Due to exceptional reactivity of 7-hydroxycoumarin, the synthesis of new coumarin derivatives of dimer and tetramer type has been carried out. The synthesis was carried out from 7-hydroxycoumarin and various aromatic aldehydes. In this way, compounds of the dimer 6,6’-(benzilidene)bis (7-hydroxycoumarin) type, as well as of the tetramer 6,6’,6”,6”’-(1,4-dimethylethenylenyl)tetra (7-hydroxycoumarin) type were prepared.

The newly synthesized derivatives contain different functional groups, and as such they could exhibit microbiological activity. Therefore, we tested the microbiological activity of these derivatives on various species of bacteria and fungi. The tested compounds have shown different activity in terms of growth inhibition of microorganisms. Newly synthesized derivatives exhibit antibacterial activities, manifested as growth inhibition on Gram-positive bacteria types (Bacillus, Staphylococcus), while the activity against Candida was much weaker. The same compound did not show any antimicrobial activity against two Gram-negative bacteria types (Escherichia coli, Pseudomonas aeruginosa). The compound 1 showed the best microbiological activity. The obtained results confirmed its good antibacterial and antymycotic activities against different microorganisms.

KEY WORDS: dimers, tetramers, derivatives of 7-hydroxycoumarin, antimicrobial activity, minimal inhibition concentration, MIC, minimal bactericidal concentration, MBC
INTRODUCTION

Studies of natural and synthetic coumarins and its derivatives have been present for a number of years. Coumarins and their derivatives are characterized by excellent chemical reactivity and different bioactivity (1-4). Their remarkable biological potential is the reason for synthesis of many new products, suitable for application in modern therapy. A great number of synthesized derivatives have shown pharmacological activity, and many of them are applied in therapy as anticoagulant (5), antibacterial (6) and antifungal agents (7). The interest in coumarins has recently increased significantly because it was found that they reduce the HIV activity (8, 9). Further, coumarin derivatives have shown cytostatic activity and therefore can be considered as potential candidates for anti-cancer therapy (10). Recently, coumarin derivatives of dimer and tetramer type were reported to possess HIV-1 integrase inhibitory activity. These facts urged the researches to undertake the synthesis of coumarin derivatives and study their biological activity.

MATERIAL AND METHODS

Syntheses of dimers of 4-hydroxycoumarin

The synthesis was carried out from 4-hydroxycoumarin and various aromatic aldehydes (8). The first step of the synthesis is aldol condensation, followed by dehydration, resulting in creation of a stable chromon. In the last step, the chromon conjugates in the presence of 4-hydroxycoumarin, the result of which is the creation of dimers of 3,3′-benzylidene)bis(4-hydroxycoumarin) type, as well as of tetramers of 3,3′,3″,3‴-(1,4-dimethylenphenyl)tetra(4-hydroxycoumarin) type.

The general procedure for the preparation of dimers and tetramers of 4-hydroxycoumarin

2 mmol for dimers synthesis or 4 mmol for tetramers synthesis of 4-hydroxycoumarin were dissolved in 6 ml of hot ethanol and 1 mmol of the corresponding aldehyde was added. The reaction mixture was heated under reflux for 24 hours. After the completion of the reaction, the reaction mixture was cooled at room temperature, which resulted in crystallization of the target compound. Pure compounds with constant melting points were obtained by recrystallization from chloroform.

Physical-chemical characteristics

Microanalyses for C, H and N were preformed on Perkin Elmer 2400 elementary analyzer (Germany), IR spectra were recorded on Perkin Elmer FT-IR 1000 (Germany) in KBr discs. The ‘H NMR spectra were recorded at 300.075 MHz, in CDCl3 and DMSO, on NMR Spectrometer, Varian Unity Plus 500 MHz and Bruker Advance DPX 300 MHz (Varian, UK).

