Antibiofilm Efficacy of Myrmecodia Pendens Methanol Extract and NaOCl Against Enterococcus Faecalis ATCC 29212

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Abstract

Enterococcus faecalis are the most common pathogen associated with failed root canal treatment. It can withstand the ecological conditions in endodontic treated canals and cause reinfection by forming biofilm. Myrmecodia Pendens rich in bioactive compounds that have antibacterial properties. Aims of this study is to evaluate and compare the antimicrobial effects of Myrmecodia pendens methanol extract with NaOCl 5% on E. faecalis biofilm.

The 48-h E. faecalis ATCC 29212 biofilm was treated by various concentrations of M. pendens methanol extract. The MBEC was determined using a serial micro dilutions method after treated for 1 minute and 30 minutes. Optical density read by ELISA reader at λ 490 nm. NaOCl 5% used as control

Statistical analysis used: Differences between samples and control were analyzed using one-way ANOVA and then Tukey's multiple comparisons using IBM SPSS ver 25 software. P values of less than 0.05 were regarded as significant.

M. pendans methanol extract has MBEC value at 100 mg/ml for 1 minute and 25 mg/ml for 30 minutes against E. faecalis biofilm ATCC 29212

M. pendans methanol extract has antibiofilm activity and can be developed as an alternative endodontic irrigant.

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Introduction

Endodontic failure often caused by persistent bacterial infection in the root canal and periradikular.[1,2] *Enterococcus faecalis* are found in a high percentage of root canal failures (22-77%). E. faecalis is able to survive in the root canal by the formation of biofilms and plays a major role in persistence periradicular infection after root canal treatment. [1,3]

Root canal disinfection help to eradicate biofilm and neutralize endotoxin on the root canal surface.[4] Sodium hypochlorite (NaOCl) is a golden standard for root canal irrigation, which has been widely used for disinfection of root canal treatments.[5] NaOCl 5.25% can kill *E. faecalis* in 30 seconds.[4]

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However, there are some disadvantages, NaOCl has an unpleasant taste, toxic, potential allergic, cannot dissolve the smear layer (inorganic material) and corrosion on the instrument.[6,7] Therefore an alternative irrigation solution is needed.

Myrmecodia pendens is an epiphyte plant that can be found in Sorong, Papua. It is traditionally used as a remedy by native.[8] Myrmecodia pendens was rich in bioactive compounds such as flavonoids, tannins, saponins, alkaloids, and terpenoids.[7,9] Several previous studies have revealed that Myrmecodia pendens extracts have antimicrobial effects on Candida albicans, Shigella dysentriae, Enterococcus. faecalis, Streptococcus mutans and Streptococcus sanguis, making them perfect candidates for alternative irrigant. [6,7]

The aim of this study was to evaluate and compare the antibacterial efficacy of M.pendens methanol extract with NaOCl 5% against *E. faecalis* biofilm.

Materials and methods

Bacterial strain and inocula preparation

The bacterial culture of *E. faecalis* strain (American Type Culture Collection, ATCC 29212) was obtained from a chemical laboratory, Faculty of Chemistry, Padjadjaran University. For the inoculum preparation, one inoculating loop of bacteria were grown in liquid Brain Heart Infusion (BHI) and incubated at 37°C for 24 hours under anaerobic condition using the anaerobic jar. The bacteria suspension was then diluted until it reached the standard of 0.5 McFarland standard (0.5 x 10^8 CFU/ml).[9]

Preparation of extracts of *M. pendens*

Bacterial strain and culture conditions

An *E. faecalis* ATCC 29212 obtained from the stock culture at Research Laboratory of Chemistry Department, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Bandung, Indonesia.

Plant Materials

M. pendans was obtained from Sorong, Papua, Indonesia. The plant species were identified taxonomically at the Department of Biology, Padjadjaran University, Indonesia. *M. pendens* was washed with running tap water to remove any adsorbed contaminant from the sample surface. The cleaned sample was chopped into smaller parts and dried.

Extraction of the Plant Materials

The extraction of *M. pendans* bulbs was performed using maceration methods with methanol as a solvent. The extraction method performed according to the procedure described by Shahraki[10] with a slight modification. *M. pendans* bulbs were cut into small pieces and soaked in a methanol solvent at room temperature for 72 hours. The resulting supernatant was filtered and concentrated on a rotatory evaporator at 40°C for removed the solvent.

