



Evaluation of Pro-Inflammatory Cytokines Expression in Mouse Splenocytes After Incubation with Biofield Treated Herbomineral Formulation: Effect of Biofield Energy Healing Treatment - The Trivedi Effect[®]

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Abstract: The use of herbomineral formulations in the healthcare sector has increased due to their high safety and better therapeutic action. A new proprietary herbomineral formulation was formulated with a mixture of the herbal root extract of ashwagandha and three minerals viz. zinc chloride, magnesium gluconate, and sodium selenate. The aim of the study was to evaluate the immunomodulatory potential of Biofield Energy Healing (The Trivedi Effect[®]) on the formulation when applied to splenocyte cells isolated from mice spleen. The formulation was divided into two parts; one was the control without any Biofield Energy Treatment, while the other part was defined as the Biofield Energy Treated sample, which received Biofield Energy Healing Treatment remotely by seven renowned Biofield Energy Healers. The test formulation was evaluated to find the expression of pro-inflammatory cytokines such as TNF- α , MIP-1 α , and IL-1 β along with non-cytotoxic concentrations by MTT assay. The splenocytes were given the Biofield Energy Treated and untreated sample at concentrations range (0.00001053 to 10.53 μ g/mL) for 48 hours and was reported with safe concentration up to 1.053 μ g/mL with percentage viability range from 76.7% to 109.2% in both samples. Biofield Energy Healing significantly enhanced the cell viability as compared with the untreated formulation. The expression of TNF- α was significantly inhibited in the Biofield Treated formulation at 0.01053, 0.1053, and 1.053 μ g/mL by 1.77%, 1.93%, and 3.55%, respectively compared with the untreated formulation. The rest of the tested concentrations of the Biofield Treated formulation showed an increase in TNF- α expression at 0.00001053, 0.0001053, and 0.001053 μ g/mL by 7.26%, 8.50%, and 8.50%, respectively compared to the vehicle control group. Similarly, the MIP-1 α expression was inhibited by the Biofield Energy Treated formulation and showed immunosuppression activity at 0.01053 μ g/mL by 18.47% ($p \leq 0.001$) compared to the untreated formulation. MIP-1 α expression was reported as 628.94 ± 13.0 pg/mL in the untreated formulation, while it decreased to 512.74 ± 1.9 pg/mL in the Biofield Treated formulation at 0.01053 μ g/mL. In addition, the IL-1 β secretion was also significantly inhibited by the Biofield Treated formulation at concentrations 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 72.02%, 50.16%, 30.68%, and 22.11%, respectively as compared with the untreated formulation. Overall, The Trivedi Effect[®] significantly down-regulated the pro-inflammatory cytokines and potentiated the immunosuppressive effect of the treated formulation, which can be better utilized in organ transplants, anti-aging, stress management, autoimmune disorders, and inflammatory disorders, etc. to modulate the immune system by improving overall health.

Keywords: Biofield Energy Healing Treatment, Biofield Energy Healers, The Trivedi Effect®, LPS, Pro-Inflammatory Cytokines, TNF- α , MIP-1 α , IL-1 β

1. Introduction

The healing properties of plant extracts have been recognized and utilized worldwide since ancient times. 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 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 immune modulating properties, anti-inflammatory, antioxidant, anti-infective, and anti-viral [4-7]. *Withania somnifera* (ashwagandha) biological activity is mainly reported due to the presence of withanolides, and it is used as complementary medicine in alternative 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]. The importance of minerals such as selenium, zinc, and magnesium is to modulate the immune system because 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 titre [11]. These formulations can be used for better therapeutic effect 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 Treatment) as a complementary and alternative approach to study the impact of Biofield Treatment on the herbomineral formulation for its immunomodulatory potential with respect to the pro-inflammatory cytokines in splenocyte cells isolated from mice.

In recent years, several scientific reports along with clinical trials have shown the useful effects of Biofield Energy Treatment, which has shown enhanced immune function in cases of cervical cancer patients with therapeutic touch [12], massage therapy [13], etc. Complementary and Alternative Medicine (CAM) are recognized therapies by The

National Center for Complementary and Integrative Health (NCCIH). 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 reported 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®) has been extensively studied with significant outcomes in many scientific fields such as cancer research [16, 17], altered antimicrobial sensitivity of pathogenic microbes in microbiology [18-21], genetics [22, 23], altered physical and chemical properties of pharmaceutical compounds [24-27], improved overall growth and yield of plants in agricultural science [28-31], and in changing the structure of the atom in relation to various metals, ceramics, polymers and chemicals in materials science [32-35].

