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TOXICITY TEST OF ROSELLE NANOHYDROXYAPATITE-GELATIN ON OSTEOBLASTS CELLS USING MTT ASSAY

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ABSTRACT
Background: Orthodontic treatment often results in a relapse of tooth position after appliance removal due to insufficient bone remodeling. Effective prevention requires controlling inflammation and enhancing osteoblast activity to promote new bone formation and stabilization. Roselle calyx extract contains flavonoids with anti-inflammatory and antioxidant properties, while nanohydroxyapatite combined with gelatin improves scaffold porosity, cell adhesion, and biodegradability, thereby enhancing bone bioactivity. Methods: This in vitro study evaluated the cytocompatibility of nanohydroxyapatite–gelatin–roselle (NanoHAp-Gel-RE) composite on osteoblasts using an MTT assay. Data were analyzed for normality using the Shapiro–Wilk test, followed by one-way ANOVA and Tukey's HSD post hoc test to determine differences between treatment groups. Results: Cell viability analysis showed that NanoHAp-Gel-RE at concentrations of 15%, 20%, 25%, and 30% resulted in viability values of 90.71%, 112.38%, 117.96%, and 117.34%, respectively. All tested concentrations demonstrated viability greater than 50%, indicating no cytotoxic effects. Conclusion: Roselle calyx extract and NanoHAp-Gel-RE composite exhibit good cytocompatibility toward osteoblast cells. Their ability to enhance cell viability suggests the potential for application as biocompatible therapeutic materials to support bone remodeling in preventing post-orthodontic relapse.

INTRODUCTION

Orthodontic treatment aims to achieve optimal tooth positioning for both aesthetic and functional purposes. Once the treatment is complete, it is essential to maintain the new position of the teeth to ensure long-term success. A common method to prevent relapse is the use of retainers. Numerous studies have been conducted on strategies to prevent relapse following orthodontic treatment. Currently, researchers are developing materials that can be applied directly to the supporting tissues of the teeth to aid in this prevention.

Hydroxyapatite is commonly used for bone defect reconstruction due to its similarity to natural bone. It offers excellent biocompatibility, bioactivity, and promotes tissue regeneration. However, its low porosity and slow biodegradability limit its use as a standalone material. Combining hydroxyapatite with materials such as gelatin can increase porosity, enhance cell adhesion, and improve biodegradability, leading to increased scaffold bioactivity (Hengky, 2011; Lian et al., 2018).

Gelatin is a natural polymer that can be obtained from the collagen found in animals. It has excellent biodegradability and biocompatibility. In engineering, gelatin functions as a matrix in various applications. Its ability to protect colloidal systems helps maintain the transition between sol and gel phases, which can enhance the properties of brittle hydroxyapatite (Barreto et al., 2023). Mixing hydroxyapatite and gelatin can be optimized using nanomaterial technology to create nano-sized hydroxyapatite particles (about 100 nm). This enhances their reactivity with gelatin, improving the physical, chemical, and mechanical properties of the mixture. This combination may serve as an effective scaffold to prevent relapse after orthodontic treatments (Bayram et al., 2024).

Roselle petals are derived from one of the most common roselle plants used for extracts due to their rich content of bioactive compounds. These petals are a good source of dietary fiber, polyphenols, vitamin C, as well as essential minerals such as iron, zinc, calcium, magnesium, and potassium. As a result, they are frequently utilized as a standard ingredient in herbal medicine (Mariod et al., 2021; Osei-Kwarteng et al., 2021; Prasetyoputri et al., 2021). The polyphenols found in roselle petals consist of sour phenolics and flavonoids. These bioactive phenolic compounds are recognized for their health benefits. They possess antioxidant, anti-inflammatory, antimicrobial, anticancer, antihypertensive, and antidiabetic properties (Prasetyoputri et al., 2021). Polyphenols have benefits for remodeling bones. They help maintain bone integrity by reducing oxidative stress and inflammation, while also influencing the differentiation of osteoblasts and osteoclasts through osteoimmunological actions (Nicolin et al., 2019). Antioxidants play a crucial role in reducing oxidative stress and free radicals. Oxidative stress occurs due to the excessive production of reactive oxygen species (ROS), leading to an imbalance between the activities of osteoclasts and osteoblasts. High levels of ROS can hinder osteoblast activity and differentiation. In contrast, antioxidants contribute to increased osteoblast differentiation, bone formation, and the survival of osteocytes, while also regulating the differentiation and activity of osteoclasts (Ekeuku et al., 2021; Marcucci et al., 2017; Nicolin et al., 2019).

