



RESEARCH ARTICLE

REVISED Effects of 3% binahong (*Anredera cordifolia*) leaf extract gel on alveolar bone healing in post-extraction tooth socket wound in Wistar rats (*Rattus norvegicus*)

[version 2; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: Binahong (*Anredera cordifolia* (Ten.) STEENIS) is a widely available herbal plant in Indonesia and has been intensely researched for its healing abilities due to its biological activities, but few have studied its capability in accelerating hard tissue healing in post-extraction tooth sockets. The purpose of this study was to analyse the effects of 3% binahong leaf extract gel on alveolar bone healing in post-extraction sockets in Wistar rats.

Methods: In this study, 48 male Wistar rats were randomly allocated to twelve groups. After the extraction of the left mandibular incisor, sockets in Group I to IV were given 3% binahong leaf extract gel, group V to VIII were given a control gel, and group IX to XII were given Gengigel® for 14 days. The residual socket volume (RSV) and fibroblast proliferation were observed on the 3rd, 7th, and 14th day post-extraction, while the osteoblast and osteocyte proliferation were observed on the 7th, 14th, and 28th day post-extraction. The RSV data were analysed using repeated measure ANOVA and one-way ANOVA, while the histopathological data were analysed using one-way ANOVA.

Results: The results showed that the binahong group had the lowest RSV and the highest fibroblast proliferation compared to the other groups on the 7th day ($p < 0.05$) and the highest osteoblast and osteocyte proliferation compared to the other groups on the 14th day ($p < 0.05$).

Conclusion: The experiment showed that 3% binahong leaf extract gel could accelerate wound closure, which was characterized by a greater decrease in the RSV value in comparison to the other treatment groups and could enhance alveolar bone healing by increasing the proliferation of fibroblasts, osteoblasts, and osteocytes.

Open Peer Review

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Any reports and responses or comments on the article can be found at the end of the article.

Keywords

Anredera cordifolia, tooth extraction, wound healing, fibroblasts, osteoblasts, osteocytes

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REVISED Amendments from Version 1

In each section, some changes have been done in the diction. In Introduction, we have added the medicinal and pharmacological activities of Binahong and have also included some previous wound healing studies using medicinal plants. In Methods, we have also added details regarding georeferencing and effect size. Our tables and figures have also been renewed and improved with new details, such as error bars, new charts, and better quality of histopathological figures, to provide the readers a clearer picture of our research methods and results. We have included two new figures (Figure 1 and 2) in regards to this. In the discussion, we have included the information regarding the safety of Binahong leaf extract and also the limitations and future perspectives of this study.

We would like to thank our reviewer Lidia Audrey Rocha Valadas, Dalia Hussein El-Rouby, and Mohd Farhan Hanif Reduan for their thoughtful inputs regarding our article. We hope this revision could better explain our research.

Any further responses from the reviewers can be found at the end of the article

Introduction

Tooth extraction will leave a socket wound and can affect a person's quality of life, especially in terms of the ability to eat and speak.^{1,2} Socket wound healing involves the healing of soft tissue that is, comprised of the gingiva and connective tissue, as well as the healing of hard tissue, the alveolar bone.³ Both soft tissue and hard tissue undergo the same healing phases, namely the inflammatory, proliferative, and remodelling phases.²

Some of the cells that play important roles during the healing of socket wounds are fibroblasts, osteoblasts, and osteocytes. At the beginning of alveolar bone healing, the cells that are more active are fibroblasts because these cells produce collagen, glycosaminoglycans, proteoglycans, and adhesive glycoproteins that will form granulation tissue.⁴ Granulation tissue is rich in blood vessels and aims to restore tissue unity and prepare for bone formation by osteoblasts, osteoclasts, and osteocytes in the remodelling phase.^{5,6} In mice, the fibroblasts in alveolar bone begin to proliferate on the 3rd day after tooth extraction and continue to increase until the 7th day.^{7,8} From the 7th day to day 14th day, there will be a decrease in the number of fibroblasts as new alveolar bone starts to fill the socket.⁸ A proper proliferation of fibroblasts will trigger good bone formation and accelerate socket wound closure because fibroblasts also take part in wound retraction.⁹ Osteoblasts are mononuclear cells that synthesize osteoid matrix and play an important role from the proliferative phase to the remodelling phase of healing.^{2,3,10,11} Based on previous research, osteoblasts in the bone healing process after tooth extraction can be found on the 7th day and will continue to differentiate for more than 21 days.^{3,7} Osteocytes are the matured form of osteoblasts.¹² When osteoblasts form bone, they can be trapped in the matrix formed by osteoblasts and differentiate into osteocytes.^{11,12} In the bone healing process, osteocytes play a role in bone remodelling by maintaining the integrity and vitality of the new bone.¹¹ Based on previous research, there are more osteocytes on the 28th day post-extraction.¹³

Currently, alternative medicine with herbal ingredients has started to be used to help wound healing. The employment of natural active ingredients in herbal medicine can also reduce the cost of wound care.¹⁴ Several plants that have previously been investigated for their effects on wound healing are Mobe (*Artocarpus lakoocha*), which can increase cell proliferation and migration; Miswak (*Salvadora persica*), which can increase the formation of alveolar bone; and Moringa (*Moringa oleifera*), which can increase the expression of bone-forming proteins.^{15–17} Another plant, namely *Aloe vera*, has even been proven effective in accelerating the socket wound healing after tooth extraction in humans.¹⁸

Binahong (*Anredera cordifolia* (Ten.) STEENIS) is a plant used in herbal medicine. Binahong is often considered as a wild plant and harmful to other plants because its growth is invasive. However, the characteristics of Binahong, which are its abilities to grow easily and rapidly in varied climates, can also be advantages because Binahong can be used as an alternative medicine that is also highly-sustainable.^{19,20} Binahong is known to have pharmacological properties, such as antiinflammatory, antibacterial, antifungal, antiviral, and antioxidant properties.^{19,21} It could also improve kidney function, counter hyperlipidemia, reduce hypertension, become anticancer, act as an analgesic, and help wound healing, including tooth socket wounds.^{19,21} Binahong also has been used in traditional medicine by placing its leaves over wounds and it has shown an affinity in accelerating wound healing.^{21,22} This is due to the presence of the secondary metabolites contained in the Binahong leaf extract such as tannins, saponins, flavonoids, alkaloids, anthraquinones, phenolic acids, triterpenes, steroids, and glycosides.²³ The flavonoids found are quercetin, rutin, apigenin, apigetrin, morin, myricetin, and vitexin.^{24–27} The phenolic acid found in Binahong leaf extract is P-coumaric acid, and the triterpenes found are ursolic acid and oleanolic acid.^{24,28} A few studies have been conducted to observe the effects of Binahong leaf extract on wound healing. Hanafiah *et al* showed that Binahong leaf extract could increase the proliferation of NIH-3T3 fibroblasts and also proved that 3% Binahong leaf extract gel could accelerate palatal mucosal wound healing in rats by increasing the proliferation of fibroblasts.^{29,30} The study of Khoswanto *et al* showed that the application of 10%

Binahong leaf extract gel could increase the expression of BMP-2 (bone morphogenetic protein-2) and osteoblasts in the tooth socket wound of rats on the 7th day post-extraction.³

The purpose of this study was to determine the effects of 3% Binahong leaf extract gel application on the acceleration of socket wound closure and the proliferation of fibroblasts, osteoblasts, and osteocytes in the healing of alveolar bone in tooth socket wounds up to 28 days post-extraction.

