Immunosuppressive effects of *Euphorbia hirta* in experimental animals

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**Abstract** *Euphorbia hirta* L. (Euphorbiaceae) (*E. hirta*) is a tree locally used as a traditional medicine in Africa and Australia to treat numerous diseases such as hypertension, respiratory ailments, tumors, wounds, antipyretic, anti-inflammatory activities, etc. Therefore, we undertook to investigate their immunomodulatory effect on T lymphocytes (CD3+, CD4+ and CD8+ receptors) and Th1 cytokines (IL-2, TNF-α, IFN-γ) in a dose-dependent manner. *E. hirta* ethanol extract at 25, 50, 100 and 200 mg/kg doses was given orally for 7 days from the day of immunization. *E. hirta* maximum inhibition at 100 and 200 mg/kg p.o. was found to significantly block the production of the cell-mediated immune response, (CD3+, CD4+ and CD8+ receptors) and (IL-2, TNF-α, IFN-γ) and also prolongs graft rejection. *E. hirta* also showed a decrease of delayed hypersensitivity (DTH) response and dose-related decrease in the primary antibody response, respectively. Based on the data, it can be suggested that *E. hirta* is a potent and non-toxic immunosuppressor, which can be further explored for the development of potent immunosuppressor.

**Keywords** Cytokines · T-lymphocyte · Cyclosporine · Levamisole · Flowcytometry

**Introduction** Activation of immune system involves coordinated interaction of various signaling molecules with several immune cells and facilitation of the cross-talk between these immune cells to evoke a desired immune response. In this respect, lymphocytes and antigen presenting cells are important parts of the immune system which switch over disease-specific cytokines and chemokines secretion during the pathological conditions (Khan et al. 2009). IFN-gamma and IL-2 secreted by Th1 cells can block the proliferation of Th2 cells, and high concentrations of IL-4 or IL-10 can block the generation of Th1 cells from naïve T cells (Kidd 2003). Cyclosporine, tacrolimus, mycophenolic mofetil—the prodrug for mycophenolic acid—and rapamycin are immunosuppressive drugs in use for organ transplantation and to treat some autoimmune diseases. However, these drugs are critical dose related, they exhibit a high degree of interindividual and intraindividual pharmacokinetic and pharmacodynamic variability, which increases the possibility of therapeutic failure if these agents are used at uniform doses in all patients (Wong 2003). There are several medicinal plants that are considered to possess immunomodulatory properties (Mathur et al. 2010). Recently, the understanding of research on immunomodulators has come up as a new field of immunopharmacology (Archana et al. 2011). As a consequence, there continues to be a high demand and challenge to the medical system for new immunosuppressants without any or less side effects (Suna et al. 2005). Medicinal plants serve as therapeutic alternatives, safer choices (Gautam et al. 2007) and a larger number of these plants and their isolated constituents have shown beneficial therapeutic effects including antioxidant, anti-inflammatory, anticancer, antimicrobial, and immunomodulatory effects (Salem 2005).
Keeping in mind the above-mentioned pharmacological benefits of medicinal plants and requirement of novel immunosuppressor, we have made an effort to identify and explore the immunosuppressive properties of *E. hirta*. The genus family *Euphorbia* (Euphorbiaceae or spurge family) is one of the largest families of plant world, with about 300 genera and 7,500 species in non-tropical areas such as the Mediterranean, the Middle East, South Africa, and southern USA (Chellaiah et al. 2006). *E. hirta* exhibits antiamoebic, antibacterial, antimalarial and antioxidant (Tona et al. 2000; Liu et al. 2007; Sharma et al. 2007; Suresh et al. 2008). *E. hirta* is also known to have anti-allergic, antipyretic, anti-inflammatory (Singh et al. 2006; Shih et al. 2010), etc. Recent studies have indicated that *E. hirta* has potent long-term antioxidant properties (Subramanian et al. 2011). The stem sap is used in the treatment of eyelid styes and a leaf poultice is used on swelling and boils (The Wealth of India 2005). *E. hirta* slows down matrix metalloproteinase’s (MMPs) and tissue inhibitors of matrix metalloproteinase’s (TIMPs) in the rat articular cartilage was investigated (Lee et al. 2008). In the present study, we have investigated whether oral administration of *E. hirta* suppresses the immune function, particularly the humoral, cell-mediated immune responses, T-lymphocytes (CD3+, CD4+ and CD8+) and Th1 cytokines (IL-2, TNF-α, IFN-γ) in a dose-dependent manner.

Materials and methods

Extraction of test material

Test material was ground to coarse powder. 500 g of the powdered material was extracted with 95 % ethyl alcohol (2 l) at room temperature by mechanical stirring for 2 h. The extraction process was repeated three times more under similar conditions. Pooled extract was concentrated under reduced pressure and the gummy residue (44 g) was stored in desiccating conditions till further use.

Markers

The marker was isolated from ethanolic extract by column chromatography. 20 g extract was subjected to Silicagel (100–200 mesh) chromatography. The column was eluted using a gradient of CHCl3–CH3OH (100:0–0:100) to afford 20 fractions. All the 20 fractions were checked on TLC (run in n-butanol:acetic acid:water 4:1:5), spots were visualized by freshly prepared borinate–PEG solution (2-aminoethylidiphenylborinate, 1 % in CH3OH:polyethylene glycol-4000, 5 % in C2H5OH, 1:1 v/v). Out of 20 fractions, fraction-8 (eluted in 15 % CH2OH in CHCl3) showed one major spot in TLC. The fraction was subjected to repeated column chromatography on silica gel to obtain a compound. The compound was identified as quercitrin (Eldahshan 2011). It was finally purified by crystallization and identified with the help of 1H, 13C NMR and in comparison with data reported in the literature.

Chemoprofiling

Equipment

The Water HPLC system comprising two Waters 515 HPLC pumps, automatic sampling unit (Waters 717 plus auto sampler), column oven, photodiode array detector (Waters 2996), Merck Rp-18 column (5 μm, 250 × 4.00 mm ID), temperature control module II and Waters Empower software was used for data analysis and data processing.

Experimental conditions

Quercitrin, was quantified in the extract at 30°C, the analysis was performed at a flow rate of 1.0 ml/min using mobile phase consisted of ACN(B):1.5 % AcOH in water (A) [gradient: time in minute (B %): 0 (12), 25 (21), 30 (25), 40 (50), 50 (75), 60 (90), 70 (12)]. The photodiode array detector was set at wavelength of 340 nm for quantification.

Sample preparation and quantification

Accurately weighed quantity of the dried extract (21 mg) was dissolved in 2 ml methanol:water (1:1 v/v mixture) HPLC grade. The sample was centrifuged and filtered through Millipore micro filter (0.45 μm) and was used for analysis. 10 μl from it was injected into the HPLC system (Fig. 1). Quercitrin (1.2 mg) was dissolved in 5 ml methanol:water (1:1 v/v mixture) HPLC grade. From the
solution 2, 4, 6, 8, 10 μl was injected in the HPLC system for plotting of calibration curve (Fig. 2). Linearity in the concentration range of 480–2,400 μg/ml was observed. The marker compounds in the extract were quantified using the calibration curve. It was found that extract contained 0.55 % quercitrin.

Animals

Female inbred Balb/c mice (20–24 g, 10–12 week old) were obtained from animal house of IIIM, Jammu. Animals were employed in groups of six for the study. All the animals were maintained in transparent polycarbonate filter top cages in animal isolator cabins at 22 ± 2 °C with 12 h light/dark cycle and free access to pellet food (Ashirwad India Ltd) and autoclaved water. All experimental protocols and the number of animals used for the experimental work were duly approved by the Institutional Animals Ethics Committee (IAEC) of Indian Institute of Integrative Medicine, CSIR, Canal Road, Jammu, J&K, India, (CPCSEA registration no. 67/99/CPCSEA) according to the Government of India accepted principles for laboratory animal use and care under No. 10/1998-99.