Antimicrobial activity

The antimicrobial activity was tested by the diffusion method (11) against various bacteria such as Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 6538P, Escherichia coli ATCC 8739, Pseudomonas aeruginosa ATCC 9027, while antifungal activity was tested against Candida albicans ATCC 10231. The results of our tests were presented as the inhibition zones, given in millimeters (mm). The compound that showed best antimicrobial activity was further tested by the dilution method (11). For determination of antimicrobial activity (diffusion method) Müller-Hinton and Sabouraud nutritious bases were used. Casein soya bean digest broth (Triptic soya buillon) was used in the dilution method. When using the diffusion method, the test samples were dissolved in dimethyl sulfoxide (99.5 % DMSO) to obtain a 1 mg/ml stock solution. The inhibition zones for bacteria were measured in millimeters at the end of an incubation period of 18 h at 37°C, and for fungi after 48 h at 25°C. As reference substances gentamycin sulphate and clotrimazole were used. Solution of gentamycin sulphate was prepared in phosphate buffer (pH 8.0) at standard concentration 0.5 I.U. (500 μg/ml). Solution of clotrimasole standard was prepared by solving 25 mg clotrimasole in 50 ml dimethylsulfoxide (125 μg/ml). For the analysis by the dilution method solution of the compound 1 was prepared, followed by formation of a series of 12 dilutions with liquid nutritious base. The 2.0 ml casein soya bean digest broth was added to the 2.0 ml starting solution of test material and thus the first dilution was formed. Subsequently, 2.0 ml of this solution was diluted with 2.0 ml casein soya bean digest broth to give the second dilution and so on until 12 dilutions were obtained. The final concentration of DMSO in liquid nutritious base was ≤ 1.25 %. After the incubation for 24 h, the last tube with no growth of microorganisms was taken to represent MIC expressed in mg/ml. The concentration of the prepared solutions were: 0.5 mg/ml, 0.25 mg/ml, 0.125 mg/ml, 0.0625 mg/ml, 0.03125 mg/ml, 0.0156 mg/ml, 0.0078 mg/ml, 0.0039 mg/ml, 0.00195 mg/ml.
The aim of this method was to determine the exact concentration of the investigated compound which will have an inhibitory effect on the growth of selected microorganisms. This concentration was considered as minimal inhibition concentration (MIC).

### Results

Derivatives of dimer and tetramer type were prepared by condensation of 4-hydroxycoumarin with corresponding aromatic aldehyde. The synthesized compounds have the same basic structure, and their structures are shown in the following tables.

#### Table 1: Structures of the prepared 4-hydroxycoumarin derivatives

| Compound No | R₁ | R₂ | R₃ | Compd. No | R₁ | R₂ | R₃ |
|-------------|----|----|----|-----------|----|----|----|
| 1           | H  | Br | OH | 10        | H  |      |    |
| 2           | OCH₃| H  | H  | 11        | OCH₃|      |    |
| 3           | H  | H  | OCH₃|         |      |      |    |
| 4           | Br | H  | H  |           |      |      |    |
| 5           | NHCOC₂H₅| H  | H  |           |      |      |    |
| 6           | H  | H  | NO₂|           |      |      |    |
| 7           | Cl | H  | H  |           |      |      |    |
| 8           | (CH₃)₂N| H  | H  |           |      |      |    |
| 9           | H  | H  | H  |           |      |      |    |

