Hepatoprotective Effect of Biofield Energy Treatment On *Tert*-Butyl Hydro Peroxide Induced Liver Injury in Hepatocellular Carcinoma Cell Line (HepG2)

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Abstract

The present study was performed to investigate the Hepatoprotective potential of the Biofield Energy (The Trivedi Effect®) Treated test item, Dulbecco's Modified Eagle Medium (DMEM) in HepG2 cells. The test item was distributed into two parts. One part received Consciousness Energy Healing Treatment by a renowned Biofield Energy Healer, Mahendra Kumar Trivedi and was labeled as the Biofield Energy Treated DMEM group and the other part referred as the untreated DMEM group, where no Biofield Treatment was provided. Results showed that more than 97% cell viability of the test items were observed by MTT assay, which indicated a safe and nontoxic nature of the test items. The Biofield Treated DMEM showed significant ($p \leq 0.001$) protection of cells by 10% against oxidative stress induced by *t*-BHP, while the untreated DMEM group showed 0.4% protection. The level of interleukin-8 (IL-8) was significantly ($p \leq 0.01$) reduced by 31.57% in Biofield Treated DMEM than untreated DMEM. The level of ALT enzyme activity was significantly ($p \leq 0.001$) reduced by 66% in Biofield Treated DMEM compared to untreated DMEM. Cholesterol level was significantly ($p \leq 0.001$) reduced by 46.23% in Biofield Treated DMEM than untreated DMEM. Besides, Biofield Treated DMEM group showed 51.18% increased the level of albumin compared to untreated DMEM group. Overall, results demonstrated that Biofield Treatment significantly protect the liver hepatocytes against oxidative stress. Therefore, Consciousness Energy Healing (The Trivedi Effect®) Treatment might be useful as a hepatoprotectant against different types of liver injuries like cirrhosis, alcohol abuse, hemochromatosis, Wilson's disease, Gilbert's disease, cholangiocarcinoma, steatosis, Budd-Chiari syndrome, etc.

Abbreviations

CAM : Complementary and Alternative Medicine

DMEM : Dulbecco's Modified Eagle Medium

FBS : Fetal Bovine Serum

Introduction

The liver is a vital organ actively involved in most of the metabolic functions. Hepatic damage is associated with distortion of these metabolic functions [1]. Liver disorders are the serious global health burdens occurring due to modern food styles, excessive pollution and intake of some drugs [2]. "As per the World Health Organization records, liver cirrhosis is the 8th leading cause of death in the United States and 13th leading cause of death globally, with worldwide mortality having increased by 45.6% from 1990 to 2013 due to alcohol-induced liver cirrhosis" [3]. Overproduction of free radical species like Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) leads to an increased lipid peroxidation end product that causes hepatic necrosis [4-6]. Human hepatoma cell lines (HepG2) have been routinely used as an alternative model to human hepatocytes *in vitro* for the evaluation of Hepatoprotective activity [7,8]. Moreover, this cell line has many advantages *viz.* easily available and cryopreserved in huge quantity, and even the drug metabolizing capability do

not decrease upon cultivation [9]. There are huge limitations for conducting liver disease research in human beings due to high cost and potential ethical issues. Therefore, the authors focused to develop an alternative treatment to investigate the potential of Consciousness Energy Healing Treatment (The Trivedi Effect®) on the test item, Dulbecco's Modified Eagle Medium (DMEM) for protection of hepatocyte cells in Human Hepatoma Cells (HepG2). Numerous scientific data supported the useful effects of Biofield Energy Treatment in cases of cancer patients *via* massage therapy [10], therapeutic touch [11], etc. Biofield Therapy or Healing Modalities is one of treatment approach under the broad category of Complementary and Alternative Medicine (CAM) therapies to enhance physical, mental, and emotional human wellness.

The National Center of Complementary and Integrative Health (NCCIH) has recognized and accepted Biofield Energy Healing as a CAM health care approach in addition to other therapies, medicines and practices such as chiropractic/osteopathic manipulation, natural products, yoga, deep breathing, Tai Chi, meditation, Qi Gong, special diets, massage, relaxation techniques, progressive relaxation, homeopathy, acupuncture, guided imagery, hypnotherapy, healing touch, movement therapy, rolfing structural integration, pilates, mindfulness, Ayurvedic medicine, acupressure, essential oils, traditional Chinese herbs and medicines, naturopathy, Reiki, aromatherapy, and cranial sacral therapy. Human "Biofield" has a subtle form of energy that has the ability to act as an effective manner [12]. CAM therapies have been practiced worldwide with reported clinical benefits in various health care system [13].

