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RESEARCH ARTICLE

Viability of 7F2 pre-osteoblast after Sulawesi stingless bee (*Tetragonula biroi*) 1% propolis nanoemulsion extraction

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ABSTRACT

Produced by a stingless bee (Tetragonula biroi) from Sulawesi Island, propolis is an active compound that requires further studies to determine its herbal-based medicinal applications. In addition, propolis is well known as a potential natural ingredient for herbal medicine with minimal side effects. The stingless honeybee, native to Sulawesi, produces propolis, a resin. Propolis from Trigona stingless bees in Sulawesi has the highest flavonoid content compared to Apis mellifera bees. Flavonoids are the most common compounds contained in propolis. Propolis from stingless bees may have the potential to stimulate osteoblast cell proliferation and be responsible for bone regeneration. The objective of this study is to investigate the viability of the 7F2 pre-osteoblast cell line after administration of the stingless bee's 1% propolis nanoemulsion extract (PNE). A 1% PNE was formulated by maceration methods and diluted into several concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78%. A particle size analysis was done to examine the particle size and zeta potential of the 1% PNE. A Tetrazolium Technique assay was carried out to examine 1% PNE application on a 7F2 pre-osteoblast cell line on days 1, 3, and 7. The highest viability of 7F2 pre-osteoblast cells was at 0.78% concentration on day 7, and the lowest viability was observed at 100% concentration on day 1, with significant differences among groups ($p < 0.05$). A 1% PNE with a particle size of 151.28 to 182.2 nm and a zeta potential of −32.76 mV was successfully formulated. The 1% PNE was non-toxic to 7F2 pre-osteoblast cells, with a cell toxicity limit at a concentration of 1.56%.

Keywords: cell line; medicine; pre-osteoblast; propolis; viability

INTRODUCTION

Propolis is a cost-effective ingredient that has minimal side effects.[1] Propolis is also a resin produced by the stingless honeybee *Tetragonula biroi (T. biroi),* which originates from Sulawesi. Propolis from Sulawesi stingless bees, *T. biroi,* has the highest flavonoid content compared to that of *Apis mellifera* bees.[2] Propolis consists of wax, resin, essential oils, pollen, and organic components.[3] Resins contain phenols, flavonoids, and various forms of acids. Flavonoids are the most common compounds contained in propolis. Flavonoids, which are compounds derived from the polyphenol group, act as antioxidants, antivirals, antibacterials, anti-inflammatories, and antifungals.[4] However, the biocompatibility of propolis from Sulawesi stingless bees still lacks further examination.

Propolis has a strong antioxidant capacity because it contains flavonoids and quercetin,

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which have strong anti-inflammatory properties. Flavonoids stimulate the release of signaling molecules; they are considered valuable as biomaterials used in bone regeneration. Propolis contains flavonoids, which inhibit cell damage caused by hydrogen peroxide by eliminating free radicals and protecting cell membranes against lipid peroxidation.[5] In the field of dentistry, propolis is an ingredient that has many benefits. Cell and tissue responses are the key to the success of biomaterial design and application in dental medicine. One method to investigate cell and tissue responses is through in vitro cytotoxicity testing by measuring cell viability in cell culture.[6] To investigate the level of toxicity of chemical substances, it is necessary to carry out a cytotoxicity test. One of the most widely used cytotoxicity tests is the Microculture Tetrazolium Technique (MTT) assay. In this method, the yellow MTT tetrazolium salt is reduced by living cells to purple formazan crystals.[7] The parameter obtained is the inhibition concentration (IC_{50}) value, which states the concentration value that results in a 50% inhibition of cell proliferation, thereby indicating the potential toxicity of a compound to cells. Osteoblast cell culture is an ideal in vitro test model for biocompatibility and osseointegration testing. In this study, 7F2 pre-osteoblast cells were used, which were cultured in vitro. 7F2 cells were used because these cells are related to mature osteoblasts, and they express alkaline phosphatase and secrete type I collagen. 7F2 cells contain a homogeneous cell population associated with mature osteoblasts and a more stable passage number compared to other osteoblast cell lines, such as mouse calvaria 3T3-E1 (MC3T3-E1).[8] A unique property of 7F2 is that it represents a new stage of osteoblast differentiation. Osteoblasts are cells that are responsible for the bone formation process. Their proliferation and differentiation play an important role in the process of osteogenesis.[9,10] The hypothesis of this study is that a 1% propolis nanoemulsion extract (PNE) has good biocompatibility and viability with a 7F2 pre-osteoblast. Based on the research questions above, the objective of this study is to analyze the viability of 1% 7F2 pre-osteoblasts cell line following the *in vitro* application of 1% PNE (*T. biroi) from Sulawesi*.