### Table 2: Molecular mass, yield, elemental analysis and spectral data of the prepared compounds

| Compd. No | Yield (%) | Molecular formula (Mᵣ) | Calcd. found | IR (KBr) (cm⁻¹) | Spectral data |
|-----------|-----------|------------------------|--------------|----------------|---------------|
|           | % C       | % H       | % N          | 1H NMR (CDCl₃) (δ, ppm) |
| 1         | 72.37     | C₂₅H₁₅O₇Br   | 507.29       | 298            | 3276(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar); 646, 492(C-Br) |
| 2         | 80.00     | C₂₆H₁₈O₇     | 442.42       | 396            | 7 (s. 3H. OCH₃-14); 6.27(s. 1H. CH-12); 6.76-7.605 (m. 4H. arH) |
| 3         | 73.50     | C₂₆H₁₈O₇     | 442.42       | 407            | 3.56 (s. 3H. OCH₃-14); 6.09 (s. 1H. CH-12); 7.10-7.90 (m. 4H. arH); 11.22 (s. 1H. OH-11) |
| 4         | 74.30     | C₂₅H₁₅O₆Br   | (491.29)     | 390            | 6.29 (s. H. CH-12); 7.10-7.90 (m. 4H. arH); 11.53 (s. H. OH) |
| 5         | 66.81     | C₂₆H₁₈O₇     | 442.42       | 43            | 2.98            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 6         | 73.50     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 7         | 82.00     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 8         | 63.50     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 9         | 77.50     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 10        | 84.01     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |
| 11        | 84.00     | C₂₅H₁₅O₆     | (497.40)     | 36            | 2.79            | 1672(OH); 1672(C=C-C=O) lactone; 1610, 1570, 1496, 1454(C=C ar) |

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while they differ in substituents at the phenyl ring. The structures of prepared dimers and tetramers of 4-hydroxycoumarin are shown in Table 1. The results of yield, elemental analysis and spectral data for synthesized compounds shown in Table 2. Antimicrobial activity data are presented in Table 3 and Table 4.

| Microorganism | S. aureus (ATCC 6538P) | B. subtilis (ATCC 6633) | P. aeruginosa (ATCC 8739) | E. coli (ATCC 9027) | C. albicans (ATCC 10231) |
|---------------|------------------------|-------------------------|---------------------------|---------------------|--------------------------|
| Compound (1 mg/ml) | INHIBITION ZONES (mm) | | | | |
| 1 | 34.5 | 26.0 | 0 | 0 | 15.2 |
| 2 | 12.0 | 12.0 | 0 | 0 | 13.1 |
| 3 | 21.0 | 16.2 | 0 | 0 | 9.0 |
| 4 | 20.0 | 11.0 | 0 | 0 | 10.0 |
| 5 | 12.0 | 0 | 0 | 0 | 12.0 |
| 6 | 14.0 | 11.0 | 0 | 0 | 10.7 |
| 7 | 23.0 | 12.0 | 0 | 0 | 10.0 |
| 8 | 24.0 | 18.4 | 0 | 0 | 18.2 |
| 9 | 24.0 | 11.0 | 0 | 0 | 10.8 |
| 10 | 22.0 | 9.6 | 0 | 0 | 11.0 |
| 11 | 20.0 | 11.0 | 0 | 0 | 10.4 |
| Control (DMSO) | 0 | 0 | 0 | 0 | 6.0 |
| Gentamycin (0.5 μg/ml) | 12.0 | 13.5 | 12.0 | 12.0 | / |

TABLE 3. Antimicrobial activity of tested dimers and tetramers, expressed as the inhibition zone (mm)

Table: Microorganism | MIC (mg/ml) | MBC (mg/ml)
|-------------------|-------------|-------------|
| Staphylococcus aureus (ATCC 6538P) | 0.0625 | 0.25 |
| Bacillus subtilis (ATCC 6633) | 0.12500 | 0.5 |
| Candida albicans (ATCC 10231) | 0.03125 | 0.0625 |

TABLE 4. Minimum Bactericidal Concentration (MBC) and Minimum Inhibitory Concentration (MIC) of tested compound 1 by the dilution method

