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Kava-241 Reduced Periodontal Destruction in a Collagen Antibody Primed *Porphyromonas gingivalis* Model of Periodontitis

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Abstract

Aim—The aim of this study was to evaluate the effect of Kava-241, an optimized *Piper methysticum* Kava compound, on periodontal destruction in a collagen antibody primed oral gavage model of periodontitis.

Methods—Experimental periodontitis was induced by oral gavage of *Porphyromonas gingivalis* (*P.gingivalis*) + type-II collagen antibody (AB) in mice during 15 days. Mice were treated with Kava-241 concomitantly or prior to *P.gingivalis* gavage and compared to untreated mice. Comprehensive histomorphometric analyses were performed.

Results—Oral gavage with *P.gingivalis* induced mild epithelial downgrowth and alveolar bone loss while oral gavage with additional AB priming had greater tissular destruction in comparison to gavage alone ($p < 0.05$). Kava-241 treatment significantly ($p < 0.05$) reduced epithelial downgrowth (72%) and alveolar bone loss (36%) in *P.gingivalis*+AB group. This Kava-241 effect was associated to a reduction of inflammatory cells counts within soft tissues and an increase of fibroblasts ($p < 0.05$).

Conclusion—Priming with type-II collagen antibody with oral gavage is a fast and reproducible model of periodontal destruction adequate for the evaluation of novel therapeutics. The effect of Kava-241 shows promise in the prevention and treatment of inflammation and alveolar bone loss associated with periodontitis. Further experiments are required to determine molecular pathways targeted by this therapeutic agent.

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Keywords

periodontitis; oral gavage; kavain; alveolar bone loss; inflammation

Introduction

Periodontitis is a chronic inflammatory disease with a high prevalence, with over 47% of the US adult population is affected (Eke et al. 2012). Bacteria from supra and sub-gingival biofilms are considered key etiological factors (Hajishengallis & Lamont 2012). Among them, *Porphyromonas gingivalis* (*P.gingivalis*) is a well-documented pathogen recognized as a predominant contributor to periodontitis (Hajishengallis 2009) able to induce disease in established animal models that have demonstrated similarities to human pathology (Graves et al. 2008; Graves et al. 2012). Recognition of bacterial surface antigens by Toll-like receptors (TLRs), TLR2 and TLR4 specifically, induces secretion of a wide range of proinflammatory cytokines such as interleukin-1 β (IL-1 β), IL-6, IL-11, IL-17, and tumor necrosis factor alpha (TNF- α) from resident or recruited inflammatory cells (Taylor 2010). This sustained inflammation is also associated to the recruitment and activation of osteoclasts through the secretion of Receptor Activator of Nuclear Factor κ B Ligand (RANKL) contributing to alveolar bone destruction (Lapérine et al. 2016) and periodontitis worsening.

Several studies demonstrated a bidirectional link between periodontitis and arthritis which led to the development of investigational murine models mainly based on type-II collagen antibody injection (AB) (Tang & Amar 2016). Interestingly, some have proposed an impact of type-II collagen injections on alveolar bone loss (Corrêa et al. 2017). However, none have evaluated the impact of AB priming on the periodontal destruction initiated by *P.gingivalis*.

Non-surgical therapy is considered the gold-standard of periodontal treatment. It consists of oral hygiene instruction and scaling and root planing aiming at disturbing bacterial biofilms. Despite good results and improvement of clinical parameters, some systemic or local risk factors may impair treatment response (Bouchard et al. 2016). For example, some susceptible sites, such as deep pockets or furcations of multi-rooted teeth, remain difficult to treat and may require surgical intervention (Heitz-Mayfield & Lang 2013). To improve non-surgical treatment outcomes and to reduce the need of surgery, several additive therapies have been developed such as the use of antiseptics, antibiotics, probiotics, or anti-inflammatory drugs (Mombelli et al. 2011; Martin-Cabezas et al. 2016; Agossa et al. 2016). However, neither are definitive in halting periodontitis and some are associated with serious side effects or poor patient compliance. Thus, there is a need to develop novel pharmaceutical alternatives of synthetic or natural origins with improved success rate for periodontal therapy (Alani & Seymour 2014).

Kava, a compound extracted from the *Piper methysticum* plant, has been suggested to be responsible for the low incidence of cancer in natives of Polynesia and Micronesia (Singh 1992). It has been credited for its relaxing, sedative, anxiolytic, antiarthritic and anti-inflammatory properties (Shimoda et al. 2012; Teschke & Lebot 2011). Kava mainly constitutes of kavalactones and, in smaller amounts, flavokavain (Folmer et al. 2006).

Recently, it was demonstrated that kavain derived compounds are able to inhibit TNF- α secretion *in vitro* and *in vivo* through ERK/LITAF related pathway modulation (Pollastri et al. 2009; Tang & Amar 2016). In its natural state, kava extract has been reported to cause adverse effects including hepatic, neurologic, and dermatological toxicity (Gounder 2006). Therefore, research has focused on identification of active compounds with low risk of toxicity (Pollastri et al. 2009), high solubility and stability *in vivo*, and a high rate of cell permeability. We reported recently the moderate but beneficial effect of kava on a conventional oral gavage model of periodontitis (Yuan et al., 2011). In light of these findings the present aimed at evaluating the impact of AB injection on periodontal destruction associated to *P.gingivalis* gavage and, secondarily, to test whether Kava-241 is effective in prevention or treatment of advanced periodontal inflammation and related alveolar bone destruction.

Materials and Methods

Kava-241 compound

Our objective in medicinal chemistry and biochemical screening efforts was to optimize the structure of the natural product kavain known for its potential anti-inflammatory properties. Our medicinal chemistry effort generated a kava analogue with optimized activity. Further optimization based on the current biological screening was performed. The synthesis of the unsaturated enone of the Kavain analogue is illustrated in Figure 1. The synthetic approach is based on the use of a highly enolizable cyclic 1,3-diketone because of their low PKa in O-acylation of the intermediate enol. Accordingly, treatment of a methylene chloride solution of cyclic 1,3-diketone with benzoyl chloride in the presence of pyridine efficiently provides the O-acylated enol derivative.

