Original Article

Evaluation of 5-Aminolevulinic acid, Methylene Blue, Toluidine Blue O Photosensitizers on Clinically Isolated *Porphyromonas gingivalis* and *Prevotella intermedia*: An *in vitro* Study

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Abstract

Objective: To evaluate the antibacterial efficacy of 5-aminolevulinic acid (5-ALA), methylene blue (MB), and toluidine blue O (TBO) photosensitizers against clinically isolated *Porphyromonas gingivalis* (*P. gingivalis*) and *Prevotella intermedia (P. intermedia)*.

Methods: Patients with a periodontal pocket depth of ≥5 mm were selected for subgingival plaque sampling. Bacterial identification was carried out using colony morphology, gram staining and then validated using the PCR technique. The photosensitizers tested were 5-ALA, MB, and TBO. The antibacterial test performed using agar well, disk diffusion and double-fold serial dilution methods for determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC).

Results: Glossy, smooth, and looked grey, light brown, or black-pigmented colonies of *P. intermedia* on Columbia agar plates were confirmed by gram staining and PCR tests. Contrary to MB and TBO, 5-ALA photosensitizers didn't have antibacterial efficacy against *P. gingivalis* and *P. intermedia*. The antibacterial test showed an increased inhibition zone with increasing the photosensitizers' concentration. The greatest inhibition zones were at 10mg/ml concentration for both MB and TBO. The MIC for MB was 2 mg/ml, and 1mg/ml, while for TBO, it was 1mg/ml and 0.5mg/ml for *P. gingivalis* and *P. intermedia*, respectively.

Conclusions: *MB and TBO have antibacterial properties against P. gingivalis and P. intermedia, and their efficacy increase with increasing concentration. TBO is more effective when compared to MB. Furthermore, P. intermedia is more sensitive to photoactivation in comparison to P. gingivalis.*

Keywords: *Antibacterial effect, Methylene blue, Porphyromonas gingivalis, Prevotella intermedia, Toluidine blue O, 5-aminolevulinic acid.*

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Introduction

Dental plaque is a main etiological factor for periodontal diseases (PDs). It develops over time, initially supragingival, with a mature subgingival plaque[1]. As the plaque accumulates, several periodontopathogens are colonized, such as *P. gingivalis* and *P. intermedia*[2-4]. The bacterial plaque would induce host response which plays a major role in tissue destruction[5,6]. *P. gingivalis* is a potential periodontal infection known to penetrate the oral epithelial cells. Therefore, the invasion may be a characteristic of virulent bacteria associated with periodontitis. It can interact with other microorganisms to create bacterial communities, colonizing and forming dental plaque. Due to its damaging effects on periodontal tissues, *P. gingivalis* benefits from its interaction and coaggregation in the subgingival plaque[7]. In addition, *P. gingivalis* can alter the host's immune response to foster the persistence of both itself and other infections[8]. Oral microbial dysbiosis, plaque formation, and coaggregation are all significantly influenced by virulence factors of *P. gingivalis.* They have the ability to either directly affect other bacteria or create an ideal habitat[9].

An essential stage in the pathophysiology of many illnesses is epithelial cell invasion. Bacteria's capacity for intracellular survival permits them to avoid immune system recognition and perhaps even spread. It has been shown that these infections' pathogenicity depends on their survival capacity inside the host cell. For example, the clinical isolates of *P. intermedia* from a human periodontal pocket invade oral epithelial cells. This invasion was mainly due to the type of fimbriae the bacteria express on the cell surface[4]. Thus Periodontitis can cause tissue destruction and results in signs such as bleeding upon probing, periodontal pockets, clinical attachment loss, bone destruction, and ultimately tooth loss[10].

Periodontal treatment aims to remove supra- and subgingival plaque to eliminate the bacteria that cause PDs[11]. Non-surgical mechanical debridement eliminates the etiologic factor on the tooth and root surfaces. Therefore, it is considered the gold standard for cause-related therapy in patients with PDs[12]. However, mechanical therapy does not remove periodontal pathogens since bacteria may survive in radicular cementum and dentinal tubules or spread from reservoirs inside the mouth to periodontal sites[6]. In addition, several anatomical locations, such as deep pockets, concavities, or furcation zones, can also limit the effectiveness of non-surgical therapy[13].

Adjunctive agents such as local or systemic antibiotics have been used in mechanical therapy to address these issues. These adjunctive agents have been shown to help eradicate pathogens still present after mechanical debridement[14]. However, systemic antibiotic administration has raised significant concerns about their side effects and developing bacterial resistance[15].

Antibacterial photodynamic therapy (aPDT) is a promising treatment option for antibiotic-resistant bacterial infections. aPDT is a bacterial eradication method based on the photosensitization of bacteria with exogenous compounds called photosensitizers (PS)[16]. aPDT is based upon the concept that bacteria can preferentially take up a PS or photoactivatable agent that absorbs light. Furthermore, exposure to visible light in the presence of molecular oxygen generates singlet oxygen and free radicals that are cytotoxic to microorganisms[17]. Therefore, adjunctive agents could be considered to enhance the outcomes of non-surgical periodontal therapy.

The aim of this *in vitro* study is to determine the efficacy of toluidine Blue O (TBO) and methylene blue (MB), and five aminolevulinic acids (5-ALA) as photodynamic therapies against clinically isolated *P. gingivalis* and *P. intermedia*.

Materials and methods

Isolation of the bacterial strains

The Ethics Committee approved the study of the College of Dentistry of the University of Sulaimani (ethical approval number: 55/21on 3rd November 2021). The patients' consent and approvals were obtained before collecting the samples. Four healthy patients with periodontitis and periodontal pocket depth of 5 mm or more, untreated for at least six months, were selected for subgingival plaque sampling. Cotton rolls were used for the isolation of selected periodontal pockets. First, a sterile periodontal curette removes any present supragingival plaque and calculus. Later, a sterile paper point (ISO 40) was carefully introduced into the periodontal pocket until tissue resistance was felt and then kept in position for 60 seconds.

