Antioxidant and Antimicrobial Activity of New Amide Compounds Containing Azo Group Using Dicyclohexylcarbodiimide (DCC) as Coupling Agent

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Abstract: *A series of amide compounds (**A2D1–A2D6**) were synthesized based on sulfathiazole by converting it to diazonium salt using $NaNO_2$ and concentrated HCl via diazotization reaction. This reaction was followed by coupling reaction with vanillic acid in alkaline medium to generated azo compound 4-hydroxy-3-methoxy-5-((4-(N-(thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-benzoic acid (**A2**). Compound **A2** was reacted with substituted aromatic amines such as 2-amino-4,6-dimethylpyridine, sulfamerazine, sulfadiazine, sulfanilamide, sulfathiazole, and sulfanilic acid to form corresponding amides using DCC as coupling reagent to be promoted condensation reaction. The structures of synthesized compounds have been diagnosed with elemental analysis, FTIR, ^{1}H-NMR, ^{13}C-NMR, and mass spectrometry. The antibacterial activities for all new synthetic compounds was estimated accurately depending on selected bacteria such as* Staphylococcus aureus *(Gram-positive) and* Escherichia coli *(Gram-negative) using different concentrations to calculate minimum inhibition concentration. The effectiveness of inhibiting fungi was also studied against* Candida albicans *by agar diffusion method. Finally, the antioxidant capacities of the prepared compounds were determined by using DPPH radical scavenging method. The results of potential radical scavenging activity were given as IC_{50}. The compounds showed strong biological activity and good antioxidant activity compared with the standard substance Vitamin C.*

Keywords: *antimicrobial; azo; DCC; sulfathiazole; vanillic acid*

■ INTRODUCTION

Dicyclohexylcarbodiimide (DCC) is one of the most frequently used coupling agents. DCC was first utilized in the mid-1950s, a discovery that greatly advanced the accepted synthetic methods at that time [1]. DCC is a valuable reagent, especially for the preparation of amides, nitriles, ketones, esters, and peptides, due to its role as an intensive dehydrating agent [2]. The coupling of amines and carboxylic acids via a coupling substance or with previous processing of the carboxylic acid typically leads to the formation of amides. The most effective route to prepare an amide bond is through the condensation of an amine with a carboxylic acid, primarily the carboxylate moiety. This moiety must be activated to react correctly with the amine group. In such reactions, reactive functional groups must often be protected.

Amide linkages are essential to living organisms as they form the backbone of proteins. Proteins are considered polymers of essential amino acids, which are typically connected through amide linkages, also known as peptide bonds. Peptide and protein formation occur when the carbon atom of the carboxylate moiety of a protected amino acid reacts with the amine moiety of another protected amino acid via an amide bond [3]. In this process, DCC reacts with the carboxyl group of a protected amino acid to generate an activated acylating reagent, which subsequently reacts with the amino group of another acid to form a new amide bond. Amide-containing compounds play a significant role in various biological functions, including the production of antibiotics like penicillin G. Additionally, amides contribute to the stinging taste of *Piper nigrum* and are present in anandamide, a fatty acid derivative found in chocolate that is known to elicit a sense of pleasure [4].

Sulfonamides, such as sulfanilamide, sulfadiazine, sulfamerazine, and sulfathiazole, are crucial in the pharmaceutical field due to their effectiveness as antimicrobial drugs. These sulfa drugs are used to treat various conditions, including epilepsy, actinobacillosis, and inflammation [5]. Azo compounds, characterized by the –N=N– group, are commonly conjugated with polycyclic aromatic rings, heteroaromatic systems, or two mono-aromatic rings. The unique structure of azo compounds makes them valuable in medical and pharmaceutical applications, including their use in foods, cosmetics, pigments, and chemical analyses. Azo compounds have also been recognized for their antibiotic, antifungal, and anti-HIV and other chemotherapeutic uses [6]. This paper evaluates these compounds' antimicrobial properties against *S. aureus, E. coli*, and *C. albicans* and their antioxidant properties.

■ EXPERIMENTAL SECTION

Materials

Each chemical used in this occupation was reagent grade and purified before use. Vanillic acid, sulfathiazole, sulfadiazine, sulfanilamide, and sulfamerazine were supplied from Sigma Aldrich. DCC, sodium nitrite ($NaNO_2$), hydrochloric acid (HCl), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and citric acid were supplied from Fluka Company.

Instrumentation

The IR spectra for all preparing compounds were recorded at Shimadzu FTIR type 8400S spectrophotometer in KBr discs within a range of 400–4000 cm^{-1}. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with Brucker instrument (400 MHz) using DMSO-d_6, which acted as a standard solvent, and trimethylsilane as an interior standard. Mass spectra were done on a 5975C spectrometer at 70 eV. C.H.N analysis was run on an Elemental Euro Vector EA-3000 A micro analyzer appliance. The purity of all compounds and completeness of a reaction was checked with thin-layer chromatography (TLC) on silica gel-protected aluminum plates.

Procedure

Synthesis of 4-hydroxy-3-methoxy-5-((4-(N-(thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-benzoic acid (A2)

