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Biological Significance of the Biofield Energy Treatment Based Test Formulation on Various Biomarkers Using Cell-Based Assays

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Abstract

The aim of the present study determined the impact of the Biofield Energy Treated test formulation using cell lines related with vital organs functioning. Different cells based assay were used based on the vital organs function of bones, heart, liver, lungs, and brain. The test formulation and cells media was divided into two parts; one untreated (UT) and other part received the Biofield Energy Treatment remotely by a renowned Biofield Energy Healer, Dimitrius Anagnos, USA and were 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 non-toxic against all the cell line. Cytoprotective action of the test formulation showed a significant maximum restoration of cell viability by 25.6% (at 63.75 µg/mL), 46.7% (at 0.1 µg/mL), and 109.5% (at 63.75 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 human cardiac fibroblasts cells (HCF) cells, while 41.3%, 22.8%, and 34.8% at 63.75 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. However, cyto-protective activity in human hepatoma cells (HepG2) showed improved cell viability by 117.7% (at 0.1 µg/mL), 61.3% (at 25.5 µg/mL), and 104% (at 0.1 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. ALP activity in MG-63 cells was significantly increased by 105.7% at 10 µg/mL in the UT-Med + BT-TI group, while in Ishikawa cells showed maximum increased ALP activity by 368% and 602% at 0.1 µg/mL in the UT-Med + BT-TI and BT-Med + BT-TI groups respectively, as compared to the untreated group. The maximum percent cellular protection of HCF (heart) cells (decreased of LDH activity) was significantly increased by 58.8% at 1 µg/mL in the UT-Med + BT-TI group, while BT-Med + UT-TI group showed increased protection by 32.6% at 25 µg/mL, and improved cellular protection by 60.4% and 109.5% at 25 and 63.75 µg/mL respectively, in the BT-Med + BT-TI group 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 35.9% (at 10 µg/mL), 84.2% (at 25.5 µg/mL), and 87.6% (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. Cellular protection of A549 (lungs) cells (increased of SOD activity) in terms of percentage was increased by 35.2% (at 0.1 µg/mL), 35.2% (at 0.1 µg/mL), and 79.7% (at 1 µg/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 at 25 µg/mL by 30.6%, 107.7%, and 89.1% 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 at 50 µg/mL by 156.1%, 158.7%, and 68.1% 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. In conclusion, 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; Organ health; Cardiac health; Liver health; Lungs health; Multiple organ failure; Bone health

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Introduction

Herbo-mineral formulations contained specific metals or minerals as major composition, along with some vital herbs that have their beneficial effects on different biological systems. On the basis of scientific literatures, minerals, vitamins, and other constituents of the formulation would be selected that would significantly improve the overall organs health and quality of life. Thus, it would be a mandate to design such as novel test formulation and develop a quality profile, which contain all vital constituents in the formulation. Considering this, it is planned to evaluate biological activities of novel test formulation against wide range of pathological diseases using various biomarkers in human bones, heart, liver, lungs, and brain cells. These are the major body organs and concern of human overall health across the globe. Besides, it has been reported that free radicals (Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)) are one of the major contributors in various pathological conditions that cause serious deleterious effects on the body. These free radicals significantly affects the body macro-molecules such as lipids, proteins and DNA results in severe cellular damage and creates an imbalance among production of ROS and the defense systems[1]. Thus, the present scenario required a novel formulation, which could regulates the proper functioning of the organs and results in improved overall health conditions. With this respect, the test formulation is designed that composed of a total of 11 important ingredients such as calcium chloride, magnesium gluconate, zinc chloride, sodium selenate, ferrous sulfate, vitamin B_{12}, vitamin D_3, ascorbic acid, vitamin B_6, *panax ginseng* extract, and beta carotene. All the constituents present in the formulation was selected based on their scientific data such as panax ginseng is considered as potent immunomodulator, improved mental and physical abilities, improved mental and cognitive health, and was effective against many pathological conditions such as lung disorder, liver disorder, breast cancer, liver cancer, aging, muscle damage, and overall health[2]. Minerals and vitamins present in the formulation was reported to be highly active against wide range of pathological conditions with respect to health of major vital organs such as bones, heart, liver, lungs, and, brain. Zinc plays major role in most of the biochemical reaction in living organism due to its enzyme catalyzing activity. Magnesium reduces the production of inflammatory cytokine through activation of NF-κB pathways, which is a novel innate immunomodulatory mechanism[3-5]. However, the acceptance rate of nutraceuticals and the significant effects in various studies reported to be a successful lead in health care sector[6-8]. The novel test formulation was designed and tested against various standard specific cell lines, for bone, liver, heart, brain, and lung health along with overall female reproductive functions[9-18]. Thus, the present study was aimed to detect the overall health status using novel test formulation. Thus, this novel test formulation would be the best alternative medicine and can be used for multiple organ health disorders. Besides, multiple organ failure, cardiovascular diseases, and related chronic disorders are supposed to be responsible for high mortality rate worldwide[19,20].

