Original Article

The Antibacterial and Antibiofilm Effect of Amoxicillin and *Mangifera indica* L. Leaves Extract on Oral Pathogens

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Abstract

Objective: This study aimed to determine the antibacterial and antibiofilm effects of amoxicillin combined with extract of *Mangifera indica* L. leaves against *Staphylococcus aureus* and *Porphyromonas gingivalis*. **Materials and Methods:** This was an experimental laboratory *in vitro* study with a posttest-only control group design. An antibacterial test using the plate count method and an antibiofilm test using the microtiter plate biofilm assay method were conducted. The research samples comprised extract of *M. indica* L. leaves with a concentration of 100%; amoxicillin and extract concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%; and amoxicillin. Dimethyl sulfoxide served as a negative control and co-amoxiclav served as a positive control. **Results:** The combination of amoxicillin and the extract exhibited an antibacterial effect against *S. aureus* at a concentration of 12.5% and higher and more effective than co-amoxiclac *P. gingivalis* at a concentration of 3.125% and higher. In the antibiofilm test, the combination of amoxicillin and the extract at a concentration of 25% after 1 h of incubation and a concentration of 6.25% after 3 h of incubation inhibited *S. aureus*. The inhibition of *S. aureus* biofilms at a concentration of 100% after 24 h of incubation was as effective as that of co-amoxiclav. The extract at a concentration of 25% over the entire incubation period showed more potent inhibition against the *P. gingivalis* biofilm than co-amoxiclav. **Conclusions:** The ethanolic extract of *M. indica* L. leaves and the combination of amoxicillin and the extract have the potential to inhibit the growth and formation of *S. aureus* and *P. gingivalis* biofilms.

Keywords: *Amoxicillin, antibiofilm, arumanis mango, ethanol extract of Mangifera indica L. leaves*

Introduction

Based on data from Basic Health Research (Riskesdas) in 2018, Indonesia's dental and oral health problems reached 57.6% of overall health-care problems.[1] The latter is influenced by poor oral hygiene, which triggers various diseases, such as dentoalveolar abscesses and periodontitis.[2,3] A dentoalveolar abscess is a pathological cavity in the oral cavity that contains pus due to secondary infection caused by caries, trauma, failure of root canal treatment, and poor oral hygiene.[3] Periodontitis is a chronic inflammation of the periodontal tissue structure, including gingiva, bone, and the periodontal ligament, which can cause pocket formation, recession, tooth mobility, or tooth loss.[2] Dentoalveolar abscesses and periodontitis are associated with bacterial pathogens in biofilms.[3,4]

A biofilm is a collection of microbial cells, especially bacteria, attached to the tooth surface and coated by an extracellular polymeric substance. This coating protects cells and enables accelerated growth rates, along with additional horizontal gene transfer between cells within the coating, which promotes additional problems, such as antibiotic resistance.[5] The formation of biofilms in the oral cavity involves complex competition between microflora for initial attachment.[6] *Staphylococcus aureus*, a Gram-positive bacterium, initiates adherence in biofilm formation and produces multiple layers of biofilm embedded in a glycocalyx layer.[7,8] *S. aureus* has several virulence factors (capsules, adhesins, coagulase, hyaluronidase, staphylokinase, enterotoxin, and leucocidin) that support biofilm formation in a dentoalveolar abscess.[6] *Porphyromonas gingivalis*, which belongs to anaerobic Gram-negative bacteria, is the second most common colonizing bacteria in biofilms. *P. gingivalis* virulence factors, such as lipopolysaccharides, capsules, fimbriae, outer membrane proteins, proteases, and enzymes, induce the destruction of periodontal tissue, causing periodontitis.[9]

How to cite this article: Soesanto S, Hepziba ER, Yasnill, Widyarman AS. The antibacterial and antibiofilm effect of amoxicillin and *Mangifera indica* L. leaves extract on oral pathogens. Contemp Clin Dent 2023;14:145-51.

