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Immunomodulatory Effects of *Calotropis procera* on Human T-lymphocytes *In Vitro*

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ABSTRACT

Calotropis procera has been used in the traditional medicinal system for treating various diseases because of its phytochemical composition. In the present study, the methanolic leaf extract of *C. procera* was investigated for its immunomodulatory potential *in vitro* using T-lymphocytes. Phytohaemagglutinin (PHA) st https://researcherslinks.com/journals imulated T-lymphocytes were treated with extract of *C. procera* (1, 2, and 4 µg/mL) for 4, 8, 12, 24, and 48 h. Levels of pro-inflammatory cytokines (Interleukin-6 and Interleukin-8) were measured after 8, 12, and 24 h while levels of anti-inflammatory cytokines (Interleukin-10) were measured after 4 and 48-h via enzyme-linked immunosorbent assay (ELISA). *C. procera* treatment exhibited a significant decline in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines on PHA stimulated T-cells in time-dependent and dose-dependent manner. These findings are suggesting the immunomodulatory potential of methanolic leaf extract obtained from *C. procera* that together with its other therapeutic properties can be used towards modulating the immune response.

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Authors' Contribution
IA and SS performed all the experimental work and wrote the article. SB and SYK supervised the work, helped in reviewing data, analysis of results and provided editorial advice. BK helped in the statistical analysis of the data.

Key words
***Calotropis procera*, Immunomodulatory, T- lymphocytes, Cytokines**

INTRODUCTION

T cells participate in the cell-mediated immunity and generate the delayed-type hypersensitivity and show an inflammatory response by regulating the synthesis of antibodies as well as direct killer cell activity (Srikumar *et al*., 2005; Chaplin, 2010). T-cells are differentiated into two types of cells i.e. cytotoxic T cells and helper T cells that carry the specific surface molecules CD4+ and CD8+, respectively. The CD4+ cells differentiate into different types of effector cells i.e. Th1 are Th2 which released different types of cytokines. Th1 cells synthesize cytokines like Interleukin-2, Interferon gamma (IFNγ), and necrosis factor-beta (TNF-β) which participate in the cell-mediated immunity, pathogenesis, and produce immune responses that are dependent on phagocytosis through activation of T cells and macrophages. IL-4, IL-10, IL-5, and IL-13 are the cytokines secreted by Th2 cells involved in humoral immunity, the B cells stimulation, and therefore produces immune responses without phagocytosis (Romagnani, 1999; Stowe *et al*., 2010; Forsey *et al*., 2003; Alvarez *et al*., 2012).

The effectiveness of the immune system can be influenced by different exogenous and endogenous factors that lead to either immunosuppression or immunostimulation. Immunomodulation elevates the individual's defense system positively or negatively with drugs or compounds (Feng *et al*., 2019). Plants-derived compounds are more useful immune-modulators (Jakhar *et al*., 2014). One of the wild-growing shrub *Calotropis procera* has been used in the traditional medicinal system (Batool *et al*., 2020) because it contains cardiotonic agents such as calactin, calotropin, amyrin, calotoxin, amyrin esters, coroglaucigenin, uscharin, corotoxigenin, voruscharine, and calotropagenin applied in the treatment (Ahmed *et al*., 2005; Meena *et al*., 2010). This project was designed to evaluate the immunomodulatory role of *C. procera* by measuring the concentration of cytokines. The stimulation or activation of the cells is necessary for the secretion of cytokines because in most cases cytokines did not secrete constitutively so stimulation induces the cytokines gene transcription and production (Gulati *et al*., 2016). Cytokines are the considerable immunoregulators of the body which are proteins or the glycoproteins released

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by the cells of the immune system (Schroder *et al*., 2004). A lot of research has been done about the production of cytokines and it is generally believed that the most leading cells which produce cytokines are the T helper cells Th1 and Th2 (Liew, 2002). In present research concentrations of two pro-inflammatory (IL-6 and IL-8) and one anti-inflammatory (IL-10) cytokines were measured through ELISA at different time points.

MATERIALS AND METHODS

Ethical approval

The Advanced Studies and Research Board of University of Sargodha, 40100 Pakistan approved this study (Ref: UOS/Acad/402/1, Date: May 17, 2018).

