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Synthesis, in vitro antioxidant activity, and physicochemical properties of novel 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives

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ABSTRACT

In this study, eight new 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**4**) compounds were synthesized by the reactions of 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3**) with 4-(4-methylbenzoxy)benzaldehyde (**1**). The eight compounds were characterized using IR, ^{1}H NMR, ^{13}C NMR, and UV spectral data. In addition, the synthesized compounds were analyzed for their potential in vitro antioxidant activities, including reducing power, free radical scavenging and metal chelating activity. These antioxidant activities were compared to those from standard antioxidants, such as EDTA, BHA, BHT and α-tocopherol. Moreover, compounds **4** were titrated potentiometrically with tetrabutylammonium hydroxide in four non-aqueous solvents (isopropyl alcohol, *tert*-butyl alcohol, acetone and *N*,*N*-dimethylformamide). Thus, the half-neutralization potential values and the corresponding pK_a values were determined in all cases. In addition, the lipophilicity of the synthesized compounds was investigated using HPLC. Kinetic parameters of the complexes were obtained for each stage of thermal degradation using the Coats–Redfern and Horowitz–Metzger methods.

1. Introduction

Antioxidants have the capacity to protect organisms and cells from damage induced by oxidative stress; therefore, considerable research has been conducted to examine this feature. Novel compounds have attracted scientists' interest in recent years. Natural sources that provide the active components for preventing or reducing the impact of oxidative stress on cells have been used [1]. Exogenous chemicals and endogenous metabolic processes in the human body or in food systems might produce highly reactive free radicals, especially oxygen, which are capable of oxidizing biomolecules that can result in cell death and tissue damage. Oxidative damage has a pathological role in serious human diseases (e.g., emphysema, cirrhosis, atherosclerosis and arthritis). Furthermore, a variety of pathophysiological processes, such as inflammation, diabetes, genotoxicity and cancer, stem from the excessive generation of reactive oxygen species (ROS) induced by various stimuli that exceed the antioxidant capacity of the organism [2].

Even several natural sources of these active components have been used to synthesize and obtain effective new antioxidative compounds. It is well-known that 1,2,4-triazole derivatives present an antitumor activity on many cancer types, such as leukemia, non-small cell lung, colon, melanoma, ovarian, renal, prostate, and breast cancers. Several in vitro studies were performed for this purpose in recent years [3–7]. The anticancer activity of 1,2,4-triazole derivatives might be associated with their antioxidant activity. Additionally, 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have a broad spectrum of biological activities [8–18]. In addition, several studies involving the synthesis of *N*-arylidenamino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are available in the literature [15–21].

In the present study, the following antioxidant activities were explored using the newly synthesized compounds: 1,1-diphenyl-2-picryl-hydrazyl (DPPH·) free radical scavenging, reducing power and metal chelating activities. Furthermore, several 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives were titrated potentiometrically with tetrabutylammonium hydroxide (TBAH) in non-aqueous solvents to determine their pK_a values [15–23]. The hydrophobicity, meaning the solubility of synthesized compounds in aqueous solutions, was also investigated. The concentration of a non-ionized compound in two different solutions is the partition coefficient. The partition coefficient (log P) of any ionizable solute could be measured in the aqueous phase in which the pH value was adjusted for its non-ionized form. The ratio of the concentrations of non-ionized solute in the solvents is calculated according to the log P values. The log P value is a measure of lipophilicity and is not pH-dependent. In the HPLC analysis, the

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capacity factor (k′), which is a measure of the migration rate of an analyte on a column, was extrapolated from the binary eluents to 100% water to find the log k'_w values. It is reported that log k'_w values could successfully replace the n-octanol–water partition coefficient (log $P_{o/w}$) [24].

Because pK_a and lipophilicity are highly related to the solubility of these compounds in different liquid systems and the ability or inability for them to cross the cell membrane, these parameters are responsible for the biological activity in the body system [25,26]. Therefore, it is of great importance to know the pK_a and lipophilicity of any proposed and potentially active compound to classify and understand its behavior with respect to the permeability in body tissues and to understand the compound's solubility in various liquid systems.

2. Experimental

2.1. Chemistry

Chemical reagents and all solvents used in this study were purchased from Merck AG (Darmstadt, Germany), Sigma (Sigma-Aldrich GmbH, Sternheim, Germany) and Fluka (Buchs, Switzerland). The starting compounds 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones **3** were prepared from the reactions of the corresponding ester ethoxycarbonylhydrazones **2** with an aqueous solution of hydrazine hydrate as described in the literature [27,28]. Melting points were determined in open glass capillaries using a WRS-2A Microprocessor melting-point apparatus (Liaoning, mainland China) and are uncorrected. The IR spectra were obtained on an ALPHA-P BRUKER FT-IR (Germany) spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in deuterated dimethyl sulfoxide with TMS as an internal standard using a Bruker (Germany) spectrometer at 400 MHz and 100 MHz, respectively. UV absorption spectra were measured in 10 mm quartz cells between 200 and 400 nm using a PG Instruments Ltd T80 UV/VIS (Leicestershire, United Kingdom) spectrometer. Extinction coefficients (ε) are expressed in L mol^{-1} cm^{-1}.

