

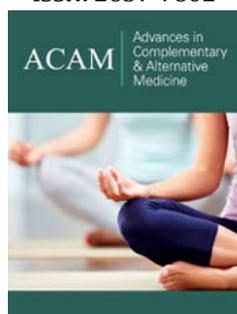
Analysis of the Biofield Energy Based Test Formulation on Vital Organ Health Specific Biomarkers Using Cell Based Assay

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Abstract

Vital organs dysfunction is one of the major concerns now-a-day, which results in high mortality rate in health care centers. Thus, growth and normal functioning of the vital organs is the major concern for better health. The aim of this study was to investigate the impact of the Biofield Energy on the test formulation and the cell line media for the function of vital organs such as bones, heart, liver, lungs, and brain using cell-based assays. Different organ-based cell lines were used in the study for testing the effects of test formulation. The test item (TI) and specific cell line media (Med) was divided into two parts; one untreated (UT-TI) and other part received the Biofield Energy Treatment remotely by a renowned Biofield Energy Healer, Maria Isabel Aguilar Tiraboschi, Uruguay and were labeled as the Biofield Energy Treated (BT) test formulation. Cell viability data suggested that the test formulation was safe and non-toxic in nature in the tested cell lines. Cytoprotective action of the test formulation showed a significant maximum restoration of cell viability by 23.7% (at 25.5µg/mL), 54.1% (at 10µg/mL), and 81.6% (at 63.75µg/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 human cardiac fibroblasts cells (HCF) cells, while 53.4% (at 63.75µg/mL), 20.2% (at 63.75µg/mL), and 43.9% (at 10µg/mL) improved cellular protection of human hepatoma cells (HepG2) cells 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 addition, cytoprotective activity in adenocarcinoma human alveolar basal epithelial cells (A549) showed improved cell viability by 121.9% (at 1µg/mL), 416% (at 25.5µg/mL), and 323% (at 25.5µg/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. ALP activity in MG-63 cells was maximum increased by 78.2% at 50µg/mL in the BT-Med + BT-TI group, while in Ishikawa cells showed maximum increased ALP activity by 67.5% at 50µg/mL in the BT-Med + UT-TI group as compared to the untreated group. The maximum percent cellular protection of HCF (heart) cells (decreased of LDH activity) was significantly increased by 26.6% (at 25.5µg/mL), 37.9% (at 10µg/mL), and 76.5% (at 25.5µg/mL) 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. Alanine amino transferase (ALT) in terms of percent protection of HepG2 (liver) cells (decreased of ALT activity) was reported by 21.1% (at 10µg/mL), 93.9% (at 63.75µg/mL), and 51.4% (at 63.75µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI groups respectively, as compared to the untreated test group. Cellular protection of A549 (lungs) cells (increased of SOD activity) in terms of percentage was increased by 57.2% (at 25.5µg/mL), 176.9% (at 10µg/mL), and 184.2% (at 10µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared to untreated group. Serotonin level was significantly increased by 34.8% (at 63.75µg/mL), 65.4% (at 63.75µg/mL), and 398.7% (at 1µg/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med+BT-TI groups, respectively compared to the untreated test group in human neuroblastoma cells (SH-SY5Y). However, the relative quantification (RQ) of vitamin D receptor (VDR) was significantly increased by 207.9% (at 0.1µg/mL), 37.1% (at 10µg/mL), and 141% (at 10µg/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, Biofield Energy Treated test formulation (The Trivedi Effect®) would be significantly useful for multiple organ health that can be used against coronary artery disease, arrhythmias, congenital heart disease, cardiomyopathy, cirrhosis, liver cancer, hemochromatosis, asthma, chronic bronchitis, cystic fibrosis, osteoporosis, etc.

Keywords: The Trivedi effect®, Biofield Energy Treatment; Cardiac health; Liver health; Lungs health; VDR receptor; Brain health; Bone health

Introduction

The use of herbal based test formulations and their related remedies are widely embraced in most of the developed countries as one of the best approach of complementary and alternative medicines (CAMs). These alternative treatment approaches are now becoming the

mainstream treatment remedies in many countries such as in the UK and the rest of Europe, as well as in North America and Australia [1]. The major reason behind accepting the herbal based therapies in developing countries is the belief that these therapies would promote overall quality of life in healthier living [2]. These therapies are accepted as a balanced and moderate healing approach for individuals as compared with the synthetic medicines or over-the-counter drugs, which are very expensive and associated with some adverse effects. Due to these drawbacks, herbal based test formulations in the global market are booming and contributing a huge percent in drug market [3]. Besides, minerals, vitamins, and other vital constituents are now added in herbal formulation in order to provide significant healing in health care systems. These unique formulations can be useful for managing high blood pressure, heart disease, asthma, other respiratory diseases, immunodeficiency diseases, aging and many more [4]. With this respect, a novel herbomineral test formulation was developed that would improve the overall functioning of multiple organs.

