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The potential of commercially available phytotherapeutic compounds as new photosensitizers for dental antimicrobial PDT: A photochemical and photobiological *in vitro* study

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ABSTRACT

The present study evaluated the effectiveness of extracts of commercially available *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* as photosensitizers in Antimicrobial Photodynamic Therapy (aPDT). Each photosensitizer (PS) was analyzed in a spectrophotometer between 350 and 750 nm to determine the ideal light source. Once the absorption bands were determined, three light sources were selected. To determine the concentration of use, the compounds were tested at different concentrations on bovine dentin samples to evaluate the risk of staining. Once the concentration was determined, the PSs were evaluated for dark toxicity and phototoxicity on fibroblast and bacteria culture. Each compound was then irradiated with each light source and evaluated for the production of reactive oxygen species (ROS). The bacterial reduction was tested on *E. faecalis* culture in planktonic form and on biofilm using an energy of 10 J and an Energy Density of 26 J/cm^2. The tested compounds exhibited light absorption in three bands of the visible spectrum: violet (405 nm), blue (460 nm) and red (660 nm). At a 1:6 concentration, none of the compounds caused tooth staining as they did not exhibit significant toxicity in the cells or bacterial suspension. Additionally, significant ROS production was observed when the compounds were irradiated at each wavelength. When aPDT was performed on the plactonic and biofilm bacteria, significant microbial reduction was observed in both cases, reaching a reduction of up to 5Logs. In conclusion, extracts of *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* exhibited potential for use as photosensitizing agents in aPDT.

1. Introduction

Light has been used as an antimicrobial agent since ancient times, and one possible use is in antimicrobial Photodynamic therapy (aPDT). Such strategy involves the use of a light source and photosensitizer (PS). When irradiated, the excited PS reacts with a substrate, mostly oxygen, and reactive oxygen species (ROS) are produced, such as singlet oxygen, hydroxyl radicals and superoxide, which induce irreversible damage to microorganisms [1]. aPDT is considered a promising method for eradicating pathogenic bacteria, with some PSs, such as methylene blue, being currently tested in combination with low-intensity light sources to produce a bactericidal effect [2]. aPDT has proved efficacious against different classes of microorganisms, such as Gram-positive, Gram-negative bacteria and yeasts [3].

Many *in vitro* and *in vivo* studies have demonstrated the efficacy of aPDT to treat localized microbial infections, and has therefore been widely used in dermatology [4] and dentistry [5], where infections are usually localized and not deep within the tissues, facilitating access to light and photosensitizers; aPDT is also recognized as a minimally invasive and non-toxic treatment strategy [6,7]. In oral infections, aPDT has demonstrated promising results for the elimination of microorganisms that cause diseases, such as endodontic infections [2,8,9], periodontitis [10,11], dental cavities [12,13] and periimplantitis [14].

Different PSs have been used in aPDT, such as Methylene blue [8], Toluidine blue [15], Rose Bengal [16], Azulene [17], Erythrosine [18] and Malachite green [19]. These agents have, however, exhibited side effects and limitations when applied to dental tissues, especially teeth staining [20], as they are all dyes.

New PSs that are safe and have fewer side effects [21] are therefore required, especially in dentistry, as teeth staining may cause aesthetic

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issues for most patients and professionals [22,23].

In the wake of growing interest in complementary and alternative medicine, herbal extracts and phytotherapeutic compounds (PCs) have recently attracted a great deal of attention. Herbal products have been used in popular medicine since ancient times, featuring in medical traditions all over the world [24]. In India and Egypt, for example, the seeds from *Psoralea corylifolia* L. have been used for the treatment of vitiligo. *Ammi majus* L. is applied to areas of skin affected by the disease before exposure to sunlight [25]. In recent years, therefore, the pharmaceutical industry has developed considerable interest in exploring plants as sources of new phytotherapeutic agents, [26].

With an increasing incidence of antibiotic overuse, many herbal compounds with medicinal and antimicrobial properties have been studied. Research into the use of PCs against multiresistant bacteria could contribute to the development of new drugs [26].

The present study investigated the photochemical, antimicrobial and cytotoxic effects of different types of PC used as a new photosensitizer in antimicrobial photodynamic therapy in dentistry.

