

RESEARCH ARTICLE

The Secondary Metabolites Screening and the Effectiveness (*Ananas comosus* (L.) Merr of the Queen Pineapple Stems in Decreasing the Number of *Enterococcus faecalis*'s Colonies

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ABSTRACT

Background: A cause of endodontic treatment failure was sterilization process error so that bacterias still left in the root canal. *Enterococcus faecalis* were resistant to sterilization medicines, it was necessary to develop natural sterilization medicines such as the Queen pineapple stems that had the antibacterial content. **Purpose:** The aim of this research was to determine the effectivity of the Queen pineapple (*Ananas comosus* (L.) Merr) stems extract in decreasing the *Enterococcus faecalis*'s colonies. **Materials and Methods:** This research was true experimental with the post test only control group design. The Queen pineapple stems extract tested its secondary metabolites contents. *Enterococcus faecalis* ATCC 29212 cultured in Brain Heart Infusion Broth synchronized with Mc Farland 0,5 solution and divided into seven groups (n=7). It used dilution series method, each BHIB tube given by the 100% extract (P1); 50% (P2); 25% (P3); 12,5% (P4) ; 6,25% (P5) ; 3,125% (P6) and without the extract (K(-)), incubated for 24 hours and streaked on Mueller Hinton Agar and incubated for 24 hours, then calculate the colonies' amount of each group. Datas analyzed by Mann-Whitney U-Test because only 50% and 25% concentration can be calculated. **Result:** The result showed that the Queen pineapple stems contained flavonoids, tannins, alkaloids, saponins, also there were significant differences among the 50% and 25% concentration groups $p < 0.05$. **Conclusion:** The Queen pineapple stems extract's 50% concentration was effective to decrease the number of *Enterococcus faecalis*'s colonies.

Keywords: Endodontic failure, *Enterococcus faecalis*, *Ananas comosus* (L.) Merr., antibacterial, sterilization.

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BACKGROUNDS

Endodontic is the part of dentistry involving the diagnosis and the treatment of disease or injury in pulp tissue and periapical tissue. Conventional endodontic divided into three main stages, there are (1) the biomechanical preparation of the root canal (cleaning and shaping), (2) disinfection of the root canal, and (3) the root canal obturation. The endodontic treatment goal is to reduce or eliminate microorganisms and their products from root canal so that teeth can be maintained as long as possible in the mouth and restore the aching teeth to be accepted biologically by the surrounding tissue.^{1,2}

The existence of endodontic treatment failure can be caused by (1) irritation apical tissue fluids that infected in root canal filled not hermetic [63,46%], (2) error while maintenance [14,42%], and (3) error while diagnosis [22,12%].³ Root canal treatment failure is also often caused by errors on the sterilization procedure. One of the sterilization procedure is eliminating the gram-positive organisms.⁴

Biomechanical preparation and root canal irrigation are very important to reduce the number of bacteria during endodontic treatment. They also need to be supported by the administration sterilization because it will help to eliminate bacteria remain after the preparation or at least inhibit the recurrent infection in root canal treatment between visit⁵, neutralize the remnants of debris in root canal, control and prevent pain.⁶

The bacteria that are commonly found in root canal are anaerobic, microaerophile, facultative and obligate anaerobic bacteria⁷, one of them is *Enterococcus faecalis* that is the most commonly found (77%).⁸ Based on research of Gomes *et al*, from the 53 species of bacteria in 60 kind of root canal, bacteria that is most commonly found in root canals with endodontic failure is *Enterococcus faecalis* (27%).⁹

One of plants that have the potential as a natural root canal sterilization medicine is pineapple (*Ananas comosus* (L.) Merr) that is widely cultivated in Indonesia subtropical, especially from Queen varieties.¹⁰ Pineapples have very complex content, rich in mineral both macro and micro, organic substances, water and vitamin. It also contains fiber, chlorine, iodine, phenols, magnesium, sodium, potassium, bromelain enzymes, flavonoids, and saponins.^{11,12,13,14} Antibacterial contents of pineapples are bromelain enzymes, flavonoids and saponins.

Bromelain enzymes are the only nutrient can only be found in pineapple plant, which is in the stem, bark, leaves, fruit or stems in different quantities.^{15,11} Bromelain bromelain enzymes in the form of condensed extract are most numerous on the stems, which is about 0,1% to 0,6%.¹⁶ Therefore, in this study, part of the pineapple plant that will be used are the stems. Based on explanation above, it's necessary to know the effects of Queen pineapple stems extract as an alternative medicine root canal sterilization in inhibiting the growth of *Enterococcus faecalis* bacteria.

