Protective Effects of Triphala on Dermal Fibroblasts and Human Keratinocytes

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Abstract

Human skin is body’s vital organ constantly exposed to abiotic oxidative stress. This can have deleterious effects on skin such as darkening, skin damage, and aging. Plant-derived products having skin-protective effects are well-known traditionally. Triphala, a formulation of three fruit products, is one of the most important rasayana drugs used in Ayurveda. Several skin care products based on Triphala are available that claim its protective effects on facial skin. However, the skin protective effects of Triphala extract (TE) and its mechanistic action on skin cells have not been elucidated in vitro. Gallic acid, ellagic acid, and chebulinic acid were deduced by LC-MS as the major constituents of TE. The identified key compounds were docked with skin-related proteins to predict their binding affinity. The IC50 values for TE on human dermal fibroblasts (HDF) and human keratinocytes (HaCaT) were 204.90 ± 7.6 and 239.13 ± 4.3 μg/mL respectively. The antioxidant capacity of TE was 481.33 ± 1.5 mM Trolox equivalents in HaCaT cells. Triphala extract inhibited hydrogen peroxide (H2O2) induced RBC haemolysis (IC50 64.95 μg/mL), nitric oxide production by 48.62 ± 2.2%, and showed high reducing power activity. TE also rescued HDF from H2O2-induced damage; inhibited H2O2 induced cellular senescence and protected HDF from DNA damage. TE increased collagen-I, involucrin and filaggrin synthesis by 70.72 ± 2.3%, 67.61 ± 2.1% and 51.91 ± 3.5% in HDF or HaCaT cells respectively. TE also exhibited anti-tyrosinase and melanin inhibition properties in a dose-dependent manner. TE increased the mRNA expression of collagen-I, elastin, superoxide dismutase (SOD-2), aquaporin-3 (AQP-3), filaggrin, involucrin, transglutaminase in HDF or HaCaT cells, and decreased the mRNA levels of tyrosinase in B16F10 cells. Thus, Triphala exhibits protective benefits on skin cells in vitro and can be used as a potential ingredient in skin care formulations.

Introduction

The skin of the human beings acts as a bridge between internal and external environment being well-equipped with an intricate network of antioxidant substances (redox-active) and enzymes, continually exposed to the oxidative environment [1, 2]. The skin is composed of...
Skin Protective Benefits of Triphala

The objective of the study was to investigate the skin protective benefits of Triphala on human dermal fibroblasts (HDF) and human keratinocytes (HaCaT) cells.

Materials and Methods

Maintenance of cell lines

Human Dermal Fibroblast (HDF), Human Keratinocytes (HaCaT), Mouse melanoma (B16F10), Human melanoma (A-375), and Mouse monocyte macrophage (RAW264.7) cells were procured from American Type Culture Collection (ATCC). HDF cells were cultured in minimum essential medium (MEM), HaCaT cells were cultured in Ham’s F-12 medium, and RAW264.7/A-375 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) high glucose medium, with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified CO2 (5%) chamber.
Chemicals and reagents

Fetal Bovine Serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulphoxide (DMSO), trichloroacetic acid (TCA), N-(1-naphthyl) ethylenediamine dihydrochloride, sulphamid acid, tannic acid, custom oligonucleotides were obtained from Sigma-Aldrich, MO, USA. Penicillin, streptomycin, potassium ferricyanide, H2O2, ferric chloride, ferrous sulphate (FeSO4), and sodium nitrite were obtained from Himedia (Mumbai, India). Cellular Senescence Assay kits were purchased from Millipore Corp. (USA). Involucrin and filaggrin ELISA kits were purchased from Cloud-Clone Corp. (USA), Collagen-Type I kits were purchased from Blue Gene Biotech, China. All molecular biology reagents for PCR were obtained from Bio Rad, USA.

Composition and preparation of Triphala extract

Triphala contains equal proportions of three fruits- Indian gooseberry (Phyllanthus emblica L.), Haritaki (Terminalia chebula Retz.), and Vibhitaki (Terminalia bellerica Roxb.). The drug regulatory authority department of AYUSH have recommended Triphala as natural medicine approved by Ministry of Health and Family Welfare, Government of India. The herbs used in the formulation were procured following good procuring practices and well-maintained in proper conditions. The plants were taxonomically classified by Dr. Kannan, a pharmacognosist and sample of plant constituents has been deposited in the herbarium of R&D, The Himalaya Drug Company, Bangalore, India. Triphala granules (100 g) (Batch No. 1308032 FD) were crushed in liquid nitrogen using mortar and pestle to obtain fine powder. It was sequestered with 1000 ml of water at room temperature for 30 min. The mixture was then extracted at 37°C for 24 h in a shaking water bath (300 rpm). The slurries were cooled down to room temperature, centrifuged at 1000 rpm for 15 min, and the supernatant was collected. The extract was lyophilized and a yield of 2.9% of the DW of Triphala was obtained. This Triphala extract (TE) was used for all the experimental studies.

Phytochemical analysis

The total phenol content, tannin, and flavonoid content in TE was estimated using spectrophotometric or colorimetric methods as described earlier [16–18]. The Folin-Ciocalteau and Folin-Denis method was followed for determination of phenol and tannin content. The total flavonoid content was measured colorimetrically in Triphala extract (TE) using quercetin as standard. Gallic acid and Quercetin were used as reference standards in the experiments.

Liquid chromatography-mass spectrophotometer (LC-MS) analysis

The extracts of Triphala (TE) and its individual constituents were subjected to LC-MS analysis. The sample was prepared in water and filtered through 0.2 μm filter prior to injection into the mass spectrometer. Sample was injected into the ES ionization source using a syringe pump at a flow rate of 30μl/min.

LC-MS conditions: The API 2000 (Applied biosystem/MDS SCIEX, Canada) mass spectrometer coupled with an (Electron spray ionization) source and a HPLC (Shimadzu LC-20AD) was used for molecular mass determination and analysis of Triphala and its individual constituents extracts. The instrument was operating in the negative mode with an ion spray voltage -4500 V and declustering potential -20 V. The analyst 1.5 version software was used for controlling batch acquisition and data analysis [19].
Molecular docking studies

Molecular docking was performed with energy minimized structures of protein and ligands. The energy-minimized structure of gallic acid, ellagic acid, and chebulinic acid were docked with AQP-3, SOD-2, Collagen, Transglutaminase, Involucrin, Filaggrin, and Tyrosinase using AutoDock Vina 1.1.2 [20]. The protein and ligand files were converted into a PDBQT file format, a modified protein data bank format having atomic charges and definitions for ligands including topological information. After receptor–ligand preparation and specifying the binding site, program for docking was run from the command prompt [21]. The binding energy was calculated for the docked complex (Receptor-ligand) and represented as affinity (kcal/mol).

Cell cytotoxicity assay

HDF and HaCaT cells were cultured in 96-well plates (1 x 10^4 cells/ml) and treated with various concentrations (15.82–1000 μg/ml) of TE respectively. The percentage cytotoxicity was estimated by MTT assay at 540 nm after 24 h incubation, [22] using the Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT). From the values obtained, the percentage cytotoxicity (IC50 value) was calculated. For further experiments, non-toxic concentrations were used in the study.

