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# Gamma delta T cell stimulatory activity of Tea and Indian herb extracts

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**Abstract:** Herb extracts have been used in Indian traditional medicine (*Ayurveda*) to treat various ailments and also shown to have immunomodulatory effects. The present study evaluated the ability of these extracts to activate pool of gamma delta (γδ) T lymphocytes that play an important role in enhancing host responses to infections and cancer. Peripheral blood mononuclear cells (PBMCs) were isolated form peripheral blood of healthy individuals and stimulated with aqueous extracts of herbs, ethylamine, IPP and BrHPP for 12 days. Phenotype of expanded γδ T lymphocytes was determined by dual color flow cytometry using subset specific ($V\delta^{2+}CD^{3+}$, $V\delta^{1+}CD^{3+}$, $\alpha\beta^{+}CD^{3+}$) fluorochrome labeled antibodies. Immunomagnetically purified γδ T lymphocytes were stimulated with herb extracts and IFN-γ, TNF-α, IL-4, IL-10 released in cell free supernatants was quantitated by ELISA.Our results demonstrate that the aqueous extracts of Jeevani (*Tricopus zeylanicus*), Black tea (*Camellia sinensis*) and American ginseng (*Panax quinquefolius*) exhibited the ability to significantly ($P < 0.05$) expand γδ T lymphocytes from PBMC during in vitro culture. The activated γδ T lymphocytes expressed activation markers CD25 and CD69 and released IFN-γ and TNF-α. Priming of γδ T lymphocytes with these herb extracts would be a promising approach to enhance immunity to bacterial infections and cancer.

**Keywords:** *Immunomodulation; γδ T lymphocytes; Interferon-γ;* Tricopus zeylanicus*; Phosphoantigens; Tea; Herb extracts*

## Introduction

Human gamma delta (γδ) T lymphocytes represent a distinct subset of T lymphocytes expressing γδ T cell receptor (TCR) on their surface. γδ T lymphocytes represent 2–5% of adult human peripheral lymphocytes and differ from classical αβ TCR expressing lymphocytes with respect to their ontogeny, tissue tropism, gene repertoire and antigen recognition.[1] Vδ2 T lymphocytes represent the majority of peripheral blood γδ T lymphocytes whereas Vδ1 are resident mainly within epithelial tissues.[2, 3] Several lines of evidence have implicated γδ T lymphocytes to play an important role during infection, autoimmunity and tumor immune surveillance.[3]

In contrast to αβ T lymphocytes, γδ T lymphocytes have the ability to recognize antigen directly without antigen presentation by MHC. γδ T lymphocytes recognize small phosphorylated nonpeptidic molecules produced by microorganisms and also by transformed eukaryotic cells.[4] These molecules have been identified as intermediates of Rohmer (4-hydroxy-3-methyl-but-2-eneyl pyrophosphate; HMBPP) or Mevalonate (isopentyl prrophosphate;IPP) pathway in microbes and humans respectively.[5]

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γδ T lymphocytes also respond to alkylamines[6] and tannins[7] present in edible plants and bacteria. Tea (*Camellia sinensis*) which is widely consumed beverage worldwide is largest dietary source of alkylamine. Tea contains L-theanine, an amino acid that is catabolized to ethylamine[8] known to stimulate γδ T lymphocytes. Reports on dietary intake of tea, vegetable and fruits that can prime γδ T lymphocytes has led to renewed interest in using bioactive food components as regulators of γδ T lymphocytes.[9]

Based on the potential of γδ T lymphocytes to adapt their TCR to recognize antigens directly, we initiated investigations on identification of novel γδ T lymphocyte stimulatory activity in aqueous herb extracts used for treatment of various ailments and known to possess immunomodulatory properties. The herbs were selected based on their medicinal use and ability to stimulate innate immune cells such as macrophages and NK cells. There are no reports on how γδ T cells, the key players of innate immunity, are stimulated by these herbs extracts described in Ayurveda (Indian traditional medicine) (Table 1). We therefore thought that it was appropriate to analyze the effects of the herb extracts on γδ T cells which play an important role in innate immunity besides the NK cells and macrophages. American ginseng (*Panax quinquefolius*) was included in the study as it is a known immunomodulator and is known to activate macrophages, NK cells and plays a role in activating

