Photodiagnosis and Photodynamic Therapy 27 (2019) 218–226

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

Synergistic effect of methylene blue and biogenic gold nanoparticles against *Enterococcus faecalis*

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ARTICLE INFO

Keywords:
Enterococcus faecalis
Methylene blue
Biogenic gold nanoparticles
Photodynamic bactericidal efficacy
Antimicrobial photodynamic therapy

ABSTRACT

This study reports successful photodynamic inactivation of planktonic and biofilm cells of *Enterococcus faecalis* using Methylene Blue (MB) in combination with gold nanoparticles synthesized using the cell-free filtrate obtained from 3-day biomass of *Trichoderma asperellum* strain. Monodispersed colloidal gold nanoparticles were characterized by UV–vis absorption, TEM and DLS to be 13 ± 3 nm spheres. Diode lasers with the peak-power wavelength λ = 660 nm (output power of 21, 41 and 68 mW; power density of 55, 108 and 179 mW·cm^{-2}, respectively, were used as a light source to study the effects of MB alone, the gold nanoparticles alone (AuNPs) and the MB + AuNPs mixture on the viability of *E. faecalis* cells. The lethal effect of planktonic cells was achieved for MB after 30 min of laser irradiation with energy fluence of 322 J·cm^{-2}. When MB + AuNPs mixture was used as photosensitizer, the lethal effect was achieved with energy fluence of 292 J·cm^{-2}. The biofilm culture was more resistant to photo-inactivation and the best bactericidal effect of MB as photosensitizer was found after light dose of 483 J·cm^{-2}. The bacterial cell viability was reduced by 99.92%. It was proved that MB + AuNPs mixture synergistically enhances the kill of the studied microorganism as the same light dose resulted in 99.991% kill.

1. Introduction

Enterococci belong to a group of microorganisms known as lactic acid bacteria (LAB) that produce bacteriocins [1] and these species have been widely used over the last decade in the food industry as probiotics or as starter cultures [2,3]. Moreover, these bacteria are an important part of the human digestive tract [4,5]. For many years *Enterococcus* species were believed to be harmless to humans and considered unimportant medically. Recently, *Enterococcus* species have become one of the most common nosocomial infections worldwide [6–8]. Hidron et al. [9] have shown that enterococci are currently the second most common cause of bacteremia associated with healthcare, an increase from the sixth most common cause in the 1980s.

The genus Enterococcus includes more than 17 species, although only a few cause clinical infections in humans. *Enterococcus faecalis* is the most common species associated with clinical infection [9]. The most frequently infections caused by enterococci occurs in the urinary tract. These bacteria were also isolated from cultures of intra-abdominal, pelvic and soft tissue infections, foot ulcers, as well as from bone and bone marrow disorders in diabetics. Other infections, less common or less frequent due to enterococcal disease, include meningitis, haemorrhagic osteomyelitis, septic arthritis, and pneumonia.

E. faecalis plays a major role in endodontic infections. This is a predominant microorganism in root-filled teeth, which is the cause of endoscopic topical periodontitis and can be isolated from the root canal in pure culture [10–12]. In addition, it has been reported that cases with ineffective endodontic treatment were associated with this bacterial strain. Several virulence factors, such as: aggregation, surface proteins, gelatinase, cytosine toxin, extracellular production of superoxide, antibiotic resistance determinants, make it difficult to remove these microorganisms [7,13,14]. In addition, *E. faecalis* resistance to conventional antimicrobial agents such as sodium hypochlorite,

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https://doi.org/10.1016/j.pdpdt.2019.05.042
Received 11 January 2019; Received in revised form 26 April 2019; Accepted 31 May 2019
Available online 01 June 2019

chlorhexidine or calcium hydroxide has been reported, emphasizing the need for better disinfection methods [15,16].

Several disinfection methods alternative to current standard endodontic methodologies have been explored. For example, the potential of ozone, a highly reactive form of oxygen that is generated by passing oxygen through high-voltage, as an antibacterial agent in endodontic infections has been recently described [17,18]. Nagayoshi et al. [19] found that ozonated water had the same activity as 2.5% NaOCl against *E. faecalis* biofilms developed on dentin. It should be noticed that elimination of these bacteria is most relevant for successful endodontic therapy.

Among disinfection methods which can be used in endodontic therapy, aPDI (Antibacterial Photodynamic Inactivation) has gained special attention. aPDI is defined as the application of a non-toxic dye known as a photosensitizer (PS), which is activated by non-thermal visible light with the appropriate wavelength(s) in the presence of oxygen to produce cytotoxic reactive oxygen species [20,21]. After the absorption of the photon, the photosensitizer is promoted from a low energy level fundamental state to a higher-energy singlet state and then, by intersystem crossing, it can convert to an excited triplet state. From the relatively long-lived triplet state it can then follow two photochemical pathways, named type I and type II reactions. In the type I mechanism, the photosensitizer reacts with molecules such as the cell membrane constituents and transfers a proton or an electron to form free radicals and radical ions. In the type II reaction, the excited PS can transfer its energy directly to molecular oxygen resulting in the production of reactive oxygen species (ROS) that are able to kill microbial cells [22]. Photo-damage caused by aPDI results in loss of enzymatic activity, protein oxidation, protein-protein cross-linking and inhibition of metabolic processes (e.g. DNA synthesis, glucose transport). Direct damage to the cell membrane leads to deactivation of the membrane transport system and to leakage of cellular content [23].

It is well known that PS is selectively bound by microbial cells [24]. The best way to achieve this goal was to provide a cationic charge of the photosensitizer because microbial cells have a more pronounced negative charge compared to mammalian cells and cationic PS bind selectively to these cells. The binding of the PS to the microbial cells is fast, while uptake of the cationic PS by mammalian cells is slow, thus giving good selectivity when a short drug-light interval (few minutes) is employed [25]. Furthermore, aPDI works equally well regardless of the antibiotic resistance status of the microbial cells [26].

Despite intensive studies, the results of the eradication of *E. faecalis* with aPDI are ambiguous. Yildirim et al [27] studied the effects of different light exposure durations on *E. faecalis* reduction and concluded that irradiation for 1 min is adequate to achieve the antimicrobial effect. On the other hand, there are some studies indicating that aPDI was not an effective disinfectant. For example, Hecker et al. compared NaOCl with aPDI, and found that aPDI was a less effective disinfectant [28]. Some authors emphasized that an effective aPDI against *E. faecalis* must be combined with traditional mechanical debridement and irrigation [29,30].

The presented paper is a continuation of our long-standing experience on the enhancement of photodynamic inactivation of pathogenic microorganisms using the biogenic metallic nanoparticles [31,32].

In this work, we report, the effect of biogenic gold nanoparticles on reducing the dose of laser radiation that effectively kills *E. faecalis* cells.

