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In vitro Evaluation of Immunomodulatory Effects of the Test Formulation by the Estimation of Natural Killer Cells and Phagocytosis Activities

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Abstract

Although the immunomodulatory effects of vitamins, minerals, and many herbs *per se* have been extensively studied, while research related to possible immunomodulatory effects of a combined formulation is relatively scarce. Here, the potential immunomodulatory effects of the combination of eight components *viz.* zinc chloride, ferrous sulfate, sodium selenate, nanocurcumin, copper chloride, magnesium gluconate, vitamin C, and vitamin D_3 were investigated. These components are widely used in therapeutic, cosmetics and dietary supplements. The current study investigated the *in vitro* immunomodulatory effect of a Consciousness Energy Healing (The Trivedi Effect®) Treated nanocurcumin-based test formulation in lipopolysaccharide (LPS)-induced natural killer (NK) cells activity in splenocytes cell-line co-incubated with mouse lymphoma cell line (Yac-1). The formulation was divided into two parts, in which one part of the formulation was received the Biofield Treatment by a renowned Biofield Energy Healer, Mr. Mahendra Kumar Trivedi and was defined as the Biofield Energy Treated sample, while the other part was denoted as the untreated test sample. Cell viability using MTT assay in mouse splenocytes showed more than 98% cells were viable upto 10.4 µg/mL of the tested concentrations, indicating that the test formulation was safe and nontoxic. The NK cells activity was measured in cell supernatants using ELISA. The NK cells activity was significantly altered by 26.73%, 11.82%, 7.64%, 0.36% and 36.36% in the untreated formulation group at 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the vehicle control (VC) group. Further, Biofield Energy Treated test formulation exhibited 39.64%, 8.18%, 17.27%, 51.93% and 42.55% alteration of the NK cells activity at the concentrations of 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the VC group. Moreover, the cell viability of the test formulation was assessed in RAW 264.7 (Mouse Macrophage Cell Line) for phagocytosis activity was safe upto 13.3 µg/mL with >90% on cell viability. The phagocytosis assay data showed that at two concentrations (1.3 and 7.6 µg/mL) the Biofield Energy Treated test formulation improved extent of phagocytosis by 6.31% and 5.91%, respectively compared to the untreated test formulation group. In summary, the Biofield Energy

Treated test formulation may have indeed immunomodulatory effect by enhanced NK cells activity and extent of phagocytosis responses. These observations indicated that the Biofield Energy Treated test formulation has the potential effects through modulating the expression of NK cells function and phagocytosis and might be developed as a useful anti-inflammatory product for various inflammatory disorders.

Keywords: Nanocurcumin; Immunomodulation; MTT; Natural Killer Cells Activity; Phagocytosis

Abbreviations: NK: Natural Killer; CAM: Complementary and Alternative Medicine; NCCAM: National Center for Complementary/Alternative Medicine; NHIS: National Health Interview Survey.

Introduction

With the concern of defense capability against infectious microbes, innate immunity evokes preexisting defense mechanisms that have specialized to specifically recognize microbes and eliminate infection [1]. The main purpose of the innate immune system is to mediate inflammation by the recognition of microbes which is expressed by innate immune cells [2]. An important class of innate immune cells that play a critical role in mediating the antitumor immune response is the natural killer (NK) cell and phagocyte cells like polymorphonuclear neutrophil [3,4]. The primary mechanism of NK cells cytotoxicity is through the granule exocytosis process upon formation of an immunological synapse. The NK cells contain preformed cytoplasmic granules that resemble secretory lysosomes and contain perforin and granzymes [5]. Besides, main mechanism of phagocytosis to the invading pathogens or at the site of infection by the process of rolling, adhesion of the cells to the vascular endothelial cells, diapedesis, phagocytosis and finally degradation of pathogen [6]. For the treatment of various ailment, synthetic products or formulation are target oriented but possess some adverse reaction. Besides, natural products, vitamins and trace elements are very effective non-selectively with less adverse effects than synthetic products. An organism can physiologically homeostat the cells by either stimulation or suppression of immune system [7,8]. The utilization of Complementary and Alternative Medicine (CAM) has been drastically increased in the universe for the both therapeutic and prophylactic management of different ailments in the human population [9]. There were various minerals and herbs have been extensively used for the modulation of the immune system. Likewise, a herbomineral formulation was designed to act on immune system. Treatments based on the herbal formulation are amongst the safest therapies due to lack of adverse-effects and cost-effective approach [10]. The novel formulation (based on herbal and mineral ingredients), was a combination of ashwagandha and trace elements *viz.* magnesium gluconate hydrate, zinc chloride, and sodium selenate. Each ingredients of this formulation are commonly used as nutraceutical supplement to improve the general health by increasing the body's immunity [11-14].