MATERIAL AND METHODS

The research was true experimental with the post test only control group design.

The parameters were seen in this study are a secondary metabolite content of qualitative and quantitative Queen pineapple stem extract, also the number of *Enterococcus faecalis* bacterial colonies that growing on *Mueller Hinton Agar* (MHA) media after being treated. The treatment group in this study were divided by the concentration of the extract stem *Ananas comosus* (L.) Merr. Queen, the group P1 is 100% concentration; P2 is 50% concentration; P3 is 25% concentration; P4 is 12.5% concentration; P5 is 6.25% concentration; P6 is 13.125% concentration; and K (-) is without giving Queen pineapple stem extract.

The procedure begins with the preparation of the stems *Ananas comosus* (L.) Merr obtained from Pineapple Plantation, Kediri. A kilogram of dried stem was extracted using 95% ethanol by maceration for 3 days (flushed every day). Then the extract is filtered using filter paper (1st filtrate) and the rest was re-extract using 95% ethanol and then filtered (2nd filtrate). Furthermore, the 1st and 2nd filtrate were collected, evaporated by the evaporator at 70°C until the volume becomes $\frac{1}{4}$ of the initial volume and followed by drying in a water bath until it becomes a condensed extract.

Condensed extract was divided into three to test the qualitative and quantitative secondary metabolites content, and also the sensitivity test of bacteria. Secondary metabolite content test was done at the Laboratory of Phytochemistry, Widya Mandala

Catholic University Surabaya, which includes examination tannins, alkaloids, saponins and total flavonoids. Qualitative secondary metabolite content test is done by inserting the sample extract stem *Ananas comosus* (L.) Merr tubes with the addition of different reagents for each compound test.^{17,18} Quantitative secondary metabolites content of total flavonoids was done using spectrophotometry.¹⁹

The next step is the extract dissolved in DMSO 1% to obtain the desired concentration. Furthermore, Queen pineapple stem extract stir until mixed with the solvent using a vortex for 10 seconds. After that, it sterilized and filtered with a 0.45 μ m diameter of syringe microporus membrane to maintain the purity and contamination of other microorganisms in the Queen pineapple stem extract.

The *Enterococcus faecalis* bacteria ATCC 29212 was obtained from the Center for Clinical Microbiology Laboratory of Health, Surabaya that cultured in Brain Heart Infusion (BHI) media liquid that has been incubated for 24 hours at 37°C in an anaerobic atmosphere. Turbidity of *Enterococcus faecalis* bacteria suspension equated with Mc Farland 0.5 standard to obtain a bacterial suspension containing 1,5x10⁸ CFU/mL with the naked eyes by holding the test tube side by side and looked at the black-white striped background. If the turbidity of the bacterial suspension is still not the same, the bacteria suspension was diluted or given additional bacteria, then homogenized with *sentrifuge*.²⁰

The next stage is *Enterococcus faecalis* inoculation from BHI liquid media that has been synchronized with a Mc Farland 0.5 solution turbidity (a

bacterial suspension containing 1.5×10^8 CFU/mL), and then wipe the bacterial culture on the entire surface of the MHA media by using a sterile swab.

The determination of the concentration from the ingredient pineapple stem extract (*Ananas comosus* (L.) Merr) Queen of the decrease in the number of *Enterococcus faecalis* bacterial colonies and bacterial sensitivity test was using dilution series/dilution.²⁰ First, prepare the sterile tube and labeled P1, P2, P3, P4, P5, P6 and K-. Each tube is filled with liquid BHI media (0.5 mL). Queen pineapple stem extract with 100% concentration on the tube inserted into the P1 tube until 1 mL. A half mL of P1 tube is inserted into the P2 tube up to 1 mL. The dilution from the second tube is $\frac{5}{10} = \frac{1}{2} = 50\%$. Furthermore, from P2 tube was taken 0.5 mL then inserted into the P3 tube so that the dilution is $\frac{1}{4} = 25\%$. In the same way, done until P6 tube, and the 0.5 mL of P6 tube removed. K- tube is the negative control containing liquid BHI media. After the serial dilution finished, put 0.01 mL *Enterococcus faecalis* inoculum on P1 tube until the K- tube. Then, each tube was incubated for 24 hours at 37°C. The reading of the results of serial dilution of material on the growth of *Enterococcus faecalis* bacteria, by visual observation by an expert observer who observes the presence or absence of growth characterized that signed with turbidity or sediment. The dark colored and turbidity of extract material occurs in all the tubes, then streak in each tube by taken 1 osse and implanted in MHA media. Incubating at 37°C for 24 hours, and then observing the growth

of bacteria from streak results and count the number of colonies grow was done manually by creating a line of boxes in petridish and expressed as CFU.

