

# Effect of Biofield Energy Healing Treatment (The Trivedi Effect<sup>®</sup>) Based Herbomineral Formulation on Pro-Inflammatory Cytokines Expression in Murine Dendritic and Splenocyte Cells

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**Abstract:** The utilization and demand of self-medication with herbomineral-based formulations have increased day-by-day across the globe over the last decade. A new proprietary herbomineral formulation was prepared with the mixture of minerals (zinc, magnesium, and selenium) and the herbal root extract of ashwagandha. The current study was undertaken to evaluate the Biofield Energy Healing (The Trivedi Effect<sup>®</sup>) on the test herbomineral formulation using murine dendritic cells (DCs) and splenocytes *in vitro*. The formulation was divided into two parts, one part was control without any Biofield Energy Treatment, while the other part was defined as the Biofield Energy Treated sample, which received the Biofield Energy Healing Treatment remotely from eighteen renowned Biofield Energy Healers. The effect of the Biofield Energy Treated formulation in murine cells was monitored with an estimation of pro-inflammatory cytokines levels such as tumor necrosis factor (TNF- $\alpha$ ), macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ) and interleukin (IL-1 $\beta$ ) in cell culture supernatants along with estimations of non-cytotoxic concentrations of the test formulation by MTT assay. The Biofield Treated formulation showed 114.2%, 122.6%, 141.2%, 127.8%, and 114.1% cell viability at concentrations 1.05, 5.2, 10.5, 25.6, and 51.2  $\mu$ g/mL, respectively in DCs. Similarly, the Biofield Energy Treated and untreated formulations showed more than 100% cell viability in mice splenocytes at 5  $\mu$ g/mL. The level of TNF- $\alpha$  in DCs was significantly ( $p \leq 0.05$ ) inhibited by 19.21% in the Biofield Treated formulation at concentration 5.2  $\mu$ g/mL as compared to the untreated test formulation. The level of MIP-1 $\alpha$  in LPS induced mice splenocyte cells was reduced by 15.35% in the Biofield Energy Treated formulation at 0.0105  $\mu$ g/mL as compared to the untreated formulation. Similarly, the level of IL-1 $\beta$  in LPS induced mice splenocyte cells was significantly ( $p \leq 0.05$ ) reduced by 31.59% in the Biofield Treated formulation at 1.05  $\mu$ g/mL as compared to the untreated formulation. Altogether, the results suggest that The Trivedi Effect<sup>®</sup> (Biofield Energy Healing Treatment) showed significant down-regulation of the tested pro-inflammatory cytokines expression and potentiated the immunosuppressive effect of the treated formulation to modulate the immune system. These data also suggest that the Biofield Treated test formulation can be used for autoimmune and

inflammatory diseases, stress management and anti-aging by improving overall health.

**Keywords:** Biofield Energy Healing Treatment, Biofield Energy Healers, The Trivedi Effect<sup>®</sup>, Immunomodulation, Dendritic Cells, Splenocytes, Cytokines

## 1. Introduction

Since ancient times, natural products have played primary roles in Complementary and Alternative Medicine (CAM) and have been used worldwide for the treatment and prevention of various diseases or ailments. Moreover, the use of alternative and complementary medicine has increased in circumstances where conventional medicine and the allopathic model of health care is ineffective, *i.e.* cases of advanced diseases like cancer or with ailments/problems that are not limited to only the brain and the body. Many medicinal plants and minerals have immunomodulatory properties. The ashwagandha (*Withania somnifera*) plant is one important herbal product that is widely used in complementary and alternative medicine for various types of diseases. There has been increased interest in the use of natural substances, especially pertaining to plants, derived herbal extracts and various essential minerals, which are collectively known as nutraceuticals [1]. The herbal extracts report various pharmacological benefits such as immunostimulation, stress reduction, appetite stimulation, growth promotion and antimicrobial properties. These effects were found to be caused by the presence of active constituents such as steroids, flavonoids pigments, alkaloids, phenolics, and terpenoids [2]. Based on the literature, a new proprietary herbomineral formulation was prepared that combined the herbal root extract of ashwagandha with three minerals *viz.* zinc, magnesium, and selenium. Ashwagandha root is a traditional Indian herb (also known as Indian Ginseng) commonly used as a constituent in herbal tonic preparation. It is considered an 'adaptogen', (a term used to describe herbs that improve physical energy, athletic ability and vitality, as well as increase sexual capacity, fertility and immunity to cold infections) [3]. Herbal preparations that contain ashwagandha root extract have shown anti-inflammatory, anti-arthritis, antibiotic, anti-tumor, and immunomodulatory effects with withanolides [4, 5]. Ashwagandha root extract has been shown to modulate the immune response *via* cytokines expression level [6]. Mazumder *et al.* (2012) reported that zinc, along with *Glycyrrhiza glabra*, had increased the leukocyte count and phagocytic index and potentiated immunomodulatory effects [7]. Sodium selenate greatly improved the process of lymphocytes proliferation to the increased level of IL-2. The literature reports that sodium selenate increased the phagocytic index and down-regulated the expression levels of pro-inflammatory cytokines *viz.* IL-1, IL-6, and TNF- $\alpha$  [8]. Selenium produces antioxidant properties by scavenging free-radicals. Selenium also plays an important role in several immunological and metabolic processes of phagocytosed bacteria during intracellular digestion [9]. Many

trace elements such as zinc, magnesium, selenium, copper and manganese etc. have strong immunomodulatory effects [10]. Different cytokines are involved in host responses with respect to the inflammation and immune activation process that lead to various diseases [11]. Therefore, anti-inflammatory phytomedicines are used as an adjunct therapy for the management of chronic inflammatory disorders [12-14].