Biofield Energy Healing Modalities are classified as Complementary and Alternative Medicine (CAM) therapies, which is supposed to be highly effective in order to enhance the physical, mental, and emotional human wellness[21,22]. In addition, National Center of Complementary and Integrative Health (NCCIH) has well defined, recognized, and accepted the Biofield Energy Healing therapies as a CAM health care approach along with other 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 worldwide in nonliving materials and living organisms. 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], improved 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

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 ethylene diaminetetraacetic 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. *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 was purchased 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 Treatment: Biofield Energy Healing was done on the test formulation, which was the combination of eleven ingredients *viz.* calcium chloride, *panax ginseng* extract, vitamin B_{12}, β-carotene, vitamin D_3, zinc chloride, magnesium gluconate, sodium selenate, ferrous sulfate, ascorbic acid, and vitamin B_6. The test formulation / media were divided into two parts, one portion was considered as the untreated group, where no Biofield Energy Treatment was provided. Further, the untreated group was treated by a "sham" healer for comparison purposes, who did not have any knowledge about the Biofield Energy Healing Treatment. Another test formulation portion received the Biofield Energy Treatment (The Trivedi Effect®) remotely by Dimitrius Anagnos, under standard laboratory conditions for ~3 minutes through healer's unique Biofield Energy Transmission process and was referred as the Biofield Energy Treated formulation. 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 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: 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 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].

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: Cytoprotective effect of the test formulation in various cells such as human cardiac fibroblasts-HCF; human hepatoma cells-$HepG_2$; 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 10mM *t*-BHP for 3.5 hours was given to the cells. The 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 = [(Absorbance$_{sample}$-Absorbance$_{t\text{-BHP}}$)]*100/ [Absorbance$_{untreated}$-Absorbance$_{t\text{-BHP}}$].............. (2)

Estimation of Alkaline Phosphatase (ALP) activity: For the estimation of ALP, 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 1 X 10^4 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 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 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 (*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 = {(X-R) / R}*100---------------------- (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 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. 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.

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

Estimation of ALT in liver cells ($HepG_2$): The human hepa-

toma cells ($HepG_2$) 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.

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

% Increase in SOD activity = ((X-R)/R)*100................ (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 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................ (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-N................ (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 percentage.

Results and Discussion

Cell viability using MTT assay: For the estimation of cell viability, MTT assay was performed in six cell-lines separately and the data were assessed as percentage of cell viability (Graph not shown). The criteria for non-cytotoxic concentration of test formulation and the positive controls by MTT cell viability assay was resulted in less than 30 % cytotoxicity or greater than 70 % cell viability were considered as non-cytotoxic concentrations. Overall, the experimental data suggested that the overall percent cell viability in different cell-lines *viz.* MG-63, Ishikawa, A549, $HepG_2$, HCF, and SH-SY5Y was more than 70% viable cells. Based on the percent cell viability data, it was observed that the test formulation and positive controls were found safe and non-toxic at the tested concentrations and were selected for other parameters.

