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Immunomodulatory effects of crude acetone and water leaf extracts from *Tulbaghia violacea* on RAW264.7 cells stimulated with lipopolysaccharide (LPS)

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ABSTRACT

Introduction: *Tulbaghia violacea* is a medicinal plant used in traditional healing practices in South Africa. This study sought to investigate if water and acetone extracts from *T. violacea* impact immunomodulation by influencing the production of cytokines and nitric oxide (NO) in macrophages stimulated by lipopolysaccharide (LPS).

Methods: The 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was performed to examine the toxicity of acetone and aqueous extracts from *T. violacea* on RAW264.7 cells treated with LPS over 24 hours. The effect of NO production in treated cells was examined using a Greiss assay, while cytokine levels were determined using a Luminex assay.

Results: The viability of RAW264.7 cells remained higher than 80% after exposure to 50 µg/mL or lower concentrations of both water and acetone extracts. The acetone extract showed potent inhibitory effects on NO production at 50 µg/mL and increased pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin-1a (IL-1α), and interleukin-6 (IL-6) but did not significantly affect interleukin-1beta (IL-1β). The water extract significantly increased IL-4 levels ($P<0.05$) at 48 hours. Both extracts increased granulocyte-colony stimulating factor (GCSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-gamma-induced protein 10 (IP10), macrophage inflammatory protein-1 alpha and beta (MIP-1α and MIP-1β), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and regulated upon activation, normal T cell expressed and secreted RANTES levels to over 10 000 pg/mL.

Conclusion: Both extracts from *T. violacea* possess immunomodulatory activities on LPS-stimulated RAW264.7 cells. Further studies should determine their toxicities and suitability as alternatives to synthetic counterparts.

Implication for health policy/practice/research/medical education:
Treatment with *Tulbaghia violacea* extracts showed immunomodulatory activities on LPS-stimulated RAW264.7 cells. These findings can inform policy promoting the use of this plant in health care, especially in areas where it is readily available and culturally acceptable as well as serve as an avenue for further research in the mechanism of action, which can potentially lead to the discovery of new anti-inflammatory and immune-boosting therapeutic agents.

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Introduction

Tulbaghia violacea is known for its antioxidant, antimicrobial, and anticancer properties. Communities relying on medicinal plants for their well-being have used *T. violacea* to boost their immunity (1). The leaves of *T. violacea* have been found to contain terpenoids, flavonoids, saponins, tannins, and several sulfur-containing compounds (2). Therapeutic formulations containing bioactive compounds with immunomodulatory capabilities attract the interest of natural product

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scientists because of their efficacy, low level of cytotoxicity towards healthy cells, and few adverse effects (3,4). The pharmacological effects of these substances on the human immune system have been associated with a range of therapeutic advantages (5). A thorough investigation of immunomodulation in *T. violacea* is crucial for the development of novel therapeutic approaches and comprehension of how natural compounds might assist in the prevention and treatment of diseases.

In humans, the immune system operates through a complex network of various immune cells (6). Inflammation is a natural response to injury or tissue damage and serves as an important component of the body's physiological and immune functions (7). Typically, this immune response occurs as the body's defence mechanism against potential harm such as invasive viruses, bacteria, and other pathogens (8). Inflammation is controlled by several extracellular mediators and regulators, including cytokines and growth factors (9-12). Among these, cytokines serve as important modulators that influence acute and chronic inflammatory responses through complex interactions (3).

Cytokines play an important role in cell signalling and are divided into pro- and anti-inflammatory, based on their biological effects on the body (13). Anti-inflammatory cytokines like interleukin (IL)-4, IL5, IL10, and IL-13 interfere with inflammatory responses, often by suppressing the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α) and IL-1 production in macrophages (13). On the other hand, pro-inflammatory cytokines, including IL-1, IL6, and TNF-α trigger systemic inflammation (14).

Macrophages are specialized immune cells that contribute to inflammation. They are found in different tissues within the body and respond to foreign agents or signals from damaged cells by migrating to the site of injury and becoming activated. Upon activation, they produce cytokines and enzymes, like cyclooxygenases (COX) and inducible nitric oxide synthase (iNOS), which help to recruit more immune cells to the site of injury (8,9,15). Activated macrophages express the iNOS enzyme responsible for catalysing the oxidative deamination of L-arginine, resulting in the production of nitric oxide (NO) (14-17). While synthetic compounds, including selective COX-2 inhibitors and nonsteroidal anti-inflammatory drugs (NSAIDs) have demonstrated efficacy, they are not without side effects, including an increased risk of cardiovascular thrombosis (18). Therefore, exploring alternative therapies, such as natural plant extracts with minimal side effects, is essential to replace or complement existing anti-inflammatory drugs.

Immunomodulation using medicinal plants offers a promising avenue for managing chronic inflammatory responses as an alternative to conventional chemotherapy (19). Certain plants are known to have immunomodulatory properties; examples include milk thistle, which has been shown to increase the activity of B cells, T cells, and macrophages (19), and garlic, which influences cell-mediated immunity (20). Different bioactive compounds, for instance, polysaccharides, resveratrol, and organosulfur are responsible for the immunomodulatory activity observed in these plants (21).

Although natural plant extracts have been explored for their anti-inflammatory properties, it is essential to consider their potential to induce inflammatory responses by activating pro-inflammatory cytokines. Certain plant extracts have been studied for their pro-inflammatory properties, including their ability to stimulate the release of cytokines (IL-1, IL-6, and TNF-α) (22). However, the effects of *T. violacea* on both pro- and anti-inflammatory cytokines (TNF-α, IL-1, IL-6, IL-10, IL-4) and NO production in macrophages remain largely unexplored. Understanding these responses could prove valuable in enhancing immune surveillance or promoting tissue repair (23). Investigating how water and acetone extracts of *T. violacea* influence key immune mediators will enhance our understanding of its role in immune modulation. Furthermore, studies on the dose-dependent effects of *T. violacea* on immune function are scarce. By using different concentrations (10, 30, and 50 µg/mL) of both extracts, this study will help establish whether the immunomodulatory effects of the plant are concentration-dependent, and at which doses the extracts stimulate or suppress immune responses. This information is crucial for determining the safe and effective therapeutic concentrations of the plant. Examining the effect of plant extracts like those from *T. violacea* on immune cells can provide important insights into their complex immunomodulatory properties and contribute to the development of new therapies for immune-related disorders.

