Brazilin Cream from *Caesalpinia sappan* Inhibit Periodontal disease : In Vivo Study (#95896)

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Brazilin Cream from *Caesalpinia sappan* Inhibit Periodontal disease : In Vivo Study

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Background: Periodontal diseases, including gingivitis, represent gingival tissue injuries caused by chronic inflammation driven by the activity of Nuclear Factor-kappa B (NF-κB), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), p38, and Tumor Necrosis Factor-α (TNF-α). Unaddressed chronic inflammation can lead to persistent disturbances in other parts of the body. Brazilin is a naturally occurring plant chemical that may have antibacterial and anti-inflammatory effects. Treatment based on the natural plant compound brazilin is developed in the form of a topical cream for easy application.

Objective: The aim is to develop the natural compound brazilin in the form of a topical cream as an anti-inflammatory agent to reduce NF-κB expression through Imunohistochemistry (IHC) methods, and the expression of pro-inflammatory genes IL-1β, IL-6, p38, and TNF-α.

Methods: Male Sprague-Dawley rats were induced with gingivitis using *P. gingivalis* bacteria. The observed groups included rats treated with a single application of brazilin cream and rats treated with two applications of brazilin cream. The treatment was administered for 15 days. On days 3, 6, 9, 12, and 15, anatomical wound observations and wound histology using hematoxylin-eosin and Masson's Trichrome staining were performed. NF-κB protein expression was analyzed using the IHC method. Gingival inflammation gene expression of NF-κB, IL-1β, IL-6, p38, and TNF-α was measured using q-RTPCR.

Results: Single and double applications of brazilin cream increased angiogenesis and decreased NF-κB protein expression, in addition to the IL-1β, IL-6, p38, and TNF-α gene expressions.

Conclusion: In a rat gingivitis model, Brazilin cream may function as an anti-inflammatory agent in the gingival tissue

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Abstract

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days 3, 6, 9, 12, and 15, anatomical wound observations and wound histology using hematoxylin-eosin and Masson's Trichrome staining were performed. NF-κB protein expression was analyzed using the IHC method. Gingival inflammation gene expression of NF-κB, IL-1β, IL-6, p38, and TNF-α was measured using q-RTPCR.

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Keywords: Anti-inflamation, Brazilin, Gene expression, Gingivitis, Periodotal

Introduction

Periodontal disease (PD) is a condition of inflammatory wounds with signs of damage to the tissues supporting the teeth, including the gingival tissue and connective tissue that attaches the teeth to the alveolar bone (periodontal ligament) (Paul et al., 2021). Disparities in the subgingival - microbial population and the human immune respone are characteristics of a dysbiotic state close to the gingival edge, knwn as periodontal disease (Abusleme et al., 2013). Gingivitis is one of these disorders, which is characterized by gingival inflammation caused by the cumulative influence of certain taxa of microbes. Gingivitis can develop into periodontitis, a condition marked by the deterioration of tissues supporting the teeth, if treatment is not received. While some gingivitis instances are benign and may be treated, gingivitis is acknowledged by science as the precursor of a number of periodontal illnesses, including periodontitis. Periodontitis occurs due to the impact of excessive microbial development in the subgingiva so that it responds to inflammation which will damage the tissue supporting the teeth, in very severe cases it can even

cause tooth loss (Petersen & Ogawa, 2012). According to records held by the World Health Organization, around 35-50% of people in the world are affected by periodontitis, with around 9-17% affected by gingivitis and this increases to 70-90% during puberty, which is around 47% of the adult population (Holm-Pedersen et al., 2015; Eke et al., 2012).

There are risk factors associated with periodontal disease, namely the habit of smoking in large and unlimited amounts, poor dental hygiene, consumption of drugs that are not recommended, age, and a disturbed mental state (stress) (Nazir et al., 2017). Oral hygiene risk factors lead to increased gingival crevicular fluid (GCF) flow due to bacterial plaque formation driven by increased pathogen-associated molecular patterns (PAMPs) (Cekici et al., 2014). This will activate the innate immune pathway which chemically stimulates cells close to the periodontal epithelium to become inflamed due to the growth of gram-negative bacteria (Fujita et al., 2018; Kumar, 2017). The main mediator of inflammation is through toll-like receptors (TLRs) which also provide inflammation to the periodontal ligament, gingival fibroblasts and dendritic cells (Song et al., 2017; Behm et al., 2019). Proinflammatory cytokines and chemokines, such as Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β), Interleukin 6 (IL-6), Interleukin 8 (IL-8), Interleukin 12 (IL-12), Interleukin 17 (IL-17), and Receptor Activator of Nuclear Factor kappa B Ligand (RANK-L), are expressed more frequently in adjacent cells found in connective tissue and alveolar bone (Duka et al., 2019).

