Discussion
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Immune-modulators are the substances that act on the immune system and have the ability to modify the immune response. Their effects may be stimulatory or suppressive. Non-specific immune stimulation is aimed to enhance state of resistance to pathogens or tumors. In this capacity, their use has been proposed along with chemotherapy to combat intracellular/chronic infections, and to eliminate the residual tumor, such as in melanoma, colon cancer and bladder cancer etc. Non specific immune suppressants reduce the capacity of the immune system to respond to antigens, either by killing the dividing cells as in the case of cytotoxic drugs and steroids or by interfering with the processes of signal transduction etc., in a more selective way as done by cyclosporin A, and rapamycin. These immunosuppressive drugs that selectively block the function of T-lymphocytes have been widely used to prevent graft rejection (also graft vs host disease) and to control autoimmune diseases.

The synthetic immunomodulatory agents have several limitations, apart from the cost of these agents, the major drawback is their side effects, the necessity to administer them parenterally, which often leads to serious and disturbing adverse reactions.
Therefore a vigorous pursuance to develop useful immunomodulators assumes a lot of importance.

The potential of Indian medicinal plants to be useful as immunomodulators appears encouraging, because they are cost effective, efficacious, non toxic, have a broad spectrum activity and may have less side effects. Literature suggests that many of the plants have immunomodulatory potential. Ayurveda, the traditional system of Indian medicine gives a lot of emphasis on the use of therapies that produce a "pro-host" effect, thus keeping a person in a state of "positive health".

The plants described as "rasayana" in charaka are given for prevention of disease and strengthening of both physical and mental health. They are commonly used following epidemics or during the convalescent period.

The present investigations revealed immunomodulatory activity in the selected Indian medicinal plants. In vitro cytotoxicity studies were done for all the selected plants on Vero cell line. The in vitro experiments were done on lymphocyte proliferation by \(^{3}H\)-thymidine uptake and induction of IL-4 and IFN-\(\gamma\) by RT-PCR. In vivo experiments were done on adjuvant induced arthritis, delayed type hypersensitivity and humoral antibody response.
Several methods for preparing an initial extract of the plant material have been reported. One of the most important and fundamental considerations in designing a phytochemical screening procedure is the selection of a proper solvent. Methanol was taken as the solvent for primary extraction. Methanolic extracts of the plants exhibit a well marked immunomodulatory activity (Simons et al., 1989). Similarly reports of Kapil and Sharma (1997) indicate that the methanol extracts are the most active extracts. Among the various methods that are available, 80% ethanol and methanol appears to be the best solvent (Farnsworth, 1966). The rational for adopting such an extraction procedure was based on the polarity of the solvents that would leach out compounds soluble in a particular solvent (Samy et al., 1998). Similar observation was found in our study where we noted the crude extracts showed an optimum immunomodulatory activity with methanol extracts.

Cytotoxicity studies was done on vero cell lines to prove that the plant extracts were not toxic. Before proceeding to in vitro or in vivo trials it is a fundamental procedure to determine cytotoxicity of any natural product in an established cell line. All the 10 extracts were studied for cytotoxicity. No observable cytotoxic effect was seen when it was analysed in vitro in Vero cell line at concentrations ranging from 2 mg/ml to 31.25 μg/ml).
Amplification of the immune response usually involves proliferation of particular sub populations of lymphoid cells that are normally in the resting state. Thus, lymphoid cell responses to antigens (Kozbor et al., 1989), super antigens (Uchiyama et al., 1989), alloantigens (Shalaby et al., 1988), autoantigens (Kozak et al., 1982), cytokines (Clark et al., 1989) and agents such as antibodies (Geppert and Lipsky, 1988; Nakagawa et al., 1988) that transduce signals at the cell membrane are primarily measured by proliferation assays.

Proliferative assays have the following applications in clinical studies: (1) Assessment of overall immunologic competence of T cells or B cells as manifested in their ability to respond to polyclonal proliferation signals such as mitogens or anti-CD3 antibodies. Defects in the proliferation may be indicative of fundamental cellular immunologic defect.

