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Synthesis, characterization and *in vitro* antimicrobial activity of the Cu(II) and Fe(III) complexes with 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid

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Abstract:1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (Ciprofloxacin, CFL) is a drug that belongs to the second generation of fluoroquinolone antibiotics with a wide range of effects on Gram-positive and Gram-negative bacteria. The bactericidal action of ciprofloxacin results from the inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV. In organism there is a possibility of interaction of CFL with biogenic elements in the blood, which could lead to the formation of complexes. This can cause change in the activity of antibiotics towards pathogenic microorganisms. The aim of this work was to investigate the interaction of CFL as ligand with the biological cations Cu(II) and Fe(III) in physiological condition. Synthesized complexes were characterized using IR spectroscopy and stereo-microscopy. Antimicrobial screening was performed on bacterial strains of *Escherichia coli*, *Enterococcus faecalis*, *Staphylococcus aureus* and *Salmonella Enteritidis*. The results of IR spectroscopy showed that the Cu(II) and Fe(III) complexes with CFL were formed through the oxygen donor atoms of carboxyl and carbonyl group of the ligand. The color and size changes of the crystal of the ligand and complexes were also clearly seen. Antimicrobial screening has shown that CFL and CFL complexes have similar antimicrobial activity against all tested strains. The $Cu(CFL)_2$ complex showed better antimicrobial activity compared to the $Fe(CFL)_2(H_2O)_2$ complex.

INTRODUCTION

1-Cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-1,4 dihydroquinoline-3-carboxylic acid (Ciprofloxacin, CFL) is a fluoroquinolone antimicrobial commercially available as the monohydrate phase of its hydrochloride. It is formulated for oral and ophthalmic use, while intravenous administration is in the form of lactate salt negative microorganisms. The structure of CFL is shown in Figure 1. The mechanism of action of CFL is based on the inhibition of the activity of enzymes of DNA gyrase (topoisomerase II) and topoisomerase IV which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination. The CFL is one of the most frequently prescribed antimicrobial drugs (19th WHO Model List of Essential Medicines, April 2015) and is the first fluoroquinolone

that has shown significant activity outside the urinary tract (Correia *et al.*, 2017). The reason for its widespread is the susceptibility of multi-resistant pathogens to ciprofloxacin. Additionally, drug efficacy of CFL is clinically proved in the treatment of nosocomial infections, such as those of the respiratory and urinary tract, skin infections and sexually transmitted diseases (Sharma *et al.*, 2010). Ciprofloxacin is the most active quinolone against *Pseudomonas aeruginosa* (LeBel, 1988).

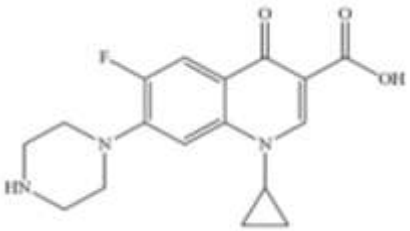

Figure 1. *The structure of CFL*

The analysis of the interaction of biogenic metal M(II) cations with O, N, S-donor atoms of ligands often used in the treatment of a wide spectrum of diseases is important for monitoring of distribution, pharmacokinetics, excretion, drug efficacy and adverse effects (Cipurković *et al.*, 2017).

The copper is present in the organism in the composition of cupro-enzymes and regulators of expression, of which the most famous are lysyl oxidase - involved in the crosslinking of collagen and elastin, tyrosinase - essential for the synthesis of melanin and superoxide-dismutase - responsible for defense against free radicals (Solioz, 2018). The most important characteristics of the active copper associated with proteins are its function in the metabolism of O_2 or N/O compounds and a frequent association with oxidative organic and inorganic radicals (Wolfgang and Jochen, 1996). Biological functions of copper are mainly related to its role as ligand for metallocenes and the biochemical role of copper is primarily catalytic (Hefnawy and El-Khaiat, 2015). Copper is in serum present in two main forms; bound to plasma-ceruloplazmin protein and to albumin (Angelova *et al*., 2011). Human gastrointestinal system can absorb 30-40% of copper introduced by ingestion. Minerals with similar chemical properties can reduce copper absorption, while proteins, soluble carbohydrates and organic acids, except for ascorbic, produce a positive effect in terms of increased solubility and copper flow in the intestines, which improves absorption and bioavailability (Wapnir, 1998).

