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Embryonic toxicology evaluation of novel *Cissus quadrangularis*, bioceramics and tendon extracellular matrix incorporated scaffolds for periodontal bone regeneration using zebrafish model

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ABSTRACT

Introduction: The development of novel scaffold incorporating *Cissus quadrangularis* extract, bioceramics and tendon extracellular matrix (ECM) for periodontal bone regeneration necessitates a thorough assessment of their embryotoxicity to ensure biocompatibility and safety. This study evaluates the embryonic toxicology of these innovative scaffold using the zebrafish model, which provides a rapid transparent and highly sensitive system for assessing development toxicity.

Materials and methods: Zebrafish embryos were exposed to scaffold containing *Cissus quadrangularis* extract (80 % ethanol), bioceramics, and tendon extracellular. Briefly, the scaffold immerged in the E3 medium for 48h and the extract (10, 50 and 100µl/ml) was evaluated against developing embryos for different developmental anomalies such as survival, malformation, heartbeat and the expression of RunX2 and Bmp2 genes.

Results: The results showed that the SEM analysis revealed that the membrane was rough in nature. FTIR analysis confirmed the presence of hydroxylate groups, collagen and hydroxyapatite in the synthesized membrane. Meanwhile, the scaffold did not show any of the developmental defects such as hatchability inhibition and neural toxicity. The mortality was comparable to negative control. Further, the study revealed that the scaffold induced the osteogenic potential by elevating the RunX2 and Bmp2 expression.

Conclusion: This study highlighted the inclusion of C. *quadrangularis* extract could be a beneficial for enhancing periodontal bone regeneration. Further, this also revealed that the extract did not impede the normal tissue development or regeneration using zebrafish embryo as a model.

1. Introduction

Periodontal bone loss is a significant issue affecting millions worldwide. It is primarily caused by periodontitis, a chronic inflammatory condition that leads to the destruction of alveolar bone and soft tissue surrounding the teeth. It untreated, periodontal disease can result in tooth loss, decreased quality of life, and increased susceptibility to systematic diseases such as cardiovascular disorders and diabetes.[1,2] Restoring lost periodontal bone remains a major challenge in regenerative medicines, necessitating the development of biomaterials that can effectively stimulate bone formation and integration. Scaffold based tissue engineering has emerged as a promising strategy for periodontal bone regeneration. Scaffold provide a three-dimensional structure that facilitates cell attachment, proliferation, and differentiation while serving as a temporary extracellular matrix (ECM) to support new tissue growth.[3] In recent years, bioactive scaffolds incorporating bio ceramics,

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natural bioextracts, and ECM components have gained significant attention due to their ability to enhance osteogenesis and improve clinical outcomes. Bioceramics such as hydroxyapatite (HAp) and bioactive glass (BG) plays a crucial role in bone tissue engineering. These materials mimic mineral composition of natural bone, providing a bioactive interface that promotes osteoblast adhesion and differentiation. Bioceramics also exhibit excellent biocompatibility and osteogenicity, making them suitable for periodontal applications. However, their brittle nature necessitates their incorporation in to composite scaffolds to improve mechanical properties and biofunctionality.[4]

Natural bioextracts derived from plants and marine sources have recently been explored for their regenerative potential. These bioactive compounds exhibit anti-inflammatory, antimicrobial, and osteogenic properties, which are critical for promoting bone healing in periodontal defects. Bioextracts such as polyphenols, flavonoids, and alkaloids have demonstrated the ability to enhance mesenchymal stem cell differentiation onto osteoblasts, modulate inflammatory pathways, and inhibit bacterial biofilm formation. Incorporating these bioextracts into scaffold can potentially enhance their regenerative capabilities while reducing inflammation and microbial infections.[5] *Cissus quadrangularis* is one of medicinal plant widely recognized for its bone-healing, anti-inflammatory, antioxidant and regenerative properties. It is traditionally used in Ayurveda and modern medicine for fracture healing, wound repair and tissue regeneration, making it a promising candidate for therapeutic applications. One of its bioactive mechanisms lies in its ability to enhance osteogenesis and fibroblast proliferation, promoting faster tissue regeneration. It is rich in flavonoids, triterpenes and phytosterols.[6]

