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Evaluation of Biological Response Elicited by Two Novel Tooth Cream Formulations of *Cocos nucifera*- Cell Line Studies and MTT Assay on Human Gingival Fibroblast

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Abstract

The current research endeavors to assess the biological reactions induced by two newly formulated tooth creams from Cocos nucifera. Freshly harvested coconut from the Tirupur district of Tamil Nadu underwent processing to create two distinct formulations: one utilizing coconut milk and the other lyophilized coconut extract. Tooth cream samples were prepared and tested against commercial tooth cream following ISO 10993-5 recommendations. Human gingival fibroblast cells were isolated and cultured according to approved protocols, and cytotoxicity evaluations were conducted through MTT assay and live/dead staining. Results indicated high cell viability in both coconut-based formulations, comparable to the commercial tooth cream. Live/dead staining revealed predominantly live cells with minimal cytotoxic effects. Novel coconut-derived tooth creams exhibit comparable biocompatibility to commercial formulations, showing high cell viability and minimal cytotoxicity with human gingival fibroblasts. This suggests coconut-based tooth creams as safe alternatives for oral care, advocating further investigation into their efficacy and tissue safety. Overall, our findings endorse natural compound utilization in dental care formulations.

Keywords: *Biocompatibility, Coconut-Derived, Dental Care, Gingival Fibroblasts, Tooth Creams.*

Introduction

Biocompatibility testing remains paramount, even when sourcing materials from nature for medical and dental applications [1]. While natural resources offer promising therapeutic potential, their intrinsic properties must undergo rigorous evaluation to ensure compatibility with the human body [2]. This scrutiny is essential to safeguard against adverse reactions and ensure patient safety in the utilization of natural remedies within healthcare practices [3–5].

The initial stage in verifying the biocompatibility of any medical substance is conducting cytotoxicity testing [6]. Through in vitro exposure to tissue cells and subsequent observation of the resultant consequences, cytotoxicity testing aims to ascertain the toxicity of medical substances and the chief constituent elements that make them. The human body has several defense mechanisms in place to shield cells against pH abnormalities and cytotoxins. Initially, biomaterials undergo in vitro laboratory testing to identify their physical and mechanical properties in a laboratory setting. Once proven to have beneficial effects, it is essential to test their effect on biological tissues. For these, the examinations are conducted in vitro using grown

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mammalian cells, such as those seen in mice. Recent advances in this field have led to biocompatibility testing on human gingival fibroblast cells. Future directions for tissue engineering and organs-on-a-chip methods in biological research on dental materials [7, 8].

White spot lesions (WSLs) are a common occurrence during orthodontic treatment, often resulting from plaque accumulation around brackets and wires. These lesions represent areas of demineralization on the enamel surface, caused by the acidic byproducts of bacterial metabolism. Prevention strategies include meticulous oral hygiene practices, fluoride application, and the use of remineralizing agents [9, 10]. Early detection and intervention are crucial to prevent the progression of WSLs into cavities, ensuring optimal oral health outcomes for orthodontic patients [9, 11, 12].

Natural resources such as fluoride, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) derived from milk, and hydroxyapatite have been explored for their ability to control white spot lesions by promoting enamel remineralization [13]. These substances have shown promising results in clinical studies, demonstrating their potential as natural remedies for managing the early stages of dental demineralization.

Cocos nucifera, or coconuts, grow on palms and begin fruiting after approximately three years. The coconut comprises six layers: exocarp, endocarp, mesocarp, testa, kernel (coconut meat), and water, all of which are edible and rich in protein and minerals. In the food industry, extracting oil from overly ripe coconut kernels is a significant use of coconuts. Furthermore, lyophilized extracts, fractions, and ethyl acetate extracts of *C. nucifera* fibre are particularly abundant in polyphenols, including tannins, flavonoids, epicatechins, and catechins [14, 15]. It not only holds a prominent place in various cuisines but also exhibits significant potential as a natural remedy for various health issues. Specifically, coconut has demonstrated remarkable capabilities in promoting remineralization, particularly in dental applications [16]. In simpler terms, mature coconut water contains approximately 15.19±0.03 mg of calcium per 100 mL, while mature coconut kernels contain around 14 mg of calcium per 100 grams [17]. Indeed, given its rich calcium content and potential remineralization properties, coconut holds promise for dental applications, potentially aiding in the restoration of demineralized tooth enamel and reinforcing its resilience against decay and erosion.

