



Immunomodulatory Effect of *Tinospora cordifolia* with Special Reference to Suppression of Cytokine Storm Induced in SARS-CoV-2

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Abstract

Background: *Tinospora cordifolia* (*T. cordifolia*) is one such plant that has been studied for its many medicinal properties.

Objective: The objective of this study was to investigate the *in vitro* exposure of human peripheral blood mononuclear cells to *T. cordifolia* plant might stimulate the induction of anti-inflammatory cytokines, interleukin-10 and interleukin-37.

Methods: *T. cordifolia* plant powder was sterilized by several methods to eliminate presence of microorganisms in plant powder. The sterilized *T. cordifolia* plant powder was exposed to peripheral blood mononuclear cells to determine the inhibitory concentration by conducting a cytotoxicity test. ELISA test was performed to check whether *T. cordifolia* stimulates the peripheral blood mononuclear cells into producing anti-inflammatory cytokines. **Results:** Pasteurization technique was a success as no bacterial or fungal growth was observed on nutrient agar, blood agar, and sabouraud dextrose agar. The optimal inhibitory concentration of *T. cordifolia* is 72 mg/ml. The results of the ELISA tests showed the production of interleukin-10 and interleukin-37 when stimulated by *T. cordifolia*. **Conclusion:** *T. cordifolia* plant powder could be a potential alternative for non-steroidal anti-inflammatory drugs as the plant induces secretion of interleukin-10 and interleukin-37 that subsidizes the interleukin-6

Key Words: Cytokine storm, ELISA, Interleukin-10, Interleukin-37, *Tinospora cordifolia*

Introduction

Immune system keeps the body in a state of equilibrium in healthy individuals. However, various endogenous and exogenous variables can either stimulate or depress the immune system. Immunomodulators are a class of drugs that can regulate or alter pathophysiological processes (Jantan et al., 2015). Biological immunomodulatory drugs can lower system or local inflammation in healthy individuals by down-regulating detrimental immune responses.

Herbal medicines that boost the immune system's performance are classified as adaptogens or immunostimulants. Adaptogens, including Ginseng, is a type of herbal product that has medicinal properties that boost the body's tolerance to stress and protect from infections. In addition, natural spices and the separated active components have been shown to target inflammatory pathways and elicit anti-inflammatory effects in various chronic conditions in various investigations (Kunnumakkara et al., 2021).

T. cordifolia is an herb that medical practitioners have used for years for its healing powers. *T. cordifolia* stems

Significance | Development of anti-inflammatory herbal drug

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have demonstrated hepatoprotective, antipyretic, cytotoxic, anti-diabetic, and immunomodulatory properties, while the whole plant was shown to have hepatoprotective, antiulcer, and antioxidant properties (Singh and Chaudhuri, 2017). Furthermore, *T. cordifolia* has been proved to contain a wide range of secondary metabolites like the tannins, alkaloids, cardiac glycosides, saponins, triterpenoids, phytosterols, and polyphenols which have proved to be of great medicinal value and act as immunomodulators, anti-diabetic medicine, anti-allergic, anti-leprotic and anti-inflammatory agents (Pruthvish and Gopinatha, 2018).

Dry stem crude extracts of *T. cordifolia* bound to macrophage with a polyclonal B cell mitogen, G1-4A, have been shown to improve immunological response in mice by stimulating interleukin-1 (IL-1) production and macrophage activation (Ghosh and Saha, 2012). In addition, the (1, 4)-alpha-D-glucan (alpha D-glucan), derived *T. cordifolia* has been shown to activate human lymphocytes with the downstream synthesis of the pro- and anti-inflammatory cytokines, *in vitro* (Koppada et al., 2009).

