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Full Length Research Paper

Effect of the Biofield Energy Treated Test Formulation on Pro-Inflammatory Cytokines Expression in Mouse Splenocytes

Mahendra Kumar Trivedi[1], Snehasis Jana[2,] *

[1]Trivedi Global, Inc. Henderson, Nevada, USA
[2]Trivedi Science Research Laboratory Pvt. Ltd., Thane (W), Maharashtra India

Corresponding Author's E-mail Address:publication@trivedieffect.com (S. Jana)

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A new proprietary formulation was designed with a mixture of the nanocurcumin along with minerals and vitamins *viz.* zinc chloride, magnesium gluconate, sodium selenate, ascorbic acid (vitamin C), cholecalciferol (vitamin D_3), iron sulfate, and copper chloride. The study aimed to evaluate the immunomodulatory activity of Biofield Energy Healing (The Trivedi Effect®) Treated test formulationby the estimation of cytokines in mice splenocyte cells. The test formulation was divided into two different parts, one part was control without any Biofield Energy Treatment, while the other part was defined as Biofield Energy Treated sample, which received Biofield Energy Healing Treatment by Mr. Mahendra Kumar Trivedi. The Biofield Energy Treated test formulation was evaluated to find the expression of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α), macrophage inflammatory protein (MIP-1α), and interleukin (IL-1β) in splenocyte cell culture supernatant along with determination of non-cytotoxic concentrations (MTT assay). MTT assay showed significant cell viability at all the tested concentrations and found safe with more than 88% cell viability. TNF-α was significantly reduced by 15.21% and 16.41% in the Biofield Energy Treated test formulation group at 0.5 and 5.2µg/mL, respectively as compared with the vehicle control group. The level of IL-1β was also significantly reduced by 13.49%, 13.82%, and 20.44% at 0.1, 8, and 10 µg/mL, respectively as compared to the vehicle control group. Overall, The Trivedi Effect®-Energy of Consciousness Energy Healing Treatment significantly down-regulated the pro-inflammatory cytokines and further potentiate the immunomodulatory effect of the test formulation, which could be better to use against many organ transplantation (for example kidney transplants, liver transplants and heart transplants) and various disease conditions such as Asthma, Ulcerative Colitis, Alzheimer's Disease, Atherosclerosis, Dermatitis, Diverticulitis, Hepatitis, Irritable Bowel Syndrome, Parkinson's Disease, stress etc. with a safe therapeutic index to improve overall health and quality of life.

Keywords: The Trivedi Effect®, Biofield Energy Healing Treatment, Pro-inflammatory cytokines, MIP-1α, TNF-α, IL-1β.

