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Article

Synthesis, characterization, antimicrobial, antioxidant, and anti-cancer activity of new hybrid structures based on benzimidazole and thiadiazole

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Hybrid structures containing multiple pharmacophore units with known activity have attracted attention due to their promising outcomes. In this study, several new hybrid structures containing benzimidazole and thiadiazole units were synthesized. The newly synthesized compounds were structurally analyzed using ^{1}H-NMR, ^{13}C-NMR, HRMS, and elemental analysis. The antimicrobial, *in vitro* anti-cancer, and antioxidant activities of all compounds were investigated. *In vitro* antimicrobial activities of the compounds were determined against Gram-positive (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212), Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) bacteria and fungi (*C. albicans* ATCC 10231) by using broth microdilution method. The compound **5g** bearing 4-methoxyphenyl derivative showed the best activity with 32 µg/mL against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853. The MTT test was used to determine the cytotoxicity of the produced compounds on the MCF-7 (human breast cancer) and L-929 (fibroblast) cell lines. FRAP method was used to determine the antioxidant properties of synthesized compounds. The Ferric Reducing Antioxidant Power of the compounds **5a**, **5b,** and **5c** showed more antioxidant properties than vitamin E. The compound **5g** stands out in the series in that it is not toxic on the healthy cell line and has promising antimicrobial activity.

Keywords: Benzimidazole. Thiadiazole. Antimicrobial. Anti-cancer. Antioxidant.

INTRODUCTION

Cancer is a major public health issue and is the leading cause of death worldwide. In 2020, 19.3 million new cases of cancer and 10 million cancer-related deaths were reported (Anastassova *et al*., 2022). According to the World Cancer Report of the WHO, the number of deaths due to cancer is estimated to more than double in the next few years (Parkin, 2001). Chemotherapy is one of the most efficient ways to treat cancer since it slows the growth and metastasis of tumors, and a variety of chemical compounds with various structures have been designed to treat different forms of cancer. The use of cytotoxic drugs is a tried-and-true treatment strategy to slow down the growth of tumors (Ismael *et al*., 2008). However, one of the drawbacks of anti-cancer treatment and the biggest challenge in cancer therapy is that anti-cancer medications are also harmful to normal cells, causing numerous side effects (Aydemir *et al*., 2003). This non-targeted drug delivery constrains the therapeutic efficacy of the current anti-cancer treatment. Thus, the hunt for medicines that are effective with fewer adverse effects is the main aim of researchers in the development of anti-cancer therapies. Reactive oxygen species (ROS) promote the growth of cancer cells. They interact with tumor initiation, propagation, tumor spread, tumor microenvironment, and therapeutic resistance to facilitate cancer development (Xing *et al*., 2022). Antioxidants

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guard organisms against these reactive oxygen species. These are chemicals that neutralize free radicals, which can damage an organism's tissues and cells, and inhibit oxidation processes that occur during chemical reactions in metabolic pathways (Sies, 1997). Thus, research into the antioxidant characteristics of synthesized compounds is extensively explored in medicinal chemistry. The search for active compounds that might stop or minimize the effects of oxidative stress on cells has become an important area of research nowadays. Benzimidazole-thiadiazole complexes have shown promising antioxidant activity against reactive oxygen species.

One of the major public health issues of the twenty-first century is antimicrobial resistance (AMR), which poses a threat to the effective prevention and treatment of an expanding number of microbial infections that are no longer susceptible to the conventional medications used to treat them (Prestinaci, Pezzotti, Pantosti, 2015). Microbial resistance is one of the most urgent issues in clinical practice, and a primary objective of current biomedical research is to discover new, powerful drugs that can combat multi-resistant bacteria. Antibiotic abuse and pharmaceutical corporations' lack of interest in investing in antibiotic development have made the discovery of new antibiotic classes inevitable. Benzimidazole is known as a fortunate structure in pharmaceutical chemistry, having a variety of biological functions. Benzimidazole has a benzene ring system in which the benzene ring is attached to a five-member imidazole ring having nitrogen atoms at positions 1 and 3 and thus is known as a heterocyclic aromatic compound (Khokra, Choudhary, 2011). Additionally, benzimidazole is a tremendous scaffold of therapeutic importance with promising pharmacological properties. The safety and efficacy profiles of benzimidazole medications in clinical use are well-established.

