https://doi.org/10.31925/farmacia.2022.5.19 **ORIGINAL ARTICLE**

THE EFFECTS OF THE COMBINATION OF ETHANOL BINAHONG LEAF (*ANREDERA CORDIFOLIA*) AND MOBE LEAF (*ARTOCARPUS LAKOOCHA*) EXTRACT GEL ON FIBROBLAST AND OSTEOCYTE PROLIFERATION IN WOUND HEALING POST TOOTH EXTRACTION SOCKET ON WISTAR RATS (*RATTUS NORVEGICUS*)

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Abstract

Herbal medicines have been widely used throughout the world because of its fewer side effects compared to chemical-based medicines. The purpose of this study was to determine the effect of the combination of ethanol extract gel from binahong leaves and mobe leaves on the proliferation of fibroblasts and osteocytes in socket wounds after tooth extraction in Wistar rats. This *in vivo* experimental study involved 32 male Wistar rats which were randomly allocated to 8 groups of observations. Tooth extraction was carried out on the lower left incisor. A combined extract gel made of, binahong leaves and mobe leaves in the ratio of 1:1, 1:3, 3:1 was applied to group I to II, III to IV and V to VI respectively, meanwhile, group VII and VIII were given Aloclair® gel and serve as the control group. On the 7th and 21st days after tooth extraction, the rats were sacrified and the proliferation of fibroblasts and osteocytes were observed with Hematoxylin-Eosin staining. Research data were analyzed using one-way ANOVA test. The results showed that the combination group of ethanol extract gel of binahong leaves and mobe leaves with a ratio of 3:1 had the highest number of fibroblast on 7th day ($p < 0.05$) and the highest number of osteocyte on 21st day compared to the control group ($p < 0.05$). The experiments showed that combination of ethanol binahong leaves and mobe leaves extract gel had an effect on increasing the proliferation of fibroblasts and osteocytes on the healing of socket wounds after tooth extraction in Wistar rats on the 7th and 21st days.

Rezumat

Medicamentele pe bază de plante au fost utilizate pe scară largă în întreaga lume datorită efectelor secundare mai puține în comparație cu medicamentele pe bază de substanțe chimice. Scopul acestui studiu a fost de a determina efectul combinației de gel cu extract etanolic din frunze de Binahong și frunze de Mobe asupra proliferării fibroblastelor și osteocitelor în rănile de la nivelul soclului după extracția dinților la șobolanii Wistar. Acest studiu experimental *in vivo* a implicat 32 de șobolani Wistar masculi care au fost repartizați aleatoriu în 8 grupuri de observații. Extracția dentară a fost efectuată pe incisivul inferior stâng. Un gel de extract combinat format din frunze de binahong și frunze de mobe în proporție de 1:1, 1:3, 3:1 a fost aplicat la grupurile I - II, III - IV și, respectiv, V - VI, între timp, la grupurile VII și VIII s-a administrat gel Aloclair® și a servit drept grup de control. În zilele 7 și 21 după extracția dinților, șobolanii au fost sacrificați și proliferarea fibroblastelor și a osteocitelor a fost observată cu colorarea cu Hematoxilină-Eozină. Rezultatele au arătat că grupul de combinație tratat cu gel de extract etanolic din frunze de binahong și frunze de mobe în raport de 3:1 a avut cel mai mare număr de fibroblaste în ziua a 7-a ($p < 0,05$) și cel mai mare număr de osteocite în ziua a 21-a în comparație cu grupul de control ($p < 0,05$). Experimentele au arătat că gelul cu extract etanolic din frunze de binahong și extract de frunze de mobe a avut un efect de creștere a proliferării fibroblastelor și a osteocitelor asupra vindecării rănilor de soclu după extracția dinților la șobolanii Wistar în zilele 7 și 21.

Keywords: *Anredera cordifolia*, *Artocarpus lakoocha*, fibroblast, osteocytes, wound healing

Introduction

Tooth extraction is a procedure of removing a tooth from the dental alveolus (socket) in the alveolar bone. The process may cause damage, both the hard and soft tissues, thus trigger the physiological response of the wound healing process [1]. This

process consists of phases that are interconnected with one another, namely haemostasis, inflammation, proliferation and tissue remodelling [2, 3].

