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

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Abstract: Due to the increased popularity of herbomineral preparations in the healthcare sector, a new proprietary herbomineral formulation was formulated consisting of ashwagandha root extract and three minerals *viz.* zinc chloride, magnesium gluconate, and sodium selenate. The objective of the study was to evaluate the *in vitro* effect of Biofield Energy Healing (The Trivedi Effect®) on the test formulation using murine splenocyte cells. The herbomineral formulation was divided into two parts; one defined as the control, while the other part was treated with the Biofield Energy Healing Treatment performed from a remote distance by twenty renowned Biofield Energy Healers (The Trivedi Effect®) and defined as the Biofield Treated formulation. The splenocyte cells were exposed to test formulations at concentration from 0.00001053 to 10.53 µg/mL and were analyzed after 48 hours for cell viability using MTT assay. The expression of the cytokines (TNF-α, IFN-γ, IL-1β, and MIP-1α) was determined using ELISA assay. The cell viability data showed that all the tested concentration ranges were found to be safe with percentage cell viability at more than 80%. Further, TNF-α expression was significantly inhibited in the Biofield Treated test formulation group with respect to the vehicle control, while at 0.001053 and 0.1053 µg/mL, the expression was suppressed by 1.70% and 8.16%, respectively in the Biofield Treated test formulation compared to the untreated formulation. However, a significant immunosuppression was reported in IFN-γ expression at 0.00001053, 0.0001053, 0.01053, 0.1053, and 1.053 µg/mL by 12.63%, 2.31%, 8.31%, 9.15%, and 7.86%, respectively in the Biofield Treated test formulation compared with the untreated test formulation. The MIP-1α expression was inhibited by 8.31%, 21.53%, and 8.70% at 0.0001053, 0.01053, and 0.1053 µg/mL, respectively in the Biofield Treated formulation compared with the untreated test formulation. However, IL-1β expression was significantly suppressed by 19.72% at concentration 0.00001053 µg/mL in the Biofield Treated test formulation compared with the untreated test formulation. Thus, the down-regulation of tested cytokines and chemokines in the Biofield Energy Healing test formulation might be applicable for controlling acute and chronic inflammation in many clinical diseases. Overall, the results demonstrated that The Trivedi Effect®- Biofield Energy Healing (TEBEH) has the capacity to potentiate the immunomodulatory activity of the test formulation, which can be useful against autoimmune disorders. Biofield Treated Test formulation may also be

useful in anti-aging, anti-inflammatory, stress management and in preventing immune-mediated tissue damage in organ transplants by improving overall health and quality of life.

Keywords: Biofield Energy Healing Treatment, The Trivedi Effect®, Herbomineral Formulation, Immune-Modulation, Pro-inflammatory Cytokines, Splenocytes

1. Introduction

Herbomineral formulations have always been a major target of scientific research for significant immunomodulatory potential. The healing properties of plants and their 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-modulatory, anti-inflammatory, antioxidant, anti-infective, and anti-viral properties [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.

According to the scientific studies and clinical trials, Biofield Energy Treatment has been reported to have significant outcome in terms of enhanced immune function of cervical cancer patients with therapeutic touch [12], massage therapy [13], etc. The National Center of Complementary and Integrative Health (NCCIH) has recognized and accepted Biofield Energy Healing as a complementary and alternative medicine (CAM) health care approach in addition to other therapies, medicines and practices such as Tai Chi, yoga, deep breathing, natural products, Qi Gong, massage, chiropractic/osteopathic manipulation, acupuncture, acupressure, meditation, mindfulness, healing touch, special diets, naturopathy, progressive relaxation, homeopathy, guided imagery, relaxation techniques, hypnotherapy, movement therapy, pilates, rolfing structural integration, Ayurvedic medicine, traditional Chinese herbs and medicines, aromatherapy, essential oils, cranial sacral therapy, applied prayer (as is common in all religions, like Buddhism, Hinduism, Christianity and Judaism), and Reiki. To this day, Biofield Energy Healing has had significant impact in the transformation of living organisms and nonliving materials including metals, ceramics, polymers, chemicals, and pharmaceutical compounds. Human Biofield Energy has subtle energy that has the capacity to work in an effective manner [14]. Reports showed 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 science [16, 17], altering microbial characteristics and features including changing the microbial sensitivity of pathogenic microbes in microbiology [18-21], genetics [22, 23], altered physical and chemical compounds in pharmaceutics [24-27], improved the overall productivity, quality and yield of crops and plants in agricultural science [28-31], and in materials science where The Trivedi Effect® has demonstrated its ability to alter the structural, thermal and physical properties of metals, polymers, chemicals and ceramics [32-35].

The authors of this study sought 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 model on mice splenocyte cells.

