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Synthesis of a New Isoxazolidine and Evaluation Anticancer Activity against MCF-7 Breast Cancer Cell Line

Wijdan Abbas Eneama, Husam Hamza Salman, Mazin Nadhim Mousa

Department of Pharmaceutical chemistry, college of pharmacy, university of Basrah, Iraq

Abstract: In the United States, breast cancer is the most common cancer among women, the second leading cause of cancer-related deaths., and a major contributor to premature mortality as indicated by the average and total number of years of life lost. According to the American Cancer Society, there will be 43,250 fatalities and 287,850 new instances of invasive breast cancer among American women in 2022(1). Heterocyclic compounds containing nitrogen and sulfur constitute over 75% of FDA-approved medications, suggesting the significance of these compounds in the development of drugs(2). of them, heterocyclic compounds containing nitrogen and oxygen, particularly isoxazolidine derivatives, have attracted attention due to their supporting antitumor efficaciousness(3). Several new isoxazolidine derivatives were produced. The MTT assay was used to evaluate for anticancer activity against human cancer cell lines such as MCF-7 and HdFn, as well as normal cells. Structures of Isoxazolidines were defined by FT-IR, 13C-NMR, 1H-NMR, and E-I mass spectroscopy were used to prove the structures of the formed compounds. The IC50 values of the synthesized compounds indicate that compound (IZ2) has a much higher IC50 value in MCF-7 cells than HdFn. Isoxazolidine derivatives bearing a p-Nitro and an m-Nitro aromatic substituent at the isoxazolidine ring showed considerable antitumor activities in Mcf-7 cell lines with IC50 values ranging from 23 µg/ml to 153 µg/ml.

Key points: Isoxazolidines, breast cancer.

1- Introduction

Isoxazolidines are a saturated five-membered heterocyclic ring having oxygen and nitrogen atoms close to each other (4). This ring can be found in a wide range of organic products., characterized as important biological agents, and plays an important part largely in the preparation of nucleosides analogues and nucleotides that are used as antiviral, antibacterial, antifungal, antioxidant, ant-mycobacterial and anticancer agents in medicinal applications(5–9). Thus, Isoxazolidine's cyclic credit can be good for Ribose, in general, plays a critical role in organic chemistry. The most useful method to construct the isoxazolidine ring is the intra and intermolecular cycloaddition of Nitrones (dipoles) with olefins (dipolarophile). The method is called 1,3-DC reactions. Despite the interest shown in Isoxazolidines. This research will concern the preparation of some isoxazolidine compounds and identifying their structures by known spectral methods(10–13). Different types of spectroscopy (FT-IR, 1H-NMR, 13C-NMR, and MS) were used.

Breast cancer is one of the most frequent malignant neoplasms in women around the world, and metastasis is the primary cause of cancer death. Breast cancer manifests itself as a tumour when breast cells proliferate uncontrollably(14). Breast carcinoma is the most prevalent malignant tumours in Iraqi women. It will account for 34.01% of all female malignant cases in 2021, according to the cancer registry division of the Iraqi Cancer Board in Baghdad/Ministry of Health (15).

2-Materials and methods:

All chemicals utilized were of a 99% to 99.9% purity and were bought from BDH or Sigma Aldrich Companies. TLC maintains a follow of the reactions' progress, and the plates are visualized by iodine and UV light ((λ=254 nm). The uncorrected melting points were estimated using electrothermal analysis—open capillary tubes on the SMP30 melting point equipment. FT-IR spectrophotometer (SHIMADZU 8100s / Japan) was used to record the infrared spectra of compounds as KBr discs ranging from 4000-400 cm^{-1}. ^{1}H-NMR spectra were taken at Bruker 400MHz Avance III in deuterated DMSO-d6 (δ=2.50 ppm). The Bruker apparatus was used to record the 125 MHz ^{13}C-NMR spectra—EI-mass spectra of the synthesized Isoxazolidines measured at the chemistry faculty at Tehran University.

