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Full Length Research Paper

Abundant CD4 Th-2 cytokine stimulation by medicinal plant *Pongamia pinnata Linn.* on human peripheral blood mononuclear cell (PBMC)

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Immune cells produce cytokines in response to various stimuli. The initial cytokine milieu at which immune cells interact have a positive impact on the outcome of the immune response generated. Modulations or polarizations of cytokines are known to affect the outcome of the disease. Immunotherapy is a novel approach to combat cytokine mediated diseases and several medicinal plants are shown to possess immune modulatory properties. One of the popular Indian medicinal plants known as *Pongamia pinnata* has been extensively studied for its bioactive properties. However, its immune modulatory property has not been studied yet. Therefore we broached on this study of the immune stimulation property of *P. pinnata* on healthy human peripheral blood mononuclear cell (PBMC). *P. pinnata* extracts were prepared using various solvents and they were used to stimulate human PBMC. We noticed that *P. pinnata* extracts induced abundant IL-10 production. Our experimental results indicated that *P. pinnata* may be a strong inducer of Th-2 cytokines and could be used to treat Th-1 cytokine mediated pathology.

Key words: Immunotherapy, cytokines, Th-1, Th-2, *Pongamia pinnata*, peripheral blood mononuclear cell (PBMC), ELISA.

INTRODUCTION

Traditional medicine systems have been practiced in various parts of the world, of which Indian traditional medicine systems namely Ayurvedha,-Siddha and Unani describe the medicinal values of several Indian medicinal plants (Savita et al., 2010). Medicinal values of *P. pinnata* have long been known especially for its use in the treatment of cutaneous disease. *P. pinnata* popularly known as 'Karanj' or Karanja in Hindi and Pungai in tamil is a medium sized glabrous tree, found throughout India and further distributed eastwards, mainly in the littoral regions of South Eastern Asia and Australia.

In the Ayurvedic literature of India, different parts of this plant have been recommended as a remedy for various human ailments such as bronchitis, whooping cough, rheumatic joints and are used to quench dipsia in diabetes (Kirtikar and Basu, 1995). The seed and seed oil have been used to treat various inflammatory and infectious diseases such as leucoderma, leprosy, lumbago, and muscular and articular rheumatism (Ballal, 2005). The leaves are used as digestive, laxative, anthelmintic and to cure piles, wounds and other inflammations (Kirtikar and Basu, 1995). A hot infusion of leaves is used in a medicated bath to relieve rheumatic pains and clean ulcers in gonorrhea (Krishnamurthi, 1969; Satyavathi et al., 1987) and scrofulous enlargement (Chopra et al., 1933; Bhattacharjee, 2001). Its different extracts of roots and seeds (ethanol, petroleum ether, benzene extracts and others) have been reported to have an anti-inflammatory activity (Singh and pandey 1996; Srinivasan et al., 2001). Various parts of the plant have been investigated for its anti HSV (Elanchezhiyan et al., 1993), antiparasitic (Baswa et al., 2001; Uddin et al., 2003), and anti-inflammatory (Srinivasan et al., 2001), antinociceptive and antipyretic (Srinivasan et al., 1993)

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properties.

In our previous report we have shown that *P. pinnata* seed extract showed anti HSV properties on vero cells (Elanchezhiyan et al., 1992). In addition, phytochemical examinations of this plant have indicated the presence of furanoflavones, furanoflavonols (Talapatra et al., 1980; Talaptra et al., 1982), chromenoflavones, flavones (Sharma et al., 1973; Ahmad et al., 2004), furanodiketones and flavonoid glucosides (Essa et al., 2005; Chopade et al., 2008). Although various phyto-chemical constituents and diverse medicinal activities have been reported, no immune stimulation studies have been carried out to shed light on the role of *P. pinnata* in immune stimulation.

Immunotherapy is now being recognized as an alternative to conventional chemotherapy for a variety of disease conditions, in which the immune response is aberrant (Richard and Bruce, 2005; Marc, 2008). Immune stimulators have been known to support T-cell function, activate macrophages and granulocytes, and complement natural killer cells apart from affecting the production of various effector molecules such as cytokines generated by activated cells (Vilcek and Feldmann, 2004). These non-specific effects is known to offer protection against different pathogens, including bacteria, fungi, viruses, etc. and constitute an alternative method to the conventional chemotherapy (Ian and kent, 2001).

