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Acetophenone benzoylhydrazones as antioxidant agents: Synthesis, *in vitro* evaluation and structure-activity relationship studies

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ABSTRACT

Acetophenone and its analogues are naturally-occurring compounds found in many foods and plants. In this study, a series of acetophenone benzoylhydrazones **5a-o** were designed and synthesized as new potential antioxidant agents. Designed molecules contain hydrazone and phenolic hydroxyl moieties which possibly contribute to antioxidant activity. The antioxidant properties of compounds **5a-o** in terms of reducing ability and radical-scavenging activity were assessed by using FRAP and DPPH tests, respectively. While the unsubstituted compound **5a** had the superior capacity in the FRAP assay, the 2,4-dihydroxyacetophenone analogue **5g** was the most potent radical scavenger in the DPPH method. The antioxidant potential of representative compounds **5a** and **5g** was further confirmed by TEAC and ORAC assays. Cell viability assays revealed that while the promising compounds **5a** and **5g** had no significant toxicity against HepG2 and NIH3T3 cells, they potently protected HepG2 cells against H_2O_2-induced oxidative damage at low concentrations. Furthermore, spectroscopic studies with different biometals demonstrated that **5g** was able to interact with Cu^{2+} to form a 1:1 complex.

1. Introduction

Oxidative stress has been described as the unbalance of reactive oxygen species' (ROS) generation and the organism's capacity to counteract their action (Persson, Popescu, & Cedazo-Minguez, 2014). High concentrations of free radicals and ROS, including hydrogen peroxide, superoxide, hydroxyl radical and peroxynitrite may cause damage to lipids, proteins and DNA in biological systems (Sugamura & Keaney, 2011). It has been estimated that oxidative stress contributes to the pathogenesis and pathophysiology of over 100 diseases, such as cardiovascular and inflammatory diseases, cataracts, carcinogenesis, Parkinson's, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Sahebkar, Panahi, Yaribeygi, & Javadi, 2018; Varghese, Patel, & Yadav, 2018). Furthermore, oxidative stress plays critical roles in the impairment of physiological functions and in the biology of aging (Franco & Vargas, 2018; Maulik, McFadden, Otani, Thirunavukkarasu, & Parinandi, 2013).

Antioxidants are currently considered to be a potential treatment for oxidative stress-related diseases and are widely used as ingredients of functional foods to prevent chronic diseases, such as cancer, atherosclerosis and heart disease (Lee, Woo, Ahn, & Je, 2014; Varadharaj et al., 2017). Also, antioxidants are beneficial as supplements in foods for maintaining redox balance and avoiding oxidative damage to protect against lipid oxidation and off-flavour development (Lönn, Dennis, & Stocker, 2012). In particular, natural antioxidants, such as vitamin C, α-tocopherol, ubiquinol, and polyphenols, are widely used to scavenge free radicals and to combat the harmful effects of ROS (Choi, Lee, Hong, & Lee, 2012). Recently, many efforts have been focussed on designing antioxidants containing phenolic hydroxyl groups (Bandgar et al., 2013). The potential antioxidant effect of polyphenols and phenolic compounds is related to their reducing activity, hydrogen-donating, and singlet oxygen-quenching properties (Prior, Wu, & Schaich, 2005).

Acetophenone and its substituted analogues are naturally occurring compounds found in many foods, such as apples, apricots, bananas, beef, cheese, and cauliflowers (Müller-Schwarze, & Houlihan, 1991), as well as in many plants, such as *Camellia sinensis* (Dong et al., 2012). Also, acetophenone has been approved by FDA for use as a flavouring agent in non-alcoholic beverages, ice creams, candies, baked goods,

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gelatins, puddings, and chewing gums (Hazardous Substances Data Bank, http://toxnet.nlm.nih.gov/).

The quantum mechanical calculation studies of hydroxyacetophenone derivatives by Bentes, Borges, Monteiro, de Macedo, and Alves (2011) postulated that the presence of a carbonyl group in phenolic derivatives may stabilize the radical formed during oxidation, extending the conjugation via resonance effects (Bentes et al., 2011). Furthermore, Rezk, Haenen, van der Vijgh, and Bast (2002) have reported the potential antioxidant activity of 2,6-dihydroxyacetophenone that is possibly explained by stabilization of the radical that is formed after hydrogen abstraction (Rezk et al., 2002).

On the other hand, several hydrazones were reported as potent antioxidants due to their free radical-scavenging activity (Mohammed Khan et al., 2012). Structurally, the hydrazones are characterized by an azomethine group, which has a critical rule in antioxidant activity (Belkheiri et al., 2010).

Based on these findings and in continuation of our works on antioxidant agents (Emami, Hosseinimehr, Shahrbandi, Enayati, & Esmaeeli, 2012; Foroumadi et al., 2007; Khoobi et al., 2011, 2011), we report here the synthesis and antioxidant properties of acetophenone benzoylhydrazones containing a phenolic group and their structure-activity relationships (Fig. 1). As depicted in Fig. 1, designed molecules contain a phenolic hydroxyl group and hydrazone moiety which possibly contribute to radical-scavenging activity and the antioxidant property.

2. Materials and methods

2.1. Chemical reagents and instruments

The required starting materials and reagents were obtained from Sigma-Aldrich and Merck. The intermediate compounds **2a,b** and **3a,b** were prepared according to the reported methods (Bhole, & Bhusari, 2011; Masunari, & Tavares, 2007; Rando, Avery, Tekwani, Khan, & Ferreira, 2008). 4-Methoxy-2-hydroxyacetophenone (**4e**) was synthesized from 2,4-dihydroxyacetophenone as described previously (Safavi et al., 2010). All reactions were monitored by thin-layer chromatography (TLC) using pre-coated silica gel plates (Kieselgel 60 F_{254}). The spots on TLC were visualized and detected by UV lamp (254 nm). Melting points were determined in glass capillary tubes on a Stuart Scientific apparatus and are uncorrected. IR spectroscopy was carried out on a FT-IR Perkin Elmer spectrometer (KBr disks). All NMR spectra were recorded on a Bruker ultrashield avance III spectrometer, working at 400 MHz and chemical shifts are expressed as ppm in respect to the internal standard tetramethylsilane (TMS). Elemental analyses were carried out on a CHN-O-rapid elemental analyzer (GmbH-Germany) for C, H and N, and the results are within ± 0.4% of the theoretical values.