Antigen

Fresh sheep red blood cells (SRBC) were collected aseptically from the jugular vein of sheep and stored in cold sterile Alsever’s solution, was washed three times with pyrogen-free sterile saline (NaCl, 0.9 % w/v) and adjusted to the concentration of 5 × 10⁹ cells/ml for immunization and challenge at the required time schedule.

Effect on general behavior and maximum dose tolerance in mice

The maximum dose tolerance in mice were carried out following (OECD 1996), guidelines No. 423. Graded doses of the test drug were administered orally to group of 8 rats and 10 mice by the method of (Singh et al. 1978). The animals were observed for first 2 h continuously and then at half-hourly interval for next 6 h for changes in reactivity, gait, motor activity, ptosis, respiration rate, writhing, etc. A high-dose toxicity effect resulting into mortality was recorded over 1-week period and the acute oral LD₅₀ was calculated.

Humoral antibody response

Mice were immunized by injecting 20 μl of 5 × 10⁹ SRBC/ml intraperitoneally (i.p.) on day 0, and the blood samples were collected on day +7 (before challenge) for primary antibody. Haemagglutination antibody titres were determined following the microtitration technique described by (Nelson and Mildenhall 1967), BSA-saline alone served as a control.

Skin allograft rejection

The modified method of Billingham and Medawar (1951) was followed to study the skin allograft rejection time in mice. Graded doses of test material were administered to the animals for 7 days and graft rejection time (GRT) was recorded by daily observation of epithelial skin layer survival. Control group was given vehicle only, and another group received cyclosporine as standard at 5 mg/kg body weight daily for 7 days.

Induction and evaluation of delayed type hypersensitivity reaction

The method of Doherty (1981) was followed. E. hirta was administered 2 h after SRBC injection and once daily on consecutive days. Six days later, the thickness of the left hind foot was measured with a spheromicrometer (pitch, 0.01 mm) and was considered as a control. The mice were then challenged by injecting the same amount of SRBC intradermally into the left hind footpad. The foot thickness was measured again after 24 h.

Lymphocyte immunophenotyping

Immunophenotyping focuses on lymphocyte populations involved in acquired immunity. A specific molecule present on the cell surface defines characteristics of lymphocytes such as state of activation or functional capabilities. Immunization of Balb/c mice was carried out by injecting 20 μl of 5 × 10⁹ SRBC/ml (i.p.) E. hirta ethanol extract was carried out for 5 days. Same amount of SRBC was then injected into the mice for the challenge on day 6 and blood was collected after 24 h of challenge in
heparinised tubes from retro-orbital plexus for estimation of CD3+, CD4+ and CD8+ surface activation markers. Murine monoclonal antibodies conjugated to a fluoro-chrome and directed against co-receptors CD3+, CD4+ and CD8+ were used in a multiparametric flowcytometric assay to quantify the lymphocyte subsets associated with the cell-mediated immune response. These antibodies were added directly to 100 μl of whole blood, which was then lysed using whole blood lysing reagent (BD Biosciences). Following the final centrifugation, samples were resuspended in phosphate buffer saline (pH 7.4) and analyzed directly on the flowcytometer (LSR, BD Biosciences) using Cell Quest Pro Software (BD Biosciences).

Intracellular cytokine estimation

Whole blood (100 μl) was pipetted directly into a 12 x 75 mm fluorescence-activated cell sorting tube containing 20 μl of monoclonal antibodies for the T-helper surface antigen CD4+ and CD8+ (BD Biosciences) and incubated at room temperature in the dark for 10 min. Then, 1 % paraformaldehyde (0.5 ml) was added for 10 min to stabilize the monoclonal antibody–surface antigen complex. RBCs were lysed using 2 ml of 1 x lysing solution (BD Biosciences) for 10 min. After centrifugation at 300g for 5 min, the supernatant was aspirated and 1 x permeabilizing solution (500 μl, BD Biosciences) was added into the pellet and incubated for 10 min at room temperature in the dark. After washing with 3 ml buffer (1 % bovine serum albumin, 0.1 % Na3, 1 x PBS), cytokine-specific antibodies (20 μl, IL-2 TNF-α and IFN-γ, BD Biosciences) were added to the cells and incubated for 30 min at room temperature in the dark. After one final wash, cells were resuspended in 1 % paraformaldehyde (500 μl) and stored at 4 °C until flowcytometry analysis. Cells were acquired using a (LSR, BD Biosciences) flowcytometer and data were analyzed using Cell Quest software. A minimum of 10,000 cells was counted from each sample.

Statistical analysis

Data represents mean ± SEM of eight animals. *p < 0.05; **p < 0.01; ***p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons).

Result

Effect on general behavior and maximum dose tolerance in mice

Mice and rats treated with *E. hirta* at a maximum oral dose of 2.500 mg/kg did not show any difference in gross general behavior compared with the control group of animals that were administered only the vehicle. No mortality was observed over an observation period of 7 days.

Humoral immune response

*Euphorbia hirta* (25–200 mg/kg p.o.) produced a dose-related decrease in the primary antibody synthesis. Maximum effect was observed at 100 mg/kg (4.7 ± 0.16 % decrease) after which the suppressive effect influence and was 4.5 ± 0.14 % at 200 mg/kg oral dose. Cyclosporine used as a standard drug showed 3.6 ± 0.13 % decrease in antibody synthesis at the dose of 5 mg/kg oral dose (Table 1).

Skin allograft rejection

Oral administration of *E. hirta* at 25, 50, 100 and 200 mg/kg delayed the skin allograft rejection time in mice (days) by 17.5 ± 1.17, 18.0 ± 1.11, 20.16 ± 1.08 and 22.12 ± 1.05, respectively. Cyclosporine at 5 mg/kg increased the rejection time by 23.2 ± 1.01 % (Table 2).

### Table 1 Effect of *E. hirta* ethanol extract or cyclosporine on humoral immune response (antibody titer) in mouse model (mean ± SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Antibody titer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized control</td>
<td>0</td>
<td>6.5 ± 0.22</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>25</td>
<td>5.6 ± 0.21*</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>50</td>
<td>5.01 ± 0.19**</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>100</td>
<td>4.7 ± 0.16***</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>200</td>
<td>4.5 ± 0.14***</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>5</td>
<td>3.6 ± 0.13***</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

### Table 2 Effect of *E. hirta* ethanol extract or cyclosporine on homologous graft rejection in mouse model (mean ± SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Mortality</th>
<th>Rejection time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized control</td>
<td>0</td>
<td>Nil</td>
<td>13.0 ± 1.25</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>25</td>
<td>Nil</td>
<td>17.5 ± 1.17*</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>50</td>
<td>Nil</td>
<td>18.0 ± 1.11**</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>100</td>
<td>Nil</td>
<td>20.16 ± 1.08***</td>
</tr>
<tr>
<td><em>E. hirta</em> extract</td>
<td>200</td>
<td>Nil</td>
<td>22.12 ± 1.05***</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>5</td>
<td>Nil</td>
<td>23.2 ± 1.01***</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)
Table 3  Effect of E. hirta ethanol extract or cyclosporine on delayed type hypersensitivity (DTH) response in mouse model (mean ± SE)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Dose (mg/kg)</th>
<th>Foot pad thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitized control</td>
<td>0</td>
<td>0.72 ± 0.08</td>
</tr>
<tr>
<td>E. hirta extract</td>
<td>25</td>
<td>0.66 ± 0.02</td>
</tr>
<tr>
<td>E. hirta extract</td>
<td>50</td>
<td>0.60 ± 0.07**</td>
</tr>
<tr>
<td>E. hirta extract</td>
<td>100</td>
<td>0.42 ± 0.04***</td>
</tr>
<tr>
<td>E. hirta extract</td>
<td>200</td>
<td>0.40 ± 0.02***</td>
</tr>
<tr>
<td>Cyclosporine</td>
<td>5</td>
<td>0.36 ± 0.02***</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