Immunotherapy involves alteration of an immune response that is detrimental to the host. In general the default pathways of immune response efficiently handle and eliminate countless numbers of pathogens and pathogenic substances that they encounter every second (Francis, 2002). However, microorganisms and tumors skew the immune response towards their benefit (Neema et al., 2000). In this context, immunotherapy serves as a powerful tool to restore the protective immune response. CD4+ T helper cells upon antigenic encounter either polarize towards Th-1 phenotype which produces IFN-γ and IL-2 and promotes cell mediated immunity, or Th-2 type which produces IL-4 and IL-10 and promotes humoral or antibody mediated immunity (Stephanie and Kim, 1997). During diseases like HIV/AIDS, the default Th-1 phenotypes are beneficial to the host but the virus induces an immune deviation towards Th-2 phenotype, which is not protective (Clerici and Shearer, 1993; Ramesh, 2005). In these situations, a methodology to restore the Th-1 response would be beneficial to the host. In this manuscript *P. pinnata* seed extracts were used to stimulate the human PBMCs and tested for various Th1 and Th2 cytokines and the results are discussed in the context of medical benefits of *P. pinnata.*

MATERIALS AND METHODS

Plant collection and extraction

P. pinnata seeds were collected at Chennai, Tamil Nadu, India, and authenticated. Shade dried *P. pinnata* plant seeds were used for the extraction. Aqueous extracts were prepared by soaking the seed powder in water and evaporated. Dry powder was collected and used for the PBMC stimulation experiments. Methanolic extract was prepared as described by Archana and Namashivayam. The filtrate was dried and final product was weighed and stored at -85ºC until use.

Isolation of Human PBMCs

Blood obtained from healthy donors (Blood Bank, Voluntary Health Services, Chennai) was used in this study. Separation of blood cells was performed using Histopaque (Sigma Aldrich Chemicals, USA) and PBMCs were isolated as per the manufacture's procedure. After isolation, PBMCs were washed three times in Hank's Balanced Salt Solution (HBSS, Himedia, India) and resuspended in RPMI 1640 supplemented with 10% FBS (Pan Biotech, Germany), 1% of 200 mM L-glutamine (Himedia, India) and antibiotics (Himedia, India).

Stimulation of PBMCs

Isolated PBMCs were plated at a concentration of 2 $x10^6$ cells /ml/well in 24 well tissue culture plates. Different concentrations of *P. pinnata* extracts were used for stimulation (test group) and concanavalin A (Con A) treated cultures served as a positive control while cells treated with distilled water as a negative control. Cultures supernatants were collected at 24, 48 and 72 h post stimulation and screened for Th-1 cytokines (IFN-γ and IL-2) and Th-2 cytokines (IL-4 and IL-10) by ELISA (BD-Pharmingen, USA). ELISA was performed as per the directions of the manufacturer and concentrations of the cytokines were derived from standard curve.

RESULTS

In order to study the cytokine stimulation property of *P. pinnata*, two different concentrations namely 50 and 100 µg/ml were evaluated. A non-specific stimulant, Con A was used as a positive control and cultures treated with sterile distilled water served as negative controls. Cultures were incubated at 37ºC with different drug preparation and culture supernatants were collected at different time points and tested for Th1 cytokines and Th2 cytokines. Aqueous *P. pinnata* seed extracts induced an overwhelming IL-10 production. As shown in Figure 1, there was a positive correlation with the IL-10 cytokine production with different concentrations of the extract i.e. 100 µg/ml concentration of the extract induced more IL-10 than 50 µg/ml. In addition IL-10 production was found to be more during the early hours (24 h post stimulation (PS)) and the levels diminished later. This data clearly showed that *P. pinnata* seed extract stimulated a potent Th-2 cytokine, IL-10.

