The Effect of Agarwood Leaves Ethanol Extract on *Porphyromonas gingivalis* Growth Inhibition and in Vitro Cytotoxicity Assay on Fibroblast

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Abstract: Agarwood leaves (Aquilaria malaccensis Lamk.) have an antibacterial activity that could be used as wound healing agent. *Porphyromonas gingivalis* were the main pathogens in periodontitis. This was a laboratory experimental study post-test only control group design. Agarwood leaves were obtained from Ibun Garden, Majalaya District, West Java. Sample identified and determined by Biology Research Center, LIPI Indonesia. Extraction and phytochemical test were conducted at Aretha Medika Utama BBRC Bandung and BPTRO Bogor. Fibroblast ATCC 3T3 Balb/C obtained and cultured at Aretha Medika Utama BBRC. Cytotoxicity test was carried out by using MTS Assay method, and the results are adjusted to ISO 10993-5. IC_{50} was obtained using PROBIT analysis. Inhibition assay was carried out by well-diffusion method and the results are adjusted to Davis and Stout criteria. Research and *P. gingivalis* (ATCC 33277) was carried out at Microbiology Laboratory Faculty of Dentistry, Universitas Padjadjaran. Results were analysed with ANOVA. The results indicate agarwood leaves had weak inhibitory ability at under concentration of 50% and moderate inhibition at a concentration of 100%. The cytotoxicity results showed no toxic effect at under concentrations of 62.5 µg/mL. The IC_{50} at a concentration of 215.54 µg/mL.

1 INTRODUCTION

Periodontal disease is defined as various types of conditions that affect the supporting structures of the teeth, including gingiva, alveolar bone, and periodontal ligament (Kinane et al., 2017). This disease is the 11th most common disease in the world and can be classified into gingivitis and periodontitis (Nazir, 2017). Periodontitis is an inflammatory disease in dental support tissue caused by specific microorganisms resulting in progressive destruction of the periodontal ligament and alveolar bone by pocket formation and recession (Newman et al., 2012).

Periodontitis has a prevalence of 10.8% or is experienced by approximately 743 million individuals in the world and is the sixth highest prevalence disease according to Global Burden Disease (Wijaksana, 2019; Séverin, 2018). The pathophysiology of periodontitis is an imbalance of microorganisms in the oral cavity that causes chronic exposure to several pathogenic bacteria periodontitis, including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans, Tannerella forsythia*, and *Treponema denticola* (How et al., 2016).

P. gingivalis is one of the main etiologic agents in the development of periodontitis with a prevalence of 92% (How et al., 2016; Liu et al., 2013). *P. gingivalis*

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is a gram-negative, anaerobic, saccharolytic, and non-motile bacteria. These bacteria are shaped like cocci or rod and belong to the group of black pigmented bacteria (How et al., 2016). The main habitat of *P. gingivalis* is plaque in the subgingival pocket of the oral cavity. The proportion of *P. gingivalis* was found to be higher in the deep periodontal pocket compared to the shallower periodontal pocket. This is due to the availability of amino acid fermentation requirements to produce bacterial energy in the periodontal pocket, such as low sugar levels, low oxygen levels, rich in blood and serum protein, and has a stable pH that is slightly alkaline (How et al., 2016)

P. gingivalis bacteria have several factors that play a role in the virulence process of human cells, namely fimbriae, lipopolysaccharide (LPS), capsular polysaccharide (CPS), hemagglutinin, and gingipain. In addition, *P. gingivalis* interacts with body tissues by adhesion and coaggregation so that these bacteria can invade the body's epithelial cells. However, the low biological activity of *P. gingivalis*, especially its endotoxicity, causes these bacteria to colonize and grow in sterile tissue without being detected by the body (Klein et al., 2012).

The treatment that has been carried out against *P. gingivalis* infection still has many drawbacks. The use of medications such as the antiseptic Chlorhexidine can cause staining of the teeth and some other disadvantages. In addition, *P. gingivalis* is also known to be resistant to antibiotics, including amoxicillin, clindamycin, and metronidazole (Gerits et al., 2017). This has led to research on new and natural substances in the treatment of periodontitis due to *P. gingivalis* infection.

