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RESEARCH ARTICLE

The antibacterial effect of Anchovy (Stolephorusinsularis) Extract Against P.aeruginosa

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ABSTRACT

Introduction: *Root canal infection is largely a continuation of the caries process that is not treated and develops so that it involves the root canal. Sterilization is a part of multivisit root canal treatment and important for the success of root canal treatment. The Anchovy (Stolephorus insularis) contains protein, vitamins (A, B1, C), and minerals (Fe, Ca, K, F). Calcium fluoride (CaF2) contained in anchovies can inhibit bacteria that cannot be removed by chemo-mechanical processes such as instrumentation and irrigation* ***Purpose:*** *The aim of this study was to determine the antimicrobial ability of anchovy extract (Stolephorus insularis) to P. aeruginosa.* ***Materials and Methods:*** *This study was a laboratory experimental research which with post test only control group design. Diffusion method were applied with 2 controls: positive control using ChKM solution, negative control using 1% DMSO and 3 concentrations of jengki anchovy extract (Stolephorus insularis) 18%, 24%, and 30% as treatment groups, where each group consisted of 5 samples. Antimicrobial was assessed by measuring the diameter of the clear zone around the discs contained the anchovy extract (Stolephorus insularis). Data were analyzed by Kruskal-Wallis test followed by Mann-Whitney test.* ***Result:*** *The results from this study showed clear zone around the discs of the anchovy extract (Stolephorus insularis). The more concentration of the extract showed the more antimicrobial zone diameter. The average inhibitory zone at 18% concentration was 6.03 mm, 24% 7.59 mm, 30% 8.69 mm, positive control of ChKM solution 31.43 mm for negative control DMSO 1% 6.03 mm. The largest diameter of the clear zone is at a concentration of 30%.* ***Conclusion:*** *The results obtained showed that the inhibition zone of the Stolephorus insularis extract concentration of 30% had the largest average among the other concentrations of 8.69 mm.*

Keywords: *Anchovy extract (Stolephorus insularis), P. aeruginosa, root canal sterilization, antimicrobial effect.*

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INTRODUCTION

Dental and oral health is an important issue and needs to be considered. The most common dental and oral health problems are caries and periodontal disease.[1] Caries can affect both adults and children. Caries process mostly progressed as root canal infection. Untreated caries will continue to grow and become a gateway for microorganisms to enter the pulp and activated inflammatory response than continue to become pulp necrosis.[1,2]In necrosis teeth, pulp tissue will be a media of bacterial products accumulation and microorganism breakdown products[3,4] Bacteria and their products and inflammatory mediators will accumulate in the root canal and exit to the periapical tissue through the apical foramen so that abnormalities occur in periapical tissue such as chronic apical periodontitis [3,5]

Chronic apical periodontitis is a polymicrobial infection, an infection caused by various types of bacteria, namely Pseudomonas aeruginosa (P.aeruginosa), S.aureus, A.faecalis, Actinomyces spp, Streptococcus spp, Porphyromonas spp and Eubacterium[6] One of them is P.aeruginosa is a bacterium found in the root canals that causes endodontic treatment failure. P. aeruginosa is an opportunistic component of root canal microbes that can be found in primary endodontic infections, in tooth necrosis, abscesses, extraction wounds, endodontic infections, sinus fluid, and injury to injury [6]

Root canal treatment can be divided into three main stages, namely root canal preparation or cleaning and forming and smoothing, root canal disinfection or sterilization and root canal filling.[7] Sterilization is a part of multivisit root canal treatment and is important for successful root canal treatment. The use of root canal sterilization agents will kill pathogenic microorganisms in the root canal. Root canal sterilization materials must meet several requirements, including effective in killing microorganisms in the root canal with a long-term effect, not toxic, does not irritate the root canal and can inhibit pain [3]

Root canal treatment is one of the dental treatment, that involves treating diseases or injuries in pulp and periapical tissue. The purpose of this treatment is to restore the condition of a tooth that is biologically acceptable to the surrounding tissue so that the tooth can be maintained as long as possible in the mouth. This means the tooth does not cause complaints and can functioning fine. [8]

Although done irrigation in the root canal, but microorganisms may still be left behind, especially in the dentine tubules. Other researchers mention that cleaning and forming root canals and irrigation of root canals significantly reduce or kill microorganisms from the root canals. But the complete elimination of microorganisms cannot always be achieved clinically, due to the anatomical shape of the root canal and the limitations of instrumentation and irrigation[6] These bacteria are mostly habitats in the mouth area especially saliva, tongue and gingival. This bacterium is quite unique and has not been isolated from other habitats. Just like Enterococcus faecalis, Actinomyces is also a gram-positive bacteria that are anaerobic and do not form spores. Aerobic bacteria such as Diphtheroids, Staphylococci, Streptococci, E.coli, Pneumoniae, P. aeruginosa are also found in root canals of teeth that experience necrosis [9,10]

