LABORATORY RESULTS ON INVESTIGATION OF LASER BEAMS INTERACTION WITH CHLORPROMAZINE, TO OVERPASS MULTIPLE DRUG RESISTANCE ACQUIRED BY BACTERIA

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The paper presents a new approach in the treatment of multiple drug resistance acquired by bacteria: generation of photoproducts by exposure of Chlorpromazine to UV laser radiation emitted at 266 nm. This compound is not an antibiotic used to treat infections, but a psychotropic medicine, developed for psychiatric treatments. The methods to evaluate the results of irradiations are: UV-VIS-NIR absorption, LIF, FTIR and LC-TOF-MS. It results that the solution which contains the parental compound and the photoproducts obtained by irradiation are efficient against Gram-positive and some Gram-negative bacteria which acquired immunity by the development of multiple drug resistance properties.

Keywords: laser spectroscopy, chlorpromazine, Gram-positive bacteria, Gram-negative bacteria, FTIR, LIF, optical absorption, LC-TOF-MS.

1. Introduction

The medical practice shows that the multidrug resistance (MDR) to treatment in the case of bacteria, infections and tumors are often reported in the literature. On the other hand, photochemistry is an already well established domain which is continuously growing [1]. The combination of the methods and procedures

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of these two domains may lead to solutions for fighting MDR resistance in patients and bacteria. This is a world-wide priority since reports of MDR acquired by known Gram-positive and Gram-negative bacteria and tumors are alarming, not to speak about new bacteria or even viruses developed by natural selection or accident, for which there are not usable treatment procedures. So, the exposure of solutions of medicines in water (water is chosen since it is biocompatible) to laser radiation may lead to generation of new photoproducts that single or in combinations with other photoproducts and parent compounds may be effective against MDR targets. The lasers used in such applications may be chosen function of the molecular structure of medicines implied in treatment. The Nd:YAG laser's emission at 1,064 nm and the harmonics of this wavelength are mostly used [2]. In this paper are reported our results about the use of Chlorpromazine (CPZ) exposed to laser radiation in destroying Gram-positive and Gram-negative bacteria.

2.Materials and methods

2.1 Medicine used for applications

The CPZ belongs to phenothiazine group. It is currently used to treat psychiatric disorders [3,4] and is member of a larger class of medicines evaluated for their properties to fight MDR after proper processing [5-10]. In general, phenothiazine derivatives have the general chemical formula $S(C_6H_4)_2NH$ which contains two heterocyclic rings united by a –NH– bond and a Sulphur atom (–S) in median position [11]. They are used as insecticides, antiseptic and tranquilizers [12] and have photosensitive properties at exposure to white/daily light. This recommends them as candidates for use in the treatment of MDR by exposure to laser beams. CPZ, or otherwise named 2-cloro-10-(3-dimetilaminopropil) phenothiazine has a Cl atom in position 2 and an alkyl-amino lateral catena in position 10. Its chemical structure is shown in Fig.1 A), and B) and C) where 2D and 3D structures are presented, respectively; the specific chemical formula is $C_{17}H_{19}ClN_2S$ and the molecular mass is 318.86 g/mol [13 -15]. Among other properties of CPZ solutions in water that are important in applications is the pH value; typically, a 5% water solution of Hydrochloride CPZ has the pH between 4 and 5.5. The CPZ absorption spectra show intense absorption peaks in the near ultraviolet, but the samples show lower absorbance in the visible and near infrared up to 1200 nm. Two absorption bands have high intensities, at 250 – 265 nm and 300 – 320 nm. The peak of 250-265 nm band is produced by $\pi \rightarrow \pi^*$ and the peak in the second band at larger wavelength, is produced by $n \rightarrow \pi^*$ transitions. This recommends the irradiation of CPZ solutions in water at wavelengths between 210 nm and 320 nm. The CPZ was purchased in powder form from Sigma, Madrid, Spain, at biological grade (over 98.9%) purity and the solvent was ultrapure water.