T. cordifolia alcoholic extract stimulated macrophages through antigen presentation and phagocytosis. Thus, *T. cordifolia* possesses immunostimulant characteristics that medical practitioners have employed for its therapeutic effects. It belongs to the *Menispermaceae* family and is known as "Guduchi" Sharma et al. (2019). It has been used in ayurvedic medicine for the treatment of skin problems, allergies, inflammation, rheumatism, and urinary disorder. Furthermore, *T. cordifolia* has been demonstrated to affect rats' tumor necrosis factor- α (TNF- α) and cyclooxygenase-2 (Cox-2) gene expression. TNF- α and Cox-2 gene expression were significantly reduced in rats treated with *T. cordifolia*. In addition, TNF- α and Cox-2 gene expression were down regulated while HAMP and toll-like receptor-4 (TLR-4) gene expression were inhibited, indicating that *T. cordifolia* has anti-inflammatory properties (Ghatpande et al., 2019).

SARS-CoV-2 is a novel coronavirus (CoV) previously unknown to mankind (Adil et al., 2021). It is designated as a beta-CoV of group 2B, and it is the cause of the coronavirus disease of 2019. Its epidemiology is linked to a seafood market in Wuhan, Hubei Province, China in December 2019 (Wu et al., 2020). Naturally occurring viral mutations can arise anywhere in the SARS-CoV-2 genome. In addition, mutations have been identified for the receptor-binding domain (RBD) on the spike protein of SARS-CoV-2 (Cennimo et al., 2021). Several of these alterations have a higher binding affinity for human angiotensin converting enzyme 2 (ACE2), which is likely due to the RBD's structural stability. Higher viral shedding, a longer infectious interval, increased infectivity, and greater environmental stability are all possible pathways for increased transmission.

Antigen-presenting cells process and display the SARS-

CoV-2 viral antigen to natural killer (NK) cells and CD8-positive cytotoxic T cells in the context of major histocompatibility (MHC) antigens once the immune system detects it (Soy et al., 2020). This can cause the activation of both innate and adaptive immune responses, resulting in the generation of high amounts of pro-inflammatory cytokines. Pro-inflammatory cytokines mediate local and systemic inflammations. This can result in multi-organ failure and, in the worst-case scenario, death. Hence, there is a clear need for a method to inhibit the cytokine storm effectively. The anti-inflammatory cytokine response is one of the immune systems many facets. Anti-inflammatory cytokines are a group of immunoregulatory molecules that regulate the response of pro-inflammatory cytokines (Sultani et al., 2012). There are many anti-inflammatory cytokines such as interleukin-4 (IL-4), interleukin-6 (IL-6), interleukin-11 (IL-11), interleukin-10 (IL-10), and interleukin-37 (IL-37) (Tabana et al., 2016). The viral spike protein of SARS-CoV-2 induces the TNF- α -converting enzyme (TACE)-dependent alteration of ACE-2 (Iannaccone et al., 2020). This allows the virus to penetrate the host cells. SARS-CoV-2 severity is linked to higher levels of inflammatory mediators including cytokines and chemokines such as IL-2, IL-7, IL-10, TNF, granulocyte colony stimulating factor (G-CSF), monocyte chemo-attractant protein-1, macrophage inflammatory protein 1 alpha, CXC-chemokine ligand 10 (CXCL10), C-reactive protein, ferritin, and D-dimers in blood (Hojo et al., 2020). When SARS-CoV-2 survivors and non-survivors are compared, the blood IL-6 levels are highly correlated with the disease mortality showing that the deadly SARS-CoV-2 is classified as a cytokine release syndrome (CRS) that is induced by a cytokine storm with high mortality. IL-6 levels above a certain threshold are linked to the cause of acute respiratory syndrome and a higher risk of mortality (Smetana & Brábek, 2020).

Inhibition of IL-1 β , IL-6 and TNF may help to reduce excessive inflammatory responses. The high viral titer accompanied by the strong inflammatory cytokine and chemokine responses observed during pathogenic human coronavirus infection is associated with significant morbidity and mortality (Ye et al., 2020). The treatment of SARS and MERS in the past has shown that lowering the viral load by early management and regulating the inflammatory responses with immunomodulators could enhance the prognosis of SARS-CoV-2 infection.