The paper point was carefully removed and streaked over Columbia agar plates. The media ingredients per 500 ml were as follows: Columbia agar base (21.5%), 5 µg/ml (1.5mg) of hemin, 1µg/ml vitamin k1 (0.5mg), 5% human blood (25 ml), and distilled water. Inoculated plates were incubated for 7–10 days at 37°C in an anaerobic environment induced by Oxoid Anaerogen (Leicestershire, England) and an anaerobic jar (BBL® GasPak system).

Identification and isolation of Bacterial strains

P. gingivalis was obtained from the stored bank of the College of Veterinary, University of Sulaimani. The samples were stored at -80°C, identified, and preserved from a previous study[18]. While *P. intermedia* identification was carried out using colony morphology, black pigmentation, gram staining, and anaerobic control and then validated using the PCR technique.

DNA extraction and molecular identification

A colony was extracted from each sample and mixed with 50 µl of sterilized ultra-pure deionized distilled water (ddH_2O) in a 0.2ml microcentrifuge tube, stirred thoroughly until homogenized (vortex mixer), and then incubated for 10 minutes at 95°C in the heat block. The samples were centrifuged after that, and the supernatant DNA was utilized as a template[19].

DNA amplification was performed using modified universal primers targeted at the 16s rRNA gene[20].

5' – AGAGTTTGATCCTGGCTCAG-3' Forward primer

5' – GTATTACCGCGGCTGCTG - 3' Reverse primer

The PCR was carried out in a final volume of 20 µl (including 2 µl of reverse primer (10pmol/l), 2µl of forwarding primer (10pmol/l), 10 µl of Add Start Taq Master (2x conc., South Korea), 1µl of nuclease-free water, and 5 µl of DNA template). The DNA sample was denatured for 10 minutes at 95°C for one cycle. The amplification step was then repeated 35 times (denaturation of the DNA template at 95°C for 30 seconds, annealing of the modified universal primer at 65°C for 30 seconds, and primer extension at 72°C for 30 seconds). The final extension was performed in the thermocycler PCR for 5 minutes at 72°C for one cycle.

The PCR product was analyzed on 2% agarose gel electrophoresis at 80V for 35 minutes. Three µl ethidium bromide was used to stain the gel. A 100 bp plus DNA ladder was used as a molecular weight marker. Gel purification was performed for the bands by using the gene JET™ Gel extraction kit (Fermentas UK)[21].

Photosensitizer's stock preparation

To attain the necessary concentration of 0.5, 1, 5, and 10 mg/ml, stock solutions of both TBO and MB (Biochem, France) were produced in sterile phosphate buffer saline (PBS) (pH 7.2) before the experiment. At the same time, 5-ALA (Sigma-Aldrich, Steinheim, Germany) stock solutions of concentrations 10, 15, 20, and 25mg/ml were prepared again with PBS solution. All were then filtered using 0.22 µm-pore-size membrane filters (CHMLAB group, Terrassa, Barcelona, Spain) and kept in the dark at 4°C. The light source in this experiment was a diode emitting light of a wavelength of 635 nm with a measured output of 1200 mW/cm^2.

Agar disk-diffusion test method

In this procedure, Mueller-Hinton (MH) agar plates were inoculated with a standardized inoculum of 100µl of the tested microorganism (*P. gingivalis* and *P. intermedia*), using 0.5 MacFarland spectrophotometry standardization (1.5 X 10^8) colony forming units (CFU/ml). Then, filter paper discs of Whatman® filter paper (Sigma-Aldrich, Steinheim, Germany) of 6mm in diameter containing the photosensitizer (TBO, MB, or 5-ALA) at the desired concentration, as mentioned previously, were placed on the agar surface. After 1 minute in the dark condition, the bacteria were irradiated with the red light emitting for one minute. Then the Petri dishes are incubated under anaerobic conditions for 24 hours at 37°C. The diameters of inhibition growth zones were measured. Chlorhexidine (CHX) was used as a positive control.

Agar-well diffusion method

The MH agar plate surface was inoculated by dispersing microbial inoculum (*P. gingivalis* and *P.intermedia*) across the whole agar surface, similar to the approach employed in the disk-diffusion method. A sterile micropipette tip, a hole with a diameter of 6 mm was punched and 60µl of the photosensitizers (MB, TBO, and 5-ALA) at the specified concentrations added into the well; after 1 minute in the dark condition, the bacteria were irradiated with the red light emitting for one minute. The agar plates were then incubated in an anaerobic condition.

MIC and MBC determination

The phenothiazine PSs (MB and TBO) solutions stocks of 16mg/ml were prepared by dissolving it in PBS (pH 7.2) and passing the solution through 0.22 µm micropore filters. The PSs were serially diluted using a two-fold serial dilution method arranged as 8, 4, 2, 1.0, 0.5,0.25, and 0.125 mg/ml in seven test tubes. Each test tube contained 900µl of the Muller-Hinton broth media, and 1000µl of the PS (MB or TBO) was added to the first test tube then double-fold serial dilution was performed. Hence, each tube contains 900µl/ml of PS and broth mixture. Finally, the bacterial suspension was adjusted on 0.5 McFarland standard (1.5 x10^8 CFU/ml), and each of those tubes was inoculated with a 100 µl of the adjusted bacterial strains (either *P.gingivalis* or *P. intermedia)* to have a total volume of 1000 µl/tube. CHX 0.12% was used as a bactericidal (negative control, no bacterial growth), and a broth solution allowed bacterial growth (positive control).