Dendritic cells (DCs) play an important role in the immune system and possess the ability to stimulate naive T cells. Murine DCs are characterized by *CD11c* marker expression, which is a common marker among plasmacytoid and myeloid DCs [15]. Thymus derived lymphocyte helper cells 1 (TH1) are the primary sources for interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), IL-2, interferon- $\gamma$  (IFN- $\gamma$ ), lymphotoxins and other factors which characterize the cell-mediated immune responses [16]. The different types of immune cells such as DCs, macrophages and spleen play an important role in stopping acute/chronic inflammation and retrieving a steady state strategy through the secretion of immuno-modulating cytokines [17]. These immune cells have been reported to be useful as cellular models for *in vitro* studies. Therefore, murine bone marrow derived dendritic cells (BMDCs) and splenocytes were used to assess the effect of the test formulation on *in vitro* cell cultures. In recent years, Biofield Energy Healing Treatment has been accepted as an alternative and complementary method of health care and has been shown to have impacts on various properties of living organisms in a cost-effective manner [18]. The use of alternative and integrative medicine approaches have increased in the facilitation of wellness and because of their minimal side-effects for cancer patients and people suffering from chronic diseases. Recent studies report that the uses of energy medicine provide the highest benefit to cancer patients as compared to other complementary and alternative forms of medicine [19].

Human Biofield Energy has subtle energy that has the capacity to work in an effective manner [20]. Reports show that CAM therapies have been practiced worldwide with reports of clinical benefits in different health disease profiles [21]. Biofield Energy comes under the category of energy medicine, which is well defined and accepted by National Center for Complementary and Integrative Health (NCCIH), a subdivision of the National Institutes of Health (NIH). This energy can be harnessed and transmitted by individuals into living and non-living things *via* the process of Biofield Energy Healing. Biofield Energy Healing Treatment (The Trivedi Effect<sup>®</sup>) have been known to transform the structural, physical, and thermal properties of various metals, chemicals, ceramics and polymers in materials science [22-25], improved the overall productivity, yield and quality of crops

[26-29], altered characteristics and features of microbes [30-33], improved excellent outcomes of various nutraceutical compounds in the fields of nutraceuticals [34-37], and improved the growth and anatomical characteristics of various medicinal plants [38, 39]. Based on the literature related to Biofield Energy Treatment, the authors of this study sought to investigate the effects of Biofield Energy (The Trivedi Effect<sup>®</sup>) on the test herbomineral formulation, which might modulate the key pro-inflammatory cytokines expression in *in vitro* murine DCs and splenocytes models.

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

3-(4, 5-diamethyl-2-thiazolyl) 2, 5 diphenyl-2H-tetrazolium (MTT), Lipopolysaccharide (LPS), L-glutamine, RPMI-1640, penicillin, HEPES, streptomycin, 2-mercaptoethanol were purchased from Sigma Chemical Company in St. Louis, MO, a subsidiary of Sigma-Aldrich Corporation. ELISA (enzyme-link immunosorbent assay) assay kits for all cytokines such as TNF- $\alpha$ , macrophage inflammatory protein-1 $\alpha$  (MIP-1 $\alpha$ ), and IL-1 $\beta$  were purchased from R&D systems, U.S.A. Fetal bovine serum (FBS) was procured from GIBCO, U.S.A. Ashwagandha root extract powder 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, U.S.A. Rapamycin, NaHCO<sub>3</sub>, and EDTA were procured from Sigma. All other chemicals used in this experiment were of analytical grade and procured from local vendors.

### 2.2. Test Formulation and Reference Standard

The test formulation contained the combination of four ingredients: zinc chloride, magnesium gluconate, sodium selenate, and ashwagandha root powder extract. Different concentrations of the test formulation were used for the study *i.e.* concentration range of 1.05 to 1052.5  $\mu\text{g/mL}$  for bone marrow DCs and 0.000105 to 10.5  $\mu\text{g/mL}$  with LPS stimulated splenocyte culture for cell viability assay. LPS was used as an immunostimulant and as an inducing agent at 0.5  $\mu\text{g/mL}$  and rapamycin (1 and 10 nM) was used as the reference standard (positive control) in splenocyte culture. TNF- $\alpha$  estimated in DCs, which was treated with 0.1% DMSO, served as the vehicle control group. MIP-1 $\alpha$  and IL-1 $\beta$  were estimated in splenocyte cells, which includes splenocyte cells treated with LPS (0.5  $\mu\text{g/mL}$ ) along with 0.005% DMSO, and was defined as the vehicle control group.

### 2.3. Biofield Energy Treatment Strategies

The herbomineral formulation was divided into two parts. One part was considered the control sample and the other part was defined as the treated sample. Both samples were kept under standard laboratory conditions at the research laboratory Dabur Research Foundation near New Delhi in

Ghaziabad, India. The treated sample was subjected to Biofield Energy Healing (also known as The Trivedi Effect<sup>®</sup>), which was remotely administered for a period of 5 minutes by a group of eighteen Biofield Energy Healers, eleven of which were remotely located in the U.S.A., four in Canada, one in the UK, one in Russia and one in Ireland. Similarly, the control sample was subjected to "sham" healers under the same laboratory conditions for 5 minutes. The sham healers did not have any knowledge about the Biofield Energy Treatment. After that, the Biofield Energy Treated and untreated samples were kept in similar sealed conditions and used for the *in vitro* study on DCs and splenocytes for cytokines estimation.