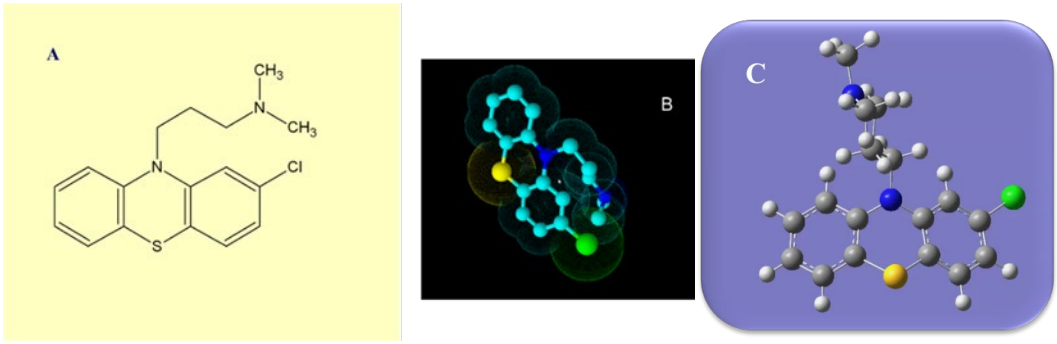

Fig. 1. Chemical structure of CPZ. A) Plane (2D) structure; B). 3D structure; C). 3D chemical structure optimized using Gaussian 09. Yellow ball - Sulphur, dark blue - Nitrogen, green - Chlorine.

2.2 Experimental set-up and samples' complex analyses

The scheme of the irradiation set – up and the equipment used to analyze irradiation results on samples is shown in Fig.2.

The samples were CPZ solutions prepared in ultrapure water at concentrations between 2 mg/mL and 20 mg/mL. They were exposed to the fourth harmonic of the Nd:YAG pulsed laser beam at 266 nm. The pulse full time width at half maximum was 6ns at 10 pps repetition rate and the mean energy per pulse was, typically, 6.5 mJ. For 10 mL cell, the optical path length was one cm and the laser beam cross section on the cell was 0.38 cm^2, showing a fluence of 17mJ/cm^2.

The pulse energy was measured by a system: beam splitter (BS) - energy meter. The status of irradiated sample was explored using measurements with standard instruments to evidence modifications which might give information about sample's evolution due to exposure to laser beam:

(i) the absorption spectra using an UV- VIS spectrophotometer to evaluate the modifications of the molecular electronic and vibrational states in the parent compound;

(ii) the FTIR spectra to observe modifications within molecular vibrational bands in infrared at longer wavelength;

(iii) the LC-TOF-MS measurements to identify by liquid chromatography coupled with mass spectrometry and time of flight detection the photoproducts generated by irradiation. One laboratory system was used to evidence differences: laser induced fluorescence (LIF) of samples measured in visible and near infrared; LIF spectra may be measured in mL cells or µL droplets in real time, or after irradiation [16].