2.2. General procedure for the synthesis of compounds *5a-o*

To a solution of benzohydrazides **3a** or **3b** (1 mmol) and acetophenone derivative **4a-h** (1 mmol) in methanol (5 ml), a few drops of glacial acetic acid were added and the reaction mixture was refluxed at 70 °C for 6–8 h. After consumption of starting materials (monitoring with TLC), the reaction mixture was cooled to room temperature and left in the refrigerator overnight. The precipitated crystals were separated by filtration and washed with cooled methanol to give pure compounds **5a-o**.

*N′-(1-Phenylethylidene)benzohydrazide (**5a**)*. Yield: 42%; mp: 147–148 °C; IR (ν_{max}, cm^{-1}): 3467, 3054, 1611, 1541, 1488, 1316, 1284, 1133, 1026, 799, 758, 693, 564; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.38 (s, 3H, CH_3), 7.38–7.48 (m, 3H, H-3′, H-4′ and H-5′), 7.50–7.62 (m, 3H, H-3, H-4 and H-5), 7.78–7.95 (m, 4H, H-2′, H-6′, H-2 and H-6), 10.79 (br s, 1H, NH). Anal. Calcd for $C_{15}H_{14}N_2O$: C, 75.61; H, 5.92; N, 11.76. Found: C, 75.49; H, 5.89; N, 11.73.

*4-Hydroxy-N′-(1-phenylethylidene)benzohydrazide (**5b**)*. Yield: 81%; mp: 246–247 °C; IR (ν_{max}, cm^{-1}): 3500, 3150, 1670, 1583, 1436, 1323, 1222, 1174, 903, 846, 773, 699, 504; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.36 (s, 3H, CH_3), 6.86 (d, 1H, J = 8.4 Hz, H-3 and H-5), 7.38–7.46 (m, 3H, H-3′, H-4′ and H-5′), 7.79 (d, 2H, J = 8.8 Hz, H-2 and H-6), 7.80–7.88 (m, 2H, H-2′ and H-6′), 10.09 (s, 1H, 4-OH), 10.52 (s, 1H, NH). Anal. Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.97; H, 5.53; N, 10.98.

*N′-(1-(2-Hydroxyphenyl)ethylidene)benzohydrazide (**5c**)*. Yield: 44%; mp: 183–184 °C; IR (ν_{max}, cm^{-1}): 3211, 1637, 1612, 1577, 1485, 1304, 1285, 1025, 931, 834, 744, 712, 690; ^{1}H NMR (400 MHz, DMSO-d_6) δ:

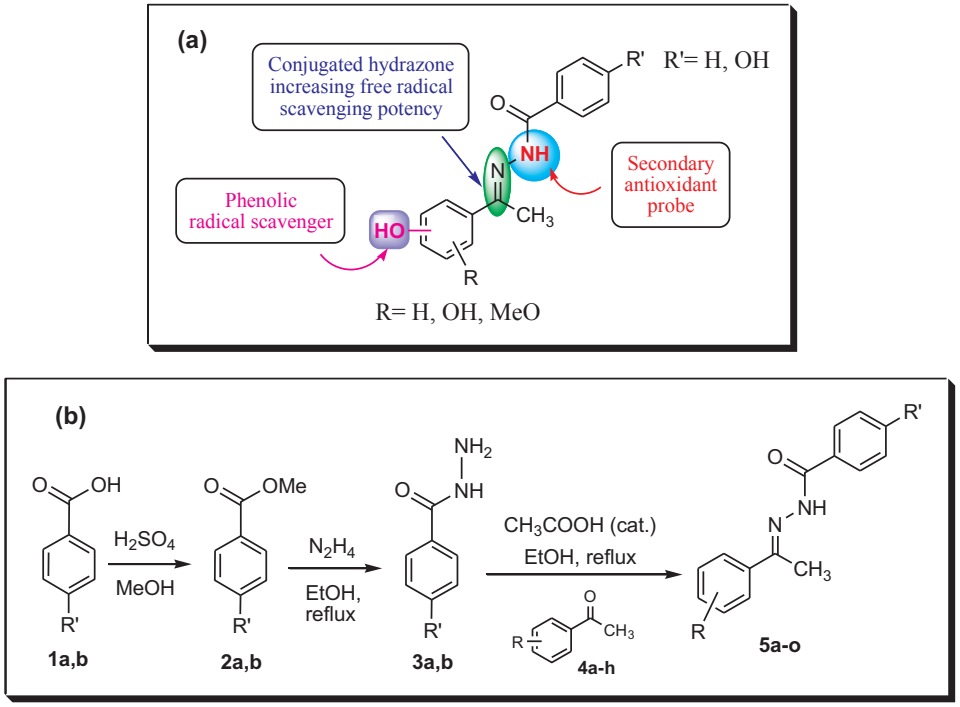


Fig. 1. (a) Design of acetophenone benzoylhydrazones as antioxidant agents; (b) Synthesis of compounds **5a-o**.

2.50 (s, 3H, CH_3), 6.88–6.94 (m, 2H, H-3′ and H-5′), 7.32 (td, 1H, J = 7.6 and 1.2 Hz, H-4′), 7.55 (dd, 2H, J = 8.0 and 7.2 Hz, H-3 and H-5), 7.60–7.67 (m, 2H, H-4 and H-6′), 7.95 (d, 2H, J = 7.2 Hz, H-2 and H-6), 11.36 (br s, 1H, NH), 13.38 (s, 1H, 2′–OH). Anal. Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.79; H, 5.54; N, 11.20.

4-Hydroxy-N′-(1-(2-hydroxyphenyl)ethylidene)benzohydrazide ***(5d)***. Yield: 57%; mp: 258–259 °C; IR (ν_{max}, cm^{-1}): 3189, 3054, 1611, 1541, 1488, 1305, 1133, 1104, 932, 892, 799, 758, 564; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.47 (s, 3H, CH_3), 6.85–6.93 (m, 4H, H-3, H-5, H-3′ and H-5′), 7.30 (td, 1H, J = 8.4 and 1.6 Hz, H-4′), 7.63 (dd, 1H, J = 8.4 and 1.6 Hz, H-6′), 7.84 (d, 2H, J = 8.4 Hz, H-2 and H-6), 10.21 (br s, 1H, 4-OH), 11.08 (br s, 1H, NH), 13.44 (br s, 1H, 2′–OH). Anal. Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.80; H, 5.23; N, 10.34.