In addition to medicament, periodontal disease treatment also varies depending on extent of the affected periodontal tissue and can be performed with and/or without surgery. Some of the most common non-surgical procedures are scaling and root planing and medication either locally or systemically with the aim of infection control, inhibition of microbial growth, and restoring the healthy state of periodontal tissue. The most common surgical procedure is the periodontal flap, aimed to restore the clinical attachment of the periodontal ligament. This surgery involves incisions and requires wound healing as well as postoperative tissue regeneration (Newman et al., 2012; Williams et al., 2016; Hudwekar et al., 2019).

Fundamentally wound healing is a complex cellular process, focuses on restoring the structure and function of damaged tissues through 3 (three) phases, namely the inflammatory phase, proliferation phase, and remodelling phase. Fibroblasts are important cells in the wound healing process, derived from undifferentiated mesenchymal cells. Fibroblasts produce mucopolysaccharides, amino-glycine, and proline acids which are the basic ingredients for linking the edges of the wound. Inflammatory signals activate the proliferation and maturation of the fibroblasts, which are responded by collagen synthesis and cross-bond initiation to form an extracellular matrix as well as differentiate into myofibroblast phenotype to facilitate wound closure (Sugiaman, 2011; Gonzales et al., 2016).

Agarwood (Aquilaria malaccensis Lamk.) is a plant that grows in the forests of Indonesia. The final product of agarwood known as gubal contain resin as a result from mushroom infection in induction process (Janshen et al., 2017). Induction process is an outcome from a long term and complex microorganism interaction, makes gubal extremely rare and high in value. Agarwood resin often used as ingredient in perfume and cosmetic industry (Musir et al., 2016; Nugraha and Ginting, 2013). The use of resin that is too dominant, make the other part of agarwood often become overlooked and resulted as waste, especially the leaf (Wangiyana, 2020).

In traditional medicine, agarwood leaves tend to be used empirically by Indonesians as a treatment for malaria, diabetes, asthma, abdominal pain (constipation), and skin care. These potencies achieved by drinking the leaf's brew or inhaling the scent of burned leaves and stems (Wangiyana, 2020; Syamsul et al., 2020). Agarwood leaf still can't reach its maximum use yet in Indonesia due to the lack of information about the goods contained (Janshen et al., 2017).

Agarwood leaf extract as an antibacterial facilitated by the presence of flavonoids as main compound that intervene in the destruction of bacterial cell membranes (Nomer et al., 2019; Warganegara and Restina, 2016). Along with alkaloids, flavonoids shall change the protein structure found on the outer surface of bacteria namely fimbriae, resulting in the decrease of its hydrophobic property and inhibits bacterial adhesion with host cell (Pratiwi et al., 2015). Furthermore, there are saponins with active substances to lower the surface tension of bacterial cell walls. The substance will bind to the cytoplasmic membrane to destabilize the bacterial cell membrane, causing cytoplasm leakage, resulted in bacterial lysis Dennis et al., 2017). Phytochemical screening of agarwood leaf extract shows presence of tannins and steroids/triterpenoids that invade the bacterial cell membrane, causing the membrane becomes brittle and easily destroyed (Sari et al., 2017).

Wound healing-wise, agarwood leaf extract proven to accelerate the inflammatory process, as well as re-epithelization in mice with DM. Main compounds that act as mentioned in agarwood are flavonoids, saponins, and tannins (Suhardiman and Juanda, 2019; Wahid and Safwan, 2019; Fauzi et al., 2017). Flavonoids act in the activation and proliferation of fibroblasts and induce the production of collagen fiber in order to accelerate wound healing process. Along with flavonoids, saponins stimulates blood vessels proliferation, while tannins act as homeostat by inhibits the production of prostaglandin and stimulating vasoconstriction (Suhardiman and Juanda, 2019; Fauzi et al., 2017; Rahmadhani et al., 2020).

Research on the comparison of total phenol levels in agarwood leaf steeping and agarwood leaf ethanol extract showed that the ethanol extract has higher flavonoids level, namely 62.9 mg GAE/gram and 28.5 GAE/gram in agarwood leaf steeping. Other studies showed that there are 8342.82 mg/100 gram flavonoids contained with antioxidant activity ranging from 28.50 – 40.30 ppm, classified as very strong (Nurmiati and Wijayanti, 2018; Komang et al., 2018; Harahao et al., 2015).