Submitted : 26-Jul-2022
Revised : 06-Jan-2023
Accepted : 21-Feb-2023
Published : 30-Jun-2023

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Access this article online

Website: https://journals.lww.com/cocd

DOI: 10.4103/ccd.ccd_399_22

Quick Response Code:

Amoxicillin from the penicillin group is often used to treat dentoalveolar abscesses and periodontitis. Amoxicillin is a broad-spectrum antibiotic that works by binding to penicillin-binding proteins in Gram-positive and Gram-negative bacteria and inhibiting the transpeptidation process in bacteria.[3,10] Overuse of amoxicillin can lead to side effects, such as hypersensitivity reactions, nausea, vomiting, diarrhea, thrombocytopenia, dermatological disorders, and resistance.[11] Resistance to amoxicillin can also occur due to the destruction of the ß-lactam ring by ß-lactamase enzymes produced by *S. aureus* and *P. gingivalis*.[2,12] Therefore, amoxicillin is often combined with clavulanic acid, known as co-amoxiclav, to reduce resistance.[13] Clavulanic acid is a ß-lactamase inhibitor that works by inactivating the pathogen's ß-lactamase, thereby increasing the antibacterial activity of amoxicillin.[11] However, the use of co-amoxiclav can cause side effects, such as itching, redness around the mouth, diarrhea, nausea, and idiosyncratic drug-induced liver injury.[11,13] Alternative medicines using herbal plants have the potential to minimize these side effects.[14]

Arumanis mango (*Mangifera indica* L) is a herbal plant from India cultivated in various tropical and subtropical regions, including Indonesia.[15,16] Indonesians cultivate Arumanis mangos for their sweetness, freshness, and fragrance. The fruit contains Vitamins A, B, and C, which are beneficial for health.[17] In addition, the seeds, skin, roots, and leaves of *M. indica* L. have various properties in traditional medicine. *M. indica* L. leaves are generally discarded and considered waste, even though these leaves contain secondary metabolites, including phenolics (mangiferin, tannins, and flavonoids), alkaloids, saponins, terpenoids, glycosides, and steroids that have potential antibacterial, antifungal, antiviral, antiparasitic, antioxidant, anti-inflammatory, antitumor, anticancer, and analgesic effects.[18]

Previous studies showed that *M. indica* L. leaf extracts reduced the number of *Streptococcus mutans* and improved the antibacterial effect of clindamycin against *S. aureus*.[19,20] However, no research has investigated the potential of combining amoxicillin with *M. indica* L. leaf extracts in combating *S. mutans* and *P. gingivalis*. Thus, to address this research gap, this study aimed to determine the effectiveness of amoxicillin and ethanolic extract of Arumanis mango (*M. indica* L.) in inhibiting the growth and formation of *S. aureus* and *P. gingivalis* biofilms.

Materials and Methods

This experimental *in vitro* study was performed at the Microbiology Center of Research and Education (MiCORE) Laboratory, Faculty of Dentistry, Universitas Trisakti, Jakarta, Indonesia. An ethanolic extract of Arumanis mango (*M. indica* L.) leaves was prepared at the Research Institute for Spices and Medicinal Plants (BALITTRO) in Bogor, West Java, Indonesia. The test solutions used were 10% dimethyl sulfoxide (DMSO) (negative control), co-amoxiclav (positive control), ethanolic extract of *M. indica* L. leaves with a concentration of 100%, and a combination of amoxicillin with extract concentrations of 3.125%, 6.25%, 12.5%, 25%, 50%, and 100%.

The sample size was calculated using Federer's formula, with n equals number of repetition, and t equals as total test group (8 groups comprised 6 treatment groups, 1 positive control, and 1 negative control). Based on this formula, each treatment group was conducted with four-time repetition for all assays.

Ethanolic extract of *Mangifera indica* L. leaves

The ethanolic extract of *M. indica* L. leaves was prepared using the maceration method. The mango leaves were washed, dried, and mashed, and the simplicial was then soaked in 70% ethanol at a ratio of 1:5. The maceration process was performed for 2–3 h, and the macerate was then allowed to stand for 24 h and filtered. The extract was diluted with 10% DMSO solution to obtain extract concentrations of 50%, 25%, 12.5%, 6.25%, and 3.125%.

Preparation of antibiotic solution

Amoxicillin and co-amoxiclav solution were prepared by crushing 500 mg of amoxicillin and 625 mg of co-amoxiclav until smooth, using a mortar and pestle. In total, 1.2 mg of amoxicillin and 1.5 mg of co-amoxiclav were each mixed with 6 ml of sterile distilled water until homogeneous to obtain 200 g/µl of amoxicillin and 250 g/µl of co-amoxiclav.

Phytochemical tests

Qualitative phytochemical tests were performed at BALITTRO to identify secondary metabolites, such as alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids, and glycosides, in the ethanolic extract of *M. indica* L. leaves.

