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In Silico Study, Synthesis and Evaluation of Cytotoxic Activity of New Sulfonamide-Isatin Derivatives as Carbonic Anhydrase Enzyme Inhibitors

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ABSTRACT

Aim: Design, molecular docking, synthesis, and evaluation of cytotoxic activity of new compounds I, II, III, and IV that have isatin-sulfonamide derivatives.

Materials and Methods: for chemical synthesis, chemical compounds such as sulfonamide, 4-amino ethyl benzoate, isatin, and its derivatives were used. For the docking study (MOE), software program version 2015.10 was used. The MTT assay was utilized to predict the cytotoxic activity.

Results: The synthesized compounds demonstrated significant inhibition of carbonic anhydrase XII activity through molecular docking, as well as significant inhibition of cancer cell viability. Compounds II and IV show higher S-scores than acetazolamide. Also, the MTT assay shows that compounds II and IV have IC50 values of 0.06 µM and 0.105 µM against MCF-7 cells, respectively, while acetazolamide has an IC50 value of 0.394 µM. While acetazolamide had an IC50 of 0.901 µM, compounds II and IV had IC50s of 0.063 µM and 0.114 µM against Hct116 cells, respectively. The MTT assay explains compounds II and IV have better cytotoxic activity compared with acetazolamide.

Conclusion: New compounds that were produced showed signs of cytotoxicity and carbonic anhydrase inhibitory qualities.

KEYWORDS: carbonic anhydrase, sulfonamide, docking study.

1. INTRODUCTION

After cardiovascular disorders, cancer is the second most common cause of mortality in the US [1, 2]. Sex and age are the most important in cancer susceptibility and treatment, and the males are more subjected to cancer than the females [3, 4]. Women die from colorectum and breast cancer, while men die from prostate, lung, and colorectum cancer [5]. Antitumor medication resistance in cancer cells is the primary cause of the increased incidence of cancer treatment failure, creating significant difficulties for the healthcare system [6]. Overcoming this barrier still presents a big issue that calls for more research and creative fixes. The family of enzymes known as carbonic anhydrases (CAs) is one of the novel targets for tumor cells, with the goal of treating the tumor microenvironment to minimize chemotherapy resistance. CAs are widely distributed members of the metalloenzymes group, with eight gene groups spanning from α- to ι encoding their genes. They are present in a variety of mammals [7]. All human CAs (hCAs) are members of the α-class; fifteen isozymes have been found so far; these vary in terms of molecular characteristics, oligomeric arrangement, cellular localization, organ and tissue distribution, expression levels, kinetic properties, and reactivity to various inhibitor classes [8]. Hypoxia increases the expression of carbonic anhydrase, which is primarily found in perineurotic tumor cell patterns. CA IX and CA XII have been linked to cancer development [9]. It has been suggested that CA XII regulates cell adhesion, proliferation, and malignant cell invasion. It's interesting that human epithelial cancers that come from places that don't normally express these isoforms, like the cervix, lungs, kidneys, prostate, and breast, overexpress CA IX [10]. Sulfonamides, or R-SO2NH2, are a significant class of compounds with the ability to inhibit CA isoenzymes, which are implicated in various physiological and pathological processes [11]. One sulfonamide-based CA inhibitor that is used in clinical practice is acetazolamide [12]. Several CAIs, such as acetazolamide, ethoxzolamide, and dichlorphenamide, have been used in clinical practice for many years as diuretics and systemically acting antiglaucoma medications. CAIs use their deprotonated sulfonamide nitrogen atom to coordinate the zinc ion at the catalytic site [13]. The diverse chemical structures of heterocyclic compounds have long been a source of interest for drug discovery due to their wide range of pharmacological potential [14]. One such biologically active heterocyclic molecule is isatin, also referred to as indole quinone, indanedione, or 1H-indole-2,3-dione [15].

2. EXPERIMENTAL

Pure raw materials and modern apparatus were used to prepare, confirm, and identify the synthesized samples. The Electro Thermal Technique (SMP30) type was used to detect the melting point. Thin Layer Chromatography 1020 GS-Silica Gel 60 type was used to follow the reaction's progression. To identify the synthesized chemicals, the Pharmacy Faculty at Kufa University used a Fourier transform infrared spectrophotometer (8400s), Shimadza (KBr), to record the spectra. Using TMS as an internal reference, nuclear magnetic resonance (^{1}H, ^{13}C-NMR) data were obtained at Teheran University, Iran, using a Bruker 400 MHz apparatus. The Molecular Operating Environment (MOE) Program was utilized in an in silico investigation to demonstrate the precise binding of the resultant compounds with the target receptors.

2.1. Chemical Section

2.1.1. Synthesis of Schiff base derivatives (Ia-Id) [16,17]

In general, isatin and its derivatives (5-Methyl Isatin, 5-Methoxy Isatin, and 5-Fluoro Isatin) were condensed with 4-amino ethyl benzoate (in equal moles) using hot 100% ethanol as a solvent to create Schiff base derivatives. Drop by drop, three to four drops of glacial acetic acid were added to the mixture. The final mixture was refluxed for 48 hours. TLC analysis was used to follow the reaction steps (hexane: ethyl acetate, 3:1 v/v). After the finished product was poured over broken ice water, the precipitate was separated, filtered, and repeatedly cleaned with hot distilled water and ethanol. It was then dried and recrystallized from ethanol.

