# Upregulation of Th1 polarization by *Taraxacum officinale* in normal and immune suppressed mice

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**The present study was undertaken to study the potential of *Taraxacum officinale* aqueous extract (aerial part; TO-10) to maintain immune homeostasis in normal and chronic restraint stress and cyclosporine-A-induced immune suppressed mice. Immune restorative effect of test drug was evaluated first in normal and then in immune-compromised mice using flow cytometer and Elisa techniques. TO-10 enhanced the expression of T-cell subsets and CD28, CD69 and CD80/CD86 co-stimulatory molecules in sheep red blood corpuscles (SRBCs)-immunized mice. Flow cytometric analysis revealed that TO-10 upregulated the expression of Th1 cytokines, IL-2, IFN-gamma and IL-12, and regularized the increased expression of IL-10 in chronically stressed animals. It also normalized the elevated corticosterone levels, and reversed the chronic stress-induced hypertrophy of adrenal glands and atrophy of spleen and thymus. The results show that TO-10 is also able to maintain immune homeostasis in normal and immune-compromised conditions. Chicoric acid, a major constituent of TO-10, seems to be responsible for skewing to Th1 immune polarization as shown by its stimulatory effect on the expression of IFN-gamma and IL-2 in phorbol 12-myristate 13-acetate + ionomycin stimulated peripheral blood mononuclear cells (PBMCs).**

**Keywords:** Cytokines, *Taraxacum officinale*, immunity, flow cytometer, mice.

THE severity of various pathological conditions is often related to the outcome of the disturbed immune functions of the body and therefore the use of immune modulators is of great remedial value in boosting the desired immune response. Decrease in immunological response has strong implications for disease susceptibility and progression, which is at least in part due to the reduction of lymphocytes and related cytokines[1]. Psychological and physical stressors influence cytokine responses[2,3], cytolytic activity, lymphocyte proliferation[4], lymphocyte number[5,6] and neuronal signal transmission[7]. Several studies have revealed that chronic stress has significant suppressive effects on the immune system[8], including innate immunity (e.g. natural killer cell lysis), T-cell responses, and antibody production *in vivo* and *in vitro*[9]. T-lymphocytes have a key role to play in the immune system, and are further divided on the basis of their cytokine secretion into Th1 and Th2 cells. This ultimately determines the induction of cellular and/or humoral immune responses. The appropriate choice of Th1 or Th2 cytokine profile is crucial and the dichotomy of this selection is regulated early in the course of the immune response. Consequently, the optimal immunotherapy should restore and/or uphold a well-balanced Th1 and Th2 response, suited to the immune challenge[10].

Now-a-days medicinal plants have become the cheapest source of drugs for majority of the world's population. *Taraxacum officinale* Weber, locally known as 'dandelion' (English), 'kanphul' (Hindi), belonging to the family Asteraceae, is widely distributed in the warmer temperate zones of the northern hemisphere. This plant has long been used as a medicinal herb around the globe. The first evidence for its therapeutic use was mentioned by Arabian physicians of the 10th and 11th centuries to treat liver and spleen ailments[11]. It is also considered to be a 'blood purifier' and is employed as a mild laxative, for treating arthritic and rheumatic complaints as well as eczema and other skin conditions in popular medicines[12]. In traditional Chinese medicine, dandelion in combination with other herbs is used to treat hepatitis, to enhance immune response to upper respiratory tract infections, bronchitis or pneumonia, and as a compress for its antimastopathy activity[13,14]. A recent study has shown the protective effect of *Taraxacum officinale* (TO) on acute lung injury induced by lipopolysaccharide (LPS) in mice[15]. In spite of being a well-known traditional herbal remedy with a long history of traditional medicinal usage, only limited scientific information is available to justify the reputed uses of TO. So, the aim of the present study was to evaluate the ability of *T. officinale* (TO-10) in augmenting T-cell response and restoring Th1/Th2 homeostasis in restraint stress and chemical-induced immune-suppressed mice, and also to identify the active compound responsible for the activity.

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## Materials and methods

### *Plant material*

Plant material was collected in Srinagar, Jammu & Kashmir, India in April 2008 and identification was done at Indian Institute of Integrative Medicine (IIIM), Jammu. A voucher specimen (accession no. 21748) has been deposited at the Herbarium of IIIM, Jammu.

### *Preparation of TO-10 extract*

The dried and powdered aerial part of plant without flowers (250 g) was extracted with *n*-hexane (750 ml) for 24 h and filtered. The marc was re-extracted in the same solvent (750 ml) and filtered. Filtrate was pooled and concentrated on a rotavapor under vacuum to give 4.85 g of the extract labelled as TO-6. The marc left after hexane extraction was further extracted with dichloromethane (DCM), ethyl acetate, methanol and water sequentially, using the same procedure as described for hexane extraction. DCM extract was labelled as TO-7 (3.6 g), ethyl acetate extract as TO-8 (3.38 g), methanol extract as TO-9 (38.55 g) and water extract as TO-10 (36.95 g) respectively. All these extracts were screened for immunomodulatory activity, among which TO-10 proved to be the best candidate.

### *Standardization of TO-10 by high performance thin layer chromatography*

Two marker compounds, namely chicoric acid (CA) and chlorogenic acid were isolated and characterized from the water extract (TO-10) of *T. officinale*. On the basis of these markers, the water extract was standardized using high performance thin layer chromatography (HPTLC) method. The percentage of chicoric acid and chlorogenic acid was found to be 6.22 and 3.068 respectively. Figure 1 shows the HPTLC chromatogram of standard chicoric acid, chlorogenic acid, mixture of chicoric acid and chlorogenic acid along with the extract of TO-10.

### *Maximum tolerable dose determination*

Maximum tolerable dose (MTD) in animals was determined using the OECD method[16]. Test material was orally administered in graded doses and animals were monitored for change in weight, general behaviour and mortality at 0.5, 2, 6 and 12 h intervals after administration of test material. The test material was found to be well tolerated up to 2500 mg/kg.

### *Animals*

For the present study, 10–12 weeks-old male Swiss albino mice, weighing about 22–24 g were employed. The animals used for experimental work were duly approved by the Institutional Animals Ethics Committee (IAEC) of IIIM, Jammu. All the animals used in the experimental work received proper human care according to ethical regulations on animal research.

### *Experiment 1: immunomodulatory studies in normal mice*

*Lymphocyte proliferation assay:* Swiss albino mice were sacrificed and spleens were excised. A single-cell suspension of splenocytes was prepared by teasing the tissue gently using a sterile needle and forceps between phosphate-buffered saline (PBS). RBC lysis was done after centrifuging the cell suspension at 1000 *g* for 5 min, by adding 1 ml of 1 × erythrocyte lysis buffer. Splenocytes ($2 \times 10^5$ cells/well) were seeded in triplicates in a 96-well culture plate after viability check with Evan's blue dye. Sub-optimal concentrations of Concavalin (Con) A (0.5 μg/ml) and lipopolysaccharide (LPS) (1 μg/ml) were added to each well separately for priming T-cells and B-cells along with different concentrations (12.5, 25, 50, 100, 200 μg/ml) of TO-10. Plates were incubated at 37°C in a humidified atmosphere of 5% $CO_2$ for 72 h. After incubation, cell proliferation was determined by MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide][17].

*In-vivo studies – Drug administration:* Test drug was prepared in distilled water and administered orally for the duration of the experiment at graded oral doses ranging from 1.56, 3.12, 6.25, 12.5, 25, 50, 100 to 200 mg/kg. Levamisole (2.5 mg/kg) was used as positive control and cyclosporine A (5 mg/kg) was used as negative control. Both controls were also administered orally.

*Cellular immune response (delayed-type hypersensitivity response):* We followed the method of Doherty to assess SRBC induced delayed type hypersensitivity (DTH) response in mice[18]. Mice were sensitized and challenged with SRBC intraperitoneally (i.p.) in the left hind. Test material was administered 2 h after SRBC injection and once daily on consecutive days. A spheromicrometer (pitch, 0.01 mm) was used to measure thickness of the left hind foot paw at 24 h (day 1), 48 h (day 2) and 72 h (day 3) after challenge, whereas the right hind paw served as a control.

*Flow cytometric study of T-cell surface markers:* Here, 200 μl of $5 \times 10^9$ SRBC/ml i.p. was injected to Swiss albino mice on day 0. The mice were than challenged on day 6 with an equal amount of SRBC and blood was collected after 48 h. FITC-labelled $CD3^+$, $CD4^+$ monoclonal antibodies and PE-labelled $CD8^+$ monoclonal antibodies were used to establish the percentage of $CD3^+$, $CD4^+$, $CD8^+$ T-cells in naive-control, sensitized-control and treated group of animals by flow cytometry[19].