Bacterial culture

S. aureus ATCC 25923 and *P. gingivalis* ATCC 33277 were obtained from the MiCORE Laboratory, Faculty of Dentistry, Trisakti University. *S. aureus* was cultured on brain heart infusion broth medium (Sigma Aldrich, St. Louis, Missouri). *P. gingivalis* was cultured on Tryptone Soya Broth (Sigma Aldrich, St. Louis, Missouri) medium enriched with hemin (5 mg/l), Vitamin K (10 mg/l), 0.5% yeast extract, and L-cystine (400 mg/l).[21] The medium was incubated in an anaerobic jar (Oxoid, Basingstoke, and Hampshire) at 37° for 24 h under anaerobic conditions. Following the incubation, the bacterial absorbance value was standardized into McFarland standard of 0.5, approximately 1.5×10^8 colony forming unit (CFU)/mL (optical density $OD_{600} \pm 0.132$), before the following assays.

Microdilution and total plate count

To evaluate the antibacterial properties of the extract combined with amoxicillin, an antibacterial test was performed on the plate count by the microdilution method. In total, 100 µL of cultured *S. aureus* ATCC 25923 and *P. gingivalis* ATCC 33277 were distributed into 96-well plates (Nest Biotech, Jiangsu, China). A test solution of 100 µl was added to each well and incubated at 37°C for 24 h under anaerobic conditions. After incubation, each mixture containing treated bacteria was diluted 10^5 times. Five microliters of diluted mixture were then spread on a petri dish containing sterile brain heart infusion agar media. The growth of bacterial colonies was calculated after incubation at 37°C for 24 h.

Microtiter plate biofilm assay

The bacterial cultures were inserted into each well of the 96-well plates and then incubated at 37°C for 24 h under anaerobic conditions. The supernatant was discarded, leaving a thin layer on the surface of the well. The wells were rinsed using phosphate-buffered saline (PBS) (Biomatics, Ontario, Canada). Each test solution (200 µl) was added to each well and then incubated for 1, 3, and 24 h at 37°C. The wells were rinsed twice with PBS and fixated over burning spirit lamp. Crystal violet (Merck, Darmstadt, Germany) (200 µl) was then added to each well and allowed to stand for 15 min, followed by rinsing twice and standing for 15 min. In the past step, 200 µl of 96% ethanol was added. The OD was measured using a microplate reader (Safas, Monaco) with a wavelength of 490 nm.

Statistical analysis

The research data were processed using the Statistical Package for the Social Sciences (SPSS) computer program, version 26 (IBM, Armonk, NY, USA). The Shapiro–Wilk method was used to test the normality of the data. If the data were normally distributed ($P > 0.05$), a one-way analysis of variance test was conducted, followed by Turkey's honestly significant difference test (significance level of $P < 0.05$) to verify the significance between the groups.

Results

Phytochemical screening

The results of the phytochemical screening qualitatively proved that the ethanolic extract of *M. indica* L. leaves contains alkaloids, saponins, tannins, phenolics, flavonoids, and steroids [Table 1].

Table 1: The results of phytochemical screening of the ethanol extract of *Mangifera indica* L. leaves

Secondary metabolites	Screening result
Alkaloids	+
Saponins	+
Tannins	+
Phenolics	+
Flavonoids	+
Triterpenoids	−
Steroids	+
Glycoside	−

Antibacterial test using microdilution and plate count methods

The antibacterial test results showed that the ethanolic extract of *M. indica* L. leaves at 100% concentration and the combination of amoxicillin with extracts of various concentrations exhibited antibacterial and antibiofilm effects against *S. aureus* and *P. gingivalis* [Figure 1] In terms of inhibition of *S. aureus*, the results obtained by the combination of amoxicillin and the extract were not significantly different from those obtained using the positive control ($P > 0.05$). The extract at a concentration of 12.5% and higher was effective against *S. aureus*, and the extract at a concentration of 3.125% and higher was effective against *P. gingivalis* [Figures 2 and 3].

