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Antibacterial and antioxidant activities of novel 2-ethoxy-4-[(4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoate derivatives

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ABSTRACT: In the present study, 3-alkyl/aryl-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**1**) reacted with 3-ethoxy-4-(3-methoxybenzoxy)-benzaldehyde (**2**) to obtain the corresponding nine new 2-ethoxy-4-[(3-alkyl/aryl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoates (**3**). The compounds **3** were also treated with 4-piperidinecarboxamide in the presence of formaldehyde according to the Mannich reaction to synthesize 2-ethoxy-4-{[3-alkyl/aryl-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]-azomethine}-phenyl 3-methoxy-benzoates (**4**). The structures of Schiff base and Mannich base derivatives were identified with spectral methods. Antimicrobial and antioxidant screening were carried out and discussed. Mannich base derivatives were found to be more active than Schiff bases in the biological screening. The effect values of Mannich Base derivatives dominate Neomycin and Streptomycin antibiotics.

KEYWORDS: Schiff base; Mannich base; 1,2,4-triazole-5-one; antimicrobial activity; antioxidant activity.

1. INTRODUCTION

Antibiotic resistance is one of the significant public health concerns worldwide. The rapid emergence and prevalence of antibiotic-resistant pathogens require a serious effort to identify, develop, and design new antibiotics [1]. Considering the importance of heterocyclic compounds in medicinal chemistry, the design of novel heterocycles can play a vital role.

Triazoles are five-membered heterocyclic compounds containing three nitrogen atoms. Some of the modern drugs which have a triazole moiety are alprazolam, triazolam, estazolam (hypnotic, sedative, tranquilizer), trazodone (antidepressant, anxiolytic), trapidil (hypotensive), terconazole (antifungal), hexaconazole (antifungal), etizolam (amnesic, anxiolytic, anticonvulsant, hypnotic, sedative, and skeletal muscle relaxant), rilmazafone (hypnotic, anxiolytic) and rizatriptan (antimigraine agent) [2]. Some 1,2,4-triazoles and 4,5-dihydro-1*H*-1,2,4-triazol-5-ones have shown various biological activities [3-8].

The classical Mannich reaction, a three-component condensation between structurally diverse substrates containing at least one active hydrogen atom, an aldehyde component, and an amine reagent, leads to a class of compounds known as Mannich bases [9]. Mannich bases have applications in the pharmaceutical and other industries, such as petroleum, cosmetics, dyes, and food. Mannich bases acquired from the 1,2,4-triazoles have been reported to have biological activities such as antioxidant, antifungal, anti-lipase, and antibacterial properties [10-14].

As a result of the increasing interest in new 1,2,4-triazole compounds, including potential biological active moieties, a series of 2-ethoxy-4-[(4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoates were obtained, and their antimicrobial and antioxidant activities were evaluated. In this

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way, nine novel **3**-type compounds were synthesized by the reactions of 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (**1**) with 3-ethoxy-4-(3-methoxybenzoxy)-benzaldehyde (**2**). In addition, **3**-type compounds were treated with 4-piperidinecarboxamide in the presence of formaldehyde according to the Mannich reaction to synthesize **4a,b,d-g** (Figure 1). Moreover, the antimicrobial activity of compounds **3** and their *N*-Mannich base derivatives **4** were determined. Furthermore, fifteen novel compounds' antioxidant activities were evaluated using reducing power, free radical scavenging, and metal chelating activities.

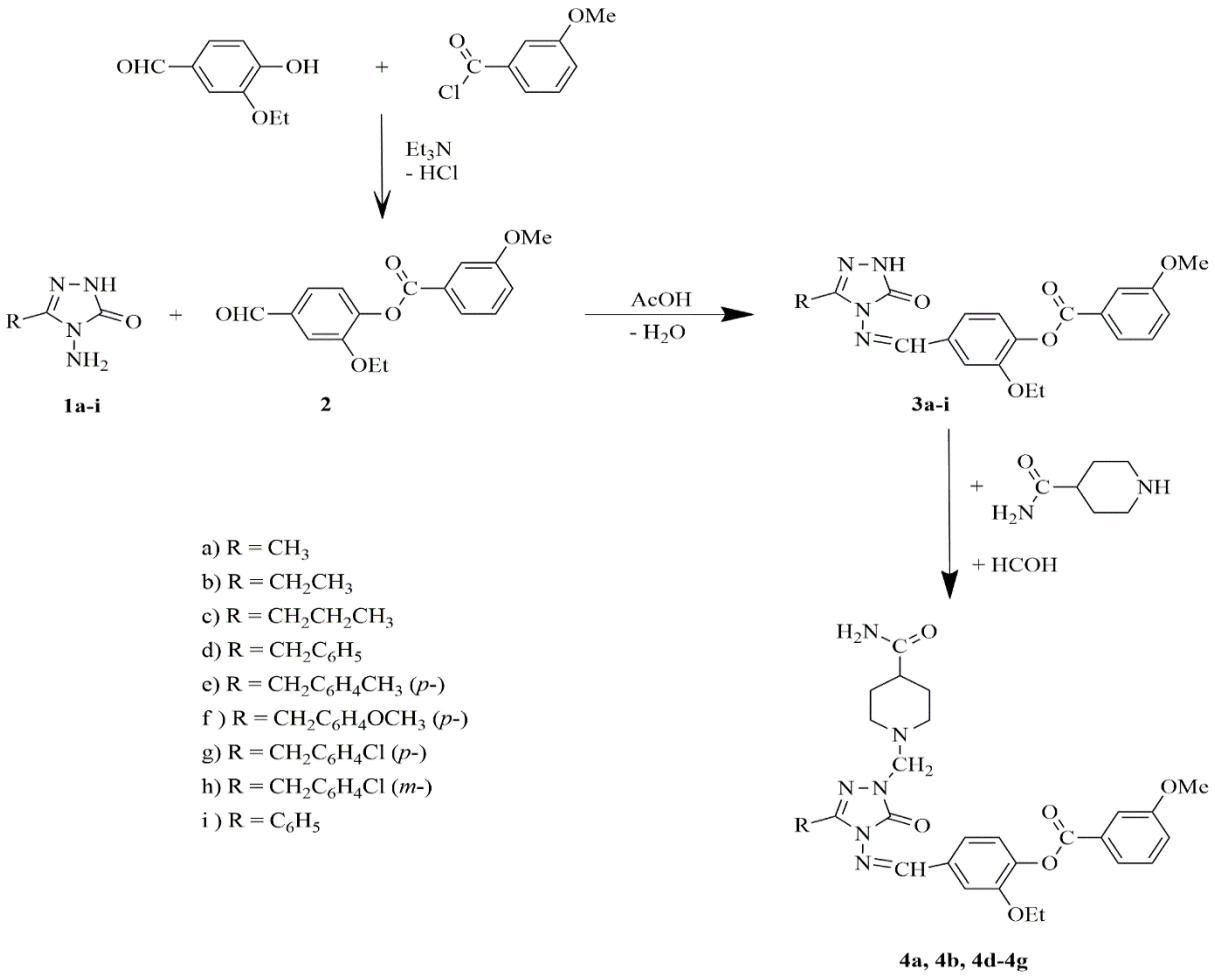

Figure 1. The reactions route of the synthesized compounds

2. RESULTS AND DISCUSSION

2.1. Chemistry

In the study, nine new 2-ethoxy-4-[(3-alkyl/aryl-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoates (**3**) and six new 2-ethoxy-4-{[3-alkyl(aryl)-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoates (**4**) were synthesized as shown in Figure 1. The structures of nine new Schiff bases (**3**) and their six new *N*-Mannich base derivatives (**4**) were characterized using IR, ^{1}H NMR, and ^{13}C NMR data.

2.2. Antimicrobial activity

The new compounds were screened for their antimicrobial activity in the present work. The antimicrobial activity was graded in terms of the diameter of the inhibition zone: – inactive (<5.5 mm); + mildly active (5.5–10 mm); ++ moderately active (11–16 mm); +++ highly active (≥17 mm) as shown in Table 1 [15].

