ORIENTAL JOURNAL OF CHEMISTRY
An International Open Access, Peer Reviewed Research Journal
www.orientjchem.org
ISSN: 0970-020 X
CODEN: OJCHEG
2025, Vol. 41, No.(6):
Pg. 1991-2002

# Synthesis, Characterization, Novel Pyrazolo [3,4-d]Pyrimidine Derivatives of Study Cytotoxcity, Antioxidant and Anticancer *In vitro*

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http://dx.doi.org/10.13005/ojc/410613

(Received: November 10, 2025; Accepted: December 11, 2025)

**ABSTRACT**

The novel synthesis of twocompounds of pyrazolo[3,4-d]pyrimidine derivatives(Gz1,Gz2) was attempted to be created. By reacting 5-methyl-2,4-dihydro-3H-pyrazol-3-one(A) with various aromatic aldehyde, thiourea, under minimal response conditions, these compounds were synthesized with acceptable yields. The newly synthesized compounds' structural characteristics were determined using C.H.N elemental Analysis (Carbon. Hydrogen. Nitrogen analyzer spectroscopy), FT-IR (Fourier-Transform Infrared Spectroscopy), $^1$H-NMR(Hydrogen Nuclear Magnetic Resonance spectroscopy) and Mass spectrometry. Determination the synthetic compounds color, physical characteristics, cytotoxicity, antioxidant activity, and anticancer activities, the synthesized compounds (Gz1, Gz2) antioxidant activity was investigated. Utilizing 1,1-diphenyl-2-picrylhydrazyl(DPPH) free radical scavenging tests, the compounds demonstrated a moderate-high antioxidant efficacy. The cellular toxicity of the synthesized compounds(Gz1, Gz2) it was investigated on MCF-7 cell lines using the MTT assay. Compound (Gz2) shows the biggest toxicity towards MCF-7 cell lines. The way method of cell death was investigated using various morphological and apoptotic strategies. (AO) Acridine Orange and (EB)Ethidium Bromide fluorescent staining were used to measure the live/dead rates of cell viability.

**Keywords:** Pyrazole, Pyrimidine, Anticancer, Antioxidant DPPH, MCF-7, Fluorescent.

## INTRODUCTION

The synthesis of novel pyrazolo[3,4-d] pyrimidine derivatives has recently attracted considerable attention due to their varied biological activities, especially their anticancer properties[1]. Cancer, being a highly aggressive and fatal illness, continues to be one of the primary medical issues worldwide[2]. It ranks as the second foremost cause of mortality globally, accounting for 9.6 million fatalities in 2018. Although there is a modest ability to mitigate the effects of infection-related malignancies, a consistent decrease in these tumors is seen with socioeconomic progress[3]. To

further reduce cancer incidence, particularly those associated with infections, immunization, screening, and treatment programs must be both economical and accessible to all demographics[4]. Cancer may be induced by genetic mutations or abnormalities in normal cell differentiation caused by extrinsic stimuli such as pharmaceuticals, infections, tobacco use, or dietary influences. Pyrazolo[3,4-d]pyrimidine derivatives exhibit diverse pharmacological activity, including antiviral, anti-inflammatory, antibacterial, antimycobacterial, antihypertensive, radioprotective, and antioxidant characteristics[5]. Considering these advantageous attributes, investigating pyrazolo[3,4-d]pyrimidine derivatives in oncological therapy is pertinent and imperative. With advances in oncology, there is an increasing need to discover and develop compounds that can effectively target the various mechanisms that drive cancer progression6. The inherent versatility of pyrazolo[3,4-d]pyrimidines as a pharmacophore provides a foundation for the synthesis of compounds that can be optimized for multiple therapeutic effects, potentially offering novel solutions to address the limitations of current cancer treatments[7]. By facilitating the development of compounds that not only inhibit cancer cell growth but also minimize adverse effects associated with conventional therapies, the study of pyrazolo[3,4-d] pyrimidine derivatives contributes to a more targeted, effective, and patient-friendly cancer treatment landscape[8]. The continued exploration and optimization of these derivatives hold promise for the future of anticancer drug development, where specificity and multimodal action will be essential for managing the complex biology of cancer[9].

## EXPERIMENTAL

### Materials and instruments

These chemical products can be purchased from Merck Company, a Germany Company, and Sigma-Aldrich Company which is based United State of America. FT-IR spectra were recorded on KBr using a frequency range of 4000-400 $cm^{-1}$ on an infrared spectrometer FT-IR(8000), single beam path laser, Fourier Shimadzu transformed. The $^1$H-NMR spectrometers are reported with the $^1$H-NMR Bruker spectrometer (500 MHz) in DMSO-$d_6$. GC-mass spectra in the block are obtained using Fisons Trio(1000) spectrometer. The C.H.N element analyses are provided using of an (EM-017) analyzer.