Cell culture, LPS stimulation and treatment procedures

RAW 264.7 cells (TIB-71, ATCC, Manassas, VA, USA) were cultured in RPMI 1640 medium (Life Technologies, NY, NY, USA) with 10% FBS at 37° in 5% CO₂ atmosphere. According to experimental design, cells were stimulated by 0.1 $\mu\text{g}\cdot\text{ml}^{-1}$ *E.coli*-LPS and/or treated with 100 to 300 $\mu\text{g}\cdot\text{ml}^{-1}$ kavain (AvaChem Scientific, San Antonio, TX, USA) or synthesized Kava-241.

Bacterial culture

The W83 *P. gingivalis* strain was used to induce periodontitis (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 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO), menadione (1 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO) and sodium bicarbonate (420 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, St. Louis, MO) in an anaerobic chamber with 85% N₂, 10% H₂, and 5% CO₂ at 37°C.

TNF- α ELISA

The supernatants from treated cells were subjected to ELISA for the detection of TNF- α concentration with an Invitrogen kit (KMC3011, ThermoFisher, Dublin, OH, USA). ELISA immunoreactivity was quantified using a microplate reader (Bio-Rad, Hercules, CA, USA).

Cytotoxicity assay

Cell viability was measured using a Cell Counting Kit-8 (CCK-8) (Sigma-Aldrich, St. Louis, MO). Briefly, cells were treated with Kava-241 ($300 \mu\text{g}\cdot\text{ml}^{-1}$) for 4h. CCK-8 was then added into the cell's medium for an incubation time of 2 hours, and absorbance was calculated at 450 nm to determine the number of viable cells.

Animals, experimental periodontitis and treatment procedures

Forty-nine six-week-old, pathogen-free DBA1/BO male mice (Taconic Farm, Rensselaer, NY) were used in this study. Mice were fed sterile food and distilled water *ad libitum*. All procedures were approved by local ethical committee. Experimental periodontitis was induced by delivery of 5×10^8 CFU *P.gingivalis* through oral gavage as described previously (Yuan et al. 2013) during 15 days. Mice were randomly assigned to one of seven groups (Figure 2): a *P.gingivalis*-infected and AB injected group; a *P.gingivalis*-infected group, AB injected and Kava-241 treatment group; a *P.gingivalis*-infected, AB injected and Kava-241 prevention group; a *P.gingivalis*-infected only group; AB only group; Kava-241 treatment only group; and a Kava-241 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., Armitville, 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 isophurane and were inoculated with an average 5×10^8 colony-forming units of *P.gingivalis* in 100 μl phosphate-buffered saline with 2% carboxymethylcellulose (Sigma-Aldrich, St Louis, MO, USA) administered by oral gavage for 15 days (1 inoculation/day). The non-*P.gingivalis*-infected group was inoculated with 2% carboxymethylcellulose only (Sigma-Aldrich, St Louis, MO, USA). Mice in the *P.gingivalis* + AB group were injected intraperitoneally with ArthritoMab (CIA-MAB-2C, MD Bioproducts) on day-1 (7 mg/mouse) and day-5 (4mg/ mouse). Mice in the Kava-241 treatment group were intraperitoneally injected with 40 mg/kg of the Kava-241 on days 5, 8, 11 and 14 of the experiment. Mice in the Kava-241 prevention groups were intraperitoneally injected with two doses (40 mg/kg) of Kava-241. In this group, the first dose was administered three and one days before the *P.gingivalis* oral gavage administration followed by injection at 2, 6, 9, and 13 days.

Tissue preparation

After 15 days of experimentation, mice were euthanized by CO₂ inhalation. Right side of palatal bone and intact surrounding tissue was fixed with 4% freshly prepared paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS (pH 7.2) for 24h at 4°C. Following fixation, 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 solution (Sigma-Aldrich, St. Louis, MO) for 14 days at 4°C. 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 and stained with hematoxylin (Fisher Scientific, Pit, IL) – eosin (ACROS Organics, Morris Plains, NJ).

Left side of mice skulls were mechanically defleshed after 15 minutes of treatment in boiling water, washed with PBS, and then exposed overnight in 3% hydrogen peroxide. Then, they were washed with PBS three times for 5 minutes, immersed in bleach for 1 minutes, washed again with PBS three times for 5 minutes, and then dried at 37°C for 1 hour. Skulls were stained at room temperature with 1% methylene blue (Sigma-Aldrich, St. Louis, MO) for 1 minute and dried for 30 minute at 37°C.

Histomorphometric and morphometric analysis

Palatal root and furcation of first and second molars were considered. Measurements included extension of the apical migration of epithelium (epithelial downgrowth) and bone resorption on hematoxylin-eosin (HE)-stained sections from images captured at 100× magnification using an image analysis software (Image-Pro Plus Version 5.0, Media Cybernetics, Silver Spring, MD). Epithelial downgrowth and bone loss measurements were performed at two sites distal of M1 and mesial of M2. Epithelial downgrowth was defined by measuring the distance from the cemento-enamel junction (CEJ) to the apical extent of the junctional epithelium. Alveolar bone loss 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 distances (µm). Alveolar bone loss was confirmed by morphological measurement on defleshed samples as previously described (Klausen et al. 1989; Yuan et al. 2011).

Inflammatory cell histomorphometric analysis

A minimum of 6 fields from each interdental area (from CEJ to root apex of the second molar) were analyzed. Analysis was performed 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 (polymorphonuclear neutrophils (PMN), mononuclear leukocytes and fibroblasts) were counted manually from images captured at 400× magnification on H&E-stained sections as described previously (Yuan et al. 2011). The data were then reported as the number of each type of cell per µm².