### 2.4. Experimental Animal

C57BL/6 male mice (8 weeks old) were purchased from M/S Vivo Bio Tech Ltd., Hyderabad, India and acclimatized for one week prior to the experiment. The mice were maintained under controlled conditions with a temperature of  $22 \pm 3^\circ\text{C}$ , humidity of 30–70% and a 12-hour light/12-hour dark cycle and rodent laboratory diet and drinking tap water were provided *ad libitum*. All the procedures were followed in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Approval was obtained from the Institutional Animal Ethics Committee prior to carrying out the animal experiments. The various concentrations of the test formulation were used from 1.05 to 51.2  $\mu\text{g/mL}$  for BMDCs and 0.000105 to 1.05  $\mu\text{g/mL}$  for LPS stimulated splenocytes culture. The respective vehicle controls (DMSO) were kept in the assay.

### 2.5. Mouse Bone Marrow-Derived Dendritic Cells (DCs) Cultures

C57BL/6 male mice were euthanized by CO<sub>2</sub> asphyxiation. Subsequent experimental steps were conducted in a laminar air flow. BMDCs were induced from bone marrow (BM) cells using the modified method of Inaba *et al.* (1992) [40]. Briefly, a single cell suspension was prepared from BM obtained from the femur. After removing all muscle tissues from the femur using gauze, the bones were placed in a 90 mm dish with 70% alcohol for 1 minute, washed twice with PBS, and then transferred into a fresh dish containing RPMI 1640 medium. Both ends of the bones were cut with scissors in the dish, and then the bone marrow (BM) was flushed out using 2 mL of RPMI 1640 medium with the help of a syringe attached with a 25-gauge needle. To remove small pieces of bone and debris, the tissue was suspended and passed through a nylon mesh and red blood cells were lysed with ammonium chloride. After washing, lymphocytes and other cells were killed with a cocktail of monoclonal antibodies (mAbs) and rabbit complement for 60 minutes at 37°C. After that, whole BM cells ( $2 \times 10^6$  cells/mL) were cultured in RPMI 1640 medium in 90 mm Petri dishes (Sigma Aldrich, St. Louis, MO) at 37°C, 5% CO<sub>2</sub>, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin G, 100 mg/mL

streptomycin, 20 ng/mL recombinant mouse GM-CSF (R & D systems Inc., U.S.A.). The cells were incubated for 24 hours. The petri-plates were then gently swirled and the medium containing non-adherent cells, and which was then removed and replaced with the nutrient medium as described above. The supplemented medium was replaced every three days. On day 6, non-adherent and loosely adherent DCs were collected, washed, and seeded in culture plates for further assays. Cell counts were performed using a hemocytometer and cell viability was determined with the help of trypan-blue dye exclusion technique, results showing  $\geq 95\%$  of viable cells. The cells were cultured in 96-well tissue culture plates with  $5 \times 10^3$  cells per well. They were incubated at  $37^\circ\text{C}$  in a humidified atmosphere with  $5\% \text{CO}_2$  for the indicated period.

### 2.6. Mouse Splenocyte Cultures

C57BL/6 male mice were sacrificed and the spleens were removed and ground by passing them through a sterile plastic strainer under aseptic conditions. After that, the cells were centrifuged twice at  $1000 \text{ g}$  for 5 minutes. Erythrocytes were lysed by lysis buffer ( $0.15 \text{ M NH}_4\text{Cl}$ ,  $0.01 \text{ M NaHCO}_3$ , and  $0.1 \text{ mM EDTA}$ , pH 7.4) and then the cell pellets were washed twice with RPMI-1640 medium. Then, the cells were resuspended in a complete RPMI-1640 medium (RPMI 1640 medium plus  $10\% \text{ FBS}$ ,  $2 \text{ mM}$  glutamine, streptomycin and  $100 \text{ IU/mL}$  of penicillin and  $15 \text{ mM}$  HEPES, and  $50 \text{ mM}$  2-mercaptoethanol). Cell counts were performed with the help of hemocytometer and cell viability was determined using trypan-blue dye exclusion technique, 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  $37^\circ\text{C}$  in a humidified atmosphere containing  $5\% \text{CO}_2$  for the specified period.

### 2.7. Cytotoxicity by MTT Assay

The number of viable cells was estimated based on the conversion of MTT to formazan dye using mitochondrial enzyme. Both cells were cultured overnight in 96-well plates, at a density of  $5 \times 10^3$  cells per well for BMDCs and  $0.2 \times 10^6$  cells per well for splenocytes. After the Biofield Energy Treatment on the test formulation with the desired incubation period, the medium was removed.  $20 \mu\text{L}$  of  $5 \text{ mg/mL}$  MTT was then added to each well and incubated for 3 hours further at  $37^\circ\text{C}$  in a humidified  $5\% \text{CO}_2$  atmosphere. The cells were centrifuged and supernatants were removed. The cell pellet in each well was resuspended in  $150 \mu\text{L}$  of DMSO to dissolve formazan crystals. The optical density of each well was read at  $540 \text{ nm}$  using Biotek Reader (SIAFRT/Synergy HT multimode reader). The effect of the test formulation on cell viability of DCs and splenocyte cells was determined as per equation 1:

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

Where;  $\% \text{ Cytotoxicity} = \{(\text{O.D. of Control cells} - \text{O.D. of cells treated with the test formulation}) / \text{OD of Control cells}\} * 100$ .

