



# Modulation of Pro-inflammatory Cytokines Expression of the Biofield Energy Healing (The Trivedi Effect<sup>®</sup>) Based Herbomineral Formulation in Mouse Splenocytes

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**Abstract:** With the increasing popularity of herbomineral preparations in healthcare, a new proprietary herbomineral formulation was formulated with ashwagandha root extract and three minerals *viz.* zinc, magnesium, and selenium. The aim of the study was to evaluate the immunomodulatory potential of Biofield Energy Healing (The Trivedi Effect<sup>®</sup>) on the herbomineral formulation using murine splenocyte cells. The test formulation was divided into two parts. One was the control without the Biofield Energy Treatment. The other part was labelled the Biofield Energy Treated sample, which received the Biofield Energy Healing Treatment remotely by twenty renowned Biofield Energy Healers. Through MTT assay, all the test formulation concentrations from 0.00001053 to 10.53 µg/mL were found to be safe with cell viability ranging from 102.61% to 194.57% using splenocyte cells. The Biofield Treated test formulation showed a significant ( $p \leq 0.01$ ) inhibition of TNF- $\alpha$  expression by 15.87%, 20.64%, 18.65%, and 20.34% at 0.00001053, 0.0001053, 0.01053, and 0.1053 µg/mL, respectively as compared to the vehicle control (VC) group. The level of TNF- $\alpha$  was reduced by 8.73%, 19.54%, and 14.19% at 0.001053, 0.01053, and 0.1053 µg/mL, respectively in the Biofield Treated test formulation compared to the untreated test formulation. The expression of IL-1 $\beta$  reduced by 22.08%, 23.69%, 23.00%, 16.33%, 25.76%, 16.10%, and 23.69% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53 µg/mL, respectively compared to the VC. Additionally, the expression of MIP-1 $\alpha$  significantly ( $p \leq 0.001$ ) reduced by 13.35%, 22.96%, 25.11%, 22.71%, and 21.83% at 0.00001053, 0.0001053, 0.01053, 1.053, and 10.53 µg/mL, respectively in the Biofield Treated test formulation compared to the VC. The Biofield Treated test formulation significantly down-regulated the MIP-1 $\alpha$  expression by 10.75%, 9.53%, 9.57%, and 10.87% at 0.00001053, 0.01053, 0.1053 and 1.053 µg/mL, respectively compared to the untreated test formulation. The results showed the IFN- $\gamma$  expression was also significantly ( $p \leq 0.001$ ) reduced by 39.16%, 40.34%, 27.57%, 26.06%, 42.53%, and 48.91% at 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53 µg/mL, respectively in the Biofield Treated test formulation compared to the VC. The Biofield Treated test formulation showed better suppression of IFN- $\gamma$  expression by 15.46%, 13.78%,

17.14%, and 13.11% at concentrations 0.001053, 0.01053, 0.1053, and 10.53  $\mu\text{g/mL}$ , respectively compared to the untreated test formulation. Overall, the results demonstrated that The Trivedi Effect<sup>®</sup>- Biofield Energy Healing (TEBEH) has the capacity to potentiate the immunomodulatory and anti-inflammatory activity of the test formulation. Biofield Energy may also be useful in organ transplants, anti-aging, and stress management by improving overall health and quality of life.

**Keywords:** Biofield Energy Healing Treatment, Biofield Energy Healers, The Trivedi Effect<sup>®</sup>, Herbomineral Formulation, Immune-Modulation, Pro-inflammatory Cytokines, Splenocytes

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## 1. Introduction

Herbomineral formulations have always been a major target of scientific research due to their significant immunomodulatory potential. Plant products and their extracts are used in both allopathic health care as well as complementary and alternative health care in order to improve overall health and the immune system [1, 2]. However, much attention has been focused on discovering herbal products with immunomodulatory activity along with low toxicity and better bioavailability [3]. Many scientific studies have identified the immunomodulatory properties of medicinal plants, which can be further potentiated with the addition of some minerals that regulate the immune cells. These types of formulations are commonly defined as herbomineral formulations and are the major target for pharmaceutical companies as phytopharmaceutical products or as dietary supplements. Based on the literature, a new proprietary herbomineral formulation was formulated with a combination of the herb ashwagandha (*Withania somnifera*) root extract and three minerals *viz.* zinc, magnesium, and selenium. All the ingredients of the formulation in this present study possess important activities such as immunomodulatory, anti-inflammatory, antioxidant, anti-infective, and anti-viral properties [4-7]. Ashwagandha has been reported to have an effect on biological activity mainly due to the presence of withanolides, and it is used as an herbal medicine in alternative and complementary therapies [8, 9]. Apart from its common attributes such as antibacterial, immunomodulatory and antitumor effects, many clinical and preclinical data have been available with respect to its immunomodulatory impact [4, 10]. Minerals such as selenium, zinc, and magnesium have been shown to have significant importance in modulating the immune system, and their synergistic impact has been well-defined [5].

