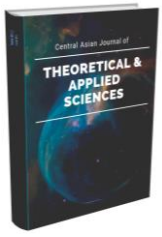

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Article

Synthesis, and Characterization of Novel Tetrazole Derivatives with Antioxidant, Anticancer, and Antibacterial Activities

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Abstract: This study focuses on the synthesis, characterization, and biological evaluation of novel tetrazole derivatives (Y8-Y14). The compounds were synthesized through the reaction of Schiff bases with sodium azide in tetrahydrofuran under reflux. The structural confirmation was performed using FT-IR and ^{1}H-NMR spectroscopy, revealing characteristic absorption bands and chemical shifts indicative of successful synthesis. The antioxidant activity of the synthesized compounds was evaluated using the DPPH radical scavenging method. Compound Y12 exhibited excellent antioxidant properties, with the highest scavenging activity (103.4%) at a concentration of 7.8125 μg/mL, comparable to ascorbic acid. The cytotoxic activity of compound Y12 was tested against breast cancer cells (MCF-7) and normal cell lines (WRL68) using the MTT assay. Y12 showed significant cytotoxic effects with an IC_{50} value of 206 μg/mL, demonstrating its potential as a therapeutic agent for breast cancer treatment. Additionally, the antibacterial activity of the synthesized compounds was assessed against ***Escherichia coli*** (Gram-negative) and ***Staphylococcus aureus*** (Gram-positive) using the well diffusion method. The compounds displayed varying inhibition zones, with activity increasing proportionally with concentration. Control antibiotics such as ***amoxicillin***, ***ampicillin***, and ***ciprofloxacin*** were used for comparison. The study highlights the promising biological activities of these tetrazole derivatives, including their antioxidant, cytotoxic, and antibacterial properties, suggesting their potential application in medical and pharmaceutical fields.

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1. Introduction

Tetrazole compounds are organic heterocyclic molecules characterized by a five-membered ring structure containing four nitrogen atoms and one carbon atom [1]. This unique chemical configuration gives tetrazoles remarkable stability and versatility, making them pivotal in medicinal and pharmaceutical chemistry [2]. Their ability to interact with diverse biological targets has established them as crucial scaffolds in drug development [3]. Tetrazoles can be synthesized through several well-established methods, tailored to specific chemical requirements and applications [4]. The most common approach is the 3+2 cycloaddition reaction, where an azide reacts with a nitrile under thermal or catalytic conditions [5], often enhanced by catalysts such as Lewis acids or transition metals [6]. Hydrothermal synthesis involves reacting hydrazoic acid or its salts (e.g., sodium azide) with suitable precursors under high-temperature and high-pressure conditions to yield high product quantities [7]. Direct substitution on aromatic precursors containing functional groups like halides is another method, often employed for specific substrates. Modern techniques [8], such as microwave-assisted synthesis, have

significantly reduced reaction times while maintaining high yields by applying microwave irradiation to mixtures of azides and nitriles [9]. Additionally, solvent-free methods, aligned with green chemistry principles, use solid-state reactions to minimize environmental impact [10]. Tetrazoles exhibit significant biological and pharmaceutical importance. As antioxidants, they effectively counteract oxidative stress caused by free radicals, major contributors to degenerative diseases such as cancer and cardiovascular disorders [11]. Tetrazoles achieve this by donating electrons or hydrogen atoms, stabilizing reactive oxygen species [12], and protecting biological systems [13]. Their anticancer potential is particularly notable against breast cancer cells [14], such as the MCF-7 line, where they inhibit cell proliferation through mechanisms like direct DNA interaction or enzyme inhibition [15]. Their selective toxicity, targeting cancer cells while sparing normal cells, makes them highly desirable in cancer therapy [16]. Beyond their roles as antioxidants and anticancer agents, tetrazoles serve as antimicrobial agents, anti-inflammatory drugs, and anticoagulants [17]. Their incorporation into bioactive molecules has proven instrumental in designing modern therapeutics for chronic and complex diseases [18]. In conclusion, tetrazole compounds are vital in medicinal chemistry due to their diverse synthesis methods and significant biological activities [19]. Their antioxidant and anticancer potential, combined with their broad pharmaceutical applications [20], underscores their importance in advancing drug development and therapeutic innovation [21].