The concentrations that resulted in  $>95\%$  viability were selected for subsequent cytokine estimation.

### 2.8. Cytokines Assays Using ELISA

The effects of the Biofield Energy Treated and untreated test samples on the production of  $\text{TNF-}\alpha$ ,  $\text{MIP-1}\alpha$  and  $\text{IL-1}\beta$  were measured by ELISA method using culture supernatants collected from the treated cells. Briefly, ELISA plates were coated overnight and kept at  $4^\circ\text{C}$  with coating buffer containing capture antibody at the recommended concentration. After washing with PBS-T (PBS with  $0.05\% \text{ Tween 20}$ ), the plates were blocked with assay diluent for at least 2 hours at room temperature.  $100 \mu\text{L}$  of culture supernatants from different experimental samples and standards were incubated overnight at  $4^\circ\text{C}$  and, after three washes, biotinylated anti-mouse cytokine ( $\text{TNF-}\alpha$ ,  $\text{MIP-1}\alpha$  and  $\text{IL-1}\beta$ ) antibodies at the recommended concentrations were incubated for 1 hour at room temperature and the plate was incubated for 45 minutes at room temperature with gentle shaking. Plates were again washed 3 times and then  $100 \mu\text{L}$  of horseradish per-oxidase (HRP)-streptavidin conjugate solution was added and the plate was incubated for 45 minutes at room temperature with gentle shaking in a shaker. Next, the plate wells were washed 3 times and  $100 \mu\text{L}$  of 3,3',5,5'-tetramethylbenzidine (TMB) was added to the wells, followed by 30 minutes of incubation at room temperature in the dark. Then  $50 \mu\text{L}$  of  $0.2 \text{ mol/L}$  sulphuric acid was added to each well to stop the reaction. After that, the plates were read for absorbance at  $450 \text{ nm}$  using a Biotek reader (SIAFRT/Synergy HT multimode reader). Standards were run in parallel to the samples. Concentrations were determined and the experiment was done in triplicates.

### 2.9. TNF- $\alpha$ Level in Mouse Dendritic Cells (DCs)

DCs were seeded in a 24-well plate at a density of  $0.5 \times 10^6$  cells per well with  $1 \text{ mL}$  of culture medium and co-incubated for 24 hours with different non-cytotoxic concentrations ranging from 25 to  $500 \mu\text{g/mL}$  of the Biofield Treated and untreated test samples. DCs treated with  $0.1\% \text{ DMSO}$  was included as the vehicle control. After 24 hours of incubation, culture supernatants were collected and assayed. The supernatants were analyzed for the estimation of  $\text{TNF-}\alpha$  using ELISA as per the manufacturer's instructions [41]. Concentrations were determined in two wells in each sample and this experiment was conducted in triplicates.

### 2.10. MIP-1 $\alpha$ and IL-1 $\beta$ in Splenocytes

For estimation of  $\text{MIP-1}\alpha$  and  $\text{IL-1}\beta$  in LPS ( $0.5 \mu\text{g/mL}$ ) induced splenocytes ( $2 \times 10^6$  cells/well in 24-well culture plates) were treated with the Biofield Treated and untreated test formulations at selected non-toxic concentrations ( $0.00005$  to  $5 \mu\text{g/mL}$ ) in triplicates. Splenocytes treated with LPS along with  $0.005\% \text{ DMSO}$ s were included as control cells. Untreated cells were included as a negative control. Rapamycin was included as a positive control. After 48 hours of incubation, the supernatants were analyzed for the secreted

levels of MIP-1 $\alpha$  and IL-1 $\beta$  using ELISA as per the manufacturer's instructions [42, 43].

**2.11. Statistical Analysis**

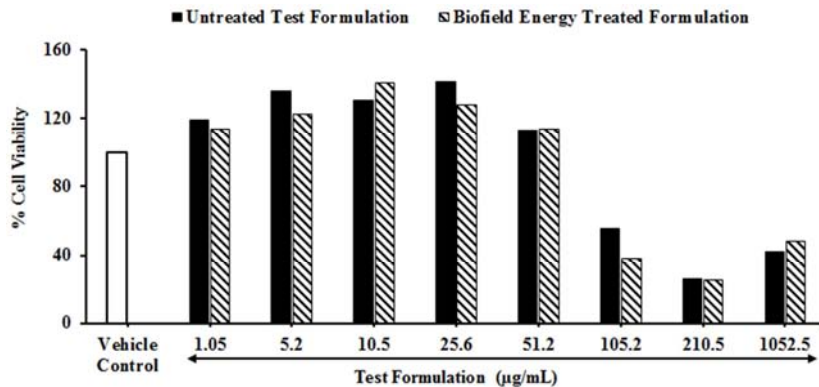
The experiment was performed in triplicates and the data are presented as mean  $\pm$  standard error of mean (S.E.M.). Comparisons of the means of control and test groups were subjected to Student's *t*-test for two group comparison. Statistical significance was considered at  $p \leq 0.05$ .

