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The effect of ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide and 2% chlorhexidine digluconate on matrix metalloproteinase-9, transforming growth factor-1 levels, osteoclast, and osteoblast cells in Wistar rats with chronic apical periodontitis

Ni Kadek Eka Widiadnyani[1*], I Nyoman Mantik Astawa[2], I Wayan Putu Sutirta Yasa[3], I Dewa Made Sukrama[3], I Made Bakta[3], Ida Bagus Putra Manuaba[4], I Wayan Suardana[2], I Made Sudarmaja[3]

[1]Postgraduate Student, Faculty of Medicine, Universitas Udayana, Bali, Indonesia;
[2]Faculty of Veterinary Medicine, Universitas Udayana, Bali, Indonesia;
[3]Faculty of Medicine, Universitas Udayana, Bali, Indonesia;
[4]Chemistry Study Program, Faculty of Math and Science, Universitas Udayana, Bali Indonesia;

*Corresponding author:
Ni Kadek Eka Widiadnyani;
Postgraduate Student, Faculty of Medicine, Universitas Udayana, Bali, Indonesia;
eka.widiadnyani@unud.ac.id

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ABSTRACT

Background: Infections of the dental pulp and periapical tissues involve bacteria which can induce and enhance the immune response of the dental pulp but are not effective in eliminating root canal bacteria. A study was conducted to determine the effect of giving ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide and 2% chlorhexidine digluconate on the chronic inflammatory response in periapical tissue by measuring MMP-9, TGF-β_1 levels, osteoclast, and osteoblast cells count.

Methods: An experimental design with a post-test-only control group design involving 24 right molars of male Wistar rats divided into 4 groups. Each group consisted of 6 rats (K-) bacteria, (K+) bacteria, calcium hydroxide and 2% chlorhexidine digluconate, (P_1) bacteria, calcium hydroxide and 10% meniran extract, (P_2) bacteria, calcium hydroxide, 2% chlorhexidine digluconate and 10% meniran extract}. Examination of MMP-9, TGF-β_1 levels, and the number of osteoclasts and osteoblasts were evaluated until day 14.

Results: The administration of ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide as an intracanal medicament (P1) which was evaluated for 14 days overall showed a significant difference between groups ($p<0.005$). This condition can be seen in the group (P1), which has a lower MMP-9 level of 2465.88 pg/mL compared to the group (P2), (K+), and (K-). The levels of TGF-β_1 in the group (P1) were 181.57pg/mL, lower than the group (P2), (K+), and (K-). The number of osteoclast cells in the group (P1) was 1.67, which was lower than the group (P2), (K+), and (K-) was 5.00. The number of osteoblast cells in the (P1) group was 52.67, higher than the 32.17 group (P2), (K+), and (K-).

Conclusion: Green meniran extract (Phyllanthus niruri Linn) and its role as an intracanal medicament in teeth with chronic apical periodontitis.

Keywords: *calcium hydroxide, chronic apical periodontitis, Phyllanthus niruri Linn*.
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INTRODUCTION

Dental caries disease with apical periodontitis is a disease whose prevalence is quite high and increases with age, especially at the age of 50 years, with a 10-15% lower prognosis than teeth without apical periodontitis.[1] Epidemiological data showed that apical periodontitis is experienced by 50% of the population aged 50 years and 62% aged over 60 years in Indonesia.[2] Diseases of pulp tissue and periapical tissue accounted for the 7th position in the list of the 10 biggest diseases in outpatient hospitals in Indonesia, which were 86,421 cases, with the percentage of male patients at 45.6% and females at 54.4%.[3]

Infections of the dental pulp and periapical tissues involve anaerobic Gram-positive and Gram-negative bacteria which can induce and enhance the immune response of the dental pulp but are not effective in eliminating root canal

bacteria. One of the anaerobic Gram-positive bacteria that is persistent and resistant is *Enterococcus faecalis* which cannot be removed by instrumentation and irrigation even though root canal treatment has been carried out.[4] The chronic inflammatory reaction will then stimulate a secondary immune response in the periapical region known as chronic apical periodontitis (CAP).[5,6] Chronic apical periodontitis can cause progressive damage to the periodontal ligament and alveolar bone resorption, resulting in tooth mobility and loss.[7]

The occurrence of pathogenic changes in CAP can indirectly stimulate high levels of the pro-inflammatory cytokine mediator matrix metalloproteinase-9 (MMP-9), and levels of the anti-inflammatory cytokine transforming growth factor (TGF-β) activated in periapical tissues by MMP-9.[8-14] Matrix metalloproteinase-9 (MMP-9) plays a role in the degradation of organic matrix dentin that contributes to periapical inflammation. Degradation of organic matrix dentin can reduce denatured collagen from the extracellular matrix (ECM), periodontal ligament damage, and bone resorption in the periapical area. Decreased denatured collagen can increase MMP-9 levels, which can destroy the gingival tissue and alveolar bone that surround the teeth. Increased levels of MMP-9 could lead to long-term periapical tissue inflammation.[10-12]