Tendon extracellular matrix (T-ECM) is another promising component for scaffold based periodontal regeneration. Tendon ECM is rich in collagen, glycosaminoglycans, and growth factors that provide a natural microenvironment conductive to bone and soft tissue healing. The structural and biochemical composition to tendon ECM closely resembles the naive periodontal ligament, making it an ideal candidate for promoting periodontal attachment and bone regeneration.[7] Additionally, tendon ECM based scaffolds have been shown to support osteoblast proliferation and differentiation while providing mechanical stability to the regenerative construct. While these bioactive materials hold great promise for periodontal bone regeneration, their safety and biocompatibility must be rigorously eventuated before clinical applications.[4] One of the most effective models for embryotoxicity and developmental toxicity screening is the zebrafish (*Danio rerio*) model. Zebrafish embryos are widely used in toxicological studies due to their genetic similarity to mammals, rapid development, and optical transparency, which allows real-time observation of developmental processes. Moreover, zebrafish models enable high-throughput screening of biomaterials, making them as invaluable tool for assessing the safety of novel scaffold formulations.[8,9]

The use of zebrafish embryos for evaluating embryotoxicity provides several advantages. The external development of zebrafish embryos allows for direct exposure to test compounds in a controlled environment, facilitating the assessment of teratogenic effects and morphological abnormalities. Additionally, zebrafish share key developmental signalling pathways with humans, including bone morphogenetic proteins (BMPs).[8] Wnt signalling, and fibroblast growth factors (FGFs), making them a relevant model for studying osteogenesis-related toxicity. Scaffold based strategies incorporating bioceramics, bioextracts, and ECM components represent a promising approach for periodontal bone regeneration. However, ensuring their safety through rigorous embryotoxicity testing is essential for successful clinical translation. The zebrafish model serves as an efficient and reliable system for evaluating the biocompatibility and potential development effects of these biomaterials.[10] Through this study, we aimed to bridge the gap between innovative scaffold design and their safe application in periodontal regenerative therapies.

2. Materials and Methods

The fabrication of plant extract, bioceramics and tendon extracellular matrix (ECM) incorporated scaffolds for periodontal bone regeneration involved multiple stages, including material preparation, scaffold synthesis, *in vitro* biocompatibility assessment, embryonic toxicity evaluation using zebrafish.[11]

To prepare the bioactive plant extract *Cissus quadrangularis,* was collected from Chennai and processed. The plant materials were washed, shade dried at 40 °C for 48h and grounded into a fine powder. The extraction was carried out using 80 % ethanol via Soxhlet extraction for 48h, followed by filtration and evaporation under reduced pressure using a rotary evaporator at 40 °C.[12] The extract was stored at −20 °C until further use. Hydroapatite (HAp) bioceramics were synthesized using the wet precipitation method, in which calcium nitrate and diammonium phosphate were mixed at a Ca/P molar ratio of molecular ratio as 1.67. The solution was stirred at 80 °C for 4h, aged for 24h, washed, dried and calcined at 800 °C to obtain crystallized Hap. The synthesized bioceramics was characterized using XRD, to determine crystallinity, SEM for morphological analysis and EDX for elemental composition.[13] Tendon ECM was obtained from bovine Achilles tendon and subjected to a decellularization process. The tendon was treated with 0.1 % Triton X – 100 and 1 % SDS for 72h followed by enzymatic digestion with DNAs and RNAs to remove the cellular debris. The processed ECM was lyophilized and ground into a fine powder for scaffold fabrication. The scaffold was prepared using freeze drying method. The composite solution containing 40 %, 30 % HAp, 10 % ECM and 10 % plant extract was homogenized and freeze dried at −80 °C for 24h. The scaffold was cross linked with 0.5 % glutaraldehyde and sterilized using UV method. The physio chemical properties after scaffold been evaluated using SEM (JEOL JSM-IT800), XRD (Bruker D8 Advance)and FTIR (PerkinElmer, USA) method.[11]

The embryonic toxicology evaluation was performed using zebrafish (*Danio rerio*) embryos-maintained 28 °C in E3 medium. The scaffold was extracted with sterile E3 medium by immerging method for 48h. Further, the scaffold extract was tested using 10, 25, 50 and 100 µl/ml. The hatching and survival rates were recorded at 24, 48 and 72h of post fertilization (pfh) respectively.[14] The morphological changes were assessed under the stereomicroscope, and gene expression analysis were conducted for osteogenic (Runx2 and Bmp2). All statistical analysis were conducted using Graphpad prism (ver 7). The data were analysed using one-way ANOVA followed by Tukey post hoc test. RT-qPCR data were normalized using the ΔΔCt method with GADPH as the house keeping gene.[15,16]