Provision of root canal sterilization is intended to kill bacteria that cannot eliminated by chemo-mechanical processes such as instrumentation and irrigation. Based on their chemical properties, root canal sterilization materials are divided into two groups, namely specific drug classes and non-specific drugs which can be in the form of one or a combination of several antibiotics. One of the root canal sterilization drugs that are often used is the phenol group, such as ChKM (Chlorophenol Kamfer Menthol) which has a disinfection ability and has a broad spectrum so that it can be used in root canal treatment [11] However, this phenol sterilization drug has

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several weaknesses that are toxic, can kill cells that are still potential, are allergens that can cause immune reactions and have a pungent odor and an unpleasant taste that causes discomfort in patients [3]

In a study conducted by Fa'izah (2016) using anchovy extract (Stolephorus insularis) has antibacterial activity against Streptococcus mutans at concentrations of 3%, 6% and 12%. In this study it can be seen that the fluoride contained in the extract of anchovy (Stolephorus insularis) has antibacterial activity against Streptococcus mutans.[12] Stolephorus insularis contains antibacterial substances such as fluoride. Besides that, Stolephorus insularis also contains energy, protein, fat, carbohydrate, calcium, iron, phosphorus, vitamin A, vitamin B and vitamin C.[13] In Indonesia, Stolephorus insularis is very easy to obtain and most people consume it.[14] This is because Stolephorus insularis is one of the most abundant resources throughtout Indonesia, especially in coastal waters.[12,15]

Antimicrobials are biological or chemical compounds that can interfere with microbial growth activities.[16,17] Fluoride in bacteria works bacteriostatically, which inhibits cell proliferation by preventing the synthesis of nucleic acids which are a vital part of cell development. The mechanism of action is by binding to the polymerase-RNA enzyme (in subunits) so that it inhibits RNA and DNA synthesis.[18]

Based on the above reasons, the researchers wanted to know the concentration of Stolephorus insularis extract which was effective as an antimicrobial against the growth of P. aeruginosa bacteria.

MATERIALS AND METHODS

This research is an experimental laboratory research. The design of this study is the post test only control group design. For the study design, group objects are divided into 5 groups. The study design group 1 (negative positive control group) using 1% DMSO ChKM, group 2 (negative control group) using 1% DMSO and groups 3, 4, and 5 were the treatment groups that were given jengki anchovy extract (Stolephorus insularis) with different concentrations, namely 18%, 24%, and 30%.

The experimental unit or sample used in this study is pure P. aeruginosa bacteria from the Microbiology Laboratory of the Surabaya Health Laboratory Center with the criteria that the number of P. aeruginosa bacterial colonies is synchronized with a Mc Farland solution of 0.5.

P. aeruginosa bacteria obtained in the form culture of blood agar, then taken as much as 2 ose then incubated on liquid BHI (Brain Heart Infusion) media for 2x24 hours at 37 ° C in an anaerobic atmosphere. The turbidity of the P. aeruginosa bacterial suspension was then synchronized with the Mc Farland standard 0.5 to obtain a bacterial suspension containing 1.5x108 CFU / ml (Colony Forming Units) with the eye carried out by holding the test tube next to each other and looked at him on a white background with black stripes. If the turbidity of the bacterial suspension is still not the same, the bacterial suspension can be diluted or given additional bacteria, then homogenized by centrifuge.[9] The process of equalizing bacteria with the Mc Farland 0.5 standard is carried out at the Oral Biology Laboratory of the Faculty of Dentistry, Hang Tuah University, Surabaya.

Antibacterial power was examined by measuring the diameter of the clear zone that appeared around the filter paper placed on the BHI agar media by the diffusion method. Measurement using a digital caliper (with a diameter of mm). In order to obtain more valid data, measurements were made three times, namely the longest, medium and shortest diameters and then taken an average of the three length measurements.

The anchovy extract (Stolephorus insularis) is a sea fish obtained from Kenjeran Beach fishermen. The anchovy (Stolephorus insularis) used for this research is fresh the anchovy. The anchovy (Stolephorus insularis) is extracted by maceration method by means of

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the anchovy (Stolephorus insularis) is roasted at <100ºC for three hours in five days, blended, then sifted until 100 mesh fineness. The anchovy fish powder (Stolephorus insularis) is taken as much as 500 grams, then soaked using 96% ethanol until submerged for 3x24 hours. Then the thick extract of the anchovy (Stolephorus insularis) with semisolida consistency made three concentrations of 18%, 24%, and 30% using 1% DMSO.