Table 1. Antimicrobial activity of the compounds **3** and **4**.

Compounds	Microorganisms and inhibition zone (mm)					
	Bs	Bc	Pa	Kp	Sa	Ec
3a	14 (++)	9 (+)	10 (+)	14 (++)	12 (++)	18 (+++)
3b	12 (++)	14 (++)	10 (+)	16 (++)	13 (++)	17 (+++)
3c	17 (+++)	15 (++)	14 (++)	15 (++)	11 (++)	15 (++)
3d	9 (+)	11 (++)	10 (+)	13 (++)	15 (++)	14 (++)
3e	13 (++)	13 (++)	13 (++)	14 (++)	10 (+)	14 (++)
3f	12 (++)	9 (+)	11 (++)	8 (+)	13 (++)	11 (++)
3g	19 (+++)	15 (++)	19 (+++)	16 (++)	12 (++)	14 (++)
3h	11 (++)	12 (++)	14 (++)	12 (++)	10 (+)	9 (+)
3i	16 (++)	8 (+)	12 (++)	15 (++)	16 (++)	19 (+++)
4a	18 (+++)	17 (+++)	15 (++)	15 (++)	17 (+++)	16 (++)
4b	16 (++)	13 (++)	19 (+++)	19 (+++)	16 (++)	11 (++)
4d	15 (++)	15 (++)	17 (+++)	17 (+++)	21 (+++)	19 (+++)
4e	15 (++)	15 (++)	13 (++)	14 (++)	19 (+++)	10 (+)
4f	20 (+++)	18 (+++)	19 (+++)	16 (++)	22 (+++)	17 (+++)
4g	18 (+++)	13 (++)	15 (++)	18 (+++)	17 (+++)	16 (++)
Amp.	33	36	36	35	37	34
Neo.	17	17	17	16	13	16
Str.	12	12	12	11	21	10

Bs: *Bacillus subtilis* (ATCC-11774), Bc: *Bacillus cereus* (ATCC-11778), Pa: *Pseudomonas aeruginosa* (ATCC-27853), Kp: *Klebsiella pneumoniae* (ATCC-4352) Sa: *Staphylococcus aureus* (ATCC-6538), Ec: *Escherichia coli* (ATCC-25922), Amp.: Ampicillin (3261), Neo.: Neomycin (3360), Str.: Streptomycin (3385).

The bacterial strain in which the synthesized **3** type Schiff bases were most effective was *E. coli*. B. *subtilis*, a gram-positive strain of bacteria, was the second most affected bacteria. The values obtained for other gram-positive bacteria, *B. cereus* and *S. aureus*, were similar. In contrast to the *E. coli* strain, the effect value obtained for *P. aureginosa* and *K. pneumoniae* was generally found in the intermediate range. As a result of the comparison of Schiff bases synthesized in the study with standard antibiotics, obtaining results that compete with Neomycin and Streptomycin was accepted as an essential effect. However, values close to the standard antibiotic Ampicillin could not be reached.

The effect value of **4** type Mannich bases obtained as the other compound group is considerably higher than the Schiff bases from which they are derived. It is stated as a promising result that the high-level effect is mainly obtained in both gram-positive and gram-negative strains. The effect values of 6 different synthesized Mannich Bases dominate Neomycin and Streptomycin antibiotics.

In a recent study, the effect of compounds containing 1,2,4-triazole ring on *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 strains was examined, and the presence of antibacterial effect was determined [16].

In the following years, a similar study was carried out on gram-negative bacteria with spores, and data on the presence of high-level effects were obtained. This study determined a significant effect value against other gram-negative bacteria [17].

In the study examining the antibacterial effects of Schiff Bases, moderate effects were observed against *E. coli*, while low effects were observed against *S. typhi*. The study supports that the antibacterial effect of Schiff Bases is not high. In addition, it has been determined that Mannich bases have antibacterial and antifungal effects even at low concentrations.

The antibacterial effect of Schiff Bases was also tested, and in one of these studies, a low impact on *E. coli* and *S. typhi* was obtained. However, Mannich bases have been found to have antibacterial and antifungal effects even at low concentrations [18, 19].

The fact that there is a very similar relationship between the studies examined in the literature and this study was considered a consistent result. The study was carried out *in vitro* to determine the presence of the antibacterial effect. After deciding on this effect, a different study will be presented in detail, selecting suitable organisms, determining MIC values, and analyzing ADME.

2.3. Antioxidant activity

The FRAP assay uses antioxidants as reductants in the redox-coupled colorimetric method and implements an oxidant system that can be easily reduced in stoichiometric excess. Figures 2 and 3 show that the compounds showed a feeble reducing activity for Fe^{+3} compared to standard antioxidants. In other words, the compounds showed a relatively poor ability of electron donors to scavenge free radicals.

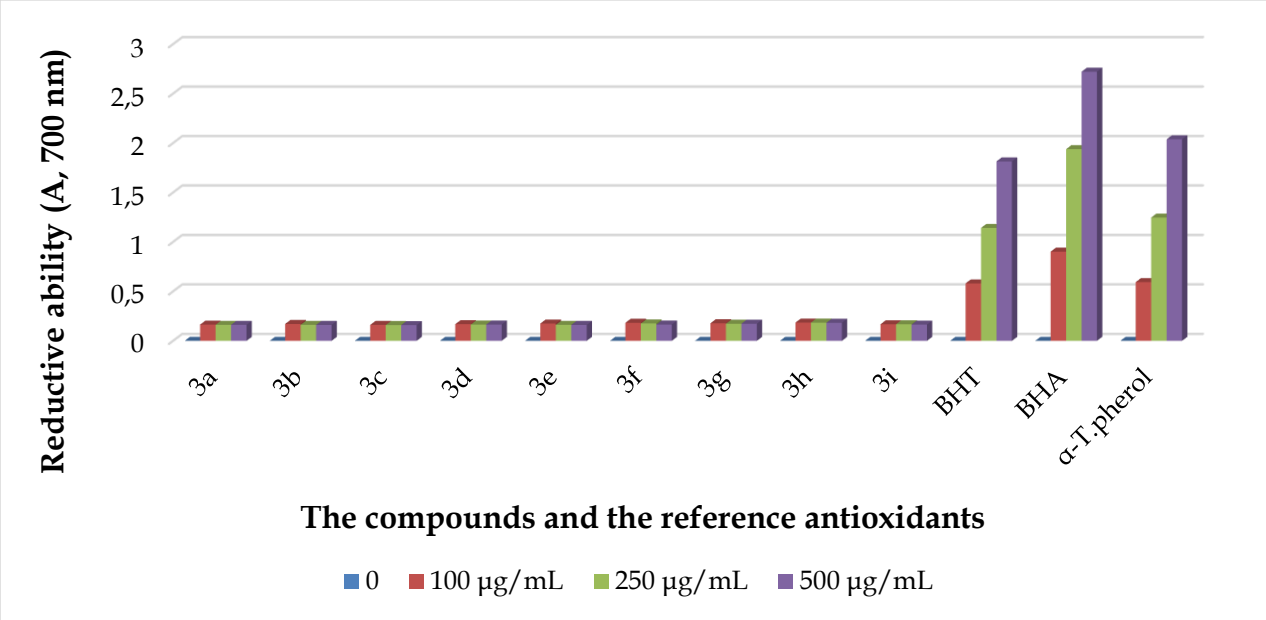

Figure 2. Total reductive potential of different concentrations of the compounds **3**, BHT, BHA and α-tocopherol