Anti-inflammatory cytokine IL-10 helps to prevent inflammation and autoimmune disorders. IL-10 inhibits the activity of Th-1 cells, NK cells and macrophages. This can help restore tissue homeostasis and avoid tissue damage. The monocytes, macrophages, Tregs, Th2 T cells, and other CD4+ T cells that produce both IL-10 and IFN- γ are the most important *in vivo* sources for many infections (Kessler et al., 2017). In a negative and positive feedback loop, macrophages generate IL-10

to reduce excessive inflammatory cytokine production during infection. IFN- γ and IL-10 itself tightly control IL-10 expression. It can either limit or positive feedback to increase its own production. IL-10 is able to suppress pro-inflammatory cytokines in HIV, HBV, influenza virus, dengue, and other viral infections (Fadhilah et al., 2021).

IL-37 is a member of IL-1 family of anti-inflammatory cytokines. It has been demonstrated to reduce histocompatibility complex molecules and inflammation in rheumatic diseases by suppressing IL-1, IL-6, TNF, and CCL2 (Conti et al., 2020). PBMCs, monocytes, plasma cells, dendritic cells, epithelial cells, carcinoma cells, testis, thymus, and uterus have all been reported to have IL-37 mRNA and protein. IL-37 is a potent anti-inflammatory cytokine that helps prevent tissue damage during infections by decreasing the duration and intensity of immune and inflammatory responses. IL-37 is usually expressed in inflamed tissues and not in tissues from healthy subjects (Tete et al., 2012). *T. cordifolia* is a plant that has shown many curative properties. Hence, this study was used to determine the cytotoxicity of *T. cordifolia* stem powder using peripheral blood mononuclear cells (PBMCs). In addition, the immunomodulatory properties of the plant in inducing IL-10 and IL-37 were studied based on the IC₅₀ value. Therefore, the objective of this study was to investigate the ability of *T. cordifolia* stem powder to stimulate the induction of IL-10 and IL-37 when exposed to PBMCs.

MATERIALS AND METHOD

Plant material:

T. cordifolia stem powder was procured from a certified source. The plant powder was grounded and filtered using a 60cc sieve. The plant stem powder was sterilized by pasteurization method. Five grams of plant stem powder was added into a beaker and microwaved for 9 minutes at 75°C and rapidly cooled at 4°C, for 3 consecutive cycles. After that it was kept under ultraviolet light for 30 minutes (González-Monroy et al., 2018).

Microbial contamination screening:

The plant powder was tested for fungal and microbial contaminations. The standard protocol for the microbial contamination screening was standardized in our laboratory. Blood agar (BA) and nutrient agar (NA) were prepared to test for bacterial contaminations, and sabouraud dextrose agar (SDA) was prepared to test for fungal contaminations. The sterilized plant powder was added into an Eppendorf tube containing double distilled water. The contents were vortex, and the plant mixture was then evenly streaked on the agar plates. The agar plates were incubated for 24 hours at 37°C. After 24 hours, the plates were examined for any bacterial or fungal growth.

Cell line preparation:

PBMCs were isolated from blood procured through blood bank after getting the appropriate ethical clearance from institutional ethics committee. The heparinized venous blood was centrifuged by using the gradient method. The blood mixture was centrifuged at 1500 rpm for 40 minutes. The PBMCs that formed was transferred to a sterile falcon tube carefully with addition of 6ml 1X PBS. The cells were suspended and centrifuged at 1000 rpm for 10 minutes. After centrifugation the supernatant was removed and the PBMCs were suspended in RPMI-1640 media and incubated at 37°C and 5% CO₂ incubator (Panda et al., 2013).