Iron as an essential bioelement in contact with oxygen forms oxides, which are highly unstable and therefore not readily available for absorption in organisms (Abbaspour, Hurrell and Kelishadi, 2014). Iron is an integral part of proteins, such as hemeproteins, which are involved in various crucial biological processes, such as reversible oxygen binding for hemoglobin and myoglobin and is responsible for transport and storage of oxygen (Souza, 2005). In an adult male body the iron content is 3000-4000 mg, and the adult female body contains only 2000-3000 mg. The difference in iron content in men and women is attributed to lower iron reserves in women, lower hemoglobin concentrations and lower vascular volume than men (Arora and Kapoor, 2012). The most important forms of iron storage are ferritin and hemosiderin and are stored in the bone marrow and liver.

EXPERIMENTAL

Synthesis of the complex

A mixture of ethanol and water in a ratio of 50/50 (v/v) was used as a solvent for the ligand and Cu (II) and Fe (III) salts. The metals (M) and the ligand (L) were mixed in a 1:2 molar ratio (n/n). The solutions of the metal (10 mL) and ligand (10 mL) were mixed in a glass and stirred on a magnetic stirrer without heating. The pH value of the solution was adjusted with 1 M NaOH. The optimum pH value for testing the Cu (II)-CFL system is 5.6 and for the Fe (III)-CFL system is 7.3. The prepared solutions were mixed on a magnetic stirrer for 30 minute, then left to stand in a darkened area for two weeks in order to precipitate the complex. The resulting products were filtered through a blue strip filter paper and then dried at room temperature, after which their characterization was performed.

FTIR characterization

In order to determine the structure of the complex Nicolet iS10 FT-IR spectrophotometer - Thermo Fisher Scientific was used.

Morphological characterization

In order to compare color, texture and ligand particle size, the synthesized solid complexes were treated with DMSO and subjected to microscopic analysis. Analysis was performed on the binocular microscope Leica DM 2500P in polarized light with and without crossed Nicole (XPL and PPL).

In vitro antimicrobial activity

Antimicrobial activity was studied by a diffusion method on reference bacterial strains (from ATCC collection) from gram positive group (*Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 51299) and gram negative bacteria (*Escherichia coli* ATCC 25922 and *Salmonella Enteritidis* ATCC 13076) by procedure form Clinical and Laboratory Standards Institute, 2009. From the bacterial strains of overnight cultures, suspensions of 0.5 McFarland turbidity were prepared (density 107-108 CFU/mL, depending on soy). The strains were then placed on the surface of the nutrient substrate-Mueller-Hinton agar (MH), dispersed in sterile Petri dishes. Substrate thickness was 4 mm. In the agar sterile drill-shaped holes were made ("wells"), into which 50 µL of CFL and Cu (II) and Fe (III) complexes solution in concentration of 1 mg/ml were added. After the plates were left at room temperature for 15 minutes, the substance was diffused into agar, incubated at 37°C/24 h. After the incubation period, the size of the inhibitory zone was measured and the sensitivity of the microorganisms was expressed as follows: if the zone for inhibition of microorganism growth was greater than 20 mm, it was labeled with three pluses (+++), representing the highest sensitivity of

the microorganisms. If the inhibitory zone ranged from 16 to 20 mm, it was marked with two pluses (++). Very weak sensitivity is marked with a plus (+) if the inhibitory zone is 10-15 mm in diameter. For the inhibitory zone less than 10 mm or if absent, the minus (-) has been used (Pirvu *et al*., 2014).