Scientific research has documented that in the presence of minerals, herbal medicines have been found to exhibit a high level of phagocytic index and improved antibody titer [11]. These formulations can be used for better therapeutic effects in immune compromised patients affected with cardiovascular diseases, age and stress related diseases, cancer, and autoimmune disorders. Along with herbomineral formulations, the Biofield Energy Healers in this study have used energy medicine (Biofield Energy Healing Treatments) as a complementary and alternative approach to study the impact of Biofield Energy Treatments on the specified herbomineral formulation for its immunomodulatory potential with respect to the pro-inflammatory cytokines in

splenocyte cells isolated from mice.

According to scientific studies and clinical trials, Biofield Energy Treatments have been reported to have significant outcome in terms of the enhanced immune function of cervical cancer patients with therapeutic touch [12] and massage therapy [13], etc. The National Center of Complementary and Integrative Health (NCCIH) has recognized and accepted Biofield Energy Healing as a Complementary and Alternative Medicine (CAM) health care approach in addition to other therapies, medicines and practices such as homeopathy, deep breathing, natural products, special diets, naturopathy, massage, chiropractic/osteopathic manipulation, acupuncture, acupressure, rolfing structural integration, progressive relaxation, guided imagery, relaxation techniques, movement therapy, hypnotherapy, pilates, Ayurvedic medicine, traditional Chinese herbs and medicines, cranial sacral therapy, aromatherapy, essential oils, applied prayer (as is common in all religions, like Buddhism, Hinduism, Christianity and Judaism), Qi Gong, healing touch, Reiki, Tai Chi, yoga, mindfulness and meditation. To this day, Biofield Energy Healing has had significant impact in the transformation of living organisms and nonliving materials including polymers, ceramics, metals, chemicals, and pharmaceutical compounds. Human Biofield Energy has subtle energy that has the capacity to work in an effective manner [14]. Reports show that Complementary and Alternative Medicine (CAM) therapies have been practiced worldwide with clinical benefits in different health disease profiles [15]. This energy can be harnessed and transmitted by individuals into living and non-living things *via* the process of Biofield Energy Healing. Biofield Energy Treatment (The Trivedi Effect<sup>®</sup>) has been extensively studied with significant outcomes in many scientific fields such as cancer science [16, 17], altering microbial characteristics and features including changing the microbial sensitivity of pathogenic microbes in microbiology [18-21], genetics [22, 23], altered physical and chemical compounds in pharmaceuticals [24-27], improved the overall productivity, quality and yield of crops and plants in agricultural science [28-31], and in materials science where The Trivedi Effect<sup>®</sup> has demonstrated its ability to alter the structural, thermal and physical properties of metals, polymers, ceramics and chemicals [32-35]. The authors of this study sought to evaluate the impact of Biofield Energy Treatment (The Trivedi Effect<sup>®</sup>) on the given herbomineral formulation, which might improve the immunomodulatory function in an *in vitro* cellular model of mice splenocyte cells.

## 2. Materials and Methods

### 2.1. Chemicals and Reagent

Lipopolysaccharide (LPS), 3-(4, 5-diamethyl-2-thiazolyl) 2, 5 diphenyl-2 H-tetrazolium) (MTT), Roswell Park Memorial Institute (RPMI-1640), L-glutamine, penicillin, streptomycin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2- mercaptoethanol, concanavalin A (Con-A), rapamycin, NaHCO<sub>3</sub>, and EDTA were purchased from Sigma Chemical Corp. (St. Louis, MO), a subsidiary of Sigma-Aldrich Corporation. ELISA (enzyme-link immunosorbent assay) assay kits for all cytokines tumor necrosis factor alpha (TNF- $\alpha$ ), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), and interleukin-1 beta (IL-1 $\beta$ ) were purchased from R&D Systems, USA. Fetal bovine serum (FBS) was purchased from GIBCO, USA. All other chemicals used were of analytical grade available in India. Ashwagandha (*Withania somnifera*) root extract powder ( $\geq 5\%$  of total withanolides) was procured from Sanat Products Ltd., India. Zinc chloride and magnesium (II) gluconate hydrate were procured from Tokyo Chemical Industry Co., Ltd. (TCI), Japan. Sodium selenate was procured from Alfa Aesar, USA.

### 2.2. Test Formulation and Reference Standard

The test formulation contained four ingredients: ashwagandha root powder extract, zinc chloride, sodium selenite, and magnesium gluconate. LPS was used as an inflammatory stimulant, while Con-A and rapamycin were used as a reference standard (positive control) for immunostimulatory and immunosuppressive action, respectively in the splenocytes assay.

### 2.3. Experimental Animal

C57BL/6 male mice (8 weeks old, 22 gm body weight) were purchased from Vivo Bio Tech Ltd., Hyderabad, India and acclimatized for one week prior to the experiments. The rodents were maintained under controlled conditions with a temperature of  $22 \pm 3^\circ\text{C}$ , humidity of 30% to 70% and a 12-hour light/12-hour dark cycle and laboratory rodent diet and drinking tap water were provided *ad libitum*. All the procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH). The approval of the Institutional Animal Ethics Committee (IAEC) was obtained prior to carrying out the animal experiment.

