Evaluating the Stability, Biocompatibility, and Efficacy of Chitosan-Phloroglucinol Composite for Wound Healing Applications

Harisankar KC[a], S Visnuvinayagam[b], Pavan Kumar Dara[c], Vinu Vijayan[b], Lekshmi RG Kumar[a], Tejpal CS[a], Dhandapani N [d], Anandan R[a*]

[a]Biochemistry & Nutrition Division, ICAR-Central Institute of Fisheries Technology, Cochin, Kerala, 682029, India; [b]Microbiology, Fermentation & Biotechnology Division, ICAR-Central Institute of Fisheries Technology, Cochin, Kerala, 682029, India; [c] Department of Biotechnology, Faculty of Science and Humanities, SRMIST, Kattankulathur 603203, Tamil Nadu; [d]RVS College of Pharmaceutical Sciences, Sulur, Coimbatore, Tamil Nadu, 641402, India

* Corresponding author: Dr. Rangasamy Anandan (kranandan@rediffmail.com)

Abstract: This study investigates the development and characterization of a chitosan-phloroglucinol composite, focusing on its size, zeta potential, cytotoxicity, and wound healing properties. The composite exhibited a higher zeta potential, indicating excellent stability. Cytotoxicity analysis in the L929 cell line revealed that phloroglucinol alone had an IC_{50} value of 27.60 ± 1.28 μg/ml, indicating significant toxicity. In contrast, the chitosan-phloroglucinol composite showed a higher IC_{50} value of 81.94 ± 4.28 μg/ml, suggesting a reduced toxicity profile compared to phloroglucinol alone. The wound healing potential of the composite was evaluated using a scratch assay, demonstrating a significant reduction in the scratch area of 93.15±4.65%, compared to 41.95±2.09% in control cells. Altogether, the present research observation indicated the wound-healing potential of chitosan-phloroglucinol composite as a safe, effective, and non-toxic material for the future experimental and clinical treatment of wounds.

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Keywords: Chitosan; phloroglucinol; L929 cell line; wound healing activity

1. Introduction

Serious skin flaws may not heal successfully without adequate treatment, despite the skin's amazing ability to regenerate itself. Wound dressings are essential for covering these regions and encouraging quicker healing (Zhang et al., 2024). Chitosan is a natural cationic polysaccharide made up of (1→4)-2-amino-2-deoxy-β-d-glucan (Fig 1). It is obtained from chitin, a structural component present in the shells of crustaceans and the cell walls of fungi. The conversion from chitin to chitosan occurs through a process called deacetylation, during which acetyl groups are removed. (Matica et al., 2019). Among the various biopolymers, Chitosan has been widely explored for its diverse medicinal properties, particularly in traditional medicine, where it is used for wound healing, cholesterol management, and weight loss. Its therapeutic applications are driven by its natural biocompatibility, antimicrobial properties, and potential to modulate biological activities (Wang et al., 2020). Chitosan possesses reactive functional groups, specifically hydroxyl (−OH) and amino ($-NH_2$) groups, that allow for the development of various derivatives. These available reactive sites enhance chitosan's properties and functionalities, enabling its application in specialized areas such as drug delivery and biomedical engineering (Perumcherry et al., 2022).

