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Biofield Energy Therapy: Role in Multiple Organ Health Specific Biomarkers in Cell-Based Assay

Sakina Aleemah Ansari[1], Mahendra Kumar Trivedi[1], Alice Branton[1], Dahryn Trivedi[1], Gopal Nayak[1], Mayank Gangwar[2] and Snehasis Jana[2]*

[1]Trivedi Global, Inc., Henderson, Nevada, USA

[2]Trivedi Science Research Laboratory Pvt. Ltd., Thane-West, Maharashtra, India

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***Corresponding author:** Snehasis Jana, Trivedi Science Research Laboratory Pvt. Ltd., Thane-West, Maharashtra, India, Email: publication@trivedieffect.com

Abstract

The major cause of high rate of mortality is the multiple organ dysfunction among critical care healthcare services. The aim of the current study was to evaluate the impact of the Biofield Energy Treated test formulation using standard and specific cell lines related with vital organs functioning. The test formulation and the specific cell media was divided into two parts; one part was untreated (UT) and other part received the Biofield Energy Treatment remotely by a renowned Biofield Energy Healer, Sakina Aleemah Ansari, USA and labeled as the Biofield Energy Treated (BT) test formulation/media. The test formulation was tested against various activities using cell line assay in their specific medium (Med). The test formulation was tested for cell viability, and the results showed that the test formulation at tested concentrations was found safe and non-toxic. Cytoprotective activity showed improved cellular restoration by 105.4% (at 25 µg/mL), 32.8% (at 25 µg/mL), and 151.8% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, as compared to the untreated test group in the human cardiac fibroblasts cells (HCF) cells, while improved restoration of cell viability by 22.4% (at 25.5 µg/mL), 67.1% (at 10 µg/mL), and 72.9% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, as compared to the untreated test group in HepG2 cells. Cellular restoration in A549 cells was improved by 9.3%, 70.4%, and 14.1% at 0.1, 1, and 25.5 µg/mL respectively, in the BT-Med + UT-TI group, while 3% and 4.6% improved cellular restoration was reported at 10 and 25.5 µg/mL respectively, at BT-Med + BT-TI groups as compared to the untreated test group. ALP activity in Ishikawa cells was significantly increased by 68.4%, 41.1%, and 18.8% at 0.1, 10, and 50 µg/mL respectively, in the UT-Med + BT-TI group, while in MG-63 cells showed maximum increased ALP activity by 92.7%, 84.5%, and 93.2% respectively in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI group respectively, at 50 µg/mL as compared to the untreated group. The maximum percent cellular protection of HCF (heart) cells (decreased of LDH activity) was significantly

increased by 51.6%, 88.7%, and 53.7% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, at 10 µg/mL as compared to the untreated group. Alanine amino transferase (ALT) activity was reported in terms of percent cellular protection of HepG2 (liver) cells. The test data showed improved HepG2 cells protection (represents decreased ALT activity) by 33%, 90.2%, and 72.1% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, at 25 µg/mL as compared to the untreated test group. Percentage cellular protection of A549 (lungs) cells (represents increased of SOD activity) was increased by 21.5% at 25.5 µg/mL in the UT-Med + BT-TI, while 33.4%, 25.8%, and 21.5% at 1, 10, and 25.5 µg/mL respectively, in the BT-Med + UT-TI group, and 12.8% increased SOD activity at 25.5 µg/mL in the BT-Med + BT-TI groups as compared to the untreated group. Serotonin level was significantly increased 137.4% (at 0.1 µg/mL), 65.4% (at 0.1 µg/mL), and 77.3% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively compared to untreated test group in human neuroblastoma cells (SH-SY5Y). However, the relative quantification (RQ) of vitamin D receptor (VDR) was significantly increased by 24.2% (at 1 µg/mL), 213.7% (at 10 µg/mL), and 328.7% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared to the untreated in MG-63 cells. Thus, the data concluded that The Trivedi Effect® would be significantly useful for improving multiple organs health, which can be used for many coronary artery diseases, arrhythmias, congenital heart disease, cardiomyopathy, cirrhosis, liver cancer, hemochromatosis, asthma, chronic bronchitis, cystic fibrosis, osteoporosis, etc.

Keywords: The Trivedi Effect®; Biofield Energy Treatment; Organ health; Multiple organ failure; Bone health

Introduction

Herbal based nutraceutical products with selected phytonutrients continue to expand rapidly across the world against treatment of various health challenges among the national healthcare settings [1]. It was observed that the use of complementary and alternative medicines has witnessed a tremendous acceptance among the public and diverted their interest in natural therapies in developing as well as developed countries. More than four billion people in the developing world supports the use of herbal based medicinal therapies, which is one of the primary sources of healthcare and traditional medical practice in various communities [2]. Along with herbal based medicinal products, major supplements such as vitamins, minerals along with many alternative treatment approaches have been found to have significant role in therapeutic approach. The reason for acceptance of these formulations is the minimal or no adverse effects compared with the synthetic drug moieties. Synthetic drugs affect the immune system and results in overall quality of life. It also affects the organs and their functions which results in multiorgan failure. However, there is no such novel herbal-based test formulation that can improve the overall organ health using cell based standard assays. Thus, a novel test formulation has been developed that would improve the overall functioning of multiple organs using standard cell line based bioassay. With this respect, the novel test formulation was designed on the basis of best scientific literature, which is the combination of herbal products *viz.* panax ginseng extract and beta carotene, minerals *viz.* calcium chloride, magnesium gluconate, zinc chloride, sodium selenate, ferrous sulfate, and vitamins *viz.* vitamin B_{12}, vitamin D_3, ascorbic acid, and vitamin B_6. This formulation is designed for overall functioning of the organs that can results in improved overall health conditions against many pathological conditions such as lung disorder, liver disorder, breast cancer, liver cancer, aging, muscle damage, and overall health. Minerals and vitamins present in the test formulation provide significant functional support to all the vital organs [3-5]. Panax ginseng has significant capacity to improve mental, physical abilities, cognitive health, and is defined as the potent immunomodulator [6,7]. The test formulation was tested against many cell lines and was evaluated for biological activities such as bone health parameters using MG-63 cells, lung health parameter using A549 cells, liver health parameter using HepG2 cells, heart health parameter using Human Cardiac fibroblasts, and neuronal health parameter using SH-SY5Y cells [8-17]. In addition, the test formulation and the cell line specific media was

treated with the complementary medicine *i.e.* Biofield Energy Treatment by a renowned Biofield Energy Healer.