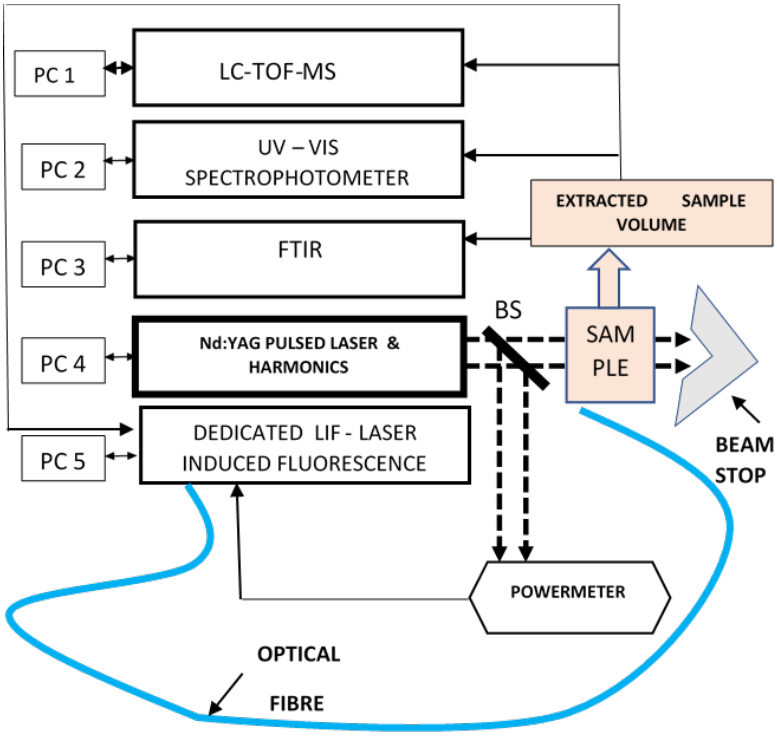

Fig. 2. Detailed scheme of the set-up containing: (i) irradiation system: Nd:YAG laser, beam splitter BS, power meter, (ii) optical fiber to collect and analyze light emitted by the sample or extracted volumes from it, (iii) equipment used to perform either in real time or on extracted samples their comprehensive analyses: UV-VIS spectrophotometer, FTIR, LIF.

3. Results

In this chapter are presented the main original results obtained on the modifications of the CPZ molecules in water solutions under laser radiation and about the photoproducts generated during this process, which in cocktails, may efficiently act on some MDR bacteria, killing them.

3.1 Measurements on Chlorpromazine

The absorption spectra of CPZ in water at 0.2 mg/mL and 2 mg/mL are shown on Fig.3. They are modified for both concentrations function of irradiation time; the overall intensity and the intensity distributions are different for the two concentrations. Two peaks are evidenced, out of which the 254 nm is the main one; both peaks show hypochromic shift during irradiation time up to 120 min and then hyperchromic shift until 240 min. The secondary peak at 307 nm has a bathochromic shift up to 344 nm. Two new bands show-up, at 503 nm (*) and 540 nm (**) for irradiation times longer than 30 min which may be due to the production of promazine associated with CPZ destruction (inset in Fig.3) [17]. This

is in agreement with Ref. [19] where the photoproducts were evaluated by Thin Layer Chromatography; five photoproducts with higher polarity than CPZ were observed.

The modification of the CPZ content in solution with increasing irradiation time is confirmed by the fluorescence spectra shown in Fig.4. LIF spectra for CPZ solution at 2 mg/mL have a broad emission spectrum with the peak at 504 nm.

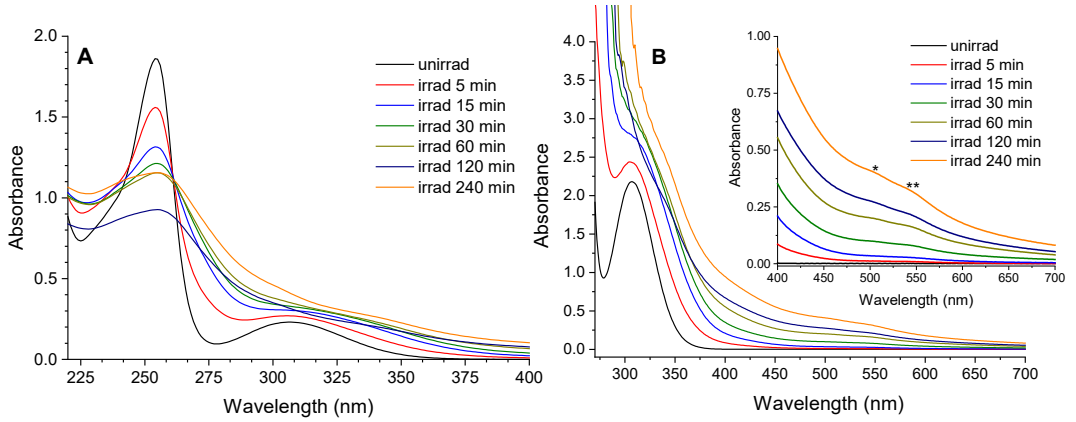

Fig. 3. The absorption spectra of CPZ in water from unirradiated to irradiated samples up to 240 min. A. Sample concentration 0.2 mg/mL (diluted from 2 mg/mL); spectra between 200 nm and 400 nm; B. Sample concentration 2 mg/mL; spectra measured between 280 nm and 700 nm.

