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ORIGINAL ARTICLE

Antimicrobial Efficacy of *Myrmecodia pendens* Extract and Fraction Combination against *Enterococcus faecalis* ATCC 29212

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ABSTRACT

Enterococcus faecalis can withstand harsh environmental conditions in the root canal and cause a secondary infection. *Myrmecodia pendens* is an herbal medicine rich in polyphenol compounds that have antibacterial, antioxidant, and anticancer properties. **Objectives:** To analyze the effects of *M. pendens* fraction combination on the sensitivity of *E. faecalis*. **Methods:** The minimum inhibitory concentration (MIC) was determined using a serial microdilution method, and the minimum bactericidal concentration (MBC) was determined by adding the test sample to sterile Mueller Hinton agar medium. **Results:** The MIC of combination 1 (hexane-ethyl acetate, HE) was 0.049 mg/mL, whereas those of combinations 2 (hexane-water, HA) and 3 (ethyl acetate-water, EA) were 0.098 and 0.390 mg/mL, respectively. The MIC of NaOCl was 0.390 mg/mL, and that of methanol extract was 0.390 mg/mL. The MBC of combination 1 (HE) was 12.50 mg/mL, whereas that of combinations 2 (HA) and 3 (EA) was 50 mg/mL. The MBC of NaOCl was 25 mg/mL and that of methanol extract was 50 mg/mL against *E. faecalis*. This study showed that fraction combinations increase the antibacterial effect of *M. pendens* against *E. faecalis* ATCC 29212. **Conclusion:** The HE fraction combination showed the best effect against *E. faecalis* and can be developed as an alternative endodontic irrigant.

Key words: antibacterial effect, *Enterococcus faecalis*, fraction combination, *Myrmecodia pendens*

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INTRODUCTION

Enterococcus faecalis is the most common pathogen associated with failed root canal treatment.¹⁻⁴ It can withstand harsh environmental conditions in the root canal and cause a secondary infection.^{1,5} *E. faecalis* is found in 4%–40% of primary endodontic infections, and its prevalence rates from persistent endodontic infections or recurrent periradicular lesion are much higher. Failed root canal treatment cases are nine times likely to be infected with *E. faecalis*, with a prevalence rate ranging from 22% to 77%.^{1,2} *E. faecalis* can remain viable in extreme pH, nutrient-deficient, and oxygen-deficient conditions.² It can form a biofilm for its defense, making it 1000 times more resistant to antibodies and antimicrobial agents than non-biofilm bacteria and avoiding destruction from intracanal medicament.^{1,2,4}

The major factors associated with endodontic treatment failure are when the procedures undertaken in root canals fail to control and eliminate bacterial infection.^{2,5} Mechanical instrumentation alone in the canal system could not eliminate the bacteria. About 35% of the root canal area is left untouched because of the complex root canal anatomy. Therefore, we should combine with irrigation for elimination and killing of bacteria from the root canals.² Root canal disinfection by endodontic irrigants disrupts bacteria and neutralizes endotoxins in root canal walls.²

Sodium hypochlorite (NaOCl) is the most potent disinfectant in endodontics because of its excellent antimicrobial activity and ability to dissolve necrotic tissues and organic component of dentin.^{2,5} It kills bacteria very rapidly even at low concentrations; 5.25% NaOCl solution can kill *E. faecalis* in 30 s.

The disadvantages of NaOCl include caustic and toxic effects on tissue.^{2,3,5}

Herbal products are a rich source of bioactive compounds and have provided important therapeutic agents. Most herbal products possess antimicrobial properties that have increased attention.⁶ The use of herbal products as irrigants is promising because of their safety, efficiency, and acceptability.⁷ *Myrmecodia pendens* rich in polyphenol compounds, such as flavonoids, tannins, saponins, alkaloids, and terpenoids, exhibits anticancer, antibacterial, and antioxidant activities.⁸⁻¹⁰ *M. pendens* lives as epiphytes, originating from Sorong, Papua, Indonesia. It is used empirically as a medical plant to treat various diseases.¹¹⁻¹³ This plant is valuable as an alternative endodontic irrigant and shows minimal side effects. Soraya (2016) found that *M. pendens* extract has an antibacterial effect against *E. faecalis*. However, whether the fraction combination of *M. pendens* can potentially inhibit *E. faecalis* remains unknown. This research aimed to analyze the effects of *M. pendens* fraction combination on the sensitivity of *E. faecalis*.

