SYNTHESIS, COMPLETE ASSIGNMENT OF $^1$H- AND $^{13}$C-NMR SPECTRA AND ANTI-OXIDANT ACTIVITY OF NEW AZINE DERIVATIVE BEARING COUMARIN MOIETY

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ABSTRACT

In this research, the synthesis of a new azine derivative with coumarin moiety was performed in three reaction steps, starting from 4-hydroxycoumarin. The first step in synthesis was the acetylation of 4-hydroxycoumarin to yield 3-acetyl-4-hydroxycoumarin and then the obtained 3-acetyl-4-hydroxycoumarin was reacted with hydrazine hydrate and give a corresponding hydrazone. Condensation of the hydrazone with 4-ethoxy-3-methoxybenzaldehyde afforded the target compound 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-ethoxy-3-methoxybenzylidene)-hydrazine in a good yield. The resulting azine derivative is fully spectrally characterized, including complete assignment of $^1$H- and $^{13}$C-NMR spectra, as well as 2D NMR ($^1$H–$^1$H COSY, NOESY, HSQC and HMBC) spectra. The antioxidant activity of corresponding hydrazone and target compound was evaluated by DPPH method where hydrazone derivative displayed a significant and target azine good antioxidant activity, with IC$_{50}$ ($\mu$M) values 11.69 and 216.60, respectively.

Keywords: Synthesis, Azines, Coumarins, Spectral analysis, $^1$H- and $^{13}$C-NMR, Antioxidant activity.

INTRODUCTION

The chemistry of organic compounds undoubtedly represents an experimental field with the greatest possibilities for research that is expanding daily. Synthesis of these compounds attracts great attention to organic chemists, due to their reactivity and a wide spectrum of both biological and therapeutic activities. A similar trend is seen for azines, compounds formed by the reaction of two different or the same carbonyl compounds (mostly aldehydes or ketones) with hydrazine (Safari & Gandomi-Ravandi, 2014; Chourasiya et al., 2019). The chemical properties and biological activities of these compounds mostly are the consequence of the presence of double bonds and nitrogen atoms. It is well known that they possess antibacterial (Veena et al., 2011; Chourasiya et al., 2015), antifungal (Kurteva et al., 2011), anticonvulsant (Gul et al., 2004), anti-neuroinflammatory (Subedi et al., 2017), anticancer (Krezel et al., 1999; Qian et al., 2010; Liang et al., 2014) and antioxidant (Li et al., 2011) activities. Also, they behave as aldo reductase inhibitors (Meanwell et al., 1991) and MDR reversal agents (Paterna et al., 2018). Due to their ability to donate the lone electron pairs, azines have found great application as chemical sensors for many metal ions (Wei et al., 2017; Tiwari et al., 2018). In addition to the above, azines represent very good synthons in synthetic chemistry, especially for the preparation of heterocyclic compounds.

Coumarin and its derivatives represent a heterocyclic system that contains condensed benzene and α-pyrone ring. These condensed systems have the ability for different substitution reactions and as a result, a variety of biologically and pharmacologically active derivatives are formed. To determine these activities, an enormous number of synthesized coumarin derivatives are subjected to various assaying. They showed a remarkably wide spectrum of antimicrobial (Al-Haiza et al., 2003; Medimagh-Saidana et al., 2015; López-Rojas et al., 2018), antifungal (Guerra et al., 2015; Forezi et al., 2018), anti-HIV (Kirkicharian et al., 2002; Su et al., 2006; Srivastav et al., 2018), anticoagulant (Manolov & Danchev, 1999), antitumor (Nofal et al., 2000; Fayed et al., 2019) and anti-inflammatory (Kontogiorgis & Hadjiapavlov-Litina, 2005). Also, many coumarin derivatives are used as optical brightening agents and food supplements (Wang et al., 2013; Zhang et al., 2016). Especially, 3-substituted-4-hydroxycoumarins are the main representatives of biologically very active coumarin derivatives. A good example of this type compound is the well-known antibiotics novobiocin and chlorobiocin. The mentioned 3-substituted coumarin core has a great pharmacological significance which is reflected in antibacterial (Laurin et al., 1999), anticoagulant (Manolov et al., 2006) and antioxidant (Kotali et al., 2016) properties.