RBC hemolysis assay

The RBC hemolysis was performed with the modified protocol originally described by Reddy et al [23]. The volume of TE samples and control with erythrocyte suspensions were adjusted to 1 ml by adding 0.9% saline. H2O2 (100 μM) was used to induce oxidative stress and the samples were incubated at 37°C for 3 h. After incubation, the mixture was centrifuged at 1200 rpm for 5 min followed by supernatant collection. The absorbance was measured at 540 nm using Synergy HT multi-detection microplate reader (Bio-Tek, Winooski, VT). RBC hemolysis by H2O2 was taken as 100% cell lysis, and hemolysis of the treated and untreated erythrocytes was calculated as percentage of this value. Ascorbic acid (100 μM) served as positive control.

Reducing power assay

Various concentrations of TE (0–400 μg/ml) in corresponding solvents were mixed with 500 μl phosphate buffer (pH 6.6) and 500 μl of 1% potassium ferricyanide. The mixture was incubated at 50°C in water bath for 20 min. After cooling, 400 μl of 10% TCA was added and centrifuged at 10,000 rpm for 20 min. The supernatant (500 μl) was mixed with 500 μl of distilled water and 100 μl of a freshly prepared 0.1% ferric chloride solution. The absorbance was read at 700 nm [24]. In a similar way, control was also prepared excluding the test samples. Ascorbic acid at various concentrations (0–400 μg/ml) was used as standard.

Antioxidant assay

HaCaT cells (1 x 10^4 cells/ml) were seeded in 40-mm petri plates and incubated for 24 h at 37°C. The cells were treated with two concentrations of TE (50 and 100 μg/ml) and incubated for 24 h at 37°C with 5% CO2. The cell lysates were collected and the antioxidant capacity was analyzed using the total antioxidant power kit (Oxford Biomedical Research, USA). The analysis was repeated thrice (n = 3) by comparing and interpolating the values with that of the standard 6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox) from the calibration curve.

NO-scavenging assay

RAW264.7 cells were seeded into 40-mm Petri plates (1 x 10^6 cells) and incubated for 24 h at 37°C. The cells were treated with TE (50 and 100 μg/ml) and lipopolysaccharide (LPS; 1 μg/ml)
and further incubated for 24 h at 37°C. NO production was measured in the culture medium and detected by the Griess reaction using sodium nitrite as the standard. Griess reagent (0.1% N-(1- naphthyl) ethylenediamide dihydrochloride, 1% sulphanilamide in 5% phosphoric acid) and an equal volume of cell supernatants were mixed and the absorbance was measured at 540 nm. The nitrite concentration was calculated and expressed as μmol/ml. Dexamethasone (100 μM) was used as standard in this experiment.

Radical scavenging assay

HDF cells were used for the radical scavenging assay and the protocol described by Murrell et al [25] was slightly modified. Fibroblasts were seeded at 1 × 10⁴ cells/well in 96-well plates and grown in MEM and incubated for 24 h at 37°C. Three different experimental sets were performed: a) Addition of TE along with H₂O₂ to cells (co-treatment); b) Pre-treatment of cells with TE for 1 h prior exposure to the H₂O₂, and c) Post-treatment with TE after H₂O₂ exposure for 1 h [26]. Ascorbic acid (100 μg/ml), a H₂O₂ scavenger was used as a reference standard. Cell control and cells treated with H₂O₂ alone were also maintained. The plates were incubated at 37°C for 24 h and MTT assay was used to assess the protection offered by TE.

DNA protection assay

For investigating the beneficial effects of TE on hydroxyl (OH) radical induced DNA damage, total DNA was isolated from HDF using the DNA isolation kit (Krishgen Biosystems, India). The reaction was performed in a tube containing 3 μl of DNA in 3 μl of PBS (pH 7.4), which was pre-treated with 2 μl of TE at two concentrations (50 and 100 μg/ml) and incubated at 37°C for 1 h. Later, 2.5 μl of 2 mM FeSO₄ and 7.5 μl of 1 mM H₂O₂ were added; the mixture was incubated at 37°C for 30 min [27]. After incubation, the mixture was loaded on to 1% agarose gel and stained with 1 mg/ml ethidium bromide before visualization. DNA bands were observed under the UV light. Densitometric analysis was carried out using Image J software (Rasband, USA) and a graph was plotted.

Cellular senescence assay

HDF cells (1 × 10⁴ cells/ml) were seeded into 40-mm Petri plates and incubated for 24 h at 37°C. The cells were co-treated with TE (50 and 100 μg/ml) and H₂O₂ (10 μM) for 1 h at 37°C. The media was aspirated and the cells were rinsed twice with 2 ml of phosphate buffered saline (PBS). The cells were stained with SA-β-gal as described by the cellular senescence assay kit. The cells were incubated overnight at 37°C without CO₂, away from light. The stained cells were rinsed twice in 2 ml of PBS and were scored under light microscope (10×).

Tyrosinase inhibition assay

The mouse melanoma cells (B16F10) were cultured in the 40 mm petri plates (1×10⁵ cells/well) for 24 h. The cells were treated with or without several concentrations of TE and further stimulated for 24 h in the presence or absence of 100 nM α-MSH. The lysis buffer (100 μL of 50 mM sodium phosphate buffer (pH 6.8) with 1% Triton X-100 and 0.1 mM PMSF) was used to lyse cells and it was further centrifuged at 20,000 × g for 30 min at 4°C. The supernatant collected was added to the plate and the ELISA plate reader was used to monitor absorbance at 492 nm [28–30]. The percentage of enzyme inhibition was computed according to the given formula: Percent inhibition (%) = [C − T/C] × 100, where C and T are the absorbance for the control and test samples, respectively [31]. For standard tyrosinase inhibitor, Kojic acid (100 μM) was used in the experiments.
Melanin spitting/inhibition assay
The mouse melanoma (B16F10) and Human melanoma (A-375) cells were seeded in the 40 mm petriplates (1×10^5 cells/well) using the DMEM medium supplemented with 10% FBS for 24 h. The cells were treated with various concentrations of TE and 100 μM concentration of forskolin for the stimulation of melanin for 72 h and detached by 0.05% trypsin-EDTA. The cell pellets were mixed in 120 μL of 1 N NaOH for 1 h at 65°C and melanin content was read at 405 nm through an ELISA reader [30–31]. For melanin content analysis, kojic acid was used as a reference standard.

Collagen-I, involucrin, and filaggrin ELISA
HDF or HaCaT cells were used for collagen-I, involucrin, and filaggrin estimation, respectively. The cells (1 × 10^4 cells/ml) were seeded into 40-mm Petri plates and incubated for 24 h at 37°C. The cells were treated with TE (50 and 100 μg/ml) and incubated for 96 h at 37°C, Ascorbic acid and Kinetin were used as positive controls for collagen-I, involucrin, and filaggrin ELISA respectively. After incubation period, the supernatant was collected and collagen-1, involucrin, and filaggrin content was estimated using commercially available ELISA kits. For visualization of collagen-I, TE treated HDF cells were stained with Sirus red [32] and the images were captured using phase contrast microscope.

