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Research Paper

Alcoholic extract of *Cicer microphyllum* augments Th1 immune response in normal and chronically stressed Swiss albino mice

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Abstract

Objective The purpose of this study was to observe the effect of an alcoholic extract of *Cicer microphyllum* (I^3M/38/A001) (whole plant without seeds and flowers) on the immunological parameters of sheep red blood cell immunized normal and chronically stressed Swiss albino mice.

Methods Estimation of T-cell subsets ($CD3^+$, $CD4^+/CD8^+$), CD80/CD86, CD28, CD 69, costimulatory molecules and Th1/Th2 cytokines was carried out using a flow cytometer. This was followed by study of the delayed type hypersensitivity response, in-vitro lymphocyte proliferation assay and measurement of Th1/Th2 cytokines in isolated peripheral blood mononuclear cells by flow cytometry. An enzyme immune assay was used to analyse corticosterone levels in the serum of chronically stressed animals.

Key findings We found that oral administration of I^3M/38/A001once daily at the graded doses of 6.25, 12.5, 25, 50, 100 and 200 mg/kg p.o. enhanced the proliferation and differentiation of T lymphocytes in sheep red blood cell normal and chronically stressed mice, as shown by flow cytometric analysis. The extract selectively induced type 1 immunity: it guided enhanced expression of Th1 cytokines, interferon-γ and interleukin-2, while no significant change in interleukin-4 (Th2 cytokine) levels was observed. Confirmation of Th1 polarization was confirmed by the augmented levels of interferon-γ and interleukin-2 in isolated peripheral blood mononuclear cells. A significant suppression of raised corticosterone levels was also observed in stressed animals, which suggests the extract's normalizing effect on the hypothalamic–pituitary–adrenal axis. Co-stimulatory molecules, CD28, CD69, CD80 and CD86, which are important secondary signals for the activation of the immune system, elicited significant expression in I^3M/38/A001 treated mice.

Conclusion Our studies show the immune potentiating and immune recuperative effect of the test drug in sheep red blood cell-immunized normal and chronically stressed mice.

Keywords chronic stress; *Cicer microphyllum*; immunophenotyping; intracellular cytokines

Introduction

Optimal immunotherapy requires the restoration of balanced Th1 and Th2 responses, suited to immune challenges, where functional integration of the immune system is accomplished mainly by cell-to-cell communication that relies on cytokines.[1] Based on the cytokines secreted, $CD4^+$ T cells can be subdivided into T helper cells, Th1 cells and Th2 cells. The relative levels of Th1 or Th2 activity can critically influence the outcome of any immune response. Th1 cells mainly produce interferon (IFN)-γ, interleukin (IL)-2 and IL-12, and are responsible for cell-mediated immunity, providing protection against intracellular pathogens. In contrast, Th2 cells chiefly produce IL-4, IL-5 and IL-13 and are responsible for the humoral immune response, promoting growth and differentiation of B-cells and neutralizing antibodies such as IgG1 in mice.[2] There are number of factors like poor diet, steroids, environmental pollution and stressful conditions that can affect the immune system function and disturb the Th1/Th2 homeostasis. Of these factors, chronic

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stress is more deleterious and leads to a dysfunctional immune system that opens the door to many health hazards. Stress inhibits many aspects of immune response, including innate immunity (e.g. natural killer cell lysis), T-cell responses and antibody production, both *in vivo* and *in vitro*,[3] and therefore makes the organism susceptible to opportunistic infections.

Currently, the importance of natural products in achieving T-cell homeostasis by selectively augmenting Th1 or Th2 responses is increasingly recognized. This is because natural products may often be used for prolonged periods without harm to the patient. Many modern drugs have their origin in traditional medicine and ethnopharmacology. Numerous molecules have emerged from Ayurvedic medicine and our present study is also an effort in this direction.

Cicer microphyllum Benth, part of the Fabaceae family, is known locally as Kukunnory or wild gram. It is a wild relative of the cultivated chickpea and is a herbaceous plant adapted to cold and high-altitude conditions that is widely distributed in the western Himalayas from Afghanistan to Tibet and western Nepal. The leaves of the plant are used locally to cure bronchitis and as an astringent. The unripened seeds are considered a stimulant, tonic and aphrodisiac. The seeds are also used to cure thirst and burning sensation in stomach, and have been used as an anti-helmintic.[4] The whole plant is used for increasing milk production and as a general tonic for cows.[5] However, no scientific studies proving the value of its traditional uses have been reported so far, therefore the objective of this study was to investigate the in-depth immunoregulatory potential of an alcoholic extract of *C. microphyllum* (whole plant, excluding seeds or flowers) in normal sheep red blood cell (SRBC) immunized normal mice. This was followed by study of its immune-restorative activity in chronically stressed mice, where prolonged activation of the hypothalamic–pituitary–adrenal (HPA) axis results in suppression of immune response.

Materials and Methods

Collection of plant material

The plant was collected from the Zanskar area of Ladakh (Jammu and Kashmir, India) in August 2007 and authenticated by Dr S.N. Sharma and Dr S. Kitchlu. A voucher sample (Accession no.21818) was retained and deposited at the Herbarium of IIIM, Jammu.

Preparation of the extract

The authenticated and shade-dried plant material of *Cicer microphyllum* (200 g; whole plant without seeds and flowers) was extracted with 95% ethanol (1500 ml) with mechanical stirring at 1000 rpm for 3 h. The plant material was filtered and the marc was further extracted with 95% ethanol by the same method. The process was repeated three times and the filtrate was pooled and concentrated in a rotary evaporator at 45°C and reduced pressure. On concentration, a dark brown gummy extract (23.5 g) was obtained, which was labeled as I^3M/38/A001.

High-pressure liquid chromatography of I^3M/38/A001 extract

The high-pressure liquid chromatography (HPLC) analysis of I^3M/38/A001 was carried out on an Agilent series 1100 instrument equipped with a binary pump, an auto sampler, an automatic electronic degasser, an automatic thermostatic column oven, a diode array detector and a computer with Chemstation software for data analysis. HPLC was used in combination with a quadrupole ion trap mass spectrometer (Esquire 3000) from Bruker, Bremen (Germany) equipped with an atmospheric pressure ionization electrospray interface. High-purity nitrogen from a nitrogen generator was used as a carrier gas.

The liquid chromatography separations were achieved using an RP-18, Merck (15 μm, 4.6 × 250 mm) column. The mobile phase, consisting of water (A) and acetonitrile (B), was delivered in a gradient at a flow rate of 0.8 ml/min with 5, 5, 60, 100, 100, 5 and 5% of acetonitrile at time intervals of 0.0, 5, 20, 40, 45, 60 and 65 min, respectively. The samples were analysed at 30°C to obtain the perfect peaks. The UV chromatograms were recorded at 215, 254 and 340 nm. Compounds like biochanin A, medicarpin, biochanin A-7-O-glucoside, formononetin and its glucosides, 3,4′,7-trihydroxy flavones, daidzein, garbanzol and petrocarpin have been reported in other *cicer* species such as *C. arietinum*.[6] Some of these compounds were also found to be present in the I^3M/38/A001 extract and these were identified on the basis of liquid chromatography mass spectrometry/Mass spectrometry (LCMS-MS). The peaks in the HPLC chromatogram at retention times of 15.5, 15.9, 16.5 and 19.1 min corresponded to the compounds formononetin glucoside, biochanin A-7-O-glucoside, 3,4′,7-trihydroxy flavones and biochanin A, respectively, and these compounds were identified on the basis of MS-MS data.[7] It is important to note here that none of these compounds or the extract of any of *cicer* species have been shown to possess the activity that we report in this paper.