METHODS

Bacterial strain and inoculum 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 was grown in liquid brain heart infusion (BHI) and incubated at 37 °C for 24 h under anaerobic conditions by using the anaerobic jar. The bacterial suspension was then diluted until it reached the standard of 0.5 McFarland standard (0.5 × 10⁸ CFU/mL).¹⁰

Preparation of extracts and fraction of *M. pendens* plant materials

M. pendens was obtained from Sorong, Papua, Indonesia. The plant species were identified taxonomically at the Department of Biology, Padjadjaran University, Indonesia.

Extraction of the plant materials

The extraction of *M. pendens* bulbs was performed using maceration methods. This method was chosen because of its simplicity and manageability.¹⁴ Extraction and fractionation were performed in accordance with the procedure described by Shahraki¹⁵ with slight modifications. As much as 3.970 kg of *M. pendens* bulbs was cut into small pieces and soaked in a methanol solution at room temperature for 48 h. The extract was concentrated on a rotatory evaporator at 40 °C for removed methanol. This process was repeated until the solvent on the last extract was colorless.

Fractionation of extract

The obtained extract was mixed with water (H₂O) and transferred to a separating funnel to prepare the fraction. The n-hexane solvent was added to the funnel, and the n-hexane fraction was extracted. The procedure was repeated until the hexane fraction in the last fraction was colorless. In the next step, the remainder of the solvent in the separating funnel was combined with ethyl acetate solvent, and the ethyl acetate fraction was extracted. The procedure was repeated until the ethyl acetate fraction in the last fraction was colorless. In the end, the remaining solvent (H₂O) from the previous steps was extracted. The n-hexane, ethyl acetate, and water fractions were prepared after omitting the solvent by a rotary evaporator at 40 °C.

Phytochemical screening

The crude methanolic extracts and fraction of *M. pendens* were screened for the presence of phytochemical compounds, such as alkaloids, flavonoids, steroids, tannins, saponins, terpenoids, and phenolics, in accordance with the procedure described by Harbone.¹⁶ Phytochemical reagent was added to the extract and fraction solution. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

Determination of the minimum inhibitory concentration (MIC)

Fraction combinations of *M. pendens* were divided to three groups: group 1, a combination of hexane and ethyl acetate fraction (HE); group 2, a combination of hexane and water fraction (HA); and group 3, a combination of water and ethyl acetate fraction (EA). Blank bacterial culture was used as a negative control, whereas methanol extract (ME) and 5% NaOCl were used as a positive control.

The MIC of fraction combination from the bulbs of *M. pendens* was determined using serial microdilution/ broth microdilution in accordance with the procedure described by Eloff¹⁷ with slight modifications. Optical density was read by an ELISA reader at λ 490 nm. Fraction combination was serially diluted in a 96-well microplate with BHI as a medium. In brief, 100 µL of the test sample (50 µL from each fraction) was added to well column 1, and all wells from column 1 to column 12 initially received 100 µL of BHI. Then, two-fold serial dilutions were performed by transferring 100 µL from column 1 to column 2 and continued through column 12. In the end, 100 µL of the excess medium was discarded from the well in column 12. The final concentration ranged from 0.012 mg/mL to 25 mg/mL (each fraction), or 0.024 mg/mL to 50 mg/mL of the fraction combination concentration was obtained. A 10 µL aliquot of the inoculum (bacterial suspension) was added to the well, and then the plate was sealed with

parafilm and incubated for 24 h at 37 °C. The MICs were considered as the lowest concentrations of the sample that prevented bacterial growth, shown by no turbidity in the well after incubation for 24 h at 37 °C. Further, it was standardized well by a microplate reader at λ 490 nm.^{10,18}

The percentage of inhibition of each seed extract was determined as follows¹⁹:

$$\% \text{ inhibition} = \left(1 - \left(\frac{OD_{\text{sample}} - OD_{\text{sampleblank}}}{OD_{\text{solvent}} - OD_{\text{solvent}}} \right) \right) \times 100$$

Information :

OD sample : sample + media + bacteria

OD sample blank : sample + media

OD solvent : media + bacteria

OD solvent blank : media

Determination of the minimum bactericidal concentration (MBC)

The MBC was measured using the method described by Upadhyay with modifications.¹⁸ For this purpose, 100 µL from each well above the MIC concentration was added to sterile Mueller Hinton agar medium and spread evenly, and then the plate was sealed with parafilm and incubated for 24 h at 37 °C.