Next set of experiments were done on IFNγ stimulation by *P. pinnata* and illustrated in the Figure 2. *P. pinnata* extract did not stimulate IFN-γ production at any of the concentration tested. There was a mild peak of IFN-γ with 100 mg/ml of methanolic extract. Positive controls showed abundant IFN-γ and negative controls failed to show any such activity. Cultures were also estimated for

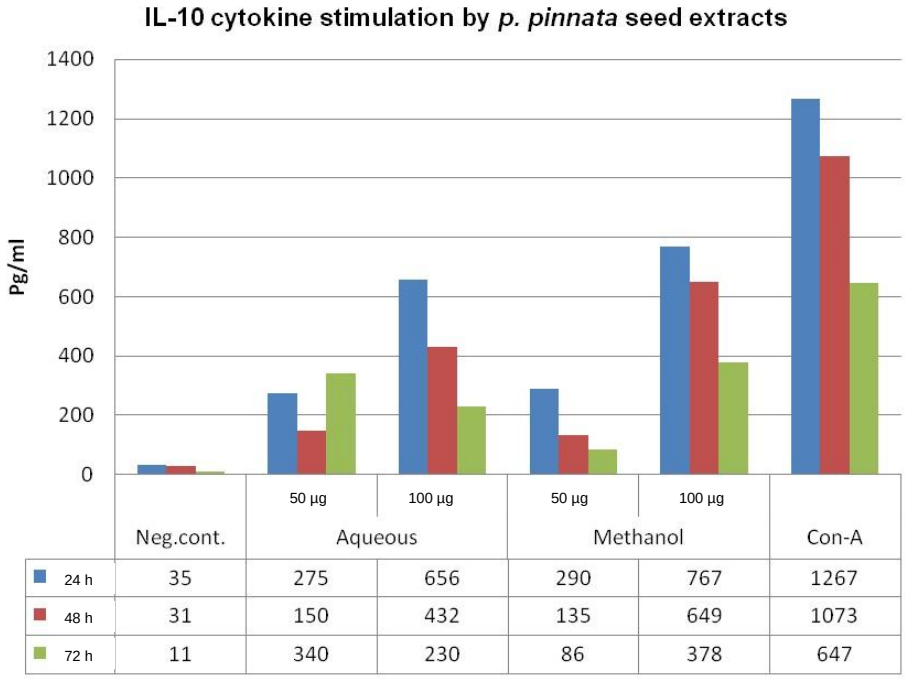

Figure 1. Human IL-10 production by *P. pinnata* extracts. 2 x10^6 PBMC/ml/well was added into 24 well tissue culture plates and the supernatants collected at different time points were tested for cytokine production by ELISA. Neg.cont. =Distilled water treated cultures (negative controls) and Con-A=Concanavalin-A treated cultures (positive controls). Cytokine concentrations were represented in pg/ml.

