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BOSTON UNIVERSITY

HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

OPTIMIZED KAVA COMPOUND TREATMENT REDUCED PORPHYROMONAS GINGIVALIS-INDUCED ALVEOLAR BONE LOSS

By

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B.D.S., King Saud University, Riyadh, Saudi Arabia, 2012

Submitted in partial fulfillment for the requirements of the degree of
Master of Science in Dentistry
In the Department of Molecular and Cell Biology

2017

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DEDICATIONS

To my adoring parents,

Khulaif Alshammari & Marifah Alshammari,

To my beloved brother,

Nasser ,Yousef ,Younis ,Ahmed ,Mohammad ,Idres,Ebrahim ,Essa ,Abdullah

To my cute sisters,

Tahani ,Amani ,Reem ,Hanna

For their love, encouragement, support enthusiasm, and sacrifice.

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I also appreciate Dr. Serge Dibart, Chairman of the Department of Periodontology, for providing such an excellent educational environment.

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ABDULSALAM ALSHAMMARI

Boston University, Henry M. Goldman School of Dental Medicine 2017
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ABSTRACT

BACKGROUND: *Porphyromonas gingivalis* (*P. gingivalis*) is a well-documented pathogen in chronic periodontitis, an inflammatory disease that results in the destruction of periodontal tissues. Kava, a compound extracted from the *Piper methysticum* plant, has been shown to have an anti-inflammatory effect in various systemic inflammatory diseases. The aim of this study was to assess the effects of a modified Kavain-derived compound, Kava-241, on periodontal destruction in a periodontitis-induced murine model.

METHODS: The study involved 49 mice divided into three groups: control, diseased, and treatment. Diseased mice were infected with *P.* gingivalis via oral gavage and a type 2 collaged antibody over a 15-day period to mimic periodontal infection. Treated mice received Kava-241 treatment before or after disease induction over the same 15-day period. Positive and negative control groups included mice that were diseased but received no treatment, and mice that received only treatment without *P.* gingivalis infection respectively. Bone loss and inflammatory cell activity was assessed by a morphometric analysis of the left mouse maxillae and a histomorphometric analysis of TRAP and H&E stained tissue sections of the right mouse maxillae.

RESULTS: The *P. gingivalis* infected group showed significantly increased alveolar bone loss, inflammatory, and osteoclastic activity throughout the experimental period in comparison to the untreated control groups. The *P. gingivalis*-infected mice that received treatment with Kava-241 showed a significant decrease in inflammatory cell activity in periodontal connective tissues as compared to mice that did not receive any treatment. In

periodontal connective tissues, Kava-241 treated mice showed significant decreases of 61.9% and 41.6% of polymorphonucleocyte and monocyte cell counts, respectively, compared to untreated mice. Furthermore, the mice that received treatment with 241 post-infection showed a statistically significant decrease in alveolar bone loss. Histomorphometric analysis demonstrated 72.7% and 37.0% reductions of epithelial down-growth and bone loss respectively. Morphometric analysis demonstrated a 46.7% reduction of bone loss in treated mice compared to controls.A similar trend of reduction of osteoclastic activity was noted between the treated group and untreated group, however, this was not statistically significant. Mice pre-treated with 241 showed similar levels of bone loss and inflammatory cell activity as compared to those treated post-infection.

CONCLUSION: Our results demonstrate that in a murine model, *P. gingivalis* induced periodontitis can be treated by 241, a modified Kava compound. The experimental model demonstrated the use of morphometric and histomorphometric analyses to accurately quantify alveolar bone loss. Further modification of Kava could yield a more effective and safer therapeutic compound in the treatment of periodontal inflammation and bone loss.

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CHAPTER ONE

INTRODUCTION

1.1. Periodontitis

Periodontitis, an inflammatory disease caused by bacterial plaque, is characterized by the destruction of the tooth supporting structure, involving epithelial downgrowth and alveolar bone loss [1, 2].

1.2. Pathogenesis of periodontitis

The shift from a normal symbiotic microbiotic state to a dysbiotic state consisting of bacterial communities forming on subgingival tooth surfaces is the initial cause of peridontitis[3, 4]. The major periodontal pathogen associated with periodontitis is the gram-negative anaerobe, *Porphyromonas gingivalis (P. gingivalis)* [5, 6] There are many potential virulence factors and enzymes that could cause the pathogenicity of *P. gingivalis*, such as lipopolysaccharide, fimbriae, and various enzymes [7, 8]. The initiation and progression of periodontitis are dependent on the mixed flora of plaque. Evidence shows that *Aggregatibacter actinomycetemcomitans* can pass through epithelial cells and into underlying connective tissue [9]. *P. gingivalis* can invade and persist in epithelial cells [10, 11]. The breakdown of the antibacterial defense barrier and subsequent launch of periodontitis may be caused by bacterial leukotoxins that can kill neutrophils directly [12, 13]. However, bacterial infection is not the only cause of periodontitis; host susceptibility may play an important role in disease [23, 24][14, 15]. The host response is variable among individuals and contributes significantly to the expression of periodontal diseases[15].

1.3. Epidemiology

Periodontitis is one of the most common chronic diseases in humans[16]. A recent epidemiolgical study of periodontal diseses estimated that 5–20% of the world's population suffers from chronic periodontitis[17]. Periodontitis is a prevalent disease that affects >47% of US adults, 8.5% of them having its most severe form [18] .

1.4. Systemic effect of periodontitis

The risk for atherosclerosis, diabetes, and adverse pregnancy could be impacted by periodontitis[19-22]. Furthermore, periodontitis has been associated with systemic diseases, such as cardiovascular complications[23], rheumatoid arthritis[24] , and Alzheimer's disease[25].

1.5. Immunology in periodontal disease

Innate immunity is considered to act as a sentinel for the immune system and is immediately activated after recognition of the diverse range of microbial pathogens. Innate immune cells recognize signature molecules of microorganisms via various pattern recognition receptors (PRRs)[26]. Toll-like receptors (TLRs) are a well-characterized class of PRRs in mammalian species [27, 28]. TLRs can detect bacteria expressing various pathogen-associated molecular patterns (PAMPs)[29]. TLR2 can identify bacterial lipoproteins, peptidoglycans, and lipoteichoic acid-[30]; TLR4 can identify lipopolysaccharide (LPS) [31]. LPS, lipoproteins, and fimbriae are surface components of *P. gingivalis* that interact with TLR2 and TLR4 expressed by host cells. This interaction stimulates the production of proinflammatory cytokines [32] such as interleukin-1B (IL-1B) , IL-6, -11, -17 and tumor necrosis factor alpha (TNF-alpha). These proinflammatory cytokines are among a broad array of biomolecules

that have consistently been reported to be elevated in gingival crevicular fluid (GCF) andperiodontium of periodontitis patients[33-38]. In addition to the inflammatory response, an increased production of anti-inflammatory cytokines [39, 40], such as IL-10, and receptor activator of NF-kB ligand (RANKL), an osteoclast differentiation factor that promotes osteoclastogenesis[41], has been observed. The resultant osteoclastogenesis leads to the alveolar bone resporption observed in patients with periodontitis. In response, alveolar bone resorption occurs with increased osteoclastogenic activity leading to catabolism.

Bone and tooth loss are the likely end result of the alveolar bone resorption observed in peridontitis. Bone loss results from the activation ofthe inflammatory process that promote interactions between the receptor activator of nuclear factor-kappa B (RANK), RANK ligand (RANKL), and the RANKL antagonist, osteoprotegerin (OPG) (Cochran 2008)[37]. These mediators are directly involved in osteoclastogenesis, activation, differentiation and osteoclast survival [42-44]. RANKL can mediate Periodontitis-related alveolar bone loss *in vivo*[45, 46] . Given this interaction, it is possible that a RANK-RANKL/ OPG signalling pathway plays a significant role in mediating the alveolar bone loss observed in periodontal disease.

Kavain has been found to inhibit LPS- induced TNF-α Factor (LITAF)-mediated TNF-α secretion both *in vitro* and *in vivo* in a murine model in which Kavain-treated mice were injected with lethal doses of LPS and found to be immune (Pollastri et al. 2009).[47] Prior *in vivo* studies have shown that *P. gingivalis*-induced bone loss resulted from a TLR2 and TNF-dependent macrophage-specific innate immune response to *P. gingivalis* infection [51].

