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Antioxidant properties and growth-inhibitory activity of coumarin Schiff bases against common foodborne fungi

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ABSTRACT

A series of coumarinylSchiff bases were examined for their antifungal, antioxidant (1,1-diphenyl-2-picryhydrazyl (DPPH) free radical and galvinoxyl radical scavenging activity) and metal chelating activity. Coumarin derivatives possessing dihydroxyphenyl moiety showed the best antioxidant activity in all antioxidant assays applied. High correlation between DPPH and galvinoxyl scavenging activity (r = 0.8238; p <0.05) was observed. Antifungal activity tests were performed against four common mycotoxin producing foodborne fungi, Aspergillusflavus, A. ochraceus, Fusariumgraminearum and F. verticillioides. Compounds bearing dihydroxyphenyl moiety were also proven to be an excellent antifungals.

Keywords: coumarin, antioxidant activity, antifungal activity, Schiff bases

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INTRODUCTION

Coumarins, consisting of fused benzene and α-pyrone rings, are an important group of low-molecular weight phenolics [1], constituting one of the most common families of green plant secondary metabolites [2].Coumarins, both natural and synthetic, are compounds with many diverse biological activities [3,4,5,6].A number of natural and synthetic coumarin derivatives have been reported as antioxidant [7,8], antimicrobial [9], antifungal [9,10]and tuberculostatic[11]agents.Natural coumarins affect the formation and scavenging of reactive oxygen species (ROS) and influence free radical-mediated oxidative damage [12].The styryl carbonyl moiety in coumarin nucleus is expected to affect scavenging of reactive substances derived from oxygen and may influence free radical mediated pathologies [13,7], but their antioxidant activity, as well as other pharmacological activities, are strongly structure dependent[14]and depend on the substituents of the coumarin core[15].According to some authors [16]coumarins should bear at least one hydroxyl group to show antioxidant activity, since it is proven that hydroxyl groups of hydroxycoumarins are potent electron/hydrogen donors to free radicals[2].Upon donation of an electron/hydrogen the radicals are formed which are stabilized through delocalization of electrons across the molecule [2,17].

Apart from being good antioxidants, coumarins are also proven to possess a good antifungal activity, since plants use them as a defense when subjected to adverse conditions[18]. A vast variety of plant extracts containing coumarins act as antifungals as well as bacteria growth inhibitors [19].Fungitoxic activity of coumarins depends on the position and the nature of substituents on coumarin core, and it is proven that substituted coumarins show better activity than unsubstitutedcoumarin[20].Phenolic, hydroxy and carboxy groups on coumarin skeleton are important for antimicrobial activity[21]and according to Brooker et al. [22,23]halogenated coumarins also possess a potent antifungal activity, especially the ones with chloro substituents. As mycotoxins cause a great damage to crops and animals [24], novel and more effective, antifungal drugs are required. Humans are also susceptible to mycotoxins, and the best way to avoid mycotoxicoses is by reducing human exposure to mycotoxins [25].Apart from this, fungi can cause damage on food through spoilage and degradation of nutrients or changed enzymatic activity.

This study is based on previously reported enhanced antimicrobial and antioxidative activity of 7-substituted coumarin derivatives[26].The aim of this work was to investigate antifungal and antioxidative activity of heterocyclic compounds, Schiff bases, bearing coumarin moiety, since the imino group (-C=N-) containing compounds form a significant class of compounds in medicinal and pharmaceutical chemistry, possessing antibacterial, antifungal and antitumor activity [27, 2].

In this study main mycotoxigenic and food spoilage fungi (*Aspergillus* and *Fusarium* species according to [28]) were tested against coumarin derivatives[29,30] and three methods for antioxidant activity determination were compared.

MATERIALS AND METHODS

The absorbance was measured on UV visible spectrophotometer Helios γ, (Thermo Spectronic, Cambridge, UK). EPR (electron paramagnetic resonance) was measured on Electron Spin Resonance ESP 300 (Bruker). Microplates were read on Sunrise absorbance reader (Tecan Group Ltd., Männedorf, Switzerland). Incubation was carried in Aqualytic AL 500-8 incubator (Aqualytic, Dortmund, Germany).

