Phytochemical Compounds and Potential anti-Osteoclastogenesis Effect of Extracted Aloe vera

Sularsih[1]*, Sarianoferni[1,2], Widyasri Prananingrum[1], Siswandono[3]

1. Department of Dental Material, Faculty of Dentistry, Universitas Hang Tuah, Surabaya, Indonesia.
2. Departement of Dentomaxillofacial Radiology,Faculty of Dentistry, Universitas Hang Tuah,Surabaya,Indonesia.
3. Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia.

Abstract

This study was identified phytochemical compounds of extracted Aloe vera and to analyze anti-osteoclastogenesis activity through the RANK expression and osteoclast cells on socket of tooth extraction of Cavia cobaya. Identification phytochemical compounds used colorimetric methods. Docking analysis to predict binding to TLR-2 receptor. Thirty-six male Cavia cobaya were divided: K (-) was not used scaffold, K (+) with chitosan and P with combination Aloe vera and chitosan. Each group divided into 7 and 14 days observation. Immunohistochemical examination was conducted to examine the RANK expression and Histopathological examination to examine osteoclast cells. The compounds identified in the extracted Aloe vera included flavonoids, saponins, tannins, and anthraquinone. There was hydrogen bonding and steric interaction between Aloe vera and TLR-2 receptor. Anova test showed RANK and osteoclast significant decreased ($p \leq 0.05$; $p=0.00$) especially in P compare to K (-) and K (+). LSD Test showed significant differences between the groups. Aloe vera's compounds have anti-osteoclastogenesis activity and its combination with chitosan decreased the RANK expression and osteoclast on socket healing of Cavia cobaya.

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Introduction

Osteoclastogenesis is complex process of osteoclast differention and maturation that requires induction of Receptor Activator of Nuclear Factor κB ligand (RANKL) which activated RANK.[1] RANK has important roles to regulate osteoclast differentiation and activation on bone healing. It is transmembrane proteins expressed in osteoclasts as activators of NF-kβ receptors and activated when binding to RANKL, promotes osteoclast precursor cells to differentiate into osteoclasts. Osteoclasts cells are known to be capable of bone resorbtion. Osteoprotegerin (OPG) decreases bone resorbtion by inhibiting interaction between RANKL and RANK. The RANK/RANK / OPG sinyaling system controlling osteoclastogenesis.[2,3]

Corresponding author:
Sularsih
Department of Dental Material, Faculty of Dentistry,
Universitas Hang Tuah, Surabaya, Indonesia.
E-mail: larsihdentist@gmail.com

Previous study reported that 40%-60% alveolar ridge resorbtion occurs post tooth extraction in period of 3 months.[4,5,6] This condition causes the failure placement of dental implant or denture treatment post tooth extraction.[6]

The application of *Aloe vera* herbal plant or it combination with xenograft concelous bone could accelerate alveolar bone healing post tooth extraction by stimulating BMSCs proliferations, osteoblast differentiation, osteopontin, VEGF, BMP2 expression, alkaline phosphatase activity and mineralization.[4] The blended *Aloe vera* and natural polymers chitosan have antibacterial and antiinflammation effect. It might be promising for ridge preservation.[7,8] Combination *Aloe vera* and chitosan scaffold have scaffold characteristic with good mechanical strength and physical properties and nontoxic that can potentially be used for alveolar bone healing.[9,10]

Aloe vera is common herb grown in tropical area including in Batu, Malang, East Java, Indonesia. Batu city is near Malang in the East Java of Indonesia. The plantation of Batu have tropical climate condition suitable for *Aloe vera* grown.[11] *Aloe vera* is species of *Aloe* belonging

to *Liliaceae* family is the most widely for medical. The active compounds in *Aloe vera* and its extracts, such as aloctin protein, acemannnan, flavonoids, saponins, tannins and anthraquinones reported to have antioxidant, antiinflammation, antiviral and bone healing activity. Due to its biological properties, *Aloe vera* potent to be tissue engineering material which promotes cell migration, proliferation and bone healing.[8,12] Varying phytochemical compounds that corelation with biological activity depend on the variation of climate and soil condition of growing.[12]

In the bone healing of tooth extraction, RANK has important roles to regulate osteoclast differentiation and bone resorbtion. Therefore, this study aim was to to identify phytochemical compounds of extracted *Aloe vera* from Batu, Malang, Indonesia and to analyze anti-osteoclastogenesis activity through the RANK expression and osteoclast cells after application of combination *Aloe vera* and chitosan scaffold on socket of tooth extraction of Cavia cobaya.

