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Proprietary Ayurvedic Medicine for Comprehensive Oral Care: An In-Vitro Analysis of Cytotoxicity and Wound Healing Potential

Author names and affiliations

(i) **Saravanan Sampoornam Pape Reddy**., **MDS, MPH, (PhD)**
Dept of Periodontology
Army Dental Centre (Research and Referral) Delhi
drsaravanan.sp@gmail.com Orcid ID – 0000-0003-1391-6125

(ii) **Delfin Lovelina Francis., MDS (Corresponding Author)**
Dept of Public Health Dentistry
Saveetha Dental College and Hospitals, SIMATS University
Chennai, Tamil Nadu
delfin_lovelina@yahoo.co.in Orcid ID – 0000-0002-3355-1322

(iii) **Prabhakaran Gopinathan**., B.Sc, MBA
Managing Director,
JP & GP Marketers and Consultants Pvt. Ltd.,
KP 8/934, Pranavam, Kochalummoodu, Chirayinkeezhu, Trivandrum.
pgprabakar@gmail.com Website: www.edinora.com
Orcid ID – 0000-0002-0315-0851

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Abstract

Introduction

There are many herbal products available for mouth rinsing, including Peppermint oil, known for its antiseptic properties, can freshen breath and kill bacteria in the mouth. Tea tree oils, another natural antiseptic, tea tree

oil can help fight oral infections and reduce inflammation. Sage is a common herb used for mouth rinses, sage has anti-inflammatory and antibacterial properties that can help soothe sore throats and freshen breath. Myrrhis often used in traditional medicine for oral health, myrrh can help fight bad breath, gum disease, and tooth decay. Another ingredient eucalyptus can help kill bacteria and freshen breath, making it a popular ingredient in many natural mouth rinses. It's important to note that while herbal products can be effective for oral health, it's always best to talk to a healthcare professional before using any new products.

Material and Methods

In adherence with protocols of in-vitro studies with the primary objective to study the in-vitro cytotoxicity and cell activity of a proprietary combination of ayurvedic medicine in (n=10) of test were compared with (n=10) control samples for one tailed hypothesis using level of confidence as 0.05 and power as 95%. The study used L929 and HaCaT cells for assessing half maximal inhibitory concentration (IC50 log and linear dose), cytotoxicity through MTT assay and cell migration through in-vitro scratch wound assay and the image analysis was done using Graphpad.

Results

The test sample which is a combination of proprietary ayurvedic components showed higher IC50 (28.19 mg/ml) than the control sample (7.13 mg/ml). In addition, there was a significant difference in in-vitro wound scratch assay with progressive cell migration and early wound closure with test samples.

Conclusion

The field of oral health as a whole need to start considering sustainable developmental ends as they relate to day-to-day work and start migrating for a greener economy. The public impact about the ecological footprint left by personal care products like dentrifrices in the form of organic products are now more acceptable and is completely secure. The results of this in-vitro study suggests that proprietary dentrifices provide a natural and environmentally favorable alternative to conventional toothpastes which are free of synthetic fragrances, preservatives, and artificial sweeteners. However, that not all organic dentifrices are created equal, and careful consideration should be given when selecting a product that suits the needs and preferences of the individual. Incorporating organic dentifrices into one's oral hygiene routine can contribute to a healthier, more environmentally conscious lifestyle.

Abbreviations

CRIS - Checklist for Reporting In-Vitro Studies

HMIC - Half-Maximal Inhibitory Concentration / IC-50 Log dose

ANOVA - Analysis of Variance

MEM - Minimum Essential Medium

MTT - 3-(4,5-dimethylthiazo1-2-y1)-2,5-diphenyltetrazolium bromide

FBS - Fetal Bovine Serum

OECD - Organization for Economic Cooperation and Development

OMOEC - Oral Mucosa Organ Equivalent Cultures

SEC - Skin Equivalent Cultures

NaF - Sodium Fluoride

ATP - Adenosine Triphosphate

SDS - Sodium dodecyl sulphate

CAPB - Cocamidopropyl betaine

SLS - Sodium lauryl sulphate

MIC - Minimum inhibition concentration

MBC - Minimum bactericidal concentration

EHOM - Engineered human oral mucosa

IL - Interleukin

TNF - Tumor Necrosis Factor

CFU - Colony Forming Unit

ECM - Extracellular matrix

IVIVE - In vitro–in vivo extrapolation

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Footnotes – Nil

Figure captions

Figure 1 – Dose response curve of L-929 cells exposed test / control in logarithmic dose range.

Figure 2 – Dose response curve of L-929 cells exposed test / control in linear dose rang

Figure 3 – Phase contrast microscopy of HaCaT cells in wound scratch assay with test / control

Figure 4 – Cell migration rate of HaCaT cells in-vitro scratch assay after 24 and 48 h.

Figure 5 – Percentage wound closure of scratch on HaCaT cells after 24 and 48 h.

Tables

Table 1 - Rate of cell migration and the percentage wound closure

Description - Mean rate of cell migration and the percentage wound closure in test and control groups after 24 and 48 h exposure.

Footnote – S.D. – Standard Deviation

Data statement – The data provided in this manuscript are true to the best knowledge of the authors.

Graphical abstract – Not provided

1.0 Introduction

Health is a state of complete well-being without discomfort or pain. The factors that influence an individual's health are both internal and external to his or her society and environment. [1] Oral health is a condition of the oral and associated tissues that allows a person to eat, speak, and socialize without active disease, discomfort, or embarrassment and contributes to overall health. [2] Oral cavity is a window into the overall health of a patient. It is the interface between the body and its environment. When proper oral hygiene is not practiced, oral health and overall health and quality of life suffer. Their relationship is multifaceted and intricate. Poor oral health has been linked to cardiovascular disease, osteoporosis, mental health, diabetes, and stroke, among others. Infections, caries, and periodontal disease may result from poor oral health. A greater number of missing teeth has been associated with a lower quality of life, and poor dentition influences dietary intake.[3] Oral hygiene practices may be mechanical, chemical, or a hybrid of the two. Mechanical oral hygiene procedures appear to be the most effective of all available oral hygiene measures, such as toothbrushes, dentifrices, and interdental aids. Toothbrushing in conjunction with dentifrices is currently the most reliable method for preventing and controlling plaque formation on an individual level.[4] Dentifrices are frequently used for both cosmetic and therapeutic purposes, and their regular use is the most effective method

for preventing and controlling oral diseases. Toothpaste is the most popular and widely accessible preventive oral health care product among dentifrices among the Indian population. Modern toothpastes, when used regularly, can help prevent dental caries and limit the regrowth of dental plaque and gingivitis, according to scientific literature.[5]