Table 1. Physical properties of Schiff bases derivatives (Ia-Id)

Symbol	Chemical formula	Color	Yield %	M.Wt (g/mol)	M.P	R_f
Ia	$C_{17}H_{14}N_2O_3$	Yellow powder	83	294.1	216-219	0.6
Ib	$C_{17}H_{13}FN_2O_3$	orange powder	92	312.30	170-172	0.5
Ic	$C_{18}H_{16}N_2O_4$	Light yellow powder	78	324.33	253-255	0.4
Id	$C_{18}H_{16}N_2O_3$	Dark Brown powder	88	308.33	95-97	0.66

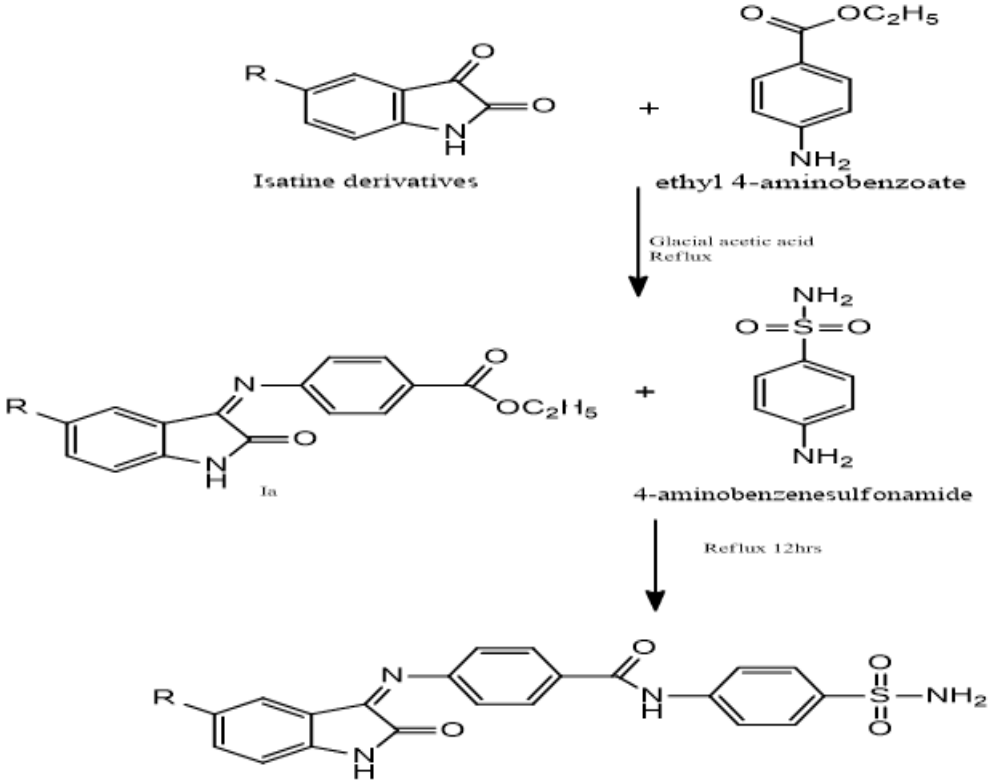
2.1.2. Synthesis of Final products (I-IV) [18,19]

In equal moles, the prepared ligands in the first step were combined with 4-aminobenzene sulfonamide using absolute ethanol: DMF (1:1 v/v) as solvent. The reaction mixture was refluxed for 12hrs. Thin Layer Chromatography (hexane: ethyl acetate (3:2 v/v)) gave its approval for the reaction to end. Crushed ice was added once the solution was reduced to half. The raw material was removed with a filter, cleaned in cold water, and then recrystallized from the water after a day.

Table 2. Physical properties of final products

Symbol	Chemical formula	Color	Yield %	M.Wt (g/mol)	M.P	R_f
I	$C_{21}H_{16}N_4O_4S$	Light yellow powder	69	420.09	205-207	0.4
II	$C_{21}H_{15}FN_4O_4S$	Yellow powder	80	438.08	198-200	0.4
III	$C_{22}H_{18}N_4O_5S$	Orange powder	59	450.47	227-230	0.42
IV	$C_{22}H_{18}N_4O_4S$	Brown-black powder	73	434.1	150-153	0.5

2.2 Chemical synthesis



R=H compound I, R=F compound II, R=OCH3 compound III, R=CH3 compound IV

Scheme (1): synthesis of intermediate and final product.

2.3 Spectroscopic analysis [20]

Compound Ia ($C_{17}H_{14}N_2O_3$) FT-IR (cm^{-1}) The FT-IR (cm^{-1}) analysis of Compound Ia ($C_{17}H_{14}N_2O_3$) reveals 3269 stretching bands of the (N-H) group of amides, 2924 stretching bands of aromatic (C-H), 1737 stretching bands of ester (C=O), 1647 stretching vibrations of the imine or Schiff base group (C=N), 1610 stretching bands of the amide (C=O), 1573 stretching bands of aromatic ring (C=C), and 1182 and 1114 stretching bands of the ester (C-O).

