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ANTI-INFLAMMATORY EFFECTS OF *Vitex trifolia* LEAVES HYDROALCOHOLIC EXTRACT AGAINST HYDROGEN PEROXIDE (H_2O_2)- AND LIPOPOLYSACCHARIDE (LPS)-INDUCED RAW 264.7 CELLS

AHMAD TAMIM GHAFARI[1,2], AISYAH HASYILA JAHIDIN[1], YUSLINA ZAKARIA[1] and MIZATON HAZIZUL HASAN[1*]

[1]*Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), 42300 Bandar Puncak Alam, Selangor Darul Ehsan Malaysia*
[2]*Department of Pharmacology, Faculty of Pharmacy, Kabul University, Kabul 1006, Afghanistan*
[*]*E-mail: mizaton_hazizul@uitm.edu.my*

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ABSTRACT

Inflammation is the human body's defensive response against harmful events and a hallmark of many chronic conditions. Commonly, pharmacological approaches to treat inflammation include the use of non-steroidal anti-inflammatory drugs (NSAIDs) that could potentially possess life-threatening side effects after prolonged use. Hence there is a need for safer alternatives with fewer possible side effects. *Vitex trifolia* is a shrub from the family Verbenaceae, which possesses potential anti-inflammatory effects and is traditionally used to treat inflammation-related diseases in several Asian countries. This study aimed to explore the antioxidant and anti-inflammatory effect of *V. trifolia* leaves hydroalcoholic extract (VT) against murine macrophages (RAW 264.7 cells) induced with hydrogen peroxide (H_2O_2) and lipopolysaccharide (LPS). The reactive oxygen species (ROS) production was evaluated in the H_2O_2-induced macrophages. On the other hand, the interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and cyclooxygenase (COX) levels were quantified in the LPS-induced macrophages. VT (25 & 50 µg/mL) showed protective effects and significantly (p<0.05) increased the cell viability and reduced the ROS production compared to that of macrophages treated with 300 µM H_2O_2 alone. Additionally, VT (50 & 100 µg/mL) significantly (p<0.05) reduced LPS-induced TNF-α and IL-6 levels and COX activity compared to the macrophages treated with LPS (1 µg/mL), alone. However, VT and diclofenac had no inhibitory effect on IL-1β induced by LPS. Moreover, a significant positive correlation was found between VT antioxidant and anti-inflammatory effects. Concisely, these outcomes showed the potential antioxidant and anti-inflammatory effect of VT with a positive correlation between these protective actions. Therefore, our results suggest that VT may serve as a source of nutraceutical compounds with impending antioxidant and anti-inflammatory activities. However, further molecular investigations on the isolated compounds of the plant and *in vivo* studies are suggested for future work.

Key words: Anti-inflammatory, antioxidant, correlation, lipopolysaccharide, reactive oxygen species, *Vitex trifolia*

INTRODUCTION

Inflammation is a physiological process triggered by different endogenous and exogenous stimuli, such as pathogens, allergens, trauma, and irradiation. Its primary role is to eradicate the stimuli from the injury site and restore the physiological state of the cells (Chen *et al*., 2017). Acute inflammation usually minimizes the injury or infection and restores the tissue's physiological homeostasis, and it usually lasts for several days. However, uncontrolled acute inflammation may become chronic which can significantly contribute to a variety of chronic inflammatory diseases, such as cancer, heart diseases, rheumatoid arthritis, type 2 diabetes, and neurodegenerative diseases (Chen *et al*., 2017; Nurul Amin *et al*., 2020; Placha & Jampilek, 2021). Acute inflammation is induced upon recognition of the invading agent by the pattern recognition receptors (PRRs) for example toll-like receptors (TLR), C-type lectin receptors (CLRs), retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs). Stimulated PRRs can further activate signalling pathways and transcription factors such as nuclear factor kappa B (NF-κB), activating protein-1 (AP-1), and mitogen-activated protein kinases (MAPKs) that further induce the pro-inflammatory cytokines and antimicrobial molecules production, also the recruitment of inflammatory cells (Biswas, 2016; Chen *et al*., 2017). Immune cells invade injurious agents and attempt to kill them by releasing toxic contents such as reactive oxygen

* To whom correspondence should be addressed

species (ROS), reactive nitrogen species (RNS), proteinase 3, cathepsin G, and elastase (Medzhitov, 2010). Moreover, TLR stimulation can induce oxidative stress by unbalancing the production of pro-inflammatory and anti-inflammatory cytokines (Biswas, 2016).

Anti-inflammatory drugs are classified into nonsteroidal anti-inflammatory drugs (NSAIDs), such as diclofenac, indomethacin, celecoxib, nimesulide, ibuprofen, naproxen, and meloxicam (Fokunang, 2018); and steroidal anti-inflammatory drugs (SAIDs), e.g., prednisone, dexamethasone, methylprednisolone, and prednisone (Samuel *et al*., 2017). These conventional drugs are widely used to treat and control inflammation and inflammatory-related diseases. By preventing the formation of pro-inflammatory prostaglandins (PGs), NSAIDs reduce inflammation, whereas SAIDs suppress the expression of pro-inflammatory genes. NSAIDs such as ibuprofen, indomethacin, and diclofenac are the most used among the other pharmacological groups with the same effect (Nunes *et al*., 2020). They are the most common anti-inflammatory and analgesic drugs responsible for 5 to 10% of all prescribed medications each year. However, the chronic use of NSAIDs should be avoided because of the risk of gastrointestinal (GI) bleeding as it can potentially contribute to the increase of hospitalization and death rates as a result of the latter drugs' long-term use side effects, including gastrointestinal, renal, and cardiovascular complications (Marcum & Hanton, 2010). Reports suggested that 30% of adverse drug reactions related to hospitalizations are because of NSAIDs use (Wongrakpanich *et al*., 2018). Hence, the need for alternatives. Medicinal plants are a good resource for developing new drugs, especially plants that are yet to be investigated and are considered important alternatives to conventional drugs (Bahadori *et al*., 2016). *Vitex trifolia* (family Verbenaceae) is a medicinal plant with multi-pharmacological properties, including potential anti-inflammatory effects. It is a shrub, also known as a three-leaf chaste tree (Wee *et al*., 2020), that can grow in tropics and subtropics regions, including Central Asia and Asia-Pacific regions (Orwa *et al*., 2009; Meena *et al*., 2011). *Vitex trifolia* is rich in phenolic and terpenoid compounds, contributing to its anti-inflammatory effect (Saklani *et al*., 2017; Fang *et al*., 2019; Wee *et al*., 2020). Traditionally, the plant treats headaches, colds, migraines, allergies, fever, inflammation, and various pain including rheumatic pain (Rani & Sharma, 2013; Tsai *et al*., 2016). Infusion and decoction of the plant are used to treat intestinal problems, tuberculosis, and amenorrhea (Dehsheikh *et al*., 2019). The inner bark of *V. trifolia* is used to treat diarrhea, cough, hypertension, sinusitis, periodontitis, and tuberculosis (Suchitra & Cheriyan, 2018). Additionally, the plant is proven to possess antibacterial (Abd Aziz *et al*., 2011; Kulkarni, 2012; Mary & Banu, 2015; Luo *et al*., 2017), antiviral (Vimalanathan *et al*., 2009; Chinsembu, 2019), antifungal (Hernandez *et al*., 1999; Devi & Singh, 2014), anthelminthic (Thenmonzhi *et al*., 2013), anticancer (Chan *et al*., 2016; Huang *et al*., 2016; Chan *et al*., 2018), hepatoprotective (Anandan *et al*., 2009), and antidiabetic (Nishina *et al*., 2017) properties. The multi-pharmacological properties of the plant are due to the existence of different secondary metabolites such as phenolics, steroids, terpenoids, glycosides, and tannins (Jangwan *et al*., 2014). Other phenolic compounds have been isolated from *V. trifolia*, such as casticin, 2',3',5-trihydroxy-3,6,7,trimethoxyflavone (Chan *et al*., 2018), luteolin, artemetin, (Nishina *et al*., 2017), 3,6,7-trimethyl quercetagetin, vitexin, 5-methyl artemetin, 7-desmethyl artemetin, luteolin-7-O-b-D-glucuronide, luteolin-3-*O*-b-D-glucuronide, isoorientin (Hernández *et al*., 1999; Kulkarni, 2012; Manaf *et al*., 2016), and vitexicarpin 1 (Abdul Hakeem *et al*., 2016). Persicogenin, penduletin, and chrysosplenol-D are also isolated from *V. trifolia*'s fruits (Aye *et al*., 2019). However, not much work has been done on the cytoprotective effect of *V. trifolia* extracts against RAW264.7 macrophages. Consequently, no clear correlation between the antioxidative action and anti-inflammatory properties were laid out to establish the impact of this plant on the development of new and safer anti-inflammatory agent.

Vitex trifolia has potent anti-inflammatory effects. Several researchers have done *in vitro* and *in vivo* studies to investigate the plant's potential anti-inflammatory and antioxidant effects (Matsui, 2009, 2011; Ankalikar & Viswanathswamy, 2017a, 2017b; Saklani *et al*., 2017; Dehsheikh *et al*., 2019; Wee *et al*., 2020). However, data are limited for the hydroalcoholic leaf extract of the plant. Water and alcohol are agro-solvent or biodegradable solvents. They possess minimum health risks to the patient's health and are eco-friendly solvents. Moreover, these solvents with high polarity can efficiently extract polar secondary metabolites such as phenolics which are reported to be the main compounds responsible for the plant's antioxidant and anti-inflammatory activities (Chemat *et al*., 2012; Mutalib & Latip, 2020). Here, we have explored the antioxidant and anti-inflammatory activities of *V. trifolia* leaves hydroalcoholic extract (VT) against hydrogen peroxide (H_2O_2)- and lipopolysaccharide (LPS)-induced RAW 264.7 cells.

MATERIALS & METHODS

Materials

RAW 264.7 cell line, Dulbecco's modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin

(100 µg/mL) were purchased from American Type Culture Collection (ATCC, USA). Diclofenac (DICL), hydrogen peroxide (H_2O_2), phosphate-buffered saline (PBS), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich Co. (USA). Ethanol was provided by Merck (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for the measurement of IL-1β, IL-6, and TNF-α were provided from eBioscience, Inc. (USA), and ROS assay kit, COX activity assay kit, cell lysis buffer, and protease inhibitor cocktail were purchased from ProCell, Inc. (Germany).