Because azines showed a similar pharmacological effect, it is expected that the synthesis of azine derivatives with 3-acetyl-4-hydroxycoumarins moiety will give a promising result. This expectation confirmed a group of compounds of this type, which showed significant CDK (cyclic-dependent kinase) inhibition and a promising anticancer effect (Abdel Latif et al., 2016). However, compounds of this type are not numerous and their literature data are very scarce, primarily in the field of NMR spectral data and biological activities with an emphasis on antioxidant activity. As part of our interest in the discovery of

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novel compounds with possible pharmacological potential and following our previous work (Dekić et al., 2010, Radulović et al., 2015; Ristić et al., 2019), here we reported the synthesis, complete assignment of $^1$H- and $^{13}$C-NMR spectra, as well as 2D NMR spectra and antioxidant activity of new azine derivative obtained by condensation of hydrazone of 3-acetyl-1-dihydroxyxoumarin and an aromatic aldehyde, 4-ethoxy-3-methoxybenzaldehyde.

EXPERIMENTAL

General

Chemicals and materials. All solvents and chemicals were purchased from Merck (Germany), Fluka (Germany), Acros Organics (Belgium), J. T. Baker (USA), Fisher Scientific (USA) and Sigma-Aldrich (USA). Before use all solvents (acetone, methanol, absolute ethanol, ethyl acetate, hexane and chloroform) were distilled while other chemicals were used as thus obtained. The flow of the chemical reaction was monitored by thin layer chromatography (TLC) on aluminium plates with a previously applied layer of silica gel 60 F$_{254}$, layer thickness 0.2 mm, Merck (Germany). Visualization of spots on TLC plates was performed using a UV lamp (254 nm) or spraying (1:1, v/v) with an aqueous solution of sulfuric acid and then the TLC plates were heated until stains appeared. Column chromatography was used for purification and performed on silica gel 60 (particle size 40-63 μm), Fluka (Germany). Melting points were determined on MPM-HV2 melting point instrument (Germany).

UV-Vis measurements. UV spectra and absorbance were done on LLG UniSPEC2 spectrophotometer (Germany).

IR measurements. IR spectra were done on Thermo Nicolet 6700 FT-IR spectrophotometer (USA).

NMR measurements. One-dimensional (1D) and two-dimensional (2D) $^1$H- and $^{13}$C-NMR spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer (Switzerland).

$^1$H- spectra were recorded at 400 MHz and $^{13}$C-NMR spectra at 100.6 MHz at 25 ºC. Deuterated dimethyl sulfoxide (DMSO-$d_6$) was used as the solvent, while tetramethylsilane (TMS) was used as the internal standard. The values of chemical shifts are given in δ (ppm) units relative to TMS ($\delta_H = 0.00$ ppm) for $^1$H- spectra or to the signal of residual solvent DMSO-$d_6$ ($\delta_H = 2.50$ ppm and $\delta_C = 40.45$ ppm) for $^{13}$C- and heteronuclear 2D NMR spectra. For recording 2D NMR spectra ($^1$H-$^1$H-COSY, NOESY/ROESY, HSQC and HMBC) as well as for multipulse DEPT95 and DEPT135 spectra, the standard pulse sequences in the instrument software (Topspin) were used. Scalar couplings (J) are expressed in hertz (Hz).

HRMS measurements. HRMS(EI) analysis of the synthesized compounds was performed on a JOEL Mstation JMS 700 mass spectrometer (Germany). The ionization energy was 70 eV, ion trap 300 μA and the temperature of the ion source was 230 ºC.

Elemental microanalysis. Microanalysis of carbon, hydrogen, oxygen and nitrogen was performed on a Carlo Erba 1106 microanalyzer (Italy).

Synthesis

Synthesis of 3-acetyl-4-hydroxy-2H-chromen-2-one (2)

To a solution of 4-hydroxy-2H-chromen-2-one (1) (1.5 g, 9.3 mmol) in acetic acid (8 ml) POCl$_3$ (2.8 ml, 30 mmol) was slowly added. For the next 35 minutes, the mixture was heated at reflux in the oil bath. After completion of the reaction, the mixture was cooled in an ice bath, and the resulting precipitate was filtered. Recrystallization from ethanol gave 3-acetyl-4-hydroxy-2H-chromen-2-one (2) as white needle crystals (yield 90%, m.p. 135-136 ºC).