### Step-I: Synthesis of 5-methyl-2,4-dihydro-3H-pyrazol-3-one(A)[10]

To synthesize the desired compound, the following procedure was followed:

A mixture of β-ketoester (ethyl acetoacetate) (1.0 equivalent) and 2-3 drops of acetic acid was stirred at 20°C. Hydrazine hydrate (1.1 equivalent) was then added to this mixture and stirred for 5-10 min while maintaining the temperature range at 15-20°C. A white solid precipitated out, which was collected by filtration using suction and washed twice with 20 mL of water. The product was further washed with a 50:50 methanol-water mixture and then dried under vacuum at 40°C for two hours, yielding an 86% product with a melting point of 122-123°C and $R_f$ value of 0.78. Elemental analysis. Calc. C, 48.97; H, 6.16; N, 28.56; Found. C, 49.09; H, 7.00; N, 28.15. FT-IR (KBr, $cm^{-1}$, stretching), υ(NH-Pyrazole) 3194.23, υ(Ar-H) 3047.63, υ(C-H, aliphatic) 2965.91, υ(C=O. of ketone), 1701.27, υ(Ar, C=C) 1607.48, υ(C-N. of aromatic-N-) 1340.57. $^1$H-NMR (300 MHz, DMSO-$d_6$, δ/ppm): δ(s, 1.96ppm, 3H, Pyrazol-$CH_3$), δ(s, 3.40ppm, 2H, Pyrazol-$CH_2$-), δ(s, 11.84ppm, 1H, NH-Pyrazole). m/z: 98 ($M^+$, R% 20), shown the important fragmentation peaks in 41*m/z*, 50*m/z*, 57*m/z*, 69*m/z*, 81*m/z*, 89*m/z* & 98*m/z*, This data supports the successful synthesis of the target compound with the desired purity and expected physical properties.

### Step-II: Synthesis of 3-Mythyl-4-(substituted-phenyl)-1,4,5,7-tetrahydro-6H-pyrazolo[3,4-d] pyrimidine-6-thione($G_{z1}$ & $G_{z2}$)[11]

To synthesize the target compound, the following procedure was conducted, A mixture containing aldehyde (1.0 equivalent), pyrazole (1.0 equivalent), and thiourea (1.5 equivalents) was stirred in 20 mL of acetonitrile until a clear solution formed. Concentrated HCl (3-4 drops) was then added as a catalyst, and the reaction mixture was refluxed for 10-16 hours. Upon completion of the reaction, the mixture was cooled. In certain cases, a solid product precipitates during reflux. The product was collected by filtration, washed with acetonitrile, and dried in an oven. This procedure yielded the desired compound, with product quality confirmed through subsequent analyses.

### 3-Methyl-4-(3-methylstyryl)-1,4,5,7-tetrahydro-6H-pyrazolo[3,4-d]pyrimidine-6-thione($G_{z1}$)

The synthesis of the target compound was

achieved by reacting 3-(m-tolyl)acryl aldehyde (1.46 g, 0.01mole) with 3-methyl-2-pyrazolin-5-one (0.98 g, 0.01 mole) and thiourea (0.76 g, 0.01mole). The reaction yielded a yellow solid product with an 82% yield, melting point of 163-161°C, and an $R_f$ value of 0.75. Eelemental analysise.Calcc.C, 64.12; H, 5.67; N, 19.70. Found.C, 63.35; H, 4.89; N, 20.02. FT-IR (KBr, $cm^{-1}$), stretching), υ(NH-Pyrimidine) 3379.40, υ(NH-Pyrimidine) 3279.10, υ(NH-Pyrazole) 3176.87, υ(Ar-H) 3047.63, υ(C-H, aliphatic) 2881.14, υ(Pyrazole, C=N) 1666.55, υ(N-H bend) 1612.54, υ(Ar, C=C) 1512.24-1469.81, υ(C=S) 1128.39; $^1$H-NMR (500 MHz, DMSO-$d_6$, δ/ppm): δ(s,2.77ppm, 3H, Pyrazole-$CH_3$), δ(s, 2.99ppm, 3H, Ph-$CH_3$), δ(s, 5.41ppm, 1H, CH-Pyrimidine), δ(d, 6.18-7.05ppm,*J* = 10, 2H, CH=CH), δ(m, 7.62-7.82ppm, 3H, Ar-H), δ(s, 7.89ppm, 1H, NH-Pyrimidine), δ(s, 8.42ppm, 1H, NH-Pyrazole). δ(s, 9.08ppm, 1H, NH-Pyrimidine). m/z: 284 ($M^+$, R% 38), shown the important fragmentation peaks in 41*m/z*, 50*m/z*, 57*m/z*, 69*m/z*, 98*m/z*, 115*m/z*, 129*m/z*, 144*m/z*, 173*m/z*, 201*m/z*, 265*m/z* & 281*m/z*, This data confirms the successful synthesis of the compound with the expected physical, elemental, and spectroscopic characteristics.

