Goji Berry Extract (Lycium barbarum L.) Efficacy on Oral Pathogen Biofilms

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Goji Berry Extract (*Lycium barbarum* L.) Efficacy on Oral Pathogen Biofilms

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***Abstract*— Lycium barbarum L. fruit, which contains flavonoids and phenolic acids, has antibacterial properties that are expected to inhibit bacterial growth. The objective of this study is to determine the antibacterial and the antibiofilm effects of L.barbarum L. fruit ethanol extract towards S. mutans and P. gingivalis. An in-vitro laboratory experiment was performed with a post-test control group design. The extract of L.barbarum L. fruit was obtained by maceration technique using 96% ethanol as a solvent. The antibacterial assay was performed by microdilution and plate count methods. The antibiofilm effect was performed using a biofilm-assay method. The results of microdilution and plate count methods showed that the most effective antibacterial concentration against S. mutans and P. gingivalis was 100 µg/mL when compared with negative control ($p < 0.05$). In the biofilm assay, the most effective concentration against S. mutans was 100 µg/mL at the 3-hours incubation time, while for P. gingivalis, the most effective concentration was 100 µg/mL at 24-hours incubation time when compared with negative control ($p<0.05$). These results indicate that ethanol extract of L. barbarum L. fruit was demonstrated to have antibacterial and antibiofilm effects against oral pathogens S. mutans and P. gingivalis with 87.15% and 97.73% of biofilms reduction respectively.**

Keywords*— antibacterial, antibiofilm, Lycium barbarum L., Porphyromonas gingivalis, Streptococcus mutans.

I. Introduction

Oral health positively affects the appearance, physical, mental, and interpersonal well-being, of an individual. Oral health is a part of overall health, contributes to the quality of life [1]. National Basic Health Research Data (Riskesdas 2018) showed dental and oral health problems in 57.6% of the Indonesian population. The prevalence of dental caries in Indonesia in 2018 was 88.8%, with an average DMF-T index of 7.1, which is a very high severity of dental caries. Moreover, 74.1% of the Indonesian population experienced periodontitis [2].

Caries are the process of the demineralization of inorganic material and the dissolution of organic material, leading to bacterial invasion through the dentin layer until it reaches the pulp [3,4]. The process of dental caries depends on the presence of fermentable sugars (substrates), the type of tooth and saliva (host), cariogenic microbial flora (biofilm), and time [5]. Periodontitis is a disease caused by microorganisms that cause inflammation of tooth-supporting tissue and causes progressive destruction of periodontal ligament and alveolar bone. The sign of periodontitis is the formation of pockets, recessions, or both [6]. Periodontitis in adults caused by numerous local factors, such as biofilms or calculus, is classified as chronic periodontitis [7].

The formation of biofilms begins when microorganisms in the planktonic state merge into bacterial colonies and wrap themselves in a self-produced extracellular polymer matrix [8]. In the initial phase of biofilm formation, there is an increase in Gram-positive cocci activity, one of which is *Streptococcus mutans*, which is able to attach to tooth surface through the formation of extracellular polysaccharides that cause biofilm matrix to have a gelatin-like consistency that facilitates attachment of bacteria to the tooth surface [4,9,10]. *Porphyromonas gingivalis*, which is a secondary bacterium, is an anaerobic Gram-negative bacterium found in periodontal pockets that causes chronic periodontitis. Various virulence factors of *P. gingivalis* such as gingipains, fimbriae, and lipopolysaccharides, play important role in periodontal disease progression and induce dysbiosis in biofilms [11,12].

Chlorhexidine mouthwash is used to prevent caries and treat periodontitis and is considered as the gold standard for controlling dental plaque and gingivitis due to its efficacy against a wide variety of microorganisms. However, chlorhexidine has various side effects, including taste disturbances, discoloration of teeth and mucosa, mucosal desquamation, salivary stone formation, irritation, dry oral cavity, and allergic reactions, such as contact stomatitis. The World Health Organization (WHO) recommends finding new natural ingredients to overcome the side effects of chemical agents [13,14].

