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Substituted imino and amino derivatives of 4-hydroxycoumarins as novel antioxidant, antibacterial and antifungal agents: Synthesis and *in vitro* assessments

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ABSTRACT

A series of imino and amino derivatives of 4-hydroxycoumarins were synthesised and evaluated for antioxidant potential, through different *in vitro* models such as (DPPH) free radical-scavenging activity, linoleic acid emulsion model system, reducing power assay and phosphomolybdenum method. Also, antimicrobial activity of obtained coumarins was evaluated against 13 bacteria and eight fungi. All prepared compounds possessed good antioxidant activity and among them a *p*-nitrophenol derivative with IC_{50} at 25.9 μM possessed radical-scavenging activity which was comparable to BHT. Observed data for antibacterial activity indicated strong activity of all tested amino derivatives, while imines showed better antifungal properties.

1. Introduction

In vivo molecular oxygen is easily converted to reactive free radicals such as superoxide anions ($O_2^{-\cdot}$) and hydroxyl radicals ($HO^{\cdot}$), which are highly reactive substances that react with lipids, proteins and DNA, provoking irreversible changes of their biomolecular structure (Halliwell, 1990). Reactive oxygen species (ROS) are continuously generated in very low amounts by the transfer of one electron to an oxygen molecule during various physiological processes, such as respiration chain, oxygenase and cellular immunization reactions (Dröge, 2002; Filomeni, Rotilio, & Ciriolo, 2006). They play an essential role in the control of cell functions. They are intermediate metabolites in several enzymatic reactions, involved in post-translational protein turnover and play a role in the control of signal transduction. Many components of the vascular system, such as leucocytes, monocytes and endothelial cells are able to release ROS upon appropriate stimulation. Thus, ROS are associated with the incidence of various diseases, such as heart diseases, thrombosis, hypertension, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Juliano, Colavita, Leo, Pratico, & Violi, 1997; Lassegue & Griendling, 2004; McIntosh, Trush, & Troncoso, 1997). Besides oxidative stress, reactive oxygen species are associated with the induction of DNA single- and double-strand breaks and are considered to be the first step in several human degenerative diseases, cancer and ageing (Festa et al., 2001). Tissues with high oxygen consumption rate and the central nervous system (CNS), in particular, are more easily susceptible to oxidative damage under conditions of oxidative stress, due to the presence of excitatory amino acids, such as glutamate, elevated iron stores, cell membranes rich in polyunsaturated fatty acids and low levels of the natural antioxidant glutathione in neurons (Barnham, Masters, & Bush, 2004). Furthermore, the blood–brain barrier reduces the permeability and the protective efficacy of most antioxidants (Gilgun-Sherki, Melamed, & Offen, 2001; Gilgun-Sherki, Rosenbaum, Meland, & Offen, 2002).

The emergence of multi-drug resistant microorganisms has made the treatment of infectious diseases difficult and has, over the last decades, become a serious medical problem. As bacteria and fungi continuously evolve mechanisms of resistance to currently used drugs, the discovery of novel and potent antimicrobial agents is the best way to overcome bacterial and fungal resistance and develop effective therapies.

The imino group (–C=N–), containing compounds typically known as Schiff bases, form a significant class of compounds in medicinal and pharmaceutical chemistry with several biological applications that include antibacterial (Pannerselvam, Nair, Vijayalakshmi, Subramanian, & Sridhar, 2005; Sithambaram Karthikeyan, Jagadesh Prasad, Poojary, & Subramanya Bhat, 2006), antifungal (Pandeya, Sriram, Nath, & Declercq, 1999) and antitumour activity (Mladenova, Ignatova, Manolova, Petrova, & Rashkov, 2002).

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From the fundamental point of view, reduction of imines to the corresponding amines represents one of the most widely used and valuable functional group transformations in synthetic organic chemistry, since amines constitute important precursors to compounds that are of much interest in the pharmaceutical and agricultural industries (Moglie, Alonso, Vitale, Yusb, & Radivoy, 2006).

Coumarin (1,2-benzopyrone) derivatives constitute one of the most common families of green plant secondary metabolites, several of them being reported to display multiple biological properties (Egan et al., 1990; Jurd, Corse, King, Bayne, & Mihara, 1971). Many products which contain a coumarin subunit exhibit biological activities, such as molluscicidal, anthelmintic, hypnotic, and insecticidal activities (Schonberg & Latif, 1954). Also, the medicinal properties of coumarins, include inhibition of platelet aggregation, cytochrome P450 and steroid 5α-reductase (Hoult & Paya, 1996; Kostova, 2005). However, the best-known compounds in this series are some 4-hydroxycoumarins, such as the drugs warfarin and acenocoumarol, which have been widely used in anticoagulation therapy for over 20 years (Hirsh et al., 2001). A number of coumarins were found to affect the formation and scavenging of ROS, exhibiting tissue-protective antioxidant properties, which may include numerous different molecular mechanisms and are probably related to their structural analogy with flavonoids and benzophenones (Beillerot, Rodríguez Domínguez, Kirsch, & Bagrel, 2008). Indeed, this structure type can bind Fe(III) and thus inhibit hydroxyl radical and hydrogen peroxide formation produced by Fenton's reactions. The hydroxyl groups of some hydroxycoumarins are potent H· donors for free radical acceptors, due to electron delocalization across the molecule (Sharma, Rajor, Chopra, & Sharma, 2005). Also, some simple hydroxylated coumarin derivatives have been reported to inhibit xanthine oxidase (Ferrari, Sgobba, Gamberini, & Rastelli, 2007).

Prompted by the above-mentioned biological properties of coumarin derivatives and in continuation of our previous work (Sukdolak, Vuković, Solujić, Manojlović, & Krstić, 2004; Vukovic, Sukdolak, Solujic, & Milosevic, 2008), we present here the preparation of some new imino and amino derivatives of 4-hydroxycoumarins and the investigation of their antibacterial, antifungal and antioxidative properties.

Table 1
Substituents and yields of obtained compounds **2b–8b**.

No.	*R*	Conventional method A		Microwave assisted method B	
		Time of synthesis[a] (h)	Yield[b] (%)	Irradiation time (min)	Yield[b] (%)
2b	Ph	9	75	3	95
3b	*p*-tolyl	9.5	73	3	97
4b	*m*-tolyl	9	84	3	94
5b	*o*-tolyl	9.5	73	3	94
6b	*p*-NO_2-phenyl	13.5	51	3	92
7b	*m*-NO_2-phenyl	12.5	62	3	97
8b	Benzyl	10	75	3	97
9b	ω-C_4H_8COOH	10	42	3	87

[a] Reflux in heating equipments.
[B] Percentage of yield calculated from practical and theoretical yields.

2. Materials and methods

2.1. Chemicals

All applied chemicals and reagents were of the highest purity available and purchased from the Sigma–Aldrich Chemical Company (St. Louis, MO), Difco and Merck Laboratory Supplies (Darmstadt, Germany).

