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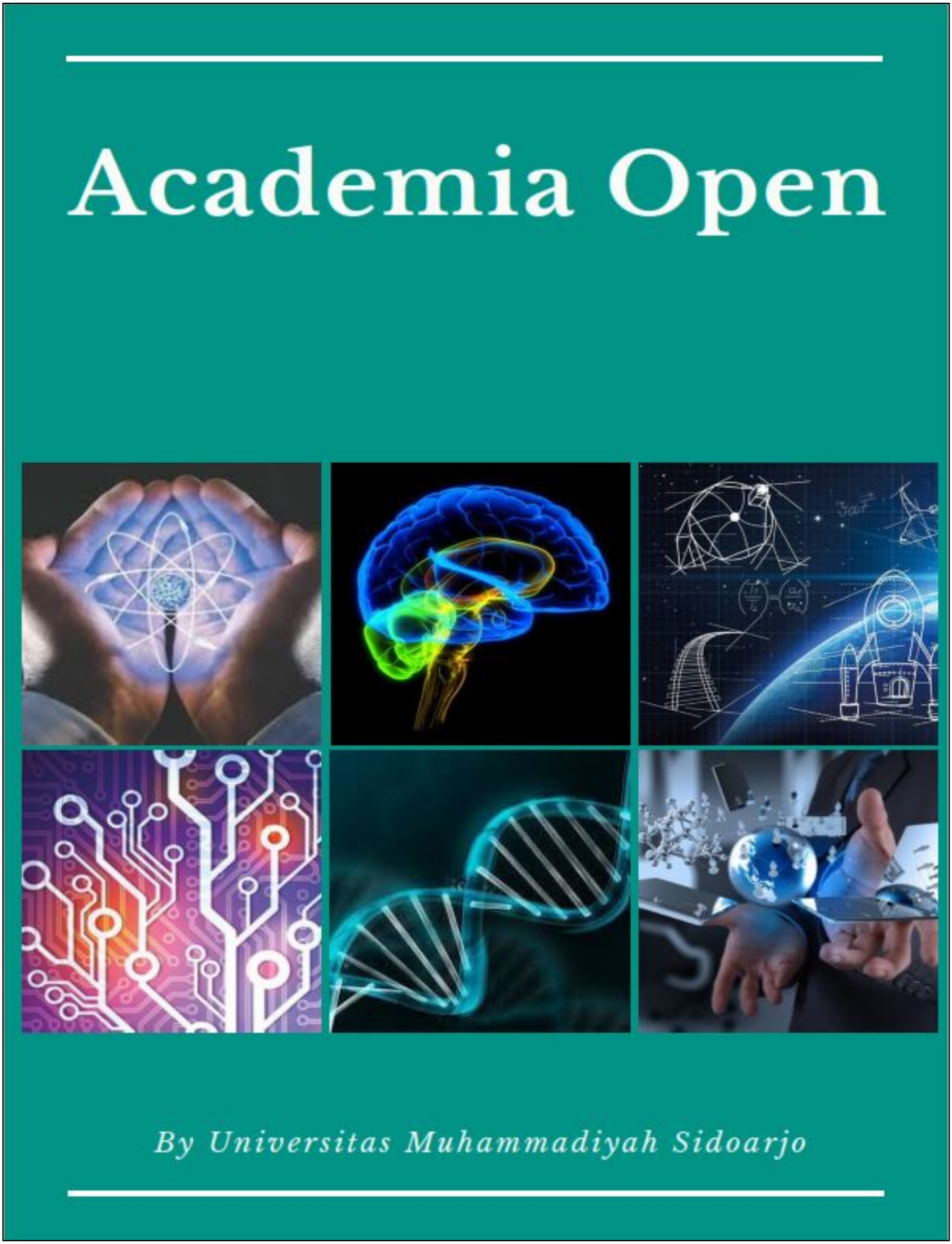

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Isoxazolidine Derivatives Exhibit Selective Antitumor Activity Against Breast Cancer Cells

Turunan Isoxazolidine Menunjukkan Aktivitas Antitumor Selektif Terhadap Sel Kanker Payudara

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Abstract

Breast cancer, a leading cause of cancer-related mortality in women, necessitates effective therapeutic interventions. Heterocyclic compounds, prevalent in FDA-approved pharmaceuticals, play a pivotal role in drug development. This study focuses on isoxazolidine derivatives, a subgroup of nitrogen and oxygen-containing heterocycles, known for their potential in antitumor applications. A series of novel isoxazolidine compounds were synthesized and evaluated for their anticancer efficacy using MTT assays against MCF-7 and HdFn cell lines, alongside normal cells. Structural elucidation employed FT-IR, 13C-NMR, 1H-NMR, and E-I mass spectroscopy. Results revealed compound (IZ3) with an IC50 value of 32.49 µg/ml, demonstrating notable antitumor activity in MCF-7 cells compared to HdFn. Notably, compounds (IZ1 and IZ2) exhibited IC50 values of 64 µg/ml and 128 µg/ml, respectively. These findings underscore the potential of isoxazolidine derivatives as promising candidates for targeted breast cancer therapies, warranting further investigation in preclinical models and clinical trials.