Some of the cells that play an important role during the healing of socket wounds are fibroblasts and osteocytes. In the healing of soft tissue, cells that play an important role are fibroblasts. While in hard tissue healing, cells that play an important role are osteocytes [4]. This is because when the tissue is inflamed, fibroblasts will migrate towards the wound, proliferate and produce collagen matrix to carry out tissue repair. When collagen and Extra Celluler Matrix (ECM) are synthesized, a new epithelium will form on the mucosa that covers the wound surface [1, 3]. The proliferation of fibroblasts begins on the 3rd day and will reach its peak on the 7th day post-injury. The proliferation of fibroblasts at the wound healing stage indicates a well wound healing process [5].

After the proliferative phase, wound healing enters a remodelling phase. The remodelling phase is characterized by tissue and collagen remodelling, epidermal maturation and wound shrinkage. In the healing of socket wounds after tooth extraction, the remodelling phase is followed by bone formation [6]. One of the largest components in bone formation is osteocyte cells. Osteocytes are osteoblasts that are confined within the new bone matrix during the bone remodelling phase. These osteocytes are the most abundant cells found in mature bone and have a longer life span compared to osteoblasts and osteoclasts which are only temporarily found on the bone surface [7, 8]. In the bone healing process, osteocytes play a role in maintaining bone integrity and vitality [6]. Based on research by Olaitan *et al*., the number of osteocytes in alveolar bone healing increased at the 4th week after tooth extraction [9].

According to WHO, 70 - 80% of the world's population uses herbal medicines as alternative treatments in wound healing [1, 3]. The herbal plants that can be used are binahong leaves (*Anredera cordifolia*) and mobe leaves (*Artocarpus lakoocha*) [4, 10]. Secondary metabolites that play a role in healing socket wounds on binahong leaves and mobe leaves include flavonoids, saponins and tannins [10, 11]. Flavonoids in binahong leaves are the main components that act as anti-inflammatory and antioxidants by increasing the process of mitogenesis, cell interactions and adhesion molecules in the cell proliferation phase [1, 11] While the main component of mobe leaves is a flavonoid known as artocarpine [12]. According to research by Hanafiah *et al*., the application of binahong leaf extract gel can heal alveolar bone in socket wounds after tooth extraction of Wistar rats against the proliferation of fibroblasts, osteoblasts and osteocytes with a concentration of 3% [4]. Khoswanto *et al*. research, using binahong leaf extract gel to treat socket wounds after tooth extraction in mice, showed that binahong leaf extract gel could increase the expression of bone morphogenetic protein-2 (BMP-2) and osteoblasts in socket wound tissue [2]. Another study by Hanafiah *et al*. showed that mobe leaf extract gel with a concentration of 3% can accelerate the healing process of socket wounds after tooth extraction of Wistar rats, both clinically and microscopically on the 7th day compared to mobe leaf extract gel with a concentration of 1% [10].

Based on the background that has been described, binahong leaves and mobe leaves alone are known to accelerate the healing of socket wounds because they have active components that play a role in wound healing. To the best of our knowledge, studies involving the combination of the two leaves are not available. Therefore, researchers are interested in conducting research on to explore the effect of the combination of ethanol extract gel of binahong leaves and mobe leaves on the proliferation of fibroblasts and osteocytes on the healing of socket wounds after tooth extraction in Wistar rats.

Materials and Methods

Ethical considerations

All experimental procedures in this study were performed in accordance with Institutional Animal Care and Usage Committee (ARRIVE) 2.0 guidelines. The protocol was approved by the Health Research Ethics Committee (KEPK) at the Department of Biology, Faculty of Mathematics and Natural Sciences, North Sumatra University, Medan, North Sumatra, Indonesia with reference numbers: 0058/KEPH-FMIPA/2022, 0059/KEPH-FMIPA/2022 and 0060 /KEPH-FMIPA/2022.

Sample size

The sample size in this study was calculated based on previous research of a similar nature [2]. The final sample size will be four animals per group (with a total of 8 groups) or 32 rats in total.

Rats

The sample in this study was Wistar rats (*Rattus novergicus*). Eligible samples were those that met the inclusion criteria, *i.e.*: male Wistar rats aged approximately three months with a body weight of 200 - 250 g and in healthy condition, which were characterized by active movement, hair was not easily separated, and there were no wounds on the body and oral cavity. For the exclusion criteria, *i.e.*: having a systemic disease or disorder, already receiving research treatment and dead mice. The rats were acquired and housed at the Animal House in the Faculty of Mathematics and Natural Sciences, University of North Sumatra, Indonesia.