2.2. Instrumentation and apparatus

The microwave-assisted reactions were carried out in a MICROSYNTH Microwave Synthesis System manufactured by Milestone Inc. (Shelton, CT). Products were identified by determination of melting points (Kofler-hot stage apparatus), using elemental analysis (Carlo Erba 1106 microanalyser), IR (Perkin-Elmer Grating Spectrophotometers Model 137 and Model 337, KBr disc, ν in cm^{-1}), ^{1}H-NMR (Varian, Palo Alto, CA; Gemini 200 spectrometer, $CDCl_3$, δ in ppm) and GC/MS (Agilent, Santa Clara, CA) techniques (HP 5MS capillary column, 30 m × 0.25 mm i.d., film thickness 0.25 μm; injector and transfer line temperatures of 250 and 280 °C, respectively; oven temperature programmed at 150 °C with an increase of 10 °C/min to 300 °C (isothermal for 10 min); carrier gas, helium; MS quadruple temperature 150 °C; mass scan range, 35–500 amu at 70 eV). Analytical TLC was performed on Merck Silica gel 60 F_{254} TLC plates (layer thickness, 0.25 mm), and monitored by UV light (254 and 365 nm) or iodine vapours. Column chromatography was carried out using Merck 7734 (60 ± 200 mesh) silica gel and monitored by TLC.

2.3. Synthesis of imino and amino derivatives of 4-hydroxycoumarins

2.3.1. Condensation of 3-acetyl-4-hydroxy-chromene-2-one ***1*** with amines ***2a–9a***

2.3.1.1. Method A – conventional method. A mixture of 3-acetyl-4-hydroxy-chromene-2-one **1** (0.01 mol), amine **2a–9a** (0.01 mol) and catalytic amount of *p*-toluenesulphonic acid (10^{-5} mol) in anhydrous toluene (50 ml) was heated with azeotropic removal of water over a period of 10–12 h. Progress of reaction was monitored by TLC (toluene:acetone = 7:3). At the end of reaction, the solvent was evaporated to one quarter of its volume; then the obtained solid products were filtered, dried, purified *via* column chromatography (benzene:acetone = 8:2) to give compounds **2b–9b**.

2.3.1.2. Method B – microwave method. Catalytic amount of *p*-toluenesulfonic acid (10^{-5} mol) was added to 50 ml toluene solution of equimolar amounts (0.01 mol) of 3-acetyl-4-hydroxy-chromene-2-one **1** and amines **2a–9a**. The mixture was heated under microwave for 3 min. After cooling, the solvent was evaporated to one

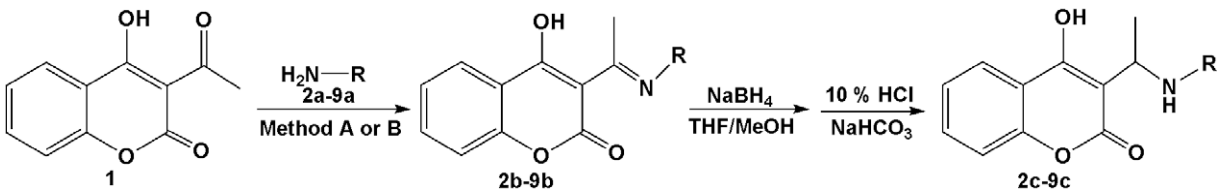

Fig. 1. Synthesis of imino and amino derivatives of 4-hydroxy coumarins.

quarter of its volume, then the obtained solid was filtered and recrystallised from methanol (Table 1, Fig. 1).

*2.3.2. Reduction of imino derivatives of 4-hydroxy-chromene-2-one **2b–9b** to amines **2c–9c***

Sodium borohydride (0.129 g, 0.0034 mol) was slowly added in several portions to a solution of 0.0034 mol of the imine **2b–9b** in 50 ml MeOH/THF (8:2). The reaction mixture was stirred at room temperature for 4 h and monitored periodically by TLC. Upon completion, the solvent was evaporated and the residue was treated with hydrochloric acid (10 w/v). Then the reaction mixture was made basic with a saturated aqueous $NaHCO_3$ solution and the organic products were extracted with dichloromethane (3×50 ml). The organic layer was dried over anhydrous sodium sulphate and was filtered. After evaporation of the solvent, the obtained residue was purified via column chromatography (benzene:acetone = 8:2) to give amines **2c–9c**.

2.4. Measurement of antioxidant activity

2.4.1. DPPH assay

The method used by Takao, Watanabe, Yagi, and Sakata, (1994), was adopted with suitable modifications. DPPH (8 mg) was dissolved in MeOH (100 ml) to obtain a concentration of 80 μg/ml. Serial dilutions were carried out with stock solutions (4 mM) of the compounds (**2b–9b**; **2c–9c**) in methanol to obtain concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01563, 0.0078 and 0.0039 mM. Diluted solutions (2 ml each) were mixed with DPPH (2 ml) and allowed to stand for 30 and 60 min for any reaction to occur. The absorbance was recorded at 517 nm using a Perkin–Elmer Lambda 25 UV/Vis spectrophotometer. The experiment was performed in triplicate and the average absorbance was noted for each concentration. The IC_{50} value, which is the concentration of the test compound that reduces 50% of the initial free radical concentration, was calculated in μM. Ascorbic acid and BHT were used as reference standards, at the same concentrations in methanol as were used for the tested compounds. Control sample was prepared containing the same volume without test compounds and reference compounds. The radical-scavenging activity of the tested samples, expressed as percentage inhibition of DPPH, was calculated according to the formula:

$$IC(\%) = [(A_0 - A_t) \setminus A_0] \times 100,$$

where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of blank sample, at a particular time. Percentage inhibition after 30 and 60 min was plotted against concentration, and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

2.4.2. Reducing power assay

The reducing power test is based on reduction of ferric to ferrous by the potent antioxidant. In the presence of cyanide ions, and adding a new amount of Fe^{3+}, blue colour of $Fe_4[Fe(CN)_6]_3$ develops. The reducing activity of prepared compounds was measured according to the method described by Oyaizu (1986). Sample of 300 μl of various dilutions (from 2 to 0.0039 mM, methanol solutions) was mixed with 300 μl of phosphate buffer (0.2 M, pH 6.6) and 300 μl of 1% potassium ferricyanide. The mixtures were incubated at 50 °C for 20 min. After incubation 300 μl of 10% trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer (0.6 ml) of solution was mixed with 0.6 ml of distilled water and 120 μl of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. The control sample contained 300 μl distilled water, 300 μl of phosphate buffer, 300 μl of 1% potassium ferrocyanide and 300 μl of 10% trichloroacetic acid. The blank sample contained 300 μl distilled water, 300 μl of phosphate buffer, 300 μl of 1% potassium ferricyanide and 300 μl of 10% trichloroacetic acid. Ascorbic acid and BHT were used as positive controls. The reducing power of samples was calculated by the following formula:

$$RP(\%) = (A_B - A_A) \times 100;$$

where: RP is reducing power; A_B is absorbance of control sample (100%); A_A is the absorbance of the tested sample. Percentage of reducing power RP (%) was plotted against concentration, and the equation for the line was used to obtain the RP_{50} value. All determinations were carried out in triplicate. A lower RP_{50} value indicates greater reducing power ability.