Fig. 3 Effect of E. hirta ethanol extract on CD3+ receptors in whole blood. Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard control. *p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

Fig. 4 Effect of E. hirta ethanol extract on CD4+ receptors in whole blood. Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard control. *p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

Fig. 5 Effect of E. hirta ethanol extract on CD8+ receptors in whole blood. Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard control. *p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

Fig. 6 Effect of E. hirta ethanol extract on intracellular IL-2 secreting CD4+ T-cell in whole blood. Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard control. *p < 0.05; ** p < 0.01; *** p < 0.001 compared to sensitized control (analysis of variance, ANOVA followed by Tukey–Kramer for multiple comparisons)

Lymphocyte immunophenotyping

Euphorbia hirta showed effect of 38.3% of CD3+, 22.36% of CD4+ and 12.36% of CD8+ surface activation markers at 200 mg/kg (p.o.) dose, respectively. The sensitized control values were 68.3% of CD3+, 33.7% of CD4+ and 16.4% CD8+ T cells. This shows a significant decrease in CD3+, CD4+ and CD8+ T cells against sensitized group (Figs. 3, 4, 5). Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard drugs.

Effect of E. hirta ethanol extract on IL-2 and TNF-α secreting CD4+ T cells

Oral administration of E. hirta at specified doses showed a significant dose-dependent down-regulation of Th1 cytokines as compared to sensitized control group. E. hirta at 25–200 mg/kg showed a significant down-regulation of IL-2 production where maximum down regulatory effect was
observed at 100 (17.5 %) and 200 mg/kg (15.1 %) dose (Fig. 6), respectively. Similar trend was observed with TNF-α maximum inhibitory effect was seen at 100 (10.04 %) and 200 mg/kg (8.98 %) dose (Fig. 7). Cyclosporine 5 mg/kg and levamisole 2.5 mg/kg were used as standard drugs.

Effect of *E. hirta* ethanol extract on IFN-γ secreting CD8+ T cells estimation

IFN-γ secreting CD8+ T cell estimation was assayed and the effect of *E. hirta* was tested on the release of selected Th1 cytokine IFN-γ. The results are shown in (Fig. 8), indicating that *E. hirta* inhibited IFN-γ production in a dose-related manner. A dose as high as 100 and 200 mg/kg was found to be the most effective for down-regulation of IFN-γ where its effect was (10.91, 8.82 %) of IFN-γ production. The sensitized control values were 15.74 % (Fig. 8), respectively.

**Discussion**

Plant-based, traditional medicine systems continues to play an essential role in health care, with about 80 % of the world’s inhabitants relying mainly on traditional medicines for their primary health care (Owolabi et al. 2007). There are several medicinal plants that are considered to possess immunomodulatory properties (Mathur et al. 2011). This research work focused on the identification of clinically useful and safe products from *E. hirta* ethanol extract that could modulate immune responses. There are now numerous examples in experimental models where modulation of the Th1 balance by administration of recombinant cytokines or cytokine antagonists alters the outcome of the diseases (Stephens et al. 2002). However, their clinical efficacy has been limited and has associated complications (Oberholzer et al. 2000; Wieland et al. 2005). The trends indicate that there is an utmost need for orally active non-peptide compounds that can modulate Th1 balance (Whelan et al. 2003). Levamisole is the only known oral clinically used immunostimulant, which restores suppressed immune function of B and T cells, monocytes and macrophages.

The antibody titre and DTH responses were determined for the assessment of the effect of *E. hirta* on humoral and cell-mediated immune responses. Humoral immunity involves the production of antibody molecules in response to an antigen and DTH reaction is triggered by antigen-specific T cells. DTH reaction is also an important in vivo manifestation of cell-mediated immune response and is characterized by the expansion of antigen-specific Th1 type CD4+ T cells during the initial phase, and an inflammation response by Th1 cytokines released from CD4+ T cells during the effectors phase. The present study evaluated the effect of the ethanol extract on humoral response; its influence was tested on sheep erythrocyte specific haemagglutination antibody titre in mice. It was found to significantly suppress the production of circulating antibodies (Table 1). Whether its suppressive effect on the antibody responses was a direct result of its action on the B cells or an indirect effect via suppression of helper T cell functions is not known. Supporting the hypothesis of T lymphocytes inhibition is the increase in the homologous skin graft rejection time in mice treated with *E. hirta* ethanol extract showing almost 69.2 % inhibition in the rejection (Table 2). In our studies, we found that *E. hirta* showed highly significant and dose-dependent inhibition of SRBC-induced antibody titre and DTH response at 100 and 200 mg/kg, p.o. (Table 3). Th1 response is considered central to regulation of antigen-specific classical cell-mediated functions such as delayed type hypersensitivity (DTH) response and B cell activation (Bourgeols and Corinne 2003). The results suggest that in normal animals,
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levamisole in comparison to *E. hirta* ethanol extract resulted in significantly suppressive humoral and cellular immune responses. Interestingly, in sensitized animals, *E. hirta* extract exhibited dose-dependent suppression of cellular and humoral immune response comparable to cyclosporine (5 mg/kg) indicating unique suppressive profile (Tables 1, 3). The basic mechanism involved in graft rejection time is the suppression of T lymphocytes.

Result suggests that *E. hirta* ethanol extract treatment showed significant decrease in CD3+, CD4+ and CD8+ T-lymphocytes count as compared to sensitized control and levamisole (standard) group (Figs. 3, 4, 5). Hence, comparative study of levamisole and *E. hirta* ethanol extract was planned where effect on Th1 cytokines was studied (in vivo) in immunized Balb/c mice. Since IL-2 is a particularly indicates T-cell activation, *E. hirta* ethanol extract showed significantly lower levels of IL-2 at 100 and 200 mg/kg compared with sensitized control group (Fig. 6). IL-2 plays a central role in the therapeutic manipulation of the immune system. Even more striking was the significantly decreased TNF-α (Fig. 7) and IFN-γ (Fig. 8) at 100 and 200 mg/kg. The results suggested that oral administration of *E. hirta* ethanol extract decreased Th1 response IL-2, TNF-α and IFN-γ cytokines levels against sensitized control. TNF-α is made by many other cells as well as macrophages, which are major source, especially after priming by IFN-γ and inhibition of TNF-α signals is inhibited by the anti-inflammatory effect of *E. hirta* extract. An important reduction of IFN-gamma in *E. hirta* treated groups compared to control indicates inhibition of important cytokine that is implicated in Th1 development by mediating IL-12Rβ2 chain expression (Szabo et al. 1997).

Conclusions

The studies on humoral immune response and cell-mediated immune response on Th1 cytokines and cell surface markers by flowcytometry analysis clearly indicates the immunosuppressive effects of *E. hirta* ethanol extract. It appears very promising in the treatment of autoimmune diseases. The findings demonstrate it to have a potent immunosuppressive potential, which is suggestive of its possible therapeutic usefulness. Its apparent safety over long-term administration is encouraging enough to warrant further studies to explore its possible role in modern clinical practice.

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Conflict of interest The authors declare that there is no conflict of interest.