### 2.4. Biofield Energy Healing Strategies

The herbomineral formulation was divided into two parts. One part of the test formulation did not receive any sort of treatment and was defined as the control group, while the Biofield Energy Treatment was given to the other part of the herbomineral formulation, which was defined as the treated formulation group. The Biofield Energy Treatment was provided by a group of twenty Biofield Energy (The Trivedi Effect<sup>®</sup>) Healers, fifteen of which were remotely located in the U.S.A., two in Canada, one in the UK, one in Australia,

and one in Germany. The test formulation was located in Dabur Research Foundation near New Delhi in Ghaziabad, India. This Biofield Energy Treatment was administered for 5 minutes through the Healers' unique Energy Transmission process remotely to the test formulation, which was kept under standard laboratory conditions. None of the Biofield Energy Healers in this study visited the laboratory in person, nor had any contact with the herbomineral samples. Similarly, the control sample was kept under the same laboratory conditions and was subjected to "sham" healers for five minutes. The sham healers did not have any knowledge about the Biofield Energy Treatment. After that, the Biofield Energy treated and untreated test formulations were kept in similar sealed conditions and used for the *in vitro* study on splenocyte cells for cytokines estimation.

### 2.5. Experimental Design

The experimental study was divided into 7 groups. Group 1 comprised of the splenocyte cells without LPS and was denoted as the negative control. Group 2 served as a stimulant group that included cells with LPS. Group 3 included the splenocyte cells with LPS along with the vehicle (0.005% DMSO) and was denoted as the vehicle control. Groups 4 and 5 were defined as the positive control, which included cells with Con-A (0.5  $\mu\text{g}/\text{mL}$ ) and rapamycin (1 nm and 10 nm), respectively. Groups 6 and 7 were denoted as the test item groups that included splenocyte cells with LPS along with the untreated and Biofield Treated formulations, respectively, at concentrations 0.00001053 to 10.53  $\mu\text{g}/\text{mL}$ . After 48 hours of incubation, supernatants were analyzed for the secreted levels of TNF- $\alpha$ , MIP-1 $\alpha$ , and IL-1 $\beta$  using ELISA as per the manufacturer's instructions. Concentrations were determined in triplicate wells of each sample.

### 2.6. Isolation of Murine Splenocytes

C57BL/6 male mice were sacrificed and their spleens were aseptically removed and grounded by passing them through a sterile plastic strainer under aseptic conditions. After the cells were centrifuged twice at 1000 g for 5 minutes, erythrocytes were lysed by a lysis buffer (0.15 M NH<sub>4</sub>Cl, 0.01 M NaHCO<sub>3</sub>, and 0.1 mM EDTA, pH 7.4) and then the cell pellets were washed twice with the RPMI-1640 medium. Further, the cells were resuspended in the complete RPMI-1640 medium (RPMI 1640 medium plus 10% fetal bovine serum, 2 mM glutamine, 100 IU/mL of penicillin and streptomycin, 15 mM HEPES and 50 mM 2-mercaptoethanol). The cell counts were performed using a hemocytometer and cell viability was determined using the trypan-blue dye exclusion technique with the results showing  $\geq 95\%$  of viable cells. The cells were cultured in 96-well tissue culture plates with  $0.2 \times 10^6$  cells per well. They were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> for the indicated period [36].

### 2.7. Cell Culture and Test Formulation Treatment

Splenocyte ( $0.2 \times 10^6$  cells per well) cells were grown in

96-well culture plates using a RPMI-1640 medium supplemented with 10% FBS, 100 units/mL of penicillin, and 100 µg/mL of streptomycin. LPS (50 ng/mL) induced splenocyte cells cultures were grown for 48 hours at 37°C in a humidified CO<sub>2</sub> incubator (5% CO<sub>2</sub>). The effect of cytotoxicity from the formulation was tested by treating cells with different concentrations of the test formulation in RPMI-1640 medium. Various concentrations of the test formulation were used *i.e.* 0.00001053 µg/mL to 10.53 µg/mL in the presence of inflammatory stimulus (LPS) for cell viability assay. The respective vehicle controls (DMSO) were kept in the assay for comparison.

### 2.8. Cytotoxicity by MTT Assay

The effects of the Biofield Treated and untreated test formulations on cell viability at the concentration range of 0.00001053 µg/mL to 10.53 µg/mL was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The number of viable cells was determined by the ability of mitochondria to convert MTT to formazan dye. Splenocyte cells were cultured overnight in 96-well plates, at a density of 0.2 x 10<sup>6</sup> cells per well. After treatment with the test formulation and incubation period, the medium was removed. 20 µL of 5 mg/mL MTT was then added to each well and incubated for 3 hours further at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. The cells were centrifuged and supernatants were removed. The cell pellets in each well was resuspended in 150 µL of DMSO to dissolve formazan crystals. The optical density of each well was read at 540 nm using a BioTek Reader (SIAFRT/Synergy HT multimode reader, US).

The effect of the test formulation on the cell viability of splenocyte cells was determined with equation (1):

$$\% \text{ Cell viability} = 100 - \% \text{ cytotoxicity} \quad (1)$$

Where; % cytotoxicity = [(O. D. of control cells – O. D. of cells treated with the test formulation)/O. D. of control cells]\*100.