Determination of correlation coefficients (Pearson method) and student t-test were performed in Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA).

3.1. Chemicals

All the chemicals were purchased from commercial suppliers.

3.2. Tested 2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)acetohydrazide derivatives (2a-z)

A series of Schiff bases (*E*)-N-2-aryliden-2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy) acetohydrazide**(2a-z)** were preparedaccording to the procedure described by [31]: A mixture of 2-((4-methyl-2-oxo-2*H*-chromen-7-yl)oxy)acetohydrazide (**1**; 0.01 mole) and a suitable aromatic aldehyde (**Ar/a-z**; 0.01 mole) was refluxed in absolute ethanol (30 mL) in presence of a catalytic amount of glacial acetic acid for 2 to 4 hours. The reaction mixture was cooled and the precipitate was filtered and recrystallized from methanol to give compounds **2a-z**. Structures of the tested compounds, Schiff bases (**2a-z)**, are shown in **Figure 3.**

3.3. Antioxidant activity

3.3.1. DPPH scavenging activity

3.3.1.1. Spectrophotometric determination of DPPH (1,1-diphenyl-2-picryhydrazyl radical) scavenging activity

Determination of antioxidant activity was performed according to [26].

3.3.1.2. Determination of DPPH (1,1-diphenyl-2-picryhydrazyl radical) scavenging activity by EPR spectroscopy

The DPPH radical scavenging activity of coumarin derivatives was also determined by EPR spectroscopy, according to [26].

3.3.2. Determination of galvinoxyl radical scavenging activity by EPR spectroscopy

The galvinoxyl radical scavenging activity of coumarin derivatives was also determined by EPR spectroscopy, according to[26].

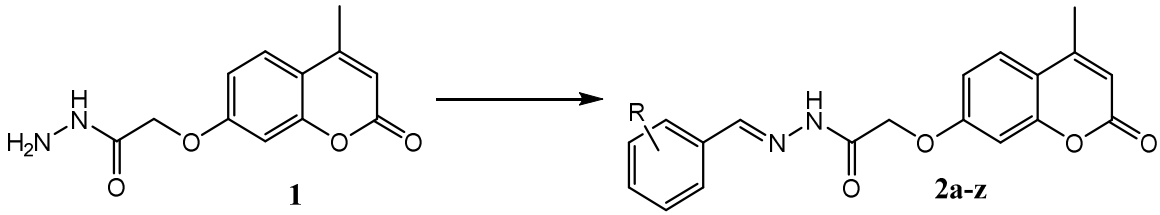
3.4. Iron chelating activity

The chelating activity of coumarin derivatives for ferrous ions Fe^{2+} was measured according to [26].

3.5. Antifungal activity

Broth microdilution assays were performed in accordance with the guidelines detailed in CLSI document M38-A[32].Fungi which were used in this experiment are major producers of mycotoxins and food contaminants [29]: *Aspergillusflavus*(NRRL 3251); *Aspergillusochraceus*(CBS 589.68); *Fusariumgraminearum* (CBS 110.250) and *Fusariumverticillioides* (CBS 119.825). Preparation of inoculum, medium and drug dilutions were preapared as described in our previous publications [26].

Following inoculation, all plates were incubated at 35 °C in an atmospheric incubator. After 48 h of incubation plates were read on microplate reader at 450 nm. Minimal inhibitory concentration for 100% cell death (MIC_{100}) was defined as the lowest concentration reducing the optical density by 100% at 450 nm compared with growth control[33,34].