2.0 Material and Methods

The study was carried out in accordance with the protocols proposed for the development of CRIS (Checklist for Reporting In-Vitro Studies) guidelines.[6] The null hypothesis was that the proprietary ayurvedic medicine combinations are not effective in oral wound healing. The alternate hypothesis stated that combination of ayurvedic medicines can be potentially beneficial to oral wound healing and act as a natural dentrifice. The primary objective was to study the in-vitro cytotoxicity and cell activity of a proprietary combination of ayurvedic medicine with secondary objective to evaluate the potential role of this combination in wound healing using wound scratch assay. The total number (n=20) of test and control samples used were ten per group which was calculated with G*Power v.3.1 for one tailed hypothesis using level of confidence as 0.05 and power as 95%. Graphpad Prism v.8 software was used to calculate the IC50 log / linear dose. Since there was no contamination in any of the samples, there was no sample loss. During the cell culture experiments, there were two independent researchers who were blinded. The prepared samples were packed in identical tubes and randomized using a table of random numbers to avoid selection and confounding bias. The allocation concealment was conducted by a first blinded researcher regarding the sample and a second blinded researcher analyzing the experimental outcomes. The outcome measures included Half-Maximal Inhibitory Concentration (IC-50 Log dose) and *in-vitro* scratch assay. The statistical analysis was carried out using parametric tests; t-tests to compare the two matched groups and ANOVA (Analysis of Variance) to compare within the groups at different timelines, with the results tabulated. A p-value below 0.05 was deemed statistically significant. The test and control sample compositions are presented in the supplementary files.

2.1 Cell Culture – L929 Cells

L929 and HaCaT cells were cultured in serum Eagle's Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) and 3% Glutamine, 7.5% Sodium bicarbonate and 1% Penicillin-Streptomycin. Cells were kept at 37 °C, 5% CO2 and 90% humidity. Confluent cells were harvested with 0.25%Trypsin and 0.2% EDTA and seeded into 96-well multi-well plates for experiments. All procedures were performed aseptically and within a laminar air flow work station.

2.2 Cell activity/Cytotoxicity by MTT-assay

2.2.1 Preparation of test samples for cytotoxicity assay

The test samples were taken aseptically from the laminar flow bench and weighed with an analytical balance (Sartorius, QUINTIX). For in vitro analysis, the test and control samples were dissolved in culture medium. To obtain the stock concentration, the test and control samples were mixed with culture medium without serum at a concentration of 1 g/ml. To obtain dilutions of the test and control samples, the stock concentration was further diluted with complete culture medium containing serum. The Half-Maximal Inhibitory Concentration (HMIC) was calculated using the activity of cells exposed to test and control concentrations in log and linear concentrations. Cells cultured in normal medium served as the cell control.

The analysis dose was calculated using the 3-(4,5-dimethylthiazo1-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay. The ability of cells to reduce MTT was used to assess the cellular activity of L929 cells exposed to test or control sample. The viable and metabolically active cells convert soluble yellow MTT to

purple formazan crystals. For the test sample, no specific dose was known. As a result, the dose of the test samples was analyzed step by step. To begin the test, a logarithmic concentration range was created and then confirmed with dose at linear dilutions.

2.2.2 Half-Maximal Inhibitory Concentration - IC50 Log Dose

The stock concentrations of test and control samples were diluted with culture medium containing serum to obtain concentrations of test and control in the logarithmic range (l00, 10, 1, 0.l and 0.01 mg per 1 ml of medium). L929 cells were seeded at a density of $1x10^4$ cells/well in 96 well plates and allowed to reach sub-confluency. The culture medium was removed and 100 µL of test and control sample dilutions were added to each well (n=6). The cells were then incubated for 24 hours. After removing the test sample and culture medium from the cells, 50µL of MTT (1 mg/ml in culture medium without serum) medium was added to the wells. Cells were incubated in a CO_2 incubator for two hours. MTT medium was removed from each well, and l00µL of isopropanol was added to each well to dissolve the formazan product formed by cell activity.

Using a Multiwell Plate reader (Biotek, USA), the color developed was quantified by obtaining optical density (OD) at 570nm. The OD expressed by cells exposed to log concentrations of test sample extract was normalized and then analyzed for non-linear regression to determine inhibitor versus response (Figure 1). IC50 log dose was calculated using Graphpad Prism V8 software. The mean IC50 logarithmic dose range for the test and control was 18.73mg/ml and 2.03mg/ml respectively.

2.2.3 Half-Maximal Inhibitory Concentration—Linear Dose

By exposing cells to a linear dose range, the HMIC was confirmed. The dose for the linear dose range was calculated using the IC50 value from the log concentration. Test and control stock solutions were diluted with complete culture medium to yield concentrations of 50, 25, 12.5, and 6.25mg/ml. The OD expressed by cells exposed to linear concentrations of test sample extract was normalised and then analysed for non-linear regression to determine inhibitor versus response (Figure 2). The mean linear IC50 for test and control was 28.19 mg/ml and 7.131mg/ml respectively.

2.3 Cell migration through in vitro scratch assay

2.3.1 Selection of dose for wound healing assay

The analysis dose was calculated using the MTT assay. The HMIC was calculated using the cellular activity of L929 cells exposed to various concentrations of test and control samples. The IC50 values for test and control was 28.19 mg/ml and 7.131 mg/ml respectively. As a result, a much lower concentration of 3.12 mg/ml was used as the dose for the wound healing assay.

2.3.2 Preparation of test samples for Scratch Wound Assay

To obtain the stock concentration, the test samples and control samples were mixed with culture medium without serum at a concentration of 1g/ml. Membrane filtration was used to sterilize the test sample extract. The test sample stock solution was further diluted with complete culture medium containing serum to achieve a final concentration of 3.12 mg/ml. Cells cultured in normal medium served as the Assay Control, while cells cultured in serum-free medium served as the Negative Control.

