged at 400 g for 5 min. The pellets were resuspended in FACS buffer. The cells were acquired using BD FACSCalibur™ (BD Biosciences, USA) and the data were analysed using FlowJo® software version v10 (Tree Star, Inc, USA).

Extracts treatment

Approximately 8×10^4 cells were seeded into a 96-well flat bottom plate. Twenty-five nM PMA was added into the wells and the cells were incubated for 72 h in 5% (v/v) CO_2 incubator. After 72 h, the media containing PMA were removed and the cells were washed twice with sterile PBS. Both ethanol and aqueous extracts were dissolved in RPMI 1640 medium to obtain a range of extract concentrations i.e. 0.25, 0.5, 1.0, 2.0, 4.0 and 8.0 mg/mL. Two hundred µL of these extracts were then added into the 96-well plate containing macrophages and the plate was incubated at 5 % (v/v) CO_2 at 37 °C for 48 h. Cells cultured in medium only (without extracts) served as the negative control. After incubation, the media supernatant was collected and transferred into a fresh 96-well round bottom plate. This plate was subjected to centrifugation at 800 g for 3 min. One hundred and fifty µL supernatant from each sample was collected and kept at -80 °C until further use. The remaining cell pellets were discarded.

Assessment of extract-treated cells viability

PrestoBlue™ Cell Viability Reagent was used to assess the cells viability after 48 h treatment of extracts on the U937 monocyte-derived macrophages. In this experiment, 10 µL of PrestoBlue™ Reagent was added directly to the cells in culture medium at the end of 48 h extract treatment. The cells were incubated for at least 20 min at room temperature. The fluorescence intensity was then recorded by using a microplate reader (BMG Labtech FLUOstar Omega, Germany) set to 450 nm and 540 nm.

Measurement of cytokine secretion

The amounts of IL-4 and IL-13 in the supernatant, after extracts incubation, were assessed by using IL-4 and IL-13 pre-coated ELISA plates. All buffers for ELISA including IL-4 and IL-13 standards were made according to the manufacturer's protocol. The standards, samples, and controls were assayed in triplicate. A 50 µL of standard, control, or sample was added into different wells. The plate was then covered with an adhesive strip and incubated for 2 h at room temperature. The plate was decanted, washed three times and blotted against clean paper towels. A 200 µL of IL-4 or IL-13 conjugate was added to each well and the plate was further incubated for another 2 hours at room temperature. After several washings, 200 µL substrate solution was added into each well. The plate was then incubated for 20 min at room temperature, protected from light, followed by the addition of 50 µL stop solution into the wells. Optical density of each well was determined within 30 min using a microplate reader (BMG Labtech FLUOstar Omega, Germany) set to 450 nm and 540 nm. The readings obtained at 540 nm were subtracted from the readings at 450 nm to correct any optical imperfection.

Statistical analysis

All experiments were conducted in triplicate and in three independent tests. The statistical analysis was performed using IBM SPSS Statistics Version 22. The significant difference between each test group and negative control group (untreated cells) was analysed using one-way analysis of variance (ANOVA) and post hoc Dunnett's *t*-test; $p<0.05$.

Results and Discussion

In this study, sequencing polarity extraction was carried out to enable different types of phytochemicals to be extracted sequentially from ETOH and water. From the ultrasound extraction technique, a small quantity of thick sticky extract was obtained with ETOH (about 2.27% yield) and about 17% for aqueous extract. In order to drive the differentiation of U937 monocytic cells to macrophages, 25 nM PMA was used. Upon addition of PMA, the U937 cells morphology changed from round-shaped, non-adherent to larger irregular-shaped cells with highly adherent characteristics. An increase in cytoplasm and nuclear ratio with pseudopodia and cytoplasmic protrusion were also observed (data not shown). In addition, the U937 cell viability dropped to 87% when treated with 25 nM PMA as compared to the unstimulated cells (viability taken as 100%). However, there was no significant cell death.

Flow cytometry was carried out to further assess the outcome of cells differentiation using PMA. This was done by measuring the changes in CD14 and CD11b expression of the cells. Flow cytometric analysis showed that PMA stimulation did not significantly affect the CD14 expression on the U937 cells as the expression was downregulated from about 7% to 4.5% upon stimulation. On the other hand, CD11b expression was upregulated from 16% in untreated cells to about 38% when treated with 25 nM PMA (Figure 1).

Figure 2 shows the effects of ETOH and aqueous extracts on the viability and proliferation of PMA induced-U937 cells. It is demonstrated that 0.5 mg/mL aqueous extract exhibited a peak of relative fluorescence units (RFU) (almost 100,000 RFU compared to around 40,000 RFU in non-treated cells), indicating an increase in the viability and proliferation of the cells compared to untreated cells. However, the cells viability dropped significantly when treated with 1 mg/mL of aqueous extract and gradually dropped as the extract concentration increased. Meanwhile, increased concentration of ETOH extract caused a gradual decline in the cells viability.