The authors of this study want to evaluate the impact of Biofield Energy Treatment (The Trivedi Effect®) on the given herbomineral formulation, which might improve the immunomodulatory function in *in vitro* cellular models on mice splenocyte cells.

2. Materials and Methods

2.1. Chemicals and Reagents

Lipopolysaccharide (LPS), 3-(4, 5-dimethyl-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, NaHCO₃, 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- α), macrophage inflammatory protein-1 α (MIP-1 α), and interleukin-1 beta (IL-1 β) 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 a combination of four ingredients: ashwagandha root powder extract, zinc chloride, sodium selenate, 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 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. Laboratory rodent diet and drinking tap water were provided *ad libitum* and 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. 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

One part of the test formulation did not receive any sort of treatment and was defined as the control group, while Biofield Energy Treatment was given to the herbomineral formulation defined as the treated group. The Biofield Energy Treatment was administered for 5 minutes by the seven Biofield Energy Healers (The Trivedi Effect[®]), six of which were remotely located in the U. S. A. and one of which was remotely located in Canada, while the test formulation was located in Dabur Research Foundation near New Delhi in Ghaziabad, India, under standard laboratory conditions. This treatment was provided through the Biofield Energy Healers' unique Energy Transmission process (The Trivedi Effect[®]) remotely to the test formulation. Further, the control group was treated by a sham healer for comparative purposes. The sham healer does 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 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 includes cells with LPS. Group 3 included the splenocyte cells with LPS along with vehicle (0.005% DMSO) denoted as the vehicle control. Groups 4 and 5 were defined as the positive control, which includes cells with Con-A (0.5 $\mu\text{g/mL}$) 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 untreated and Biofield Treated formulations, respectively, at concentration 0.00001053 to 10.53 $\mu\text{g/mL}$. After 48 hours of incubation, supernatants were analyzed for the secreted levels of TNF- α , MIP-1 α , and IL-1 β 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 the spleens were aseptically removed and grounded by passing 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_4Cl , 0.01 M NaHCO_3 , 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 0.2×10^6 cells per well. They were incubated at 37°C in a humidified atmosphere of 5% CO_2 for the indicated period [36].

2.7. Cell Culture and Test Formulation Treatment

Splenocyte (0.2×10^6 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 $\mu\text{g/mL}$ of streptomycin. LPS (50 ng/mL) induced splenocyte cells cultures were grown for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2). The effect of cytotoxicity of the formulation was tested by treating cells with different concentrations of the test formulation in RPMI-1640 medium. The various concentrations of the test formulation were used *i.e.* 0.00001053 $\mu\text{g/mL}$ to 10.53 $\mu\text{g/mL}$ 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 effect of the Biofield Treated and untreated formulations at the concentration range of 0.00001053 $\mu\text{g/mL}$ to 10.53 $\mu\text{g/mL}$ were tested for cell viability 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 0.2×10^6 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_2 atmosphere. The cells were centrifuged and supernatants were removed. The cell pellet 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 BioTek Reader (SIAFRT/Synergy HT multimode reader, US).

The effect of the formulation on cell viability of splenocyte cells was determined as per 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 concentration that resulted in >75% viability was selected for subsequent cytokine estimation.

2.9. Determination of Cytokines (TNF- α and IL-1 β) and Chemokine (MIP-1 α) Using ELISA

The *in-vitro* activity of the Biofield Treated and untreated test formulations were estimated on the mice splenocytes for the production of TNF- α , MIP-1 α , and IL-1 β 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 μ L culture supernatant from different experimental samples and standards were incubated overnight at 4°C and, after three washes, biotinylated anti-mice cytokine (TNF- α , MIP-1 α , and IL-1 β) 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. The plates were again washed 3 times and then 100 μ L of horseradish peroxidase (HRP)–streptavidin conjugate solution was added and the plate was incubated for 45 minutes at room temperature with gentle shaking. Next, the plate wells were washed 3 times as previous and 100 μ L 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. Further, 50 μ L of 0.2 mol/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 triplicate for each sample [37].