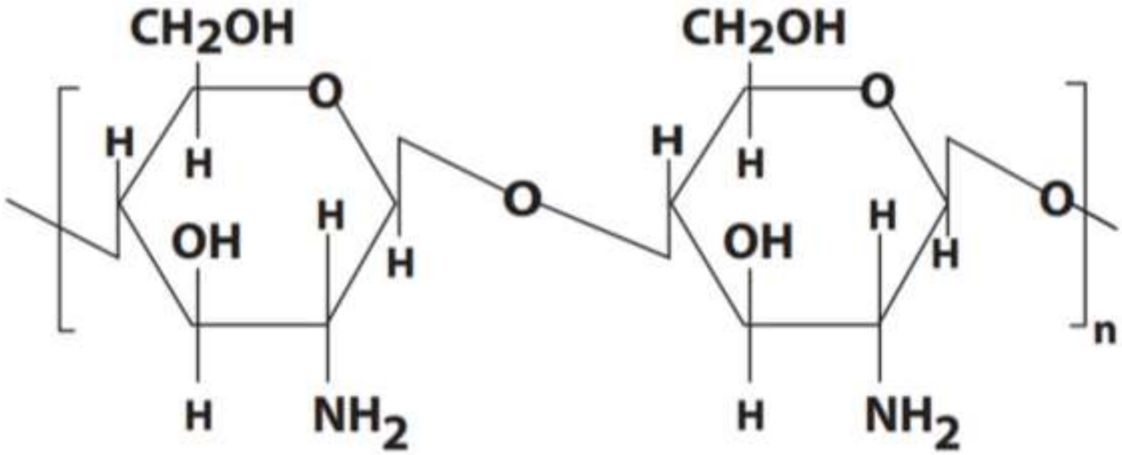

Fig. 1. Structural Overview of Chitosan

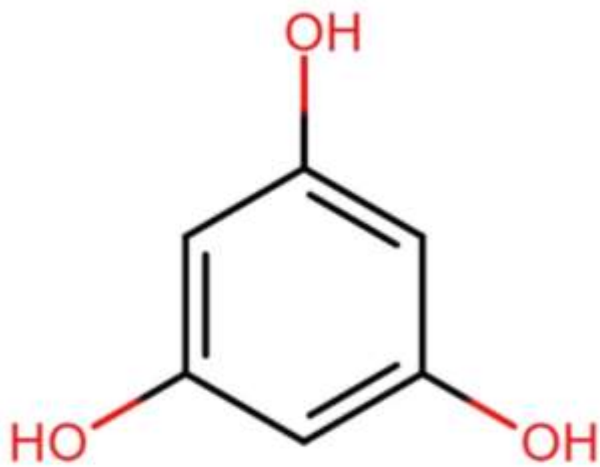

Fig. 2a. Structural Overview of Phloroglucinol

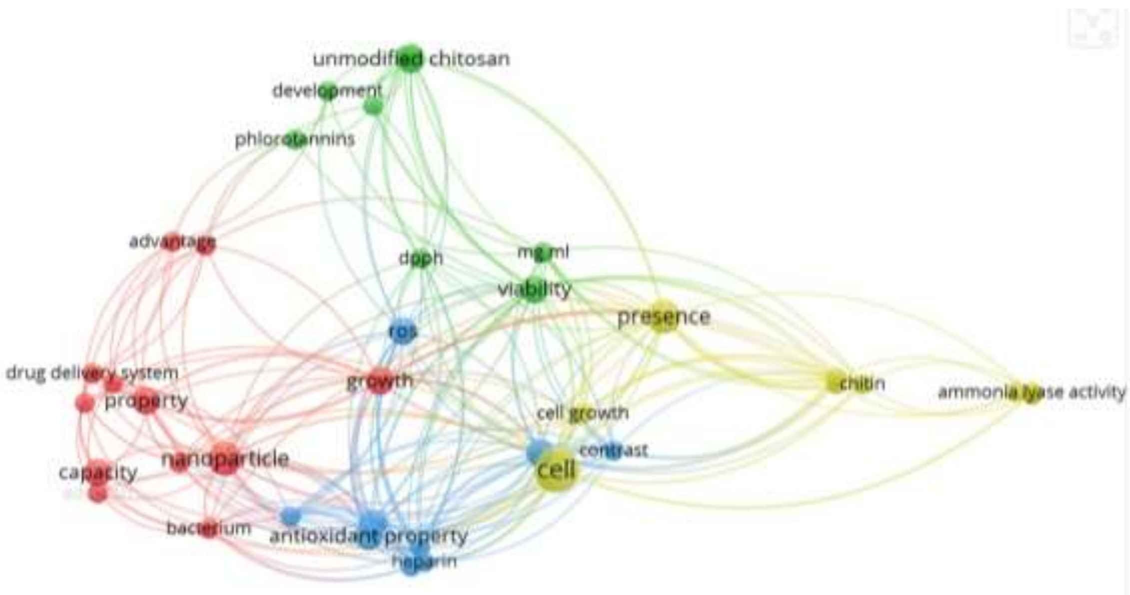

Fig. 2b. Application of chitosan-phloroglucinol membrane in tissue engineering applications (VOS viewer (11-12-2024)

Phloroglucinol (Fig.2a), a polyphenolic compound of plant origin, consists of an aromatic phenyl ring with three hydroxyl groups (1,3,5-trihydroxybenzene) and is known to exhibit various biological activities, including antimicrobial, anticancer, anti-allergic, anti-inflammatory, and antioxidant effects (Faezeh et al., 2022). It is mostly obtained from marine brown seaweeds and a variety of plants. It is a key component in the biosynthesis of many secondary metabolites; studies show that it is involved in the synthesis of about 700 various compounds. Notably, a number of these metabolites are present in marine algae and species in the *Myrtaceae* family (Khan et al., 2022). The encapsulation of phloroglucinol with chitosan represents a promising strategy to combat infections associated with biofilm-forming pathogens. In the present study, an attempt has been made to prepare a chitosan-phloroglucinol composite and the wound healing potential of composite on L929 cells was examined using scratch assays by assessing the migration of cells as an indicator of wound healing process.