Compound Ib ($C_{17}H_{13}FN_2O_3$) FT-IR (cm^{-1}) The compound Ib ($C_{17}H_{13}FN_2O_3$) exhibits 3417 stretching bands in the (N-H) group of amides, 3068 stretching bands in the aromatic (C-H) group, 1720 stretching bands in the ester (C=O), 1662 stretching vibrations in the imine or Schiff base group (C=N), 1610 stretching bands in the amide (C=O), 1610 and 1487 stretching bands in the aromatic ring (C=C), and 1278 and 1201 stretching bands in the ester (C-O).

Compound Ic ($C_{18}H_{16}N_2O_4$) FT-IR (cm^{-1}) The FT-IR analysis of Compound Ic ($C_{18}H_{16}N_2O_4$) reveals 3271 stretching bands of the (N-H) group of amides, 2980 stretching bands of aromatic (C-H), 1710 stretching bands of ester (C=O), 1676 stretching vibrations of the imine or Schiff base group (C=N), 1604 stretching bands of the amide (C=O), and 1492 stretching bands of aromatic ring (C=C).

Compound Id ($C_{18}H_{16}N_2O_3$) FT-IR (cm^{-1}) The compound Id ($C_{18}H_{16}N_2O_3$) exhibits 3280 stretching bands in the (N-H) group of amides, 2983 stretching bands in the aromatic (C-H) group, 1708 stretching bands in the ester (C=O), 1602 stretching vibrations in the imine or Schiff base group (C=N), 1523 stretching bands in the aromatic ring (C=C), and 1276 and 1174 stretching bands in the ester (C-O).

Compound I ($C_{21}H_{16}N_4O_4S$) exhibits **FT-IR (cm^{-1})** 3468 and 3404 stretching bands of the (N-H) group of the primary amines, 3230 stretching band of the (N-H) group of amides, 2983 stretching band of aromatic (C-H), 1716 stretching band of the newly formed amide (C=O), 1656 stretching vibration of the imine or Schiff base group (C=N), 1602 stretching band of the amide (C=O). **^{1}H NMR (ppm)** The amide's 1H appeared as a singlet at 7.5-8 ppm, and the hydrogens of the three aromatic rings appeared as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. **^{13}C-NMR (ppm):** 129 as a singlet of carbonyl C=O and 118-163 as singlet of aromatic C.

Compound II ($C_{21}H_{15}FN_4O_4S$) **FT-IR (cm^{-1})** The FT-IR (cm^{-1}) analysis reveals the presence of 3421 and 3473 stretching bands in the (N-H) group of the primary amines, 2985 stretching bands in the aromatic (C-H) group, 1705 stretching bands in the newly formed amide (C=O), 1660 stretching vibrations in the imine or Schiff base group (C=N), 1620 stretching bands in the amide (C=O),

and 1473 stretching bands of the aromatic ring (C=C).**^{1}H NMR (ppm)** The amide's 1H appeared as a singlet at 7.5-8 ppm, and the hydrogens of the three aromatic rings appeared as a multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. **^{13}C-NMR (ppm):** 129 as a singlet of carbonyl C=O and 118-163 as a singlet of aromatic C.

Compound III ($C_{22}H_{18}N_4O_5S$) **FT-IR (cm^{-1})** 3468 and3435 stretching bands of the (N-H) group of the primary amines, 2927 stretching band of aromatic (C-H), 1668 stretching vibration of the imine or Schiff base group (C=N), 1602 stretching band of the amide (C=O). **^{1}H NMR (ppm)** The 1H of the amide appeared as a singlet at 7.5-8 ppm and the hydrogens of the three aromatic rings showed as multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. Three hydrogens of methyl appeared as singlet at 2.5 ppm. **^{13}C- NMR (ppm):** 129 as singlet of carbonyl C=O, 118-163 as singlet of aromatic C and 55 as singlet of C of OCH_3.

Compound IV ($C_{22}H_{18}N_4O_4S$) FT-IR (cm^{-1}) The FT-IR (cm^{-1}) spectrum of Compound IV ($C_{22}H_{18}N_4O_4S$) shows 3427 and 3468 stretching bands of the primary amines' (N-H) group, 3244 stretching bands of the amides' (N-H) group, 2983 stretching bands of the aromatic (C-H) group, 1718 stretching bands of the newly formed amide (C=O), and 1604 stretching vibrations of the imine or Schiff base group (C=N). ^{1}H NMR (ppm) The amide's ^{1}H appeared as a singlet at 7.5-8 ppm, and the hydrogens of the three aromatic rings appeared as a multiplet at 7-7.5 ppm. The sulfonamide group was observed as a singlet at 6 ppm. Three hydrogens of methoxy appeared as singlet at 2.8 ppm. ^{13}C- NMR (ppm): 129 as a singlet of carbonyl C=O, 118-163 as a singlet of aromatic C, and 21 as a singlet of C of CH_3.