The term "benzimidazole" refers to the ring structure in which the benzene ring is fused to the 4,5-positions of the imidazole ring (Debus, 1858). It is one of the earliest nitrogen heterocycles initially created by Hobrecker in 1872. Benzimidazole has become an extensively employed heterocyclic moiety in modern times (Wright, 1951). Because of its active pharmacophore, it has applications as antitubercular (Mohapatra, Ganguly, 2024; Ranjith *et al*., 2013), anti-inflammatory (Jahan, *et al.,* 2024; Vasantha *et al*., 2015), antitrichinellosis (Keri *et al*., 2015), anti-cancer (Acar Çevik *et al.,* 2024; Yadav, Narasimhan, 2016), antioxidant (Işık *et al.,* 2022; Arora *et al*., 2014), antihistaminic (Sridevi *et al*., 2010), and antimicrobial agent (Celik *et al.*, 2022; Ansari, Lal, 2009). The purine base and the benzimidazole core's structural resemblance make it easy for benzimidazole to interact with living systems. A wide range of pharmacological activities of this compound draws attention to the inclusion of this beneficial structure in the design and synthesis of this molecule with other structures to make complex compounds.

1,3,4-Thiadiazoles is a poly-heteroatomic system containing a five-membered heterocyclic ring associated with conjugated p electrons and distinct regions of positive and negative charges (Karki *et al*., 2011). Due to its dense and highly polarizable structure and net neutral electron charge, it can traverse cellular membranes and interact with biological targets. The 1,3,4-thiadiazole ring's inductive action and the comparatively high aromaticity make this compound behave as a modestly weak base. The (S-C=N) toxophoric units that are present in substituted thiadiazols give rise to a wide spectrum of pharmacological actions, including anti-cancer, antibacterial, anti-inflammatory, antifungal, anticonvulsant, antiviral, and antiparasitic properties (Khadri *et al*., 2020).

Since cancer drugs suppress the immune system of patients, they become more susceptible to microbial infections. When microbial infections occur in cancer patients, they have to use multiple drugs. The development of antimicrobial and anti-cancer drugs is very important to protect cancer patients from microbial infections. Considering the effect of oxidative stress on cancer, it is very important to synthesize compounds with both anti-cancer, antimicrobial, and antioxidant effects. The main objectives of this study include the synthesis, characterization, and *in vitro* testing of seven distinct derivatives with benzimidazole and thiadiazole rings,

which are believed to have antioxidant, antibacterial, antifungal, and anti-cancer properties.

MATERIAL AND METHODS

Chemistry

All compounds were purchased from Sigma-Aldrich (Sigma-Aldrich Corp., St. Louis, MO, USA) or Merck (Merck KGaA, Darmstadt, Germany). The synthesized compounds' melting points (ºC, uncorrected) were determined using a Mettler-Toledo MP 90 melting point instrument (Columbus, Ohio, OH, USA). With Bruker 75 MHz and 300 MHz digital FT-NMR spectrometers (Bruker Bioscience, Billerica, MA, USA), ^{13}C-NMR and ^{1}H-NMR, spectra were captured in DMSO-d_6 solvent with TMS acting as an internal standard. The units of coupling constants (J) are Hertz. Shimadzu LC/MS ITTOF equipment was used to determine M+1 peaks (Shimadzu, Tokyo, Japan). On Silica Gel 60 F254 TLC plates, thin-layer chromatography (TLC) experiments were conducted (Merck KGaA, Darmstadt, Germany).

Synthesis of Benzaldehyde derivative (1) sodium metabisulfite salt:

Ethanol was used to dissolve of methyl 4-formyl benzoate (5g, 0.03 mol). Drop by drop, ethanol-dissolved sodium metabisulfite (6.84 g, 0.036 mol) was added to the benzaldehyde solution. The reaction's components were mixed for an hour at room temperature once the dripping process was finished. It was filtered to remove the precipitated product.

Synthesis of methyl 4-(5-chloro-1H-benzo[d]imidazole-2-yl) benzoate (2):

4-Chlorobenzene-1,2-diamine (0.022 mol) was dissolved in DMF, and sodium metabisulfite salt of benzaldehyde derivative (7.09 g, 0.026 mol) was added. By adding the reaction's contents to iced-water at the end, the result was precipitated. From the ethanol, the precipitated product was removed and crystallized.

Synthesis of 4-(5-chloro-1H- benzo[d]imidazole-2-yl) benzohydrazide derivatives (3):

Compound 2 (0.018 mol) and excess of hydrazine hydrate (5 mL) were placed in the same vial and ethanol (15 mL) was added. The mixture was refluxed for 12h. When the reaction was completed, the mixture was poured into iced-water, the product was filtered.

N-substituted-2-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)benzoyl)hydrazine-1-carbothioamide (4):

The appropriate quantity of isothiocyanate (1.1 mmol) was added after the hydrazide derivative compound 3 (1 mmol) had been dissolved in 10 ml of ethanol, and the mixture was then refluxed for three hours. It was filtered to remove the precipitated product.