The novel test formulation was 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 B₁₂, vitamin D₃, ascorbic acid, and vitamin B₆. Minerals and vitamins used in the novel formulation were added due to their significant use to support the organ health and its functioning [5-8]. Panax ginseng overall improve wellness and thinking, memory, concentration, physical stamina, work efficiency, preventing muscle damage, Alzheimer's disease, athletic endurance, improve mental and cognitive health, and is a potent immunomodulator [9,10]. This formulation was tested using standard organ functioning specific cell line based assays for different biological activities. The cell-based activities included bone health study using MG-63 cells, lung health study using A549 cells, liver health study using HepG2 cells, heart health study using Human Cardiac fibroblasts, and neuronal health study using SH-SY5Y cells [11-20]. In addition, the test formulation and the cell based specific media was treated with the one of the complementary medicine i.e. Biofield Energy (The Trivedi Effect®-Consciousness Energy Healing) by a renowned Biofield Energy healer. Biofield Energy healing practice has wide existence from thousands of years in various forms, which purport to sense and modulate the subtle energies of the body. Various form of CAM based biofield therapies regulates the energy fields and interact with energy fields with the information that surrounds living systems in order to improve the healing process. This treatment approach is reported to have significant clinical importance in terms of improved overall physical, mental, and emotional human wellness [21-24] without any invasive procedures.

Thus, Biofield Energy Treatment is one of the best unifying concepts between the traditional and contemporary explanatory energy models. The Trivedi Effect®-Consciousness Energy Healing has been accepted worldwide with significant results in the field of metal science [25,26], agriculture science [27], microbiology [28,29], biotechnology [30,31], and improved compounds bioavailability [32,33], dermatology [34,35], nutraceuticals [36],

cancer research [37], bone health [38-40], human health and wellness. Due to the continued clinical and preclinical applications of Biofield Energy Healing Treatments, the novel proprietary 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 standard cell-lines.

Material and Methods

Chemicals and reagents

Ferrous sulfate, vitamin B₆, vitamin D₃, vitamin B₁₂, calcium chloride, naringenin, trimetazidine (TMZ), 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). Zinc chloride, magnesium gluconate, β-carotene, and calcitriol were purchased from TCI chemicals, Japan. Panax ginseng extract obtained from panacea Phytoextracts, India. Sodium selenate and ascorbic acid were obtained from Alfa Aesar, India. Silymarin and curcumin were obtained from Sanat Chemicals, India and quercetin obtained from Clearsynth, India. 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 strategy

The test formulation was the combination of eleven ingredients *viz.* calcium chloride, panax ginseng extract, vitamin B₁₂, β-carotene, vitamin D₃, zinc chloride, magnesium gluconate, sodium selenate, ferrous sulfate, ascorbic acid, and vitamin B₆. This test formulation and cell media was divided into two parts. One portion was considered as the untreated group, where no Biofield Treatment was provided. Further, the untreated group was treated with a "sham" healer for comparison purposes. The "sham" healer did not have any knowledge about the Biofield Energy Healing Treatment. The other portion of the test formulation/media received the Biofield Energy Treatment (The Trivedi Effect®) remotely by Maria Isabel Aguilar Tiraboschi, under laboratory conditions for ~3 minutes through healer's unique Biofield Energy transmission process and is referred as the Biofield Energy Treated test formulation/media. The Biofield Energy healer was located in the Uruguay; however, the test items 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 samples. 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.

Assessment of cell viability using MTT assay

Cells were counted using hemocytometer and plated in 96-well plates at the specific density described in Table 1. The cells were then incubated overnight under growth conditions to allow cell recovery and exponential growth. Following overnight incubation, cells were treated with different concentrations of test formulations (BT/UT). Following respective treatments, cells were incubated in a CO₂ incubator at 37 °C, 5% CO₂, and 95% humidity and incubated for

time period mentioned in Table 1. After incubation, the plates were taken out and 20µL of 5mg/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 150µL of DMSO was added to each well to dissolve formazan crystals. The absorbance of each well was read at 540nm using Synergy HT microplate reader. The percentage cytotoxicity at each tested concentration of TI was calculated using Equation 1:

$$\%Cytotoxicity = \left[\frac{(R - X)}{R} \right] * 100 \quad (1)$$

Where,

X = Absorbance of treated cells;

R = Absorbance of untreated cells

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

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

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

Evaluation of the cytoprotective effect of the formulation

Cells (human cardiac fibroblasts-HCF; human hepatoma cells-HepG2; and adenocarcinomic human alveolar basal epithelial cells-A549) 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, oxidative stress was given to the cells using 10 mM *t*-BHP for 3.5 hours. The untreated cells served as a control that did not receive any treatment and was maintained in cell growth medium only. Cells treated with 10mM 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:

$$\%Protection = \left[\frac{Absorbance_{sample} - Absorbance_{t-BHP}}{Absorbance_{untreated} - Absorbance_{t-BHP}} \right] * 100 \quad (2)$$

