Scientific Report

regarding the implementation of the project between May - December 2018 (Stage I)

The objective of the project is to generate antimicrobial agents by exposing aqueous solutions of TZ and CIP to 266 nm laser radiation for time intervals ranging from 1 minute to 240 minutes. For unirradiated and irradiated medicines solutions, the antimicrobial activity will be determined against CIP sensitive and resistant Gram-positive/-negative bacteria. The optimal irradiation time for the best antimicrobial activity will be established. Samples will be investigated using an HPTLC densitometry system in order to determine laser induced fluorescence (LIF) and fluorescence lifetime (FLT) for the separated photoproducts. The results will be correlated with mass spectrometry measurements.

Within this stage, the objectives were the development of the three experimental set-ups (for the irradiation of TZ and CIP, for LIF measurements and for LIF life time measurements) the optical characterization of the irradiated samples and the testing of irradiated solutions against bacteria strains.

Within this stage the following were realized:

1. Development of the irradiation set-up.
2. Irradiation of TZ and CIP aqueous solutions for various time intervals.
3. Development of the experimental set-ups used for LIF and LIF lifetime measurements.
4. Optical characterization of unirradiated and irradiated solutions of TZ and CIP by UV-Vis and FTIR absorption, LIF and LIF lifetime.
5. Selection of Gram-positive/-negative bacteria sensitive and resistant to CIP.
6. Susceptibility test of Gram-positive/-negative bacteria to unirradiated and irradiated TZ and CIP.
7. Selection of the best irradiation conditions in order to obtain agents with enhanced photo-antimicrobial effect.

1. Development of the irradiation set-up.

The samples were irradiated with a pulsed laser beam having 6.5 mJ average pulse energy at 266 nm (the fourth harmonic of a Nd:YAG laser), 6-ns FTWHM and 10-Hz pulse repetition rate. The optical path length was 1 cm, the beam spot area on the cuvette was 0.38 cm^2, and the beam fluence 17.1 J/cm^2. LIF signal was collected using an optical fiber positioned at 45° with respect to beam propagation direction. Signals were recorded using a spectrograph with 2.6 nm optical resolution.

2. Irradiation of TZ and CIP aqueous solutions for various time intervals.

For photochemical study of TZ and CIP solutions in ultrapure water, 2 mg/mL was irradiated with a pulsed laser beam having 6.5 mJ average pulse energy at 266 nm. The solutions were continuously stirred at 700 rpm to permanently homogenize the solution. Irradiation was made at different time intervals between 1 and 240 min.

3. Development of the experimental set-up used for LIF and LIF lifetime measurements.

For the LIF studies an experimental system consisting of a laser diode, droplet generation system, optical fiber and spectrograph was developed. The unirradiated and irradiated solutions were excited with a laser emitting diode at 375 nm for different frequencies (1 - 50 MHz). LIF signals were collected using an optical fiber positioned at 45° with respect to beam propagation direction. Signals were recorded using a spectrograph with 2.6 nm optical resolution.

For LIF lifetime studies an experimental system consisting of a laser diode, droplet generation system, optical fiber, photosensor module and oscilloscope was developed. The unirradiated and irradiated solutions were excited with a laser emitting diode at 375 nm at 40 MHz. LIF signal was collected using an optical fiber positioned at 45° with respect to beam propagation direction. A photosensor module having 0.78 ns rise time, was used for LIF lifetime measurements and its output was fed to a digital oscilloscope.

4. Optical characterization of unirradiated and irradiated solutions of TZ and CIP by UV-Vis and FTIR absorption, LIF and LIF lifetime.

4.1.UV-Vis absorption spectroscopy

The UV-Vis absorption spectra of unirradiated and 1-240 min irradiated TZ and CIP are shown in Figure 1. Two absorption bands with peaks at 262 nm and 315 nm characterized the absorption spectra of unirradiated TZ; CIP was characterized by four bands with peaks at 207, 223, 277, 316 and 331 nm.