Highlight:

- Novel Isoxazolidine Compounds: Synthesized and Evaluated
- Selective Antitumor Activity: Demonstrated in MCF-7 Breast Cancer Cells
- Promising Therapeutic Candidates: Isoxazolidine Derivatives for Targeted Breast Cancer Therapy

Keyword: Breast cancer, Isoxazolidine Derivatives, Antitumor Activity, Heterocyclic Compounds, Drug Development

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Introduction

1. Cancer

Cancer refers to a group of diseases that mostly afflict organisms with more than one multicellular layer [1, 2]. It is characterized by alterations in the expression of several genes, which leads to disruption of the normal cellular program for cell division and differentiation. This is the defining characteristic of the condition [3]. This leads to an imbalance between the rate of cell reproduction and the rate of cell death, which favors the growth of the tumor cell population. [4, 5]. Cancer starts with one mutated cell that starts growing out of control. Progression and invasion of the surrounding connective tissues are the outcome of more mutations and the selection of changed cells within the population that proliferate more quickly Cell masses can metastasis, or spread to other parts of the body, as they enlarge and harm the nearby healthy tissues [6, 7]. A critical stage in the development of cancer that results in a more advanced stage and a poorer prognosis is metastasis. It can be divided into five main steps: Increased cell motility brought on by changes in cell-matrix and cell-cell interactions is a hallmark of invasion. relationships between the elements of the extracellular matrix. Tumor cells that have spread beyond their original site and into circulatory systems are known as extravasation. Malignant cells go to a capillary bed through the circulation systems in the third step, known as dissemination, where they adhere to the vessel walls or are prevented from spreading because of physical limitations. During the extravasation phase, cancer cells penetrate the arteries and reach their intended organs. The last phase, known as colonization, is when metastatic cells proliferate and form little or large metastases. [8].

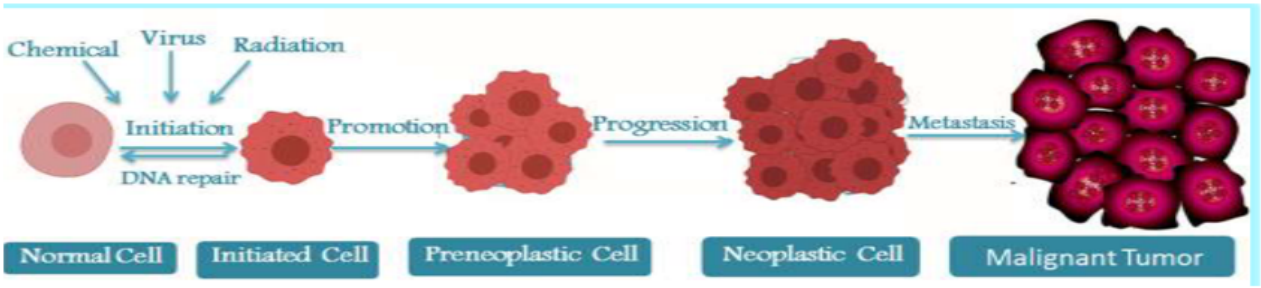

Figure 1. *Tumor growth progresses from normal cells to metastasis [9].*

2. Breast cancer

Breast cancer is the most recurrent type of cancer in women around the world., and metastasis is the primary cause of cancer death. When breast cells proliferate uncontrolled, they form a tumor, which is what causes breast cancer [8]. When proliferating cells enter neighboring tissues and organs and develop faster than the surrounding cells, the tumor is considered malignant. Malignant breast cancer grows irreversibly in the absence of treatment. If the malignant tumor is not removed, the patient may die in the early stages. Tumor growth varies widely amongst individuals, with younger women experiencing greater growth [9]. The Iraqi Cancer Board in Baghdad/Ministry of Health's cancer registry section [10] states that breast carcinoma is the most frequent malignant tumor in Iraqi women and will represent 34.01% of all female malignant cases in 2021. The second most common cause of cancer-related deaths in the US and a major factor in premature mortality as determined by the average and cumulative number of years lost to cancer in women is breast cancer. In 2022, 287,850 new cases of invasive breast cancer will be identified, and 43,250 women will lose their lives to the disease, according to the American Cancer Society [11].

3. Treatment of Breast Cancer

Breast cancer treatment is mostly decided by the stage of the tumor. Surgery (removal of cancer tissue), radiation therapy, and systemic therapies are all treatments that can be performed alone or in combination. Breast cancer is a complex disease with numerous treatment choices based on clinical factors such as age, cancer type, size, stage of diagnosis, and metastasis [12]. Chemotherapy is a pharmacological treatment that is administered to cancer patients before surgery to reduce the size of the malignancy. Chemotherapies are used to treat cancer in many ways, depending on tumor size, lymph node status, patient health, and age [13].

4. Heterocyclic compounds

Within organic chemistry, heterocyclic compounds have shown themselves as a vital topic. The definition given by IUPAC [10] is "cyclic compounds having as ring member's atoms of at least two different elements." Heterocycle ring structures are primarily composed of atoms other than carbon, with the most prevalent substituents being sulfur, nitrogen, and oxygen [11, 12]. Depending on which heteroatom(s) are present in the ring formations, heterocycles can be classified as oxygen, nitrogen, or sulfur. Compounds are grouped within each class according to the size of the ring structure, which is determined by the total number of atoms [13]. The physicochemical properties are significantly influenced by the substituent groups of the core scaffold as well as the kind and size of

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ring structures [14]. Heterocyclic compounds have key roles as antibiotics [15, 16] and antivirals [17, 18]. antifungals [19], anti-inflammatory drugs [20], and cancer therapies [21]. Readers are advised to examine additional in-depth literature on this topic as the wide range of uses of heterocycles is as varied as they are and cannot be fully explored in the scope of this review [22].