Cell proliferation in response to external stimuli is a very complex process often involving delivery of a signal or set of signals to cell membrane, activation of intracellular enzymatic pathways that are not well understood, activation and transcription of multiple genes, DNA and protein synthesis and finally cell division. A number of facts can influence the lymphocyte proliferation assay - the extraction methods, lymphocyte preparation, culture medium
composition, concentration of mitogen and extracts, pH and incubation temperatures. The viability of the lymphocytes was ensured before proceeding to the proliferative assay. A significant percentage of dead or damaged cells may inhibit the proliferative response.

PHA was used as a mitogen and different concentrations of the PHA was tested 10 µg/ml, 20 µg/ml, 30 µg/ml and 40 µg/ml. Among all these concentrations tested, 20 µg/ml was found to give optimum proliferation. For PHA induced proliferation, incorporation of thymidine peaks after 2 to 3 days of culture.

The present study was focussed on the influence of plant extract on mitogen-induced lymphocyte proliferation. Among the 10 different medicinal plants tested for lymphocyte proliferation Alternanthera sessilis has shown a higher percentage (67%) stimulation of lymphocyte proliferation at a concentration of 100 µg/ml. Three of the other plants Polygala chinensis, Oxalis corniculata and Tribulus terrestris showed stimulation of lymphocyte proliferation ranging from 63% to 64%.

Eventhough all the four above mentioned plants, showed more or less similar stimulatory activity on lymphocyte proliferative, we have selected the plant Alternanthera sessilis which has showed the maximum stimulation on lymphocyte proliferation which has been
used to study the active fraction involved in the stimulation of lymphocyte proliferation. Whether the therapeutic efficacy of this herb may, in part, be mediated via its influence on the immune response is not known. No immunomodulatory study has been reported for either *Alternanthera sessilis* or other *Alternanthera* species. The results obtained in our study indicated that *Alternanthera sessilis* has the capacity of increasing the cellular immune response. The extract showed no spontaneous stimulatory activity on the human lymphocytes but when the cells were cultured with the extract in the presence of a suboptimal dose of PHA, a strong enhancement in the cell proliferation was observed. A possible explanation for this might be the presence of same components similar to lectins present in the extract which can bridge cells by binding to surface proteins on stimulated cells and may co-stimulate and facilitate cellular interactions (Peacock *et al.*, 1990).

In our study the extract of *Alternanthera sessilis*, *Polygala chinensis*, *Oxalis corniculata* and *Tribulus terrestris* have shown a maximum activity at a concentration of 100 µg/ml. This result correlates with the results of Wong and Tan (1996), where the extract of *Rhaphidophora korthalsii* was found to stimulate the lymphocyte proliferation at similar concentrations.
In the present study, the initial extraction of the plants was done with the solvent methanol. The methanolic extracts have shown a significant stimulatory effect on lymphocyte proliferation. Similar observations were made in the study of Sharma et al. (1994), where the methanolic leaf extract of Picrorhiza kurroa have been found to stimulate the proliferation of lymphocytes.

The mitogenic activity of Alternanthera sessilis correlates with the results of Tinospora cordifolia, which has been extensively explored for its immunostimulant effects. In several distinct studies, the capacity of herbal plants on lymphocyte proliferation in the presence of mitogens, has been studied. In this regard, the immunostimulatory activity of garlic on cell mediated immunity has been reported (Lamm and Riggs, 2000). Green tea has been proven to increase humoral and cellular activity (Klein et al., 2000). Echinacea is being tried as an agent for immune stimulation (Bauer et al., 1999), and Acanthospermum hispidium, a tropical plant, has been shown to enhance the proliferation of T lymphocytes after stimulation with Con A or allogenic stimulator cells in the mixed lymphocyte culture (Summerfield and Saalmuller 1998).

