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Original Research Article

The Effectiveness of Ethanol Extract of Neem Leaf (*Azadirachta indica*) Mouthwash Against the Growth of *Streptococcus sp.*

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Abstract

Background: *Streptococcus sp.* is a bacteria group in the oral cavity that plays a significant role in initiating plaque biofilm formation on teeth. One way to control plaque is gargling with mouthwash. Neem leaves contain antibacterial compounds such as alkaloids, steroids, glycosides, and flavonoids that can be formulated as mouthwash ingredients.

Objective: The study aimed to determine the effectiveness of Ethanol Extract of Neem Leaf (EENL) mouthwash on the growth of *Streptococcus sp*.

Methods: The research design was a pretest-posttest control group. The 28 samples of *Streptococcus sp*. were divided into 7 treatment groups (4 repetitions). The treatment group was given EENL mouthwash with concentrations of 2.5%, 5%, 7.5%, 10%, and 12.5%, a negative control was given aquadest and positive control was given 0.2% chlorhexidine gluconate. Bacterial growth is known through the absorbance values. Data analysis used one-way Anova test and post hoc LSD test at $p<0.05$

Results: EENL mouthwash has antibacterial activity against *Streptococcus sp*. One way ANOVA test on the difference in absorbance values shows a p=0.00. LSD post hoc test between EENL concentration of 2.5% and chlorhexidine 0.2% showed equivalent antibacterial activity ($p>0.05$). Meanwhile, the post hoc LSD test between concentrations of 5% - 12.5% with 0.2% chlorhexidine showed a significant difference in antibacterial power ($p<0.05$). This indicates a stronger antibacterial activity at a concentration of 5% - 12.5% compared to 0.2% chlorhexidine.

Conclusion: EENL mouthwash effective against the growth of *Streptococcus sp*

Keywords: *Ethanol extract of neem leaf; Streptococcus sp; Mouthwash*
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INTRODUCTION

According to the Indonesian Ministry of Health, 2018 Basic Health Research Data showed that 57.6% of Indonesians have dental and oral health problems due to a lack of awareness regarding the importance of dental and oral health.[1] It can lead to the formation of dental plaque if not paid attention to oral hygiene, which is the precursor to other oral diseases.[2]

Dental plaque is a deposit that is tightly attached to the tooth surface, consisting of microorganisms that multiply in an intercellular matrix and will continue to accumulate if not cleaned adequately.[3] Adequate and accumulated plaque can form an acidic environment (lactic acid) from the fermentation of sucrose by Streptococcus species which eventually starts enamel decalcification until the formation of subsequent caries.[4] The initial colonization process is dominated by *Streptococcus* oral, which constitutes more than 80% of the initial biofilm constituents.[5] The effective way to reduce dental plaque is by controlling plaque.[6]

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Table 1. *The results of the inhibition test with UV-Vis Spectrophotometry with a wavelength of 600 nm*

No.	Treatment	Rep 1		Rep 2		Rep 3		Rep 4		Mean		Result
		Before	After	Before	After	Before	After	Before	After	Before	After	
1	**2,5%**	**0.188**	**0.102**	**0.169**	**0.085**	**0.152**	**0.071**	**0.137**	**0.041**	**0.162**	**0.075**	**Decrease**
2	5%	0.231	0.027	0.191	0.087	0.170	0.024	0.229	0.063	0.205	0.050	**Decrease**
3	7,5%	0.460	0.116	0.428	0.203	0.468	0.275	0.474	0.223	0.458	0.204	**Decrease**
4	10%	0.605	0.293	0.593	0.282	0.647	0.233	0.545	0.219	0.598	0.257	**Decrease**
5	12,5%	2.710	0.281	2.809	0.345	2.715	0.300	2.703	0.311	2.734	0.309	**Decrease**
6	Negative Control	0.252	0.251	0.271	0.270	0.156	0.159	0.165	0.171	0.211	0.213	**Increase**
7	Positive Control	0.143	0.096	0.205	0.131	0.210	0.043	0.175	0.067	0.183	0.084	**Decrease**

Note: "Increase" indicates the absorbance value after incubation> the absorbance value before incubation, which means that there is bacterial growth; whereas "Decrease" indicates the absorbance value after incubation ≤ the absorbance value before incubation, which means that bacterial growth is inhibited.

