



## Research article

# Synergistic effect of carboxypterin and methylene blue applied to antimicrobial photodynamic therapy against mature biofilm of *Klebsiella pneumoniae*



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## ABSTRACT

The control of multidrug-resistant (MDR) bacteria is a growing public health problem, and new strategies are urgently needed for the control of the infections caused by these microorganisms. Notoriously, some MDR microorganisms generate complex structures or biofilms, which adhere to surfaces and confer extraordinary resistance properties that are fundamental challenges to control infections. One of the promising strategies for the control of MDR bacteria is antimicrobial photodynamic therapy (aPDT), which takes advantage of suitable photosensitizers (PS), oxygen and radiation to eradicate microorganisms by the generation of highly reactive species, including reactive oxygen species (ROS) that cause cytotoxic damage and cell death. Habitual aPDT treatments use only methylene blue (MB), but MDR microorganism eradication is not completely achieved. The key result of this study revealed that a combination of two known PSs, 6-carboxypterin (Cap, 100  $\mu\text{M}$ ) and MB (2.5–10  $\mu\text{M}$ ) exposed to ultraviolet and visible radiation, presents a synergistic effect on the eradication of a MDR *Klebsiella pneumoniae* strain. Similar effect was observed when the treatment was performed either with planktonic or biofilm growing cells. Moreover, it was found that after treatment the killing action continues in the absence of irradiation leading to the eradication of the microorganisms growing in biofilm. Therefore, the combined aPDT represents a promising strategy for the management of clinical contact surfaces, disinfection of surgical instruments, biofouling and even antimicrobial wastewater treatment.

## 1. Introduction

In the last years conventional antimicrobial therapies have been failing due to (i) the emergence of microbial resistance to antibiotics, known as multidrug resistance (MDR) and/or (ii) the ability of bacteria to form biofilms that avoid conventional antimicrobial action [1], causing serious infections called biofilm-associated persistent infections (BAPI). Therefore, the development of new strategies to eliminate this kind of microorganisms is urgent [2]. Antimicrobial Photodynamic Therapy (aPDT) emerges as a new methodology in the fight against persistent infections.

Antimicrobial photodynamic therapy is a minimally invasive technique based on a photochemical process in which a non-toxic photosensitizer (PS) can be activated by low doses of radiation of a specific

wavelength [3, 4]. The absorbed energy generates excited states of the PS which participates in electron-transfer reactions, generating radicals on biomolecules and reactive oxygen species (ROS), such as superoxide anion and hydroxyl radicals (Type I reactions) or singlet oxygen ( $^1\text{O}_2$ , type II reactions). These reactive species are cytotoxic and damage cellular components, such as proteins, nucleic acids, and phospholipids (membranes) [5]. In consequence, bacterial cells are affected by “photodynamic action” caused by aPDT. Moreover, after the treatment, the elimination of microorganisms and its virulence factors occurs with no significant side effects for the host [6].

The selection of PSs is the key point to get successful results in the aPDT treatment. Normally, an efficient PS must have high absorption coefficient in the whole excitation wavelength range and high triplet state quantum yields. The triplet excited state must have high energy and

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long lifetime. Additionally, sometimes strategies for disinfection in outdoor conditions are necessary, where sun is the cheapest source of radiation. Efficient PSs with absorption ranges within the solar spectrum could be used for sterilization of surfaces, water, etc., in outside areas.

A widely used PS is methylene-blue (MB, 3,7-bis(dimethylamino)-phenothiazin-5-ium chloride), which under light activation may act as a strong oxidizer to destroy targeted cells through cellular damage, alteration of membrane permeability, and protein inactivation [7, 8]. The wavelength range for MB photodynamic action is in the orange-yellow wavelength range (550–700 nm), with maximum molar absorptivity of  $85,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 660 nm, and with a quantum yield for triplet excited states formation of 0.52, and for singlet oxygen production about 0.5 [9]. Under radiation, MB shows bactericidal effects against both Gram-positive and Gram-negative bacteria [10, 11]. The low cost and availability of MB, as well as the possibility of using non-laser polychromatic radiation sources, makes it a suitable PS that can be used in underserved populations for the treatment of a variety of diseases [9].