2.4.3. Inhibition (%) of lipid peroxidation in a linoleic acid emulsion

The total antioxidant activity of synthesised compounds was carried out by use of a linoleic acid system (Masude, Isibe, Jitoe, & Naramati, 1992). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid, 0.2804 g of Tween 20 as emulsifier and 50 ml of phosphate buffer (0.2 M, pH 7.0), and then the mixture was homogenised. A 0.5-ml ethanol solution of different concentrations of imines and amines (1000, 500, 250 and 125 μM) was mixed with a linoleic acid emulsion (2.5 ml, 0.02 M, pH 7.0) and phosphate buffer (2 ml, 0.2 M, pH 7.0). The reaction mixture was incubated at 37 °C in the dark to accelerate the peroxidation process. Aliquots of 100 μl were taken at different intervals during incubation. The degree of oxidation was measured by sequentially adding ethanol (4.7 ml, 75%), an ammonium thiocyanate sample solution (100 μl, 30%) and ferrous chloride (100 μl, 0.02 M in 3.5% HCl). After 3 min, the absorbance at 500 nm was read. Ascorbic acid and BHT were used as reference compounds. A control was performed with linoleic acid but without the tested compounds. All data reported are the average of triplicate analyses. Percentage inhibition of lipid peroxide generation was calculated using the formula:

$$\%\text{Inhibition} = [(A_0 - A_t)/A_0] \times 100,$$

where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of the control sample.

2.4.4. Determination of total antioxidant capacity

The antioxidant activity of the tested compounds was evaluated by the phosphomolybdenum method according to the procedure of Prieto, Pineda, and Aguilar, (1999), with some modification. The assay is based on the reduction of Mo(VI) to Mo(V) by the test compounds and subsequent formation of a green phosphate/Mo(V) complex at acid pH. One hundred microlitres of the solution of tested compounds (15.6–250 μg/ml) was combined with 1 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95 °C for 90 min. Then the absorbance of the solution was measured at 695 nm against a blank after cooling to room temperature. Ethanol (100 μl) in the place of the solution of the tested compounds was used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid. Determinations of total antioxidant capacity were carried out in triplicate.

2.5. Determination of antibacterial and antifungal activity

2.5.1. Culture of microorganisms

Test bacteria which were used in this experiment are: (a) Gram-positive bacteria: *Bacillus mycoides* (FSB 1), *Bacillus subtilis* (FSB 2), *Micrococcus lysodeikticus* (ATCC 4698), *Staphylococcus aureus* (ATCC 25923) and *Staphylococcus aureus* (FSB 30); (b) Gram-negative

bacteria: *Azotobacter chroococcum* (FSB 14), *Enterobacter cloaceae* (FSB 22), *Erwinia carotovora* (FSB 31), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (FSB 26), *Pseudomonas fluorescens* (FSB 28), *Pseudomonas glycinea* (FSB 40) and *Pseudomonas phaseolicola* (FSB 29). All tested bacteria are from the Faculty of Agriculture, Beograd, University of Beograd, Serbia. The Laboratory for Microbiology, Department of Biology, Faculty of Science, Kragujevac, University of Kragujevac, Serbia confirmed identification of all tested bacteria.

Fungi which were used in this experiment are: *Aspergillus glaucus* (FSB 32), *Aspergillus niger* (FSB 31), *Candida albicans* (ATCC 10259), *Fusarium oxysporum* (FSB 91), *Penicillium verrucosum* (FSB 21), *Trichoderma longibrachiatum* (FSB 13), *Trichoderma harzianum* (FSB 12) and *Trichoderma viride* (FSB 11). All tested fungi are from the Laboratory for Micology, Department of Biology, Faculty of Science, University of Kragujevac.

Bacteria were cultured for 24 h at 37 °C in Mueller–Hinton broth. Suspensions of fungal spores were prepared from two-day old cultures which were grown on SDA plates at 30 °C. The final inoculum size was 10^6 CFU/ml (according to 0.5 McFarland standard; turbidimetric method was applied) for the antibacterial assay and 10^4 CFU/ml for the antifungal assay.

2.5.2. Assay for in vitro antibacterial activity

The minimal inhibitory concentration (*MIC*) of the prepared compounds against tested bacteria was determined based on a microdilution method in 96 multi-well microtitre plates (Sarker, Nahar, & Kumarasamy, 2007). The dissolved compounds were first diluted to the highest concentration to be tested (500 mg/ml); 50 μl of Mueller–Hinton broth was distributed from the 2nd to the 12th well, a volume of 50 μl from each of the compounds initially prepared was pipetted into the 1st test wells of each microtitre line, and then 50 μl of scalar dilution were transferred from the 2nd to the 12th well. To each well 10 μl of resazurin indicator solution (prepared by dissolving a 270-mg tablet in 40 ml of sterile distilled water) and 30 μl of Mueller–Hinton broth were added. Finally, 10 μl of bacterial suspension (10^6 CFU/ml) were added to each well, to achieve a concentration of 10^5 CFU/ml. The final concentrations of the compounds adopted to evaluate antibacterial activity ranged from 0.244 to 500 mg/ml. Two columns in each plate were used as controls: one column with a broad-spectrum antibiotic as a positive control (streptomycin in a serial dilution of 0.244–500 mg/ml) and one column containing the solvent ethanol as negative control. Plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and prepared in triplicate, and then they were placed in an incubator at 37 °C for 18–24 h. Colour change was then assessed visually. Any colour change from purple to pink or colourless was recorded as positive. The lowest concentration at which colour change occurred was taken as the *MIC* value. The average of 3 values was calculated to give the *MIC* for the tested compounds and standard drug.

2.5.3. Assay for in vitro antifungal activity

Broth microdilution assays were performed in accordance with the guidelines in CLSI document M27-A2 (National Committee for Clinical Laboratory Standards, 2002). Stock solutions were prepared in water for ketoconazole and ethanol for the compounds in the present study. A final dilution was carried out in an RPMI 1640 medium, buffered to pH 7.0 with 0.165 M morpholenepropanesulphonic acid buffer. Each microdilution well containing 100 μl of the twofold concentrations of prepared compounds or standard drug ketoconazole (0.244–500 mg/ml) was inoculated with 100 μl of the diluted twofold inoculum suspension (the final volume in each well was 200 μl and inoculum size of 10^3 CFU/ml). Growth (drug free) and sterility controls were also included. Microdilution trays were incubated in ambient air at 35 °C. *MIC* values were determined visually after 48 h of incubation, as the lowest concentration of drug that caused no detectable growth. The average of 3 values was calculated to give the *MIC* for the tested compounds and standard drug.

3. Results and discussion

3.1. Synthesis of imino and amino derivatives 4-hydroxycoumarins

For the synthesis of imino derivatives of 4-hydroxy-chromene-2-one (**2b**–**9b**), the requisite starting material, 3-acetyl-4-hydroxy-chromene-2-one (**1**), was prepared from commercially available 4-hydroxy-chromene-2-one by the previously reported procedure (Sukdolak et al., 2004). Preparation of imino derivatives **2b**–**9b** by conventional condensation method with azeotropic removal of water was characterised with lower yields of desired compounds (not more than 84%) followed by time-consuming purifications by column chromatography. Thus, we reported improvements in the synthesis of these compounds using a microwave-promoted reaction. Homogenous heating under microwave exposure resulted in increasing yields of desired compounds **9b**–**16b** (up to 87%), decrease in time of reaction and use of the purification procedure only from the point of recrystallisation from appropriate solvents. As presented in Table 1, the yield has been significantly increased using reaction under microwave heating conditions, particularly in the case of imino derivatives with *p*-nitrophenyl (92%), *m*-nitrophenyl (97%) and pentanoic acid substituents (87%). Their further transformation to the corresponding amines (**2c**–**9c**) was performed by reduction with sodium borohydride in a methanol:tetrahydrofuran mixture (8:2). After reduction, the solvent mixture was removed on a rotary evaporator at 40 °C and the residue was treated with 10% HCl for a period of 15 min. Then a solution of $NaHCO_3$ was added to pH 7 and an extraction with methylene chloride was performed; the organic layer was separated, dried under anhydrous sodium sulphate and removed under reduced pressure to form amines **2c**–**9c**.