In our prior investigation, we observed that both coconut milk extract and lyophilized coconut extract demonstrated significant potential for enamel remineralization. [18]. Building upon the favourable outcomes of coconut pulp extracts, two tooth cream formulations were developed utilizing these extracts. This decision stems from their observed effectiveness in promoting enamel remineralization, suggesting their potential as active ingredients in dental care products aimed at improving oral health. The current research endeavours to assess the biological reactions induced by two newly formulated tooth creams from Cocos nucifera. This evaluation will be conducted through MTT assay and live/dead assay techniques, utilizing human gingival fibroblasts as the experimental model.

Materials and Methods

Freshly harvested Cocos nucifera from the Tirupur district of Tamil Nadu was utilized to create a novel toothpaste formulation. The mature coconut was processed by grating and splitting the kernel into halves. One half was used to prepare coconut milk, while the other underwent lyophilization. Subsequently, the coconut milk and lyophilized coconut extract were separately incorporated with other toothpaste ingredients to

develop two distinct novel toothpaste formulations.

Sample Preparation

Tooth cream samples were prepared in the laboratory and subjected to comparative testing against a commonly used commercial tooth cream. Following ISO 10993-5 recommendations, eluates of these materials were prepared. Each tooth cream (0.2 g) was mixed with 1 mL of DMEM culture medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA, USA), centrifuged at 4200 rpm, and the supernatant was collected and filtered. This conditioned medium underwent sterilization via exposure to ultraviolet light for two hours. Subsequent experiments utilized samples at varying concentrations: undiluted (1:1), half-diluted (1:2), and quarter-diluted (1:4).

Isolation and Culture of Human Gingival Fibroblasts (Figure 1).

The study protocol received approval from the Institutional Ethics Committee of Saveetha Dental College and hospitals, with approval ID SRB/SDC/PhD/ORTHO-2007/23/049. Human gingival fibroblast cells, previously collected from patients aged 18–25 undergoing tooth extractions, were obtained from the research laboratory. Isolation involves enzymatic digestion using collagenase (900 u/mL) and dispase (400 u/mL) at 37 ℃ for 1 hour. Primary cultures of HGF were established using RPMI 1640 supplemented with 20% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 ℃ with 5% CO2. Medium was changed every three days, and cells were subcultured at 80% confluence. Cells in passage 2 were utilized for all in vitro experiments in this study.