2. Materials and Methods

A. Material

All chemicals used in this work were purchased from Fluka, Aldrich, and BDH and used without further purification.

B. Devices Used

The melting points were measured using Electrothermal Melting Apparatus 9300. Shimadzu FT-IR 8400S spectrophotometer with a scale of (400-4000) cm^{-1} by KBr disc. ^{1}H-NMR and ^{13}C-NMR spectra on Bruker instruments running at 400 MHz. Thin Layer Chromatography (TLC) was performed using Fluka silica gel plates with 0.2 mm thickness, activated with fluorescent silica gel G, and visualization was achieved using UV light. Microbiological media used in the study were sterilized using an autoclave from Raypa steam sterilizer (Spain) in the Advanced Microbiology Research Laboratory, University of Tikrit. Petri dishes used in the microbiological study were incubated using a Heraeus D-63450 incubator (Germany) in the same laboratory. Visible laser experiments employed a 2010 Helium-Neon laser with a power output of 1 milliwatt and a wavelength of 808 nanometers, Tikrit University.

C. Preparation of Tetrazole Derivatives (Y14-Y8)

A mixture of 0.005 mol of the prepared Schiff bases dissolved in 25 mL of tetrahydrofuran and 0.005 mol (0.325 g) of sodium azide dissolved in 15 mL of the same solvent was stirred under reflux for 6–9 hours. The completion of the reaction was monitored using thin-layer chromatography (TLC). Afterward, the reaction mixture was cooled, and the resulting precipitate was filtered, washed with cold water, and recrystallized using ethanol [22], 23]. Table (1) presents the physical properties of the synthesized tetrazole derivatives.

D. Biological activity study

Two types of pathogenic bacteria were used in this study, one of which is Gram-positive, which is ***Staphylococcus aureus***, and one of which is Gram-negative, which is ***Escherichia coli***. Chemicals with medicinal uses. It is used to measure and determine the minimum inhibitory concentration (MIC), and chemical solutions of (Y8-Y14) were prepared in concentrations (25, 50, 75) mg/ml and using a solvent Dimethyl sulfoxide (DMSO) [24], [25]. The sensitivity test for the bacteria isolates used in the study was carried

out by diffusion method in the nutrient medium Mueller-Hinton agar, which is a transparent food medium with a dark yellow color that is useful in testing the sensitivity of microorganisms to antibiotics because it contains on an animal infusion, casein and starch are extracted [26]. It supports the growth of most microbes and microorganisms [27]. The medium was prepared and sterilized by autoclave, then distributed in dishes and left to harden, then small pits were made at a rate of four holes in each plate [28]. Then it was incubated at (37 ^{0}C) for a period of (24 hours) [29]. The results were read on the next day to show the derivatives sensitivity derivatives used, which depends on the diameter of the inhibition evident in the dishes around the holes used [30], as the increase in diameter Inhibition means the increase in the biological activity of the prepared compounds and compare that with the diameter of inhibition for antibiotics [31].

E. Assessment of Antioxidant Activity of Synthesized Compounds In Vitro

The DPPH radical (2,2-Diphenyl-2-Picryl Hydrazyl) was used to evaluate the free radical scavenging activity of the synthesized compounds (Y12). DPPH exhibits a violet color in methanol, which turns yellow upon reaction with antioxidants [32]. A 0.1 mM DPPH solution was prepared by dissolving 4 mg in 100 mL methanol [33]. Then, 3 mL of the DPPH solution was mixed with 1 mL of compounds at concentrations of 7.8125–500 μg/mL for Y12, 15.625–500 μg/mL for (Y12), and 125–1000 μg/mL for ascorbic acid (positive control). After 30 minutes in the dark, absorbance was measured at 516 nm [34].