Biofield Energy as a Complementary and Alternative Medicine (CAM) includes both putative and subtle energy fields that have the ability to regulate the living organisms homeodynamic function's along with a substantial role in perceptive and guiding various health processes [18]. Besides, many outstanding results have been reported worldwide with the cancer science application of Biofield Energy Healing Treatment [19,20]. Human Biofield Energy is recently reported that it is capable of suppression of mouse lung carcinoma growth besides significant immune function and anti-inflammatory activity [21]. Thus, Biofield Energy Healing therapies have gained popularity because of improved immunological response, while its current status and future perspectives has been reported worldwide [22]. Besides, CAM therapies have been recommended by The National Center for Complementary/Alternative Medicine (NCCAM) and there therapies exist in various therapies such as external qigong, Johrei, Reiki, therapeutic touch, yoga, Qi Gong, polarity therapy, Tai Chi, pranic healing, deep breathing, chiropractic/osteopathic manipulation, guided imagery, meditation, massage, homeopathy, hypnotherapy, progressive relaxation, acupressure, acupuncture, special diets, relaxation techniques, Rolfing structural integration, healing touch, movement therapy, pilates, mindfulness, Ayurvedic medicine, traditional Chinese herbs and medicines in biological systems [23,24]. The Trivedi Effect®-Consciousness Energy Healing therapies have been widely accepted and popular worldwide healing approach with significant results in many scientific field. The Trivedi Effect® has been reported with significant results in the metal physicochemical properties, agriculture science, microbiology, biotechnology, and changed bioavailability of many compounds, skin health, nutraceuticals, cancer science research, improved bone health, human health and wellness [25-40]. Due to the continued clinical and preclinical applications of Biofield Energy Healing Treatments, the test formulation was studied for impact of the Biofield Energy Healing Treated test formulation on the function of vital organs such as bones, heart, liver, lungs, and brain specific biomarkers in different cell-lines.

Materials and Methods

Chemicals and Reagents

Chemicals such as panax ginseng extract were obtained from panacea Phytoextracts, India. Sodium selenate and ascorbic acid were obtained from Alfa Aesar, India. Quercetin was purchased from Clearsynth, India, while silymarin and curcumin were obtained from Sanat Chemicals, India. Ferrous sulfate, vitamin B_6, vitamin D_3, vitamin B_{12}, calcium chloride, naringenin, trimetazidine (TMZ), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), and ethylenediaminetetraacetic acid (EDTA) were procured from Sigma Chemical Co. (St. Louis, MO). Zinc chloride, magnesium gluconate, β-carotene, and calcitriol were purchased from TCI chemicals, Japan. Reverse Transcription Kit, RNeasy Mini Kit, and Syber Green PCR kits were procured from Qiagen, India. All the other chemicals used in this experiment were analytical grade procured from India.

Biofield Energy Healing Treatment

Biofield Energy Healing Treatment was performed on the test formulation and the specific cell line medium. Eleven ingredients combination were used for the test formulation such as panax ginseng extract, β-carotene, zinc chloride, calcium chloride, magnesium gluconate, sodium selenate, ferrous sulfate, ascorbic acid, vitamin B_{12}, vitamin D_3, and vitamin B_6. The test formulation and the medium were divided into two parts, one portion was considered as the untreated group, where no Biofield Energy Treatment was provided (UT-TI and UT-Med). Further, the untreated group was treated with a "sham" healer for comparison purposes, who did not have any knowledge about the Biofield Energy Healing Treatment. Another test formulation portion and the medium received the Biofield Energy Treatment (The Trivedi Effect®) remotely by Sakina Aleemah Ansari under standard laboratory conditions for ~3 minutes through healer's unique Biofield Energy Transmission process and were referred as the Biofield Energy Treated formulation (BT-TI) and Biofield Energy Treated medium (BT-Med). The Biofield Energy Healer was located in the USA, however the test formulation constituents were located in the research laboratory of Dabur Research Foundation, New Delhi, India. Biofield Energy Healer in this experiment did not visit the laboratory, nor had any contact with the test sample and the medium. After that, the Biofield Energy Treated and untreated test items were kept in similar sealed conditions and used for the study as per the study plan.

Cell Viability Testing using MTT Assay

All the experimental cells used in this study were counted for cell viability using hemocytometer in 96-well plates at the specific density as mentioned in the Table 1. The cells were then incubated overnight under standard growth conditions to allow cell recovery and exponential growth. Following overnight incubation, cells were treated with different concentrations of test formulations

(BT/UT). After respective treatments, the cells were incubated in a CO_2 incubator at 37°C, 5% CO_2, and 95% humidity. After incubation, the plates were taken out and 20 µL of 5 mg/mL of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution was added to all the wells followed by additional incubation for 3 hours at 37°C. The supernatant was aspirated and 150 µL of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was read at 540 nm using Synergy HT microplate reader. The percentage cytotoxicity at each tested concentration was calculated using Equation 1:

% Cytotoxicity = [(R-X)/R] *100..... (1)

Where, X = Absorbance of treated cells; R = Absorbance of untreated cells

The concentrations exhibiting percentage cytotoxicity <30% was considered as non-cytotoxic [41].

S. No.	Cell Line	Plating	Time Point
1	MG-63 (Bone)	$3x10^4$ cells/ well, 96-well plate	5 days
2	Ishikawa (Uterus)	$3x10^4$ cells/ well, 96-well plate	5 days
3	A549 (Lung)	$10x10^4$ cells/ well, 96-well plate	24 hours
4	HepG2 (Liver)	$1x10^4$ cells/ well, 96-well plate	24 hours
5	Human Cardiac fibroblasts (Heart)	$1x10^4$ cells/ well, 96-well plate	24 hours
6	SH-SY5Y (Neuronal cell)	$10x10^4$ cells/ well, 96-well plate	24 hours

Table 1: Information related to six cell lines with their plating density and time-point.

