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An Efficient Synthesis and Antioxidant Properties of Novel Imino and Amino Derivatives of 4-Hydroxy Coumarins

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Series of imino and amino derivatives of 4-hydroxy coumarins were synthesized via conventional and microwave promoted procedure 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. All prepared compounds possess good antioxidant activity and among them *p*-nitro-phenyl derivative **6c** with IC_{50} at 25.9 μM possesses radical scavenging activity which is comparable to standard BHT, while the best reducing power was observed in a case of benzyl amino compound **8c** (RP_{50} 255.6 μM). Also, observed data indicated that compounds may serve as inhibitors of lipid peroxidation process.

Key words: 4-Hydroxy coumarin, Imines, Amines, Antioxidant activity

INTRODUCTION

In vivo molecular oxygen is easily converted to reactive free radicals, which contain the superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($HO^{\bullet}$) and 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 et al., 2006). They play an essential role in the control of cell functions. They are intermediate metabolites in several enzymatic reactions, involved in the post-translational protein turnover and play a role in the control of signal transduction. Many components of the vascular system, such as leukocytes, monocytes and endothelial cells are able to release ROS upon the appropriate stimulation. Thus, ROS are associated with incidence of various diseases such as heart diseases, thrombosis, hypertension, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Juliano et al., 1997; Lassegue and Griendling, 2004; McIntosh et al., 1997). Besides oxidative stress, reactive oxygen species is associated with the induction of DNA single- and double-strand breaks and is considered to be the first step in several human degenerative diseases, cancer and aging (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 et al., 2004). Furthermore, blood-brain barrier reduces the permeability and the protective efficacy of most antioxidants (Gilgun-Sherki et al., 2001, 2002).

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 et al., 1971). Many products which contain a coumarin subunit exhibit biological activities such

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as molluscicides, anthelmintic, hypnotic, insecticidal activity (Schonberg and Latif, 1954). Also, the medicinal properties of coumarins include inhibition of platelet aggregation, cytochrome P450 and steroid 5α-reductase (Hoult and 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 et al., 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 $H^{\cdot}$ donors for free radical acceptors, due to electron delocalization across the molecule (Sharma et al., 2005). Also, some simple hydroxylated coumarin derivatives have been reported to inhibit xanthine oxidase (Ferrari et al., 2007).

Prompted by the above-mentioned biological properties of coumarin derivatives, we present here the preparation of some new imino and amino derivatives of 4-hydroxy coumarins and the investigation of their antioxidative properties.

MATERIALS AND METHODS

The microwave assisted reactions were carried out in the MICROSYNTH Microwave Synthesis System (Milestone Inc. 25 Controls Dr. Shelton), the microwaves are generated by magnetron at the frequency of 2450 MHz having an output energy range of 100-500 watts. 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, ν in cm^{-1}), NMR (Varian Gemini 200 spectrometer, ^1H at 200 MHz, ^{13}C at 50 MHz) and GC/MS (Agilent 6890N/5975B) techniques.

Reaction of condensation of 3-acetyl-4-hydroxy-chromene-2-one 1 with amine 2a-9a

Method A-Conventional method: Mixture of 3-acetyl-4-hydroxy-chromene-2-one **1** (0.01 mol), amine **2a-9a** (0.01 mol) and catalytic amount of *p*-toluene sulfonic acid in anhydrous toluene (50 mL) was heated with azeotropic removal of water in the period of 10-12 h. Progress of reaction was monitored by TLC (toluene: acetone = 7:3). At the end of reaction, solvent was removed under reduced pressure. The solid products were filtered, dried, purified via column chromatography (benzene: acetone = 8:2) to give compounds **2b-9b**.

Method B-Microwave method: Catalytic amount of *p*-toluene sulfonic acid was added to 50 mL toluene solution of equimolar amounts (0.01 mol) of 3-acetyl-4-hydroxy-chromene-2-one **1** and amine **2a-9a**. The mixture was heated under microwave for 3 minutes. After cooling, the solvent was removed, and then the obtained solid was filtered and recrystallized from methanol (Table I, Scheme 1).

Reduction of imino derivatives of 4-hydroxy-chromene-2-one 2b-9b to amine 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). Reaction mixture was stirred at room temperature in the period of 4h and monitored periodically by TLC. Upon the 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 sulfate and was filtered. After evaporation of the solvent, the obtained residue was purified via column chromatography (benzene:acetone = 8:2) to give amine **2c-9c**.

Physical and spectroscopic data of the synthesized compounds

4-Hydroxy-3-(1-(phenylimino)ethyl)-2H-chromen-2-one 2b

Anal. calcd. for $C_{17}H_{13}NO_3$ (Mol. Wt. 279.29): C, 73.11; H, 4.69; N, 5.02: found: C, 73.09; H, 4.71; N, 4.97. IR (KBr, cm^{-1}): 3415 (OH), 3073 and 3037 $(=CH)_{ar}$, 2929 and 2853 (CH_3), 1704 (lactone C=O), 1609 (imino C=N), 1592, 1561 and 1480 $(C=C)_{ar}$. ^1H-NMR ($CDCl_3$, 200 MHz): δ 2.72 (s, 3H, CH_3-C=N), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, J = 8.3 Hz, J = 1.1 Hz, C_8-H), 7.6 (dd, J = 7.8 Hz, J = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 6.9-7.21 (m, 5H, phenyl), 16.15 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.9 (CH_3-C=N), 97.4 (C_3), 116.2 (C_{10}), 116.8 (C_8), 119.1 (C_5), 119.8 ($C_{2''}$, $C_{6''}$), 125.9 (C_6), 126.9 ($C_{4''}$), 129.4 (C_7), 131.5 ($C_{3''}$, $C_{5''}$), 138.1 ($C_{1''}$), 152.4 (C_9), 163.4 (C_2), 180.3 ($C_{1'}$), 180.5 (C_4). M.P. 169-171°C.

