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Peperomia pellucida extracts stimulates bone healing in alveolar socket following tooth extraction

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1. Introduction

Peperomia pellucida (草胡椒 cǎo hújiāo) is a plant that has been widely used and shown to have several pharmacological activities, such as fracture and wound healing activities.[1] Its effect on bone is promoted by its oestrogenic effect, its ability to induce calcium homeostasis, anabolic effects on osteoblasts, inhibition of osteoclastogenesis, antioxidant effects, and bone-related nutritional content.[2–8] Additionally, the analgesic, anti-inflammatory and antibacterial effects of this plant may be beneficial in wound treatment, including healing tooth sockets at post-extraction. The anti-inflammatory, antihemorrhagic, and wound healing properties of this plant have been mentioned in Ayurveda records.[1,9]

Tooth extraction is a frequent maintenance treatment in dentistry. Postoperative pain and discomfort, lost workdays and healing complications have been well reported for the immediate postoperative period. Therefore, the maintenance and/or acceleration of the healing process of the alveolar bone after extraction is something that needs to be considered.

Alveolar bone is characterized by distinctive features, such as continuous and rapid remodelling in response to stimulation by force.[9] Although *P. pellucida* has potency in the healing process, no reports have documented the effect of the plant in healing the alveolar socket after tooth extraction. Hence, the present study was planned to evaluate the beneficial effects of *P. pellucida* on the healing of the alveolar socket after tooth extraction in rats.

This research included the examination of alveolar healing parameters using micro-computed tomography (μCT) scanning and histology. Phytochemical tests were also performed to confirm the plant's potency.

2. Materials and methods

2.1. Sample preparation

P. pellucida plants (wild) were collected from the Ciater and Cagak Region, West Java Province, Indonesia, in March and April 2016. The plant was authenticated at the Herbarium Bandungense, Institut Teknologi Bandung, Indonesia with document number 705/I1·CO2.2/PL/2016 by a botanist. The plants were extracted with n-hexane, ethyl acetate, and 96% ethanol (CV Fadillah, Bandung Kulon, Indonesia) using the sequential maceration method for 3 × 24 h. The filtrate was collected and evaporated using a rotary evaporator. Only ethyl acetate and ethanol extracts were used in this research.

2.2. Test animals

The experimental animal groups were comprised of 8-week-old male Wistar rats. After 14 days of acclimatization, the rats were randomized and put into four different groups (control, alendronate, ethyl acetate extract and ethanol extract groups). Throughout the study period, sterile water ad libitum was provided, and sterile standard solid rat chow rats were given except 24 h after tooth

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List of abbreviations

GAE	Gallic acid equivalents
μCT	micro-computed tomography
EDTA	Ethylenediamine tetraacetic acid
BV	Bone volume
TV	Tissue volume
BV/TV	Bone volume/tissue volume
Tb.N	Trabecular number
Tb.Th	Trabecular thickness
PMN	Polymorphonuclear
UV	Ultraviolet
GC-MS	Gas chromatography-mass spectrometry
IUPAC	International union of pure and applied chemistry

extraction, in which the diet was crumbled.

2.3. Ethics statement

This experimental study was conducted at the Animal Teaching Hospital of the Faculty of Veterinary Medicine, Bogor Agricultural Institute, Bogor, Indonesia. Ethical clearance from the Animal Medical Research Ethics Committee was obtained prior to the start of the study with certificate number 067/KEH/SKE/VII/2017. All procedures were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The test animals were anaesthetized by the intramuscular administration of 87 mg/kg bw ketamine chloride and 13 mg/kg bw xylazine chloride. The test animals were thus humanely killed with an excessive dose of anaesthetic (ketamine and xylazine) at the end of the experimental periods.