Real time PCR
To further validate the role of TE on skin differentiation and/or youthful markers, HDF and HaCaT cells (1 × 10^6 cells/ml) were cultured in 60-mm dishes. The cells were treated with two concentrations of TE (50 and 100 μg/ml) and incubated for 24 h at 37°C. Cell control was also maintained. Total RNA extraction was performed on both the cells using RNA isolation kits (Krishgen Biosystems). The total RNA was treated with DNase to avoid contamination from the genomic DNA. For real-time quantitative PCR (qPCR), 1 μg of total RNA was reverse transcribed into first-strand cDNA using SuperScript III (invitrogen) and an oligo-dT primer. The PCR amplification occurred in a reaction volume of 20 μl containing 2 μL cDNA, 10 μl SYBR Green supermix (Bio Rad, USA), and CFX96 real-time system (Bio Rad, USA). The primers used for collagen-I, elastin, AQP-3, SOD-2, involucrin, filaggrin, transglutaminase (TGM1), and tyrosinase are listed in Table 1. The mRNA expression levels were normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. The Ct values of TE treated cells were calculated and the data was expressed in terms of fold change over cell control. Also, the amplified product was run in 1.5% agarose gel electrophoresis and gel picture captured under UV light.

Statistical analysis
All the experiments were performed in triplicates and every data point indicates the standard deviation of three samples. Graphpad Software QuickCalcs Student’s t-test was used for data evaluation and also for computing the significance level of P < 0.05 and P < 0.01.

Results and Discussion
Over the years, several skin protecting agents of natural and synthetic origin have been introduced [33]. Natural herbal compounds like phenolic acids, flavonoids, and high molecular weight polyphenols have attracted huge attention as beneficial protective agents [34]. Triphala, the magical wonder from the Ayurveda is conventionally used for treating various skin conditions like skin aging, since antiquity [35]. A potential skin protecting compound can control
the ROS and oxidative stress, promote exfoliation, skin turnover, and help in maintaining consistent synthesis and replacement of molecules associated with extracellular matrix [36]. It is well-known fact that free radicals play a crucial role in the progression of some diseases including aging. It is also clear that supplementing the human body with antioxidants helps in oxidative stress reduction and slows down problems related to diseased conditions [37]. Several plant phytoactives demonstrate free radical-scavenging or antioxidant activity [38].

The present study explored the natural antioxidant and skin protective properties of Triphala (TE) in quenching the reactive oxygen and nitrogen radicals, delaying cellular senescence and regulating marker genes involved in various biological process of aging. In addition, the key constituents of Triphala responsible for its skin protective role were also identified by mass spectrometry and their functions were predicted by molecular docking prior to in vitro studies.

Triphala (TE) and its individual constituents were subjected to LC-MS for identifying the key compounds contributing to its biological activity. The presence of gallic acid, and ellagic acid were detected in both TE (Fig 1) and its individual constituentsTable 2(S1–S3 Figs). Recently, the metabolite profiling for polyphenols in T.chebula showed the presence of several compounds including chebulinic acid [39]. Also, the HPLC profile of Triphala showed gallic acid, ellagic acid, and chebulinic acid to be the predominant compounds [40]. The compounds identified above were docked with various skin-related proteins and represented as binding affinities in Kcal/mol (S1 Table). In addition, the docking studies of major compounds of TE with involucrin, filaggrin, and collagen-I are shown in (S4 Fig). The binding affinity closer to 10 and above indicated efficient binding. It is predicted that binding of ligand with the protein could either inhibit or enhance its activity.

A preliminary phytochemical analysis revealed TE as a rich source of polyphenols (34.6 ± 3%) and tannins (32.5 ± 2%). It also indicated the negligible flavonoid content in TE. Previously, also the phytochemical analysis of the Triphala revealed polyphenols and tannins to be the major constituents [18]. In order to establish the protective role of TE on skin, cellular cytotoxicity was performed using MTT assay, a technique to monitor the cellular health of the cells. The IC50 values for TE on HDF and HaCaT were 204.90 ± 7.6 and 239.13 ± 4.3 μg/mL respectively. Only higher concentrations of TE were cytotoxic to both HDF and HaCaT cells (S5 Fig). Thus the non-toxic concentrations were further used for subsequent in vitro experiments.

A hemocompatibility study was conducted to validate the cytotoxic effects of TE on normal RBC cells. TE dose dependently inhibited RBC haemolysis with 57.87 ± 0.8% inhibition at 100 μg/ml with IC50 values of 64.95 μg/ml (Fig 2A), whereas ascorbic acid (100 μg/ml)

### Table 1. List of primers used in RT-PCR studies.

| Gene         | Primer sequence               | Annealing Temperature |
|--------------|-------------------------------|-----------------------|
| Col-I        | For 5’-TGACGAGACCAAGAACTG-3’ | 60°C                  |
|              | Rev 5’-TACCAGGGTTTGAICTCAGA-3’|                       |
| Elastin      | For 5’- GCCCCTGGATAAAAAGACTCC-3’ Rev 5’- GTCTCTCTGCTCTGCTGT-3’ | 60°C                  |
| AQP-3        | For 5’- GTTTGATGGTGAGGAAACCA-3’ Rev 5’- GGGACCTCCTACCTGCTGT-3’ | 60°C                  |
| SOD-2        | For 5’- CGACCTGCCCTAGCAGTACG-3’ Rev 5’- TGACACACACCATTGAAGT-3’ | 60°C                  |
| TGM1         | For 5’- CCCCGCCATGAGACTCTACA-3’ Rev 5’- ATCTCTATGGTCCACGATACAA-3’ | 60°C                  |
| Filaggrin    | For 5’- TTTCGTGTTTCTGCTTGTC-3’ Rev 5’- CTGGCAACTCAGGTTCCCAT-3’ | 60°C                  |
| Tyrosinase   | For 5’- GGCCACGTCTTCCAGGAG-3’ Rev 5’- TGCTGCTTCTGATGGGCAAAATC-3’ | 60°C                  |
| Involucrin   | For 5’- ACTGAGGGCGGAGGAG-3’ Rev 5’- TCTGCCTACGGCTACTGGTG-3’ | 60°C                  |
| GAPDH (Human)| For- 5’ AAGGTGAAAGTCGGAGTCAA 3’ Rev- 5’ AATGAAGGGTCATTTGATGG 3’ | 60°C                  |
| GAPDH (Mouse)| For- 5’ ACCACAGCTCATGCCATCAC 3’ Rev- 5’ TCCACCACCTTTGCTGTA 3’ | 60°C                  |

doi:10.1371/journal.pone.0145921.t001
inhibited RBC haemolysis by 63.60 ± 0.9%. H2O2 (control) showed complete (100%) haemolysis. Erythrocytes become frangible in adverse conditions of H2O2 exposure; it triggers a multitude of changes at cellular level like alteration in membrane deformation, hemolysis, rearrangement of phospholipids, cell surface morphology, and erythrocytes alteration [41–43]. Thus, RBC hemolysis assay demonstrated the effectiveness of TE where it displayed no significant lysis of normal RBC cells compared to the control. TE established to be an effective antioxidant by protecting RBCs from membrane damage due to increasing H2O2-induced oxidative stress.