4-Hydroxy-3-(1-(p-tolylimino)ethyl)-2H-chromen-2-one 3b

Anal. calcd. for $C_{18}H_{15}NO_3$ (Mol. Wt. 293.32): C, 73.71;

H, 5.15; N, 4.78; found: C, 73.72; H, 5.12; N, 4.79. IR (KBr, Cm^{-1}): 3421 (OH), 3073 $(=CH)_{ar}$, 2985, 2922 and 2852 (CH_3), 1709 (lactone C=O), 1611 (imino C=N), 1597, 1569, 1513 and 1483 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.41 (s, 3H, CH_3-$C_{4''}$), 2.69 (s, 3H, CH_3-C=N), 7.09-7.65 (ABq, 4H, *J* = 8.43 Hz, $C_{2'',3'',5'',6''}$-H), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C-7-H), 16.07 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.6 (CH_3-$C_{4''}$), 20.8 (CH_3-C=N), 97.3 (C_3), 116.6 (C_{10}), 116.8 (C_8), 119.3 (C_5), 119.9 ($C_{2''}$, $C_{6''}$), 125.9 (C_6), 129.3 (C_7), 129.9 ($C_{4''}$), 131.7 ($C_{3''}$,$C_{5''}$), 138.4 ($C_{1''}$), 163.5 (C_2), 152.4 (C_9), 180.4 (C_4), 180.5 ($C_{1'}$). M.P.147-149°C.

4-Hydroxy-3-(1-(m-tolylimino)ethyl)-2H-chromen-2-one 4b

Anal. calcd. for $C_{18}H_{15}NO_3$ (Mol. Wt. 293.32): C, 73.71; H, 5.15; N, 4.78: found: C, 73.72; H, 5.14; N, 4.79. IR (KBr, Cm^{-1}): 3417 (OH), 3067 $(=CH)_{ar}$, 2982, 2929 and 2853 (CH_3), 1697 (lactone C=O), 1606 (imino C=N), 1600, 1566 and 1484 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.42 (s, 3H, CH_3-$C_{3''}$), 2.70 (s, 3H, CH_3-C=N), 7.01 (dd, 1H, *J* = 7.58 Hz, *J* = 1.14 Hz, $C_{4''}$-H), 7.04 (dd, 1H, *J* = 8.12 Hz, *J* = 1.14 Hz, $C_{6''}$-H), 7.12 (s, 1H, $C_{2''}$-H), 7.25 (m, 1H, $C_{5''}$-H), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 15.9 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.9 (CH_3-C=N), 21.0 (CH_3-$C_{3''}$), 97.2 (C_3), 116.7 (C_8), 116.9 (C_{10}), 118.9 ($C_{6''}$), 119.3 (C_5), 121.9 ($C_{2''}$), 124.0 ($C_{4''}$), 125.9 (C_6), 129.3 (C_7), 130.7 ($C_{5''}$), 139.1 ($C_{1''}$), 139.1 ($C_{3''}$), 163.6 (C_2), 152.1 (C_9), 180.3 ($C_{1'}$), 180.6 (C_4). M.P. 109-110°C.

4-Hydroxy-3-(1-(o-tolylimino)ethyl)-2H-chromen-2-one 5b

Anal. calcd. for $C_{18}H_{15}NO_3$ (Mol. Wt. 293.32): C, 73.71; H, 5.15; N, 4.78: found: C, 73.75; H, 5.21; N, 4.73. IR (KBr, Cm^{-1}): 3467(OH), 3072.51 $(=CH)_{ar}$, 2935 and 2856 (CH_3), 1712 (lactone C=O), 1611 (imino C=N), 1594, 1563 and 1486 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.30 (s, 3H, CH_3-$C_{1''}$), 2.81 (s, 3H, CH_3-C=N), 7.23 (dd, 1H, *J* = 8.01 Hz, *J* = 1.12 Hz, $C_{6''}$-H), 7.13 (m, 1H, $C_{4''}$-H), 7.28 (dd, 1H, *J* = 7.62 Hz, *J* = 1.11 Hz, $C_{3''}$-H), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.37 (m, 1H, $C_{5''}$-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 15.8 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.2 (CH_3-C=N), 20.9 (CH_3-$C_{2''}$), 97.8 (C_3), 116.1 (C_{10}), 116.8 (C_8), 118.9 ($C_{6''}$), 119.3 (C_5), 122.7 ($C_{2''}$), 124.1 ($C_{4''}$), 125.8 (C_6), 129.1 (C_7), 129.8 ($C_{5''}$), 138.9 ($C_{1''}$), 139.1 ($C_{3''}$), 163.5 (C_2), 152.5 (C_9), 179.6 ($C_{1'}$), 180.4 (C_4). M.P. 138-139°C.