Research conducted by Daliva et al. (2017) who used more than 70,000 participants to describe genetic variants in genes such as IL-1α, IL-1β, IL-6, IL-10 and matrix metalloproteinase (MMP-3) which were systematically reviewed and meta-analyzed on the extent of the affected genome by periodontal genetic factors shows a significant relationship between MMP-9 and the risk of contracting periodontitis. In addition, the nuclear factor-kappa B (NF-κB) is essential for the

inflammatory processes that affect the tooth's gingiva and alveolar bone, particularly when periodontal disorders like gingivitis and periodontitis are present (Ghafouri et al., 2022). In certain conditions, elevated NF-κB expression can exacerbate inflammation (Arabachi et al., 2014).

The plant *Caesalpinia sappan* L. also referred to as brazilian wood or sappan wood, is found across Southeast Asia and is a member of the leguminous plant family. Secang wood is known to contain natural compounds, one of which is brazilin, so that dry secang wood has been widely used as food or traditional medicinal drink (Toegel et al., 2012). Following studies, the potential of the substance known as brazilin to protect cells from oxidative stress as well as its anti-inflammatory, antibacterial, and hypoglycemic qualities were discovered, and have biological effects on a variety of pathologies, involving fibrillogenesis, osteoarthritis, Parkinson's disease, Alzheimer's disease, and others or the majority of meranic's precursors are linked to anticancer properties (Nava et al., 2022).

Topical creams and gels enable the targeted delivery of active ingredients to specific areas, reducing the risk of impacting beneficial bacteria in the oral microbiome (Amiri et al., 2021). The use of cream-based applications on the gums has been widely explored in the pharmaceutical industry due to the biodegradable and biocompatible properties of creams. Cream is widely used because it provides better tolerance to patients, has fewer side effects, tends to be non-toxic, there are few complaints that cause allergies due to use of the cream, it does not irritate the eyes or skin, and production costs are lower (Deogade et al., 2012). Some creams and gels contain anti-inflammatory properties, such as calendula and aloe vera, which can help reduce inflammation and enhance healing. Thus, this study aims to apply cream brazilin to

prevent periodontitis in gigivitis animal models by measuring gene expression of NF-κB, IL-1β, IL-6, p38, and TNF-α.

Materials & Methods

Preparation of brazilin cream sample

Brazilin isolation was obtained from Secang (*Caesalpinia sappan* L.) wood powder through a modified maceration process (Widowati et al., 2014). Secang wood was extracted using 96% ethanol for 24 hours which was soaked three times. While the extraction process took place twice, the macerate will be separated from the residue which is then evaporated at room temperature. Washing the macerate using hot water and then dried. the crude is added with methylene chloride 1: 1 which is then stirred and filtered. the residue which is separated from the filtrate is added with chloroform 1: 2 which is then used filtrate after being separated from the residue. the filtrate is evaporated and the isolate is obtained. In the process of separation and purification of brazilin, fractions from secang wood extract were isolated using vacuum column chromatography and gravity column chromatography. The separated fractions were then further purified using preparative thin layer chromatography (KLT) (Sari et al., 2018). The resulting isolates were then tested for purity using one-dimensional and two-dimensional KLT before elucidating the structure of brazilin using instrument analysis techniques such as NMR-1H. (Kim et al., 2014).

The brazilin isolate preparation that is owned will be used to make a cream that has been modified from Ahmad et al. (2019) cream making is carried out in two stages which are divided into two phases. Phase one is dissolving the formulation into 70 grams of pure coconut oil (VCO) assisted by using a sonicator for 60 minutes with a maximum temperature of 60oC. The

second phase is to dissolve the surfactant (sucrose monoester 1750) and cosurfactant (glycerol) into a beaker and stir until cream is formed. Finally, the first and second phases are mixed little by little while continuing to stir on a hot plate for 30 to 40 minutes (Widowati et al., 2023; Ahmad et al., 2019).

Induction of gingivitis in rats

Animal experiments were conducted at the iRATco Animal Laboratory Services facility in Bogor, Indonesia. All testing protocols described have been approved by Animals Ethics Committee of iRATco VLS with number 4.2.012-3/KEHI//I/2023. This research was carried out on male Sprague Dawley rats obtained from the animal facility, aged 18-20 weeks with a body weight of around 150-200 g. The animals selected were animals that met the inclusion criteria (male, Sparague Dawley breed, weighing 150-200 g, and 18-20 weeks old). The exclusion criterion in this study was the presence of visible signs of disease during other experimental activities. Animals will be allowed to acclimatize for a week placed in *Individually Ventilated Caged* (IVC) cages measuring 30.5 x 20.5 x 15.5 cm and with a 2 cm high sawdust mattress which is replaced every 2-3 days (Hidayat et al. al., 2022). The temperature provided was individually controlled and ventilated at 22°C and 40% relative humidity with a 12 h light/dark cycle. Rats were given free access to food containing 5% crude fiber, 18% crude protein, and 50% crude fat (PT Indoofeed) and water ad libitum after they acclimated (Kurniati et al., 2021; Widowati et al, 2022). Six groups of rats were used: vehicle control group (single oral base cream), positive control group (bacteria-induced rats), and negative control group (normal rats), drug control (single patent drug), test group I (single Brazilin cream) and test group II (double Brazilin cream). The drug used in the drug group is Kenalog Triacinolone Acetonide ointment