Plaque control is an effort to prevent the accumulation of plaque on the tooth surface.[7] It is known that mechanical plaque control is the most effective way to prevent dental caries and diseases such as gingivitis and periodontitis.[8] Mechanical plaque control is by brushing teeth.[9,10] In addition, plaque control can be performed chemically in addition to increasing the performance and duration of plaque control using anti-plaque agents.[11] Control plaque chemically is by gargling with antibacterial liquid.[6,9] Gargling using antibacterial liquid can kill bacteria that stick to the surface of the teeth.[9]

The most frequently prescribed antiseptic agents in dentistry is chlorhexidine gluconate mouthwash. However, the most common side effects associated with long-term chlorhexidine gluconate use were increased staining of teeth and other oral surfaces, increased calculus formation, altered taste perception, and gingival irritation.[12]

Currently, many mouthwashes with medicinal plant-based ingredients have been studied to have antibacterial effects with minimal side effects.[13] The neem plant whose leaves were studied for having antibacterial compounds.[14] The content of antibacterial compounds found in the ethanol extract of neem leaves such as alkaloids, steroids, saponins, tannins and flavonoids can be used as one of the active ingredients in making mouthwash.[15]

Based on the explanation above, the aim of this study is to increase the herbal potency of neem leaves as an alternative to mouthwash by conducting research on the effectiveness of EENL mouthwash on the growth of *Streptococcus sp.*

MATERIALS AND METHODS

This research was conducted at the Laboratory of Microbiology, Faculty of Medicine, Universitas Diponegoro and Integrated Laboratory of Universitas Diponegoro, Semarang. This type of research is a laboratory experiment with a pretest-posttest control group design, in accordance with the KEPK (Komisi Etik Penelitian Kesehatan) Universitas Diponegoro No. 15/EC/KEPK/FK-UNDIP/1/2021. There were 28 samples consisting of a positive control group with chlorhexidine gluconate 0.2%, a negative control group with distilled water, and a treatment group with a concentration of 2.5%, 5%, 7.5%, 10%, and 12,5% EENL mouthwash. Neem leaf extract was obtained from the immersion process of 500 gr dried neem leaf in 96% ethanol for 24 hours, then filtered and evaporated to produce 100% pure neem leaf ethanol extract. The extract was made into a mouthwash formulation with a concentration of 2.5%, 5%, 7.5%, 10%, and 12.5% with the addition of PEG 40 hydrogenated castor oil, menthol, sodium benzoate, citric acid, glycerin, and saccharin.

Streptococcus sp. subject obtained by culture from an oral swab which was implanted aseptically on blood agar medium and incubated at 37°C for 24 hours. The results of the incubation were carried out with gram staining to identify the morphology of *Streptococcus sp*. with a microscope. Morphology of *Streptococcus sp.* was round or oval and elongated or lined up like a chain. The suspension was made by culturing *Streptococcus sp* isolates on 28 mL of NaCl solution and compared with 0.5 Mc Farland's solution.

The treatment was carried out by mixing the *Streptococcus sp.* and an EENL mouthwash with a concentration of 2.5%, 5%, 7.5%, 10%, and 12.5% in a test tube of 1 mL each and homogenized with vortex. The sample measured the absorbance value on spectrophotometry with a wavelength of 600 nm. Then, the sample was incubated at 37ºC for 24 hours, and the absorbance value was measured again. Bacterial growth is known from the mean difference in absorbance values before and after incubation.