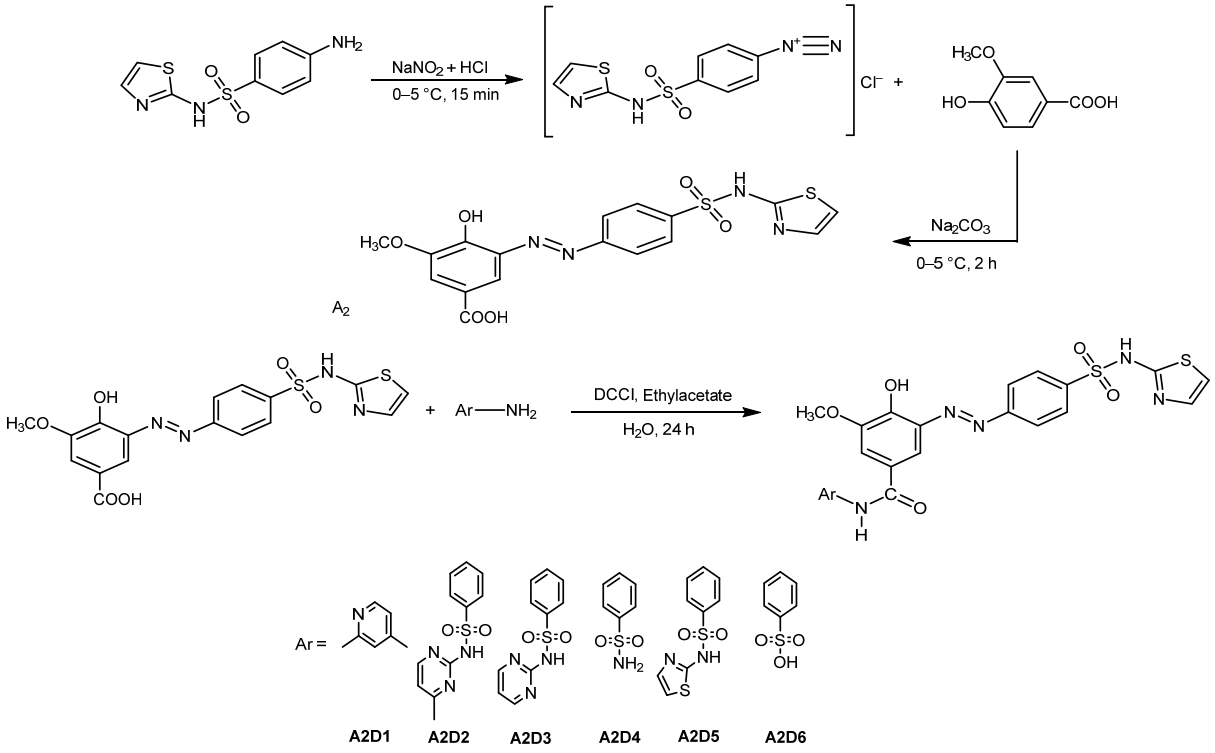
The compound was prepared according to a previously reported procedure [7-8]. Sulfathiazole (0.45 g, 2 mmol) was dissolved in 10 mL of 2 M HCl in an ice-cold solution, stirring until a clear mixture was obtained while maintaining the temperature at 0–5 °C. A solution of $NaNO_2$ (0.4139 g, 6 mmol) in 4 mL of water was then prepared and added dropwise to the sulfathiazole solution with continuous stirring, ensuring that the temperature remained between 0–5 °C throughout the addition. The reaction mixture was stirred for an additional 30 min. Vanillic acid (0.336 g, 2 mmol) was dissolved in 15 mL of an aqueous sodium bicarbonate solution (1.05 g, 10 mmol). The vanillic acid solution, maintained at 0–5 °C, then was gradually added to the cold diazonium salt solution. The resulting mixture was stirred gently while, maintaining the temperature at 0–5 °C. The precipitated solid was collected by filtration and washed thoroughly with cold water. The product was recrystallized from absolute ethanol to afford the azo compound. The purity of the prepared azo compound was confirmed by TLC using a methanol-acetone (3:7) mixture as the eluent.

Darkness brown crystals were procured in 91% yield, R_f: 0.51, m.p. 100 °C. Elemental analysis for $C_{17}H_{14}N_4O_6S_2$, Calc.: C 47.00; H 3.25; N 12.90, Found: C 47.32; H 3.27; N 12.95. IR (KBr) cm^{-1}: 3553 (O–H), 3417 (N–H), 3101 ($CH_{Arom.}$), 2916 ($CH_{Aliph.}$); 1635 (C=O), 1589 (N=N), 1527 ($C=N_{Sulfa}$), 1388 ($SO_{2\ Asym.}$), 1141 ($SO_{2\ Sym.}$), 1084 (C–O), 941 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 10.12 (s, 1H$_c$, COOH), 8.74 (s, 1H$_s$, NH), 8.24 (s, 1H$_d$, Arom-H), 8.13 (s, 1H$_e$, Arom-H), 7.97 (dd, 2H$_{ff'}$, Arom-H), 7.81 (dd, 2H$_{gg'}$, Arom-H), 7.46 (s, 1H$_h$, Arom-H), 7.35 (s, 1H$_i$, Arom-H), 6.93 (s, 1H$_b$, OH), 3.92 (s, 3H$_a$, OCH_3). ^{13}C-NMR (DMSO-d_6 400 MHz): δ/ppm 182.71 (COOH), 176.27 (S–C=N), 145.59, 140.24, 138.24, 132.47, 131.57, 129.69, 124.95 ($CH=C_{Arom.}$), 43.55 (OCH_3). The EI-MS (*m/z*): 434 $[M]^+$, 389 $[C_{16}H_{13}N_4O_4S_2]^+$, 343 $[C_{15}H_{11}N_4O_2S_2]^+$, 256 $[C_9H_{10}N_3O_2S_2]^+$, 185 $[C_6H_7N_3O_2S]^+$, 149 $[C_3H_3NO_2S_2]^+$, 69 $[C_4H_7N]^+$.

General procedure for the synthesis of amide derivatives

The new compounds (**A2D1**–**A2D6**) were synthesized by reacting **A2** with various amines, following a literature procedure [9]. **A2** (0.533 g, 1 mmol) was mixed with 1 mmol of different amines, specifically 2-amino-4,6-dimethylpyridine (0.122 g), sulfamerazine (0.264 g), sulfadiazine (0.250 g), sulfanilamide (0.172 g), sulfathiazole (0.255 g), and sulfanilic acid (0.173 g), in 50 mL of ethyl acetate. The reaction mixture was stirred until a clear solution was obtained. DCC solution was prepared by dissolving DCC (0.206 g, 1 mmol) in 5 mL of ethyl acetate. This solution was added dropwise over 20 min to the amine mixture with continuous stirring, which led to the formation of a white precipitate. The resulting precipitate was filtered, and the filtrate was washed thoroughly with 15 mL of 5% aqueous citric acid. After the extraction, the aqueous layer was discarded, and the organic layer was carefully collected. The solvent was evaporated to yield the corresponding amide. The crude amides were recrystallized from absolute methanol. The reaction progress and purity of the newly synthesized amides were monitored by TLC using an ethanol-chloroform (3:7) solvent system.