F. 2.6. Cytotoxic Activity of Synthesized Compounds Against Breast Cancer Cells (MCF-7)

Breast cancer cells (MCF-7) were obtained, stored in liquid nitrogen, and cultured at Kashan University. The WRL 68 normal cell line was used as a control. Essential solutions included antibiotics, sodium bicarbonate, PBS, trypsin, and EDTA, alongside culture media such as RPMI-1640 and serum-free media [35]. Cells were thawed, cultured in Falcon flasks with RPMI-1640 medium containing 10% FBS, and incubated at 37°C with 5% CO_2. Once growth was confirmed, cells were washed with PBS, treated with trypsin-EDTA to detach them, centrifuged, and resuspended in fresh medium [36]. Cell viability was assessed using Trypan Blue and a Haemocytometer [37]. The MTT assay was performed to evaluate the cytotoxic effects of synthesized compounds (Y12) on MCF-7 and WRL 68 cells. Breast cancer cells (1×10^4–1×10^6 cells/mL) were seeded into 96-well plates, treated with varying concentrations of Y12 (12.5–400 μg/mL), and incubated for 24 hours [38]. MTT solution was added, followed by incubation for 4 hours. DMSO was used to dissolve formazan crystals, and absorbance was measured at 570 nm using an ELISA reader. IC50 values were calculated to determine cytotoxicity [39].

3. Results

In this study, tetrazoles derivatives (Y14-Y8) were prepared by the reaction of equal moles of Schiff base derivatives with sodium azide in the presence of tetrahydrofuran as a solvent, as depicted in Scheme 1.

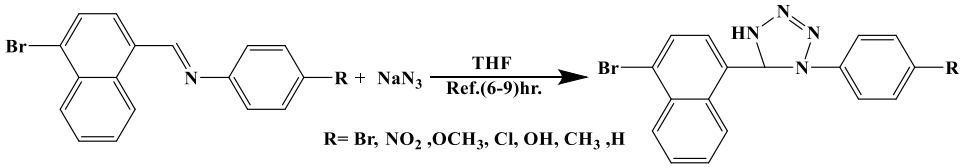

Figure 1. Route of prepared compounds (Y8-Y14).

4. Discussion

A. Characterization of Schiff bases derivatives (Y8-Y14) by FT-IR, ^{1}H-NMR

When analyzing the infrared (IR) spectra of tetrazole derivatives, several characteristic absorption bands were observed, confirming the structural modifications during synthesis. The disappearance of the azomethine (C=N) stretching band associated

with Schiff bases indicates successful tetrazole formation. In its place, a new absorption band in the range of 3195–3232 cm^{-1} was detected, attributed to the stretching vibration of the (N-H) bond in the tetrazole ring [40]. Additionally, aromatic (C-H) stretching vibrations appeared in the range of 3076–3014 cm^{-1}, while asymmetric and symmetric aliphatic (C-H) stretching vibrations were observed at 2951–2912 cm^{-1} and 2889–2850 cm^{-1}, respectively [41]. The presence of the tetrazole ring was further confirmed by an absorption band in the range of 1457–1444 cm^{-1}, corresponding to the (N=N) stretching vibration [42]. Aromatic (C=C) stretching vibrations were evident with absorption bands in the ranges of 1552–1519 cm^{-1} and 1512–1479 cm^{-1}. The (N-C) stretching vibrations within the tetrazole ring were identified in the range of 1260–1228 cm^{-1}, and the (N-N) stretching vibrations were observed at 1089–1062 cm^{-1} [43]. These spectral features, detailed in Table (2), provide strong evidence of the structural integrity and successful synthesis of tetrazole derivatives, as shown in and Figures (1,2).

Upon examining the ^{1}H-NMR spectrum of compound [Y8], several distinct signals were observed, confirming the molecular structure. A multiple in the range of 6.88–8.01 ppm was attributed to the protons of the aromatic rings. A singlet at 5.25 ppm corresponded to the proton of the CH group in the tetrazole ring [44]. Another singlet at 2.25 ppm was assigned to the proton of the NH group. Additionally, a signal at 2.53 ppm was observed, representing the protons of the solvent DMSO-d^{6} [45]. These chemical shifts and signal patterns are illustrated in Figure (3).

