The Pharmaceutical and Chemical Journal, 2017, 4(4):91-101

Available online www.tpcj.org

Research Article

ISSN: 2349-7092
CODEN(USA): PCJHBA

Investigation of Antioxidant, Biological and Acidic Properties of New 3-Alkyl(Aryl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones

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Abstract Some novel 3-alkyl(aryl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4a-h**) were synthesized from the rections of 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**2a-h**) with 3-acetoxy-4-methoxybenzaldehyde (**3**). The new eight compounds were characterized using by elemental analyses and IR, ^{1}H-NMR, ^{13}C-NMR and UV spectral data. In addition, the synthesized compounds were analyzed for their in vitro potential antioxidant activities in three different methods. Antibacterial activity of the compounds **4a-h** were also evaluated aginst six bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus* and *Klebsiella pneumoniae*. Moreover, **4** type compounds were titrated potentiometrically with tetrabutylammonium hydroxide in four non-aqueous solvents such as isopropyl alcohol, tert-butyl alcohol, acetone, N,N-dimethylformamide and the half-neutralization potential values and the corresponding pK_a values were determined for all cases.

Keywords 4,5-Dihydro-1*H*-1,2,4-triazol-5-one, Schiff base, antioxidant activity, antibacterial activity, pK_a, potentiometric titrations.

Introduction

1,2,4-Triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are reported to possess a broad spectrum of biological activities such as antifungal, antimicrobial, hypoglycemic, antihypertensive, analgesic, antiviral, antiinflammatory, antitumor, antioxidant and anti-HIV properties [1–9]. In addition, several articles reporting the synthesis of some *N*-arylidenamino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have been published [8,9].

On the other hand, it is known that 1,2,4-triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one rings have weak acidic properties, so some 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, and the pK_a values of the compounds were determined [7–9].

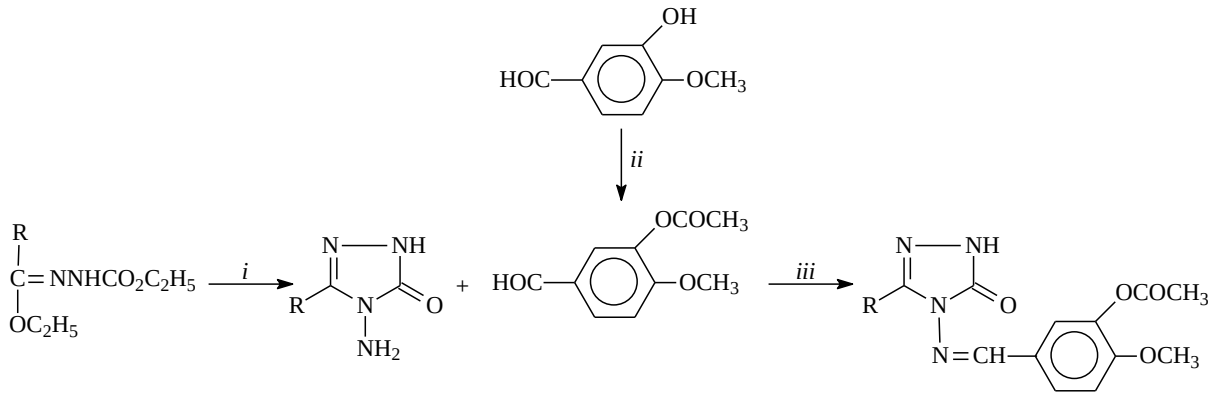
Furthermore, antioxidants are extensively studied for their capacity to protect organisms and cells from damage induced by oxidative stress. Scientists in various disciplines have become more interested in new compounds, either synthesized or obtained from natural sources, which could provide active components to prevent or reduce the impact of oxidative stress on cells [10]. Exogenous chemicals and endogenous metabolic processes in human body or in food system might produce highly reactive free radicals, especially oxygen derived radicals, which are capable of oxidizing biomolecules, resulting in cell death and issue damage. Oxidative damages play a significant

pathological role in human diseases. For example, cancer, emphysema, cirrhosis, atherosclerosis and arthritis have all been correlated with oxidative damage. Also, excessive generation of ROS (reactive oxygen species) induced by various stimuli and which exceeds the antioxidant capacity of the organism leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer [11].

