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RESEARCH ARTICLE

Biofilm Effectivity of Ethanol Extracts (Casuarina equisetifolia) Leaves of the Bacteria Enterococcus faecalis

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ABSTRACT

Background: *Enterococcus faecalis is one of the bacteria that can form biofilms that can cause persistent endodontic infections. Biofilm is the unity of microbial cell surface surrounded by a matrix of extracellular polymeric substances (EPS). The ethanol extracts of Casuarina equisetifolia leaves are known to have potentially bioactive compounds as antibacterials such as flavonoids, saponins, tannins, phenols, and alkaloids.* ***Objective:*** *To determine the power antibiofilm ethanol extracts of Casuarina equisetifolia leaves at multiple concentrations of the bacteria Enterococcus faecalis.* ***Methods:*** *This study is an experimental research design post-test only control group design. The sample divided into 5 groups consisted of positive control Enterococcus faecalis in media Trypticase Soy Broth (TSB)+DMSO 1%, and 4 treatment groups of ethanol extracts of Casuarina equisetifolia leaves a concentration of 0,5%, 1%, 1,5%, and 2%. Biofilm is made using Enterococcus faecalis ATCC 29212 bacteria were cultured on TSB media were incubated for 1x24 hours. 0,1 ml of the bacteria Enterococcus faecalis with a concentration of* 10^6 *put on a plate micro titter then done using crystal violet staining. Biofilm-checked by measuring optical density (OD) values using the ELISA reader. Data analysis used Kruskal-Wallis followed by Mann-Whitney test.* ***Results:*** *The ethanol extracts Casuarina equisetifolia leaves on the growth of Enterococcus faecalis bacteria indicate the presence of power antibiofilm. The results mean % mortality of bacteria ($p<0.05$) were significant in all groups that have the power antibiofilm effectiveness as against the growth of bacteria Enterococcus faecalis.* ***Conclusion:*** *The ethanol extracts Casuarina equisetifolia leave have antibiofilm power against bacterial growth Enterococcus faecalis.*

Keywords: *Enterococcus faecalis, Enterococcus faecalis antibiofilm, Casuarina equisetifolia leaves*

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INTRODUCTION

Dental caries is the process of crushing or softening enamel or dentin. The crushing process continues until the underlying tissue, and this is the beginning of the formation of holes in the teeth. Caries is a progressive, localized deterioration of the tooth structure, and most commonly causes pulp disease. It is currently generally accepted that for the development of caries, specific bacteria are required on the tooth surface.[1] Bacteria play a major trigger for inflammatory responses in the dental pulp.[2] Several studies have shown that obligate anaerobes are the predominant species in infected root canals. The failure of a root canal treatment can be caused by the presence of microorganisms left in the root canal. The bacterium that often causes infections resulting in root canal treatment failure is Enterococcus faecalis.[3]

Enterococcus faecalis is a gram-positive and fermentative non-spore-positive anaerobic facultative bacterium. The prevalence of infections caused by Enterococcus faecalis ranges from 24%-77%. This is due to various virulence resistance factors of Enterococcus faecalis, including its ability to compete with other microorganisms in their invasion of dentinal tubules and its ability to withstand low nutritional conditions.[1,4] Resistance of Enterococcus faecalis is due to its ability to form a biofilm layer that enables these bacteria to be 1000 times more resistant to phagocytosis, antibodies, and antimicrobials than organisms that do not produce biofilms.

A biofilm is a unit of the microbial cell surface that is covered by an extracellular polymeric substance matrix. A biofilm is a community of bacteria that is organized, communicating with each other, and attached to an inert or living surface.[5,6] The formation of a biofilm starts with several bacteria (planktonic cells) attached to a surface, then reproduces themselves to form a thin layer (monolayer) of the biofilm. Biofilms show very high resistance to antimicrobials, because of the adhesion of the EPS matrix and protection from barriers that inhibit the infiltration of the antimicrobial agent.

Root canal drugs are used to eliminate bacteria that cannot be eliminated by chemo-mechanical processes, such as the Enterococcus faecalis bacteria.[1] deficiency such as the Chlorophenol Kamfer Menthol (ChKM) class of drugs which are toxic, can cause irritation and soft tissue necrosis, pungent odor, and can cause allergic reactions.