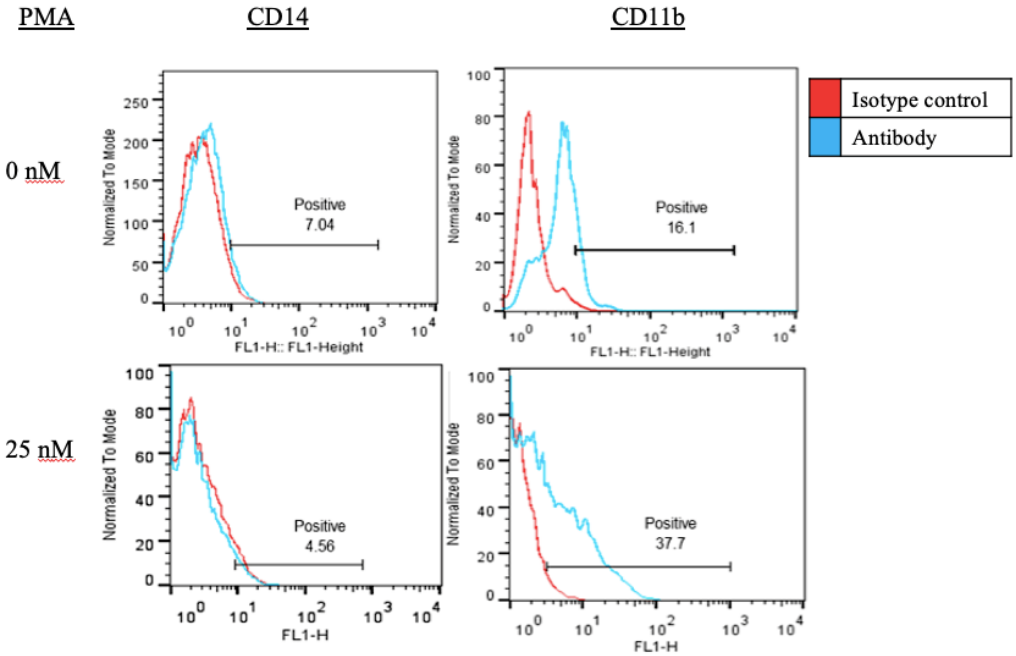

Figure 1. Flow cytometric analysis on CD14 and CD11b expression of U937 cells. U937 cells were incubated with 25 nM PMA for 72 h and stained with mouse anti-human CD14 and anti-CD11b antibodies, including the respective isotype control antibodies. Upon PMA stimulation, CD14 expression was downregulated to approximately 2-fold level while the CD11b expression level was upregulated from 16 % in untreated monocytes to about 38% after stimulation.

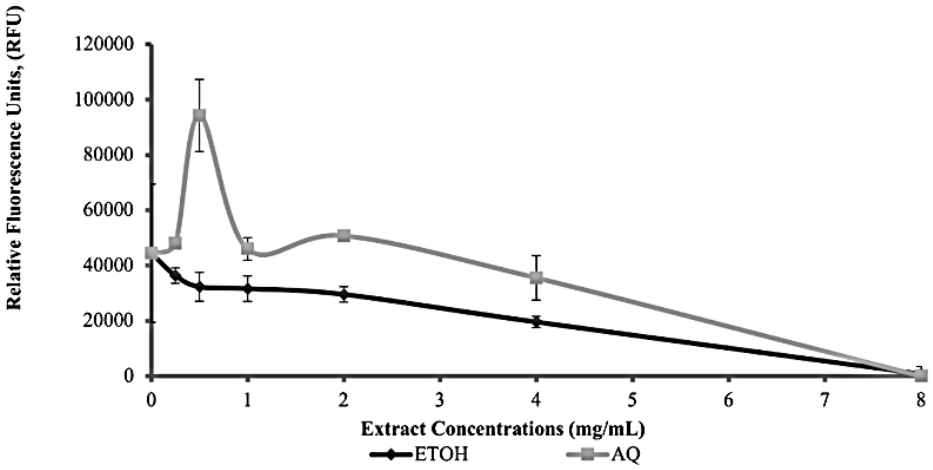

Figure 2. Effects of ETOH and aqueous extracts on PMA induced-U937 cells viability and proliferation. Aqueous extract at the concentration of 0.5 mg/mL exhibited an RFU peak, and then gradually declined to baseline as it reached maximum concentration, 8 mg/mL. ETOH extracts caused gradual decrease on cells viability. At maximum concentration of the extracts (8 mg/mL), both ETOH and aqueous extracts caused a baseline drop in cell viability. The experiment was done in triplicate; the data represents the mean ± SE, and is a representative of three independent experiments.