The tubes were sealed with cotton and vortexed for 30 seconds for proper mixing of the bacteria and the photosensitizers and left in the dark for two minutes to let the photosensitizers be absorbed by the bacterial strains(22). Later, each tube was irradiated with a red light-emitting diode (635nm) for one minute. Finally, the test tube was incubated for 24 hrs at 37.5°C in an anaerobic jar using Anaeropack-Anaero and checked for turbidity (bacterial growth). The tubes with no turbidity were considered MIC, and a concentration above the MIC is considered MBC, where there is no bacterial growth(23). These tests were performed in triplicate and later confirmed by culturing the tubes' content on the MH agar plates.

Statistical analysis

Mean and the standard deviation is used to present the descriptive statistics. The normality of the data was checked using a Shapiro-Wilk analysis. The t-test for independent samples was performed to compare two means of the normally distributed groups, While the non-parametric data were compared using the Mann-Whitney U test. All groups' levels of significance (p-values) were set at 0.05 for all statistical analyses. Statistical Package for Social Sciences (SPSS) Version 25 was employed to conduct the descriptive and inferential analysis.

Results

Bacterial isolation and identification

In the current study, bacterial colonies were found in the glossy, smooth, and looked grey, light brown, or black-pigmented colonies on Columbia agar plates. These colonies were morphologically apparent after 7-10 days of bacterial culturing on the supplemented Columbia agar (Figure1 A and B). Later, the gram staining procedures, under X100 power, showed gram-negative, rod-shaped bacteria that may assume coccobacilli forms, as shown in Figure1 C and D. DNA sequencing showed purified colonies of *P. intermedia*, as shown in Figure 2.

Antibacterial susceptibility tests

5-aminolevulinic acid

The results of the photoactivation of the PSs (test groups) and the CHX (control group) against both bacterial species applied on the MH agar media showed that the 5-ALA group didn't show any inhibition zone up to 25mg/ml concentration for both disk diffusion and agar-well methods. However, both MB and TBO show inhibition zones (Figures 3 and 4).

Methylene blue

The results of both agar-well and disk diffusion tests of MB against *P. gingivalis* show statistically significant differences compared to the CHX control group (except 5mg/ml MB concentration in the disk diffusion test). In addition, the inhibition zone's diameter increased with increasing the MB concentration of up to 10mg/ml. The inhibition zones for the agar well and disk diffusion tests were 12.2 and 14.03 mm, respectively (Figure 3 and Table 1).

Regarding the results of the agar well and disk diffusion test of MB against *P. intermedia*, the result show statistically significant differences compared to the control group, except 10mg/ml group for the agar well diffusion and 5 mg/ml group for the disk diffusion tests where the result was comparable to the positive control. The maximum inhibition zones were 12.0 and 14.3 mm for agar well and disk diffusion tests, respectively (Figure 4 and Table 1).

Toluidine Blue O

Similarly, the results of the agar well and disk diffusion test of TBO against *P. gingivalis* showed a statistically significant difference compared to the control group except in 10 mg/ml concentration in the agar well diffusion test and the 5 mg/ml group for the disk diffusion tests. In addition, the results showed an increase in the inhibition zones' diameter with TBO concentration. The greatest diameters were 12.2 and 16.3 mm inhibition zones for agar well and disk diffusion tests, respectively (Figure 3 and Table 1).

Likewise, the results of the agar well and disk diffusion test of TBO against *P. intermedia* bacteria show statistically significant differences except in the 1mg/ml group for both the agar well and disk diffusion tests. The largest inhibition zones were 16.13- and 17.1-mm inhibition zones for agar well and disk diffusion tests, respectively (Figure 4 and Table 1).

The minimum inhibitory concentration of MB and TBO for P. *gingivalis* and *P. intermedia*

The MIC test of the PS was determined using the macro broth dilution test. In addition, PS' antibacterial activity was evaluated against both isolated bacterial species. The MICs for the MB against both *P. gingivalis* and *P. intermedia* were 2mg/ml and 1mg/ml, respectively. While the MICs for the TBO photosensitizer against *P. gingivalis* were 1mg/ml, and for *P. intermedia*, they were 0.5mg/ml. (Figure 5 A and B).

MBC was determined by selecting the concentration that revealed no bacterial growth during the MIC assessment. The MBC for the MB was 4mg/ml and 2mg/ml, While the MBC for the TBO was 2mg/ml and 1mg/ml against *P. gingivalis* and *P. intermedia*, respectively. (Table 2 and Figure 5 A and B).

