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Immunomodulatory properties of Indian medicinal plants in Kolli hills: Stimulation and suppression of lymphocyte proliferation - *In vitro*

K. PERINBAM[1], S. AROKIYARAJ[1], ALEX MATHEW[1], O.S. AYSHA[2], K. BALARAJU[3] and P. AGASTIAN[3]

[1] PG & Research, Department of Biomedical Engineering, Sathyabama University, Chennai - 119 (India)
[2] Elite Bioscience Foundation, Chennai - 119 (India)
[3] Department of Plant Biology and Biotechnology, School of Life Sciences, Loyola College, Chennai - 34 (India)

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ABSTRACT

The present study was carried out to evaluate the immunomodulatory activity of four Indian medicinal plants. *Solanum nigrum*, *Indigofera tinctoria*, *Euphorbia hirta* and *Azima tetracantha* were tested for stimulation and inhibition of lymphocyte proliferation via lymphocyte proliferation assay by ^3H thymidine uptake. RT-PCR was investigated to detect *in vitro* induction of cytokines such as IL-4 and IFN-γ for all the extracts. Among the plants tested *Azima tetracantha* (100 µg/ml) showed the highest rate of lymphocyte proliferation (76%), *Indigofera tinctoria* (100 µg/ml) showed maximum inhibition of lymphocyte proliferation (70%). *A. tetracantha* and *S. nigrum* showed IFN-γ induction while *I. tinctoria* and *A. tetracantha* showed IL-4 induction. This *in vitro* study suggests that *A. tetracantha* and *I. tinctoria* are potent to have immunomodulatory agents.

Key words: Immunomodulation, Lymphocyte proliferation, RT-PCR, Vero cell.

INTRODUCTION

Medicinal plants which form the backbone of traditional medicine, have in the last few decades been the subject for very intense pharmacological studies; this has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutics value and as a sources of lead compounds in the drug development[1]. Medicinal plants are commonly used for the treatment of various ailments, as they are considered to have advantages over the conventionally used drugs that are expensive and known to have harmful side effects. Since, there is an ever-growing interest in investigating different species of plants to identify their potential therapeutic applications. This increasing interest is due to a tremendous historical legacy in folk medicine use of plants as medicine. Several number of plants used in traditional medicines for rejuvenation therapy and chronic ailments have been shown to stimulate immune responses and several active substances had been isolated[2]. In clinical medicine both facts of immunomodulation *viz.* stimulation and suppression are of equal importance. The study of immunomodulation has a role to play in various fields varying from opportunistic infections to medical oncology[3]. Usage of plants as a source of immunomodulators presents a promising future, since they are cost effective, have a broad spectrum activity and may have no side effects[4-5]. Several studies have previously attempted the immunomodulating effects of medicinal plants on lymphocyte proliferation in the presence of mitogen, allogenic cells and specific antigen[6-8]. The present

study was undertaken to test the methanol extract of four different medicinal plants *Solanum nigrum* L. (Solanaceae), *Indigofera tinctoria* L. (Fabaceae), *Euphorbia hirta* L. (Euphorbiaceae) and *Azima tetracantha* L. (Salvadoraceae) for their immunomodulatory activity *in vitro*.

MATERIALS AND METHODS

Plant materials

The plant materials as a whole were collected during the month of March 2006 from Kolli hills, Tamil Nadu, India, through interviews and questionnaire among the tribal practitioners for various ailments like skin diseases, ulcer, asthma, cough and hydrophobia. The plant materials were taxonomically identified and voucher specimen deposited in the department herbarium, Loyola College, Chennai (India). Fresh plant materials were washed under running water, shade dried and the parts were coarsely powdered and stored in air tight bottles.

Extraction

Ten grams of dried plant material were extracted with 100 ml of methanol kept on a rotary shaker for 48 h. Thereafter, it was filtered and centrifuged at 5000 g for 15 min. The supernatant was separated and the solvent was evaporated to make the final volume one-fifth of the original volume[9]. The extracts were filter sterilized and stored at 4^0C until use.