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Abstract
Stone fruits have been reported to possess various health promoting activities due to their high antioxidant levels. In the current study eight plum, six sweet cherry, five apricot and five peach cultivars grown in Kashmir valley of India were harvested at maturity stage and evaluated for their total antioxidants in terms of total phenols, carotenoids, anthocyanin and vitamin C content as well as their respective antioxidant activities on fresh weight basis (fw). Among the plum cultivar, “Green Gauge”, demonstrated the highest phenolic content (210mg/100g fw) as well as total antioxidant activity (476µmole/g) and in case of sweet cherries, “Siah Gole” cultivars exhibited the highest total phenol content (350mg/100g fw) and respective antioxidant activity (446µmole/g). In case of apricot, though “Quetta” variety showed the highest phenol content but “Hercott” demonstrated the highest antioxidant activity. Likewise in peach “July Elberta” possessing lesser phenol content than “Saharanpuri” cultivar showed highest antioxidant activity. Such type of results suggests that quantity of phenolic content alone does not impart high antioxidant activity to such fruits but the type of phenolic compound equally governs such characteristics. The current investigation suggests that such type of stone fruits rich in phenolic antioxidants comprises a promising functional food group to be used against various oxidative stress related diseases.

Key words: Stone fruits, Phenolics, Vitamin C, Anthocyanin, Carotenoids, Antioxidant activity.

Introduction
Functional Food are foods or dietary components that may provide a health benefit or desirable physiological effects beyond basic nutrition and thus allows us to take greater control of our health through the food choices we make (1). Currently, functional attributes of many traditional foods are being discovered, while new food products are being developed with beneficial components. Rapid advances in science and technology, increasing healthcare costs, changes in food laws affecting label and product claims, an aging population and rising interest in attaining wellness through diet are among the factors fueling whole world’s interest in such functional foods (2). Credible scientific research indicates that there are many clinically demonstrated potential health benefits from food components, specifically to reduce the risk of oxidative stress related diseases and help the body combat such metabolic processes that lead to degenerative conditions (3,4). Examples of foods possessing such active ingredients include fruits and vegetables, whole grains, fortified or enhanced foods and beverages, and some dietary supplements that usually, look, smell and taste the same as their regular counterparts and supplementing the diet with such beneficial phytonutrients may reduce the risk of degenerative diseases during aging.

Historically, fruits and vegetables have been used as medicinal agents and until recently the practice of western medicine involved the prescription of specific plants and foods (5). It is
now well established fact that fruit and vegetable products especially those that are rich in secondary metabolites (frequently called phytochemicals) are gaining increasing interest (6). Epidemiological studies have shown that diet rich in such foods significantly reduce the incidence and mortality rates of degenerative diseases caused by oxidative stress. The protective effect of such fruit and vegetable based foods has been attributed to the fact that they may provide an optimal mix of phytochemicals including natural antioxidants that comprise one of the most common components in functional food production. During recent past in food industry, these antioxidants are widely being used to prevent rotting and help long storage, transportation and easy marketing without deteriorating the quality of the product. Thus, interest on use of antioxidants from natural sources in the form of phytochemicals is remarkably increasing due to their important role in disease prevention of both plants as well as animals (7). In humans, these naturally occurring compounds act by scavenging harmful free radicals implicated in the most common cancers as well as in other degenerative diseases including poor brain function. (8,9). In addition, they are also responsible for induction of enzymes that detoxify carcinogens and also block the progression of cancer by deactivating at least 30 types of agents that may cause cancer (10). In contrast, the synthetic forms of antioxidants have been seen to have entirely different role to play with most of them possessing toxic and carcinogenic effects (11).

During recent years, stone fruits are acquiring new interest mainly due to the fact that they contain some components in different quantities in the form of phenolic compounds and carotenoids with potent antioxidative effects and are thus known to play an important role in human health. Phenolic compounds from such fruits are becoming of great interest as researchers are discovering their functional activities in the form of drugs, colorants, flavors, and antioxidants. Some phenolics share certain biological and chemical properties that might be effective inhibitors of chemical mutagens and/or carcinogenesis. Thus, one of the best approaches to increase the intake of such beneficial compounds is to screen the potent cultivars and increase their concentration inside these fruits by breeding and selection. To best of our knowledge, none of the stone fruit cultivar till date from the Kashmir valley has been analyzed for such important compounds. Thus, in the current study, an attempt has been made to evaluate health promoting secondary metabolites as well as antioxidant activities in different cultivars of plum, cherry, apricot and peach grown in Kashmir valley of India.

Materials and Methods

Chemicals: All chemicals and reagents used in the current study were of analytical grade and mostly purchased from Sigma chemicals (India).

Sample collection: In this study eight plum, six cherry, five apricot and five peach cultivars were procured from various fields of SKUAST (K), Shalimar, Srinagar, Kashmir as well as from local market at fresh maturity stage. The fruits were selected according to uniformity of size, shape and colour and then transported to the Biochemistry and Molecular Biotechnology Laboratory, Division of Post Harvest Technology, SKUAST (K), Shalimar Campus, Srinagar within an hour for analysis. All the measurements were conducted in triplicates.

Estimation of Vitamin C, Anthocyanin and Carotenoids: Estimations were performed as per methods of Rangana (12). For Vitamin C estimation 10 ml of sample extract was taken in volumetric flask and made upto 100ml volume with metaphosphoric acid and filtered. 10 ml of filtrate was pipetted into conical flask and titrated against the standard dye solution to a pink end point. Total anthocyanin content in samples was determined by extracting with ethanolic HCl and measurement of colour determined at the wavelength of maximum absorption. The content was calculated by making use of the $e_{max}$ (molecular extinction coefficient) value as 98.2.
For estimation of carotenoids, the samples were extracted in acetone and transferred to petroleum ether phase. Total carotene was read colorimetrically using petroleum ether for baseline correction.

**Total phenolic content analysis:** Total phenolic content was determined according to Singleton and Rossi method (13). Ten grams of sample was thoroughly crushed and homogenized in mortar pestle with 10 ml of 80% ethanol. The extract was centrifuged at 10,000 rpm for 15 min at 4°C and supernatant preserved. The pellet following was resuspended in 5 ml of 80% ethanol, centrifuged and the resulting supernatant combined with initial extract. Triplicate supernatant extractions were made for each sample. The pooled ethanolic extracts were evaporated to dryness. The evaporated extracts were solubilised in 5ml distilled water and used for the estimation of total phenolics. 500ìl of the sample extract was combined with 2.5ml of double distilled water and 0.5ml of Folin cioculateau reagent. After 3min of incubation period, 20% sodium carbonate was added to each sample, vortexed and boiled in a water bath for exactly one min. The absorbance was measured at 650nm against reagent blank A standard curve was established using catechol. Absorbance values were converted to milligram of phenolics per 100g of fresh tissue. For each cultivar three replicates were analyzed.

**Determination of total antioxidant activity :** The total antioxidant potential of samples were determined using using FRAP assay of Benzie and Strain (14) as a measure of antioxidant power and extracts were prepared as described above for estimation of total phenols. The assay was based on the reducing power of a compound (antioxidant). A potential antioxidant reduces the ferric ion (Fe³⁺) to the ferrous ion (Fe²⁺) that forms a blue complex (Fe³⁺/TPTZ). FRAP reagent consisted of 10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40mM HCL, 300 mM sodium acetate buffer (pH 3.6) and 20mM ferric chloride in distilled water in the ratio of 1:1: 10 (v/v). A 100 ml extract was added to 3ml of FRAP reagent and mixed thoroughly. After standing at ambient temperature (20°C) for 4 min, absorbance at 593 nm was noted against reagent blank. Calibration was against a standard curve (50-1000 mmol ferrous ion) produced by the addition of freshly prepared ammonium ferrous sulfate. Values were obtained from three replications and expressed as mmol FRAPg⁻¹ fresh weight

**Statistical analysis:** Three replicates of each sample were used for statistical analysis. Analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) and regression analysis. Differences at P < 0.05 were considered to be significant.