Assessment of Alkaline Phosphatase (ALP) Activity

The cells (human bone osteosarcoma cells-MG-63 and human endometrial adenocarcinoma cells-Ishikawa) were

counted using a hemocytometer and plated in 24-well plates at the density corresponding to 1x10⁴ cells/well in phenol-free DMEM supplemented with 10% CD-FBS. Following the respective treatments, the cells in the above plate were incubated for 48 hours in CO₂ incubator at 37°C, 5% CO₂, 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 1xPBS 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, 50µL of substrate solution i.e., 5mM of *p*-nitrophenyl phosphate (*p*NPP) in 1M diethanolamine and 0.24mM magnesium chloride (MgCl₂) solution (pH 10.4) was added to all the wells followed by incubation for 1 hour at 37 °C. The absorbance of the above solution was read at 405nm using Synergy HT microplate reader. The absorbance values obtained were 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:

$$\%Increase\ in\ ALP = \left\{ \frac{(X - R)}{R} \right\} * 100 \quad (3)$$

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)

The human cardiac fibroblasts (HCF) Cells were counted and plated at the density of 0.25X10⁶ cells/ well in 24-well plates in cardiac fibroblast specific medium 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 10mMt-BHP for 3.5 hours. The untreated cells were served as control that did not receive any treatment and were maintained in cell growth medium only. Cells treated with 10mM 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.

$$\%Increase = \left[\frac{LDH\ activity_{sample} - LDH\ activity_{t-BHP}}{LDH\ activity_{untreated} - LDH\ activity_{t-BHP}} \right] * 100 \quad (4)$$

Estimation of ALT in liver cells (HepG2)

The human hepatoma cells (HepG2) were counted and plated at the density of 5X10⁴ 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 400µM *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

400µM 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.

$$\%Increase = \left[\frac{(ALT\ activity_{sample} - ALT\ activity_{t-BHP})}{ALT\ activity_{untreated} - ALT\ activity_{t-BHP}} \right] * 100 \quad (5)$$

Estimation of superoxide dismutase (SOD) in lung (A549) cells

The adenocarcinomic human alveolar basal epithelial cells (A549) were counted and plated at the density of 1×10^4 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 100µM 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:

$$\%Increase\ in\ SOD\ activity = \left(\frac{X - R}{R} \right) * 100 \quad (6)$$

Where,

X = SOD activity corresponding to Test Item or Positive Control

R = SOD activity corresponding to Control group.

Estimation of serotonin in neuronal cells (SH-SY5Y)

The human neuroblastoma (SH-SY5Y) cells were counted and plated at the density of 10×10^4 cells/well in 96-well plates followed by overnight incubation. The cells were then treated with the test items/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.

$$\left[\frac{(X - R)}{R} \right] * 100 \quad (7)$$

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 human bone osteosarcoma (MG-63) cells were counted using the hemocytometer were plated at a density of 2×10^5 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.

The untreated cells that 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 and VDR expression was determined by Q-PCR 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-CACGTCAGTACGCGGTACTT-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:

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

Where,

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

Statistical Analysis

All the values were represented as mean±SD (standard deviation) of three independent experiments. The statistical analysis was performed using Sigma Plot statistical software (v11.0). For two groups 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 $p \leq 0.05$.

Result and Discussion

Cell viability using MTT assay

MTT assay was used for the determination of non-cytotoxic concentration of the formulation, test media of different cell lines, and positive controls in terms of percent viable cells in six (6) different cell-lines viz. MG-63, Ishikawa, A549, HepG2, HCF, and SH-SY5Y. Based on the percent cell viability data, it was observed that the formulation and positive controls were safe and non-toxic at the tested concentrations in six different cell lines and selected for other parameters analysis.

Evaluation of cytoprotective effect of the test formulation

Cytoprotective effect of the test formulation was screened and the data was presented in terms of percentage cellular protection against *t*-BHP induced cell damage (Figure 1). Cell line-based assays for identification of cryoprotection action in different cells can be best determined using *tert*-butyl hydroperoxide (*t*-BHP) method [41,42]. In addition, cryoprotection data in any cell line system in presence of test compound represents cellular injuries and oxidative stress, which is one of the reasons to induce cell death [43-47]. 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 25µM, 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 16.3%, 23.7%, and 18.7% at 10, 25.5, and 63.75µg/mL respectively,

