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INHIBITION OF *Asystasia gangetica* ETHANOLIC EXTRACT ON *Porphyromonas gingivalis* AND *Fusobacterium nucleatum* BIOFILM FORMATION

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ABSTRACT

Periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* develop a biofilm on the surface of the dental apex, causing inflammation in the teeth' supporting tissues. *Asystasia gangetica* has been observed for its analgesic, anti-inflammatory, and antimicrobial effect. The ethanolic extract of *A. gangetica* may be developed as a root canal sterilization dressing due to its benefits. This study aims to determine the inhibitory effect of ethanolic extract of *A. agangetica* on *P. gingivalis* and *F. nucleatum* from forming biofilm. Antibacterial activity was determined using Kirby-Bauer disk diffusion. The inhibition of biofilm formation was evaluated using microtiter plate biofilm assay with various concentrations of extract. Base on the classification of inhibition zone, ethanolic extract of *A. gangetica* had moderate to strong antibacterial effects on *P. gingivalis* at 12.5-50 mg/mL, and moderate effect on *F. nucleatum* at 25-50 mg/mL. Results also showed that the extract at 50 mg/mL, inhibited biofilm formation by more than 72% against *P. gingivalis* ($p<0.05$) and up to 75% against *F. nucleatum* ($p<0.05$). The value of $MBIC_{50}$ was determined at 5.19 mg/mL and 7.44 mg/mL, respectively. Ethanolic extract of *Asystasia gangetica* has a potential inhibitory effect on *P. gingivalis* and *F. nucleatum* biofilm formation. Studies suggest more detailed approaches to a better understanding of the interactions between lead compounds and bacterial cells within the biofilm structure.

Keywords: *Asystasia gangetica, Fusobacterium nucleatum, Porphyromonas gingivalis*

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INTRODUCTION

Porphyromonas gingivalis and *Fusobacterium nucleatum* exist in a biofilm formation on the surface of dental apex, which induced inflammatory response causing chronic periodontal disease (Newman et al., 2021; Tavares et al., 2018; Thurnheer et al.,

2019). *Porphyromonas gingivalis* and *Fusobacterium nucleatum* are anaerobic Gram-negative bacteria that have been identified as the major oral bacteria in sub-gingival and contribute to root canal infection pathogenesis (Dashper et al., 2010; Duran-Pinedo et al., 2014; Gigi et al., 2019; Tavares et al., 2018). The mechanisms of bacterial colonization of natural and artificial surfaces, as well as the surrounding periodontal tissues, including direct attachment to saliva proteins and epithelial cell receptors, as well as interactions with early bacterial colonizers (Atsushi Saito, 2008; Periasamy & Kolenbrander, 2009). Because of its vast ability to co-aggregate with other microorganisms, this bacteria was recognized as a crucial organism for dental biofilm formation.

Currently, one of periodontal disease treatment management is by doing rooting canal/ endodontic treatment that can be accomplished by eliminating all sources of infection furthermore antibiotics and anti-inflammatories are given as adjuvant therapy(Hammami & Nasri, 2021; Newman et al., 2021). Additionally, mouthwash, the main ingredient of which is chlorhexidine, can help to reduce plaque formation (Kumar, 2017; Van Winkelhoff et al., 1996). Unfortunately, long-term antibiotic treatment may develop in bacteria resistance; also, the adverse effects of chlorhexidine gluconate in mouthwash, such as taste problems, oral irritation, and local allergic symptoms, necessitate an innovative therapeutic approach (Kumar, 2017).

Plant extracts have been studied for alternative herbal therapy due to their low toxicity, minimal risk of side effects, and cheap cost. *Asystasia gangetica* (L.) T. Anderson, also known as Chinese violet, is one of the *Acanthaceae* plants noted for these features. *Asystasia gangetica* is a straggling herb that is commonly found among short grasses and along walkways in tropical areas (Suzuki et al., 2019). In Indonesia, *Asystasia gangetica* was found in oil palm and rubber plantations which was grouped as invasive weeds in agro-ecosystems. *Asystasia gangetica* is used traditionally for several different ailments, diseases, and was known to contain secondary metabolites (Akah et al., 2003; Janakiraman & Jeeva, 2014; Sama et al., 2014; Tillo et al., 2012). Previous studies have demonstrated that *Asystasia gangetica* extracts exhibit significant antibacterial activity against various pathogenic bacteria, including *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, indicating its potential as a natural antimicrobial agent (Pulungan, et al., 2023; Daffodil, et al., 2013; Hamid, et al., 2011). Ethanol was selected as the extraction solvent due to its effectiveness in extracting both polar and nonpolar bioactive compounds, including flavonoids and phenolic acids, which are responsible for the antimicrobial properties of medicinal plants (Alara, et al., 2021). The particular goals of this study were to measure the effectiveness inhibitory activity of ethanolic extract of *Asystasia gangetica* against *Porphyromonas gingivalis* and *Fusobacterium nucleatum* biofilm formation.