Based on previous research about the effectiveness of hydroxyapatite as a biomaterial, this study aims to evaluate the toxicity of a combination of roselle extract with nanohydroxyapatite and gelatin on osteoblasts.

METHOD

Roselle Extract

A 1 kg sample of dried roselle was extracted using the maceration method with 5000 ml of ethanol over three separate 24-hour periods. Following each interval, the mixture was filtered to obtain the extract. The resulting filtrate was then concentrated using a rotary evaporator under vacuum at approximately 40°C, yielding 34.37 grams of a thick roselle ethanol extract.

Scaffold NanoHAp-Gel-Roselle Extract

Dissolve 1.5 grams (15%), 2 grams (20%), 2.5 grams (25%), and 3 grams (30%) of roselle extract in 5 ml of 96% alcohol. Weigh out 10 grams of NanoHAp-Gel-HMPC 2% granules. Mix the NanoHAp-Gel-HMPC 2% into the roselle solution and stir until the mixture is homogeneous. Next, place the combined mixture of NanoHAp-Gel-HMPC 2% and roselle extract into a food dehydrator, and dry it at 40°C for 24 hours. Once dried, crush the NanoHAp-Gel-HMPC 2% and roselle extract until it becomes a fine powder.Add 12 ml of NaCl to the fine powder of NanoHAp-Gel-HMPC 2% along with 10 grams of roselle extract. Stir the mixture using a magnetic stirrer for 1 hour until it forms an injectable solution.

Toxicity Test

In the cytotoxicity test using the MTT Assay, osteoblast cell cultures were prepared in a laminar flow hood. Osteoblasts were cultured in a monolayer form using Eagle's media with 5% Fetal Bovine Serum (FBS) in Roux culture bottles and incubated at 37°C for 48 hours. After incubation, the cultures were washed five times with Phosphate Buffered Saline (PBS) to remove residual serum. Trypsin-EDTA was added to detach the cells, which were then resuspended in 100 µL of media (86% Eagle's media, 1% penicillin-streptomycin, and 100 units/mL fungizone) and transferred to a 96-well microplate. Each sample was sterilized with UV light, and a solution of 0.05 grams dissolved in 1 mL of ethanol was added (50 µL per well). The plate underwent 24 hours of incubation at 37°C, followed by the addition of 10 µL of MTT reagent (5 mg/mL) per well and a further 4-hour incubation. Finally, 50 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals, and the optical density was measured using an ELISA reader. The cell viability and inhibition percentages were determined using the equation below (Kamiloglu et al., 2020):

$$\%\ \text{Viability} = \frac{\text{Mean OD of sample}}{\text{Mean OD of blank}}\ x\ 100$$

Data analysis

Statistical analysis was performed using IBM SPSS Statistics (Version 29.2). Data normality was assessed using the Shapiro–Wilk test. If the data were normally distributed ($p > 0.05$), homogeneity of variance was examined using Levene's test. When both assumptions were met, differences between groups were analyzed using one-way ANOVA followed by Tukey's post hoc test. If the homogeneity assumption was violated, Welch's ANOVA and Games–Howell post hoc test were used instead. When data were not normally distributed, the Kruskal–Wallis test followed by Dunn's post hoc test was applied. A significance level of $p < 0.05$ was considered statistically significant.

RESULTS

The absorbance values obtained from the ELISA reader for osteoblast cell cultures are presented in Table 4.1, demonstrating the outcomes of the MTT assay performed on cells treated with varying concentrations of NanoHAp-Gelatin-Roselle extract (NanoHAp-Gel-RE). Based on these absorbance data, cell viability percentages were calculated. The results showed that the 15% concentration yielded the lowest viability value at 90.71%, while the 20% concentration resulted in a viability of 112.38%. The highest viability was observed at the 25% concentration, reaching 117.96%, followed by the 30% concentration with a viability of 117.34%.