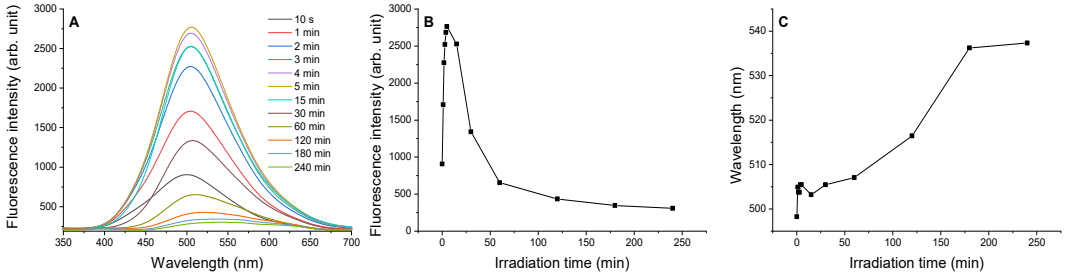

Fig. 4. LIF spectra of CPZ at 2 mg/mL when exposed to 266 nm: A). Recordings made in real time between 350 and 750 nm, during irradiation of samples, when irradiation time is increased from 10 s to 240 min; B). Fluorescence intensity variation during the irradiation; C) Bathochromic effect on central peak in LIF spectra at different irradiation time intervals.

The fluorescence intensity increases in the first 5 minutes of irradiation and then decreases with the increasing of exposure time. This shows that the beam energy transferred to CPZ molecules in a first instance is higher than the lowest binding energy within the molecule and, consequently, the molecules deplete [20]. The bathochromic effect increases with exposure time (Fig.4B) suggesting that the ratios between photoproducts' concentrations change during irradiation.

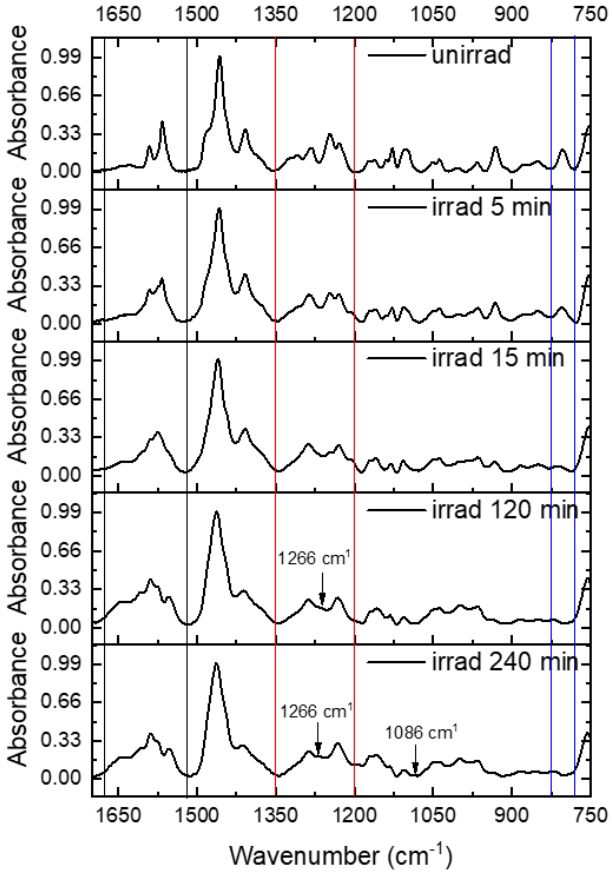

Fig. 5. FTIR spectra of CPZ sample that contains unirradiated and 5, 15, 120 and 240 min irradiated solution, recorded in the spectral range 3600-750 cm^{-1}.