2.2. General procedure for the synthesis of compounds **4**

4-Hydroxybenzaldehyde (0.01 mol) dissolved in ethyl acetate (100 mL) was treated with 4-methylbenzoyl chloride (0.01 mol); triethylamine (0.01 mol) was added slowly with stirring at 0–5 °C. Stirring was continued for 1 h, followed by refluxing for 3 h and filtering. The filtrate was evaporated in vacuo and the crude product was washed with water and recrystallized from ethanol to afford compound **1**. The corresponding compound **3** (0.01 mol) was dissolved in acetic acid (20 mL) and treated with 4-(4-methylbenzoxybenzaldehyde) **1** (0.01 mol). The mixture was refluxed for 2 h and subsequently evaporated at 50–55 °C in vacuo. Several recrystallizations of the residue from ethanol gave pure compounds 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one **4** as colorless crystals.

2.2.1. 3-Methyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4a**)

Yield: 3.26 g (97%); mp: 233 °C; IR (KBr, υ, cm^{-1}): 3179 (NH), 1726, 1703 (C═O), 1599 (C═N), 1274 (COO), 833 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.30 (s, 3H, CH_3), 2.44 (s, 3H, $PhCH_3$), 7.43–7.45 (m, 4H, Ar-H), 7.95 (d, 2H, Ar-H), 8.05 (d, 2H, Ar-H), 9.77 (s, 1H, N═CH), 11.86 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 11.61 (CH_3), 21.76 ($PhCH_3$), 123.17 (2C), 126.36 (arom-C), 129.48 (2C), 130.06 (2C), 130.41 (2C), 131.80, 144.78, 153.15 (arom-C), 145.23 (triazole C_3), 151.69 (N═CH), 153.37 (triazole C_5), 164.78 (COO); UV λ_{max} (ε): 290 (16.151), 254 (19.504), 220 (19.412) nm.

2.2.2. 3-Ethyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4b**)

Yield: 3.40 g (97%); mp: 180–182 °C; IR (KBr, υ, cm^{-1}): 3160 (NH), 1739, 1700 (C═O), 1595 (C═N), 1264 (COO), 832 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.23 (t, 3H, $CH_2\underline{CH_3}$), 2.44 (s, 3H, $PhCH_3$), 2.70 (q, 2H, $\underline{CH_2}CH_3$), 7.43–7.46 (m, 4H, Ar-H), 7.93–7.96 (m, 2H, Ar-H), 8.04–8.07 (m, 2H, Ar-H), 9.77 (s, 1H, N═CH), 11.88 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 10.57 ($CH_2\underline{CH_3}$), 19.02 ($\underline{CH_2}CH_3$), 21.76 ($PhCH_3$), 123.20 (2C), 126.37 (arom-C), 129.45 (2C), 130.06 (2C), 130.41 (2C), 131.84, 145.28, 153.21 (arom-C), 148.54 (triazole C_3), 151.84 (N═CH), 153.38 (triazole C_5), 164.79 (COO); UV λ_{max} (ε): 292 (13.503), 252 (15.579) nm.

2.2.3. 3-n-Propyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4c**)

Yield: 3.50 g (96%); mp: 171–173 °C; IR (KBr, υ, cm^{-1}): 3207 (NH), 1731, 1689 (C═O), 1593 (C═N), 1259 (COO), 835 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 1.02 (t, 3H, $CH_2CH_2\underline{CH_3}$), 1.76 (sext, 2H, $CH_2\underline{CH_2}CH_3$), 2.50 (s, 3H, $Ph\underline{CH_3}$), 2.72 (t, 2H, $\underline{CH_2}CH_2CH_3$), 7.48–7.51 (m, 4H, Ar-H), 7.99 (d, 2H, Ar-H), 8.11 (d, 2H, Ar-H), 9.82 (s, 1H, N═CH), 11.94 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 13.96 ($CH_2CH_2\underline{CH_3}$), 19.40 ($CH_2\underline{CH_2}CH_3$), 21.75 ($PhCH_3$), 27.19 ($\underline{CH_2}CH_2CH_3$), 123.20 (2C), 126.36 (arom-C), 129.43 (2C), 130.05 (2C), 130.41 (2C), 131.83, 145.22, 153.23 (arom-C), 147.39 (triazole C_3), 151.78 (N═CH), 153.38 (triazole C_5), 164.78 (COO); UV λ_{max} (ε): 294 (15.098), 256 (18.553) nm.

2.2.4. 3-Benzyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4d**)

Yield: 4.04 g (98%); mp: 197–198 °C; IR (KBr, υ, cm^{-1}): 3155 (NH), 1738, 1693 (C═O), 1594 (C═N), 1265 (COO), 815 (1,4-disubstituted benzenoid ring), 764 and 701 (monosubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.44 (s, 3H, $PhCH_3$), 4.08 (s, 2H, CH_2Ph), 7.22–7.34 (m, 5H, Ar-H), 7.42–7.45 (m, 4H, Ar-H), 7.91 (d, 2H, Ar-H), 8.05 (d, 2H, Ar-H), 9.73 (s, 1H, N═CH), 12.00 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.75 ($PhCH_3$), 31.54 (CH_2Ph), 123.16 (2C), 126.37 (arom-C), 127.21 (arom-C), 128.94 (2C), 129.28 (2C), 129.48 (2C), 130.05 (2C), 130.41 (2C), 131.77, 136.28, 145.22, 152.98 (arom-C), 146.72 (triazole C_3), 151.69 (N═CH), 153.39 (triazole C_5), 164.78 (COO); UV λ_{max} (ε): 292 (18.884), 256 (23.052) nm.