The IR spectral data of the compounds **2b**–**9b** confirmed the presence of coumarine –OH (at 3406–3467), lactone –C=O (at 1697–1712) and –C=N– group (at 1606–1614). Similarly NMR broadened singlets in the range of 15.8–16.15 ppm also confirmed the presence of coumarine –OH, while multiplets in the range of 7.3–7.7 ppm indicated the presence of protons from the benzenoid part of the molecule. Observed singlets at 2.61–2.81 ppm revealed the presence of methyl protons from imino groups. The imino derivatives **3b**, 4b and **5b** showed additional singlets (2.30–2.42 ppm) from methyl protons attached at the aromatic part, while compound **8b** possesses a singlet at 3.25 ppm from –C=N–CH_2 group. In contrast to other prepared imino derivatives, compound **9b** revealed triplets from two methylene protons at 2.46 and 2.70 ppm, a multiplet at 1.82 ppm from two protons at C″-2 and a broadened singlet from COOH group at 11.3 ppm, which indicated presence of the *n*-pentanoic acid part of molecule.

In general, the IR spectral data of the amines **2c**–**9c** revealed bands at 3429–3455 cm^{-1} (coumarine –OH), 3179–3193 cm^{-1} (–NH group) and 1664–1681 cm^{-1} (lactone –C=O). Additional bands for compound **9c** from –COOH group were observed in a region of 2590–3611 cm^{-1} (–OH) and at 1710 cm^{-1} (–C=O). In the ^{1}H-NMR spectral data, all the compounds showed a quartet of one proton at around 1.33 ppm and a doublet of three protons at around 3.78 ppm, accounted for by the –CH–CH_3 group. Broadened singlets in a region at 3.87–4.14 ppm were assigned to NH groups, while singlets at low field (16.55–17.19 ppm) confirmed presence of C-4–OH protons. Characteristic resonances from methylene groups at 1.82, 2.46 and 3.60 ppm, were observed for compound **9c**, and indicated C_4 fragment of molecule.

3.2. Free radical-scavenging activity on 2,2-diphenyl-1-picrylhydrazyl radical

This assay is based on the measurements of the scavenging ability of compounds towards the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH**.**).The disappearance of this commercially available radical is measured spectrophotometrically at 517 nm in a methanolic solution. The antioxidant activity was expressed as the 50% inhibitory concentration (IC_{50}) based on the amount of compound required for a 50% decrease of the initial DPPH radical concentration.

As presented in Table 2, the imines **2b**–**9b** with IC_{50} values in the range of 304.1–446.9 μM showed lower radical-scavenging activities in comparison to butylated hydroxytoluene (BHT) and ascorbic acid. Among them, compounds **5b** and **4b** with *o*-tolyl and *m*-tolyl substituents at imino nitrogen showed highest hydrogen donor ability to DPPH radical (IC_{50} values were 304.1 and 334.6 μM, respectively). Observed data for prepared compounds **2c**–**9c** indicated more effective scavenging activity (IC_{50} values were in the range of 25.9–138 μM) related to imines **2b**–**9b**, which was attributed to presence of hydroxyl and amino groups, as well as formation of hydrogen bonds between them as the main factor of increased acidity of protons from both groups or their ability to be scavenged by DPPH. Compound **6c** as *p*-nitrophenyl derivative with IC_{50} at 25.9 μM possesses highest radical-scavenging activity, which is comparable to BHT. On the other hand, better antiradical activity was observed in the case of phenyl derivative **2c** and *m*-nitrophenyl derivative **7c** (IC_{50} values were 34.1 and 40.9 μM, respectively) in comparison to ascorbic acid (IC_{50} 42.4 μM). In a series of compounds with tolyl fragment, compound **5c** as *o*-methyl isomer with IC_{50} 72.3 μM showed best antiradical activity, while *m*-tolyl derivative **4c** with IC_{50} 131.8 μM showed least activity. This indicated the influence of methyl substituent as an electron donor group at positions C″-2 and C″-6 of phenyl ring to increase the capability of NH protons to scavenge the DPPH radicals. Almost equal antiradical potency of compounds **8c** and **9c** (IC_{50} were 62.6 and 63.4 μM, respectively) could be attributed to similar electron donating natures of $C_6H_5CH_2$ and C_4COOH substituents bonded to the NH group. Since the corresponding IC_{50} values for all synthesised compounds after 60 min were lower than after 30 min, DPPH antiradical scavenging activity was also time-dependent.

3.3. Evaluation of reducing power

The reducing power of prepared coumarin derivatives, which may serve as a significant reflection of the antioxidant activity, was determined using the iron(III) to iron(II) reduction assay. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of compounds. The presence of reductants in the solution causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, the Fe^{2+} ion can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. Table 2 showed the reducing power of imino and amino derivatives of 4-hydroxy-chromene-2-one compared to BHT and ascorbic acid. All tested compounds showed some degree of reducing power; however, as anticipated, their reducing power was inferior to ascorbic acid, which is known to be a strong reducing agent. Assayed compounds were able to reduce the ferric ions to corresponding ferrous ions, reaching 50% of reduction, with RP_{50} values ranking from 278.8 to 350.3 μM for imines **2b**–**9b** and 255.6 to 435.7 μM for amines **2c**–**9c**. In the series of imines, compounds **9b** as *n*-pentanoic acid derivative and **4b** with *m*-tolyl group attached to imino nitrogen showed the best reducing power (RP_{50} values were 278.8 and 279.7 μM, respectively). Low reducing power was gained in the cases of compounds **3b**, 5b and **8b** (RP_{50} values were 350.3, 371.2 and 324.4 μM, respectively). Comparison of these results with results obtained in DPPH assay clearly indicated an opposite effect of substituent bonded to imino nitrogen and position of methyl or nitro group bonded to phenyl ring to reducing power. In the series of tolyl and phenyl derivatives the reducing power followed the following order: ascorbic acid > *m*-tolyl > phenyl > *p*-tolyl > *o*-tolyl > BHT. Obviously, presence of a methyl group on the phenyl ring and type of substitution affected decrease in reducing power. Also, from the point of substitution, similar results were gained in cases of nitrophenyl derivatives **6b** as *para* and **7b** as *meta* isomer (RP_{50} values were 307.3 and 296.0 μM, respectively). In the group of amines, benzyl amino derivative **8c** with RP_{50} value of 255.6 μM showed the best reducing power, while phenyl derivative **2c** and

Table 2
Antioxidant activity of synthesised imino and amino derivatives of 4-hydroxy coumarins.