1.6. Rheumatoid ARITHRITIS

Rheumatoid arthritis (RA) is an autoimmune disease involving joint inflammation. RA has also been associated with systemic conditions such as cardiovascular disease. . The disease pathogenicity is due to an imbalance in inflammation-related cytokines, such as IL-1, IL-6, int-γ, and TNF-α. Current methods of diagnosing rheumatoid arthritis in patients by ACR and EULAR include four factors: the number of joints involved, the concentration of specific autoantibodies (RF and ACPA), the levels of general inflammation markers (C-reactive protein and ECR), and the duration of the symptoms. These methods present with a significant error rate, and an accurate method to identify the disease and its pathogenesis remains unknown. Furthermore, long-term treatment is unavaiable. In order to study the effects of RA, this study used a collagen-induced arthritis mouse model that closely mimics rheumatoid arthritis.

1.7. 241 KAVA COMPOUND

Kavain is a compound found in a beverage prepared from the roots of *Piper methysticum,* originating in Polynesia and Micronesia. It has been suggested to be responsible for the low incidence of cancer in natives of this island[52] as well as its analgesic[53] , relaxant[54] , and anticonvulsant effects[55]. Recently, it was demonstrated that Kavain inhibits LITAF-mediated TNF-α secretion both *in vitro* and *in vivo* where Kavain-treated mice were immune to lethal doses of LPS, Given the immunity to lethal doses of LPS described earlier, Kavain can be said to be mediating TNF-α

suppression[47]. Kava-derived compounds appear to be a promising anti-inflammatory agent that may also aid in the prevention and/or treatment of localized bone loss observed in periodontitis and in the joints affected by RA .

1.8. Collagen antibody-induced arthritis (CAIA)

The collagen antibody-induced arthritis (CAIA) murine model of rheumatoid arthritis can be used to identify pathogenic mechanisms and screen for potential therapeutic agents. To induce arthritis, a cocktail of monoclonal antibodies is given to the mice. These antibodies are directed to conserve auto-antigenic epitopes in collagen type II and endotoxins. This CAIA model offers several key advantages which are lacking in the classic collagen-induced arthritis (CIA) model, including rapid disease onset, a high uptake rate, synchronicity, and the capacity to use genetically modified mice. This protocol takes 1–2 weeks to be completed.[56]

1.9. Study Model

Murine Murine models have been widely used to further understand the pathogenesis and theraputic modalities in periodontitis because the periodontal anatomy and the histopathology of periodontal diseases in mice are similar to those found in humans[61].Several other benefits to use a murine model for studies of periodontitis includeknown genetics, controllable microflora, low cost, ease of handling, and a well- defined immune system.[62, 63]

The purpose of this study was to test the ability of Kavain to alleviate the symptoms of CAIA and *P. gingivalis*- gavage-induced periodontitis in a murine model. Bone and connective tissues parameters were quantified and analyzed to determine the role of treatment and prevention in experimental periodontitis.

1.10. Hypothesis

Periodontitis is an inflammatory disease caused by dysbiotic microbial infection, and Rheumatoid arthritis is an autoimmune disease that primarily causes inflammation. Recent publications demonstrate that the Kava compound inhibits LITAF-mediated TNF-α secretion *in vitro* and *in vivo.* We hypothesize that a modified Kava-derived compounds may help to prevent or treat localized bone loss and inflammation observed in periodontitits and rheumatoid arthritis.

1.11. Objectives

I. To evaluate the ability of a modified Kava compound in reducing the activity involved in the inflammatory process in a murine model of *P. gingivalis*-gavage-induced periodontitis.and,

II. To evaluate the ability of the modified Kava compound to alleviate the symptoms of CAIA *in vivo* in a murine model of RA.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Mice

This study was carried out in a pathogen-free unit of the animal facility located at Boston University Medical Campus. To minimize any potential estrogen effects, only six-week-old, pathogen-free DBA1/BO male mice (Taconic Farm, Rensselaer, NY) were used. The mice were fed sterile food and distilled water *ad libitum*. All of the experimental procedures in this study were approved by the Institutional Animal Care and Use Committee at the Boston University Medical Center.

2.2. Bacteria

The specific *P. gingivalis* strain W83 was used (BAA-308; ATCC, Manassas, VA, USA). This pathogen was cultured and maintained in Schaedler anaerobe broth (Oxoid Ltd., Basingstoke, Hampshire, England), supplemented with hemin (5 μg/ml, Sigma-Aldrich, St. Louis, MO), menadione (1 μg/ml, Sigma-Aldrich, St. Louis, MO), and sodium bicarbonate (420 μg/ml, Sigma-Aldrich, St. Louis, MO) in an anaerobic chamber with 85% N_2, 10% H_2, and 5% CO_2 at 37°C. Bacteria at early logarithmic-phase growth were used for inoculation procedures. An average 5×10^8 colony-forming units of W83 (BAA-308; ATCC, Manassas, VA, USA) in 100 μl phosphate-buffered saline with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA). This was administered by oral gavage for 15 days.

.

Hemin solution

25.0 mg Hemin

0.5 ml 1M NaOH

49.5 ml Distilled water

The solution was mixed well and filtered with a sterile 0.22 µm filter.

Menadione (Vitamin K) solution

150.0 mg Menadione

30.0 ml 95% Ethanol

The solution was mixed well and filtered with a sterile 0.22 µm filter.

4.2% Sodium Bicarbonate ($NaHCO_3$)

2.1 g $NaHCO_3$

50.0 ml Distilled water

The solution was mixed well and filtered with a sterile 0.22 µm filter.

Schaedler anaerobe broth

14.2 g Schaedler anaerobe broth

500.0 ml Distilled water

The broth was autoclaved for 30 min at 121°C.

The broth was cooled to room temperature.

5 ml hemin and 100 µl menadione were added to 500 ml broth.

2.3. Induction of periodontitis lesion

Forty-seven mice were randomly assigned to one of seven groups: a *P. gingivalis*-infected and antibody injected group (periodontitis)(positive control); a *P. gingivalis*-infected group, antibody injected and Kava compound treatment group; a *P. gingivalis*-infected, antibody injected and Kava compound prevention group; a *P. gingivalis*-infected only group; antibody only group; Kava compound treatment only group; and a Kava compound preventive only group. Mice were given sulfamethoxazole at 0.87 mg/ml and trimethoprim at 0.17 mg/ml ADD (Hi-Tech Pharmacal Co. Inc., Armityville, NY, USA) in milli-Q water ad libitum for 10 days, followed by 3 days without antibiotics. Mice in the *P. gingivalis*-infected group were anesthetized with Isophrane and, mice were inoculated with an average 5×10^8 colony-forming units of *P. gingivalis* strain W83 (BAA-308; ATCC, Manassas, VA, USA) in 100 µl phosphate-buffered saline with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA) and were administered by oral gavage for 15 days. With an average 5×10^8 colony- forming units of *P. gingivalis* strain W83 (BAA-308; ATCC, Manassas, VA, USA) in 100 µl phosphate-buffered saline with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA) and was administered via oral topical application for a total of 15 inoculations. The non-*P. gingivalis*-infected groups were anesthetized with Isophrane and inculated with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA). This was administered by oral gavage for 15 days. Mice were euthanized by CO_2 inhilation at scheduled

time points 15 days after gavage inculation. In histomorphometry: N=7 in each group; in morphometry: N=7 in each group and in cell counting: N=7 in each group.

