Scientific and technical report

Financing contract

Project title: The synthesis of new hybrid [2.2]paracyclophane-flavonoids systems with potential antimicrobial activity

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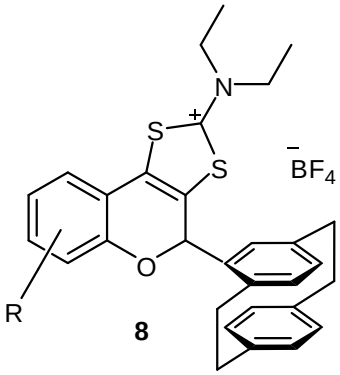
Acronym: [2.2]PC-Flav

No. 48/2018

Evaluation of antibacterial properties for the synthesized flavonoids

Stage 2 2019



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Testing the activity of 1,3-dithiolium flavonoids on a wide spectrum of bacteria.

Determination of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC values determined for the four flavonoids, tested against two bacterial strains (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922), are presented in Table 1. Although antimicrobial activity is important in both bacterial strains, it should be noted that the effect is much more

important against the Gram-positive bacteria. The four flavonoids showed much more important antibacterial properties compared to other synthetic flavonoids: 2´,4´,3-trihydroxychalcone (MIC = 46 mg/l against *E. coli*), 3-*O*-alkyl-(+)-catechine derivatives (MIC = 0.5-2 and 32-128 mg/l), 7-*O*-genisteine derivatives (MIC = 1,7 and > 50 mg/l against some Gram positive bacteria and > 50 mg/l against some Gram negative bacteria), etc. It should also be noted that three of the synthesized compounds (R = Cl, Br, I) proved to be more effective compared to pandurantine A (MIC = 0.5-1 mg/l against *S. aureus*) and izobavachalcone (MIC = 0.3-0.6 and 0.3 - >39.1 mg/l)- two of the most active natural flavonoids with antibacterial properties known to date.

Tabel 1 – The MIC and MBC values for the four investigated flavonoids

Flavonoid	Bacterial strain			
	MIC (µg/ml)		MBC (µg/ml)	
	S. aureus	***E. coli***	***S. aureus***	***E. coli***
R = F	**0.97**	**15.62**	**1.95**	**15.62**
R = Cl	**0.24**	**3.9**	**0.48**	**3.9**
R = Br	**0.24**	**3.9**	**1.95**	**1.95**
R = I	**0.24**	**1.95**	**0.48**	**0.97**

MIC = minimum inhibitory concentration

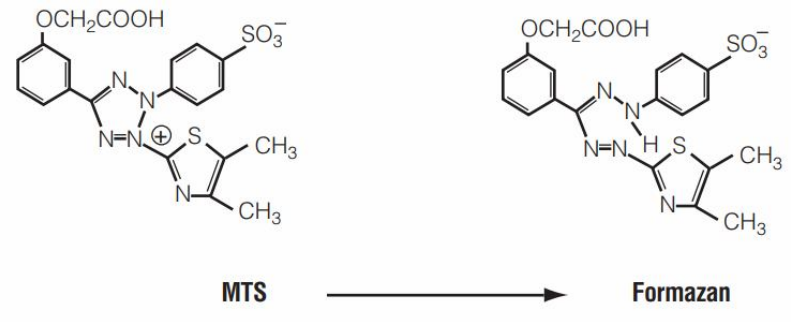
MBC = minimum bactericidal concentration

Evaluation of the influence of synthetic flavonoids on bacterial

The four tested flavonoids have significantly inhibited the growth of all bacteria in the exponential growth phase (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 13883). The presence of flavonoids in the culture medium determined the extension of the lag phase by up to 9 hours for *S. aureus* and *B. subtilis* at a concentration of 0.24 µg/ml compared to the control. Higher concentrations (3.9 µg/ml and 15.62 µg/ml) have induced the extension of the lag phase by up to 12 hours for *E. coli* and *K. Pneumoniae*. Throughout the experiments there was no growth noticed for 2 × MIC concentrations.