2.3.3 In vitro scratch assay

HaCaT cells were used for in-vitro scratch assay. Trypsinized exponentially growing cells were seeded at a density of $2x10^4$ cells per well in a 24-well plate. The cells were incubated for 24 hours to achieve 80% confluence. Using a 200 µL pipette tip, scratch wounds were made in a straight line on the cell monolayer.

Cellular debris were removed by rinsing three times with Phosphate Buffer Saline (PBS). In triplicate, test / control medium containing 3.12mg/ml of of the samples were added. Parallel to the test system, the assay control and negative control were also run. Cells were examined immediately after scratching and images were captured at a magnification of l0X. The cells were incubated in a CO^2 incubator for 24 to 48 hours. The wound closure was calculated using Image J Software and images taken at 24 and 48 hours. The width of the remaining wound and area after 24 and 48 hours were used to calculate the rate of cell migration. The percentage of wound closure was calculated using the wound's remaining area at 24 and 48 hours. The rate of cell migration was calculated using the formula;

$$\text{Rate of cell migration} = \frac{W_i - Wf}{t}$$

where W_i is the initial wound width, *Wf* is the final wound width and t is the time in hours.

The percentage of wound closure was calculated as follows;

$$\text{Wound closure \%} = \left[\frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=0h}}\right] \times 100$$

where *At = 0h* is the initial wound area and *At=Ah* is the final wound area.

Observations with an inverted phase-contrast light microscope revealed that HaCaT cells treated with 3.12 mg/ml of test/control samples had healthy morphology, progressive cell migration, and wound closure (Figure 3).

The rate of cell migration and the percentage of wound closure calculated by Image J analysis are shown in Table 1. The assay control representing cells treated with serum-containing medium showed progressive cell migration and wound closure, and the negative control with cells in serum-free medium showed poor cell migration and wound closure as anticipated. (Figure 4). The rate of cell migration (µm/h) and percentage of wound closure at which cells migrated when exposed to test sample at 3.15 mg/ml concentration was statistically significantly higher than the controls indicating good cell migration ($p< 0.05$). The serum-starved negative control showed a lower cell migration compared to assay control. The sample control at a similar concentration showed a lesser migration rate compared to the test.

2.3.4 Image Analysis

The rate of wound closure was similar in image analysis of wound closure on HaCaT monolayer to various samples (Figure 5). The test samples wound closure was comparable to control samples. The negative control with no serum had very little wound coverage, whereas the assay control had the highest percentage of wound closure.

3.0 Discussion

Ayurvedic medicine offers a holistic approach to healthcare that has been utilized for centuries in India. Recently, there has been an increase in interest in the use of Ayurvedic oral hygiene products as an alternative to conventional oral care. Using in-vitro analysis, this paper will evaluate the cytotoxicity and wound healing potential of a selection of Ayurvedic oral hygiene products. Through this analysis, we hope to determine if these products can provide alternatives for comprehensive oral care that are both effective and safe. The first activities in alternative testing can be traced back to the "Three Rs (3R principle, i.e. replacement, reduction, refinement)," which served as the foundation for William Russell and Rex Burch's 1958 book The Principles of Humane Experimental Technique. [1]

The 3Rs principle advocates the search for animal replacement with non-living models, reduction in animal use, and refinement of animal use practices. As an example, the Draize in vivo rabbit eye and skin irritation assay could be used to test chemicals or cosmetic products. The Draize test was developed in 1940 and is now used by the Organization for Economic Cooperation and Development (OECD). To replace this test, several alternative methods have been developed over the years. As a result, these experiments were limited to cell culture and cell monolayers.[2,3,4]

To that end, oral mucosa organ equivalent cultures (OMOEC) and skin equivalent cultures (SEC) derived from human tissue were tested for their reactivity to dentifrices with varying degrees of irritation potential. Histopathology, cell viability (MTT assay), and cytotoxicity were used to evaluate the responses of OMOEC and SEC to test dentifrices. In response to the test dentifrices, cell viability in OMOEC and SEC was reduced in a dose- and time-dependent manner. These findings in an in vitro model of oral mucosa irritation allow for direct comparison of in vitro responses with those obtained in an in vivo model, laying the groundwork for a tiered approach to assessing the irritation potential of oral care products.[3] To test the irritation of oral-care products and to provide systems for studying the pathology of the oral cavity, three-dimensional models of human oral epithelia have been developed. Using normal oral epithelial cells and serum-free medium, in-vitro tissue models acquire a buccal or gingival phenotype. The use of the MTT assay and the release of cytokines clearly distinguished the formulations from the oral care products. It was determined that the oral tissue models represent a highly reproducible, non-animal method for evaluating the irritation potential of newly developed oral care products, and that they should be useful for studying the innate immunity, biology, and pathology of the oral mucosa.[5] It was discovered that contradictory data in the literature for Sodium Fluoride (NaF) induced aberrations in-vitro, and that cell-cycle kinetic studies indicated that aberrations were induced in cells exposed to NaF, with the greatest increases in aberrant cells occurring in cells exposed at metaphases. Experiments confirmed the sensitivity of G 2 cells to the induction of aberrations by NaF.[7]

Plant extracts, phenolic compounds, and metal salts are among the more recent agents that have been successfully incorporated into dentifrices despite their adverse effects. Currently, numerous products contain the phenolic compounds Triclosan and Bis-biguanide, Chlorhexidine. Triclosan has antifungal and antibacterial activity against yeasts and oral bacteria. Triclosan has been combined with a co-polymer or another compatible antimicrobial agent, zinc citrate, to increase its clinical efficacy. The co-polymer enhances the oral retention of Triclosan, resulting in additional reductions of salivary bacterial counts in vivo. In addition to possessing antimicrobial properties, zinc salts can inhibit glycolysis and bacterial proteases at low concentrations. A zinc citrate/Triclosan dentifrice reduced plaque accumulation and gingivitis in an experimental study of human gingivitis; the ratio of anaerobic to aerobic bacteria and the proportions of Actinomyces species in plaque were also reduced. The prolonged use of a zinc citrate/Triclosan toothpaste did not significantly alter the ecology of supragingival plaque, nor did it result in the selection of Triclosan-resistant bacteria.[8] In vitro biofilm models (Hydroxyapatite Disc Biofilm Models), developed supra-gingival plaques (Modified Drip-flow Biofilm Reactors), and multiple devices were used to compare the antimicrobial effects of a triclosan-containing dentifrice with a stannous fluoride and zinc lactate combination. This multi-model approach to determining the efficacy and specificity of dentifrices against compositionally distinct plaques revealed that both formations reduced bacterial viability and plaque accumulation, and their effects could be differentiated in nascent and deep plaques, with the triclosan dentifrice causing greater viability reductions.[9]