Statistical Analysis

All measurements were performed blinded 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. An ANOVA (one-way) followed by Tukey post-hoc analysis was performed for multiple comparison. A *p*-value <0.05 was considered to be statistically significant. All *in vitro* experiments have been performed at least in triplicate.

Results

In vitro evaluation of Kava-241 anti-inflammatory properties and cytotoxicity

The cytotoxic and anti-inflammatory effects of Kava and Kava-241 was evaluated. Cell viability and LPS-induced TNF-α secretion of RAW cells exposed to either kava, Kava-241 or left untreated have been measured. Kava-241 and kava reduced significantly LPS-induced TNF-α secretion (85% and 72% respectively) after 1h (Figure 3A) relative to untreated cells.

The anti-TNF- α effect obtained by Kava-241 was significantly greater than the one obtained with kava parent molecule ($p < 0.05$) while the same trend was observed after 4h of treatment. Regarding cytotoxicity, Kava-241 treatment was associated to reduced cell death than kava treatment ($p < 0.05$) (Figure 3B). These results emphasized the safe usage of Kava-241 as a potential therapeutics.

Periodontal destruction associated to *P.gingivalis* is increased by collagen-AB injection

In an attempt to develop a new model of shorter but more advanced experimental periodontitis, injections of AB have been performed in the early phase of *P.gingivalis* gavage. Epithelial downgrowth was measured by the distance of apical migration of epithelial attachment relative to the CEJ (Figure 4). *P.gingivalis* + AB procedure leads to greater epithelial downgrowth and alveolar bone loss than *P.gingivalis* gavage model ($p < 0.05$) (Figures 4A and 4B). Interestingly, injection of AB alone did not induce periodontal destruction, neither at the soft tissue level (Figure 5A) nor at the alveolar bone level (Figure 5B) where *P.gingivalis* + AB demonstrated the greatest alveolar bone loss compared to other groups (Figure 6) highlighting the synergism between *P.gingivalis* gavage and AB injections. Negative control groups which received only *P.gingivalis* or only AB also demonstrated significantly less alveolar bone loss (50.72% and 36.5% respectively).

Effect of Kava-241 treatment on epithelial downgrowth and alveolar bone loss

To evaluate the therapeutic effect of Kava-241, the *P.gingivalis* + AB model was selected as it displays more severe periodontal destruction and allows more efficient evaluation of potential therapeutic effects of a drug. Both Kava-241 approaches, either treatment or prevention, significantly reduced epithelial downgrowth. Compared to the *P.gingivalis* + AB group, the Kava-241 treatment and Kava-241 preventive groups demonstrated 72.7% and 84% less epithelial downgrowth respectively ($p < 0.05$) (Figures 4 and 5A). The same trend was observed regarding alveolar bone loss as quantified by morphometric analysis (Figures 4, 5B and 6). Both Kava-241 treatment and prevention reduced alveolar bone loss (by 36.98% and 39.05% respectively) in comparison with *P.gingivalis* + AB group ($p < 0.05$).

Effect of treatments on inflammatory cell infiltrate

The number of inflammatory cells including PMN, mononuclear leukocytes and connective tissue fibroblasts was evaluated in each group. *P.gingivalis* + AB group exhibited the highest PMN and mononuclear leukocyte cell counts per μm^2 , and the least number of fibroblasts per μm^2 compared to the other groups (Figure 7). Mice that received Kava-241 treatment or preventive treatment demonstrated 61.96% and 67.35% fewer PMN's respectively compared to *P.gingivalis* + AB group. The untreated groups that received only *P.gingivalis* or AB demonstrated 61.24% and 81.65% fewer PMN's compared to positive controls ($p < 0.05$). The same results were observed regarding mononuclear leukocytes as Kava-241 treatment and Kava-241 preventive treatment demonstrated 41.58% and 62.01% fewer mononuclear leukocytes respectively. Conversely, the number of fibroblasts increased in Kava-241 groups (8.92% in Kava-241 treatment group and 27.47% in preventive treatment group).

Discussion

This study demonstrated the relevance of a new model of experimental periodontitis occurring in a shorter period of time (10-14 days) while producing advanced periodontal bone loss. and the potential therapeutic effects of a kavain derived compound, Kava-241, used as a treatment or in a preventive manner. To induce epithelial downgrowth and alveolar bone loss, several models of experimental periodontitis can be used including oral gavage or use of infected ligatures that can display differential trend of tissular destruction (Graves et al. 2012). The oral gavage model is a well-described model of experimental periodontitis. Here, an optimized oral *P.gingivalis* gavage model, primed by AB injections, was used to evaluate the Kava-241 therapeutic effect on induced epithelial downgrowth, alveolar bone loss as well as inflammatory cells recruitment.

Herein, the injections of AB concomitant to administration of *P.gingivalis* through oral gavage synergistically increased the epithelial downgrowth and alveolar bone loss in comparison to *P.gingivalis* oral gavage alone. This approach induced periodontal lesions in a shorter period of time compared with *P.gingivalis* gavage alone. The magnitude of the periodontal destruction allowed a significant effect size amenable for the evaluation of novel anti-inflammatory therapeutics with minimal risk of complications such as increased period of disease induction or increased risk of mouse death that may occur in case of surgical approach or repeated anesthesia. AB is a well described method to induce arthritis in mice (Sim et al. 2016; Tang & Amar 2016). Rheumatoid arthritis and periodontitis shared several aetiopathogenic mechanisms related to the immune host response and to cytokines secretion (Culshaw et al. 2011). Recently, in a rat model of experimental periodontitis, the bidirectional interaction between these diseases was illustrated by an increase of alveolar bone loss in rats affected by both periodontitis and arthritis induced by type-2 collagen injection (Corrêa et al. 2017). Underlying mechanisms explaining the increase of periodontal destruction are still unknown, however recent reports suggested that arthritis-related cytokines IL-1, TNF- α or IL-17 known for their role in OPG/RANKL expression and bone homeostasis (Takahashi et al. 2014; Chen et al. 2014) as potential contributor also to periodontal tissue destruction (Corrêa et al. 2017). Therefore, this new model of periodontitis induction may be useful to evaluate drug effect on severe form of murine periodontitis.

