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An Impact of the Trivedi Effect® - Biofield Energy Healing on Herbomineral Formulation for Immunomodulation of Pro-inflammatory Cytokines in Biofield Treated Mouse Splenocytes

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Abstract: Herbomineral formulations have increased in recognition and popularity 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, magnesium, and selenium. The aim of the study was to evaluate the immunomodulatory potential of Biofield Energy Healing (The Trivedi Effect®) on the test formulation when applied to splenocyte cells isolated from the Biofield Treated mice. The test formulation was divided into two parts. One part was denoted as the control without any Biofield Energy Treatment. The other part was defined as the Biofield Energy Treated sample, which received the Biofield Energy Healing Treatment remotely by seven renowned Biofield Energy Healers. A wide concentration range (0.00001053 to 10.53 μg/mL) of the test formulation was used to determine non-cytotoxic concentrations using MTT assay. Further, the expression of pro-inflammatory cytokines (TNF-α, MIP-1α, and IL-1β) was determined by ELISA method. The test formulation was evaluated and found to be safe up to 1.053 μg/mL with a percentage cell viability range of 73% to 97% using MTT assay. The Biofield Treated formulation improved the cell viability up to 6.61% compared with the untreated test formulation. TNF-α expression was significantly inhibited by 16.72% at 0.1053 μg/mL compared with the untreated test formulation, however expression was significantly altered by 53.67% and 25.62% at 0.01053 and 1.053 μg/mL, respectively compared to the untreated test formulation. TNF-α expression was also suppressed in the Biofield Treated test formulation at 0.001053 and 0.1053 μg/mL by 4.0% and 8.56%, respectively as compared with the vehicle control. MIP-1α suppression was reported in the Biofield Treated test formulation at 0.00001053 to 1.053 μg/mL by 8.43%, 22.02%, 21.92%, 20.54%, 5.40%, and 19.82%, respectively compared with the vehicle control. However, the Biofield Treated formulation further exhibited substantial suppression of MIP-1α at 0.0001053, 0.001053, 0.01053, and 0.1053 μg/mL by 13.50%, 7.38%, 36.83% ($p \leq 0.001$), and 2.53%, respectively compared with the untreated test formulation. In addition, significant inhibition of IL-1β secretion was reported in the Biofield Treated formulation at 0.0001053, 0.001053, 0.01053, and 0.01053 μg/mL by 32.40%, 14.99%, 60.42%, and 15.15%, respectively compared with the untreated test formulation. The Biofield Energy Healing Treatment significantly potentiated the immunosuppressive effect of the test formulation in Biofield Treated mouse splenocytes, which 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®, Immune-Modulation, Pro-inflammatory Cytokines, Splenocytes, TNF-α, MIP-1α, IL-1β

1. Introduction

The traditional systems of medicine widely use herbal drugs for many biological activities, but there are limited experimental studies based upon herbomineral formulations that combine herbs or plant extracts with minerals. Medicinal plants and minerals have been widely reported to have many healing properties including anti-inflammatory, anti-diabetic, and anti-stress activities, as well as improving overall health and the immune system [1, 2]. The immunomodulatory activity of herbal medicine can be potentiated with the presence of important minerals. These combination products are rapidly gaining attention due to their low toxicity and better bioavailability [3]. A newly formulated herbomineral formulation with improved immunomodulatory activity could be an advancement for pharmaceutical companies with respect to nutritional supplements. However, significant anti-inflammatory activity is always an important target for any new herbomineral formulation [4]. Although the global market has different anti-inflammatory, analgesic, and immunomodulatory potential formulations with high efficacy, they are unfortunately unsuitable for many patients due to their limited potency, less tolerance, and adverse effects, etc. In the search of some novel formulation, a new proprietary herbomineral formulation was formulated using the root extract of the important medicinal plant *Withania somnifera* (ashwagandha), along with three minerals *viz.* zinc chloride, magnesium gluconate, and sodium selenate. The beneficial effects of the test formulation might be attributed to the immunomodulatory potential of each of the individual components [5-8]. For example, ashwagandha belongs to the family *Solanaceae* and is commonly used in alternative therapies for its immunomodulatory, antitumor, and antibacterial effects, and much more. This is due to the presence of pharmacologically active molecules like withanolides [9-11]. Additionally, it has been well-documented that minerals such as selenium, zinc, and magnesium have significant importance in the modulation of the immune system and have been found to have strong immunomodulatory potential [6]. 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 the 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 Energy Treatment on the herbomineral formulation for its immunomodulatory potential with respect to the pro-inflammatory cytokines in splenocyte cells isolated from the biofield energy treated mice.

