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In Vitro Evaluation of Cissus Quadrangularis Infused with Platelet Rich Fibrin and Methacrylated Hyaluronic Acid Based Scaffold for Periodontal Regeneration

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ABSTRACT

Introduction

Regeneration of the periodontal tissues is a challenging endeavor due to the complex anatomy and function of these structures. Cissus quadrangularis (CQ), a medicinal plant with known anti-inflammatory and osteogenic properties, has shown promising results in various tissue engineering applications. There has been growing interest in utilizing bioactive materials and advanced techniques to enhance periodontal regeneration.Therefore, the aim of the study is to fabricate and evaluate the novel cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold for periodontal regeneration.

Materials and methods

The hydrogel scaffold samples were prepared by mixing platelet-rich fibrin extract, methacrylated hyaluronic acid extract, and the natural product cissus quadrangularis, and then solidifying them in sample wells. The study groups consisted of the following scaffold types: methacrylated hyaluronic acid (HA) scaffold, methacrylated hyaluronic acid + cissus quadrangularis (HA+CQ) scaffold, methacrylated hyaluronic acid + platelet-rich fibrin (HA+PRF) scaffold, and methacrylated hyaluronic acid + platelet-rich fibrin + cissus quadrangularis (HA + PRF + CQ) scaffold. Scanning electron microscope (SEM) analysis was performed for the scaffold characterization. Swelling analysis, Compression strength analysis, MTT Compatibility Assay, Differentiation analysis and Osteogenesis Assay was also conducted.

Results

Morphology of the novel HA_PRF_CQ scaffold was analyzed using SEM which showed uniformly dispersed circular shapes. There was minimal swelling of 18% and maximum compressive strength of 29.24 N was observed in cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold. Maximum differentiation of cells was seen cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold. In MTT analysis, the highest value of cell viability was shown in HA_PRF_CQ scaffold (80%). The highest rate of osteogenesis was shown by the HA_PRF_CQ scaffold sample.

Conclusion

Within the limits of this in vitro study, cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold was highly biocompatible, has good compressive strength, low swelling and enhanced osteogenesis.

Keywords: Platelet rich fibrin, Cissus quadrangularis, Scaffold, Periodontal regeneration, Intrabony defects

INTRODUCTION

Periodontal disease is a prevalent oral health issue marked by the breakdown of the periodontium, which includes the gingiva, periodontal ligament, and bone structure (1).Regeneration of the periodontal tissues is a challenging endeavor due to the complex anatomy and function of these structures. Traditional treatments aim to manage the disease and prevent its progression, but achieving complete and predictable regeneration remains a clinical challenge(2). In recent years, tissue engineering strategies, particularly the use of scaffolds, have emerged as a promising treatment option for periodontal regeneration(3). Scaffolds provide a three-dimensional framework that supports cell attachment, proliferation, and differentiation, facilitating the regeneration of periodontal ligament, cementum, and alveolar bone(4). These scaffolds serve as temporary matrices that guide the formation of new tissue, ultimately leading to the restoration of periodontal architecture and function(5). Various scaffold materials, including synthetic polymers, natural polymers, and composite materials, have been investigated for their suitability in periodontal regeneration. Additionally, the design and fabrication of scaffolds mimic the native extracellular matrix of periodontal tissues(6) .

There has been growing interest in utilizing bioactive materials and advanced techniques to enhance periodontal regeneration. Among these materials, Cissus quadrangularis (CQ), a medicinal plant with known anti-inflammatory and osteogenic properties, has shown promising results in various tissue engineering applications (7). CQ is a perennial climber of the Vitaceae family that is typically found in India's drier regions. It is most often used in India where it is known as "hadjod" and is used to improve bone health (8). It exhibits anti-osteoporotic action through various pathways and mechanisms. Comprising superior natural matrices with well-known bioactivity, it has been extensively researched using cell lines and animal models, demonstrating its efficacy in combating a wide range of illnesses, including osteoporosis, arthritis, and gastric ulcers. (9).

Hyaluronic acid (HA) which occurs naturally in human body plays a important role in the extracellular matrix (ECM) (10). HA has been extensively used in periodontal and guided bone regeneration and has become a major focus, particularly in dentistry and maxillofacial surgery(11). The demand for bone regenerative therapy is high, ranging from maxilla augmentation to repairing cranial bone damage(12). HA-incorporated scaffolds and carriers play

a significant role in bone regeneration, available in either hard or colloidal forms, serving as cell-seeding scaffolds or carriers for bioactive components(13).

Platelet-rich fibrin (PRF) is another biocompatible material that has gained attention for its ability to stimulate tissue regeneration(14). PRF is produced from the patient's own blood, it has a high concentration of platelets, various growth factors and cytokines that will stimulate the soft tissue healing and periodontal tissue regeneration(15). A matrix composed of platelet-rich fibrin (PRF) contains all the cellular and molecular components necessary for optimal healing, representing a physiological concentrate that remains unaltered(16). Since PRF membranes are entirely specific to the donor and cannot be considered allogeneic graft tissue, they retain all circulating immune cells and highly allergenic plasmatic chemicals(17). Choukroun outlines the fundamental healing components of PRF when performing maxillary defect surgery. Immediately after ablation, the cavity is filled with blood, which constitutes a physiological or "lighter" form of PRF(18). Studies suggest that a cystic cavity typically takes between six and twelve months to heal; however, when filled with PRF, the healing process accelerates, likely due to the PRF's superior organization. PRF can effectively harness stem cells and promote recovery. Moreover, PRF is more homogeneous, stable, and easier to handle and apply locally compared to a genuine blood clot. When utilized as a resorbable membrane for guided tissue regeneration, platelet-rich fibrin can prevent undesirable cells (such as epithelial cells) from migrating into the bony defect, create space for osteogenic and angiogenic cells to infiltrate, and facilitate mineralization of the underlying blood clot (19). Therefore, the aim of the study is to fabricate and evaluate the novel cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold for periodontal regeneration.

