



Viability study of antimicrobial photodynamic therapy using curcumin, hypericin and photogem photosensitizers in planktonic cells of *Streptococcus mutans*

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Abstract

Aims: Investigate the potential of curcumin, hypericin and photogem as antimicrobial agents in the antimicrobial photodynamic therapy (APDT) in *Streptococcus mutans* planktonic cultures.

Methods and Results: Susceptibility of *S. mutans* (ATCC 25175) to APDT using three concentrations of curcumin (250, 750 and 1000 mg l⁻¹), hypericin (1, 5 and 10 mg l⁻¹) and photogem (1, 5 and 10 mg l⁻¹) was examined by colony-forming units method. Separated specimens of each group were irradiated using specific wavelengths (450 ± 10 nm, 590 ± 10 nm and 630 ± 10 nm). Results demonstrate absence of antibacterial effects for the groups that didn't have the association of light with photosensitizers. Antibacterial effects were observed in the groups using photogem and hypericin. When compared with the positive control, the observed reductions were in the order of 6-logs up to the eradication of the treated cells. It is possible to co-relate the increase of photosensitizer concentration and irradiation time with higher antibacterial effect.

Conclusions: APDT have potential to be used as an oral disinfectant method.

Significance and Impact of the Study: APDT can be used prior to the execution of any oral treatment to decrease the dentist and staff exposure to a big bacterial load during the subsequent treatments and to lower the potential of environmental cross-contamination.

Introduction

From 1930's onwards, antibiotics became the medicine of first choice on the treatment of several bacterial infections.^[1] At the beginning of its usage, these medicines were so efficient that some "incurable diseases," like tuberculosis, were completely eradicated in U.S. at that moment in time. In the late 60's some claimed "it was about time to stop with all research related to bacterial infectious diseases,"^[2] but its indiscriminate usage led to the appearance of resistant bacterial strains and frequent reports of antibiotic treatment failure.^[3,4] Hence in function of this current problem, it becomes important and, at the same time necessary, the development of new medicines, techniques or

strategies to overcome the development of resistant strains due to the usage of antibacterial treatments.

Photodynamic therapy (PDT) is a technique often used at the medical field to treat several types of cancers. Its mechanisms of action are based on the generation of reactive oxygen species (ROS) due to the interaction between visible light (at specific wavelengths) with non-toxic photosensitizers and tissue oxygen.^[5] The free radicals generated by this interaction will promote the oxidation of organic molecules without the need of a specific metabolic pathway or target structure.^[6] This non-specific mechanism of action places the PDT as a potential technique to inactivate numerous bacteria without the development of new resistant strains.

In the dentistry field, PDT can also be known as antimicrobial PDT (APDT)^[5] or as photodynamic antimicrobial chemotherapy.^[7] This technique has been used with some degree of success in the treatment of oral infections such as dental caries and periodontal diseases. Usually the photosensitizers used to this end are methylene blue, toluidine blue and Bengal rose. However, depending on the concentrations and types of the photosensitizer used, this treatment can induce to the occurrence of severe discoloration of the dental elements submitted to the treatment. This adverse effect makes the wide spreading of such technique difficult or even impossible because it can alter significantly the patient's esthetics.

In that way, it is important to assess the potential of novel photosensitizers such as curcumin and hypericin to be used as a viable alternative to the current photosensitizers. The objective of this study was to assess the antimicrobial potential of the photosensitizers hypericin, photogem and curcumin in planktonic cultures of *S. mutans*. The APDT protocols (named as "treatments") used in this study were arbitrarily determined in function of the maximum wavelength absorption of each one of the photosensitizers assessed and in function of their individual dilution needs. The null hypothesis is that none of the antimicrobial photodynamic treatments investigated will be able to reduce the initial microorganism population and that all photosensitizers herein investigated have the same antimicrobial efficacy.

Materials and Methods

A controlled *S. mutans* strain (ATCC-25175) was acquired from the Adolfo Lutz institute (Sao Paulo, SP). This was reactivated in 5 ml of BHI (Brain Heart Infusion [BHI], Lansing, MI, USA) in microaerophilic jar at 37°C (Microbial Incubator - 410 NDRE, Vargem Grande Paulista, SP, Brazil) during the period of 18 h. After the growth, the suspension was centrifuged (Centrifuge - Revan Ciclo, CI 014117, Campinas, Sao Paulo, Brazil) using 3000 rpm for 5 min, and immediately after, the cells were washed 2 times with sterile phosphate buffer saline.