RESULT

The results of the qualitative and quantitative secondary metabolites test and a sensitivity test *Enterococcus faecalis* bacteria to the Queen pineapple (*Ananas comosus* (L.) Merr) stem extract with 100%; 50%; 25%; 12.5%; 6.25 and 3.125% concentration and 1% DMSO solution as a control. They were analyzed descriptively that aims to obtain a picture of the distribution and summarizing data to clarify the presentation of the results, then the hypothesis test using *Mann-Whitney U* test with 95% significance level ($p=0,05$) by using SPSS 16.0 version.

Table 1. The results of the secondary metabolites Queen pineapple (*Ananas comosus* (L.) Merr) stem extract.

Compound	Note	Presentage (%)
Flavonoids	Positive	0,00033525
Alkaloids	Positive	*
Saponins	Positive	*
Tannins	Positive	*

Note: *not tested

Based on table 1, Queen pineapple (*Ananas comosus* (L.) Merr) stem extract has the antibacterial content that can decrease the number of *Enterococcus faecalis* bacterial colonies.

Table 2. Mean and deviation standard of Queen pineapple (*Ananas comosus* (L.) Merr) stem extract concentration in decrease the number of *Enterococcus faecalis* bacterial colonies

Concentration of Extract	Replication	Mean (CFU/mL) $\pm$ Deviation Standard
100% (P1)	7	*
50% (P2)	7	2076.29 $\pm$ 1.512
25% (P3)	7	1.95E4 $\pm$ 1.329
12,5% (P4)	7	∞
6,25% (P5)	7	∞
3,125% (P6)	7	∞
DMSO 1% (K(-))	7	∞

Note: *there is not a bacterial colonies growth

∞ : Can't be calculated

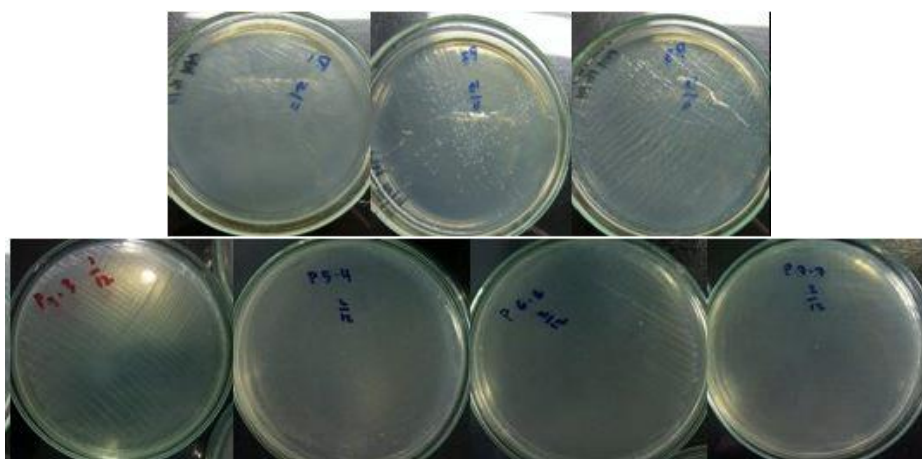


Figure 1. The number of *Enterococcus faecalis* bacterial colonies in MHA media of P1 group (100%) is not found the growth of bacterial colonies, in P2 group (50%) were found the growth of bacterial colonies that far fewer than in P3 group (25%), and in P4 group (12,5%), P5 group (6,25%), P6 group (3,125%), K(-) group (DMSO 1% can not be calculated manually or with Quebec Colony Counter (QCC) because the number of bacterial colonies are too many.