2. Materials and methods

This research was conducted in accordance with the ethical principles of animal experimentation. The Animal Research and Ethics Committee of the São Leopoldo Mandic Research institute, Brazil, evaluated and approved the study.

2.1. Phytotherapeutic compounds

Four commercial types (herbal extracts) of *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* were purchased from a local market (EBPM Comercial Ltda) and further standardized at a concentration of 0.4 mol/L. As control, Methylene blue (MB) was purchased from Sigma-Aldrich and prepared as an aqueous solution. Following preparation, all the compounds were stored in darkness and refrigerated until use.

2.2. Optical characteristics of the phytotherapeutic compounds

The optical characteristics of the PCs were obtained *via* absorbance spectra in a spectrophotometer (Spectrum SP 2000 UV) using 1.0 cm optical pathlength quartz cuvettes. Their absorption spectra were recorded at wavelengths of 350–700 nm.

2.3. Maximal compound concentration

To determine the maximal concentration that could be used on dental tissue, serial dilutions of each PC was tested on bovine teeth. Samples of dentine and enamel measuring 5 × 5 x 5 mm were prepared from the crown surface of the teeth and cleaned with EDTA-T 17% (Fórmula e Ação, Brazil) for 1 min and then 1% NaOCl (Biodinamica, Brazil).

Prior to staining, the teeth samples were evaluated with a portable spectrophotometer (Color digital sensor – Easyshade Advanced 4.0 Vita-Wilcos) to identify their initial shade using the Vitapan Classical dental shade scale.

After establishing the initial shade, the samples were submerged into each tested compound solution with concentrations varying from 1:1 to 1:10 (n = 5). All samples were placed in contact with the PC for 15 min, simulating the clinical application of aPDT in an endodontic or periodontal context.

The samples were then cleaned in distillated water for 1 min, dried for 10 min at room temperature and evaluated for tooth staining using the portable spectrophotometer.

The maximal concentration without any sign of tooth staining was selected for use in subsequent experiments.

2.4. Dark toxicity and phototoxicity

Fibroblasts from the gingival mucosa were obtained from explants of healthy human attached gingiva. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St Louis, MO, USA) supplemented with 1% antimycotic solution (Sigma) containing 10% fetal bovine serum (Cultilab, Campinas, SP, Brazil). The cultures were incubated at 37 °C in a humidified environment (5% CO_2 / 95% air) and assessed every 24 h. Once the cells reached subconfluence, they were harvested with trypsin and subcultured. The fibroblast cultures used in the following procedure were from passages three to six for all experiments. The cells were maintained in a cell culture bank and used according to the previously described cell culture method [27].

The cells were then seeded onto 60-mm diameter plastic culture dishes (110 cells/mm^2) and incubated under standard cell culture conditions (37 °C, 100% humidity, 95% air and 5% CO_2). Once 100% confluence was reached, the cells received 100 µl of each PC (at 0.4 M) in dark conditions and were also irradiated with a total of 10 J of energy (using 3 different light sources), as described below. Dark toxicity and phototoxicity were measured after 30 min and 24 h, for both experiments.

PBS solution (negative control group), alcohol solution at 60% (PC vehicle – positive control) and Chlorhexidine solution at 0.12% (Colgate, Brazil) or sodium hypochlorite at 1% (Biodinâmica, Brazil) were also tested to provide a comparative analysis of irrigation solutions commonly used in dental treatments.

2.5. MTT assay

Cell viability was assessed using the 3-[4,5-dimethylthiazol- 2-yl]-2,5- diphenyl tetrazolium bromide (MTT, Sigma) method at 30 min and after 24 h of culture. The cells were incubated with MTT at 10% in culture medium (5 mg/mL) at 37 °C for 4 h. The MTT solution was then discarded using aspiration, and 200 µL of Dimethyl Sulfoxide (Sigma) was added to each well, followed by gentle agitation for 5 min on a plate shaker. Subsequently, 150 µL from each sample solution was transferred onto a fresh 96-well plate. Optical density was assessed on a spectrophotometer at 570 to 650 nm (Epoch; Bio-Tek), and the data were expressed as absorbance levels.

2.6. Light sources

Once the absorption band data of each compound from the previous experiments was available, the light sources were selected.