2. Materials and Methods

2.1. Chemicals and Reagents

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. 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. All other chemicals used were of analytical grade available in India.

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. The mice were maintained under controlled conditions with a temperature of 22 ± 3°C, humidity of 30% to 70% and a 12-hours light/12-hours dark cycle and laboratory rodent diet and drinking tap water were provided *ad libitum*. All the procedures were in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH). The approval of the Institutional Animal Ethics Committee (IAEC) was obtained prior to carrying out the animal experiment.

2.4. Biofield Energy Healing Strategies

The herbomineral formulation was divided into two parts. One part of the test formulation did not receive any sort of treatment and was defined as the control group, while the Biofield Energy Treatment was given to the herbomineral formulation defined as the treated formulation group. The Biofield Energy Treatment was provided through a group of twenty Biofield Energy Healers (The Trivedi Effect®), eighteen of which were remotely located in the U.S.A. and two of which were remotely located in Canada, while the test formulation was located in the research laboratory of Dabur Research Foundation near New Delhi in Ghaziabad, India. This Biofield Treatment was administered for 5 minutes through the Healers' unique Energy Transmission process remotely to the test formulation under standard laboratory conditions. None of the Biofield Energy Healers in this study visited the laboratory in person, nor had any contact with the herbomineral samples. Further, the control group was treated by a "sham" healer for comparative purposes. The sham healer did not have any knowledge about the Biofield Energy Treatment. After that, the Biofield Energy treated and untreated test formulations were kept in similar sealed conditions and used for the *in vitro* study on splenocyte cells for cytokines estimation.

2.5. Experimental Design

The experimental study was divided into 7 groups. Group 1 comprised of the splenocyte cells without LPS and was denoted as the negative control. Group 2 served as a stimulant group that includes cells with LPS. Group 3 included the splenocyte cells with LPS along with vehicle (0.005% DMSO) and was denoted as the vehicle control. Groups 4 and 5 were defined as the positive control, which includes cells with Con-A (0.5 µg/mL) and rapamycin (1 nm and 10 nm), respectively. Groups 6 and 7 were denoted as the test item groups that included splenocyte cells with LPS along with the untreated and Biofield Treated formulations, respectively, at concentration 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 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 them through a sterile plastic strainer under aseptic conditions. After the cells were centrifuged twice at 1000 *g* for 5 minutes, erythrocytes were lysed by a lysis buffer (0.15 M NH_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 re-suspended 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 ≥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 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 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 Biofield Treated and untreated formulations at the concentration range of 0.00001053 µg/mL to 10.53 µg/mL were tested for cell viability 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 were cultured overnight in 96-well plates, at a density of 0.2 x 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 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 in 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, IFN-γ, and IL-1β) and Chemokine (MIP-1α) Using ELISA

The *in vitro* activity of the Biofield Treated and untreated test formulations were estimated on the mice splenocyte cells for the production of TNF-α, IFN-γ, 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 4ºC. 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 4ºC and, after three washes, biotinylated anti-mice cytokine (TNF-α, IFN-γ, MIP-1α, and IL-1β) 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 µL of horseradish per-oxidase (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 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 ± standard error of mean (SEM) and were subjected to one-way analysis of variance (ANOVA) followed by Dunnett's test and Student's *t*-test for two groups comparison. Statistical significance was considered at $p \leq 0.05$.

3. Results and Discussion

3.1. In Vitro Splenocyte Cells Viability by MTT Assay

In vitro splenocyte cells viability was performed after 48 hours using MTT assay, and the results were presented in Figure 1 with respect to the positive control, vehicle control, and the test formulation at different tested concentrations. The results showed a significant change in percentage of cell viability after the Biofield Energy Treatment in the tested concentrations of the formulation. Con-A and rapamycin showed immunostimulatory and immunosuppressive action, respectively, and used as the positive control in the experiment. The untreated cells, LPS, and Con-A group showed 100%, 187.7%, and 94.9% cell viability, respectively. The vehicle control group reported with 100% cell viability and the rapamycin group showed values of 136.8%, 132.3%, 136.5%, and 120.5% at concentrations 0.01, 0.1, 1 and 10 nM, respectively. With respect to the vehicle control, the percentage of cell viability was increased, which might be due to proliferation in the cell culture. The tested concentration range of the herbomineral test formulation was selected as 0.00001053 to 10.53 µg/mL on splenocyte cells. The test formulation was found safe at all the tested concentrations, with percentage viability ranging from 88.6% to 187.2%. Based upon this result, all the tested concentrations of the herbomineral formulation were selected for the estimation of cytokines. The maximum cell viability in cases of the untreated and treated test formulations was reported as 187.2% (at 0.1053 µg/mL) and 152% (at 0.0001052 µg/mL), respectively. However, the percentage of cell viability in the Biofield Treated test formulation was increased by 29%, 52%, 13.5%, and 13.7% at concentrations 0.00001053, 0.0001053, 0.001053, and 1.053 µg/mL, respectively in comparison to the vehicle control group. Overall, it can be concluded that the Biofield Treated test formulation showed increased cell viability with respect to the vehicle control group.