Prepare BHI media so that it is sterile for 5 research samples, each sample consisting of 3 treatment groups and 2 control groups. Taking P. aeruginosa culture from liquid BHI (Brain heart Infusion) media that has been equaled with turbidity with Mc Farland 0.5 solution (bacterial suspense containing 1.5x108 CFU / ml), then rub the bacterial culture on the entire surface of BHI media (Brain Heart Infusion)) so that by using a sterile cotton swab.[9]

In the positive control group, the disc was immersed in 1 ml of ChKM solution for 10 seconds. In the negative control group, the disc was immersed in 1 ml DMSO 1 ml for 10 seconds. In the treatment group, the disc was immersed in Stolephorus insularis extract with a concentration of 18%, 24% and 30% for 10 seconds, then placed the disc in one of the BHI media zones using sterile tweezers and was slightly pressed. Insert petridish into anaerobic jar with gaskit and anaerobic indicator, then incubated in an incubator for 2x24 hours at 37ºC.

Inhibited zones formed were measured and the data obtained were analyzed by statistical tests using SPSS version 23.

RESULTS

The research data were analyzed descriptively to get a picture of the distribution and summation of data in order to clarify the presentation of research results.

Table 1shows that there is an antibacterial power of Stolephorus insularis extract at a concentration of 18% (6.03 mm); 24% (7.59 mm); 30% (8.69 mm); and in the positive control, ChKM (31.43 mm) against P. aeruginosa. While the negative control of 1% DMSO did not show any antibacterial power against P. aeruginosa.

Table 1. Average diameter of inhibition zone and standard deviation of Stolephorus insularis extracts against the growth of P. aeruginosa bacteria.

Group	Replication	Average	Std. Deviation
K+	5	31,43	2,68
K-	5	6,03	0,01
P1	5	6,03 7,59	0,01
P2	5	8,69	0,43
P3	5		0,77
Total	25		

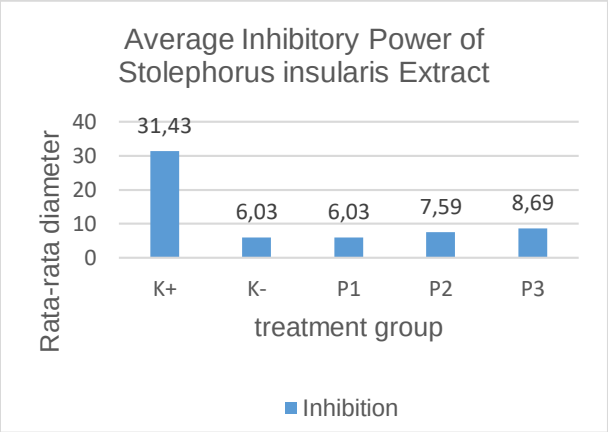

Figure 1. Graph of average diameter of inhibition zone

In Figure 1, the results of the study showed that there were inhibitory zones of Stolephorus insularis extract on the growth of Pseudomonas aeruginosa in the K +, P2 and P3 groups, whereas in the K- and P1 groups there was no inhibitory zone. The treatment was then tested for significance with an error rate of 5% (p = 0.05).

Normality test is done before the research hypothesis test. The normality test is one of the One Way ANOVA test requirements. Furthermore, the results of the study were tested for normalitydistribution using Shapiro-Wilk, because the number of research subjects is less than 50. This test aims to determine whether the data obtained are normally

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distributed or not with a significance level of 0.05 (p = 0.05). If the data is normally distributed (p> 0.05) it can proceed with homogeneity test and if the data is not normally distributed (p <0.05) then the data transformation is carried out.

Table 2.*Shapiro-Wilk test* results

Group	Shapiro-Wilk	Sig.
K+	0,896	0,391
K-	0,833	0,146
P1	0,833	0,146
P2	0,974	0,902
P3	0,967	0,854

Based on the results of the normality test it can be seen that the Stolephorus insularis inhibition zone variable in each treatment group is normally distributed because it has a p value > 0.05. Then it can be continued with homogeneity test and if the data is not normally distributed (p<0.05) then the data transformation is carried out.