N′-(1-(4-Hydroxyphenyl)ethylidene)benzohydrazide ***(5e)***. Yield: 48%; mp: 241–242 °C; IR (ν_{max}, cm^{-1}): 3300, 1626, 1569, 1486, 1372, 1268, 1152, 1027, 979, 838, 708, 694, 666; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.30 (s, 3H, CH_3), 6.82 (d, 2H, J = 8.0 Hz, H-3′ and H-5′), 7.45–7.60 (m, 3H, H-3, H-4 and H-5), 7.72 (d, 2H, J = 7.6 Hz, H-2′ and H-6′), 7.88 (d, 2H, J = 6.4 Hz, H-2 and H-6), 9.83 (s, 1H, 4′–OH), 10.67 (s, 1H, NH). Anal. Calcd for $C_{15}H_{14}N_2O_2$: C, 70.85; H, 5.55; N, 11.02. Found: C, 70.74; H, 5.70; N, 10.88.

4-Hydroxy-N′-(1-(4-hydroxyphenyl)ethylidene)benzohydrazide ***(5f)***. Yield: 75%; mp: 290–291 °C; IR (ν_{max}, cm^{-1}): 3300, 1625, 1534, 1502, 1440, 1286, 1248, 1172, 845, 764, 621, 538; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.28 (s, 3H, CH_3), 6.80 (d, 2H, J = 8.4 Hz, H-3′ and H-5′), 6.84 (d, 2H, J = 8.4 Hz, H-3 and H-5), 7.63–7.73 (m, 2H, H-2′ and H-6′), 7.77 (d, 2H, J = 8.4 Hz, H-2 and H-6), 9.78 (s, 1H, 4′–OH), 10.06 (s, 1H, 4-OH), 10.39 (s, 1H, NH). Anal. Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.83; H, 5.20; N, 10.51.

N′-(1-(2,4-Dihydroxyphenyl)ethylidene)benzohydrazide ***(5g)***. Yield: 90%; mp: 221–222 °C; IR (ν_{max}, cm^{-1}): 3491, 3216, 1606, 1509, 1485, 1304, 1254, 1100, 991, 848, 796, 713, 651; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.42 (s, 3H, CH_3), 6.29 (d, 1H, J = 2.4 Hz, H-3′), 6.35 (dd, 1H, J = 8.6 and 2.4 Hz, H-5′), 7.46 (d, 1H, J = 8.8 Hz, H-6′), 7.54 (t, 2H, J = 8.0 Hz, H-3 and H-5), 7.61 (t, 1H, J = 7.4 Hz, H-4), 7.92 (d, 2H, J = 7.2 Hz, H-2 and H-6), 9.91 (s, 1H, 4′–OH), 11.18 (s, 1H, NH), 13.57 (br s, 1H, 2′–OH). Anal. Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.87; H, 5.21; N, 10.22.

4-Hydroxy-N′-(1-(2,4-dihydroxyphenyl)ethylidene)benzohydrazide ***(5h)***. Yield: 48%; mp: 292–293 °C; IR (ν_{max}, cm^{-1}): 3447, 1608, 1502, 1456, 1266, 1146, 1109, 990, 844, 789, 605, 527; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.39 (s, 3H, CH_3), 6.27 (d, 1H, J = 2.0 Hz, H-3′), 6.33 (dd, 1H, J = 8.8 and 2.4 Hz, H-5′), 6.87 (d, 2H, J = 8.4 Hz, H-3 and H-5), 7.43 (d, 1H, J = 8.8 Hz, H-6′), 7.81 (d, 2H, J = 8.4 Hz, H-2 and H-6), 9.86 (br s, 1H, 4′–OH), 10.13 (br s, 1H, 4-OH), 10.90 (br s, 1H, NH), 13.61 (s, 1H, 2′–OH). Anal. Calcd for $C_{15}H_{14}N_2O_4$: C, 62.93; H, 4.93; N, 9.79. Found: C, 62.68; H, 5.11; N, 9.87.

N′-(1-(2-Hydroxy-4-methoxyphenyl)ethylidene)benzohydrazide ***(5i)***. Yield: 25%; mp: 216–217 °C; IR (ν_{max}, cm^{-1}): 3400, 1650, 1617, 1602, 1508, 1265, 1205, 1133, 828, 715, 678; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.45 (s, 3H, CH_3), 3.78 (s, 3H, OCH_3), 6.45–6.52 (m, 2H, H-3′ and H-5′), 7.50–7.58 (m, 3H, H-3, H-5 and H-6′), 7.62 (t, 1H, J = 7.2 Hz, H-4), 7.93 (d, 2H, J = 7.2 Hz, H-2 and H-6), 11.25 (s, 1H, NH), 13.68 (s, 1H, 2′–OH). Anal. Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.83; H, 5.68; N, 9.71.

4-Hydroxy-N′-(1-(2-hydroxy-4-methoxyphenyl)ethylidene)benzohydrazide ***(5j)***. Yield: 89%; mp: 271–272 °C; IR (ν_{max}, cm^{-1}): 3323, 1623, 1605, 1499, 1290, 1164, 1110, 1027, 847, 786, 652, 635, 559; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.42 (s, 3H, CH_3), 3.77 (s, 3H, OCH_3), 6.43–6.50 (m, 2H, H-3′ and H-5′), 6.88 (d, 2H, J = 8.8 Hz, H-3 and H-5), 7.54 (d, 1H, J = 8.8 Hz, H-6′), 7.82 (d, 2H, J = 8.4 Hz, H-2 and H-6), 10.18 (s, 1H, 4-OH), 10.98 (s, 1H, NH), 13.74 (s, 1H, 2′–OH). Anal. Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 63.81; H, 5.21; N, 9.35.