1	R	1	R
a	H	**n**	3-phenoxy
b	2-OH	**o**	2-OH-5-NO_2
c	3-OH	**p**	3,4,5-$(OCH_3)_3$
d	4-OH	**q**	2-Cl
e	2-OCH_3	**r**	3-Cl
f	3-OCH_3	**s**	2-Br
g	4-OCH_3	**t**	3-Br
h	2,3-$(OH)_2$	**u**	4-Br
i	2,4-$(OH)_2$	**v**	2-F
j	2,5-$(OH)_2$	**w**	3-F
k	3,4-$(OH)_2$	**x**	4-F
l	3,5-$(OH)_2$	**y**	styryl
m	3-OCH_3-4-OH	**z**	4-$N(CH_3)_2$

Figure 3. Structures of tested coumarinyl Schiff bases

RESULTS AND DISCUSSION

Antioxidant activity

Synthesis, as well as the spectral data for all coumarin derivatives subjected to this research werepreviously published [31].

Data in **Table 1.**indicate that substituents on phenyl ring have a great influence on antioxidant activity expressed as DPPH radical scavenging activity. Schiff base bearing 2,3-dihydroxyphenyl moiety (**2h**) on benzene ring was proven to be the best DPPH radical scavenger. This result was expected, since it is well known that catechol moiety influences the antioxidant activity [17].Roussaki et al. [17], have proven that catecholicmoiety (phenolic ring bearing two hydroxy groups in *ortho* position) is crucial for antioxidant activity, as well as other authors [35,36] who were investigating coumarin derivatives with *o*-dihydroxy phenolic groups. According to the same authors[35,36] compounds bearing methoxy or one hydroxy group did not show significant antioxidative activity, which was also confirmed in our research. In fact, compounds bearing methoxy group/s (**2e**, **2f**, **2g**) and one hydroxyl group (**K**, **2b**, **2c**, **2d**) did not show any significant antioxidant activity (**Table 1**). Compound **2k**,possessing two hydroxy groups in *ortho* position as well, exhibited good antioxidant activity, but much lower than compound **2h**. This indicates that hydroxy groups in *ortho* position are not sufficient for a high antioxidant activity, the position of the groups on phenyl ring is crucial. When two hydroxy groups are in position 1,4 of the

phenyl ring, a stable phenoxyl radical, which allows an oxygen atom to share a positive charge, causing stabilization through delocalization, is formed (**Figure 1**). When two hydroxyl groups are in position 1,3 of the phenyl ring an oxygen cannot share a positive charge which influences the DPPH scavenging activity (**Figure 2**)[37].

Table 1.DPPH and galvinoxyl radical scavenging activity of coumarinyl Schiff bases[1]

Compound	% DPPH radical scavenging[a]	% DPPH radical scavenging[b]	% galvinoxyl radical scavenging
ascorbic acid[2]	85.2 ± 6.1	88.6 ± 0.9	82.6 ± 1.4
7-hydroxy-4-methylcoumarin	2.4 ± 0.82	4.9 ± 4.6	4.2 ± 3.2
1	14.4 ± 4.1	10.2 ± 0.6	32.9 ± 2.5
2a	22.9 ± 3.4	9.7 ± 1.6	33.8 ± 2.9
2b	15.3 ± 5.9	7.3 ± 2.2	47.6 ± 6.1
2c	3.6 ± 0.5	3.9 ± 0.5	22.6 ± 2.1
2d	4.4 ± 0.7	5.0 ± 1.7	18.5 ± 2.8
2e	3.1 ± 0.2	-3.5 ± 4.4	16.4 ± 0.4
2f	0.2 ± 1.0	-0.2 ± 2.3	18.9 ± 1.5
2g	3.7 ± 1.1	-6.1 ± 2.9	23.3 ± 2.1
2h	75.4 ± 0.5	88.7 ± 1.9	98.4 ± 0.2
2i	7.0 ± 3.5	2.5 ± 1.1	17.4 ± 4.9
2j	33.8 ± 6.0	51.4 ± 1.2	98.3 ± 0.3
2k	42.6 ± 3.4	50.4 ± 2.9	98.4 ± 0.1
2l	4.6 ± 3.9	-1.6 ± 16.5	1.5 ± 2.2
2m	32.0 ± 2.4	38.2 ± 5.2	20.9 ± 1.6
2n	3.9 ± 3.2	-4.0 ± 2.9	19.3 ± 2.8
2o	2.3 ± 2.3	3.3 ± 4.9	4.4 ± 4.3
2p	3.5 ± 0.1	-7.3 ± 6.4	30.5 ± 3.4
2q	12.5 ±3.3	3.9 ± 0.5	43.9 ± 0.9
2r	11.7 ± 6.5	1.9 ±1.5	43.7 ± 0.6
2s	6.6 ± 6.3	-2.0 ± 8.2	20.2 ± 8.8
2t	3.0 ± 0.5	-5.5 ± 3.4	4.6 ± 3.5
2u	5.0 ± 0.4	0.8 ± 3.0	21.4 ± 0.7
2v	3.0 ± 0.5	5.2 ± 2.3	22.8 ± 1.0
2w	2.3 ± 0.4	5.5 ± 0.4	23.8 ± 3.9
2x	7.4 ± 1.0	14.6 ± 6.1	19.4 ± 2.4
2y	3.9 ± 3.5	2.4 ± 6.1	19.3 ± 7.5
2z	7.7 ± 3.1	3.6 ± 3.7	0.7 ± 0.6