**Table 1: Herb Extracts Used in the Study**

| Herbs | Botanical Name | Known Medicinal Use | Modulation of Innate Immunity |
|---|---|---|---|
| Licorice | *Glycyrrhiza glabra* | Mouth & peptic ulcers (Edward, 1991) | Macrophage (La *et al.*, 2011)<br>NK cells (Bhat *et al.*, 2010) |
| Basil | *Ocimum sanctum* | Stomach disorders (Khosla, 1995) | NK cells (Bhat *et al.*, 2010)<br>Macrophages (Bhattacharya *et al.*, 2009) |
| Shankhpushpi | *Evolvulus alsinoides* | Antiepileptic (Dandekar *et al.*, 1992) | Macrophages (Ganju *et al.*, 2003) |
| Vidarikand | *Pureraria tuberosa* | Revitalizer (Rani *et al.*, 1997) | Macrophage (Pravin *et al.*, 2012) |
| Cardamom | *Elettaria cardamom* | Stomach disorder (Gilani *et al.*) | NK cells (Bhat *et al.*, 2010;<br>Majdalawieh *et al.*, 2010) |
| Ginger | *Zingiber officinalis* | Antiepileptic (Langner *et al.*, 1998) | NK cells (Bhat *et al.*, 2010) |
| Shatavari | *Asparagus racemosus* | Adaptogen (Bopana *et al.*, 2007) | NK cells (Thakur *et al.*, 2012) |
| Ashwagandha | *Withania somnifera* | Adaptogen (Bhalla *et al.*, 2011) | NK cells (Bhat *et al.*, 2010) |
| Jeevani | *Trichopus zeylanicus* | Adaptogen (Pushpangadan *et al.*, 1988) | Macrophages (Pushpangadan *et al.*, 1995) |
| American ginseng | *Panax quinquefolius* | Common cold (Seida *et al.*, 2011) | NK cells (Miller, 2012)<br>Macrophage (Ichikawa *et al.*, 2009) |
| Black tea | *Camellia sinensis* | Antioxidant activity (Patel, 2005) | Macrophages (Monobe *et al.*, 2012) |

innate immune functions (Table 1). Black tea extracts in the present study was used as a positive control as earlier reports have demonstrated its ability to expand γδ T cells and enhance antibacterial immunity.[6] In the present study, more in depth investigations were conducted with respect to ability of Black tea extracts to activate γδ T cells.

In the present investigations, we have analyzed the in vitro ability of herb extracts to expand the pool of γδ T lymphocytes from PBMCs of healthy individuals. We further explored the activation status of expanded γδ T lymphocytes and their ability to produce IFN-γ and TNF-α. Our results demonstrate that extracts of Black tea, Jeevani and American ginseng have the ability to expand Vδ2 T lymphocytes. The expanded γδ T lymphocytes expressed increased levels of CD25 and CD69 activation markers. The activated γδ T lymphocytes showed marked release of cytokines IFN-γ and TNF-α.

An important implication of our observations is that herb extract based approach as dietary supplement may enhance the effectiveness of innate immune responses. Priming of γδ T lymphocytes with these herb extracts will enhance immunity to bacterial infections and against tumors.

## Methodology

### Chemicals Used

IPP (Isopentenylpyrophosphate) was purchased from Sigma-Aldrich, MO, USA. BrHPP (Bromohydrin pyrophosphate), synthetic analogue of IPP was a gift from Innate Pharma, Marseilles. Ethylamine was purchased from Sigma-Aldrich, MO, USA.

### Preparation of Herb Extracts

2 gm of the plant part of each herb (Table 1) was suspended in 20 ml of distilled water and kept at room temperature for 2 hr with continuous mixing. This suspension was boiled for 2 hr. The contents were centrifuged at 1,000*g* for 10 min and the supernatant was collected and stored at 4°C. Black tea was purchased (Brooke Bond Red Label tea, Hindustan Unilever Limited, Mumbai, India) and a 10% solution was boiled for 5 min. The contents were centrifuged at 1,000*g* for 10 min and the supernatant was collected and stored at 4°C. American ginseng capsules (200 mg) capsules manufactured by Afexa Life Sciences Inc. (formerly called CV Technologies Inc.), Edmonton, Alberta, Canada, were purchased and dissolved to make 10% solution. The contents were centrifuged at 1,000*g* for 10 min, and the supernatant was collected and stored at 4°C. The 10% stock solution was used at concentrations 1% to 0.0001% in the MTT assay to determine the maximum noncytotoxic concentration of each herb (Table 2). Henceforth in the text, the herb will be referred to by their common names.

### Separation of Lymphocytes

Peripheral blood (20–30 ml) was collected from healthy individuals by vein puncture. Lymphocytes were separated from heparinized peripheral blood using FiColl Hypaque (Sigma-Aldrich, MO, USA) density gradient centrifugation. The Tata Memorial Centre institutional

**Table 2: Preparation of Herb Extracts and Concentrations Used in the Study**

| Herbs | Botanical Name | Plant Part Used | Concentration Used in Assay (%)[a] |
|---|---|---|---|
| Licorice | *Glycyrrhiza glabra* | Roots | 0.1 |
| Basil | *Ocimum sanctum* | Aerial parts | 0.1 |
| Shankhpushpi | *Evolvulus alsinoides* | Whole plant | 0.1 |
| Vidarikand | *Pureraria tuberosa* | Tuber | 0.1 |
| Cardamom | *Elettaria cardamom* | Fruits | 0.1 |
| Ginger | *Zingiber officinalis* | Rhizome | 0.1 |
| Shatavari | *Asparagus racemosus* | Tuber | 0.5 |
| Ashwagandha | *Withania somnifera* | Roots | 0.5 |
| Jeevani | *Trichopus zeylanicus* | Leaves | 0.5 |
| American ginseng | *Panax quinquefolius* | Roots | 0.5 |
| Black tea | *Camellia sinensis* | Leaves | 0.5 |

[a]The highest concentration that was noncytotoxic on PBMCs as analysed by the MTT assay.

review board approved the study protocol, and informed written consent was obtained from each volunteer before blood collection.