Based on the absorption bands of each PC, three different common light sources, regularly found in a dental office, were selected: a dental cure LED – Radii (SDI, Australia) with 1200 mW output power emitting at 460 nm, a dental bleaching arch (Brightmax - MMOptics, Brazil) with 700 mW, emitting at 405 nm and an LED light (Hellen - Coxo, China) with 120 mW, emitting at 660 nm.

The irradiation time was calculated according to the power output of each light source to provide a total of approximately 10 J of energy (Table 1).

Table 1
Light parameters.
Source: own authorship.

Parameters	BrightMax MMOptics	Radii-SDI	Hellen-Coxo
Wavelength	405 nm (± 10 nm)	460 nm (± 20 nm)	660 nm (± 20 nm)
Power output	700 mW	1200 mW	120 mW
Energy	10 J	10 J	10 J
Irradiation time	15 s	9s	90s

2.7. Reactive oxygen species detection

The aPDT method is based on the production of reactive oxygen species, which causes damage to microorganisms; the mechanism of action occurs when the photosensitizer agent absorbs the light, transfers its energy to oxygen, and produces highly reactive oxygen species.

To quantify the reactive oxygen species generated by aPDT, the following *in vitro* experiment was performed. Each of the PC and MB solutions were tested individually over time. In a quartz cuvette (1 cm optical path), 3 mL of each PC and MB at 0.4 M in distillate water was irradiated using the three light sources. The optical density of *N,N*-dimethyl-4-notrosoaniline (RNO) at 13.3 μM in the presence of 15-mM L-histidine was analyzed in a spectrophotometer (Spectrum SP 2000 UV) at 440 nm, following each irradiation (energy of 10 J for each light source). For the irradiation the tip of the light source was positioned over the top of the cuvette, parallel to its long axis [28].

2.8. Bacterial strain and growth conditions

Enterococcus faecalis ATCC 29212 was used in the present study. Microorganisms were maintained through subculturing on brain heart infusion (BHI; Difco, Detroit, MI). Culture was aerobically grown in BHI broth at 37 °C for 24 h. Test tubes with 0.5 mL BHI were contaminated with 0.5 mL of *E. faecalis* in suspension and incubated at 37 °C for 24 h, giving a final cell concentration of 4×10^7 CFU/mL.

2.9. Planktonic bacterial reduction

Test tubes containing 0.5 mL BHI broth were contaminated with 0.5 mL of *E. faecalis* suspension and incubated at 37 °C for 24 h, giving a final cell concentration ranging from 10^8 to 10^9 CFU/mL. The samples were centrifuged and the bacteria were resuspended in PBS solution.

The test tubes containing the bacterial suspension were incubated with 1 mL of each PC, MB solution (each at 0.4 M) or saline solution for 10 min in dark conditions. Irradiation was performed from the bottom to the top, in test tubes using each of the light sources as described in Table 1.

After the pre-irradiation period and irradiation, 100 μL aliquots were collected for serial dilution and streaking on BHI agar plates for the counting of colony forming units (CFUs). All the surveys were performed using three test tubes in each group.

2.10. Bacterial reduction in biofilm

To grow the biofilm, dental composite discs measuring 10 mm in diameter and 1 mm in thickness were made using orthodontic elastomeric rings (Orthometric, Brazil) as a mold. The composite discs were then sterilized in a steam autoclave (121 °C for 15 min).

The sterile samples were submerged into a petri dish containing 20 mL of BHI broth contaminated with 1 mL of the bacterial suspension for 7 days at 37 °C. The BHI broth was replaced every 24 h, allowing the formation of biofilm. The samples were then rinsed with saline solution to remove nonadherent cells from the biofilm and randomly divided into each group: G1 - Curcumin, G2 - Lemon, G3 – Hamamelis, G4 – Hypericum and G5 – Methylene Blue.

The samples from each group (n = 5) received 100 μl of photosensitizer for 1 min and were irradiated with each light source, receiving a total of 10 J of energy.

After irradiation, the samples were placed inside a sterile 1.5 mL microcentrifuge tube, which was subsequently sealed and vortexed for 1 min. It was then challenged with low-output ultrasonication to unbind the biofilm. One-hundred-μL aliquots were added to the wells of a 96-well plate for serial dilution and streaking on square BHI agar plates. The plates were incubated using standard aerobic conditions at 37 °C for 24 h for colony-forming unit (CFU) counting. The mean values from the samples in each group were then computed.