Before being given treatment, rats were acclimatized for one week as an adaptation to their environment [13].

Study design and groups

The study conducted was an *in vivo* experiment with a post-test only control design. The rats were randomized by simple random sampling and divided into 8 groups by the Animal House lab technicians. The socket wounds in treatment: groups (I to VI) were given a combination of ethanol extract gel of binahong leaves and mobe leaves with different ratios (1:3 for group I and II, 1:1 for group III and IV, 3:1 for group V and VI). Socket wounds in group VII and VIII were given Aloclair® gel (Sinclair Pharma, Italy) and served as positive control groups. The proliferation of fibroblast and osteocytes were observed on the 7th and 21st day post-extraction.

Tooth extraction

The acclimatized rats were given a combined general anaesthetic at a dose of 91 mg/kg bw of ketamine (Agrovet market, Peru) and 9.1 mg/kg bw of xylazine (Interchemie, Netherlands) intraperitoneally. After that, the left mandibular incisor of the Wistar rat was extracted using an artery clamp (Wells Spencer, London) with a luxation motion. Then the post-extraction socket was cleaned of blood and debris by irrigating it using aquadest solution [13]. The extraction was performed by the same veterinarian who was blinded to the group allocation.

Binahong leaf and mobe leaf ethanol extract gel preparation

The ethanol extract of binahong leaves and mobe leaves used in this study was obtained from the Pharmacognosy Laboratory of the Faculty of Pharmacy, University of North Sumatra, Indonesia. The method used is the maceration method with 80% ethanol (Smart Lab Indonesia, Indonesia). Binahong leaves and mobe leaves were washed under running water to get rid of dirt. Binahong leaves and mobe leaves were dried and weighed. The leaves were grinded into powder using with an electric blender (Philips HR2115, Indonesia) and then soaked in 80% ethanol in a closed container and distilled for five days at room temperature. After five days, the 80% ethanol solvent was replaced with new solvent and soaked again for two days. Subsequently, a final filtering process was carried out. Then, a water bath was used to evaporate the solvent until the extract dried [14]. Next, the gel was made by adding 10 mL of hot distilled water to a mortar, then 0.125 g of carbopol (Merck, Germany) was added and the mixture was stirred with a pestle. Next, 1.5 g of triethanolamine (TEA) (Merck, Germany) and 2 g of glycerin (Merck, Germany) were added and the mixture was stirred until it was homogeneous. In a second mortar, a mixture of 10 mL distilled water, 0.125 g of hydroxypropyl methylcellulose (HPMC) (Merck, Germany), nipagin (Merck, Germany) and nipasol (Merck, Germany) were stirred until homogeneous. The second mortar mixture was poured into the first mortar and the mixture was stirred until it was homogeneous [4]. The gel used for the treatment groups in this study was made from the combination of ethanol extract gel of binahong leaves and mobe leaves with a total concentration of 3%. The 3% gel was made of and prepared in three different ratios, *i.e.*: 1:3 (0.15 g binahong leaves + 0.45 g mobe leaves), 1: 1 (0.3 g binahong leaves + 0.3 g mobe leaves) and 3:1 (0.45 g binahong leaves + 0.15 g mobe leaves).

Application of gels

The socket wounds in the treatment groups (I-VI) were applied with combination gel of binahong leaf and mobe leaf with the ratio of 1:3, 1:1, 3:1, while the socket wounds in the control groups (groups VII and VIII) were applied with Aloclair®. In each application, 0.1 mL of gel was applied directly on the socket wound using a 1 mL syringe (One Med Health Care, Indonesia) and an irrigation tip with a bent needle (Ivoclar Vivadent, Ø: 1.2 mm, Liechtenstein) until all surfaces of the socket were covered by the gel. The application begins when after a tooth extraction and is counted as day 1 and was carried out twice a day (08.00 a.m. - 10.00 a.m. and 16.00 a.m. - 18.00 a.m.). After the gel was applied, the rats were not given water for about an hour to prevent the gel from coming out of the socket. The gel was applied for 14 days after tooth extraction because the socket wound took approximately 14 days to heal.