Plant materials and extraction

Vitex trifolia leaves were gathered in Subang Jaya, Selangor Darul Ehsan, Malaysia. With a voucher specimen number 0215USJ, the plant was verified and deposited at the Atta-ur-Rahman Institute for Natural Product Discovery (AuRINs), Universiti Teknologi MARA, Puncak Alam, Selangor Darul Ehsan, Malaysia. The collected leaves were air-dried under the shade for five days before grounded into a fine powder using a steel-jar Waring blender (E8000, USA). The powdered leaves (150 g) were combined with water and ethanol with a 1:1 (v/v) ratio. They were macerated under sonication for 30 min according to the method described by Celenghini *et al*. (2017). The solvent extraction from the sample was done using a rotary evaporator (40 °C, 100 mbar). The remaining sample underwent lyophilization to obtain a dried powder. The final extract was kept in a -20°C freezer until further use.

Cell culture

RAW 264.7 cells were cultured according to the ATCC protocol. Briefly, cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) containing 25 mM HEPES buffer, 4.5 g/l D-glucose, 0.2% sodium bicarbonate, 1 mM sodium pyruvate, and 2 mM L-glutamine, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin (100 IU/mL) and streptomycin (100 µg/mL). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C and the culture medium was changed every three days. The cells were then subcultured when 70-80% of confluency was reached.

Stock solutions preparation

The stock solutions of extract and the standard drug were prepared by dissolving accurately weighed 200 mg of VT and DICL in 1 mL of 100% DMSO and diluted to the required working concentrations before adding to the cells. The concentration of DMSO was maintained below 0.05% in the working solutions to avoid any possible effect of DMSO on cells. Exactly 1 mg of LPS was dissolved directly in DMEM (10 mL) to achieve a 100 µg/mL stock solution of LPS. To avoid possible contamination, all stock solutions were filtered using a 0.22 µm syringe filter and stored at a 4 °C chiller before use. All stock solutions were prepared fresh.

Cell viability assay

The cell viability assay was run to determine the cytotoxicity of VT by determining their half-maximal inhibitory concentration (IC_{50}). Cell viability was measured using the MTT assay described by Liou *et al*. (2014). The RAW264.7 cells were seeded at 1×10^4 cells/well in 96-well plates and incubated for 24 h in the CO_2 incubator. Next, the media was aspirated from the wells, and 100 µL of various concentrations of VT (0-2000 µg/mL) was added to each well. About 100 µL of H_2O_2 with a final concentration of 500 µM was used as a positive control. The cells were then incubated for 24 h. Next, the media was aspirated, and the cells were washed with 100 µL of PBS, twice. Then, each well was added 100 µL of MTT solution with 0.5 mg/mL of final concentration in media and incubated for a period of 4 h. After incubation, the MTT solution was aspirated from the wells, and to each well, 100 µL of DMSO was added to dissolve the formazan crystal. A microplate reader (InfiniteM200, Tecan, Switzerland) spectrophotometer was used to measure the absorbance at 570 nm. Cell viability was determined using Equation 1, and the IC_{50} was calculated from the viability plot.

Equation 1:

$$\text{Cell viability \%} = \frac{\text{Sample}_{\text{absorbance}} - \text{Blank}_{\text{absorbance}}}{\text{Control}_{\text{absorbance}} - \text{Blank}_{\text{absorbance}}} \times 100$$

Cytoprotective effect assay

The cytoprotective effects of VT were determined against H_2O_2-induced cytotoxicity using the MTT cell viability assay. Briefly, RAW 264.7 cells were seeded at 1×10^4 cell/well in 96-well plates and incubated for 24 h. Next, cells were induced, before the treatment, with 100 µL of H_2O_2 with a final concentration of 300 µM for 1 h. The media was then aspirated, and cells were treated with 100 µL of VT (25-200 µg/mL) and then incubated for another 24 h. Next, the cell viability assay was carried out.

ROS measurement

The intracellular ROS was measured using a commercial ROS detection kit from PromoCell, Inc. (Germany). The kit utilizes dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), a fluorogenic agent that can quickly enter the cell. Upon entry, $DCFH_2$-DA is converted to dichlorodihydrofluorescein ($DCFH_2$) by intracellular esterase. The latter compound is oxidized by intracellular ROS and yields a highly fluorescent

product detected by a microplate reader. The intensity of the fluorescent is directly related to the intracellular ROS level.

Briefly, the macrophages were seeded in a 96-well plate at 5×10^4 cells/well and were incubated for 24 h to obtain 70-80% confluency. Next, cells were labeled with 100 µL of ROS Label diluted in ROS assay buffer and incubated for 45 min in the dark. The ROS Label was then aspirated in the cells were induced with H_2O_2 with a final concentration of 300 µM for 1 h followed by treatment with 100 µL of VT (25-100 µg/mL) and DICL (100 µg/mL). Later, the supernatant was removed, and 100 µL of PBS was added to each well. The fluorescence was read using a microplate reader (InfiniteM200, Tecan, Switzerland) equipped with a fluorescent (Ex=488 nm, Em=520 nm) detector.

Pro-inflammatory cytokines measurement

The levels of pro-inflammatory cytokines were assessed by an enzyme-linked immunosorbent assay (ELISA), as described by Liou *et al*. (2014). Briefly, RAW 264.7 cells were seeded in 6-well plates at the density of 1×10^6 cells/well and were incubated for 24 h. Next, the macrophages were induced with a final concentration of 1 µg/mL of LPS for 1 hr, followed by VT (25, 50, & 100 µg/mL) for 24 h. DICL (100 µg/mL) was used as a positive control.

Next, the cells were detached using a cell scraper and centrifuged at 10,000 ×*g* for 5 min at 4 °C. The supernatants were immediately used for the measurement or were kept at -80 °C freezer until further analysis. The levels of IL-1β, IL-6, and TNF-α were measured using a specific ELISA pre-coated kit (eBioscience, USA) by the manufacturer's protocols. The pre-coated plates were washed twice with 400 µL of wash buffer. Exactly 50 µL of the sample was added to each well in duplicate, and with sample diluent, the volume was adjusted to 100 µL and 50 µL of Biotin-Conjugate was added to each well. The plate was covered with an adhesive film and incubated at room temperature (18-25 °C) for 2 h on a microplate shaker (Stuart, UK) set at 400 rpm. The wells were then drained and washed four times with 400 µL of wash buffer. Following the addition of streptavidin-HRP (100 µL) to each well, the plate was sealed with an adhesive film and incubated for 1 h (18–25 °C) on a microplate shaker (Stuart, UK) set to 400 rpm. Afterward, the wells were again emptied and washed four times with 400 µL of wash buffer. TMB Substrate Solution (100 µL) was pipetted to all wells, and plates were incubated at room temperature for 10 min in the dark. Exactly 100 µL of Stop Solution was quickly pipetted into each well to stop the enzyme reaction. The absorbance was immediately read at 450 nm using a microplate reader (InfiniteM200, Tecan, Switzerland).

Cyclooxygenase activity measurement

The total COX activity was measured using a fluorometric assay kit (PromoCell, Germany). The COX activity was measured by the amount of resorufin produced over time due to the reaction between hydroperoxy endoperoxide and 10-acetyl-3,7-dihydroxyphenoxazine in the cell lysate.

Briefly, $1x10^6$ RAW 264.7 cells/well were planted in 6-well plates and cultured for 24 h. Then, cells were treated with VT (25, 50, & 100 µg/mL) and DICL (100 µg/mL) as a positive control for a further 24 h after being stimulated with LPS (1 g/mL) for 1 h. Then, the cells were detached from the plate and centrifuged at 2,000 ×*g* for 5 min. The supernatant was discarded, and the cell pellet was washed with cold PBS before the cells were resuspended in 0.3 mL of lysis buffer with protease inhibitors. The samples were later vortexed and incubated on ice for 5 min. In duplicates, exactly 20 µL of each sample was added to a 96-well plate. Next, to each well, 1 µL of COX probe, 2 µL of COX cofactor, 2 µL of DMSO, and 85 µL of COX assay buffer was added and mixed. Approximately 10 µL of arachidonic acid was put into all wells at once using a multichannel pipette, and immediately the fluorescence was measured (Ex/Em = 535/587 nm) in a kinetic mode once every 15 sec for 30 min using a microplate reader (InfiniteM200, Tecan, Switzerland). Precisely 100 µL of standard resorufin was added in duplicate in a 96-well plate with final concentrations of 0, 4, 6, 12, 16, and 20 pmol, and the fluorescence was measured at ex/mm = 535/587 nm at an endpoint using a microplate reader (InfiniteM200, Tecan, Switzerland). The COX activity was quantified using Equation 2:

$$\Delta RFU_{535/587nm} = RFU_{T_2} - RFU_{T_1}$$

where RFU_{T1} is the sample reading at time T_1, and RFU_{T2} is the sample reading at T_2. ΔRFU was used to find B pmol from the resorufin calibration curve. The COX activity was calculated using Equation 3:

$$\text{COX activity} = \frac{B}{\Delta M} \times \text{Sample Dilution} = \mu U$$

where B is the pmol of resorufin generated in the sample by COX coenzyme activity and ΔM is the reaction time.

Data analysis

The data were presented as mean ± standard deviation (S.D.). The statistical analyses were done using analysis of variance (ANOVA) followed by Duncan's posthoc test using SPSS (Statistical Package for the Social Sciences) software 21 (IBM, USA). Duncan's test was chosen to analyze the different sets of means. To determine the correlation coefficient

between antioxidant and anti-inflammatory activities, Pearson's correlation analysis was employed. The value of $p<0.05$ was considered statistically significant. The IC_{50} values were calculated using Microsoft Office Excel 365 (Microsoft, USA).

RESULTS

Cell viability assay

The viability of the RAW264.7 cells was assessed using the MTT cell viability assay, and the IC_{50} value was calculated for VT The extract showed no toxicity until 100 µg/mL. However, their cytotoxicity was evident at higher concentrations (Figure 1). The IC_{50} value of VT was calculated to be 355.00 ± 15.32 µg/mL. For the positive control, 500 µM of H_2O_2 was used, and it was found to reduce the cell viability to 51.59 ± 4.32%.

Cytoprotective effect of VT

The cytoprotective effect of VT was assessed against H_2O_2 cytotoxicity. Cells were treated with H_2O_2 (300 µM) for 1 h and followed by treatment with VT in different concentrations (Figure 2). H_2O_2 at 300 µM significantly ($p<0.05$) decreased cell viability to 71.08 ± 1.25% compared to non-treated cells. VT at 25 and 50 µg/mL significantly improved the cell viability of H_2O_2-treated cells to 85.46 ± 3.4 and 88.95 ± 5.56%, respectively. However, no significant increase or decrease was recorded in the cell viability of cells treated with 100 µg/mL (78.12 ± 0.92%), and 200 µg/mL (71.11 ± 1.81%) of VT On the other hand, DICL (47.78 ± 3.63%) decreased cell viability significantly compared to the untreated cells and the control cells (pre-treated with H_2O_2).