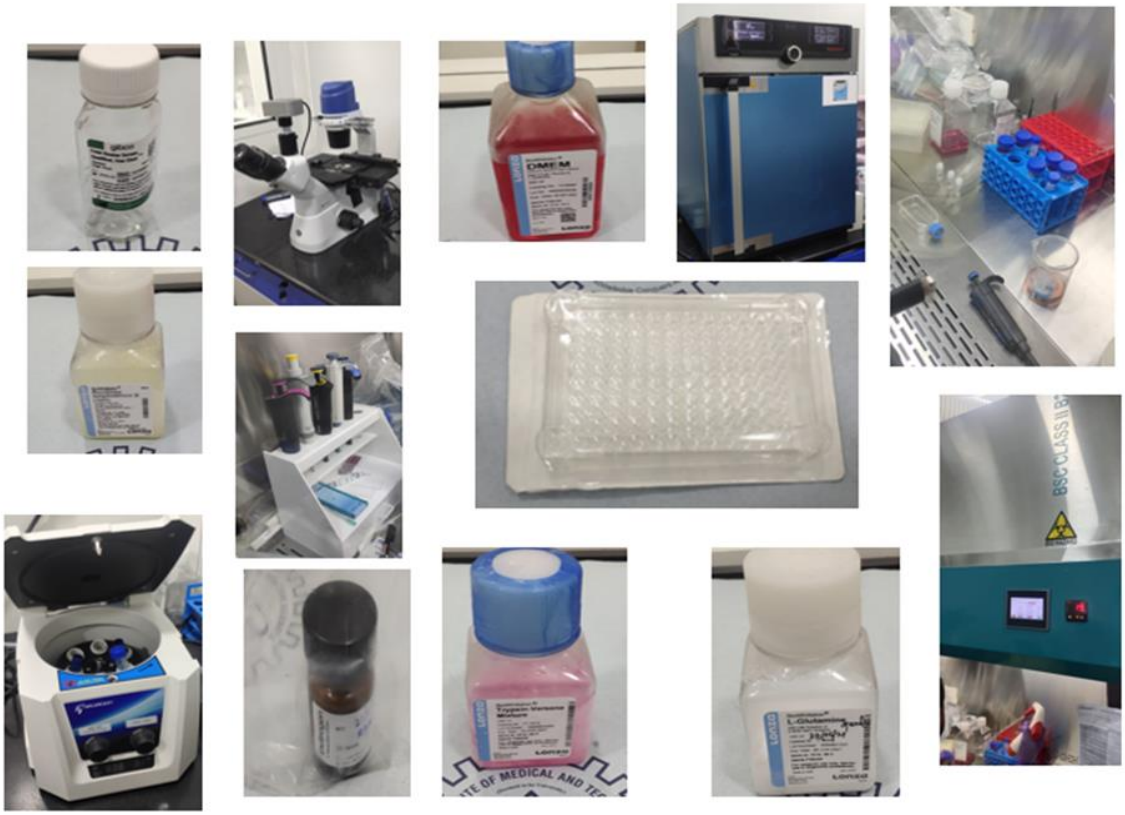

Figure 1. Armamentarium to Assess Biocompatibility of Toothpaste

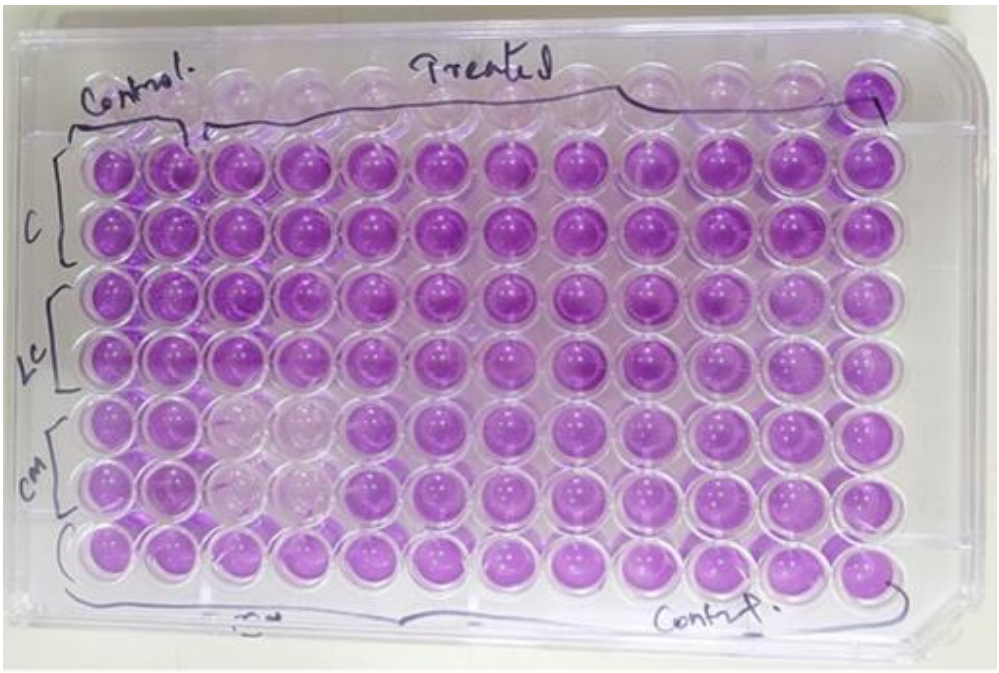

Figure 2. Cell Viability of the Various Toothpaste Assessed by MTT Assay Using Human Gingival Fibroblast

Cytotoxicity Evaluation

Cell Viability/ MTT Assay

The biocompatibility of Coconut Milk tooth cream, Lyophilized coconut tooth cream, ACP CCP tooth cream, and Control groups on HGF primary cells was assessed over 24 hours using the MTT assay, as outlined in a previous study by Koka P et al, 2018. Briefly, various ratios of each group were incubated with gingiva-derived primary cells seeded on a 96-well culture plate for 24 hours. To determine cell viability, post-incubation cells were treated with 10 μl of stock MTT dye (10 mg/ml) per well and further incubated at 37 ℃ for 4 hours. Formazan crystals were dissolved with 100 µl DMSO in each well, and absorbance was measured at 570 nm using a Synergy hybrid Multi-Mode Reader (BioTek, Winooski, VT, US). Cell viability percentage was calculated using the provided equation (Figure 2).