Amidst the broad field of Complementary and Alternative Medicine (CAM), there have been a substantial amount of scientific reports that show the beneficial effects of Biofield Energy Healing Therapy. However, the effect of Biofield Energy Treatment showed beneficial results to enhance the immune function of cervical cancer patients using therapeutic touch [12], massage therapy [13], etc. Biofield Energy Therapies have been practiced worldwide recently with significant therapeutic outcomes, such as enhanced personal well-being in the case of cancer patients [14], improved functional ability in arthritis patients [15], and decreased pain and anxiety [16]. 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 naturopathy, natural products, homeopathy, mindfulness, meditation, yoga, deep breathing, Tai Chi, Reiki, Qi Gong, chiropractic/osteopathic manipulation, massage, special diets, progressive relaxation, guided imagery, cranial sacral therapy acupressure, acupuncture, relaxation techniques, hypnotherapy, rolfing structural integration, movement therapy, pilates, Ayurvedic medicine, traditional Chinese herbs and medicines, healing touch, essential oils, aromatherapy, and applied prayer (as is common in all religions, like Buddhism, Christianity Hinduism, Judaism and Buddhism). Human Biofield Energy has subtle energy that has the capacity to work in an effective manner [17, 18]. Biofield Energy Healing Treatment (The Trivedi Effect®) had significant impact in the transformation of living organisms and nonliving materials such as medical science [19, 20], microbiology [21-24], genetics and biotechnology [25, 26], nutraceuticals [27-28], agricultural science and livestock [29-32], and materials science [33-35].

This experiment was designed to evaluate the impact of Biofield Energy Healing (The Trivedi Effect®) Treatment on the new herbomineral formulation for immunomodulatory potential after co-incubation with the isolated splenocyte cells from the Biofield Energy Treated mice.

2. Materials and Methods

2.1. Chemicals and Reagents

The test formulation component ashwagandha (*Withania somnifera*) root extract powder (≥ 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. Other experimental chemicals such as 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, $NaHCO_3$, 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-1α (MIP-1α), and interleukin-1 beta (IL-1β) were purchased from R&D systems, USA. Fetal bovine serum (FBS) was purchased from GIBCO, USA. All other chemicals used in the experiment were of analytical grade available in India.

2.2. Test Formulation and Reference Standard

The test formulation comprised of a combination of four ingredients *viz.* 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 the reference standard (positive control) for immunostimulatory and immunosuppressive action, respectively in the splenocyte 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 mice were maintained under controlled conditions with a temperature of 22 ± 3°C, humidity of 30% to 70% and a 12 hours of light or 12 of hours dark cycle and rodent laboratory 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. The approval of the Institutional Animal Ethics Committee was obtained prior to carrying out the animal experiment.

2.4. Biofield Energy Healing Strategies

The herbomineral test formulation was divided into two parts. One part of the herbomineral formulation did not receive any sort of treatment and was defined as the control group, while another part received the Biofield Energy Treatment and was defined as the Biofield Treated test formulation. Further, one group of mice received the Biofield Energy Treatment *per se* by the Biofield Energy Healers under similar conditions, which were used to isolate the splenocyte cells as per the study design (Figure 1). These isolated splenocyte cells were known as the Biofield Treated splenocyte cells. The Biofield Energy Healing Treatment (The Trivedi Effect®) was provided by the group of seven Biofield Energy Healers, 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 the research laboratory of Dabur Research Foundation near New Delhi in Ghaziabad, India, and kept under standard laboratory conditions. This treatment was provided for 5 minutes through the Biofield Energy Healers' unique Energy Transmission process (The Trivedi Effect®), administered remotely to the test formulation. Similarly, the control sample was subjected to "sham" healers under the same laboratory conditions for 5 minutes. The sham healer 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 splenocyte cells for cytokines estimation.