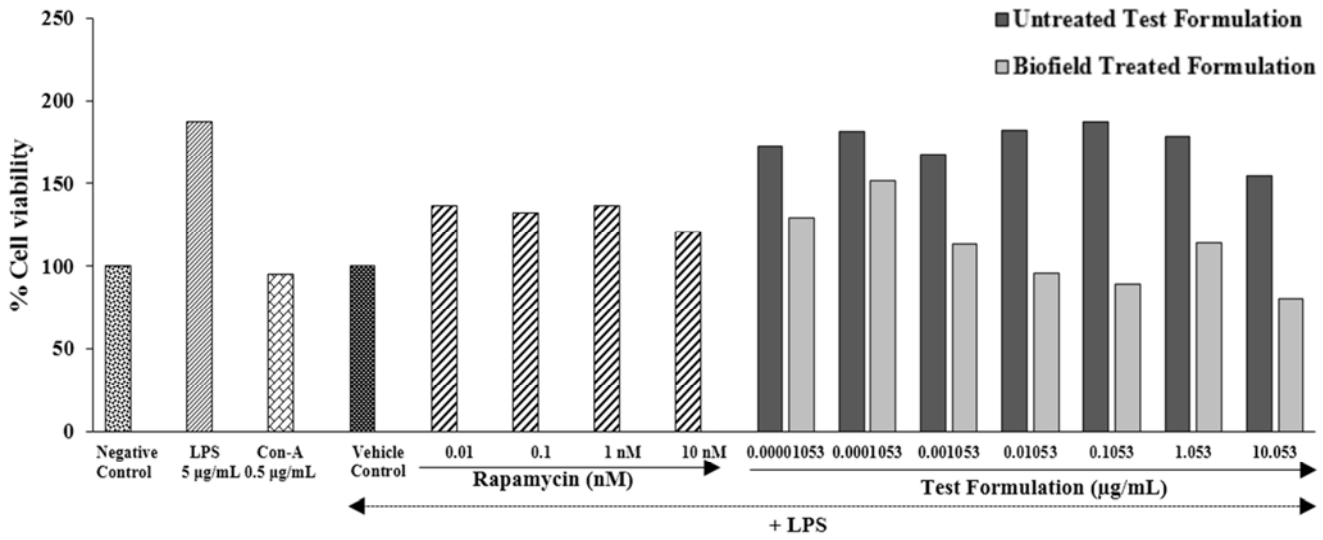

Figure 1. *MTT assay in mice splenocyte cells after 48-hours of treatment with different Biofield Treated and untreated test formulation concentrations in the presence of 0.5 µg/mL LPS. The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader. Cell viability was defined as the absorbance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

Overall, the results of MTT assay recommend that the test formulation was safe at all the tested concentration ranges (*i.e.* from 0.00001053 to 10.53 µg/mL) on the basis of percentage *in vitro* viability of splenocyte cells. With respect to the vehicle control, all the tested herbomineral formulation groups showed increased cell viability. This assay defines the metabolic activity by evaluating the activity of succinate dehydrogenase, a mitochondrial enzyme.

However, the percentage of cell viability was significantly increased after the Biofield Energy Treatment was provided to the test formulation. MTT assay was regarded as the standard test for evaluating cell viability [38]. This assay is widely used in the *in vitro* evaluation of the cell toxicity for any formulations and is regarded as a more rapid, less costly, less time-consuming, and nonradioactive method as compared with the other assays. This assay shows cell proliferation results on the basis of cell growth and metabolic activity [39].

3.2. Effect of Biofield Treated Formulation on the Expression of Pro-inflammatory Cytokines (TNF-α, IFN-γ, and IL-1β) and Chemokine (MIP-1α)