Therefore, agarwood leaf has the potential sources of antibacterial, antioxidant and anti-inflammatory that in addition to inhibiting the growth of *P. gingivalis*, shall be effective in wound healing process.

2 METHODS AND MATERIALS

2.1 Agarwood Leaf Extraction

Agarwood leaves was obtained from Ibun Garden, Majalaya District, West Java and had been identified by Biology Research Center, Lembaga Ilmu Pengetahuan Indonesia, Bogor. Extraction was carried out by maceration method and ethanol as solvent. For cytotoxicity assay, 5 mg total of extract was dissolved in 1 mL DMSO 10% and become seven different extract concentration (500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL, and 7.81 µg/mL). As for inhibition assay, serial dilution method used to make working concentration. 100% extract was dissolved with 10 mL aquadest to 50%, 25%, 12.5%, 6.25%, 3.13%, 1.56% agarwood leaf extract. Both final concentrations were filtered using 0.22 µm tissue culture pore syringe resulted in sterile sample.

2.1.1 Phytochemical Test

The qualitative phytochemical assay was carried out by Farnsworth method. Results showed Agarwood leaf ethanol extract indeed contain flavonoid, saponin, tannin, alkaloid, triterpenoid, steroid, and phenol.

2.2 Cytotoxicity Assay (Viability Test using MTS Assay)

Fibroblast cell (3T3 Balb/C) ATCC CCL-163 was obtained from Aretha Medika Utama BBRC Bandung as collection. Thawing and subculture process was conducted, and cells were cultured in a complete medium, contained of 10% FBS (Biowest, S81B-500), 1% ABAM (Biowest, L0010-100), 1% Amphotericine B (Biowest, L0009-050), 1% MEM Vitamins (Biowest, X0556-100), 1% L-Glutamine (Biowest, X0551-100), 0.2% Nanomycopulitine (Biowest, LX16-100), 0.1% Gentamicin (Gibco, 15750060) and basal medium DMEM High Glucose (Biowest, L0103-500).

Cells were harvested and calculated using hemacytometer after it reached the confluency of 70%, then implanted with 5 x 10^3 density in a 96 well-plate. After incubated for 24 h, the old medium was replaced with 200 µL new medium and 20 µL agarwood leaf ethanol extract, then proceeded to be incubated under 37˚C with 5% CO_2. Furthermore, 20 µL of MTS reagent was added on each well and was incubated for 3 h. Absorbance was measured using spectrophotometer with 490 nm. Cells death was calculated based on the absorbance and integrated to standard curve of 3T3 Balb/C.

2.2.1 Statistical Analysis

Results that obtained as data processed with IBM SPSS 21.0 ver. Normality test was carried out then proceeded to One-way ANOVA and Tuckey HSD (Post-Hoc). IC_{50} value was obtained using PROBIT analysis.

2.3 Inhibitory Assay

P. gingivalis which had been made into a suspension were taken with a cotton swab and spread into the blood agar medium. Then a hole is made using a perforator in the inoculated agar, forming a well. Furthermore, the wells will be filled with each treatment, specifically positive control with antiseptic chlorhexidine solution, negative control with aquadest, and ethanol extract of agarwood leaves with

various concentrations. The filled media were then incubated for 24 hours at 37^0C.

The same procedure will be repeated three times. The inhibition zone that formed around the well was measured using a calliper with units of mm as research data. Results were further categorized according to inhibition zone category according to Davis and Stout.

2.3.1 Statistical Analysis

Results that obtained as data processed with IBM SPSS 21.0 ver. Normality test was carried out using Shapiro-Wilk then proceeded to One-way ANOVA and Dunnett T3 (Post-Hoc).

3 RESULTS AND DISCUSSION

3.1 Results

3.1.1 Cytotoxicity Assay

Phytochemical analysis of agarwood leaf ethanol extract showed positive result from the presence of flavonoid, saponin, tannin, alkaloid, triterpenoid, and phenol as shown in Table 1.