2. Materials and methods

2.1. Reagents

All chemicals agents including tetrachloroauric acid and Methylene Blue (MB) were obtained from POCH Poland. Methylene Blue was prepared by dissolving the powdered dye in phosphate buffered saline (PBS, pH 7.4) and sterilized by filtration through 0.22-μm pore diameter membranes (Millex®-HP syringe-driven filter unit, Millipore). The solution of MB was stored in the dark at room temperature.

2.2. Synthesis and characterization of gold nanoparticles

The biogenic gold nanoparticles (AuNPs) were synthesized by the cell-free filtrate obtained from 3-day biomass of *Trichoderma asperellum* according to the procedure described previously [33]. The formation of AuNPs was monitored by observing the changing color of the cell-free filtrate after incubation with the gold ions. Absorption spectra in the range of 200–800 nm were obtained with a spectrophotometer (Shimadzu UV-1650PC) to confirm the formation of AuNPs in the cell-free filtrate. The size and morphology of the obtained AuNPs were determined using a transmission electron microscope TEM (TEM, Zeiss EM 900). The sample was prepared by placing a drop of AuNPs nanoparticles on a carbon-coated copper grid and subsequently drying in air before transferring it to the microscope. From electron micrographs the particle size distribution was found for at least 150 particles. The surface charge of the AuNPs was studied using a Zeta potential/particles sizer, NICOMP 380 ZLS, PSS.NICOMP Particle Sizing System at 25 °C. The AuNPs sample was freeze-dried and powder was analyzed using a X-ray Philips material research diffractometer (Philips, Eindhoven, Netherlands).

Biogenic AuNPs solutions were dialyzed against water to remove free gold ions. The concentration of the studied nanostructures was approximately 20 ppm.

2.3. Light source

Diode lasers with the peak-power wavelength $\lambda = 660$ nm (output power of 21, 41 and 68 mW; light intensity of 55, 108 and 179 $mW \cdot cm^{-2}$) were used in this study.

2.4. Bacterial strain and culture conditions

An amount of 5 mL of an overnight culture of *Enterococcus faecalis* PCM 2673 (grown aerobically at 37 °C, with shaking, in Mueller broth) was centrifuged at 6000 *g* for 5 min and the supernatant was discarded. The pellet was re-suspended in 5 mL phosphate buffered saline (PBS). For each experiment, fresh inocula with an optical density of ˜0.08 (OD_{550}) containing approximately 1×10^6 of colony-forming units (CFU/mL) was used throughout the study.

2.5. Development of bacterial biofilm

Biofilms were formed on commercially available pre-sterilized black polystyrene flat-bottom 96-well plates (Thermo Scientific™). At first, 150 μL of a standardized *E. faecalis* PCM 2673 cell suspension was transferred into each well of a microtiter plate and incubated for 4 h at 37 °C in a shaker (50 rpm). After this period, the supernatant (containing non-adhered cells) was removed from each well and plates were washed using 150 μL phosphate buffered saline (PBS). Then, 150 μL of fresh medium was added to each well and the plates were further incubated for 24 h. After 24 h biofilm formation, the supernatant was again removed and each well was washed twice with 100 μL of PBS to remove loosely adherent cells.

Biofilms before and after photosensitization treatment were stained using a SYTOX® Green dead cell stain (ThermoFisher Scientific, USA) and incubated at room temperature in the dark for 15 min. Visualization of the biofilm was performed under Olympus 60BX light microscope with appropriate excitation/emission filter cubes (excitation bandpass filter 460–490 nm, emission long pass filter > 515 nm).

2.6. The effect of MB, AuNPs and MB + AuNPS on the growth of E. faecalis

A twofold serial dilution of the MB (initial concentration 1.0 $g\,L^{-1}$) was performed. A 150 μL aliquot of the standardized suspension of *E.*

faecalis was added to each well of a 96-well flat-bottom microtiter plate (Thermo Scientific™) and MB was added to obtain final concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg L^{-1}. The plate was then incubated at 37 °C, without shaking for 120 min (in dark). After incubation, the number of colony forming units per mL (CFU/mL) was determined.

The biofilm formed was immersed in 150 µL of MB (at concentrations given above) for 120 min (without shaking). Then, the cells in the biofilm were scraped off the well wall using a sterile toothpick and were transferred to Falcon tubes containing 10 mL of PBS. To disrupt the biofilms, the contents of the tubes were homogenized for 30 s using an ultrasonic homogenizer (VC505, Sonics, USA) with an output power of 50 W. Then, serial dilutions were prepared and 100 µL aliquots of each dilution were seeded in duplicate onto Mueller agar and incubated for 24 h at 37 °C. After incubation, the number of colony forming units per well (CFU/well) was determined.

The dark toxicity of MB was expressed as a $\log_{10}$ unit reduction of bacterial cells calculated by comparing the initial population size with that after incubation. The culture of the studied microorganism was incubated under the same reaction conditions and was used as a control.

The effect of the biogenic AuNPs (20 ppm) and MB + AuNPs mixture on the growth of *E. faecalis* (in planktonic and biofilm cultures) was studied under the same conditions as described above.

2.7. Photodynamic inactivation of planktonic cells

Photodynamic inactivation of planktonic culture *E. feacalis* was evaluated according to the method described previously with slight modifications [31,32]. A 100 µL aliquot of the standardized suspension of the studied bacterium was added to each well of a 96-well flat-bottom microtiter plate. These assays were divided into the following groups: treatment with the gold nanoparticles only (AuNPs-L, n = 5); treatment with the gold nanoparticles and laser irradiation (AuNPs + L, n = 5); treatment with MB at the concentration of 6.25 mg L^{-1} only (MB−L, n = 5); treatment with MB at the concentration of 6.25 mg L^{-1} and laser irradiation (MB + L, n = 5); treatment with MB at the concentration of 6.25 mg L^{-1} and the gold nanoparticles only (MB + AuNPs-L, n = 5); treatment with MB at the concentration of 6.25 mg L^{-1} and the gold nanoparticles with laser irradiation (MB + AuNPs + L, n = 5). The assay groups AuNPs-L and AuNPs + L received 100 µL of the biogenic gold nanoparticles (the final concentration of the gold nanoparticles was 20 ppm), whilst the assay groups MB−L and MB + L received 100 µL of Methylene Blue. The assay groups MB + AuNPs-L and MB + AuNPs + L received 100 µL of the Methylene Blue-gold nanoparticles mixture. The plate was then shaken for 120 min in an orbital shaker (in dark) at the temperature of 37 °C. The wells containing the assay groups + L were then exposed to laser(s) light for various periods of time (5, 10, 15, 30 and 45 min). Ten additional wells containing the bacterial suspension (100 µL) and PBS (100 µL) were prepared. Five of these wells were exposed to laser light to determine the effect of light alone on cell viability, while the remaining five were stored in the dark as an overall control and to determine the initial concentration of cells in the suspensions. After irradiation, serial dilutions were prepared and 100 µL aliquots of each dilution were seeded in duplicate onto Mueller agar (Difco) and incubated for 24–48 h at 37 °C. After incubation, the number of colony forming units per mL (CFU/mL) was determined.