0.1% (Taisho, DKL1124400130A1). The test, drug and positive groups will be given local anesthesia to induce gingivitis using *P. gingivalis* bacteria at a concentration of 2 x 10^8 per rats. The volume of bacteria injected was 50 µl with a ratio of 1:1. Bacteria are injected into the gingiva using a Nichimate stepper. The inducer was applied every 3 days for 14 days or until gingivitis occurred in all samples. All groups received the same housing and feeding regimen. For subjects in this group we applied a protocol to induce experimental periodontitis using incisor ligatures and gingival tissue harvesting. Gum inflammation, swelling and unnatural redness are signs of gingivitis. After 14 days the rats were euthanized using an overdose of isoflurane via inhalation (Elhaieg et al., 2023). then the rat's jaw was removed and soaked in 10% formalin for two days. The rat jaws were then soaked again in a weak acid solution for a decalcification process for 10 days before analysis. Rats that are sick, die, or do not gain weight during acclimatization will be executed. At the end of the probationary period, those who are still alive will still be executed.

Wound Histopathological Examination

The procedure for Masson Trichome staining involved soaking the tissue in Weigert iron hematoxylin for ten minutes, followed by a distilled water rinse and 10-15 minutes of staining with Bieblich Scarlet Acid Fucin solution. After another rinse rith distilled water, the slides are stained for 10-15 minutes usiang a phosphomolybdenum-phosphotungstic acid solution. After being dyed for between 5 and 10 minutes with aniline blue solution, the tissues was submerged in a 1% acetic acid and 95% absolute ethanol solution. The examination was carried out under a microscope covered with a cover glass. The parameters measured were the area of inflammation, angiogenesis, and collagen tissue area (Laksmitawati et al., 2023; Suvik et al., 2012).

182

183 **Immunohistochemistry Assay (IHC)**

184 The gingival tissue to be fixed begins with preservation in paraffin. Deparaffinization was
185 carried out for 15 minutes at a temperature of 56oC and then the glass object was rinsed with
186 xylene. After deparaffinization, proceed to the rehydration stage using various ethanol (absolute,
187 90%, and 70%) with additional soaking for 30 minutes after rinsing with phosphate buffer saline
188 (PBS). At a temperature of 121oC for 10 minutes antigen retrieval was carried out in citrate
189 buffer (pH 6 Abcam ab208572). To block endogenous peroxidase, samples were treated in
190 menthanol (Merck, 106009) with 3% H2O2 (Merck, 107209). Primary antibody reactions were
191 carried out overnight at room temperature after incubation with 5% Bovine serum albumin for 10
192 minutes. Visualization of target proteins using the HRP/DAB detection rabbit specific IHC Kit
193 (ABC) (Abcam, ab64261). After making a hematoxylin counterstain, the stained tissue was
194 examined under a Primostar microscope (Zeiss). Photography was accomplished with Lumenera
195 Infinity 1-3c. Qualitative comparison of IHC results was based on the number of positive cells
196 and expression intensity. ImageJ software was utilized for quantification of the positive index on
197 immunohistochemistry slides (Rosni et al., 2021, Widowati et al., 2022).

198

199 **Quantification of Gingival Tissue TNF-α, IL-1β, IL-6, and p38 by q-RTPCR**

200 Total rat gingival RNA was extracted and purified using the Direct-zol RNA Miniprep Plus Kit
201 (Zymo, R2073) in accordance with the manufacturer's instructions. Utilizing iScript Reverse
202 Transcription Supermix for RT-PCR (Bio-Rad, 170-8841), complementary DNA synthesis was
203 performed. We used the Agilent AriaMx 3000 real-time PCR technology to evaluate gene
204 expression quantitatively. Evagreen Master Mix (Bio-Rad, 1725200) was the qPCR reaction mix

used (Widowati, et al., 2020; Widowati et al., 2021). Table 1 displays the primer sequences (Macrogen), and Table 2 displays the RNA's concentration and purity, which were measured spectrophotometrically at 260/280 nm.

Statistic analysis

Statistical analysis was carried out with the help of SPSS 22.0 software. One-way analysis of variance (ANOVA) and post Hoc Tukey dam value *p<0.05 to be considered significant.

Results

Makrogingiva

Rat that have been given induced gingivitis after 15 days are checked for gingival tissue to see the effect of treatment given qualitatively. Rat gingival tissue (Figure 1) shows a rat gingivitis model using bacterial induction of *P. gingivalis* successfully made starting from day 3 shows damage to rat teeth from the onset of dental plaque to day 15 where it can be seen that rat teeth are increasingly damaged due to tartar on the teeth. In the brazilin cream treatment group showed macro improvements in the gingival condition of the gingivitis model rats, there was a gradual improvement from day to day that could treat the condition of the gums or gingival tissue in rats, although plaque remained but did not cause tartar (Figure 1). This shows that brazilin cream treatment has an antibacterial effect on the cause of gingivitis and can be effective in the treatment of the condition of the gums and teeth.