ROS measurement

The antioxidant effect of VT was evaluated on H_2O_2-induced RAW 264.7 cells, and ROS production was measured using a commercial ROS detection kit (PromoCell, Germany). RAW 264.7 cells were induced with 300 µM of H_2O_2 for 1 hr and followed by treatment with different concentrations of VT H_2O_2 at 300 µM significantly ($p<0.05$) produced an excessive

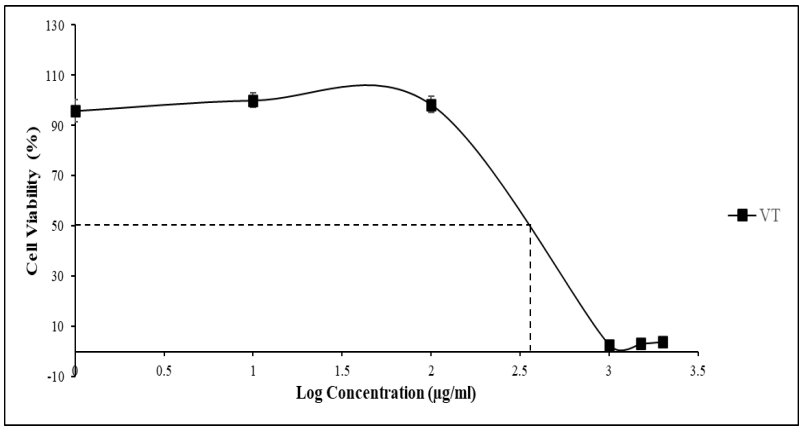

Fig. 1. The cytotoxicity effect of *V. trifolia* leaves hydroalcoholic extract (VT) against RAW 264.7 cells via MTT assay.

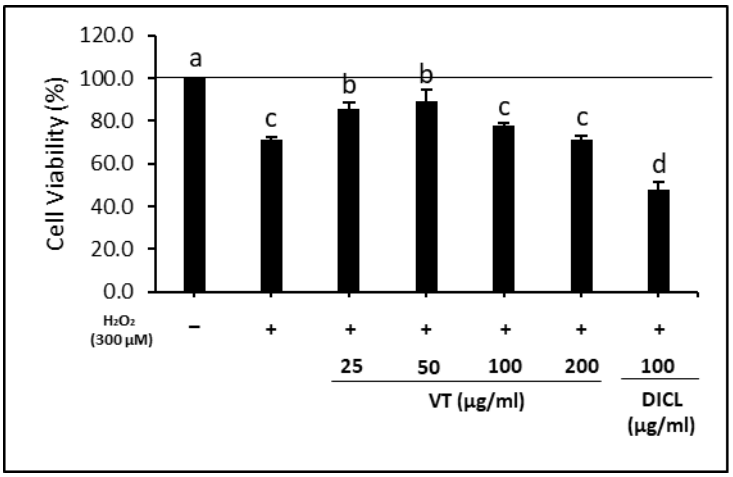

Fig. 2. Cytoprotective effect of *V. trifolia* leaves hydroalcoholic extract (VT) on H_2O_2-induced cytotoxicity. Different letters indicate statistically significant differences ($P\leq0.05$). Duncan posthoc test ($n=3$).

amount of ROS compared to the non-treated cells. VT at 25, 50, and 100 µg/mL significantly (p<0.05) inhibited the production of H_2O_2-induced ROS to 10.6 ± 2.81%, 20.54 ± 1.68%, and 13.36 ± 1.58%, respectively in contrast to the cells treated with H_2O_2 alone. Meanwhile, the same effect was observed with DICL (100 µg/mL) on the H_2O_2-induced cells. DICL was found to reduce the ROS production significantly (p<0.05) to 31.13 ± 0.13% but, not as low as VT-treated cells (Figure 3).

Pro-inflammatory cytokines measurement

The effect of VT on pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α was evaluated using a specific ELISA commercial kit (eBiosciece, USA). As shown in Figures 4, 5, and 6, in the absence of any treatment, RAW 264.7 cells produced very low levels of TNF-α, IL-6, and IL-1β. However, the production of TNF- α, IL-6, and IL-1β were significantly (p<0.05) increased in RAW 264.7 cells with LPS (1 µg/mL) to 4.15 ± 0.15, 13.08 ± 1.07, and 15.1 ± 1.08 folds, respectively compared to non-treated cells. VT (25, 50, and 100 µg/mL) inhibited the production of TNF-α induced by LPS and significantly (p<0.05) diminished the TNF-α level to 3.13 ± 0.19, 3.31 ± 0.01, and 3.19 ± 0.09 folds, respectively, compared to cells treated with LPS alone (4.15 ± 0.15 folds) (Figure 4). A similar effect of VT was observed on IL-6 levels when cells were treated with VT At 25 and 50 µg/mL, VT significantly (p<0.05) reduced IL-6 levels to 7.81 ± 0.55 and 8.78 ± 2.36 folds, respectively compared to that of cells treated with LPS (1 µg/mL) alone (Figure 5). Contrastingly, treatment of cells with 100 µg/mL DICL significantly reduced the levels of TNF-α and IL-6 to 2.65 ± 0.13 and 6.37 ± 1.06, respectively. No inhibitory effects of VT and DICL were seen on IL-1β production as the extract at 25, 50, and 100 µg/mL increased the LPS-induced production of IL-1β to 21.44 ± 2.83, 22.4 ± 2.45, and 18.8 ± 3.31 folds, respectively (Figure 6), however, the increases were not statistically significant.

COX activity measurement

The effect of VT (25, 50, & 100 µg/mL) was evaluated on LPS-induced COX activity using a COX fluorometric assay kit (PromoCell, Germany) (Figure 7). Cells treated with LPS (1 µg/mL) alone were found to have a significantly (p<0.05) high level of COX activity (0.83 ± 0.11 µU) compared to cells with no LPS (0.063 ± 0.01 µU). Treatment with 25, 50, and 100 µg/mL of VT were found to significantly (p<0.05) inhibit COX activity to 0.49 ± 0.16, 0.51 ± 0.11, and 0.3 ± 0.07 µU, respectively. DICL (100 µg/mL) managed to significantly (p<0.05) reduced COX activity to 0.023 ± 0.02 µU, comparable to the untreated cells.

Correlation between antioxidant and anti-inflammatory activities of *V. trifolia*

Pearson's correlation analysis was performed to calculate the correlation coefficient between the inhibition of ROS and the inhibition of IL-6, TNF- α, and COX activity to assess the relationship between the antioxidant and anti-inflammatory properties of VT (Table 1).

As shown in Table 1, ROS inhibition showed a significant positive correlation to IL-6, TNF-α, and COX activity with Pearson's correlation coefficients of 0.670, 0.969, and 0.827, respectively. Moreover, a significant positive correlation was also found between TNF-α inhibition and COX activity inhibition with Pearson's correlation coefficients of 0.853.

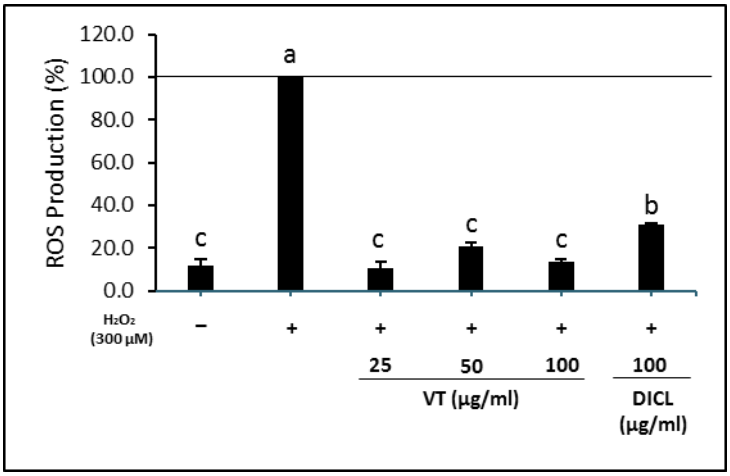

Fig. 3. Antioxidant effect of V. trifolia leaves hydroalcoholic extract (VT) on H_2O_2-induced ROS production. Different letters indicate statistically significant differences (P≤0.05). Duncan posthoc test (n=3).

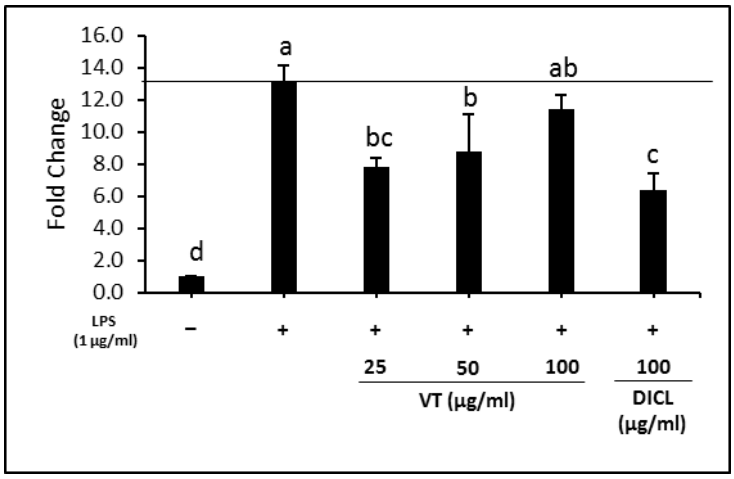

Fig. 4. Vitex trifolia leaves hydroalcoholic extract (VT) effect on LPS-induced TNF-α production. Different letters indicate statistically significant differences (P≤0.05). Duncan posthoc test (n=3).

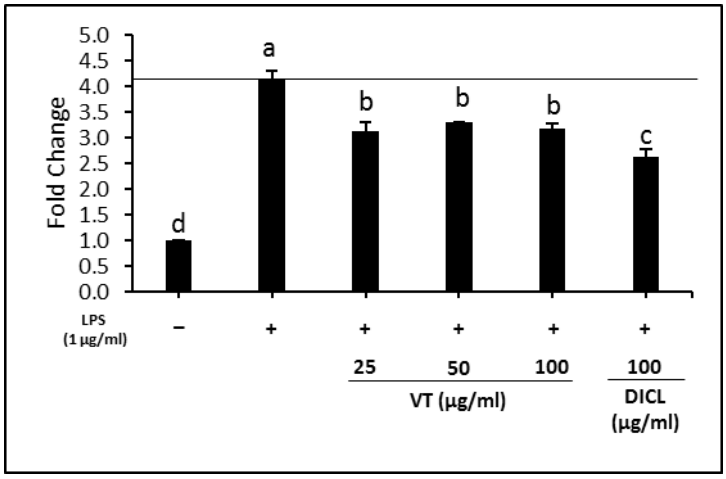

Fig. 5. Vitex trifolia leaves hydroalcoholic extract (VT) effect on LPS-induced IL-6 production. Different letters indicate statistically significant differences (P≤0.05). Duncan posthoc test (n=3).