3-((2-Amino-5-(N-(thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-*N*-(4,6-dimethylpyridin-2-yl)-4-hydroxy-5-methoxybenzamide (A2D1). Light yellow solid was acquired in 91% yield. R_f: 0.57, m.p. 132–130 °C. Elemental analysis for $C_{24}H_{22}N_6O_5S_2$, Calc.: C 53.52, H 4.12, N 15.60, Found: C 53.80, H 4.14, and N 15.64. IR (KBr) cm^{-1}: 3537 (O–H), 3260 (N–H), 3151 ($CH_{Arom.}$), 2870 ($CH_{Aliph.}$), 1782 ($C{=}O_{AmideI}$), 1750 ($NH_{AmideII}$), 1593 (N=N), 1269 ($SO_{2\ Asym.}$), 1126 ($SO_{2\ Sym.}$), 891 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 11.26 (s, 1H$_e$, NH–CO), 10.10 (s, 1H$_k$, NH), 8.76 (s, 1H$_d$, Arom-H), 8.57 (s, 1H$_b$, Arom-H), 8.30 (d, 1H$_l$, Arom-H), 8.07 (d, 1H$_m$, Arom-H), 7.81 (dd, 1H$_{j,j'}$, Arom-H), 7.44 (dd, 1H$_{i,i'}$, Arom-H), 7.03 (s, 1H$_n$, Arom-H), 6.68 (s, 1H$_f$, Arom-H), 5.47 (s, 1H$_h$, OH), 3.97 (s, 3H$_g$, OCH_3), 1.85 (s, 3H$_a$, CH_3), 1.59 (s, 3H$_c$, CH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 175.94 (C=O), 171.98 (C=N), 148.04, 143.86, 129.94, 126.08, 120.54, 113.60, ($CH{=}C_{Arom.}$), 47.99 (OCH_3), 21.61, 19.52 (CH_3).



Scheme 1. General pathways for synthesis of compound **A2** and **A2D1**-**A2D6**

3((2-Amino-5-(*N*-(thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-4-hydroxy-5-methoxy-*N*-(4-(*N*-(1-methylpyrimidin-2-yl)sulfamoyl)phenyl)benzamide (A2D2). Yellowish solid was acquired in 77% yield. R_f: 0.69, m.p. 158–160 °C. Elemental analysis for $C_{28}H_{24}N_8O_7S_3$, Calc.: C 49.40, H 3.55, N 14.66, Found: C 49.62, H 3.56, N 16.51. IR (KBr) cm^{-1}: 3498 (O–H), 3309 (N–H), 3705 ($CH_{Arom.}$), 2928 ($CH_{Aliph.}$), 1782 ($C=O_{AmideI}$), 1750 ($NH_{AmideII}$), 1593 (N=N), 1222 ($SO_{2\ Asym.}$), 1153 ($SO_{2\ sym.}$) 952 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 12.37 (s, 1H$_g$, NH–CO), 10.09 (s, 1H$_d$, NH), 10.06 (s, 1H$_n$, NH), 9.78 (d, 1H$_b$, Arom-H), 8.31 (d, 1H$_c$, Arom-H), 8.23 (dd, 2H$_{e,e'}$, Arom-H), 8.14 (dd, 2H$_{f,f'}$, Arom-H), 7.91 (s, 1H$_h$, Arom-H), 7.89 (s, 1H$_i$, Arom-H), 7.74 (dd, 2H$_{m,m'}$, Arom-H), 7.62 (dd, 2H$_{l,l'}$, Arom-H), 6.89 (s, 1H$_p$, Arom-H), 6.56 (s, 1H$_o$, Arom-H), 5.16 (s, 1H$_j$, OH), 3.93 (s, 1H$_k$, OCH_3), 1.72 (s, 3H$_a$, CH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 175.00 (C=O), 171.74 (C=N), 148.61, 141.29, 138.09, 138.07, 131.34, 124.04, 118.68, 112.35, 118.71, ($CH=C_{Arom.}$), 43.15 (OCH_3), 24.91 (CH_3). The EI-MS (*m/z*): 680 $[M]^+$, 663 $[C_{28}H_{23}N_8O_6S_3]^+$, 424 $[C_{19}H_{16}N_6O_4S]^+$, 368 $[C_{18}H_{16}N_4O_3S]^+$, 293 $[C_{12}H_{13}N_4O_3S]^+$, 224 $[C_9H_6NO_2S_2]^+$.

3((2-Amino-5-(*N*(thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-4-hydroxy-5-methoxy-*N*-(4-(*N*-pyrimidin-2-yl)surfamoyl)phenyl)benzamide (A2D3). Yellow solid was acquired in 74% yield. R_f: 0.69, m.p. 139–141 °C. Elemental analysis for $C_{27}H_{22}N_8O_7S_3$, Calc.: C 48.64, H 3.33, and N 16.18, Found: C 48.90, H 3.34, and N 16.86. IR (KBr) cm^{-1}: 3502 (O–H), 3225 (N–H), 3105 ($CH_{Arom.}$), 2978 ($C–H_{Aliph.}$), 1739 ($C=O_{AmideI}$), 1729 ($NH_{AmideII}$), 1423 (N=N), 1195 ($SO_{2\ Asym.}$), 1060 ($SO_{2\ Sym.}$), 949 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 10.89 (s, 1H$_g$, NH–CO), 9.73 (s, 1H$_d$, NH), 9.49 (s, 1H$_n$, NH), 7.35 (dd, 2H$_{a,a'}$, Arom-H), 7.29 (t, 1H$_b$, Arom-H), 7.23 (dd, 2H$_{e,e'}$, Arom-H), 7.10 (dd, 2H$_{f,f'}$, Arom-H), 6.96 (s, 1H$_h$, Arom-H), 6.82 (s, 1H$_i$, Arom-H), 6.73 (dd, 2H$_{m,m'}$, Arom-H), 6.65 (dd, 2H$_{l,l'}$, Arom-H), 6.52 (d, 1H$_p$, Arom-H), 6.45 (s, 1H$_o$, Arom-H), 5.45 (s, 1H$_j$, OH), 3.95 (s, 3H$_k$, OCH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 175.10 (C=O), 171.74 (C=N), 131.66, 126.45, 121.79, 119.94, 114.26, ($CH=C_{Arom.}$), 47.97 (OCH_3). The EI-MS (*m/z*): 666 $[M]^+$, 551 $[C_{24}H_{19}N_6O_6S_2]^+$, 424 $[C_{20}H_{18}N_5O_4S]^+$, 279 $[C_{11}H_{11}N_4O_3S]^+$, 224 $[C_{13}H_{10}N_3O]^+$, 143 $[C_4H_3N_2O_2S]^+$, 56 $[C_2H_4N_2]^+$.