This study aimed to examine how acetone and water leaf extracts derived from *T. violacea* affect the production of NO and cytokine in RAW264.7 cells treated with lipopolysaccharide (LPS). RAW 264.7 cells are the most used model for evaluating anti-inflammatory and immunomodulatory drug candidates. Specifically, the RAW 264.7 cell line exhibits a strong and widely recognized inflammatory reaction, particularly when exposed to inflammatory triggers like LPS. LPS, the prominent outer membrane component of gram-negative bacteria, is crucial in the development of gram-negative bacterial infections and has been a significant active component in research on macrophage inflammation generated by pathogens (24). By varying the concentrations of *Tulbaghia* leaf extracts, the study assessed RAW264.7 cell viability, determined the amount of NO produced, and assessed how the extracts affect cytokine production within the cells.

Methods

Sourcing of plant material

Fresh *Tulbaghia violacea* leaves were collected from a

plant nursery in General Froneman Street, situated in South East 7, Vanderbijlpark in Gauteng province of South Africa, and were maintained according to the procedures described by Takaidza et al (25). The plant was authenticated by Professor Stefan Seibert, a botanist at North-West University, Potchefstroom, South Africa. For reference, a voucher specimen (ST0008) was deposited in the AP Goossens Herbarium.

Plant extract preparation

The protocol described by Takaidza et al (25) was followed while preparing water and acetone extracts from *T. violacea*. Briefly, 10 g of freshly collected *T. violacea* leaves were used to prepare crude leaf extracts. The leaves were homogenized in sterile distilled water or 100 mL of absolute acetone. After letting the acetone homogenate stand for 24 hours, it was filtered using No. 1 Whatman filter paper. The filtrate was then left to evaporate in a fume hood, and the resultant extract was stored at 4 °C until it was analysed. To prepare the water homogenate, a water bath was brought to a boil at 100 °C for 10 minutes, then the mixture was cooled and filtered using a No. 1 Whatman filter paper. The filtered solution was stored at -20 °C for 24 hours, then lyophilized and kept at 4 °C in an airtight container until needed.

Preparation and culture of RAW 264.7-C macrophage cell line

A RAW 264.7-C macrophage adherent cell line from Abelson Murine leukaemia was obtained from Cellonex (Johannesburg, South Africa). The cells were cultivated in a culture medium comprising phenol-free DMEM (GE Health Life Sciences, Logan, UT), supplemented with 10% fasting blood sugar (FBS) (Thermo-Fisher Scientific, Waltham, MA) and 1% Pen-Strep. Cell culture was conducted in a humidified 5% CO_2 incubator (ESCO, Singapore) at 37 °C (25).

Determination of viability of RAW 264.7-C cells

The RAW264.7 cells (1×10^5 cells/mL) were seeded using a phenol-free DMEM complete culture media in a 96-well plate, then incubated for 24 hours at 37 °C in a humidified 5% CO_2 incubator (25). Following incubation, the media was aspirated, and the cells were washed twice with phosphate-buffered saline (PBS). Next, the cells were treated with each extract prepared in DMEM complete media and added in 10 to 50 µg/mL concentrations. LPS was introduced to each treatment until 1 µg/mL concentration was reached. The controls included cells treated with LPS (1 µg/mL) alone and a group without treatment. The previous incubation and PBS-washing steps were repeated. Then, 10 µL of MTT solution prepared in PBS at a concentration of 5 mg/mL was added to a fresh plate containing 100 µL of the cell culture media. The plates were then incubated for another 4 hours before removing 85 µL of media from each well and replacing it with 100 µL of DMSO. The plates were slightly agitated to dissolve the formazan, while the amount of formazan produced was determined using a microplate reader (Epoch 2, BioTek, Winooski, VT) at 570 nm. The following formula was used to calculate the percentage viability of RAW264.7 cells:

Cell Viability (%) = [1- (absorbance of untreated control – absorbance of treated Sample) / (absorbance of untreated control)] × 100.

NO production

RAW 264.7 cells (2.0×10^5 cells/well) were seeded into 24-well plates containing phenol-free DMEM culture media supplemented with 1 µg/mL LPS (26). These plates were then incubated for 24 hours at 37 °C in a humidified 5% CO_2 incubator. Following incubation, 0.2 mL of the media containing the water or acetone extracts was added to the cell culture media at 10, 30, and 50 µg/mL concentrations. LPS was added to each well until reaching a final concentration of 1 µg/mL. After this, the plates were incubated for 24 hours in an incubator with 5% CO_2 at 37 °C. Next, the media was carefully placed in 2 mL tubes and centrifuged for 5 minutes at 500 × g, and the resulting supernatant was kept in 100 µL aliquots at -20 °C. Cells treated with LPS only served as positive controls, while supernatants from cells not treated with LPS served as negative controls. To determine the precise NO levels, 50 µL of the supernatant was used. The nitrite content in the culture medium indicated NO production was measured using the Griess Reagent Kit for Nitrite Determination (G-7921, Thermofisher). In brief, the cell culture medium (50 µL) was mixed with an equal volume of Griess reagent consisting of 0.1% (w/v) naphthyl ethylenediamine-HCl and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid. After 15 min incubation at room temperature, absorbance was measured at 540 nm using an Epoch 2 microplate reader (BioTeK). To estimate the amounts of nitrite in the samples, a standard curve was generated using the serial dilutions of $NaNO_2$ (1-100 µM).

Immunomodulatory activity

To determine how both extracts used in the study affected the expression of cytokines in the stimulated cells, we used the MILLIPLEX® MAP kit. Sample preparation followed the procedure outlined in section 2.5. The MILLIPLEX® MAP kit comprised a Mouse Cytokine/Chemokine Magnetic Bead Panel (MYCTMAG70PMX25BK, Merck, Darmstadt, Germany). Cytokine concentration in each sample was confirmed using the Luminex Multiple Assay as per the manufacturer's guidelines. Thereafter, a Millipore Luminex 200™ system (Merck) was then used to run the samples.

Data analysis

For this study, data was based on biological triplicates

unless otherwise indicated, and GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla, CA) was used to calculate the means. Heat maps were generated using normalized data and the CIMMiner software package (27). Statistical significance was determined using t-tests for two groups and ANOVA for multiple groups.

Results

Effect of acetone and water extracts on RAW264.7 cell viability

In the study, the MTT test was used to examine how water and aqueous extracts from *T. violacea* at varying concentrations (10, 30, and 50 µg/mL) affected the viability of RAW264.7 cells stimulated with LPS after a 24-hour incubation period. Although there were minor variations across the various plant extract concentrations, the difference in viability between the treated and untreated samples was not statistically significant ($P > 0.05$). Interestingly, the cells remained consistently viable at 80% or higher (Figure 1). The results showed similar trends in the 48-hour treatment (data not shown).

