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RESEARCH ARTICLE

# Wound Simulator: An in vitro study of the effect of PLAY on Chemotherapy induced Oral Mucositis

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## Abstract

Chronic wounds, a significant healthcare issue, cause morbidity and financial burdens for patients. Oral mucositis, a condition causing inflammation and ulceration of the oral mucosa, affects over 10% of cancer patients in India. This devastating condition increases the length and costs of cancer treatment, impacts the patient's lifestyle, and increases susceptibility to infections. Treatment options like oral hygiene, chemoprotective agents, and cryotherapy are not uniformly effective. Conversely, Platelet-derived biomaterials are revolutionizing wound healing due to their anti-inflammatory and wound repair properties, cost-effectiveness, and ethical production. We hypothesize PLAY, a human blood derivative rich in natural growth factors and bioactive mediators, will mitigate the inflammatory response induced by 5-fluorouracil (5FU) and phorbol myristate acetate (PMA) and accelerate the wound closure in an invitro model of chemo-induced oral mucositis. To study this was stimulated using MCF-7 breast cancer cell lines. They were treated with 5FU (5-fluorouracil; chemotherapeutic drug) and PMA (Phorbol 12-myristate 13-acetate; protein kinase C activator) was stimulated to mimic inflammatory response associated with chemotherapy to build an invitro oral mucositis model. The key markers for inflammation like, like Cyclooxygenase 2(COX-2), Metalloproteinase 1(MMP-1), Interleukin-6 (IL-6), and Interleukin-8 (IL-8) were assessed, to evaluate PLAY's modulatory effects. Our findings showed that PLAY significantly downregulates COX-2 and MMP-1 expression, highlighting its anti-inflammatory properties. However, IL-6 and IL-8 levels increased, indicating a complex interplay in maintaining inflammatory balance. Additionally, scratch assays with human fibroblast cells demonstrated the PLAY's enhanced wound healing properties when treated with the conditioned media from chemo-treated cancer cells. These results highlight PLAY's anti-inflammatory and wound-healing properties, which would alleviate oral mucositis symptoms and improve patient outcomes.

**Keywords:** Wound healing, Chemotherapy induced oral mucositis, 5-Flurouracil, Inflammation.

## Introduction:

The Oral mucositis Oral mucositis is one of the prevalent side effects of chemotherapy-treated cancer patients (1). The symptoms include painful sores, erythema, edema, and ulcerations in the oral mucosa (2). This condition leads to a worse prognosis, reduced quality of life, and increased disease management costs (3). It affects approximately 30-70% of breast cancer patients depending on chemotherapy dosage and regimen, 40% of cancer patients with solid tumors receiving chemotherapy, and 76% of bone marrow transplant patients (4,5). This problem is relevant to India due to rise of breast cancer patients in Indian women (6). Oral mucositis in chemo-treated patients common side effect and chemotherapy is widespread a primary treatment modality in cancer patients (7). Socio-economic disparities, limited access to supportive care, and cultural dietary habits further exacerbate the impact of chemo-induced oral mucositis on treatment compliance and quality of life (8). Our research aims to provide accessible and cost-effective treatment options suitable for the Indian populations to address this issue.

Currently, the common treatment methods for oral mucositis include Basic Oral Care, drugs like Palifermin (9), topical products like Orajel™, and mucosal protectants like Gelclair® and Zilactin®. More recently, oral cryotherapy (a method of cooling the oral mucosal to reduce the blood flow to reduce the severity of damage due to chemo drugs) is an emerging method, but it is not useful in treatments using radiotherapy or a combination of chemo and radiotherapy (10). While all these methods are effective to some extent, their efficacy highly depends on factors like type of cancer treatment, tumor location, dosage of chemotherapy, etc., making it necessary to explore alternative solutions (5).

5-fluorouracil(5-FU) is an anti-metabolic chemo-drug usually used in treatment of breast, oral, and head and neck cancer. It affects the rapidly dividing cancer cells by disrupting DNA synthesis but the mucosal cells, which have a high turnover rate are also affected leading to tissue damage in 80% of chemotherapy patients. This disruption leads to reactive oxygen species (ROS) production, activating inflammatory pathways (11) and upregulating pro-inflammatory cytokines and genes like cyclooxygenase-2 (COX-2), involved in prostaglandin synthesis via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (12,13). Additionally, Phorbol 12-myristate 13-acetate (PMA), is a known mitogen to induce COX-2 expression. This is used in our study as a positive control to induce COX-2 gene expression simulating an inflammatory model invitro.