RESEARCH METHOD

This in vitro laboratory experimental study applied the post-test only control group design. The experimental protocols were approved by the Ethics Review Committee of the Faculty of Medicine, Jenderal Soedirman University. Extract of *Asystasia gangetica* was obtained from the Research Centre for Pharmaceutical Ingredients and Traditional Medicine BRIN, Indonesia. The ethanolic extract was prepared from fresh leaves of *Asystasia gangetica* using maceration method with 70% ethanol as the solvent. This

research used extract concentrations of 50 mg/mL, 25 mg/mL, 12.5 mg/mL, 6.25 mg/mL, and 3.125 mg/mL. A metronidazole antibiotic with concentration 30 mg/mL was used as a positive control. In contrast, 1% DMSO was used as a negative control. All experiments were performed in triplicate to ensure reproducibility of results. The bacterial used in the present study were *Porphyromonas gingivalis* (ATCC 33277) and *Fusobacterium nucleatum* (ATCC 25586). Each bacterial was cultivated on Mueller-Hinton Agar (MHA) plates (Thermo Fisher Scientific Inc, Oxoid, UK) in an anaerobic chamber at 37°C for 48 hours using an anaerocult® gas pack (Merck, Darmstadt, Germany). The colony was re-cultured in 10 mL of Mueller-Hinton Broth (MHB) (Thermo Fisher Scientific Inc, Oxoid, UK) under the same condition then transferred into aqueous medium and compared to McFarland 0.5 (1 x 10^8 CFU/mL), which was subsequently used for antibacterial testing.

Antibacterial activity

The antibacterial activity testing was done using Kirby-Bauer disk diffusion method with some modifications (Hudzicki, 2016). Briefly, blank paper disc was filled for 10 minutes with the extract with various concentration. The disc was placed in a space equidistant from each other into MHA plate with a streak of *P. gingivalis* and *F. nucleatum* in it gently touched with sterile forceps. Following the incubation for 24 hours, diameter of each zone of inhibition was estimated using a caliper with a precision of 0.05 mm.

Antibiofilm activity

The inhibition of biofilm formation was completed using microtiter plate biofilm (MtPB) assay, with some modifications (Kırmusaoğlu, 2019). Each culture was then diluted at 1:100 in a fresh MHB medium at room temperature. Each diluted culture was transferred amount 100 µl into individual wells of sterile 96-well flat-bottom polystyrene tissue culture-treated plates (Sigma Aldrich, Costar, USA) and 100 µl of ethanolic extract of *A. gangetica* was added into each well at various concentrations. They were further incubated in an anaerobic atmosphere for 24 hours at 37°C using an anaerocult® gas pack.

To eliminate non-adherent bacteria, each well was washed three times using Phosphate Buffer Saline (PBS) (Merck, Darmstadt, Germany). For staining, 200 µL of 1% crystals violet (CV) was added and incubated for 30 minutes. Furthermore, each well was washed three times with PBS after the excess CV solution was removed. The CV were extracted by soaking bacteria in 100 µL of 5% acid isopropanol for 15 minutes at room temperature. Finally, the optical density (OD) at 590nm (A_{590}) of the stained adherent bacterial biofilms were quantified using a microplate reader (Bio-Rad Laboratories Inc., CA, USA) and analyzed for percentage of inhibition of biofilm formation and Minimum Biofilm Inhibition Concentration ($MBIC_{50}$).

Data analysis

The significance of differences between the groups was analyzed using a One-Way analysis of variance (ANOVA). The *p*-value of <0.05 with a 95% confidence interval was considered statistically significant. Minimum Biofilm Inhibition Concentration ($MBIC_{50}$) was defined as the lowest concentration required to inhibit 50% of biofilm formation and was then analyzed using linear regression.