**3. Results**

**3.1. In Vitro Immune Cells Viability Assay by MTT Assay**

The effects of the Biofield Energy Treated and untreated test formulations on the proliferation of mouse BMDCs and splenocyte cells were examined after 24 hours and 48 hours, respectively using MTT cell viability assay. The effects of

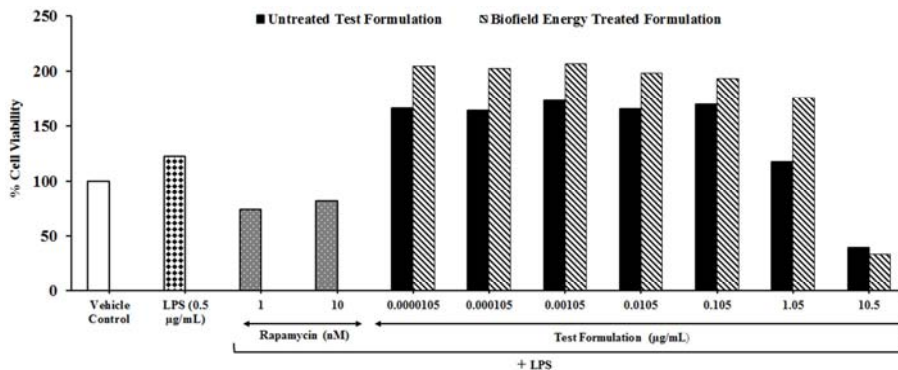
the test formulation on the viability of DCs and splenocytes are shown in Figure 1 and Figure 2, respectively. The cell viability in the untreated test group showed 136.30%, 141.90%, and 113.00% at concentrations of 5.2, 25.6, and 51.2  $\mu\text{g/mL}$ , respectively in DCs. Further, increasing the dose of the untreated sample resulted in reduced cell viability up to 26% at 210.5  $\mu\text{g/mL}$ . So, the dose ranges from 1.05 to 51.2  $\mu\text{g/mL}$  were selected for the cytokine (TNF- $\alpha$ ) estimation. Similarly, the Biofield Energy Treated formulation showed 114.2%, 122.6%, 141.2%, 127.8%, and 114.1% cell viability at concentrations of 1.05, 5.2, 10.5, 25.6, and 51.2  $\mu\text{g/mL}$ , respectively in DCs. The increased cell viability with respect to the vehicle control might be due to proliferation in the cell culture. Further, increasing the dose of the Biofield Treated formulation resulted in decreased cell viability up to 25% at concentration 210.5  $\mu\text{g/mL}$ .



**Figure 1.** MTT Assay in mouse bone marrow dendritic cells (DCs) after 24 hours of treatment with various concentrations of the test formulation in the absence of LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of the Biofield Energy Treated cells relative to the untreated vehicle cells.

LPS induced splenocytes were treated with the test formulation at the concentration ranges from 0.0000105 to 1.05  $\mu\text{g/mL}$  for 48 hours. The result of percent cell viability is presented in Figure 2. The cytokines analysis was also conducted using the same concentration range. The cell viability of the LPS group was 122.8%, while rapamycin (the reference standard of the immunosuppressive agent) showed

74.2% and 81.6% cell viability at 1 and 10 nM, respectively. Both the Biofield Energy Treated and untreated test formulations showed more than 100% cell viability at 5  $\mu\text{g/mL}$  concentration. Therefore, the dose range from 0.0000105 to 1.05  $\mu\text{g/mL}$  of the test formulation was selected for the cytokines (MIP-1 $\alpha$  and IL-1 $\beta$ ) estimation.



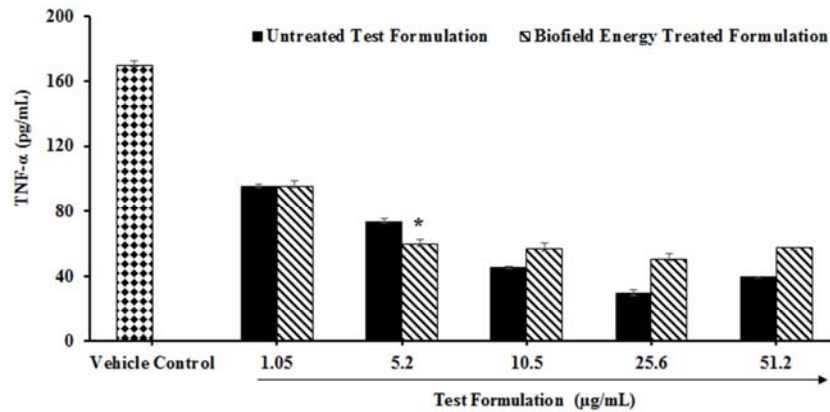
**Figure 2.** MTT Assay in splenocyte cells after 48 hours of treatment with various concentrations of the test sample in the presence of 0.5  $\mu\text{g/mL}$  of LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of the Biofield Treated cells relative to the untreated vehicle cells.

**3.2. Effect of the Test Formulation on the Expression of Pro-inflammatory Cytokines TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$  in Immune Cells**

Lymphocyte proliferation and the activation of natural killer (NK) cells are cytokine dependent [44]. The type of cytokine up/down regulation affects the course of immune response and the whole network of immune regulation. The pro-inflammatory cytokines TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$  play a pivotal role in inflammation, immune modulation, and lymphocyte activation. Various herbomineral formulations have the potential to modulate the expression and activation of cytokines. Therefore, we examined the effect of the test formulation on the levels of TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$  expression at two different immune cells: dendritic and spleen cells. The production of the cytokines by these immune cells was tested in the culture supernatants using the commercial ELISA kits.