2.1. General Method of Synthesis of Maleanilinic Acids (M1, M2 and M3):

In a round bottom flask (100 ml), 8 ml of ethyl acetate was mixed with 6 mmol of maleic anhydride and 6 mmol of aniline derivative in separate beakers. The maleic anhydride solution was mixed into the aniline derivative solution with an extra 5 mL of ethyl acetate and stirred for about 5 minutes. Maleanilinic acid has been obtained without further purification(16–18). (Table 1).

Table 1: Physical properties of maleanilinic acid and components.

Compd.	X	The name	M. p. (ºC)	Yield (%)	Appearance
M1	**3-Cl**	4-((3-chloromethyl)phenyl)amino)-4-exobut-2-anoic acid	194-196	75	White powder
M2	**3-NO_2**	4-((3-nitrophe)amino)-4-exobut-2-anoic acid	203-205	83	Yellow powder
M3	**4-NO_2**	4-((4-nitrophe)amino)-4-exobut-2-anoic acid	196-198	80	Dark Green powder

2.2 Synthesis of *N*-Substituted phenyl maleimide (MD1, MD2, and MD3):

5.85 mmol of maleanilinic acid (**M1, M2, or M3**), 1.83 mmol of anhydrous sodium acetate, and 31.7 mmol of acetic anhydride were combined to produce a suspension in a 100 ml flask with a round bottom. The reaction mixture was maintained between 60 and 70 °C while being shaken. The reaction mixture was completed by adding 100 cc of cold water. The product was collected by vacuum filtering and re-crystallized in ethanol (19).

Table 2: Physical properties of Maleimide derivatives

Compd.	**X**	**The name**	**M. P. (ºC)**	**Yield (%)**	**Appearance**
MD1	3-Cl	1-(3-chlorophe)-1H-pyrrole-2,5-dion	88-90	70	White
MD2	3-NO_2	1-(3-nitrophe)-1H-pyrrole-2,5-dion	122-124	68	Light yellow
MD3	4-NO_2	1-(4-nitrophe)-1H-pyrrole-2,5-dion	169-171	73	Light green

2.3 Synthesis of N-Substituted Phenyl hydroxylamine (H1 and H2):

In an Erlenmeyer flask (250 ml), nitrobenzene or 4-chloronitrobenzene (40 mmol), ammonium chloride (46 mmol), and water (100 ml) were mixed and vigorously agitated for 1 hour. Slowly adding 90 mmoles of zinc dust to the mixture while it was being stirred very well raised the temperature to 65–70 °C. The stirring continued for a further 15 minutes(20). The hot mixture was filtered, and the filtrate was NaCl-saturated. An ice-salt water mixture was used to cool the saturated solution. The required *N*-phenyl hydroxylamine (H1) was obtained by vacuum suctioning and re-crystallizing from a toluene and petroleum ether mixture (M. P. = 81–83, yield = 90%). In the case of *N*-(4-Clorophenyl) hydroxylamine (**H2**) (M. P. = 88–89, yield 75%), methanol and water (1:3) mixture was used instead of water(20–22).

2.4 General Procedure of Synthesis of Nitrones (NT1-NT4):

An ethanolic solution (15 ml) containing a suitable aldehyde (10 mmol) was introduced into a 100 ml roundbottom flask, along with a stirring ethanoic solution (15 ml) containing an appropriate hydroxylamine (10 mmol). The mixture was agitated at ambient temperature for 24 hours. The nitrone required for the experiment was obtained through the application of suction. Subsequently, the substance was re-crystallized using 100% ethanol(23–25)... **Table 3: Data for the synthesized Nitrones.**

Compd.	X1	X2	The name	M. P. (°C)	Yield (%)	Appearance
NT1	H	4-NO_2	1-(4-nitrophenyl)-N-phenylmethanimine oxide	187-189	72	Yellow
NT2	4-Cl	4-NO_2	N-(4-chlorophenyl)-1-(4-nitrophenyl)methanimine oxide	197-199	68	Yellow
NT3	H	4-CH_3O	1-(4-methoxyphenyl)-N-phenylmethanimine oxide	116-119	70	White
NT3	4-Cl	4-CH_3O	N-(4-chlorophenyl)-1-(4-methoxyphenyl)methanimine oxide	165-167	74	White

