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The effect of Indian jujube leaves extract in inhibiting the growth of *Porphyromonas gingivalis*

Gracia Yodianvi Pratiwi[1], Henry Yonatan Mandalas[1], Vinna Kurniawati Sugiaman[2*]

[1]Department of Periodontology, Faculty of Dentistry Maranatha Christian University, Indonesia
[2]Department of Oral Biology, Faculty of Dentistry Maranatha Christian University, Indonesia

ABSTRACT

Introduction: Periodontitis is an inflammatory disease that destroys tooth-supporting tissues and is associated with increased risk factors for systemic diseases. The main pathogen of periodontitis is the bacteria *P. gingivalis*, a Gram-negative, anaerobic, pleomorphic, coccobacillus, non-motile, and saccharolytic. The leaves of Indian jujube (*Ziziphus mauritiana* Lam.) have the main bioactive compounds such as saponins, tannins, and flavonoids which have antimicrobial activities against pathogenic microorganisms. This study aims to analyse various concentrations of the ethanol extract of Indian jujube leaves against *P. gingivalis*. **Methods:** The method used in this study was the disc diffusion test based on the Clinical and Laboratory Standard Institute. *P. gingivalis* preparation and the fresh leaves of Indian jujube collected from one of the plantations in Probolinggo, East Java. In this study, tests used various concentrations of Indian jujube leaves extract, namely 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. Data antibacterial activity was classified according to David and Stout's inhibition zone classification. **Results:** Indian jujube leaves extract with a concentration between 10-30% had weak antibacterial activity, 40-60% had moderate antibacterial activity, and 70-100% had strong antibacterial activity. The largest inhibitory zone diameter against *P. gingivalis* was found at a concentration of 100%. **Conclusion:** Indian jujube leaves extract starting from a concentration of 70% can inhibit the growth of *P. gingivalis* with strong antibacterial activity.

Keywords: Indian jujube leaves; *Porphyromonas gingivalis*; periodontitis

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INTRODUCTION

Chronic periodontitis is one type of periodontal disease. It is a long-lasting inflammatory disease affecting the soft and hard tissues around the teeth. Chronic periodontitis is related to common and preventable biological risk factors such as high blood pressure, high blood cholesterol, diabetes mellitus, genetic factors, obesity, and behavioural risk factors such as an unhealthy diet,

*Corresponding author: Vinna Kurniawati Sugiaman, Department of Oral Biology, Faculty of Dentistry Maranatha Christian University, Indonesia. 65, Surya Sumantri Street, Bandung, West Java, 40164, Indonesia; Phone: +62 812-2115-650; e-mail: vinnakurniawati@yahoo.co.id

physical inactivity, and tobacco use. These risk factors can cause disease progression to become more aggressive.

Chronic periodontitis most often occurs in adulthood, although it can occur in children and adolescents due to the accumulation of plaque and calculus.[1] The bacteria most often found in chronic periodontitis are *Fusobacterium spp.*, *Bacteroides fragilis*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Prevotella spp.*[2] It is known that the high prevalence of chronic periodontitis in adolescents, adults, and the elderly makes chronic periodontitis a severe problem for the community's oral health.[3]

P. gingivalis is a gram-negative, anaerobic, asakarolytic, and black-pigmented bacteria associated with periodontal and systemic disease development.[4] *P. gingivalis* appears as the primary etiological agent in periodontal disease's pathogenesis and inflammatory processes.[5] Virulence factors such as lipopolysaccharide (LPS), fimbriae, protease, capsule, adhesive domain, and outer membrane vesicle owned by *P. gingivalis* can invade local periodontal tissues, take nutrients for growth, and defend themselves from the body's defence mechanisms.[5,6] These pathogenic bacteria appear as much as 85.75% in the sample subgingival plaque in patients with chronic periodontitis, and about 40-100% of adults with chronic periodontitis are infected by these opportunistic bacteria.[6]

Several classes of antibiotics are recommended to treat chronic periodontitis, such as clindamycin, doxycycline, amoxicillin, and metronidazole.[7] Improper administration of antibiotics can lead to antibiotic resistance. Therefore, microbiologists are trying to find new antimicrobial agents using natural plant products.[8] Compared to global biodiversity, Indonesia is the second-largest centre of biodiversity, which consists of medicinal plant biodiversity with 2500–7500 species. These medicinal plants are economically valuable to various communities.[9] Farmakope Indonesia[10] reported that many plants can be used as substitutes for chemical antimicrobial agents, such as fennel fruit, African leaves, Bidara leaves, Binahong leaves, Ceremai leaves, Moringa leaves, and many more.