4-Hydroxy-3-(1-(4-nitrophenylimino)ethyl)-2H-chromen-2-one 6b

Anal. calcd. for $C_{17}H_{12}N_2O_5$ (Mol. Wt. 324.29): C, 62.96; H, 3.73; N, 8.64: found: C, 62.97; H, 3.77; N, 8.69. IR (KBr, Cm^{-1}): 3414 (OH), 3081 and 3046 $(=CH)_{ar}$, 2992, 2947 and 2849 (CH_3), 1707 (lactone C=O), 1608 (imino C=N), 1580, 1519 and 1483 $(C=C)_{ar}$, 1557 and 1340 (NO_2). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.76 (s, 3H, CH_3-C=N), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.43 (d, 2H, *J* = 8.9 Hz, $C_{3'',5''}$-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 8.39 (d, 2H, *J* = 8.9 Hz, $C_{2'',6''}$-H), 15.95 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.8 (CH_3-C=N), 97.9 (C_3), 116.4 (C_8), 116.9 (C_{10}), 119.6 (C_5), 123.2 ($C_{2''}$, $C_{6''}$), 125.3 (C_6), 129.1 (C_7), 133.2 ($C_{3''}$, $C_{5''}$), 136.1 ($C_{1''}$), 145.3 ($C_{4''}$), 152.4 (C_9), 163.1 (C_2), 177.5 ($C_{1'}$), 180.7 (C_4). M.P. 212-215°C.

4-Hydroxy-3-(1-(3-nitrophenylimino)ethyl)-2H-chromen-2-one 7b

Anal. calcd. for $C_{17}H_{12}N_2O_5$ (Mol. Wt. 324.29): C, 62.96; H, 3.73; N, 8.64; found: C, 62.96; H, 3.73; N, 8.64. IR (KBr, Cm^{-1}): 3416 (OH), 3089 and 3062 $(=CH)_{ar}$, 2980, 2936 and 2853 (CH_3), 1705 (lactone C=O), 1609 (imino C=N), 1590, 1538 and 1491 $(C=C)_{ar}$, 1562 and 1353 (NO_2). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.61 (s, 3H, CH_3-C=N), 7.26 (dd, 1H, *J* = 8.02 Hz, *J* = 1.12 Hz, $C_{6''}$-H), 7.3 (m, 1H, C_6-H), 7.34 (s, 1H, $C_{2''}$-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.54 (m, 1H, $C_{5''}$-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 8.09 (dd, 1H, *J* = 8.5 Hz, *J* = 1.12 Hz, $C_{4''}$-H), 16.02 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 19.2 (CH_3-C=N), 97.9 (C_3), 116.4 (C_8), 116.5 (C_{10}), 117.8 ($C_{2''}$), 119.4 (C_5), 121.4 ($C_{4''}$), 125.5 (C_6), 127.3 ($C_{6''}$), 129.3 (C_7), 131.5 ($C_{5''}$), 141.9 ($C_{1''}$), 163.5 (C_2), 148.2 ($C_{3''}$), 152.1 (C_9), 177.4 ($C_{1'}$), 180.1 (C_4). M.P. 209-210°C.

3-(1-(Benzylimino)ethyl)-4-hydroxy-2H-chromen-2-one 8b

Anal. calcd. for $C_{18}H_{15}NO_3$ (Mol. Wt. 293.32): C, 73.71; H, 5.15; N, 4.78; found: C, 73.73; H, 5.13; N, 4.73. IR (KBr, Cm^{-1}): 3406 (OH), 3032 and 3012 $(=CH)_{ar}$, 2930 (CH_2), 1698 (lactone C=O), 1612 (imino C=N), 1586, 1572 and 1485 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 2.64 (s, 3H, CH_3-C=N), 3.25 (s, 2H, CH_2-N=C), 7.29 (m, 2H, $C_{3'',5''}$-H), 7.26 (m, 1H, $C_{4''}$-H), 7.39 (dd, 2H, *J* = 7.7 Hz, *J* = 1.1 Hz, $C_{2'',6''}$-H), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.6 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 16.01 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 17.9 (CH_3-C=N), 57.4 (C=N-CH_2), 97.7 (C_3), 116.7 (C_{10}), 116.8 (C_8), 119.3 (C_5), 125.9 (C_6), 126.7 ($C_{4''}$), 127.0 ($C_{2''}$, $C_{6''}$), 128.3 ($C_{3''}$, $C_{5''}$), 129.3 (C_7), 138.7 ($C_{1''}$), 163.5 (C_2), 167.1 ($C_{1'}$),

152.5 (C_9), 180.3 (C_4). M.P. 151-152°C.