2.8. Photodynamic inactivation of biofilm cells

The biofilm formed was immersed in 200 µL of MB (50 mg L^{-1}), or AuNPs or the MB + AuNPs mixture for 120 min (incubation time) in an orbital shaker (in dark) at 37 °C. Subsequently, the suspended plates were irradiated according to the protocol described above (see 2.6). The energy fluencies of 288 J cm^{-2} (41 mW diode laser with light intensity of 108 mW·cm^{-2}; 45 min of irradiation), 322 J·cm^{-2} and 483 J ·cm^{-2} (68 mW diode laser with light intensity of 179 mW·cm^{-2}; 30 or 45 min of irradiation) were used in this experiment. After the treatments, the number of colony forming units per well (CFU/well) was determined according to the protocol described in 2.5.

2.9. Statistical analysis

All experiments were run in triplicate and the data are presented as a mean ± standard deviation (SD). The difference between two means was compared by a two-tailed unpaired Student's test. The values of $p < 0.05$ were considered as a significant.

3. Results

The presented study describes proposed photo-inactivation of *E. faecalis* viable cells in planktonic and biofilm cultures, which included MB as a photosensitizer and low energy light source (diode laser) and this process was effectively enhanced by the biogenic gold nanoparticles synthesized by *T. asperellum*.

3.1. Synthesis and characterization of the biogenic gold nanoparticles

The biogenic gold nanoparticles were synthesized by exposure of gold ions to the cell-free filtrate of *Trichoderma asperellum* and after 2 h of incubation at room temperature the change of colour of the cell-free filtrate from yellow to red was observed (*inset in* Fig. 1A). The presence of gold nanoparticles (AuNPs) was confirmed by UV–vis spectrum which showed a strong resonance centred at around 531–533 nm (Fig. 1A). The shape and the size of the synthesized AuNPs were studied by transmission electron microscopy (TEM) and dynamic light scattering (DLS measurements). Fig. 1B shows a representative TEM micrograph of the gold nanoparticles synthesized by the cell-filtrate of *T. asperellum*. The particles were spherical in shape and uniformly distributed without any significant agglomeration. The particle size histogram (Fig. 1C) showed that the studied nanoparticles sizes ranged from 10 to 21 nm and possess an average size of 13 ± 3 nm. The size distribution given by the histogram indicates that almost 60% of the gold particles re in the 13 to 15 nm size range. The particles size obtained from dynamic light scattering measurements is higher than that estimated from TEM measurements and is 20 ± 3 nm. Zeta potential of the biogenic gold nanoparticles showed the value of -17 ± 3 mV, indicating the great stability of the particles in aqueous suspension. To prove the presence of elemental gold in the cell-free filtrate after incubation with gold ions, XRD analysis was performed. Characteristic packs were observed on the obtained diffractogram at about 2θ = 38.2°, 44.4° and 64.6°, which corresponds to the crystal planes [111], [200] and [220] of *face center cubic* (fcc) crystal structure of metallic gold (Fig. 1D).

3.2. Dark toxicity studies

In our initial experiments, it was necessary to establish the dark toxicity of MB and MB + AuNPs for the studied bacteria. We have examined the effect of MB in the range of concentrations from 3.125 mg L^{-1} to 100 mg L^{-1} on viability of planktonic and biofilm cells of *E. faecalis*. The effect of MB + AuNPs mixture was tested at varying concentrations of MB (3.125–100 mg L^{-1}) and constant concentration of AuNPs (20 ppm).

As shown in Fig. 2A, MB was non-toxic to the bacteria and 18.7%, and 12.9% reduction was observed for MB concentrations of 6.25 mg L^{-1} and 3.125 mg L^{-1}, respectively. Higher concentrations of MB inhibited the growth of the studied microorganism and it was found reduction of the viability of the *E. faecalis* planktonic culture by 45%, 85%, 90% and 98% for MB concentrations of 12.5 mg L^{-1}, 25 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, respectively ($p < 0.05$). The biogenic AuNPs