Materials and methods

The chemicals used were all bought from (BDH) or (Sigma Aldrich) Companies and ranged in purity from 99% to 99.9%. TLC keeps track of the developments of the responses. and the plates are viewed using UV light (λ= 254 nm) and iodine [23]. Electro thermal analysis was used to estimate the uncorrected melting points. The melting point apparatus on the {SMP30} has open capillary tubes [24]. Infrared spectra of compounds were recorded using an FT-IR spectrophotometer (SHIMADZU 8100s / Japan) with a range of {4000-400 cm^{-1}} for KBr discs. 1H-NMR spectra were obtained at Bruker 400MHz [25] Avance III in deuterated DMSO-d6 (δ~2.50 ppm). The ^{13}C-NMR spectra at 125 MHz were recorded using the Bruker equipment [26]. The synthetic Isoxazolidines' EI-mass spectra were measured at Tehran University's Chemistry Faculty.

1. General Method of Synthesis of Maleanilinic Acids (M1, M2 and M3): [24–26].

6 mmol of maleic anhydride, 6 mmol of aniline derivative, and 8 ml of ethyl acetate were combined in separate beakers and placed in a round-bottom flask (100 ml). After adding 5 mL of ethyl acetate to the aniline derivative solution, the maleic anhydride solution was combined and agitated for approximately five minutes. It has proven possible to obtain maleanilinic acid without additional purification. (Table: 1).

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

Table 1. *Physical properties of maleanilinic acid and components.*

2. Synthesis of N-Substituted phenylmaleimide (MD1, MD2, and MD3): [27].

In a 100 ml flask with a round bottom, 5.85 mmol of maleanilinic acid (M1, M2, or M3), 1.83 mmol of anhydroius sodium acetate, and (31.7) mmol of acetic anhydrid were mixed to make a suspension. While shaking, the reaction mixture was kept between 60 and 70 °C. To complete the reaction mixture, 100 cc of cold Water was used.. The product was collected by vacuum in ethanol, filtration and recrystallization were performed.

Compd.	X	The name	M. P. (o C)	Yield (%)	Appearance
MD1	3-Cl	1-(3-chlorophe)-1 H-pyrrole-2,5-dion	88-90	70	White
MD2	3-NO2	1-(3-nitrophe)-1H-pyrrole-2,5-dion	122-124	68	Light yellow
MD3	4-NO2	1-(4-nitrophe)-1H-pyrrole-2,5-dion	169-171	73	Light green

Table 2. *Physical properties of Maleimide derivatives [32]*

3. Synthesis of N-Substituted Phenyl hydroxylamine (H1 and H2): [28–32].

Nitrobenzene or 4-chloronitrobenzene (40 mmol), ammonium chloride (46 mmole), and water (100 ml) have been combined in a 250 ml Erlenmeyer flask and rapidly stirred for one hour. The liquid was rapidly agitated while 90

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mmole of zinc dust was added, bringing the temperature up to 65–70 °C. There was more stirring for fifteen minutes [21]. The heated mixture was filtered, and NaCl was added to the filitrate. The saturated solution was cooled using a solution of ice and salt water. Suction with vacuum was used to extract the necessary N-phenyl hydroxylamine (H1), which was then recrystallized from a combination of petroleum ether and toluene (M. P. = 81–83, yield = 90%) [35]. The N-(4-Clorophenyl) hydroxylamine (H2) (M. P. = 88–89, yield = 75%) was treated with a combination of methanol and water (1:3) in place of water.

4 General procedure of Synthesis of Nitrones (NT1-NT4): [33–35]:

In a 100 ml round-bottom flask, an ethanoic solution (15 ml) of a suitable aldehyde (10 mmol) was combined with a stirring ethanoic solution (15 ml) of a suitable hydroxylamine (10 mmole). For a full day, the mixture was mixed at room temperature. After obtaining the necessary nitrone through suction, pure ethanol was used to re-crystallize it.

Compd.	X1	X2	The name	M. P. (o C)	Yield (%)	Appearance
NT1	H	4-NO2	1-(4-nitrophenyl)-N-phenylmethanimine oxide	187-189	72	Yellow
NT2	4-Cl	4-NO2	N-(4-chlorophenyl)-1-(4-nitrophenyl)methanimine oxide	197-199	68	Yellow
NT3	H	4-CH3O	1-(4-methoxyphenyl)-N-phenylmethanimine oxide	116-119	70	White
NT3	4-Cl	4-CH3O	N-(4-chlorophenyl)-1-(4-methoxyphenyl)methanimine oxide	165-167	74	White

Table 3. *Data for the synthesized Nitrones.*

5. General procedureof synthesize of isoxazolidine (IZ1-IZ3). [36–41].

Equimolar volumes of Maleimide (M1-M3) and nitrone (N1-N4) were refluxed in toluene for the prescribed amount of time in a 100 ml round-bottom flask (Table 4). The reaction was seen using TLC (hexane: ethyl acetate: 2:1). The required isoxazolidine were filtered and re-crystallized with toluene once the reaction result cooled.