3. DOCKING STUDY

The research includes building the protein and ligand structures for a molecular docking analysis using the 2015.10 MOE (Molecular Operating Environment) software. Using ChemDraw Professional 12.0, the ligand structures were precisely drawn. After that, the ligands were protonated in the Molecular Operating Environment (MOE) in three dimensions. Partial charges were added, and energy minimization was performed before the results were saved. The genetically modified carbonic anhydrase XII crystal structure (PDB: 1JCZ/chain A) was acquired from the PDB website and integrated into MOE for the receptor. To prepare the target protein, the remaining chains and small molecules were removed, leaving only the chain sequences essential to the protein's function. Additionally, water molecules were eliminated. The protein's atom potentials were modified, and hydrogen bonds were added before the active site was found. Lastly, MOE was used to load the previously produced ligands from the stored data, and the docking procedure was carried out.

4. STUDY OF CYTOTOXIC CELL LINES

The Iranian National Cell Bank (Pasteur Institute) provided the human normal cell line HUVEC, the human colorectal cancer cell line Hct116, and the human breast cancer cell line MDA-MB-231. Gibco's RPMI-1640 medium, supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) was used to cultivate the cells. Trypsin/EDTA (Gibco) and phosphate-buffered saline (PBS) solution were used to passage the cells, which were then maintained at 37 °C in humidified air with 5% CO_2. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Sigma-Aldrich) assay was used to measure the proliferation and vitality of the cells. Trypsin was used to digest the cells, after which they were extracted and adjusted to a density of 1.4 × 104 cells/well. The cells were then planted into 96-well plates containing 200 µl of new media per well and allowed to grow for a full day. The cells were treated to 600–7.4 µg/ml of the compounds for a whole day at 37 °C and 5% CO_2 after forming a monolayer. Following a 24-hour treatment period, 200 µl/well of MTT solution (0.5 mg/ml in phosphate-buffered saline [PBS]) was added, and the supernatant was disposed away. After that, the plate was incubated for a further four hours at 37 °C, all the while maintaining the monolayer culture in the initial plate MTT solution: The collected cell supernatant was followed by the addition of 100 µl of dimethyl sulfoxide to each well. The cells were cultured at 37 °C on a shaker plate until the crystals completely dissolved. The vitality of the cells was measured by measuring absorbance at 570 nm using an ELISA reader (Model wave xs2, BioTek, USA). The concentration of the compounds that induced 50% of cell death (IC_{50}) was determined using the appropriate dose-response curves.

5. RESULT OF DOCKING STUDY

A reference compound must be identified when working in a docking study, like acetazolamide. Use reference acetazolamide to compare the binding properties of designed compounds to the active site of the carbonic anhydrase XII enzyme with the binding properties of acetazolamide to the active site of the same enzyme. Two factors depend on docking study results to assess the activity and selectivity of compounds: the S-score and RMSD (root mean square deviation). The S-score measures the binding affinity between the desired enzyme and newly designed compounds, with a lower S-score value indicating more binding properties. RMSD refers to the distance between atoms of the active site of designed compounds and the posed ligand. In the preparatory stages, hydrogen atoms are added to the structure of the carbonic anhydrase XII enzymes to correspond to the natural PH. Table (3) displays

the docking compound results, indicating that the newly designed compounds exhibit strong binding affinities with the carbonic anhydrase XII enzyme. The binding energy of synthesized compounds with the active site of selected enzymes ranges from -6.64 to -7.12 Kcal/mol compared with the -5.82 Kcal/ mol binding energy of acetazolamide. Acetazolamide forms three bonds with amino acids of the active site of the enzyme, while newly synthesized compounds show more than three bonds forming with the active site of the enzyme. So, when comparing the results of the energy of binding of synthesized compounds with acetazolamide, it shows more selectivity of synthesized compounds to the carbonic anhydrase XII enzyme (1JCZ code in PDB).

Table 3. Binding properties of newly synthesized compounds with CA XII (PDB: 1JCZ/chain A)

Compound	R group	Docking S-scores in ΔG (Kcal/mol)	RMSD	Number of binding sites	Molecules that involve in binding
ACTAZOLAMIDE	----	-5.82	2.094	3	Zn 3:901, Thr199, Lys67
I	H	-6.64	1.321	4	Zn 3:901, Thr199, His96, His94
II	F	-6.89	1.678	5	Zn 3:901, Thr199, His96, His94, Ser132
III	OCH_3	-7.12	1.921	6	Zn 3:901, Thr199, His119, His96, Ser132, Leu198
IV	CH_3	-6.75	1.211	7	Zn 3:901, Thr200, Thr199, His94, Lys67, Ser132, Leu198