The ability of Sodium dodecyl sulphate (SDS), a major component of modern dentifrices, to completely lyse cells may have repercussions when this detergent is used for oral hygiene. Even at low concentrations, SDS diminished the protective barrier function of the oral epithelium. In cases of gingival or periodontal inflammation, it is well known that the concentration of neutrophils in gingival crevicular fluid is elevated. If neutrophils are lysed by SDS from dentrifice, their collagenase, elastase, and cathepsin G contents will be released. Recent research has demonstrated that collagen fibrils exposed to SDS are susceptible to non-specific proteolytic cleavage. Even some microbial proteases are enhanced by SDS.[10] In a double-blind cross-over study, the effect of seven dentrifice formulations that differed only in detergent concentration was evaluated. 78% of participants experienced visible oral epithelial desquamation during the test periods. All reactions were linked to toothpastes containing detergents. SLS and Cocamidopropyl betaine (CAPB)-containing dentifrices were linked to 42 and 3 desquamative reactions, respectively, out of 45 desquamative reactions. Since the detergent-free paste did not result in desquamation, it seems likely that the detergents caused these reactions.[11] Using human gingival epithelial cells and fibroblasts, the in-vitro cytotoxicity of triclosan and SLS alone and in combination was determined. For gingival epithelial cells, the 24-h midpoint cytotoxicity values were 0.052 mM triclosan and 0.0075% SLS, while for GF fibroblasts, the respective values were 0.09 mM triclosan and 0.0127% SLS. Co-exposure of epithelial cells and fibroblasts to triclosan and SLS resulted in a synergistic increase in cytotoxicity.[12] Another study concluded that concentrations of SLS have dual effects on reconstituted human oral mucosa; although some cell death was observed within the epithelium, the increased epithelial thickness, proliferation, and E-cadherin expression induced at lower concentrations may be associated with a protective mucosal response, whereas at higher concentrations a more destructive type of reaction predominated.[13] SLS is a surfactant that is used to lower the surface tension of water. Most commercially available dentifrices contain 0.5-2.0% SLS, which inhibited human gingival fibroblasts in a dose- and time-dependent manner; and thereby suggested using SLS-free dentifrices to avoid the potential delay in wound healing.[14]

The effect of NaF concentrations on human oral mucosal fibroblasts was investigated. NaF was cytotoxic to oral mucosal fibroblasts at concentrations of 4 mmol/L or higher, according to the results. In a dose-dependent manner, exposure of cells to NaF for 2h inhibited protein synthesis, cellular adenosine triphosphate (ATP) level, and functional mitochondrial activities. On the cellular ATP level, the IC50 of NaF was approximately 5.75 mmol/L. After 2 hours of incubation at 4 mmol/L, 8 mmol/L, and 12 mmol/L, NaF inhibited 31%, 56%, and 57%, respectively, of mitochondrial functions. NaF was not inhibited significantly at concentrations below 2 mmol/L. (40 ppm). These findings suggest that NaF can be toxic to oral mucosal fibroblasts in vitro by inhibiting protein synthesis, mitochondrial function, and ATP depletion.[14]

Using normal human palatal fibroblasts and epithelial cells, three-dimensional engineered human oral mucosa (EHOM) was created. They were treated with AquafreshA or CrestA for 1, 4, 8, and 24 h, or left untreated, and then their viability and morphology were evaluated. At 24 hours after contact, the desquamation was substantial but restricted to the uppermost layers of the treated tissues. There was no increase in cell death in these tissues, indicating that the dentifrice accelerated desquamation of layers containing differentiated cells. Dentifrices upregulated Interleukin (IL)-1 while downregulating IL-8 and Tumor Necrosis Factor TNF- secretion, indicating a disruption in the cascade of inflammatory responses as measured by cytokine levels. As suggested by the upregulation of gelatinase activities, these dentifrices may also impair normal repair mechanisms.[15] Multiple human studies have demonstrated that xylitol products

significantly reduce the incidence of dental caries. A recent study revealed that the amount of Colony Forming Units (CFU) of Streptococci Mutans after tooth brushing with xylitol-containing toothpaste is less than that of toothpaste without xylitol.[16]

In culture medium, samples of whitening toothpastes (Colgate Whitening (CW) and Oral-B Whitening (OBW)) and common (non-whitening) toothpastes (Colgate and Oral-B) were extracted for an in-vitro study evaluating the cytotoxicity and genotoxicity of whitening and common toothpastes. Human gingival fibroblasts were exposed to various dilutions of culture media that had been previously exposed to such substances. Using the micronucleus formation assay, genotoxicity was evaluated. Colgate was significantly less cytotoxic than CW, Oral-B, and OBW across all dilutions (p 0.01), while CW was the most cytotoxic toothpaste (p 0.01). Compared to the untreated control, the number of micronuclei was highest in the whitening toothpastes (p 0.01). Comparatively to UC, Colgate and Oral-B toothpastes were not genotoxic (P= 0.326). The whitening toothpastes and Oral-B were more cytotoxic and genotoxic to cells in vitro than the common toothpastes, with the OBW toothpaste exhibiting the most pronounced genotoxicity. [16] In an intriguing study, only one of seven tested toothpastes exhibited the most desirable characteristics for the world's ageing population, including low cytotoxicity and low abrasiveness, demonstrating the need for a safer alternative.[17]