2.10. Statistical Analysis

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

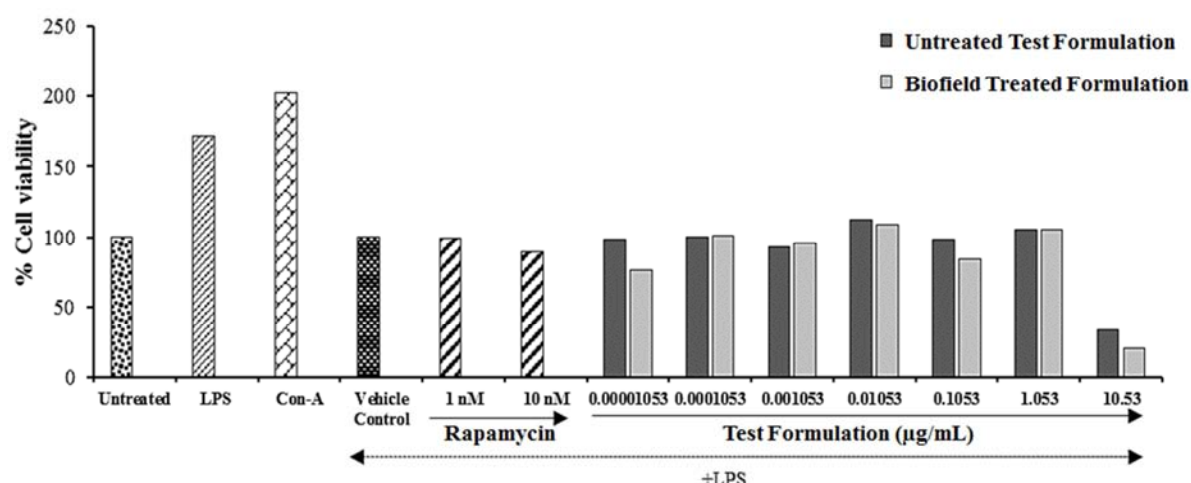


Figure 1. MTT assay in the splenocyte cells after the 48-hours of treatment with different test formulation concentrations in the presence of 0.5 μ g/mL LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of the test formulation treated cells relative to the untreated vehicle control group.

3. Results

3.1. MTT Assay on Splenocyte Cells

The effect of the Biofield Treated and untreated test formulations on the proliferation of mice splenocyte cells was examined after 48 hours through MTT cell viability assay. The effect of the test formulation on the viability of the splenocytes is shown in Figure 1. The results showed the % cell viability was altered after Biofield Treatment in the tested concentration of the test formulation. The untreated, LPS, and Con-A group showed 100%, 171.7%, and 201.9% cell viability, respectively. The vehicle control group reported with 100% and the rapamycin group as 98.9% and 89.9% at

concentrations 1 and 10 nm, respectively. The increased cell viability with respect to vehicle control might be due to proliferation in cell culture. Con-A and rapamycin showed immunostimulatory and immunosuppressive action, respectively, as used as positive control in the experiment. Concentration range of 0.00001053 to 10.53 μ g/mL was selected for comparison of cell viability after adding the Biofield Treated and untreated test formulations to the splenocyte cells. The test formulation was found safe at concentration up to 1.053 μ g/mL with percentage viability range from 76.7% and 109.2%. The cell viability was greatly decreased at the concentration of 10.53 μ g/mL in both the Biofield Treated (20.9%) and the untreated formulations (33.9%), so this concentration was not selected for the

estimation of cytokines. However, at three tested concentrations the Biofield Treated test formulation showed increased cell viability, *i.e.* at 0.0001053, 0.01053, and 1.053 $\mu\text{g/mL}$, while the rest of the three concentrations showed decreased cell viability *i.e.* at 0.00001053, 0.001053, and 0.1053 $\mu\text{g/mL}$ with respect to the vehicle control group. The percentage of increased cell viability in the Biofield Treated test formulation was 1.00%, 2.89%, and 0.29% at 0.0001053, 0.001053, and 1.053 $\mu\text{g/mL}$ respectively as compared with the untreated test formulation. Similarly, the concentrations 0.0001053, 0.01053, and 1.053 $\mu\text{g/mL}$ showed increased cell viability as 0.9%, 9.2%, and 5.5%, respectively as compared with the vehicle control group. The percentage of decreased cell viability reported at 0.00001053, 0.01053, and 0.1053 $\mu\text{g/mL}$ was 22.36%, 2.58%, and 14.54%, respectively in comparison to the untreated test formulation. Overall, the results showed that the cell viability was significantly altered after the Biofield Energy Treatment.

3.2. Effect of the Test Formulation on the Expression of Pro-Inflammatory Cytokines (TNF- α and IL-1 β) and Chemokine (MIP-1 α) in the Splenocyte Cells

The effect of the Biofield Treated herbomineral formulation was observed on pro-inflammatory cytokines TNF- α , MIP-1 α , and IL-1 β . All play a major role in inflammation, immune modulation, and lymphocyte activation, so it might be expected that the herbomineral formulations can modulate the expression and activation of cytokines. Therefore, the expression of TNF- α , MIP-1 α and IL-1 β at six concentrations was examined in the spleen cells. The effect of the test formulation on pro-inflammatory cytokines was estimated by incubating the formulation with various concentrations of the treated and untreated test formulations for 48 hours using ELISA assay.