Effect of *Tulbaghia violacea* extracts on the production of NO

NO production by RAW264.7 cells followed a decreasing trend with every increase in the concentrations of both extracts (Figure 2). Nevertheless, the difference in the mean NO levels detected in RAW264.7 cells treated with the water extracts was not significant ($P > 0.05$) compared to those of the control group. Aside from the cells treated with 50 µg/mL, similar results were noted for the crude acetone extract treatments. In addition, the 50 µg/mL acetone extract was found to significantly ($P < 0.05$) reduce NO production by 46% compared to the control (cells treated with the LPS alone) (Figure 2).

Effect of *Tulbaghia violacea* water and acetone extracts on inflammatory cytokines

Effect of water and acetone extracts on pro-inflammatory cytokines

Treating RAW264.7 cells with various concentrations of acetone extracts significantly increased ($P < 0.05$) IL-1α production after 24 hours (Figure 3A), with the highest secretion recorded at 30 µg/mL. However, after 24 hours, IL-1α was significantly decreased ($P < 0.05$) at 10 µg/mL. Contrarily, IL-1β secretion did not show any difference between samples treated with LPS and other treatments after 24 and 48 hours (Figure 3B). After 24 and 48 hours, the levels of IL-6 in all samples treated with different concentrations of the acetone extracts surpassed

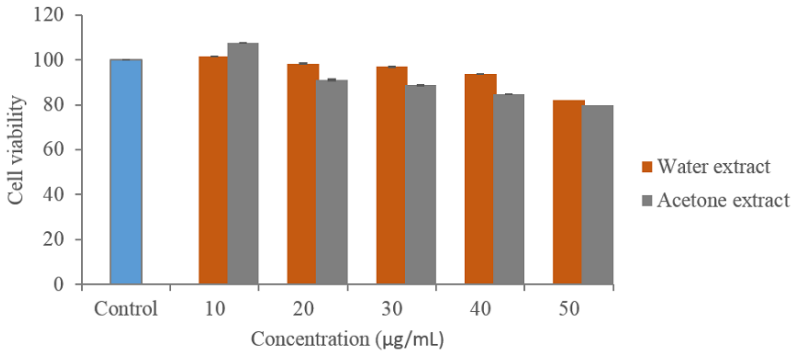

Figure 1. Effect of *Tulbaghia violacea* acetone and water extracts on RAW264.7 cell viability. Results are presented as the mean ± standard deviation (SD) from three biological replicates.

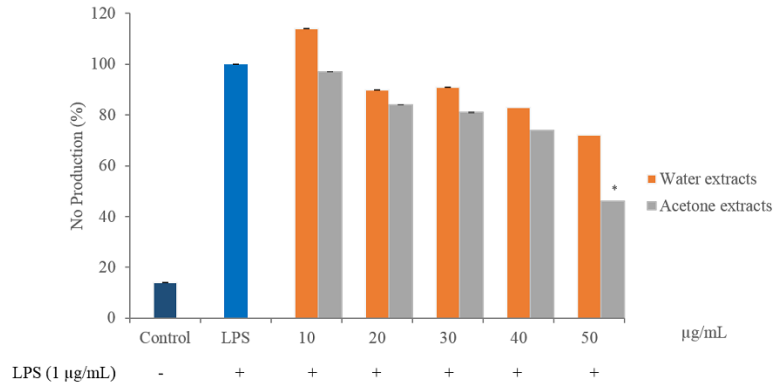

Figure 2. Effect of *Tulbaghia violacea* acetone and water extracts on nitric oxide (NO) production in RAW264.7 cells treated with lipopolysaccharide (LPS). Results are presented as the mean ± standard deviation (SD) of three independent experimental runs. * Denotes significance at $P < 0.05$ compared to the cells treated with LPS alone.

the detection limit of 15 000 pg/mL (Figure 3C). After stimulation with 30 µg/mL of crude acetone extracts for 24 hours, a substantial increase ($P<0.05$) in TNF-α production was observed. In contrast, applying 10 and 50 µg/mL of the crude acetone extracts did not significantly reduce TNF-α production after 48 hours (Figure 3D).

In comparison to the LPS control, IL-1α increased significantly ($P<0.05$) after 24 hours at all three water extract concentrations. However, as observed in Figure 4A, the water extracts did not significantly ($P>0.05$) affect IL-1α levels after 48 hours (Figure 4A). Figure 4B shows that the differences in the average IL-1β production observed between cells treated solely with LPS and those treated with the three concentrations of water extract at 24 and 48 hours were not statistically significant ($P<0.05$). All treatments, including the crude water extract, produced more IL-6 above the detection limit of 15 000 pg/mL after 24 and 48 hours, except the cells that received 10 µg/mL of the extract after 48 hours (Figure 4C). Intriguingly, TNF-α production increased at 24 hours when samples were treated with crude water extract (Figure 4D), whereas only the samples containing 50 µg/mL of the extracts and LPS demonstrated an increase in TNF-α production above the upper detection limit after 48 hours (Figure 4D).

Effect of Tulbaghia violacea on anti-inflammatory cytokines

At a concentration of 30 µg/mL, treatment with acetone extract significantly ($P<0.05$) increased IL-4 production after 24 hours though, IL-4 concentration remained lower than 5 pg/mL at this time (Figure 5A). In contrast, IL-4 secretion significantly ($P<0.05$) decreased at 50 µg/mL after 48 hours. Regarding IL-5, a significant ($P<0.05$) increase was only observed at the 30 µg/mL concentration of this extract after 24 and 48 hours. The difference between the treatments and the cells treated with LPS alone was not significant (Figure 5B). There was no significant difference in acetone extracts, although a trend of increased IL-10 production was observed after 24 hours treatment with the higher extract concentrations (Figure 5C). Similarly, the levels of IL-10 did not differ significantly after 48 hours ($P>0.05$). A significant ($P<0.05$) increase in the production of IL-13 was noted after treating the cells with 30 and 50 µg/mL of the extracts at 24 hours. Regarding IL-13, no difference was observed between the treatments and cells treated with LPS after 48 hours.

For the cells treated with the water extracts, the levels of IL-4 in the treatment groups did not differ ($P>0.05$) from the cells treated solely with LPS after 24 hours. However, at 50 µg/mL, a significant ($P<0.05$) rise in IL-4 levels was seen after 48 hours, even though the concentration was below 4 pg/mL (Figure 6A). There was no apparent difference ($P>0.05$) in the levels of IL-5, IL-10, and IL-13 between the cells treated with LPS alone and those treated with the extracts after 24 and 48 hours (Figures 6B, 6C, and 6D).