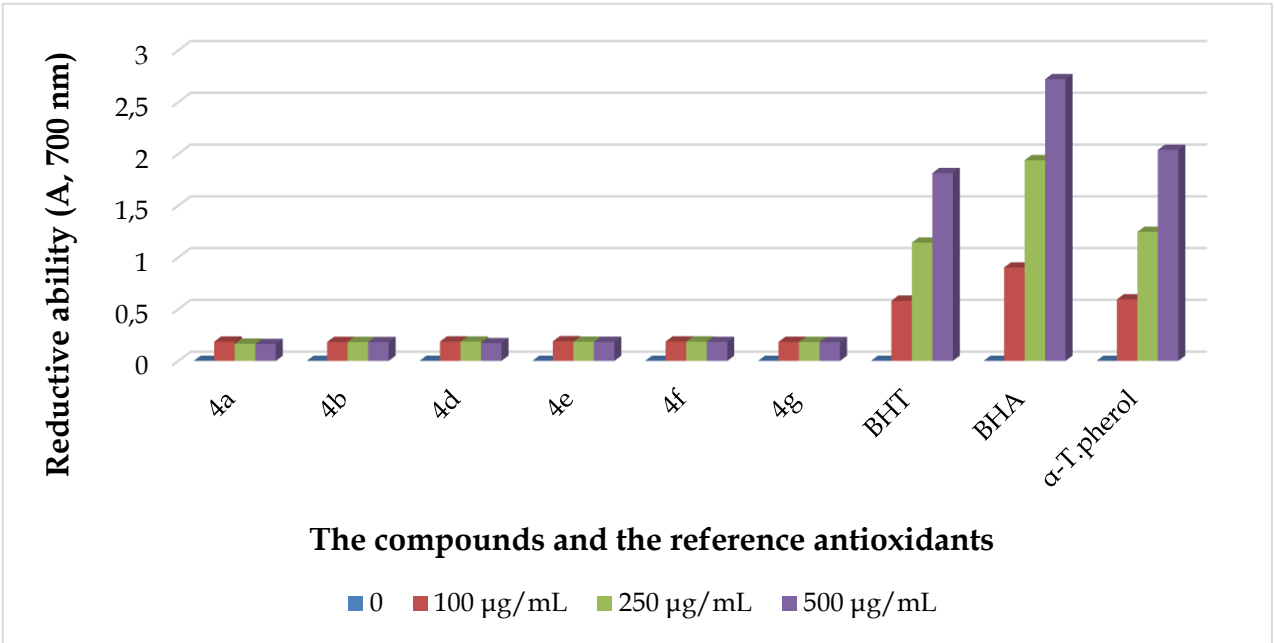

Figure 3. Total reductive potential of different concentrations of the compounds **4**, BHT, BHA and α-tocopherol

All the compounds tested with this method showed higher absorbance than the standard antioxidant reactions and nearly the same as the control reaction's absorbance. Antioxidants cause a decrease in the absorbance of DPPH radicals because the reaction between antioxidant molecules and radicals progresses, which results in the scavenging of the radical by hydrogen donation. Figures 4 and 5 illustrate a decrease in the concentration of DPPH radicals due to the scavenging ability of the standard antioxidants, but the same

results were not observed for compounds **3** and **4**. These results indicate that the newly synthesized compounds showed no activities as radical scavengers.

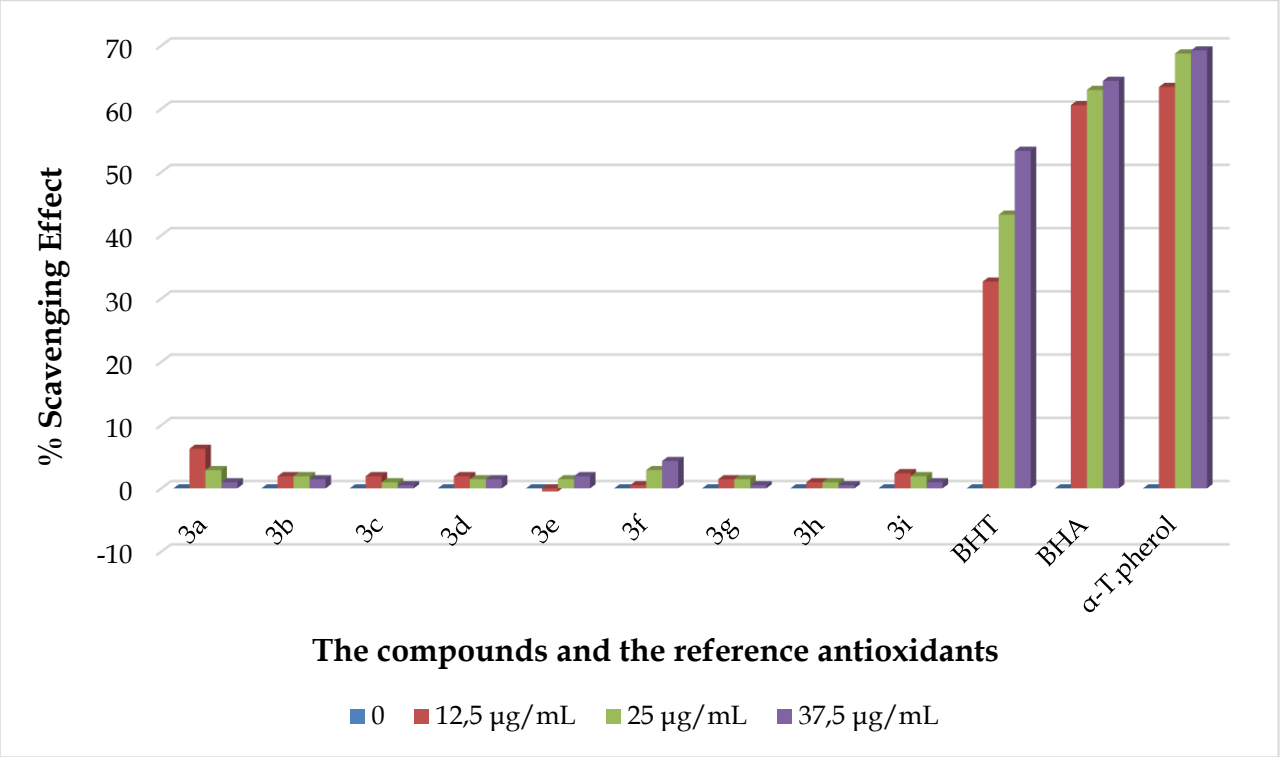

Figure 4. Scavenging effect of the compounds **3**, BHT, BHA and α-tocopherol at different concentrations

Ferrous ion chelating activities of compounds **3** and **4**, EDTA, and α-tocopherol are shown in Figures 6 and 7, respectively. In this study, metal chelating capacity was vital as it reduced the concentrations of the catalyzing transition metal. Chelating agents that form σ-bonds with the metal have been reported to be effective as secondary antioxidants because they stabilize the oxidized form of the metal ion by reducing the redox potential [20]. The data in Figures 6 and 7 reveal that the compounds exhibit a significant iron-binding capacity; this suggests that their effects as peroxidation protectors may be related to their iron-binding capacity. However, the chelating activity of newly synthesized Schiff bases does not depend on concentration. As a result, it was seen that the new Mannich base derivatives were more active than the new Schiff base derivatives.