Cell culture

Vero cells (African green monkey kidney cell) were obtained from National Centre for Cell Science, Pune, India, grown in EMEM (Eagle's minimum essential medium) supplemented with Earle's salts and 10 % heat inactivated NBCS (New born calf serum), 100 IU/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamycin. The cells were maintained at 37^0C in a humidified atmosphere with 5 % CO_2 and were subcultured twice a week.

Cytotoxicity assay

Each methanol extracts were dissolved separately in 1 ml of 20 % DMSO (Dimethyl sulphoxide), filter sterilized and further diluted to attain concentration of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.062 mg/ml. 100 µl of cell suspension containing $5x10^6$ cells seeded onto a 96-well microtitre plate. 100 µl of different of concentrations of extracts (2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml and 0.062 mg/ml) were added after 24 h of seeding. Control consisted of cells without extract, and with DMSO. The microtitre plates were incubated at 37^0C in a humidified incubator with 5% CO_2 for period of 72h. The morphology of the cells were inspected daily and observed for microscopically detectable alterations.

Isolation of Peripheral blood mononuclear cells (PBMC)

PBMC were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over Ficoll/Hypaque solution (Histopaque, Sigma, St. Louis MO). Mononuclear cells were collected from the interphase, washed three times in RPMI-1640. Their viability was determined by trypan blue exclusion test[10]. Cell suspensions were adjusted to $5x10^6$ cell/ml and suspended in RPMI-1640 supplemented with 10% FCS. 100 µl of cell suspension solution was placed in each well of a 96 well flat bottom microtitre plate (Nunc 167008, Nuncton, Roskilde, Denmark). The cells were incubated for 24 h. After incubation, phytohaemagglutinin (PHA) (Gibco BRL, Gaithersberg MD) were added to the control wells (20 µg/ml). The extracts at different concentrations (50, 100, 150µg/ml) were co-cultured with or without PHA. The plates were incubated in 5% CO_2 air humidified atmosphere at 37^0 C for 72 h. Subsequently ^{3}H-Thymidine (50 ci/µmol, 1µci/well, Amersham USA) was added into each well. After 16 h incubation, the cells were harvested on glass fiber filters. Radioactivities in the filters were measured by a scintillation counter (Packard TRI CARB 2100 TR USA) in Counts Per Minute (CPM). Controls consisted of PBMC with PHA (100 % activity) and PBMC with medium (0 % activity). The enhancement and inhibition activity of each extract on lymphocyte proliferation was calculated by the following equation[11].

$$\text{Enhancement activity (\%)} = \frac{\text{Extract treated (CPM)-control group(CPM)}}{\text{Control group (CPM)}} \times 100$$

Percentage inhibition of lymphocyte proliferation was calculated by the following

equation.

$$\text{Inhibition (\%)}=1-\frac{\text{CPM(PHA)+CPM (extract treated)}}{\text{CPM (PHA)}}\times 100$$

RT-PCR analysis for expression of IL-4 and IFN- γ

PBMC were isolated, washed twice and resuspended in RPMI – 1640 supplemented with 100µg/ml penicillin, 50 µg/ml gentamycin, 2mM L-glutamine, 10% heat inactivated fetal calf serum (FCS). Non activated PBMC and PBMC activated with PHA (GIBCO BRL, Gaithersberg, MD) at 20 µg/ml were adjusted to a final concentration of 5 x 10^6 cells/ml. Plant extracts at different concentrations (50, 100, 150µg/ml) were added to the PBMC, cells were cultured in flat bottom 96 well micro titer plates in duplicates and incubated in 5 % CO_2 air humidified atmosphere at 37^{0}C for 72 hr. RNA were extracted using the guanidium isothio cyanate-phenol chloroform method[12] washed twice in 80% ethanol-diethyl pyrocarbonate (DEPC) treated water and dried. The samples were resuspended in 8 µl – DEPC treated water and reverse transcribed using Moloney Murine Leukemia Virus Reverse Transcriptase (GIBCO BRL, Gaithersburg, MD) and Oligo d(T) priming in a total reaction volume of 20 µl [13]. Amplification of cDNA was carried out using cytokine specific primer pairs with the following 5' and 3' sequences[14].

