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EVALUATION OF WOUND HEALING & ANTIAGING POTENTIAL OF AYURVEDIC DENTIFRICES USING HUMAN FRIBROBLAST - *IN-VITRO*

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ABSTRACT

The oral cavity is under continues exposure to hot & cold drinks & junk foods, substances such as alcohol, tobacco products and nicotine etc. which contribute towards production of free radicals. Presence of bacteria and other disease-causing agents further aggravates the oxidative damage.[1] Oxidative stress in oral disease is related to other systemic diseases in the body such as periodontitis, cardiovascular, pancreatic, gastric, and liver diseases.[2] There is significant attention of researchers to the potential benefit from the use of antioxidants in the field of dental medicine. In this study antioxidant and antiaging potential of TIs (Test Items) were investigated using two cell system: WI-38 (Human lung fibroblast) & HGF (Human Gingival fibroblast). Since the oral mucosa is constituted of fibroblasts, these cell types are highly relevant for testing oral care products. Two marketed ayurvedic dentifrices (Dabur Dant Rakshak Paste & Dabur Red Paste) were selected for the study based on presence of antioxidant & antiaging ayurvedic ingredients in the formulation. e.g., *Cinnamonum zeylanicum* & *Syzygium aromaticum* are proven antioxidant ayurvedic herbs. *Piper longum*,[3] *Salvadora persica*,[4] & *Glycyrrhiza glabra* [5] are ayurvedic rasayanas *(Rejuvenators)* which commonly used for antiaging & cell rejuvenation benefits in ayurveda. Dabur Red Paste, Dabur Dant Rakshak Paste demonstrated antioxidant potential via cytoprotection and inhibition of reactive oxygen species (ROS) generation in both WI-38 and HGF cells system. In addition, both the ayurvedic toothpaste exhibited wound healing potential which confirms their antiaging properties when compared against placebo.

KEYWORDS: *ROS, Antiaging, Antioxidant activity, Wound Healing, Dabur Red Paste, Dabur Dant Rakshak Paste, Ayurveda, Rasayanas.*

1. INTRODUCTION

Free radicals are highly reactive molecular species contains an unpaired electron and imbalance between free radicals & antioxidant called as oxidative stress. Treatment of oral and dental health problems may include natural antioxidant remedies that are available in topical oral applications such as mouth rinse, gel, paste, gum, or lozenge compositions. These topical antioxidant remedies help reduce free-radical or reactive-oxygen species, which are causative inflammatory factors in the progression of gingival and periodontal diseases. Antioxidants neutralize damaging free radicals that produce disease states. Antioxidants are available from different sources, including vitamins, minerals, enzymes, and hormones, food, as well as herbs such as ayurvedic rasayanas. The rapidly advancing field of dental pharmacotherapeutics has paved the way for the development of a wide array of antioxidants that have beneficial clinical effects.[6]

Human fibroblasts (WI-38) & HGF (Human Gingival fibroblast) have been reported to investigate the antioxidant potential of test agents against oxidative damage.[7,8,9] Aging may result in delaying the wound healing process in oral cavity. There are reports that aging may negatively affect gingival wound-healing.[10] Wound healing promoting potential of test compounds has been tested using scratch assay in human fibroblasts. [11,12] Hence, fibroblasts were used as model system to determine antioxidant & wound healing potential of TIs along with actual gingival fibroblast which more relevant to dental & oral health, which reflects their antiaging or regenerative properties.

Cellular aging is generally defined as the progressive cellular damages, causing a gradual loss of cellular functions, and resulting eventually in cell death. The exposure to genotoxic agents i.e., free radicals, which may induce damage to DNA (Deoxyribonucleic acid) in general or more extensively at telomeres, can increase cancer risk and pace of aging. The dosage of cigarette smoking is shown to negatively correlate with telomere length. The telomere attrition caused by smoking one pack of cigarettes a day for a period of 40 years is equivalent to 7.4 years of life. [13] Babizhayev *et al.* have proposed that telomere length can serve as a biomarker for evaluation of the oxidative damage caused by smoking and may also predict the rate at which an individual is aging. The smoking increases oxidative stress, expedites telomere shortening, and may increase the pace of aging process. Antioxidants can potentially protect telomeric DNA from oxidative damage caused by extrinsic and intrinsic DNA damaging agents.[14]