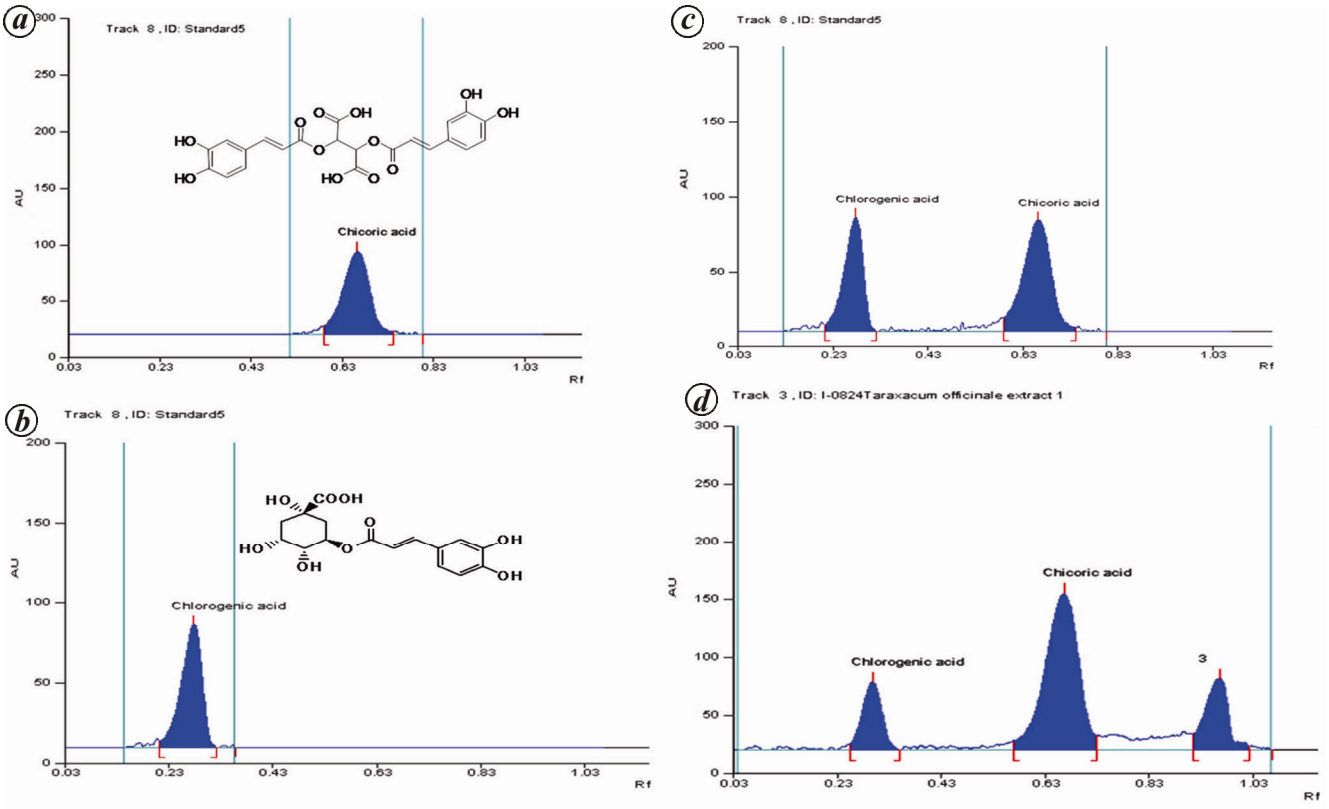


**Figure 1.** HPTLC chromatograms: ***a***, Standard chicoric acid at (Rf = 0.66 ± 0.01); ***b***, Standard chlorogenic acid (Rf = 0.29 ± 0.02); ***c***, Mixture of chicoric acid (Rf = 0.66 ± 0.01) and chlorogenic acid (Rf = 0.29 ± 0.02); ***d***, Water extract of *Taraxacum officinale* showing the presence of chlorogenic acid and chicoric acid at Rf = 0.29 ± 0.02 and 0.66 ± 0.01 respectively.

*Flow cytometric study of co-stimulatory molecules:* Splenic cells from treated and untreated Swiss albino mice were suspended in RPMI-1640 medium after removing the red blood cells by RBC lysis buffer. Briefly, 100 µl of splenocytes at $2.0 \times 10^6$ cells/ml was stained with FITC labelled anti-CD3 and PE-labelled anti-CD28 in one set, while FITC-labelled anti-CD3 and PE-labelled anti-CD69 were used in the second set. Similarly, FITC-labelled CD14 was added with PE conjugated anti-CD80 (B7-1) in one set, while FITC-labelled CD14 was added with PE-conjugated anti-CD86 (B7-2) monoclonal (mAbs) in another set for antigen presenting cells (APCs) (macrophages) after erythrocyte lysis with fluorescence-activated cell sorting (FACS) lysis solution. Cells were then kept in the dark for 30 min at 4°C, washed twice with PBS after staining, and subjected to FACS analysis[20].

*Intracellular cytokine estimation:* For this, 100 µl of whole blood was taken in different falcon tubes. FITC-labelled anti-mouse $CD4^+$ T-cell marker and phycoerythrin (PE)-labelled IL-2 monoclonal antibodies were used in one set, while FITC-labelled anti-mouse $CD4^+$ and PE-labelled IFN-$\gamma$ monoclonal antibodies were used in the second set; FITC-labelled anti-mouse $CD4^+$ and PE-labelled IL-4 monoclonal antibodies were used in the third set of experiments. Tubes were incubated in the dark for 30 min at room temperature and acquired on flow cytometer (BD Biosciences).

### *Experiment 2 – immunomodulatory studies in immune-compromised animals*

*Chronic restraint stress – Stress induction and antigenic stimulus* – Here, 50 ml conical polypropylene tubes was used to induce restrain in mice for 12 h during the dark cycle (2000–0800 h) for 14 days. Next 0.2 ml of SRBC was injected intra-peritoneally on day 5, and thereafter test drugs were administered at dose levels ranging from 12.5 to 200 mg/kg from day 5 to the next seven consecutive days. On day 12, animals were challenged by the same volume of SRBC. On day 14, blood was collected from retro-orbital plexus in EDTA-coated tubes for the estimation of T- and B-cell surface receptors and Th1 and Th2 cytokines using flow cytometry. Korean ginseng (KG), a known anti-stress agent, at the dose level of 100 mg/kg was used as positive control.

*Flow cytometric analysis of T-cell surface markers and Th1/Th2 cytokines in peripheral blood of chronically stressed mice:* $CD3^+$, $CD4^+$ and $CD8^+$ (cytotoxic cells) were estimated in peripheral blood of SRBC-immunized,

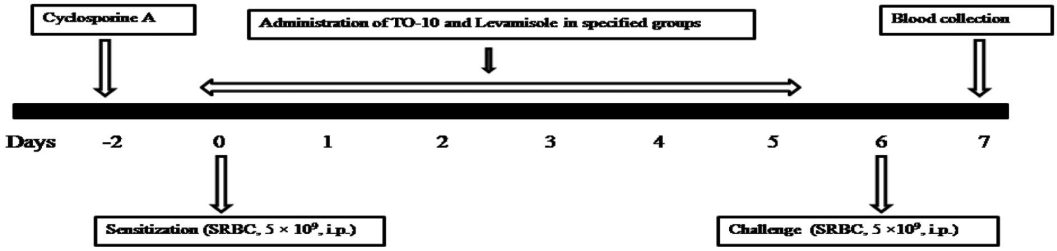


**Figure 2.** Schematic representation of the study carried out for evaluation of the effect of TO-10 at graded doses in cyclosporine A-induced immune suppressed mice.

chronically stressed mice using the method described above in case of normal mice[19]. Similarly, assessment of $CD4^+$/IL-2, IFN-gamma (Th1 cytokines) and IL-4 (Th2 cytokine) and whole lymphocytes IL-12 and IL-10 was also carried out.

*Corticosterone assay:* Corticosterone in serum was estimated by competitive immunoenzymatic method (EIA kit Cayman Chemicals) at a wavelength of 450 nm. All samples were assayed in triplicate.

*Body organ weights:* Following the last stress session, we evaluated the body weights of all animals and thereafter all the animals were sacrificed and their thymus, spleen and adrenal glands were removed and weighed. The organ weight ratio of the animals from all the groups was determined.

*Cyclosporine A-induced immunosupression:* This model was used to test the ability of the test material to restore immune response in chemical-induced immune suppressed conditions. Figure 2 provides a schematic representation. The following group configurations were maintained: normal control (NC), immune-suppressed control (ISC), TO-10 (12.5, 25, 50, 100 and 200 mg/kg) and Levamisole (2.5 mg/kg).

*Flow cytometric estimation of T-cell subsets and intracellular cytokines:* Flow cytometric estimations of $CD3^+$, $CD4^+$, $CD8^+$ T-cell surface markers and CD4-specific IL-2, IFN gamma and IL-4 were carried out by the same method as described above.