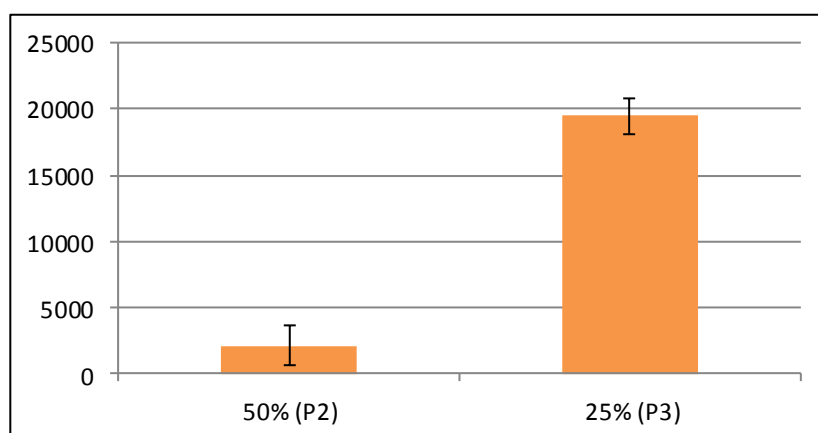


Figure 2. The concentration mean bar chart of Queen pineapple (*Ananas comosus* (L.) Merr) stem extract concentration that were the most effective in decreasing the number of *Enterococcus faecalis* bacterial colonies.

Based on figure 1 and figure 2, it appears the decline in the number of *Enterococcus faecalis* bacterial colonies that is quite dramatically in the 50% compared to 25% Queen pineapple (*Ananas comosus* (L.) Merr) stem extract.

Test of normality is using the Shapiro-Wilk because the samples used are ≤ 50 with significant value 0,05.²¹

Shapiro-Wilk test results show that the data distribution is not normal ($p < 0,05$). Therefore, it followed by non-parametric tests using the Mann-Whitney U Test, as this study has two groups, unpaired and have a numerical measurement scale.

The results of Mann-Whitney U test show a significant value different that is 0,002 ($p < 0,05$). This shows a significant difference between the treatment groups. It can be concluded "there is a significant difference in the mean reduction in the number of the *Enterococcus faecalis* bacterial colonies between the 50% and 25% concentration group".

DISCUSSION

The main cause of endodontic failure is the ability of microorganisms to survive in the apical root canals that have been treated the endodontic treatment.²² Root canal sterilization medicine administration is an important step in killing the bacteria in the root canals.²³ *Enterococcus faecalis* is a bacteria that can survive in the root canals after endodontic treatment because of the factors virulent.²⁴ The samples used in this study is the *Enterococcus faecalis* ATCC 29212 bacteria, because it is based on the research of Subbiya et.al. that have similar virulence to the

Enterococcus faecalis that isolated from the root canal.²⁵ Because of the virulence of *Enterococcus faecalis* ability in root canals, it is necessary to develop the materials sterilization from the natural ingredients such as Queen pineapple.

The test materials used in this study is Queen pineapple (*Ananas comosus* (L.) Merr) stem obtained in Pineapple Plantation Kediri that were ripe and fresh because it contains the bromelain enzyme which is more than the pineapple fruit that still green or unripe. Election on the pineapple stems as the test material because in the form of condensed extract, the content of bromelain enzymes are most commonly found in pineapple stems those are as much as 0,1-0,6%.¹⁶ Queen *Ananas comosus* (L.) Merr stems extracted by maceration method using ethanol 95%. It aims to release antibacterial compounds both polar or non-polar.²⁶ After obtained the 100% Queen *Ananas comosus* (L.) Merr stems extract with thick and dense consistency, extract divided to secondary metabolite content of qualitative and quantitative test, and then the rest will be split into several concentration by using DMSO 1% solvent.

The secondary metabolite content test which done are a qualitative tests contain tannins, alkaloids and saponins, and also the quantitative test contain total flavonoids. The qualitative test of tannins, alkaloids and saponins showed the positive results, as well as the quantitative test of total flavonoids obtained 0,00033525 %.

The initial concentration of Queen *Ananas comosus* (L.) Merr stems extract studied were 100%; 50%; 25%; 12,5%; 6,25% and 3,125%

refers to research of Anwari and Azizah using the dilution series/dilution method.^{27,28,29} Dilution method can determine the Minimal Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC).²⁹

In this study can not be determined the limit of MIC and MBC for the Queen *Ananas comosus* (L.) Merr stems extract were too thick and turbid. So that in BHI broth media can not be seen the turbidity in front of the black line visually, which is to determine the limits of MIC. From the results of streak from BHI broth media to MHA media, the concentration of Queen *Ananas comosus* (L.) Merr stems extract that was not found the growth of *Enterococcus faecalis* bacterial colonies was at 100% concentration group. However, 100% can not be determined as the limit concentratin of MBC because the concentration distance is too far from 50% to 100%. To be more precise in determining the limits of MBC, 100% concentration thinned back into some concentration to reach 50% concentratin.