The HPLC chromatogram (at wavelengths of 215, 254 and 340 nm) for I^3M/38/A001 is shown in Figure 1.

Safety study

The acute oral safety study was carried out following Organization for Econonomic Co-operation and development (OECD) guideline No.423.[8] A single dose of the test material was administered up to a dose level of 2500 mg/kg, once. The animals were observed for any gross behavioral changes for 6 h after treatment with the test drug, and daily thereafter for a total of 14 days.

Animals

The animal protocols used for the experimental work were approved by our Institutional Animals Ethics Committee. The National Institute of Health (NIH) 'Guide for care and use of laboratory animals' was strictly followed (NIH Publication No.85-23, revised 1985).

Swiss albino mice (male, 10–12 weeks old) were randomly distributed in groups as per the experimental protocols ($n = 6$). They were kept in air-conditioned and pathogen-free isolators with a temperature of 23 ± 2°C and humidity of 55.6 ± 10% on a regulated 12-h light and dark cycle.

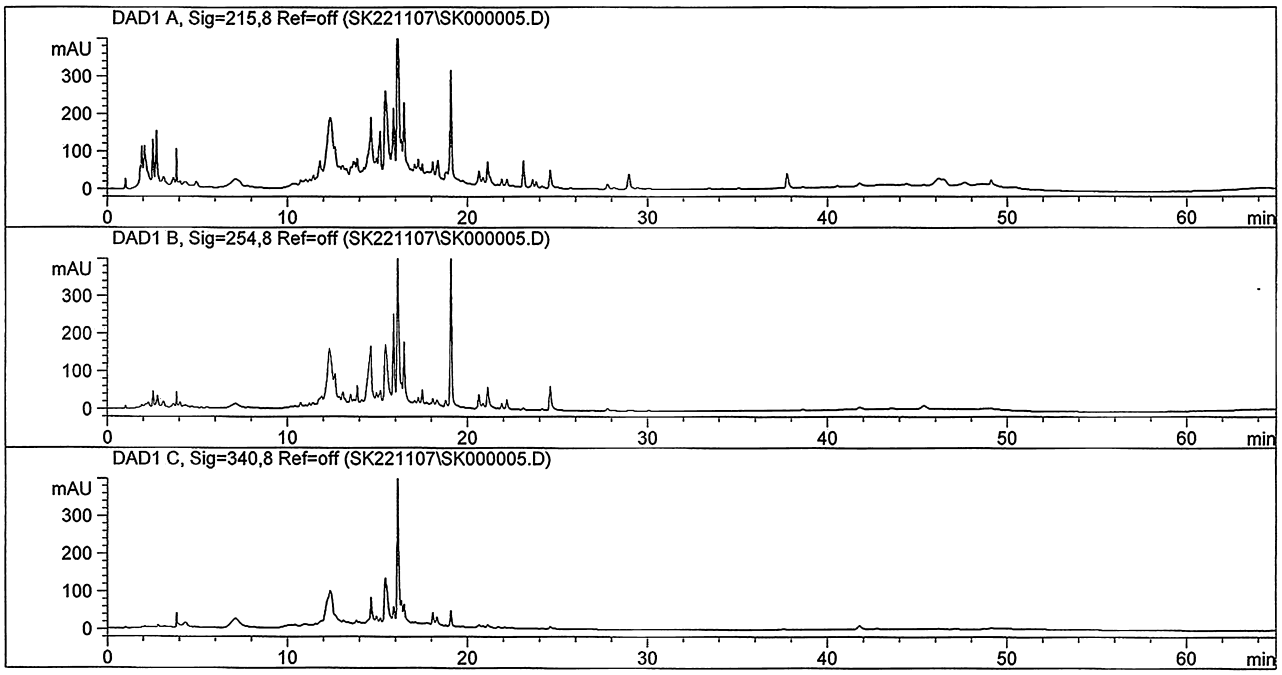

Figure 1 The HPLC chromatogram (at wavelengths of 215, 254 and 340 nm) for I3M/38/A001.

Chemicals

Concanavalin A (Con A), lipopolysaccharide (LPS), 3-(4,5-diamethyl-2-thiazolyl)-2,5 diphenyl-2H-tetrazolium) (MTT), Rosewell Park Memorial Institute (RPMI)-1460, L-glutamine, penicillin, streptomycin, 4-(2-hydroxy ethyl)-1-piperazineethane sulphonic acid (HEPES), phorbol 12-myristate 13-acetate (PMA) and ionomycin (I) were purchased from Sigma Chemical Co. (St Louis, MO). Fluorescienisothiocyanate (FITC)-labeled $CD4^+$, $CD3^+$, $CD80^+$, $CD28^+$ anti-mouse monoclonal antibody (mAbs), phycoerythrin (PE)-labeled $CD8^+$, $CD86^+$ and $CD69^+$ anti-mouse monoclonal antibody, Golgi plug, IFN-γ monoclonal antibody, IL-2, IL-4 monoclonal antibody, flourescence activated cell sorter (FACS) lysing solution and FACS permeabilizing solution were all obtained from BD Biosciences, USA. The corticosterone enzyme immune assay (EIA) kit was obtained from Cayman Chemicals.

Experimental design

The study was carried out in two phases:

- phase I, consisting of immunomodulatory studies in normal mice
- phase II, consisting of testing of the restorative effect of the drug on the immune profile of chronically stressed animals.

Immunomodulatory study in normal animals

In-vivo study

Grouping of animals and drug administration

Animals were divided into 10 groups with $n = 6$. Group I served as normal control and group II was the SRBC control, where animals were sensitized with SRBC, 5×10^9 cells per ml, i.p. on day 0. Groups III, IV, V, VI, VII and VIII were the treated groups, to which the test drug was administered at a dose level ranging from 6.25 to 200 mg/kg p.o., daily for the duration of the experiment. Group IX was the positive standard, and was administered with levamisole (2.5 mg/kg), a known immunostimulant that restores suppressed immune function.. Group X was as the negative standard and was administered with cyclosporine A (5 mg/kg), a known immunosuppressive agent.

Antigenic stimulus

The animals were sensitized with SRBC (5×109 cells per ml, i.p.) on day 0 and divided in seven groups, each group comprising six mice. Animals were then administered different doses of $I^3M/38/A001$ orally, for seven consecutive days. On day 7, animals were challenged by same amount of SRBC and after 48 h blood was collected from all the groups for the estimation of various T-cell and B-cell surface markers and Th1/Th2 cytokine estimation.