Methods

Ethical considerations

All experimental procedures in this study were carried out in accordance with the Institutional Animal Care and Usage Committee (ARRIVE) guidelines 2.0. Ethical clearance had been approved by the Health Research Ethics Committee (KEPK) at the Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, North Sumatra, Indonesia with letter numbers: 0206/KEPH-FMIPA/2021 and 0202/KEPH-FMIPA/2021.

Animals

Sample size

The sample size in this study was calculated based on previous research of a similar nature.³ The effect size (Cohen's d) between the experimental group and the control group from the previous research was 3.31.³ The minimum sample size obtained from the calculations was three animals and the final sample size was adjusted to four animals per group (with a total of 12 groups) or 48 animals in total. There was a 10% addition from the minimum sample size to anticipate sample exclusion during the experiment.

Rats

Animals used in this study were 48 male Wistar rats (*Rattus norvegicus*) of two to three months old and with an average body weight of 200-250 grams. The rats should also be in good health and had never received any research treatment previously. Rats with abnormalities or those that died before the experiment ended were excluded from the study. The rats that died were not replaced as long as the minimum sample size was still maintained. The rats were acquired and housed at the Animal House in the Faculty of Mathematics and Natural Sciences in Universitas Sumatera Utara, Medan, North Sumatra, Indonesia. Before administering the treatment, the rats were acclimatized for seven days to assure that the rats could adapt to their surroundings and were allowed *ad libitum* feeding and drinking.

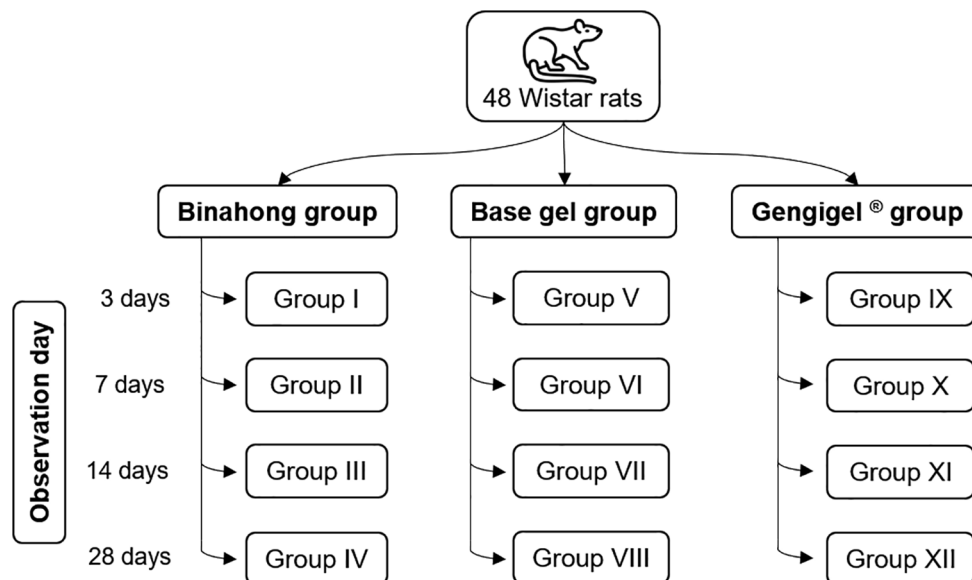


Figure 1. Group allocation; each group is differentiated from one another based on its treatment and observation day and is comprised of four Wistar rats.

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 allocated into twelve groups by the Animal House lab technicians: group I to IV were given 3% Binahong leaf extract gel, group V to VIII were given base gel as the negative control, and group IX to XII were given Gengigel® as the positive control (Figure 1). The treatments were given for 14 days as that was the approximate amount of time needed for the socket wound to close completely.² The residual socket volume (RSV) and fibroblast proliferation were observed on the 3rd, 7th, and 14th day post-extraction, while the osteoblast and osteocyte proliferation (cells) were observed on the 7th, 14th, and 28th day post-extraction. All researchers were blinded to the box assignment of the rats, except for the researchers who were in charge of applying the gel to the socket wounds.

Tooth extraction

Wistar rats were anesthetized intraperitoneally using a combination of 91 mg/kgBW ketamine (Agrovet market, Peru) and 9.1 mg/kgBW xylazine (Interchemie, Netherlands) with an anesthetic dose of 0.1 mL/100 g rat weight to alleviate the pain induced by the procedure.³¹ After that, the mandibular left incisor was extracted using an artery clamp (Wells Spencer, London) with a luxation movement.³² After the extraction, the socket was irrigated with distilled water to clean the socket from debris. The extraction was performed by the same veterinarian who was blinded to the group allocation.

Binahong leaf extract gel and base gel preparation

The Binahong leaf extract used in this study was obtained from the Pharmacognosy Laboratory, Faculty of Pharmacy, Universitas Sumatera Utara, Medan, North Sumatra, Indonesia. A total of 400 g fully opened Binahong leaves and aged approximately 12 weeks were selected and obtained from Simpang Perdagangan village, Tigabinanga district, Karo regency, North Sumatra, Indonesia in April 2017. Tigabinanga district is located at coordinate 3.0729, 98.2415. It has an altitude of 490 to 750 meter above sea level.³³ Karo regency has a tropical climate, with rainy season (August-January, March-May) and dry season (February, June, July).³⁴ Binahong leaves were extracted by the maceration method using 80% ethanol (Smart Lab Indonesia, Indonesia) as the solvent. The Binahong leaves were initially grinded into powder 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.³⁵

The base 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.³⁰ The second mortar mixture was poured into the first mortar and the mixture was stirred until it was homogeneous. To obtain 20 g of 3% Binahong leaf extract gel, 0.6 g of Binahong leaf extract was added to the base gel and the mixture was stirred until it was homogenous (Figure 2). The gel was reproduced every 3 to 4 days to ensure its freshness.

Application of gels

The socket wounds in group I to VI were applied with 3% Binahong leaf extract gel; group V to VIII were given base gel; and group IX to XII were given Gengigel® (Ricerfarma, Italy). In every application, 0.1 mL of gel was applied directly to the socket wound using a 1 mL syringe (One Med Health Care, Indonesia) with a bent needle irrigation tip (Ivoclar Vivadent, Ø: 1.2 mm, Liechtenstein) until the gel covered the entire wound surface to make sure that the gel would be directly in contact with the wound. Applications were made twice a day in the morning at 8.00 a.m.-10.00 a.m. and in the afternoon at 4.00 p.m.-6.00 p.m. for 14 days. The time was chosen to ensure consistent schedule for the gel application each day and the application was conducted by a researcher who was aware of the group allocation.