Cytotoxicity test:

The 3-(4, 5-dimethylthiazol-2-yl)-5-diphenyl tetrazolium bromide assay (MTT assay) was conducted to determine the effects of *T. cordifolia* on PBMCs (Al-Suede et al., 2014a). The colorimetric assay was conducted to determine the cell viability after the treatment with the plant powder. Five different concentrations (150 mg/ml, 75 mg/ml, 37.5 mg/ml, 18.75 mg/ml, and 9.37 mg/ml) of *T. cordifolia* was prepared by two-fold dilution. The isolated PBMCs were seeded in a 96 well plate. The cells were then exposed to the different concentrations of the *T. cordifolia* plant. The assay was performed in triplicates and incubated for 24 hours at 37°C in 5% CO₂ incubator. After 24 hours of exposure of the cells with the plant concentrations, 10 μ l of MTT solution was added and incubated for another 4 hours at 37°C in 5% CO₂ incubator. The purple formazan crystals that were formed after 4 hours were dissolved by adding 100 μ l of Solubilization. The plate was incubated for another 30 minutes at 37°C in 5% CO₂ incubator. The intensity of the dissolved formazan crystals were quantified at 570 nm.

Cytokine evaluation:

A. Treatment of PBMCs

PBMCs were treated with *T. cordifolia* plant powder 24 hours before the cytokine evaluation. Five concentrations of *T. cordifolia* plant was prepared according to the IC₅₀ value obtained from the cytotoxicity test. The concentrations were 288 mg/ml, 144 mg/ml, 72 mg/ml, 36 mg/ml and 18 mg/ml. PBMCs were freshly harvested and cells were counted prior to cell seeding in 96 well plate. The isolated PBMCs were seeded in a 96 well plate at a concentration of 1×10^6 cells per well. The cells were then treated with the five different concentrations of the plant solution. Each of the concentrations and controls were done in triplicates. The plate was then incubated for 24 hours at 37°C in 5% CO₂ incubator.

B. IL-10

The IL-10 level was conducted according to manufacturer instruction (Thermo Fisher Scientific). ELISA plate

was coated with 100 μL /well of capture antibody in Coating Buffer and blocked with 200 μL of ELISA/ELISAPOT Diluent (1X) as described in the product manual. Two-fold serial dilution of the standard was performed to make the standard curve. 100 μL of supernatant was added to the appropriate wells. ELISA/ELISAPOT diluent (1X) was added to the blank well. The plate was sealed and incubated at room temperature for 2 hours. The wells were washed for four times. 100 μL /well of diluted Detection Antibody was added to all wells. The plate was sealed and incubated at room temperature for an hour. The wells were washed four times after the incubation period. 100 μL diluted Avidin-HRP was added to all the wells. The plate was sealed and incubated for 30 minutes at room temperature. The wells were again washed for six times. 100 μL /well of Substrate Solution was added to each well. The plate was incubated at room temperature for 15 minutes. 100 μL /well of Stop Solution was added, and absorbance was read at 450 nm.

C. IL-37

The protocol of IL-37 study was adopted from Human IL-37/IL-1F7 uncoated ELISA kit manufactured by Thermo Fisher Scientific. ELISA plate was coated with 100 μL /well of Coating Buffer and blocked 250 μL of Blocking Buffer as described in the product manual. The assay plate was prepared and incubated at room temperature for 2 hours. The wells were washed twice to remove the remaining blocking buffer. Two-fold serial dilution of the standard was performed with Sample Diluent Z to make the standard curve. 50 μL /well of the supernatant was added to the appropriate wells. 50 μL /well of antibody was added to all wells. The plate was sealed and incubated at room temperature for 2 hours. After 2 hours the wells were washed four times. After that 100 μL /well of Streptavidin-HRP was added to all the wells. The plate was sealed and incubated for one hour at room temperature. The wells were washed four times. 100 μL /well of Substrate Solution was added to each well. The plate was incubated at room temperature for 15 minutes. 100 μL /well of Stop Solution was added and absorbance was read at 450 nm.