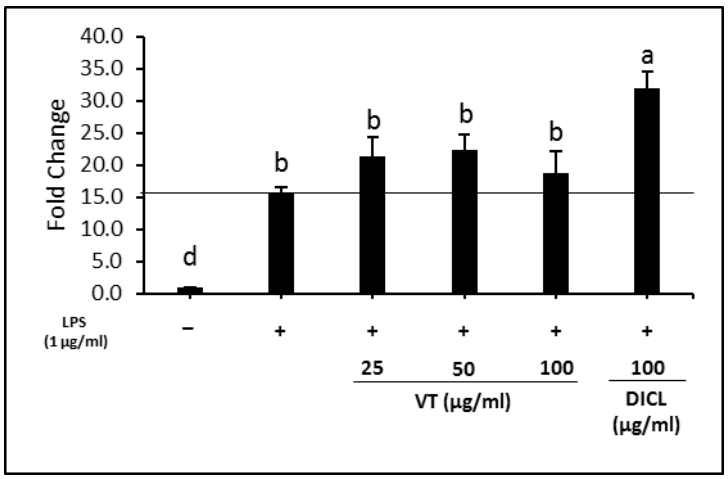

Fig. 6. Vitex trifolia leaves hydroalcoholic extract (VT) effect on LPS-induced IL-1β production. Different letters indicate statistically significant differences (P≤0.05). Duncan posthoc test (n=3).

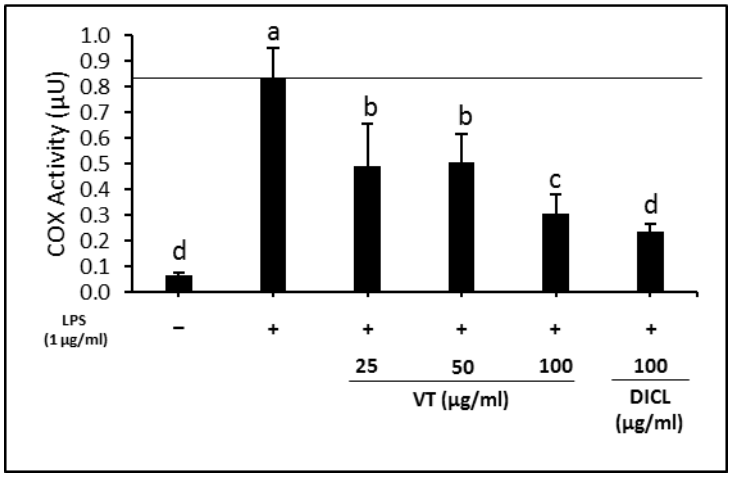

Fig. 7. *Vitex trifolia* leaves hydroalcoholic extract (VT) effect on LPS-induced total COX activity. Different letters indicate a statistically significant difference ($P \leq 0.05$). Duncan posthoc test ($n=3$).

Table 1. Correlation study between antioxidant and anti-inflammatory effects of VT

	TNF-α Inhibition	IL-6 Inhibition	Total COX Activity Inhibition	ROS Inhibition
TNF-α Inhibition		.667*	.869**	.980**
IL-6 Inhibition	.667*		0.354	.682*
Total COX Activity Inhibition	.869**	0.354		.846**
ROS Inhibition	.980**	.682*	.846**	

* ($p<0.05$)
**($p<0.01$)

DISCUSSION

Inflammation and inflammation-related chronic diseases rank among the greatest threats to human health (Pahwa *et al*., 2020), affecting millions of people worldwide with a high prevalence of mortality (Naghavi, 2019). Furthermore, conventional drugs, such as NSAIDs, are usually taken to treat and control inflammation-related diseases. Despite NSAIDs' life-threatening side effects, the prevalence of their use is high among patients (Bjarnason *et al*., 2018).

Natural products with pharmacological effects, particularly medicinal plants, are thought to be a source of possible novel therapeutic agents with effects that are safer than those of conventional pharmaceuticals now on the market. As per the WHO reports, 65% of the world population uses herbal medicines as healthcare supplements (Nunes *et al*., 2020). *V. trifolia* is a traditional medicinal plant of tropical and sub-tropical regions that can possess several pharmacological effects, including antioxidant and anti-inflammatory properties (Kulkarni, 2012; Saklani *et al*., 2017; Wee *et al*., 2020). In Chinese and Unani traditional medicines, the plant is used for the same effects (Suchitra & Cheriyan, 2018). Several researchers have attempted to explore different types of *V. trifolia* extracts with various phytochemical compositions using other *in vivo* and *in vitro* studies on antioxidant and anti-inflammatory activities. However, data are limited for the hydroalcoholic extract, considered a green extract, and preferred herbal medicinal supplements.

The IC_{50} value for VT against RAW 264.7 cells was 355.00 ± 14.32 µg/mL, which shows that the plant extract is classified as weak cytotoxic against RAW 264.7 cells. According to the National Cancer

Institute (NCI), the reference value for cytotoxicity are classified as strong cytotoxic effects (IC_{50} of < 21 µg/mL), moderate cytotoxic effects (IC_{50} of 21–200 µg/mL), and weak cytotoxic effects (IC_{50} of 201–500 µg/mL). The IC_{50} values of > 501 µg/mL are considered to be non-cytotoxic (Grever *et al*., 1992). To our knowledge, there was no published data on the cytotoxicity of VT against the RAW 264.7 cell line. However, in a recent study, Wee and his colleagues (2020) managed to determine the IC_{50} values of different extracts of the *V. trifolia* leaves (hexane, methanol, ethanol, and aqueous extracts) as 12.2 ± 2.5, 120.3 ± 12, 185 ± 33.3, and 172 ± 26.4 µg/mL, respectively against U937 macrophages (Wee *et al*., 2020). In another study, the aqueous leaves extract of *V. trifolia* showed a significant cytotoxicity effect on RAW 264.7 cells only at a very high concentration of 5000 µg/mL (Matsui *et al*., 2009). These variations of findings could be because of different extracts and extraction methods that can affect the chemical composition and the pharmacological effects of a final extract. Additionally, cell type and methods used for the cell viability assay can also be the reason for different results. Our findings are corroborated by the positive control used for the cytotoxicity assay. H_2O_2 (500 µM), which serves as a positive control was able to reduce the cell viability to 51.59 ± 4.32%, similar to several studies (Al-Sheddi *et al*., 2016; Bach *et al*., 2018; Kwon *et al*., 2019; Lin *et al*., 2019).

According to Kwon *et al*. (2019), RAW 264.7 cells treated with 500 µM of H_2O_2 showed a cell viability of 60% after 24 hr of incubation (Kwon *et al*., 2019). Similarly, 500 µM of H_2O_2 decreased the cell viability of MIN6 cells, a murine pancreatic β-cell line, to 44% (Bach *et al*., 2018). Moreover, 500 µM of H_2O_2 inhibited the growth of HepG2 cells by 45-60% (Chen *et al*., 2011; Al-Sheddi *et al*., 2016). However, the LC_{50} of H_2O_2 for RAW 264.7 was reported to be about 360 µg/mL, using Alamar blue assay instead of the MTT assay to evaluate the cell viability, where MTT assay is the gold standard for cytotoxicity testing (Nkala *et al*., 2020).

Next, VT's cytoprotective and antioxidant activity was evaluated against H_2O_2-induced cytotoxicity and oxidative stress, which is a common model for assessing the cytoprotective of natural substances against oxidative stress stimuli. H_2O_2 is one of the most important free radicals that can easily diffuse into the cells through aquaporin or peroxiporin (Nakao, 2008; Lennicke *et al*., 2015; Wragg *et al*., 2020). It acts as a pro-oxidant at high concentrations and causes oxidative stress (akao *et al*., 2008; Lennicke *et al*., 2015). Moreover, H_2O_2 directly contributes to producing other potent ROS such as O^{-2}, hydroxyl radical, and hypochlorous acid radical, further worsening the oxidative stress state (Coyle *et al*., 2006; Keshari *et al*., 2015; Sies, 2017). The overproduction of ROS, including H_2O_2, can cause oxidative damage to DNA and RNA and induce lipid and protein oxidation leading to cell injury and cell death (Kasote *et al*., 2015). Cell death by H_2O_2 can occur by activating apoptosis. H_2O_2-induced apoptosis occurs via sustained aviation of c-jun N-terminal kinase (JNK) through MAPKs pathways and death receptors such as TNF receptor-1 (TNFR1). The activation of JNK further activates BCL/BAD and caspase pathways leading to cell death (Ryter *et al*., 2007; Xiang *et al*., 2016).

The current study showed the cytoprotective effect of VT against H_2O_2-induced cytotoxicity. VT (25 & 50 µg/mL) increased cell viability of H_2O_2-induced cells to 85.46 ± 3.4% and 88.95 ± 5.56%, respectively, in contrast to the cells treated with H_2O_2 (300 µM) alone which decreased the cell viability to 78.12 ± 0.92%. VT (25 & 50 µg/mL) showed significant differences in comparison to untreated cells (+H_2O_2) on cell viability. This suggested that VT at those concentrations can protect against oxidative stress induced by H_2O_2. However, VT at 100 µg/mL was unable to protect the cells. Yet, the ROS production was significantly reduced in all concentrations of VT as opposed to the untreated cells which signifies the ability of VT to inhibit oxidative stress. The cell viability decrement experienced by cells treated with VT (100 µg/mL) may be due to the increment of the concentration of the extract (additive effect). Thus, the cytoprotective effect of VT could be because of the remarkable antioxidant activity of the compounds in the extract that showed inhibition of H_2O_2-induced ROS after 6 hr of incubation at low concentrations (25-100 µg/mL). As we reported previously, a high concentration of phenolic compounds can indeed contribute to the cytoprotective action of the plant (Ghafari *et al*., 2021).