Histopathology of inflammation and angiogenesis of gingival tissue in gingivitis model rats by HE method

Histopathological evaluation of inflammation and angiogenesis in rat gingival tissue can be seen using HE staining. The application of brazilin creams on rat gingiva is effective in increasing the number of fibroblast cells, increasing new blood vessels and reducing the inflammatory process, so it can help the healing process of rat gingival wounds seen in (Figure 1). Based on the results of histopatholgy of gingival tissues at 40x magnification of the field of view, the number of cells undergoing inflammation and angiogenesis is calculated and then averaged. Based on Figure 3, the inflammation score graph shows that groups V & VI reduced inflammation and had a significant difference ($P < 0.05$) with the positive control group which was induced by bacteria. The angiogenesis score also showed that brazilin application (V&VI group) was able to increase the angiogenesis score compared to the PC group (Figure 2B).

Gingival NF-κB protein expression in gingivitis rat model using IHC methods

Evaluation of NF-κB expression using the IHC method in Figure 4 shows that there is a significant effect of using brazilin cream on rat gingival tissue. All treatment groups were able to reduce NF-κB expression levels compared to the positive group (group II). The most effective application of brazilin cream in reducing Nf-KB expression levels is two applications of brazilin cream (group VI).

Histopathology of collagen scores in rat gingival models using MT method

The collagen value was determined by the Masson trichome method (Figure 5). Treatment with 1 and 2 times apply of brazilin cream was able to increase the collagen score, although visually there wasn't much difference. However, the 2 times apply of brazilin cream group (group VI) was more effective in increasing the collagen score (Figure 6).

Gene Expression IL-1β, IL-6, p-38, and TNF-α toward brazilin cream

The impact of using brazilin cream on the levels of p-38, TNF-α, IL-1β, and IL-6 gene expression in the gingival tissue of rats suffering from gingivitis is displayed in Figure 7 (A,B,C,D). The outcomes demonstrated that, in comparison to the positive control group (Group II), the use of brazilin cream decreased the levels of p-38, TNF-α, IL-1β, and IL-6 ($P<0.05$). Brazilin cream applied twice a day is the most effective therapy to reduce TNF-α, p-38, IL-1β, and IL-6 levels. (2x).

Discussion

The main reason of tooth loss is periodontitis, an inflammatory condition brought on by bacterial infections in the mouth (Chen et al., 2021). Inflammation that occurs due to a lack of antioxidants results in an excessive inflammatory response. Efforts to prevent inflammation can be treated with natural or synthetic ingredients that are antioxidants.In this case, treatment was carried out using the natural compound brazilin. Treatment with the natural compound Brazilin in the form of a cream is used in this case. Brazilin cream was administered as treatment because brazilin reportedly has antioxidant, anti-inflammatory, and antibacterial properties that reduce proinflammatory gene expression (Nirmal et al., 2015). The quality of the cream produced meets standards with a homogeneous texture (not lumpy and the particles are evenly distributed), the resulting spreadability is 4.89 with a pH of 8 and the resulting viscosity is 208,000 (Vinna et al., 2024 under review). The research evaluation began with an examination of the gingival tissue of rats that had been induced by bacteria. The results were shown after 15 days of inflammation caused by bacteria in the gingival tissue of rats (Figure 1). The swelling and inflammation of the

274 gingival tissue that occurred was reported in previous studies to be caused by plaque bacteria
275 such as P. gingivalis (Rani et al., 2022).
276 Macrogingiva in rats refers to observable outcomes or changes at a gross or macroscopic level in
277 the gingival tissue of rats (Table 3). This involves the observation of changes visible at a larger
278 scale, such as alterations in color, swelling, or bleeding in the rat's gums. Macrogingival
279 outcomes in rats often serve as the initial basis for assessing gum health and identifying early
280 signs of periodontal issues. However, for a more in-depth evaluation, microscopic examinations,
281 such as histology or assessment of gene expression at the cellular level, are often required to
282 understand tissue changes and immune responses that may occur at a smaller scale. Standard
283 microscopic examination of tissue that has been fixed is hematoxylin and eosin (HE) staining.
284 There are two methods for staining this material: manual and automated. However, the manual
285 method is more common due to its low sample volume requirements, which reduces the need for
286 facilities (Cardiff et al., 2014). Collagen density examination can be measured by MT method.
287 The MT method is an approach to staining that is capable of distinguishing between smooth
288 muscle fibers (SMF) and extracellular matrix (ECM) in a single tissue. It can also be employed
289 to identify specific kinds of collagen. Observations were conducted under a microscope with 6
290 fields of view and a magnification of 400 times (Figure 5) (Hernández-Morera et al., 2016).
291 Our study highlights the application of brazilin cream on the gingiva of rats, exhibiting a
292 decrease in the inflammatory process, the creation of new blood vessels, and a rise in fibroblast
293 cells. This phenomenon proves beneficial in facilitating the healing process of gingival wounds
294 in rats. This result is consistent with a research by Baru et al. (2021), which found that the
295 development of new blood vessels during gingivitis therapy might be interpreted as an indication
296 of gingivitis healing. According to Smith et al. (2015), the development of these blood vessels

occurs due to reduced inflammation, which, in turn, supplies oxygen and nutrients to promote tissue regeneration. Another study also reported that the use of pomegranate gel can reduce gum inflammation and promote tissue regeneration (Somu et al., 2012).

