



Synthesis of New Azo Dyes of Uracil *via* Ecofriendly Method and Evaluation For The Breast, Liver and Lung Cancer Cells *In vitro*

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ABSTRACT

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Uracil derivatives have been the focus of many researchers examining their potential as a cancer-fighting agent. Human breast, liver, and lung cancer cells were used to test the target compounds and compared to cisplatin. There was a wide range of activity in the newly synthesized compounds against breast and lung cancer, but none were effective against liver cancer. Nevertheless, the most effective substances were 4d and 4j, which were quite effective against both kinds of cell lines. It was shown that the 4d compound is more effective against breast cancer than 4a, 4b, and 4c compounds, and 4j showed higher cytotoxic activity than 4e, 4f, and g compounds. We have been told that further research into the toxicity of these potent substances, as well as in-vivo testing, are necessary.

1. Introduction

A class of organic molecules with significant industrial utility is known as the aromatic azo dyes. A vast range of products, including dyes, pigments, food additives, and pharmaceuticals, can be made from it [1, 2]. The vast range of colors, brightness, simplicity, and ease with which these dyes can be manufactured, as well as their decent dyeing performance, have all drawn increasing interest. As a result, lasers and nonlinear optical systems are just one of their many high-tech uses, similar to thermal transfer printing and fuel cells [3], as well as dye-sensitized solar cells and photodynamic treatment.

Furthermore, the medical value of azo dyes is well-established, as are their functions in a number of biological processes, such as the inhibition of protein synthesis, RNA, and DNA production, as well as in the development of cancerous tumors and the fixation of nitrogen [4]. The azo dyes, taken as a whole, are the most diversified class of synthetic colorants.

Aromatic azides are synthesized employing stoichiometric and often ecologically unfriendly catalysts, such as metal salt oxidation of aromatic amino compounds and metal ion reduction of aromatic nitrogen

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compounds [5]. In order to make aromatic azo dyes without using solvents, it was possible to self-catalyze the diazotization of acidic salts of amino compounds, followed by coupling with electron-rich aromatic compounds [6]. For the first time, organic molecules may be synthesized without the use of a solvent, thanks to Grindstone Chemistry [7]. As a result, organic synthesis using grinding at room temperature is a step toward a greener context of chemistry [8-9]. Aim: In this study, we describe a new, solvent-free, three-component synthesis of azo dyes of uracil in a single pot at ambient temperature that is both efficient and environmentally friendly.

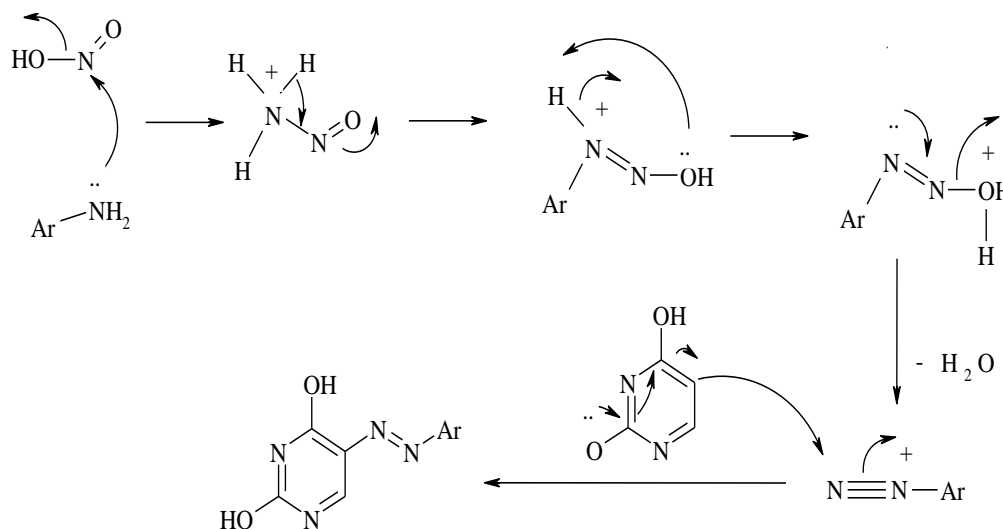
2. Material and methods

Electrothermal IA 9100 apparatus was used to measure all melting points (Shimadzu, Japan). Perkin-Elmer 1650 spectrophotometer (USA) was used to record the infrared spectra (IR) of potassium bromide pellets. Tetramethyl silane was used as an internal standard to calculate the chemical shifts (ppm) of $^1\text{H-NMR}$ spectra in dimethyl sulfoxide (DMSO) on a Varian Mercury 300 MHz NMR spectrometer. Apart from that, EI Ms-QP 1000 (70 eV) was used to record mass spectra (Shimadzu, Japan). Vario, Elementar device was used for microanalyses (Shimadzu). To track the development of all reactions,