The antioxidant activity of *V. trifolia* can be exhibited through its radical scavenging ability and modulation of pro-oxidant enzymes and signaling pathways. Ankalikar and Viswanthswam (2017b) reported the H_2O_2 scavenging activity of VT However, the extract was not tested on cells but tested for H_2O_2, hydroxyl radical, and NO scavenging activities. Another study revealed that the IC_{50} value for the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity of the *V. trifolia* methanol leaves extract was 16.8 µg/mL (Shah *et al*., 2013). An IC_{50} value of 81.72 µg/mL for the DPPH-scavenging activity of *V. trifolia*'s leaves was observed in a related study (Saklani *et al*., 2017). Furthermore, the plant's ethanol leaves extract's ferrous ion chelating activity was seen with an IC_{50} value of 40 µg/mL (Saklani *et al*., 2017). The antioxidant activity of *V. trifolia* can also be achieved through its regulatory effect on related pathways and transcription factors. Casticin, the main flavonoid of *V. trifolia*, can enhance the expression of nuclear factor-erythroid factor 2-related factor 2 (Nfr2) (Liou *et al*., 2014). Under an oxidative

stress state, Nrf2 is disassociated from Keap1, leading to an increase in its translocation into the nucleus, where the expression of more than 500 antioxidant genes can be stimulated (Bellezza *et al*., 2018).

The antioxidant activity of *V. trifolia* can be seen through modulation of the NF-κB pathway, which plays both pro-oxidant and antioxidant roles during oxidative stress. Activated NF-κB under oxidative stress can translocate into the nucleus and regulate the expression of antioxidant genes (Vrankova *et al*., 2016). The NF-κB pathway, according to some findings, may also function in a pro-oxidant mechanism. By competing with Nrf2 for the transcriptional co-activator CBP (CREB-binding protein)-p300 complex, ROS-activated NF-κB can suppress Nrf2. Alternatively, the NF-κB pathway can induce the expression of pro-oxidant genes such as NADPH oxidase (NOX), NOX2 subunit gp91phox, and nitric oxide synthase (NOS) (Lingappan, 2018). *V. trifolia* was reported to inhibit the activation of the NF-κB signaling pathway by several reports. In another study, ethanol fruits extract of *V. trifolia* was reported to inhibit the TNF-α induced activation of NF-κB. Viterotulin C and vitetrifolin B, isolated from the extract, inhibited the activation of NF-κB by 68.86% and 62.44%, respectively (Fang *et al*., 2019). Another active compound of *V. trifolia*, pyronopyran-1,8-dione (PPY), was also reported to inhibit the activation of NF-κB (Lee, G. *et al*., 2017).

The aqueous extract of *V. trifolia* leaves was reported to significantly inhibit the expression of NF-κB p65 and p50 subunits in LPS-induced RAW 246.7 cells (Matsui *et al*., 2012). The regulatory effect of *V. trifolia* on MAPKs can also contribute to its antioxidant activity as ROS can activate MAPKs signaling pathways through the inhibition of MAPKs phosphatase, which further leads to cell injury and cell death (Zhang *et al*., 2016).

Casticin and other polyphenols from *V. trifolia* had demonstrated the ability to inhibit MAPKs signaling pathways, including p38, extracellular signal-regulated kinase (ERK)1/2, and JNK (Liou *et al*., 2014). Furthermore, *V. trifolia* or its chemical constitutes inhibited the pro-oxidant enzymes such as COX-2, lipoxygenase (LOX), xanthine oxidase (X.O.), and NOX. *V. trifolia* leaves aqueous extract has been reported to have an inhibitory effect on inducible-NOS (iNOS) when tested on LPS-induced murine macrophages (Matsui *et al*., 2009). It has been proven that polyphenols can inhibit the activity of NOS and X.O., LOX, COX (Hussain *et al*., 2016), iNOS (Liou *et al*., 2014), and NOX (Yousefian *et al*., 2019).

Further investigation was carried out to determine the anti-inflammatory properties of VT. The extract was tested on LPS-induced RAW 264.7 cells, and its inhibitory effects on pro-inflammatory cytokines and COX activity were assessed. The cytokine levels (TNF-α & IL-6) in the LPS-induced cells treated with VT displayed significant differences in comparison to the untreated cells but no significant differences were observed between concentrations of VT Evidently, VT can reduce pro-inflammatory cytokine levels triggered by LPS providing optimum effect even at the lowest concentration (50 µg/mL).

IL-1β, IL-6, and TNF-α are pro-inflammatory cytokines released during acute inflammation. These cytokines initiate the primary immune response against invading agents by increasing the production of other pro-inflammatory mediators. Instead, COX-2 is the main enzyme of the arachidonic acid pathway that produces pro-inflammatory P.G.s. The latter mediators are involved in most acute inflammation signals (Borish & Steinke, 2003; Tsuge *et al*., 2019; Jang *et al*., 2020). In this study, 1 µg/mL of LPS significantly ($p<0.05$) induced RAW 264.7 cells and increased the levels of the pro-inflammatory cytokines, and COX activity. Similar results with 1 µg/mL of LPS were reported by several studies (Dong *et al*., 2018; Kim *et al*., 2018; Zhang *et al*., 2021). According to Zhang *et al*. (2021), 1 µg/mL of LPS significantly increased the levels of TNF-α, IL-6, and COX activity after 12 h of incubation in RAW 264.7 cells (Zhang *et al*., 2021). In a similar study, pre-treatment of RAW 264.7 cells with 1 µg/mL of LPS for 1 h significantly induced the production of IL-6 and TNF-α (Kim *et al*., 2018). Moreover, an elevated level of COX-2 was observed in RAW 264.7 cells post-incubation with 1 µg/mL of LPS (Dong *et al*., 2018).

VT at lower concentrations (25-100 µg/mL) exhibited a potential anti-inflammatory effect by significantly reducing the LPS-induced production of inflammatory mediators such as TNF-α, IL-6, and COX-2. However, the extract has not exerted any significant inhibitory effect on the LPS-induced production of IL-1β. Here, the anti-inflammatory effect of the extract on inflammatory mediators such as IL-6, TNF-α, and COX was reported for the first time. However, according to Ankalikar and Viswanathswamy (2017a), the anti-inflammatory effect of the same extract of the plant on cotton-pellet-induced granuloma and carrageenan-induced paw edema in rats was observed. The extract (100 mg/mL) reduced the paw edema volume to 0.67 ± 0.03 mL in comparison to the control group after 1 hr. It was also seen that the extract at 100 mg/kg prevented the formation of dry and wet exudate by 36.45% and 36.94%, respectively. Nonetheless, there was no report on the anti-inflammatory action of the plant extract on specific cytokines and COX (Ankalikar & Viswanathswamy, 2017a).

In another study, *V. trifolia* leaves hydroalcoholic extract (50-800 µg/mL) was reported to inhibit albumin denaturation and heat-induced hemolysis with inhibition rates of 17.01 - 78.18% and 29.45 - 82.43%, respectively (Ankalikar & Viswanathswamy,

2017b). The ethanolic and aqueous extracts of *V. trifolia* leaves significantly reduced the paw edema volume to 200 mg/kg. The ethanolic extract showed a more potent effect than the aqueous extract with 43.00 and 36.07% inhibition rates, respectively (Kulkarni, 2011). Furthermore, the inhibitory effect of *V. trifolia* leaves aqueous extract on IL-6 and TNF-α evaluated by Matsui *et al*. (2009) was observed. Significant inhibitions to IL-6, TNF-α, and COX-2 at higher concentrations such as 2500 and 5000 µg/mL in LPS-induced RAW 264.7 cells were demonstrated; however, no significant difference was found in the effect was observed at lower concentrations (Matsui *et al*., 2009, 2012).

VT has a more potent inhibitory effect on IL-6, TNF-α, and COX production at low concentrations such as 25, 50, and 100 µg/mL in the present study. However, no significant inhibitory effect was observed on IL-1β production. Nevertheless, Matsui *et al*. (2009) reported that the inhibitory effect on LPS-induced IL-1β production was found at 2500 µg/mL of the aqueous extract. In a recent study, Wee *et al*. (2020) demonstrated the inhibitory effect of ethanol extract of *V. trifolia* leaves on LPS-induced TNF-α and IL-1β production with IC_{50} values of 43.6 ± 3.9 and 29.2 ± 2.3 µg/mL, respectively. However, in the same study, the plant dichloromethane extract was reported to have a more potent inhibitory effect on LPS-induced TNF-α and IL-1β production with IC_{50} values of 4.7 ± 0.9 and 1.2 ± 0.2 µg/mL, respectively (Wee *et al*., 2020). Moreover, isolated compounds of *V. trifolia* such as PPY (Lee, G. *et al*., 2017) and casticin (Chan *et al*., 2018) were also reported to have a significant inhibitory effect on IL-1β. This difference can be due to different extracts, working concentrations, and evaluation methods. The inhibitory effect of VT on cytokine and COX-2 can be attributed to various secondary metabolites. Additionally, the abundance of phenolic compounds in the extract can also contribute to their potent anti-inflammatory effect (Ghafari *et al*., 2021).

On a molecular basis, the effect of *V. trifolia* on IL-6, TNF-α, and COX-2 may be due to its ability to modulate inflammatory-related signaling pathways, such as NF-κB and MAPKs signaling pathways. As discussed earlier, upon stimulation, the activated NF-κB transcription factor induces the expression of inflammatory mediators, including cytokines and COX-2 (Lawrence & Fong, 2010; Chen *et al*., 2018; Wang *et al*., 2019). On the other hand, cytokines and COX-2 production can also be induced by activating the MAPKs pathway through PRRs stimulation (Tasneem *et al*., 2019). ERK is involved in IL-6, and TNF-α synthesis, while p38 regulates IL-1 stimulated production of IL-6 and induction of COX-2 (Thalhamer *et al*., 2008). VT can impede the production of inflammatory mediators *via* a diminution of NF-κB translocation into the nucleus. This occurs by inhibition of the expression of the NF-κB p50 and p65 subunits (Matsui *et al*., 2012). Casticin of *V. trifolia* has also been shown to act *via* the blockade of NF-κB (Chan *et al*., 2018). The vascular inflammation in the human umbilical vein was inhibited by casticin past the blockade of the NF-κB transcription factor (Lee *et al*., 2012). Also, casticin has been shown to have an inhibitory effect on p38 and ERK1/2 in LPS-induced murine macrophages by enhancing their inhibitors (Liou *et al*., 2014).

Other active compounds of *V. trifolia*, namely viterolutin C, vitexilacton D, and vitexilactone, were reported to block TNF-α-induced NF-κB activation in the HEK 293 cell line (Fang *et al*., 2019). The inhibitory effect of *V. trifolia* on the MAPKs pathway has been described by several studies. PPY isolated from *V. trifolia* can also block ERK1/2 phosphorylation (Lee, G. *et al*., 2017). Furthermore, inhibition of COX-2 can occur by the suppression of cytokine production as IL-1β and TNF-α are potent inducers of COX-2. Moreover, this inhibition can also be seen through direct antagonism.

