Research Paper

Immunosuppressive effect of medicinal plants of Kolli hills on mitogen-stimulated proliferation of the human peripheral blood mononuclear cells *in vitro*

S. Arokiyaraj, K. Perinbam, P. Agastian*, K. Balaraju*

PG and Research, Sathyabama University, Chennai - 600 119; *Department of Plant Biology and Biotechnology, School of Life Sciences, Loyola College, Chennai - 600 034, India

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Correspondence to:
K. Perinbam
E-mail: sistperinbam@yahoo.com

ABSTRACT

Four medicinal plant species were collected from the Kolli hills of Tamil Nadu and were screened for their immunosuppressive effect. The plants were shade dried and extracted with methanol. The crude methanol extracts were tested for inhibition of lymphocyte proliferation via lymphocyte proliferation assay by ^{3}thymidine uptake. The test plants were *Justicia gendarussa, Plumbago indica, Aloe vera*, and *Aegle marmelos*. Among the plants tested *J. gendarussa* (100 µg/ml) showed the highest lymphocyte inhibition (84%). Sequential extraction of *J. gendarussa* in various solvents (n-hexane, benzene, ethyl acetate, chloroform, acetone, ethanol, and water) confirmed that all of the above extracts at 50 µg/ml, aqueous extract inhibited lymphocyte proliferation. Further, 17 high performance liquid chromatography fractions were collected for the aqueous extract and fraction no. 15 showed maximum inhibition of lymphocyte proliferation. The present study indicates that these extracts should be investigated further for the possible presence of immunosuppressive components.

KEY WORDS: Cytotoxic, immune system, mitogen, secondary metabolites

Modulation of immune response, as a possible therapeutic measure, by using medicinal plant products has become a subject of scientific investigation. Indian medicinal plants are commonly used for the treatment of various ailments and are considered, by many, to have advantages over the conventionally used drugs, which are expensive and known to have harmful side effects.[1] Our immune system plays a vital role in protecting us from various infections. Its integrity and efficiency is important during the treatment of many diseases.[2] Besides primary metabolites with immunomodualtory activity, several plant have secondary metabolites that have been found to interfere with different immune system functions, including the activation of cell-mediated immunity.[3,4] Several studies have previously demonstrated the immunomodulating effects of medicinal plants on lymphocyte proliferation in the presence of mitogens, allogenic cells, and specific antigens.[5-7] Knowing the potential of plant extracts as immunomodulators, the medicinal plants of the Kolli hills of Tamil Nadu were selected for this study. Although the local traditional healers have ethnomedical knowledge of the value of these plants, there have been no biological studies on the immunomodulatory activity of these plants. The present study was undertaken to test the extracts of four different medicinal plants for their immunomodulatory property on mitogen-induced lymphocyte proliferation *in vitro*.

Material and Methods

Local traditional healers

Ten local traditional healers who had practical experience with medicinal plants were interviewed in Kolli hills, Tamil Nadu, India, in the month of October 2006. They were interviewed separately and asked to collect the plants that they traditionally used for treating ailments such as rheumatism, cough, inflammation, diabetes, ulcers, and diarrhea. Of the collected plants, four were selected by the interviewer for the study. These plants were taxonomically identified and deposited in the department herbarium. The voucher specimens in duplicate were deposited in the department herbarium, Loyola College, Chennai (India).

Preparation of crude extracts

Fresh plant materials were washed under running water and shade dried. The parts were coarsely powdered and extracted with methanol after 48 h. The solvent was removed by rotary evaporation under reduced pressure at <45°C temperature. The resulting crude extracts were stored at 4°C until use. Subsequent extraction was carried out in a soxhlet extraction unit (Borosil, Mumbai) using n-hexane, ethyl acetate, chloroform, ethanol 96%, benzene, acetone, and water. Organic solvents were removed under reduced pressure. Water was removed by freeze drying. The resulting extracts were stored in vials and assayed.

High performance liquid chromatography (HPLC) analysis

A Shimadzu model LC-64 liquid chromatograph with C18 reversed-phase column was used to separate the major components of the aqueous extracts. After the column was washed to achieve the baseline, the gradient parameters were set and the run was started by injecting 250 μl of the extract. A flow rate of 1.5 ml/min was employed. Peaks were detected by monitoring their absorbance at 405 nm and 280 nm. The chromatogram was recorded on a Shimadzu-C-R 3 A chromatopac recording integrator. Chromatographic patterns were plotted for the extracts. Fractions were collected in a collector, evaporated in a rotary vacuum pump, and lyophilized.