Pterins are heterocyclic compounds widespread in living systems, which participate in relevant biological processes, such as metabolic redox reactions, and can photoinduce the oxidation of biomolecules through both type I and type II mechanisms [12]. The excitation wavelength range for pterins is in the UV-A (300–400 nm), with maximum molar absorptivity of  $\sim 7,000 \text{ M}^{-1} \text{ cm}^{-1}$  at  $\sim 350 \text{ nm}$  and a quantum yield for triplet excited states between 0.2–0.5, depending on the given pterin derivative [13]. It was previously demonstrated that pterin derivatives can be efficiently used as PS. Particularly, the photodynamic action of 6-carboxypterin (2-amino-4-hydroxypteridine-6-carboxylic acid, Cap), was tested under artificial UV-A radiation and sunlight exposure, proving to be efficient for the elimination of planktonic bacteria in drinking water and in preventing the growth of *Staphylococcus aureus* biofilms [14]. The corresponding quantum yield of singlet oxygen production for Cap is about 0.3, in the experimental conditions [13].

In this work, we have used a *Klebsiella pneumoniae* MDR strain, since represents a serious cause of healthcare related infections, including septic shock and mortality [15]. This bacteria is an opportunistic Gram negative pathogen, responsible of several diseases, and is extremely resistant to antibiotic treatments due to their capacity to generate biofilms [16]. Moreover, *K. pneumoniae* has been found as contaminating microorganism in roll boards, anesthesia equipment, doors, nurses area, beds, IV pumps and pole and floors in operating rooms (ORs) and/or intensive care units (ICUs) [17]. Frequently, the removal of *K. pneumoniae* is very difficult, even sometimes impossible. Thus, the development of new strategies to eliminate them is urgent since, as *K. pneumoniae*, there are many MDR-microorganisms and BAPI represents a serious healthcare problem.

The aim of the present work was to develop an aPDT based in the combination of two PSs able to eradicate MDR *K. pneumoniae* biofilms under ultraviolet and visible artificial radiation (denoted UV + Vis hereinafter). Cap and MB were chosen as PSs because of their excellent individual photodynamic inactivation properties, and more important, due to their complementary wavelengths range of absorption (Figure 1), which improves the efficiency in the use of polychromatic radiation. Finally, the combined aPDT proposed in this work could be applied to clinical contact surfaces, disinfection of surgical instruments, biofouling and even for the antimicrobial treatment of wastewater. To the best of our knowledge, it is the first time that aPDT combining Cap and MB was used to eradicate pathogenic planktonic bacteria and biofilms.

## 2. Materials and methods

### 2.1. Materials

Methylene Blue (MB, purity >90%) from Sigma Aldrich; pterin (Ptr, purity >99%) pterin-6-carboxylic acid (Cap, purity >99%) from Schircks

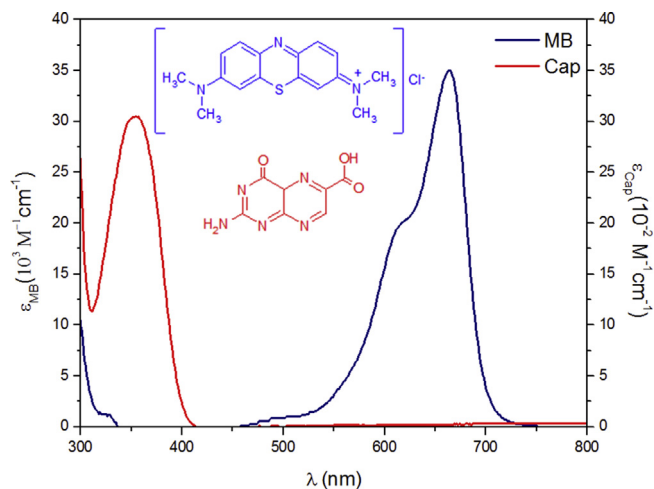


Figure 1. Molecular structures and absorption spectra of Cap and MB.

Laboratories, Switzerland. Acetonitrile (ACN) and ammonium acetate ( $\text{NH}_4\text{Ac}$ ) were purchased from J. T. Baker. Aqueous solutions were prepared with MilliQ water ( $\sim 10 \text{ M}\Omega \text{ cm}$  resistivity purified in a Milli Q Reagent Water System apparatus) and the pH was adjusted by adding drops of HCl and NaOH solutions from a micropipette using a pH-meter sensION pH31 GLP combined with microelectrode XC161 (Radiometer Analytical). Nutrient Broth (NB) (Britania, Argentina) containing pluri-peptone (5.0 g/l), meat extract (3.0 g/l) at a final pH 6.9; and Nutrient Agar (Britania, Argentina) containing pluri-peptone (5.0 g/l), meat extract (3.0 g/l), sodium chloride (8.0 g/l) and agar (15 g/l) at a final pH 7.3, were used to growth microorganism. Mueller-Hinton agar (Difco, France) meat extract (2.0 g/l), acid digest of casein (17.5 g/l), starch (1.5/l) and agar (17 g/l) at a final pH 7.3 was used in disk diffusion assays.

