Int J Pharm Bio Sci 2015 July.; 6(3): (B) 929 - 936

Research Article **Immunology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

CYTOKINE AND LEUKOTRINE (LTB_4) STIMULATION PROPERTIES OF *PONGAMIA PINNATA* BARK AQUEOUS EXTRACTS

MANIKANNAN MATHAIYAN[1] AND DINESH SUBRAMANIYAM*[2]

[1]Centre for Drug Discovery and Development, Sathyabama University, Chennai
[2]Dr. ALM PG Institute of Basic Medical Sciences, University of Madras, Chennai

ABSTRACT

Immunotherapy is a novel approach to combat cytokine mediated diseases. Immunomodulatory drugs are known to play a vital role in altering the cytokine profile and the outcome of disese. Several medicinal plants were shown to possess immune modulatory properties. One of the popular Indian medicinal plant known as *Pongamia pinnata* (*P. pinnata*) has been extensively studied for its bioactive properties. We have reported in our previous study for *P. pinnata's* immune modulatory properties however this study extended to investigate further its immunomodulatory property of bark extracts. In this study it was found that bark extracts of *P. pinnata* profoundly increased the human IFN- γ and IL-10 which also support our previous findings with seeds extracts. These findings strongly suggest that *P. pinnata's* immunomodulatory role on human immune cells.

KEYWORDS: PBMCs, Leukotriene B_4, Cytokines, *Pongamia pinnata*, Immunotherapy

*Corresponding author

DINESH SUBRAMANIYAM
Dr. ALM PG Institute of Basic Medical Sciences,
University of Madras. *Chennai*

INTRODUCTION

Medicinal plants and their active components have been shown to be an important source of antimicrobial drugs and immune modulators[1]. Thus the development of drugs with immunomodulation activity from natural resources has become an attractive area of research. *Pongamia pinnata* (L.) Pierre (Leguminosae, papilionaceae;synonym *Pongamia glabra* Vent.), popularly known as 'Karanj' or Karanja in Hindi, is a medium sized glabrous tree, found throughout India, which has a long traditional use in Ayurvedic, Siddha and Unani systems of medicine treating various human diseases and ailments[2]. Various parts of this plant are used in Ayurvedic formulation to treat bronchitis, whooping cough, and to treat skin diseases[3]. The leaves are used as digestive, laxative, anthelmintic and to cure piles, wounds and other inflammations[4]. Hot infusions of leaves are used as a medicated bath for relieving rheumatic pains and for cleaning ulcers in gonorrhea and scrofulous enlargement[5]. Different extracts of roots and seeds (ethanol, petroleum, ether, benzene extracts and others) of *P. pinnata* have been reported to have an anti-inflammatory activity[6,7] and immunomodulatory[8,9] properties. Immunotherapy involves around 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. However, microorganisms and tumors skew the immune response towards their benefit[9,10]. In this context, immunotherapy serves as a powerful tool to restore the protective immune response. Cytokines and Leukotrienes are important molecules in innate immunity. CD4+ T helper cells upon antigenic encounter either polarize towards Th-1 phenotype (that produces IFN γ and IL-2) which promotes cell mediated immunity or Th-2 type (which produces IL-4 and IL-10) which promotes humoral or antibody mediated immunity. 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[11]. In situations like this immunomodulatory compounds play a vital role in restoring the Th-1 response and this approach would be beneficial to the host. Leukotriene B_4 (LTB_4; 5[S], 12[R]-dihydroxy-6,14-cis-8,10-transeicosatetraenoic acid) is a lipid mediator, enzymatically metabolized from arachidonic acid and it activates the macrophages, granulocytes and the immune effector cells[12,13]. Therefore, a key role has been proposed for LTB_4 in the pathogenesis of a variety of inflammatory diseases. LTB_4 also induces a migration of both CD4+ and CD8 + T cells to tissues in the early phase of the immune response. It drives IL-4 and IL-5 production and promotes CD4+ cell proliferation while inhibiting CD8+ cell expansion[14]. In general, LTB_4 has been found to be pro-inflammatory in healthy people and has been found in higher concentration among allergic individuals. It is in this context, an attempt was made to evaluate the cytokine and leukotriene stimulation property of *P. pinnata* bark aqueous extracts on human PBMCs and RAW 264.7 cells respectively. In addition, we have previously reported the *invitro* cytokine stimulation property of seed extracts on human PBMCS. And this study is conducted to evaluate the *P. pinnata*'s bark extracts immunomodulation potential.

