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StemForte Plus® for stem cell activation and telomerase activity for anti-aging purposes

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Background

Cellular aging is a natural phenomenon that results in a progressive decline in the resistance to stress and other cellular damages, causing a gradual loss of cellular functions and resulting eventually in cell death. Further, due to stress on the cell, wear and tear effect on the cell as well as due to cell injury, cell death process initiated. To replace dead cells with new cells, inside our body, we have stem cells, which undergo differentiation and convent themselves to the required cell as per the need of the body [1].

Various types of CD markers like CD1 to CD247 are available on different cell types inside the body. Few important cell surface markers available on stem cells are CD7, CD15, CD34, CD45, CD73, CD117, CD133, CD184, CD191, CD326, CD338, and like. Any chemical, which can able to bind with any one of such CD markers present on the stem cells, and modulate its function as an activator, can result in faster proliferation and differentiation of stem cells. This results in faster replacement of new cells in place of injured cells or dead cells. That means activation of stem cells is an important phenomenon for faster repairing and rejuvenation of body cells [2–4].

Various chemicals has been screened for their stem cells stimulatory role which includes, plerixafor [5], stem reginin- R (SRI) [5], G-CSF [6], (https://www.accessdata.fda.gov/drugsatfda_docs/label/2012/103353s5147lbl.pdf), GM-CSF, etc. (https://www.accessdata.fda.gov/drugsatfda_docs/label/2017/103362s5237lbl.pdf). But all of them possess minor to moderate adverse drug reaction. Hence there is a need for a safer and effective treatment option for stem cell modulators for tissue regeneration and growth.

Our StemForte Plus® is a patented and proprietary nutritional formulation comprising various natural powerful antioxidants like grape seed skin and fruit extract, bovine colostrum, brown seaweed, kino tree, proprietary super blend, and many more powerful ingredients. The antioxidant potential of StemForte Plus® was checked using ORAC assay and ORAC value calculated observed was 1867.15 umol/TE/g [7].

StemForte Plus® supports the natural release of adult stem cells. To scientifically prove this claim, in the present study, it has been tested using human bone marrow stem cells by quantifying CD34 and CD45 markers using the Flow cytometry method [8–12].

Further, telomerase, a ribonucleoprotein, catalyzes the addition of TTAGGG repeats to the ends of vertebrate chromosomes, using a complementary sequence of its intrinsic RNA component as a template. Telomerase restores short bits of DNA known as telomeres, which are otherwise shortened when a cell divides via mitosis. Activation of telomerase leads to an increase in the life span of cells and retard the cell aging process. Chemicals that can activate telomerase enzyme can act as an anti-aging agent.

In the present study, StemForte Plus® has been tested for its potential for telomerase activation activity. Photometric enzyme immunoassay is designed for the detection of telomerase activity, utilizing the Telomeric Repeat Amplification Protocol (TRAP). Telomerase PCR ELISA is designed for use in life science research studies for the highly sensitive qualitative detection of telomerase activity in cell extracts from cell cultures and other biological samples. Telomerase PCR ELISA utilizes a single-tube reaction mixture that simplifies the amplification reaction. The ELISA technique uses a biotinylated primer to immobilize the TRAP reaction products within a streptavidin-coated microplate, and a specific DIG-labeled probe for detection [13].

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Both above studies were done using a non-cytotoxic concentration of StemForte Plus®. To determine this non-cytotoxic concentration of StemForte Plus®, an in-vitro cytotoxicity test was performed using Caco2 cell line using neutral red update assay as per OECD TG129 [14, 15].

Material and methods

Test system details (bone marrow cells and cell line)

For cytotoxicity test, Caco2 cell line (ATCC®HTB-37™, human colon epithelial cells) purchased from National Centre for Cell Sciences was used. For rest two in-vitro studies, human bone marrow cell (Cat No. 2S-101D, Lonza, USA) was used.

Test item extract and dilution preparation

For the in-vitro cytotoxicity assay, the culture media with serum was used as an extraction vehicle as it does support cellular growth as well as extract both polar and non-polar substances. Five hundred milligram of StemForte Plus® powder was incubated with 5 ml of complete medium (DMEM medium + 10% FBS+ 1% Antibiotic Solution) (Undiluted concentration of 100 mg/ml) in shaking incubator at 37 °C for overnight. The extract was spun down at 10000 rpm for 10 min to sediment the particle matter. The extract was filtered through 0.22 μM syringe filters for sterilization. The eight concentrations of the test item by diluting the primary extract using 10-fold dilutions (e.g. 1:10, 1:100, and 1:1000 up to 7th dilution) were prepared. The culture medium alone was used as the vehicle control and was treated in the same manner as that of the test item extract [15, 16].