**3.3. Modulation of TNF- $\alpha$  Expression in Mouse Bone Marrow Dendritic Cells (BMDCs)**

DCs were treated with the test formulation at non-cytotoxic concentrations for 24 hours. The effect of the formulation on TNF- $\alpha$  secretion in DCs is represented in Figure 3. Both the untreated and Biofield Energy Treated test formulation groups demonstrated decreased levels of TNF- $\alpha$  secretion at different concentrations *i.e.* 1.05, 5.2, 10.5, 25.6, and 51.2  $\mu\text{g/mL}$  as compared to the vehicle control group. The maximum down-regulation effect was observed at 25.6  $\mu\text{g/mL}$  of the Biofield Treated and untreated formulations with respect to the vehicle control group. The Biofield Energy Treated and untreated formulations showed 59.3 and 73.4  $\text{pg/mL}$  of TNF- $\alpha$  levels at 5.2  $\mu\text{g/mL}$  as compared to the control group. Therefore, the level of TNF- $\alpha$  in the Biofield Treated group was decreased by 19.21% as compared to the untreated group, which was statistically significant ( $p \leq 0.05$ ) (Figure 3).

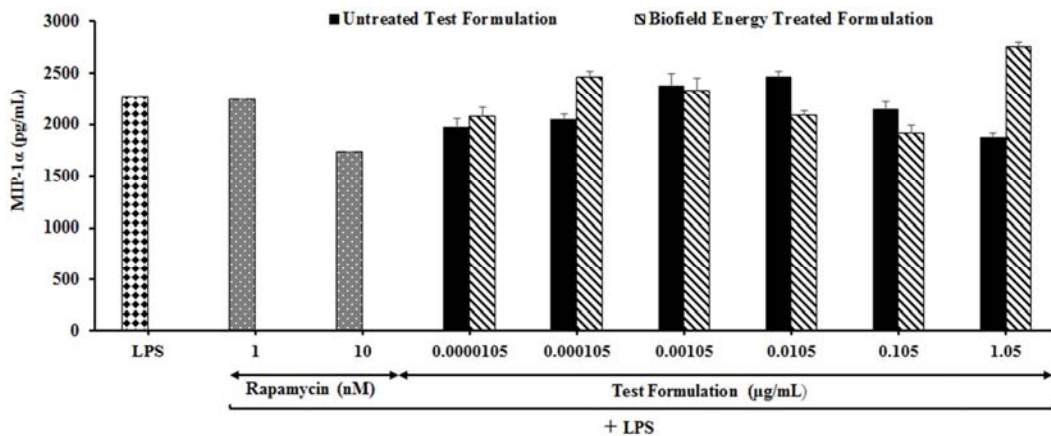


**Figure 3.** Dose-dependent inhibition of TNF- $\alpha$  by the test formulation. For each concentration treatment, the level of TNF- $\alpha$  release was measured after 24 hours of treatment. The values are represent as means  $\pm$  SEM of independent ELISA experiment, \* $p \leq 0.05$  as compared to the untreated test formulation.

**3.4. Modulation of MIP-1 $\alpha$  and IL-1 $\beta$  Expression in Mouse Splenocytes**

Splenocytes were cultured in RPMI-FBS (10%) with LPS and the levels of MIP-1 $\alpha$  and IL-1 $\beta$  cytokines were measured by ELISA in the culture supernatants. The effect of the test

formulation on each cytokine production was observed after 48 hours of incubation at various concentrations of the Biofield Energy Treated and untreated test formulations. Rapamycin was used as the positive control.



**Figure 4.** Dose-dependent inhibition of LPS mediated production of MIP-1 $\alpha$  by 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 of independent ELISA experiments.