Materials and methods

Thirty-six male Cavia cobaya, aged 3 - 3.5 months and weighed 300- 375 grams were used. Cavia cobaya were acquired from experimental animal Laboratory of Biochemical laboratory, Medical Faculty, Universitas Airlangga Indonesia. Cavia cobaya were divided into six groups: K (-) without scaffold for 7 days, K (+) with chitosan scaffold for 7 days, P1 with *Aloe vera* and chitosan scaffold for 7 days, K (-) without scaffold for 14 days, K (+) with chitosan scaffold for 14 days, P2 with *Aloe vera* and chitosan scaffold for 14 days. This research was obtained ethical approval from the Ethical committee of Dentistry Faculty, Airlangga University with certificate number: 012 / HRECC.FODM / III / 2018.

Preparation *Aloe vera* Extract

Aloe vera plant obtained from Batu plantation, Malang, East Java, Indonesia. *Aloe vera* leave was identified in Pharmaceutical Laboratory of Wijaya Kusuma University, Surabaya, East Java, Indonesia. *Aloe vera* was cleaned, the inner gel was taken and blended. The smooth gel was dried using Freeze dryer and then dissolved in 70 % ethanol with ratio of 1:4, stirrer for 30 minutes with magnetic stirrer and allowed for 48 hours. The supernatant was filtered with filter paper and Erlenmeyer. It evaporated with vacuum rotary evaporator and dissolved using Sodium carboxymethyle cellulose 3.5%.[9]

Phytochemical Screening of *Aloe vera* Extract and Docking Analysis

Phytochemical compounds of *Aloe vera* determined by phytochemical screening. The secondary metabolite including flavonoids, saponins, tannins, anthraquinones and lignins were identified using coloration reaction. The phytochemical analyses of *Aloe vera* Extract were carried out to identify secondary metabolites. The analyses were identified for flavonoids using aluminium chloride test, for saponins using froth test, for tannins using ferric chloride test, for anthraquinones using sodium hydroxide test and for lignins using phloroglucinol hydrochloride test.[13] Docking Analysis to predict binding between *Aloe vera* and TLR-2 receptor using *Molegro Virtual Docker* (MVD) program.[14]

Preparation of Scaffold

Chitosan powder with a deacetylation degree of > 75-85% and a molecular weight of 50,000-190,000 Da (Sigma, Product number: 448869, Lot number: MKBH7256V) was used in this study. One gram of chitosan powder was dissolved in 100 mL of acetic acid 2% then stirred using a magnetic stirrer, neutralized with NaOH solution, centrifuged at a speed of 2000 rpm for 30 minutes, and filtered with filter paper. Chitosan scaffold was made by putting chitosan 1 % gel into the scaffold mold and frozen at a temperature of -80 degrees for 24 hours and dried used freeze dryer at a temperature of 95-103 degrees for 72 hours.[9]

Combination chitosan and *Aloe vera* scaffold was made by mixing chitosan 1 % gel and *Aloe vera* Extract 50 % gel in a ratio of 1: 1 then put it into the scaffold mold and frozen at a temperature of -80 degrees for 48 hours. Afterwards, freeze drying was carried out at a temperature of 100 degrees for 6 hours. The scaffold then was removed from the mold and sterilized with a UV clean bench sterilizer.[9]

Application of Scaffold

The left mandibular incisor of Cavia cobaya of treatment group was extracted and scaffold was applicated into the socket then sutured with non resorbable sutures. The left mandibular incisor of Cavia cobaya of control group was extracted and then sutured with non resorbable sutures. Cavia cobaya were sacrificed

on 7 and 14 days post tooth extraction. RANK expression examined by immunohistochemistry and Histopathological examination to examine osteoclast cells in the apical third of teeth. Immunohistochemical staining was conducted using RANK monoclonal antibodies (Santa Cruz Biotechnology Inc, [64C1385.1]: sc-59981). Hematoxylin Eosin (HE) staining was conducted to analysize osteoclast cells. The examination of RANK expression and osteoclast cells in the apical third of teeth were carried out by two observers in 5 different visual fields by using a microscope (CKX41; Olympus, Japan) at 400x magnification.