MATERIALS AND METHODS

All methods in this research were carried out in accordance with the relevant guidelines and regulations by the Ethics Committee, Faculty of Dental Medicine, Universitas Airlangga Surabaya, East Java, Indonesia, with appointment number 0651/HRECC.FODM/VII/2024. The instrument, glassware, and materials were prepared; then, the propolis ingredients were measured as a whole. The materials were sorted off stuck-on dirt. Propolis was cut into smaller sizes. The propolis was measured and divided into three parts: 233.58, 235.52, and 233.42 grams. Two point five liters of 96% ethanol solvent per jar were measured for maceration (three jars). The propolis was put into the three jars and then macerated with 96% ethanol. Maceration was stored and stirred daily for seven days. Tools and glassware were prepared for filtering. The propolis extract was filtered after seven days of maceration, and the propolis extract filtrate was placed in an Erlenmayer (OneMed, Sidoarjo, Indonesia). The propolis extract was evaporated and filtrated with a rotary evaporator (Sigma Aldrich, USA) at a temperature of 45 °C – 50 °C until the solvent had completely evaporated. After evaporation, it produced a thick propolis extract, which was then ovened. Next, ethanol in one volume of extract (1:1) was added to obtain a thick propolis ethanol extract (100% concentration), which was used as a test material. Propolis extract with a concentration of 100% was diluted using artificial saliva (Sigma Aldrich, USA) to obtain concentrations of 100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.78%. The formula used to dilute the extract was $M1 \times V1 = M2 \times V2$.[11]

The propolis solution was put into a beaker as much as 100 mL at each concentration. Then, a solution of 1 mL of polysorbate 80 was added and stirred thoroughly via a homogenizer (Model 300, 131 V/T, USA) at a speed of 1,000 rpm for 10 minutes. After that, a 0.1% sodium

tripolyphosphate (Na-TPP) solution of as much as 20 mL was added to the mixture, which was then stirred in the homogenizer at a speed of 4,000 rpm for 60 minutes. During the process of mixing the solution, the aim was to increase the mixing speed to reduce particle size. Therefore, smaller particle sizes were obtained. This examination produced nanoparticle formulations. Finally, all formulas were stored at room temperature for further use.[11]

The 1% PNE was diluted 1:100 with distilled water and preconcentrated to measure the transmittance value using a UV-Vis spectrophotometer (Shimadzu UV 1800, Japan) at a wavelength of 650 nm with distilled water as a blank. Determination of particle size was carried out using a particle size analyzer (PSA) (Horiba SZ-100, Indonesia). The sample solution was put into a cuvette and read by a PSA. The particle size corresponding to the proportional size of the niosome preparation ranged from 151 to 182 nm. The research was carried out by preparing a sample of 0.05 grams, hydrated using 5.0 ml of pH 6.0 phosphate buffer and sonicated for 20 minutes. Then, it was put into a cuvette, and the size was determined. PSA is a tool used to analyze particle size, polydispersity index, and zeta potential of 1% PNE (*T. biroi*).[11]