2. Materials and Methods

Materials

L929 fibroblast cells were obtained from the National Centre for Cell Science (NCCS), Pune, Maharashtra, India. For cell culture, Dulbecco's Modified Eagle Medium (DMEM), Trypsin, and Fetal Bovine Serum (FBS) were sourced from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). Low molecular weight chitosan and phloroglucinol were obtained from Sigma-Aldrich, St. Louis, Missouri, USA.

2.1 Preparation of chitosan–phloroglucinol composite:

The chitosan–phloroglucinol composite was synthesized in our laboratory as per the following procedure. Briefly, 0.5 g of low molecular weight chitosan (deacetylated chitin – poly D-glucosamine) was dissolved in 100 ml of 1% lactic acid. The mixture was thoroughly stirred using a magnetic stirrer until complete dissolution was achieved. Following this, 10 mg of phloroglucinol was added to the solution to form the chitosan–phloroglucinol composite. The resulting mixture was then homogenized at 12,000 rpm for 30 min. The stability and size were confirmed using Zeta Sizer.

2.2. Particle size and Zeta potential

The Zetasizer Nano ZS (Malvern Instruments) was used to measure the zeta potential and particle size distribution. The analysis was performed at a temperature of 25°C. Zetasizer software was used to process each measurement.

2.3 Assessment of Cytotoxicity of Chitosan-Phloroglucinol Composites on L929 Cells via MTT Assay

MTT assay was conducted to assess the cytotoxic effects of chitosan, lactic acid, phloroglucinol, and their composite on L929 mouse fibroblast cells. L929 cells were treated with various concentrations of chitosan, phloroglucinol and chitosan-phloroglucinol composite in triplicate to ensure statistical validity. Cells were incubated for 24 h in a CO_2 incubator to allow for treatment effects. Following the incubation period, 100 µl of 0.5 mg/ml MTT solution in 1X phosphate-buffered saline (PBS) was added to each well. The cells were then incubated for an additional 3 h to facilitate the reduction of MTT to formazan crystals by metabolically active cells. After the incubation, the MTT solution was carefully discarded. The purple formazan crystals formed within the cells were dissolved in 100 µl of 100% dimethyl sulfoxide (DMSO) and incubated for 10 minutes at room temperature. The absorbance of the resulting purple solution was measured at 570 nm using a Biotek Synergy HT Microplate reader. Cytotoxicity of test samples was calculated using the equation, (Herdiana et al., 2022).

$$Cell\ viability(\%) = \frac{OD\ OF\ TEST * 100}{OD\ OF\ CONTROL}$$

2.4 Evaluation of Wound Healing Potential of Chitosan-Phloroglucinol Composite using Scratch Assay in L929 Cells

The wound healing potential of chitosan-phloroglucinol composite was evaluated in L929 cells using scratch Assay (Vijayan and Kiran, 2023). Cells were seeded in 6-well plates and allowed to reach confluence. A sterile 10 µl pipette tip was then employed to create a uniform scratch, resulting in a cell-free area. Following this, a gentle wash with phosphate-buffered saline (PBS) was performed to remove any residual cell debris. Subsequently, the culture medium was supplemented with phloroglucinol and chitosan- phloroglucinol composite. Images of the scratched areas were captured at the 0^{th} hour. The cells were then incubated for 24 hours to facilitate migration. At the end of the incubation period, the images of the scratched regions were examined. The gap sizes were analyzed using ImageJ software. To quantify the migration capability of the cells, the cell-free area observed at 24 hour was subtracted from the initial scratch area measured at 0^{th} hour.

2.5 Statistical analysis

Results are expressed as mean+S.D. and Student's t-test was used to assess statistical significance.

3. Results and discussion

Phloroglucinol, a polyphenolic compound, exhibits a wide range of biological activities, including antimicrobial, anticancer, anti-allergic, anti-inflammatory, and antioxidant properties. Chitosan, possessing both amino ($-NH_2$) and hydroxyl (-OH) groups, is capable of interacting with a variety of compounds. These non-covalent modifications enhance its biological properties, contributing to its versatility in biomedical applications. The focus of the present is to investigate the wound healing potential of chitosan-phloroglucinol composite on L929 cell line.