This energy can be harnessed and transmitted by the experts into living and non-living things *via* the process of a unique Energy Transmission process (The Trivedi Effect®). The Trivedi Effect®- Consciousness Energy Healing Treatment has been reported with a significant revolution in the field of cancer research [14,15], nutraceuticals [16,17], skin health [18,19], bone health [20-22], materials science [23-25], agriculture [26,27], microbiology [28-30], biotechnology [31,32]. Apart from this, The Trivedi Effect® also tremendously improved bioavailability of various low bioavailable compounds [33-35] and improved overall human health and wellness. Based on the above information and to minimize the existed limitations in liver research in human beings, authors intend to develop a new treatment strategy to study the impact of the Biofield Energy Healing Treatment (The Trivedi Effect®) on the test item (DMEM) for liver hepatocyte cells growth in Hepatocellular Carcinoma Cell Line (HepG2).

Materials and Methods

Chemicals and Reagents

Antibiotics solution (Penicillin-Streptomycin) was purchased from HiMedia. Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were obtained from Gibco, India. Alanine Aminotransferase (ALT) 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and Ethylenediaminetetraacetic Acid (EDTA) were obtained from Sigma Chemical Co. (St. Louis, MO). The positive controls silymarin and mevinolin were procured from Sanat products ltd., India and Zliesher Nobel, respectively. All the other chemicals used in this experiment were analytical grade procured from India.

Biofield Energy Healing Strategy

The test item (DMEM) was used in this experiment and one portion was considered as the untreated DMEM group, where no Biofield Treatment was provided. Further, the untreated group was treated with "sham" healer for comparison purpose. The sham healer did not have any knowledge about the Biofield Energy Healing Treatment. The other portion of the test item was received Biofield Energy Treatment and defined as the Biofield Energy Treated DMEM group. Biofield Energy Healing Treatment (known as The Trivedi Effect®) was received under laboratory conditions for ~3 minutes through Mahendra Kumar Trivedi's unique Biofield Energy Transmission process. Biofield Energy Healer was located in the USA, 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

The cell viability was performed by MTT assay in HepG2 cell line. The cells were counted and plated in a 96-well plate at the density corresponding to 10×10^3 cells/well/180 μL in DMEM + 10% FBS. The cells in the above plate(s) were incubated for 24 hours in a CO_2 incubator at $37^{\circ}C$, 5% CO_2, and 95% humidity. Following incubation, the medium was removed and the following treatments were given. In the Biofield Treated test item (DMEM) group, 200 μL of the Biofield Energy Treated test item (DMEM) was added to wells, and in the untreated DMEM group, added 200 μL of untreated DMEM. Besides, in the positive control groups, added 180 μL of DMEM with 20 μL of positive controls were added from the respective 10X stock solutions.

After incubation for 48 hours, the effect of test items on cell viability was assessed by MTT assay. 20 μL of 5 mg/mL of MTT was added to all the wells and incubated at $37^{\circ}C$ for 3 hours. The supernatant was aspirated and 150 μL of Dimethyl Sulfoxide (DMSO) was added to all wells to dissolve formazan crystals. The Optical Density (OD) of each well was read at 540 nm using Biotek Reader.

Effect of the test items on viability of HepG2 cells was determined using Equation (1):

$$\% \; Cell \; viability \; = (100 - \% \; Cytotoxicity) \ldots\ldots\ldots\ldots\ldots (1)$$

Where, % Cytotoxicity = {(O.D. of cells of untreated DMEM – O.D. of cells Biofield Treated DMEM/positive controls)/ O.D. of cells of untreated DMEM}*100 For test items and positive controls, concentrations resulting ≥70% cell viability were taken as safe/non-cytotoxic concentration.