Cytoprotective Action of the Test Formulation

Cytoprotective effect was evaluated on the human cardiac fibroblasts-HCF; human hepatoma cells-HepG2; and adenocarcinomic human alveolar basal epithelial cells-A549. These cells were counted and plated in suitable medium followed by overnight incubation. The cells were then treated with the test items/positive control at the non-cytotoxic concentrations for 24 hours. After 24 hours, the oxidative stress using 10 mM *t*-BHP for 3.5 hours was given to the cells. The cells treated with 10 mM of *t*-BHP alone served as negative control. After 3.5 hours of incubation with *t*-BHP the above plates were taken out and cell viability was determined by MTT assay. The percentage protection corresponding to each treatment was calculated using equation 2:

$$\% \text{ Protection} = [(\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{t-BHP}})]*100 / [\text{Absorbance}_{\text{untreated}} - \text{Absorbance}_{\text{t\_BHP}}] \quad (2)$$

Estimation of Alkaline Phosphatase (ALP) activity

ALP was estimated in two cells *viz.* human bone osteosarcoma cells-MG-63 and human endometrial adenocarcinoma cells-Ishikawa, which were counted using a hemocytometer and plated in 24-well plates at the density corresponding to 1 X 10^4 cells/well in phenol-free DMEM supplemented with 10% CD-FBS. After the respective treatments, the cells in the above plate were incubated for 48 hours in CO_2 incubator at 37°C, 5% CO_2, and 95% humidity. After 48 hours of incubation, the plates were taken out and processed for the measurement of ALP enzyme activity. The cells were washed with 1 X PBS and lysed by freeze-thaw method *i.e.,* incubation at -80°C for 20 minutes followed by incubation at 37°C for 10 minutes. To the lysed cells, 50 µL of substrate solution *i.e.*5 mM of *p*-nitrophenyl phosphate (*p*NPP) in 1M diethanolamine and 0.24 mM magnesium chloride ($MgCl_2$) solution (pH 10.4) was added to all the wells followed by incubation for 1 hour at 37°C. The absorbance was measured at 405 nm using Synergy HT microplate reader (Biotek, USA). The absorbance values were obtained and normalized with substrate blank (*p*NPP solution alone) absorbance values. The percentage increase in ALP enzyme activity with respect to the untreated cells (baseline group) was calculated using Equation 3:

$$\% \text{ Increase in ALP} = \{(X-R)/R\}*100 \quad (3)$$

Where, X = Absorbance of cells corresponding to positive control and test groups

R = Absorbance of cells corresponding to baseline group (untreated cells)

Estimation of Lactate Dehydrogenase (LDH) in Human Cardiac Fibroblasts (HCF) Cells

HCF cells were used for the estimation of LDH activity. The cells were counted and plated at the density of 0.25 X 10^6 cells/ well in 24-well plates in cardiac fibroblast specific mediumfollowed by overnight incubation. The cells were then treated with the test formulation combinations/positive control at the non-cytotoxic

concentrations for 24 hours. After 24 hours, oxidative stress was given to the cells using 10 mM *t*-BHP for 3.5 hours. The untreated cells were served as control group, which did not receive any treatment and were maintained in cell growth medium only. The cells were treated with 10 mM of *t*-BHP alone served as the negative control. After 3.5 hours of incubation with *t*-BHP, the above plates were taken out and LDH activity was determined using LDH activity kit as per manufacturer's instructions. The percent increase in LDH activity was calculated using equation 4.

$$\% \text{ Increase} = [(\text{LDH activity}_{\text{sample}} - \text{LDH activity}_{\text{t-BHP}})]*100 / [\text{LDH activity}_{\text{untreated}} - \text{LDH activity}_{\text{t\_BHP}}] \quad (4)$$

Estimation of ALT in Liver Cells (HepG2)

The human hepatoma cells (HepG2) were used for the estimation of ALT activity. The cells were counted and plated at the density of 5 X 10^4 cells/well in 48-well plates in DMEM media followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations for 24 hours. After 24 hours, oxidative stress was given to the cells using 400 µM *t*-BHP for 3.5 hours. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. Cells treated with 400 µM of *t*-BHP alone served as negative control. After 3.5 hours of incubation with *t*-BHP, the above plates were taken out and ALT activity was determined using ALT activity kit as per manufacturer's instructions. The percent increase in ALT activity was calculated using equation 5.

$$\% \text{ Increase} = [(\text{ALT activity}_{\text{sample}} - \text{ALT activity}_{\text{t-BHP}})]*100 / [\text{ALT activity}_{\text{untreated}} - \text{ALT activity}_{\text{t\_BHP}}] \quad (5)$$

Estimation of Superoxide Dismutase (SOD) in Lung (A549) Cells

The adenocarcinomic human alveolar basal epithelial cells (A549) were used for the estimation of SOD activity. The A549 cells were counted and plated at the density of 1 X 10^4 cells/well in 24-well plates in DMEM followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations along with 100 µM *t*-BHP to induce oxidative stress. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. Cells treated with 100 µM of *t*-BHP alone served as negative control. After 24 hours of incubation with *t*-BHP the above plates were taken out and SOD activity was determined using SOD activity kit as per manufacturer's instructions. The percent increase in SOD activity was calculated using equation 6:

$$\% \text{ Increase in SOD activity} = ((X-R)/R)*100 \quad (6)$$

Where, X = SOD activity corresponding to test item or positive control

R = SOD activity corresponding to the control group.

Estimation of Serotonin in Neuronal Cells (SH-SY5Y)

The human neuroblastoma (SH-SY5Y) cells were used for the estimation of serotonin level. The cells were counted and plated at the density of 10 X 10^4 cells/well in 96-well plates followed by overnight incubation. The cells were then treated with the test formulation/positive control at the non-cytotoxic concentrations. The untreated cells served as control that did not receive any treatment and were maintained in cell growth medium only. The treated cells were incubated for 24 hours. Serotonin release was determined by ELISA as per manufacturer's protocol. The percent increase in serotonin levels was calculated using equation 7-

$$[(X-R)/R]*100 \quad (7)$$

Where, X = Serotonin levels corresponding to test item or positive control,

R = Serotonin levels corresponding to control group.

Effect of Test Formulation on Vitamin D Receptor (VDR) in Bone (MG-63) Cells

The effect of test formulation on vitamin D receptor (VDR) activity in bone (MG-63) cells were counted using the hemocytometer at density 2 X 10^5 cells/well in 6-well plates followed by overnight incubation. The cells were then sera starved for 24 hours and treated with the test formulation/positive control at the non-cytotoxic concentrations, while control group did not receive any treatment, which were maintained in cell growth medium only. The treated cells were incubated for 24 hours and VDR expression was determined by qPCR using VDR specific primers. Cells were harvested by scrapping and washed with PBS. Cell pellets obtained were analyzed for VDR gene expression using human VDR specific primers: Forward: 5'-GCTGACCTGGTCAGTTACAGCA-3', Reverse: 5'-CACGTCACTGACGCGGTACTT-3'.VDR gene expression was normalized using House-keeping (HK) reference. Relative quantification (RQ) of VDR gene in Biofield Energy Treated cells was calculated with respect to the untreated cells using equation 8:

$$RQ = 2\text{-}N \quad \text{..... (8)}$$

Where, N is the relative Threshold Cycle (C_T) value of treated sample with respect to the untreated sample.