Upon examining the ^{1}H-NMR spectrum of compound [Y10], several distinct signals were observed, confirming the molecular structure. A multiple in the range of 6.94–8.03 ppm was attributed to the protons of the aromatic rings. A singlet at 4.88 ppm corresponded to the proton of the CH group in the tetrazole ring [46]. Another singlet at 3.07 ppm was assigned to the proton of the CH3 group. A singlet at 2.40 ppm was attributed to the proton of the NH group. Additionally, a signal at 2.51 ppm was observed, representing the protons of the solvent DMSO-d^{6} [47]. Figure (4) illustrates these chemical shifts and signal patterns.

B. Measurement of antioxidant activity for certain compounds synthesized ex vivo

The qualitative evaluation of antioxidant activity for free radical scavenging is performed using the TLC method to determine the ability of the synthesized compounds (Y12) to reduce oxidative stress, along with a study of their quantitative free radical scavenging activity. The effect of a compound is observed when it changes the purple color of DPPH (2,2-Diphenyl-2-Picryl Hydrazyl) to yellow [48]. DPPH is soluble in methanol and turns yellow when exposed to antioxidants. This process occurs through the donation of a hydrogen atom from the phenolic hydroxyl group to the DPPH● radical [49]. The intensity of the yellow color indicates a positive result. The findings indicate that the synthesized compounds (Y12) under study have excellent scavenging activity, with a noticeable yellow color change [50]. Six concentrations of the synthesized compound (Y12) were used, along with the control sample (15.625, 31.25, 62.5, 125, 250, 500 μg/mL). The study of the antioxidant efficacy of compound (Y12) revealed that it is a good source of antioxidants, with scavenging activity comparable to ascorbic acid (control sample). The data collected showed that the free radical scavenging activity of compound (Y12) reached its highest value (103.4%) at a concentration of 7.8125 μg/mL, and the lowest free radical scavenging activity (18.7%) at a concentration of 500 μg/mL [51], as shown in Figure (5).

C. Cytotoxicity Assay Results for Breast Cancer (MCF-7) Cells

The cytotoxic potential of the synthesized compound (Y12) was evaluated against MCF-7 breast cancer cells using the MTT assay. The assay was conducted in vitro, treating MCF-7 cells with six concentrations of (Y12) in sextuplicate. The assay also compared the effects of (Y12) on MCF-7 and WRL68 normal cell lines [52]. Experimental setup involved preparing a cell suspension in a 96-well plate, treating it with 200 μg/mL of (Y12), and incubating it at 37°C in a 5% CO_2 environment for 24 hours. Post-incubation, 10 μL of MTT solution and 100 μL of DMSO were sequentially added [53]. Absorbance readings were

taken at 570 nm using an ELISA plate reader. Data analysis yielded IC_{50} values, indicating the concentration of (Y12) required to inhibit 50% of cell viability [54]. (Y12) exhibited significant cytotoxic activity against MCF-7 cells, with an IC_{50} value of 206 μg/mL. Statistical analysis revealed a significant difference ($P \leq 0.0001$) between the effects on MCF-7 and WRL68 cell lines [55]. These findings are illustrated in Figures (6,7). The results underscore the potential of (Y12) as a lead compound for further development in breast cancer therapeutics.

D. Evaluation of the Biological Activity of Prepared Compounds

Heterocyclic compounds exhibit varied biological activity against Gram-positive and Gram-negative bacteria. The biological activity of certain compounds synthesized in this thesis was evaluated against two bacterial strains: ***Escherichia coli*** and ***Staphylococcus aureus***. These bacteria were selected due to their medical significance, as they cause numerous diseases. Furthermore, they differ in their resistance to antibiotics. The biological activity of the synthesized compounds was assessed using the well diffusion method [56], and by measuring the inhibition zone. The results indicate that the synthesized compounds have varying abilities to inhibit the growth of both Gram-positive and Gram-negative bacteria at different levels [57]. ***Amoxicillin***, ***ampicillin***, and ***ciprofloxacin*** were used as control antibiotics, selected based on their common use in laboratories and their alignment with World Health Organization (WHO) testing standards. These antibiotics are broad-spectrum, particularly effective against the two studied bacterial strains, along with many others [58]. These antibiotics also have large inhibition zones, offering high selectivity when studying bacterial sensitivity to the synthesized compounds. Since these antibiotics are used to treat various infections, including urinary tract infections caused by *E. coli* and *S. aureus*, as well as simple bladder infections, chronic bacterial prostatitis, lower respiratory tract infections, sinusitis, and joint and bone inflammation, their effectiveness is critical [59]. The compounds in the study demonstrated varying inhibitory activities against ***E. coli***, and similarly, they showed varying inhibitory effects against ***S. aureus***. A positive correlation was found between concentration and inhibition, with inhibition increasing as the concentration increased. The highest inhibition rates were observed at a concentration of 100 mg/mL [60], as shown in Table (3) and Figures (8).