[a]spectrophotometrically determined ; [b]EPR determined; [1]data are means± standard deviation of three replicates
[2]ascorbic acid was used as standard

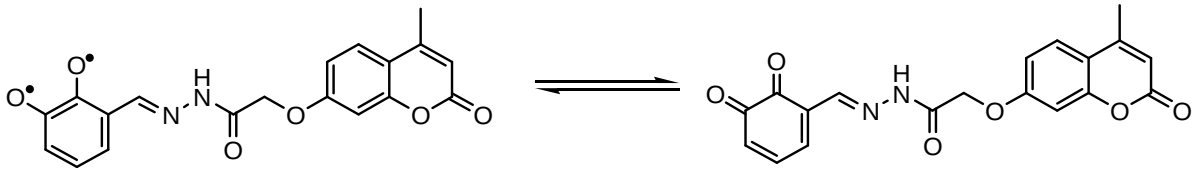


Figure 1. Stable quinoid structure of compound 2h stabilized through electron delocalization

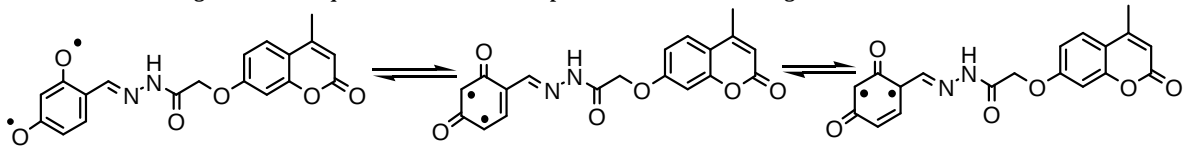


Figure 2. Unstable structure of compound 2i after electron/hydrogen donation, with no possibility of stabilization through delocalization

The substitution radical in position 1,2 and 1,4 of the phenyl ring donates an electron to the aromatic ring to activate it, while the substitution in position 1,3 inactivates the ring, which influences the ability of the compound to form a stable radical upon scavenging DPPH radicals[37].This was detected for the compounds **2i** and **2l**, which did not show significant antioxidant activity, since they cannot form stable quinoid structures.