## MTT Assay

PBMCs were incubated with serial dilutions of each extract starting from 5% (maximum concentration) for 24 hr. The cells were treated with 100 µg of MTT dye (Sigma-Aldrich, MO, USA) {(3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)} for 4 hr at 37°C with 5% $CO_2$. The crystals formed were dissolved in 100 µl DMSO (Sigma-Aldrich, MO, USA). The absorbance was read at 540 nm with reference wavelength 690 nm on ELISA reader (Sunrise, Tecan, Grodig, Austria).

## Stimulation of PBMCs with Antigens and Herb Extracts

PBMCs were incubated with medium alone, herb extracts or IPP (40 µM), BrHPP (200 nM) and ethylamine (0.5 mM, 3mM and 7.5 mM) in the presence of rIL-2 (30 IU/ml) in RPMI medium (Gibco, Invitrogen, NY, USA) supplemented with 10% heat inactivated human AB serum and antibiotics. On the 3rd, 6th and 9th day of culture, rIL-2 (30 IU $ml^{-1}$) was added. On Day 12, the cells were harvested and stained with fluorochrome labeled antibodies. For herb extracts, PBMCs were stimulated with aqueous extracts of each herb at concentrations shown in Table 1.

## Flow Cytometry

For dual color flow cytometry, PBMCs ($0.5 \times 10^6$) or purified γδ T lymphocytes ($1 \times 10^5$) were incubated with fluorochrome FITC- or PE-conjugated mouse anti-human MAb isotype IgG, CD3, Vδ2, αβ, CD25, CD69 (BD Biosciences, San Diego, CA, USA), Vδ1 (Pierce Biotech, IL, USA) for 45 min at 4°C in FACS buffer (1×PBS, 0.01% sodium azide, 1% FCS). The stained cells were acquired on BD FACSCalibur (BD Biosciences, NJ, USA) with 10,000 total events recorded for each sample. Cellquest software (BD Biosciences, NJ, USA) was used for analysis.

## Isolation of γδ T lymphocytes

PBMCs were stimulated with anti CD3MAb (1 µg $ml^{-1}$) and rIL-2 (100 IU $ml^{-1}$) (Peprotech, NJ, USA) as described earlier.[10] γδ T lymphocytes were isolated by positive separation using MACS (Miltenyi Biotec, Bergisch, Germany). The isolated γδ T lymphocytes were analysed for their purity on flow cytometer.

## Cytokine Assay

Purified γδ T lymphocytes were incubated with varying dilutions of Black tea, Jeevani and American ginseng as indicated for 24 hr at 37°C. In parallel, γδ T lymphocytes were stimulated with ethylamine (0.5, 3, 7.5 and 15 mM) which served as positive control. After incubation, the cell free supernatants were collected. The cytokines (IFNγ, TNFα, IL-4 and IL-10) were assayed using a commercially available Opt-EIA sandwich ELISA Kit (BD Biosciences, San Diego, CA, USA) as per the manufacturer's instructions.

## Statistical Analysis

All results were analysed using GraphPad Prism soft-

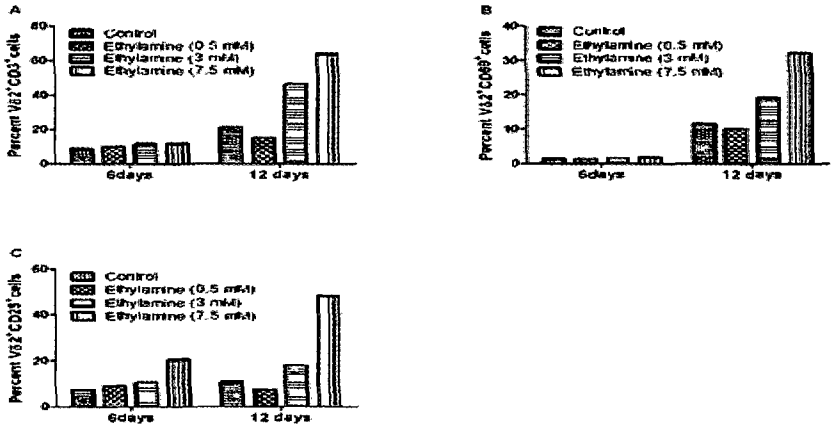


**Figure 1.** Comparison of 6-day *vs.* 12-day stimulation of lymphocytes with ethylamine—Comparison of $V\delta2^+CD3^+$ expansion and expression of activation markers on γδ T cells from 6-day *vs.* 12-day stimulation of PBMCs with ethylamine. PBMCs were stimulated with ethylmaine at concentration 7.5, 3 and 0.5 mM for 6 and 12 days. **(A)** Percent expansion of $V\delta2^+CD3^+$ γδ T cells. **(B)** Percent expression of early activation markers CD69 and **(C)** late activation marker CD25 on $V\delta2^+$ γδ T cells. The figure shows representative data of γδ T cells activation seen in a healthy individual.