Table 1. Some physical properties of tetrazole derivatives.

Comp.	R	Molecular Formula/ M.Wt g/mol	Color	Time (h)	M.P 0C	Rf	Yield %
Y8	Br	C17H12N4Br2 / 432.12	Light orange	9	190-192	0.70	65
Y9	NO2	C17H12N5O2Br / 398.22	Brown	8	175-177	0.72	72
Y10	OCH3	C18H15N4OBr / 383.25	Yellow	8	243-245	0.44	71
Y11	Cl	C17H12N4BrCl / 387.67	Dark Brown	7	200-202	0.46	72
Y12	OH	C17H13N4OBr / 369.22	Orange	6	263-265	0.52	66
Y13	CH3	C18H15N4Br / 367.25	Dark yellow	8	182-184	0.59	70
Y14	H	C17H13N4Br / 353.22	Dark Brown	8	208-209	0.81	67

Table 2. FT-IR absorption results for tetrazole derivatives by KBr (cm^{-1}).

Comp.	R	νN-H & νC-H Arom.	νC-H Aliph.	νN=N	νC=C Arom.	νC-N & νN-N	Others
Y8	Br	3225 & 3064	2932, 2885	1448	1532, 1482	1233 & 1072	ν (C-Br) 548
Y9	NO2	3232 & 3014	2918, 2875	1444	1545, 1479	1228 & 1089	ν(NO2) 1510, 1357
Y10	OCH3	3197 & 3069	2912, 2889	1452	1542, 1494	1235 & 1081	ν(C-O) 1341
Y11	Cl	3213 & 3076	2920, 2850	1450	1552, 1512	1249 & 1078	ν (C-Cl) 759
Y12	OH	3220 & 3043	2943, 2868	1453	1537, 1493	1234 & 1084	ν(OH) 3341
Y13	CH3	3195 & 3038	2951, 2880	1451	1533, 1506	1260 & 1062	---
Y14	H	3217 & 3041	2923, 2853	1457	1519, 1487	1246 & 1072	---

Table 3. Biological effectiveness of prepared compounds and control treatments (inhibition in mm).

Comp. No.	*Escherichia coil*			*Staphylococcus aureus*		
Conc. mg/ml	**25**	**50**	**100**	**25**	**50**	**100**
Y8	0	0	0	0	0	1
Y9	0	0	5	0	1	2
Y10	0	1	1	1	2	4
Y11	0	0	0	0	0	0
Y12	2	4	5	0	0	0
Y13	0	0	0	0	0	5
Y14	0	1	1	0	0	0
Amoxicillin	2	4	5	2	5	7
Ampicillin	2	3	5	2	4	6
Ciprofloxacin	1	2	5	2	3	5
Blank disk	0	0	0	0	0	0