Evaluation of cytoprotective effect of the test formulation: The test formulation was tested for initial screening of cytoprotective activity and 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 µg/mL, respectively as compared to the *t*-BHP induced group. Besides, the maximum restoration of cell viability among the tested groups by the test formulation was reported as 25.6% (at 63.75 µg/mL), 46.7% (at 0.1 µg/mL), and 109.5% (at 63.75 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 (UT-Med + UT-TI group). Similarly, silymarin was used as positive control in $HepG_2$ 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 30.1% and 41.3% at 25.5 and 63.75 µg/mL respectively, in the UT-Med + BT-TI group. Similarly, 14.1% and 22.8% improved cellular restoration was reported at 25.5 and 63.75 µg/mL respectively, at BT-Med + UT-TI groups as compared to the UT-Med + UT-TI group. However, 20.1% and 34.8% improved cellular restoration was reported at 25.5 and 63.75 µg/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 56.8% and 66.4% at 10 and 25 µg/mL, respectively compared to the *t*-BHP induced group. Besides, the test formulation showed maximum restoration of cell viability by 117.7%, 39.3%, and 58% at 0.1, 25.5 and 63.75 µg/mL respectively, in the UT-Med + BT-TI group. Similarly, 36.3% and 61.3% improved cellular restoration was reported at 10 and 25.5 µg/mL respectively, at BT-Med + UT-TI groups as compared to the UT-Med + UT-TI group. However, 104%, 90.5%, 53.9%, and 72.7% improved cellular restoration was reported at 0.1, 10, 25.5 and 63.75 µg/mL respectively, at BT-Med + BT-TI groups as compared to the UT-Med + UT-TI group. Overall, it can be assumed that significant improved cellular restoration was reported due to Biofield Energy Treatment in all the three tested cell lines. However, *in vitro* cell-based assays under the stimulation of oxidative stress using *tert*-butyl hydroperoxide (*t*-BHP) is considered as the standard method of analysis for cytoprotection[41,42], which also reflects the cellular protection of vital organs and their functioning *viz*. heart, liver, and lungs. Cytoprotection using various methods is now considered as one the vital tool to protect the cell against injuries due to free radicals and many other factors such as oxidative stress[43,44]. This also results in many immune related disorders such as cardiovascular diseases, aging, cancer, diabetes, and many more[45-47]. The present study revealed that Biofield Energy Healing Treatment (The Trivedi Effect®) significantly protects the *t*-BHP induced oxidative stress against in HCF, $HepG_2$, and A549 cells with respect to the cardiotoxicity, hepatotoxicity, and lung cell toxicity. Therefore, the Biofield Energy Healing Treatment could be successfully used for the management of various pathological etiologies against cardiovascular, liver, and various lung diseases.