RESULTS AND DISCUSSION

Microbial contamination screening:

T. cordifolia was tested for fungal and microbial contaminations. The results showed that non-sterilized *T. cordifolia* plant powder had bacterial colony growth on NA, BA which indicates the presence of fastidious bacteria in the plant powder and fungal growth was observed on SDA. Another batch of the plant powder was sterilized by pasteurization method. Following the sterilization, plant powder was streaked onto NA, BA and SDA. There were no bacterial or fungal growth observed on the NA, BA and SDA plates, respectively. This indicates that sterilization of *T. cordifolia* plant powder by pasteurization was a

success, and it killed most of the microorganisms. The absence of microorganisms in NA, BA and SDA plates indicates that *T. cordifolia* plant powder is sterile and free from microorganisms (Figure 1).

Cytotoxicity test:

MTT assay was conducted to determine the effects of *T. cordifolia* on PBMCs. The MTT cell proliferation assay measures the rate of cell proliferation and, conversely the decline in cell viability caused by the metabolic processes such as apoptosis and necrosis. The IC_{50} represents the concentration at which a substance exerts half of its maximal inhibitory effect. The IC_{50} of *T. cordifolia* stem powder towards PBMCs was 72 mg/ml. Thus, a linear relationship between the cell quantity and signal produced is established for the PBMCs. This allowed for the reliable assessment of the changes in the PBMC proliferation rate (Bahuguna et al. 2017).

Cytokine evaluation:

Cytokines are potent soluble immune mediators that can be used as target biomarkers of inflammation (Koelman et al., 2019). Under normal physiological conditions, the human immune system consists of several redundant pathways and immunoregulatory control elements that work together to coordinate the immunological response triggered by an external signal. Anti-inflammatory cytokines are a group of immunoregulatory molecules that regulate the response of pro-inflammatory cytokines.

IL-10 is an anti-inflammatory cytokine that helps to avoid inflammation and autoimmune diseases. Th-1 cells, NK cells and macrophages are all inhibited by IL-10. This can help to restore tissue homeostasis and avoid tissue damage (Conti et al., 2020). IL-37 is a member of IL-1 family of anti-inflammatory cytokines. It can reduce both innate and acquired immunity. It has been shown to suppress IL-1 β , IL-6, TNF, and CCL2, which inhibits histocompatibility complex molecules and inflammation in rheumatic diseases. Considering IL-37 and IL-10 inhibit several pro-inflammatory cytokines and cells that stimulate pro-inflammatory cytokines, they may be able to suppress the cytokine storm triggered by SARS-CoV-2.

A. IL-10

PBMCs were treated with different concentrations of the standard (pg/ml). Figure 2a represents the standard and average absorbance concentrations of the IL-10 secreted by PBMCs after exposure with the standard. *T. cordifolia* stem powder concentrations that were chosen to determine the concentration of IL-10 were 18 mg/ml, 36 mg/ml, 72 mg/ml and 144 mg/ml. The concentrations of IL-10 secreted by the unstimulated and stimulated PBMCs are shown in figure 2b. There was no secretion of IL-10 in the control, which is the unstimulated PBMCs. IL-10



Figure 1: Microbial contamination screening of non-sterilized and sterilized *T. cordifolia* plant powder. (a) Presence of bacteria on NA; (b) Absence of bacteria on NA; (c) Presence of fastidious bacteria on BA; (d) Absence of fastidious bacteria on BA; (e) Presence fungus on SDA; (f) Absence of fungus on SDA.