In research carried out by Song et al. (2018), gingivitis inflammation is associated with some regulatory T cells and CD4+ T cells that produce the bone-resorptive cytokine, Receptor Activator of Nuclear Factor-κB (NF-κB), which causes dental bone resorption. Patients suffering gingivitis and periodontitis will have proinflammatory cytokines like TNF-α and IL-1β in their gingival tisssue and gingival crevicular fluid (Papathanasiou et al., 2020;). Gingival fibroblast will express more IL-6 and p-38 in respons to IL-1β andd TNF-α, which will aid in the development of gingivitis (Gündogar & Uzunkaya et al., 2021; Cavalli et al., 2021). Conversely, Stadler et al. (2016) observed a drop in IL-1β periodontitis patients following theraphy is indicating signs of healing. In contrast, increased IL-1β worsened the condition in patients with experimental mucositis.

Based on our findings, brazilin cream applied twice was able to inhibit NF-κB levels, and reduce the expression of pro-inflammatory genes (IL-1β, IL-6, P38, TNF-α, and NF-κB) (Figure 7 A,B,C,D). This result in consistent with studies done by Kircheis et al. (2020), who reported that partial inhibition of NF-κB expression can inhibit highly pro-inflammatory cytokines and chemokines. Nirmal et al. (2015) also stated that brazilin can inhibit the primary regulator pro-inflammatory cytokine production, the NF-κB signaling pathway. The pro-inflammatory cyrokines IL-1β, IL-6, and TNF-α can be inhibited by brazilin through the NF-κB signaling pathway. This is further supported by Lee et al. (2022) in his research showing that brazilin showed a protective effect against the loss of fibroblast cell viability caused by UVB and

319 significantly blocked ROS in UVB by inhibiting MMP-1/3 expression, which is a side effect of
320 suppressing NF-κB activation.
321 Gingivitis is characterized by an inflammatory response involving immune cell infiltration, such
322 as neutrophils, triggered by the presence of bacteria and byproducts from their metabolic
323 processes, leading to disturbances in blood flow and increased collagenolytic activity resulting in
324 extracellular matrix degradation, particularly collagen (Chandran et al., 2021). Brazilin has been
325 linked to a decrease in the expression of genes that promote inflammation. There are several
326 biological actions associated with brazil, including as antibacterial and anti-inflammatory effects
327 (Olanwanit & Rojanakorn, 2019). According to Vij et al. (2023), brazilin's anti-inflammatory
328 qualities can counteract reactive oxygen species (ROS), which lowers oxidative stress and
329 inflammation. Numerous growth factors mediate the whole angiogenesis process, including
330 VEGF, which is regulated in gingivitis. Concentrated growth factors and human gingival
331 fibroblasts have been proven to enhance gingival regeneration through angiogenesis (Roi et al.,
332 2022; Wang et al., 2022). This aligns with the statement by Ghosh and Gaba (2013) that the
333 primary effects of active compounds in plant extracts have the potential for anti-inflammatory
334 healing due to antimicrobial activity, antioxidant benefits, and active components that can
335 enhance cell proliferation, angiogenesis, and collagen production.
336

337 ## Conclusions

338 The application of brazilin cream treatment has the potential to improve gingival tissue by
339 suppressing inflammation, enhancing angiogenesis, as indicated by the increased growth of new
340 blood vessels, and promoting collagen activity. The downregulation of pro-inflammatory
341 cytokines, such as IL-1β, IL-6, p-38, TNF-α, and NF-κB is indicative of this.

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Figure 1

Effect of brazilin cream toward inflammation and angiogenesis of gingival tissue in gingivitis model rats using HE method (40x magnification)

*I: Negative control (normal rat), II: Positive control (bacterial-induced rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream

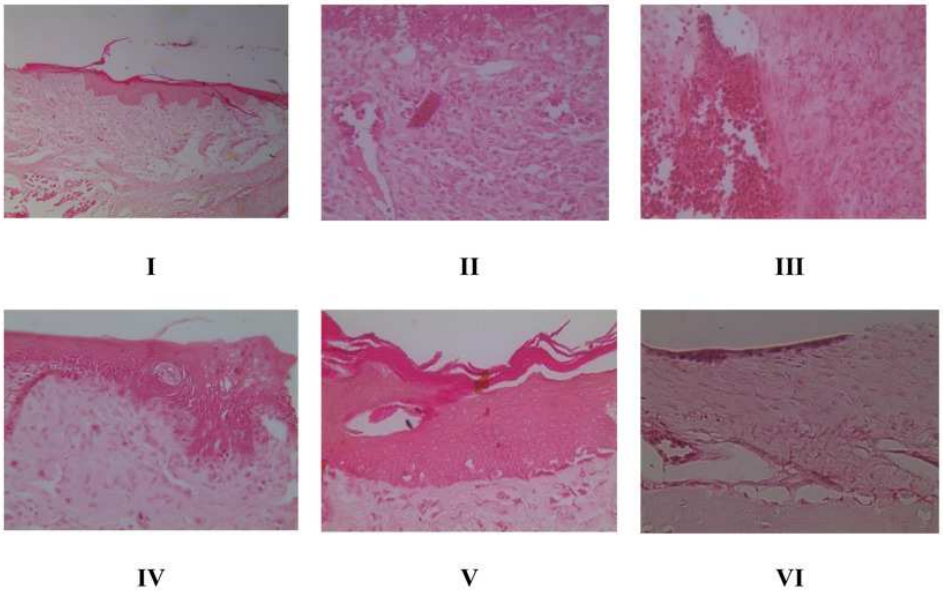

I
II
III
IV
V
VI

Figure 2

Effect brazilin cream toward histopathology of gingival tissue gingivitis rats model.

A) Inflamation. B) Angiogenesis.

*Data are presented as mean ± standard deviation. For each treatment, the assay was performed in four repetitions I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream. Different superscripts (a,ab,bc,c) for inflammation score and different superscript (a,bc,c) mark significant differences among treatment groups based on Kruskal Wallis and Mann-Whitney tests ($P < 0.05$).

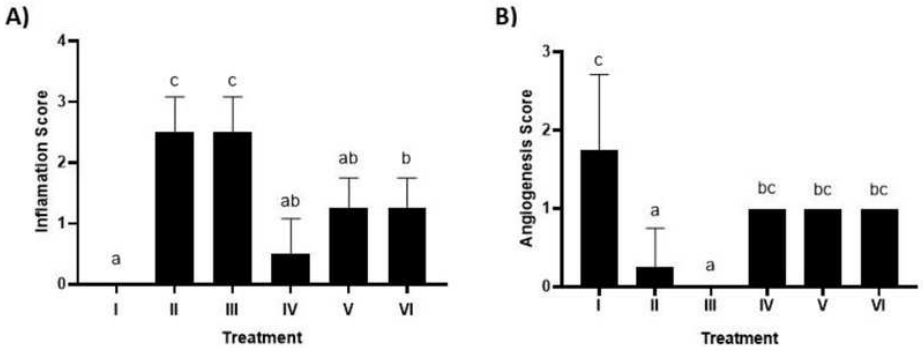

A)
Inflamation Score
0
1
2
3
4
a
c
c
ab
ab
b
I
II
III
IV
V
VI
Treatment
B)
Angiogenesis Score
0
1
2
3
c
a
a
bc
bc
bc
I
II
III
IV
V
VI
Treatment

Figure 3

Effect of brazilin cream toward gingival NF-κB protein expression using IHC methods (40x magnification)

*I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream

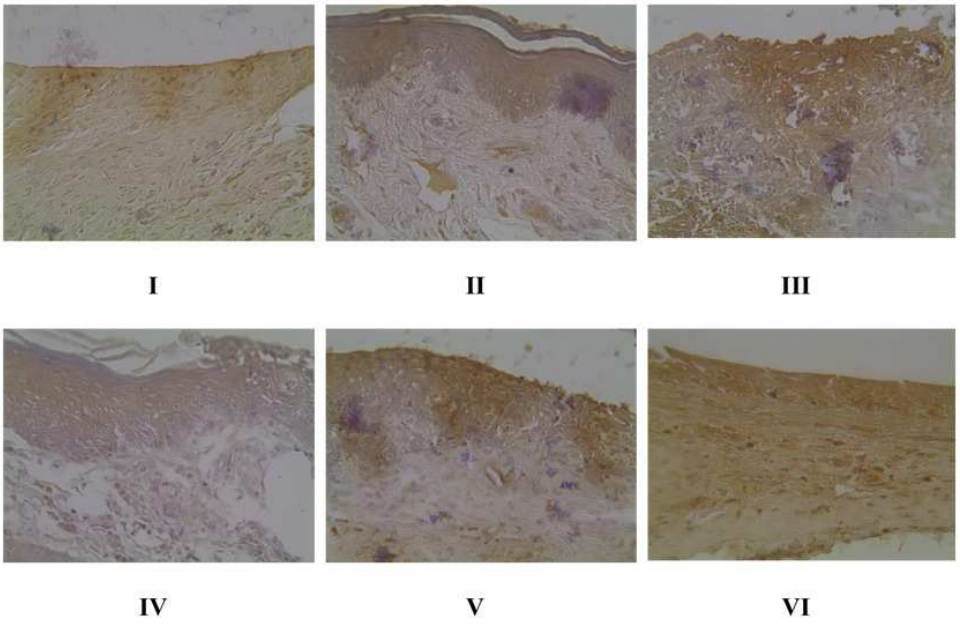

I
II
III
IV
V
VI

Figure 4

Effect of brazilin cream toward gingival NF-κB protein expression in gingivitis rats model

*Data are presented as mean ± standard deviation. For each treatment, the assay was performed in four repetitions. I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream. Different superscripts (a,b,c,d,e) mark significant differences between treatment groups ($P < 0.05$, One way ANOVA and Tukey HSD test).