N-substituted-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl) phenyl) 1,3,4-thiadiazole-2-amine (5a–5g):

The appropriate thiosemicarbazide derivative was stirred in 10 ml of H_2SO_4 in an ice bath. Then it was stirred for another 10 minutes at room temperature, at the end of the time it was poured slowly on ice, adjusted to pH=8 with aqueous ammonia and filtered. It is washed with water and crystallized from ethanol.

N-ethyl-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5a): Yield: 72 %. M.p. 311.5 ºC. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.21 (3H, t, *J*=7.17 Hz, -CH_3), 5.33-5.35 (2H, m, -CH_2), 7.24 (1H, dd, J_1=3.03 Hz, J_2=9.21 Hz, Benzimidazole CH), 7.64-7.67 (2H, m, Benzimidazole CH), 7.93 (2H, d, *J*=8.52 Hz, 1,4-disubstituted benzene), 8.25 (2H, d, *J*=8.43 Hz, 1,4-disubstituted benzene). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 14.65, 35.53, 114.47, 117.31, 119.64, 123.59, 126.19, 126.65, 128.36, 128.77, 129.04, 130.79, 134.80, 135.98, 155.41. HRMS (m/z): $[M+H]^+$ calcd for $C_{17}H_{14}N_5SCl$: 356.0731; found: 356.0732. Anal. calcd. For $C_{17}H_{14}N_5SCl$, C, 57.38; H, 3.97; N, 19.68. Found: C, 57.46; H, 3.96; N, 19.72.

N-(2-chloroethyl)-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine

(5b): Yield: 65 %. M.p. 197.3 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.47-3.60 (4H, m, -CH_2), 7.24-7.27 (1H, m, Aromatic C-H), 7.62-7.68 (2H, m, Aromatic C-H), 7.97-7.99 (1H, m, Aromatic C-H), 8.09-8.12 (1H, m, Aromatic C-H), 8.23-8.27 (2H, m, Aromatic C-H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 25.46, 45.61, 112.63, 117.51, 121.57, 121.98, 123.85, 125.92, 127.28, 128.53, 129.31, 130.71, 138.29, 152.22, 154.82. HRMS (m/z): $[M+H]^+$ calcd for $C_{17}H_{13}N_5SCl_2$: 391.0329; found: 391.0334. Anal. calcd. For $C_{17}H_{13}N_5SCl_2$, C, 52.32; H, 3.36; N, 17.94. Found: C, 52.48; H, 3.35; N, 17.98.

N-phenyl-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5c): Yield: 62 %. M.p. 310.3 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 7.52-7.55 (3H, m, Aromatic CH), 7.62-7.65 (2H, m, Aromatic CH), 7.93-7.96 (2H, m, Aromatic CH), 8.21-8.28 (3H, m, Aromatic CH), 8.35-8.40 (2H, m, Aromatic CH).^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 105.67, 109.51, 112.94, 114.71, 119.18, 121.67, 124.53, 126.82, 128.13, 129.10, 129.33, 130.15, 132.24, 134.77, 135.07, 148.68, 151.18. HRMS (m/z): $[M+H]^+$ calcd for $C_{21}H_{14}N_5SCl$: 404.0731; found: 404.0721. Anal. calcd. For $C_{21}H_{14}N_5SCl$, C, 62.45; H, 3.49; N, 17.34. Found: C, 62.64; H, 3.48; N, 17.39.

N-(2-chlorophenyl)-5-(4-(5-chloro-1H-benzo[d] imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5d): Yield: 89 %. M.p. 307.4 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 7.68 (2H, d, J=8.46 Hz, Aromatic C-H), 7.73 (2H, s, Aromatic C-H), 8.06 (3H, d, J=8.76 Hz, Aromatic C-H), 8.28-8.31 (4H, s, Aromatic C-H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 115.75, 122.79, 123.67, 124.50, 125.17, 126.84, 127.25, 127.64, 127.79, 127.98, 128.47, 128.86, 129.37, 130.28, 130.60, 131.32, 132.40, 148.83, 151.89. Anal. calcd. For $C_{21}H_{13}N_5SCl_2$, C, 57.54; H, 2.99; N, 15.98. Found: C, 57.72; H, 2.98; N, 15.93.