iodine vapors, UV light irradiation, or a mobile phase of chloroform-methanol (3:1) was used in Thin Layer Chromatography (TLC) with silica gel 60 for TLC (Merck)(254nm). Scheme I was used to synthesize the target chemical compounds.

It is noteworthy that azo dyes constitute a significant category of industrial dyes that were synthesized through a two-stage process. The initial step involved the diazotization reaction between nitrous acid and an aromatic amine, while the subsequent stage involved the coupling reaction with another electron-rich aromatic system.

The first stage of the procedure comprised the interaction of the aromatic amine with nitrous acid, which produced a highly reactive diazonium cation with an electron-deficient nitrogen atom and an incomplete octet. As a result, the nearest electron-rich aromatic system, in this case uracil, would come under attack and quickly undergo substitution by the diazonium cation. The highly reactive diazonium molecule completed the reaction by coupling with the electron-rich aromatic system, on the other hand, which was extremely susceptible to electrophilic aromatic substitution. As seen in Scheme 1, the azo dye was the final outcome of this reaction.



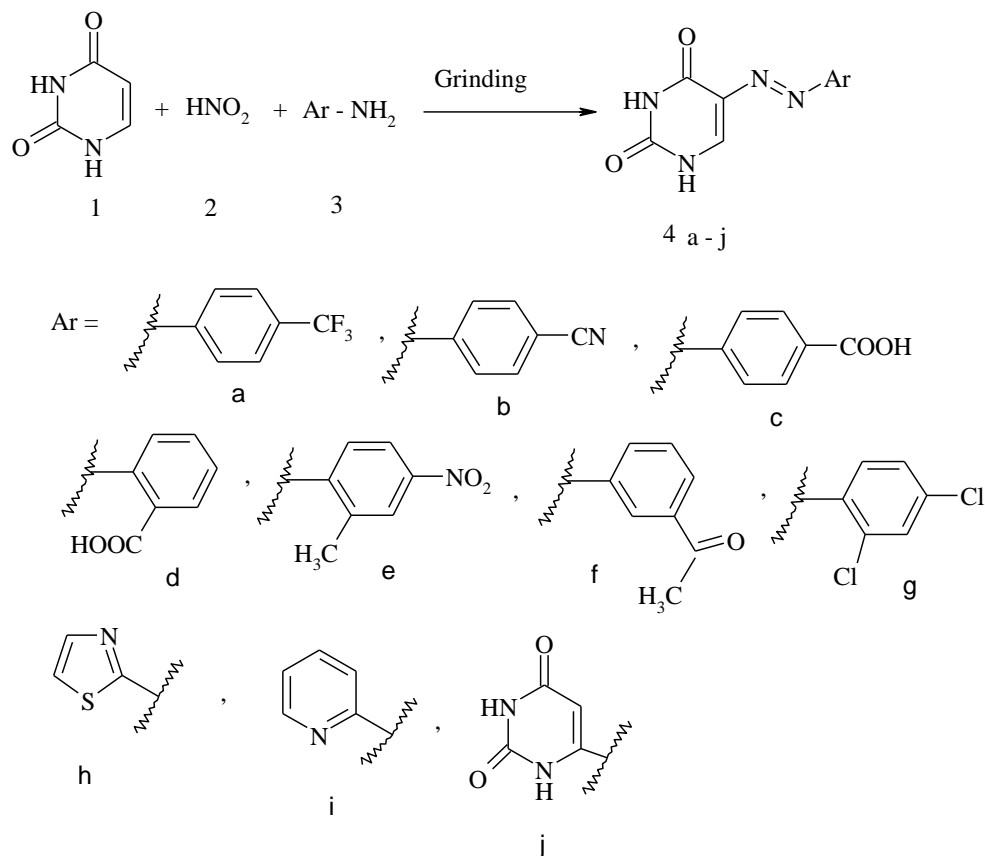
Scheme 1: Speculated Azo Dye Formation Mechanism.