1. INTRODUCTION

Many herbal active molecules have been scientifically studies and identified for their immunomodulatory properties and that can be enhanced with the addition of some minerals that regulate the immune cells. Based on the literature, authors formulated a new proprietary test formulation including nanocurcumin, zinc chloride, magnesium (II) gluconate hydrate, sodium selenate, ascorbic acid (vitamin C), cholecalciferol (vitamin D_3), iron (II) sulfate, and copper chloride. The components used in this formulation havingvarious pharmacological activities like immune modulating properties, antioxidant, anti-infective, anti-inflammatory, and anti-viral (Lukác and Massányi, 2007; Galland, 1998; Wintergerst et al. 2007). These types of multi component formulations are the major target for various phytopharmaceutical products as the dietary supplements (Rishton, 2008). The novel test formulation was treated with Biofield Energy Healing Treatment as Complementary and Alternative Medicine (CAM) approach by a renowned Biofield Energy Healer and was evaluated for its antioxidant potential in male *Sprague Dawley* rats. Conventionally, curcumin was being used as a nutritional spice, coloring agent, and food preservative in many countries, but after its extensive research all round the world, its use has been widespread against many biological functions. This bioactive agent, apart from its condimental activities, has a surprisingly wide range of pharmacological properties such as anticancer, antiviral, antifungal, antioxidant, antiangiogenic, and anti-inflammatory properties (Aggarwal and Harikumar, 2009; Maheshwari et al. 2006). The healing property of curcumin has been well known and practiced worldwide as complementary and alternative health care to improve overall health and the immune system (Gupta, 2013). The major issue in curcumin is its low absorption, fast metabolism and fast systemic elimination from the body (Anand, 2007). In order to improve the curcumin bioavailability, curcumin nano particles was formed, which showed improved oral and systemic absorption (Nabavi et al. 2014). However, scientific reports suggested that *in vivo* bioavailability can be improved significantly in nanocurcumin as compared with the curcumin by 60-folds (Ma et al. 2007). Biofield Energy Healing has been reported as a CAM with significant results with respect to immunity (Jain et al. 2015). National Institute of Health/National Center for Complementary and Alternative Medicine (NIH/NCCAM) suggested the significance of various energy therapies in order to promote the health and healing such as deep breathing, Qi Gong, natural products, yoga, Tai Chi, chiropractic/osteopathic manipulation, meditation, massage, special diets, homeopathy, progressive relaxation, acupressure, guided imagery, relaxation techniques, acupuncture, healing touch, hypnotherapy, pilates, movement therapy, rolfing structural integration, traditional Chinese herbs and medicines, Ayurvedic medicine, naturopathy, essential oils, aromatherapy, Reiki, mindfulness, cranial sacral therapy and applied prayer (Rubik, 2002). In addition, many basic and clinical reports have been published and reported the importance of Biofield Energy Healing Treatment on the immune system of cancer patient using cancer therapy (Lutgendorf et al. 2010) and massage therapy (Ironson et al. 1996). However, energy can exists in various forms that can be harnessed and transmit it into living and non-living things by the process of Biofield Energy Treatment. The Trivedi Effect® had been expansively reported with significant results in different scientific fields like cancer research ([a, b]Trivedi et al. 2015), microbiology ([c,d,e,f]Trivedi et al. 2015), genetics ([g,h]Trivedi et al. 2015), pharmaceutical science ([i,j,k,l]Trivedi et al. 2015), agricultural science ([m,n,o,p]Trivedi et al. 2015), skin health (Holmlund et al. 2017; Peoples et al. 2017; Smith et al. 2017), nutraceuticals ([a,b]Trivedi et al. 2017), and materials science ([q,r,s,t]Trivedi et al. 2015). Thus, the study has been designed to

evaluate the impact of the Biofield Energy Treated test formulation on the level of pro-inflammatory cytokines in the *in vitro* cellular models on mice splenocyte cells.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents

Roswell Park Memorial Institute (RPMI-1640), lipopolysaccharide (LPS), 3-(4, 5-dimethyl-2-thiazolyl) 2, 5 diphenyl-2 H-tetrazolium) (MTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), L-glutamine, rapamycin, penicillin, streptomycin, and 2- mercaptoethanol were purchased from Sigma Chemical Corp. (St. Louis, MO). Fetal bovine serum (FBS) was purchased from GIBCO, USA. ELISA (enzyme-link immunosorbent assay) assay kits for macrophage inflammatory protein-1α (MIP-1α), interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α) were purchased from R&D Systems, USA. Copper chloride, Iron sulfate, and cholecalciferol (vitamin D_3) were obtained from Sigma Chemical Co. (St. Louis, MO). Zinc chloride and magnesium (II) gluconate hydrate were obtained from TCI, Japan. Nanocurcumin was purchased from Sanat Products Ltd., India. Sodium selenate and ascorbic acid were procured from Alfa Aesar, USA. Rest of the chemicals used in this experiment were analytical grade available in India.

2.2. Test Formulation and Reference Standard

The test formulation contained a combination of nanocurcumin along with minerals *viz.* iron sulfate, copper chloride, zinc chloride and magnesium (II) gluconate hydrate, vitamins *viz.* cholecalciferol (vitamin D_3) and ascorbic acid (Vitamin C). Rapamycin was used as a reference standard (positive control) and LPS was used as an inflammatory stimulantin cytokines assay in splenocyte cells.

2.3. Experimental Animal

C57BL/6 male mice (8 weeks old) were purchased from Vivo BioTech Ltd., Hyderabad, India. Normal pellet diet (NPD) and drinking water were given *ad libitum* with a controlled temperature (22 ± 3°C), humidity (30% to 70%) and a 12-hour light/12-hour dark cycle. Before initiation of animal experiment approval was taken from the Institutional Animal Ethics Committee (IAEC).