Statistical Analysis

All the experimental values were presented as mean ± SD (standard deviation) of three independent experiments. The statistical analysis was performed using SigmaPlot statistical software (v11.0). For two group comparison, student's *t*-test was used. For multiple group comparison, one-way analysis of variance (ANOVA) was used followed by post-hoc analysis by Dunnett's test. Statistically significant values were set at the level of $p \leq 0.05$.

Results and Discussion

Cell Viability using MTT Assay

MTT assay for cell viability testing was used in each cell lines for testing the safe concentrations of test formulation. The concentrations of the test formulation were found safe on the basis of percentage of cell viability data. The criteria for selection of non-cytotoxic test formulation concentration and the positive controls were found to be less than 30% cytotoxicity or greater than 70% cell viability. All the results were considered and represented as safe and non-cytotoxic concentrations. The experimental data suggested that the overall percent cell viability in different cell-lines *viz.* MG-63, Ishikawa, A549, HepG2, HCF, and SH-SY5Y were found safe, which were tested for other activities.

Evaluation of Cytoprotective Effect of the Test Formulation

Cytoprotective activity of the test formulation was screened against three cell lines *viz.* HCF, HepG2, and A549 cells. The results were presented in terms of percentage cellular protection against *t*-BHP induced cell damage (Figure 1). Cytoprotective activity in the cell line based assay after induction of oxidative stress using *tert*-butyl hydroperoxide (*t*-BHP) is considered as the gold standard [41,42]. Trimetazidine (TMZ) was used as a positive control group in human cardiac fibroblasts cells (HCF) for cytoprotective effect which showed significant restoration of cell viability by 48%, 57.2%, and 87.2% at 5, 10, and 25 µg/mL, respectively as compared to the *t*-BHP induced group. Besides, the restoration of cell viability among the tested groups by the test formulation was reported as 59.5% and 105.4% at 10 and 25 µg/mL respectively, in the UT-Med + BT-TI group, while 32.8% improved cellular restoration at 25 µg/mL in the BT-Med + UT-TI, and 151.8% and 97.4% improved cellular restoration at 10 and 25 µg/mL respectively, in the BT-Med + BT-TI group as compared to the untreated test group (UT-Med + UT-TI group). Similarly, silymarin was used as positive control in HepG2 cells, which resulted in significant cellular restoration by 40%, 65.9%, and 86.6% at 5, 10 and 25 µg/mL respectively, as compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 6.1% and 22.4% at 0.1 and 25.5 µg/mL respectively, in the UT-Med + BT-TI group, while 54.6% and 67.1% improved cellular restoration at 1 and 10 µg/mL respectively, in the BT-Med + UT-TI, and 55.1%, 72.9%, and 6.7% improved cellular restoration at 0.1, 10 and 25.5 µg/mL respectively, in the BT-Med + BT-TI group as compared to the untreated test group (UT-Med + UT-TI group). In addition, quercetin was used as positive control in adenocarcinomic human alveolar basal epithelial cells (A549) resulted, restoration of cell viability by 47.3%, 56.8%, and 66.4% at 5, 10 and 25 µg/mL, respectively compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 1.6% at 10 µg/mL in the UT-Med + BT-TI group, while 9.3%, 70.4%, and 14.1% increased cell viability at 0.1, 1, and 25.5 µg/mL respectively in the BT-Med + UT-TI group. Similarly, 3% and 4.6% improved cellular restoration was reported at 10 and 25.5 µg/mL respectively, at BT-Med + BT-TI groups as compared to the UT-Med + UT-TI group. Cytoprotection action is directly correlated with the protection of cells against injuries due to stress and free radicals [43,44]. This cell damage is one of the leading causes of immune related disorders such as cardiovascular diseases, aging, cancer, diabetes, and many more [45-47]. Overall, it can be assumed cellular restoration was achieved due to Biofield Energy Treatment (The Trivedi Effect®) after induction of *t*-BHP induced oxidative stress against the HCF, HepG2, and A549 cells with respect to the cardiotoxicity, hepatotoxicity, and lung cell toxicity. Therefore, the Biofield Energy Healing Treatment could be used against many pathological etiologies such as cardiovascular, liver, and lung diseases.

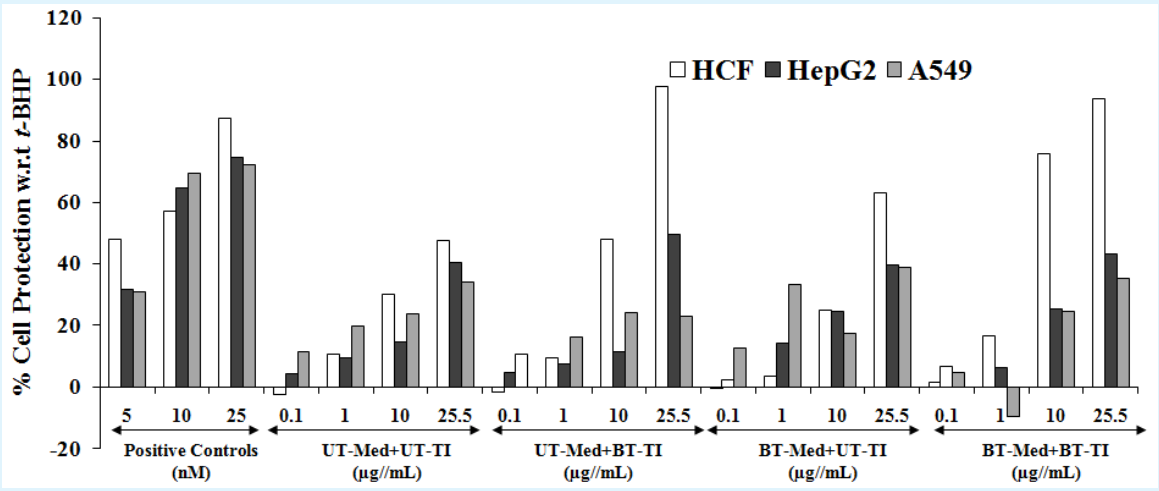

Figure 1: Cytoprotective action of the test formulation in human cardiac fibroblasts cells (HCF), human hepatoma cells (HepG2), and adenocarcinomic human alveolar basal epithelial cells (A549) against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. Trimetazidine (µM), silymarin (µg/mL), and quercetin (µM) were used as positive control in HCF, HepG2, and A549 cells, respectively. UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of Alkaline Phosphatase (ALP) Activity

ALP is one of the important bone health biomarker, it correlation has been reported in controlling various bone disorders [48,49] such as low bone density and osteoporosis, osteogenesis imperfect and Paget's disease, which makes bones brittle. ALP activity was evaluated against two cell lines, MG-63 and Ishikawa cells after treatment with the test formulation. Naringenin (nM) was used as positive control in Ishikawa cells, and the results suggested significant increased ALP level by 18.3%, 35.8%, and 109.4% at 0.1, 1, and 10 nM respectively as presented in the Figure 2.