Comp.	DPPH assay [a] IC_{50} (μM)		Reducing power assay	Inhibition (%) of lipid peroxidation in a linoleic acid emulsion[c]			
	30 min	60 min	[b]RP_{50} (μM)	1000 μM	500 μM	250 μM	125 μM
2b	446.9 ± 12.1	281.5 ± 9.1	285.3 ± 2.3	74.5 ± 1.1	50.5 ± 1.8	38.5 ± 2.1	36.6 ± 2.3
3b	352.6 ± 22.2	266.6 ± 12.2	350.3 ± 3.3	94.4 ± 1.3	77.4 ± 1.6	60.4 ± 2.3	53.9 ± 2.1
4b	334.6 ± 21.9	170.1 ± 8.7	279.7 ± 2.1	88.6 ± 2.1	75.4 ± 1.4	46.2 ± 2.1	29.9 ± 2.4
5b	304.1 ± 13.2	237.8 ± 18.3	371.2 ± 4.4	78.5 ± 1.9	66.5 ± 1.2	35.9 ± 2.9	25.4 ± 2.5
6b	371.6 ± 12.7	152.1 ± 17.4	307.3 ± 3.5	81.3 ± 1.5	73.4 ± 1.1	55.8 ± 3.5	43.6 ± 2.5
7b	498.7 ± 13.2	441.5 ± 7.6	296.0 ± 4.3	76.4 ± 1.6	72.8 ± 1.4	49.2 ± 1.1	31.8 ± 2.7
8b	424.9 ± 14.3	402.8 ± 11.2	324.4 ± 3.2	63.2 ± 1.4	52.9 ± 1.5	39.3 ± 1.4	30.8 ± 3.5
9b	394.2 ± 21.8	377.4 ± 24.6	278.8 ± 2.6	62.6 ± 2.4	51.8 ± 1.7	39.0 ± 1.8	15.8 ± 3.1
2c	34.1 ± 13.4	33.1 ± 11.3	435.7 ± 4.7	57.6 ± 2.2	34.9 ± 1.7	25.6 ± 2.7	17.9 ± 1.5
3c	93.9 ± 13.3	58.8 ± 11.5	365.1 ± 2.3	57.7 ± 2.0	37.6 ± 1.8	29.5 ± 2.4	22.4 ± 2.7
4c	131.8 ± 21.6	56.0 ± 15.3	428.1 ± 3.3	59.4 ± 2.5	31.5 ± 1.5	21.5 ± 2.3	15.9 ± 2.4
5c	72.3 ± 13.4	45.0 ± 19.8	293.4 ± 4.3	58.9 ± 1.6	35.6 ± 1.9	27.6 ± 2.1	15.0 ± 2.6
6c	25.9 ± 11.8	25.0 ± 14.6	338.2 ± 4.4	67.8 ± 1.7	47.5 ± 2.1	29.7 ± 1.5	20.9 ± 1.7
7c	40.9 ± 13.9	37.2 ± 7.7	310.5 ± 3.3	58.3 ± 1.1	37.8 ± 2.2	27.6 ± 1.5	15.6 ± 1.8
8c	62.6 ± 14.2	56.9 ± 21.2	255.6 ± 3.3	60.3 ± 1.1	48.7 ± 2.0	27.2 ± 1.3	18.1 ± 2.0
9c	63.4 ± 12.7	58.4 ± 14.5	279.5 ± 2.2	52.9 ± 1.4	41.7 ± 2.3	22.8 ± 1.2	16.8 ± 1.7
BHT	25.4 ± 22.7	12.78 ± 7.6	447.1 ± 4.4	90.5 ± 1.3	90.1 ± 2.1	89.7 ± 1.1	89.6 ± 1.3
Asc	42.4 ± 22.7	27.22 ± 14.3	142.3 ± 4.5	27.8 ± 1.4	20.5 ± 2.5	16.0 ± 1.7	8.8 ± 1.5
α-Toc				69.2 ± 1.9	67.3 ± 2.9	63.6 ± 1.8	60.2 ± 2.1

[a] IC_{50} values were determined by linear regression analysis. Results are mean values ± SD from at least three experiments.
[b] RP_{50} values were determined by linear regression analysis. Results are mean values ± SD from at least three experiments.
[c] Data of percentage are reported as means ± SD of three measurements.

m-tolyl derivative **4c** with RP_{50} values of 435.7 and 428.1 μM, respectively, possess low reducing power. Also, RP_{50} values are influenced by the substituent bonded to amino group, as well as the type of substitution of phenyl moiety. The reducing power of tolyl and phenyl derivatives was decreased in order: *o* > *p* > *m* > phenyl, which clearly demonstrated influence of position and electron donor nature of methyl group attached to phenyl ring, as well as low reducing power of phenyl derivative **2c**. Also, *m*-nitro compound **6c** showed better activity than the corresponding *p*-nitro derivative **7c** (RP_{50} values were 338.2 and 310.5 μM, respectively).

3.4. Inhibition (%) of lipid peroxidation in a linoleic acid emulsion

In the present study, the antioxidant activities of imines and amines of 4-hydroxychromene-2-one, determined by peroxidation of linoleic acid using the thiocyanate method at 37 °C, after addition of different concentration of prepared compounds, were determined. During the linoleic acid peroxidation, peroxides are formed and these compounds oxidise Fe^{2+} to Fe^{3+}, the latter Fe^{3+} ion forms a complex with SCN^-, which has a maximum absorbance at 500 nm. High absorbance (or low value of% of inhibition) is an indicator of high concentration of peroxide formed during the emulsion incubation. As presented in Table 2, the antioxidant activity of synthesised imines and amines exhibited an amount-dependent manner. In the control sample without tested compounds, the absorbance at 500 nm increased up to the maximal value in 72 h, and then it decreased. The reason for this was that linoleic acid hydroperoxides, generated from the peroxidation of linoleic acid, decomposed to many secondary oxidation products, or the intermediate products may be converted to stable end-products and the substrate was exhausted. All tested concentrations added to linoleic acid emulsion were able to reduce the formation of hydroperoxide. Generally, imines **2b**–**9b** showed better ability to inhibit lipid peroxidation than the corresponding prepared amines **2c**–**9c**. Compound **3b** at a concentration of 1000 μM showed better antioxidant activity than BHT, while in cases of other compounds and other tested concentrations BHT was superior in the inhibition of peroxidation. All imines possess better antioxidant activity than ascorbic acid, while at the concentration of 1000 and 500 μM, compounds **3b**–**7b** showed high level inhibitor properties to lipid peroxidation in contrast to α-tocopherol. Other two tested imines **8b** as benzyl imino and **9b** as *n*-pentanoic acid derivatives showed lower activities than α-tocopherol, which clearly indicated that the phenyl moiety attached to imino nitrogen has influence on formation of stable end-products during the process of lipid peroxidation. Among the amines **2c**–**9c**, only *p*-nitrophenyl derivative **6c** at a concentration of 1000 μM showed 67.8% inhibition, an antioxidant activity a few percentage lower than that of 1000 μM α-tocopherol (74.8%). Inhibitor activities of other amines are similar. All abovementioned confirmed that the reduction of imino group led to decrease of antioxidant activity, as well as that, in a series of tested amines, only benzopyran moiety was responsible for the inhibition of lipid peroxidation.