After the reactivation of the cells, these were cultivated overnight in an appropriate incubator (Microbial Incubator - 410 NDRE, Vargem Grande Paulista, SP, Brazil) in temperatures of 37°C during a period of 16 h. Following, the resultant material had its turbidity adjusted visually in order to obtain a turbidity of half of the first grade of McFarland's scale, which can be understood as an initial population of 3×10^8 UFC ml⁻¹.

To all photosensitizers studied (curcumin, hypericin and photogem), the Groups 1 through 3 were constituted by the following controls: G1 Positive growth control Planktonic suspension of *S. mutans* without any treatment; G2 Alcohol control-50 µl of alcohol 98° (mandatory to dilute the hypericin photosensitizer); G3-500 µl of cloridrate of clorhexidine 2%. The "treatments" were then divided into 15 experimental groups (G4-G18) to each photosensitizer as follows

Following, each one of the selected photosensitizers were prepared individually into three different

concentrations, as follows: (i) curcumin-1500 mg l⁻¹; 750 mg l⁻¹ and 250 mg l⁻¹; (ii) Hypericin-10 mg l⁻¹; 5 mg l⁻¹ and 1 mg l⁻¹ and (iii) Photogem[®]-10 mg l⁻¹; 5 mg l⁻¹ and 1 mg l⁻¹. After the completion of the dilution step, the photosensitizers have reached its therapeutic concentrations and were stored in test tubes wrapped with aluminum foil (to prevent photosensitizers' photobleaching), after that, the photosensitizers were stored in a refrigerator at the temperature of 4°C for 10 min. Each photosensitizer was removed from refrigeration only immediately before its use in the experiment.

The irradiation of the samples was possible through the usage of a prototype device based on the light-emitting diodes (LED) technology, this device was named as biotable (technology support laboratory-IFSC-USP, Sao Carlos, SP, Brazil) and it was designed and built specifically for this project. This Biotable had one control unit [Figure 1a] and three interchangeable irradiation heads (schematic drawing - [Figure 1c]). The wavelengths used were selected in accordance with the maximum absorption spectra of each one of the photosensitizers, as follows: Curcumin (450 ± 10 nm - Blue light), hypericin (590 ± 10 nm - Amber light) and photogem (630 ± 10 nm - Red light). The output power used for each photosensitizer were, respectively, 45 mw/cm², 55 mW/cm² and 35 mW/cm² and the final energy dose (J/cm²) in each experimental situation was obtained as being the product of the power output by the irradiation time divided by the irradiation area.

With the basic experimental requirements completed, 500 µl aliquots were separated from the overnight cultures, each 500 µl aliquot were then individually placed into separated wells of a 24 wells plate. After that, separated aliquots of 100 µl of the photosensitizers as described in Table 1 were added to the wells containing the microorganisms. Immediately after that, the plates were individually placed within the specific irradiation head [Figure 1b], until the completion of the irradiation time proposed in each one of the experimental situations here investigated.

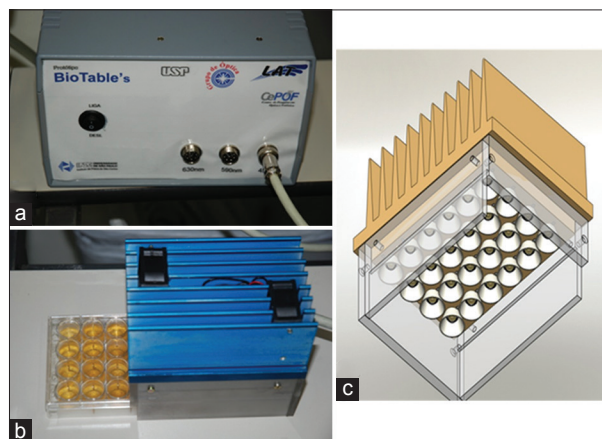


Figure 1: (a) Controller unit of the prototype irradiation device; (b) Irradiation head with samples being positioned and (c) Solidworks® drawing of the irradiation head

Table 1: Distribution of samples in function of each one of the photosensitizers used