Various scientific literatures reported the beneficial effect of the Energy Therapy (Biofield Energy Treatment) on immune systems, i.e., preservation of immune function in cervical cancer patients after therapeutic touch, massage therapy in enhancing immune system, and many more [15,16]. The National Center for Complementary/Alternative Medicine (NCCAM) has elaborated and given preference to the energy therapies, as it works by manipulating the energy fields that theoretically surround and penetrate the body [11,17]. As per the National Health Interview Survey (NHIS) 2012, which comprised that about ~18% Americans has been used the dietary supplement as complementary health approaches as compared with other practices. The NCCAM has recognized and accepted Biofield Therapy as a CAM approach in addition to other therapies and practices such as Tai Chi, natural products, Qi Gong, deep breathing, yoga, homeopathy, chiropractic/osteopathic manipulation, massage, meditation, healing touch, special diets, progressive relaxation, guided imagery, relaxation techniques, acupressure, acupuncture, hypnotherapy, mindfulness, aromatherapy, movement therapy, pilates, naturopathy, rolfing structural integration, essential oils, Ayurvedic medicine, traditional Chinese herbs and medicines, Reiki, cranial sacral therapy and applied prayer (as is common in all religions, like Buddhism Christianity, Judaism and Hinduism). Human Biofield Energy has subtle form of energy that has the capacity to work in an effective manner [18]. The CAM therapies have been extensively practiced worldwide with reported

positive outcomes in different disease profiles [19]. This subtle form of energy can be harnessed and transmitted by individuals into living and inanimate object *via* the process of unique Energy Transmission process (The Trivedi Effect®) and the data has been published in numerous peer-reviewed science journals with significant outcomes in cancer research, microbiology, genetics, pharmaceutical science, agricultural science, and materials science [20-37]. In this regards, authors intend to evaluate the impact of Biofield Energy Healing Treatment (The Trivedi Effect®) on the herbomineral-based formulation for immunomodulatory action in two different cell-lines (splenocytes co-incubated with Yac-1 and macrophage) with the estimation of NK cells activity and phagocytosis responses.

Materials and Methods

Requirement

Antibiotics (penicillin and streptomycin) and DMEM (phenol-red free) were procured from HiMedia. DMEM was procured from GIBCO, USA. Direct Red 80, 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium) (MTT), and β-Estradiol (positive control) were purchased from Sigma Chemical Co. St. Louis, MO. Iron sulfate, copper chloride, cholecalciferol, and sodium carboxymethyl cellulose were obtained from Sigma Chemical Co. (St. Louis, MO). Nanocurcumin was purchased from Sanat Products Ltd., India. Zinc chloride and magnesium (II) gluconate hydrate were obtained from TCI, Japan. Sodium selenate and ascorbic acid were procured from Alfa Aesar, USA. Tetrahydrocurcumin (THC) was procured from Novel Nutrients, India. All the other chemicals used in this experiment were analytical grade procured from India.

Experimental Design

The experiment was designed into eleven groups. Group 1 contained the defined cells without lipopolysaccharides (LPS), denoted as the normal control (NC). Group 2 included cells with LPS. Group 3 and 4 contained cells with concanavalin A (ConA) and (ConA + LPS), respectively. Group 5 included cells, vehicle (DMSO) along with LPS defined as vehicle control (VC). Group 6, 7, 8, and 9 defined as positive controls of rapamycin (RAP), dexamethasone (DEX), curcumin (CUR), and tetrahydrocurcumin (THC), respectively. Group 10 and 11 denoted as the nanocurcumin-based untreated test formulation and Biofield Energy Treated test formulation group, respectively that included cells along with LPS at various concentrations.