In the present paper, the antioxidant and antimicrobial activities of eight new 3-alkyl(aryl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4a-h**), which were synthesized by the reactions of 3-alkyl-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3a-h**) with 3-acetoxy-4-methoxy-benzaldehyde were determined (Scheme 1).

Moreover, we also examined the potentiometric titrations of the synthesized compounds **4** with TBAH in four non-aqueous solvents; isopropyl alcohol, *tert*-butyl alcohol, acetone and N,N-dimethylformamide (DMF) to determine the corresponding half-neutralization potentials (HNP) and the corresponding pK_a values. The data obtained from the potentiometric titrations was interpreted, and the effect of the C-3 substituent in 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring as well as solvent effects were studied [7–9].

Scheme 1:



1 **2** **3** **4**

i) $N_2H_4.H_2O$, reflux, 6h; ii) Ac_2O, reflux, 1h; iii) AcOH, reflux, 1h

a) R = CH_3, b) R = CH_2CH_3, c) R = $CH_2CH_2CH_3$, d) R = $CH_2C_6H_5$, e) R = $CH_2C_6H_4CH_3$ (*p*-), f) R = $CH_2C_6H_4OCH_3$ (*p*-), g) R = $CH_2C_6H_4Cl$ (*p*-), h) R = C_6H_5

Materials and Methods

Chemical reagents and all solvents used in this study were purchased from Merck AG, Aldrich and Fluka. Melting points which were uncorrect were determined in open glass capillaries using an Electrothermal 9100 digital melting point apparatus. The IR spectra were obtained on a Perkin-Elmer Instruments Spectrum One FT-IR spectrometer. ^{1}H and ^{13}C NMR spectra were recorded in deuterated dimethyl sulfoxide with TMS as internal standard using a Bruker Ultrashield spectrometer at 200 MHz and 50 MHz, respectively. UV absorption spectra were measured in 10 mm quartz cells between 200 and 400 nm using a Schimadzu-1201 UV/VIS spectrometer. Extinction coefficients (ε) are expressed in $L \cdot mol^{-1} \cdot cm^{-1}$.

The starting compounds **2a-h** were prepared from the reactions of the corresponding ester ethoxycarbonylhydrazones (**1a-h**) with an aqueous solution of hydrazine hydrate as described in the literature [12,13].

General procedure for the synthesis of 3-alkyl(aryl)-4-(3-acetoxy-4-methoxybenzyliden-amino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (4a-h)

3-Hydroxy-4-methoxybenzaldehyde 3 (0.01 mol) was refluxed with acetic anhydride (15 mL) for 0.5 h. After addition of absolute ethanol (50 mL), the mixture was refluxed for 1 h more. Evaporation of the resulting solution at 40-45 °C in vacuo and several recrystallizations of the residue from EtOH gave pure compound **3**. White solid;

M.P:86-88°C; IR (ATR)2862 and 2750 (CHO), 1764, 1678 (C=O) cm^{-1}. The corresponding compound **2** (0.01 mol) was dissolved in acetic acid (15 ml) and treated with 3-acetoxy-4-methoxybenzaldehyde (**3**) (0.01 mol). The mixture was refluxed for 1.5 h and then evaporated at 50-55 °C *in vacuo*. Several recrystallizations of the residue from an appropriate solvent gave pure compounds **4a-h** as colourless crystals.

3-Methyl-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4a): Yield 96%, m.p. 206-208°C. IR (KBr): 3179 (NH), 1757, 1710 (C=O), 1608 (C=N), 1273 (COO) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, CH_3), 2.28 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 7.24 (d, 1H, ArH, *J*=8.60 Hz), 7.61 (s, 1H, ArH), 7.67 (d, 1H, ArH, *J*=8.60 Hz), 9.60 (s, 1H, N=CH), 11.57 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 11.57 (CH_3), 20.83 ($CO\underline{C}H_3$), 56.57 (OCH_3), 113.35, 121.21, 126.75, 128.60, 140.18, 154.82 (arom-C), 144.70 (triazole C_3), 151.73 (N=CH), 153.44 (triazole C_5), 168.95 (C=O). UV λ_{max} (ε): 302 (16800), 272 (14980), 234 (16540) nm. Anal. Calcd. For $C_{13}H_{14}N_4O_4$: C, 53.79; H, 4.86; N, 19.30. Found: C, 53.89; H, 4.97; N, 19.50.