Interleukin-6 (IL-6) cytokine is an upstream marker of COX-2 in the inflammatory signaling pathway (14). It is produced by different cell types including keratinocytes, monocytes, fibroblasts, and cancer cells (15). In acute wounds, IL-6 promotes leukocyte chemotaxis and fibroblast proliferation, aiding in wound re-epithelialization through the Janus kinase/signal transducers and activators of transcription Janus kinase/signal transducer (JAK/STAT) and mitogen-activated protein kinase (MAPK) signaling pathways (16). However, prolonged IL-6 upregulation in chronic wounds, often due to infection or tumors, leads to an accumulation of mononuclear cells and pro-inflammatory macrophages, damaging surrounding tissues (17). Interleukin-8 (IL-8) is a pro-inflammatory cytokine that attracts neutrophils to wound sites. It promotes endothelial cell proliferation and angiogenesis in both acute and chronic wounds

(18). Studies show IL-8 enhances cell migration and adhesion in keratinocytes, suggesting similar effects in fibroblasts and other healthy cell lines (19).

Furthermore, to understand the mechanisms that regulate cellular proliferation during wound healing, the extracellular matrix (ECM) remodeling plays a vital role. The ECM provides structural support and regulates cell behavior which plays an important role in tissue remodeling through degradation of ECM and in wound re-epithelialization. This is mainly mediated by matrix metalloproteinases (MMPs), a family of zinc-dependent proteases. Primarily, MMP-1, produced by basal keratinocytes known to play a major role by degrading collagen facilitating the migration of proliferative cells, which promotes tissue repair (20,21). Studies have shown that in acute wounds MMP activity is balanced by tissue inhibitors of metalloproteinases (TIMPs) mediating the ECM remodeling. Whereas in chronic wounds, elevated and prolonged levels of MMPs disrupt the balance, resulting in excessive ECM degradation thereby increasing the tissue damage. Which leads to persistent inflammation, and cell death (21).

To replicate a wound model(2D), the scratch assay is commonly used where a uniform scratch is made in a confluent cell monolayer, and the migration of cells is assed to measure the wound closure rate over time (22). This method is useful for assessing the wound healing potential of therapeutic interventions, including those with anti-inflammatory and regenerative properties at invitro conditions.

Platelet-derived biomaterials are an emerging and promising method of treating chronic wounds like oral mucositis, pressure ulcers, diabetic foot ulcers, etc., because of the tissue regeneration and anti-inflammation properties of growth factors present in them. Recent studies show that platelets are not only the drivers of wound healing, but also contain several growth factors, chemokines, and cytokines aiding tissue repair (23). PLAY, an in-house developed product of International Stem Cell Services Limited (iCREST), is sourced from human blood and contains natural growth factors like platelet-derived growth factor (PDGF), hepatocyte growth factor (HGF), transforming growth factor-beta (TGF-β), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), etc., known for their therapeutic potential in wound healing studies. It is also known to contain bioactive mediators such as stromal cell-derived factor 1 (SDF-1) and monocyte chemoattractant protein-1 (MCP-1) that enhance its effectiveness. These components are known for their anti-inflammatory properties, as they modulate the wound bed microenvironment from a pro-inflammatory state to an anti-inflammatory state, which accelerates the wound to shift to the next proliferative and remodeling phase, ultimately healing the wound faster. This indicates PLAY can serve as a possible intervention for oral mucositis.

We hypothesize PLAY, a human blood derivative rich in natural growth factors and bioactive mediators, will mitigate the inflammatory response induced by 5-fluorouracil (5-FU) and phorbol myristate acetate (PMA) and accelerate the wound closure in an invitro oral mucositis model. This hypothesis will be tested by analyzing gene and cytokine expression and evaluating the extent of wound closure, assessed for the key markers of inflammation—COX-2, IL-6, MMP-1, and IL-8—and scratch assay using human fibroblast cells. Our study showed that PLAY downregulated COX-2 and MMP-1 inflammatory genes, and also promoted cell migration and wound closure. This provides insights into the potential therapeutic

mechanisms of PLAY in managing chemo-induced oral mucositis.