### 2.2. Bacterial culture and biofilm formation

*K. pneumoniae*, isolated from a male patient with recurrent urinary tract infection (UTI), was selected as representative multidrug resistant Gram-negative bacteria forming biofilm to mimic surfaces colonized by bacterial pathogen in hospital environments. The antibiotic susceptibility of this strain was performed by the disk diffusion using standard antibiotic disks and Mueller-Hinton agar plates (Difco, France) [18]. The antibiotic assayed were: nitrofurantoin (300  $\mu\text{g}$ ), ampicillim-sulbactam (10 $\mu\text{g}$ /10 $\mu\text{g}$ ), ofloxacin (5  $\mu\text{g}$ ), norfloxacin (10  $\mu\text{g}$ ), ciprofloxacin (5  $\mu\text{g}$ ), levofloxacin (5  $\mu\text{g}$ ), cephalotin (30  $\mu\text{g}$ ), ceftazidime (30  $\mu\text{g}$ ), gentamicin (10  $\mu\text{g}$ ), amikacin (30  $\mu\text{g}$ ) and meropenem (10  $\mu\text{g}$ ) (Britania, Argentina). The isolated bacteria were grown in NB at 35 °C with shaking (150 rpm) overnight. Then, *K. pneumoniae* suspension was adjusted to  $\sim 10^6$  colony-forming units (CFU)  $\text{mL}^{-1}$  in fresh 1/10 diluted NB (dNB). Bacterial suspensions were confirmed by viable count method and used for microbiological assays. Biofilm formation was carried out on microtiter plates as previously described with minor modifications [14]. After resuspension in fresh growth medium, aliquots of bacterial suspension were added to the wells of sterile flat-bottom 96-well microtiter test plates (96-well, Greiner Bio-One, Germany) and incubated for 24 h at 35 °C to allow biofilm formation (24h-biofilm). The wells were washed with sterile distilled water in order to remove planktonic and sessile cells weakly attached to the surface. Quantification of viable bacteria in the biofilm was carried out by serial dilution method and plate counting after their detachment. A triplicate series of experiments and two replicates were carried out in each case. These values were used as the “initial number of bacteria in the biofilm” to which the reduction of viable cells after antimicrobial treatment was referred.

### 2.3. Steady-state irradiation

The irradiation system employed consists in two tube lamps located over the samples at 1 cm: a) UV lamp (8 W UV tube, maximum wavelength 365 nm) and b) white fluorescent (8 W tube, wavelength range 350–750 nm). Biofilms were exposed to both lamps at the same time. Pre-irradiated PS solutions were irradiated in fluorescence quartz cells of 0.4 cm of path length, at room temperature (distance from the lamp 1 cm). It is important to highlight that the spectral irradiance of the UV lamp is  $31 \pm 4 \text{ Wm}^{-2}$ , which is lower than the solar spectral irradiance measured at solar noon ( $37 \pm 4 \text{ Wm}^{-2}$ ) during the spring period [14]. Moreover, the dose of UV-A radiation used in this work was  $14.88 \text{ J/cm}^2$  while the normal minimal erythema dose is greater than  $20 \text{ J/cm}^2$  [19,20]. Therefore, the UV-A dose applied in this study would be considered safe for clinical therapy.

### 2.4. Photodynamic inactivation of biofilm using single and combined photosensitizers

The antimicrobial effects of aPDT against 24h-biofilm of *K. pneumoniae* were evaluated with each PS alone, MB or Cap, and with a combination of both PSs at a final pH 7.3. The concentration range used was  $2.5\mu\text{M}$ – $10 \mu\text{M}$  for MB and  $100 \mu\text{M}$  for Cap. When both PSs were combined solutions were prepared to reach MB/Cap concentrations of  $2.5 \mu\text{M}/100 \mu\text{M}$ ,  $5 \mu\text{M}/100 \mu\text{M}$  and  $10 \mu\text{M}/100 \mu\text{M}$ . PSs were added to pre-existing 24h-biofilms, incubated for 15 min in the dark and then were exposed to radiation during 80 min keeping the temperature at  $35 \text{ }^\circ\text{C}$ . Afterwards, a set of wells treated by aPDT were washed and enumerated by the plate count method and other identical set remained in darkness at  $35 \text{ }^\circ\text{C}$  for 20 h, in order to quantify persisting viable sessile bacteria to determine if killing action continued during the dark phase (aPDT dark phase assay).