The correlation analysis showed a positive correlation between ROS production and cytokines activity. This means that the antioxidant activity of VT can contribute to its anti-inflammatory effect and vice versa. ROS are free radicals produced by endogenous and exogenous sources (Khanna *et al*., 2014; Adwas *et al*., 2019). The endogenous ROS are produced during the inflammatory process, metabolic reaction, severe exercise, ischemia, infection, and aging. On the other hand, pollution of water, alcohol consumption, smoking, some drugs, and radiation, could be their exogenous sources (Pizzino *et al*., 2017; Adwas *et al*., 2019).

Enzymatic and non-enzymatic antioxidants normally eliminate ROS (Adwas *et al*., 2019). However, uncontrolled production of ROS can be directly involved in triggering signaling pathways that lead to the onset of pathological states. They cause damage to deoxyribonucleic acid (DNA), protein modification, lipid oxidation, cell injury, and mutagenic activity. Other than that, ROS are also involved in apoptosis, necrosis, cell proliferation, and carcinogenesis activities (Mittal *et al*., 2014; Adwas *et al*., 2019). There is an interdependent relationship between oxidative stress and the inflammatory process. Inflammation as a primary abnormality has the overproduction of ROS as its consequence. At the same time, prolonged oxidative stress by itself can cause inflammation and inflammation-related diseases (Biswas, 2016), and it is the leading cause of the progression of many chronic inflammatory disorders (Mittal *et al*., 2014).

ROS are produced by immune cells as part of the inflammatory process to enhance the clearance of stimuli throughout inflammation. But, their prolonged production can lead to chronic inflammation

and oxidative stress (Pizzino *et al*., 2017). Pro-inflammation cytokines, such as IL-6 and TNF-α, can induce the production of ROS through the overexpression of NADPH oxidase (Mittal *et al*., 2014; Biswas, 2016).

Instead, ROS by themselves can induce the production of inflammatory mediators. Generally, ROS are recognized by PRRs such as TLR, NLR, and RLR (Chen, Y. *et al*., 2018). They are reported to activate transcription factors such as NF-κB, AP-1, and hypoxia-inducible factor 1 alpha (HIF-1α), which push the expression of the pro-inflammatory genes causing the production of proteins such as monocyte chemoattractant protein-1 (MCP-1), TNF-α, IL-1, and transforming growth factor-beta (TGF-β). Also, ROS can alter tyrosine kinases, such as Src, Ras, phosphatidylinositol 3-kinase (PI3K), epidermal growth factor receptor (EGFR), p38, JNK, and ERK. Numerous biological responses are triggered when these redox-sensitive pathways are activated (Nakao *et al*., 2008; Schieber & Chandel, 2014). Moreover, mitochondrial ROS can contribute to the LPS-induced production of IL-1β, IL-6, and TNF-α (Mittal *et al*., 2014; Forrester *et al*., 2019) and amplify its effect (Ko *et al*., 2017). Additionally, they are reported to increase the activation of NALP3 inflammasome, an inflammatory complex that activates caspases and IL-1β (Mittal *et al*., 2014; Forrester *et al*., 2019) through the activation of NLR (Biswas, 2016). The induction of COX-2 production by ROS was also reported. In a study, the inhibition of ROS in LPS-stimulated zebrafish remarkably decrease the levels of NO, iNOS, and COX-2 (Ko *et al*., 2017). Meanwhile, ROS inhibitors were found to reduce the production of IL-6 and TNF-α (Ranneh *et al*., 2017). This can indicate and justify the correlation between ROS production and pro-inflammatory cytokines levels. It shows that the plant's antioxidant effects can directly contribute to its anti-inflammatory effects.

CONCLUSION

V. trifolia leaves hydroalcoholic extract was discovered to have substantial anti-inflammatory and antioxidant properties with a notable positive correlation between the effects. Furthermore, the extract has been proven to have weak cytotoxicity. Secondary metabolites including phenols, flavonoids, and terpenoids may be responsible for the results as these metabolites work synergistically to influence the signaling pathways related to the antioxidant and anti-inflammatory properties of the extract. However, further molecular investigations on the isolated compounds of the plant and *in vivo* studies are suggested for future work.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Abd Aziz, S.M., Low, C.N., Chai, L.C., Abd Razak, S.S.N., Selamat, J., Son, R., Sarker, M.Z.I. & Khatib, A. 2011. Screening of selected Malaysian plants against several food borne pathogen bacteria. *International Food Research Journal*, **18(3)**: 1141–1147.

Abdul Hakeem, N.R.S., Md Yusof, N., Jahidin, A.H., Hasan, M.H., Mohsin, H.F. & Abdul Wahab, I. 2016. Vitex Species: Review on Phytochemistry and Pouch Design for Nutritional Benefits. *Scientific Research Journal*, **13(2)**: 15. https://doi.org/10.24191/srj.v13i2.9374

Adwas, A., Elsayed, A.S.I., Azab, A.E. & Quwaydir, A. 2019. Oxidative stress and antioxidant mechanisms in human body. *Journal of Applied Biotechnology & Bioengineering*, **6(1)**: 43–47. https://doi.org/10.15406/jabb.2019.06.00173

Al-Sheddi, E.S., Farshori, N.N., Al-Oqail, M.M., Musarrat, J., Al-Khedhairy, A.A. & Siddiqui, M.A. 2016) Protective effect of *Lepidium sativum* seed extract against hydrogen peroxide-induced cytotoxicity and oxidative stress in human liver cells (HepG2). *Pharmaceutical Biology*, **54(2)**: 314–321. https://doi.org/10.3109/13880209.2015.1035795

Anandan, R., Jayakar, B., Karar, B., Babuji, S., Manavalan, R. & Kumar, R.S. 2009. Effect of ethanol extract of flowers of *Vitex trifolia* Linn. on CCl_4-induced hepatic injury in rats. *Pakistan Journal of Pharmaceutical Sciences*, **22(4)**: 391–394.

Ankalikar, A. & Vishwanathswany, A.H. 2017a. Effect of leaves of *Vitex trifolia* linn on different stages of inflammation. *Indian Journal of Pharmaceutical Education and Research*, **51(3)**: 461–471. https://doi.org/10.5530/ijper.51.3.74

Ankalikar, A. & Vishwanathswany, A.H. 2017. Effect of *Vitex trifolia* Linn and *Solanum nigrum* Linn on oxidative. *Indian Journal of Health Sciences and Biomedical Research*, **10(3)**: 269–275. https://doi.org/10.4103/kleuhsj.kleuhsj

Aye, M.M., Aung, H.T., Sein, M.M. & Armijos, C. 2019. A review on the phytochemistry, medicinal properties and pharmacological activities of 15 selected Myanmar medicinal plants.

Molecules, **24(2)**: 1–34. https://doi.org/10.3390/molecules24020293

Bach, L.T., Dung, L.T., Tuan, N.T., Phuong, N.T., Kestemont, P., Quetin-Leclercq, J. & Hue, B.T.B. 2018. Antioxidant activity against hydrogen peroxide-induced cytotoxicity of *Euphorbia hirta* L. *AIP Conference Proceedings*, **2049(December)**: 1–6. https://doi.org/10.1063/1.5082519

Bahadori, M.B., Dinparast, L. & Zengin, G. 2016. The Genus *Heracleum*: A Comprehensive Review on Its Phytochemistry, Pharmacology, and Ethnobotanical Values as a Useful Herb. *Comprehensive Reviews in Food Science and Food Safety*, **16***:* 1018-1038.

Bellezza, I., Giambanco, I., Minelli, A & Donato, R. 2018. Nrf2-Keap1 signaling in oxidative and reductive stress. *Biochimica et Biophysica acta (BBA) - Molecular Cell Research,* **1865(5)**: 721-733

Biswas, S. K. 2016. Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox?. *Oxidative Medicine and Cellular Longevity*, **2016**: 5698931.

Bjarnason, I., Scarpignato, C., Holmgren, E., Olszewski, M., Rainsford, K.D. & Lanas, A. 2018. Mechanisms of Damage to the Gastrointestinal Tract From Nonsteroidal Anti-Inflammatory Drugs. *Gastroenterology*, **154(3)**: 500–514. https://doi.org/10.1053/j.gastro.2017.10.049

Borish, L.C. & Steinke, J.W. 2003. Cytokines and chemokines. *Journal of Allergy and Clinical Immunology*, **111(2)**: 460–475. https://doi.org/10.1067/mai.2003.108

Celenghini, R.M.S., Vilegas, J.H.Y. & Lancas, F.M. 2001. Extraction and quantitative HPLC analysis of coumarin in hydroalcoholic extract of Mikania glomerata Spreng. (“guaco”) Leaves. *Journal of the Brazilian Chemical Society*, **12(6)**: 706–9.

Chan, E.W.C., Wong, S.K. & Chan, H.T. 2018. Casticin from *Vitex* species: a short review on its anticancer and anti-inflammatory properties. *Journal of Integrative Medicine*, **16(3)**: 147–152. https://doi.org/10.1016/j.joim.2018.03.001

Chemat, F., Vian, M.A. & Cravotto, G. 2012. Green extraction of natural products: Concept and principles. *International Journal of Molecular Sciences*, **13(7)**: 8615–8627. https://doi.org/10.3390/ijms13078615

Chen, Linlin, Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J., Li, Y., Wang, X. & Zhao, L. 2017. Inflammatory Responses and Inflammation-associated Diseases in Organs. *Oncotarget*, **9(6)**: 7204–7218.

Chen, X., Zhong, Z., Xu, Z., Chen, L. & Wang, Y. 2011. No protective effect of curcumin on hydrogen peroxide-induced cytotoxicity in HepG2 cells. *Pharmacological Reports*, **63(3)**: 724–732. https://doi.org/10.1016/S1734-1140(11)70584-9

Chen, Y., Zhou, Z. & Min, W. 2018. Mitochondria, oxidative stress and innate immunity. *Frontiers in Physiology*, **9(OCT)**: 1–10. https://doi.org/10.3389/fphys.2018.01487

Chinsembu, K.C. 2019. Chemical diversity and activity profiles of HIV-1 reverse transcriptase inhibitors from plants. *Brazilian Journal of Pharmacognosy*, **29(4)**: 504–528. https://doi.org/10.1016/j.bjp.2018.10.006

Cooper, C., Chapurlat, R., Chapurlat, R., Al-Daghri, N., Herrero-Beanumont, G., Bruyere, O., Rannou, F., Roth, R., Uebelhart, D. & Reginster, J.Y. 2019. Safety of Oral Non-Selective Non-Steroidal Anti-Inflammatory Drugs in Osteoarthritis: What Does the Literature Say?. *Drugs & Aging*, **26(Suppl 1)**: 15-24.

