Original Research Article

Synthesis and screening of anticancer potentials of some new terephthaldehyde-derived nitrone compounds

Husam Hamza Salman[1]*, Munther Abduljaleel Mohammed Ali[1], Eman Tariq Ali[2]
[1]Department of Pharmaceutical Chemistry, [2]Department of Laboratories Clinical Science, College of Pharmacy, University of Basrah, Basrah, Iraq

*For correspondence: **Email:** hussam_712003@yahoo.com*

Abstract

Purpose: To synthesize and screen some new nitrone compounds derived from terephthaldehyde for their anticancer potential.
Methods: Six new compounds (H, p-Me,p-Br, p-Cl, o-Cl and m-Me) were synthesized via a condensation reaction between terephthaldehyde and a variety of aryl hydroxylamine compounds derived from nitrobenzene and its derivatives. The chemical structures of these compounds were identified using elemental CHN analysis and were elucidated using Fourier Transform infra-red (FT-IR), ^{1}H-nuclear magnetic resonance (^{1}H NMR), mass spectrometry (MS), and elemental analysis. The anticancer effects of the compounds were screened in vitro with respect to their cytotoxicity on MCF7 human cancer cells line. The IC_{50} values were obtained by MTT assay and their effects on apoptosis of MCF-7 cells were assessed using Acridine orange-ethidium bromide AO/EtBr staining method under a fluorescence microscope.
Results: Only four compounds (2b, 2d, 2e, and 2f) inhibited more than 50 % of the growth of MCF-7 cells. The strongest anti-proliferation effect against MCF-7 cells was exhibited by 2f (m-Me), producing more apoptosis which increased membrane disruption and consistency of lysosome vacuoles; it also exhibited higher cytotoxic effects on human cancer cell lines ($IC_{50} < 7.5$) than the other synthesized compounds.
Conclusion: The new nitrone compounds (2b, 2d, 2e, and 2f) synthesized from terephthaldehyde exhibit some anticancer properties, and so are potential anticancer agents.

Keywords: Terephthaldehyde, Nitrone, Cytotoxicity, Anticancer, MCF-7 cells

INTRODUCTION

Nitrones are 1,3-dipolar compounds which contain the azomethine group (-C=N^{+}-O^{-}) [1] and exert biological and chemical effects. In addition, the azomethine group possesses an oxygen atom bonded to the nitrogen atom (-CH=NO-) by co-ordinate bond [2]. These compounds possess numerous beneficial and pharmacological effects such as anticancer [3], antifungal [4], neuroprotective [5], antibacterial [6] and antioxidant [4] properties. The most effective method used in the synthesis of nitrones involves a condensation reaction between hydroxylamines and carbonyl

compounds. This reaction smoothly proceeds with high yield [7,8].

Cancer is an ailment where numerous anomalous cells grow wildly, paying little mind to the customary standards of cell division [9]. Cancer ranks second after heart disease among diseases that result in the high death rate of patients, Cancer represents about 23 % of all demise cases in the united kingdom with breast cancer MCF-7 being the most wide-spread [10]. The exact cause of cancer has not yet been determined, in spite of the fact that the hereditary viewpoint is engaged with 5 to 10 % of cancer [11]. Different causes incorporate diseases, unhealthiness, weight, absence of physical movement, contamination and tobacco use. These variables influence the movement of biogenes legitimately or by implication that can prompt cancer [11]. The target of the present examination was to investigate the potential anticancer impacts of some new nitrones derived from terephthaldehyde against the MCF-7 cell line.

EXPERIMENTAL

Materials

Terephthaldhyde, absolute ethanol, m-nitrotoluene, p-nitrotoluene, o-chloronitrobenzene, p-bromonitrobenzene and p-chloronitrobenzene were obtained from Sigma-Aldrich Company. Nitrobenzene, ammonium chloride and zinc dust were obtained from BDH Company.

Instruments

The melting points of the synthesized nitrone compounds were determined on the Gallenkimp apparatus, while CHN analysis was carried out on UoroVector model/EA3000A. The FT-IR 8400S SHIMADZU apparatus was used to record the IR spectra using KBr disks. Bruker model ultra-shield 500 MHz (Switzerland) was used to obtain ^{1}H-NMR spectra at Tehran University, Iran. Deuterated dimethyl sulfoxide used as a solvent, with TMS (tetra-methyl silane) as the internal standard.