<u>Isophrane</u>

0.74 ml Phosphate-buffer saline (PBS)

0.16 ml Ketamine (100 mg/ml)

0.10 ml Xylazine (20 mg/ml)

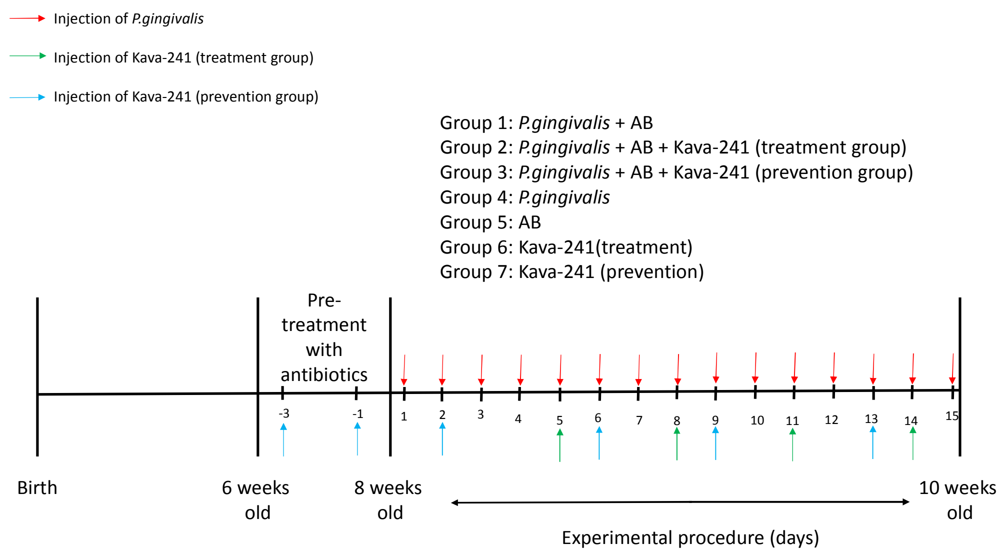


Figure 1:Overview for experimentally induced periodontitis model

2.4. Induction of Arthritis lesion

Forty seven mice were randomly assigned to one of seven groups: a *P. gingivalis*-infected and antibody injected group (periodontitis); *P. gingivalis*-infected group; antibody injected and Kava compound treatment group; *P. gingivalis*-infected, antibody injected and Kava compound prevention group; *P. gingivalis*-infected only group, antibody only group; Kava compound treatment only group; and Kava compound preventive only group. Mice in the antibody injected group were

anesthetized with Isophrane and were intraperitoneally injected with 2 mg of CAIA one day before the *P.gingivalis* oral gavage admenestration .

2.5. 241 Kava Compound treatment and prevention

Forty seven mice were randomly assigned to one of seven groups: a *P. gingivalis*-infected and antibody injected group (periodontitis); *P. gingivalis*-infected group, antibody injected and Kava compound treatment group; *P. gingivalis*-infected, antibody injected and Kava compound prevention group; *P. gingivalis*-infected only group; antibody only group; Kava compound treatment only group; and Kava compound preventive only group. Mice in the Kava treatment group were intraperitoneally injected with 40 mg/kg Kava compound on days 5, 8, 11 and 14 of the experiment. However, mice in the Kava prevention groups were intraperitoneally injected with two doses of 40 mg/kg Kava compound. The first dose was given three days before the *P.gingivalis* oral gavage admenestration. Followed by second dose one day before the *P.gingivalis* oral gavage admenestration followed by the following adminstration timing days 2, 6, 9, and 13 of the experiment.

2.6. Tissue specimen preparation for periodontal analysis

The palatal bone and intact surrounding tissue from each euthanized animal was dissected and fixed with 4% freshly prepared paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS (pH 7.2) for 24 hr at 4°C. After fixation, the specimens were consecutively washed with 5%, 10%, and 15% glycerol (American Bioanalytical, Natick, MA) in PBS, each for 15 min at 4°C, and decalcified in an Ethylenediaminetetraacetic Acid Tetrasodium Salt Dihydrate EDTA(Sigma-Aldrich, St. Louis, MO)for 14 days at 4°C. Once a day during this period, the specimens were

gently stirred and the solutions refreshed. The samples were then immersed in 30% sucrose (Sigma-Aldrich, St. Louis, MO) in PBS until embedding. The tissue block was embedded with a HISTO PREP® compound (Fisher Scientific, Hanover Park, IL) for cryostat sectioning. Serial mesiodistal sections (5 µm) parallel to the long axis of the teeth were made. The correctly oriented sections were stained with hematoxylin and eosin for histomorphometric analysis.

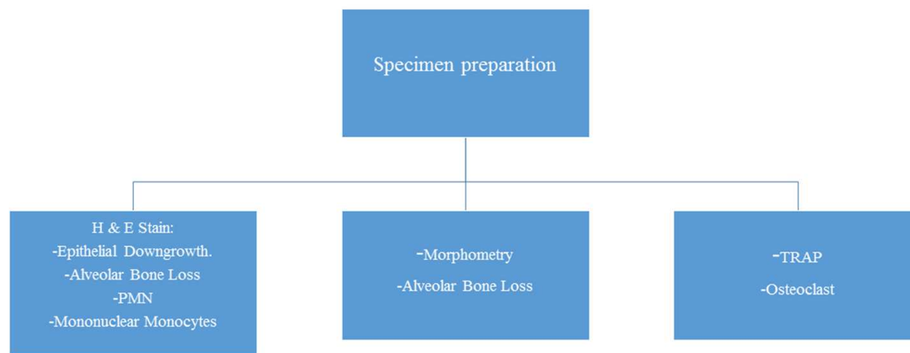


Figure 2:Overviw of Periodontal Analysis

2.7. Hematoxylin and Eosin (H&E) stain

The sections were fixed by immersing the slides in acetone (ACROS Organics, Morris Plains, NJ) for 5 min, then air drying them for 5 min at room temperature. The slides were rinsed for three times every 5 min in PBS to remove the tissue-freezing matrix, and then immersed in Hematoxalin solution (Fisher Scientific, Pit, IL) for 3 min. They were then washed with tap water for 3 min, fast-dipped in acid ethanol (Sigma-Aldrich, St. Louis, MO) 8 to 12 times, washed with tap water two times for 1 min, and then cleansed with de-ionized water for 2 min. The slides were then placed in an eosin solution (ACROS Organics, Morris Plains, NJ) for 30 sec and dehydrated for 3 min in each solution of alcohol series: 95%, 95%, 95%, 100%, 100%, and 100%. Finally, the slides were bathed in Xylene (Fisher Scientific,

Pit, IL) three times for 5 min and coverslipped with Permount (Fisher Scientific, Pit, IL).

2.8. Histomorphometric analysis

Interdental areas between the first and second molars of the right maxillary quadrants were examined. The measurements included extension of the apical migration of epithelium (epithelial downgrowth) and bone resorption on H&E-stained sections from images captured at 100X magnification using an image analysis system (Image-Pro Plus Version 5.0, Media Cybernetics, Silver Spring, MD). Epithelial downgrowth was defined by measuring the distance from the cementoenamel junction (CEJ) to the apical extent of the junctional epithelium. Bone resorption was measured as the distance between the CEJ and the alveolar bone crest (ABC). Sections from different specimens were evaluated in a random sequence. The measurements were repeated 2 times per site, and the results were presented as the distances (μm).

A minimum of 6 fields from each interdental area (from CEJ to root apex of the second molar) were analyzed. To ensure that the region examined represents the buccal-lingual midpoint, the analysis was done only for sections where the root canal systems of the adjacent teeth were visible and properly oriented. In each area of interest, the total number of inflammatory cells was counted manually from images captured at 400X magnification on H&E-stained sections. The inflammatory cells included polymorphonuclear neutrophils (PMN), mononuclear leukocytes, and fibroblasts; all were identified by their characteristic morphologies. The data were then reported as the number of each type of cell per mm^2.

2.9. Morphometric analysis

The alveolar bone around the left maxillary second molars was measured by the morphometric method as previously described.[64] The skulls were mechanically defleshed after 15 min of treatment in boiling water, washed with PBS, and then exposed overnight in 3% hydrogen peroxide. Next, they were washed with PBS three times for 5min, immersed in bleach for 1 min, washed again with PBS for three times for 5min, and then dried in an incubator at 37°C for 1 hr. The skulls were then stained at room temperature with 1% methylene blue (Sigma-Aldrich, St. Louis, MO) for 1 min, after which, they were dried in an incubator for 30 min at 37°C. The measurement of distances from cemento-enamel junction (CEJ) to alveolar bone crest (ABC) were made under a dissecting microscope fitted with a video image marker measurement system (Image-Pro Plus version 6.0) standardized to give measurements in μm. The measurements were repeated 2 times per site. All measurements were made on images taken with the 3 molar teeth in the same occlusal plane.

The distance from the CEJ to the ABC was measured at 6 sites per tooth mesio-buccal (MB), mid-buccal (MidB), disto-buccal (DB), distopalatal (DP), midpalatal (MidP) and mesio-palatal (MP) was done with the assistance of an image analysis system.