## Materials and Methods

**Cell Culture:** MCF-7 breast cancer cell line was procured from National Centre of Cell Science in Pune, India. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Hi media) supplemented with 10 % in 10% Fetal bovine serum (FBS) and maintained at 37°C with 5% CO2 at standard cell culture conditions. At 80% confluency adhered cells were detached using 0.25% trypsin-EDTA (Hi Media) and were maintained for further studies.

**Drug treatment:** In a 6 well plate, 1.5 x $10^5$ cells were seeded and cultured in DMEM supplemented with 10% FBS. The treatment of cells was as follows:

**Experimental Groups:** *Control Group* - Cells were maintained in DMEM containing 10% FBS without any treatment for 48hours. *10% PLAY Group* - Cells were treated with 10% PLAY, prepared by adding 100 µL of PLAY to 900 µL of DMEM supplemented with 10% FBS for 48hours. *PMA Group* - Cells were treated with 3.048 ng/mL of phorbol 12-myristate 13-acetate (PMA), prepared in DMEM with 10% FBS for 48hours. *5-FU Group* - Cells were treated with 2 µg/mL of 5-fluorouracil (5-FU) (sigma), prepared in DMEM with 10% FBS for 48hours. Note: Both drugs of concentrations were diluted with plain DMEM (Gibco). PMA + 10% PLAY Group - Cells were first treated with 3.048 ng/mL PMA for 48 hours. After 48 hours, PMA-containing media was removed, and cells were subsequently treated with 10% PLAY in DMEM with 10% FBS. *5-FU + 10% PLAY Group*-Cells were first treated with 2 µg/mL 5-FU for 48 hours. After 48 hours, 5-FU-containing media was removed, and cells were subsequently treated with 10% PLAY in DMEM with 10% FBS.

**Semi-quantitative polymerase chain reaction :** After drug treatment, total RNA was extracted using the TRIzol® method (Takara Bio Inc., Japan) according to the manufacturer's instructions. Reverse transcription was performed using 1 µg of total RNA and the Prime Script™ cDNA Synthesis Kit (Cat. #6110A, Takara Bio Inc., Shiga, Japan) to generate complementary DNA (cDNA).

Semi-quantitative PCR was carried out to assess COX-2 gene expression. The reaction mix contained 1 µL of synthesized cDNA as template in a 25 µL PCR reaction volume. Primer sequences for COX-2 and the housekeeping gene GAPDH were obtained from Applied Biosystems. PCR amplification was performed for 35 cycles, with the following cycling conditions: initial denaturation at 95°C for 3 min, followed by 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 45 sec, with a final extension at 72°C for 5 min. The PCR products were resolved on a 1.5% agarose gel stained with ethidium bromide and visualized under UV illumination. Band intensities were quantified using ImageJ software (NIH, USA) and normalized to GAPDH expression.

The primer sequences used are as follows: GAPDH: FGGTCGGAGTCAACGGATTTGGTCG; R-CCTCCGACGCCTGCTTCACCAC, COX-2: F-CCACTTCAAGGGATTTTGGA, R-GAGAAGGCTTCCAGCTTTT, MMP-1: F-GAGCAAACACATCTGAGGTACAGGA, R-TTGTCCCGATGATCTCCCCTGACA;

**ELISA- Enzyme Linked Immunosorbent Assay**: IL-6 and IL-8 cytokines levels were measured using ELISA method. Firstly, the supernatants were collected after the drug treatment from the cells after 48 hours of drug exposure and centrifuged at 1500 rpm for 10

mins. The supernatant was stored at -20°C and the cytokines levels for IL-6 and IL-8 was measured by following the manufacturer's procedure BD optia (Human Il-6 and Human IL-8) in duplicates with a standard (positive control) and a blank.

**Scratch Assay:** In a 24-well plate, Human fibroblast cells-HFS (primary cells) of seeding density 3000 cells/cm² were seeded and allowed to adhere for 24 hours in 10% FBS supplemented DMEM media. Using a sterile 200 µL micropipette tip, a scratch was made and the wells were washed with saline to remove dead cells. The following treatments were done to the respective groups: Control (HFS) – plain media, HFS + IC–50 CM - Conditioned media was prepared with 5FU (IC-50 concentration) treated either MCF-7 cells and 40% of it was treated to HFS cells in plain media. HFS + IC-50 CM + PLAY - 10 % PLAY was added along with 40% IC-50 CM and 10 % PLAY alone group. Using a 10X magnification inverted microscope images were captured.