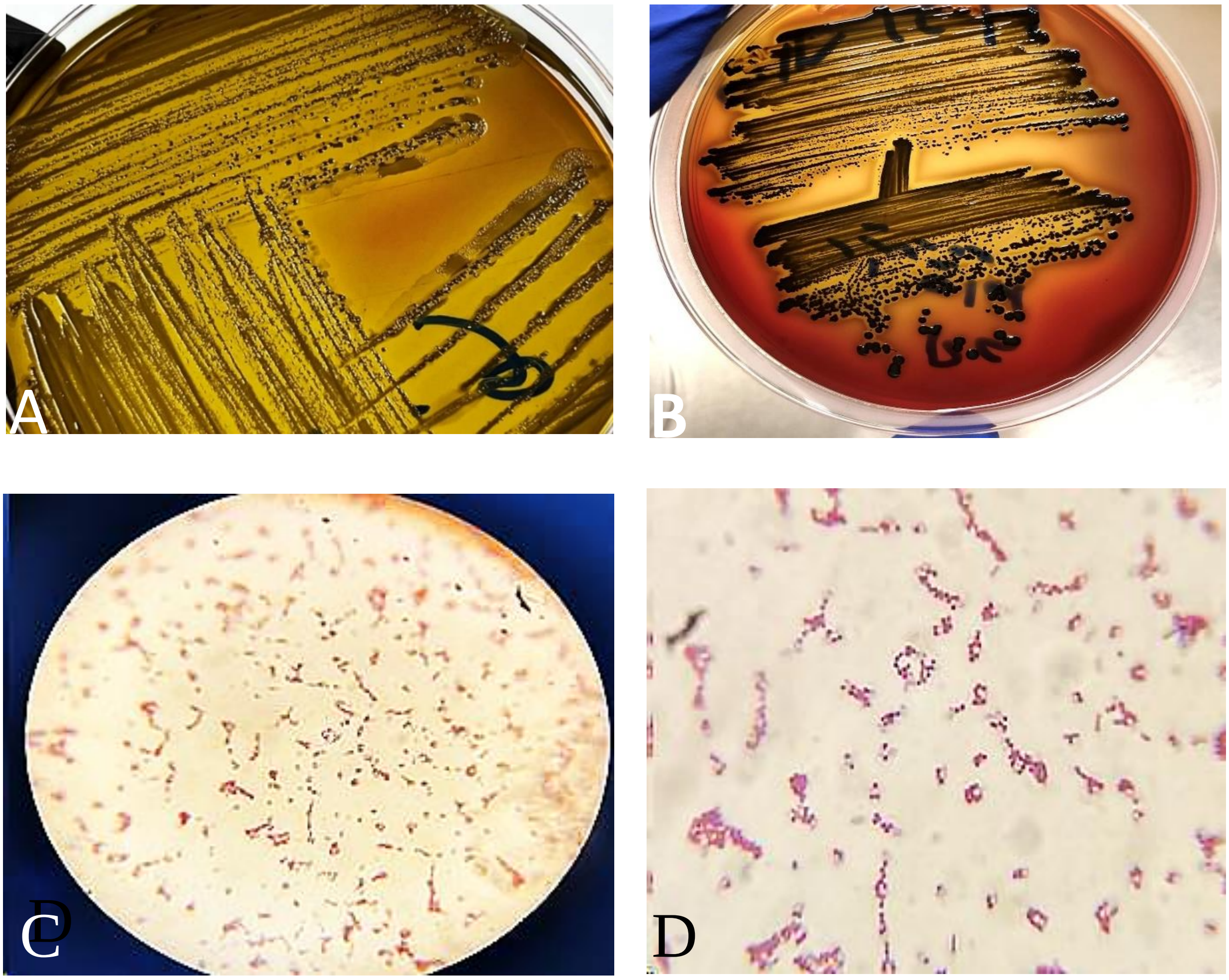

Figure 1: A. Isolated bacterial colonies of *P. intermedia* after 7 days. B. Revised bacterial colonies of *P. gingivalis* after 7 days C. Microscopic feature of the short rods of *P. intermedia* D. Magnified short rod-shaped *P. intermedia* colonies.

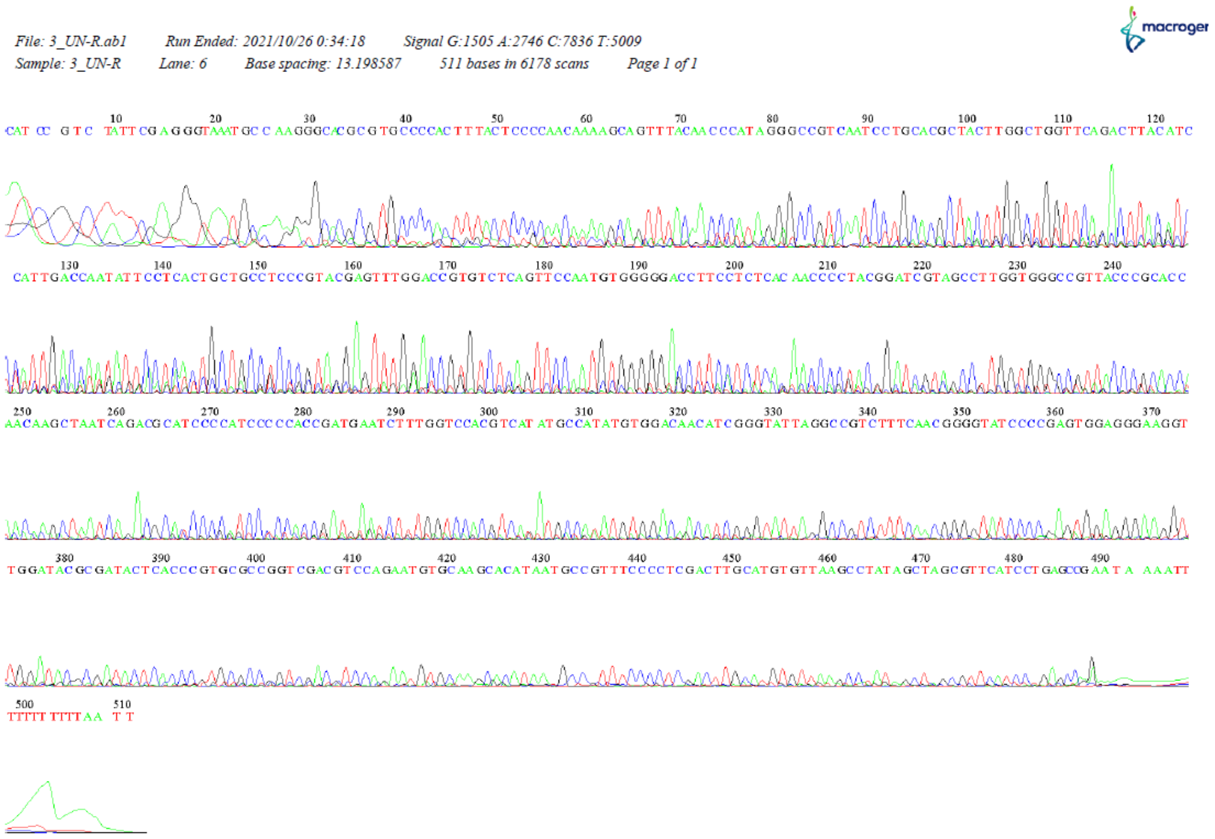

Figure 2: DNA sequencing results of the purified colony of *P. intermedia* conducted in Macrogen, South Korea.