**4-(3,5-Dihydroxyphenyl)-3-methyl-1,4,5,7-tetrahydro-6H-pyrazolo[3,4-d]pyrimidine-6-thione($G_{z2}$)**

The compound was synthesized by reacting 3,5-dihydroxybenz aldehyde (1.38 g, 0.01mole) with 3-methyl-2-pyrazolin-5-one (0.98 g, 0.01mole) and thiourea (0.76 g, 0.01mole). The reaction produced a white solid with a yield of 79%, melting point of 224-225°C, and an $R_f$ value of 0.68. Elemental analysis. Calc. C, 52.22; H, 4.380; N, 21.28. Found. C, 53.23; H,4.340; N, 21.19.FT-IR-(KBr, $cm^{-1}$, stretching), υ.(Ar-OH) 3443.05, υ(NH-Pyrimidine) 3336.96, υ(NH-Pyrimidine) 3228.95, υ(NH-Pyrazole) 3120.93, υ(Ar-H) 3064.99,ῡ(C-H, aliphatic) 2908.75,ῡ(PyrazoleC=N) 1666.55, υ(N-H bende) 1641.48,ῡ(Ar, C=C) 1600.97-1541.18,ῡ(C=S) 1132.25, $^1$H-NMR (500 MHz, DMSO-$d_6$, δ/ppm): δ(s, 2.70ppm, 3H, Pyrazole-$CH_3$), δ(s, 5.04ppm, 1H, CH-Pyrimidine), δ(m, 6.98-7.35ppm, 3H, Ar-H), δ(s, 7.88ppm, 1H, NH-Pyrimidine), δ(s, 8.80ppm, 1H, NH-Pyrazole).ῡ(s, 9.86ppm, 1H, NH-Pyrimidine), δ (s, 13.85ppm, 1H, Ar-OH). m/z: 276 ($M^+$, R% 40), shown the important fragmentation peaks in 41*m/z*, 51*m/z*, 57*m/z*, 76*m/z*, 69*m/z*, 98*m/z*, 115*m/z*, 104*m/z*, 109*m/z*, 128*m/z*, 149*m/z*, 167*m/z*, 265*m/z* & 220*m/z*, These analytical results confirm the successful synthesis and characterization of the compound, with properties consistent with its expected structure.

**Hemolysis Assay of Pyrazolo[3,4-d]Pyrimidine Compounds $G_{z1}$ & $G_{z2}$[12,13]**

The hemolytic ratio (HR) was calculated to quantify the extent of hemolysis in the sample. The formula used to determine HR is as follows:

$$HR(\%) = \frac{\text{Absorbance of sample} - \text{Absorbance of negative control}}{\text{Absorbance of positive control} - \text{Absorbance of negative control}} \times 100$$

Where:

- **Absorbance of sample:** The absorbance at 540 nm of the supernatant from the sample with tested compounds ($G_{z1}$ & $G_{z2}$).
- **Absorbance of negative control:** The absorbance at 540nm of the supernatant from the 2% RBC suspension in normal saline (without compounds).
- **Absorbance of positive control:** The absorbance at 540nm of the supernatant from the 2% RBC suspension in distilled water, which causes complete hemolysis.

This formula gives the percentage hemolysis, where 100% represents complete hemolysis, and 0% represents no hemolysis, helping to evaluate the hemolytic potential of the tested compounds ($G_{z1}$& $G_{z2}$) at various concentrations.

**Antioxidant activityof Pyrazolo[3,4-d]Pyrimidine Compounds $G_{z1}$ & $G_{z2}$[14]**

A newly created using (0.004w/v) DPPH methanol solution The quantity of the synthesised pyrazolo[3,4-d]pyrimidine derivatives with ascorbic acid (a standard) was examined in terms of their ability to neutralise 1,1-diphenyl-2-picry hydroxyl (DPPH) radical method. It was diluted in 1 mL of pyrazolo[3,4-d]pyrimidine. If there was disagreement about which one of the two should be diluted then the choice of diluent depends on the type of challenge. Samples with varying test sample solution concentrations (25ppm, 50ppm, 75ppm, 100ppm, 125ppm, 150ppm, 175ppm ppm and 200ppm standard concentration) were prepared. The reaction solution was allowed to warm at 25°C for half an hour in dark condition. The optical density at 517nm was determined by using UV spectro

photometer. Ascorbic acid can be considered as positive regulator because of its antioxidant activity. The actual inhibited free radicals are given as a per cent of the total of inhibited free radicals. The following equation was used to get the percentage of inhibition.

The DPPH scavenging activity was calculated to evaluate the antioxidant potential of the synthesized mono and bis oxazolone derivatives compared to ascorbic acid. The equation used for determining the percentage of DPPH radical inhibition is:

$$\text{DPPH Scavenging Activity (\%)} = \frac{(\text{A control} - \text{A test sample})}{\text{A control}} \times 100$$

Where:

**-A control:** The absorbance at 517nm of the DPPH methanol solution without any test sample (this represents 0% inhibition or full radical activity).

**-A test sample:** The absorbance at 517nm of the DPPH solution after adding the oxazolone derivatives at various concentrations (25, 50, 75, 100, 125, 150, 175, and 200ppm).The DPPH solution was kept in the dark at 25°C for 30 min to allow for complete interaction with the antioxidants. Ascorbic acid, with known antioxidant activity, was used as a positive control to validate the assay. This formula calculates the percentage of DPPH radicals scavenged, indicating the antioxidant efficiency of the test samples compared to the control.