Histopathological analysis

Wound healing in the socket was observed on day 7 (groups I, III, V and VI) and day 21 (groups II, IV, VI and VIII). After reaching the 7th and 21st days, the rats were euthanized by neck dislocation. Then, the jaws of the mice were excised and the alveolar bone tissue was removed from the socket and then immersed in 10% EDTA solution (Merck, Germany) for 10 days. Then Haematoxylin-Eosin (HE) (Merck, Germany) staining was performed and the centre of the tissue was cut transversely and observed using a microscope. The process of making histological preparations was done by fixation, dehydration, clearing, embedding, blocking sectioning, staining and mounting [15]. The calculation of the proliferation of fibroblasts and osteocytes by mean cell count was observed under an electric microscope with a magnification of 400 times in 5 fields of view. The calculation of the mean cell count was carried out by two observers blinded to avoid bias and was aided with a tally

counter and a calculator. The results obtained from each field of view are averaged [16].

Statistical analysis

The data were first analysed with Shapiro-Wilk normality test to ascertain the distribution of the data. Data that were normally distributed would be analysed with parametric tests. The histopathological data of the mean cell count of fibroblasts and osteocytes were analysed using one-way ANOVA test. The results were considered as significant if the p-value was below 0.05. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS), version 21 (IBM® Inc., USA) by a researcher who was aware of the group allocation.

Results and Discussion

Based on the results of the Shapiro-Wilk normality test, all data on proliferation of fibroblasts and osteocytes in socket wound healing after tooth extraction of male Wistar rats on the 7th and 21st days were normally distributed ($p > 0.05$) and the data analysis continued with statistical tests one-way ANOVA.

Based on the one-way ANOVA test, there were significant differences in the number of fibroblasts and osteocytes between the 7th and 21st days in the gel treatment group with the combination of ethanol extract of binahong leaves and mobe leaves 1:3, 1:1, 3:1 and the positive control group of Aloclair gel after tooth extraction of male Wistar rats ($p < 0.05$) (Table I).

Based on the one-way ANOVA test, there were significant differences in the number of fibroblasts and osteocytes between the treatment group with the gel combination of binahong leaf extract and mobe leaf 1:3, 1:1, 3:1 and the positive control group Aloclair gel on the 7th and 21st days after tooth extraction of male Wistar rats ($p < 0.05$) (Table II).

Table I

The difference in the average number of fibroblasts and osteocytes in each treatment group between the 7th and 21st days after tooth extraction of male Wistar rats

Treatment	Observation (day)	Fibroblas (Mean ± SD)	Osteocyte (Mean ± SD)
Combination 1:3	7	160.27 ± 9.00	59.13 ± 1.67
	21	77.40 ± 2.27	67.80 ± 2.20
Combination 1:1	7	132.87 ± 8.33	60.67 ± 5.58
	21	92.67 ± 2.08	74.07 ± 1.33
Combination 3:1	7	176.47 ± 2.13	65.60 ± 5.10
	21	120.47 ± 1.10	83.80 ± 2.83
Aloclair	7	107.33 ± 5.10	48.20 ± 4.42
	21	69.67 ± 4.50	60.47 ± 1.85

Table II

The difference in the average number of fibroblasts and osteocytes between the treatment groups on the 7th and 21st days after tooth extraction of male Wistar rats

Observation (day)	Treatment	Fibroblast (Mean ± SD)	Osteocyte (Mean ± SD)
7	Combination 1:3	160.27 ± 9.00	59.13 ± 1.67
	Combination 1:1	132.87 ± 8.33	60.67 ± 5.58
	Combination 3:1	176.47 ± 2.13	65.60 ± 5.10
	Aloclair	107.33 ± 5.10	48.20 ± 4.42
21	Combination 1:3	77.40 ± 2.27	67.80 ± 2.20
	Combination 1:1	92.67 ± 2.08	74.07 ± 1.33
	Combination 3:1	120.47 ± 1.10	83.80 ± 2.83
	Aloclair	69.67 ± 4.50	60.47 ± 1.85

Fibroblasts can be seen among the blue-stained collagen fibre (Figure 3). The results of the mean cell count of fibroblast proliferation (Figure 1) show that the number of fibroblasts in each group decreased from day 7 to day 21. The number of fibroblasts in the gel treatment group with the combination of ethanol extract of binahong leaves and mobe leaves with a ratio of 3:1 was higher than the other groups.