*Screening of different fractionations of TO-10:* To mark out the active constituent responsible for the immunomodulatory profile TO extract, TO-10 was fractionated using two different methods, by flash chromatography and then by liquid–liquid extraction method to yield 11 different fractions. These fractions were subjected to estimation of IL-2 in isolated PBMCs. Cells (PBMCs) were separated from venous blood by density gradient method on histopaque. We collected PBMCs at the interface and washed them three times with PBS. A concentration of $2 \times 10^6$ cells/ml was maintained in 96 deep-well plates. Cells were stimulated for cytokine production with 10 ng/ml phorbol 12-myristate 13-acetate in combination with 1 mg/ml ionomycin (PMA + I; Sigma, St Louis, MO, USA). Golgi plug (BD Biosciences) at a concentration of 1 µl/ml was added. All the test drugs were added at a concentration 10 µg/ml and incubated for 4 h at 37°C. After adding permeabilizing agent, phycoerythrin (PE)-labelled antimouse IL-2, IFN-gamma and IL-4 were added to the samples in separate falcon tubes and again incubated for 30 min, resuspended in PBS (pH 7.4) and acquired directly on the flow cytometer (BD LSR; Becton Dickinson)[21].

*Effect of chicoric acid on Th1/Th2 cytokines in isolated PBMCs:* The same protocol as discussed above was followed. CA was added at concentrations of 0.25, 0.5, 1 and 2 µg/ml and incubated for 4 h at 37°C. PE-labelled antimouse IL-2, IFN-gamma and IL-4 were added to the samples, resuspended in PBS (pH 7.4) and acquired directly on the flow cytometer (BD LSR; Becton Dickinson)[21].

*Statistics:* Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons.

## Results and discussion

### *Lymphocyte proliferation assay*

The main aim of this study was to elucidate the lymphocytes proliferative potential of TO-10 in Swiss albino mice splenocytes using MTT assay. Results showed that TO-10 elicited a momentous increase in the proliferative response of lymphocytes stimulated with Con A. However, the effect was concentration-dependent with optimum results observed at higher dose level of 100 µg/ml. LPS-stimulated splenocytes showed only marginal increase in proliferation. TO-10-treated splenocytes cultured in the absence of mitogens did not show any significant proliferative effect (Figure 3).

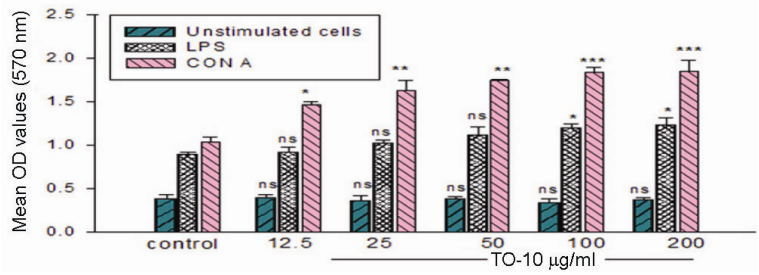


**Figure 3.** Effect of TO-10 (µg/ml) on proliferation of B and T lymphocytes *in vitro*. Splenocytes were isolated and stimulated with sub-optimal doses of mitogens Con A 0.5 µg/ml and LPS 1 µg/ml for T and B-cell respectively. Proliferation was measured by MTT reduction assay.

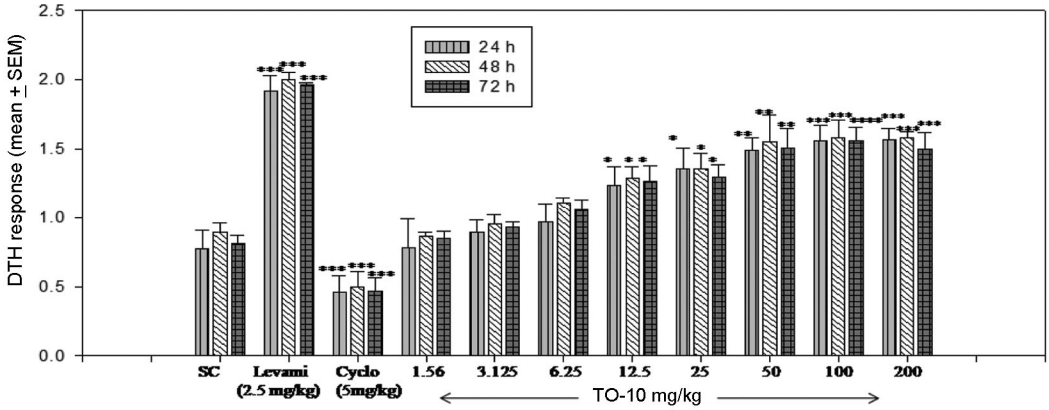


**Figure 4.** Effect of graded doses of TO-10 on delayed type hypersensitivity response in sheep red blood corpuscles-immunized mice. All values shown as mean ± SEM ($n = 6$). Bonferroni test for multiple comparisons was used to compute $P$ values. ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

### *Delayed-type hypersensitivity response*

The swelling of the left hind food was measured with a spheromicrometer (pitch, 0.01 mm) at 24 h (day 1), 48 h (day 2) and 72 h (day 3) after challenge with SRBCs. TO-10 modulated the cellular immune responses by increasing the delayed-type hypersensitivity response in a dose-dependent manner. The most significant effect was observed at dose levels of 100 and 200 mg/kg (Figure 4). This further confirms its cell-mediated immune upregulatory potential as DTH immune reaction is usually represented by T-cell response.

### *Flow cytometric analysis of T-cell subsets*

Intracellular estimation of total ($CD3^+$) and differential *T*-cells ($CD4^+/CD8^+$) was carried out using flow cytometer (BD-LSR). PE-labelled anti-CD19, PE-labelled anti-CD8, FITC-labelled anti-CD4 and FITC-labelled anti-CD3 were added directly to 100 µl of whole blood and incubated. After the last centrifugation, samples were resuspended in PBS (pH, 7.4) and subjected to flow cytometric evaluation. Results showed that TO-10 enhanced the expression of $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocytes in a dose-related manner, as depicted by flow cytometric analysis. However, maximum effect was observed at dose level of 100 mg/kg, where the percentage of increase in $CD3^+$ T-cell population was 64.87 ± 0.44 (mean ± SEM), $CD4^+$ T-cell population was 40.46 ± 0.45 (mean ± SEM) and $CD8^+$ T-cell population was 25.44 ± 0.26 (mean ± SEM) (Figure 5).

### *Flow cytometric evaluation of co-stimulatory molecules*

Co-stimulatory molecules such as CD28, CD69 and CD80/86 are important signalling molecules responsible for the activation of immune response. They were estimated in splenic cells isolated from treated and untreated Swiss albino mice. TO-10 markedly upregulated the expression $CD28^+$ and $CD69^+$ T-cells, and CD80 and CD86 expression in macrophages in a dose-dependent manner. Optimum enhancement was observed at a dose of 100 mg/kg, where almost two-fold increase was found in the number of cells expressing $CD28^+$ and $CD69^+$, and $CD80^+/CD86^+$ markers (Table 1 and Figure 6 *a* and *b*).

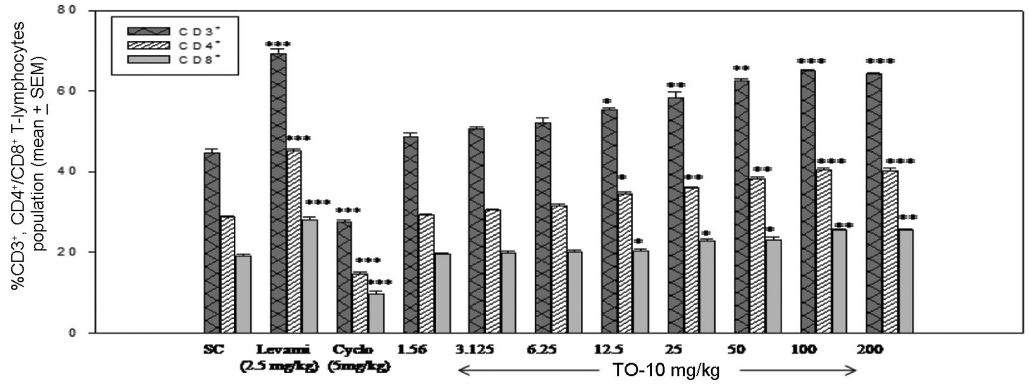


**Figure 5.** Effect of different doses of TO-10 (mg/kg, p.o.) on $CD3^+$, $CD4^+$ and $CD8^+$ T-cell population in SRBC-immunized mice. Each column represents mean ± SEM of six mice per group. SC, Sensitized control; Levami, Levamisole; Cyclo, Cyclosporine-A. Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons). ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$.