5-(1-(4-Hydroxy-2-oxo-2H-chromen-3-yl)ethylideneamino)pentanoic acid 9b

Anal. calcd. for $C_{16}H_{17}NO_5$ (Mol. Wt. 303.11): C, 63.36; H, 5.65; N, 4.62: found: C, 63.27; H, 5.68; N, 4.63. IR (KBr, Cm^{-1}): 3418 (coumarine OH), 3602-2811 (OH form COOH), 2947, 2930 and 2875 (CH_3, CH_2), 1721 (C=O from COOH), 1703 (lactone C=O), 1614 (imino C=N), 1600, 1560 and 1487 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.82 (m, 4H, $C_{2''}$,$_{3''}$-H), 2.46 (t, 2H, J = 7.07 Hz, $C_{4''}$-H), 2.70 (s, 3H, CH_3-C=N), 3.60 (t, 2H, J = 7.1 Hz, $C_{1''}$-H), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, J = 8.3 Hz, J = 1.1 Hz, C_8-H), 7.6 (dd, 1H, J = 7.8 Hz, J = 1.7 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 11.3 (bs, 1H, OH from COOH), 16.11 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 18.9 (CH_3-C=N), 22.9 ($C_{3''}$), 30.4 ($C_{2''}$), 33.5 ($C_{4''}$), 53.9 ($C_{1''}$), 98.1 (C_3), 116.5 (C_8), 116.7 (C_{10}), 119.3 (C_5), 125.4 (C_6), 129.2 (C_7), 152.5 (C_9), 163.4 (C_2), 169.0 ($C_{1'}$), 178.5 ($C_{5''}$), 180.4 (C_4). M.P. 169-171°C.

4-Hydroxy-3-(1-(phenylamino)ethyl)-2H-chromen-2-one 2c

Anal. calcd. for $C_{17}H_{15}NO_3$ (Mol. Wt. 281.31): C, 72.58; H, 5.37; N, 4.98: found: C, 72.64; H, 5.51; N, 5.03. IR (KBr, cm^{-1}): 3429 (OH), 3183 (NH), 3072 $(=CH)_{ar}$, 2970 and 2933 (CH), 1669 (C=O), 1614, 1602, 1567 and 1497 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.34 (d, 3H, J = 6.68 Hz, $C_{1'}$-CH_3), 3.91 (q, J = 6.68 Hz, 1H, $C_{2'}$-CH), 3.96 (bs, 1H, NH), 7.3 (m, 1H, C_6-H), 7.64 (dd, 1H, J = 8.4 Hz, J = 1.1 Hz, C_8-H), 7.84 (dd, J = 7.8 Hz, J = 1.67 Hz, 1H, C_5-H), 7.29 (m, 1H, C_7-H), 6.87-7.23 (m, 5H, phenyl), 17.05 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.3 ($C_{2'}$), 45.4 ($C_{1'}$), 91.8 (C_3), 117.3 (C_8), 117.4 (C_{10}), 117.6 ($C_{2''}$, $C_{6''}$), 121.8 ($C_{4''}$), 123.7 (C_5), 124.1 (C_6), 129.4 ($C_{3''}$, $C_{5''}$), 131.4 (C_7), 145.6 ($C_{1''}$), 152.8 (C_9), 161.3 (C_2), 163.8 (C_4). Yield 89%. M.P. 189-190°C.

4-Hydroxy-3-(1-(p-tolylamino)ethyl)-2H-chromen-2-one 3c

Anal. calcd. for $C_{18}H_{17}NO_3$ (Mol. Wt. 295.33): C, 73.20; H, 5.80; N, 4.74; found: C, 73.12; H, 5.67; N, 4.77. IR (KBr, cm^{-1}): 3436(OH), 3191 (NH), 3071(=CH)$_{ar}$, 2989 and 2966 (CH), 1671 (C=O), 1612, 1601, 1566 and 1499 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.33 (d, 3H, J = 6.69 Hz, $C_{1'}$-CH_3), 2.26 (s, 3H, $C_{4''}$-CH_3), 3.93 (q, 1H, J = 6.69 Hz, $C_{2'}$-CH), 4.09 (bs, 1H, NH), 6.73-7.11 (ABq, 4H, J = 8.43 Hz, $C_{2''}$,$_{3''}$,$_{5''}$,$_{6''}$-H), 7.29 (m, 1H, C_6-H), 7.63 (dd, 1H, J = 8.3 Hz, J = 1.11 Hz, C_8-H), 7.84 (dd, 1H, J = 7.81 Hz, J = 1.69 Hz, C_5-H), 7.29 (m, 1H, C_7-H), 17.09 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.8 ($C_{2'}$), 21.2 (CH_3-$C_{4''}$), 45.3 ($C_{1'}$), 91.9 (C_3), 113.6 ($C_{2''}$, $C_{6''}$), 117.3 (C_{10}), 117.4 (C_8), 123.7 (C_5), 124.1 (C_6), 129.4 ($C_{4''}$), 129.8 ($C_{3''}$, $C_{5''}$), 131.3 (C_7), 145.2 ($C_{1''}$), 152.9 (C_9), 161.4 (C_2), 163.8 (C_4). Yield 86%. M.P.167-169°C.