2.2.5. 3-p-Methylbenzyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4e**)

Yield: 4.08 g (96%); mp: 171–173 °C; IR (KBr, υ, cm^{-1}): 3154 (NH), 1739, 1692 (C═O), 1595 (C═N), 1263 (COO), 820 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.25 (s, 3H, $Ph\underline{CH_3}$), 2.44 (s, 3H, $PhCH_3$), 4.02 (s, 2H, CH_2Ph), 7.12 (d, 2H, Ar-H), 7.22 (d, 2H, Ar-H), 7.42–7.45 (m, 4H, Ar-H), 7.91 (d, 2H, Ar-H), 8.05 (d, 2H, Ar-H), 9.72 (s, 1H, N═CH), 11.98 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.08 ($CH_2Ph\underline{CH_3}$), 21.76 ($PhCH_3$), 31.15 (CH_2Ph), 123.19 (2C), 126.38 (arom-C), 129.14 (2C), 129.50 (2C), 129.50 (2C), 130.06 (2C), 130.41 (2C), 131.79, 133.16, 136.27, 145.23, 152.98 (arom-C), 146.88 (triazole C_3), 151.68 (N═CH), 153.38 (triazole C_5), 164.80 (COO); UV λ_{max} (ε): 292 (12.014), 256 (15.021) nm.

2.2.6. 3-p-Methoxybenzyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4f**)

Yield: 4.36 g (99%); mp: 196–197 °C; IR (KBr, υ, cm^{-1}): 3157 (NH), 1738, 1695 (C═O), 1594 (C═N), 1243 (COO), 828 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.44 (s, 3H, $PhCH_3$), 3.71 (s, 3H, $PhOCH_3$), 4.00 (s, 3H, CH_2Ph), 6.88 (d, 2H, Ar-H), 7.26 (d, 2H, Ar-H), 7.43–7.45 (m, 4H, Ar-H), 7.93 (d, 2H, Ar-H), 8.06 (d, 2H, Ar-H), 9.73 (s, 1H, N═CH), 11.96 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.76 ($PhCH_3$), 30.68 (CH_2Ph), 55.47 (OCH_3), 114.34 (2C), 123.21 (2C), 126.37 (arom-C), 128.03, (arom-C), 129.51 (2C), 130.07 (2C), 130.35 (2C), 130.42 (2C), 131.80, 145.24, 153.00, 158.55

(arom-C), 147.05 (triazole C_3), 151.70 (N═CH), 153.39 (triazole C_5), 164.81 (COO); UV λ_{max} (ε): 292 (16.212), 254 (19.374) nm.

*2.2.7. 3-p-Chlorobenzyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4g**)*

Yield: 4.28 g (96%); mp: 210 °C; IR (KBr, υ, cm^{-1}): 3169 (NH), 1729, 1705 (C═O), 1605 (C═N), 1259 (COO), 840 (1,4-disubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.44 (s, 3H, $PhCH_3$), 4.09 (s, 2H, CH_2Ph), 7.36–7.45 (m, 8H, Ar-H), 7.91 (d, 2H, Ar-H), 8.05 (d, 2H, Ar-H), 9.73 (s, 1H, N═CH), 12.03 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.76 ($PhCH_3$), 30.85 (CH_2Ph), 123.20 (2C), 126.36 (arom-C), 128.87 (2C), 129.52 (2C), 130.06 (2C), 130.41 (2C), 131.24 (2C), 131.73 (arom-C), 131.90, 135.25, 145.24, 153.06 (arom-C), 146.41 (triazole C_3), 151.67 (N═CH), 153.41 (triazole C_5), 164.80 (COO); UV λ_{max} (ε): 292 (17.171), 256 (21.127) nm.

*2.2.8. 3-Phenyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-one (**4h**)*

Yield: 3.80 g (95%); mp: 216 °C; IR (KBr, υ, cm^{-1}): 3140 (NH), 1738, 1692 (C═O), 1604 (C═N), 1260 (COO), 760 and 686 (monosubstituted benzenoid ring); ^{1}H NMR (400 MHz, DMSO-d_6): δ 2.34 (s, 3H, $PhCH_3$), 7.43–7.46 (m, 4H, Ar-H), 7.54–7.56 (m, 3H, Ar-H), 7.91–7.94 (m, 4H, Ar-H), 8.05 (d, 2H, Ar-H), 9.70 (s, 1H, N═CH), 12.41 (s, 1H, NH); ^{13}C NMR (100 MHz, DMSO-d_6): δ 21.76 ($PhCH_3$), 123.30 (2C), 126.33 (arom-C), 127.10 (arom-C), 128.42 (2C), 129.03 (2C), 129.71 (2C), 130.05 (2C), 130.41 (2C), 130.60, 131.59, 145.07, 156.18 (arom-C), 145.24 (triazole C_3), 151.82 (N═CH), 153.58 (triazole C_5), 164.78 (COO); UV λ_{max} (ε): 302 (12.935), 254 (24.627) nm.

2.3. Antioxidant activity: chemicals

Butylated hydroxytoluene (BHT) was purchased from E. Merck (Darmstadt, Germany). Ferrous chloride, α-tocopherol, 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$), 3-(2-pyridyl)-5,6-bis(phenylsulfonic acid)-1,2,4-triazine (ferrozine), butylated hydroxyanisole (BHA), ethylenediaminetetraacetic acid (EDTA) and trichloroacetic acid (TCA) were bought from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany).