Clinical measurement of residual socket volume

Measurements of the socket volume were made with a digital caliper (Digital Caliper, China), a pair of compasses (Joyko®, Indonesia), and a periodontal probe (Kohler, NR-3182, Germany) and were performed on the rats in group III, VII, XI, so that repeated measurements could be made on the same samples. The measurements were performed by the same researcher blinded. The average socket volume was calculated for each male Wistar rat. To determine the residual socket volume for each rat, the socket volume obtained from the measurements on the 3rd day, 7th day, and 14th day were each divided by the socket volume on the 1st day. Socket volume = mesiodistal width × buccolingual width × probing depth.³⁶

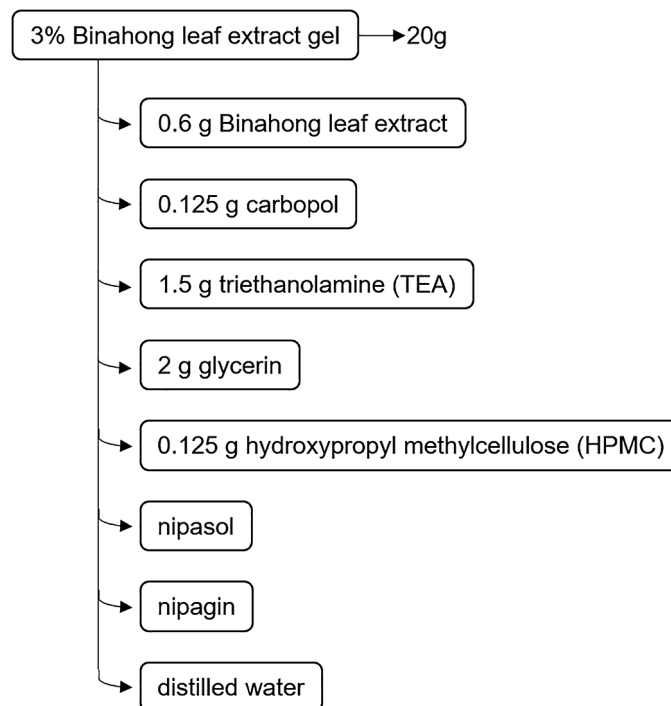


Figure 2. The composition of 3% Binahong leaf extract gel.

Histopathological analysis

After reaching the 3rd day (group I, V, IX), the 7th day (group II, VI, X), the 14th day (group III, VII, XI), and the 28th day (group IV, VIII, XII) post-extraction, the rats from the respective groups were sacrificed by cervical dislocation. This method was chosen to ensure rapid termination and lower chance of tissue contamination. The mandible of the rats was removed from the skull and the socket wound tissue was excised. Fresh tissue was fixed in 10% Buffered Neutral Formalin (BNF) solution (Milestone Medical, Italy) with a pH of 6.8-7.0 and the ratio of tissue to the BNF solution was 1:10.³⁷ The tissue containers were labelled and fixation was carried out for 12-48 hours, after which the tissue was immersed in 10% EDTA solution (Merck, Germany) for 10 days for decalcification.³⁸ The tissue was then cut using a scalpel with a thickness of 4 mm and the tissue was placed in tissue cassettes and put into a basket.³⁷ The baskets were loaded into an automatic processor machine and then transferred to a dehydration machine for tissue dehydration.³⁷ The next stage was embedding, in which the tissue was placed in a mould and submerged in liquid paraffin.³⁷ The cooled paraffin blocks were cut with a thickness of 4-5 μ m using a microtome machine and the slices were placed in a water bath at 45°C.³⁷ The tissue slices would be collected on clean glass slides. The slides were labelled and placed in an incubator at 37°C to dry overnight.³⁷ The slides were then stained with haematoxylin-eosin (Merck, Germany) to see osteoblasts and osteocytes and with Masson's Trichrome (Merck, Germany) to see fibroblasts microscopically.³⁷ The stained tissue slides were then observed under an electric microscope (Primo Star, Carl Zeiss, Germany) with a magnification of 400 times in 10 viewing fields. Fibroblasts when observed under microscope are spindle-shaped with an ovoid nucleus and are commonly found in connective tissue.⁴ Osteoblasts when active are spindle-shaped or cuboidal, large and broad with abundant basophilic cytoplasm. When inactive, osteoblasts are flat, spindle-shaped, and lined up along the bone matrix surface.^{10,11,39} Meanwhile, osteocytes are smaller than osteoblasts, branched out, and are trapped in lacunae among the matrix formed by osteoblasts.^{11,39}

The calculation of the mean cell count was carried out by two observers blinded to avoid bias and was aided with a tally counter (SXH, 5136, China) and a calculator (Casio, fx-82MS, Japan). The results of the fibroblast, osteoblast, and osteocyte cell counts from 10 viewing fields were averaged.⁴⁰

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 RSV data were analysed using repeated measures ANOVA and one-way ANOVA test, while the histopathological data of the mean cell count of fibroblasts, osteoblasts, and osteocytes were analysed using one-way ANOVA test. The multiple comparison tests were performed with Least

Significant Difference (LSD) 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

Clinical measurement of residual socket volume

The residual socket volume (RSV) was measured on the 3rd, 7th, and 14th day post-extraction. The RSV in all treatment groups decreased over time and based on the repeated measures ANOVA test, there were significant differences in the RSV on the 3rd, 7th, and 14th day in the Binahong group, the Gengigel® group, and the base gel group ($p < 0.05$) (Figure 3A). In the Binahong group, significant RSV differences were observed between the 3rd and 7th day, between the 3rd and 14th day, and between the 7th and 14th day, while in the Gengigel® group and the base gel group, significant RSV differences were only observed between the 3rd and 14th and 7th and 14th day ($p < 0.05$). There was no difference in the RSV between the 3rd and 7th day in the Gengigel® group and the base gel group (Table 1).

Based on the one-way ANOVA test, there was a significant difference in the RSV among the treatment groups on the 7th and 14th day ($p < 0.05$). On the 7th day, there were significant RSV differences between the Binahong group and the Gengigel® group, between the Binahong group and the base gel group, and between the Gengigel® group and the base gel group, while on the 14th day, the significant RSV differences were only between the Binahong group and the base gel group and between the Gengigel® group and the base gel group (Table 2).

Histopathological analysis

Fibroblast proliferation was examined on the 3rd, 7th, and 14th day post-extraction. There was an increase trend in the number of fibroblasts from the 3rd to 7th day and a decrease from the 7th to 14th day (Figure 3B). Fibroblasts can be seen among the blue-stained collagen fibre (Figure 4). Based on the one-way ANOVA test, there were significant differences in the number of fibroblasts on the 3rd, 7th, and 14th day in the Binahong, Gengigel®, and base gel groups ($p < 0.05$). In the Binahong group, there were significant differences in the number of fibroblasts between the 3rd and 7th day and between the 7th and 14th day, while in the Gengigel® group, there were significant differences in the number of fibroblasts between the 3rd and 7th day and between the 3rd and 14th day. In the base gel group, the significant differences were only observed between the 3rd and 7th day (Table 1).