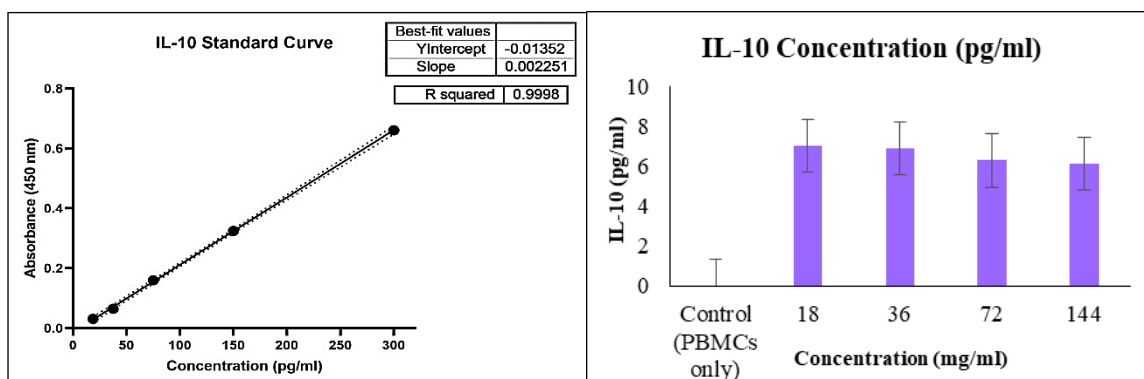


Figure 2: Secretion of IL-10 by PBMCs. (a) IL-10 concentration (pg/ml) secreted by PBMCs exposed with different concentrations of standard and (b) IL-10 concentration (pg/ml) secreted by PBMCs exposed with different concentrations of *T. cordifolia* plant powder.

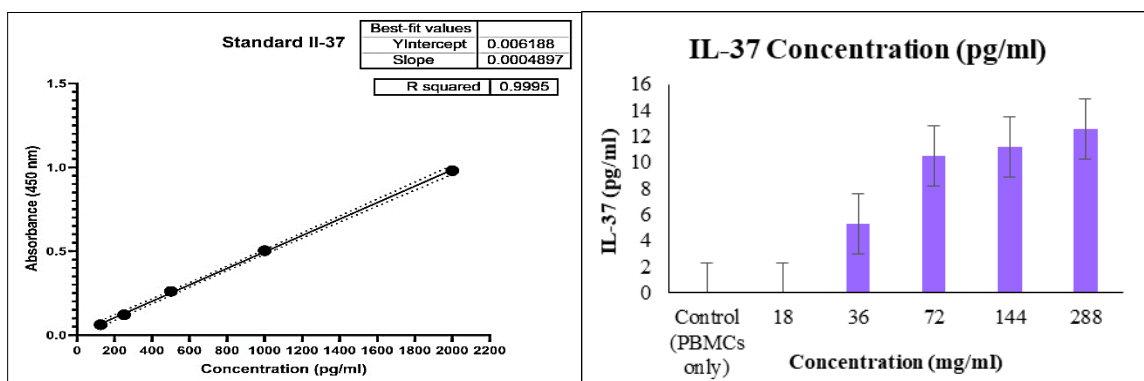


Figure 3: Secretion of IL-37 by PBMCs. (a) IL-37 concentration (pg/ml) secreted by PBMCs exposed with different concentrations of standard and (b) IL-37 concentration (pg/ml) secreted by PBMCs exposed with different concentrations of *T. cordifolia* plant powder.

secreted by PBMCs when treated with 18 mg/ml of *T. cordifolia* plant powder was 7.045 pg/ml. The treatment of PBMCs with 36 mg/ml of *T. cordifolia* plant powder stimulated secretion of IL-10 (6.897 pg/ml) slightly lower than the control. When the concentration is increased to 72 mg/ml the secretion of IL-10 was 6.304 pg/ml. As the concentration of the plant increased, the concentration of IL-10 kept on decreasing gradually. The concentration of IL-10 secreted kept reducing (6.156 pg/ml). The result indicates that treatment of PBMCs with *T. cordifolia* stem powder stimulates the cells to secreting IL-10, since there was no secretion of IL-10 in the control. However, decline in the concentration of IL-10 could indicate that a lower concentration of *T. cordifolia* plant powder is sufficient to stimulate the secretion of IL-10 (Figure 2).

IL-10 also inhibited the release of IL-1 β from PBMCs. It was demonstrated that IL-10 has a potent ability to inhibit human PBMC responses to opportunistic fungal pathogens. IL-10 suppressed the synthesis of IL-6 by T-cells via a monocyte- and IL-2-independent mechanism. All these studies prove that IL-10 has the capabilities to inhibit pro-inflammatory cytokines. However, in this study the PBMCs were not induced in secreting IL-10. This could be due to *T. cordifolia* stem powder not having the immunomodulation's to induce the PBMCs into secreting IL-10 or IL-10 is not secreted at the concentrations that were tested in this study.

