

***In Vitro* Cell-Based Biomarkers Study of Vital Organs: Impact of the Biofield Energy Based Test Formulation**

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Abstract

The present study was undertaken to evaluate the impact of Biofield Energy Treated test formulation using multiple cell-lines. The test formulation and cell media (Med) was divided into two parts; one part was untreated (UT) and other part received Biofield Energy Treatment remotely by a renowned Biofield Energy Healer, Krista Joanne Callas, USA and labeled as Biofield Energy Treated (BT) test item (TI)/Med. Based on cell viability, test formulation was found safe. Cytoprotective action of test formulation showed significant restoration of cell viability by 89.9% and 106.4% in human cardiac fibroblasts cells (HCF) cells, while improved restoration of cell viability by 77.3% and 69% in HepG2 cells compared to untreated. Cellular restoration in A549 cells was also improved by 141.2% and 157.1% compared to untreated. ALP activity was significantly increased by 118.7% and 140.7% in UT-Med + BT-TI and BT-Med + UT-TI, respectively at 0.1 µg/mL than untreated. Percent cellular protection of HCF (heart) cells (decreased of LDH activity) was significantly increased by 89.9% and 106.4% in UT-Med + BT-TI and BT-Med + BT-TI, respectively than untreated. HepG2 cells protection (decreased ALT activity) was increased by 59.8% in BT-Med + BT-TI than untreated. Superoxide dismutase (SOD) level was increased by 22.8% in BT-Med + BT-TI than untreated. Serotonin level was significantly increased by 361.7% and 197.6% in BT-Med + UT-TI and BT-Med + BT-TI, respectively than untreated in human neuroblastoma cells (SH-SY5Y). However, relative quantification (RQ) of vitamin D receptor (VDR) was significantly increased by 116.5%, 214.7%, and 241.5% in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI, respectively than untreated in MG-63 cells. Overall, data showed a significant improvement of organ-specific functional enzyme biomarkers. Thus, Biofield Energy Treated Test formulation (the Trivedi Effect[®]) would be useful for multiple organs health that can be beneficial against coronary artery disease, arrhythmias, congenital heart disease, cardiomyopathy, cirrhosis, liver cancer, hemochromatosis, asthma, chronic bronchitis, cystic fibrosis, osteoporosis, etc.

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Keywords: The Trivedi Effect[®], Biofield Energy Treatment, Organ health, Bone health, Functional biomarker

Received: Jun 14, 2019

Accepted: July 18, 2019

Published: July 18, 2019

Editor: Jong In Kim, Wonkwang University, Korea.

Introduction

Alternative system of medicine includes treatments different from mainstream therapeutic approach denoted as integrative or complementary medicine. Herbal treatment-based approach and their related remedies are widely accepted in most of the developed countries which is one of the best approaches of complementary and alternative medicines (CAMs) [1]. Herbal based nutraceutical test formulations along with the selected phytonutrients continued throughout the country, which is likely to be expanding with very rapid speed across the Globe against various health challenges treatments among the national healthcare settings. Various CAM treatment approaches are now becoming the mainstream treatment remedies in many countries such as in the UK, Europe, as well as in North America and Australia [2] due to the huge accepting rate in all the developing countries as they are promoting overall quality of life in healthier living [3]. Along with herbal based medicinal products, most of the vital 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. These unique alternative treatment approach using some novel test formulations can be useful for managing high blood pressure, heart disease, asthma, other respiratory diseases, immunodeficiency diseases, aging and many more [4]. Owing to the recent literature and availability of the database with respect to herbal drug formulation, a novel herbomineral test formulation was developed for overall functioning of multiple organs. This test formulation included *Panax ginseng* extract, beta carotene, calcium chloride, magnesium gluconate, zinc chloride, sodium selenate, ferrous sulfate, vitamin B₁₂, vitamin D₃, ascorbic acid, and vitamin B₆. Minerals and vitamins used in the novel formulation for overall support of organ health and its functioning [5-8]. *Panax ginseng*, a potent immunomodulator was reported to have improved wellness and thinking, memory,

concentration, physical stamina, work efficiency, preventing muscle damage, Alzheimer's disease, athletic endurance, improve mental and cognitive health [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 Treatment is an ancient healing art (CAM approach), which include a life force energy transmitted by a renowned Biofield Energy Healer. This treatment stimulates body's own auto-healing response and has the ability to regulate the living organisms homeodynamic function's along [21]. This treatment addressing and balance the physical, mental, emotional and spiritual state simultaneously, and The Trivedi Effect[®]-Consciousness Energy Healing has many outstanding results reported worldwide. 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 [22]. Thus, Biofield Energy Healing therapies have gained popularity because of improved immunological response, while its current status and future perspectives has been reported worldwide. 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, Tai Chi, pranic healing, deep breathing, chiropractic/osteopathic manipulation, meditation, massage, homeopathy, hypnotherapy, progressive relaxation, acupressure, acupuncture, special diets, relaxation techniques, Rolfing movement therapy, pilates, mindfulness, and many more [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 [25, 26], agriculture science [27], microbiology [28, 29], biotechnology [30, 31], and changed bioavailability of many compounds [32, 33], skin health [34, 35], nutraceuticals [36], cancer science research [37], improved bone health [38-40], human health and wellness. 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