Significant differences in the number of fibroblasts among the Binahong group, Gengigel® group, and the base gel group were observed on the 3rd, 7th, and 14th day ($p < 0.05$). On the 3rd day, there were significant differences in the

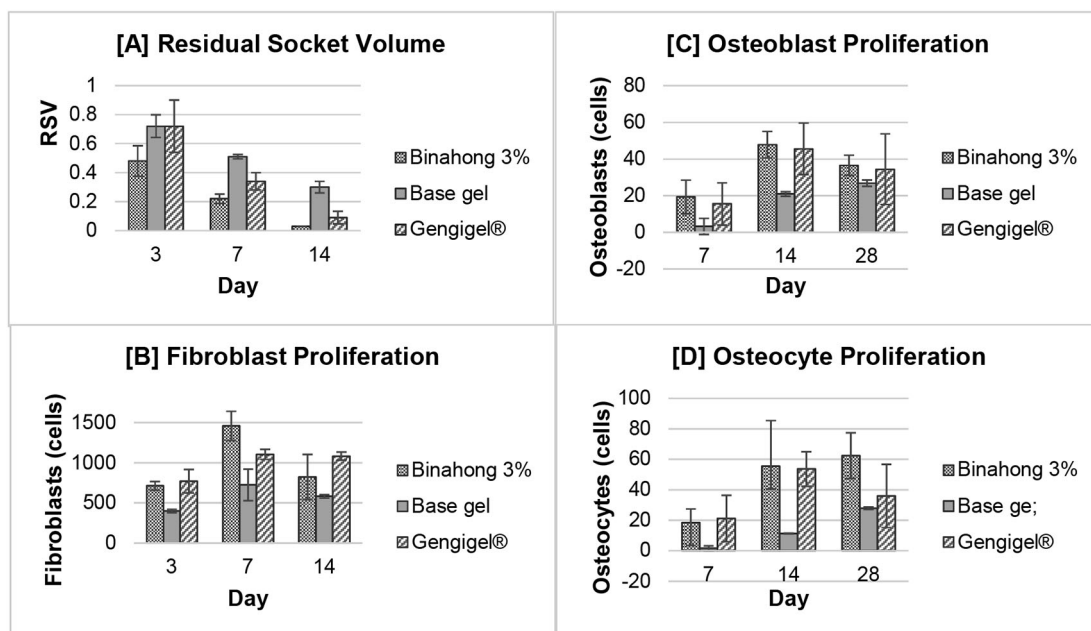


Figure 3. Clinical and histopathological analysis of alveolar bone healing between different treatment groups. Results were shown as the means of the residual socket volume (clinical observation) and the number of fibroblast, osteoblast, and osteocyte cells (histopathological observation).

Table 1. Effects of each treatment on socket wounds for different time periods.

Treatment	Observation (day)	RSV (Mean $\pm$ SD)	Fibroblast (cells) (Mean $\pm$ SD)	Observation (day)	Osteoblast (cells) (Mean $\pm$ SD)	Osteocyte (cells) (Mean $\pm$ SD)
Binahong 3%	3	0.48 $\pm$ 0.105 ^a	713.40 $\pm$ 54.896 ^a	7	19.33 $\pm$ 9.129 ^a	18.38 $\pm$ 9.068 ^a
	7	0.22 $\pm$ 0.032 ^b	1458.95 $\pm$ 182.654 ^b	14	47.78 $\pm$ 7.208 ^b	55.50 $\pm$ 29.928 ^b
	14	0.03 $\pm$ 0.000 ^c	824.27 $\pm$ 280.527 ^a	28	36.49 $\pm$ 5.545 ^b	62.44 $\pm$ 14.987 ^b
Base gel	3	0.72 $\pm$ 0.078 ^a	399.85 $\pm$ 20.977 ^a	7	3.25 $\pm$ 4.356 ^a	1.60 $\pm$ 1.709 ^a
	7	0.51 $\pm$ 0.015 ^a	724.87 $\pm$ 196.289 ^b	14	20.88 $\pm$ 1.239 ^b	11.37 $\pm$ 0.252 ^b
	14	0.30 $\pm$ 0.040 ^b	582.07 $\pm$ 22.830	28	26.78 $\pm$ 1.728 ^c	27.93 $\pm$ 0.861 ^c
Gengigel®	3	0.72 $\pm$ 0.180 ^a	771.70 $\pm$ 146.452 ^a	7	15.48 $\pm$ 11.487	21.18 $\pm$ 15.229
	7	0.34 $\pm$ 0.060 ^a	1105.40 $\pm$ 62.366 ^b	14	45.52 $\pm$ 14.056	53.63 $\pm$ 11.388
	14	0.09 $\pm$ 0.044 ^b	1082.87 $\pm$ 50.217 ^b	28	34.42 $\pm$ 19.269	35.93 $\pm$ 20.740

The parameters examined to show the effects of each treatment were the residual socket value (RSV) in the clinical observation and the number of fibroblasts, osteoblasts, and osteocytes in the histopathological observation. The values shown were the mean $\pm$ standard deviation of at least three replicates. Different alphabets indicated significant statistical differences ($p < 0.05$) between the time periods analysed with the LSD test. The lack of alphabets or the same alphabets indicated no statistical differences ($p > 0.05$) between the time periods analysed with the LSD test.

Table 2. Comparison of the effects of different treatments on socket wounds.

Observation (day)	Treatment	RSV (Mean $\pm$ SD)	Fibroblast (cells) (Mean $\pm$ SD)	Observation (day)	Osteoblast (cells) (Mean $\pm$ SD)	Osteocyte (cells) (Mean $\pm$ SD)
3	Binahong 3%	0.48 $\pm$ 0.105	713.40 $\pm$ 54.896 ^a	7	19.33 $\pm$ 9.129	18.38 $\pm$ 9.068
	Base gel	0.72 $\pm$ 0.078	399.85 $\pm$ 20.977 ^b		3.25 $\pm$ 4.356	1.60 $\pm$ 1.709
	Gengigel®	0.72 $\pm$ 0.180	771.70 $\pm$ 146.452 ^a		15.48 $\pm$ 11.487	21.18 $\pm$ 15.229
7	Binahong 3%	0.22 $\pm$ 0.032 ^a	1458.95 $\pm$ 182.654 ^a	14	47.78 $\pm$ 7.208 ^a	55.50 $\pm$ 29.928 ^a
	Base gel	0.51 $\pm$ 0.015 ^b	724.87 $\pm$ 196.289 ^b		20.88 $\pm$ 1.239 ^b	11.37 $\pm$ 0.252 ^b
	Gengigel®	0.34 $\pm$ 0.060 ^c	1105.40 $\pm$ 62.366 ^c		45.52 $\pm$ 14.056 ^a	53.63 $\pm$ 11.388 ^a
14	Binahong 3%	0.03 $\pm$ 0.000 ^a	824.27 $\pm$ 280.527	28	36.49 $\pm$ 5.545	62.44 $\pm$ 14.987 ^a
	Base gel	0.30 $\pm$ 0.040 ^b	582.07 $\pm$ 22.30 ^a		26.78 $\pm$ 1.728	27.93 $\pm$ 0.861 ^b
	Gengigel®	0.09 $\pm$ 0.044 ^a	1082.87 $\pm$ 50.217 ^b		34.42 $\pm$ 19.269	35.93 $\pm$ 20.740

The parameters examined to show the effects of each treatment were the residual socket value (RSV) in the clinical observation and the number of fibroblasts, osteoblasts, and osteocytes in the histopathological observation. The values were shown as average $\pm$ standard deviation of at least three replicates. Different alphabets indicated significant statistical differences ($p < 0.05$) between the treatments analysed with the LSD test. The lack of alphabets or the same alphabets indicated no statistical differences ($p > 0.05$) between the treatments analysed with the LSD test.