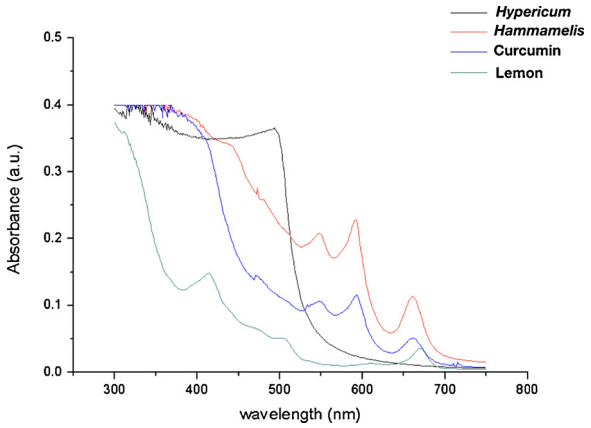

Fig. 1. Absorption spectra of Lemon, Hypericum, Hamamelis and Curcumin in 0.4 M alcoholic solution.

The medians and means of the L, b, a and Vitapan scale values for tooth shade, cell viability and bacterial count were calculated with the corresponding standard deviations. The mean tooth shade values and bacterial counts (in CFU per milliliter) from each group were tested for significant differences by ANOVA followed by the Tukey test. For cell viability, two-way ANOVA was applied followed by the *post hoc* Bonferroni test. $P < 0.05$ was considered statistically significant. Statistical comparisons between the means were performed on Graphpad Prism 5 software package (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Absorption spectra and light sources

The absorption spectra of *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* are shown in Fig. 1. The analysis revealed two main absorption bands, which match the light sources most commonly found in a dental office. It was possible to identify a large absorption band covering the UV and blue wavelengths and another covering the red to IR spectrum.

According to the absorption bands, the three light sources selected were: a dental bleaching arc with LEDs emitting at 405 nm, an LED based curing appliance emitting at 460 nm and an LED photobiomodulation device emitting at 660 nm.

3.2. PC concentration

The maximum concentration assay showed that concentrations of all the PCs lower than 1:6 or around 0.4 M did not result in any significant staining to dentine or enamel tissue, when evaluated using the Vitapan scale or L, B and A values (data not shown). As this was the maximum concentration that did not stain the tooth tissue, all subsequent experiments used this concentration for photochemical or microbiological analysis.

3.3. Dark toxicity - fibroblasts cells

After both periods of contact, 30 min or 24 h, all the PCs at a 0.4 M concentration exhibited lower cytotoxicity when compared to 1% sodium hypochlorite, 60% alcohol or 0.12% Chlorhexidine. Furthermore, *Hamamelis* and *Hypericum* exhibited similar or higher cell viability than the saline solution used as a control after 30 min. The results therefore showed that the PCs are safe to use intraorally, as seen in Fig. 2

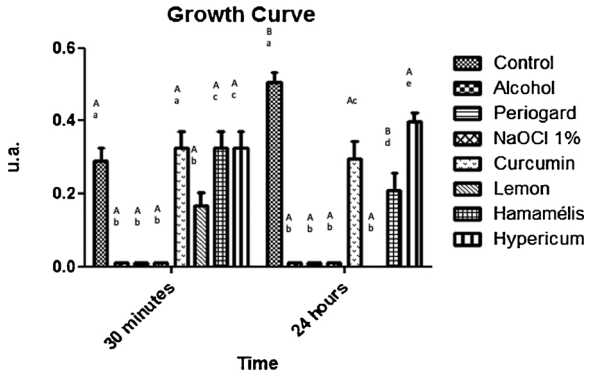

Fig. 2. Viability of fibroblast cells when exposed to chlorhexidine 0.12%, alcohol 60%, sodium hypochlorite 1%, Lemon, Curcumin, Hamamelis and Hypericum compounds at 70 μM in dark conditions.

Table 2
Dark toxicity in planktonic bacteria.
Source: own authorship.