2.10. Osteoclast Activity Analysis

Activity of osteoclasts along the alveolar bone crests were measured between first, second, and third molars in 5μm histological slices. Osteoclasts were detected by

staining for TRAP. 40mL of 0.2M Sodium Acetate buffer solution (pH 5.0) was mixed with 56mg of Fast Red Violet LB Salt, 38.8mg Tartaric Acid, and 160µL of 10% $MgCl_2$. In a separate container in an opaque container to block light. Then, 6.4mg of Naphthol was mixed thoroughly with 0.4mL of N,N-dimethylformamide. The sodium acetate and naphthol solution were then mixed together. The solution was filtered through a 22µL filter into a new container that was also opaque. The solution was kept at 37^0C before, during, and after staining slides. 5µm thick histological slides from mice were then exposed to the TRAP solution for 10 minutes at 37^0C. The slides were then washed for 30 minutes before being counter-stained with hematoxilline for 5 seconds. Osteoclasts were identified as being positively stained for TRAP, present along the alveolar bone crest, possessing and possessing a ruffled border with an underlying lacunae. The number of osteoclasts present was determined by x400 magnification using image analysis software. Data are presented as the number of osteoclasts per µm of bone.

2.11. Statistical analysis

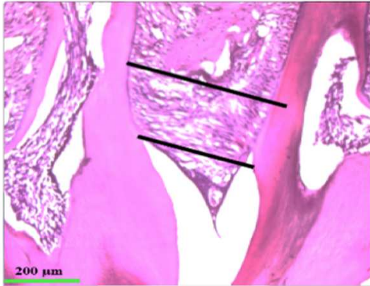
All measurements were performed in a blind fashion and double-checked at a one-week interval. Intra-examiner variation was found to be less than 5%. All values were expressed as mean ± SEM. A two-tailed Student's *t*-test for comparison between two groups. A P value of <0.05 was considered to be statistically significant.

CHAPTER THREE

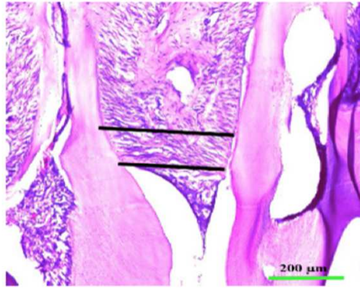
RESULTS

3.1. Epithelial downgrowth

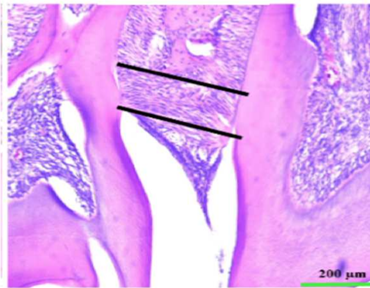
The apical migration of epithelium is a sign of the pathological progress of periodontitis. Epithelial downgrowth was measured by the distance of apical migration of epithelial attachment relative to the CEJ. The positive control (antibody and *P.gingivalis)* group exhibited the most epithelial downgrowth when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment and the antibody, *P.gingivalis* and Kava compound prevention groups exhibited significantly less epithelial downgrowth (72.7% and 84% respectively), when compared with positive control group (antibody and *P.gingivalis*)., The mice in the *P.gingivalis*-only and antibody-only groups showed a statistically significant smaller epithelial downgrowth (61.1% and 86.2% respectively) when compared with the positive control group (antibody and *P.gingivalis)*.. Mice in the antibody, *P.gingivalis* and Kava compound prevention group performed better than those in the antibody, *P.gingivalis* and Kava compound treatment group, particularly in regards to reducing epithelial downgrowth. Mice treated with the antibody only, Kava treatment only and Kava preventive only exhibited significantly less epithelial downgrowth , as compared with the positive control group (antibody and *P.gingivalis*) (86.3% and 91.9% respectively).