The three components were present in our current method, and the reaction steps were carried out simultaneously via one-pot synthesis. The reactants were first put through a simple, solvent-free synthesis in which they were combined with an agate mortar and pestle. The reaction's progression was watched, and a quick experiment yielded

the azo colors that were produced. Remarkably, the grinding process, in such a way, embedded gentle activation energy in the reaction. This friction energy was adequate activation for such reactions to be accomplished. Correspondingly, it introduced a proficient chance for the three components of the synthesis to be reacted. The

susceptibility of the electron-rich aromatic system was shown to have a substantial impact on how long the grinding process lasted. Moreover, the structural variation that interned to electrophilic substitution governed the reaction completion, hence, the grinding time. When compared to conventional methods, there was generally a

discernible decrease in reaction time, resulting in an efficient green process. The results further supported the environmentally benign character of the synthesis by demonstrating excellent conversion and high yield approaches that improved the experiment's atom economy.



Scheme 2: Synthesized compounds

3. Experimental

3.1. General Procedure for synthesis:

The provided phenoxide salt of uracil 1 and sodium nitrite (0.002 moles) were combined in an equimolar ratio with amino compound hydrochloride acidic salts 3 (0.001 moles) and subjected to solvent-free condition at room temperature using an agate mortar and pestle. Using thin layer chromatography (TLC) and alumina sheets, the reaction's completion was tracked. After being properly cleaned with distilled water, filtered, and dried, the colored paste that had resulted. Then, the produced solid was re-crystallized from DMF/water.

5-[(E)-[4-(trifluoromethyl)phenyl]diazinyl]pyrimidine-2,4(1H,3H)-dione: 4a

Yield: 75%; mp: 270-272°C, Grinding time: 20 min. Color: orange powder, IR (KBr cm⁻¹): 3228 (NH), 3150

(CH, aromatic), 1673,1691, (2C=O), 1480 (N=N), ¹H-NMR (DMSO-d₆), 7.1-7.4,7,6 ppm (4H, *dd*, Ar-H), 9.5,10.2 ppm (2H, *s*, 2NH, exchange with D₂O), ppm (1H,*s*,uracil), MS: m/z (%), 284.19 (M⁺,18.3%), Anal.Calcd., for C₁₁H₇F₃N₄O₂: C, 46.49; H, 2.48; N, 19.71. Found: C,46.22; H,2.47; N,19.66.

4-[(E)-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)diazinyl]benzonitrile: 4b

Yield: 72%; mp: 256-258°C, Grinding time: 22 min. Color: yellow powder, IR (KBr cm⁻¹): 2225 (CN),3245 (NH), 3173 (CH, aromatic), 1675,1681, (2 C=O), 1477 (N=N), ¹H-NMR (DMSO-d₆), 7.2-7.5 ppm (4H,*dd*,Ar-H), 9.6,10.1 ppm (2H,*s*, 2NH, exchange with D₂O), 8.2 ppm (1H, *s*, uracil). MS: m/z (%), 241.20 (M⁺,11.4%), Anal.Calcd., for C₁₁H₇N₅O₂: C, 54.77; H, 2.93; N, 29.03. Found: C,54.62; H,2.97; N,29.11.

4-[(E)-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)diazinyl]benzoic acid: 4c

Yield: 71%; mp: 263-265°C, Grinding time: 24 min. Color: brown powder, IR (KBr cm⁻¹): 3300 (OH), 3255 (NH), 3178 (CH, aromatic), 1674, 1682, 1735 (3C=O), 1479 (N=N), ¹H-NMR (DMSO-d₆), 7.3-7.6 ppm (4H, *dd*, Ar-H), 9.2, 10.3, 11.7 (3H, *s*, 2NH, 1OH, exchange with D₂O), 8.1 ppm (1H, *s*, uracil). MS: *m/z* (%), 260.20 (M⁺, 15.8%), Anal. Calcd., for C₁₁H₈N₄O₄: C, 55.77; H, 3.10; N, 21.53. Found: C, 55.68; H, 3.18; N, 21.61.