Several therapeutic agents targeting key inflammatory markers such as COX-2 and TNF- α have already been proposed to treat periodontitis and to promote regeneration of *periodontium* such as NSAIDs or omega-3 fatty acid (Morand et al. 2016; Kesavalu et al. 2007). However, none is considered to be a key element of the periodontal treatment. In this study, we evaluated the impact of a specific kava derived compound on periodontal destruction induced by *P.gingivalis*. Kava-241 has been selected to be tested *in vivo* based on its *in vitro* properties. Indeed, we showed that Kava-241 had a significantly less cell cytotoxic effect than kava, a possible reason behind hepatotoxicity (Olsen et al. 2011; Martin et al. 2014). These properties may promote the use of Kava-241 in the safe management of periodontal diseases. Furthermore, Kava-241 cell treatment reduced more efficiently LPS-induced TNF- α than parent kava making it a good candidate as a therapeutic agent and clearly optimizing its effect compared to parent kava. Anti-microbial properties of kava have

also been proposed, however, our *in vitro* evaluation did not show significant effect of Kava-241 on *P.gingivalis* growth (data not shown).

In vivo, Kava-241 treatment resulted in a reduction of periodontal breakdown, inflammatory cell infiltrate, and increased the number of fibroblasts. These observations are consistent with findings of our prior study where parent kava compound was tested in an experimental periodontitis mouse model and was shown to reduce inflammatory infiltrate measured by PMN and mononuclear cells counts and increased fibroblasts (Yuan et al. 2011). Regarding the specific effects of Kava-241 on the cell count, our hypothesis was that the Kava-241 would also reduce the inflammation and promote wound healing resulting in an increase of connective tissues. Indeed, Kava-241 promoted greater reduction of periodontal inflammation (decrease of PMN, leukocytes, key mediators of inflammation and immune response) and improved significantly parameters of wound healing (i.e fibroblast counts) than parent Kava compound. However, further experimentation is required to determine the cell-specific effects of Kava-241.

To date, only a small number of studies have evaluated the potential therapeutic interest of kavain or related compounds in the context of infectious or inflammatory diseases (World Health Organization 2007). Therefore, the precise mechanisms activated or inhibited by kava are not very well described. However, some recent data suggest that kavain or other derived molecules, such as flavokavains, act on molecular pathways that are activated by lipopolysaccharide (LPS) such as NF- κ B, AP-1 and ERK (Tang & Amar 2016; Kwon et al. 2013). *In vitro*, it was demonstrated in macrophages stimulated by LPS, that flavokavain-A inhibits iNOS and COX-2 expression (Kwon et al. 2013). iNOS is a free radical associated with the pathogenesis of several inflammatory diseases and its expression is increased in periodontitis tissues indicating a possible involvement in tissular destruction related to the inflammation (Shaker et al. 2013). COX-2 is a key enzyme associated to inflammatory response and to PGE₂ synthesis, a key mediator of host-immune response associated with periodontitis (Schaefer et al. 2010). It has been also demonstrated in a rat model of experimental periodontitis that *P.gingivalis* induces COX-2 expression emphasizing the role of this pathway in periodontal tissue destruction (Oliveira et al. 2008).

In another study, kavain was able to inhibit TNF- α secretion induced by LPS (Tang & Amar 2016). TNF- α is a hallmark of periodontal inflammation and its increase has been documented in periodontitis (Özer Yücel et al. 2015) and in response to infection with periodontal pathogens including *P.gingivalis* and its virulence factors in several cell types (Kocgozlu et al. 2009; Huck et al. 2017). Interestingly, Folmer *et al.* showed that Kava compounds are able to inhibit TNF- α induced NF- κ B activation, a key transcription factor involved in immune and inflammatory response (Folmer et al. 2006). NF- κ B activation has been observed in response to periodontal infection and recognition of *P.gingivalis* through Toll-like Receptors (TLRs) and activation of NF- κ B is associated to osteoclastogenesis modulation (Zhang et al. 2011). This pathway, targeted by Kava, may explain the decrease of alveolar bone loss observed in our model. *In vivo*, the anti-inflammatory properties of Kava were measured in an induced inflammatory paw model where its injection reduced from 90% the swelling induced by *E.Coli*-LPS (Tang & Amar 2016) demonstrating the promising anti-inflammatory properties of Kava.

Conclusion

Our results provide evidence that injection of AB concomitant with *P.gingivalis* oral gavage aggravates periodontal destruction and may be used as a faster and more reliable disease model. Furthermore, Kava-241 compound could effectively reduce *P.gingivalis* associated periodontal destruction suggesting its use as a promising therapeutic agent of periodontal diseases in the future. However, some future studies should focus on determining the most effective dosing regimen and its full mode of action in comprehensive structure-function experiments especially regarding the impact of Kava-241 on bone remodeling.

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Clinical relevance

Scientific rationale for study

There are currently no definitive therapeutic approaches for treatment of periodontitis. We sought to demonstrate the efficacy of a novel anti-inflammatory agent in the treatment of periodontitis in a murine model.

Principal findings

The present study confirmed that administration of Kava-241 and optimized Kava compound in an advanced periodontitis murine model, resulted in a reduced tissue breakdown, decreased inflammatory cell infiltrate and alveolar bone loss and increased of fibroblasts.

Practical implications

Identification of novel therapeutics for periodontitis can be useful to improve our success rate in the treatment and prevention of periodontal diseases.

