Antibacterial Activity of Red Pine (*Pinus densiflora*) and Sumatran Pine (*Pinus merkusii*) Leaf Extracts against Oral Pathogens

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Abstract

The aim of the experiment was to find out the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of P. densiflora and P. merkusii leaf extracts. Dental and oral diseases are potentially caused by some oral pathogens. Various antimicrobial agents have been developed to prevent dental diseases because they have more minimal side effects than synthetic ones. Red pine (Pinus densiflora) and Sumatran pine (Pinus merkusii) have active compounds such as Triterpenoid, Limonene, Benzoic acid, Cinnamic acid, Tannin, Flavonoid, and Saponin that potentially develop natural antibacterial agents. P. densiflora and P. merkusii leaf extracts were tested for their antibacterial activity against Enterococcus faecalis, Streptococcus mutans, and Phorymonas gingivalis which grew in BHI (Brain Heart Infusion) Broth. The MIC and MBC values were determined by calculating the growth of bacterial colonies on NA media in CFU/ml. The data were analyzed using ANOVA and Tukey with SPSS for windows. The result showed a very significant different ($P<0.05$) with MIC of P. merkusii was at 0.39%, 0.39%, and 0.78%, and MBCs of 0.78%, 0.78%, and 1.56%. While P. merkusii were at the MICs of 1.56%, 1.56%, and 3.125%, and at the MBCs of 3.125%, 3.125%, and 6.25% against E. Faecalis, S. mutans, and P. gingivalis. The result indicates that P. densiflora and P. merkusii had the antibacterial activity against E. faecalis, S. mutans, and P. gingivalis.

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Introduction

Dental caries and periodontitis are the most common oral diseases and major causes of tooth loss[1]. Dental and oral diseases can result in severe infection, and thus antibacterial agents in many forms are necessary for the prevention or treatment of the diseases. Antibacterial agents consist of synthetic and natural substances. The use of synthetic antibacterial agents has been reported to cause some side effects, including bacterial resistance and tooth discoloration. The natural antibacterial agents have been developed because they have more effective antibacterial effects and minimal side effects[2].

Medicinal plants that can produce natural antibacterial agents include *P. densiflora* and *P. merkusii*. Some studies have reported active compounds in pine leaf extract have antibacterial, antifungal, anti-inflammatory, and antioxidant effects[3,4]. Both types of pines have been proven to have antibacterial properties against some positive and negative gram bacteria[3,5].

Therefore, the present study aimed to investigate whether or notred pine and Sumatran pine leaf extracts can inhibit and kill oral bacteria.

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Materials and methods

This present study has been approved by Ethics Commission of the Faculty of Dental Medicine, Universitas Airlangga, Surabaya (No.241/HRECC.FODM/V/2019). The sample used was a stock of bacteria obtained from

patients examined by the Research Center Faculty of Dental Medicine, Universitas Airlangga.

P. densiflora leaf extract at a 100% concentration was obtained from Seoul National University, Korea. It was then diluted at BPKI (Indonesian Institute of Research and Consultation) Surabaya. As much as 2 ml of the extract was taken and then diluted with 1 ml of aquadest and 2-3 drops of glycerin to accelerate dissolution. The extract was diluted in several concentrations, i.e., 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.195%, and 0.0975%.

The *P. merkusii* leaves were collected from PTPN XII Mumbul Garden, Jember, East Java, Indonesia. The leaves were dried in 6 hours, then cut 0.5-1 cm long. One kilogram of leaves was mashed, soaked in 2 L of 70% ethanol, stirred several times, and stored in an Erlenmeyer flask in 24 hours. The leaves were soaked at a room temperature on a shaker at a speed of 120 rpm continuously for 24 hours. After 24 hours, the solution was filtered using the Whatman filter paper No. 41 to obtain brown mass. The solvent (ethanol) in the macerate was then evaporated with a rotary vacuum evaporator at a temperature of 50-60°C. Finally, thick ethanol-free liquid (100% pure extract) was obtained, and 2 ml of the extract was then diluted in several concentrations, i.e.,12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.195%, and 0.0975%[6].