2.4. Biofield Energy Healing Treatment Strategies

The test formulation was divided in to two parts. One part of the test formulation did not receive any treatment and was defined as the control group. Another part received Biofield Energy Treatment by a renowned Biofield Energy Healer, Mr. Mahendra Kumar Trivedi remotely under standard laboratory conditions for ~3 minutes and known as Biofield Energy Treated Test formulation. Further, the control group was treated with a “sham” healer for comparison purpose.

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.

2.5. Experimental Design

Splenocytes isolated from animals were divided into various experimental groups *viz.* vehicle control, positive control, untreated test formulation, and Biofield Energy Treated test formulation at various concentrations. All the groups were treated with lipopolysaccharides (LPS) at the rate of 50 ng/mL as inflammatory stimulant. After incubation for 48 hours cytokines like MIP-1α, IL-1β, and TNF-α using ELISA as per the manufacturer’s instructions. Concentrations were determined in triplicate wells of each sample.

2.6. Cell Culture and Test Formulation Treatment

Splenocyte (0.2 X 10^6 cells per well) cells were isolated from the animals and 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 cell cultures were grown in a humidified CO_2 incubator (5% CO_2) for 48 hours at 37^oC. The effect of cytotoxicity of the test formulation was tested by treating cells with different concentrations of the test formulation in RPMI-1640 medium.

2.7. Cytotoxicity by MTT Assay

The effect of the Biofield Energy Treated and untreated test formulation sat the concentration range of 0.01 to 10.4 µg/mL were tested for cell viability using 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The details procedure was followed as per Trivedi et al. 2016 (Trivedi et al. 2016). The cell viability was determined using equation 1 and results showed more than 75% cell viability were selected s for cytokine estimation.

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

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

2.8. Evaluation of Cytokines

ELISA was used for the evaluation of TNF-α, MIP-1α, and IL-1β. 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 cell culture supernatant from different experimental samples and standards were incubated overnight at 4ºC 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 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. Further, 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. In addition, 50 µL 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). The standards were run in parallel to the samples, and the concentrations were determined in triplicate for each sample [Madaan et al. 2015).

2.9. Statistical Analysis

Data were expressed as mean of three replicates ± SEM and were subjected to one-way analysis of variance (ANOVA) for multiple groups 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. MTT Assay on Splenocyte Cells

The results of the Biofield Energy Treated and untreated test formulations on the proliferation of mice splenocyte cells were examined after 48 hours using MTT assay. The effect of the test formulation on the splenocyte cells viability are shown in Figure 2. The results showed the % cell viability in all the tested concentrations (upto 10 µg/mL) of the test formulation were found safe and hence was used in estimation of different cytokines. The vehicle control group was reported with 100% cell viability in presence of LPS. However, the positive control group rapamycin showed cell viability greater than 88%.The test formulation at various concentration range *i.e.* 0.01 to 10 µg/mL was tested on the splenocyte cells. The Biofield Energy Treated and untreated test formulation was found safe at all the tested concentration with percentage cell viability range from 98% and 115% upto 10 µg/mL. Thus, these concentrations were selected for the estimation of cytokines. The increased cell viability in different groups with respect to the vehicle control group might be due to the proliferation in cell culture. Cells viability assay using MTT method suggested that the test formulations concentration was found safe with respect to the *in vitro* cell viability of splenocytes. The results evaluated through MTT assay suggested the metabolic activity by measuring the activity of succinate dehydrogenase, a mitochondrial enzyme. Cell viability using MTT assay is widely used for most of the i*n vitro* cell based activity of any test drug. MTT assay is regarded as rapid, inexpensive, time effective, and as a non-radioactive method in order to judge the cell proliferation with respect to cell growth and metabolic activity (Seo et al. 2005).