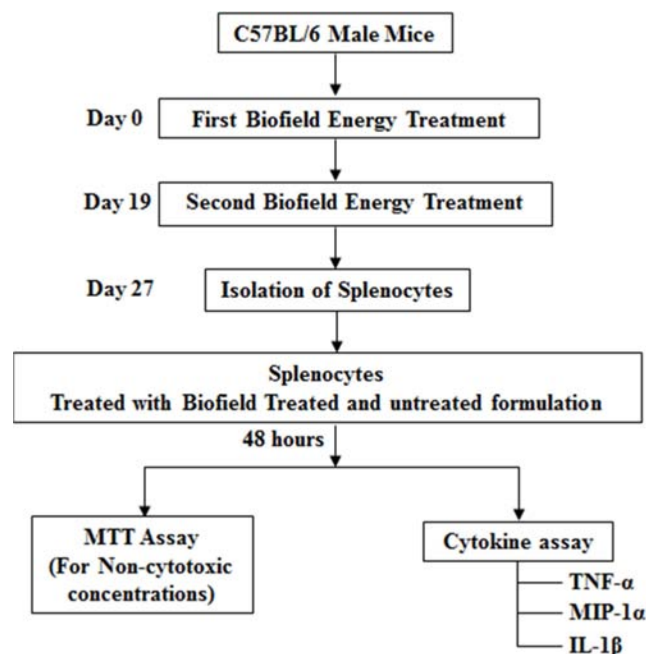

Figure 1. *Schematic experimental design of the Biofield Energy Healing Treatment.*

2.5. Experimental Design

The experimental study was divided into 7 groups. Group 1 consisted of splenocyte cells isolated from the Biofield Energy Treated animal without LPS and was denoted as the negative control. Group 2 served as a stimulant group that included similar cells with LPS. Group 3 included the same isolated 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 controls *i.e.* Con-A (0.5 µg/mL) and rapamycin (1 nM and 10 nM), respectively. Group 6 and 7 were denoted as the test item groups that included splenocyte cells (isolated from the Biofield Energy Treated animal) with LPS along with the untreated and Biofield Energy Treated test formulation, respectively at concentrations 0.00001053 to 10.53 µg/mL. After 48 hours of incubation, supernatants were analyzed for the secreted levels of TNF-α, MIP-1α, and IL-1β using ELISA (enzyme-linked immunosorbent assay) as per the manufacturer's instructions. Concentrations were determined in triplicate wells of each sample.

2.6. Isolation of Murine Splenocytes

The Biofield Energy Treated C57BL/6 male mice were sacrificed and their spleens were aseptically removed and ground 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 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 RPMI-1640 medium. Further, the cells were resuspended in 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 ≥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 Item Treatment

The splenocyte (0.2×10^6 cells per well) cells isolated from the Biofield Energy Treated mice were grown in 96-well culture plates using 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_2 incubator (5% CO_2). The effect of cytotoxicity of the test 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 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 effect of the test formulation at the concentration range of 0.00001053 µg/mL to 10.53 µg/mL was tested for cell viability assay using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The number of viable cells were determined by the ability of mitochondria to convert MTT to formazan dye. Splenocyte cells isolated from the Biofield Energy Treated mice 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 37ºC in a humidified 5% CO_2 atmosphere. The cells were centrifuged and supernatants were removed. The cell pellets in each well were 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 test formulation on the cell viability of splenocyte cells was determined as 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 >72% viability was selected for subsequent cytokine estimation.

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

The *in-vitro* activity of the test formulations was estimated on the mice splenocyte cells for the production of TNF-α, MIP-1α, and IL-1β 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-mice cytokine (TNF-α, MIP-1α, and IL-1β) 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 previous 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. Further, 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 ± SEM and were subjected to Student's *t*-test for two group comparison. Statistical significance was considered at $p \leq 0.05$.

3. Results and Discussion

3.1. MTT Assay on Splenocyte Cells

The splenocyte cells isolated from the Biofield Energy Treated mice were studied for viability assay after exposure of the test formulation using MTT cell viability assay after 48 hours of incubation. The cell viability results are summarized in Figure 2. The results showed the % cell viability was altered after the Biofield Energy Healing Treatment in all the tested concentrations of the herbomineral test formulation.