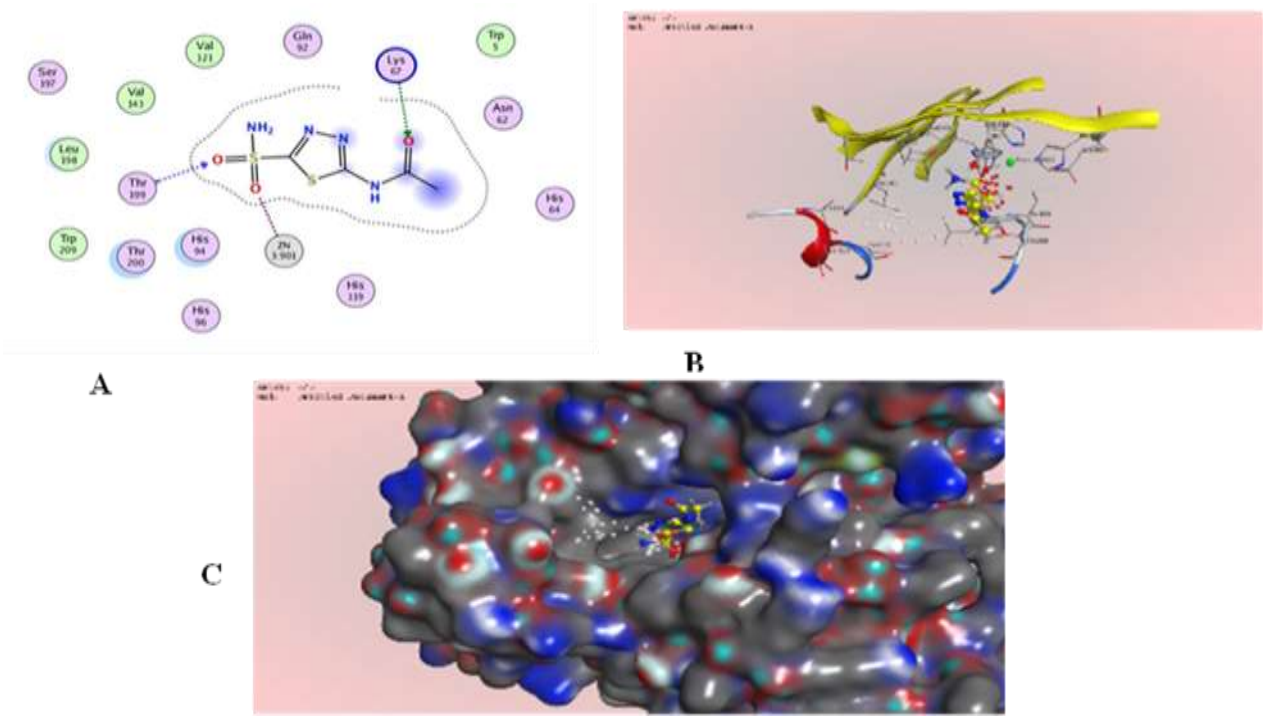


Figure (1): Acetazolamide with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Acetazolamide with active site, (B) explain the 3D picture of binding Acetazolamide with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

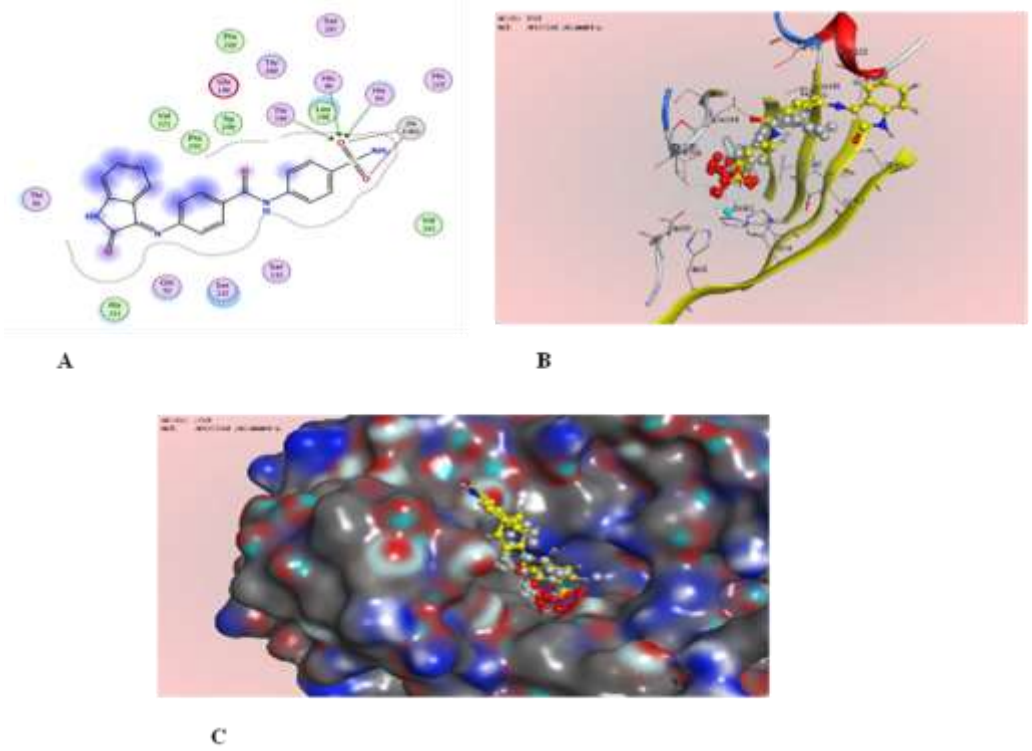


Figure (2): Compound I with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound I with active site, (B) explain the 3D picture of binding Compound I with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