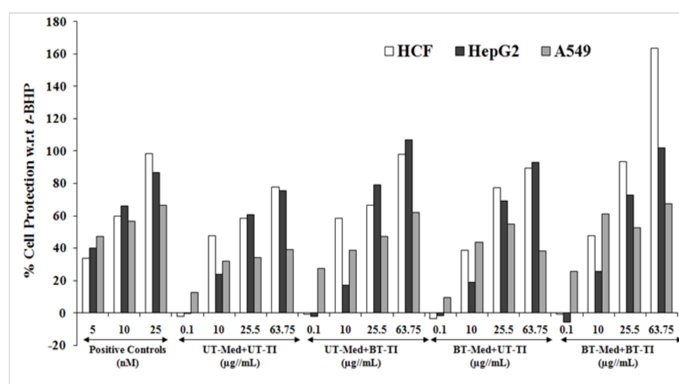

Figure 1: Cytoprotective action of the test formulation in human cardiac fibroblasts cells (HCF), human hepatoma cells ($HepG_2$), and adenocarcinomic human alveolar basal epithelial cells (A549) against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. Trimetazidine (µM), silymarin (µg/mL), and quercetin (µM) were used as positive control in HCF, $HepG_2$, and A549 cells, respectively. UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of Alkaline Phosphatase (ALP) Activity: The test formulation was tested for the estimation of ALP activity against two cell lines, MG-63 and Ishikawa cells. In case of MG-63 cells, calcitriol (nM) was used as positive control and the results suggested significant increased ALP level by 12%, 23%, and 53.5% at 0.1, 1, and 10 nM respectively as presented in Figure 2. However, the experimental test groups showed maximum increased ALP activity by 105.7%, 50.5%, and 99% at 10 µg/mL in UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, as compared to the UT-Med + UT-TI group in MG-63 cells. Similarly, naringenin was used as positive control for Ishikawa cells, and the data showed significant improved level of ALP by 35.8%, 69.5%, and 109.4% at 1, 5, and 10 nM, respectively. In the experimental tested groups, the ALP percent was significantly increased by 368%, 164.2%, and 137.2% at 0.1, 1, and 10 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 161%, 30.4%, and 52.8% at 0.1, 1, and 10 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 602%, 196.3%, and 189.6% at 0.1, 1, and 10 µg/mL, respectively in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Thus, the overall result showed significant improved ALP level because of Biofield Energy Healing Treated, which is reported as one of the significant bone health biomarker responsible for many bone related disorders[48,49] such as low bone density and osteoporosis, osteogenesis imperfect and Paget's disease of bone that makes the bones brittle.

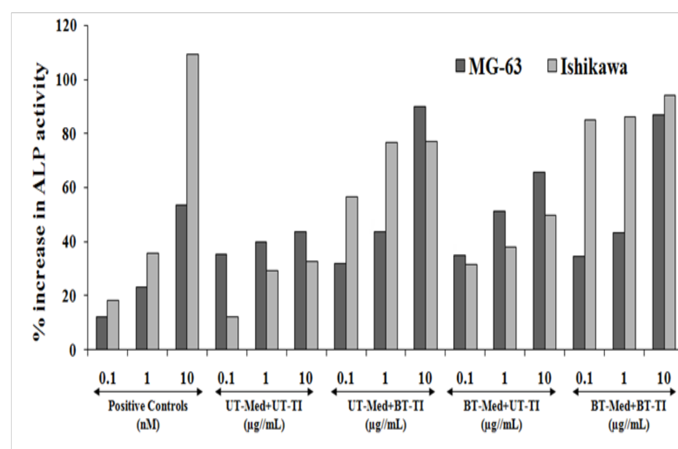

Figure 2: Alkaline phosphatase (ALP) activity in human bone osteosarcoma cells (MG-63) and human endometrial adenocarcinoma cells (Ishikawa) after treatment of the test formulation. Calcitriol and naringenin were used as positive control in MG-63 and Ishikawa cells, respectively. UT: Untreated; Med: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

Identification of lactate dehydrogenase (LDH) activity in human cardiac fibroblasts (HCF): LDH is an enzyme that is present in nearly all the living cells and responsible for anaerobic cellular respiration, while it has been extensively expressed in most of the body tissues, such as blood cells, skeletal muscle,

and heart muscle. LDH play a vital role in tissue injury, necrosis, hypoxia, hemolysis, or malignancies. In addition, LDH is one of the common and best biomarker for heart disease or tissue injuries. HCF cells are used for the estimation of LDH activity because 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]. Decreased LDH activity would be represented by increased cellular protection of HCF cells after treatment with the test formulation 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 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 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 58.8%, 22.4%, 14.2%, and 25.6% at 1, 10, 25, and 63.75 µg/mL concentrations respectively, in the UT-Med + BT-TI group, while 32.6% and 14.6% improved cellular protection (decreased of LDH activity) at 25 and 63.75 µg/mL respectively in the BT-Med + UT-TI group, and 60.4% and 109.5% improved cellular protection (decreased of LDH activity) at 25 and 63.75 µg/mL respectively, in BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. The experimental data suggested that there was a significant reduction of LDH level after Biofield Energy Treatment and protect HCF cells, which might be helpful to resist against various pathological conditions such as tissue injury, necrosis, hypoxia, hemolysis or malignancies.