2.4. Experimental protocol and rat tooth extraction model

The upper right incisor of test animals was extracted. The animals were given the carrier solution only (0.5% Tween 80 and 0.5% CMC Na), Alovell (alendronate; Novell Pharmaceutical Laboratories) 100 μg/kg bw, ethyl acetate and ethanol extract of *P. pellucida* at 100 mg/kg bw once a day for seven days. At the end of the experimental periods, the maxillae were collected. The maxillae were fixed in 10% neutral-buffered formalin (pH 7.4) at room temperature for 24 h, subsequently washed overnight in running water and maintained in alcohol fixative (70% ethanol) until the conclusion of μCT analysis. The maxillae were then decalcified in 4.13% EDTA (pH 7.2) and submitted to histological processing.

2.5. Micro-computed tomography analysis

Rat maxilla bones were tested with high resolution using a Bruker SkyScan1173® μCT scanner. In the scanner, specimens were aligned with the vertical axis and 70% ethanol was used as a medium. The instrument was set at 50 kV voltage, 160 μA current, 0.2° rotation step and 1150 ms exposure time. Frame averaging and random movement with a value of 10 and a 1.0 mm aluminium filter were performed during the scanning process. A set of projection images (±1406 images) of the specimen was successfully obtained with a resolution of 13.895463 μm per pixel with dimensions of 2240 × 2240 pixels. The projection images were reconstructed using NRecon® with the GPUReconServer reconstruction kernel. As many as one-third of the total tooth sockets were analysed for the region of interest. The image qualities were checked further with DataViewer®, and analysis of the reconstructed greyscale images was performed using CTAn®. The parameters measured were bone volume (BV), tissue volume (TV), bone volume/tissue volume (BV/TV), trabecular number (Tb. N), and trabecular thickness (Tb.Th). The 3D images of the skull are presented in Supplemental data 1.

2.6. Histological analysis

EDTA was used as the decalcified solution. Staining was performed using haematoxylin and eosin. The bone microarchitecture profiles of the preparations were observed using a binocular microscope (Olympus®) at 100x magnification and the Optilab Viewer® application. The numbers of osteoblasts, osteoclasts, osteocytes, polymorphonuclear (PMN) cells, blood vessels, and fibroblasts were counted.

2.7. Determination of total phenolic content

The total phenolic content was determined using the Folin-Ciocalteu reagent as described in a previous study with slight modifications.[10] In this study, the mixture of test extracts and Folin-Ciocalteu reagent was vortexed for 10 s and then kept at room temperature (±26 °C) for 5 min before adding 4 mL of sodium carbonate solution. Absorbance was measured at λ 760 nm after 45 min of incubation at room temperature. Total phenolic content was expressed as mg of gallic acid equivalents per gram of extract (g GAE/100 g extract).

2.8. Gas chromatography-mass spectrometry analyses

GC-MS analyses were carried out using Agilent (US) gas chromatography with an ALS injector. The GC device was equipped with an HP-5MS column (30 m × 0.25 mm x 0.25 μm) coated with a nonpolar phase of 5% phenyl methyl silox. The conditions were as follows: temperature programming from 70 to 320 °C held at 70 °C for 2 min, and at 300 °C for 3 min (rate 3 °C/min), column oven temperature of 7 °C, injection temperature 250 °C, injection mode was splitless, flow control initial rate was 1 mL/min and post-run flow rate was 0.57353 mL/min, carrier gas pressure was 8.8085 psi, total flow was 104 mL/min, column flow was 3 mL/min, linear velocity was 36.796 cm/s, purge flow was 100 mL/min and split ratio was 1.0 min. Additionally, solvent cut time was 3.5 min, ion source temperature was 230 °C, interface temperature was 250 °C, detector gain mode was 0.50 and the threshold was 150. For the mass spectra, the event time was 0, the start time was 3.5 min, the end time was 650 min, the start m/z was 30, and the end m/z was 600. Analytical grade solvents were used and procured from Merck, Germany.

2.9. Statistical analysis

The data was analysed statistically using SPSS software version 22.0 for Windows. The analyses were performed by one-way analysis of variance (ANOVA) followed by LSD analysis or Kruskal–Wallis test using Bonferroni adjustment. Values of samples were considered significant at $p < 0.05$. All values are expressed as the mean ± standard deviation (SD).