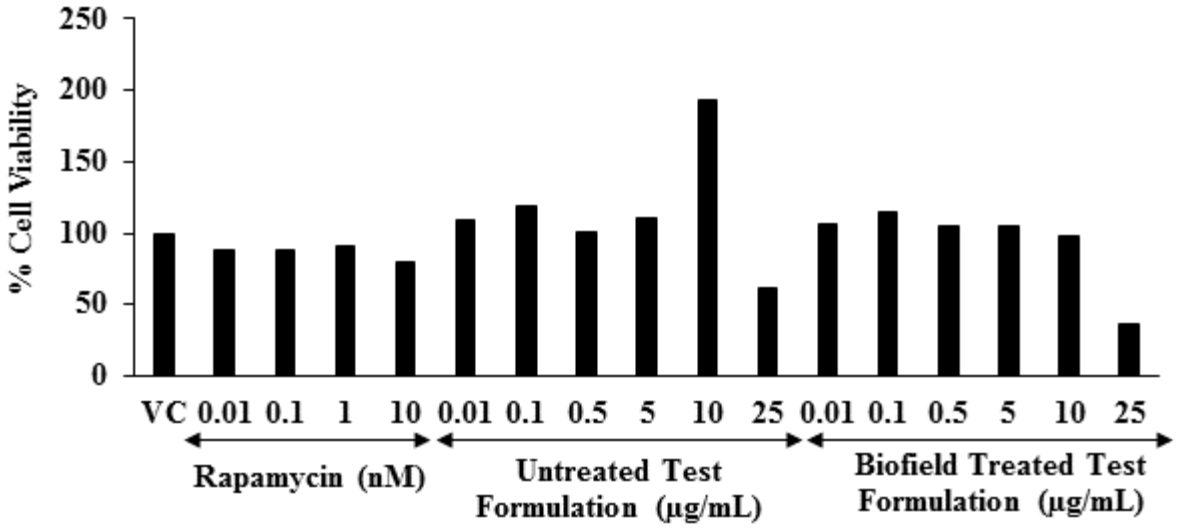

Figure 1: *Cell viability assay using MTT in the splenocyte cells after 48-hourtreatment with different concentrations of the test formulation in the presence of LPS (50 ng/mL). The absorbance of the MTT formazan was determined at 540 nm in an ELISA reader.*

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

The effect of the Biofield Energy Treated test formulation on pro-inflammatory cytokines TNF-α, MIP-1α, and IL-1β was observed and compared. All inflammatory cytokines plays a major role in inflammation, immune modulation, and lymphocyte activation, so it might be expected that the novel test formulations can modulate the expression and activation of tested cytokines. The effect of the test formulation on pro-inflammatory cytokines was estimated with various concentrations for 48 hours using ELISA assay.

3.2.1. Evaluation of TNF-α

The expression of TNF-α in splenocyte cells after treatment with the Biofield Energy Treated and untreated test formulations are represented in Figure 2. The results suggested that both the untreated and Biofield Energy Treated groups suppressed the TNF-α secretions at different tested concentrations as compared with vehicle control group. The vehicle control group showed TNF-α values as 380.4 ± 12.74 pg/mL. The level of TNF-α was reduced by 4.12%, 15.21%, and 16.41% at 0.1, 0.5, and 5.2 µg/mL, respectively in the Biofield Energy Treated test formulation as compared to the vehicle control group. Besides, the untreated test formulation was reduced the level of TNF-α by 19.67%, 12.63%, and 19.25% at 0.1, 0.5, and 5.2 µg/mL, respectively as compared to the vehicle control group.