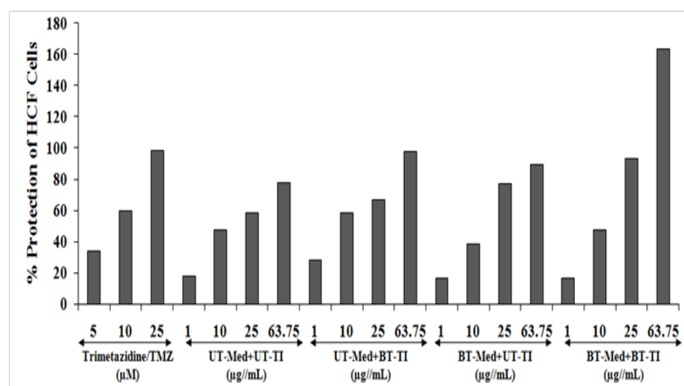

Figure 3: The effect of the test formulation on the percent protection of HCF cells in terms of decreased lactate dehydrogenase (LDH) activity against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. TMZ: Trimetazidine; UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of alanine amino transferase (ALT) activity in $HepG_2$ cells: ALT is an enzyme found mostly in the liver and kidney cells, along with heart and muscles in smaller amounts. This is one of the important that have vital function in cellular energy production, thus alteration in the level results in hepatocellular injury and death[53]. Various liver disorders are linked with high increased level of ALT that results in cellular damage and reduced cell viability[54]. ALT activity was evaluated using $HepG_2$ cells protection and the results are presented in terms of decreased ALT activity (Figure 4). The positive control, silymarin was selected in ALT activity and the data suggested increased percentage cellular protection (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 $HepG_2$ cells (decreased of ALT activity) by 35.9% and 10% at 10 and 25.5 µg/mL respectively, in the UT-Med + BT-TI group, while increased cellular protection of $HepG_2$ cells (decreased of ALT activity) by 56.5%, 84.2%, and 65% at 10, 25.5, and 63.75 µg/mL respectively, in the BT-Med + UT-TI group, and increased cellular protection of $HepG_2$ cells (decreased of ALT activity) by 87.6% and 81.8% at 10 and 63.75 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 4). Therefore, the experimental data suggested Biofield Energy Treatment significantly protects the liver hepatocytes with reduced ALT enzyme as compared to the *t*-BHP inducing group. This can be significantly useful in liver cancer, liver cirrhosis, hepatomegaly, liver failure, and hepatitis.

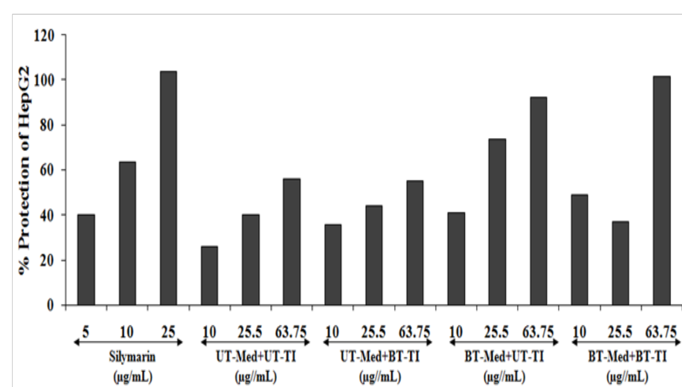

Figure 4: The effect of the test formulation on the percent protection of human liver cancer ($HepG_2$) cells in terms of decreased alanine amino transaminase (ALT) activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item.

Estimation of superoxide dismutase (SOD) activity in adenocarcinomic human alveolar basal epithelial cells (A549): Most of the body cells have their own defense system and high quantity of antioxidants, which prevent or repair the cellular damage caused by free radicals, reactive oxygen species (ROS) and many other factors that cause cell death. SOD is one of the important antioxidant defense mechanism of body that can reduce ROS generation, oxidative stress along with inhibition of endothelial activation[55]. The study was designed to identify the level or change on SOD level in the A549 cells and improved activity represents increased cellular protection and the data was presented in Figure 5. The positive control, quercetin showed improved percentage increase in the SOD activity with respect to the *t*-BHP by 68.4%, 83.9%, 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 35.2% at 0.1 µg/mL in the UT-Med + BT-TI group, while increased SOD activity by 35.2% and 4.2% at 0.1 and 1 µg/mL in the BT-Med + UT-TI group, and increased SOD activity by 79.7% and 5.5% at 1 and 10 µg/mL in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 5). The experimental data showed that Biofield Energy Treat-

ment has significantly improved the SOD activity that can be used in various respiratory diseases such as pneumonia, asthma, pulmonary fibrosis, and lung cancer.