Control assays experiments were carried out with biofilms grown in dNB, were performed in triplicate series and with two replicates as follows:

- (1) Dark control: biofilms were kept in darkness after the addition of Cap or/and MB (radiation-free experiment);
- (2) UV + Vis radiation control: biofilms were incubated in fresh diluted nutrient broth and irradiated with UV-A and/or visible radiation for 80 min (PSs-free experiment);
- (3) Growth control: biofilms were incubated in fresh diluted nutrient broth for 80 min (UV + Vis radiation and PSs free experiment). The growth control represents the maximum number of sessile cells expected in biofilms after 80 min growth.

### 2.5. Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) test to evaluate differences between groups. A p value of  $<0.05$  was considered statistically significant.

### 2.6. Analysis of irradiated solutions in microtiter plates

**UV-Vis spectrophotometry.** Electronic absorption spectra were recorded on a Shimadzu UV-1800 spectrophotometer, using quartz cells of 0.4 cm optical path length. Absorption spectra of the irradiated aqueous or dNB solutions, containing Cap, MB or Cap/MB, were recorded before and after irradiation (UV + Vis radiation during 80 min).

### 2.7. High-performance liquid chromatography

A high-performance liquid chromatography (HPLC) equipment Prominence from Shimadzu (solvent delivery module LC-20AT, on-line degasser DGU-20A5, communications bus module CBM-20, auto sampler SIL-20A HT, column oven CTO-10AS VP and photodiode array (PDA)

detector SPD-M20A) was employed for monitoring the reaction. A Luna C5 column (reverse phase,  $5\mu\text{m}$ , Phenomenex) was used for product separation. ACN/ $\text{NH}_4\text{Ac}$  (pH = 6) were used as mobile phase. A gradient between 5% to 40% of ACN was used. The flow rate was  $0.6 \text{ ml/min}$  and the run time 35 min. The injection volume was  $50 \mu\text{L}$  for all samples. The simultaneous estimation of Cap and MB was carried out at a detection wavelength of 346 and 660 nm, respectively. Aqueous solutions of MB (pH 5), Cap (pH 7.5) and pterin (Ptr) (pH 7.5) standards were employed to obtain the corresponding calibration curves.

## 3. Results and discussion

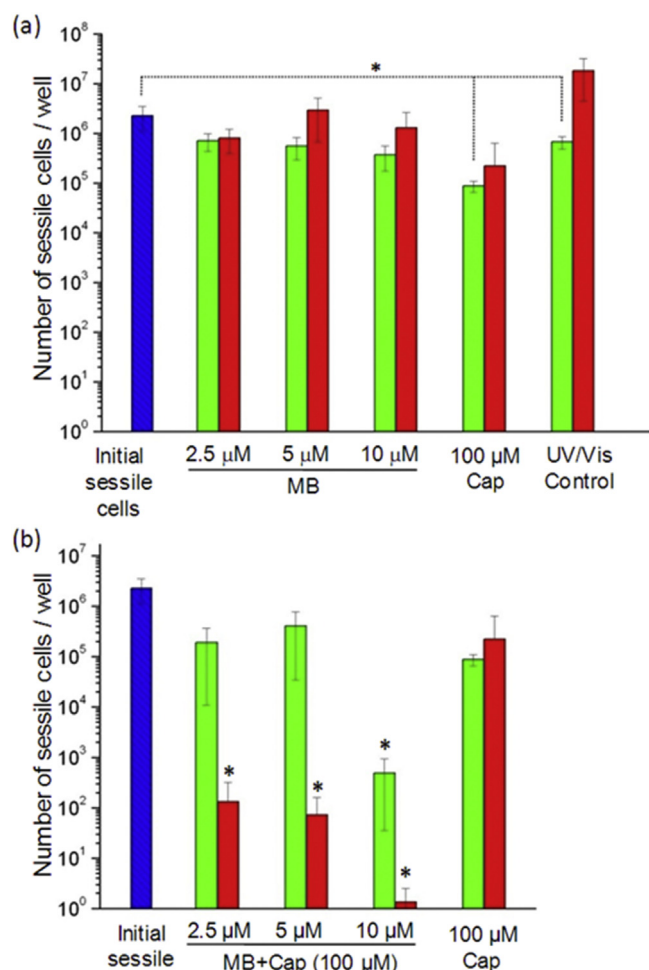
### 3.1. Inhibition of pre-existing biofilm proliferation and bacterial killing