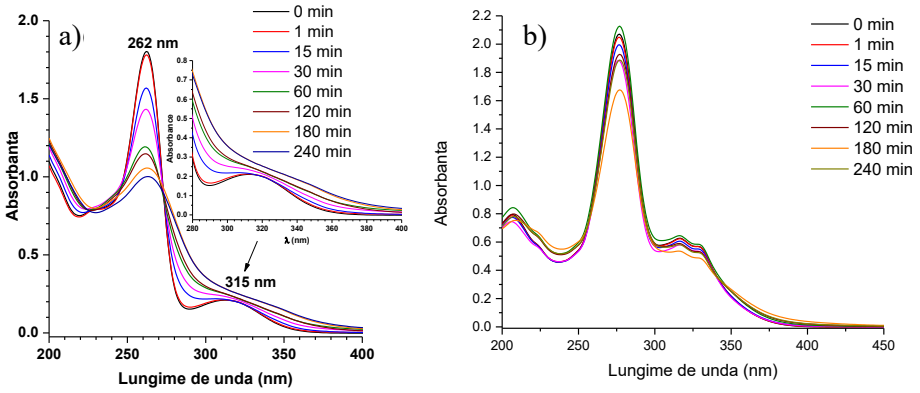

Figure 1. a) Absorption spectra of 0.2 mg/mL TZ unirradiated and irradiated with 266 nm between 1 min and 240 min. b) Absorption spectra of 0.2 mg/mL CIP unirradiated and irradiated with 266 nm between 1 min and 240 min.

During 240 min irradiation of aqueous solutions, the UV-Vis absorption spectra suffered hypochromic, hypsochromic, and bathochromic shifts when compared with that of unirradiated solutions.

The time-stability study of TZ solutions suggests that their stability was linear with exposure time at 266 nm. Longer irradiation times produce photoproducts that are stable for shorter periods of time. For CIP, the samples irradiated 15 min or 60 min have presented the longest time stability, respectively four weeks.

The dynamics of the peaks identified in absorption spectra shows an absorption dependence on irradiation time and all changes in spectra suggest photodegradation.

4.2. FTIR absorption spectroscopy

The unirradiated and irradiated TZ and CIP solutions were investigated by FTIR spectroscopy and the vibrations corresponding to various molecular bonds were identified. The IR spectra of unirradiated and irradiated TZ and CIP are presented in Figure 2.

When comparing the spectra of unirradiated and irradiated TZ it was observed that sulfoxide and phenol groups are formed.

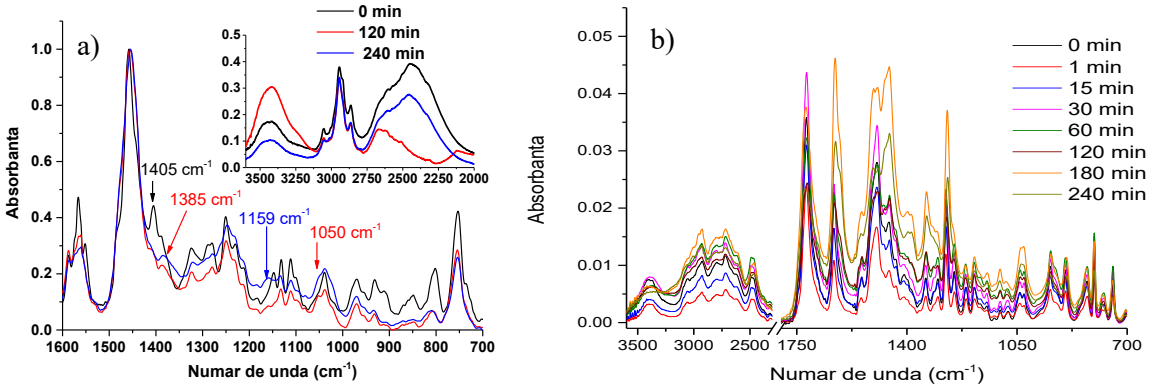

Figure 2. FTIR spectra of unirradiated and irradiated a) TZ and b) CIP.

For CIP, the IR spectra modification suggested cleavage of piperazine and formation of ketone.

4.3. LIF measurements

The LIF spectra obtained during irradiation of 2 mg/ml TZ solution up to 240 min with a 266 nm laser beam were characterized by the presence of a single band with peak at 504 nm (Figure 3). The intensity of fluorescence increased in the first minute of irradiation and afterwards decreased. At the same time, the peak intensity undergone bathochromic shifts by the end of irradiation time, suggesting formation of new photoproducts.

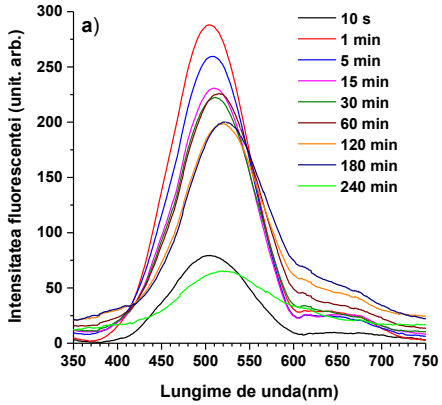

Figure 3. LIF spectra of 2 mg/ml TZ solution irradiated up to 240 min.