RESULTS AND DISCUSSION

Structure of the complex

Due to the presence of oxygen donor atoms and one nitrogen atom, CFL can act as a ligand in the reactions with metals ion. Spectral studies suggest that ciprofloxacin behaves as a bidentate ligand in these complexes, the carboxylic oxygen and carbonyl oxygen atoms participating in these bonds. In this case the results obtained indicate the formation of complexes like: $[M(CFL)_2(H_2O)_2]$, where M = Fe^{3+}, and $[M(CFL)_2]$, where M = Cu^{2+}. The proposed structures of metal complexes CFL with Cu^{2+} and Fe^{3+} are shown in Figure 2. These structures show that the complex with Fe^{3+} contains two molecules of CFL per unit metal atom and each molecule is directly coordinated to the Fe^{3+} ion along with two water molecules giving an octahedral complex, similar to the molecular structures of the complexes of the other fluoroquinolone (Gao *et al*, 1995**)**. Based on the IR spectra of the Cu(II) complex with CFL, its square-planar configuration is proposed (Chohan, Supuran and Scozzafava, 2005).

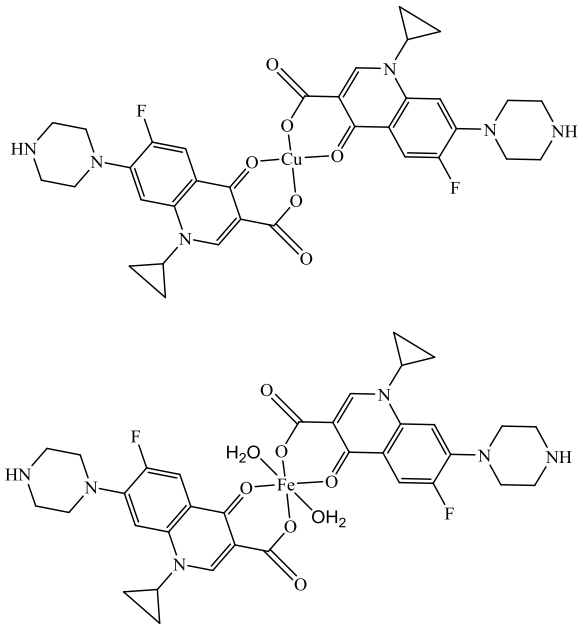

Figure 2. *Proposed structure of $Cu(CFL)_2$ and $Fe(CFL)_2(H_2O)_2$ complexes*

Synthesized complexes differ in color from the parent ligand. The resulting complex $Fe(CFL)_2(H_2O)_2$ is orange, while the complex $Cu(CFL)_2$ is colored light blue.

Spectral characterization

Figure 3 shows the FTIR spectra of CFL and CFL complexes with Fe(III) and Cu(II).

At the IR spectrum of ciprofloxacin, the strong, sharp peak was detected at 1703.2 cm^{-1}, indicating the vibration of the carbonyl group from CFL.

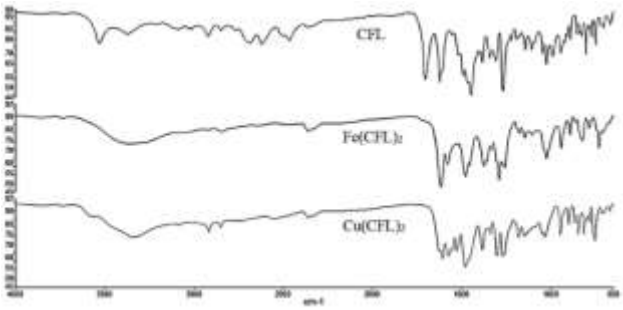

Figure 3. *FTIR spectra of CFL and complexes $Fe(CFL)_2$ and $Cu(CFL)_2$*

At 3376.4 cm^{-1}, a wide peak was detected which corresponds to the N-H bond vibrations. Also, the wide peak of the O-H group is present at 3529 cm^{-1} in the ligand spectrum. The strong peaks at 1622.8 and 1443.8 cm^{-1} confirm the presence of a double bond C=C. Vibrations of the C-F bond cause the appearance of a strong, sharp peak at 1023.7 cm^{-1} and the weak peak at the interval of 3085.8-3013.2 cm^{-1} is most likely due to the vibration of the Ar-H bond. Peaks of medium to low intensity were observed at 1106.3 and 1143.6 cm^{-1}, which were caused by stretching of the C-N bond. Due to the appearance of a strong intensity peak at 1268.4 cm^{-1}, the presence of the C-O group was confirmed.