Antibiofilm test using the microtiter plate biofilm assay method

The results of the antibiofilm test showed that the addition of extracts to amoxicillin in inhibiting *S. aureus* biofilms had a significantly lower OD value compared to the positive control ($P < 0.05$), starting at a concentration of 25% after 1 h of incubation [Figure 4] and at a concentration of 6.25% after 3 h of incubation [Figure 5]. After 24 h of incubation, following OD measurement, the group with the combination of extract concentration of 100% and amoxicillin proved not to be significantly different from OD of the positive control ($P > 0.05$) [Figure 6]. In the *P. gingivalis* biofilm, the OD values of the addition of amoxicillin and the extract group, starting at a concentration of 25% after 1, 3, and 24 h of incubation, were smaller than the OD values of the positive control amoxiclav. The OD values of the treatment group were significantly different from those of the positive control amoxiclav [Figures 7-9].

Discussion

As shown by our results, the ethanol extract of *M. indica* L. leaves contains secondary metabolites, including alkaloids, saponins, tannins, phenolics, flavonoids, and steroids. Different mechanisms of action of each compound account for the antibacterial and antibiofilm properties of the extract. Alkaloids inhibit the formation of peptidoglycan in bacterial cells. The alkaloids can disrupt the amino acid structure of bacterial DNA, leading to bacterial lysis.[22] Saponins damage the cell membrane and cell wall permeability in the diffusion process, resulting in the release of enzymes, amino acids, nutrients, and water, leading to cell destabilization and cell death.[23] Tannins form a complex with protein in the cell wall, namely,

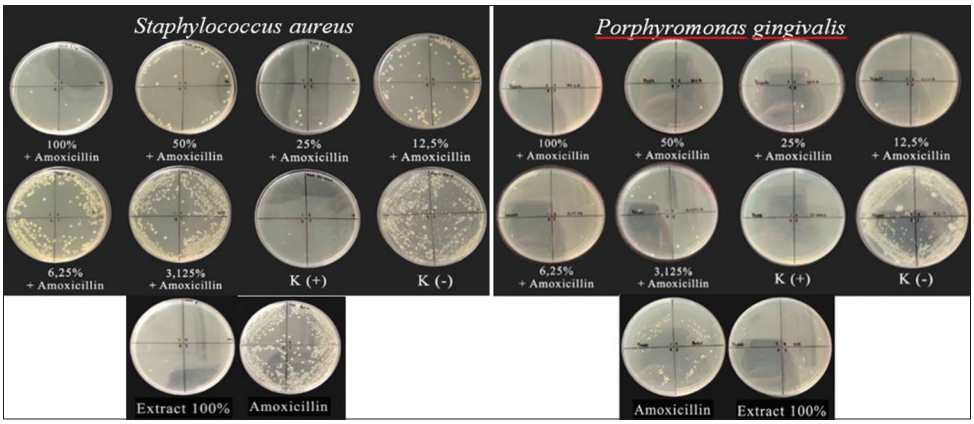

Figure 1: The results of the inhibition test of *Staphylococcus aureus* and *Porphyromonas gingivalis* using the plate count method

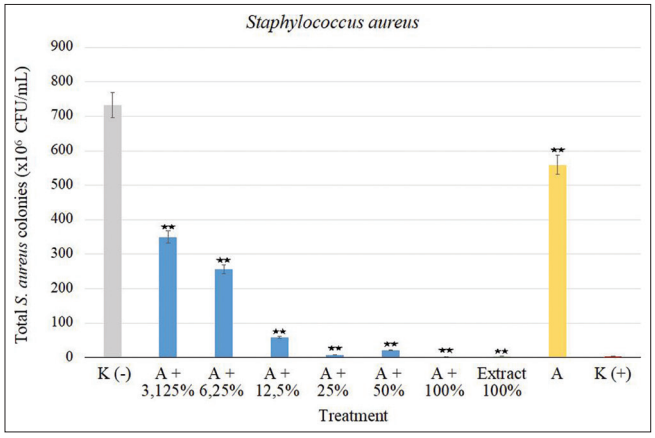

Figure 2: Graph of the average total colony of *Staphylococcus aureus* (*$P < 0.05$, **$P < 0.01$)

