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Synthesis, Antioxidant and Antimicrobial Activities of Some 1-Acetyl-3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones

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ABSTRACT

*3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**3a, b, d, e, g**) reacted with acetic anhydride to afford correspondig 1-acetyl-3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**4a, b, d, e, g**). The newly synthesized 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, including reducing power, free radical scavenging and metal chelating activity. Moreover, antibacterial activity of these five new compounds and eight recently reported 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**3a–h**) were screened agaist seven bacteria such as Bacillus subtilis, Yersinia enterocolitca, Bacillus cereus, Staphylococcus aureus, Escherichia coli, Pasterulla multicida and Klebsiella pneumoniae.*

Keywords: 4,5-Dihydro-1*H*-1,2,4-triazol-5-one, Acetylation, Antioxidant activity, Antibacterial activity.

INTRODUCTION

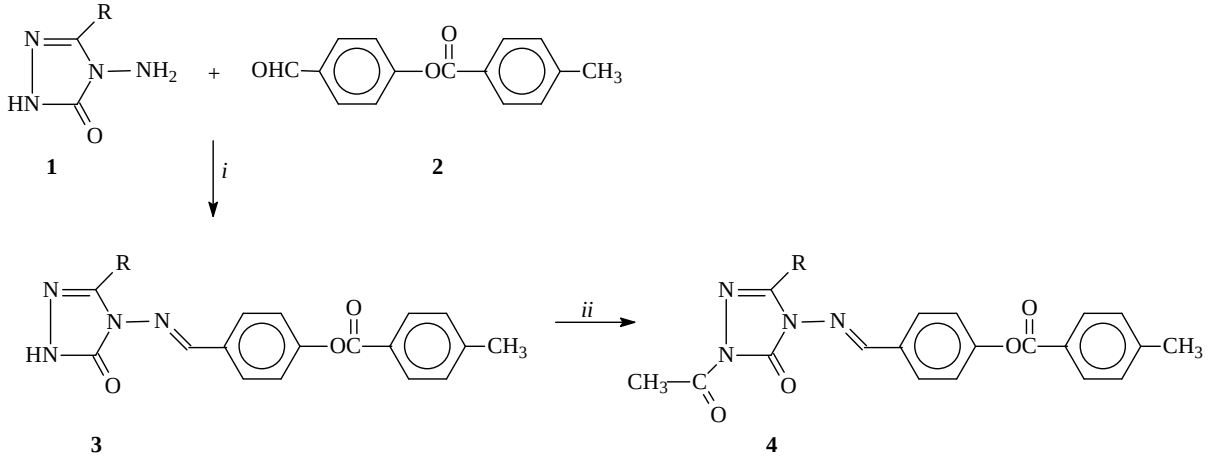
Antioxidants have extensively been studied for their capacity to protect organism and cell from damages that are induced by oxidative stress. Scientists have become more interested in new compounds; they have either synthesized or obtained them from natural sources that could provide active components for preventing or reducing the impact of oxidative stress on cell [1]. Exogenous chemicals and endogenous metabolic processes in human body or in food system might produce highly reactive free radicals, especially oxygen, derived from radicals which are capable of oxidizing biomolecules that result in the cell death and issue damage. Oxidative damages significantly play a pathological role in serious human diseases such as cancer, emphysema, cirrhosis, atherosclerosis and arthritis which have all been correlated with oxidative damage. Also, the excessive generation of ROS induced by various stimuli which exceeds the antioxidant capacity of the organism leads to variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer [2].

In addition, in the past 25 years, the incidence of microbial infection has increased on alarming levels all over the world as a result of antimicrobial resistance. A growing number of immuno-compromised patients are as a result of cancer chemotherapy, organ transplantation and HIV infection which are the major factors contributing to this increase. The health problem demands to search and synthesize a new class of antimicrobial compounds effective against pathogenic microorganisms that developed resistance to the antibiotics used in the current regiment [3-6].