Indian jujube (*Ziziphus mauritiana* Lam.) is a tree that grows in tropical and subtropical areas in various world regions. The Indian jujube leaves are believed to be able to treat asthma, fever, accelerate wound healing, and overcome infection.[11] Therefore, several researchers conducted phytochemical tests of the extract of Indian jujube leaves, and the results showed that these leaves extract contains major bioactive components which can inhibit the growth of microorganisms.[12] The results of phytochemical analysis of the Indian jujube leaves extract to contain flavonoids, tannins, saponins, resins, quinones, glucose, terpenoids, and polyphenols.[11,12] According to previous research, the bioactive compounds in Indian jujube leaves extract effectively overcome bacterial infections and can be used as an antifungal, antioxidant, and cancer treatment.[13]

Based on these fundamentals, researchers were interested in studying the effect of Indian Jujube (*Ziziphus mauritiana* Lam.) leaves extract in inhibiting the growth of *P. gingivalis*. Therefore, this study aims to analyse various concentrations of the ethanol extract of Indian jujube leaves against *P. gingivalis*.

METHODS

The sample used in this study was *P. gingivalis* ATCC 33277, obtained from the Microbiology Laboratory of the Faculty of Dentistry Universitas Padjadjaran, Bandung. Indian jujube leaves were taken from the plantations in Probolinggo, East Java. This plant has been identified by the Biology Research Center of the Indonesian Institute of Sciences, Cibinong, Bogor (Certification approval number of 514/T/ LAB/X/20).

Instruments and Materials

Instruments: Evaporator, grinder, Analytical Balance (AXIS, AGN220C), Autoclave (HiClave, H-50), Microwave (SHIVAKI, SMW 103), Biosafety Cabinet (BSC) level II (Telstar Bio II Advance Plus), Whatman Filter Paper no. 3 (GE Healthcare, 1003-090), Sterile cotton swab (OneMed, 67723), Caliper (MODERN, SIGMA 6"), Disposable Petri dish (ThermoFisher Scientific, 101VR20), Incubator (Binder, CB53), Micropipette (20- 200 µl, 100-1000 µl) (Eppendorf), Tip (20-200 µl, 100-1000 µl) (Borosil), 1.5 ml Eppendorf tube (SPL, 60015-1), Vortex (WiseMix, VM-10), Falcon tube 15 ml

(SPL, 50015), Falcon tube 50 ml (SPL, 50050), Serological Pipet (SPL, 91005).

Materials: *P. gingivalis* ATCC 3277, Indian Jujube leaves extract (*Ziziphus mauritiana* Lam.), 0.9% NaCl solution, Mueller Hinton Agar (MHA) (Himedia, M173), Mueller Hinton Broth (MHB) (Himedia, M391), 100% DMSO (Merck, 1.02952. 1000), Chlorhexidine 0.2% (Minosep, 0101220-C065), ddH2O.

Extract of Indian jujube leaves

The extract method used was maceration with 70% ethanol as the solvent. First, the Indian Jujube leaves were separated from the whole plant. Then, the Indian Jujube leaves were being dried. Dried Indian Jujube leaves weigh as much as 2,500 grams or 2.5 kilograms. Next, the simplicia was put into a maceration vessel, and 3 litres of 70% ethanol for soaking was added. The immersion was carried out for 24 hours, stirring occasionally. Maceration was repeated several times.