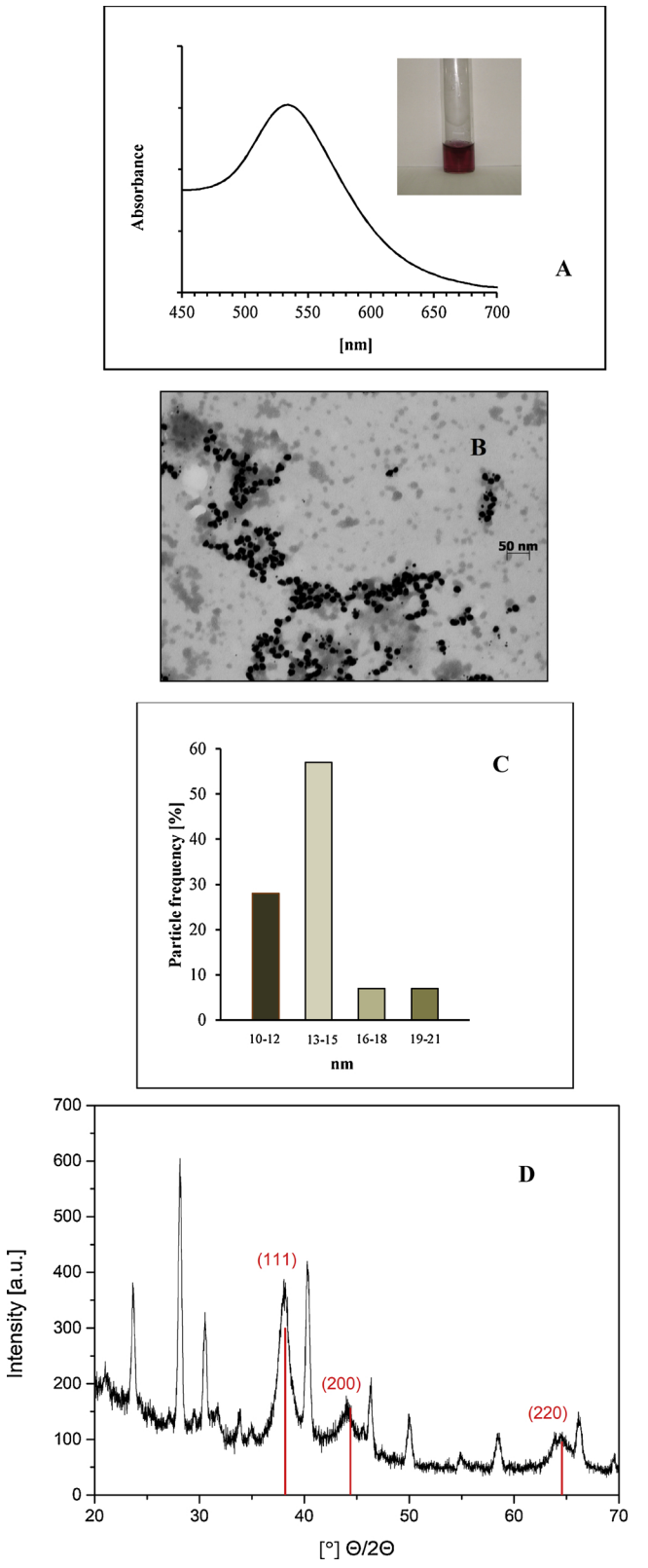

Fig. 1. Absorption spectrum of the biogenic AuNPs; the *inset* shows the colour of the cell-free filtrate of *T. asperellum* turning red after incubation with $AuCl_4^-$ ions (**A**); TEM micrographs of AuNPs (**B**); the particle size histogram of AuNPs (**C**).

at the concentration of 20 ppm did not affect the number of bacteria cells (*Supplementary Information,* Fig. S1). Changes in the value of CFU/well were insignificant and were within the measurement error.

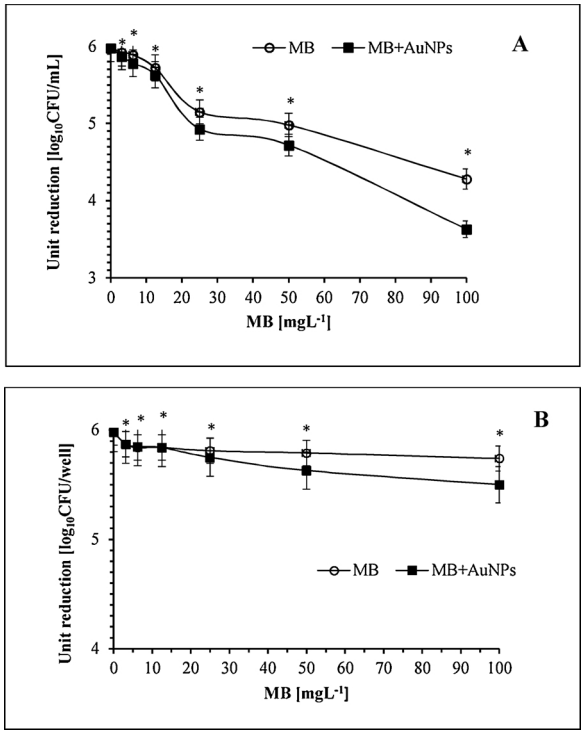

Fig. 2. The effect of concentration of MB and MB + AuNPs mixture on viability of planktonic cells (**A**) and biofilm cells (**B**) of *E. faecalis.* Mean and SEM, *t* test; asterisks indicate the statistical differences $p < 0.05$.

The MB + AuNPs mixtures have a higher antibacterial activity than MB alone and the reduction in viability of planktonic cells was 22.4%, 36.9%, 55.3%, 91%, 94.5% and 99.55% for MB concentrations ranged between 3.125 mg L^{-1} and 100 mg L^{-1} ($p < 0.05$).

In the case of biofilm of *E. faecalis,* a significantly lower unit of reduction in viability of cells, compared to planktonic culture was observed up to the concentration of 100 mg L^{-1} of MB (mortality of bacterial cells did not exceed 31%). A higher mortality rate was found when MB + AuNPs mixtures were used in experiment and the reduction in viability was 46.3% 60.1% for MB concentration 50 mg $L^{-1}$and 100 mg L^{-1}, respectively ($p < 0.05$) (Fig. 2B). 6.25 mg L^{-1} concentration of MB and 20 ppm of AuNPs were used in photosensitizing experiments with planktonic culture. All experiments with biofilm culture were carried out using MB at concentration of 50 mg L^{-1} with 20 ppm of AuNPs.

3.3. Photo-inactivation of planktonic cells of E. Faecalis

To exclude the influence of any endogenous photosensitizers activatable by illumination, the effect of laser light alone on the viability of *E. faecalis* was carried out. Bacterial cells were irradiated with 68 mW diode lasers with energy fluency 322 and 483 J·cm^{-2} and no effect on growth has been found (data not shown). Changes in the value of CFU/mL were insignificant and were within the measurement error.

Next, the MB or AuNPs, or MB + AuNPs mixture were studied as photosensitizers in photodynamic treatment against planktonic *E. faecalis* cells. First, high power of 68 mW with light intensity of 179 mW cm^{-2} was used to inducing a biocidal effect of MB. Fig. 3 shows the mean CFU/mL (log_{10}) and standard deviation of planktonic cells after the different treatments.

The AuNPs indicated insignificant reduction of 22.6%, 26.1%, 27.8% and 34.2% in the viability of the *E. faecalis* planktonic culture after 5 (energy fluence of 54 J cm^{-2}), 10 (108 J cm^{-2}), 15 (162 J cm^{-2}), 30 (322 J cm^{-2}) and 45 min (483 J·cm^{-2}) of irradiation, respectively ($p < 0.05$).

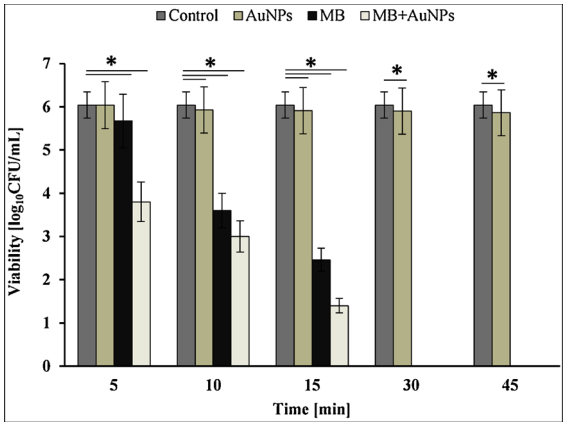

Fig. 3. Effect of AuNPs, MB and MB + AuNP mixture on viability of the planktonic cells of *E. faecalis* following the exposure to diode laser with light intensity of 179 mW cm^{-2} for 5 min, 10 min, 15 min, 30 min and 45 min. Mean and SEM, *t* test; asterisks indicate the statistical differences $p < 0.05$.