Synthesis of N-substitutedphenylhydroxyl-amine (1a, 1b, and 1f)

In a 500-mL conical flask, a mixture of the corresponding nitrobenzene (0.205 moles), NH_4Cl (0.235 moles) and water (400 ml) was stirred vigorously. Zinc dust powder (31 g) was added portion-wise for 20 min. The stirring continued for 20 min. The mixture was filtered off

then washed with hot water. Thereafter, to saturate the filtrate, NaCl was added. Then by using an ice-bath for cooling the reaction mixture. The resultant hydroxylamine was filtered and recrystallized from toluene and petroleum ether [12].


Substituted nitrobenzene → 1a-1f Arylhydroxylamine

X= H (1a), p-Me (1b), p-Br (1c), p-Cl (1d), o-Cl (1e), m-Me (1f)
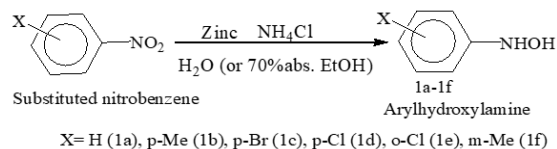
Synthesis of N-substitutedphenylhydroxyl-amine (1c and 1d)

The above method was modified using 70 % ethanol. In 200 ml of 70 % ethanol, NH_4Cl (0.115 moles) and the appropriate halonitrobenzene (0.1 mole) was dissolved. The mixture was stirred with heating at 60 $^{\circ}$C for 45 min. Thereafter, the mixture was saturated with NaCl then purified while hot. Desired hydroxylamine was filtered and recrystallized from toluene and petroleum ether[12].

Synthesis of nitrones (2a-2d and 2f)

A mixture of terephthaldehyde (0.02 moles) and arylhydroxylamine i.e. **1a-1d** and **1f** (0.081 moles) in 20 ml of absolute EtOH was stirred overnight. The desired nitrone was filtered and purified from ethanol[13]**.** The procedures used for the synthesis of nitrones are summarized in Table 1.

X, X'= H (2a), p-Me (2b), p-Br (2c), p-Cl (2d), o-Cl (2e), m-Me (2f)
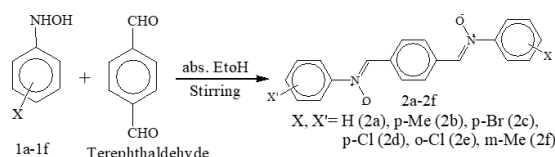
Synthesis of nitrone (2e)

To avoid decomposition of N-(o-chlorophenyl) hydroxylamine, the previous procedure was modified as follows: A mixture of o-chloronitrobenzene (0.036 moles) and ammonium chloride (0.046 moles) in 25 ml of 60 % ethanol was stirred at 10 $^{\circ}$C. While stirring at 10 $^{\circ}$C, 0.065 mol of zinc dust was added portion-wise over a period of 1h. The precipitate formed was filtered and washed three times with 8 ml of boiling ethanol, and 0.018 moles of terephthaldehyde were added to the alcoholic filtrate. At room temperature, the mixture was stirred for 20 hour, next the precipitated nitrone was filtered and recrystallized from absolute ethanol.

Reagents and chemicals

The breast cancer cell line (MCF-7) was gotten from the Iraqi Center of Cancer and Medical Genetics Research (ICCMGR). RPMI-1640 medium was purchased from Gibco (USA). Sodium salicylate was purchased from Fluka Company (Switzerland), while Phosphate buffer saline was provided by OXOID (England). Furthermore, crystal violet stain, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT).

Acridine orange (AO), fetal bovine serum, trypsin-EDTA, ethidium bromide (EB) and dimethyl sulfoxide (DMSO) were given from Sigma-Aldrich (St Louis, MO, USA). Kapa Biosystems, Inc. BioSource International (Belgium) provided a penicillin-streptomycin solution. All chemicals applied were of the diagnostic evaluation in this examination. All solutions and buffers were set up as substantive previously[14].

Determination of anticancer activity

Cell viability

The effects of the six chemical compounds (H, p-Me, p-Br, p-Cl, o-Cl, and m-Me) on the viability of MCF-7 cell line were evaluated using SUNRICE Tecan absorbance reader (Schooler) for measuring the absorbance at 620 nm. The growth medium was decanted and the cell culture was washed once with 2 ml trypsin-versene solution.