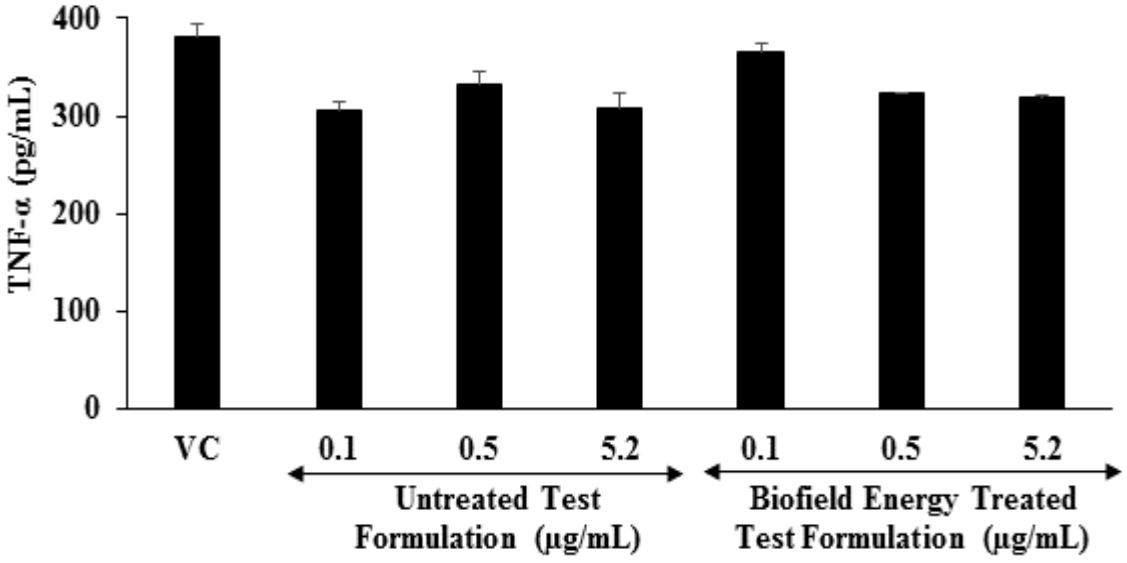

Figure 2: *The LPS mediated production of TNF-α by the Biofield Treated and untreated test formulations. For each concentration treatment, the level of TNF-α release was measured 48 hours after receiving treatment. The values are represented in pg/mL as mean ± SEM.*

TNF-α plays a major role in immune disorders, and is the controlling factor for many diseases (Bemelmans et al. 1996). TNF-α is regarded as a potential therapeutic target due to its important role in many inflammatory processes, however data suggested that an increased TNF-α level are found to have various acute and chronic inflammatory conditions such as trauma, sepsis, infection, rheumatoid arthritis (Popa et al. 2007).Overall, it can be suggested that the Biofield Treated test formulation has significant immunomodulatory action by inhibiting the concentration of TNF-α as compared with the vehicle control.

3.2.2. Evaluation of MIP-1α

The effect of the Biofield Energy Treated and untreated test formulations on the level of MIP-1α is shown in Figure 3. The level of MIP-1α in the vehicle control group was 1337.4 ± 36.6 pg/mL. The positive control, rapamycin was significantly ($p \leq 0.001$) reduced the level of MIP-1α level by 26.09%, 40.27%, and 40.40% at 0.01, 0.1, and 1 nM, respectively as compared to the vehicle control group. The untreated test formulation showed inhibition of MIP-1α secretion at the tested concentrations *i.e.* at 0.1, 0.5, and 1 µg/mL by 8%, 4.66%, and 15%, respectively as compared to the vehicle control group. However, the MIP-1α expression was reduced by 3.79% at 0.5 µg/mL in the Biofield Energy Treated test formulation group as compared to the vehicle control group.