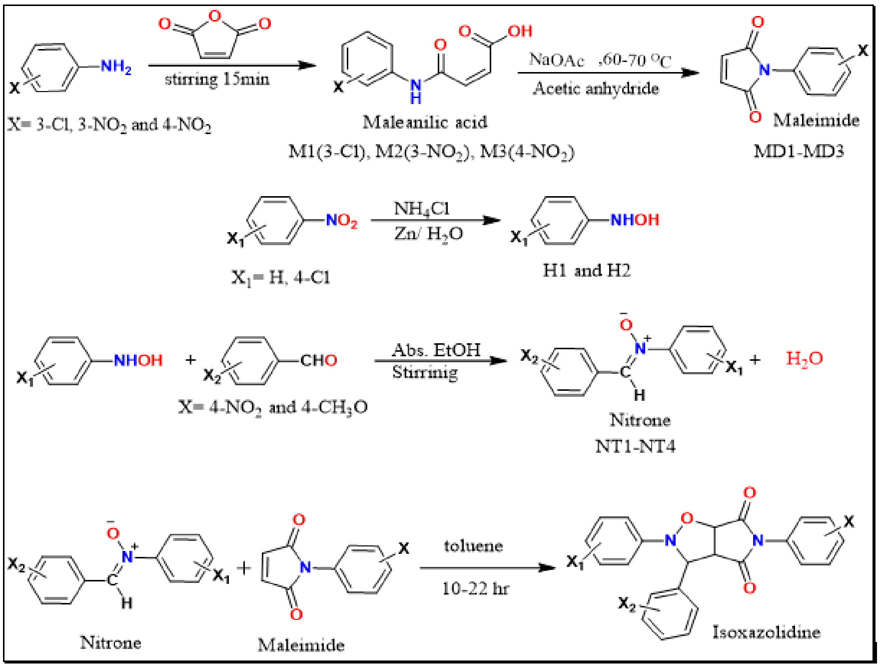
2.5 General procedure of synthesis of Isoxazolidines (IZ1-IZ4).

In a round-bottom flask, 100 ml, Equimolar amounts of nitrone (**N1-N4**) and Maleimide (**M1-M3**) were refluxed in toluene for the appropriate time (Table 2-6). TLC was used to monitor the reaction (hexane: ethyl acetate: 2:1). After the reaction product cooled down, Isoxazolidines were filtered and re-crystallized with toluene(26–29).

Table 4: data of isoxazolidine derivatives

Compd.	X	X1	X2	Time (hr.)	M. P. (°C)	R_f	Yield (%)	Appearance
IZ1	3-Cl	H	4-NO_2	11	238-240	0.5	58	yellow
IZ2	3-NO_2	H	4-NO_2	13	240-242	0.7	52	white
IZ3	3-Cl	H	4-OCH_3	10	192-194	0.6	58	yellow
IZ4	3-Cl	4-Cl	4-NO_2	14	199-201	0.7	55	yellow

Scheme 1: general synthesis of Isoxazolidines



2.5.1: 5-{3-Chloro-ph}-3-{4-Nitro-ph}-2-Ph-tetraahydro-4*H* –Pyrolo {3,4-D} Isoxazol-4,6-{5H}-Dion *(IZ$_1$):*

Yield: 58%, mp. 238-240°C. IR (KBr): ν 1350 (-N-O) (-C =C)1489, 1726 (-C=O), 2976(-C-H), 3076 cm-^{1} (Ar-H)). ^{1}H NMR{400 MHz, DMSO} δ~ 8.33 – 8.25 (m:2H), 7.89 – 7.83 (m:2H), 7.51 – 7.39 (m:2H), 7.28 (*J* = 6.7 Hz, d, 4H), 6.99 (*J* = 6.6, 2.0 Hz, tt, 1H), 6.69 (*J* = 7.3, 1.8 Hz, dt, 1H), 6.44 (*J* ~ 2.0 Hz, d, 1H), 6.17 (s:1H), 5.42 (*J* ~ 7.3 Hz, d, 1H), 4.20 (*J* ~7.3 Hz, d, 1H).