Cell culture

Vero cells (African green monkey kidney cell) were obtained from the National Centre for Cell Science, Pune, India. Vero cells were grown in EMEM (Eagle's minimum essential medium) supplemented with Earle's salts and 10% heat-inactivated NBCS (newborn 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% CO_2 and were subcultured twice a week.

Cytotoxicity assay[8]

Each extract (crude methanol extract; the six different solvents such as n-hexane, benzene, ethyl acetate, chloroform, acetone, ethanol, and aqueous residues; and the 17 HPLC fractions) was dissolved separately in 1 ml of 20% DMSO (dimethyl sulphoxide), filter sterilized, and then further diluted to attain concentrations of 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, and 0.062 mg/ml. Cell suspension (100 μl) containing 5 $\times 10^6$ cells was seeded onto a 96-well microtiter plate. After 24 h of seeding, 100 μl of the different concentrations of the 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. Control consisted of cells without extract and with DMSO. The microtiter plates were incubated at 37°C in a humidified incubator with 5% CO_2 for period of 72 h. The morphology of the cells was 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 in the interphase and washed three times in RPMI-1640. Their viability was determined by trypan blue exclusion test.[9] Cell suspensions were adjusted to 5 $\times$ 10^6 cell/ml and suspended in RPMI-1640 supplemented with 10% FCS.

Lymphocyte proliferation assay by ^{3}H thymidine uptake

The *in vitro* cellular proliferation was performed as previously described by Lie-Chwen Lin.[10] The cell suspension (100 μl) was seeded in each well of a 96-well flat-bottomed microtiter plate (Nunc 167008, Nuncton, Roskilde, Denmark). The cells were incubated for 24 h. After incubation, 100 μl of the extracts at different concentrations (50, 100, 150 μg/ml) were added. The cultures were stimulated with 20 μg/ml of PHA (Gibco BRC, Gaithersberg, MD). Controls consisted of PBMC with PHA (100% stimulation activity), PBMC with RPMI (0% activity), and sample with methanol (0% activity) as background sample. The plates were incubated in 5% CO_2 air humidified atmosphere at 37°C for 72 h. Lymphocyte proliferation was estimated by adding ^{3}H-thymidine (50 ci/mmol, 1 μci/well; Amershan USA). After 16 h of incubation, the cells were harvested on glass fiber filters. Radioactivity in the incorporated cells was measured by using a liquid scintillation counter (Packard TRI CARB 2100 TR USA).

Percentage of inhibition of lymphocyte proliferation for each extract was calculated by the following equation: [10]

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

Statistical analysis

Each experiment with PBMC was run in triplicate. Significance was calculated by Student's t test, through the ANOVA model. $P<0.05$ was taken to indicate statistical significance. Results are reported as mean ± S.D

Results

Cytotoxicity assay

None of the tested extracts (crude methanol extract; six different solvents such as n-hexane, benzene, ethyl acetate, chloroform, acetone, ethanol and aqueous residues; and the 17 HPLC fractions) of the four different medicinal plants showed any signs of cytotoxicity on the Vero cell line, even at the highest concentration of 2 mg/ml, i.e., there was no loss of monolayer, granulation, or vacuolization in the cytoplasm [Figures 1 and 2].

Lymphocyte proliferation assay by ^{3}H thymidine uptake

Inhibition of lymphocyte proliferation: The methanol extract of the four medicinal plants (belonging to different families) used in the traditional system of medicine were tested for their ability to inhibit PBMC proliferation at a concentration between 50-150 μg/ml. After stimulation with PHA and incubation for 72 h, all the four species inhibited PBMC proliferation, as measured by ^{3}H-thymidine incorporation by DNA from the cells. Extract of *J. gendarussa* showed the maximum inhibitory activity, when compared to *P. indica, Aloe vera* and. *Aegle marmelos*, at a concentration of 100 μg/ml [Table 1].

Sequential extraction of *J. gendarussa* showed more inhibitory effect on lymphocyte proliferation in aqueous, chloroform, and ethanol than in n-hexane, ethyl acetate, and benzene at a concentration of 50 μg/ml [Table 2].

