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Synthesis and Bioactivities of 3-Alkyl(aryl)-4-Amino-1,2,4-Triazol-5-ones Based Succinamide Derivatives: Amide and *N*-Mannich Compounds

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3-Alkil(aril)-4-Amino-1,2,4-Triazol-5-on Temelli Suksinamid Türevlerinin Sentezi ve Biyoaktiviteleri: Amid ve *N*-Mannich Bileşikleri

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Abstract

1,2,4-Triazoles, recognized for their remarkable therapeutic potential, play a significant role in drug development due to their chemical stability and interaction with various biological targets. In this study, 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-one compunds were reacted with Succinyl Chloride to produce six *N*,*N*'-di[3-alkyl(aryl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]succinylamide derivatives. These derivatives were further modified via the Mannich reaction with formaldehyde and morpholine, yielding three *N*-Mannich derivatives. Structural characterization of nine synthesized compounds was performed using IR, ^{1}H-NMR and ^{13}C-NMR spectroscopy. Biological evaluations focused on antioxidant properties, including reducing power, free radical scavenging capacity, and metal chelating activity. The antioxidant effects of the compounds were found to be generally weak. Antimicrobial activity tests, performed against Bacillus cereus, Klebsiella pneumoniae, Staphylococcus aureus, Serratia marcescens, and Candida albicans using the agar well diffusion method, revealed that some compounds exhibited strong antimicrobial properties. This study emphasizes the significant biological potential of triazole derivatives, suggesting that their bioactivity can be further enhanced through functional group modifications.

Keywords: *4,5-Dihydro-1H-1,2,4-triazol-5-one; Amide; Mannich base; Antioxidant activity; Antimicrobial activity.*

Öz

1,2,4-Triazoller, yüksek terapötik potansiyelleriyle tanınmakta ve kimyasal stabiliteleri ile çeşitli biyolojik hedeflerle etkileşim kurma özellikleri sayesinde ilaç geliştirmede önemli bir rol oynamaktadır. Bu çalışmada, 3-alkil(aril)-4-amino-4,5-dihidro-1*H*-1,2,4-triazol-5-on bileşikleri Suksinil Klorür ile reaksiyona sokularak altı adet *N*,*N*'-di[3-alkil(aril)-4,5-dihidro-1*H*-1,2,4-triazol-5-on-4-il]suksinamid türevi sentezlenmiştir. Bu türevler, formaldehit ve morfolin kullanılarak Mannich reaksiyonu ile modifiye edilmiş ve üç adet *N*-Mannich türevi elde edilmiştir. Sentezlenen dokuz bileşiğin yapısal karakterizasyonu IR, ^{1}H-NMR ve ^{13}C-NMR spektroskopisiyle gerçekleştirilmiştir. Biyolojik değerlendirmeler sırasında, indirgeme gücü, serbest radikal giderme kapasitesi ve metal şelatlama aktivitesi gibi antioksidan özellikler incelenmiştir. Bileşiklerin antioksidan etkilerinin genelde zayıf olduğu belirlenmiştir. Agar kuyucuk yöntemi kullanılarak Bacillus cereus, Klebsiella pneumoniae, Staphylococcus aureus, Serratia marcescens ve Candida albicans gibi patojen mikroorganizmalara karşı yapılan antimikrobiyal aktivite testleri, bazı bileşiklerin güçlü antimikrobiyal özelliklere sahip olduğunu göstermiştir. Bu çalışma, triazol türevlerinin önemli biyolojik potansiyele sahip olduğunu ve fonksiyonel grup modifikasyonlarıyla biyolojik aktivitelerinin artırılabileceğini ortaya koymaktadır.

Anahtar Kelimeler: *4,5-Dihidro-1H-1,2,4-triazol-5-on; Amid; Mannich bazı; Antioksidan aktivite; Antimikrobiyal aktivite.*

1. Introduction

In the fields of chemistry and biotechnology, heterocyclic compounds are of great importance due to their rich biological activities and structural diversity. 1,2,4-triazole derivatives, one of these compounds, constitute a group that attracts attention thanks to their chemical stability, broad spectrum of biological activity and easily modifiable structures (Sumrra et al. 2020). Especially succinamide derivatives are widely used in pharmaceutical research together with triazoles because they contain an amide group and this structure allows strong interactions with biological targets. These compounds are considered as an effective building block in drug discovery by offering various therapeutic effects such as antifungal (Dong et al. 2023), antimicrobial (Gandham et al. 2024), antioxidant (Alkan et al. 2007; Gokce et al. 2013), anticancer (Emami et al. 2022) and anti-HIV (Feng et al. 2021). These studies show that the biological activities of triazole and succinamide derivatives can be optimized and a broader spectrum of action can be achieved, especially if they are modified to contain functional groups such as amides and Mannich bases (Kumar et al. 2021). Such structural modifications increase both the chemical stability of the compounds and enable them to establish stronger and

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more selective interactions with biological targets (Matin et al. 2022).

Mannich bases are β-aminoketone derivatives obtained through the Mannich reaction, which involves formaldehyde, an amine, and a carbonyl compound containing an active methylene group (Yamali et al. 2016). These compounds hold significant importance in pharmaceutical chemistry, exhibiting a wide range of biological activities such as anticancer, antimicrobial, antifungal, anti-inflammatory, and antiparasitic effects (Gul H.I. et al. 2005; Geethapriya and Elumalaiim 2021; Raoof and Sadiq 2022). Other potential applications include interactions with enzymes involved in the antioxidant defense system, inhibition of the mitochondrial respiratory chain (Kucukoglu et al. 2014), and suppression of topoisomerase enzymes (Mete et al. 2010). In particular, C-Mannich bases refer to structures in which the amino group is directly bonded to a carbon atom, offering greater stability and broader functional diversity compared to classical Mannich bases. These structures exert biological effects through mechanisms such as DNA topoisomerase inhibition, suppression of cyclooxygenase-2 (COX-2) activity, disruption of microtubule dynamics, and induction of apoptosis (Gul M. et al. 2005; Yamali et al. 2023). These multifaceted biological properties further highlight the critical need for novel antimicrobial agents, especially in the fight against drug-resistant microorganisms.

The overuse and inappropriate use of antibiotics has become one of the biggest threats to public health due to antimicrobial resistance. The World Health Organization emphasizes that infections caused by antibiotic-resistant bacteria are becoming increasingly difficult to treat and this situation is a major burden on the global health system (Frieri et al. 2017). Studies on triazole and succinamide derivatives have shown that these compounds offer broad-spectrum effects against both gram-positive and gram-negative bacteria (Ge and Xu 2021; Xie et al. 2022). In particular, Mannich bases have been reported to enhance antimicrobial activities and strengthen the therapeutic potential of such compounds (Bishoyi et al. 2021; Akyıldırım et al. 2023). Therefore, the development of more effective and selective forms of triazole and succinamide derivatives against resistant microorganisms is considered as a priority issue in pharmaceutical research.