In vitro cytotoxicity

The caco-2 cells were detached from the culture flask by enzymatic digestion (trypsin/EDTA) to cell suspension having a population of 10×10^4 cells/mL in complete medium. A volume of 100 μL of culture medium was added in the peripheral wells of a 96-well tissue culture microtiter plate to be considered as a blank. In the remaining wells, 100 μL of a cell suspension was added. The cells were incubated for 24 h ± 2 (5.0 ± 0.5% CO_2, 37 ± 1 °C ≥ 90% humidity) to form a half-confluent monolayer to ensure cell recovery and growth by using microscopy for each well across the microtiter plate. The Sodium Lauryl Sulphate, high-density polyethylene (HDPE) granules, and vehicle control were kept as positive, negative control, and blank control respectively similar to the test item extraction. After the duration of incubation for test extract preparation, the culture medium was aspirated from each well of the culture plate. Per well, 100 μL of treatment medium containing either the appropriate concentration of reference item or test item extract was added in duplicates. The cell was incubated further for 48 ± 2 h. (5 ± 0.5% CO_2, 37 ± 1 °C, ≥ 90% humidity). This test was performed as per OECD TG129 Guideline [15, 16].

For qualitative observations, the cells were observed after 48 hrs. treatment, each well was examined under a phase-contrast microscope to identify systematic cell seeding errors and growth characteristics of control and treated cells and categorized in reactivity grades. For quantitative analysis, after incubation, the plate was inverted to remove the medium from the wells adhered cells were carefully rinsed with 250 μL/well pre-warmed Dulbecco's Phosphate Buffered Saline (DPBS).

The rinsing solution was removed by inversion of the plate and blot dry on paper towels. 250 μL of 25 μg/mL neutral red (NR) dye was added in complete medium to all wells (including the blanks) and incubated at (37°C ± 1°C, ≥90% humidity, 5.0% ± 0.5 % CO_2/air) for 3.0 hours ± 0.1 h (37°C ±1°C, ≥90% humidity, 5.0% ± 0.5 % CO_2/air). After incubation, the NR medium was removed and cells were rinsed carefully with 250 μL/well pre-warmed D-PBS. 100 μL NR desorb solution (freshly prepared 49 parts water + 50 parts ethanol + 1-part glacial acetic acid) was added to all wells (including blanks) to extract the dye [15–17].

The microtiter plates were shacked rapidly on a microtiter plate shaker for 20 – 45 minutes. The plates were protected from light while shaking. Plates were kept at a resting position for at least five minutes after removal from the plate shaker/ mixer. There was no bubble observed in the plate. The light absorption was measured at 540 nm in a microtiter plate reader using the blanks as a reference [18].

A decrease in the number of living cells results in a decrease in metabolic activity in the sample. This decrease directly correlates to the amount of neutral red detected as monitored by the optical density at 540 nm. The cell viability percentage was calculated using % cell viability. Half-maximal inhibitory concentration (IC_{50}) was calculated which corresponds to the IC_{50} to measure the potency of a substance in inhibiting a specific biological or biochemical function.

In-vitro stem (primary) cell stimulation

Human bone marrow stem (primary) cells were cultured in a controlled environment followed by stimulation with non-cytotoxic concentrations of the test item along with the single concentration of controls were tested for various time points. The bone marrow stem cell suspension was adjusted to attain the population of 5×10^4 cells/mL in a complete medium. 500 μL of a cell suspension was added 12-well tissue culture plates. The cells were incubated for 24 hrs (5.0 ±0.5 % CO_2, 37±1°C, ≥ 90 % humidity) to form a half-confluent monolayer as well as suspension. This incubation period ensures cell

recovery, adherence, and progression to the exponential growth phase. Each well was examined under a phase-contrast microscope to ensure that cell growth should be relatively even across the culture plate. Culture media with serum was used as an extraction vehicle and HDPE granules were used as a negative control.

The extract of Stem Forte Plus® prepared in culture media with serum was used to treat the cells for analyzing the effect on stem cell-specific with respective to the dose level over the specified time. Five non-cytotoxic concentrations (1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml) of StemForte Plus® extract were used to stimulate the cells for four different time points ranging from 8 hrs, 24 hrs, 48 hrs and 72 hrs time interval for reasonable outcomes at specified culture conditions (5±0.5% CO_2, 37±1°C, ≥ 90% humidity). The culture medium alone was used as the vehicle control. After the completion of incubation for variable time points, the cell suspension was collected in a sterile centrifuge tube followed by centrifugation at 1000 rpm for 5 minutes at 4 °C. The culture medium was aspirated from each tube and washed with chilled PBS followed by centrifugation at 1000 rpm for 5 minutes at 4 °C.

The Flow Cytometry method was designed to detect the change in surface receptor expression in stimulated cells. After *in vitro* stimulation, the cell pellet was resuspended in chilled FACS buffer (PBS with 5 % FBS- Sterile) and kept in 2-8 °C if not processed immediately for a maximum of 60 min. The cell suspension was centrifuged at 1000 rpm for 5 minutes at 4°C. To block non-specific interactions, the cells were pre-incubated with 100 μL of fetal bovine serum for 10-20 minutes at 25°C before staining. The cells were washed with gentle handling by adding four-volume of FACS buffer followed by centrifugation at 1000 rpm for 5 minutes at 4°C. The cell pellet was suspended in 100 μL of Fixation Buffer (6 % PFA-paraformaldehyde in PBS) and incubated at 2-8 °C for 30 min.