The effective antioxidant capacity of TE was evaluated using reducing power assay. In reducing power assay, transfer of electrons occurs wherein a ferric salt is utilized as an oxidant [44]. The reducing ability of TE highlighted its antioxidant potential (Fig 2B). Throughout the concentration range (0–400 μg/ml), TE and Ascorbic acid showed nearly the same trend in their reductive capability. The reducing power of TE at 400 and 25 μg/ml was equivalent to that of ascorbic acid at 216.86 ± 5.1 μg/mL and 18.01 ± 0.5 μg/mL respectively. Antioxidant
substances are present as reductant in the antioxidant samples leading to reduction of the Fe3+/ferricyanide complex to the ferrous form. The formation of Fe2+ can be measured by observing the development of Perl’s Prussian blue color at 700 nm [44]. The reducing capacity of TE demonstrates its potential as an antioxidant.

Table 2. LC-MS/MS characteristics of Triphala and its constituents.

| Compound Name          | Molecular formula | Molecular weight (g/mol) | Obtained molecular ion (m/z) |
|------------------------|-------------------|--------------------------|----------------------------|
| Terminalia chebula     |                   |                          |                            |
| Gallic acid            | C7H6O5            | 170.12                   | 168.92                     |
| Ellagic acid           | C14H6O9           | 302.19                   | 300.96                     |
| Chebullagic acid       | C41H30O27         | 954.66                   | 953.1                      |
| Chebulinic acid        | C41H32O27         | 956.67                   | 954.97                     |
| Emblica officinalis    |                   |                          |                            |
| Gallic acid            | C7H6O5            | 170.12                   | 168.83                     |
| Emblicanin A & B       | C34H22O22         | 782.52                   | 781.2                      |
| Geraniin               | C41H30O27         | 952.64                   | 952.67                     |
| Terminalia bellerica   |                   |                          |                            |
| Gallic acid            | C7H6O5            | 170.12                   | 168.78                     |
| Friedelin              | C30H50O           | 426.71                   | 424.9                      |
| Ellagic acid           | C14H6O9           | 302.19                   | 300.81                     |
| Triphala extract (TE)  |                   |                          |                            |
| Gallic acid            | C7H6O5            | 170.12                   | 168.69                     |
| Ellagic acid           | C14H6O9           | 302.19                   | 300.91                     |
| Chebulinic acid        | C41H32O27         | 956.67                   | 955.3                      |
| Emblicanin A & B       | C34H22O22         | 782.52                   | 781.03                     |
| Friedelin              | C30H50O           | 426.71                   | 424.8                      |

doi:10.1371/journal.pone.0145921.t002

Fig 2. (A) Effect of TE on H2O2-induced RBC hemolysis. RBC cells were treated with different concentrations of TE and ascorbic acid (AA) (25–100 μg/mL) and the cells were induced with 100 μM H2O2 for cell lysis. Data was expressed as percentage of control. Results are shown as mean ± SEM of three experiments. (B) Reducing power activity of TE at different concentrations (0–400 μg/ml). Absorbance was read at 700 nm and the data are represented as ascorbic acid equivalents. Results expressed as mean ± SEM (n = 3).

doi:10.1371/journal.pone.0145921.g002
The problem in measurement of individual antioxidant substances and possible synergy among antioxidants, has led to the development of methods to assess the total antioxidant capacity of biological specimens [45]. The total cellular antioxidant capacity of TE was evaluated using HaCaT cells. The antioxidant capacity of TE was found to be 481.33 ± 1.5 mM Trolox equivalents in HaCaT cells. It was earlier reported that human epidermis has more antioxidant capacity compared to dermis [46]. The total antioxidant activity of the TE was evaluated by comparing with the standard solution of Trolox.

Nitric oxide (NO), a bioactive molecule plays vital roles in physiological and pathological processes [47]. To evaluate the effect of TE in curbing NO production, nitric oxide assay was performed. After 24 h of stimulation, NO levels of LPS treated RAW264.7 cells were significantly higher. TE decreased NO production to 48.62 ± 2.2 and 35.47 ± 1.6% at 100 μg and 50 μg/ml concentration respectively (Fig 3). LPS induced NO was reduced to 62.24 ± 0.7% by dexamethasone at 100 μM concentration. In addition to ROS and RNS, NO is a signalling molecule implicated in widespread of pathological events like apoptosis gene expression, inflammation, enzyme regulation [48–49]. In response to inflammatory stimuli, high levels of NO are secreted that impart pro-inflammatory effects [48] and could act as an oxidant [46]. Plant derived products have intrinsic properties to combat the harmful effects of excessive NO production and thus have substantial interest in its prevention in humans. In this study, TE inhibited NO production in LPS-induced RAW264.7 cells. Previous studies have shown that TE has strong NO-scavenging properties [50].

![Fig 3. Effect of TE on NO production in LPS-stimulated RAW264.7 cells. Cells were treated with TE (50 and 100 μg/mL) and dexamethasone (Dexa) (100 μM), with/without LPS (1 μg/mL) and incubated for 24 h at 37°C. After incubation, the cell supernatant was used to determine NO level by Griess reagent method. The values are expressed as mean ± SEM of three experiments (p<0.01, p<0.05).](https://doi.org/10.1371/journal.pone.0145921.g003)
H$_2$O$_2$ is an oxidant that induces the generation of free radicals. In order to check the concentration of H$_2$O$_2$ required inducing reversible damage on fibroblasts, a modification of the protocol reported by Murrell et al [25] was implemented. MTT assay was employed to study the protection rendered by TE at different concentrations. Ascorbic acid and TE extract at the concentration of 100 $\mu$g/mL showed a significant protection of fibroblasts against hydrogen peroxide-induced cell membrane damage. The fibroblasts co-treated with the extract showed more protection than that of pre-treatment of TE and hydrogen peroxide (Fig 4A and 4B). However, TE could not regain the viability of the fibroblasts post-treatment (data not shown).

Oxidation is generally considered to play an important role in various disorders in cells [51]. In this study, TE exhibited antioxidant activity and protected HDF cells from H$_2$O$_2$ damage. The results of the MTT assay indicated that TE could rescue HDF cells from oxidative injury induced by H$_2$O$_2$. The fibroblasts pre-treated and co-treated were protected from the damage suggesting that TE might protect the cell membrane of HDFs, thus limiting the damage induced by H$_2$O$_2$.

The skin of humans is amenable to different DNA-damaging factors of the environment and hence, needs several in-house functions to reduce, repair, and protect from such deleterious effects. Skin pigmentation, antioxidant enzymes, epidermal thickness, DNA repair and apoptosis are the mechanisms involved in offering skin protection. [52]. In the present study, TE showed strong scavenging effects on hydroxyl radicals and was further examined for its protective role against DNA damage due to generation of hydroxyl ions via Fenton reaction. The DNA treated with 2 mM FeSO$_4$ and 1 mM H$_2$O$_2$ lead to oxidation of DNA generating free hydroxyl ions from the fenton’s reaction. However, TE treatment showed a dose-related inhibition of DNA oxidation against OH radical induced damage at 100 and 50 $\mu$g/mL.