3-Ethyl-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4b): Yield 98%, m.p. 258-260°C. IR (KBr): 3190 (NH), 1760, 1710 (C=O), 1605 (C=N), 1270 (COO) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.20 (m, 3H, $CH_2\underline{CH_3}$), 2.30-2.60 (m, 5H, $\underline{CH_2}CH_3$ + $COCH_3$), 3.86 (s, 3H, OCH_3), 6.98-7.51 (m, 3H, ArH), 9.49 (s, 1H, N=CH), 11.74 (s, 1H, NH). UV λ_{max} (ε): 318 (17690), 308 (17280), 238 (18940), 214 (13080) nm.

3-*n*-Propyl-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4c): Yield 99%, m.p. 172-174°C. IR (KBr): 3178 (NH), 1765, 1705 (C=O), 1604, 1590 (C=N), 1276 (COO) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 0.95 (t, 3H, $CH_2CH_2CH_3$, *J*=7.40 Hz), 1.68 (sext, 3H, $CH_2\underline{CH_2}CH_3$, *J*=7.40 Hz), 2.30 (s, 3H, $COCH_3$), 2.63 (t, 3H, $\underline{CH_2}CH_2CH_3$, *J*=7.30 Hz), 3.83 (s, 3H, OCH_3), 7.24 (d, 1H, ArH, *J*=8.60 Hz), 7.59 (s, 1H, ArH), 7.68 (d, 1H, ArH, *J*=8.60 Hz), 9.60 (s, 1H, N=CH), 11.80 (s, 1H, NH). UV λ_{max} (ε): 316 (16020), 302 (16650), 296 (16760), 236 (16520) nm.

3-Benzyl-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4d): Yield 94%, m.p. 191-193°C. IR (KBr): 3158 (NH), 1758, 1705 (C=O), 1606, 1592 (C=N), 1276 (COO), 760 and 704 (monosubstituted benzenoid ring) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 4.05 (s, 2H, CH_2Ph), 7.17-7.31 (m, 6H, ArH), 7.55 (s, 1H, ArH), 7.65 (d, 1H, ArH, *J*=8.60 Hz), 9.58 (s, 1H, N=CH), 11.90 (s, 1H, NH). UV λ_{max} (ε): 306 (17990), 234 (21870), 208 (14050) nm. Anal. Calcd. For $C_{19}H_{18}N_4O_4$: C, 62.29; H, 4.95; N, 15.29. Found: C, 62.12; H, 4.45; N, 14.30.

3-(*p*-Methylbenzyl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4e): Yield 99%, m.p. 194-196°C. IR (KBr): 3159 (NH), 1768, 1706 (C=O), 1604, 1590 (C=N), 1278 (COO), 802 (1,4-disubstituted benzenoid ring) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.20 (s, 3H, CH_3), 2.30 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 3.98 (s, 2H, CH_2Ph), 7.09 (d, 2H, ArH, *J*=8.00 Hz), 7.17-7.25 (m, 3H, ArH), 7.54 (s, 1H, ArH), 7.65 (d, 1H, ArH, *J*=8.60 Hz), 9.55 (s, 1H, N=CH), 11.90 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.66 (CH_3), 20.85 ($CO\underline{C}H_3$), 31.18 (CH_2Ph), 56.58 (OCH_3), 113.39, 121.47, 126.63, 128.38, 129.14 (2C), 129.48 (2C), 133.17, 136.21, 140.13, 153.97 (arom-C), 146.81 (triazole C_3), 151.71 (N=CH), 153.10 (triazole C_5), 168.94 (C=O). UV λ_{max} (ε): 302 (19620), 296 (19300), 234 (19700), 226 (18220) nm.

3-(*p*-Methoxybenzyl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4f): Yield 94%, m.p. 189-191°C. IR (KBr): 3158 (NH), 1752, 1705 (C=O), 1614, 1591 (C=N), 1275 (COO), 802 (1,4-disubstituted benzenoid ring) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, $COCH_3$), 3.70 (s, 3H, *p*-OCH_3), 3.85 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2Ph), 6.85 (d, 2H, ArH), 7.15-7.25 (m, 3H, ArH), 7.56 (s, 1H, ArH), 7.66 (d, 1H, ArH, *J*=8.60 Hz), 9.58 (s, 1H, N=CH), 11.60 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.85 (CH_3), 20.85 ($CO\underline{C}H_3$), 30.71 (CH_2Ph), 55.45 (*p*-OCH_3), 56.58 (OCH_3), 113.40, 114.33 (2C), 121.49, 126.65, 128.04, 128.38, 130.59 (2C), 140.15, 153.97, 158.52 (arom-C), 146.97 (triazole C_3), 151.80 (N=CH), 153.13 (triazole C_5), 168.95 (C=O).