3.2.1. Assessment of TNF- α Expression

The effect of the Biofield Treated and untreated test

formulations on TNF- α secretions in splenocyte cells are represented in Figure 2. Results suggest that both the untreated and Biofield Treated groups demonstrated suppression of TNF- α secretions at different tested concentrations *i.e.* at 0.00001053 to 1.053 $\mu\text{g/mL}$. The negative control (untreated cells), LPS, Con-A, and vehicle control group showed TNF- α values as 87.60, 504.65, 271.32, and 501.55 pg/mL, respectively. However, the untreated test formulation demonstrated suppression of TNF- α from LPS stimulated levels at five tested formulation concentrations out of six *i.e.* at 0.0001053, 0.001053, 0.01053, 0.1053, and 1.053 $\mu\text{g/mL}$ by 12.51%, 7.50%, 6.49%, 5.80%, and 16.46%, respectively as compared with the vehicle control. The maximum decreased value of TNF- α of the untreated test formulation was reported at 1.053 $\mu\text{g/mL}$ *i.e.* 418.99 pg/mL. At three tested concentrations, the Biofield Treated test formulation showed an inhibition of TNF- α *i.e.* at 0.01053, 0.1053, and 1.053 $\mu\text{g/mL}$ by 1.77%, 1.93%, and 3.55%, respectively as compared to the vehicle control group. The maximum inhibition of TNF- α in the Biofield Energy Treated test formulation was reported (483.72 pg/mL) at the test formulation concentration 1.053 $\mu\text{g/mL}$ as compared to the vehicle control. On the other hand, the Biofield Energy Treated test formulation demonstrated an increase in TNF- α at three tested formulation concentrations *i.e.* 0.00001053, 0.0001053, and 0.001053 $\mu\text{g/mL}$ by 7.26%, 8.50%, and 8.50%, respectively as compared to the vehicle control group. Overall, it can be suggested that the test formulation has immunosuppressive effects by inhibiting the concentration of TNF- α as compared with the vehicle control, while the Biofield Energy Treatment has increased the concentration of TNF- α in all the concentrations as compared with the untreated formulation. The Biofield Energy Treatment showed a significant effect on altering the level of TNF- α as compared to the untreated test formulation.

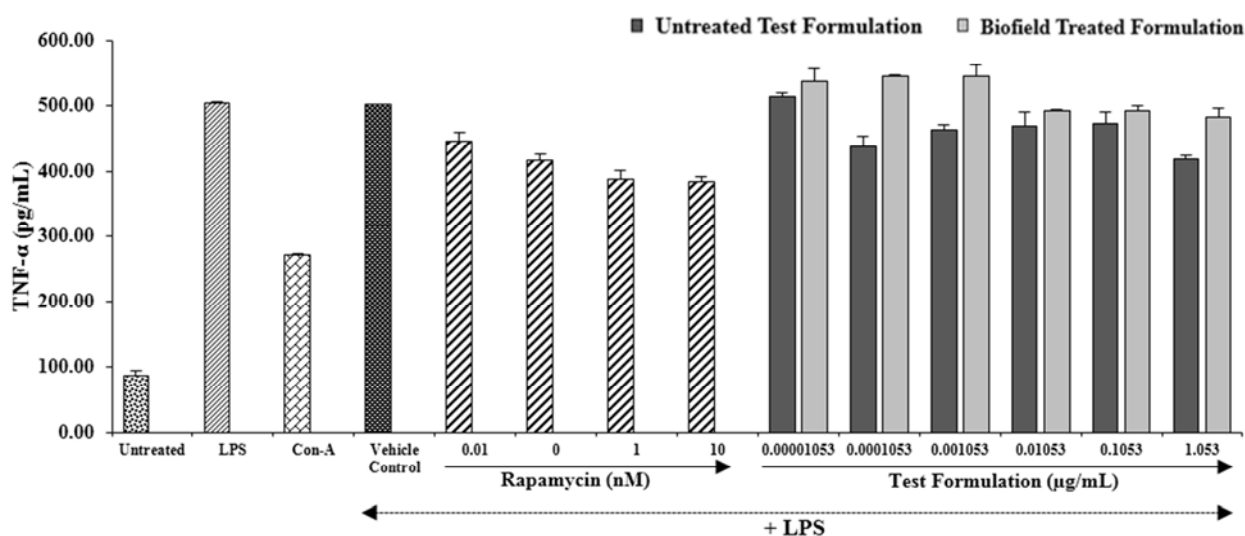


Figure 2. Concentration-dependent effect on TNF- α by the Biofield Treated and untreated test formulations. For each concentration treatment, the level of TNF- α release was measured after receiving 48-hours of treatment. All values are represented (in pg/mL) as mean \pm SEM.