The cells were washed with FACS buffer thrice the volume by centrifugation for 5 minutes at 4 °C. The quantity of each direct fluorochrome-conjugated antibody for CD34 and CD45 was added in staining buffer in an appropriate volume (diluted up to 1:2500). The final staining volume of 100 μL was added to the cells pellet for staining [19]. Multiple antibodies were combined as per the requirement to be analyzed in the same set of the population for comparison. The cell was resuspended properly to ensure a single cell population by gentle tapping and incubated for at least 60 minutes at 2-8 °C along with Protected from light. The cells were washed with FACS buffer thrice the volume by centrifugation for 5 minutes at 2-8°C. For storage of samples before analysis, cells were suspended in 100 μL of Flow Cytometry Staining Buffer. The data were acquired on a flow cytometer following the manufacturer's recommended method and on the principle of gating.

The population was separated based on their fluorochromes associated with cell receptors. At least 1000 cells were analyzed to segregate the population and a comparison was done for their amount of receptor expression. Percentage increase or decrease was calculated based on the receptor expression by using BD Diva Software. The untreated cells were considered as a major population to be gated as a control for analysis. The CD marker expression within the untreated cell was considered as the base unit and in comparison, to the control, we have to find our no. of fold increase or decrease in the expression level of CD34 and CD45 in case of StemForte Plus® incubated cells at different concentration (1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml) as well as after incubation at different time points viz. 8 hrs, 24 hrs, 48 hrs, and 72 hrs.

In vitro telomerase activation protocol

To perform *in-vitro* telomerase stimulation activity of Stem-forte Plus®, TRAP method was used. The stimulated bone marrow stem (primary) cells were harvested in chilled PBS followed by centrifugation at 1000 rpm for 5 minutes at 4 °C. The pellet was preserved at -80 °C to be analyzed further. The frozen pelleted cells were thawed and resuspended in 200 μL Lysis reagent pre-cooled on ice by retro- pipetting at least 3 times and incubate on ice for 30 min. The lysate was centrifuged for 20 min at 2-8 °C. The supernatant was recovered and transfer to a fresh tube. To ensure that no cellular debris of the pelleted cells was transferred, only 175 μL of the cell extract was collected. For each test and control group sample, 25 μL reaction mixtures were transferred into a tube suitable for PCR amplification followed by the addition of 3 μL cell extract per tube and 50 μL of sterile water. Tubes were transferred to a thermal cycler and a combined primer elongation/ amplification reaction was performed Viz. 1 cycle at 25 °C for 10 min, 1 at cycle 94 °C for 5 min, 30 cycles for the 30s (94 °C), 30s (50 °C), 90s (72°C), 1 cycle at 25 °C for 10 min, and hold at 4 °C. The 5 μL amplified product was used from tube mix with 20 μL of denaturation reagent. After incubation at 25 °C for 10 minutes, 225 μL hybridization buffer was added per tube and mix thoroughly by vortexing briefly. 100 μL were transferred of the mixture per well of the pre-coated microplate and incubated at 37°C on a shaker for 2 hrs. The wells were covered with the self-adhesive cover foil and incubate for another 1 hr at 37°C. The Hybridization solution was removed completely followed by washing with 250 μL of Washing buffer per well for a minimum of 30 s each and the washing buffer was removed carefully. 100μL Anti-DIG-POD working solution was added in each well. The

microplate was incubated at 18 - 22°C for 30 min while shaking. The solution was removed completely. Rinse 5 times with 250 μL of Washing buffer per well for a minimum of 30 s each, and remove the Washing buffer carefully. 100 μL TMB substrate solutions were added per well to room temperature. The wells were covered with foil and incubate for color development at 15 to 25°C for 10–20 min while shaking. Without removing the reacted substrate, 100 μL Stop reagent was added per well to stop color development. Using a Microplate (ELISA) reader, the absorbance was measured for each of the samples at 450 nm within 30 min after the addition of the Stop reagent.

The average of every duplicate reading was calculated. The reading of blank was subtracted from each average experimental value of all controls and test to be considered as corrected value. The reading of untreated cell was considered as the base unit and in comparison, to