Another experiment involved measurement of LIF spectra of TZ irradiated solutions using as excitation source a picoseconds laser diode at 375 nm. The liquid volume generated as a droplet and was 15 µL. LIF spectra were recorded at different laser diode frequencies between 1 and 50 MHz during 1000 µs.

For the unirradiated TZ solution, LIF spectrum in the first 10 µs of exposure at 375 nm with a frequency of 10 MHz presented a band with peak at 478 nm. For the irradiated TZ solution the following bands resulted: 1 min - 484 nm, 15 min - 501 nm, 30 min - 503 nm, 60 min - 502 nm, 120 min - 500 nm, 180 min - 485 and 240 min 504 nm. With increasing frequency, the fluorescence intensity increases linearly by up to 91%. By increasing the exposure time (at 375 nm) to 1000 µs, bathochromic and hyperchromic shifts could be observed.

The LIF spectrum of 2 mg/mL CIP solution irradiated 240 min with a laser beam at 266 nm was characterized by the presence of a single band with a maximum at 458 nm (Figure 4). During irradiation, the intensity of fluorescence suffers a 65% hypochromic shift. For CIP solutions, peak wavelength did not change during CIP exposure to UV radiation. These changes in the fluorescence spectrum suggest the formation of photoproducts as a result of CIP interaction with the laser beam emitted at 266 nm.

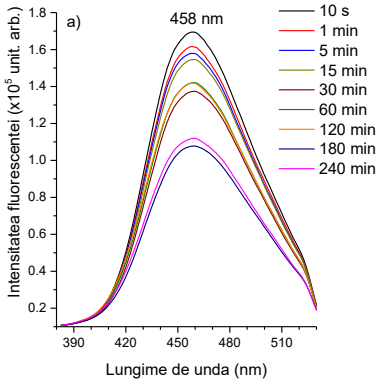

Figure 4. LIF spectra of 2 mg/ml CIP solution irradiated up to 240 min.

As for the measurement of LIF spectra of CIP irradiated solutions using as excitation source a picoseconds laser diode emitting at 375 nm, the volume generated as a droplet was of 15 µL and LIF spectra were recorded at different laser diode frequencies (1-50 MHz) for 1000 µs.

For the unirradiated CIP solution, LIF spectrum in the first 10 µs of laser exposure to 375 nm at a frequency of 10 MHz presented a band with peak at 456 nm. For irradiated TZ solution the following bands resulted: 1 min - 458 nm, 15 min - 458 nm, 30 min - 459 nm, 60 min - 458 nm, 120 min - 462 nm, 180 min - 461 and 240 min 465 nm. With increased frequency, the fluorescence intensity increases linearly by up to 85%. Correspondingly, bathochromic and hyperchromic shifts were observed. For the rest of the samples, no changes in the wavelengths of the peaks were observed.

By increasing the irradiation time to 1000 µs, bathochromic, hyperchromic and hyperchromic shifts occurred.

4.4. LIF lifetime (FLT) measurements

FLT values were determined by fitting the time-resolved fluorescence signals with an exponential decay function. The transient fluorescence signals for unirradiated and irradiated TZ and CIP are presented in Figure 5.

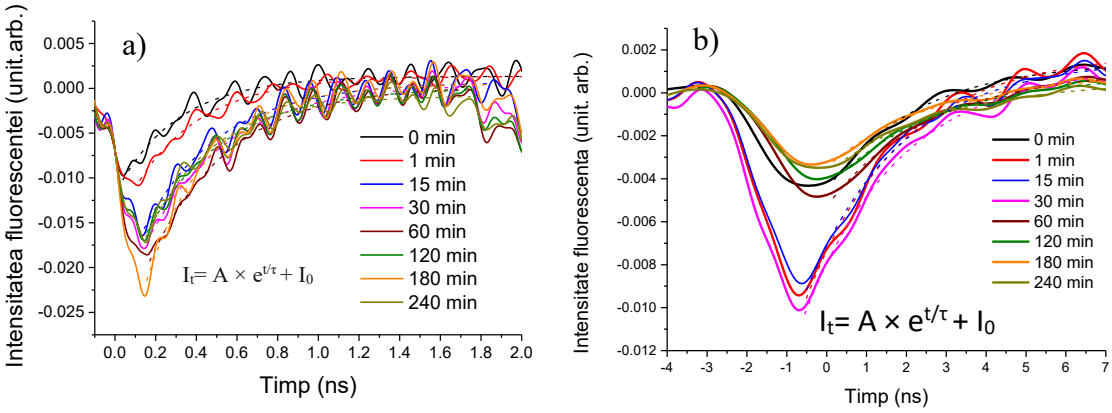

Figure 5. Transient fluorescence signal for a) unirradiated and irradiated TZ and b) unirradiated and irradiated CIP

For unirradiated TZ, a FLT value of 3.02 ns was obtained, while for TZ irradiated 240 min a value of 3.24 nm. The FLT resulted values did not increase linearly with exposure time of 266 nm irradiated TZ solutions, suggesting that different photoproducts are formed for each exposure time. For unirradiated CIP, a FLT of 1.58 ns was obtained, while for irradiated CIP 240 min a value of 1.96 nm was obtained. The obtained FLT values increase linearly with the exposure time of 266 nm irradiated CIP solutions, suggesting the formation of photoproducts.