3.2.2. Estimation of MIP-1 α Expression

The effect of the Biofield Energy Treated and untreated test formulations on MIP-1 α secretion is shown in Figure 3. The figure demonstrates that the Biofield Treated and untreated test formulations inhibit the expression of MIP-1 α as compared with the vehicle control group. However, the comparative effect of the Biofield Energy Treated and untreated test formulations on MIP-1 α secretion in splenocyte cells showed significant alterations at all the tested concentrations. The untreated cells, LPS, Con-A, and vehicle control group showed values of MIP-1 α as 42.8 ± 14 , 598.6 ± 11.2 , 285.5 ± 10.3 , and 768.0 ± 10.2 pg/mL, respectively. The untreated test formulation showed significant inhibition of MIP-1 α secretion at all the tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 24.27%, 41.30%, 45.72%, 18.11%, 19.18%, and 36.15%, respectively as

compared to the vehicle control group. The Biofield Energy Treatment further enhanced the immunosuppressive property of the test formulation by 18.47%. The enhanced activity of MIP-1 α expression was reported at concentration 0.01053 μ g/mL *i.e.* 512.74 ± 1.9 pg/mL in the Biofield Treated test formulation and 628.94 ± 13.0 pg/mL in the untreated formulation. It showed significant decreased level of MIP-1 α by 18.47% ($p \leq 0.001$) as compared to the untreated formulation. The rest of the other Biofield Treated test formulation concentrations showed an increased level of MIP-1 α as compared with the untreated test formulation. However, the Biofield Energy Treated test formulation group reported an inhibition of MIP-1 α secretion at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 25.41%, 25.71%, 35.55%, 33.24%, 19.18%, and 27.78%, respectively as compared with the vehicle control group.

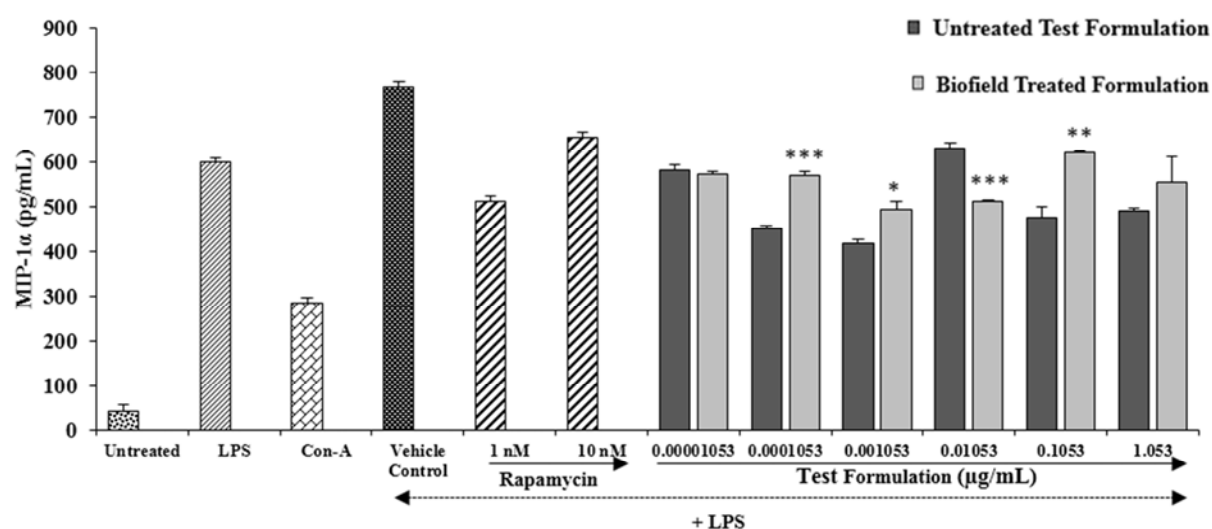


Figure 3. Concentration-dependent inhibition of LPS mediated production of MIP-1 α by the Biofield Treated and untreated test formulations. For each concentration treatment, the level of MIP-1 α release was measured after receiving 48-hours of treatment. The values are represented in pg/mL as mean \pm SEM (** $p \leq 0.001$, * $p \leq 0.01$, and $p \leq 0.05$, as compared with the untreated test formulation).