Currently, many plants uses as an alternative are being developed, because root canal medicines have several shortcomings. One of the plants that can be used is Cemara Shrimp (Casuarina equisetifolia) which is a plant that is easy to grow and easy to find on the coast of Indonesia. Casuarina equisetifolia has no economic value and benefits, so far it is only used as an aesthetic plant, as a bonsai, and can be found along the coast and used as greening on critical land.[1]

Casuarina equisetifolia is a plant used in traditional medicine for the treatment of diarrhea, coughs, wounds, toothaches, and can be used as a lotion for swelling and diabetes.[7,8] Studies on the antibacterial activity of Casuarina equisetifolia conducted by Nehad [7], shows that Casuarina equisetifolia extract has antimicrobial effectiveness, at a concentration of 2.5% using ethanol and methanol extract can inhibit bacteria, a concentration of 10% shows the result in ethanol extract is 30.33 ± 0.33, and the result of methanol extract shows a result of 36.67 ± 0.33. The methanol extract of the leaves of Cemara Udang (Casuarina equisetifolia) has better results when compared to the ethanol extract, but it is widely reported that methanol has toxic properties.[9] So the researchers chose to use an ethanol solution to make the extract of the leaves of Cemara Udang (Casuarina equisetifolia).

Besides functioning as an antibacterial, Casuarina equisetifolia can be used as an antifungal, one of which is Candida albicans at a concentration of 10% which can inhibit Candida albicans. Research conducted by Nehad using leaf extract of Shrimp Cemara (Casuarina

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equisetifolia) on pathogenic microorganisms with concentrations of 2.5%, 5%, 7.5%, 10%, 15%, and 20% against Staphylococcus aureus bacteria.[7] At very low concentrations 2.5% can function as antibacterial, but there are no research data on the power of antibiofilm (Casuarina equisetifolia) against Enterococcus faecalis bacteria. This shows that the higher the extract concentration used, the higher the level of the extract's toxicity. [7]

Based on this background, this study determined the antibiofilm potency of the leaf extract of Cemara Udang (Casuarina equisetifolia) as an alternative drug for root canal sterilization to inhibit the growth of Enterococcus faecalis bacteria which is often found in failed root canal treatments.

MATERIALS AND METHODS

This study used experimental laboratory research. Research on Enterococcus faecalis bacteria was carried out in vitro using the microtiter plate assay method.

The sample used in this study was the bacterium Enterococcus faecalis ATCC 29212 obtained from the Surabaya Health Laboratory Center. Then, the Enterococcus faecalis stock was cultured on TSB media. Trypticase Soy Broth (TSB) media is used as a medium for biofilm formation by Enterococcus faecalis bacteria, because this medium is the most effective medium for biofilm formation by bacteria.

The number of samples used in this study was 45 samples, then divided into 5 groups, namely group 1 positive control using Enterococcus faecalis bacteria on Trypticase Soy Broth (TSB) + 1% DMSO media, group 2,3,4, and 5 treatment groups given an extract of Shrimp Cemara (Casuarina equisetifolia) leaves from Madura with a concentration of 0.5%, 1%, 1.5%, and 2%.

The method used to test biofilms is the microtiter plate biofilm assay method. The initial test suspension was made equal to the turbidity of 0.5 Mc Farland and diluted to reach a bacterial concentration of 106. The suspension of Enterococcus faecalis bacteria was cultured into the microplate and then incubated at 37°C for 6x24 hours. After 6 days, 0.1 ml of suspension from the ethanol extract of the leaves of Cemara Udang (Casuarina equisetifolia) was added at a concentration of 0.5%, 1%, 1.5%, and 2% into each microplate well. Then on the 7th day, the optical density value was measured using an ELISA reader.[10]

RESULTS

Data obtained from the measurement of optical density values, then calculated using the formula for % bacterial mortality:

% Bacterial mortality = $1-(\frac{OD7-KB-OD0}{PC7-PC0}) \times 100\%$

Explanation :

OD7: *Optical density (596nm) microtiter samples on 7th day of incubation*

OD0: Optical density (596nm) microtiter samples at 0 incubation day

KB: Optical density (596nm) control of materials on the 7th day of incubation

PC7: Optical density (596nm) positive control on 7th day of incubation

PC0: Optical density (596nm) positive control at 0 incubation days.

Tabel 1. Results% of mortality by the biofilm Enterococcus faecalis

Group	Mean ± Std. Deviation
K1	21,03±14,58
K2	94,38± 5,33
K3	92,94± 6,79
K4	73,83±11,55
K5	90,48± 8,75

Information: K1: K + (Enterococcus faecalis bacteria on TSB + DMSO 1% media), K2: (0.5% extract), K3: (1% extract), K4: (1.5% extract), K5: (Extract 2%) in%.