Compounds with halogen substituents, as expected[4]did not show any significant antioxidant activity. It is important to point out that there is statistically significant difference ($p < 0.05$), between the antioxidant activity of novel compounds derived from 7-hydroxy-4-methylcoumarin and the antioxidant activity of 7-hydroxy-4-methylcoumarin itself. Thus, the modification of a starting compound (7-hydroxy-4-methylcoumarin) by substitution in position 7 contributed to the achievement of higher antioxidant activity, in contrast to the results of [38] who claimed that hydroxyl group in position 7 of simple coumarins is crucial for antioxidant activity. Results for DPPH scavenging activity determined by EPR spectroscopy are in accordance with the above mentioned results for spectrophotometrically determined DPPH scavenging activity. When comparing those results a high correlation

coefficient is gained ($r = 0.97$, $p < 0.05$). This was expected, since both methods employ the same radical and sample preparation, the only difference is that in the spectrophotometric method there is a chance that the residual color of reaction products interferes with the results. This disadvantage is overcome in the EPR method which directly measures free radicals and is more sensitive than the spectrophotometric one.

Results obtained for galvinoxyl radical (phenoxy type radical) scavenging are also in accordance with the results obtained for DPPH scavenging activity. Oxygen centered radicals (galvinoxyl radical) and nitrogen centered radicals (DPPH) react with phenols via two different mechanisms, hydrogen atom transfer (HAT) and single-electron transfer (SET) and contribution of the first or second mechanism depends on the solvent and/or redox potential of the investigated compounds[39]. This indicates, that, the mechanism of galvinoxyl and DPPH radical scavenging is the same at the same conditionsbutgalvinoxyl radical is much more reactive towards phenolic compounds than DPPH radical[40,41,42].In our investigation where polar solvent DMSO was used, there is a high correlation between these two methods, but the mechanisms were not elucidated. Both galvinoxyl and DPPH radicals are colored which enables spectrophotometric determination, but the products which are formed in the reaction can also be colored and therefore absorb to a certain extent at the same wavelength as radicals, thus interfering with the results[36]. To avoid this kind of interference, EPR method was employed.

Schiff bases **2h**, **2j**, **2k** showed the best galvinoxyl scavenging activity, even better than ascorbic acid which was used as standard. All of these Schiff bases bear two hydroxyl groups on phenyl ring, compound **2h** in position 2,3, **2j** in position 2,5 and compound **2k** in position 3,4. All of these compounds are able to form stable quinoid structures upon electron/hydrogen donation, unlike the compound **2i** with two hydroxyl groups in position 2,4 of the phenyl ring, whose antioxidant activity is much lower. There is a high correlation between DPPH and galvinoxyl scavenging activity ($r = 0.8238$; $p < 0,05$) and this investigation shows that thegalvinoxyl radical is more reactive towards investigated compounds than DPPH radical in the polar solvent like DMSO. Also, there is statistically significant difference ($p < 0,05$) between the antioxidant activity of Schiff bases derived from 7-hydroxy-4-methylcoumarin and 7-hydroxy-4-methylcoumarin itself. Schiff bases derived from 7-hydroxy-4-methylcoumarin possess higher antioxidant activity than 7-hydroxy-4-methylcoumarin.

Table 2. Chelating activity of coumarinyl Schiff bases

Compound	% chelating activity
EDTA	96.9 ± 0.9
K	10.2 ± 1.5
1	2.8 ± 3.8
2a	-
2b	-
2c	5.6 ±2.6
2d	0 ± 3.8
2e	13.4 ± 3.4
2f	13.1 ± 4.0
2g	0 ± 3.1
2h	-
2i	1.9 ± 1.8
2j	0 ± 8.0
2k	0 ± 6.9
2l	12.4 ± 1.0
2m	0 ± 1.9
2n	6.8 ± 4.5
2o	**0 ± 2.0**
2p	**8.2 ± 1.0**
2q	**10.8 ± 2.4**
2r	**7.4 ± 2.8**
2s	**11.2 ± 2.2**
2t	**2.8 ± 2.6**
2u	**0 ± 0.5**
2v	**0 ± 3.0**
2w	**10.1 ± 2.9**
2x	**-**
2y	**0 ± 3.8**
2z	**0 ± 1.1**

- indicated compounds are not completely soluble in the solvent used, thus not possible to determine chelating activity under these conditions

Considering data for antioxidant activity, it is clear that the presence of two hydroxyl groups has a great influence on radical scavenging activity. Lin et al. [1] reported a correlation of radical-scavenging effects of coumarins with the number of hydroxyl groups, which is also observed in this work.