Furthermore, the immersed simplicia was filtered with filter paper to separate the pulp and filtrate, and the obtained filtrate was collected and put into a glass bottle. The filtered simplicia was then concentrated in a vacuum rotary evaporator with a temperature of 500°C and a pressure of 40 rpm, then evaporated using a water bath to obtain a thick ethanol extract of Indian jujube leaves. The Indian jujube leaves extract was then carried out by phytochemical tests and the dilution process using DMSO 10% to obtain concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%.

Preparation of suspension and culture of P. gingivalis

10.5 grams of MHB medium was dissolved in 500 mL ddH_2O. The *P. gingivalis* ATCC 33227 colonies that had been cultured on Mueller Hinton Agar (MHA) medium was inoculated into Mueller Hinton Broth (MHB) medium. The suspension in the test tube was homogenised using a vortex mixer. The turbidity of the solution was then adjusted to the McFarland 0.5 standard solution to obtain an inoculum with a number of bacteria around 1-2x10^8 CFU/mL. The standard density accuracy of bacterial suspension with McFarland 0.5 can also use a spectrophotometer by inserting 1ml of suspension into six-well plates with a light length of 1cm. The McFarland standard of 0.5 has an absorbance reading of 0.08 to 0.1 at a 620-625 nm wavelength.

The steps to culturing the *P. gingivalis* bacterial suspension into Mueller Hinton Agar (MHA) were initiated by taking 19 grams of MHA medium dissolved in 500 mL ddH_2O. The medium was heated using a microwave until boiling and homogeneous. Next, the medium was sterilised using an autoclave at a temperature of 121°C for 20 minutes. The MHA medium was poured on a petri dish to made an agar plate. A sterile cotton swab was dipped in a bacterial suspension whose turbidity has been pre-adjusted with the standard solution of McFarland 0.5. The cotton swab was rubbed onto the surface of the MHA evenly and left at room temperature for 3-5 minutes until the suspension was absorbed into the agar.

Disc diffusion test procedure

Paper discs with a diameter of 6 mm were immersed in 1 mL of 10% DMSO solution as a negative control, Indian jujube leaves were extracted with various concentrations and chlorhexidine 0.2% as a positive control for 5 minutes. In each medium cultured with bacteria, a paper disc was placed. Each medium was incubated for 24 hours at 37°C. The inhibition zone for the growth of the *P. gingivalis* bacteria that formed around the paper disc was measured using a calliper with units of mm as research data. Then, repeat the same procedure with three replications based on Federer's Formula.

Antibacterial inhibitory tests procedure

The research was conducted in December 2020 at the Microbiology Laboratory of Aretha Medika Utama, Bandung, with three repetitions according to the calculation of Federer's formula. Twelve treatments were carried out for each repetition, namely disc paper immersed with 0.2% chlorhexidine as a positive control, 10% DMSO as a negative control, and Indian jujube leaves extract, which had been diluted into ten concentrations, namely 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%. Each repetition used two Petri dishes containing agar planting media and cultured bacteria. Each petri dish, in one repetition, received six different treatments. Then the incubation was carried out for 24 hours in a CO_2 incubator at a temperature

of 37°C. Based on the data obtained, the measurement of the inhibition zone produced by each treatment is continued using a calliper. The results of the measurement of the inhibition zone of Indian jujube leaves extract will be grouped into the inhibition category according to Davis and Stout.

RESULTS

Based on the results of qualitative phytochemical tests, the Indian jujube leaves extract used in this study contained bioactive compounds presented in Table 1.

Table 1. Qualitative phytochemical screening results of Indian jujube leaves extract

Bioactive compounds	Results
Alkaloids	+
Saponin	+
Tannin	+
Phenolic	+
Flavonoids	+
Triterpenoid	+
Steroid	+
Glycoside	+