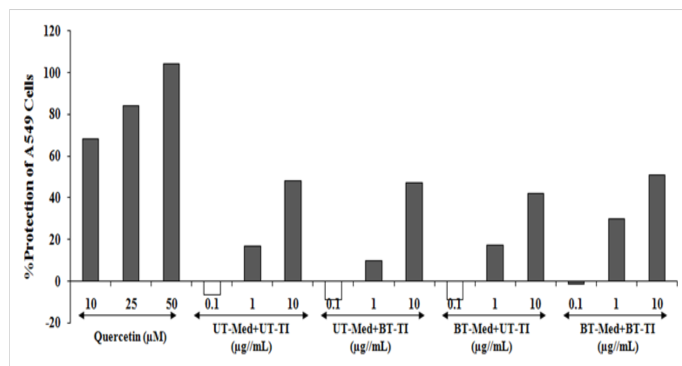

Figure 5: The effect of the test formulation on the percent protection of lungs cells (A549) in terms of increased SOD activity under the stimulation of *tert*-butyl hydroperoxide (*t*-BHP). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item. Data are expressed as mean ± SD of three independent experiments.

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. Serotonin activity was tested and the effect of Biofield Energy Treated test formulation is presented in Figure 6. The positive control, curcumin showed 66.3%, 115.1%, and 143.4% increased the level of serotonin at 0.1, 1, and 5 µg/mL respectively, compared to the vehicle control (VC) group. The data showed significant increased serotonin level by 30.6% and 3.5% at 25 and 63.75 µg/mL respectively, in the UT-Med + BT-TI, while significant increased serotonin by 16.5%, 46.1%, 107.7%, and 46.6% at 1, 10, 25, and 63.75 µg/mL respectively, in the BT-Med + UT-TI, and 29.7%, 89.1%, and 44% improved serotonin level at 10, 25, and 63.75 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group (Figure 6). Serotonin role has been well defined and was present in the brain, bowels, and blood platelets. Besides, 5-HT role has been predefined in many neuronal functions such as sleep, feeding, pain, sexual behavior, cardiac regulation, and cognition. Serotonin imbalance results in many neuropsychiatric disorders such as Alzheimer's disease, cognitive health, loss of ability of thinking, depression, memory loss, *etc.*[56]. Our research study showed significant improved level of serotonin after treatment with the Biofield Energy Healing Treated test formulation that would be highly useful against various neurodegenerative diseases and improved brain functioning. Besides, it can have various applications utilizing the measurement of antioxidant in cells and tissue are highly diverse and span in various disciplines like genetics, biochemistry, physiology, neuroscience, and molecular biology.

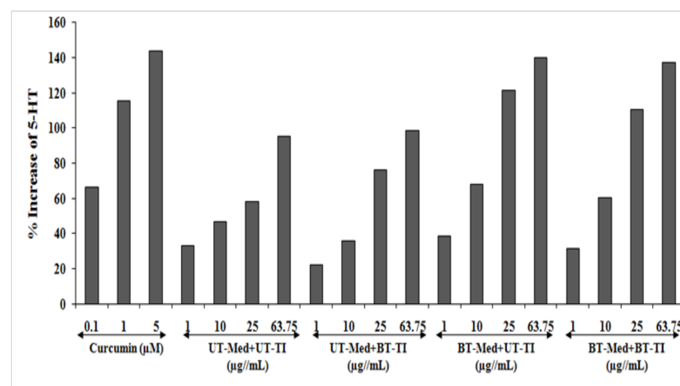

Figure 6: The effect of the test formulation on percent increase in 5-hydroxy tryptamine (5-HT) or serotonin in human neuroblastoma cells (SH-SY5Y). UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

Effect of test formulation on vitamin D receptors (VDRs): Human bone osteosarcoma cells (MG-63) was used for the estimation of VDR activity, which is the ideal *in vitro* testing method for any test compound. The expression of VDRs was studies 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-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 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 24.1%, 136.3%, and 156.1% in the UT-Med + BT-TI group at 1, 10, and 50 µg/mL respectively, while 26.6%, 98.8%, and 158.7% 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 26.8% and 68.1% at 10 and 50 µg/mL respectively, in the BT-Med + BT-TI group as compared to the UT-Med + UT-TI group. Overall, the data concluded that VDR expression was significantly improved in MG-63 after treatment with the test formulation. 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].