All the test chemicals were procured from standard specifications such as Panax ginseng extract was 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 was purchased from Clearsynth, India. 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 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

The test formulation constituents and the specific cell line media was used for the treatment with the Biofield Energy. The test formulation was the combination of eleven ingredients such as panax ginseng extract, β-carotene, zinc chloride, calcium chloride, magnesium gluconate, sodium selenate, ferrous sulfate, ascorbic acid, vitamin B₁₂, vitamin D₃, and vitamin B₆. The test formulation constituents and the cell line media 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 portion of the test formulation and the medium received the Biofield Energy Treatment (The Trivedi Effect[®]) remotely by Krista Joanne Callas, 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 CO₂ incubator at 37°C, 5% CO₂, 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:

$$\% \text{ Cytotoxicity} = [(R-X)/R] * 100 \dots \dots \dots (1)$$

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

Table 1. Information related to six cell lines with their plating density and time-point [41].

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

The concentrations exhibiting percentage cell viability >70% was considered as non-cytotoxic.

Cytoprotective Action of the Test Formulation

Cytoprotective effect of the test formulation in selected cells such as 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. Further, 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:

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

Estimation of Alkaline Phosphatase (ALP) Activity

For the estimation of ALP, two cells such as 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 1 X 10⁴ 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 a 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 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 (pNPP) in 1 M diethanolamine and 0.24 mM 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 405 nm using Synergy HT microplate reader (Biotek, USA). The absorbance values obtained were normalized with substrate blank (pNPP solution alone) absorbance values. The percentage increase in ALP enzyme activity with respect to the untreated cells (baseline group) was calculated using Equation 3:

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

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 10⁶ cells/ well in 24-well plates in cardiac fibroblast specific medium followed 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. Cells 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.

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

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 10⁴ 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.

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

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 10⁴ 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.

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

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 used for the estimation of serotonin level. The cells were counted and plated at the density of 10 X 10⁴ 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.

$$\frac{(X-R)}{R} * 100 \dots \dots \dots (7)$$

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 10⁵ 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'-CACGTCCTGACGCGGTACTT-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:

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

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 percentage. 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

The tested cell lines viz. MG-63, Ishikawa, A549, HepG2, HCF, and SH-SY5Y were screened for cell viability using MTT assay and was found safe with the tested concentrations. All the tested test concentrations

of the test formulation were found safe on the basis of percentage of cell viability. The test criteria for 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. Overall, the experimental data suggested that the overall percent cell viability in different cell-lines 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, while the data was presented in terms of percentage cell protection against *t*-BHP induced cell damage (Figure 1). 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 34%, 60%, and 98.3% at 5, 10, and 25 μ M, respectively as compared to the *t*-BHP induced group. The restoration of cell viability among the tested groups by the test formulation was reported as 53.3%, 35.9%, 23.4%, and 89.9% at 0.1,