Table 1: Phytochemical Analysis Results.

Compound	Results
Flavonoids	(+)
Saponins	(+)
Tannins	(+)
Terpenoids	(+)
Triterpenoids/Steroids	(+) Triterpenoid
Phenols	(+)
Alkaloids	(+)

The outcomes of cytotoxicity assay included mean of absorbance, corrective absorbance, number of viable cells, percentage of viability, and percentage of inhibition obtained through calculations and spectrophotometer measurements.

Parameters used in this study were the percentage of viability cell and IC_{50}. Cell viability was obtained and calculated from the number of cells that are still alive (viable) after treated, while IC_{50} value was obtained through PROBIT analysis with the aim of knowing the concentration of agarwood leaf extract which can inhibit fibroblasts growth by as much as half the population. Results of cytotoxicity assay shown in Appendix.

In vitro cytotoxicity assay according to ISO 10993-5: Biological Evaluation of Medical Devices states that, if the relative cell viability for the extract concentration of a sample is more than equal to 70%, then the material must consider as non-toxic (International Standard Organization, 2009; International Standard Organization, 2012). Based on these standards in this study, the concentration of agarwood leaf extract that did not toxic on 3T3 Balb/C fibroblasts were concentrations of 62.5 µg/mL, 31.25 µg/mL, 15.63 µg/mL and 7.81 µg/mL. Meanwhile, concentrations of 500 µg/mL, 250 µg/mL, and 125 µg/mL were toxic.

Standard curve of 3T3 Balb/C cells was created (Figure 1) as a reference for calculating the number of cells based on the absorbance obtained. The curve made by regressing the absorbance value and the number of cells into the standard curve line equation y = ax + b. The y-axis is the absorbance value, while the x-axis is the number of cells.

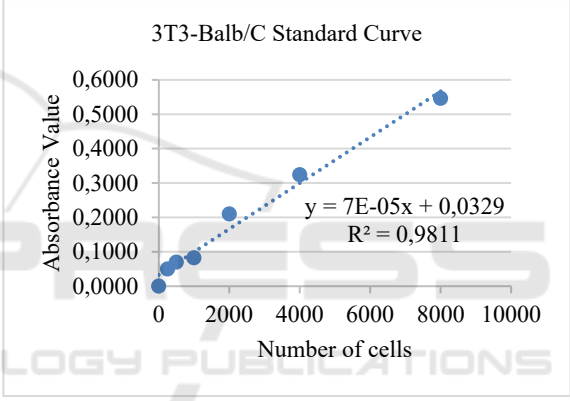

Figure 1: 3T3 Balb/C Standard Curve.

The regression curve shows linear relationship between the number of cells and the absorbance value. This could also be seen in observations on well plates. The darker the color produced on the well plate, the higher the absorbance value and the number of viable cells. This curve is then used as a reference for calculating the number of viable cells in each treatment.

Furthermore, statistical analysis conducted towards these data resulting in normality distributed and homogenous data. Analysis proceeded to ANOVA and Post-Hoc Tuckey HSD. Results showed that each treatment significantly affecting the difference in cell viability by significance (p)<0.5. The IC50 value obtained was at a concentration of 215.54 µg/mL using PROBIT analysis.

3.1.2 Inhibitory Assay

In this study, 9 treatments were repeated three times on *P. gingivalis*. There were 7 concentrations of agarwood leaf ethanol extract, namely 100%, 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56%. Other treatments given were positive control with antiseptic chlorhexidine solution and negative control with aquadest.

Parameter used in this study was the diameter of the inhibition zone produced in each treatment as the form of a clear zone on agar media. The results of this inhibition zone measurement will then be interpreted into the inhibition category according to Davis and Stout.

As shown in appendix and Figure 2, measurements of inhibition zone diameter showed that the largest diameter of the inhibition zone was in group 9, namely chlorhexidine as positive control. Then followed by a group of 8, namely the ethanol extract of gaharu leaves with a concentration of 100%. Meanwhile, the smallest diameter of the inhibition zone was in group 1, namely the negative control of aquadest.