**Results and Discussion**

Recent reports have shown that a healthy diet containing high plant based antioxidants could prevent approximately 30% of all cancers (15). No doubt genetic manipulations can increase such antioxidant constituents of fruits and vegetables, however there is a limit beyond which increased concentrations may cause undesirable levels of astringency in these crops (16). Therefore, plant breeders and food producers are increasingly identifying specific genotypes and varieties of fruits and vegetables rich in functional ingredients comprising of nutritive and non-nutritive antioxidants e.g. anthocyanin, carotenoids, phenols etc. Among different types of fruits, stone fruits such as plums, peaches and apricots, have been found to be successful in killing cancer cells and are known to play an important role in human health due to the range of antioxidant rich phytochemicals especially phenolic compounds. However, before recommending such fruits as functional foods, further studies including rapid selection procedures, secondary effects of phenolics on fruit quality and postharvest traits, and the bioactive properties of selected fruit genotypes are needed. (17). In the current study different cultivars of stone fruits grown in Kashmir valley of India, were screened for their health promoting effects in terms of their antioxidant activities as well as various antioxidants present in their respective extracts. As previous reports
have shown (18) that phenol extraction from plant tissue in ethanol is preferred over extraction in water due to the presence of water-soluble antioxidant vitamins and sugars that may mask the antioxidant activity of polyphenols therefore, all the extracts in the current study were prepared in ethanol.

Amoung the stone fruits large number of plum cultivars have been introduced into India and it has been found that European plums perform better in the hills, while as Japanese plums adopted more in sub-mountainous lower elevations. In order to develop a database of plums grown in Kashmir valley of India, eight different cultivars were analyzed for their secondary metabolites including total phenols, vitamin C, carotenoids and anthocyanin in addition to their respective antioxidant activities. As per previous reports the anthocyanin content of plums range from 4.41 to 23.12 mg/100g fresh weight (f.w.) and total phenolic content from 298 to 563 mg/100g f.w.. Further, anthocyanin content and phenolic content were reported to be well correlated with the antioxidant activities (19). In the current study the anthocyanin and phenolic content were found to be variable in red and yellow flesh plum varieties. It was observed that anthocyanin content slightly increased with the red colour intensity, which ranged from 15.25 mg/100g f.w for “Wickson” to 45.8 for “Satsuma” (Table 1). Interestingly, our results clearly demonstrated that most of the plum varieties evaluated in this study possess higher levels of anthocyanin content than those reported previously and thus put them in the category of other fruits rich in anthocyanin content.

Previously it has been reported that total phenol content of different fruits and vegetables, can vary from 2-500 mg/100g f.w. and in case of plum it falls in the range of 125.0 to 372.6 mg/100 g f.w. (20). Interestingly, in this current study the total free phenolic content of most of the selected cultivars was found to fell in the range of that found in apple varieties i.e. 117- 430 mg/100g f.w (21). It was noteworthy that “Green Gauge” variety that is very unpopular in Kashmir valley recorded significantly highest phenol content (210 mg/100gm fw) followed by “Santa Rosa” (205 mg/100gfw), Burbank (200 mg/100gfw), Grand Duke (198mg/100gfw), Wickson (180 mg/100gfw), Satsuma (165g/100gfw), Warwick (112mg/100gfw) and Reine Claude-de-Bary (78 mg/100gfw) respectively.

As large number of reported scientific data indicates a strong correlation between total phenol content and antioxidant activity of fruits and vegetables (18, 22). Therefore, rather than measuring only the antioxidant contents, there is an increasing interest in the measurement of total antioxidant activity of crops by using various assay methods like ferric reducing antioxidant

<table>
<thead>
<tr>
<th>Plum Cultivar</th>
<th>Vitamin C (mg/100g)</th>
<th>Total carotenoids μg/100g</th>
<th>Anthocyanin (mg/100g)</th>
<th>Total Phenols (mg/100g)</th>
<th>Antioxidant activity (μmole/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Burbank</td>
<td>5.01</td>
<td>320</td>
<td>17.8</td>
<td>200</td>
<td>442</td>
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<tr>
<td>Green Gauge</td>
<td>7.2</td>
<td>433</td>
<td>22.9</td>
<td>210</td>
<td>476</td>
</tr>
<tr>
<td>Wickson</td>
<td>4.8</td>
<td>560</td>
<td>15.25</td>
<td>180</td>
<td>382</td>
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<tr>
<td>Satsuma</td>
<td>5.30</td>
<td>820</td>
<td>45.8</td>
<td>165</td>
<td>326</td>
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<tr>
<td>Grand Duke</td>
<td>3.8</td>
<td>520</td>
<td>35.6</td>
<td>198</td>
<td>441</td>
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<tr>
<td>Warwick</td>
<td>6.2</td>
<td>300</td>
<td>29.5</td>
<td>112</td>
<td>191</td>
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<tr>
<td>Reine Claude-de-Bary</td>
<td>4.9</td>
<td>400</td>
<td>20</td>
<td>78</td>
<td>209</td>
</tr>
<tr>
<td>Santa Rosa</td>
<td>6.3</td>
<td>850</td>
<td>33.5</td>
<td>205</td>
<td>335</td>
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</table>

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Table 2. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh cherry cultivars

<table>
<thead>
<tr>
<th>Cherry Cultivar</th>
<th>Vitamin C (mg/100g)</th>
<th>Total carotenoids (μg/100g)</th>
<th>Anthocyanin (mg/100g)</th>
<th>Total Phenols (mg/100g)</th>
<th>Antioxidant activity (μmole/g)</th>
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</thead>
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<tr>
<td>Makhmali</td>
<td>3</td>
<td>1400</td>
<td>159</td>
<td>190</td>
<td>307</td>
</tr>
<tr>
<td>Awal No</td>
<td>2</td>
<td>1650</td>
<td>134</td>
<td>190</td>
<td>288</td>
</tr>
<tr>
<td>Siah Gole</td>
<td>5</td>
<td>1800</td>
<td>182</td>
<td>350</td>
<td>446</td>
</tr>
<tr>
<td>Double</td>
<td>4</td>
<td>2100</td>
<td>134</td>
<td>180</td>
<td>300</td>
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<tr>
<td>Misri</td>
<td>2.3</td>
<td>1700</td>
<td>122</td>
<td>325</td>
<td>394</td>
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<td>Tontal</td>
<td>1.9</td>
<td>1870</td>
<td>102</td>
<td>400</td>
<td>201</td>
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Table 3. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh Apricot cultivars

<table>
<thead>
<tr>
<th>Apricot cultivars</th>
<th>Vitamin C (mg/100g)</th>
<th>Total carotenoids (μg/100g)</th>
<th>Anthocyanin (mg/100g)</th>
<th>Total Phenols (mg/100g)</th>
<th>Antioxidant activity (μmole/g)</th>
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</thead>
<tbody>
<tr>
<td>Charmagz</td>
<td>8</td>
<td>840</td>
<td>9.2</td>
<td>300</td>
<td>149</td>
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<tr>
<td>Gilgiti sweet</td>
<td>10</td>
<td>600</td>
<td>12</td>
<td>303</td>
<td>202</td>
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<tr>
<td>Hercot</td>
<td>8.5</td>
<td>450</td>
<td>8</td>
<td>379</td>
<td>276</td>
</tr>
<tr>
<td>Quetta</td>
<td>7.6</td>
<td>620</td>
<td>6.9</td>
<td>385</td>
<td>165</td>
</tr>
<tr>
<td>Halman</td>
<td>6.12</td>
<td>110</td>
<td>6.3</td>
<td>346</td>
<td>240</td>
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</table>

