

Antibacterial effects of *Pluchea indica* Less leaf extract on *E. faecalis* and *Fusobacterium nucleatum* (in vitro)

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ABSTRACT

Background: *Enterococcus faecalis* (*E. faecalis*) and *Fusobacterium nucleatum* (*F. nucleatum*) are the most common bacteria found in infected tooth root canal. Most of these bacteria often cause failure in endodontic treatments. *Pluchea indica* Less leaf is a species of plants that has several chemical properties. It consists of flavonoids, tannins, polyphenols, and essential oils which have been reported as antibacterial agents. Because of its benefits, the extract of *Pluchea indica* Less leaves may be potentially developed as one of root canal sterilization dressing. **Purpose:** This study aimed to determine antibacterial activity of *Pluchea indica* Less leaves extract against *E. faecalis* and *F. nucleatum* bacteria. **Method:** Dilution method was conducted first to show Minimum Inhibitory Concentration (MIC) of the extract against *E. faecalis* and *F. nucleatum*. The antibacterial activity test on *Pluchea indica* Less leaves extract was performed on *E. faecalis* and *F. nucleatum* bacteria using agar diffusion method. The *Pluchea indica* Less leaves extract used for antibacterial activity test was at a concentrations of 100%, 50%, 25%, 12.5%, and 6.25%. Thirty-five petridiscs were used and divided into five groups based on the extract concentration. **Result:** The results showed strong and moderate antibacterial effects of the *Pluchea indica* Less leaves extract on *E. faecalis* at the concentrations of 100% and 50%, while on *F. nucleatum* only at the concentration of 100% with moderate effect. **Conclusion:** *Pluchea indica* Less leaves extract has antibacterial activity against *E. faecalis* and *F. nucleatum* bacteria with strong-moderate effect.

Keywords: *Pluchea indica* Less leaves extract; *Enterococcus faecalis*; *Fusobacterium nucleatum*; antibacterial

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INTRODUCTION

Microorganisms play an important role in causing inflammation of pulp and periapical tissues. Several researches have reported the existence of microorganisms in infected pulp and root canals, 90% of which are facultative anaerobic and gram-positive bacteria, followed by gram-negative bacteria and some fungal groups.^{1,2} Teeth with necrotic pulp tissues usually show a large number of combinations bacteria. Some species of bacteria, such as *Peptostreptococcus provetii*, *Actinomyces odontolyticus*, *Porphyromonas endodontalis*, and *Streptococcus salivarius*, also are commonly found in infected root canals. In failed treatment of root canals, *Enterococcus faecalis* (*E. faecalis*)

bacteria are mostly found with a prevalences of 67% to 77% in persistent endodontic infection cases.³ In addition to *E. faecalis* bacteria, *Fusobacterium nucleatum* (*F. nucleatum*) bacteria also are mostly found in root canals, as many as 60% to 70% of periodontal lesion cases.¹

Good root canal treatment can be achieved by removing all sources of infection through preparation, sterilization, and filling of the root canal. However, there is still a failure in root canal treatment. One of the main causes of root canal treatment failure is the presence of microorganisms that can survive both in root canal and in apical region of the teeth. It may be because the microorganisms still have an ability of resistance to medicaments used during root canal treatment.²⁻⁴

Giving intra-canal sterilization medicine as one of antimicrobial agents, therefore, becomes an important stage in root canal treatment, because it can kill microorganisms in root canal teeth.^{5,6} Root canal sterilization drugs that have been used in dentistry since the past, nevertheless, are known to cause irritation in the periapical region and to be cytotoxic, because these contain active ingredients and toxic chemicals. These root canal sterilization drugs are largely classified into phenols, including formokresol, camphorated parachlorophenol, thymol, metakresilasetat, and halides (iodine-potassium iodide).⁷ In addition to phenols, compounds that also used as root canal medicament are calcium hydroxide, N₂, halogen, such as sodium hypochlorite and iodide, and quaternary ammonium (quats).⁸ However, a research conducted by Grossman et al.⁸ shows that the use of formokresol can generate a high degree of irritation and can cause necrosis for 2-3 months. Hydrogen peroxide and sodium hypochlorite also are known to generate less irritation than most intra-canal medicaments. Meanwhile, cresatin is known to generate little inflammation.