Composition of Test Formulation

The composition of each ingredients in the test formulation consisted of nanocurcumin majorly (200 mg/mL), in combination with five trace metals and two vitamins were defined as zinc chloride (0.397 mg/mL), ferrous sulfate (0.569 mg/mL), sodium selenate (0.00137 µg/mL), copper (II) chloride (0.121 mg/mL), magnesium gluconate (6.5 mg/mL), vitamin C (0.572 mg/mL), and cholecalciferol (0.0191 mg/mL).

Cell Culture and Maintenance

Mouse was used for the isolation of splenocytes as per standard protocol [6]. The LPS (0.5 µg/mL) induced splenocyte cells cultures were grown for 48 hours at 37°C in a humidified CO_2 incubator (5% CO_2) [23]. The single cell suspension of splenocytes co-incubated with mouse lymphoma cell line (Yac-1) for the estimation of natural killer (NK) cells activity in RPMI medium containing 10% FBS was plated at a density of 2 x 10^4 cells per well in 96-well culture plates. The mouse macrophage, RAW 264.7 (5 x 10^4 cells per well) cells were grown in 24-well culture plates using RPMI-1640 medium supplemented with 10% FBS, 100 µg/mL of streptomycin, and 100 units/mL of penicillin for phagocytosis activity. The LPS (0.5 µg/mL) induced macrophage cells cultures were grown as per Yac-1 culture condition [38].

Biofield Energy Healing Approach

The test formulation was a combination of eight ingredients *viz.* zinc chloride, ferrous sulfate, sodium selenate, nanocurcumin, copper chloride, magnesium gluconate, vitamin C (ascorbic acid) and vitamin D3 (cholecalciferol). Each ingredient of the test formulation was divided into two parts. One part of each ingredient was considered as control, where no Biofield Energy Healing Treatment was provided to these ingredients. Further, the control groups were treated with "sham" healer for comparison purpose. The sham healer did not have any knowledge about the Biofield Energy Healing Treatment. Second part of each ingredients received Biofield Energy Healing Treatment (known as The Trivedi Effect®) under laboratory conditions for 3 minutes through the Healer's unique Energy Transmission process to the test formulation. Biofield Energy Healer in this study did not visit the laboratory, nor had any contact with the herbomineral samples. After that, the Biofield

Energy Treated and untreated ingredients were kept in similar sealed conditions and used for the study as per the study plan.

MTT Assay

Cytotoxicity was determined by exposing cells (splenocytes, Yac-1, and macrophage) to different concentrations of test formulation in RPMI. The respective vehicle control kept in the assay was DMSO with LPS. The single cell suspension of splenocytes in medium containing 10% FBS was plated at a density of 0.2 x 10^6 cells/well in 96-well culture plates. Cells were treated with the untreated and Biofield Energy Treated test formulation along with LPS at concentrations ranging from 0.01 µg/mL to 104.1 µg/mL. After treatment, cells were incubated in a CO_2 incubator at 37ºC and 5% CO_2 for 48 hours. Effect of the test formulation on the viability of splenocytes was estimated by 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. 20 µL of 5 mg/mL of MTT was added to all the wells and incubated at 37ºC for 3 hours. Cells were centrifuged and supernatant was removed. The cell pellet in each well was resuspended in 150 µL of DMSO to dissolve formazan crystals. The O.D. of each well was read at 540 nm using Biotek Reader. The number of viable cells was estimated based on the conversion of MTT to formazan dye using a mitochondrial enzyme. The effect of the test formulation on cell viability of splenocytes was determined with the help of the following equation 1:

$$\% \text{ Cell viability} = (100 - \% \text{ cytotoxicity}) \quad \text{(Eq. 1)}$$

Where; % Cytotoxicity = {(O.D. of Control cells – O.D. of cells treated with test formulation)/ OD of Control cells}*100 [39].