The cells used were the 7F2 pre-osteoblast cell line (ATCC, CRL-12557, UK) obtained from the Research Center of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya, East Java, Indonesia. The 7F2 pre-osteoblast cell line was cultured in Dulbecco's Modified Eagle Medium (DMEM) without ribonucleosides,

Figure 2. Nanoemulsion propolis (*T. biroi*) extract ready to use

deoxyribonucleosides, and sodium bicarbonate (M0894, Sigma Aldrich, USA), with 10% FBS and 1% penicillin/streptomycin. Cells were incubated at 37 °C, 5% CO_2. Phosphate Buffer Saline (PBS) (20012027, Gibco, USA) was regularly used as a washing buffer. Cells were routinely passaged in 0.25% Trypsin-EDTA (T4049, Sigma Aldrich, USA) until they reached 85%–90% confluence.[9,10]

7F2 cells were cultured in T25 flasks (Sigma Aldrich, USA) at a seeding density of 1.25×10^5 cells per well. Cells were incubated at 37 °C (5% CO_2) for three to four days until they reached 85%–90% confluence. Confluent cells were washed using PBS (20012027, Gibco, USA). A volume of 2 ml was used in each well. Then, the washing buffer was removed and replaced with 1 mL of Trypsin-EDTA 0.25% (T4049, Sigma Aldrich, USA). Cells were placed in an incubator (37 °C, 5% CO_2) (Esco Lifesciences, USA) for the release process within three to five minutes until

(A) (B)

Figure 1. (A) Chopped propolis; (B) Measured propolis extract

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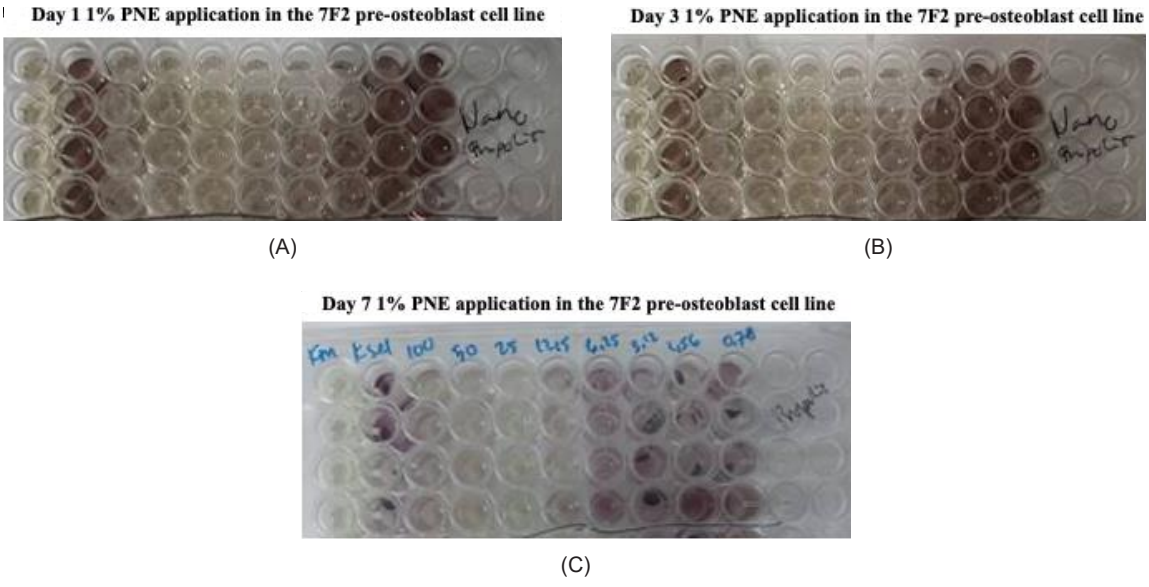
completely released. When the cells had detached completely, 2 ml of culture medium was added to the cells to inhibit further tryptic activity. The cell suspension was then centrifuged at 100 g for five minutes. The supernatant was discarded, and the pellet was resuspended in a culture medium. The number of cells was counted using 1x Trypan blue (T6146, Sigma Aldrich, USA) and a hemocytometer counting chamber (BR717810, Sigma Aldrich, USA). Suspension cells were harvested by centrifugation. Adherent cells were released from the substrate through trypsinization or scraping. Next, 10 ml of the cells that had been harvested were taken and put in a hemacytometer.[9,10]