2.3.1. Reducing power

The reducing power of the synthesized compounds was determined. Different concentrations of the samples (50–250 μg/mL) in ethanol (1 mL) were mixed with phosphate buffer (2.5 mL, 0.2 M, pH = 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min and afterwards a portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was centrifuged for 10 min at 1000 ×*g*. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%) and then the absorbance at 700 nm was measured in a spectrophotometer. A higher absorbance of the reaction mixture indicated a greater reducing power.

2.3.2. Free radical scavenging activity

The free radical scavenging activity of the synthesized compounds was determined. Briefly, a 0.1 mM solution of $DPPH^{\cdot}$ in ethanol was prepared, and this solution (1 mL) was added to sample solutions in ethanol (3 mL) at various concentrations (50–250 μg/mL). The mixture was shaken vigorously and allowed to remain at room temperature for 30 min. Next, the absorbance was measured at 517 nm in a spectrophotometer. The lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The $DPPH^{\cdot}$ concentration (mM) in the reaction medium was calculated from the following calibration curve and determined by linear regression (R^2: 0.997): Absorbance = $(0.0003 \times DPPH^{\cdot}) - 0.0174$.

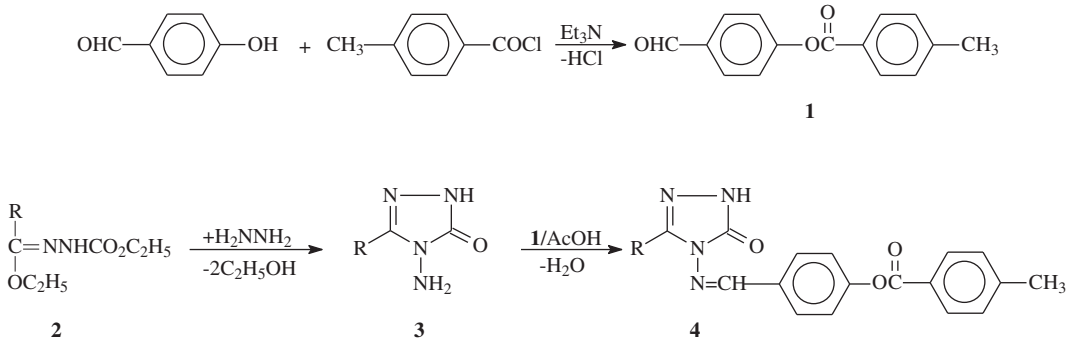
The capability to scavenge the DPPH radical was calculated by using the following equation: $DPPH^{\cdot}$ scavenging effect (%) = $(A^0 - A^1 / A^0) \times 100$, where A^0 is the absorbance of the control reaction, and A^1 is the absorbance in the presence of the samples or standards.

2.3.3. Metal chelating activity

The chelation of ferrous ions by the synthesized compounds and standards was estimated. In the study, all of the compounds and the standard antioxidants were dissolved in ethanol. Briefly, the synthesized compounds (30–90 μg/mL) were added to a 2 mM solution of $FeCl_2 \cdot 4H_2O$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL), and then the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was measured at 562 nm in a spectrophotometer. All tests and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine–Fe^{2+} complex formation was given by the formula: % inhibition = $(A^0 - A^1 / A^0) \times 100$, where A^0 is the absorbance of the control, and A^1 is the absorbance in the presence of the samples or standards. The control did not contain compound or standard.

2.4. Potentiometric titrations

A Jenco model ion analyzer (Jenco, USA) and an Ingold pH electrode (Mettler Toledo, Spain) were used for potentiometric titrations. For each compound that would be titrated, the 0.001 M solution was separately prepared in each non-aqueous solvent. The 0.05 M solution of



a) R= CH_3, **b**) R= CH_2CH_3, **c**) $CH_2CH_2CH_3$, **d**) $CH_2C_6H_5$, **e**) R= $CH_2C_6H_4.CH_3$ (*p*-), **f**) R= $CH_2C_6H_4.OCH_3$ (*p*-), **g**) R= $CH_2C_6H_4.Cl$ (*p*-), **h**) R= C_6H_5

Scheme 1. Synthetic route for compounds **1**, **3** and **4**.

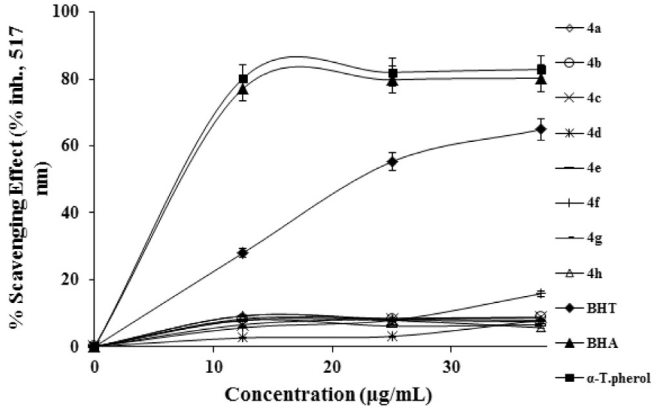


Fig. 1. Scavenging effect of compounds **4a–h**, BHT, BHA and α-tocopherol at different concentrations (12.5–25–37.5 μg/mL).

TBAH in isopropyl alcohol, which is widely used in the titration of acids, was used as titrant. The mV values that were obtained by the pH-meter were recorded. Finally, the HNP values were determined by plotting the mL (TBAH)-mV graphic.