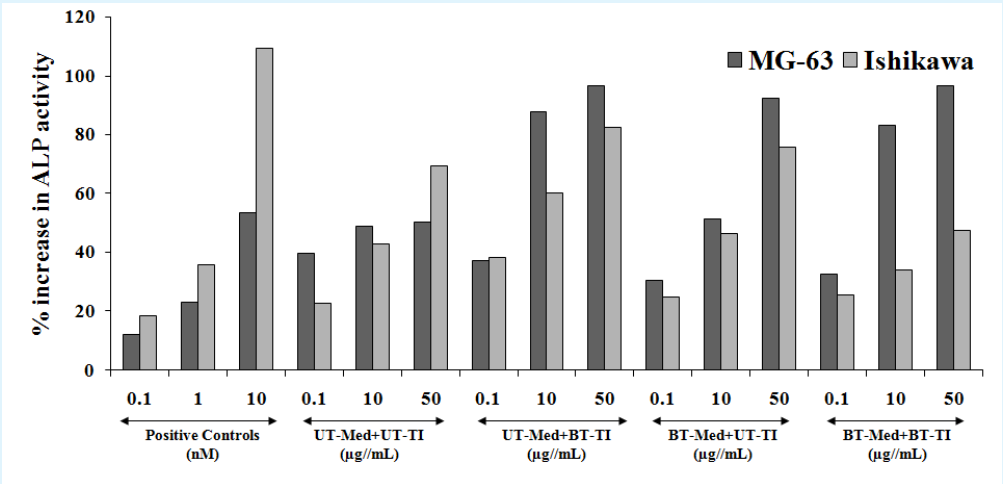

Figure 2: Alkaline phosphatase (ALP) activity in human bone osteosarcoma cells (MG-63) and human endometrial adenocarcinoma cells (Ishikawa) after treatment of the test formulation. Calcitriol and naringenin were used as positive control in MG-63 and Ishikawa cells, respectively. UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

However, the experimental test groups showed maximum increased ALP activity by 68.4%, 41.1%, and 18.8% at 0.1, 10, and 50 µg/mL respectively, in the UT-Med + BT-TI, while 9.6%, 8.2%, and 9.2% increased ALP activity at 0.1, 10, and 50 µg/mL respectively, in the BT-Med + UT-TI group, and 12.3% improved ALP level was found at 0.1 µg/mL in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group in Ishikawa cells. Similarly, calcitriol was used as positive control for MG-63 cells, and the data showed significant improved level of ALP by 12%, 23%, and 53.5% at 0.1, 1, and 10 nM, respectively. In the experimental tested group of MG-63 cells, the ALP percent was significantly increased by 79.5% and 92.7% at 10 and 50 µg/mL, respectively in the UT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Similarly, ALP percent was significantly increased by 5.1% and 84.5% at 10 and 50 µg/mL, respectively in the BT-Med + UT-TI group as compared to the UT-Med + UT-TI group. However, ALP percent was significantly increased by 69.8% and 93.2% at 10 and 50 µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group in the MG-63 cells. Overall, it can be concluded that after treatment with the Biofield Energy Healing Treatment significant level of ALP was increased. Thus, Biofield Energy Treated Test formulation would be highly recommended option in various bone disorders without any adverse effects.

Lactate Dehydrogenase (LDH) Activity in Human Cardiac Fibroblasts (HCF)

LDH is an enzyme found in all the living cells and found to be responsible for anaerobic cellular respiration. LDH is extensively expressed in most of the body tissues, such as blood cells, skeletal muscle, and heart muscle and play a vital role in tissue injury, necrosis, hypoxia, hemolysis, or malignancies. LDH is one of the ideal biomarker for heart disease or tissue injuries [50]. LDH activity was estimated in HCF cells, and the data was presented as increased HCF cellular protection, which represents decreased LDH activity in various groups. The effect of test formulation in different groups with respect to the percent protection of HCF cells in terms of decreased level of lactate dehydrogenase (LDH) activity is presented in the Figure 3.

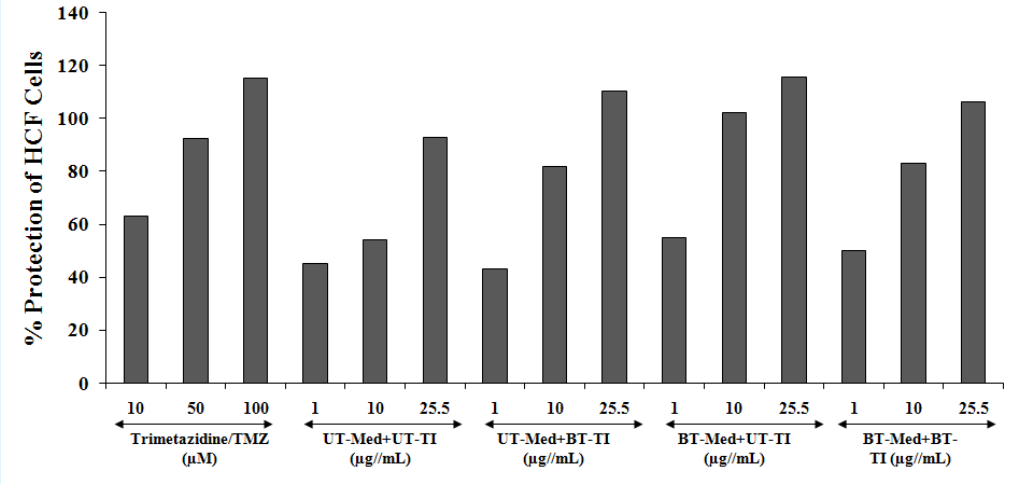

Figure 3: The effect of the test formulation on the increased percent protection of HCF cells (i.e. decreased lactate dehydrogenase) activity against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. TMZ: Trimetazidine; UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