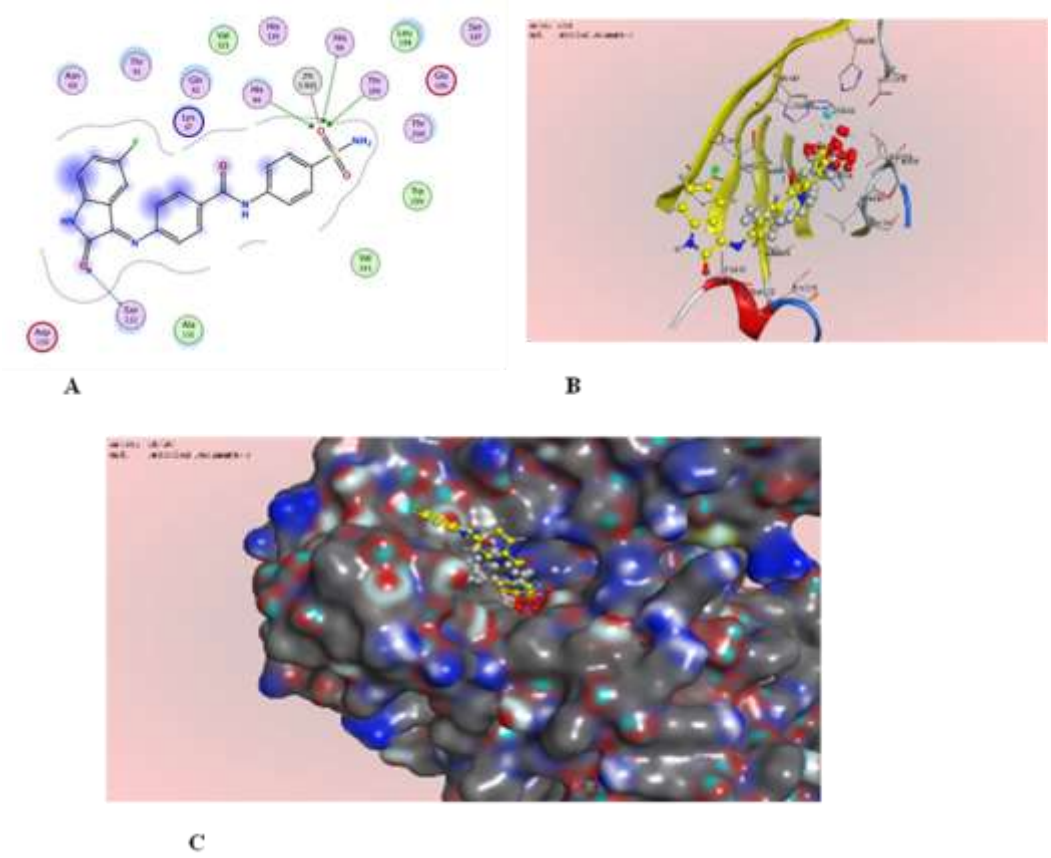


Figure (3): Compound II with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound II with active site, (B) explain the 3D picture of binding Compound II with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