Another type of measurements made to characterize sample's composition after irradiation is FTIR which allows to identify changes in the IR vibrational structures of the molecules generated by exposure to laser beam. In Fig.5 is shown a comparison of FTIR spectra obtained for a CPZ sample at 2 mg/mL in water that is either unirradiated or irradiated 5, 15, 120 and 240 min. Modifications of the vibrations that correspond to different chemical bonds in the CPZ molecule have been identified showing the generation of photoproducts, starting with 5 min exposure time. The peaks at 1590 and 1566 cm^{-1} (marked between the black vertical lines) responsible for stretching vibration of the aromatic ring C=C–C are modified continuously during irradiation process. Changes can be observed even after 5 min irradiation. In the same way, peak disappearance is observed in the domain 1340-1200 cm^{-1} (marked between the red vertical lines). The peaks at 1313 and 1286 cm^{-1} (in-plane bending vibration for four H atoms adjacent to the ring) and at 1248 and 1229 cm^{-1} (in-plane bending vibration of C-H from aromatic ring) which are present in the unirradiated spectrum disappear after 15 min irradiation and are

replaced with peaks at 1289 cm^{-1} and 1232 cm^{-1}. This suggests modification at the ring structure of the parental molecule, possibly the addition of functional groups to it. At 120 min exposure a new band appears at 1266 cm^{-1}. This is also visible for 240 min irradiated CPZ, which is assigned to the stretching vibration of the C-O bond within a phenol group. So, the formation of hydroxyl group is indicated. The new band at 1086 cm^{-1} that appears in the 240 min irradiated CPZ spectrum is assigned to the stretching vibration of S-O sulfoxide group indicating the formation of oxidative photoproducts. The band at 804 cm^{-1} (marked between the blue vertical lines) characteristic to the C-Cl stretching vibration disappears after 15 min irradiation, which indicates the formation of promazine (PZ) that is another phenothiazine. The observed modifications suggest the complete degradation of the parental CPZ and the generation of photoproducts that contain phenol and sulfoxide group [14].

These measurements may be completed by liquid chromatography coupled with mass spectrometry and time of flight (LC-TOF-MS) studies. The accurate measurements of the molecular mass (< 5 ppm) allow to identify photoproducts, as shown in Fig.6, where LC-TOF-MS spectra of 2 mg/mL CPZ unirradiated and irradiated water solutions are shown.

The photoproducts were identified from the plot which shows the intensity of the measured signals function of the m/z ratios (the mass to charge ratio for each produced ionized fragments or ions).

The chromatograms were obtained on CPZ solution irradiated between 1 min and 240 min and suggest the generation of 7 photoproducts accompanied by the constant decrease of CPZ presence. In Fig.6 the LC-TOF/MS plots show modifications of intensity (number of ions) function of the m/z ratio for CPZ, out of which the molecular ion at 319 u.a.m. is identified, which corresponds to CPZ ions. One may observe that after 120 min exposure, CPZ does not exist anymore in solution and the concentrations of the photoproducts vary during irradiation due to exposure time and to the competitivity between photoreactions triggered by the irradiation process.

The identified photoproducts are: promazine (PZ), promazine sulfoxide (PZ-SO), 2-hydroxy promazine (PZ-OH), 2-hydroxy promazine sulfoxide (PZ–OH–SO), chlorpromazine sulfoxide (CPZ–SO) and other 3 compounds found at the values of m/z 292, 308 and 300 u.a.m, respectively.

Out of the overall chromatogram, the percentual concentration was extracted for each compound and each irradiation time. Although it is the parent compound, CPZ disappeared from the sample after 120 min irradiation.