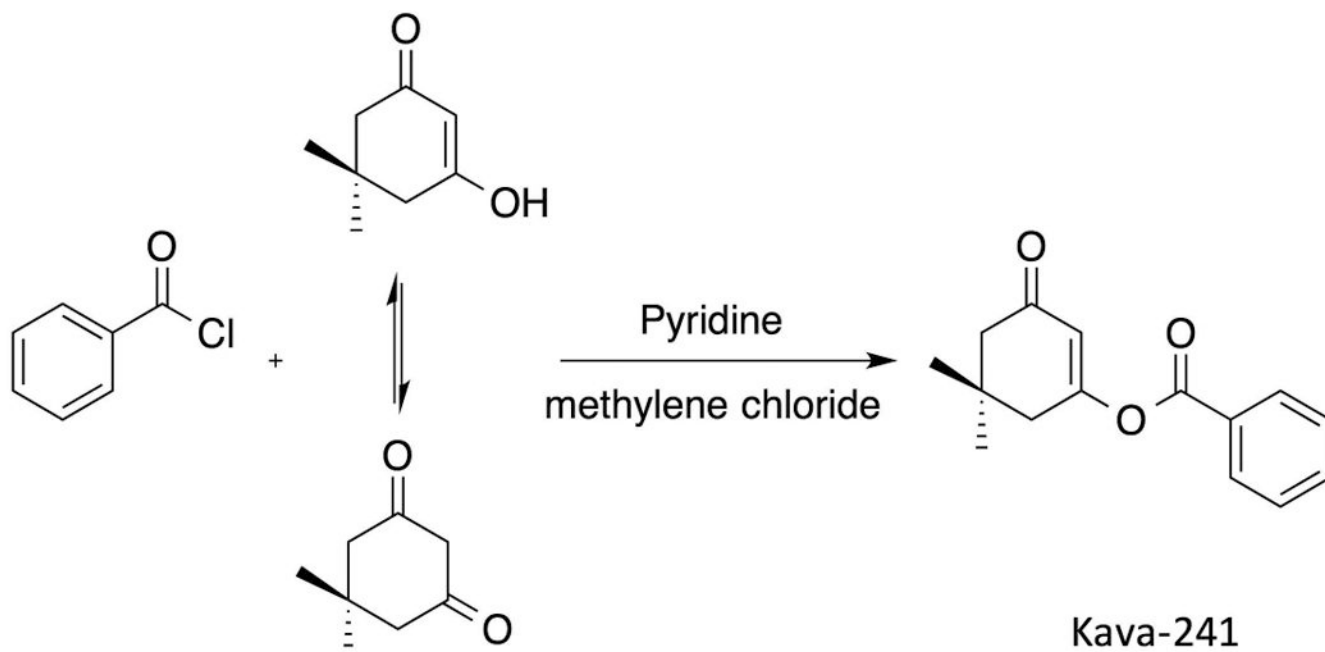


Figure 1. Synthesis and structural characteristic of Kava-241

The synthetic approach is based on the use of a highly enolizable cyclic 1,3-diketone that because of their low PKa smoothly participate in O-acylation of the intermediate enol. Accordingly, treatment of a methylene chloride solution of cyclic 1,3-diketone with benzoyl chloride in the presence of pyridine efficiently provides the O-acylated enol derivative Kava-241.

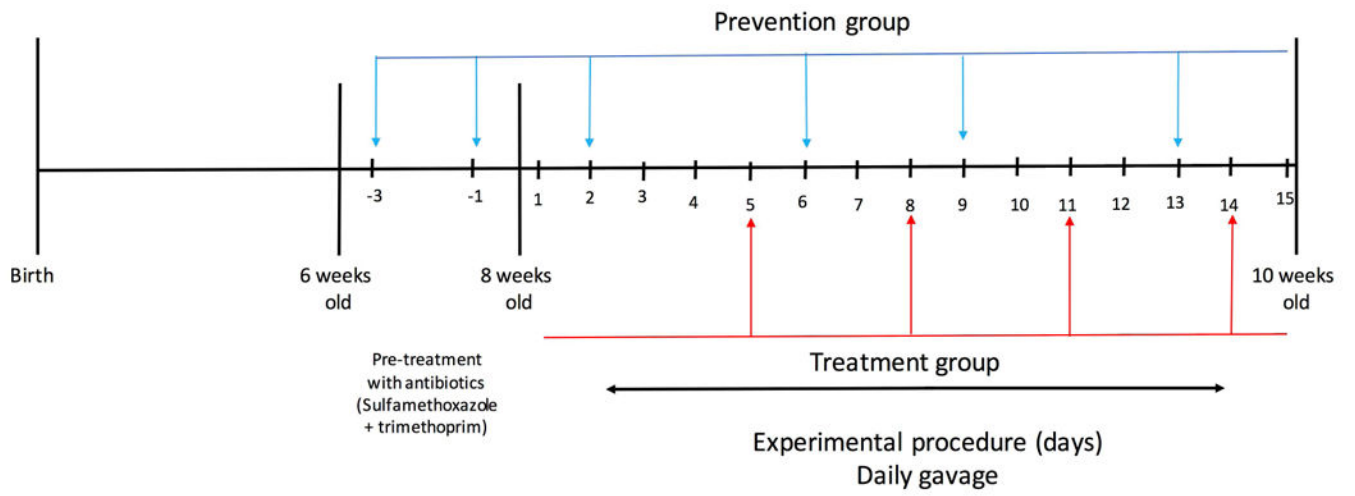


Figure 2. Flow-chart of the study

Blue arrows indicate days of injection of Kava-241 in the preventive group. Red arrows indicate days of injection of Kava-241 in the treatment group. In all groups, *P.gingivalis* oral gavage was performed daily.