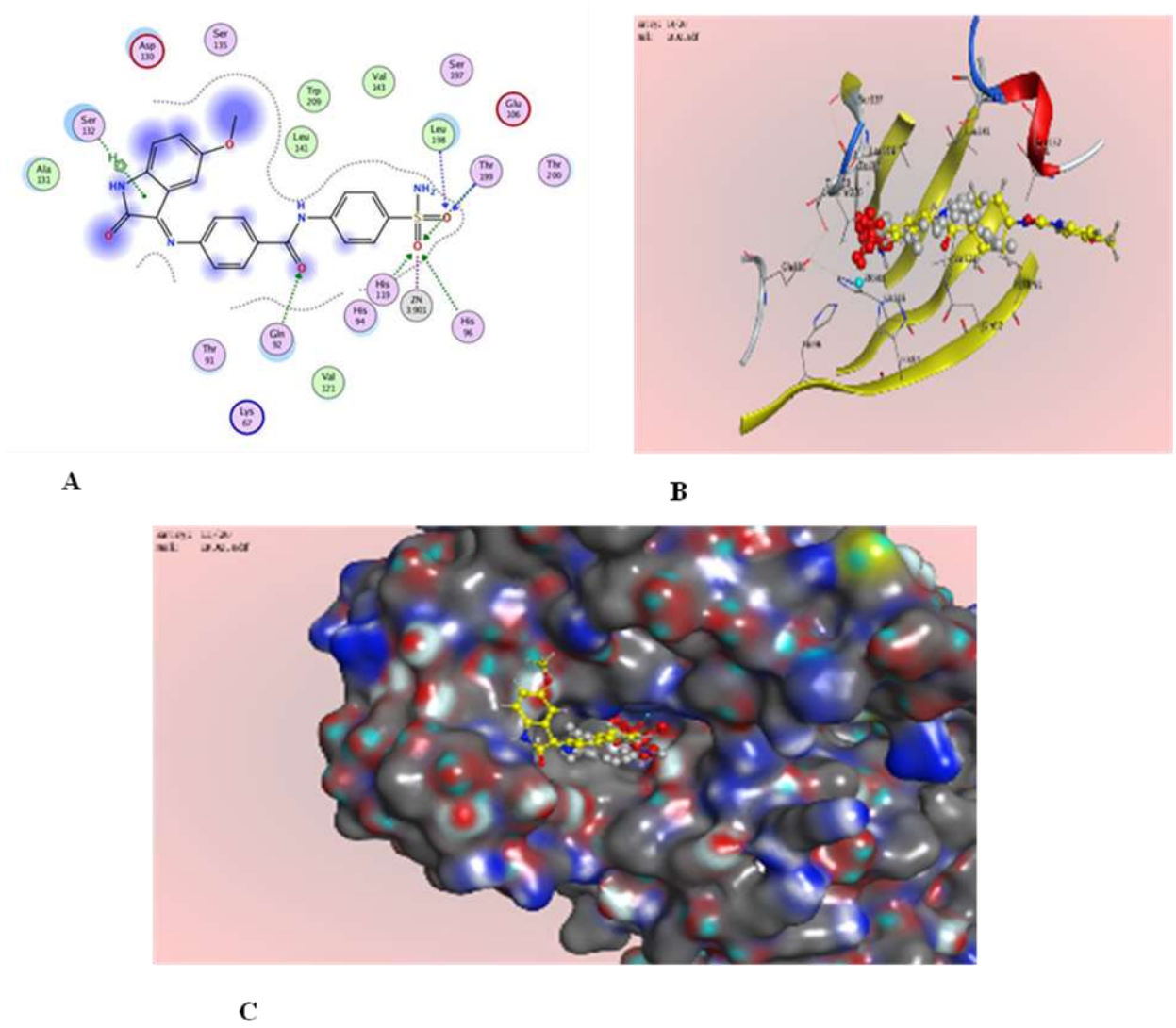


Figure (4): Compound III with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound III with active site, (B) explain the 3D picture of binding Compound III with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

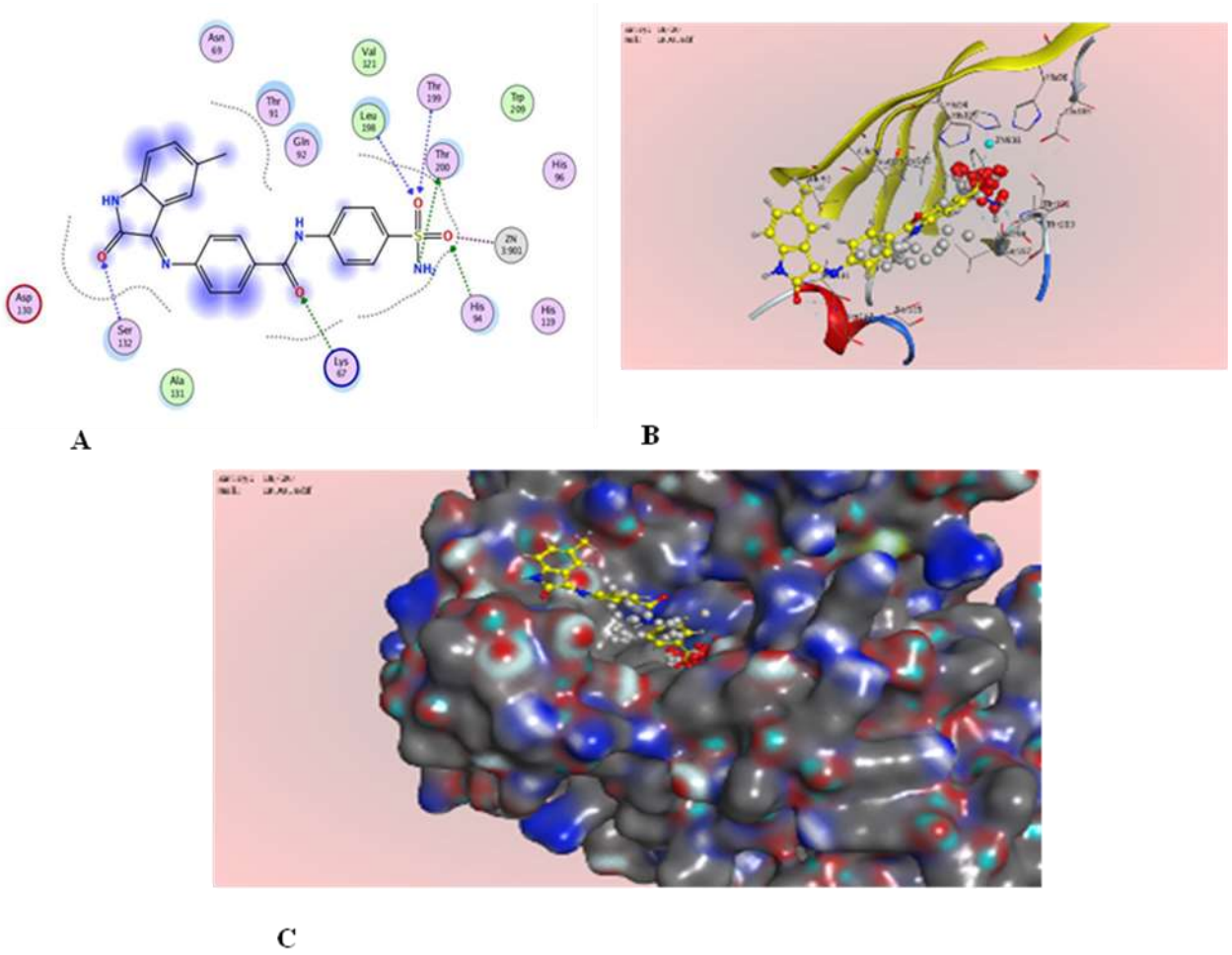


Figure (5): Compound IV with Carbonic anhydrase XII (PDB code:1JCZ). Where (A) explain the 2D picture of binding Compound IV with active site, (B) explain the 3D picture of binding Compound IV with an active site and (C) explains the 3D picture of entrance and binding with whole protein.