B. IL-37

Concentrations of the standard used to treat PBMCs and average absorbance of IL-37 secreted by PBMCs are shown in figure 3a. *T. cordifolia* stem powder concentrations that were chosen to determine the concentration of IL-37 were 18 mg/ml, 36 mg/ml, 72 mg/ml, 144 mg/ml and 288 mg/ml. The concentrations of IL-37 secreted by the unstimulated and stimulated PBMCs are shown in figure 3b. There was no secretion of IL-37 in control, which is the unstimulated PBMCs. The exposure of PBMCs with 18 mg/ml of *T. cordifolia* plant powder did stimulate the secretion of IL-37. As the concentration increased to 36 mg/ml, the concentration of IL-37 secreted by PBMCs was 5.301 pg/ml. The concentration of IL-37 surged two times higher (10.508 pg/ml) when the concentration of plant powder increased to 72 mg/ml. As the concentration of *T. cordifolia* plant powder increased to 144 mg/ml and 288 mg/ml, concentration of IL-37 slightly increased (11.2 pg/ml) and (12.5 pg/ml), respectively. Although a slight increase in the concentration of IL-37 was observed when the concentration of *T. cordifolia* plant powder increased to 144 mg/ml and 288 mg/ml, the increase was almost constant. This means the concentration of 72 mg/ml of plant powder is optimal to stimulate the secretion of IL-37. The study indicates that treatment of PBMCs with *T.*

cordifolia stem powder does stimulate the PBMCs into secreting IL-37, since there was no secretion of IL-37 in control (Figure 3).

SARS-CoV-2 activates IL-10 which in turn stimulates the production of pro-inflammatory cytokines such as IL-6 and TNF. This eventually results in the formation of a cytokine storm which has detrimental effects on the body, specially the lungs Al-suedeet al., 2021). *In vivo* investigations with rodents and transfected cells have shown that IL-37 has anti-inflammatory properties as it inhibited IL-1. It has been reported that IL-37 is a very effective anti-inflammatory protein and that inhibiting it could be a viable therapeutic technique. IL-37 is a natural suppressor of inflammation generated through a caspase-1 that cleaves pro-IL-37 into mature IL-37 that translocate to the nucleus and inhibits the pro-inflammatory gene transcription.

CONCLUSION AND RECOMMENDATION

T. cordifolia is known for its medicinal properties such as hepatoprotective, antioxidant, antipyretic, cytotoxic, antiulcer, anti-diabetic, and immunomodulatory. *T. cordifolia* stem powder was chosen for this study because it is known to have immunostimulant properties. Based on this research, the sterilization method that worked best for *T. cordifolia* was pasteurization at 75°C for 9 minutes and rapidly cooled at 4°C, for 3 consecutive cycles. After which it was kept under ultraviolet light for 30 minutes. With this method, there was minimal to no growth observed. However, further research is required with different parts of the plant and different types of plant products to optimize the results. *T. cordifolia* stem powder has the immunomodulatory properties to induce the PBMCs into secreting anti-inflammatory cytokines, IL-10 and IL-37. This could offer a new avenue for suppressing IL-6, TNF, and IL-1 associated with the cytokine storm in SARS-CoV-2 and the treatment of other viral and inflammatory diseases. It could be used as a non-steroidal anti-inflammatory agent for the treatment of inflammation. Therefore, it can be used as an adjuvant and an alternative to steroidal anti-inflammatory agents. This could be beneficial to the individuals that cannot tolerate the steroidal anti-inflammatory agents.

Author Contributions

ATW, TA, KM, and AG have designed the experiments. ATW, KM, TA, FSRA, and AG have conducted the experiments, collected and analyzed the data. AG, FSRA, ATW, TA, and KM have drafted the manuscript. All the authors have read and approved the final manuscript.

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Competing financial interests

The author(s) declare no competing financial interests.

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