3.5. Total antioxidant activity

The phosphomolybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds and the formation of green Mo(V) complexes with a maximal absorption at 695 nm. Using this method the results from Table 3 indicate that the prepared amines **2c**–**9c** have better antioxidant capacity than the corresponding imines **2b**–**9b**. In a series of imino derivatives of 4-hydroxycoumarins, and at the highest tested concentration (250 μg/ml), the best reducing activities were found for compounds with nitro-phenyl substituents **6b** and **7b** (12.58 and 12.33 μg ascorbic acid/ml, respectively), while in the case of tolyl derivatives **3b**, 4b and **5b** were the lowest values measured (11.70, 11.83 and 11.71 μg ascorbic acid/ml, respectively). On the other hand, comparing the antioxidant capacity within the range of all tested concentrations, best results were observed for compounds **9b**, 8b and **2b**. In the group of amines **2c**–**9c**, the highest antioxidant capacity was observed for *p*-nitrophenyl derivative **6c**. Also, good reducing activities for all tested concentrations were found for compounds **8c** and **9c**, while phenyl derivative **2c**, as well as, *o*-tolyl compound **5c** showed the lowest reducing activity.

Table 3
The total antioxidant activity expressed as equivalents of ascorbic acid (μg/ml).

Comp.	250 μg/ml	125 μg/ml	62.50 μg/ml	31.25 μg/ml	15.60 μg/ml
Equivalents of ascorbic acid (μg/ml)[a]					
2b	12.03 ± 0.25	8.64 ± 0.29	5.29 ± 0.31	3.18 ± 0.27	2.29 ± 0.17
3b	11.70 ± 0.54	9.25 ± 0.32	6.14 ± 0.32	3.96 ± 0.35	2.92 ± 0.19
4b	11.83 ± 0.13	9.47 ± 0.38	5.99 ± 0.58	3.44 ± 0.47	2.18 ± 0.45
5b	11.71 ± 0.24	9.37 ± 0.27	5.18 ± 0.24	2.92 ± 0.44	1.92 ± 0.11
6b	12.58 ± 0.29	8.84 ± 0.41	4.77 ± 0.22	3.18 ± 0.41	2.70 ± 0.26
7b	12.33 ± 0.27	8.82 ± 0.47	4.68 ± 0.35	3.25 ± 0.38	1.73 ± 0.11
8b	12.11 ± 0.57	9.18 ± 0.39	5.92 ± 0.41	4.10 ± 0.33	1.95 ± 0.38
9b	12.18 ± 0.65	9.22 ± 0.36	6.22 ± 0.11	3.73 ± 0.35	3.14 ± 0.24
2c	45.50 ± 0.68	20.30 ± 0.34	7.96 ± 0.14	4.25 ± 0.51	0.55 ± 0.25
3c	46.98 ± 0.29	23.15 ± 0.37	12.77 ± 0.25	4.77 ± 0.23	1.77 ± 0.28
4c	47.09 ± 0.35	20.89 ± 0.38	11.14 ± 0.31	4.55 ± 0.21	0.95 ± 0.21
5c	47.53 ± 0.31	20.63 ± 0.25	8.07 ± 0.24	3.51 ± 0.18	0.84 ± 0.17
6c	54.57 ± 0.13	27.26 ± 0.27	11.83 ± 0.36	7.63 ± 0.25	4.66 ± 0.29
7c	45.90 ± 0.11	24.26 ± 0.28	11.29 ± 0.38	8.96 ± 0.38	5.40 ± 0.33
8c	49.16 ± 0.25	23.56 ± 0.21	11.59 ± 0.13	5.25 ± 0.24	3.51 ± 0.39
9c	49.35 ± 0.12	18.81 ± 0.51	10.94 ± 0.17	4.62 ± 0.35	3.70 ± 0.34

[a] Data of percentage are reported as means ± SD of three measurements.

3.6. Antimicrobial activity of tested compounds

The newly prepared coumarin derivatives **2b**–**9b** and **2c**–**9c**, together with streptomycin and ketoconazole as references, were tested against a panel of Gram-positive and Gram-negative bacteria and fungi (Tables 4 and 5).

3.6.1. Antibacterial activity of tested compounds

As shown in Table 4, the amino compounds **2c**–**9c** were more potent than the corresponding imino compounds **2b**–**9b**. In the series of imino derivatives, high antibacterial activities (*MIC* values were 31.25 μg/ml) were observed for compounds with *n*-pentanoic acid (**9b**) and benzyl fragments (**8b**) bonded to imino nitrogen against seven of 13 test bacteria. Also, the compound **6b** as *p*-NO_2-phenyl derivate of 4-hydroxycoumarin showed activity, with *MIC* values of 15.6–31.25 μg/ml against six test bacteria. On the other hand, bacteria *K. pneumoniae* and *P. glycinea* were most sensitive against tested imino derivatives (*MIC* values were in the range of 7.8–31.25 μg/ml, except in the case of compound **4b**, since the observed *MIC* values were 31.25 and 62.5 μg/ml, respectively), while *S. aureus* (ATCC 25923) and *E. cloaceae* were the most resistant tested bacteria with observed *MIC* values in the range of 62.5–125 μg/ml. Corresponding amino compounds **2c**–**9c** showed excellent antibacterial activities, especially *n*-pentanoic acid derivative **9c** with observed *MIC* values in the range of 3.9–15.6 μg/ml, which are for bacteria *S. aureus* (FSB 30), *E. coli* and *P. fluorescens* are two times lower and for *S. aureus* (ATCC 25923) four times lower than the *MIC* values of standard drug streptomycin. Also, compounds with phenyl (**2c**) and *p*-tolyl (**3c**) residues at NH group inhibited the growth of all bacteria at the concentrations of 3.9–7.8 μg/ml (except in a case *P. phaseolicola*, MIC values were 62.5 and 31.25 μg/ml, respectively), which indicated their antibacterial potency to be similar to streptomycin. The introduction of *o*-tolyl

Table 4
Antibacterial activity of tested imino and amino derivatives of 4-hydroxycoumarins.