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

3.2.1. Estimation of TNF-α Expression

The cytokine analysis on TNF-α secretion in splenocyte cells in the presence of the Biofield Treated and untreated test formulations are represented in the Figure 2. Data suggested that both the untreated and Biofield Treated test formulation groups demonstrated a significant suppression of TNF-α secretion at different tested concentrations *i.e.* at 0.00001053 to 10.53 µg/mL. The negative control (untreated cells), LPS, Con-A, and vehicle control groups showed TNF-α values as 74.04 ± 5.40, 154.49 ± 3.06, 381.09 ± 36.24, and 323.08 ± 10.60 pg/mL, respectively. However, the untreated test formulation demonstrated a significant suppression of TNF-α from LPS stimulated levels at all the tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53 µg/mL by 23.51%, 22.12%, 12.80%, 13.69%, 22.22%, 24.31%, and 30.46%, respectively as compared with the vehicle control. Further, the Biofield Treated test formulation also showed significant inhibition of TNF-α at all concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53 µg/mL by 16.87%, 17.36%, 14.29%, 10.62%, 28.57%, 20.84%, and 24.90%, respectively as compared to the vehicle control group. In addition, at two tested concentrations *i.e.* at 0.001053 and 0.1053 µg/mL, the Biofield Treated test formulation showed suppression by 1.70% and 8.16%, respectively as compared with the untreated test formulation. On the other hand, the Biofield Treated test formulation demonstrated an increase in TNF-α levels at five tested concentrations *i.e.* 0.00001053, 0.0001053, 0.01053, 1.053, and 10.53 µg/mL by 8.69%, 6.12%, 3.56%, 4.59%, and 7.98%, respectively as compared to the untreated test formulation.

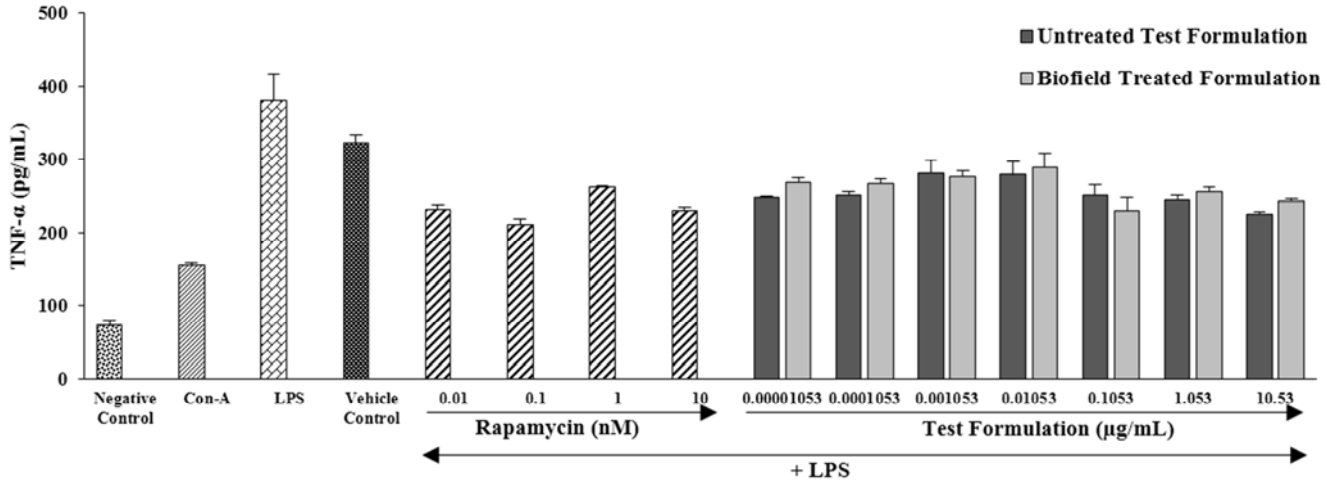

Figure 2. *Concentration-dependent effect on TNF-α by the Biofield Treated and untreated test formulations. For each concentration treatment, the level of TNF-α release was measured after 48 hours of treatment. All the values are represented in pg/mL as mean ± SEM.*

Overall, it can be suggested that the Biofield Treated formulation has significant immunosuppressive activity by inhibiting the concentration of TNF-α as compared with the vehicle control, while the Biofield Treatment has also shown an alteration in the concentration of TNF-α as compared with the untreated formulation. The Biofield Treatment showed significant effect on altering the level of TNF-α as compared to the untreated test formulation. For most immune disorders, TNF-α is the major factor that controls many disease pathologies [40, 41]. The role of TNF-α and its alterations have been significantly reported to improve insulin resistance, lipid profiles, etc. in patients' chronic inflammatory diseases [42]. So, it can be suggested that the Biofield Treated test formulation can be used in many inflammatory disorders by controlling the expression of TNF-α.