MATERIALS AND METHODS

(i) Plant collection and extraction

P. pinnata (L.) barks were collected at Chennai, Tamil Nadu, India and was authenticated. Shade dried *P. pinnata* plant barks were used for the extraction. Aqueous extract was prepared as per standard procedure[15]. Briefly, 100g of shade dried *P. pinnata* bark powder was added to 500ml of sterile double distilled water and the suspension was kept in an airtight container at room temperature for 7 days and shaken 5–6 times daily. For seven days, the supernatant was collected at regular interval and filtered. The filtrate was lyophilized and stored at -85^{0}C until use.

(ii) Human PBMCs isolation and stimulation

Healthy donor's Buffy coat was procured from VHS Adyar, Chennai. Separation of blood cells was performed using Histopaque, (Sigma Aldrich Chemicals, India) 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 200mM L-glutamine (Himedia, India) and antibiotics penicillin with streptomycin (Himedia,India). 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 Phytoheamagglutinin (PHA) treated cultures served as positive control while cells treated with distilled water as negative control. Cultures supernatants were collected at 6, 12, 18 and 24 hours of post stimulation and screened for Th-1 cytokines namely IFN-γ and IL-2 and Th-2 cytokines (IL-4 andIL-10) by ELISA (BD-Pharmingen, USA). ELISA was performed as per the directions of the manufacturer and concentrations of the cytokines were calculated by linear regression analysis.

(iii) Leukotriene B_4 (LTB_4) stimulation

RAW 264.7 cells, a mouse macrophage cell line, was obtained from the National Centre for Cell Sciences (NCCS), Pune, India, and were cultured in Dulbecco's Minimum Essential Medium (DMEM, Sigma, USA Cat. No. D6046) supplemented with 10% FBS and antibiotics (penicillin 100 IU/ml and streptomycin 100 µg/ml) in a 5% CO2 incubator at 37°C. Cultures for leukotriene B_4 measurement were set up as previously described.[16] Briefly, cells were plated in a 24-well flat bottom tissue culture plate at a concentration of 2 x 10^6cells/well. These cells were treated with different concentrations (100 µg/ml to 500 µg/ml) of extracts. Lipopolysaccharide (LPS)-(Sigma-Aldrich, USA) treated culture served as positive control and cells with medium were considered as negative control. Culture supernatants were collected at 6 hours of post stimulation. Collected supernatants were frozen at -80°C until testing. LTB_4 production in culture was determined by competitive ELISA using a commercial kit (Enzo Life Sciences, Cat. No. ADI - 900 - 068). The ELISA was performed according to the manufacturer's instructions. Briefly, 100 µl of samples or standards were added to the plate coated with anti-LTB_4 antibody and incubated at room temperature for 2 hours. Following this, the plate was washed 3 times and 200 µl pNPP substrate was added to the wells and incubated for 2 hours at room temperature. Finally, 50 µl of stop solution was added and the enzyme reaction was stopped. The yellow color produced was measured using an ELISA reader (BioTek, USA) at 405nm. The intensity of the bound yellow color is proportional to the concentration of LTB_4 in the sample. Leukotriene levels in the unknown samples were calculated using Logit-Log paper plot. All assays were repeated 3 times and the results represent the average and standard deviation (SD) of 3 experiments.

Toxicity assays on P. pinnata bark extracts

Extracts or Triton-X (Positive control for toxicity) or negative control were mixed with human PBMC and they were incubated at 37^{o}C for 24 hours. Then the cells were tested for their viability as a direct measure for extract induced cytotoxicity. This was done by MTT (dimethyl trizoldiphenyltetrasolium bromide) (Sigma-Aldrich, Cat. No. M2003) assay as described by Mosmann T, 1983[17]. For this PBMCs and RAW 264.7 cells were plated at a concentration of 2 x10^6cells/ml in a 24 well culture plate. At the end of 24 hours the cells were harvested and treated with 250 µl of MTT solution/well and incubated at 37ºC for 2 hours. Then 200 µl of DMSO was added and further incubated at 37ºC for 2 hours. The plates were read at 540 nm and the percentage of viability was calculated using the following formula as described elsewhere[18]. Cellular viability was also tested by trypan blue dye exclusion technique as described elsewhere[19].