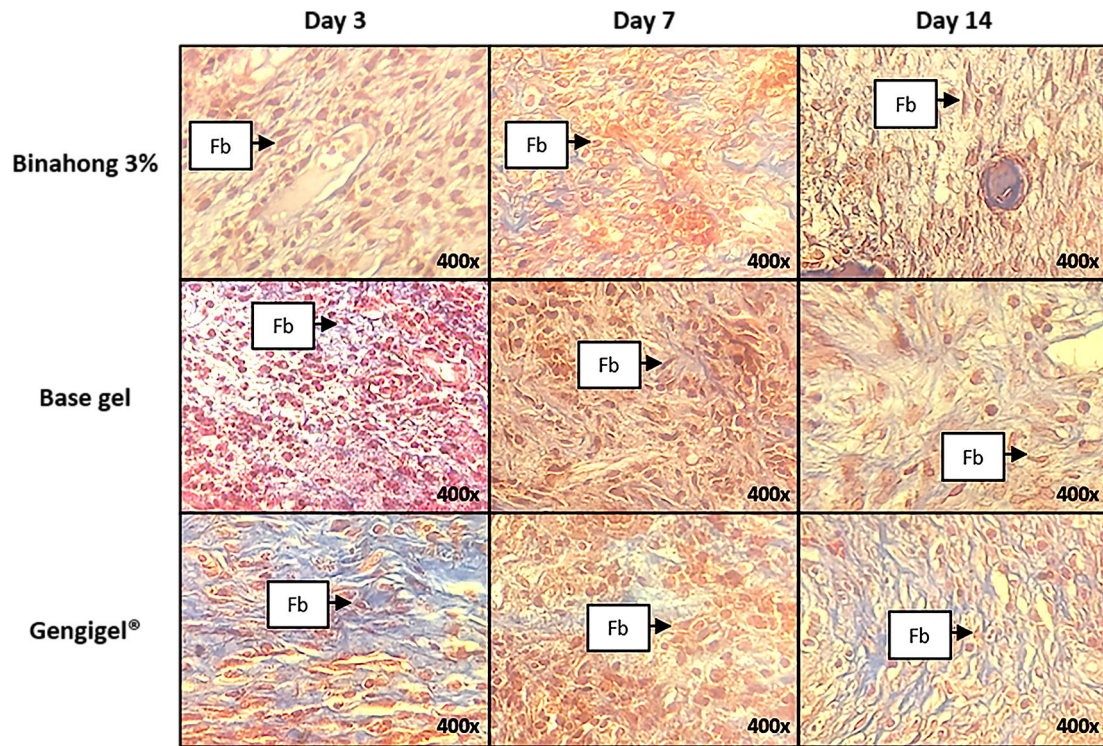


Figure 4. Histopathological observation of fibroblasts in the alveolar bone healing of sockets. The observation was conducted on the 3rd, 7th, and 14th day post-extraction socket wounds stained with Masson's Trichrome. (Abbreviation: Fb: Fibroblasts (×400)).

number of fibroblasts between the Binahong group and the base gel group and between the Gengigel® group and the base gel group. On the 7th day, there were significant differences between the Binahong group and the Gengigel® group, between the Binahong group and the base gel group, and between the Gengigel® group and the base gel group. On the 14th day, a significant difference was only observed between the Gengigel® group and the base gel group (Table 2).

The proliferation of osteoblasts was examined on the 7th, 14th, and 28th day post-extraction. In the Binahong group and the Gengigel® group, there was an increase trend in the number of osteoblasts from the 7th to 14th day and a decrease from the 14th to 28th day, although in the base gel group, the number of osteoblasts kept rising over time (Figure 3C). Osteoblasts can be seen along the alveolar bone matrix (Figure 5). Based on the one-way ANOVA test, there were significant differences in the number of osteoblasts among the 7th, 14th, and 28th day in the Binahong group and the base gel group ($p < 0.05$), while the Gengigel® group showed no differences in the number of osteoblasts among those days. In the Binahong group, there were significant differences between the 7th and 14th day and between the 7th and 28th day, while in the base gel group, there were significant differences between the 7th and 14th day, between the 7th and 28th day, and between the 14th and 28th day (Table 1).

The differences in the number of osteoblasts among the Binahong group, Gengigel® group, and the base gel group were only observed on the 14th day post-extraction ($p < 0.05$). On the 14th day, significant differences were observed between the Binahong group and the base gel group and between the Gengigel® group and the base gel group (Table 2).

Osteocyte proliferation was examined on the 7th, 14th, and 28th day post-extraction. In the Binahong group and the base gel group, there was a trend of increase in the number of osteocytes over time, although in the Gengigel® group, the increase only happened from the 7th to 14th day and was followed by a decrease from the 14th to 28th day (Figure 3D). Osteocytes can be seen in the alveolar bone matrix inside lacunae (Figure 5). Based on the one-way ANOVA test, there were significant differences in the number of osteocytes among the 7th, 14th, and 28th day post-extraction only in the Binahong group and the base gel group ($p < 0.05$). No difference was observed in the Gengigel® group among those days. In the Binahong group, there were significant differences between the 7th and 14th day and between the 7th and 28th day,