Ayurvedic rasayanas offer benefits of Rasadi Dhatus. Ayurveda described various plants as Rasayanas such as Pippali, and Mulethi. These drugs enhance nutritional intake and qualities of Dhatus which leads longevity, improve strength and Ojabala. The antioxidant activity of Rasayana is due to the presence of constituents such as vitamin C, carotene, riboflavin, with anolide, tanins, gallic acid and polyphenols. It is believed that Rasayana drugs increases collagen fibrin synthesis, absorption of iron and levels of natural antioxidants; dismutase, Catalase, Glutathione peroxidase therefore reduces risk of oxidative stress. The antioxidant potential of Rasayana drugs possess benefits like; Vayasthapana (delaying aging), Balakara (strengthen the body) and Roga-apaharana (improve immunity). Antioxidants play significant role towards the delaying aging, prevention of disease and decreases risk of cancer.

Rasayana drugs are used as rejuvenators and nutritional supplements in various clinical indications. Rasayana drugs also possess antioxidant activity and suppress activity of oxidative stressors and thus reduce production of free radicals. Therefore, Rasayana drugs offer therapeutic potential against the diseases associated with oxidative stress; auto immune diseases, cancer and tumor.[15] Dabur Red Paste was found effective in reducing the free radical, indicating that Paste possessing the antioxidant activity.[16]

Research has established that free radical production plays a role in the aging; studies are conducted to examine the potential relationship between varying free radical and antioxidant concentrations related to cell life and longevity.[17] The World Congress 2015 adopted the Tokyo Declaration on Dental Care and Oral Health for Healthy Longevity for recognition that maintenance of oral and dental health throughout life is a fundamental factor for improving quality of life, helping to protect against noncommunicable diseases and contributing towards preventing the aggravation of such diseases - it can also contribute to longer healthy life expectancy.

The antioxidant potential of multiherbal ayurvedic dentifrice was determined as indicated by cytoprotective and ROS inhibition potential against oxidative stress induced by an agent t-butylhydroperoxide (t-BHP). Increase in cell survival and inhibition of ROS was investigated against oxidative damage conditions in cell lines. Further, the antiaging & regenerating effects of ayurvedic dentifrice were determined as indicated by the wound healing potential in scratch assays. These end point parameters were selected considering their relevance in oral health. Table 1; represents relevance & significance of selected method for evaluation of antiaging potential & its relevance to oral health.

Table 1: Cell based assays & its relevance to Antiaging & Cell protection in Oral Cavity.

S. No.	Parameter	Method	Relevance	Significance
1	Cytoprotection	MTT Assay-Determination of cell viability against oxidative damage[8,9]	Enhanced cell viability/reduction in cytotoxicity upon treatment with TIs suggests their antioxidant potential via combating t-BHP induced oxidation and cell damage.	This indicates that the TIs have the capability to reduce the oxidative stress and restore the cell damage caused by oxidative agent by enhanced cell survival.
2	ROS generation	ROS generation was determined using DCFDA dye based fluorometric method[8,9]	Inhibition of intracellular formation of ROS by TIs upon exposure to t-BHP.	This indicates that the TIs have the capability to reduce oxidative stress and restore the cell damage caused by oxidative agent exposure by quenching the ROS production.
3	Wound healing	Determination of cell migration by wound healing in scratch assay[10,11,12]	A mechanical scratch is created in a confluent layer of fibroblasts that represents the wound. Cells tend to migrate towards the empty space inside the scratch area, which reflects the extent of cell migration.	An increase in the extent of cell migration upon treatment with TIs suggests their regenerative/anti-aging potential.

2. MATERIALS AND METHODS

2.1. Materials: The composition details of the Ayurvedic toothpaste are given in Table 2A & 2B.

Table 2A: Ingredients of Dabur Dant Rakshak Paste Product Manufacturer – Dabur India Ltd.