Coyle, C.H., Martinez, L.J., Coleman, M.C., Spitz, D.R., Weintraub, N.L. & Kader, K.N. 2006. Mechanisms of H_2O_2-induced oxidative stress in endothelial cells. *Free Radical Biology and Medicine*, **40(12)**: 2206–2213. https://doi.org/10.1016/j.freeradbiomed.2006.02.017

Dehsheikh, A.B., Sourestani, M.M., Dehsheikh, P.B., Vitalini, S., Iriti, M. & Mottaghipisheh, J. 2019. A comparative study of essential oil constituents and phenolic compounds of Arabian lilac (*Vitex trifolia* var. Purpurea): An evidence of season effects. *Foods*, **8(2)**: 1–14. https://doi.org/10.3390/foods8020052

Devi, W.R. & Singh, C.B. 2014. Chemical composition, anti-dermatophytic activity, antioxidant and total phenolic content within the leaves essential oil of *Vitex trifolia*. *International Journal of Phytocosmetic and Natural Ingredients*, **1(1)**: 1-5. https://doi.org/10.15171/ijpni.2014.05

Dong, J., Li, J., Cui, L., Wang, Y., Lin, J., Qu, Y. & Wang, H. 2018. Cortisol modulates inflammatory responses in LPS-stimulated RAW264.7 cells via the NF-KB and MAPK pathways. *BMC Veterinary Research*, **14(1)**: 1–10. https://doi.org/10.1186/s12917-018-1360-0

Fang, S.M., Liu, R., Li, L., Yao, J.L., Liu, E.W., Fan, G.W., Zhang, H. & Gao, X.M. 2019. Anti-inflammatory diterpenes from the fruits of *Vitex trifolia* L. var. simplicifolia Cham. *Journal of Asian Natural Products Research*, **21(10)**: 985–991. https://doi.org/10.1080/10286020.2018.1482881

Fokunang, C. 2018. Overview of non-steroidal anti-inflammatory drugs (nsaids) in resource limited countries. *MOJ Toxicology*, **4(1)**: 5–13. https://doi.org/10.15406/mojt.2018.04.00081

Forrester, S J., Kikuchi, D.S., Hernandes, M.S., Xu, Q. & Griendling, K.K. 2019. Reactive oxygen species in metabolic and inflammatory signaling. *Circulation Research*, **122(6)**: 877-902.

Ghafari, A.T., Jahidin, A.H., Zakaria, Y. & Hasan, M.H. 2021. phytochemical screening and high-

performance thin-layer chromatography of *Vitex trifolia* leaves hydroalcoholic extract: Potential anti-inflammatory properties. *Journal of Pharmaceutical Science International,* **33(28A)**: 111-121. https://doi.org/10.9734/jpri/2021/v33i28A31515

Grevet, M.R., Schepartz, S.A. & Chabner, B.A. 1992. The national institute of cancer: Cancer drug discovery and development program. *Seminars in Oncology*, **19(6)**: 622-638.

Hernández, M.M., Heraso, C., Villarreal, M.L., Vargas-Arispuro, I. & Aranda, E. 1999. Biological activities of crude plant extracts from *Vitex trifolia* L. (Verbenaceae). *Journal of Ethnopharmacology*, **67(1)**: 37–44. https://doi.org/10.1016/S0378-8741(99)00041-0

Huang, M., Zhang, L., Zhou, F., Ma, X., Li, Z., Zhong, T. & Zhang, Y. 2016. A new ursane triterpenoid possessing cytotoxicity from the fruits of *Vitex trifolia* var. simplicifolia. *Chemistry of Natural Compounds*, **52(4)**: 660–663. https://doi.org/10.1007/s10600-016-1733-1

Jang, Y., Kim, M. & Hwang, S.W. 2020. Molecular mechanism underlying the actions of arachidonic acid-derived prostaglandins on peripheral nociception. *Journal of Neuroinflammation*, **17(30)**: 1-30.

Jangwan, J.S., Aquino, R.P., Mencherini, T., Picerno, P. & Singh, R. 2014. Chemical constituents of ethanol extract of leaves and molluscicidal activity of crude extracts from *Vitex trifolia* linn. *Herba Polonica*, **59(4)**: 19–32. https://doi.org/10.2478/hepo-2013-0021

Kasote, D.M., Surendra, Katyare, S.S., Hegde, M.V. & Bae, H. 2015. Significance of antioxidant potential of plants and its relevance to therapeutic applications. *International Journal of Biological Sciences*, **11(8)**: 982–991.

Keshari, A.K., Verma, A.K., Kumar, T. & Srivastava, R. 2015. Oxidative stress: A review. *The International Journal of Science & Technoledge*, **3(7)**: 155-162.

Khanna, R., Karki, K., Pende, D. & Khanna, R. 2014. Inflammation, Free radical damage, oxidative stress and cancer. *Interdisciplinary Journal of Microinflammation*, **1(1)**: 1–5. https://doi.org/10.4172/ijm.1000109

Kim, Y.J., Lee, J.Y., Kim, H.J., Kim, D.H., Lee, T.H., Kang, M.S. & Park, W. 2018. Anti-inflammatory effects of *Angelica sinensis* (Oliv.) Diels water extract on RAW 264.7 induced with lipopolysaccharide. *Nutrients*, **10(5)**: 647. https://doi.org/10.3390/nu10050647

Ko, E.Y., Cho, S.H., Kwon, S.H., Eom, C.Y., Jeong, M.S., Lee, W.W., Kim, S.Y., Heo, S.J., Ahn, G., Lee, K.P., Jeon, Y.J. & Kim, S.Y. 2017. The roles of NF-kB and ROS in regulation of pro-inflammatory mediators of inflammation induction in LPS-stimulated zebrafish emryos. *Fish & Shellfish Immunology*, **68(2017)**: 525-529.

Kulkarni, L.A. 2011. Anti-inflammatory activity of *Vitex trifolia* Linn. (Verbaneacae) leaves extracts. *International Journal of Pharmaceutical Sciences and Research*, **2(8)**: 2037-2040.

Kulkarni, L.A. 2012. *Vitex trifolia* Linn. (Verbaneaceae): A review on pharmacological and biological effects, isolated and known potential phytoconstituents of therapeutic importance. *International Journal of Research in Pharmaceutical Sciences*, **3(3)**: 441–445.

Kwon, D.H., Cha, H.J., Lee, H., Hong, S.H., Park, C., Park, S.H., Kim, G.Y., Kim, S., Kim, H.S., Hwang, H.J., & Choi, Y.H. 2019. Protective effect of glutathione against oxidative stress-induced cytotoxicity in RAW 264.7 macrophages through activating the nuclear factor erythroid 2-related factor-2/heme oxygenase-1 pathway. *Antioxidants*, **8(4)**: 82. https://doi.org/10.3390/antiox8040082

Lawrence, T. & Fong, C. 2010. The resolution of inflammation: Anti-inflammatory roles for NF-κB. *International Journal of Biochemistry and Cell Biology*, **42(4)**: 519–523. https://doi.org/10.1016/j.biocel.2009.12.016

Lee, G., Jung, K.H., Ji, E.S. & Bae, H. 2017. Pyranopyran-1,8-dione, an active compound from vitices fructus, attenuates cigarette-smoke induced lung inflammation in mice. *International Journal of Molecular Sciences*, **18(7)**: 1–10. https://doi.org/10.3390/ijms18071602

Lee, S.M., Lee, Y.J., Kim, Y.C., Kim, J.S., Kang, D.G. & Lee, H.S. 2012. Vascular protective role of vitexicarpin isolated from *Vitex rotundifolia* in human umbilical vein endothelial cells. *Inflammation*, **35(2)**: 584–593. https://doi.org/10.1007/s10753-011-9349-x

Lennicke, C., Rahn, J., Lichtenfels, R., Wessjohann, L.A. & Selinger, B. 2015. Hydrogen peroxide–production, fate and role in redox signaling of tumor cells. *Cell Communication and Signaling*, **13(39)**: 2015. https://doi.org/10.1186/s12964-015-0118-6

Lingappan, K. 2018. NF-κB in Oxidative Stress. *Current Opinion in Toxicology*, **7**: 81-86

Liou, C.J., Len, W. Bin, Wu, S.J., Lin, C.F., Wu, X.L. & Huang, W.C. 2014. Casticin inhibits COX-2 and iNOS expression via suppression of NF-κB and MAPK signaling in lipopolysaccharide-stimulated mouse macrophages. *Journal of Ethnopharmacology*, **158(2014)**: 310–316. https://doi.org/10.1016/j.jep.2014.10.046

Luo, P., Yu, Q., Liu, S. N., Xia, W. J., Fang, Y. Y., An, L. K., Gu, Q. & Xu, J. 2017. Diterpenoids with diverse scaffolds from *Vitex trifolia* as potential topoisomerase I inhibitor. *Fitoterapia*, **120(May)**: 108–116. https://doi.org/10.1016/j.

fitote.2017.06.006

Manaf, S.R., Daud, H.M., Alimon, A.R., Mustapha, N.M., Hamdan, R.H., Muniandy, K.M., Mohamed, N.F.A., Razak, R. & Hamid, N.H. 2016. The Effects of *Vitex trifolia*, *Strobilanthes crispus* and *aloe vera* herbal-mixed dietary supplementation on growth performance and disease resistance in red hybrid tilapia (*Oreochromis* sp.). *Journal of Aquaculture Research & Development*, **7**: 425. https://doi.org/10.4172/2155-9546.1000425

Marcum, Z.A., & Hanlon, J.T. 2010. Recognizing the risk of chronic nonsteroidal anti-inflammatory drug use in older adult. *Annals of Longterm Care*, **18(9)**: 24-27.

Matsui, M., Adib-Conquy, M., Coste, A., Kumar-Roiné, S., Pipy, B., Laurent, D. & Pauillac, S. 2012. Aqueous extract of *Vitex trifolia* L. (Labiatae) inhibits LPS-dependent regulation of inflammatory mediators in RAW 264.7 macrophages through inhibition of Nuclear Factor kappa B translocation and expression. *Journal of Ethnopharmacology*, **143(1)**: 24–32. https://doi.org/10.1016/j.jep.2012.05.043

Matsui, M., Kumar-Roine, S., Darius, H.T., Chinain, M., Laurent, D. & Pauillac, S. 2009. Characterisation of the anti-inflammatory potential of *Vitex trifolia* L. (Labiatae), a multipurpose plant of the Pacific traditional medicine. *Journal of Ethnopharmacology*, **126(3)**: 427–433. https://doi.org/10.1016/j.jep.2009.09.020

Medzhitov, R. 2008. Origin and physiological roles of inflammation. *Nature*, **454(7203)**: 428–435. https://doi.org/10.1038/nature07201

Meena, A.K., Niranjan, U.S., Rao, M.M., Padhi, M.M. & Babu, R. 2011. A review of the important chemical constituents and medicinal uses of *Vitex* genus. *Asian Journal of Traditional Medicines*, **6(2)**: 54–60.