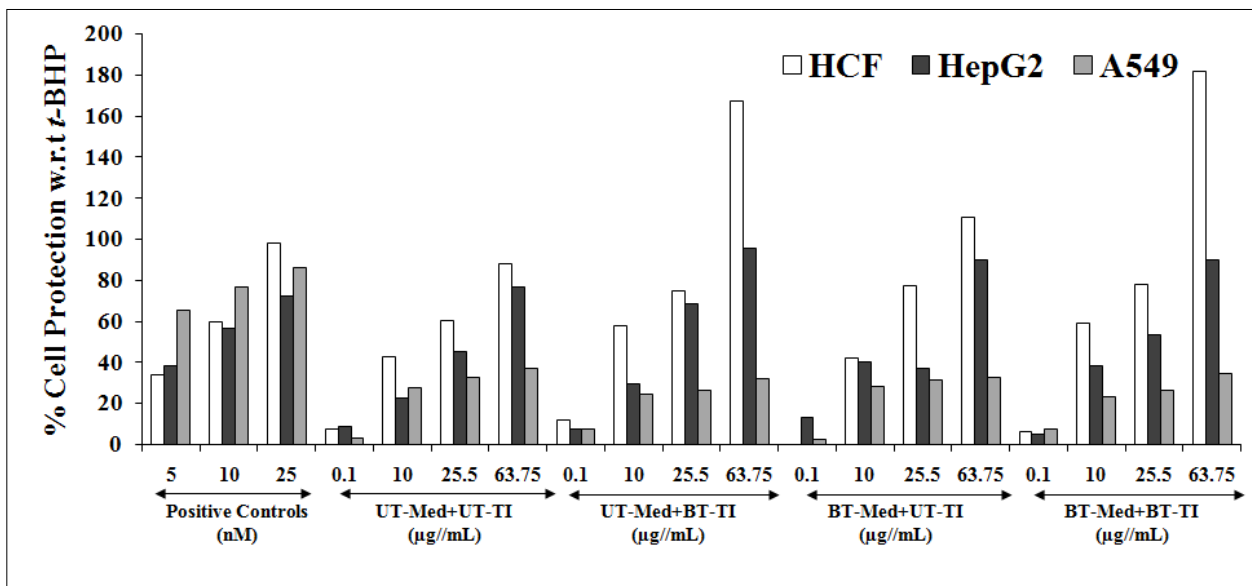


Figure 1. Cytoprotective action of the test formulation in human cardiac fibroblasts cells (HCF), human hepatoma cells (HepG2), and adenocarcinomic human alveolar basal epithelial cells (A549) against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. Trimetazidine (μ M), silymarin (μ g/mL), and quercetin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

10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group as compared to the untreated group. Moreover, 27.3% and 25.7% improved cellular restoration at 25.5 and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-TI group as compared to the untreated. Further, 38.5%, 28.6%, and 106.4% improved cellular restoration at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the untreated. Similarly, silymarin was used as positive control in HepG2 cells, which resulted in significant cellular restoration by 38.4%, 56.6%, and 72.6% at 5, 10 and 25 $\mu\text{g}/\text{mL}$, respectively as compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 30.4%, 50.7%, and 24.5% at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group than untreated. Moreover, 44.1%, 77.3%, and 16.7% improved cellular restoration at 0.1, 10, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-TI than untreated. Further, 69%, 17.7%, and 16.7% improved cellular restoration at 10, 25.5, and 63.75 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + BT-TI group as compared to the untreated. Additionally, quercetin was used as positive control in adenocarcinomic human alveolar basal epithelial cells (A549) resulted, restoration of cell viability by 65.7%, 76.7%, and 86.1% at 5, 10 and 25 $\mu\text{g}/\text{mL}$, respectively compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 141.2% at 0.1 $\mu\text{g}/\text{mL}$ in the UT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Similarly, 157.1% improved cellular restoration was reported at 0.1 $\mu\text{g}/\text{mL}$ in the BT-Med + BT-TI group as compared to the untreated. The overall data clearly signifies significance of Biofield Energy healing Treatment with respect to the cytoprotective activity after oxidative stress using *tert*-butyl hydroperoxide (*t*-BHP). However, this method has been considered as the gold standard for testing the cytoprotective action by stimulation in cell based assay [41, 42]. Cytoprotection action is defined as a tool to protect the cells against injuries [43, 44], which could protect against many immune related disorders such as cardiovascular diseases, aging, cancer, diabetes, and many more [45-47]. Thus, Biofield Energy Treatment (The Trivedi Effect[®]) can be significantly used to protect the *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.

Estimation of Alkaline Phosphatase (ALP) Activity

The test formulation and the test media was evaluated for ALP activity against two cell lines, MG-63 and Ishikawa cells after Biofield Energy Treatment. Naringenin (nM) was used as positive control in Ishikawa cells, and the results suggested significant increased ALP level by 9.5%, 23.7%, and 130.2% at 0.1, 1, and 10 nM respectively as shown in the Figure 2. However, the experimental test groups showed maximum increased ALP activity by 118.7% and 21.3% at 0.1 and 50 $\mu\text{g}/\text{mL}$, respectively in the UT-Med + BT-TI group; while, 140.7%, 72.9%, and 48.9% increased ALP activity at 0.1, 10, and 50 $\mu\text{g}/\text{mL}$, respectively in the BT-Med + UT-TI group as compared to the untreated. Further, 59.5% and 56.9% improved ALP level was found at 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 in Ishikawa cells. Similarly, calcitriol was used as positive control for MG-63 cells, and the data showed significant improved level of ALP by 13.2%, 21.4%, and 35.4% at 0.1, 1, and 10 nM, respectively. The ALP percent activity in MG-63 cells was significantly increased by 7%, 59.8%, and 69.4% at 0.1, 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 5.7%, 63.5%, and 71.8% 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 13.9%, 70.3%, and 71.4% 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 in the MG-63 cells. ALP is one of the important bone health biomarker responsible for controlling various bone disorders [48, 49] such as low bone density and osteoporosis, osteogenesis imperfect and Paget's disease, which makes bones brittle. Biofield Energy Treatment would be highly recommended option in bone disorders without any adverse effects, because ALP level was significant improved after treatment with the Biofield Energy Healing Treatment.