. ^{13}C-NMR (125 at MHz; DMSO): 13C NMR (101 at MHz, DMSO) 174.20,173.16, 148.19, 147.52, 146.83, 132.60, 131.01, 129.64, 124.16, 123.34, 121.63, 114.80, 78.33, 68.20, 56.86, 40.51,39.98, 39.77, 39.56, 39.35ppm. EI-MS for C23H16ClN3O5 ([M+H]) calculated.449, Found 449,1

2.5.2: 5-(3-Nitro-ph)-3-(4-Nitro-ph)-2-Ph-tetr-ahydro-4H-Pyrolo-[3,4-D] Isoxazol-4,6(5H)-Dion (IZ$_2$):

Yield: 52%, mp. 240-242°C. IR (KBr): ν 1350 (-N-O) (-C =C)1487, 1726 (-C=O), 2974(-C-H), 3074cm-^{1} (Ar-H). ^{1}H NMR (400 at MHz; DMSO) δ ¬8.27 (m, 3H), 7.87 (m: 2H), 7.73 (J ~ 8.2 Hz, t, 1H), 7.45 (*J* = 2.1 Hz, t, 1H), 7.28 (*J* = 4.2 Hz, d, 4H), 7.14 (ddd, *J* = 8.1, 1.9, 1.0 Hz, 1H), 6.97 (hept, *J* ~ 4.5 Hz, 1H), 6.21 (s, 1H), 5.48 (d, *J* ~7.4 Hz, 1H), 4.24 (m, 1H).

. ^{13}C NMR (101 at MHz, DMSO) δ 173.27, 149.00, 147.52, 146.81, 132.60, 131.01, 129.62, 124.16, 123.32, 121.03, 115.80, 78.31, 68.21, 56.66, 41.51. EI-MS for C23H16N4O7([M+H]) calculated 460 Found 460.2.

3.5.3: 5-(3-Chloro-ph)-3-(4-Methoxy-ph)-2-Ph-tetra-hydro-4H-Pyrolo-[3,4-D] Isoxazol-4,6(5H)-Dion (IZ3):

Yield: 58%, m.p. = 192-194ºC. IR (KBr): ν 1384 (-N-O) (-C =C) 1485, 1724 (-C=O), 2956 (-C-H), 3070cm-^{1} (Ar-H), (C-Cl) 765, (C-O-CH3) 1251cm^{-1}. ^{1}H NMR (400 at MHz, DMSO) δ¬ 7.52 (*J* = 7.1 Hz, d, 2H), 7.29 (m, 4H), 7.17 (m: 5H), 6.92 (*J* = 8.3 Hz, d, 2H), 5.41 (*J* = 7.7 Hz, d, 1H), 5.00 (*J* = 9.1 Hz, d,1H), 4.16 (m:1H), 3.74 (s, 3H).

^{13}C NMR (101 at MHz, DMSO) δ 174.42, 172.25, 159.53, 148.03, 133.48, 133.42, 131.25, 129.33, 129.23, 129.06, 127.48, 126.80, 125.81, 125.07, 119.55, 114.43, 77.84, 70.53, 55.51, 54.92. EI-MS for C$_{24}$H19ClN2O4([M+H]) calculated. 434 Found 434,4

2.5.4: 5-(3-chloro-ph)-2-(4-chloro-ph)-3-(4-nitro-ph) tetra-hydro-4H-pyrolo[3,4-d] isoxazol-4,6(5H)-Dion(IZ4).