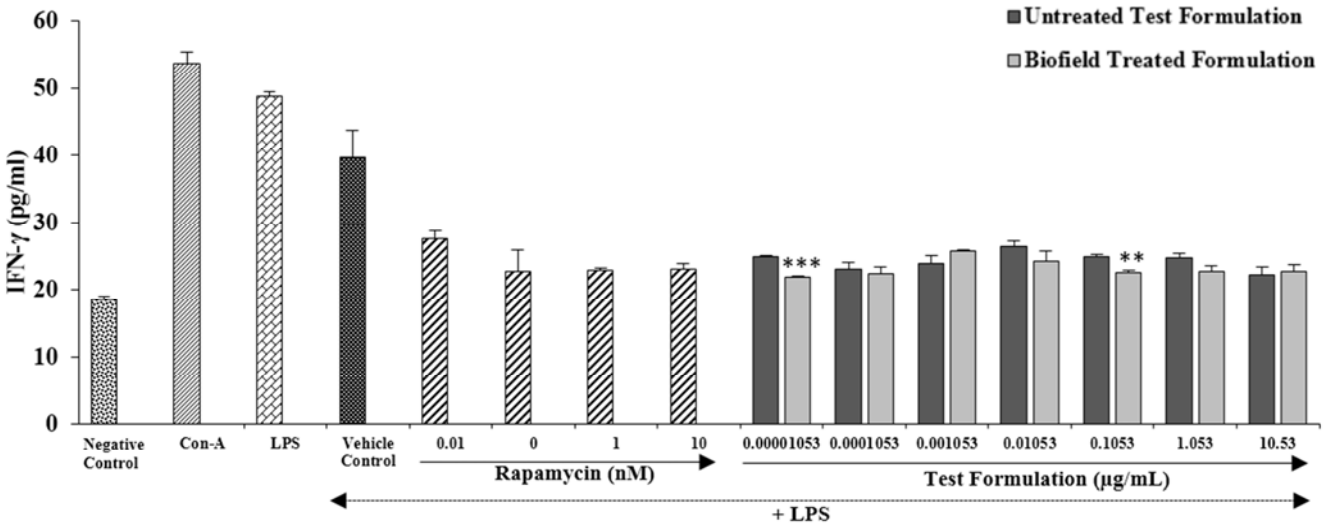

Figure 3. *Concentration-dependent effect of LPS mediated production of IFN-γ by the Biofield Treated formulation. For each concentration treatment, the level of IFN-γ release was measured after 48-hours of treatment. The values are represented in pg/mL as mean ± SEM (*** $p \leq 0.001$ and ** $p \leq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

3.2.2. Estimation of IFN-γ Expression

Estimation of IFN-γ expression in mice splenocyte cells after treatment with the Biofield Treated and untreated test formulations are represented in Figure 3. The results show that in the test formulation groups there was significant inhibition of IFN-γ expression as compared with the vehicle control group, while the Biofield Treated test formulation further enhanced the immunosuppression at most of the concentrations. The negative control (untreated cells), Con-A, LPS, and vehicle control group showed IFN-γ values as 18.47 ± 0.44, 53.73 ± 1.73, 48.80 ± 0.64, and 39.67 ± 4.04 pg/mL, respectively. However, the untreated test formulation demonstrated significant suppression of IFN-γ from LPS stimulated levels at all the tested formulation concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053 and 10.53 µg/mL by 37.66%, 42.20%, 40.01%, 33.28%, 37.48%, 37.81%, and 44.21%, respectively as compared with the vehicle control. Further, the Biofield Treated test formulation also showed significant inhibition of IFN-γ at all concentrations *i.e.* at 0.00001053, 0.0001053,

0.001053, 0.01053, 0.1053, 1.053 and 10.53 µg/mL by 45.22%, 43.53%, 35.14%, 38.82%, 43.21%, 42.70%, and 42.85%, respectively as compared to the vehicle control group. At five tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.01053, 0.1053, and 1.053 µg/mL, the Biofield Treated test formulation showed further suppression of IFN-γ by 12.63%, 2.31%, 8.31%, 9.15%, and 7.86% as compared with the untreated test formulation. However, the Biofield Treated test formulation demonstrated increase in IFN-γ levels at two tested formulation concentrations *i.e.* 0.001053 and 10.53 µg/mL by 8.11% and 2.44%, respectively as compared to the untreated test formulation.

Various literature suggests that IFN-γ expression plays a key role in the regulation of visceral adipose tissue inflammatory response [43], inflammation, and glucose homeostasis [44], as well as in the inhibition of the inflammatory response of macrophages cells (in IFN-γ deficiency condition) [45] and many other important inflammatory disorders. So, it can be concluded that the Biofield Treated test formulation would be a better alternative and complementary herbomineral supplement with respect to inflammatory disorders.