3. Results

3.1. Micro-computed tomography analysis

Three-dimensional images of the maxillae from the μCT (Fig. 1) reveal the alveolar healing result of the sockets on day 7. Thicker bone trabeculae with larger amounts of hyperdense areas than the

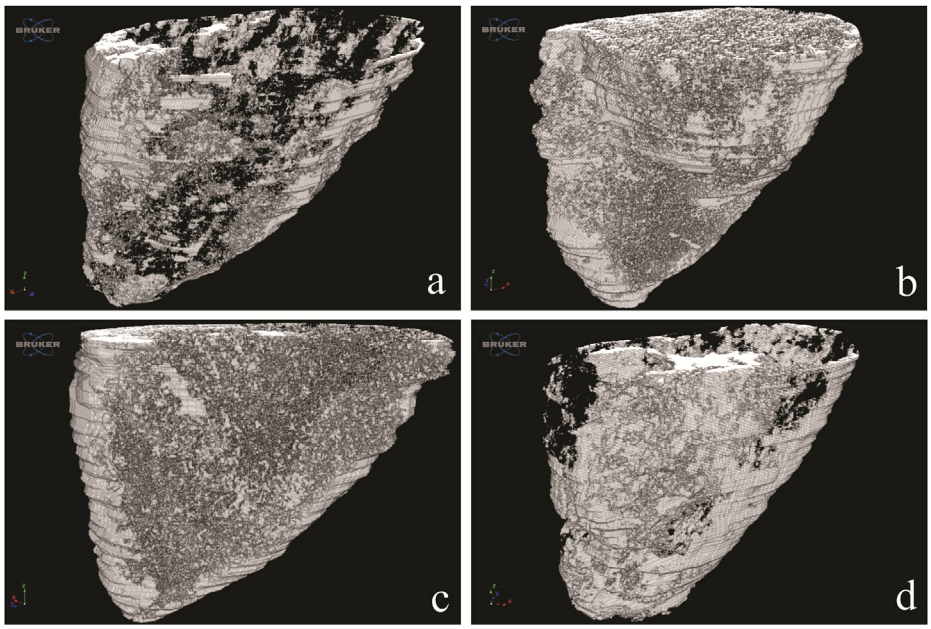

Fig. 1. Three-dimensional image of the alveolar socket from micro-computed tomography (μCT) analysis (ventral view). The hyperdense areas show mineralized bone. a = control, b = alendronate, c = ethyl acetate extract, d = ethanol extract.

control were observed in the alendronate and *P. pellucida* extract groups. This area is where bone formation starts, centripetal from the apical and lateral walls of the sockets towards the coronal and centre regions of the alveolus.

The quantitatively evaluated values of the bone micro-architecture features (Table 1) confirmed the morphological observations derived from the μCT. Alendronate gave higher improvement for all of the bone parameters compared to the control. The same was also shown by the extracts. However, the improvement was not statistically significant, except for Tb.Th, which was significantly higher in the ethanol extract group.

3.2. Histological analysis

The histological analysis showed that the healing process occurred sequentially in all groups. To some extent, there were overlapping phases characterized by the presence of clots, granulation tissue formation with inflammatory cell infiltration, angiogenesis, proliferation of fibroblasts and collagen synthesis, bone formation and remodelling (Fig. 2). This descriptive analysis was supported by the semiquantitative analysis of each parameter (Table 2).

3.3. Determination of total phenolic content

Total phenolic content was determined using gallic acid. The calibration curve of gallic acid was obtained using a linear fit (r^2 = 0.9978). The results demonstrated that the ethanol extract and ethyl acetate extract of the plants contained 3.90 ± 0.43 g GAE/100 g extract and 5.17 ± 1.02 g of GAE/100 g extract respectively.