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Fig 4. Radical scavenging effect of TE on HDF cells against H$_2$O$_2$-induced cell damage. (A) HDF cells were pre-treated with various concentrations of TE for 1 h and exposed to 10 $\mu$M H$_2$O$_2$, and further incubated for 24 h at 37°C. (B) HDFs were co-treated with various concentrations of TE and 10 $\mu$M H$_2$O$_2$, incubated for 24 h at 37°C. Cell viability was determined by MTT assay and the results expressed as mean ± SEM (n = 3). (C) Protective effect of TE on DNA damage in HDF cells by release of OH radicals from Fenton reaction. The DNA were isolated from HDF cells and treated with two concentrations of TE (50 and 100 $\mu$g/mL), FeSO$_4$ (2mM) and H$_2$O$_2$ (1 mM), incubated for 1 h at 37°C. DNA bands were resolved in 1% agarose gel stained with ethidium bromide. Densitometry analysis showing the protective effect of TE on H$_2$O$_2$-induced DNA damage. Values shown depict arbitrary units. Data is expressed as mean ± SEM (n = 3).

doi:10.1371/journal.pone.0145921.g004
concentrations respectively (Fig 4C). Tannic acid also protected HDF from DNA damage (Fig 4C). Thus, TE ameliorated hydroxyl radical-stimulated DNA damage. The precursors and catalysts for Fenton’s reaction co-exist in the mitochondrial matrix that makes mitochondria an important site for sustained hydroxyl radical production during oxidative stress [53]. Exposure of DNA to Fenton reaction leads to DNA strand breaks and base modifications [54–55]. In the experimental study, the rate of DNA damage was typically reduced by TE, showing its protective effects against H$_2$O$_2$ damage.

Cellular senescence plays a vital role in regulating cellular aging both in vitro and in vivo [56]. The aging cells are identified with increased lipofuscin granules and a distinct phenotype, which includes flat morphology and bulged cell size [56–57]. Significant changes are also observed at protein and gene levels [57]. Accumulation of β-galactosidase occurred in H$_2$O$_2$ induced HDF cells in the fibroblasts, appeared as bluish green cells under the microscope (Fig 5A–5G). TE exhibited protective effect on H$_2$O$_2$ induced HDF cells with a significant decrease in β-galactosidase stained cells. The cells treated with tannic acid, used as standard also protected the cells from H$_2$O$_2$ induced cellular senescence (Fig 5C). Cellular senescence is an indication of age-associated etiology [58]. H$_2$O$_2$ induces senescence-associated beta-galactosidase activity in HDF cells [59–60]. Thus TE protected the cells from H$_2$O$_2$-induced cellular senescence that proved its anti skin aging property.

Melanogenesis involves the enzyme tyrosinase for catalyzing the oxidative reaction of tyrosine to dihydroxy-phenylalanine (DOPA) and Dopaquinone, a common intermediate to both eumelanogenic and pheomelanogenic pathways [61–63]. Conventionally, L-tyrosine and L-DOPA are regarded as positive regulators of melanogenesis [64]. Melanin possesses photoprotective properties; the cumulation of abnormal amounts of melanin in various skin parts leads to darkening or skin pigmentation that hampers the skin beauty. To evaluate the skin-whitening properties of TE, melanin spitting/inhibition, and anti-tyrosinase assays were performed. The mouse melanoma cells (B16F10) is widely used for investigating anti-tyrosinase and melanin inhibition properties of the plant extracts [65–67]. TE showed tyrosinase

![Fig 5. Effect of TE on cellular senescence in HDF cells.](image-url)
inhibition 26.17 ± 1.0% and 16.16 ± 2.3% at 100 μg/ml and 50 μg/mL respectively in B16F10 cells. Kojic acid (KA) was used as a positive control, showed 44.63 ± 1.2% at 100 μM concentration (Fig 6A). Earlier, ellagic acid inhibited UV irradiation induced skin pigmentation [65–67]. Previous literature on the inhibition of tyrosinase activity by several compounds led to the use of various inhibitors in cosmetics for the treatment of hyperpigmentation [68–69]. Similarly, in melanin inhibition assay, TE displayed significant melanin inhibition at 100 μg/ml (59.08 ± 0.7%) and 50 μg/mL (45.55 ± 2.0%) respectively in B16F10 cells. Kojic acid (KA) was used as a reference standard and it showed 66.71 ± 0.6% at 100 μM concentration (Fig 6B). In addition, the melanin inhibition assay was also performed in A-375 (Human melanoma) cells. TE showed dose-dependent melanin inhibition (Fig 6C). Several plant extracts like *Morus alba* L. (Moraceae) or *Glycyrrhiza glabra* (Leguminosae) have found their applications as skin lightening agents in cosmeceuticals [70–72].

Human dermal fibroblasts continuously produce products related to extracellular matrix and collagen-I, is one such classical product. To evaluate the effect of TE on collagen-I production in fibroblast cells, ELISA was performed. HDF treated with TE showed an increase in collagen-I production by 70.72 ± 2.3% and 58.24 ± 4.6% at 100 μg and 50 μg/mL respectively. Ascorbic acid increased collagen-I production by 73.67 ± 2.2% in HDF cells (Fig 7A). In addition, TE treated HDF cells producing collagen-I stained pink as detected by Sirus red staining (S6 Fig). Keratinocytes, apart from providing the structural integrity, also secrete out various substances like cytokines, hormones, and neurotransmitters [3]. Differentiating keratinocytes express cytokeratins and accessory proteins such as involucrin, filaggrin, loricrin, cystatin, envoplakin, periplakin etc. in progressing layers to build the epidermal barrier [3]. ELISA measurements revealed a steady increase in involucrin content in TE treated HaCaT cells after 4 days. TE increased involucrin content by 67.61 ± 2.1% and 41.95 ± 1.9% at 100 and 50 μg/mL.
respectively (Fig 7B). Kinetin increased the involucrin content by 30.30 ± 2.8% at 100 μM concentration. TE increased filaggrin by 51.91 ± 3.5% and 41.01 ± 2.1% at 100 and 50 μg/mL respectively (Fig 7C). Kinetin increased the filaggrin content by 34.38 ± 3.0% at 100 μM concentration.

In dermis, the reduction of skin structural proteins can lead to several signs of aging [73–74]. To further establish the skin protective role of TE, the mRNA levels for skin-related proteins/markers were checked. TE at 100 and 50 μg/ml increased the gene expression of collagen-I, elastin, SOD-2, AQP-3, transglutaminase, involucrin, filaggrin in HDF or HaCaT cells. However, the tyrosinase gene expression decreased upon treatment with TE at 100 and 50 μg/ml in B16F10 cells. The Ct values were normalized to that of GAPDH and given in the (Fig 8A–8I). The amplified products stained with ethidium bromide confirmed the results of qPCR (Fig 8). Collagen, one of the structural proteins of the skin, is the major constituent of connective tissue, hair, and nails [75]. It helps in maintaining the elasticity, firmness and flexibility of the skin. Elastin, a predominant protein in the connective tissue provides elasticity to the skin and lungs [75, 76]. Involucrin one of the important markers of the skin helps in protecting the skin corneocytes by formation of the cell envelope [77]. TE-treated HDF or HaCaT cells increased the synthesis of collagen-I and involucrin respectively. The expression profile of collagen and elastin were up-regulated in TE treated HDF cells. The results highlight the role of TE in increasing collagen and elastin gene expression. AQP-3 in the skin of human beings is age-related and decreases in level occur with aging [78]. In dermal pathophysiological course, AQP-3 can transport water and glycerol to epidermis, so AQP-3 is significant to epidermal barrier and water retention. AQP-3 gene expression was up-regulated by TE in HaCaT cells, indicating that TE can function as a skin protectant by hydrating the skin cells. The molecules such as thiols, DNA, and mitochondrial proteins are regarded most responsive to modification by oxidative stress [79]. Stress on epidermal skin leads to generation of several metabolites that
scavenge free radicals and stimulate antioxidative enzymes, including superoxide dismutase [80]. SOD-2 is a cellular antioxidant present in mitochondrial matrix and offers defence against superoxide anions, which are the by-products of oxidative phosphorylation [81]. The present study showed that TE stimulates SOD-2 expression in HDF cells, thus enabling them to combat oxidative stress.