2-[(E)-(2,4-dioxo-1,2,3,4-tetrahydropyrimidin-5-yl)diazinyl]benzoic acid: 4d

Yield: 76%; mp: 278-280°C, Grinding time: 22 min. Color: yellowish-brown powder, IR (KBr cm⁻¹): 3305 (OH), 3258 (NH), 3183 (CH, aromatic), 1672, 1678, 1738 (3C=O), 1482 (N=N), ¹H-NMR (DMSO-d₆), 7.2-7.5 ppm (4H, *m*, Ar-H), 9.1, 10.4, 11.8 (3H, *s*, 2NH, 1OH, exchange with D₂O), 8.1 ppm (1H, *s*, uracil). MS: *m/z* (%), 260.20 (M⁺, 15.8%), Anal. Calcd., for C₁₁H₈N₄O₄: C, 55.77; H, 3.10; N, 21.53. Found: C, 55.73; H, 3.28; N, 21.51.

5-[(E)-(2-methyl-4-nitrophenyl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4e

Yield: 67%; mp: 260-262°C, Grinding time: 18 min. Color: red powder, IR (KBr cm⁻¹): 3247 (NH), 3178 (CH, aromatic), 1672, 1683, (2C=O), 1467 (N=N), ¹H-NMR (DMSO-d₆), 7.1-7.3 ppm (3H, *m*, Ar-H), 9.6, 10.1 ppm (2H, *s*, 2NH, exchange with D₂O), 8.2 ppm (1H, *s*, uracil). MS: *m/z* (%), 275.22 (M⁺, 9.7%), Anal. Calcd., for C₁₁H₉N₅O₄: C, 48.01; H, 3.30; N, 25.45. Found: C, 48.12; H, 3.47; N, 25.36.

5-[(E)-(3-acetylphenyl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4f

Yield: 68%; mp: 275-277°C, Grinding time: 16 min. Color: orange powder, IR (KBr cm⁻¹): 3292 (NH), 3178 (CH, aromatic), 1662, 1673, 1715 (3C=O), 1468 (N=N), ¹H-NMR (DMSO-d₆), 2.6(3H, *s*, CH₃CO), 7.2-7.4 ppm (4H, *m*, Ar-H), 9.5, 10.2 ppm (2H, *s*, 2NH, exchange with D₂O), 8.2 ppm (1H, *s*, uracil). MS: *m/z* (%), 258.23 (M⁺, 7.9%), Anal. Calcd., for C₁₂H₁₀N₄O₃: C, 55.81; H, 3.90; N, 22.70. Found: C, 55.72; H, 3.87; N, 22.76.

5-[(E)-(2,4-dichlorophenyl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4g

Yield: 70%; mp: 284-286°C, Grinding time: 15 min. Color: orange red powder, IR (KBr cm⁻¹): 3265 (NH), 3182 (CH, aromatic), 1663, 1670, (2C=O), 1468 (N=N), ¹H-NMR (DMSO-d₆), 7.1-7.3 ppm (3H, *m*, Ar-H), 9.4, 10.3, (2H, *s*, 2NH, exchange with D₂O), 8.2 ppm (1H, *s*, uracil). MS: *m/z* (%), 285.08 (M⁺, 12.9%), 287.07 (M+2, 4.3%) Anal. Calcd., for C₁₀H₆Cl₂N₄O₂: C, 42.13; H, 2.12; N, 19.65. Found: C, 42.22; H, 2.27; N, 19.76.

5-[(E)-5-(1,3-thiazolyl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4h

Yield: 64%; mp: 294-296°C, Grinding time: 20 min. Color: yellow powder, IR (KBr cm⁻¹): 3264 (NH), 3173 (CH, aromatic), 1665, 1672, (2C=O), 1484 (N=N), ¹H-NMR (DMSO-d₆), 7.6-7.9 ppm (3H, *m*, Ar-H), 9.5, 10.4 ppm (2H, *s*, 2NH, exchange with D₂O), 8.1 ppm (1H, *s*, uracil). MS: *m/z* (%), 223.21 (M⁺, 17.2%), Anal. Calcd., for C₇H₅Cl₂N₅O₂S: C, 37.67; H, 2.26; N, 31.38. Found: C, 37.52; H, 2.28; N, 31.46.