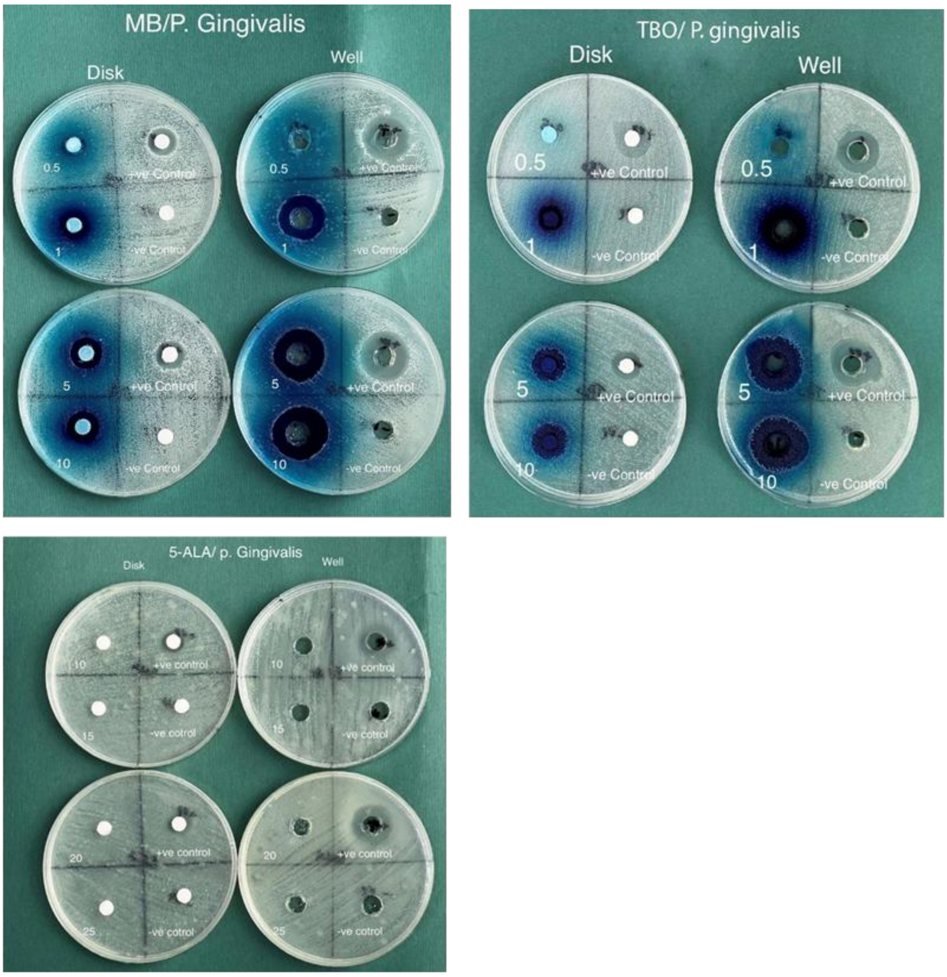

Figure 3: Result of antibacterial susceptibility test for MB, TBO and 5-ALA against *P. gingivalis* by both agar well and disk diffusion methods.