Natural Killer (NK) Cells Assay

The effect of the test formulation on NK cell activity was performed in culture supernatants (mouse splenocytes) by ELISA method using Biotek reader (SIAFRT/Synergy HT multimode reader) according to the manufacturer's instructions [40, 41]. The single cell suspension of Yac-1 in medium containing 10% FBS was plated at a density of 20,000 cells/well in 96-well culture plates. After incubation for 24 hours, splenocytes were added to each well at density of 1 x 10^6 cells/well. After incubation for 4 hours, cells were treated with the untreated and Biofield Energy Treated test formulation along with LPS at concentrations ranging from 0.104 µg/ml to 10.4 µg/mL. The resultant immunomodulatory effect of the test formulation on NK cells activity in presence of LPS was determined with the help of the following equation 2:

$$\% \text{ NK cells activity} = [(\text{OD of Yac} - 1 \text{ cells alone}) - \{(\text{OD of NK} + \text{Yac} - 1 \text{ cells}) - (\text{NK alone})\}]/\text{OD of Yac} - 1 \text{ cells alone}] * 100 \quad \text{(Eq. 2)}$$

Phagocytosis Assay

This assay was used the quantitative colorimetric detection of engulfed prelabeled zymosan particles by mouse macrophages cell line (RAW264.7). The phagocytosis assay was measured in culture supernatants using CytoSelect™ 96-Well Phagocytosis Assay kit (Zymosan, Colorimetric Format) as per manufacturer's instructions (Cell Biolabs Inc. USA). For the estimation of extent of phagocytosis in LPS (0.5 µg/mL) induced macrophage, the cells were exposed to the test formulation at selected non-toxic concentrations. After 24 hours of incubation, supernatants were analyzed for the assessment of extent of phagocytosis colorimetrically as per manufacturer's instructions [42,43]. The percent increase phagocytosis in presence of LPS was determined by the following equation 3:

$$\% \text{ Increase in phagocytosis} = [\{\text{O. D at 405 nm in LPS} + \text{Test formulation treated cells} - \text{O. D. at 405 nm in LPS treated control cells}\} / \text{O. D. at 405 nm in LPS treated control cells}] * 100 \quad \text{(Eq. 3)}$$

Results and Discussion

Cells Viability by MTT in Splenocytes

The cell viability in terms of percentage after treatment with the test formulation and positive controls are shown in Figure 1. The normal splenocyte cells (NC), LPS alone, and ConA alone groups showed 100%, 149%, and 245.2% cell viability, respectively. However, LPS with ConA and vehicle control (VC) group showed 214% and 100% cell viability, respectively. The cell viability of rapamycin (RAP) was 88.7%, 88.1%, 90.3%, and 79.8% at 0.01, 0.1, 1, and 10 nM, respectively. Dexamethasone (DEX) showed 110.6%, 86.7%, 79.8%, and 84.3% cell viability. Further, curcumin (CUR) showed more cell viability as 105.7%, 181.6%, 158.94%, and 39.6% at 7.4, 10, 25, and 50 µg/mL, respectively. The percent cell viability was observed by 108.8%, 175%, 151.6%, and 140% in case of tetrahydrocurcumin (THC) group at the concentration of 7.4, 10, 25, and 50 µg/mL, respectively. Besides, the nanocurcumin-based untreated test formulation exhibited 110.9%, 119.3%, 108.89%, 193.3%, and 61.9% at 5.2, 7.7, 10.4, and 26 µg/mL, respectively. While, Biofield Energy Treated test formulation exhibited 105.4%, 100.1%, 98.1%, and 35.9% cell viability at 5.2, 7.7, 10.4, and 26 µg/mL, respectively. Among these, more than 80% cell viability at different concentrations were selected for subsequent cytokines estimation (Figure 1).