Pluchea indica Less (L.) is a plant that has long been known by the people of Indonesia for its benefits. These plants are often used as hedge plants, has special smell and bitter taste. Part of these plants that always used are its leaves and roots, which is traditionally function as efficacious fever, appetite enhancer, and sweat bullets. *Pluchea indica L.* leaves contain chemical properties such as tannins, flavonoids, polifenolat, and essential oils that are known to have antibacterial effect.^{9,10} Antibacterial effects of *Pluchea indica L.* leaves has been reported by Purnomo,¹¹ which is said that *Pluchea indica L.* leaves has antibacterial effect against *Staphylococcus sp.*, *Propionobacterium sp.*, dan *Corynebacterium*. Antibacterial test results on *Pluchea indica L.* leaves extract against *Methicillin Resistant Staphylococcus aureus* indicate a minimum inhibitory concentration (MIC) of 20%,¹² while antibacterial test results against *Streptococcus mutans* showed a MIC at concentration of 25%.¹³

Alternative materials from plant extracts currently have been considered as antimicrobial agents since these alternative materials have natural effects, so that the side effects are expected to be lower than from chemical drugs. One of those herbs that has antibacterial properties is *Pluchea indica L.* leaf.⁹ For those reasons, in this research we aimed to determine the antibacterial activity of *Pluchea indica L.* leaves extract against *E. faecalis* and *F. nucleatum* bacteria, which are commonly found as the cause of infection in the pulp and periapical tissues as well as failure in root canal treatment. Consequently, the results of this research are expected to be developed in dentistry as one of alternative to root canal sterilization drug.

MATERIALS AND METHOD

This research was a true experiment research with *post test only-control-group* design. This research used *Pluchea*

indica L. leaves at a concentration of 100%, 50%, 25%, 12.5%, and 6.25%. The number of samples in each group was seven. The tools used in this research were blender, funnel cups, stirrers, measuring cups 500 ml, erlenmeyer tube 500 ml, beaker glass 600 ml, rotary evaporator, filter paper, analytical balance (Analytical Balance CPA 423S Sartorius), oese, spiritus burner, test tubes (BD Falcon), micropipette (Eppendorf), petridiscs, paper disc 5mm, and incubator (500 Memmer). The materials used in this research include *Pluchea indica L.* leaves fresh obtained from UPT Materia Medika Batu, ethanol 80%, sterile distilled water, brain heart infusion (BHI) medium, stock of *E. faecalis* bacteria, stock of *F. nucleatum* bacteria, Mc Farland 0.5, Mueller Hinton Agar (MHA) media, and *Pluchea indica L.* leaves extract with concentration of 100%, 50%, 25%, 12.5%, 6.25%.

Pluchea indica Less leaves were washed under running water. Second, they were dried, aerated, and protected from the sun for 14 days until the leaves were dried and easily crushed. Third, they were crushed in a blender and sieved to obtain the leaves powder. Fourth, 500 grams of the powder was weighed using an analytical balance, and then macerated with 80% ethanol as much as 2 liters shielded from sunlight. The maceration was performed for 3 x 24 hours. But, every 1 x 24 hours, each extract was filtered and macerated back with 800 ml of new ethanol. Finally, the results of the filtrate were then combined and evaporated with a rotary evaporator at temperature 60°C for two hours.¹⁴

Pluchea indica L. leaves extracts were made in five concentration levels, namely 100%, 50%, 25%, 12.5%, and 6.25% w/v (g/ml) by weighing each extract as much as 1 g, 0.5gr, 0.25gr, 0.125gr, and 0.0625gr and then diluted with sterile distilled water as much as 1 ml.

Phytochemical screening was conducted on *Pluchea indica L.* leaves extract. The screening procedure was performed flavonoid test, tannin test, poliphenols test, and phenols test. Flavonoid test was done by mixing 1 mg sample of extracts with 0.5 mg magnesium powder and 5 drops hydrochloric acid (HCL) 2 N. The mixture then was heated over a water bath at temperature 60° C for five minutes in test tube, and then filtered. Next, the filtrate in a test tube was added with three drops of amyl alcohol and then shaken vigorously. The presence of flavonoids was characterized by the formation of yellow to red colour that could be drawn by amyl alcohol. Tannin test was done by mixing 1 mg sample of the extract with five drops solution of 1% gelatin in test tube. The presence of tannin then was marked with a white precipitate. Poliphenols test was done by mixing 1 mg sample of extract with three drops solution of reagent FeCl₃ 1% in test tube. The presence of poliphenols compound was marked with blue-black color. Phenols test was done by mixing 1mg sample of extract with 3 drops solution of FeCl₃ 1% and three (tetes) of K₄Fe(CN)₆. The presence of phenol compound was marked with purple, blue, or green color.¹²