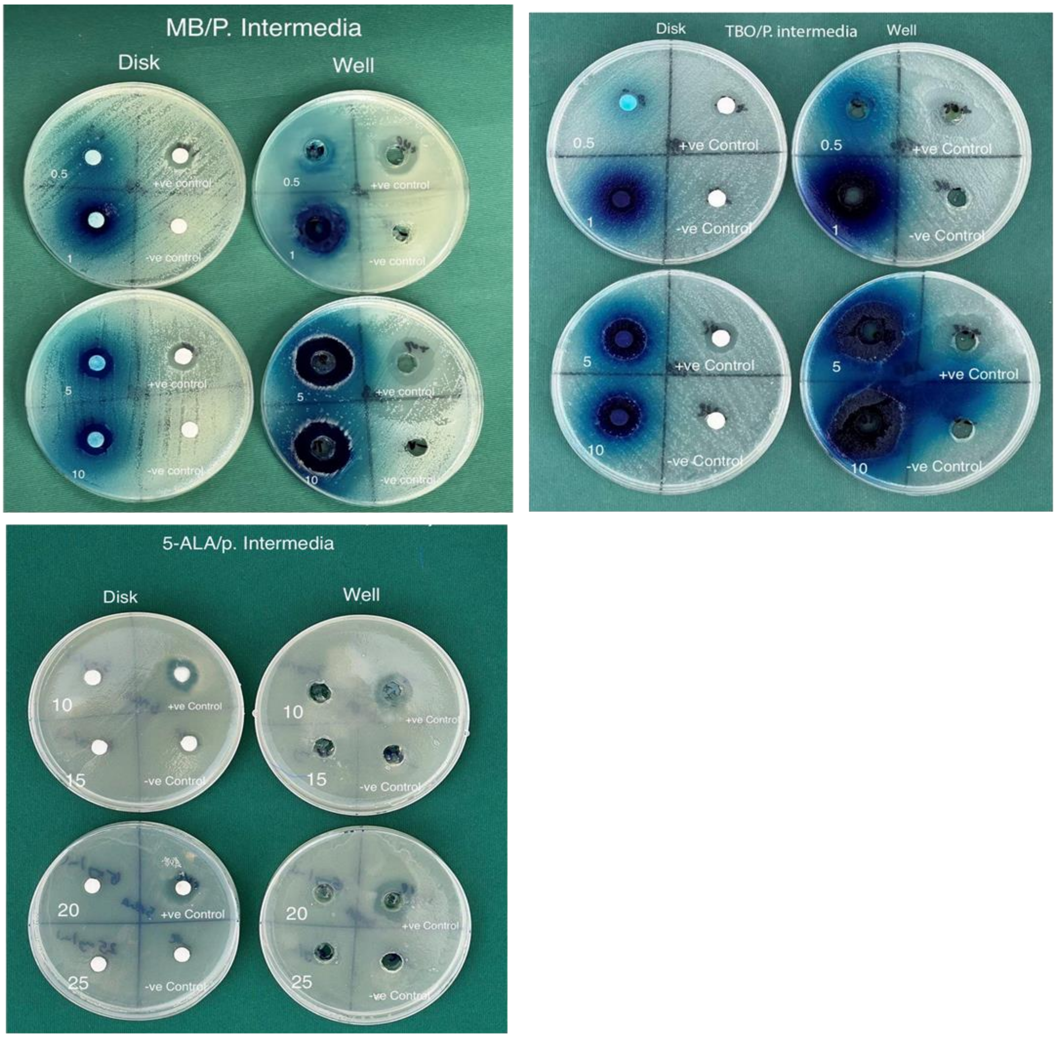

Figure 4: Result of antibacterial susceptibility test of all of MB, TBO and 5-ALA against *P. intermedia* by both agar well and disk diffusion methods.

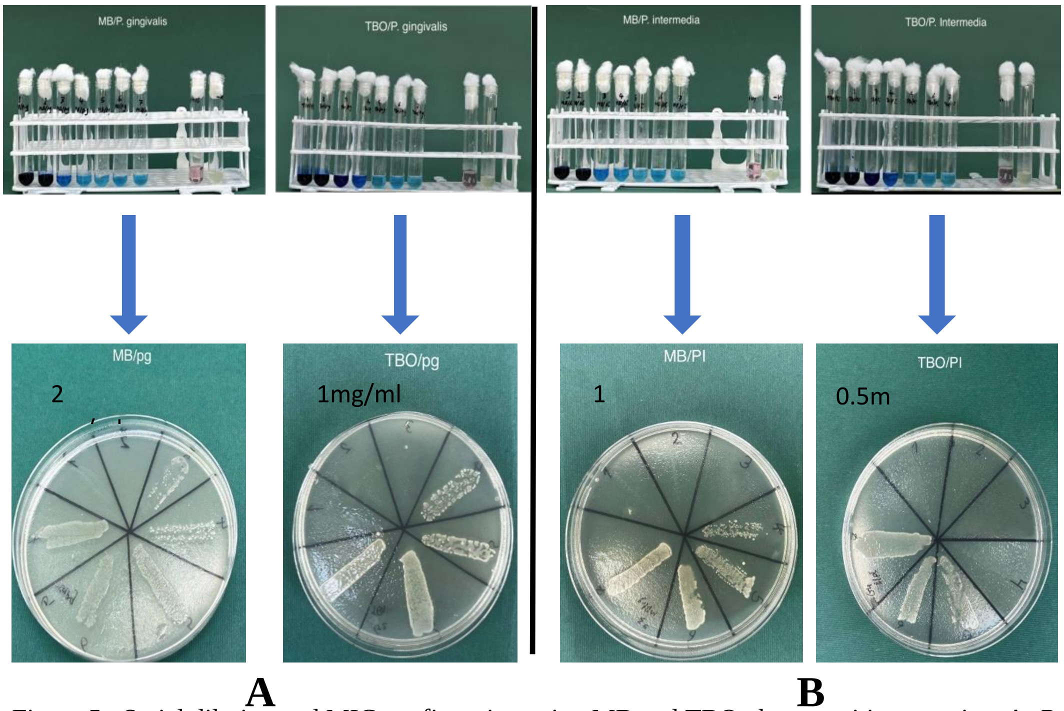

Figure 5: Serial dilution and MIC confirmation using MB and TBO photosensitizers against A. *P. gingivalis.* B. *P. intermedia*

Table 1: Agar well and disk diffusion tests for MB and TBO PSs against *P. gingivalis* and *P. intermedia*.

PS	Co	Positive control *(P.g)* CHX (Mean±SD)		*P. g* (Mean±SD)		p-value *(P.g Vs. CHX)*		Positive control *(P.i)* CHX (Mean±SD)		*P. I* (Mean±SD)		p-value *(P. i)*	
		Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk	Well	Disk
MB	0.5	1.1±0.28	12.2±0.3	0.00±0.00	0.00± 0.00	0.034	0.034	10.8±0.2	13.2±0.2	0.00±0.00	0.00±0.00	0.000	0.000
	1			9.1± 0.28	10.3± 0.28	0.043	0.043			9.5±0.50	11.3±0.26	0.014	0.001
	5			11.3±0.28	12.4± 0.20	0.043	0.369			11.5±0.50	13.4±0.20	0.088	0.288
	10			12.2±0.20	14.03± 0.05	0.046	0.043			12±1	14.3±0.10	0.111	0.001
TBO	0.5	2.07±0.12	14.33±1.52	0.00±0.00	0.00± 0.00	0.034	0.000	11.9±0.1	13.1±0.23	6.16±0.28	8.03±0.05	0.046	0.043
	1			9.83±0.76	12± 0.5	0.046	0.066			11.9±0.10	12.9±0.11	1	0.197
	5			11.4±0.20	15.16±0.28	0.046	0.507			14.43±0.40	16.06±0.11	0.000	0.043
	10			12.2±0.26	16.3± 0.28	0.487	0.072			16.13±0.23	17.1	0.046	0.043

Co: Concentration, P. g; P. gingivalis, P. i: P. intermedia, CHX: Chlorhexidine.

Table 2: MIC and MBC results for MB and TBO PSs against tested bacteria P. gingigvalis and P. intermedia.