3-(*p*-Chlorolbenzyl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4g): Yield 98%, m.p. 208-210°C. IR (KBr): 3159 (NH), 1770, 1705 (C=O), 1603, 1590 (C=N), 1276 (COO), 801 (1,4-disubstituted benzenoid ring) cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, $COCH_3$), 3.85 (s, 3H, OCH_3), 3.95 (s, 2H, CH_2Ph), 7.24 (d, 1H, ArH, *J*=8.60 Hz), 7.33-7.39 (m, 4H, ArH), 7.55 (s, 1H, ArH), 7.66 (d, 1H, ArH, *J*=8.60 Hz), 9.60 (s, 1H, N=CH), 11.95 (s, 1H, NH). UV λ_{max} (ε): 306 (21690), 232 (26100) nm.

3-Phenyl-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4h): Yield 91%, m.p. 182-184°C. IR (KBr): 3192 (NH), 1763, 1689 (C=O), 1611, 1595 (C=N), 1278 (COO), 769 and 689 (monosubstituted benzenoid ring) cm^{-1}. ^1H NMR (DMSO-d_6): δ 2.38 (s, 3H, $COCH_3$), 3.86 (s, 3H, OCH_3), 7.26 (d, 1H, ArH, *J*=8.60 Hz), 7.50-7.55 (m, 4H, ArH), 7.71 (d, 1H, ArH, *J*=8.60 Hz), 7.86-7.90 (m, 2H, ArH), 9.50 (s, 1H, N=CH), 12.30 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.83 ($CO\underline{C}H_3$), 56.65 (OCH_3), 113.53, 121.94, 123.01, 125.83, 126.45, 128.28 (2C), 128.76 (2C), 130.55, 140.13, 156.83 (arom-C), 147.51 (triazole C_3), 151.62 (N=CH), 154.21 (triazole C_5), 169.10 (C=O). UV λ_{max} (ε): 312 (19330), 304 (19900), 292 (19930), 260 (19900), 24 (20430), 222 (16840) nm.

Antioxidant Activity

Chemistry

Butylated hydroxytoluene (BHT) was purchased from E. Merck. 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) and trichloracetic acid (TCA) were bought from Sigma (Sigma–Aldrich GmbH, Sternheim,Germany).

Reducing power

The reducing power of the synthesized compounds was determined according to the method of Oyaizu [14]. Different concentrations of the samples (50-250 µg/mL) in DMSO (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. after which a portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged for 10 min at 1000 x g. The upper layer of 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 spectrophometer. Higher absorbance of the reaction mixture indicated greater reducing power.

Free radical scavenging activity

Free radical scavenging activity of compounds was measured by $DPPH^{\cdot}$, using the method of Blois [15]. Briefly, 0.1 mM solution of $DPPH^{\cdot}$ in ethanol was prepared, and this solution (1 mL) was added to sample solutions in DMSO (3 mL) at different concentrations (50-250 µg/mL). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm in a spectrophometer. Lower absorbance of the reaction mixture indicated 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: 0.997):

$$\text{Absorbance} = 0.0003 \text{ x } DPPH^{\cdot} - 0.0174$$

The capability to scavenge the DPPH radical was calculated using the following equation:

$$DPPH^{\cdot} \text{ scavenging effect (\%)} = (A_0 - A_1/A_0) \text{ x } 100$$

where A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the samples or standards.

Metal chelating activity

The chelation of ferrous ions by the synthesized compounds and standards were estimated by the method of Dinis *et al* [16]. Briefly, the synthesized compounds (50-250 µg/mL) were added to a 2 mM solution of $FeCl_2$ (0.05 mL). The reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and 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 then measured at 562 nm in a spectrophotometer. All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given by the formula: % Inhibition = $(A_0 - A_1/A_0)$ x 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.