N-cyclohexyl-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5e): Yield: 91 %. M.p. 272.2 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.18-1.32 (6H, m, cyclohexyl CH), 1.61-1.74 (3H, m, cyclohexyl CH), 1.97-1.99 (2H, m, cyclohexyl CH), 7.33 (1H, dd, J1=1.68 Hz, J2=8.64 Hz, Benzimidazole CH), 7.69 (1H, d, J=8.55 Hz, Benzimidazole C-H), 7.73-7.74 (1H, m, Benzimidazole C-H), 7.97 (2H, d, J=8.52 Hz, 1,4-disubstituted benzene), 8.25 (2H, d, J=8.73 Hz, 1,4-disubstituted benzene). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 21.09, 23.59, 25.35, 30.03, 32.42, 57.56, 113.14, 114.29, 121.19, 122.91, 124.68, 125.17, 126.28, 127.01, 127.74, 128.22, 129.88, 136.63, 155.05. Anal. calcd. For $C_{21}H_{20}N_5SCl$, C, 61.53; H, 4.92; N, 17.08. Found: C, 61.71; H, 4.93; N, 17.11.

N-isopropyl-5-(4-(5-chloro-1H-benzo[d]imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5f): Yield: 92 %. M.p. 294.4 °C. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 1.22-1.25 (6H, m, -CH_3), 3.86-3.91 (1H, m, -CH), 7.25 (1H, dd, J1=2.10, Hz, J2=8.52 Hz, Benzimidazole CH), 7.61-7.67 (1H, m, Aromatic CH), 7.92-7.95 (2H, m, Aromatic C-H), 8.01-8.03 (1H, m, Aromatic C-H), 8.23-8.27 (2H, m, Aromatic C-H). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 22.55, 47.59, 116.58, 118.66, 119.90, 123.96, 127.26, 127.69, 130.61, 133.83, 140.68, 142.24, 144.22, 152.33, 155.44. HRMS (m/z): $[M+H]^+$ calcd for $C_{18}H_{16}N_5SCl$: 370.0888; found: 370.0882. Anal. calcd. For $C_{18}H_{16}N_5SCl$, C, 58.45; H, 4.36; N, 18.93. Found: C, 58.63; H, 4.37; N, 18.97.

N-(4-methoxyphenyl)-5-(4-(5-chloro-1H-benzo[d] imidazole-2-yl)phenyl)-1,3,4-thiadiazole-2-amine (5g): Yield: 89 %. M.p. Semi-solid. ^{1}H-NMR (300 MHz, DMSO-d_6): δ = 3.76 (3H, s, -OCH_3), 7.28-7.31 (3H, m, Aromatic C-H), 7.60 (2H, d, J=9.03 Hz, Aromatic C-H), 7.66-7.69 (2H, m, Aromatic C-H), 8.04 (2H, d, J=8.55 Hz, 1,4-disubstituted benzene), 8.30 (2H, d, J=8.55 Hz, 1,4-disubstituted benzene). ^{13}C-NMR (75 MHz, DMSO-d_6): δ(ppm): 55.69, 113.14, 114.82, 115.60, 120.00, 123.31, 123.41, 127.08, 127.64, 127.81, 128.58, 130.67, 132.29, 134.39, 150.43, 155.25, 156.54, 165.60. Anal. calcd. For $C_{22}H_{16}N_5OSCl$, C, 60.90; H, 3.72; N, 16.14. Found: C, 61.08; H, 3.73; N, 16.18.

Antimicrobial Activity

In vitro antimicrobial activities of the compounds were evaluated against Gram-positive (*Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212), Gram-negative (*Escherichia coli* ATCC 25922,

Pseudomonas aeruginosa ATCC 27853) bacteria and fungi (*Candida albicans* ATCC 10231). For this purpose, minimum inhibitory concentrations (MIC) of the compounds along with reference antimicrobials were determined against these microorganisms. The study was conducted according to the Clinical Laboratory Standards Institute (CLSI) M100-S28 protocol for bacteria (Wayne, 2017) and CLSI M27-A3 protocol for fungi (Wayne, 2008) Mueller Hinton Broth (MHB) for bacteria and RPMI-1640 for fungi were used in this research.

The serial dilutions of each compound at the range of 2-512 µg/mL were prepared in 96-well microplates after all compounds dissolved in DMSO. The McFarland 0.5 standard was used to prepare suspensions of each microbe, and as a result, densities of 10^5 cfu/ml were achieved. Bacteria and fungus were placed in microplates and incubated for 24 and 48 hours, respectively, at 37 and 35 degrees Celsius. These microorganisms were evaluated against the reference antimicrobials. Growth control of microorganisms and sterilization control of the mediums were tested at the same time. Besides, DMSO which is used as a solvent in this test was also tested for antimicrobial activity. The wells with the lowest concentration without microbial growth were determined as MICs of each compound, after the incubation period. The detection was made by macroscopic evaluation using dye MTT (Shi *et al*., 2007). The experiment was repeated three times.