Curcumin			Hypericin			Photogem		
Group	Concentration (mg/l)	Energy dose (j/cm ²)	Group	Concentration (mg/l)	Energy dose (j/cm ²)	Group	Concentration (mg/l)	Energy dose (j/cm ²)
G4	-	40	G4	-	1	G4	-	40
G5	-	80	G5	-	3	G5	-	80
G6	-	120	G6	-	6	G6	-	120
G7	250	-	G7	1	-	G7	1	-
G8	750	-	G8	5	-	G8	5	-
G9	1500	-	G9	10	-	G9	10	-
G10	250	40	G10	1	1	G10	1	40
G11	250	80	G11	1	3	G11	1	80
G12	250	120	G12	1	6	G12	1	120
G13	750	40	G13	5	1	G13	5	40
G14	750	80	G14	5	3	G14	5	80
G15	750	120	G15	5	6	G15	5	120
G16	1000	48	G16	10	1	G16	10	48
G17	1000	80	G17	10	3	G17	10	80
G18	1000	120	G18	10	6	G18	10	120

After that, the content of each well, from each one of the 24 wells plates used, were individually removed and placed in separated test tubes and were then homogenized for 1 minute using a vortex (Quimis-SS Labor Equipamentos e produtos medicos, Diadema, SP, Brazil). Following the homogenization step, 50 µl aliquot of each one of the test tubes were plated in Petri dishes containing culture medium (BHI with Bacto Casitone-Becton, Dickinson and Company, Sparks, MD - USA) to promote the growth of *S. mutans* colonies.

The inoculated petri dishes were then placed in microaerophilic microbiological jars that were incubated at 37°C in a controlled incubator during the period of 48 h. At the end of that time, the jars containing the petri dishes were removed from the incubator and the antimicrobial effect of the PDT was assessed by visual analysis by counting the colonies that grew on the Petri dishes. The experiment was performed in duplicate following standard and consecrated microbiological procedures described elsewhere.

Results

The Graphs 1 through 3 present the mean and standard deviation values of the observed bacterial growth for all the situations proposed in this study (G1-G18 for each photosensitizer). The obtained results were plotted comparatively with the control groups (G1 - positive control - no treatment; G3 - negative control - 2% Clorhexidine) for better observation of the performance of each one of the APDT treatments herein investigated [Graph 1].

It can be observed from the chart displayed above that the treatment using cloridrate of clorhexidine 2% (G3 - negative

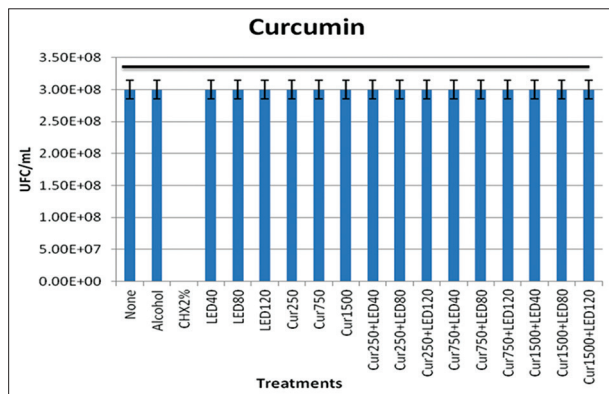
control group - 2% clorhexidine) was the only effective treatment against the studied microorganisms. All the other treatments proposed were not effective and presented growth values comparable with the values obtained for the positive control group (G1 - positive control - no treatment) [Graph 2].

The results obtained for the hypericin photosensitizer demonstrate clear evidences of the antimicrobial action of the proposed "treatments." It is possible to be observed that all APDT treatments were able to reduce significantly the initial bacterial population. It is also possible to observe that the hypericin treatments with concentrations of 5 mg/l and 10 mg/l presented comparable growth values with the negative control group (G3 - negative control group - 2% clorhexidine) [Graph 3].

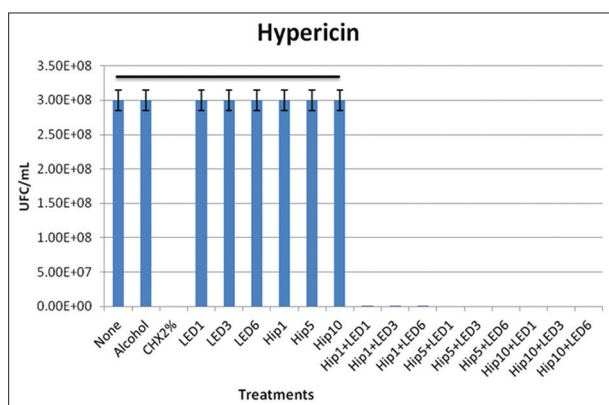
The results obtained for the groups that used photogem as the photosensitizer demonstrated that, with exception of the most aggressive APDT treatment (highest concentration and highest energy dose) used, no antibacterial action was possible to be noticed for the remaining treatments. Significant reduction levels of the initial population were observed with the treatment G18 (Pho10+LED120).