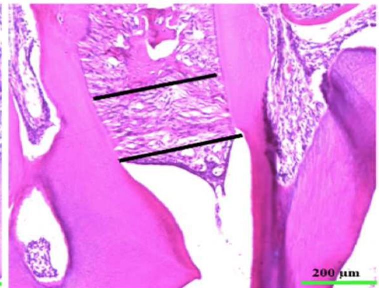
P.gingivalis + Ab



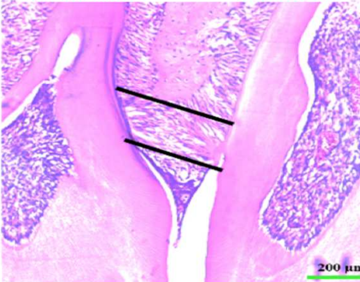
P.gingivalis + Ab+ Kava Treatment



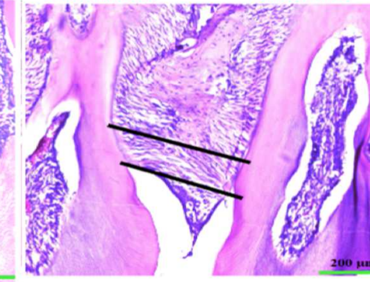
P.gingivalis + Ab+ Kava Preventive



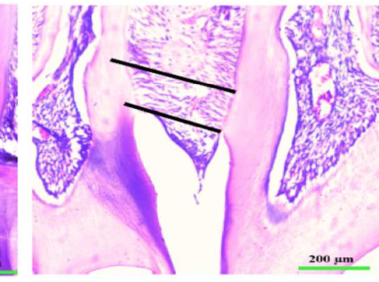
P.gingivalis



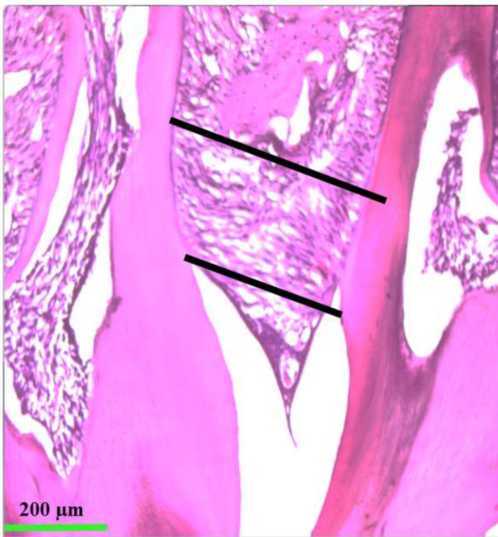
Ab



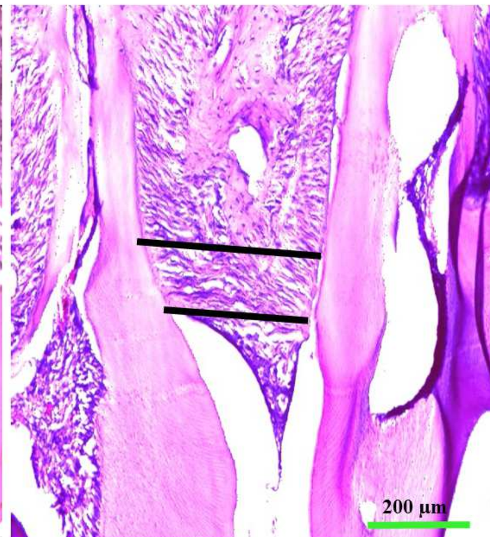
Kava Preventive



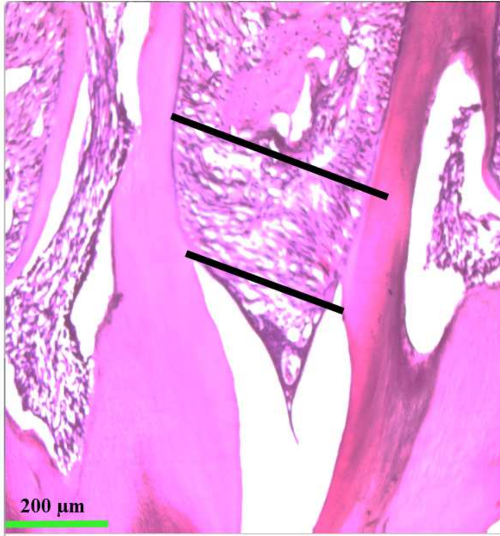
Kava Treatment



Pg+Ab



Pg+Ab+Kava tx



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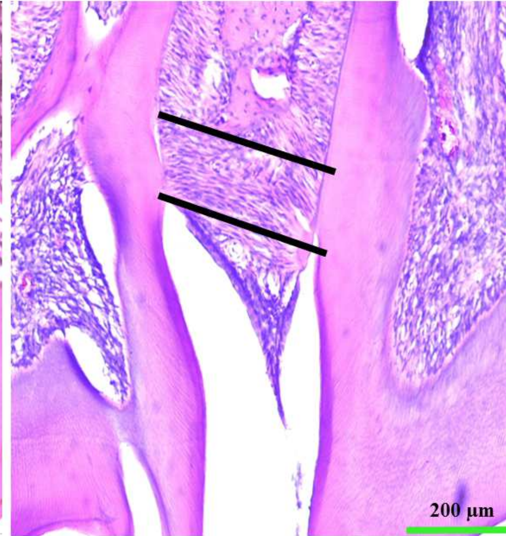
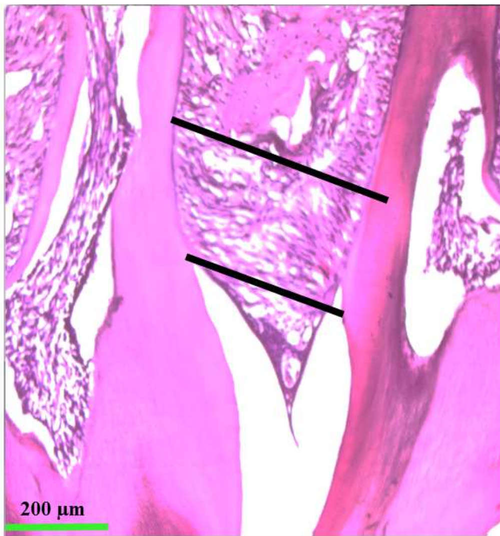
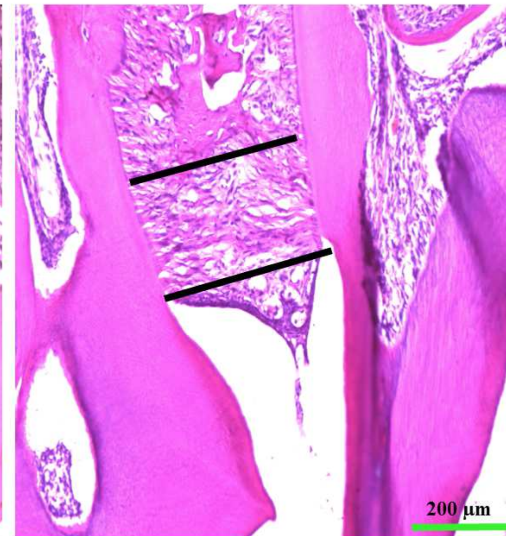


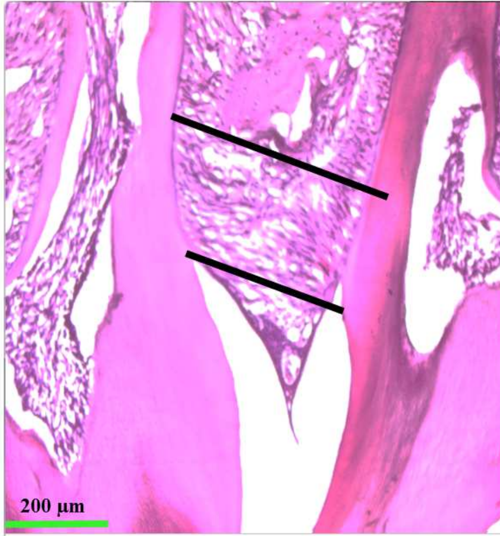
Pg+Ab+Kava preventive



Pg+Ab



Pg only



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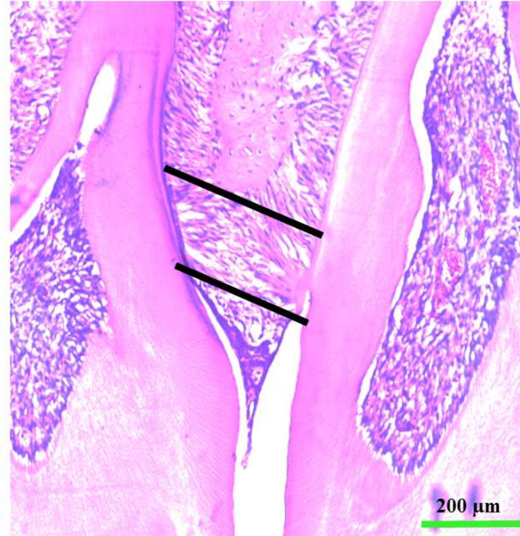
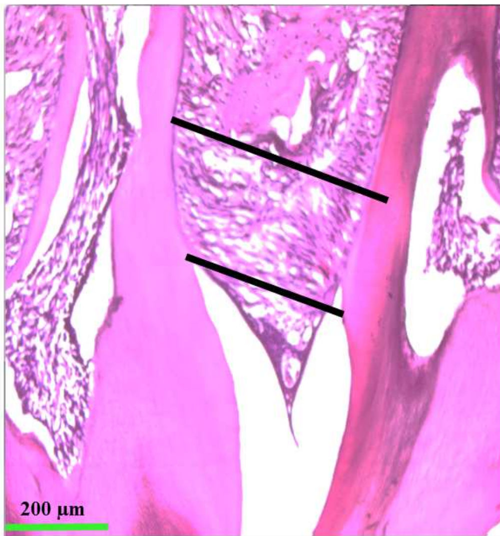


Ab only



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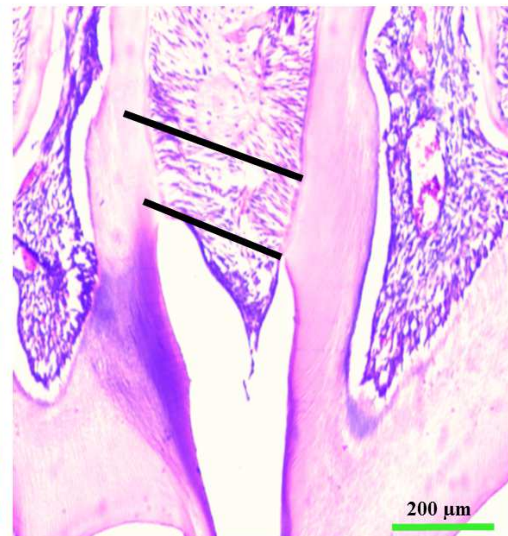

Kava Tx Only

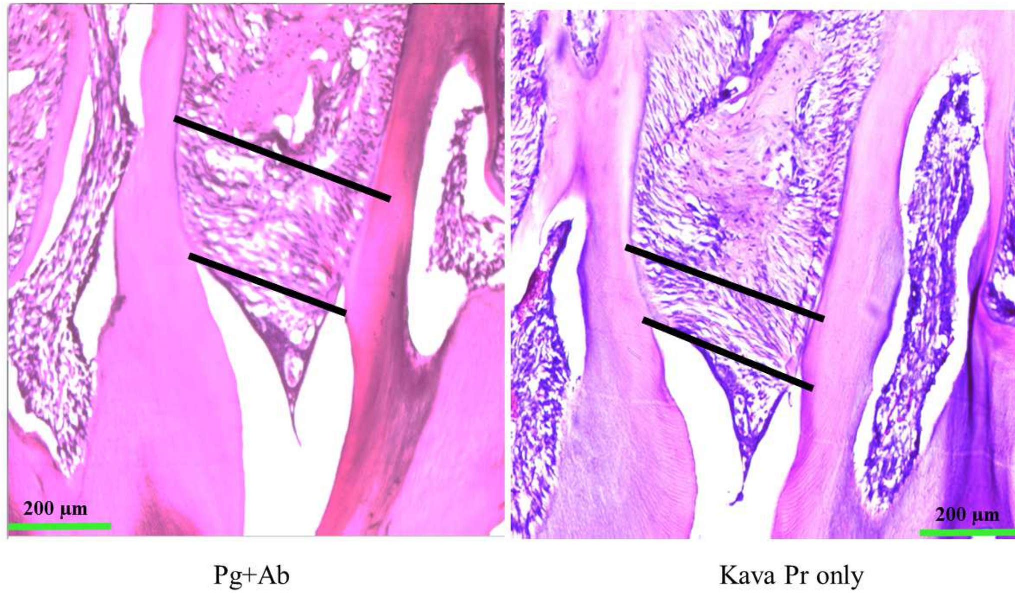

Figure 3:Histomorphometric Analysis Epithelial Downgrowth and Bone Loss.