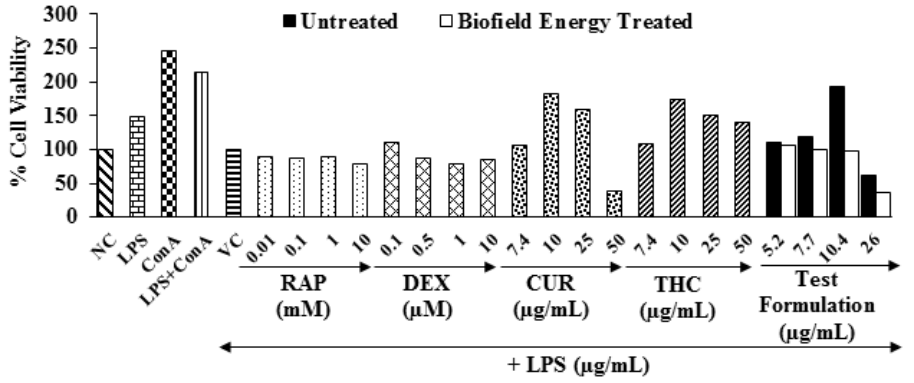

Figure 1: Measurement of cell viability by MTT assay in splenocyte cells. Values are represented as percentage. NC: Normal control; LPS: Lipopolysaccharide; ConA: Concanavalin; VC: Vehicle control; RAP: Rapamycin; DEX: Dexamethasone; CUR: Curcumin; THC: Tetrahydrocurcumin; UNT: Untreated; BET: Biofield Energy Treated.

Assessment of Natural Killer Cells Activity

The natural killer (NK) cells are the large granular cytotoxic lymphocytes in the part of innate immune system. However, it lack of antigen-specific cell surface receptors and possess the ability to identify and kill the transformed cells through the release of granzyme and perforin granules, which induce cell death *via* apoptosis [44,45]. The NK cells functions are mainly screened by monitoring the NK cell cytotoxicity (NKCC) [46]. In this experiment, after treatment with The Trivedi Effect® - Consciousness Energy Healing Treatment to the nanocurcumin based test formulation the cell killing function of NK cells were altered significantly. The response of the novel formulation on NK cells activity is presented in Figure 2. From figure 2, it was observed that the untreated cells and vehicle control (VC) groups showed 36.03% and 55% of NK cells activity, respectively. Positive control rapamycin (RAP) showed 38.80%, 44.43%, and 51.66% NK cells response in a concentration-dependent manner at 0.1, 1, and 10 nM, respectively. Moreover, curcumin (CUR) was also exhibited a dose dependent NK cells activity by 34%, 44.34%, and 48.37% at 1, 5, and 10 µg/mL, respectively. Besides, dexamethasone (DEX) and tetrahydrocurcumin (THC) showed different pattern of dose-response phenomenon like at lower concentration showed more efficiency of NK cell activity and at higher concentration possess low efficiency of NK cells activity. Further, the NK cells activity was significantly altered by 26.73%, 11.82%, 7.64%, 0.36% and 36.36% in the untreated test formulation group at 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the vehicle control (VC) group. Further, the Biofield Energy Treated test formulation significantly altered the NK cells activity by 39.64%, 8.18%, 17.27%, 51.93% and 42.55% at the concentrations

of 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the VC group. At 0.5 µg/mL, the Biofield Energy Treated test formulation showed better NK cells activity (4.12%) compared to the untreated test formulation group (Figure 2).

Like T cells, NK cells are effectors of the innate immune system that do not require prior sensitization to kill the target cells. According to the "missing self" hypothesis, NK cells can directly lyse the target cells with deficient or aberrant self-major histocompatibility complex (MHC) class I molecules [47,48]. Multiple articles reported the closed relationship between various tumor cell growth *viz.* melanoma, myeloma, lymphoma etc. and NK cells function. Tumor growth is associated with a progressive impairment of NK cell function, which govern by reduced expression of activating receptors and decreased effector functions of NK cells activity [49-52].