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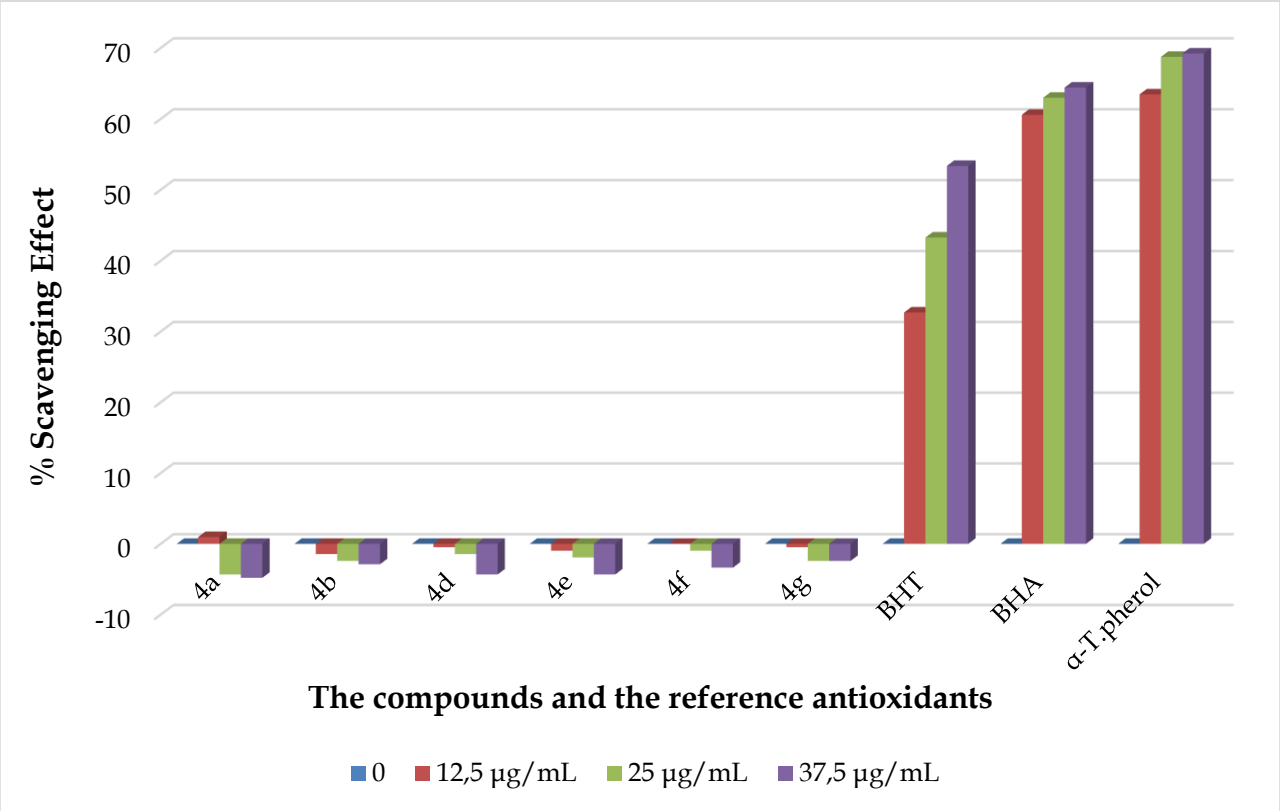

Figure 5. Scavenging effect of the compounds **4**, BHT, BHA and α-tocopherol at different concentrations

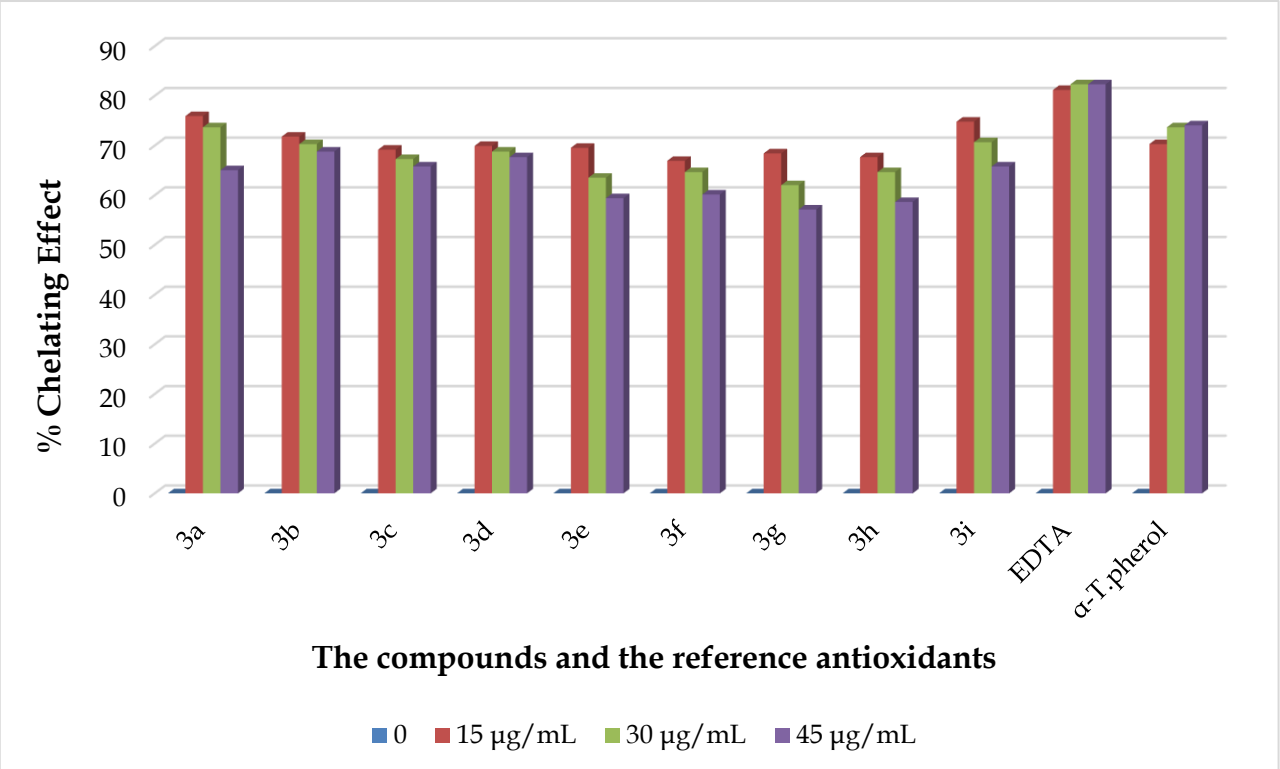

Figure 6. Metal chelating effect of different amount of the compounds **3**, α-tocopherol and EDTA

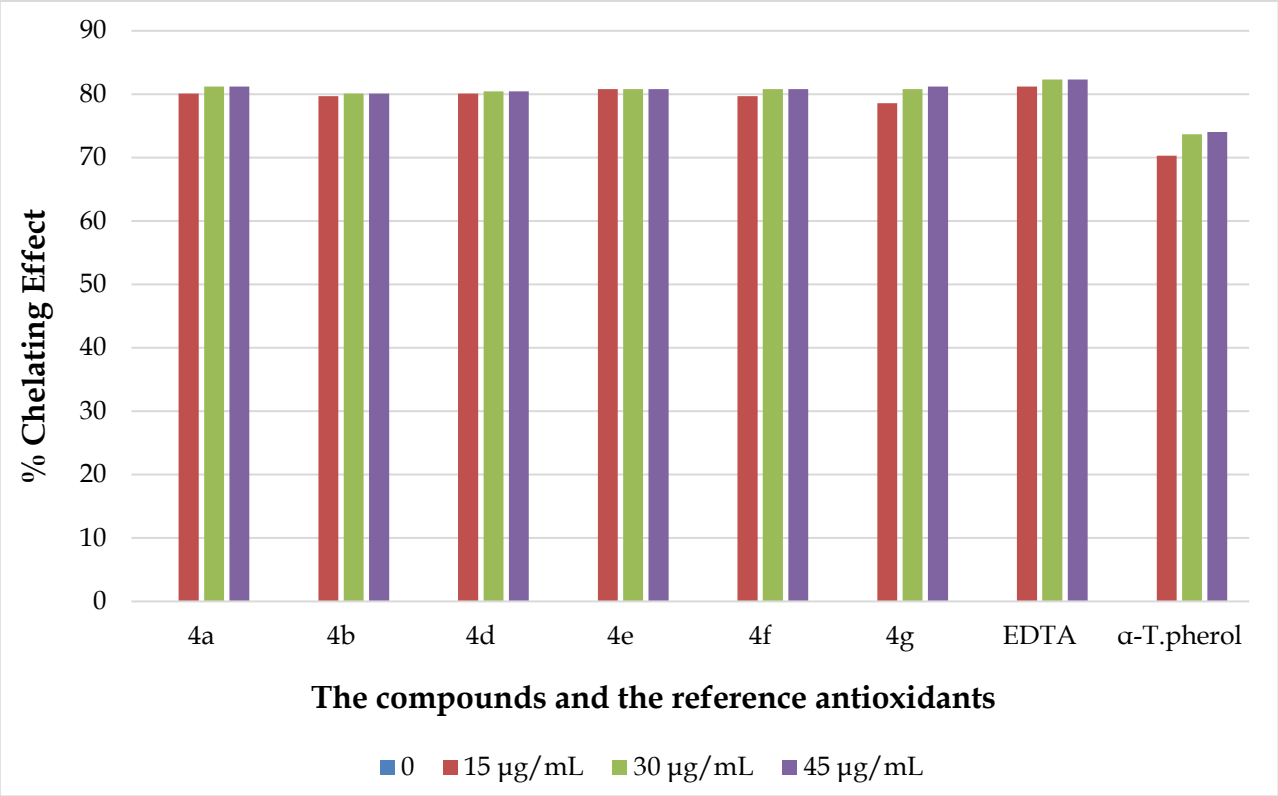

Figure 7. Metal chelating effect of different amount of the compounds **4**, α-tocopherol and EDTA