RESULTS

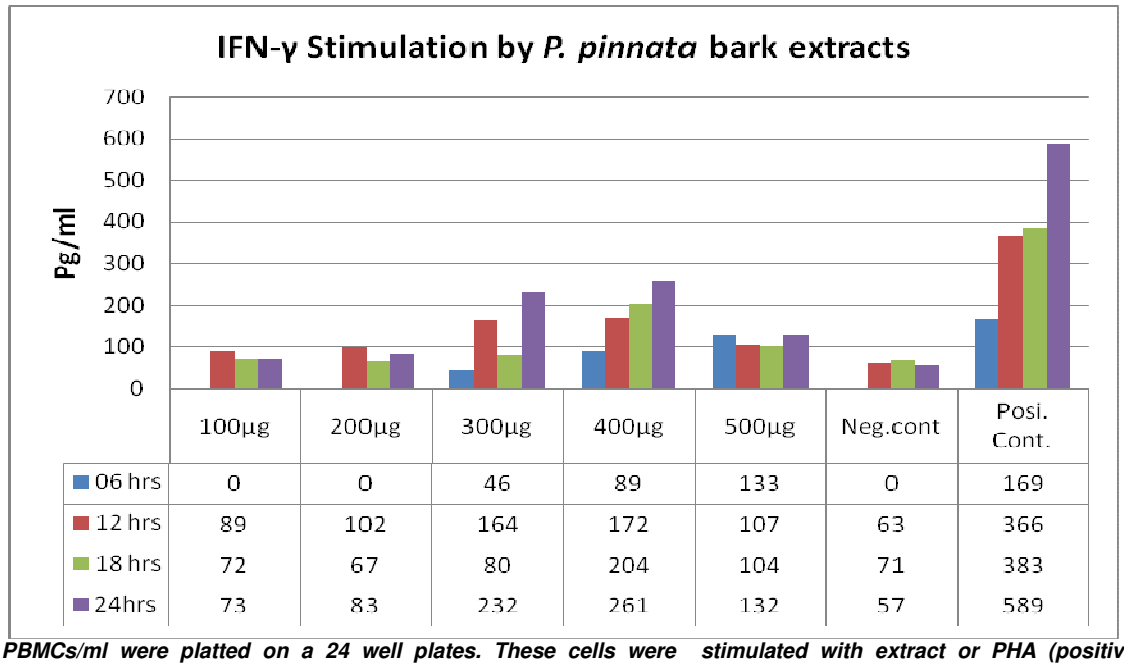
Cultures were incubated at 37^{o}C and culture supernatants were collected and tested for Th1 cytokines namely, IFN-γ, IL-2 and Th2 cytokines namely IL-4 and IL-10. Cytokines were measured after stimulation of 6 hours to 24 hours. IFN-γ production were minimal or at basal level after 6 hrs, but the notable amount IFN-γ secreted at 12 hrs, 18hrs and 24hrs of time points. Of the five concentrations were tested, 100µg/ml extract did not show any significant increase in IFN-γ production. However 400µg/ml extract showed about two fold increased cytokine production in comparison to the 6 hrs sample (Graph-I).