Oxidative stress is seen as one of the basic mechanisms of many diseases as reactive chemical molecules formed during cell metabolism disrupt cellular balance (Pisoschi et al. 2021). The condition caused by such reactive molecules leads to permanent damage to biomolecules such as DNA, proteins and lipids, paving the way for serious health problems such as cancer, cardiovascular disorders and neurodegenerative diseases (Apel and Hirt 2004; Jomova et al. 2023). Triazole and succinamide derivatives are considered as an effective tool in combating oxidative stress thanks to their free radical scavenging capacity and metal chelating ability (Gulcin and Alwasel 2022). Transition metals (e.g., Fe^{2+} and Cu^{2+}) are known to increase cellular damage by catalyzing oxidative processes. Therefore, both triazole and succinamide derivatives have been shown to be able to inhibit oxidative chain reactions by forming complexes with these metals (Zafar et al. 2021).

In this study, 3-alkyl(aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-one derivatives were synthesized and their biological activities were extensively investigated. Accordingly, the enrichment of triazole derivatives with functional groups such as amide and Mannich bases increased the potential to optimize the antimicrobial and antioxidant properties of these compounds. The results obtained shed light on the development of new therapeutic agents in the fight against microorganisms with antibiotic resistance and oxidative stress-induced diseases (Ikizler and Yüksek 1994). The main aim of the study was to synthesize 1,2,4-triazole derivatives as novel compounds with antimicrobial and antioxidant potential and to comprehensively evaluate their biological activities. Accordingly, six different 2-type *N,N*′-di[3-alkyl(aryl)-4,5-dihydro-1*H*-1,2,4-triazol-5-one-4-yl]-succinamide derivatives were synthesized by reaction of Succinyl Chloride with 1-type compound. In the next step of the study, the synthesized 2-type compounds 2a, 2d and 2e were subjected to Mannich reaction using formaldehyde and morpholine and three 3-type compounds (3a, 3d, 3e) *N,N*′-di[1-(morpholine-4-yl-methyl)-3-alkyl(aryl)-4,5-dihydro-1*H*-1,2,4-triazol-5-on-4-yl]-succinamide derivatives were obtained. In line with the aim of the study, both the structure-activity relationships of the synthesized compounds were evaluated and the molecular mechanisms of their biological activities were investigated. The results of the study provide data that can be evaluated in drug development processes.

2. Materials and Methods

Considering the quality and consistency of the materials used in the experiments of this research, the chemicals used were obtained from "Aldrich", "Merck" and "Fluka" suppliers.

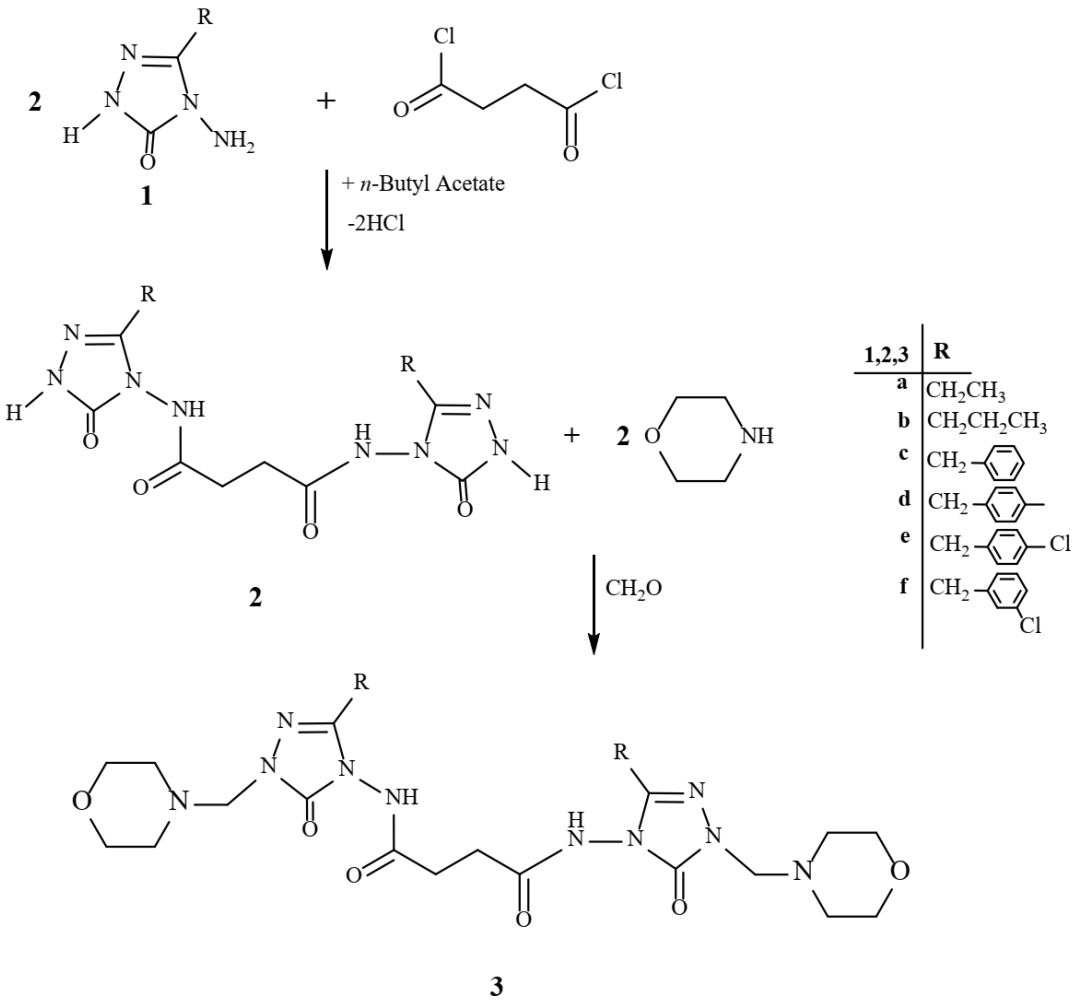

Figure 1. Synthesis methods of compounds **1–3** (Only **3a**, **3d** and **3e** were obtained)

On the other hand, the solvents required in the experiments were obtained from local and internationally known distributors. The melting points of the synthesized compounds were precisely measured using "WRS-2A Microprocessor Melting Point Device". This analysis provides important data to verify the purity and chemical stability of the obtained compounds. On the other hand, in order to determine the functional groups and structural properties of these compounds, their IR spectra were calculated using "ALPHA-P BRUKER FT-IR" spectrometer. This device provides detailed structural information by detecting intermolecular vibrations with high sensitivity.