Flow cytometric analysis of T-cell surface markers

The analyses of subsets, namely $CD3^+$ (total T cell), $CD4^+$ (T-helper cells) and $CD8^+$ (cytotoxic cells), were performed on peripheral blood. FITC-labeled $CD3^+$ and $CD4^+$, and PE-labeled $CD8^+$ monoclonal antibodies were added directly to 100 μl of whole blood, which was then lysed using FACS lysing solution. Following the final centrifugation, samples were resuspended in phosphate buffer saline (pH 7.4)[9] and analysed directly on a flow cytometer using Cell Quest Pro Software. FITC-labelled $CD3^+$ and $CD4^+$ events were analysed on FL-1 Height and PE-labelled $CD8^+$ on FL-2 Height.

Th1/Th2 cytokines estimation

The blood was collected from the retro-orbital plexus and was placed in falcon tubes containing EDTA, to which Golgi plug

(1 μl/ml) was then added. A total of 100 μl of whole blood was taken and 500 μl of permeabilizing solution was added to each tube. IFN-γ monoclonal antibodies were used in one set, PE-labeled IL-2 monoclonal antibodies in the second set and PE-labeled IL-4 monoclonal antibodies in the third set of experiments. The tubes were incubated in the dark for 30 min at room temperature. Two millilitres of 1X FACS lysing solution was then added with gentle mixing, followed by incubation for 10 min at room temperature. The samples were centrifuged (300–400*g*) for 10 min. The supernatant was aspirated and the sample was resuspended in 500 μl of phosphate buffer saline (PBS).[10] Acquisition and analysis were performed directly on a flow cytometer using Cell Quest Pro software.

Delayed type hypersensitivity response

The method of Doherty was followed for determination of the SRBC-induced delayed-type hypersensitivity (DTH) response in normal mice.[11] Mice were immunized by injecting 200 μl of 5×10^9 SRBC/ml i.p. The animals were treated with graded doses of I^3M/38/A001 for the next six consecutive days. On day 7, they were challenged by injecting the 20 μl of 5×10^9 SRBC/ml intradermally into the left hind footpad. The footpad thickness was measured at 24 h (day 1), 48 h (day 2) and 72 h (day 3) after challenge.

In-vitro study

Medium

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), l-glutamine (2 mM), penicillin (50 mg/ml), streptomycin (50 mg/ml) and 2-mercaptoethanol (ME) (0·05 mM).

Lymphocyte proliferation assay

I^3M/38/A001 was assayed for lymphocyte proliferative responses using naïve Swiss albino mice splenocytes.[12] The spleens were excised aseptically and a single cell suspension was prepared by teasing the tissue between two glass slides. This suspension was centrifuged at 400*g* for 10 min at 4°C. The viability of splenocytes was checked using Evan's blue dye. Splenocytes (2×10^5 cells/well) were seeded in triplicate in a 96-well culture plate. Sub-optimal concentrations of Con A (0.5 μg/ml) and LPS (1 μg/ml) were added to each well separately for priming T-cells and B-cells, respectively. 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. Absorbance was measured in an ELISA plate reader (Meglan, USA) at 570 nm.

Isolation, treatment and measurement of Th1/Th2 cytokines in murine PBMCs by flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by density gradient centrifugation on histopaque. PBMC at the interface were collected and washed three times with PBS. After washing, the cells were adjusted to a concentration of 2×10^6 cells/ml cells were stimulated for cytokine production with 10 ng/ml phorbol 12-myristate 13-acetate in combination with 1 mg/ml ionomycin (PMA + I). I^3M/38/A001 was added at concentrations of 6.25, 12.5, 25, 50 and 100 μg/ml and incubated for 4 h at 37°C.

Further processing was performed by the addition of FACS permeabilizing solution (Becton Dickinson), followed by the addition of PE-labeled antimouse IL-2, IFN-γ and IL-4. The cells were incubated in the dark, and after being washed with sterile PBS, samples were resuspended in PBS (pH 7.4) and acquired directly on the flow cytometer (BD LSR; Becton Dickinson).[13]

Immunomodulatory study in stressed animals

Method of stress induction

Male Swiss albino mice, 10–12 weeks old and weighing about 22–24 g, were employed for the study. The animals used for the experimental work were approved by our Institutional Animals Ethics Committee after verifying the protocols that were followed for carrying out the experiments. According to ethical regulations on animal research, all the animals used in the experimental work received proper humane care. Polypropylene tubes (50 ml) having proper ventilation were used to induce stress in the animals during the experimental period. Mice were restrained in these 50-ml conical polypropylene tubes for 12 h during the dark cycle (2000–0800 h) for 14 days.[14] After each 12-h restrained session mice were given free access to pellet food and water.

Antigenic stimulus

To study immune responses in chronically stressed mice, stress induction was carried out for a period of 14 days as described above. On day 5, animals were sensitized by injecting 0.2 ml of SRBC i.p. and administration of different doses of the test drug was carried out from day 5 for the next consecutive 7 days. Korean gingeng (KG) (100 mg/kg), a known antistress, agent was used as the positive control. On day 12 animals were challenged by same volume of SRBC and on day 14 blood was collected from the retro-orbital plexus in EDTA-coated tubes for the estimation of T-cell and B-cell surface receptors and Th1 and Th2 cytokines using flowcytometry.[9,10]

Flow cytometric analysis of T-cell surface markers in SRBC-immunized chronically stressed mice

For normal mice, estimation of $CD3^+$ (total T cell), $CD4^+$ (T-helper cells) and $CD8^+$ (cytotoxic cells) was performed on the peripheral blood of SRBC-immunized chronically stressed mice by the method described above.[9]

Th1/Th2 cytokines estimation in peripheral blood

Flow cytometric estimation of IL-2, IFN-γ (Th1 cytokines) and IL-4 (Th2 cytokine) was carried out by the method described above. Briefly, 100 μl of whole blood was taken in falcon tubes. PE-labeled IFN-γ monoclonal antibodies were used in one set of experiments, PE-labeled IL-2 monoclonal antibodies in the second set and PE-labeled IL-4 monoclonal antibodies in the third set.[8] Finally, acquisition and analysis were performed directly on a flow cytometer using Cell Quest Pro software (BD Biosciences).

Flow cytometric analysis of co stimulatory molecules

Splenic cells from the treated and untreated mice were suspended in RPMI-1640 medium after removing the red blood cells with RBC lysis buffer. Briefly, 100 μl of splenocytes at 2.0×10^6 cells/ml were stained with FITC-labeled anti-CD28, anti-CD69 for T cells, and with FITC conjugated anti-CD80 (B7-1), anti-CD86 (B7-2) monoclonal (mAbs) for macrophages, after erythrocyte lysis with FACS lysis solution. Thereafter the cells were kept in the dark for 30 min at 4°C. After staining, the cells were washed twice with PBS, and the volume was made up to 500 μl for FACs analysis.[15]

Delayed type hypersensitivity response in stressed animals

The method of Doherty,[11] was followed with some modification for determination of the SRBC-induced DTH response in stressed mice. Chronic stress was induced over 14 days, as described above. On day 5, mice were immunized by injecting 200 μl of 5×10^9 SRBC/ml i.p. They were then treated with graded doses of I^3M/38/A001 for the next six consecutive days. On day 12, the mice were challenged by injecting 20 μl of 5×10^9 SRBC/ml intradermally into the left hind footpad. The footpad thickness was measured at 24 h, 48 h and 72 h (day 3) after challenge.