5-[(E)-(pyridine-2-yl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4i

Yield: 71%; mp: 288-290°C, Grinding time: 17 min. Color: orange-red powder, IR (KBr cm⁻¹): 3272 (NH), 3186 (CH, aromatic), 1668, 1673, (2C=O), 1481 (N=N), ¹H-NMR (DMSO-d₆), 6.8-7.2 ppm (4H, *m*, Ar-H), 9.3, 10.5 ppm (2H, *s*, 2NH, exchange with D₂O), 8.2 ppm (1H, *s*, uracil). MS: *m/z* (%), 217.18 (M⁺, 13.6%), Anal. Calcd., for C₉H₇N₅O₂: C, 49.77; H, 3.25; N, 32.25. Found: C, 49.82; H, 3.29; N, 32.16.

5-[(E)-(2,6-dioxo-1,2,3,6-tetrahydropyrimidin-4-yl)diazinyl]pyrimidine-2,4(1H,3H)-dione: 4j

Yield: 75%; mp: 295-297°C, Grinding time: 22 min. Color: red powder, IR (KBr cm⁻¹): 3277 (NH), 3187 (CH, aromatic), 1669, 1678, (4C=O), 1481 (N=N), ¹H-NMR (DMSO-d₆), 6.8-7.2 ppm (4H, *m*, Ar-H), 9.2-10.6 ppm (2H, *s*, 4NH, exchange with D₂O), 8.0, 8.1 ppm (2H, *s*, uracil). MS: *m/z* (%), 250.17 (M⁺, 11.6%), Anal. Calcd., for C₈H₆N₆O₄: C, 38.41; H, 2.42; N, 33.59. Found: C, 38.32; H, 2.59; N, 33.46.

3.2. Cultivation of cell lines

Three cancer cell lines, namely HepG2 liver, MCF-7 breast, and A549 lung, were donated by the American Type Culture Collection (ATCC) for the screening of the examined chemicals (Rockville, MD, USA). Tumor cells were cultured in DMEM supplemented with 10% heat-inactivated GIBCO fetal calf serum, 100 units per milliliter of penicillin, and 100 micrograms per milliliter of streptomycin at 37°C, in a humid environment with 5% carbon dioxide. Apart from that, cells were cultured in a 25 cm² flask with 5 mL of rich culture media at a concentration of 0.50 x 10⁶.

3.3. Proliferative test in a cell culture

SulfoRhodamine-B stain (SRB) assay was used to assess the antiproliferative activity *in vitro*, in accordance with the previously described standard protocol [9]. To determine the IC₅₀, a formula was developed, and the results are shown in Table 1.

3.4. Biochemistry assessment

At 37°C and 5% CO₂, the cells were treated for 24 h with 20 L of either the reference medication, cisplatin, or the compounds. Using a tight pestle homogenizer, the cells were extracted and fully killed in saline before being used for subsequent biochemical analysis. In order to determine the following parameters, cell homogenates were centrifuged, and the supernatants were collected. Cell homogenates were used to precipitate and analyze nucleic acids (RNA and DNA), including total protein. [10].

3.5. Statistical analysis

The findings for at least three independent experiments are presented as the mean standard error (SE). One-way ANOVA was used to analyze the statistical differences, and a significance level of $p < 0.05$ was used.

4. Results

4.1. In vitro antiproliferative activity

The measurement of growth inhibitory concentration (IC₅₀) was employed to examine the antiproliferative

activity of novel substances against three cell lines, MCF-7, HepG2, and A549 cell lines (breast, liver, and lung carcinoma, respectively), in comparison to cisplatin as the reference medication. Our finding showed that non-new synthesis compounds have inhibitory activity against HepG2 cells; four of them had minimum inhibitory concentration against the breast cancer cell line MCF-7. Additionally, the human lung cancer's cell line A549 was severely inhibited by four compounds (Table 1, Figure 1). The MCF-7 and A549 cell lines grow normally when we use our culture method. The novel compounds (4a, 4c, 4d, and 4b) had IC₅₀ values for the MCF-7 cell line of 5.40, 9.50, 3.30, and 6.90 g/mL, respectively. In addition, it was demonstrated that compound 4d (IC₅₀: 3.30 g/mL) is more potent and effective than the reference drug, cisplatin (IC₅₀: 4.90 g/mL). Apart from that, the IC₅₀ of the novel compounds (4f, 4e, 4j, and 4g) was 6.10, 7.50, 4.40, and 7.50 g/mL, correspondingly, for the A549 cell line. In contrast to cisplatin, which has an IC₅₀ of 3.85 g/mL, compound 4j has an IC₅₀ of 4.40 g/mL.