2.5. HPLC analysis for determination of log k'_w values

The LC system consisted of a Spectra-SYSTEM P2000 gradient pump, a Spectra SYSTEM SCM 1000 degasser, a Rheodyne manual injector with a 20 μL injection loop and a Spectra SYSTEM UV2000 detector (Thermo Separation Products, USA). The detector was set at 260 nm. A Phenomenex Luna 5 mm C18 100 A° LC column (250 × 4.6 mm) was used for elution. The logk′ values were determined for each compound. The mobile phases were prepared by mixing methanol with water in the proportions 100:0, 95:5, 90:10, 85:15, and 80:20 (v/v). The flow rate was 0.8 mL/min. All measurements were made at least in duplicate. The average reproducibility of each determination was better than 1.0% relative. The capacity factors (k′) were determined using $k' = (t_R - t_0) / t_0$, where t_R is the retention time of the compound, and t_0 is the void volume or the dead time. An aqueous solution of uracil was used for the measurement of void volume. All the chemical components were dissolved in methanol and diluted with the mobile phase before injection into the HPLC system for a final concentration of 10 μg mL^{-1}.

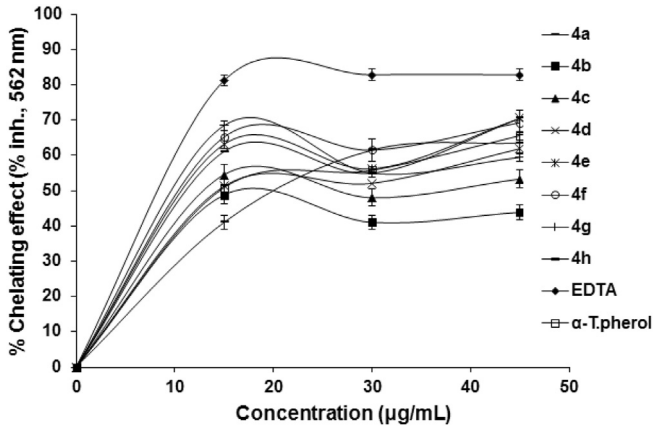


Fig. 2. Metal chelating effect from different amounts of compounds **4a–h**, EDTA and α-tocopherol on ferrous ions.

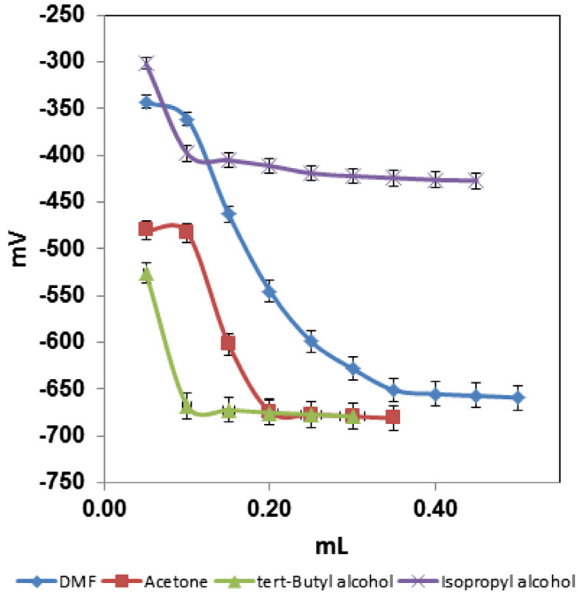


Fig. 3. Potentiometric titration curves of 0.001 *M* solutions of compound **4b** titrated with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, DMF and acetone at 25 °C.

3. Results and discussion

3.1. Chemistry

4-(4-Methylbenzoxy)benzaldehyde **1**, which was recently reported [29], was obtained from the reaction of 4-hydroxybenzaldehyde with 4-methylbenzoyl chloride by using triethylamine. The 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones **4a–h** were synthesized by the reactions of compounds 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones **3a–h** with 4-(4-methylbenzoxy)benzaldehyde **1** (Scheme 1).

The structures of eight new 3-alkyl(aryl)-4-[4-(4-methylbenzoxy) benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one **4a–h** compounds were identified by using IR, ^{1}H NMR, ^{13}C NMR and UV data.

3.2. Biological activity

3.2.1. Antioxidant activity

The antioxidant activities of eight new compounds **4a–h** were identified. In the study, all of the compounds and the standard antioxidants were dissolved in ethanol. If the compounds were not soluble in ethanol, DMSO was used for the compounds and the standards to compare their activities in the same conditions. Several methods are used to determine antioxidant activities. The methods used in the present study are given below.

Table 1
The HNP and the corresponding pK_a values of compounds **4a–h** in isopropyl alcohol, *tert*-butyl alcohol, DMF and acetone.

Comp.	Isopropyl alcohol		*tert*-Butyl alcohol		DMF		Acetone	
	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a
4a	–	–	–	–	−477	18.37	−380	15.83
4b	–	–	–	–	−352	15.33	−480	12.18
4c	—	–	–	–	–	–	–	–
4d	–	–	–	–	–	–	−99	8.91
4e	–	–	–	–	−415	16.84	–	–
4f	–	–	−364	13.49	–	–	−350	15.17
4g	−308	14.32	–	–	−366	15.72	–	–
4h	–	–	–	–	−269	13.33	–	–

Table 2
Measured log k′$_w$ values for the synthesized compounds.

Compound	k_w[b]	S[a]
4a	16.89499	−0.17899
4b	25.35267	−0.27037
4c	35.74553	−0.38391
4d	41.41959	−0.44683
4e	63.47511	−0.68821
4f	38.31554	−0.41304
4g	64.50168	−0.70133
4h	39.05277	−0.42058

[a] S is the slope.
[b] log k′$_w$ is the intercept of the plot of log k′ versus φ (the volume percentages are of methanol in the mobile phase).