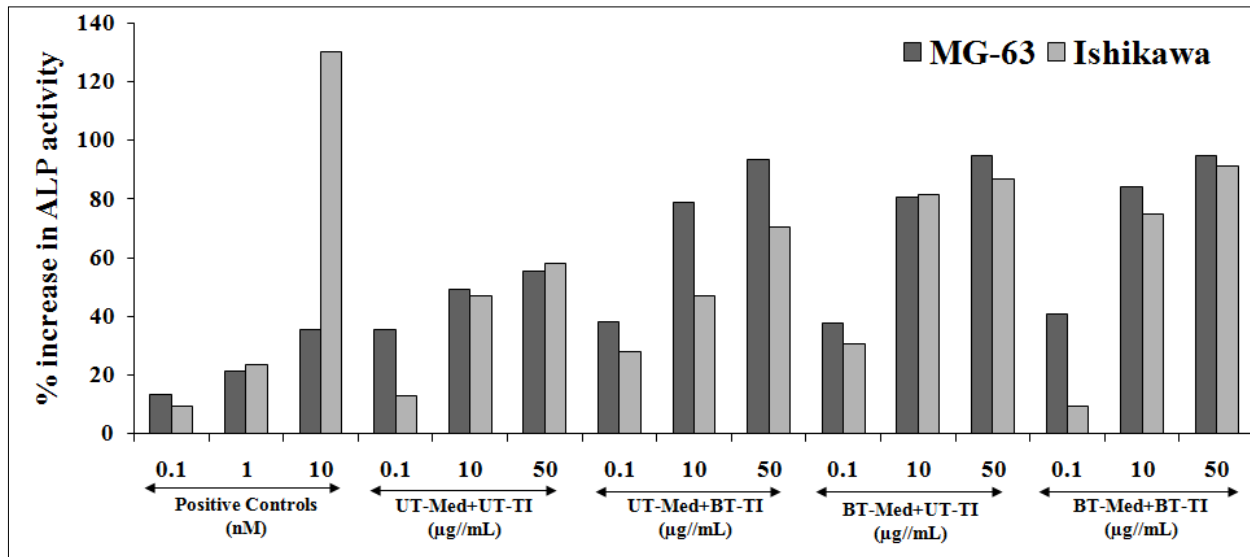


Figure 2. Alkaline phosphatase (ALP) activity in human bone osteosarcoma cells (MG-63) and human endometrial adenocarcinoma cells (Ishikawa) after treatment of the test formulation. Calcitriol and naringenin were used as positive control in MG-63 and Ishikawa cells, respectively. UT: Untreated; Med: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

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

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 (i.e. improved HCF cellular protection) is presented in the Figure 3. The positive control, trimetazidine (TMZ) showed 34%, 60%, and 98.3% increased cellular protection of HCF cells (decreased of LDH activity) at 5, 10, and 25 μ M 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 35.9%, 23.4%, and 89.9% at 10, 25.5, and 63.75 μ g/mL, respectively in the UT-Med + BT-TI group; while, 27.3% and 25.7% improved cellular protection (decreased of LDH activity) at 25.5 and 63.75 μ g/mL, respectively in the BT-Med + UT-TI group than untreated group. Further, 38.5%, 28.6%, and 106.4% improved cellular protection (decreased of LDH activity) at 10, 25.5, and 63.75 μ g/mL, respectively in the BT-Med + BT-TI group as compared to the untreated group. 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 along with synthesis of growth factors and cytokines [50-52]. LDH

activity was estimated in HCF cells, as 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. In conclusion, 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. Besides, LDH is the best biomarker for heart disease or tissue injuries.

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

ALT is one of the important liver health enzymes along with kidney, heart, and muscles. Up and down regulation of this enzyme may results in hepatocellular injury and death [53]. Hepatic cellular damage has been linked with high level of ALT, which affects the cell viability and damage to the cells [54]. The level of ALT activity was estimated with the help of HepG2 cell and the results are presented in terms of increased percentage cellular protection (which represents decreased ALT activity) in the Figure 4. The positive control, silymarin was in HepG2 cells for ALT activity and