Discussion

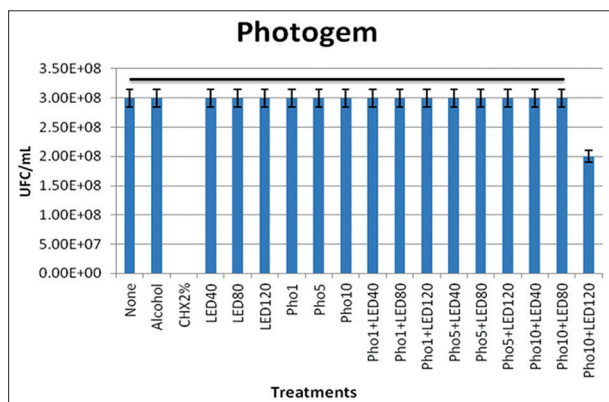
It is estimated that the oral microcosm holds from 700 to over a 1000 different microorganisms in its normal bacterial flora.^[8] *S. mutans* is one of the most investigated bacterial strains in the field of dentistry, and currently, these microorganisms are considered as one of the main pathogens agents related to the process of dental decay development.



Graph 1: Effect of the antimicrobial photodynamic therapy on the growth of *Streptococcus mutans* (UFC/ml) using the photosensitizer curcumin in function of concentration (mg/ml) and energy dose (j/cm^2). Horizontal bar denote no significant differences among treatments



Graph 2: Effect of antimicrobial photodynamic therapy on the growth of *Streptococcus mutans* (UFC/ml) using the photosensitizer hypericin in function of concentration (mg/ml) and energy dose (j/cm^2). Horizontal bar denote no significant differences among treatments



Graph 3: Effect of antimicrobial photodynamic therapy on the growth of *Streptococcus mutans* (UFC/mL) using the photosensitizer Photogem® in function of concentration (mg/mL) and energy dose (J/cm^2). Horizontal bar denote no significant differences among treatments

The world health organization reports that dental caries holds the third position in the ranking of diseases that most affect human beings (incidence around of 90% worldwide).^[9] The treatment of such affection consists on the removal of the affected tissue and the replacement of it using restorative materials like amalgam or resin composites. It is estimated that billions of dollars are spent annually in the U.S. Alone in the treatment of dental decay. Therefore, is of critical importance for the modern dentistry, the development of ultraconservative and minimally invasive strategies capable of promoting oral decontamination and prevent biofilm formation.

In this context, arises as an interesting alternative the use of APDT.^[10,11] Its mechanism of action is based on unspecific oxidation of organic molecules mediated by a generation of ROS.^[12,13] The literature reports that is unlikely to observe the appearance of resistant bacteria strains resulting from the use of this treatment because its mechanisms of action does not depend on a target metabolic pathway or of an electronic affinity.^[13]

The objective of this work was to assess the antibacterial efficacy of APDT using three photosensitizers in planktonic cultures of *S. mutans*. The results clearly demonstrate that independently of the concentrations tested, the photosensitizers curcumin, hypericin and photogem when used without light irradiation presented growth values similar to those observed on the positive control group (G1-positive control-no treatment), and therefore did not present enough toxicity that could promote bacterial death.

These findings are corroborated by Wilson *et al.*,^[14] that in a similar study assessed the bactericidal effect of the PDT using 27 types of photosensitizers in different concentrations over the microorganisms *Streptococcus sanguis* (NCTC 10904), *Porphyromonas gingivalis* (W50), *Fusobacterium nucleatum* (NCTC 10562) e *Actinobacillus actinomycetemcomitans* (Y4) using visible light ($632.8 \pm 5 \text{ nm}$) with low power intensity ($7.3 \text{ mW}/\text{cm}^2$) that was emitted by a HeNe laser system. In this study, the authors reported that with exception of the phthalocyanine photosensitizer, all others photosensitizers tested, did not showed noticeable bactericidal effect when used without the irradiation with the proper wavelengths, in addition, the authors also reported that the best levels of bacterial death were attained with the protocols that used the toluidine blue and Azure A chloride photosensitizers, once that these have their maximum absorptions peaks at 632.2 nm and 632.4 nm respectively, and are closely located to the wavelength emitted by the HeNe laser system.

The results obtained for the groups that used only light as the treatment to promote bacterial death were comparable with the values observed for the positive control group (G1-positive control-no treatment), which means that the wavelengths tested, in the conditions described in the materials and methods section (output power and irradiation time), did not have enough energy to promote cell damage and therefore cannot be used by themselves to promote oral decontamination.