S. No.	Ayurvedic Ingredient
1.	*Glycyrrhiza glabra*
2.	*Trachyspermun ammi*
3.	*Mentha* species
4.	*Mentha piperita*
5.	*Acacia arabica*
6.	*Rubia cordifolia*
7.	*Cinnamonum zeylanicum*
8.	*Acacia catechu*
9.	*Caesalpinia sappan*
10.	*Piper cubeba*
11.	*Syzygium cumini*
12.	*Quercus infectoria*
13.	*Zizypus jujuba*
14.	*Barleria prinoitis*
15.	*Zanthoxylum alatum*
16.	*Syzygium aromaticum*
17.	*Hemidesmus indicus*
18.	*Mimusops elengi*
19.	*Terminalia bellirica*
20.	*Punica granatum*
21.	*Salvadora persica*
22.	*Eucalyptus globulus*
23.	*Foeniculum vulgare*
24.	*Anacylus pyrethrum*
25.	*Azadirachta indica*
26.	*Piper nigrum*
27.	*Piper longum*
28.	*Zingiber officinale*
29.	*Juglans regia*
30.	*Prunus amygladus*
31.	*Elettaria cardamomum*
32.	*Red Ochre*

Table 2B: Ingredients of Dabur Red Paste Product Manufacturer – Dabur India Ltd.

S. No.	Ayurvedic Ingredient
1	*Mentha* species
2	*Zanthoxylum alatum*
3	*Syzygium aromaticum*
4	*Cinnamomum camphora*
5	*Piper nigrum*
6	*Piper longum*
7	*Zingiber officinale*
8	*Red Ochre*

Placebo: Placebo was made without herbal ayurvedic actives in calcium carbonate base.

Positive Control: α- tocopherol (Sigma, Catalog No. C6763, Batch/Lot No. SLBD577V). **(Positive control was used only for validation purpose of the method and not for comparing results with test item).*

Test System

WI-38 (Human Lung Fibroblasts)

Name: WI-38 (Human Lung Fibroblast cell line)
Growth Medium: EMEM+ 12% FBS+ 2mM L-glutamine
Source: American Type Culture Collection (ATCC), USA
Antibiotics: Penicillin (100 U/ml), Streptomycin (100 µg/ml) Growth Conditions: 5% CO2, 37 °C, 95% Humidity.

HGF (Human Gingival Fibroblasts)

Name: HGF (Human Gingival Fibroblast cells)
Growth Medium: Gingival fibroblast medium + 20% FBS
Source: American Type Culture Collection (ATCC), USA
Growth Conditions: 5% CO2, 37 °C, 95% Humidity

2.2. Method & Experimental Procedure

Based on the above information, the present study was conducted to evaluate the antioxidant & antiaging potential of toothpaste using WI-38 & HGF cell lines.

Ayurvedic Dentifrices was determined on following parameters:

A) Protection of cell viability/survival against oxidative damage by MTT assay.
B) Anti-apoptotic potential against oxidative damage by ROS generation.
C) Wound healing potential by scratch assay.

A) Protection of Cell Viability/Survival Against Oxidative Damage by MTT Assay

Cultured fibroblasts insulted with oxidative stress/damage inducing agent such t-BHP was used as a model system. t-BHP treatment resulted in cell death/loss of cell viability. Cells were treated with toothpaste in the presence of oxidative agent.

Cells were trypsinized and a single cell suspension of WI-38 was prepared. Cells were counted on a hemocytometer. WI-38 were seeded at a density of 10,000 cells/well/180µl in respective medium in 96-well plates. Cells were incubated in a CO2 incubator for 24 h at 37 °C, 5% CO2 and 95% humidity.

The assays were conducted as follows
The protective effect of TIs on cell survival was determined by Cell viability was assessed by 3-(4, 5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

The survival of cells against t-BHP induced cytotoxicity was calculated as:
% Cell viability = 100-% Cytotoxicity

B) Anti-Apoptotic Potential Against Oxidative Damage by ROS Generation

Recovery of cell viability and reduction in apoptosis (programmed cell death) by inhibition of intracellular ROS generation suggest the beneficial role of TIs in managing and preventing the oxidative stress in oral cavity. Cells were trypsinized and a single cell suspension of WI-38 was prepared. Cells were counted on a hemocytometer. WI-38 were seeded at a density of 20,000 cells/well/180µl in respective medium in 96-well plates. Cells were incubated in a CO2 incubator for 24 h at 37 °C, 5 % CO2 and 95 % humidity. After 24 h, medium was removed, and treatments provided with TIs. After incubation for 24 h, cells were stained with DCFDA (2′,7′- Dichlorofluorescin diacetate). After staining, cells were exposed to oxidative damage with t-BHP, after incubation for 30 min. & 1hr, fluorescence (FL) of each well was read at excitation 485/20, emission 528/20.