1,2,4-Triazole and 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have been found to have a broad spectrum of biological activities [6-11]. In addition, several articles about the synthesis of some *N*-arylidenamino-4,5-dihydro-

1*H*-1,2,4-triazol-5-one derivatives have been published [9-11]. The acetylation of 4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives have also been reported [9,10,12].

In the present paper, five new 1-acetyl-3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzyliden-amino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4a, b, d, e, g**) were synthesized by the reactions of 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3 a, b, d, e, g**) with acetic anhydride (Scheme **1**). In addition, due to a wide range of applications to find their possible antioxidant activity, the newly synthesized compounds were investigated by using different antioxidant methodologies: 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\cdot}$) free-radical scavenging, reducing power and metal chelating activities. Furthermore, the antimicrobial activity of five new 1-acetyl-3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzyliden-amino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**4a,b,d,e,g**) and eight recently reported 3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**3a-h**)**,** which were synthesized according to reference [11] were determined**.**



i) AcOH, reflux, 1.5 h; ii) Ac_2O, reflux, 1 h

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

Scheme 1

EXPERIMENTAL SECTION

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 a WRS-2A Microproceeor Melting-point apparatus. The IR spectra were recorded on a Alpha-P Bruker FT-IR spectrometer. ^1H and ^{13}C NMR spectra were obtained in deuterated dimethyl sulfoxide with TMS as internal standard using a Bruker Ultrashield 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 spectrometer. Extinction coefficients (ε) are expressed in $L \cdot mol^{-1} \cdot cm^{-1}$.

The starting compounds **1a-h** were prepared from the reactions of the corresponding ester ethoxycarbonylhydrazones with an aqueous solution of hydrazine hydrate as described in the literature [12,13]. The compounds **3a-h** were synthesized from the reactions of the corresponding compounds **1a-h** with compound **2** according to the literature [11].

General Method for the Preparation of 1-Acetyl-3-alkyl(aryl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (4)

The corresponding compound **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 compounds **4a,b,d,e** and **f** as colourless crystals.

1-Acetyl-3-methyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4a): Yield: 79%, m.p. 231-3°C. IR (υ, cm^{-1}): 1766, 1733, 1696 (C=O), 1600 (C=N), 1259 (COO), 837 (1,4-disubstituted benzenoid ring). ^1H NMR (DMSO-d_6): δ 2.33 (s, 3H, CH_3), 2.37 (s, 3H, $PhCH_3$), 2.44 (s, 3H, $COCH_3$), 7.43-7.48

(m, 4H, ArH), 8.00 (d, 2H, ArH), 8.06 (d, 2H, ArH), 9.65 (s, 1H, N=CH). UV λ_{max} (ε): 290 (11230), 252 (11820) nm. Anal. Calcd. For $C_{20}H_{18}N_4O_4$: C, 63.49; H, 4.79; N, 14.81. Found: C, 62.93; H, 4.85; N, 14.46.

1-Acetyl-3-ethyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4b): Yield: 82%, m.p. 183-4°C. IR (υ, cm^{-1}): 1766, 1736, 1694 (C=O), 1601 (C=N), 1258 (COO), 834 (1,4-disubstituted benzenoid ring). ^{1}H NMR (DMSO-d_6): δ 1.27 (s, 3H, $CH_2\underline{CH_3}$), 2.45 (s, 3H, $PhCH_3$), 2.51 (s, 3H, $COCH_3$), 2.79 (s, 3H, $\underline{CH_2}CH_3$), 7.43-7.49 (m, 4H, ArH), 7.99 (d, 2H, ArH), 8.06 (d, 2H, ArH), 9.65 (s, 1H, N=CH). ^{13}C NMR (DMSO-d_6): δ 10.02 ($CH_2\underline{CH_3}$), 19.11 ($\underline{CH_2}CH_3$), 21.77 ($PhCH_3$), 23.99 ($CO\underline{C}H_3$), 123.33 (2C), 126.33, 129.83 (2C), 130.08 (2C), 130.44 (2C), 131.31, 145.28, 153.78 (arom-C), 148.61 (triazole C_3), 150.71 (N=CH), 155.40 (triazole C_5), 164.77 (COO), 166.48 ($\underline{C}OCH_3$). UV λ_{max} (ε): 286 (16990), 250 (19350) nm. Anal. Calcd. For $C_{21}H_{20}N_4O_4$: C, 64.28; H, 5.14; N, 14.28. Found: C, 63.49; H, 5.16; N, 13.96.