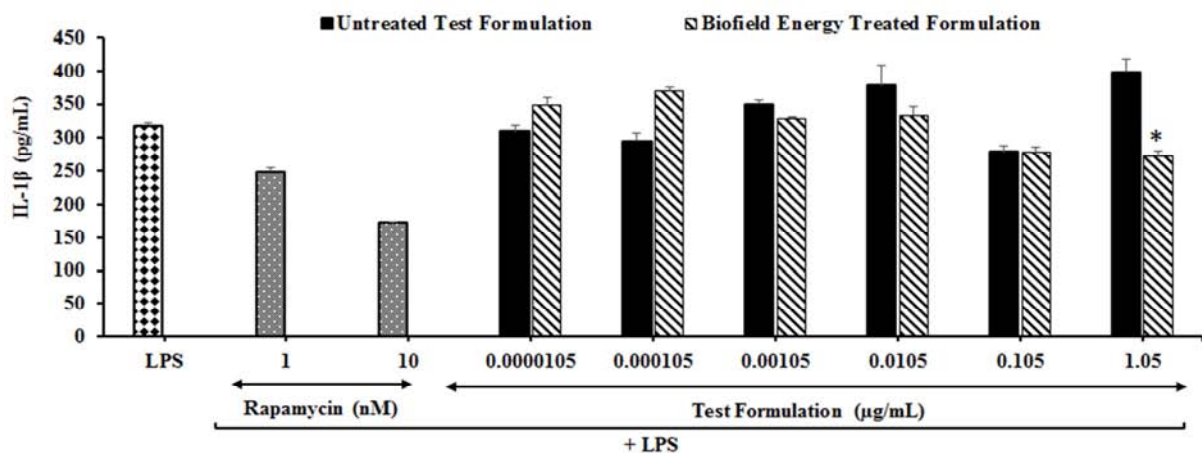
### 3.4.1. MIP-1 $\alpha$ Expression

The effect of the test formulation on MIP-1 $\alpha$  secretion in splenocytes is shown in Figure 4. This figure demonstrates the comparative expression profile of MIP-1 $\alpha$  secretion in splenocytes of the Biofield Treated and untreated groups. Both the untreated and Biofield Treated formulations demonstrated inhibition of MIP-1 $\alpha$  expression as compared to the LPS stimulated cells (vehicle control). However, the Biofield Energy Treated formulation group enhanced the down-regulation of MIP-1 $\alpha$ . At concentration 0.105  $\mu\text{g/mL}$ , the level of MIP-1 $\alpha$  was observed as 1915.5 and 2144.1 pg/mL in the Biofield Treated and untreated formulation groups, respectively. Moreover, there was 10.66% reduction of MIP-1 $\alpha$  in the Biofield Treated group as compared to the untreated group. The level of MIP-1 $\alpha$  was observed as 2087.8 pg/mL and 2466.4 pg/mL in the Biofield Treated and untreated test formulation groups, respectively at 0.0105  $\mu\text{g/mL}$ . There was 15.35% reduction of MIP-1 $\alpha$  in the Biofield Treated group as compared to the untreated group.

### 3.4.2. IL-1 $\beta$ Expression

Figure 5 demonstrates the comparative effect of the Biofield Energy Treated and untreated formulation on IL-1 $\beta$  secretion

in splenocytes. Both the untreated and the Biofield Treated test formulations demonstrated inhibition of IL-1 $\beta$  as compared to the LPS stimulated cells (vehicle control). The Biofield Energy Treated formulation exhibited better inhibition of IL-1 $\beta$  secretion than the untreated formulation group at most of the tested concentrations. The level of IL-1 $\beta$  was observed as 329.5 pg/mL and 349.9 pg/mL in the Biofield Treated and untreated groups, respectively at 0.00105  $\mu\text{g/mL}$ . There was 5.83% reduction of IL-1 $\beta$  in the Biofield Treated group as compared to the untreated group. Moreover, at concentration 0.0105  $\mu\text{g/mL}$ , the level of IL-1 $\beta$  was observed as 332.3 pg/mL in the case of the Biofield Energy Treated group, whereas 378.4 pg/mL was found in the untreated group. There was 12.18% reduction of IL-1 $\beta$  in the Biofield Treated group as compared to the untreated group. Additionally, at concentration 0.105  $\mu\text{g/mL}$ , level of IL-1 $\beta$  was unchanged between the Biofield Treated and untreated test formulation groups. However, at concentration 1.05  $\mu\text{g/mL}$  the level of IL-1 $\beta$  was found at 272.4 pg/mL and 398.2 pg/mL in the Biofield Treated and untreated group, respectively. There was statistically significant ( $p \leq 0.05$ ) reduction of IL-1 $\beta$  by 31.59% in the Biofield Treated group as compared to the untreated group.



**Figure 5.** Dose-dependent inhibition of LPS mediated production of IL-1 $\beta$  by test formulation. For each concentration treatment, the level of IL-1 $\beta$  release was measured after 48 hours of treatment. The values are represented as mean  $\pm$  SEM of independent ELISA experiment, \* $p \leq 0.05$  as compared to the untreated test formulation.

## 4. Discussion

The use of herbomineral products to maintain or improve overall health has gradually increased across the globe over the last couple of years. Herbal extracts have also been shown to modulate immune responses during inflammation. A new proprietary herbomineral formulation was developed, consisting of four ingredients *viz.* ashwagandha, zinc, magnesium and selenium. Ashwagandha was shown to inhibit TNF- $\alpha$  induced NF- $\kappa$ B activation in human myelomonoblastic leukemia cells in a study conducted by Singh and Aggrawal (1995) [45, 46]. Zinc deficiency influences the generation of cytokines, including IL-1 $\beta$ , IL-2, IL-6, and TNF- $\alpha$ , and in response to zinc supplementation, plasma cytokines exhibit a

dose-dependent response. The mechanism of action may reflect the ability of zinc to either induce or inhibit the activation of NF- $\kappa$ B. The major involvement of zinc in the immune system [47] includes an ability to influence the production and signaling of numerous inflammatory cytokines in a variety of cell types [48, 49]. Magnesium sulphate reduced cytokine production in intrapartum women and term and preterm neonates, demonstrating effectiveness in those at risk for inflammation associated with adverse perinatal outcomes, whereby magnesium plays a critical regulatory role in NF- $\kappa$ B activation, cytokine production, and disease pathogenesis [50].

Recently, Chen Y. *et al.* (2009), reported that the supplementation of MC3T3-E1 with methylseleninic acid (MSA) (0.5 mM to 4 mM) reduced the activation of NF- $\kappa$ B

leading to a decrease in IL-6, MCP-1, COX-2 and iNOS in response to MDA-MB-231 conditioned medium [51]. The authors of this study developed a product which showed immunostimulating effects that could contribute to the maintenance of the immune system, which may prevent the progression of acute or chronic diseases. 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, and by eicosanoid such as PGE2. Thus, inhibitors of these cytokines have been considered as a candidate for anti-inflammatory drugs. The immune cells, especially the bone marrow cells such as dendritic and splenocyte cells, are always used to screen the potential immunomodulatory effect of a substance/product. This is because the thymus is the major primary lymphoid organ for T cell development, while bone marrow is for B and NK cells. These immune cells then migrate to the spleen, which is the secondary lymphoid tissue, and respond to antigens in there. Therefore, the regulation of thymus and spleen cell proliferation, which is closely related to maintaining immune homeostasis, can be considered as an important marker for immune response control. Lymphocytes/monocytes/macrophages are key mediators of inflammation and are widely distributed in the body. Therefore, mouse bone marrow derived DCs and splenocytes, which represent appropriate model systems to study the immune responses, were utilized to investigate the anti-inflammatory effects of the newly developed proprietary herbomineral formulation.