ware (Version 5.01, GraphPad Software, Inc., CA, USA). Significance was assessed by unpaired Student's *t* test. The difference between groups was considered statistically significant when the *P* value was <0.05.

## Results

### Expansion of γδ T Lymphocytes after Stimulation with Herb Extracts

In the present investigations, we examined the ability of the herb extracts to stimulate γδ T lymphocytes. Ethylamine, IPP and BrHPP are known antigens that stimulate γδ T lymphocytes and were therefore used as positive controls in the experiments. PBMCs isolated from healthy individuals were incubated with various concentration of ethylamine (0.5, 3, 7.5 mM) for 6 and 12 days at 37°C. At the end of incubation period, the lymphocytes were immunophenotyped for analysing the expanded γδ T cell phenotype and expression of activation markers (CD25 and CD69) on γδ T lymphocytes.

As shown in Figure 1A, stimulation of PBMCs with ethylamine for 12 days caused a significant expansion of $V\delta^{2+}CD^{3+}$ phenotype at concentrations of 3 mM and 7.5 mM compared to control (unstimulated cells). The expression of early activation markers CD69 was seen on ethylamine stimulated γδ T lymphocytes at 3 and 7.5 mM concentrations (Fig. 1B). Late activation marker CD25 was expressed maximally on PBMCs stimulated with 7.5 mM ethylamine (Fig. 1C). A representative flow cytometry graph showing dual expression of CD3 and Vδ2 phenotype on PBMCs stimulated with ethylamine (7.5 mM) is shown in Figure 2.

Stimulation of PBMC with IPP (40μM) and BrHPP (200 nM), used as positive controls, showed 52.8% and 55.8% expansion of $V\delta^{2+}CD^{3+}$ phenotype, respectively. which was comparable to that observed with ethylamine (42.8%). Upregulation of expression of activation markers CD25 and CD69 were observed on IPP, BrHPP and ethylamine expanded Vδ2 subset of γδ T lymphocytes over control (unstimulated cells).The extracts of Licorice, Basil, Shankhapushpi, Vidarikhand, Cardamom, Ginger, Shatavari, Ashwagandha, Jeevani, American ginseng and Black tea were pretitrated at concentration ranging from 1% to 0.0001% on PBMCs and viability assessed by MTT assay. The maximum concentration that was noncytotoxic for each extract was used in all further assays (Table 2).

PBMCs of healthy individuals were incubated in vitro for 12 days with ethylamine, Black tea and American ginseng which served as positive control and extracts of nine herbs Licorice, Basil, Shankapushpi, Vidarikhand, Cardamom, Ginger, Shatavari, Ashwagandha and Jeevani to analyze their ability to stimulate outgrowth of γδ T lymphocytes.

As seen in Figure 3A, immunophenotyping of PBMCs isolated from healthy individual incubated with extracts of Black tea (0.1%) showed an increase in the expression of $V\delta^{2+}CD^{3+}$ subset of γδ T lymphocytes (*P*

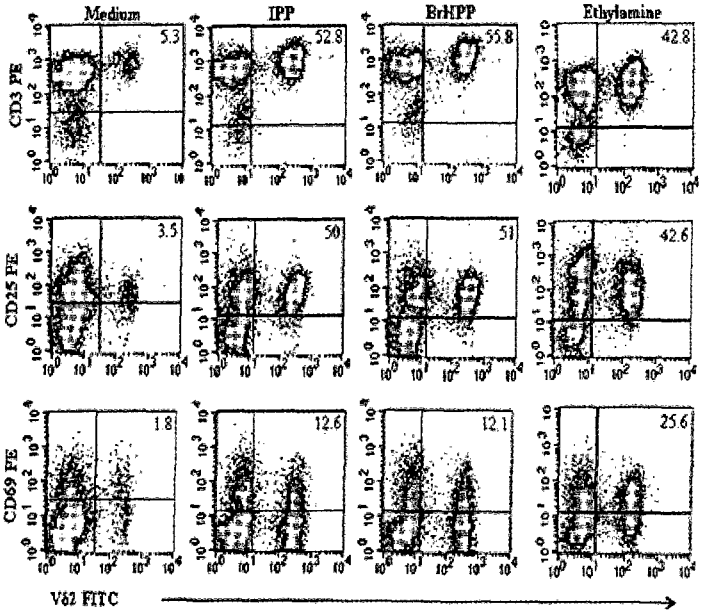


**Figure 2.** Stimulation of PBMCs with Phosphoantigens and ethylamine for 12 days—Dual colour flow cytometry on PBMCs stimulated with IPP, BrHPP and ethylamine for 12 days. PBMCs were stained with Vδ2FITC and CD3PE (1st row), Vδ2FITC and CD25PE (2nd row) and Vδ2FITC and CD69PE (3rd row). The graph is representative of four independent experiments with four different donors. The percentages of dual positive cells are indicated in upper-right quadrant of each figure.