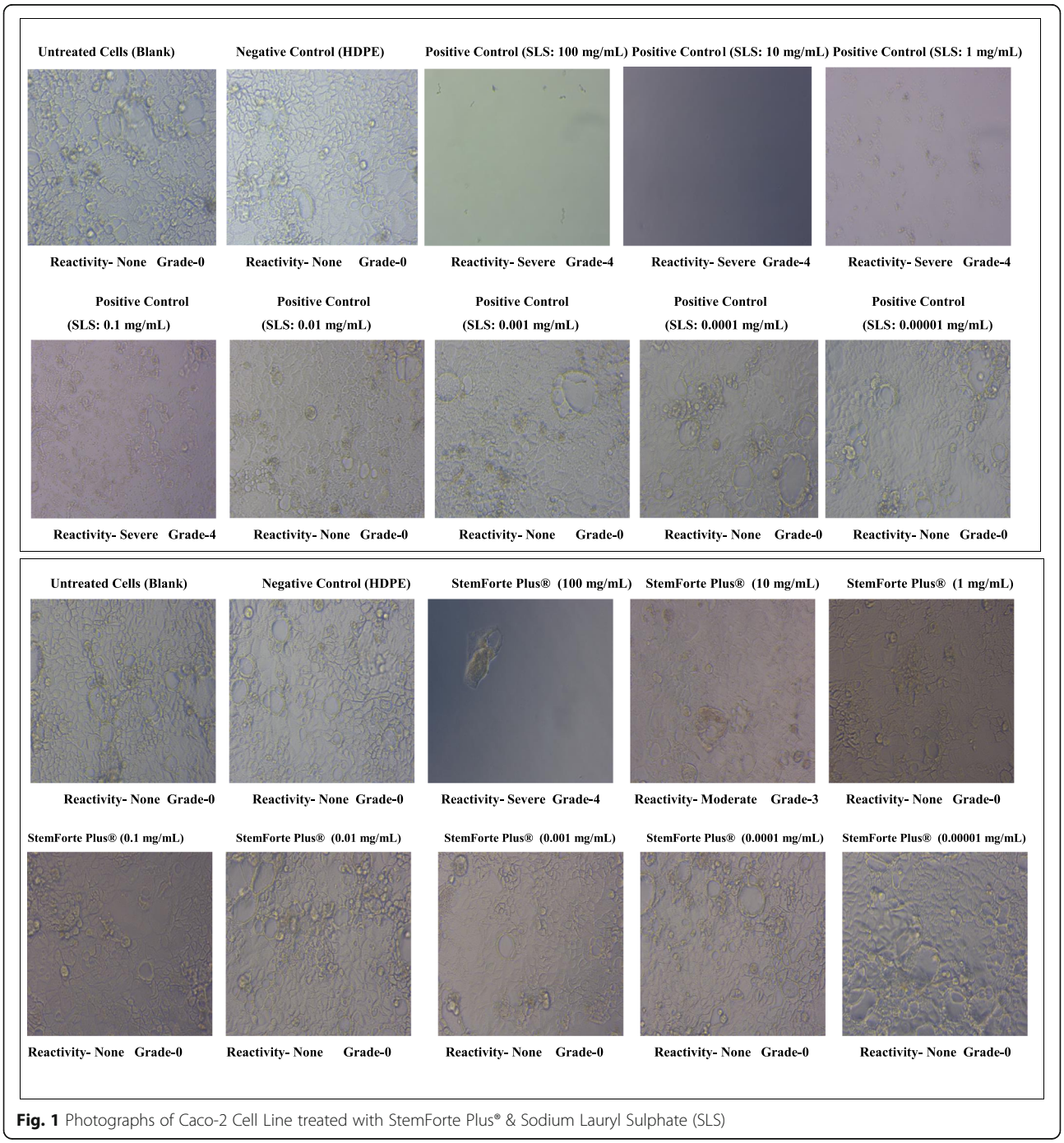
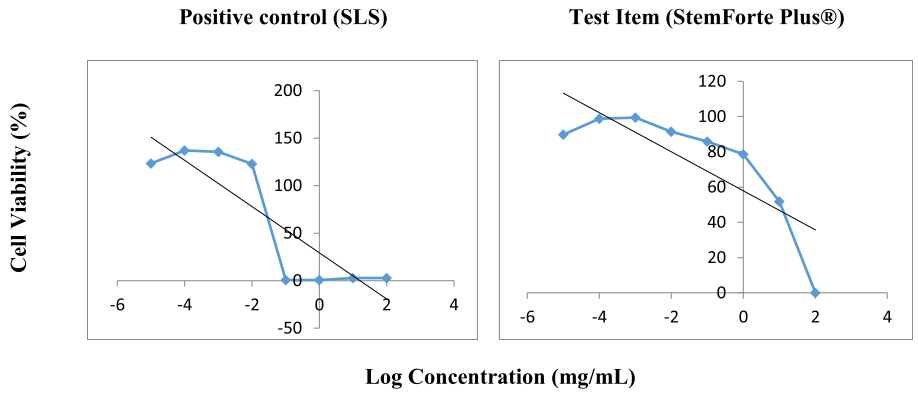


Fig. 1 Photographs of Caco-2 Cell Line treated with StemForte Plus® & Sodium Lauryl Sulphate (SLS)