Table 4. Quality characteristics in terms of antioxidant composition and antioxidant activity of fresh Peach cultivars

<table>
<thead>
<tr>
<th>Peach cultivars</th>
<th>Vitamin C (mg/100g)</th>
<th>Total carotenoids (μg/100g)</th>
<th>Anthocyanin (mg/100g)</th>
<th>Total Phenols (mg/100g)</th>
<th>Antioxidant activity (μmole/g)</th>
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</thead>
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<td>Saharanpuri</td>
<td>8</td>
<td>600</td>
<td>6</td>
<td>952</td>
<td>160</td>
</tr>
<tr>
<td>July Elberta</td>
<td>7.5</td>
<td>652</td>
<td>5</td>
<td>361</td>
<td>452</td>
</tr>
<tr>
<td>Elberta</td>
<td>6.90</td>
<td>430</td>
<td>7</td>
<td>287.4</td>
<td>241</td>
</tr>
<tr>
<td>Quetta</td>
<td>6</td>
<td>940</td>
<td>5</td>
<td>538</td>
<td>150</td>
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<tr>
<td>Awal No</td>
<td>4</td>
<td>441</td>
<td>7</td>
<td>309</td>
<td>132</td>
</tr>
</tbody>
</table>

power (FRAP) assay developed by Benzie and Strain (14). In this study the antioxidant activity measured by FRAP assay in red/purple-flesh varieties of plum was found to be higher as compared to light colored yellow flesh plums. Interestingly, Green Gauge cultivar possessing highest phenolic content also demonstrated maximum value of antioxidant activity (476 mmol/g f.w.) followed by Burbank (442mmol/g f.w.), Grand Duke (441mmol/ 100g f.w.), Wickson (382mmol/ g f.w.), Santa Rosa (335mmol/ g f.w.), Satsuma (326mmol/ g f.w.), Reine Claude-de-Bary (209mmol/ g f.w.) and Warwick (191mmol/ g f.w.). The correlation developed between total antioxidant activity (Y) and total phenolic content (X) of these selected plum varieties had a high

Antioxidant power of stone fruits
correlation coefficient of $R^2=0.790$. Whereas in contrast to earlier reports, total anthocyanin and total antioxidant activity showed least correlation with the correlation coefficient of only $R^2=0.013$. It can be suggested that green guage cultivar of plums is a promising fruit with rich antioxidant composition (especially phenolic content) as well as antioxidant activity and can be better exploited to be used as functional food.

Likewise, sweet cherries have been reported to possess many health promoting activities due to presence of many secondary metabolites including phenols. Prvulovic et al (23) reports that phenolic composition of sweet cherries is genotype dependent as well as influenced by climatic conditions. In the Kashmir Agricultural market, fresh sweet cherries represent an important, but fragile, commodity. As evident from table 2, the antioxidant composition and antioxidant activities of selected six cherry cultivars viz Makhmali, Awal Number, Siah Gole, Double, Misri and Tontal were found to be highly variable. It was observed that total phenol content in the extracts of the freshly harvested cherry samples varied from ~180 - 350 mg/100 g fw. Among the selected cultivars “Siah Gole” cultivar exhibited the highest antioxidant activity (446 ì mole/g f w) as compared to other varieties. In this type of stone fruit also strong correlation ($R^2 = 0.782$) was observed between total phenolic content and total antioxidant activities. These results are very well in accordance to previous reports that indicate anthocyanins did not seem to be the only important antioxidant to influence the antioxidant activity of the fruits, when correlating with DPPH data (23).

Among stone fruits apricot possesses very high carotene content that plays an important role in maintenance of human health. Due to presence of carotene and lycopene this fruit has been reported to have antipyretic, antiseptic, emetic, and ophthalmic properties and also protect heart and eyes (24). As the levels of antioxidant compounds especially phenolic compounds have been reported to be different in apricot varieties (25). Therefore, genotype variations in terms of antioxidant composition and antioxidant activities of five apricot cultivars grown in Kashmir valley were also evaluated in the current study. The ethnolic extracts from these cultivars demonstrated variable levels of antioxidant composition with Gilgit Sweet demonstrating the highest Vitamin C content and Halman the lowest (Table 3). Likewise Charmagz showed the highest Carotenoid content and Halman the lowest. Highest phenolic content was found in Quetta and lowest in Charmagz variety. Interestingly, in this type of fruit the Hercot variety possessing intermediate phenol content showed the highest antioxidant activity as compared to “Quetta” and though a good correlation ($R^2 = 0.782$) was found between total phenol content and total antioxidant activity in almost all the fresh apricot varieties. Such type of results suggests that quantity of phenolic content alone does not impart high antioxidant activity to such fruits but the type of phenolic compound equally governs such characteristics.

Previous reports have shown that carotenoid content in yellow-flesh peaches is higher (2-3 mg carotene/100 g fw-fresh weight) than as found in white or red-flesh one (0.01-1.8 mg carotene/100 g fw). In contrast antioxidant activity has been reported to be about 2-fold higher in red-flesh varieties than in white/yellow-flesh peach varieties that correlated best with their respective phenolic content (19). In our study as shown in table 4, Saharanpuri peach cultivar showed the highest Vitamin C content and Quetta the highest carotenoid content. Saharanpuri cultivar possessed the highest phenol content and Elberta the lowest. Interestingly, in these selected peach cultivars also, inspite of demonstrating a good correlation ($R^2 = 0.648$) between total phenolic content and total antioxidant activities, “July Elberta” cultivar possessing intermediate phenol content demonstrated the highest antioxidant activity as found in case of apricot cultivars.

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Conclusion

It is suggested from the present study that these stone fruits grown in Kashmir valley comprise a promising group with wide range of genetic variability in terms of antioxidant composition (especially phenolic content) as well as antioxidant activities. The fruits were found to show better correlation between total phenolic content and their respective antioxidant activities. In plum it is Green Gauge, in cherry it is Siah Gole, in apricot it is Hercott and in peach it is July Elberta that are recommended to all age groups for better consumption per day due to their highest antioxidant activities. The current study signifies that such fruits due to high antioxidant power can be further exploited to be used as strong antidegenerative food as well as health promoting functional foods. Commercial value added powder can be formulated from them for designer foods that can also be included in breakfast cereals, snacks, confectioneries, backed goods and pet foods as health promoting food. In addition, it is suggested that such cultivars could be incorporated into the breeding programmes for development of antioxidant rich germplasm of stone fruits in the area.

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References


Antioxidant power of stone fruits


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In vivo comparison of probiotic bacteria’s with their DNA: 
As augmenter of immune efficacy

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ABSTRACT

The recent increase in the incidence of infectious diseases or immunological disorders raises a need to find new immunomodulators. Probiotics have been shown to be one of the alternative agents which strengthen the immune response of the body. But recently bacterial DNA is also being explored as an immune enhancer. Present study was conducted to compare the in vivo immunomodulating capacity of three probiotic strains as live bacteria with their genomic DNA. Probiotic bacteria’s ($10^9$ cells mL$^{-1}$) were administered orally whereas their extracted DNA’s (75 µg mL$^{-1}$) were injected into the tibialis anterior muscle in 3 doses over a span of 17 days. The animals were sacrificed after the completion of experimental period i.e. 17 days. Immune status of the treated animals was assessed by employing the tests for Humoral Immune Response and Cell Mediated Immune Response as Delayed Type Hypersensitivity, Nitroblue Tetrazolium Reduction test, Inducible Nitric Oxide Synthase and Bactericidal activity was studied in SRBC immunized mice. Levamisole (25mgkg$^{-1}$) was used as the standard drug. Overall, these results demonstrated that a substantial augmentation in immune efficacy was observed in the animals receiving genomic DNA over the group receiving viable bacteria. It is concluded that genomic DNA of probiotics should be exploited as a potent immune enhancer and as a biotherapeutic agent.