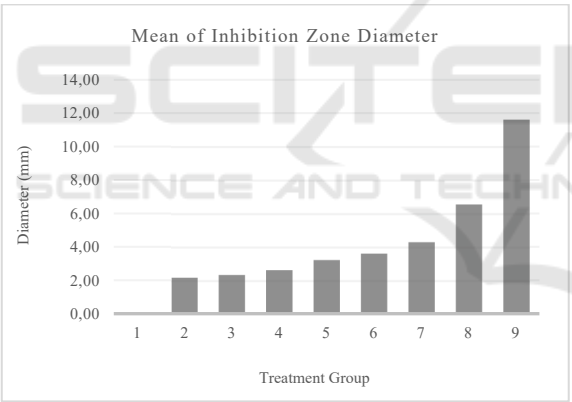

Figure 2: Mean of inhibition zone diameter.

Note:
1 treatment group for (-) control (aquadest)
2 treatment group for agarwood leaf ethanol extract 1.56%
3 treatment group for agarwood leaf ethanol extract 3.13%
4 treatment group for agarwood leaf ethanol extract 6.25%
5 treatment group for agarwood leaf ethanol extract 12.5%
6 treatment group for agarwood leaf ethanol extract 25%
7 treatment group for agarwood leaf ethanol extract 50%
8 treatment group for agarwood leaf ethanol extract 100%
9 treatment group for (+) control (chlorhexidine)

According to the Davis and Stout inhibition category, the result will be categorized as weak if the inhibition zone formed is 5 mm or less. Followed by moderate inhibitory ability if 5-10 mm inhibition zone formed. While strong inhibitory ability when 10-20 mm zone is formed. Lastly, inhibitory ability will be categorized as very strong if more than 20 mm zone formed (Rastina et al., 2015). Based on these categories, ethanol extract of agarwood leaf has weak inhibitory ability at concentrations of 50%, 25%, 12.5%, 6.25%, 3.13%, and 1.56%. While the concentration of 100% has moderate inhibitory ability against the growth of *P. gingivalis*.

Normality test was performed resulting in normally distributed data by significance of $p<0.05$. Data analysis was proceeded to One-way ANOVA and it was found to support H_0, states that agarwood leaf extract has significance effect on *P. gingivalis* growth. Further analysis was carried out by using Post-Hoc Dunnett T3 method. Results showed that each concentration of agarwood leaf extract has different significance effect toward *P. gingivalis* growth.

Table 2: Post-Hoc Dunnett T3 Analysis.

Treatment	Inhibition Zone
(-) Control	0 ± 0.00^{a}
1,56%	2.18 ± 0.08^{b}
3,13%	2.35 ± 0.15^{bc}
6,25%	2.63 ± 0.08^{bdc}
12,50%	3.23 ± 0.34^{cde}
25%	3.62 ± 0.10^{def}
50%	4.30 ± 0.39^{ef}
100%	6.55 ± 0.23^{g}
(+) Control	11.62 ± 0.60^{h}

3.2 Discussion

3.2.1 Cytotoxicity Assay

This study results showed that the highest percentage of cell viability was in the group with concentration of 7.81 µg/mL, and the lowest was in the group with concentration of 500 µg/mL. These data indicate that there is decrease in cell viability which is inversely proportional to the increase in concentration. The differences in cell viability at each concentration could indicate that the cell response towards each concentration was also different, which is thought to be due to the variance in the amount of active compound content at each concentration.

Qualitative phytochemical testing carried out in this study did not prove the exact amount and proportion of active compounds contained in agarwood leaf extract. In addition, the dilution of agarwood leaf extract is also thought to result in a decrease in the number of active compounds along with the smaller the concentration, since the amount

of extract keep reduced during the dilution process. Referring to several previous studies, the active compounds that play a role in this research are saponins, flavonoids, and tannins.

Saponins are derivatives of glycosides that can act as antioxidant agents capable of neutralizing free radicals by binding to active oxygen. The lyobipolar properties possessed by saponins allows their interaction with cell membranes by reducing the surface tension of cells (Yildirim and Kutlu, 2015). Saponins also could increase the expression and activation of TGF-β, FGF, and VEGF via monocyte proliferation. Activation of FGF will increase fibroblast proliferation and stimulate fibronectin synthesis. Fibronectin synthesis will induce fibroblast migration. The fibroblasts will then be responsible for the synthesis of collagen in the extracellular matrix (Suharto and Etika, 2019; Ardiana et al., 2015).