A light dose-dependent reduction of viability by 95.44%, 99.2% and 99.93% was observed corresponding to 54 J cm^{-2}, 108 J cm^{-2}, 162 J cm^{-2} energy fluences when MB was used as photosensitizer (Fig. 3). A lethal effect of MB (the count was below the detection limit) was achieved after photosensitization of *E. faecalis* with MB when activated by laser light was achieved after 30 and 45 min of irradiation (energy fluence was 322 J cm^{-2} and 483 J cm^{-2}).

Mixture of MB with AuNPs greatly enhanced photoinactivation of *E. faecalis* as 5 min irradiation (54 J cm^{-2}) resulted in viability reduction by 99.43% (Fig. 3).Prolonged exposure time to 10–15 minutes caused the reduction in viability by 99.728% and 99.998%. Complete inactivation of *E. faecalis* (the count was below the detection limit) in suspension was achieved after exposing bacteria to laser light for 30 min (322 J cm^{-2}). The decrease in bacterial cell viability in all groups was statistically significant ($p < 0.05$).

When the 41 mW diode laser with light intensity of 108 mW·cm^{-2} was used, the AuNPs showed an negligible reduction in *E. faecalis* culture viability and the largest reduction in cell viability was observed after 45 min of treatment with energy fluence of 288 J cm^{-2} and was 22.6% ($p < 0.05$) (Fig. 4).

A significant destruction of planktonic cells of *E. faecalis* was achieved using MB as the photosensitizer. The MB showed 70.6% and 97.7% reduction in *E. faecalis* viability after 5 (energy fluence was 32 J cm^{-2}) and 10 min (energy fluence was 64 J cm^{-2}) of irradiation. A longer expose time, *i.e.* 15 min (96 J cm^{-2}), 30 min (192 J cm^{-2}) and 45 min (288 J cm^{-2}) resulted in a reduction in cfu by 98.53%, 99.953%

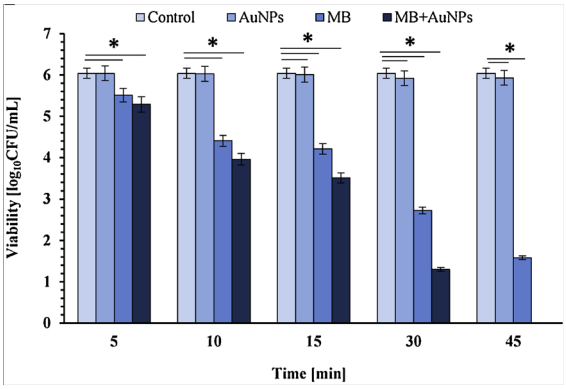

Fig. 4. Effect of AuNPs, MB and MB + AuNPs mixture on viability of planktonic cells of *E. faecalis* following the exposure to diode laser with light intensity of 108 mW cm^{-2} for 5 min; 10 min, 15 min, 30 min and 45 min. Mean and SEM, *t* test; asterisks indicate the statistical differences $p < 0.05$.

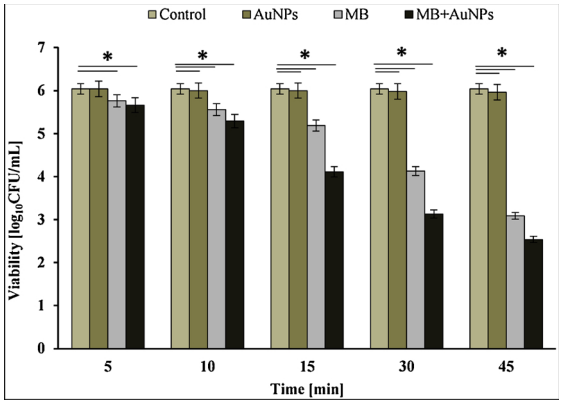

Fig. 5. Effect of AuNPs, MB and MB + AuNPs mixture on viability of planktonic cells of *E. faecalis* following the exposure to diode laser with light intensity of 55 mW cm^{-2} for 5 min, 10 min, 15 min, 30 min and 45 min. Mean and SEM, *t* test; asterisks indicate the statistical differences $p < 0.05$.

and 99.997% ($p < 0.05$). When the MB + AuNPs mixture was used in our experiments, after 5, 10, 15 and 30 min of laser irradiation the viable count showed a reduction by 82.3%, 99.17%, 99.71% and 99.9982%, respectively. After 45 min of irradiation (288 J cm^{-2}) a lethal effect of MB + AuNPs was observed after photosensitization of *E. faecalis* (the count was below the detection limit). The decrease in bacterial cell viability in all groups was statistically significant ($p < 0.05$).

When the 21 mW diode laser with radiation power density of 55 mW cm^{-2} was used, the AuNPs showed an negligible reduction in *E. faecalis* culture viability and the largest reduction in cell viability was observed after 45 min of treatment with energy fluence of 153 J cm^{-2} and was 17.1% ($p < 0.05$) (Fig. 5).

MB as photosensitizer after 5 (17 J cm^{-2}), 10 (34 J cm^{-2}), 15 (51 J cm^{-2}), 30 (102 J cm^{-2}) and 45 min (153 J cm^{-2}) of irradiation showed a reduction in *E. faecalis* viability by 47.7%, 67%, 86%, 87.7% and 99.89%, respectively ($p < 0.05$).

As can be seen in Fig. 5, the kill achieved by the MB + AuNPs mixture was also light dose-dependent with the kill increasing as the exposure time was increased from 5 to 45 min and after 5, 10, 15, 30 and 45 min of laser treatment, the viable count showed a reduction by 58.5%, 82.3%, 98.83%, 99.88% and 99.969% respectively. The decrease in bacterial cell viability in all groups was statistically significant ($p < 0.05$).

3.4. Photo-inactivation of biofilm cells of E. Faecalis

In this set of experiments, the AuNPs, MB and MB + AuNPs mixture were studied as photosensitizers in photodynamic treatment against biofilm cells of *E. faecalis*. As can be seen in Fig. 6, the AuNPs showed a negligible reduction in biofilm of *E. faecalis* viability and the largest reduction in cell viability was observed after laser light treatment with energy fluence of 483 J cm^{-2} and was 34.1% ($p < 0.05$). The bactericidal effect achieved by the MB was light dose-dependent with the kill increasing as the energy fluence was increased from 288 J cm^{-2} to 483 J cm^{-2} and the viable count showed a reduction by 94.1%, 99.476% and 99.92%, respectively ($p < 0.05$). Mixture of MB with AuNPs greatly enhanced photo-inactivation of biofilm of *E. faecalis* as energy fluence of 288 J cm^{-2} resulted in reduction of viability by 99.51% (Fig. 6). Laser treatment with energy fluence of 322 J cm^{-2} and 483 J cm^{-2} caused the reduction in viability by 99.92% and 99.991%, respectively. The decrease in bacterial cell viability in all groups was statistically significant ($p < 0.05$).