**Cell lines and culture[15]**

MCF-7 (a human breast cancer cell line) was purchased from the National Cell Bank of Iran at Pasteur Institute Iran. Cells were maintained in RPMI-1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco) and 100 U/mL penicillin and 100 µg/mL streptomycin. The cells were cultured at 37°C under 5% $CO_2$ and 95% humidity and subcultured using trypsinized 0.25% trypsin solution with 0.02% EDTA (Gibco) and PBS. The nutrient media and conditions under which the cells were maintained as three-dimensional colonies were similar to two-dimensional monolayer culturing.

**MTT cell viability assay in MCF-7 Cells[16]**

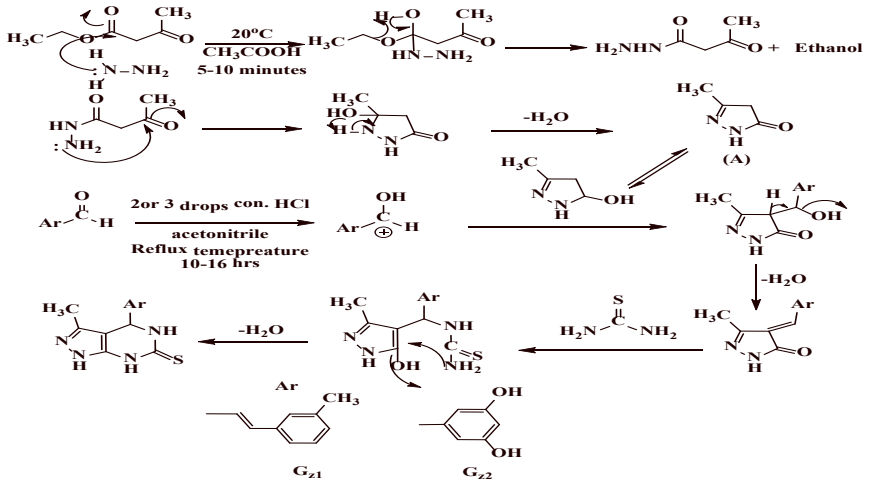
Cell proliferation and cell viability were measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (Sigma, Aldrich) assay. In brief, for monolayer culture, MCF-7 cells were exposed to trypsin digestion, counted, and re-plated at a deensity of $1.4 \times 10^4$ cells/well in 96-well plates containing 200 µL of freshes mediuim peir well for 24 hours. Whene celles reached 80-90% confluence, they were treated with 100–6.25 µg/mL of the compounds for 24 h at 37°C in 5% $CO_2$. For the monolayer culture, the plate was left undisturbed in the original position after the end of the treatment (24 h), in which 200 µL/well of supernatant was removed and replaced with 200 µL/well of freshly prepared MTT solution (0.5 mg/mL in PBS), and the plate was incubated at 37°C for 4 h more. MTT solution (the culture medium was removed from the wells and replaced with dimethyl sulfoxide; 100 µL per well was required). Afterwards, cells were incubated on a shaker at 37°C until the crystals were dissolved. A cell viability assay was performed using an ELISA reader (Model wave xs2, BioTek, USA) to determine absorbance at 570 nm. The $IC_{50}$ value was calculated from the respective dose-response curves of the compounds for their concentration causing 50% of cell death.

**Fluorescent Staining[17]**

For the determination of live or dead cells, fluoroorescence staining of ethidium bromide (Sigma-Aldrich) and acridine orange (AO) simultaneously was done. First, MCF-7 cells were cultured in 6-well cell culture plates and exposed to the $IC_{50}$ of the compounds for 24 hours. Then cells were washed with PBS and then added with a solution of EB/AO. The stained cells were immediately observed and photographed using a fluorescence microscope (Axioskop 2 Plus, Ziess, Germany). The distinction between viable, apoptotic, and necrotic cells is made on the basis of the difference between dye uptake and compromised membrane. The green coloration means that the cells are viable and stained only with AO; green and orange colorations indicate early and late apoptosis of cells with condensed chromatin and with moderate alteration of membrane permeability, stained with both AO and EB; the orange coloration indicates necrosis of cells stained only with EB. Each of the five samples was representative of a stained slide since five photos were taken of randomly chosen regions of the slides.

## RESULTS AND DISCUSSION

Pyrazolo[3,4-d]pyrimidine and its derivatives are a significant class of heterocyclic compounds, known for their diverse biological activities in both medicine and agriculture. These compounds are synthesized through the reaction of 3-methyl-2-pyrazolin-5-one with an aldehyde and thiourea, yielding pure pyrazolo[3,4-d]pyrimidine. The key step in the synthesis of pyrazolo[3,4-d] pyrimidine derivatives ($G_{z1}$ and $G_{z2}$) involves treating 3-methyl-2-pyrazolin-5-one with an aldehyde and thiourea in acetonitrile, resulting in the formation of $G_{z1}$ and $G_{z2}$. The general reaction mechanism for the synthesis of pyrazolo[3,4-d]pyrimidine is illustrated in Scheme 1, as referenced in sources[18,19].