Signaling performed by FGF plays an important role in the regulation of cell pluripotency through interaction with FGFRs. FGF/FGFRs will then be responsible for cellular processes such as proliferation, migration, embryonic development, tissue regeneration, and cell metabolism. This entire process is mediated by activation of RAS - mytogen-activates protein kinase (MAPK), Phospholipase C Gamma, and signal transducers and activators of transcription (STAT). These various signaling pathways will also work together with signaling from other growth factors, such as TGF-β (Mohammadi et al., 2020).

In molecular level, flavonoids could induce extracellular signal-regulated kinase (ERK). ERK can specifically recognize various growth factor receptors on the cell surface such as FGFRs, KGFR, and EGFR also significantly activate these rexeptors (Etika et al., 2017). Arginine, which is a derivative of flavonoids, has been shown to affect the proliferation of human gingival fibroblasts and fibroblasts through activation of amino acid receptors and cyclic-AMP response element binding (CREB). This activation will then stimulate the secretion of various growth factors and also the extracellular matrix (Kurahashi and Fujii, 2015).

Flavonoids contributed in macrophage activation which will stimulate the synthesis of several growth factors such as PDGF, FGF, EGF, TGF-β, and TGF-α. Macrophages together with neutrophils can synthesize Reactive Oxygen Species (ROS) which is a chemically reactive molecule, formed due to the acceptance of electrons by the O_2 molecule. ROS produced in the wound healing process is superoxide radical anion which will then be broken down into H_2O_2 (hydrogen peroxide) and oxygen molecules through the superoxide dismutase mechanism. The disintegration of superoxide radical anion aims to prevent the formation of destructive ROS with high concentrations such as peroxynirite (ONOO--) or hydroxyl radical (-OH) Jiang et al., 2018; Ningrum and Kurniawaty, 2019).

Tannins contributed to the differentiation of fibroblasts into myofibroblasts along with the production of an extracellular matrix which important in wound contraction. But along with this process, tannins can also control the differentiation and proliferation of fibroblasts through the expression of genes involved in extracellular matrix production. This is performed by induction of Smad2 and Erk proteins on the TGF-β1 signaling pathways. This regulation of fibroblast differentiation and proliferation aims to prevent fibrosis forming in the wound. The molecular mechanism of the anti-proliferative effect of fibroblasts by tannins is thought to be due to the regulation of cyclin gene expression All these mechanisms suggest that both differentiation and inhibition of fibroblast proliferation depend on inhibition of Smad2 and Erk activation on the TGF-β1 signaling pathways (Pattarayan et al., 2018).

Toxicity to fibroblasts in this study not only could be caused by the level of the active compound at each concentration, mechanism of action, and structure of the active compound in the agarwood leaf extract. In vitro, the mechanism of cytotoxicity can be in the form of cell membrane destruction, prevention of protein synthesis, binding with irreversible receptors, prolonged inhibition of polydeoxynucleotide and enzyme reactions (Aslanturk, 2018). It has also been shown that toxic agents can induce excessive nitric oxide production, ROS followed by oxidative stress, and mitochondrial dysfunction as a result of oxidative stress. Toxic agents can also potentially release components that can directly result in DNA damage, followed by apoptosis (Stammenković-Radak and Andjelković, 2016; Zhang, 2018; Spindola et al., 2018).

The determination of IC_{50} value is important in determining and understanding the pharmacological and biological characteristics of a chemotherapeutic agent. The IC_{50} value is a measurement of drug efficacy indicating the amount of concentration of a chemotherapeutic agent required to inhibit half the biological processes. This suggests a description of the antagonistic potential of chemotherapeutic agents in a study Aykul and Martinez-Hackert, 2016; He et al., 2016).

In this study, the IC_{50} value obtained was at a concentration of 215.54 µg / mL, which indicates that

this concentration can inhibit fibroblast proliferation by up to 50% of the population. These results can be used as a reference for further research regarding the potential of agarwood leaf extract as a wound healing agent by using the IC_{50} value as the minimum concentration of agarwood leaf extract.

