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 (CLR), retinoic acid-inducible gene (RIG)-I-like receptors (RLR), and nucleotide-binding and oligomerization domain (NOD)-like receptors (NLR). 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 species (ROS).

ANTI-INFLAMMATORY EFFECTS OF *Vitex trifolia* LEAVES HYDROALCOHOLIC EXTRACT AGAINST HYDROGEN PEROXIDE (H\textsubscript{2}O\textsubscript{2})- AND LIPOPOLYSACCHARIDE (LPS)-INDUCED RAW 264.7 CELLS

AHMAD TAMIM GHAFARI\textsuperscript{1,2}, AISYAH HASYILA JAHIDIN\textsuperscript{1}, YUSLINA ZAKARIA\textsuperscript{1} and MIZATON HAZIZUL HASAN\textsuperscript{1*}

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

Accepted 7 October 2022, Published online 31 October 2022

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\textsubscript{2}O\textsubscript{2}) and lipopolysaccharide (LPS). The reactive oxygen species (ROS) production was evaluated in the H\textsubscript{2}O\textsubscript{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\textsubscript{2}O\textsubscript{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*
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 subtropical 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 & Cheriyyan, 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), anthelmintic (Thenmonzhi et al., 2013), anticancer (Chan et al., 2016; Huang et al., 2016; Chan et al., 2018), hepatoprotective (Anandan et al., 2009), and anti-diabetic (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 quercetagenin, 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 (Abd Hakeem et al., 2016). Persicogenin, pendauletin, 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,O,)- 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
leaves were gathered in Subang.

50 mg of LPS was dissolved directly in DMEM (10%

avoid any possible effect of DMSO on cells. Exactly

maintained below 0.05% in the working solutions to

diluted to the required working concentrations before

200 mg of VT and DICL in 1 mL of 100% DMSO and

drug were prepared by dissolving accurately weighed

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$_2$O$_2$ 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
(0.5 mg/mL) for 24 h. Next, cells were induced, before the treatment,
with 100 µL of VT (0-2000 µg/mL) was added to each well. About
100 µL of H$_2$O$_2$ 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$_{50}$
calculated from the viability plot.

Equation 1:

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$_2$O$_2$-induced cytotoxicity using the MTT cell
viability assay. Briefly, 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
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 × 10^5 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 supernatant was removed, and 100 µL of PBS was added to each well. The fluorescence was measured at ex/mm = 535/587 nm at an endpoint 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:

$$ΔRFU_{T_2} - RFU_{T_1}$$

where RFU_{T_1} is the sample reading at time T_1, and RFU_{T_2} 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:

$$COX \text{ activity} = \frac{B}{ΔM} × \text{Sample Dilution} = \mu \text{U}$$

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 \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \) cytotoxicity. Cells were treated with \( \text{H}_2\text{O}_2 \) (300 µM) for 1 h and followed by treatment with VT in different concentrations (Figure 2). \( \text{H}_2\text{O}_2 \) 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 \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \)).

#### ROS measurement

The antioxidant effect of VT was evaluated on \( \text{H}_2\text{O}_2 \)-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 \( \text{H}_2\text{O}_2 \) for 1 hr and followed by treatment with different concentrations of VT. \( \text{H}_2\text{O}_2 \) at 300 µM significantly \( (p<0.05) \) produced an excessive
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 \(\text{H}_2\text{O}_2\)-induced ROS to 10.6 ± 2.81%, 20.54 ± 1.68%, and 13.36 ± 1.58%, respectively in contrast to the cells treated with \(\text{H}_2\text{O}_2\) alone. Meanwhile, the same effect was observed with DICL (100 µg/mL) on the \(\text{H}_2\text{O}_2\)-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 (eBioscience, 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 \((1 \mu\text{g/mL})\) 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 \((1 \mu\text{g/mL})\) 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 \((1 \mu\text{g/mL})\) alone were found to have a significantly \((p<0.05)\) high level of COX activity \((0.83 ± 0.11 \mu\text{U})\) compared to cells with no LPS \((0.063 ± 0.01 \mu\text{U})\). 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.
ANTI-INFLAMMATORY EFFECTS OF *Vitex trifolia* LEAVES

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).
ANTI-INFLAMMATORY EFFECTS OF Vitex trifolia LEAVES

**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$_2$O$_2$ (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$_2$O$_2$ showed a cell viability of 60% after 24 hr of incubation (Kwon et al., 2019). Similarly, 500 µM of H$_2$O$_2$ decreased the cell viability of MIN6 cells, a murine pancreatic β-cell line, to 44% (Bach et al., 2018). Moreover, 500 µM of H$_2$O$_2$ inhibited the growth of HepG2 cells by 45-60% (Chen et al., 2011; Al-Sheddi et al., 2016). However, the LC$_{50}$ of H$_2$O$_2$ 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$_2$O$_2$-induced cytotoxicity and oxidative stress, which is a common model for assessing the cytoprotective of natural substances against oxidative stress stimuli. H$_2$O$_2$ is one of the most important free radicals that can easily diffuse into the cells through aquaporin or peroxiporin (Nakao, 2008; Lennnicke et al., 2015; Wragg et al., 2020). It acts as a pro-oxidant at high concentrations and causes oxidative stress (akao et al., 2008; Lennnicke et al., 2015). Moreover, H$_2$O$_2$ 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$_2$O$_2$, 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$_2$O$_2$ can occur by activating apoptosis. H$_2$O$_2$-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$_2$O$_2$-induced cytotoxicity. VT (25 & 50 µg/mL) increased cell viability of H$_2$O$_2$-induced cells to 85.46 ± 3.4% and 88.95 ± 5.56%, respectively, in contrast to the cells treated with H$_2$O$_2$ (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$_2$O$_2$) on cell viability. This suggested that VT at those concentrations can protect against oxidative stress induced by H$_2$O$_2$. 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$_2$O$_2$-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$_2$O$_2$ scavenging activity of VT. However, the extract was not tested on cells but tested for H$_2$O$_2$, 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 constituents inhibited the pro-oxidant enzymes such as COX-2, lipoygenase (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 inflammatory mediators was observed. In another 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).

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
The inhibitory effect of VT on cytokine and COX-2 was reported to have a more potent inhibitory effect on IL-6, TNF-α, and IL-1β production with IC₅₀ 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 (Thalhammer 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-inflammatory 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 & Chandle, 2014). Moreover, mitochondrial ROS can contribute to the LPS-induced production of IL-1β, IL-6, and TNF-α (Mittal et al., 2014; Forrestor 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; Forrestor 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.

ACKNOWLEDGEMENT

The authors acknowledge the Higher Education Development Program (HEDP), the Ministry of Higher Education of Afghanistan, and the Faculty of Pharmacy, Kabul University, for funding the scholarship recipient and also, the Faculty of Pharmacy, Universiti Teknologi MARA (UiTM), for approving and supporting this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


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


fitote.2017.06.006


https://doi.org/10.1016/j.ear.2017.06.006