A cytotoxic test was conducted by taking cells from the CO_2 incubator and observing the condition of the cells under an inverted microscope (Olympus, Japan). Then, the number of cells was counted using a hemocytometer and according to the cell counting protocol. The number of cells required for cytotoxic testing was determined via an MTT assay using the MTT reagent 3-(4.5-dimethylthiazol-2-yl). 2.5-diphenyl tetrazolium bromide was used to clearly detect metabolic changes with 5×10^3 cells/well. Cells were planted into 96-well plates, 100 l/well each. Two wells were left empty for media control. The cells were incubated in a CO_2 incubator overnight or until they were 80% confluent at 37 °C. After overnight, a 96-well plate was taken from the incubator to observe the condition of the cells under an inverted microscope. All wells were washed using PBS, 100 l/well each. The test solution concentration series of 1% PNE (*T. biroi*) (100%, 50%, 25%, 12.5%, 6.25%, 3.125%, 1.56%, and 0.75%) of as much as 20 liters was entered into the wells according to the MTT map that had been made.

Cells were incubated in a CO_2 incubator overnight. The cell media were discarded, the cells were washed using PBS 1x, and 100 l of MTT reagent was added to each well, including the media control. The cells were incubated for two to four hours in a CO_2 incubator until formazan was formed. To dissolve the formazan crystals formed after incubation, 100 ml DMSO was added. The condition of the cells was checked under an inverted microscope. If formazan was clearly formed, a stopper of 100 l of 10% SDS was added in 0.1 N HCl. The plate was wrapped in aluminum foil and incubated in a dark place at room temperature overnight. Next, the plate wrapper was opened, and the plate was closed. The 96-well plate was inserted into an Enzyme-Linked Immunosorbent Assay (ELISA) reader with <0xC8> = 540 nm to read the absorbance of each well and make observations on days 1, 3, and 7.[9,10] Data were analyzed using the statistical package



(A) (B)

(C)

Figure 3. Color change to purple after administration of MTT in 96-well plates. A: On day 1, B: On day 3, C: On day 7

for social sciences version 20.0 for Windows, which includes normality and homogeneity tests ($p < 0.05$), one-way analysis of variance (ANOVA) tests with a different significance value of $p < 0.05$ (IBM Company, Illinois, Chicago, US).

RESULTS

In this study, a 7F2 pre-osteoblast cell line that can be induced and differentiated into osteoblast cells was used. These 7F2 pre-osteoblast cells were treated with a 1% PNE with the aim of determining cytotoxicity with varying concentrations used. If the viability of pre-osteoblast cells is maintained, the yellow MTT fluid will turn purple; this can be seen in Figures 3–6.

If the percentage of cell viability was less than 70%, then the material exposed to the cells would be toxic. The results of the MTT assay on the viability of 7F2 pre-osteoblast cells are shown in Table

Table 1. MTT test results of 1% PNE on 7F2 pre-osteoblast cells line on day 1.

Day	Treatment Group	Mean absorbance (Optical density)	Stadandard deviation	Cell viability (%)
1	Medium only control group	0.06	0.09	0
	Cell only control group	0.75	0.06	100
	100%	0.06	0.07	0.83
	50%	0.07	0.07	1.34
	25%	0.17	0.05	15.81
	12,5%	0.18	0.05	17.71
	6.25%	0.29	0.02	33.16
	3.125%	0.31	0.01	36.2
	1.56%	0.58	0.03	75.71
	0.78%	0.62	0.005	81.05