The untreated cells isolated from the Biofield Treated mice, LPS, and Con-A group showed 100%, 187.44%, and 160.47% cell viability, respectively, while the positive control (rapamycin) group showed percentage cell viability as 81.30% and 78.70% at concentrations 1 and 10 nM,

respectively in the presence of LPS (0.5 µg/mL). Con-A and rapamycin showed immunostimulatory and immunosuppressive action, respectively, and were used as positive controls in the experiment. The test formulation concentrations range from 0.00001053 to 10.53 µg/mL were selected for the cell viability assay on the Biofield Treated splenocyte cells, and concentrations up to 1.053 µg/mL were found to be safe with percentage viability ranging from 72.91% to 97.54%. Out of the six tested concentrations, the cell viability was increased by 3.17%, 5.07%, 2.77%, and 6.61% in the Biofield Treated test formulation *i.e.* at 0.0001053, 0.01053, 0.1053, and 1.053 µg/mL, respectively, while two concentrations showed decreased cell viability at 0.00001053 and 0.01053 µg/mL with respect to the untreated test formulation.

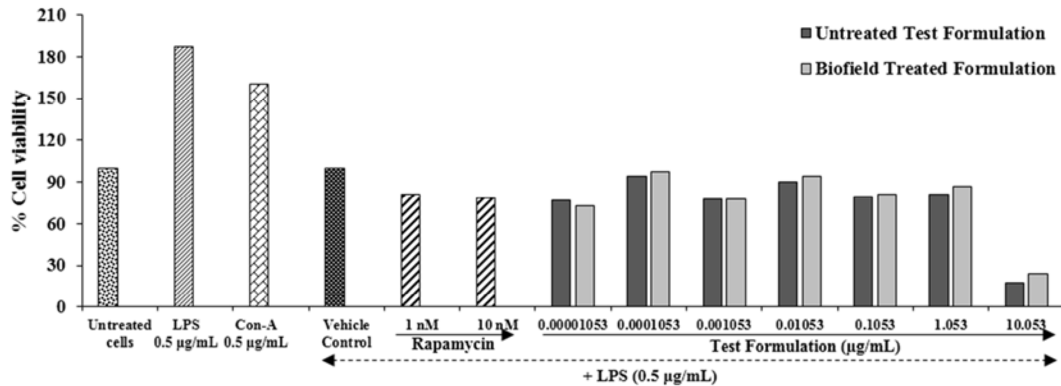

Figure 2. *MTT assay in splenocyte cells (isolated from the Biofield Treated mice) after 48 hours of treatment with different test formulation concentrations in the presence of 0.5 µg/mL LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance ratio (expressed as a percentage) of the test formulation treated cells relative to the untreated vehicle control group.*

MTT assay suggests that the concentrations of the test formulation were found safe up to 1.053 µg/mL with respect to the viability of splenocyte cells. However, the viability percentage was significantly increased after the Biofield Energy Treatment on the test formulation. This cell viability assay defines metabolic activity by evaluating the activity of succinate dehydrogenase, a mitochondrial enzyme. MTT assay is widely used in the *in vitro* evaluation of the cell toxicity for any test formulations, and is regarded as a more rapid, less costly, less time consuming, and non-radioactive method as compared with the other assays. This assay displays cell proliferation results on the basis of cell growth and metabolic activity [38].

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

The effect of the test formulation was evaluated for pro-inflammatory cytokines and chemokines levels in the Biofield Treated splenocyte cells. Pro-inflammatory cytokines and chemokines play important roles in inflammation, immune modulation, and lymphocyte activation. Therefore, six concentrations were examined for the expression of TNF-α, MIP-1α and IL-1β in the splenocyte cells isolated from the Biofield Treated animals. The effect of the test formulation on pro-inflammatory cytokines was estimated after 48 hours of incubation with the test formulation using ELISA assay.