Approximately 2-3 ml of trypsin-Bersin was added to the cell culture, then culture flask was rocked gently, then the culture was incubated at 37 °C until the cells separated from the flask. The cells were dispensed in a 5ml growth medium and redistributed at the required density into the culture. The cells were brooded in a humidified climate containing 5% CO_2 at 37 °C [14].

MTT assay

This examine was finished by a prior qualified technique [15]. The cells were seeded in 96-well microtiter plates with RPMI medium at a concentration of 1×10^5 cells/ml, then were incubated overnight for attachment. At that point, (100 µg/mL) from each compound were added in triplicate to the wells at various concentrations (25, 50, 100, 200, and 300 µg/mL), followed by incubation for 72 hr. Thereafter, MTT was added to the cells at a concentration of 2 µg/mL, followed by further incubation for 3 hr. Next the

incubation time, the MTT was aspirated from the wells then supplanted with DMSO to break up the formazan crystals formed. The cells were additionally incubated for 15 min before deciding the absorbance of each well at 620 nm. The percentage of cytotoxicity was determined using Eq 1.

$$H (\%) = \{(A - B)/A\}100 \ldots\ldots\ldots (1)$$

where **A** and **B** are the absorbance of the control, and test samples, respectively.

Acridine orange-ethidium bromide (AO/EtBr) double recolouring

The capability of the compounds to stimulate the death of the MCF-7 cells was tried utilizing the AO/EtBr recolouring method [16]. The cells were seeded into 96-well microtiter plates in RPMI medium at a concentration of 1×10^5 cells/ml and were incubated overnight for attachment. Then, the cells were incubated by (100 µg/mL) with each of (p-Me, p-Cl, o-Cl and m-Me) for 24 h, followed by washing using PBS. Thereafter, 100 µL of dual fluorescent dye was added to the wells prior to the visualization of the cells under a fluorescence microscope.

Determination of IC_{50}

The concentration of compounds which produced 50 % inhibition of cell development (IC_{50}) was resolved from cell survival (%) versus drug concentration (µg) curves. yielding an equation used to compute the concentration of substance required to produce a half decrease in cell viability (IC_{50}) as past outlined [17,18].

Statistical analysis

Both unpaired *t*-tests with Graph Pad Prism 6 were used statistically analyzed the data obtained. The values are introduced presented as mean ± standard deviation (SD) of triplicate estimations. Both Bonferroni's post-test and One-way ANOVA were utilized for factual investigation [19]. Analysis of the non-linear regression curve for IC_{50} determination was done with Graph Pad PRISM version 6 (Graph Pad Software, Inc., La Jolla, CA, USA)

RESULTS

The results of the mass spectral characterization of the synthesized compounds are listed in Table 1, along with other physical data.

Table 1: Physical data of the synthesized nitrones

Compound	X, X'	Name	Mol. formula	m.p. (°C)	Yield (%)
2a	H	1,4-phenylene-bis(*N*-phenylmethanimine*N*-oxide)	$C_{20}H_{16}N_2O_2$	261-263	94
2b	p-Me	1,4-phenylene-bis[*N*-(4-methylphenyl)methanimine*N*-oxide]	$C_{22}H_{20}N_2O_2$	267-268	86
2c	p-Br	1,4-phenylene-bis[*N*-(4-bromophenyl)methanimine*N*-oxide]	$C_{20}H_{14}Br_2N_2O_2$	278-280	75
2d	p-Cl	1,4-phenylene-bis[*N*-(4-chlorophenyl)methanimine*N*-oxide]	$C_{20}H_{14}Cl_2N_2O_2$	251-252	78
2e	o-Cl	1,4-phenylene-bis[*N*-(2-chlorophenyl)methanimine*N*-oxide]	$C_{20}H_{14}Cl_2N_2O_2$	217-219	68
2f	m-Me	1,4-phenylene-bis[*N*-(3-methylphenyl)methanimine*N*-oxide]	$C_{22}H_{20}N_2O_2$	214-216	89

Table 2 shows the elemental (CHN) data for the synthesized nitrones (**2a-2d**). The outcome supports the framework of the nitrone compounds.