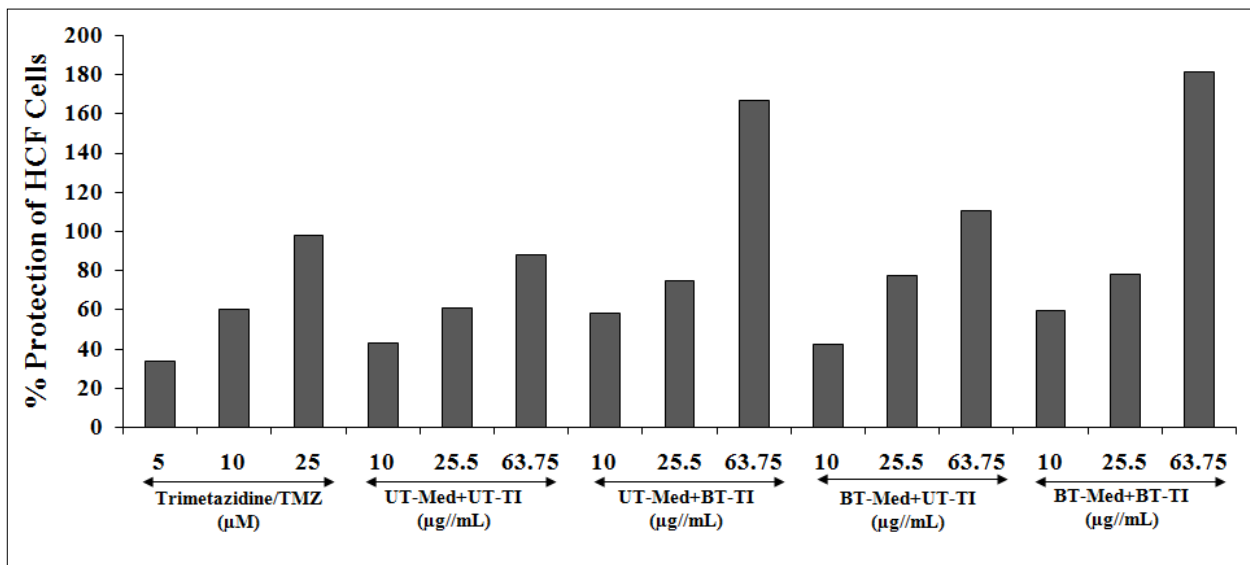


Figure 3. The effect of the test formulation on the increased percent protection of HCF cells, which represents decreased lactate dehydrogenase (LDH) activity against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. TMZ: Trimetazidine; UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

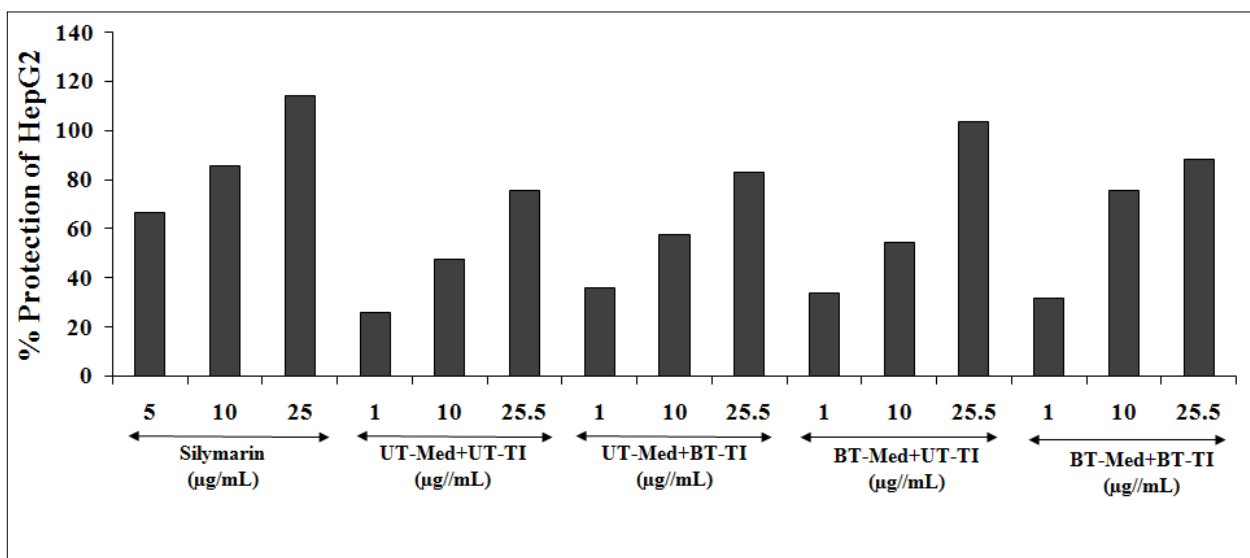


Figure 4. The increased percentage protection of liver cells (HepG2) that represents decreased (ALT) Alanine amino transaminase activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

the data suggested increased percentage cellular protection of HepG2 cell (decreased ALT activity) by 66.4%, 85.8%, and 114.4% at 5, 10, and 25 $\mu\text{g/mL}$, respectively. Similarly, the test formulation group showed improved cellular protection of HepG2 cells (*i.e.*, decreased of ALT activity) by 39.2%, 21.2%, and 9.9% at 1, 10, and 25.5 $\mu\text{g/mL}$, respectively in the UT-Med + BT-TI group; while, 31.3%, 15.2%, and 37.3% at 1, 10, and 25.5 $\mu\text{g/mL}$, respectively in the BT-Med + UT-TI group as compared to the untreated group. Further, cellular protection of HepG2 cells (decreased of ALT activity) was increased by 23%, 59.8%, and 17.1% at 1, 10, and 25.5 $\mu\text{g/mL}$, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 4). Overall, the results showed significant activity after treatment with the Biofield Energy Healing Treatment. 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.