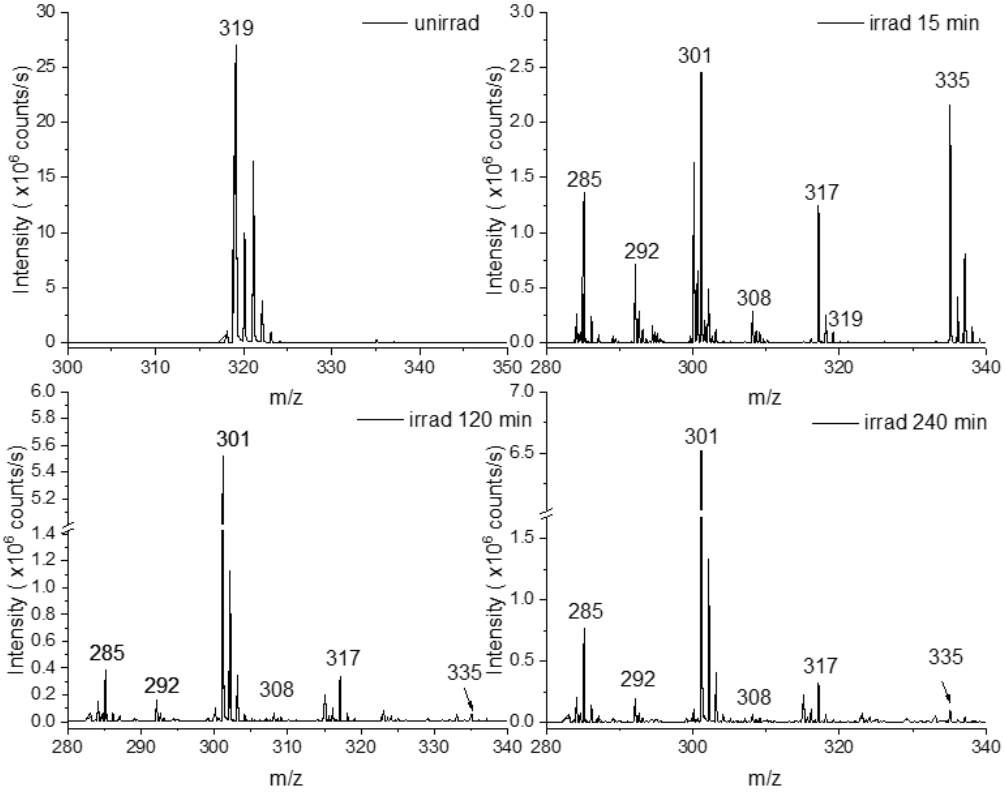

Fig. 6. LC-TOF-MS spectra of 2mg/mL CPZ unirradiated and irradiated water solutions.

The time dependence of CPZ-SO shows a maximal concentration of 6.84% after 5 min exposure and then, decreases down to 1.1% at the end of exposure (240min). PZ–OH–SO shows maximum concentration of 5.41% after 120 min which decreases to 4.26% at the end. For PZ, the maximum concentration is 46.58% after 60 min irradiation and 28.31% at the end. These data show that the maximum abundance of products is obtained after 60 minutes irradiation; the parent compound CPZ disappears from the solution after 120 min.

At longer time intervals, the dominating product is PZ-OH/PZ–SO that shows a constant increase in concentration with irradiation time, up-to the maximum of 63.41% after 240 min.

In accordance to these data, during the irradiation of the parent compound solution, the sample has an inner evolution extremely dynamic which is characterized by the disappearance of the parent compound and formation of photoproducts whose concentrations vary during irradiation, so that at the end the mixture is dominated by PZ-OH/PZ–SO.

3.2 Applications on bacteria cultures

The applications of the solutions were made on cultures of Gram-positive and -negative bacteria such as several strains of the Gram-positive *Staphylococcus aureus*, which are not absolutely immune to the treatment with "standard" antibiotics, but show a high resistance to unirradiated CPZ.

The schemes for bacterial samples preparations and for evaluation of results are described in detail in [21,22].