The data were statiscally analyzed using Shapiro-Wilk test to analyze the normally distributed data. Data Homogeneity was analyzed using Levene test and the differences of the groups were analyzed using One-way Analysis of Variance ANOVA) and Multiple comparison LSD test ($p<0.05$) were performed based on Shapiro-Wilk and Levene's test ($p>0.05$).

Results

Aloe vera leave was identified in Pharmaceutical Laboratory of Wijaya Kusuma University, Surabaya, Indonesia. *Aloe vera* was identified as *Aloe vera L* species (family: *Liliaceae*). The identification and phytochemical compounds of *Aloe vera* can be seen di table 1.

Classification of Aloe vera plant	
Kingdom	: Plantae
Division	: Spermatophyta
Subdivison	: Angiospermae
Class	: Monocotyledoneae
Ordo	: Liliales
Family	: Liliaceae
Genus	: Aloe
Species	: *Aloe vera L*

Metabolites	Result
Flavonoids	+
Saponins	+
Tannins	+
Anthraquinones	+
Lignins	-

Note: (+): presence; (−): absence

Table 1. The identification and phytochemicals detected of *Aloe vera* obtain from Batu plantation.

Aloe vera Extract in this study contain secondary metabolite including flavonoids, saponins, tannins and anthraquinones. Figure 1 shown there was hydrogen bonding and steric interaction between the compound of *Aloe vera* (acemannan polysaccharides) to TLR-2 reseptor.

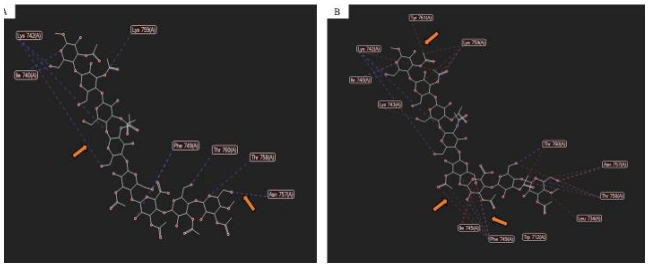

Figure 1. The interaction of Aloe vera (acemannan polysaccharides) with amino acids at TLR-2 receptor through hydrogen bonding (A), hydrogen bonding and steric interaction (B).

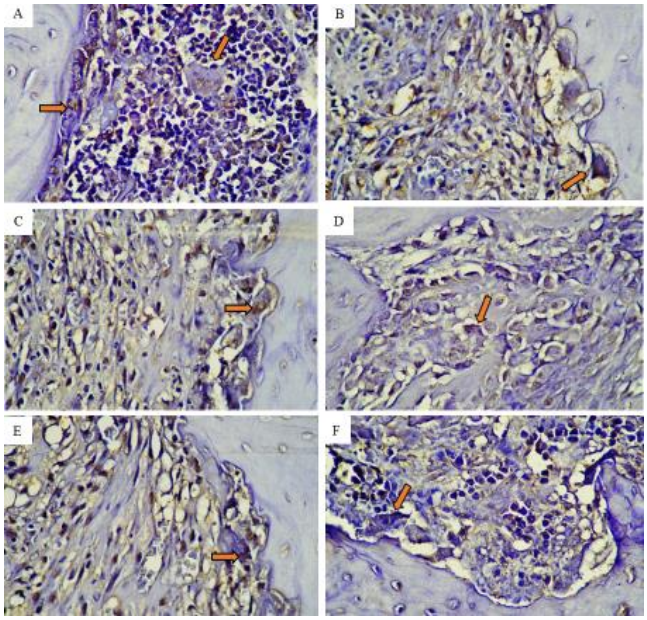

Figure 2. Imunohistochemical images of the expression of RANK with magnification 400x on 7 days observation in each group: (A) K (-) group, (C) K (+) groups, (E) P1 group; on 14 days observation:(B) K (-) group, (D) K (+) groups, (F) P2 group.

Based on Figure 2 and 3, it shown the expressions of RANK and osteoclast cells for 7 and 14 days observation on bone healing process of tooth extraction. The expressions of RANK and osteoclast cells on bone healing process post dental extraction using combination *Aloe vera* and chitosan scaffold (P1 and P2 groups) was more decreasing compared to the control group.