< 0.0001) over control (unstimulated cells). American ginseng (0.5%) also induced an expansion of $V\delta^{2+}CD^{3+}$ phenotype ($P < 0.01$). Amongst the herb extracts, only Jeevani at 0.5% concentration showed a significant increase in $V\delta^{2+}CD^{3+}$ subset of γδ T lymphocytes from PBMCs ($P < 0.01$). Interestingly, we observed a statistically significant expansion of $V\delta^{1+}CD^{3+}$ subset of γδ T lymphocytes with Jeevani ($P < 0.05$) and black tea ($P < 0.05$) compared to control (unstimulated cells). $V\delta^{1+}CD^{3+}$ did not expand when PBMCs were incubated with extracts of American ginseng or rest of the herbs (Fig. 3A and 3B). Ethylamine which significantly expanded $V\delta^{2+}CD^{3+}$ (Figs. 1 and 2) showed no statistically significant expansion of $V\delta^{1+}CD^{3+}$ subset (data not shown).

Extracts of Licorice, Basil, Shankhapushpi, Vidarikhand, Cardamom, Ginger, Shatavari, Ashwagandha, did not induce expansion of γδ T lymphocytes from PBMCs over that observed with control (unstimulated cells) (Fig. 3B).

## Expression of Activation Markers on γδ T Lymphocytes Stimulated with Herb Extracts

As can be observed from Figure 4, significant increase in early activation marker CD69 was observed on γδ T lymphocytes stimulated with Jeevani ($P < 0.05$) and Black tea ($P < 0.05$). CD25, the late activation marker was significantly increased on γδ T lymphocytes after stimulation with Jeevani ($P < 0.05$), Black tea ($P < 0.01$) and American ginseng ($P < 0.05$). The upregulation of expression of activation markers was not seen with rest of the eight herb extracts.

It was interesting that significant expansion of $\alpha\beta^{+}CD^{3+}$ T lymphocytes was not observed after stimulation of PBMCs with all extracts included in the study under the same assay conditions (Fig. 5).

## Cytokine Profiles of Purified γδ T Lymphocytes Stimulated with Herb Extracts

γδ T lymphocytes were isolated from PBMCs by immunomagnetic purification. Figure 6 shows that γδ T lymphocytes of >95% purity were obtained from *ex-vivo* anti CD3 MAb stimulated PBMCs of healthy donors.

Purified γδ T lymphocytes were stimulated with Ethylamine, Black tea, Jeevani and American ginseng at various dilutions as indicated in Figure 7.

The cytokines IFN-γ and TNF-α released in the culture supernatants were quantitated by ELISA. Ethylamine was initially titrated at concentration of 0.5, 3, 7.5 and 15 mM to observe its ability to induce IFN-γ release from γδ T lymphocytes. Stimulation of purified γδ T