The structural elucidation of the compounds was supported by the acquisition of ^{1}H-NMR and ^{13}C-NMR spectra. These analyses were carried out at the "Mersin University Central Research Laboratory" using a "Bruker 400 MHz NMR" device and the chemical environments of the protons and carbon atoms in the compounds were precisely determined. In addition, within the scope of the evaluation of antioxidant properties, various test methods such as reducing power (RA), free radical scavenging (FRE) and metal chelating (MCA) were applied to determine the electron-donating capacity of the compounds. These analyses comprehensively reveal the antioxidant capacity of the compounds (Guidea et al. 2020).

Experimental studies were carried out meticulously in the Organic Chemistry Research Laboratory of the Department of Chemistry, Faculty of Arts and Sciences, Kafkas University. All analyses were carried out within the framework of standard procedures to ensure the reliability of the data obtained. In order to determine the optical and spectroscopic properties, analyses were carried out using "PG Instruments Ltd T80 UV/VIS Spectrometer". In addition, the antimicrobial activities of the synthesized compounds were examined in detail in the "Microbiology Research Laboratory, Faculty of Education, Kafkas University".

2.1 Synthesis of type 2-3 compounds

Using a round bottom flask, the compound 3-(alkyl/aryl)-4-amino-4,5-dihydro-1*H*-1,2,4-triazol-5-one (**2a-2f**) (0.01

mol) was weighed and 20 mL *n-butyl acetate* was added and the mixture was stirred with a magnetic stirrer until dissolved. Succinyl Chloride (0.01 mol) was added to the solution in a controlled manner. The resulting reaction mixture was heated under reflux conditions for 5-6 hours at a constant temperature using a reflux system.

After completion of the reaction time, the solid product formed was cooled to room temperature and separated by vacuum filtration. The filtered crude product was dried under a vacuum in a desiccator containing calcium chloride ($CaCl_2$) to remove moisture. Then crystallization was performed several times using ethanol for purification. The crystals whose purity was improved and dried under vacuum were identified as compounds of type 2 (2a, 2b, 2c, 2d, 2e, 2f).

Then, compounds 2a, 2d and 2e were weighed in amounts determined as 0.01 mol and transferred to the reaction vessel. 100 mL of ethanol was added to completely dissolve these compounds. To the homogeneous solution, morpholine (0.015 mol) and then formaldehyde (0.02 mol) were carefully added in certain amounts. The reaction mixture was heated by refluxing at a constant temperature for 3 hours using a back cooling system. After heating was completed, the resulting mixture was allowed to rest overnight at room temperature and the solid product was allowed to slowly precipitate. The solid precipitate was carefully collected by vacuum filtration and crystallized several times in ethanol to increase its purity. The purified crystals were characterized as 3 types of compounds (3a, 3d, 3e) after drying under a vacuum.

As shown in Table 1, the synthesized compounds exhibited melting points ranging from 75 °C to 287 °C, with yields between 71.34% and 82.27%. All compounds were obtained as crystalline solids, indicating high purity and consistent solid-state characteristics.

Table 1. Physical properties of type **2-3** compounds

Compounds	Melting Point (°C)	Yield (%)	Appearance
2a	222	75.12	Crystal
2b	244	73.38	Crystal
2c	263	73.82	Crystal
2d	245	77.81	Crystal
2e	277	78.11	Crystal
2f	287	71.34	Crystal
3a	75	80.51	Crystal
3d	100	82.27	Crystal
3e	160	81.13	Crystal

Table 2 summarizes the characteristic IR absorption bands of the synthesized type **2** and type **3** compounds. The spectra display NH stretching vibrations in the range of 3111–3317 cm^{-1}, C=O stretching vibrations between 1673–1745 cm^{-1}, and C=N stretching vibrations around 1579–1594 cm^{-1}. Aromatic ring substitution patterns were identified based on specific out-of-plane C–H bending vibrations, such as monosubstituted (760, 701 cm^{-1}), 1,4-disubstituted (800–810 cm^{-1}), and 1,3-disubstituted (888, 784 cm^{-1}) aromatic rings.

Table 2. IR spectrum data of type **2-3** compounds

Compounds	Functional Group (υ, cm^{-1})			Aromatic Rings (υ, cm^{-1})
	NH	C=O	C=N	
2a	3201, 3111	1723, 1673	1594	-
2b	3264, 3119	1717, 1690	1592	-
2c	3279, 3196	1742, 1680	1590	**Monosubstitue:** 760, 701
2d	3317, 3220	1720, 1683	1587	**1,4-disubstitue**: 802
2e	3316, 3189	1721, 1681	1586	**1,4-disubstitue**: 800
2f	3278, 3199	1745, 1675	1591	**1,3-disubstitue**: 888, 784
3a	3195	1720, 1689	1590	-
3d	3214	1704	1579	1,4-disubstitue: 810
3e	3200	1721, 1698	1586	1,4-disubstitue: 807

Table 3. ^{1}H-NMR spectrum data of type **2** compounds

Compound No	Proton Type	Chemical Shift (δ/ppm)	Multiplicity	Coupling Constant (*J*, Hz)
2a	$2CH_3$	1.13	Triplet (t)	7.60
	$2CH_2$	2.29	Quartet (q)	7.20
	CH_2CH_2	2.61	Multiplet (m)	-
	2NH	10.82; 11.50	Singlet (s); Singlet (s)	-
2b	$2CH_3$	0.87	Triplet (t)	7.20
	$2CH_2$	1.52; 2.25	Sextet (sext); Triplet (t)	7.20, 7.20
	CH_2CH_2	2.61	Multiplet (m)	-
	2NH	10.80, 11.54	Singlet (s)	-
2c	CH_2CH_2	2.57	Multiplet (m)	-
	$2CH_2Ph$	3.67	Singlet (s)	-
	Aromatik H	7.22-7.33	Multiplet (m), 10H	-
	2NH	10.85; 11.68	Singlet (s); Singlet (s)	-
2d	$2CH_3$	2.26	Singlet (s)	-
	CH_2CH_2	2.56	Multiplet (m)	-
	$2CH_2Ph$	3.79	Singlet (s)	-
	Aromatik H	7.09-7.15	Multiplet (m), 8H	-
	2NH	10.81; 11.64	Singlet (s); Singlet (s)	-
2e	CH_2CH_2	2.59	Multiplet (m)	-
	$2CH_2Ph$	2.68	Singlet (s)	-
	Aromatik H	7.24-7.30; 7.35-7.39	Multiplet (m), 4H; Multiplet (m), 4H	-
	2NH	10.79; 11.71	Singlet (s); Singlet (s)	-
2f	CH_2CH_2	2.58	Multiplet (m)	-
	$2CH_2Ph$	3.70	Singlet (s)	-
	Aromatik H	7.18-7.20; 7.29-7.34	Multiplet (m), 2H; Multiplet (m), 6H	-
	2NH	10.87; 11.73	Singlet (s); Singlet (s)	-