1-Acetyl-3-benzyl-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4d): Yield: 84%, m.p. 149-50°C. IR (υ, cm^{-1}): 1728 (C=O), 1604 (C=N), 1257 (COO), 833 (1,4-disubstituted benzenoid ring), 739 and 688 (monodisubstituted benzenoid ring). ^{1}H NMR (DMSO-d_6): δ 2.44 (s, 3H, $PhCH_3$), 2.52 (s, 3H, $COCH_3$), 4.17 (s, 2H, CH_2Ph), 7.26-7.47 (m, 9H, ArH), 7.95 (d, 2H, ArH), 8.05 (d, 2H, ArH), 9.61 (s, 1H, N=CH). ^{13}C NMR (DMSO-d_6): δ 21.76 ($PhCH_3$), 24.04 ($CO\underline{C}H_3$), 31.49 (CH_2Ph), 123.29 (2C), 126.33, 127.47, 129.01 (2C), 129.48 (2C), 129.86 (2C), 130.07 (2C), 130.43 (2C), 131.26, 135.17, 145.27, 153.77 (arom-C), 148.52 (triazole C_3), 148.80 (N=CH), 155.04 (triazole C_5), 164.76 (COO), 166.46 ($\underline{C}OCH_3$). UV λ_{max} (ε): 290 (17240), 252 (20680) nm. Anal. Calcd. For $C_{26}H_{22}N_4O_4$: C, 68.71; H, 4.88; N, 12.33. Found: C, 67.61; H, 4.92; N, 12.13.

1-Acetyl-3-(4-methylbenzyl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4e): Yield: 85%, m.p. 156-8°C. IR (υ, cm^{-1}): 1734 (C=O), 1607 (C=N), 1257 (COO), 827 (1,4-disubstituted benzenoid ring). ^{1}H NMR (DMSO-d_6): δ 2.26 (s, 3H, $CH_2Ph\underline{CH_3}$), 2.44 (s, 3H, $PhCH_3$), 2.51 (s, 3H, $COCH_3$), 4.11 (s, 2H, CH_2Ph), 7.14 (d, 2H, ArH), 7.27 (d, 2H, ArH), 7.44-7.47 (m, 4H, ArH), 7.95 (d, 2H, ArH), 8.05 (d, 2H, ArH), 9.60 (s, 1H, N=CH). ^{13}C NMR (DMSO-d_6): δ 21.10 ($CH_2Ph\underline{C}H_3$), 21.76 ($PhCH_3$), 24.05 ($CO\underline{C}H_3$), 31.11 (CH_2Ph), 123.31 (2C), 126.32, 129.36 (2C), 129.57 (2C), 129,86 (2C), 130.07 (2C), 130.43 (2C), 131.27, 132.01, 136.58, 145.28, 153.76 (arom-C), 148.51 (triazole C_3), 148.95 (N=CH), 155.00 (triazole C_5), 164.77 (COO), 166.47 ($\underline{C}OCH_3$). UV λ_{max} (ε): 290 (25580), 252 (29950) nm. Anal. Calcd. For $C_{27}H_{24}N_4O_4$: C, 69.22; H, 5.16; N, 11.96. Found: C, 68.51; H, 5.19; N, 11.82.