Comparing FTIR spectra of the parent ligand with the spectra of synthesized complexes, some differences were registered. Due to the absence of strong bands at ~ 1700 cm^{-1} characteristic of C=O and ~ 3500 cm^{-1} from O-H group, it was concluded that the bonds between metal and ligand were realized via the oxygen donor atoms of the carbonyl and carboxyl groups of the ligand. The characteristic vibrations of the ligand and complexes are shown in Table 1.

Table 1. Characteristic vibrations of the ligand and complexes (cm^{-1})

Functional group	Ligand/Complex		
	CFL	$Cu(CFL)_2$	$Fe(CFL)_2(H_2O)_2$
C=O	1703.2	-	-
C-O	1268.4	1268.9	1291.2
O-H	3529	3511.2	-
N-H	3376.4	3337.3	3351.5
C=C	1622.8	1606.2	1615.4
C-F	1023.7	1030.5	1022.9
C-N	1143.6	1339.2	1103.2
Ar-H	3085.8	-	-

Morphological characterization

The morphology of CFL crystals and its complexes with Cu(II) and Fe(III) are shown in Figures 4-6.

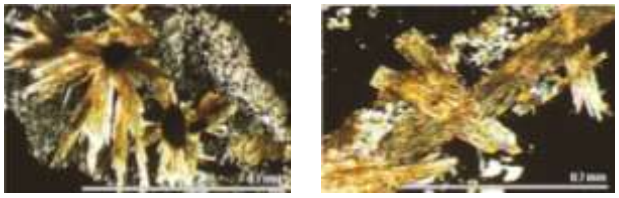

Figure 4. *Morphology of CFL crystals*

Regarding the size of the crystals of the ligand, they are divided into small crystals (below 1 mm) and medium crystals (1-10 mm). Interferential colors are live of the first order. These crystals are characterized with radially shape originated from the same center (Figure 4 - left) and prismatic forms (Figure 4 - right).

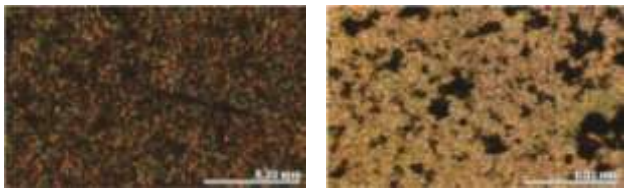

Figure 5. *Morphology of $Fe(CFL)_2(H_2O)_2$ crystals*

Regarding the size of the $Fe(CFL)_2(H_2O)_2$ complex crystals presented at Figure 5 they are very small crystals called microcrystals (below 1 mm). Interferential colors are live of the first order.

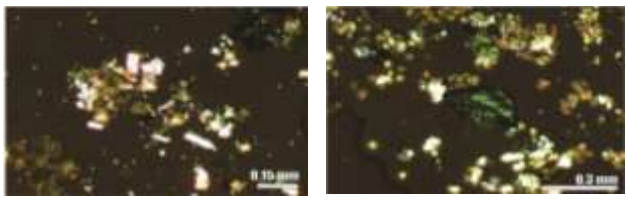

Figure 6. *Morphology of $Cu(CFL)_2$ crystals*

Figure 6 shows $Cu(CFL)_2$ complex crystals. Crystals are small (below 1 mm). Interferential colors are poorly expressed and crystals are mostly transparent. The morphology of crystals points to prismatic forms (Figure 6 - left). Crystals with pronounced interferential colors appear in a different way (Figure 6 - right). Also, at Figure 6 - right a crystal that could be classified as euhedral (idiomorphic) and indicates an irregular hexagram. The color of the crystal indicates presence of Cu and is probably a consequence of the thickness of the crystal.