RESULTS AND DISCUSSION

The antimicrobial activity of *A. gangetica* ethanolic extract against planktonic *P. gingivalis* and *F. nucleatum* was evaluated using Kirby Bauer method. As shown in Table I, *A. gangetica* ethanolic extract exhibited moderate to strong growth inhibition against both bacteria pathogens in a concentration-dependent manner. At 50 mg/mL concentration, extract generated an inhibition zone of 15.58±0,41 mm against *F. nucleatum,* indicating strong antimicrobial effects. In contrast, only moderate inhibition (zone of 9.22±0.30 mm) was observed against *P. gingivalis* at the same concentration. No growth inhibition was detected at extract concentration below 12.5 mg/mL for both bacteria.

Table 1. Inhibitory zones of *Asystasia gangetica* on the growth of *P. gingivalis* and *F. nucleatum*, as well as their resistance response

Isolates	**Extract concentration (mg/mL)**	**Diameters of inhibition zones (mm)**	**Classification inhibition response**(Davis & Stout, 1971)
P. gingivalis	50	9.22±0.30	Moderate
	25	6.14±0.36	Moderate
	12.5	0	n/a
	6.25	0	n/a
	3.125	0	n/a
F. nucleatum	50	15.58±0.41	Strong
	25	12.77±0.38	Moderate
	12.5	7.25±0.46	Moderate
	6.25	0	n/a
	3.125	0	n/a

To examined the efficacy of minimum inhibitory concentration of *A. gangetica* ethanolic extract in inhibiting biofilm formation by *P. gingivalis* and *F. nucleatum* using a microtiter plate biofilm assay. As shown in Table II and Figure 1, the extract significantly inhibited biofilm formation in a dose-dependent manner compared to the vehicle control for both bacteria. At 50 mg/mL concentration, extract inhibit 72.06% and 76.74% of biofilm formation by *P. gingivalis* and *F. nucleatum*, respectively. At the lowest concentration (3.125 mg/mL), extract inhibited biofilm formation significantly by 22.37% for *P. gingivalis* and 31.80% for *F. nucleatum* compared to the 1% DMSO as control.

Table 2. Biofilm inhibition upon treatment with *Asystasia gangetica*

Extract concentration	**Inhibition Biofilm (%)**	
(mg/mL)	***P. gingivalis***	***F. nucleatum***
50	72.06	76.74
25	66.69	73.18
12.5	57.63	66.22
6.25	44.32	55.93
3.125	22.37	31.80
Metronidazole 30 mg/mL	81.32	86.32
DMSO 1%	3.79	4.51

The $MBIC_{50}$ values, defines as the minimum biofilm inhibitory concentration required to inhibit 50% of biofilm formation, were determined by linear regression analysis. A lower $MBIC_{50}$ indicates greater potency. Extract was slightly more potent againt *P. gingivalis* biofilms, with an MBIC50 of 5.19 mg/mL compared to 7.44 mg/mL for *F. nucleatum* (Figure 1). However, the $MBIC_{50}$ values were relatively close, varying by only 2,25 mg/mL between the two bacteria.

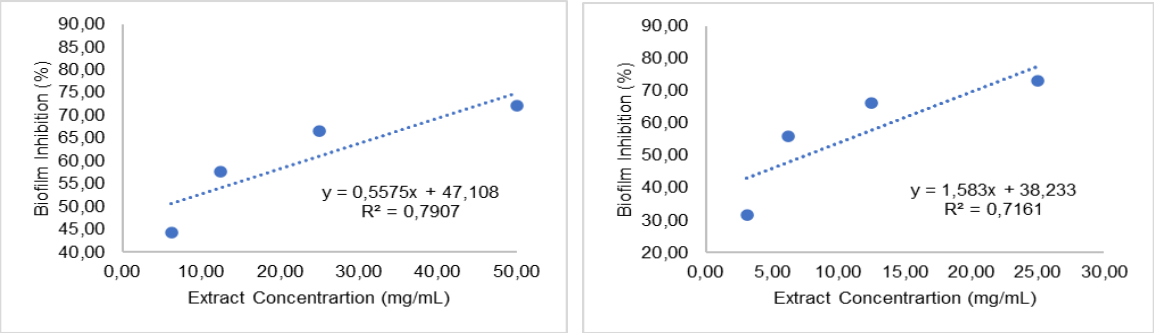

Figure 1. Linear regression curve for $MBIC_{50}$ determination of ethanolic extract of *A. gangetica* against *P. gingivalis* and *F. nucleatum* biofilm formation using MtPB assay. $MBIC_{50}$ of *P. gingivalis* was 5.19 mg/mL and $MBIC_{50}$ of *F. nucleatum* was 7.44 mg/mL.