Table 2: Elemental (CHN) analysis of nitrones (2a-2f)

Compound	Calculated (%)			Found (%)		
	C	*H*	*N*	*C*	*H*	*N*
2a	75.93	5.10	8.86	77.13	5.42	8.66
2b	76.72	5.85	8.13	76.88	5.81	8.28
2c	50.66	2.98	5.91	50.88	3.19	5.93
2d	62.35	3.66	7.27	62.65	3.87	7.53
2e	62.35	3.66	7.27	62.88	4.16	7.67
2f	76.72	5.85	8.13	77.20	5.52	8.01

FT-IR spectra

The medium absorption band observed at (1673 - 1593 cm^{-1}) for all nitrone compounds (Table3) was due to the stretching vibration of the C=N group. The FT-IR spectra of nitrones showing a very strong band at regions 1180-1215 cm^{-1} and 1068 - 1095 cm^{-1} can are due to the stretching frequencies of C-N and N-O bonds, respectively.

Stretching frequencies of the aromatic C-H occurred at 3047 - 3128 cm^{-1}. Out-of-plane bending (o.o.p bend) and in-plane bending (in. pl. bend) of aromatic C-H appeared at regions 837 - 860 cm^{-1} and 1393 - 1420 cm^{-1}, respectively. All infrared spectra of nitrones showed forceful bands at 1454 - 1481 cm^{-1} and at 1500 - 1558 cm^{-1} due to the stretching frequencies of the aromatic C=C group. Nitrones **2b** and **2f** showed weak bands for aliphatic C-H bond at 2920 and 2924 cm^{-1}, respectively [20,21].

^{1}H-NMR spectra

The synthesized nitrones exhibited single signals at 8.218-8.621 ppm which are attributable to protons H-13 and H-13'. A singlet signal for all spectra of synthesized compounds appeared at 8.481-8.561 ppm, due to the equivalent protons H-8, H-9, H-11, and H-12. On the other hand, nitrones **2a** and **2e** exhibited multiple signals at the ranges 7.545-7.952 ppm and 7.515-7.776 ppm, respectively.

In the case of compounds **2b** and **2f,** their ^{1}H-NMR spectra showed a singlet signal for the methyl group at 2.395 ppm and 2.417 ppm, respectively. In the other compounds (**2a-2c**), the ^{1}H-NMR spectra exhibited doublet signals for equivalent protons (H-3,5 and H-3',5') and equivalent protons (H-2,6 H2',6')[20-22], as shown in Table 4. The signals of a the ^{1}H-NMR spectrum of compound **2f** are summed up in Table 4.

Table 3: FT-IR (cm^{-1}) spectral data for nitrones (2a-2f)

Compound	C=N	C=C	C-N	N-O	C-H aromatic			C-H aliphatic
					Str.	*o.o.p.bend.*	*in-pl..*	
2a	1589	1485 1458 1558	1192	1068	3093 3051	856 763	1404	-
2b	1593	1454 1500 1554	1192	1068	3053	856 767	1415	2920
2c	1581	1477 1550	1192	1072	3094	837 706	1416	-
2d	1589	1481 1554	1180	1072	3093 3057	833 705	1420	-
2e	1573	1496 1469	1195	1095	3047	860 756	1408	-
2f	1585	1481 1462 1554	1215	1068	3128 3074	845 779	1393	2924

Str: stretching, O.O.P: Out of plane, in –pl. bend: in-plane bending

Table 4: ^{1}H-NMR spectra data for compounds **2a-2f**

Compound	x, x'	^{1}H-NMR, δ (ppm), ^{n}J H-H (Hz) 2H (13, 13')	4H (8,9,11,12)	6H 2CH$_3$	(C-H) aromatic
2a	H	8.587	8.561	-----	7.545-7.952(m, 10 H, 2-6, 2'-6')
2b	p-Me	8.549	8.539	2.395	7.836(d, 4H, 2, 6, 2', 6', ^{3}J= 8.5) 7.364(d, 4H, 3, 5, 3', 5', ^{3}J= 8)
2c	p-Br	8.621	8.550	-----	7.928(d, 4H, 3, 5, 3', 5', ^{3}J= 9) 7.778(d, 4H, 2, 6, 2', 6', ^{3}J= 9)
2d	p-Cl	8.617	8.551	-----	7.997(d, 4H, 3, 5, 3', 5', ^{3}J= 8.5) 7.645(d, 4H, 2, 6, 2', 6', ^{3}J= 9)
2e	o-Cl	8.218	8.481	-----	7.515-7.776(m, 8H, 2-5, 2'-5') 7.767(s, 2H, 6, 6')
2f	m-Me	8.550	8.550	2.417	7.440(t, 2H, 3, 3', ^{3}J= 7.5) 7.723(d, 2H, 2, 2', ^{3}J= 8) 7.352(d, 2H, 4, 4', ^{3}J= 7.5)