IC_{50}	0.140 mg/mL	5.090 mg/mL
LD_{50}	663.743 mg/Kg	2529.298 mg/Kg

Fig. 2 Quantitative estimation of cell viability based on NRU assay

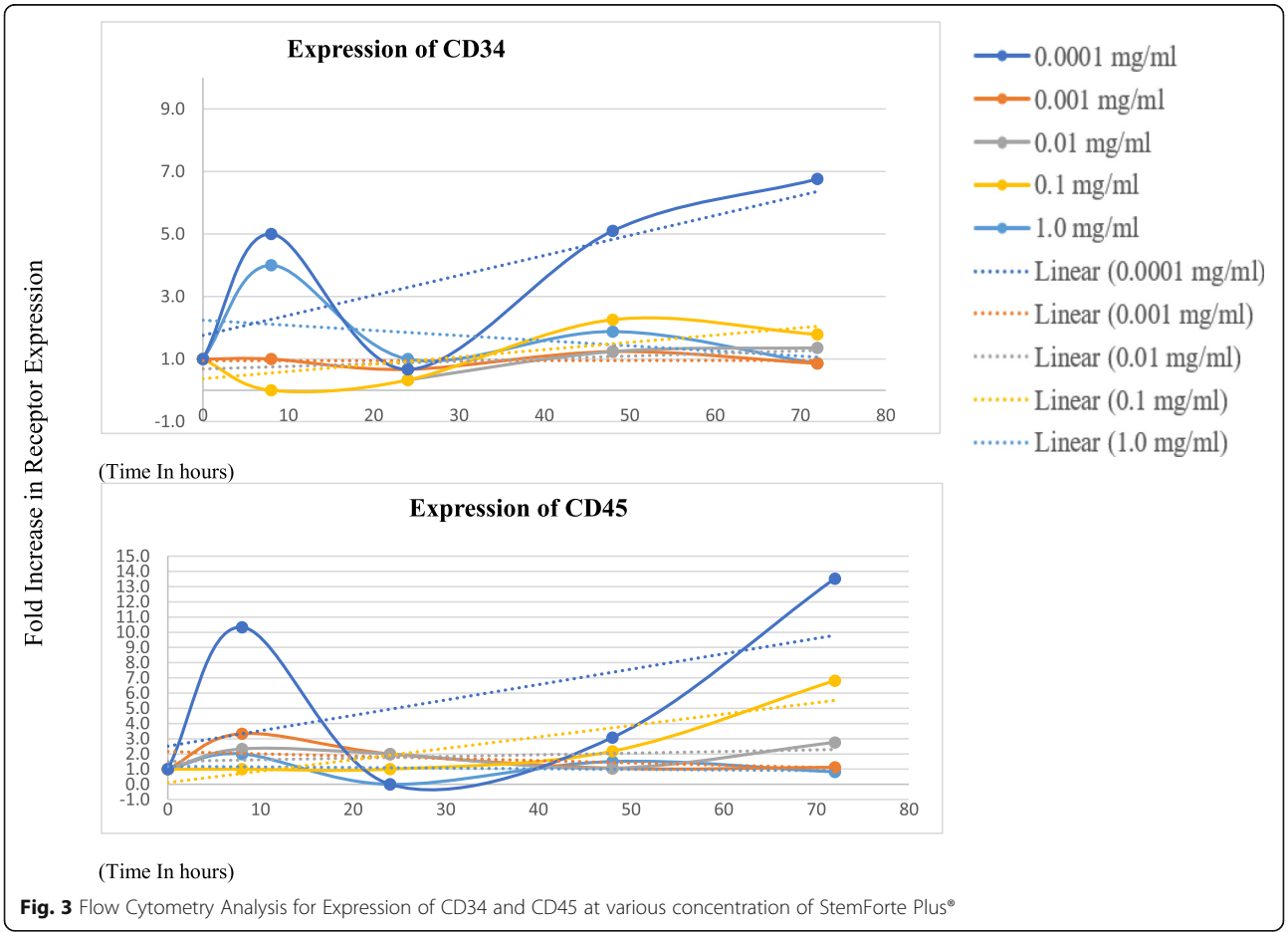


Fig. 3 Flow Cytometry Analysis for Expression of CD34 and CD45 at various concentration of StemForte Plus®

the control, we have to find our no. of fold increase or decrease in expression. The reading of untreated cell was considered as the base unit and in comparison to the control, we have to find out fold increase or decrease in the activity level of telomerase by StemForte Plus® incubated cells at different concentration (1 mg/ml, 0.1 mg/ml, 0.01 mg/ml, 0.001 mg/ml and 0.0001 mg/ml) as well as after incubation at different time points viz. 8 hrs, 24 hrs and 48 hrs [13–15].

Results

In vitro cytotoxicity

Microscopic examination was done for changes in cell general morphology, vacuolization, detachment, cell lysis, and membrane integrity. The assignment of reactivity grades and determination of cell viability percentage was done concerning the characteristics of control groups. The extract of StemForte Plus® has shown no reactivity and acceptable cell viability (> 70% viability) at the concentration of 1 mg/ml and below. Hence 1 mg/ml was taken as the highest non-cytotoxic dose in the next two in-vitro experiments using human bone marrow cells.

Based on the concentration-response of the cytotoxicity level, the IC_{50} was calculated which is a measure of the effectiveness of a substance in inhibiting cell growth. The IC_{50} was estimated at 5.090 mg/mL when treated with direct exposure for Caco-2 cell-line under the experimental condition of in vitro assay.