The zeta potential of the chitosan-phloroglucinol composite was recorded at 75.9±3.8 mV (Fig 3), which implies good stability, indicating a strong electrostatic interaction between chitosan and phloroglucinol. In comparison, the individual components showed lower zeta potentials. Chitosan had a zeta potential of 62.7±0.2 mV, while phloroglucinol was significantly lower at 2.32±0.15 mV. The size of chitosan-phloroglucinol composite observed was 3187±94 nm (Table 1). The size of phloroglucinol was 678.5±11.5 nm and the size of chitosan was 4025±4.8 nm.

Table 1. Characterization of Size, Polydispersity Index (PDI), and Zeta Potential of Chitosan-Phloroglucinol Composites

Sample	Z-average size(nm)	PDI	Zeta Potential(mV)
Chitosan	4025±4.8	0.721±0.04	62.7±0.2
Phloroglucinol	678.5±11.5	0.81±0.04	2.32±0.15
Chitosan-Phloroglucinol Composite	3187±94	0.437±0.06	75.9±3.8

The size of the composite is intermediate between that of the two components, suggesting that phloroglucinol has been effectively integrated into the chitosan matrix. Similarly, Pang et al. (2024) encapsulated chitosan with whey protein isolate, revealing that the resulting composite particles were larger than those formed by whey protein isolate alone. The PDI of chitosan was measured at 0.721±0.04, indicating a relatively broad distribution of particle sizes. Similarly, phloroglucinol exhibited a PDI of 0.81±0.04, which also suggests a considerable degree of size variability among its particles. In contrast, the chitosan-phloroglucinol composite demonstrated a significantly lower PDI of 0.437±0.06. This reduction in PDI indicates a more uniform particle size distribution within the composite compared to the individual components. A lower PDI is often desirable in composite materials, as it can enhance stability and improve the homogeneity of the material, potentially leading to more consistent performance in applications such as drug delivery or wound healing (Almalik et al.,2017). The relatively smaller size of phloroglucinol may contribute to the overall reduction in the chitosan-phloroglucinol composite size, which could enhance its dispersion and stability. Additionally, the larger size of chitosan compared to the composite supports the idea that the interaction between chitosan and phloroglucinol modifies the aggregation behaviour of the particles.

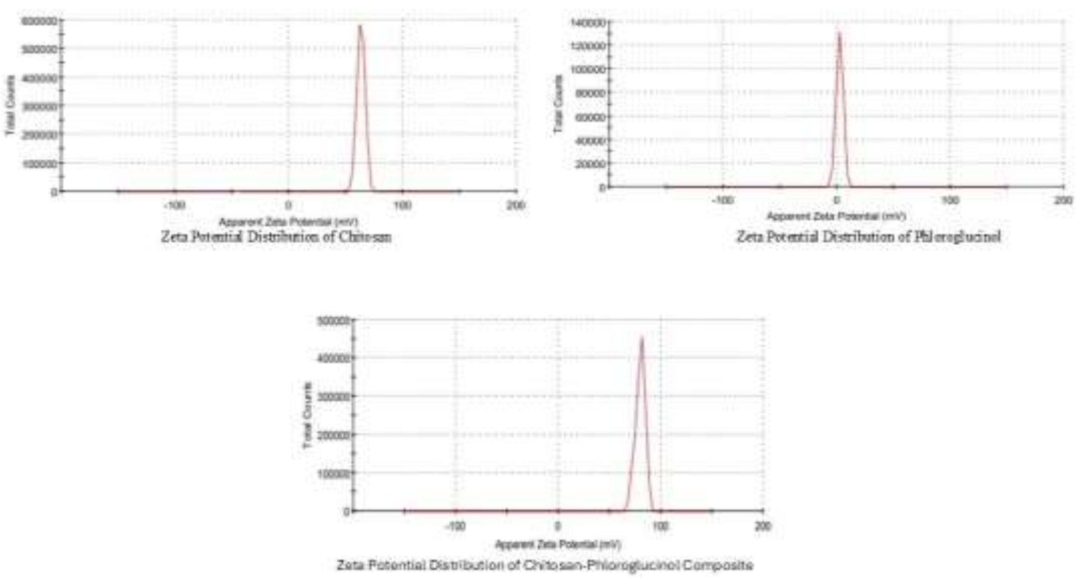

Fig. 3. Stability Assessment of Chitosan-Phloroglucinol Composite via Zeta Potential