Preparation of plant extract

Plant species was identified by Dr. Muhammad Amin Ullah Shah, Department of Botany, University of Sargodha. Type specimen were submitted to herbarium University of Sargodha with voucher number IA-6030. Fresh leaves of *Calotropis procera* were collected from local fields of Sargodha in June 2019. Fine powder of air-dried leaves was mixed with the solvent (methanol) in a ratio of 1:10 in a conical flask and the flasks were kept in an orbital shaker for 8 h at 200 rpm. The solvent was evaporated using a rotary evaporator at 75°C. The extract was stored in amber bottles at 4°C and the stock solution was prepared from dried extract with a concentration of 3 mg/mL in 10% dimethyl sulfoxide for further use.

Isolation and propagation of T lymphocytes

T-lymphocytes were isolated using Lymphopure (Biolegend, USA) having a density of 1.077±0.001 g/mL. Fresh human blood was collected from a healthy donor into EDTA coated tubes and diluted with an equal amount of phosphate buffer saline PBS with 1:1 dilution. For every 1mL of diluted blood 0.5 mL of lymphopure is added in a separate 15 mL tube. Diluted blood was layered over the lymphopure carefully to avoid mixing of two layers and centrifuged at 500 g for 45 min at 15°C. Blood components are migrated into several layers showing 4 layers: plasma layer on the top, second cloudy layer had lymphocytes with monocytes cells which were removed carefully to hoover up the cells and transferred gradually to a 15 mL centrifuge tubes. The separated layer was diluted with Park Memorial Institute 1640 Media (RPMI) and was centrifuged at 500g for 10 min. The remaining supernatant was dumped and PBS was used to wash the obtained cell pellet twice and re-suspended with media.

Cells suspension was shifted to the T-25 cell culture flask which has the 5 mL of RPMI 1640 media supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 µg/mL), and amphotericin (0.25 µg/mL). The PHA (1 µg/mL) was added as a mutagen for T-cells. Cells in the medium were incubated at 37°C for 24 h in a CO_2 incubator (5% carbon dioxide and 95% humidity). This incubation allows T-cells to be separated in suspension and monocytes were remained attached to the surface of the flask. T-cells pellet obtained after centrifugation for 10 min at 500 g at the bottom was suspended with RPMI-1960 media. The cells were counted using hemocytometer.

Immunomodulation of T-lymphocytes

For stimulation of T-lymphocytes, cells were seeded in 96 wells cell culture plate according to plan in the absence or presence of PHA. For dose dependent immunomodulation study of plant extract, 3 different concentrations were made from the stock solution of plant extract i.e 1, 2, and 4 µg/mL by diluting it with media RPMI 1640 (Table I). Cultured T cells were treated 3 times with each dose 1, 2, and 4µg/mL dilution alone on ELISA plate valves. After that, the cells were treated with these three doses and PHA. Some of cultured T cells were untreated while some were treated only with PHA to observe the difference of cytokines amount released from the treated T cells with different doses of extract.

Table I. Concentrations of plant leaf extract.

S. No.	Stock conc.	Working conc.	Dilution for 2x conc.	Stock used
CP1	3mg/mL	1µg/mL	1-1500	0.66 µL/mL
CP2	3mg/mL	2 µg/mL	1-750	1.32 µL/mL
CP3	3mg/mL	3 µg/mL	1-375	2.64 µL/mL

CP, Calotropis procera.

For dose-dependent immune-modulation, cultured T-cells cells were treated with 1, 2 and 4 µg/mL concentration of extract. After each time points, samples were collected for quantification of cytokines.

Quantification of cytokines

The T lymphocytes stimulated with PHA in presence of *C. procera* leaf extract and extract alone without PHA. Different concentrations of cytokines (pro-inflammatory and anti-inflammatory) i.e. interleukin-6, interleukin-8, and interleukin -10 were produced by T-cells. A human ELISA kit was used to quantify the concentrations of cytokines released after stimulation with PHA in the presence or absence of three different doses of leaf extract. No of cells/mL were calculated using the following formula described

by Hudson and Hay (1980) No of cells/mL= N/5 × 25 × 104 × 5(dilution), N= Number of cells counted in 5 groups of 16 small squares.

Statistical analyses

Statistical analysis of obtained data was done by One Way Analysis of Variance (ANOVA) followed by Tukey's post hoc analysis. GraphPad Prism version 8.00 was used to analyze the data.

RESULTS

Effect of Calotropis procera *on IL-6 and IL-8*

Stimulation of T-cells with PHA led to enhance the secretion of IL-6 IL-8 and IL-10 in time-dependent manner when compared with control. When PHA stimulated T-cells were treated with different doses of plant extract for 8 and 12 h, only 4µg/mL dose prevented the increase in pro-inflammatory cytokines (IL-6 and IL-8) production significantly while the other two doses i.e. 1 and 2 µg/mL did not produce any significant result. While for 24 h all doses significantly prevented the IL-6 and IL-8 production in PHA stimulate T-cells. The *C. procera* leaf extract without PHA stimulation did not produce a significant concentration of cytokines by T-cells (Figs. 1, 2).