1-Acetyl-3-(4-chlorobenzyl)-4-[4-(4-methylbenzoxy)benzylidenamino]-4,5-dihydro-1*H*-1,2,4-triazol-5-one (4g): Yield: 78%, m.p. 173-4°C. IR (υ, cm^{-1}): 1728 (C=O), 1602 (C=N), 1258 (COO), 832 (1,4-disubstituted benzenoid ring). ^{1}H NMR (DMSO-d_6): δ 2.44 (s, 3H, $PhCH_3$), 2.46 (s, 3H, $COCH_3$), 4.18 (s, 2H, CH_2Ph), 7.41-7.46 (m, 8H, ArH), 7.94 (d, 2H, ArH), 8.05 (d, 2H, ArH), 9.61 (s, 1H, N=CH). ^{13}C NMR (DMSO-d_6): δ 21.77 ($PhCH_3$), 24.03 ($CO\underline{C}H_3$), 30.80 (CH_2Ph), 123.31 (2C), 126.33, 128.93 (2C), 129.88 (2C), 130.07 (2C), 130.43 (2C), 131.24, 131.45 (2C), 13 2.17, 134.17, 145.28, 153.79 (arom-C), 148.52 (triazole C_3), 148.52 (N=CH), 155.02 (triazole C_5), 164.76 (COO), 166.44 ($\underline{C}OCH_3$). UV λ_{max} (ε): 292 (20750), 252 (25130) nm. Anal. Calcd. For $C_{26}H_{21}N_4O_4Cl$: C, 63.87; H, 4.33; N, 11.46. Found: C, 62.62; H, 4.39; N, 11.34.

Antioxidant Activity

Chemicals: 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), ethylenediaminetetraacetic acid (EDTA) 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] as explained in the literature [11].

Free radical scavenging activity: Free radical scavenging activity of compounds was measured by $DPPH^{\cdot}$, using the method of Blois [15] as explained inthe literature [11].

Metal chelating activity: The chelation of ferrous ions by the synthesized compounds and standards were estimated by the method of Dinis *et al*. [16] as explained in the literature [11].

Antimicrobial Activity

Simple susceptibility screening test using agar-well diffusion method [17] as adapted earlier [18] was used for determination of antimicrobial activities of **3a-h, 4a, 4b, 4d, 4e** and **4g** compounds. All test microorganisms were obtained from the Microbiologics Environmental Protection Laboratories Company in France and are as follows; *Escherichia coli* (ATCC-25922), *Klebsiella pneumoniae* (ATCC-4352), *Staphylococcus aureus* (ATCC-6538),

Bacillus subtilis (ATCC-11774), *Bacillus cereus* (ATCC-11778), *Yersinia enterocolitica* (ATCC-27729), *Pasteurella multocida* (ATCC-12945). 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.

RESULTS AND DISCUSSION

In this study, the structures of five new 1-acetyl-3-alkyl(aryl)-4-[4-(4-methyl-benzoxy)benzylidenamino]-4,5-dihydro-1H-1,2,4-triazol-5-ones (**4a**,**b**,**d**,**e**,**g**) were identified using by elemental analyses and IR, ^{1}H-NMR, ^{13}C-NMR and UV spectral data.

Antioxidant Activity

The compounds **4a**,**b**,**d**,**e**,**g** 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]. In this study, all of the amounts of the compounds showed lower absorbance than blank. 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, compounds did not show the reductive activities.