This study investigated the inhibitory effects of As*ystasia gangetica* ethanolic extract on the growth and biofilm formation of two key oral pathogens, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*. This study demonstrate that the extract exhibits moderate to strong antimicrobial activity against both planktonic bacteria and significantly inhibits their biofilm formation in a dose-dependent manner.

The antimicrobial activity of *A. gangetica* extract against planktonic *P. gingivalis* and *F. nucleatum* showed varying degrees of effectiveness. At 50 mg/mL, the extract demonstrated strong inhibition against *F. nucleatum* (15.58±0.41 mm inhibition zone) but only moderate inhibition against P. gingivalis (9.22±0.30 mm inhibition zone). This differential susceptibility aligns with recent findings by Karygianni et al. (2020), who reported varied antimicrobial efficacy of natural extracts against different oral pathogens. However, our results show stronger inhibition compared to those reported by Santos et al. (2019) for *Eugenia uniflora* L. extract against the same bacterial species, suggesting potentially higher antimicrobial potency of *A. gangetica*.

Regarding biofilm inhibition, results revealed that *A. gangetica* extract significantly reduced biofilm formation by both *P. gingivalis* and *F. nucleatum*, with maximum inhibition rates of 72.06% and 76.74%, respectively, at 50 mg/mL. These findings are particularly promising when compared to a study by Chen et al. (2021), where a novel synthetic compound achieved maximum biofilm inhibition rates of 65% against *P. gingivalis* at similar concentrations. The potent anti-biofilm activity of *A. gangetica* extract, even at lower concentrations, suggests its potential as a natural alternative for oral biofilm control.

This study found that *A. gangetica* extract was slightly more effective in inhibiting *P. gingivalis* biofilm formation (MBIC50 = 5.19 mg/mL) compared to F. *nucleatum* (MBIC50 = 7.44 mg/mL), despite showing stronger antimicrobial activity against planktonic *F. nucleatum.* This discrepancy highlights the complex nature of biofilm

formation and the potential for differential mechanisms of action against planktonic and biofilm-associated bacteria. Similar observations were reported by Liu et al. (2022) when studying the effects of quercetin on oral biofilms, emphasizing the importance of evaluating both planktonic and biofilm forms when assessing antimicrobial agents.

The potent antimicrobial and anti-biofilm activities of *A. gangetica* extract observed in this study likely stem from the synergistic effects of various bioactive compounds present in the plant. While the exact mechanisms remain to be elucidated, recent research on plant-derived antimicrobials provides insights into potential modes of action. Polyphenols, flavonoids, and terpenes, which are commonly found in plant extracts, have been shown to disrupt bacterial cell membranes, inhibit essential enzymes, and interfere with quorum sensing systems (Slobodníková et al., 2016). Karygianni et al. (2021) demonstrated that epigallocatechin gallate (EGCG), a polyphenol found in green tea, can inhibit the growth of oral pathogens by binding to peptidoglycan, thereby compromising cell wall integrity. It is plausible that similar compounds in *A. gangetica* extract may exert comparable effects on *P. gingivalis* and *F. nucleatum*, explaining the observed growth inhibition of planktonic cells.

Regarding biofilm inhibition, the mechanisms may be more complex and multifaceted. The ability of *A. gangetica* extract to inhibit biofilm formation at concentrations lower than those required for planktonic growth inhibition suggests that the extract may interfere with specific biofilm-associated processes. Recent work by Rumbaugh et al. (2022) has shown that certain plant-derived compounds can disrupt extracellular polymeric substances (EPS) production, a critical component of biofilm matrix. Additionally, some phytochemicals have been found to downregulate genes associated with adhesion and biofilm formation. Liu et al. (2023) reported that baicalein, a flavonoid compound, inhibited *Streptococcus mutans* biofilm formation by suppressing gtfB, gtfC, and gtfD genes, which are crucial for extracellular polysaccharide synthesis.

This study provides evidence that *A. gangetica* ethanolic extract has potential inhibitory activities against the periodontal pathogens *P. gingivalis* and *F. nucleatum*. Further studies needed to identify the active compounds responsible for the antibacterial properties, as well as the potential synergistic effects of the bioactive components. These findings emphasize *A. gangetica's* potential as a novel therapeutic alternative in the management of periodontal diseases.

CONCLUSION

Based on the results of the research that has been done, it can be concluded that the ethanolic extract of *Asystasia gangetica* has the ability to inhibit the growth of *Porphyromonas gingivalis* and *Fusobacterium nucleatum.*

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