The data were analyzed using normality test and homogenity test. The result of analysis was homogen and have a normal distribution. Table 2 shown ANOVA test value $p=0.00$ ($P\leq0.05$) of RANK expression and osteoclast cells on bone healing process post dental

extraction showed significantly differences. There was significant differences of RANK expression and osteoclast cells on P1 and P2 groups compare to K (+) and K (-) for 7 and 14 days. LSD Test showed that P1 and P2 groups decreasing The RANK expression and Osteoblast cell compare to K (+) and K (-).

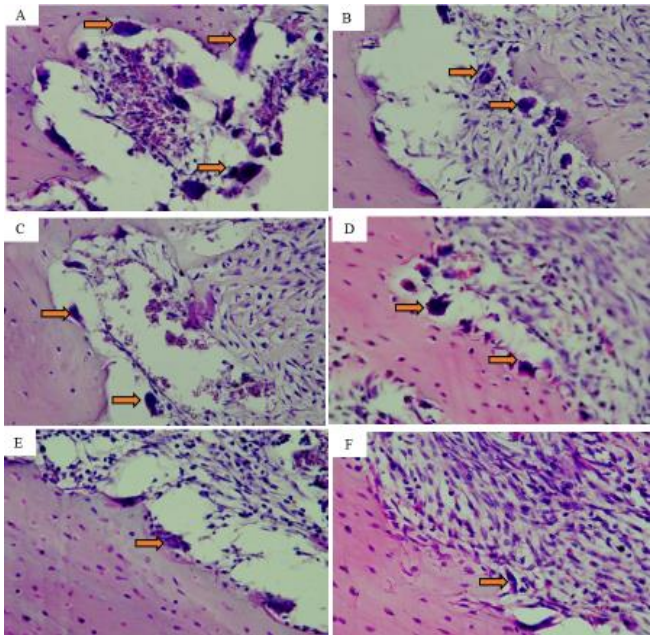

Figure 3. Histopatological images of osteoclast cells with magnification 400x on 7 days observation in each group: (A) K (-) group, (C) K (+) groups, (E) P1 group; on 14 days observation:(B) K (-) group, (D) K (+) groups, (F) P2 group.

Groups	N	RANK Expressions (cells/LP)	Osteoclast Cells	P
		$\bar{x}$ ± SD	$\bar{x}$ ± SD	
K (-) on days 7	6	13,45 ± 2,62 ^{d}	15,89 ± 1,27 ^{d}	0.000*
K (-) on days 14	6	14,22 ± 1,44 ^{d}	8,55 ± 2,15 ^{bc}	
K (+) on days 7	6	10,13 ± 1,11 ^{c}	10.11 ± 1,24 ^{c}	
K (+) on days 14	6	11,42 ± 1,39 ^{c}	8,84 ± 1,47 ^{bc}	
P1 on days 7	6	7,88 ± 2,25 ^{b}	8,00 ± 1,77 ^{b}	
P2 on days14	6	5,21 ± 1,05 ^{a}	5,23 ± 1,42 ^{a}	

Table 2. The mean and standard deviation of RANK expression and osteoclast cells in all groups.
ote:*significant at α=0.05 (Oneway Anova).
abc different superscripts show that there were differences between groups (multiple LSD comparisons).

Discussion

Many studies have demonstrated that *Aloe vera* has been concidered as medical plant that could be used in tissue engineering. The bioactive components of *Aloe vera* has anti-inflamatory, antioxidant, antibacterial and bone healing properties.[8,12] In this study *Aloe vera* leaves from Batu plantation, Malang, East Java, Indonesia was identified as *Aloe vera L* species (family: *Liliaceae*), which contain secondary metabolite including flavonoids, saponins, tannins and anthraquinones. The variance of *Aloe* species would have different phytochemical compounds due to the differences of climate, temperature and air condition. The Biological activity would depend on the compounds.[12] *Aloe vera* contains flavonoid and antraquinones which strong anti-inflammmatory and antioxidant effects.[8,15] Saponin and tannins component have antibacterial and anti-inflamatory effects. *Aloe vera* also contains potentially active compounds including lectin protein (aloctin), amino acid, enzymes, healing hormone, vitamin, mineral, organic or inorganic compound and polysaccrides mannan that can reduce inflammation caused by tooth extraction, enhance bone healing process and stimulate new bone formation.[4,8,15] The hydrogen bonding and steric interaction between the compound of *Aloe vera* to the TLR-2 receptor as target receptor shown that *Aloe vera* have anti osteoclastogenesis activity. TLR-2 receptor as target receptor regulate complex signal by activating macrophages. Macrophages stimulate the signal transduction process in the inflammatory process.[1,4] The synergistic action of the compounds have correlation with pharmacological and physiological activities.[12,15,16]