Iron chelating activity

This investigation is based upon complex formation between ferrozine and Fe^{2+}, which is red in color. In the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Therefore, the chelating activity of the coexisting chelator is estimated by measurement of color reduction at 562 nm.

The formation of red colored complex was partially inhibited by compounds **2e**, **2f**, **2l** and **2s (Table 2)**. Chelating activity of these compounds was moderate, but not comparable with EDTA, which was used as standard compound. All of these compounds possess one or two electron donating groups in *o*- and *m*- position, with the exception of **2s**, which possesses electron withdrawing –Br group in *o*- position. Addition of electron donating groups has been proven to enhance iron chelating activity[43]. Other compounds showed low or no chelating activity under these conditions.

Antifungal activity of tested compounds

Antifungal activity of novel synthesized compounds was tested in accordance with NCCLS document M38-A procedure, and results were shown in **Table 3**. Interpretation of mold MICs has long been known to be problematic, so single colonies at the surface and skip wells (additional growth above determined MIC) were ignored[44].

Table 3.Antifungal activity of tested coumarinyl Schiff bases

Compound	MIC_{100} (μg mL^{-1})			
	A. flavus	*A. ochraceus*	*F. graminearum*	*F. verticillioides*
K[1]	1-10	1 - 10	< 0.1	> 10
1	> 10	0.1-1	> 10	> 10
2a	> 10	1 – 10	> 10	> 10
2b	0.1 - 1	1 – 10	> 10	> 10
2c	< 0.1	< 0.1	1 – 10	1 - 10
2d	1 - 10	> 10	1 – 10	1 - 10
2e	< 0.1	< 0.1	> 10	> 10
2f	1 – 10	> 10	< 0.1	> 10
2g	> 10	> 10	> 10	> 10
2h	< 0.1	> 10	< 0.1	> 10
2i	< 0.1	> 10	< 0.1	< 0.1
2j	< 0.1	> 10	< 0.1	< 0.1
2k	1 – 10	< 0.1	< 0.1	> 10
2l	1 – 10	1 – 10	< 0.1	> 10
2m	> 10	0.1 – 1	< 0.1	> 10
2n	< 0.1	> 10	1 – 10	< 0.1
2o	1 – 10	> 10	> 10	> 10
2p	-	-	> 10	-
2q	> 10	0.1 – 1	> 10	> 10
2r	> 10	0.1 – 1	> 10	< 0.1
2s	1 – 10	< 0.1	1 – 10	< 0.1
2t	1- 10	> 10	> 10	1 – 10
2u	> 10	> 10	> 10	> 10
2v	1 – 10	> 10	> 10	1 – 10
2w	> 10	> 10	1 – 10	> 10
2x	1 – 10	> 10	< 0.1	> 10
2y	< 0.1	< 0.1	1 – 10	1 – 10
2z	< 0.1	> 10	1 – 10	< 0.1

[1]*7-hydroxy-4-methylcoumarin*

Antifungal activity for the tested compounds against *A. flavus* was observed for Schiff bases **2c**, **2e**, **2h**, **2i**, **2j**, **2n**, which showed the best antifungal activity at all concentrations applied. Compounds **2c**, **2h**, **2i** and **2j** bear hydroxyl groups on phenyl ring which, according to some authors[45,46] can contribute to the antifungal activity. The rest of the compounds bearing one or two hydroxyl groups also showed very good antifungal activity (**Table 3**). Although the presence of halogens on phenyl ring was proven to enhance the antifungal activity[22,23,46] in this investigation compounds with hydroxyl groups were proven to be better antifungals than those bearing halogens. Coumarinyl Schiff bases have proven to be excellent antifungals on *A. ochraceus* as well, especially compounds **2c**, **2e**, **2k**, **2s** and **2y**. The presence of hydroxy and methoxy groups, again, was of the great importance for antifungal activity, as well as the position of these groups on phenyl ring, considering the fact that compounds **2f**, **2i**, **2j**, also bearing hydroxy groups exhibited lower antifungal activity than above mentioned ones. Coumarinyl Schiff bases bearing halogen substituents, as well as those with hydroxyl groups, have shown antifungal activity depending on the position of halogen on phenyl ring. Compounds with chloro substituents **2q**, **2r** showed an excellent antifungal activity, while among the brominated ones only compound **2s** acted as good antifungal agent. Compounds with fluoro substituent did not show any significant antifungal activity. This is in accordance with other authors [22,23]who found that chlorinated coumarin derivatives show better antifungal activity than the ones bearing bromo