3. Results

3.1. Scanning electron microscopy (SEM) analysis

The surface morphology of the sample was analysed using SEM. The imaging was performed at an accelerating voltage of 3 kV, with a working distance of approximately 5.06 mm. The magnification was set at 150x, providing a detailed view of the sample's microstructural characteristics within a field of view of 853 × 640 µM. The SEM image, captured using scan time of 7.383 s ensured high-resolution imaging, emailing precise analysis of the surface morphology (Fig. 1). The microstructural examination showed well-defined surface features with distinct morphological patterns. The observed texture suggested that the synthesized membrane was rough in surface to the maximum extent and the integrated with the nanoparticles. This result supported its potential application in biomedical research.

3.2. X- ray diffraction (XRD) analysis

The X-ray diffraction (XRD) analysis was conducted to evaluate the crystalline nature and phase composition of the *C. quadrangularis*

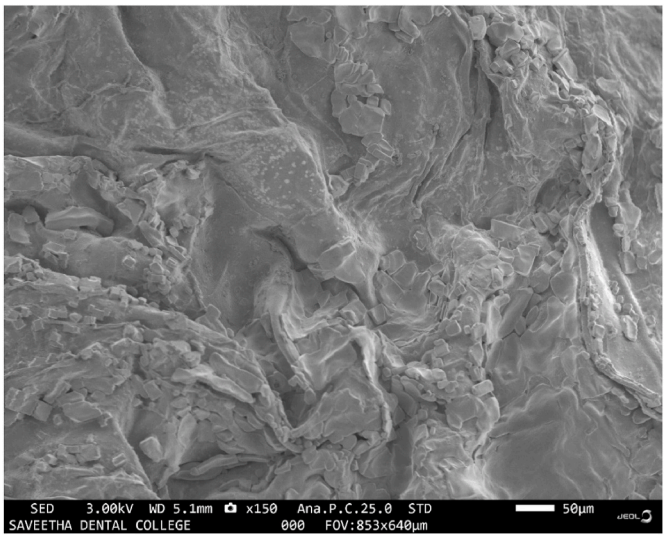

Fig. 1. SEM image of synthesized scaffold with *Cissus quadrangularis* extract, and tendon extracellular matrix (ECM).

incorporated bioceramics and TECM incorporated scaffolds. The diffraction pattern revealed characteristic peaks at 17.3°, 21.148°, 23.713°, 20.222°, 29.901°, 24.238° and 47.48° (2θ), confirming the presence of both organic and inorganic phases within the scaffold. The peaks at 17.3° and 21.148° suggested an amorphous or semi-crystalline structure, likely arising from compounds of *C. quadrangularis* extract (Fig. 2). The extract might be included with characteristic antioxidant, anti-inflammatory, and anti-regenerative properties. Peaks at 23.713° and 20.222° are indicative of collagenous or proteinaceous structures, confirming the incorporation of tendon ECM components into the scaffold matrix. The presence of these organic biomolecules enhanced the biocompatibility, cell adhesion and mechanical resilience of the scaffold, which was essential for tissue engineering applications. µThe most prominent peak at 29.901° corresponded to the hydroxyapatite (HA), a key mineral phase in bio-ceramics known for its osteo-conductivity and structure similarity to natural bone. This confirmed the successful formation and integration of HA nanocrystals within the scaffold, supporting its potential application in bone tissue engineering. Additionally, the peak at 24.238° suggested the presence of calcium phosphate phases, which further enhanced the biocompatibility of the scaffold by promoting cell proliferation and differentiation. A high-intensity peak at 47.483° suggested well-formed crystalline structures,