Yield: 55%, mp 199-201ºC. IR (KBr): ν 1348(-N-O), 1722 (-C=O), 2978(-C-H), 3072cm-^{1} (Ar-H), (C-Cl)776, (-C =C)1485. ^{1}H NMR (400 at MHz, DMSO) δ 8.28 (m:2H), 7.84 (*J* = 8.8 Hz, d, 2H), 7.48 ~ (m, 2H), 7.34 (m, 2H), 7.56~ (m, 2H), 6.82 (*J* = 6.8, 2.0 Hz, dt, 1H), 6.53 (*J* = 2.1 Hz, d, 1H), 6.19 (s, 1H), 5.47 (*J* = 7.3 Hz, d, 1H), 4.21 (*J* = 7.3 Hz, d, 1H).

^{13}C NMR (101 MHz, DMSO) δ 174.17, 173.11, 147.85, 147.54, 146.49, 133.51, 133.01, 131.19, 129.44, 129.40, 129.08, 127.32, 126.49, 125.58, 124.18, 116.69, 78.33, 68.17, 56. 73. EI-MS for C23H15Cl2N3O5 ([M+H]) calculated 383 Found 383,3.

3. Cell Lines Studies

The MTT colourimetric assay evaluates the anticancer activity of the newly created and produced compounds [IZ1-IZ4] on the MCF-7 human breast cancer cell line. (30).

3.1.MCF-7 cell line.

The Michigan Cancer Foundation-7 (MCF-7) human breast cancer cell line is widely utilized in breast cancer research and experimental experiments. In 1970, a pleural effusion from a lady with metastatic breast cancer was used to create this cell line. Tissue Culture Technique was employed in the solutions and media. (31).

3.2. HdFn Cell Line.

The Human Dermal Fibroblast of Neonatal (HdFn) is a normal human cell line derived from the neonatal foreskin. It has scientific uses in scleroderma, skin ageing, wound healing, and gene delivery(32).

3.3 Maintenance of Cell Lines

The following procedure was carried out once a confluent monolayer had formed in the vessel: The cell sheet was washed with phosphate-buffered saline (PBS), and the growing media was aspirated. The cell was treated with a trypsin/EDTA solution of two to three millilitres. The ship was slowly rocked for an hour to cover the monolayer. The cells could detach from the vessel after being incubated for 1–2 minutes at 37°C. Pipetting was used to transfer cells from the wedding surface into the growing medium, which had been refreshed with a complete RPMI medium (15-20 ml). Culture jars, flasks, or plates were used to redistribute the cells at the appropriate concentration before being placed in a 37°C, 5% CO2 incubator. Haemocytometer counts were entered into the following calculation to determine cell concentration. Multiply the cell count by the dilution factor (sample volume) and then multiply by 10^4 to get the total cell count in each milliliter(33).

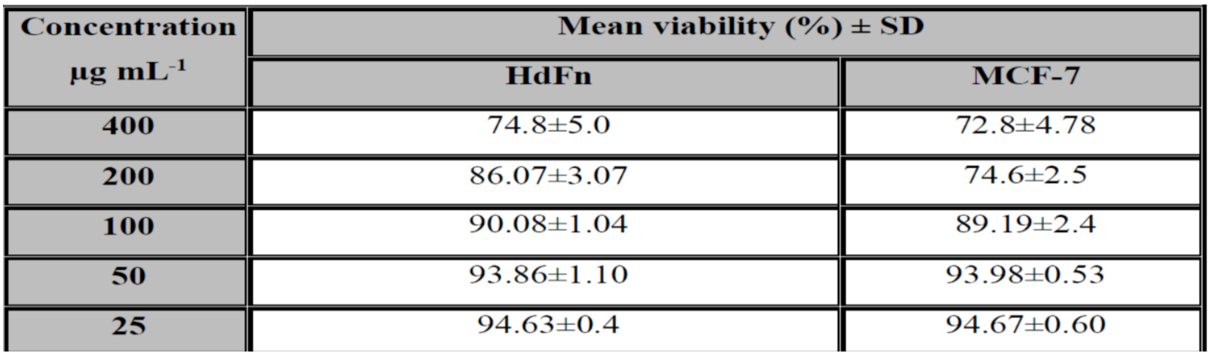
4. MTT cytotoxicity assay:

Follow the manufacturer's instructions for agreement (34): In 96 flat well micro-titer plates, tumour cells (1x104–1x106 cells/ml) were cultured in a final amount of 200 ml of complete culture media each well. Sterilized parafilm was placed over the microplate, and it was gently shaken. The plates were incubated for 24 hours at 37°C with 5% CO2. Following the incubation period, the medium was withdrawn, and the wells were refilled with two-fold serial dilutions of the Isoxazolidines (400, 200, 100, 50, and 25 mg/ml). Each concentration was employed in triplicate, along with the controls, cells cultured in a serum-free medium. The plates were incubated for four hours of exposure at 37°C and 5% CO2. 50 mg/ml of Isoxazolidines was administered to each well for a full day. After adding isoxazolidine derivatives, ten millilitres of the MTT solution were applied to each well. The plates were incubated at 37°C and 5% CO2 for four hours. After the media were properly removed, a 5-minute addition of 100 ml of Solubilization solution was made to each well. The absorbance was measured at a wavelength of 575 nm using an ELISA reader. The following formula was used to determine the concentration of chemicals needed to cause a 50% loss in cell viability for each cell line based on a statistical analysis of the optical density data: $Y= D+ A-D / 1+10^{(x-\log C)\ B}$ (35).

5. Statistical Evaluation:

A one-way analysis of variance ANOVA (Duncan) was used to determine whether or not the group variance was significant; statistical significance was assessed as p 0.05. Graph Pad Prism version 9.4 (Graph Pad Software Inc., La Jolla, CA) was used to calculate statistical significance and to express data as mean standard deviation. (36).

Table 1: IZ1 cytotoxic effect on HdFn and MCF-7 cell lines.



Concentration μg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	74.8±5.0	72.8±4.78
200	86.07±3.07	74.6±2.5
100	90.08±1.04	89.19±2.4
50	93.86±1.10	93.98±0.53
25	94.63±0.4	94.67±0.60

Table 2. IZ2 cytotoxic effect on HdFn and MCF-7 cell lines.

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	73.148±1.13	39.96±2.75
200	84.02±1.2	49.76±6.65
100	92.2±0.99	70.795±5.26
50	96.33±0.40	89.73±4.82
25	96.29±0.8	94.36±0.67

Table 3: IZ3 cytotoxic effect on HdFn and MCF-7 cell lines

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	71.33±0.57	61.96±4.2
200	76.33±2.51	68.32±2.49
100	84±3	82.02±3.90
50	85.66±2.08	92.4±3.81
25	94.6±0.57	94.71±0.43

Table 4. IZ4 cytotoxic effect on HdFn and MCF-7 cell lines.

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	72.64±1.9	41.39±5.7
200	80.20±3.11	51.81±0.4
100	85.64±3.3	61.96±4.38
50	94.17±0.77	73.22±2.7
25	96.18±0.23	84.14±0.83

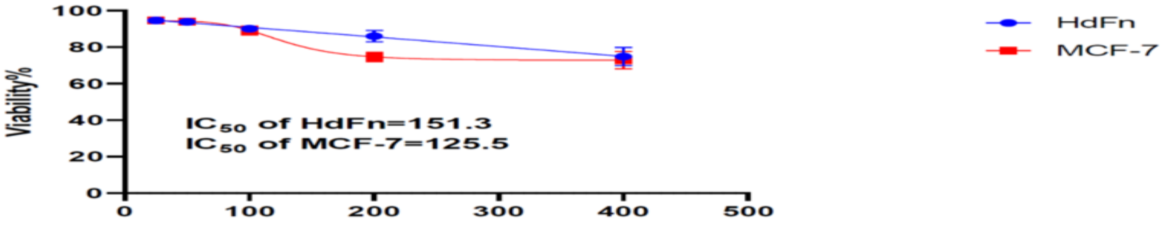
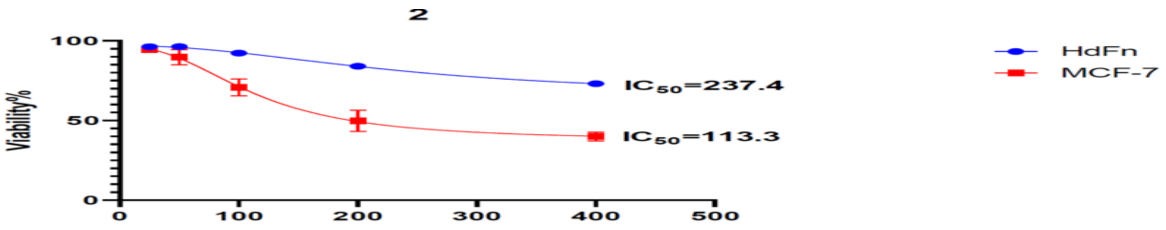