The concentrations that resulted in >75% viability were selected for subsequent cytokine estimation.

### 2.9. Determination of Cytokines (TNF- $\alpha$ , IFN- $\gamma$ , and IL-1 $\beta$ ) and Chemokine (MIP-1 $\alpha$ ) Using ELISA

The *in vitro* activity of the Biofield Treated and untreated test formulations were estimated on the mice splenocyte cells for the production of TNF- $\alpha$ , IFN- $\gamma$ , MIP-1 $\alpha$ , and IL-1 $\beta$  using enzyme-linked immunosorbent assay (ELISA). The ELISA plates were coated with an antibody in a coating buffer at the recommended concentration and kept overnight at 4°C. After washing with PBS-T (PBS with 0.05% Tween 20), the plates were blocked with assay diluent for at least 2 hours at room temperature. A total of 100 µL culture supernatant from different experimental samples and standards were incubated overnight at 4°C and, after three washes, biotinylated anti-mouse cytokine (TNF- $\alpha$ , IFN- $\gamma$ , MIP-1 $\alpha$ , and IL-1 $\beta$ ) antibodies at the recommended concentrations were incubated for 1

hour at room temperature and the plates were incubated for 45 minutes at room temperature with gentle shaking. The plates were again washed 3 times and then 100 µL of horseradish per-oxidase (HRP)–streptavidin conjugate solution was added and the plates were incubated for 45 minutes at room temperature with gentle shaking. Next, the plate wells were washed 3 times as previously done and 100 µL of 3,3',5,5'-tetramethylbenzidine (TMB) one-step substrate reagent was added, followed by a 30-minute incubation at room temperature in the dark. Next, 50 µL of 0.2 mole/L sulphuric acid was added to each well to stop the reaction and the plates were read for absorbance at 450 nm using a BioTek Reader (SIAFRT/Synergy HT multimode reader). Standards were run in parallel to the samples, and the concentrations were determined in triplicates for each sample [37].

### 2.10. Statistical Analysis

Data were expressed as mean  $\pm$  standard error of mean (SEM) and subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparison and Student's *t*-test for two groups comparison. Statistical significance was considered at  $p \leq 0.05$ .

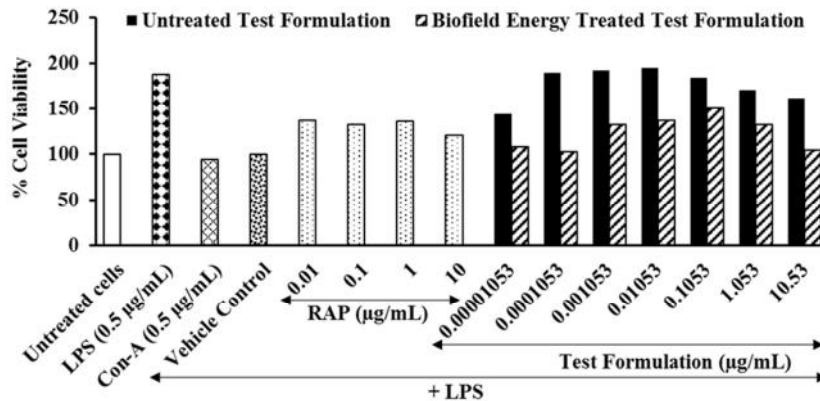
## 3. Results and Discussion

### 3.1. In vitro Splenocyte Cells Viability by MTT Assay

*In vitro* splenocyte cells viability was performed after 48 hours using MTT assay, and the results are presented in Figure 1 with respect to the positive control, vehicle control, and the test formulation at different tested concentrations. The results showed a significant change in the percentage of cell viability in the tested concentrations of the test formulation after receiving the Biofield Energy Treatment. Con-A and rapamycin showed immunostimulatory and immunosuppressive action, respectively, and were used as the positive control in the experiment. The untreated cells, LPS, and Con-A group showed 100%, 187.7%, and 94.9% cell viability, respectively. The vehicle control group reported with 100% cell viability and the rapamycin group showed values of 136.8%, 132.3%, 136.5%, and 120.5% at concentrations 0.01, 0.1, 1 and 10 nM, respectively. With respect to the vehicle control, the percentage of cell viability was increased, which might be due to proliferation in the cell culture. The tested concentration range from 0.00001053 to 10.53 µg/mL of the herbomineral test formulation was selected for the splenocyte cells. The test formulation was found safe at all the tested concentrations, with percentage viability ranging from 102% to 195%. Based upon this result, all the tested concentrations of the herbomineral formulation were used for the estimation of cytokines. The % of cell viability with LPS alone at 0.5 µg/mL was significantly increased by 87.74%, while con-A decreased the cell viability at the same concentration as compared to the untreated cells. The percentage of cell viability was significantly increased by 10.09%, 8.90%, and 10.30% at

0.0001053, 0.001053, and 0.01053  $\mu\text{g/mL}$ , respectively in the Biofield Energy Treated test formulation as compared to the vehicle control (Figure 1). Overall, both the Biofield

Energy Treated and untreated test formulations showed more than 100% cell viability up to 10.53  $\mu\text{g/mL}$ .