Phytotherapeutic compounds	Bacterial reduction (log10)
PBS	0.5
Alcohol	1
Curcumin	1.5
Lemon	0.8
Hamamelis	0.5
Hypericum	0.8

3.4. Dark toxicity - E. faecalis

Fig. 2: Viability of fibroblasts when exposed to 0.12% chlorhexidine, 60% alcohol, 1% sodium hypochlorite and Lemon, *Hypericum*, *Hamamelis* and curcumin compounds in alcohol solution at 0.4 M.

3.5. Dark toxicity – E. faecalis

After the 10-minute period that simulated the total aPDT time in periodontal or endodontic treatment, none of the PCs alone (at 0.4 M) caused a significant reduction in bacteria (Table 2).

Phototoxicity - Fibroblasts

When irradiated with 10 J of energy, most of PC showed a decrease in cell count after 30 min and also after 24 h. When compared to phototoxicity in *E. faecalis*, however, none of the PC tested showed a cytotoxic effect that resulted in significant cell death.

On average, the bacterial reduction was greater than 3 log and, in some cases, such as *Hypericum* or *Hammamelis* when irradiated at 405 nm, the antimicrobial effect reached a 5-log reduction. Conversely, regarding the fibroblasts, cell toxicity was lower than 1 log. Even Methylene Blue, photosensitizer with the greatest phototoxicity, did not cause cell reduction greater than 1 log when irradiated at 660 nm. (Fig. 3)

3.6. ROS production

Reactive oxygen species were formed from each PC excitation with violet, blue or red light sources. RNO degradation occurred in the presence of L-histidine, which was confirmed by its absorption band decay during the irradiation period. As expected, when the light source emission was coincident with the absorption band of the compound, ROS production increased significantly. Fig. 4 shows the RNO degradation for each compound when irradiated by a 405, 460 or 660 nm light source. Also, good ROS production correlation was achieved with each PC when associated with 405, 460 and 660 nm and biofilm reduction ($r = 0.678$, $P = 0.02$).

3.7. Bacterial reduction in planktonic culture

The results of bacterial reduction from planktonic culture are presented in Table 3. As expected, when irradiated with 405 or 460 nm, MB exhibited the lowest reduction. Moreover, irradiation with red emission LED resulted in higher bacterial killing rates for almost all the compounds.

3.8. Biofilm reduction

As observed for the planktonic bacteria, biofilm reduction is dependent on light – PC absorption. When irradiated with 405 or 460 nm, MB again showed the lowest antimicrobial effect. The highest reduction was once more observed with irradiation at 660 nm (Fig. 5).

4. Discussion

Interest in phytomedicine has increased in recent years. Many herbal drugs have potential properties that can be used as alternative to classic PS compounds. The literature is however scarce regarding information on the quality, safety and efficacy of herbal plants for use in antimicrobial photodynamic therapy [24]. In a recent literature review, Marrelli et al. [25] examined different herbal compounds tested in the photodynamic treatment of skin cancer. There was no discussion about aPDT, however.

The hypothesis tested in the present study was whether commercial Phytotherapeutic compounds could be used as a PS in aPDT. The absorption spectra of Curcumin, Lemon, *Hamamelis* and *Hypericum* were obtained, identified and the light source for irradiation was chosen

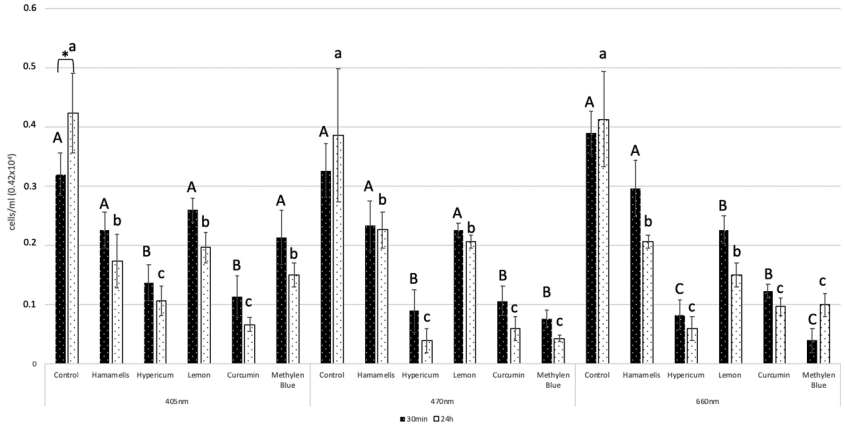

Fig. 3. photoxicity of each compounds at 70 μM to fibroblast cells after 30 min and 24 h, after irradiation with 405, 460 and 660 nm ligh source at 10 J of energy.