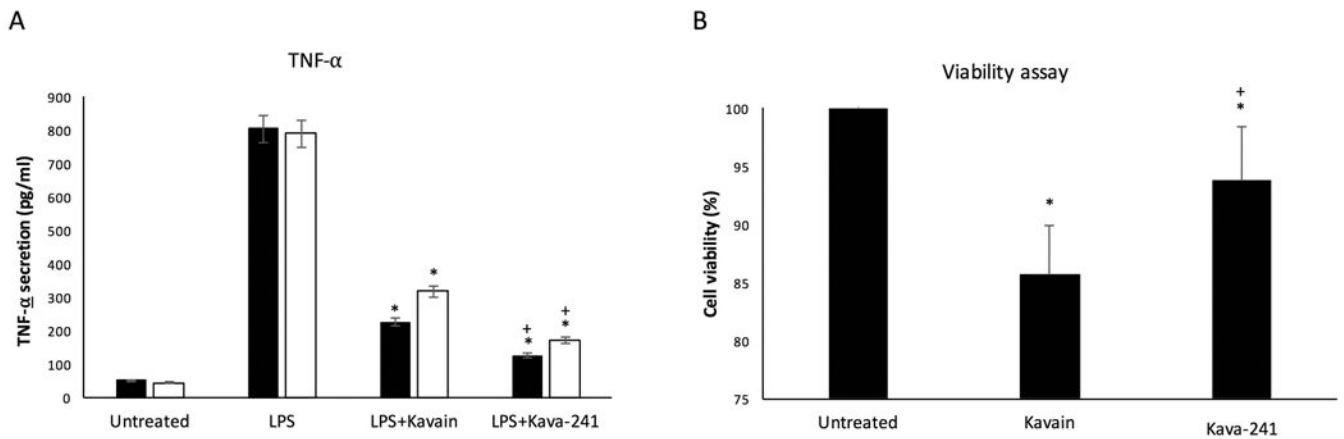


Figure 3. TNF- α secretion and cell viability in cells treated with Kavain and Kava-241

A. TNF- α secretion: RAW 264.7 cells were stimulated with $0.1 \mu\text{g}\cdot\text{ml}^{-1}$ *E.coli*-LPS and treated with Kavain or Kava-241 for 1 (white columns) to 4h (black columns). TNF- α secretion was measured from supernatants (*: $p<0.05$ vs LPS; +: $p<0.05$ vs Kavain). **B.**

Cytotoxicity: RAW 264.7 cells were treated with Kavain or Kava-241 ($300 \mu\text{g}\cdot\text{ml}^{-1}$) for 4h. Cell viability has been evaluated using CCK8 kit. Results are expressed in percentage of viable cell according to untreated control (*: $p<0.05$ vs LPS; +: $p<0.05$ vs Kavain). All experiments have been performed at least in triplicate.

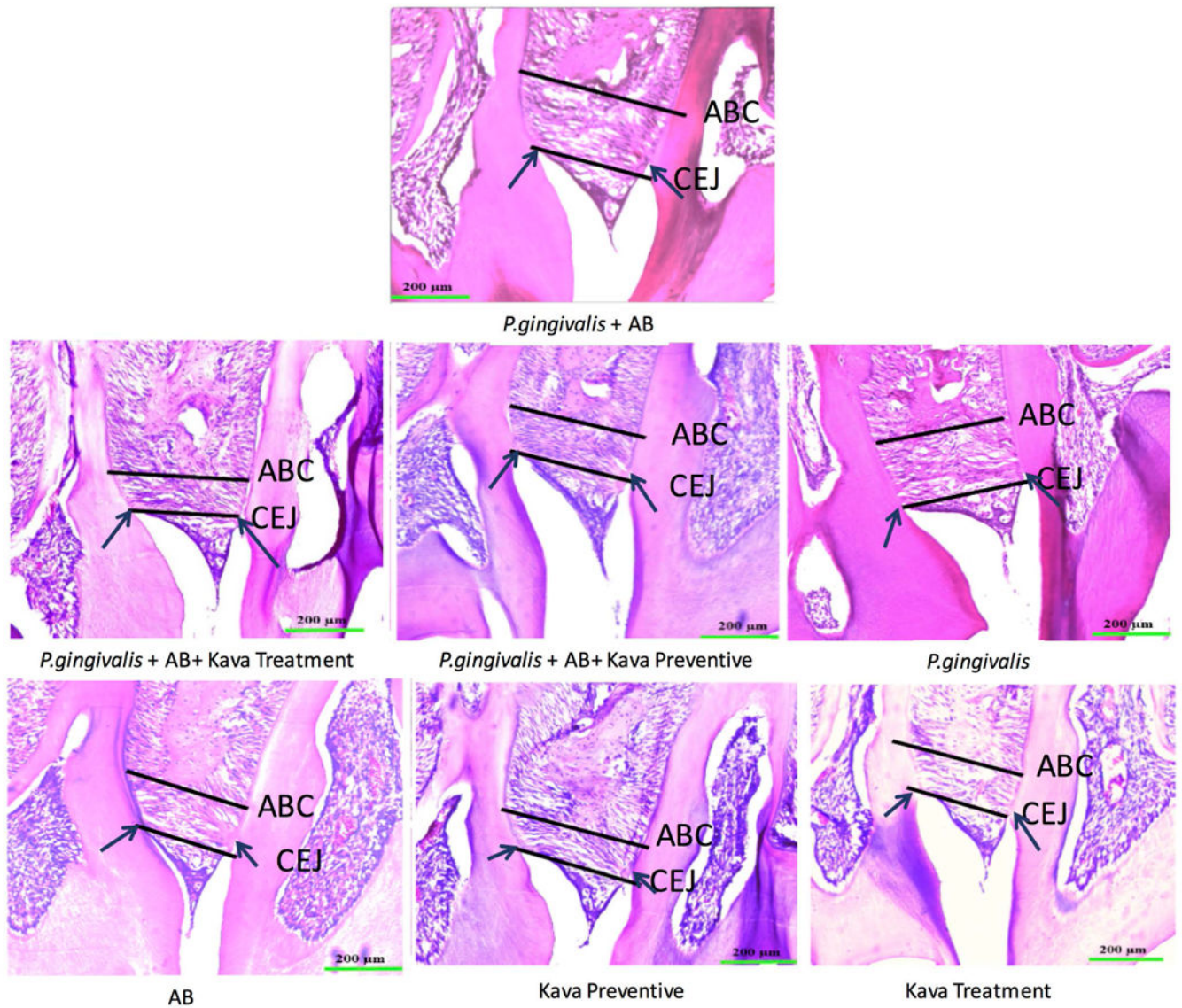


Figure 4. Histomorphometric analysis

Representatives of histological section from each group. Images were captured at 100× magnification after hematoxylin-eosin staining. Lines represent alveolar bone crest (ABC) and cemento-enamel junction (CEJ) levels. Black arrows show apical extent of the epithelial downgrowth.