Antimicrobial Activity

Simple susceptibility screening test using agar-well diffusion method [17] as adapted earlier [18] was used for determination of antimicrobial activities of **4a-h** compounds. All test microorganisms were obtained from the Microbiologics Environmental Protection Laboratories Company in France and are as follows; *Escherichia coli* ATCC 259222, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 4352, *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 11774, *Bacillus cereus* ATCC 11778. All the newly synthesized compounds were weighed and dissolved in dimethylsulphoxide (DMSO) to prepare extract stock solution of 1 mg/ml.

Each microorganism was suspended in Mueller-Hinton Broth and diluted to 106 colony forming unit (cfu) per ml. They were "flood-inoculated" onto the surface of Mueller Hinton Agar and then dried. Five-millimeter diameter wells were cut from the agar using a sterile cork-borer and 250–5000 μg/50 μl of the chemical substances were delivered into the wells. The plates were incubated for 18 h at 35 °C. Antimicrobial activity was evaluated by measuring the zone of inhibition against the test organism. Dimethylsulphoxide was used as solved control.

Potentiometric Titrations

A Jenway 3040-model ion analyzer and an Ingold pH electrode 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 TBAH in isopropyl alcohol, which is widely used in the titration of acids, was used as titrant. The mV values that were obtained in pH-meter were recorded. Finally, the HNP values were determined by drawing the mL (TBAH)-mV graphic.

Results and Discussion

In this study, the structures of eight new 3-alkyl(aryl)-4-(3-acetoxy-4-methoxybenzylidenamino)-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4a-h**) were identified using by elemental analyses and IR, ^{1}H-NMR, ^{13}C-NMR and UV spectral data.

Antioxidant Activity

The compounds **4a-h** were screened for their *in-vitro* antioxidant activities. Several methods are used to determine antioxidant activities. The methods used in this study are discussed below.

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. 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 for its potential antioxidant activity [19]. The antioxidant activity of a putative antioxidant has been attributed to various mechanisms, among which are prevention chain initiation, binding of transition metal ion catalyst, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [20].

In this study, all the amount of the compounds showed lower absorbance than standard antioxidants. Hence, no activities were observed to reduce metal ions complexes to their lower oxidation state or to take part in any electron transfer reaction. In other words, synthesized compounds did not show the reductive activities, but compound **4b** showed higher activities than blank and its reductive ability was concentration-dependent as seen in Figure **1**. Reducing power of the compound and the standards were found as following order: BHT > α-tocopherol > BHT > **4b**.

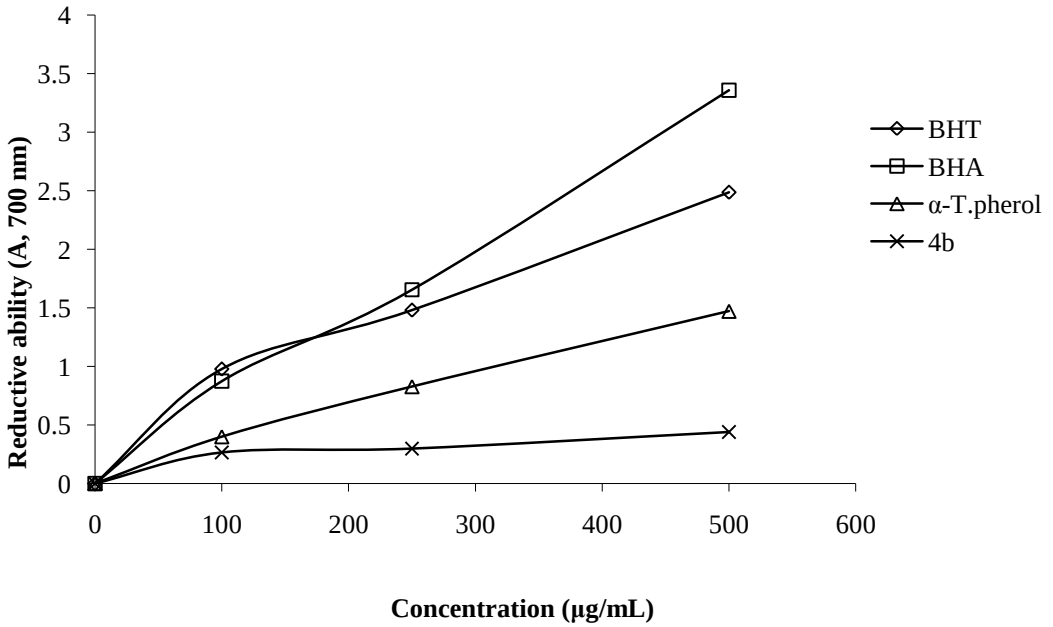


*Figure 1: Total reductive potential of different concentrations of compound **4b**, BHT, BHA and α-tocopherol.*