Anti-cancer Activity

Cell Culture

MCF-7 and L929 cell lines were purchased from ATCC (American Type Culture Collection) and studied. Cells were mixed with 88% DMEM (Dulbecco's modified Eagle's medium; Gibco, Thermo Fisher Scientific), 10% FBS (Fetal Bovine Serum; Sigma Aldrich), 1% glutamine (Sigma Aldrich), and 1% penicillin (Sigma-Aldrich) solutions. The cells to which the medium was added were allowed to grow by incubating at 37 °C in an environment containing 95% humidity and 5% CO_2. All syntheses were dissolved with DMSO solution and it was ensured that their final concentration did not exceed 0.5% at the time of introduction into the cell.

Cell Viability Assay

The cytotoxic effects of all compounds synthesized by MTT analysis on L929 and MCF7 cell lines were investigated. 96-well plates were used for seeding cells. Approximately 10000 cells were seeded in each well. Cells were allowed to adhere for 24 hours and then 20 mM solution was prepared (with DMSO) for each synthesis. The syntheses were given to each well in triplicate, ensuring that the DMSO ratio in the final well concentration did not exceed 0.5% and the final concentration was 100µM. The wells were then incubated for 48 hours. All wells without synthesis were used as positive controls. After the incubation, the wells were treated with MTT solution to determine metabolically active cells and incubated at 37 °C for 3 hours. After the MTT interaction, the wells were emptied and DMSO solution was placed in them. The formazan crystals formed were dissolved with this solution and the number of viable cells in each well was determined by color change. The absorbance values were read at 540 nm with the help of a microplate and the values found were represented as mean ± standard deviation (± SD). The experiment was repeated two times.

Antioxidant Activity

This method, in which the reducing capacity of iron (III) is determined by antioxidants, was developed by Benzie and Strain in 1996. Fe(III)-TPTZ complex is formed as a result of the reaction of Fe(III) with tripyridyltriazine (TPTZ), and this complex is reduced to Fe(II)-TPTZ complex with the antioxidant in the environment. The color of this complex is dark blue and gives maximum absorbance at 593 nm (Benzie, Strain, 1996). Incubation is performed for up to 30 minutes to complete the reaction and read the correct absorbance values. The results obtained can be given as Trolox equivalent or as IC_{50} values. The experiment was repeated two times.

RESULTS AND DISCUSSION

Chemistry

The synthesis phase of the new structures was carried out in five stages, as shown in Figure 1. The aldehyde portion of the methyl 4-formyl benzoate compound was treated with sodium metabisulfite in ethanol. Thus, the sodium metabisulfite addition product of the aldehyde was obtained. In the second step, as a result of the condensation reaction of benzaldehyde sodium metabisulfite product and 4-chlorobenzene-1,2-diamine under reflux and methyl 4-(5-chloro-1*H*-benzo[*d*]imidazole-2-yl)benzoate (2) was obtained. Compound 3 was synthesized by treating compound 2 with hydrazine hydrate in ethanol in the following procedure (3). The suitable isothiocyanate derivatives and the hydrazide derivative were refluxed in ethanol, and the precipitated product was filtered out. The next step involved cyclizing the thiosemicarbazide molecule in the presence of strong sulfuric acid to produce the thiadiazole derivatives (**5a-g**).