in the UT-Med + BT-TI as compared with the untreated test group. Similarly, restoration of cell viability was increased in BT-Med + UT-TI group was 54.1%, 24.1%, and 32.1% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, while increased cellular restoration was reported by 37.6%, 66.8%, and 81.6% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$ respectively in the BT-Med + BT-TI group as compared with the untreated test group. Similarly, silymarin was used as positive control in HepG2 cells, which resulted in significant cellular restoration by 31.6%, 64.6%, and 74.6% at 5, 10 and 25 $\mu\text{g}/\text{mL}$, respectively as compared to the *t*-BHP induced group. Besides, test formulation groups such as in the UT-Med + BT-TI group showed increased cellular restoration by 21.9%, 37%, and 53.4% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$ respectively, as compared to the untreated test group. Besides, the test formulation showed maximum restoration of cell viability by 20.2% at 63.75 $\mu\text{g}/\text{mL}$ in the BT-Med + UT-TI group. Similarly, 43.9% and 12.7% improved cellular restoration was reported at 10 and 25.5 $\mu\text{g}/\text{mL}$ respectively, at BT-Med + BT-TI groups as compared to the 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 69.3% and 72.2% at 10 and 25 μM , respectively compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 121.9% at 1 $\mu\text{g}/\text{mL}$ in the UT-Med + BT-TI group. Similarly, 416% and 163.9% improved cellular restoration was reported at 25.5 and 63.75 $\mu\text{g}/\text{mL}$ respectively, at BT-Med + UT-TI groups as compared to the UT-Med + UT-TI group. However, 323% and 61.7% improved cellular restoration was reported at 25.5 and 63.75 $\mu\text{g}/\text{mL}$ respectively, by BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. The experimental data showed significant improved cellular protection after Biofield Energy healing treatment against vital organs and their functioning viz. heart, liver, and lungs. Thus, Biofield Energy healing treatment (The Trivedi Effect®) can be assumed to be useful clinically for the management of oxidative stress induced by various external and internal factors. Therefore, the Biofield Energy healing treatment could be successfully used for the management of various pathological etiologies against cardiovascular, liver, and various lung diseases.

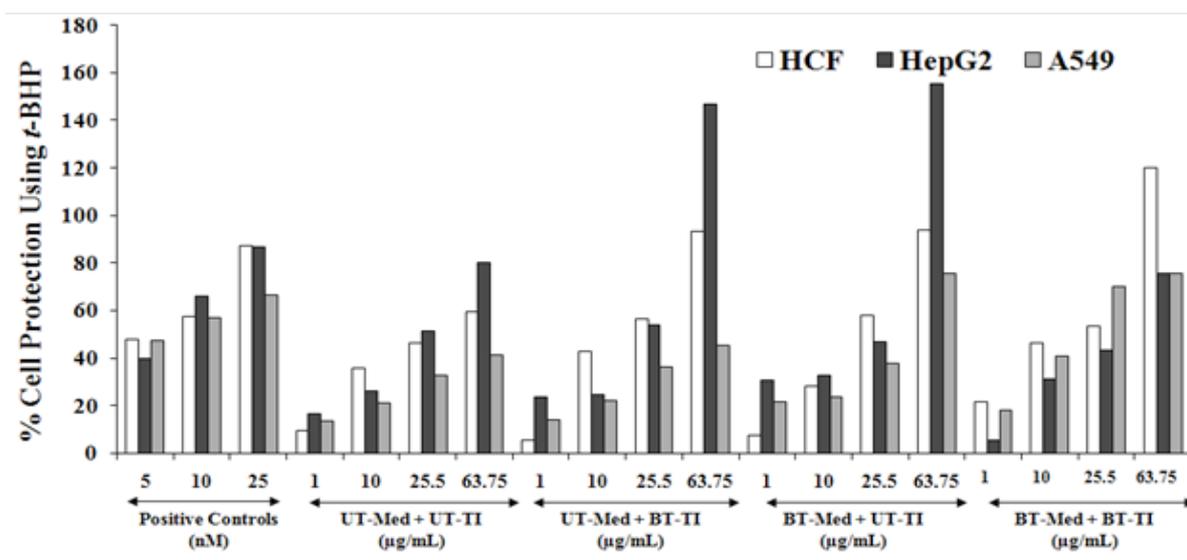


Figure 1: Cytoprotective action of the test formulation in human cardiac fibroblasts cells (HCF), human hepatoma cells (HepG2), and adenocarcinomic human alveolar basal epithelial cells (A549) against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. Trimetazidine (μM), silymarin ($\mu\text{g}/\text{mL}$), 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 activity of test formulation and the test media of specific cell lines were tested for MG-63 and Ishikawa cells. ALP level represents the bone health biomarker of bone related disorders such as for the assessment of osteoporosis [48,49]. The ALP results can provide the comprehensive information about the bone health as it represents the biochemical biomarkers of bone turnover. Calcitriol (nM) was used as positive control in the MG-63 cells, and the results suggested significant increased ALP level by 20%, 22.7%, and 36.8% at 0.1, 1, and 10nM respectively as presented in Figure 2. However, the experimental test groups showed increased ALP activity by 31.7% and 77.6% at 10 and 50 $\mu\text{g}/\text{mL}$ respectively, in the

UT-Med + BT-TI group as compared to the UT-Med + UT-TI group in MG-63 cells. Similarly, ALP activity was increased by 10.5%, 67.3%, and 78.2% at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$ respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Similarly, naringenin was used as positive control for Ishikawa cells, and the data showed significant improved level of ALP by 25.9%, 49.2%, and 151.9% at 0.1, 1, and 10nM respectively. In the experimental tested groups, the ALP percent was significantly increased by 17.2% and 62.7% at 10 and 50 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Similarly, ALP percent was significantly increased by 18.5%, 33.2%, and 67.5% at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-

TI group as compared to the UT-Med + UT-TI group. However, ALP percent was significantly increased by 19.7%, 5.9%, and 36.3% at 0.1, 10, and 50µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Overall, the experimental

results revealed significant improved bone ALP level after Biofield Energy healing treatment that has an important application in low bone density, osteoporosis, osteogenesis imperfect and Paget's disease of bone that makes the bones brittle.