Effect of 2a-2f on cell viability of (MCF-7) cell line

At first, the cytotoxic effects of the six compounds (H, p-Me, p-Br, p-Cl, o-Cl and m-Me) on the viability of the (MCF-7) cell line for 72 h was investigated. The outcome appeared significant suppression of cell propagation after 72 h, there was a decrease in cytotoxicity on MCF-7 cell line when handling 2e (o-Cl) at a concentration of 100 µg/mL, while the cells treated with both bands exhibited moderate cytotoxic impact on the cells at the same concentration. Nitrone **2f,** at the grouping of 100 µg/mL killed > 57.53 % of the cells.

Conversely, neither compound **2a** nor compound **2c** was able to induce inhibition of cell growth in MCF-7 cell line after 72 h at the concentration of 100 µg/ml used in the assay (Figure 1). Consequently, these compounds were not used in subsequent studies.
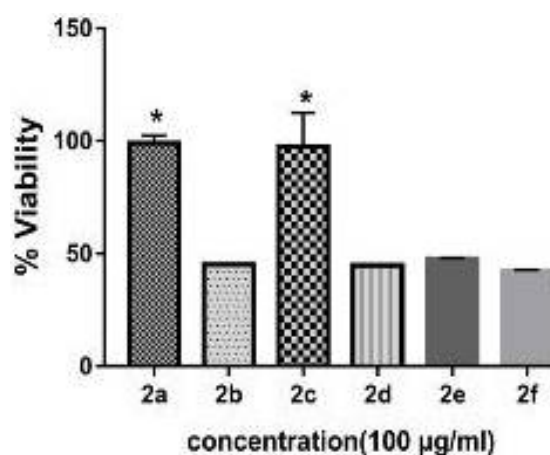

Figure 1: Effect of compounds 2a-2f on cell viability of MCF-7 cells. 2b: cells treated with p-Mel; **2d:** treated with p-Cl; **2e:** treated with o-Cl, and **2f:** cells treated with m-Me. Each nitrone was used at a concentration of 100 µg/ml; *$p < 0.0001$ (one-way ANOVA and Bonferroni's post-test)

Cytotoxic effect of the compounds on MCF-7 cells

The cytotoxic impacts of p-Me, p-Cl, o-Cl and m-Me on the feasibility of (MCF-7) cell line were studied for 72 h. Only 4 of these compounds were able to inhibit more than 50 % of the growth of MCF-7 cells. Two compounds (**2b** and **2f**) exhibited high anti-proliferative effects on cells. The IC_{50} values for were 7.298 µg for **2b** (p-Me), 8.960 µg for **2d** (o-Cl), and 10.74 and 7.5 µg, respectively for **2e** and **2f**. The strongest anti-proliferation effects on MCF-7 cells lines were produced by **2b** and **2f** (IC_{50} of 7.298 and 7.5 µg, respectively). A table 4 showing the above results.
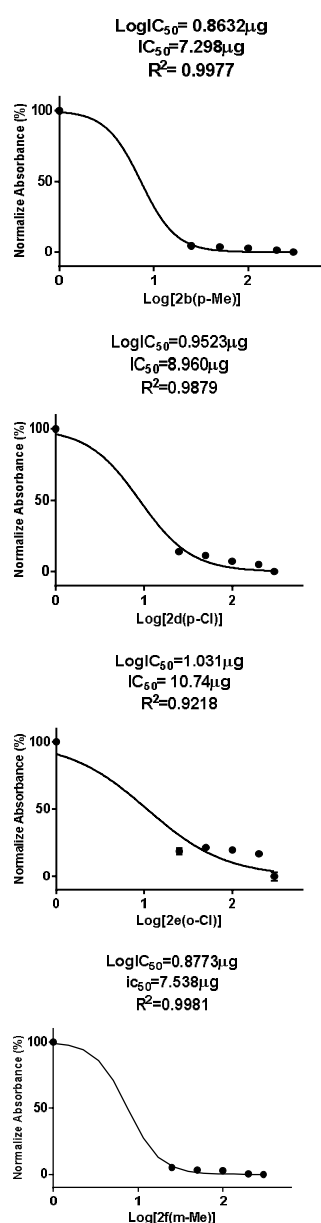
for determinations of IC_{50} values which depicted the cytotoxic effects of the compounds. Each data point is the mean of triplicate determinations.
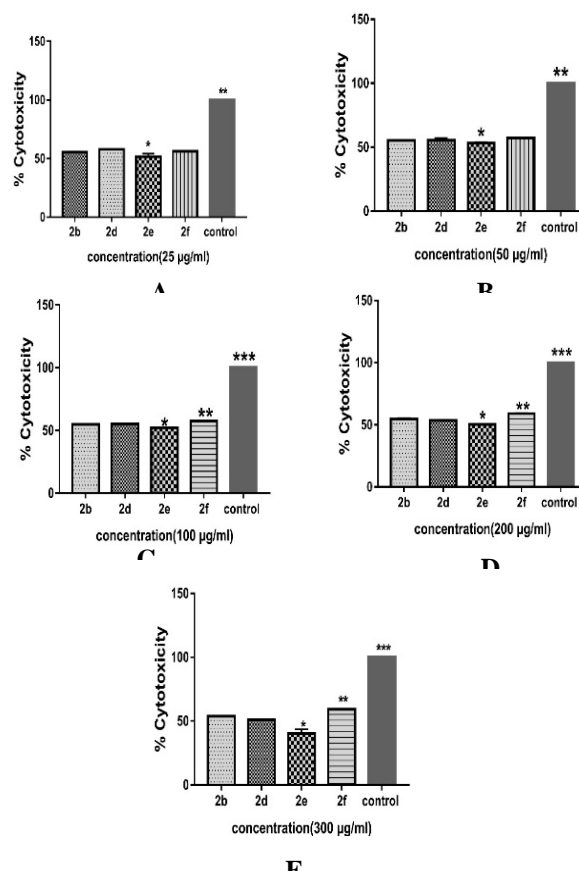