DPPH• radical scavenging activity

The scavenging of the stable DPPH radical model is a widely used method to evaluate antioxidant activities in a relatively short time compared with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability [21]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [22]. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517 nm induced by antioxidants. The absorption maximum of a stable DPPH radical in ethanol was at 517 nm.

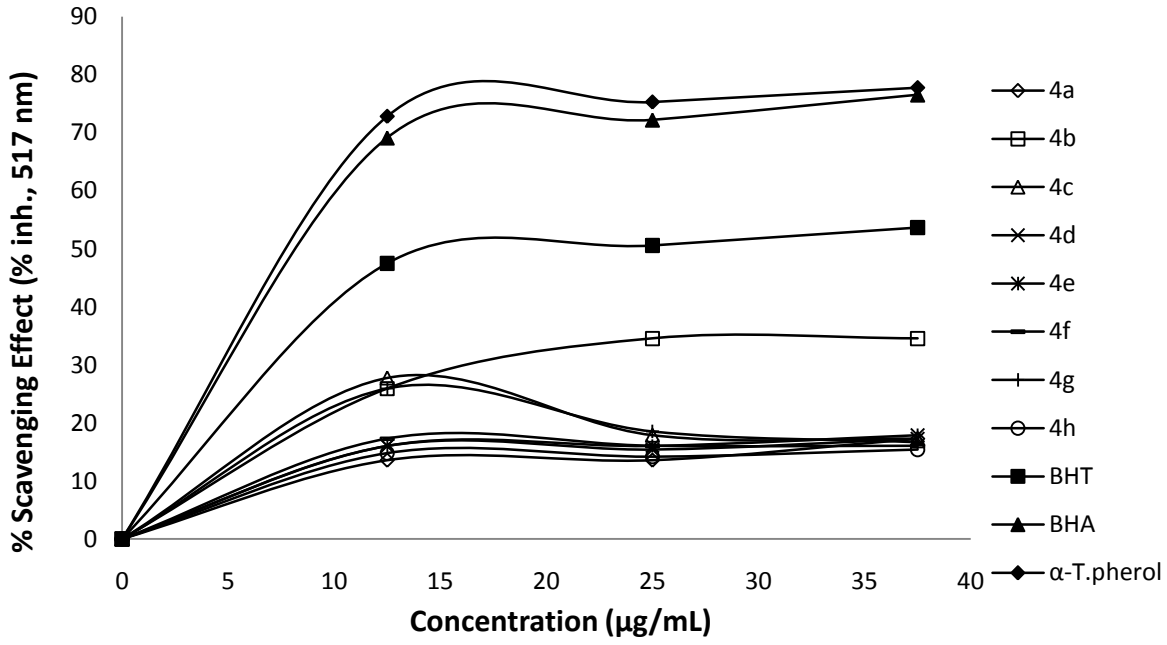


*Figure 2: Scavenging effect of compounds **4a-h**, BHT, BHA and α-tocopherol at different concentrations*

The decrease in absorbance of DPPH radical was caused by antioxidants, because of reaction between antioxidant molecules and radical, progresses, which result in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Hence, DPPH is usually used as a substrate to evaluate antioxidative activity of antioxidants [23]. BHT, BHA and α-tocopherol were used as a reference to antioxidant compounds. Compounds **4a**, **4b**, **4e** and **4h** tested with this method exhibited DPPH free radical scavenging activity in a concentration-dependent manner. Figure **2** illustrates a decrease in the concentration of DPPH radical due to the scavenging ability of these compounds. These results indicate that the newly synthesized **4b** compound showed moderate activity as a radical scavenger, indicating that it has good activities as hydrogen donors. The other compounds showed low scavenging activity. The radical scavenging effect of the compounds and standards decreased in the order of α-tocopherol > BHA > BHT > **4b** > **4e** > **4a** > **4h**.