Even though there was a minimal detectable level of IL-4 observed at various concentration (data not shown) there is no significance with time similarly IL-2 levels were minimal or below the detection limit (Graph-II). Interestingly as shown in Graph-III there was a striking increase in IL-10 production was noticed at 400µg/ml of the extract and the levels increased up to 18 hrs of time points. In order to find *P. pinnata* bark extract's efficiency of modulating innate immune systems, the LTB_4 stimulation assay was performed. For the stimulation RAW 264.7 cells were treated with bark aqueous extracts. Cells treated with sterile distilled water served as negative control, cells treated with extracts are test group and lipopolysaccharide served as Positive control and cells were incubated for 6 hours to 24 hours at 37ºC. Culture supernatants were collected and evaluated for LTB_4 concentration and the concentrations are illustrated in Graph-IV. LTB_4 level of unstimulated group shows 47 ± 3.5 Pg/ml. Upon treatment with *P. pinnata* the LTB_4 levels were increased to 126 ±5.1 Pg/ml at 400 µg/ml. When the unstimulated group was compared with the extract treated group 78 Pg/ml increase was noticed and this increase was found to be statistically significant ($P < 0.001$). As shown in the Graph IV, LPS treated group showed a concentration of 276 ± 17.3 pg/ml. Based on this it is concluded that *P. pinnata* seed extracts had stimulated those cells produced abundant LTB_4. Thus the data showed that *P. pinnata* bark extract (400µg/ml) was a potent stimulator of innate immune molecule such as LTB_4. In the above experiments it was seen that *P. pinnata* bark extracts were found to exhibit several immune modulatory activities. These extracts upto 500 µg/ml were treated with human PBMC and the toxicity was evaluated by MTT assay as described elsewhere[19]. The toxicity is represented as percentage of viable cells upon extract treatment or positive control (Triton-X) or negative control (Distilled water). As shown in the Graph-V the *P. pinnata* bark aqueous extract failed to show any toxicity as revealed by 96.1±1.4 percent viability on 24 hours post treatment. Thus from this study it could be concluded that *P. pinnata* bark extracts at 400 µg/ml concentration was non-toxic. Almost similar levels of viable cells were found by trypan blue dye exclusion technique.

DISCUSSION

P. pinnata bark extracts showed immune modulation properties on human PBMCs and RAW 264.7 cells respectively. The current work is an extension of the previous work and currently *P. pinnata* barks were used to test cytokine stimulation property on human PBMC and Leukotriene (LTB_4) potential on RAW 264.7 cells and their results are presented. Results clearly indicated that *P. pinnata* bark extracts profoundly increased the IFN-γ and IL-10 production. It indicates that *P. pinnata* extracts are promoters of Th1 and Th2 response. Though there was a detectable level of other cytokines tested, the levels were minimal. We have compared these findings with our previous study results[8,9] and other studies conducted similarly for immune modulation properties[20]. In a study conducted by Mare et al., *Boswellia carterii* extracts used to stimulate spleen cells and both Th1 and Th2 cytokine levels were measured and they noticed that *Boswellia carterii* extracts induced a strong Th2 response and inhibited Th1 cells[21]. In our study we noticed bark extracts stimulated both Th1 and Th2 cytokines. In our study levels of IL-2 and IL-4 cytokine levels after stimulation were very low and IL-10 produced in higher amount than other cytokines tested. Though there was a significant increase of Th2 cytokine (IL-10) the level of IL-4 was too low. This could be due to the time line at which we were tested the sample collection up to 24 hours may not be optimal to detect higher IL-4 levels. In our study, we have tested different concentrations of the extract and these concentrations were chosen 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* bark extracts on the cytokine stimulation.

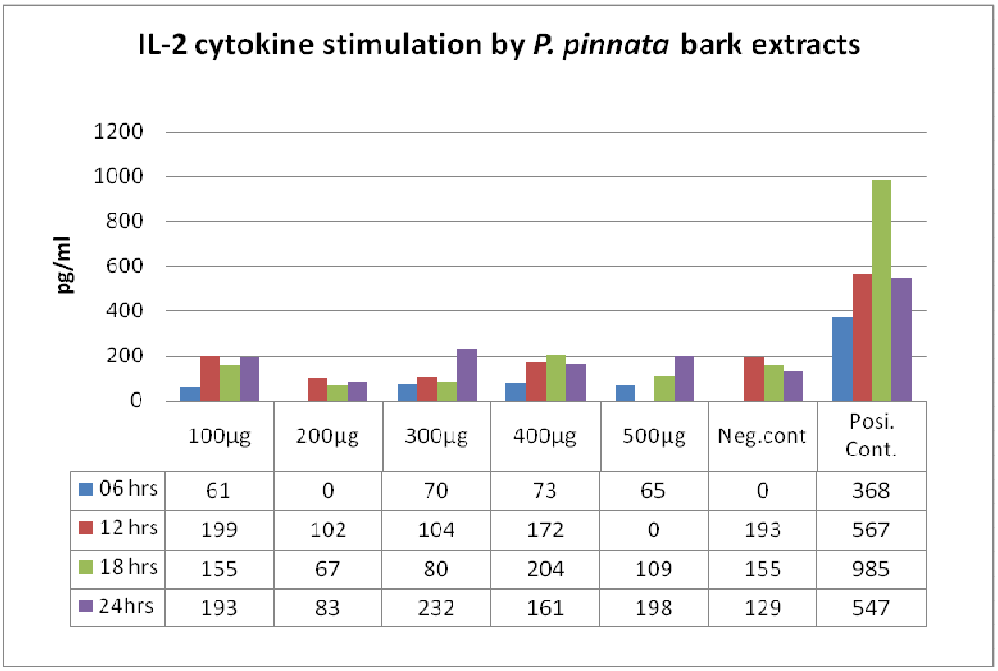
Graph I
IFN-γ cytokine stimulation by P. pinnata bark extracts