The phytochemical test results of *P. densiflora* and *P. merkusii* leaf extracts were conducted first at BPKI Surabaya before the antibacterial activity test. The results can be seen in Table 1.

Phytochemical compounds	*Pinus densiflora* concentration	*Pinus merkusii* concentration
Triterpenoid	4. 80%	3.61%
Alpha-pinene	2.44%	1.22%
Beta-pinene	2.36%	2.38%
Limonene	3.19%	2.88%
Benzoic acid	0.21%	0.38%
Cinnamic acid	0.55%	0.41%
Tannin	1.08%	6.05%
Flavanoid	3.18%	2.48%
Saponin	2.18%	1.42%

Table 1. Phytochemical analysis results (in %).

Bacteria culture was taken using sterile osse, planted on BHI Broth, and incubated within 24 hours at 37^{0}C. The culture was adjusted with the Mc. Farland standard which resulted in 0.5 or equivalent to 1.5x108 CFU/ml to obtain bacteria with a specific concentration. After obtaining the same turbidity as the standard, the suspension is diluted[7].

This study employed a dilution method for the antibacterial test. Twenty sterile tubes were prepared. They consisted of 8 tubes for the administration of *P. densiflora* leaf extract and 2 tubes for control, and 8 tubes for the administration of *P. merkusii* leaf extract with various concentrations, and 2 tubes for control. The 0.05 ml bacterial suspension standardized with 0.5 Mc Farland (1.5x108 CFU/ml) was planted in a tube of BHIB and *P. densiflora* and *P. merkusii* leaf extracts at various concentrations, i.e., 12.5%, 6.25%, 3.125%, 1.56%, 0.78%, 0.39%, 0.195%, and 0.0975%. The 0.05 ml bacterial suspension and BHIB without the mixture of *P. densiflora* and *P. merkusii* leaf extracts were added to K + (positive control) test tube. While the K- (negative control) test tube only contained BHI Broth without additional *S. mutans*, *P. densiflora*, and *P. merkusii* leaf extracts to ensure no bacterial contamination in the media. Each group consisted of 4 samples. Then, all test tubes were incubated in an anaerobic incubator at 37ºC for 1 x 24 hours.

The growth of bacteria from *P. densiflora* and *P. merkusii* extracts could be observed by counting the number of colonies growing in 0.1 ml bacterial subculture from each test tube. Moreover, positive and negative control groups on nutrient agar were isolated using the spreader method and were incubated in an anaerobic environment at 37ºC for 2 x 24 hours.

The MIC and the MBC were determined by counting the number of colonies growing on nutrient agar manually and comparing the number with that under the positive control, expressed as CFU/ml. The results showed the lowest concentrations of *P. densiflora* and *P. merkusii* leaf extracts could inhibit the growth of the bacteria known as MIC. While MBC was taken from the lowest concentration that results in 99.9% bacterial death compared to the positive control[8]. The oral pathogens test results of the MIC and MBC can be seen in table 2.

The Kolmogorov-Smirnov test was employed to identify the normal distribution of data. Besides, this study also utilized the

Levene test to examine the homogeneity of the data and the One-way ANOVA to identify the significance of all treatment groups.

Bacteria	MIC	MBC
Enterococcus faecalis	1.56%	3.125%
Streptococcus mutans	1.56%	3.125%
Pheromones gingvalis	3.125%	6.25%

Table 2. MIC and MBC of *Pinus merkusii* (in %).

Results

P. densiflora leaf extract could inhibit *E. faecalis* and *S. mutans* when its MIC and MBC was at 0.39% and 0.78%, respectively. Meanwhile, the MIC and MBC of *P. merkusii* against the bacteria were at 1.56% and 3.125%, respectively. *P. densiflora* leaf extract inhibiting *P. gingivalis had* the MIC and MBC at 0.78% and 1.56%, respectively. The MIC and MBC of *P. merkusii* to inhibit the bacteria were at 3.125% and 6.25%, respectively. The One-Way ANOVA test showed a significance value of 0.000 ($p<0.05$), meaning there were significant differences in the number of bacterial colonies between groups.