Filaggrin, a protein that binds to keratin fibers in epithelial cells plays a vital role in epidermal homeostasis, release of free amino acids, and helps in water retention [82]. The filaggrin gene expression was up-regulated upon treatment with different concentrations of TE in HaCaT cells, indicating the role of TE in skin moisturization. Transglutaminase, a calcium-dependent enzyme catalyzes an intermolecular peptide bond formation between proteins and has an essential role in formation of cornified cell envelope [83]. The transglutaminase gene (TGM1) expression was up-regulated in HaCaT cells after treatment with TE at two different concentrations indicating pore reduction/or skin tightening. Tyrosinase catalyzes tyrosine to DOPA and Dopaquinone thus playing a vital role in melanogenesis, which, is explored by researchers for screening various melanin inhibitors [68–72]. The tyrosinase gene expression was down-regulated by TE in B16F10 cells, suggesting its role in skin-whitening.

Triphala (TE) has become a potential candidate for skin care and protection through its antioxidant and anti-inflammatory properties. However, no studies have demonstrated the skin protecting benefits of TE using in vitro skin models. The results highlight the benefits of Triphala (TE) associated with its potential antioxidant property, radical-scavenging ability, delaying cellular senescence, building structural proteins such as collagen and elastin, restoring skin barrier function along with providing hydration to the skin.

Fig 8. Effect of TE on expression of selected genes involved in biological functions related to aging. HDF and/or HaCaT cells were treated with different doses of TE for 24 h at 37°C. RNA was then isolated and qPCR carried out using oligo-dT primers for first strand synthesis followed by gene specific primers as described in the section 2.14. The gene expression was quantified and normalized to GAPDH and the data represented as mean ± SD (n = 2). The image shown is a representative from among two replicates. * indicate P < 0.05 and ** indicate P < 0.01. (A) Collagen-I gene expression (B) Elastin gene expression (C) SOD-2 gene expression (D) AQP-3 gene expression (E) Filaggrin gene expression (F) Transglutaminase (TGM1) gene expression (G) Tyrosinase gene expression (H) GAPDH gene expression (I) Involucrin gene expression.

doi:10.1371/journal.pone.0145921.g008
Conclusion
This article for the first time reports the protective effects of Triphala (TE) on Human skin cells in vitro. Triphala has potential antioxidant property and it acts as a skin-protective ingredient by re-building skin structural proteins and stimulating selective youth genes. The results obtained however, needs further detailed clinical investigation.

Supporting Information

S1 Fig. LC-MS spectra of Terminalia chebula extract. The arrow indicates the identified compounds.
(TIF)

S2 Fig. LC-MS spectra of Emblica officinalis extract.
(TIF)

S3 Fig. LC-MS spectra of Terminalia bellerica extract.
(TIF)

S4 Fig. Docking studies of major constituents of Triphala (Gallic acid, Ellagic acid, and Chebulinic acid) with involucrin, filaggrin, & collagen-I. a-c) Involucrin d-f) Filaggrin g-i) Collagen-I. The black arrow indicates the docked compound.
(TIF)

S5 Fig. Effect of TE on HaCaT and HDF cells a) Cytotoxicity of Triphala (TE) on HaCaT and HDF cells b) Untreated HaCaT cells (Control) c) TE (50 μg/ml) treated HaCaT cells d) TE (100 μg/ml) treated HaCaT cells e) HDF cells without treatment (Control) f) TE (50 μg/ml) treated HDF cells g) TE (100 μg/ml) treated HDF cells.
(TIF)

S6 Fig. Collagen-I staining in HDF cells a) Control b) Ascorbic acid c) TE (50 μg/ml) treated HDF cells d) TE (100 μg/ml) treated HDF cells. The pink color indicates the collagen-I production in HDF cells.
(TIF)

S1 Table. Protein-Ligand binding affinity in Kcal/mol.
(DOC)

S1 Text. Raw/Experimental data for protective effects of TE on HDF and/or HaCaT cells a) Cytotoxicity of TE on HaCaT and HDF cells b) RBC hemolysis assay c) Reducing power assay d) Radical scavenging assay e) Collagen-I ELISA assay.
(XLSX)

Acknowledgments
We thank Ms. Jayashree B Keshav, Senior Manager, The Himalaya Drug Company, Bangalore for editing the manuscript. Also we thank Pavan Kumar Bellamakondi, Research Associate, Department of Cell biology for his help in performing the collagen-I staining.

Author Contributions
Conceived and designed the experiments: SRV TOS SP SR. Performed the experiments: TOS AM LMSK SRV NSP. Analyzed the data: SRV TOS AM LMSK NSP. Contributed reagents/materials/analysis tools: SR SP. Wrote the paper: SRV AM TOS.
References

1. Slominski A, Zmijewski MA, Skobowiat C, Zbytek B, Slominski RM, Steketee JD. Sensing the environment: regulation of local and global homeostasis by the skin’s neuroendocrine system. Adv. Anat. Embryol. Cell Biol. 2012; 212:1–115.

2. Robinson MK, Binder RL, Griffiths CE. Genomic-driven insights into changes in aging skin. J Drugs Dermatol. 2009; 8: s8–s11. PMID: 19623778

3. Slominski A, Zmijewski MA, Semak I, Zbytek B, Pisarchik A, Li W, et al. Cytochromes P450 and skin cancer: role of local endocrine pathways Anti-cancer Agents Med. Chem. 2014; 14(1): 77–96.

4. Tiwari AK. Antioxidants: New-generation therapeutic base for treatment of polygenic disorders. Curr Sci. 2004; 86: 1092–1102.

5. Sivanandham V. Free radicals in health and diseases. Pharmacol Online. 2011; 1: 1062–1077.

6. Gopaul R, Knaggs HE, Lephart J. Salicin regulates the expression of functional ‘youth gene clusters’ to reflect a more youthful gene expression profile. Int J Cosmet Sci. 2011; 33: 416–420. doi: 10.1111/j.1468-2494.2011.00645.x PMID: 21449910

7. Prota G. Melanins and melanogenesis. Cosmetics Toiletries. 1996; 111(55): 43–51.

8. Mora PC, Baraldi PG. Dermocosmetic applications of polymeric biomaterials. In: Dumitru S, ed., Polymeric Biomaterials, 2nd ed. New York and Basel, Marcel Dekker. 2000; pp. 459–490.