MATERIALS AND METHODS

Cissus quadrangularis and methacrylated hyaluronic acid extract preparation

50 grams of powdered cissus quadrangularis were combined with 250 mL of ethanol and shaken for 24 hours at 120 rpm. The mixture was allowed to settle for another 24 hours, after which to obtain the extract of cissus quadrangularis the collection of supernatant was done and then subjected to flash evaporation. 2g of hyaluronic acid powder was dissolved in 100 mL of distilled water separately and stirred until a homogeneous viscous solution of hyaluronic acid was achieved.

Preparation of PRF

Venipuncture was used to take 10ml of intravenous blood from the antecubital fossa, which was then transferred to a sterile 10 ml tube without anticoagulants. The tube was then centrifuged at around 3000 rpm for 10 minutes. The acellular plasma from the tube's upper part was removed. The fibrin clot in the middle was then scraped away from the red blood cells in the lower area. Natural and gradual polymerization resulted in the creation of a fibrin clot containing considerable amounts of platelets and leukocyte growth factors. This clot can be squeezed between two pieces of gauze to create a autologous fibrin membrane.

Study groups

The preparation of hydrogel samples was done by mixing platelet-rich fibrin extract, methacrylated hyaluronic acid extract, and cissus quadrangularis, and then solidifying them in sample wells. The study groups consisted of the following scaffold types: methacrylated hyaluronic acid (HA) scaffold, methacrylated hyaluronic acid + cissus quadrangularis (HA+CQ) scaffold, methacrylated hyaluronic acid + platelet-rich fibrin (HA+PRF) scaffold, and methacrylated hyaluronic acid + platelet-rich fibrin + cissus quadrangularis (HA + PRF + CQ) scaffold.

Scanning Electron Microscopy (SEM) Analysis

To fix the hydrogel samples, they were submerged in a 4% paraformaldehyde solution overnight. Dehydration of the samples are done using a series of graded ethanol baths (varying from 10% to 100%). After dehydration, the samples are placed on aluminum stubs and gold-coated with a sputter coater at 37°C before being viewed under a scanning electron microscope (JEOL JSM-IT800 FE-SEM, JEOL Ltd., Tokyo, Japan) to take micrographs of the samples.

Swelling Analysis

The scaffold samples were first dry weighed. The scaffold samples were immersed in 5 mL of 10% PBS solution and incubated at 37 °C. After an incubation period of one hour, the prepared scaffold samples were removed from the solution. The extra fluid on the scaffold sample surface

was removed by a wipe. Swelling and fluid absorption of the scaffold was evaluated by first wet weighing the samples. The swelling analysis ratio was determined using the below mentioned formula, by comparing the dry weight (W0) and the wet weight (Ww):

Swelling ratio = ((Ww - W0) / W0) x 100%

Compression Strength Analysis

The mechanical strength of the scaffold samples was assessed by applying compressive forces using a universal testing machine (Instron E3000 universal testing machine, USA). A 0.2 mm thick piece of tin foil was inserted between the testing machine piston and the scaffold sample. A spherical steel tip with a diameter of 4 mm was then positioned on the scaffold sample's surface, and compressive strength was measured at a cross head speed rate of 1 mm/min. The compressive strength or maximum force values in newtons were tabulated and compared

MTT Compatibility Assay

In 96-well plates, scaffold samples were seeded (100 µL per well). After this, another 10 µL of MTT solution was added to each well. Then these scaffold samples were incubated for about 3.5 hours at 37°C temperature. Once the incubation time is over, to each scaffold sample well, 100 µL of DMSO solubilization solution was added , which will dissolve and following crystalline formazan product was formed.The cell viability and level of proliferation of different prepared samples was measured with the final absorbance at 570 nm .

Differentiation Analysis

In 24-well plates coated with 0.3% poly2-hydroxyethyl methacrylate the prepared scaffold samples were placed . This was done to avoid cell adhesion. The scaffold samples were then incubated at 37°C for one week. The samples were exposed and then treated with 2.5 mL of treated cell induction medium containing Dulbecco′s Modified Eagle′s Medium supplemented with ten percent fetal bovine serum and one percent penicillin and streptomycin. The differentiation degree of the scaffold samples was monitored using a contrast microscope (Labomed LB232 Contrast Microscope, USA).

Osteogenesis Assay (picrosirius red staining)

Seven days of induction period of scaffold samples were followed. Then, once the time period was over the conditioned media was withdrawn and the cells rinsed with PBS. They were then fixed in 70% ethanol solution for about 30 minutes and then rinsed for 3 times. The collagen was dyed with 0.1% picrosirius red in a saturated aqueous solution of picric acid. To quantify the stained nodules, dissolve the stain in 0.5 mL of 1:1 (vol/vol) 0.1% NaOH and absolute methanol for 30 minutes at room temperature. Then, 0.1 mL of the solubilized dye was added to each well of a 96-well plate, and the absorbance was measured at 540 nm.

RESULTS

Scanning Electron Microscopy (SEM) Analysis

The molecular morphology characteristics of the hydrogel samples are observed using scanning electron microscopy (JEOL JSM-IT800 FE-SEM, Tokyo, Japan) at 3.00 kV.