Bacteria cultures are harvested and cultivated in Agar cells and nourished in standard conditions; they are exposed to CPZ unirradiated solution in water at 20 mg/mL, on one hand and to irradiated CPZ solutions from time intervals between one min and 20 min, on the other.

In Fig.7 are shown results of the exposure of *Staphylococcus aureus* ATCC 25923 to irradiated solution which is applied on the discs placed in bacteria environment in quantities between 100 μg and 600 μg.

This kind of qualitative results is obtained for the interaction of solutions with other Gram-positive bacteria, as well [23,24].

In Fig.7 is shown that the unirradiated water solution of CPZ does not have any effect on the bacteria culture. For CPZ irradiated 1 min, starting from 500 μg on, the bacteria are killed, as results from the transparent discs 5 and 6. This effect is even stronger for CPZ solution irradiated 240 min, where the action is observed from 400 μg on, and the quantities of killed bacteria are slightly higher than for CPZ irradiated 1 min.

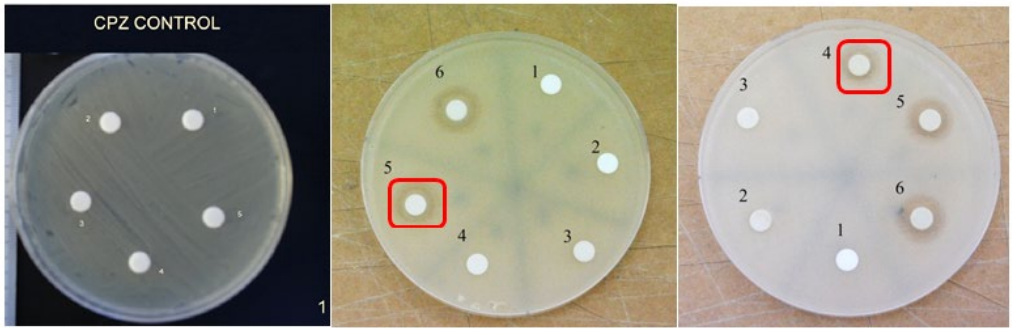

Figure 7. Qualitative data about the biological activity of CPZ exposed at 266 nm, against ATCC 25923 Staphylococcus aureus strain. Numbers 1 to 5 in each disc correspond to the quantities of solution placed on discs: 1 (100 μg); 2 (200 μg); 3 (300 μg); 4 (400 μg); 5 (500 μg); 6 (600 μg).

These results may be completed with quantitative data obtained about the minimum inhibitory concentration (MIC) for *Staphylococcus aureus* ATCC 25923

and *Escherichia coli* 8735 (which is a Gram-negtive bacterium) when CPZ irradiated solution is applied. For the solution exposed 240 min at 266 nm, MIC values expressed in μg/mL are 32 times lower. This demonstrates that the use of CPZ irradiated solutions for application on the two strains is efficient, which is in agreement with data reported in Ref. [22, 24].

5. Conclusions

The reported results are ground breaking in one of the most actual domains in biomedicine and, in particular, in post-surgery care and epidemiology. The MDR fight can be made by preparing new medicines which remains anyway a quite costly and time-consuming alternative, or by finding new ways of acting on existing medicines which leads to the development of associated treatment procedures. One of the most recent methods is based on the modification of molecular structures of some medicines and generation of photoproducts starting from a parent compound exposed to laser radiation. We demonstrated that the successful fight against MDR of some Gram-positive and Gram-negative bacteria may be achieved by exposure of CPZ to laser beam emitted at 266 nm. When exposed to UV laser radiation the CPZ molecules generate new photoproducts which were identified and the resulting solutions contain cocktails of photoproducts which were applied on Gram-positive and Gram-negative bacteria such as *Staphylococcus aureus ATCC 25923* or *Escherichia coli* 8735, respectively, and proved to be able to kill them. This result opens new and promising perspectives in fighting MDR acquired by some bacteria by enlarging the potential candidates amongst existing medicine.

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