Effect of *Tulbaghia violacea* on MCP-1 and MIP-1α

Monocyte chemoattractant protein-1 (MCP-1) production

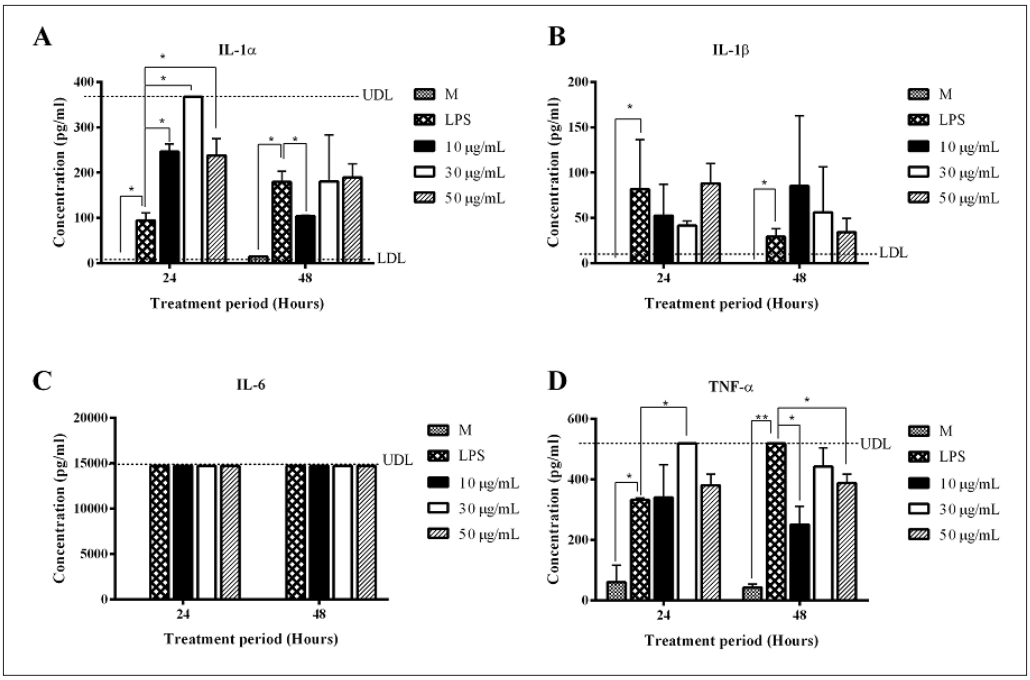

Figure 3. Effect of acetone extract of *Tulbaghia violacea* on the production of interleukin-1 alpha (IL-1α) (A), interleukin-1 beta (IL-1β) (B), interleukin-6 (IL-6) (C), and tumor necrosis-factor-alpha (TNF-α) (D) of RAW264.7 cells. M: Cells treated with medium only (control). Data are presented as mean ± standard deviation (SD); * and ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively compared to LPS-only treated cells. LPS: Lipopolysaccharide; UDL: Upper detection limit; LDL: Lower detection limit.

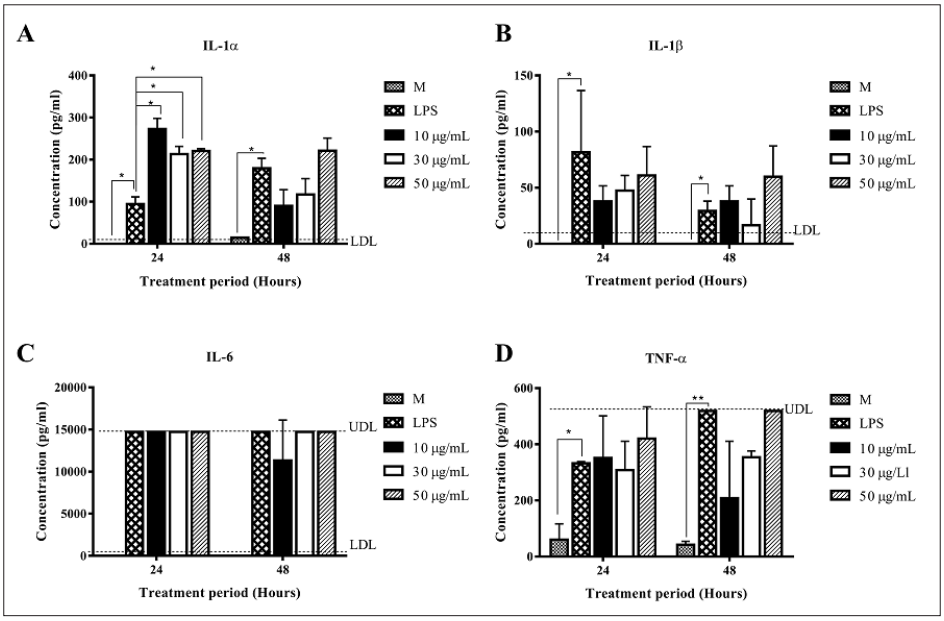

Figure 4. Effect of water extract of *Tulbaghia violacea* on the production of interleukin-1 alpha (IL-1α) (A), interleukin-1 beta (IL-1β) (B), interleukin-6 (IL-6) (C), and tumor necrosis-factor-alpha (TNF-α) (D) of RAW264.7 cells. M: Cells treated with medium only (control). Data are presented as mean ± standard deviation (SD); * and ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively compared to LPS-only treated cells. LPS: Lipopolysaccharide; UDL: Upper detection limit; LDL: Lower detection limit.