The positive control, trimetazidine (TMZ) showed 63.1%, 92.3%, and 115.2% increased cellular protection of HCF cells (decreased of LDH activity) at 10, 50, and 100 µM concentration as compared to the *t*-BHP group. The test formulation showed maximum percent protection of HCF cells (decreased of LDH activity), which was significantly increased by 51.6% and 19% at 10 and 25.5 µg/mL concentrations respectively, in the UT-Med + BT-TI group, while 21.1%, 88.7%, and 24.5% improved cellular protection (decreased of LDH activity) at 1, 10, and 25.5 µg/mL respectively, in the BT-Med + UT-TI group, and 10.8%, 53.7%, and 14.6% improved cellular protection (decreased of LDH activity) at 1, 10, and 25.5 µg/mL respectively, in BT-Med + BT-TI group as

compared to the UT-Med + UT-TI group. Thus, the results suggested that significant reduced level of LDH activity in term of percentage cellular protection after treatment with the test formulation. LDH activity can be best depicted using HCF cells, as these cells play a central role in the extracellular matrix maintenance of the normal heart functioning [51,52]. Thus, overall data suggested that LDH activity using HCF cells was significantly reduction after Biofield Energy Treatment that could be useful against various pathological conditions such as tissue injury, necrosis, hypoxia, hemolysis or malignancies.

Estimation of Alanine Amino Transferase (ALT) Activity in HepG2 Cells

ALT is one of the important liver health enzymes and its alteration may results in hepatocellular injury and death. ALT is also present in kidney, heart, and muscles along with liver [53]. High level of ALT enzyme may results in decreased cell viability and cell death [54]. ALT activity was estimated using HepG2 cell and the results are presented in terms of increased percentage cellular protection (represents decreased ALT activity) in the Figure 4. The positive control, silymarin was in HepG2 cells for ALT activity and the data suggested increased percentage cellular protection of HepG2 cell (decreased ALT activity) by 40.2%, 63.4%, and 103.7% at 5, 10, and 25 µg/mL concentrations, respectively. Similarly, the test formulation groups showed improved cellular protection of HepG2 cells (*i.e.* decreased of ALT activity) by 27.7%, 33%, and 10.3% at 10, 25.5, and 63.75 µg/mL respectively, in the UT-Med + BT-TI group, while increased cellular protection of HepG2 cells (decreased of ALT activity) by 15.5%, 90.2%, and 57.1% at 10, 25.5, and 63.75 µg/mL respectively, in the BT-Med + UT-TI group, and increased cellular protection of HepG2 cells (decreased of ALT activity) by 72.1% and 70.2% at 25.5 and 63.75 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 4). Overall, the data of this activity showed that Biofield Energy Treatment significantly improved the cellular protection with reduced ALT enzyme, which suggests its application in the liver cancer, liver cirrhosis, hepatomegaly, liver failure, and hepatitis.

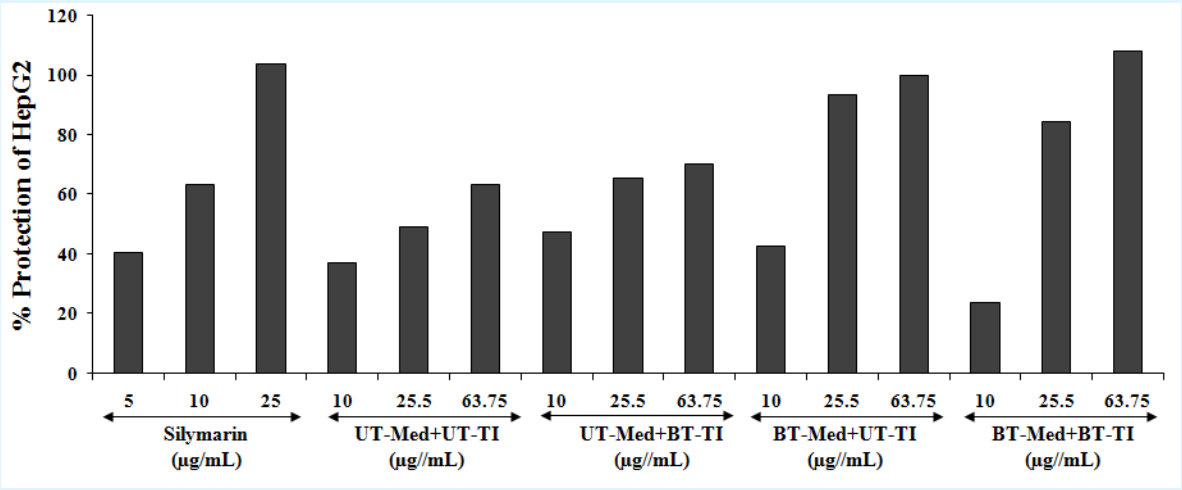

Figure 4: Increased percentage protection of liver cells (HepG2) representing decreased (ALT) Alanine amino transaminase activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Superoxide Dismutase (SOD) Activity in Adenocarcinomic Human Alveolar Basal Epithelial Cells (A549)

SOD is one of the best antioxidant defense systems in nearly all living cells that are exposed to oxygen. It also prevents the cellular damage against various types of stress and free radicals, which results in cell death [55]. The SOD activity was estimated using A549 cells and improved activity represents the increased cellular protection (Figure 5). The positive control, quercetin showed improved percentage of SOD activity with respect to the *t*-BHP by 68.4%, 3.9%, and 104.2% at 10, 25, and 50 µg/mL concentration respectively. However, the percent protection of A549 (lungs) cells (increased of SOD activity) was significantly increased by 5.5% and 21.5% at 1 and 25.5 µg/mL respectively, in the UT-Med + BT-TI group, while increased SOD activity by 33.4%, 25.8%, and

21.5% at 1, 10, and 25.5 µg/mL respectively, in the BT-Med + UT-TI group, and increased SOD activity by 5.5% and 12.8% at 1 and 25.5 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group(Figure 5). Overall, the experimental data revealed that the Biofield Energy Treatment has significantly improved the SOD antioxidant defense activity, which could protect from many respiratory diseases such as Alzheimer's disease, pneumonia, asthma, Down's syndrome, familial amyotrophic lateral sclerosis (FALS), Parkinson's disease, dengue fever, cancer, cataract, and several neurological disorders.