Comp.	Bacteria											
	Gram-positive					Gram negative						
	B.m.	*B.s.*	*M.l.*	*S.a.*	*S.a.(i)*	*A.c.*	*En.cl.*	*Er.ca.*	*Es.co.*	*K.p.*	*P.fl.*	*P.gl.*	*P.ph.*
MIC values of tested compounds (μg/ml)													
2b	31.25	62.5	62.5	62.5	62.5	125	62.5	125	62.5	15.6	62.5	62.5	62.5
3b	125	125	125	125	62.5	62.5	62.5	125	62.5	31.25	62.5	31.25	62.5
4b	31.25	62.5	62.5	62.5	31.25	62.5	62.5	62.5	62.5	31.25	62.5	62.5	62.5
5b	62.5	31.25	62.5	62.5	125	62.5	125	62.5	62.5	15.6	62.5	31.25	62.5
6b	62.5	15.6	125	62.5	31.25	31.25	62.5	31.25	125	15.6	62.5	31.25	125
7b	62.5	62.5	62.5	125	62.5	62.5	62.5	62.5	125	7.8	125	31.25	125
8b	62.5	31.25	62.5	125	31.25	125	125	31.25	31.25	15.6	62.5	15.6	31.25
9b	31.25	31.25	31.25	125	62.5	31.25	125	62.5	31.25	31.25	62.5	62.5	31.25
2c	15.6	7.8	3.9	3.9	7.8	3.9	3.9	7.8	7.8	7.8	3.9	7.8	62.5
3c	7.8	7.8	3.9	3.9	7.8	3.9	3.9	7.8	7.8	7.8	3.9	7.8	31.25
4c	15.6	15.6	3.9	7.8	7.8	7.8	3.9	3.9	7.8	7.8	3.9	7.8	31.25
5c	62.5	62.5	3.9	15.6	7.8	7.8	3.9	3.9	7.8	15.6	3.9	7.8	62.5
6c	15.6	31.25	3.9	15.6	15.6	3.9	7.8	7.8	7.8	7.8	3.9	7.8	15.6
7c	15.6	7.8	3.9	31.25	15.6	3.9	7.8	7.8	15.6	15.6	7.8	15.6	7.8
8c	15.6	31.25	3.9	3.9	3.9	7.8	7.8	7.8	31.25	7.8	3.9	7.8	15.6
9c	7.8	7.8	7.8	3.9	3.9	7.8	7.8	7.8	15.6	7.8	3.9	7.8	7.8
*S**	7.8	7.8	1.95	15.6	7.8	7.8	1.95	7.8	31.25	1.95	7.8	7.8	3.9

*S** Streptomycin; *B.m.* – *Bacillus mycoides* (FSB 1); *B.s.* – *Bacillus subtilis* (FSB 2); *M.l.* – *Micrococcus lysodeikticus* (ATCC 4698); *S.a.* – *Staphylococcus aureus* (ATCC 25923); *S.a.(i)* – *Staphylococcus aureus* (FSB 30); *A.c.* – *Azotobacter chroococcum* (FSB 14); *En.cl* – *Enterobacter cloaceae* (FSB 22); *Er.ca* – *Erwinia carotovora* (FSB 31); *Es-co* – *Escherichia coli* (ATCC 25922); *K.p.* – *Klebsiella pneumoniae* (FSB 26); *P.fl.* – *Pseudomonas fluorescens* (FSB 28); *P.gl* – *Pseudomonas glycinea* (FSB 40); *P.ph.* – *Pseudomonas phaseolicola* (FSB 29).

Table 5
Antifungal activity of tested imino and amino derivatives of 4-hydroxycoumarins.

Comp.	Fungi							
	A.gl.	*A.n.*	*C.a.*	*F.o.*	*P.ve.*	*T.l.*	*T.h.*	*T.v.*
MIC values of tested compounds (μg/ml)								
2b	62.5	125	31.25	31.25	62.5	31.25	31.25	31.25
3b	62.5	125	31.25	62.5	62.5	31.25	125	125
4b	62.5	125	31.25	62.5	62.5	31.25	62.5	31.25
5b	125	31.25	31.25	125	31.25	31.25	31.25	31.25
6b	31.25	31.25	125	125	62.5	62.5	62.5	62.5
7b	62.5	31.25	125	125	31.25	62.5	62.5	31.25
8b	31.25	125	31.25	62.5	62.5	31.25	62.5	31.25
9b	31.25	62.5	31.25	62.5	62.5	31.25	125	125
2c	62.5	125	125	125	125	62.5	62.5	125
3c	125	125	125	62.5	125	62.5	62.5	125
4c	62.5	125	125	62.5	125	62.5	62.5	62.5
5c	125	250	250	125	125	62.5	62.5	62.5
6c	62.5	62.5	62.5	62.5	62.5	125	125	62.5
7c	62.5	125	62.5	125	125	125	125	125
8c	62.5	250	62.5	125	62.5	250	125	62.5
9c	62.5	250	125	250	125	250	125	62.5
*K**	7.8	7.8	1.95	3.9	3.9	7.8	7.8	7.8

*K** Ketoconazole; *A.gl* – *Aspergillus glaucus* (FSB 32); *A.n* – *Aspergillus niger* (FSB 31); *C.a.* – *Candida albicans* (ATCC 10259); *F.o.* – *Fusarium oxysporum* (FSB 91); *P.ve* – *Penicillium verrucosum* (FSB 21); *T.l.* – *Trichoderma longibrachiatum* (FSB 13); *Trichoderma harzianum* (FSB 12); *T.v.* – *Trichoderma viride* (FSB 11).

fragment at NH group (compound **5c**) decreased the potency against *B. mycoides*, *B. subtilis* and *P. phaseolicola* (*MIC* values were 62.5 μg/ml, respectively). The most sensitive bacteria against synthesised amino compounds **2c**–**9c** were *S. aureus* (ATCC 25923), *A. chroococcum*, *E. carotovora*, *E. coli*, *P. fluorescens* and *P. glycinea*, while the resistant bacteria against tested compounds were *B. mycoides*, *B. subtilis*, *M. lysodeikticus* and *P. phaseolicola*.

3.6.2. Antifungal activity of tested compounds

Antifungal activity of prepared imines **2b**–**9b** was in the range of 31.25–125 μg/ml, with high potency noted for compound **5b** as *o*-tolyl imino derivative against six of eight fungi (*MIC* values were 31.25 μg/ml, respectively). Also, good antifungal activity was observed for compound **2b**, which possesses a phenyl fragment at the imino nitrogen and at the concentration of 31.25 μg/ml inhibited growth of five test fungi, while at the same *MIC* value benzyl imino compound **8b** showed inhibitor activity against four tested fungi. The most sensitive fungi were *C. albicans* and *T. longibrachiatum* and their growth was inhibited by compounds **2b**–**5b**, 8b and **9b** at 31.25 μg/ml. Based on Table 5, prepared amines **2c**–**9c**, in contrast to imines **2b**–**9b**, showed lower potency against tested fungal strains, with *MIC* values in the range of 62.5–250 μg/ml. Among them, *p*-nitrophenyl derivative **6c** at a concentration of 62.5 μg/ml showed potency against six of eight fungi. The most resistant fungus was *A. niger*, whose growth was inhibited by compounds **5c**, 8c and **9c** at 250 μg/ml, which is not comparable to the antifungal potency of the standard drug ketoconazole.

4. Conclusions

In this study we presented the antioxidant and antimicrobial activities of imino and amino derivatives of 4-hydroxycoumarins. The synthesised compounds scavenged the DPPH radical, reduced Fe^{3+} cations and inhibited lipid peroxidation in a concentration and time-dependent manner. Also, prepared compounds showed excellent antimicrobial activity against all test microorganisms.

These experiments show that introduction of new pharmacophores on the 4-hydroxycoumarin moiety has a significant influence on its antimicrobial properties, resulting in potential antimicrobial agents. On the other hand, their antioxidative activity and the fact that they are structurally modified derivatives of naturally-occurring compounds make them potential dietary antioxidants.

References

Barnham, K. J., Masters, C. L., & Bush, A. (2004). Neurodegenerative diseases and oxidative stress. *Nature Reviews Drug Discovery, 3*, 205–214.