In particular, studies of MMP-9, TGF-β1 levels and the number of osteoblast cells with periapical lesions are rarely performed. Some studies have discussed the relationship between TGF-β and MMP-9. It was stated that TGF-β could regulate the regulation of matrix induction of MMP-9 by TNF-α in monocytes/macrophages through medicaments.[15-21] Other studies stated that TGF-β expression had the opposite pathway in CAP infection, and TGF-expression was activated by MMP-9 in periapical tissue damage due to induction of *Enterococcus faecalis* bacteria for 21 days.[16] A research demonstrated that there would be a positive effect on the application of calcium hydroxide (Ca(OH)2)-based intracanal medicament on the reduction of MMPs levels in teeth with failed root canal treatment and apical periodontitis.[19] This is in accordance with research by Charles *et al*. regarding the role of MMPs in periodontitis and their management.[20] Research by Maria, who used triantibiotic paste as an intracanal medicament, stated that there was a decrease in MMP-9 levels in the periapical area with cases of CAP, as evidenced by a decrease in the number (Colony Forming Unit) of bacterial CFU in the teeth.[21]

In the field of dentistry, especially endodontics, cases involving necrotic teeth with periapical lesions can be cured as quickly as possible through root canal treatment. The success of root canal treatment is strongly influenced by intracanal medicament materials. The purpose of intracanal medicament is to reduce pain, eliminate all bacteria remaining in the root canal, reduce periradicular inflammation, and prevent alveolar bone resorption and reinfection. The main ingredient of intracanal medicaments has been proven, and until now, the most widely used is calcium hydroxide $Ca(OH)_2$.[7,22]

In general, one of the mixing materials $Ca(OH)_2$ commonly used and proven to kill bacteria *Enterococcus faecalis* is chlorhexidine digluconate 2%.[23] As a synthetic sterilizing agent, the disadvantages of 2% chlorhexidine digluconate are that it is not able to dissolve the remnants of necrotic tissue, does not have the ability to inhibit endotoxin from Gram-negative bacteria, and is still not effective in removing bacterial biofilm *Enterococcus faecalis*.[21-23]

The increasing problem of resistance from microorganisms causes the search for medicinal plant materials for sterilization and anti-inflammation to continue. Based on the phytochemical research, green meniran (*Phyllanthus niruri Linn*) contains secondary metabolites of flavonoids, terpenoids, alkaloids, steroids, tannins, and saponins.[24-27] Other studies stated that many flavonoids are reported to have properties such as antibacterial, antiviral, antifungal, anti-inflammatory and several other activities.[24-27] The results of several studies using herbal plants stated that polyphenol compounds could stimulate osteoblast cell differentiation and inhibit the induction of NF-κB Ligand (RANKL) in osteoclast differentiation.[24-27]

The herbal ingredient of green meniran extract (*Phyllanthus niruri Linn*) and its role as a clinical application in tooth periapical inflammation have never been studied and published.[24-28] Root canal treatment (PSA) therapy strategy through -based intracanal medicaments ($Ca(OH)_2$) by giving ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) to chronic apical periodontitis (CAP) it is certain that it will stop within 14 days. This process can reduce the development of progenitors and recruitment of osteoclasts, stimulate apoptosis of mature osteoclasts and suppress the occurrence of apoptosis in osteoblasts.[25,27] The aim of this study was to know the effect of ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide and 2% chlorhexidine digluconate on matrix metalloproteinase-9, transforming growth factor-1 levels, osteoclast, and osteoblast cells in Wistar rats with chronic apical periodontitis.

METHODS

This research is a true experimental study with a randomized posttest-only control group design. This research was carried out at the Pharmacology Laboratory of the Faculty of Mathematics and Natural Sciences, Universitas Udayana and the Veterinary Medicine Laboratory of Universitas Udayana in January - June 2021. The samples in this study were white male rats of the Wistar strain aged 24-25 weeks, weight 300-350g chronic apical periodontitis induced by *Enterococcus faecalis* ATCC 29212 bacteria to the floor of the pulp cavity with an amount of 10^9 CFU as much as 0.01 ml using a micropipette. Then the cavity was closed with RMGIC Fuji II LC restoration material and left for 21 days until chronic apical periodontitis occurred in the periapical tissues of the right molar teeth of RA Wistar rats. The sample size was obtained using the independent mean difference formula, which is 6 samples for each group.

The dried leaves of green meniran (*Phyllanthus niruri Linn*) were obtained from sugarcane farmers in the Batu Malang area through a drying process to obtain 1000 grams of coarse powder, which was carried out at the UPT Laboratory of Herbal Materia Medika in Batu Malang

city. The research was started by using 300 grams of fine powder of meniran leaves for extract preparation by maceration with 96% ethanol solution, which was carried out at the Pharmacognosy Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Udayana. After obtaining the Meniran extract, preparations were made for qualitative and quantitative phytochemical tests and the LCMS test to determine the number of compounds and components of green meniran bioactive substances. The formulation of meniran extract was made by dilution method to obtain meniran extract with a concentration of 10%.