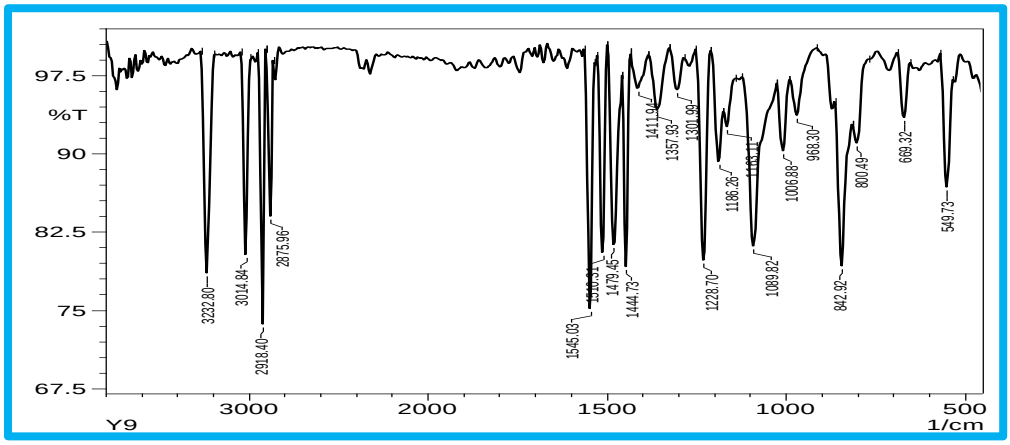

Figure 2. FT-IR spectrum of the compound (Y9).

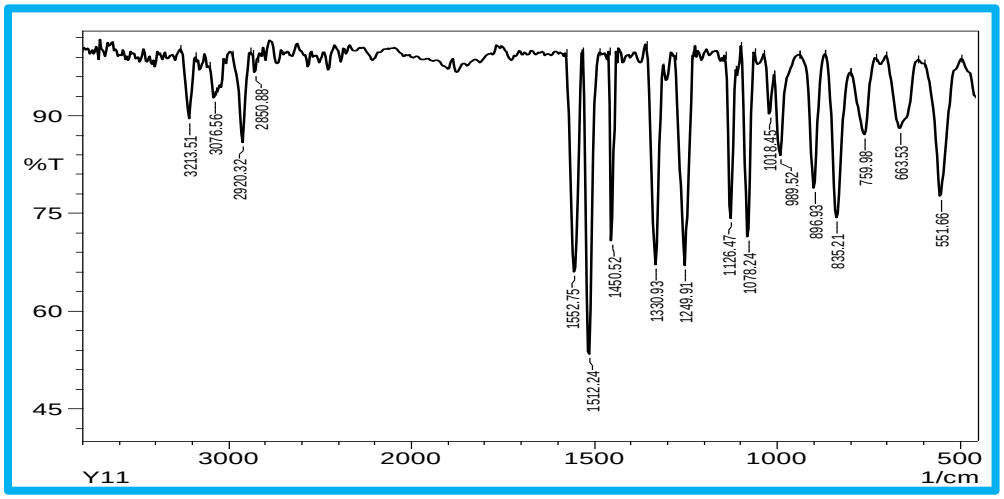

Figure 3. FT-IR spectrum of the compound (Y11).

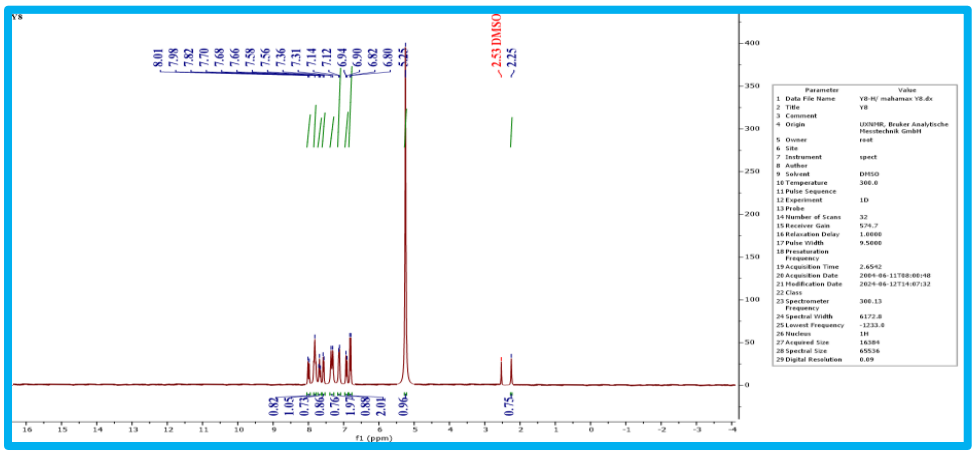

Figure 4. [1]H-NMR spectrum of the compound (Y8).