Osteocytes can be seen in the alveolar bone matrix inside lacunae (Figure 4). The results of the mean cell count of osteocyte proliferation (Figure 2) show that the number of osteocyte cells in each group increased from day 7 to day 21. The number of osteocytes in the gel treatment group with the combination of ethanol extract of binahong leaves and mobe leaves with a ratio of 3:1 was higher than the other groups.

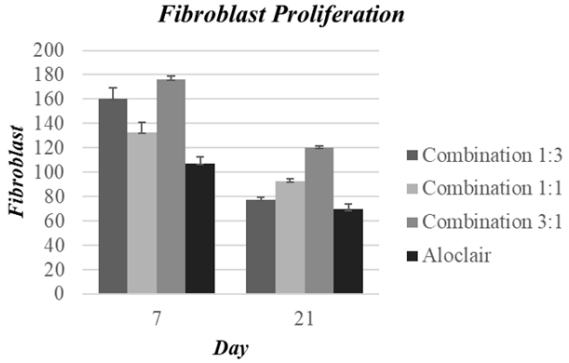

Figure 1.
Mean cell count diagram of fibroblast on day 7 and 21

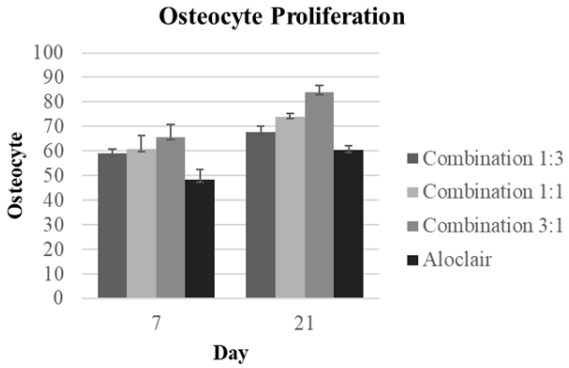

Figure 2.
Mean cell count diagram of osteocytes on day 7 and 21

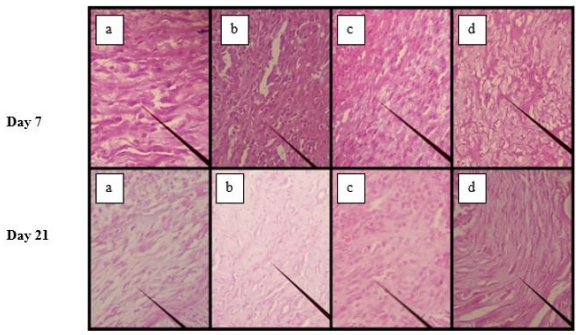

Figure 3.
Histopathological observation of fibroblast in the alveolar bone healing of sockets
The observation was conducted on 7th and 21st day post-extraction socket wounds stained with Haematoxylin-Eosin after the application of gel: (a) combination 1:3; (b) combination 1:1; (c) combination 3:1; (d) aloclair (x400)

Binahong leaf and mobe leaf extracts have an effect on healing socket wounds which are characterized by an increase in fibroblast and osteocyte proliferation. Based on the results of the research that has been carried out, the combination of ethanol extract gel of binahong leaves and mobe leaves was proven to be able to increase the proliferation of fibroblasts and osteocytes in the healing of socket wounds after tooth extraction of Wistar rats. Based on research by Hanafiah *et al*. in 2021, that the application of 3% binahong leaf extract gel was able to accelerate the healing process of alveolar bone in post-extraction tooth socket Wistar rats by increasing the proliferation of fibroblasts, osteoblasts and osteocytes [4]. While on mobe leaves, another study by Hanafiah *et al*. in 2020, that mobe leaf extract gel with a concentration of 3% gave a better effect than other concentrations in accelerating socket wound closure both clinically and microscopically [10].