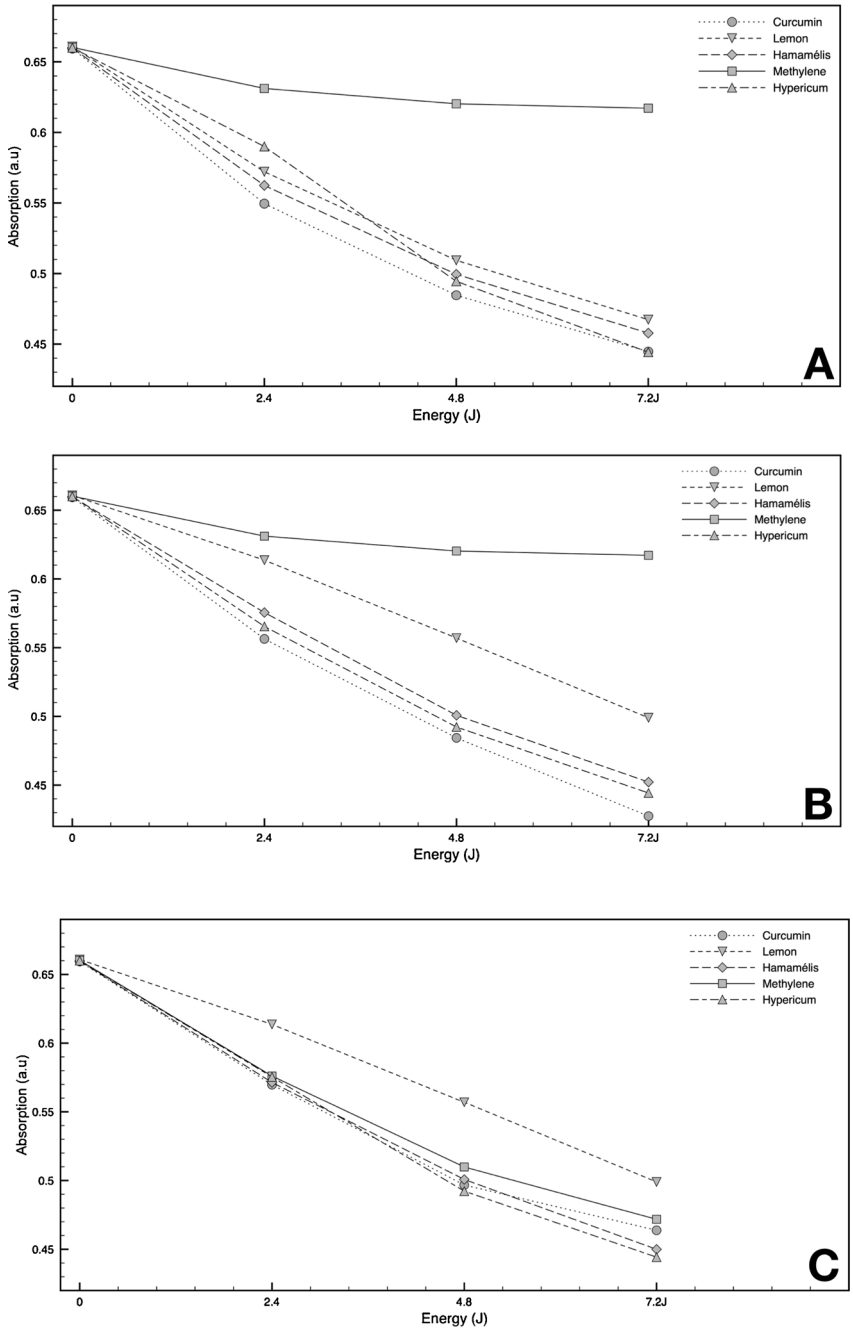

Fig. 4. ROS production using MB at different concentrations after irradiation with following ligh sources: 405 nm (A), 460 nm (B) or 660 nm (C). Indirect measurement of total ROS formation by reduction of RNO absorbance at 440 nm.