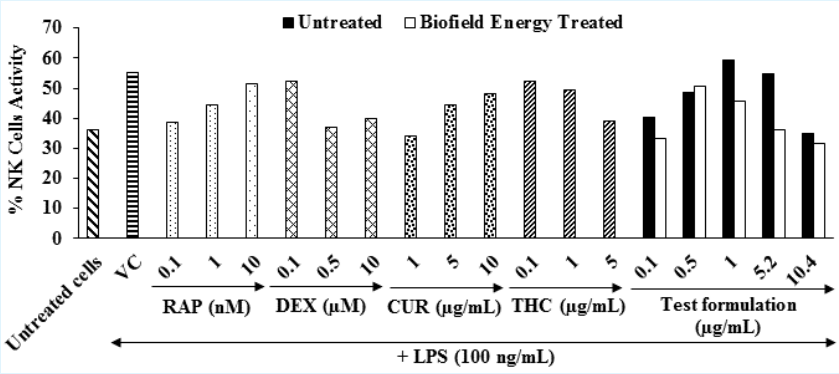

Figure 2: Effect of the nanocurcumin-based test formulation on natural killer cells activity in LPS mediated splenocytes co-incubated with Yac-1. *LPS: Lipopolysaccharide; VC: Vehicle control; RAP: Rapamycin; DEX: Dexamethasone; CUR: Curcumin; THC: Tetrahydrocurcumin.*

Assessment of Phagocytosis

Cells Viability by MTT Assay in RAW 264.7 (Mouse Macrophage Cell Line)

The normal control (NC), LPS stimulant, and vehicle control (VC) groups showed 100%, 94.5%, 100% cell viability, respectively. The test formulation (untreated and Biofield Treated) showed >90% on cell viability upto the concentration of 13.3 µg/mL as compared to VC group. The cell viability was found high in few tested concentrations than NC group, which could be due to the more proliferation in cells (Figure 3). Moreover, the positive control groups also showed more than 70% viable cells with respect to the VC group.

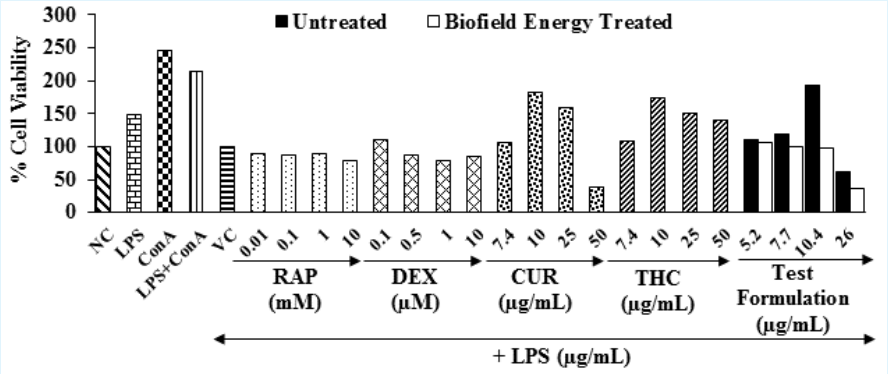

Figure 3: Measurement of cell viability by MTT assay in mouse macrophage cell line (RAW264.7). NC: Normal control; LPS: Lipopolysaccharide (µg/mL); ConA: Concanavalin; VC: Vehicle control; RAP: Rapamycin; DEX: Dexamethasone; CUR: Curcumin; THC: Tetrahydrocurcumin; UNT: Untreated; BET: Biofield Energy Treated.

Extent of Phagocytosis

The phagocyte cells (*i.e.,* polymorphonuclear neutrophil) is the first line defense of innate immune system against invading pathogens or at the site of infection. After invasion of pathogens or infection of a particular site the phagocyte cells immediately migrate to that site through different steps *viz.* rolling, adhesion of the cells to the vascular endothelial cells, diapedesis, phagocytosis and finally degradation of pathogen [6,53,54]. The effect of the novel formulation on the extent of phagocytosis activity in depicted. The LPS and vehicle control (VC) group showed 292.73% and 276.36% increased of phagocytosis, respectively compared to the untreated cells group. The untreated test formulation group showed 10.63%, 11.59%, and 14.49% of phagocytosis at the concentration of 2.5, 4.9, and 6.7 µg/mL, respectively compared to the VC group. Further, the extent of phagocytosis was increased by 5.8% and 21.26% in the Biofield Energy Treated test formulation at 1.3 and 6.7 µg/mL, respectively compared to the VC group (Figure 4). At two concentrations (1.3 and 6.7 µg/mL) the Biofield Energy Treated test formulation improved extent of phagocytosis by 6.31% and 5.91%, respectively compared to the untreated test formulation group.