**Table 1.** Effect of graded doses of TO-10 (mg/kg, p.o.) on CD28, CD69 present on activated T-cells and CD80 and CD86 present on antigen presenting cells in sheep red blood corpuscles immunized mice

| Treatment | Dose (mg/kg, p.o.) | $CD28^+$ T-cells (mean ± SEM) | $CD69^+$ T-cells (mean ± SEM) | $CD80^+$ APCs (mean ± SEM) | $CD86^+$ APCs (mean ± SEM) |
|---|---|---|---|---|---|
| SC | – | 9.06 ± 0.32 | 7.43 ± 0.29 | 12.13 ± 0.27 | 15.43 ± 0.30 |
| TO-10 | 1.56 | 9.32 ± 0.47 (2.86↑) | 8.24 ± 0.27 (10.90↑) | 12.67 ± 0.44 (4.45↑) | 16.13 ± 0.35 (4.53↑) |
| TO-10 | 3.12 | 9.75 ± 0.21 (7.61↑) | 8.88 ± 0.38 (19.51↑) | 13.18 ± 0.38 (8.65↑) | 16.77 ± 0.26 (8.68↑) |
| TO-10 | 6.25 | 9.98 ± 0.29 (10.15↑) | 9.03 ± 0.30 (21.53↑) | 13.79 ± 0.42 (13.68↑) | 17.02 ± 0.22 (10.30↑) |
| TO-10 | 12.5 | 10.22 ± 0.26 (12.80↑) | 9.52 ± 0.34* (28.12↑) | 14.09 ± 0.44 (16.15↑) | 17.80 ± 0.37* (15.35↑) |
| TO-10 | 25 | 10.88 ± 0.43* (20.08↑) | 10.13 ± 0.22* (36.33↑) | 14.68 ± 0.32* (21.02↑) | 18.02 ± 0.21* (16.78↑) |
| TO-10 | 50 | 11.79 ± 0.32** (30.13↑) | 10.94 ± 0.45* (47.24↑) | 15.05 ± 0.39* (24.07↑) | 18.72 ± 0.38* (21.32↑) |
| TO-10 | 100 | 12.23 ± 0.28** (34.98↑) | 11.75 ± 0.34** (58.14↑) | 16.98 ± 0.22** (39.98↑) | 19.49 ± 0.32** (26.31↑) |
| TO-10 | 200 | 12.30 ± 0.31** (35.76↑) | 11.67 ± 0.48** (57.06↑) | 17.12 ± 0.39** (41.13↑) | 19.54 ± 0.30** (26.63↑) |
| Levamisole | 2.5 | 16.11 ± 0.26*** (77.81↑) | 13.57 ± 0.38*** (82.63↑) | 19.89 ± 0.45*** (63.97↑) | 23.79 ± 0.29*** (54.18↑) |
| Cyclosporine | 5 | 4.79 ± 0.31*** (47.13↓) | 3.79 ± 0.30*** (48.99↓) | 7.01 ± 0.33*** (42.20↓) | 9.88 ± 0.28*** (35.96↓) |

Each value represents mean ± SEM of six mice per group. Post-ANOVA (Bonferroni test for multiple comparisons); ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. ↑, Increase. Values in parenthesis represent percentage of activity against control.

### *Intracellular cytokine estimation*

Th1 (IL-2, IFN-gamma) and Th2 (IL-4) cytokines were estimated to study the effect of test drug on cytokine level. After 48 h of SRBC challenge, blood was collected from all the treated and non-treated groups and subjected to flow cytometric analysis of intracellular cytokines. Treatment with TO-10 resulted in dose-dependent increase of $CD4^+$-specific Th1 (IL-2, IFN-gamma) and a non-significant suppression of $CD4^+$/IL-4 cytokine, which is the cytokine of type 2 immunity. Again, maximum effect was observed at 100 mg/kg (Table 2). Thus, our results suggest a specific Th1 upregulatory potential of the test material.

### *Experiment 2*

*Chronic restraint stress:* After verifying the Th1 immune-stimulatory potential of the test drug in normal mice, its possible ability to restore immune response in

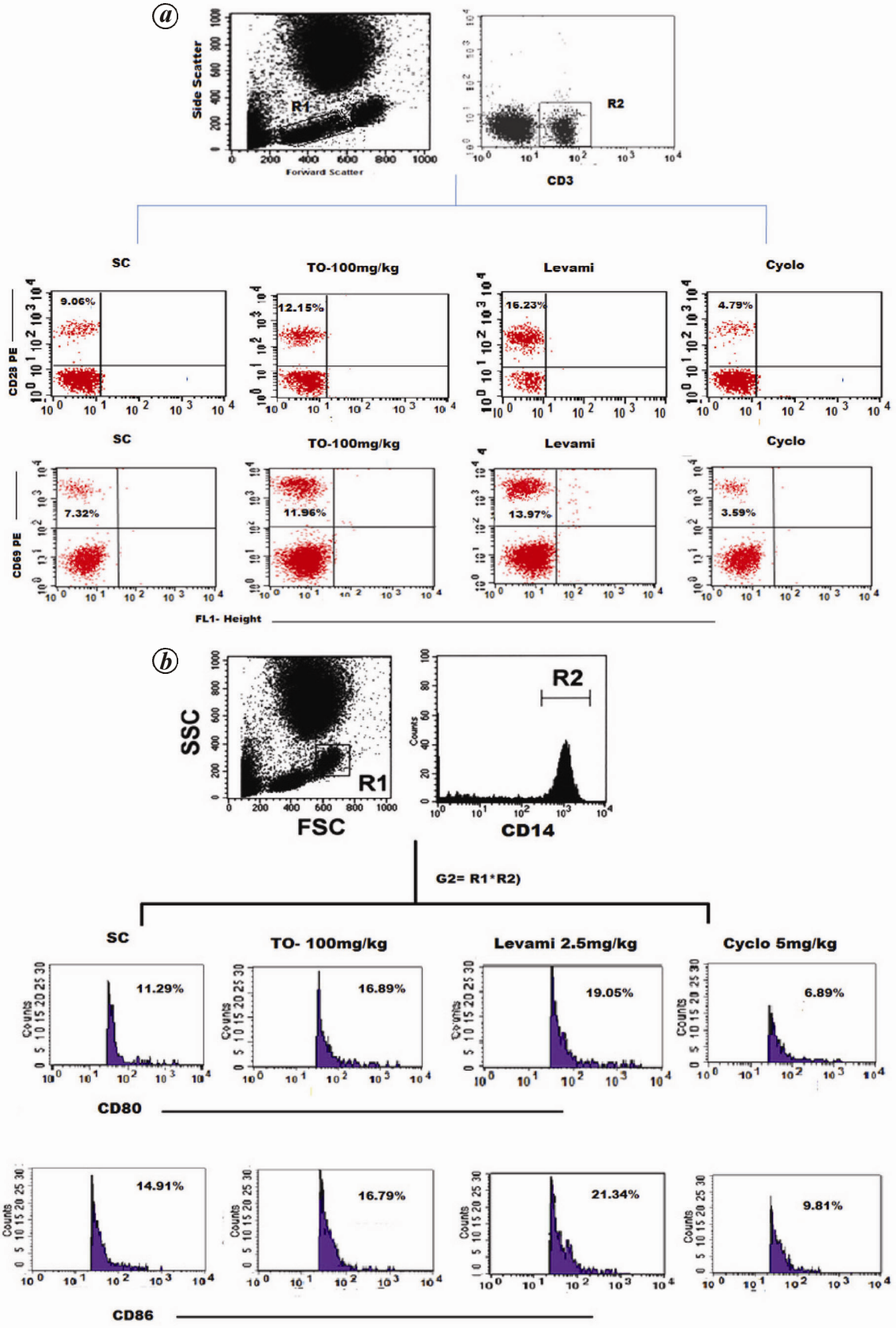


**Figure 6** ***a***, ***b***. Flow cytometric representaion of the effects of graded doses of TO-10 (mg/kg, p.o.) on CD28, CD69 present on activated T-cells CD80 and CD86 present on macrophages, in SRBC-immunized mice. The dot plots represent counts for one representative mouse from each group.