The cytotoxic effects of chitosan, phloroglucinol, and their composite were evaluated in L929 mouse fibroblast cells using the MTT assay. This assay serves as a reliable method for quantifying cell viability based on the metabolic activity of viable cells. The IC_{50} value for chitosan was determined to be 94.64 ± 4.73 µg/ml (Table 2). This indicates that a concentration of approximately 94.64 ± 4.73 µg/ml is required to inhibit cell viability by 50%. Phloroglucinol demonstrated a markedly lower IC_{50} value of 27.60±1.32 µg/ml. This result indicates that phloroglucinol has a higher potency in exerting cytotoxic effects on L929 cells, requiring much less concentration to inhibit cell viability. The IC_{50} value for the chitosan-phloroglucinol composite was recorded at 81.94±4.09 µg/ml. This value indicates that the composite maintains a level of cytotoxicity, although it is less potent than phloroglucinol alone. The higher IC_{50}

compared to phloroglucinol suggests that the presence of chitosan may reduce the overall toxicity of the composite when compared to phloroglucinol alone.

Table 2: IC_{50} Values of Chitosan-Phloroglucinol Composite Against L929 Cells

Samples	**IC_{50}Value (µg/ml)**
Chitosan	94.65± 4.73
Phloroglucinol	27.60 ± 1.28
Chitosan-phloroglucinol	81.94 ± 4.28

At a concentration of 50 µg/ml, chitosan exhibited a cell viability of 90.86% (Fig 4). This suggests that during treatment with chitosan, a significant portion of the cells remains viable. In contrast, phloroglucinol displayed a dramatically reduced cell viability of only 5.71% at a concentration of 50 µg/ml. This result indicates a high degree of cytotoxicity, revealing that phloroglucinol significantly impairs cell viability at this concentration. Notably, the chitosan-phloroglucinol composite exhibited a cell viability of 94.38%, indicating that the combination of these two components markedly reduces the toxicity associated with phloroglucinol alone. This present observation has suggested that chitosan may offer a cytoprotective effect by enhancing cell survival even in the presence of phloroglucinol. At the lowest tested concentration of 3.12 µg/ml, chitosan exhibited a cell viability of 139.37%. These results indicate that chitosan may have a stimulatory effect on cell viability at this concentration, suggesting potential benefits for cellular growth and maintenance. At a concentration of 3.12 µg/ml, phloroglucinol showed a cell viability of 108.32%. This finding suggests that phloroglucinol, similar to chitosan, may promote cell viability at lower concentrations, indicating a potential positive effect on cell metabolism or growth. Notably, the chitosan-phloroglucinol composite also exhibited a cell viability of 108.32% at the same concentration of 3.12 µg/ml. This result further supports the idea that the combination of these two components does not detract from their individual beneficial effects on cell viability. The findings from this study have revealed that chitosan phloroglucinol composite at higher concentration is capable of maintaining cell viability at a notable level as compared to that of per se effects phloroglucinol. The remarkable cell viability noted in cells treated with chitosan-phloroglucinol has suggested that it can improve the cell viability and biocompatibility, which are essential prerequisites required for wound healing process.

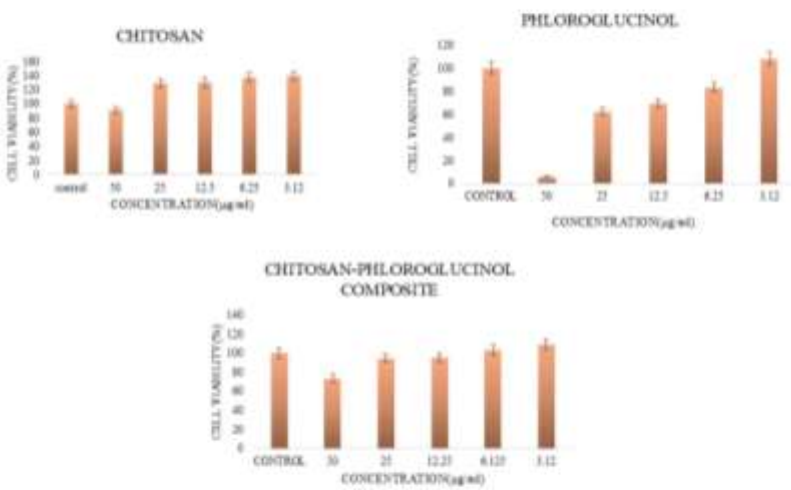

Fig 4 Effect of Chitosan Phloroglucinol Composite Against L929 Cell Line Using MTT Assay