Effects of Calotropis procera *on IL-10*

IL-10 produced from PHA stimulated T-cells when treated with extract for 4 and 8 h give different results as compared to IL-6 and IL-10. 1 µg/mL dose did not show any significant result for both time points while 2 µg/mL leaves extract exhibited a significantly increased IL-10 production. The treatment of stimulated cells with 4 µg/mL resulted in an intensified concentration of IL 10 significantly (Fig. 3).

DISCUSSION

T-cells are the major players of immune system mainly through the production of cytokines as reported earlier that *in vitro* stimulation of lymphocytes with appropriate mitogens like CoA, PHA activates the cells to produce appropriate cytokines to mount an immune response (Xu *et al*., 2008; Zhou *et al*., 2016; Wattrang *et al*., 2012; Yong *et al*., 2017; Ghafourian *et al*., 2014). Therefore, to evaluate the immunomodulatory effects of *C. procera* among PHA stimulated T lymphocytes three doses of leaf extract i.e. 1, 2, and 4 µg/mL were used. There are several reports wherein PHA has been tested for stimulation of T-lymphocytes to study immune activation (Ghafourian *et al*., 2014; Norian *et al*., 2015). PHA is a mitogen that can stimulate the proliferation of T-lymphocytes and its cells stimulation activity is being measured by the production of different cytokines (Lin *et al*., 2018). In the present study pro-inflammatory cytokines i.e. IL-6, IL-8, and anti-inflammatory cytokines IL-10 were quantified as the readout of immunomodulation in PHA stimulated T-cells in the presence or absence of leaf extract of *C. procera*.

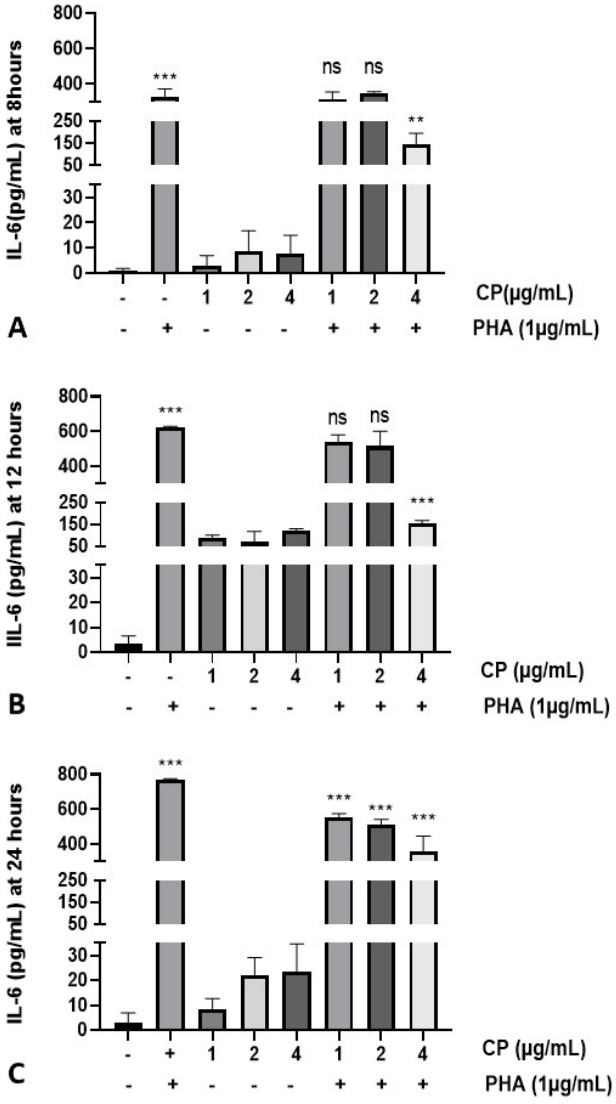

Fig. 1. Effect of *Calotropis procera* on IL-6 production. At 8 (A), 12 (B) and 24 h (C) PHA stimulation of T-cells produced the IL-6 significantly (P<0.001) up to 357.1±47.48 pg/mL, 625.7 ± 8.08 pg/mL, and 772.9 ± 7.07 pg/mL respectively. While different doses of *C. procera* extract prevent the production of IL-6 by PHA stimulated T-cells. Data represents Mean±SD and analyzed by ANOVA with post-Tukey's test (**P<0.01, ***P<0.001).