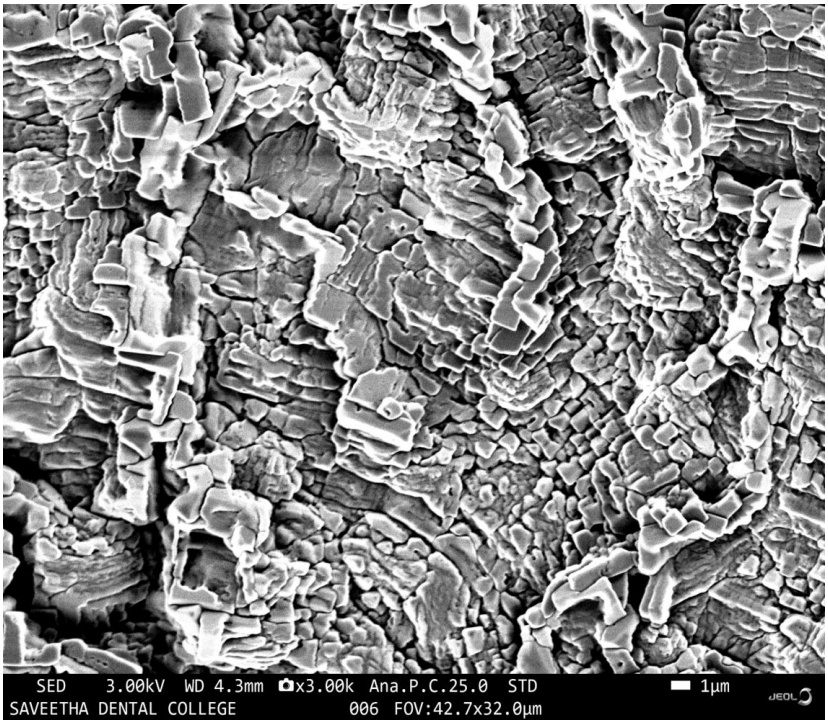

Figure 1: Scanning electron microscopy image showing non uniformly dispersed rectangular shape of HA scaffold

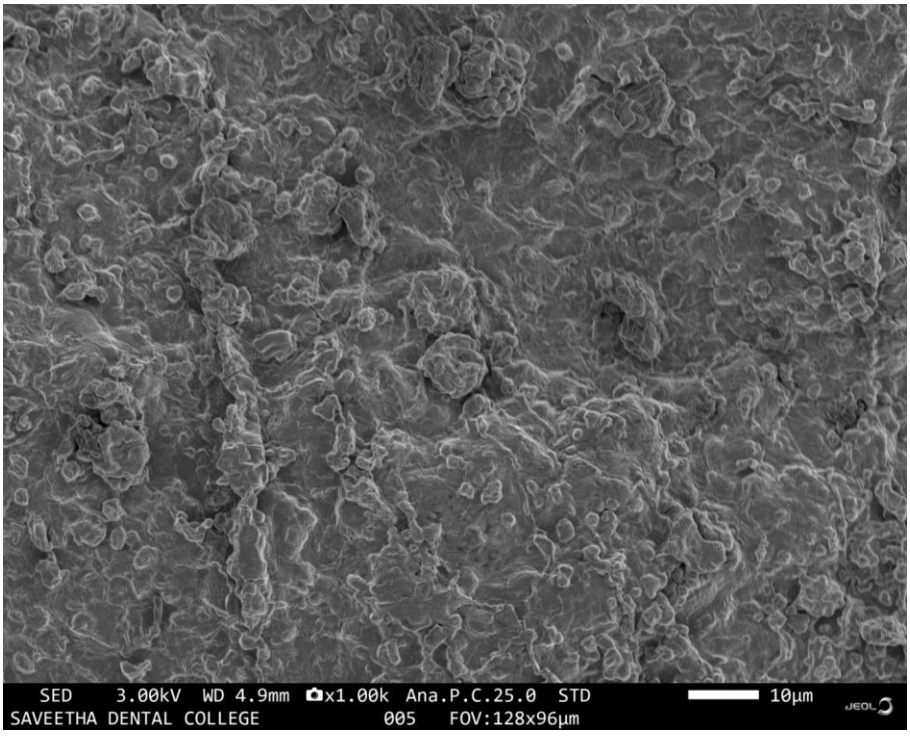

Figure 2: Scanning electron microscopy image showing non uniformly dispersed rhomboid shape of HA + CQ scaffold