Phytochemicals screening

The crude methanolic extracts of *M. pendans* were screened for the presence of alkaloids, flavonoids, steroids, tannins, saponins, terpenoid and phenolic using standard methods.[11] The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Biofilm formation

E. faecalis biofilm formation was grown according to the procedure described by Cai[12] with a slight modification. *E. faecalis* biofilms were grown in a sterile flat-bottom 96-well microtiter plate. In detail, a single colony of *E. faecalis ATCC 29212* strain was inoculated into BHIB for 24 hours. The preculture was diluted to approximately 0.5 *Mc Farland* standard (1 x 10^8 CFU/ mL) in fresh BHIB. Then, transferred 100 µl to each well and incubated for 48 h at 37°C anaerobically with a gaspac.

Determination of the minimum biofilm eradication concentration (MBEC)

The ability of methanol extract of *M. pendans* to eradicate *E. faecalis* biofilm was determined using a serial micro dilutions method according to the procedure described by Mohsenipour[13] with a slight modification. The plates containing *E. faecalis* biofilm washed three times with sterile PBS to remove the nonadherent cell, followed by aliquoting 100 µL of methanol extract to a final concentration 12.5 mg/ml - 100 mg/ml in the well. The wells containing bacteria were used as a negative control, and NaOCl 5% were used as a positive control. The 96 well plates were left to stand for between 1 and 30 min. The disruption of biofilm biomass was determined by safranin staining. The plates were washed three times with sterile PBS, then stained with 150 µl of safranin 0.1% and incubated at room temperature for 15 min after which the plates were washed three times with sterile distilated water to remove an unadsorbed stain. The semi-quantitative assessment of biofilm formation was performed by adding 150 µl of ethanol 96% to destain the wells. Absorbance determined at λ 490 nm using a microplate reader. The mean absorbance (OD_{490nm}) of the samples was determined. Optical absorbance represents the actual bacterial biofilm mass.[14] If the negative value for optical density (OD) was obtained, it was presented as zero.[15] the percentage inhibition obtained using Eq.1.[16]

$$\text{Eradication \%} = \frac{(OD\ negative\ control) - OD\ sample}{OD\ negative\ control} \text{ x } 100$$

The experiment was performed with three replicates. The MBEC value was the lowest concentration that was able to eradicate the biofilm.

Statistical analysis

Differences between samples and control were analyzed using one-way analysis of variance (ANOVA) and then Tukey's multiple comparisons using IBM SPSS ver 25 software. *P* values of less than 0.05 were regarded as significant.

Results

Phytochemical screening

The phytochemical screening of crude methanolic extracts of *M. pendans* revealed the presence of some bioactive compounds such as alkaloid, tannin, saponin, flavonoid, triterpenoid and phenolic compound. The phytochemical test of the extract is shown in table 1.

No	Phytochemical test	Extract MeOH
1	Tanin	+
2	Saponin	+
3	Alkaloid	+
4	Fenolik	+
5	Flavonoid	
	Flavonoid A	+
	Flavonoid B	+
	Flavonoid C	+
6	Triterpenoid	+

Table 1. Phytochemical compounds found in the extract and fraction of M. pendans.
Legends : absent; + : present; MeOH : Methanol; EtOAc : Ethyl acetate.

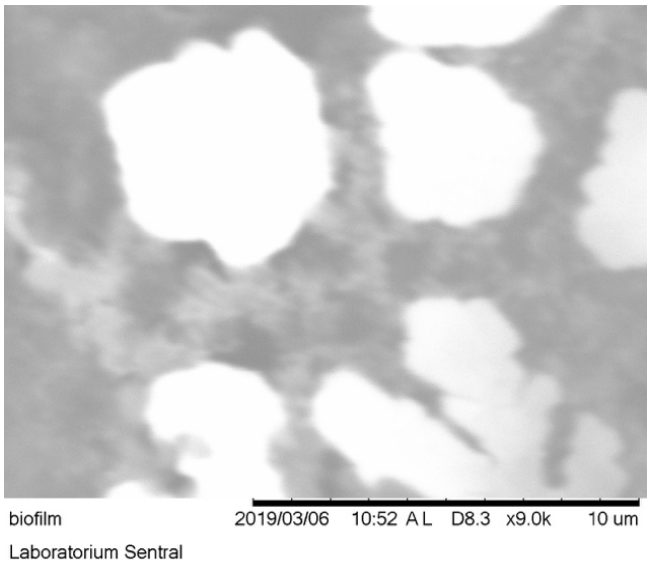

Figure 1. *E. faecalis* biofilm formation seen by SEM at 9000x.