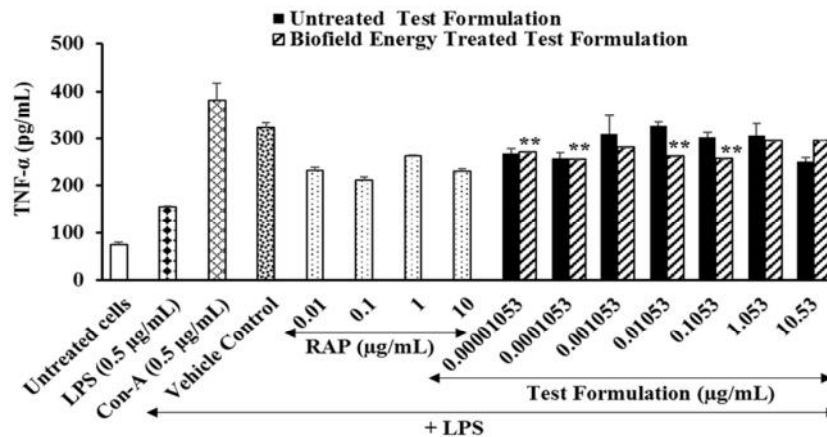


**Figure 1.** MTT assay in mouse splenocyte cells after 48 hours of treatment with various concentrations of the test formulation in the presence of 0.5  $\mu\text{g/mL}$  of LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was denoted as the absorbance ratio (expressed as a percentage) of the test formulation treated cells relative to the untreated vehicle cells. Values are represented as mean  $\pm$  SEM. RAP: Rapamycin; LPS: Lipopolysaccharides; Con-A: Concanavalin-A.

### 3.2. Estimation of TNF- $\alpha$ Expression

An estimation of IFN- $\gamma$  expression in mouse splenocyte cells after treatment with the test formulation is represented in Figure 2. The level of TNF- $\alpha$  was 232.05%, 211.54%, 262.50%, and 229.81% in the positive control group (rapamycin) at 0.01, 0.1, 1, and 10  $\mu\text{g/mL}$ , respectively. The expression of TNF- $\alpha$  was significantly reduced by 17.16%, 20.14%, 4.07%, 6.15%, 5.26%, and 22.32% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53  $\mu\text{g/mL}$ , respectively in the untreated test formulation as compared to the VC group. The Biofield Energy Treated test formulation showed a significant reduction of TNF- $\alpha$  expression by 15.87% ( $p \leq 0.01$ ), 20.64% ( $p \leq 0.01$ ), 12.80%, 18.65% ( $p \leq 0.01$ ), 20.34% ( $p \leq 0.01$ ), 8.63%, and 8.24% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53  $\mu\text{g/mL}$ , respectively as compared to the vehicle control (VC) group. Further, the Biofield Energy Treated test formulation also showed significant inhibition of TNF- $\alpha$  at 0.001053, 0.01053, and 0.1053  $\mu\text{g/mL}$  by 8.73%, 19.54%, and 14.19%, respectively as compared to the untreated test

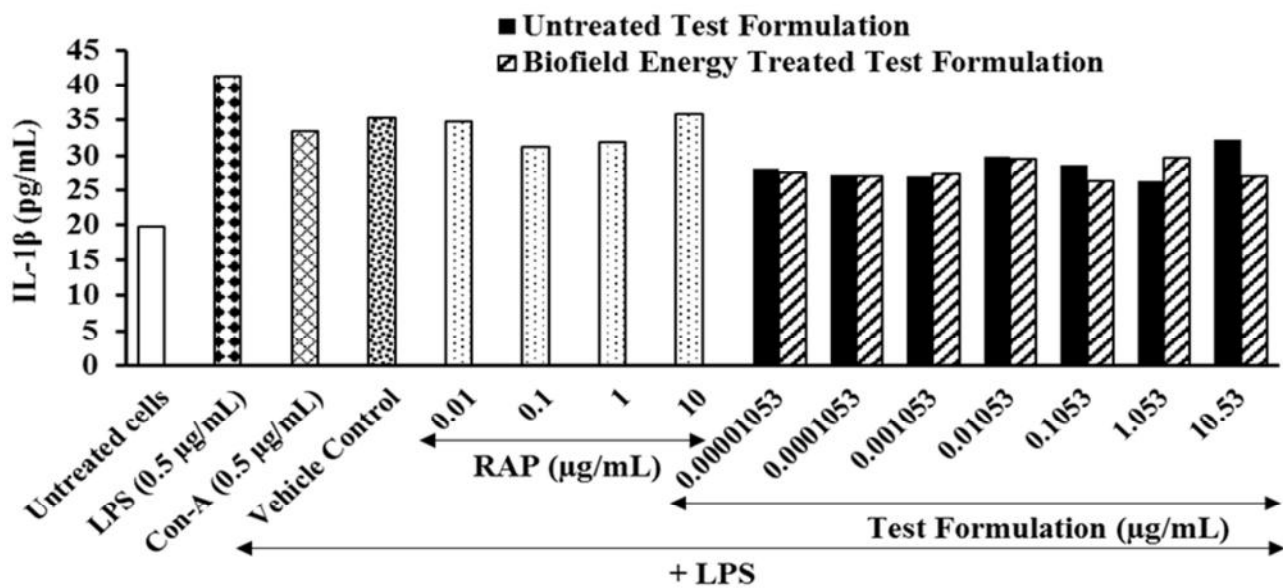
formulation. The expression of TNF- $\alpha$  in the rest of the concentrations were also altered (Figure 2). TNF- $\alpha$  is a circulating factor and is also identified as a key regulator of the inflammatory response. Literature shows that it interacts with two different type of receptors, tumor necrosis factor receptor 1 and 2 (TNFR1 and TNFR2), which are differentially expressed in cells and tissues and initiate both distinct and overlapping signal transduction pathways. These diverse signaling cascades lead to a range of cellular responses, which include cell death, survival, differentiation, proliferation and migration [38]. Since the expression of TNF- $\alpha$  was inhibited in the Biofield Energy Treated test formulation group at all the concentrations as compared to vehicle control group, it is assumed that the down-regulation of cellular response might be accelerated due to the Biofield Energy Healing administered to the test formulation. This down-regulation of TNF- $\alpha$  expression might be helpful in controlling inflammatory disorders like rheumatoid arthritis, multiple sclerosis, etc.



