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

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

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(Received: April 05, 2008; Accepted: May 26, 2008)

**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 \(^3\)H thymidine uptake. RT-PCR was investigated to detect *in vitro* induction of cytokines such as IL-4 and IFN-\(\gamma\) for all the extracts. Among the plants tested *Azima tetracantha* (100 \(\mu\)g/ml) showed the highest rate of lymphocyte proliferation (76%), *Indigofera tinctoria* (100 \(\mu\)g/ml) showed maximum inhibition of lymphocyte proliferation (70%). *A. tetracantha* and *S. nigrum* showed IFN-\(\gamma\) 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.

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A 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. The extracts were filter sterilized and stored at 4°C 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°C in a humidified atmosphere with 5% CO₂ and were subcultured twice a week.

**Cytotoxicity assay**

Each methanol extracts were dissolved separately in 1 ml of 20% DMSO (Dimethyl sulfoxide), 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 5×10⁶ cells seeded onto a 96-well microtitre plate. 100 µl of different 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°C in a humidified incubator with 5% CO₂ for period of 72 h. 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/Hyphaque 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. Cell suspensions were adjusted to 5×10⁶ 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% CO₂ air humidified atmosphere at 37°C for 72 h. Subsequently ³H-Thymidine (50 cì/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.

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

Percentage inhibition of lymphocyte proliferation was calculated by the following

\[
\text{Percentage inhibition} = \frac{\text{Control group (CPM)} - \text{Extract treated (CPM)}}{\text{Control group (CPM)}} \times 100
\]
equation.

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

**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 10⁶ 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% CO₂ air humidified atmosphere at 37°C for 72 hr. RNA were extracted using the guanidium isothiocyanate-phenol chloroform method 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, Gaithersberg, MD) and Oligo d(T) priming in a total reaction volume of 20 µl. Amplification of cDNA was carried out using cytokine specific primer pairs with the following 5’ and 3’ sequences:

**IL-4**

5’ - ATG GGT CTC ACC TCCCAA 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 CCG TGG ACG ATG GAG GG - 3’

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Botanical name</th>
<th>Part used</th>
<th>% Stimulation (50 µg/ml)</th>
<th>% Stimulation (100 µg/ml)</th>
<th>% Stimulation (150 µg/ml)</th>
</tr>
</thead>
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<tr>
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<td>Leaf</td>
<td>49±2</td>
<td>64±3</td>
<td>61±3</td>
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<td>Indigofera tinctoria</td>
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<td>23±4</td>
<td>38±1</td>
<td>32±2</td>
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<td>3.</td>
<td>Euphorbia hirta</td>
<td>Leaf</td>
<td>31±3</td>
<td>47±2</td>
<td>41±5</td>
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<td>Leaf</td>
<td>58±2</td>
<td>76±1</td>
<td>70±3</td>
</tr>
</tbody>
</table>

% of stimulation of lymphocyte at 50, 100 & 150µg/ml, measured by ³H-thymidine incorporation in DNA, after 72 h incubation in 37°C at 5% CO₂ atmosphere. Data are reported as the mean ± S.D in triplicates. P <0.01 when compared to control (Student t-test).

<table>
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<th>S. No.</th>
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<th>% inhibition (150 µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>1.</td>
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<td>47±2</td>
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<td>Indigofera tinctoria</td>
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<td>68±1</td>
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<td>3.</td>
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<tr>
<td>4.</td>
<td>Azima tetracantha</td>
<td>Leaf</td>
<td>40±4</td>
<td>48±4</td>
<td>43±3</td>
</tr>
</tbody>
</table>

% of inhibition of lymphocyte at 50, 100 & 150µg/ml, measured by ³H-thymidine incorporation in DNA, after 72 h incubation in 37°C at 5% CO₂ 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 bromide15.

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 3H 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
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-α 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.

**DISCUSSION**

The present report showed that the methanol extract of leaves from four tested plants, exerts stimulatory and inhibitory potential of lymphocyte proliferation by 3H 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 wilfordii* 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., and seed extracts of Aeginetia indica induced IL-2, IFN-γ, IL-6 production and lymphocyte proliferation invitro. 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. Among the four plants, IFN-γ induction observed in Azima tetracantha and Solanum nigrum. While Indigofera tinctoria and Euphorbia hirta did not show IFN-α 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. 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, or Th, 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.

REFERENCES