Ferrous ion chelating activity

The chelating effect towards ferrous ions by the compounds and standards was determined. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator [24]. Transition metals have pivotal role in the generation oxygen free radicals in living organism. The ferric iron (Fe^{3+}) is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on condition, particularly pH [25] and oxidized back through Fenton type reactions with the production of hydroxyl radical or Haber-Weiss reactions with superoxide anions. The production of these radicals may lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may not activate metal ions and potentially inhibit the metal-dependent processes [26].

Also, the production of highly active ROS such as $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ is also catalyzed by free iron though Haber-Weiss reactions:

$$O^{\cdot}_2 + H_2O_2 \rightarrow O_2 + OH^- + OH^{\cdot}$$

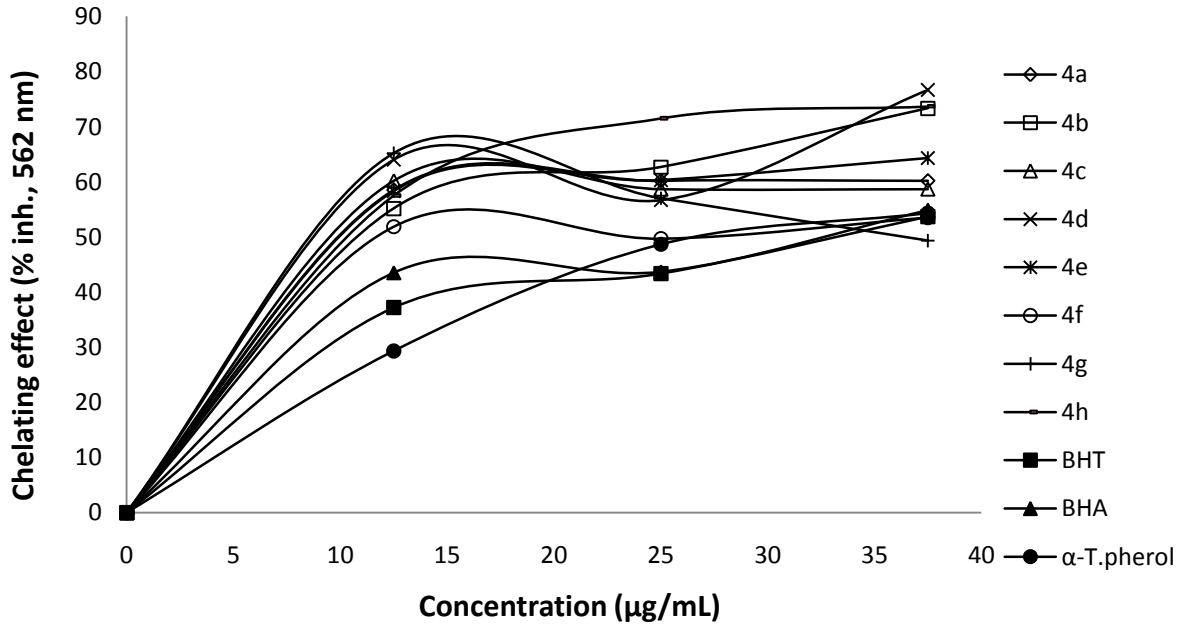


Figure 3: *Metal chelating effect of different amount of the compounds **4a-h**, BHT, BHA and α-tocopherol on ferrous ions.*

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 reactive free radicals via the Fenton reactions:

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

Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion, which is the most powerful pro-oxidant among the various types of metal ions [27]. Ferrous ion chelating activities of the compounds, BHT, BHA and α-tocopherol are shown in Figure **3**. In this study, metal chelating capacity was significant since it reduced the concentrations 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 metal ion [28].

The data obtained from Figure **3** reveal that the compounds demonstrate a marked capacity for iron binding, suggesting that their action as peroxidation protectors may be related to their iron binding capacity. On the other hand, the metal chelating effects of the compounds **4a**, **4b**, **4e** and **4h** were concentration-dependent, the other compounds were not. The metal chelating effect of the compounds and standards decreased in the order of **4h** > **4b** > **4e** > **4a** > BHA > α-tocopherol > BHT. On the other hand, free iron is known to have low solubility and a chelated iron complex has greater solubility in solution, which can be contributed solely by the ligand. Furthermore, the compound-iron complex may also be active, since it can participate in iron-catalyzed reactions.