3.2.3. Estimation of IL-1 β Expression

The effect of the Biofield Treated and untreated test formulations on IL-1 β expression is shown in Figure 4. The figure demonstrates the inhibition of IL-1 β after treatment with the Biofield Energy Treated and untreated test formulations as compared with the vehicle control group. However, the comparative effect of the treated and untreated test formulations on IL-1 β secretion in splenocyte cells showed significant inhibition at 5 tested concentrations out of 6. The untreated cells, LPS, Con-A, and vehicle control group showed values of IL-1 β as 7.67 ± 1.9 , 78.86 ± 5.8 , 19.48 ± 4.5 , and 54.90 ± 3.4 pg/mL, respectively. The untreated test formulation showed a significant inhibition of IL-1 β secretion at all the tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 77.21%, 40.13%, 31.73%, 36.62%, 43.24%, and 42.78%, respectively as compared with the vehicle

control group. The Biofield Treatment showed further significant improvement in the immunosuppressive property of the test formulation at all the concentrations, except at 0.00001053 μ g/mL, as compared to the untreated formulation. The significant inhibition of IL-1 β secretion after the Biofield Treatment was reported at the concentrations *viz.* 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 72.02%, 50.16%, 30.68%, and 22.11%, respectively as compared with the untreated formulation. The maximum IL-1 β secretion inhibition was reported in the Biofield Treated test formulation at 0.001053 μ g/mL. While 60.17 ± 7.7 was reported in the untreated test formulation, the Biofield Treated sample reported 16.84 ± 1.8 pg/mL ($p \leq 0.01$). However, the Biofield Treated group reported an inhibition of IL-1 β secretion at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, and 1.053 μ g/mL by 35.36%, 43.24%, 80.89%, 68.41%, 60.65%, and 55.42%, respectively as compared with the vehicle control group.

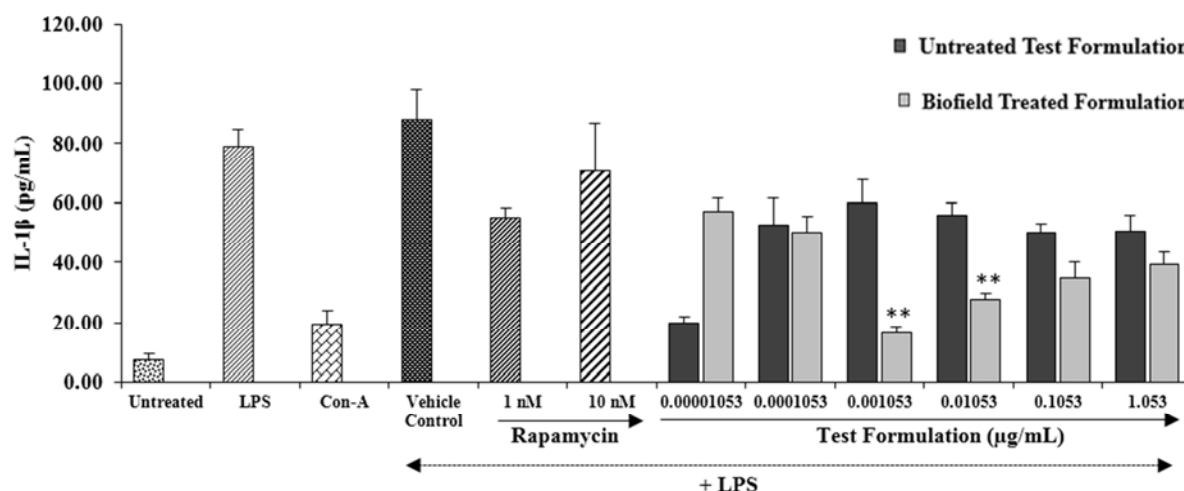


Figure 4. Concentration-dependent inhibition of LPS mediated production of IL-1 β by the test formulations. For each concentration treatment, the level of IL-1 β release was measured in cell supernatant after receiving 48-hours of treatment. All values are represented in pg/mL as mean \pm SEM (** $p \leq 0.01$, as compared with the untreated test formulation).