3.2.1.1. Total reductive capability using the potassium ferricyanide reduction method. The reductive capabilities of compounds are assessed by the extent of conversion of the Fe^{3+}/ferricyanide complex to the Fe^{2+}/ferrous form using the method of Oyaizu [30]. The reducing powers of the compounds were observed at different concentrations, and results were compared with BHA, BHT and α-tocopherol. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [31]. The antioxidant activity has been attributed to various mechanisms, among which are the prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [32]. In this study, all of the compounds had a lower absorbance than the standard antioxidants. Hence, no activity was observed for reducing metal ion complexes to their lower oxidation state or for any electron transfer reaction. Therefore, the compounds did not exhibit a reductive activity.

3.2.1.2. DPPH· radical scavenging activity. The free radical scavenging activity of compounds was measured via DPPH· using the method of Blois [33]. The stable DPPH radical scavenging model is a widely used method to evaluate an antioxidant activity in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to the antioxidant's hydrogen-donating ability [34]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [35]. The reduction capability of DPPH radicals was determined by a decrease in absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was 517 nm. Because of the reaction between antioxidant molecules and radicals, the absorbance of the DPPH radical caused by antioxidants decreased, which results in radical scavenging by hydrogen donation. This process is visually noticeable as a change from purple to yellow. Hence, DPPH· is usually used as a substrate to evaluate an antioxidative activity [36]. In this study, antiradical activities of compounds and standard antioxidants such as BHT, BHA and α-tocopherol were determined by using the DPPH· method. Scavenging effect values of compounds **4** with BHT, BHA and α-tocopherol at different concentrations are given in Fig. 1. The newly synthesized compounds showed no radical scavenger activity.

3.2.1.3. Ferrous ion chelating activity. The chelating effect towards ferrous ions by the compounds and standards was determined according to the method of Dinis [37]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, complex formation is disrupted, resulting in the loss of the complexes' red color. Therefore, color reduction measurements can serve as an estimate of the chelating activity of the coexisting chelator [38]. Transition metals have a pivotal role in the generation of free oxygen radicals in living organisms. Ferric iron (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+} form, depending on such conditions as pH [39], and then oxidized again. Products of Fenton-type reactions or Haber–Weiss reactions can interact with superoxide anions resulting in hydroxyl radicals. The production of these highly active compounds may lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may not activate the metal ions and in turn potentially inhibit metal-dependent processes [40]. Additionally, the production of highly active ROS, such as $O_2^{\bullet-}$, H_2O_2 and $OH^{\bullet}$, is also catalyzed by free iron though the Haber–Weiss reaction as shown below:

$$O_2^{\bullet} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}.$$

Among the transition metals, iron is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down the hydrogen and lipid peroxides to form reactive free radicals via the Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}.$$

The Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of the Fe^{2+} ion, which is the most powerful pro-oxidant among the various types of metal ions [41]. Ferrous ion-chelating activities of the compounds **4** with EDTA and α-tocopherol are shown in Fig. 2. In this study, metal chelating capacity was significant because it reduced the concentration of the catalyzing transition metal. It was reported that chelating agents that form σ-bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion [42]. The data obtained from Fig. 2 reveal that the compounds, especially

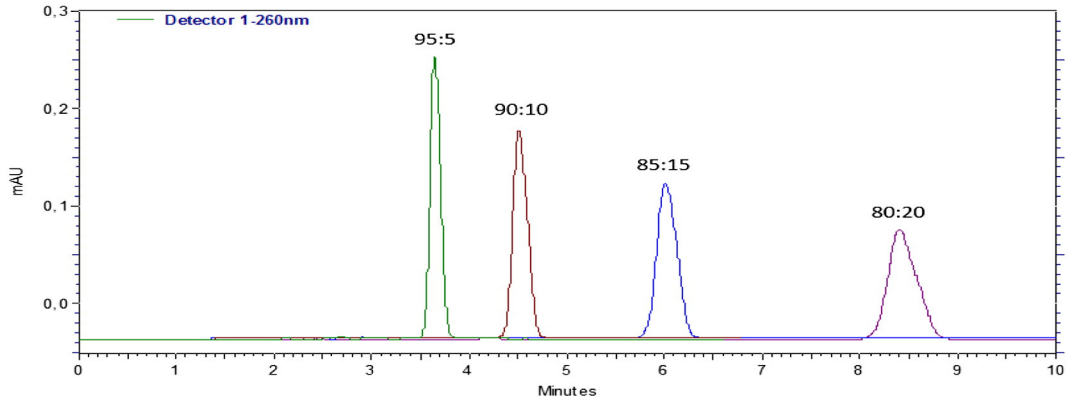


Fig. 4. Overlapping chromatograms of **4a** obtained by using different mobile phases (95:5, 90:10, 85:15, 80:20 MeOH/water (v/v)): Experimental conditions: flow rate = 1 mL min^{-1}, detection wavelength = 260 nm, injection volume = 20 μL.

4d and **4h**, demonstrate a marked capacity for iron binding, suggesting that their action as peroxidation protectors may be related to their iron-binding capacity. The metal-chelating effect of the compounds and standards decreased in the order of EDTA > α-tocopherol > **4h** > **4d**. On the other hand, free iron is known to have low solubility and a chelated iron complex has greater solubility in solution. Furthermore, the compound-iron complex may also be active because it can participate in iron-catalyzed reactions.