**Table 2.** Effect of graded doses of TO-10 (mg/kg, p.o.) on intracellular CD4-specific IL-2, IFN-gamma and IL-4 expression in SRBC immunized mice

| Treatment | Dose (mg/kg, p.o.) | $CD4^+$ IL-2 (mean ± SEM) | $CD4^+$ IFN-gamma (mean ± SEM) | $CD4^+$ IL-4 (mean ± SEM) |
|---|---|---|---|---|
| SC | – | 9.30 ± 0.28 | 9.48 ± 0.25 | 8.06 ± 0.20 |
| TO-10 | 1.56 | 9.73 ± 0.28<br>(4.62↑) | 9.86 ± 0.22<br>(4.00↑) | 7.90 ± 0.42<br>(1.98↓) |
| TO-10 | 3.12 | 10.03 ± 0.33<br>(7.84↑) | 10.13 ± 0.33<br>(6.85↑) | 7.79 ± 0.2<br>(3.34↓) |
| TO-10 | 6.25 | 10.15 ± 0.44<br>(9.13↑) | 11.09 ± 0.28<br>(16.98↑) | 7.47 ± 0.30<br>(7.32↓) |
| TO-10 | 12.5 | 10.75 ± 0.22 *<br>(15.59↑) | 11.36 ± 0.33 *<br>(19.83↑) | 7.10 ± 0.35<br>11.91↓ |
| TO-10 | 25 | 11.01 ± 0.21*<br>(18.38↑) | 11.87 ± 0.27 **<br>(25.21↑) | 6.87 ± 0.32<br>(14.76↓) |
| TO-10 | 50 | 12.83 ± 0.23**<br>(37.95↑) | 12.08 ± 0.22b**<br>(27.42↑) | 6.62 ± 0.43<br>(17.86↓) |
| TO-10 | 100 | 13.98 ± 0.13***<br>(50.32↑) | 12.34 ± 0.16 ***<br>(30.16↑) | 6.55 ± 0.24 *<br>(18.73↓) |
| TO-10 | 200 | 13.69 ± 0.23 ***<br>(47.20↑) | 12.42 ± 0.14***<br>(31.01↑) | 6.57 ± 0.35 *<br>(18.48↓) |
| Levamisole | 2.5 | 17.36 ± 0.23***<br>(86.66↑) | 15.93 ± 0.19***<br>(68.03↑) | 11.68 ± 0.31***<br>(44.91↑) |
| Cyclosporine | 5 | 3.84 ± 0.33***<br>(58.70↓) | 5.67 ± 0.32***<br>(40.18↓) | 5.06 ± 0.27***<br>(37.22↓) |

Each value represents mean ± SEM of six mice per group. Post-ANOVA (Bonferroni test for multiple comparisons); ***$P \le 0.001$, **$P \le 0.01$, *$P \le 0.05$. ↑, Increase; ↓, Decrease. Value in parenthesis represents percentage of activity against control.

**Table 3.** Effect of TO-10 (mg/kg, p.o.) on intracellular IL-12 and IL-10 expression in chronically stressed mice

| Treatment | Dose (mg/kg, p.o.) | IL-12 (mean ± SEM) | IL-10 (mean ± SEM) |
|---|---|---|---|
| SC | | $7.39 \pm 0.39^{a,***}$ | $5.97 \pm 0.29^{a,***}$ |
| RSC | | $4.44 \pm 0.31^{b,***}$<br>(39.91↓) | $8.01 \pm 0.26^{b,**}$<br>(34.17↑) |
| RS + SC + TO-10 | 6.25 | $4.94 \pm 0.27^{cns}$<br>(11.26↑) | $7.63 \pm 0.30^{cns}$<br>(4.74↓) |
| RS + SC + TO-10 | 12.5 | $5.38 \pm 0.26^{c,*}$<br>(21.17↑) | $7.09 \pm 0.34^{cns}$<br>(11.48↓) |
| RS + SC + TO-10 | 25 | $6.19 \pm 0.21^{c,**}$<br>(39.41↑) | $6.84 \pm 0.25^{cns}$<br>(14.60↓) |
| RS + SC + TO-10 | 50 | $6.56 \pm 0.28^{c,**}$<br>(47.74↑) | $6.60 \pm 0.23^{cns}$<br>(17.60↓) |
| RS + SC + TO-10 | 100 | $7.06 \pm 0.22^{c,***}$<br>(59.00↑) | $6.23 \pm 0.21^{c,**}$<br>(22.22↓) |
| RS + SC + TO-10 | 200 | $6.92 \pm 0.21^{c,***}$<br>(55.85↑) | $6.15 \pm 0.27^{c,**}$<br>(23.22↓) |
| RS + SC + KG | 100 | $8.92 \pm 0.17^{c,***}$<br>(100.90↑) | $5.92 \pm 0.23^{c,***}$<br>(26.18↓) |

Each value represents mean ± SEM of six mice per group. Post-ANOVA (Bonferroni test for multiple comparisons); ***$P \le 0.001$, **$P \le 0.01$, *$P \le 0.05$. Astericks with $P$ value 'a' indicate significant difference of RSC vs NC and 'b' indicates TO-10 treated groups vs RSC group. ↑, Increase; ↓: Decrease. Value in parenthesis represents percentage of activity against control.

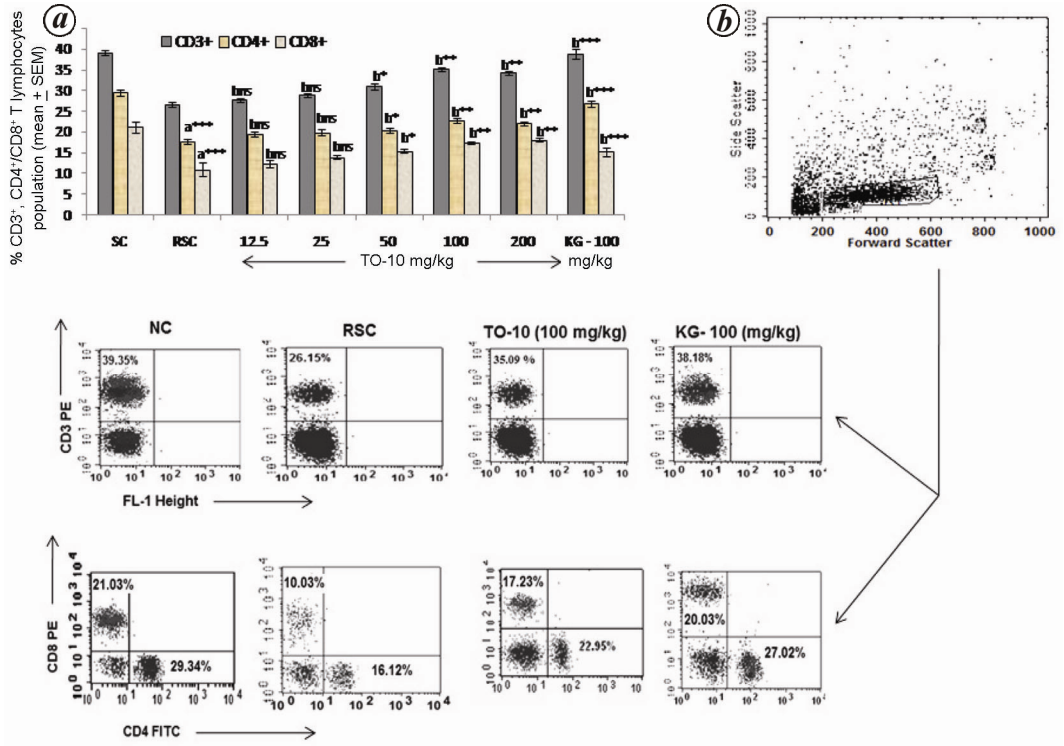


**Figure 7.** ***a***, Effect of graded doses of TO-10 (mg/kg) on $CD3^+$ and $CD4^+/CD8^+$ T-cell population in chronically stressed mice. Each column represents mean ± SEM of six mice per group. NC, Normal control; RSC, Restraint stress control; KG, Korean ginseng (positive standard). Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. Astericks with $P$ value 'a' indicate significant difference of RSC vs NC, and 'b' indicates TO-10-treated groups vs RSC group. ***b***, Flow cytometric quadrant plot representation showing the effect of TO-10 (effective dose only) on $CD3^+$ and $CD4^+/CD8^+$ T-cell population in chronically stressed mice. The histograms represent counts for one representative mouse from each group.

immune-suppressed mice was evaluated. Here we used two different methods for inducing immunosuppression. One is the natural method, chronic restraint stress, which is known to have suppressive effects on many aspects of immune response. The second is chemical-induced immunosuppression, wherein we suppressed the immune system by administering cyclosporine A (5 mg/kg).

*Effect of TO-10 on T-cell subsets in chronically stressed mice:* Quantification of T-cell surface markers ($CD3^+$ and $CD4^+/CD8^+$) was also carried out in mice subjected to chronic restraint stress for 14 days. On day 14 after the last stress session, blood was taken from all the groups, treated as well as non-treated, and subjected to estimation of T-cell surface markers by flow cytometer. Population of $CD3^+$, $CD4^+/CD8^+$ T-cells was considerably down-regulated in SRBC-immunized restraint stress control (RSC) group compared to SC group ($P < 0.001$). Treatment with graded doses of TO-10 restored $CD3^+$ and $CD4^+/CD8^+$ T-cell population to normal levels. However, significant effect was obtained at the dose level of 100 mg/kg, where $CD3^+$ T-cell population was 35.11 ± 0.40 (mean ± SEM), while $CD4^+/CD8^+$ T-cell population was 22.83 ± 0.56 (mean ± SEM) and 17.34 ± 0.33 (mean ± SEM) respectively (Figure 7).