Compd.	X	X1	X2	Time (hr.)	M. P. (O C)	Yield (%)	Appearance
IZ5	3-Cl	4-Cl	4-OCH3	11	190-192	52	white
IZ6	4-NO2	4-Cl	4-NO2	12	228-230	54	white
IZ7	3-NO2	4-Cl	4-OCH3	20	191-194	61	white

Table 4. *Data for the synthesized isoxazolidine :*

6. Chemical synthesis:

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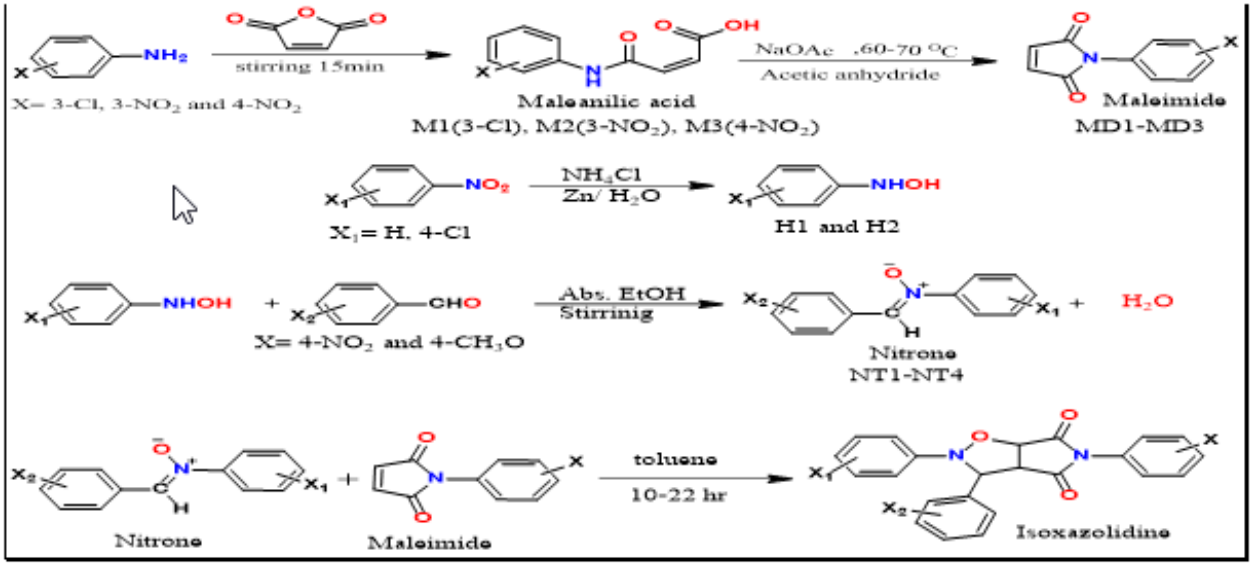

Figure 2. *Scheme synthesis of new isoxazolidine compounds (IZ $_1$ -IZ $_3$) is illustrated below (Scheme1).*

5-(3-chlorophenyl)-2-(4-chlor- phenyl)-3-(4-methoxyphenyl) tetra h ydro-4H-pyrolo[3,4-d] isoxazol -4,6(5H)-Dion (IZ 1).

Yeild: 52%, mp. 190-192^{o}C. IR {KBr}: ν 1384(-N-O), 1722 (-C=O), 2962(-C-H), 3099cm-^{1} (Ar-H), (C-Cl) 736, (-C =C)1487, (C-O-CH3)1255 cm^{-1}. ^{1}H NMR (400 MHz, DMSO) δ 7.49 (s, 1H), 7.32 (s, 2H), 7.07 (s, 2H), 6.92 (s, 1H), 5.43 (s, 1H), 5.01 (s, 1H), 4.16 (s, 1H), 3.74 (s, 2H), 3.35 (s, 2H), 2.49 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 174.22, 172.12, 159.61, 146.94, 133.48, 133.38, 131.25, 129.34, 129.18, 129.09, 129.03, 127.13, 126.81, 125.82, 121.12, 114.49, 77.90, 70.53, 55.52, 54. 86. EI-MS for C24H18Cl2N2O4 ([M+H]) calculated 468 Found 468,1.