3-((2-amino-5-(*N*-thiazol-2-yl)sulfamoyl)phenyl)diazenyl)-4-hydroxy-5-methoxy-*N*-(4-sulfamoylphenyl)benzamide (A2D4). Light yellow solid was acquired in 96%. R_f: 0.87, m.p. 120–122 °C. Elemental analysis for $C_{23}H_{20}N_6O_7S_3$, Calc.: C 46.93, H 3.42, N 14.28, Found: C 47.13, H 3.44, N 14.33. IR (KBr) cm^{-1}: 3518 (O–H), 3225 (N–H), 3105 ($CH_{Arom.}$), 2928 ($C–H_{Aliph.}$), 1732 ($C=O_{AmideI}$), 1627 ($NH_{AmideII}$), 1415 (N=N), 1220 ($SO_{2\ Asym.}$), 1168 ($SO_{2\ Sym}$), 902 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 11.95 (s, 1H$_g$, NH–CO), 10.28 (s, 1H$_j$, OH), 9.52 (s, 1H$_n$, NH), 8.41 (dd, 2H$_{b,b'}$, Arom-H), 8.23 (dd, 2H$_{c,c'}$, Arom-H), 7.96 (s, 1H$_h$, Arom-H), 7.86 (s, 1H$_i$, Arom-H), 7.74 (dd, 2H$_{m,m'}$, Arom-H), 7.46 (dd, 2H$_{l,l'}$, Arom-H), 7.18 (d, 1H$_p$, Arom-H), 6.98 (d, 1H$_o$, Arom-H), 6.58 (s, 2H$_a$, NH_2), 3.93 (s, 3H$_k$, OCH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 175.14 (C=O), 171.76 (C=N), 148.87, 144.62, 141.33, 137.22, 127.86, 122.01, 118.06, ($CH=C_{Arom.}$), 43.25 (OCH_3). The EI-MS (*m/z*): 588 $[M]^+$, 449 $[C_{17}H_{17}N_6O_5S_2]^+$, 389 $[C_{16}H_{13}N_4O_4S_2]^+$, 225 $[C_{13}H_{11}N_3O]^+$, 185 $[C_6H_7N_3O_2S]^+$, 149 $[C_7H_7N_3O]^+$, 56 $[C_2H_4N_2]^+$.

3-((2-Amino-5(*N*-(thaizol-2-yl)sulfamoyl)phenyl)diazenyl)-4-hydroxy-5-methoxy-*N*-(4-(*N*-thaizol-2-yl)sulfamoyl)phenyl)benzamide (A2D5). Pale yellowish solid was acquired in 68%. R_f: 0.84, m.p. 147–150 °C. Elemental analysis for $C_{26}H_{21}N_7O_7S_4$, Calc.: C 46.49, H 3.15, N 46.60, Found: C 46.60, H 3.17, N 14.65. IR (KBr) cm^{-1}: 3502 (O–H), 3325 (N–H), 3100 ($CH_{Arom.}$), 2931 ($CH_{Aliph.}$), 1730 ($C=O_{AmideI}$), 1728 ($NH_{AmideII}$), 1581 (N=N), 1226 ($SO_{2\ Asym}$), 1195 ($SO_{2\ Sym}$), 941 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 12.06 (s, 1H$_f$, NH–CO), 11.23 (s, 2H$_{c,c'}$, NH–SO_2), 9.32 (dd, 2H$_{a,a'}$, Arom-H), 9.01 (dd, 2H$_{b,b'}$, Arom-H), 8.63 (dd, 2H$_{d,d'}$, Arom-H), 8.51 (dd, 2H$_{e,e'}$, Arom-H), 8.33 (s, 1H$_g$, Arom-H), 7.76 (s, 1H$_h$, Arom-H), 7.37 (dd, 2H$_{k,k'}$, Arom-H), 6.91 (dd, 2H$_{l,l'}$, Arom-H), 5.32 (s, 1H$_i$, OH), 3.93 (s, 3H$_j$, OCH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 174.99 (C=O), 171.74 (C=N), 148.06, 145.40, 143.51, 141.32, 137.45, 129.70, 124.37, 118.28, 112.85, ($CH=C_{Arom.}$), 43.14 (OCH_3). The EI-MS (*m/z*): 671 $[M]^+$, 446

$[C_{17}H_{14}N_6O_5S_2]^+$, 349 $[C_{14}H_{13}N_4O_5S]^+$, 285 $[C_{10}H_{11}N_3O_3S_2]^+$, 224 $[C_{13}H_{10}N_3O]^+$, 186 $[C_6H_8N_3O_2S]^+$, 99 $[C_3H_3N_2S]^+$, 56 $[C_2H_4N_2]^+$.

4-(3-((2-Amino-5-(*N*-(thiazol-2-yl)sulfamoyl)phenyl)-4-hydroxy-5-methoxy-benzenesulfonic acid (A2D6). Yellowish white solid was acquired in 81%. R_f: 0.46, m.p. 120–121 °C. Elemental analysis for $C_{23}H_{19}N_5O_8S_3$, Calc.: C 46.85, H 3.25, N 11.88, Found: C 47.09, H 3.26, N 11.93. IR (KBr) cm^{-1}: 3502 (O–H), 3325 (N–H), 3125 ($CH_{Arom.}$), 2931 ($CH_{Aliph.}$), 1743 ($C=O_{AmideI}$), 1708 ($NH_{AmideII}$), 1577 (N=N), 1230 ($SO_{2\ Asym}$), 1192 ($SO_{2\ Sym}$), 945 (S–N). ^{1}H-NMR (DMSO-d_6 400 MHz) δ/ppm: 13.81 (s, $1H_g$, OH), 12.44 (s, $1H_d$, NH–CO), 9.72 (s, $1H_k$, NH), 8.14 (dd, $2H_{b,b'}$, Arom-H), 7.47 (dd, $2H_{c,c'}$, Arom-H), 6.92 (s, $1H_f$, Arom-H), 6.70 (s, $1H_e$, Arom-H), 6.49 (dd, $2H_{i,i'}$, Arom-H), 6.35 (dd, $2H_{j,j'}$, Arom-H), 6.21 (d, $1H_l$, Arom-H), 5.86 (d, $1H_m$, Arom-H), 5.21 (s, $1H_a$, OH), 3.90 (s, $3H_h$, OCH_3). ^{13}C-NMR (DMSO-d_6 400 MHz) δ/ppm: 175.02 (C=O), 171.74 (C=N), 146.73, 141.51, 135.52, 128.58, 122.19, 114.45, ($CH=C_{Arom.}$), 43.16 (OCH_3). The EI-MS (*m/z*): 589 $[M]^+$, 509 $[C_{23}H_{19}N_5O_5S_2]^+$, 410 $[C_{17}H_{16}O_4N_5S_2]^+$, 325 $[C_{16}H_{13}N_4O_2S]^+$, 279 $[C_{15}H_{11}N_4S]^+$, 224 $[C_{13}H_{10}N_3O]^+$, 204 $[C_9H_8N_4S]^+$, 99 $[C_3H_3N_2S]^+$, 56 $[C_2H_4N_2]^+$.