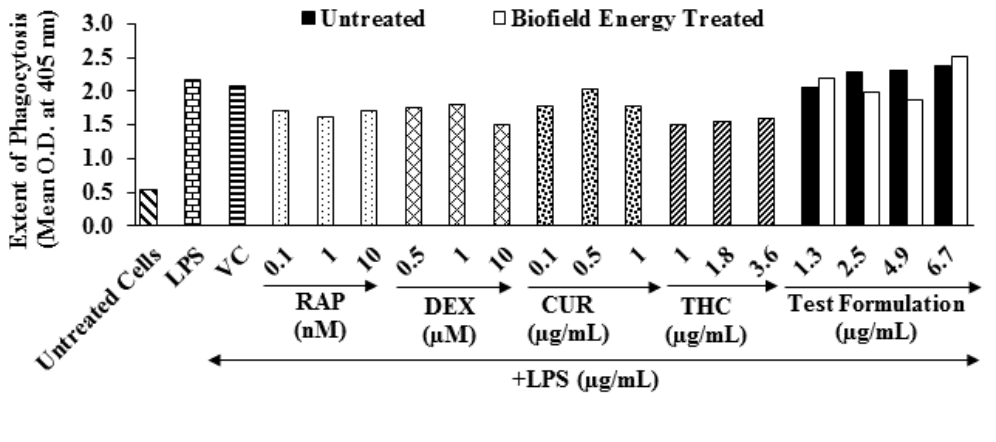

Figure 4: Effect of the test formulation on phagocytosis in LPS mediated mouse macrophage cell line. *LPS: Lipopolysaccharide; VC: Vehicle control; RAP: Rapamycin; DEX: Dexamethasone; CUR: Curcumin; THC: Tetrahydrocurcumin; Test formulation: Nanocurcumin-based formulation.*

Conclusion

The cell viability of the nanocurcumin-based test formulation was found as safe at the tested concentrations upto 10.4 µg/mL in mouse splenocytes. Besides, the untreated test formulation altered NK cells activity by 26.73%, 11.82%, 7.64%, 0.36% and 36.36% at 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the vehicle control (VC) group. However, the NK cells activity was significantly altered by 39.64%, 8.18%, 17.27%, 51.93% and 42.55% at 0.1, 0.5, 1, 5.2, and 10.4 µg/mL, respectively compared to the VC group. Further, the cell viability of the test formulation was assessed in RAW 264.7 (Mouse Macrophage Cell Line) for phagocytosis activity was safe upto 13.3 µg/mL with >90% on cell viability. The phagocytosis assay data showed that at two concentrations (1.3 and 6.7 µg/mL) the Biofield Energy Treated test formulation improved extent of phagocytosis by 6.31% and 5.91%, respectively compared to the untreated test formulation group. The study results summarized that the nanocurcumin-based formulation showed significant modulation of NK cells expression and extent of phagocytosis responses in mouse splenocyte cells co-incubate with mouse lymphoma cell line (Yac-1) and mouse macrophage cell line (RAW264.7), respectively. Overall, data indicated a significant improvement of natural killer cells activity and phagocytosis upon exposure with the nanocurcumin-based test product on tested cell-lines. Thus, The Trivedi Effect® - Consciousness Energy Healing Treatment has the significant antioxidant and immunomodulatory activities *in vitro*. It is then anticipatated that the Biofield Energy Treated herbomineral formulation could be a more useful as an immunomodulatory formulation for healthy human

and in patients in the near future. The immunomodulatory activity of the Biofield Treated novel formulation could be expanded to other therapeutic arena such as organs (kidney, liver, and heart) transplant, autoimmunedisorders (Graves' Disease, Addison Disease, Multiple Sclerosis, Myasthenia Gravis, Rheumatoid Arthritis, Systemic Lupus Erythematosus, etc.), inflammatory disorders (Crohn's Disease, Irritable Bowel Syndrome, Ulcerative Colitis, Vasculitis, etc.), aging, and strees.

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Conflicts of interest

The authors declare no conflicts of interest.

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