IL-4	5'- ATG GGT CTC ACC TCC CAA CTG CT - 3'
	5'- CGA ACA CTT TGA ATA TTT CTC TCT CAT - 3'
IFN-γ	5' - TGC ATC TTG GCT TTG CAG CTC TCC CTC ATG GC - 3'
	5' - TGG ACC TGT GGG TTG TTG ACC TCA ACC TTG GC - 3'
β-actin	5' - TGA CGG GGT CAC CCA TGT GCC CAT CTA CTA - 3'
	5' - GAA GCA TTG CGG TGG ACG ATG GAG GG - 3'

Table 1: Effect of stimulation of methanol extract on PBMC

S. No.	Botanical name	Part used	% Stimulation (50 µg/ml)	% Stimulation (100 µg/ml)	% Stimulation (150 µg/ml)
1.	*Solanum nigrum*	Leaf	49±2	64±3	61±3
2.	*Indigofera tinctoria*	Whole	23±4	38±1	32±2
3.	*Euphorbia hirta*	Leaf	31±3	47±2	41±5
4.	*Azima tetracantha*	Leaf	58±2	76±1	70±3

% of stimulation of lymphocyte at 50, 100 & 150µg/ml, measured by ^{3}H-thymidine incorporation in DNA, after 72 h incubation in 37^{0}C at 5% Co_2 atmosphere. Data are reported as the mean ± S.D in triplicates. P <0.01 when compared to control (Student *t*- test).

Table 2: Effect of inhibition of methanol extract on PBMC

S. No.	Botanical name	Part used	% inhibition (50µg/ml)	% inhibition (100 µg/ml)	% inhibition (150 µg/ml)
1.	*Solanum nigrum*	Leaf	28±3	47±2	42±4
2.	*Indigofera tinctoria*	Whole	60±2	70±1	68±1
3.	*Euphorbia hirta*	Leaf	21±4	37±2	36±5
4.	*Azima tetracantha*	Leaf	40±4	48±4	43±3

% of inhibition of lymphocyte at 50, 100 & 150µg/ml, measured by ^{3}H-thymidine incorporation in DNA, after 72 h incubation in 37°C at 5% Co_2 atmosphere.
Data are reported as the mean ± S.D in triplicates.
P <0.01 when compared to control (Student *t*- test).

5 µl of cDNA was amplified on a thermocycler PTC-100 (MJ Research Inc., Water Town MA). The PCR products (10µl) for IL-4 (656 bp), IFN-γ (456) and β-actin (358 bp) were separated on 1% agarose gel using electrophoresis and visualized by staining with ethidium bromide[15].

Statistical Analysis

All measurements were evaluated statically using student *t*- test through ANOVA model, taking $P < 0.05$ as significance.

RESULTS

Cytotoxicity assay

All the tested methanol extracts of four different medicinal plants tested for cytotoxicity on Vero cell line did not show any cytotoxicity even at the highest concentration of 2 mg/ml. i.e. loss of monolayer, granulation and vacuolization in the cytoplasm.

Lymphocyte proliferation of ^{3}H thymidine uptake Stimulation of Lymphocyte proliferation

Out of four different medicinal plant extracts tested for stimulation of lymphocyte proliferation, *A. tetracantha* (100µg/ml) showed a maximum of 76%, whereas *S. nigrum* showed 64% followed by *E. hirta* 47% and *I. tinctoria* 38%. 100 µg/ml was taken as an ideal concentration, because the activity was high when compared to 50 µg/ml. 150 µg/ml showed similar to 100 µg/ml (Table 1).
Inhibition of Lymphocyte proliferation

Among four different extracts tested for inhibition on mitogen induced lymphocyte proliferation at three different concentrations, *I. tinctoria* showed maximum inhibition of lymphocyte proliferation of 70% followed by *A. tetracantha* 48%, *S. nigrum* 47% and least in *E. hirta* 37%. At 100µg/ml, it was maximum inhibition of lymphocyte proliferation when compared to 50 and 150 µg/ml (Table 2).