	100µg	200µg	300µg	400µg	500µg	Neg.cont	Posi. Cont.
06 hrs	0	0	46	89	133	0	169
12 hrs	89	102	164	172	107	63	366
18 hrs	72	67	80	204	104	71	383
24hrs	73	83	232	261	132	57	589

Two million PBMCs/ml were platted on a 24 well plates. These cells were stimulated with extract or PHA (positive control) or unstimulated (negative control). And evaluated for FN-γ cytokine stimulation

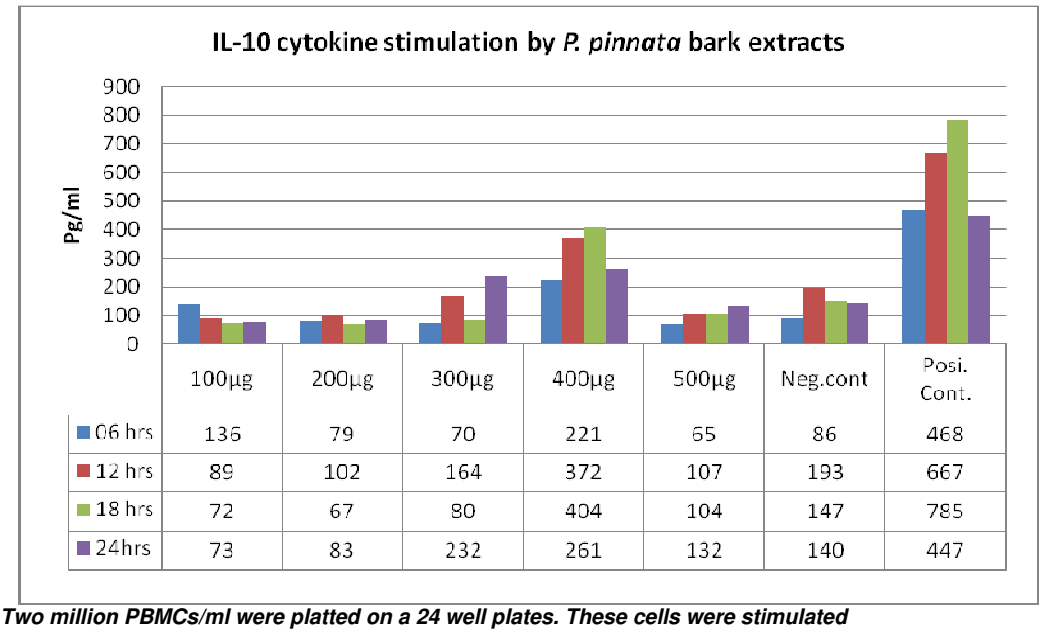
Graph II
IL-2 cytokine stimulation by P. pinnata bark extracts



	100µg	200µg	300µg	400µg	500µg	Neg.cont	Posi. Cont.
06 hrs	61	0	70	73	65	0	368
12 hrs	199	102	104	172	0	193	567
18 hrs	155	67	80	204	109	155	985
24hrs	193	83	232	161	198	129	547

Two million PBMCs/ml were platted on a 24 well plates. These cells were stimulated with extract or PHA (positive control) or unstimulated (negative control) and evaluated for IL-2 cytokine stimulation.