4. Discussion

Traditional, alternative and complementary medicines and therapies play a major role worldwide with respect to the current human wellness and traditional healthcare sectors, including overall healthcare management, longevity, anti-aging and quality of life. However, more attention is required to understand the use of alternative energy medicine and its utilization in current healthcare systems. The scope of traditional medicine has only been improved in developing countries [38], but the use of Biofield Energy as a Complementary and Alternative Medicine (CAM) approach still requires implementation in the practice by many scientific research. The herbal medicine industry is increasing worldwide more than 10% annually, and it may reach up to \$5 trillion by the year 2050 [39]. Many herbomineral products have shown significant effect in curing many diseases with minimal adverse effects. One widely used example of an herbomineral formulation is the *Naga bhasma* in the Indian system of medicines, which is used for various disorders such as diabetes, indigestion, inflammation of the intestine, osteomalacia, blood disorders, infertility, regenerate germinal epithelium of the testes, as a potent revitalizer, and many more uses within our bodies [40]. The Trivedi Effect[®]-Biofield Energy Healing (TEBEH) is a new proprietary method that can be used to enhance the impact of herbomineral formulations, which can be further used to modulate the immune function with long-term effectiveness and minimal toxicity at a lower cost, which might improve overall health and quality of life.

The individual ingredients of the herbomineral formulation, including ashwagandha and zinc chloride, have already been reported in many scientific reports to modulate the inflammatory response using cytokines estimation [4-7]. Lymphocyte proliferation and the activation of NK cells are cytokine dependent [41], and its up and down regulation

could affect the course of immune response and the whole network of immune regulation. The individual components, such as ashwagandha, have been reported to inhibit the NF- κ B and AP-1 transcription factors in human peripheral blood and synovial fluid mononuclear cells [42]. With respect to the minerals present in the formulation, the deficiency of zinc directly influenced the cytokines generation such as IL-2, IL-6, IL-1 β , and TNF- α , and influenced its generation in a concentration depended manner. It was reported that zinc also alters the cytokines generation by inhibiting the activation of NF- κ B [43].

Magnesium also plays an important role in the activation of NF- κ B, and affects the generation of cytokines, and would be effective in the risk situations associated with inflammation or its related diseases pathogenesis [44]. However, selenium from the diet mostly incorporates into selenoproteins, and plays an important role in inflammation and for initiating immunity. Selenium influences different leukocytes effector functions such as cytokines secretion, migration, adherence, and phagocytosis. It plays an important role in cytokine production using calcium flux and oxidative pathway [45-47].

Study results suggest that the Biofield Treated test formulation showed an anti-inflammatory effect and suppressed the level of tested cytokines as compared with the vehicle control group. However, the Biofield Energy Treated formulation further potentiated the effect as compared with the untreated test formulation, which may better prevent the progression of acute or chronic infection, because inflammation is regarded as the first response that releases cytokines. Thymus and spleen cell proliferation are considered as vital parameters to maintain immune homeostasis [48]. Inhibition of cytokines such as TNF- α , IL-1, and IL-6, and eicosanoid such as PGE2 from any formulation can be considered to have anti-inflammatory properties, and the Biofield Energy Treated test formulation

can be better utilized as an anti-inflammatory agent in comparison to the untreated test formulation.

Cells viability assay (MTT) suggest that the Biofield Treated and untreated test formulations' concentration was found safe with respect to the *in vitro* viability of splenocytes until concentration 1.053 µg/mL, while the viability percentage was increased after the Biofield Energy Treatment. The MTT assay evaluates the metabolic activity by measuring the activity of succinate dehydrogenase, a mitochondrial enzyme. This test is widely used in the *in vitro* evaluation of the cell toxicity of any test drug. MTT assay is regarded as more rapid, less costly, less time consuming, and as a non-radioactive method to show the cell proliferation results on the basis of cell growth and metabolic activity [49]. Further, the level of TNF-α was inhibited at three tested higher concentrations *i.e.* 0.01053, 0.1053, and 1.053 µg/mL by 1.77%, 1.93%, and 3.55%, respectively by the Biofield Treated test formulation. Thus, it can be concluded that the Biofield Energy Treatment potentiated the inhibition and showed immunosuppressive activity at higher concentrations. TNF-α plays a major role in immune disorders, and is the controlling factor for many diseases [50]. However, the level of MIP-1α was inhibited at all of the Biofield Treated test formulation concentrations with respect to the vehicle control, while showing significant inhibition at 0.01053 µg/mL *i.e.* 18.74% with respect to the untreated test formulation. Reduction of MIP-1α levels might be useful in decreasing the inflammatory responses [51].