Discussion

The obtained results have shown that the newly synthesized derivatives possess antibacterial and antymycotic activities, tested on various bacteria species (B. subtilis, S. aureus) and Candida. The same compounds did not show any antimicrobial activity against two Gram-negative bacteria types (E. coli, P. aeruginosa). 3,3’- (5-bromobenzylidene-2-hydroxy)bis(4-hydroxycoumarin) compound 1 has shown the best activity against S. aureus, with the inhibition zone of 34.5 mm. The same compound has also shown good activity against B. subtilis, with the inhibition zone of 24 mm. 3,3’-(4-bromobenzylidene)bis(4-hydroxycoumarin) compound 4, the dimer with only bromine in structure at C16 did not have as good activity against Gram-positive bacteria, (inhibition zone of 24.0 mm against S. aureus and inhibition zone of 10.0 mm against B. subtilis) as compound 1. Possible explanation lies in the fact that oxygenation of the benzilidene ring contributes to an increase of antibacterial activity (3). Already published studies have shown that the introduction of chlorine as a substituent contributes to the antimicrobial activity. Thus, the compound with chlorine at the position C16 compound 7, has shown a good activity against S. aureus with the inhibition zone of 23.0 mm, while the activity against B. subtilis, with the inhibition zone of 12.0 mm, was significantly weaker. The compound with the methoxy group at the position C7 compound 3, shows good activity against S. aureus with the inhibition zone of 21.0 mm, while the compound with the same group at the position of C16 compound 5, shows a considerably weaker effect (inhibition zone of 2.0 mm). Again, this could be explained in terms of oxygenation of the coumarin ring. Another compound that has shown a very good activity against the Gram-positive bacteria (S. aureus, inhibition zone of 25.0 mm) and against B. subtilis, (inhibition zone of 18.4 mm) has dimethylamino group at the position C16 compound 8. The tetramer structures have given relatively good inhibition zone against S. aureus, (compound 10 and compound 11 with inhibition zone of 22.0 mm and 20.0 mm). Both tetramers have shown much weaker effect against B. subtilis (inhibition zone of 11.0 and 9.6 mm). The obtained results do not suggest that the increase of the size of molecules entails the increase in antimicrobial activity. The inhibition zones of most of the analyzed compounds are greater than the inhibition zone of the gentamycin sulphate (inhibition zone of 12.0 mm).
known that the Gram-negative bacteria are much more resistant than the Gram-positive bacteria. In view of the fact that the Gram-negative bacteria membrane is also more lipophytic than the Gram-positive bacteria membrane, it was to be expected that the compounds, which are lipophytic in their nature, should penetrate the cell membrane of these bacteria. However, the Gram-negative bacteria remained resistant to the analyzed compounds. The results of antimycotic activity show that almost all synthesized derivatives have similar activity. However, the dimers with bromine, hydroxy group on benzilidene ring (compound 1) and dimethylamino group on benzilidene ring (compound 8) (inhibition zones of 14, 2 and 18, 2 mm, respectively) have shown the best activity. For other tested compounds (compounds 5, 6, 7) zones of inhibition of growth of fungus Candida albicans are much smaller (inhibition zone of 9, 0 mm – 13, 1 mm, respectively). All tested compounds showed weaker activity than clotrimazole standard (inhibition zone of 24, 0 mm). The compound, which showed the best microbiological activity according to the diffusion method (compound 1) was analyzed also by the dilution method. Using the dilution method the same compound showed very good activity in a very small concentration against previously tested microorganisms:

- MIC for Staphylococcus aureus of 0,25 mg ml⁻¹, MBC 0,0625 mg ml⁻¹
- MIC for Candida albicans of 0,0625 mg ml⁻¹, MBC 0,03125 mg ml⁻¹
- MIC for Bacillus subtilis of 0,5 mg ml⁻¹, MBC 0,12500 mg ml⁻¹

CONCLUSION

The tested compounds have shown different activity in terms of growth inhibition of microorganisms. Newly synthesized derivatives have shown antibacterial activities, manifested as growth inhibition of various Gram-positive bacteria (B. subtilis, S. aureus), while the activity against Candida was much weaker. The same compound did not show any antimicrobial activity against two Gram-negative bacteria types (E. coli, P. aeruginosa). The compound 1 showed the best microbiological activity when tested by the diffusion method, and was tested also by the dilution method. Obtained results confirmed its antibacterial and antymycotic activities against various microorganisms.

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