Cytotoxicity studies. Materials and methods

The cytotoxicity of the studied samples for the evaluation of the cellular metabolic activity was measured using the colorimetric test (CellTiter 96® Aqueous One Solution Cell Proliferation Assay, Promega). The CellTiter 96® AQueous One Solution agent contains a tetrazolium [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS] and an electron coupling agent, phenazine ethosulfate. This MTS in cell mitochondria is enzymatically transformed into a colored formazan, which absorbs at 490 nm, this absorbance being able to be read on a microplate reader. The absorbance value will depend on the amount of formazan produced, which in turn depends on the number of active mitochondria. Practically, indirectly, the MTS test reflects the number and state of health of the tested cells.



https://www.promega.ro/products/cell-health-assays/cell-viability-and-cytotoxicity-assays/celltiter-96-aqueous-one-solution-cell-proliferation-assay-_mts_/?catNum=G3582

The cells – normal human dermal fibroblasts – NHDF were purchased from PromoCell and cultivated in alfa-MEM (Lonza) medium supplemented with 10% fetal bovine serum (FBS, Biochrom GmbH) and 1% mixture of penicillin-streptomycin-amphotericin B (10K / 10K / 25 µg/100 ml, Lonza). For cultivation and expansion flasks with an area of 25 and 75 cm^2 were used, treated for tissue cultures until a sufficient number of cells is obtained. The cultures were performed in an incubator at 37 °C, 5% CO_2, wet atmosphere. A solution of 0.25% trypsin in EDTA (Lonza) was used to detach the adherent cells at the time of the passages. Once the required amount of cells was obtained (in about 1-2 weeks), the cells were detached with trypsin, washed in the medium, centrifuged and counted, after which they were seeded in 96 well microplates, treated for cell cultures; the concentration was 5000 cells/well.

The next day the solutions with the tested compounds were prepared (LB1-5). Because of the low solubility in water of these compounds, they were dissolved in DMSO. Thus starting with a 3200 µg/ml concentration in DMSO, several serial dilutions were performed in DMSO, after which they were further diluted 100 times, this time in culture medium, finally obtaining a range of concentrations 32 – 1.64 µg/ml. The cells cultured the day before were treated by replacing the existing culture medium with 100 µl/well of

these concentrations. The control cell wells were treated with 100 µl culture medium with 1% DMSO. Once the treatment was performed the cell plates were returned to the incubator.

After 44 hours of treatment, using a multichannel micropipette 20 µl/well of MTS solution was administered with and the plates were incubated for another 4 hours. Finally, the absorbance at 490 nm was read using a microplate reader (Ensight, PerkinELmer).

The experiments were repeated 3 times, and the data was interpreted using the program GraphPad Prism 6.04 (GraphPad Software, San Diego, CA). To calculate the concentration at which cell viability decreases to 50% (IC_{50}) from the program a nonlinear regression model with 4 parameters was applied and a logarithmic graph was obtained from concentration against effect (viability).

Results

The cytotoxic effect of the compounds described in Table 1 was evaluated by treating normal human dermal fibroblast cells for 48 hours with a range of their concentrations. For IC_{50} determination a logarithmic curve was obtained from the concentration and effect from which the IC_{50} value was extrapolated. The vertical values in the graph were deduced based on the MTS test, a colorimetric test that indirectly estimates the degree of cell viability.

Biocompatible polymeric membranes

The incorporation of tricyclic flavonoids in polymeric membranes was performed in a biocompatible and biodegradable polyurethane substrate. The study of active substance release was performed in relation to a calibration curve determined on a freshly prepared solution of $5x10^{-3}$ M in water. The spectral determinations for the release of the active ingredient indicate that the membrane used can be successfully used as a controlled transporter/releaser, especially if it is biocompatible and biodegradable.

Project leader,

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