Tables 3 and 4 summarize the characteristic ^{1}H-NMR spectral data of type **2** and type **3** compounds. The observed chemical shifts, multiplicities, and coupling constants are fully consistent with the expected proton environments of the synthesized molecules. Signals corresponding to aliphatic protons (δ 0.87–2.68 ppm) were observed as triplets, quartets, sextets, or multiplets, indicating the presence of methyl and methylene groups in various chemical environments. Characteristic singlets for $–CH_2Ph$ protons appeared in the range of δ 2.68–3.84 ppm, while aromatic protons resonated between δ 7.09–7.39 ppm, consistent with mono- or disubstituted phenyl rings identified in the IR analysis. The distinct singlet signals of the NH protons (δ 10.79–11.73 ppm) further confirmed the presence of the triazole-linked amide moieties. In type 3 compounds, additional signals for $–CH_2NCH_2–$, $–CH_2OCH_2–$, and $–NCH_2N–$ groups were observed between δ 2.53–4.49 ppm, matching the expected Mannich base framework.

Table 4. ^{1}H-NMR spectrum data of type **3** compounds

Compound No	Proton Type	Chemical Shift (δ/ppm)	Multiplicity	Coupling Constant (*J*, Hz)
3a	$2CH_3$	1.08	Triplet (t)	7.20
	$2CH_2$	2.34	Quartet (q)	7.20
	$2CH_2NCH_2$	2.53	Triplet (t)	4.40
	CH_2CH_2	2.62	Multiplet (m)	-
	$2CH_2OCH_2$	3.54	Triplet (t)	4.40
	$2NCH_2N$	4.48	Singlet (s)	-
	2NH	10.95	Singlet (s)	-
3d	$2CH_3$	2.26	Singlet (s)	-
	$2CH_2NCH_2$	2.54	Multiplet (m)	-
	CH_2CH_2	2.58	Multiplet (m)	-
	$2CH_2OCH_2$	3.51-3.54	Multiplet (m)	-
	$2CH_2Ph$	3.84	Singlet (s)	-
	Aromatik H	7.10-7.17	Multiplet (m), 8H	-
	2NH	11.00	Singlet (s)	-
3e	$2CH_2NCH_2$	2.54	Multiplet (m)	-
	CH_2CH_2	2.59	Multiplet (m)	-
	$2CH_2OCH_2$	3.51	Multiplet (m)	-
	$2CH_2Ph$	3.72	Singlet (s)	-
	$2NCH_2N$	4.49	Singlet (s)	-
	Aromatik H	7.25; 7.37	Doublet (d), 4H; Doublet (d), 4H	8.40; 8.80
	2NH	10.95	Singlet (s)	-

Table 5. ^{13}C-NMR spectrum data of type **2** compounds

Compound No	Carbon Type	Chemical Shift (δ/ppm)
2a	2CO	170.89
	2Triazol C_5	152.69
	2Triazol C_3	148.89
	CH_2CH_2	27.59
	Alifatik C	17.79($2\underline{CH_2}CH_3$), 9.80($2CH_2\underline{CH_3}$)
2b	2CO	170.85
	2Triazol C_5	152.65
	2Triazol C_3	147.35
	CH_2CH_2	27.59
	Alifatik C	26.09($2\underline{CH_2}CH_2CH_3$), 18.78($2CH_2\underline{CH_2}CH_3$), 13.32($2CH_2CH_2\underline{CH_3}$)
2c	2CO	170.95
	2Triazol C_5	152.57
	2Triazol C_3	147.14
	C-3 Aromatik C	135.08 (2C), 128.83 (4CH), 128.49(4CH), 126.79 (2CH)
	CH_2CH_2	27.58
	Alifatik C	30.70($2CH_2Ph$)
2d	2CO	170.93
	2Triazol C_5	152.58
	2Triazol C_3	147.27
	C-3 Aromatik C	135.82 (2C), 131.99 (2C), 129.05 (4CH), 128.70 (4CH)
	CH_2CH_2	27.57
	Alifatik C	30.13($2CH_2Ph$), 20.61($2PhCH_3$)
2e	2CO	171.01
	2Triazol C_5	152.53
	2Triazol C_3	146.83
	C-3 Aromatik C	134.07 (2C), 131.54 (2C), 130.63 (4CH), 128.42 (4CH)
	CH_2CH_2	27.57
	Alifatik C	29.96($2CH_2Ph$)
2f	2CO	171.01
	2Triazol C_5	152.50
	2Triazol C_3	146.65
	C-3 Aromatik C	137.52 (2C), 133.00 (2C), 130.24 (2CH), 128.67 (2CH), 127.61 (2CH), 126.85 (2CH)
	CH_2CH_2	27.58
	Alifatik C	29.95($2CH_2Ph$)

Table 5 presents the characteristic ^{13}C-NMR data for type 2 compounds. The carbonyl carbons (δ 170.85–171.01 ppm) and triazole ring carbons C5 (δ 152.50–152.69 ppm) and C3 (δ 146.65–148.89 ppm) were consistently observed, confirming the presence of the triazole–amide core. Aromatic carbons resonated between δ 126.79 and 137.52 ppm, with substitution patterns matching those inferred from IR spectra and ^{1}H-NMR aromatic proton signals. The aliphatic methylene carbons of the –CH_2CH_2– linker appeared around δ 27.57–27.59 ppm, while methyl carbons and benzyl methylene carbons resonated between δ 9.80 and 30.70 ppm, depending on the substitution type.

Table 6 summarizes the ^{13}C-NMR data for type 3 compounds. The carbonyl carbons were observed at δ 170.92–171.04 ppm, while triazole C5 and C3 carbons resonated between δ 151.89–152.01 ppm and δ 145.80–147.18 ppm, respectively, confirming the heterocyclic core. Characteristic signals for Mannich-base-specific carbons were detected, including –NCH_2N– (δ 66.10–66.19 ppm), –CH_2OCH_2– (δ 65.99–66.02 ppm), and –CH_2NCH_2– (δ 49.87–49.90 ppm). The –CH_2CH_2– linker carbons appeared at δ 27.52–27.57 ppm, while aliphatic methyl and benzyl methylene carbons were recorded between δ 9.77 and 29.92 ppm. Aromatic carbons resonated in the δ 128.50–135.92 ppm range, matching the substitution patterns identified from the IR and ^{1}H-NMR data.