Homogeneity test

Table 3.*Levene test* results

Levene Test	Sig.
8,48	0,000

From the results of the homogeneity test (Levene Test) it is known that the significance value of 0,000 has a significance value of less than 0.05 (p <0.05). , namely the Kruskal-Wallis test to determine the difference between the positive control with the treatment group concentrations of 18%, 24%, and 30% of the Stolephorusinsularis extract in each sample.

Hypothesis testing

Table 4.*Kruskal-Wallis Test*

Chi-Square	21,610
Df	4
Asymp. Sig	0,000

Table 4 obtained a significance value of 0,000 (p <0.05). This shows the difference in meaning between positive controls with each treatment group that has different concentrations. Based on this, the Mann-Whitney test was continued.

Mann-Whitney Test

Post Hoc analysis to see a significant difference in the inhibition of Stolephorus insularis on the growth of Pseudomonas aeruginosa bacteria from each group. To do the Post Hoc analysis of the Kruskall-Wallis test is to use the Mann-Whitney test

Table 5.*Mann-Whitney test results(LSD)*

	K+	K-	P1	P2	P3
K+		0,009*	0,009*	0,009*	0,009*
K-			1,000	0,009*	0,009*
P1				0,009*	0,009*
P2					0,047*
P3					

From the results of the Mann-Whitney test it is known that all treatments compared with K + (positive control / ChKM) showed significant differences. In P1 (Stolephorus insularis extract 18%) when compared to K- (negative control), there was no significant difference because the significance value was greater than 0.05 (p> 0.05), in P2 (Stolephorus insularis extract 24%) showed significant differences when compared with K- (negative control / DMSO1% solution), whereas in P3 (Stolephorus insularis extract 30%) showed significant differences when compared with K- (negative control / DMSO1% solution) To determine a significant difference determined by the average antimicrobial extract of Stolephorus insularis.

Based on table 5 the LSD test results there was a significant difference in inhibition of P. aeruginosa bacteria (p <0.05) in the K + group with the K-, P1, P2 and P3 groups, the K- group with the P2 and P3 groups. While K- with group P1 there was no significant difference because the significance value was greater than 0.05 (p> 0.05). While between group P1 with group P2, P3 and group P2 with group P3 there were significant differences.

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DISCUSSION

This study aims to determine the antibacterial power of Stolephorus insularis extract against Pseudomonas aeruginosa. Stolephorus insularis contains between 15.7-38.3 ppm fluoride, mostly in the form of CaF2 compounds which are shortly decomposed in liquid form, so they can provide sufficient quantities of F ions in their environment.[19] CaF2 provides the best fluoridation results because of its ability to release fluoride gradually and also acts as a fluoride reserve.[15]In a previous study using Stolephorusinsularis extract, it had antibacterial activity against Streptococcus mutans at concentrations of 3%, 6%, and 12%.[12] Based on these studies it can be seen that the fluoride content contained in the extract of Stolephorus insularis has antibacterial activity against the bacterium Streptococcus mutans.

In this study, the extract of Stolephorus insularis was tested for its inhibition against the Pseudomonas aeruginosa bacteria which are obligate gram-negative aerobic bacteria. The prevalence of infection in cases of failure after root canal treatment caused by Pseudomonas aeruginosa ranges from 24%-77%. This is due to various virulence and resistance factors of Pseudomonas aeruginosa.[19]

The inhibitory study of Stolephorus insularis extract on the growth of Pseudomonas aeruginosa bacteria was carried out by a diffusion method that used BHI agar media, because it was quite practical to do and was an effective media used to determine the growth of Pseudomonas aeruginosa bacteria which is a gram-negative aerobic obligate. [16] Diffusion method used in this study because researchers can see the level of sensitivity of Pseudomonas aeruginosa against Stolephorus insularis extract. In addition, the diffusion method is a simple and easy procedure and can be used to test aerobic and facultative anaerobic bacteria.[10]

DMSO 1% is used as a dilute extract of Stolephorus insularis as well as a negative control (K-) because the researchers conducted a comparison test of the homogeneity of the extract first using diluent aquades and 1% DMSO. From these results it is known that the extract of Stolephorus insularis is more homogeneous in 1% DMSO solution compared to distilled water. The use of 1% DMSO is also due to DMSO being one of the solvents that can dissolve almost all compounds both polar and non-polar.[20] DMSO does not show any influence on bacteria to 2% DMSO concentration, even there is no visible effect on 3% DMSO concentration. New bacteria showed a decrease in the number when exposed to a DMSO concentration of 4% or more.[17] The statistical test results also showed that 1% DMSO had a significant difference compared to all positive concentrations and controls.