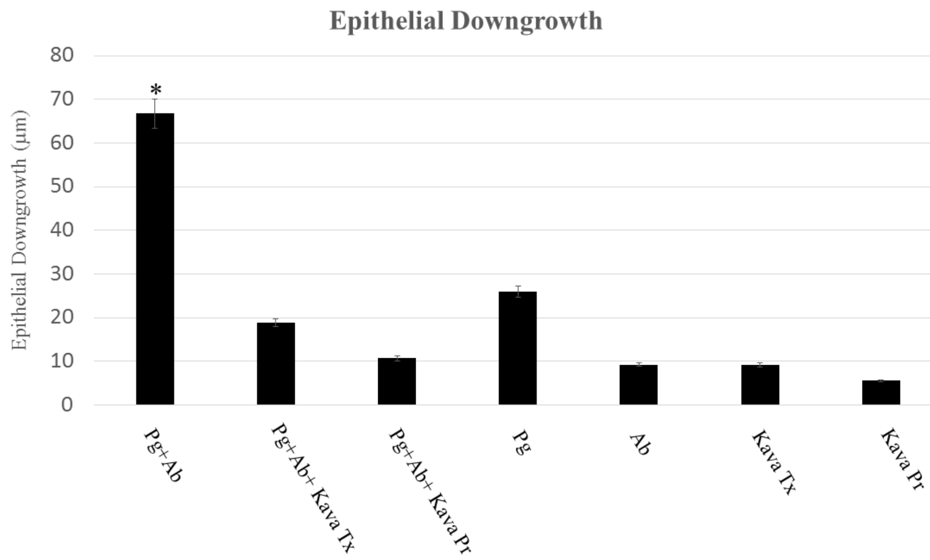

Figure 4:Histomorphometric Epithelial Downgrowth graph.

groups	N	Subset for alpha = 0.05		
		1	2	3
7.00	14	5.4959		
6.00	14	9.1042		
5.00	14	9.2119		
Ab+Pg+KavaPr	14	10.6415	10.6415	
Ab+Pg+KavaTr	14	18.8144	18.8144	
4.00	14		25.9193	
Ab+Pg	14			66.7598
Sig.		.151	.062	1.000

Table 1: Histomorphometric Epithelial Downgrowth .

3.2. Alveolar Bone Loss Histomorphometry

To assess alveolar bone loss, distances were measured from the CEJ to the ABC on histomorphometry sections. The positive control (antibody and *P.gingivalis)* group exhibited the highest amount of alveolar bone loss when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited significantly less alveolar bone loss (36.98% and 39.05% respectively) when compared with the positive control group.Mice in the *P.gingivalis* only and antibody only groups showed statistically less alveolar bone loss (36.5% and 50.72% respectively) when compared with positive control group. This finding indicates that the combined effect of *P. gingivalis* and antibody is required in order to achieve maximum bone loss. Mice in the antibody, *P.gingivalis* and Kava compound prevention group showed a further decreased alveolar bone loss compared to those in the antibody, *P.gingivalis*

and Kava compound treatment group. Mice treated with the antibody only, Kava treatment only, or Kava preventive only exhibited significantly less alveolar bone loss compared with the positive control group (58.5% and 43.18% respectively).

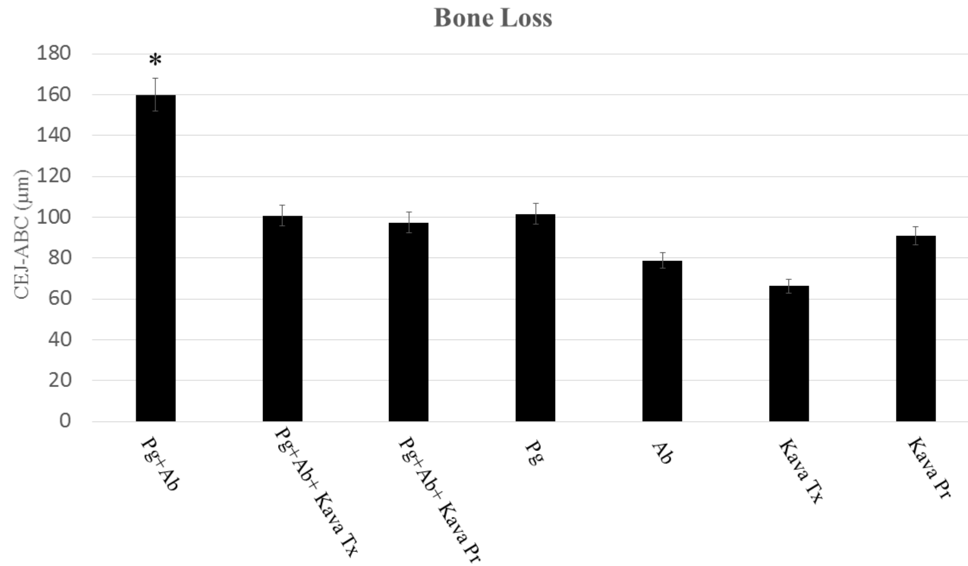

Figure 5:Histomorphometric Bone Loss Graph.

BoneGroup	N	Subset for alpha = 0.05	
		1	2
KavaTx only	7	66.2266	
Ab only	7	78.7434	
KavaPr only	7	90.7986	
Ab+Pg+KavaPr	7	97.4041	
Ab+Pg+KavaTx	7	100.7126	
Pg only	7	101.4574	
Ab+Pg	7		159.8137
Sig.		.312	1.000

Table 2: Histomorphometric Bone Loss.

3.3. Alveolar Bone Loss Morphometry

To measure alveolar bone loss, the distance from the CEJ to the ABC was measured at 6 sites per tooth mesio-buccal (MB), mid-buccal (MidB), disto-buccal (DB), distopalatal (DP), midpalatal (MidP) and mesio-palatal (MP) with the assistance of the Image Pro system. All measurements were made on images taken with the 3 molar teeth in the same occlusal plane. The positive control (antibody and *P.gingivalis)* group exhibited the highest amount of alveolar bone loss when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited significantly less alveolar bone loss (46.68% and 48.49% respectively), when compared with positive control (antibody and *P.gingivalis)* group. The mice in the *P.gingivalis*.only and antibody only groups showed a statistically significant lower amount of alveolar bone loss (22.07% and 40.15% respectively) when

compared with positive control group (antibody and *P.gingivalis)*. This finding indicates that the combined effect of *P.gingivalis* and antibody is required to achieve maximum bone loss. Mice in the antibody, *P.gingivalis* and Kava compound prevention group showed a significantly greater decrease in alveolar bone loss than the antibody, *P.gingivalis* and Kava compound treatment group. The mice treated with the antibody only, Kava treatment only and Kava preventive exhibited significantly less alveolar bone loss compared to the positive control group (antibody and *P.gingivalis*) (46.68% and 43.07% respectively).