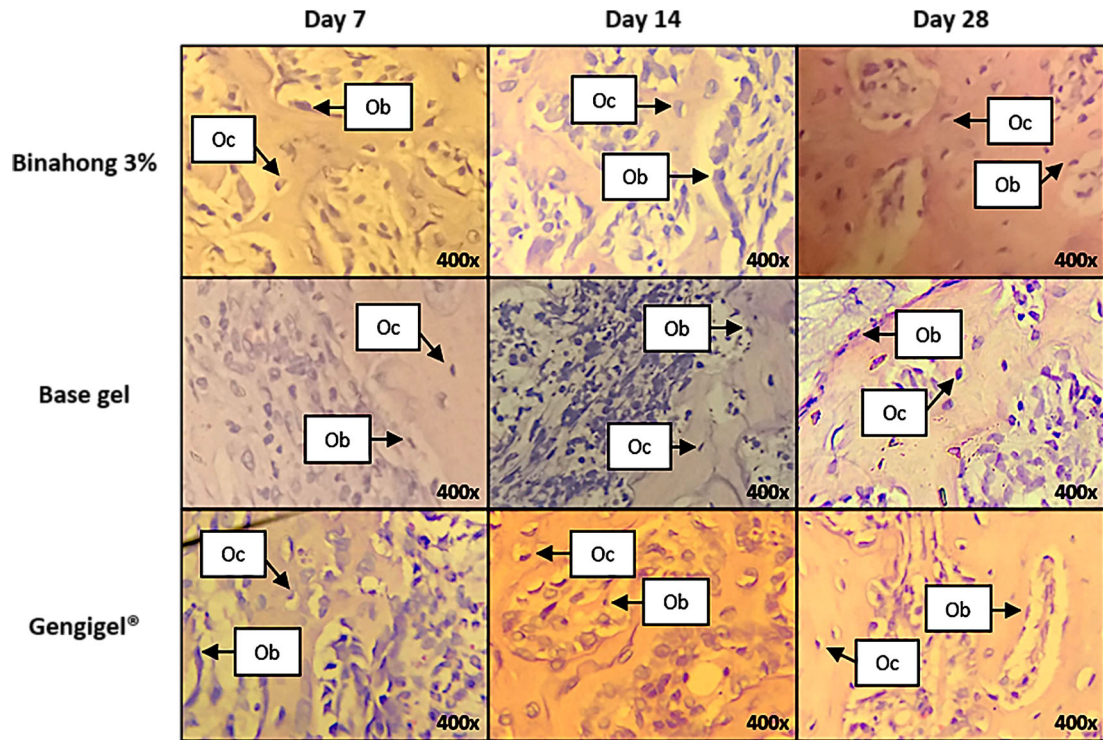


Figure 5. Histopathological observation of osteoblasts and osteocytes in the alveolar bone healing of sockets. The observation was conducted on the 7th, 14th, and 28th day post-extraction socket wounds stained with haematoxylin-eosin. (Abbreviations: Ob: osteoblasts; Oc: osteocytes ($\times 400$)).

while in the base gel group, significant differences were observed between the 7th and 14th day, between the 7th and 28th day, and between the 14th and 28th day (Table 1).

Differences in the number of osteocytes among the treatment groups were only observed on the 14th and 28th day post-extraction ($p < 0.05$). On the 14th day, there were significant differences between the Binahong group and the base gel group and between the Gengigel® group and the base gel group. On the 28th day, significant differences were observed only between the Binahong group and the base gel group (Table 2).

Discussion

The concentration of the Binahong leaf extract gel in this study was chosen based on a previous research by Hanafiah *et al*, which examined the effects of Binahong leaf extract gel on the healing of palatal mucosal wounds.³⁰ Among the various Binahong leaf extract gel concentrations in the research, 3% Binahong leaf extract gel showed the best affinity in increasing fibroblast proliferation.³⁰ In this study, the RSV in every treatment group decreased over time and the Binahong group showed a lower RSV in comparison with the other treatment groups on the 7th and the 14th day ($p < 0.05$). A lower RSV implied a decrease in socket wound size, so it can be assumed that the Binahong group showed a better affinity in accelerating wound closure compared to the positive and negative control groups. Fibroblast is one of the cells that affects wound retraction and it can be assumed that the acceleration of wound socket closure is influenced by the number of fibroblasts.³⁵

Aside from the ones in Gengigel® group, fibroblasts in this study increased from the 3rd to the 7th day and decreased from the 7th to the 14th day. According to Vieira *et al*, who examined the physiologic socket wound healing in mice, the fibroblasts in the alveolar bone would proliferate and would continue to increase until the 7th day post-extraction. The number of fibroblasts would decrease from the 7th day to the 14th day following the formation of new alveolar bone.⁸ On the 7th day, the highest fibroblast proliferation was in the Binahong group. In the Gengigel® group, there was no significant difference in the number of fibroblasts between the 7th day and the 14th day because the hyaluronic acid in Gengigel® had more effects in the early phase of wound healing in fibroblast proliferation compared to alveolar bone formation.⁴¹ Fibroblast can secrete collagen and extracellular matrix, which will make up the granulation tissue.

Granulation tissue will then be replaced by the provisional matrix, which has fewer inflammatory cells and more matrix and new blood vessels.⁴²

In this study, osteoblasts increased from the 7th day to the 14th day and decreased from the 14th day to the 28th day. Osteoblast proliferation reached its peak on the 14th day and started to decrease until the 28th day, in which the osteoblasts had matured.⁸ In the base gel group, osteoblast proliferation continued to increase until the 28th day. It could be assumed that in the base gel group, the socket wound healing was still on the early phase of bone formation, which was shown by the high number of undifferentiated osteoblasts. Osteoblasts will produce a matrix, which is called osteoid, and osteoid will be mineralized, forming woven bone.⁴² Osteoblasts that become trapped in the matrix will become osteocytes. Osteocytes have a role in managing bone remodelling because they can influence the activities of osteoblasts and osteoclasts.⁴ In the study, there was an increase in the number of osteocytes from the 7th day until the 28th day in the Binahong group and the highest osteocyte number was on the 28th day. The osteocytes were found among the alveolar bone matrix. This finding concurred with the experiment of Olaitan *et al* that showed a higher osteocyte proliferation in the 4th week compared to the 2nd week post-extraction.¹³

Several studies on the toxicity of *Anredera cordifolia* leaf extract have been carried out. Research conducted by Salasanti CD, *et al.* proved that *Anredera cordifolia* ethanolic leaf extract did not cause mortality in rats.⁴³ The study showed no significant changes in body weight formation, organ weight, hematology, or blood biochemistry in rats.⁴³ Another study conducted by Sukandar EY, *et al.* showed that *Anredera cordifolia* ethanolic leaf extract could be administered up to 1000 mg/kg bw with no teratogenic effect.⁴⁴

The Binahong plant contains various secondary metabolites that function in the proliferation of fibroblasts, osteoblasts, and osteocytes, optimising wound healing and in turn lowering the value of RSV. In the Binahong leaf extract, there is saponin, which can increase the expression of TGF- α (transforming growth factor-alpha) and TGF- β (transforming growth factor-beta).^{19,45} TGF- β can activate fibroblasts and TGF- α can activate Osterix, which will function in osteoblast differentiation.^{19,45} Saponin is also antiseptic and it can affect cell membrane integrity and cause the lysis of pathogens, especially fungi.⁴⁶

Apigenin, a flavonoid found in Binahong, can increase the expression of TGF- β and PDGF (platelet-derived growth factor) that also function in fibroblast activation so that it can migrate towards the clot.^{47,48} PDGF is also a protein that affects fibroblast proliferation.⁴⁸ Apigenin also has anti-inflammatory properties because it can inhibit the activation of NF- κ B (nuclear factor kappa B).⁴⁹ Inhibited NF- κ B can prevent the production of inflammatory mediators that can increase inflammation.⁴⁹ The other flavonoids in the Binahong plant that function in socket wound healing are quercetin and vitexin.^{50–52} Quercetin has the ability to reduce osteoclast formation through inhibiting IL-17 (interleukin-17), which is induced by RANKL (receptor activator of nuclear factor kappa-B ligand), and quercetin can also increase osteogenesis, angiogenesis, and function as an antioxidant.^{50,51} Vitexin can affect bone formation by increasing osteoblast differentiation through p-Smad (phosphorylation-small mother againts) and Runx2 (runt-related transcription factor 2).⁵²

The tannin in the Binahong plant functions in fibroblast migration because it can increase VEGF (vascular endothelial growth factor) in the early phase of wound healing.⁵³ VEGF is a protein that plays a role in fibroblast migration.⁴⁸ Tannin is also an antioxidant. Antioxidant is needed to neutralise free radicals that are produced during wound healing. Free radicals can damage cell protein structure, which will prevent cell proliferation.⁵⁴