The limitations of the in vitro NRU methods are largely due to the differences between whole animal and cell culture systems. Therefore, the data generated through in vitro experimentation can be used for the determination of starting doses for acute oral systemic toxicity tests. The LD_{50} was estimated to 2529.298 mg/kg (for rat) for StemForte Plus® as per the formula mentioned in OECD TG129 for the test item with unknown molecular weight [15, 16].

The photographs of cell morphology for the treated cells have been provided in Fig. 1. The graph of % cell viability has been shown in Fig. 2.

Effect of StemForte plus® on CD marker expression

It was observed that there were concentration-dependent increases in the expression of CD34 and CD45 after single-dose administration of StemForte Plus®. The highest increase was observed at the concentration of 1 mg/ml. Further, it was also observed that after 72 hrs of incubation there was 7 folds increase in the case of the CD34 marker whereas there were 14 folds increase in CD45 marker in the cells treated with StemForte Plus® (See Figs. 3, 4 and 5).

Effect of StemForte plus® on telomerase activity

Out of the five different concentrations of StemForte Plus® extract, a medium concentration (0.01 mg/ml) causes increased telomerase activity by 7.2 folds after 8 hrs of incubation. Thereafter there was a decline in telomerase activity as the time of incubation increases (See Fig. 6).

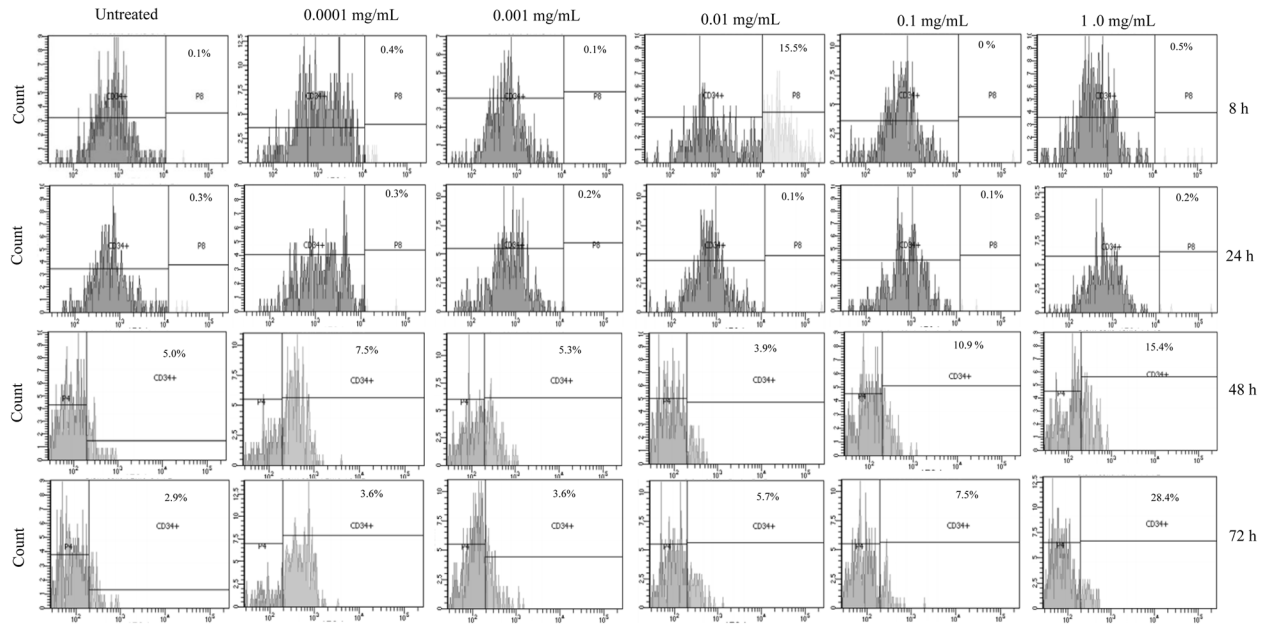


Fig. 4 In vitro CD34 expression stimulated by 'StemForte Plus®' by Flow Cytometry Method