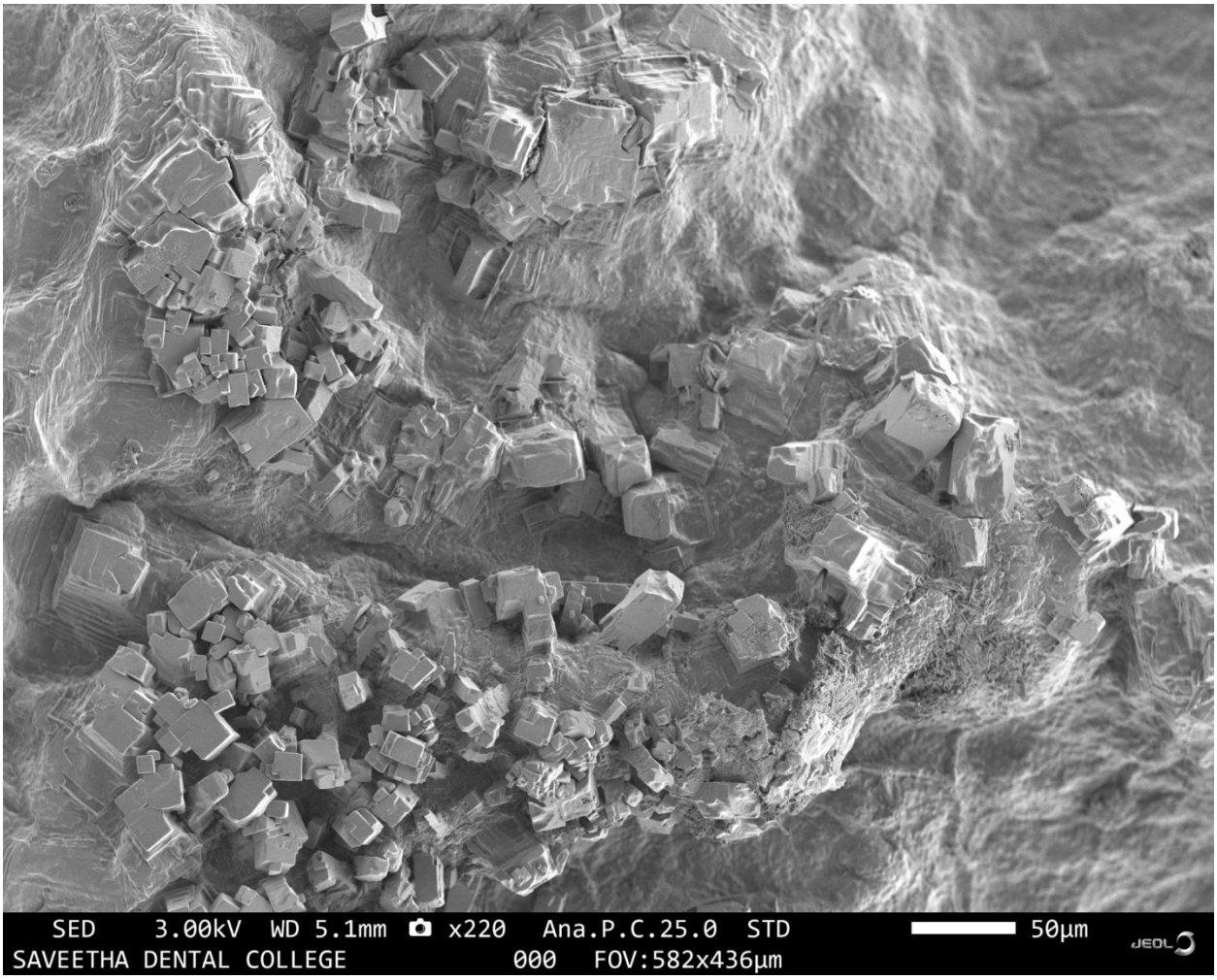

Figure 3: Scanning electron microscopy image showing irregularly shape of HA + PRF scaffold

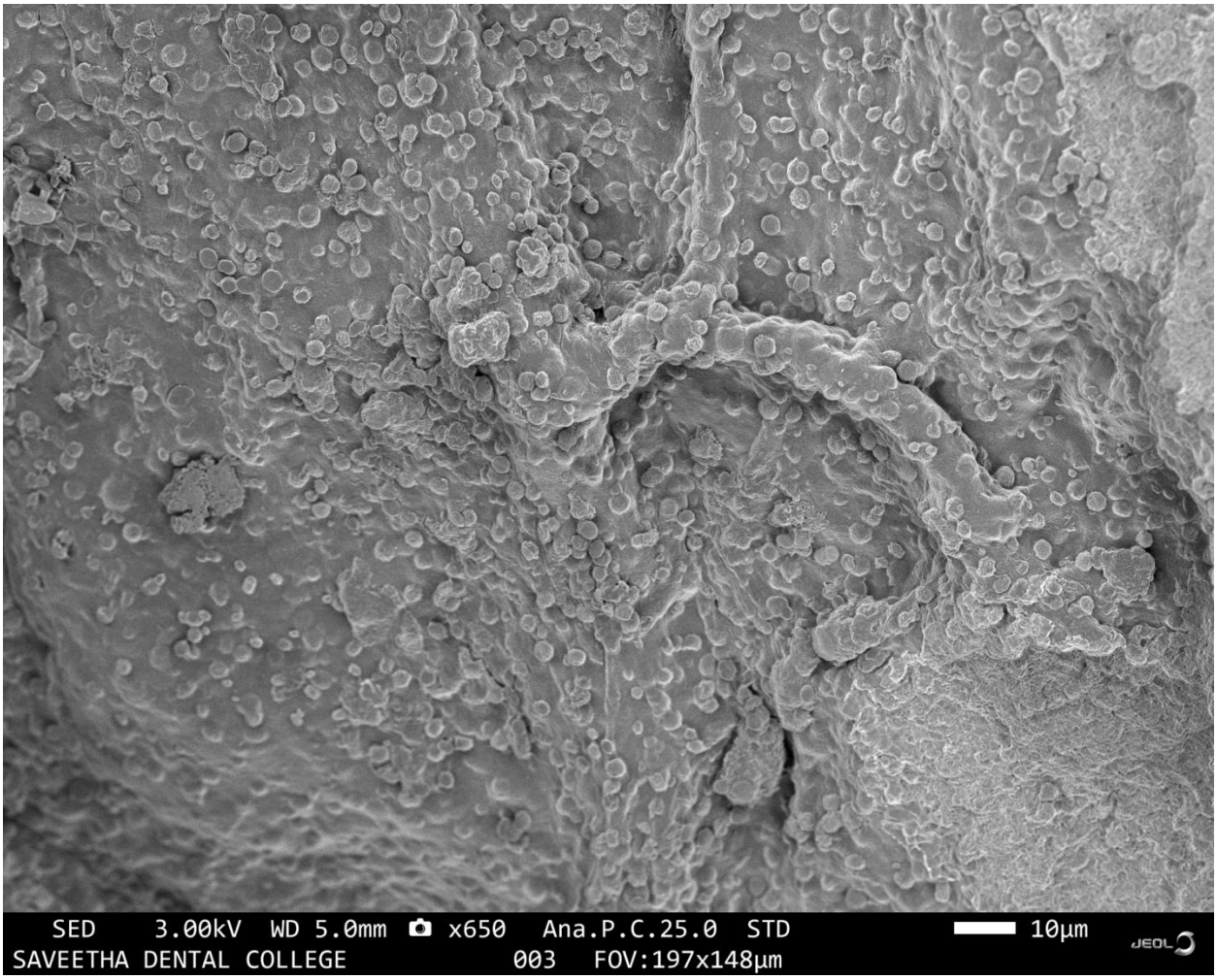

Figure 4: Scanning electron microscopy image showing uniformly dispersed circular shape of HA + PRF + CQ scaffold

SWELLING ANALYSIS

The fluid absorbed by each sample is quantified, and the values are compared as depicted. The HA + CQ + PRF scaffold exhibited a swelling of 18%, indicating its potential as a biomaterial for periodontal regeneration due to its minimal tissue swelling, allowing for proper nourishment of the tissues (Figure 5).