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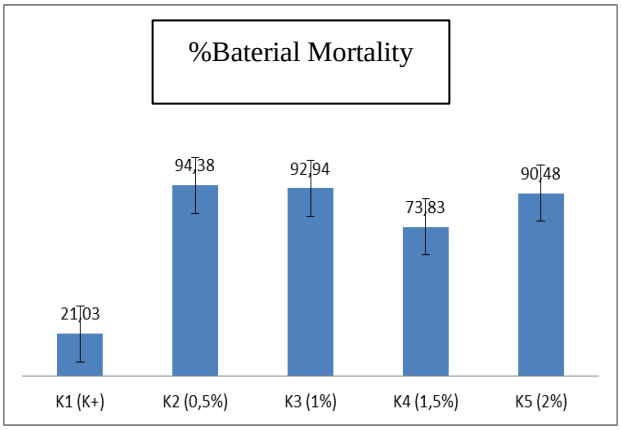
Tabel 2. Antibiofilm power of ethanol extract of shrimp pine leaves (Casuarina equisetifolia) graph of mean% mortality of Enterococcus faecalis bacteria. Information: K1 (Enterococcus faecalis bacteria on TSB + DMSO 1% media, K2: 0.5% extract, K3: 1% extract, K4: 1.5% extract, and K5: 2% extract.



Based on the table and graph of the average antibiotic power of Enterococcus faecalis, it shows that at a concentration of 0.5% it has the greatest effectiveness against Enterococcus faecalis biofilm compared to ethanol extract of shrimp pine leaves in various concentrations. This is evidenced by the results of% bacterial mortality, concentrations of 0.5%, 1%, 1.5%, and 2% that have antibiofilm power against Enterococcus faecalis bacteria. Positive control for DMSO 1% had the lowest percentage compared to the ethanol extract concentration of Cemara Udang leaves.

Tabel 3. Results of normality test with Shapiro-Wilk

Group	Mean ± Std. Deviation
K1	0,245
K2	0,007
K3	0,004
K4	0,208
K5	0,047

Based on the results of the normality test it can be seen that the antibiofilm power of the ethanol extract of the leaves of Cemara Udang (Casuarina equisetifolia) in the K1, K2, K3, K4, K5 groups have an abnormal distribution because it has a value (p <0.05). Then the non-parametric test was carried out, the hypothesis test used was the Kruskal-Wallis test, then the Mann-Whitney test was performed.

Tabel 4. Kruskal-Wallis Test Results

Group	Sig
K1	0,000

In Table 4, the significance value is obtained of 0.000 (p <0.05). This shows that there are differences between treatment groups.

Subsequently, a Post Hoc analysis was carried out to see a significant difference in the antibiofilm power of the ethanol extract of Cemara Shrimp (Casuarina equisetifolia) leaves against the growth of Enterococcus faecalis bacteria from each group. To perform Post Hoc analysis of the Kruskal-Wallis test is to use the Mann-Whitney test.

Tabel 5. Mann Whitney result

Kelompok	K2	K3	K4	K5
K1	.000*	.000*	.000*	.000*
K2		.340	.000*	.387
K3			.001*	.436*
K4				.003*

Remarks *: there is a significant difference.

The Mann-Whitney test results show that there is a difference between the K1 group as a positive control and the K2, K3, K4, and K5 groups as the treatment group. In the K2 group when compared with the K3 group, there was no significant difference. Likewise between groups K2 with K5, and K3 with K5 because they have a significant value> 0.05.

DISCUSSION

Biofilm is a unity of the microbial cell surface covered by a matrix of extracellular polymeric substances.[5]

The selection of Enterococcus faecalis ATCC 29212 is based on the research of Subbiya[8], where the Enterococcus faecalis ATCC 29212 bacteria have the same virulence

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as the Enterococcus faecalis bacteria isolated from root canals.

In this study, a concentration of 0.5% was used because based on the results of preliminary research, the concentration of 0.5% had the power of antibiofilm against the growth of the Enterococcus faecalis bacteria.