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 and the data was presented in Figure 5. SOD is one the best antioxidant defense mechanism of the body, which prevent the cellular damage against various types of stress and free radicals, which results in cell death [55]. The positive control, quercetin showed improved percentage increase in the SOD activity with respect to the *t*-BHP by 12.5%, 53.6%, and 62.1% at 0.1, 1, and 10 μM concentration respectively. However, the percent protection of A549 (lungs) cells (increased of SOD activity) was significantly increased by 22.1% at 10 $\mu\text{g/mL}$ in the BT-Med + UT-TI group, and increased SOD activity by 22.8% and 22.1% at 1 and 10 $\mu\text{g/mL}$ respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 5). The present 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 pneumonia, asthma, pulmonary fibrosis, and lung cancer due to significant increased cellular protection of A549 cells and improved level of SOD enzyme.

Estimation of Serotonin Level in Human Neuroblastoma (SH-SY5Y) Cells

Serotonin assay was performed using SH-SY5Y cells and the effect of test formulation and cell line media was assessed after 24 hours of treatment using ELISA assay. Serotonin 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. Curcumin was used a positive control, showed 112.8%, 127.2%, and 160.2% increased the level of serotonin at 0.1, 1, and 5 μM , respectively compared to the vehicle control (VC) group. The data showed significant increased serotonin level by 8.3% and 5.3% at 10 and 25 $\mu\text{g/mL}$, respectively in the UT-Med + BT-TI group; while, 361.7%, 272.9%, and 133.4% at 10, 25, and 63.75 $\mu\text{g/mL}$, respectively in the BT-Med + UT-TI group as compared to the untreated group. Moreover, serotonin level was significantly increased by 155.8%, 197.6%, and 106.5% at 10, 25, and 63.75 $\mu\text{g/mL}$, respectively in the BT-Med + BT-TI group as compared to the untreated (Figure 6). The present serotonin experiment showed that the serotonin level was significantly improved in the entire tested group. The significant improved level of serotonin after treatment with the Biofield Energy Healing Treated would be useful against various neurodegenerative diseases.

Effect of Test Formulation on Vitamin D Receptors (VDRs)

Human bone osteosarcoma cells (MG-63) was used for evaluation of the VDR activity against the Biofield Energy Treated test formulation. The expression of VDRs was studies using the phenomenon of ligand binding through vitamin D active molecule, which was estimated using quantitative-polymerase chain reaction (qPCR) amplification. Using 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

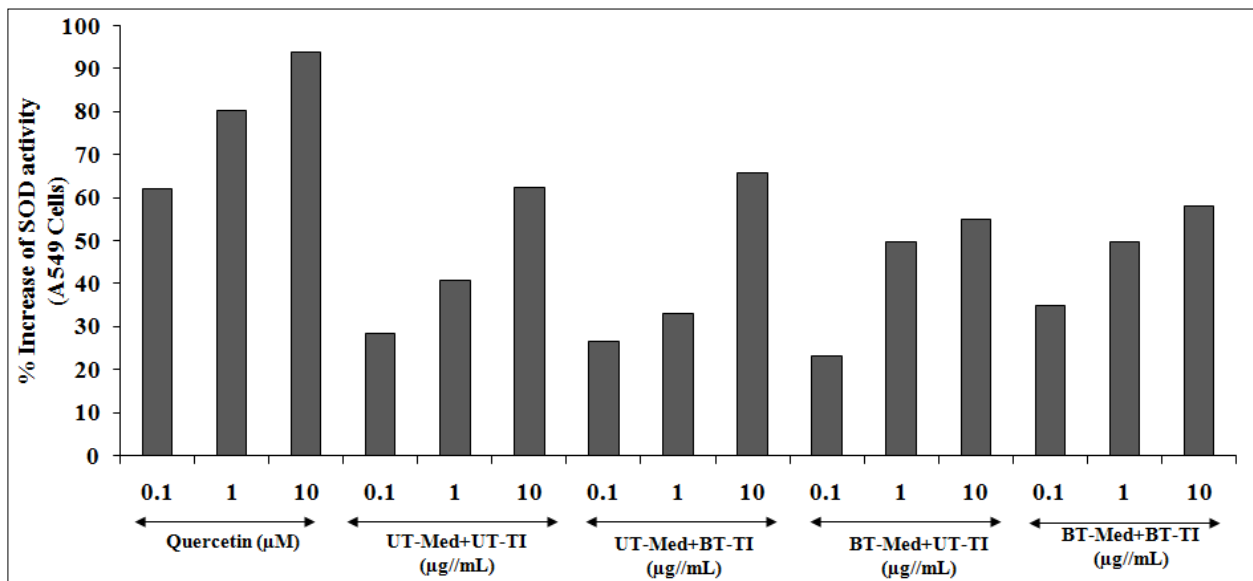


Figure 5. The improved percent protection of lungs cells (A549) in terms of increased SOD activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