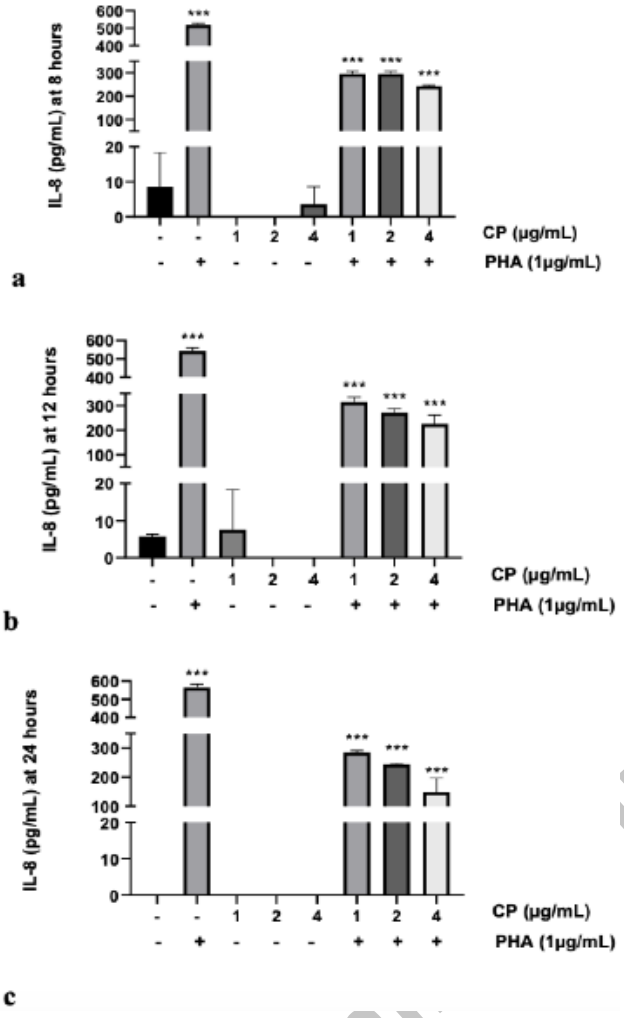

Fig. 2. Effects of *Calotrpis procera* on IL-8 production. Methanolic leaf extracts of *Calotrpis procera* prevent the IL-8 (pro-inflammatory cytokines) pro-duction in PHA-stimulated T-lymphocytes at 8 hours (a), 12 hours (b) and 24 hours (c). The cells were stimulated with or without PHA (1µg/mL) in the presence or absence of leaf extract (1,2 and 4µg/mL) for 8, 12, and 24 hours. Conditioned media was collected to quantify IL-8 using ELISA assay. Data represent mean ±SD and are analyzed by one way ANOVA followed by post Tukey's test (***p<0.001). CP, *Calotrpis procera*; PHA, phytohaemagglutinin; IL-8, interleukin 8; pg/mL, pictogram per milliliter; µg/mL, microgram per milliliter; ns, non-significant.

Stimulation of T-cells with PHA for 8, 12, and 24 h increased the production of IL-6, IL-8, and IL-10 in a time-dependent manner. However, the production of pro-inflammatory cytokine IL-6 decreased significantly in the PHA stimulate T-cells in the presence of 4 µg /mL leaf extract for four and eight h while at 24 h' time point, all doses decreased the production of IL-6 significantly in a dose-dependent manner (Fig. 1). In the case of IL-8 all doses prevented the production of cytokines for all time points studied, in dose-dependent manner (Fig. 2). However, the production of anti-inflammatory cytokines (IL-10) significantly intensified among PHA stimulated T lymphocytes treated with leaf extract in the dose and time-dependent manner (Fig. 3).

As it was observed that during normal immune response Th0 cells activated when they were exposed to mitogen or antigen and can differentiate into Th1 or Th2 effectors (Franciotta *et al*., 2003). It is possible that this plant may have switched the immune system towards Th2 cells signaling.