N′-(1-(2,5-Dihydroxyphenyl)ethylidene)benzohydrazide ***(5k)***. Yield: 44%; mp: 210–211 °C; IR (ν_{max}, cm^{-1}): 3500, 1655, 1641, 1538, 1488, 1319, 1289, 1204, 1172, 931, 821, 786, 710, 614; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.42 (s, 3H, CH_3), 6.71–6.79 (m, 2H, H-3′ and H-4′), 6.98 (d, 1H, J = 2.0 Hz, H-6′), 7.55 (t, 2H, J = 7.2 Hz, H-3 and H-5), 7.63 (t, 1H, J = 7.2 Hz, H-4), 7.93 (d, 2H, J = 7.2 Hz, H-2 and H-6), 8.95 (s, 1H, 5′–OH), 11.30 (s, 1H, NH), 12.60 (s, 1H, 2′–OH). Anal. Calcd for $C_{15}H_{14}N_2O_3$: C, 66.66; H, 5.22; N, 10.36. Found: C, 66.49; H, 5.30; N, 10.18.

N′-(1-(2-Hydroxy-5-methoxyphenyl)ethylidene)benzohydrazide ***(5l)***. Yield: 35%; mp: 151–152 °C; IR (ν_{max}, cm^{-1}): 3466, 3233, 1601, 1577, 1510, 1488, 1340, 1219, 1147, 1040, 921, 814, 715, 690; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.49 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 6.86 (d, 1H, J = 8.8 Hz, H-3′), 6.95 (dd, 1H, J = 8.8 and 2.8 Hz, H-4′), 7.14 (d, 1H, J = 2.8 Hz, H-6′), 7.55 (t, 2H, J = 7.4 Hz, H-3 and H-5), 7.63 (t, 1H, J = 7.4 Hz, H-4), 7.95 (d, 2H, J = 7.2 Hz, H-2 and H-6), 11.34 (s, 1H, NH), 12.83 (s, 1H, 2′–OH). Anal. Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.80; H, 5.66; N, 9.91.

4-Hydroxy-N′-(1-(2-hydroxy-5-methoxyphenyl)ethylidene)benzohydrazide ***(5m)***. Yield: 75%; mp: 260–261 °C; IR (ν_{max}, cm^{-1}): 3367, 2985, 2831, 1670, 1609, 1507, 1439, 1374, 1254, 1042, 921, 847, 784, 619; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.47 (s, 3H, CH_3), 3.75 (s, 3H, OCH_3), 6.84 (d, 1H, J = 8.8 Hz, H-3′), 6.89 (d, 2H, J = 8.4 Hz, H-3 and H-5), 6.93 (dd, 1H, J = 8.8 and 2.8 Hz, H-4′), 7.12 (d, 1H, J = 2.8 Hz, H-6′), 7.84 (d, 2H, J = 8.4 Hz, H-2 and H-6), 10.21 (br s, 1H, 4-OH), 11.07 (br s, 1H, NH), 12.89 (br s, 1H, 2′–OH). Anal. Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 64.21; H, 5.59; N, 9.35.

N′-(1-(4-Hydroxy-3-methoxyphenyl)ethylidene)benzohydrazide ***(5n)***. Yield: 30%; mp: 150–151 °C; IR (ν_{max}, cm^{-1}): 3400, 1637, 1509, 1423, 1311, 1274, 1226, 1097, 1026, 890, 713, 635; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.31 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 6.82 (d, 1H, J = 8.0 Hz, H-5′), 7.29 (d, 1H, J = 7.2 Hz, H-6′), 7.43–7.61 (m, 4H, H-3, H-4, H-5 and H-2′), 7.88 (d, 2H, J = 6.8 Hz, H-2 and H-6), 9.45 (br s, 1H, 4′–OH), 10.70 (br s, 1H, NH). Anal. Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.51; H, 5.48; N, 10.16.

4-Hydroxy-N′-(1-(4-hydroxy-3-methoxyphenyl)ethylidene)benzohydrazide ***(5o)***. Yield: 38%; mp: 207–208 °C; IR (ν_{max}, cm^{-1}): 3400, 1639, 1609, 1501, 1453, 1313, 1279, 1176, 1029, 850, 618; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.29 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 6.81 (d, 1H, J = 8.4 Hz, H-5′), 6.85 (d, 1H, J = 8.4 Hz, H-3 and H-5), 7.18–7.32 (m, 1H, H-6′), 7.38–7.49 (m, 1H, H-2′), 7.78 (d, 2H, J = 8.8 Hz, H-2 and H-6), 9.40 (s, 1H, 4′–OH), 10.06 (s, 1H, 4-OH), 10.43 (s, 1H, NH). Anal. Calcd for $C_{16}H_{16}N_2O_4$: C, 63.99; H, 5.37; N, 9.33. Found: C, 64.01; H, 5.29; N, 9.36.

2.3. DPPH free radical-scavenging assay

The DPPH radical-scavenging activity of compounds **5a-o** was determined according to the reported method (Nur Alam, Bristi, & Rafiquzzaman, 2013). Briefly, the test compounds at various concentrations (30, 60, 100, and 150 µl) were added to 3 ml of DPPH solution (0.1 mM in ethanol) and the reaction mixture was shaken vigorously. After incubation at room temperature for 10 min, the absorbance of this solution was determined at 517 nm after 10, 30, and 60 min, using a spectrophotometer. The percent of scavenging activity was obtained from the following equation:

$$\text{Radical scavenging}\,(\%) = [OD_{(DPPH)} - OD_{(DPPH+sample)}]/OD_{(DPPH)} \times 100$$

The radical-scavenging activity was expressed as IC_{50} value obtained from the regression analysis.

2.4. FRAP assay

The total antioxidant capacity of compounds **5a-o** was measured by using the FRAP assay according to the method of Benzie and Strain with some modifications (Benzie & Strain, 1999). This method measures the

ability of the test compound to reduce Fe^{+3}-TPTZ (2,4,6-tripyridyl-*s*-triazine) to a ferrous form (Fe^{+2}) that absorbs light at 593 nm. The FRAP solution, prepared freshly, contained 25 ml of acetate buffer (300 mM, pH 3.6) plus 2.5 ml of TPTZ solution (10 mM) in 40 mM HCl and 2.5 ml of ferric chloride ($FeCl_3{\cdot}6H_2O$, 20 mM). This solution was used as blank. The sample was incubated at 37 °C throughout the monitoring period. Absorbancies of the blank and samples were measured after 10 min at 593 nm, and the results were compared with the standard curve provided using different concentrations of $FeSO_4$. The final FRAP value of each sample was the mean value of three replications. The linearity of the relationship between Fe^{+2} concentration and absorbance was very good within this concentration range ($r^2 = 0.997$). Quercetin was used as standard antioxidant in this method.