3.2.2 Inhibitory Assay

Based on the mean of inhibition zone measured, it can be concluded that the diameter of the inhibition zone is directly proportional towards the concentration of agarwood leaf extract.

Phytochemical test results showed that agarwood leaf extract contain several active compounds that can inhibit the growth of *P. gingivalis*. The main compounds contained in the ethanol extract of gaharu leaves are flavonoids. These compounds contributed in the destruction of bacterial cells due to damage to the permeability of their cell membranes (Nomer et al., 2019; Warganegara and Restina, 2016). The action mechanism of flavonoids as antimicrobial is divided into 3, that is nucleic acid synthesis inhibition, inhibiting the function of bacterial cell membranes, and inhibiting energy metabolism from amino acids (Nomer et al., 2019).

Flavonoids can inhibit nucleic acid synthesis in bacterial cells because of their A and B rings. These rings contributed in the intercalation process or the process of hydrogen bonding by accumulating nucleic acid bases so that the formation process of DNA and RNA is inhibited (Nomer et al., 2019). These flavonoid compounds can also cause damage to the permeability of bacterial cell walls so that bacterial-toxic substances can enter these bacterial cells. In addition, flavonoids also form complex compounds with extracellular proteins that can damage cell membranes owned by bacteria resulting in leakage of intracellular compounds. Flavonoids are also able to inhibit the energy metabolism process of bacteria by interfering with the macromolecular biosynthesis process of these bacteria. Due to inhibited metabolic processes, these molecules cannot develop properly to meet the needs of these bacteria (Nomer et al., 2019; Sapara and Waworuntu, 2016).

There are various types of flavonoids, such as genkwanin, apigenin, and luteolin. Some of these compounds are a class of flavonoids which are found in agarwood leaves (Wangiyana, 2020). Genkwanin, apigenin, and luteolin are known to have antibacterial activity. Apigenin can affect the cytoplasmic membrane of bacteria, this compound will interfere with the metabolic process of bacteria and ultimately inhibit energy production in bacteria (Xie et al., 2014).

Other active compounds found in agarwood leaf extract are alkaloids, tannins, saponins, and triterpenoids/steroids. These compounds are involved in the destruction of bacterial cell membranes (Sari et al., 2017). Alkaloids penetrate the bacterial lipopolysaccharide membrane, causing depolarization of the cytoplasmic membrane. This compound will then affect the production of enzymes in bacteria and cause leakage in the cytoplasm of the bacteria (Cushnie et al., 2014). Meanwhile, tannins invade the polypeptides present in the cell walls, these compounds can denature proteins and eventually lead to bacterial lysis (Zeniusa et al., 2019).

Saponins would bind water molecules and dissolve fat so that it could disrupt the surface tension of bacterial cells and eventually cause cell destruction. In addition, there are triterpenoids/steroids that can bind to lipid molecules on the bacterial cell membrane. These compounds will disrupt the integrity of the bacterial cell membrane and can change the morphology of the cell membrane. In the end, bacterial cells will be fragile and will undergo lysis (Sari et al., 2017).

Previous research states that agarwood leaf extract produced moderate to strong inhibition of Staphylococcus aureus bacteria, a gram-positive bacterium (Liana, 2014). While research conducted upon gram-negative bacteria showed that the inhibition power of agarwood leaf extract was weaker when compared to gram-positive bacteria. This is caused by 3 layers of the wall owned by gram-negative bacteria. Those are the outer lipoprotein layer, the middle lipopolysaccharide layer, and the outer peptidoglycan layer (Septiani et al., 2017).

In this study, the inhibitory ability produced towards *P. gingivalis* was weak to moderate. This bacterium is an encapsulated gram-negative which makes it more resistant to antibacterial activity compared to other bacteria. This is supported by other research which states that agarwood leaf extract has weak to moderate inhibitory power against several other encapsulated gram-negative bacteria such as *Escherichia coli, Klebsiella pneumoniae* and *Vibrio mimicus* (Hendra et al., 2016; Begum, 2016; Jihadi et al., 2020).