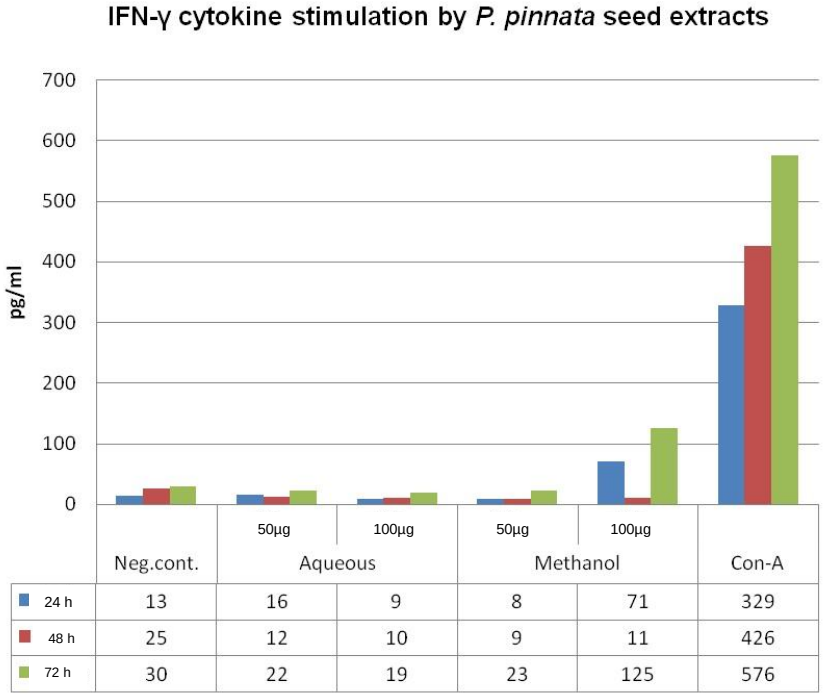

Figure 2. Human IFN *γ* production by *P. pinnata* extracts. 2 x10^6 PBMC/ml/well was added into 24 well tissue culture plates and the supernatants collected at different time points were tested for cytokine production by ELISA. Neg.cont.=Distilled water treated cultures (negative controls) and Con-A=Concanavalin-A treated cultures (positive controls). Cytokine concentrations were represented in pg/ml.

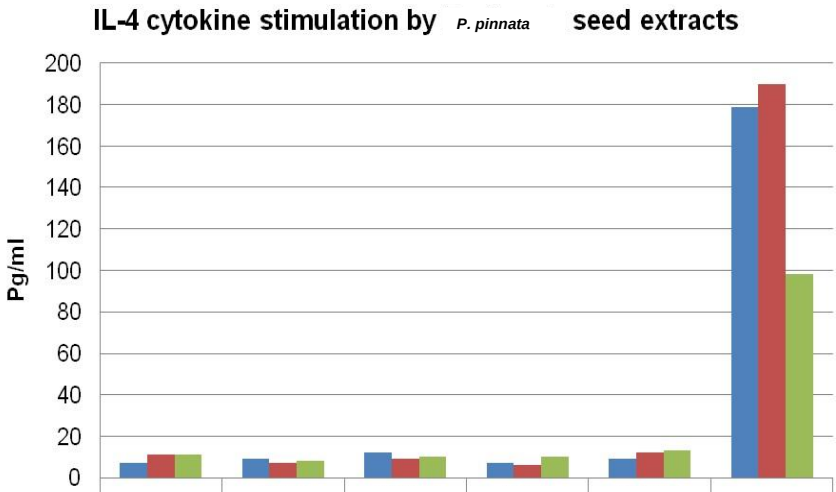

Figure 3. Human IL-4 production by *P. pinnata* extracts. 2 x10^6 PBMC/ml/well was added into 24 well tissue culture plates and the supernatants collected at different time points were tested for cytokine production by ELISA. Neg.cont.=Distilled water treated cultures (negative controls) and Con-A=Concanavalin-A treated cultures (positive controls). Cytokine concentrations were represented in pg/ml.

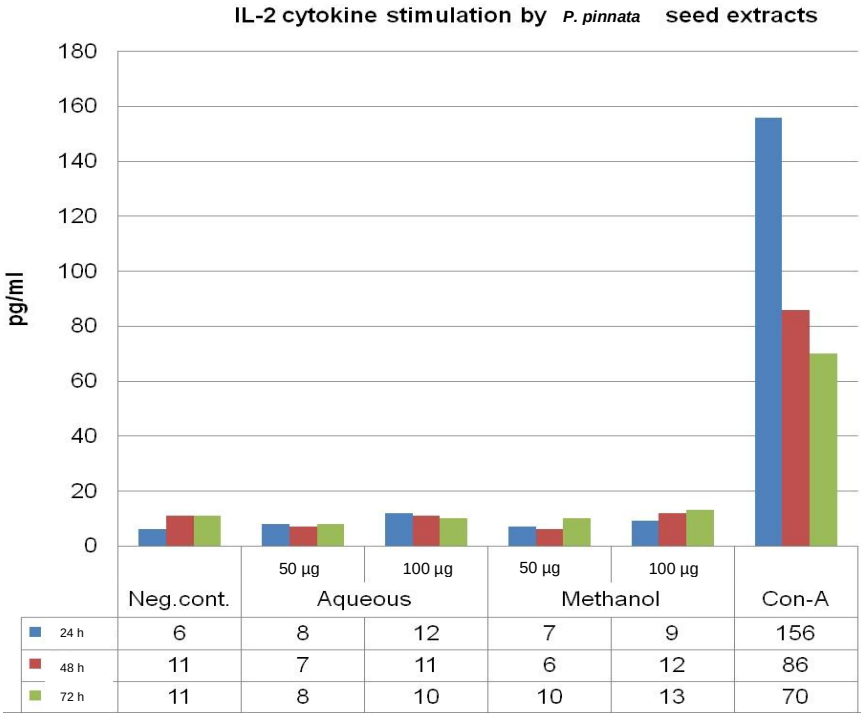

Figure 4. Human IL-2 production by *P. pinnata* extracts. 2 x10^6 PBMC/ml/well was added into 24 well tissue culture plates and the supernatants collected at different time points were tested for cytokine production by ELISA. Neg.cont.=Distilled water treated cultures (negative controls) and Con-A=Concanavalin-A treated cultures (positive controls). Cytokine concentrations were represented in pg/ml.