2.5. Trolox equivalent antioxidant capacity (TEAC) assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS) radical-scavenging activity of selected compounds (**5a**, **5g**, **5k** and **5o**) was determined, based on trolox equivalent antioxidant capacity (TEAC). This assessment was according to the method of Re et al. (1999). Briefly, ABTS powder (54.2 mg) was dissolved in 10 ml of phosphate buffer (5 mM, pH 7.0) and mixed with 1 g of MnO_2 and incubated at room temperature within 15 h for generation of $ABTS^{\cdot+}$ radicals. The prepared solution was filtered and the filtrate was diluted with phosphate buffer until the absorbance of solution equals 0.70 ± 0.01 at 734 nm. Different concentrations of desired compounds (**5a**, **5g**, **5k** or **5o**) were mixed with 4 ml of ABTS solution and incubated for 10 min at room temperature. The decrease of absorbance was monitored at 734 nm after 10 min. The percentage of radical inhibition activity (%) was calculated according to the following equation:

$$\text{Scavenging of } A_{734nm}(\%) = \frac{1 - A_f}{A_o} \times 100$$

where A_o is the absorbance of the untreated radical and A_f is the absorbance measured 10 min after addition of the samples.

Different concentrations of trolox (0.05–1 mM) were used to obtain a standard curve. The ABTS radical-scavenging activity of samples was calculated and plotted against the concentration of trolox and results were expressed as TEAC (trolox equivalent antioxidant capacity).

2.6. Oxygen radical absorbance capacity (ORAC) assay

ORAC assay was performed following the method of Kim, Jang, & Kim (2007) with some modifications. First, solutions of 0.1 μM fluorescein and 90 mM AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride), both in 75 mM phosphate buffer (pH 7.4), were prepared. Then, 1.5 ml of fluorescein solution and 250 μl of sample (**5a**, **5g**, **5k** or **5o**) were mixed and incubated for 15 min at 37 °C in the dark. After the addition of 250 μl of AAPH, the decrease in the fluorescence intensity per min (λ_{ex} = 485 nm and λ_{em} = 535 nm) was monitored for 60 min in a spectrofluorometer (Jasco, FP-750 spectrofluorometer, Kyoto, Japan). Several concentrations of trolox (2–16 μM) were used to find a calibration curve. The area under the curve (AUC) was calculated for each; the difference in the area under the curves between each sample and the blank were calculated and results were expressed as TEAC value. The results were expressed as micromolar equivalents of trolox for each microgramme of desired compounds (μM TE/μg).

2.7. Metal binding studies

The UV absorption spectra of selected compound **5g** and its related acetophenone **2,4-DHA** (2,4-dihydroxyacetophenone), in the absence or presence of sulfate salts of Cu^{2+}, Zn^{2+}, Fe^{2+} and Al^{3+} cations were recorded with wavelength ranging from 200 to 400 nm after incubating in methanol at room temperature for 30 min, using a JASCO V-630 spectrophotometer (Tokyo, Japan). The final volume of reaction mixture was 5 ml, and the final concentrations of tested compound and metals were 20 μM. The stoichiometry of the **5g**–Cu^{2+} complex was determined using the molar ratio method (Meyer & Ayres, 1957), as follows: the final concentration of **5g** was 20 μM, and the final concentration of Cu^{2+} (ranging from 2 to 32 μM), and the absorption spectra of the solutions were recorded after 30 min. The UV spectra were recorded and treated by numerical subtraction of $CuSO_4$ and **5g** at corresponding concentrations, plotted versus the mole fraction of tested compound. The absorbance of the absorption peak at 375 nm was plotted against the molar concentration of Cu^{2+}. The breakpoint revealed the stoichiometry of the **5g**–Cu^{2+} complex.

2.8. Cell lines and cytotoxicity assay

The normal fibroblast cell line NIH3T3 (mouse embryo fibroblast) and Human Hepatoma HepG2 cell line were purchased from Pasture institute, Tehran, Iran. The cells were seeded at 10^4 cells/well in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 unit/ml of penicillin-G, 100 μg/ml of streptomycin in each well of a 96-well plate. The cells were cultured in an incubator containing 5% CO_2 for 24 h in 37 °C.

The selected compounds **5a** and **5g** were dissolved in dimethyl sulfoxide (DMSO) as a 100 μM stock solution and then diluted to the different concentrations in the RPMI 1640 medium. The final concentration of DMSO was 0.4% in medium culture. After 24 h, the cells were treated with various concentrations of compounds (200, 100, 50, 10 and 1 μg/ml). After 24 h of incubation, 10 μl of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (5 mg/ml) was added to each well, and incubated for 4 h. Then the culture medium from each well was carefully removed, and DMSO (200 μl) was added into each well, gently shaken for 15 min and the absorbance was determined with an ELISA reader (Bioteck) at 570 nm (Eghtedari et al., 2017). Two independent experiments in triplicate were done for determination of cell viability inhibition for each compound. The IC_{50} values were calculated from concentration–response curves by using the Prism 6, GraphPad Software and expressed as means ± SD.

2.9. Viability assay against H_2O_2-induced cell death in HepG2 cells

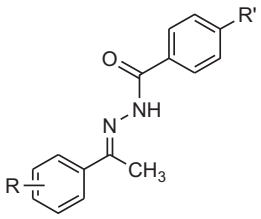
The HepG2 cells were seeded at 10^4 cells/well in RPMI 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 unit/ml of penicillin-G and 100 μg/ml of streptomycin in each well of a 96-well plate. The cells were cultured in an incubator containing 5% CO_2 for 24 h at 37 °C. After 24 h, the cells were treated with various concentrations (10 and 1 μg/ml) of selected compounds **5a** and **5g**. Curcumin was used as positive control, at the same concentrations. Then, two hours later H_2O_2 (200 μM) was added to the culture medium for induction of cell death in HepG2 cells. The cell viability was measured after 24 h of incubation by using the MTT assay as described previously (Jiang et al., 2014).