3. CONCLUSION

Novel 4,5-dihydro-1*H*-1,2,4-triazol-5-ones were synthesized and screened for their *in vitro* antimicrobial and antioxidant capacity. From the results, all the Mannich base derivatives showed great activity against the tested microorganisms. Besides, all the new compounds demonstrate a remarkable ability for the chelating effect, but Mannich bases were more active than Schiff bases. In this regard, the observed biological activity of the new compounds could improve the new triazole-based therapeutic target.

4. MATERIALS AND METHODS

4.1. Chemistry

Chemicals used for the study were purchased from Aldrich, Fluka and Merck. Melting point was taken on Electrothermal Melting-point Apparatus in open capillary tube and was not corrected. The IR spectra were determined on Alpha-P Bruker FT-IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in deuterated dimethyl sulfoxide (DMSO-d_6) with tetramethylsilane (TMS) using Bruker Ultrashield Plus Biospin spectrophotometer at 400 MHz and 100 MHz. 3-Alkyl/Aryl-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-ones (1) were synthesized as explained in the literature [21,22].

4.2. General procedure for the synthesis of the compounds 3

3-Ethoxy-4-hydroxybenzaldehyde (0.01 mol) dissolved in ethyl acetate (100 mL) was treated with 3-methoxybenzoyl chloride (0.01 mol). Then triethylamine (0.01 mol) was slowly added to this solution by stirring at 0-5 °C. The stirring process was continued for two hours; afterward, the mixture was refluxed for three hours and filtered. After evaporation *in vacuo* of the filtrate, the crude product was washed with water. Finally, recrystallization from ethyl alcohol gave the novel compound **2**. Yield: 95; m.p. 92°C; IR (cm^{-1}) υ_{max}: 2883 and 2790 (CHO), 1749, 1725 (C=O), 1269 (COO), 793 and 745 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.34 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.88 (s, 3H, OCH_3), 4.15 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.18-7.21 (m, 1H, Ar-H), 7.34-7.36 (m, 1H, Ar-H), 7.41-7.45 (m, 1H, Ar-H), 7.47-7.53 (m, 2H, Ar-H), 7.71 (d, 1H, Ar-H; *J*=1,20 Hz), 7.80 (d, 1H, Ar-H; *J*=0,80 Hz), 10.00 (s, 1H, CHO) ppm; ^{13}C NMR (DMSO-d_6,

100 MHz) δ: 14.54 ($OCH_2\underline{CH_3}$), 55.52 (OCH_3), 64.76 ($O\underline{CH_2}CH_3$), [112.09, 114.67, 120.33, 122.74, 123.50, 124.53, 129.65, 130.33, 135.26, 145.60, 151.53, 159.76] (Ar-C), 164.08 (COO), 191.08 (CHO) ppm. Afterward, the compound **1** (0.01 mol) was dissolved in 20 mL acetic acid, then treated with 0.01 mol 3-ethoxy-4-(3-methoxybenzoxy)-benzaldehyde (**2**). After the obtained mixture was refluxed for 1.5 hours and evaporated at 50-55 °C *in vacuo*, the residue was recrystallized several times from ethanol to yield pure compounds **3a-i** as colorless crystals.

4.2.1. 2-Ethoxy-4-[(3-methyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]phenyl 3-methoxy-benzoate (3a)

White solid; yield: 98%; mp: 160 °C; IR (cm^{-1}) υ_{max} 3191 (NH), 1723, 1693 (C=O), 1600, 1583 (C=N), 1264 (COO), 799 and 700 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.34 (s, 3H, CH_3), 1.34 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.89 (s, 3H, OCH_3), 4.13 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.18-7.19 (m, 1H, Ar-H), 7.20-7.26 (m, 1H, Ar-H), 7.40-7.47 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2.80, 1,60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.80 (s, 1H, N=CH), 9.95 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 11.51 (CH_3), 14.65 ($OCH_2\underline{CH_3}$), 55.53 (OCH_3), 64.71 ($O\underline{CH_2}CH_3$), [112.15, 114.60, 120.25, 121.40, 122.74, 123.33, 129.59, 130.58, 132.53, 145.76, 151.97, 159.73] (Ar-C), 143.10 (Triazol-C_3), 151.12 (Triazol-C_5), 153.91 (N=CH), 164.38 (COO) ppm. Anal. Calcd for $C_{20}H_{20}N_4O_5$: C, 60.60; H, 5.09; N, 14.13. Found: C, 60.99; H, 5.12; N, 14.05.

4.2.2. 2-Ethoxy-4-[(3-ethyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]phenyl 3-methoxybenzoate (3b)

White solid; yield: 98%; mp: 138 °C; IR (cm^{-1}) υ_{max} 3175 (NH), 1728, 1694 (C=O), 1594 (C=N), 1273 (COO), 794 and 720 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 6H, $2CH_2CH_3$; *J*=7,20 Hz), 2.80 (q, 2H, CH_2CH_3; *J*=7,60 Hz), 3.89 (s, 3H, OCH_3), 4.13 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.18-7.21 (m, 1H, Ar-H), 7.23-7.26 (m, 1H, Ar-H), 7.40-7.46 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2.80, 1,60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.45 (s, 1H, NH), 9.80 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 10.10 ($CH_2\underline{CH_3}$), 14.64 ($OCH_2\underline{CH_3}$), 19.22 ($\underline{CH_2}CH_3$), 55.53 (OCH_3), 64.69 ($O\underline{CH_2}CH_3$), [112.17, 114.61, 120.24, 121.32, 122.75, 123.34, 129.59, 130.58, 132.60, 149.81, 151.95, 159.74] (Ar-C), 143.08 (Triazol-C_3), 151.12 (Triazol-C_5), 153.78 (N=CH), 164.37 (COO) ppm.

4.2.3. 2-Ethoxy-4-[(3-n-propyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]phenyl 3-methoxy-benzoate (3c)

White solid; yield: 97%; mp: 140 °C; IR (cm^{-1}) υ_{max} 3179 (NH), 1730, 1695 (C=O), 1595 (C=N), 1275 (COO), 793 and 720 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.05 (t, 3H, $CH_2CH_2CH_3$; *J*=7,20 Hz), 1.35 (t, 3H, OCH_2CH_3; *J*=6,80 Hz), 1.81 (sext, 2H, $CH_2\underline{CH_2}CH_3$; *J*=7,60 Hz), 2.75 (t, 2H, $\underline{CH_2}CH_2CH_3$; *J*=7,60 Hz), 3.89 (s, 3H, OCH_3), 4.13 (q, 2H, OCH_2CH_3; *J*=6,80 Hz), 7.20-7.21 (m, 1H, Ar-H), 7.24-7.26 (m, 1H, Ar-H), 7.40-7.46 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2.40, 1,60 Hz), 7.81-7.84 (m, 1H, Ar-H), 9.75 (s, 1H, NH), 9.80 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 13.71 ($CH_2CH_2\underline{C}H_3$), 14.64 ($OCH_2\underline{CH_3}$), 19.47 ($CH_2\underline{C}H_2CH_3$), 27.45 ($\underline{C}H_2CH_2CH_3$), 55.53 (OCH_3), 64.67 ($O\underline{CH_2}CH_3$), [112.20, 114.61, 120.24, 121.27, 122.75, 123.35, 129.59, 130.59, 132.63, 148.71, 152.02, 159.73] (Ar-C), 143.07 (Triazol-C_3), 151.10 (Triazol-C_5), 153.73 (N=CH), 164.39 (COO) ppm.