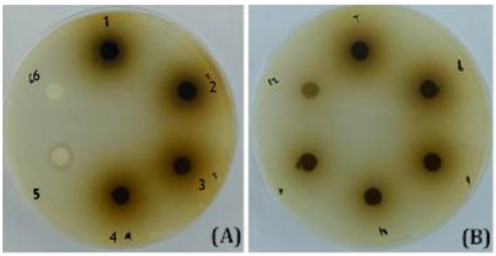

Figure 1. Inhibitory zone observation of Indian jujube leaves extract on *P. gingivalis*: (A) Inhibitory zone of concentrations of 100; 90; 80; and 70% of Indian jujube leaves extract and DMSO 10% as negative control; (B) Inhibitory zone of concentrations of 60; 50; 40; 30; 20; and 10% of Indian jujube leaves extract

Figure 1A: label number 1 was the inhibitory zone of the 100% concentration; label number 2 was the inhibitory zone of the 90%; label number 3 was the inhibitory zone of the 80%; label number 4 was the inhibitory zone of the 70%; label number 5 was the inhibitory zone of 0.2% chlorhexidine; label number 6 was the inhibitory zone of 10% DMSO negative control.

Figure 1B: label number 7 was the inhibitory zone of the 60% concentration; label number 8 was the inhibitory zone of the 50%; label number 9 was the inhibitory zone of the 40%; label number 10 was the inhibitory zone of the 30%; label number 11 was the inhibitory zone of the 20%; label number 12 was the inhibitory zone of the 10%.

Table 2. Inhibitory zone measurement results of indian jujube leaves extract

Treatments (Concentrations %)	Inhibitory zone (mm)			Mean	Standard deviation
	1	2	3		
Positive control (CHX 0.2%)	10.90	11.33	10.96	11.06	0.23
Negative control (DMSO 10%)	0.00	0.00	0.00	0.00	0.00
10%		0.00	0.00	0.00	0.00
20%		0.00	0.00	0.00	0.00
30%		0.00	0.00	0.00	0.00
40%		8.38	8.35	8.70	0.59
50%		8.73	8.57	9.39	1.28
60%		9.58	9.20	9.86	0.84
70%		10.90	11.09	11.01	0.10
80%		11.09	11.08	10.88	0.36
90%		11.64	11.14	11.31	0.29
100%		12.60	11.41	11.94	0.61

David and Stout categorised the inhibitory zone into four categories: less than 5mm was weak; 5mm-10mm was moderate; 10mm-19mm was strong; more than 20 mm was very strong.[14] Based on these categories, the measurement results of the inhibitory zone of Indian jujube leaves extract were categorised in Table 3.

Table 3. Inhibitory zone classification of Indian jujube leaves extract

Indian jujube leaves extract concentration (%)	Mean (mm)	Antibacterial activity classification
10%	0.00	Weak
20%	0.00	Weak
30%	0.00	Weak
40%	8.70	Moderate
50%	9.39	Moderate
60%	9.86	Moderate
70%	11.01	Strong
80%	10.88	Strong
90%	11.31	Strong
100%	11.94	Strong

All data generated were tested first for normality distribution with the Lilliefors normality test, a non-parametric normality test, and the results suggested that the data were normally distributed.

Therefore, it was proper to conduct the statistical analysis in-advance with the one-way ANOVA test. Prior to the analysis, the data homogeneity test was carried out using the Bartlett homogeneity test, and the results suggested that the data was not homogeneous. In the present study, the data being analysed were only the inhibitory zone produced by the positive control and the 40%-100% concentrations of Indian jujube leaves extract because the negative control and the 10%-30% concentrations of Indian jujube leaves extract did not produce any inhibitory zone.

Table 4. The results of one-way ANOVA test

Descriptive results			
Mean	n	Std. Dev	Treatment
11.06	3	0.23	K+
8.70	3	0.59	40%
9.39	3	1.28	50%
9.86	3	0.84	60%
11.01	3	0.10	70%
10.88	3	0.36	80%
11.31	3	0.29	90%
11.94	3	0.61	100%
10.52	24	1.17	Total

Analysis of variance					
Source	SS	df	MS	F	p-value
Treatment	24.93	7	3.56	8.57	0.0002
Error	6.64	16	0.42		
Total	31.57	23			

The one-way ANOVA test results generated the p-value=0.0002, indicating a significant difference in the average inhibitory zone of the eight concentrations seen from the average inhibitory zone ($p<0.05$). Because the data was not homogeneous, after a one-way ANOVA test was carried out, the post-hoc analysis t-test was conducted to determine which groups had significant differences, with results presented in Table 5. The post-hoc analysis results suggested that the 70% Indian jujube leaves extract was the minimum concentration that could inhibit the growth of *P. gingivalis*, with a similar ability as the positive control (0.2% chlorhexidine).