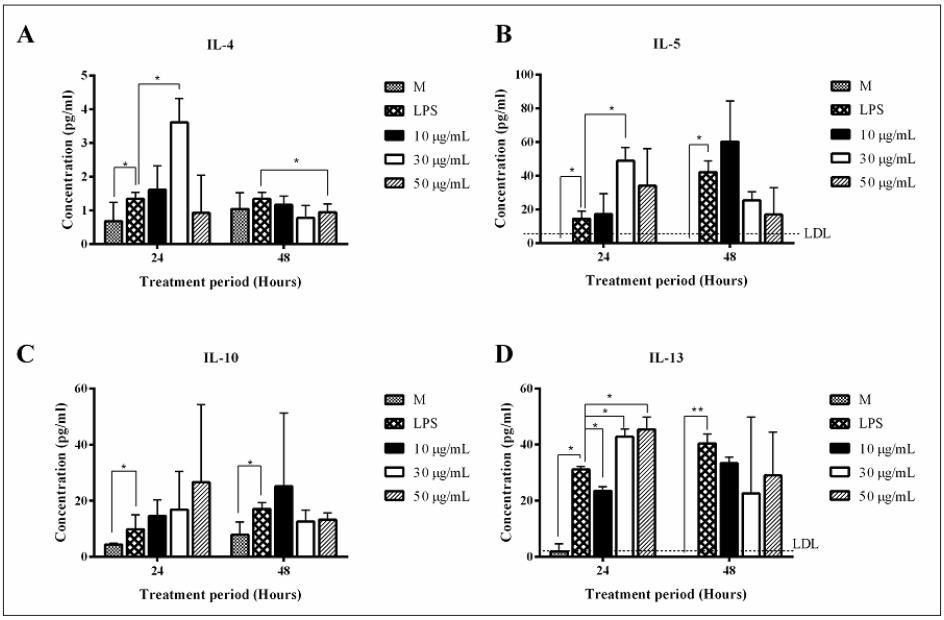

Figure 5. The effect of acetone extract of *Tulbaghia violacea* on the production of interleukin-4 (IL-4) (A), interleukin-5 (IL-5) (B), interleukin-10 (IL-10) (C), and interleukin-13 (IL-13) (D) of RAW264.7 cells. M: Cells treated with medium only (control). Data are presented as mean±standard deviation (SD); * and ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively compared to LPS-only treated cells. LPS: Lipopolysaccharide; UDL: Upper detection limit; LDL: Lower detection limit.

increased for each concentration with the acetone extract at 24 and 48 h, surpassing the detection limit (200 000 pg/mL) (Figure 7A). Similarly, MCP-1 concentrations surpassed the detection limit after 24 and 48 hours following treatment with the water extract (Figure 7C). For MIP-1α, treatment with acetone extract did not alter MIP-1α levels ($P > 0.05$) compared to cells treated with only LPS and the untreated control after 24 and 48 hours (Figure 7B). In addition, treating the cells with the water extract did not affect MIP-1α production in both treated and untreated samples, although results showed higher MIP-1α levels (10 000 pg/mL) after 48 hours (Figure 7D).

Analysis of the 25-plex cytokine panel

For an in-depth examination of the cytokine responses in this study, heatmaps were generated using data from all the 25-plex cytokine panels. Both crude extracts stimulated high pro-inflammatory cytokine secretion after 24 and 48 hours (Figures 8a and 8b). Contrarily, anti-inflammatory cytokine production was comparatively low after both time intervals (Figures 8a and 8b). Two distinct cytokine clusters, A and B, were noted after treatment with both extracts at 24 and 48 hours. Cluster A included IL-2, IL-β, IL-4, IL-9, IL-15, IL-6, RANTES (regulated upon activation, normal T cell expressed and secreted), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage chemotactic protein-1

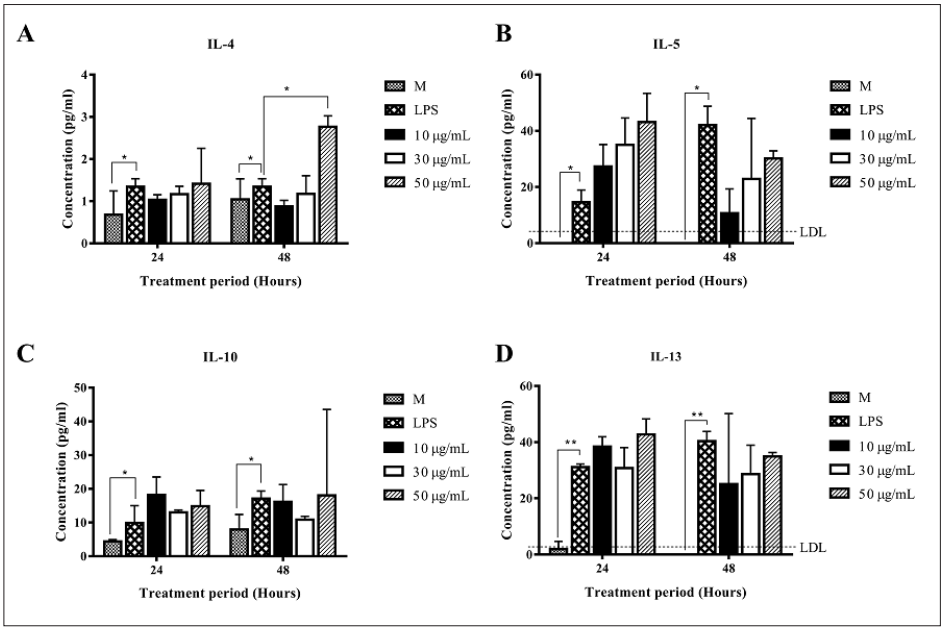

Figure 6. Effect of water extract of *Tulbaghia violacea* on the production of interleukin-4 (IL-4) (A), interleukin-5 (IL-5) (B), interleukin-10 (IL-10) (C), and interleukin-13 (IL-13) (D) of RAW264.7 cells. M: Cells treated with medium only (control). Data are presented as mean ± standard deviation (SD); * and ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively compared to LPS-only treated cells. LPS: Lipopolysaccharide; UDL: Upper detection limit; LDL: Lower detection limit.

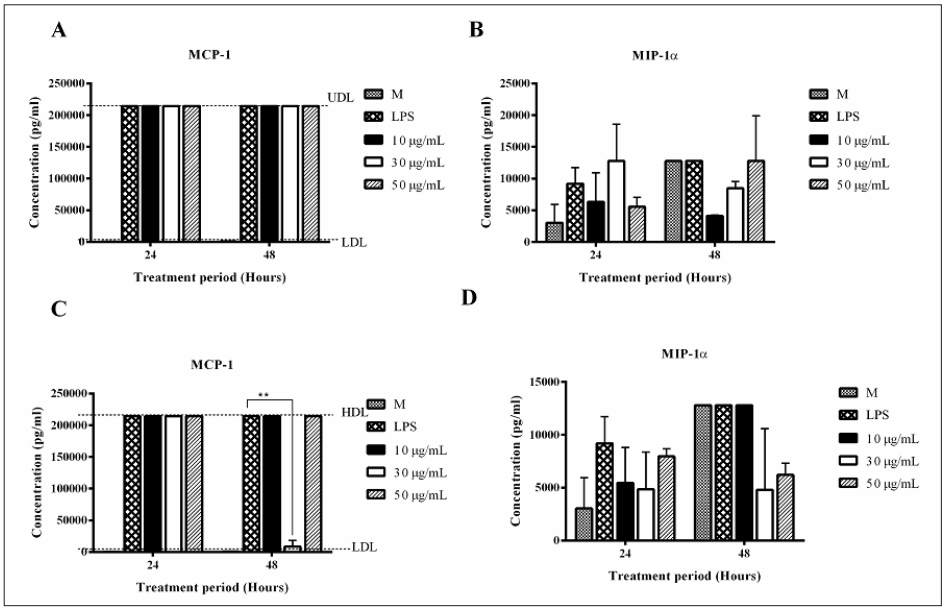

Figure 7. Effects of acetone (A and B) and water (C and D) extracts of *Tulbaghia violacea* on the production of macrophage chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α) of RAW264.7 cells treated with lipopolysaccharide (LPS). Results are presented as mean ± standard deviation (SD) of biological replicates. M: Cells treated with medium only (control). * and ** indicate significance at $P < 0.05$ and $P < 0.01$, respectively compared to LPS-only treated cells. UDL: Upper detection limit; LDL: Lower detection limit.