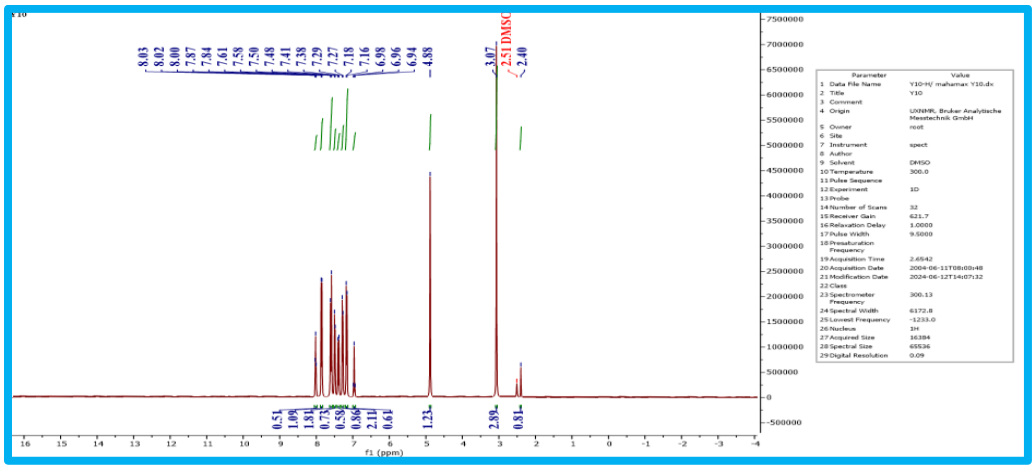

Figure 5. [1]H-NMR spectrum of the compound (Y10).

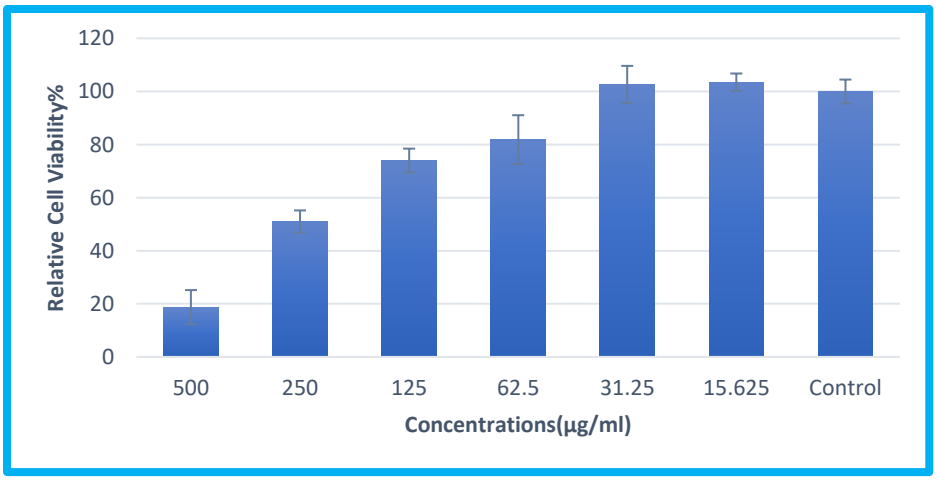

Figure 6. Antioxidant activity of compound (Y12).