4.2.4. 2-Ethoxy-4-[(3-benzyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]phenyl 3-methoxy-benzoate (3d)

White solid; yield: 98%; mp: 164 °C; IR (cm^{-1}) υ_{max} 3200 (NH), 1738, 1703 (C=O), 1583 (C=N), 1266 (COO), 820 and 729 (*meta*-disubstituted aromatic ring), 746 and 700 (monosubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.89 (s, 3H, OCH_3), 4.11 (s, 2H, CH_2Ph), 4.12 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.20-7.22 (m, 1H, Ar-H), 7.24-7.27 (m, 1H, Ar-H), 7.30-7.45 (m, 8H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,80, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.35 (s, 1H, NH), 9.75 (s, 1H, N=CH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.64 ($OCH_2\underline{CH_3}$), 32.07 (CH_2Ph), 55.53 (OCH_3), 64.64 ($O\underline{CH_2}CH_3$), [111.75, 114.61, 120.24, 121.65, 122.75, 123.70, 129.60, 130.57, 132.51, 147.70, 151.71, 159.74] (Ar-C), [127.18, 128.68 (2C), 128.91 (2C), 135.07] (Ar-C linked C_3), 143.09 (Triazol-C_3), 151.08 (Triazol-C_5), 153.62 (N=CH), 164.38 (COO) ppm.

4.2.5. 2-Ethoxy-4-[(3-p-methylbenzyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoate (3e)

White solid; yield: 99%; mp: 183 °C; IR (cm^{-1}) υ_{max} 3190 (NH), 1738, 1704 (C=O), 1583 (C=N), 1268 (COO), 827 (*para*-disubstituted aromatic ring), 798 and 712 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.36 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 2.33 (s, 3H, $PhCH_3$), 3.89 (s, 3H, OCH_3), 4.07 (s, 2H, CH_2Ph), 4.11 (q, 2H, OCH_2CH_3; *J*=6,80 Hz), 7.12 (d, 2H, Ar-H; *J*=8,00 Hz), 7.18-7.21 (m, 1H, Ar-H), 7.23 (d,

2H, Ar-H; *J*=8,00 Hz), 7.24-7.26 (m, 1H, Ar-H), 7.40-7.45 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.80-7.83 (m, 1H, Ar-H), 9.75 (s, 2H, N=CH + NH); ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.68 ($OCH_2\underline{CH_3}$), 21.05 ($PhCH_3$), 31.65 (CH_2Ph), 55.53 (OCH_3), 64.63 ($O\underline{CH_2}CH_3$), [111.76, 114.62, 120.25, 121.66, 122.75, 123.29, 129.60, 130.58, 131.96, 147.83, 151.92, 159.74] (Ar-C), [128.82 (2C), 129.38 (2C), 132.58, 136.79] (Ar-C linked C_3), 143.06 (Triazol-C_3), 151.07 (Triazol-C_5), 153.59 (N=CH), 164.40 (COO) ppm.

4.2.6. 2-Ethoxy-4-[(3-p-methoxybenzyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azo-methine]phenyl 3-methoxy-benzoate (3f)

White solid; yield: 97%; mp: 141 °C; IR (cm^{-1}) υ_{max} 3185 (NH), 1737, 1701 (C=O), 1584 (C=N), 1263 (COO), 826 (*para*-disubstituted aromatic ring), 801 and 716 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.36 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.78 (s, 3H, *p*-OCH_3), 3.89 (s, 3H, *m*-OCH_3), 4.05 (s, 2H, CH_2Ph), 4.12 (q, 2H, OCH_2CH_3; *J*=6,40 Hz), 6.85 (d, 2H, Ar-H; *J*=8,80 Hz), 7.20-7.28 (m, 3H, Ar-H), 7.34-7.36 (m, 1H, Ar-H), 7.41-7.45 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.56 (s, 1H, NH), 9.75 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.65 ($OCH_2\underline{CH_3}$), 31.20 (CH_2Ph), 55.26 (*p*-OCH_3), 55.53 (*m*-OCH_3), 64.65 ($O\underline{CH_2}CH_3$), [111.84, 114.62, 120.25, 121.59, 122.75, 123.32, 129.60, 130.57, 132.55, 147.98, 151.83, 159.74] (Ar-C), [114.15 (2C), 126.98, 129.99 (2C), 158.77] (Ar-C linked C_3), 143.08 (Triazol-C_3), 151.09 (Triazol-C_5), 153.66 (N=CH), 164.40 (COO) ppm.

4.2.7. 2-Ethoxy-4-[(3-p-chlorobenzyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoate (3g)

White solid; yield: 99%; mp: 173 °C; IR (cm^{-1}) υ_{max} 3180 (NH), 1739, 1704 (C=O), 1582 (C=N), 1265 (COO), 824 (*para*-disubstituted aromatic ring), 797 and 714 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.89 (s, 3H, OCH_3), 4.08 (s, 2H, CH_2Ph), 4.10 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.18-7.21 (m, 1H, Ar-H), 7.23-7.75 (m, 5H, Ar-H), 7.41-7.45 (m, 3H, Ar-H), 7.71 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.71 (s, 1H, NH), 9.75 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.65 ($OCH_2\underline{CH_3}$), 31.44 (CH_2Ph), 55.53 (OCH_3), 64.67 ($O\underline{CH_2}CH_3$), [111.85, 114.62, 120.26, 121.58, 122.75, 123.37, 129.61, 130.53, 133.15, 147.14, 151.81, 159.74] (Ar-C), [128.65 (2C), 130.27 (2C), 132.36, 133.50] (Ar-C linked C_3), 143.20 (Triazol-C_3), 151.13 (Triazol-C_5), 153.93 (N=CH), 164.37 (COO) ppm. Anal. Calcd for $C_{26}H_{23}ClN_4O_5$: C, 61.60; H, 4.57; N, 11.05. Found: C, 61.77; H, 4.54; N, 11.34.

4.2.8. 2-Ethoxy-4-[(3-m-chlorobenzyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]-phenyl 3-methoxybenzoate (3h)

White solid; yield: 97%; mp: 175 °C; IR (cm^{-1}) υ_{max} 3190 (NH), 1737, 1703 (C=O), 1592 (C=N), 1270 (COO), 817 and 711 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), 3.89 (s, 3H, OCH_3), 4.08 (s, 2H, CH_2Ph), 4.11 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 7.18-7.21 (m, 1H, Ar-H), 7.22-7.38 (m, 5H, Ar-H), 7.41-7.45 (m, 3H, Ar-H), 7.71 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.69 (s, 1H, NH), 9.76 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.64 ($OCH_2\underline{CH_3}$), 31.72 (CH_2Ph), 55.53 (OCH_3), 64.68 ($O\underline{CH_2}CH_3$), [111.65, 114.61, 120.26, 121.72, 122.75, 123.35, 129.60, 130.55, 132.35, 146.90, 151.78, 159.74] (Ar-C), [127.00, 127.44, 129.13, 129.92, 134.50, 137.05] (Ar-C linked C_3), 143.19 (Triazol-C_3), 151.15 (Triazol-C_5), 153.91 (N=CH), 164.37 (COO) ppm.