Wistar rats preparation

Wistar strain male rats that had been prepared according to the inclusion criteria were randomized to be used as research samples. The teeth of chronic apical periodontitis rats were made, which were previously anesthetized by injection of ketamine HCl i.m (Ketalar®, Warner-Lambert, Irlandia) on the hamstrings at a dose of 0.24 ml/300-350BB, then bacteria were applied *Enterococcus faecalis* ATTC 29212 bacteria to the bottom of the pulp chamber of the maxillary right molar which has been exposed by an amount of 10^9 CFU as much as 0.01 ml. Then the cavity was closed with RMGIC-LC and left for 21 days.

On day 22, the condition of the supporting tissues of the teeth was evaluated with reddish gingiva and tooth mobility. Then the rats were again anesthetized using 0.24 ml of ketamine HCL to apply intracanal medicament into the pulp chamber. In the positive control group (K+), chronic apical periodontitis rats have applied calcium hydroxide medicament and 2% chlorhexidine digluconate. In treatment group one (P1), chronic apical periodontitis rats have applied calcium hydroxide medicament and 10% green meniran leaf ethanol extract. In the treatment group two (P2), chronic apical periodontitis rats were applied with calcium hydroxide medicament, 2% chlorhexidine digluconate and 10% green meniran leaf ethanol extract in the negative control group (K-) rats with chronic apical periodontitis. The application of the medicament material was allowed to remain until the 14th day in the pulp chamber. Then, on the 14th day in each group, the levels of MMP-9 and TGF-β1 with Rat ELISA were examined, and the number of osteoclast cells and osteoblast cells by histopathological examination.

The data analysis tests carried out in this study were the Shapiro-Wilk test for normality and Levene's test for homogeneity. The comparative test used by Oneway ANOVA was followed by a Post-Hoc test to assess which groups differ from one another. The limit for each test is 0.05.

RESULT

In the study of MMP-9 levels from the serum of chronic apical periodontitis Wistar rats, the overall difference in MMP-9 levels between groups showed, in treatment group one (P1) with the application of calcium hydroxide paste and 10% green meniran leaf ethanol extract which was applied intracanal was 2465.88 pg/mL. These levels were lower when compared to the other three groups, namely in the second treatment group (P2) of 2657.62pg/mL, the positive control group (K+) of 4099.55pg/mL and the negative control group (K-) of 4914.75pg/mL. mL, and found a significant difference between the intervention groups through the One-Way ANOVA test ($p<0.001$). Post Hoc test to see the difference between study groups in pairs, there was a significant difference in the average levels of MMP-9 in the K- group and P1 (mean difference = 2448.86; p <0.001*), in the K- and P1 groups (mean difference = 2257.13; p<0.001*), in the K+ group and P1 (mean difference = 1633.67; p= <0.003*), in the K+ group and P2 (mean difference = 1441.93;<0.007*), in the P1 group with K- (mean difference = -2448.86; p<0.001*), group P1 and K+ (mean difference = -1633.67; p<0.003*), group P2 and K- (mean difference = -2257.13; p<0.001*), P2 group with K+ (mean difference = -1441.93; p<0.007*). The P1 and P2 groups were better at reducing MMP-9 levels than K+ with K-, but there was no significant difference between P1 and P2.

In the study of TGF-1 levels from the serum of chronic apical periodontitis Wistar rats, the overall difference in TGF-β1 levels between groups showed that TGF-β1 levels in treatment group one (P1) with the application of calcium hydroxide paste and 10% green meniran leaf ethanol extract, which was applied intracanal was 181.57pg/mL. These levels are lower when compared to the other three groups, namely in the second treatment group (P2) at 235.77pg/mL, positive control (K+) at 321.14pg/mL, and negative control (K-) at 404.94pg/mL was found a significant difference between the intervention groups through the One-Way ANOVA test ($p < 0.001$). Post Hoc test to see the difference between study groups in pairs, there was a significant difference in the average levels of TGF-β1 in the K- and K+ groups (mean difference = 83.80; p<0.022*), in the K- and P1 groups (mean difference = 223.37; p<0.001*), in the K+ group with K- (mean difference = -83.80; p<0.022*), in the K+ group and P1 (mean difference = 139.57; p<0.001*), in group K+ and P2 (mean difference = 85.37; p <0.020*), group P1 and K- (mean difference = -223.37; p<0.001*), group P1 and K+ (mean difference = -139, 57; <0.001*), group P1 and P2 (mean difference = -54.19; 0.123*). Group P2 and control- (difference in mean = -169.17; p<0.001*), group P2 and control+ (difference in mean = -85.37; p<0.020*), group P1 and P2 were better at lowering TGF- levels 1 was compared with K+ with K-, but there was no significant difference between P1 and P2.