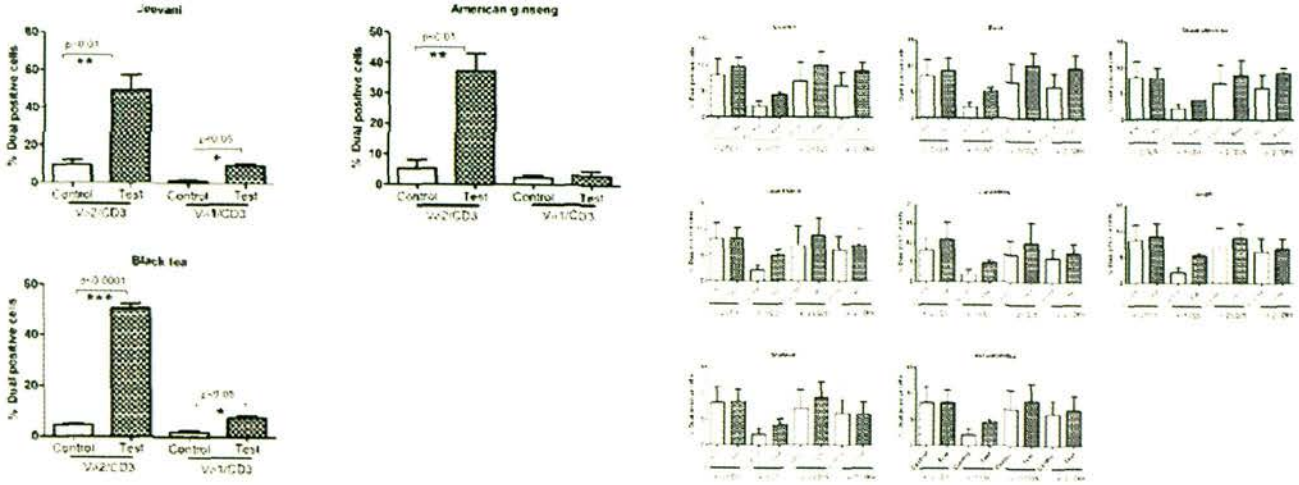


**Figure 3.** Immunophenotypic analysis of PBMCs stimulated with herb extracts—Immunophenotyping of PBMCs stimulated with herb extracts (Test) and unstimulated PBMCs (Control). **(A)** PBMCs ($n = 3$) were stimulated with extracts of Jeevani (0.5%) American ginseng (0.5%) and Black tea (0.1%) and analysed for expansion of Vδ2CD3 and Vδ1CD3. Statistically significant expansion of $V\delta2^+CD3^+$ phenotype was observed with Jeevani ($P<0.01$), American ginseng ($P<0.01$) and Black tea ($P<0.0001$) over control. Expansion of $V\delta1^+CD3^+$ phenotype was observed after stimulation of PBMC with Jeevani ($P<0.05$) and Black Tea ($P<0.05$) compared to control. **(B)** PBMCS ($n = 3$) were stimulated with extracts of Licorice, Basil, Shankhapushpi, Vidarikhand, Cardamom, Ginger, Shatavari and Ashwagandha. No significant expansion of phenotype was observed in test samples over control. The results are shown as mean ± SE of three independent experiments.

lymphocytes with ethylamine (15 mM) for 24 hr at 37°C showed a significant increase in release of IFN-γ over control (unstimulated cells). Aqueous extract of Black tea, Jeevani and American ginseng was titrated at dilutions ranging from 0.5% to 0.00005% on purified γδ T lymphocytes. Higher dilutions of the tea extacts showed increased ability to release IFN-γ from γδ T lymphocytes. Jeevani ($P < 0.05$) and American ginseng at 0.5% concentration were able to stimulate significant IFN-γ release from γδ T lymphocytes. The levels of IFN-γ released after stimulation with 0.5% Jeevani were higher than those observed after stimulation with ethylamine. γδ T lymphocytes stimulated with all the concentrations of Black tea and Jeevani also released TNF-α. However TNF-α was not released after stimulation with American ginseng. Stimulation of γδ T lymphocytes with Ethylamine also showed TNF-α production.

The purified γδ T lymphocytes upon stimulation with Black tea, Jeevani and American ginseng did not produce IL-4 or IL-10 at all the concentrations analysed (data not shown).

## Discussion

γδ T lymphocytes function as a bridge between innate and adaptive immune system (11). Human γδ T lymphocytes expressing Vγ9Vδ2 phenotype recognize non-peptide compounds such as prenyl pyrophosphates (12) aminobisphosphonates[13] and alkylamines.[14] Prenyl pyrophosphates and alkylamines are natural antigens present in bacteria and also in tea, apples and wine. Vδ2 T lymphocytes recognize prenyl pyrophosphates that are intermediates of the mevalonate pathway of isoprenoid synthesis in eukaryotes and of the 2-C-methyl-D erythritol 4-phsophate pathway in prokaryotes. IPP is less potent compared to HMBPP in stimulating γδ T lymphocytes, both being intermediates of the mevalonate and 2-C-methyl-D erythrilol 4- phosphosphate pathway. IPP is known to be increased by dietary (L-theanine) and pharmacologic (bisphosphonate) intervention.[15, 16] In the present study, we used IPP and BrHPP as positive controls to stimulate γδ T lymphocytes. A marked increase in expansion of γδ T lymphocytes (Vδ2 phenotype) was observed when PBMCs of healthy individuals were stimulated with IPP or BrHPP. We also showed that these expanded γδ T lymphocytes expressed CD69 and CD25 the early and late activation markers, respectively.

Ethylamine is a breakdown product of tea digestion in humans and is produced by acid hydrolysis of L-theanine in the gut and by enzymatic hydrolysis mediated by amidases in the liver. Significant levels of L-theanine