In the present study, scratch assay in the L929 cell line was carried out to assess the impact of chitosan phloroglucinol composite on cell migration to confirm the wound healing activity. The scratched area decreased by 41.95±2.09% (Fig 5) in control cells, which did not receive any therapy, suggesting a baseline degree of recovery. Phloroglucinol-treated cells only displayed a slight decrease of 8.39±0.42%, indicating that it was not very effective at encouraging cell migration. The treatment with chitosan resulted in a 50.25±2.51% reduction in scratch area. The chitosan demonstrates some capability to promote healing. The chitosan-phloroglucinol combination, on the other hand, significantly reduced the scratched area by 93.15±4.65%. This finding is consistent with a previously published study (Kumbhar et al., 2023) that revealed that chitosan-treated scratches had more cells migrating towards the wound site and indicated greater wound closure than curcumin alone. Our present results also confirm the same pattern. In contrast to cell lines treated with phloroglucinol alone, the injection of a chitosan-phloroglucinol composite showed potential wound healing properties in our investigation. In vitro investigations by Felice et al. (2015) have demonstrated that thiolated quaternary ammonium-chitosan conjugates are viable therapeutic options for the clinical management of wounds. Previous reports by Huang et al. (2022) have shown that phloroglucinol derivative carbomer hydrogel accelerates MRSA-infected wound healing. Experimental studies by Bridi et al. (2021) validated the ability of phloroglucinol derivatives to stimulate in vitro cellular proliferation and reaffirmed the significance of *Hydricum* species as possible sources of chemicals that aid in wound healing. The synergistic effect of phloroglucinol and chitosan is most likely responsible for the observed improved wound healing performance.

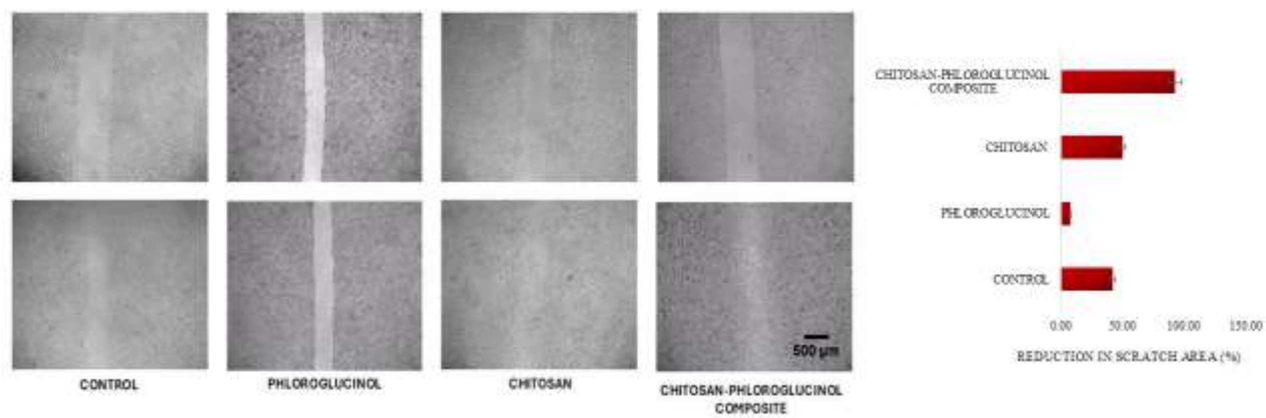

Fig. 5. Evaluation of Wound Healing Potential of Chitosan-Phloroglucinol Composite Using Scratch Assay in L929 Cells

4. Conclusion

In conclusion, the present *in-vitro* study shows that phloroglucinol and chitosan-phloroglucinol composite exerted a notable capacity to enhance cell viability at low concentrations in L929 cells. The chitosan-phloroglucinol composite can be considered as a therapeutic option for the wound healing process through enhanced cell migration. It is noticed that chitosan-phloroglucinol composite is not only confirming its non-toxic property but also indicating the capable to maintain the cellular growth, which is particularly promising aspect to be studied in detail for potential applications in tissue engineering and regenerative medicine. These findings demonstrate that the combination of chitosan and phloroglucinol significantly enhances wound healing potential and reduces its toxicity, highlighting its promising application in therapeutic interventions, subject to further investigation.

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