In this study, the diffusion method used was agar well diffusion method which is widely used to evaluate the antimicrobial activity of extracts derived from plants. In this method, a hole with a diameter of 8 mm is made which will then be filled with agarwood leaf extract. The agar well diffusion method is used with the aim that the results obtained directly reflecting the agarwood leaf extract inhibitory ability. This is ought due to the diffusion process is better and

the volume of material placed is more than the agar disk diffusion method Balouiri et al., 2016).

There are several factors that can affect the diameter of the bacterial growth inhibition zone. The first factor is the temperature used at the time of incubation. If during the incubation process the agar media are stacked, there is a possibility that there is a temperature difference between the plates. In addition, the thickness of the agar also affects the diameter of the inhibition zone. The best agar thickness is 4 mm. More than that, the diffusion process of the extract will be slower (Zeinusa et al., 2019). The dilution of the extract could also affect the diffusion ability. Higher concentration means decreasing solubility or thicker extract. Therefore, the extract diffusion process with higher concentrations will be slower.

Another factor that can affect the diameter of inhibition zone is the turbidity of the bacterial suspension. In this study, the measurement of the turbidity level was only visually performed by comparison with the 0.5 Mc Farland solution. If the bacterial suspension is too turbid, the resulting inhibition zone diameter will be smaller and vice versa.

The phytochemical test carried out in this study was a qualitative test. Hence, it is difficult to determine with certainty the number of compounds contained in agarwood leaf extract which can inhibit *P. gingivalis* growth. In addition, the solvent used in diluting the ethanol extract of gaharu leaves was distilled water (aquadest) which was also used as a negative control in this study. It was found that this treatment did not produce a clear zone on the agar medium, meaning that distilled water confirmed had no inhibitory ability and did not affect *P. gingivalis* growth.

In this study, the concentration of agarwood leaf extract was made in percent units makes the range of concentrations that could be made as a treatment was less than optimal. Making the concentration in units of PPM (parts per million) might be better because this method refers more to the unit of concentration. This is a way to measure the concentration of a substance that is both very low and high, which 1 ppm is equivalent to 1 milligram per liter, or the concentration in percent is 0.0001%.

4 CONCLUSIONS

This study showed that there was a cytotoxicity effect of the agarwood leaf extract towards fibroblasts in vitro at concentrations of 500 µg/mL, 250 µg/mL, and 125 µg/mL, and there was no cytotoxicity effect of agarwood leaf extract towards fibroblasts in vitro at concentrations of 62.50 µg/mL, 31.25 µg/mL, 15.63 µg/mL, and 7.81 µg/mL. Agarwood leaf extract also had effect towards *P. gingivalis* growth with the maximum inhibition at a concentration of 100% which is classified into the moderate inhibition category.

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APPENDIX

Cytotoxicity Assay

Treatment	Results				
	XA	XC	Number of Cells	%Viability	%Inhibition
Cell Control	1,7049	1,2395	17238	100,00	0,00
DMSO 10%	1,6521	1,2337	17154	99,51	0,49
500 µg/mL	0,8455	0,4081	5360	31,10	68,90
250 µg/mL	1,0178	0,6374	8636	50,10	49,90
125 µg/mL	1,0430	0,6675	9065	52,59	47,41
62.5 µg/mL	1,4046	1,0277	14211	82,44	17,56
31.3 µg/mL	1,5046	1,1200	15530	90,09	9,91
15.6 µg/mL	1,6080	1,2064	16765	97,26	2,74
7.81 µg/mL	1,9290	1,5217	21269	123,39	-23,39

XA : Mean of absorbance
XK : Mean of corrective absorbance
ALE : Agarwood leaf extract

Inhibitory Assay

Treatment	Inhibition Zone			Mean
	1	2	3	
- Control	0,00	0,00	0,00	0,00
1,56%	2,20	2,10	2,25	2,18
3,13%	2,35	2,20	2,50	2,35
6,25%	2,65	2,55	2,70	2,63
12,50%	2,85	3,50	3,35	3,23
25%	3,50	3,70	3,65	3,62
50%	3,85	4,50	4,55	4,30
100%	6,50	6,35	6,80	6,55
+ Control	10,95	12,10	11,80	11,62