In the study of osteoclast cells, what was measured was the number of osteoclast cells around the lumen of the trabeculae in the apical teeth of chronic apical periodontitis Wistar rats. The number of osteoclast cells in the first treatment group (P1) with the application of calcium hydroxide paste and 10% green meniran leaf ethanol extract, which was applied intracanal, was 1.67 field of view. This number is lower when compared to the other three groups, namely in the second treatment group (P2) with a 2.17 field of view, positive control (K+) with a 4.67 field of view and negative control (K-) with a 5.00 field of view. , and found a significant difference between the intervention groups through the One-Way ANOVA test ($p<0.001$). Post Hoc test by looking at the differences between groups, there was a significant difference in the average number of

osteoclast cells in the K- group with P1 (mean difference = 3.33; p<0.001*), in the K- and P2 groups (mean difference = 2, 83; p<0.001*), group K+ with P1 (mean difference = 3.00; p<0.001*), in group K+ and P2 (mean difference = 2.50; p<0.001*), in group P1 and K - (mean difference = -3.33; p <0.001*), group P1 and K+ (mean difference = -3.00; p<0.001*), group P1 and P2 (mean difference = -0.50; <0.413), group P2 with K- (mean difference = -2.83; 0.001*), group P2 with control+ (mean difference = -2.50; p<0.001*). The K+ group with K- was better at reducing the number of osteoclast cells than P1 and P2, but there was no significant difference between K- and K-.

In the study of osteoblast cells, the number of osteoblast cells around the endosteum/trabeculae was measured in the apical teeth of chronic apical periodontitis Wistar rats. The number of Osteoblast cells in treatment group one (P1) with the application of calcium hydroxide paste and 10% green meniran leaf ethanol extract, which was applied intracanal, was 52.67 wide field of view. This number is higher when compared to the second treatment group (P2) with 32.17, positive control (K+) with 16.83 fields of view, negative control group (K-) with 11.17, and found a significant difference between the intervention group through One Way ANOVA test (p<0.001). Post Hoc test by looking at the differences between groups, there was a significant difference in the average number of osteoblast cells in the K- and P1 groups (mean difference = -21.00; p<0.001*), K- and P2 groups (mean difference = -15.33; p<0.013*), in the K+ group with P1 (mean difference = -35.83; p<0.001*), in the K+ group with P2 (mean difference = -20.50; p <0.002*), P1 group with K- (difference in mean = 21.00; p<0.001*), group P1 and K+ (difference in mean = 35.83; <0.001*), group P1 and P2 (difference in mean = 41.50; <0.001*), group P2 with K- (difference in mean = 15.33; <0.013*), group P2 and K+ (difference in mean = 20.50; <0.002*), Group P1 and P2 were better at increasing the number of osteoblast cells than K+ with K -, and found a significant difference between P1 and P2 (Figure 1).

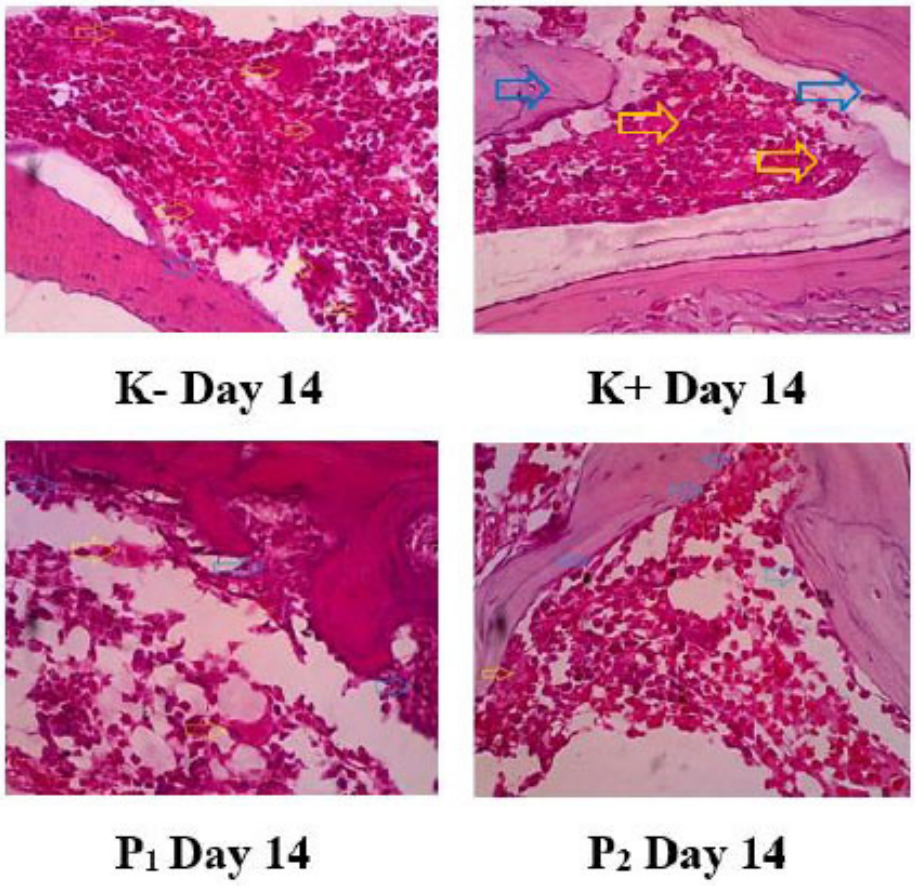

Figure 1. Histological description of the number of osteoclasts and osteoblasts on day 14 in the negative control group, positive control group, treatment 1 and treatment 2 with Haematoxylin – Eosin staining (100X magnification). Osteoblast cells are shown by blue arrows, and osteoclast cells are shown by yellow arrows.