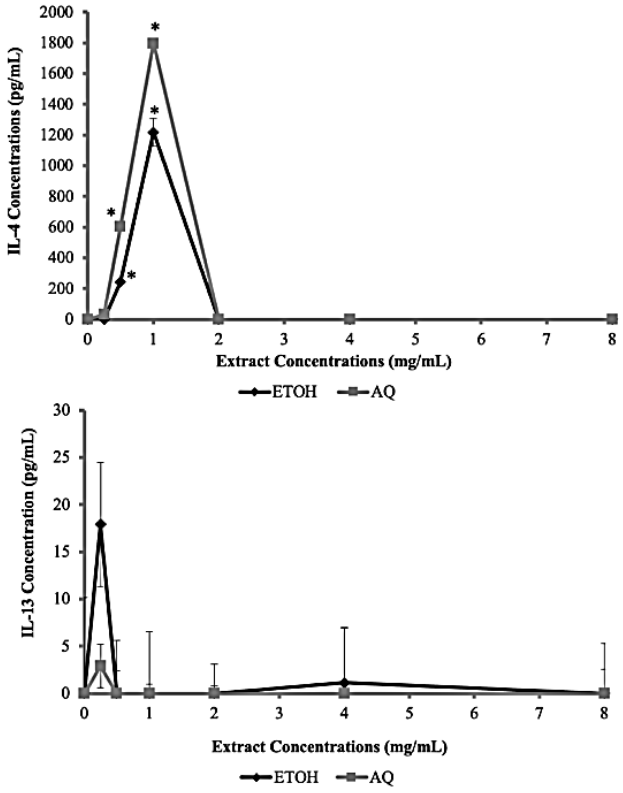

Figure 3. IL-4 and IL-13 secretion by PMA-induced U937 macrophages treated with ETOH and aqueous extracts. The untreated cells secretion was taken as negative control. For both extracts, 1 mg/mL significantly produced the highest secretion of IL-4, approximately 1200 pg/mL (ETOH) and 1800 pg/mL (aqueous). The IL-13 secretion was much lower than that of IL-4 but none of the secretion was significant when compared to untreated cells. The experiment was done in triplicate; the data represents the mean ± SE, and is a representative of three independent experiments. Significant difference between extract-treated cells and control group: *$p<0.05$

From ELISA results, it was shown that 1 mg/mL of ETOH and aqueous extracts significantly stimulated the highest secretion of IL-4, approximately 1200 and 1800 pg/mL, respectively ($p<0.05$) (Figure 3). At the concentration of 0.5 mg/mL, AQ extract caused significant IL-4 secretion of approximately 600 pg/mL, whereas, ETOH extracts at 0.5 mg/mL, significantly stimulated the secretion of 245 pg/mL IL-4 ($p<0.05$). In addition, we found that neither of the extracts induced significant release of IL-13. In this study, only about 18.0 pg/mL and 3.0 pg/mL of IL-13 release was stimulated by 0.25 mg/mL ETOH and aqueous extracts, respectively.

Previously, Sriwanthana et al. reported 15% yield of ethanol extract using soxhlet method [8]. It was indeed a much higher yield compared to the yield obtained in this study (2.27%). The difference may possibly be due to different methods used, as one of the advantages of soxhlet extraction is the ability to produce large amount of extracts with much smaller quantity of solvent. However, this method is not suitable for thermolabile compounds as prolonged heating may lead to degradation of compounds [18]. Therefore, ultra-sonication is feasible and useful for the extraction of thermosensitive and unstable compounds [19].

THP-1 and U937 are the most widely used myeloid cell line models for investigating monocytic differentiation and biological functions of differentiated cells [20]. The U937 cell suspension appeared to be round in shape, non-adherent, and grew rapidly in suspension within 2–3 days, consistent with the observation obtained by several other studies [21,22]. PMA, which is a protein kinase C activator, stimulates macrophage functions including phagocytosis and immunomodulatory cytokines secretion. It is commonly suggested as the most potent differentiation agent, compared to other agents like DMSO, retinoic acid, vitamin D3 and cytokines [23]. In this study, 25 nM PMA [24] was used to drive the differentiation of U937 monocytic cells to macrophages. The stimulation caused the morphological transformation of the cells, similarly as reported by others [25,26]. As PMA has the ability to differentiate monocyte into macrophage, it also inhibits cell proliferation [26]. Thus, in this study, in order to avoid PMA-associated toxicity to the cells [27], we used low concentration of PMA i.e.25 nM (a similar concentration used by Paulsen et. al. [24]) to stimulate the differentiation, although higher concentrations have also been used previously [28-30].