Herbal products were researched for incorporation into dentrifrices in light of the wide range of adverse effects caused by allopathic compounds. In vitro examination of the effect of Salvadora persica in toothpaste on the inhibition of bacterial growth determined the inhibition zone, minimum inhibition concentration (MIC), and minimum bactericidal concentration (MBC). The tested microorganisms consisted of six strains of Streptococcus mutans isolated from Jakarta, Indonesia schoolchildren. They concluded that Salvadora persica in toothpaste has antimicrobial activity against schoolchildren-isolated local strains of Streptococcus mutans. Consequently, it may possess anti-caries properties.[18] In a clinical study, the commercially available bleaching effect paste (Oral B) had the highest frequency of irritation reports, tooth sensitivity, taste discomfort, and texture discomfort. It had the lowest pH, the highest abrasivity, and the lowest cell viability ($p<0.01$), causing adverse reactions in patients. The properties of toothpaste should be well known for patient indication, thereby minimising discomfort reports.[19] The cytotoxicity of Systemp Desensitizer, Aqua-Prep F, Isodan, and Gluma to human gingival and pulpal fibroblast cells did not decrease with dilution, and these products were found to be more cytotoxic than the other products ($p<0.05$).[20] In an experiment, 16 different commercial toothpastes were prepared to see how they affected oral epithelial cells and HeLa cells. The toxicity of these materials increased significantly as exposure time with toothpaste increased ($p<0.005$).[21] The antimicrobial efficacy of fluoridated and herbal dentifrices with Meswak was compared. Salivary bacterial counts decreased steadily with herbal dentifrices containing Aloe vera and Meswak, which can be safely recommended as an antimicrobial-effective alternative to fluoridated dentifrices.[22] The cytotoxicity was concentration and time dependent; as the concentration of the materials increased, so did their cytotoxicity over time. More than 90% cytotoxicity were exhibited by SLS. For human gingival fibroblasts, the cytotoxicity of NaF varied from 25% to 70%, while the cytotoxicity of all concentrations of zinc lactate and sodium benzoate was 50%. The study suggested that these compounds should be replaced with alternatives in commercially available toothpastes.[23]

Increasingly, compounds of natural origin are used as dental hygiene aids. We evaluated the antibacterial activity of dentifrices containing natural active ingredients against oral bacteria in multiple test systems using four distinct methods. Corsodyl Daily (CD), Kingfisher Mint (KM), and Parodontax fluoride (PF) were compared to Colgate Cavity Protection (CCP) and triclosan-containing toothpaste (Colgate Total [CT]). KM caused the greatest reductions in viability in simulated supragingival plaques, while CT caused the greatest reductions overall. CD caused moderate reductions following single exposures, while PF had no effect. The DNA profiles of eubacteria were not significantly altered by any herbal formulation in dense plaques, but KM significantly decreased the number of viable streptococci. While both non-herbal comparators exhibited antibacterial activity, the formulation containing triclosan caused greater reductions in bacterial viability than the herbal and non-herbal formulations.[24]

A study evaluating the antimicrobial activity of an experimental dentifrice containing Ricinus Communis for denture hygiene against Staphylococcus aureus, Escherichia coli, Streptococcus mutans, Enterococcus faecalis, Candida albicans, and Candida glabrata. The MIC test was conducted with R. communis in 2.5% pure oil. R. communis did not inhibit E. coli, but the minimum inhibitory concentration (MIC) (0.0781%) was effective against the other microorganisms. For comparative purposes, the commercial toothpastes Colgate, Trihydral, and Corega Brite were evaluated. The commercial and experimental toothpastes were both ineffective against E. coli. The experimental toothpastes containing R. communis at concentrations of 2, 5, and 10% were effective against S. mutans, S. aureaus, and E. faecallis. Comparing the experimental dentifrices, the product containing 10% R. communis produced the largest zones of bacterial growth inhibition and had comparable antimicrobial activity to commercial dentifrices, with the exception of S. aureus.[25] The gingival inflammation reducing efficacy of a miswak extract-containing dentrifice (Salvadora Persica) was compared to that of a herbal and a conventional dentrifice. The approximal plaque index decreased significantly in all groups, with no significant differences between them. The miswak extract-containing toothpaste had the same effect as the herbal toothpaste and can be used safely for domestic oral hygiene in gingivitis patients, which is comparable to the findings of the current study. The rosemary extract-containing dentrifice was able to inhibit the growth of S. mutans, Streptococcus Oralis and Lactobacillus Rhamnosus demonstrating antimicrobial activity comparable to that of commercially available toothpastes for inhibition of S. mutans and S. oralis.[26] Aloe vera is renowned for its remarkable medicinal qualities. These plants are one of the most abundant natural sources of health for humans. Aloe vera is an ancient, natural substance that would be hailed as a significant scientific breakthrough if it emerged from a modern pharmaceutical lab. It has been utilised in dentistry for its effect on wound healing, gingivitis, plaque control, and the treatment of oral mucosal lesions. It has potent antiviral effects both indirectly and directly due to stimulation of the immune system and aloe emodin, respectively. Aloe vera contains glutathione peroxide activity, superoxide dismutase enzymes, and a phenolic antioxidant, which may be responsible for its antioxidant and antitumor effects. The same has now been incorporated into oral formulations alongside dentifrices.[27] Numerous plants and herbs possess potent antimicrobial activity against a wide range of microorganisms. They presumably act against and modulate the factors that are essential for microbial survival or activity and have no side effects. Mango branches, eucalyptus branches, pudina leaves, and garlic bulbs were examined for their antibacterial properties. At comparable concentrations, test compounds derived from mango and eucalyptus twigs exhibited significant antibacterial effects, whereas Pudina and garlic extracts exhibited no significant antibacterial effects.[28]

Tocosh is a potato that has undergone a process of hydraulic oxidation that enhances its antimicrobial properties. It was evaluated in order to determine the antibacterial and cytotoxic activity of a new experimental dentrifice derived from Solanum tuberosum. Solanum tuberosum was not cytotoxic because it had only one CC50 at 0.26927 mg/mL and 0.26845 mg/mL, respectively, for the cell lines 3T3 and DU145. Tocosh toothpaste has an antibacterial effect against S. aureus and S. mutans, making it a new ecological dentifrice that is non-cytotoxic because it does not alter the viability of cells.[29] According to the agar well diffusion method, the antimicrobial efficacy of dentifrices is directly proportional to the size of the obtained zone of inhibition. Among the herbal combinations, dentifrices containing akarkara, neem, babool, tomar, pudina, lavang, pippli, vajradanti, bakul, vidang, haldi, pilu, majuphal, and meswak exhibited the greatest zone of inhibition. Herbal dentifrices are more effective than chemical dentifrices, have no adverse side effects, and have potential therapeutic effects on a variety of oral lesions, according to the findings of this study which was similar to the results of the present study.[30] The evaluation of antimicrobial efficacy of Parodontax dentifrice revealed antimicrobial activity at all dilutions ($p < 0.05$) demonstrating a bactericidal effect at all concentrations of the extract. Pure forms of propolis exhibited antimicrobial activity against S. mutans, but not as much as a dentrifice containing herbal extracts and sodium bicarbonate which also supports the results of the current experimental in-vitro study.[31]