3.2.1. Estimation of TNF-α Expression

The results of TNF-α expression in the splenocyte cells of the Biofield Treated mice with respect to the test formulation are represented in Figure 3. At all the tested concentrations, both the untreated and Biofield Energy Treated formulation groups showed altered expressions of TNF-α. The untreated cells, LPS, Con-A, and vehicle control groups showed values of TNF-α as 100.14, 191.24, 255.60, and 208.19 pg/mL, respectively. At two different concentrations *i.e.* at 1 and 10 nM, the rapamycin group showed TNF-α expression as 158.48 and 184.91 pg/mL, respectively.

However, the Biofield Energy Treated test formulation showed increases in the expression of TNF-α at five concentrations as compared with the untreated formulation. The significant alterations ($p \leq 0.01$) were reported in the Biofield Energy Treated formulation at concentrations 0.01053 and 1.053 µg/mL, and the levels were increased by 53.67% and 25.62%, respectively in comparison to the untreated test formulation. At concentration 0.1053 µg/mL, TNF-α was found to be suppressed by 16.72% in the Biofield Energy Treated formulation as compared with the untreated test formulation. On the other hand, the decreased expression of TNF-α in the untreated formulation group with respect to the vehicle control was reported at 0.00001053, 0.001053, 0.01053, and 1.053 µg/mL by 7.18%, 19.05%, 6.90%, and 19.18%, respectively. The Biofield Energy Treated formulation group showed suppression of TNF-α expression by 4.0% and 8.56% at 0.001053 and 0.1053 µg/mL, respectively as compared with the vehicle control. Overall, the test formulation showed immunosuppressive effect by inhibiting the concentration of TNF-α as compared with the vehicle control at four out of six tested concentrations. The Biofield Energy Treatment showed significant effect in altering the level of TNF-α as compared to the untreated test formulation.

Overall, it can be concluded that the Biofield Energy

Healing Treatment potentiated the TNF-α inhibition, and showed immunosuppressive activity mainly at higher concentrations. TNF-α plays a major role in immune disorders and is also defined as a controlling factor for many other diseases [39]. Thus, it can be suggested that the Biofield Treated test formulation can be used in many inflammatory disorders and autoimmune diseases.

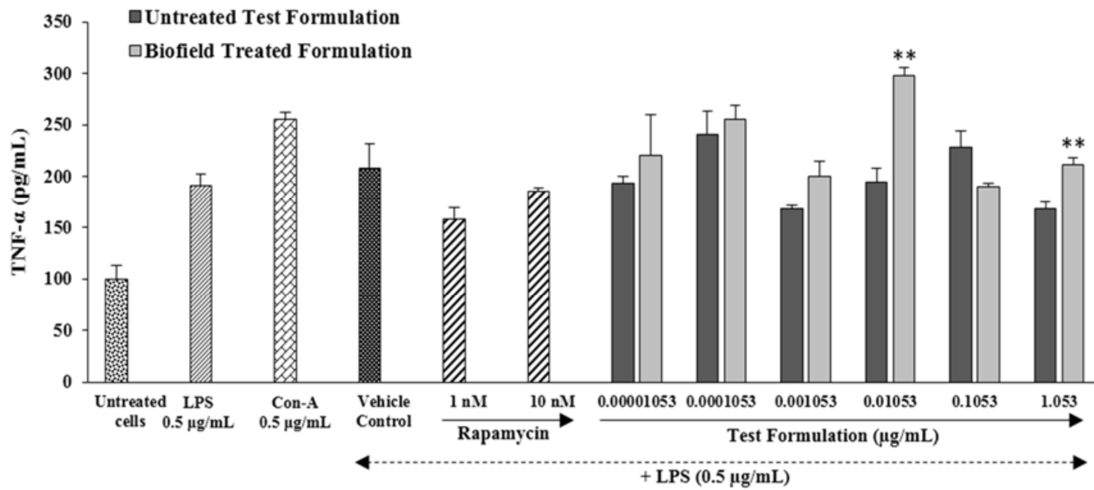

Figure 3. *Concentration-dependent effect on TNF-α by test formulation using splenocyte cells isolated from the Biofield Treated animals. For each concentration treatment, the level of TNF-α release was measured after 48-hours of treatment. All values are represented in pg/mL as mean ± SEM (**$p \leq 0.01$ as compared with the untreated test formulation).*