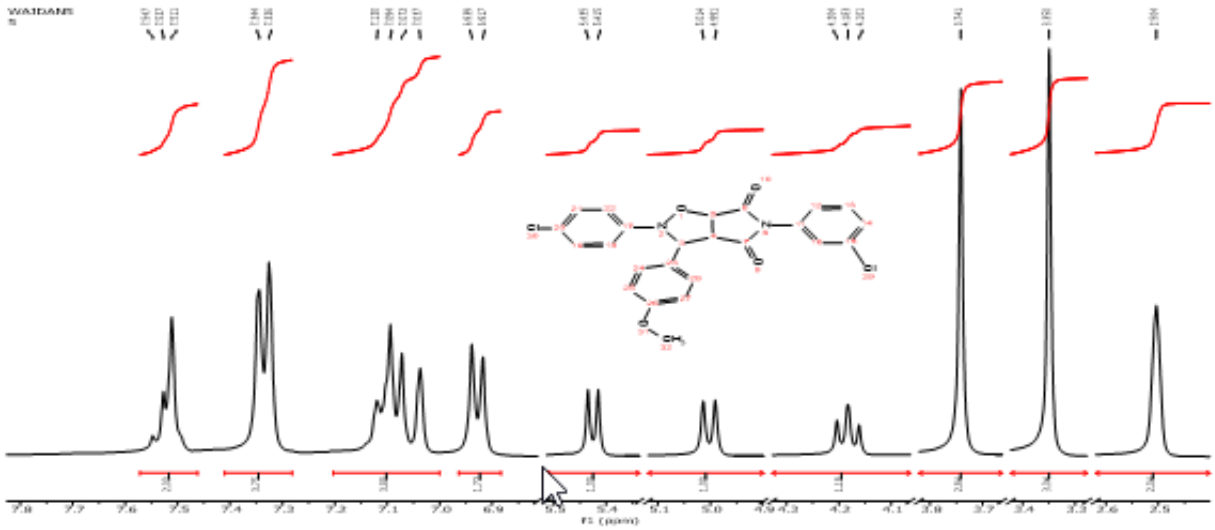

Figure 3. *^{1}H NMR of IZ1*

2-(4-chlorophenyl)-3,5-bis(4-nitrophenyl) tetr-hydro-4H-pyrrol[3,4-d] isoxazol-4,6(5H)-dion (IZ2):

Yeild: 54%, mp 228-230^{o}C. IR (KBr): ν 1346(-N-O), 1726 (-C=O), (-C =C)1490,2858(-C-H), 3113cm-^{1} (Ar-H), (C-Cl) 771. (^{1}H NMR (400 MHz, DMSO) δ 7.84 (d, *J* = 11.6 Hz, 1H), 7.31 (s, 1H), 7.27 (s, 1H), 7.25 (s, 1H), 7.18 (s, 1H), 7.16 (d, *J* = 1.8 Hz, 1H), 5.53 (1H s,), 4.24 (s, 1H), 2.30 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 173.95, 172.98, 147.58, 147.46, 146.17, 137.81, 129.39, 128.66, 127.56, 127.20, 125.77, 124.76, 124.14, 116.95, 78.13, 68.29, 56.81, 21.51. EI- MS for $C_{23}H_{15}ClN_4O_7$ ([M+H]) calculated 494 Found 494,1.

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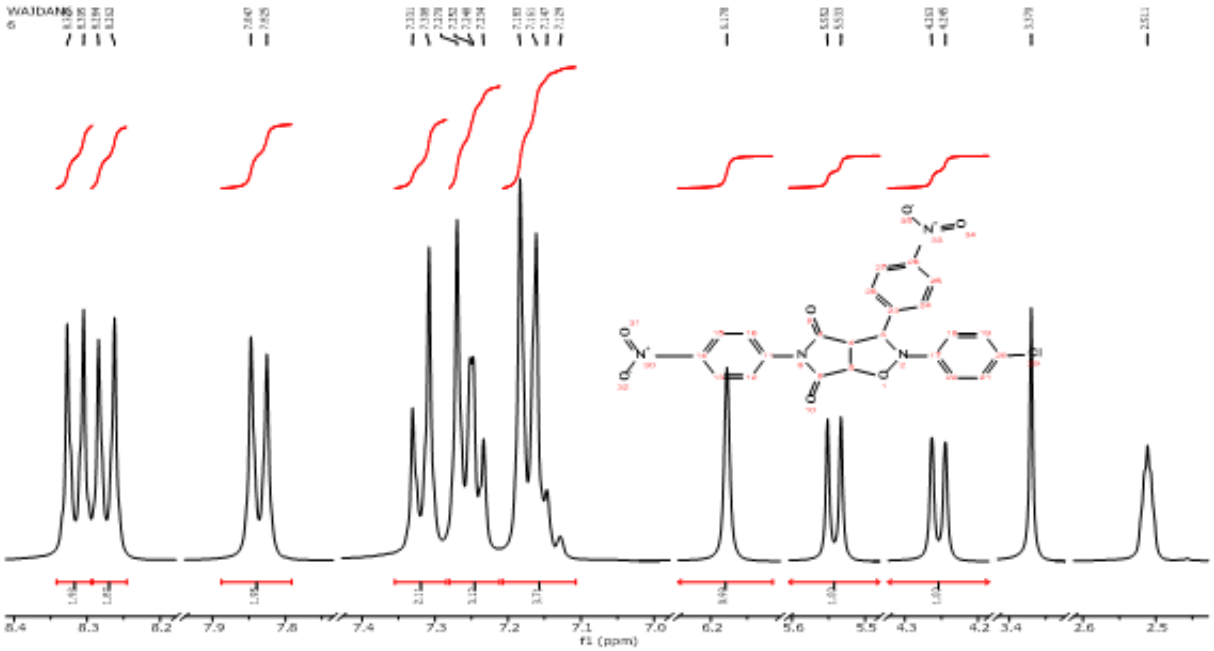

Figure 4. ***[1]H NMR of IZ2***

2-(4-chlorophenyl)-3-(4-methoxyphenyl)-5-(3-nitrophenyl) tetrhydro-4H-pyrolo[3,4-d] isoxazol-4,6(5H)-dion:

Yeild61%, m, p 202-204 .[1]H NMR (400 MHz, DMSO-d_6) δ 8.41 – 8.32 (m, 2H), 7.48 – 7.40 (m, 2H), 7.37 – 7.29 (m, 4H), 7.25 (t, J ~ 7.5 Hz, 1H), 7.15 (dd, J = 15.3, 7.4 Hz, 1H), 7.10 – 7.02 (m, 2H), 6.97 – 6.88 (m, 2H), 5.45 (d, J = 7.7 Hz, 1H), 4.94 (d, J = 9.2 Hz, 1H), 4.22 (dd, J ~ 9.2, 7.7 Hz, 1H), 3.74 (3H s).[13]C NMR (101 MHz, DMSO-d_6) δ 174.32, 172.01, 159.66, 147.23, 146.70, 137.62, 129.38, 129.30, 129.27, 129.17, 128.69, 127.89, 126.91, 124.92, 121.59, 114.65, 77.84, 70.73, 55.53, 54. 98. EI- MS for C24H18ClN3O6 ([M+H]) calculated 479.09. Found 479.2.

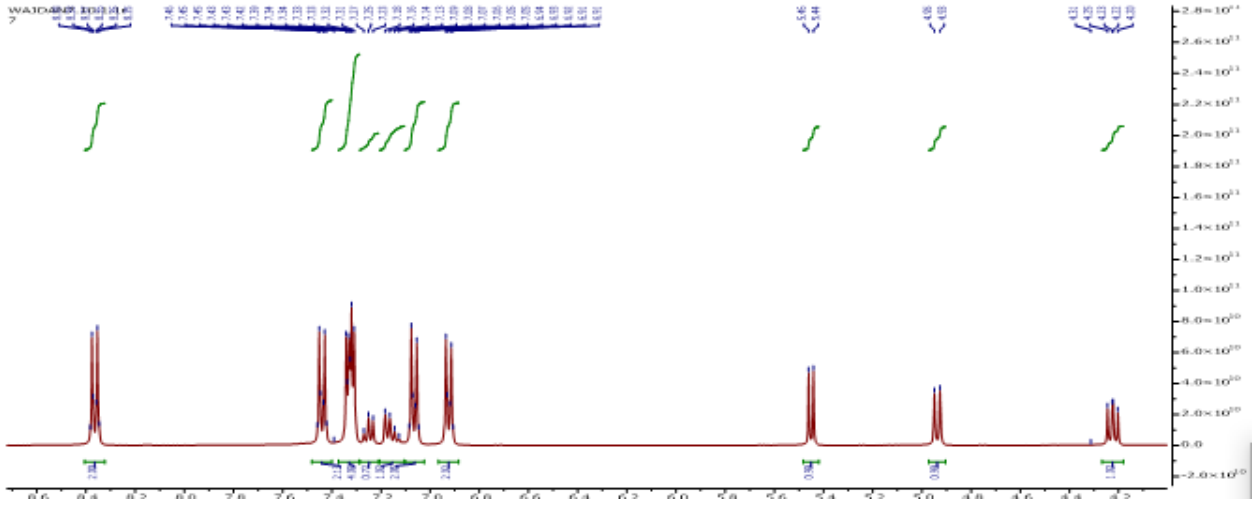

Figure 5. *[1]H NMR of IZ3*

7. Materials and methods

Equipment and Apparatus

Equipment	Company (Origin)
Centrifuge Cooling	Eppendorf (Germany).
(Inverted Microscope)	Olympus (Japan)
CO2 incubator.	Gallenkamp (England).

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Laminar Air Flow	K and K (Korea).
Micro~titer Plate Reader	Bio-Rad (Germany)
Sensitive Balance	Sartorius (Germany)
Haemocytometer	Sigma (USA)
ELISA reader	Thermofisher(Japan)

Table 5. *The following tools and apparatus were employed during the investigation:*

Biological and Chemical Materials

Chemicals and biological materials used in this study were classified according to their manufacturer as follow:

Material	Company (Origin)
2,2-diphenyl-1-picrylhydrazyl(DPPH), EDTA, Dimethyl~ sulfoxde (DMSO), Trypsin, Fatal bovine Serum, RPMI-1640 Media, Phosphate Buffer Saline, Sodium bicarbonat.	Sigma (USA)
Benzyl Penicillin, Streptomycin	Ajanta Pharm (India)

Table 6. *Classification of research producers*

Kits

The following kits were used in the study:

Kit	Company (Origin)
MTT Kit	Intron Biotech {Korea}

Table 7. *Kit used in the research*

Cell Lines Studies :

The anticancer activity of the newly designed and manufactured compounds [IZ_1-IZ_3] on the MCF-7 human breast cancer cell line is assessed using the MTT colorimetric test [42].

MCF-7 cell line .

The Michigan Cancer Foundation-7 (MCF-7) is a human breast cancer cell line that is often used in breast cancer research and experiments. In 1970, a woman with metastatic breast cancer had pleural effusion that was used to make this cell line. Solutions and Media Used for Tissue Culture [43].

HdFn Cell Line .

The Human Dermal Fibroblast of Neonatal (HdFn) is a human normal cell line that was derived from neonatal foreskin and has scientific uses in scleroderma, skin aging, wound healing, and gene delivery[44].