Antimicrobial activity

The antimicrobial activity of compounds **A2D1**–**A2D6** was tested *in vitro* using the agar diffusion method with nutrient agar (NA) as the culture medium [10]. The pathogens used for testing included *S. aureus* (ATCC 25923) as a Gram-positive bacterium, *E. coli* (ATCC 25922) as a Gram-negative bacterium, and the fungal strain *C. albicans* (ATCC 14053). Dimethyl sulfoxide (DMSO) served as the solvent and was used as a negative control disc. The experiment aimed to assess the zone of inhibition produced by each compound against the tested microorganisms. Bacterial and fungal inocula (0.2 mL each) were spread evenly onto the surface of the Sabouraud dextrose agar and NA medium using an L-shaped glass rod. Filter paper discs impregnated with the test compounds were placed onto the agar plates and allowed to diffuse for 10 min. All compounds, along with sulfathiazole, amoxicillin, and nystatin as reference drugs, were dissolved in DMSO at different concentrations (100, 50, and 25 mg) to determine their minimum inhibitory concentration (MIC). The synthesized azo compounds were applied to the central wells of the agar plates in 0.1 mL aliquots. Fungal plates were incubated at 25 ± 2 °C, while bacterial plates were incubated at 37 ± 2 °C for 24 h. After incubation, the zones of inhibition were measured in millimeters for each microorganism. Additionally, the MIC values of the compounds were calculated to assess their antimicrobial potency [11].

Antioxidant activity

The radical scavenging activity of the new amides was evaluated using the DPPH free radical scavenging assay. This method measures the ability of compounds to act as free radical scavengers or hydrogen donors by reacting with DPPH radicals. At room temperature, the DPPH solution appears violet, with a characteristic absorption peak at 515 nm. Upon interaction with a radical scavenger, the color of the solution changes to pale yellow or colorless, indicating the reduction of the radical. The progress of the reaction can be visually monitored through this color change, and the degree of radical scavenging can be quantified by measuring the reduction in absorbance at 515 nm. This allows for the determination of the initial amount of free radicals present, providing insight into the antioxidant potential of the tested compounds.

The antioxidant activity of the synthesized compounds was evaluated using the Baba and Malik procedure [12]. For each compound, 0.2 mL at varying concentrations (0.78, 1.56, 3.17, 6.25, 12.5, 25, 50, and 100 μg/mL) was added to 3.8 mL of a 100 μg/mL DPPH solution. The resulting mixtures were incubated in the dark at 25 °C for 30 min. A negative control solution containing all reagents except the samples was prepared, and methanol was used as a blank. The DPPH radical scavenging efficiency was measured by UV-vis spectroscopy at 515 nm, and the results were reported as IC_{50} values. Additionally, the reduction efficiency of **A2** and the newly synthesized amides was assessed with some modifications to the procedure [12]. The scavenging activity of ascorbic acid (Vitamin C) was also measured for comparison. The inhibition percentage of the prepared compounds was calculated using Eq. (1);

$$IC_{50}\ (\%) = \frac{A_b - A_m}{A_b} \times 100\% \quad (1)$$

where, A_b represents the control solution absorption, and A_m represents the sample absorption.

■ RESULTS AND DISCUSSION

The **A2** compound was synthesized from sulfathiazole via a diazotization reaction. This involved the reaction of sulfathiazole with $NaNO_2$ and HCl to generate the corresponding diazonium salt, which was then coupled with vanillic acid at 0–5 °C to form the target compound, as shown in Scheme 1. The synthesized **A2** was subsequently reacted with various amines, including 2-amino-4,6-dimethylpyridine, sulfamerazine, sulfadiazine, sulfanilamide, sulfathiazole, and sulfonic acid, to yield compounds **A2D1**, **A2D2**, **A2D3**, **A2D4**, **A2D5**, and **A2D6**, respectively, with good yields.