(MCP-1), IL-5, IL-10, IL-13, TNF-α, and IL-α whereas cluster B comprised IL-12P70, IL-7, MIP-1β (macrophage inflammatory protein-1β), IFNG (interferon gamma), MIP-2 (macrophage inflammatory protein-2), IL-12P40 (interleukin-12 subunit beta), KC (keratinocyte chemoattractant), IP-10 (interferon gamma-induced protein 10), MIP-1α (macrophage inflammatory protein-1α), IL-17, and granulocyte-colony stimulating factor (GCSF) (Figure 8a). Interestingly, after 48 hours treatment, a shift in the composition of cytokine was witnessed in both clusters (Figure 8b). Anti-inflammatory cytokines, such as IL-12P70, IL-7, IL-12P40, IFNG, IL-17, IP-10, and KC, assembled within cluster A, whereas pro-inflammatory cytokines, including IL-6, IL-13, IL-9, MCP-1, TNF-α, RANTES, and IL-α, clustered with cluster B (Figure 8b). Moreover, pro-inflammatory cytokines were predominant in cluster A after 48 hours, except for IL-13. In addition, after 24 and 48 hours treatments, the samples were separated into two clusters, C and D. After 24 hours treatment, a sample (Mac-untreated macrophages) remained isolated within its node, but the acetone and water extracts grouped with LPS-treated samples (Figure 8a). However, the cluster containing the Mac (untreated macrophages) was combined with W30 (water extract at 30 µg/mL) after 48 hours.

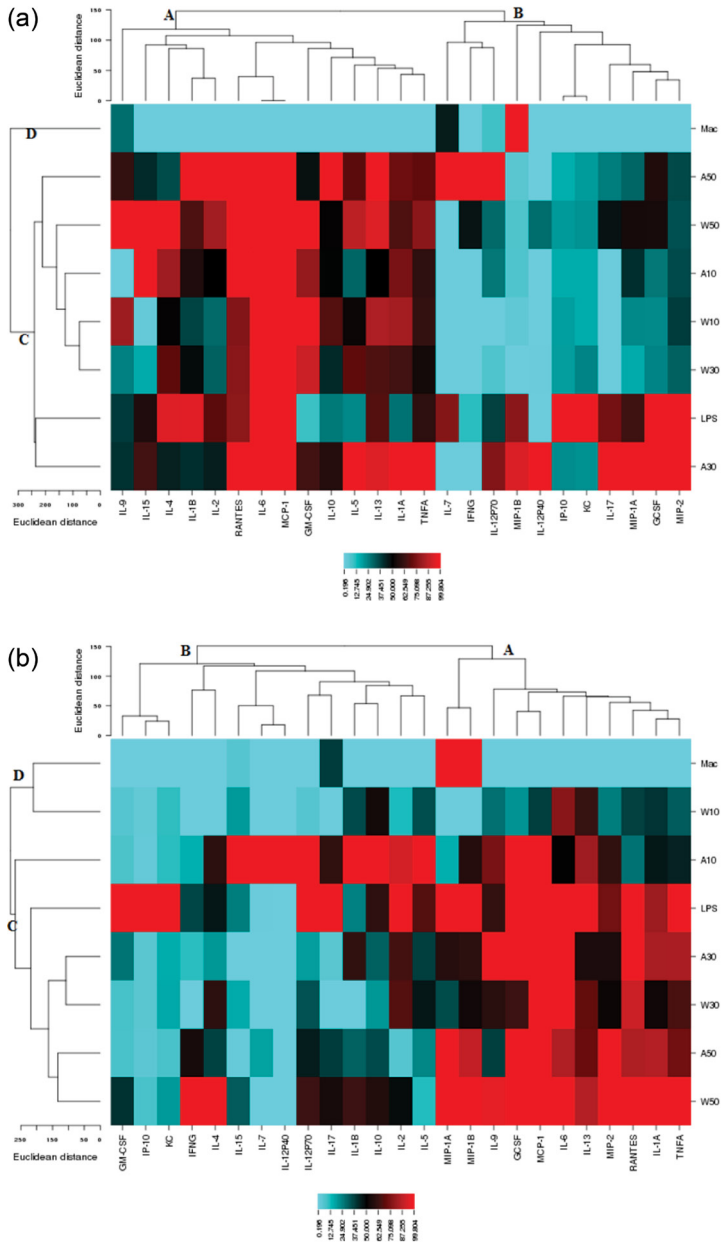

Figure 8. Reconstruction of heat maps depicting the analysis of 25 cytokines in serum samples from RAW264.7 cells treated with *Tulbaghia violacea* water and acetone extracts for 24 hours (a) and 48 hours (b) at 10, 30, and 50 µg/mL extract concentrations. Results are presented as mean ± standard deviation (SD) of biological replicates. The mean concentrations of cytokines in pg/mL are shown on a logarithmic scale, where blue, black, and red represent low, medium, and high levels, respectively, as shown on the color bar. A one-matrix CIM was done using CIMMINER.

Discussion

This study evaluated the immunomodulatory effects of water and acetone extracts of *T. violacea* and provided insights into its potential therapeutic uses. Both extracts demonstrated immuno-stimulative properties to varying degrees. Immunomodulators are important compounds capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response (27). They influence various immune cell types, cytokine networks, and signalling pathways, essential for maintaining immunological balance (28). The importance of immunomodulators arises when the immune system faces challenges in its optimal functioning, especially in susceptible groups like children, the elderly, and individuals with weakened immune systems (28). Hence, the search for effective immunomodulatory agents, including both immune-stimulating and immunosuppressive agents, obtained from nature is of utmost importance (29).

The primary purpose of this study was to examine how water and acetone extracts obtained from *T. violacea* influenced the immune system. Therefore, NO production, as well as cytokine and chemokine secretions in macrophage cells (RAW264.7), stimulated with LPS, were evaluated in the study. Previous studies have reported the cytotoxic effects of *T. violacea* both *in vitro* and *in vivo* (1), highlighting the need to determine the lowest concentrations upon which the extracts could become cytotoxic to the cells. The viability tests conducted on RAW264.7 cells exposed to water and acetone crude extracts from *T. violacea* revealed that concentrations up to 50 µg/mL of both extracts maintained cell viability at more than 80% (Figure 1). Hence, concentrations within this range were considered in subsequent experiments, as they were not detrimental to the cells.