Mittal, M., Siddiqui, M.R., Tran, K., Reddy, S.P. & Malik, A.B. 2014. Reactive oxygen species in inflammation and tissue injury. *Antioxidants and Redox Signaling*, **20(7)**: 1126–1167. https://doi.org/10.1089/ars.2012.5149

Mutalib, N.A., & Latip, N.A. 2020. Antagonistic Drug-herb Interactions between Clinacanthus nutans and Cyclophosphamide on WRL 68 Cell Line. *Pharmaceutical Sciences and Research*, **7(2)**: 81–89. https://doi.org/10.7454/psr.v7i2.1093

Naghavi, M. 2019. The global, regional, and national burden of inflammatory bowel disease in 195 countries and territories, 1990–2017: A systematic analysis for the Global Burden of Disease Study 2017. *The Lancet*, **5**: 17-30.

Nakao, N., Kurokawa, T., Nonami, T., Tumurkhuu, G., Koide, N. & Yokochi, T. 2008. Hydrogen peroxide induces the production of tumor necrosis factor-α in RAW 264.7 macrophage cells via activation of p38 and stress-activated protein kinase. *Innate Immunity*, **14(3)**: 190–196. https://doi.org/10.1177/1753425908093932

Nishina, A., Itagaki, M., Sato, D., Kimura, H., Hirai, Y., Phay, N. & Makishima, M. 2017. The rosiglitazone-like effects of Vitexilactone, a constituent from *Vitex trifolia* L. in 3T3-L1 preadipocytes. *Molecules*, **22(11)**: 1–13. https://doi.org/10.3390/molecules22112030

Nkala, B.A., Mbongawa, H.P. & Qwebani-Qgunley, T. 2020. The evaluation of cytotoxicity and anti-inflammatory effects of selected South African medicinal plants against C2C12 cells and RAW 264.7 cells. *International Journal of Scientific and Research Publications (IJSRP)*, **10(2)**: 207–220. https://doi.org/10.29322/ijsrp.10.02.2020.p9830

Nunes, R., Arantes, M.B., Menezes, S., Pereira, D.F., Leandro, L., Passos, M.D.S., & Moraes, L.P.D. 2020. Plants as Sources of Anti-Inflammatory Agents. *Molecules*, **25(3726)**: 1–22.

Nurul Amin, M., Siddiqui, S.A., Ibrahim, M., Lukman Hakim, M., Ahammed, Kabir, A. & Sultana, F. 2020. Inflammatory cytokine in the pathogenesis of cardiovascular disease and cancer. *Medicine*, **8**: 1-12.

Orwa, C., Mutua, A., Kindt, R., Jamnadass, R. & Anthony, S. 2009. Agroforestree Database: a tree reference and selection guide version 4.0 (http://www.worldagroforestry.org/sites/treedbs/treedatabases.asp)

Pahwa, R., Goyal, A., Bansal, P. & Jialal, I. 2020. Chronic inflammation. StatPearls [Internet]. Treasure Island (F.L.): StatPearls Publishing; 2021 Jan.

Pizzino, G., Irrera, N., Cucinotta, M., Pallio, G., Mannino, F., Arcoraci, V., Squadrito, F., Altavilla, D. & Bitto, A. 2017. Oxidative Stress: Harms and Benefits for Human Health. *Oxidative Medicine and Cellular Longevity*, **2017**: 1–13.

Placha, D. & Jampilek, J. 2021. Chronic Inflammatory Diseases, Anti-Inflammatory Agents and Their Delivery Naosystem. *Pharmaceutics*, **13(1)**: 64. https://doi.org/ 10.3390/pharmaceutics13010064

Rani, A. & Sharma, A. 2013. The genus Vitex: A review. *Pharmacognosy Reviews*, **7(14)**: 188–198. https://doi.org/10.4103/0973-7847.120522

Ranneh, Y., Ali, F., Akim, A.M., Abd Hamid, H., Khazaai, H. & Fadal, A. 2017. Crosstalk between reactive oxygen species and pro-inflammatory markers in developing various chronic diseases: a review. *Applied Biological Chemistry*, **60(3)**: 327-338

Ryter, S.W., Hong, P.K., Hoetzel, A., Park, J.W., Nakahira, K., Wang, X. & Choi, A.M.K. 2007. Mechanisms of cell death in oxidative stress. *Antioxidants and Redox Signaling*, **9(1)**: 49–89. https://doi.org/10.1089/ars.2007.9.49

Saklani, S., Mishra, A.P., Chandra, H., Atanassova,

M.S., Stankovic, M., Sati, B., Shariati, M.A., Nigam, M., Khan, M.U., Plygun, S., Elmsellem, H. & Suleria, H.A.R. 2017. Comparative evaluation of polyphenol contents and antioxidant activities between ethanol extracts of *Vitex negundo* and *Vitex trifolia* L. Leaves by different methods. *Plants*, **6(4)**: https://doi.org/10.3390/plants6040045

Samuel, S., Nguyen, T. & Choi, H.A. 2017. Pharmacologic characteristics of corticosteroids. *Journal of Neurocritical Care*, **10(2)**: 53–59. https://doi.org/10.18700/jnc.170035

Schieber, M. & Chandel, N.S. 2014. ROS function in redox signaling and oxidative stress. *Current Biology*, **24***:* R453-R462.

Sies, H. 2017. Hydrogen peroxide as a central redox signaling molecule in physiological oxidative stress: Oxidative eustress. *Redox Biology*, **11(2017)**: 613-619.

Suchitra, M. & Cheriyan, B.V. 2018. *Vitex trifolia*: An ethnobotanical and pharmacological review. *Asian Journal of Pharmaceutical and Clinical Research*, **11(4)**: 12–14. https://doi.org/10.22159/ajpcr.2018.v11s4.31689

Tasneem, S., Liu, B., Li, B., Choudhary, M.I. & Wang, W. 2019. Molecular pharmacology of inflammation: Medicinal plants as anti-inflammatory agents. *Pharmacological Research*, **139**: 126–140. https://doi.org/10.1016/j.phrs.2018.11.001

Thalhamer, T., McGrath, M.A. & Harnett, M.M. 2008. MAPKs and their relevance to arthritis and inflammation. *Rheumatology*, **47(4)**: 409–414. https://doi.org/10.1093/rheumatology/kem297

Thenmozhi, S., Vibha, S., Dhanalakshmi, M., Manjuladevi, K., Diwedi, S. & Subasini, U. 2013. Evaluation of anthelmintic activity of *Vitex trifolia* Linn. leaves against *Pheretima posthuma*. *International Journal of Pharmaceutical & Biological Archives*, **4(5)**: 878 – 880.

Tsai, T.Y., Li, C.Y., Livneh, H., Lin, I.H., Lu, M.C. & Yeh, C.C. 2016. Decreased risk of stroke in patients receiving traditional Chinese medicine for vertigo: A population-based cohort study. *Journal of Ethnopharmacology*, **184(2016)**: 138–143. https://doi.org/10.1016/j.jep.2016.03.008

Tsuge, K., Inazumi, T., Shimamoto, A. & Sugimoto, Y. 2019. Molecular mechanisms underlying prostaglandin E2-exacerbated inflammation and immune diseases. *International Immunology*, **31(9)**: 597–606. https://doi.org/10.1093/intimm/dxz021

Vimalanathan, S., Ignacimuthu, S. & Hudson, J.B. 2009. Medicinal plants of Tamil Nadu (Southern India) are a rich source of antiviral activities. *Pharmaceutical Biology*, **47(5)**: 422–429. https://doi.org/10.1080/13880200902800196

Vrankova, S., Barta, A., Klimentova, J., Dovinova, I., Liskova, S., Dobesova, Z., Pechanova, O., Kunes, J. & Zicha, J. 2016. The regulatory role of nuclear factor kappa B in the heart of hereditary hypertriglyceridemic. *Oxidative Medicine and Cellular Longevity*, **2016**: 9814 038.

Wang, T., Fu, X., Chen, Q., Patra, J.K., Wang, D., Wang, Z. & Gai, Z. 2019. Arachidonic acid metabolism and kidney inflammation. *International Journal of Molecular Sciences*, **20(15)**: 1–28. https://doi.org/10.3390/ijms20153683

Wee, H.N., Neo, S.Y., Singh, D., Yew, H.C., Qiu, Z.Y., Tsai, X.R.C., How, S.Y., Yip, K.Y.C., Tan, C.H. & Koh, H.L. 2020. Effects of *Vitex trifolia* L. Leaf extracts and phytoconstituents on cytokine production in human u937 macrophages. *BMC Complementary Medicine and Therapies*, **20(1)**: 1–15. https://doi.org/10.1186/s12906-020-02884-w

Wongrakpanich, S., Wongrakpanich, A., Melhado, K. & Rangaswami, J. 2018. A comprehensive review of non-steroidal anti-inflammatory drug use in the elderly. *Aging and Disease*, **9(1)**: 143–150. https://doi.org/10.14336/AD.2017.0306

Wragg, D., Leoni, S. & Casini, A. 2020. Aquaporin-driven hydrogen peroxide transport: A case of molecular mimicry?. *RSC Chemical Biology*, **1(5)**: 390-394.

Xiang, J., Wan, C., Guo, R. & Guo, D. 2016. Is hydrogen peroxide a suitable apoptosis inducer for all cell types?. *BioMed Research International*, **2016**: Article ID 7343965.

Yousefian, M., Shakour, N., Hosseinzadeh, H., Hayes, A.W., Hadizadeh, F. & Karimi, G. 2019. The natural phenolic compounds as modulators of NADPH oxidases in hypertension. *Phytomedicine*, **55**: 200–213. https://doi.org/10.1016/j.phymed.2018.08.002

Zhang, H., Guo, Q., Liang, Z., Wang, M., Wang, B., Sun-Waterhouse, D., Waterhouse, G.I.N., Wang, J., Ma, C. & Kang, W. 2021. Anti-inflammatory and antioxidant effects of Chaetoglobosin Vb in LPS-induced RAW264.7 cells: Achieved via the MAPK and NF-κB signaling pathways. *Food and Chemical Toxicology*, **147**: 111915. https://doi.org/10.1016/j.fct.2020.111915

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y. & Dong, W. 2016. ROS and ROS-mediated cellular signalling. *Oxidative Medicine and Cellular Longevity*, **2016**: Article ID 4350965