Tested bacteria	MB		TBO	
	MIC	MBC	MIC	MBC
P. gingivalis	2mg/ml	4mg/ml	1mg/ml	2mg/ml
P. intermedia	1mg/ml	2mg/ml	0.5mg/ml	1mg/ml

Discussion

Scaling and root planing are the gold standard treatment for periodontal disease. However, the treatment outcome varies between patients and sites in the same patient. Hence, adjunctive antimicrobial therapy is prescribed in certain situations[11,12,14]. Furthermore, owing to side effects and susceptibility to bacterial resistance, other adjunctive agents, such as PS, may address these issues[16,17]. Therefore, this study aimed to evaluate the efficacy of TBO, MB, and 5-ALA as photodynamic therapy against clinically isolated *P. gingivalis* and *P. intermedia*.

P. gingivalis and *P. intermedia* belong to the red and orange complexes associated with periodontal diseases and periodontitis in particular[4,7,9]. Thus, these bacteria isolated from clinical samples were evaluated. To the best of our knowledge, this is the first time that TBO, MB, and 5-ALA were examined against clinically isolated *P. gingivalis* and *P. intermedia*.

The result of this study shows that 5-ALA, either by disk diffusion or agar well diffusion tests, did not have antibacterial effects against examined species. These results are in line with another study examining the effects of 5-ALA against gram-negative bacteria[24].

On the contrary, other studies showed that 5-ALA PS is effective against numerous Gram-positive and Gram-negative bacteria, parasites, and fungi[25,26]. The efficiency of 5-ALA is determined by various parameters, including the nature of bacteria cell walls, cell membrane transport mechanism, pH value, concentration, and time of PS exposure[25-27]. This might explain the ineffectiveness of 5-ALA in the current study. Taking that into consideration, the MIC and MBC tests were not conducted for 5-ALA as the maximum dose tested in both disk and agar well diffusion (25mg/ml) did not yield any inhibition zone against both *P. gingivalis* and *P. intermedia*.

Both MB and TBO are effective against *P. gingivalis* and *P. intermedia*. However, in both the agar well and disk diffusion tests, the results consistently reveal that MB and TBO application in a low concentration of 0.5mg/ml against *P. gingivalis* did not induce any inhibitory zones. Furthermore, with increasing PSs concentrations, greater inhibitory zones were produced compared to CHX.

The results of the current study demonstrated that *P. intermedia* has shown to be more sensitive to both MB and TBO photosensitizers when compared to the *P. gingivalis*; the MIC test results further confirm this. This result agrees with the previous studies, which demonstrated that the *P. intermedia* was more sensitive to the catastrophic effects of the ROS species when compared to the *P. gingivalis*(28,29). Additionally, bacteria might produce enzymes needed to balance the redox inside cells and eliminate excess ROS through redox metabolism(28). It was revealed that *P. intermedia* encoding genes lack the superoxide dismutase (SOD) gene; this means that *P. intermedia* may have fewer key ROS scavenging enzymes and poorer ROS removal capabilities than *P. gingivalis*(28,29). This perspective may also explain why *P. intermedia* was more sensitive to photoactivation than *P. gingivalis*.

On the other hand, the results of the current study showed that the disk diffusion method had greater inhibitory zones when compared to the agar well diffusion test for the same concentration of the same PS type against the same bacterial species. Diffusion of the hydrophilic MB and TBO molecules on the agar surface depends on the physicochemical properties of these macromolecules(30). The better surface tension property of MB and TBO in the disk diffusion method and slower diffusion of the PSs through the agar medium in the agar well diffusion method might explain this finding.

The result of MIC and MBC of the current study showed that a lower concentration of TBO (1mg/ml for *P. gingivalis* and 0.5mg/ml for *P. interemedia*) was needed to have MIC when compared with MB photosensitizer (2mg/ml for *P. gingivalis* and 1mg/ml for *P. intermedia*). These findings are commensurate with a previous study conducted by Usacheva et al.(31), which reported that TBO is more active against gram-negative bacteria when compared to MB photosensitizers. They suggested that the solubility of TBO was higher in the hydrophilic region of the membrane; thus, TBO could interact more easily with the bacterial membrane than MB photosensitizers. As a result, the TBO concentration within the bacterial cell will be higher than that of MB. This may explain the greater antibacterial activity of TBO while they were photoactivated.

Finally, this study had some limitations, such as profuse settings of the photosensitizer's activation, time, the distance of the light source, type of the light source, properties of the photosensitizers, and biological conditioning for bacterial growth. All these factors were challenging in predicting the results.

Conclusions

The result of this study shows the effectiveness of MB and TBO as photodynamic therapies against clinically isolated *P. gingivalis* and *P. intermedia*. On the contrary, 5-ALA is not effective. Furthermore, TBO was more effective than PS than MB. And *P. intermedia* was more sensitive than *P. gingivalis* toward MB and TBO. Therefore, clinical studies are highly recommended to show the effect of MB and TBO on clinical periodontal parameters.