As model of MDR microorganism it was used a *K. pneumoniae* strain exhibiting resistant to multiple antibiotics, such as nitrofurantoin, fluoroquinolones (ofloxacin, norfloxacin, ciprofloxacin and levofloxacin), ampicillin-sulbactam, cephalosporins (cephalotin and ceftazidime), aminoglycosides (gentamicin). The only efficient antibiotics to eliminate the current *K. pneumoniae* strain were meropenem or amikacin. Therefore, conventional options to fight against this pathogen are limited. As a consequence, it is observed a high incidence of diseases, frequently in-hospital urinary tract infections, caused by *K. pneumoniae*, indicating that the control of this microorganism deserves the development of alternative antimicrobial strategies. According to these observations, the aim of this work is the development of a new and more efficient aPDT. The combination of two PSs could improve the treatment and therefore, the use of single PS (MB or Cap) or mixtures of both PSs under irradiation by UV + Vis light, in a safe dose for clinical therapy, was evaluated. The safety of the irradiation and the selection of non-toxic PSs allow the treatment to be used for disinfection of skin areas under medical manipulation and not only for disinfection of hospital material.

#### 3.1.1. Direct irradiation of pre-existing biofilm

*K. pneumoniae* mature biofilms (24 h-biofilms) formed on multiwells plates and preincubated with Cap ( $100 \mu\text{M}$ ) or/and MB ( $2.5$ ,  $5$  and  $10 \mu\text{M}$ ) were exposed to UV + Vis radiation during 80 min. After irradiation, the number of viable bacteria was determined immediately (Figure 2a, green bars). Results showed that for biofilms pre-incubated with Cap ( $100 \mu\text{M}$ ) alone the bacterial survival immediately after irradiation was  $1.3 \log_{10}$  and  $0.9 \log_{10}$  lower compared with the initial number of sessile cells in biofilms (initial inoculums, blue bar) and with UV + Vis control, respectively. These values indicate a bacteriostatic action on *K. pneumoniae*, as it was previously observed for *Staphylococcus aureus* [14]. In the same way, biofilms pre-incubated with MB alone and at different concentrations ( $2.5$ ,  $5$  and  $10 \mu\text{M}$ ), were not significantly affected by the treatment. In addition, these results do not differ significantly from that observed in the UV + Vis control. It is important to remark that the usual concentrations of MB for a typical aPDT treatment is in the range  $20$ – $50 \mu\text{M}$  [11], higher than the concentrations tested in this work.

On the other hand, when biofilms were pre-incubated with solutions containing both Cap and MB the decrease of the viability immediately after irradiation was improved (Figure 2b). For those samples pre-incubated with the higher concentration of MB tested ( $10 \mu\text{M}$ ) in combination with Cap ( $100 \mu\text{M}$ ), the decrease in viability immediately after irradiation was at least  $3 \log_{10}$  lower than the initial bacterial number (bactericidal action). The lowest MB concentrations tested ( $2.5 \mu\text{M}$ ) in combination with Cap ( $100 \mu\text{M}$ ), immediately after irradiation, did not enhance the antimicrobial action in comparison with Cap pre-incubated biofilms ( $p > 0.05$ ). However, after the treatment with MB ( $10 \mu\text{M}$ ) and Cap ( $100 \mu\text{M}$ ) and 80 min of UV + Vis irradiation, a great increase on the antimicrobial action was observed (Figure 2b, green bars). The observed decrease could be defined as synergistic, according to the Bonapace's criteria [21], since the increase in bacterial killing by the combined aPDT (MB,  $10 \mu\text{M}$  and Cap  $100 \mu\text{M}$ ) was more than  $2 \log_{10}$  in comparison to



**Figure 2.** aPDT effect with single and mixtures of PSs on biofilms. (a) single photosensitizer and (b) the mixture Cap + MB; (green bars) immediately after irradiation and; (red bars) after irradiation followed by 20 h of dark incubation \* indicates significant differences ( $p < 0.05$ ) and antimicrobial synergistic effect.

single aPDT with the highest concentration of Cap (100  $\mu$ M). For all the conditions tested, PSs dark toxicity was evaluated, determining the absence of toxicity under dark incubation, as it was observed in previous works [14, 22].