Based on the literature, it has been shown that human emotions such as loneliness are directly correlated with the down-regulation of genes bearing anti-inflammatory response elements and the up-regulation of genes bearing response elements for pro-inflammatory NF-kappaB/Rel transcription factors [52]. In this study, the cytotoxic effect of the Biofield Energy Treated formulation was screened by assessing the metabolic activity of immune cells through MTT assay. MTT assay was previously proven to show the similar result in cell proliferation as the bromodeoxyuridine (BrdU) assay, because its accurate correlation between the metabolic activity and cell growth was proven [53]. The anti-inflammatory effects of both the Biofield Energy Treated and untreated test samples were evaluated here in two different immune cells from the mice. The metabolic activity is evaluated by measuring the activity of a mitochondrial enzyme succinate dehydrogenase using the MTT test. This test is widely used in the *in vitro* evaluation of the toxicity of any test item. Both DCs and splenocytes (immune cells) were exposed to various concentrations (1.05 to 51.2  $\mu\text{g}/\text{mL}$  for DCs and 0.000105 to 1.05  $\mu\text{g}/\text{mL}$  for splenocytes) of the test formulation for 24 hours and 48 hours, respectively. There was a significant reduction in the DCs viability at concentration  $>51.2 \mu\text{g}/\text{mL}$  and splenocyte cells showed cytotoxic at 10.5  $\mu\text{g}/\text{mL}$  of the test formulation.

The Biofield Treated test formulation demonstrated the greatest potential for modulating the inflammatory response of DC and splenocyte cells *in vitro* assay. There was a significant reduction of TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$  levels in the

supernatants of all tested concentrations. Importantly, these effects were not attributed to a decrease in cellular viability, indicating that this formulation was not adversely affecting these cultured murine DCs and splenocytes. It is worthwhile to note that the anti-inflammatory effects of the test formulation were observed in both DCs and mouse splenocytes with the suppression of pro-inflammatory cytokines (TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$ ) production. Several cytokines are deeply associated with most inflammatory diseases. In particular, TNF- $\alpha$  and IL-1 $\beta$  are prominent contributors to chronic inflammatory disorders. Our data suggests that the test formulation modulates DC and splenocytes function. The Biofield Energy Treated formulation showed significant inhibition of pro-inflammatory cytokines expression like TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$  as compared to the untreated formulation. Therefore, The Trivedi Effect<sup>®</sup> may act *via* the specific inhibition of nuclear factor-kappa B (NF- $\kappa\text{B}$ ), a transcription factor involved in the activation of many inflammatory mediator genes.

## 5. Conclusions

Overall, results suggest that the Biofield Energy Treated formulation exerts better and significant inhibition of pro-inflammatory cytokines (TNF- $\alpha$ , MIP-1 $\alpha$  and IL-1 $\beta$ ) expression levels as compared to the untreated group in both immune cells *viz.* of DCs and splenocytes. The level of TNF- $\alpha$  was significantly suppressed ( $p \leq 0.01$ ) by 19.21% in DCs in the Biofield Treated test group at 5.2  $\mu\text{g}/\text{mL}$  as compared to the untreated test formulation group. The level of MIP-1 $\alpha$  in LPS induced splenocyte cells was reduced by 15.35% in the Biofield Energy Treated test formulation at 0.0105  $\mu\text{g}/\text{mL}$  as compared to the untreated test formulation group. Moreover, the level of IL-1 $\beta$  in LPS induced splenocyte cells was significantly ( $p \leq 0.05$ ) reduced by 31.59% in the Biofield Treated test group at the concentration of 1.05  $\mu\text{g}/\text{mL}$  as compared to the untreated group. In conclusion, the Biofield Energy Treated test formulation could be used as an effective complementary and alternative treatment and/or method of prevention for various types of autoimmune and inflammatory diseases, stress management and anti-aging as an effective anti-inflammatory and immunomodulatory product without any adverse effects. The Biofield Energy Treated test formulation can be a better complementary and alternative medicine to prevent the immune-mediated tissue damage in cases of organ transplants (for example kidney transplants, liver transplants and heart transplants), various autoimmune disorders such as Lupus, Addison Disease, Celiac Disease (gluten-sensitive enteropathy), Dermatomyositis, Graves' Disease, Hashimoto Thyroiditis, Multiple Sclerosis (MS), Myasthenia Gravis, Pernicious Anemia, Aplastic Anemia, Reactive Arthritis, Rheumatoid Arthritis, Sjogren Syndrome, Systemic Lupus Erythematosus, Type 1 Diabetes, Alopecia Areata, Crohn's Disease, Fibromyalgia, Vitiligo, Psoriasis, Scleroderma, Chronic Fatigue Syndrome and Vasculitis, as well as inflammatory disorders such as Asthma, Ulcerative Colitis, Alzheimer's Disease, Atherosclerosis, Dermatitis, Diverticulitis, Hepatitis, Irritable Bowel Syndrome, and



Parkinson's Disease etc. along with autoimmune and inflammatory diseases, stress management and anti-aging to improve 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; DC: Dendritic cell; BM: Bone marrow; ELISA: Enzyme-linked immunosorbent assay

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