The test was continued by culturing the post-incubation sample on media nutrient agar (NA) to determine whether the sample had the ability to kill bacteria. The test sample was streaked on NA media using an inoculating loop that had been sterilized with bunsen. NA media that had been cultured were incubated at 37ºC for 24 hours. The growth of *Streptococcus sp*. is characterized by the presence of colonies growing on NA media.

Table 2. *The result of Post hoc LSD test*

	EENL 5%	EENL 7,5%	EENL 10%	EENL 12,5%	Negative control	Positive control
EENL 2,5%	.020*	.000*	.000*	.000*	.000*	.736
EENL 5%		.046*	.000*	.000*	.000*	.040*
EENL 7,5%			.026*	.000*	.000*	.000*
EENL 10%		.		.007*	.000*	.000*
EENL 12,5%					.000*	.000*
Negative control						.000*

Note: (*) indicates a significant difference between treatment groups (p <0.05)

The data were analyzed using the *Sapphiro-Wilk test* to see the normality of the data. The analysis was continued with the *one-way Anova* test and the *post hoc LSD* test at $p<0.05$.

RESULTS

The inhibition test of the EENL mouthwash against the growth of *Streptococcus sp*. of the dilution method with spectrophotometry can be seen in Table 1. Inhibition of bacterial growth by spectrophotometric tests occurred in the EENL mouthwash treatment with a concentration of 2.5%, 5%, 7.5%, 10%, 12.5%, and a positive control because there was a decrease in the absorbance value after incubation. Meanwhile, the negative control treatment with distilled water was due to an increase in the absorbance value so that it indicated the growth of *Streptococcus sp*.

The data obtained was tested for normality using the Shapiro-Wilk test, for all treatments showed p = 1,000, which means p> 0.05, so it can be said that the data is parametric or normally distributed. One way Anova test results showed p = 0.000, so p <0.05, which means that there is a difference in the effectiveness of bacterial inhibition.

The results of the Post hoc LSD test in Table 2 show that there is a significant difference between the positive control group and the ethanol extract of neem leaves with a concentration of 5%, 7.5%, 10%, and 12.5%. The comparison between EENL mouthwash with a concentration of 2.5% and 0.2% chlorhexidine gluconate has a p > 0.05, which means that there is no significant difference. Meanwhile, the concentration of EENL mouthwash 2.5% - 12.5% each had a significant difference (p < 0.05), indicating an increase in antibacterial activity as the EENL concentration increased.

Table 3. *The results of the bactericidal test at NA*

No	Treatment	Rep 1	Rep 2	Rep 3	Rep 4
1	EENL 2,5%	-	-	-	-
2	EENL 5%	-	-	-	-
3	EENL 7,5%	-	-	-	-
4	EENL 10%	-	-	-	-
5	EENL 12,5%	-	-	-	-
6	Negative control	+	+	+	+
7	Positive control	-	-	-	-

The research was continued by testing the bactericidal activity of the EENL mouthwash against *Streptococcus sp*. on NA media which can be seen in Table 3. In the mouthwash concentration of 2.5%, 5%, 7.5%, 10%, 12.5%, and positive control, there was no growth of *Streptococcus sp*. bacteria, while in negative control there was growth of *Streptococcus sp*.

DISCUSSION

The results of the minimum inhibitory concentration test in Table 1 using UV-Vis spectrophotometry indicate that there is an inhibition of bacterial growth at the lowest concentration of 2.5%, and the higher the extract concentration value, the difference in the absorbance value will increase. This is due to the higher content of active ingredients in EENL, such as nimbin and nimbidin, so the reaction is stronger.[16] Research continued to assess the growth of bacteria on NA medium. The observation on NA media showed that the concentration of 2.5% mouthwash EEDM there is no growth of bacteria *Streptococcus sp*. These results indicate that the MIC and MBC of EENL mouthwash are at a concentration of 2.5%.