Biofilm formation

Enterococcus faecalis biofilm formation after incubation for 48 h at 37°C anaerobically and stained with safranin seen by scanning electron microscope (SEM) shown at figure 1.
Figure 1. *E. faecalis* biofilm formation seen by SEM at 9000x

Determination of MBEC values

The activities of *M. pendes* methanol extract on reduction of biofilm biomass in 1 min and 30 min treatment are presented in fig 2. Antibiofilm activity of the *M. pendens* methanol extract towards the *E. faecalis* biofilm tended to increase over time. Biofilm formation was decreased with *M. pendens* methanol extract at 100 mg/ml for 1-minute treatment and at 12.5 mg/ml for 30-minute treatment.

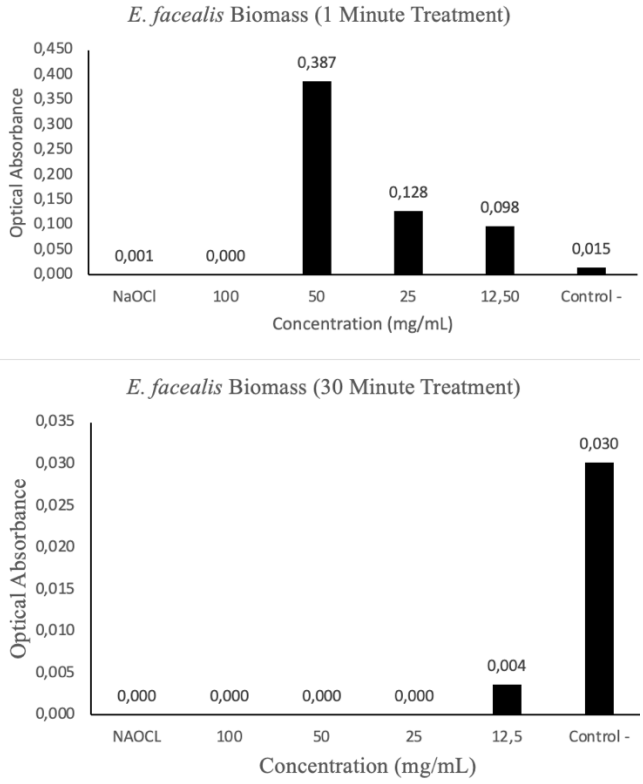

Figure 2. Effect of *M. pendens* extract on *E. faecalis* biofilm biomass after (A) 1 minute and (B) 30-minute treatment.

Furthermore, the percentage inhibition of *M. pendens* methanol extract were not significantly different (table 2). *M. pendens* methanol extract for 1 min of treatment showed MBEC value at 100 mg/mL and for 30 min showed MBEC value at 25 mg/mL(table 3).

NO	Concentration (mg/ml)	% INHIBITION	
		1 min	30 min
1	100	3320.83±6758.84*	5731.03±1145.37*
2	50	-2316.67±2386.30	149.43±72.52
3	25	−702.08±263.42	229.88±128.35
4	12.5	−510.42±1259.59	87.36±88.20
5	NaOCl	95.83±3.61	101.15±1.2

Table 2. Eradication of *M. pendens* methanol extract against *E. faecalis* biofilm.

Bacteria	MBEC 1 MIN (mg/mL)	MBEC 30 MIN (mg/mL)
E. faecalis	100	25

Table 3. MBEC of *M. pendens* methanol extract against *E. faecalis* biofilm.

Discussion

The methanol extract of Myrmecodia pendens was obtained using the maceration method, this method is the most commonly used method for extracting bioactive compounds because of its simplicity.[11] Phytochemical screening results showed that methanol extract of Myrmecodia pendens contained tannins, saponins, alkaloids, phenolics, flavonoids, and triterpenoid compound.