**Scheme 1. Mechanism of formation of Pyrazolo[3,4-d]Pyrimidine ($G_{z1}$ & $G_{z2}$)**

### Structurie determiniation

The chemicals strructures of the synthesized compoundes were determined basis on the FT-IR, $^1$H-NMR, GC-Mass, spectroscopic techniques and C.H.N analysis. The The spectra of $G_{z1}$ and $G_{z2}$ are also distinguished by the appearance of the wide middle band because of the stretching oscillation of (N-H)(Pyrimidine and Pyrazole) at (3120.93-3379.40) cm$^{-1}$. The spectra of compound $G_{z2}$ contain broad bands at 3443.05 cm$^{-1}$, which arose from the stretching vibration of the O-H group. The FT-IR spectra[20] have demonstrated new absorption bands by stretching vibrations of the Pyrazole C=N moieties on compound $G_{z1}$ and $G_{z2}$ at (1666.55) cm$^{-1}$. That the core of compounds $G_{z1}$ and $G_{z2}$ has the pyrazolo[3,4-d]pyrimidine structure has been confirmed by the $^1$H-NMR spectrum data[21]. N-H(pyrimidine and pyrazole) group protons were at (7.88-9.86)ppm for $G_{z1}$ and $G_{z2}$, respectively. The signal of the proton of the methyl group (CH-Pyrimidine) was obtained at $\delta$ (5.30-5.31)ppm. A singlet at $\delta$(13.85)ppm assigned for the proton of the O-H group in compounds $G_{z2}$ was observed, respectively. Some resonance signals at (2.50 and 3.10-3.30)ppm were attributed to the DMSO-d6 solvent present in the samples. The synthesized pyrazolo[3,4-d]pyrimidine derivatives were further elucidated by the GC-mass spectra. The GC-Mass[22] spectra of the pyrazolo[3,4-d]pyrimidine compounds revealed the correct molecular ion peaks. The molecular ion peaks appeared at m/z: 284 (M+, R%38) and m/z: 276 (M+, R%40) for compounds $G_{z1}$ and $G_{z2}$, respectively.

### Hemolysis Study

The hemolysis study was selected as a straightforward and reliable method for the preliminary evaluation of the cytotoxic effects of new compounds on erythrocytes. Since increased hemolysis can harm the human body, understanding how an intravenous compound interacts with red blood cells is crucial. The extent of damage to erythrocytes can be assessed by measuring the hemoglobin released[23] from lysed cells. A hemolysis ratio below 10% indicates that the compound is safe for intravenous use. According to the results in Table 01, at a concentration of 8 mg/mL, the average hemolysis rates for $G_{z1}$ and $G_{z2}$ were 5.78% and 5.40%, respectively. Across all tested concentrations, hemolysis remained below 6%, suggesting that the pyrazolo[3,4-d]pyrimidine compounds can be administered intravenously without significant risk[24].

**Table 1: The results of the hemolysis ratio (%) for compounds $G_{z1}$ & $G_{z2}$. at different concentration**

| Compound code | HR(%) 2 mg/mL | 4 mg/mL | 6 mg/mL | 8 mg/mL | 10 mg/mL |
|---|---|---|---|---|---|
| Gz1 | 0.28 | 0.77 | 1.67 | 2.82 | 5.78 |
| Gz2 | 0.21 | 0.73 | 1.14 | 2.66 | 5.43 |

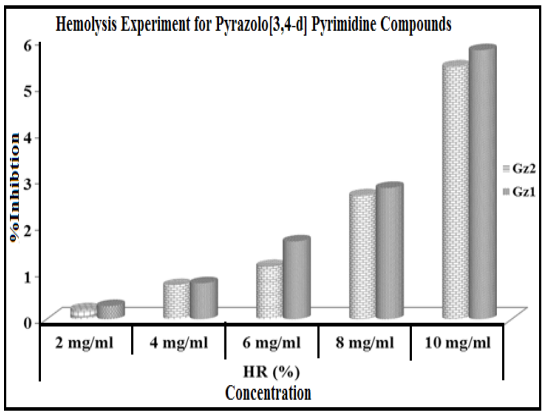



**Fig. 1. Measurement of hemolysis for Pyrazolo[3,4-d] Pyrimidine compounds towards blood cells at different concentrations**

## Antioxidant Activity

A condition arises when the rate of free radical generation surpasses the body's ability to counteract oxidative stress, leading to harmful effects on lipids, proteins, and DNA. Therefore, protective strategies that neutralize free radicals are crucial for preventing damage to biological molecules. Free radicals are unstable molecules that contribute to various diseases, while antioxidants are compounds that neutralize these radicals[25]. The antioxidant potential of synthesized compounds is often assessed using the DPPH assay, which involves a stable free radical, DPPH. The reduction of DPPH occurs through the acceptance of an electron or a hydrogen atom. *In vitro* DPPH radical scavenging assays are employed to evaluate the ability of pyrazolo[3,4-d] pyrimidine compounds to neutralize free radicals, thereby determining their antioxidant capacity. The reduction in sample activity was determined by the change in color of the DPPH solution from a deep purple to yellow. In relation to Table 2, the samples' antioxidant activity is evaluated and expressed by comparing it with the standard antioxidant ascorbic acid[26]. The DPPH radical was used to assess the free radical-scavenging effects of all synthesized compounds at various doses (25ppm, 50 ppm, 75ppm, 100ppm, 125ppm, 150ppm, 175ppm, and 200ppm). Conversely, it is well recognized that organic molecules containing electron-donating groups, such as amine, methoxy, and hydroxy, serve as excellent free radical scavengers. The inhibition of the samples ranges from 89.13% to 92.89%. Succus mustae, derived from a 90% vitamin C content, had the maximum cytotoxic inhibition of 99.08% at 1000ppm. Among all test substances at a concentration of 1000ppm, compound ($G_{z2}$) had the greatest percent inhibition at 92.89%, attributed to the presence of the electron-donating group OH. Furthermore, it is significant that all samples below 1000ppm exhibited a concentration-dependent reduction in inhibition[27]. In a concentration of 1000ppm, chemical ($G_{z1}$) exhibited the lowest inhibition at 89.13%