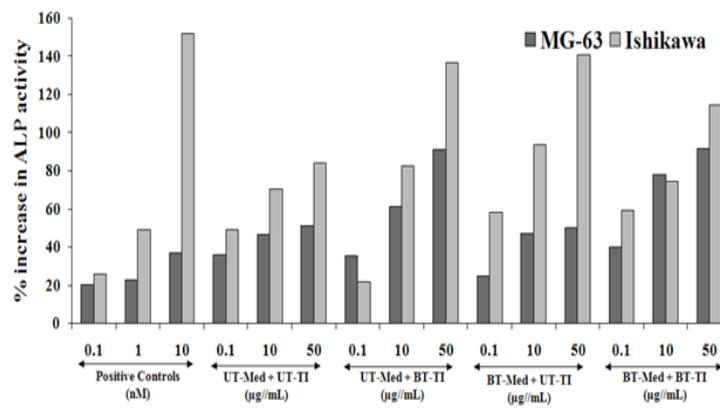


Figure 2: Alkaline phosphatase (ALP) activity in human bone osteosarcoma cells (MG-63) and human endometrial adenocarcinoma cells (Ishikawa) after treatment of the test formulation. Calcitriol and naringenin were used as positive control in MG-63 and Ishikawa cells, respectively.

UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Identification of lactate dehydrogenase (LDH) activity in human cardiac fibroblasts (HCF)

LDH activity of test formulation and test media was evaluated in the HCF cells, while the results were concluded in terms of decreased LDH activity, which represents increased cellular protection of HCF cells. LDH play a vital role in tissue injury, necrosis, hypoxia, hemolysis, or malignancies. HCF cells are one the best cell culture model for the estimation of LDH activity as it plays a central role in the extracellular matrix maintenance of the normal heart functioning [50-52]. The results are presented in Figure 3. The positive control, trimetazidine (TMZ) showed 69.4%, 80.1%, and 104.2% increased cellular protection of HCF cells (decreased of LDH activity) at 10, 50, and 100µM, respectively 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 24.4% and 26.6% at 10 and 25.5µg/mL concentrations respectively, in the UT-Med + BT-TI group, while 30%, 37.9%, and 9.9% improved cellular protection (decreased of LDH activity) at 1, 10, and 25.5µg/mL respectively in the BT-Med + UT-TI group, and 36.4%, 9.1%, and 76.5% improved cellular protection (decreased of LDH activity) 1, 10, and 25.5µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Overall, the LDH activity results suggested improved cellular protection of HCF cells, which would be clinically useful in the cells of skeletal muscle, heart muscle, and blood cells, which can be beneficial for overall heart health. The present data concluded a significant reduction of LDH level after Biofield Energy Treatment and protection of the HCF cells, which would be useful in different pathological conditions.

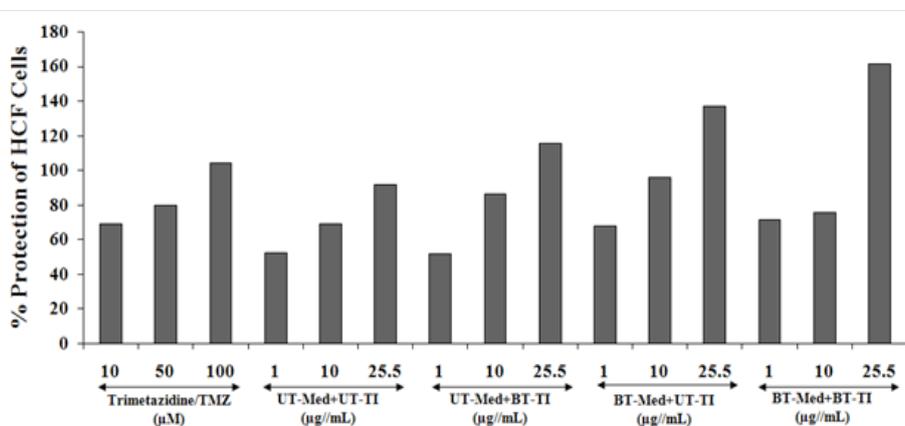


Figure 3: The effect of the test formulation on the percent protection of HCF cells in terms of decreased lactate dehydrogenase (LDH) activity against *tert*-butyl hydroperoxide (*t*-BHP) induced damage.