Antibacterial effect of Myrmecodia pendens methanol extract on *E. faecalis* biofilm measured by a microtiter-plate test method. This is one of the most often used methods for measure total biofilm biomass because it has good objectivity and accuracy in reading the data obtained. In this method, the turbidity of stained bacteria is measured by a spectrophotometer.[15,17,18]

Safranin used to staining of biofilm biomass and 96% ethanol to dissolve biofilms attached to the surface of 96 well. The absorbance value obtained illustrates biofilm biomass. The decrease in absorbance value is proportional to the decrease in biofilm biomass.[14,18]

Enterococcus faecalis, facultative anaerobic gram-positive cocci, found in a high percentage of root canal failures. *E. faecalis* is able to survive in the root canal by the formation of biofilms and plays a major role in persistence periradicular infection after root canal treatment.[3]

This bacteria has the ability to form biofilms by producing enzyme sortase, autolysin, extracellular DNA (eDNA) and enterococcal surface proteins (esp).[1,19] Biofilms are aggregation of microorganisms enclosed in matrix consisting of a mixture of polymeric compound, primarily consisting of polysaccharides, generally referred to as extracellular polymeric substance (EPS)[3,20,21]

Biofilms make bacteria become 1000 times more resistant to antibacterial than organisms that cannot form biofilms, with restrict antibacterial diffusion, maintain pH homeostasis and suppress lymphocyte activities. [19,22] Biofilms are bacteria strategy to survive from an antimicrobial agent and harsh condition by slowing down the rate of growth, reducing metabolic activity and producing a protective extracellular matrix.[23,24] Biofilm protect bacteria and reduce antibiotic susceptibility, contributing to the persistence of biofilm infection.[13]

Antibiofilm activity of *Myrmecodia pendens* methanol extract against *E. faecalis* biofilm has not been previously known. This study shows that *M. pendens* methanol extract has the ability to eradicate *E. faecalis* biofilms. *Myrmecodia pendens* methanol extract can eradicate *E. faecalis* biofilm that had been formed at a concentration of 100 mg/ml after incubation for 1 minute and at a concentration of 25 mg/mL after incubation for 30 minutes. The abilities of *M. pendens* methanol extract for eradicating biofilm was increased according to concentration (concentrations dependent) and increase according to the time (time-dependent). In a comparison with NaOCl 5% which is used as a standard irrigation solution, NaOCl 5% can eradicate biofilms by 95.83 ± 3.61 % after incubation for 1 minute and can eradicate all biofilms after 30 minutes. NaOCl 5% prove its ability as a potent irrigation solution for root canal treatment. There were statistically significant differences ($P < 0.05$) between the activity of *M. pendens* variation concentration and NaOCl.

Bacterial eradication by active compounds could be reached by several mechanisms such as disruption of cell wall biosynthesis and permeability of cell membranes, inhibition of protein synthesis, inhibition of nucleic acid metabolism, inhibition of the enzyme activity.[9] For an irrigant to be effective against biofilm, besides eliminating the bacteria it should have the ability to eliminate extracellular polysaccharide matrix (EPS) as a source of nutrient and/or as a suitable surface for bacterial

growth.[25] *M. pendens* has the ability to eradicate biofilm because it is rich with bioactive compounds that have antibiofilm activity such as flavonoids, tannins, and triterpenoids.[20] The previous study shows that flavonoids (Xanthohumol) have the ability to disrupt formed biofilms by inhibiting lipid metabolism and causing damage to the stability of the bacterial cytoplasmic membrane.[20] The other *M. pendens* bioactive compound, terpenoids also has antibiofilm activity by affect cell membrane integrity and destroy bacterial biofilms.[9] This study confirmed the ability of *M. pendens* extracts to eradicate *E. faecalis* biofilm. According to the results of this research, the antimicrobial potential of this plant was confirmed and the extracts of this plant are suitable choices as an alternative irrigant.

Conclusions

The results of this study provide a scientific basis for the antibacterial activity of *M. pendans* extract against *E. faecalis* biofilm. *M. pendans* methanol extract has MBEC values at 100 mg/ml for 1-minute treatment and at 25 mg/ml for 30-minute treatment. The results of the present investigation suggest that the *M. pendans* extract can be used as a potential alternative as an endodontic irrigant.

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Declaration of Interest

All authors declare has no conflict of interest on the publication of the research.

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