Figure 3: Comparison of degrees of inhibition of MCF-7 cells amongst the various nitrones at different concentrations (25, 50, 100, 200 and 300 µg/mL). **(2b)** cells treated with p-Mel, **(2d)** cells treated with p-Cl, **(2e)** cells treated with o-Cl, and **(2f)** cells treated with m-Me at different concentrations. Each data point represents mean ± SD; *p < 0.01 (one-way ANOVA and Bonferroni's post-test)

The results in Figure 5, Table 5 were revealed that the ability of these compounds to inhibit MCF-7 proliferation was more than 50 % at all concentrations tested. Bar chart E shows that the compound **2f** had the highest inhibition (59.27 %) at (300 µg/ml) concentration when compared to the other compounds and MCF-7 untreated cells (p < 0.0001).

Morphological changes in MCF-7 cell nucleus

AO/EtBr recolouring technique was utilized to study the changes in MCF-7 nuclear morphology after treatment with the different compounds (**2b, 2d, 2e** and **2f**). This method is applied to recolour explicit pieces of the cell and to decide the particular apoptotic markers of atomic alterations.

Figure 2: IC_{50} values of compounds 2b, 2d, 2e, and 2f, as obtained from MTT assay. The curves were used

Table 5: Comparison of compounds in the degree of inhibition of MCF-7cell growth

Conc. (µg/ml)	Nitron compound					$P \leq$	2b vs2d	2b vs2e	2b vs2f	2d vs 2e	2e vs 2f
	2b	**2d**	**2e**	**2f**	**Control**						
25	55.57	57.93	51.52	56.17	100	0.0001	Ns	0.01	Ns	0.0005	0.005
50	55.23	55.52	53.17	57.17	100	0.0001	Ns	0.04	Ns	0.02	0.0006
100	54.77	55.17	52.13	57.53	100	0.0001	Ns	0.0001	0.0001	0.0001	0.0001
200	54.47	53.45	50.27	58.93	100	0.0001	Ns	0.0001	0.0001	0.0001	0.0001
300	53.7	51.03	40.4	59.27	100	0.0001	Ns	0.0001	0.004	0.0001	0.0001

As shown in Figures (6), all of the nitrones compounds, caused cell death but the **2f** (m-Me) compound produces the most apoptosis when exposure of MCF-7 cells to the compounds led to increased membrane disruption and the formation of lysosome vacuoles, as As shown in (Figure 4) when compared to untreated control cells (A, B, C, and D).
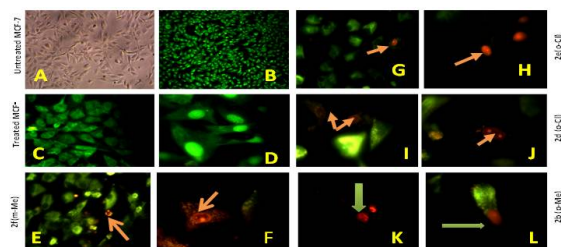