Cell culture systems are indispensable to a vast array of biomedical and clinical investigations. Two-dimensional cell culture models (2D) provide fundamental information on the cytotoxicity, penetration, and accumulation of drugs in cells and are of the utmost importance when selecting new compounds with the desired biopharmaceutical properties as potential candidates for novel drugs. Three-dimensional (3D) tissue models that more accurately mimic in vivo conditions and the functions of living tissue are an improvement over 2D growing cells. These models reduce the cost of drug development, make drug screening more efficient, reduce the failure rate in medicine discovery, and eliminate the use of animals in experiments. Hence in this study, animal models were not used and cell culture experiments were preferred. The development of 3D organoid techniques led to the creation of models with highly complex organotypic cytoarchitectures, including cell–cell and cell–extracellular matrix (ECM) interactions.[32] Organoids enable not only molecular but also functional analysis, enhancing the clinical and toxicological relevance of the method. However, as complexity increases, batch-to-batch variability, reproducibility, and testing throughput decrease. In addition, organoid culture is technically difficult and more costly. Additionally, advanced pharmaco- and toxicokinetic, including computational modelling are required to predict internal exposure as well as in vitro kinetics combining both by IVIVE (in vitro–in vivo extrapolation).[33] Although it is known that the predictability of animal studies is limited, the one-year experience provides a sense of security in contrast to the use of alternative approaches, which are viewed as carrying greater uncertainty due to their novelty.

In an effort of reviewing ‘Green Dentistry’ it was concluded that, 46 organic toothpastes with a total of 156 ingredients, of which 89.1% were considered active and 10.9% were considered inactive. Twenty-two percent of the components had previously documented adverse effects which confirmed previous findings that organic toothpastes are safe for use in the mouth. By choosing products with caution, patients can reduce their exposure to chemicals like polymers (like polyethylene glycol) and carbomers, detergent agents (like sodium lauryl sulfate), and triclosan.[34] Inadequate clinical evidence prevents making recommendations for everyday use to consumers based on efficacy and biocompatibility in addition to the lack of proper CRIS guideline based reporting of in-vitro studies on such materials.

4.0 Conclusion

The test samples showed an IC50 value much higher (Mean = 28.19 mg/ml) than the Controls (Mean = 7.131 mg/ml). The in vitro scratch assay suggested that the HaCaT cells treated with test samples exhibit healthy morphology and progressive cell migration and wound closure as a function of time. The cells treated with serum-containing medium showed progressive cell migration and wound closure, whereas, the cells treated with serum-free medium showed poor cell migration and wound closure as anticipated. Since the present study was intended to reduce the exposure of animal experimentation and yielded optimally better results in terms of biocompatibility and cell cytotoxicity, the clinical extrapolations can be further performed using a properly designed randomized controlled clinical trials with a longitudinal follow up in future.

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Figure captions

Figure 1 – Dose response curve of L-929 cells exposed test / control in logarithmic dose range.

Figure 2 – Dose response curve of L-929 cells exposed test / control in linear dose rang

Figure 3 – Phase contrast microscopy of HaCaT cells in wound scratch assay with test / control

Figure 4 – Cell migration rate of HaCaT cells in-vitro scratch assay after 24 and 48 h.

Figure 5 – Percentage wound closure of scratch on HaCaT cells after 24 and 48 h.

Tables

Table 1 - Rate of cell migration and the percentage wound closure

Description - Mean Rate of cell migration and the percentage wound closure in test and control groups after 24 and 48 h exposure.

Footnote – S.D. – Standard Deviation

Appendices – Nil

Footnotes

L929 cells (ATCC USA), HaCaT cells (NCCS, Pune)

MTT (3-(4, 5-dimethylthiazo1- 2-y1)-2, 5-diphenyltetrazolium bromide, Cat No. M5655 Sigma)

Minimum Essential Medium (M0759, Sigma)

Foetal Bovine Serum (FBS, Cat No10270-106, Thermo Fischer)

Glutamine (Cat No. G7513), Phosphate Buffered Saline (PBST900, TaKaRa)

Trypsin EDTA 0.25% (T4049, Sigma)

96 well, 24 well plates, Cell culture flask (T25cm^2) (Nunc, Thermofischer)

CO2 incubator (Thermo, Japan),

Biosafety cabinet Classll (Esco Class II Singapore)

Water bath (Julabo, Germany)

Analytical Balance (Sartorius, Germany)

Shaker Incubator (Orbitek, India)

Mutliwell plate reader (Biotek,USA)

Inverted phase contrast microscope (Motic USA)

Supplementary Data

Composition of test sample (Edinora™ Dentrifice)

1. Miswak (Salvadora Persica)
2. Licorice – Glycyrrhiza Glabra
3. Chebulic Myrobalan
4. Beleric Myrobalan
5. Dry ginger – Zingiber Officinale
6. Honey
7. Virgin coconut oil
8. Aloe Vera Juice
9. Black Pepper
10. Clove Bud
11. Cardamom
12. Cinnamon
13. Eucalyptus Oil
14. Peppermint
15. Rosemary Officinalis
16. Neem
17. Turmeric

Composition of Control Sample (Commercial Sample)

1. Glycerin
2. Aqua
3. Hydrated Silica
4. Sodium Lauryl Sulfate
5. Arginine
6. Aroma
7. Cellulose Gum
8. Zinc Oxide
9. Poloxamer 407
10. Zinc Citrate
11. Tetrasodium Pyrophosphate
12. Xanthan Gum
13. Benzyl Alcohol
14. Cocamidopropyl Betaine
15. Sodium Fluoride
16. Sodium Saccharin
17. Phosphoric Acid
18. Sucralose
19. CI 77891