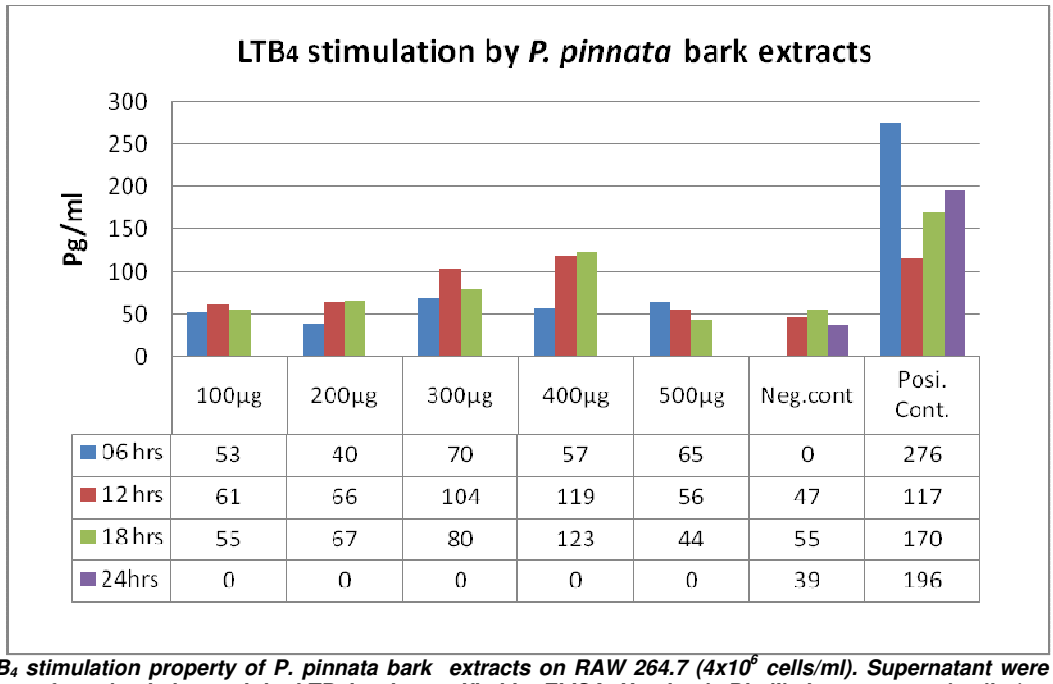
Graph III
IL-10 cytokine stimulation by P. pinnata bark extracts



	100µg	200µg	300µg	400µg	500µg	Neg.cont	Posi. Cont.
06 hrs	136	79	70	221	65	86	468
12 hrs	89	102	164	372	107	193	667
18 hrs	72	67	80	404	104	147	785
24hrs	73	83	232	261	132	140	447

Two million PBMCs/ml were platted on a 24 well plates. These cells were stimulated with extract or PHA (positive control) or unstimulated (negative control).