TMZ: Trimetazidine; UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of alanine amino transferase (ALT) activity in HepG2 cells

ALT activity was tested in HepG2 cells and the results are presented in terms of decreased ALT activity, which showed increased cellular protection of HepG2 cells. ALT enzyme in liver has significant importance in various physiological processes; it helps the body to metabolize protein. ALT is the major enzyme used for metabolic energy production and vital role in hepatocellular injury and death. However, it is also present in the kidney cells and heart muscles [53]. High level of ALT may be linked with the liver disorder or cellular damage with cellular injury [54]. The results of ALT are presented in Figure 4. The positive control, silymarin was selected in ALT activity and the data suggested increased percentage cellular protection (decreased ALT activity) by 56%,

85%, and 118.9% at 5, 10, and 25 μ M concentrations, respectively. Similarly, the test formulation groups showed improved cellular protection of HepG2 cells (decreased of ALT activity) by 21.1%, 15.2%, and 14.1% at 10, 25.5, and 63.75 μ g/mL respectively, in the UT-Med + BT-TI group, while increased cellular protection of HepG2 cells (decreased of ALT activity) by 93.9% at 63.75 μ g/mL in the BT-Med + UT-TI group, and increased cellular protection of HepG2 cells (decreased of ALT activity) by 10.4% and 51.4% at 25.5 and 63.75 μ g/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 4). Overall, the ALT results showed a significant increased cellular protection of HepG2 cells after treatment with the Biofield Energy Treatment (The Trivedi Effect®), which significantly protects the liver hepatocytes that can be useful in liver cancer, liver cirrhosis, hepatomegaly, liver failure, and hepatitis.

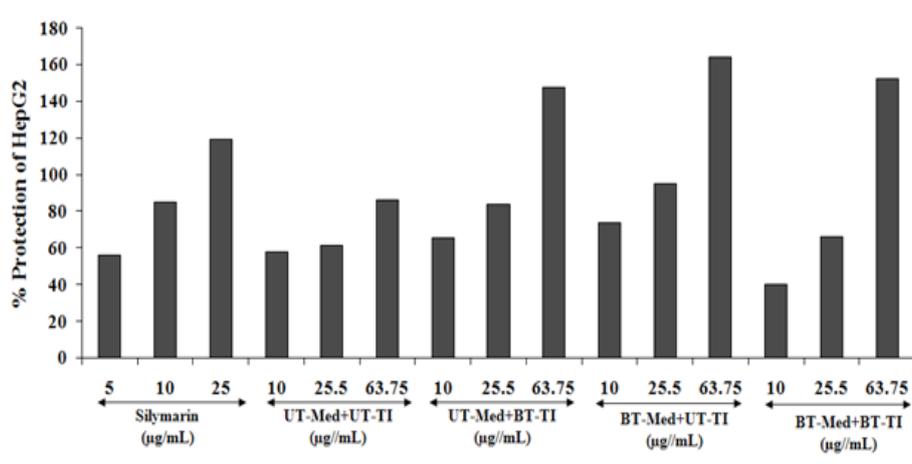


Figure 4: The effect of the test formulation on the percent protection of human liver cancer (HepG2) cells in terms of decreased alanine amino transaminase (ALT) activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP).

UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of superoxide dismutase (SOD) activity in adenocarcinomic human alveolar basal epithelial cells (A549)

SOD activity was evaluated in A549 cells in terms of increased cellular protection. SOD protect from highly active molecules known as free radicals, which are present in almost all the body. SOD represents high antioxidant activity known as body defense system that showed repair of the cellular damage caused due to free radicals, reactive oxygen species (ROS), and many other factors causing cell death [55]. The detailed data of SOD in different groups was presented in Figure 5. The positive control, quercetin showed improved percentage increase in the SOD activity with respect to the *t*-BHP by 74%, 89.8%, and 129.9% at 10, 25, and 50 μ M concentration respectively. However, the percent protection of A549 (lungs) cells (increased of SOD activity) was significantly increased by 57.2% and 5.3% at 25.5 and 63.75 μ g/mL, respectively in the UT-Med + BT-TI group, while increased SOD activity by 176.9%, 124.7%, and 44.2% at 10, 25.5, and 63.75 μ g/mL respectively, in the BT-Med + UT-TI group, and increased SOD activity by 184.2%,

117.8%, and 46.1% at 10, 25.5, and 63.75 μ g/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 5). Thus, experimental data showed significant improved cellular protection of A549 cells and improved level of SOD enzyme. Thus, Biofield Energy healing treatment has significantly improved the SOD activity that can be used in various respiratory diseases such as pneumonia, asthma, pulmonary fibrosis, and lung cancer.

Estimation of serotonin level in human neuroblastoma (SH-SY5Y) cells

Serotonin level was estimated in SH-SY5Y cells after treatment with the test formulation using standard cell-based assay after 24 hours of treatment using ELISA method. Serotonin an important neurotransmitter has been reported to regulate the mood and social behavior, appetite and digestion, sleep, memory, and sexual desire and related functions. Serotonin imbalance results in neuropsychiatric disorders such as emesis, irritable bowel syndrome (IBS), and pulmonary and systemic hypertension, Alzheimer's disease, cognitive health, loss of ability of thinking,

migraine, depression, memory loss, etc. [56-59]. Serotonin activity was reported, and the effect of Biofield Energy Treated test formulation is presented in Figure 6. The positive control, curcumin showed 112.8%, 127.2%, and 160.2% increase the level of serotonin at 0.1, 1, and 5µM respectively, compared to the vehicle control (VC) group. The data showed significant increased serotonin level by 3.3% and 34.8% at 25.5 and 63.75µg/mL in the UT-Med +

BT-TI, while significant increased serotonin was reported by 42.1% and 65.4% at 25.5 and 63.75µg/mL respectively, in the BT-Med + UT-TI, and 398.7%, 202.1%, and 198.3% improved serotonin level at 1, 25.5, and 63.75 respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 6). Biofield Energy healing treated test formulation can be used against various neurodegenerative diseases and improved brain functioning.