Pathway analysis of osteoblast enhancement mechanism through MMP-9, TGF-β1, and osteoclast mechanism

In order to assess whether MMP-9, TGF-β1, osteoclasts, and osteoblasts work in one pathway, a multivariate analysis was performed with pathway analysis, as shown in Figure 2.

DISCUSSION

Levels of matrix metalloproteinase-9 (MMP-9) rats with chronic apical periodontitis

The bacterium *Enterococcus faecalis* is one of the etiologic factors of chronic infection in response to various inducers that can stimulate the production of gelatinases B from MMP-9. Matrix metalloproteinase-9 (MMP-9) is detected in endothelial cells, osteoblasts, fibroblasts, and inflammatory cells and is released during inflammatory reactions that are directly induced by bacterial endotoxins and their synthesis is controlled by inflammatory mediators such as IL-1, IL-6, TNF. -α, a prostaglandin (PG) that is also involved in MMP-9 expression.[17-19] MMP-9 release proves its role in inflammatory pathogenesis.[17-19]

The low levels of MMP-9 in the first treatment group (P1) indicated the high alkalinity of the medicament paste produced by the release of hydroxyl ions from the active compound of meniran extract and calcium hydroxide for 14 days. Based on research results, Blobe *et al.* stated that the high alkalinity resulting from the medicament paste could inhibit the damage process in the periapical tissue by immediately carrying out various cellular functions of TGF-β1 to regulate proliferation, growth, differentiation, adhesion, cell survival, production of extracellular matrix (ECM) proteins and

apoptosis of various cell types.[29,30] This research is also in accordance with the research Paula-Silva *et al.*, Dezerega *et al.*, and Lundmark *et al.*, which showed that calcium hydroxide medicament paste could act in MMP-9 inhibition in vivo.[28,31,32]

The high alkalinity produced in the dentin environment of the root canal will directly inhibit the activation of bacterial virulence (LPS/LTA), which is the main inducer of MMP-9 production so that it indirectly blocks the production of pro-inflammatory cytokine expression (TNF-α, IL-1β) and inhibits the release of pro-inflammatory cytokines such as growth factor (EGF, TGF-β) and phorbol ester.[32,33]

In the second treatment group (P2) where the MMP-9 levels were higher than in the first treatment (P1). This condition indicates that the reactive oxygen species (ROS) involved in MMP-9 activity are still higher around the apical lesion, resulting in oxidative stress. Based on the research Vianna study, the chemical interaction that occurs between $Ca(OH)_2$ powder and 2% chlorhexidine digluconate produces calcium digluconate salt, which can reduce the ionization process.[34] According to Signoretti *et al.,* the mechanism for releasing 2% chlorhexidine digluconate in calcium hydroxide is slow, so this condition can affect the rate of penetration of meniran ions into the dentinal tubules.[27] In addition, for 14 days, there was a decrease in the antibacterial effectiveness of calcium hydroxide, which could affect the alkanes of the root canal dentin environment. The slow penetration rate of hydroxyl ions cannot run well, thus affecting the tissue repair mechanism.[22-27] According to Athanassiadis *et al.,* in order to obtain its antimicrobial effect, chlorhexidine must be present for up to 12 weeks for the chlorhexidine molecule to be incorporated into the root canal dentin.[35]

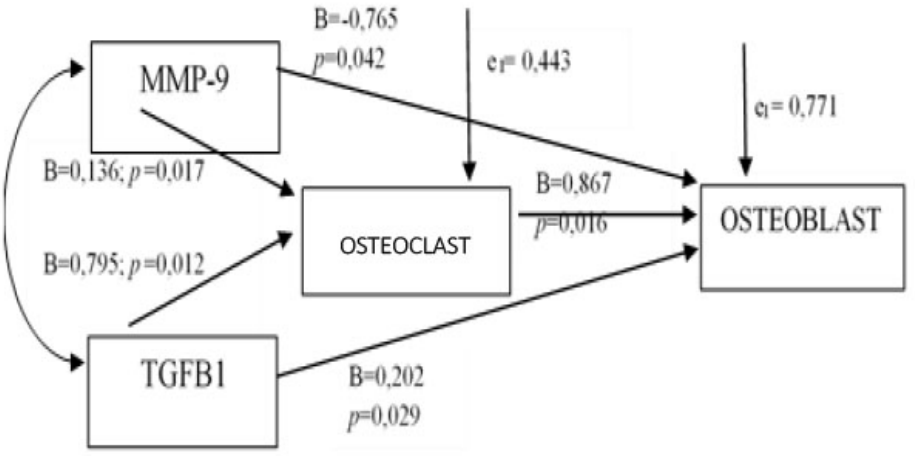

Figure 2. Pathway analysis of MMP-9, TGF-β1 against osteoblasts either directly or through osteoclasts and osteoclasts directly on osteoblasts.