After the treatment, biofilms were kept in the dark condition during 20 h (Figure 2b, red bars). Unexpectedly, bacterial cell death was enhanced for all conditions of combined aPDT. The treatment of biofilms by irradiation and by a combined aPDT, with the highest MB concentration (10  $\mu$ M) and Cap (100  $\mu$ M), and after 20 h of dark incubation results in the early complete killing of bacteria. Thus, an eradication effect for *K. pneumoniae* biofilms was achieved. Consequently, a residual antimicrobial action improves the total killing action.

### 3.1.2. Effect of the addition of preirradiated PSs solutions to a pre-existing biofilm

Taking into account the results described above, the question that arises is “which process causes bacterial death during the dark incubation of PS + UV-Vis pretreated biofilms?” Bacterial killing during 20 h dark incubation may be a consequence of i) the damage produced during irradiation, that leads to alterations in metabolic processes that causes death after the light exposure; or ii) toxic substances formed in the culture media during irradiation (TS-effect) able to kill cells in the dark. In this sense, to differentiate these two options, *K. pneumoniae* biofilms were pre-incubated with protein (dNB) plus Cap + MB solutions and irradiated

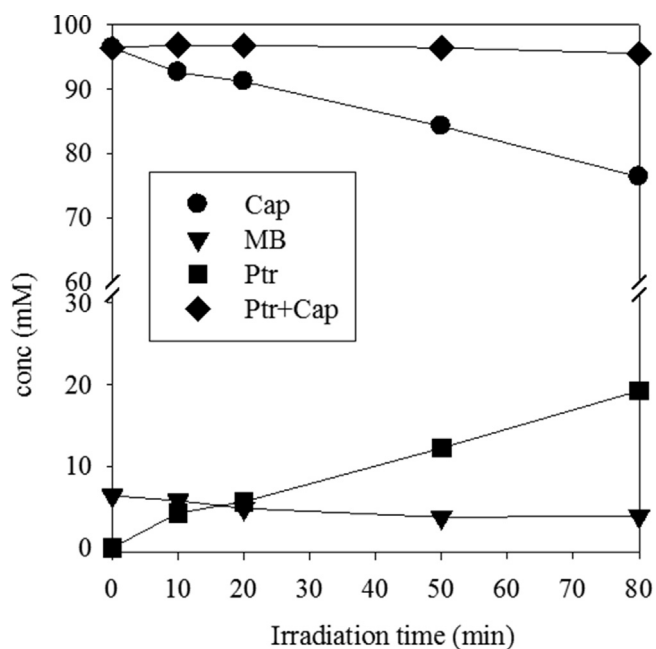
(UV + Vis) for 80 min. Then immediately after the irradiation was turned off, the culture media was discarded and replaced by fresh dNB with PSs and, after that, incubated in the darkness to evaluate possible TS-effect. These results were compared with pre-irradiated dNB solutions containing Cap + MB combinations that were added to pre-existing biofilms of *K. pneumoniae* as shown in Figure 2b (red bars). Interestingly, this assay showed that during the dark exposure with fresh dNB plus PSs, the growth of biofilms samples was not inhibited, and biofilm recovers and grows, leading to the formation of a new planktonic population (evidenced by turbidity in the medium). These results indicate that fresh media allows bacteria to recuperate, and that combined aPDT does not markedly affect the bacterial viability. On the contrary, maintaining the medium after irradiation leads to nearly total killing of cells, indicating that there is a residual effect of the products of PSs irradiation that inhibits the growth of bacteria.

According to these results, it can be noticed that eradication condition could be achieved by the high antimicrobial action during the aPDT followed by the dark exposure that allows the action of the residual effect of the new species generated during irradiation. In this sense, we may hypothesize that dark phase inhibition may be attributed to: a) the formation of a toxic photo-product/s by reaction with susceptible targets of excited states of MB and/or Cap during irradiation; and/or b) oxidation of proteins in the broth medium by both PSs, MB and/or Cap leading to the consumption of the medium nutrients or the generation of toxic products for bacteria. To elucidate these hypotheses we performed the experiments described in the next section.