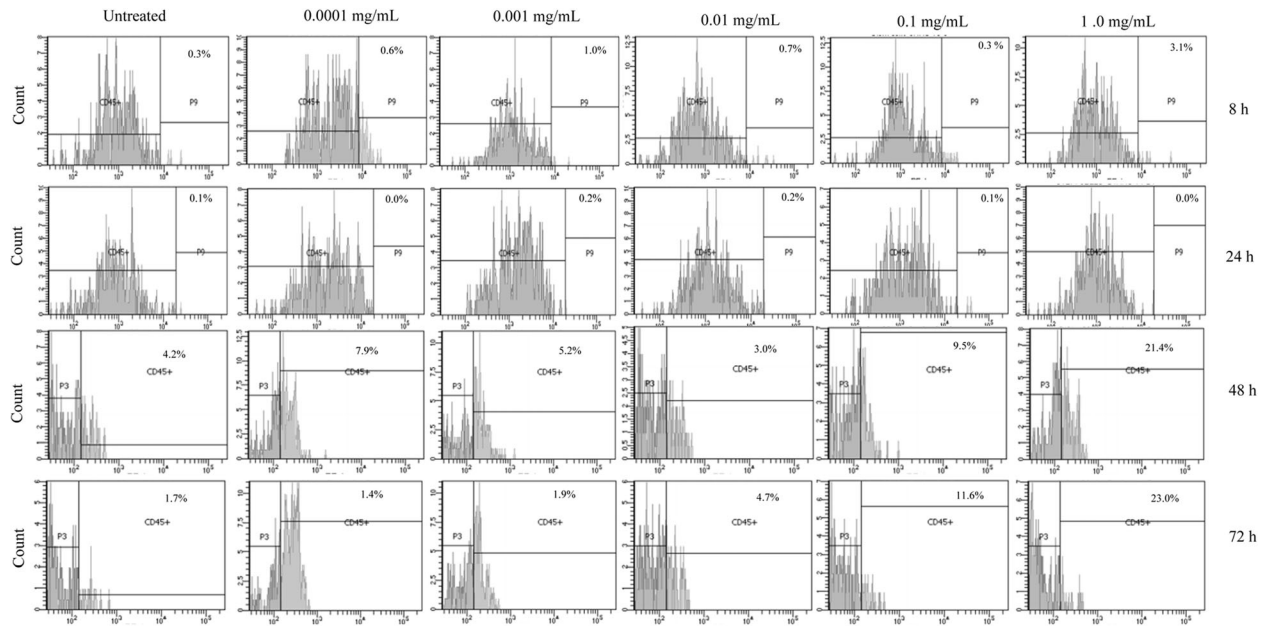


Fig. 5 In vitro CD45 expression stimulated by 'StemForte Plus®' by Flow Cytometry Method

Discussion

In vitro cytotoxicity testing of Stemforte Plus® was performed using the Caco2 cell line and as per the method described under OECD TG129 using neutral red uptake (NRU) method.

Out of tested eight different concentrations of Stemforte Plus®, it was found non-cytotoxic at 1 mg/ mL and below concentration. The IC_{50} and LD_{50} calculated based on criteria provided in OECD TG129 guidance document were 5.090 mg/ml and 2529.298 mg/kg for rat respectively. If we convert Therapeutic dose (In rat) = LD_{50} /10 = 2529.298/10 = 252.93 mg/kg (for rat). Using this data, calculated Human therapeutic dose (In Human) = Rat dose (mg/kg) /6.2 = 252.93/6.2 = 40.80 mg/kg in Human.