The bactericidal effect of aPDI was confirmed by SYTOX Green using fluorescence microscopy. The obtained micrographs showed that the number of nonviable cells depended on the photosensitizer used

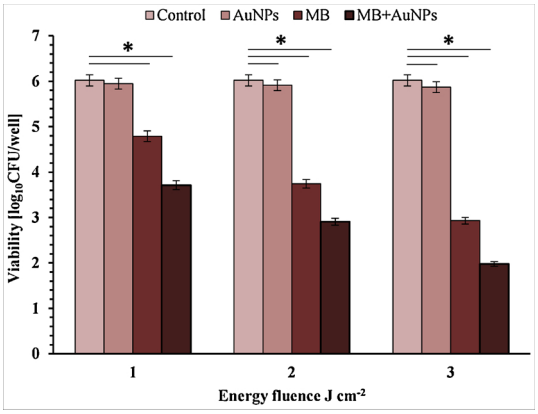

Fig. 6. Effect of AuNPs, MB and MB + AuNPs mixture on viability of biofilm cells of *E. faecalis* following the exposure to laser light with energy fluence of 292 J cm^{-2} (**1**), 322 J cm^{-2} (**2**) and 483 J cm^{-2} (**3**). Mean and SEM, *t* test; asterisks indicate the statistical differences $p < 0.05$.

(Fig. 7A–D). The highest bactericidal effect was observed after laser light treatment with energy fluence of 483 J cm^{-2} with mixture of MB and AuNPs mixture as a photosensitizer (Fig. 7D).

3.5. Studies on the type of effect of enhancement of photo-bactericidal activity of MB

From the results shown above it was concluded that the biogenic gold nanoparticles effectively enhanced the photobactericidal activity of MB. An important issue in this work is to understand the nature of this process. For determining the type of effect that is operative, the number of bacteria remaining alive was measured after the dose of each component, f(a) and f(b), alone and in combination, f(a + b) (Table S2-S5; *Supplementary Information*). It was previously shown [34,35] that the effect is synergistic when the number of live bacterial cells after the reaction of combined agents f(a + b) is less than the sum of the two components f(a) + f(b) reacting by themselves (**1**)

$$f(a+b) < f(a) + f(b) \quad (1)$$

For the effect to be synergistic, the number of live bacteria after the treatment with the combined agents f(MB + AuNPs), must be less than the sum of bacteria remaining alive after the two agents f(MB) + f (AuNPs) reacted alone. A set of data recorded in one experiment is shown below (the laser energy fluence was 16.6 J cm^{-2}). Bacteria remaining alive after f(a) (MB at concentration of 6.25 mg L^{-1}) reaction with 1.1×10^6 ($\log_{10} = 6.04$) bacteria: $f(a) = 5.72 \times 10^5$; bacteria remaining alive after f(b) (AuNPs at concentration of 20 ppm) reaction with 1.1×10^6 ($\log_{10} = 6.04$) bacteria: $f(b) = 1.09 \times 10^6$. Therefore, $f(a)+f(b) = 1.662 \times 10^6$ remaining alive after reacting with 2.2×10^6 bacteria, which corresponds to 8.31×10^5 live bacterial cells after

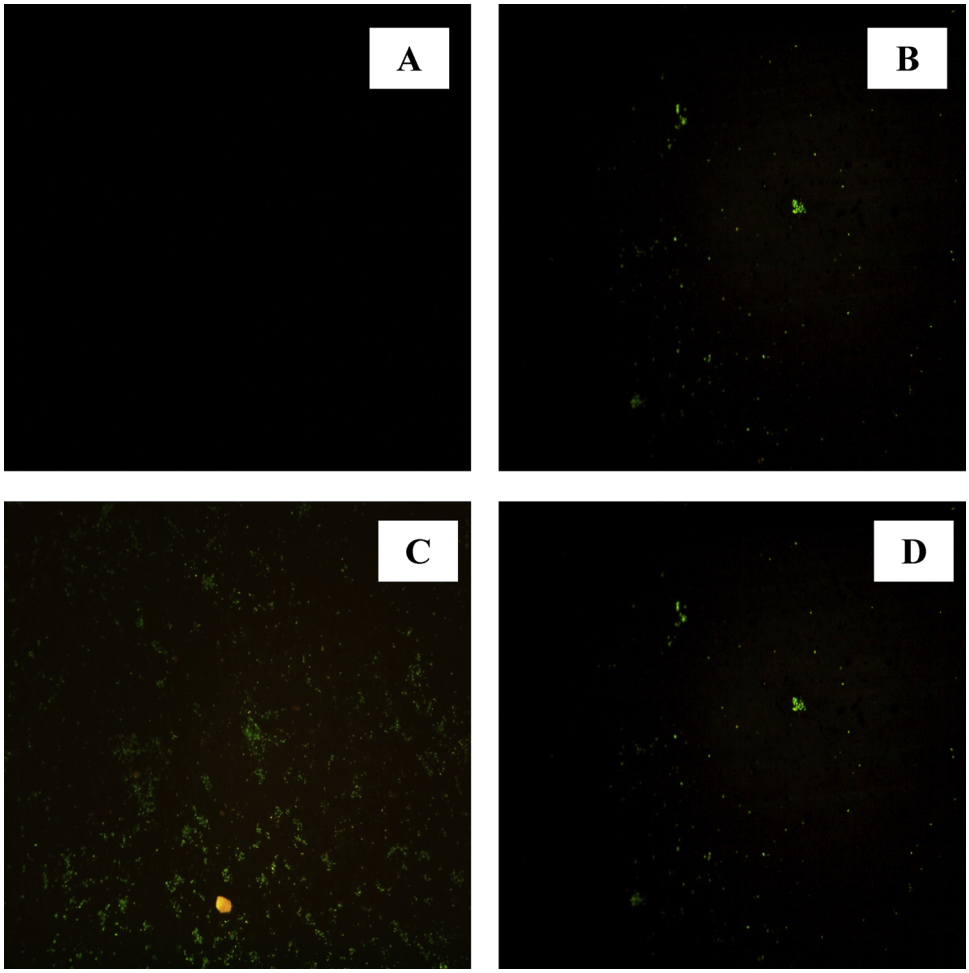

Fig. 7. Fluorescence microscopy images of biofilms before and after photo-inactivation. Nonviable bacteria are accessible to SYTOX Green and appear green. *E. faecalis* biofilm: (**A**) cells before treatment; cells after treatment with laser light with energy fluence of 483 J cm^{-2} and (**B**) AuNPs as a photosensitizer; (**C**) MB as a photosensitizer ; (**D**) MB + AuNPs mixture as a photosensitizer; images were obtained at a magnification of 20.

reacting with 1.1×10^6 bacteria. Similarly, f(a + b) = 4.55×10^5 remaining alive bacteria after reacting with 1.1×10^6 bacteria. Therefore, f(a + b) (MB + AuNPs) left 4.55×10^5 living cells, while f (a) (MB) + f(b) (AuNPs) = 8.31×10^5 bacterial cells and these data showed that the reaction of the combined MB and AuNPs left less bacteria than the sum of the same doses of MB and AuNPs acting alone on *E. feacalis*. It is worth noticing that 376,000 fewer *E. feacalis* cells remained alive by the two combined components than the sum of two agents reacting alone with the same bacterial concentration. From these results, it was concluded that the enhancement of the bactericidal effect of MB by biogenic gold nanoparticles is synergistic.