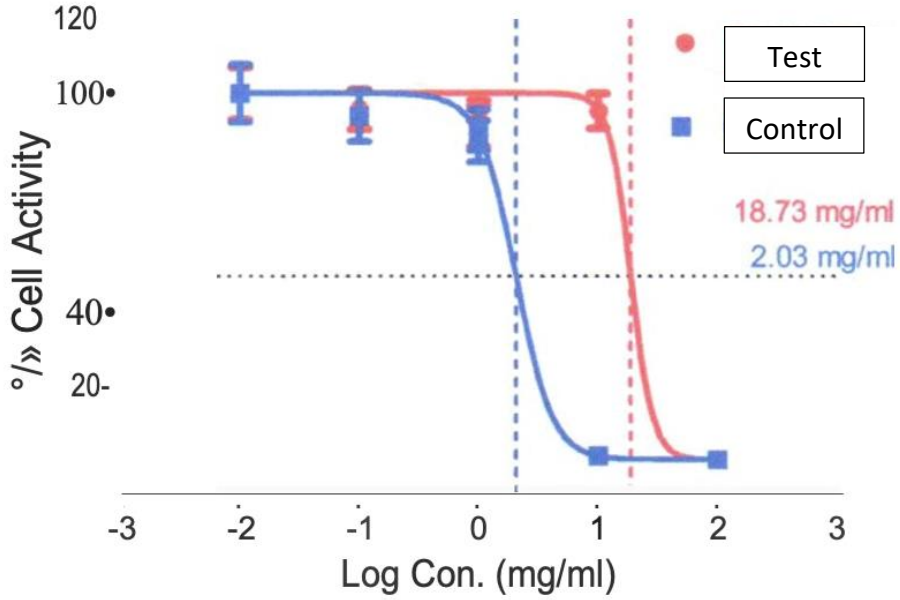

Figure 1 – Dose response curve of L-929 cells exposed test / control in logarithmic dose range.

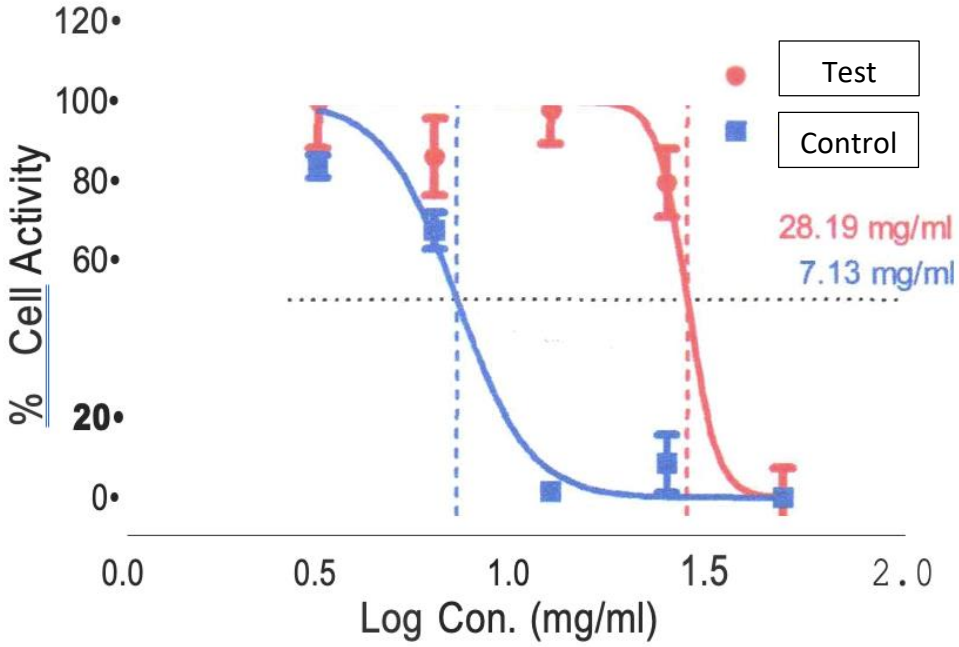

Figure 2 - Dose response curve of L-929 cells exposed test / control in linear dose range.