The triterpenes in the Binahong leaf extract, such as ursolic acid and oleanolic acid, have anti-inflammatory, antiseptic, and antioxidant properties.^{19,55} Ursolic acid can also influence the differentiation and proliferation of osteoblasts and improve the activity and mineralisation of ALP (alkaline phosphatase).⁵⁶

Previous studies have shown the antimicrobial and antiinflammatory activities in Binahong leaves in relation to its effectivity in some fields in dentistry, such as periodontal therapy, but this study has further proved that Binahong leaves have the potential to be utilised in other fields in dentistry, especially in terms of wound healing acceleration after surgical procedures.^{24,57,58} More effort should be put in investigating the mechanism of how the plant works in healing injured tissue. The limitation of this study was that it simply showed the effects on 3% Binahong leaf extract gel on the proliferation of fibroblasts, osteoblasts, and osteocytes without studying the mechanism of how the substance could affect the proliferation. The 3% Binahong leaf extract gel used in this study also had to be constantly reproduced to ensure its freshness, and so there might be variability between the batches, despite the same composition. Future studies can be focused on the effects of 3% Binahong leaf extract gel on the proteins involved in fibroblast, osteoblast, and osteocyte proliferation. Further experiments can also be done to obtain a more stable and durable 3% Binahong leaf extract gel formulation or even to create a nanogel formulation as a more effective mode of delivery. The substance's effectiveness should also be tested on humans in clinical trials.

Conclusion

The study concluded that the application of 3% Binahong leaf extract gel could enhance alveolar bone healing, which could be shown through the decreasing value of residual socket volume and the increasing proliferation of fibroblasts, osteoblasts, and osteocytes.

Data availability

Underlying data

Zenodo: The dataset of 'The effects of 3% Binahong (*Anredera cordifolia*) leaf extract gel on the alveolar bone healing in post-extraction tooth socket wound in Wistar rats (*Rattus norvegicus*)'. <https://doi.org/10.5281/zenodo.5189362>.⁵⁹

This project contains the following underlying data:

- Raw data of fibroblast mean cell count.csv (Raw data of fibroblast mean cell count)
- Raw data of osteoblast mean cell count.csv (Raw data of osteoblast mean cell count)
- Raw data of osteocyte mean cell count.csv (Raw data of osteocyte mean cell count)
- Raw data of residual socket volume.csv (Raw data of residual socket volume)
- Readme.txt (Explanations about the raw data in the dataset)

Zenodo: Rat tooth extraction and 3% Binahong (*Anredera cordifolia* (Ten.) STEENIS) leaf extract gel. <https://doi.org/10.5281/zenodo.5202954>.⁶⁰

This project contains the following underlying data:

- Rat anesthesia.tiff (Intraperitoneal anesthesia on the rat)
- Rat tooth extraction.tiff (The extraction of the rat's mandibular left incisor)
- The Binahong leaf extract gel.tif (3% Binahong leaf extract gel)
- The Binahong leaf extract.tif (Binahong leaf extract)

Reporting guidelines

Zenodo: ARRIVE checklist for 'The effects of 3% Binahong (*Anredera cordifolia*) leaf extract gel on the alveolar bone healing in post-extraction tooth socket wound in Wistar rats (*Rattus norvegicus*)'. <https://doi.org/10.5281/zenodo.5203068>.⁶¹

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We have checked that the authors followed my suggestions. We suggest Approved

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Dentistry, Pharmacology; Biofilms; Natural products

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 21 April 2022

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I have checked the revised manuscript and found that the authors have addressed all the comments and the revisions are appropriate.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

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Mohd Farhan Hanif Reduan 

Department of Clinical Studies, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan, Kelantan, Malaysia

The manuscript and the study is worth for indexing on this platform as it highlights the use of local plants available for the alveolar bone healing.

Few considerations should be taken to improve the quality of the article:

1. Introduction - to include all pharmacological activities of the plant
2. Methodology - to cite for the H&E and Masson Trichome procedures. It is good if the authors can explain how to differentiate the various type of cells; fibroblasts, osteocytes, osteoclasts and osteoblasts
3. Results - the good quality of histopathology figures must be included in the article, referring to Figure 2: the bluish stain fibroblasts cannot be seen clearly by the reviewers and readers. Same comment for the Figure 3.
4. Discussion - to include future direction of the present study, the limitation of the current study can be added and explained.
5. Conclusion - Well concluded

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Histopathology, natural product development, animal study, veterinary

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Mar 2022

Olivia Avriyanti Hanafiah

We thank the reviewer for reviewing our manuscript and offering their suggestions. Please find below a point-by-point response to the comments. We hope that the reviewer would find the modifications satisfactory.

Comment: Introduction - to include all pharmacological activities of the plant

Response: Thank you for the insight, we have added this in Paragraph 4 of Introduction.

Comment: Methodology - to cite for the H&E and Masson Trichome procedures. It is good if the authors can explain how to differentiate the various type of cells; fibroblasts, osteocytes, osteoclasts and osteoblasts

Response: Citations have been added in the "Histopathological analysis" section in Methods. Details about cells have also been added in the same section. Thank you for the input.

Comment: Results - the good quality of histopathology figures must be included in the article, referring to Figure 2: the bluish stain fibroblasts cannot be seen clearly by the reviewers and readers. Same comment for the Figure 3.

Response: Following the suggestion, we have uploaded improved versions of Figure 2 and Figure 3 (Now referred to as Figure 4 and Figure 5 in the new version)

Comment: Discussion - to include future direction of the present study, the limitation of the current study can be added and explained.

Response: Following the suggestions, we have added these details in the last paragraph in Discussion.

Comment: Conclusion - Well concluded

Response: We thank you for this input.

Competing Interests: No competing interests were disclosed.

Reviewer Report 17 January 2022

<https://doi.org/10.5256/f1000research.76599.r101384>

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Dalia Hussein El-Rouby

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Effects of 3% binahong (*Anredera cordifolia*) leaf extract gel on alveolar bone healing in post-extraction tooth socket wound in Wistar rats (*Rattus norvegicus*)

The points that should be addressed to make the article scientifically sound:

Introduction:

'The healing of soft tissue, which are comprised of the gingiva and connective tissue...' - Please revise the language style

Binahong: the letter B should always be capitalised

Methods:

Sample size calculation: 48 male Wistar rats were randomly allocated to twelve groups. Please mention the effect size obtained from the previous study (Khoswanto et al, 2019)

Rats with abnormalities or those that died before the experiment ended were excluded from the study: how did you replace rats who died before the end of the study to maintain the sample size?

Results

Please provide figures showing the 95% confidence interval in error bars

The residual socket volume (RSV): Please specify the unit of measurement (mm³ ?) in the graph, table 2 and text

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Oral tumors, use of natural products for chemo-prevention and improving oral condition, use of bone substitutes

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Mar 2022

Olivia Avriyanti Hanafiah

We thank the reviewer for reviewing our manuscript and offering their suggestions. Please find below a point-by-point response to the comments. We hope that the reviewer would find the modifications satisfactory.