HPLC analysis

Aqueous extract of *J. gendarussa* showed the maximum inhibitory activity on lymphocyte proliferation; HPLC fractionation of this extract was carried out. 17 HPLC fractions were collected and tested for inhibition of lymphocyte proliferation. Out of 17 fractions tested, fraction no.15 showed the maximum inhibition of 88% at a concentration of 10 μg/ml [Table 3].

Discussion

On comparison of the cell proliferation in nontreated and extract-treated cultures, the methanol extracts of *J. gendarussa*, *P. indica, Aloe vera*, and *A. marmelos* showed the ability to cause

Figure 1: Vero cell monolayer

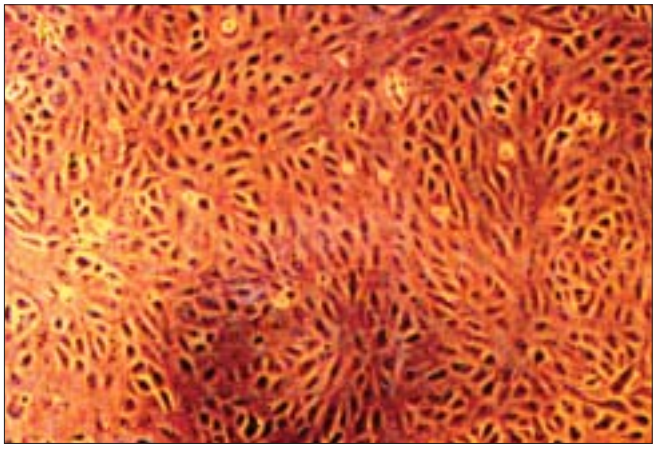

Figure 2: Vero cell treated with plant extract

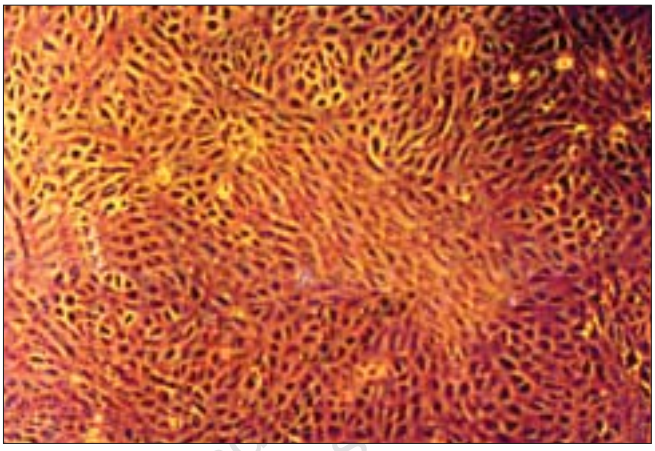

Table 1

Methanol extract of medicinal plants of Kolli hills

Botanical name	*Part used*	*% Inhibition (50 µg/ml)*	*% Inhibition (100 µg/ml)*	*% Inhibition (150 µg/ml)*
Justicia gendarussa	Leaf	64 ± 4	84 ± 3	81 ± 6
Plumbago indica	Root	60 ± 0	80 ± 2	80 ± 1
Aloe vera	Leaf	61 ± 6	79 ± 1	78 ± 2
Aegle marmelos	Leaf	59 ± 2	77 ± 2	76 ± 3

% of inhibition of lymphocyte at 50, 100, and 150 µg/ml, measured by ^{3}H-thymidine incorporation in DNA, after 72 h incubation at 37°C in 5% CO2 atmosphere. Data are reported as the mean ± S.D in triplicates. *P* <0.01 when compared to control (Student's t test)

Table 2

Sequential extraction of *Justicia gendarussa*

Solvent	*% Inhibition (25 µg/ml)*	*% Inhibition (50 µg/ml)*	*% Inhibition (100 µg/ml)*
n-hexane	60 ± 2.0	73 ± 3.6	65 ± 4.0
Benzene	50 ± 5.0	61 ± 2.5	50 ± 1.5
Ethyl acetate	52 ± 3.5	66 ± 2.6	60 ± 6.0
Chloroform	64 ± 3.6	84 ± 2.3	70 ± 3.0
Acetone	61 ± 1.7	76 ± 1.5	65 ± 1.5
Ethanol	61 ± 2.0	81 ± 2.5	77 ± 5.3
Aqueous	65 ± 5.1	90 ± 2.6	80 ± 1.0

% of inhibition of lymphocyte at 25, 50, and 100 µg/ml, measured by ^{3}H-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's t test)