The results of the post hoc LSD test showed that 2.5% concentration of EENL mouthwash did not have a significant difference in effectiveness in inhibiting *Streptococcus sp* bacteria with 0.2% chlorhexidine gluconate mouthwash, it thus be said to have an equivalent antibacterial effect. Apart from acting as an antibacterial in mouthwash, EENL can also act as an astringent, antiseptic, antiulcer, anti-inflammatory and antigingivitis.[15] Balappanavar et al study showed the same thing that 2% neem extract had better effectiveness than 0.2% chlorhexidine gluconate on plaque and gingival indices.[17] Thus, the EENL 2.5% mouthwash has the potential as an alternative to herbal mouthwash because it can act as an antiseptic, antiulcer, anti-inflammatory and anti-gingivitis.

Neem leaves have antibacterial activity which has been investigated for their great potential for phytochemical compounds and the use of this plant is very useful in health care.[18] Neem exhibits a therapeutic role as it is rich in valuable active compounds such as azadirachtin, nimbolinin, nimbin, nimbidine, nimbidol, salannin, and quercetin.[19] Azadirachtin as the main compound in neem leaves can interfere with the oxidative phosphorylation process in bacterial mitochondria, causing the bacterial cell respiration chain inhibited.[20,21] Other active compounds such as nimbolinin, nimbin,

nimbidine, nimbidol, salannin, and quercetin show an antimicrobial role through an inhibitory effect on microbial growth and damage the cell walls so that bacteria die.[19] The antibacterial activity of neem leaves is also influenced by chemical compounds found in the EENL such as alkaloids, flavonoids, glycosides and steroids.[22] The phytochemical composition of neem leaves is quantitatively dominated by alkaloid compounds of 14.5%, 2.10% of flavonoids, 0.27% glycosides and 0.03% of steroids.[23]

The mechanism of antibacterial action on alkaloid compounds works by inhibiting nucleic acid synthesis, because they inhibit the enzyme dihydrofolate reductase which has a role in cell growth and division in bacteria.[24,25] Flavonoids act as bactericidal and bacteriostatic by damaging the cytoplasmic membrane, inhibiting energy metabolism and inhibiting nucleic acid synthesis against microorganisms.[26] The antibacterial activity of glycosides is to disrupt the permeability of the cytoplasmic membrane which causes leakage of cellular and cell components in lysed bacteria.[27] Steroids can interact with cell membrane phospholipids which are permeable to lipophilic compounds, causing decreased membrane integrity and altered cell membrane morphology which causes brittle cells and lysis.[28]

In this study the data showed that the ethanol extract of neem leaves mouthwash was effective in inhibiting the growth of *Streptococcus sp*. These results are in line with previous studies where the ethanol extract of neem leaves was effective in inhibiting the growth of *S. mutans*.[29] The results of this study are supported by Bijauliya et al. which suggests that the antibacterial activity of neem leaves is effective against pathogenic bacteria such as *E. coli, Corynebacterium bovis* and *Staphylococcus aureus*.[30] Results of research by El-Mahmood et al. also showed that the pure extract, ethanol, methanol and acetone from neem leaves had an effective antimicrobial effect against *E. coli, B. subtitles, Salmonella typhus, Pseudomonas, Staphylococcus aurous, Klebsiella pneumonia* and *Staphylococcus epidermitis.*[30]

In this study, EENL mouthwash at high concentrations had a dark color, this can be a difficulty when absorbing light with UV-Vis spectrophotometry. These deficiencies can be minimized by chromatography techniques usingor High Performance Liquid Chromatography (HPLC).[31] Chromatography technique is a biophysical technique that allows the separation, identification and purification of components of a mixture so that the concentration of the solution can be reduced to facilitate qualitative and quantitative analysis.[32]

CONCLUSION

EENL mouthwash at a concentration of 2.5% had an antibacterial effect equivalent to 0.2% chlorhexidine gluconate and at a concentration of 5% - 12.5% had a stronger antibacterial effect. The conclusion of this study was that the ethanolic extract of neem leaf mouthwash was effective against the growth of *Streptococcus sp*.

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