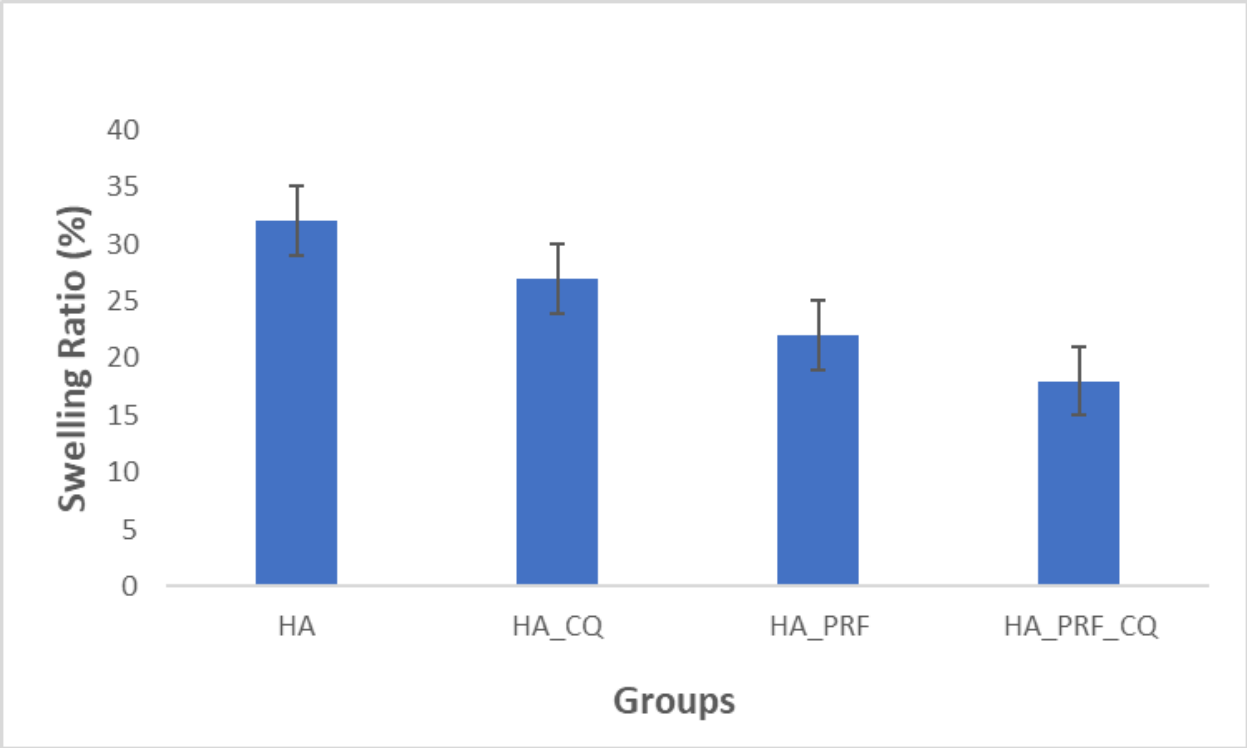

Figure 5: Swelling analysis showing maximum swelling of 32% for HA scaffold, 27% swelling for HA_CQ scaffold, 22% swelling for HA_PRF scaffold and minimal swelling of 18% was observed for the HA_PRF_CQ scaffold.

COMPRESSION STRENGTH ANALYSIS

The compressive strength of the scaffold samples was assessed by exposing them to compressive forces in a universal testing machine and their compressive limits were measured. The values are compared as illustrated.

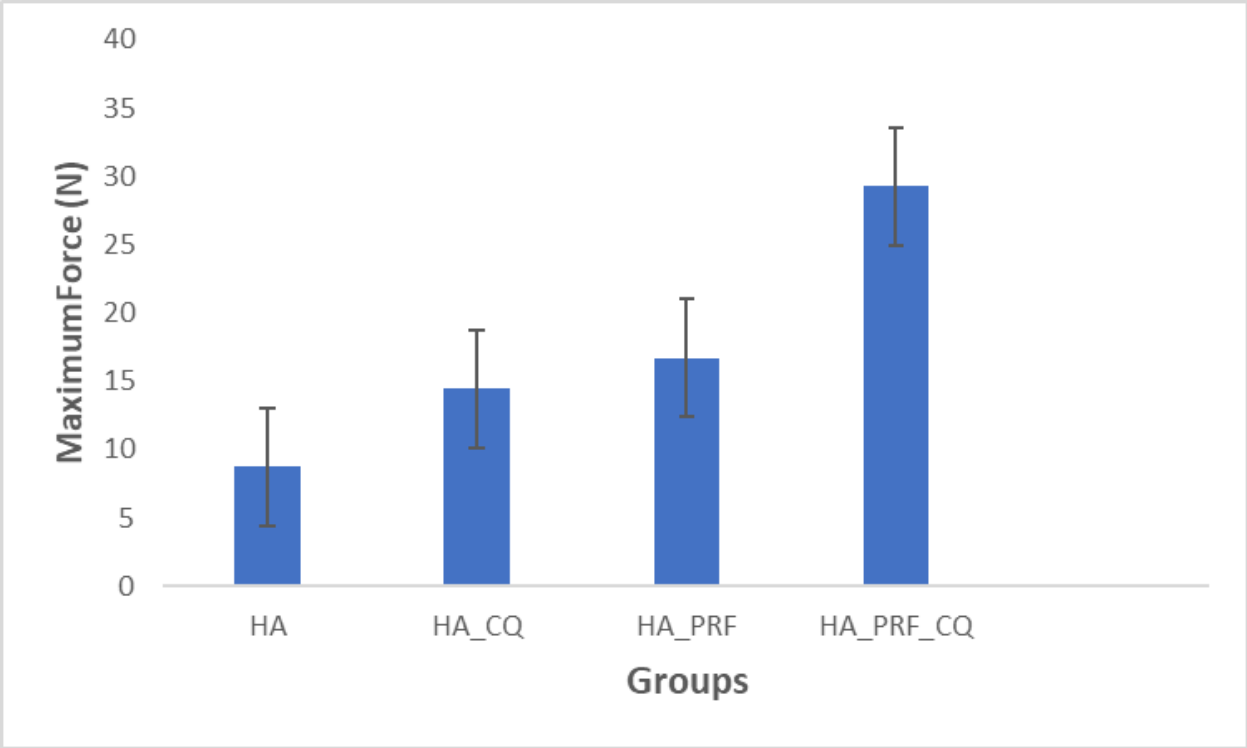

Figure 6: Compression analysis showing strength of 8.73 N for HA scaffold, 14.46 for HA_CQ scaffold, 16.72 N for HA_PRF scaffold and maximum strength of 29.24 N was observed for HA_PRF_CQ scaffold.