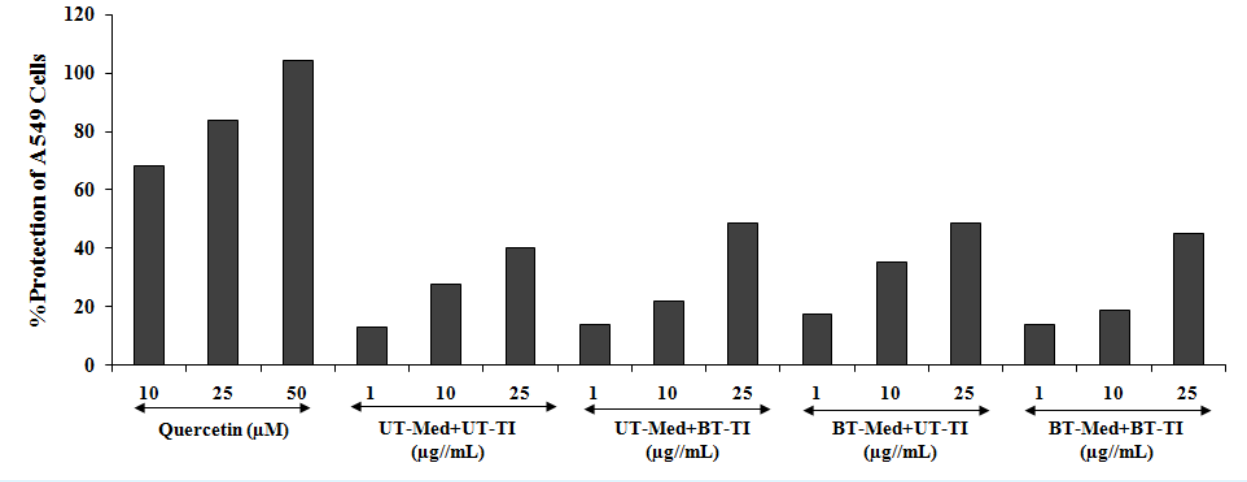

Figure 5: Percent cellular protection of lungs cells (A549) in terms of increased SOD activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of Serotonin Level In Human Neuroblastoma (SH-SY5Y) cells

Serotonin assay was performed using SH-SY5Y cells and the effect of test formulation was assessed after 24 hours of treatment using ELISA assay. Low level of serotonin represents depression because it is important neurotransmitter. In addition, it is supposed to be responsible for many neuropsychiatric disorders (*viz.* Alzheimer's disease, cognitive health, loss of ability of thinking, depression, memory loss, etc.) along with various neuronal disorders like sleep, feeding, pain, sexual behavior, cardiac regulation, and cognition [56]. Serotonin activity was tested and the data is presented in the Figure 6.

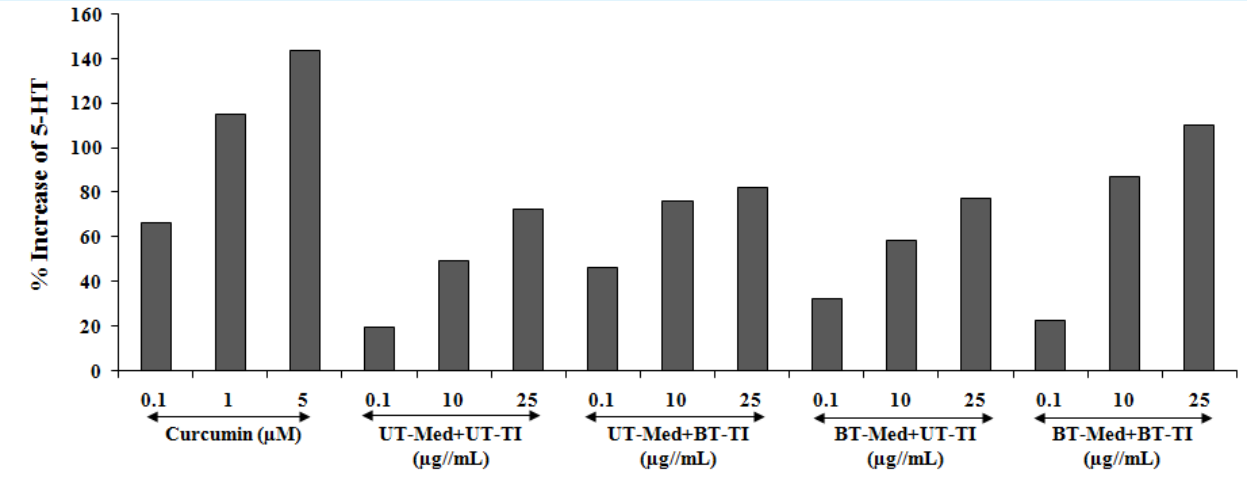

Figure 6: The effect of the test formulation on percent increase in 5-hydroxy tryptamine (5-HT) in human neuroblastoma cells (SH-SY5Y). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Curcumin was used a positive control, showed 66.3%, 115.1%, and 143.4% increased level of serotonin at 0.1, 1, and 5 µg/mL respectively, compared to the vehicle control (VC) group. The experimental data showed significant increased serotonin level by 137.4%, 5.1%, and 13% at 0.1, 10, and 25 µg/mL respectively, in the UT-Med + BT-TI, while significant increased serotonin by 65.4%, 18.8%, and 6.4% at 0.1, 10, and 25 µg/mL respectively, in the BT-Med + UT-TI, and 16.9%, 77.3%, and 51.8% improved serotonin level at 0.1, 10, and 25 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 6). Thus, serotonin level was significantly improved in the entire tested group. Our data revealed significant improved level of serotonin after treatment with the Biofield Energy Healing Treated test formulation that would be highly useful against various neurodegenerative diseases, improved brain functioning, social behavior, appetite and digestion, sleep, memory, sexual desire, and associated functions.

Effect of Test Formulation on Vitamin D Receptors (VDRs) in MG-63 Cells

Human bone osteosarcoma cells (MG-63) was used for the estimation of VDR activity. VDR expression was studies using ligand binding through vitamin D active molecule, which was estimated using quantitative-polymerase chain reaction (qPCR) amplification. With the use of real time PCR, different VDR-relative threshold cycle (VDR-C_T) values were obtained after complete amplification cycles using specific primer probes. Relative quantification (RQ) was calculated from the VDR-C_T and house-keeping (HK)-C_T values in MG-63 cells. The values after treated with the Biofield Energy Treated and untreated test formulation and positive control are represented in the Figure 7. Calcitriol was used as a positive control and the RQ of VDR was found to be increased in concentration-dependent manner by 55.8%, 65.9%, and 109.9% at 0.1, 1, and 10 nM, respectively. The experimental test groups showed increased RQ of VDR expression by 24.2% and 4.4% in the UT-Med + BT-TI group at 1 and 50 µg/mL respectively, while 64.9%, 213.7%, and 127% increased RQ of VDR at 1, 10, and 50 µg/mL respectively, in the BT-Med + UT-TI group, and increased RQ of VDR by 166.6%, 328.7%, and 205.1% at 1, 10 and 50 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Calcitriol was reported to bind with the VDRs and extensively regulates the calcium homeostasis, immunity, overall cellular growth, and differentiation [57]. Calcitriol controls various calcium metabolisms and play a vital role in improving quality of life and overall bone cell growth and development [58,59]. In conclusion, VDR expression was significantly improved in MG-63 after treatment with the Trivedi Effect®, which could be the best alternative treatment approach for bone related disorders.