Synthesis of 3-(1-hydrazoneoethyl)-4-hydroxy-2H-chromen-2-one (3)

3-Acetyl-4-hydroxy-2H-chromen-2-one (2) (2.05 g, 10 mmol) was suspended in methanol (10 mL). After stirring for 10 minutes at room temperature, hydrazine hydrate (0.5 g, 10 mmol) was added and the obtained mixture was refluxed in a water bath for 5 hours. The reaction was monitored by TLC using ethyl acetate/hexane (2:1, v/v) as eluent. After completion of the reaction the mixture was cooled to room temperature, the resulting precipitate was filtered and washed with methanol. After air drying, the precipitate was recrystallized from ethanol to give 3-(1-hydrazoneoethyl)-4-hydroxy-2H-chromen-2-one (3) as a pure light-greenish yellow powder (yield 89%, m.p. 222-224 ºC). IR (KBr, cm$^{-1}$): 3458, 3281, 3190, 1688, 1605, 1552, 1172, 1102. $^1$H-NMR (400 MHz, DMSO-$d_6$): 15.17 (brs, HO–C(4)); 7.94 (dd, $J = 8.0, 1.6$, H–C(5)); 7.60, (ddd, $J = 8.4, 8.0, 1.6$, H–C(7)); 7.34 (td, $J = 8.0, 0.8$, H–C(6)); 7.25 (dd, $J = 8.4, 8.0$, H–C(8)); 6.16 (brs, NH$_2$); 2.64 (s, Me). $^{13}$C-NMR (100.6 MHz, DMSO-$d_6$): 178.3 (C(4)); 166.4 (C=N); 162.2 (C(2)); 153.4 (C(8a)); 133.8 (C(7)); 125.8 (C(5)); 123.9 (C(6)); 120.7 (C(4a)); 116.6 (C(8)); 94.1 (C(3)); 16.5 (Me). HRMS(EI): (M$^+$) 218.0673 (C$_{11}$H$_{12}$N$_2$O); requires 218.0691 (A = 1.8 mmu). Anal. calc. for C$_{11}$H$_{10}$N$_2$O: C, 60.55; H, 4.62; N, 12.83; O, 22.00; found: C, 60.76; H, 4.73; N, 12.78; O, 21.73.

Synthesis of 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethyliendyl]-2-(4-ethoxy-3-methoxybenzylidene)-hydrazine (5)

3-(1-Hydrazoneoethyl)-4-hydroxy-2H-chromen-2-one (3) (1.1 g, 5 mmol) was dissolved with stirring at room temperature in absolute ethanol (10 ml). Subsequently 4-ethoxy-3-methoxybenzaldehyde (4) (0.9 g, 5 mmol) was added and obtained mixture was refluxed in a water bath for 3 hours. The reaction was monitored by TLC using ethyl acetate/hexane (3:2, v/v). When the reaction was complete, reaction mixture was cooled to room temperature. The resulting precipitate was filtered and washed with chloroform/methanol (1:1, v/v). After purification by SiO$_2$ column chromatography (ethyl acetate/hexane (1:1, v/v) the target compound (5) was obtained.
as brightness yellow powder (yield 83%, m.p. 186-188 °C). IR (KBr, cm⁻¹): 3389, 3092, 2975, 1697, 1601, 1555, 1176, 1111. ¹H-NMR (400 MHz, DMSO-d₆) 16.39 (brs, HO–C(4)); 8.68 (s, N=CH); 7.95 (dd, J = 7.2, 1.6, H–C(5)); 7.61, (dd, J = 8.4, 7.2, 1.6, H–C(7)); 7.48 (d, J = 1.6, H–C(2)); 7.41 (d, J = 8.2, H–C(6')); 7.29 (dd, J = 8.4, 7.2, 0.8, H–C(6)); 7.25 (dd, J = 8.4, 0.8, H–C(8)); 7.10 (d, J = 8.2, H–C(5')); 4.11 (q, J = 6.8, CH₂CH₂O–C(4')); 3.85 (s, OMe–C(3')); 2.96 (s, Me); 1.37 (t, J = 6.8, CH₂CH₂O–C(4')). ¹³C-NMR (100.6 MHz, DMSO-d₆): 178.3 (C(4)); 171.8 (C=N); 161.3 (C(2)); 157.1 (N=CH); 153.4 (C(8a)); 152.1 (C(4')); 149.6 (C(3')); 133.8 (C(7)); 126.1 (C(1')); 125.8 (C(5)); 124.7 (C(6')); 123.9 (C(6)); 120.7 (C(4a)); 116.6 (C(8)); 112.8 (C(5')); 109.7 (C(2')); 94.1 (C(3)); 64.4 (CH₂CH₂O–C(4')); 55.9 (OMe–C(3')); 17.9 (Me); 15.0 (CH₂CH₂O–C(4')). HRMS(EI): (M⁺) 380.1384 (C₂₁H₂₀N₂O₃); requires 380.1372 (Δ = +1.2 mnu). Anal. calc. for C₂₁H₂₀N₂O₃: C, 66.12; H, 5.48; N, 7.47; O, 20.93.