Bacteria used in this research were derived from Special Infection Hospital (RSKI) Airlangga University

Surabaya. The use of bacteria in this research was to create a suspension of *E. faecalis* and *F. nucleatum* bacterial colonies with BHIB media in test tubes, then incubated at 37°C for 48 hours anaerobically. Turbidity of the suspension of *E. faecalis* and *F. nucleatum* bacteria was equated with *Mc Farland* 0.5, which is equivalent to the number of bacteria of 1.5×10^8 CFU/ml. After obtaining the same turbidity, the suspension was diluted to reach a bacterial infectious dose of 1×10^6 CFU/ml (for *E. faecalis*) and 1×10^8 CFU/ml (for *F. nucleatum*).^{15,16}

The antibacterial activity test on *Pluchea indica* L. leaves extract against the growth of *E. faecalis* and *F. nucleatum* bacteria was performed using agar diffusion method and paper discs with a diameter of 5 mm.¹⁶ The paper discs were dipped in *Pluchea indica* L. leaves extract with a concentration of 100%, 50%, 25%, 12, 5%, and 6.25% as much as 50 µl. For the control group, sterile distilled water as much as 50 µl was used. Each paper disc then was implanted in petridisc containing muller hinton agar (MHA) solid media with *E. faecalis* bacterial colonies as much as 1×10^6 CFU/ml and *F. nucleatum* bacterial colonies as much as 1×10^8 CFU/ml. After that, each petridisc was incubated in incubator at 37°C for 24 hours anaerobically. Inhibition zones formed around the paper discs then were measured using a caliper with a precision of 0.05mm. This procedure was repeated seven times for each group of *E. faecalis* and *F. nucleatum* bacteria.^{15,16} The inhibition zones formed around the paper discs on a group of *E. faecalis* and *F. nucleatum* bacteria are classified based on the response of bacterial growth inhibition (Table 1).¹⁷

Analysis of the data used Kolmogorov-Smirnov test to determine whether the distribution of the data was normal. Next, one way Anova test was performed to determine the significance of differences between the groups.

RESULTS

The test results showed that *Pluchea indica* L. leaves extract contained several metabolites, such as flavonoids, tannins, polifenolat, and phenol. The metabolites contained at most are tannins (Table 2).

The antibacterial activity test on the *Pluchea indica* L. leaves extract against *E. faecalis* and *F. nucleatum* bacteria

was performed using agar diffusion method. Previously, dilution method or serial thinning method was conducted to determine the MIC of the extract. Based on the results, it is known that MIC value of the *Pluchea indica* L. leaves extract against *E. faecalis* bacteria was at a concentration of 12.5%, while against *F. nucleatum* bacteria was at a concentration of 50%. Then we made concentration of the extract above and below the MIC, that was 100%, 50%, 25%, 12,5%, dan 6,25%. The results of antibacterial activity test using agar diffusion method can be known based on the inhibition zones formed around the paper discs on *E. faecalis* and *F. nucleatum* bacteria. The mean diameter of inhibition zones formed around the paper disc then was classified in response to bacterial growth inhibition as shown in Table 3.

Table 3 shows the mean diameter of inhibition zones formed around the paper discs in the group of *E. faecalis* bacteria at the extract concentrations of 100% and 50% with medium to strong inhibition responses. Meanwhile, at the concentrations below 50%, there was no inhibition zone. On the other hand, in the group of *F. nucleatum* bacteria, the diameter of the inhibition zone was seen only at the concentration of 100% with medium inhibition response,

Table 1. Classification of inhibition response to the bacterial growth

Diameters of inhibition zones	Inhibition response
≥20 mm	Very Strong
11 – 19 mm	Strong
5 – 10 mm	Moderate
< 5 mm	Weak

Table 2. Metabolites in *Pluchea indica* Less leaves extract

Metabolites	Test results
Flavonoids	++
Tannins	+++
Polifenolat	++
Phenol	++

Note: The sign (+) indicates the tested extracts containing metabolites

Table 3. The mean diameter of the inhibition zones of the *Pluchea indica* Less leaf extract on the growth of *E. faecalis* and *Fusobacterium nucleatum* bacteria along with their resistance response