Collectively, the spectral data confirm the presence of all expected structural elements and functional groups in the synthesized molecules. The observed chemical shifts are in good agreement with the proposed molecular structures, supporting the intended substitution patterns. Together with the IR and ^{1}H-NMR results, these findings validate that the target compounds were correctly and successfully synthesized.

Table 6. ^{13}C-NMR spectrum data of type **3** compounds

Compound No	Carbon Type	Chemical Shift (δ/ppm)
3a	2CO	170.92
	2Triazol C_5	152.01
	2Triazol C_3	147.18
	2NCH_2N	66.10
	2CH_2OCH_2	66.02
	2CH_2NCH_2	49.90
	CH_2CH_2	27.57
	Alifatik C	17.64(2$\underline{CH_2}CH_3$), 9.77(2$CH_2\underline{CH_3}$)
3d	2CO	170.96
	2Triazol C_5	151.95
	2Triazol C_3	146.23
	C-3 Aromatik C	135.92 (2C), 131.81 (2C), 129.06 (4CH), 128.62 (4CH)
	2NCH_2N	66.15
	2CH_2OCH_2	66.02
	2CH_2NCH_2	49.89
	CH_2CH_2	27.54
	Alifatik C	29.92(2CH_2Ph), 20.60(2PhCH_3)
3e	2CO	171.04
	2Triazol C_5	151.89
	2Triazol C_3	145.80
	C-3 Aromatik C	133.91 (2C), 131.63 (2C), 130.65 (4CH), 128.50 (4CH)
	2NCH_2N	66.19
	2CH_2OCH_2	65.99
	2CH_2NCH_2	49.87
	CH_2CH_2	27.52
	Alifatik C	29.56(2CH_2Ph)

2.2 Biological activities of synthesized compounds

In the determination of the biological properties of the assay, the antioxidant and antimicrobial activities of the synthesized compounds of type **2 (2a-2f)** and type **3 (3a, 3d, 3e)** were investigated. Three basic mechanisms (RA, FRE, MCA) were used to determine antioxidant properties, while antimicrobial activity was analyzed by Agar Well Diffusion Method (AWDM) against different microorganisms and drugs (Erhonyota et al. 2023).

RA is an important parameter determining the antioxidant capacity of the compounds and was analyzed according to the Oyaizu method (Oyaizu 1986). The basic principle of this method is based on measuring the absorbance of the Prussian blue complex formed by the reaction of the test compounds with $K_3Fe(CN)_6$ to reduce Fe^{2+} ions at a wavelength of 700 nm. The increase in absorbance values represents the electron-donating capacity of the compounds and, hence, the RA (Benzie and Strain 1996).

FRE was analyzed using the Blois method (Blois 1958). In this method, the stable free radical DPPH (1,1-diphenyl-2-picrylhydrazyl) is reduced by antioxidant compounds and the color change occurs. The color change from red to yellow was measured at 517 nm wavelength with the help of a UV-spectrophotometer and the decrease in absorbance values was evaluated in direct proportion to the FRE activity. Measurement of FRE capacity is especially important in the prevention of oxidative stress-induced biomolecular damages such as lipid peroxidation, protein oxidation and DNA damage (Kotha et al. 2022).

MCA was tested using the Dinis method (Dinis et al. 1994). In this method, inhibition of the formation of the complex of Fe^{2+} ions with ferrozine indicates the capacity of the compounds to bind metal ions. Absorbance values were measured at a wavelength of 562 nm and the chelating efficiency of metal ions was analyzed. Chelating plays a critical role in the inhibition of mechanisms that catalyze free radical formation, especially the Fenton reaction (Winterbourn 2018).

The antimicrobial activity of the synthesized compounds was evaluated using AWDM (Perez et al. 1990). This method is a reliable technique that makes the inhibitory effects of compounds on target microorganisms visual and measurable.

In the antimicrobial efficacy study, *Candida albicans* (CA, ATCC10231), a yeast-like fungal species, *Staphylococcus*

aureus (SA, ATCC6538) and *Bacillus cereus* (BC, ATCC11778), Gram-positive bacteria, *Serratia marcescens* (SM, ATCC13880) and *Klebsiella pneumoniae* (KP, ATCC4352), Gram-negative bacteria were used. These microorganisms are frequently preferred standard test strains to evaluate the efficacy of antimicrobial agents (Chavez-Esquivel et al. 2021). In particular, SA and KP are among the pathogens that show antibiotic resistance and are widely discussed in the literature because they cause serious infections (Frieri et al. 2017). CA, which is used as a fungal species, is the main agent of common opportunistic infections in humans and is an important model organism for evaluating the efficacy of antifungal agents (Liu et al. 2014).

Microorganism cultures were grown overnight in Mueller Hinton liquid medium and diluted to a density of 10^6 cfu/mL. The prepared cultures were inoculated on Mueller Hinton agar medium, and then 50 µL stock solutions of the test compounds (250-500 µg/mL) were added to the wells using a sterile technique. Ampicillin (A, 3261, 10 µg), Neomycin (N, 3360, 10 µg) and Streptomycin (S, 3385, 5 µg) were used as a standard antibiotics for antimicrobial activity comparisons. In addition, Fluconazole (F, FCA-25, 5 µg) was preferred as a reference drug in antifungal activity assays (Zalevskaya and Gur'eva 2021). DMSO was used as solvent control and recorded as a negative control.

3. Results and Discussions

In the analyses of the study, the antioxidant capacities of type **2-3** compounds were evaluated via RA, FRE and MCA. As a standard reference, α-tocopherol (1 mg/mL), BHT (1 mg/mL) and BHA (1 mg/mL) were used in RA and FRA analyses, while EDTA (1 mg/mL) and α-tocopherol (1 mg/mL) were used in MCA analyses. In addition, antimicrobial activities against Gram-positive and Gram-negative bacteria and a yeast-like fungus were compared using AWDM. The choice of these microorganisms increases the comparability of the findings of the study in accordance with the literature and contributes to the evaluation of the broad-spectrum antimicrobial activity of the synthesized compounds. Furthermore, the findings of the analysis reveal both the antioxidant and antimicrobial potential of the synthesized compounds and serve as a reference for biomedical applications.