To ensure that the true reaction mechanism is synergistic, the values of 2f (a) and 2f (b) should also be established and compared with the value of f (a + b) to show that:

$$2f(a) > f(a+ b) \text{ and } 2f(b) > f(a+ b) \quad (2)$$

For the selected example, the calculations are as follows:

2f(a) = 1.144×10^6 > f (a + b) = 4.55×10^5 and 2f (b) = 2.18×10^6 > f (a + b) = 4.55×10^5

These results show that both 2f(a) and 2f(b) doses left a larger number of live bacteria than f(a + b); therefore, it is proved that the combined effect of MB + AuNPs mixture is synergistic.

From the perspective of bacteria killed, the effect is synergistic when the number of killed bacteria after the reaction of the combined agent f(a + b) is more than the sum of the two components f(a) + f(b) acting alone. Bacteria killed after MB reaction with 1.10×10^6 ($\log_{10}$ = 6.04) bacteria: f(a) = 5.28×10^5; bacteria killed after AuNPs reaction with 1.10×10^6 ($\log_{10}$ = 6.04) bacteria: f(b) = 1.00×10^4. Therefore, f(a) + f(b) = 5.38×10^5 killed cells after reacting with 2.20×10^6 bacteria, which corresponds to 2.69×10^5 killed cells after reacting with 1.10×10^6 bacteria. Similarly, f(a + b) = 6.45×10^5 was killed after reacting with 1.10×10^6 bacteria. Therefore, f(a + b) (6.45×10^5) > f(a) + f(b) (5.38×10^5) and it was concluded that this effect is synergistic.

Analysis of the data collected in Tables S1-S4 revealed that, irrespective of the energy fluence used in the experiments, the effect of each component (MB or AuNPs) reacting alone against the studied planktonic and biofilm cells of *E. faecalis* is additive, whereas their combined reaction (MB + AuNPs mixture) effect is synergistic.

4. Discussion

Enterococcus faecalis is resistant to most of the intracanal medicaments, probably due to its ability to regulate internal pH with an efficient proton pump [36]. It is well known that this bacterium can survive under harsh environmental conditions such as alkalinity, or malnutrition in root canal environment [37,38] and therefore it is justified to look for alternative methods of destroying this microorganism. The present study proposed an efficient photo-inactivation of *E. faecalis* living cells in planktonic and biofilm cultures using MB as a photosensitizer and a diode laser as a source of light (660 nm). Methylene blue has been chosen as a photosensitizer because it is a well-known synthetic non-porphyrin compound (containing phenothiazine) compatible with the wavelength of visible light (up to 685 nm) and has a high rate of generation of oxygen reactive species [39]. MB has been used as an intrinsic antimicrobial compound in conventional (non-light-mediated) antimicrobial therapy research for over 100 years [40]. It is known that photosensitization reactions induced by MB excitation mainly concern damage to nucleic acids, proteins and lipids. It has been reported that photodynamic activity of MB occurs mostly via the type I mechanism [41]. In the type I reaction, the excited photosensitizer reacts directly with the substrate, in a one-electron transfer reaction, to produce a radical or radical ion in both the photosensitizer and the substrate. Although electron transfer can proceed in either direction, usually the substrate donates an electron to the sensitizer, resulting in a substrate radical cation and a sensitizer radical anion. In the presence of oxygen, both of these radicals can further react to produce oxygenated products. Several studies have reported of its *in vitro* activity and in animal models of infection, and MB has received regulatory approval to mediate aPDI of dental infectious diseases, such as periodontitis and caries [42–44]. In addition, previous studies [45] have reported that MB, when used in concentrations up to 100 mg L^{-1}, minimizes the chances of dental discoloration.

In 2006, Tegos and Hamblin showed for the first time that phenothiazinium-based PSs are substrates of MDRs (multidrug resistance pumps) in bacteria [46]. It is recognized that it is a general phenomenon applicable to all photoactive phenothiazinium dyes. Kishen et al. [47] believed that this problem can be solved by using a phenothiazine dye with an MDR inhibitor. These authors studied the role of a specific microbial EPI, verapamil hydrochloride, in the MB-mediated aPDI of *E. faecalis* biofilms and it was found that that photo-inactivation of biofilm was enhanced by EPI. Drawing on these data, we have made an attempt to resolve the problem of enhancing phototoxic activity of MB against planktonic and biofilm cells of *E. faecalis*. In our studies the biogenic gold nanoparticles, synthesized by exposure of gold ions to the cell-free filtrate of *T. asperellum*, were applied to improve the efficiency of the photo-inactivation of *E. faecalis* by MB. The synthesis of the AuNPs was monitored by the change in colour of the reactive mixture. The colour arises by the excitation of surface plasmon vibrations in the metal nanoparticles. This phenomenon is known as the surface plasmon resonance [48]. UV–vis spectrum exhibits an intense peak at 531–532 nm corresponding to the surface plasmon resonance frequency of nanocrystalline gold particles. The TEM technique was used to determine the morphology and size of the nanoparticles. TEM images indicated that the gold nanoparticles were relatively uniform in diameter and spherical in shapes. The average size of the particles was found to be 13 ± 3 nm. The size of these particles determined by DLS was estimated to be 20 ± 3 nm. This is due to the fact that the particle size is increased significantly by contributions from the hydrated capping agents and also from solvation effects. As shown in Figs. 3–6, the studied AuNPs did not induce a significant death of *E. faecalis* on exposure to laser light. The highest reduction in planktonic cell viability of 34.2% was found when the cells were treated with a high energy fluence of 483 J cm^{-2}. We believe that this insignificant photo-bactericidal effect of AuNPs was due to the generation of singlet oxygen what was previously described by Chadwick et al. [49].

In all our experiments it was observed that bactericidal effect of MB was light dose-dependent with the kill increasing as the energy fluence was increased from 17 J cm^{-2} to 483 J cm^{-2}. A high reduction in viability of planktonic cell culture in the presence of MB after exposure of 108 J cm^{-2}, 153 J cm^{-2}, 162 J cm^{-2}, 192 J cm^{-2} and 288 J cm^{-2} was obtained. The reduction in the number of viable cells reached 99.2%, 99.89%, 99.926%, 99.953% and 99.997%. A lethal inactivation of the planktonic culture (the count was below the detection limit) was found after exposure of 322 J cm^{-2} and 483 J cm^{-2}.