3.3. Potentiometric titrations

To determine the pK_a values of the compounds **4a–h**, they were titrated potentiometrically with TBAH in four non-aqueous solvents: isopropyl alcohol, *tert*-butyl alcohol, acetone and *N*,*N*-dimethyl formamide (DMF). The mV values read in each titration were plotted against the 0.05 M TBAH volumes (mL) added, and potentiometric titration curves were obtained for all the cases. From the titration curves, the half-neutralization potential (HNP) values were measured, and the corresponding pK_a values were calculated. The data obtained from the potentiometric titrations were interpreted, and the effect of the C-3 substituent on the 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring, as well as solvent effects, were studied [15–23,43].

As an example, the potentiometric titration curves for 0.001 M solutions of compounds **4b** titrated with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, DMF and acetone are shown in Fig. 3.

When the solvent dielectric permittivity is taken into consideration, the acidity order can be given as follows: DMF (ε = 36.7) > acetone (ε = 36) > isopropyl alcohol (ε = 19.4) > *tert*-butyl alcohol (ε = 12). As seen in Table 1, the acidity order for compound **4a** is: acetone > DMF, for compound **4b** it is: DMF > acetone, for compound **4f** it is: acetone > *tert*-butyl alcohol, and for compound **4g** it is: isopropyl alcohol > DMF. Moreover, as seen in Table 1, for compounds **4a–4f** and **4h** in isopropyl alcohol, for compounds **4c**, **4d**, and **4f** in DMF, for compounds **4a–4e**, **4g**, and **4h** in *tert*-butyl alcohol and for compounds **4c**, **4e**, **4g**, and **4h** in acetone, the HNP values and the corresponding pK_a values were not obtained.

When the acidity power of compounds in solvents was researched considering the dielectric constant, HNP and pK_a values in *tert*-butyl alcohol and isopropyl alcohol were not assigned because the titration curves were not obtained in amphiprotic solvents. Therefore, the acidity order was not conducted. It was determined that compound **4b** was more acidic than acetone in the DMF environment and was appropriate with the theoretical order when studying dipolar aprotic solvents.

Table 3
The parameters for the decomposition of compounds **4a–h**.

Compounds	E_a (kJ/mol)		r^2	
	H–M	C–R	H–M	C–R
4a	133.2	139.2	0.998	0.999
4b	139.5	141.6	0.997	0.998
4c	136.8	135.3	0.999	0.997
4d	143.5	146.8	0.995	0.997
4e	149.3	152.3	0.996	0.995
4f	151.9	153.6	0.996	0.999
4g	159.5	161.5	0.997	0.998
4h	157.3	160.8	0.995	0.996

It is well known that the acidity of a compound depends on certain factors, the most important of which are the solvent effect and the molecular structure [15–23,43]. Table 1 and Fig. 3 show that the HNP values and corresponding pK_a values obtained from the potentiometric titrations depend on the non-aqueous solvents used and the substituents at C-3 on the 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring.

3.4. HPLC analysis for determination of log k'_w values

In the reverse phase HPLC system, the lipophilic molecules tend to be distributed in the lipophilic stationary phase instead of hydrophilic mobile phase. The more hydrophobic the molecule, the more time it will spend on the stationary phase and the higher the concentration of organic solvent will be required to promote elution.

The relationship between log k′ and methanol concentration in the mobile phase is known in HPLC theory, and it is described with the formula: $\log k' = \log k'_w - S\varphi$, where k_w represents the k value for a compound if the pure water is used as eluent, S is the slope of the regression curve, and φ is the volume percentage of methanol in the mobile phase [44,45]. For each studied compound, a linear correlation was found between log k′ and φ, and the correlation coefficients were all >0.99. The values of S and the extrapolated log k'_w are given in Table 2.

Octanol–water partition coefficients are the most widely used measures of lipophilicity in modeling biological partition/distribution. The partition coefficient can be estimated by a classical shake-flask method. However, it has many disadvantages, such as requiring relatively large amounts of solutes and solvents and it is time-consuming.

It has long been recognized that the retention of a compound in reversed-phase liquid chromatography is governed by its lipophilicity/hydrophobicity, and thus correlates with the octanol–water partition coefficient [46].

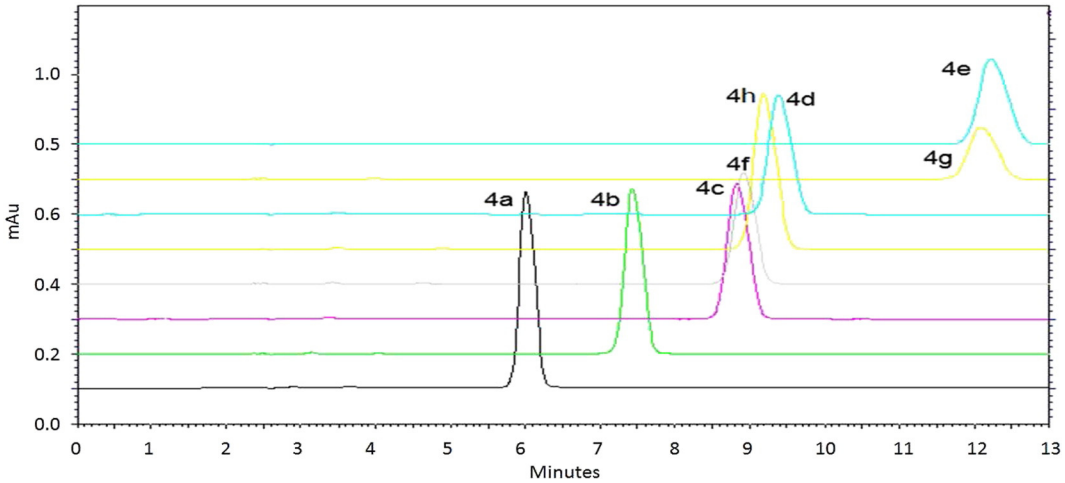


Fig. 5. Overlapping chromatograms of **4a–h** by using 85:15 MeOH/water (v/v) mixture as the mobile phase: Experimental conditions: flow rate = 1 mL min^{-1}, detection wavelength = 260 nm, injection volume = 20 μL.