6. CYTOTOXIC EVALUATION

The discovery of new anticancer medications with fewer side effects and more selectivity against cancer cells is essential. Normal cells surround cancerous cells in the human body [21]. Therefore, substances meant to be used in clinical settings should be more lethal to tumor cells than normal ones. In a cell culture environment, the cytotoxicity effect of the substances (I-IV) was evaluated using cell line MTT tests. The effects of the chemicals were investigated using cell types: MCF7, which represents breast cancer cells; HCT-116, which represents colorectal cells; and MCF10, which represents normal cells. Using MTT assays, in vitro studies show superior cytotoxic activity of newly synthesized compounds. Examine the compounds' capacity to inhibit the growth of cancerous cells in order to evaluate their effectiveness. The results shown in Table 4 explain good anti-cancer activity toward the carbonic anhydrase XII enzyme. Table (4): Anti-cancer activity toward CA XII enzyme

compound	MCF-7 IC_{50} µm	Hct116 IC_{50} µm	HCF10a IC_{50} µm
ACETAZOLAMIDE	**0.394**	**0.901**	**1.313**
Compound I	**0.147**	**0.144**	**16.535**
Compound II	**0.060**	**0.063**	**0.578**
Compound III	**1.553**	**1.660**	**0.558**
Compound IV	**0.10.5**	**0.114**	**0.503**

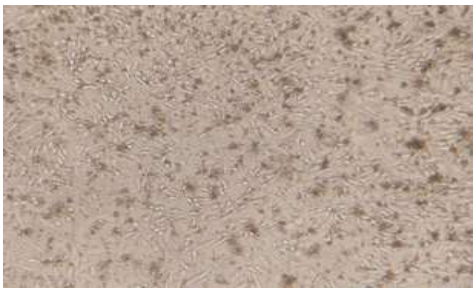
Control Hct-116

Hct-116 cells treated with acetazolamide

Hct-116 cells treated with compound I

Hct-116 cells treated with compound II

Hct-116 cells treated with compound III



Hct-116 cells treated with compound IV

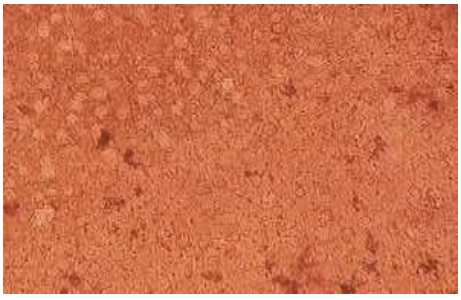
Figure (6): Show the morphology of the Hct-116 control cells and the morphology of Hct-116 cells treated with acetazolamide and newly synthesized compounds I, II, III, and IV at IC_{50}; note the number of living cells is less when treated with newly synthesized compounds.

Control MCF-7

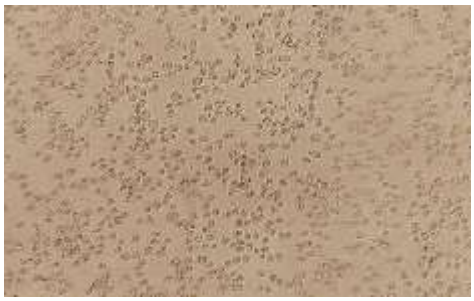
MCF-7 cells treated with acetazolamide

MCF-7 cells treated with compound I

MCF-7 cells treated with compound II



MCF-7 cells treated with compound III



MCF-7 cells treated with compound IV

Figure (7): This picture shows the shape of the MCF-7 control cells and the shape of the MCF-7 cells treated with acetazolamide and newly synthesized compounds I, II, III, and IV at IC_{50}. It is important to note that the newly synthesized compounds have fewer living cells.

7. CONCLUSION

This study shows that it has effective anti-cancer compounds acting on the Carbonic Anhydrase XII enzyme (PDB code: 1JCZ). Three main steps act on it: design, synthesis, and evaluation of biological activity. Use ^{1}H NMR, ^{13}C NMR, and FT-IR to identify and characterize compounds. The anti-cancer efficiency of the compounds was tested against MCF-7 and Hct116 cell lines using the MTT assay This research elucidates why two out of four compounds exhibit superior S.score and RMSD values in MOE docking compared to acetazolamide. Also, compounds II and IV have good cytotoxic activity IC_{50} (0.06 µM and 0.105 µM) respectively, in MCF-7 when compared with the IC_{50} of acetazolamide (0.394 µM) and compounds II and IV have better cytotoxic activity IC_{50} (0.063 µM and 0.114 µM), respectively, in Hct116 when compared with the IC_{50} of acetazolamide (0.901 µM).

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