## 3.2. Analysis of irradiated PSs solutions

### 3.2.1. Analysis of irradiated solution of PSs

Aqueous solutions containing each PSs alone or combined were exposed to UV + Vis irradiation and then analyzed by spectrophotometry and HPLC. Solutions containing both Cap (100  $\mu$ M) and MB (10  $\mu$ M) exhibited important changes in the absorption spectra upon irradiation. HPLC analysis showed a strong decrease in Cap concentration and a slight decrease in MB concentration during irradiation (Figure 3). A new product with higher retention time ( $t_R$ ) was detected and identified as pterin (Ptr), since the  $t_R$  and the absorption spectra



**Figure 3.** Concentrations of Cap (●), MB (▼) and Ptr (■) in irradiated aqueous solution of Cap + MB ([Cap] = 100  $\mu$ M; [MB] = 10  $\mu$ M) as a function of irradiation time. Squares (◆) represent the sum of Ptr + Cap concentrations.

were equal to those registered for a control solution of Ptr. This result was expected since it was previously demonstrated that photo-degradation of Cap yields Ptr [23]. The total concentration of pterins ([Cap] + [Ptr]) was constant, within the experimental error, indicating that Cap was quantitatively converted into Ptr. Comparison of the photolysis of Cap in the presence and in the absence of MB showed that MB does not affect the photochemistry of Cap. Therefore, it can be assumed that no additional photo-product emerged as consequence of reaction between PSs.

### 3.2.2. Analysis of aqueous solution of Cap + MB during UV + Vis irradiation in the presence of nutrient broth (NB)

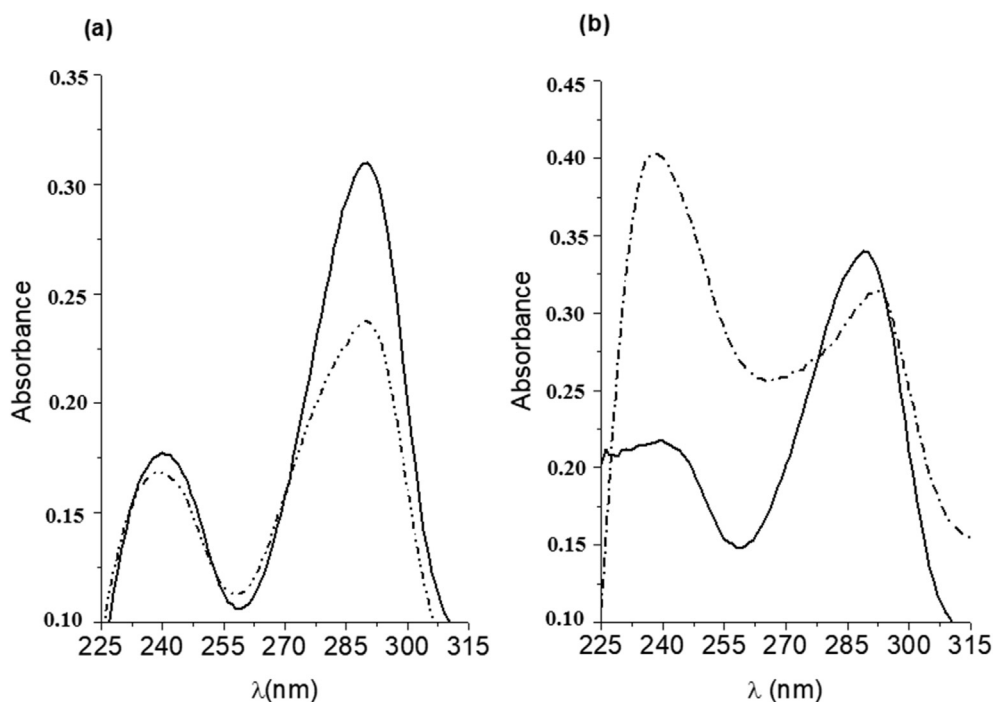
Experiments similar to those described in the previous section (3.1.3), were made with the addition of protein (NB) after and before UV + Vis irradiation of Cap + MB solution ([Cap] = 100  $\mu$ M and [MB] = 10  $\mu$ M). The culture medium NB was added to PSs aqueous solutions to reach the protein content (0.8 mg/ml), corresponding to dNB used in the microbiological assays. Several changes in the absorption spectra of the Cap + MB solutions were observed during irradiation. Proteins present typical absorption spectra with maximum at 220 and 260 nm, due to peptidic bonds and the presence of aromatic amino-acids, respectively [24]. When proteins were added to Cap + MB aqueous solution that was previously irradiated, the change in the absorption spectra was limited to a decrease in the intensity at 280 nm, in comparison with the control (without irradiation) (Figure 4a). However, a strong absorbance increase in the wavelength range corresponding to proteins absorption (230 nm maximum) was observed when the solution with proteins plus the mixture Cap + MB was irradiated, compared with the control condition (without irradiation) (Figure 4b). These results would indicate that proteins are transformed together with Cap + MB photosensitized oxidation that leads to a nutrient deficiency in the culture media. Consequently, after aPDT treatment, bacteria damaged by the photosensitized oxidation could not recover their healthy state due to the protein transformation. All these events may be considered to interpret the killing action that continues under dark condition (Figure 2b) that will be analyzed in the following section.