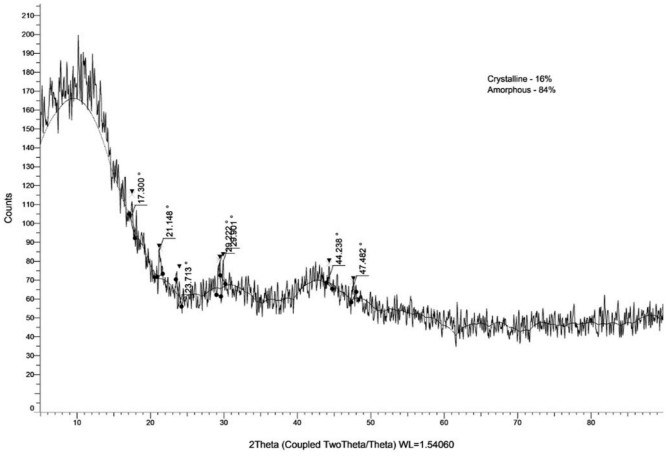

Fig. 2. XRD image of synthesized scaffold with *Cissus quadrangularis* extract, and tendon extracellular matrix (ECM).

likely associated with mineralized components that contributed to the scaffold's mechanical strength and stability.

3.3. Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR analysis was performed to identify the functional groups present in the composition of the *C. quadrangularis* incorporated bioceramics and TECM incorporated scaffolds (Fig. 3). The characteristic absorption bands observed at 3398 cm^{-1}, 2881.1 cm^{-1}, 1644.3 cm^{-1}, 1453.7 cm^{-1}, 1350.8 cm^{-1}, 1248 cm^{-1}, 1064.8 cm^{-1}, 1034.7 cm^{-1}, 923.07 cm^{-1}, 842.70 cm^{-1}, 698.54 cm^{-1}, 600.7 cm^{-1}, 570.6 cm^{-1} and 442.65 cm^{-1} provide insights into the chemical composition and successful integration of bioactive compounds within the scaffold. The peak at 3398 cm^{-1} corresponded to the O-H stretching vibration of hydroxyl groups, indicating the presence of water molecules, polyphenols or hydroxylated bioceramic components such as hydroxyapatite. The band at 2881.1 cm^{-1} indicated the presence of C-H stretching vibrations of aliphatic hydrocarbons, confirmed the lipid or carbohydrate components from both plant-based and ECM-derived proteins. 1644.3 cm^{-1} reflected due to the amide I band, characteristics of proteinaceous materials like collagen from tendon ECM. 1453.7 cm^{-1} corresponded to the C-H bending vibrations, often associated with proteins, carbohydrates and lipids due to organic matrix components. 1350.8 cm^{-1} showed the presence of C-N stretching from amide II vibrations, further validating the presence of collagen and ECM proteins in the scaffold. 1248 cm^{-1} was attributed to phosphate stretching vibrations (PO_4^{3-}), indicating the presence of bioceramic components like hydroxyapatite or calcium phosphate. 1064.8 cm^{-1} and 1034.7 cm^{-1} corresponded to C-O stretching from polysaccharides or phosphate vibrations. The FTIR confirmed the successful incorporation of plant bioactive compounds and ECM derived proteins into bioceramic scaffold.

3.4. Survival and hatching rates

Embryos exposed to lower scaffold extract concentrations (1–10 µL mL^{-1}) exhibited survival rates above 90 % while higher concentrations (50–100 µL mL^{-1}) led to a decline in viability, with significant mortality observed at 100 µL mL^{-1}, whereas control group exhibited a 100 % (Fig. 4). The hatching rate followed a similar trend with embryos exposed to 1 µL mL^{-1} showing a 95 % hatch rate, while at 100 µL mL^{-1}, only 40 % of embryos successfully hatched by 72hpf. Statistical analysis using one-way ANOVA and post hoc Tukey's test confirmed significant differences between groups, emphasizing the impact of increasing concentrations on embryonic development. The findings suggested that while lower concentrations of the extracts exhibited negligible toxicity, higher concentrations significantly showed reduction in viability and hatching rate (Fig. 5).