Evaluation of Cytoprotective Effect of the Test Item

Cells were trypsinized and a single cell suspension of HepG2 was prepared. Cells were counted on an hemocytometer and seeded at a density of 10×10^3 cells/well/180 μL in DMEM + 10% FBS in a 96-well plate. Cells were incubated in a CO_2 incubator for 24 hours at 37°C, 5% CO_2 and 95% humidity. After 24 hours, the medium was removed and the following treatments were given. In the test item groups, 180 μL of the test items were added to wells. In the positive control group, 160 μL of serum free medium and 20 μL of positive control from the respective 10X stock solution was added to wells. After 24 hours of treatment, cells were treated with *t*-BHP at 250 μM (20 μL from the respective 10X stock) for 4 hours. After 4 hours, the protective effect of the test items on cell viability was assessed by MTT assay as per study protocol.

Estimation of Interleukin-8 (IL-8)

HepG2 cell suspension in DMEM containing 10% FBS was plated at a density of 0.3×10^6 cells/well/1 mL in a 12-well plate. Cells were incubated in a CO_2 incubator for 24 hours at 37°C, 5% CO_2, and 95% humidity. Cells were sera starved by replacing the medium with DMEM + 10% FBS for 24 hours. After 24 hours of sera starvation, medium was removed and pre-treatment were provided to the different treatment groups. After 24 hours of treatment, cells were stimulated with inflammatory stimulus TNF-α at a final concentration of 10 ng/mL. After treatment, cells were incubated in a 5% CO_2 incubator for 24 hours. After 24 hours of incubation, culture supernatants were collected from each well and stored at -20°C until analysis. The level of cytokine (IL-8) in culture supernatants of HepG2 cells was determined using ELISA as per manufacturer's instructions.

Estimation of ALT

Cells were trypsinized and a single cell suspension of HepG2 was prepared and counted on an hemocytometer. Cells were seeded at a density of 10×10^3 cells/well/180 μL in DMEM + 10% FBS in a 96-well plate. Cells were incubated in a CO_2 incubator for 24 hours at 37°C, 5% CO_2, and 95% humidity. After 24 hours, medium was removed and different treatments were given as per study plan. After incubation for 24 hours, cells were treated with 250 μM of *t*-BHP. After 4 hours of incubation, culture supernatants were collected from each well and stored at -20°C until analysis. The level of ALT in culture supernatants of HepG2 cells was determined using commercial kit as per manufacturer's instructions.

Estimation of Cholesterol

Cells were trypsinized and a single cell suspension of HepG2 was prepared. Cells were counted using an hemocytometer and seeded at a density of 1 million cells/well/mL in DMEM + 10% FBS in a 6-well plate. Cells were incubated in a CO_2 incubator for 24 hours at 37°C, 5% CO_2 and 95% humidity. After 24 hours, medium was removed and treated with different treatment groups. After 24 hours of incubation, cell lysates were prepared in the following manner. Lysis buffer containing chloroform: isopropanol: IGEPAL CA630 in the ratio of 7:11:0.1 was prepared. Medium was removed from each well and 400 μL of the above buffer was added to each well, which led to detachment of cells and formation of white layer. Cells were scrapped off and transferred into a labeled centrifuge tubes. The cells were homogenized in ice using a tissue homogenizer for 4-5 minutes until the solution was turned turbid in appearance. After homogenizing, the cells were centrifuged at 13000*g* for 10 minutes. The supernatant was collected in a prelabeled centrifuge tube and the pellet was discarded. The tube containing the supernatant was kept at 37°C for 24 hours for evaporation of buffer. After 24 hours, the tube was removed from 37°C and the dried lipids (small yellow colored pellet) were obtained, which was stored at -20°C until analysis. The level of cholesterol in cell lysates of HepG2 cells was determined using a commercial kit as per manufacturer's instructions.

Estimation of Albumin

Cells were trypsinized and a single cell suspension of HepG2 was prepared. Cells were counted using an hemocytometer and seeded at a density of 0.25 million cells/well/1 mL in DMEM + 10 % FBS in a 24-well plate. Then, the cells were incubated in a CO_2 incubator for 24 hours at 37°C, 5% CO_2, and 95% humidity. Further, the cells were sera starved by replacing the medium with DMEM + 10% FBS for 24 hours. After 24 hours, medium was removed and various treatments were given. After 48 hours of incubation, culture supernatants were collected from each well and stored at -20°C until analysis. The level of albumin in culture supernatants of HepG2 cells were determined using a commercial kit as per manufacturer's instructions.

Statistical Analysis

All the values were represented as Mean ± SEM (standard error of mean) of three independent experiments. 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 $p \leq 0.05$.