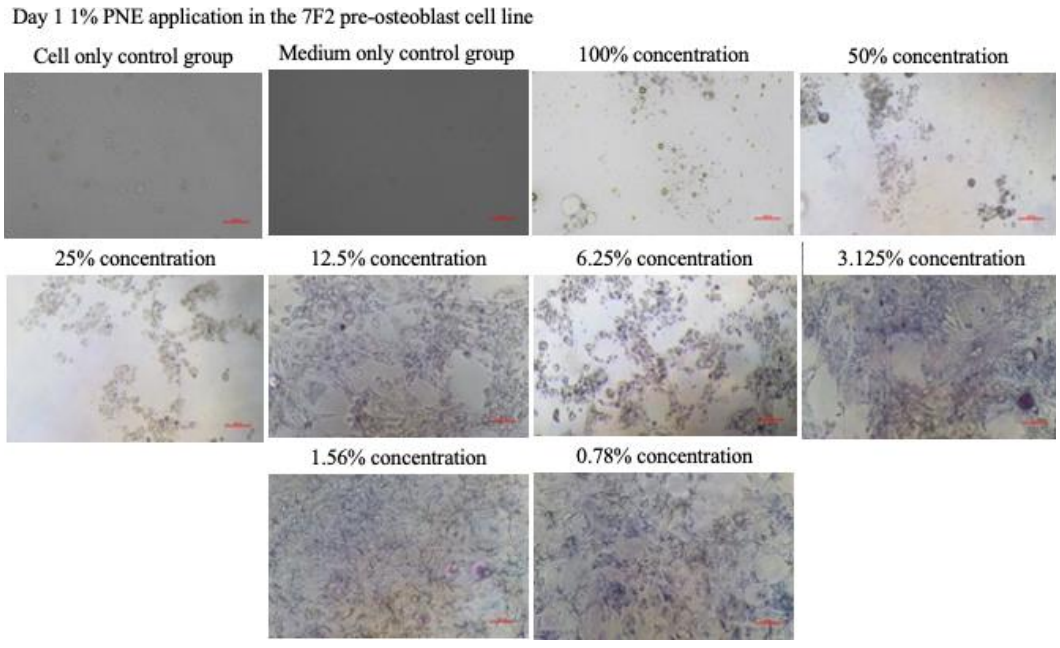


Figure 4. Morphology of 7F2 pre-osteoblast cell line after administration of 1% PNE on day 1. Living cells are polygonal and flat, while dead cells are rounded and narrow with 100× magnification

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1.The results of cell viability, as observed in Table 1, showed that the highest viability on day 1 of 7F2 pre-osteoblast cells was at a concentration of 0.78% (81.05%), while the lowest was at a concentration of 100% (0.83%). There was significant different between groups in the day 1 ($p < 0.05$).

After the application of MTT, readings were carried out using an ELISA reader with a wavelength of 540 nm. Then, the reading results in the form of absorbance values (OD) were expressed in percentages using this formula:

$$\% \text{ viability} = \frac{\text{AER Treatment Group x 100\%}}{\text{AER Control Cell}}$$