4.2.9. 2-Ethoxy-4-[(3-phenyl-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl)azomethine]phenyl 3-methoxy-benzoate (3i)

White solid; yield: 99%; mp: 188 °C; IR (cm^{-1}) υ_{max} 3162 (NH), 1739, 1701 (C=O), 1584 (C=N), 1278 (COO), 797 and 722 (*meta*-disubstituted aromatic ring), 763 and 686 (monosubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.33 (t, 3H, OCH_2CH_3; *J*=6.40 Hz), 3.89 (s, 3H, OCH_3), 4.09 (q, 2H, OCH_2CH_3; *J*=6,40 Hz), 7.18-7.26 (m, 2H, Ar-H), 7.38-7.49 (m, 6H, Ar-H), 7.72 (m, 1H, Ar-H), 7.81-7.83 (m, 1H, Ar-H), 7.97-7.99 (m, 2H, Ar-H), 9.79 (s, 1H, N=CH), 9.94 (s, 1H, NH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.62 ($OCH_2\underline{CH_3}$), 55.53 (OCH_3), 64.59 ($O\underline{CH_2}CH_3$), [112.04, 114.61, 120.26, 121.76, 122.76, 123.38, 129.60, 130.54, 132.50, 146.31, 152.03, 159.73] (Ar-C), [126.53, 128.36 (2C), 128.56 (2C), 130.41] (Ar-C linked C_3), 143.22 (Triazol-C_3), 151.11 (Triazol-C_5), 155.31 (N=CH), 164.41 (COO) ppm. Anal. Calcd for $C_{25}H_{22}N_4O_5$: C, 65.49; H, 4.84; N, 12.22. Found: C, 65.80; H, 4.82; N, 12.13.

4.3. General procedure for the synthesis of the compounds 4

The compound 3 (5 mmol) was dissolved in absolute ethyl alcohol. Then formaldehyde (% 37, 10 mmol) and 4-piperidinecarboxamide (6 mmol) were added to this solution. The reaction mixture was refluxed for four hours and filtered. The solution was stored at room temperature for one overnight. Then, the solid formed was gathered by filtration and then washed with cold ethanol. The pure compounds collected after a few ethanol crystallizations of the crude product.

4.3.1. 2-Ethoxy-4-{[3-methyl-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4a)

White solid; yield: 78%; mp: 172 °C; IR (cm^{-1}) υ_{max} 3370 and 3183 (NH_2), 1740, 1702, 1648 (C=O), 1602, 1581 (C=N), 1276 (COO), 801 and 730 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.34 (t, 3H, OCH_2CH_3; *J*=6,40 Hz), [1.73-1.77 (m), 1.90 (m), 2.10-2.15 (m), 2.40-2.43 (m), 3.09-3.12 (m)] (piperidine 9H), 3.89 (s, 3H, OCH_3), 4.12 (q, 2H, OCH_2CH_3; *J*=6,40 Hz), 4.65 (s, 2H, NCH_2N), 5.49 (m, 2H, NH_2), 7.18-7.27 (m, 2H, Ar-H), 7.38-7.45 (m, 3H, Ar-H), 7.71 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.83 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 11.44 (CH_3), 14.64 ($OCH_2\underline{C}H_3$), 28.88 ($2CH_2$), 42.13 (CH), 50.16 ($2CH_2$), 55.52 (OCH_3), 64.69 ($O\underline{C}H_2CH_3$), 66.88 (NCH_2N), [112.03, 114.62, 120.23, 121.39, 122.73, 123.34, 129.60, 130.56, 132.56, 143.77, 151.12, 159.74] (Ar-C), 143.09 (Triazol-C_3), 151.12 (Triazol-C_5), 153.49 (N=CH), 164.36 (COO), 177.02 ($CONH_2$) ppm. Anal. Calcd for $C_{27}H_{32}N_6O_6$: C, 60.44; H, 6.01; N, 15.66. Found: C, 59.84; H, 5.91; N, 15.31.

4.3.2. 2-Ethoxy-4-{[3-ethyl-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4b)

White solid; yield: 80%; mp: 155 °C; IR (cm^{-1}) υ_{max} 3345 and 3186 (NH_2), 1739, 1703, 1651 (C=O), 1585 (C=N), 1266 (COO), 797 and 737 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.31-1.36 (m, 6H, $2CH_2CH_3$), [1.70-1.76 (m), 1.88-1.92 (m), 2.12-2.15 (m), 2.42-2.43 (m), 3.10-3.12 (m)] (piperidine 9H), 2.78 (q, 2H, CH_2CH_3; *J*=7,20 Hz), 3.89 (s, 3H, OCH_3), 4.11-4.13 (m, 2H, OCH_2CH_3), 4.67 (s, 2H, NCH_2N), 5.52 (s, b, 1H, NH), 5.75 (s, b, 1H, NH), 7.20-7.27 (m, 2H, ArH), 7.38-7.45 (m, 3H, Ar-H), 7.71 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.82 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 10.44 ($CH_2\underline{C}H_3$), 14.63 ($OCH_2\underline{C}H_3$), 19.15 ($\underline{C}H_2CH_3$), 28.87 ($2CH_2$), 42.18 (CH), 49.95 ($2CH_2$), 55.52 (OCH_3), 64.67 ($O\underline{C}H_2CH_3$), 66.85 (NCH_2N), [112.06, 114.63, 120.22, 121.30, 122.72, 123.34, 129.60, 130.55, 132.65, 147.69, 151.28, 159.73] (Ar-C), 143.04 (Triazol-C_3), 151.11 (Triazol-C_5), 153.36 (N=CH), 164.38 (COO), 177.34 ($CONH_2$) ppm.

4.3.3. 2-Ethoxy-4-{[3-benzyl-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4d)

White solid; yield: 83%; mp: 193 °C; IR (cm^{-1}) υ_{max} 3394 and 3208 (NH_2), 1740, 1709, 1661 (C=O), 1601 (C=N), 1265 (COO), 805 and 739 (*meta*-disubstituted aromatic ring), 762 and 710 (monosubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), [1.72-1.76 (m), 1.91 (m), 2.10-2.14 (m), 2.43-2.44 (m), 3.11 (m)] (piperidine 9H), 3.89 (s, 3H, OCH_3), 4.06-4.10 (m, 4H, CH_2Ph + $O\underline{CH_2}CH_3$), 4.69 (s, 2H, NCH_2N), 5.40-5.44 (m, 2H, NH_2), 7.20-7.37 (m, 7H, Ar-H), 7.41-7.45 (m, 3H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,80, 1.60 Hz), 7.80-7.82 (m, 1H, Ar-H), 9.75 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.64 ($CH_2\underline{C}H_3$), 28.91 ($2CH_2$), 31.96 (CH_2Ph), 42.16 (CH), 50.18 ($2CH_2$), 55.53 (OCH_3), 64.60 ($O\underline{C}H_2CH_3$), 66.98 (NCH_2N), [111.58, 114.63, 120.22, 121.65, 122.73, 123.29, 129.60, 130.55, 132.58, 145.44, 151.15] (Ar-C), [127.09, 128.66 (2C), 128.74 (2C), 135.46] (Ar-C linked to C_3), 143.03 (Triazol-C_3), 151.06 (Triazol-C_5), 153.16 (N=CH), 164.37 (COO), 176.96 ($CONH_2$) ppm. Anal. Calcd for $C_{33}H_{36}N_6O_6$: C, 64.69; H, 5.92; N, 13.72. Found: C, 64.62; H, 5.87; N, 13.48.