3.2.2. Estimation of MIP-1α Expression

The expression of MIP-1α on splenocyte cells isolated from the Biofield Energy Treated animals after exposure to the test formulation is shown in Figure 4. The results showed that MIP-1α secretion was inhibited in the presence of the test formulation as compared with the vehicle control group. However, the comparative effect of the test formulation on MIP-1α secretion in splenocyte cells showed significant alteration at all the tested concentrations. The untreated cells, LPS, Con-A, and vehicle control group showed values of MIP-1α as 88.32 ± 9.5, 988.32 ± 52.16, 337.93 ± 40.90, and 1548.21 ± 54.73 pg/mL, respectively. However, the rapamycin at two different concentrations *i.e.* at 1 and 10 nM showed MIP-1α expression as 1083.43 and 1168.78 pg/mL, respectively. The untreated test formulation showed significant inhibition of MIP-1α secretion at 5 tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.1053, and 1.053 µg/mL by 9.89%, 9.89%, 15.69%, 2.94%, and 36.21%, respectively as compared to the vehicle control group. However, the Biofield Energy Treated test formulation showed suppression of MIP-1α in all the tested concentration *i.e.* 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053 and 1.053 µg/mL by 8.43%, 22.02%, 21.92%, 20.54%, 5.40%, and 19.82%, respectively as compared with the vehicle control group. The Biofield Energy Healing Treatment enhanced the immunosuppressive property of the test formulation in comparison with the untreated test formulation. Data suggest that the Biofield Energy Treated test formulation showed significant suppression of MIP-1α in 4 tested concentrations out of 6, *i.e.* 0.0001053, 0.001053, 0.01053, and 0.1053 µg/mL by 13.50%, 7.38%, 36.83% ($p \leq 0.001$), and 2.53%, respectively as compared with the untreated test formulation.

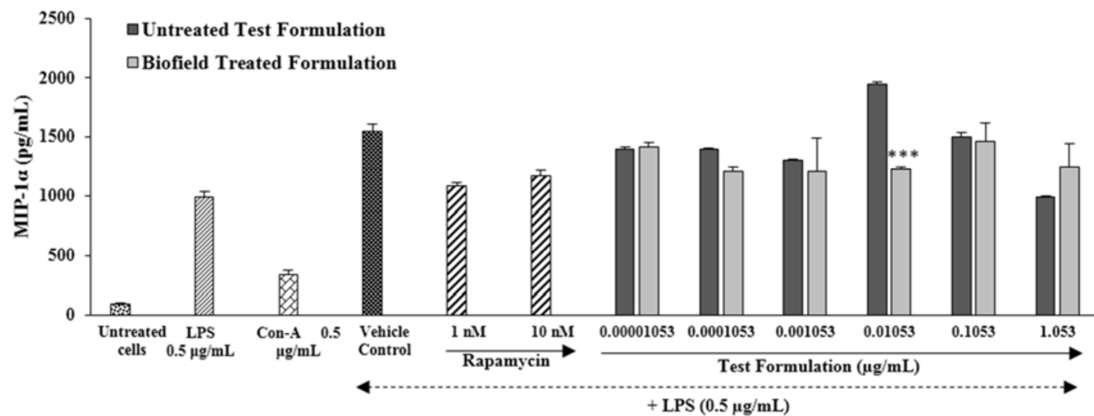

Figure 4. *Concentration-dependent inhibition of LPS mediated production of MIP-1α by the test formulation. For each concentration treatment, the levels of MIP-1α were measured after 48 hours of treatment. The values are represented in pg/mL as mean ± SEM (***$p \leq 0.001$ as compared with the untreated test formulation).*

This suggests that the level of MIP-1α was inhibited at all the Biofield Energy Treated test formulation concentrations with respect to the vehicle control and showed significant inhibition up to 36.83% with respect to the untreated test formulation. The scientific reports suggest that the reduction of MIP-1α would be beneficial in minimizing the inflammatory responses in several diseases [40].

3.2.3. Estimation of IL-1β Expression

The expression of IL-1β in the presence of the test formulation is presented in Figure 5. The results demonstrated the inhibition of IL-1β after treatment with the Biofield Treated and untreated test formulations as compared with the vehicle control group. However, the comparative effect of the Biofield Treated and untreated test formulations on IL-1β secretion in the Biofield Treated splenocytes showed significant inhibition at 4 out of 6 tested concentrations. The untreated cells, LPS, Con-A, and vehicle control group showed values of IL-1β as 8.18 ± 0.89, 51.65 ± 4.04, 15.40 ± 2.75, and 38.23 ± 3.5 pg/mL, respectively.