Corticosterone assay

Corticosterone is a major stress hormone in rodents and increases during periods of stress. All samples were assayed for corticosterone in serum by the competitive immunoenzymatic method (EIA kit Cayman Chemicals) at a wavelength of 450 nm. Measurements were taken in triplicate.

Body and organ weights

After the last stress session, the body weights of the animals were taken, after which the animals were sacrificed and their thymus, spleen and adrenal glands were removed and weighed.

Statistical analysis

Data are expressed as mean ± SEM. The statistical significance of the differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons).

Results

Safety studies

The test material was found to be well tolerated up to 2500 mg/kg. No change in general behavior or any mortality was observed in the group of animals treated with I^3M/38/A001 over a period of 14 days.

Effect of I^3M/38/A001 on T-cell surface markers

I^3M/38/A001 upregulated T cells, $CD3^+$ and $CD4^+/CD8^+$ percentages, as compared to control, in a dose-dependent manner. However, the most significant increase was obtained at dose levels of 25 and 50 mg/kg (Table 1).

Effect of I^3M/38/A001 on Th1/Th2 cytokines

Treatment with I^3M/38/A001 resulted in a dose-dependent increase in the expression of intracellular IL-2 and

Table 1 Effect of I^3M/38/A001 on $CD3^+$, $CD4^+/CD8^+$ T-cell population and intracellular IL-2, IFN- gamma and IL-4 expression in unsensitized and sensitized Swiss albino mice

	Treatment	Dose (mg/kg p.o.)	$CD3^+$ T cells (mean ± SEM)	$CD4^+$ T cell (mean ± SEM)	$CD8^+$ T cells (mean ± SEM)	IL-2 (mean ± SEM)	IFN-gamma (mean ± SEM)	IL-4 (mean ± SEM)
1	Normal control	–	34.21 ± 1.22	20.92 ± 0.78	14.29 ± 1.08	5.45 ± 0.18	4.89 ± 0.28	4.02 ± 0.19
2	SRBC control	–	40.15 ± 0.98^{a***}	29.21 ± 0.56^{a***}	21.25 ± 1.27^{a***}	8.92 ± 0.25^{a***}	8.10 ± 0.14^{a***}	6.81 ± 0.11^{a***}
3	SRBC + I^3M/38/A001	6.25	42.39 ± 0.34^{bns}	29.65 ± 0.67^{bns}	22.02 ± 0.44^{bns}	9.40 ± 0.14^{b*}	8.61 ± 0.24^{bns}	6.90 ± 0.14^{bns}
4	SRBC + I^3M/38/A001	12.5	45.86 ± 1.08^{b*}	32.14 ± 1.13^{bns}	24.82 ± 1.09^{bns}	10.18 ± 0.17^{b**}	9.58 ± 0.28^{b*}	7.02 ± 0.15^{bns}
5	SRBC + I^3M/38/A001	25	51.95 ± 0.69^{b**}	38.65 ± 0.87^{b*}	26.59 ± 0.33^{b*}	10.71 ± 0.29^{b**}	9.98 ± 0.11^{b**}	7.06 ± 0.23^{bns}
6	SRBC + I^3M/38/A001	50	52.24 ± 0.41^{b**}	39.42 ± 0.47^{b**}	25.55 ± 1.08^{b**}	9.18 ± 0.19^{b**}	9.93 ± 0.37^{b**}	7.09 ± 0.16^{bns}
7	SRBC + I^3M/38/A001	100	50.14 ± 0.67^{b**}	39.12 ± 1.22^{b**}	25.14 ± 0.78^{b**}	9.11 ± 0.29^{b*}	9.34 ± 0.45^{b*}	7.03 ± 0.28^{bns}
8	SRBC + I^3M/38/A001	200	49.34 ± 0.78^{b*}	35.51 ± 1.05^{b*}	25.09 ± 1.36^{b*}	9.03 ± 0.17^{b*}	9.24 ± 1.09^{b*}	7.04 ± 0.16^{bns}
9	Levamisole	2.5	68.11 ± 0.31^{b***}	45.20 ± 1.11^{b***}	29.84 ± 0.59^{b***}	12.15 ± 0.13^{b***}	11.11 ± 0.64^{b***}	9.79 ± 0.26^{b***}
10	Cyclosporin	5	26.24 ± 1.34^{b***}	15.26 ± 0.98^{b***}	10.15 ± 1.22^{b***}	5.04 ± 0.14^{b***}	4.88 ± 0.34^{b***}	4.79 ± 0.32^{b***}

Each column represents mean ± SEM of six mice per group. I^3M/38/A001, *Cicer microphyllum* alcoholic extract. Levamisole is positive standard, cyclosporin is negative standard. Asterisks with *P*-value 'a' indicate a significant difference of SRBC control versus normal control; 'b' indicates I^3M/38/A001 versus SRBC control. The statistical test employed is ANOVA followed by Bonferroni test for multiple comparisons. $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$.