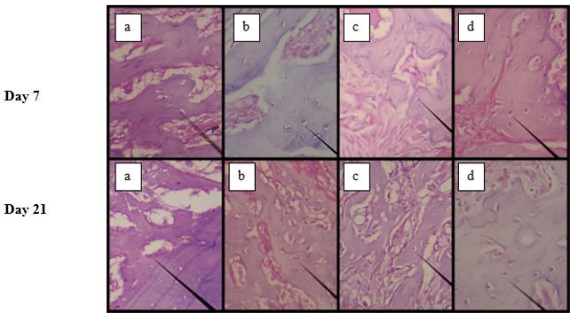

Figure 4.
Histopathological observation of osteocytes in the alveolar bone healing of sockets
The observation was conducted on 7th and 21st day post-extraction socket wounds stained with Haematoxylin-Eosin after the application of gel: (a) combination 1:3; (b) combination 1:1; (c) combination 3:1; (d) aloclair (x400)

The results showed that the highest number of fibroblasts was found on day 7 and decreased the number of fibroblasts on day 21 (Figure 1). This is in accordance with the physiological wound healing process, where the formation of fibroblasts peaks on the 7th day and decreases because the collagen and ground substance produced by fibroblasts will form granulation tissue to maintain the integrity of the wound tissue and prepare for the formation of alveolar bone. As alveolar bone tissue begins to form, the number of fibroblasts decreases and collagen production is replaced by osteoblasts [17].
The results showed that the number of osteocytes increased from day 7 to day 21 and the highest number of osteocytes was found on day 21 (Figure 2). This is in accordance with the research of Olaitan *et al*. in 2019, that the number of osteocytes in alveolar bone healing increased until the 4th week after tooth extraction, because the peak of differentiation and proliferation of osteoblasts occurred on the 14th day after tooth extraction and the process of maturation of osteoblasts into osteocytes will last until the 28th day [9].
Based on Tables I and II, each group showed a significant difference in the number of fibroblasts and osteocytes after tooth extraction of male Wistar rats ($p < 0.05$). The highest number of fibroblasts and osteocytes was found in the group that was applied the combination gel of binahong leaves and mobe leaves 3:1 compared to the control group of

Aloclair® gel. It can be assumed that the combination of binahong leaf extract gel and mobe leaf extract had a better effect on fibroblast and osteocyte proliferation on socket wound healing, thus showing a significant difference compared to Aloclair® gel.

Based on research by Hanafiah and Yuniarti in 2017, the results of phytochemical screening in binahong leaf extract contain many secondary metabolites, namely saponins, tannins, alkaloids, terpenoids, steroids, glycosides and flavonoids [11, 15]. The role and the most commonly found as drugs in curing various diseases are flavonoids, saponins and tannins. While in mobe leaves, based on research by Bhattacharya E *et al*. in 2019, that the results of phytochemical screening in mobe leaf extracts contain secondary metabolites such as flavonoids, saponins and tannins [19]. Hakim *et al*. research showed that the most abundant metabolites found in mobe plants were flavonoids [20].

The flavonoids contained in binahong leaves and mobe leaves play a role in the tissue remodelling phase, by increasing vascularization so that the supply of oxygen and nutrients to the injured tissue and cells takes place maximally. This then resulted in the increase of collagen synthesis which will accelerate the wound healing process [11]. In addition, mobe leaves also contain artocarpine, a flavonoid that has a role in the wound healing process, by stimulating collagen formation, reepithelization and the formation of new blood vessels [21]. Artocarpine is also able to accelerate the wound healing process by accelerating the inflammatory phase, stimulating collagen formation, myofibroblast differentiation, stimulating fibroblast proliferation and migration, forming new blood vessels, increasing wound contraction during the remodelling phase and increasing the production of Transforming Growth Factor-β (TGF-β) [22]. Saponins can accelerate wound healing by increasing the ability of TGF-β1 receptors. The increase in the TGF-β1 receptor, can accelerate the proliferation and migration of fibroblasts towards the wound [11]. In addition, saponins have a function as an antiseptic that can prevent infections in wounds by killing and inhibiting the growth of microorganisms [18]. Tannins function as astringents that can work as vasoconstrictors and stimulate protein release and protein deposition. In addition, tannins can prevent further infections caused by bacteria by blocking the synthesis of nucleic acids and the action of the ATPase enzyme in blocking metabolic pathways [11, 16]. According to Septiana *et al*., tannins have antioxidant properties that are able to bind unstable free radicals so as to prevent cell membrane damage. A well-formed cell membrane will accelerate the proliferation process [23].

Conclusions

Combination of ethanol extract gel of binahong leaves (*Anredera cordifolia*) and mobe leaves (*Artocarpus lakoocha*) had an effect on increasing the proliferation of fibroblasts and osteocytes on the healing of socket wounds after tooth extraction in Wistar rats on the 7th and 21st days.

Conflict of interest

The authors declare no conflict of interest.

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