**Table 2: The inhibitory values displayed by the test Pyrazolo[3,4-d]Pyrimidine compounds $G_{z1}$ & $G_{z2}$**

| Compound code | 25ppm | 50ppm | 75ppm | 100ppm | 125ppm | 150ppm | 175ppm | 200ppm |
|---|---|---|---|---|---|---|---|---|
| Gz1 | 11.78 | 31.98 | 38.99 | 48.79 | 51.57 | 62.46 | 78.35 | 89.13 |
| Gz2 | 23.76 | 35.32 | 40.49 | 50.97 | 63.06 | 74.62 | 87.99 | 92.89 |
| Sorbic | 33.02 | 39.48 | 46.81 | 57.28 | 70.70 | 88. 61 | 97.77 | 99.08 |

## *In vitro* Anti-breast Cancer Activity

The cells were treated with manufactured pyrazolo[3,4-d]pyrimidine compounds $G_{z1}$ and $G_{z2}$ at doses of 6.25, 12.5, 25, 50, and 100 µg/mL for 24 h[28], as shown in Fig. 3, MCF-7% cytotoxicity of compounds $G_{z1}$ and $G_{z2}$ compared to standard medication tamoxifen[29]. The MTT experiment demonstrated that at 100 µg/mL, both $G_{z1}$ and $G_{z2}$ reduced the MCF-7 cell growth to a maximum of 78.78±1.51 and 85.96±2.87 after 24 hours. Overall, compound $G_{z2}$ was more powerful against[30] MCF-7 cells than compound $G_{z1}$. This speculates that it may potentially bind to these receptors and their pathways that lead

to MCF-7 cell inhibition. Fig. 5 shows instances of MCF-7 cells treated to various treatments with chemicals $G_{z1}$ and $G_{z2}$. Doxorubicin, $G_{z1}$ and $G_{z2}$ likewise revealed $IC_{50}$ values of 15.28, 29.42, and 21.93 µg/mL, respectively[31]. The toxicity of doxorubicin, $G_{z1}$ and $G_{z2}$ in the MCF-7 cell line is displayed in Fig. 4, further revealing that the MCF-7 cell line was more susceptible to $G_{z2}$ compared to other chemicals. According to our results, $G_{z1}$ and $G_{z2}$ may slow the development of MCF-7 cells, and they operate as an anti-breast agent[32].

**Fluorescence microscopy by Acridine orange and ethidium bromide (AO & EB) staining**

In the context of fluorescence emission, apoptosis was disclosed using fluorescence microscope after treating the cells with AO & EB. Firstly, we evaluated the impact of the $G_{z1}$ and $G_{z2}$ compounds in triggering apoptosis in MCF-7 cells where the cells were originally sowed on 6 well culture plate and treated with chemicals at $IC_{50}$ for 24 hours[33]. However, via the AO/EB fluorescent labeling approach, more detailed research on the apoptotic impact of $G_{z1}$ and $G_{z2}$ in MCF-7 cells were performed[34]. Living cells had fluoresced green consistently and exhibited a specific extremely well organized structure in the nucleus. Early apoptotic cells which had damaged membrane but intact membrane and their DNA was broken while the peri-nuclear chromatin condensation was clearly obvious by brilliant green patches or tiny pieces[35]. Late apoptotic cells have the orange to red nucleus with constricted or broken chromatin. Nonviable cells displayed orange to red luminous nuclei without any indication of chromatin characteristic of apoptosis. As indicated in Fig. 6, control cells Treatment with the vehicle had all the live green cells with normal morphological appearance[36]. In contrast, the compounds $G_{z1}$ and $G_{z2}$ treated cells exhibited a large increase in EB stained cells, showing the cells were in early and/or late apoptotic stages as stated by[37]. Bright green spots were detected in cell therefore showing the cells in early apoptosis while orange stained cells verify them as in the late stage of apoptosis Fig.6. Some developed compounds $G_{z1}$ and $G_{z2}$ affected the MCF-7 cells viability and had high potential inhibition from the MTT assay. Moreover, the quantitative evaluation of expression apoptotic markers indicated that breast cancer MCF-7 cells were treated with chemicals $G_{z1}$ and $G_{z2}$ which more efficiently triggered apoptosis at lower concentrations[38].