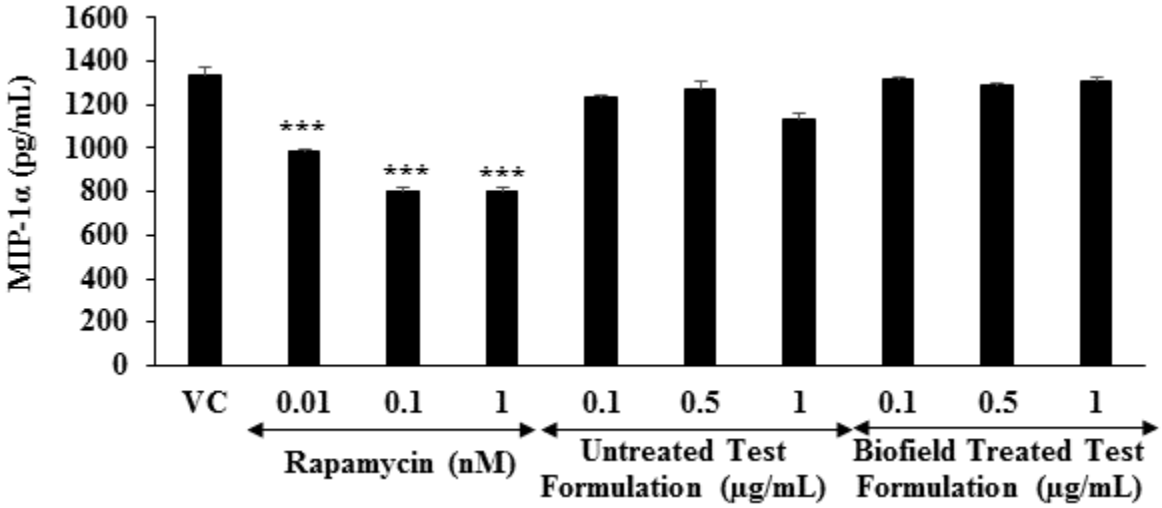

Figure 3: *The LPS mediated production of MIP-1α by the Biofield Treated and untreated test formulations. For each concentration treatment, the level of MIP-1α release was measured 48 hours after receiving treatment. The values are represented in pg/mL as mean ± SEM (***p≤0.001 vs. vehicle control)..*

Scientific studies suggested that macrophage inflammatory proteins 1 alpha and beta (MIP-1 alpha and beta) are the heparin binding proteins, which are responsible for variety of inflammatory and immunoregulatory activities (Driscoll, 1994). In addition, the reduction of MIP-1α levels might be useful in decreasing the inflammatory responses (Hsieh et al. 2008). However, the level of MIP-1α was inhibited at the Biofield Energy Treated test formulation with respect to the vehicle control group. Hence, it was suggested that the Biofield Energy Healing based test formulation would be useful against many inflammatory disease.

3.2.3.Estimation of IL-1β Expression

The effect of the test formulation on IL-1β expression in splenocytes is shown in Figure 4. The figure demonstrates the inhibition of IL-1β after treatment with the Biofield Energy Treated test formulation as compared with the vehicle control group. In addition, Biofield Energy Healing Treatment further suppressed the level of IL-1β in all the three concentrations as compared with the untreated test formulation. The level of IL-1β in the vehicle control group was 45.8 ± 3.82 pg/mL. The level of IL-1β was reduced by 6.88% and 9.52% at 0.1 and 8 µg/mL, respectively as compared to the vehicle control group. Besides, the Biofield Energy Treated test formulation group was significantly suppressed the level of IL-1β by 13.49%, 13.82%, and 20.44% at 0.1, 8, and 10 µg/mL, respectively as compared to the vehicle control group. The positive control rapamycin showed 7.38%, 14%, and 29.04% ($p \leq 0.05$) reduction of IL-1β at 0.1, 1, and 10 nM, respectively as compared to the vehicle control group.