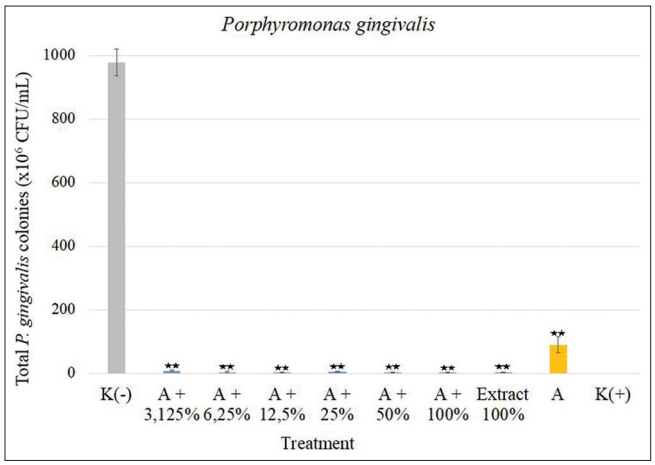

Figure 3: Graph of the average total colony of *Porphyromonas gingivalis* (*$P < 0.05$, **$P < 0.01$)

proline, which can damage the cell wall.[22] The most abundant phenolic compound in *M. indica L* leaves is mangiferin. Mangiferin belongs to the xanthone C-glucosyl group, which can damage cell structure and cell membranes and inhibit bacterial protein synthesis.[24] Previous research reported that mangiferin compounds interfere with the mechanism of drug resistance; thus, restoring bactericidal and bacteriostatic effects of nalidixic acid, ampicillin, tetracycline, and sulfamethoxazole/trimethoprim.[25] However, the exact mechanism of how mangiferin interferes with the mechanism of drug resistance is yet to be elucidated. Flavonoids, as antibacterial, play a role in disrupting the activity of cell wall formation by suppressing cytoplasm function, interrupting the nutrient exchange process, and thereby inhibiting the energy supply of bacteria.[26] In addition, flavonoids inhibit enzymes from producing quorum-sensing signals, thereby disrupting the communication process between cells during biofilm formation.[27] Steroids can cause leakage of lysosomes and membrane phospholipids that reduce the integrity of cell membranes and lead to cell lysis.[28]

Thus far, only a few studies have focused on antibacterial and antibiofilm properties of ethanolic extract of *M. indica* L. leaves.[19,20] The concentration of the extract used in this study ranged from 3.125% to 100%. As a positive control, co-amoxiclav, a combination of amoxicillin and clavulanic acid, was used. Amoxicillin, a ß-lactam antibiotic, works by inhibiting the synthesis of bacterial cell walls.[11] The release of ß-lactamase enzymes by *S. aureus* and *P. gingivalis* decrease the antibacterial effect of amoxicillin.[2,12] The addition of clavulanic acid binds to ß-lactamase enzymes from bacteria, inhibiting the enzyme thereby unable to cleave ß-lactam ring in amoxicillin, so the amoxicillin can still exhibit its antibacterial activity.[11]

In the microdilution and plate count tests, the addition of the ethanolic extract of *M. indica* L. leaves to amoxicillin inhibited the growth of *S. aureus* and *P. gingivalis*, and the combination of the ethanolic extract and amoxicillin was as effective as that of co-amoxiclav. In terms of antibacterial activity, a combination of ethanolic extract at a concentration of 12.5% and amoxicillin was as effective as co-amoxiclav against *S. aureus*, and a concentration as low as 3.125% was as effective as co-amoxiclav against *P. gingivalis*. Therefore, ethanolic extract of *M. indica* L. leaves may be a potential ß-lactamase inhibitor equivalent to clavulanic acid. The results of this study are in line with the research of Hartanto *et al*., who showed that adding

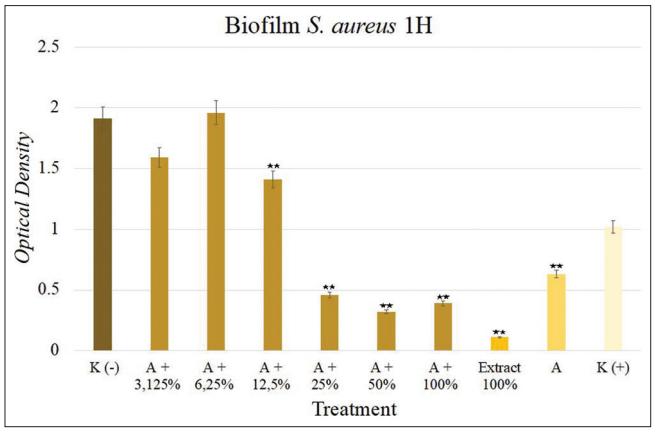

Figure 4: Graph of the average OD value of *Staphylococcus aureus* biofilm after 1 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