The untreated test formulation showed significant inhibition of IL-1β secretion in four tested concentrations *i.e.* at 0.00001053, 0.001053, 0.1053, and 1.053 µg/mL by 22.94%, 23.07%, 38.71%, and 56.08%, respectively as compared with the vehicle control group. However, the Biofield Energy Treated test formulation group reported with inhibition of IL-1β secretion at four tested concentrations by 22.94%, 23.07%, 38.71%, and 54.65% at 0.00001053, 0.001053, 0.1053, and 1.053 µg/mL, respectively as compared with the vehicle control group. The comparative results suggest that the Biofield Energy Treatment significantly improved the immunosuppressive property of the test formulation at four concentrations as compared to the untreated test formulation. The significant inhibition of IL-1β secretion after the Biofield Treatment was reported at concentrations 0.0001053, 0.001053, 0.01053, and 0.01053 µg/mL by 32.40%, 14.99%, 60.42%, and 15.15%, respectively as compared with the untreated formulation.

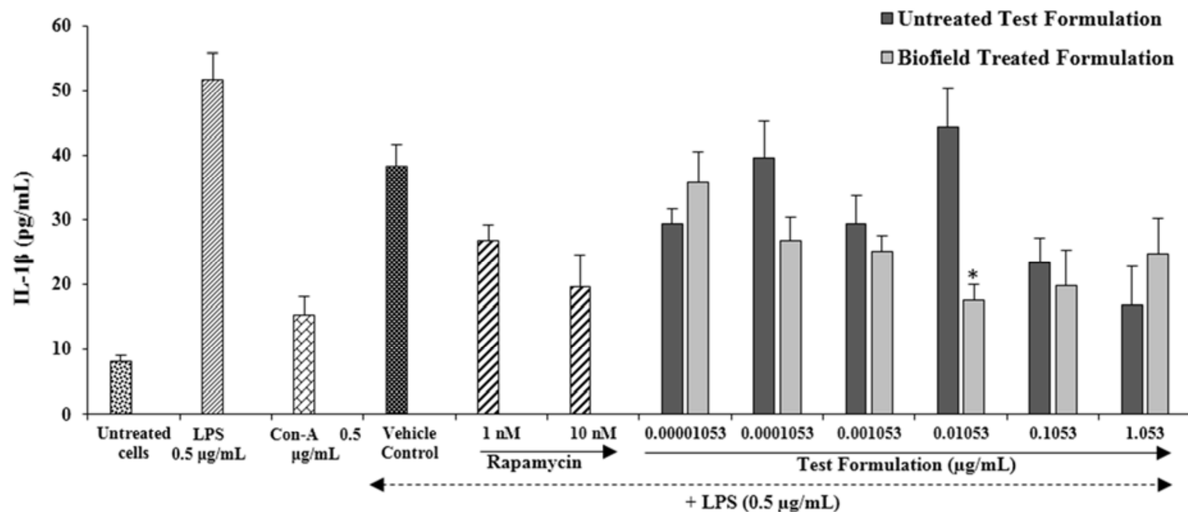

Figure 5. *Concentration-dependent effect of LPS mediated production of IL-1β by the test formulation. For each concentration treatment, the level of IL-1β release was measured in cell supernatant after 48-hours of treatment. All values are represented in pg/mL as mean ± SEM (*p≤0.05, as compared with the untreated test formulation).*

Overall, the results suggest that better immunosuppressive activity was reported at higher concentrations as compared to lower concentrations of the test formulation. The expression of IL-1β was decreased in the Biofield Energy Treated formulation at all the concentrations, except at concentration 0.00001053 µg/mL. The immunological and inflammatory functions of IL-1β in controlling the immune response during infections are well-defined [41, 42]. 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.