Figure 1: **IZ1 cytotoxicity on Mcf-7 cells in conc. (µg/ml).**



Fgure2: IZ2 cytotoxicity on Mcf-7 cells in conc.(µg/ml)

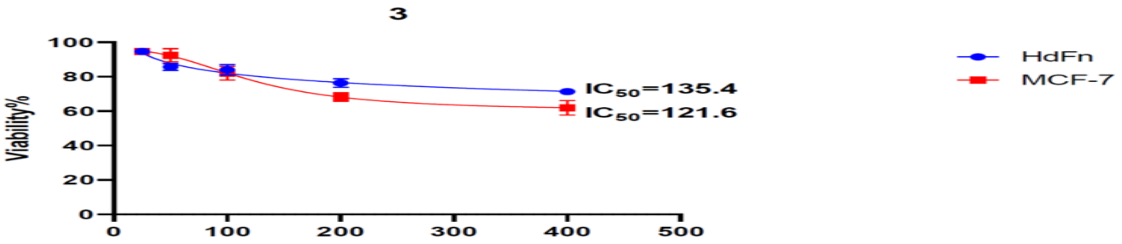


Figure 3: IZ3 cytotoxicity on Mcf-7 cells in conc. (µg/ml).

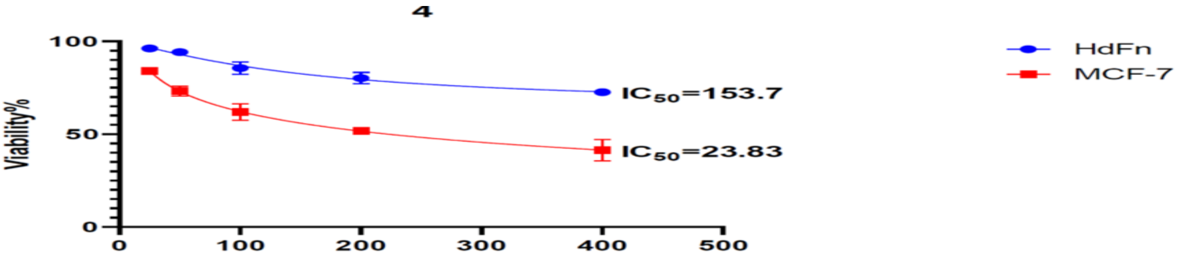
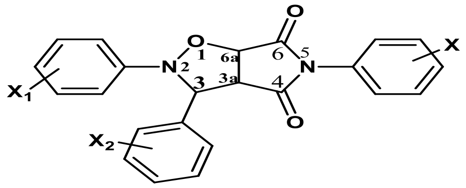


Figure 4: IZ4 cytotoxicity on Mcf-7 cells in conc. (µg/ml).

4. Results & Discussion.

Isoxazolidines chemical cytotoxicity in vitro against the mcf~7 cell line compared to the normal cell (HdFn) line.