9. Nakayama H, Ebihara T, Satoh N, Jinnai T. Depigmenting agents In: Elsner P, Maibach HI, eds., Cosmeceuticals Drugs vs. Cosmetics. New York and Basel, Marcel Dekker 2000; pp. 123–144.

10. Jagetia GC, Baliga MS, Malagi KJ, Klamath M. The evaluation of the radioprotective effect of Triphala (an ayurvedic rejuvenating drug) in the mice exposed to gamma-radiation. Phytomedicine. 2002; 9: 99–106. PMID: 11995956

11. Singh DP, Govindarajan R, Rawat AK. High-performance liquid chromatography as a tool for the chemical standardisation of Triphala-an Ayurvedic formulation. Phytochem Anal. 2008; 19: 164–168. PMID: 1787225

12. Pawar V, Lahorkar P, Ananthanarayana DB. Development of a RP-HPLC method for analysis of Triphala churma and its applicability to test variations in Triphala churna preparations. Ind J Pharm Sci. 2009; 71: 382–386.

13. Tierra M. The Wonders of Triphala: Ayurvedic Formula for Internal Purification. 1996; Available:http://www.planetherbs.com/specific-herbs/the-wonders-of-triphala.html

14. Baliga MS, Meera S, Mathai B, Rai MP, Pawar V, Palatty PL, et al. Scientific validation of the ethnomedicinal properties of the Ayurvedic drug Triphala: A review. Chin J Integ Med. 2012; 18: 946–954.

15. Deep G, Dhiman M, Rao AR, Kale RK. Chemopreventive potential of Triphala (a composite Indian drug) on benzo (a) pyrene induced for stomach tumorigenesis in murine tumor model system. J Exp Clin Cancer Res. 2005; 24: 555–563. PMID: 16471319

16. Wolfe K, Wu X, Liu RH. Antioxidant activity of apple peels. J Agric Food Chem. 2003; 51: 609–614. PMID: 12537430

17. Jia Z, Tang M, Wu J. The determination of flavonoid content in mulberry and their scavenging effect on superoxide radicals. Food Chem. 1999; 64: 555–599.

18. Naik GH, Priyadarsini KI, Mohan H. Free radical scavenging reactions and phytochemical analysis of Triphala, an ayurvedic formulation. Current Sci. 2006; 90:1100–6.

19. Sandeep RV, Sharath kumar LM, Satyakumar V, Patki PS. Water Soluble Components of ‘Osteoecare’ Promote Cell Proliferation, Differentiation, and Matrix Mineralization in Human Osteoblast-Like SaOS-2 Cells. Sci Pharm. 2014; 82: 375–391. doi: 10.3797/scipharm.1310-25 PMID: 24959407

20. Trott O, Olson AJ. AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization and multithreading. J Comput Chem. 2010; 31: 455–461. doi: 10.1002/jcc.21334 PMID: 19499576

21. Mishra A, Gauri SS, Mukhopadhyay SK, Chatterjee S, Das SS, Mandal SM, et al. Identification and structural characterization of a new pro-apoptotic cyclic octapeptide cyclospadin from somatic seedlings of Santalum album L. 2014; Peptides 54:148–158. doi: 10.1016/j.peptides.2014.01.023 PMID: 24503375

22. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Meth. 1983; 65: 55–63.

23. Reddy CSSS, Subramaniam MVV, Vani R, Devi SA. In vitro models of oxidative stress in rat erythrocytes: Effect of antioxidant supplements. Tox In vitro. 2007; 21:1355–1364.
24. Anusuya N, Gomathi R, Manian S, Sivaram V, Menon A. Evaluation of Basella rubra L., Rumex nepalensis Spreng and Commelina benghalensis L. for antioxidant activity. Int J Pharm and Pharmaceut Sci. 2012; 4: 714–720.

25. Murrell GAC, Francis MJO, Bromley L. Modulation of fibroblast proliferation by oxygen free radicals. Biochem J 1990; 265:659–665. PMID: 2154966

26. Mensah AY, Sampson J, Houghton PJ, Hyland J, Dunn M, et al. Effects of Buddleia globosa leaf and its constituents relevant to wound healing. J Ethnopharmacol. 2001; 77:219–226. PMID: 11535367

27. Qian ZJ, Jung WK, Kim SK. Free radical scavenging activity of a novel Antioxidative peptide purified from hydrolysate of bullfrog skin, Rana catesbeiana Shaw. Bioresearch Technol. 2008; 99:1690–8. PMID: 17512726

28. Hearing V, Tsukamoto K. Enzymatic control of pigmentation in mammals. FASEB J 1991; 5: 2902–2909. PMID: 1752358

29. Azhar-ul-Haq, Malik A, Khan MTH, Anwar-ul-Haq, Khan SB, et al. Tyrosinase inhibitory lignans from the methanol extract of the roots of Vitex negundo Linn and their structure-activity relationship. Phytotherapy 2006; 13: 255–260. PMID: 16492528

30. Kim YJ, No JK, Lee JS, Kim MS, Chung HY Anti-melanogenic activity of 3, 4-Dihydroxyacetophenone: Inhibition of tyrosinase and MITF. Biosci Biotechnol Biochem. 2006; 70: 532–534. PMID: 16495675

31. Kumar KJS, Yang JC, Chu FH, Chang ST, Wang SY, Lucidone, a novel melanin inhibitor from the fruit of Linderia erythrophaca Makino. Phytother. Res. 2010; 243:1158–1165.

32. Mias C, Lareo E, Trouche E, Roncalli J, Calise D, Seguelas MH, et al. Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction. Stem Cells. 2009; 27 (11): 2734–2743. doi: 10.1002/stem.169 PMID: 19591227

33. Manayi A, Saeidnia S, Gohari AR, Abdollahi M. Methods for the discovery of new anti-aging products - targeted approaches. Expert Opin Drug Discov. 2014; 9: 383–405. doi: 10.1517/17460441.2014.885014 PMID: 24494592

34. Svobodova A, Psojava T, Walterova D. Natural phenolics in the prevention of UV- induced skin damage. A review Biomed. Pap. Med. Fac. Univ. Palacky Olomouc. Czech Repub. 2003; 147:137–145.

35. Baq A, Bhattacharya SK, Pal NK. Antibacterial potential of hydroalcoholic extracts of Terminalia chebula Retzius auyurvedic decoction and evaluation of its chemopreventive activity. J Ethnopharmacol. 2013; 179:1977–1980. PMID: 23069922

36. Lu K, Chakroborty D, Sarkar C, Lu T, Xie Z, Zhongfa L, et al. Triphala and its active constituent chebulinic acid are natural inhibitors of vascular endothelial growth factor-mediated angiogenesis. PLOS ONE. 2012; 7: e30954. doi: 10.1371/journal.pone.0030954 PMID: 22937129

37. Snyder LM, Fortier NL, Trainor J, Jacobs J, Lob B, Lubin B, et al. Effect of hydrogen peroxide exposure on normal human erythrocyte deformability, morphology, surface characteristics and spectrin-hemoglobin cross-linking. J Clinical Invest. 1985; 76:1971–1977.