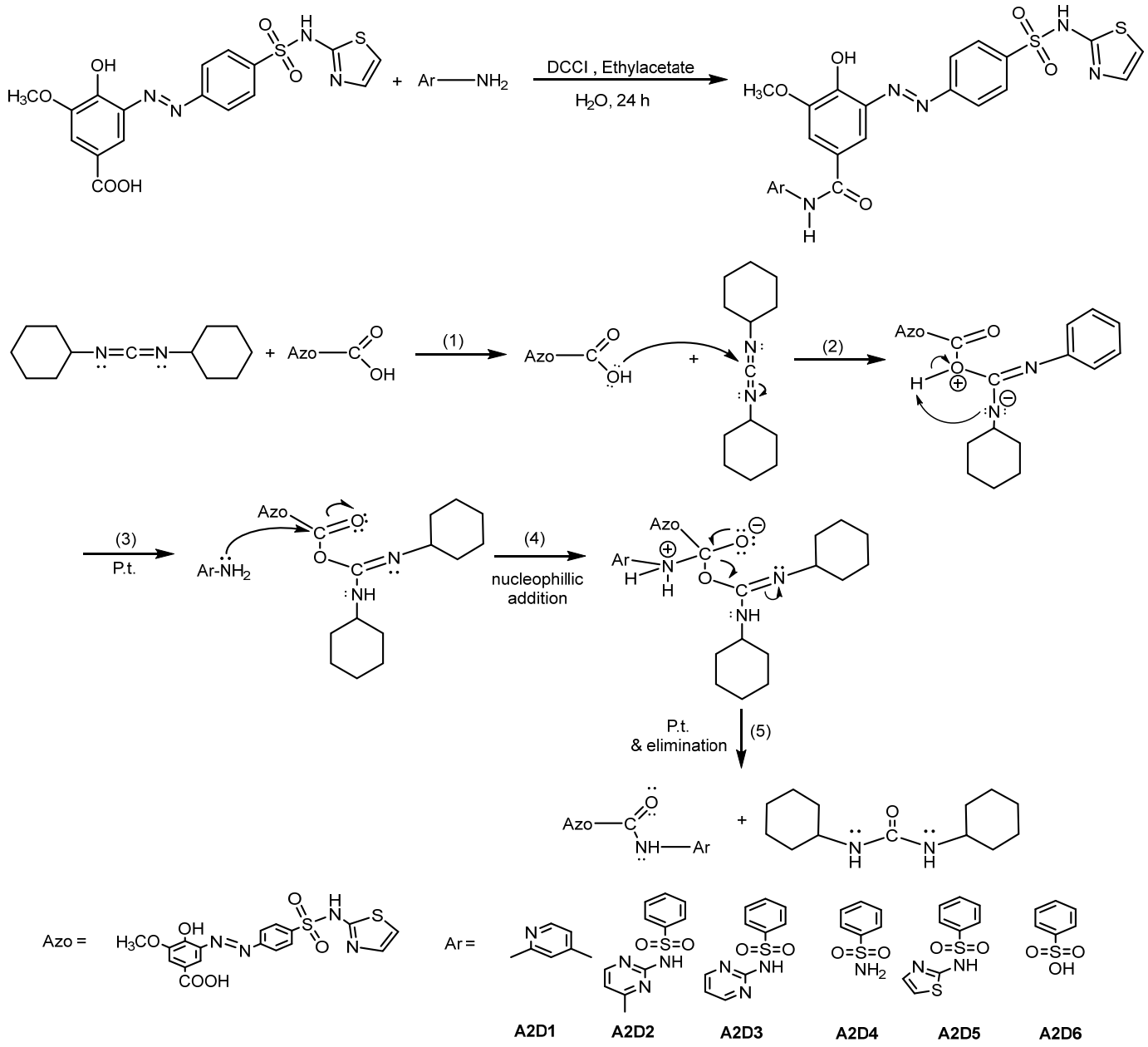
This work focuses on the synthesis of amides through the reaction of equimolar amounts of compound **A2** with different amines. The formation of amides from carboxylic acids is thermodynamically favorable [13]. DCC was used as a catalyst to promote this reaction under mild conditions [14]. The mechanism of the coupling between the amine and carboxyl groups (from azo-vanillic acid) under DCC catalysis proceeds through a few key steps. First, protonation of DCC occurs (step 1), followed by the addition of the carboxylate anion from the azo-vanillic acid to the protonated imide (step 2), forming an O-acylisourea intermediate. In step 3, the aromatic amine undergoes nucleophilic addition to the carbonyl group of the intermediate, with proton transfer, to form the amide. Finally, the amide product is formed, and dicyclohexylurea, a by-product, precipitates as a white solid (leaving group), as illustrated in Scheme 2.

FTIR Spectra

The IR spectra of the synthesized compounds (Fig. S1) revealed a broad band in the range of 3537–3498 cm^{-1}, corresponding to the stretching vibration of the hydroxyl group (ν(OH)). A medium band between 3340–3225 cm^{-1} was attributed to the stretching vibration of the amine group (ν(NH)). The formation of the amide bond was confirmed by the presence of strong bands corresponding to the carbonyl stretching vibration (amide I, ν(C=O)) observed between 1782–1730 cm^{-1}, and a medium band assigned to ν(NH) stretching vibration (amide II) appearing in the range 1750–1627 cm^{-1}. Additionally, all compounds exhibited clear absorption bands in the range 1269–1195 cm^{-1}, alongside bands between 1195–1126 cm^{-1}, corresponding to the asymmetric and symmetric stretching vibrations of the sulfonyl group (ν(SO_2)) [15-16]. The spectra also showed absorption bands in the range 1593–1415 and 949–891 cm^{-1}, which were assigned to the stretching vibrations of the azo group (ν(N=N)), as indicated in the experimental section.

^{1}H- and ^{13}C-NMR Spectra

The ^{1}H- and ^{13}C-NMR spectra were summarized in Fig. S2 and S3. The ^{1}H-NMR spectrum of the synthesized compounds exhibited a singlet signal in the range of δ 6.93–5.06 ppm, which is attributed to the protons of the phenolic group (–OH). The acidic protons in compounds **A2** and **A2D6** were characterized by singlet signals at 10–12 and 13.81 ppm, respectively [17]. The spectra of **A2D1**–**A2D6** revealed singlet signals in the range of 12.44–10.89 ppm, corresponding to the (NH–CO) protons [14]. The new amide compounds also displayed a singlet signal at 11.23–9.72 ppm, attributed to the NH protons associated with the sulfa moiety. The methoxy group (OCH_3) in all synthesized compounds was identified by a singlet signal at 3.97–3.78 ppm [7]. Moreover, the signals for all aromatic ring protons appeared within their expected ranges. The ^{13}C-NMR spectra of the new amide compounds showed signals in the range of 175.94–174.99 ppm, attributed to the (NHC=O) moiety. In contrast, the signal for the carboxylic acid (COOH) in compound **A2** appeared at 182.71 ppm, confirming the formation of the amide bond in the synthesized compounds [18]. Furthermore, signals in the range of δ 47.99–13.14 ppm were assigned to the carbon atom of the OCH_3 group. The methyl groups in compounds **A2D1** and **A2D2** appeared at 19.52 and 24.91 ppm, respectively. Additionally, the carbon atoms of the aromatic rings showed various signals in their expected regions.



Scheme 2. Mechanism preparation of amide

EI-MS

Mass spectrometry is a pivotal analytical technique employed across various scientific disciplines, including chemistry, pharmacy, biochemistry, and medicine. This technique enables the identification and quantification of unknown compounds within a sample, as well as the determination of molecular weight and molecular formula of the molecules present. The process involves bombarding gaseous molecules with high-energy electrons, which results in the ejection of an electron from either a bond or a non-bonding electron, thereby converting the molecule into a positively charged ion known as a molecular ion ($[M]^+$). Subsequently, fragmentation occurs, generating a variety of charged and neutral fragments that traverse a magnetic field. This movement allows for the separation of the resulting ions based on their distinct mass-to-charge ratios ($[m/z]$) [19].