*Effect of TO-10 on CD4-specific IL-2, IFN-gamma and IL-4 levels:* Flow cytometric evaluation of CD4-specific Th1 (IL-2, IFN gamma) and Th2 (IL-4) cytokines was also carried out in blood of animals subjected to chronic restraint stress. TO-10 showed considerable dose-related upregulation of intracellular $CD4^+$/IL-2 and IFN-gamma (Th1 cytokines) in SRBC-immunized mice subjected to restraint stress compared to SC control group. The optimum effect was obtained at 100 mg/kg dose. However, no restitution of suppressed $CD4^+$/IL-4 (Th2 cytokine) level was found on treatment with $I^3M/38/A001$, thereby showing the specific Th1 upregulating potential of TO-10 (Figure 8 *a* and *b*). This suggests that TO-10 selectively stimulates the Th1 pathway.

*Effect of TO-10 on whole-blood IL-12 and IL-10 levels in chronically stressed mice:* IL-12 and IL-10 play an imperative role in determining Th1/Th2 pathway. IL-12 has a key role in the initiation of cell-mediated immunity and favours Th1 polarization[22]. IL-10 is well recognized as a suppressor cytokine that plays a regulatory role in controlling the activity of T- and B-cells[23,24]. We estimated the whole blood percentage of IL-12 and IL-10 in chronically stressed animals to verify the effect of stress as well as the test extract on the levels of these two regulatory

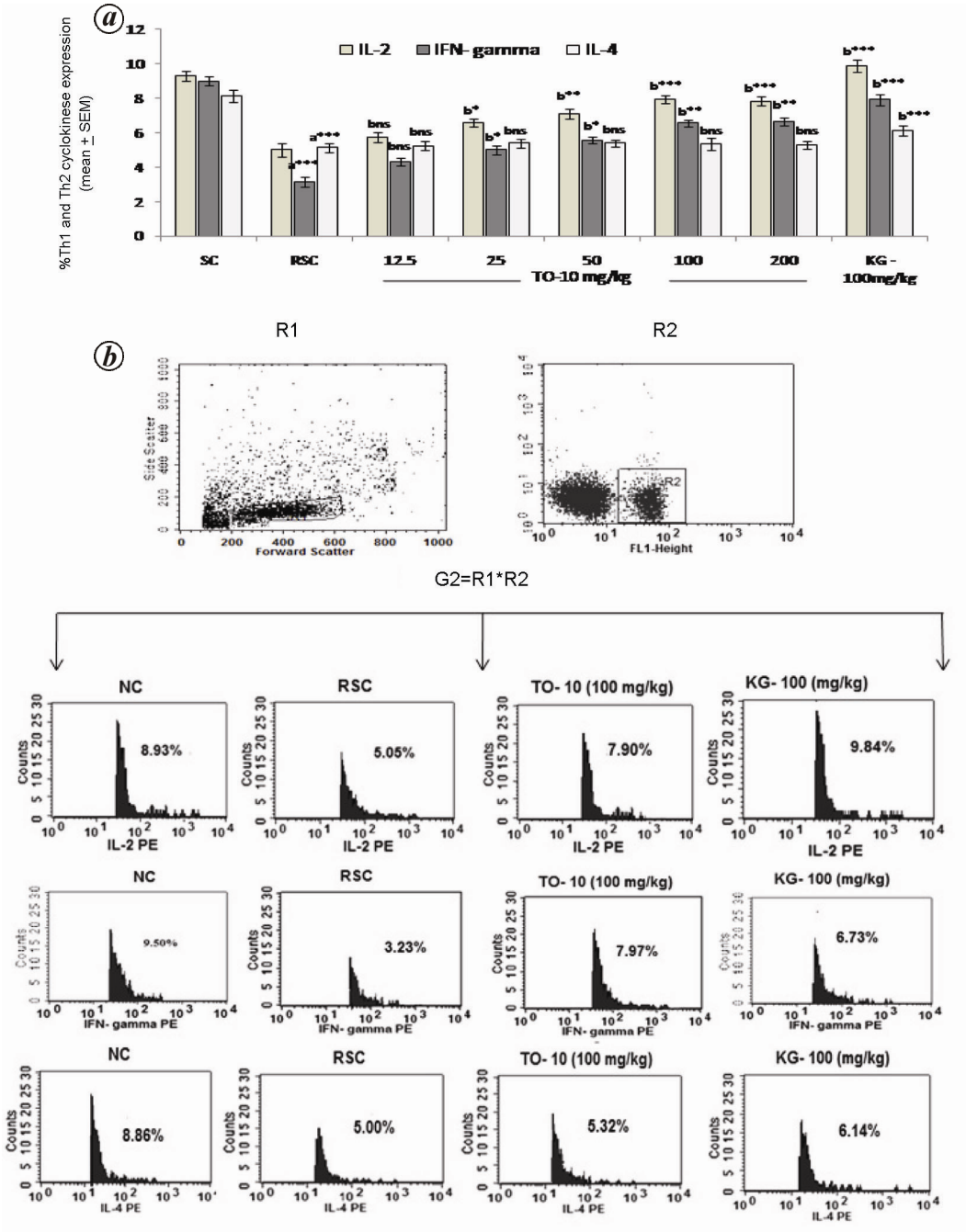


**Figure 8.** ***a***, Bar graphs showing the effect of graded doses of TO-10 on CD4-specific IL-2, IFN-gamma and IL-4 levels in chronically stressed mice. Each column represents mean ± SEM of six mice per group. NC, Normal control; RSC, Restraint stress control; KG, Korean ginseng (positive standard). Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); $***P \leq 0.001$, $**P \leq 0.01$, $*P \leq 0.05$. Each value represents mean ± SEM of six mice per group. Post-ANOVA (Bonferroni test for multiple comparisons). ***b***, Flow cytometric histrogram showing the effect of TO-10 (effective dose only) on CD4-specific IL-2, IFN-gamma and IL-4 levels in chronically stressed mice. The histograms represent counts for one representative mouse from each group.

cytokines. A significant suppression of IL-12 and an increase in IL-10 were observed in chronically stressed mice. This further supports the possibility that unbalanced IL-12/IL-10 may be one of the main factors responsible for stress-induced immunosuppression[25]. Whereas TO-10 caused considerable increase in the expression of whole-blood IL-12 along with suppression of raised IL-10 levels in stressed animals (Table 3 and Figure 9), thus showing the anti-stress activity of the extract.

*Corticosterone assay:* Corticosterone is the main stress hormone in rodents and its concentration increases during stressful conditions. Serum was separated from the blood collected in falcon tubes and estimated by competitive

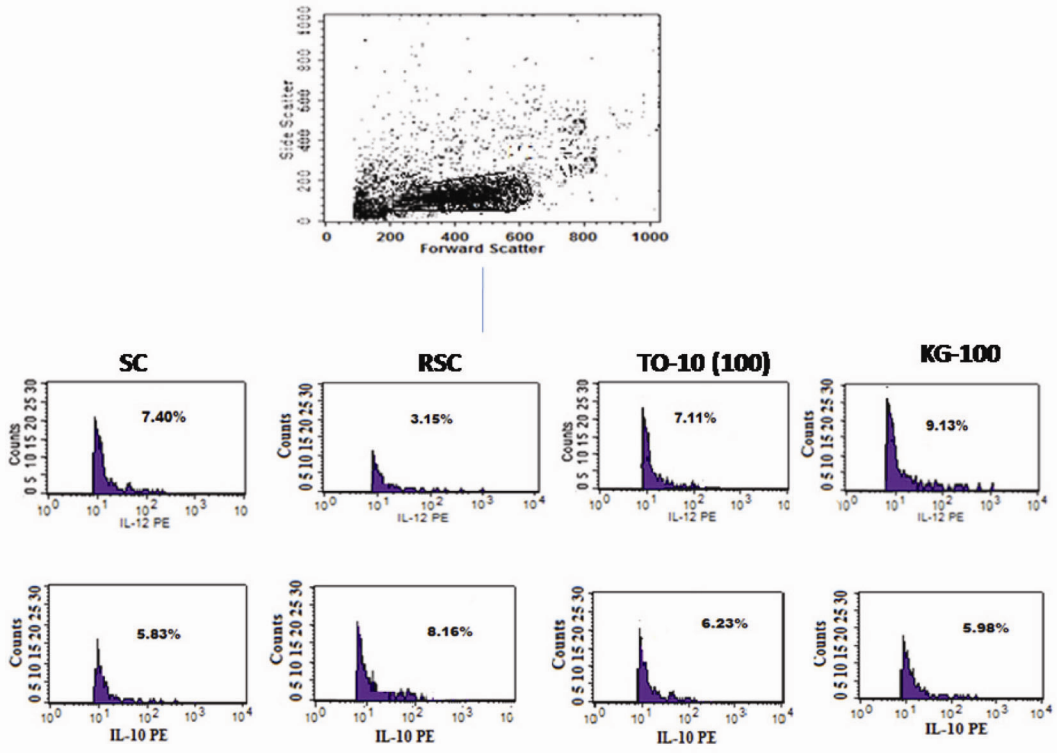


**Figure 9.** Flow cytometric histogram of the effect of TO-10 (mg/kg, p.o.) on intracellular IL-12 and IL-10 expression in chronically stressed mice. Histograms represent counts for one representative mouse from each group.