3.5. Heart rate analysis

Heart rate measurements at 48 and 72 hpf demonstrated no significant alterations at low scaffold extract concentrations. However, exposure to 100 µL mL^{-1} resulted in a 10 % reduction in heartbeats. The zebrafish embryo mortality test was conducted over a 72-h period, and the results are summarized in Fig. 6. The control group exhibited a gradual increase in mortality, starting from 0.2 at 24 h and rising to 1.5 by 72 h. At the lowest concentration (1 µL mL^{-1}), mortality was slightly higher than the control at 24 h (0.4), with a minor increase at 48 h (1.2) but a decrease at 72 h (1.1), indicating potential adaptation or delayed toxicity. At 10 µL mL^{-1}, mortality followed a similar trend, increasing from 0.6 at 24 h to 1.3 at 48 h, but declining to 1.o at 72 h. Higher concentrations (50 and 100 µL mL^{-1}) resulted in more pronounced effects. At 50 µL mL^{-1}, mortality was 0.8 at 24 h, remained at 1.2 at 48 h and significantly increased to 2.2 at 72 h. The highest concentration (100 µL mL^{-1}) showed the greatest mortality, with 0.8 at 24 h, 1.4 at 48 h, and the peak of 2.9 at 72 h. This dose-dependent increase in mortality

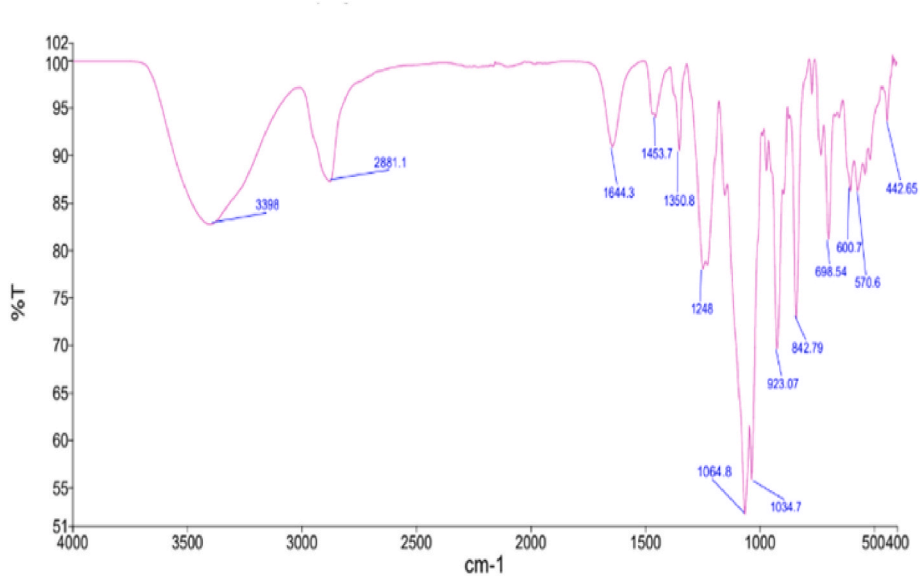

Fig. 3. FTIR image of synthesized scaffold with *Cissus quadrangularis* extract, and tendon extracellular matrix (ECM).

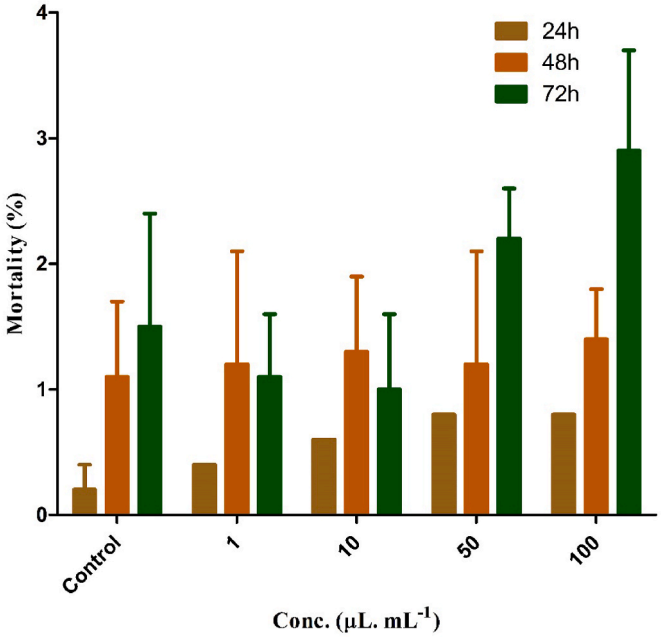

Fig. 4. Effect of scaffold extract in mortality ratio in zebrafish embryos.

at higher concentrations suggests acute toxicity over time. Overall, the results indicate that while lower concentrations exhibit moderate toxicity, higher doses (50 and 100 µL mL^{-1}) lead to increased mortality over time, highlighting potential developmental toxicity in zebrafish embryos.[17]

3.6. Lordosis

The zebrafish embryo test for lordosis was conducted over a 72 h period, and the results indicate that no lordotic deformities were observed in any of the tested concentrations. The control group showed zero instances of lordosis, confirming normal development under standard conditions. Similarly, embryos exposed to increasing concentrations (1,10,50, and 100 µL mL^{-1}) also exhibited no lordotic abnormalities, suggesting that the tested compound did not induce spinal curvature defects at any concentration within the experimental timeframe.