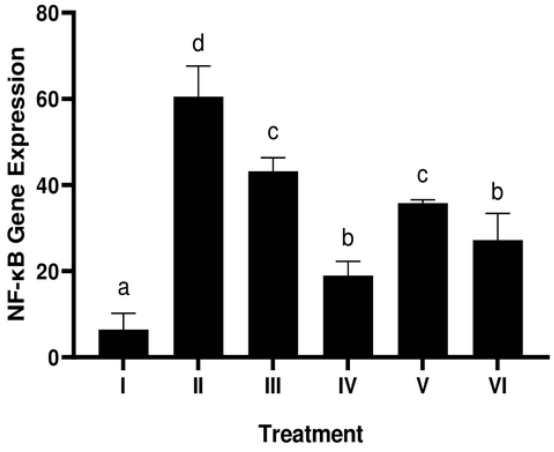

NF-κB Gene Expression
80
60
40
20
0
a
d
c
b
c
b
I
II
III
IV
V
VI
Treatment

Figure 5

Effect of brazilin cream toward collagen scores in rat gingival with MT methods (40x magnification)

*I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream.

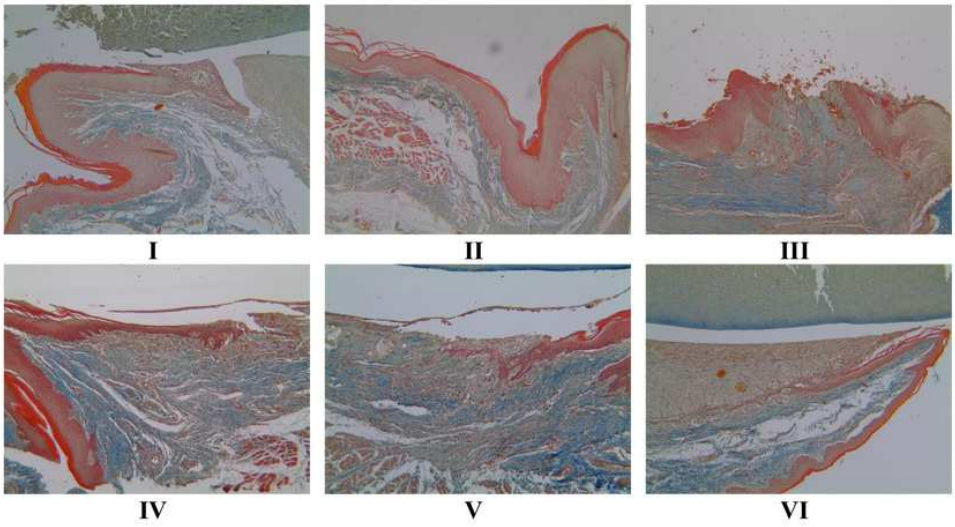

I
II
III
IV
V
VI

Figure 6

Effect of brazilin cream toward collagen in gingivitis rats model

*Data are presented as mean ± standard deviation. For each treatment, the assay was performed in four repetitions. I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream. Different superscripts (a,ab,abc) mark significant differences between treatment groups ($P < 0.05$, One way ANOVA and Tukey HSD test).

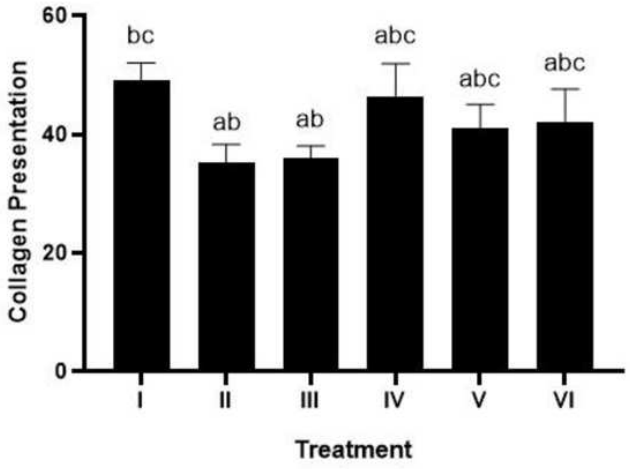

60
40
20
0
Collagen Presentation
bc
ab
ab
abc
abc
abc
I
II
III
IV
V
VI
Treatment

Figure 7

Effect of brazilin cream toward gene expression in gingivitis rats model

A) IL-1β gene expression B). IL-6 C) P-38 dan D) TNF-α

*Data are presented as mean ± standard deviation. For each treatment, the assay was performed in four repetitions. I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream. Different superscripts (a,b,c,d) mark significant differences between treatment groups ($P < 0.05$, One way ANOVA and Dunnet T3 test).