The use of natural ingredients as antimicrobial agents has become an alternative because of their low cost and lower toxicity [15]. According to WHO, traditional medicine has been used globally and can be a major source of health for millions of people and sometimes the only source of care and is also culturally acceptable, affordable, and trusted by community[16]. Goji berry (*Lycium barbarum* L.) has been widely used as a traditional medicine by people in Asia, especially in the northwestern part of China, for more than 2000 years. Recently, *L. barbarum* L. has been gaining popularity as a highly nutritious food used to improve health in North America, Europe, and Asia [17]. *Lycium barbarum* L. has a red, oblong fruit with a length of 6–20 mm and a diameter of 3–10 mm. *Lycium barbarum* L. fruit is harvested when it is ripe and is then dried for later use [18]. The fruit, roots, tree bark, and flowers of *L. barbarum* L. are used as medicine [19].

The polysaccharides of *L. barbarum L.* exhibited properties that improve eye health and reproductive system; reduce fat and blood sugar; regulate immunity. It's also anticancer, anti-tumor, antioxidant, anti-fatigue, antiviral, anti-aging, hepatoprotective, neuroprotective, and cardioprotective properties [17,20]. The flavonoids and phenolic acids of *L. barbarum* L. have potential as antioxidants and antimicrobials [21].

Lycium barbarum L. fruit is effective against Gram-negative bacteria (e.g. *Escherichia coli*) and Gram-positive bacteria (e.g. *Staphylococcus aureus*)[22]. However, there have been no studies regarding the antibacterial effect of *L. barbarum* L. fruit against *S. mutans* and *P. gingivalis* as causing bacteria of dental caries and chronic periodontitis. Thus, this is the first study that analyzed the antibacterial and antibiofilm efficacy of goji berry (*L. barbarum* L.*)* ethanol extract towards *S. mutans* and *P. gingivalis* as oral pathogens.

II. Materials and Methods

A. Ethanol extract production from L. barbarum L. fruit

Dried *L. barbarum* L. fruit (100 g) from Chinese medicine store "Lancar Jaya" at Teluk Gong Raya No. 43, Jakarta Utara (produced in Zhongning, Ningxia, China) was ground in a blender until it became powder. It was then immersed in 96% ethanol with a ratio of 1:8 for 72 hours, stirring every 15 minutes. Furthermore, filtration was performed using Whatman No. 1 filter paper and evaporated with a rotary evaporator at 40°C temperature, 60 rpm speed, and 20 atm pressure so a thick and solvent-free extract was obtained with a concentration of 100 µg/mL. Furthermore, extracts were diluted using sterile distilled water until concentrations of 50, 25, 12.5, and 6.25 µg/mL were obtained.

B. Phytochemical Assay

Phytochemical assays were performed qualitatively to determine whether the ethanol extract of *L. barbarum* L. fruit contained flavonoids, phenols, quinones, steroids, terpenoids, and alkaloids. The qualitative results are expressed as (+) for the presence and (-) for the absence of phytochemicals.

C. Bacterial Cultures

Streptococcus mutans ATCC 25175 and *Porphyromonas gingivalis* ATCC 33277 from Microbiology Center of Research and Education (MiCORE) Laboratory, Faculty of Dentistry, Trisakti University, were cultured on brain heart infusion (BHI) (Oxoid, Hampshire) broth medium and incubated at 37°C for 24 hours in anaerobic atmosphere. Furthermore, the absorbance measurements were performed to obtain the McFarland standard of 0.5 = 1.5 × 10^8 CFU/mL (OD_{600} = 0.132).

D. Microdilution

Each well of a 96-well plate was distributed 100 µL of either an *S. mutans* or *P. gingivalis* culture. Subsequently, 100 µL of the following solutions was used as a treatment: ethanol extracts of *L. barbarum* L. fruit at 100, 50, 25, 12.5, and 6.25 µg/mL concentrations, 0.2% chlorhexidine gluconate as a positive control, and sterile distilled water as a negative control. The measurement of bacterial cell density was performed using microplate reader at 600 nm wavelength before and after the 96-well plates were incubated for 24 hours. All treatments were done in triplicate.

E. Total Plate Count

Bacterial growth was measured by re-diluting contents in 96-well plates for 10,000 times and cultured on BHI agar medium and incubated for 24 hours at 37°C.