### 3.3. Bacterial damage by aPDT

The mechanisms proposed for bacterial inactivation involve both direct oxidation (Type I mechanism) of biological targets (lipid membranes, proteins and DNA), and oxidation mediated by singlet oxygen ( $^1\text{O}_2$ ) (Type II mechanism). Cap absorbs in the UV-A region (310–400 nm) and MB has strong absorption in the visible region (550–700nm). Both PSs, under suitable radiation, produces triplet excited states ( $^3\text{Cap}^*$  and  $^3\text{MB}^*$ , respectively) and participates in both type I and type II photosensitized oxidations [5].

For MB, it has been reported that type I process may be favored by the binding of positive charged MB to proteins which facilitate protein–MB electron transfer reactions and formation of PS aggregates [25]. Moreover, type II reactions are amplified in membranes by the binding of PSs because of the decrease in dimerization of PSs and the increase of oxygen concentration in the less polar media, which favor the  $^1\text{O}_2$  yield. On the other hand, the bactericidal action of Cap under aPDT is attributed also to both type I and type II mechanisms under UV-A excitation. Interestingly, the addition of Cap to MB leads to an enhanced killing effect, in agreement with previous results which demonstrated that Cap is efficient in the prevention of *S. aureus* growth, either as planktonic cells or as pre-existing biofilms [14].

Overall, the reduction in the number of viable sessile of *K. pneumoniae* observed might be the result of a combination of both photosensitized mechanisms, that is the participation of  $^1\text{O}_2$  (type II mechanism) and also all the chemical reactions derived from both  $^3\text{Cap}^*$  and  $^3\text{MB}^*$  (type I mechanism), that leads to the injury of different bacterial biological targets. The combination of two different PSs (Cap + MB) resulted in a bactericidal synergic action, and a more efficient use of radiation. In our opinion, it is possible that each PS acts preferably with a different target, and that the greater variety of the resulting damaged biomolecules leads to a condition of difficult recovery for the microorganism. In addition to the damage caused to the bacteria, the consumption of nutrients necessary for their recovery or the generation of oxidation products causes the damage to be prolonged preventing bacterial recovery and thus achieving bacterial eradication and improving the efficiency of the treatment.



**Figure 4.** (a) Absorption spectra of mixture of non irradiated proteins and pre-irradiated PSs (Cap + MB) (dash-dot line) and non-irradiated PSs (Cap + MB) (solid line); (b) Solution containing proteins and PSs (Cap + MB), irradiated (dash-dot line) and non-irradiated (solid line).

#### 4. Conclusions

In this work it has been demonstrated that a treatment of aPDT based on the combination of the photosensitizers (PSs) 6-carboxypterin (Cap) and methylene blue (MB) is efficient for control, and even eradication, of multidrug resistant (MDR) *K. pneumoniae* mature biofilms. Synergistic effect was observed by combination of the two photosensitizers and, interestingly, bactericidal activity was exhibited on preexisting mature biofilm after aPDT treatment followed by overnight-dark incubation. The results presented in this work, clearly show that a combination of two known PSs in aPDT treatment, is able to complete the eradication of MDR bacteria biofilms, unachievable through conventional antimicrobial treatment or an aPDT treatment mediated by a single PS. Therefore, although this study was performed in a single strain, the combined aPDT represents a promising strategy for the disinfection of clinical surfaces prone to bacterial contamination, indwelling devices and biofouling.

#### Declarations

##### Author contribution statement

Maira Gaspar Tosato, Carolina Lorente, Alejandro Miñán: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Patricia Schilardi, Mónica Fernández Lorenzo de Mele, Andrés H. Thomas: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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##### Competing interest statement

The authors declare no conflict of interest.

##### Additional information

No additional information is available for this paper.

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