Keywords: Lactobacillus, Bifidobacterium, Immunomodulatory activity, Bacterial DNA, Humoral Immune Response, Cell Mediated Immune Response.

INTRODUCTION

Probiotics are the live microorganisms that confer health benefits to the host animal when administered in adequate amounts [1]. Probiotics have nutritional benefits, they improve lactose utilization, have anti-cholesterol, anti-carcinogenic activities, anti-mutagenic, anti-infectious, immunomodulating activities, assists in preservation and in single cell protein production [2-7].

The immune system is known to be involved in the etiology as well as pathophysiologic mechanism of many diseases. Modulation of immune response to alleviate the disease has been interest for many years [8]. The function and efficacy of immune system may be influenced by many exogenous factors like food and pharmaceuticals, physical and psychological stress and hormones etc. resulting in either immune stimulation or immunosuppression
Some bacterial cell components such as peptidoglycans, lipoteichoic acid, secreted soluble substances [9,10] and genomic DNA [11] reportedly play role in immunomodulation responses but primary component is yet to be identified.

Structural difference between bacterial and eukaryotic DNA apparently account for the ability of bacterial DNA to serve as an immune activating agent. Specifically, bacterial DNA is thought to activate inflammatory cells because of its high content of short sequences with unmethylated CpG dinucleotides [12]. In mammalian DNA, CpG containing sequences occur at a much lower frequency than in bacterial DNA, and the cytosine present in CpG dinucleotide of mammalian DNA is usually methylated [13,14]. However, unmethylated bacterial DNA motifs comprising cytosine linked to guanine by a phosphate bond (CpG motifs), also known as immunostimulatory sequence (ISS) oligodeoxynucleotides (ODNs), are reportedly mitogenic for murine B cells [12]. Bacterial DNA and immunostimulatory CpG-ODNs activate Antigen Presenting Cells (APCs) such as macrophages and dendritic cells. Cell activation occurs upon DNA endosomal uptake, resulting within minutes in activation of the Stress Kinase pathway and NF-kB. As a consequence, APCs produce cytokines including IL-12, IL-6 and IL-1 and upregulate coreceptor molecules [15].

The importance of present study lies in the fact that probiotic bacteria are used as immunomodulators but a literature survey revealed that DNA of probiotics have not been studied for immune response. The purpose of current study was to compare in vivo, immunomodulatory activity of probiotic viable bacteria with its isolated genomic DNA.

**MATERIALS AND METHODS**

2.1 **Bacterial strain and culture condition**

The strain of *Lactobacillus delbrueckii* 405 (LB 405), *Lactobacillus brevis* 403 (LB 403), *Bifidobacterium bifidium* BD4 234 (Bif 234) was procured from National Dairy Research Institute, Karnal, Haryana. The cultures so obtained were given two revival cycles in de Man–Rogosa–Sharpe broth (MRS broth) at 37 °C. Bacterial cultures were grown and maintained for further use. For genomic DNA preparation, cells were grown in the corresponding medium containing 1 to 1.5 % glycine to facilitate cell lysis [16].

2.2 **Preparation of genomic DNA of bacterial strain**

Genomic DNA was isolated and purified with several modifications [16]. Briefly, an overnight culture (1.5 ml) was pelleted at 14000 rev min⁻¹ (microcentrifuge) 25°C for 5 minutes and resuspended in 500µL EDTA (50mM⁻¹). 100 µL of 30mgml⁻¹ Lysozyme was added to cell suspension and incubated for 60 minutes at 37°C. Cell lysis was achieved using NaOH/SDS solution (pH 12.5) and incubation 20 min at 37°C followed by 10 min incubation on ice. Protein removal was carried out with phenol followed by chloroform:isoamyl alcohol (24:1) extraction. DNA was precipitated by addition of isopropanol and washed with 70% ethanol to remove residual contamination. DNA was then resuspended in 20-30 µL of TE (Tris 10mM, EDTA 1mM pH 8.0). The concentration and purity of DNA were analyzed spectrophotometricaly (Shimadzu, UV-1650 PC spectrometer) by measuring OD₂₆₀/OD₂₈₀. Only the DNA with OD₂₆₀/OD₂₈₀ ratio ranging between 1.8 and 2.0 respectively was used. The quality of DNA was further analyzed on 1 % agarose gel (100V for 20-40 min) containing 0.5 µgm⁻¹ ethidium bromide. The endotoxin level in the DNA preparation were <0.001 ngµg⁻¹ of DNA according to Limulus amebocyte lysate assay.

2.3. **Mice**

Swiss albino male mice (18-22gm) maintained on standard laboratory diet (Kisan Feeds Ltd., Mumbai, India) and water *ad libitum* were employed in the present study. The animals were divided into respective groups each of minimum six animals, housed individually in the departmental animal house and were exposed to 12 hr cycle of light and dark. The experimental protocol was approved by Institutional Animal Ethical Committee (Registration No: 107/99/CP-CSEA-2010-40) were carried out as per the guidelines of committee for Purpose of Control and Supervision on Experimental on Animals (CPCSEA) Ministry of Environment and Forest, Government of India.

2.3.1 **Experimental animal design: Animals were divided into nine major groups:**

- **Group I:** Untreated Control group (not subjected to any treatment i.e. kept only on diet)
- **Group II:** Positive control (25mgkg⁻¹ Levamisole i.p for 17 consecutive days)
- **Group III:** Immunized control (mice sensitized with SRBC and kept on normal diet)
- **Group IV:** Lactobacillus delbrueckii 405 (LB 405) for 17 consecutive days at the rate of 10⁹ cells day⁻¹ mouse⁻¹ as oral dose.
Group V: DNA of Lactobacillus delbrueckii 405 (DNA LB 405) three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL⁻¹ mouse⁻¹.

Group VI: Lactobacillus delbrueckii 403 (LB 403) for 17 consecutive days at the rate of 10⁶ cells day⁻¹ mouse⁻¹ as oral dose.

Group VII: DNA of Lactobacillus delbrueckii 403 (DNA LB 403) three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL⁻¹ mouse⁻¹.

Group VIII: Bifidobacterium bifidium BD4 234 (Bif 234) for 17 consecutive days at the rate of 10⁹ cells day⁻¹ mouse⁻¹ as oral dose.

Group IX: DNA of Bifidobacterium bifidium BD4 234 (DNA Bif 234) three injections in left tibialis anterior muscle [17] after 6 days at the rate of 75µg mL⁻¹ mouse⁻¹.

2.3.2 Immunization
Sheep blood was collected in Alsever’s solution in the ratio 1:2 and was centrifuged at 400 × g for 10 min at 4 ° C. The erythrocyte pellet obtained was washed and suspended in PBS (0.1 M, pH 7.2) for further use as per [18]. All mice were antigenically challenged intraperitoneally with a single dose (100µl ml⁻¹ of 1 × 10⁷ cells/ml) of sheep red blood cells (SRBC).