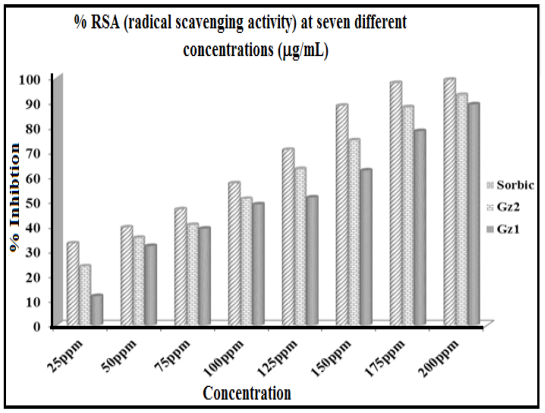



**Fig. 2. DPPH radical scavenging activity of Pyrazolo[3,4-d] Pyrimidine compounds at different concentrations compared with ascorbic acid**

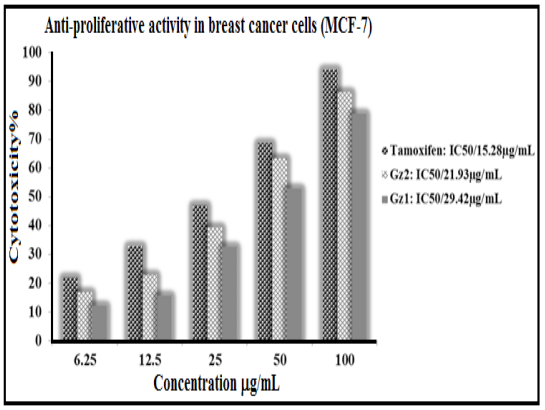



**Fig. 3. Anti-proliferative activity in breast cancer cells (MCF-7) cultured with various conentrations of tamoxifen, $G_{z1}$ and $G_{z2}$ after 24 hours**

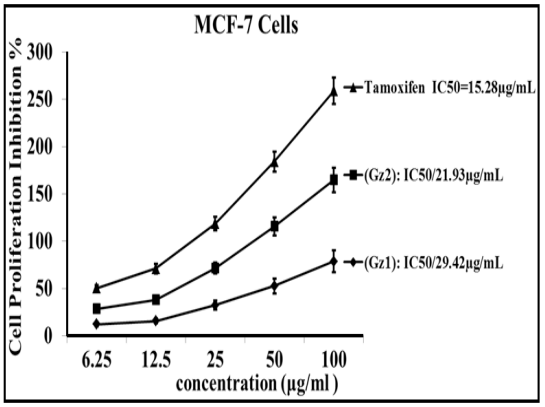



**Fig. 4. Proliferation inhibition effect of tamoxifen, $G_{z1}$ and $G_{z2}$ in MCF-7 cell line**

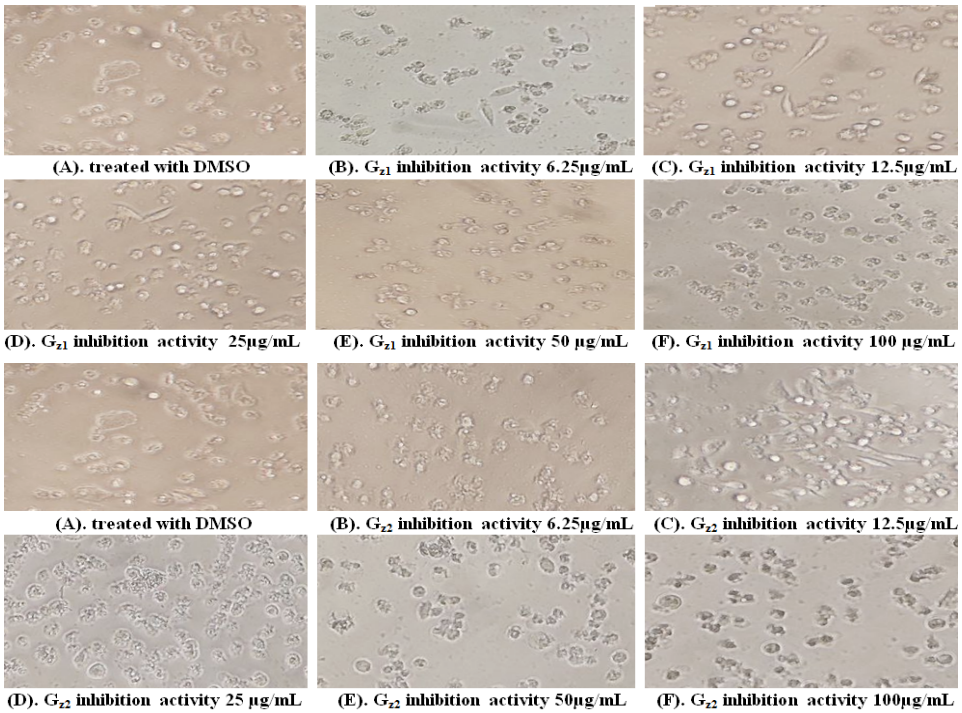



**Fig. 5. Representative images of untreated MCF-7 cells and treated MCF-7 cells with compounds(Gz1 & Gz) in various concentrations after 24 hours**