Results and Discussion

Assessment of Cell Viability Using MTT Assay

The results of the cell viability of the test items and positive

controls using MTT assay in HepG2 cells are shown in (Figure 1). Silymarin (a known potent hepatoprotectant) showed more than 136% at the concentrations between 5 to 25 µg/mL and mevinolin, "a highly potent competitive inhibitor of Hydroxymethylglutaryl-Coenzyme A (HMG-CoA) reductase and a cholesterol-lowering agent" showed greater than 97% cell viability upto 20 µg/mL. Besides, the untreated DMEM and Biofield Energy Treated DMEM showed 113% and 108.9% cell viability, respectively. Overall, the positive controls and the Biofield Energy Treated test items found more than 97% cell viability, which indicated that the test items and positive controls were safe and nontoxic at the tested concentrations.
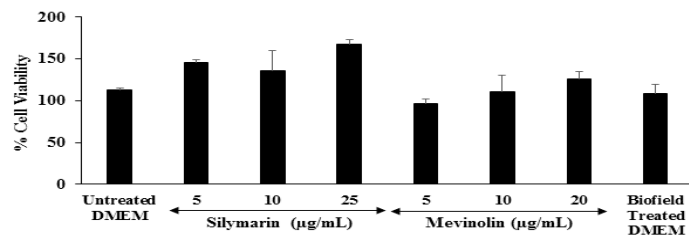

Figure 1: Effect of the test items and positive controls on cell viability in HepG2 cells after 48 hours of treatment. All the values are represented as mean ± SEM of three independent experiments.

Evaluation of Cytoprotective Effect of the Test Items

tert-Butyl hydroperoxide (*t*-BHP) has been extensively used as a chemical inducer for the preparation of liver-injured animal model [36]. In hepatocyte cultures and in the liver, *t*-BHP is metabolism by liver cytochrome-P450 to form free radicals and subsequently initiate lipid peroxidation, causes cell injury [37]. The impact of the Biofield Energy Treated test item on the restoration of cell viability of HepG2 cells was determined against *t*-BHP induced cell damage. The cytoprotective effect of the test item against *t*-BHP induced damage is shown in (Figure 2). Silymarin restored cell viability by 4.9%, 38.4% (*p*≤0.001), and 66.1% (*p*≤0.001) at 1, 5, and 25 µg/mL, respectively compared to the *t*-BHP induced group. Further, the Biofield Energy Treated DMEM showed 10% and the untreated DMEM exhibited 0.4% restoration of cell viability under the influence of *t*-BHP. The results showed that Consciousness Energy Healing has significantly protect hepatocyte cells induced by *t*-BHP, which could be due to The Trivedi Effect®. Therefore, The Trivedi Effect® - Treated DMEM could be more useful for the management liver disorders.
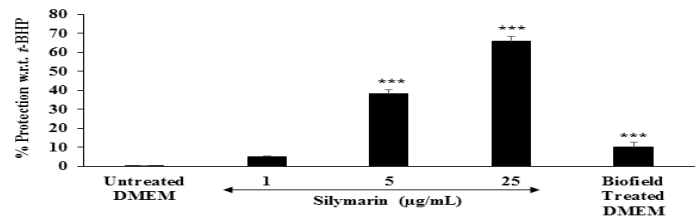

Figure 2: Evaluation of cytoprotective effect of the test items in HepG2 cells against *tert*-butyl hydroperoxide (*t*-BHP) induced damage. All the values are represented as mean ± SEM of three independent experiments. ***p≤0.001 *vs*. untreated DMEM group.

Estimation of Interleukin-8 (IL-8)

Interleukin-8 (proinflammatory chemokine) is a potent chemoattractant for neutrophils [38], and causes acute liver inflammation. Increased level of oxidative stress causes increased secretion of IL-8, and ultimately recruit the inflammatory cells causes' localized inflammation [39]. From literatures indicated that the level of IL-8 was high in case of chronic liver disease (alcoholic liver disease and hepatitis C) [40]. The effect of the test items on the interleukin-8 (IL-8) is shown in (Figure 3). Moreover, the Biofield Energy Treated DMEM showed 31.57% reduction of IL-8 compared to the untreated DMEM group.
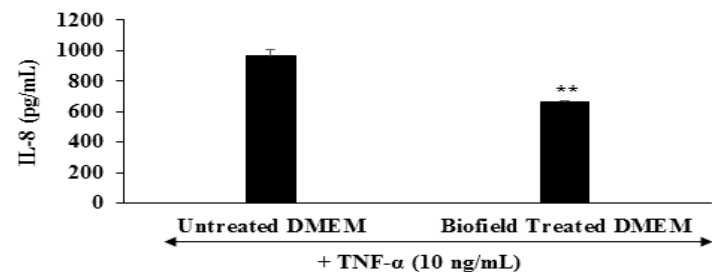