Alternanthera sessilis was extracted with different solvents such as n-hexane, benzene, ethyl acetate, chloroform, acetone, ethanol and aqueous. The extracts were tested for stimulatory
potential of lymphocyte proliferation. The aqueous extract have shown a higher stimulation of 69% of lymphocyte proliferation where as ethanol, chloroform, benzene, acetone, n-hexane and ethylacetate have shown 67.5%, 67%, 52.8%, 55%, 45% and 42.3% respectively. This study revealed that the highest amount of active principle responsible for the stimulation of lymphocyte proliferation may be present in the aqueous, chloroform and ethanol extracts, compared to the extracts of n-hexane, benzene, acetone and ethyl acetate.

HPLC is a well developed chromatographic technique which is more advantages compared to other column chromatographic techniques. Separation of compounds using HPLC is specific, accurate, reduces the purification time, gives high resolution, high quality of compounds were obtained by this separation method.

The aqueous extract of * Alternanthera sessilis* was further fractionated with high pressure liquid chromatography to separate the fractions. Twenty fractions were collected and tested for lymphocyte proliferation. Among the 20 fractions tested, fraction No.4, fraction No.11, fraction No.15, fraction No.16, fraction No.18 and fraction No.20 showed significant stimulation (72%, 65.8%, 62.3%, 68%, 71.5% and 64% respectively) of lymphocyte proliferation.

The fractions have shown a highest activity at 10 μg/ml which agrees with the result of the ethanolic compounds of *Dichrocephala*
bicolor which showed enhancement of lymphocyte proliferation at a concentration of 10 μg/ml (Lie-Chwen Lin et al., 1999). The fraction 4, fraction 18, fraction 16 have shown highest enhancement on the proliferation of lymphocytes. This indicates the mitogenic activity of the fractions of Alternanthera sessilis.

The present study revealed that among the 10 crude medicinal plants tested for lymphocyte proliferation, Justicia gendarussa showed 65.2% inhibition of lymphocyte proliferation and the other extracts Aloe vera, Plumbago indica and Aegle marmelos showed 62.3%, 60% and 59% respectively at a concentration of 100 μg/ml. Among the 10 plants tested for lymphocyte proliferation Justicia gendarussa has shown a higher inhibition of lymphocyte proliferation. The leaves of Justicia gendarussa were used tropically in oedema of beriberi and rheumatism. The impaired incorporation of tritiated thymidine into cellular DNA was not due to a toxic effect of Justicia gendarussa since we observed that the number of viable cells in treated and control cultures remained constant according to trypan blue exclusion method. This result agree with those obtained for other plants such as Azadirachta indica Cedrela lilloi and Trichilia elegans (Vandernat et al., 1987; Labadie et al., 1989; Nores et al., 1997). The extract of Justicia gendarussa was successively extracted with different solvents with increasing polarity such as n-hexane, Benzene, Ethyl acetate, Chloroform, Acetone, Ethanol and
Aqueous. The extracts obtained were tested for inhibitory potential of lymphocyte proliferation. The aqueous extract have shown a higher inhibition of lymphocyte proliferation 66% and the other extracts ethanol, chloroform, n-hexane, Benzene and Ethylacetate showed 64%, 63.5%, 55.6% and 43% inhibition of lymphocyte proliferation at a concentration of 50 µg/ml. The results of our study correlated with the finding of Benencia et al. (2000) who has reported a significant inhibition in cellular proliferation was observed for *Trichilla glabra*. The aqueous extracts of fresh green leaves from *Cedrella lilloi* and *Trichilia elegans* exert inhibitory effects on several components of the mouse immune system (Nores et al., 1997).

The aqueous extract of *Justicia gendarussa* was further fractioned with high pressure liquid chromatography. A total of 17 fractions were collected from the HPLC fractionation and tested for lymphocyte proliferation. Among the 17 fractions tested, fraction No.6, fraction No.7, fraction No.10, fraction No.14 and fraction No.15 showed 60%, 61%, 64%, 65% inhibition on lymphocyte proliferation at a concentration of 10 µg/ml.