The results show that:

1. The direct effect of MMP-9 on Osteoclasts is statistically significant, indicated by the value of p = 0.017 (<0.05).
2. The direct effect of TGF-β1 on osteoclasts is significant, indicated by the value of p = 0.012 (<0.05).
3. The effect of MMP-9 on osteoblasts directly through osteoclasts was significant, indicated by the value of p = 0.042 (<0.05).
4. The effect of TGF-β1 on Osteoblasts directly through Osteoclasts was significant, indicated by the value of p = 0.029 (<0.05).
5. The direct effect of Osteoclasts on Osteoblasts is significant, indicated by the value of p = 0.016 (<0.05)
6. The effect of MMP-9 through Osteoclasts on Osteoblasts is expressed by the magnitude of the B coefficient obtained by multiplying the B coefficient of its effect on MMP-9 with the B coefficient of the osteoclast effect on Osteoblasts = (0.136 x 0.867) and the path coefficient is 0.118. So the overall B coefficient value is 0.118 + 0.765 = 0.883. In conclusion, the role of MMP-9 in its effect on Osteoblasts either directly or through Osteoclasts is only 88.30%, while 11.70% is through other pathways due to other variables not examined in this study.
7. The effect of TGF-β1 through Osteoclasts on Osteoblasts is expressed by the magnitude of the B coefficient obtained by multiplying the B coefficient of its effect on TGF-β1 by the B coefficient of Osteoclast effect on Osteoblasts = (0.795 x 0.867) and the path coefficient is 0.689. So the overall B coefficient is 0.689 + 0.202 = 0.891. In conclusion, the role of TGF-β1 in its effect on Osteoblasts either directly or through osteoclasts is only 89.10%, while as much as 10.87% is through other pathways due to other variables not examined in this study.

In the second treatment group (P2) where the MMP-9 levels were higher than in the first treatment (P1). This condition indicates that the reactive oxygen species (ROS) involved in MMP-9 activity are still higher around the apical lesion, resulting in oxidative stress. In general, this condition can be caused by the antioxidant mechanism produced from the mixture of these three medicament ingredients that do not interact in a balanced way. According to Signoretti *et al.*, the mechanism for releasing 2% chlorhexidine digluconate in calcium hydroxide is slow, so this condition can affect the rate of penetration of meniran ions into the dentinal tubules.[27] In addition, for 14 days, there was a decrease in the antibacterial effectiveness of calcium hydroxide, which could affect the alkanes of the root canal dentin environment. The slow penetration rate of hydroxyl ions cannot run well, thus affecting the tissue repair mechanism.[22-27] According to Athanassiadis *et al.,* in order to obtain its antimicrobial effect, chlorhexidine must be present for up to 12 weeks for the chlorhexidine molecule to be incorporated into the root canal dentin.[35]

MMP-9 levels were higher in the positive control group (K+) at 4099.55pg/mL ± 671.51 compared to treatment group one (P1) and treatment two (P2) but lower than the negative control group (K-). This condition probably occurs

because the mixture of medicament materials between $Ca(OH)_2$ powder and 2% chlorhexidine digluconate produces a liquid consistency. The resulting interaction is a calcium digluconate salt which can reduce the ionization process due to the precipitation and coagulation of the cytoplasm due to cross-linking of proteins.[36-41] Chlorhexidine digluconate will deprotonate the guanidine group at pH >10, which is produced by $Ca(OH)_2$ in a liquid medium.[42-45]

Chlorhexidine digluconate 2% produces a slow and continuous ion release mechanism so that conditions can affect resistance factors and bacterial pathogenesis, including interactions with other bacteria in the root canal, forming synergies or negative associations with other bacteria, and also has the ability to evade the host immune system, and pathogenic bacterial resistance factors. The etiological factor of endodontic bacterial sequelae will trigger an exaggerated immune response that causes the release of pro-inflammatory cytokines and the emergence of oxidative stress.[41-45]

In the differences between groups, where treatment group one (P1) and treatment group two (P2) proved better in lowering MMP-9 levels than K+ with K-, there was no significant difference between P1 and P2. This condition could be due to the interaction between medicament paste mixtures which both have strong alkaline properties with high alkalinity and synergize between medicament ingredients with each other to prevent oxidative stress due to inducers of root canal bacterial endotoxins.