The lowest concentration that yielded no visible growth after the sub-culturing was considered as the MBC. NaOCl and methanol extracts were used as positive controls.

RESULTS

Extract condition

The maceration method was performed because it is the safest and simplest extraction method. By using maceration, we avoided the loss of the thermolabile active compound. After 2 days of extraction using 26.520 L of 70% methanol as solvent, 93.620 g of the extract was obtained from 3.970 kg of *M. pendens* bulbs. The characteristic of the extract was red-brownish powder and odorless.

Phytochemical screening

The phytochemical screening of crude methanolic extracts of *M. pendens* revealed the presence of some bioactive compounds, such as alkaloids, tannins, saponins, flavonoids, triterpenoids, and phenolic compounds. The phytochemical test results of the extract and fraction are shown in Table 1. The maceration method is the most common method for the extraction of bioactive components because of its simplicity and manageability.¹⁴

Methanol was effective to extract alkaloids, tannins, saponins, flavonoids, triterpenoids, and phenolic

Table 1. The results of phytochemical test of the extract and fraction of *M. pendens*

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

- : absent; + : present; MeOH : Methanol; EtOAc : Ethyl acetate

compounds. Aquadest could dissolve tannins, phenolics, flavonoids, and triterpenoid compounds. Ethyl acetate is a semipolar compound that could dissolve alkaloids, tannins, saponins, flavonoids, triterpenoids, and phenolic compounds, the same with methanol extract. Less chemical compounds of *M. pendens* had non-polar properties; only triterpenoid was dissolved in hexane.

The total bioactive compounds with regard to the different solvents used for fractionation were as follows: ethyl acetate > water > hexanes.

Determination of MIC and MBC

The MIC of the fraction combination was observed by reading the optical density with an ELISA reader at 490 nm. The percentage of inhibition increased with increasing concentration (Table 2).

The growth rate of *E. faecalis* was difficult to be observed with the naked eyes because the fraction has a solid color and settles in the well. Hence, subculture from the microtiter plate onto the surface of Mueller Hinton agar medium was performed to detect bacterial growth. Combination 1 (HE) showed the lowest MIC at 0.049 mg/mL and the highest activity against *E. faecalis*, whereas combinations 2 (HA) and 3 (EA) had MICs at 0.098 and 0.390 mg/mL, respectively. The positive control NaOCl showed the same MIC of 0.390 mg/mL against *E. faecalis* as the methanol extract (Figure 1).

The activity of plant extracts was considered significant if MICs were below 100 µg/mL, moderate when 100 < MIC ≤ 625 µg/mL, or weak when MIC > 625 µg/mL. Therefore, the activity recorded for the *M. pendens* fraction combination can be considered significant for the HE and HA fraction combinations and moderate for the EA fraction combination .

The lowest MBC was found in combination 1 (HE) at 12.50 mg/mL against *E. faecalis*. Similarly,

Table 2. Inhibition percentage of *Myrmecodia pendens* fraction combination against *Enterococcus faecalis*

NO	Concentration (mg/ml)	% INHIBITION				
		HA	HE	EA	ME	NaOCl
1	50.00	105.83±6.36	-2800.48±443.80	106.83±45.72		90.61±2.64
2	25.00	88.10±0.14	-147.02±7.29	182.72±43.02		91.78±6.73
3	12.50	157.11±1.95	24.39±9.21	108.13±62.66	120.37±10.68	47.12±3.38
4	6.250	156.75±12.93	106.25±50.81	188.45±12.38	128.59±40.15	43.11±4.02
5	3.125	152.95±33.31	149.24±42.87	97.47±7.98	124.21±39.60	23.93±1.49
6	1.563	131.63±9.38	64.00±1.59	61.28±27.22	163.82±0.31	28.16±10.85
7	0.781	89.28±2.76	62.39±0.82	26.87±17.74	116.49±1.32	6.33±32.88
8	0.390	66.18±1.22	50.60±8.38	0.23±5.68	39.37±3.89	-4.99±13.48
9	0.195	37.53±18.98	33.96±52.82	-6.15±2.34	-0.85±9.05	-9.18±9.25
10	0.098	28.90±21.50	60.48±4.34	-6.94±6.25	13.82±0.79	9.72±13.42
11	0.049	-4.14±1.01	28.01±34.02	-25.57±7.28		11.23±11.36
12	0.024	3.91±5.81	-18.36±12.14	4.61±6.22		-27.17±12.38