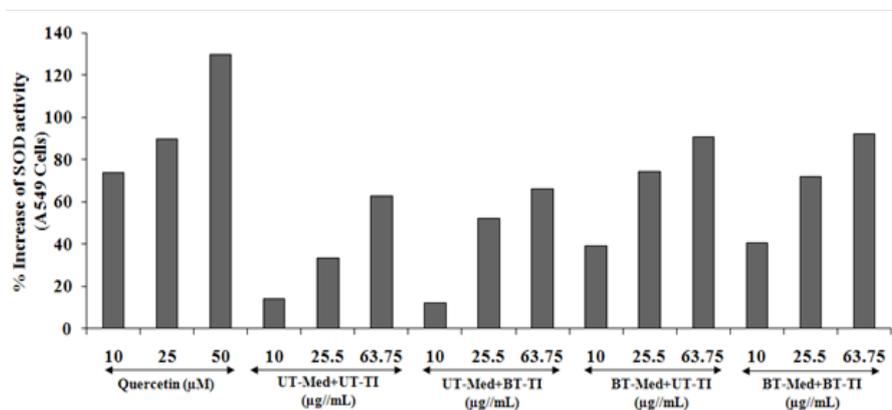


Figure 5: The effect of the test formulation on the percent protection of lungs cells (A549) in terms of increased SOD activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP).

UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item. Data are expressed as mean ± SD of three independent experiments.

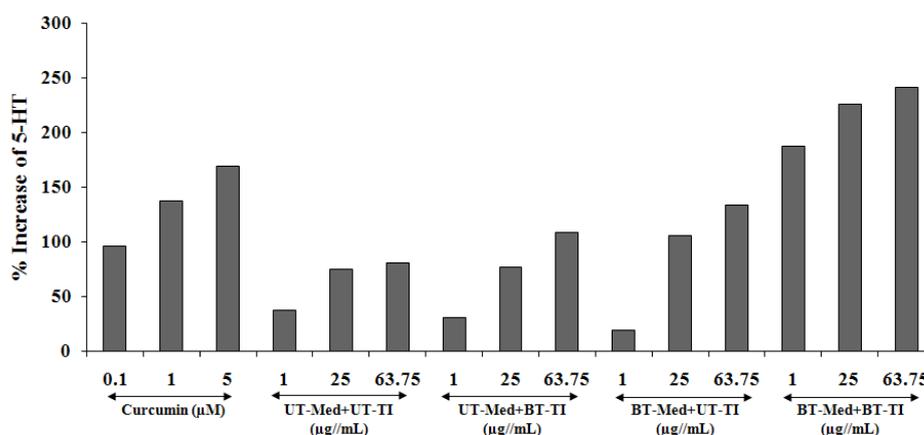


Figure 6: The effect of the test formulation on percent change in 5-hydroxy tryptamine (5-HT) or serotonin in the human neuroblastoma cells (SH-SY5Y).

UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Evaluation of vitamin D receptors (VDRs) activity

VDR activity was estimated using human bone osteosarcoma cells (MG-63) and its expression was studied using the phenomenon of ligand binding through vitamin D active molecule that can be estimated using quantitative-polymerase chain reaction (qPCR) amplification. With the help of real time PCR, different VDR-relative threshold cycle (VDR-CT) values were obtained after complete amplification cycles using specific primer probes. Relative quantification (RQ) was calculated from the VDR-CT and

housekeeping (HK)-CT values in MG-63 cells. The VDR-CT values of different experimental test groups are represented in Figure 7. Calcitriol was used as a positive control and the RQ of VDR was found to be increased in concentration-dependent manner by 59.1%, 93.2%, and 131.3% at 1, 10, and 100nM, respectively. The experimental test groups showed increased RQ of VDR expression by 207.9%, 122.7%, and 203.5% in the UT-Med + BT-TI group at 0.1, 1, and 10µg/mL respectively, while 37.1% increased RQ of VDR at 10µg/mL in the BT-Med + UT-TI group, and increased RQ of VDR

by 78%, 122.7%, and 141% at 0.1, 1, and 10 μ g/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. In conclusion, the results showed significant increased RQ-VRD expression in the MG-63 cells after treatment in various groups. Calcitriol was reported to bind with the VDRs and reported

to regulate the calcium homeostasis, immunity, overall cellular growth, bone growth, and cell differentiation [60-61]. The results were well collaborated and can be concluded that after Biofield Energy Treatment the activity of VDR expression was significantly improved.