8. The cytotoxic impact of compounds containing Isoxazolidines on cancer cell lines when isoxazolidine are present in varying amounts.

Upkeep of Cell Lines [17].

1) The following procedure was carried out when the cells in the vessel created a confluent monolayer:

2) The cell sheet was cleaned with PBS after the growing medium was aspirated.

3) A trypsin\\EDTA solution of two to three millilitres was applied to the cell. With gentle rocking, the vessel was flipped over to completely cover the monolayer. The cells were allowed to separate from the vessel after one to two minutes of incubation at 37°C.

4) After adding 15–20 ml of fresh complete RPMI media, cells were pipetted into the growing medium from the wedding surface.

5) After redistributing the cells to the appropriate concentration in culture vessels, flasks, or plates as needed, they

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were incubated at 37°C in an incubator with 5% CO2.

6) Using the haemocytometer to count the cells and the following calculation, the cell concentration was obtained:

Total Cell Count/ml = cell count multiplied by sample volume (dilution factor) * 10^4

9. The MTT Protocol

MTT ready-to-use kit (Intron Biotech) was used to test the cytotoxicity of different concentrations of isoxazolidine:

Kit components:

• 10 vials of 1 mL MTT solution.

• 2 bottles of 50 mL Solubilization solution.

MTT cytotoxicity assay:

For agreement [19], follow the manufacturer's instructionsOn 96 flat well micro-titer plates, tumor cells (1x104–1x106 cells/ml) were cultivated with a final volume of 200 ml of complete culture media per well. After sterilizing the parafilm, the microplate was carefully shackled. The plates were incubated with 5% CO2 at 37°C for of 24 hours. Following incubation, the medium was removed and two-fold serial dilutions of isoxazolidine (400, 200, 100, 50, and 25 mg/ml) were added to the wells. Each concentration was used in triplicate, and controls (cells fed with serum-free medium) were also included. For the course of the exposure, plates were incubated at 37°C with 5% CO2. (4 hours). For 24 hours, 50 mg/ml of Isoxazolidines was administered to each well. After the exposure to isoxazolidine derivatives, 10 millilitres of MTT solution were added to every well. After that, plates were incubated for four hours at 37°C with 5% CO2. Each well received 100 ml of the Solubilization solution, which was left in place for five minutes after the medium was gently removed. An ELISA reader was used to measure the absorbance at a wavelength of 575 nm. Using the formula Y= D+ A-D / 1+10 (x-logC) B (20), the optical density data was statistically analysed to determine the concentration of chemicals needed to result in a 50% loss in cell viability for each cell line.

10. Statistical Analysis :

To determine if group variance was significant or not, a one-way analysis of variance ANOVA (Duncan) was utilized; statistical significance was assessed as $p < 0.05$. The statistical significances were determined using Graph Pad Prism version 9.4 (Graph Pad Software Inc., La Jolla, CA), and the data were presented as mean± standard deviation.).[45]

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	62.26±4.62	27.6±2.27
200	65.97±1.10	39.12±3.15
100	71.142±1.8	54.012±3.23
50	86.304±3.74	64.699±4.7
25	95.37±0.9	75.03±4.98

Figure 6. *The cytotoxic effect of IZ $_1$ on HdFn and MCF-7 cell line.*

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Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	72.14±5.12	53.47±1.13
200	85.57±2.82	64.39±4.02
100	92.78±1.58	78.39±0.75
50	94.5±0.40	94.09±2.20
25	95.33±0.63	94.86±1.3

Figure 7. *The cytotoxic effect of IZ $_2$ on HdFn and MCF-7 cell line.*

Concentration µg mL^{-1}	Mean viability (%) ± SD	
	HdFn	MCF-7
400	67.47±2.09	37.11±3.82
200	75.50±1.28	44.63±3.41
100	83.87±1.37	54.128±0.48
50	90.085±0.9	65.43±2.38
25	94.753±0.7	77.39±2.54