3.4. Gas chromatography-mass spectrometry analyses

GC-MS analysis showed the different chemical compounds in both extracts. The major peaks are summarized in Supplemental Data 2 and 3. The chemical structures of the detected compounds are presented in Supplemental Data 4.

4. Discussion

The healing process of alveolar bone is a complex sequential process. The process involves different tissues and is also linked with the inflammatory immune response of the host for effective bone healing. According to traditional knowledge and recent research studies about its potency, *P. pellucida* is a candidate for post-tooth extraction bone healing.

Our data demonstrated that all of the treatments accelerated the bone healing process compared with the control at day 7 post-extraction. Based on the 3D image of the tooth socket, the healing process was almost complete. Under normal conditions, the bone healing process will happen in the same way but reach its completion only on day 28[9].

Micro-CT data provided the bone parameters for the physical analysis. Alendronate, as a standard drug, raised the value of all

Table 1
Bone microarchitecture parameters analysed by microcomputed tomography.

Parameters	BV (mm^3)	TV (mm^3)	BV/TV(%)	Tb.N (1/mm)	Tb.Th (mm)
Control	1.57 ± 0.67	9.23 ± 1.72	18.05 ± 11.50	3.67 ± 3.12	0.06 ± 0.01
Alendronate	3.07 ± 2.00	8.87 ± 0.14	34.47 ± 22.23	5.34 ± 3.53	0.07 ± 0.01
Ethyl Acetate Extract	2.74 ± 1.78	9.40 ± 0.78	28.90 ± 17.93	4.84 ± 3.46	0.06^{b} ± 0.01
Ethanol Extract	1.94 ± 0.63	8.44 ± 1.49	23.61 ± 8.02	2.96 ± 1.14	0.08^{a} ± 0.01**

There were three samples per condition that were analysed in triplicates. $^{a-b}$ Significantly different from each other ($p < 0.05$). *, $p < 0.05$ versus control; **, $p < 0.01$ versus control.