according to each spectrum. Using curcumin salt, Panhoca et al. [29] also selected a teeth bleaching arc as a light source, while Cieplik et al. [30] used an LED emitting at 600–700 nm to irradiate MB and Fekrazad et al. [31] used a polychromatic tungsten light for activated lemon essential oil. These authors used a purified compound instead of a commercially available PCs. The choice of a teeth bleaching arc (emitting at 405 nm), LED-based curing appliance (460 nm) and LED photobiomodulation device (660 nm) was based on the fact that in most cases, dentists already have these appliances in their office for use in other treatments. With the correct PS, therefore, these light sources could be used for aPDT with no or only minor adaptations and generally low additional costs.

The tested compounds demonstrated little tooth staining (enamel or dentine) potential, even at higher concentrations (0.4 M), in contrast to phenothiazinium dyes such as Methylene blue, which must be used at low molar concentrations to avoid staining or additional procedures to remove it from tooth tissue after aPDT, as described by Figueredo et al. [22] and Carvalho et al [23].

Some reports have identified a certain toxicity with PCs, especially *Hypericum* when used against tumor cells [32,33]. In the present study, none of the PCs demonstrated significant dark toxicity or phototoxicity when in contact with fibroblasts, when evaluated by MTT assay. Additionally, most of the PCs were less toxic to cells than 0.12% chlorhexidine or sodium 1%, hypochlorite, the most common irrigation solutions in dentistry. Conversely, after being irradiated with the appropriated wavelength, all the PCs exhibited phototoxicity against planktonic bacteria and biofilm. Qiao et al. [34] demonstrated that even after irradiation of MB with 17 J, this parameter was not toxic to

Table 3
Planktonic bacterial reduction (log10).
Source own authorship.

Light source	PC	Reduction (log10)
405	Curcumin	5.2
405	Lemon	2.5
405	Hamamelis	2.7
405	Hypericum	5.0
405	Methylene Blue	0.28
460	Curcumin	4.0
460	Lemon	0.93
460	Hamamelis	2.3
460	Hypericum	3.4
460	Methylene Blue	0.16
660	Curcumin	4.0
660	Lemon	0.7
660	Hamamelis	4.0
660	Hypericum	5.2
660	Methylene Blue	4.0

fibroblasts from the periodontal ligament or gingiva confirming the results of this study. Since the energy used in this study was 10 J, it can be confirmed that the energy necessary to produce bacterial reduction is safe for fibroblasts. The energy used for PC irradiation in this study was based on the parameters recommended by Garcez and Hamblin [1], who reported on a real-time method to analyze the energy response to the antimicrobial effect of PDT. The analysis found that 10 J is the minimal energy necessary for biofilm reduction in endodontic aPDT, and this energy was therefore used herein.

E. faecalis was the microorganism tested since this bacteria is an important pathogen in endodontic infection [35] and can be associated with refractory periodontitis [36]. Moreover, *E. faecalis* is developing high levels of antibiotic resistance and can cause a wide range of diseases in humans [37]. Due to its multiple mechanisms of action and the non-specificity of ROS when acting in different biological molecules, aPDT has demonstrated good results *in vitro* [38] and *in vivo* [2] against antibiotic resistant bacteria strains.

Antimicrobial or Oncologic PDT are based on ROS production, such as singlet oxygen, hydroxyl radicals and superoxide, which induce injury or death to microorganisms or tumor cells [4,5]. The production of ROS by the tested PC is therefore crucial to aPDT efficiency. When irradiated, at the same molar concentration, by 405 or 460 nm light, most of the PCs showed good ROS production, except for lemon extract activated by blue light. As expected, MB did not exhibit significant ROS production when irradiated at these short visible wavelengths. Yet, when activated by 660 nm, Curcumin, *Hypericum* and *Hamamelis* extracts showed similar ROS production to MB, the gold standard for aPDT with a red light source [39].