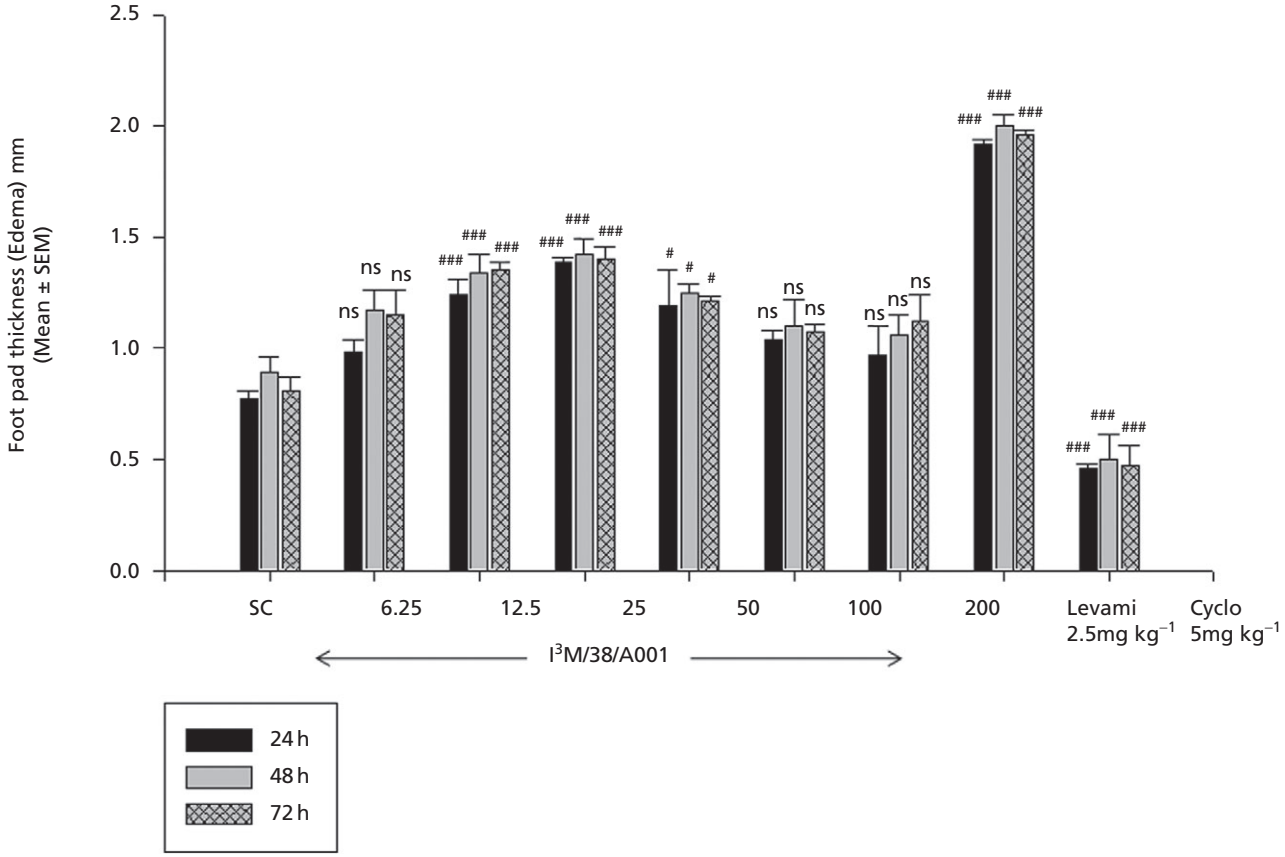

Figure 2 Effect of graded doses of $I^3M/38/A001$ on delayed-type hypersensitivity response in SRBC-sensitized mice. All values are shown as mean ± SEM of six mice. SC, sensitized control; $I^3M/38/A001$, *Cicer microphyllum* alcoholic extract; Levami, levamisole (positive standard); Cyclo, cyclosporine (negative standard). Statistical test employed is ANOVA followed by Bonferroni test for multiple comparisons. Asterisks with *P*-value 'b' indicate I3M/38/A001 versus SRBC control; ns, non-significant; $^{###}P \leq 0.001$, $^{##}P \leq 0.01$, $^{#}P \leq 0.05$.

IFN-gamma (Th1 cytokines) with no significant effect on IL-4 levels. The most significant effect was obtained at a dose level of 25 mg/kg (Table 1).

Delayed-type hypersensitivity response

The immune reaction in DTH response is characterized by swelling at the site of challenge, and by an infiltration of monocytes/macrophages and lymphocytes into the epidermis and dermis.[14] Oral administration of $I^3M/38/A001$ significantly upregulated the DTH response in SRBC-induced mice. The most significant effect was elicited at a dose level of 25 mg/kg, and 48 h after the SRBC challenge (Figure 2).

Lymphocyte proliferation assay

$I^3M/38/A001$ elicited a considerable increase in proliferative response in the Con A-stimulated T lymphocytes and LPS-stimulated B lymphocytes. The increase in proliferation was observed in a dose-dependent manner, the optimal effect being at 25 μg/ml. Cells grown in the absence of mitogens did not show any significant proliferative effect (Figure s1).

$I^3M/38/A001$ elicited Th1 cytokine expression in murine PBMCs *in vitro*

$I^3M/38/A001$ enhanced the intracellular expression of Th1 cytokines (IL-2 and IFN-gamma) in PMA + I-activated murine PBMCs in a dose-dependent manner. In contrast, the expression of IL-4, a Th2 cytokine, did not show any significant change on treatment (Figure S2).

Flow cytometric analysis of T-cell surface markers in SRBC-immunized chronically stressed mice

$CD3^+$ and $CD4^+/CD8^+$ T-cell population was significantly reduced in the restraint-stress control group as compared to the SRBC control group. Treatment of SRBC-immunized restraint-stressed animals with graded doses of $I^3M/38/A001$ restored $CD3^+$ and $CD4^+/CD8^+$ T-cell populations to normal levels. The most marked effect was obtained at a dose level of 25 mg/kg (Table 2).

Th1/Th2 cytokine estimation

$I^3M/38/A001$ produced a significant dose-related upregulation of intracellular IL-2 and IFN-gamma (Th1 cytokines) in SRBC-immunized restraint-stressed animals as compared to the RS + SRBC control group. The optimum effect was obtained at a 25 mg/kg dose. However, no restoration of suppressed IL-4 (Th2 cytokine) levels was found on treatment with $I^3M/38/A001$, thereby further confirming the specific Th1 upregulating effect of the test material (Table 3).

Table 2 Effect of $I^3M/38/A001$ on $CD3^+$, $CD4^+$/ $CD8^+$ T-cell population of SRBC-immunized chronically stressed Swiss albino mice

	Treatment	Dose (mg/kg p.o.)	$CD3^+$ T cells (mean ± SEM)	$CD4^+$ T cells (mean ± SEM)	$CD8^+$ T cells (mean ± SEM)
1	Normal control	–	32.29 ± 0.71	21.25 ± 1.24	15.54 ± 1.32
2	SRBC control (without stress)	–	39.92 ± 0.33^{a***}	27.56 ± 0.98^{a**}	24.78 ± 1.29^{a***}
3	RS + SRBC	–	26.12 ± 0.43^{b***}	18.12 ± 0.79^{b***}	10.98 ± 1.67^{b***}
4	RS + SRBC + $I^3M/38/A001$	6.25	28.56 ± 0.63^{cns}	19.56 ± 1.23^{cns}	11.78 ± 1.27^{cns}
5	RS + SRBC + $I^3M/38/A001$	12.5	30.56 ± 0.22^{c*}	19.67 ± 0.89^{c*}	12.56 ± 0.89^{c*}
6	RS + SRBC + $I^3M/38/A001$	25	32.17 ± 0.39^{c**}	21.49 ± 0.32^{c**}	14.84 ± 0.44^{c*}
7	RS + SRBC + $I^3M/38/A001$	50	32.02 ± 1.01^{c**}	20.34 ± 1.24^{c*}	15.59 ± 1.11^{c*}
8	RS + SRBC + $I^3M/38/A001$	100	30.11 ± 0.52^{c*}	19.13 ± 1.00^{cns}	13.34 ± 1.23^{cns}
9	RS + SRBC + $I^3M/38/A001$	200	29.01 ± 1.02^{cns}	19.03 ± 1.34^{cns}	13.12 ± 0.45^{cns}
10	RS + SRBC + KG	100	36.32 ± 1.11^{c***}	21.46 ± 0.45^{c***}	15.23 ± 0.98^{c**}
11	$I^3M/38/A001$ (without stress and SRBC)	200	33.67 ± 0.56^{cns}	23.45 ± 0.71^{cns}	17.78 ± 0.89^{cns}

Each column represents mean ± SEM of six mice per group. $I^3M/38/A001$, *Cicer microphyllum* alcoholic extract; RS, restraint-stress control; KG, Korean ginseng (positive standard). Asterisks with *P*-value 'a' indicate significant difference of SRBC control versus normal control; 'b' indicates RS + SRBC control versus. SRBC control group; 'c' indicates $I^3M/38/A001$ versus RS + SRBC control; $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. The statistical test employed is ANOVA followed by Bonferroni test for multiple comparisons.