$$Cell\ viability\ (\%) = \frac{\left(OD_{\{\text{test sample}\}} - OD_{\{\text{Blank}\}}\right)}{\left(OD_{\{\text{PC}\}} - OD_{\{\text{Blank}\}}\right)} \times 100$$

Live/Dead Assay

To visualize live and dead cells, live/dead staining was conducted within 24 hours post-incubation with control and experimental groups on HGF cells. A Live/Dead Viability/Cytotoxicity kit (Calcein-AM dye, Invitrogen, USA) was employed following the manufacturer's guidelines with slight adjustments. Initially, HGF cells were seeded in 6-well plates at a density of 1x106 cells per well. After 24 hours of culture, Calcein-AM dye was added and incubated for 30 minutes, followed by washing with 1xPBS. Subsequently, cells were examined using inverted Phase contrast fluorescence microscopy (Invitrogen, evos). Viable cells, emitting green fluorescence, were stained by Calcein-AM alone. Live and dead

stained cells were manually counted, and the ratio of live to dead cells was calculated for each cell condition. Cellular aspect ratios were determined from thresholded LIVE/DEAD images using the Analyse Particles measurement in Fiji (Figure 4).

Results

The MTT test was used to assess the 24-hour cell survival of human gingival fibroblast cells that had been exposed to elutes at varying concentrations (1:1, 1:2, 1:3, and 1:4) in the experimental group (commercial toothpaste, LC toothpaste, and CM-Toothpaste) and control group. Means ± SD (n = 3) are displayed for the data (Figure 3). One way ANOVA and Kruskal Wallis statistical testing were used to analyze the obtained values. The values in comparison to the control group were found to be statistically significant $p<0.05$.

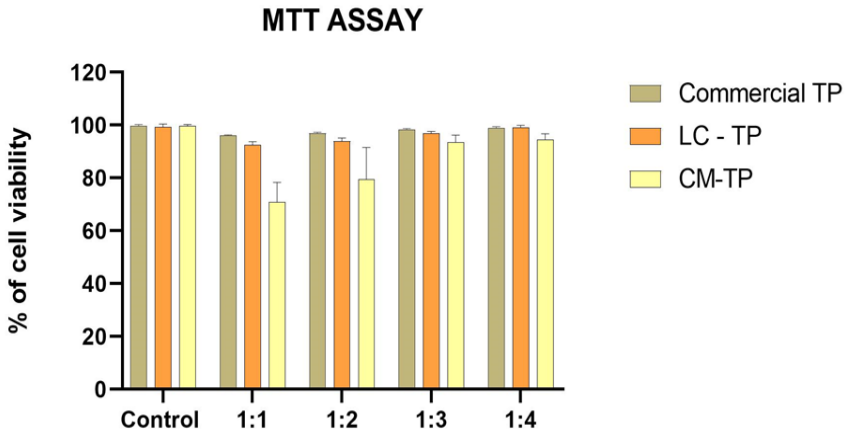

Figure 3. The Viability of Human Gingival Fibroblast Cells Following Exposure to Eluates of Different Concentrations (1:1, 1:2, 1:3, And 1:4)

The HGF morphological assessment and live/dead assay were performed after being treated for 24 hours with untreated control and experimental group toothpaste (commercial toothpaste, LC toothpaste, and CM-Toothpaste), Live cells were stained with calcein-AM dye and allowed to incubate for 15 minutes. Phase contrast fluorescence microscopy with a 20x objective and an inverted phase contrast microscope with a 10x objective were used to examine the cells (Figure 4).