Figure 8. *The cytotoxic effect of IZ $_3$ on HdFn and MCF-7 cell line.*

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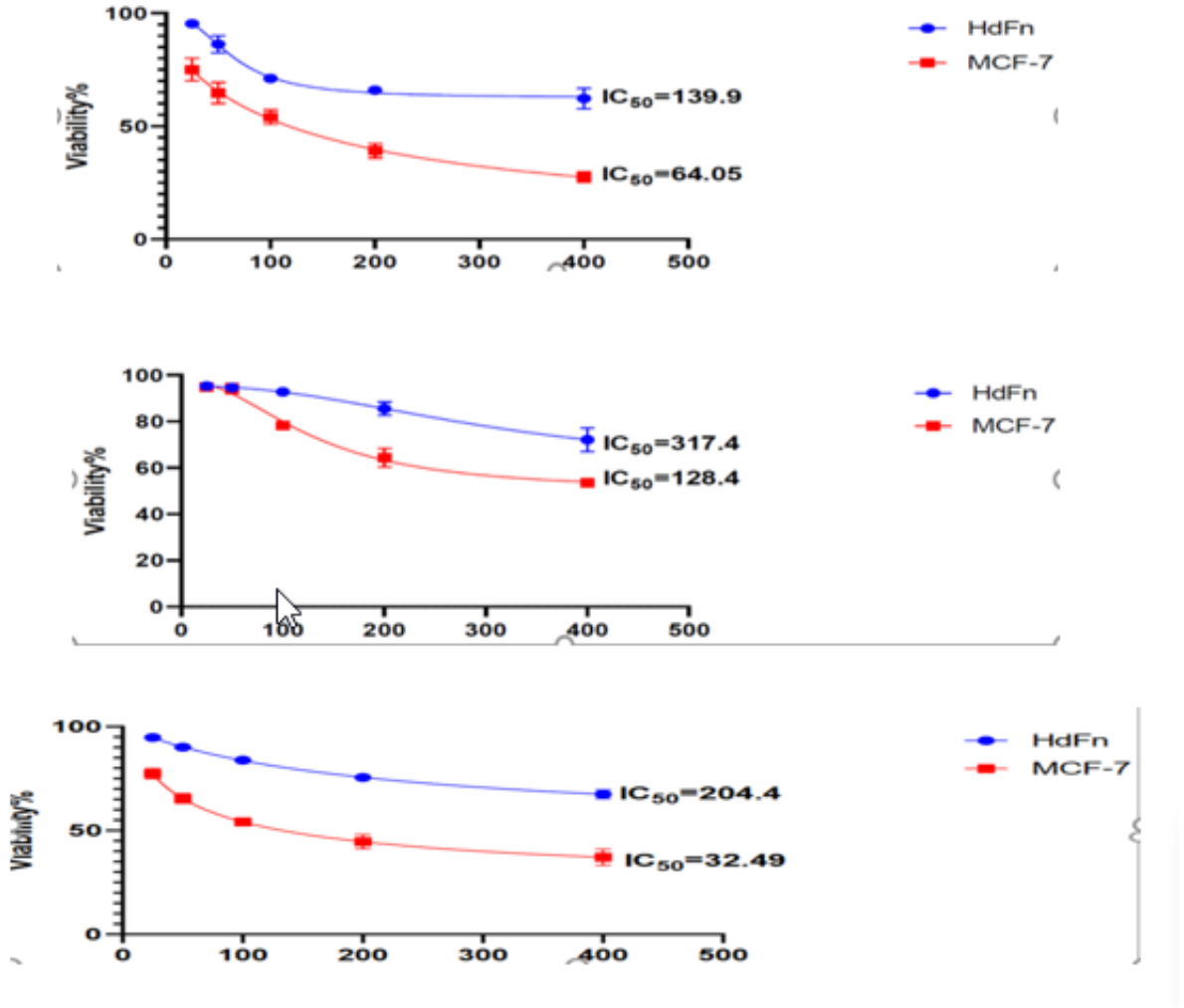

Figure 9. *figure 1 (Top). cell toxicity of IZ1 on Mcf-7 cells in conc.(µg/ml), figure 2 (Center). cell toxicity of IZ 2 on Mcf-7 cells in conc.(µg/ml), figure 3 (Bottom). cell toxicity of IZ3 on Mcf-7 cells in conc. (µg/ml*

Result and Discussion

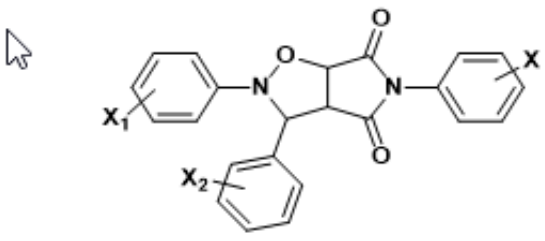

Figure 10. *General structure of isoxazolidines compounds*

Compd.	X	X 1	X 2	IC 50 M g/ml	
				McF-7 cells	HdFn
IZ1	3-Cl	4-Cl	4-OCH3	64.05	139.9
IZ2	4-NO2	4-Cl	4-NO2	128.4	317.4
IZ3	3-NO2	4-Cl	4-OCH3	43.5	159

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Table 8. ***Isoxazolidines chemical cytotoxicity in vitro against the mcf-7 cell line compared to the normal cell (HdFn) cell line.***

In this work, the MTT assay was used to examine the cytotoxic effect of (IZl-IZ3) against cancer cell lines, specifically MCF-7 and the normal cell line HdFn. The investigation of (IZl-IZ3)'s anticancer activity involved calculating its ICs (Hall maximal inhibitory concentration), a quantitative measure of the amount needed to halt the growth of a particular biological process (such as an enzyme, cell, cell receptor, or microorganism) by half. As seen in Figures (1-3), the study's findings demonstrated the compound's (IZ3) highly substantial cytotoxic activity against human cancer cell lines, with variations evident as a result of various substitutions.

Results of initial tests on the anticancer activity of Isoxazolidines compounds show that compounds (IZ3) have the highest effectiveness of inhibition on the MCF-7 IC_{50}*M*g/ml (43.5) %. This increased activity IZ3 due to can be linked to the para-methyl group's role as an electron-donating group in the phenyl moiety and (No2) roles as electron withdrawing group. While the substitution of (No2) on para position two aromatic rings and (Cl^-) on para-position reduces effectiveness.

Conclusion

It shows that the substitution on the aromatic rings plays very little role in the bioactivity of these compounds, which means that the bioactivity is determined by the core structure of the molecules. Since all the compounds contain the same pharmacophore, i.e., isoxazolidine ring, Maleimide, the compounds showed similar range of cytotoxicity[45].

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