4.3.4. 2-Ethoxy-4-{[3-(p-methylbenzyl)-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4e)

White solid; yield: 73%; mp: 178 °C; IR (cm^{-1}) υ_{max} 3395 and 3202 (NH_2), 1741, 1707, 1658 (C=O), 1600, 1582 (C=N), 1264 (COO), 847 (*para*-disubstituted aromatic ring), 805 and 717 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=6,40 Hz), [1.73-1.78 (m), 1.89-1.92 (m), 2.06-2.08 (m), 2.39-2.45 (m), 3.10-3.12 (m)] (piperidine 9H), 2.31 (s, 3H, $PhCH_3$), 3.88 (s, 3H, OCH_3), 4.06 (s, 2H, CH_2Ph), 4.11 (q, 2H, OCH_2CH_3; *J*=6,40 Hz), 4.68 (s, 2H, NCH_2N), 5.57 (s, 1H, NH), 5.80 (s, 1H, NH), 7.10-7.12

(m, 2H, Ar-H), 7.18-7.31 (m, 5H, Ar-H), 7.38-7.44 (m, 2H, Ar-H), 7.71 (m, 1H, Ar-H), 7.80-7.82 (m, 1H, Ar-H), 9.75 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.65 ($CH_2\underline{C}H_3$), 21.04 ($PhCH_3$), 28.89 ($2CH_2$), 31.55 (CH_2Ph), 42.22 (CH), 50.19 ($2CH_2$), 55.52 (OCH_3), 64.59 ($O\underline{C}H_2CH_3$), 66.95 (NCH_2N), [111.57, 114.64, 120.21, 121.68, 122.72, 123.28, 129.61, 130.55, 132.64, 145.64, 151.15, 159.74] (Ar-C), [128.63 (2C), 129.35 (2C), 132.34, 136.70] (Ar-C linked to C_3), 143.00 (Triazol-C_3), 151.05 (Triazol-C_5), 153.08 (N=CH), 164.39 (COO), 177.30 ($CONH_2$) ppm.

4.3.5. 2-Ethoxy-4-{[3-(p-methoxybenzyl)-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4f)

White solid; yield: 75%; mp: 167 °C; IR (cm^{-1}) υ_{max} 3310 and 3194 (NH_2), 1743, 1710, 1649 (C=O), 1603, 1585 (C=N), 1278 (COO), 851 (*para*-disubstituted aromatic ring), 810 and 739 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=6,40 Hz), [1.73-1.76 (m), 1.90-1.93 (m), 2.05-2.08 (m), 2.42-2.43 (m), 3.10 (m)] (piperidine 9H), 3.77 (s, 3H, *p*-OCH_3), 3.89 (s, 3H, *m*-OCH_3), 4.04 (s, 2H, CH_2Ph), 4.10 (q, 2H, OCH_2CH_3; *J*=7,20 Hz), 4.68 (s, 2H, NCH_2N), 5.50 (m, 2H, NH_2), 6.84 (d, 2H, Ar-H; *J*=8,40 Hz), 7.20-7.31 (m, 5H, Ar-H), 7.39-7.45 (m, 2H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.81-7.83 (m, 1H, Ar-H), 9.76 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.65 ($CH_2\underline{C}H_3$), 28.90 ($2CH_2$), 31.09 (CH_2Ph), 42.18 (CH), 50.18 ($2CH_2$), 55.26 (*p*-OCH_3), 55.53 (*m*-OCH_3), 64.61 ($O\underline{C}H_2CH_3$), 66.95 (NCH_2N), [111.68, 114.64, 120.22, 121.58, 122.73, 123.31, 129.60, 130.55, 132.62, 145.75, 151.16, 159.74] (Ar-C), [114.11 (2C), 127.38, 129.80 (2C), 158.71] (Ar-C linked to C_3), 143.03 (Triazol-C_3), 151.07 (Triazol-C_5), 153.18 (N=CH), 164.39 (COO), 177.05 ($CONH_2$) ppm.

4.3.6. 2-Ethoxy-4-{[3-(p-chlorobenzyl)-1-(4-piperidinecarboxamide-1-yl-methyl)-4,5-dihydro-1H-1,2,4-triazol-5-one-4-yl]-azomethine}phenyl 3-methoxybenzoate (4g)

White solid; yield: 78%; mp: 160 °C; IR (cm^{-1}) υ_{max} 3359 and 3186 (NH_2), 1743, 1717, 1646 (C=O), 1585 (C=N), 1276 (COO), 847 (*para*-disubstituted aromatic ring), 803 and 742 (*meta*-disubstituted aromatic ring); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.35 (t, 3H, OCH_2CH_3; *J*=7,20 Hz), [1.73-1.76 (m), 1.90-1.93 (m), 2.05-2.09 (m), 2.42-2.44 (m), 3.10-3.12 (m)] (piperidine 9H), 3.89 (s, 3H, OCH_3), 4.05-4.09 (m, 4H, OCH_2CH_3 + CH_2Ph), 4.69 (s, 2H, NCH_2N), 5.40-5.44 (m, 2H, NH_2), 7.18-7.33 (m, 8H, Ar-H), 7.41-7.43 (m, 1H, Ar-H), 7.72 (dd, 1H, Ar-H; *J*=2,40, 1.60 Hz), 7.80-7.83 (m, 1H, Ar-H), 9.76 (s, 1H, N=CH) ppm; ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 14.65 ($CH_2\underline{C}H_3$), 28.89 ($2CH_2$), 31.34 (CH_2Ph), 42.11 (CH), 50.14 ($2CH_2$), 55.53 (OCH_3), 64.63 ($O\underline{C}H_2CH_3$), 67.03 (NCH_2N), [111.71, 114.63, 120.24, 121.55, 122.73, 123.37, 129.61, 130.53, 132.08, 144.95, 151.09, 159.74] (Ar-C), [128.83 (2C), 130.10 (2C), 132.44, 133.87] (Ar-C linked to C_3), 143.14 (Triazol-C_3), 151.04 (Triazol-C_5), 153.44 (N=CH), 164.36 (COO), 176.90 ($CONH_2$) ppm.

4.4. Antimicrobial activity

In the study, bacterial and yeast strains were purchased from the company of Microbiological Environmental Protection Laboratories (France): *Bacillus substilis* (ATCC 11774), *Bacillus cereus* (ATCC 11778), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 4352), *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 25922). A simple susceptibility screening test using an agar well diffusion technique was used [23,24]. All new compounds were measured and dissolved in DMSO to obtain 1 mg/ml of extract stock solution.

Each microorganism was suspended in Mueller-Hinton Broth and diluted to 106 colony-forming units (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. Ampicillin, neomycin, and streptomycin were used as positive controls, and DMSO was used as the solved control.

4.5. Antioxidant activity

The antioxidant activities of new compounds and standard antioxidant compounds, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), α-tocopherol and ethylenediaminetetraacetic acid

(EDTA) were determined by several antioxidant tests, such as reducing power, free radical scavenging activity and metal chelating activity.

The reducing power of compounds **3** and **4** was determined using Oyaizu's method [25]. Different concentrations of the samples (100-500 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 minutes after the incubation period; a portion of trichloroacetic acid (2.5 mL, 10%) was added to the mixture and then centrifuged for 10 minutes 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 was measured at 700 nm in a spectrophotometer. Higher absorbance of the reaction mixture indicated greater reducing power.

The free radical scavenging effect of compounds **3** and **4** was estimated by DPPH· using the Blois method [26]. Briefly, 0.1 mM DPPH· solution was prepared in ethanol, and this solution (1 mL) was added to the sample solutions in DMSO (3 mL) at different concentrations (50-250 µg/mL). The mixture was shaken vigorously and kept at room temperature for 30 minutes. The absorbance was then measured at 517 nm in a spectrophotometer. The lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The DPPH· concentration (mM) in the reaction medium was calculated from the following calibration curve and determined by linear regression (R: 0.997):

$$\text{Absorbance} = 0.0003 \times \text{DPPH} - 0.0174$$

The DPPH· radical scavenging capacity was calculated using the following equation:

$$\text{DPPH· scavenging effect (\%)} = (A_0 - A_1/A_0) \times 100$$

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

The chelation of ferrous ions by compounds **3** and **4** was measured according to the method of Dinis *et al* [27]. Briefly, the synthesized compounds (15–45 µg/mL) were added to 2 mM $FeCl_2.4H_2O$ (0.05 mL) solution. The reaction was initiated by adding 5 mM ferrozine (0.2 mL), and then the mixture was shaken vigorously and left at room temperature for 10 minutes. After the mixture reached equilibrium, the absorbance of the solution was measured at 562 nm in a spectrophotometer. The formula gave the percentage inhibition of ferrosine-Fe^{2+} complex formation: % 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 samples. The control did not contain a compound.

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