MTT COMPATIBILITY ASSAY

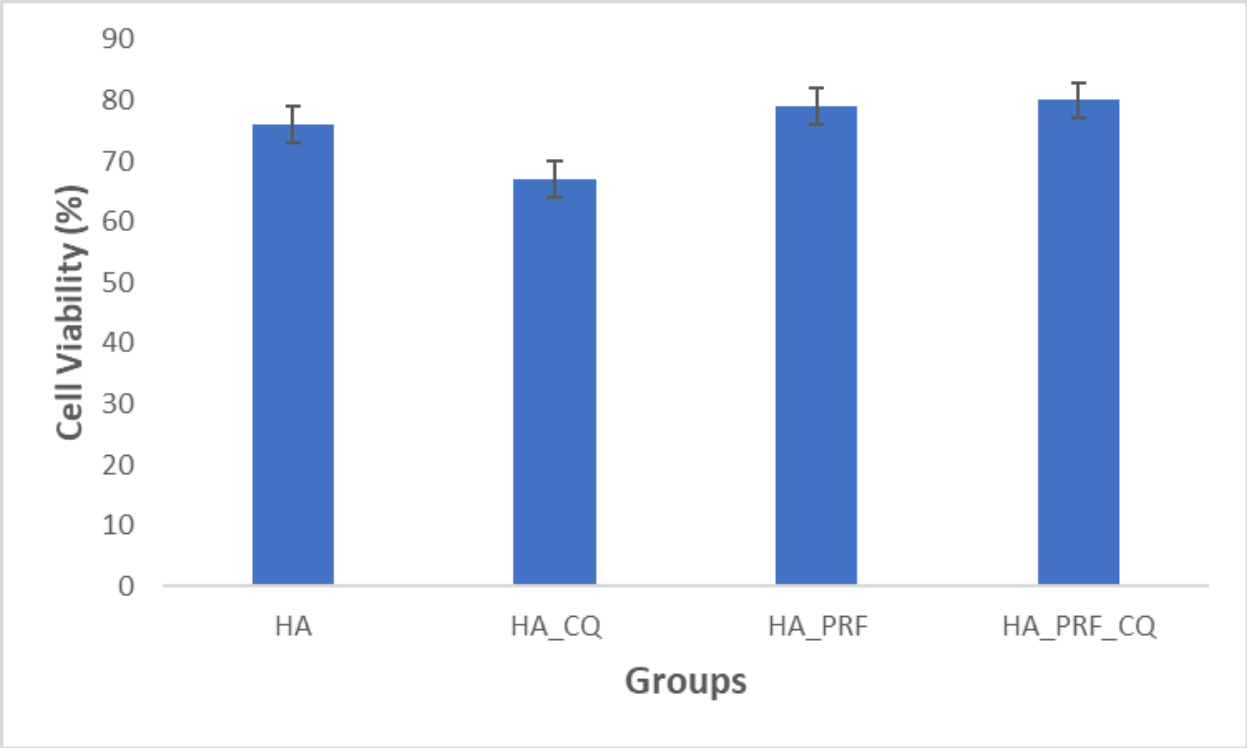

Figure 7: The MTT assay indicates comparable cell viability values for all samples, ranging from 65% to 75%. Highest value of cell viability was shown in HA_PRF_CQ scaffold (80%)

DIFFERENTIATION ANALYSIS

Differentiation analysis reveals the formation of tendon tissue from the scaffold, visualized at 20x magnification using a contrast microscope. Maximum differentiation was observed in the HA_PRF_CQ scaffold (Figure 10).

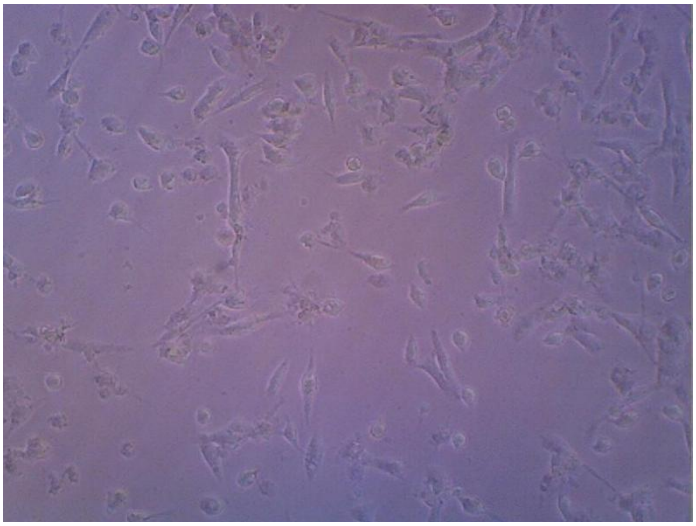

Figure 8: Contrast microscopy image of hyaluronic acid + Cissus quadrangularis (HA_CQ) scaffold