5. Selection of Gram-positive/-negative bacteria sensitive and resistant to CIP.

The quantitative assays of antimicrobial activity of photoproducts mixtures were performed on Gram-positive bacterial strains: *S. aureus* ATCC 25923 (MSSA), *Staphylococcus aureus* ATCC 25923 (MSSA) adapted to ethidium bromide (EtBr), *Staphylococcus aureus* SM1 (resistant la CIP), *Staphylococcus epidermis ATCC* 12228, *Staphylococcus epidermis* ATCC 12228 adapted to la EtBr, *Enterococcus faecalis ATCC* 29212 and Gram-negative bacterial strains: *Escherichia coli* ATCC 25922, *Escherichia coli* 20398 (resistant la CIP), *Salmonella enterica* NCTC 133349 and *Klebsiella aerogenes* ATCC 15038.

6. Susceptibility test of Gram-positive/-negative bacteria to unirradiated and irradiated TZ and CIP.

In the case of irradiated TZ samples, the antimicrobial effect was increased up to 16 times when compared to with that of the unirradiated TZ. Thus, the best MIC values obtained for *S. aureus* ATCC 25923 (MSSA) and *S. aureus* ATCC 25923 (MSSA) adapted to EtBr, *S. aureus* SM1 (MRSA) and *S. epidermidis* ATCC 12228 was of 3.125 mg/L, for *S. epidermidis* ATCC 12228 EtBr of 1.56 mg/L and for *E. faecalis* ATCC 29212 of 6.25 mg/L.

Irradiated TZ does not show improved antimicrobial effects against Gram-negative bacteria. The same was observed for irradiated CIP, where no improvement of antibacterial activity against Gram-positive and Gram-negative bacteria was observed.

For *S. aureus* ATCC 25923 (MSSA), *S. aureus* ATCC 25923 (MSSA) adapted to EtBr, *S. epidermis* ATCC 12228 and *S. epidermis* ATCC 12228 adapted to EtBr unirradiated or irradiated TZ do not act as efflux pump inhibitors and do not facilitate the activity of the CIP antibiotic.

7. Selection of the best irradiation conditions in order to obtain agents with enhanced photo-antimicrobial effect.

The susceptibility studies showed that the best irradiation conditions of TZ to obtain improved antimicrobial photoproducts implied an exposure lifetime of 60 min for *S. aureus* ATCC 25923 (MSSA), 120 min for *S. aureus* and 30 min for *S. aureus* ATCC 25923 (MSSA) EtBr, *S. epidermis* ATCC 12228, *S. epidermis* ATCC 12228 EtBr and *E. faecalis* ATCC 29212.

From MIC analysis, TZ irradiated solutions can be considered antimicrobial agents that do not act on the efflux pumps of tested bacteria. Thus, their mechanism of action on bacteria is still unknown, but possible effects that can be mediated by these irradiated solutions could be: direct inhibition of bacterial replication, inhibition of bacterial motility or elimination of plasmids.

Conclusions

During this stage, the experimental set-ups were developed. Photo-degradation studies of CIP and TZ, both at a concentration of 2 mg/mL, were conducted in order to highlight the photochemical changes resulted during 266 nm laser irradiation. The mixture of photoproducts was analyzed by UV-Vis and FTIR absorption spectroscopy, laser-induced fluorescence, LIF.

From the absorption and LIF spectra one can deduce the photo-dissociation of TZ and CIP molecules into photoproducts, photoproducts that were confirmed by the FT-IR spectra.

In vitro susceptibility testing of microorganisms to antimicrobial agents showed a strong antimicrobial action of irradiated TZ solutions for 30, 60, 120, 180 and 240 min. These solutions are not efflux pump inhibitors, acting against bacteria by other mechanisms of action. As expected, irradiated TZ solutions can destroy MDR bacteria, being 82 times more efficient than the antibiotic CIP.

In the case of irradiated CIP solutions, the modification of the molecular structure under the action of laser radiation emitted at 266 nm generated photoproducts that presented the same antimicrobial effect as the parental compound. In this way the project objectives for this stage have been fulfilled and the estimated results have been obtained.

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