Konopka and Goslinsk^[5] published an interesting review paper that also corroborates our results. In that particular study,

the authors evaluated several different aspects of the use of PDT in dentistry. The authors concluded that the photodynamic reaction can only be observed when the photosensitizers are used together with the proper wavelengths in each specific situation.

Different reduction levels of the bacterial growth could be observed on the experimental PDT groups (light irradiation + photosensitizers) when compared with the positive control group (G1 - positive control - no treatment). Independently on photosensitizer concentration and of the energy dose used, all photodynamic groups that assessed the use of Curcumin as photosensitizer, presented results comparable with the values observed for the positive control group (G1 - positive control - no treatment). Two hypotheses are possible to occur when we consider the basic mechanism of action of PDT. The first one is that small amounts of singlet oxygen may be being generated during the irradiation of the samples, simply because this photosensitizer can present low quantum yield. The second hypothesis is that the concentrations and energy doses chosen in this investigation were located under the workable threshold. We believe that in this situation the cell death will depend on a probabilistic relation between the amount of singlet oxygen generated and the amount of cells in the inoculum.

This hypothesis can be sustained by the work published by Dahl^[15] that investigated the oxidation capability of singlet oxygen on Gram-positive and Gram-negative bacteria. The authors concluded that this extremely reactive free radical presents a very short lifetime (in order of 0,04 μ s) and low diffusion capacity. According with the authors, these two factors acting together limit significantly the activity action radius of this free radical. The authors also concluded that despite this important characteristic this radical is able to oxidize bacteria in both of the gram classifications and showed lower efficacy for the Gram-negative species.

In order to increase the antibacterial behavior of the PDT, some reports in the literature described the use of a pre-irradiation incubation time of 5-10 min to promote a higher uptake of the drug by the bacteria.^[16] According with these authors, these incubation times were able to increase significantly the levels of the observed bacterial death when compared with the situation when no pre-irradiation time was used.

On the other hand, the photodynamic treatments that used hypericin as photosensitizer showed the best results of bacterial inactivation and were able to reach reduction levels comparable with the results found on the negative control group (G3 - negative control - 2% Chlorhexidine). These findings suggest that the success of APDT treatments in planktonic cultures of *S. mutans* depends directly on the type and concentration of the photosensitizer used.

These results are corroborated by Paulino *et al.*^[17] That by using a metabolic assay observed the influence of this therapy on the viability of *S. mutans* and on fibroblasts cell cultures. The photosensitizer used was Bengal rose and the irradiation device was a hand held light curing unit (with spectral band of 400-500 nm). Their results demonstrated the eradication of the studied microorganism without affecting the fibroblast culture of cells.

Bevilacqua *et al.*^[18] furthermore sustains the findings of the present study. The authors investigated the bactericidal effect of the PDT over planktonic cultures of *S. mutans* using toluidine blue as photosensitizer and a LED irradiation device emitting light at 640 ± 20 nm in low power intensity regimes. Their findings demonstrated that the PDT was capable to eradicate the studied microorganisms.

More recently Zou *et al.*^[19] Investigated the bactericidal potential of the APDT in the decontamination of *S. mutans* biofilms using a hematoporphirin derivative hematoporphyrin monomethyl ether as photosensitizer and visible light generated by a diode laser in low energy density levels. In this paper, the authors concluded that PDT can be a viable alternative to being used in the treatment and control of dental plaque related diseases.

Our findings demonstrated that hypericin photosensitizer had the highest antibacterial efficacy against *S. mutans* planktonic cells. Although some promising results were presented, we believe that more studies are still necessary to assess possible biocompatibility issues of the use of such photosensitizer and irradiation protocols before this therapy can be used as a co-adjuvant treatment in the daily clinical routine. In addition, we anticipate that once these kinds of tests have been completed, the APDT using hypericin as photosensitizer will have the necessary potential to be used as a professional technique to promote oral decontamination prior to any other dental treatment. We also believe that further work have to be done in order to investigate the bactericidal effect of this photosensitizer in *S. mutans* biofilms and in multi-species biofilms.

Conclusions

From the photosensitizers and APDT treatments used, only hypericin showed significant inhibitory effect against the *S. mutans* in planktonic phase, which demonstrates that, the irradiation of such photosensitizer with visible wavelength ($590 \text{ nm} \pm 5 \text{ nm}$) during the periods of 1'33" and 3'15" has potential to be used in the promotion of the microbial control of *S. mutans* present in saliva. Also, we conclude that both null hypotheses presented are false.

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