References

1. Jakubovics NS, Goodman SD, Mashburn-Warren L, Stafford GP, Cieplik F. The dental plaque biofilm matrix. Periodontol 2000. 2021;86(1):32-56.
2. Zhang Z, Liu D, Liu S, Zhang S, Pan Y. The role of Porphyromonas gingivalis outer membrane vesicles in periodontal disease and related systemic diseases. Front Cell Infect Microbiol. 2020;10:585917:1-12.
3. Cheng WC, Van Asten SD, Burns LA, Evans HG, Walter GJ, Hashim A, et al. Periodontitis-associated pathogens P. gingivalis and A. actinomycetemcomitans activate human CD14(+) monocytes leading to enhanced Th17/IL-17 responses. Eur J Immunol. 2016;46(9):2211-21.
4. Naito M, Belvin BR, Shoji M, Gui Q, Lewis JP. Insertional inactivation of Prevotella intermedia OxyR results in reduced survival with oxidative stress and in the presence of host cells. Microorganisms. 2021;9(3):551.
5. Pan W, Wang Q, Chen Q. The cytokine network involved in the host immune response to periodontitis. Int J Oral Sci. 2019;11(3):1-13.
6. Colombo APV, Magalhães CB, Hartenbach FaRR, Do Souto RM, Da Silva-Boghossian CM. Periodontal-disease-associated biofilm: A reservoir for pathogens of medical importance. Microb Pathog. 2016;94(1):27-34.
7. Kuboniwa M, Houser JR, Hendrickson EL, Wang Q, Alghamdi SA, Sakanaka A. Metabolic crosstalk regulates Porphyromonas gingivalis colonization and virulence during oral polymicrobial infection. Nat Microbiol. 2017;2(11):1493-9.
8. Hajishengallis G, Lambris JD. Microbial manipulation of receptor crosstalk in innate immunity. Nat Rev Immunol. 2011;11(3):187-200.
9. Xu W, Zhou W, Wang H, Liang S. Roles of Porphyromonas gingivalis and its virulence factors in periodontitis. Adv Protein Chem Struct Biol. 2020;120:45-84.

10. Könönen E, Gursoy M, Gursoy UK. Periodontitis: A multifaceted disease of tooth-supporting tissues. J Clin Med. 2019;8(8):1135.
11. Hartenbach F, Silva-Boghossian CM, Colombo APV. The effect of supragingival biofilm re-development on the subgingival microbiota in chronic periodontitis. Arch Oral Biol. 2018;85(1):51-7.
12. Tariq M, Iqbal Z, Ali J, Baboota S, Talegaonkar S, Ahmad Z, et al. Treatment modalities and evaluation models for periodontitis. Int J Pharm Investig. 2012;2(3):106-22.
13. Van Der Weijden G, Dekkers GJ, Slot DE. Success of non-surgical periodontal therapy in adult periodontitis patients: A retrospective analysis. Int J Dent Hyg. 2019;17(4):309-17.
14. Barca E, Cifcibasi E, Cintan S. Adjunctive use of antibiotics in periodontal therapy. J Istanb Univ Fac Dent. 2015;49(3):55-62.
15. Heta S, Robo I. The side effects of the most commonly used group of antibiotics in periodontal treatments. Med Sci (Basel). 2018;6(1):1-6.
16. Liu Y, Qin R, Zaat SA, Breukink E, Heger M. Antibacterial photodynamic therapy: overview of a promising approach to fight antibiotic-resistant bacterial infections. J Clin Transl Res. 2015;1(3):140-67.
17. Liu C, Mo L, Niu Y, Li X, Zhou X, Xu X. The Role of Reactive Oxygen Species and Autophagy in Periodontitis and Their Potential Linkage. Front Physiol. 2017;8(1):439.
18. Sha AM, Garib BT. Antibacterial effect of curcumin against clinically isolated porphyromonas gingivalis and connective tissue reactions to curcumin gel in the subcutaneous tissue of rats. Biomed Res Int. 2019;2019:6810936:1-14.
19. Hovhannisyan GG. Fluorescence in situ hybridization in combination with the comet assay and micronucleus test in genetic toxicology. Molecular cytogenetics. 2010;3(1):17.
20. Myer PR, Kim M, Freetly HC, Smith TPL. Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. J Microbiol Methods. 2016;127(1):132-40.
21. Lee PY, Costumbrado J, Hsu C-Y, Kim YH. Agarose gel electrophoresis for the separation of DNA fragments. JoVE. 2012;20(62):e3923:1-5.
22. Hasegawa GK, Morais JLD, Soares M, Freitas AMD. Photodynamic inactivation of Escherichia coli by methylene blue and malachite green under red LED light. Revista Ambiente & Água. 2015;10(1):318-26.
23. Nakonechny F, Barel M, David A, Koretz S, Litvak B, Ragozin E, et al. Dark Antibacterial Activity of Rose Bengal. Int J Mol Sci. 2019;20(13):3196.
24. Nitzan Y, Salmon-Divon M, Shporen E, Malik Z. ALA induced photodynamic effects on gram positive and negative bacteria. Photochem Photobiol Sci. 2004;3(5):430-5.
25. Harris F, Pierpoint L. Photodynamic therapy based on 5-aminolevulinic acid and its use as an antimicrobial agent. Med Res Rev. 2012;32(6):1292-327.
26. Fotinos N, Convert M, Piffaretti JC, Gurny R, Lange N. Effects on gram-negative and gram-positive bacteria mediated by 5-aminolevulinic Acid and 5-aminolevulinic acid derivatives. Antimicrob Agents Chemother. 2008;52(4):1366-73.
27. Lauritano D, Moreo G, Palmieri A, Vella FD, Petruzzi M, Botticelli D, et al. Photodynamic Therapy Using 5-Aminolevulinic Acid (Ala) for the Treatment of Chronic Periodontitis: A Prospective Case Series. Appl Sci. 2022;12(6):3102:1-12.
28. Mccord JM, Edeas MA. SOD, oxidative stress and human pathologies: a brief history and a future vision. Biomed Pharmacother. 2005;59(4):139-42.
29. Zou P, Cao P, Liu J, Li P, Luan Q. Comparisons of the killing effect of direct current partially mediated by reactive oxygen species on Porphyromonas gingivalis and Prevotella intermedia in planktonic state and biofilm state - an in vitro study. J Dent Sci. 2022;17(1):459-67.
30. Floury J, El Mourdi I, Silva JV, Lortal S, Thierry A, Jeanson S. Diffusion of solutes inside bacterial colonies immobilized in model cheese depends on their physicochemical properties: a time-lapse microscopy study. Front Microbiol. 2015;6(1):366.
31. Usacheva MN, Teichert MC, Biel MA. Comparison of the methylene blue and toluidine blue photobactericidal efficacy against gram-positive and gram-negative microorganisms. Lasers Surg Med. 2001;29(2):165-73.