Table 3 Data showing the restorative effect of different doses of $I^3M/38/A001$ on IL-2, IFN- gamma and IL-4 expression in SRBC immunized chronically stressed animals

S.NO	Treatment	Dose (mg/kg p.o.)	IL-2 (mean ± SEM)	IFN – gamma (mean ± SEM)	IL-4 (mean ± SEM)
1	Normal control	–	6.54 ± 0.27	5.78 ± 0.22	5.02 ± 0.38
2	SRBC control (without stress)	–	9.56 ± 0.34^{a***}	8.99 ± 0.18^{a**}	8.45 ± 0.25^{a**}
3	RS + SRBC	–	4.01 ± 0.36^{b***}	2.98 ± 0.23^{b***}	3.14 ± 0.20^{b***}
4	RS + SRBC + $I^3M/38/A001$	6.25	4.88 ± 0.36^{c*}	3.39 ± 0.25^{cns}	3.39 ± 0.26^{cns}
5	RS + SRBC + $I^3M/38/A001$	12.5	5.14 ± 0.25^{c*}	4.73 ± 0.32^{c*}	3.45 ± 0.17^{cns}
6	RS + SRBC + $I^3M/38/A001$	25	6.89 ± 0.28^{c**}	5.80 ± 0.15^{c**}	3.49 ± 0.26^{cns}
7	RS + SRBC + $I^3M/38/A001$	50	6.50 ± 0.31^{c**}	5.91 ± 0.29^{c**}	3.55 ± 0.18^{cns}
8	RS + SRBC + $I^3M/38/A001$	100	5.67 ± 0.26^{c*}	5.03 ± 0.14^{c*}	3.32 ± 0.34^{cns}
9	RS + SRBC + $I^3M/38/A001$	200	5.12 ± 0.22^{c*}	4.89 ± 0.32^{c*}	3.24 ± 0.16^{cns}
10	RS + SRBC + KG	100	8.72 ± 0.26^{c***}	7.79 ± 0.21^{c***}	4.50 ± 0.38^{c*}
11	$I^3M/38/A001$ (without stress and SRBC)	200	6.87 ± 0.29^{cns}	5.57 ± 0.30^{cns}	5.23 ± 0.12^{cns}

Each column represents mean ± SEM of six mice per group. I3M/38/A001, *Cicer microphyllum* alcoholic extract; RS, restraint-stress control; KG, Korean ginseng (positive standard). Asterisks with *P*-value 'a' indicate significant difference of SRBC control versus normal control; 'b' indicates RS + SRBC control versus SRBC control group; 'c' indicates I3M/38/A001 versus RS + SRBC control; $^{***}P \leq 0.001$, $^{**}P \leq 0.01$, $^{*}P \leq 0.05$. The statistical test employed is ANOVA followed by Bonferroni test for multiple comparisons.

Flow cytometric analysis of co-stimulatory molecules

CD80 and CD86 are secondary signal molecules present on antigen-presenting cells (APCs). CD28 and CD69 are present on activated T lymphocytes. Treatment with $I^3M/38/A001$ markedly upregulated the expression of CD80 and CD86 molecules on splenic APCs in chronically stressed animals as compared to the control group. The most significant increase was found at dose levels of 25 and 50 mg/kg (Table 4).

Delayed-type hypersensitivity response in stressed animals

Chronic stress significantly reduced the DTH response in SRBC-immunized mice. However, treatment with $I^3M/38/A001$ augmented the DTH response in a dose-dependent manner. The largest increase was observed at dose levels of 25 and 50 mg/kg (Figure 3).

Corticosterone assay

Corticosterone is a marker of stress response in rodents and prolonged secretion of this hormone during chronic stress is responsible for suppression of various immune parameters. Its concentration increased at least three times in mice subjected to restraint stress (RS + SRBC-control) as compared to the normal control group. However, a significant decrease in the level of corticosterone was observed in case of the groups treated with $I^3M/38/A001$ (Figure 4)

Body and organ weights

The body weights of the animals decreased considerably in restraint stress conditions. However, in the case of animals treated with graded doses of $I^3M/38/A001$, the increase in the body weight was found to be within the normal range (Table S1).

Exposure to chronic restraint stress resulted in a significant increase in adrenal gland weight. The decrease in the thymus

Table 4 Effect of doses of I^{3}M/38/A001 on CD28$^+$, CD69$^+$ activated T cells and CD80/CD86$^+$ antigen-presenting cells in chronically stressed Swiss albino mice

	Treatment	Dose (mg/kg p.o.)	CD28$^+$ T cells	CD69$^+$ T cells	CD 80$^+$ cells	CD86$^+$ cells
1	Normal control	–	6.06 ± 0.13	4.08 ± 0.06	5.36 ± 0.23	6.13 ± 0.39
2	SRBC control (without stress)	–	9.06 ± 0.34^{a***}	7.23 ± 0.22^{a***}	9.89 ± 0.30^{a***}	10.24 ± 0.24^{a***}
3	RST + SRBC	–	4.72 ± 0.33^{b***}	2.72 ± 0.08^{b***}	5.05 ± 0.44^{b***}	4.20 ± 0.14^{b***}
4	RST + SRBC + I^{3}M/38/A001	6.25	5.58 ± 0.23^{cns}	3.07 ± 0.11^{cns}	5.98 ± 0.12^{cns}	5.45 ± 0.22^{cns}
5	RST + SRBC + I^{3}M/38/A001	12.5	5.89 ± 0.12^{c*}	3.54 ± 0.10^{c*}	6.45 ± 0.39^{c*}	5.89 ± 0.29^{c*}
6	RST + SRBC + I^{3}M/38/A001	25	6.08 ± 0.11^{c**}	3.95 ± 0.14^{c**}	7.02 ± 0.22^{c**}	6.19 ± 0.22^{c**}
7	RST + SRBC + I^{3}M/38/A001	50	6.00 ± 0.11^{c**}	3.97 ± 0.08^{c**}	6.78 ± 0.29^{c**}	6.24 ± 0.36^{c**}
8	RST + SRBC + I^{3}M/38/A001	100	5.72 ± 0.10^{c*}	3.46 ± 0.03^{c*}	6.24 ± 0.15^{c*}	6.03 ± 0.32^{c*}
9	RST + SRBC + I^{3}M/38/A001	200	5.46 ± 0.11^{cns}	3.37 ± 0.19^{cns}	6.03 ± 0.45^{cns}	5.89 ± 0.42^{cns}
10	RST + SRBC + KG	100	7.22 ± 0.16^{c***}	5.00 ± 0.21^{c***}	7.62 ± 0.20^{c***}	8.08 ± 0.40^{c***}
	I^{3}M/38/A001 (without stress and SRBCs)	200	6.00 ± 0.26^{cns}	4.13 ± 0.22^{cns}	5.41 ± 0.18^{cns}	6.04 ± 0.31^{cns}

Values are shown as mean ± SEM of six mice per group. I^{3}M/38/A001; *Cicer microphyllum* alcoholic extract; RS, restraint-stress control, KG, Korean ginseng (positive standard). Asterisks with *P*-value 'a' indicate significant difference of SRBC control versus normal control; 'b' indicates RS + SRBC control versus SRBC control group; 'c' indicates I^{3}M/38/A001 versus RS + SRBC control. Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons). $^*P < 0.05$, $^{**}P < 0.01$,$^{***}P < 0.001$.