4-Hydroxy-3-(1-(m-tolylamino)ethyl)-2H-chromen-2-one 4c

Anal. calcd. for $C_{18}H_{17}NO_3$ (Mol. Wt. 295.33): C, 73.20; H, 5.80; N, 4.74; found: C, 73.18; H, 5.66; N, 4.78. IR (KBr, cm^{-1}): 3455 (OH), 3179 (NH), 3069 $(=CH)_{ar}$, 2970, 2932 and 2870(CH), 1670 (C=O), 1615, 1601, 1567, 1497 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.33 (d, 3H, J = 6.69 Hz, $C_{1'}$-CH_3), 2.21 (s, 3H, $C_{3''}$-CH_3), 3.87 (q, 1H, J = 6.69 Hz, $C_{2'}$-CH), 3.94 (bs, 1H, NH), 7.01 (dd, 1H, J = 7.58 Hz, J = 1.12 Hz, $C_{4''}$-H), 6.77 (dd, 1H, J = 7.99 Hz, J = 1.12 Hz, $C_{6''}$-H), 6.71 (s, 1H, $C_{2''}$-H), 7.19 (m, 1H, $C_{5''}$-H), 7.3 (m, 1H, C_6-H), 7.63 (dd, 1H, J = 8.3 Hz, J = 1.11 Hz, C_8-H), 7.86 (dd, 1H, J = 7.8 Hz, J = 1.7 Hz, C_5-H), 7.28 (m, 1H, C_7-H), 17.09 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.8 ($C_{2'}$), 21.1 (CH_3-$C_{3''}$), 45.2 ($C_{1'}$), 91.4 (C_3), 110.4 ($C_{6''}$), 113.9 ($C_{2''}$), 117.2 (C_{10}), 117.3 (C_8), 118.4 ($C_{4''}$), 123.6 (C_5), 124.3 (C_6), 129.1 ($C_{5''}$), 131.2 (C_7), 138.7 ($C_{3''}$), 146.2 ($C_{1''}$), 152.7 (C_9), 161.5 (C_2), 163.7 (C_4). Yield 91%. M.P. 119-121°C.

4-Hydroxy-3-(1-(o-tolylamino)ethyl)-2H-chromen-2-one 5c

Anal. calcd. for $C_{18}H_{17}NO_3$ (Mol. Wt. 295.33): C, 73.20; H, 5.80; N, 4.74; found: C, 73.28; H, 5.76; N, 4.79. IR (KBr, cm^{-1}): 3433 (OH), 3193 (NH), 3066 $(=CH)_{ar}$, 2985, 2970, 2932 (CH), 1670 (C=O), 1615, 1602, 1568, 1499 $(C=C)_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.31 (d, 3H, J = 6.67 Hz, $C_{1'}$-CH_3), 2.19 (s, 3H, $C_{3''}$-CH_3), 3.82 (q, 1H, J = 6.67 Hz, $C_{2'}$-CH), 3.87 (bs, 1H, NH), 6.91 (dd, 1H, J = 8 Hz, J = 1.11 Hz, $C_{6''}$-H), 6.99 (m, 1H, $C_{4''}$-H), 7.21 (dd, 1H, J = 7.62 Hz, J = 1.11 Hz, $C_{3''}$-H), 7.29 (m, 1H, C_6-H), 7.65 (dd, 1H, J = 8.29 Hz, J = 1.1 Hz, C_8-H), 7.11 (m, 1H, $C_{5''}$-H), 7.86 (dd, 1H, J = 7.8 Hz, J = 1.7 Hz, C_5-H), 7.27 (m, 1H, C_7-H), 17.08 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 17.7 (CH_3-$C_{2''}$), 19.5 ($C_{2'}$), 44.1 ($C_{1'}$), 91.9 (C_3), 111.3 ($C_{6''}$), 117.4 (C_8), 117.5 (C_{10}), 121.1 ($C_{4''}$), 122.5 ($C_{2''}$), 123.3 (C_5), 124.2 (C_6), 126.1 ($C_{5''}$), 128.6 ($C_{3''}$), 131.4 (C_7), 144.5 ($C_{1''}$), 152.7 (C_9), 161.2 (C_2), 163.9 (C_4). Yield 82%. M.P. 147-149°C.

4-Hydroxy-3-(1-(4-nitrophenylamino)ethyl)-2H-chromen-2-one 6c

Anal. calcd. for $C_{17}H_{14}N_2O_5$ (Mol. Wt. 326.30): C, 62.57; H, 4.32; N, 8.59: found: C, 62.67; H, 3.97; N, 8.61. IR (KBr, Cm^{-1}): 3433 (OH), 3184 (NH), 3076 $(=CH)_{ar}$, 2971 and 2931 (CH), 1673 (C=O), 1613, 1601, 1567 and 1497 $(C=C)_{ar}$, 1528 and 1344 (NO_2). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.33 (d, 3H, J = 6.68 Hz, $C_{1'}$-CH_3),

3.78 (q, 1H, *J* = 6.68 Hz, $C_{2'}$-CH), 4.14 (bs, 1H, NH), 7.3 (m, 1H, C_6-H), 7.4 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 6.81-8.13 (ABq, 4H, *J* = 8.9 Hz, $C_{2'',3'',5'',6''}$-H), 7.6 (dd, 1H, *J* = 7.79 Hz, *J* = 1.71 Hz, C_5-H), 7.7 (m, 1H, C_7-H), 17.1 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.1 (C_2), 43.5 ($C_{1'}$), 91.5 (C_3), 113.4 ($C_{2''}$, $C_{6''}$), 117.1 (C_8), 117.6 (C_{10}), 123.8 (C_5), 124.1 (C_6), 127.2 ($C_{3''}$, $C_{5''}$), 131.3 (C_7), 136.3 ($C_{4''}$), 151.1 ($C_{1''}$), 152.3 (C_9), 161.4 (C_2), 163.9 (C_4). Yield 74%. M.P. 222-223°C.