HA: hexane-water; HE: hexane-ethyl acetate; EA: ethyl acetate - water; ME: methanol extract; NaOCl: sodium hypochlorite

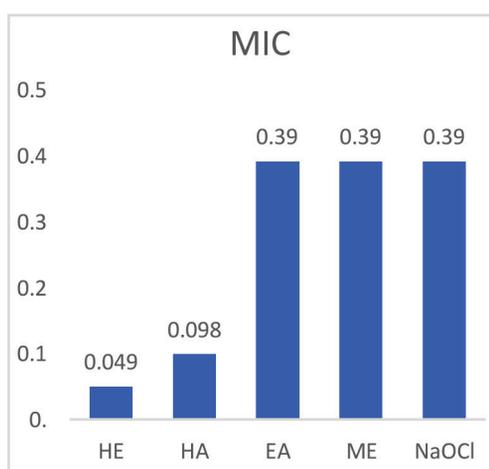


Figure 1. Comparison of MICs of fraction combination and positive control. HE: hexane-ethyl acetate; HA: hexane-water; EA: ethyl acetate - water; ME: methanol extract; NaOCl: sodium hypochlorite

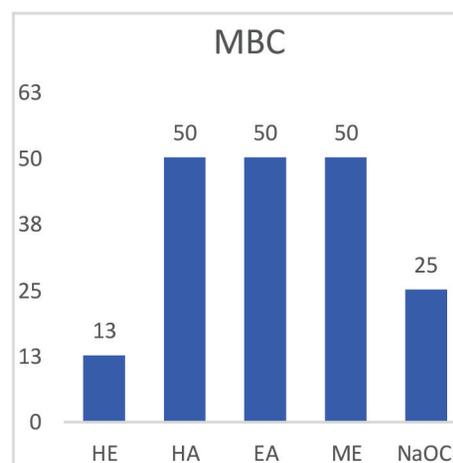


Figure 2. Comparison of MBCs of fraction combination and positive control. HE: hexane-ethyl acetate; HA: hexane-water; EA: ethyl acetate - water; ME: methanol extract; NaOCl: sodium hypochlorite

combinations 2 (HA) and 3 (EA) had the same MBC at 50 mg/mL against *E. faecalis*. The MBC of the positive control NaOCl was 25 mg/mL, and that of the methanol extract was 50 mg/mL against *E. faecalis* (Figures 2).

DISCUSSION

M. pendens is rich in bioactive compounds that have antibacterial, antifungal, anticancer, and antioxidant properties.⁸ Phytochemical test showed that methanol extract of *M. pendens* contains tannins, saponins, alkaloids, phenolics, flavonoids, and triterpenoids. This result agrees with the previous study by Sudiono et al.⁸ Methanol is known as a magic solvent because

it can attract most bioactive compounds. Crude extracts contain complex mixtures of some classes of bioactive compounds that are selectively soluble in different solvents. In this sense, solvent polarity plays a key role in increasing bioactive compound solubility.²⁰

The various chemical structures and polarities result in a wide range of fractionation solvents (water, methanol, ethyl acetate, and hexane).²⁰ Fractionation could separate the bioactive compound from the extract and increase its purity.²¹ Therefore, fractionation is expected to increase biological activities. Fractionation solvent affects phytochemical content and biological activities.²⁰ The present study revealed that fractionation separated certain parts of *M. pendens* secondary

metabolite extract according to its polarity. Aquadest could dissolve several compounds, such as tannins, phenolics, flavonoids, and triterpenoids. The most bioactive compounds were dissolved in ethyl acetate fraction, indicating that most of them are semipolar and the least bioactive compounds were dissolved in the non-polar hexane fraction, and triterpenoids were dissolved only in the hexane fraction. Phytochemical data informed that that the components contained in *M. pendens* are mostly semipolar.²²

A previous study reported that methanol can dissolve polar compounds, such as sugar, amino acid, glycoside compounds, phenolic compounds with low and medium molecular weights and medium polarity, aglycon flavonoids, anthocyanins, terpenoids, saponins, tannins, xantoxilin, totarol, quacinoind, lactones, flavones, phenones, and polyphenols. Aquadest is effective to extract glycoside compounds, amino acids, sugar, aglycon compounds, and vitamin C. Ethyl acetate is effective to extract alkaloids, aglycons, glycoside compounds, sterols, terpenoids, and flavonoids. Hexanes can solve non-polar compounds or lipophilic compounds, which are generally lignin, wax, lipids, and aglycon, sterols, and terpenoids.²²