Figure 3: The effect of the test items on the level of Interleukin-8 (IL-8) against TNF-α stimulation. TNF-α: Tumor Necrosis Factor Alpha. All the values are represented as mean ± SEM of three independent experiments. **p≤0.01 *vs*. untreated DMEM group.

Estimation of Alanine Aminotransferase (ALT)

The effect of the test items on Alanine Aminotransferase (ALT) is shown in (Figure 4). Silymarin (positive control) 8.4%, 25.6%, and 79.2% (*p*≤0.01) reduction of ALP level at 1, 5, and 25 µg/mL, respectively with respect to the untreated DMEM group. Additionally, the Biofield Energy Treated DMEM group

showed a significant ($p \leq 0.01$) reduction of ALT by 66% compared to the untreated DMEM group (Figure 4). Aminotransferases are an excellent marker of hepatocellular injury. There were various factors responsible for liver disorders *viz.* alcohol, medication, viral hepatitis, autoimmune hepatitis, Wilson's disease etc. in which the transaminases levels were raised. Thus, an elevation of serum ALT enzyme chances of clinically significant liver disorders [41-43]. In this experiment, the Biofield Energy Treatment significantly protect the liver hepatocytes in terms of reducing the level of ALT under the stimulation of *t*-BHP.
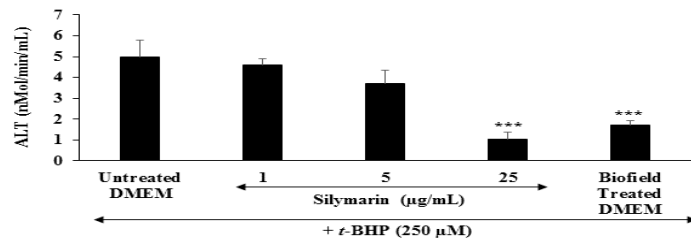

Figure 4. The effect of the test items on Alanine Amino-Transaminase (ALT) enzyme activity against *Tert*-Butyl Hydroperoxide (*t*-BHP) induced cell damage after 4 hours of treatment. All the values are represented as mean ± SEM of three independent experiments. ***$p \leq 0.01$ *vs.* untreated DMEM group.

Estimation of Cholesterol

The effect of the test items on the level of cholesterol is shown in (Figure 5). The positive control (mevinolin) showed 17.45%, 25%, and 80.19% ($p \leq 0.001$) reduction of cholesterol at the concentration of 5, 10, and 20 μM, respectively compared to the untreated DMEM group. Further, cholesterol level was significantly reduced by 46.23% in the Biofield Energy Treated DMEM group with respect to the untreated DMEM group (Figure 5). Optimum level of cholesterol is important for good health. One of the function of liver is to produce and clear cholesterol in the body. Most of the cases, a high level of cholesterol is always harmful; however it is necessary for the synthesis of hormones, vitamin D, and various enzymes [44]. Overall, liver plays a central role in cholesterol homeostasis. Due to abnormality of liver functions lead to excess levels of lipid profile that ultimately leads to heart disease [45]. Here, The Trivedi Effect® has significantly reduced cholesterol level, which could be beneficial for hepatic and cardiac patients and to improve overall health.
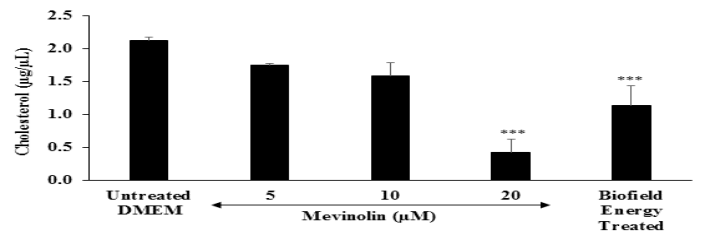

Figure 5: The effect of the test items on cholesterol synthesis in HepG2 cells after 24 hours of treatment. Values are represented as mean ± SEM of three independent experiments. ***$p \leq 0.001$ *vs.* Untreated DMEM group.