Group	**1**	**2**	**3**	**4**	**5**	**Average**
NanoHAp-Gel-Roselle extract 15%	0.220	0.274	0.354	0.307	0.311	0.293
NanoHAp-Gel-Roselle extract 20%	0.339	0.360	0.366	0.354	0.395	0.363
NanoHAp-Gel-Roselle extract 25%	0.354	0.400	0.384	0.400	0.368	0.381
NanoHAp-Gel-Roselle extract 30%	0.401	0.361	0.389	0.382	0.362	0.379
Control Cells (cell + DMEM)	0, 337	0.313	0.317	0.311	0.337	0.323

Table 1. Results of MTT assay on osteoblast cells after NanoHAp-Gel-Roselle Extract application.

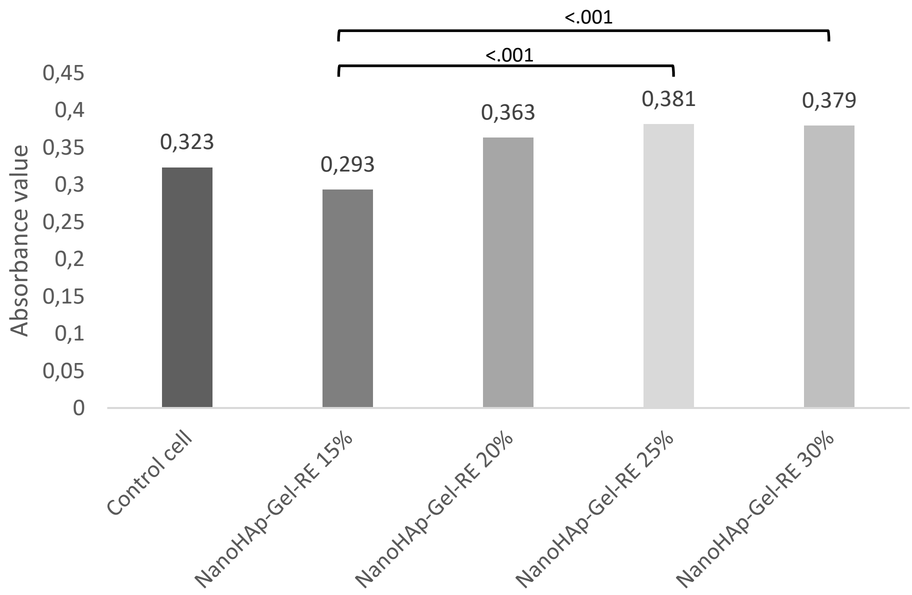

Figure 1. The absorbance value between concentrations of NanoHAp-Gel-Roselle extract.

Discussion

This experimental research focuses on extracting dried roselle flowers using the maceration method with 96% ethanol, based on Badaring et al. This cold extraction technique avoids heating, making it ideal for heat-sensitive compounds (Badaring et al., 2020). In a study conducted by Handoyo (2020), the maceration technique that avoids high temperatures is particularly beneficial for natural materials due to their tendency to decompose or get damaged when exposed to heat (Handoyo, 2020). Research by Yunida and Khodijah (2020) indicated that higher concentrations of ethanol solvent resulted in increased levels of quercetin (Yunita & Khodijah, 2020). Additionally, the findings of Kurniawati et al. (2016) suggested that 96% ethanol was the most effective type of solvent (Kurniawati et al., 2016).

After conducting the MTT assay test, the percentage of cell viability was calculated using Microsoft Excel. This was followed by a normality test using the Shapiro-Wilk test in the SPSS application. The data for the hydroxyapatite-gelatin-roselle extract at concentrations of 15%, 20%, 25%, and 30% yielded $p > 0.05$. This result indicates that the data distribution is normal. Furthermore, the results from the MTT assay demonstrate that the hydroxyapatite-gelatin-roselle extract samples are not toxic, as the percentage of viable cells is above 50%. The findings align with the research conducted by Pakpahan et al. (2024), which indicated that concentrations with a viability percentage above 70% are considered non-toxic. The study demonstrated that roselle flower extract has potential applications in bone tissue engineering and the regeneration of bone tissue, showing no cytotoxic effects on osteoblasts (Pakpahan, Bagus Narmada, et al., 2024).