3.1 Antioxidant mechanisms

The RA of the obtained compounds was analyzed at different concentrations and 700 nm wavelength using UV spectrophotometer. It was observed that the absorption values of type 2 (2a, 2b, 2c, 2d, 2e and 2f) and type 3 (3a, 3d, 3e) compounds were at similar levels with the control group and were significantly lower than the standard antioxidants α-tocopherol, BHT and BHA.

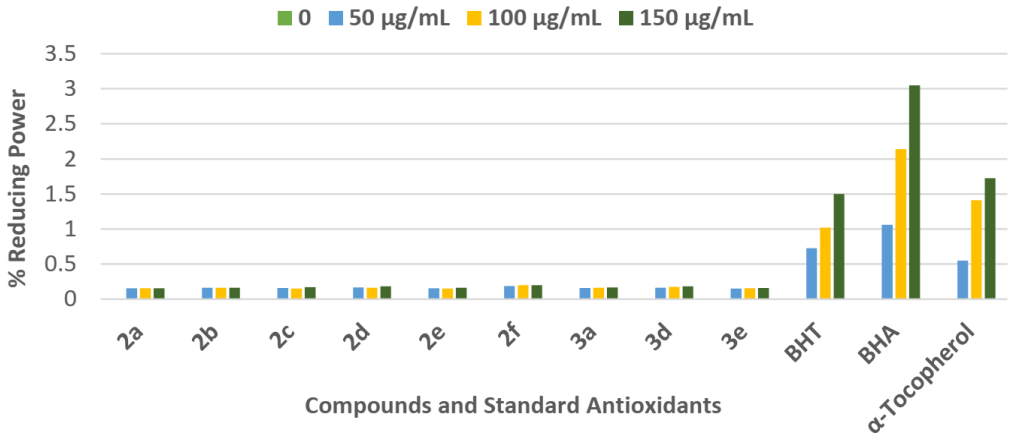

Figure 2. % RA of type **2** and **3** compounds

The measurement results are presented in Figure 2 and the change in absorption values depending on different concentrations is given in detail. These findings provide important data for the evaluation of the synthesized compounds in terms of RA as well as for the investigation of structure-activity relationships. The data reveal that the RA of the synthesized compounds is limited and they do not exhibit strong antioxidant properties. In particular, the lack of electron-donating capacity indicates that these compounds are not effective in combating oxidative stress through the reduction mechanism. However, it should not be ruled out that the compounds may exhibit potential activity through other antioxidant mechanisms (e.g., FRE or MCA).

In the FRE activity tests performed on the synthesized **2**-type amide derivative compounds (**2a, 2b, 2c, 2d, 2e** and **2f**) and **3**-type *N*-Mannich-based compounds (**3a, 3d,** and **3e**), it was observed that the measured inhibition values were not significant. The values obtained as a result of UV

spectrophotometric analysis at 517 nm wavelength were quite low compared to both the control group and the reference antioxidants α-tocopherol, BHA and BHT Amide derivatives (type **2** compounds) gave unstable and insufficient results in terms of FRE activity. The weak electron-donating capacity of these compounds and the absence of functional groups in their structures to support antioxidant activity were considered the main reasons for their low inhibition values. Although the amide derivatives (type **2** compounds) showed a very limited improvement compared to the *N*-Mannich base derivatives (type **3** compounds), this increase was not statistically significant. Despite the potential of the morpholine group to contribute to the FRE activity, this effect was not evident in the compounds tested.

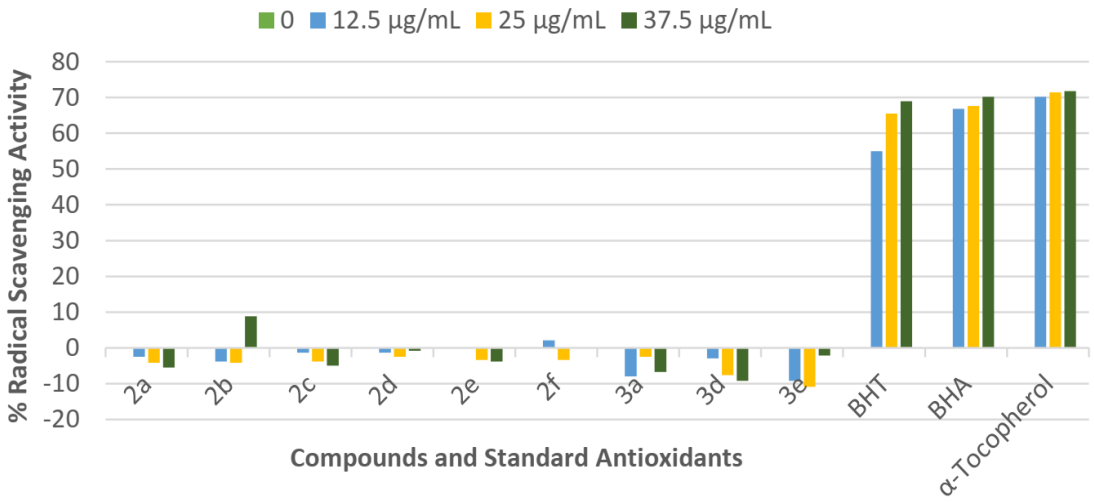

Figure 3. % FRE activites of type **2** and **3** compounds

In general, the findings reveal that both type **2** and type **3** compounds do not exhibit significant antioxidant properties through the FRE mechanism. This indicates that the structure-activity relationships of the compounds are weak and structural modifications are needed to increase the antioxidant activity in order to optimize the FRE activities. In the RA and FRE activity analyses, both amide derivatives (type **2**) and *N*-Mannich-based derivatives (type **3**) did not present significant inhibition values and were insufficient in terms of activity against standard antioxidants. From a medicinal chemistry perspective, the absence of classical radical-scavenging groups such as hydroxyl or phenolic moieties in the structure of these compounds may be the main reason for the observed low activity. In addition, the insufficient electron density and limited resonance stabilization on the triazole ring may have reduced the ability to stabilize free radicals. These findings suggest that targeted modifications involving electron-donating groups could enhance the antioxidant capacity of these compounds in future studies. Therefore, the antioxidant potential of the compounds should be investigated through an alternative mechanism such as MCA activity.