the other T cell cytokines namely IL-4 and IL-2. As noticed in the Figures 3 and 4, both cytokines were found to be at the basal level indicating that *P. pinnata* was selectively stimulating only IL-10.

DISCUSSION

Previously we reported that 100 mg/ml (w/v) of *P. pinnata* seed aqueous extracts did not show any toxicity in both *in vitro* and *in vivo* models tested (Elanchezhiyan et al., 1992). We also reported that aqueous extract of *P. pinnata* seeds inhibited HSV viral replication on the vero cells (Elanchezhiyan et al., 1993). Currently *P. pinnata* seeds were used to test cytokine stimulation property on human PBMCs. Results clearly indicated that *P. pinnata* seed extracts profoundly stimulated the IL-10 production, indicating that *P. pinnata* extracts are promoters of Th-2 response. Though there was a detectable level of other cytokines tested, the levels were minimal and thus appear to be not involved in bioactivities.

A careful overview of literature indicated that the observation of IL-10 stimulation by *P. pinnata* extract was first of a kind and there were no similar studies available to compare and discuss directly. Hence we compared similar activity produced by other medicinal plants. In a study conducted by Mare et al. (2005), *Boswellia carterii* extracts were used to stimulate spleen cells and they found that the plant extracts also produced large amount of IL-10.

In our study, levels of Th1 cytokines after stimulation were very low and that could be due to anti-Th1 property of the higher amount of the IL-10 that was produced in the culture. Though there was a significant increase in IL-10 level, the level of IL-4 (another Th-2 cytokine) was too low. This could be due to the reason that 72 h could be too short to measure IL-4. This warrants additional experiments to narrow down to optimum time points to measure IL-4. In our study, we have tested two different concentrations of the extract and these concentrations may not be optimal enough to induce the other cytokines. Experiments are under way to study the dose response pattern of *P. pinnata* on the cytokine stimulation.

Our study clearly indicated that *P. pinnata* seed extract strongly induced IL-10 production which is a strong Th-2 cytokine. CD4 cells (T helper cells) can be classified into 4 major groups and they are Th-1, Th-2, Th-17 and T regs (Linda et al., 2011). During autoimmune disorders Th-1 cells and Th-17 cells are involved in the pathological role while T regs are involved in the suppression of autoreactive T cells. IL-10 is normally produced by Th-2 cells and T regs (Charles and Arno, 2010) and it has strong activity to inhibit Th-1 cytokines. During autoimmune disorders Th-1 cells cause the pathology (Hans and Richard, 2004). Hooks et al., 1979 described that IFN-γ in the sera of patients with systemic lupus erythematosus (SLE) and showed a good correlation between IFN-γ titers and disease activity. Moreover, IFN-γ titers correlated posi-tively with antibodies to DNA and negatively with serum levels of third components of complement. Current therapy for SLE includes treatment with immune suppressants (e.g., prednisolone, cyclophosphamide, and tacrolimus) or NSAIDs (non-steroidal anti-inflam-matory drugs). However these therapies are known to cause adverse side effects such as infections, infertility, amenorrhea, and metabolic abnormalities (Hayashi, 2010). In a condition like this cytokine therapy to suppress IFN-γ without eliciting side effects would be beneficial. In this context our finding is of paramount importance. Patients could be administered *P. pinnata* drug which could induce abundant IL-10 and alleviate the disease. Almost all autoimmune disorders are caused by autoreactive Th-1 CD4 cells and *P. pinnata* therapy could be beneficial to such patients. In addition *P. pinnata* therapy also would stimulate antibody-mediated immunity.

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