Bacterial isolates	Mean diameter of inhibition zone (mm)					
	Control	at the concentration of 100%	at the concentration of 50%	at the concentration of 25%	at the concentration of 12.5%	at the concentration of 6.25%
<i>E. faecalis</i>	0	16.2 mm (Strong)	7.76 mm (Medium)	0	0	0
<i>Fusobacterium nucleatum</i>	0	5.73 mm (Medium)	0	0	0	0

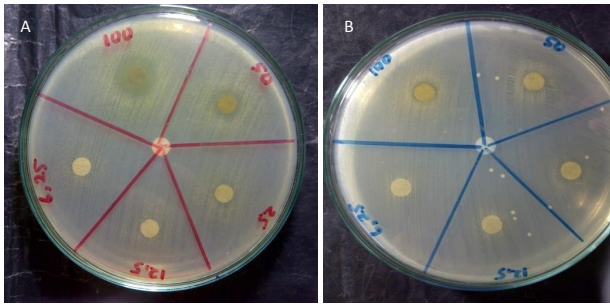


Figure 1. Diameter of inhibition zone that seen around the paper disc in agar diffusion method (A) *E. faecalis*, (B) *Fusobacterium nucleatum*.

while at concentration below 100%, there was no inhibition zone. The size of diameter of inhibition zones formed around the paper discs in the group of *E. faecalis* and *F. nucleatum* bacteria can be seen in Figure 1. The highest diameter of inhibition zone in the group of *E. faecalis* and *F. nucleatum* bacteria was seen at a concentration of 100%. This was consistent with the statement of Pelczar dan Chan,¹⁸ which states that the higher concentration of the extract is, the greater antibacterial effect will be produced.

The results of one way Anova statistical test conducted on the group of *E. faecalis* bacteria, moreover, showed that there was a significant value (p) less than 0.05 at the concentration of 100% and 50% when compared with the extract concentrations of 25%, 12.5%, 6.25%, and without the extract (control). It means that there was a significant difference in the mean diameter of the inhibition zone at the extract concentrations of 100% and 50% when compared with the concentrations of 25%, 12.5%, 6.25%, and without the extract (control). Similarly, there was also a significant value (p) less than 0.05 in the group of *F. nucleatum* bacteria at the extract concentration of 100% when compared with the extract concentrations of 50%, 25%, 12.5%, 6.25%, and without the extract (control). It indicates that there was a significant difference in the mean diameter of the inhibition zone at the concentration of 100% when compared with at the concentrations of 50%, 25%, 12.5%, 6.25%, and without the extract (control).

DISCUSSION

Inhibition responses of the *Pluchea indica L.* leaves extract on the growth of *E. faecalis* bacteria were moderate and strong responses. At the concentration of 50%, the mean diameter of inhibition zone generated the smallest inhibition response, while at the concentration of 100%, the mean diameter of inhibition zone generated the largest inhibition response, relatively strong (Table 3). On the other hand, the inhibition response of the *Pluchea indica Less leaf* extract on the growth of *F. nucleatum* bacteria was only seemed at a concentration of 100% with moderate response (Table 3).

The *Pluchea indica L.* leaves extract has ability to inhibit the growth of *E. faecalis* and *F. nucleatum* bacteria. This was because *Pluchea indica L.* leaves extract contains some compounds that act as antibacterial, such as tannins, flavonoids, and essential oils. In the chemical structure of tannin contains gallo and pirogallo groups, which can react with the bacterial membrane protein. Ester aromatic ring from the gallo and pirogallo groups will bind to protein transport cell envelope of the bacteria, which then will caused protein leakage so that causing damage to cell wall of bacteria and caused bacteria dead.¹⁹⁻²¹ In addition, non-specific bonding also occurs through hydrogen bonding of the groups, which can caused damage to the cytoplasmic membrane of the bacteria, so the membrane functions as a selective permeability barrier, carrier active transport function, as well as control of the internal composition of the cell, will be disrupted. Therefore, if the function of the cytoplasmic membrane integrity is damaged, macromolecules and ions will be out of the cell, and then the cell will be damaged and dead.²²