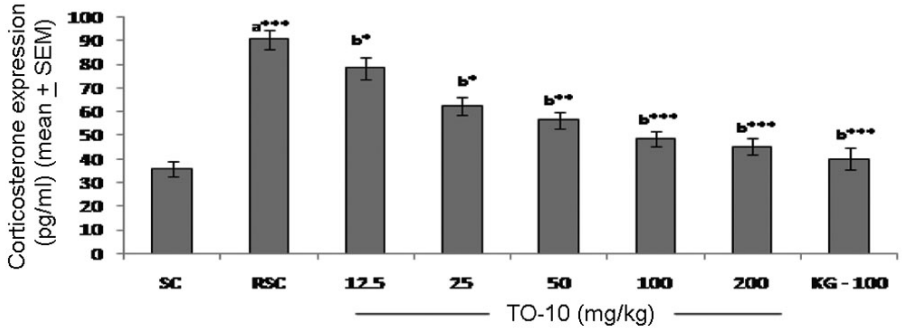


**Figure 10.** Effect of TO-10 (mg/kg) on raised corticosterone levels in restaint stress-induced chronically stressed mice. Each column represents mean ± SEM of six mice per group. NC, Normal control; RSC, Restraint stress control; KG, Korean ginseng (positive standard). Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. Astericks with *P* value 'a' indicate significant difference of RSC vs NC, and 'b' indicates TO-10 treated groups vs RSC group.

EIA method. A significant enhancement in its levels was observed in RSC group compared to control group. Treatment with TO-10 at graded oral doses encountered the harmful effects of restraint stress by normalizing the raised corticosterone levels with effective results obtained at dose levels of 100 and 200 mg/kg (Figure 10).

*Body and organ weights ratio:* After taking the body weight of the animals, they were sacrificed and immune-specific organs like thymus and spleen removed and weighed to see the effect of chronic stress as well as test drug on these parameters. It was observed that weight of thymus and spleen decreased noticeably in restraint stress conditions along with significant enhancement in adrenal gland weight. However, TO-10 significantly reversed the conditions in a dose-dependent manner, showing maximum effect at higher dose levels of 100 and 200 mg/kg (Table 4).

**Table 4.** Effect of TO-10 (mg/kg) on body and organ weight ratio of chronically stressed mice

| Variables | SC (mean ± SEM) | RSC (mean ± SEM) | TO-10 (12.5 mg/kg) (mean ± SEM) | TO-10 (25 mg/kg) (mean ± SEM) | TO-10 (50 mg/kg) (mean ± SEM) | TO-10 (100 mg/kg) (mean ± SEM) | TO-10 200 mg/kg) (mean ± SEM) | KG-100 mg (mean ± SEM) |
|---|---|---|---|---|---|---|---|---|
| Body wt (g) (day 14) | $23.78 \pm 3.87^{a,**}$ | $17.37 \pm 4.11^{b,**}$ | $20.27 \pm 5.01^{c,*}$ | $22.69 \pm 4.0^{c,**}$ | $23.77 \pm 3.4^{c,**}$ | $24.05 \pm 3.0^{cns}$ | $24.61 \pm 4.5^{cns}$ | $23.92 \pm 4.00^{c,***}$ |
| Thymus (mg) | $657.45 \pm 3.61^{a,***}$ | $309.24 \pm 4.02^{b,***}$ | $413.20 \pm 3.02^{c,*}$ | $488.40 \pm 3.5^{c,**}$ | $510.65 \pm 3.6^{c,**}$ | $551.21 \pm 3.21^{c,***}$ | $521.35 \pm 3.9^{c,***}$ | $601.29 \pm 4.40^{c,***}$ |
| Thymus wt/body wt | $23.08 \pm 4.11^{a,***}$ | $14.34 \pm 3.49^{b,***}$ | $18.24 \pm 5.62^{c,*}$ | $19.24 \pm 3.51^{c,**}$ | $21.64 \pm 4.2^{c,**}$ | $22.40 \pm 3.79^{c,***}$ | $20.11 \pm 4.04^{c,***}$ | $22.55 \pm 3.89^{c,***}$ |
| Spleen (mg) | $758.87 \pm 4.01^{a,**}$ | $375.28 \pm 3.51^{b,***}$ | $490.35 \pm 5.11^{c,*}$ | $512.45 \pm 4.03^{c,**}$ | $545.49 \pm 3.0^{c,**}$ | $621.45 \pm 3.9^{c,***}$ | $639.23 \pm 4.7^{c,***}$ | $730.23 \pm 3.56^{c,***}$ |
| Spleen wt/body wt | $21.34 \pm 4.50^{a,***}$ | $15.03 \pm 3.32^{b,***}$ | $17.29 \pm 3.77^{c,**}$ | $19.02 \pm 3.5^{c,**}$ | $19.82 \pm 4.7^{c,*}$ | $20.11 \pm 3.0^{cns}$ | $21.93 \pm 4.69^{cns}$ | $24.21 \pm 5.32^{c,***}$ |
| Adrenal glands (mg) | $14.01 \pm 4.61^{ans}$ | $45.88 \pm 3.98^{b,***}$ | $39.76 \pm 4.12^{c,*}$ | $35.80 \pm 3.53^{c,**}$ | $31.12 \pm 5.02^{c,**}$ | $29.34 \pm 3.75^{c,*}$ | $29.90 \pm 4.1^{cns}$ | $18.56 \pm 3.71^{c,***}$ |
| Adrenal glands wt/body wt | $0.69 \pm 2.09^{ans}$ | $2.87 \pm 3.03^{b,**}$ | $2.01 \pm 1.87^{c,*}$ | $1.75 \pm 0.42^{c,**}$ | $1.31 \pm 0.44$ | $0.98 \pm 2.0^{c,*}$ | $1.06 \pm 3.01^{cns}$ | $0.83 \pm 2.50^{c,***}$ |

Each value represents mean ± SEM of six mice per group. Post-ANOVA (Bonferroni test for multiple comparisons); $***P \leq 0.001$, $**P \leq 0.01$, $*P \leq 0.05$. Astericks with $P$ value 'a' indicate significant difference of RSC vs NC and 'b' indicates TO-10-treated groups vs RSC group.

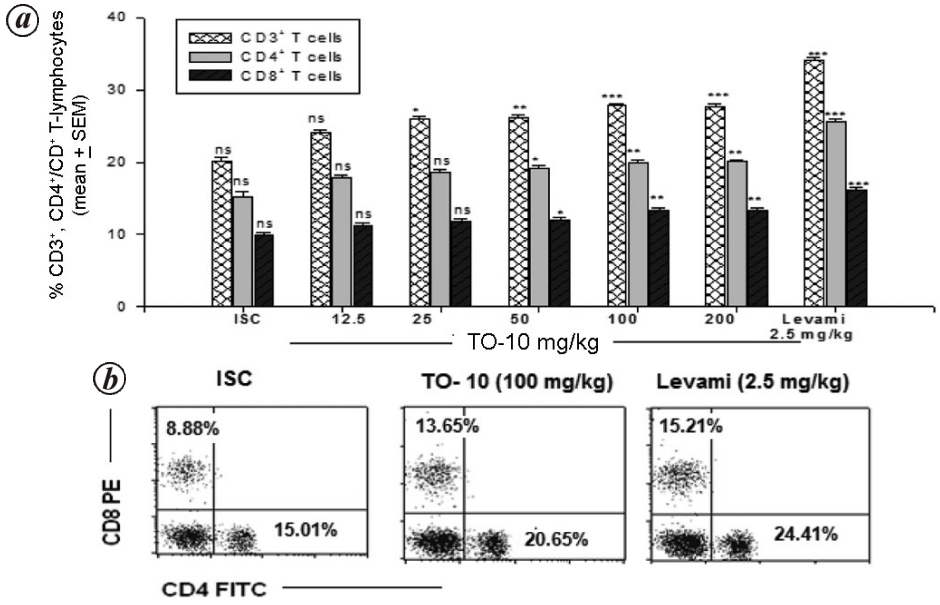


**Figure 11.** ***a***, Bar graph representing the effect of different doses of TO-10 (mg/kg, p.o.) on $CD3^+$, $CD4^+$ and $CD8^+$ T-cell population in cyclosporine-A-induced immune suppressed mice. Each column represents mean ± SEM of six mice per group. ISC, Immune-suppressed control and Levami, Levamisole (2.5 mg/kg). Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); $***P \leq 0.001$, $**P \leq 0.01$, $*P \leq 0.05$. ***b***, Flow cytometric quadrant plot representation showing the effect of TO-10 (effective dose only) on $CD3^+$ and $CD4^+/CD8^+$ T-cell population in cyclosporine-A-induced immune-suppressed mice. The histograms represent counts for one representative mouse from each group.