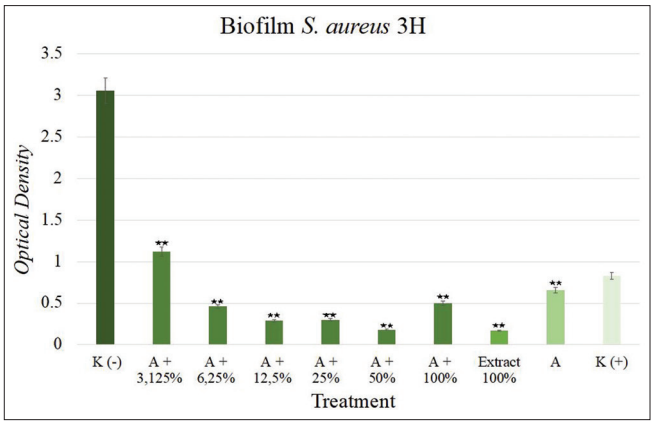

Figure 5: Graph of the average OD value of *Staphylococcus aureus* biofilm after 3 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

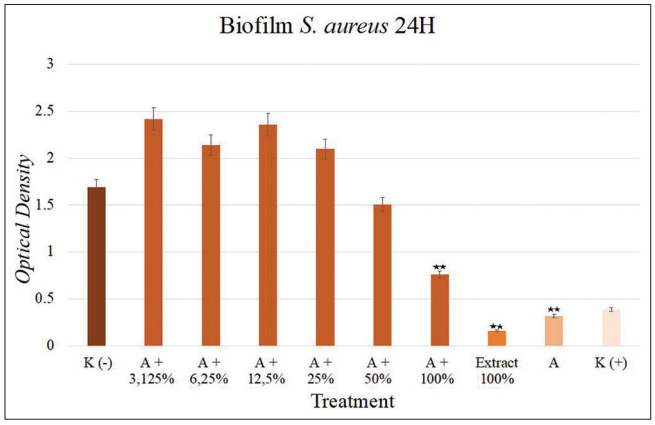

Figure 6: Graph of the average OD value of *Staphylococcus aureus* biofilm after 24 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

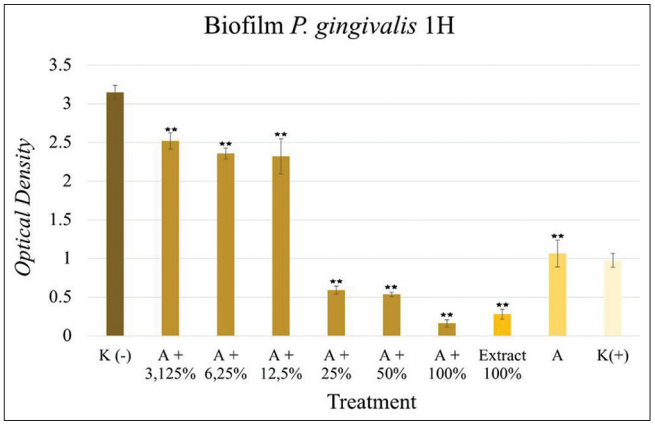

Figure 7: Graph of the average OD value of *Porphyromonas gingivalis* biofilm after 1 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

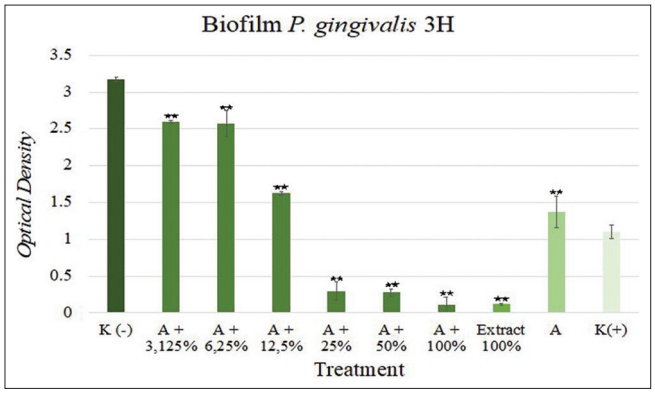

Figure 8: Graph of the average OD value of *Porphyromonas gingivalis* biofilm after 3 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