Compd.	X	X$_1$	X$_2$	Log *p*	IC$_{50}$*Mg*/ml	
					McF-7 cells	HdFn
IZ1	3-Cl	H	4-NO$_2$		125.5	151.3
IZ2	3-NO$_2$	H	4-NO$_2$		113.3	237.4
IZ3	3-Cl	H	4-OCH$_3$		121.6	135.4
IZ4	3-Cl	4-Cl	4-NO$_2$		23.83	153.7



The general structure of isoxazolidine derivatives

The isoxazolidine derivatives were synthesized using a 1,3-DC reaction using Nitrones compound and Maleimide. The experiment utilized reflux conditions with toluene used as the solvent. Nitrones were prepared by the reaction involving an aromatic aldehyde and N-phenyl hydroxylamine. The Fourier-transform infrared (FT-IR) spectra exhibited several bands employed for characterization. Specifically, the stretching band of the dicarbonyl amide group's carbonyl (C=O) functionality was observed at a range of 1722-1726 cm^{-1} (37). The vibrational frequency associated with the carbon-

carbon double bond (C=C) occurs within the wavenumber ranges of 1485-1591 cm^{-1} (38). The stretching vibration of (N-O), the (C-N), and (C-O) bonds, which make up the isoxazolidine ring, was inferred from the observed absorption in the range of (1178-1384) cm^{-1} (39). The vibration of a methoxy group in IZ3 is indicated by the appearance of characteristic peaks in the infrared spectra at approximately 1180-1251 cm^{-1} (40). About NMR spectra, ^{1}H-NMR spectra of the Isoxazolidines (IZ1-IZ4) show a doublet signal at region (5.00-5.54 ppm) assigned for proton H-6a. Isoxazolidines (IZ1, IZ2, IZ4) show a doublet signal at (4.20-4.25 ppm) attributed to proton H-3a while in Isoxazolidines IZ3, the proton H-3a appeared as triplet signal at 4.16 ppm. Proton H-3 of compounds IZ1, IZ2, and IZ4 appeared at (6.17-6.21 ppm) as a singlet signal, while of IZ3, the proton H-3 appeared as a doublet signal at 5.41 ppm (37)(38). ^{13}C-NMR spectra have characteristic resonance signals of carbon atoms. The signals of carbon-carbonyl groups (C-4 and C-6) of all compounds appeared at ranges (173.95- 174.42 ppm) and (172.24- 173.19 ppm), respectively. The 13- carbon spectra showed three significant signals at regions (55.50-56.86 ppm), (68.17-70.52 ppm) and (77.83- 78.33 ppm) assigned to the carbon atoms C-3a, C-6a and C-3, respectively. The appearance of the three signals of carbon atoms C-3a, C-6a, and C-3 support the formation of isoxazolidine rings for the compounds (IZ1-IZ4). This study showed that the compound (IZl-IZ4) has highly significant cytotoxic activity against human cancer cell lines, with visible changes due to different substitutions, as shown in Figures (1-4). Results of initial tests on the anticancer activity of Isoxazolidine compounds show that compounds (IZ2) have the highest inhibition effectiveness on the MCF-7 (60% for IZ2). This increased activity is linked to bearing a *p*-Nitro and an *m*-Nitro aromatic substituent at the isoxazolidine ring. While the substitution of (No2) in the para position and (Cl^{-}) in the m- position reduces effectiveness as observed in compounds IZ1. It shows that the substitution of the aromatic rings plays very little role in the bioactivity of these compounds, which means that the core structure of the molecules determines the bioactivity. Since all the compounds contain the same pharmacophore, i.e., Isoxazolidines ring, Maleimide, the compounds showed a similar range of cytotoxicity(39).

5. Conclusions:

This study aimed to explore the generation of novel chemical entities possessing biological functions. Four isoxazolidine derivatives (Iso1-Iso4) were produced by the intermolecular nitrone N-phenyl Maleimide cycloaddition process. The generated compounds underwent comprehensive characterization by meticulous spectroscopic investigation. The produced compounds underwent biological evaluation by an in vitro experiment utilizing MTT to assess their anticancer activities. The results obtained from the initial assessment of the antiproliferative activity of isoxazolidine compounds indicate that all the compounds exhibit a modest level of antiproliferative activity(40). The compounds, specifically Iso2, show the greatest efficacy in inhibiting the growth of MCF-7 and HdFn normal cell lines, representing breast cancer, with IC50 ranging from 23 µg/ml to 153 µg/ml.

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