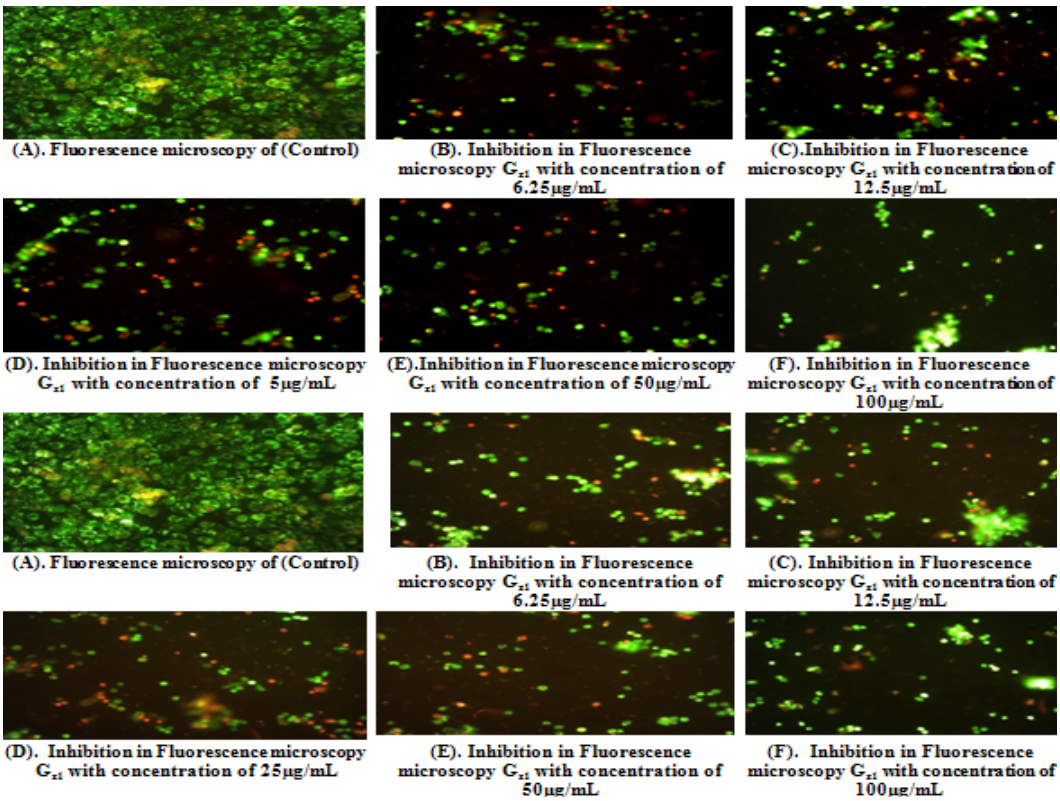



**Fig. 6. Evaluation of apoptosis in MCF-7 cells. Representative acridine (AO/EB) staining in MCF-7 cells treated at $IC_{50}$ concentration of compounds $G_{z1}$ and $G_{z2}$. Green cells represent viable cells and orang cells dead**

## CONCLUSION

The main focus of the current work is the synthesis of new derivatives of pyrazolo[3,4-d] pyrimidinein good yields (79-82%), that have anti-proliferative activity against MCF-7 breast cancer *in vitro*. FT-IR, $^1$H-NMR, GC-Massand C.H.N were the spectral techniques used to confirm the chemical structures of $G_{z1}$ and $G_{z2}$. A hemolysis study demonstrated the safety use within the body of the manufactured compounds. And MTT findings demonstrated the good cytotoxicity of $G_{z1}$ and $G_{z2}$ in the MCF-7 cell line. that was most hazardous to MCF-7 cells was compound $G_{z2}$.The high antioxidant action of $G_{z2}$ against DPPH radicals was validated by antioxidant experiments conducted on synthetic compounds. In conclusions, we have shown that the chemical $G_{z2}$ significantly suppresses cell growth and causes apoptosis and, to a lesser degree, necrosis in breast cancer cells.Which can be applied in the treatment of cancer.

**Highlights:**

- There were novel Pyrazolo[3,4-d]Pyrimidine compounds made.
- Compounds $G_{z1}$ and $G_{z2}$ at a dose of 10 mg/mL shown (5.43% to 5.78%) in vitro hemolysis research activity; this suggests that their use within the body is safe.
- $G_{z2}$ exhibited Antioxidant activity that was comparable to Sorbic acid.
- Tested for their *in vitro* cytotoxic activity (a human breast cancer cell line) was purchased from national cell bank of iran. The best hit was $G_{z2}IC_{50}$= 21.93 µg/mL comparison with doxorubicin $IC_{50}$ = 15.28 µg/mL.
- The apoptotic effect for derivatives Pyrazolo[3,4-d]Pyrimidine in MCF-7 cells was detected using fluorescent staining method.

**Rights of Humanand Animal**

The research that forms the basis of this work did not involve the use of humans or animals.

**Availability of Information and Data**

Confirmation to the authors. The findings of the study, and the data supporting it, are given in the paper and its appendices.

**Finance**

A prominent finding of this research is that no funding was sourced from the public, private or non-profit funding agency for this study.

## ACKNOWLEDGEMENT

The authors would like to thank the college of veterinarymedicine and college of sciences at Al-Muthanna University for their partial support.

**Conflict of interest**

The writers say they have no financial or other conflicts of interest.

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