MCA activity tests performed on the synthesized 2 types (2a, 2b, 2c, 2d, 2e) of amide derivative compounds and 3 types (3a, 3d, 3e) of *N*-Mannich-based compounds revealed a strong antioxidant potential of these compounds. The analyses showed that their chelating capacity for Fe^{2+} ions was comparable to the reference chelating agents EDTA and α-tocopherol. Among the amide derivatives, compounds 2b, 2c, and 2d exhibited particularly notable MCA activity, surpassing α-tocopherol. This is attributed to the amide group's ability to form strong complexes with metal ions, which enables effective inhibition of Fe^{2+} ion complexation. Such inhibition may play a key role in preventing the Fenton reaction, a major pathway for free radical generation. In this context, compound 2d demonstrated activity levels close to EDTA, highlighting its potential as a competitive antioxidant agent. These findings suggest that optimizing the structure-activity relationship of amide derivatives could further enhance their antioxidant capacity. Considering the principles of medicinal chemistry, the high affinity of the amide group for metal ions through its oxygen atom may offer an advantage in designing structures with effective chelating properties. Moreover, the planar structure and electron density of the amide group can enhance the stability of metal complexes, potentially enabling the development of more effective radical-scavenging systems in biological environments.

3 type of compounds (*N*-Mannich bases) gave even stronger results in terms of MCA. The morpholine group in the *N*-Mannich structure increased the binding capacity to metal ions thanks to nitrogen atoms and this contributed to the high chelating activity of the

compounds. When the MCA activity levels of compounds **3a, 3d** and **3e** were compared with EDTA, they were found to be very close. Therefore, compounds **3a, 3d** and **3e** were found to have high chelating capacity with similar performance to the reference compounds. Nitrogen-containing heterocyclic structures are generally considered as pharmacophores with high metal ion coordination ability in pharmaceutical chemistry; this explains the contribution of the morpholine group to the chelation capacity.

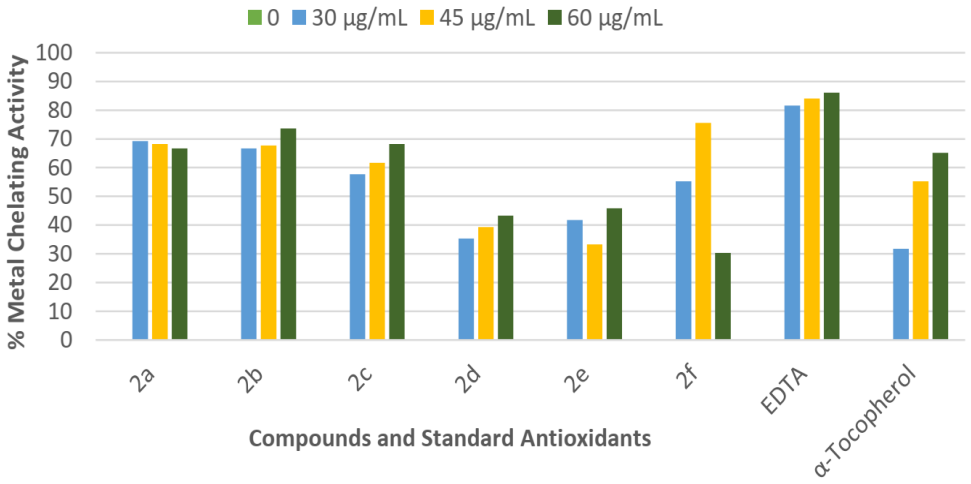

Figure 4. % MCA activities of type **2** compounds

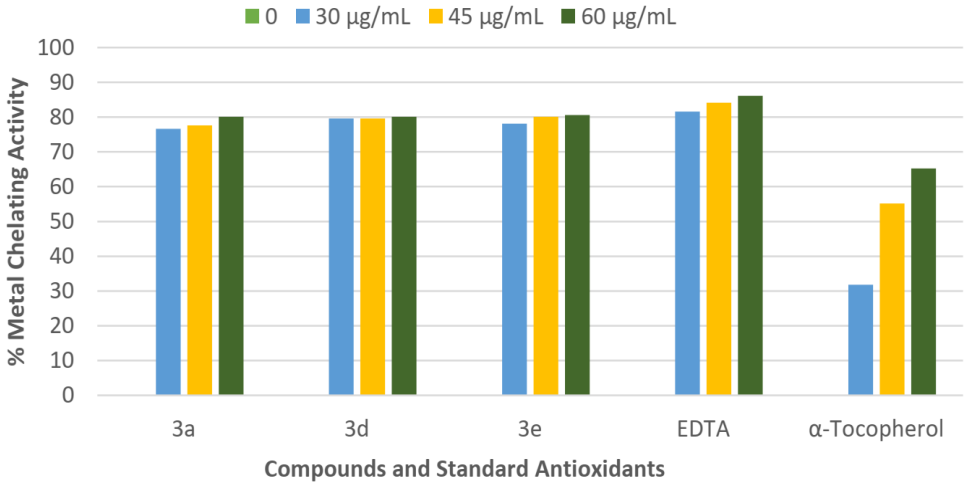

Figure 5. % MCA activities of type **3** compounds

These positive results obtained from MCA tests indicate that the synthesized compounds are candidates for the prevention of oxidative stress-induced diseases and for evaluation as a potential antioxidant agent in biomedical applications.

These findings indicate that the metal ion binding potential of the synthesized **2-3**-type compounds offers an effective mechanism to reduce oxidative stress by inhibiting free radical formation. The high MCA capacity is a factor that increases the antioxidant potential of the compounds and provides a distinct advantage in structure-activity relationships. Future structural modifications aiming to optimize the position and number of these pharmacophore groups may enable the development of more potent and selective antioxidant candidates.

3.2 Antimicrobial activities

When the antibacterial effects of compounds 2a–2f synthesized as 1,2,4-triazol-5-one derivatives were examined, it was concluded that the effect against BC was significant. Compound 2d had a high effect, while compounds 2b, 2c, 2e and 2f had a medium effect. For SA, low effect was obtained from compounds 2b, 2c, while moderate effect was obtained from compound 2d. The fact that these values obtained for BC are at par with the standard antibiotics N and S reveals the significance of the effect.

For gram-negative KP, compounds 2b, 2c, 2e and 2f showed low activity, while compound 2d was close to these compounds and was in the intermediate range and contained a value equal to S. The values obtained for

gram-negative SM were better, and all compounds synthesized in this group were found to be moderately effective. It should be considered important that the values obtained especially for SM were close to all standard antibiotics.

On the other hand, the efficacy values obtained against fungi were negligibly low. Only compound 2c showed a low effect. Although these results demonstrate that the amide derivatives are effective against Gram-positive bacteria, their limited antifungal activity may be attributed to the absence of suitable functional groups in their structures capable of interacting with fungal cell walls. Additionally, the interaction capacity of the amide group with potential binding sites that target bacterial cell wall synthesis should also be considered.