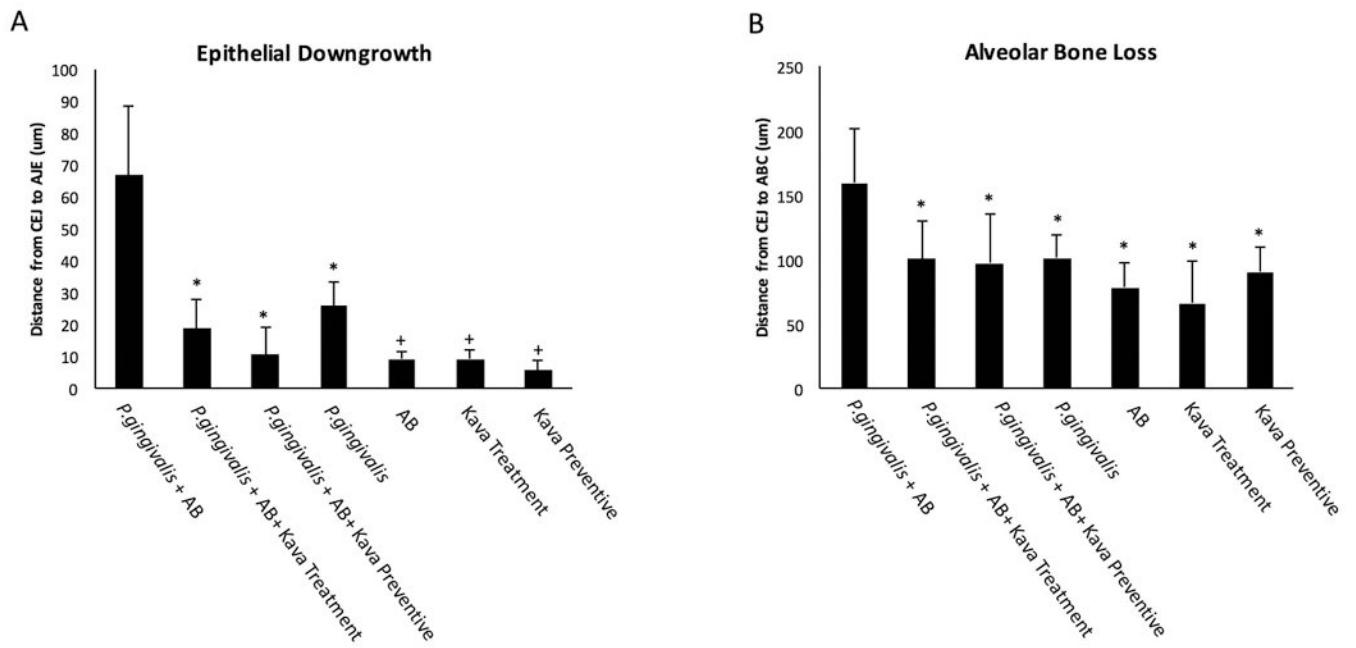


Figure 5. Epithelial downgrowth (A) and alveolar bone loss (B)

Epithelial downgrowth was defined by measuring the distance from the cemento-enamel junction (CEJ) to the apical extent of the junctional epithelium. Alveolar bone loss was measured as the distance between the CEJ and the alveolar bone crest (ABC). Results are expressed in μm (*: $p < 0.05$ vs *P.gingivalis* + AB; +: $p < 0.05$ vs *P.gingivalis*)