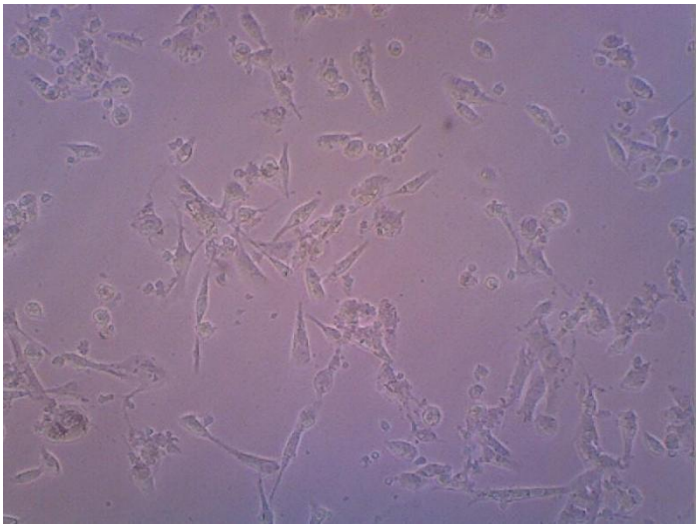

Figure 9: Contrast microscopy image of hyaluronic acid + platelet rich fibrin (HA_PRF) scaffold

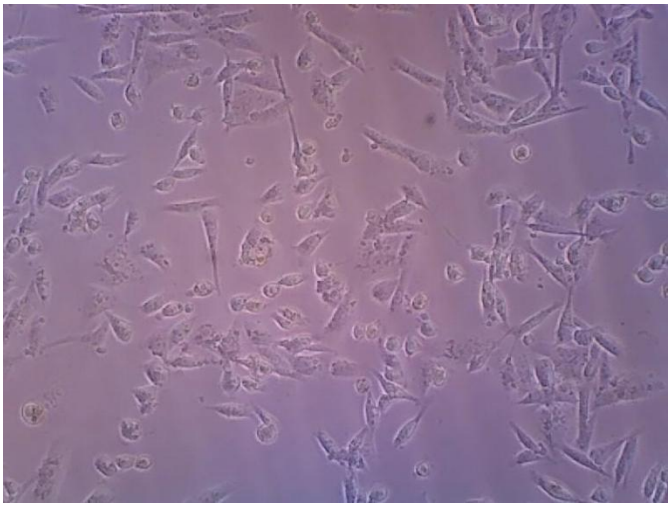

Figure 10: Contrast microscopy image of hyaluronic acid+ platelet rich fibrin + cissus quadrangularis (HA_PRF_CQ) scaffold

OSTEOGENESIS ASSAY

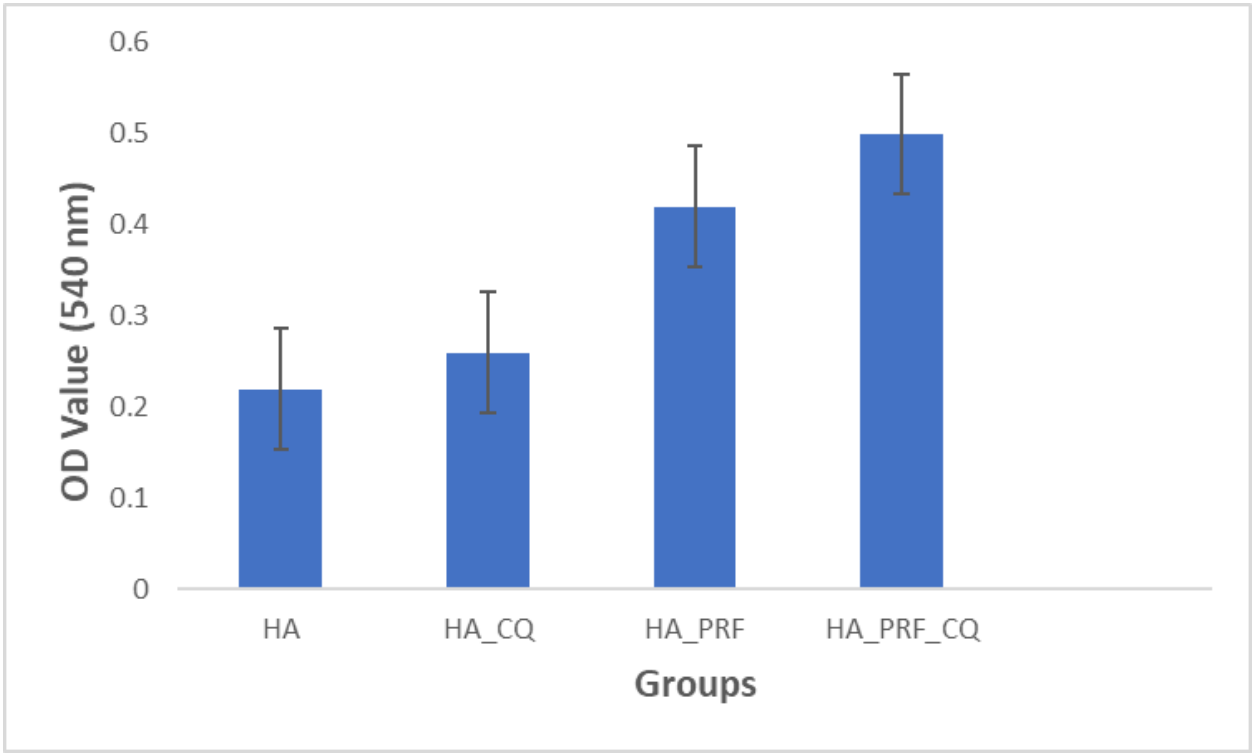

Figure 11: Osteogenesis assay by picrosirius red staining. The highest rate of osteogenesis is shown by the HA_PRF_CQ scaffold sample.

DISCUSSION

The choice of material is crucial in determining a scaffold's biological capabilities. HA derivatives or composite scaffolds containing HA have shown considerable promise in enhancing osteogenesis and mineralization. Such advanced biomaterial holds great potential as a tool in bone regeneration(11). Cissus quadrangularis has shown promising potential in the treatment of periodontitis. As an herbal remedy, Cissus quadrangularis, also known as "Hadjod" or "Veld grape," has been traditionally used in Ayurvedic medicine for various health conditions, including bone fractures and joint disorders(20). Cissus quadrangularis contains compounds with anti-inflammatory properties, which can help reduce inflammation, promote bone healing and regeneration, have antimicrobial properties, and stimulate collagen production(21).