Figure 4: A comparison of the level of apoptosis following treatment with the different compounds. This figure explains the impact of nitrone compounds on apoptosis of MCF-7 cell line by using fluorescence microscop after 24 h of treatment compared to untreatedMCF-7 cells. 2f was the most compound produced more apoptosis. (A) 10x fluorescence image of untreated, unstained control; (B) x10, (C) x40 and (D) x100 fluorescence images of untreated control stained with AO/EtBr. (E) x40 image showing apoptotic cells recolored orange, while the non-apoptotic and viable cells were stained green (arrowed). (F) x100 fluorescence image of MC7 cells treated with 2f compound, showing stain in certain parts of the cell indicating special apoptotic signs of nuclear alterations. (G) x40 and (H) x100 showing stain in specific parts of the cell after treated with 2e (o-Cl) compound. Also (I) x40 and (J) x100 when treated with 2d (p-Cl) compound showing stain in specific parts of the cell. Moreover (K) x40, (L) x100 image indicating special apoptotic signs of nuclear alterations when treated with 2b (p-Me) compound

DISCUSSION

Generally, the outcome exhibit differences in anti-proliferative, apoptosis and cytotoxic effects amongst the tested compounds. The present study confirmed through the results that these nitrone compounds synthesized from terephthaldehyde (2b, 2d, 2e, and 2f) are effective and have the capability for inhibiting the growth of breast cancer MCF-7 cell by 50 %, but there was variance in the degree of inhibition.

More interesting, the study confirmed that nitrone compounds synthesized are new of their kind and there are no previous studies to indicate and support our results. It was found that the potency of cell growth inhibition correlated with the position of the electron-withdrawing nitro group on the tested compounds.

Figures 2 shows that all of the nitrone compounds caused cell death. These results proved that the compounds **2b, 2d, 2e** and **2f** are potential sources of efficacious and cytotoxic materials. In any case, the 2f nitrone compounds were discovered the best exacerbates that delivered more apoptosis when contrasted with the other. This compound showed high cytotoxicity through restraint of MCF-7 multiplication, relative to different mixes and untreated MCF-7 cells. Furthermore, the study assumed the effects of **2f** and **2b** might be due to the existence of the methyl group at 4-and 3-positions, which may increase their lipophilicity. This is most likely because of the presence of the azomethine group (-CH=N-) which possesses biological and chemical properties. Moreover, the azomethine group possesses an oxygen atom bonded. to the nitrogen atom (-CH=NO-) by co-ordinate bond [2].

These chemical groups are important due to their anticancer effects. Through the results, it turns out that the most significant inhibition at 300 µg/ml produced by **2f** compound, showing stain in certain parts of the cell indicating specials apoptotic signs of nuclear alterations. The study assumed the mechanism action of this compound when the introduction of compounds to the cells prompted expanded layer disturbance and the development of lysosome vacuoles when contrasted with untreated control cells (Figure 5). This might be because of their capacity to enter the cell layer and influence capability to penetrate the cell membrane and affect the RNA levels of *p53, Bax, bcl-2, caspase-3, and caspase-9* [23,24].

CONCLUSION

The new nitrones compounds (**2b, 2d, 2e,** and **2f**) synthesized from terephthaldehyde exhibit

some anticancer properties, and so are potential anticancer agents. The strongest anti-proliferation effect against MCF-7 cells was exhibited by **2f** (m-Me) which produced more apoptosis than the other compounds. Thus, **2f** possesses the potential for further development into an effective anticancer and antioxidant agent.

DECLARATIONS

Acknowledgement

Conflict of interest

The authors declare that no conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Husam Hamza was project administrator. Husam Hamza and Munther Abduljaleel took part in preparation and identification of the nitrone compounds. Husam Hamza and Eman Tariq Ali conceived and designed the study. Husam Hamza and Eman Tariq Ali performed the experimental work. Husam Hamza and Eman Tariq carried out data collation and statistical analyses. Husam Hamza and Eman Tariq prepared the original draft of the manuscript and did review and editing.

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