The treatment group with using chitosan and *Aloe vera* scaffold could decrease RANK expression in bone healing post tooth extraction. This can be seen in this study, statistically there was significant differences of expression of RANK on treatment group with combination *Aloe vera* and chitosan scaffold compared with control group on 7 and 14 days observation. The lowest RANK expressions were found on combination *Aloe vera* and chitosan scaffold group on 7 and 14 days observation. RANK is a cell surface receptor as an activator of the NFkB receptor, a transmembrane protein that activates osteoclast precursor cells to differentiate into osteoclasts when activated by RANKL.[2,3] If there is binding between RANK and RANKL, it activates osteoclast formation and bone resorption process will remain. Macropages will release pro-inflammatory cytokines which regulate osteoclast

activity.[1,2,3,17] The interaction of lectin protein (aloktin) bonds with polysaccharides and bradykinase enzymes in *Aloe vera* component will stimulate macrophages as a key to tissue healing and increase anti-inflammatory activity. The anthraquinones component have antibacterial anti-inflammatory activity thus accelerating bone healing.[15,18,19] C-glycosides from anthraquinone increase anti-inflammatory activity through the cyclooxygenase inhibition pathway and reduce prostaglandin E2 arachidonic acid, thus accelerating cell proliferation.[19,20,21]

Aloe vera can reduce the number of RANK expressions through the signaling pathways ERK 1/2 and JNK which are the superfamily of MAPK. Macrophages through the ERK 1/2 pathway and JNK activate M1 through decreased NFkB activation as a pathway for the formation of cytokines and growth factors pro-inflammatory cytokines such as inetrleukin-1 (IL-1), IL-6 and tumor necrosis factor α. The inhibition of pro-inflammatory cytokines thereby inhibiting the stimulation of pre-osteoclast differentiation into osteoclasts and decreasing RANK expression.[22,23]

The RANKL/OPG/RANK signaling system was the key of the Basic Multicellular Unit (BMU), achieving a balance of resorption and bone formation. Previous study reported that the RANKL/OPG/RANK optimum signaling activity oocured after the 14 days on alvolar bone defects of tooth extraction because in this periode there was high protein synthesis activity and the amount of metabolic activity of cells during the bone healing process.[24,25] On the 28 day after tooth extraction, RANKL will decrease inhibited by OPG to control bone formation. This can be seen in the results of this study that between days 7 compared to days 14, RANK expression was not significant difference in both the control groups. In the period until the 14 days post tooth extraction, the metabolic activity of the cells was still not optimum yet.

The aplication of chitosan scaffold shown average number of RANK expression was lower than control group on 7 and 14 days. Chitosan containing glycosaminoglycan components, a natural polymer which able to stimulate macrophage cells inhibiting the secretion of pro-inflammatory cytokines. Proinflammatory cytokines can stimulate bone resorption by regulating osteoclast formation. Chitosan activates macrophage and dendritic cells, significantly down regulation of pro-inflammatory markers CD86 and MHCII, decreases IL-1 and TNF α expression.[26] Chitosan scaffold has low osteoinductivity compared to chitosan combined with collagen or hydroxyapatapatite and growth factor.[9,27] Scaffold with single material used as chitosan scaffolds are less able for the requirements of graft materials.[9] Therefore, RANK expression of group with chitosan scaffold is higher compared to the treatment group with combination chitosan and *Aloe vera* scaffold. Thus, chitosan and *Aloe vera* scaffold in our study shown decreasing RANK expression and were statistically significantly different compared to the control groups. *Aloe vera* tends to inhibit bone resorption in bone healing post tooth extraction.

Conclussions

It could be concluded that *Aloe vera*'s phytochemical compounds have anti-osteoclastogenesis activity and its combination with chitosan could accelerate alveolar bone healing of tooth socket by decreasing RANK expression and osteoclast cells.

Declaration of interest

The authors declare that there is not conflict of interest.

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