F. Biofilm Assay

Bacterial culture (200 µL) was dispensed into each 96-well plate and incubated at 37°C for 48 hours in an anaerobic atmosphere. Furthermore, the supernatant was removed until a thin layer of biofilm was left on the bottom surface of the well. Then, wells were rinsed with a solution of phosphate-buffered saline (PBS). The ethanol extracts of *L. barbarum* L. fruit at a concentration of 100, 50, 25, 12.5, and 6.25 µg/mL, 0.2% chlorhexidine gluconate as positive control, and sterile distilled water as negative control were added 200 µL to wells using a micropipette and incubated at 37°C for 1, 3, and 24 h in an anaerobic atmosphere. The well was rinsed twice using PBS and then fixated over a flame. Crystal violet dye (200 µL; 0.05% w/v) was added to each well and left for 15 minutes. The well was rinsed twice using PBS and left for 15 minutes. Then, 200 µL of 96% ethanol was inserted, and optical density was measured using a microplate reader (SAFAS MP96, Monaco) at 595 nm wavelength.

G. Statistical Analysis

Data were processed by using Statistical Product and Service Solution (SPSS) software version 25.0 and the normality test was performed by using the Shapiro-Wilk method. Normally distributed data ($p > 0.05$) was analyzed by one-way analysis of variance (ANOVA) test. Significant data ($p < 0.05$) were analyzed with a posthoc test using Tukey's test with a significance level of $P < 0.05$ to determine which groups were significantly different.

III. Results

A. Phytochemical Assay Results

The phytochemical test qualitatively showed that the ethanol extract of *L. barbarum* L. fruit contained flavonoids, phenols, steroids, and terpenoids (Table I.).

Table I . THE QUALITATIVE PHYTOCHEMICAL TEST RESULTS OF ETHANOL EXTRACT OF *LYCIUM BARBARUM* L. FRUIT

Extract	Test	Result
Ethanol extract of *L. barbarum* L. fruit	Flavonoids	+
	Phenols	+
	Quinones	-
	Steroids	+
	Terpenoids	+
	Alkaloids	-

B. Microdilution Results and Total Plate Count Results

Results of this study showed that ethanol extract of *L. barbarum* L. fruit has antibacterial and antibiofilm effects against *S. mutans* and *P. gingivalis.* The most effective antibacterial effect was at 100 µg/mL concentration, with an optical density value of 0.358 ± 0.002 (Fig. 1) and the total number of *S. mutans* colonies of 3 ± 3.46 x 10^6 CFU/mL (Fig.

3). The optical density value of *P. gingivalis* was 0.458 ± 0.024 (Fig. 2) with a total number of *P. gingivalis* colonies of 41 ± 4.58 x 10^6 CFU/mL (Fig. 4).

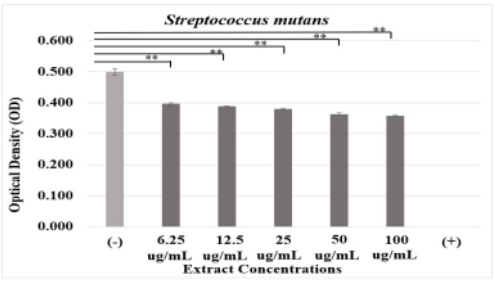


Fig. 1. Streptococcus mutans (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL). Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

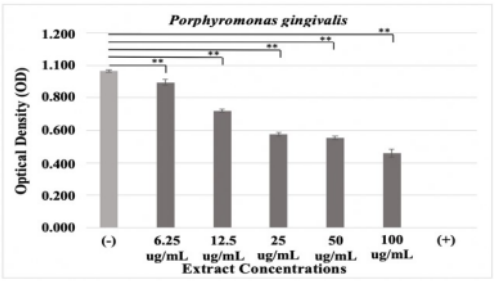
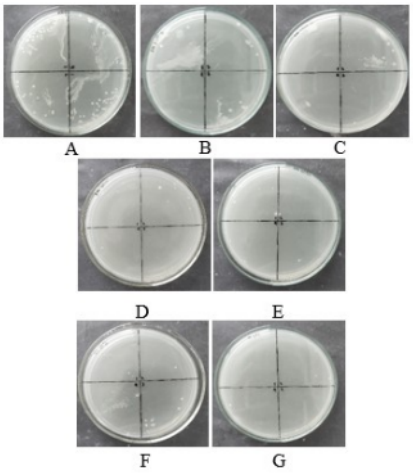


Fig. 2. Porphyromonas gingivalis (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL). Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.