Table 5. The results of post-hoc analysis (2-tail p-values for pairwise)

Independent groups t-test									
		BA-40%	BA-50%	BA-60%	BA-80%	BA-70%	K+	BA-90%	BA-100%
		8.70	9.39	9.86	10.88	11.01	11.06	11.31	11.94
BA-40%	8.70	-							
BA-50%	9.39	0.4474	-						
BA-60%	9.86	0.1212	0.6199	-					
BA-80%	10.88	0.0054	0.1240	0.1252	-				
BA-70%	11.01	0.0026	0.0934	0.0771	0.5704	-			
K+	11.06	0.0029	0.0891	0.0742	0.4935	0.7334	-		
BA-90%	11.31	0.0023	0.0642	0.0472	0.1823	0.1673	0.3193	-	
BA-100%	11.94	0.0027	0.0355	0.0253	0.0600	0.0593	0.0804	0.7197	-

DISCUSSION

The main bioactive compounds in Indian jujube leaves extract with antibacterial functions are flavonoids, saponins, and tannins. These saponins and tannins have been tested in previous studies by being injected into the blood vessels and proven to have antibacterial properties.[15,16] The tannins contained in the Indian jujube leaves extract to have a way of working by the tannins'

properties. They can bind macromolecules such as proteins so that tannins can inhibit the formation of cell walls or outer membrane vesicles in *P. gingivalis*.[16] Then, the tannins will penetrate the bacterial internal membrane and bind to bacterial DNA, disrupting the bacterial metabolic process and absorption of nutrients, which will cause the bacteria to experience cell death.[17]

Saponins, having surface-active properties, might insert into the lipid bilayer, bind to cholesterol, form domains enriched with cholesterol-saponin complexes, and finally, lyse cells. The previous research stated that it disturbs the permeability of the bacterial outer membrane.[18] Saponin can inhibit the process of hemolysis by causing a decrease in erythrocyte permeability, but at high concentrations, it will cause damage to erythrocytes.[19] *P. gingivalis* carry out hemolysis to obtain nutrients by destroying erythrocytes and binding heme. When this process is inhibited, bacterial metabolic disorders occur, and absorption of nutrients causes a decrease in the functional ability of bacteria and the death of bacteria.[5] Apart from saponins and tannins, other active compounds such as flavonoids and alkaloids also have antibacterial properties.[11]

Flavonoids have been tested in-vitro to show the resulting antibacterial activity.[20] Previous research states that several types of flavonoids have antibacterial activity, including apigenin, galanin, naringenin, epigallocatechin, errors, and their derivatives of flavanones and isoflavones.[21] The mechanism of action of flavonoids is to inhibit the secretion of bacterial enzymes such as the protease enzyme or gingipain produced by *P. gingivalis*.[22] Gingipain functions to degrade fibrinogen and heme host proteins, which inhibit blood clotting so that bleeding worsens.[6] When the protease or gingipain enzymes are inhibited, the bacteria lose their source nutrition and decreased function, such as difficulty adhering to target cells and cell death.[23]

Flavonoids can also increase the secretion of inflammatory cytokines such as interleukin 1ß, interleukin 6, interleukin 8, and tumour necrosis factor or TNFα. These inflammatory cytokines are glycoproteins that play an essential role in the body's response to infection.[20,21] Alkaloids showed antibacterial activity and inhibited ATP-dependent transport of compounds across the cell membrane. These alkaloids may serve as potential courses of compounds that can act as lead compounds to develop plant-based antibacterials and/or their adjunct compounds.[24] After the bacterial cell wall is damaged, alkaloids damage the lipopolysaccharide composition, which causes depolarisation and cytoplasmic leakage. So that it disrupts homeostasis or the balance of bacterial cells and causes bacterial cell death.[25]