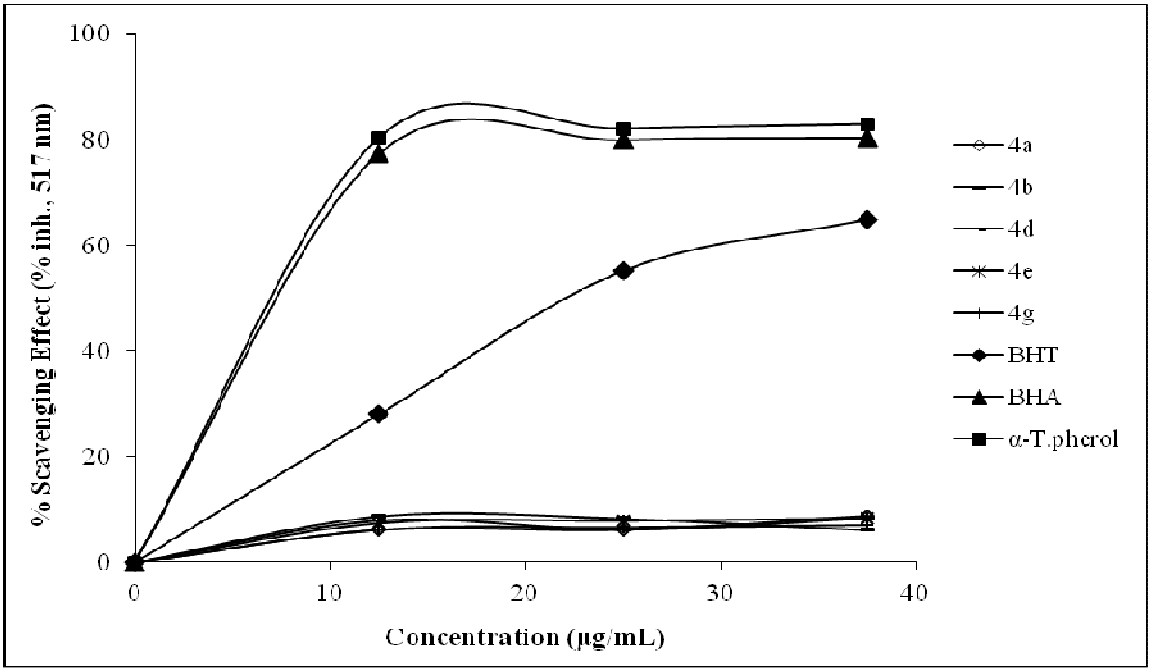


Figure 1. Scavenging effect of compounds 4a,b,d,e,g, BHT, BHA and α-tocopherol at different concentrations (12,5-25-37.5 µg/mL)

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 [20]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [21]. The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517 nm induced by antioxidants.

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 [22]. BHT, BHA and α-tocopherol were used as a reference to antioxidant compounds. All the compounds tested with this method exhibited low DPPH free radical scavenging activity as seen in Figure **1**.

Ferrous ion chelating activity: 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 [23]. 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 [24] 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 [25]. 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:

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

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 [26]. Ferrous ion chelating activities of the synthesized compounds, EDTA and α-tocopherol are shown in Figure **2**. The data obtained from Figure **2** reveal that the compounds, except **4b** and **4e**, demonstrate a marked capacity for iron binding in a concentration-dependent manner, 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 > **4d** > **4a** > α-tocopherol > **4g**.