3. Results and discussion

3.1. Synthesis

The synthetic route to acetophenone benzoylhydrazones **5a-o** is illustrated in Fig. 1. The benzoic methyl esters **2a,b** were prepared from appropriate benzoic acid derivatives **1a,b** in refluxing methanol and in the presence of H_2SO_4 as catalyst. The reaction of esters **2a,b** with hydrazine hydrate in ethanol afforded hydrazides **3a,b** (Bhole & Bhusari, 2011; Masunari & Tavares, 2007; Rando et al., 2008). Finally, condensation of hydrazides **3a,b** with different acetophenones **4a-h** in methanol under reflux produced the desired acetophenone benzoylhydrazones **5a-o**.

Table 1
Antioxidant activity of compounds **5a-o**, and cytotoxicity of selected compounds against normal fibroblast cell line (NIH3T3) and human hepatoma cell line (HepG2).



Compound	R	R'	FRAP value (mmol Fe^{2+}/l)	DPPH (IC_{50}, μg/ml)	NIH-3T3 Cytotoxicity (IC_{50}, μg/ml)	HepG2 Cytotoxicity (IC_{50}, μg/ml)
5a	H	H	1.26 ± 0.11	160 ± 8.9	283.8 ± 2.1	136.0 ± 2.8
5b	H	OH	0.54 ± 0.01	> 200	–	–
5c	2-OH	H	0.51 ± 0.02	> 200	–	–
5d	2-OH	OH	0.38 ± 0.06	55 ± 1.1	–	–
5e	4-OH	H	0.62 ± 0.5	133 ± 3.8	–	–
5f	4-OH	OH	0.67 ± 0.01	131 ± 1.0	–	–
5g	2,4-$(OH)_2$	H	0.01 ± 0.00	19 ± 0.03	189 ± 2.6	121 ± 1.4
5h	2,4-$(OH)_2$	OH	0.06 ± 0.05	136 ± 11.2	–	–
5i	2-OH-4-OMe	H	0.19 ± 0.10	194 ± 17.1	–	–
5j	2-OH-4-OMe	OH	0.24 ± 0.11	> 200	–	–
5k	2,5-$(OH)_2$	H	0.75 ± 0.09	84 ± 14.3	–	–
5l	2-OH-5-OMe	H	0.33 ± 0.04	145 ± 4.0	–	–
5m	2-OH-5-OMe	OH	0.18 ± 0.06	97 ± 1.2	–	–
5n	3-OMe-4-OH	H	0.68 ± 0.11	158 ± 21.3	–	–
5o	3-OMe-4-OH	OH	0.66 ± 0.07	87 ± 6.2	–	–
Quercetin			1.04 ± 0.01	2.7 ± 0.1	–	–
2,4-DHA[a]			–	1321 ± 74.2	–	–
Etoposide					118 ± 0.1	9.0 ± 0.1

[a] 2,4-DHA: 2,4-Dihydroxyacetophenone.

3.2. Antioxidant activity

3.2.1. General

The antioxidant properties of compounds **5a-o** were determined *in vitro* by evaluating their radical-scavenging activity and reducing ability. There are different methods for evaluation of the antioxidant potential of compounds (Nur Alam et al., 2013). In this study, two distinct methods which are commonly used for antioxidant evaluation were utilized: the FRAP (ferric reducing antioxidant power) assay and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging test.

3.2.2. Ferric-reducing antioxidant power (FRAP)

The FRAP assay is based on the reduction of the ferric 2,4,6-tripyridyl-*s*-triazine complex to the coloured ferrous form at low pH and monitoring the change in absorption at 593 nm. Accordingly, The FRAP value of test compound is obtained by comparing the absorbance changes in test reaction mixtures with those containing known concentrations of Fe^{2+} ions (Benzie & Strain, 1999; Schlesier, Harwat, Böhm, & Bitsch, 2002). The obtained data from FRAP assay of compounds **5a-o** are expressed as mmol Fe^{2+}/l in Table 1.

Based on the FRAP values listed in Table 1, the unsubstituted compound **5a** showed the highest reducing power, being more effective than quercetin as a polyphenolic antioxidant. Furthermore, the 2,5-dihydroxyacetophenone derivative **5k** with FRAP value of 0.75 mmol Fe^{2+}/l exhibited remarkable capacity for reducing of Fe^{3+} ions. Also, compounds **5e**, **5f**, **5n** and **5o,** bearing a 4-hydroxyacetophenone framework, displayed FRAP values of 0.62–0.68 mM, close to that of compound **5k**. The FRAP value of 4-hydroxybenzoyl analogue **5b** was significantly less than that of parent unsubstituted compound **5a**. Thus, the introduction of the 4-hydroxy group on the benzoylhydrazone diminishes the reducing power of the molecule. Surprisingly, the presence of oxygenated functional groups (OH or MeO) on the acetophenone part of the molecules could not improve the reducing capacity of the compounds **5c-o** compared to their congeners **5a** and **5b**.

3.2.3. Radical-scavenging activity on DPPH

The DPPH radical-scavenging activities of compounds **5a-o,** along with 2,4-dihydroxyacetophenone, are presented in Table 1. Most of acetophenone benzoylhydrazones, with the exception of **5b**, **5c** and **5j,** showed significant radical-scavenging activity. The 2,4-dihydroxyacetophenone derivative **5g** with IC_{50} value of 19 μg/ml was the most potent compound. Besides **5g**, compound **5d** (R = 2-OH and R' = OH) showed respectable activity in the DPPH test (IC_{50} = 55 μg/ml).

The comparison of 4-hydroxybenzoyl and 2-hydroxyacetophenone derivatives (**5b** and **5c**, respectively) with the parent acetophenone benzoylhydrazone (**5a**) revealed that the hydroxyl substituent decreased the radical scavenging activity. Surprisingly, the simultaneous substitution of R = 2-OH and R' = OH resulted in compound **5d** with increased activity with respect to **5b** and **5c**. Also, sole substitution of 2- or 4-OH on the acetophenone system had no positive effect on the potency but concurrent substitution of 2,4-dihydroxy dramatically improved the radical-scavenging activity. The 2,4-dihydroxy derivative **5g** was 4-fold more potent than its 2,5-dihydroxy analogue **5k**; thus the 2,4-dihydroxy pattern was better than the 2,5-dihydroxy one. The effect of a 4-hydroxy substituent on the benzoyl moiety was dependent on the substitution pattern in the acetophenone system.