Table 7. Antimicrobial activity results of type **2** and **3** compounds

	Microorganisms and zone diameter values* (mm)				
	Gram positive		**Gram negative**		**Mushroom**
Compound No	***BC*** **ATCC11778**	***SA*** **ATCC6538**	***KP*** **ATCC4352**	***SM*** **ATCC13880**	***CA*** **ATCC10231**
2a	-	-	-	12 (++)	-
2b	15 (++)	9 (+)	9 (+)	11 (++)	-
2c	16 (++)	9 (+)	9 (+)	13 (++)	10 (+)
2d	**20 (+++)**	11 (++)	11 (++)	12 (++)	-
2e	12 (++)	-	7 (+)	14 (++)	-
2f	12 (++)	-	7 (+)	13 (++)	-
3a	**28 (+++)**	15 (++)	15 (++)	**24 (+++)**	15 (++)
3d	**25 (+++)**	13 (++)	13 (++)	**22 (+++)**	15 (++)
3e	**24 (+++)**	13 (++)	13 (++)	**22 (+++)**	15 (++)
A (3261)	**36 (+++)**	**37 (+++)**	**35 (+++)**	15 (++)	-
N (3360)	**17 (+++)**	13 (++)	16 (++)	13 (++)	-
S (3385)	12 (++)	**21 (+++)**	11 (++)	12 (++)	-
F (FCA-25)	-	-	-	-	**25 (+++)**

* In the evaluation according to the inhibition diameter: <5.5 mm negative effect (-); 5.5-10 mm low effect (+); 11-16 mm moderate effect (++); ≥17 mm high effect (+++) (Demirbas et al. 2004).

In antimicrobial activity assays, both groups of compounds were tested on a wide range of microorganisms. Compounds of type 2 showed significant activity, especially against BC. Compound 2d exhibited the highest activity in both BC and SM strains, achieving comparable results with standard antibiotics (N and S). Compounds 2b, 2c, 2e and 2f also showed moderate activity against these strains. However, these compounds exhibited low activity against KP and fungal strain CA. The low activity against fungal strains may be explained by the inability of these compounds to effectively interact with fungal cells, which possess a different membrane structure.

Moreover, the significant increase in antibacterial activity observed in compounds 3a–3e, from which morpholine derivatives were obtained, was a consistent result. The value obtained against BC, where all these compounds showed high levels of activity, was much higher than the values recorded for antibiotics N and S. For Gram-positive SA, all compounds showed moderate activity, with values close to that of antibiotic N. The electron-rich nitrogen atom present in the structure of the morpholine ring may enhance binding to biological targets by enabling stronger interactions with metal ions or enzymes in the bacterial cell membrane. This may be associated with the potential of morpholine-containing compounds to inhibit bacterial cell wall synthesis or DNA replication. These 3 type of *N*-Mannich-based derivatives were particularly remarkable in terms of their antibacterial properties. Especially against Gram-positive SA and BC strains, they exhibited higher levels of activity than standard antibiotics (N and S). The activity against SM was also significantly higher than all reference antibiotics.

On the other hand, while N-Mannich derivatives showed moderate activity on CP, their antifungal efficacy was limited, with only low to moderate effects observed against the CA strain. These findings suggest that N-Mannich derivatives are more effective in combating bacterial infections, but require further optimization to enhance their antifungal potential. These findings indicate that N-Mannich derivatives are more effective in combating bacterial infections but require structural optimization to enhance their antifungal potential. In particular, integrating pharmacophore groups capable of selectively binding to fungal cell wall structures or

targeting ergosterol biosynthesis may improve antifungal efficacy.

The increase in antibacterial effect was also repeated for Gram-negative bacteria in morpholine derivatives. The moderate antibacterial effect value obtained against KP was high for SM. The value obtained against SM was higher than all standard antibiotics, while the values obtained from KP were similar to N and S. When the antifungal effect of this group of compounds was examined, it was determined that there was a moderate effect even if it was not close to standard antifungal drugs. The high affinity of the synthesized morpholine derivatives for metal ions may have contributed to their antimicrobial activity by disrupting ion homeostasis or altering redox balance within the cell membrane.

In this study, 2 types of amide derivatives (2a, 2b, 2c, 2d, 2e, 2f) and 3 types of *N*-Mannich-based derivatives (3a, 3d, 3e) synthesized within the scope of this study were evaluated comprehensively in terms of both the antioxidant and antimicrobial activities. The antioxidant activities based on FRE and RA mechanisms revealed that the antioxidant potential of these compounds is limited. In contrast, in MCA, both groups of compounds exhibited high capacity and showed significant antioxidant activity through this mechanism. Especially considering the role of metal ions in reactions catalyzing free radical formation, the chelating capacity of the compounds was found to have an important potential in combating oxidative stress. *N*-Mannich-based compounds (3 types) presented stronger results in terms of both MCA activity and antimicrobial potential. These compounds containing morpholine group showed high MCA activity comparable to EDTA due to their increased binding capacity to metal ions. In particular, compounds 3a, 3d and 3e were effective in both binding Fe^{2+} ions and inhibiting free radical formation. These findings suggest that *N*-Mannich derivatives contribute to the antioxidant potential by increasing the binding capacity of nitrogen atoms in the structure.

4. Conclusion

The results of this study show that the amide derivatives of type **2** and *N*-Mannich-based derivatives of type **3** offer significant antioxidant activity, especially through the MCA mechanism. In terms of antimicrobial activity, both groups were effective against certain Gram-positive and Gram-negative bacteria, but their antifungal effects were limited. Future studies should aim to increase both the antioxidant and antimicrobial potentials by optimizing structure-activity relationships. In particular, modifications to enhance antifungal activity may enable these compounds to be used in a wider range of biomedical applications.

Declaration of Ethical Standard

This study is derived from the doctorate thesis (thesis number: 716181) titled "Potansiyel Biyolojik Aktif Bazı Yeni N-(3-Alkil/Aril-4,5-Dihidro-1H-1,2,4-Triazol-5-on-4-il)-Amid Türevlerinin Sentezi, Karakterizasyonu ve Bazı Özelliklerinin İncelenmesi", completed by Songül ULUFER BULUT under the supervision of Prof. Dr. Haydar YÜKSEK on February 4, 2022.

Credit Authorship Contribution Statement

Author 1: Conceptualization, investigation, methodology and software, visualization and writing – original draft.

Author 2: Conceptualization, investigation, methodology and software, supervision and writing – review and editing.

Declaration of Competing Interest

The authors have no conflicts of interest to declare regarding the content of this article.

Data Availability

All data analyzed or discussed in this study are included in this article. The raw datasets supporting these findings are available from the corresponding author upon reasonable request.

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