2.4. Humoral Immune Response
To assess the humoral immune response, blood was withdrawn from retro-orbital plexus of all SRBC antigenically challenged animals on day 0 (pre-immunized), 8th and 13th (post immunization). The serum was separated and assayed by direct haemagglutination [19]. Titer was described as highest dilution capable of visible agglutination. The results were expressed as mean ± S.E.M. log titer of individual animals.

2.5. Cell mediated immune response
2.5.1. Delayed Type Hypersensitivity assay
Delayed Type Hypersensitivity response (DTH) was checked by foot pad swelling method [20]. All SRBC primed groups were challenged intradermally on day 15th with SRBC suspension (1 × 10⁷ 100µl saline⁻¹) in the hind footpad. The control lateral paw was given equal volume of saline. Paw thickness was measured with micro-caliper at 24h interval up to 72h. The difference in paw thickness compared to control was taken as a measure of DTH and expressed in millimeter. Results are expressed as mean ± S.E.M. of footpad thickness up to 72h.

2.5.2. Total lymphocyte isolation from the spleen
Spleen was excised aseptically and lymphocytes were isolated by teasing the tissue. Cells were centrifuged (400 × g for 10 min at 4 ° C) and lysed by ACK lyses solution (0.5M NH₄Cl, 10mM KHCO₃ and 0.1 mM disodium EDTA, pH 7.2). Lymphocytes obtained were washed thrice in PBS, counted and adjusted to desired concentration in RPMI for further use.

2.5.3. Nitroblue Tetrazolium Reduction assay
NBT reduction test was evaluated by employing the method described by [21]. Briefly, the lymphocyte suspension was incubated with NBT and formazion formed was extracted in dioxan. The reduction in NBT was measured spectrophotometrically at 520 nm (Shimadzu, UV-1650 PC) against dioxan as blank. The results were expressed as mean ± S.E.M. of percentage dye reduced to formazion.

2.5.4. Inducible Nitric Oxide Synthase activity
Inducible nitric oxide synthase activity in lymphocyte suspension was evaluated by a previously described procedure by [21] using arginine. The color developed (indicating presence of citrulline) was measured spectrophotometrically at 540nm against RPMI and Griess reagent as blank. The results were expressed as mean ± S.E.M. of percentage enzyme produced.

2.5.5. Bactericidal activity
Bactericidal activity was determined by [21]. Briefly, the lymphocyte suspension was incubated with bacterial suspension (Escherichia coli) at 37° C for 60 min. The lymphocytes were lysed with sterile distilled water spread on agar plate and incubated at 37 °C for 24 h. Bacterial suspension was spread in the control plate. Number of colony forming units (CFU) developed in control and test plates were counted and results were expressed as mean ± S.E.M. of bactericidal activity.
2.6. Statistical Analysis

All the results were expressed as mean ± S.E.M. Data of tests were statistically analyzed using one-way ANOVA followed by Turkey’s multiple range test, applied for post hoc analysis. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

RESULTS

3.1. Humoral Immune Response

In all groups i.e. Positive treated, Immunized Control, LB 405, DNA LB 405 ,LB 403, DNA LB 403, Bif 234 and DNA Bif 234 no anti SRBC antibody titer was observed on day 0. DNA LB 405 had a significantly higher antibody titer as compared to LB 405 (neat culture) as depicted in (Figure 1). The anti SRBC antibody titer of DNA LB 405 was found to be comparable to that of levamisole treated group which is an immune enhancer. Similarly, DNA of LB 403 and Bif 234 showed higher antibody titer as compared to neat cultures.

![Figure 1. Effect of different groups on production of anti- SRBC antibody titer on pre-immunization(0th day) and post-immunization (8th and 13th day).](image)

* * p<0.05 in comparison to sensitized control

Table 1 Delayed type hypersensitivity response

<table>
<thead>
<tr>
<th>Animal Groups</th>
<th>Time periods (h) after SRBC challenge</th>
<th>0</th>
<th>24</th>
<th>48</th>
<th>72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated Control</td>
<td>1.69 ± 0.01</td>
<td>1.69 ± 0.01</td>
<td>1.69 ± 0.01</td>
<td>1.69 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Positive Control</td>
<td>1.70 ± 0.02</td>
<td>1.86 ± 0.02</td>
<td>2.01 ± 0.04</td>
<td>1.81 ± 0.03</td>
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</tr>
<tr>
<td>Immunized Control</td>
<td>1.68 ± 0.01</td>
<td>1.72 ± 0.01</td>
<td>1.70 ± 0.01</td>
<td>1.67 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>LB 405</td>
<td>1.69 ± 0.01</td>
<td>1.77 ± 0.03</td>
<td>1.82 ± 0.01</td>
<td>1.73 ± 0.03</td>
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</tr>
<tr>
<td>DNA LB 405</td>
<td>1.70 ± 0.02</td>
<td>1.87 ± 0.02</td>
<td>2.11 ± 0.01</td>
<td>1.77 ± 0.01</td>
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</tr>
<tr>
<td>LB 403</td>
<td>1.67 ± 0.01</td>
<td>1.74 ± 0.02</td>
<td>1.80 ± 0.01</td>
<td>1.70 ± 0.01</td>
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</tr>
<tr>
<td>DNA LB 403</td>
<td>1.69 ± 0.01</td>
<td>1.83 ± 0.02</td>
<td>2.06 ± 0.02</td>
<td>1.73 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Bif 234</td>
<td>1.66 ± 0.02</td>
<td>1.70 ± 0.02</td>
<td>1.71 ± 0.01</td>
<td>1.52 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>DNA Bif 234</td>
<td>1.67 ± 0.01</td>
<td>1.72 ± 0.01</td>
<td>1.74 ± 0.02</td>
<td>1.60 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

The results are presents as mean ± S.E.M (n=6)

*p<0.001 in comparison to untreated control
**p=0.05 in comparison to sensitized control
3.2. Cell mediated immune response

3.2.1. Delayed type hypersensitivity

Effect of viable bacteria and DNA on T-cell response was studied by assessing the footpad swelling as a measure of Delayed type hypersensitivity. In untreated control group, no rise in footpad thickness was observed. However, DNA treated groups showed significant (p< 0.05) elicitation of the T-cells response as evident by an increase in footpad thickness as compared to antigen sensitized control group and groups receiving neat cultures. It was found that DNA of LB 405 and LB 403 showed comparable rise in footpad thickness as to that of levamisole treated group after 48 hours (Table 1).

3.2.2. iNOS activity

Cell mediated immune response is indicated in Figure 2. It was seen that DNA LB 405 treated group showed maximum activity which was significantly higher in comparison to control (p< 0.001) and orally treated group (LB 405). In, DNA treated group of LB 405 iNOS activity was 27.9 % higher than LB405 group (neat culture). Similarly DNA treated groups of LB 403 and Bif 234 showed higher iNOS activity than neat culture groups of LB 403 and Bif 234 respectively.

3.2.3. NBT reduction

LB 405 neat culture and its genomic DNA treated group significantly increased (p<0.05) NBT reduction as compared to immunized control group. Similar to iNOS activity, NBT reduction activity of DNA LB 405 treated group was 24.05 % higher than LB 405 neat culture group (Figure 2).

3.2.4. Bactericidal activity

The effect of Probiotic DNA on bactericidal activity was studied in terms of number of colony forming units (CFU). The treatment of animals with DNA treated groups (DNA LB 405, DNA LB403, DNA Bif 234) reduced the number of colonies and thus enhanced the bactericidal activity as compared to neat culture groups (LB 405, LB 403, Bif 234) (Figure 2).

![Graph showing influence of different groups on iNOS activity, NBT reduction and Phagocytic activity. The results are expressed as mean ± S.E.M (n=6)](image)

* p< 0.001 in comparison to untreated control
** p<0.05 in comparison to sensitized control