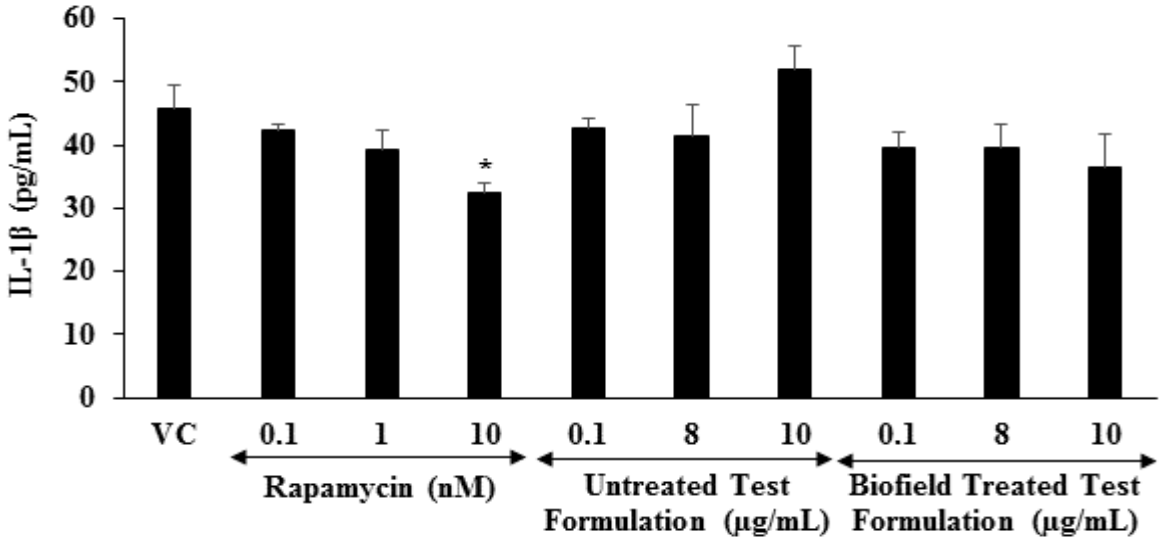

Figure 4: *Concentration-dependent inhibition of LPS mediated production of IL-1β by the test formulation. For each concentration treatment, the level of IL-1β release was measured in cell supernatant 48 hours after receiving treatment. All values are represented in pg/mL as mean ± SEM (*p≤0.05 vs. vehicle control).*

Immunological and inflammatory functions of IL-1β in controlling the immune response during infections are well defined in many studies (Dinarello, 2009; Schultz et al. 2002). Research suggests that glial, immune, and neuronal cells and their interactions play a vital role in cytokine cascade, which is the main pathway of pain and inflammatory processes. However, IL-1β is defined as a pro-inflammatory cytokine, which has been implicated in pain, inflammation and autoimmune conditions (Ren and Torres, 2009). The data suggest that the expression of IL-1β was further significantly suppressed after the Biofield Energy Treatment on the test formulation at all concentrations, implicating the importance of Biofield Energy in blockade of IL-1β, which can be considered as a therapeutic opportunity against many inflammatory conditions. The scope of natural medicine has only been improved day-by-day in the developing countries (Banerjee, 2009), however the use of Biofield Energy as a CAM approach has been reported its use successfully in many scientific research and industries, which are increasing worldwide (Clarke et al. 2015). Biofield Energy Healing has been succesfully reported in the case of cancer cell lines with respect to the inhibition of cytokine expression such as IL-1α and IL-1β levels (Gronowicz et al. 2015). Many autoimmune disorders such as rheumatoid arthritis, stress, and asthma, the best choice of treatment approach for the suppression of the cytokines production (Farrar et al. 2002). The Trivedi Effect®-Energy of Consciousness Healing Treatment, a new proprietary test formulations, which can be further used to modulate the immune function by suppression of inflammatory cytokines with long-term effectiveness to improve overall health and quality of life.

4. CONCLUSIONS

MTT assay showed more than 88% cell viability in all the groups, which suggested that the test formulation was safe. TNF-α levels were significantly inhibited by 15.21% and 16.41% at 0.5

and 5.2 µg/mL, respectively as compared with the vehicle control group. However, the level of IL-1β secretion was significantly decreased in the Biofield Energy Treated test formulation group by 13.49%, 13.82%, and 20.44% at 0.1, 8, and 10 µg/mL, respectively as compared to the vehicle control group. On the basis of experimental results of novel test formulation, it can be concluded that The Trivedi Effect®- Energy of Consciousness Healing Treatment on the test formulation significantly inhibited the activity of pro-inflammatory cytokines, which might prevent the over-activation of the immune system. On the basis of experimental results of various tested cytokines and their expression levels, significant immunomodulatory activity was observed in the new proprietary formulation after treated with The Trivedi Effect®- Biofield Energy Healing by a renowned Biofield Energy Healer, Mr. Mahendra Kumar Trivedi. Biofield Energy Treated test formulation can be used as a Complementary and Alternative Medicine (CAM) to prevent the immune-mediated diseases such as Irritable Bowel Syndrome, Rheumatoid arthritis, Ulcerative colitis and Crohn's disease, Stress, Asthma, and many more with safe therapeutic index. It can also be used for organ transplants, autoimmune disorders like Diabetes, Addison Disease, Multiple Sclerosis, Myasthenia Gravis, Pernicious Anemia, Aplastic Anemia, Systemic Lupus Erythematosus, Alopecia Areata, Vitiligo, Psoriasis, and Vasculitis, etc.

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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