**Figure 2.** The effect of the test formulation on TNF- $\alpha$  secretion in LPS mediated splenocyte cells was measured after 48 hours of treatment. The values are represented as mean  $\pm$  SEM. \*\* $p \leq 0.01$  vs vehicle control. RAP: Rapamycin; LPS: Lipopolysaccharides; Con-A: Concanavalin-A.

### 3.3. Estimation of IL-1 $\beta$ Expression

The expression of IL-1 $\beta$  in mouse splenocytes in the presence of the test formulation is demonstrated in Figure 3. The positive control rapamycin group showed up to  $35.85 \pm 2.85$  pg/mL of IL-1 $\beta$ . The untreated test formulation showed significant reduction of IL-1 $\beta$  expression by 20.24%, 23.46%, 23.69%, 15.64%, 18.86%, 25.53%, and 8.98% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53  $\mu$ g/mL, respectively as compared to the VC group. Moreover, the Biofield Energy Treated test formulation showed significant reduction of IL-1 $\beta$  secretion by 22.08%, 23.69%, 23.00%, 16.33%, 25.76%, 16.10%, and 23.69% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53  $\mu$ g/mL, respectively as compared to the VC group.



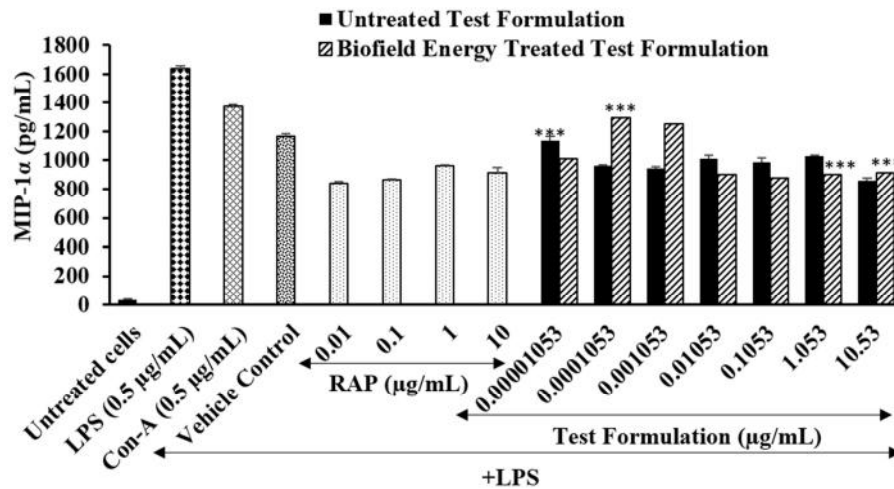
**Figure 3.** The effect of the test formulation on IL-1 $\beta$  secretion in LPS mediated splenocyte cells was measured after 48 hours of treatment. The values are represented as mean  $\pm$  SEM. RAP: Rapamycin; LPS: Lipopolysaccharides; Con-A: Concanavalin-A.

### 3.4. Estimation of MIP-1 $\alpha$ Expression

The effect of the Biofield Energy Treated test formulation on MIP-1 $\alpha$  secretion is shown in Figure 4. The results show that the Biofield Energy Treated test formulation significantly ( $p \leq 0.001$ ) reduced the MIP-1 $\alpha$  expression by 13.35%, 22.96%, 25.11%, 22.71%, and 21.83% at 0.00001053, 0.0001053, 0.01053, 1.053, and 10.53  $\mu$ g/mL, respectively as compared to the vehicle control (VC) group. The expression of MIP-1 $\alpha$  was decreased by 17.72%, 19.31%, 13.43%, 15.53%, 11.84%, and 26.41% at 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53  $\mu$ g/mL, respectively in the untreated test formulation as compared to the VC group. The data indicates that there was a significant down-regulation of MIP-1 $\alpha$  expression at 0.00001053, 0.01053, 0.1053 and 1.053  $\mu$ g/mL by 11.03%, 11.00%, 11.33%, and 12.33%, respectively in the Biofield Energy Treated test formulation as compared to the untreated test formulation (as shown in Figure 4). This indicates that the Biofield Energy Treated test formulation showed more