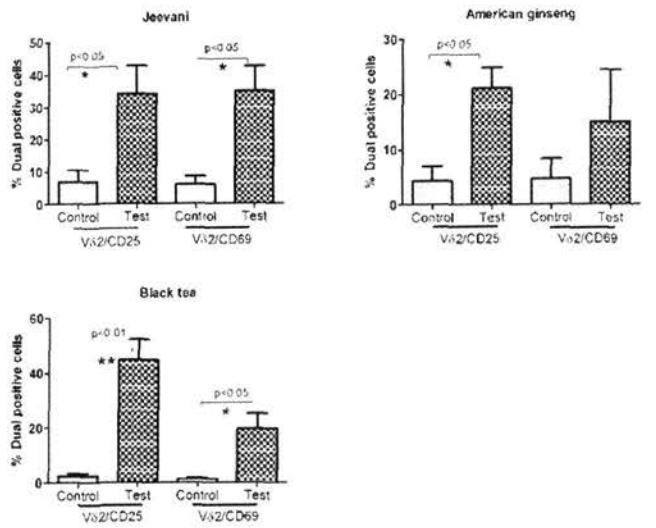


**Figure 4.** Activation status of γδ T cells stimulated with herb extracts—Expression of activation markers on γδ T cells expanded from PBMCs stimulated with Jeevani (0.5%), American ginseng (0.5%) and Black tea (0.1%). Significant increase in expression of CD25 was observed on γδT cells (Vδ2$^+$) stimulated with Jeevani ($P$<0.05), American ginseng ($P$<0.05) and black tea ($P$<0.01) as compared to control (Unstimulated PBMCs). Increase in CD69 was observed on Vδ2$^+$ T cells after stimulation with Jeevani ($P$<0.05) and black tea ($P$<0.05) compared to control. The results are shown as mean ± SE of three independent experiments.

are found in green tea, oolong tea and Black tea.[8] We stimulated the PBMCs with ethylamine to monitor the expansion of γδ T lymphocytes and the expression of activation markers on the expanded γδ T lymphocytes. We showed ethylamine to possess potent γδ T cell stimulatory activity comparable to that observed with IPP or BrHPP.

In the present investigations, we analysed the ability Black tea, American ginseng and herbs (Licorice, Basil, Shankhapushpi, Vidarikhand, Cardamom, Ginger, Shatavari, Ashwagandha and Jeevani) used in India as beverages, additives in beverages or as ayurvedic formulations (rasayanas) as home remedies for improving immunity. Amongst all the herbs we tested, only Jeevani showed potent γδ T cell stimulatory properties. Jeevani (*Tricopus zeylanicus*) is a rare plant species that grows in unique geographic location in Agastyar hills of Kerala (India). It was found that local tribe (*Kani tribe*) traditionally consumed fruits and leaves of this plant. It is reported to have adaptogenic effects,[17] aphrodisiac properties[18] and immunomodulatory effects.[19] Pushpangadan group have shown that Jeevani stimulates proliferation of macrophages and lymphocytes. In the present study, *ex vivo* treatment of PBMCs with 0.5% Jeevani showed a significant outgrowth Vδ2 subset of γδ T lymphocytes. The expanded γδ T lymphocytes expressed early and late activation markers CD69 and CD25, respectively, and released high levels of IFN-γ and TNF-α upon activation.

Similarly aqueous extracts of Black tea and American ginseng also exhibited potent γδ T cell stimulatory activity. American ginseng is reported to stimulate cells of innate immunity such as NK and macrophages (Table 1). In the present study, we observed that American ginseng also has γδ T cell stimulatory activity. Black tea showed stimulatory effects on γδ T lymphocytes expansion as well as activation. Randomised, double blind, placebo controlled intervention study demonstrated that tea extracts containing proprietory *Camellia sinensis* L-theanine and a formulation *Camellia sinensis* formulation (CSF) with defined amounts of EGCG (epigallocatechin gallate) prevented cold and flu symptoms in about one third of the study subjects by enhancing γδ T cell proliferation and IFN-γ secretion, in response to antigen.[20] Our study showed that Black tea induced cytokine release by γδ T lymphocytes and expansion of Vδ2 subset with upregulated expression of activation markers.

γδ T cell stimulatory activity has also been earlier reported in other plant extracts. Mistletoe (*Viscum album*)

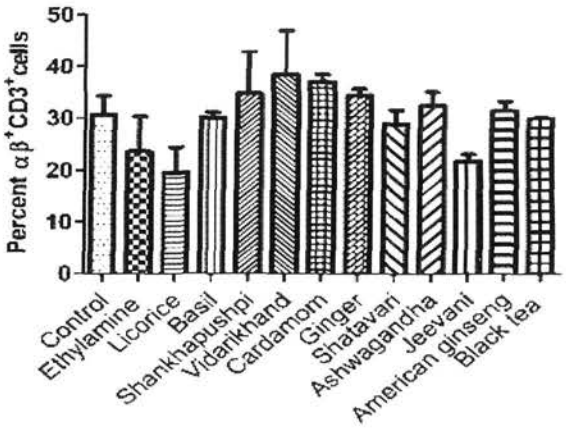


**Figure 5.** αβ T cells are not activated by herb extracts—Herb extracts do not stimulate expansion of αβ T cells. PBMCs were stimulated with ethylamine and all herb extracts for 12 days. Expansion of αβ$^+$CD3$^+$ T cells was analyzed by dual color flow cytometry using αβ FITC and CD3PE MAb. Unstimulated PBMC served as control. The results are shown as mean±SE of two independent experiments.