Reversed phase chromatography involves the adsorption of hydrophobic molecules onto a hydrophobic solid support in a polar mobile phase. Decreasing the mobile phase polarity by adding more organic solvent reduces the hydrophobic interaction between the solute and the solid support resulting in desorption. The more hydrophobic the molecule, the more time it will spend on the solid support and the higher the concentration of organic solvent will be required to promote desorption. Fig. 4 is given as an example to clarify this application. The higher methanol content in the mobile phase makes **4a** elute from the HPLC column easily. The k′ value of **4a** for different methanol compositions is directly related with its lipophilicity. If the log k′ values were extrapolated from binary eluents to 100% aqueous solutions, one could estimate the log k_w values of the synthesized compounds. In that way, the linear relationship between log k′ and methanol composition as described above could be used to measure the synthesized molecules' lipophilicity.

Fig. 5 presents overlapping chromatograms of eight compounds (**4a–h**) using an 85:15 MeOH/water (v/v) mixture as the mobile phase. As seen in Fig. 5, the most hydrophilic compound was **4a**, which has a $-CH_3$ group, and the most hydrophobic compound was **4e**, which has a $-CH_2C_6H_4CH_3$ group, as expected. It is well known that adding one more carbon to the aliphatic chain of the substituent improves the lipophilicity of a compound in a homologue series. Therefore, **4c** was more lipophilic than **4a** and **4b**. On the other hand, the electronegative substituent on the phenyl group, $-OCH_3$, strongly reduced the lipophilicity of **4f**, causing it to elute at almost the same retention time as **4c**. It is known that at the molecular level, solubility is controlled by the energy balance of intermolecular forces between solute–solute, solvent–solvent and solute–solvent molecules. Recall from general chemistry that intermolecular forces come in different strengths, ranging from very weak induced dipole–induced dipole interactions to much stronger dipole–dipole forces (including the important special case, hydrogen bonding). The experimental data in our study strongly suggest that modifying the substituents on a homologue series could dramatically change the molecular structure and the lipophilicity of the compounds [47,48], and in that way it could also change their solubility in aqueous media and the passive diffusion through cell membranes [49]. The solubility of compounds could be used also when trying to purify (e.g., recrystallization and choice of solvent) or isolate them from a multi-component reaction mixture (e.g., via extraction), or when extracting a molecule from a matrix.

3.5. Kinetic analysis

The activation energy values (Ea) for the decomposition of the compounds were calculated by employing the Horowitz–Metzger [50] and Coats–Redfern [51] equations. The Horowitz–Metzger equation is given as follows:

$$\ln\left[\frac{\ln\left(W_o - W_t^f\right)}{W - W_t^f}\right] = \frac{E_a\theta}{RT_s^2} \quad (1)$$

where W_o is the initial weight of the sample, W_t^f is the final weight of the sample, W is the weight remaining at a given temperature T, Ea is the activation energy, and T_s is the peak temperature of DTG.

The Coats–Redfern equation is as follows:

$$\log\left\{-\log\left[\frac{1-\alpha}{T^2}\right]\right\} = \log\left\{\left[\frac{AR}{aE_a}\right] - \left[\frac{E_a}{2.303RT}\right]\right\} \quad (2)$$

where α is the fraction of the sample decomposed at time t, T is the derivative peak temperature, A is the frequency factor, and R is the gas constant. The kinetic data obtained for the nonisothermal decomposition of compounds are given in Table 3. As seen in Table 3, the apparent activation energy values calculated by the Horowitz–Metzger (H–M) and Coats–Redfern (C–R) equations are close to each other for the compounds. The values from the two models are in close agreement with each other.

4. Conclusions

The identification of new compounds for protecting cells from oxidative stress is a rapidly rising area of scientific interest. Even several natural sources that provide active components have been used to synthesize and obtain effective new antioxidative compounds. It is well-known that 1,2,4-triazole derivatives present an antitumor activity on many cancer types, such as leukemia, non-small cell lung, colon, melanoma, ovarian, renal, prostate, and breast cancers. Several in vitro studies were performed for this purpose in recent years. The anticancer activity of 1,2,4-triazole derivatives might be associated with their antioxidant activity. There have been many reports about the biological activities and synthesis of 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives. In addition, several studies on the synthesis of *N*-arylidenamino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are available in the literature. However, the physicochemical properties of the synthesized substances and biological activity have rarely been discussed. In this study, the synthesis of eight new compounds was performed and they were characterized by using IR, ^{1}H NMR, ^{13}C NMR and UV spectral data. In addition, the pK_a and lipophilicity of these compounds were determined by using potentiometric titration and HPLC, respectively. Kinetic parameters were also investigated. The compounds were determined to possess an antioxidant activity and could be recommended for further clinical investigations because such compounds have already been reported to possess antitumor and antibacterial activities in earlier studies.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgments

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