**Table 5.** Screening of different fractions of TO-10 on IL-2 in PBMCs isolated from normal Swiss albino mice

| Fraction | Concentration (μg/ml) | Chicoric acid | % IL-2 expression (mean ± SEM) | Activity against control | Inference |
|---|---|---|---|---|---|
| Control | 10 | | 4.01 ± 1.12 | – | Na |
| TO-1 | 10 | NP | 4.11 ± 0.87 | 2.49↑ | Inactive |
| TO-2 | 10 | NP | 4.24 ± 0.90 | 5.73 ↑ | Inactive |
| TO-3 | 10 | NP | 5.03 ± 1.24 | 25.43↑ | Inactive |
| TO-4 | 10 | NP | 4.52 ± 1.34 | 12.71↑ | Inactive |
| TO-5 | 10 | NP | 5.15 ± 1.13 | 27.93↑ | Inactive |
| TO-6 | 10 | NP | 4.78 ± 0.95 | 19.20↑ | Inactive |
| TO-7 | 10 | NP | 5.13 ± 1.27 | 27.93↑ | Inactive |
| TO-8 | 10 | NP | 4.97 ± 0.78 | 23.94↑ | Inactive |
| TO-9 | 10 | Abundantly present | 6.47 ± 1.16 | 61.34↑ | Active |
| TO-10 | 10 | In traces | 5.86 ± 1.24 | 46.10↑ | Active |
| TO-11 | 10 | NP | 4.29 ± 0.34 | 6.98↑ | Inactive |

↑, Increase; NP, Not present. Samples showing more than 30% desired activity.

*Cyclosporine A chemical-induced immune suppressed mice – Flow cytometric analysis of T-cell surface markers and Th1/Th2 cytokines in whole blood:* Cyclosporine A is an effective immune-suppressive agent which inhibits T-cell function[26]. After 48 h of SRBC challenge, blood was taken for estimation of T-cell surface markers as well as CD4-specific IL-2, IFN gamma and IL-4. Cyclosporine-A at 5 mg/kg dose suppressed the T-cell count as shown by flow cytometric analysis. TO-10 resulted in a significant increase in $CD3^+$, $CD4^+$ and $CD8^+$ T cell count when compared to immune-suppressed control (ISC) group. However, the most significant increase was observed at higher dose levels of 100 and 200 mg/kg (Figure 11). Similarly, significant restoration in the percentage of CD4-specific Th1 cytokines was observed in TO-10-treated groups compared to ISC group. Considerable effect was observed at the dose level of 100 mg/kg p.o. However, there was no significant effect on IL-4 expression in TO-10-treated, cyclosporine-induced immune compromised mice (Figure 12).

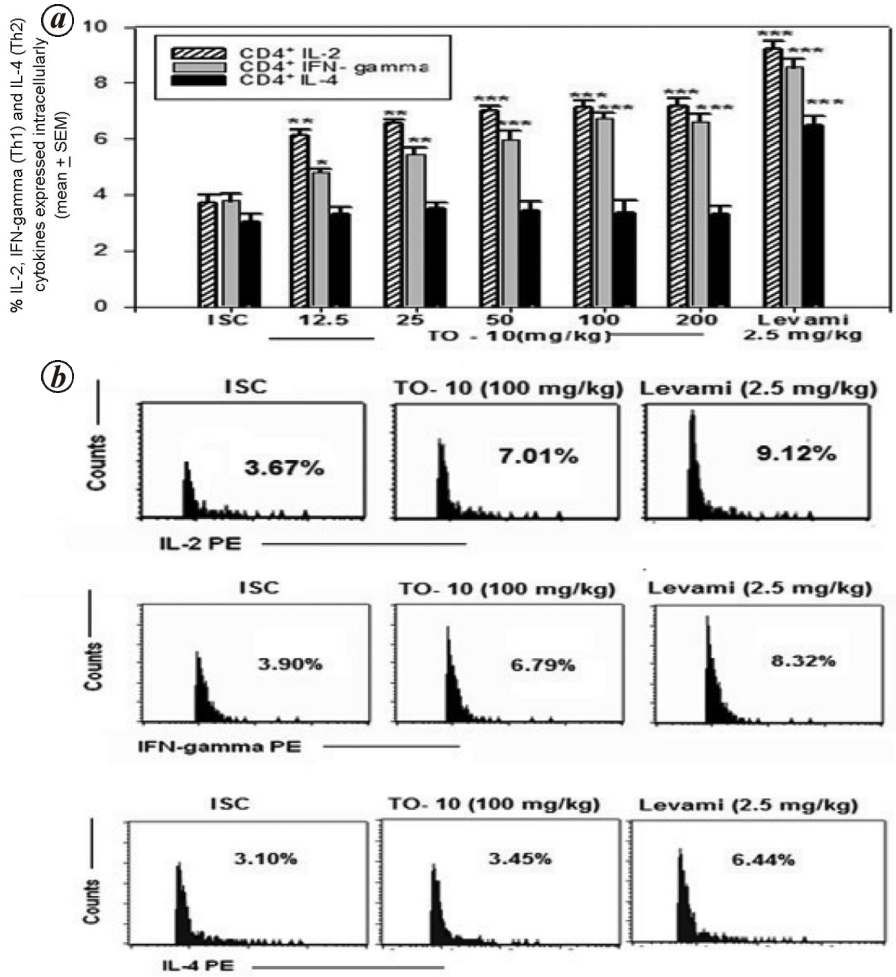


**Figure 12.** ***a***, Effect of TO-10 (mg/kg, p.o.) on CD4 specific IL-2, IFN-gamma and IL-4 in cyclosporine-A-induced immune-suppressed mice. Each column represents mean ± SEM of six mice per group. ISC, Immune-suppressed control and Levami, Levamisole (2.5 mg/kg). Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$. ***b***, Flow cytometric histogram showing the effect of TO-10 (effective dose only) on CD4-specific IL-2, IFN-gamma and IL-4 in cyclosporine-A induced immune-suppressed mice. The histograms represent counts for one representative mouse from each group.

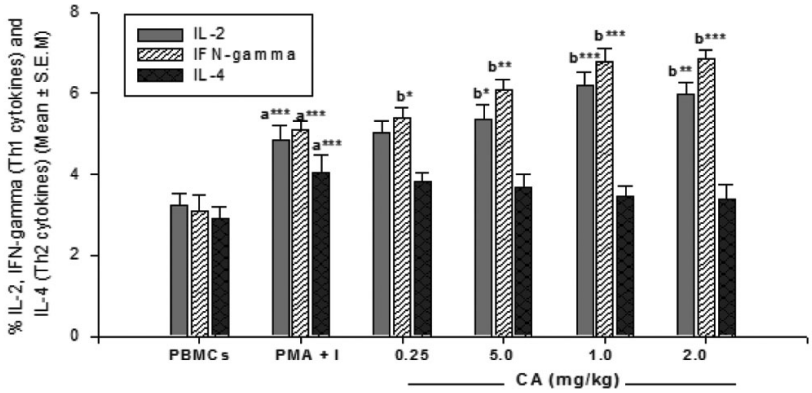


**Figure 13.** Effect of chicoric acid (CA) on IL-2, IFN-gamma and IL-4 expression in ionomycin (PMA + I) stimulated PBMCs isolated from normal Swiss albino mice. Data are represented as mean ± SEM ($n = 6$). Also, $2 \times 10^6$ cells/ml were stimulated for cytokine production with 10 ng/ml phorbol 12-myristate 13-acetate in combination with 1 mg/ml PMA + I. Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons); ***$P \leq 0.001$, **$P \leq 0.01$, *$P \leq 0.05$ vs PMA control.

*Screening of different fractions of TO-10:* To delineate the active constituent responsible for Th1 immune-stimulatory activity of TO-10, it was further fractionated by different polarity extracting procedures and column chromatography to obtain 11 different fractions. Each fraction was subjected to initial screening of IL-2 in (PMA + I)-stimulated PBMCs by flow cytometry. Fractions exhibiting more than 30% upregulation were considered active. Results showed that fraction TO-9, which contained maximum concentration of CA, displayed the most significant activity (Table 5).

*Effect of CA on Th1/Th2 cytokines in isolated PBMCs:* We further evaluated the effect of CA, a main constituent of the extract, on intracellular Th1/Th2 cytokines in (PMA + I) PBMCs. CA showed significant upregulation of intracellular IL-2 and IFN-gamma in (PMA + I) stimulated PBMCs, as depicted by flow cytometric analysis. A marginal decrease in IL-4 was observed, but it was not statistically significant (Figure 13).

## Conclusion

The present results show that TO-10 is an effective immune stimulatory agent which stimulates cell-mediated immunity and selectively supports Th1 polarization in normal and immune compromised conditions. This can be attributed to the presence of CA, a major constituent of the test extract. CA has already been reported by our group to augment immune response through the modulation of CD28/CTLA-4 and Th1 pathway in mice subjected to chronic stress[27]. This further supports our recent findings.


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