Beillerot, A., Rodríguez Domínguez, J. C., Kirsch, G., & Bagrel, D. (2008). Synthesis and protective effects of coumarin derivatives against oxidative stress induced by doxorubicin. *Bioorganic and Medicinal Chemistry Letters, 18*, 1102–1105.

Dröge, W. (2002). Free radicals in the physiological control of cell function. *Physiological Reviews, 82*, 47–95.

Egan, D., O'Kennedy, E., Moran, E., Cox, D., Prosser, E., & Thornes, R. D. (1990). The pharmacology metabolism, analysis, and applications of coumarin and coumarin-related compounds. *Drug Metabolism Reviews, 22*, 503–529.

Ferrari, A. M., Sgobba, M., Gamberini, M. C., & Rastelli, G. (2007). Relationship between quantum-chemical descriptors of proton dissociation and experimental acidity constants of various hydroxylated coumarins. Identification of the biologically active species for xanthine oxidase inhibition. *European Journal of Medicinal Chemistry, 42*, 1028–1031.

Festa, F., Aglitti, T., Duranti, G., Ricordi, R., Perticone, P., & Cozzi, R. (2001). Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by comet assay. *Anticancer Research, 21*, 3903–3908.

Filomeni, G., Rotilio, G., & Ciriolo, M. R. (2006). *Cell death and differentiation, 12*, 1555–1563.

Gilgun-Sherki, Y., Melamed, E., & Offen, D. (2001). Oxidative stress induced-neurodegenerative diseases: The need for antioxidants that penetrate the blood brain barrier. *Neuropharmacology, 40*, 959–975.

Gilgun-Sherki, Y., Rosenbaum, Z., Meland, E., & Offen, D. (2002). Antioxidant therapy in acute central nervous system injury: Current state. *Pharmacological Reviews, 54*, 271–284.

Halliwell, B. (1990). How to characterize a biological antioxidant. *Free Radical Research, 9*, 1–32.

Hirsh, J., Dalen, J. E., Anderson, D. R., Poller, L., Bussey, H., Ansell, J., et al. (2001). Oral anticoagulants: Mechanism of action, clinical effectiveness, and optimal therapeutic range. *Chest, 119*, 8S–21S.

Hoult, J. R. S., & Paya, M. (1996). Pharmacological and biochemical actions of simple coumarins: Natural products with therapeutic potential. General Pharmacology. *The Vascular System, 27*, 713–722.

Juliano, L., Colavita, A. R., Leo, R., Pratico, D., & Violi, F. (1997). Oxygen free radicals and platelet activation. *Free Radical Biology and Medicine, 22*, 999–1006.

Jurd, L., Corse, J., King, A. D., Bayne, H., & Mihara, K. (1971). Antimicrobial properties of 6,7-dihydroxy-, 7,8-dihydroxy, 6-hydroxy- and 8-hydroxycoumarins. *Phytochemistry, 10*, 2971–2974.

Kostova, I. (2005). Synthetic and natural coumarins as cytotoxic agents. *Current Medicinal Chemistry – Anti-Cancer Agents, 5*, 29–46.

Lassegue, B., & Griendling, K. K. (2004). Reactive oxygen species in hypertension: An update. *American Journal of Hypertension, 17*, 852–860.

Masude, T., Isibe, D., Jitoe, A., & Naramati, N. (1992). Antioxidant curcuminoids from rhizomes of *Curcuma zanthorrhiza*. *Phytochemistry, 33*, 3645–3647.

McIntosh, L. J., Trush, M. A., & Troncoso, J. C. (1997). Increased susceptibility of Alzheimer's disease temporal cortex to oxygen free radical-mediated processes. *Free Radical Biology and Medicine, 23*, 183–190.

Mladenova, R., Ignatova, M., Manolova, N., Petrova, T., & Rashkov, I. (2002). Preparation, characterization and biological activity of Schiff base compounds derived from 8-hydroxyquinoline-2-carboxaldehyde and Jeffamines EDÑ®. *European Polymer Journal, 38*, 989–999.

Moglie, Y., Alonso, F., Vitale, C., Yusb, M., & Radivoy, G. (2006). New active-iron based reducing system for carbonyl compounds and imines. Stereoselective reduction of cyclic ketones. *Tetrahedron, 62*, 2812–2819.

National Committee for Clinical Laboratory Standards (2002). Reference method for broth dilution antifungal susceptibility testing of yeasts. Approved standard M27-A2 (2nd ed.). Wayne, PA: National Committee for Clinical Laboratory Standards.

Oyaizu, M. (1986). Antioxidative activities of browning reaction prepared from glucosamine. *Japanese Journal of Nutrition, 44*, 307–315.

Pandeya, S. N., Sriram, D., Nath, G., & Declercq, E. (1999). Synthesis, antibacterial, antifungal and anti-HIV activities of Schiff and Mannich bases derived from isatin derivatives and N-[4-(4′-chlorophenyl)thiazol-2-yl] thiosemicarbazide. *European Journal of Pharmaceutical Sciences, 9*, 25–31.

Pannerselvam, P., Nair, R. R., Vijayalakshmi, G., Subramanian, E. H., & Sridhar, S. K. (2005). Synthesis of Schiff bases of 4-(4-aminophenyl)-morpholine as potential antimicrobial agents. *European Journal of Medicinal Chemistry, 40*, 225–229.

Prieto, P., Pineda, M., & Aguilar, M. (1999). Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: Specific application to the determination of vitamin E. *Analytical Biochemistry, 269*, 337–341.

Sarker, S. A., Nahar, L., & Kumarasamy, Y. (2007). Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the in vitro antibacterial screening of phytochemicals. *Methods, 42*, 321–324.

Schonberg, A., & Latif, N. (1954). Furochromones and coumarins. XI. The molluscicidal activity of bergapten, isopimpinillin and xanthotoxin. *Journal of the American Chemical Society, 76*, 6208.

Sharma, S. D., Rajor, H. K., Chopra, S., & Sharma, R. K. (2005). Studies on structure activity relationship of some dihydroxy-4-methylcoumarin antioxidants based on their interaction with Fe(III) and ADP. *Biometals, 18*, 143–154.

Sithambaram Karthikeyan, M., Jagadesh Prasad, D., Poojary, B., & Subramanya Bhat, K. (2006). Synthesis and biological activity of Schiff and Mannich bases bearing 2,4-dichloro-5-fluorophenyl moiety. *Bioorganic and Medicinal Chemistry, 14*, 7482–7489.

Sukdolak, S., Vuković, N., Solujić, S., Manojlović, N., & Krstić, Lj. (2004). Hantzsch reaction of 3-(2-bromoacetyl)-4-hydroxy-chromen-one. *Journal of Heterocyclic Chemistry, 41*, 593–596.

Takao, T., Watanabe, N., Yagi, I., & Sakata, K. (1994). A simple screening method for antioxidants and isolation of several antioxidants produced by marine bacteria from fish and shellfish. *Bioscience, Biotechnology and Biochemistry, 58*, 1780–1783.

Vukovic, N., Sukdolak, S., Solujic, S., & Milosevic, T. (2008). Synthesis and antimicrobial evaluation of some novel 2-aminothiazole derivatives of 4-hydroxy-chromene-2-one. *Archiv der Pharmazie – Chemistry in Life Science, 341*(8), 491–496.