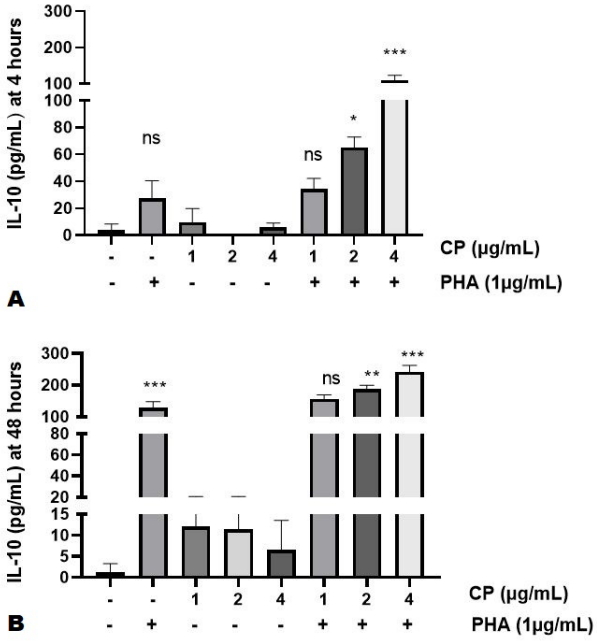

Fig. 3. Effects of *Calotropis procera* on IL-10 production. T-cells stimulated with PHA (1 µg/mL) for 4 h did not a show significant increase in the concentration of IL-10 i.e. 36.55±12.16 pg/mL whereas stimulation for 48 h resulted in a considerable increase (P<0.01) up to 142.9±20.13 pg/mL in time-dependent manner as shown in (B). Methanolic leaf extracts of *C. procera* stimulates the production of anti-inflammatory cytokines (IL-10) in dose-dependent manner. Data represent Mean±SD and analyzed by ANOVA followed by post-Tukey's test (*P<0.05, **P<0.01, ***P<0.001). CP, *Calotropis procera*; PHA, phytohaemagglutinin; IL-10, interleukin 10; pg/mL, pictogram per milliliter; µg/mL, microgram per milliliter; ns, non-significant.

Th1 cells characteristically produce pro-inflammatory cytokines while Th2 cells produce anti-inflammatory cytokines i.e. IL-4 and IL-10 (Franciotta *et al*., 2003).

In the present study, there is also a possibility of Th0 cells differentiation to Th1 only when stimulated with PHA mitogen which, therefore, produced the pro-inflammatory cytokines (IL-6 and IL-8). However, when PHA stimulated T-cells were treated with plant extract they were differentiated in Th2 pathways and, therefore, the production of anti-inflammatory cytokines (IL-10) increased significantly and pro-inflammatory cytokines decreased as the Th1 pathway was inhibited to produce the pro-inflammatory cytokines.

There are several reports that *C. procera* contains the flavonoids and these flavonoids are reported to work as suppressors of pro-inflammatory cytokines production, therefore having an immunomodulatory potential (Yehuda *et al*., 2009; Leyva *et al*., 2016; Michael *et al*., 2011). Hence, there is the likelihood of differentiation of Th0 into Th2 cells leading to a decline in IL-6 and IL-8 concentration. In present research, 4 µg/ml of leaf extract has effectively decreased the production of pro-inflammatory cytokines reflecting that decreased cell proliferation of T cell and synergistic inhibition of cytokines production shows the immunosuppressive potential of this extract (Freeman *et al*., 2014) signifying the immunomodulatory role of *C. procera.*

Over activation of the Th1 pathway could result in various autoimmune diseases such as diabetes, multiple sclerosis, rheumatoid arthritis, and inflammatory bowel diseases (Mkaddem *et al*., 2009; Yoshioka *et al*., 2017; Zoghi *et al*., 2011). The results of this work showed that plant extract inhibits the Th1 production of cytokines and increasing Th2 response leads to a decrease in the risk of these diseases with this *C. procera* leaf extract doses. IL-10 released under the extract influence proved as cytokine synthesis inhibitory factor. Th2 response also plays an important role in the protection of the host against pathogenic agents such as bacteria, helminths, and some other parasites (Taylor *et al*., 2012). The results of this study showed that increased production of IL-10 along with shifting of the immune response towards Th2 stimulatory effect, suggest the protective potential of IL-10 and Th2 in presence of this extract. Additionally, this study may encourage the ordinary use of *C. procera* for overwhelming disorders.

CONCLUSION

The results of this study suggest that *C. procera* leaf extract can modulate the PHA stimulated T lymphocytes and on one hand exhibit immune-inhibitory effects by preventing the production of IL-6 and IL-8 whereas showing immuno-stimulatory effects by increasing the production of IL-10 suggesting the shift of immune response from Th1 towards Th2 pathway. Therefore, research about recognizing the phytochemicals responsible for immunomodulation and affirming the shifting of a signaling pathway from Th1 to Th2 may be helpful to further clarify the potential usefulness of this plant as a potent immunomodulator.

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Statement of conflict interest

The authors have declared no conflict of interest.

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