3.2.3. Estimation of MIP-1α Expression

The effect of the Biofield Treated test formulation on MIP-1α secretion levels is shown in Figure 4. The figure demonstrates that the Biofield Treated and untreated test formulations inhibit the expression of MIP-1α in all the tested concentrations as compared with the vehicle control group. The untreated cells, Con-A, LPS, and vehicle control group showed values of MIP-1α as 32.84 ± 7.32, 1639.71 ± 15.10, 1374.02 ± 15.71, and 1167.65 ± 16.32 pg/mL, respectively. The untreated test formulation showed significant inhibition of MIP-1α secretion at six tested concentrations out of seven *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.1053, 1.053, and 10.53 µg/mL by 7.45%, 1.51%, 11.84%, 5.92%, 17.73%, and 14.74%, respectively as compared to the vehicle control group. However, the Biofield Treated test formulation group reported inhibition of MIP-1α secretion at 0.00001053, 0.0001053, 0.01053, 0.1053, 1.053, and 10.53 µg/mL by 6.59%, 9.69%, 13.22%, 14.11%, 7.13%, and 9.78%, respectively as compared with the vehicle control group. The Biofield Treatment further enhanced the immunosuppressive property of the test formulation at three tested concentrations *i.e.* at 0.0001053, 0.01053, and 0.1053 by 8.31%, 21.53%, and 8.70%, respectively as compared with the untreated test formulation. The rest of the other Biofield Treated test formulation concentrations showed increased levels of MIP-1α as compared with the untreated test formulation. The comparative effect of the Biofield Treated and untreated test formulation showed altered levels of MIP-1α in splenocyte cells at all the tested concentrations.

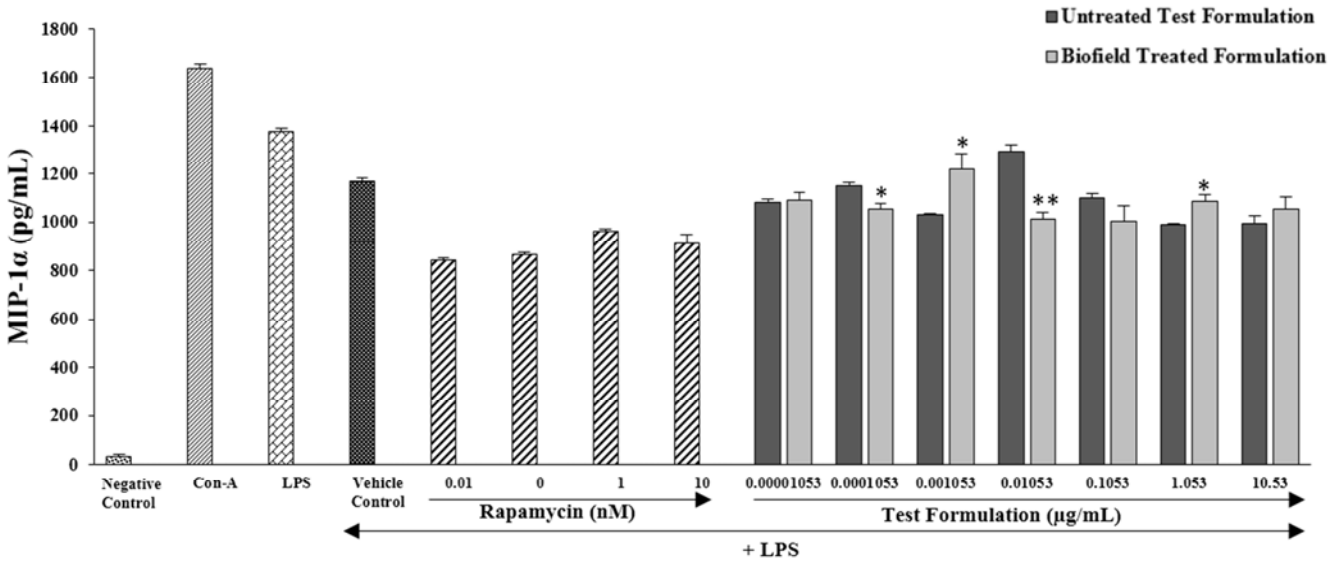

Figure 4. *Concentration-dependent effect of LPS mediated production of MIP-1α by the Biofield Treated test formulation. For each concentration treatment, the level of MIP-1α release was measured after 48-hours of treatment. The values are represented in pg/mL as mean ± SEM (**p≤0.01 and *p≤0.05, as compared with the untreated test formulation).*

Literature reports suggest that MIP-1α reduction could be beneficial in minimizing the inflammatory responses in several diseases [46]. Additionally, MIP-1α also plays an important role in mediating the acute inflammatory response in trauma hemorrhages [47]. Overall, in respect to the suppression of MIP-1α in the Biofield Treated test formulation group, the data suggest the importance of the Biofield Treated formulation in many clinical conditions.