The choice of ChKM as a positive control (K +) because it is one of the root canal sterilizers. The mechanism of action of ChKM in inhibiting bacterial growth is the same as the mechanism of action of fluorine in inhibiting bacterial growth ie inhibiting cell proliferation by inhibiting nucleic acid synthesis which is a vital part for cell development, by binding to the polymerase-RNA enzyme (in sub units) thereby inhibiting RNA synthesis and DNA.[18] In addition, ChKM is included in the derivatives of phenol compounds, the mechanism of action of phenol compounds in inhibiting bacterial growth, namely by denaturing bacterial cell proteins, inhibits cell membrane function (transporting substances from one cell to another) and inhibits nucleic acid synthesis so that bacterial growth can be inhibited.[11]

In this study Stolephorus insularis extract was examined at concentrations of 18%, 24% and 30%. The choice of concentration was based on research conducted by Fa'izah (2016), where Stolephorus insularis extract was shown to have antibacterial activity against Streptococcus mutans at concentrations of 3%, 6% and 12%. Stolephorus insularis extract with a concentration of 12% is the most effective concentration to inhibit the growth of Streptococcus mutans bacteria.[12] In this case

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researchers feel the need to increase the concentration to a concentration of 18%, 24% and 30%, this is due to various factors of virulence resistance and resistance which is found in the bacterium Pseudomonas aeruginosa. In this study it was seen that Stolephorus insularis extract had antimicrobial power against P. aeruginosa bacteria in the treatment group with concentrations of 24%, and 30%. At 18% concentration the antimicrobial power of Stolephorus insularis extract did not show any inhibitory zone with the antimicrobial power of the ChKM solution as a positive control, or with a negative control.

This is caused by anchovy (Stolephorus insularis) containing antibacterial substances, namely fluorine. The mechanism of action of fluorine in inhibiting bacteria, namely inhibiting cell proliferation by inhibiting the synthesis of nucleic acids which is a vital part of cell development. The mechanism of action of fluorine is by binding to the polymerase-RNA enzyme (in subunits) so that it inhibits RNA and DNA synthesis. [18]

In addition, fluorine also limits the supply of reserves for acid production in the synthesis of polysaccharides. The making of this acid is carried out by cariogenic bacteria that are metabolizing carbohydrates at low pH (5.5 or <5.5). However, at low pH conditions, fluoride can easily diffuse into Hydrofluoric Acid thereby inhibiting carbohydrate metabolism carried out by the cariogenic bacteria.[21] Even bacteria can experience death due to the inhibition of the effects of glycolysis in their cells so that the bacteria do not get energy.[22]

In this study it was seen that the greater the concentration of Stolephorus insularis extract, the greater the diameter of the inhibition zone. Stolephorus insularis extract with a concentration of 30% has the largest average inhibition zone of 8.69 mm, compared with an concentration of 18% with an average inhibition zone of 6.03 mm and a concentration of 24% with an average inhibition zone of 7.59 mm. However, ChKM has a greater inhibition zone compared to the whole concentration of Stolephorus extract insularis that is equal to 31.43 mm. This was also seen in statistical tests which showed that ChKM had a significant difference with the entire concentration of Stolephorus insularis extract and DMSO 1% (6.03 mm). This happens because ChKM has a fairly large phenol content consisting of 60% chloro-phenol, 40% camphor and 6% menthol. The camphor content contained in ChKM can extend its antimicrobial effect.[11] So that the inhibition of ChKM can be greater than that of Stolephorus insularis extract. Pseudomonas aeruginosa is a gram-negative bacterium that has the characteristics of a thick cell wall structure in the form of peptidoglycan.[23] Because of the characteristics of the thick bacterial cell wall, it will be very difficult for the active substance extract of Stolephorus insularis to penetrate the cell wall. In addition, gram-negative bacteria also have a defense against acidic conditions in the form of a proton pump mechanism so that it is able to balance the pH in cells and the antimicrobial substrate cannot penetrate into the cytoplasmic membrane.[24]

This study is still qualitative in nature because it only shows differences in the antibacterial power of Stolephorus insularis fish extracts against Pseudomonas aeruginosa at concentrations of 18%, 24%, 30% and research cannot be carried out in the form of quantity to measure the presence of antibacterial compounds. The results obtained showed that the inhibition zone of the Stolephorus insularis extract concentration of 30% had the largest average among the other concentrations of 8.69 mm.

CONCLUSION

Based on the results of this study, it is known that the extract of Stolephorus insularis at a concentration of 24% and 30% has an antibacterial power against Pseudomonas aeruginosa. The 30% concentration has the most effective antibacterial power to inhibit the growth of Pseudomonas aeruginosa bacteria

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