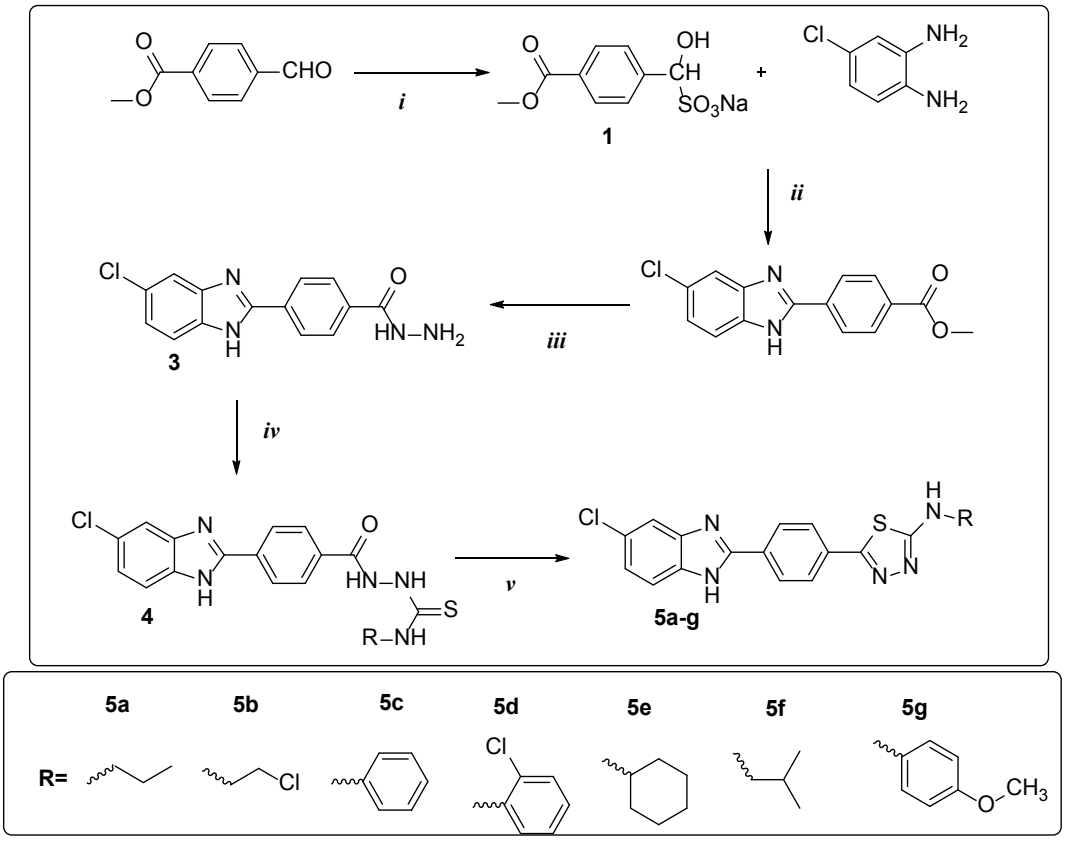

FIGURE 1 - Synthesis pathway of 1,3,4-thiazole derivative target benzimidazole compounds **5a-5g.** Reagent and conditions: (***i***) $Na_2S_2O_5$/EtOH, (***ii***) DMF/120 °C, (***iii***) NH_2NH_2/EtOH, (***iv***) RNCS/EtOH and (**v**) H_2SO_4.

Antimicrobial Activity

By employing the broth microdilution method, the compounds were examined for their *in vitro* antimicrobial activities against Gram-positive (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212), Gram-negative (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) bacteria and fungi (*C. albicans* ATCC 10231). As reference antimicrobial medications, this study used ampicillin, gentamycin, and vancomycin for their antibacterial activity and fluconazole for their antifungal activity. The MIC values determined for each substance and reference antimicrobial agents because of the experiment were presented in Table I.

TABLE I - *In vitro* MICs (μg/mL) of the compounds and reference antimicrobial drugs

Comp.	Bacteria				Fungi
	S. a.	E. f.	E. c.	P.a.	C. a.
5a	256	256	128	64	256
5b	256	256	256	64	256
5c	256	256	256	128	256
5d	256	256	256	128	256
5e	64	256	256	128	256
5f	256	256	256	128	256
5g	**32**	64	64	**32**	128
Ampicilin	2	2	16	-	-
Gentamycin	1	2	1	1	-
Vancomycin	1	2	-	-	-
Fluconazole	-	-	-	-	1

S.a.: *Staphylococcus aureus* ATCC 29213; E.f.: *Enterococcus faecalis* ATCC 29212; E.c.: *E. coli* ATCC 25922; P.a.: *Pseudomonas aeruginosa* ATCC 27853; C.a: *Candida albicans* ATCC 10231

According to result of the antimicrobial study, MICs ranged from 32 to 256 μg/mL against Gram-positive bacteria. In terms of antibacterial activity against Gram-positive bacteria, compound 5g showed the best activity with 32 μg/mL on *S. aureus* ATCC 29213. Although this MIC value did not reach those of the reference antibacterial agents, it stands out as a promising value.

MICs ranged from 32 to 256 μg/mL against Gram-negative bacteria. Compound 5g has the lowest MIC value on Gram-negative bacteria as well as Gram-positive bacteria. This MIC value on *P. aeruginosa* ATCC 27853 is 32 μg/mL which is higher than the MIC of gentamicin (1 μg/mL) on this microorganism. However, it can still be considered promising in terms of antibacterial activity.

The MIC values of the compounds in the study on *C. albicans* ATCC 10231 were determined as 256 μg/mL, except for compound 5g which had MIC at 128 μg/mL.