Lordosis, a spinal deformity often associated with developmental toxicity, can result from chemical exposure, oxidative stress, or genetic alterations. The absence of lordosis in all groups indicates that the compound, even at the highest dose (100 µL mL^{-1}), does not cause visible structural abnormalities in the zebrafish spine within 72 h. This suggests that, under the tested conditions, the compound does not interfere with axial development or musculoskeletal integrity. Further studies with extended exposure periods, histological analysis, and additional morphological assessments could provide deeper insights into potential long-term effects. Additionally, evaluating other skeletal deformities such as scoliosis or kyphosis could help determine if the compound affects vertebral integrity beyond the 72-h period.

3.7. Gene expression analysis

The RT-PCR results indicate a dose-dependent upregulation of Runx2 and BMP2, key osteogenic markers involved in bone formation (Fig. 7a and b). The control group maintained a baseline expression of 1.0 for both genes. At 1 µL mL^{-1}, Runx2 and BMP2 expression showed a slight increase (1.2 fold and 1.1 fold, respectively), suggesting early osteogenic activation. At 10 µL mL^{-1}, the expression levels increased further (Runx2: 1.5 fold, BMP2: 1.3 fold), indicating enhanced bone differentiation. At higher concentrations (50 and 100 µL mL^{-1}), Runx2 reached 2.1 fold and 2.8 fold expression, while BMP2 showed a similar trend (1.8 and 2.3 fold, respectively), suggesting a significant pro-osteogenic effect (Fig. 7a and b). These findings suggest that the compound positively regulates osteogenic differentiation in zebrafish embryos, potentially promoting bone development. Further validation through protein analysis and histological examination will help confirm these molecular findings.

4. Discussion

The embryonic toxicity assessment revealed critical insights onto the safety and biological impact of the bioextract, bioceramics, and tendon-ECM-incorporated scaffolds. The survival rate of zebrafish embryos remained above 98 % (approximately) at low and moderate scaffold extract concentrations, indicating the biocompatibility of the tested materials. The integration of plant extracts, bioceramics, and tendon-derived extracellular matrix (ECM) into scaffolds for periodontal bone

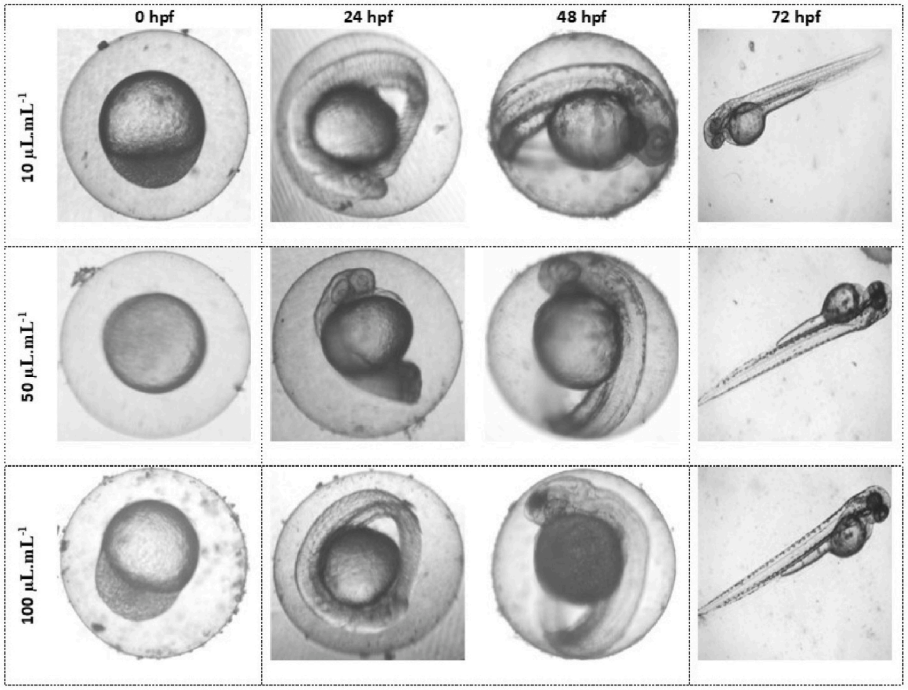

Fig. 5. Scaffold extracts showed no significant developmental defects in morphology.