Table 3

High performance liquid chromatography fractions of aqueous extract of *Justicia gendarussa* on human lymphocyte proliferation in response to PHA

Fractions	*% Inhibition (5 µg/ml)*	*% Inhibition (10 µg/ml)*	*% Inhibition (15 µg/ml)*
1	50 ± 2.0	69 ± 1.5	65 ± 5.0
2	56 ± 1.0	74 ± 2.0	70 ± 1.5
3	46 ± 0.0	52 ± 1.0	49 ± 1.0
4	58 ± 1.5	81 ± 1.5	76 ± 1.0
5	48 ± 2.0	66 ± 2.5	59 ± 1.5
6	59 ± 1.0	80 ± 1.0	71 ± 0.5
7	58 ± 1.0	81 ± 0.5	70 ± 0.5
8	54 ± 2.0	71 ± 0.5	67 ± 2.0
9	47 ± 1.0	58 ± 0.5	51 ± 1.5
10	61 ± 1.5	83 ± 0.5	77 ± 1.5
11	58 ± 2.5	65 ± 1.1	60 ± 3.0
12	54 ± 3.0	70 ± 1.0	67 ± 2.5
13	57 ± 1.5	65 ± 0.5	61 ± 0.5
14	61 ± 2.0	85 ± 1.0	77 ± 2.5
15	62 ± 3.0	88 ± 0.5	81 ± 2.0
16	43 ± 1.5	55 ± 0.5	49 ± 0.0
17	41 ± 2.0	59 ± 0.5	51 ± 2.5

Effect of HPLC fractions of aqueous extract of *Justicia gendarussa* (1-17) on lymphocyte proliferation. % of inhibition of lymphocyte at 5, 10, and 15 µg/ml, measured by ^{3}H-thymidine incorporation in DNA, after 72 h incubation at 37°C in 5% CO2 atmosphere. Data are reported as the mean ± S.D in triplicates. *P* <0.01 when compared to control (Student's t test)

significant decrease in mitogen (PHA)-induced lymphocyte proliferation at a concentration of 100 μg/ml, suggesting that they affected the T-cell proliferation in a dose-dependent manner. The results from this preliminary investigation provide evidence of the importance of ethnopharmacology as a guide to the screening of biologically active plant materials.[11] *J. gendarussa*, as a methanol extract, showed the maximum inhibition of 85% . Similarly, crude methanolic extract obtained from leaves of Meliacea were capable of inhibiting the *in vitro* proliferation of lymphocytes and in inhibiting several immune responses in which these cells are involved.[12] Rayward *et al*.[13]

showed that the inhibitory effect of the alcoholic extract of the fern *Polypodium leucotomos* in his assay was related to a direct effect on T-lymphocytes and/or macrophages. Similarly, water and ethanol extracts of *Cypreus rotundus* and *Eclipta alba* strongly decreased PBMC proliferation in a dose-dependent manner.[14] Before performing the assay, trypan blue viability test was performed to check 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 was >90%. Similarly, in the present study, we have observed that the inhibitory effects could not be considered as the toxic effect of the plants.

The present study indicated that proliferation of PHA-activated mononuclear cells was significantly inhibited by 100 μg/ml and 50 μg/ml; in comparison, concentrated lime juice extract inhibited PHA-stimulated MNC at 250 and 500 μg/ml.[15]

It is also important to note that a beneficial effect was observed; the traditional aqueous extract of *J. gendarussa* showed a strong immunosuppressive effect on mitogen-stimulated lymphocytes at a low concentration of 50 μg/ml.

J. gendarussa is common throughout India in waste places, hedges, and village shrubberies. It is an effective medicinal plant for chronic rheumatism, cephalalgia, cough, and bronchitis. The chemical constituents in the plant's leaves includes 0-disubstiuted aromatic amines, 2-amino benzyl alcohol, 2(2'-amino benzyl amino) benzyl alchol, and their respective 0-methyl ethers, friedelin, lupeol, and β-sitosterol.[16]

This study revealed the inhibitory activity of *J. gendarussa* extract on nonspecific cellular immune response. Although the exact mechanism of this effect is not known, it may be mediated by the interaction between the active components of the extracts and cell-surface molecules or growth factors involving mitogen activation, and it is possible that identification and elucidation of the active constituents in this plant may provide useful leads to the development of new and effective immunosuppressant drugs.

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