Table 1: IC₅₀ activity (µg/mL) of new compounds on three cell lines (MCF-7, HepG2, and A549)

Compounds/cell line	Cell lines		
	MCF-7	HepG2	A549
Control (DMSO)	-	-	-
Cisplatin	4.90 ± 1.20	4.20	3.85
4a	5.40 ± 1.02	-	-
4b	6.90 ± 2.04	-	-
4c	9.50 ± 3.21	-	-
4d	3.30 ± 2.21	-	-
4e	-	-	7.50 ± 1.93
4f	-	-	6.10 ± 1.45
4g	-	-	7.90 ± 2.32
4j	-	-	4.40 ± 2.34

Values are mean ± S.E

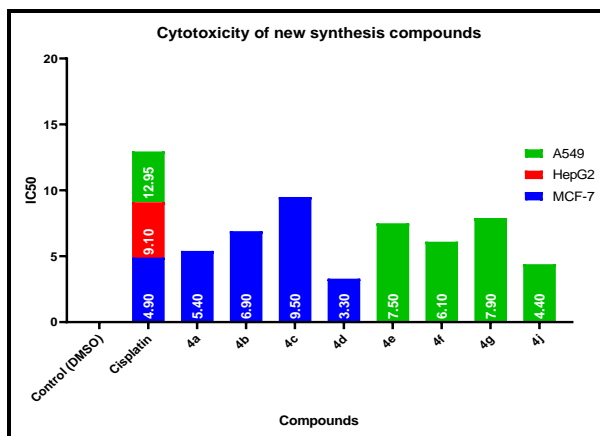


Figure 1: Cytotoxicity of newly synthesized compounds, cisplatin against three cancer cell lines as a reference.

4.2. Evaluating of antioxidants

By measuring the amounts of catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and hydrogen peroxide (H_2O_2) in cancer cells, we were able to determine the antioxidant activity of the examined substances. We also looked into how these drugs affected the quantities of nucleic acids and total proteins. Our findings revealed a significant rise ($p < 0.05$) in SOD activity and H_2O_2 levels, as well as a significant drop ($p < 0.05$) in GSH-Px and CAT activities, as well as GSH levels when compared to the control group, which indicates an increase in cellular levels of ROS. As a result, the excess H_2O_2 generated by the compounds in tumor cells cannot be eliminated. To put it another way, the accumulation of H_2O_2 and other free radicals in tumor cells ought to be a contributing factor in tumor cell death. In the case of the MCF-7 cell line, compound 4d has a higher SOD activity and H_2O_2 levels as compared with cisplatin, 4a, 4b, and 4c, respectively. However, we found 4d has lower CAT and GSH-Px activity, as well as GSH levels, as compared with

cisplatin, 4a, 4b, and 4c, accordingly. Between the newly synthesized compounds and cisplatin, there is no significant difference ($p > 0.05$). However, there is a substantial difference ($p < 0.05$) between the control and newly synthesized compounds. Additionally, we discovered a significant difference ($p < 0.05$) between compounds 4a, 4d, 4b, and 4c. The newly synthesized compounds in the case of the A549 cell line were 4j, 4f, 4e, and 4g, respectively, less potent than cisplatin. Comparing compound 4j to the other compounds under study, it had the highest activity among the novel compounds, the highest H_2O_2 activity, the lowest GSH-Px and CAT activities, the highest SOD activity, as well as the lowest GSH level. Note that the anticancer impact of the current drugs may be at least partially mediated by ROS production, according to the biochemical assay and antiproliferative activity results, which were both consistent (Table 2).

Table 2. Effect of new compounds on the activities of SOD, catalase CAT, GSH-Px, and GSH and hydrogen peroxide (H_2O_2) in MCF-7 and A549 cancer cell lines.