AER: Absorbance using the ELISA Reader

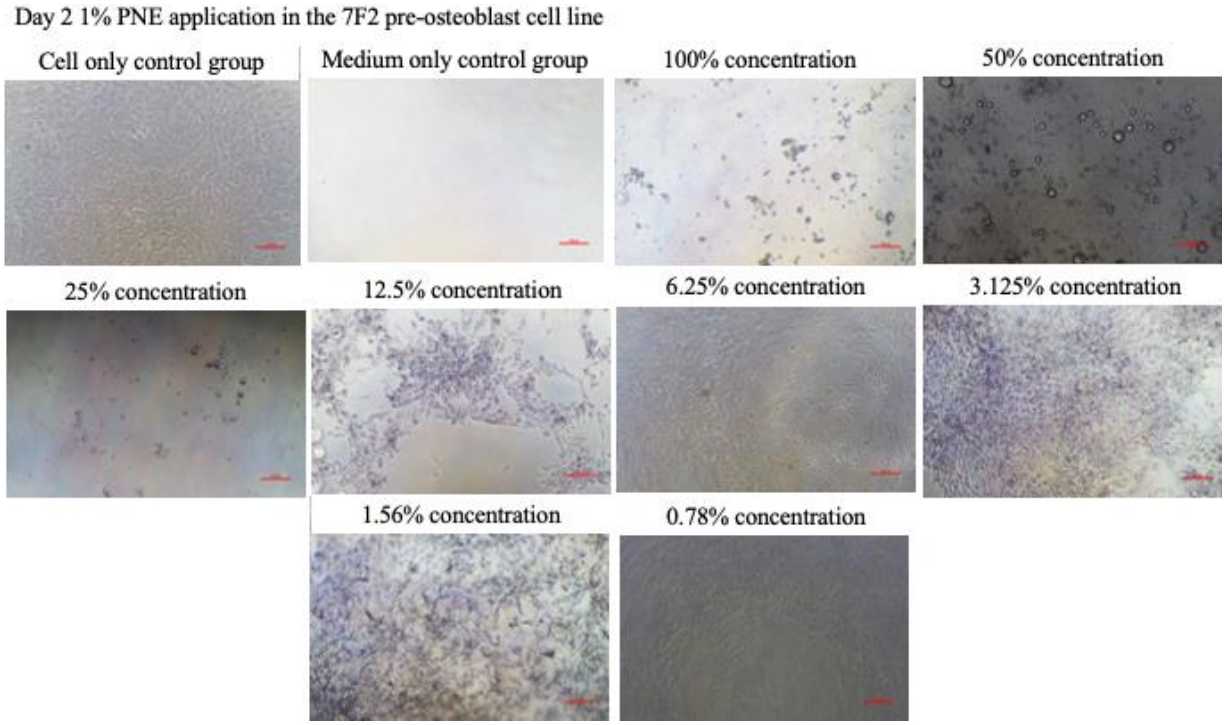

Figure 5. Morphology of 7F2 pre-osteoblast cell line after administration of 1% PNE on day 3. Living cells are polygonal and flat, while dead cells are rounded and narrow with 100× magnification

Table 2. MTT test results of 1% PNE on 7F2 pre-osteoblast cell line on day 3

Day	Treatment group	Mean absorbance (Optical density)	Stadandard deviation	Cell viability (%)
	Medium only control group	0.05	0.06	0
	Cell only control group	0.8	0.07	100
	100%	0.06	0.03	1.4
	50%	0.12	0.014	9.19
3	25%	0.23	0.07	24.02
	12.5%	0.3	0.043	34.30
	6.25%	0.38	0.048	44.79
	3.125%	0.47	0.004	56.21
	1.56%	0.65	0.09	81.13