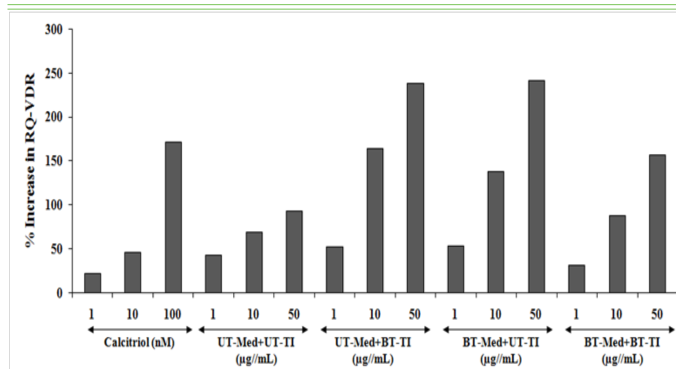

Figure 7: Effect of the test formulation on percent increase in relative quantification (RQ) of vitamin D receptors (VDRs) gene in human bone osteosarcoma cells (MG-63).UT: Untreated; Med: Medium; BT: Biofield Treated; TI: Test item

Conclusions

The test formulation was tested in different cell lines for multiple organ health analysis using combinations with the Biofield Energy Treated and untreated test formulation groups. The MTT assay 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 25.6% (at 63.75 µg/mL), 46.7% (at 0.1 µg/mL), and 109.5% (at 63.75 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 $HepG_2$ cells the maximum restoration of cell viability was 41.3% at 63.75 µg/mL in the UT-Med + BT-TI group, and the test formulation in A549 cells showed maximum restoration of cell viability by 117.7% at 0.1 µg/mL in the BT-Med + BT-TI group as compared with the untreated test group. Similarly, ALP activity in MG-63 cells showed significantly increased ALP activity at 10 µg/mL by 105.7%, 50.5%, and 99% in UT-Med + BT-TI, BT-Med + UT-TI group, and BT-Med + BT-TI groups respectively, as compared to the UT-Med + UT-TI group. Similarly, ALP activity in Ishikawa cells with maximum cellular protection was reported at 0.1 µg/mL by 368% and 602% in UT-Med + BT-TI and BT-Med + BT-TI group test groups, respectively, as compared with the untreated test group. The LDH activity was significantly decreased and the data was presented in increased percentage cellular protection data, which showed maximum cellular protection by 58.8% (at 1 µg/mL), 32.6% (at 25 µg/mL), and 60.4% (at 25 µg/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 $HepG_2$ cells (decreased of ALT activity) by 35.9% (at 10 µg/mL), 84.2% (at 25.5 µg/mL), and 87.6% (at 10 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 35.2% (at 0.1 µg/mL), 35.2% (at 0.1 µg/mL), and 79.7% (at 1 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 30.6% (at 25 µg/mL), 107.7% (at 25 µg/mL), and 89.1% (at 25 µg/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 136.3%, and 156.1% in the UT-Med + BT-TI group at 10 and 50 µg/mL respectively, while 98.8%, and 158.7% increased RQ of VDR at 10 and 50 µg/mL respectively, in the BT-Med + UT-TI group, and increased RQ of VDR by 26.8% and 68.1% at 10 and 50 µg/mL respectively, in the BT-Med + BT-TI group as compared to the untreated test control group. Thus, this experimental study concluded that the Biofield Energy based test formulation can improve the overall functioning of heart, liver, bones, neuronal, and lungs parameters against any oxidative stress or damage induced by free radicals. Thus, 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*. 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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