Cell lines	Compounds	H_2O_2 nmol/mg protein	SOD U/mg Protein	CAT U/mg protein	GSH-Px U/mg protein	GSH nmol/mg protein
MCF-7	Control (DMSO)	15.70 ± 1.60	35.00 ± 3.80	7.60 ± 0.70	9.30 ± 1.00	40.00 ± 5.00
	Cisplatin	65.50 ± 7.30 *	135.0 ± 13.80 *	2.96 ± 0.22 *	4.20 ± 0.40 *	18.60 ± 2.00 *
	4a	55.40 ± 5.65 *	130.00 ± 13.30 *	2.60 ± 0.30 *	4.80 ± 0.60 *	22.50 ± 2.60 *
	4b	43.30 ± 4.80 *,#	110.00 ± 12.00 *	3.20 ± 0.28 *	5.20 ± 0.60 *	26.80 ± 2.90 *,#
	4c	37.50 ± 4.00 *,#	85.20 ± 8.60 *,#	5.20 ± 0.50 *	7.20 ± 0.80 *	30.50 ± 3.30 *,#
	4d	77.50 ± 7.80 *	140.0 ± 14.50 *	2.26 ± 0.22 *	3.30 ± 0.38 *	17.80 ± 0.20 *
A549	Control (DMSO)	14.50 ± 1.50	39.70 ± 4.60	8.40 ± 0.80	13.20 ± 1.30	42.60 ± 5.00
	Cisplatin	65.80 ± 6.70 *	220.00 ± 24.00 *	2.30 ± 0.16 *	4.20 ± 0.38 *	13.60 ± 1.70 *
	4e	52.00 ± 5.00 *,#	90.60 ± 8.70 *,#	4.20 ± 0.460 *,#	6.50 ± 0.60 *	20.50 ± 2.20 *,#
	4f	55.20 ± 6.10 *,#	120.00 ± 13.20 *,#	3.85 ± 0.40 *	5.90 ± 0.60 *	18.20 ± 1.90 *
	4g	36.80 ± 3.60 *,#	85.60 ± 8.20 *,#	4.60 ± 0.47 *,#	7.00 ± 0.80 *,#	26.50 ± 2.50 *,#
	4j	60.20 ± 5.80 *	180.00 ± 12.20 *,#	3.30 ± 0.36 *,#	5.20 ± 0.78 *	16.80 ± 1.80 *

Data are expressed in terms of ± S.E. where * and # denotes significant difference between control and cisplatin groups, accordingly at ($p < 0.05$)

5. Discussion

In MCF-7 cells, compound 4d was more powerful than cisplatin, while in A549 cells, compound 4j was more effective than cisplatin. Similar to the widely used

anticancer drug cisplatin, some synthesized compounds exhibit antitumor activity against the human A549 lung cancer cell line and the human MCF-7 breast cancer cell line. These two compounds (4d and 4j) demonstrated significant anticancer activity and raised cellular reactive

oxygen species levels, hydrogen peroxide (H₂O₂) in the cancer cell lines A541 and MCF-7. For this discovery, there are a number of possible interpretations. There are a number of ways in which these compounds may affect the cell's antioxidant systems and hence increase the formation of free radicals, which may lead to apoptosis.

The second point is that researchers who focus on antioxidants understand that many of these substances exhibit paradoxical pro-oxidation or anti-oxidation behaviors as per their concentration, the particular metals' presence or absence, and the co-antioxidant absence or presence under oxidative stress (9, 11). The cisplatin used by Kandemir *et al.* surprisingly suppressed the generation of hydroxyl radicals at doses of 60–70 g/mL despite paradoxically raising it from 1 to 50 g/mL. Furthermore, it is very challenging to measure the whole scope of antioxidant benefit due to the multistage nature of carcinogenesis, as oxidative processes may play a variety of poorly understood functions (12, 13). Free radicals have a crucial role in cisplatin's toxicity, as evidenced by the extensive scholarly literature on the subject and this study (14). As a result, compounds 4d and 4j, in particular, may be less harmful than the standard medication cisplatin. Cisplatin's adverse side effects are due to the drug's pro-radical action (9, 11). Hence, our findings may lead to the development of non-toxic, new active molecules for chemical substances adopted in cancer therapy, having a deeper comprehension of their operating mode.

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