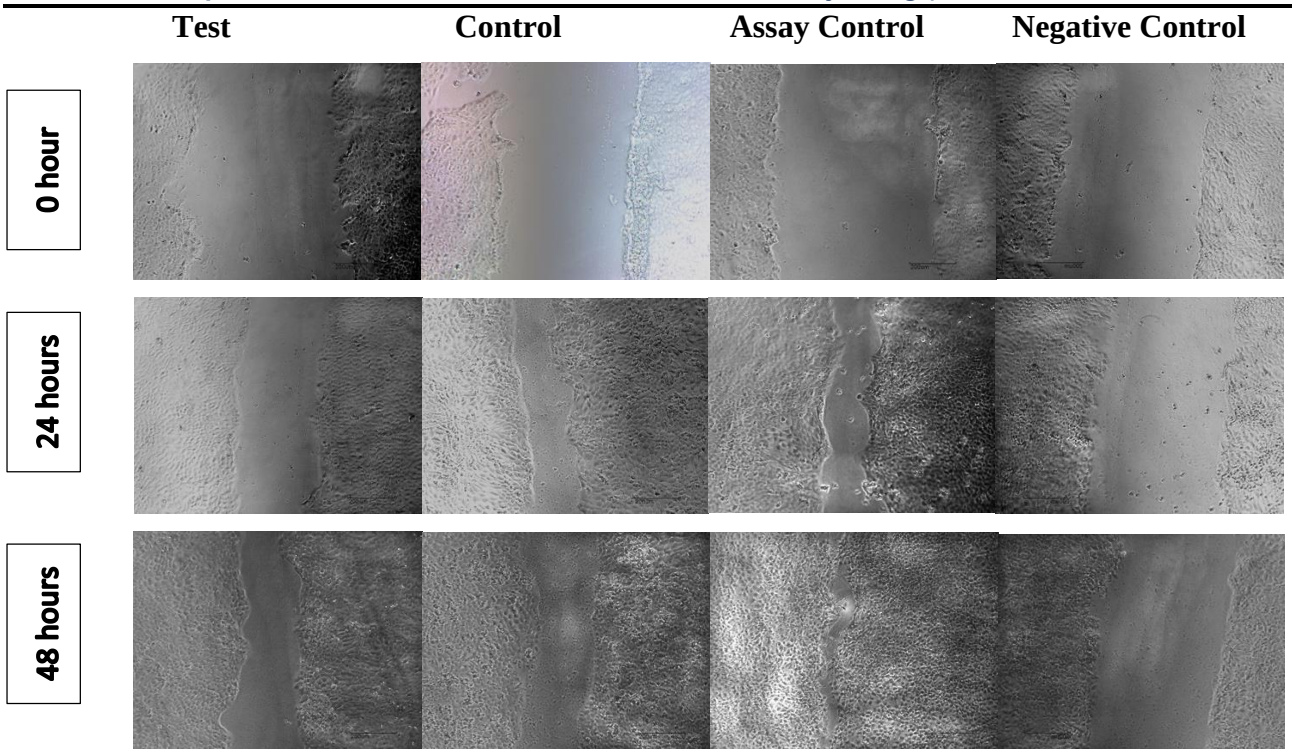

Figure 3 – Phase contrast microscopy of HaCaT cells in wound scratch assay with test / control

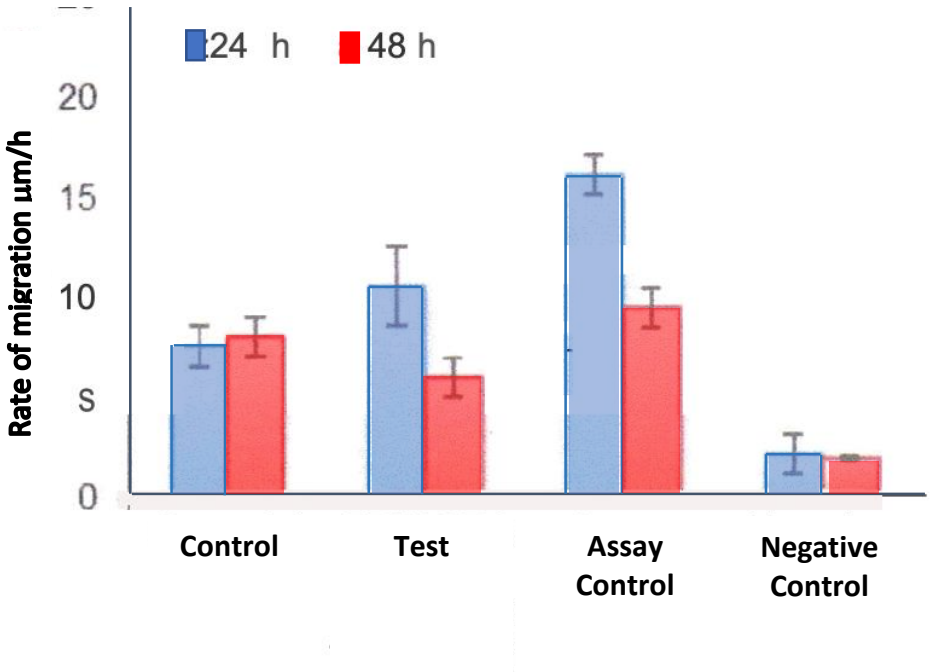

Figure 4 - Cell migration rate of HaCaT cells of test / control in-vitro scratch assay after 24 and 48 h.

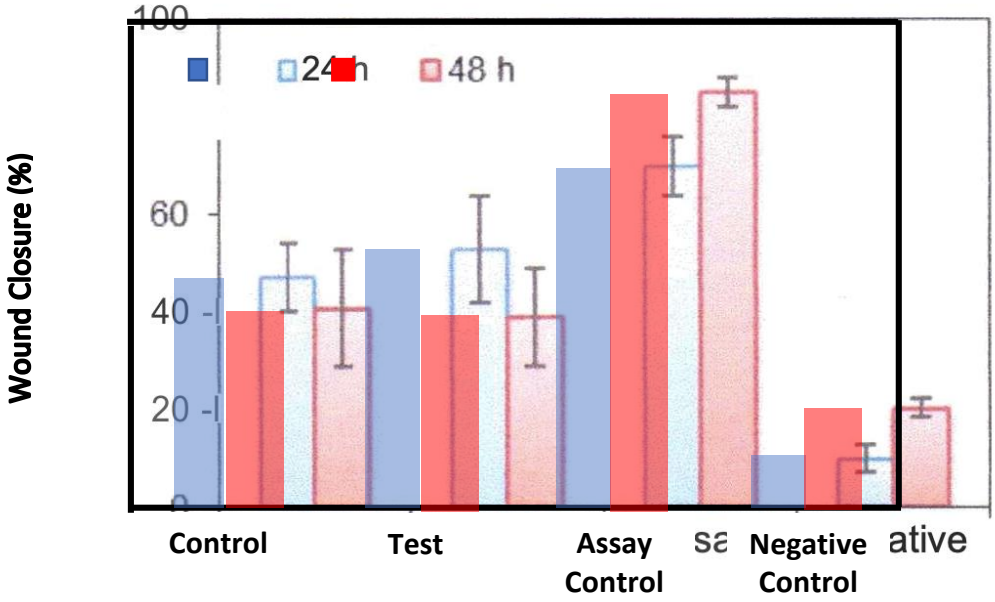

Figure 5 - Percentage wound closure of scratch on HaCaT cells of test / control after 24 and 48 h.

Tables

Samples	Rate of cell migration (pin/h)		Wound closure (%)	
	24 h	48 h	24 h	48 h
Control	7.6±1	8.1±1	47.l±7	40.70±12
Test	10.6±2	6.0±l	52.91±11	39.0±10
Assay Control	16.16±1	9.53±0.97	69.9±6	85.1±3
Negative Control	2.2±1	2.0±0.1	9.9±3	20.25±2

Table 1 - Rate of cell migration and the percentage wound closure

Description - Mean ± S.D. of rate of cell migration and the percentage wound closure in test and control groups after 24 and 48 h exposure.

Footnote- S.D. – Standard Deviation