The findings indicate that the compounds in this study showed generally modest levels of antibacterial activity and fell short of the MIC values of the reference antimicrobials. However, compound 5g outperformed all the other compounds in the series in terms of antibacterial activity.

In the study by Işık *et al.* (2024) compounds bearing benzimidazole-thiadiazole structure were investigated and their antimicrobial effects were evaluated. Promising results were obtained for the compounds. In this study, a nonsubstituted benzimidazole derivative was used. In general, it was concluded that the antibacterial effect was enhanced by substituting the 5th position of the thiadiazole ring with an alkyl group in the compounds. The presence of a methoxy group on the phenyl ring attached to the thiadiazole ring was found to increase the antimicrobial effect (Işık *et al*., 2024). Celik *et al.* (2022), synthesized 5,6-dimethyl-1*H*-benzimidazole derivatives containing thiadiazole ring and evaluated their antimicrobial effects. In this study, high antimicrobial effect was obtained by 4-methoxyphenyl substitution of thiadiazole ring. The electron-withdrawing -Cl group at the 2-position of the ethyl group was found to enhance both antibacterial and antifungal activity (Celik *et al.,* 2022).

Çevik *et al.* (2022), synthesized 5-methyl-1*H*-benzo[*d*]imidazole derivative compounds bearing thiadiazole structure and investigated their antimicrobial effects. Many of the compounds were found to have promising antibacterial and antifungal effects. In this study, compound **5g** bearing 4-methoxyphenyl ring showed promising antibacterial effect. However, when compared with other studies, it was observed that nonsubstituted, 5-methyl or 5,6-dimethyl substitution of the benzimidazole ring had a positive effect on the antimicrobial effect, while the antimicrobial effect decreased with the introduction of the -Cl substituent, which is an electron withdrawing group on the benzimidazole ring (Figure 2).

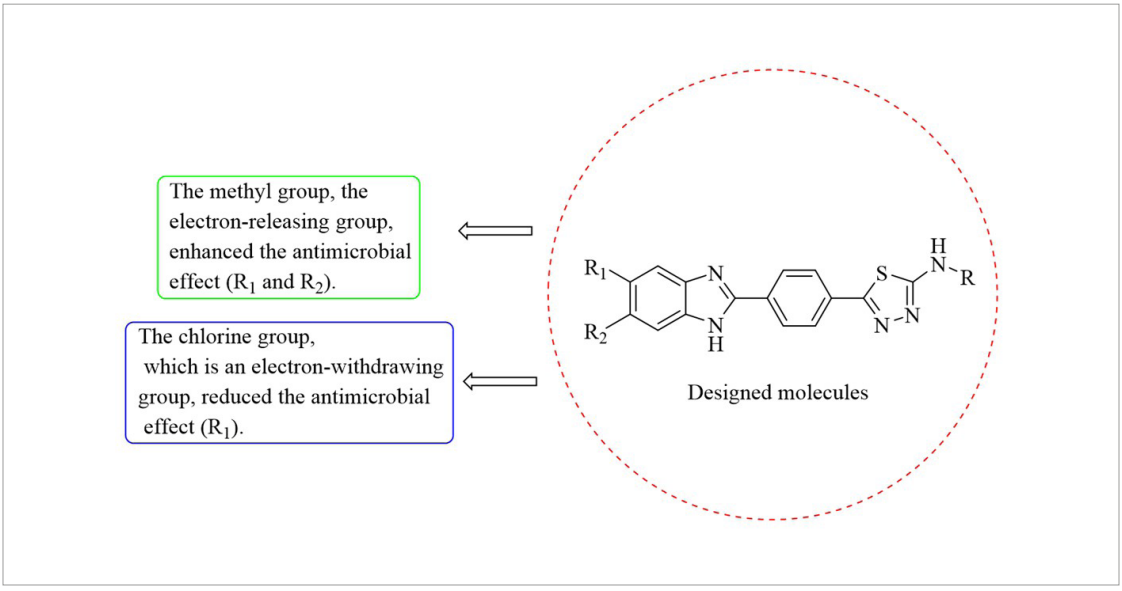

FIGURE 2 - SAR outline for benzimidazole-thiadiazole derivatives.