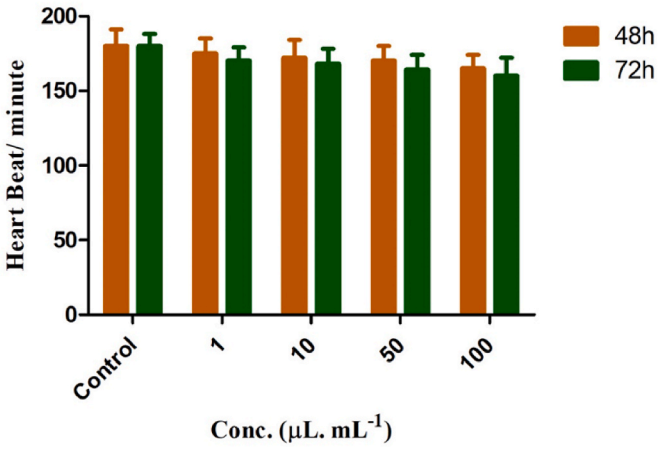

Fig. 6. Effect of scaffold extract in heart beat per minute in zebrafish embryos.

regeneration represents a multiphase approach aimed at for enhancing tissue repair and regeneration.[18] This strategy leverages the bioactive properties of natural compounds, structural support for bioceramics and biological clues provided by ECM components. The discussion herein delves into the implications of these components supported by recent literatures, to elucidate the role and synergistic effects in periodontal regeneration.[19]

The plant extracts are rich in bioactive compounds such as polyphenols, flavonoids, and alcoholics, which have been documented to exhibit the anti-inflammatory, antioxidant, anti-microbial properties. These properties are particularly beneficial in the context of periodontal regeneration, where inflammation and microbial activity can impede healing processes. Incorporating plant extract in to scaffolds can modulate the local microenvironment, promoting the tissue regeneration. For instance, Nambiar et al. (2022) emphasized the role of natural product-based scaffolds in various tissue regeneration related applications, further their potential in enhancing angiogenesis and providing necessary clues for tissue repair.[20] This aligns with the current approach of interacting plant extracts to harness their bioactive potentials in promoting the periodontal tissue regeneration.

Bioceramics, particularly calcium phosphate-based materials like hydroxyapatite (HAp) is extensively utilized in bone tissue engineering due to their excellent biocompatibility and osteoconductivity.[21,22] They provided scaffold that not only support the cell attachment and proliferation, but also, participates in bone remodelling process. Recent advancements have seen significant progress in scaffold design and their incorporation due to dynamic and bio-responsive features reflecting a shift towards scaffold capable of adopting to and mimicking the natural extracellular matrix of bone. This perspective underscores the importance of designing the scaffold that not only provide the structural support but, also, actively engaged in the biological process of bone regeneration.[11,22]

The ECM derived from tendon tissue offers a rich source of collagen and matrix proteins that are crucial for tissue regeneration. The decellularized tendon- ECM retains the native biochemical clues and structural integrity necessary for cell attachment, proliferation, and differentiation. Incorporating the tendon- ECM in to scaffolds can enhance the bioactivity, promoting the regeneration of periodontal tissues.[23] In the context of embryonic toxicology evaluation for novel bioextract, bioceramics, and tendon, extracellular matrix (ECM)-incorporated scaffold for periodontal bone regeneration, a thorough investigation into the safety profile of these materials is imperative. The design of scaffolds for tissue engineering applications, particularly for proliferation, requires an intricate balance between promoting cell proliferation and differentiation while minimizing any potential toxic effects on the surrounding tissue, including those of the developing embryos. The zebrafish model with its transparent embryos and rapid development, provides a unique in vivo system for evaluating the embryonic toxicity of scaffold materials and their counterparts. The safety of plant extracts, bioceramics and tendon ECM materials when incorporated into scaffolds, must be rigorously assessed to ensure their biocompatibility and avoid adverse effects on embryonic development and tissue formation.[24] In conclusion, the embryonic toxicology evaluation of bioextract, bioceramics, and tendon ECM-incorporated scaffolds for periodontal bone regeneration using the zebrafish model is an essential step in ensuring the safety and efficacy of these materials. A