The results on NO production in stimulated macrophages showed that plant extracts reduced LPS-induced NO production; however, this reduction was not statistically significant ($P > 0.05$) across most groups. An exception was noted in the group treated with 50 µg/mL of acetone extract (Figure 2). The superior impact of the acetone extract on NO production suggests a potential inhibitory effect of *T. violacea* crude acetone extract on iNOS, as reported in previous studies (8,18). Since iNOS is a key enzyme that produces significant amounts of NO during inflammatory responses, its regulation is important for maintaining balanced immune function. It is known that significant amounts of NO produced by iNOS contribute to vasodilation and hypotension, as observed in conditions like septic shock and inflammation. Therefore, inhibitors targeting iNOS could offer promising treatment options for inflammatory disorders linked to excessive NO production (30).

The ability of the acetone extract to significantly increase NO levels could suggest a complex interaction with iNOS, which may either be through the induction of NO synthesis or by modulating the inflammatory cascade that regulates the expression of iNOS (31). The acetone extract's greater efficacy than the water extract could be due to bioactive compounds targeting iNOS pathways more effectively. Depending on the concentration and cellular environment, these compounds may enhance or inhibit iNOS. This attribute makes the acetone extract potentially useful for therapeutic applications where selective modulation of NO production is required, especially in conditions where NO-mediated vasodilation or hypotension is prevalent. Investigating the specific mechanism through which acetone extract affects iNOS and evaluating its potential in treating inflammatory conditions associated with excessive NO production will be useful.

Further analysis focused on pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α, essential for controlling inflammation and reestablishing tissue homeostasis (14). Stimulating macrophages with bacterial LPS enhances the release of TNF-α, which in turn releases IL-6 and IL-1β. An important prerequisite for cytokine induction is the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (32). Our results revealed a dynamic immunomodulatory response induced by the acetone extract on the stimulated macrophages. Initially, the extract synergized with LPS to enhance IL-1α production in a concentration-dependent manner within the first 24 hours, demonstrating an increase in the immune response. In this period, the extract significantly increased TNF-α secretion, surpassing the levels observed in macrophages treated with LPS alone, highlighting its potent stimulatory effect on TNF-α production. However, by 48 hours, there was a notable decrease in IL-1α levels in the LPS-stimulated macrophages treated with the extract, indicating a suppression of IL-1α production below baseline levels seen with LPS alone. This suggests the extract's potential to dampen prolonged IL-1α secretion in response to LPS. A similar trend was observed with TNF-α secretion. After 48 hours of treatment with the extract, TNF-α levels in LPS-stimulated macrophages dropped from their initial high levels. This contrasted with the significant increase in TNF-α observed in macrophages stimulated with LPS alone, which reached levels above the detection threshold. In contrast, the extract initially antagonized the secretion of IL-1β during the first 24 hours, mitigating the LPS-induced secretion. However, this antagonistic effect was brief as IL-1β production increased after 48 hours, differing from the reduced levels observed in macrophages stimulated with LPS alone. Similarly, the water extracts triggered IL-1α and IL-6 production but did not significantly ($P > 0.05$) affect the levels of TNF-α and IL-1β. These results suggest the potential anti-inflammatory properties of *T. violacea* extracts by pointing to their complex regulatory effects on inflammatory cytokines.

TNF-α effectively moderates inflammation and contributes significantly to inflammatory responses

of the innate immune system by inducing cytokine production, activating or expressing adhesion molecules, and stimulating growth. It exhibits various functions, including normal cell proliferation, inflammatory, immunoregulatory, and antiviral effects induction, as well as cytostatic or cytolytic activity against tumor cells. TNF-α also plays roles in coagulation, lipid metabolism, endothelial function, and insulin resistance, highlighting its significance in the activation of immune and inflammatory responses (33). Similarly, IL-6 plays a critical role in the final maturation of B-cells into antibody-producing plasma cells (33). The observed increase in the levels of pro-inflammatory cytokines, including TNF-α, IL-1α, IL-6, and IL-1β, even at low concentrations, demonstrates that the *T. violacea* crude extracts can effectively stimulate immune response. These cytokines contribute significantly to initiating and sustaining inflammation (34). Therefore, their observed increase in the study suggests that the extracts may be potent enough to activate macrophages and stimulate the production of these cytokines even at low concentrations.

The study also examined the effects of *T. violacea* extracts on chemokines and anti-inflammatory cytokines, which are important immune response regulators. IL-4, IL-5, IL-10, and IL-13 are examples of IL receptor antagonists that specifically balance the body against pro-inflammatory cytokines (35). Treating LPS-induced cells with acetone extract resulted in increased production of IL-4, IL-5, and IL-13 after 24 hours, while IL-10 levels remained unaffected ($P>0.05$) (Figure 5). On the other hand, the water extract could only induce the production of IL-4 (Figure 6). In addition, low levels (below 100 pg/mL) of anti-inflammatory cytokines were observed after treatment with both extracts, suggesting weak anti-inflammatory activity of the extracts. These results are consistent with those of Adebayo et al (36), who used the anti-15 LOX model to determine the anti-inflammatory activity of acetone extracts of *T. violacea*, which showed a limited inhibition of -15 LOX.

The study included chemokines essential for immune modulation, including MIP-1α, MIP-1β, and MCP-1 (37,38). The study also highlighted the pro-inflammatory properties of *T. violacea* extracts through the analysis of chemokines. Treating cells with both extracts resulted in high (>5000 pg/mL) MCP-1 and MIP-1α concentrations (Figures 7A and 7C), indicating a strong pro-inflammatory response of the extracts. The heatmaps in Figures 8a and 8b further confirmed this observation and highlighted the strong pro-inflammatory activity of both extracts *in vitro*. Furthermore, the secretion of several chemokines was examined in the study (Figures 8a and 8b), with results showing increased cytokine levels. These cytokines are important for regulating inflammatory responses and include GM-CSF, G-CSF, MIP-2, and RANTES. These chemokines play specific roles in mediating and controlling immune response. For example, MIP-2 is an essential inducible chemokine that attracts neutrophils to inflammation sites (39). Conversely, GM-CSF is released by activated leukocytes and acts as a pro-inflammatory cytokine that influences the body's immune response (40). It also interacts with receptors on granulocytes, monocytes, and macrophages to promote chemotaxis, cell proliferation, and differentiation.