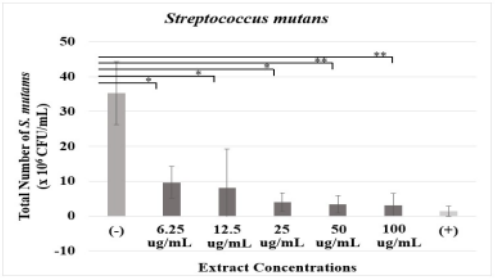
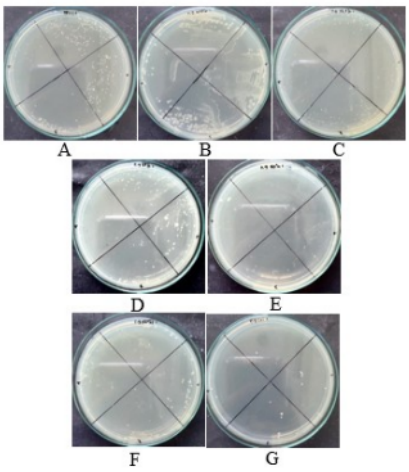


Fig. 3. Streptococcus mutans (colony forming unit) concentration response for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. A. Distilled water as negative control; B. Concentration of 6.25 µg/mL; C. Concentration of 12.5 µg/mL; D. Concentration of 25 µg/mL; E. Concentration of 50 µg/mL; F. Concentration of 100 µg/mL; G. Chlorhexidine gluconate (0.2%) as positive control.. **Significant difference at $p < 0.01$ and * $p < 0.05$



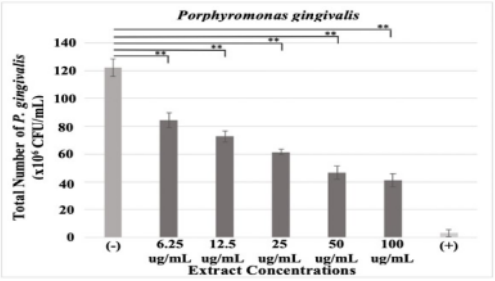


Fig. 4. Porphyromonas gingivalis (colony forming unit) concentration response for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. A. Distilled water as a negative control; B. Concentration of 6.25 µg/mL; C. Concentration of 12.5 µg/mL; D. Concentration of 25 µg/mL; E. Concentration of 50 µg/mL; F. Concentration of 100 µg/mL; G. Chlorhexidine gluconate (0.2%) as a positive control. **Significant difference at $p < 0.01$.

C. Biofilm Assay Results

In biofilm assay, the concentration of 100 µg/mL with 3 hours of incubation was the most effective in inhibiting the formation of *S. mutans* biofilm with an optical density value of 0.042 ± 0.002 (Fig. 6), whereas for *P. gingivalis* biofilm, the concentration of 100 µg/mL with 24 hours of incubation was the most effective (optical density value: 0.007 ± 0.003; Fig. 10). Statistical analysis showed that all ethanol extract concentrations of *L. barbarum* L. fruit were significantly different from negative control (Fig. 5-10) ($p < 0.05$).

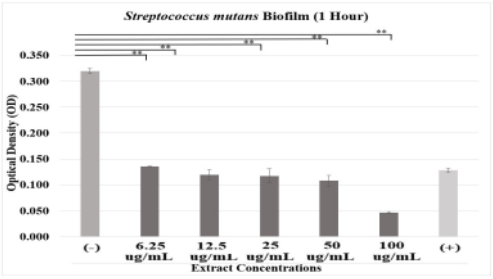


Fig. 5. Streptococcus mutans (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 1 h incubation time. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

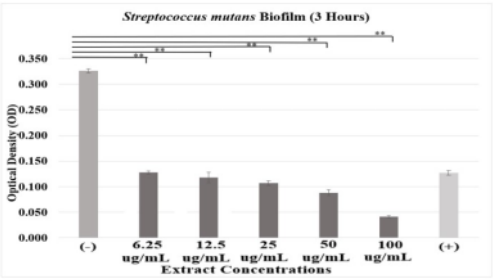


Fig. 6. Streptococcus mutans (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 3 h incubation time. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

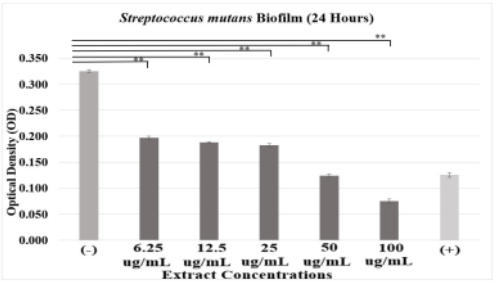


Fig. 7. Streptococcus mutans (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 24 h incubation time. Chlorhexidine gluconate (0.2 %) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