**Statistical Analysis:** All the data shown are of a minimum of 2 independent sets. Bar graphs display the mean +/- standard error of the data. GRAPHPAD Prism software was used to determine the significance and unpaired two-tailed t-test was used. Significant differences were defined as *p < 0.05, **p < 0.01 and ***p < 0.001

## Results and Discussion

To study the effect of 5FU and PMA on COX-2 Gene Expression and the Modulatory Role of PLAY in MCF-7 Cel, an in-vitro inflammatory response was stimulated using 5FU and PMA and the potential mitigating effect of PLAY was evaluated. MCF-7 (estrogen receptor-positive) cells was treated with 5FU and PMA and COX-2 gene expression was assessed using semiquantitative PCR. A significant increase in COX-2 levels was observed in MCF-7 cells following treatment with 5FU, reflecting the inflammatory and cellular stress associated with chemotherapy (*Figure 1*). Notably, in the cell groups treated with phorbol myristate acetate (PMA) increase in COX-2 expression was not as significant. Furthermore, both groups containing PLAY, showed a substantial decrease in COX-2 expression nearing basal levels suggesting PLAY's anti-inflammatory properties that could be beneficial in reducing damage to the oral mucosa.

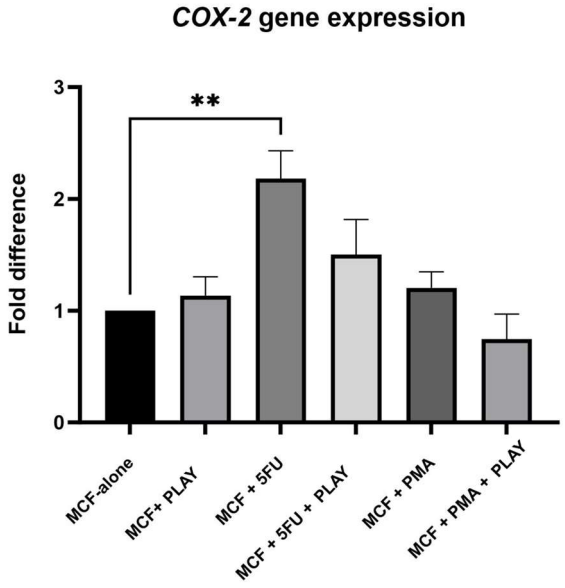


Figure 1: Effect of 5FU and PMA on COX-2 Gene Expression in MCF-7 cell lines. COX-2 levels shown as mean ± SE. *p < 0.05. (N=3)

This reflecting the inflammatory and cellular stress associated with chemotherapy. 5FU disrupts DNA synthesis (24), generates reactive oxygen species (25), and activates pathways like p53(26), p38 mitogen activated protein kinase (MAPK), NF-kB (11,27). Several studies also indicate that the upregulation of COX-2 is associated with activation of NF-kB pathways (27) and cellular death caused by them. Nuclear factor-kB (NF-kB) is a key molecule involved in mucositis and inhibition of NF-kB improved mucosal damage by 5-FU (28). Thus, PLAY's

ability to reduce COX-2 expression, validates its anti-inflammatory properties and suggests that PLAY can be used to reduce damage to the oral mucosa.

Furthermore, to study the proteolytic and tissue remodeling in oral mucositis, gene expression of MMP-1 was studied when cancer cells were treated with chemotherapy drug. MCF-7 cells treated with 5FU and PMA exhibited increased MMP-1 expression, indicating elevated proteolytic activity *(Figure 2)*. When PLAY was administered 48 hours after PMA treatment, a downregulation of MMP-1 expression to basal levels was observed, closely to untreated MCF-7 cells. However, in the group treated with 5FU+ PLAY, MMP-1 expression was not significantly reduced by PLAY, suggesting that DNA damage induced by 5FU may override the regulatory effects of PLAY.