The mass spectra of the prepared compounds exhibited $[M]^+$ peaks that align perfectly with their respective structures. The proposed fragment ions and their intensities within the spectra reveal important structural characteristics of these compounds. Specifically, the mass spectra for compounds **A2**, **A2D2**, **A2D3**, **A2D4**, **A2D5**, and **A2D6** showed $[M]^+$ at 434, 680, 666, 588, 671, and 589, respectively (Fig. S4). Notably, the mass spectrum of azo compound **A2** displayed significant fragment peaks at *m/z* of 389, 343, 256, 185, and 269, which can be attributed to the following ions: $[C_{16}H_{13}N_4O_4S_2]^+$, $[C_{15}H_{11}N_4O_2S_2]^+$, $[C_9H_{11}N_3O_2S_2]^+$, $[C_6H_7N_3O_2S]^+$, and $[C_7H_7N]^+$, respectively.

The mass spectrum of amide compound **A2D2** reveals significant peaks at *m/z* 663, 424, 293, and 56, corresponding to the ions $[C_{28}H_{23}N_8O_6S]^+$, $[C_{19}H_{16}N_6O_4S]^+$, $[C_{12}H_{13}N_4O_3S]^+$, and $[C_2H_4N_2]^+$, respectively. The mass spectrum of amide **A2D3** exhibits six fragmentation peaks at *m/z* 551, 424, 279, 224, 143, and 56, which can be assigned to the ions $[C_{24}H_{19}N_6O_6S_2]^+$, $[C_{20}H_{18}N_5O_4S]^+$, $[C_{11}H_{11}N_4O_3S]^+$, $[C_{13}H_{10}N_3O]^+$, $[C_{11}H_3N_2O_2S]^+$, and $[C_2H_4N_2]^+$, respectively. Furthermore, the mass spectra of compounds **A2D5** and **A2D6** provide crucial structural information through various fragment peaks. For compound **A2D5**, important peaks at *m/z* 446, 349, 285, 163, 99, and 56 correspond to the ions $[C_{17}H_{14}N_6O_5S_2]^+$, $[C_{14}H_{13}N_4O_5S]^+$, $[C_{10}H_{11}N_3O_3S_2]^+$, $[C_3H_3N_2O_2S_2]^+$, $[C_3H_3N_2S]^+$, and $[C_2H_4N_2]^+$. In contrast, compound **A2D6** exhibits fragmentation peaks at *m/z* 509, 810, 325, 204, 99, and 56, attributed to the ions $[C_{23}H_{19}N_5O_5S_2]^+$, $[C_{17}H_{16}O_4N_5S_2]^+$, $[C_{16}H_{13}N_4O_2S]^+$, $[C_9H_8N_4S]^+$, $[C_3H_3N_2S]^+$, and $[C_2H_4N_2]^+$, respectively. The suggested fragmentation patterns for compounds **A2**, **A2D4**, and **A2D5** are illustrated in Fig. S4. All prepared compounds displayed satisfactory elemental analyses and were characterized as non-hygroscopic colored solids.

Antibacterial and Antifungal Screening

In vitro activities of three human pathogenic microorganisms—*S. aureus*, *E. coli*, and *C. albicans*—were evaluated for **A2** and **A2D1**–**A2D6** using the agar-well diffusion method. Commercial antibacterial agents, including amoxicillin and nystatin, were used for comparison. The compounds were screened at various concentrations to determine the MIC, which serves as a standard reference for the lowest concentration of antimicrobial agents that inhibits microorganism growth after 24 h of incubation.

The concentrations used to assess the biological activity of the synthesized compounds ranged from 25 to 100 mg/mL. The antibacterial screening results indicated that the prepared compounds exhibited moderate effectiveness against the studied bacteria, particularly at a 50 mg/mL concentration. Notably, all new compounds demonstrated greater efficacy against the Gram-negative bacterium *E. coli* compared to the Gram-positive *S. aureus*. Among the synthesized compounds, **A2D6** showed the most promising activity against *E. coli*, with inhibition zones measuring 20.6, 21.2, and 21.20 mm at 25, 50, and 100 mg/mL, respectively. Additionally, compounds **A2D5**, **A2D4**, and **A2D3** exhibited good efficacy against *E. coli* at the same concentrations.

The variations in biological effectiveness among the compounds can be attributed to the distinct characteristics of the bacterial types. Each bacterial species has a unique outer wall structure, with differences in the thickness and composition of the outer membrane. Notably, the outer membrane of Gram-negative bacteria primarily consists of lipopolysaccharides, which facilitates the penetration of compounds through the lipophilic layer, thereby enhancing membrane permeability. Antibiotics function by either killing or inhibiting bacterial growth, primarily through the prevention or disruption of cell wall biosynthesis [20].

The antifungal activity of the investigated compounds against *C. albicans* revealed that the amide compounds **A2D4** and **A2D3** exhibited the highest antifungal activities at a concentration of 100 mg/mL. In contrast, compounds **A2D2** and **A2D5** demonstrated moderate activity at this concentration, while their effectiveness remained relatively stable at lower concentrations of 25 and 50 mg/mL. The MIC values for the studied compounds against the tested microorganisms were established at concentrations of 25, 50, and 100 mg/mL, as summarized in Table 1.

Table 1. Antimicrobial activity results of prepared azo and amide

Compound	*S. aureus*			*E. coli*			*C. albicans*		
	Concentration of compound (mg/mL)								
	100	50	25	100	50	25	100	50	25
A2	7	10	8	11.5	15.3	12	6	6	6
A2D1	6.5	16	11	7	15	12.5	16	6	6
A2D2	10	15	8	14	17	16.5	17	6	6
A2D3	13	14	7.5	17	18	14	20	6	6
A2D4	15	15	8	18	19	15	21	6	6
A2D5	16	15	12	19	20	17.4	9	6	6
A2D6	17	19	15	20	21	20.6	9.5	6	6
Sulfathiazole	14	15	13.5	18	13.2	9	7	6	6
Amoxicillin	30	28	20	42	25	18	9	6	7
Nystain	0	0	0	0	0	0	35	22	12

High MIC values against microorganisms indicate low efficacy of antimicrobial agents, while low MIC values signify high efficiency. *E. coli* exhibited the greatest resistance in this study, with the lowest MIC value of 25 mg/mL recorded for compound **A2D6**. Compounds **A2D5**, **A2D2**, and **A2D4** also demonstrated higher activity than the other synthesized compounds.

The synthesized compounds in this work prominently feature sulfonamide derivatives, commonly referred to as sulfa drugs. Numerous studies have highlighted these compounds' biological and medical significance in inhibiting bacterial growth, classifying them as bacteriostatic agents. Previous research has established that sulfa drugs exert their effects on microbial cells through competitive inhibition of dihydropteroate synthase, effectively blocking and disrupting the biosynthesis of folate coenzymes [21].