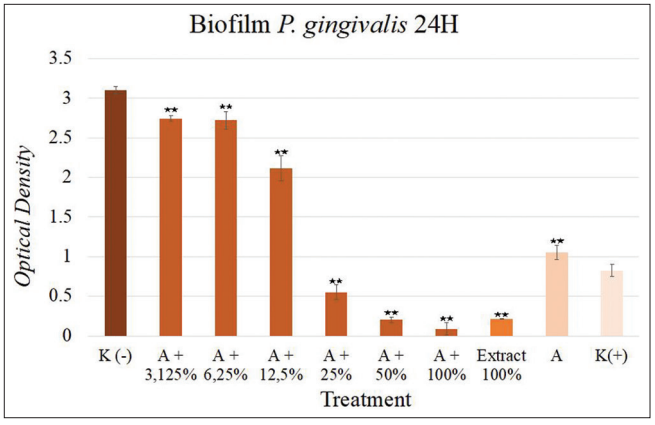

Figure 9: Graph of the average OD value of *Porphyromonas gingivalis* biofilm after 24 h of incubation (*$P < 0.05$, **$P < 0.01$). OD: Optical density

methanolic extract of *M. indica* L. leaves to clindamycin has antibacterial effects against *S. aureus*, especially at a concentration of 100%.[19] The bioactive component of *M. indica* L. leaves, namely, mangiferin, is known to have a synergistic effect on tetracycline, ampicillin, nalidixic acid, and trimethoprim in inhibiting the growth of *S. aureus*.[25]

The biofilm formation phase begins with pellicle formation, which occurs in the first few seconds to the first min from the initial contact, with the initial adhesion phase occurring 2–4 h later. After 24 h, the biofilm enters the maturation phase, becoming 1.000 - 1.500 times more resistant than planktonic bacteria.[29] The incubation time used in the antibiofilm test in this study was adjusted to the stage of biofilm formation, namely 1, 3, and 24 h. This timing aimed to determine at which stage of biofilm formation amoxicillin and the ethanolic extract of *M. indica* L. leaves

most effectively inhibited *S. aureus* and *P. gingivalis*. In the antibiofilm test, the OD from the combination of amoxicillin with ethanolic extract of *M. indica* L. leaves starting at a concentration of 25% after 1 h of incubation and 6.25% after 3 h of incubation had a significantly lower OD value compared to co-amoxiclav. This result proved that the ethanolic extract of *M. indica* L. leaves at a concentration of 25% after 1 h of incubation and 6.25% after 3 h of incubation was more effective at inhibiting *S. aureus* biofilms than co-amoxiclav. The OD values for the combination of amoxicillin and the extract at a concentration of 100% after 24 h of incubation were lower than those obtained for co-amoxiclav, but the difference was not statistically significant. Therefore, the combination of amoxicillin and an extract concentration of 100% has antibiofilm properties equivalent to those of co-amoxiclav after 24 h of incubation.

In terms of inhibiting *P. gingivalis* biofilm, the extract at a concentration of 25% and higher during the entire incubation period showed a more potent antibiofilm effect than that of co-amoxiclav. The combination of amoxicillin and the ethanolic extract of *M. indica* L. leaves inhibited the formation of *S. aureus* biofilms at a concentration of 6.25% after 3 h of incubation and *P. gingivalis* biofilms at a concentration of 25% after 1 h of incubation. This study supports the findings of previous research, which reported that ethanolic extract of *M. indica* L. leaves reduced *S. aureus* attachment and the number of *S. aureus* biofilms.[30]

This study has several limitations. First, the ethanolic extract used in this study was a crude extract. The use of a more refined extract, such as an extract exposed to extraction chromatography, would have resulted in an extract with fewer impurities. Second, only two of the many known oral pathogens were used in this study. Other oral pathogens can also be tested to expand the antibacterial activity of amoxicillin combined with *M. indica* L. leaves ethanolic extract. Further research is needed to determine the toxicity of this combination. Preclinical and clinical tests should also be conducted before the combination can be used as an alternative treatment for dentoalveolar abscesses and periodontitis.

Conclusions

Ethanolic extract of *M. indica* L. leaves contains alkaloids, saponins, tannins, phenolics, flavonoids, and steroids that have the potential to inhibit the growth and formation of *S. aureus* and *P. gingivalis* biofilms *in vitro*. Within the limitations of this preliminary study, we conclude that the addition of ethanolic extract of *M. indica* L. leaves to amoxicillin could potentially increase the antibacterial and antibiofilm properties of amoxicillin against *S. aureus* and *P. gingivalis*.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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