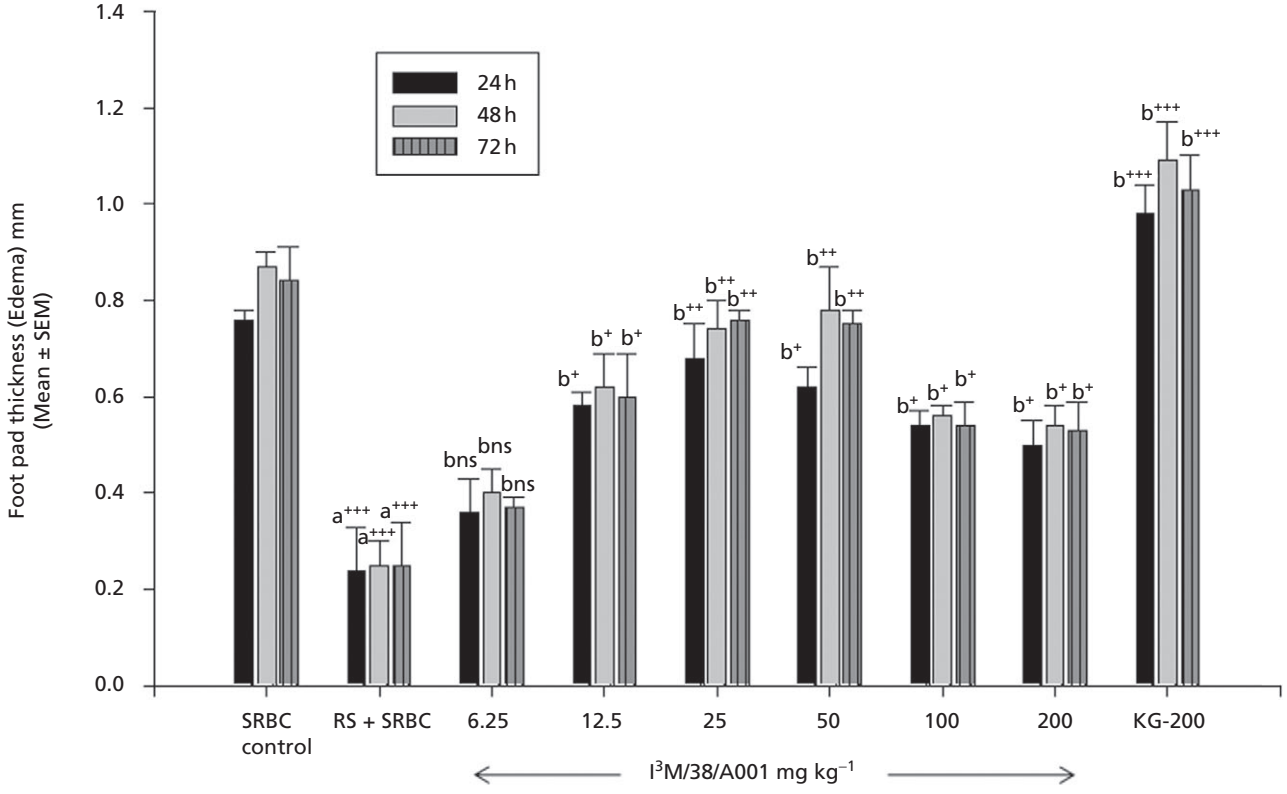

Figure 3 Effect of I^{3}M/38/A001 on delayed type hypersensitivity response (DTH) in chronically stressed mice. Asterisks with *P*-value 'a' indicate significant difference of RS + SRBC control versus SRBC control; 'b' indicates I^{3}M/38/A001 versus RS + SRBC control. Statistical significance of differences was assessed by post-ANOVA (Bonferroni test for multiple comparisons). $^+P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$.

and spleen weight thus showed the immunosuppressive effect of chronic stress. However, treatment of animals with graded doses of I^{3}M/38/A001 attenuated chronic–stress-induced atrophy of the spleen and thymus glands, and hypertrophy of the adrenal glands (Table S2).

Discussion

Modulation of the immune response in various immune-compromised situations is mediated by a complex network of signals that operate via bi-directional communication among