Antimicrobial Activity

The observed data for the antimicrobial activity of **4a-h** compounds were given in Table **1**. The data reveal that, the highest zone diameter was obtained from **4a** compound against Ec: *Escherichia coli* (ATCC 25922). The screening data also indicate that all of the compounds were found to be effective against *Klepsiella pneumoniae* (ATCC 4352) and *Escherichia coli* (ATCC 25922) strains.

Table 1: Screening for antimicrobial activity of the **4** type compounds

Compound	Microorganisms and inhibition zone (mm)					
	Bs	**Bc**	**Pa**	**Kp**	**Sa**	**Ec**
4a	-	13	-	18	-	26
4b	-	9	-	16	-	13
4c	-	11	-	19	-	14
4d	-	7	-	13	-	16
4e	9	8	-	13	-	17
4f	-	12	-	15	-	13
4g	-	-	-	15	-	11
4h	-	-	-	15	-	14

Bs: *Bacillus subtilis* (ATCC 10978), Bc: *Bacillus cereus* (ATCC 11778), Pa: *Pseudomonas aeruginosa (*ATCC 43288), Kp: *Klepsiella pneumoniae* (ATCC 4352), Sa: *Staphylococcus aureus* (ATCC 6538), Ec: *Escherichia coli* (ATCC 25922).

Potentiometric Titrations

As a separate study, newly synthesized compounds **4** were titrated potentiometrically with TBAH in four non-aqueous solvents: isopropyl and *tert*-butyl alcohol, acetone and DMF. The mV values read in each titration were plotted against 0.05 M TBAH volumes (mL) added, and potentiometric titration curves were obtained for all the cases. From the titration curves, the HNP values were measured, and the corresponding pK_a values were calculated.

The HNP values and the corresponding pK_a values of compounds **4a-h**, obtained from the potentiometric titrations with 0.05 M TBAH in isopropyl alcohol, *tert*-butyl alcohol, acetone and DMF, are presented in Table **2**. As seen in Table **2**, for compound **4a** in isopropyl alcohol, *tert*-butyl alcohol and DMF p*K*a values have not been obtained. Also, for compounds **4e** and **4g** in *tert*-butyl alcohol p*K*a values have not been obtained.

As it is well known, the acidity of a compound depends on some factors. The two most important factors are the solvent effect and molecular structure [7–9,29]. Table **2** shows that the HNP values and corresponding pK_a values obtained from the potentiometric titrations depends on the non-aqueous solvents used and the substituents at C-3, in 4,5-dihydro-1*H*-1,2,4-triazol-5-one ring.

Table 2: *The HNP and the corresponding pKa values of compounds* ***4a-h*** *in isopropyl alcohol, tert-butyl alcohol, DMF and acetone at 25 °C*

Compd.	Isopropyl alcohol		*tert*-Butyl alcohol		DMF		Acetone	
	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a	HNP (mV)	pK_a
4a	-	-	-	-	-	-	-372	15,74
4b	-198	11,64	-316	12,25	-279	13,55	-380	15,72
4c	-262	10,27	-381	12,68	-355	15,45	-277	10,90
4d	-349	15,18	-414	17,77	-352	14,86	-369	15,54
4e	-343	15,12	-	-	-322	14,65	-341	14,58
4g	-366	16,68	-	-	-293	13,87	-151	9,95
4h	-411	16,91	-493	17,93	-373	15,84	-417	16,70

Conclusion

The synthesis and *in-vitro* antioxidant and antimicrobial evaluation of new 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives are described. Compound **4b** demonstrates a marked capacity for antioxidant activity. The data reported with regard to the observed radical scavenging and metal chelating activities of the studied compounds could prevent redox cycling. From the screening results, all of the compounds, especially compound **4a**, were found to be effective against *Klepsiella pneumoniae* (ATCC 4352) and *Escherichia coli* (ATCC 25922) strains. Design and synthesis of novel small molecules can play specifically a protective role in biological systems and in modern medicinal chemistry. These results may also provide some guidance for the development of novel triazole-based therapeutic target.

Acknowledgments

This study was supported by a grant (Project Number: 2008.116.006.1) from Scientific Research Projects Coordination Unit of Karadeniz Technical University. The authors thank Fevzi Aytemiz for the antimicrobial activities.

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