This study demonstrates an increase in the number of osteoblast cells following the MTT assay test at a concentration of 30% in each experiment. This suggests that the flavonoids present in roselle can promote the bone growth process. This finding aligns with the research conducted by Majeed and Ghani (2018), which investigated the potential of applying flavonoid extract from roselle topically on fractured rabbit legs. Their study found that the flavonoid extract could accelerate the healing process in bone defects in mice compared to those that did not receive the topical flavonoid extract (Sura et al., 2018). Flavonoids exhibit various therapeutic potentials, including anti-inflammatory effects and the ability to stimulate osteogenesis. Notable flavonoids, such as anthocyanins, flavonols, isoflavones, cyanidin, daidzein, cladrin, calycosin, icariin, and petunidin, have been shown to activate osteoblasts and inhibit osteoclasts (Bellavia et al., 2021). According to Salim et al. (2015), flavonoids can help reduce inflammation by lowering the levels of IL-6. IL-6 stimulates the differentiation of osteoclasts, promotes bone resorption, and inhibits osteogenesis, which ultimately leads to a decrease in the number of osteoblasts (Salim & Kuntjoro, 2015).

Roselle flowers contain additional compounds, such as alkaloids and tannins, which can influence bone repair. Research by Lin et al. (2022) indicates that these alkaloids have significant anti-osteoporotic effects, particularly by inhibiting bone resorption (Lin et al., 2022). The findings of the study are supported by research conducted by Kusparmanto et al. (2024). The study revealed that roselle flower extract contains various phytochemical compounds, including flavonoids, tannins, saponins, phenolic compounds, and alkaloids. Among these compounds, flavonoids, tannins, and saponins are highlighted for their potential in preventing relapse after orthodontic treatment due to their anti-inflammatory and antioxidant properties (Kusparmanto et al., 2024).

Quercetin, a type of flavonoid, can influence bone formation. Research by Narmada et al. (2023) indicates that quercetin found in roselle flowers can enhance bone regeneration by increasing biomarkers associated with osteoblastogenesis (Narmada et al., 2023). Research conducted by Triwardhani et al. (2023) supports the statement that quercetin can stimulate bone resorption inhibition, influence pro-inflammatory cytokines, and affect virulence factors. Additionally, quercetin promotes increased bone formation activity, enhances antioxidant properties, reduces inflammatory cytokines, and boosts the levels of growth factors (Triwardhani et al., 2023).

In addition to the benefits associated with roselle, hydroxyapatite-gelatin has also been shown to promote bone growth. Research conducted by Samadikuchaksaraei et al. (2016) demonstrated through in vivo studies that hydroxyapatite-gelatin can enhance biocompatibility and osteoconductivity. It also accelerates collagen production during the bone healing process, and within three months, new bone nearly fills the area of the defect (Samadikuchaksaraei et al., 2016). According to research by Pakpahan et al. (2024), adding roselle extract to a hydroxyapatite-gelatin composite does not alter the hydroxyapatite crystallization phase. This indicates that the hydroxyapatite content remains suitable for use as a biomaterial in bone remodeling. Furthermore, the inclusion of roselle petal extract can enhance the crystallinity of the nanohydroxyapatite-gelatin composite without affecting the functionality of the hydroxyapatite (Pakpahan, Narmada, et al., 2024).

In a study conducted by Franzen et al. (2014), it was found that alveolar bone remodeling plays a significant role in orthodontic relapse (Franzen et al., 2014). This remodeling process involves replacing old bone with new bone and is regulated by several types of cells: osteoclasts, which dissolve bone; osteoblasts, which form new bone; and bone mesenchymal stem cells. These cells interact and collaborate to achieve effective bone remodeling. Additionally, research by Yuliana and Suharto (2020) indicates that bone remodeling not only enhances bone stability after orthodontic treatment but also helps maintain the long-term outcomes of the treatment (Prameswari et al., 2020; Rosyida et al., 2023).

The material that can be utilized will be injected into the periodontal ligament, which plays a crucial role in preventing relapse. This increased remodeling of the periodontal ligament occurs through the elevation of fibroblast growth factor-2 (FGF-2), which aids in synthesizing the extracellular matrix (ECM) produced by fibroblast cells and enhances collagen stability (Prameswari et al., 2020).

CONCLUSION

Based on the results of the study, the composite of nano-hydroxyapatite, gelatin, and roselle extract were found to be non-toxic to osteoblast cells, maintaining cell viability above 50%, which is considered the cytotoxicity threshold. The nano-hydroxyapatite–gelatin–roselle composite demonstrated a higher percentage of cell viability, indicating greater potential as a natural biomaterial to prevent orthodontic relapse. Further research is recommended to evaluate its biological performance through *in vivo* studies, including assessments of bone remodeling markers and relapse distance, to validate its clinical applicability as a local therapeutic agent in orthodontic treatment.

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