3.2.4. Estimation of IL-1β Expression

The expression of IL-1β in mice splenocytes in the presence of the Biofield Treated test formulation and the untreated test formulation is demonstrated in Figure 5. The comparative effect of the Biofield Treated and untreated test formulations on IL-1β secretion in splenocyte cells showed significant inhibition at all the tested concentrations with respect to the vehicle control. The untreated cells, Con-A,

LPS, and vehicle control group showed values of IL-1β as 19.76 ± 1.13, 41.30 ± 1.06, 33.50 ± 1.55, and 35.37 ± 3.94 pg/mL, respectively. The untreated test formulation showed significant inhibition of IL-1β secretion at all the tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53 µg/mL by 6.67%, 15.63%, 25.75%, 25.75%, 37.23%, 26.91%, and 34.94%, respectively as compared with the vehicle control group. The Biofield Treated formulation also showed significant inhibition of IL-1β secretion at all the tested concentrations *i.e.* at 0.00001053, 0.0001053, 0.001053, 0.01053, 0.1053, 1.053, and 10.53 µg/mL by 25.07%, 7.38%, 8.06%, 18.86%, 17.25%, 22.53%, and 31.34%, respectively as compared with the vehicle control group. However, at a lower concentration (0.00001053 µg/mL), the Biofield Treatment formulation further improved the immunosuppressive property and showed significantly decreased IL-1β secretion by 19.72% as compared with the untreated test formulation, while with the rest of the tested concentrations, the percentage was increased with respect to the untreated test formulation in all the concentrations of the test formulation.

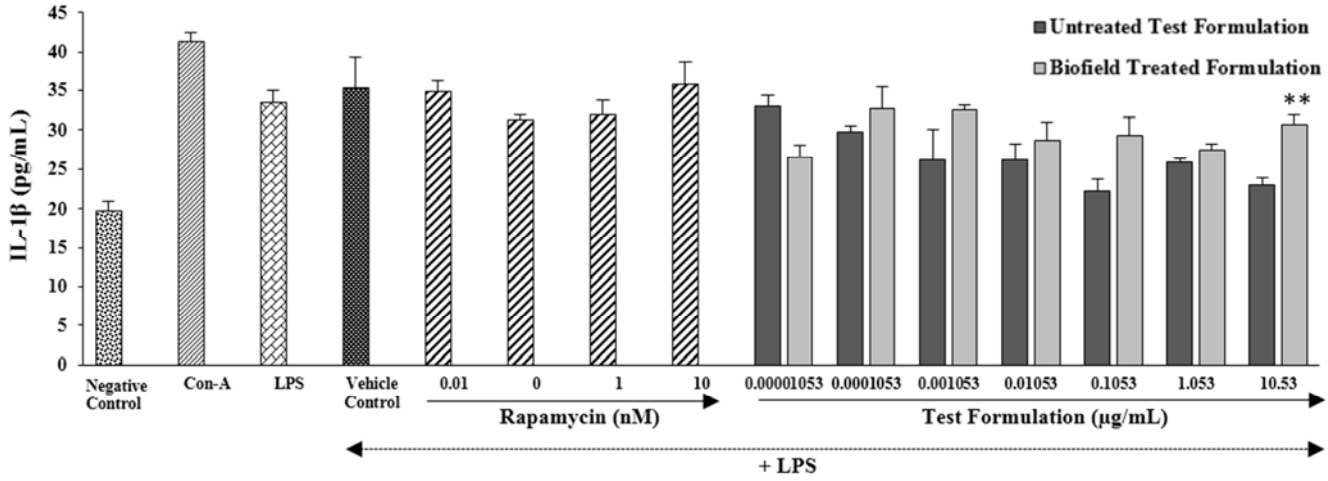

Figure 5. *Concentration-dependent effect of LPS mediated production of IL-1β by the Biofield Treated formulation. For each concentration treatment, the level of IL-1β release was measured in cell supernatant after 48 hours of treatment. All values are represented in pg/mL as mean ± SEM (**p≤0.01, as compared with the untreated test formulation).*

Overall, the results suggest that higher concentrations of the test formulation showed better immunosuppressive activity with respect to lower tested concentrations of the herbomineral test formulation. The importance of IL-1β expression in immunological and inflammatory functions during infections are well established [48, 49]. The inhibitory effect of the Biofield Treated test formulation might play an important role in mediating autoinflammatory diseases as a Complementary and Alternative Medicine (CAM) approach.

The scope of herbal and traditional medicine has been continuously increasing in developing countries [50], but the Biofield Energy Healing model has shown to be a novel therapeutic intervention approach that must still be incorporated and utilized in conventional as well as complementary and alternative medicine. The herbomineral formulation treated with Biofield Energy Healing showed significant immunomodulatory action and the results suggest that the Biofield Treated formulation can be the best alternative medicine for many inflammatory disorders. The individual components of the test formulation (ashwagandha, zinc, magnesium, and selenium) have already been reported to have immune modulatory properties. Ashwagandha has been reported to regulate the immune system by inhibiting the NF-κB and AP-1 transcription factors in human peripheral blood and synovial fluid mononuclear cells [49]. Further, zinc deficiency plays an important role in cytokines generation, such as IL-2, IL-6, IL-1β, and TNF-α, and acts in a concentration dependent manner by inhibiting the activation of NF-κB [50]. Similarly, magnesium also regulates the immune system through the activation of NF-κB and generation of cytokines, which can be effectively applicable in inflammatory conditions or their related disease pathogenesis [51]. However, selenium regulates various leukocytes effector functions such as cytokines secretion, migration, adherence, and phagocytosis with the help of calcium flux and oxidative pathway in innate immunity [52-54].