M. pendens features antimicrobial properties, and a previous study showed that the extract of *M. pendens* can inhibit the biofilm formation of *E. faecalis*, *Streptococcus sanguinis*, and *Porphyromonas gingivalis*, whereas the aqueous fraction exerts antifungal properties against *Candida albicans*.²³⁻²⁶ Kurnia et al.²⁷ reported that its flavonoid isolate is effective against *E. faecalis*, and Gartika et al.¹⁰ revealed that the terpenoid isolate of *M. pendens* has antibiofilm activities against *Streptococcus mutans*. Fraction combination may result in enhanced, reduced, or loss of activity.²⁸ In the present investigation, the fraction combinations of *M. pendens* activity against *E. faecalis* were evaluated. The antibacterial activities of the fraction combinations were tested by serial microdilution, and the MIC and MBC were determined. The fraction combinations of *M. pendens* showed an antibacterial activity against *E. faecalis* in comparison to NaOCl and methanol extract. The HE fraction combination exhibited the lowest MIC of 0.049 mg/mL, which was lower than that of the positive control methanol extract (i.e., 0.390 mg/mL) and NaOCl (0.390 mg/mL). Meanwhile, the HA and EA fraction combinations had MICs of 0.098 and 0.390 mg/mL, respectively (Table 3). Further, the antibacterial activity of the HE fraction combination was strengthened by low MBCs against *E. faecalis*, i.e., 12.5 mg/mL, which was lower than that of the positive control methanol extract (50 mg/mL) and NaOCl (25 mg/mL). The HA and EA fraction combinations had the same MBC of 50 mg/mL.

The best fraction combinations were obtained in the HE fraction combination with an MIC of 0.049

mg/mL and an MBC of 12.50 mg/mL. Most of the bioactive compounds in *M. pendens* that had antibacterial properties were semipolar and non-polar. The combination of these compounds increased the antibacterial activity.

The abilities are related to the role of active flavonoid, tannin, and triterpenoid compounds to inhibit the growth of *E. faecalis*.²³ Bioactive compounds have multiple mechanisms of antibacterial activity.²⁹ Ethyl acetate as a semipolar solvent could dissolve flavonoid compounds with antibacterial properties. A previous study showed that the flavonoid isolate from the ethyl acetate fraction exhibits antibacterial properties against *E. faecalis* with an MIC of 156 µg/mL and an MBC of 625 µg/mL.³⁰ Flavonoids contained in the *M. pendens* fraction are very important to inhibit nucleic acid synthesis (caused by topoisomerase inhibition), cytoplasmic membrane function (membrane permeability and leakage), and inhibition energy metabolism.^{6,29} Flavonoids such as rutin inhibit bacterial growth by topoisomerase inhibition, which is important for DNA synthesis; quercetin could reduce bacterial membrane permeability and antibacterial resistance.⁶ Ethyl acetate can also dissolve tannins that possess antibacterial activity. Tannins inhibit the synthesis of bacterial cell walls and cell membranes through the hydrolysis of ester bonds in gallic acid.²⁴ Catechin, a subclass of tannins, penetrates and interacts with lipid bilayers and caused membrane fusion.²⁹ Meanwhile, terpenoids can inhibit lipid formation and change the structure of cell membranes by inhibiting ergosterol synthesis.²⁴ In addition to ethyl acetate, hexane as a combination component also plays a role in HE antibacterial activity. Hexane dissolves triterpenoid compound, and terpenoid can inhibit lipid formation and influence the membrane integrity.¹⁰

This study showed that the fraction combinations can increase the antibacterial effect of *M. pendens* against *E. faecalis*. Thus, *M. pendens* is a potential alternative irrigant for endodontic treatment.

CONCLUSION

This study provides a scientific basis for the antibacterial activity of *M. pendens* fraction combination against *E. faecalis*. The HE fraction combination was the best combination with an MIC of 0.049 mg/mL and an MBC of 12.50 mg/mL. The results of the present investigation suggest that the fraction combination of *M. pendens* can be used as a potential alternative irrigant for endodontic treatment.

CONFLICT OF INTERESTS

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

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