The kinetics behaviour of selected compound **5k** in the DPPH free radical-scavenging process was determined in comparison to quercetin. To follow the kinetics behaviours, the methanolic solution of the compounds were added to the reaction medium and the absorbance was measured until it became constant. The obtained results are given in Fig. S1 (Supplementary material). Indeed, immediately after adding compound **5k** to the reaction medium, the absorbance at 517 nm dropped, due to the decrease of DPPH radicals in the medium. Similar to quercetin, compound **5k** reacted within a very short time, and a steady state was reached almost after 150 s.

3.2.4. Antioxidant capacity determined by TEAC and ORAC assays

As reviewed by Prior et al. (2005), among the available methods for the measurement of antioxidant capacity, three assays have been

Table 2
Antioxidant capacity of representative compounds evaluated by the TEAC and ORAC methods.[a]

Compound	TEAC values μM/μg	ORAC values μM TE/μg
5a	28 ± 0.6	33 ± 2.0
5g	25 ± 0.5	31 ± 1.1
5k	26 ± 0.5	18 ± 0.4
5o	27 ± 1.3	22 ± 1.4

[a] The average values (± SD) of TEAC are expressed as antioxidant capacity equivalent to micromolar of trolox per microgramme of compounds (TEAC μM/μg). The average values (± SD) of ORAC are expressed as micromolar trolox equivalents per microgramme of test compounds (μM TE/μg).

mostly considered for standardization: oxygen radical absorbance capacity (ORAC), trolox equivalent antioxidant capacity (TEAC) and Folin-Ciocalteu methods. While ORAC represents a hydrogen transfer reaction mechanism, the TEAC and Folin-Ciocalteu methods are electron transfer-based assays. In this study, two out of these methods were taken for the evaluation of antioxidant capacity. Therefore, the representative compounds **5a**, **5g**, **5k** and **5o** were examined for their antioxidant capacity by means of $ABTS^{\bullet+}$ cation radical (TEAC) and ORAC assays (Table 2). As can be seen, the antioxidant capacity obtained for compounds **5a** and **5g** by the ORAC assay was higher than the value obtained by TEAC, while compounds **5k** and **5o** showed higher capacity in the TEAC assay. Compound **5a,** with TEAC value of 28 ± 0.6 μM/μg and ORAC value of 33 ± 2.0 μM TE/μg, was the best one among the tested compounds.

3.3. Metal chelating properties of selected compound 5g

The ability of promising compound **5g** to chelate biometals such as Zn(II), Fe(II), Cu(II) and Al(III) was studied by UV–visible spectrometry and compared with its corresponding acetophenone **2,4-DHA**. The results are shown in Fig. 2. New optical bands were observed in the UV–vis spectra of compound **5g** upon the addition of $CuSO_4$, suggesting that **5g** was able to interact with Cu^{2+} to form a complex. It could be seen that the spectrum of **5g** was slightly changed upon addition of $FeSO_4$, $ZnSO_4$ and $Al_2(SO_4)_3$ (Fig. 2). Moreover, no significant shift or enhancement in absorption band occurred after addition of Zn(II), Fe (II), Cu(II) and Al(III) to **2,4-DHA**. The chelating ability could be attributed to the imine and 2-hydroxyl groups in compound **5g**.

Spectrophotometric titration was used for the Cu^{2+} binding. A series of UV − vis spectra of compound **5g** titrated by Cu^{2+} was recorded. In the absence of Cu^{2+}, the UV − vis spectrum of compound **5g** showed an absorption maximum at 327 nm. When Cu^{2+} was added, a new band at 375 nm appeared which was associated with the copper complex. The presence of an isosbestic point revealed the formation of a unique **5g**–Cu^{2+} complex. Therefore, the binding stoichiometry of compound **5g** with $CuSO_4$ was determined by studying the absorption changes at 375 nm. The molar ratio method was performed to determine the stoichiometry of the **5g**–Cu^{2+} complex, by preparing the methanol solutions of **5g** with ascending amounts of $CuSO_4$. The UV spectra were used to obtain the absorbance of the **5g**–Cu^{2+} complex and different concentrations of $CuSO_4$ at 375 nm. The results showed that absorbance increased linearly initially and then plateaued (Fig. 3). The points for the straight lines to intersect were determined to be at a mole proportion of 1.0, revealing a 1:1 stoichiometry for the **5g**–Cu^{2+} complex.

3.4. Cytotoxicity of promising compounds 5a and 5g

In order to check the safety profile of promising antioxidants, the effect of compounds **5a** and **5g** on the viability of normal cells (NIH3T3) and Human Hepatoma cells (HepG2) was investigated by

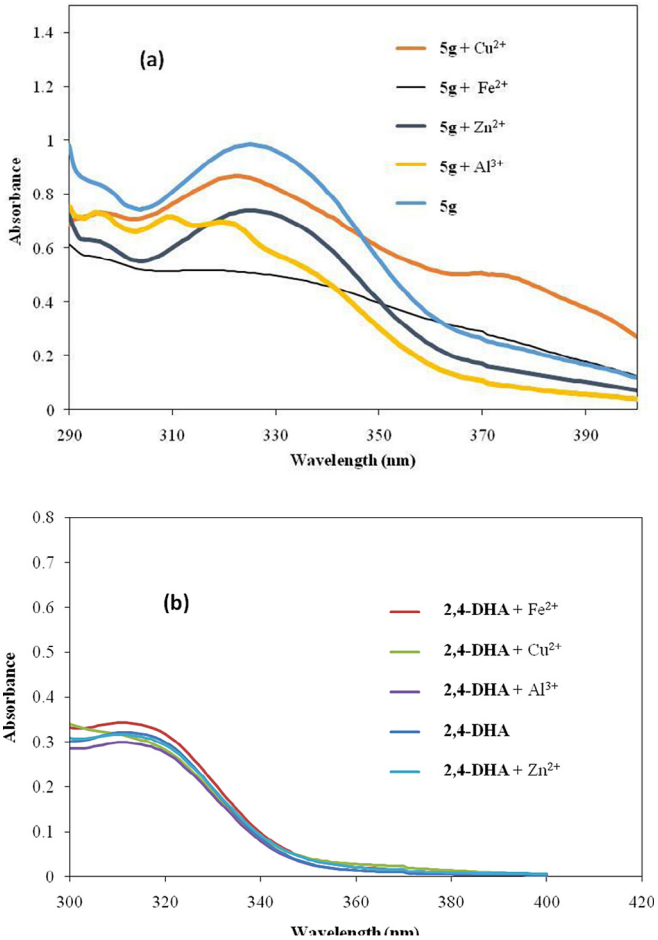


Fig. 2. The UV spectra of (a) compound **5g** and (b) **2,4-DHA** (20 μM) alone and in the presence of $FeSO_4$, $ZnSO_4$, $Al_2(SO_4)_3$ or $CuSO_4$ in methanol.