Discussion

Antibacterial activity is influenced by several factors, such as the concentration of the extract, the content of antibacterial compounds, the diffusion power of the extract, and the types of bacteria that are inhibited[9]. The antibacterial activity test on Gram-positive (*E. faecalis* and *S. mutans*) was better than Gram-negative (*P. Gingivalis*) due to the nature of the bacteria's cell walls. In Gram-positive bacteria, the cell wall structure is simpler and layered with low lipid content (1-4%), allowing bioactive materials to enter the cell and find targets to work according to its mechanism more easily[10]. Additionally, the phytochemical test showed that the two pines had the antibacterial activity from the contained of the Triterpenoids, Alpha-pinene, Beta-pinene, Limonene, As. Benzoate, As. Cinammic, Tannin, Flavonoids, and Saponins.

Alpha-pinene and beta-pinene are the main terpenoids in pine trees. Terpenoids work by forming lipid monolayer in cell walls, causing damage to the porin structure. Porin damage results in decreased cell permeability and inhibits the nutrients entry to bacteria[11].

Benzoic acid in pine trees plays a role in the acid molecular diffusion. Low pH level of the mouth causes interference with cellular activities such as glycolysis and active transport. Benzoic acid also causes damage to several enzymatic components which affect the acetic acid metabolic activity, oxidative phosphorylation, and the citric acid cycle[12].

In the bacterial activities, the bacteria need ATP derived from glucose. Cinnamic acid inhibits enzymes that play a role in glucose intake, resulting in decreased the amount of ATP. Decreased ATP inhibits the bacterial activities[13].

Meanwhile, tannins are antibacterial in three mechanisms. First, tannins can prevent bacterial adhesion in the host, and thus no colonies and bacterial replication occur. Moreover, tannins react on cell walls, and then protein denaturation occurs. Denatured proteins cause bacterial cell walls to lyse easily and kill the bacteria. Finally, tannins can inhibit the reverse transcriptase and DNA topoisomerase that can prevent the growth bacterial cells due to strong iron-binding capacity[14].

Flavonoid compounds have an antibacterial activity in three mechanisms. Initially, extracellular protein complexes formed by flavonoids on bacterial cell walls allow phenolic compounds to enter and damage the cell membranes leading to bacterial death[15]. Besides, flavonoids inhibit the topoisomerase activity, DNA replication process, and DNA synthesis[16,17]. Eventually, flavonoids can inhibit the reduction of cytochrome C which causes reduced oxygen and disrupts bacterial metabolism and biomolecules[15].

Saponins are glycoside (polar) and triterpenoid (non-polar) compounds. Saponins are classified as surfactants that can dissolve polar and non-polar compounds[16]. Saponins diffuse across the cell wall membranes and are bound with the plasma membranes, leading to instability of permeability in the cell walls and cytoplasmic leakage. These results cause interference with some bacterial physiological activities[18]. The research trials of *P. densifora* and *P. merkusii* leaf extracts showed that the higher the concentration was, the more effective the extracts reduced the number of oral bacterial colonies. Observed from the mechanism of the active compounds, both

Pinus densiflora and *Pinus merkusii* are quite effective both in inhibiting and killing oral pathogens. As a result, further examinations are required to develop natural antibacterial agents from alternative materials in pine trees. The findings of study can also be references for PERHUTANI Indonesia to explore the potentials of *Pinus merkusii* optimally.

Conclusions

In conclusion, *P. densiflora* and *P. merkusii* have an antibacterial activity against *E. faecalis, S. mutans,* and *P. gingivalis*.

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Declaration of Interest

The authors report no conflict of interest.

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