Graph IV
LTB_4 stimulation by P. pinnata bark extracts

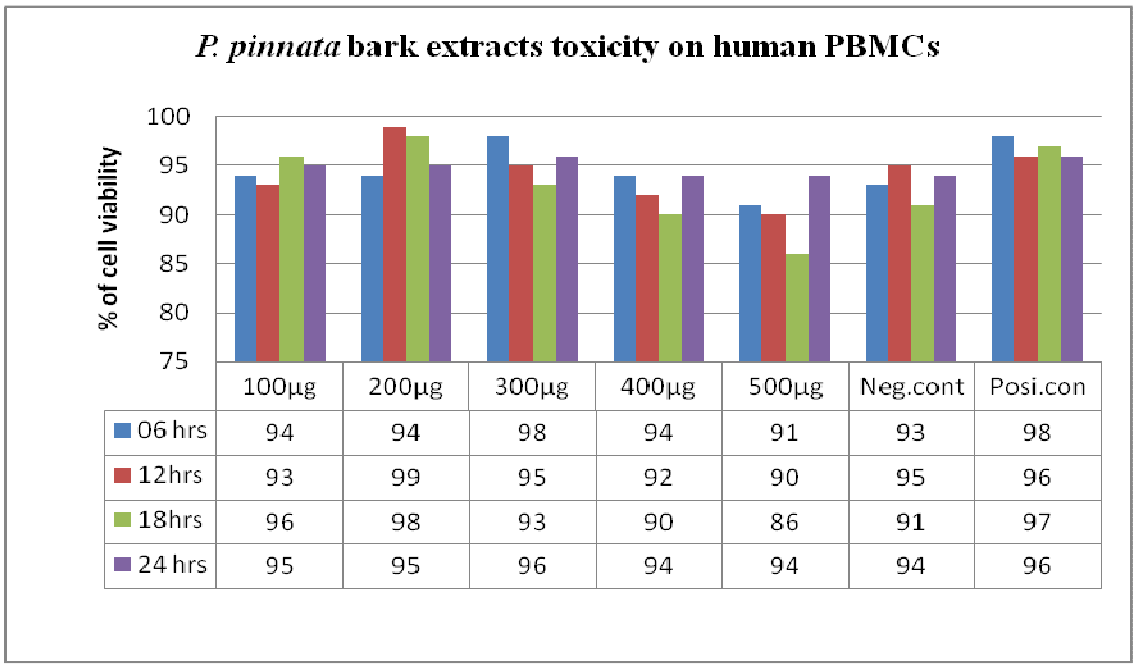


	100µg	200µg	300µg	400µg	500µg	Neg.cont	Posi. Cont.
06 hrs	53	40	70	57	65	0	276
12 hrs	61	66	104	119	56	47	117
18 hrs	55	67	80	123	44	55	170
24hrs	0	0	0	0	0	39	196

Illustrate the LTB_4 stimulation property of P. pinnata bark extracts on RAW 264.7 ($4x10^6$ cells/ml). Supernatant were collected from 6 hours to 24 hours after stimulation and the LTB_4 level quantified by ELISA. Unstimul.=Distilled water treated cells (unstimulated group) and LPS=Lipopolysaccharide (100 ng/ml) treated cultures (positive control). LTB_4 levels were represented in pg/ml.

Graph V

Cytotoxicity studies of P. pinnata bark extracts on human PBMCs



	100µg	200µg	300µg	400µg	500µg	Neg.cont	Posi.con
06 hrs	94	94	98	94	91	93	98
12hrs	93	99	95	92	90	95	96
18hrs	96	98	93	90	86	91	97
24 hrs	95	95	96	94	94	94	96

Illustrates the toxicity of P. pinnata bark extracts on human PBMCs. 2x106 PBMCs were stimulated with 100µg to 500µg concentration of P. pinnata bark extracts. After 24 hours of post stimulation cells were treated with 250 µl of MTT solution/well and incubated at 37ºC for 2 hours. Plate was read at 540 nm and the percentage of viability was calculated. Cells viability was represented in percentages. Negative-distilled water treated culture served as negative control, Triton-X treated culture served as positive control.

CONCLUSION

Pongamia pinnata also known as *Millettia pinnata* has a long history of being used as a medicine by Indian traditional medicine system such as Siddha, Ayurvedha and Unani. And in our previous study we have reported the immune modulation properties of *P. pinnata* seeds extracts. With this background the current study was conducted to elucidate the cytokine stimulating and LTB_4 stimulation property of *P. pinnata* bark extracts on human PBMCs and RAW 264.7 cells respectively. This experiment showed bark extract of *P. pinnata* stimulated both IL-10 and IFN-γ significantly. Major role of these cytokines is to control antibody mediated and cell mediated immunity. LTB_4 is one of the pro-inflammatory lipid mediator and a part of innate immunity and it was measured in RAW 264.7 cells by ELISA. LTB_4 level increased upon 400µg/ml extract treatment from 57 to 123 Pg/ml (Graph-IV). Throughout the study *P. pinnata* aqueous bark extract was treated with human PBMCs and RAW 264.7cells. A separate MTT assay was performed to find toxicity of *P. pinnata* bark extract. In none of the occasions we found any observable toxicity. The experiment showed more than 96% of viable cells suggesting the *P. pinnata* extract was not toxic (Graph-V). Our study clearly showed that *P. pinnata* bark extracts possess cytokine and LTB_4 stimulation potential.

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