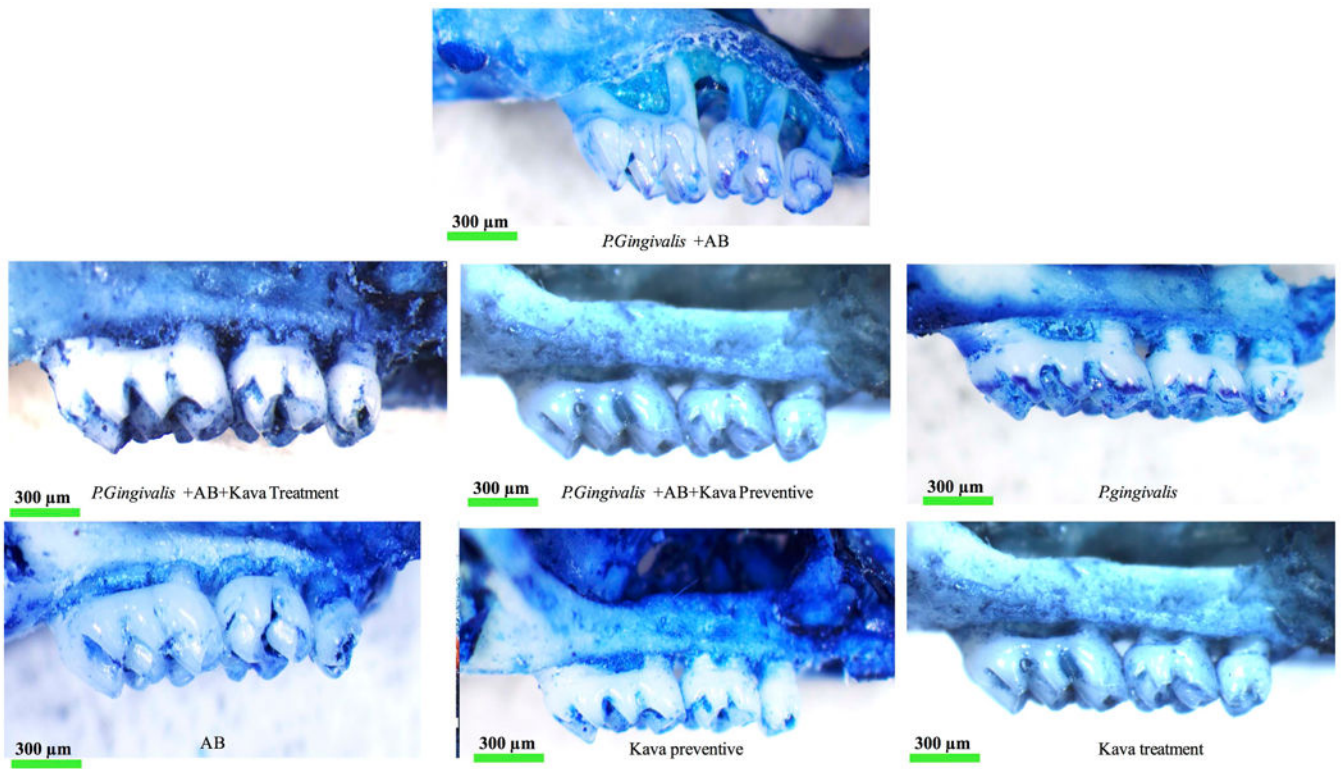


Figure 6. Morphometric analysis of alveolar bone loss
Representatives of the alveolar bone loss in each group. Images were captured at 3.5× after methylene blue staining.

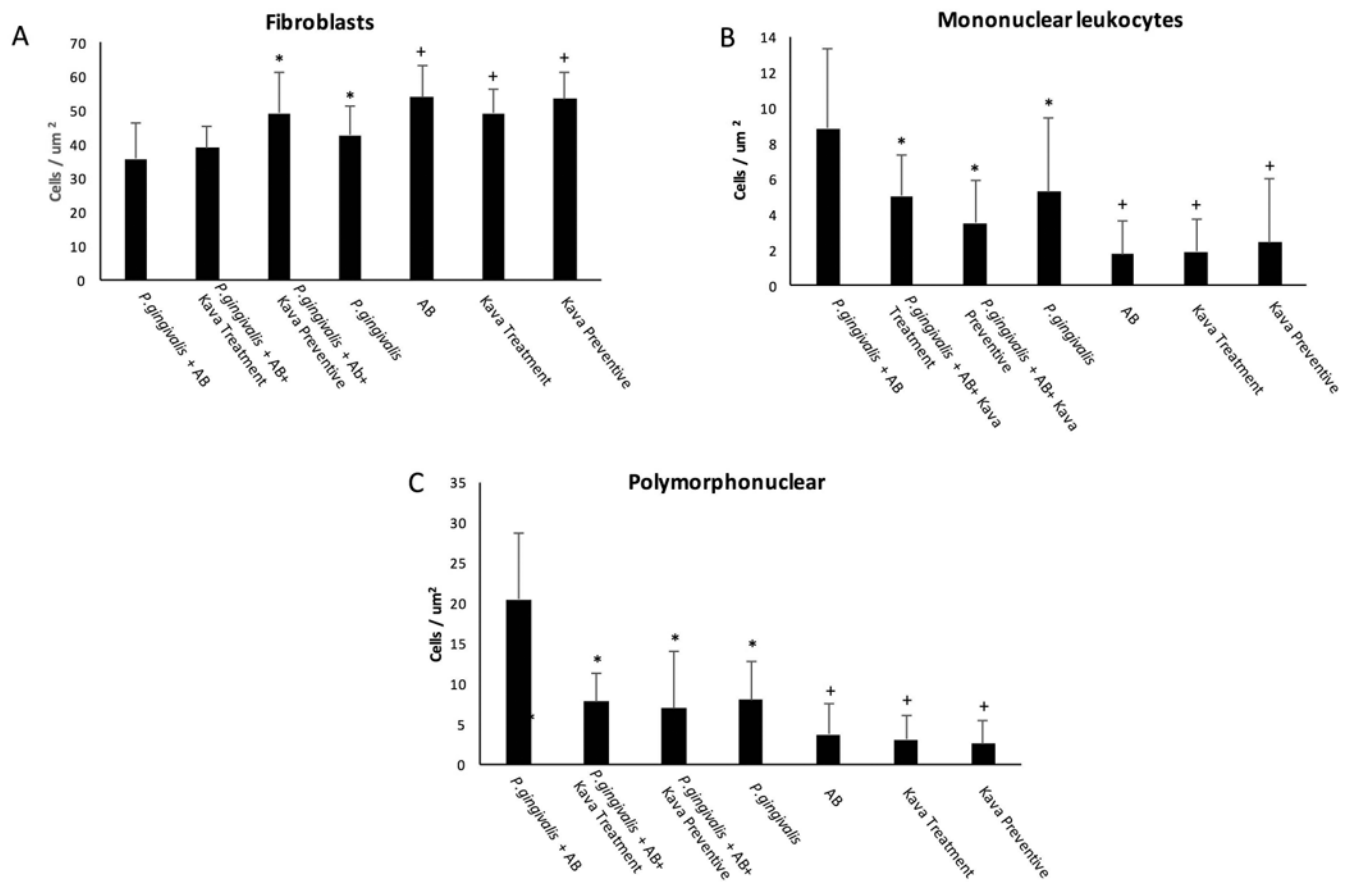


Figure 7. Inflammatory cell counts

A) Fibroblasts; B) Mononuclear leukocytes; C) Polymorphonuclear (*: $p < 0.05$ vs *P.gingivalis* + AB; +: $p < 0.05$ vs *P.gingivalis*). Cell count was expressed as number of cells per μm^2 . A minimum of 6 fields from each interdental area (from CEJ to root apex of the second molar) were analyzed and cells were counted manually from images captured at 400 \times magnification on HE-stained sections.