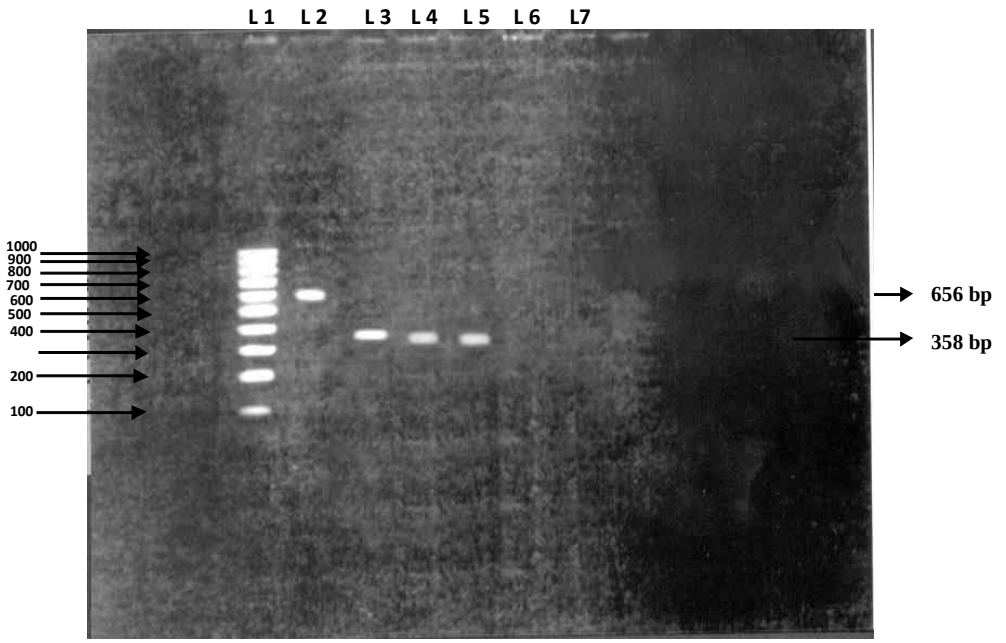

Fig. 1: RT-PCR for induction of cytokines IL-4

Agarose gel visualizing RT-PCR products (IL 4). L1: Molecular weight marker (1000 bp Ladder), L 2: β-actin control, L 3: PHA control, L 4: *Azima tetracantha*, L 5: *Indigofera tinctoria*, L 6:*Solanum nigrum,* L 7: *Euphorbia hirta.* L – Lane

RT-PCR analysis for expression of IL-4 and IFN- γ

RT-PCR techniques were used to test for induction of cytokines IL-4 and IFN-γ for the above mentioned plants. *A. tetracantha* showed both IL-4 and IFN-ã induction (Fig. 1) and *I. tinctoria* showed IL-4 induction and *S. nigrum* showed IFN-γ induction (Fig. 2). *E. hirta* showed neither IL-4 nor IFN-γ induction.