Herbomineral products have been reported to have beneficial results with minimal side effects in various diseases such as diabetes, indigestion, inflammation of the intestine, osteomalacia, blood disorders, infertility, potent revitalizer, etc. [43]. Due to its high safety profile and therapeutic effect, the scope of herbal and alternative and complimentary medicine has vastly increased worldwide [44]. Each of the individual components comprising the test formulation have been scientifically reported to have immunomodulatory effects. Ashwagandha is reported to inhibit the NF-κB and AP-1 transcription factors [45]. Minerals such as zinc directly influence the cytokines (IL-2, IL-6, IL-1β, and TNF-α) generation, in cases of deficiency [46]. Magnesium also affects the cytokines generation by activation of *NF-κB*, which has proven to be effective in inflammatory diseases [47]. Selenium plays an important role in inflammation by modulating the leukocytes effector functions like cytokines secretion, migration, adherence, and phagocytosis [48-50]. When applied to herbomineral formulations, Biofield Energy Healing Treatments can be a novel approach for immunosuppressive action. Overall, the

effect of the Biofield Energy Treated herbomineral formulation showed immunosuppressive effect on the level of tested cytokines and chemokines (TNF-α, IL-1β, and MIP-1α) in splenocyte cells isolated from the Biofield Energy Treated mice, which supports the use of Biofield Energy Treated test formulation for various types of autoimmune disorders.

4. Conclusions

Based on the current findings of this study, the splenocyte cells isolated from the Biofield Energy Treated mice showed significant immunosuppressive effect on the tested cytokines (TNF-α, MIP-1α, and IL-1β) after administration of the Biofield Treated test formulation as compared with the untreated test formulation. MTT assay in the Biofield Treated splenocyte cells suggest that the Biofield Energy Treated formulation increased the cell viability by 3.17%, 5.07%, 2.77%, and 6.61% at 0.0001053, 0.01053, 0.1053, and 1.053 µg/mL, respectively as compared with the untreated test formulation. Further, the levels of cytokines were significantly suppressed in the Biofield Energy Treated test formulation group. TNF-α level was significantly inhibited by 16.72% at 0.1053 µg/mL in the Biofield Treated test formulation as compared with the untreated test formulation. However, the Biofield Energy Treated formulation group also showed low levels of TNF-α expression by 4.0% and 8.56% at 0.001053 and 0.1053 µg/mL, respectively as compared with the vehicle control group. In the case of MIP-1α, the Biofield Energy Treated test formulation showed significant suppression by 13.50%, 7.38%, 36.83% ($p \le 0.001$), and 2.53% at 0.0001053, 0.001053, 0.01053, and 0.1053 µg/mL, respectively as compared with the untreated test formulation. Besides, the levels of MIP-1α were also downregulated in all the tested concentrations of the test formulation by 8.43%, 22.02%, 21.92%, 20.54%, 5.40%, and 19.82% at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053 and 1.053 µg/mL, respectively as compared with the vehicle control group. Suppression of IL-1β expression was also reported in the Biofield Energy Treated test formulation group by 32.40%, 14.99%, 60.42% ($p \le 0.05$), and 15.15% at 0.0001053, 0.001053, 0.01053, and 0.01053 µg/mL, respectively as compared with the untreated formulation.

On the basis of the experimental results of the various tested cytokines and their expression, significant immunosuppressive activity was reported in the new herbomineral formulation after treatment with The Trivedi Effect® - Biofield Energy Healing (TEBEH) by the group of seven renowned Biofield Energy Healers. The Biofield Energy Treated test formulation can be used as an effective Complementary and Alternative Medicine (CAM) approach to prevent and treat immune-mediated diseases such as Irritable Bowel Syndrome, Rheumatoid arthritis, Ulcerative colitis and Crohn's disease, Stress, Asthma, and many more, with a safe therapeutic index. Biofield Energy Healing Treatments can also be utilized in 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, Sjogren Syndrome, Systemic Lupus Erythematosus, Alopecia Areata, Fibromyalgia, Vitiligo, Psoriasis, Scleroderma, Chronic Fatigue Syndrome, Vasculitis, and Type 1 Diabetes. Biofield Energy Healing can also be utilized for the anti-inflammatory disorders, stress prevention and management, anti-aging, and for 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; NCCIH: National Center of Complementary and Integrative Health; CAM: Complementary and Alternative Medicine

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