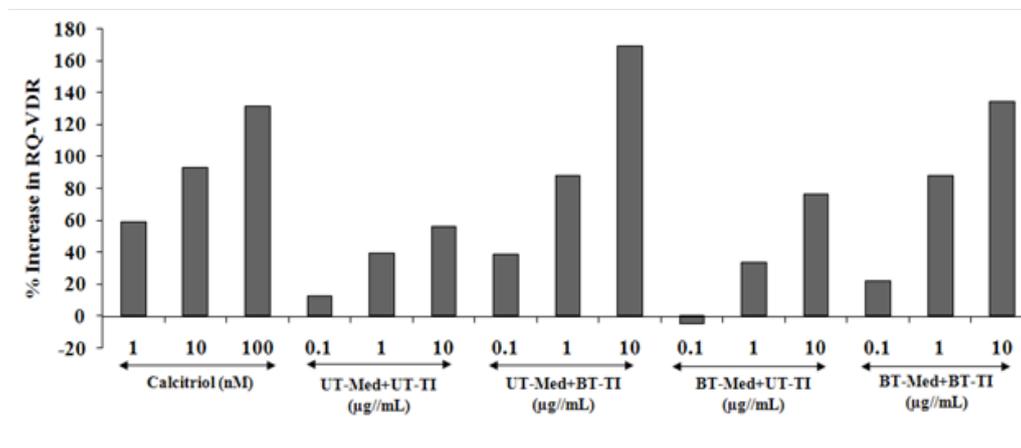


Figure 7: Effect of the test formulation on percent increase in relative quantification (RQ) of vitamin D receptors (VDRs) gene in human bone osteosarcoma cells (MG-63).

UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Conclusion

The novel proprietary Biofield Energy Treated test formulation was found safe and non-toxic based on MTT cell viability assay in all the six tested cells. Cytoprotective activity against t-BHP induced cell damage was tested using human cardiac fibroblasts cells (HCF), which showed restoration of cell viability by 23.7% (at 25.5 μ g/mL), 54.1% (at 10 μ g/mL), and 81.6% (at 63.75 μ g/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 by 53.4% (at 63.75 μ g/mL), 20.2% (at 63.75 μ g/mL), and 43.9% (at 10 μ g/mL) in the UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI groups respectively, as compared to the untreated test group. Similarly, the test formulation in A549 cells showed maximum restoration of cell viability by 121.9% (at 1 μ g/mL), 416% (at 25.5 μ g/mL), and 323% (at 25.5 μ g/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. ALP activity in MG-63 cells showed significantly increased ALP activity by 77.6% and 78.2% in the UT-Med + BT-TI and BT-Med + BT-TI groups respectively, at 50 μ g/mL as compared to the untreated test group. Similarly, ALP activity in Ishikawa cells with maximum cellular protection at 50 μ g/mL by 62.7%, 67.5%, and 36.3% in the UT-Med + BT-TI, BT-Med + UT-TI, BT-Med + BT-TI groups respectively, as compared to the untreated test group. LDH data was presented in terms of increased percentage cellular protection data, which suggested significant decreased activity, which showed maximum cellular protection by 26.6% (at 25.5 μ g/mL), 37.9% (at 10 μ g/mL), and 76.5% (at 25.5 μ g/mL) 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 was studied and data showed maximum improved cellular protection of HepG2 cells (decreased of ALT activity) by 21.1% (at 10 μ g/mL), 93.9% (at 63.75 μ g/mL), and 51.4% (at 63.75 μ g/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 57.2% (at 25.5 μ g/mL), 176.9% (at 10g/mL), and 184.2% (at 10 μ g/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 34.8% (at 63.75 μ g/mL), 65.4% (at 63.75 μ g/mL), and 398.7% (at 1 μ g/mL) in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups respectively, as compared with the untreated test group. However, VDR expression was tested in MG-63 cells, which showed increased RQ of VDR by 207.9%, 122.7%, and 203.5% in the UT-Med + BT-TI group at 0.1, 1, and 10 μ g/mL respectively, while 37.1% increased RQ of VDR at 10 μ g/mL in the BT-Med + UT-TI group, and increased RQ of VDR by 78%, 122.7%, and 141% at 0.1, 1, and 10 μ g/mL respectively, in the BT-Med + BT-TI group as compared to the untreated test control group. Thus, Biofield Energy Treatment (The Trivedi Effect[®]) can be used for improving overall health such as significant role in 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. Besides, it would also protect against many hepatic disorders (cirrhosis, liver cancer, hemochromatosis, Wilson disease), lungs disorders (asthma, chronic bronchitis, emphysema, cystic fibrosis, and pneumonia), and many immune system related 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, Hashimoto Thyroiditis, Multiple Sclerosis, Dermatitis, Diabetes, Parkinson's Disease, Myasthenia Gravis, Ulcerative Colitis (UC), Atherosclerosis, etc. to improve overall health and Quality of Life.

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