The lymphocyte proliferation activity may also be due to the direct effect of the plant extracts or may be mediated through activated release of cytokines such as IL-1, IL-2 and IFN-γ. In several studies increase in cytokine production due to herbal plants
has been shown (Haq et al., 1999; Chai et al., 1994). Large quantities of IL-10 have been secreted in culture of non-activated peripheral blood mononuclear cells and allogenic cells with whole Nigella sativa proteins (Haq et al., 1999). Seed extracts of Aegineta indica induced IL-2, IFN-γ, TNF and IL-6 production and lymphocyte proliferation in vitro (Chai et al., 1994). These reports highlight the need for further study in particular to investigate the possible action of plant extracts and its components in interfering with cytokine production.

The above mentioned 10 different crude medicinal plants were tested for induction of cytokines IFN-γ and IL4 by reverse transcription polymerase chain reaction (RT-PCR). Among the 10 plant extracts tested for IFN-γ induction, four of the extracts Polygala chinensis, Alternanthera sessilis, Plumbago indica and Aloe vera showed IFN-γ induction while the other six extracts Tribulus terrestris, Oxalis corniculata, Evolvulus alsinoides, Aegle marmelos, Vitex negundo and Justicia gendarussa did not show IFN-γ induction. The four plants Alternanthera sessilis, Plumbago indica, Polygala chinensis and Aloe vera were found to be inducers of gamma-interferon which could explain its antitumour and antiviral properties. Among 10 extracts tested for IL4-induction, four of the extracts Alternanthera sessilis, Vitex negundo and Justicia gendarussa and Aegle marmelos showed IL4 induction. While the other extracts did not show IL4 induction. Alternanthera sessilis has shown both IL4 and IFN-γ induction, hence the HPLC fractions of this extract was tested for IFN-γ and
IL4 induction. Out of the 20 fractions tested for IFN-γ induction, fraction No.4, fraction No.11, fraction No.16, fraction No.18, fraction No.19 and fraction No.20 have shown IFN-γ induction. Out of the 20 fractions tested for IL4 induction, the fraction No.4, fraction No.11, fraction No.16 and fraction No.18 have shown IL4 induction.

The extract *Justicia gendarussa* has shown significant inhibition of lymphocyte proliferation. It has not induced the cytokine IFNγ but it has induced IL4. The inhibitory action of this plant on lymphocyte proliferation and IFNγ cytokine induction may be considered for anti-inflammatory effect of this herb. The leaves of this plant is reported to be used in rheumatism (Kirthikar and Basu, 1933).

Adjuvant induced arthritis (AIA) has been very widely adapted in pharmaceutical screening programmes, being generally considered over the past two decades as an appropriate model of rheumatoid arthritis. In AIA, the heat killed mycobacterium tuberculosis cells are not degraded and remain in the synovium as perpetual antigens. Hence, the cells involved in the cell mediated immune response are in a state of perpetual activation leading to an inflammatory responses. The current concept is that inflammation and tissue destruction in the rheumatoid synovium result from complex cell-cell
interaction (Arend, 1997). In AIA the degree of inflammation was measured in terms of paw swelling.

In the present study an attempt was therefore made to assess the antiarthritic property of the crude extract *Justicia gendarussa*. Measurement of the paw volume of rats with adjuvant-induced arthritis revealed an increase in ankle diameter from day 4 which further increased up to day 19. Increased paw diameter in the arthritic animals were significantly suppressed to near normal levels in rats treated with 600 mg/kg of crude *Justicia gendarussa* aqueous extract and 3 mg/kg indomethacin. The effect of *Justicia gendarussa* extract was almost the same as that of indomethacin. In a similar study the aqueous and chloroform extracts of the *Aloe vera* gel have shown anti-inflammatory properties (Vazquez et al., 1996).