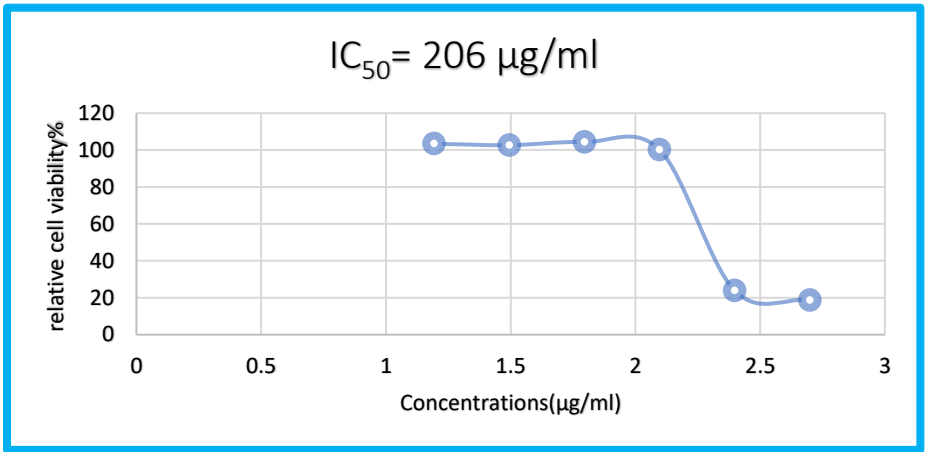

Figure 7. The effect of compound (Y12) on MCF-7 cells and HdFn cells using the MTT assay.

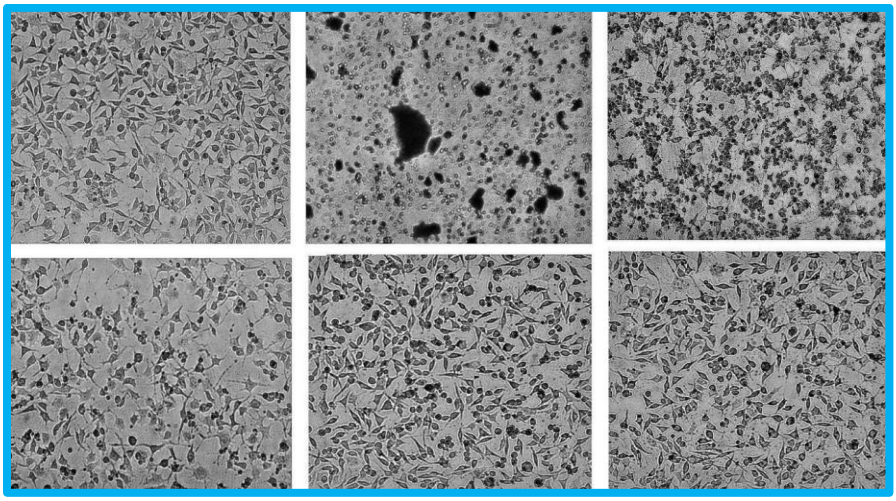

Figure 8. The efficacy of compound (Y12) against breast cancer cells.

Figure 9. Inhibitory activity of some synthesized compounds against *E. Coli* bacteria.

E. Recommendations

Further structural modifications of the synthesized compounds should be explored to enhance their biological activities and selectivity. Detailed mechanistic studies are recommended to understand the interaction of these compounds with cellular and bacterial targets. In vivo studies should be conducted to evaluate the safety, bioavailability, and efficacy of the synthesized compounds. Future research should explore the potential of combining these compounds with existing antibiotics or chemotherapeutic agents to assess synergistic effects. Expanding the scope of biological testing to include additional

bacterial strains and cancer cell lines could provide a more comprehensive evaluation of the compounds' therapeutic potential.

5. Conclusion

The synthesized tetrazole derivatives (Y8-Y14) were successfully prepared and characterized using FT-IR and ^{1}H-NMR spectroscopy, confirming the structural integrity and successful synthesis. The antioxidant activity of the synthesized compounds, particularly (Y12), demonstrated excellent free radical scavenging capabilities, with results comparable to ascorbic acid. The activity was concentration-dependent, with the highest scavenging activity observed at lower concentrations. The cytotoxicity study of (Y12) against MCF-7 breast cancer cells revealed significant cytotoxic effects with an IC_{50} value of 206 μg/mL. The compound exhibited selective activity, showing a notable difference between its effects on cancerous (MCF-7) and normal (WRL 68) cell lines. The synthesized compounds showed promising antibacterial activity against *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). The inhibition zone was positively correlated with the concentration, and the compounds demonstrated varied inhibitory effects at different concentrations. The findings suggest that the synthesized compounds have potential applications in pharmaceutical development, particularly as antioxidants, anticancer agents, and antibacterial agents.

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