Transforming growth factor-b_1 (TGF-b_1) level

This study resulted in evidence that low levels of TGF-β_1 are an indicator to determine the occurrence of decreased expression of MMP-9 in suppressing chronic inflammatory processes in apical teeth with periodontitis cases. The osteoclastic process that occurs from MMP-9 will be able to activate TGF-β, which is not stored in the tissue.[45-50] Transforming Growth Factor-β_1 (TGF-β_1) regulates various secreted cellular functions to regulate proliferation, growth, differentiation, adhesion, cell survival, production of extracellular matrix protein (ECM) and death (apoptosis) of various cell types.[46] Based on the results of many studies, it is proven that TGF- β_1 functions as an immunomodulator that can inhibit the activity of IL-2 (interleukin-2), which has the potential to activate T cells, natural killer cells, and other cell types of the immune response system as well as regulation of T lymphocytes and multifunctionality of growth factor lymphocyte proliferation to prevent oxidative stress in inflammatory areas.[47-50]

The alkaline properties possessed by flavonoids and alkaloids can replace mineral bases in maintaining ionic balance in cells. Cushnie *et al.* stated that the flavonoids contained in meniran could inhibit the formation of free radicals due to their antioxidant properties, inhibit and reduce the fat peroxidation process, and change the structure of cell membranes.[46] Lower levels of pro-inflammatory cells can increase the fibroblast cells that make up the extracellular matrix (ECM) by forming collagen fibers in the apical lesion and facilitating the healing process.[47,48] Green meniran bioactive substance (*Phyllanthus niruri Linn*) as an immunomodulator can inhibit the release of pro-inflammatory mediators (TNF-α, IL-β, IFN-γ and PGE2) which can inhibit the activation of osteoblast cells thereby suppressing osteoclastogenesis. The presence of TGF-β1 in periapical lesions suggests an important role in the inhibition of tissue maintenance and tissue development.[51-55]

Based on the results of statistical tests, the overall difference in serum levels of TGF-β1 was found to be a significant difference between the intervention groups. This condition was probably due to all treatment groups on the 14th day of the start of a continuous process of tissue repair, arranged sequentially with several stages of complex biological processes.[52,53] This phase includes the inflammatory process, cell chemotaxis, cell mitosis, and extracellular matrix (ECM) protein synthesis and leads to tissue remodeling involving pro-inflammatory cytokines, growth factors (GF), hormones and proteases.[54]

There was a significant difference in the average levels of TGF-β1 between groups where the P1 and P2 groups were better at reducing TGF-β1 levels than K+ and K-, but there was no significant difference between P1 and P2. This condition may occur because the alkalinity produced in the intracanal medicaments of the three groups is a strong base that can increase the pH of root canal dentin. The resulting hydroxyl ion can trigger the activation of the immunomodulatory TGF-β1 to promote tissue proliferation and repair.

Osteoclast cell count

The results of this study indicate that the mean and standard deviation of differences between groups of osteoclast cell numbers in treatment group one (P1), namely the application of calcium hydroxide paste and 10% green meniran leaf ethanol extract, which was applied intracanal had a lower number of osteoclast cells, namely as much as 1.67 field of view ± 0.33 compared to the other three groups, namely in the second treatment group (P2) as much as 2.17 field of view + 0.37, the positive control group (K+) as much as 4.67 fields of view ± 0.49 and at the negative control group (K-) was 5.00 with a field of view ± 0.51. The occurrence of lower osteoclastic processes in treatment group one (P1) was probably due to the consistency of the mixture of medicament materials between $Ca(OH)_2$ and 10% ethanol extract of meniran green leaves, which produced a semi-solid (thick) paste. The thick paste can cause the paste to remain on the dentin surface area of the root canal for a long time with slow and gradual ion penetration to reach the periapical area.[30,41,42] The alkalinity of this mixture of medicament materials can stay in the root canal for a long time so that it can increase the mineral base of the root canal and maintain ion balance in tissue cells.[16,27]

The number of osteoclast cells in the second treatment group (P2) was higher than the first treatment group (P1), possibly because during the osteoclastogenesis process, a mixture of $Ca(OH)_2$ paste, 2% chlorhexidine digluconate and 10% green meniran leaf ethanol extract, had ion diffusion. Gradually and slowly, the hydroxyl begins to penetrate into the dentinal tubules of the root canal. The antimicrobial effectiveness of calcium hydroxide will decrease on the 14th day, but the 2% chlorhexidine digluconate

mixture and the bioactive substances of green meniran leaves will still maintain their strong alkaline properties.

The high number of osteoclast cells in the positive control group (K+) compared to the treatment group two (P2) and treatment one (P1) could be due to a mixture of medicament paste produced from $Ca(OH)_2$ and 2% chlorhexidine digluconate, which functions as a physical barrier. In the root canal, for a long period of time results in a slow ion release mechanism. The high pH produced by $Ca(OH)_2$ will precipitate chlorhexidine digluconate molecules, resulting in a reduction in the effectiveness of chlorhexidine antimicrobials.[51,52] The slow release of ions from 2% chlorhexidine digluconate will not be able to inhibit gram-negative bacterial endotoxins and dissolve necrotic tissue.[27]

Overall, there was a significant difference in the average number of osteoclast cells between the intervention groups, where one treatment group (P1) was lower than the other three groups. However, there was a significant difference in the average number of osteoclast cells where the positive control group (K+) and the negative control group (K-) were better at reducing the number of osteoclast cells compared to P1 and P2, but not found a significant difference between K- and K+. This condition is probably due to different cellular and adaptive host immune responses that can directly suppress the inflammatory process. The medicament paste quickly suppresses the chronic inflammatory response so that inflammation does not spread and is persistent.