This study has shown that adverse changes of paw oedema in arthritic rats were substantially reversed by oral administration of *Justicia gendarussa* leaf extract. This provides evidence for its anti-inflammatory property. The soft swelling seen around the ankle joints was found to be a result of oedema of periarticular tissues. An increase in granulocytes and monocytes has been found to be associated with changes in ankle diameter (Keweifo Okai and Carroll, 1992). Paw swelling was significantly reduced in arthritic rats treated with *Justicia gendarussa*. Among herbal drugs such as
Acalypha indica Linn., Solanum nigrum Linn., Hibiscus populnea, Allium sativum Linn., Asparagus officinalis Linn., Citrus berigamia, Moringa pterygosperma have been shown to contain anti-arthritic properties (Nadkarani and Nadkarni, 1976). Our results agree with the other results of Mahaboobkhan Rasool et al. (2000) in which the Withania somnifera has promising anti-arthritic activity.

A characteristic feature of adjuvant-induced arthritis in rats is the correlation between the development of inflammation and the release of lysosomal enzymes into the extracellular compartment (Weissmann, 1972). Extensive infiltration of leucocytes in the adjuvant-injected paw leads to an increase in the levels of lysosomal enzymes (Anderson, 1970), which in turn initiates the synthesis of inflammatory mediators such as thromboxanes, prostaglandins and leukotrienes. It is likely that reduction of the release of such enzymes would prove beneficial and this indirectly confirms the protective effect of the herbal drug. Drugs capable of stabilizing the lysosomal membrane can reduce inflammation (Agha and Gad, 1995).

Taking into account the results obtained, we decided to evaluate the effect of the plant extract Justicia gendarussa on Delayed Type Hypersensitivity response in rats. The effect of the plant extract on the antigen specific cellular immune response in experimental animals was measured by determining the degree of
DTH reaction using foot pad swelling test. Different medicinal plants were studied for their action on DTH response. The administration of *Trichilia glabra* extract caused a significant reduction in footpad swelling (Benencia *et al.*, 2000). A report revealed that *Picrorhiza kurroa* increased the DTH reaction (Atal *et al.*, 1986). The extract *Nyctanthes arboristis* L. enhanced the DTH response to sheep RBC (Puri *et al.*, 1994).

In the present study, we found that the aqueous extract of *Justicia gendarussa* to inhibit DTH reactivity. The extract was able to diminish in a dose dependent way, the inflammation occurring during the DTH response. The administration of leaf extract at different concentration of (400 mg/kg, 600 mg/kg) caused a significant reduction in foot pad swelling (p<0.05). The interaction of sensitised T-cells with presented antigen is known to be associated with the release of mediators such as histamine products of arachidonic acid metabolism and eventually interferon-γ leading to DTH. Therefore the inhibitory action could be due to an influence of the extract on the biological mediators. The presence of immuno suppressive activity in the aqueous extract suggest that the immunomodulating substances of leaves are essentially in a higher concentration in the aqueous extract which may be responsible for the observed immunomodulatory activity.
In addition to the inhibitory effects on cellular immunity, Justicia gendarussa showed an inhibitory effect on humoral antibody response in a dose dependent way. The effect at 600 mg/kg was found to be higher than that at 400 mg/kg and the difference was statistically significant p<0.05. Alterations in antibody synthesis have been reported for other herbal plants. Achillea talagonica markedly decreased the anti-SRBC antibody titre in a dose dependent manner (Rezacipoor et al., 1999). Holarrhena antidysenterica, Hemidesmus indicus and Tylophora indica inhibited the haemagglutination antibody response in mice (Atal et al., 1986). T and B lymphocytes helps in the production of antibodies to T-cell dependent antigen SRBC. The extract may inhibit phagocytosis on antigen presenting cells or influence the relative amount of different cytokines produced at the stimulation site of T and B cell leading to the inhibition of humoral response to SRBC (Gregg and Denis, 1991; Vos et al., 2000; Luzzati et al., 1997). It should be noted that different dosage schedules, timings and routes of administration of the antigen and the extract may influence different types of antibody response. In our study, the immuno suppression of humoral response was observed when the rats were treated orally 1, 2 and 3 days before and after injection of the SRBC.