CD14 is a differentiation antigen on the surface of myeloid lineage cells [31] and serves as an important marker for monocytes [20]. Although the basal CD14 expression on U937 cells is quite low, we observed downregulation of its expression when stimulated with PMA. This observation is supported by reports showing CD14 downregulation in monocyte-macrophage differentiation and on LPS-induced macrophages [32]. The low level of CD14 on U937 cells is expected as blood monocytes can express either high or low level of CD14 as reported before [33]. Conversely, CD11b surface expression is a key marker for macrophages [34] and we have shown one-fold upregulation of CD11b in PMA-stimulated cells. Together with the morphological changes observed, we established that 25 nM PMA is adequate to differentiate the monocytes to macrophages without causing significant cells death. However, it is worth noting that few other surface markers, such as CD16, CD64, and CD68, could be included to better differentiate between monocytes and macrophages.

The extract-treated cells viability and proliferation were assessed by using PrestoBlue reagent, which quantitatively measure the proliferation of cells using the reducing power of living cells. The RFU is proportional to the number of cells. We demonstrated that low concentration of aqueous extract promoted cell proliferation to almost double the rate compared to that of the untreated cells. However, higher concentration of extracts caused gradual reduction in the cells viability. Similar finding was observed in ethanol extract-treated cells suggesting possible cell toxicity of the extracts. This is consistent with a report that showed gradual decrease in lymphocyte proliferation at high concentration of ethanol extract [8].

IL-4 and IL-13 are among the important cytokines produced when macrophages undergo alternative activation. They belong to the same a-helix superfamily, and their genes are located 12 kb apart on 5q31. Interestingly, with only 25% homology between them, IL-13 and IL-4 have many overlapping functions, including upregulation of major histocompatibility complex class II, regulation of macrophage activity, mediation of immune responses typically

characterised by eosinophilia and basophilia, as well as induction of IgE production in B cells [35].

Our study showed that 1 mg/mL of both extracts stimulated the highest secretion of IL-4, approximately 1200 pg/mL (ETOH) and 1800 pg/mL (AQ). Similar amount of secretion was reported when 5 mg/mL extract was used to treat PBMC [8], compared to 1 mg/mL extract used in our study. Higher potency of our extracts could be due to different growth environment conditions, leading to variation in the secondary metabolites content of plants [36,37]. Similar outcome was reported by Fonseca et al., whom observed that changes in environmental conditions such as different water and light conditions, significantly affected the secondary metabolites content in feverfew plant [38].

IL-13 has been reported to have redundant effects to those of IL-4 and at the same time possesses distinct effector functions from IL-4 [39, 40]. IL-13 inhibits the production of pro-inflammatory cytokines and chemokines and has been shown to be the key mediator of allergic inflammation independent of IgE and eosinophil [12,41].

Interestingly, it has been reported that the anti-inflammatory effects of *C. nutans* extracts are related to inhibition of TLR-4 activation and that the inhibition is related to its phenolic compounds and flavonoids [9]. Based on the past research in the ETOH and aqueous characterisations, compounds such as alkaloids, saponins and flavonoids were found in the ETOH extract while alkaloids, saponins, flavonoids, steroids and tannins were found in the aqueous extract of *C. nutans* [15]. Thus, a detailed isolation and profiling of active metabolites from our aqueous and ETOH extracts are necessary in order to explore potential active compounds that contributed to these effects.

Conclusion

We have demonstrated that both *C. nutans* ethanol and aqueous extracts potently stimulated PMA-induced U937 macrophages to produce IL-4, with the aqueous extract showing superiority over ETOH extract. This outcome suggests that the extracts may have potential in modulating M2 macrophage activation and thus, could possibly have anti-inflammatory activity. However, this study only looked at IL-4 and IL-13 secretions. Thus, further studies, which include assessment on other pro- inflammatory and anti-inflammatory cytokines secretion, are warranted in order to further elucidate the immunomodulatory role of these extracts.

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Author contributions

Ooi Swee Hong performed the experiments, analysed the data and drafted the manuscript. Nur Mazidah Noor Mohamed and Ravi Kumar Kalaichelvam contributed to the revision of the manuscript. Vuanghao Lim and Ida Shazrina Ismail contributed substantially to the conception and design of the study and revision of the manuscript. All authors contributed substantially to its critical revision and approved the final version submitted for publication.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

PMA: phorbol-12-myristate-13-acetate; IL-4: interleukin-4; IL-13: interleukin-13; ETOH: ethanol; ELISA: enzyme-linked immunosorbent assay; RPMI 1640: Roswell Park memorial institute 1640 medium; FBS: foetal bovine serum; DMSO: dimethylsulfoxide; PBS: phosphate buffered saline.