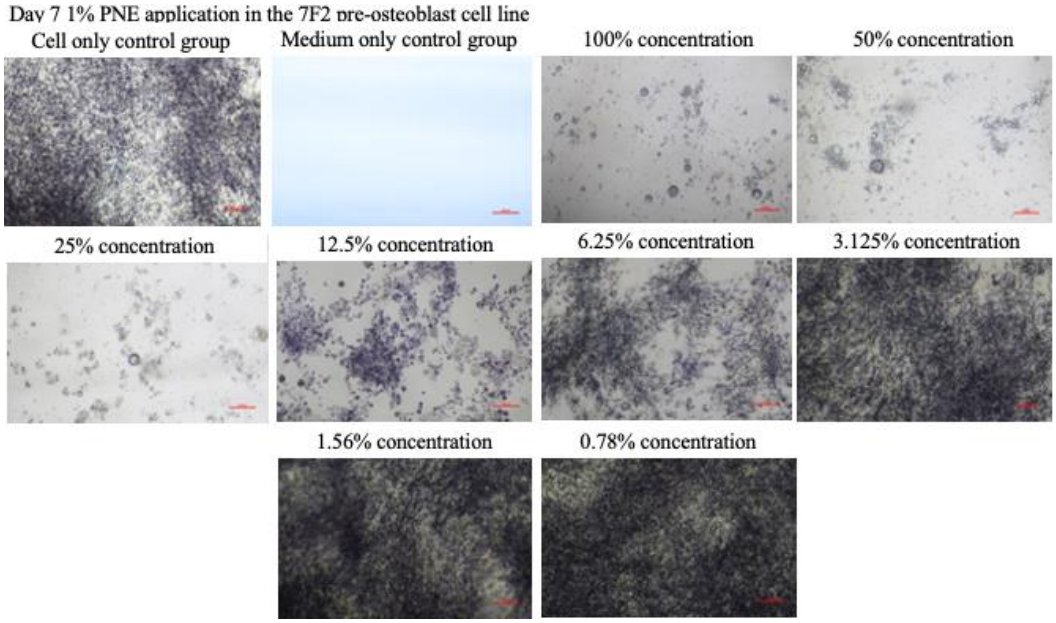

Figure 6. Morphology of 7F2 pre-osteoblast cell line after administration of 1% PNE on day 7. Living cells are polygonal and flat, while dead cells are rounded and narrow with 100× magnification

Table 3. MTT test results of 1% PNE on 7F2 pre-osteoblast cells on day 7.

Day	Treatment Group	Average Absorbance (OD)	Stadandard Deviation	Cell Viability (%)
7	Medium only control group	0.05075	0.061	0
	Cell only control group	0.418	0.107	100
	100%	0.065	0.708	3.94
	50%	0.106	0.032	15.16
	25%	0.111	0.027	16.45
	12.5%	0.131	0.006	21.96
	6.25%	0.263	0.002	57.72
	3.125%	0.287	0.002	64.11
	1.56%	0.407	0.015	96.94
	0.78%	0.418	0.002	99.80

The results of cell viability, as seen in Table 2, showed that the highest viability on day 3 of 7F2 pre-osteoblast cell was at a concentration of 0.78% (92.15%), while the lowest was at a concentration of 100% (1.4%). There was significant different between concentration groups in the day 3 ($p < 0.05$).

The results of cell viability, as shown in Table 3, revealed that the highest viability of 7F2 pre-osteoblast cell was at a concentration of 0.78% (99.8%), while the lowest was at a concentration of 100% (3.94%). There was significant different between concentration groups in the day 7 ($p < 0.05$).

DISCUSSION

In this study, 1% PNE administration in the 7F2 pre-osteoblast cell line was analyzed. The formulation of 1% PNE was successfully obtained. Nanoemulsion of PNE was formulated because nano-sized preparations can also increase the absorption of active compounds, such as active

constituents of propolis from stingless bees (*T. Biroi*). The application of nanotechnology to food and medicine shows an increasing trend nowadays.[11] This technology offers advantages in increasing the bioavailability of active ingredients, controlling the release of active ingredients, and improving sensory properties.[12] Absorption of active ingredients increases due to increased particle solubility and large particle surface area. The absorption rate of nanoherbs in the human body can almost reach 100%, whereas at the micron size, it is only 50%. In this study, the particle size test showed a value of 151.28 to 182.2 nm; this size is good for a nanoparticle because it is in the 10–1000 nm range. Apart from that, the zeta potential test showed a value of −32.76 mV; this value exceeds (+/−) 30 mV, which means that the nanoparticle solution is a colloidal solution that is quite stable toward negative charges.[13] Zeta potential is one of the main parameters in determining the stability of a nanoparticle.[11]