Overall, the results suggest that the Biofield Energy Treated test formulation can be used to treat many inflammatory disorders with little-to-no toxicity. Literature reports the significant outcomes of Biofield Energy Healing with respect to cytokines inhibition in cancer cell lines [55]. It might be suggested that the Biofield Treated test formulation can significantly inhibit the T and B lymphocytes, which might be used to improve immune/autoimmune disorders, stress, and asthma.

4. Conclusions

Based on the obtained results, it was concluded that the Biofield Energy Treated test formulation modulates the splenocyte cells function with respect to the pro-inflammatory cytokines TNF-α, IFN-γ, MIP-1α, and IL-1β. However, the Biofield Treated test formulation significantly

inhibited the activity of pro-inflammatory cytokines with respect to the untreated test formulation. *In vitro* cells viability assay showed that all the tested concentrations of the herbomineral formulation were found safe with respect to the vehicle control group. The percentage cell viability ranged from 88.9% to 187.2% in different concentration ranges of the test formulation, so concentration ranges from 0.00001053 to 10.53 µg/mL of the test formulation were selected for the splenocyte cells for the cytokines estimation.

TNF-α levels were significantly inhibited at 0.001053 and 0.1053 µg/mL, in which the Biofield Treated test formulation group showed suppression by 1.70% and 8.16% as compared with the untreated test formulation. Similarly, significant suppression of IFN-γ expression was reported at five concentrations *i.e.* at 0.00001053, 0.0001053, 0.01053, 0.1053, and 1.053 µg/mL of the Biofield Treated test formulation by 12.63%, 2.31%, 8.31%, 9.15%, and 7.86%, respectively as compared with the untreated test formulation. This might help in inflammatory tissue response and can be used in many inflammatory disorders. In the case of MIP-1α secretion, the expression was inhibited by the Biofield Treated test formulation at three tested concentrations *i.e.* at 0.0001053, 0.01053, and 0.1053 µg/mL by 8.31%, 21.53%, and 8.70%, respectively as compared with the untreated test formulation. IL-1β secretion was significantly suppressed only at a very low concentration *i.e.* 0.00001053 µg/mL by 19.72% as compared with the untreated test formulation.

On the basis of the experimental results of the various tested cytokines showed significant immunosuppressive activity in the new herbomineral formulation after treatment with The Trivedi Effect® - Biofield Energy Healing (TEBEH) by the group of twenty renowned Biofield Energy Healers. Overall, The Trivedi Effect® - Biofield Energy Healing Treated test formulation significantly inhibited the activity of pro-inflammatory cytokines and showed immunosuppressive activity with a safe therapeutic index as compared with the untreated test formulation. Biofield Energy Healing shows great promise as a complementary and alternative medicine therapy and might play an important role in autoimmune and inflammatory diseases like Chronic peptic ulcers, Tuberculosis, Hepatitis, Celiac Disease (gluten-sensitive enteropathy), Addison Disease, Irritable Bowel Syndrome, Multiple Sclerosis (MS), Parkinson's Disease, Graves' Disease, Rheumatoid arthritis, Chronic periodontitis, Ulcerative colitis, Crohn's disease, Chronic sinusitis, Lupus, Systemic Lupus Erythematosus, Hashimoto Thyroiditis Type 1 Diabetes, Asthma, Chronic active hepatitis, Myasthenia Gravis, Rheumatoid Arthritis, Reactive Arthritis, Pernicious and Aplastic Anemia, Sjogren Syndrome, Alopecia Areata, Psoriasis, Scleroderma, Fibromyalgia, Chronic Fatigue Syndrome, Vitiligo, Vasculitis, Alzheimer's Disease, Atherosclerosis, Dermatitis, Diverticulitis, stress, etc. Biofield Energy Healing may also be useful in the prevention of immune-mediated tissue damage in cases of organ transplants (for example heart transplants, kidney transplants and liver transplants), stress prevention and management, for anti-aging and in improving overall health and quality of life.

Abbreviations

LPS: Lipopolysaccharide; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS: Phosphate buffer saline; ELISA: Enzyme-linked immunosorbent assay; NCCIH: National Center of Complementary and Integrative Health; CAM: Complementary and Alternative Medicine

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