Platelet-rich fibrin (PRF) is a biocompatible material that has garnered attention for its capacity to stimulate tissue regeneration. Derived from the patient's own blood, PRF contains a high concentration of platelets, growth factors, and cytokines that promote wound healing and tissue regeneration. (14). Collagen-based scaffolds have long been used in tissue engineering due to their biocompatibility and ability to mimic the extracellular matrix. These scaffolds provide structural support and promote cell adhesion, proliferation, and differentiation, making them ideal candidates for periodontal regeneration (22).Combining Cissus quadrangularis with PRF and a collagen-based scaffold presents a novel approach to enhance periodontal regeneration. The synergistic effects of these components, including anti-inflammatory, osteogenic, and regenerative properties, hold great potential for improving treatment outcomes in periodontal therapy(7).The study demonstrates that the combination of Cissus quadrangularis, PRF, and methacrylated hylauronic based scaffold has the potential to enhance periodontal regeneration.

Morphology of the novel HA_PRF_CQ scaffold was analyzed using SEM which showed uniformly dispersed circular shapes. Swelling ratio analysis is performed to evaluate how much the prepared scaffold expands upon absorbing tissue fluid. Ideally, the swelling ratio should be low to prevent the scaffold from expanding excessively in tissue fluid, which could potentially harm surrounding tissues or disrupt nearby tissue structures' functionality. From Figure 5, the swelling analysis shows a 32% increase for the HA scaffold, 27% for the HA_CQ scaffold, 22% for the HA_PRF scaffold, and minimal swelling of 18% for the HA_PRF_CQ scaffold (23).

To assess the mechanical strength and physical durability of the scaffold samples and ensure their ability to withstand physical activity or external impacts when implanted in the body, mechanical compression analysis is conducted. From Table 6, it can be seen that the maximum compressive stress is developed. The analysis reveals a strength of 8.73 N for the HA scaffold, 14.46 N for the HA_CQ scaffold, 16.72 N for the HA_PRF scaffold, and a maximum strength of 29.24 N for the HA_PRF_CQ scaffold (24). The survivability of the hydrogel samples is assessed using the MTT compatibility assay to determine the scaffolds' efficacy, appropriateness, and biocompatibility for tissue regeneration in the human body. Figure 7 shows that the highest cell viability is observed in the HA_PRF_CQ scaffold (80%), followed by 76% for the HA scaffold, 67% for the HA_CQ scaffold, and 79% for the HA_PRF scaffold. This indicates that incorporating platelet-rich fibrin and cissus quadrangularis into the hyaluronic acid hydrogel scaffolds, along with the extracellular matrix of the tendon, creates a highly viable and biocompatible scaffold (25) .

Picrosirius red staining reveals the improved osteogenic activity of cissus quadrangularis on periodontal ligament stem cells (PDLSCs) by measuring the absorbance (optical density (OD) value) at 540 nm. Figure 11 shows that the HA_PRF_CQ scaffold sample demonstrates the highest osteogenic rate, with an OD value of 0.5 at 540 nm. Comparing the values, it is evident that the addition of cissus quadrangularis and platelet-rich fibrin enhances the osteogenic potential (25).The bioactive properties of Cissus quadrangularis, including its anti-inflammatory and osteogenic effects, synergize with the regenerative properties of PRF and collagen, leading to improved tissue repair and regeneration. This suggests that the treatment has the potential to accelerate the healing process and promote the formation of new periodontal tissue.

Furthermore, it was found that PRF preparations are a significant source of growth factors, held in place by fibrin strands(26). Angiogenic efficacy was evaluated in the chorioallantoic membrane (CAM) test and endothelial cell cultures, showing that PRF/CGF preparations are slightly more effective at promoting angiogenesis than PRP preparations(27). When comparing the growth factor contents in four different types of PRP (PRP, PRGF, A-PRF, and CGF) made from similar donors, A-PRF and CGF formulations included TGF-β1, IL-1, IL-6, VEGF, PDGF-BB, and VEGF at levels comparable to or higher than PRP preparations(28). In another study comparing scaffold-free platelet-rich fibrin (PRF) to unactivated PRP (nPRP) as an adjuvant for

bone regeneration, similar osteogenic effects were observed. In a cranial defect model in New Zealand White rabbits, PRF showed comparable efficacy to nPRP in promoting bone regeneration. These findings suggest that PRF could be used as an alternative to PRP for bone regeneration, eliminating the need for a scaffold (29). Furthermore, the study investigated the effects of hyaluronic acid (HA) on periodontal ligament (PDL) cells' inflammatory response. Native high molecular weight (HMW) HA molecules do not trigger inflammatory cells, while low molecular weight (LMW) HA does. Treatment with HA-oligo increased the production of MMP-1 in cultured human PDL cells, suggesting a potential contribution to the degeneration of periodontal tissues.

Despite numerous studies demonstrating the growth factor contents and bioactivities of PRF and CGF preparations, some medical professionals still believe that fibrin clots alone are responsible for the regenerative benefits. This may be due to the lower concentration of certain growth factors in PRF preparations and variations in preparation procedures across different studies. Overall, these findings offer valuable insights into the potential applications of platelet concentrates and hyaluronic acid in periodontal regeneration. From this study, we see that the Cissus Quadrangularis Infused with Platelet Rich Fibrin and Methacrylated Hyaluronic Acid Based Scaffold can be used for periodontal regeneration. Because this is only an in vitro study, more research, including animal trials and extensive studies, is required to determine the feasibility of employing these hydrogels as a safe therapy option for treating periodontal intrabony defects and for furcation management.

CONCLUSION

Within the limits of this in vitro study, cissus quadrangularis infused with platelet rich fibrin and methacrylated hyaluronic acid based scaffold was highly biocompatible, has good compressive strength, low swelling and enhanced osteogenesis. This study concludes that natural bioactive compounds like cissus quadrangularis infused with autologous platelet derived concentrate along with naturally occurring linear polysaccharide like methacrylated hyaluronic acid may offer promising results promoting periodontal regeneration.

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