Anti-cancer Activity

L929 and MCF-7 cell lines were used to test the cytotoxic effects of compounds **5a–5g**. For the first screening, the MTT test was used to assess the *in vitro* cytotoxic bioactivity of produced compounds against the L929 and MCF-7 cell lines. Both cell lines were exposed to the target substances at a constant concentration of 100 **µ**M to assess their cytotoxic potential. After the cells had been treated for 48 hours, cell viability percentages were calculated. Preliminary Cytotoxic Effect results of

compounds **5a-5g** against L929 and MCF-7 cell lines are presented in Table II and Figure 3. The data obtained at the end of the study showed that all compounds between **5a-5g** do not have an anti-cancer effect. As a result of the maximum dose applied, almost all compounds showed IC_{50} values which is higher than 100 µM except compound **5e** for the L929 cell line (which is 97,52 µM). Apart from this, L929 fibroblast cells showed lower cell viability than MCF-7 breast cancer cells with the maximum dose applied.

TABLE II - IC_{50} values (µM) and percent of vitality of compounds **5a-5g** and reference drug cisplatin at 100 µM concentration for MCF-7 and L929 cell lines

	MCF7		L929	
	IC_{50}	**Vitality % (100µM)**	**IC_{50}**	**Vitality % (100µM)**
5a	>100µM	90,6 ± 3,98	>100µM	68,9 ± 8,69
5b	>100µM	89,1 ± 7,25	>100µM	59,1 ± 7,09
5c	>100µM	79,5 ± 2,02	>100µM	66,1 ± 8,29
5d	>100µM	82,7 ± 6,48	>100µM	68,6 ± 1,7
5e	>100µM	52 ± 8,31	97,52µM	49 ± 7,47
5f	>100µM	74,1 ± 4,96	>100µM	59,6 ± 8,46
5g	>100µM	89,2 ± 8,83	>100µM	63,6 ± 4,98
Cisplatin	69,75mM	28,32 ± 3,78	>100µM	53,7 ± 4,83

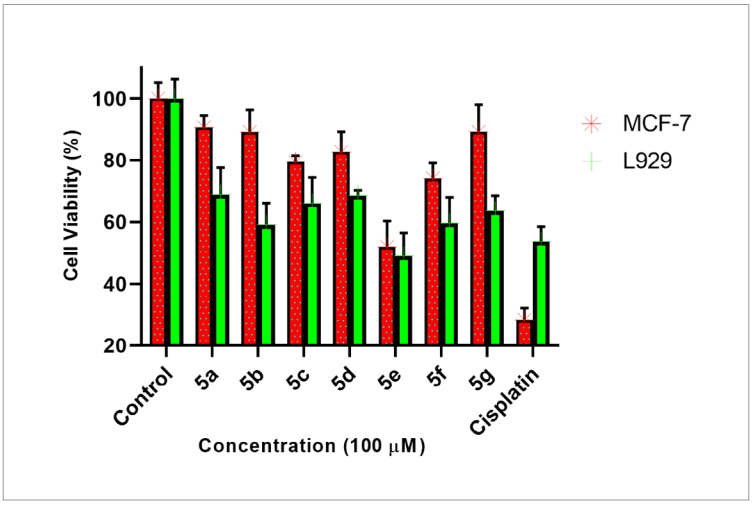

FIGURE 3 - Cell Viability of L929 and MCF-7 cell lines at 100 µM concentration.

Antioxidant Activity

Like some reducing agents, antioxidants also cause the Fe^{+3} ferricyanide complex to be reduced to Fe^{+2}. In this method, the color of the test solution changes from yellow to green, depending on the reducing power of the sample tested. This green color gives maximum absorbance at 700 nm and increasing absorbance indicates increasing reduction strength. According to this method, trolox was used as the standard antioxidant compound, and measurements were made by the procedure determined by Benzie and Strain in 1996. The Ferric Reducing Antioxidant Power values greater than or equal to 1.0 mmol Trolox Equiv./L are considered as high and desired levels. The Ferric Reducing Antioxidant Power of the compounds 5a, 5b, and 5c showed more antioxidant properties than vitamin E for iron reduction which is shown in Table III.

CONCLUSION

In this study, we designed and synthesized for the first time a series of novel benzimidazole-thiadiazole hybrids as antimicrobial, anti-cancer agents, and antioxidants using a molecular hybridization protocol. The antibacterial activity was carried out against four different bacterial strains (*S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) and one fungi strain (*C. albicans*) by using broth microdilution method. The synthesized compound was analyzed for its *in vitro* anti-cancer activity on the MCF7 cell line by using an MTT assay. According to the results, the compound **5g** showed moderate antibacterial activity against *S. aureus* ATCC 29213 and *P. aeruginosa* ATCC 27853. The compound was also analyzed for its antioxidant capacity using the FRAP method. Compounds were found to be ineffective against cancer cells, while compounds **5a**, **5b** and **5c** were found to have promising antioxidant effects.

Declaration of interest statement:

The authors declare no conflict of interest.

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