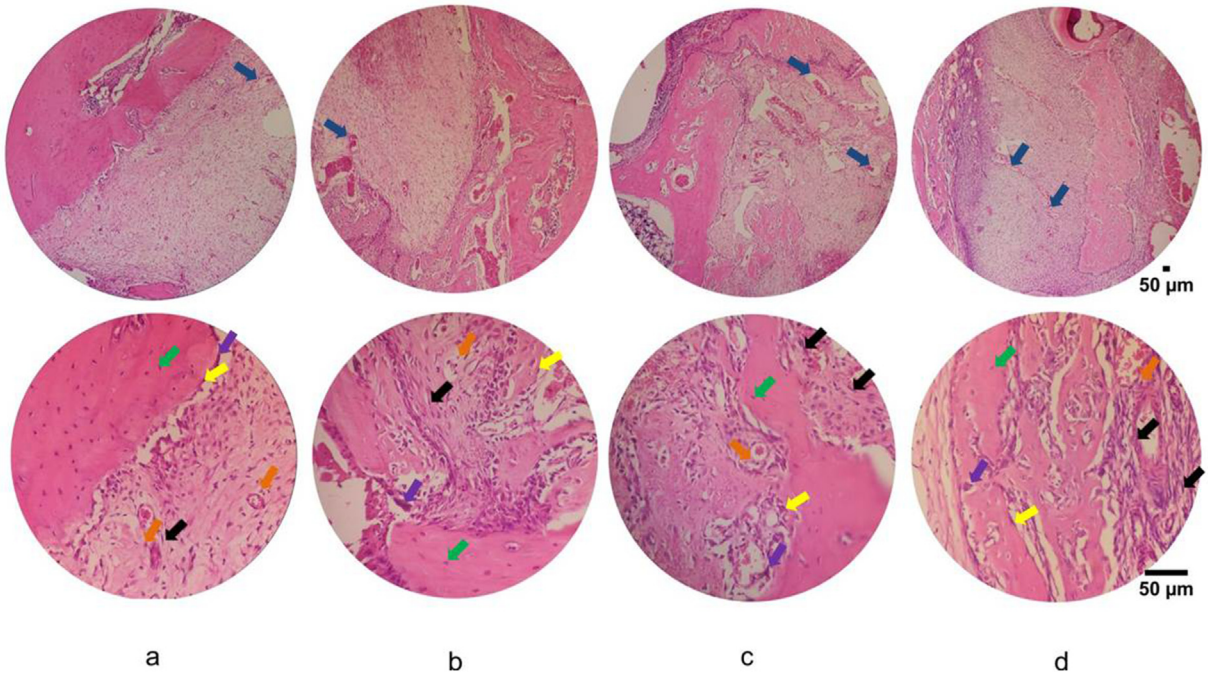

Fig. 2. Histological aspects of the apical thirds from sockets. a = control, b = alendronate, c = ethyl acetate extract, d = ethanol extract. Arrows: blue, blood vessels; purple, osteoclasts; yellow, osteoblasts; green, osteocytes; black, fibroblasts; orange, PMN. Haematoxylin-eosin staining, original magnification 10x (upper) and 40x (bottom).

Table 2
Histological analysis data of tooth socket.

Parameters	Osteoclast	Osteoblast	Osteocyte	Blood Vessel	Fibroblast	PMN
Control	1.89 ± 0.73	31.37 ± 3.36	22.03 ± 2.97	3.50 ± 0.78	23.18 ± 3.34	31.54 ± 5.25
Alendronate	1.67 ± 0.15	35.00[a] ± 6.37	26.53 ± 5.73	3.63 ± 0.65	58.77 ± 16.07*	20.87 ± 5.78*
Ethyl Acetate Extract	1.97 ± 0.42	28.39 ± 5.50	24.94 ± 7.07	3.49 ± 0.43	70.31 ± 17.71**	20.39 ± 0.78*
Ethanol Extract	1.77 ± 0.32	24.40[b] ± 2.54	24.97 ± 2.45	4.40 ± 1.47	71.00 ± 22.56**	18.77 ± 3.02**

The number of samples per condition was 3 and number of replicas was at least 4. [a–b] significantly different to each other ($p < 0.05$). *, $p < 0.05$ versus control; **, $p < 0.01$ versus control.

parameters compared to the control. The parameters analysed in this study normally increased after the extraction process. TV, Tb.Th., and Tb.N. were usually significantly improved on day 7, whereas BV and BV/TV demonstrated significantly higher values on day 14[9]. Both of the extracts could also increase the bone parameters.

Histological analysis was used to examine the improvement of other crucial factors in the bone healing process that cannot be inspected using µCT. All of the treatment groups had an increased number of osteocytes and vascularization, as shown by the increase in the number of blood vessels. Even though the values were not significantly higher than those of the control, the improvement itself is a sign of good bone healing. Osteocytes, the most abundant cell type among bone cells embedded in the matrix of calcified bone, produce various factors that regulate the onset of both bone resorption and formation, play a pivotal role in maintaining bone remodelling and homeostasis in response to mechanical stimuli and function as major mechanosensitive cells, involving the regulation of osteoclastic bone resorption.[11] At day 7, a bone matrix developed with a high proportion of osteocytes on the lining of the socket walls, which then expanded to the central region of the socket following a centripetal pattern, leading to the confluence of bone trabeculae.[9] Over this period, numerous newly formed small blood vessels indicated the presence of granulation tissue. Blood vessels are important in the bone healing process because they can support the transport of nutrients that are needed by bone cells. These nutrients include calcium and phosphate for building the mineralized bone matrix in which osteocytes are embedded.