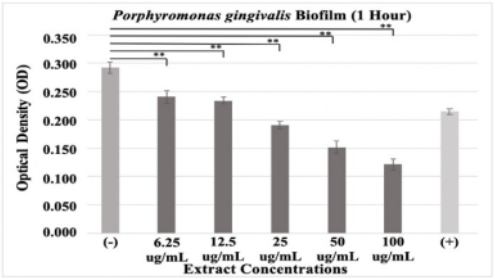


Fig. 8. Porphyromonas gingivalis (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 1 h incubation time. Chlorhexidine gluconate (0.2%) was used a as positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

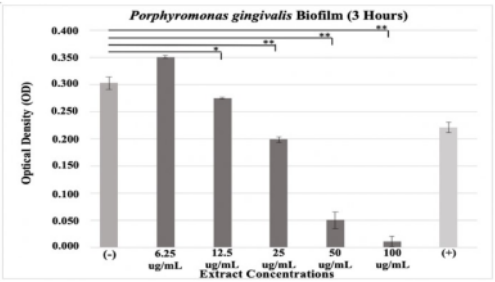


Fig. 9. Porphyromonas gingivalis (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 3 h incubation time. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. *Significant difference at $p < 0.05$; **Significant difference at $p < 0.01$.

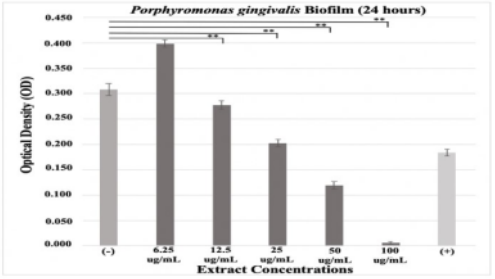


Fig. 10. Porphyromonas gingivalis (optical density) concentration response curves for treatment with ethanol extract of Lycium barbarum L. fruit in different concentration (100 µg/mL, 50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) after 24 h incubation time. Chlorhexidine gluconate (0.2%) was used as a positive control and sterile distilled water was used as a negative control. **Significant difference at $p < 0.01$.

IV. Discussion

The ethanol extract of *L. barbarum* L. fruit has various secondary metabolite such as flavonoids, phenols, steroids, and terpenoids. These metabolites have important role in inhibiting the bacterial growth of *S. mutans* and *P. gingivalis* biofilms. Flavonoid compounds can damage bacterial cell walls by removing bacterial proteins, nucleic acids, and nucleotides so that bacterial cell lysis occurs [23]. Flavonoids also interfere with the quorum sensing mechanism, which inhibits bacterial adhesion and biofilms formation on tooth

surface. The formation of biofilms is inhibited by the reduction of glucans, which are a medium for bacterial attachment, due to the inactivity of the glucosyltransferase enzyme by flavonoids [24].The ability of bacterial cell protein denaturation by phenol compounds through the formation of bonds between phenols and proteins causes damage to protein structures. The cell wall and the cytoplasmic membrane are composed of these proteins. The disruption of permeability in the cell wall and cytoplasmic membrane causes irreversible damage and leads to lysis of the bacterial cell [25,26].

Steroid compounds interaction with cell phospholipid membranes are also capable of causing lysosome leakage for the lysis of bacterial cells. Terpenoids are lipophilic and can bind to carbohydrates and fats, causing disruption of the permeability of bacterial cell walls, denaturation of cytoplasmic proteins, and inactivation of cellular enzymes, causing lysis of bacterial cells [25,27,28].

There were fewer studies about the concentration of *L. barbarum* L. as antibacterial and antibiofilm in vitro. Therefore, as a preliminary study, the concentration of *L. barbarum* L. was used from 6.25 µg/mL to 100 µg/mL. Chlorhexidine gluconate (0.2%), a broad-spectrum antimicrobial biguanide, was used as a positive control due to its potent antiplaque agent. Chlorhexidine leads to cell death by penetrating into the cell and causes leakage of intracellular component. It is considered as a gold standard mouth rinse against gingivitis and periodontitis. Chlorhexidine is indicated to reduce pocket depth in periodontitis as an adjuct therapy to dental scaling and root planning procedure.