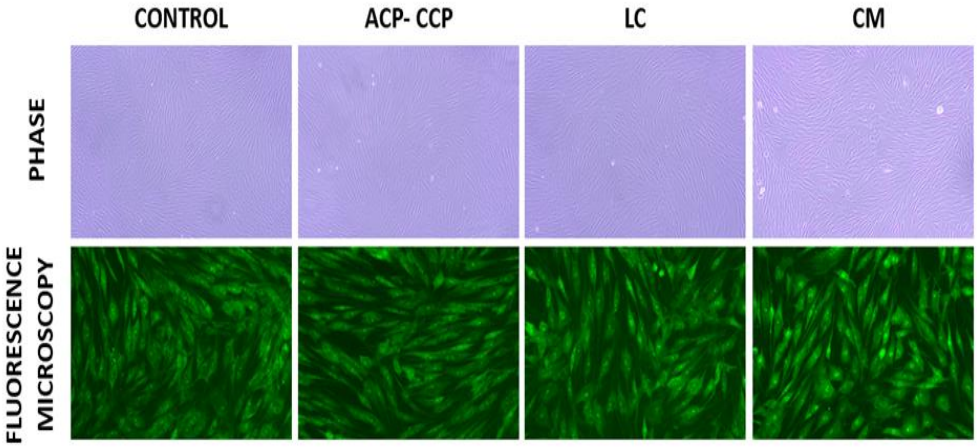

Figure 4. Morphological Assessment and Live/Dead Assay of Hgf Cells

Discussion

A colourimetric method for determining the metabolic activity of cells is the MTT test [19]. In live cells, the yellow tetrazole MTT is converted to purple formazan. Tetrazolium dye tests can also be used to quantify the cytostatic activity (the transition from proliferation to quiescence) or cytotoxicity (the loss of viable cells) of possibly harmful chemicals and treatments [20]. MTT assays at concentrations of 1:1, 1:2, 1:3 and 1:4 were done, and it was found that the toothpaste with the lyophilized coconut extract demonstrated a similar effect when compared to the commercially available ACP CPP toothpaste and the control group. Wherein the coconut milk group, a reduction in cell viability was observed in 1:1 concentration which was then restored close to other group values in other concentrations. Hence it is evident that both the toothpaste materials appear to be biologically compatible and exhibit very little effect on the viable cells.

Live/dead staining was carried out on HGF cells with control and experimental groups within 24 hours of post-incubation to visualize living and dead cells. Calcein-AM was the only staining agent utilized on viable cells that showed green fluorescence. For every cell state, the ratio of living to dead cells was computed by manually counting the labelled cells, both live and dead. In our present study, green-stained fluorescence was mostly noted among all the groups. This indicates that all the toothpaste samples tested were biocompatible, with no cytotoxic activity on the viable cells.

Advancements in orthodontic materials have revolutionized practice, emphasizing shorter treatment times, and improved biomechanics research. Yet, alongside efficiency, the evolving assessment of biocompatibility must now integrate biological material properties for future orthodontic practice [21,22].

Several toothpastes have been previously formulated and studied for their toxic properties. In a study by Camargo et al., it was shown that whitening dentifrices and a few other commercial dentifrices also exhibited significant cytotoxicity levels [23,24]. Previously, studies have been performed to identify ways of improving the biocompatibility of toothpaste, and they have shown that, to mitigate the cytotoxic impacts of toothpaste, it is advisable to substitute sodium lauryl sulfate and cocamidopropyl betaine with gentler detergents and to reduce the fluoride concentration to 400 parts per million (ppm). Alternatively, exploring the substitution of fluoride with alternative antibacterial and cariostatic agents could offer a solution [25]. Given the growing preference for natural resources over synthetic alternatives, exploring their suitability for dental applications could lead to more sustainable and effective oral care solutions. By thoroughly testing these natural resources for dental usage, we can harness their inherent benefits while ensuring safety and efficacy in oral healthcare practices.

Conclusion

In summary, both newly formulated tooth creams derived from *Cocos nucifera* are comparable to those of commercially available ACP-CCP formulations and demonstrate high biocompatibility with human gingival fibroblasts. MTT assay and live/dead assay techniques confirm minimal cytotoxicity and cell death, indicating safety for oral use. These findings highlight the potential of coconut-based tooth creams as safe alternatives for oral care. Further research can explore their effectiveness in maintaining oral health while ensuring tissue safety. Overall, our study supports the viability of utilizing natural compounds for dental care formulations.

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Conflict of interest

Nil

Author Contributions

NB was involved in the research conception, design, data collection, analysis, drafting of the manuscript, interpretation of results, and critically revising the manuscript. AS participated in the research conception, design, interpretation of data, and critical revision of the manuscript. Both authors reviewed and approved the final manuscript and agreed to take responsibility for all aspects of the work.

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