Comment: 'The healing of soft tissue, which are comprised of the gingiva and connective tissue...' - Please revise the language style

Response: Thank you for the suggestion, we have revised the diction of this sentence.

Comment: Binahong: the letter B should always be capitalised

Response: Thank you for the insight, we have thus made the changes.

Comment: Sample size calculation: 48 male Wistar rats were randomly allocated to twelve groups. Please mention the effect size obtained from the previous study (Khoswanto et al, 2019)

Response: We have added this detail in "Sample Size" section in Methods, thank you.

Comment: Rats with abnormalities or those that died before the experiment ended were

excluded from the study: how did you replace rats who died before the end of the study to maintain the sample size?

Response: The rats were not replaced as long as the minimal sample size were still maintained. Elaboration on this can be found in "Animals" section in Methods.

Comment: Please provide figures showing the 95% confidence interval in error bars

Response: Following the suggestion, we have added these details in the improved Figure 3.

Comment: The residual socket volume (RSV): Please specify the unit of measurement (mm³?) in the graph, table 2 and text

Response: Thank you for the suggestion. We have thus added the unit of measurement in the graph, table, and text for fibroblast, osteoblast, and osteocyte proliferation. Unfortunately, there is no unit of measurement for RSV as it is the ratio of end volume and beginning volume (end volume: beginning volume).

Competing Interests: No competing interests were disclosed.

Reviewer Report 03 November 2021

<https://doi.org/10.5256/f1000research.76599.r96681>

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Lidia Audrey Rocha Valadas

Department of Preventive and Community Dentistry, University of Buenos Aires, Buenos Aires, Argentina

This research is an experimental study on the use of *Anredera cordifolia* in gel form to be used after extraction.

The study is innovative especially in the area, with few works in the literature about it. In addition, the methodology has reproducibility.

A) Introduction:

1 - I suggest a paragraph about the medicinal properties reported in the literature and not just isolated constituents.

2 - There was a gap between the 2nd and 3rd paragraphs, before starting the third, I should start by addressing the use of medicinal plants for this purpose.

3 - I suggest checking the articles of these authors, it may help (some are from medicinal plants): Alexandre *et al.* 2018¹, Guimarães *et al.* 2016², Lima MDR *et al.* 2017³, Lima V *et al.* 2020⁴

, Ribeiro *et al.* 2018⁵, and Teixeira *et al.* 2017⁶.

B) Methodology:

4 - If it is a study with natural products, I suggest improving the details of georeferencing, with the addition of coordinates and season.

5 - I suggest a flowchart for the division of groups and a chart with the constituents of the gel.

C) Discussion:

6 - In the discussion, I suggest reviewing the second and third paragraphs so as not to repeat information that is already in the results.

7 - I also believe it is important to talk about the cytotoxicity and safety of the species, since the intention is that the gel will one day be clinically applicable.

8 - To further address the importance of investigating properties of natural products in Dentistry, especially in surgery, since most studies are related to cariogenic or periodontal biofilm.

9 - Finally, add limitations and future perspectives.

References

1. Alexandre JTM, Sousa LHT, Lisboa MRP, Furlaneto FAC, et al.: Anti-inflammatory and antiresorptive effects of *Calendula officinalis* on inflammatory bone loss in rats. *Clin Oral Investig.* 2018; **22** (6): 2175-2185 [PubMed Abstract](#) | [Publisher Full Text](#)
2. Guimarães MV, Melo IM, Adriano Araújo VM, Tenazoa Wong DV, et al.: Dry Extract of *Matricaria recutita* L. (Chamomile) Prevents Ligature-Induced Alveolar Bone Resorption in Rats via Inhibition of Tumor Necrosis Factor- α and Interleukin-1 β . *J Periodontol.* 2016; **87** (6): 706-15 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Lima MDR, Lopes AP, Martins C, Brito GAC, et al.: The Effect of *Calendula officinalis* on Oxidative Stress and Bone Loss in Experimental Periodontitis. *Front Physiol.* 2017; **8**: 440 [PubMed Abstract](#) | [Publisher Full Text](#)
4. Lima V, Melo IM, Taira TM, Buitrago LYW, et al.: *Uncaria tomentosa* reduces osteoclastic bone loss in vivo. *Phytomedicine.* 2020; **79**: 153327 [PubMed Abstract](#) | [Publisher Full Text](#)
5. Ribeiro DDSF, Freire JMO, Teixeira AH, Val DRD, et al.: *Tocoyena sellowiana* extract decreases bone loss in an experimental model of periodontitis in rats: Putative role for cyclooxygenase-2 and IL-1 γ inhibition. *Biomed Pharmacother.* 2018; **98**: 863-872 [PubMed Abstract](#) | [Publisher Full Text](#)
6. Teixeira AH, Freire JMO, de Sousa LHT, Parente AT, et al.: *Stemodia maritima* L. Extract Decreases Inflammation, Oxidative Stress, and Alveolar Bone Loss in an Experimental Periodontitis Rat Model. *Front Physiol.* 2017; **8**: 988 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Dentistry, Pharmacology; Biofilms; Natural products

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 29 Mar 2022

Olivia Avriyanti Hanafiah

We thank the reviewer for reviewing our manuscript and offering their suggestions. Please find below a point-by-point response to the comments. We hope that the reviewer would find the modifications satisfactory.

Comment: I suggest a paragraph about the medicinal properties reported in the literature and not just isolated constituents.

Response: We have elaborated on the medicinal properties of Binahong in paragraph three in Introduction.

Comment: There was a gap between the 2nd and 3rd paragraphs, before starting the third, I should start by addressing the use of medicinal plants for this purpose.

Response: We have followed this suggestion, thank you for the insight.

Comment: I suggest checking the articles of these authors, it may help (some are from medicinal plants): Alexandre et al. 20181, Guimarães et al. 20162, Lima MDR et al. 20173, Lima V et al. 20204, Ribeiro et al. 20185, and Teixeira et al. 20176.

Response: Thank you for the suggestions by the reviewer. We have read the articles and they have provided us with some helpful insights.

Comment: If it is a study with natural products, I suggest improving the details of georeferencing, with the addition of coordinates and season.

Response: We have added these details in "Binahong leaf extract gel and base gel preparation" section in Methods.

Comment: I suggest a flowchart for the division of groups and a chart with the constituents of the gel.

Response: Following the suggestion by the reviewer, we have added these details in Figure 1 and Figure 2.

Comment: In the discussion, I suggest reviewing the second and third paragraphs so as not to repeat information that is already in the results.

Response: Thank you for the suggestion, we have made some changes in paragraph two and three in Discussion.

Comment: I also believe it is important to talk about the cytotoxicity and safety of the species, since the intention is that the gel will one day be clinically applicable.

Response: Thank you for this insight, we have thus added information about the toxicity of Binahong, which can be found in the fourth paragraph in Discussion

Comment: To further address the importance of investigating properties of natural products in Dentistry, especially in surgery, since most studies are related to cariogenic or periodontal biofilm.

Response: As suggested by the reviewer, we have added this in the last paragraph in Discussion

Comment: Finally, add limitations and future perspectives.

Response: As suggested by the reviewer, we have added this in the last paragraph in Discussion

Competing Interests: No competing interests were disclosed.

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