Osteoblast cell count

The increase in the number of osteoblast cells in treatment one (P1) was probably due to the mixture of this medicament material with high hydroxyl ions, causing osteoprotegerin (OPG) to function as a balancing receptor that could inhibit the process of osteoclastogenesis, which was activated rapidly by the induction of the paste hydroxyl ion-molecule medicament.[21-25]

In the second treatment group (P2), the number of osteoblast cells was higher than the positive control group (K+) and negative control group (K-), possibly due to the high alkalinity produced from the three medicament paste ingredients that can directly activate local pluripotent mesenchymal stem cells from osteoblasts thus stimulating to proliferate and differentiate into preosteoblasts.[31-34] Proliferation of osteoblast cells will differentiate again, resulting in osteoid tissue becoming mature osteoblasts (immature bone consisting of type I collagen) and secreting large amounts of alkaline phosphatase, which plays an important role in depositing calcium and phosphate into the bone matrix in the matrix maturation phase.[35]

In the positive control (K+) with a number of osteoblast cells as much as 16.83, the field of view is lower than the treatment group one (P1) and treatment two (P2) because 2% chlorhexidine digluconate as a mixing agent has a slow diffusion of hydroxyl ions over time. Thereby affecting the rate of penetration of hydroxyl ions. The increase in the number of osteoblasts is strongly influenced by the consistency and viscosity of the mixing material, which will determine the release of hydroxyl ion diffusion and the rate of ion penetration into the dentinal tubules and infected periapical tissue.[12-17]

The number of osteoblast cells in the negative control group (K-) was lower than the other three groups due to the persistent bacterial endotoxin (LPS/LTA), so the endotoxin would induce osteoblasts to express nucleotide-binding oligomerization domain-containing protein-1 (NOD1) and NOD2, which contains a group of nod-like receptor proteins (NLRs), which act as intracellular sensors for bacterial peptidoglycan and initiate the production of pro-inflammatory mediators.[37-41] NOD1 and NOD2 are associated with adapter proteins, namely apoptosis-associated speck-like protein (ASC), to stimulate caspase-1 and caspase-8 activation, enzymes that show increased osteoblast activity after bacterial induction.[5-7,17,18]

There was a significant difference in the average number of osteoblast cells where the P1 and P2 groups were better at increasing the number of osteoblast cells than K+, and K-and found a significant difference between P1 and P2. The results of this study prove the fourth hypothesis that the administration of 10% ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide as an intracanal medicament in the teeth of chronic apical periodontitis Wistar rats resulted in a higher number of osteoblast cells than the control.[12-18,51,52]

This study provides test results for the presence of active chemical compounds in the ethanol extract of meniran leaves through the isolation stage of the phytochemical test followed by the LC-MS analysis stage. The results of the test of the highest active compound content of meniran extract were chemical fragments of the polyphenol component of the flavonoid group - flavanols with theaflavin chemical compounds and chemical fragments of the flavone group with 5,6,7-trimethoxyflavone chemical compounds.[12-18]

The results of this study support the researcher's hypothesis that the administration of 10% ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) in a mixture of calcium hydroxide as an intracanal medicament paste for 14 days can reduce oxidative stress through bacterial eradication so that it inhibits osteoclastogenesis differentiation and stimulates osteoblastic activity, which can be proven by reducing oxidative stress. Levels of MMP-9, TGF-β1 and the number of osteoclast cells can increase the number of osteoblast cells.[12-18]

The novelty of the study, according to the results of the study, proves the theoretical mechanism of therapy for chronic apical periodontitis by ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) through the ability to suppress TLR4 signals and stimulate interleukin 10, which can suppress the formation of the pro-inflammatory cytokine TNF-β, resulting in a decrease in MMP-9 activity. This study has a limitation that only appears in the Wistar rats setting experiment and is necessary to conduct further research that can implement the effectiveness test as an initial sterilization material at the cleaning stage and root canal shaping of human teeth with cases of chronic apical periodontitis.

CONCLUSION

Green meniran extract (*Phyllanthus niruri Linn*) and its role as an intracanal medicament in teeth with chronic apical periodontitis have not been studied and published. Overall, the results of this study prove that intracanal medicament paste from 10% ethanol extract of green meniran leaves (*Phyllanthus niruri Linn*) can be used as a calcium hydroxide admixture in chronic apical periodontitis teeth and can be used as root canal sterilization with an application time of up to 14 days without having to replace the paste.

ETHICAL CONSIDERATIONS

Ethics approval has been received prior to the study being conducted, and also already following cope and ICJME guidelines.

CONFLICT OF INTEREST

None declared.

AUTHOR'S CONTRIBUTION

All the authors were responsible for the data gathering and analysis until the published article.

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