Estimation of Albumin

The effect of the test items on the level of albumin in shown in (Figure 6). Albumin level was significantly increased by 29.65%, 69.51%, 100.21% ($p \leq 0.001$), and 142.78% ($p \leq 0.001$) at 0.5, 1, 5, and 20 μM, respectively in the positive control (silymarin) group compared to the untreated DMEM group. Besides, the Biofield Energy Treated DMEM group showed 51.18% increase the level of albumin compared to the untreated DMEM group (Figure 6). Literature suggest that due to liver disease the albumin level become low. It is a protein synthesized by liver. Apart from liver disease severe malnutrition and kidney disease also responsible for low albumin. It regulates the osmolarity and oncotic pressure of interstitial fluid in the liver extravascular space, which is influenced by various hormonal factors such as insulin, cortisol, and growth hormone [46]. It act as modulator of fluid distribution of whole body compartments [47]. Besides, it act as an antioxidant by scavenging reactive oxygen species [48]. Overall, in this experiment Biofield Treatment significantly elevated the level of albumin, which is required for maintenance of bodies osmotic and oncotic pressure.
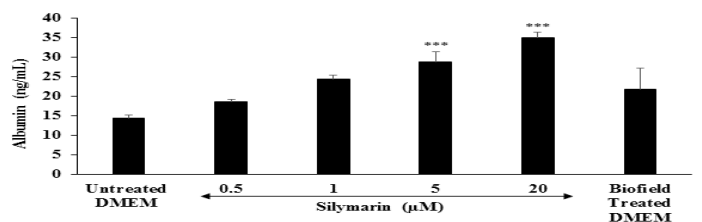

Figure 6: Effect of the test items on albumin levels assessed in HepG2 cells after 48 hours of treatment. Data are represented as mean ± SEM of three independent experiments. ***$p \leq 0.001$ *vs.* untreated DMEM group.

Conclusions

In summary, the study results Showed That the Test Items (DMEM) were found as safe and non-toxic on the basis of MTT cell viability assay. The Biofield Energy Treated test item (DMEM) showed a significant ($p \leq 0.001$) protection of cells by 10% from the oxidative damage induced by *t*-BHP, while the untreated DMEM group showed 0.4% protection. IL-8 level was significantly ($p \leq 0.01$) reduced by 31.57% in the Biofield Energy Treated DMEM group compared to the untreated DMEM group. Moreover, ALT enzyme activity was significantly ($p \leq 0.001$) reduced by 66% in the Biofield Energy Treated DMEM group compared to the untreated DMEM group. Cholesterol level was significantly ($p \leq 0.001$) reduced by 46.23% in the Biofield Energy Treated DMEM group compared to the untreated DMEM group. Further, Biofield Energy Treated DMEM group showed 51.18% increased the level of albumin compared to the untreated DMEM group. It is therefore concluded that, The Trivedi Effect® - Consciousness Energy Healing Treatment significantly protect hepatocytes and could be used as an alternative treatment approach for the management of various types of hepatobiliary disorders *viz*. cirrhosis, acute hepatitis A, B, C, D, and E, cholestasis, chronic viral hepatitis, portal hypertension in schistosomiasis, necrosis, toxoplasmosis, hepatosplenic schistosomiasis, liver abscess, autoimmune hepatitis, granulomatous hepatitis, primary biliary cholangitis (primary biliary cirrhosis), phlebitis of the portal vein, etc. In broad perspective, it could be useful to improve cell-to-cell messaging, normal cell growth and differentiation, cell cycling and proliferation, neurotransmission, skin health, hormonal balance, immune and cardiovascular functions. Moreover, it can also be utilized in aging, and various inflammatory and immune-related disease conditions like Ulcerative Colitis (UC), Alzheimer's Disease (AD), Dermatitis, Graves' Disease, Asthma, Irritable Bowel Syndrome (IBS), Hashimoto Thyroiditis, Pernicious Anemia, Sjogren Syndrome, Diabetes, Multiple Sclerosis, Systemic Lupus Erythematosus (SLE), Aplastic Anemia, Hepatitis, Dermatomyositis, Parkinson's Disease, Myasthenia Gravis, stress, etc.

Acknowledgements

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