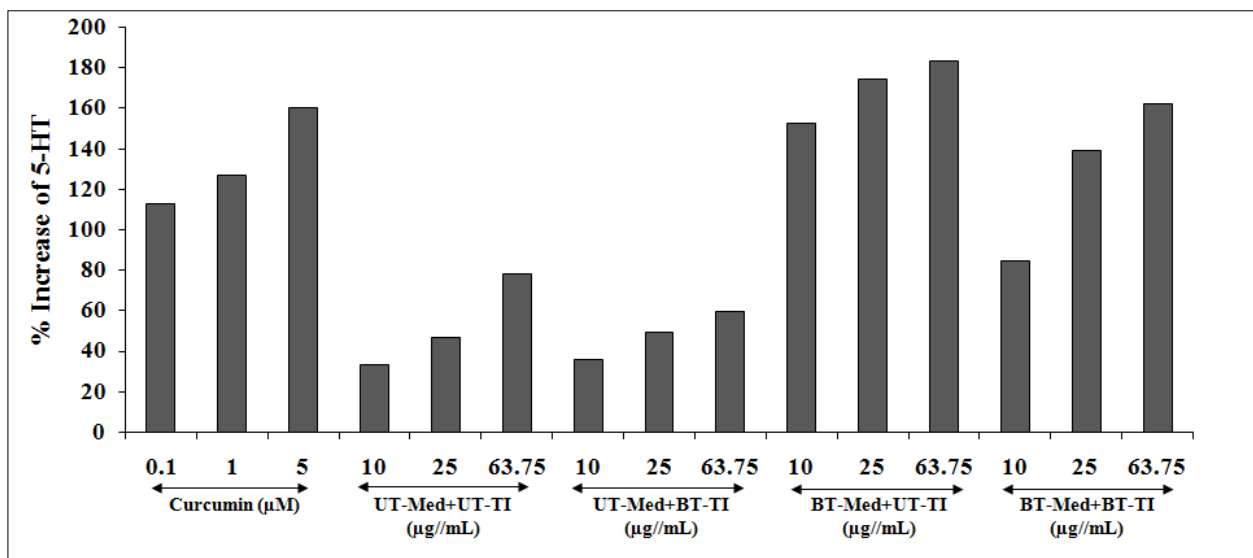


Figure 6. The effect of the test formulation on percent increase in 5-hydroxy tryptamine (5-HT) or serotonin in human neuroblastoma cells (SH-SY5Y). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

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 22.3%, 46.4%, and 171.3% at 1, 10, and 100 nM, respectively. The experimental test groups showed increased RQ of VDR expression by 75.6%, 73.6%, and 116.5% in the UT-Med + BT-TI group at 1, 10, and 50 µg/mL, respectively; while, 197%, 214.7%, and 198.8% increased RQ of VDR at 1, 10, and 50 µg/mL, respectively in the BT-Med + UT-TI group as compared to the untreated group. Further, RQ of VDR was significantly increased by 241.5%, 221%, and 235.8% at 1, 10, and 50 µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. VDR expression was significantly improved in the MG-63 cells after treatment with the test formulation and test media. 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]. Biofield Energy Healing Treatment would be the alternate treatment approach for bone related disorders such as low bone density, osteoporosis, and many more.

Conclusions

The safe concentrations of the test formulation was analyzed using MTT assay and was found as safe and non-toxic against all the tested cell lines. Cytoprotective activity against *t*-BHP induced cell damage using human cardiac fibroblasts cells (HCF) showed 89.9% (63.75 µg/mL) and 106.4% (63.75 µg/mL) restoration of cell viability in UT-Med + BT-TI and BT-Med + BT-TI groups, respectively as compared to untreated. Moreover, in HepG2 cells restoration of cell viability was observed as 77.3% and 69% in BT-Med + UT-TI and BT-Med + BT-TI groups, respectively as compared to untreated at 10 µg/mL. In A549 cells, cellular restoration was improved by 141.2% and 157.1% at 0.1 µg/mL in UT-Med + BT-TI and BT-Med + BT-TI groups, respectively as compared to untreated. ALP activity in Ishikawa cells showed significantly increased by 118.7% and 140.7% in UT-Med + BT-TI and BT-Med + UT-TI groups, respectively at 0.1 µg/mL as compared to untreated. Similarly, ALP activity in MG-63 cells was reported by 69.4%, 71.8%, and 71.4% cellular protection in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively at 50 µg/mL compared with the untreated. The percent protection of HCF cells (decreased of LDH activity) was significantly increased by 89.9% (63.75 µg/mL) and 106.4% (63.75 µg/mL) in