The hematopoietic growth factor G-CSF stimulates the production of stem cells and granulocytes in the bone marrow. It plays a significant role in tissue healing, regeneration, and defending the body against infections. Importantly, G-CSF is being explored as a potential therapy for cancer treatment (41). RANTES, secreted by activated platelets, help attract various immune cells, including eosinophils, mast cells, dendritic cells, NK cells, basophils, and T-cells to inflammation and infection sites. It may also exacerbate inflammatory conditions in certain disorders like atopic dermatitis, colitis, nephritis, and arthritis and may induce antimicrobial activity by enhancing NO production in macrophages (42,43).

Two distinct patterns of clustering (Clusters A and B) were observed in the 25-plex cytokine panel in the study after treatment with both acetone and water extracts. The clustering provides valuable information on the differential immune responses at 24 and 48 hours. It reflects the interaction between pro- and anti-inflammatory signals elicited by the extracts with potential implications for immune modulation. The stimulation of pro-inflammatory cytokines secretion by both extracts at 24 hours suggests an initial inflammatory response, which may serve as a mechanism to activate immune defense. The cytokines in Cluster A (Figures 8a and 8b) are known to be principal mediators of immune activation, and their clustering indicates coordinated regulation. On the other hand, Cluster B (Figures 8a and 8b) also contained cytokines critical for immune signalling, particularly in the stimulation of chronic or systemic inflammatory responses. The differences in the two clusters suggest that the immune response may involve multiple pathways or activation stages.

A shift in cytokine composition in both clusters was observed after 48 hours, indicating a temporal immune response regulation. Cluster A became enriched with anti-inflammatory cytokines (Figures 8a and 8b), while Cluster B contained mostly pro-inflammatory cytokines. This change may reflect a transition from an early pro-inflammatory phase to a more balanced immune modulatory phase. The dominance of anti-inflammatory cytokines in Cluster A at this stage suggests that the extracts could contribute to resolving inflammation, which is critical in preventing chronic inflammation or tissue damage (44). The persistence of pro-inflammatory cytokines in Cluster B after 48 hours could be an indication of sustained immune activation, which could also result in tissue damage. Previous studies have linked prolonged secretion of cytokines

like TNF-α, IL-6, and MCP-1 to chronic inflammatory diseases (45,46). Therefore, it is important to investigate this sustained response further by evaluating the potential risks associated with the long-term use of these extracts. The clustering of samples into groups C and D, where untreated macrophages (Mac) remained isolated at 24 hours but clustered with water extract (W30) after 48 hours, suggests that the water extracts may have delayed but significant immunomodulatory effects. The delayed clustering may indicate that though initial responses may differ, prolonged exposure to the water extract may induce similar responses to those elicited by LPS-stimulated immune activation. The clustering patterns show dynamic cytokine responses, which suggests that *T. violacea* may show a biphasic response, whereby it initially elicits an inflammatory response and later shifts to resolve the inflammation. This mechanism highlights the potential of the extracts in both immune activation and modulation, as well as the importance of concentration and time-dependent studies to avoid prolonged inflammation. Therefore, further studies are needed to determine the molecular mechanisms causing these changes and their importance in therapeutic applications.

The results showed a significant reduction of IP-10 expression in LPS-stimulated RAW264.7 cells, suggesting a significant dampening effect of the extracts on IP-10 expression. IP-10 interacts with the CXCR3 receptor in immune cells to trigger immune responses, such as cell growth inhibition, chemotaxis, angiostasis, and apoptosis. These functions of pro-inflammatory cytokines suggest that both extracts of *T. violacea* are potential immune stimulants. A study by Aremu and van Staden (1) also supports the traditional therapeutic potential of the plant. The variety of bioactive compounds found in both extracts may be responsible for the observed immunomodulatory activity, although the precise mechanisms are not properly understood (25). These compounds may play vital roles in modulating cytokine and chemokine responses (13), hence, impacting immunomodulation.

The ability of *T. violacea* to activate macrophages and initiate pro-inflammatory cytokines production could be the reason for its historical usage in traditional medicine as an immune system stimulant. Similar immune-modulating effects, such as increased NO and cytokine production in macrophages have been observed in a popular medicinal mushroom called *Ganoderma lucidum* (30,47). This mushroom is well-known for its immune-boosting properties described earlier. Consequently, the pro-inflammatory activity observed in *T. violacea* extracts may stimulate macrophages and increase resistance to microbial infections in host cells. In addition, pro-inflammatory cytokines have been reported to display anti-tumour effects by inhibiting the growth of tumor cells or stimulating the death of tumor cells (48). Among cancer patients, TNF- α, IL-β, and IL-6 have demonstrated promise in tumor shrinkage and prolonged median survival time (49). In our previous study, *T. violacea* also demonstrated anti-cancer activities (50), which may correlate with the extracts' pro-inflammatory properties. The therapeutic potential of *T. violacea* extracts against immune-related ailments is further demonstrated by their effects on cytokines, suggesting they may have anti-inflammatory properties.

The immunomodulatory activity of acetone and aqueous leaf extracts of *T. violacea* against LPS-induced RAW 264.7 cells was effectively demonstrated in the study. A standardized approach was employed to evaluate the effects of the extract with a treatment concentration of ≤50 µg/mL applied in the study. The generalizability of the findings to other cell types or *in vivo* models was restricted by the study's focus on a specific cell line (RAW 264.7 cells). The study did not identify potential active components accountable for the immunomodulatory activity.

Conclusion

This study showed that the water and acetone extracts of *T. violacea* had significant immunostimulatory effects. The results are consistent with the traditional medicinal applications of the plant and demonstrate the significance of determining whether the immune-regulating compounds in the plant alone or in combination are responsible for this effect. Further research into how solvent extraction impact immune cell activity is essential. Such studies can help identify novel immunomodulatory compounds and simplify the process of developing extracts designed to elicit specific immunological responses. In addition, further investigation in the identification of specific bioactive compounds and the elucidation of their underlying mechanisms improve our understanding of the immunomodulatory properties of *T. violacea* and provide avenues for the development of novel immune-targeted therapies. This encompasses potential treatments for allergies, autoimmune diseases, and even as adjuvants in vaccines. The study provided more insights into the immunomodulatory potential of *T. violacea* leaf extracts by revealing their ability to influence cytokine and NO production in immune cells, modulating both pro-inflammatory and anti-inflammatory cytokines, indicating their potential in both applications that require immune activation, including infections and cancer immunotherapy and in treating diseases, such as chronic inflammation.

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Authors' contribution

Conceptualization: Samkeliso Takaidza, Michael Pillay, and Cornelius Cano Ssemakalu.
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Availability of data and materials

All data generated or analysed during this study are included in this published article. The corresponding author can provide further information.

Conflict of interests

The authors declare they have no conflict of interests.

Ethical considerations

The Central Research Committee of the Vaal University of Technology approved the protocols (Approval number: FACSREC-15092020-0002).

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