Both the Biofield Energy Treated and untreated test formulations demonstrated inhibition of IL-1 $\beta$  as compared to the LPS stimulated group. However, the Biofield Energy Treated test formulation exhibited better suppression than the untreated test formulation at two concentrations (0.1053 and 10.53  $\mu$ g/mL) by 6.90% and 14.71%, respectively as compared to the untreated test formulation group. This indicates that the Biofield Energy Healing enhanced the herbomineral test formulation's immunosuppressive property. Literature indicates that the decreased production of IL-1 $\beta$  is helpful in the reduction of inflammation [39]. This Biofield Energy Treatment mediated down-regulation of IL-1 $\beta$  might be helpful in the reduction and control of inflammatory disorders like chronic pain.

inhibition of MIP-1 $\alpha$  than the untreated test formulation group. Both the untreated and Biofield Energy Treated test formulation groups demonstrated inhibition of MIP-1 $\alpha$  as compared to the LPS stimulated group. The expression of MIP-1 alpha mRNA in macrophages is induced by a variety of stimuli [40]. Additionally, the decrease in MIP-1 alpha mRNA levels might be due to transcriptional inactivation and post-transcriptional destabilization [41]. Based on the outcome of this study, the level of MIP-1 $\alpha$  expression was significantly inhibited in the Biofield Energy Treated test formulation group as compared to the untreated test formulation group at four tested concentrations. This decreased production of MIP-1 $\alpha$  in the Biofield Energy Treated test formulation might be due to the down-regulation of MIP-1 $\alpha$  mRNA expression and protein synthesis *via* the generation of reactive oxygen species (ROS). Thus, the down-regulation of MIP-1 $\alpha$  through application of Biofield Energy Healing to the test formulation might be a promising treatment for controlling and preventing the inflammation in various disorders.

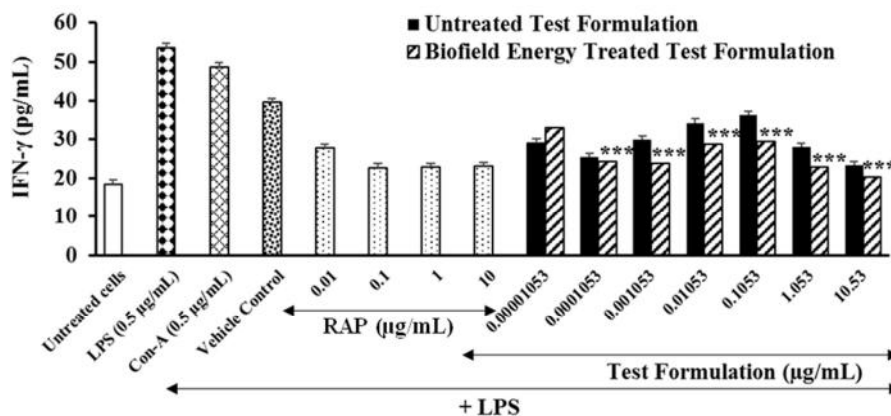


**Figure 4.** Concentration-dependent effect of LPS mediated production of MIP-1 $\alpha$  by the test formulation. For each concentration treatment, the level of MIP-1 $\alpha$  release was measured after 48 hours of treatment. The values are represented as mean  $\pm$  SEM. \*\*\* $p$ <0.001 vs vehicle control. RAP: Rapamycin; LPS: Lipopolysaccharides; Con-A: Concanavalin-A.

### 3.5. Estimation of IFN- $\gamma$ Expression

IFN- $\gamma$  is an extraordinarily pleotropic cytokine. It acts both in the innate and adaptive immune responses against pathogens and tumors, and also has the ability to maintain immune homeostasis [42]. The estimation of IFN- $\gamma$  expression in splenocyte cells after treatment with the Biofield Energy Treated and untreated test formulations is represented in Figure 5. The expression of IFN- $\gamma$  was decreased by 26.90%, 35.97%, 24.88%, 13.79%, 8.92%, 29.42%, and 41.18% at 0.0001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53  $\mu$ g/mL, respectively in the untreated test formulation as compared to the VC group. The Biofield Energy Treated test formulation showed significant ( $p \leq 0.001$ ) inhibition of the IFN- $\gamma$  expression by 39.16%, 40.34%, 27.57%, 26.06%, 42.53%, and 48.91% at 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53

$\mu$ g/mL, respectively as compared to the VC group. These data indicate that there was a significant down-regulation of IFN- $\gamma$  expression at 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53  $\mu$ g/mL by 5.00%, 15.46%, 13.78%, 17.14%, 18.57%, and 13.11%, respectively in the Biofield Energy Treated test formulation as compared to the untreated test formulation group (demonstrated in Figure 5). The level of IFN- $\gamma$  expression was significantly inhibited at six concentrations in the Biofield Energy Treated test formulation group as compared to the untreated test formulation. From science literature, it was reported that the decreased production of IFN- $\gamma$  is due to the suppression of inflammatory molecules, through the apoptotic mediators, and *via* cell growth arrest by the downregulation and nuclear translocation of STAT1 [43].