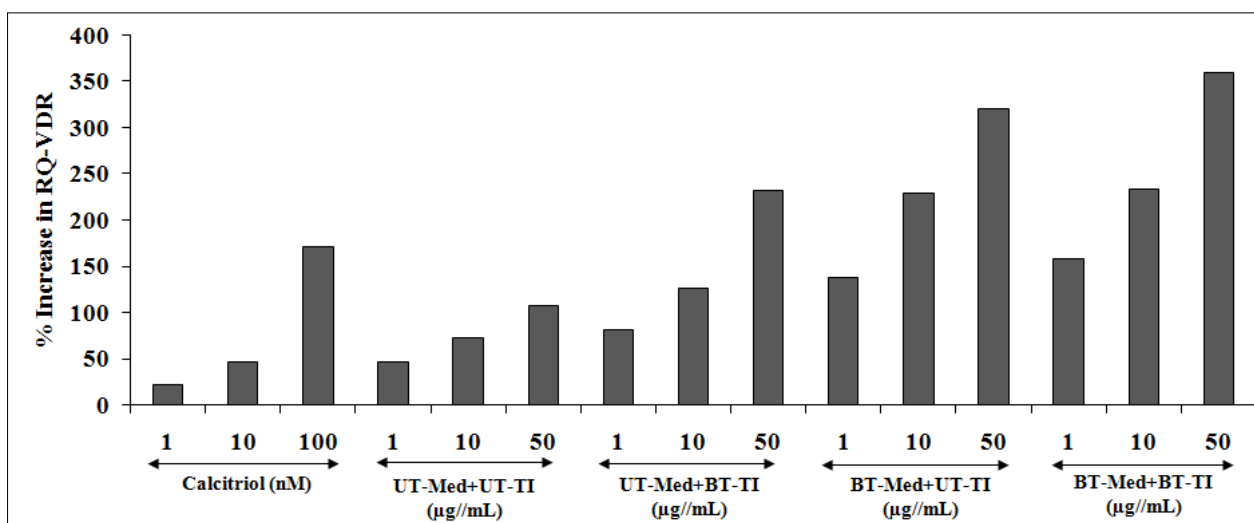


Figure 7. Relative quantification (RQ) of vitamin D receptors (VDRs) gene in human bone osteosarcoma cells (MG-63). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

UT-Med + BT-TI and BT-Med + BT-TI groups, respectively as compared to untreated. The percent protection of HepG2 cells (decreased of ALT activity) was significantly increased by 39.2% (1 µg/mL), 37.3% (25.5 µg/mL), and 59.8% (10 µg/mL) in UT-Med + BT-TI, BT-Med + UT-TI, and BT-Med + BT-TI groups, respectively as compared with the untreated. The percent protection of A549 (lungs) cells (increased of SOD activity) was significantly increased by 22.1% at 10 µg/mL in BT-Med + UT-TI group, while 22.8% and 22.1% at 1 and 10 µg/mL, respectively in BT-Med + BT-TI as compared to untreated. Serotonin level was significantly increased in SH-SY5Y cells by 361.7% (10 µg/mL) and 197.6% (25 µg/mL) in BT-Med + UT-TI and BT-Med + BT-TI groups, respectively compared with the untreated. The relative quantification (RQ) of vitamin D receptors (VDRs) level was significantly increased by 197%, 214.7%, and 198.8% at 1, 10, and 50 µg/mL, respectively in BT-Med + UT-TI group; while, 241.5%, 221%, and 235.8% at 1, 10, and 50 µg/mL, respectively in BT-Med + BT-TI as compared to untreated. Therefore, this study concluded that Biofield Energy based test formulation significantly improved the overall functioning of heart, liver, bones, neuronal, and lungs parameters against *t*-BHP induced oxidative stress. Thus, the Biofield Energy Treatment (The Trivedi Effect[®]) can be used for the prevention of various types of cardiac disorders such as stroke, congestive heart failure, congenital heart disease, rheumatic heart disease, valvular heart disease, venous thrombosis, etc. Besides, 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, Graves' Disease, Dermatitis, Diabetes, Parkinson's disease, Myasthenia Gravis, Ulcerative Colitis (UC), Atherosclerosis, etc. to improve overall health and Quality of Life.

Acknowledgements

Authors gratefully acknowledged to Trivedi Global, Inc., Trivedi Science, and Trivedi Master

Wellness for their support. In addition, authors are thankful for the support of Dabur Research Foundation for conducting this study.

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