In microdilution method, smaller OD value corresponds to higher antibacterial activity. Results of this study showed that ethanol extract of *L. barbarum* L. in all concentration were significantly lower than negative control. Total plate count results also showed reduction of total bacteria number as the concentration of the extract increased. It means that ethanol extract of *L. barbarum* L has antibacterial effect against *S. mutans* and *P. gingivalis.* Ethanol extract of *L. barbarum* L. fruit at 100 µg/mL concentration was the most effective concentration in inhibiting *S. mutans* and *P. gingivalis* bacteria and biofilms. The results of this study are supported with research by Alassadi et al (2015) towards *L. barbarum* L. fruit, which showed that alcohol group (-OH) in flavonoid structure increased the ability of the extract to inhibit microbial growth by increasing the permeability of bacterial cell membranes, and the highest concentration, at 100 µg/mL, possessed the most effective antibacterial activity compared to other concentrations, due to less flavonoid content at lower concentrations [18]. The results of this study are following with previous study regarding inhibition effect of *L. barbarum* L. extracts towards *S. aureus* and *E. coli* using disc diffusion method. Based on the results of these studies, there is an antibacterial effect against *E. coli* [29]. The results of other studies using the well diffusion method have stated that the ethanol extract of *L. barbarum* L. fruit at concentrations of 10 µg/mL and 20 µg/mL had inhibitory effects against *S. aureus* and *E. coli* [22]

This study used three different incubation times in the biofilm assay, namely 1, 3, and 24 hours, to determine the most effective phase for inhibiting *S. mutans* and *P. gingivalis* biofilms. The difference in incubation time was similar to the biofilm formation phase, starting with the pellicle formation phase in a few minutes to 1 hour, the initial adhesion phase at 2 to 4 hours, and the maturation phase after 24 hours [30].

Biofilm assay results against *S. mutans* showed that all extract concentration in 1, 3, and 24-hours incubation time had significantly lower OD than negative control. It means that ethanol extract of *L. barbarum* L. can inhibit the formation of *S. mutans* biofilm starts from 6.25 ug/mL to 100 ug/mL. In 1 and 24-hours incubation time, 100 ug/mL extract had significantly higher OD than the positive control. Hence, the antibiofilm effect of 100 ug/mL extract was more effective than positive control. This result is similar to extract concentration of 25 ug/mL, 50 ug/mL, and 100 ug/mL in 3-hours incubation.

The formation of *P. gingivalis* biofilm was inhibited from concentration of 12.5 ug/mL to 100 ug/mL in 3 and 24-hours incubation time and all concentration in 1 hour incubation time. Therefore, the antibiofilm effect of 50 ug/mL and 100 ug/mL extract in 3 and 24-hours incubation were more effective than positive control. This result is similar to extract concentration of 25 ug/mL, 50 ug/mL, and 100 ug/mL in 1-hour incubation.

The results of the antibiofilm assay showed that the most effective incubation time for inhibiting the formation of *S. mutans* biofilms was at 3-hours of incubation time, and for *P. gingivalis*, it was at 24-hours of incubation time. The most effective times for inhibiting biofilm formation were at the initial adhesion and maturation phases, respectively. The antibiofilm effect depends on the inhibition of polymer matrix formation and quorum sensing, or communication, between bacterial cells in biofilms by inhibiting autoinducer peptides, signaling molecules in Gram-positive bacteria, and acylhomoserine lactones (AHLs) in Gram-negative bacteria so bacterial virulence factors and biofilm development may be inhibited [31].

This is proven by the lowest optical density value found at a 100 µg/mL concentration in *S. mutans* (0.042 ± 0.002) and *P. gingivalis* (0.007 ± 0.003). This antibiofilm assay also showed that a concentration 100 µg/mL had a lower optical density value and was significantly different from the positive control, which means that at a 100 µg/mL concentration, the antibiofilm effect was more effective than the positive control.

V. Conclusion

The ethanol extract of *L. barbarum L. fruit*, containing flavonoids, phenols, steroids, and terpenoids, have antibacterial and antibiofilm effects against S. mutans and P. gingivalis. The most effective concentration is 100 µg/mL for both bacteria with 87.15% and 97.73% of biofilms reduction fo S. mutans and P. gingivalis, respectively. L. barbarum L. might be a promising natural-therapeutic agent as an alternative therapy. However, further research using toxicity, preclinical, and clinical tests is needed to determine whether L. barbarum L. fruit ethanol extract can be used as alternative mouthwash for preventing caries and treating chronic periodontitis.

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