Antioxidant activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was used to determine the antioxidant activity of the synthesized compounds 3 and 5, with some modification (Kadhum et al., 2011). Briefly, test samples were dissolved in methanol (12.5-400 μM) and then 2 ml of sample and 1 ml of methanolic DPPH solution (0.1 mM) were placed in a test tube. The tubes were shaken and then incubated in a dark place for 40 minutes at room temperature. For ascorbic acid, which was used as a positive control, in this case, the same procedure was repeated. The percentage inhibition of DPPH radical was determined spectrophotometrically measuring the absorbance at 517 nm against a blank (methanol) by using equation (1):

\[
inhibition(\%) = \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \times 100
\]

where \(A\) is absorbance of control (2 ml of methanol and 1 ml of DPPH), \(A_s\) is absorbance of the test samples (2 ml of samples and 1 ml of DPPH). Based on the relationship between the percentage inhibition of DPPH radical and the concentration of the tested samples, the results are performed in triplicate and presented in IC₅₀ values (μM).

RESULTS AND DISCUSSION

Chemistry

The target azine derivative 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-ethoxy-3-methoxybenzylidene)-hydrazine (5) was prepared according to sequence of reactions presented in the Scheme 1. In the first step of reaction, the acetylation of 4-hydroxycoumarin (1) was performed with glacial acetic acid in the presence of POCl₃ as a catalyst to yield 3-acetyl-4-hydroxycoumarin (2). The reaction of compound (2) with hydrazine hydrate (molar ratio 1:1) in methanol, resulted the corresponding hydrazone of 3-acetyl-4-hydroxycoumarin (3). Finally, the target azine (5) was synthesized in a good yield (83%) by condensation of obtained hydrazone (3) and 4-ethoxy-3-methoxybenzaldehyde in absolute ethanol (Scheme 1).

Spectral data (HRMS(EI), IR, ¹H- and ¹³C-NMR) of synthesized azine (5) were in complete agreement with the proposed structures. The IR spectra of compound (5) showed strong absorption bands at 3389 cm⁻¹ which corresponding to the OH group, characteristic vibrations at 3092 cm⁻¹ attributed to the Ar–H, and band at 1697 cm⁻¹ concerning C=O (lactone carbonyl) of coumarin moiety. Characteristic vibrations at 1601 cm⁻¹ are attributed to C=O. Intensity bands corresponding to C=C stretching vibration of the aromatic rings appeared at 1555 cm⁻¹.

The IR spectra at 1176 cm⁻¹ showed a characteristic C-O stretching.

High-resolution mass spectroscopy (HRMS-EI) confirmed the structure of the synthesized azine (5) with molecular formula C₂₁H₂₀N₂O₃ (M⁺) at m/z 380.1384, Δ = +1.2 mnu. In ¹H-NMR spectra of compound (5) exist seven signals with chemical shifts characteristic for aromatic methine protons which appeared as three doublets, two doublets of doublets, and two doublets of doublets of doublets. It is noticeable that in ¹H-H COSY and NOESY spectra these protons are separated into two groups of signals (Figure 1). In ¹H-NMR spectra (Table 1) three signals that appeared as doublets at 7.48, 7.41, and 7.10 ppm constitute the first group of protons, while the second group consisted of two sets of doublets of doublets at 7.95 ppm and 7.25 ppm and two doublets of doublets of doublets at 7.61 and 7.29 ppm.

![Figure 1. The NOE correlations of 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-ethoxy-3-methoxybenzylidene)-hydrazine (5) in the NOESY spectrum.](image-url)
long-range (H-7 and H-6) protons, respectively. Analyzing of 
given correlations, it was concluded that proton on position H-5 
in HMBC spectrum, which interacts through three chemical 
bonds, assigns the quaternary carbons C-4 (178.3 ppm) and C-8a 
(153.4 ppm). This spectrum also showed that the carbon at 133.8 
ppm is C-7 by the presence of a cross peak with H-5, which then 
allowed the assignment of H-7 as a peak at 7.61 ppm in HSQC. 