. When the MB + AuNPs mixture was used in experiments, after 5, 10, 15 and 30 min of laser irradiation (energy fluence of: 108 J cm^{-2}, 153 J cm^{-2}, 162 J cm^{-2} and 192 J cm^{-2}) the viable count showed a reduction by 99.728%, 99.969%, 99.998% and 99.9982%, respectively ($p < 0.05$).

A light dose of 292 J cm^{-2} was sufficient to achieve a lethal effect (the count was below the detection limit).

As shown above, the use of planktonic cultures for antimicrobial testing has produced very effective killing results that usually do not correlate with clinical findings. Compared to bacteria in suspension, the behaviour of microorganisms in biofilms is notably different and should be accounted for in laboratory tests. This is of particular importance as evidence has accumulated over the past few years that most chronic and recalcitrant bacterial infections in endodontic infections involve biofilms. Therefore, in the next set of our experiments, biofilm cells of

E. faecalis were studied. It is known that biofilms are more resistant to the photo-inactivation, hence high doses of laser light were applied (292 J cm^{-2}, 322 J cm^{-2} and 483 J cm^{-2}). A significant enhancement of photo-bactericidal activity was also found when the gold nanoparticles were combined with MB. The MB after irradiation of 292 J cm^{-2} showed a reduction in cfu of 94.1% kill compared with 99.91% kill for MB + AuNPs mixture of the same concentration. Higher light doses of 322 J cm^{-2} and 483 J cm^{-2} with MB as photosensitizer resulted in a reduction in cfu by 99.476% and 99.92%, respectively. At the same conditions, MB + AuNPs mixture was appeared to be more active as 99.92% and 99.991% kill was achieved.

SYTOX Green stain was used for a biofilm cells viability assessment. It is well known that SYTOX Green stain is a high-affinity nucleic acid stain that does not cross the membranes of live cells and yet easily penetrates cells with compromised plasma membranes. Nonviable bacteria are accessible to SYTOX Green and appear green (Fig. 7A–D). The micrographs of biofilm before and after irradiation with laser light (483 J cm^{-2}) showed that the highest mortality was observed with MB + AuNPs mixture as a photosensitizer, which is consistent with the results obtained by a serial dilution method.

The analysis of the obtained data according to Eqs. (1) and (2) confirmed that the biogenic nanogold synergistically enhances the kill of the bacteria studied, whilst having no intrinsic bactericidal activity.

A direct comparison of our results with those obtained by other authors is impossible due to different options of sensitizers and different concentrations as well as different light wavelengths, output power, irradiation time and application protocols [44]. For example, Perni et al. [50] found that gold nanoparticles of 2 nm size in combination with MB resulted in 2.2 $\log_{10}$ unit reduction of *S. epidermidis* in 10 min of laser irradiation, whilst only a reduction of 1.2 $\log_{10}$ resulted with MB and gold nanoparticles of 5 nm after the same irradiation time. No reduction in viability was observed when the largest gold nanoparticles (20 nm) were used. Those authors [51] described the formation of polysiloxane polymer capsules containing MB and gold nanoparticles. These capsules show a significant antibacterial properties against methicillin-resistant *Staphylococcus aureus* and *Escherichia coli* with up to a 3.5 $\log_{10}$ reduction in the viable count, when exposed for 5 min to light from a 660 nm laser. In another study, Naik et al. [52] demonstrated that polyurethane polymer sheets embedded with MB and 2 nm gold nanoparticles exhibited bactericidal activity against *Staphylococcus aureus* resulted in a reduction in cfu of 3.8 $\log_{10}$ unit. Our previous paper [31] showed a lethal photosensitization of *S. epidermidis* with a mixture of methylene blue and gold nanoparticles after 5 and 10 min exposure to a laser or Xe lamp.

The detailed mechanism responsible for enhancement of photo-bactericidal effect by gold nanoparticles has not yet been discovered, although many hypotheses have been discussed in the literature. Most often it is suggested that the presence of AuNPs changes the relative distribution of ROS agents or increases their production. It should be noted that various mechanisms can be involved in the killing of bacteria, e.g. local increase in the concentration of the photosensitizer through targeted delivery of nanoparticles, selective interaction with bacterial cell wall, and resonant heating of AuNPs in irradiation with laser light [53].

In our studies it has been shown that MB + AuNPs mixture exhibits a bactericidal activity against planktonic cells of *E. faecalis* higher than MB alone and it was determined that the MBC value was 173 mg L^{-1} and 86.4 mg L^{-1} for MB and MB + AuNPs, respectively *(see: Supplementary Information,* Fig. S6). On the other hand, it seems that the highest doses of laser light (292 J cm^{-2}, 322 J cm^{-2} and 483 J cm^{-2}) can cause the heating of the gold nanoparticles resulting in the enhanced bacteria kills. Worth noting is the fact that the light wavelength range exploited for illumination does not fit the plasmonic absorption band of the gold nanoparticles thus the heat production was not very effective. The explanation of this phenomenon requires additional studies which are currently carried out in our laboratory.

5. Conclusions

In conclusion, the biogenic AuNPs formed by the cell-free filtrate of *T. asperellum* were applied as successful enhancers of planktonic and biofilm cultures of *E. faecalis* photosensitization. The lethal effect of planktonic cells was found for MB after 30 min of laser irradiation with light dose of 322 J cm^{-2}. When the mixture of MB and AuNPs was used as photosensitizer the lethal effect was achieved with light dose of 292 J cm^{-2}. The biofilm culture was more resistant to photo-inactivation and the best bactericidal effect for MB was found after light dose of 483 J cm^{-2} and it was a reduction in cell viability of 99.92%.

The MB + AuNPs mixture synergistically enhances the kill of the studied microorganism as the same light dose resulted in 99.991% kill. It should be noticed that the American Society of Microbiology has decreed that any antimicrobial technique is required to kill a minimum of 3 logs of CFU (99.9%) in order to be accepted as “antimicrobial”. Thus, we have proved a synergistic efficacy of the *E. faecalis* killing when the MB + AuNPs mixture was applied as photosensitizing agent.

Conflicts of interest

The authors declare that they have no competing interests.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

This work was partially financed by a statutory activity subsidy from the Polish Ministry of Science and Higher Education (PMSHE) for the Faculty of Chemistry of Wrocław University of Science and Technology.

We gratefully acknowledge professor Marek Samoć (Wrocław University of Science and Technology, Faculty of Chemistry, Advanced Materials Engineering and Modelling Group) for review of this paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.pdpdt.2019.05.042.

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