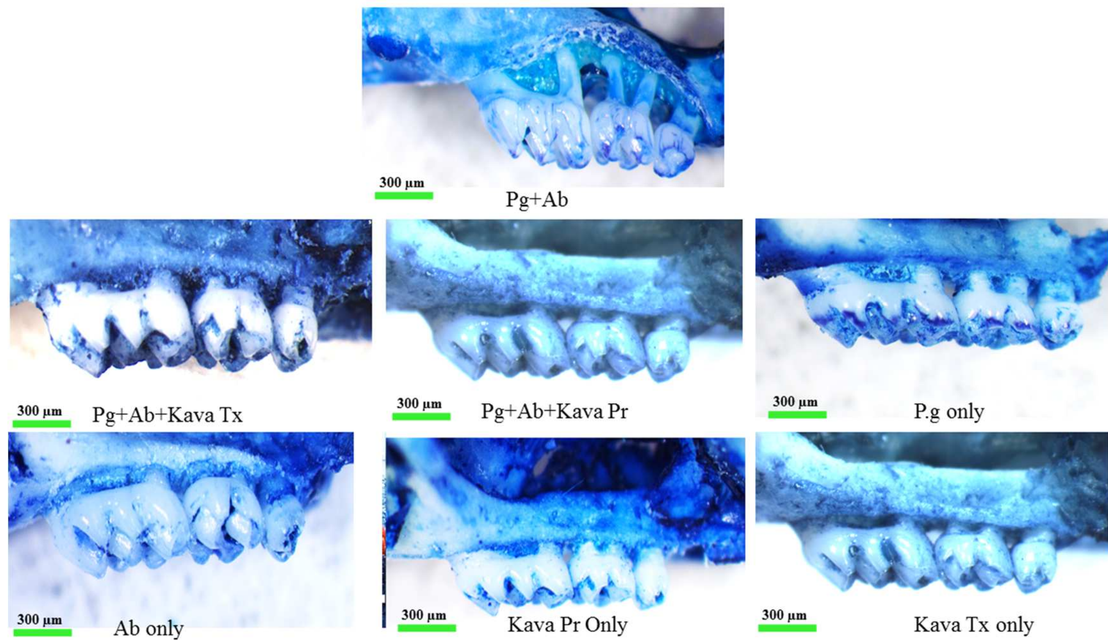

Figure 6:Morphometric Analysis

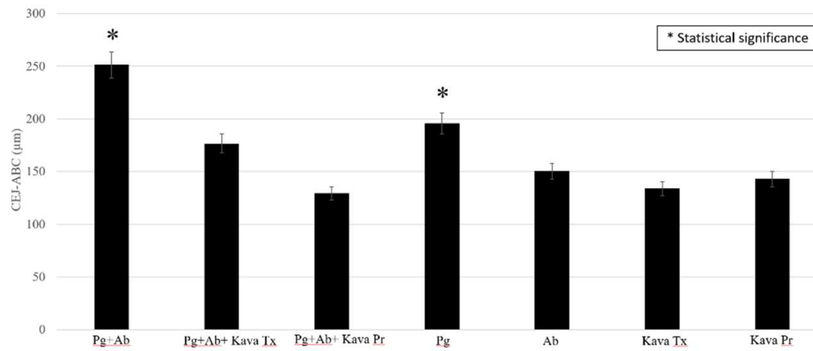

Figure 7: Morphometric Analysis Graph.

Groups	N	Subset for alpha = 0.05	
		1	2
Ab+Pg+KavaPr	7	129.3831	
KavaTx Only	7	133.9307	
KavaPr Only	7	143.0142	
Ab	7	150.3326	
Ab+Pg+KavaTx	7	176.7119	
Pg	7	195.7723	195.7723
Ab+Pg	7		251.2178
Sig.		.056	.169

Table 3:Morphometric Bone Loss

3.4. Presence of inflammatory cells in periodontitis

A minimum of 6 fields from each interdental area (from CEJ to root apex of the second molar) were analyzed. To ensure that the region examined represents the buccal-lingual midpoint, the analysis was done only for sections where the root canal systems of the adjacent teeth were visible and properly oriented. In each area of interest, the total number of inflammatory cells was counted manually from images captured at 400X magnification on H&E-stained sections. The inflammatory cells of

interest included polymorphonuclear neutrophils (PMN), mononuclear leukocytes, and fibroblasts; all were identified by their characteristic morphologies. The data were then reported as the number of each type of cell per μm^2. The positive control (antibody and *P.gingivalis)* group exhibited the maximum amount of mononuclear leukocytes when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited significantly less mononuclear leukocytes (41.58 % and 62.01% respectively), when compared with positive control (antibody and *P.gingivalis)* group. The mice in the *P.gingivalis*.only and antibody only groups showed significantly fewer mononuclear leukocytes (39.9% and 80.28% respectively) when compared with positive control group (antibody and *P.gingivalis)*. Mice in the antibody, *P.gingivalis* and Kava compound prevention group showed a significantly greater decrease in mononuclear leukocytes than the antibody, *P.gingivalis* and Kava compound treatment group. The mice treated with Kava treatment only and Kava preventive only exhibited significantly fewer mononuclear leukocytes compared to the positive control group (antibody and *P.gingivalis*) (78.36% and 72.35% respectively).

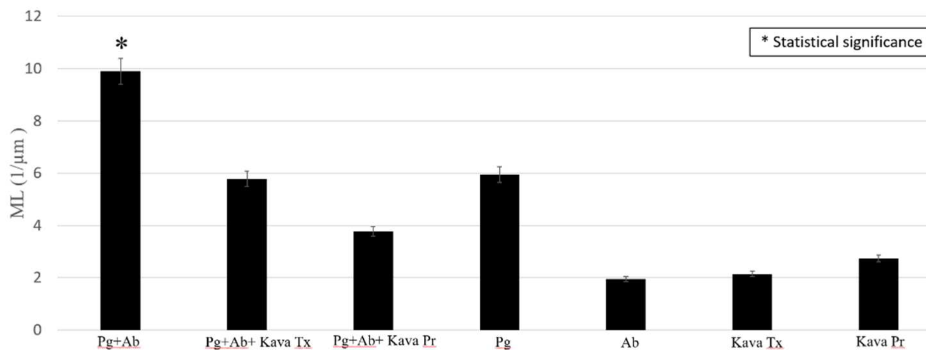

Figure 8:Mononuclear Leukocytes Count.

AllGroups	N	Subset for alpha = 0.05		
		1	2	3
5.00	42	1.9524		
6.00	42	2.1429		
7.00	42	2.7381		
3.00	42	3.7619	3.7619	
2.00	42		5.7857	
4.00	42		5.9524	
1.00	42			9.9048
Sig.		.223	.070	1.000

Table 4:Mononuclear Leukocyte Count.

The positive control (antibody and *P.gingivalis)* group exhibited the highest number of polymorphonuclear neutrophils (PMN) when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited significantly fewer mononuclear leukocytes (61.96% and 67.35% respectively), when compared with positive control (antibody and *P.gingivalis)* group. The mice in the *P.gingivalis*.only and antibody only groups showed significantly fewer polymorphonuclear neutrophils (PMN) (61.24% and 81.65% respectively) when compared with positive control group (antibody and *P.gingivalis)*. Mice in the antibody, *P.gingivalis* and Kava compound prevention group showed significantly greater decrease in mononuclear leukocytes than the antibody, *P.gingivalis* and Kava compound treatment group. The mice treated with Kava treatment only and Kava preventive only exhibited significantly fewer polymorphonuclear neutrophils (PMN)

compared to the positive control group (antibody and *P.gingivalis*) (84.97% and 87.66% respectively).

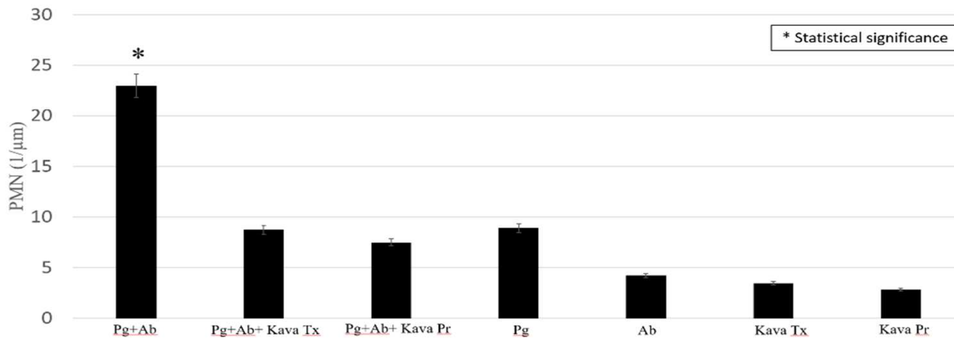

Figure 9: Polymorphonuclear Neutrophils Count

AllGroups	N	Subset for alpha = 0.05			
		1	2	3	4
7.00	42	2.8333			
6.00	42	3.4524			
5.00	42	4.2143	4.2143		
3.00	42		7.5000	7.5000	
2.00	42			8.7381	
4.00	42			8.9048	
1.00	42				22.9762
Sig.		.932	.136	.927	1.000

Table 5:Polymorphneuclear Neutrophils

3.5. Presence of fibroblast in periodontitis

The positive control (antibody and *P.gingivalis)* group exhibited the minimum amount of fibroblast when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited more fibroblasts (8.92 % and 27.47% respectively), when compared with positive control (antibody and *P.gingivalis)* group. The mice in the *P.gingivalis*.only and antibody only groups

showed significantly more fibroblasts (16.17% and 33.72%) when compared with positive control group (antibody and *P.gingivalis)*. The mice treated with Kava treatment only and Kava preventive exhibited significantly more fibroblast compared to the positive control group (antibody and *P.gingivalis*) (27.41% and 33.22% respectively).