4-Hydroxy-3-(1-(3-nitrophenylamino)ethyl)-2H-chromen-2-one 7c

Anal. calcd. for $C_{17}H_{14}N_2O_5$ (Mol. Wt. 326.30): C, 62.57; H, 4.32; N, 8.59: found: C, 62.62; H, 3.99; N, 8.60. IR (KBr, cm^{-1}): 3431 (OH), 3181 (NH), 3069 (=CH)$_{ar}$, 2970 and 2931 (CH), 1671 (C=O), 1615, 1601, 1569 and 1499 (C=C)$_{ar}$, 1524 and 1357 (NO2). ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.35 (d, 3H, *J* = 6.67 Hz, $C_{1'}$-CH_3), 3.75 (q, 1H, *J* = 6.67 Hz, $C_{2'}$-CH), 7.16 (dd, 1H, *J* = 8 Hz, *J* = 1.1 Hz, $C_{6''}$-H), 7.5 (m, 1H, C_6-H), 7.44 (s, 1H, $C_{2''}$-H), 7.65 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.44 (m, 1H, $C_{5''}$-H), 7.86 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 3.91 (bs, 1H, NH), 7.27 (m, 1H, C_7-H), 7.61 (dd, 1H, *J* = 8.45 Hz, *J* = 1.1 Hz, $C_{4''}$-H), 17.07 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 19.8 ($C_{2'}$), 44.9 ($C_{1'}$), 91.5 (C_3), 108.4 ($C_{2''}$), 113.3 ($C_{4''}$), 117.3 (C_8), 117.6 (C_{10}), 118.3 ($C_{6''}$), 123.4 (C_5), 124.3 (C_6), 130.1 ($C_{5''}$), 131.5 (C_7), 146.7 ($C_{3''}$), 148.7 ($C_{1''}$), 152.5 (C_9), 161.1 (C_2), 163.4 (C_4). Yield 76%. M.P. 215-217°C.

3-(1-(Benzylamino)ethyl)-4-hydroxy-2H-chromen-2-one 8c

Anal. calcd. for $C_{18}H_{17}NO_3$ (Mol. Wt. 295.33): C, 73.20; H, 5.80; N, 4.74; found: C, 73.21; H, 5.76; N, 4.73. IR (KBr, cm^{-1}): 3445 (OH), 3190 (NH), 3069 (=CH)$_{ar}$, 2978 and 2966 (CH), 1679 and 1664 (C=O), 1621, 1603, 1567, 1496 (C=C)$_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.29 (d, 3H, *J* = 6.7 Hz, $C_{1'}$-CH_3), 3.45 (q, 1H, *J* = 6.7 Hz, $C_{2'}$-CH), 3.67-3.81 (ABq, 2H, *J* = 12 Hz, N-CH_2-phenyl), 3.96 (bs, 1H, NH), 7.22 (m, 2H, $C_{3'',5''}$-H), 7.24 (m, 1H, $C_{4''}$-H), 7.26 (dd, 2H, *J* = 7.75 Hz, *J* = 1.12 Hz, $C_{2'',6''}$-H), 7.53 (m, 1H, C_6-H), 7.64 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.85 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.28 (m, 1H, C_7-H), 16.55 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.2 ($C_{2'}$), 42.4 ($C_{1'}$), 52.2 (C-N-CH_2), 91.8 (C_3), 117.3 (C_{10}), 117.5 (C_8), 123.8 (C_5), 124.2 (C_6), 126.6 ($C_{4''}$), 128.1 ($C_{2''}$, $C_{6''}$), 128.4 ($C_{3''}$, $C_{5''}$), 131.4 (C_7), 140.1 ($C_{1''}$), 152.5 (C_9), 161.6 (C_2), 163.7 (C_4). Yield 91%. M.P. 171-173°C.

5-(1-(4-Hydroxy-2-oxo-2H-chromen-3-yl)ethylamino) pentanoic acid 9c

Anal. calcd. for $C_{16}H_{17}NO_5$ (Mol. Wt. 305.33): C, 62.94; H, 6.27; N, 4.59: found: C, 63.01; H, 6.26; N, 4.61. IR (KBr, cm^{-1}): 3611-2590 (OH from COOH group), 3433 (OH from coumarine moiety), 3189 (NH), 3067 (=CH)$_{ar}$, 2993, 2987 and 2974 (CH), 1710 (C=O from COOH group), 1672 (C=O), 1615, 1600 and 1497 (C=C)$_{ar}$. ^{1}H-NMR ($CDCl_3$, 200 MHz): δ 1.25 (d, 3H, *J* = 6.65 Hz, $C_{1'}$-CH_3), 1.82 (m, 4H, $C_{2'',3''}$-H), 2.46 (t, 2H, *J* = 7.08 Hz, $C_{4''}$-H), 3.44 (q, 1H, *J* = 6.65 Hz, $C_{2'}$-CH), 3.60 (t, 2H, *J* = 7.05 Hz, $C_{1''}$-H), 4.02 (bs, 1H, NH), 7.54 (m, 1H, C_6-H), 7.64 (dd, 1H, *J* = 8.3 Hz, *J* = 1.1 Hz, C_8-H), 7.56 (dd, 1H, *J* = 7.8 Hz, *J* = 1.7 Hz, C_5-H), 7.27 (m, 1H, C_7-H), 11.8 (bs, 1H, OH from COOH), 17.19 (bs, 1H, C_4-OH). ^{13}C-NMR ($CDCl_3$, 50 MHz): δ 20.1 ($C_{2'}$), 21.9 ($C_{3''}$), 29.9 ($C_{2''}$), 34.7 ($C_{4''}$), 42.3 ($C_{1'}$), 54.5 ($C_{1''}$), 91.8 (C_3), 117.1 (C_8), 117.4 (C_{10}), 124.0 (C_6), 123.8 (C_5), 131.4 (C_7), 152.3 (C_9), 161.4 (C_2), 163.9 (C_4), 178.1 ($C_{5''}$). Yield 68%. M.P. 231-233°C.