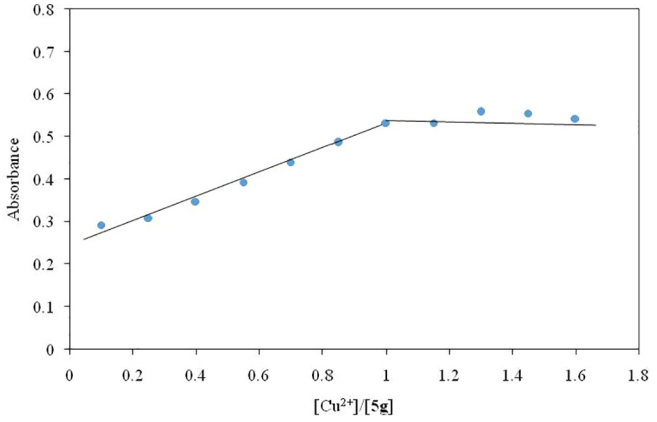


Fig. 3. Determination of the stoichiometry of **5g** (20 μM)-Cu^{2+} complex by molar ratio method.

MTT assay. The IC_{50} values of tested compounds are listed in Table 1. Neither of the compounds showed any significant activity against HepG2 or NIH3T3 cells (IC_{50} values > 120 μg/ml). These results revealed that the prototype compounds can display significant antioxidant activity at low concentrations without inducing cytotoxicity.

3.5. Protection against H_2O_2-induced cell death in HepG2 cells

HepG2 cells with a high degree of differentiation have been widely used as a cellular model to investigate protective activity of

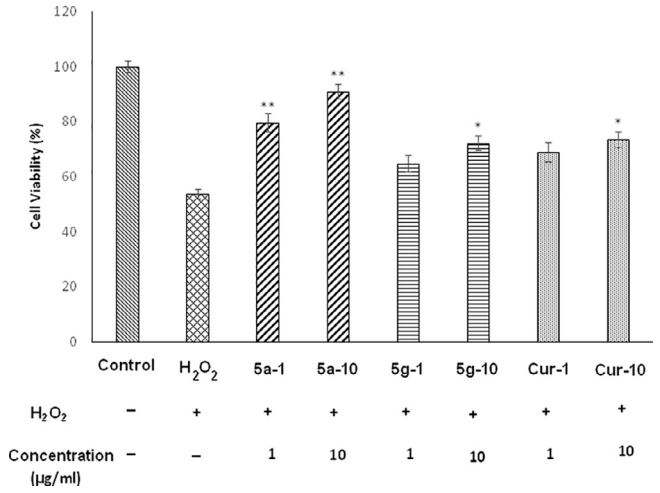


Fig. 4. Cytoprotective activity of compounds **5a** and **5g**, in comparison to curcumin (Cur) at the concentrations of 1 and 10 μg/ml against H_2O_2-induced cell death in HepG2 cells. Data are expressed as means ± SD (n = 3), **: $P < 0.01$, *: $P < 0.05$ all vs. control group.

antioxidants against oxidative injury of the living cell by H_2O_2 (Deferme et al., 2013; Dou et al., 2013; Song et al., 2010; Wolfe, & Liu, 2007). HepG2 cells reserve many biological characteristics of hepatocytes and retain the activity of many phase I, phase II, and antioxidant enzymes. Thus this cell line is a good tool for studying the cytoprotective effects of compounds (Kim, Choi, Ham, Jeong, & Lee, 2013). Here, we evaluated the protective effect of selected compounds **5a** and **5g** on H_2O_2-induced oxidative injury in HepG2 cells in comparison to curcumin, a naturally occurring cytoprotective agent.

The cells were treated with test compounds at the concentrations of 1 and 10 μg/ml and then exposed to H_2O_2. The cell viability was measured in comparison to untreated cells by using the MTT assay and the obtained results are depicted in Fig. 4. It should be noted that compounds **5a** and **5g** had no cytotoxic effect on HepG2 cells at the 1 and 10 μg/ml concentrations. As seen in Fig. 4, H_2O_2 significantly reduced the HepG2 cell viability compared with the negative control (without H_2O_2). The pretreatment of HepG2 cells with the test compounds at the concentration of 10μg/ml protected hepatocytes against cell death and significantly increased cell viability. Notably, the protecting effect of compound **5a** at the concentration of 1 μg/ml was much higher than that of curcumin. Furthermore, the protective effect of compound **5g** was comparable to curcumin at each concentration.

4. Conclusion

We have designed and synthesized a number of polyoxygenated acetophenone benzoylhydrazones **5a-o** as new potential antioxidant agents. The *in vitro* antioxidant properties of compounds **5a-o** in terms of reducing ability and radical scavenging activity were assessed by using FRAP and DPPH tests, respectively. While the unsubstituted compound **5a** had a superior capacity in the FRAP assay, the 2,4-dihydroxyacetophenone analogue **5g** was the most potent radical scavenger in the DPPH method. Furthermore, cytotoxicity assay showed that compounds **5a** and **5g** had no toxicity against HepG2 and NIH3T3 cell lines. The promising antioxidant compounds **5a** and **5g** significantly protect HepG2 cells against H_2O_2-induced oxidative damage at low concentrations. Furthermore, spectroscopic studies with different cations revealed that compound **5g** was able to interact with Cu^{2+} to form a complex. The obtained data from 2,4-dihydroxyacetophenone-derived benzoylhydrazone **5g** and its parent actophenone compound indicated that the introduction of a hydrazone moiety offers potent antioxidant potency and metal binding ability to the acetophenone scaffold. The SAR study revealed that the pattern of hydroxy and methoxy substituents on the acetophenone benzoylhydrazone framework can modulate the antioxidant properties of the prototype compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.083.

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