Enterococcus faecalis bacteria on TSB + DMSO 1% media was used as a positive control in this study because, DMSO 1% was used as a diluent for ethanol extract of leaves of Cemara Shrimp (Casuarina equisetifolia), the concentration of DMSO 1% did not have antibacterial properties that would affect the antibiofilm power of ethanol extract Leaf Cemara Shrimp (Casuarina equisetifolia) on the growth of Enterococcus faecalis.[11] This is proven based on the results of research that the ethanol extract of leaves of Cemara Shrimp (Casuarina equisetifolia) with a concentration of 0.5% has the greatest average value, namely 94.38% when compared to other concentrations. . DMSO 1% has a mean value of 21.03% which is the smallest mean value compared to other concentrations, so the addition of 1% DMSO as a diluent does not affect the effectiveness of the ethanol extract of Cemara Udang (Casuarina equisetifolia) leaves.

The ability of antibiofilm against the formation of Enterococcus faecalis bacteria is influenced by a concentration. The greater the concentration, the higher the inhibition of biofilm formation,[12] but in this study, it was shown that the higher the concentration of the leaf extract of Cemara Shrimp (Casuarina equisetifolia), the smaller the antibiofilm's power, on the contrary, the smaller the concentration, the greater the antibiofilm power produced. This research shows that 2% concentration has a mean value of 90.48%, a concentration of 1.5% has a mean value of 73.83%, a concentration of 1% has a mean value of 92.94%, and a concentration of 0.5% has a mean of 94.38%. . Concentration of 1.5% has a mean value of 73.83%, this shows a decrease in the power of antibiofilm when compared with concentrations of 0.5%, 1%, and 2%, this decrease is due to the concentration of 1.5% has a higher OD7 value. large when compared with a concentration of 2% so that the resulting mean value is small.

The ethanol extract of the leaves of Cemara Udang (Casuarina equisetifolia) contains flavonoids, saponins, tannins, phenols, and alkaloids.[7] Tannin compounds can inhibit the formation of biofilms by interfering with enzymes, inhibiting enzyme production, and reducing the concentration of calcium ions needed in the coagulation process of bacterial plasma. Bacterial plasma coagulation is needed by bacteria to form fibrin-rich biofilms so that biofilms do not form.[13]

Flavonoids are a phenol group and can kill bacteria by forming complex compounds with proteins through hydrogen bonds. At high levels of phenol, it causes protein coagulation and cell membranes to undergo lysis.[14] Saponins are characterized by their ability as a surfactant that can reduce surface tension to disrupt the integrity of bacterial cell membranes.[15] Alkaloids have alkaline groups that contain nitrogen. The presence of this base group when in contact with bacteria will react with amino acid compounds that make up the bacterial cell wall and also bacterial DNA. This reaction results in a change in the arrangement of the amino acid chains in DNA and will cause a change in the genetic balance of the DNA acid so that the bacterial DNA will be damaged. With the damage to the DNA, the bacterial cell nucleus will be damaged. This DNA damage in the bacterial cell nucleus will also encourage lysis of the bacterial cell nucleus. [16]

This research needs to be developed to find an effective concentration that can be applied as an alternative to root canal sterilization materials. In this study, the concentration of the extract used should not be more than 20% because the characteristic of the leaf extract of Cemara Udang (Casuarina equisetifolia) is sticky and dark brown, this can cause interference with the ELISA reader reading. The higher the concentration used can cause toxicity to the cells in the periapical area and around the non-vital teeth, causing further

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complications.[17] The next research can use another method to determine the antibiofilm power of the ethanol extract of Cemara Udang leaves (Casuarina equisetifolia) on the growth of Enterococcus faecalis bacteria. This research is preliminary, so it is necessary to develop research on the use of ethanol extract of Cemara Udang (Casuarina equisetifolia) leaves as an alternative material for root canal sterilization. It is feared that the color density of the extract can cause discoloration of the teeth so that further research is needed on the tooth discoloration test after applying the ethanol extract of the leaves of Cemara Udang (Casuarina equisetifolia).

CONCLUSION

Based on the results of this study, it is known that the ethanol extract of the leaves of Cemara Udang (Casuarina equisetifolia) has antibiofilm power against the growth of Enterococcus faecalis bacteria. The ethanol extract of Shrimp Cemara (Casuarina equisetifolia) leaves at concentrations of 0.5%, 1%, 1.5%, and 2% can inhibit the growth of the bacteria Enterococcus faecalis. The concentration of 0.5% is the most effective as an antibiofilm and inhibits the growth of the bacteria Enterococcus faecalis.

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