In this study, the MTT assay was used to test the reaction of 7F2 pre-osteoblast cells to 1% PNE, as shown in the figure (Figures 3–6). On the first day, it was seen that the cells on all surfaces were round with a prominent nuclear area, indicating delayed spreading. After three days, the cells became flat and spread out, indicating better adhesion. After days 3 and 7, cells formed more bridges that helped them cross the groove and spread over it, resulting in a larger number of cells and thus leading to better cell proliferation. This shows that there are fewer toxicity properties in cells. The cell response to biomaterials occurs through the adsorption of serum proteins, as well as the attachment and spreading of cells in the sample. Furthermore, at the attachment stage, a physicochemical relationship occurred between the cells and the material surface by ionic and van der Waals forces. This shows good cell distribution and cell viability in all samples. Therefore, it can be concluded that the 1% PNE is less toxic to 7F2 per-osteoblast cell line. This is in accordance with previous research indicating that propolis promotes osteoblast proliferation.[14] The findings of the study showed that the toxicity limit for propolis nanoemulsion is at a concentration of 1.56% with a viability of 96.9% because the viability is more than 70%, so it has no cytotoxic potential.[15] Based on the results, the highest percentage of cell viability was at a concentration of 0.78% on day 7, and the lowest percentage was at a concentration of 100% on day 1. Cell death is caused by the biochemical mechanisms of cell death, namely the depletion of Adenosine Triphosphate (ATP) and cell membrane rupture or defects. This ATP depletion is caused by the presence of the succinate dehydrogenase enzyme, which functions as an ATP producer yet is less than optimal in producing ATP. When this enzyme is not or is less active in producing ATP, the functional activity of the cell will be hampered, which can reduce cell performance and even lead to cell death.[16] This defect can affect the activity of mitochondria in cells, which produces ATP, thus lowering its production and leading to cell death. This cell damage causes cells to become non-viable, which will then cause cell death. Damage or lack of membrane permeability is a common feature of cell membrane injury. There are two general principles of cell injury: (1) the intracellular system is sensitive; and (2) the cellular response is dependent on the type of cell injury, such as drugs and chemicals, the length of the simulation, such as the time of exposure to cell injury, and the severity of the stimuli, such as dose or concentration.[17]

Osteoblast apoptosis is also caused by increased production of nitric oxide (NO) due to lipopolysaccharide induction. Lipopolysaccharide and proinflammatory cytokines stimulate an increase in inducible nitric-oxide synthase (iNOS).[18] Tumor necrosis factor-α, interleukin-1β, and interferon-γ cause osteoblast cell death mediated by apoptosis, not necrosis. Cytokines have been shown to produce an increase in iNOS messenger ribonucleic acid (mRNA) and NO in the cells.[19] In addition, NO causes suppression of cell viability, mitochondrial membrane potential, and ATP synthesis, which results in disruption of mitochondrial functions, reactions of intracellular oxygen species, and the Bcl-2 protein, which plays

an important role in apoptosis.[20] The results of one-way ANOVA data analysis showed that there was a significant difference in the concentration of 1.56% on day 7. The results of the research data analysis using Tukey's HSD showed a significant difference in the percentage of living cells. The percentage of living cells exposed to 1% PNE was at a concentration of 1.56% on day 1, 3, 7. The 1% PNE contains flavonoids that have the potential to stimulate osteoblast cell proliferation and maturation.[21] A substance or material can be said to be biocompatible if the material does not cause irritation to living tissue, is free from ingredients that can trigger allergic reactions, does not cause a toxic response, and does not have carcinogenic potential.[22] Determining the biocompatibility of a material can be done through a biocompatibility or toxicity test.[23] Nevertheless, this study only examined the viability of the 7F2 pre-osteoblast cell line post the administration of 1% PNE. However, the biocompatibility of herbal biomaterial, allergic reaction, toxic response, and carcinogenic potential should be examined in an animal model study in vivo.

CONCLUSION

A 1% PNE with a particle size of 151.28 to 182.2 nm and a zeta potential of −32.76 mV was utilized. The 1% PNE was found to be non-toxic to 7F2 pre-osteoblast cells with a cell toxicity limit at a concentration of 1.56%. However, further studies are still needed to investigate the osteoinductivity and the proliferation of a pre-osteoblast cell line after administration of 1% PNE.

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CONFLICT OF INTEREST

There is no conflict of interest.

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