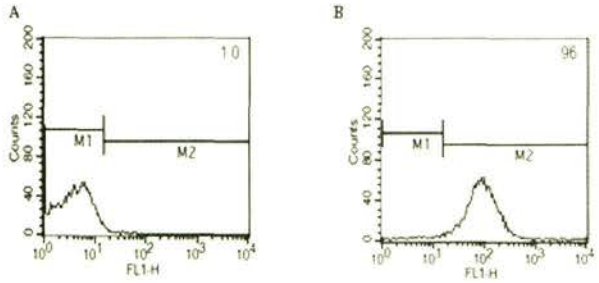


**Figure 6.** Isolation of γδ T cells from PBMCs by immunomagnetic purification—Immunomagnetic separation of γδ T cells from anti CD3 MAb stimulated PBMCs. **(A)** Negative fraction and **(B)** Positive fraction after immunomagnetic purification on MACS column. Numbers indicate % positive γδ T cells in negative and positive fractions.

is a semi-parasitic plant and a herbal remedy in the ancient Chinese Pharmacopoeia. It has been used to treat gonorrhea, syphilis, hypertension and rheumatism. The aqueous extract of European Mistletoe (EM) has been used in conventional cancer therapy for decades. Therapeutic efficacy of mistletoe extracts has been attributed to the presence of lectins that represent ribosome deactivating proteins class II.[21] Immunomodulatory effects of EM has been observed on cytotoxic T lymphocytes and NK cells and has the ability to induce release of TNF-α, IL-1, IL-6 from human PBMCs *in vitro*.[22] It was interesting to note that Chinese mistletoe lectin also showed ability to stimulate human γδ T cell cytotoxicity, apoptosis and modulation of the cytokine network.[23, 24]

The ability of the extracts of Jeevani, Black tea and American ginseng to release significant amounts of IFN-γ and TNF-α but not IL-10 and IL-4 from γδ T lymphocytes of healthy individuals is of significance. IFN-γ and TNF-α are two cytokines that are required for monocyte mediated bacterial killing.[25] IFN-γ particularly, is a pleiotropic cytokine that plays an important role in regulating the immune responses.[26] It was demonstrated that live bacterial infection *in-vivo* SCID mice results in IFN-γ production that was contributed by Vγ9Vδ2 T lymphocytes.[27] The data suggested that IFN-γ production by Vδ2 T lymphocytes may be important in mediating the antibacterial activity.

In summary, the results demonstrate that the extracts of Jeevani (*Tricopus zeylanicus*) showed potent γδ T cell stimulatory activity. The activated γδ T lymphocytes release IFN-γ and TNF-α. These data provide a rationale for further analysing the antibacterial and anti-tumorigenic potential of γδ T lymphocytes stimulated with herb extracts.

## Conclusions

Our results show that the extracts of Jeevani (*Tricopus zeylanicus*) induce expansion of γδ T lymphocytes and these activated γδ T lymphocytes produce proinflammatory cytokines IFN-γ and TNF-α. The herb can be further studied to evaluate the role it might serve to develop preventive as well as therapeutic approach to enhance

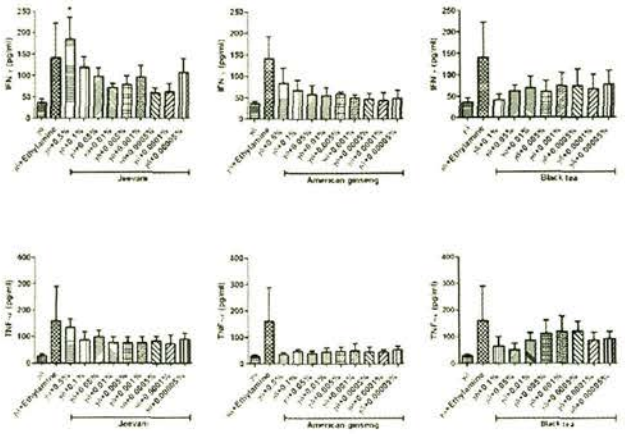

**Figure 7.** Cytokine profile of MACS purified γδ T cells stimulated with herb extracts- Cytokine profiles of γδ T cells isolated from three healthy individuals and stimulated with ethylamine and herb extracts. IFN-γ (upper row) and TNF-α (lower row) released in cell free supernatant of γδ T cells stimulated with various dilutions of Jeevani, American ginseng, Black tea, Ethylamine and unstimulated γδ T cells (control) after culture for 24 hr were quantitated by ELISA. Results are shown as mean ± SE of three independent experiments.

the immunity conferred by γδ T lymphocytes against microbial infections and cancer. Further studies on the characterization bioactive compounds present in the herb extracts that possess γδ T lymphocyte stimulatory activity are warranted.

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