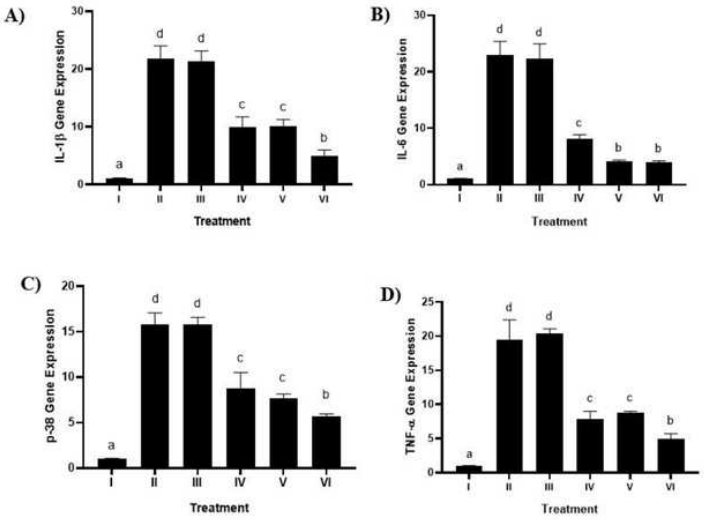

A)
IL-1β Gene Expression
Treatment
B)
IL-6 Gene Expression
Treatment
C)
p-38 Gene Expression
Treatment
D)
TNF-α Gene Expression
Treatment
I
II
III
IV
V
VI

Table 1(on next page)

Primer sequences used in qRT-PCR

Gen	Forward (5'-3')	Reverse (5'-3')	Product Length (bp)	Suhu Annealing (C)	Reference
IL-1β	AGAATACCACTTGTTGGCT	GTGTGATGTTCCCATTAGAC	134	55	NM_031512.2
IL-6	GAGCATTGGAAGTTGGGGTA	TGATGGATGCTTCCAAACTG	230	53	NM_012589.2
TNF-α	GAAGACAATAACTGCACCCA	AACCCAAGTAACCCTTAAAGTC	138	54	NM_012675.3
P-38	AGATAATGCGTCTGACGGG	AGGGGATTGGCACCAATAAA	139	58	NM_031020.3

1

Table 2(on next page)

Purity of gingival RNA treated with brazilin cream

I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream.

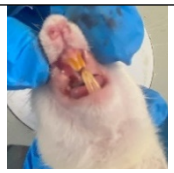
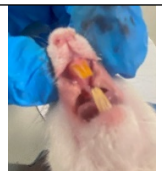
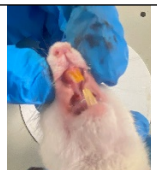
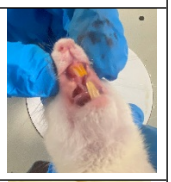
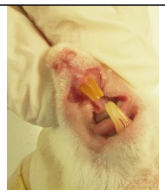
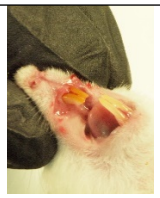
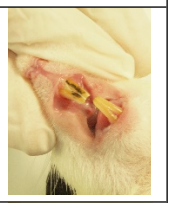
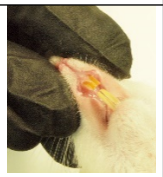
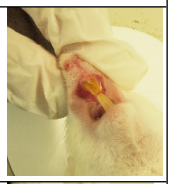
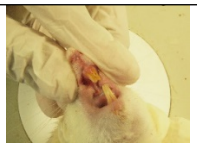
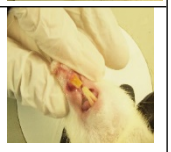
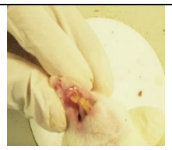
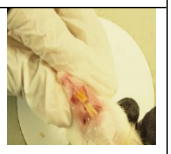
No.	Sampel	Konsentrasi (ng/ µl)	Purity (λ260/ λ280 nm)
1	I	47.8	2.211
2	II	54.875	2.204
3	III	60.65	2.441
4	IV	16.225	2.045
5	V	90.525	2.203
6	VI	41.375	2.219

1

Table 3(on next page)

Macrogingival results in gingivitis model rats with brazilin cream treatment group

I: Negative control (normal rat), II: Positive control (bacterial induction rat), III: Vehicle control (1 x oral base cream), IV: Drug control (1 x patent medicine), V: II + 1 x brazilin cream, VI: II + 2 x brazilin cream.

Group of Treatment	Day to-				
	3	6	9	12	15
I					
II					
III					
IV					
V					
VI					

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