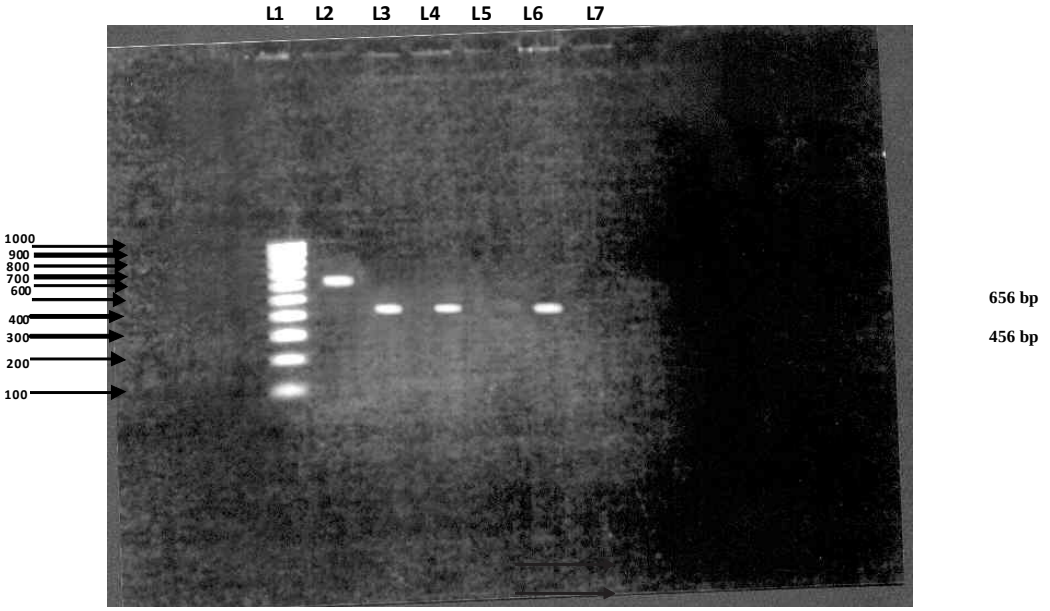

Fig. 2: RT-PCR for induction of cytokines IFN-γ

Agarose gel visualizing RT-PCR products (IFNγ). L1: Molecular weight marker (1000 bp Ladder), L2: β-actin control, L3: PHA control, L4: *Azima tetracantha*, L5: *Indigofera tinctoria*, L6:*Solanum nigrum,* L7: *Euphorbia hirta.* L - Lane

DISCUSION

The present report showed that the methanol extract of leaves from four tested plants, exerts stimulatory and inhibitory potential of lymphocyte proliferation by ^{3}H thymidine incorporation. Similarly methanol extracts from the leaves of Meliaceae were capable of inhibiting the *in vitro* proliferation of lymphocytes and several immune responses in which these cells are involved [16]. Davis and Kuttan [17] reported *Viscum album, Panax ginseng* and *Tripterygium wilfordi* were known to have immunomodulatory activity. A number of facts can influence the lymphocyte proliferation assays – the extraction methods, lymphocyte proliferation, culture medium composition, pH, concentration of mitogen and incubation temperature.

For immunostimulation, *A. tetracantha* showed higher percentage 76% and lowest in *I. tinctoria* 38%. Similar results were observed by Wong and Tan [18]. Whereas the extracts of *Rhaphidophora korthalsi* found to stimulate the lymphocyte proliferation at similar concentration. Its chemical constituents include flavonoids, triterpenoids and alkaloids [19].

I. tinctoria showed maximum inhibition (70%) on mitogen induced lymphocyte proliferation. Almeida *et al.,* [20] showed that an enriched saturated fatty acid fraction from *Kalanchoe pinnata* presents

inhibitory activity on lymphocyte proliferation and traditionally it has been used as antioxidant and hepatoprotective agent. Before performing the assay trypan blue viability test were performed for 100 % viability of PBMC. After 24, 48 and 72 h all the extract treated cells were checked for viability and in each case their viability percentage was more than 85%. So in the present study we have observed that the inhibitory effects could not be considered as the toxic effect of the plants.

Lymphocyte proliferation activity may also be due to the direct effect of plant extracts or may be mediated through activated release of cytokines such as IL-4, IFN-γ. In several studies increase in cytokine production due to herbal plants has been shown by Haq *et al.,* [21] and seed extracts of *Aeginetia indica* induced IL-2, IFN-γ, IL-6 production and lymphocyte proliferation *invitro*[22]. Immunostimulatory activity of andrographolide is evidenced by increased IL-2 and TNF-2 production and enhancement of lymphocyte proliferation, resulting in strengthened responses and cytotoxic activity of lymphocytes against cancer cells[23, 24]. Among the four plants, IFN-γ induction observed in *Azima tetracantha* and *Solanum nigrum*. While *Indigofera tinctoria* and *Euphorbia hirta* did not show IFN-ã activity. Plant derived natural products such as flavonoids, terpenoids and steroids etc. have received considerable attention in recent years due to their diverse pharmacological properties[25]. The reported activity of these plants may be due to the presence of secondary metabolites. From the above studies we suggested that immunomodulatory agents present in these plants can enhance the immunological responsiveness by interfering with its regulatory mechanisms. These agents may selectively activate either cell mediated or humoral immunity by stimulating either Th_1 or Th_2 type of T cell response respectively. Further investigation should be considered in the effects of extracts and its components in interfering with cytokine production by *in vivo* models.

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