The inhibitory zone formed can be caused by the presence of antibacterial activity produced by the active compound derived from the extract of the Indian jujube leaves extract. Table 2 presents the research results on measuring the inhibitory zone produced by administering Indian jujube leaves extract, positive control, and negative control measured using a calliper after being incubated for 24 hours with a temperature of 37°C. The results showed that the inhibitory zone formed starting with the concentration of 40-100% Indian jujube leaves extract and 0.2% chlorhexidine (positive control). While negative control (10% DMSO) and Indian jujube leave extract concentrations of 10-30% did not form an inhibitory zone.

The absence of an inhibitory zone in the concentration of 10-30% Indian jujube leaves extract can be caused by the structure of the bacterial cell wall. The microbial cell wall is the first barrier that an antimicrobial agent must overcome when interacting with its target.[32] Gram-negative bacteria possess a complex cell envelope that consists of a plasma membrane, a peptidoglycan cell wall and an outer membrane. The envelope is a selective chemical barrier that defines cell shape and allows the cell to sustain large mechanical loads such as turgor pressure.[10]

In the case of Gram-negative bacteria, an outer membrane (OM) is the outermost barrier. The OM has an asymmetric distribution of the lipids with phospholipids and lipopolysaccharide (LPS) located in the inner and outer leaflets, respectively.[32,33] Gram-positive bacteria have a single lipid membrane surrounded by a cell wall composed of a thick layer of peptidoglycan and lipoteichoic acid, anchored to the cell membrane by diacylglycerol; the structure of the cell wall is more uncomplicated than Gram-negative bacteria.[34] Therefore, the bioactive compounds contained in the extract are more difficult to

damage the cell walls of Gram-negative bacteria, which implicates the bacteria to be still able to multiply cells and not have their growth stunted.

Based on the research results, the resulting wide inhibitory zone was more extensive when the concentration was also higher because of the dilution carried out in the extract of Indian jujube leaves, where the smaller the concentration, the ratio of the extract was less than the solvent. When the amount of solvent is more than the extract, the number of bioactive compounds is less so that the antibacterial activity is not as good as the concentration of an enormous extract. So that the higher the concentration of the inhibitory power, the stronger it will be, which is then supported by Table 3, where there is an increase in the strength of the inhibitory power. Strong inhibitory was initiated from the extract concentration of 70%, supported by the results of the one-way ANOVA statistic, which suggested the inhibiting ability equivalent to 0.2% chlorhexidine in inhibiting the growth of *P. gingivalis*.

However, the size of the inhibitory zone is influenced by bioactive compounds and several other influential factors, such as the time of absorption of bacteria into the agar.[35] Other factors such as turbidity of the suspension, incubation temperature, incubation time, and thickness of the media for growth can also affect.[36] Suspension turbidity using the McFarland 0.5 or equivalent to a colony of $1.5x10^8$ and equivalent to the examination results using a spectrophotometer, namely 0.08 to 0.1 with a wavelength of 625 nm.[37] The incubation temperature used was around 35°C-37°C. The thickness of the media must also be considered, which is about 4 mm.[35,36]

When the turbidity of the bacterial suspension is less than the Mc Farland standard, the resulting diameter will be larger and vice versa. Then when the temperature used is below 35°C, the resulting diameter will be smaller, but when the temperature used is more than 37°C, the diameter will be larger because the extract diffusion is not sound. When the thickness of the agar medium is less than 4mm, the extract diffusion is faster so that the diameter will be more extensive and vice versa.

These factors as a whole can be controlled during the testing procedure. This inhibitory zone testing uses the disc diffusion method according to the Clinical and Laboratory Standard Institute. Since the study is not contained a quantitative phytochemical test, this study cannot identify the specific compound that can inhibit the growth of *P. gingivalis*.

CONCLUSION

Indian Jujube leaves extract starting from a concentration of 70% can inhibit the growth of *Porphyromonas gingivalis* bacteria with strong antibacterial activity.

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