Analogously, the proton at position H-8 showed interactions with 
C-4a (120.7 ppm) and C-6 (123.9 ppm) and thus the shift of the 
proton H-6 was deduced to be 7.29 ppm from HSQC. Also, a 
characteristic interaction was observed through two chemical 
bonds between the proton H-8 and the quaternary carbon C-8a, 
which is assigned at 153.4 ppm (Figure 2). In the HMBC 
spectrum, the assignment of carbons C-4a and C-8a were also 
confirmed by protons interactions at position H-6 and H-7, 
respectively. The signal at 2.96 ppm in 1H-NMR spectrum 
assigned as a singlet of methyl protons attached at the 3-C=N, in 
HMBC spectrum showed interactions with carbon C-3 (94.1 
ppm), through three bonds, and with the signal at 171.8 ppm, 
through two bonds, which corresponded to the carbon of imine 
group (MeC=N). The largest chemical shift at 16.39 ppm in the 
1H-NMR spectrum which appeared as a broad singlet, was 
assigned to the proton of OH group at position C-4 on coumarin moiety. The only remaining signal in 13C-NMR spectra 
at 161.3 ppm was assigned to lactone carbonyl carbon (C-2), 
since no interaction was observed in HSQC and HMBC spectra.

Scheme 1. Synthesis of target azine derivative 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-etoxy-3-methoxybenzylidene)-hydrazine (5).

Reagents and condition: (a) CH₃COOH, POCl₃, reflux (35 min.), (b) NH₂NH₂·H₂O, MeOH, reflux (5 h), (c) abs. EtOH, reflux (3 h)

Aromatic protons on the substituent side which appeared at 
7.48, 7.41 and 7.10 ppm in the 1H-NMR spectrum as a doublet, 
were attributed to H-2', H-6' and H-5', respectively. The signals 
of carbons C-2', C-6', and C-5' (Table 1) of the substituent ring, 
were readily connected to the aforementioned 1H-NMR signals 
according to correlations in the HSQC spectrum, and further 
corroborated by HMBC data. The proton of OH group on coumarin moiety in NOESY spectrum showed interaction with 
the signal at 8.68 ppm which appeared as singlet and 
corresponded to the proton of azomethine group (N=CH) on the 
substituent side. The mentioned proton in the HMBC spectrum 
have two more interactions which are accomplished through three 
chemical bonds, whereby assigned a carbons C-2' (109.7 ppm) 
and C-6' (124.7 ppm), (Figure 2). Also, the position of the carbon 
C-1' (126.1 ppm) is confirmed by the interactions of the proton 
H-5' in the HMBC spectrum. In NOESY spectrum proton H-5'

Figure 2. The HMBC correlations of 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-etoxy-3-methoxybenzylidene)-hydrazine (5).
showed interaction with a peak that appeared as a quartet at 4.11 ppm in the $^1$H-NMR spectrum and corresponded to the methylene protons of the ethoxy group. Also, methylene protons from the ethoxy group in the NOESY spectrum are in correlations with methyl protons (Figure 1) of the mentioned group which appeared as a triplet at the smallest chemical shifts of 1.37 ppm. Further, the aforementioned correlation between the methyl and methylene protons is confirmed by their interaction through two chemical bonds with the carbon atoms to which they are attached, in the HMBC spectrum. The last remaining signal (appeared as singlet) in the $^1$H-NMR spectrum at 3.85 ppm belong to the protons of a methoxy group at position 3', which is confirmed by their interaction in the NOESY spectrum with a proton at position H-2'. The chemical shift of the remaining quaternary carbon C-4' (152.1 ppm) on the substituent side was determined from HMBC interactions, through three chemical bonds (H-2' and H-6').

The data provided by the NOESY spectrum were used for the determination of the spatial relationship between the coumarin core and the aryl substituent. The proton of the OH group on coumarin moiety showed a cross peak with the hydrogen of the azomethine group (N=CH, Figure 1), which makes it possible to determine the orientation of the aryl substituent (Figure 3).

**Table 1.** $^1$H- and $^{13}$C-NMR chemical shifts ($\delta$, ppm), integrals, multiplets, coupling constants ($J$, Hz), crucial NOESY and HMBC correlations of 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-etoxy-3-methoxybenzylidene)-hydrazine (5), obtained by energy minimization using the MM2 force field in the ChemBio3D Ultra 12.0 software package.