or iodo substituents. Other authors also emphasize the importance of presence of chloro substituents for antifungal activity[47-49].

In this investigation, among the tested molds *Fusarimgraminearum* has proven to be the most sensitive to the tested compounds. More than half of the tested compounds were proven to have an excellent antifungal activity at the lowest concentration investigated. On the other side, *F. verticillioides*, was proven to be the most resistant among all the tested fungi. In this case it is clear that the substitution of hydroxyl group of 7-hydroxy-4-methylcoumarin contributed to the antifungal activity enhancement, for the compounds **2i**, **2j**, **2n**, **2r**, **2s**, **2z**. Compounds **2i** and **2j** bear dihydroxyphenyl group in their structure, and **2r** and **2s** halogen substituents on phenyl ring, so their antifungal activity was expected and is in accordance with other authors[22,23,45,46],with some exceptions. Compounds **2b**, bearing one hydroxyl group, and **2h** and **2l**, bearing two hydroxyl groups did not show significant antifungal activity, which once more points out the importance of, not only the type of substituents, but their position too. Derivatives with halogen substitution **2r** and **2s** have also demonstrated great antifungal activity.

Other authors have also proven that alkylation of hydroxyl group in position 7 of 7-hydroxy-4-methylcoumarin can influence the antifungal activity[6].In this work it was proven that synthetic modification of 7-hydroxy-4-methylcoumarin can contribute to the antifungal and antioxidant enhancement of the starting compound. There are some compounds which show both potent antioxidant and antifungal properties, like Schiff bases **2h**, **2k** and **2j**, which could be beneficiary for potential application in agriculture, food industry or even medicine.

In this study antifungal and antioxidant properties of new synthesized Schiff bases (*E*)-*N*-2-aryliden-2-(4-methyl-2-oxo-2*H*-chromen-7-yloxy)acetohydrazide were examined. The best antioxidants were proven to be compounds bearing dihydroxyphenyl moiety, **2h**, **2j** and **2k**, which showed greater antioxidant activity than the starting compound, 7-hydroxy-4-methylcoumarin, itself. When comparing two antioxidant assays, DPPH and galvinoxyl scavenging activity, there is an excellent correlation between them. The best antifungals, observing the antifungal activity towards all four molds, were proven to be compounds **2i**, **2j** and **2s**, but the overall investigation shows there are few compounds possessing both great antifungal and antioxidant activity, **2h**, **2k** and **2j**,namely.

CONCLUSION

In this study antifungal and antioxidant properties of coumarin Schiff bases were examined. Compounds bearing two hydroxyl groups on phenyl ring were proven to possess the best antioxidant activity, determined both by DPPH and galvinoxyl radical method. In this case the modification of a starting compound (7-hydroxy-4-methylcoumarin) by substitution in position 7 contributed to the achievement of higher antioxidant activity. Antifungal activity tests were performed against four common mycotoxin producing foodborne fungi, *Aspergillusflavus*, *A. ochraceus*, *Fusariumgraminearum* and F. *verticillioides*, where compounds bearing dihydroxyphenyl moiety were also proven to be an excellent antifungals. Some of these compounds, possessing both antioxidant and antifungal activity will be subjected to some further investigation on their biological activity.

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