Antimicrobial activity

Results of *in vitro* antimicrobial activity on selected bacterial strains are shown in Table 2. As already mentioned, the mechanism of CFL action is based on the inhibition of DNA enzyme activity (topoisomerase II) and topoisomerase IV, which are necessary for DNA replication. Before binding to these enzymes, CFL is chelated with metal ions (most commonly Mg^{2+}). Only after the chelation, CFL can bind to any of these two enzymes and block DNA chains synthesis.

Table 2. Antimicrobial activities of CFL and $M(CFL)_2$ complexes

Microorganism	Inhibition zone of L/ML_2 [mm]		
	CFL	$Cu(CFL)_2$	$Fe(CFL)_2(H_2O)_2$
Escherichia coli	40	40	40
Staphylococcus aureus	37	35	34
Salmonella Enteritidis	39	39	36
Enterococcus faecalis	27.5	26	23

In this regard, the results of antimicrobial *in vitro* screening test show that the tested $Cu(CFL)_2$ and $Fe(CFL)_2(H_2O)_2$ complexes have almost the same antimicrobial activity as the antibiotic itself. It is very important that formation of copper and iron complexes does not reduce antimicrobial effect of CFL, since the forming of complexes with some of biometals is an indispensable process of antibiotic.

CONCLUSIONS

The analysis has shown that in physiological conditions, the selected bioelements interact with CFL through the oxygen donor atoms of carbonyl and carboxyl groups. The morphology of the ligand and complexes are significantly different, primarily in the $Fe(CFL)_2(H_2O)_2$ complex. Relative to the parent ligand, $Cu(CFL)_2$ and $Fe(CFL)_2(H_2O)_2$ complexes shows similar antimicrobial activity as the CFL alone. Forming of complexes does not disturb the mechanism of action of antibiotics in the bacterial cell.

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Summary/Sažetak

1-ciklopropil-6-fluoro-4-okso-7-(piperazin-1-il)-1,4-dihidrokinolin-3-karboksilna kiselina (Ciprofloksacin, CFL) je lijek koji pripada drugoj generaciji fluorokinolonskih antibiotika, a ima širok spektar efekata na Gram-pozitivne i Gram-negativne bakterije. CFL sprečava diobu bakterijskih ćelija, inhibiranjem DNA giraze, topoizomeraze tipa II, te topoizomeraze IV. U organizmu postoji mogućnost interakcije CFL sa biogenim elementima iz krvi, što vodi nastanku kompleksa. Ovo može uzrokovati promjenu aktivnosti antibiotika prema patogenim mikroorganizmima. Cilj ovog rada je istražiti interakciju CFL sa biološkim kationima koji su inače prisutni u krvi, Cu(II) i Fe(III), u približno fiziološkim uslovima. Sintetizirani kompleksi su karakterizirani upotrebom IR spektroskopije i stereo-mikroskopije. Antimikrobni skrining je obavljen na bakterijskim sojevima: Escherichia coli, Staphylococcus aureus, Enterococcus faecalis i Salmonella Enteritidis. Rezultati IR spektroskopije su pokazali da su kompleksi Fe(III) i Cu(II) sa CFL formirani preko O-donorskih atoma karbonilne i karboksilne grupe liganda. Takođe, promjene boje i veličine kristala matičnog liganda i kompleksa su jasno vidljive. Antimikrobni skrining je pokazao da CFL i kompleksi imaju sličnu antimikrobnu aktivnost u slučaju svih bakterijskih sojeva, pri čemu kompleks $Cu(CFL)_2$ pokazuje bolju antimikrobnu aktivnost u poređenju sa kompleksom $Fe(CFL)_2(H_2O)_2$.