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<th>$J$ (Hz)</th>
<th>$\delta ^{13}$C (ppm)</th>
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<td>1H</td>
<td>d</td>
<td>8.2</td>
<td>112.8</td>
<td>H-6', 4'-OCH$_2$CH$_3$</td>
<td>C-1', C-3'</td>
</tr>
<tr>
<td>6'</td>
<td>7.41</td>
<td>1H</td>
<td>d</td>
<td>8.2</td>
<td>124.7</td>
<td>H-5'</td>
<td>C-2', C-4'</td>
</tr>
</tbody>
</table>

**Figure 3.** Spatial orientation of coumarin moiety and aryl-substituent in the molecule of 1-[1-(4-hydroxy-2-oxo-2H-chromen-3-yl)-ethylidene]-2-(4-etoxy-3-methoxybenzylidene)-hydrazine (5), obtained by energy minimization using the MM2 force field in the ChemBio3D Ultra 12.0 software package.
On the other hand, the proton of the azomethine group also showed simultaneous interactions with proton H-2’. All mentioned interactions indicated that the diimine bridge was created, whereby coumarin moiety and ring of the substituent, toward each other at a specific angle (Figure 3).

Antioxidant activity

Antioxidants are compounds that slow down the oxidation processes in the human body. They act as "scavengers" of free radicals, or electron donors, and thus prevent chain reactions in which other molecules in cells are damaged. In addition to natural antioxidants, synthetic antioxidants have been developed and used in practice as additives, supplements, and drugs. Due to a lack of antioxidants, many health diseases occur in humans, such as cardiovascular, carcinogenic, and inflammatory (Nojiri et al., 2004). The DPPH assay is based on monitoring the color change of a purple-colored solution of a stable DPPH radical, into a reduced yellow-colored form (DPPH-H). DPPH is a stable free radical with a delocalized free electron on nitrogen so that the molecule does not form dimers, which is often the case with most other free radicals. Delocalization allows the appearance of a purple color, with maximum absorption at 517 nm. Receiving a proton from a potential antioxidant he is reduced to hydrazine, whereby the intensity of absorption is decreased since hydrazine is not absorbed in that wavelength. A higher degree of discoloration means a higher reduction ability.

Substitution, the position of substitution, and the nature of the substituent significantly affect antioxidant activity. Thus, for example, compounds that have electron-donating groups on the phenolic ring contribute to increases in antioxidant activity, while electron-withdrawing groups usually decrease antioxidant activity (Lee et al., 2015).

As can be seen (Table 2), compound (3) showed significant antioxidant activity with IC₅₀ value of 11.69 µM, while compound (5) showed slightly more moderate antioxidant activity (216.60 µM). In our case, ascorbic acid was used as a criterion to compare the ability to neutralize DPPH radicals with the tested compounds, i.e. as a positive control, since it is known that represented an exceptional antioxidant.

Table 2. DPPH free radical scavenging activity (IC₅₀, µM) of compound (3), (5), and ascorbic acid

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>11.69</td>
</tr>
<tr>
<td>5</td>
<td>216.60</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>7.82</td>
</tr>
</tbody>
</table>

As both tested compounds on the coumarin part of the molecule have an OH group that participates in the formation of a strong hydrogen bond with the nitrogen atom, indicates that the OH group probably does not have any effect on the antioxidant action. The significant antioxidant activity of hydrazone of 3-acetyl-4-hydroxycoumarin (3) is a probable consequence of the electron-donating ability of the amino group and the high resonance stabilization of the formed radical. Similar to previous, although the tested compound (5) does not have an OH group on the aryl core that would certainly affect the increases in antioxidant activity, there are two electron-donating groups, methoxy and ethoxy group, which probably have influenced on the mentioned compound to exhibit a good antioxidant activity.

CONCLUSION

A new mixed azine derivative bearing coumarin moiety was synthesized and fully spectrally assigned using 1D- and 2D-NMR spectral data. The resulting azine was obtained in a good yield by condensation of hydrazine of 3-acetyl-4-hydroxycoumarin and 4-ethoxy-3-methoxybenzaldehyde in a simple, rapid, and very effective way. Synthesized compounds (3) and (5) were monitored for their antioxidant activity. The antioxidant activity of the compound (3) against the stable free radical DPPH showed that this compound possessed a significant antioxidant power, while compound (5) demonstrated somewhat smaller, but still good antioxidant potential. The results obtained in this study will primarily contribute to organic synthesis and better knowledge of the structure of this group of compounds, formed by the reaction of hydrazones of coumarin and aldehydes. The obtained target azine represents a very interesting starting material for further biological and pharmacological research.

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