Although it can not be determined the MIC and MBC limits of Queen *Ananas comosus* (L.) Merr stems extract, from the results of streak at BHI broth media to MHA media n be found a decrease in the number of *Enterococcus faecalis* bacterial colonies quite dramatically at 50% to 25% concentration. At 12,5%; 6,25%; 3,125% concentration and negative controls can not be calculated manually or with QCC because the amount is too many.

The results show that the greater the concentration of Queen *Ananas comosus* (L.) Merr stems extract, the greater the decrease in the number of

Enterococcus faecalis bacterial colonies. This is expected because of the greater the concentration used, the content of antibacterial in the extract even greater.

Infected root canal is a condition where the nutrition is inadequate, there are toxins from other bacteria and invasion of endodontic sterilization medicine. In this condition, *Enterococcus faecalis* lost the ability to grow and develop but remain alive, and pathogenic or often called *Viable but Nonculturable* (VBNC) phase. *Enterococcus faecalis* form the protein-collagen (Ace) bonding which act as an adhesion in root canals.^{30,31} The bromelain enzymes as a proteolytic enzyme, capable of breaking the salivary proteins bond in the collagen that used by bacteria as an attachment media becomes binding peptides and amino acids,^{12,32,33} which can lower the surface tension of the bacteria cell wall that cause the cell wall are not selective in passing solutes and other substance that may change physical and chemical properties of the cell membrane and preventing their normal functioning so as to inhibit and kill the bacteria.¹²

The flavonoid compound has the ability to inhibit bacterial growth by causing damage to the bacterial wall permeability, microsomes and lysosomes as a results of the interactions of flavonoids with bacterial DNA.³⁴ Inhibit ability of these flavonoid compounds can prevent *Enterococcus faecalis* resistance. These resistance genes obtained from DNA mutations or acquisition of new genes through the transfer of plasmids and transposons, and stored in the plasmid that can be transferred at any time.³⁵

Although saponins do not attack *Enterococcus faecalis* virulence directly, they can inhibit the growth of bacteria by destroying the cell wall surface. Saponins can cause the leakage of proteins and enzymes from within cell, lowering the surface tension of bacterial cell wall and membrane permeability damage with insert the aglycon into the lipid bilayer so that creating pores with diameter of 40 to 50 Å. Saponin also bind the cytoplasmic membrane that disturb and reduce the stability of the cell membrane that eventually led to the cytoplasm to leak, so that substances inside the cell were out and lead to cell death.^{36,37}

Tannins work by interfering with synthesis of peptidoglycan so that the forming of cell walls become less perfect. This condition will cause damage to the cell wall or cell membrane permeability loss, so that the entry and exit of substances such as water, nutrients, enzymes were unselected. If the enzymes out of the cells, there will be inhibition of the cell metabolism and the formation of ATP needed for the growth and proliferation of cells. Disruption of permeability causes the cell can not perform life activities that are inhibited or even die.^{38,39} Tannins can also bind with lipoteichoic acid (LTA) in the peptidoglycan of gram-positive bacteria that is a virulent factor that cause bacteria can adhere to the surface of the root canal, tissue inflammation and tissue damage. Tannins which bind with LTA cause bacterial growth will be more easily inhibited by antibacterial component.^{40,24}

Action mechanism of alkaloids which disturb the constituent component of peptidoglycan in the

bacterial cell.³⁶ Gram-positives bacteria have a cell wall composed of peptidoglycan thick layer (20-40 layers with thickness from 0,02-0,06 µm) and teichoic acid containing alcohol (gliserol atau ribitol).⁴¹ Teichoic acid consist of two kinds, these are teichoic acid bonded to the peptidoglycan covalently, and membrane teichoic acid (lipoteichoic acid/LTA) bonded to the membrane glycolipids covalently.⁴² cell wall synthesis disturbance caused LTA release which normally regulates the activity of hydrolytic enzymes that play a role in the dynamic changing of the cell wall in the growth and division of cell. When the LTA as a regulator is lost, these enzymes hydrolyze cell wall and causing cell death.^{43,36}

CONCLUSION

In this study, it can be concluded that the Queen *Ananas comosus* (L.) Merr stems extract have the effectivity to decrease the number of *Enterococcus faecalis*'s colonies at 50% concentration. The qualitative secondary metabolite content of Queen *Ananas comosus* (L.) Merr stems extract contain flavonoids, tannins, alkaloids and saponins. The quantitative secondary metabolite content of Queen *Ananas comosus* (L.) Merr stems extract contain 0,00033525 % flavoid total.

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