DPPH assay

The method used by Takao et al. (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 the stock solutions (4 mM) of the compounds (**2b**-**9b**; **2c**-**9c**) in methanol to obtain concentrations of 2-0.0039 mM. Diluted solutions (2 mL each) were mixed with DPPH (2 mL) and allowed to stand for 30 min 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 as μM. Ascorbic acid and BHT were used as reference standards. 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 (%) = $[(A_0 - A_t)/A_0] \times 100$, where A_t is the absorbance value of the tested sample and A_0 is the absorbance value of blank sample, in particular time. Percent inhibition after 30 min 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.

Reducing power assay

The reducing power test is based on reduction of ferric to ferrous by the potent antioxidant. In 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 methanol plant extract was measured according to the method described by Oyaizu, 1986. A sample of 300 μL of various dilutions (from 2 to 0.0039 mM) was mixed with 300 μL of phosphate buffer (0.2 mol/L, 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% trichloracetic acid was 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% trichloracetic 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% trichloracetic acid. Ascorbic acid and BHT were used as positive control. The reducing power of samples was calculated by the following formula: RP (%) = $(A_B - A_A) \times 100$; RP: reducing power; A_B: absorption of controlling sample (100%); A_A: absorption of tested sample. Percent inhibition 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. The lower RP_{50} value indicates greater reducing power ability.

Inhibition (%) of lipid peroxidation in linoleic acid emulsion

The total antioxidant activity of synthesized compounds was carried out by use of a linoleic acid system (Masude et al., 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 homogenized. A 0.5 mL ethanol solution of different concentration of imines and amines (1000, 500, 250 and 125 μM) was mixed with 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%), ammonium thiocyanate sample solution (100 μL, 30%) and ferrous chloride (100 μL, 0.02 M in 3.5% HCl). After 3 min, the peroxide values were determined by reading the absorbance at 500 nm. Ascorbic acid, BHT and α-tocopherol were used as reference compounds. Control was performed with linoleic acid but without the tested compounds. All data reported are the average of triplicate analyses. Percent inhibition of lipid peroxide generation was calculated using formula % 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.

Determination of total antioxidant capacity

The antioxidant activity of the tested compounds was evaluated by the phosphomolybdenum method according to the procedure of Prieto et al., 1999, with some modification. The assay is based on the reduction of Mo (VI)-Mo (V) by the test compounds and subsequent formation of a green phosphate/Mo (V) complex at acid pH. 100 μL of the solution of tested compounds (250-15.6 μg/mL) was combined with 1 mL of reagent solution (0.6 M sulfuric 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 using spectrophotometer against blank after cooling to room temperature. Ethanol (100 μL) in the place of 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.

RESULTS AND DISCUSSION

Synthesis of imino and amino derivatives 4-hydroxy coumarins

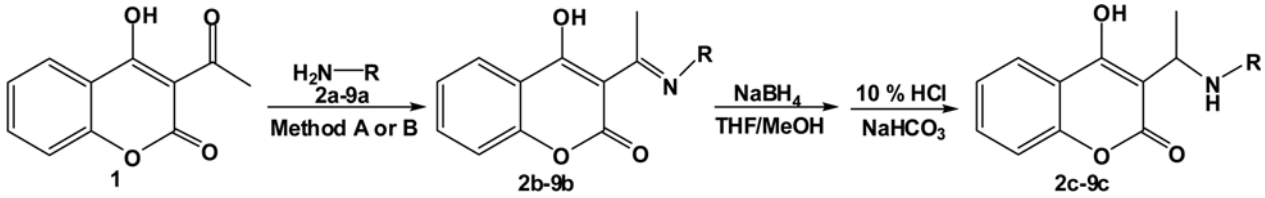
Preparation of imino derivatives **2b**-**9b** by conventional condensation method with azeotropic removal of water was characterized with lower yields of desired compounds (no more than 84%) followed by time consuming purifications by column chromatography. Thus, we reported improvements in the synthesis of these compounds from the aspect of microwave promoted reaction. Due to homogenous heating under microwave exposures, nus reactions were particularly eliminated, which 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 recrystallization from appropriate solvents. As presented in Table I, the yield has been significantly increased using reaction under microwave heating conditions particularly in case of imino derivatives with *p*-nitro-phenyl (92%), *m*-nitrophenyl (97%) and pentanoic acid substituent (87%). Their further transformation to the corresponding amines (**2c**-**9c**) was performed by reduction with sodium borohydride in a mixture of solvents methanol:tetrahydrofurane (8:2).