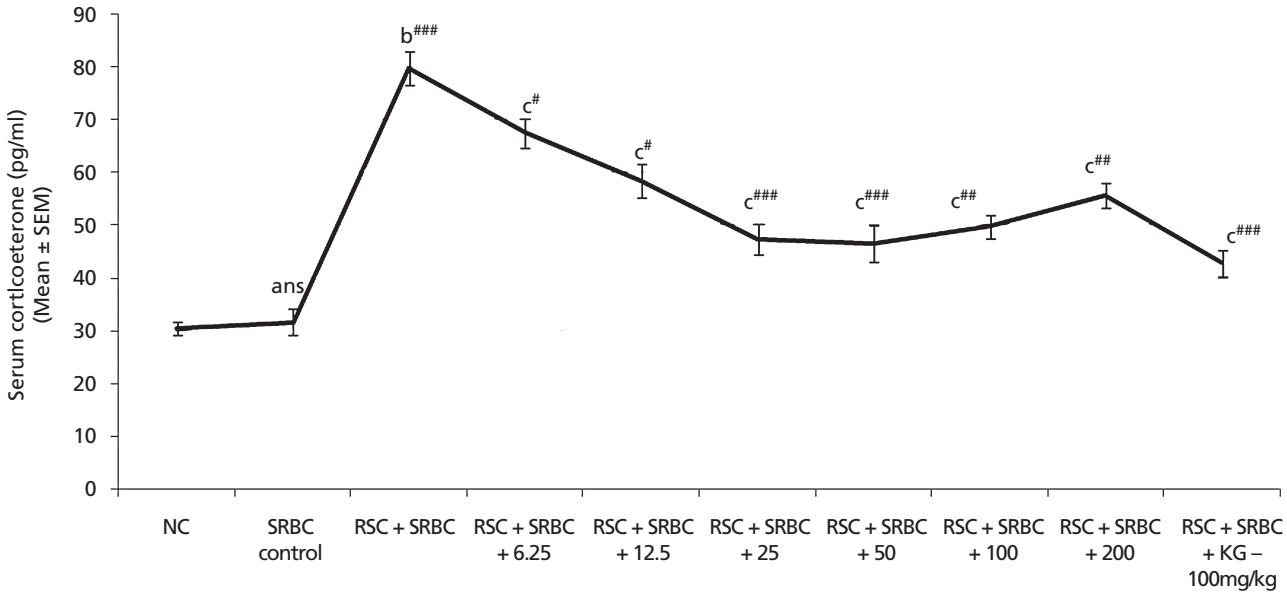

Figure 4 Effect of I[3]M/38/A001 on raised corticosterone levels in chronically stressed mice. Values are shown as mean ± SEM of six mice per group. NC, normal control; I[3]M/38/A001, *Cicer microphyllum* alcoholic extract; RS, restraint-stress control; KG, Korean ginseng (+ve standard). Asterisks with *P*-value 'a' indicate significant difference of SRBC control versus normal control; 'b' indicates RS + SRBC control versus SRBC control group; 'c' indicates I[3]M/38/A001 versus RS + SRBC control; ***$P \leq 0.001$, **$P \leq 0.01$, * $P \leq 0.05$; Bonferroni test for multiple comparisons was used to calculate statistical significance.

the nervous, endocrine and immune systems, and also involves interactions of various signaling molecules with immune cells, thereby facilitating the cross-talk between these immune cells to evoke a desired immune response. T lymphocytes play a major role in immunity and are subdivided into Th1 and Th2 helper cells on the basis of the cytokine secretion pattern, which eventually determines the induction of cellular and/or humoral immune responses. The 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. Hence, the optimal immunotherapy should restore or maintain a well-balanced Th1 and Th2 response, which is suited to the immune challenge.[16] In the present study an attempt has been made to evaluate the immune regulatory potential of *Cicer microphyllum* alcoholic extract.

Our results suggests that I[3]M/38/A001 is highly efficient in augmenting the immune response, which it does by increasing $CD3^+$ and $CD4^+/CD8^+$ T-cell populations. This is followed by an increase in the expression of related Th1 cytokines, such as IFN-gamma and IL-2 in SRBC-immunized normal mice. The specific Th1 upregulatory potential of the test drug was further supported by its stimulatory effect on Th1 cytokines in (PMA + I)-stimulated murine PBMCs. It also enhanced the DTH response in a dose-dependent manner, thereby further confirming its cell-mediated immune upregulatory potential, as the DTH immune reaction is usually represented by T-cell response.[17]

The significant Th1 upregulating potential of the test extract in normal mice prompted us to look for its therapeutic potential in chronically stressed conditions as stress is perhaps the most debilitating of all factors affecting the immune system,[18] leading to it becoming dysfunctional and opening the door to many health risks. In chronic stress, prolonged activation of the HPA axis results in an enhanced release of glucocorticoids, together with the activation of sympathetic mechanism, which is mainly responsible for the inhibition of cellular and humoral immune responses after chronic stress exposure.[19] During chronic stress, nerve terminals accelerate the recruitment of lymphocytes from the spleen, the major storage pool of lymphocytes, into the blood.[20] This results in a squeezing of the spleen, causing the reduction in its weight that is observed in cases of chronic stress exposure.

Moreover, persistent high levels of corticosterone during chronic stress causes apoptosis and necrosis in immature T and B cells, resulting in a decline in the thymus weight.[21] Treatment of chronically stressed animals with I[3]M/38/A001 significantly reduces these raised corticosterone levels, thereby significantly reversing the atrophy of the spleen and thymus, and the hypertrophy of the adrenal glands. This shows the drug's normalizing effect on the activated HPA axis. Accordingly, flow cytometric analysis of T-lymphocyte surface markers ($CD3^+$, $CD4^+/CD8^+$) in chronically stressed animals indicates significant restoration of the lymphocyte population, along with an increase in the expression of IL-2 and IFN-gamma, thereby further confirming the immune restorative effect of the test drug in chronic stressful conditions. Co-stimulatory molecules CD28, CD69, CD80 and CD86 are important secondary signals for the activation of the immune system. Signals generated by the binding of CD28 to its ligands, CD80 or CD86, which are found on antigen-presenting cells, cooperate with TCR-dependent signals, leading to cell proliferation and Th1/Th2 cytokine production.[22] Expression of these co-stimulatory molecules was also found to increase in I[3]M/38/A001-treated chronically stressed mice.

Conclusion

In conclusion, our results demonstrate that $I^3M/38/A001$ is a potent immune-stimulatory agent. It increases cell-mediated Th1 immune response as evidenced by enhanced secretion of Th1 cytokines in both normal as well as chronic stressed conditions, where compromised Th1 immunity is responsible for a dysfunctional immune system. This suggests there is potential for the use of the test material in T-cell function restoration where normal Th1 immune therapy is required to restore homeostasis.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Effect of $I^3M/38/A001$ on lymphocyte proliferation. Splenocytes (5×10^6 viable cells/ml/well)) were cultured with different concentrations of $I^3M/38/A001$ in either presence or absence of mitogens (LPS or Con A) stimulus. Bar graphs represent Mean ± S.E.M. absorbance units. Bonferroni test for multiple comparisons was used to compute *P-values.* $*P \leq 0.05$, $**P \leq 0.01$ vs. paired control.

Figure S2 Data shows the effect of different doses of $I^3M/38/A001$ on Th1/Th2 cytokines expression in murine PBMCs. 2×10^6 cells/ml cells were stimulated for cytokine production with 10 ng/ml phorbol 12-myristate 13-acetate in combination with 1 mg/ml ionomycin (PMA + I). Bonferroni test for multiple comparisons was used to compute *P*-values. $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$. Astericks with *P*-value 'a' indicate significant difference of (PMA + I) control vs. PBMC control and 'b' indicate $I^3M/38/A001$ vs. (PMA + I) control.

Table S1 Data showing the effect of $I^3M/38/A001$ on body weights of chronically stressed Swiss albino mice. Values are shown as Mean ± S.E.M. of 6 mice per group. $I^3M/38/A001$ – *Cicer microphyllum* alcoholic extract, RS- Restraint stress control, KG- Korean ginseng (+ve standard). Astericks with

P-value 'a' indicate significant difference of SRBC control vs. NC and 'b' indicate RS + SRBC control vs. SRBC control group and 'c' indicates $I^3M/38/A001$ vs. RS + SRBC control.

Table S2 Effect of $I^3M/38/A001$ on weights of immune specific organs of chronically stressed Swiss albino mice. Values are shown as Mean ± S.E.M. of 6 mice per group. $I^3M/38/A001$ – *Cicer microphyllum* alcoholic extract, RS- Restraint stress control, KG- Korean ginseng (+ve standard). Astericks with *P*-value 'a' indicate significant difference of SRBC control vs. Normal control and 'b' indicate RS + SRBC control vs. SRBC control group and 'c' indicate $I^3M/38/A001$ vs. RS + SRBC control.