Activities of flavonoids, moreover, are related to their ability to form complexes with proteins from the cell wall, which will result in damage to the permeability of the bacterial cell wall. Flavonoids have an antibacterial effect because of its ability to interact with DNA of bacteria.²³ Each flavonoid compound has an ability to damage the hydrogen bridge bonding of the strands of the DNA double chain, resulting in the disruption of the stability of the double chain structure of bacterial DNA later influencing the whole process of bacterial growth and metabolism. Flavonoids are also capable of producing energy transduction that will affect the bacterial cytoplasm and slowly motility of bacteria. It is known based on the presence of hydroxyl ions in flavonoids that can chemically alter organic compounds and nutrient transport that can cause toxic effects on the bacterial cells.^{23,25}

Essential oils in the *Pluchea indica L.* leaves extract, furthermore, play a role in damaging cell membranes and bacterial protein denaturation. The main content of essential oils is sinamaldehyda, benzyl alcohol, and eugenol compounds. Benzyl alcohol has solvent properties of fat and protein denaturation, which can cause damage to the bacterial cell membrane. Protein denaturation process involves changes in molecular protein stability and causes both changes in protein structure and protein coagulation. Proteins that undergo denaturation will lose its physiological activity and ability to function properly. Changes that occur in the protein in the cell wall will lead to increased cell permeability. Damage and increased permeability of the cell then will damage the bacterial cells.²⁴

Based on this research, it required high concentration of the extract to be able to inhibit the growth of *E. faecalis* and *F. nucleatum* bacteria. *F. nucleatum* bacteria required higher extract concentration of 100% compared to *E. faecalis* bacteria (50%). The results of this research differ from previous research that used the same extract. Research by Fadhli²⁶ that used *Pluchea indica L.* leaves extract

against *E. faecalis* bacteria, showed MIC at a concentration of 25%. The difference of MIC that was obtained from this research with previous research was likely to be caused by difference in methods used. Previous research by Fadhlia²⁶ used dilution method to show antibacterial activity of *Pluchea indica* L. leaves extract. MIC obtained through dilution method was different with MIC obtained from agar diffusion method. This difference was likely to be caused by media used for bacterial seeding. In dilution methods or a serial number thinning methods, BHIB media containing rich in nutrients are used, so the bacteria can grow rapidly and obtain maximum results.¹⁶ The difference of MIC in this research with previous research also could be caused due to the amount of compounds contained in the extract, so that it could influence the ability of the extract in inhibit the growth of bacteria. In this research we used whole extract of *Pluchea indica* L. leaves, so that we could not know certainty the mechanism of antibacterial activity of each of metabolite compounds with contain in the extract. However, it could be suspected that tannin, flavonoids, and essential oils work synergistically in inhibiting the growth of *E. faecalis* and *F. nucleatum* bacteria.

This research showed high concentration of *Pluchea indica* L. leaves extract to inhibit the growth of *E. faecalis* and *F. nucleatum* bacteria. This can be caused by the type of bacteria used in the research. *F. nucleatum* bacteria is a Gram-negative bacteria that have high phospholipids on its cell wall, making them more permeable than the Gram-positive bacteria. Gram-negative bacteria, on the other hand, have double membranes, and there is a unique periplasm space between them, that is not found in Gram-positive bacteria. In the periplasm space, there are enzymes that are capable of damaging foreign molecules that come from outside the bacterial cell.²⁷ Gram-negative bacteria also have a hydrophilic coating on the outer membrane rich in lipopolysaccharide molecules, which serve as a barrier against the entry of antimicrobial substances. Meanwhile, *E. faecalis* is Gram-positive bacteria, which have outer membrane structure and different cell wall of Gram-negative bacteria. Gram positive bacteria only have a single plasma membrane, and the majority of Gram-positive bacteria are more sensitive to antimicrobial or antibacterial materials.²⁷

Because of the high concentration of *Pluchea indica* L. leaves extract that was obtained from this reserach to inhibit the growth of *E. faecalis* and *F. nucleatum* bacteria, it needs to be examined further about the toxicity and biocompatibility of the extract. So, it can be developed in dentistry as one of alternative to root canal sterilization drug derived from plant extracts. Besides that, with the high concentration of the extract obtained in this research, the Minimum Inhibitory Concentration (MIC) could not be determined, so more researches are needed with smaller concentration range to determine the exact Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of *Pluchea indica* L. leaves extract against *E. faecalis* and *F. nucleatum* bacteria.

Based on the results of this research, it can be concluded that *Pluchea indica* L. leaves extract can inhibit the growth of *E. faecalis* and *F. nucleatum* bacteria with moderate to strong response of growth inhibition, but the MIC of the extract against *E. faecalis* and *F. nucleatum* bacteria could not be determined.

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