The IR spectral data of the compounds **2b**-**9b** con-

Table I. Reaction conditions, substituents and yields of obtained compounds **2b**-**8b**

No	R	Conventional method A		Microwave assisted method B			
		Time of Synthesis[a] (Hr.)	Yield[b] (%)	Pw (W)	Irradiation time (min.)	T (°C)	Yield[b] (%)
2b	C_6H_5-	9	75	500	3	109	95
3b	*p*-tolyl	9.5	73	500	3	109	97
4b	*m*-tolyl	9	84	500	3	109	94
5b	*o*-tolyl	9.5	73	500	3	109	94
6b	*p*-NO_2-C_6H_4-	13.5	51	500	3	109	92
7b	*m*-NO_2-C_6H_4-	12.5	62	500	3	109	97
8b	benzyl	10	75	500	3	109	97
9b	-$CH_2(CH_2)_2CH_2COOH$	10	42	500	3	109	87

[a] Reflux in heating equipments, [b] % of yield calculated from practical and theoretical yields.



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

firmed the presence of –C=N– group (at 1614-1606). In ^{1}H NMR spectra, observed singlets at 2.81-2.61 ppm revealed the presence of methyl protons from imino groups.

In general, the IR spectral data of the amines **2c**-**9c** revealed bands at 3455-3429 cm^{-1} (coumarine –OH), 3193-3179 cm^{-1} (–NH group) and 1681-1664 cm^{-1} (lactone –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 4.14-3.87 ppm were assigned to NH groups, while singlets at low field (17.19-16.55 ppm) confirmed presence of C-4-OH protons.

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 radical 2,2-diphenyl-1-picrylhydrazyl (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 II, the imines **2b**-**9b** with IC_{50} values in the range of 304.1 to 446.9 μM showed lower radical scavenging activities in comparison to standards - butylated hydroxytoluene 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 μM 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 bond 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*-nitro-phenyl derivative with IC_{50} at 25.9 μM possesses highest radical scavenging activity, which is comparable to standard BHT. On the other hand, better antiradical activity was observed in case of phenyl derivative **2c** and *m*-nitro-phenyl derivative **7c** (IC_{50} values were 34.1 and 40.9 μM, respectively) in comparison to standard compound, ascorbic acid (IC_{50} 42.4 μM). In a series of compounds with tolyl fragment, compound **5c** as ortho 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 influence of methyl substituent as electron donor group at positions $C_{2''}$ and $C_{6''}$ of phenyl ring on better 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

Table II. Antioxidant activity of synthesized imino and amino derivatives of 4-hydroxy coumarins. IC_{50}, the concentration of coumarin required to inhibit radical formation by 50%; RP_{50}, the concentration of coumarin required to reduce ferric to ferrous ions by 50%; Inhibition (%) of lipid peroxidation in a linoleic acid emulsion.

Comp.	DPPH assay [a]IC_{50} (μM)		Reducing power assay [b]RP_{50} (μM)	[c]Inhibition (%) of lipid peroxidation in a linoleic acid emulsion			
	30 min	60 min		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} and [b]RP_{50} values were determined by linear regression analysis. Results are mean values ± S.D. from at least three experiments. [c]Data of percentage are reported as means ± S.D. of three measurements.

Table III. The total antioxidant capacity expressed as equivalents of ascorbic acid (μg/mL)

Comp.	[a]Equivalents of ascorbic acid (μg/mL)				
	250 μg/mL	125 μg/mL	62.50 μg/mL	31.25 μg/mL	15.60 μg/mL
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 ± S.D. of three measurements.

of $C_6H_5CH_2$ and C_4COOH substituents bonded to NH group. Since the corresponding IC_{50} values for all synthesized compounds after 60 min were lower than after 30 min, DPPH antiradical scavenging activity was also time dependent.

Evaluation for reducing power

Reducing power of prepared coumarine 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+} can be monitored by measurement of the formation of Perl's Prussian blue at 700 nm. Table II 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-pentanatoic 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 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 showed the following order: ascorbic acid > *m*-tolyl > phenyl > *p*-tolyl > *o*-tolyl > BHT. Obviously, presence of methyl group at phenyl ring and type of substitution affect decrease in reducing power. Also, from the point of substitution, similar results were gained in cases of nitro-phenyl derivatives **6b** as para and **7b** as metha 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 *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).

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

In the present study, the antioxidant activities of imines and amines of 4-hydroxy-chromene-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 oxidize 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 II, the antioxidant activity of synthesized 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 the concentration of 1000 μM showed better antioxidant activity than the standard 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 the ascorbic acid, while at the concentration of 1000 and 500 μM, compounds **3b**, **4b**, **5b**, **6b** and **7b** showed high level inhibitor properties to lipid peroxidation in contrast to standard α-tocopherol. Other two tested imines **8b** as benzyl imino and **9b** as n-pentatnoic acid derivatives showed lower activities than the standard 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*-nitro-phenyl derivative **6c** at the concentration of 1000 μM showed 67.8% inhibition, an antioxidant activity a few percent lower than that of 1000 μM α-tocopherol (74.8%). Inhibitor activities of other amines are similar. All above mentioned confirmed

that the reduction of imino group lead to decrease of antioxidant activity, as well as, that in a series of tested amines only benzopyrane moiety was responsible for the inhibition of lipid peroxidation.

Total antioxidant capacity

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 III 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-hydroxy coumarine, 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 cases of tolyl derivatives **3b**, **4b** and **5b** lowest values were 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*-nitro-phenyl 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.

The synthesised compounds scavenged the DPPH radical, reduced Fe^{3+} cations and inhibited lipid peroxidation in a concentration and time-dependent manner.

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