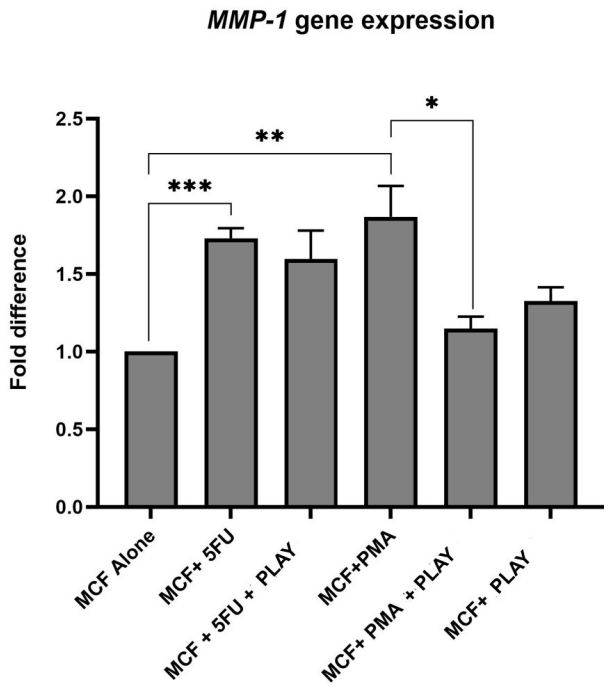


Figure 2: Effect of 5FU and PMA on MMP-1 Gene Expression in MCF-7 cell lines. MMP-1 levels shown as mean ± SE. *p < 0.05, ** p < 0.001. (N=3)

MMP-1 is upregulated through a multitude of activators such as mRNA and protein expression, activator protein (AP-1) promoters and p38 kinase (29,30) which could explain why the decrease in expression is not as significant as COX-2. PMA stimulates the transcription factor AP-1, which is crucial for the expression of MMP-1 genes, and protein kinase C, which also upregulates MMP-1 levels (31). Thus, PLAY's ability to reduce MMP-1 expression significantly in PMA groups could allude to inhibition of these pathways. However, 5FU-induced DNA damage may override PLAY's regulatory effects. Thus, PLAY may be able to prevent excess extracellular matrix (ECM) degradation and promote proliferation at the wound site.

However, through there was a downregulation observed in COX-2 and MMP-1 gene expression, IL-6 and IL-8 cytokine levels increased (*Figure 3 & 4*) after treatment of PLAY in 5FU + PLAY and PMA + PLAY groups. The upregulation of these inflammatory cytokines may be a part of the cellular response to maintain the inflammatory balance. At 48-hour treatment with 5FU resulted in a 0.2-fold increase in IL-6 expression in MCF-7 was observed, when compared to untreated controls; however, these changes were not statistically significant (*Figure 3*). Following treatment with PLAY after 5FU induction, IL-6 levels were found to be further elevated, however no significant induction in IL-6 was observed in the PMA-treated groups.

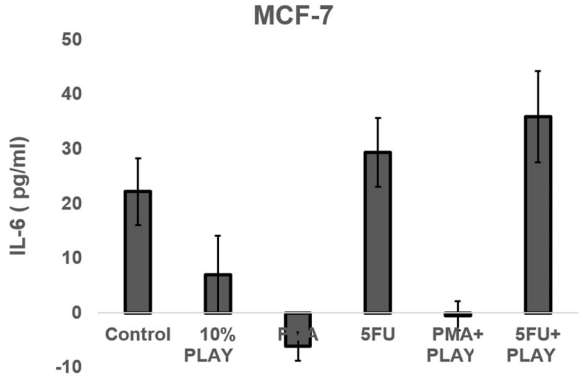

Figure 3: IL-6 cytokine analysis in response to 5FU and PMA treatment in MCF-7 cell line. IL-6 levels shown as mean ± SE. Data: ns (non-significant) p> 0.05. (N=2)

Similarly, in MCF-7 cells were treated with 5FU for 48hrs, a 0.7-fold increase in IL-8 expression was observed when compared to control. Interestingly, in the 5FU treated groups, treatment with PLAY resulted in additional increase in IL-8 expression. 5FU is shown to promote c-Jun N-terminal kinase (JNK) activation which is an important factor in inflammation and IL-8 production (32,33) Moreover, in PMA treated groups of both cell lines IL-8 levels were the same as the control group and an increase was observed in PMA with PLAY groups *(Figure 4)*. These findings highlight the complex molecular signaling in chemotherapy-treated cancer cells and reflect the modulatory influence of PLAY on IL-8 expression.