Antioxidant Activity

Antioxidants are stable molecules that play a crucial role in inhibiting or preventing cellular damage caused by reactive oxygen species by donating and neutralizing electrons. These substances mitigate the harmful effects of free radicals, thereby protecting the body's cells from oxidative stress. Free radicals are generated in the body due to various external factors, including exposure to ultraviolet rays, environmental pollutants, stress, smoking, diseases, and inflammation. Additionally, they arise from internal processes, such as digestion and metabolism, particularly during the respiratory chain when oxygen is consumed, leading to the oxidation of certain compounds and the formation of radicals like hydroxyl (•OH), superoxide ($\bullet O_2^-$), peroxyl (ROO•), and alkoxyl (RO•).

Antioxidants are essential for counteracting the increase in free radicals and maintaining cellular integrity. An imbalance between the levels of antioxidants and free radicals lead to oxidative stress, resulting in various diseases, including aging, cardiovascular disorders, skin diseases, cancer, and autoimmune conditions. Key antioxidants that mitigate oxidative damage include vitamins C and E, selenium, dark chocolate, and flavonoids. Numerous natural foods, particularly fruits and vegetables, are recognized for their antioxidant properties due to their chemical composition, which often features OH groups directly attached to aromatic rings, known as phenols or polyphenols, when multiple OH groups are present. The antioxidant action of phenols is based on their ability to donate hydrogen atoms to free radicals or stabilize them through resonance or dimerization, effectively terminating chain reactions. The DPPH method for assessing the antioxidant efficacy of synthesized substances relies on the reduction of DPPH free radicals, with DPPH serving as an electron acceptor that interacts with donor molecules such as the synthesized antioxidants [22]. The antioxidant capacity of the synthetic compounds may be attributed to the presence of

Table 2. DPPH scavenging capacities of the synthesized compounds

Compound	Concentration (μg/mL)								R^2	IC_{50} (μg/mL)
	0.78	1.56	3.12	6.25	12.5	25	50	100		
A2	3.6	7.0	8.2	17.1	27.9	44.9	69.5	84.5	0.9071	192.45
A2D1	14.2	14.5	28.3	31.5	46.9	51.5	52.1	52.7	0.8616	44.57
A2D2	3.8	6.9	11.1	13.6	21.4	28.6	30.6	59.9	0.8947	895.70
A2D3	4.8	9.0	11.6	16.6	26.7	32.7	36.4	49.3	0.9668	1380.93
A2D4	5.4	15.5	15.9	15.0	19.1	30.9	41.9	72.0	0.7815	562.79
A2D5	1.4	5.7	11.5	14.4	16.6	24.2	43.0	55.6	0.8889	1086.88
A2D6	1.4	5.0	9.5	16.2	21.1	29.4	42.3	48.8	0.9684	1191.92
Vitamin C	7.2	24.1	41.4	64.5	89.3	94.8	95.2	95.2	0.8982	4.37

the phenolic hydroxyl moiety in their chemical structures, enabling them to stabilize or scavenge the existing free radicals.

The effectiveness of the newly synthesized compounds as antioxidant agents is presented in Table 2, summarized the effectiveness values of these synthesized substances as free radical scavengers, comparing them with Vitamin C. All synthesized compounds contain phenolic OH groups, which enable them to trap or neutralize free radicals. The results indicate that compound **A2D1** demonstrates a notable ability to reduce DPPH free radicals at various concentrations (0.78, 1.56, 3.12, 6.25, 12.59, 25, 50, and 100 μg/mL), achieving reductions of 14.2, 14.5, 28.3, 31.5, 46.9, 51.5, 52.1, and 52.7%, respectively. This suggests that higher concentrations exhibit excellent antioxidant efficacy. In contrast, compound **A2** shows moderate effectiveness relative to the other compounds studied.

The assessment of IC_{50} values and the correlation coefficient (R^2) for each compound is summarized in Table 2. Notably, the new amide **A2D1** exhibits a higher antioxidant potential (lower IC_{50} value) than the other synthesized compounds, while also surpassing the standard antioxidant Vitamin C. The results indicate that **A2** and the amide compounds can function as proton donors by donating hydrogen atoms to DPPH radicals, utilizing resonance processes or conjugation systems that stabilize free radicals on aromatic rings [23]. From this study, the antioxidant efficiency of all synthetic compounds follows the order: Vitamin C > **A2** > **A2D1** > **A2D4** > **A2D2** > **A2D5** > **A2D6** > **A2D3**.

■ CONCLUSION

A novel series of azo compounds, designated as **A2**, **A2D1**, **A2D2**, **A2D3**, **A2D4**, **A2D5**, and **A2D6**, has been synthesized starting from sulfathiazole. Elemental analysis confirmed the chemical compositions of the synthesized compounds, while FTIR, MS, and both ^{1}H- and ^{13}C-NMR spectroscopy verified the presence of functional groups, particularly the N=N and NH–C=O moieties. The biological activities of these compounds were assessed *in vitro*, focusing on antibacterial, antifungal, and antioxidant properties. The antibacterial data revealed a commendable antibacterial potential across the series. Among the synthesized compounds, **A2D6** exhibited the highest activity against Gram-negative bacteria, achieving the lowest MIC of 25 mg/mL. All compounds demonstrated good to moderate efficacy at a concentration of 100 mg/mL, with compounds **A2D3** and **A2D4** showing particularly noteworthy antifungal effects against *C. albicans*. All compounds displayed significant antioxidant activity. Compound **A2D1** emerged as the most effective, with an IC_{50} value of 44.57 μg/mL, followed by compound **A2**. However, all synthesized compounds exhibited lower activity than the standard antioxidant, Vitamin C, which had an IC_{50} of 4.37 μg/mL. Future studies will aim to optimize the structural characteristics of these azo compounds to enhance their biological activities further. Additionally, investigations need to be conducted into the mechanisms underlying their antibacterial, antifungal, and antioxidant effects to better understand their potential therapeutic applications.

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■ CONFLICT OF INTEREST

The author declares no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Dania Mohammed Saleh conducted the experiments. Dania Mohammed Saleh and Bushra Kamel Aal-Salami performed the calculations. Bushra Kamel Al-Salami was responsible for writing and revising the manuscript. All authors have agreed to the final version of the manuscript.

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