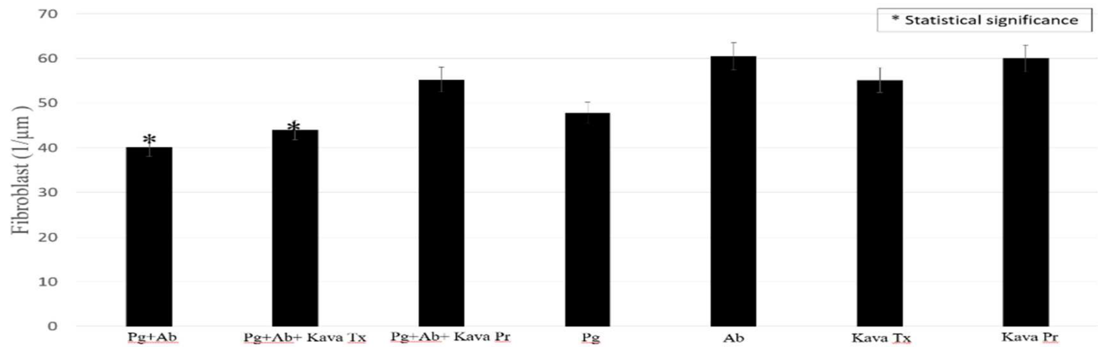

Figure 10:Fibroblast Count.

AllGroups	N	Subset for alpha = 0.05		
		1	2	3
1.00	42	40.0952		
2.00	42	44.0238	44.0238	
4.00	42		47.8333	
6.00	42			55.1905
3.00	42			55.2857
7.00	42			60.0476
5.00	42			60.5000
Sig.		.586	.621	.220

Table 6:Fibroblast Count.

3.6. Presence of Osteoclastic Activity

Osteoclastic activity showed a similar trend of reduction between the treatment group and the no-treatment group, however, this was not statistically significant. The positive control (antibody and *P.gingivalis)* group exhibited the highest amount of osteoclastic activity when compared with other groups. Mice in the antibody, *P.gingivalis* and Kava compound treatment group and the antibody, *P.gingivalis* and Kava compound prevention group exhibited less osteoclastic activity (56.3% and 25.5% respectively), when compared with positive control (antibody and *P.gingivalis)* group. The mice in the *P.gingivalis*.only and antibody only groups showed less osteoclastic activity (33.8% and 7%) when compared with positive control group (antibody and *P.gingivalis)*. The mice treated with Kava treatment only and Kava preventive only exhibited less osteoclastic activity compared to the positive control group (antibody and *P.gingivalis*) (46.4% and 46.4% respectively) .

CHAPTER FOUR

DISCUSSION

In this study, *P. gingivalis*-oral gavage was successfully used to induce experimental periodontitis in mice. The results demonstrated site-specific, time-dependent epithelial downgrowth, alveolar bone loss, and inflammation in this model. The use of histomorphometric and morphometric analysis for charting the progression of periodontitis in this model was also confirmed. Both methods yielded comparable results for detecting alveolar bone loss. Moreover, the osteoclast activity observed in

this study was consistent with the progression of an inflammatory front as demonstrated in earlier studies using a monkey model [65].

Several methods have been presented in the literature for inoculating periodontal pathogens into the oral cavity of experimental animals, including diet, ligature, and oral infection by gavage [61, 66, 67]. Most methods demonstrated an obvious periodontal breakdown. However, the *P. gingivalis*-oral gavage approach provides a simple and direct method to deliver enough bacteria into the murine gingival sulcus to colonize and to initiate pathogenesis of periodontitis. In this study, the oral gavage was administered every day to maintain a constant specific bacterial infection. After infection, *P. gingivalis* was only recovered in the Kava treatment mice (14 days) and not recovered in untreated control mice. This data suggested *P. gingivalis*-oral gavage can successfully deliver *P. gingivalis* into mouse mouth and thereby contribute to the induction of experimental periodontitis. For all these reasons, we elected to use *P. gingivalis*-oral gavage to promote alveolar bone loss in hopes of documenting a reproducible model of murine experimental periodontal disease approximating human periodontal disease.

Inflammation was clearly induced in the infected group, as evidenced by the infiltration of leukocytes, especially PMNs, 14 days after administration of P. gingivalis- oral gavage. This is consistent with observations made in ferrets, dogs, rats, monkeys, and humans ([6, 68-71]. Periodontal tissue destruction increased with increasing experimental time and corresponded to obvious inflammatory infiltration. The number of osteoclasts around the alveolar bone surface also increased after infection. The inflammatory lesion was associated with numerous osteoclasts,

resulting in alveolar bone loss. Additionally, bone at the alveolar bone crest was rapidly resorbed by 14 days after the administration of *P. gingivalis*-oral gavage, findings that are consistent with the current literature in other models [72]. Moreover, the periodontal destruction with bone resorption and epithelial downgrowth observed in the model used in this study was in agreement with data in monkeys, rodents, dogs, and humans [71, 73-75].The present finding indicates that the methods used in this study could cause rapidly destructive experimental periodontitis in mice.

Histological analysis is an accurate and widely accepted technique for measuring alveolar bone loss [76-78]. Although necessary tissue preparation steps require substantial effort, histological measurements can simultaneously provide alveolar bone loss quantification and other histology/immunohistochemistry measurements. Morphometric techniques are also widely used in measuring the alveolar bone loss in animal models of periodontitis [62, 79]. This method is precise in measuring horizontal bone loss and is reproducible [61, 64]. However, a disadvantage of morphometry is the need to remove soft tissues overlying the bone prior to making measurements

In this study, evidence is presented that mice infected with *P. gingivalis* suffer severe inflammation and periodontal tissue breakdown leading to alveolar bone loss compatible with other known animal models of periodontal disease. The histomorphometric analytical approach is recommended because both soft and hard tissue is captured.

The results of this study demonstrate that 241, a modified Kava in periodontitis resulted in a reduction of periodontal breakdown, inflammatory cell infiltrate,

osteoclastogenesis, and fibroblasts. These observations are consistent with a previous report using Kavain shows significantly reduced experimental

periodontal bone loss [80].

An additional aim of this study was to evaluate the role 241, a modified Kava in the management of inflammatory lesions, given that an important feature of periodontitis is the inflammatory lesion preceding bone loss. The use of well-defined boxes helped capture the progression of inflammatory cell counts which showed gradual decrease, while steady increase for fibroblasts from superficial to deeper structures respectively. In all three regions, 241, a modified Kava significantly reduced the inflammatory infiltrate equally affecting PMN and mononuclear cells. These results are consistent with a previous report using Kavain significantly reduced inflammatory cell count,[80]. Altogether the present data support a beneficial role of these compounds in reduction of inflammation.

CONCLUSION

The distinct roles of 241, modified Kava compound to involve the pathogenesis of periodontitis and to the direct regulation of the inflammation were determined by using a *P-gingivalis*-induced mouse periodontitis model. The high correlation of inflammation *vs.* osteoclastogenesis, inflammation *vs.* periodontal tissue breakdown, and osteoclastogenesis *vs.* alveolar bone loss were confirmed. Administration of 241, modified Kava compound in periodontitis resulted in a reduction of periodontal breakdown, inflammatory cell infiltrate, osteoclastogenesis, and increase for fibroblasts. Results of this study demonstrate that in a murine model,

P. gingivalis induced periodontitis can be treated by 241. In addition, the experimental model demonstrated the use of morphometric and histomorphometric analyses to accurately quantify alveolar bone loss. Further modification of Kava could yield a more effective and safer therapeutic compound in the treatment of periodontal inflammation and bone loss.

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