Regarding planktonic bacterial reduction, when irradiated with 405 or 460 nm, only Curcumin and *Hypericum* reached the minimal log reduction usually recognized for asepsis procedures [40] (3-log reduction). When irradiated with a red light source, however, only the lemon extract did not achieve a 3-log reduction. Similar results were found for the biofilm challenge. Once more, the red light source achieved the highest average biofilm reduction. Surprisingly, when irradiated with 460 nm, Curcumin only achieved a 1log reduction, which differed from the findings by Panhoca et at [29], in which the authors combined the photosensitizer with a surfactant (1% SDS). This may have facilitated the penetration of the PS into the biofilm and improved the antimicrobial effect of Curcumin.

Considering the main light sources normally found in a dental office, namely an LED-based curing light, dental bleaching arch and a red low-power laser/LED for photobiomodulation, the results of the present study suggest that the commercial phytotherapeutic compounds of *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* may be a viable alternative to conventional photosensitizers, such as Methylene blue, used in antimicrobial Photodynamic Therapy in dental treatment. These compounds presented a low risk of tooth staining, low dark toxicity, good ROS production and an adequate antibacterial effect, even in a biofilm challenge.

In addition to the positive results achieved in the present analysis, further studies should be performed using Gram negative bacteria and yeasts, in both a planktonic suspension and biofilm. Moreover, a direct

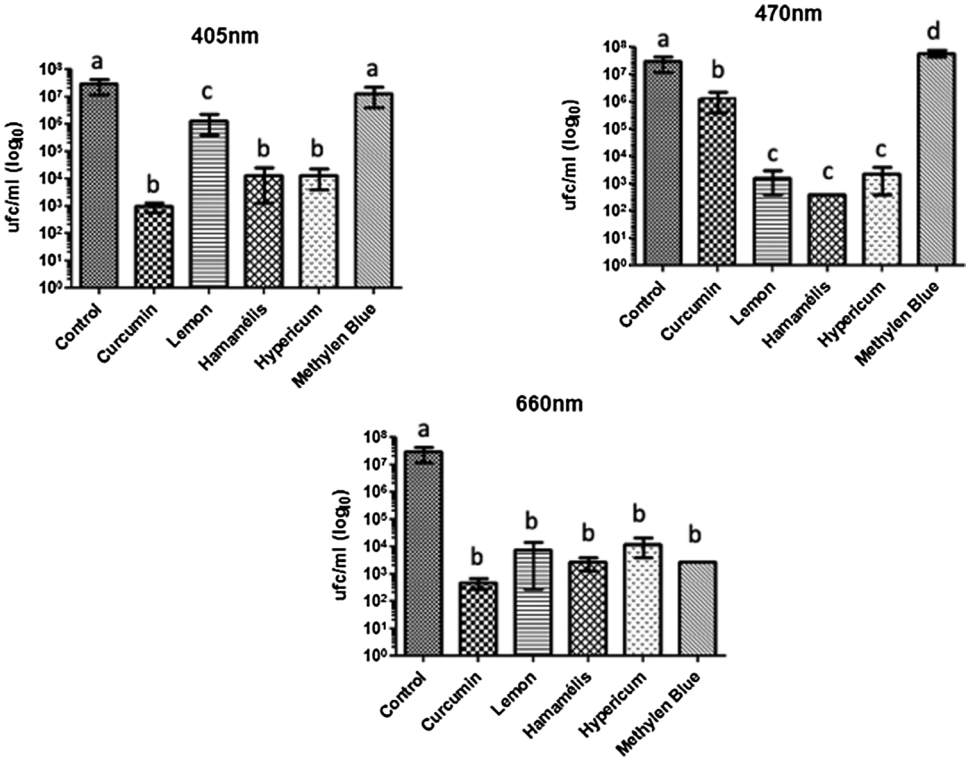

Fig. 5. Reduction of biofilm (log10) after irradiation of each PC with the light sources. Different letters indicate statistical significance ($p < 0.05$).

comparison of ROS production, especially singlet oxygen production, between pure compounds and commercial PCs should be tested.

5. Conclusion

The results of the present study demonstrated that extracts from *Curcuma longa, Citrus lemon, Hamamelis virginiana* and *Hypericum perforatum* are suitable for use in dentistry as photosensitizers in aPDT, effectively reducing bacteria, both in suspension and biofilm. Additionally, when used at the correct concentration, they offer low risk of dental staining, low dark toxicity and effective ROS production.

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