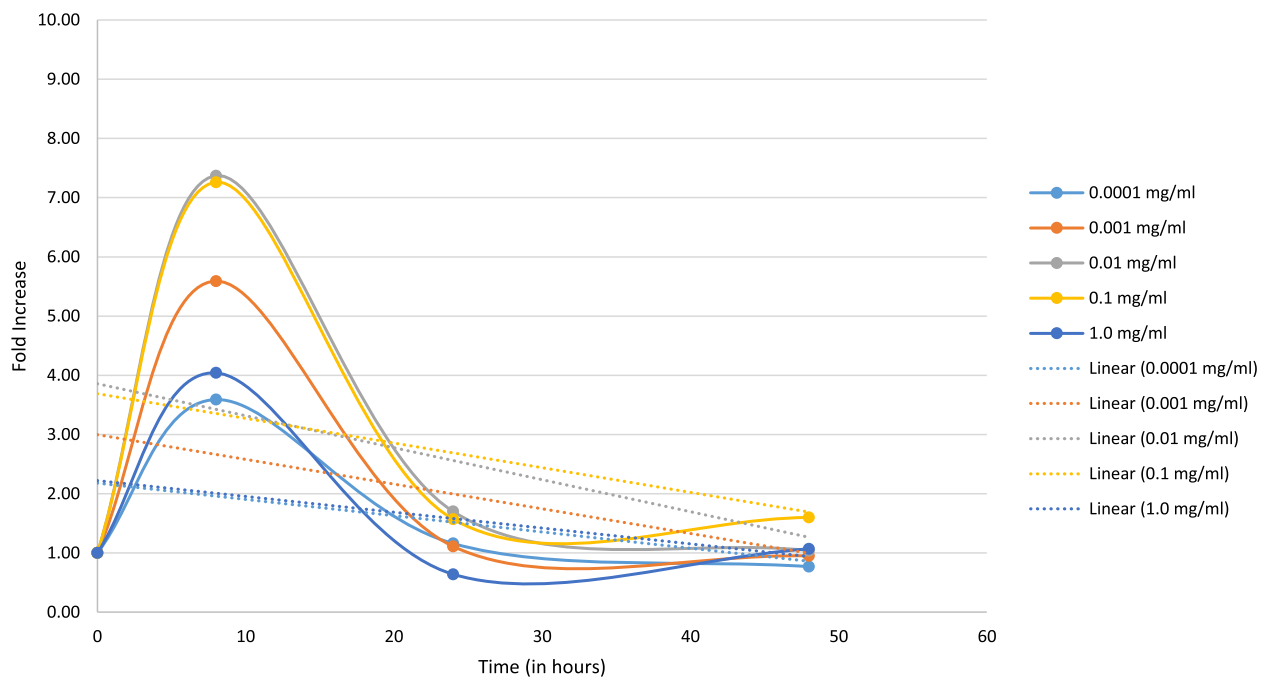


Fig. 6 Effect of StemForte Plus® on Telomerase Activity at different Time Points

Considering Average body weight of Human (60 kg), Daily human dose (HDD) = 40.80 × 60 = 2448 mg = 2.448 g of Stemforte Plus®, (Appx. 5 capsules of 500 mg each) per day daily dose in Human and calculated toxic dose in humans (HTD), it will be HDD × 10 = 2.448 × 10 = 24.48 GM per day (appx 49 capsules of 500 mg each per day).

The stem (primary) cells stimulated with Stemforte Plus® were categorized as a modulator of the immune system which shows upregulation of expression of CD34 and CD45. Both these CD markers are modulators of the process of differentiation of hematopoietic stem cells, mesenchymal stem cells, and stromal stem cells that originated in the bone marrow.

Stemforte Plus® has shown 7 folds increase in CD34 expression which was deciphered for pluripotency which acts as the origin for hematopoietic cell lineage contributing to myeloid lineage including erythroid lineage and immune specific lymphoid lineage followed by reduction upon maturation. Similarly, Stemforte Plus® has shown 14 folds increase in expression of CD45 which was reported for cell lineage contributing to erythroid lineage only and immune specific lymphoid lineage. The upregulation of CD34 and CD45 markers by clauses, which may differentiation of hematopoietic stem cells as well as somatic stem cells and thereby speeds up tissue regeneration and growth [20–22]. Hence Stemforte Plus® can be an effective natural nutritional formula to stimulate stem cells and thereby speed up the process of cellular and tissue regeneration and growth. However, functional studies will be planned and executed for the same separately.

Stemforte Plus® also produces 7 folds increase in telomerase activity. Stimulation of telomerase enzyme, linked with an increase in production of telomeres, which result in an extension of the life span of any somatic cells [23, 24]. This indicates that Stemforte Plus® likely to acts as a powerful anti-aging natural nutritional formula. However, functional studies will be planned and executed for the same separately.

Conclusions

Qualitative and quantitative evaluation of *in vitro* cytotoxicity study of Stemforte Plus® was found non-cytotoxic after 48 hours treatment at the concentration of 1 mg/mL and below. Stemforte Plus® also increases the expression of CD34 and CD45 markers and thereby likely to increase differentiation and proliferation of hematopoietic stem cells as well as somatic stem cells and thereby speed up tissue regeneration and growth. Further, Stemforte Plus® also increases telomerase activity and thereby likely to be act as powerful anti-aging natural nutritional formulation. In brief, Stemforte Plus® can be useful for tissue regeneration as well as anti-aging natural nutritional formula. However, further functional studies will be performed to determine pharmacodynamics effects of Stemforte Plus®.

Abbreviations

≥: Greater than equal to; °C: Degree centigrade; µg: Microgram; µL: Micro litre; 2S-101D: Catalog Number of Lonza for Cryopreserved ampule of Human Bone Marrow Mononuclear Cells containing ≥5 million cells; Anti-DIG-POD: Anti-digoxigenin-Peroxidase; ATCC: American type culture collection; Caco2: The immortalized cell line of human colorectal adenocarcinoma cells; CD: Cluster of differentiation; CXCR4: Chemokine receptor type 4; DMEM: Eagle's Minimal Essential Medium; DNA : Deoxyribonucleic acid; DPBS: Dulbecco's Phosphate Buffered Saline; ELISA : Enzyme-Linked Immunosorbent Assay; FACS: Fluorescence-activated cell sorting; FBS: Fetal bovine serum; FDA: Food and Drug Administration; GCSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; G-rich CSF: Guanine-rich Deoxyribonucleic acid; hrs: Hours; HDD: Human Daily Dose; HDPE: High-density polyethylene; HSC : Human hematopoietic stem cell; HTD: Human Therapeutic Dose; IC_{50}: Half maximal inhibitory concentration; LD_{50}: Median lethal dose; mg: Milligram; ml: Milliliter; nm: Nano meter; NRU: Neutral Red Uptake; OECD TG129: Organization for Economic Co-operation and Development Test Guideline 129; PCR : Polymerase chain reaction; PFA: Paraformaldehyde; RPM: Rotation per minute; s: Second; SIg: Surface Immunoglobulin; SR1: StemRegenin1; TMB: 3,3′,5,5′-Tetramethylbenzidine; TRAP: Telomeric Repeat Amplification Protocol; TTAAGGG: DNA sequence - Pattern of Tandem Repeat; USA : United States of America

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Author's contributions

Mr. Victor Chavez has designed the experiments, interpreted data, and conceptualized this manuscript. The author(s) read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Yes.

Competing interests

The authors declare that they have no competing interests.

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