C) Wound Healing Potential by Scratch Assay

The beneficial effect of TIs on cell migration against mechanical wound generated in WI-38 and HGF cells was determined using scratch assay. Mechanical wounds were created by scratching the confluent layers of cells. Subsequently, cells were treated with TIs for 24 h and allowed to migrate. The extent of cell migration was calculated in presence of TIs, which suggested their wound healing potential.

Cells were trypsinized and a single cell suspension of WI-38 was prepared. Cells were counted on a hemocytometer. Cells were seeded in respective medium in 24-well plates respectively. Cells were incubated in a CO2 incubator for 24 h at 37 °C, 5 % CO2 and 95 % humidity. Cells were sera starved with 0.25% FBS and incubated in a 5% CO2 incubator for 24h.After 24 h, treatments were provided with TIs. After treatment, cells were incubated in a 5% CO2 incubator for 24 h. The photoimaging of the scratches was done using inverted microscope. The photoimaging of the scratches was done using inverted microscope. The photomicrographs obtained were analyzed for quantitative assessment of area of wound closure using image (wound healing) analysis software.

Percentage migration at 24 h with respect to 0 h was determined as follows: [(A-B)/A]*100; where A = Distance at 0h, B = Distance at 24h

Percent increase in migration was determined as follows: (% migration in dentifrice treated) – (% migration in untreated cells)

3. RESULTS AND DISCUSSION

The present study was conducted to evaluate the antioxidant and antiaging potential of ayurvedic toothpastes using cell-based assays. To mimic the oxidative stress conditions, t-BHP was used, which is routinely employed in cell- based studies as an oxidative agent.

The protective effect of dentifrice on cell viability was used as a measure of cytoprotection, Inhibition of intracellular ROS generation suggested their antioxidant effects. Dabur Red Paste & Dabur Dant Rakshak Paste demonstrated 57.8% & 55.7% cytoprotection respectively in WI38 cell system whereas 46.5% & 43.0% respectively in HGF cell system. Dabur Red Paste & Dabur Dant Rakshak Paste demonstrated 37.5% & 77.9% inhibition of intracellular ROS respectively in WI38 cell system whereas 70.6% & 70.2% respectively in HGF cell system.

Wound healing potential was measured in a scratch assay which suggest their antiaging & regenerative potential. Dabur Red Paste & Dabur Dant Rakshak Paste demonstrated 27.3% & 57.5% wound healing respectively in WI38 cell system whereas 48.7% & 43.2% respectively in HGF cell system. Table 3A & 3B represents the summarized results of this study. The maximum beneficial effect exerted by ayurvedic toothpastes & placebo for individual parameter in WI-38 & HGF cells is shown.

Table 3A: Results for WI 38.

Parameters	Placebo	Dabur Red Paste	Dabur Dant Rakshak Paste
Cytoprotection (%)	8.1	57.8	55.7
ROS Inhibition (%)	15.6	37.5	77.9
Wound Healing (%)	21.6	27.3	57.5

Table 3B: Results for HGF.

Parameters	Placebo	Dabur Red Paste	Dabur Dant Rakshak Paste
Cytoprotection (%)	8.2	46.5	43.0
ROS Inhibition (%)	1.3	70.6	70.2
Wound Healing (%)	17.9	48.7	43.2

CONCLUSION

Based on above findings, it can be concluded that both the ayurvedic dentifrices demonstrated an overall antioxidant and antiaging potential by modulation of all the parameters in cell culture assays into both cell system (lung fibroblast & gingival fibroblast). Ayurvedic

toothpastes exhibited wound healing activity and antiaging properties when compared against placebo. Hence, Dabur Dant Rakshak Paste & Dabur Red Paste have beneficial effects as overall antioxidant, cytoprotective and antiaging properties to combat and fight oral diseases caused by oxidative stress when compared against placebo & these results can be further confirmed in In-vivo studies.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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