The limitation of this study is the lack of identification of osteocalsts and osteblasts by specific immunostaining. Additionally, the activity of extracts was not compared with a specific standard drug for bone healing after tooth extraction; instead, the comparison was carried out with an antiosteoporosis agent, alendronate. Examination of the bone cell profile in this study (Fig. 2 and Table 2) demonstrated that alendronate is also beneficial for tooth socket healing, even though the positive effect on bone cells was not significant. This finding supports previous studies that confirmed that the drug can support bone formation. However, it is not fully accepted that alendronate has a positive effect on bone formation. Other studies have found that it can have an inhibitory effect.[12–15] Alendronate, a nitrogen-containing bisphosphonate, is well known to selectively inhibit farnesyl pyrophosphate synthase within osteoclasts, which leads to osteoclast apoptosis.[16] It has been shown that alendronate can prevent alveolar bone loss caused by postmenopausal osteoporosis.[17] Other studies found that alendronate has an effect on bone formation in a dose-dependent manner. Some studies indicated that at low doses, this drug can increase proliferation, metabolic activity and deposition of calcium

by osteoblasts, but is toxic to both osteoblasts and osteoclasts at high doses.[18,19]

The number of fibroblasts was significantly accelerated by the treatment. The positive effect of the *P. pellucida* extract on fibroblast cells is reported for the first time in this paper. Fibroblasts play an essential role in the synthesis of the provisional matrix of the granulation tissue in the socket during the healing process.

Both the alendronate and the *P. pellucida* extracts also significantly decreased the number of PMN cells in this study. This finding suggests that the treatment successfully accelerated the bone healing process. Because the inflammation process proceeds faster, the recruitment of bone cells proceeds earlier. This also explains why the number of osteocytes in all of the treatment groups was higher than that in the control group. According to the normal physiology of bone healing in tooth extraction sockets, bone formation followed by subsequent remodelling starts with overlapping phases to some extent, including the presence of clots, migration and proliferation of mesenchymal stem cells, granulation tissue formation with inflammatory infiltrate, angiogenesis, proliferation of fibroblasts, and collagen synthesis.[9]

The potency of *P. pellucida* extracts in tooth socket healing was confirmed further by phytochemical tests. The total phenolic content equivalent to gallic acid found in the ethyl acetate extract was higher than that in the ethanol extract.

The ethyl acetate extract of *P. pellucida* was detected to have some beneficial compounds. In addition to the direct effect of beta-sitosterol on bone cells, the antioxidant and anti-inflammatory effects provided by the other compounds can support bone anabolic effects and decrease bone resorption.[20] The bone healing effect of this extract was also supported by its high phenolic content.

Plant-derived polyphenol compounds are also well known to have antioxidant and anti-inflammatory effects. Gallic acid – which represents the phenolic compounds in this study – has been reported to be useful for the treatment or prevention of osteoporosis.[21] Its presence is considered to play an important role in the anti-osteoporotic properties of the plant.[22] It was able to inhibit osteoclastogenesis induced in vitro by RANKL in RAW 264.7 cell lines.[23]

The bone healing effect of ethanol extract was supported by some beneficial compounds detected by GC-MS analysis, i.e., hexadecanoic acid methyl ester, 9,12,15-octadecatriene-1-ol, (Z,Z,Z)-, and phytol because of their antioxidant and anti-inflammatory effects.

Among the compounds detected using GC-MS analysis in this study, only beta-sitosterol, hexadecanoic acid methyl, and phytol have been reported before in previous studies. All parts of the plant were used to isolate beta-sitosterol from the neutral ether fraction of the methanol extract.[24] The other compounds were detected present in the leaf part of *P. pellucida* using GC-MS analysis.[25]

The beneficial effect of *P. pellucida* on bone healing events was supported by its high-value mineral composition comprising iron, calcium, and potassium as the main elements.[26] The antibacterial and analgesic activities of this plant[1] also offer benefits for the use of *P. pellucida* for human consumption, especially after tooth extraction.

5. Conclusion

This study demonstrated that oral administration of *P. pellucida* extracts promotes the healing process in the tooth socket after extraction to proceed more rapidly, particularly by increasing fibroblast proliferation and trabecular thickness. Future research warrants identification of the actual bioactive metabolites present in *Peperomia pellucida* responsible for its bone healing property and subsequent understanding their mechanisms of action at the molecular level.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtcme.2021.08.010.

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