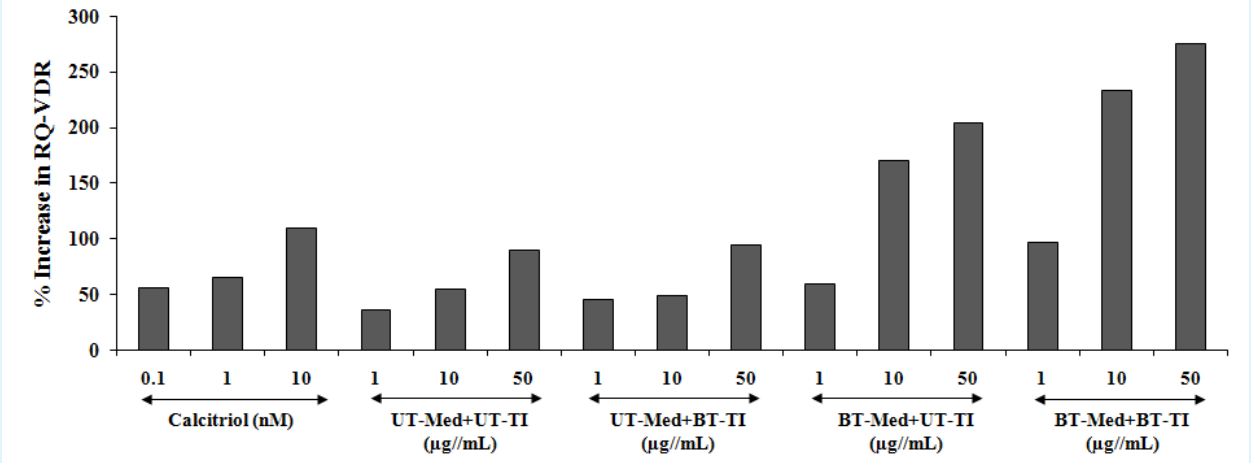

Figure 7: Relative quantification (RQ) of vitamin D receptors (VDRs) gene in human bone osteosarcoma cells (MG-63).UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Conclusion

MTT assay was used for estimation of safe concentrations of the test formulation and results showed that the test formulation was found safe and non-toxic against all the tested cell lines. Cytoprotective activity against *t*-BHP induced cell damage was tested using human cardiac fibroblasts cells (HCF), which showed

restoration of cell viability by 105.4% (at 25 µg/mL), 32.8% (at 25 µg/mL), and 151.8% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI groups respectively, as compared to the untreated test group, while in HepG2 cells the maximum restoration of cell viability was by 22.4% (at 25.5 µg/mL), 67.1% (at 10 µg/mL), and 72.9% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI groups respectively, as compared to the untreated test group. In A549 cells, cellular restoration was improved by 70.4%, and 14.1% at 1 and 25.5 µg/mL respectively, in the BT-Med + UT-TI group, while 3% and 4.6% improved cellular restoration was reported at 10 and 25.5 µg/mL respectively, at BT-Med + BT-TI groups as compared to the untreated test group. Similarly, ALP activity in Ishikawa cells showed significantly increased ALP activity by 68.4%, 9.6%, and 12.3% in the UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, at 0.1 µg/mL as compared to the untreated test group. Similarly, ALP activity in MG-63 cells with maximum cellular protection was reported at 50 µg/mL by 92.7%, 84.5%, and 93.2% in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI group test groups, respectively, as compared with the untreated test group. LDH activity was significantly decreased and the data was presented in increased percentage cellular protection data, which showed maximum cellular protection at 10 µg/mL concentration by 51.6%, 88.7%, and 53.7% in the UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, as compared to the untreated test group. ALT activity showed maximum improved cellular protection of HepG2 cells (decreased of ALT activity) by 33%, 90.2%, and 72.1% at 25.5 µg/mL in the UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, as compared with the untreated test group. SOD activity was significantly increased by 21.5% (at 25.5 µg/mL), 33.4% (at 1 µg/mL), and 12.8% (at 25.5 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, as compared with the untreated test group. Serotonin level was significantly increased in SH-SY5Y cells by 137.4% (at 0.1 µg/mL), 65.4% (at 0.1 µg/mL), and 77.3% (at 10 µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, as compared with the untreated test group. VDR expression was tested in MG-63 cells, which showed increased RQ of VDR by 24.2% at 1 µg/mL in the UT-Med + BT-TI group, while 64.9%, 213.7%, and 127% increased RQ of VDR at 1, 10, and 50 µg/mL respectively in the BT-Med + UT-TI group, and 166.6%, 328.7%, and 205.1% increased RQ of VDR at 1, 10 and 50 µg/mL respectively, in the BT-Med + BT-TI groups as compared to the untreated test control group. Thus, it can be concluded that Biofield Energy Treatment (The Trivedi Effect®) can be used for the prevention of various types of cardiac disorders such as stroke, thromboembolic disease, congestive heart failure, congenital heart disease, peripheral artery disease, rheumatic heart disease, valvular heart disease, and venous thrombosis, etc. Biofield Energy based test formulation can improve the overall functioning of heart, liver, bones, neuronal, and lungs parameters against wide range of oxidative stress or damage induced by free radicals. In addition, it would also protect against many hepatic disorders (cirrhosis, liver cancer, hemochromatosis, and Wilson disease), lungs disorders (asthma, chronic bronchitis, emphysema, cystic fibrosis, and pneumonia), and many immune disorders. In addition, this novel test formulation can also be utilized for organ transplants (*i.e.,* kidney, liver, and heart transplants), hormonal imbalance, aging, and various inflammatory and immune-related disease conditions like Asthma, Aplastic Anemia, Graves' Disease, Dermatitis, Diabetes, Parkinson's Disease, Myasthenia Gravis, Ulcerative Colitis (UC), Atherosclerosis, etc. to improve overall health and Quality of Life.

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Conflicts of Interest

The authors declare no conflicts of interest.

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