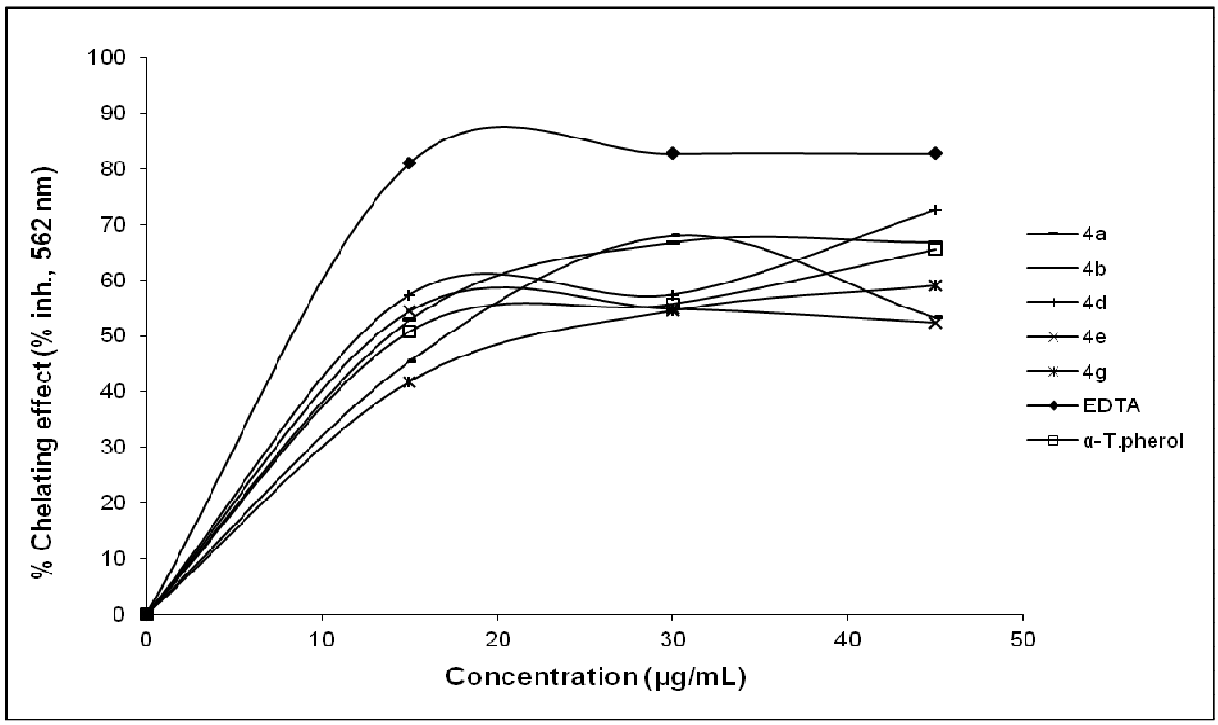


Figure 2. Metal chelating effect of different amount of the compounds 4a,b,d,e,g, EDTA and α-tocopherol on ferrous ions

Antimicrobial Activity

The observed data for the antimicrobial activity of **3** and **4** type compounds were given in Table **1**. The data reveal that, the highest zone diameter was obtained against *Bacillus subtilis* (ATCC 10978) for all the compounds. The screening data also indicate that compound **3a** was found to be effective against *Klebsiella pneumoniae* (ATCC-4352) and compound **4d** was found to be effective against *Pasteurella multocida* (ATCC-12945) strains.

Table 1. Screening for antimicrobial activity of the 3 and 4 type compounds

Compound	Microorganisms and inhibition zone (mm)						
	Bs	**Ye**	**Bc**	**Sa**	**Ec**	**Pa**	**Kp**
3a	12	10	9	11	8	-	18
3b	11	12	8	10	8	-	11
3c	14	-	-	10	8	-	14
3d	11	10	8	10	8	-	-
3e	11	-	-	9	7	-	-
3f	11	-	7	11	9	-	-
3g	11	-	-	-	-	-	12
3h	10	11	10	12	9	-	-
4a	14	14	-	10	-	-	12
4b	19	13	-	-	11	14	-
4d	17	12	9	-	8	22	-
4e	17	9	-	-	8	-	-
4g	14	-	9	-	-	10	-

Bs: Bacillus subtilis (ATCC-11774), Ye: Yersinia enterocolitica (ATCC-27729), Bc: Bacillus cereus (ATCC-11778), Sa: Staphylococcus aureus (ATCC-6538), Ec: Escherichia coli (ATCC-25922), Pm: Pasteurella multocida (ATCC-12945), Kp: Klepsiella pneumoniae (ATCC-4352).

CONCLUSION

The synthesis and *in-vitro* antioxidant and antimicrobial evaluation of new 4,5-dihydro-1H-1,2,4-triazol-5-one derivatives are described. Compound **4d** demonstrates a marked capacity for iron binding. From the screening results, the highest zone diameter was obtained against *Pasteurella multocida* (ATCC-12945) strain for compound **4d**. Design and synthesis of novel small molecules can play specifically a protective role in biological systems and in modern medicinal chemistry. In this regard, these results may also provide some guidance for the development of novel triazole-based therapeutic target.

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