Similarly, the expression of IL-1β was further significantly potentiated after the Biofield Treatment on the test formulation at all concentrations, except lower concentration values *i.e.* 0.0001053 µg/mL. The percentage of inhibition ranged from 22.11% to 72.02% in the Biofield Treated test formulation as compared with the untreated test formulation. Immunological and inflammatory functions of IL-1β in controlling the immune response during infections are well defined [52, 53]. Overall, the inhibitory effect might be the result of specific inhibition of NF-κB, a transcription factor involved in the activation of many inflammatory mediator genes. The results also reflect that the Biofield Treated test formulation showed immunosuppressive activity in LPS stimulated splenocyte cells on pro-inflammatory cytokines (TNF-α, MIP-1α, and IL-1β) secretion, which can be concluded that the Biofield Treated test formulation has the potential to act on the innate immune system. LPS was used in the study to induce the immune system to depict the disease state. The effect was significant with respect to all cytokines as they are deeply associated with inflammatory disorders (such as TNF-α and IL-1β for chronic inflammation). In the case of many autoimmune disorders, such as rheumatoid arthritis, steroids (prednisolone and dexamethasone) are preferred to suppress the cytokines production. However, this study suggests that a Biofield Treated formulation would be a better choice of treatment and has no toxicity. Besides, it can also be preferred for the inhibition of T and B lymphocytes, which could help in immune-mediated disorders such as autoimmune disorders,

stress, and asthma [54].

Biofield Energy Healing has been successfully reported in the case of cancer cell lines with respect to the inhibition of cytokine expression such as IL-1α and IL-1β levels [55]. Complementary and alternative medicine (energy therapy) has been reported with various benefits as compared with the conventional treatment approach. The National Center for Complementary and Integrative Health (NCCIH) under the National Institutes of Health (NIH) have recognized the significant outcomes of energy medicine [56].

5. Conclusions

The *in vitro* results of the Biofield Energy Treated and untreated test formulations showed significant modulatory effects on splenocyte cells isolated from mice with respect to the suppression of tested cytokines. All the tested cytokines (TNF-α, MIP-1α, and IL-1β) were reported with immunosuppressive activity with respect to the vehicle control group, while the Biofield Energy Treatment further potentiated the immunomodulation as compared with the untreated test formulation. The results of *in vitro* splenocyte cells' viability assay suggest that most of the tested concentration of the Biofield Treated and untreated test formulations showed improved cell viability, except at the higher concentration of 10.53 µg/mL. The cell viability after the Biofield Energy Treatment significantly improved with the Biofield Treated test formulation at concentrations 0.0001053, 0.001053, and 1.053 µg/mL with respect to the untreated test formulation. The results of the cytokines estimation revealed that the Biofield Treated group suppressed the TNF-α, MIP-1α and IL-1β. TNF-α levels, which were significantly inhibited at three tested concentrations of 0.01053, 0.1053, and 1.053 µg/mL by 1.77%, 1.93%, and 3.55%, respectively in the Biofield Treated test formulation as compared with the untreated test formulation. In the case of MIP-1α, both the untreated and Biofield Treated test formulation showed significant suppression, while the Biofield Energy Treatment enhanced the immunosuppressive activity at the concentration 0.01053 µg/mL by 18.47% ($p \leq 0.001$) as compared to the untreated formulation. The values of MIP-1α reported in the case of the untreated formulation was 628.94 ± 13.0 pg/mL, while it was decreased to 512.74 ± 1.9 pg/mL in the Biofield Energy Treated test formulation. Similarly, significant inhibition ($p \leq 0.001$) of IL-1β secretion was reported in the Biofield Treated test formulation at concentration 0.001053, 0.01053, 0.1053, and 1.053 µg/mL by 72.02%, 50.16%, 30.68%, and 22.11%, respectively as compared with the untreated formulation.

On the basis of the above estimation, it can be concluded that after receiving the Biofield Energy Healing Treatment (The Trivedi Effect®), administered remotely by the seven Biofield Energy Healers to the new herbomineral formulation significantly inhibited the activity of pro-inflammatory cytokines that might prevent the over-activation of the immune system. 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, Parkinson's Disease and stress etc. with a safe therapeutic index to improve overall health and quality of life. Further, the Biofield Energy Healing Treated test formulation can also be used in the prevention of immune-mediated tissue damage in cases of organ transplants (for example heart transplants, kidney transplants and liver transplants), for anti-aging, stress prevention and management, and in the improvement of 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; NCCAM: National Center for Complementary and Alternative Medicine; CAM: Complementary and alternate medicine

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