Studies show that increase in IL-8 levels would an indicator of cell migration and chemotaxis. Studies demonstrate that IL-8 behaves as a chemoattractant for keratinocytes and could potentially be linked to accelerated cell migration. Moreover, in chronic wounds IL-8RA and IL-8RB levels are lower than acute wounds (34), indicating that the increased expression induced by PLAY could promote cell migration and wound closure in mucositis

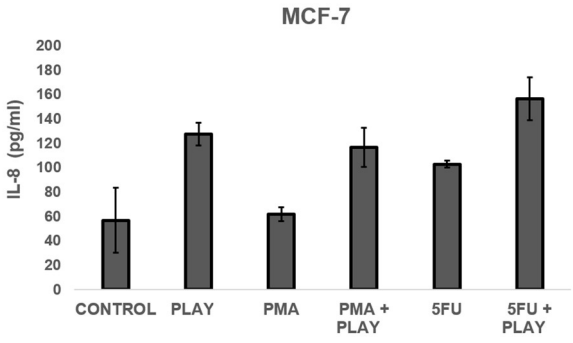


Figure 4: IL-8 cytokine analysis in response to 5FU and PMA treatment in MCF-7 Cell line. IL-8 levels shown as mean ± SE. Data: ns (non-significant) p> 0.05. (N=2)

Finally, to study the effect of the chemotherapy treated cancer cells on cell migration of human fibroblasts HFS cells cell migration assay was performed. The qualitative results demonstrate that the cell migration and density at the wound site was lower in the HFS cells treated with the CM compared to the control. However, both the CM+PLAY and 10%PLAY groups exhibit higher migration rates and better wound closure at 48hrs than both the control and CM treated groups. This could correlate to the action of PLAY in mediating factors like MMP-1 and IL-8 to promote cell migration at the molecular level and strengthens PLAY's effectiveness as an intervention in chemotherapy-treated cancer cell- induced inflammation.

Overall, platelet derived biomaterials are gaining increased popularity in regenerative medicine including for chronic wounds. They

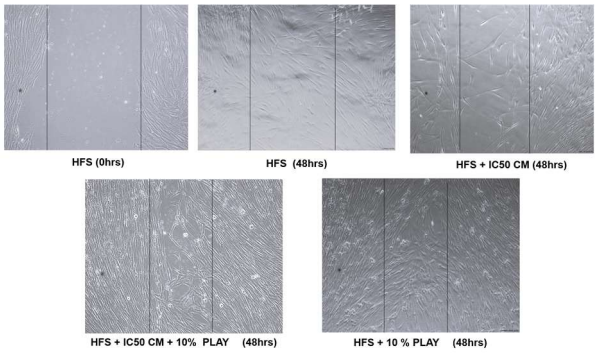


Figure 5: Enhanced cell migration in Human Fibroblast Cells by PLAY in 5FU-Conditioned Media

through anti-inflammatory and antimicrobial roles effective on endothelial cells, monocytes, fibroblasts, and keratinocytes. These indicated anti-inflammatory and wound healing properties of PLAY possibly stem from its composition of natural growth factors and bioactive mediators like VEGF, EGF, FGF, PDGF, etc. offering promise in mitigating the inflammation and pain associated with oral mucositis, significantly impacting the quality of life for patients undergoing cancer therapy.

However, these properties are yet to be studied in relation to other inflammatory markers like IL-10 NF-kappa B. TNF-Alpha, etc. We aim to investigate the same in future studies as well as profile their roles in the onset of oral mucositis. Moreover, future studies will include, studying effect of the chemotherapy drug treated MCF-7 cells on oral mucosal cells like gingival cells and to take forward for in vivo animal studies determining the significance of PLAY in treating chemo-induced oral mucositis wounds.

**Conclusion:** This research highlights the potential of alternative therapeutic approaches, such as PLAY, for the management of chronic wounds such as oral mucositis. By supporting the tissue repair process, PLAY may contribute to accelerated healing in cancer patients. Compared with conventional supportive therapies, which may have limitations in efficacy, cost, and accessibility, PLAY represents a potentially cost-effective, ethically derived, and animal cruelty-free approach. These attributes support its potential as an alternative therapeutic strategy for improving wound healing in patients with cancer-associated oral mucositis.

**Author Contributions:** Keerthana Rajesh is high school student involved in learning and performing the experiments, and contributed to the preparation of the manuscript. Bhavana N V provided mentorship, supported the experimental design and data interpretation, and contributed to manuscript preparation and review. Mercy Jennis Pramod was responsible for the production of PLAY. Jyothsna Rao supervised the study, provided scientific guidance, and contributed to the conceptualization and review of the manuscript. Gururaj Rao provided the laboratory facilities, resources, and institutional support necessary for conducting the study.

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