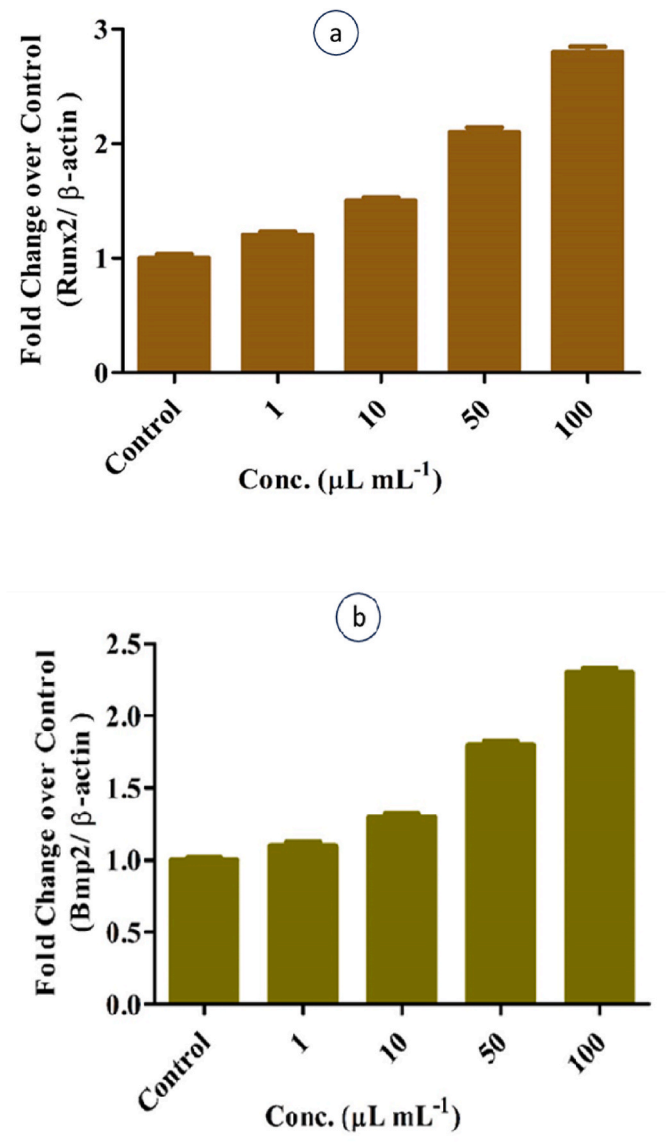

Fig. 7. The gene expression analysis of the wound healing responsible genes in the treated and control groups. (a) Runx2, (b) Bmp2 genes.

multifaced approach that includes the assessment of developmental, systemic, and oxidative stress responses, along with careful consideration of scaffold degradation, is necessary to guarantee the materials' bicompactibility. This comprehensive evaluation lays the foundation for the design of scaffolds that are not only effective in promoting tissue regeneration but also safe for use in clinical applications.

5. Conclusion

This study demonstrated a dose-dependent effect of the *Cissus quadrangularis* extract, bioceramics and tendon extracellular matrix (ECM) on zebrafish embryos. Higher concentrations slightly increased mortality, hatchability as lesser than 1 % compared with the controls. No lordosis was observed suggested that no immediate skeletal deformation, RT PCR analysis revealed an upregulation of Runx2 and Bmp2, highlighting its osteogenic potential. SEM, FTIR and XRD analyses confirmed morphology and stability. These results should that *Cissus quadrangularis* extract, bioceramics and tendon extracellular matrix (ECM) scaffold could be an excellent candidate for osteogenic therapeutics by mean of biosafety.

Patient's/Guardian's consent

The authors declare that the above-mentioned manuscript has no need of Patient's/Guardian's consent since, it is a non-clinical research manuscript.

Declaration of ethical clearance

The authors declare that the above-mentioned manuscript had not used any living materials. The manuscript was written on zebrafish embryo toxicity assay, which requires no ethical permission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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