**Figure 5.** The effect of the test formulation of IFN- $\gamma$  secretion in LPS mediated splenocyte cells was measured after 48 hours of treatment. The values are represented as mean  $\pm$  SEM. \*\*\* $p \leq 0.001$  vs vehicle control. RAP: Rapamycin; LPS: Lipopolysaccharides; Con-A: Concanavalin-A.

Inflammation is the first response of the immune system to infection or irritation. It is caused by cytokines such as TNF- $\alpha$ , IL-1, and IL-6 by eicosanoids such as prostaglandin E2 (PGE2) [44]. Thus, products, herbs, medicines and

supplements that inhibit these cytokines have been widely sought after in the development of anti-inflammatory drugs. The evaluation of the immune cells, especially the splenocyte cells, is always used when screening the potential

immunomodulatory effect of a substance/product. These immune cells migrate to the spleen, which is the secondary lymphoid tissue, and respond to antigens there. Therefore, the regulation of thymus and spleen cell proliferation is closely related to maintaining immune homeostasis and can be considered an important marker for immune response control. Furthermore, Biofield Therapies (such as Reiki, therapeutic touch, healing touch and other types of Energy Medicine) are complementary and alternative medicine modalities that are often utilized by a significant number of cancer patients. These therapies show strong evidence in reducing pain intensity in pain population, and moderate evidence in reducing pain intensity in cancer populations [45, 46]. The Biofield Energy Treated (The Trivedi Effect<sup>®</sup>) test formulations strongly inhibited the tested pro-inflammatory cytokines *viz.* TNF- $\alpha$ , IL-1 $\beta$ , MIP-1 $\alpha$ , and IFN- $\gamma$ .

## 4. Conclusions

Cell viability was assessed using MTT assay and the herbomineral formulation was found safe at all the tested concentrations. The level of TNF- $\alpha$  was significantly decreased by 19.54% and 14.19% at 0.01053 and 0.1053  $\mu\text{g/mL}$ , respectively in the Biofield Energy Treated test formulation as compared to the untreated test formulation. Moreover, the level of IL-1 $\beta$  was significantly reduced by 6.90% and 14.71% at 0.1053 and 10.53  $\mu\text{g/mL}$ , respectively in the Biofield Energy Treated test formulation as compared to the untreated test formulation. The expression of MIP-1 $\alpha$  was significantly reduced by 10.75%, 9.53%, 9.57%, and 10.87% at 0.0001053, 0.01053, 0.1053 and 1.053  $\mu\text{g/mL}$ , respectively in the Biofield Energy Treated test formulations as compared to the untreated test formulation. Furthermore, the Biofield Energy Treated test formulations showed 15.46%, 13.78%, 17.14%, and 13.11%, down-regulation of IFN- $\gamma$  expression at 0.001053, 0.01053, 0.1053 and 10.53  $\mu\text{g/mL}$ , respectively as compared to the untreated test formulation.

Therefore, The Trivedi Effect<sup>®</sup> Biofield Energy Healing (TEBEH) administered remotely by the twenty Biofield Energy Healers enhanced the herbomineral test formulation's anti-inflammatory and immunomodulatory properties. Overall, Biofield Energy Treated test formulation significantly inhibited the activity of pro-inflammatory cytokines and showed immunosuppressive activity with a safe therapeutic index as compared with the untreated test formulation. Biofield Energy Healing shows great promise as a complementary and alternative medicine therapy and might play an important role in autoimmune diseases and inflammatory disorders like Celiac Disease (gluten-sensitive enteropathy), Irritable Bowel Syndrome, Parkinson's Disease, Graves' Disease, Chronic peptic ulcers, Hepatitis, Addison Disease, Multiple Sclerosis (MS), Tuberculosis, Rheumatoid arthritis, Chronic periodontitis, Crohn's disease, Ulcerative colitis, Lupus, Systemic Lupus Erythematosus, Vitiligo, Hashimoto Thyroiditis, Chronic sinusitis, Type 1 Diabetes, Asthma, Chronic active hepatitis, Pernicious and

Aplastic Anemia, Vasculitis, Myasthenia Gravis, Rheumatoid Arthritis, Reactive Arthritis, Sjogren Syndrome, Alopecia Areata, Scleroderma, Dermatitis, Psoriasis, Fibromyalgia, Diverticulitis, Chronic Fatigue Syndrome, Alzheimer's Disease, Atherosclerosis, stress, etc. Biofield Energy Healing may also be useful in the prevention of immune-mediated tissue damage in cases of organ transplants (for example heart transplants, liver transplants and kidney transplants), for anti-aging and in improving overall health and quality of life.

## Abbreviations

LPS: Lipopolysaccharide; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate buffer saline; ELISA: Enzyme-linked immunosorbent assay

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