38. Ajila CM, Prasada Rao UJS. Protection against hydrogen peroxide induced oxidative damage in rat erythrocytes by Mangifera indica L. peel extract. Food Chem Toxicol. 2008; 46: 303–309. PMID: 17919903

39. Rajesh IFF, Strain JJ. The ferric reducing ability of plasma as a measure of ‘antioxidant power’: the FRAF assay. Anal Biochem. 1996; 239: 70–76. PMID: 8660627

40. Chung YC, Chang CT, Chao WW, Lin CF, Chou ST. Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by Bacillus subtilis IMR-NK1. J Agri Food Chem. 2002; 50: 2454–2458.
46. Shindo Y, Witt E, Han D, Epstein W, Packer L. Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin. J Invest Dermatol. 1994; 102(1): 122–124. PMID: 8288904
47. Coleman JW. Nitric oxide: a regulator of mast cell activation and mast cell-mediated inflammation. Clin Exp Immunol. 2002; 129(1): 4–10. PMID: 12100016
48. Korhonen R, Lahti A, Kankaanranta H, Moilanen E. Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Aller. 2005; 4: 471–479.
49. Salvesen GS, Dixit VM. Caspase activation: the induced proximity model. Proc Nat Acad Sci USA. 1999; 96: 10964–10967.
50. Jagetia GC, Rao SK, Baliga MS, Babu KS. The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: a preliminary study. Phytother Res. 2004; 18: 561–565. PMID: 15305317
51. Salomon DR, Phillips D. Oxidative DNA damage mediated by copper II, iron II and nickel II Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intra-strand cross-links. Mutation Res. 1999; 424: 243–256. PMID: 10064847
52. Duncan EL, Wadhwa R, Kaul SC. Hydroxyl radical is produced via the Fenton reaction in sub mitochondrial particles under oxidative stress: implications for diseases associated with iron accumulation. Redox Rep. 2009; 14(3):102–108. doi: 10.1179/135100009X392566 PMID: 19490751
53. Vyas S, Tomita Y, Brown LF, Kocher O, Dvorak HF. Differential expression of thymosin beta-10 by senescent and immortalized human cells in culture and in aging skin in vivo. Pro Nat Acad Sci USA. 1995; 92: 9833–9837.
54. Slominski A, Zmijewski MA, Pawelek J. L-Tyrosine and L-dihydroxyphenylalanine as hormone-like regulators of melanocyte functions. Pigment Cell Melanoma Res. 2012; 25:14–27. doi: 10.1111/j.1755-148X.2011.00898.x PMID: 21834848
55. Shimogaki H, Yanagawa T (1996) Inhibitory effect of ellagic acid on tyrosinase activity. American Academy of Dermatology 54th Annual Meeting, February 10–15, Washington, DC p. 280.
56. Shimogaki H, Tanaka Y, Tamai H, Masuda M. In vitro and in vivo evaluation of ellagic acid on melanogenesis inhibition. Int J Cosmet Sci. 2000; 22: 291–303. doi: 10.1046/j.1467-2494.2000.00023.x PMID: 18503416
57. Tanaka Y (2001) Ellagic acid. Pigment Cell Res 14(6): 495.
58. Sung JH, Park SH, Seo DH, Lee JH, Hong SW, Hong SS. Antioxidative and skin-whitening effect of an aqueous extract of Salicornia herbacea. Biosci Biotechnol Biochem. 2009; 73:552–556. PMID: 19270393
59. Zheng ZP, Chen S, Wang S, Wang XC, Cheng KW, Wu JJ, et al. Chemical components and tyrosinase inhibitors from the twigs of Artocarpus heterophyllus. Journal of Agricultural and Food Chemistry. 2009; 57:6649–6655. doi: 10.1021/jf9014685 PMID: 19588925
70. Lee KT, Kim BJ, Kim HJ, Heo MY, Kim HP. Biological screening of 100 plant extracts for cosmetic use (I): inhibitory activities of tyrosinase and DOPA auto-oxidation. Int J Cosmet Sci. 1997; 19: 291–298. doi: 10.1046/j.1467-2494.1997.171725.x PMID: 1850483

71. Bernard P, Berthon Y. Resveratrol: An original mechanism on tyrosinase inhibition. Int J Cosmet Sci. 2000; 22: 219–226. doi: 10.1046/j.1467-2494.2000.00019.x PMID: 18503477

72. Baurin N, Arnault E, Sciortino T, Do QT, Bernard P. Preliminary screening of some tropical plants for antityrosinase activity. J Ethnopharmacol. 2002; 82: 155–158. PMID: 12241990

73. Zheng Q, Chen S, Chen Y, Lyga J, Wyborski R, Santhanam U. Investigation of age-related decline of microfibril-associated glycoprotein-1 in human skin through immunohistochemistry study. Clin Cosmet Investig Dermatol. 2013; 6: 317–323.

74. Mukherjee PK, Maity N, Nema NK, Sarkar BK. Bioactive compounds from natural resources against skin aging. Phytotherapy. 2011; 19: 64–73. doi: 10.1016/j.phymed.2011.10.003 PMID: 22115797

75. Fulop T, Khalil A, Larbi A. The role of elastin peptides in modulating the immune response in aging and age-related diseases. Pathol Biol. 2012; 60: 28–33. doi: 10.1016/j.pathbio.2011.10.006 PMID: 22099332

76. Kurtz A, Oh SJ. Age related changes of the extracellular matrix and stem cell maintenance. Prevent Med. 2012; 54: s50–s56.

77. Watt FM. Involucrin and other markers of keratinocytes terminal differentiation. J Invest Dermatol. 1983; 81: 100s–103s. PMID: 6345687

78. Li J, Tang H, Chem M, Xie H. Aquaporin-3 gene and protein expression in sun-protected human skin decreases with skin aging. Australas J Dermatol. 2010; 51: 106–112. doi: 10.1111/j.1440-0960.2010.00629.x PMID: 20546216

79. Slominski AT, Kleszczyński K, Semak I, Janjetovic Z, Zmijewski MA, Kim TK et al. Local melatoninergic system as the protector of skin integrity. Int. J. Mol. Sci. 2014; 15: 17705–17732. doi: 10.3390/ijms151017705 PMID: 25272227

80. Janjetovic Z, Nahmias ZP, Hanna S, Jarrett SJ, Kim TK, Reiter RJ et al. Melatonin and its metabolites ameliorate UVB-induced damages in human epidermal keratinocytes. J Pineal Res 2014; 57: 90–102 doi: 10.1111/jpi.12146 PMID: 24867336

81. Triebner N, Maity P, Singh K, Ferchiu F, Wlaschek M, Kochanek SK. The role of manganese superoxide dismutase in skin aging. Dermat Endocrinol. 2012; 4: 232–235.

82. Brown SJ, McLean WhI. One remarkable molecule: filaggrin. J. Invest. Dermatol. 2012; 132: 751–762. doi: 10.1038/jid.2011.393 PMID: 22158554

83. Hitomi K. Transglutaminase in skin epidermis. Eur J Dermatol 2005; 15: 313–319. PMID: 16172037